Phenological Change in palmate (*Lissotriton helveticus*), smooth (*L. vulgaris*) and great crested (*Triturus cristatus*) newts at Llysdinam Pond in mid-Wales

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Submitted for the Higher Degree of Doctor of Philosophy

March 2009
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ACKOWLEDGEMENTS

I would like to thank the Llysdinam Trust for funding this research and my supervisor Fred Slater for advice on amphibian ecology, critical appraisal of my chapters and the opportunity to attend several conferences in the UK and overseas. Many people have given me snippets of PhD advice over the years... but there are many that deserve particular mentions....

- All my co-workers at the Llysdinam Field Centre over the past four years. Specific thanks go to Simone Lowthe-Thomas for on hand statistics advice, Rob Luxton for tips on setting up the outdoor breeding tank study. Julie for scanning documents. Liz Chadwick and Emma Durward for passing on many of the amphibian references they had accumulated. Amy Tibble and Rachel Smith for many chats in between field work and encouragement! Everyone who has checked the pitfall traps when I've been away at weekends! Also to those who got involved with non-PhD related ecology work including toad patrol, NARRS and butterfly surveys to make it a more rounded experience.

- The Professional Year students (PTYS) since without their help, many aspects of field work would have not been possible: Amy Tibble, Kate Davidson, Fiona Ross, Eleanor Sherrard-Smith, Clare James, James Chen, Annabelle Phillips, Victoria Tombs, Emily Howe, Kate McCrum, Alexandria Hale and Helen Pitt.

- The PTYS Kathryn, Fiona, El and Clare helped with torchlight surveys ...which made working in the dark a much less scary experience! They also assisted with the huge task of digging in the woodland pitfall traps and fences in the winter of 2005.

- PTYS who contributed to the many egg searches in Llysdinam Pond either as an egg searcher or as a scribe! But especially Anni who carried out a project on egg phenology for her PTY Project that contributes to this study. Anni remained cheerful even when we were in the pond in chest waders searching for eggs in snow, ice and heavy rain! She also worked determinedly on molecular analysis of eggs with advice from Geoff Hobbs, Mike Bruford and Andy King.

- James, Anni, Vicky, Emily and Kate who assisted me with netting for invertebrates and newt larvae regularly in 2007, whatever the weather and often taking longer than the 'prescribed' day! Also Helen Pitt and Alexandria Hale for spending time matching many newt photographs.

- Liz Chadwick and my advisor Hefin Jones for critical appraisal of my second year report. Rob Thomas, Jo Lello and Pete Randerson for the useful new statistics lectures.

- Darren and my friends with whom I have enjoyed many weekends when having a break from field work and writing up. I've really appreciated the many encouraging emails and texts while I've been writing up. Jen Spencer, who despite living in Australia always replied to my emails about formatting and Endnote glitches when my documents developed a mind of their own! Abigail Dando, Sian Moore, Christina White and Amy for providing a place to stay in Cardiff when I've needed. Also everyone who has given me a place to stay whilst I attended courses and meetings across the UK.

- My Mum, Dad and sister Gemma for their lasting support, and especially Darren who has been full of encouragement, a good listener, has put up with me working on many weekends, and has done far more than his fair share of the driving from Lincolnshire in recent months to visit me. **Thank you!**

ABSTRACT

Some amphibians respond to climate change by advances in phenology. Previous work at Llysdinam found significant advances in *Lissotriton* arrival dates in 1997-2005 compared with the 1980s. This study investigated newt migration phenology and whether early arrival reflected earlier breeding. A temporal difference was found in the size of *Lissotriton* newts arriving to the pond. Large newts made up a greater proportion of early arrivals. Photographic identification of ventral markings of great crested and male smooth newts was used to monitor individual movements.

Breeding was not synchronous with arrival because there was a variable delay before breeding. *Lissotriton* eggs in Llysdinam Pond were detected ten weeks earlier in 2007 than 2006. In contrast great crested females advanced oviposition by four weeks in 2007 and prolonged oviposition by five weeks. The effect of arrival time on breeding for *Lissotriton* newts was studied in outdoor tanks. There was a greater delay between arrival and egg-laying for earliest *Lissotriton* arrivals in 2006 than 2007. There was a significant decline in the length of delay between *Lissotriton* arrival and egg-laying over the season, with late arrivals breeding soon after arrival. The delay between arrival and egg-laying was reduced if mean weekly air temperatures were consistently over 2°C.

Lissotriton larvae and predatory aquatic invertebrates were surveyed by netting, and data compared with two studies in the 1980s. Invertebrates and newt larvae showed similar advances in phenology, but anuran spawning had not advanced indicating possible asynchronous interactions. In the absence of predators, there were no significant differences in *Lissotriton* hatching success or hatchling size over the season. Larvae grew faster later in the year, releasing them from predation risk by the smallest predatory invertebrates. Further research on the synchrony between species in pond habitats is suggested. If synchrony is maintained, other implications of climate change, including changes in range, susceptibility to disease and length of hydroperiod will have greater impacts on amphibian populations and possibly amphibian declines than phenological change.

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Chapter 1: Introduction

1.1 Summary

The global climate is currently going through a period of warming caused by the increase in atmospheric greenhouse gases from human activities. Wildlife is responding to climate change in a variety of ways including changes in range, morphology and phenology (the timing of events in the life-cycle). There have been concerns about a global amphibian declines crisis since the First World Congress of Herpetology in 1989. A range of factors have been implicated in amphibian declines worldwide, but one is thought to be climate change. Amphibians are thought to be particularly sensitive to changes in environmental conditions such as climate, due to being poikilotherms, having a bi-phasic lifestyle, permeable skins and unshelled eggs. One consequence of climate change has been an advance in the phenology of events in the life cycle of amphibians such as arrival at breeding ponds. In Britain, six amphibian species are widely recognised as being native, three anurans and three urodeles. This study focused on the migration and breeding phenology of the two British *Lissotriton* newts, the palmate newt (*L. helveticus*) and smooth newt (*L. vulgaris*), and the great crested newt *Triturus cristatus*. In previous research, migrations of *Lissotriton* newts to Llysdinam Pond were found to occur significantly earlier in the period 1997-2005 than 1981-1987 but it was not known if this was reflected in an earlier breeding phenology.

1.2 Climate change

Climate change is generally considered to be one of the most important environmental issues for the next century. Mean temperatures have risen by about 0.6°C over the past 100 years (Hulme et al., 2002), and 11 of the years from 1995 to 2006 were classed amongst the 12 warmest years since instrumental records began in 1850. The majority of the increase in globally-averaged temperatures that has been recorded since the mid-20th century is very likely due to increase in anthropogenic greenhouse gas (GHG) concentrations. Human activities led to a 70% increase in GHGs between 1970 and 2004 (IPCC, 2007). Concerns about climate change led to a scientific body named the 'Intergovernmental Panel on Climate Change' (IPCC) being established in 1988 to provide decision makers and those interested in climate change with objective information (IPCC, 2008). Centres for climate change research such as the Tyndall Centre at the University of East Anglia and the Hadley Centre at the Meteorological Office have looked into future

scenarios for the UK, and suggested the average annual temperature may increase between 2°C and 3.5°C by the 2080s. It is believed that higher summer temperatures will become more frequent and winters will become less cold and much wetter (Hulme et al., 2002), although there is real uncertainty concerning changes in precipitation (Beerling and Woodward, 1994).

1.3 Wildlife response to climate change

The ecological consequences of climate change have been reviewed both globally (McCarty, 2001; Walther et al., 2002) and for British wildlife specifically (Beerling and Woodward, 1994). Some studies have investigated the impacts of climate change on communities rather than individual species using microcosms for terrestrial (Jones, 2003) and freshwater ecosystems (McKee et al., 2000). Individual species may respond to global warming in a number of ways, including changes in timing of events (phenology) which will be discussed in more detail later, shifts in range and morphological changes (Hopkins, 2007; Parmesan and Yohe, 2003; Root et al., 2003).

Ranges of species may shift either pole ward or to higher altitudes as they move to occupy habitats within their metabolic temperature tolerances (Parmesan and Yohe, 2003), and therefore areas may experience changes in migrant species (Sparks et al., 2005). Climatic models have shown that plant species will shift their range north-east by 2050 to areas within their climatic suitability (Bakkenes et al., 2002). Sea surface temperature has increased leading to changes in the distribution and abundance of species, and long-term trophic changes in the northeastern North Atlantic Ocean (Beaugrand and Reid, 2003). In the freshwater environment, the trend in average temperature has had profound impacts on species compositions of lakes (Burgmer et al., 2007). In freshwater habitats, physical and ecological barriers would have to be overcome for some species, including freshwater fish to alter their range as a response to climate change (Chu et al., 2005). Distributional limits of reptiles in Switzerland were found to be mainly affected by temperature-related factors rather than topography (Guisan and Hofer, 2003).

Morphological change such as a change in body size in relation to climate change has also been documented (Chamaille-Jammes et al., 2006; Post et al., 1997; Tryjanowski et al., 2006b; Wikelski and Romero, 2003; Yom-Tov, 2001; 2006; 2008). Studies showing a change in body size in relation to temperature are more common for aquatic than terrestrial species. For example, growth rates of the freshwater amphipod *Hyalella azteca* (Panov and

McQueen, 1998) and sockeye salmon *Oncorhynchus nerka* (Pyper and Peterman, 1999) have been negatively associated with water temperature. Climate change may lead to a change in growth rates either directly or indirectly, for example Short and Neckles (1999) predicted that changes in water turbidity, salinity, water depth and species distributions due to climate change will alter growth rates of sea grasses.

Most terrestrial studies on body size have involved endothermic species, for example red deer *Cervus elaphus* hinds born following warm snowy winters were smaller as adults, produced significantly lighter sons and more male offspring, than hinds born after cold dry winters (Post et al., 1999; 1997). There was an increase in the body size of the American marten *Martes americana* (between 1949-1998) and the otter *Lutra lutra* (between 1975-2004) (Yom-Tov et al., 2006; 2008). This increase in size could have been caused directly by global warming or indirectly by warmer temperatures increasing food sources. In contrast, a decline in the size of passerine birds in Israel occurred from 1950-1999 (Yom-Tov, 2001). Recently there have been studies on changes in morphology in relation to climate change for ectothermic species. Using data from 1905 on Galapagos marine iguanas, *Amblyrhynchus cristatus* Wikelski and Romero (2003) predicted that global warming trends would cause an evolutionary increase in maximum body size. In an 18 year study in France, the body size in four populations of common lizards *Lacerta vivipara* increased for all age classes and yearling body size increased by 28%. The increase in body size appeared to be related to the higher temperatures experienced during the first month of life (Chamaille-Jammes et al., 2006). Tryjanowski et al. (2006b) found that male body size of two water frogs species (*Rana ridibunda* and *Pelophylax lessonae*) increased significantly in Poland between 1963-2003. They found a significant relationship between the body size of frogs and the North Atlantic Oscillation index. Thermal conditions during pregnancy were found to affect reproduction in a viviparous temperature snake, the aspic viper *Vipera aspis*, with temperature affecting gestation length, embryo viability and phenotype. (Lourdais et al., 2004).

1.4 Amphibian declines

The possibility of a global pattern in declines and loss of amphibian populations became apparent in 1989 at the First World Congress of Herpetology (Alford and Richards, 1999; Barinaga, 1990). The global decline scenario has received mixed reactions because the declines that have been reported may be only downward cycles of natural population fluctuations (Pechmann et al., 1991). Despite the mixed reaction, in 1991 The Declining

Amphibian Populations Task Force (DAPTF) was established to investigate the nature and extent of amphibian declines that had been reported around the world (Halliday, 2005). The Global Amphibian Assessment (GAA) was completed in 2004 and the key findings were that 32% of the world's amphibian species were threatened, in comparison to 12% of all bird species and 23% of all mammal species. The extinction of 165 amphibian species may have already occurred, and at least 43% of amphibians were exhibiting a population decline, with less than one percent of species showing evidence of a population increase. Latin America contained the largest number of threatened amphibian species, with those in the Caribbean under highest threat. 80% of amphibians were threatened in the Dominican Republic, Cuba, and Jamaica and 92% in Haiti (IUCN et al., 2006). The only amphibian to be down listed from 'critically endangered' to 'vulnerable' was the Mallorcan midwife toad or ferreret (*Alytes muletensis*) after an intense conservation programme (Garcia et al., 2005). In September 2005 a decision was made to merge the DAPTF, GAA and the Global Amphibian Specialist Group (GASG) into a unified body devoted to global amphibian conservation: The International Union for the Conservation of Nature and Species Survival Commission (IUCN/SSC) Amphibian Specialist Group (ASG). It was hoped it would be more effective to tackle the amphibian crisis (Amphibian Specialist Group, 2008).

Declines and losses of amphibian populations are a global problem and have been occurring since the late 1950s (Houlahan et al., 2000). A range of causal factors have been studied (Alford and Richards, 1999; Collins and Storfer, 2003). The GAA identified habitat loss to be the greatest threat (Gallant et al., 2007), although a newly recognised fungal disease was found to be seriously affecting an increasing number of species (IUCN et al., 2006). Other threats include ultraviolet radiation (Trudeau et al., 2005), predation (Alford and Richards, 1999), habitat modification (Becker et al., 2007), chemical contaminants (Davidson et al., 2002; Relyea, 2005), diseases (Daszak et al., 2005a; 2003; Parris and Beaudoin, 2004), and changes in climate and weather patterns (Alexander and Eischeid, 2001; Araújo et al., 2006; Carrier and Beebee, 2003; Daszak et al., 2005b; Donnelly, 1998; Pounds, 2001; Pounds and Crump, 1994; Reading, 2007). Many species are declining for unknown reasons, complicating efforts to design and implement effective conservation strategies (IUCN et al., 2006). Often there has been more than one explanation for amphibian losses, and studies to date suggest that multiple stressors may be responsible (Boone et al., 2005; Davidson et al., 2002; La Marca et al., 2005; Lips et al., 2008; Pounds et al., 2006; Rohr et al., 2004). Some of the mysterious declines in frog populations in Central America and Australia have been accompanied by equally

mysterious lizard declines (Czechura, 1991). Most research has been carried out in rich developed countries, and large areas of the world such as South America, which holds the greatest amphibian biodiversity, have more limited information.

1.5 Amphibians and climate change

The three extant Orders of Class Amphibia are the Anura (frogs and toads), the Caudata or Urodela (salamanders) and the Gymnophiona (caecilians). The frogs, toads and salamanders are well known, but caecilians are a primitive group resembling earthworms which live only in the tropics, where they burrow through damp soil (Beebee, 1996; Beebee and Griffiths, 2000). Amphibians are likely to be particularly sensitive to subtle changes in the environment such as climate change; they are poikilotherms, have permeable skins, unshelled eggs and a biphasic lifecycle. Both temperature and moisture impact directly on amphibian biology (Carey and Alexander, 2003; Duellman and Trueb, 1995). Biochemical, cellular and physiological functions generally vary two-to three fold for each 10°C change in temperature (Carey et al., 2001), and amphibian reproductive is probably the most vulnerable of all to changes in precipitation (Carey and Alexander, 2003).

The life histories of amphibians are highly diversified. Most species of frogs have external fertilization, whereas internal fertilization occurs in the majority of salamanders. The classic amphibian life history of aquatic eggs and larvae is typical of many frogs and salamanders, but there are many other modes of reproduction, including direct development of eggs on land, ovoviviparity and even viviparity (Duellman and Trueb, 1995). Few data are available on two-thirds of caecilian species (Baker, 1997; Gower, 2005), but the majority have severed their reproductive ties with the aquatic environment in favour of terrestrial reproduction (Kupfer, 2005).

Although in their 350 million years of existence, amphibians have been exposed to considerable environmental variation (Carey et al., 2001), human-induced climate change has meant that during the second half of the 20th century, the average northern hemisphere temperatures were likely the highest in at least the past 1300 years (IPCC, 2007). Climate change can have a range of implications for amphibians including changes in phenology, morphology, range, water availability and exposure to infectious disease. Water availability can affect the hydroperiod of freshwater habitats, the number of freshwater sites for breeding and therefore the length of time available to the amphibian larval period.

For the UK, climatic modelling showed that the great crested newt had an unchanging northern range margin in Britain and Ireland. Unfortunately for the natterjack toad *Epidaelea calamita*, there was no overlap between its current distribution and the modelled potential future climate space (Berry et al., 2002). Therefore, possible mitigation for climate change impacts on species may need to include the facilitation of a shift in biological range to new, suitable areas (Beebee and Griffiths, 2005).

Recently there has been growing interest in infectious diseases such as chytridiomycosis, saprolegniosis and ranavirus, and their role in amphibian declines (Daszak et al., 2003). Temperature variability can increase susceptibly of amphibians to infection (Raffel et al., 2006), and it is thought that climate change may exacerbate the outbreaks of certain pathogens (Carey and Alexander, 2003; Pounds and Crump, 1994). Saprolegnia, an aquatic phycomycetes, has been reported to cause egg mortality and the effects were exacerbated by UV-B radiation (Kiesecker et al., 2001). Annual epidemics of ranavirus result in deaths of large numbers of common frogs *Rana temporaria* in UK each year (Cunningham et al., 1996). Chytridiomycosis provides the clearest link between disease and declines (Berger et al., 1998; 2004; Bosch et al., 2007; Daszak and Cunningham, 1999; Daszak et al., 2003; Skerratt et al., 2007). Chytridiomycosis is a skin disease of amphibians caused by a zoosporic, chytrid fungus *Batrachochytridium dendrobatidis*. It was first discovered in captive amphibians during the 1990s, and was reported as the proximal cause of mortality events in declining amphibian populations of Panama and Queensland in 1998 (Daszak et al., 2005a). Pounds et al. (2006) reported with 'very high confidence that large-scale warming was a key-factor in the disappearances' of the Monteverde harlequin frog (*Atelopus* sp.) and the golden toad (*Bufo periglenes*). Their study implied that climate change increased the growth of chytridiomycosis which led to higher amphibian mortality and population declines.

1.6 Phenological change

Phenology is defined as 'the study of the times of recurring natural phenomena, especially in relation to climate' (UK Phenology Network, 2007). Robert Marsham (1708-1797) is considered to be the founding father of phenology and is best known for his 'Indications of Spring', the phenology notes which he made for 60 years (Sparks and Lines, 2008). Successive generations of his family added to the recordings and this information now provides valuable data to the UK phenology database (Sparks and Carey, 1995; UK Phenology Network, 2007). The Royal Meteorological Society formed a phenological

committee in the late 19th Century that, until 1947, collated data sent in by observers (Sparks and Collinson, 2002). Concerns regarding the threat of global climate change stimulated renewed interest in phenology so the 'UK Phenology Network' (UKPN) was created in 2000 which coordinates collection of phenological data by the public (Sparks and Collinson, 2002; Sparks et al., 2000).

One of the major requirements to identify whether there is phenological change in an organism is to have a good long-term data set. Good phenological records tend to consist of data collected consistently over an extended period of time by a well trained team of volunteers or scientists. Ornithology has many good long-term data sets, and has provided some of the best examples of the impacts of recent climate change (Crick, 2004). Probably the longest and largest data set of breeding timings for birds comes from the British Trust for Ornithology's (BTO) Nest Record Scheme, indicating significant trends towards earlier egg-laying for 20 out 65 species (Crick et al., 1997). Crick and Sparks (1999) found that laying date in 31 out of the 36 bird species analysed was related to temperature or rainfall. Several other studies have found an advance in the arrival and breeding phenology of birds (Both and te Marvelde, 2007; Crick, 2004; Møller et al., 2006; Przybylo et al., 2000; Sparks and Mason, 2004), and in some cases differences in phenological advancement have led to a change in the order of arrival of migrant species (Sparks and Tryjanowski, 2007). Additionally, evidence indicates that warmer spring weather in Europe has disrupted the synchrony between winter moth (*Operophtera brumata*) hatching and oak *Qurcus robur* bud burst, leading to a mismatch between the peak in insect availability and the peak food demands of great tit *Parus major* nestlings (Buse and Good, 1996; Visser and Holleman, 2001; Visser et al., 2006). In contrast there are few studies on mammalian species with most focusing on changes in fecundity rather than phenology. For example, red deer (Post et al., 1997) and Soay sheep *Ovis aries* (Forchhammer et al., 2001) born after warm winters were smaller than those born after cold winters and the variability persisted into adulthood.

From the Class Insecta, there have been thorough monitoring programmes of butterflies with earlier appearance found in all 17 analysed species from the Mediterranean (Stefanescu et al., 2003). In the UK, trends in mean first appearance, peak appearance and length of flight period of butterflies led to predictions that a 1°C rise in temperature would lead to an advancement in phenology by 2-10 days (Roy and Sparks, 2000). Hassall et al. (2007) analysed biological records of British dragonflies (Odonata), and found they used

climatic warming to extend their flight period by a three day advancement per 1°C rise in temperature. Phenological studies on invertebrates are important since they are vital for pollination, are prey for animals higher up the food chain and some are pest species. Much of the debate about the effect that climate change may have on insect abundance has been concerned with its possible differential effect on bud burst in plants and egg hatch in insect herbivores (Dixon, 2003).

A phenological review of 542 plant species from 1971-2000 in Europe indicated that 78% had an advancement in leafing, flowering and fruiting records (Menzel et al., 2006). Analysis of phenological observations on the early dog violet *Viola reichenbachiana* and horse chestnut *Aesculus hippocastanum* in the UK and Poland from 1970-1995 found that plants in the UK showed a greater response to climate change. It was suggested that adaptation to the local environment may lead to different responses to warming (Tryjanowski et al., 2006a). The pollen season has advanced in the Netherlands by three to 22 days, depending on the plant species, in a 30 year period (van Vliet et al., 2002), and there has been an increase in length of the growing season by 10.8 days since the 1960s (Menzel and Fabian, 1999). Despite the end to the growing season being significantly delayed, autumnal phenological events have received far less attention than spring events partially because they are harder to define (Sparks and Menzel, 2002). Gange et al. (2007) investigated 52000 individual fungal fruiting records from 315 autumnal fruiting fungal species, and found the length of the average fruiting period had more than doubled to 75 days since the 1950s. The increase in late summer temperatures and autumnal rain has caused early fruiting species to fruit earlier and late season species to fruit later, which suggests a longer period of mycelial activity, and therefore an increase in decay rate in forests. Although most studies have investigated early spring phenomena, some species require a period of chilling and may respond with later spring events (Jeger et al., 2006).

1.7 Amphibian phenology

Factors influencing reproductive cycles of amphibians are complex, and probably involve interactions between temperature, rainfall, photoperiod and endogenous circannual rhythms (Griffiths et al., 1986). There has been considerable variation in amphibian breeding phenology across the UK (Beebee and Griffiths, 2000) with earlier phenology in the south than north (Beebee and Griffiths, 2000; Chadwick, 2003). Differences in the phenological response of the common toad *Bufo bufo* and common frog between the UK and Poland suggests species are adapted to local conditions (Sparks et al., 2007). Altitude

can provide a good natural model for climate change since common frog spawning was found to be delayed by 6 days for every 100 m increase in altitude (Beattie, 1985), with a 1°C drop in temperature found for every 270 m increase in altitude. Beattie (1985) suggested that the stimulus causing frogs to emerge in the spring may be different from the one controlling spawning, since he recorded either a short or long delay between the arrival of frogs at ponds and subsequent spawning (Beattie, 1985).

In a review, McCarty (2001) cited just one publication documenting a change in amphibian phenology by Beebee (1995), which demonstrated the bias towards publications on the phenology of birds, insects and plants. Beebee (1995) studied the start of breeding activities for the natterjack toad, common frog and introduced edible frog (*Rana esculenta*) for 16 years in southern England. The natterjack toad and the introduced edible frog spawned progressively earlier with an advance of 2-3 weeks from 1978-82 to 1990-94. First spawning dates of natterjack toads and edible frogs were negatively correlated with average minimum temperature in March and April and maximum temperatures in March respectively. Although the spawning date of the common frog did not change significantly, it was correlated with the winter average maximum temperature. Days of first sighting of the three British newt species were all significantly earlier. There was a strong correlation between arrival of smooth newts and the average maximum temperature in the month preceding arrival. Beebee concluded that amphibians were breeding earlier in temperate countries, in response to warming trends in winter and early spring temperatures.

Subsequent to Beebee's study there has been an increase in research of amphibian courtship and egg-laying phenology (Chadwick et al., 2006; Corn and Muths, 2002; Gibbs and Breisch, 2001; Hartel, 2008; Reading, 1998; Scott et al., 2008; Tryjanowski et al., 2003). Meta-analyses of 143 studies on a range of species showed that overall amphibians had a greater advancement in phenology (5 days per decade) than birds and insects (Root et al., 2003). Parmesan (2007) conducted meta-analysis on 203 species from the northern hemisphere, and found that amphibians had a significantly stronger shift towards earlier breeding phenology than the other groups analysed. There was a mean advancement of 7.6 days per decade, although the largest differences were due to very strong changes for just a few amphibian species so results may not be able to be generalised.

Earliest dates of calling frogs were compared between two periods 1900-1912 and 1990-1999 in Ithaca, New York and showed a trend towards earlier courtship behaviour. Four of

the six anuran species, spring peeper (*Pseudacris crucifer*), wood frog (*Rana sylvatica*), grey tree frog (*Hyla versicolor*) and bullfrog (*Rana catesbeiana*) were calling 10-13 days earlier in the second period. The first calling dates of the green frog (*Rana clamitans*) and American toad (*Bufo americanus*) was unchanged while no species were calling later (Gibbs and Breisch, 2001). Blaustein et al. (2001) also conducted a study in North America and found that only one of four amphibian species were breeding earlier but the result was not significant, although at three of four sites there was a negative association between time to first breeding and temperatures. The locations of the weather stations used by Blaustein et al. were criticised, and reanalysis of the data for boreal toads *Bufo boreas* found significant negative regressions of breeding with air temperature and snow accumulation (Corn, 2003). In mountainous regions such as North-west America, phenology has often been determined by snow melt. In Colorado, Corn and Muths (2002) found that earlier breeding phenology for boreal chorus frogs *Pseudacris maculata* was positively associated with snow accumulation on dry years.

In Europe, Tryjanowski et al. (2003) found a 8-9 day shift in first spawning date of common frogs and common toads from 1978-2002. First spawning dates were not associated with precipitation but they were correlated with temperature. In the UK, Scott et al. (2008) found correlations between temperature and breeding of the common frog, with trends for earlier congregation and spawning but not earlier hatching. Hartel (2008) found no earlier extension to the breeding season of *Rana dalmatina* in central Romania between 1997-2007. No significant correlations were found between year of study and temperature or precipitation, although mean air temperature in February was a good predictor of the commencement of male calling activity and spawning. In the UK, the initiation of breeding in the common toad did not show a significant advancement during a 19 year study from 1980-1998. Despite this, the arrival of common toads to ponds was highly correlated with mean daily temperature over the 40 days preceding arrival. There were typically early, average and late years for adult toad arrival which could be predicted from mean winter temperatures, and five of the six early years occurred between 1990 and 1998. The earliest recorded arrival was Day 33 suggesting a threshold day length of about nine hours (Reading, 1998).

Chadwick et al. (2006) investigated median arrival dates (when 50% of the January-April total had arrived) of palmate and smooth newts at Llysdinam Pond between two periods 1981-1987 and 1997-2005. Within sexes, male palmate newts showed the greatest

advancement in arrival date (18 days) while female palmate arrival was advanced by 12 days and smooth newt arrival was advanced by 13 and 4 days for males and females, respectively. A significant advance in arrival of 2-5 days was found with every 1°C rise in February minimum temperature. 74% of the between year variability was explained by spring temperatures. Median arrival dates of males were similar in 1981-1987 but in 1997-2005 male palmate newts arrived on average 5 days earlier than male smooth newts. The average delay between the median arrival of protandrous males and the females in 1981-1987 was 8.1 days and 4.4 days for palmate and smooth newts, respectively. In 1997-2005 there was an increase in the average delay to 12.2 days and 13.4 days for palmate and smooth newts, respectively. Median departure dates were 11-14 days later in 1997-2005 for all groups except male smooth newts. The aquatic phase for breeding adults was therefore 3-4 weeks longer in the second period.

Of the British amphibians, frog spawning phenology has received the highest attention, probably because its visibility makes it ideal for phenological record keeping by the public and for large scale studies by scientists. In Wales, frog spawn was one of 22 indicators chosen by experts in 1998 to assist in monitoring climate change (Buse et al., 2001). The spawn of common toads and particularly the eggs of *Lissotriton* and *Triturus* newts are less visible. Amplexed toads wrap their spawn stings around vegetation (Beebee and Griffiths, 2000), and newt eggs are highly cryptic since they are wrapped within aquatic vegetation (Miaud, 1994). Published data on phenology of the European newts have concentrated solely on newt migration dates (Beebee, 1995; Chadwick et al., 2006).

Earlier phenology can increase the time available until metamorphosis (Reading and Clarke, 1999), although early breeders may be affected by cold spells increasing spawn mortality (Reading and Clarke, 1999). Earlier breeding may also limit exposure of eggs to UV-B radiation (Cummins, 2003), although European newts minimise exposure by wrapping eggs (Marco et al., 2001). Changes in breeding patterns not only affect species, but also the way they interact, leading to alterations in competition, predation pressure, competition and food availability. For example, early arrival of newts to breeding ponds increases the exposure of frog spawn and tadpoles to newt predation (Zahn, 1997). Species that become established early may have a greater chance of being competitively dominant than late arrivals (Alford and Wilbur, 1985; Ryan and Plague, 2004).

1.8 Amphibians in Britain

In Britain six amphibian species are widely recognised as being native. The three anurans are the common toad *Bufo bufo*, the natterjack toad *Epidaelea calamita* (previously *Bufo calamita*) and the common frog (*Rana temporaria*). The three urodeles are the great crested newt *Triturus cristatus*, the palmate newt *Lissotriton helveticus* (previously *Triturus helveticus*) and the smooth newt *Lissotriton vulgaris* (previously *Triturus vulgaris*). There has been considerable debate over the native status of the pool frog *Pelophylax lessonae* (previously *Rana lessonae*). The pool frog was thought to be introduced but recent genetic evidence showed that while some populations of pool frogs in the UK were introduced from southern Europe, there were also native pool frogs that went extinct very recently. A re-introduction from Scandinavia took place in 2005 in Norfolk to restore the distinct northern clade to the UK (Foster and Buckley, 2005; Herpetological Conservation Trust, 2008).

All British amphibians follow a two-phase life cycle with an aquatic egg and larval phase, followed by metamorphosis and dispersal onto land, where they live before returning to the water as breeding adults. In the UK, frogs, toads and newts are oviparous animals; little or no development of their egg occurs within the female. For frogs and toads there is intense competition between males in the breeding season to clasp onto a female (amplexus). The eggs are fertilised externally by the amplexed male and are laid in clumps by frogs or as strings by toads (Beebee and Griffiths, 2000). In contrast, European newts perform an intricate courtship dance (Arntzen and Sparreboom, 1989; Halliday, 1977a) leading to uptake of a spermatophore, a sperm package, by the female cloaca. After mating European newts lay and wrap their eggs individually in vegetation (Miaud, 1994; Norris and Hosie, 2005b). As the embryo develops it passes through several stages documented by Gallien and Bidaud (1959), with the rate of development dependent on factors including temperature and water quality. A developmental problem associated with chromosomes in the eggs of the great crested newt, means that 50% of eggs abort development at the tail bud stage (Beebee and Griffiths, 2000). Newt larvae become free swimming a few days after hatching and are predatory on small invertebrates in the natal pond. Adult newts are also opportunistic predators and exhibit a low degree of food selection in the pond. Despite a high degree of feeding niche overlap, the two *Lissotriton* species may be able to co-exist without competing in a eutrophic environment with abundant food, such as Llysdinam Pond (Griffiths, 1986). A few months after hatching newt larvae undergo metamorphosis and emerge from the pond as small metamorphs often referred to as 'efts' (Griffiths, 1995).

Little is known about the 'eft' stage of the life cycle, but after two or three years in the terrestrial environment they return to the pond to breed, with females generally reaching sexual maturity a year after males. Unlike *Lissotriton* newts, immature great crested newts sometimes return to the pond annually (Beebee and Griffiths, 2000).

Palmate and smooth newts are similar in morphology and colouration. This can lead to problems in identification, particularly with the females. Male palmate newts tend to be the smallest British *Lissotriton* newt, on average measuring a total length of 67 mm and male smooth newts the largest, measuring 83 mm at Llysdinam Pond in this study. Female palmate newts were larger than their male conspecifics at 76 mm while female smooth newts were smaller than conspecific males at 80 mm. The key species identification feature is that palmate newts have a pink unspotted chin while smooth newts have a cream throat with spots or speckles. Males are distinguished from females by a black cloaca which is swollen during the breeding season. Male smooth newts usually have dark pronounced ventral spots while female smooth newts have a speckled brown belly; palmate newts often have a yellow belly with no or few spots. Male newts have secondary sexual characteristics which become particularly pronounced during the aquatic period of the breeding season. The male palmate secondary sexual characters are a tail filament and webbed feet, while male smooth newts develop toe spurs and a dorsal crest in the aquatic stage (Beebee and Griffiths, 2000; Griffiths and Mylotte, 1988). Although palmate and smooth metamorphs look very similar they can often be distinguished by the length of line on their backs (Roberts and Griffiths, 1992). Great crested newts are morphologically distinct from *Lissotriton* newts, being much larger (often 15 cm or more in length) and black in colour with granular skin (Beebee and Griffiths, 2000). They have striking yellow and black belly markings which can be useful in differentiating between individual newts within a pond (Arntzen and Teunis, 1993). Breeding males have a white tail flash and a large dorsal crest (Beebee and Griffiths, 2000).

All native species of amphibians are protected under Schedule 5 of the Wildlife and Countryside Act (1981) but, with the exception of the great crested newt and natterjack toad, the protection extends only to trade in those species. The common toad, great crested newt and natterjack toad are on the UK list of Priority Species, the common frog is listed in Annex 5 and great crested newt and natterjack toad in Annex 4 of the EU Habitats and Species Directive. The two latter are also listed in Appendix 2 of the Bern Convention. For the natterjack toad and great crested newt, the Wildlife and Countryside Act (1981)

protects them from being intentionally killed, injured, taken possession of, or sold or having their place of shelter damaged or disturbed without licence. The natterjack toad is the rarest British amphibian and has been the subject of considerable conservation efforts (Buckley and Beebee, 2004). Within the UK, several conservation bodies are dedicated to amphibian conservation, including the 'Herpetological Conservation Trust' (HCT), the 'British Herpetological Society' (BHS) and 'Froglife'. The smooth newt is the most widely distributed newt in Britain whereas the palmate is typically an upland species common in Wales and Scotland but more patchily distributed throughout England. The great crested newt is declining across its range, with the UK considered a stronghold of the species, even though it may be decreasing faster than any other British amphibian or reptile (Beebee and Griffiths, 2000).

Although the cause of some amphibian declines in the UK remain unclear (Carrier and Beebee, 2003), little habitat in the UK remains unaffected by human interference so amphibian declines can usually be attributed to anthropogenic interference particularly through habitat destruction and modification (Baker, 1997; Beebee, 1973; 1977; 1997; Cooke, 1972). The protected status of great crested newt has led to an exponential increase in mitigation projects with about £1.5 million pounds spent annually (Edgar et al., 2005; Griffiths, 2004). A survey by Edgar et al. (2005) found that 27% of great crested newt habitat and half of all ponds were destroyed in developments, of which 50% were building developments and the remainder were mainly associated with mining and transport.

The classification of European newts has changed over the years based on morphological and more recently genetic discoveries. Formerly, the genus *Triturus* included all 13 species of European newts, and was informally divided into two groups based on morphological similarities. The nomenclature of genus *Triturus* was re-evaluated in 2005 after research by París (2004), and during the past year the changes have been reflected in literature. *Triturus* was split into four genera: *Lissotriton* for the small bodied species (previously *T. boscai*, *T. helveticus*, *T. italicus*, *T. montandoni* and *T. vulgaris*), *Ommatotriton* for the banded newts (previously *T. ophryticus* and *T. vittatus*), *Mesotriton* (containing only the alpine newt, formerly *T. alpestris*) and the rest remain in *Triturus*. For the purpose of this thesis when referring to the three British newt species collectively the term European newts will be used. When referring to the two small bodied newt species, the palmate and smooth newt collectively, *Lissotriton* will be used. When referring to one of the three British newt species the common name (palmate, smooth or great crested) will be used.

1.9 Research at Llysdinam Pond

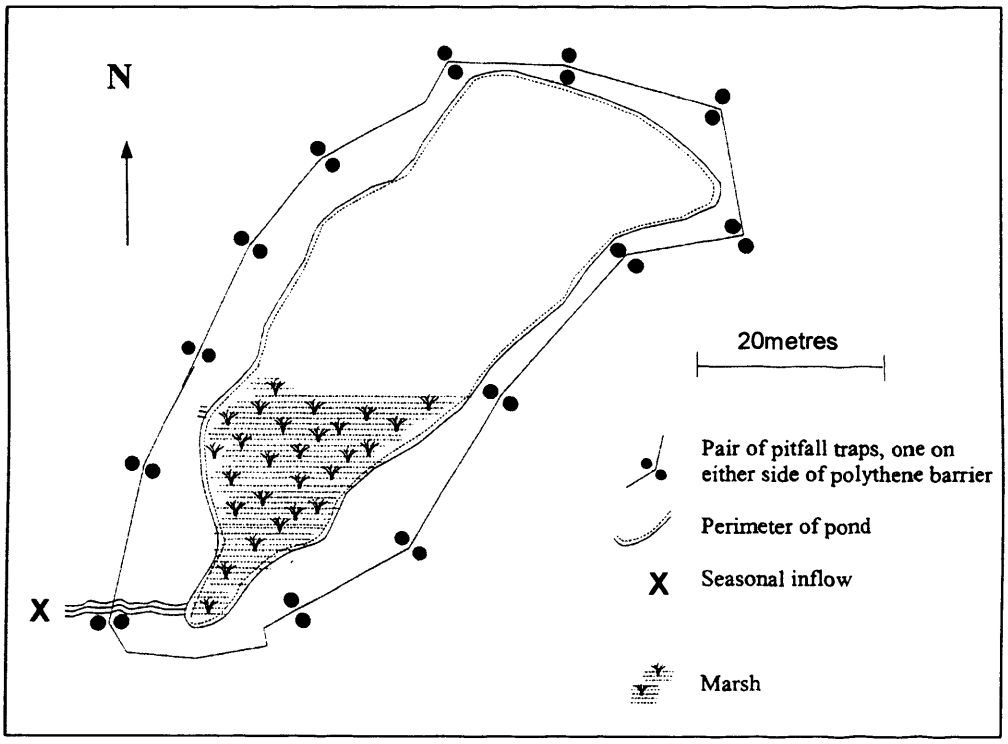
Llysdinam Pond has a history of amphibian research on toad migrations (Gittins, 1983) and various aspects of newt ecology (Chadwick et al., 2006; Griffiths, 1987; Griffiths et al., 1987; Harrison, 1985; Harrison et al., 1983; 1984). The pond is situated near the village of Newbridge-on-Wye in mid-Wales (53°12'59" N 3°27'3" W) at an altitude of 200 m (Figure 1.1). The pond has been a breeding site for a large population of palmate and smooth newts since at least the 1970s, although the number of adults arriving to breed has fluctuated annually. A smaller number of common frogs and common toads, and an increasing population of great crested newts also breed at the pond. The toad population in the late 1970s was around 1000 but from 2004-2008 was much lower with 70-250 toads arriving annually to breed. Llysdinam Pond has a surface area of approximately 900 m^2 and an average depth of 0.6 m (Harrison et al., 1983). The pond's surface area and depth varies with rainfall throughout the year, with over a 50% reduction in surface area in dry summers such as 2006 (pers. obs.). During the study period Llysdinam Pond was mainly vegetated by reed sweet grass (*Glyceria maxima*), water forget-me-not, (*Myosotis scorpioides*), floating sweet-grass (*Glyceria fluitans*), creeping bent (*Agrostis stolonifera*), water mint (*Mentha aquatica*) and bog-bean (*Menyanthus trifoliata*). From the late spring, common duckweed, (*Lemna minor*) dominated the pond surface which reduced the amount of sunlight received by the pond (Figure 1.1). An increase in *Lemna* spp. and a decrease in broad leaved pond weed (*Potamogeton natans*) and hornwort (*Ceratophyllum demersum*) have done little to change the physical character of the pond (F.M. Slater, pers. comm.). The banks were lined with grey willow (*Salix cinerea*), ash (*Fraxinus excelsior*) and beech (*Fagus sylvatica*).

Figure 1.1 **Photographs of Llysdinam Pond**

(i) In winter (ii) In summer

In February 1981 a polythene drift fence was constructed to encircle Llysdinam Pond (Harrison et al., 1983). The fence was 30 cm high and was buried 5 cm deep into the soil. Thirteen pairs of pitfall traps (approximately 22 cm in diameter and depth) were located along the drift fence at approximately 15 m intervals, one on either side of the fence (Figure 1.2). Recorders have monitored the pitfall traps since 1981. Normally the pitfall traps were checked on a daily basis between 0900 and 1000 GMT and the species, sex and direction of movement recorded for each amphibian. Amphibian movement during the preceding 24 hours was therefore recorded. It was assumed that amphibians caught outside the fence were moving towards the pond and those caught within the fence were moving away from the pond. After recording the number of newts by species and sex, they were released on the side of the fence opposite to where they were captured.

Figure 1.2 **Diagram of Llysdinam Pond**
The study site of Llysdinam Pond was surrounded by a polythene drift fence with 13 pairs of pitfall traps. The southern end of the pond has a shallow marshy area and a seasonal water inflow dependent on the amount of rainfall. Map adapted from Harrison et al. (1983) and Chadwick, (2003).



When the fence plastic sheet became worn or damaged, it was repaired or replaced, although since 2003 green 'Newt-guard' polythene has been used with a guaranteed life of at least five years (Figure 1.3). Bamboo sticks and ladders made of chicken wire mesh have been used intermittently in the past to assist with mammal escapes. In winter 2005, mammal ladders constructed from a plastic mesh were placed in each pitfall trap (Figure

1.3) and since then the mortality of small mammals has dropped considerably to an average of less than one mortality per year. It was not thought that the ladders affect newt captures since newts appeared to climb the pitfall trap sides more readily than the ladders (pers. obs. during torching surveys), and the highest number of newts ever recorded at Llysdinam arrived in the breeding season of 2006 following the addition of the mammal ladders.

Figure 1.3 The amphibian drift fence and pitfall traps at Llysdinam Pond

(i) The drift fence that encircles Llysdinam Pond. It is made from a green UV resistant plastic called 'Newt guard'

(ii) Pair of pitfall traps with mammal ladders made from plastic garden mesh

1.10 Aims of this study

Analysis of the long-term data set on the migrations of adult *Lissotriton* newts to Llysdinam Pond found an advancement in arrival phenology in 1997-2005 compared to 1981-1987, particularly of palmate newts (Chadwick et al., 2006). Records of pitfall captures from 1986-1996 were not analysed by Chadwick et al. because the data were poor with less records made less than four times per week. This research continued from Chadwick et al. (2006) to investigate whether early arrival was reflected in earlier breeding, the differences in breeding phenology between early and late newt arrivals and the consequences of earlier breeding.

Due to the more cryptic nature of urodeles, there has been less research on, and public awareness of urodeles than anurans although it was estimated that 50% of urodeles were threatened or extinct compared to 32% of anurans in the Global Amphibian Assessment of 2004 (IUCN Red List of Endangered Species, 2008). *Lissotriton* newts were an ideal study species for a site specific study at Llysdinam Pond because there was a large population in the study period of 2004-2008. Also in addition to the long-term migration data there were some limited data on *Lissotriton* larval and metamorph phenology from the 1980s (de Wijer, 1990; Harrison, 1985). Although research has taken place on anuran spawning

phenology in Britain (Beebee, 1995; Reading and Clarke, 1999; Scott et al., 2008), published studies have only been conducted on newt arrival phenology (Beebee, 1995; Chadwick et al., 2006). The overall aims of this site-specific study are to investigate the differences in *Lissotriton* morphology between arrival times, study phenology of great crested and *Lissotriton* newts in Llysdinam Pond, determine whether *Lissotriton* larval and metamorph phenology have altered between the 1980s and present, and determine whether early migration phenology leads to early breeding phenology and the consequences of this. Due to the protected status of great crested newts and the relatively small breeding population at Llysdinam, experimental work was not conducted on the species.

Chapter 2 investigates the differences in body size of *Lissotriton* newts arriving throughout the breeding season. Although Chadwick et al. (2006) found that *Lissotriton* newts were arriving earlier it was not known whether there were differences in newt morphology between arrivals times. Large females have usually been shown to have higher fecundity than smaller females (Baker, 1992; Bell, 1977; Verrell and Francillon, 1986). Additionally, male newts have been shown to prefer large females than small females (Verrell, 1986). Chapter 3 explores the possibility of an autumnal sub-migration towards the pond for palmate, smooth and great crested newts as found by Sinsch (1988) for common toads. The individual natural ventral markings of great crested newts and male smooth newts were photographed when they were captured to monitor individual migration patterns. The photographic technique for great crested newt belly markings has been used in several studies (Arntzen and Teunis, 1993; Hagström, 1973; Hagström, 1977), although it has been rarely used for male smooth newts (Hagström, 1977). Repeatability of arrival dates have been monitored in a previous study on *Ambystoma talpoideum*, although non-natural markers were used (Semlitsch et al., 1993). Chapter 4 investigates the breeding phenology of *Lissotriton* and great crested newts at Llysdinam Pond from 2006-2007 and utilises molecular analysis of morphologically indistinguishable *Lissotriton* eggs to identify differences between the egg-laying phenology of palmate and smooth newts. Although newts were arriving earlier it was not known if this was reflected in earlier breeding so egg searches were conducted in Llysdinam Pond throughout the breeding season in 2006 and 2007. Although breeding phenology was monitored in Llysdinam Pond, it was not known what affect arrival time had on breeding phenology since the commencement of egg-laying could not be linked to individual newts arriving on specific dates. Chapter 5, therefore, uses outdoor breeding tanks to assess the influence of arrival date on courtship and egg-laying phenology of *Lissotriton* newts. Outdoor tanks allowed the separation of newts by

arrival date to monitor courtship and egg-laying phenology in two breeding seasons. Chapter 6 studies *Lissotriton* hatching, larval and metamorph phenology. Some of the newt eggs laid in the outdoor breeding tank study (Chapter 5) were monitored to investigate hatching success and larval growth. Additionally, data available from research in the 1980s (Harrison, 1985 and de Wijer, 1990) allowed comparison between phenology of larvae and aquatic predatory invertebrates in the 1980s and present (Harrison, 1985). Netting surveys for newt larvae were conducted in order to compare larval phenology in Llysdinam Pond with data collected in the 1980s (de Wijer, 1990; Harrison, 1985), and predatory invertebrate abundance and phenology between the years 1984 and 2007. Data on size of newt metamorphs captured at the pond drift fence were also compared with data from Harrison (1985). Effective conservation can not be undertaken without sound ecological understanding of the species in question (Foster and Beebee, 2004), so phenological studies such as this may play an important role in guiding conservation efforts.

Chapter 2: Migration phenology of *Lissotriton* newts: differences in body size

2.1 SUMMARY

Over a two year period from January 2005, data on mass, snout-vent length and total length were recorded for *Lissotriton* newts that were captured migrating to Llysdinam Pond. Palmate newts mainly arrived between October and April, whereas smooth newts had a less protracted arrival season, between January and April. A small proportion of newt arrivals (on average 15%) were captured on departure which occurs mainly in June.

There was a wide range in body size and mass for *Lissotriton* newts arriving at the pond over the two years of data collection. Morphological data of *Lissotriton* newts arriving for the breeding season of 2006 were statistically analysed. Regression analyses showed that day of arrival and temperature accounted for only a small proportion of the variability. Categorising arrivals by month, however, showed that for the period of October 2005 until April 2006, palmate newts that arrived earlier to Llysdinam Pond were significantly larger in mass for males, and in all parameters for females. For male smooth newts, average snout-vent length declined significantly each month from January until April, mass declined significantly from January to March, but there were no significant differences in total length. Significantly more large female smooth newts (using measures of mass and length) arrived in February in 2006 than the remainder of the 2006 breeding season, although in 2005 there was a consistent gradual decline in average body size of female smooth newts between January and April. Average body condition declined significantly over the migration season for palmate and male smooth newts, whereas female smooth newts that arrived in February 2006 had a better body condition.

The differences in body size of newts arriving at Llysdinam Pond were better explained by the factor of arrival time than by temperature. Although the temporal separation in size of newts showed a general decline within the two spring migration seasons of 2005 and 2006, there were occasional periods which did not fit the general trend. The arrival of large males before large gravid females enabled males to develop secondary sexual characteristics to be ready to perform courtship displays when females arrived. Larger newts may be able to cope with the costs of arriving earlier and take advantage of earlier breeding.

2.2 INTRODUCTION

The phenomenon of migration has been well documented throughout the animal kingdom and it has been categorised into many types, usually based on the reason for the movement. Many animals undergo a seasonal or periodic migration to a site that is suitable for breeding. Specialised breeding requirements such as those of amphibians are among the strongest selecting factors for migration. Amphibian migrations occur when adults head to water to breed, and when newly metamorphosed young disperse onto land (Dingle, 1996).

Ims (1990) noted three types of environmental factors that may affect the timing of reproductive events: (i) climatic conditions such as rainfall, photoperiod and temperature, (ii) internal cues from endogenous rhythms, and (iii) biological interactions. The timing of reproduction in many amphibian species is influenced by a combination of these but environmental conditions seem to be a strong influence (Stebbins and Cohen, 1995).

In the UK, newts tend to over winter in refugia on land, and undergo a protracted migration towards breeding ponds when conditions are favourable (Beebee and Griffiths, 2000). Organisms living in seasonal environments often have limited time to complete development. Particularly in northern populations, constraints are placed on development time before the end of the available season (Laurila et al., 2001). Climate change has lead to warmer autumn and early winter temperatures (IPCC, 2007; McCarty, 2001), which amphibian species have recently exploited by an advancement in first and median arrival dates (Beebee, 1995; Chadwick et al., 2006).

Protandry, the emergence or arrival of males before females, is widespread among both plants and animals and occurs in *Lissotriton* and *Triturus* species (Chadwick et al., 2006; Griffiths et al., 1986). A number of hypotheses to explain protandry have been put forward by Morbey and Ydenberg (2001), and categorised into direct and indirect selection. They suggest that the 'Mate opportunity' hypothesis explains earlier arrival of male amphibians to breeding grounds. The 'Waiting cost' hypothesis may also explain the protandrous behaviour of males because male *Lissotriton* newts require time in the aquatic habitat to develop secondary sexual characteristics (crest and toe spurs in smooth newts, and webbed feet and filament in palmate males). It may be beneficial for females to arrive when there is a high number of searching males that are ready to mate so that they can be more selective in mate choice.

Protandry should be subject to stabilizing selection because early emerging males risk death prior to mating, and late arriving males may miss opportunities to mate (Holzapfel and Bradshaw, 2002). The possible benefits of early arrival include access to the better territories, high mating success, mating with high-quality females, and high offspring survival or growth rate (Dickerson et al., 2005; Einum and Fleming, 2000; Landa, 1992; Seamons et al., 2004; Verhulst and Tinbergen, 1991). In seasonal environments, most studies on birds have found that high quality males often arrive at breeding sites earlier and gain the highest reproductive success (Dittmann and Becker, 2003; Kipper et al., 2006; Lozano et al., 1996; Møller, 1994). Arriving early enables establishment of the most successful territories in birds (Aebischer et al., 1996). Great crested newts have particular areas for courtship displays in ponds equivalent to a 'lek' system, although *Lissotriton* newts do not tend to display territoriality in the pond (Griffiths, 1995).

For many species the possible costs of arriving early include low food availability, reduced survivorship due to predation and adverse weather, and reduced offspring survival and growth if the offspring emerge too early (Møller, 1994). Generally, high-quality males are assumed to be better able to pay the costs of early arrival than low-quality males (Verhulst et al., 1995). Dickerson et al. (2002) found that for male pink salmon *Oncorhynchus gorbusha*, access to reproductive females was positively influenced by body size, but that early arrival was beneficial to large males and later arrival was advantageous to small males. In contrast males in poor condition may arrive first if they benefit from avoiding competition with high quality males for territories and mates. In some insect species, small males of poor competitive ability emerge before large males of high competitive ability (Alcock, 1997; Eberhard, 1982). Small males of the three-spine stickleback, *Gasterosteus aculeatus*, arrived at breeding grounds before larger males. This was thought to be because although predation risk was highest early in the season, arriving early was beneficial for territory establishment (Candolin and Voigt, 2003).

Differences in arrival times between or within species can be due to different temperature thresholds. In Sweden, earlier emergence of male *Gonepteryx rhamni* butterflies seemed to be due to its lower temperature threshold for flight than the females (Wiklund et al., 1996). Timing of arrival may also depend on distance an animal has to travel from its over wintering site to the breeding grounds. Bregnballe et al. (2006) found that great cormorants *Phalacrocorax carbo* that overwintered under 300 km from the breeding ground returned 2-3 weeks earlier than those that had overwintered further away.

Several herpetological studies have investigated differences in amphibian body size between age classes (Cvetkovic et al., 1996; Halliday and Verrell, 1988), between years (Chadwick, 2003; Reading, 2007), between habitat or area (Karraker and Welsh, 2006; Lowe et al., 2006; Marangoni and Tejedo, 2008; Nobili and Accordi, 1997), for differences in reproductive success (Mathis and Britzke, 1999; Nobili and Accordi, 1997; Tejedo, 1992; Verrell and Francillon, 1986), and as a consequence of global warming (Reading, 2007; Tryjanowski et al., 2006b). In contrast, few studies have investigated size differences between arrival times within the season. Semlitsch et al. (1993) found snout-vent length had no effect on the date of return for the female salamander *Ambystoma talpoideum*, but small males returned earlier than large males. They suggest smaller males may have overwintered nearer to the pond and therefore arrived first, since they had less distance to migrate. Lode et al. (2005) found the largest *R. dalmatina* frogs arrived first, and both the males and female lost weight significantly during the breeding season.

Female body size in amphibians has been directly related to egg size and number of eggs laid (Semlitsch et al., 1993). The reproductive success of natterjack toads was found to be positively related to body size. Although egg size was related to female body size, it showed great variation (Tejedo, 1992). Verrell (1986) demonstrated that large female smooth newts contain and lay more eggs. Bell (1977) found that the size and age of female smooth newts was positively correlated with the number of eggs oviposited, while other studies found only size to have an effect on fecundity (Baker, 1992; Nobili and Accordi, 1997).

The influence of body size on reproduction in male newts remains less clear. Teasing out the importance of body size, secondary sexual characters and amount of displaying has been difficult (Griffiths, 1995). It has been suggested that large male amphibians produce more spermatophores (Halliday, 1998), however, Baker (1990a) found no relationship between number of spermatophores deposited and male body size. Additionally, female palmate newts have shown preference for male traits uncorrelated with body size such as a long caudal filament, and even a preference for small males. The longer filament may be a handicap and an honest indicator of condition of males, while body size may be less important in selection of males (Haerty et al., 2007). Indicators of good body condition, regardless of size, are displayed by crests, webbed feet and caudal filaments (Halliday, 1977a) which were found to be preferred by females (Haerty et al., 2007), and increased courtship display times (Beebee and Griffiths, 2000). Time in the aquatic environment is

required for development of secondary sexual characters (Griffiths and Mylotte, 1988), and earlier arrival can increase this.

In newts, if early breeding follows early arrival then the time for egg and larval development will be increased, which may alter the risk of predation. Fewer egg and larval predators have been found earlier in the season (Chapter 6), and egg wrapping behaviour greatly increases survival (Miaud, 1993; Orizaola and Braña, 2003a). However, fewer newt eggs were laid earlier in the year, and predators within the pond may be less satiated, causing a greater proportion of eggs to be predated than during the later peak egg-laying period (Bell and Lawton, 1975). A risk of early breeding is pond freezing leading to high mortality of eggs or larvae in cold conditions. The time when fungal growth poses greatest threat has been found to be variable, Griffiths (1995) found eggs laid earliest seem particular prone to attack, although other studies found the that warmer temperatures or exposure to UV increase susceptibility (Marco et al., 2001).

2.2.1 Aims and hypotheses

Previous work by Chadwick et al. (2006) investigated the differences in newt population size, structure, migration season and timing of arrivals at Llysdinam Pond from 1981-1987 and 2007-2005. This study aimed to investigate similarities and differences in newt migration numbers and phenology during the period 2004-2008, and in comparison with the long-term data set analysed by Chadwick at al. (2006). Chadwick et al. found a change in phenology between the periods 1981-1987 and 1997-2005 with male palmate newts in particular arriving increasingly earlier. It was not known, however, whether there were differences in morphology between early and late arrivals. Few studies have investigated size differences in amphibians between arrival times over the season. The size of newts as well as their arrival time may have implications for reproductive success. Larger newts have a lower surface area to volume ratio so would take longer to warm and cool during periods of temperature changes. Larger newts may make up a larger proportion of early arrivals because if arrival was delayed, then temperatures may have been too low to provide energy required for newts to move towards the pond. Also larger individuals may be more ready to breed, especially gravid females. Therefore it was hypothesised that (i) there is a significant difference in the morphology of newts on arrival at their breeding pond, with early arrivals being significantly larger than late arrivals. Amphibians are poikilotherms and sensitive to changes in environmental temperature. Chadwick et al. (2006) found that male palmate newts were the earliest arrivals, had the greatest advance in

arrival dates between the two periods analysed, and showed a stronger response to temperature than the other *Lissotriton* newts. Therefore for male palmate newts it was hypothesised that (ii) there is a significant relationship between the size of newts on arrival and temperature parameters.

2.3 METHODS

2.3.1 Drift fence and pitfall traps

The pitfall traps along the drift fence encircling around Llysdinam Pond were checked for amphibian captures on a daily basis, usually between 0900 and 1000 GMT. The species, sex and direction of movement were recorded for each amphibian. Amphibian movements during the previous 24 hours were therefore recorded. It was assumed that amphibians caught outside the drift fencing were moving towards the pond (arrivals), and those caught within the drift fencing were moving away from the pond (departures). After recording the amphibians by species and sex, they were released on the side of the fence opposite to where they were captured.

2.3.2 Morphological data

For two years from January 2005, morphological data on *Lissotriton* newt morphology were recorded for all newts migrating to and from Llysdinam Pond. Individuals were placed on laminated graph paper and the total length (snout to tip of tail) and body length (or snout-vent length, recorded from the snout to posterior of cloaca swelling) were recorded to the nearest 0.5 mm. The length of tail filament in male palmate newts was excluded from the total length measurement. The mass of each animal was recorded to 0.01 g using a four decimal place electronic balance. Additionally, data on gravidness of females were recorded by measuring width at widest part of head (G1) and width at widest part of belly (G2) in female newts using digital callipers. Gravidness was calculated as G2/G1. Body condition was calculated for each species and sex (Data analysis: 2.3.4.6 and Results 2.4.2). Data collection on male smooth newt morphology was extended until April 2007, since individuals were being used for photographic identification (Chapter 3, Section 3.3.2). After measurements were taken, the newts were released.

2.3.3 Weather

Weather data were recorded daily at 0900 GMT at the standard Meteorological Office weather station situated 50 m south-west of Llysdinam Pond. Data recorded therefore represent the previous 24 hours. Full weather parameters were recorded at the Llysdinam

weather station from 1988, but prior to this only rainfall was recorded. Data taken from within the Stevenson screen included minimum air temperature (°C), maximum air temperature (°C) and the 0900 GMT air temperature (°C). Ground minimum temperature (°C) was recorded from a thermometer positioned at ground level 2 m from the Stevenson screen. Data were processed by the Meteorological Office and provided for research access on the British Atmospheric Data Centre (BADC) website (UK Meteorological Office, 2008b).

2.3.4 Data analysis

2.3.4.1 Newt morphological data

The morphological data of newts captured at the Llysdinam Pond drift fence were assessed on scatter graphs, and small newts that were exceptional outliers due to small measurements of mass and length were removed from the data, since they were likely to be juveniles (16 newts in total).

2.3.4.2 Weather data

Minimum and maximum air temperature and ground minimum temperature were downloaded from the BADC website, and used for analyses (UK Meteorological Office, 2008b). Daily mean air temperature (°C) was calculated from the data as the mean of the daily maximum and minimum temperature air temperature.

2.3.4.3 Relationship between migration time, temperature and male palmate body size

The association between the morphological data of male palmate arrivals and temperature data were assessed by regression analysis. Male palmate newts were selected because they were consistently the earliest arrivals, had the greatest advance in arrival dates and showed a stronger response to temperature than the other *Lissotriton* newts (Chadwick et al., 2006). Mass was the only data set that would normalise by transformation (square root) for analysis. Minimum air temperature, maximum air temperature, daily mean air temperature (°C) and ground minimum were regressed against male palmate mass data. Regression analyses of male palmate newt mass against migration time used the factor of day of year (Day 1 was 1/10/2005).

2.3.4.4 Differences in male palmate mass between migration time and temperature

Since regression analyses showed a large scatter in the data, and temperature explained only a small amount of the variation in mass data, General Linear Model (GLM) was used

to investigate differences in male palmate mass between temperatures. Male palmate mass was analysed with temperature and arrival month as factors. Daily mean temperatures were used, and coded as 1, 2, 3 and 4 (1 = <3°C, 2 = 3.1-5°C, 3 = 5.1-7°C, 4 = >7.1°C).

2.3.4.5 Differences in Lissotriton newt morphology between months

Newt morphological data were categorised by arrival month to investigate the differences in newt morphology on arrival over the migration season. One-way Analysis of Variance (ANOVA) in Minitab 15 was used to investigate the differences in newt body size between months (Bowker and Randerson, 2008). The data used were from newts arriving at the drift fence for the breeding season of 2006 (therefore from autumn 2005 to spring 2006). This provided a full set of measurements for the whole autumn to spring migratory period for both palmate and smooth newts of both sexes. For the arrival period autumn 2005 to spring 2006, each species and sex was considered separately for data analysis. Each type of morphological data (e.g. snout-vent length, mass) was analysed separately for each species and sex. For each species and sex, data were categorised by month and ANOVA performed for months (i) that had at least six arrivals and (ii) where to minimise recaptures and replicate measurements, there were more newts arriving than departing. Due to variability in migration times, male palmate data were analysed between October 2005 and April 2006, female palmate newts between October 2005 and May 2006, and both male and female smooth newts between January and April 2006.

Square root and arcsinh transformations were used to transform some data to meet the assumptions of ANOVA. Kruskal-Wallis tests were used where data could not be transformed to meet the assumptions of parametric tests. Post-hoc multiple comparisons by Tukey-Kramer posteriori tests compared means between months after ANOVA. Pair wise comparisons by Mann-Whitney U tests in SPSS (Dytham, 2003) were carried out after Kruskal-Wallis tests. For multiple tests the Bonferroni correction was applied which provides a stricter threshold of significance in multiple tests. Bonferroni corrections differed between species due to the number of monthly comparison required. For male palmate data 21 comparisons were made between months October-April (Bonferroni correction for 21 tests $p = <0.0024$). Female palmate data had 28 comparisons between October-May (Bonferroni correction for 28 tests $p = <0.0018$) and smooth newt data had six comparisons between January-April (Bonferroni correction for six tests $p = <0.0083$).

Bar charts were used to display the data for remaining months of migration where inward bound migrations were higher than outward migrations: January-April 2005 and September 2006-January 2007. Morphological data for each species and sex are represented in separate interval charts and tables (Section 2.4.2.4-2.4.2.7). The results are displayed as three periods: Period 1: the 2005 migration season from January-April, Period 2: the migration season from October 2005-April/May 2006 (January-April 2006 in smooth newts) and Period 3: the start of the migration season from September 2006-January 2007. Morphological data collection was extended until April 2007 for male smooth newts since data were being collected for Chapter 3. Total length data of *Lissotriton* arrivals are displayed as proportions of total arrivals in stacked bar charts.

2.3.4.6 Defining Body condition

A measure of body condition of *Lissotriton* newts was required to assess differences between arrival months. Body condition can be assessed by the equation: mass/snout-vent length. This equation has been used by herpetologists such as Arntzen et al. (1999) for assessing the condition of great crested newts after marking and tissue sampling. Body condition may also be assessed by mass/total length as used by Karraker and Welsh (2006) to compare salamander body condition in different forest habitats.

The results from the two above methods for calculating body condition correlated highly with mass, and marginally with measures of length, so residuals from a regression analysis of mass against length were used calculated as a comparison. The use of residuals as an body condition index has been discussed by Green (2001a) and Schulte-Hostedde et al. (2005). Correlation analyses of the regression residuals against mass and length data showed a weaker correlation, and therefore residuals were considered of higher validity for representing body condition. Positive residuals indicated a high mass to length ratio (good body condition) and negative residuals indicated a low mass to length ratio (poorer body condition). Residuals for body indices from regression analyses of (i) mass against snout-vent length, and (ii) mass against total length were calculated in Minitab 15. No suitable transformations could be found to normalise the body condition data (residuals) so differences in body condition between months were compared by Kruskal-Wallis tests.

2.4 RESULTS

2.4.1 Newt population size, structure and migration phenology

2.4.1.1 Population size and structure

Captures of newts during the study period 2004-2008 were consistent with arrival numbers in previous years. The years 1981-1987 and 1997-2005 were analysed for changes in *Lissotriton* phenology by Chadwick et al. (2006). Reliable pitfall checks were made at least five times per week during those periods and annual arrival numbers fluctuated between 775 and 3311 newts. Morphological data were collected during 2005-2006, and these years had a large newt population, with over 3300 newts captured annually between January-April (Figure 2.1 and Table 2.1). There was a large decline in the newt population in 2007, with a 40% drop in numbers in comparison with the previous two years.

Figure 2.1 Newt arrivals and departures at Llysdinam Pond in 2004-2008
Bars on the positive axis represent number of newts that were captured arriving at Llysdinam Pond each year between January-April. Bars on the negative axis represent number of newts that were captured departing from Llysdinam Pond in June and July. All captures were made in pitfall traps at Llysdinam Pond drift fence. Data are displayed separately by newt species and sex (Table 2.1 for total newt capture numbers).

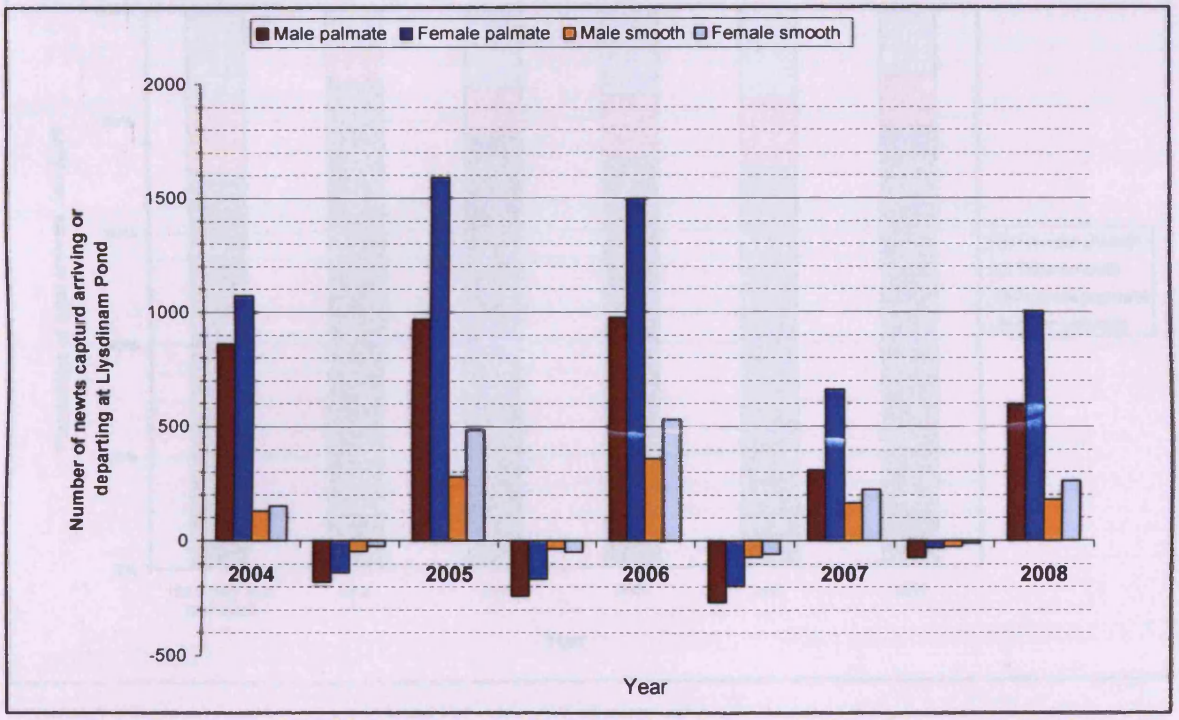


Table 2.1 Newts captures at pond drift fence (2004 -2008)

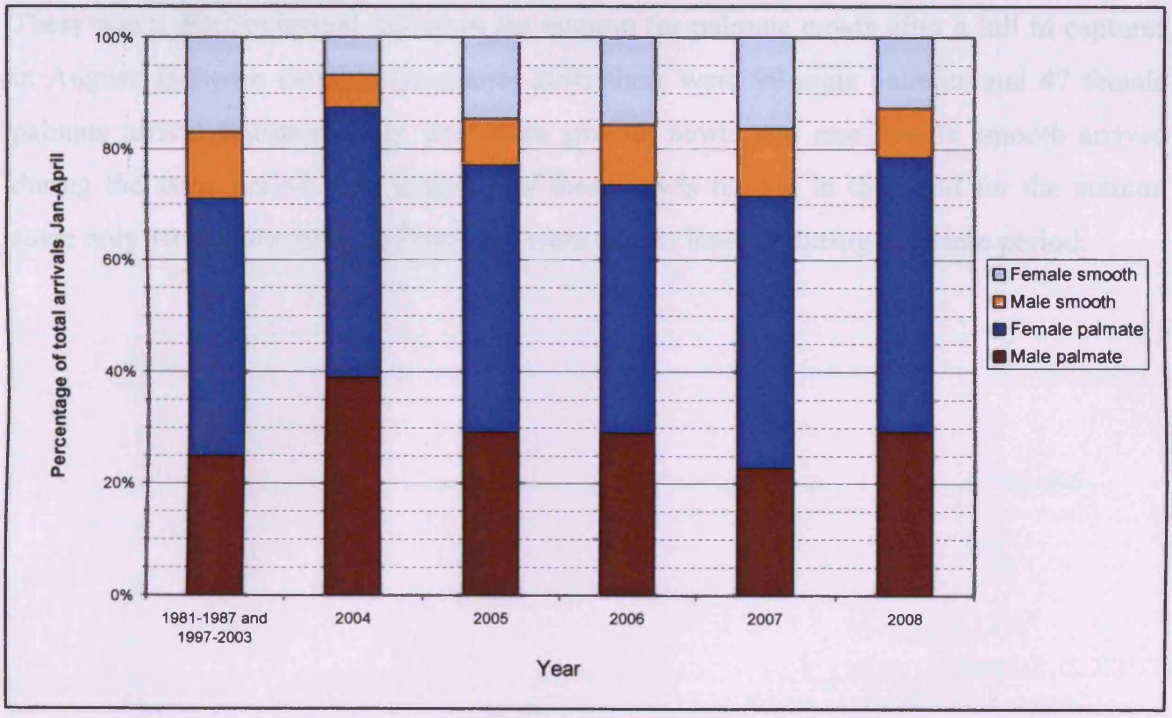
Table shows total arrival and departure captures of palmate and smooth newts at the Llysdinam Pond drift fence (Figure 2.1 for captures by species and sex).

	2004	2005	2006	2007	2008
Total capture of newts arriving Jan-April	2210	3318	3362	1359	2035
Total captures of newts departing June-July	393	492	593	139	* (data not analysed)

Llysdinam Pond consistently had a much larger palmate than smooth newt population (Figure 2.1 and 2.2). In recent years the smooth newt population made up as little as 15% (2004) of the total number of *Lissotriton* newts captured arriving, but usually contributed around 25% of total arrival captures.

Figure 2.2 Proportion of total captures for palmate and smooth newts at Llysdinam Pond drift fence

Proportion of total arrival newt captures at Llysdinam Pond drift fence by species and sex from 2004-2008. Data collected prior to this study are collectively displayed for years 1981-1987 and 1997-2003. During those two periods pitfall captures were monitored regularly, while pitfall records from 1986-1996 were poor.



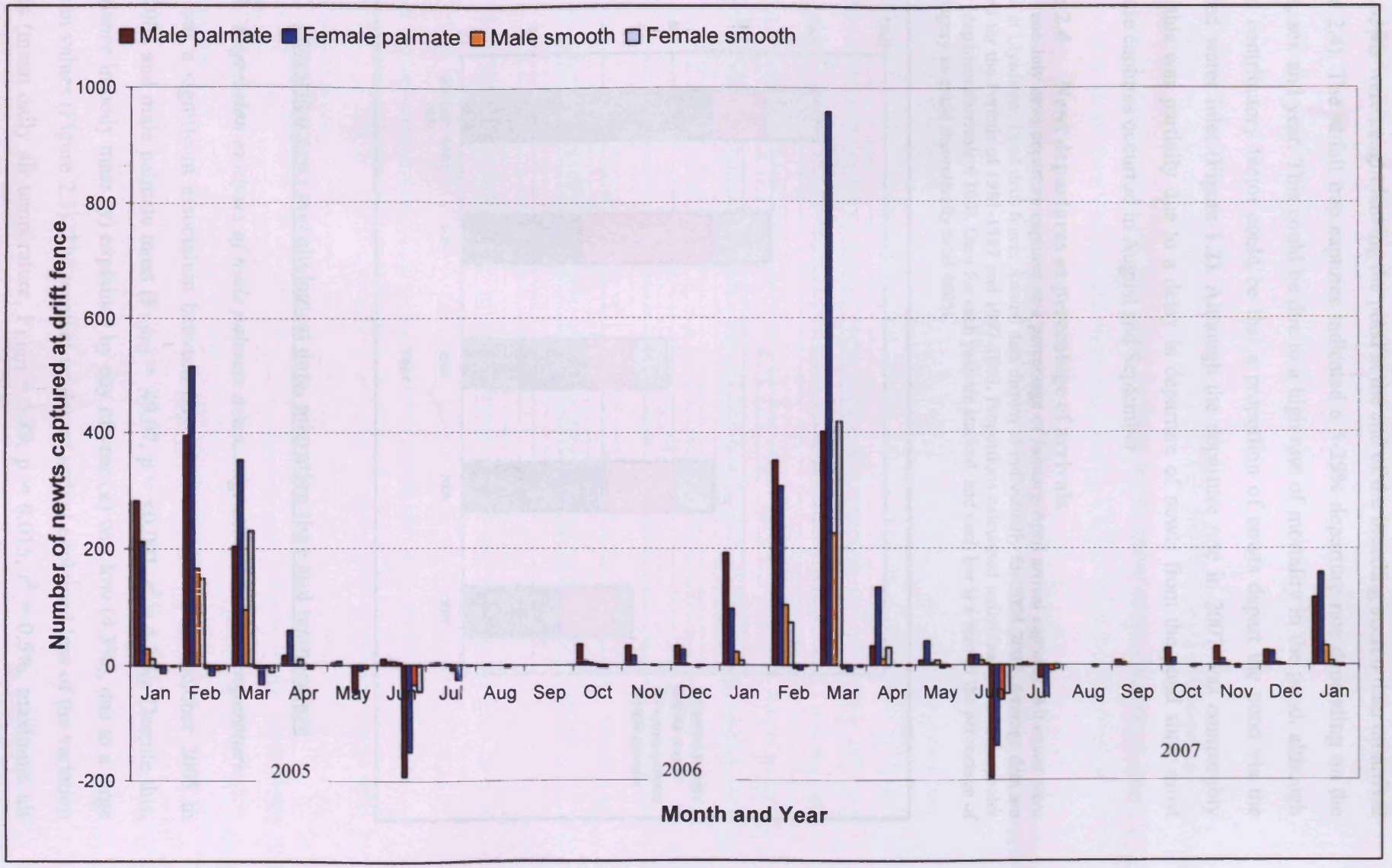
2.4.1.2 The migration season

In all years the bulk of movement towards the pond occurred from January until April, peaking during February and March. Captures were multi modal, and capture rates varied greatly between days and between months (Figure 2.3). There were low or no captures on days near or below 0°C, even during the peak migration season. The highest daily catch was 459 newts on 9/3/2006 (Day 68), and contributed a large proportion to the January-April total. On this date 122 male palmate newts were captured (12.5% of January-April total), 151 female palmate newts (10%), 95 male smooth newts (26%) and 91 females smooth newts (17%). It was the highest daily catch for all species and sexes in 2006 and ever recorded in the long-term Llysdinam migration data set. Emigration from the pond was less protracted and mainly occurred in June and July. 2006 had the highest number of newt arrivals for the entire long-term data set since 1981. In comparison with 2004 numbers, there was an increase by 210 male palmate newt (24% increase), 445 females palmate newt (40.5%), 79 male smooth newt (28%) and 127 female smooth newts (32%).

There was a distinct arrival period in the autumn for palmate newts after a lull in captures in August. Between October-December 2005 there were 99 male palmate and 47 female palmate arrival captures. Only four male smooth newts and one female smooth arrived during the same period. The majority of these newts remain in the pond for the autumn since only 10 palmate newts of each sex were caught leaving during the same period.

Figure 2.3 Monthly captures of newts at Llysdinam Pond (January 2005-2007)

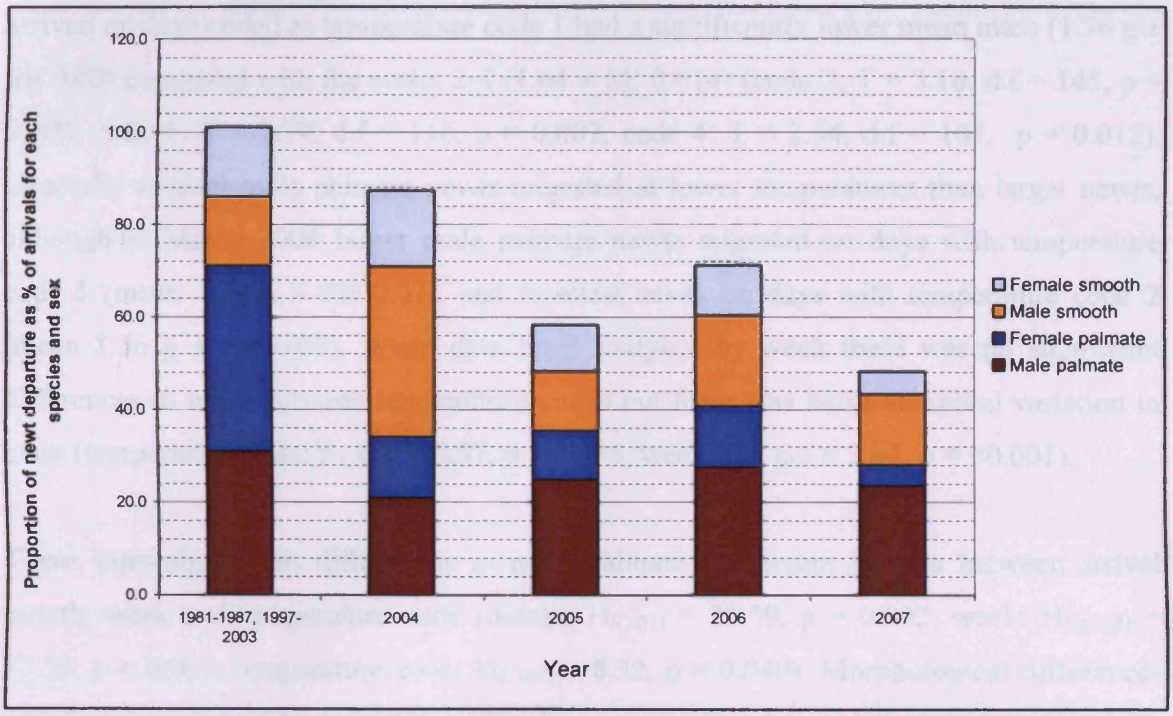
Bars represent number of newts captured monthly at the Llysdinam Pond drift fence between January 2005-2007. Bars on the positive axis represent number of newts captured on arrival and bars on negative axis represent departure captures. Data are displayed separately by newt species and sex (Table 2.1 for total newt capture numbers).



Fewer newts were caught leaving the pond at the end of the breeding season than on arrival (Figure 2.4). The pitfall trap captures indicated a 5-29% departure rate depending on the species, sex and year. This could be due to a high rate of mortality in the pond, although another contributory factor could be that a proportion of newts depart the pond via the unfenced water inlet (Figure 1.2). Although the departure rate in 2007 was comparably lower, this was partially due to a delay in departure of newts from the pond since most departure captures occurred in August and September.

Figure 2.4 Newt departures as percentage of arrivals

Annual June-July newt departure captures as a percentage of January-April arrival captures. All newts were captured at Llysdinam Pond drift fence. Annual data displayed individually for 2004-2007. Average data are displayed for the Periods of 1981-1987 and 1997-2003. Proportions calculated individually for each species and sex (departures/arrivals x 100). Data for each year are stacked, and each bar is a sum of the percentage of each category so could theoretically total 400%.



2.4.2 *Lissotriton* newt morphological data, migration time and temperature

2.4.2.1 Regression analyses of male palmate mass, migration time and temperature

There was a significant association between day of newt arrival from October 2005 to April 2006 and male palmate mass ($F_{1,1073}$ = 49.07, p = <0.001, r^2 = 4.3%). Despite this, the variance in body mass (y) explained by day of year (x) was low (4.3%), due to a large scatter in values (Figure 2.5). The weather on day of arrival explained less of the variation in mass (mean daily air temperature, $F_{1,1073}$ = 5.89, p = 0.015, r^2 = 0.5%, maximum air temperature: $F_{1,1073}$ = 12.22, p = <0.001, r^2 = 1%). There were no significant associations

between male palmate mass and minimum air temperature or ground minimum temperature. It was decided not to pursue regression analysis of body size since normality of the data for other morphological characteristics could not be achieved by transformation.

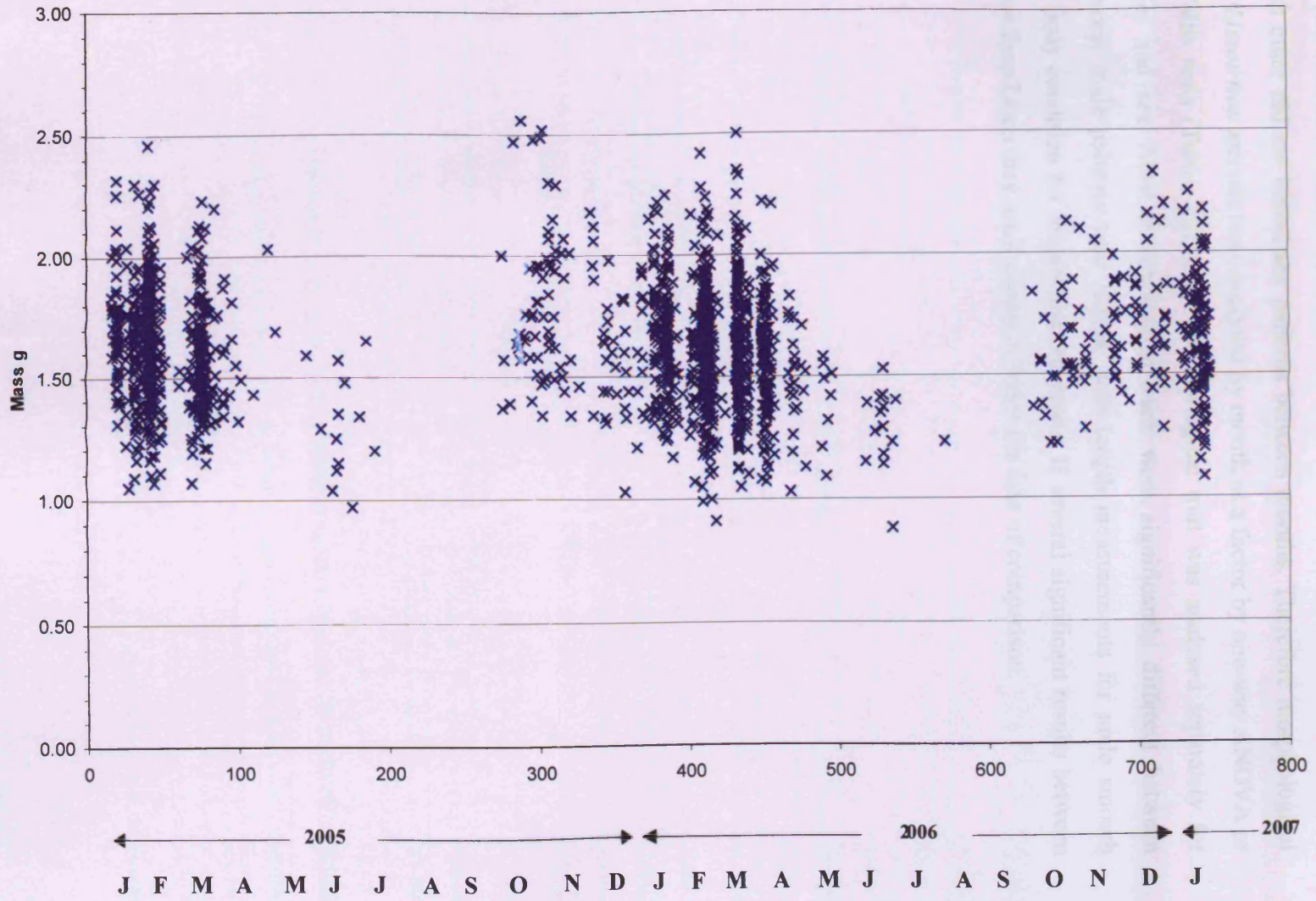
2.4.2.2 Differences in male palmate morphological data between temperature codes

Differences in mass of male palmate newts between temperature codes (Section 2.3.4.4) and time as a factor (week or month) were analysed by GLM. Mass was the only male palmate data set that could be transformed (square root transformation) to meet the assumptions of parametric tests. For data analysis by month (October-April), there was a significant difference in mass of male palmate arrivals between temperature code ($F_{3,1065}$ = 4.75, p = 0.003) and month of arrival ($F_{6,1065}$ = 8.89, p = <0.001). Male palmate newts that arrived on days coded as temperature code 1 had a significantly lower mean mass (1.56 g ± SE 0.03) compared with the codes 2-4 (1.64 ± SE 0.014) (code 2, T = 3.10, d.f = 145, p = 0.002; code 3: T = 2.77, d.f = 116, p = 0.007, code 4: T = 2.54, d.f = 107, p = 0.012). Generally smaller male palmate newts migrated at lower temperatures than larger newts, although in March 2006 larger male palmate newts migrated on days with temperature code 1 (mean 1.70 g ± SE 0.07), and smallest newts on days with temperature code 2 (mean 1.56 g ± SE 0.05). When data were analysed by week there was no significant differences in mass between temperature codes but there was still a temporal variation in mass (temperature code: $F_{3,1041}$ = 0.57, p = 0.636, week: $F_{30,1041}$ = 2.87, p = <0.001).

There were significant differences in male palmate snout-vent lengths between arrival month, week and temperature code (month: $H_{6,1071}$ = 20.59, p = 0.002, week: $H_{30,1071}$ = 52.39, p = 0.007, temperature code: $H_{3,1071}$ = 8.32, p = 0.040). Morphological differences were better explained by arrival time than temperature. October and April had no days with a mean air temperature below 5°C, yet April had a higher proportion of small newts than October, and differences in morphology between the two months were sometimes significant (Figure 2.8, Table 2.3). There were no significant differences in male palmate total length by arrival month ($H_{6,1071}$ = 9.96, p = 0.126) or week ($H_{30,1071}$ = 40.4, p = 0.097) or between temperature codes ($H_{3,1071}$ = 5.85, p = 0.119).

Figure 2.5 Mass of male palmate newts that arrived at Llysdinam Pond over a two year period

Each cross represents the mass (g) of each male palmate newt that arrived at Llysdinam Pond over the two years of data collection. On the x axis days continue from Day 17 (17/1/2005) to Day 747 (17/1/2007). Months are coded by their initial letter. Scatter graph shows large range and noise in values.



2.4.2.3 Differences in morphology of Lissotriton newts between migration time

Principal Components Analysis (PCA) in Minitab 15 revealed that Principal Component 1 (PC1) was snout-vent length, and PC2 was mass for both species of male *Lissotriton* newts, while for female *Lissotriton* newts PC1 was snout-vent length, and PC2 was gravidness (Bowker and Randerson, 2008; Townend, 2007). Plotting the scores from PCA against each other did not reveal any patterns between months. Therefore morphological data for all *Lissotriton* arrivals were analysed by month as a factor by one-way ANOVA or Kruskal-Wallis tests (Table 2.2). Each morphological trait was analysed separately for each species and sex. Most morphological traits were significantly different between months, except male palmate total length, both length measurements for male smooth newts and body condition for female smooth newts. If several significant results between months were found then they are reported in tables for ease of comparison.

Table 2.2 Differences in morphological data between months

One-way ANOVA (F values) and Kruskal-Wallis (H values) with d.fs are displayed separately for each species, sex and category of morphological data. * = significant at p = 0.05, ** = significant at p = 0.01 and *** = p significant at 0.001. P values in bold indicate a significant difference in the morphological trait of newts on arrival at Llysdinam Pond between the months analysed. Body condition (s-v length) = body condition calculated from residuals of mass v. snout-vent length. For female newts: G1 = width of newt at widest part of head, G2 = width of newt at widest part of belly measured by digital calipers.

Species, sex, time	Morphological data	Transformation for parametric test	F or H Value and d.fs	P value
Male Palmate Oct 2005-April 2006	Snout-vent length mm**		$H_{6,1068} = 20.27$	**0.002**
	Total length mm		$H_{6,1068} = 9.96$	0.126
	Mass g***	Square root	$F_{6,1068} = 8.20$	**<0.001**
	Body condition (s-v length)***		$H_{6,1068} = 47.67$	**<0.001**
	Body condition (total length)***		$H_{6,1068} = 38.84$	**<0.001**
Female Palmate Oct 2005-May 2006	Snout-vent length mm***		$H_{7,1575} = 60.27$	**<0.001**
	Total length mm***		$H_{7,1575} = 43.64$	**<0.001**
	Mass g***	Arcsinh	$F_{7,1575} = 8.38$	**<0.001**
	Width at belly mm (G2)***	Square root	$F_{7,1575} = 7.11$	**<0.001**
	Gravidness mm (G2/G1)***		$H_{7,1575} = 74.79$	**<0.001**
	Body condition (s-v length)***		$H_{7,1575} = 29.59$	**<0.001**
	Body condition (total length)***		$H_{7,1575} = 31.17$	**<0.001**
Male Smooth Jan-April 2006	Snout-vent length mm		$H_{3,357} = 6.37$	0.095
	Total length mm	None	$F_{3,357} = 2.00$	0.114
	Mass g**	Square root	$F_{3,357} = 4.19$	**0.006**
	Body condition (s-v length)**		$H_{3,357} = 15.15$	**0.002**
	Body condition (total length)*		$H_{3,357} = 10.33$	**0.016**
Female Smooth Jan-April 2006	Snout-vent length mm***		$H_{3,525} = 48.11$	**<0.001**
	Total length mm***		$H_{3,525} = 26.23$	**<0.001**
	Mass g***	None	$F_{3,525} = 12.59$	**<0.001**
	Width at belly mm (G2)***	None	$F_{3,523} = 18.13$	**<0.000**
	Gravidness mm (G2/G1)***		$H_{3,525} = 15.64$	**<0.001**
	Body condition (s-v length)		$H_{3,525} = 3.38$	0.337
	Body condition (total length)*		$H_{3,525} = 7.86$	**0.049**

2.4.2.4 Male palmate newt morphological data

For the migration period September 2005-April 2006, a decline male palmate length and mass occurred over Period 2. In contrast to male palmate mass which peaked in October-November 2005, snout-vent lengths were greater in January 2006. Significant differences in snout-vent lengths were only found between January and March 2006 (U = 64658, d.f = 591, p = <0.001). There were similar patterns in snout-vent lengths in Period 1, but Period 3 showed variability (Figure 2.6). A larger proportion of male palmate newts with a total length of over 72 mm arrived in October 2005 than later in the season (Figure 2.7), but there were no significant differences (Table 2.2). In Period 2 significant differences in male palmate mass were found between early arrivals (October-November 2005) and late arrivals (March-April 2006, Table 2.3). A lower proportion of male palmate newts of low mass (<1.40 g) and a larger proportion with a high mass (>1.9 g) arrived earlier in the season. There was inter-annual variation since there was an increase in mass of male palmate newts over time in the autumn of Period 3 (Figure 2.8). Male palmate body condition was higher earlier in Period 2 and the results were highly significant between November 2005 and months in spring 2006 (Table 2.4).

Figure 2.6 Snout-vent lengths of male palmate newts

Snout-vent lengths (mm) of male palmate newts captured at the drift fence on arrival at Llysdinam Pond. Data were recorded from three inward migration periods: Period 1 (January-April 2005), Period 2 (October 2005-April 2006) and Period 3 (September 2006-January 2007). Data are categorised by month. Variation in migration phenology meant that months used in analyses sometimes differed between species, sex and years. Bars represent means. Whiskers represent 95% confidence intervals around the mean. Diamond symbols represent medians.

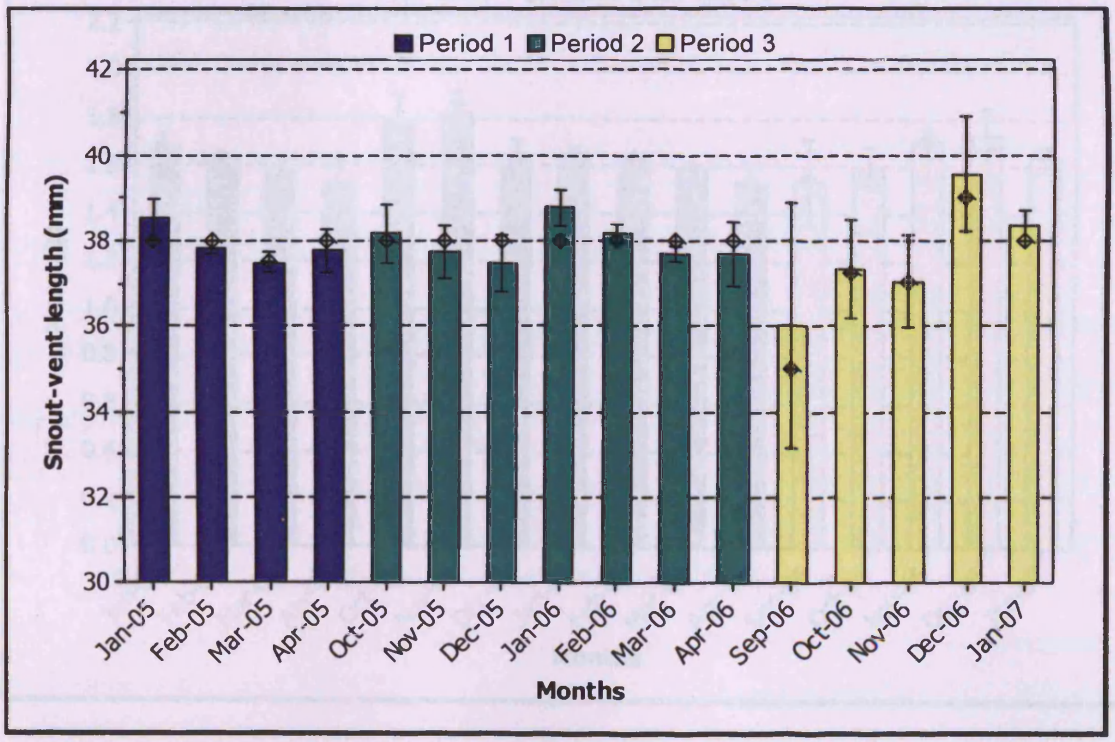


Figure 2.7 Proportion of male palmate newts arrivals at Llysdinam Pond categorised by total length

The proportion of male palmate newt arrivals, categorised by total length (mm) are displayed for each month in Period 2 (October 2005-April 2006). Newts were captured at the drift fence on arrival to Llysdinam Pond. Maximum and minimum lengths for each size category are shown in the key.

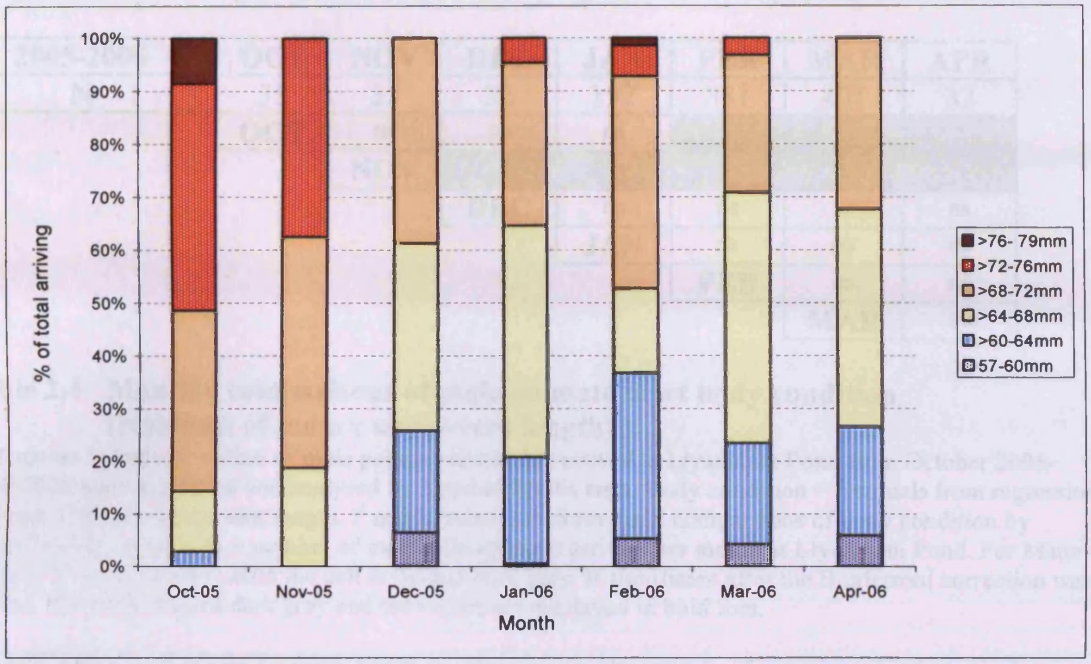


Figure 2.8 Mass of male palmate newts

Mass data (g) for male palmate newts captured at the drift fence on arrival at Llysdinam Pond. Data were recorded from three inward migration periods: Period 1 (January-April 2005, Period 2 (October 2005-April 2006) and Period 3 (September 2006-January 2007). Data are categorised by month. Variation in migration phenology meant that months used in analyses sometimes differed between species, sex and years. Bars represent means. Whiskers represent 95% confidence intervals around the mean. Diamond symbols represent medians.

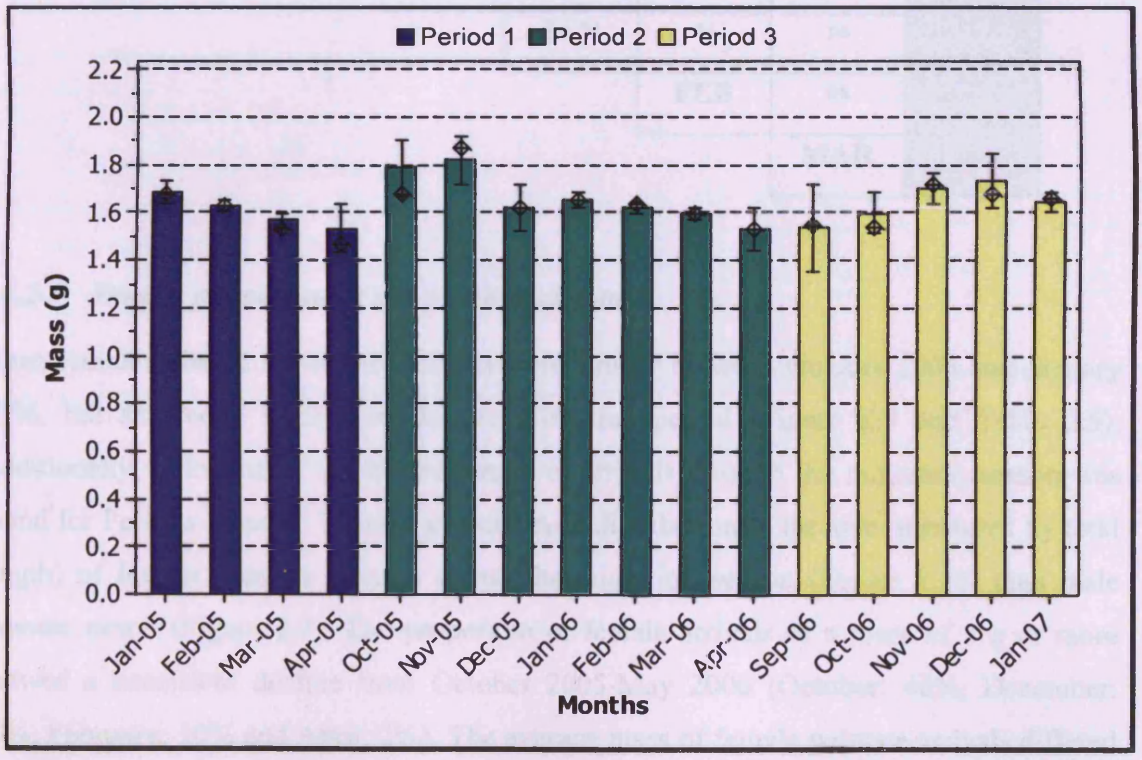


Table 2.3 Monthly comparisons of male palmate newt mass

Differences in mass data (g) of male palmate newts that arrived at Llysdinam Pond from October 2005-April 2006 were analysed by one-way ANOVA. P values are from Tukey-Kramer post-hoc tests, after one-way ANOVA on square root transformed mass data. N = number of male palmate newt arrivals per month. For Tukey-Kramer tests if the p is <0.05 the cell is shaded dark grey and value displayed in bold font.

2005-2006	OCT	NOV	DEC	JAN	FEB	MAR	APR
N	35	32	32	192	351	401	32
	OCT	ns	ns	ns	**0.003**	**<0.001**	**<0.001**
		NOV	**0.019**	**0.008**	**<0.001**	**<0.001**	**<0.001**
			DEC	ns	ns	ns	ns
				JAN	ns	ns	ns
					FEB	ns	ns
						MAR	ns

Table 2.4 Monthly comparisons of male palmate newt body condition (residuals of mass v snout-vent length)

Differences in body condition of male palmate newts that arrived at Llysdinam Pond from October 2005-April 2006 were calculated and analysed by Kruskal-Wallis tests. Body condition = residuals from regression analyses of mass v snout-vent length. P and U values are from multi comparisons of body condition by Mann-Whitney U tests. N = number of male palmate newt arrivals per month at Llysdinam Pond. For Mann-Whitney U tests if p was <0.05 the cell is shaded light grey. If significant after the Bonferroni correction was applied, the cell is shaded dark grey and the values are displayed in bold font.

2005-2006	OCT	NOV	DEC	JAN	FEB	MAR	APR
N	35	32	32	192	351	401	32
	OCT	ns	ns	U=2556.5 p=0.05	**U=3913.5 p=<0.001**	**U=4411.5 p=<0.001**	**U=235.5 p=<0.001**
		NOV	**U=280.5 p=0.002**	**U=1530.5 p=<0.001**	**U=2522.5 p=<0.001**	**U=2828.5 p=<0.001**	**U=146.55 p=<0.001**
			DEC	ns	ns	ns	U=338 p=0.019
				JAN	ns	ns	U=2209.5 p=0.011
					FEB	ns	U=4278 p=0.026
						MAR	U=4762 p=0.015

2.4.2.5 Female palmate newt morphological data

Mean female palmate snout-vent lengths were similar between October 2005 and January 2006, but showed a significant decline after that period (Figure 2.9 and Table 2.5). Additionally, a decline in snout-vent length of arrivals through the migration season was found for Periods 1 and 3. There was more even distribution in the size (measured by total length) of female palmate arrivals across the migration season (Figure 2.10) than male palmate newts (Figure 2.7). The proportion of female arrivals of a mass of 3 g or more showed a consistent decline from October 2005-May 2006 (October: 40%, December: 20%, February: 10% and April: 2%). The average mass of female palmate arrivals differed significantly between months from November onwards (Figure 2.11 and Table 2.6), and

was highly correlated with gravidness (r_s = 0.499, d.f = 1582, p = <0.001) probably due to females of high mass carrying a larger number of oocytes. The average mass of female palmate newts on arrival declined more consistently over the season than measures of length, and this was consistent across the all three time periods. There was a gradual significant decline in gravidness (G2/G1) and body condition from November 2005 to May 2006 (Table 2.7 and 2.8). Average measures of belly width (G2), generally declined from January (10.0 ± SE 0.3 mm) to May 2006 (9.3 ± 0.3 mm). Mean G2 width of female palmate arrivals was highest in November 2005 (10.1 ± 0.3 mm), but low in October and December 2005 (8.8 ± 0.5 mm and 9.5 ± 0.2 mm). Therefore gravid female palmate newts in good condition arrived at the pond from November 2005 onwards (Figure 2.12, Table 2.8), coinciding with presence of male palmate newts of high body condition. In contrast to Period 2, gravidness increased over the migration season in Period 1, and showed little variability in Period 3.

Figure 2.9 Snout-vent lengths of female palmate newts

Snout-vent lengths (mm) of female palmate newts captured at the drift fence on arrival at Llysdinam Pond. Data were recorded from three inward migration periods: Period 1 (January-April 2005), Period 2 (October 2005-May 2006) and Period 3 (September 2006-January 2007). Data are categorised by month. Variation in migration phenology meant that months used in analyses sometimes differed between species, sex and years. Bars represent means. Whiskers represent 95% confidence intervals around the mean. Diamond symbols represent medians.

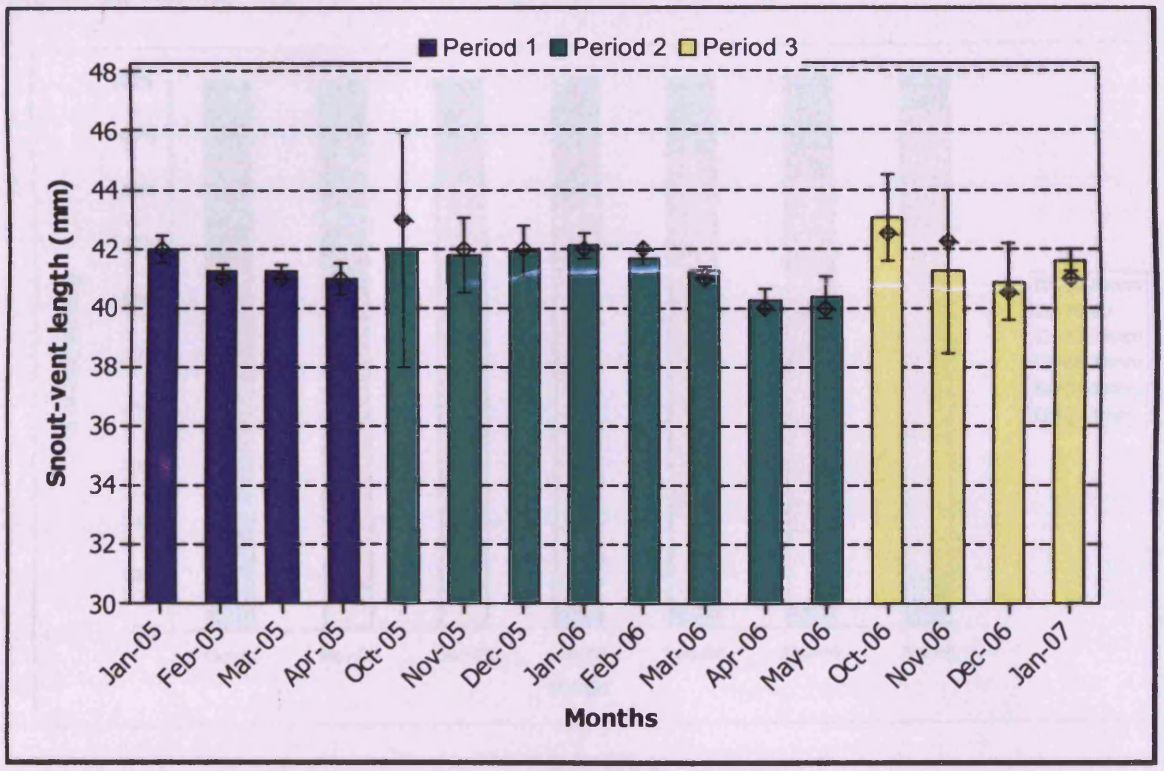


Table 2.5 Monthly comparisons of female palmate newt snout-vent lengths

Differences in snout-vent lengths (mm) of female palmate newts that arrived at Llysdinam Pond from October 2005-May 2006 were analysed by Kruskal-Wallis tests. P and U values are from multi comparisons by Mann-Whitney U tests. N = number of female palmate newt arrivals per month. For Mann-Whitney U tests if p was <0.05 the cell is shaded light grey. If significant after the Bonferroni correction was applied, the cell is shaded dark grey and the values are displayed in bold font.

2005-2006	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY
N	35	15	26	97	307	958	194	40
	OCT	ns	ns	ns	ns	ns	ns	ns
		NOV	ns	ns	ns	ns	U=668.5 p=0.031	ns
			DEC	ns	ns	ns	**U=1026.5 p=0.001**	U=334.5 p=0.014
				JAN	U=12888 p=0.044	**U=35843 p=<0.001**	**U=3451 p=<0.001**	**U=1117.5 p=<0.001**
					FEB	U=132353.5 P=0.008	**U=13126 p=<0.001**	**U=4169 p=0.001**
						MAR	**U=47493 p=0.001**	U=14960 p=0.017
							APR	ns

Figure 2.10 Proportion of female palmate newt arrivals at Llysdinam Pond categorised by total length

The proportion of female newt arrivals, categorised by total length (mm) are displayed for each month in Period 2 (October 2005-April 2006). Newts were captured at the drift fence on arrival to Llysdinam Pond. Maximum and minimum lengths for each size category are shown in the key.

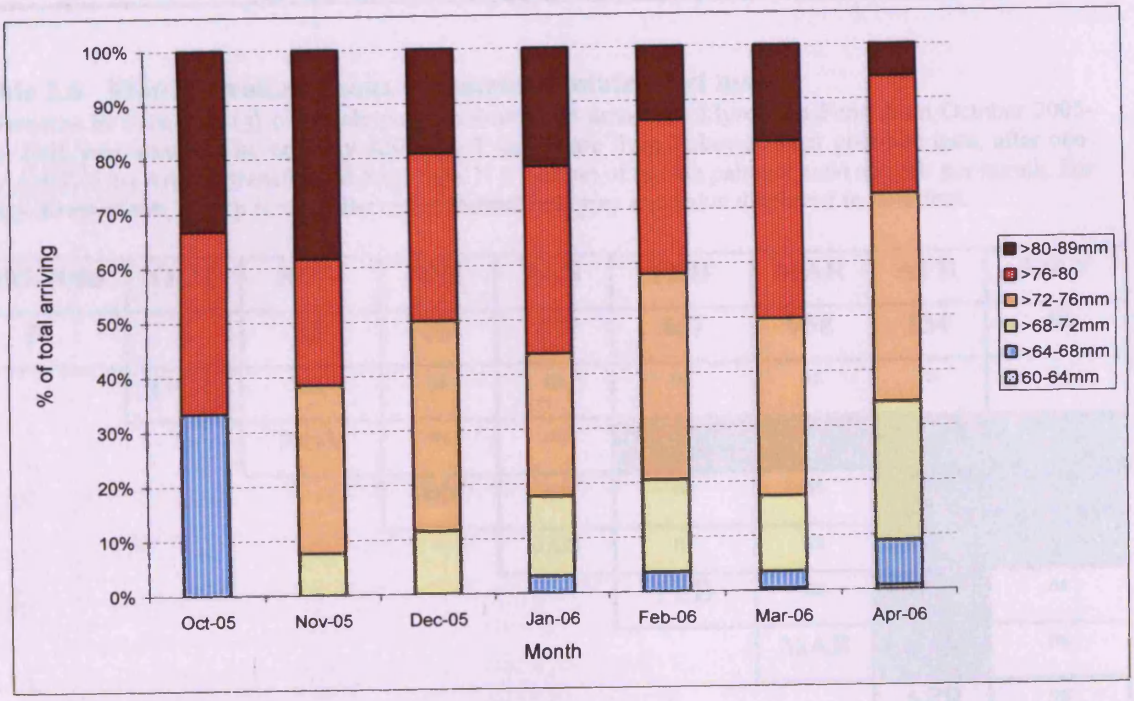


Figure 2.11 Mass of female palmate newts

Mass data (g) for female palmate newts captured at the drift fence on arrival at Llysdinam Pond. Data were recorded from three inward migration periods: Period 1 (January-April 2005), Period 2 (October 2005-May 2006) and Period 3 (September 2006-January 2007). Data are categorised by month. Variation in migration phenology meant that months used in analyses sometimes differed between species, sex and years. Bars represent means. Whiskers represent 95% confidence intervals around the mean. Diamond symbols represent medians.

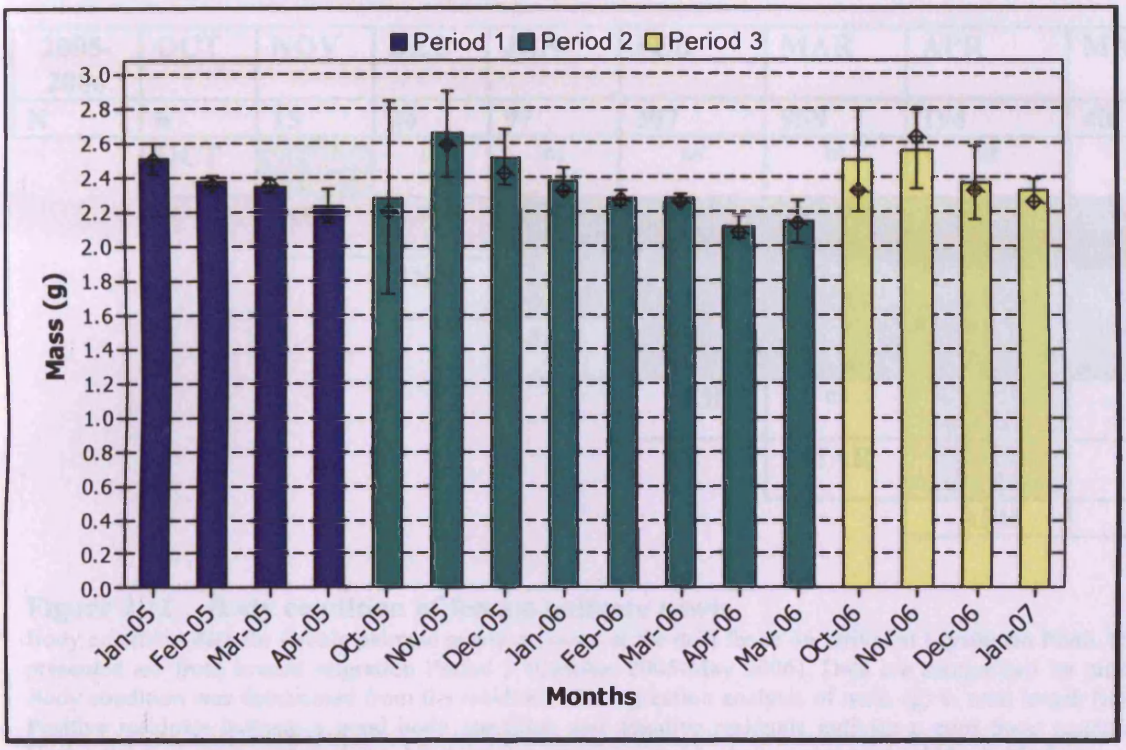


Table 2.6 Monthly comparisons of female palmate newt mass

Differences in mass data (g) of female palmate newts that arrived at Llysdinam Pond from October 2005-May 2006 were analysed by one-way ANOVA. P values are from Tukey-Kramer post-hoc tests, after one-way ANOVA on Arcsinh transformed mass data. N = number of female palmate newt arrivals per month. For Tukey-Kramer tests if the p is <0.05 the cell is shaded dark grey and value displayed in bold font.

2005-2006	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY
N	6	15	26	97	307	958	134	40
	OCT	ns	ns	ns	ns	ns	ns	ns
		NOV	ns	ns	**0.022**	**0.014**	**<0.001**	**0.001**
			DEC	ns	ns	ns	**<0.001**	**0.005**
				JAN	ns	ns	**<0.001**	**0.021**
					FEB	ns	**<0.001**	ns
						MAR	**<0.001**	ns
							APR	ns

Table 2.7 Monthly comparisons of female palmate newt gravidness

Differences in gravidness of female palmate newts that arrived at Llysdinam Pond from October 2005-May 2006 were calculated and analysed by Kruskal-Wallis tests. Gravidness was calculated as G2/G1. G1 = width of newt at widest part of head, G2 = width of newt at widest part of belly measured by digital calipers. P and U values are from multi comparisons by Mann-Whitney U tests. N = number of female palmate newt arrivals per month. For Mann-Whitney U tests if p was <0.05 the cell is shaded light grey. If significant after the Bonferroni correction was applied, the cell is shaded dark grey and the values are displayed in bold font.

2005-2006	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY
N	6	15	26	97	307	958	194	40
	OCT	U=18 p=0.036	ns	ns	ns	ns	ns	ns
		NOV	ns	**U=336.5 p=0.001**	**U=695.5 p=<0.001**	**U=2304.5 p=<0.001**	**U=292.5 p=<0.001**	**U=81 p=<0.001**
			DEC	U=924 p=0.037	**U=2004 p=<0.001**	**U=6030 p=<0.001**	**U=714 p=<0.001**	**U=204.5 p=<0.001**
				JAN	**U=10852.5 p=<0.001**	**U=32272 p=<0.001**	**U=3772 p=<0.001**	**U=1173.5 p=<0.001**
					FEB	ns	U=17160 p=0.006	ns
						MAR	U=55721.5 p=0.013	ns
							APR	ns

Figure 2.12 Body condition of female palmate newts

Body condition data for female palmate newts captured at the drift fence on arrival at Llysdinam Pond. Data presented are from inward migration Period 2 (October 2005-May 2006). Data are categorised by month. Body condition was determined from the residuals of a regression analysis of mass (g) v. total length (mm). Positive residuals indicate a good body condition and negative residuals indicate a poor body condition. Horizontal lines represent medians, black circles represent means, boxes represent middle half of data, and vertical lines (whiskers) represent the lower and upper limits, while asterisks represent outliers.

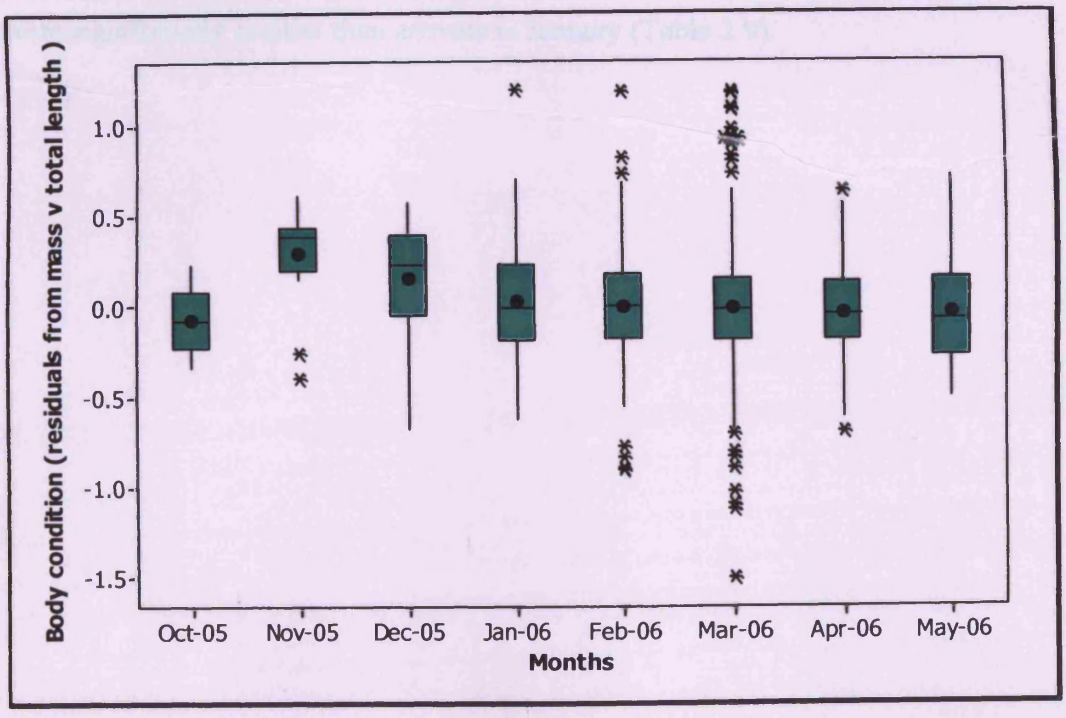


Table 2.8 Monthly comparisons of female palmate newt body condition (residuals of mass v total length)

Differences in body condition of female palmate newts that arrived at Llysdinam Pond from October 2005-May 2006 were calculated and analysed by Kruskal-Wallis tests. Body condition = residuals from regression analyses of mass v total length. P and U values are from multi comparisons by Mann-Whitney U tests. N = number of female palmate newt arrivals per month. For Mann-Whitney U tests if p was <0.05 the cell is shaded light grey. If significant after the Bonferroni correction was applied, the cell is shaded dark grey and the values are displayed in bold font.

2005-2006	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY
N	6	15	26	97	307	958	194	40
	OCT	U=13 p=0.011	ns	ns	ns	ns	ns	ns
		NOV	ns	U=357 p=0.002	U=971 p=<0.001	U=2779.5 p=<0.001	U=374 p=<0.001	U=124 p=0.001
			DEC	U=885.5 p=0.022	U=2599.5 p=0.003	U=7554.5 p=0.001	U=1102 p=0.001	U=303.5 p=0.017
				JAN	ns	ns	ns	ns
					FEB	ns	ns	ns
						MAR	ns	ns
							APR	ns

2.4.2.6 Male smooth newt morphological data

Although there was a decline in snout-vent length and total length of male smooth newt arrivals during each migration period, (Figure 2.13 and 2.14), the differences between months for January-April 2006 were not significant (Table 2.2). Mass of male smooth newt arrivals declined over all three periods (Figure 2.15) and arrivals in February and March 2006 were significantly smaller than arrivals in January (Table 2.9).

Figure 2.13 Snout-vent lengths of male smooth newts

Snout-vent lengths (mm) of male smooth newts captured at the drift fence on arrival at Llysdinam Pond. Data were recorded from three inward migration periods: Period 1 (January-April 2005), Period 2 (January-April 2006) and Period 3 (January-April 2007). Data are categorised by month. Variation in migration phenology meant that months used in analyses sometimes differed between species, sex and years. Bars represent means. Whiskers represent 95% confidence intervals around the mean. Diamond symbols represent medians.

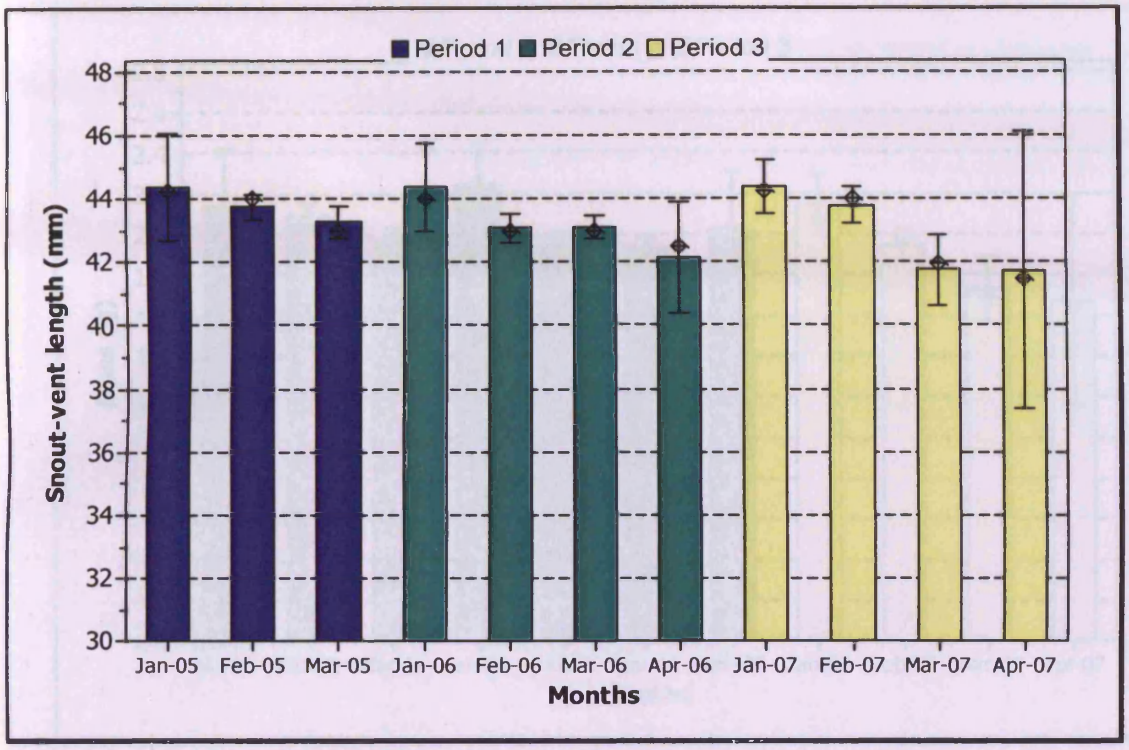


Figure 2.14 Proportion of male smooth newt arrivals at Llysdinam Pond categorised by total length

The proportion of male smooth newt arrivals, categorised by total length (mm) are displayed for each pond in Period 2. Newts were captured at the drift fence on arrival at Llysdinam Pond. Maximum and minimum lengths for each size category are shown in the key.

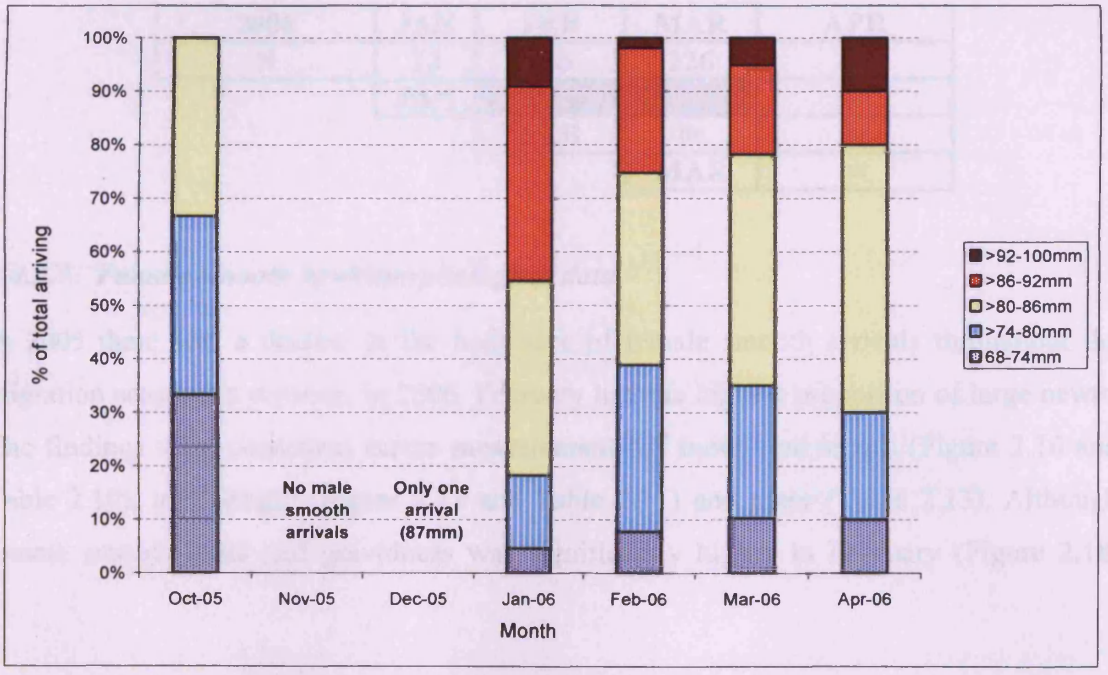


Figure 2.15 Mass of male smooth newts

Mass data (g) for male smooth newts captured at the drift fence on arrival at Llysdinam Pond. Data were recorded from three inward migration periods: Period 1 (January-April 2005, Period 2) (January-April 2006) and Period 3 (January-April 2007). Data are categorised by month. Variation in migration phenology meant that months used in analyses sometimes differed between species, sex and years. Bars represent means. Whiskers represent 95% confidence intervals around the mean. Diamond symbols represent medians.

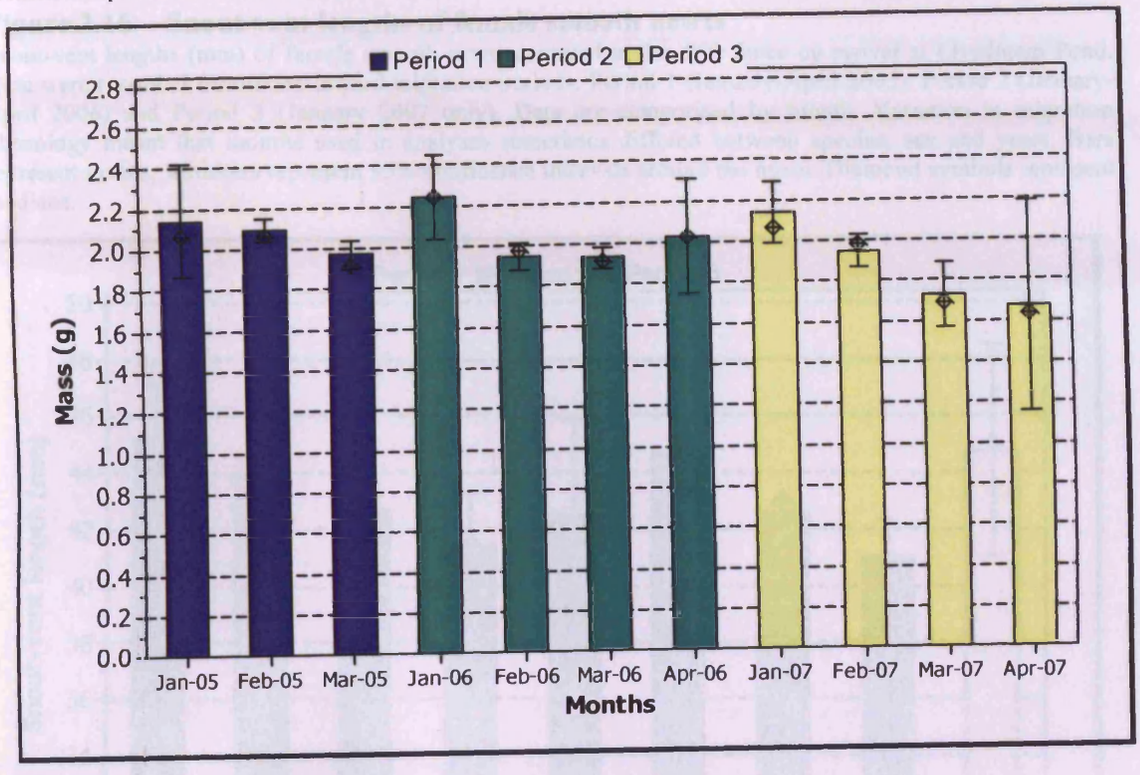


Table 2.9 Monthly comparisons of male smooth newt mass

Differences in mass data (g) of male smooth newts that arrived at Llysdinam Pond from January-April 2006 were analysed by one-way ANOVA. P values are from Tukey-Kramer post-hoc tests, after one-way ANOVA on Arcsinh transformed mass data. N = number of male smooth newt arrivals per month. For Tukey-Kramer tests if the p is <0.05 the cell is shaded dark grey and value displayed in bold font.

2006	JAN	FEB	MAR	APR
N	22	103	226	10
	JAN	**0.012**	**0.003**	ns
		FEB	ns	ns
			MAR	ns

2.4.2.7 Female smooth newt morphological data

In 2005 there was a decline in the body size of female smooth arrivals throughout the migration season. In contrast, in 2006, February had the highest proportion of large newts. The findings were consistent across measurements of snout-vent length (Figure 2.16 and Table 2.10), total length (Figure 2.17 and Table 2.11) and mass (Table 2.13). Although female smooth mass and gravidness was significantly higher in February (Figure 2.18,

Table 2.13), there was no significant difference in body condition over the period of migration (Table 2.2).

Figure 2.16 Snout-vent lengths of female smooth newts

Snout-vent lengths (mm) of female smooth newts captured at the drift fence on arrival at Llysdinam Pond. Data were recorded from three inward migration periods: Period 1 (January-April 2005), Period 2 (January-April 2006) and Period 3 (January 2007 only). Data are categorised by month. Variation in migration phenology meant that months used in analyses sometimes differed between species, sex and years. Bars represent means. Whiskers represent 95% confidence intervals around the mean. Diamond symbols represent medians.

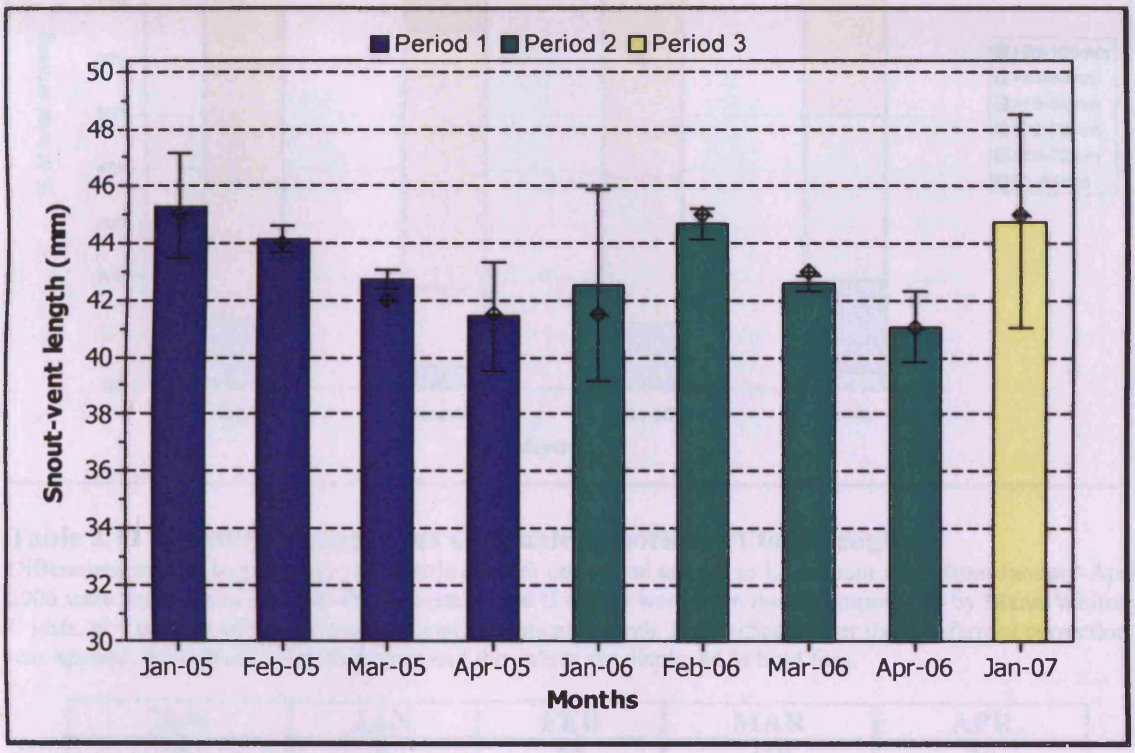


Table 2.10 Monthly comparisons of female smooth newt snout-vent lengths

Differences in snout-vent lengths (mm) of female smooth newts that arrived at Llysdinam Pond from January-April 2006 were analysed by Kruskal-Wallis tests. P and U values were from multi comparisons by Mann-Whitney U tests. N = number of female smooth newt arrivals per month. If significant after the Bonferroni correction was applied, the cell is shaded dark grey and the values are displayed in bold font.

2006	JAN	FEB	MAR	APR
N	8	73	418	30
	JAN	**U=119 p=0.006**	ns	ns
		FEB	**U=8441.5 p=<0.001**	**U=399.0 p=<0.001**
			MAR	**U=4407.5 p=0.006**

Figure 2.17 Proportion of female smooth newt arrivals at Llysdinam Pond categorised by total length

The proportion of female smooth newt arrivals, categorised by total length (mm) are displayed for each pond in Period 2. Newts were captured at the drift fence on arrival at Llysdinam Pond. Maximum and minimum lengths for each size category are shown in the key.

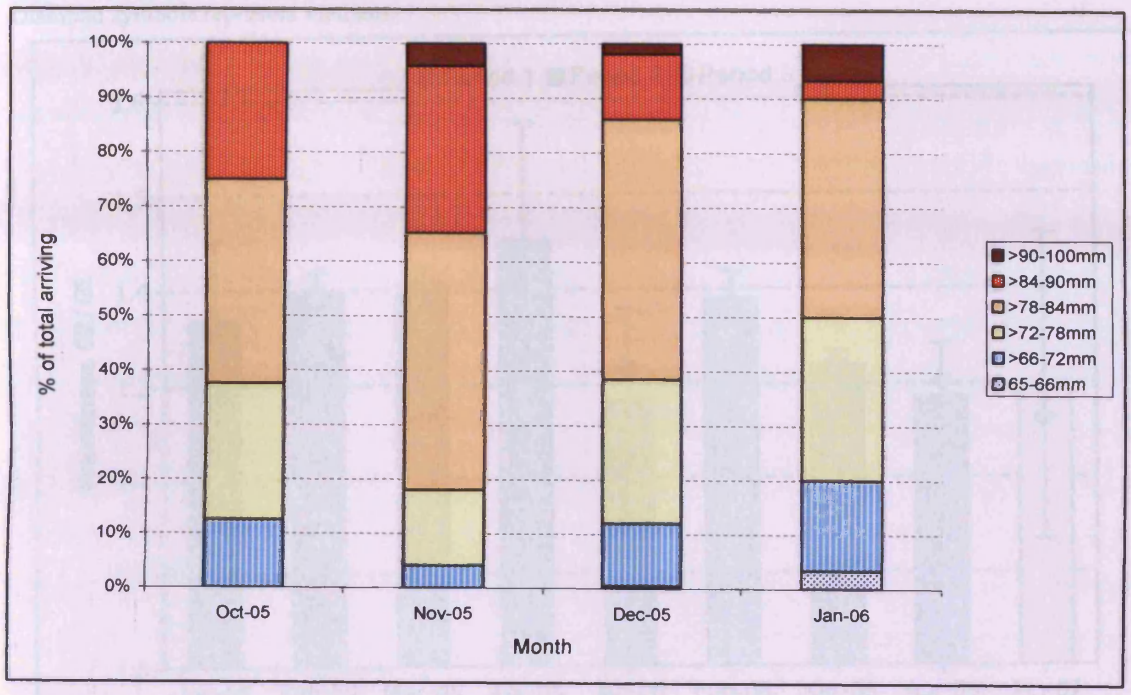


Table 2.11 Monthly comparisons of female smooth newt total lengths

Differences in total lengths (mm) of female smooth newts that arrived at Llysdinam Pond from January-April 2006 were analysed by Kruskal-Wallis tests. P and U values were from multi comparisons by Mann-Whitney U tests. N = number of female smooth newt arrivals per month. If significant after the Bonferroni correction was applied, the cell is shaded dark grey and the values are displayed in bold font.

2006	JAN	FEB	MAR	APR
N	8	73	418	30
	JAN	ns	ns	ns
		FEB	**U=9782.5 p=<0.001**	**U=608.5 p=<0.001**
			MAR	ns

Table 2.12 Monthly comparisons of female smooth newt mass

Differences in mass data (g) of female smooth newts that arrived at Llysdinam Pond from January-April 2006 were analysed by Kruskal-Wallis tests P values from Tukey-Kramer post-hoc tests, after one-way ANOVA on Arcsinh transformed mass data. N = number of female smooth newt arrivals per month. For Tukey-Kramer tests if the p is <0.05 the cell is shaded dark grey and value displayed in bold font.

2006	JAN	FEB	MAR	APR
N	8	73	418	30
	JAN	ns	ns	ns
		FEB	**<0.001**	**<0.001**
			MAR	Ns

Figure 2.18 Female smooth newt gravidness

Gravidness of female smooth newts captured at the drift fence on arrival at Llysdinam Pond. Data were recorded from three inward migration periods: Period 1 (January-April 2005), Period 2 (January-April 2006) and Period 3 (January 2007 only). Gravidness was calculated as G2/G1. G1 = width of newt at widest part of head, G2 = width of newt at widest part of belly measured by digital calipers. Data are categorised by month. Variation in migration phenology meant that months used in analyses sometimes differed between species, sex and years. Bars represent means. Whiskers represent 95% confidence intervals around the mean. Diamond symbols represent medians.

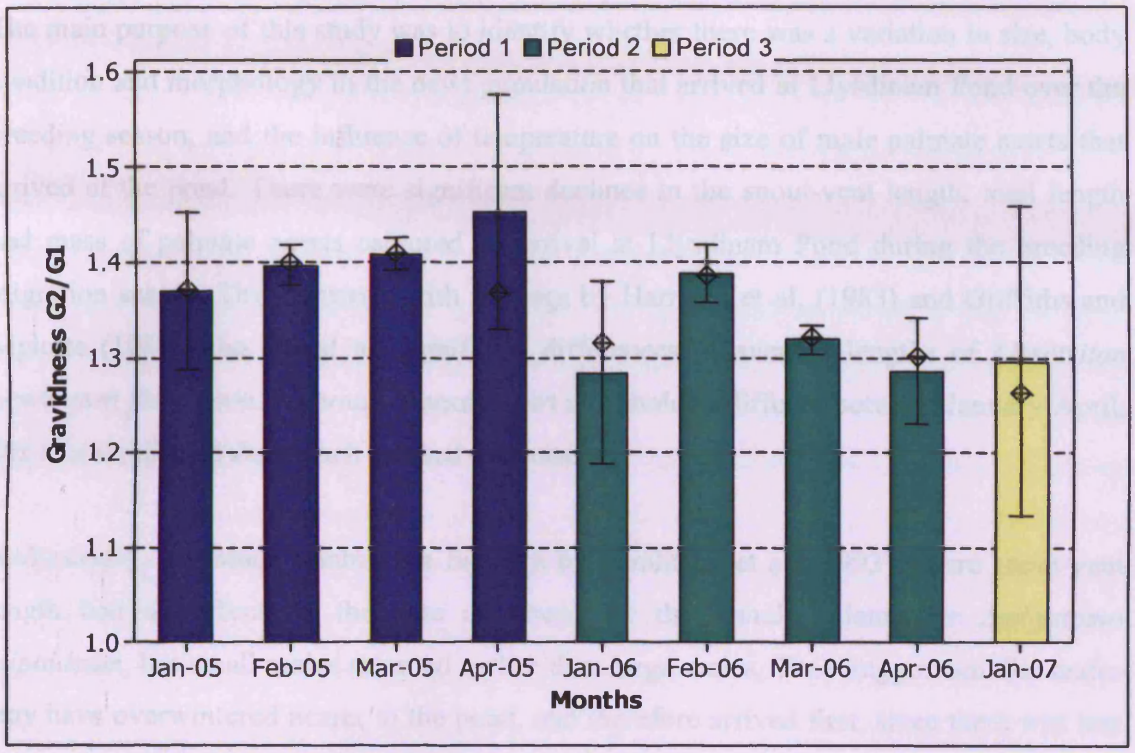


Table 2.13 Monthly comparisons of female smooth newt gravidness

Differences in gravidness of newts that arrived at Llysdinam Pond from January-April 2006 were calculated and analysed by Kruskal-Wallis tests. Gravidness was calculated as G2/G1. G1 = width of newt at widest part of head, G2 = width of newt at widest part of belly measured by digital calipers. P and U values are from multi comparisons by Mann-Whitney U test. N = number of female smooth newt arrivals per month. For Mann-Whitney U tests if p was <0.05 the cell is shaded light grey. If significant after the Bonferroni correction was applied, the cell is shaded dark grey and the values are displayed in bold font.

2006	JAN	FEB	MAR	APR
N	8	73	418	30
	JAN	U=155 p=0.033	ns	ns
		FEB	**U=11112 p=<0.001**	**U=637.5 p=0.002**
			MAR	ns

Significant differences between months were found for all morphological data of palmate newts, with the exception of male palmate total lengths. A higher proportion of larger newts in better condition arrived earlier. In male palmate newts differences in early and late arrivals were most apparent for body condition and mass. Female palmate results paralleled those of male palmate, and there was a significant decline in gravidness over

time. There were less significant differences in smooth newt morphology, although male smooth newts that arrived in January were in the best body condition and female smooth newts that arrived in February had the highest means for all morphological data.

2.5 DISCUSSION

The main purpose of this study was to identify whether there was a variation in size, body condition and morphology in the newt population that arrived at Llysdinam Pond over the breeding season, and the influence of temperature on the size of male palmate newts that arrived at the pond. There were significant declines in the snout-vent length, total length and mass of palmate newts captured on arrival at Llysdinam Pond during the breeding migration season. This contrasts with findings by Harrison et al. (1983) and Griffiths and Mylotte (1988) who found no significant differences in average lengths of *Lissotriton* newts over the season. Although smooth newt morphology differed between January-April, this species did not show such marked declines.

Additionally, this study contradicts findings by Semlitsch et al. (1993) where snout-vent length had no effect on the date of return for the female salamander *Ambystoma talpoideum*, but small males returned earlier than large males. They suggest smaller males may have overwintered nearer to the pond, and therefore arrived first, since there was less distance to migrate. *Ambystoma* species have a short stocky body so they may not be strictly comparable with the genus *Lissotriton*. Semlitsch et al. collected 12 years worth of data, and it could be the case that a prolonged study at Llysdinam study would show that the temporal changes detected in this study are not consistent between years. Most of the morphological data from spring 2005 and autumn 2006 had similar trends to the analysed migration data set from October 2005 to May 2006. Male palmate size did not show a similar trend, and increased over the migration season of autumn 2006 rather than declined as in previous years. The trend in gravidness of female palmate newts also differed in each of the three periods demonstrating annual variability.

There were some differences in male smooth morphology between months, but they were not as pronounced as the differences for palmate newt and female smooth newts. Research has shown male smooth newts prefer large females (Verrell, 1986) which was consistent with arrival times found in this study. Largest male smooth newts arrived in January and mean female size was highest in February, allowing male smooth newts to develop crests and be in good breeding state for the arrival of highest quality females. The first hypothesis

that morphology of *Lissotriton* newts on arrival at Llysdinam Pond differs significantly over the migration season was therefore accepted for palmate newts, as there were a higher proportion of larger palmate newts migrating to Llysdinam Pond earlier in the migration season. There was support for the hypothesis for smooth newts but the differences in morphology on arrival were less pronounced.

Although there was not a strong relationship between temperature and size of male palmate arrivals, when mean air temperature was categorised as four temperature codes there were some significant differences in newt morphology. There was an indication that smaller male palmate newts migrated at lower temperatures, but time of year rather than temperature accounted for a greater variation in the differences in sizes over the breeding season. Amphibians are poikilotherms and therefore their body temperature closely matches their surroundings (Duellman and Trueb, 1995). Palmate newts are an upland species (Beebee and Griffiths, 2000), and therefore may be adapted to a lower temperature threshold than smooth newts. Due to lower surface area to volume ratios, large newts would take longer to warm up as the temperature of their surroundings increases (Olalla-Tarraga and Rodriguez, 2007) so may have adapted to migrate earlier in autumn before temperatures decrease. In contrast, small individuals may be able to migrate on cold nights due to their larger surface area to volume ratio enabling more rapid warming and them to be active (Olalla-Tarraga and Rodriguez, 2007). The second hypothesis that there was a significant relationship between size of male palmate newts on arrival and temperature was rejected. It was concluded that arrival month rather than temperature explained a greater amount of the variability in newt size on arrival.

It was difficult to determine whether the larger newts were arriving earlier due to temperature thresholds or constraints or due to readiness to breed. There are a number of possible benefits of early arrival for newts. Early arrival provides more time for development of secondary sexual characteristics in males (Griffiths and Mylotte, 1988), it could lengthen the mating period for males and provide a longer egg-laying period for females. There were fewer aquatic predators very early in the season (Chapter 6 and Harrison (1985)), eggs may be exposed to less UV radiation although this only affects unwrapped eggs (Marco et al., 2001) and there was a longer period of time for larval growth before metamorphosis (Griffiths and de Wijer, 1994, May, 1993 and Chapter 6). The possible costs of arriving early include exposure to adverse weather and low prey availability (Reading, 1998). A larger proportion of larger newts arrived early to

Llysdinam Pond, and it may be the case that they were better able to gain benefits of early arrival and overcome the costs. The primary mate of female spotted salamanders *Ambystoma maculatum* sired a significantly larger number of offspring than later males (Tennessen and Zamudio, 2003) so early arrival may increase reproductive success. However, Sever (2002) investigated the histology of the female spermathecae of salamanders and found its structure enables female newts to store and use the most recently received spermatophore to fertilise eggs.

Egg-laying earlier in the year may confer benefits in avoiding animals that are predatory on eggs, such as adult great diving beetles *Dytiscus marginalis*, which were proficient at taking even unwrapped eggs (Miaud, 1993). Newts, particularly females are oophagus, hence arriving before the majority of newts, may confer survival advantages for eggs (Griffiths, 1995). Females that arrive early will lay a proportion of eggs earlier, and although the cooler water temperatures earlier in the year will lengthen development time (Bradford, 1990), some eggs will hatch successfully. These larvae would have a longer time for growth so that by summer once predatory invertebrate abundance has increased, they will at least be too large for gape-limited predators. Small larvae were found to decrease activity in the presence of predators, which could possibly limit feeding capabilities and growth (Mathis et al., 2003). Additionally, amphibians that develop at lower temperatures, metamorphose at a larger size (Walsh et al., 2008) which provides reserves for the first winter, and this metamorphic size advantage is maintained at maturity (Smith, 1987).

Newt eggs are prone to attack by fungi such as *Saprolegnia,* and such eggs are distinguished by an opaque swollen appearance. There has been a lack of consistency about when eggs are of greatest susceptibility to fungal attack. Griffiths (1995) found eggs laid earliest in cool water seem particularly prone to attack, although other studies on fungal infections found that warmer temperatures or exposure to UV increased susceptibility (Daszak et al., 2003; Gomez-Mestre et al., 2006; Kiesecker and Blaustein, 1997). The mould probably acts synergistically with environmental stressors.

Large male size has usually been thought to confer reproductive advantages (Kingsolver and Huey, 2008), although research found that large male newts did not have a mating advantage over small males, since there was no relationship between number of spermatophores deposited and male body size (Baker, 1990a). Additionally, in palmate

newts, females have been shown to have preference for traits uncorrelated with body size such as a long caudal filament and prefer small males. The longer filament may be a handicap and an honest indicator of body condition, while body size may be less important in male selection (Haerty et al., 2007).

Assumed mortality over the breeding season is thought to be greater for females than males due to reproductive costs in newts (Beebee and Griffiths, 2000). Early arrival, late departure and thus a lengthened aquatic period as shown by female palmate newts (particularly in 2007) may increase reproductive success within the year but also lead to increased mortality. Although survival varies between years, fewer smooth newts arrive in autumn and they have not shown the same decline in survivorship as have female palmate newts. Lower survival after early arrival has been found for other species, including female barn swallows *Hirundo rustica* (Møller, 2007).

A decrease in body size, age or condition on arrival at breeding sites over the season, and the benefits of early arrival has been supported by studies on a wide range of taxa, especially birds. Male common nightingales *Luscinia megarhynchos* that arrived earlier were heavy and in better conditions than late arrivals (Kipper et al., 2006). In the white stork *Circonia ciconia*, older individuals arrived at breeding grounds earlier, had larger clutch sizes and reared more chicks. Birds arriving early were more likely to lay eggs during the breeding season than those arriving late (Vergara et al., 2007). Lozano et al. (1996) determined that adult American redstarts *Setophaga ruticilla* arrived on breeding grounds before sub adults. Within the age classes, males that mated had arrived significantly earlier than those that did not mate. Young male red-breasted flycatchers *Ficedula parva* arrived significantly later to their breeding grounds than did older males. Later arrival could be due to lack of experience or avoidance of competition with older males (Mitrus, 2007). The benefits of arriving earlier could alter for different size classes; Dickerson et al. (2002) found arrival date favoured large male pink salmon early in the season and small males later in the season.

Using a drift fence with pitfall traps is a common sampling technique used in herpetofauna research. Several authors have discussed the associated biases and limitations, for example variations in numbers evading captures between body size and even reproductive condition (Heyer et al., 1994). Newts may burrow under the fence or climb over, although the earth around the fence was maintained to minimise newts burrowing under. Male smooth newts

were found to be good climbers (pers. obs. in small tanks in the laboratory and when torching for courtship behaviour), and since the fence lip was only on the inward side it would have been easier to breach the fence on inward migration than outward migration. The proportion of trespass of the fence might be considered consistent between sizes through the year at Llysdinam Pond, especially on nights with similar temperatures, but this may warrant further investigation. Overall drift fencing with pitfall traps has been argued to be suitable for collecting large amounts of data over long periods of time (Gibbons and Semlitsch, 1981).

Research has shown morphology has changed between years due to climate change. In a laboratory study, Reading and Clarke (1995) found toads prevented from hibernating by mild temperatures had decreased growth rates, matured at a smaller size and had increased mortality compared with those that hibernated. A field study following this up with data from 1983 to 2005 showed a clear relationship between a decline in body condition in female toads and the occurrence of warmer than average years (Reading, 2007). This was thought to be because they use up body fat too quickly due to a higher metabolism in warmer conditions. Declines in body mass and condition have also been shown in passerines in relation to climate change (Yom-Tov, 2001). Effects of climate on morphology may differ between species, as an increase in body size was found for edible frogs, *Rana esculenta* (Tryjanowski et al., 2006b), common lizards, *Lacerta vivipara* (Chamaille-Jammes et al., 2006), and two mammal species (Yom-Tov et al., 2006; 2008) in relation to temperature.

Given that the long term data set for Llysdinam Pond consisted of count data, and morphological data from the 1980s was limited, comparisons with earlier years were not possible. Only with past long term data or future long term data collection could investigation take place of whether trends found in this study show consistency or variability between years. The earlier arrival of large newts may be a more recent phenomenon, since such differences were not found in the 1980s (Griffiths and Mylotte, 1988; Harrison et al., 1983), and could possibly be due to mild autumn and early winter temperatures (IPCC, 2007; McCarty, 2001) being exploited, particularly by large palmate newts.

Chapter 3: Migration phenology of individual great crested and male smooth newts

3.1 SUMMARY

Photographic records were made of great crested and male smooth newt belly markings to investigate individual recognition in studying repeatability in migration dates between years, and length of the aquatic period. Great crested newts have distinctive natural markings on the ventral surface which has been used in several studies because it enables identification of individuals. Male smooth newts also have a distinctive pattern of spots on the ventral surface which has been rarely used for individual identification. Only 26 great crested newts (17 females and nine males) and eight male smooth newts were identified as repeat captures and capture frequency varied between two to seven times. For great crested females, the limited data suggest there was some repeatability in arrival dates between years, with a significant positive correlation in arrival dates between 2006 and 2007. Initial findings indicate that earlier arrival led to a longer aquatic period in most individuals due to less variation in departure than arrival dates.

Drift fences with pitfall traps were established in the mixed deciduous and coniferous woodland to the north-west of Llysdinam Pond in the winter of 2005, and monitored regularly for two years from January 2006. The main aim was to monitor individual newt movements and investigate whether a sub-migration of newts towards the pond occurred prior to the main breeding migration. Unfortunately it was not possible to study individual movements of newts in the woodland, since only three individual adult great crested newts and two male smooth individuals were captured there. In the two year period, 99 *Lissotriton* adults and 42 metamorphs were captured at the woodland drift fences which allowed some investigation of newt movement patterns within the woodland. Although data were limited, the proportion of captures indicated that adult *Lissotriton* newts and metamorphs migrated through different areas of the woodland and that a higher proportion of palmate than smooth newts migrated through the woodland habitat on the way to and from the pond.

3.2 INTRODUCTION

Newt breeding migrations are usually protracted over several weeks or months (Chadwick et al., 2006; Griffiths, 1995). Some species live in very close proximity to their breeding pond for most of their lives, while others disperse over considerable distances, and therefore have a long breeding migration each year (Griffiths, 1995). Jehle (2000) radio-tracked *Triturus* newts at three ponds and found that 95% of all great crested summer refuges fell within 63 m of the pond where most radio-tracking took place, while at the two other ponds, summer refuges were within 26-32 m of the water. *Triturus* and *Lissotriton* species prefer woodland and hedgerow habitat to open areas for migration, and tend to orientate migration to such habitats (Jehle and Arntzen, 2000; Malmgren, 2002). Sinsch (1988) found that common toads underwent an autumnal sub-migration prior to hibernating at sites closer to the pond. Although Chadwick (2006) found earlier arrival phenology of *Lissotriton* newts in the period 1997-2005 than in 1981-1987 it was not known whether an earlier autumn sub-migration towards the pond occurred in the surrounding habitat. Autumn arrival of *Lissotriton* newts at Llysdinam Pond happened more frequently in the 2000s than in the 1980s, but there may also have been a high frequency of undetected movement within the woodland habitat surrounding Llysdinam Pond.

Newts use a range of orientation cues (Sinsch, 2006), and this has been studied in arenas situated near to ponds (Diego-Rasilla, 2003; 2007; 2008; Joly and Miaud, 1993). One cue that newts are thought to use is the Earth's magnetic field (Diego-Rasilla, 2003; 2005; 2008) which is a particularly useful form of navigation for nocturnal migratory behaviour (Schlegel and Renner, 2006). Radio tracking could assist with monitoring migratory patterns and habitat use, but may be more suitable for large bodied *Triturus* newts due to body mass limitations for transmitters (Jehle and Arntzen, 2000). The elongated body shape of newts also makes fitting transmitters difficult, and therefore migration patterns of urodeles are poorly known (Jehle and Arntzen, 2000). *Lissotriton* newt migratory patterns have mainly been monitored with the use of drift fences (Chadwick et al., 2006; Harrison et al., 1983; Malmgren, 2002) and refuge searches (Griffiths, 1984).

Amphibians are difficult to mark because of their small size, potential for limb regeneration and sensitive skin (Murray and Fuller, 2000). Nevertheless, a wide variety of marking techniques have been used to mark adult amphibians in mark-recapture studies to gain information on a wide range of parameters including population size and movement

patterns (Brown, 1997; Donnelly et al., 1994). Marking techniques include numbered rings (Andreone, 1986), tags (Elmberg, 1989; Watson et al., 2000), dyes via panjet inoculators (Chadwick, 2003), acrylic polymers as visible implant elastomers (Bailey, 2002) and passive integrated transponders (PIT) or microchips (Chadwick, 2003; Watson et al., 2000). Since microchips are only suitable for newts over 2 g body mass this excludes use for the majority of *Lissotriton* newts (Fasola et al., 1993). Other invasive techniques include toe clipping (Denton and Beebee, 1993a; Golay and Durrer, 1994; Griffiths, 1984; Parris and McCarthy, 2001; Rothermel, 2004) and auto-transplantation. Auto-transplantation is where under anaesthetic, a piece of coloured skin is removed from the belly to the back, where a piece of skin had been previously removed, providing a recognisable marker (Andreone, 1986).

Some studies using invasive marking techniques have checked the effects of the marking on body condition and mortality with mixed findings. No negative effects were found from pit tagging on the Oregon spotted frog *Rana pretiosa* (Watson et al., 2000), captive and released fire salamanders *Salamandra salamandra* (Schulte et al., 2007) or of tail clipping on the body condition of individual great crested newts (Arntzen et al., 1999). Mixed findings were reported for toe clipping (Halliday, 1995) with reduced returns rates (Parris and McCarthy, 2001) and inflammation to the toes, which may not be apparent initially but delayed by up to a month (Golay and Durrer, 1994). Knee-tags resulted in serious lacerations in a third of the 12 frogs that were recaptured by Watson et al. (2000). At the Llysdinam Field Centre, harnesses used to attach transmitters or beta lights to toads resulted in some chafing damage (Durward, 2001; Parker and Gittins, 1979).

Unknown stress and later injury after release from artificial markers makes natural markings a non-intrusive alternative (Bradfield, 2004). The use of natural markings in identifying individual animals has been most frequently used for long lived mammalian species, including cheetahs *Acinonyx jubatus* (Kelly, 2001) and marine mammals (Hiby and Lovell, 2001), and such studies have used sketches, photographs and video footage. There have been concerns about analysing large data sets with over 100 images but computer aided matching has proved useful for large populations (Gamble et al., 2008; Hiby and Lovell, 2001; Kelly, 2001). Accuracy of natural marking identification has even been checked by microsatellite markers where from 1410 images of humpback whales (*Megaptera novaeangliae*) no false positive and 14 false negative matches were identified.

A false negative match was not noticing two images match, while a false positive was mistakenly matching images from two individuals (Stevick et al., 2001).

Amphibian studies have used skin patterns to identify individuals. In anurans, skin markings on the dorsal surface (Bowechop et al., 2006; Pittman et al., 2008), belly (Bowechop et al., 2006; Golay and Durrer, 1994) and throat (Denton and Beebee, 1993a) have been used to identify individuals. One study on the Archey's frog *Leiopelma archeyi* used six images taken from multiple angles (Bradfield, 2004). For urodele species some individual identification has been carried out using dorsal markings (Gill, 1978) but most have used ventral markings (Arntzen and Teunis, 1993; Gamble et al., 2008; Harris and Gill, 1980; Harris et al., 1995; Harris and Ludwig, 2004; Nijhuis and Kaplan, 1998; Slater, 1992). Although in the past sketches and photocopying were used to record skin patterns, now, with digital technology, individual identification using photographic images is easier and no longer an expensive technique.

Marks have often been used in recapture studies where the assumption was that there was no mark-induced mortality and that marks were not lost (Bailey, 2002). The belly patterns of *Ambystoma californiense* could change over time (Reaser, 1995), although for great crested newts the pattern was virtually stable by the end of the juvenile stage (Arntzen and Teunis, 1993). Consistency in skin markings over time was also found by Denton and Beebee (1993a) for natterjack toads. Hagström (1973) first described the belly pattern method for identifying great crested newts by their distinct yellow and orange belly markings. Several studies have used the great crested belly pattern as a sole method or to assist in great crested identification (Arntzen and Teunis, 1993; Hagström, 1977; Ortmann et al., 2006). Male smooth newts also have a distinctive pattern of spots on the ventral surface which has been rarely used for individual identification (Hagström, 1977).

3.2.1 Aims and hypotheses

Chadwick et al. (2006) found that male palmate newts showed the most pronounced change in phenology with an advance in arrival of 11.5 days for the first 10% of the January-April migration population, and a 17.7 day shift in median arrival day from the 1980s to 1997-2005. Male smooth newts had a smaller advance of 10.8 days for first 10% of the population and a 4.2 day shift in median arrival day. With just tally data of pitfall captures it was not known whether there was individual repeatability in arrival times between years with some individuals arriving consistently early. Individual arrival times

could not be studied for the earlier arrivals, male palmate newts, since they do not possess distinct natural markings to identify individuals. However, photographic identification of the unique belly markings of great crested and male smooth new could be used to investigate individual arrival phenology. It was hypothesised that (i) there is individual repeatability in arrival and departure dates between years. Chadwick (2003) found that the average aquatic period had increased from 14-15 weeks in 1981-1987 to 16-20 weeks in 1997-2004. The change in length of the aquatic period was highly significant for palmate newts and near significance for smooth newts. Since the period of time over which newt departures tend to occur is shorter, it was hypothesised that (ii) the length of aquatic period of individual newts is longer for earlier arrivals. Sinsch found that toads underwent a sub-migration towards their breeding pond in autumn. Therefore drift fencing constructed in woodland adjacent to Llysdinam Pond was used to test the hypothesis that (iii) there is an earlier sub-migration towards the pond prior to main amphibian breeding season that has not been detected by the pond drift fence. Using photographic records it could be determined whether individual newts are responding consistently between years in migrating through the woodland. Previous studies have detected patterns in amphibian dispersal from ponds after metamorphosis or after breeding. Therefore at Llysdinam it was hypothesised that (iv) there is directional dispersal patterns into the woodland habitat by adult newts and newt metamorphs.

3.3 METHODS

3.3.1 Great crested newt photographic identification

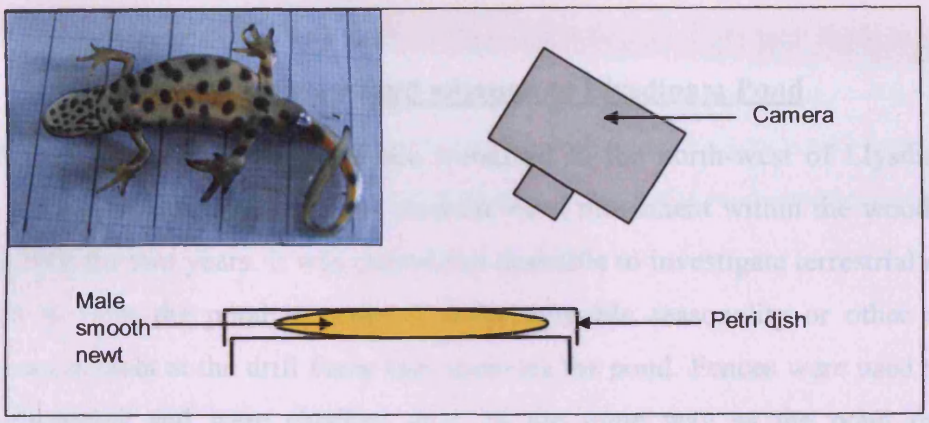
Adult great crested newts captured at the drift fence encircling Llysdinam Pond were photographed with a digital camera to keep a record of the individual belly markings. Great crested newts were held between the forefingers and a photograph taken of the ventral surface (see Appendix I). Afterwards newts were released on the opposite side of drift fence to which they were captured. Photographic records were taken from September 2004 to July 2008. Some photographic and photocopied records from 2003 and earlier in 2004 were also available. 83 male and 96 female photographs were printed, dated and matched by hand.

3.3.2 Male smooth newt photographic identification

For two years from January 2006 photographic records were also made of the ventral spot patterns of male smooth newts captured at the drift fence (Figure 3.1). Since male smooth newts are much smaller than great crested newts a different method was used to

photograph them. Each male smooth newt was placed in the lid of a Petri dish, the other half placed on top and the newt turned upside down to display the belly pattern. The belly markings were photographed at close range on macro setting, and then the newts were released.

Figure 3.1 Method for photographing male smooth newts and an example image
Each male smooth newt was placed in the lid of a Petri dish, the other half placed on top. The newt and dish were turned upside down to display the belly pattern, which was photographed at close range on macro setting.



The method did not harm the newts since a Petri dish is light in mass and just served to immobilise the newt for a brief time while the photograph was taken. A piece of laminated graph paper under the Petri dish provided a scale. The date of each photograph was recorded with direction of the newt's movement. Body length and mass measurements were also taken from January 2006-January 2007 (to provide data for Chapter 2). The photographic method was attempted for female smooth newts for two weeks before being abandoned since spot patterns were much less distinct.

There was a much larger set of male smooth photographs (690 photographs) so they were printed as small dated pictures and separated into descriptive categories before being matched by hand (Appendix II). Categories were: (i) many small spots, (ii) few small spots, (iii) many large spots, (iii) medium sized spots (iv) line of spots on belly (v) at least two spots joined and (vi) distinct other marking. Distinct uncommon markings included a newt with a circular pattern, newts with black patches rather than spots and newts with no spots on central belly (Figure 3.2). There were therefore between 10 and 120 photos in each category. Some newts fell into more than one category and were checked against both. Potential matches were checked with larger digital images on the computer to avoid any false positive matches.

Figure 3.2 Distinctive male smooth newt belly patterns
Photograph of distinctive circular marking and a male with no spot markings on central belly.

3.3.3 Newt movements in woodland adjacent to Llysdinam Pond

10 drift fences were constructed in the woodland to the north-west of Llysdinam Pond during winter 2005 (Appendix III) to measure newt movement within the woodland from January 2006 for two years. It was considered desirable to investigate terrestrial movement up to 60 m from the pond in order to detect possible seasonality or other movement patterns not evident at the drift fence that encircles the pond. Fences were used to monitor newt movements and were checked daily in the same way as the pond drift fence. Amphibians found were identified by species and sex (male smooth and great crested newts were photographed), and released 5 m away from the fence at which they were captured, in the direction of movement. The fences were constructed from 'newt guard' plastic sheets, lengths of wood and wooden stakes to which the 'newt guard' was stapled into place. Three plastic flowerpots were installed as pitfall traps on each side of the fence with a small mammal ladder in each to prevent accidental casualties. Each flower pot measured 19 cm diameter and 17 cm depth. Each fence was constructed with a two-way lip on the top to prevent newts climbing over the top and the plastic sheet was dug into the ground to approximately 5 cm depth (Figure 3.3).

Each fence was 5 m long with angled ends to direct newts at the end of the fence inwards. Three lines of fences with three fences in each were installed, these being at 20, 40 and 60 m from Llysdinam Pond drift fence. The three fences in line were spaced at 15 m intervals, parallel to the pond drift fence which created three Transects A-C (Transect A was Fences 1, 2 and 3, Appendix III). The three pots on each side of the woodland fence were numbered 1-3 from south-north. The initial site selected for the central front fence proved to be too waterlogged, so although this fence remained (Fence 10), the fence layout was shifted 5 m to avoid the main rainfall runoff in the woodland and consequential water logging of the traps.

The woodland to the north-west of the pond was chosen as the location for the fences because more newts migrated out from the pond to the woodland area (pers. obs.) so captures would be maximised. Transects A and B were in deciduous and coniferous mixed woodland with good undergrowth cover dominated by nettle (*Urtica dioica*), bramble (*Rubus fruticosus*), holly (*Ilex aquifolium*), ferns (*Dryopteris* sp.) and mosses (Bryophyta) in wetter areas (Durward, 2001). Transect C was in the coniferous dominated side of the woodland with less undergrowth and a covering of coniferous needles, although Fence 7 had more undergrowth than 8 and 9 (Figure 3.3). Constructing pitfall fences in the woodland was relatively difficult, due to large numbers of rocks and thick tree roots. In some areas digging was impossible and instead the soil level was raised around the fences to make the pitfall pots flush with the ground. To enable this one tonne of quarry soil was brought onto the site to build up the ground around each fence. Flowerpot saucers were used as lids for the traps to enable the traps to be closed when daily checking was not possible.

Figure 3.3 A woodland drift fence

10 drift fences were constructed in the woodland to the north-west of Llysdinam Pond to enable newt movement patterns within the woodland to be monitored over a two year period. Each fence was 5 m long with angled ends to direct newts inwards at the end of the fence. Fences were constructed from 'newt guard' plastic sheets, lengths of wood and wooden stakes to which the 'newt guard' was stapled. Fence 8 of Transect C is pictured. There were three transects (A-C), and each included three fences plus there was an additional fence (Fence 10) that was constructed within a boggy area of woodland. Each fence had three pitfall traps on either side to capture amphibians and detect directional movement away from or towards Llysdinam Pond. Woodland pitfalls were usually checked on a daily basis and when this was not possible then the traps were covered with flowerpot saucers.

3.3.4 Data analysis

Recaptures rates were low from 690 male smooth newts that were photographed over the two year period. Therefore intended repeatability arrival analysis (Semlitsch et al., 1993) was not possible. Instead a basic assessment of arrival repeatability using correlation analysis was conducted for great crested newts only. Spearman's rank correlation was used to correlate arrival dates in male and female great crested newts (Bowker and Randerson, 2008). Low recaptures in male great crested newts meant that years were combined for correlation analysis. Captures in the woodland pitfalls were analysed by Chi squared analysis (χ^2) to determine whether movements patterns of adult newts and metamorphs was directional rather than random (Bowker and Randerson, 2008). All analyses were conducted in Minitab 15.

3.4 RESULTS

3.4.1 Photographic data for great crested newts

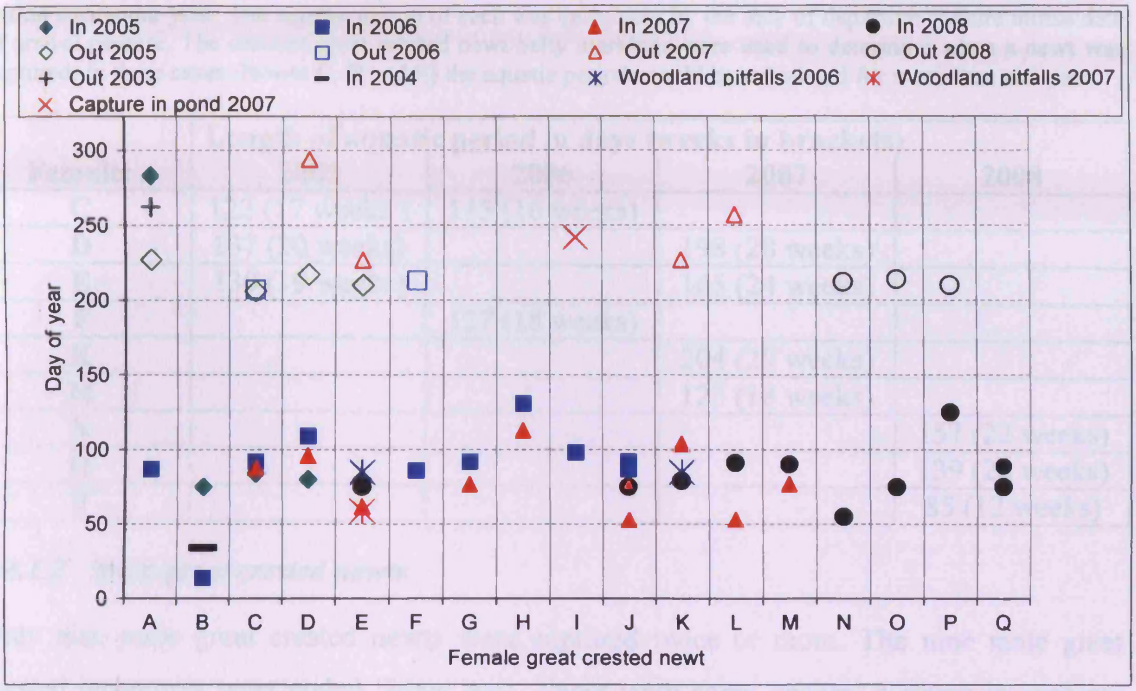
Matching of great crested newt photographs was relatively straight forward due to the smaller number of photographs and the very distinct ventral markings (Appendix I for examples of great crested ventral markings). In total there were 83 adult male and 96 female photographs (or photocopies) to match. This corresponds to 73 individual males (nine with repeat captures) and 59 individual females (17 with repeat captures).

3.4.1.1 Female great crested newts

There were 17 great crested females captured twice or more over the four year period. The 17 female great crested recaptures were code as 'Female A-Q'. There were some general patterns in newt capture dates (Figure 3.4), but some newt arrival dates were particularly interesting and showed repeatability. Female C arrived on Day 84, 92 and 88 for 2005, 2006 and 2007, respectively. Departure dates were Day 206 and 207 in 2005 and 2006, respectively. Female J was captured arriving at the drift fence twice in 2006 (on Day 87 and 92) and 2007 (Day 55 and 77). Female Q was captured arriving at the drift fence twice in 2008. There was an interval of 5-22 days between recaptures for Female J and 14 days for Female Q. It was not known whether they entered the pond between the recaptures, but the captures indicated possible roaming behaviour by some newts before entry to the pond.

Figure 3.4 Female great crested newt arrival and departure dates

The 17 female great crested newts that were captured in pitfall traps more than once from 2003-2008 are represented as letters A-Q. Captures labelled as 'In' or 'Out' were made at the Llysdinam Pond drift fence. Arrival captures are labelled as 'In' and departure captures are labelled as 'Out'. The capture in the pond was made during netting surveys undertaken for Chapter 6. Woodland pitfall captures were made at drift fences constructed in the woodland to the north-west of Llysdinam Pond. Each capture date at Llysdinam Pond drift fence, in the woodland traps or in the pond is represented with a different symbol.



Only two individual female great crested newts were recaptured in the woodland pitfalls. They were both captured at end of March in 2006 on Day 85 (Female E at Fence 8, Pot 3 and Female K at Fence 1). They were not captured at the main pond drift fence in 2006. Interestingly in 2007, Female E was recaptured again at Fence 8 in the same pot at the end of February (Day 59) three days before reaching Llysdinam Pond drift fence, a distance of 40 m. This suggested that at least some great crested newts follow similar migratory routes annually. Newts recaptured between 2006 and 2007, all arrived earlier in 2007 which had higher mean monthly temperatures early in the year (January: 5.9°C, February: 4.8°C) than 2006 (January: 3.9°C, February: 3.1°C).

Arrivals dates showed highly non-significant correlations and so a lack of repeatability between years, except for 2006 and 2007 ($r_s = 0.975$, d.f = 3, $p = 0.005$, Females C, D, G, H, J). Departure dates showed no significant correlation ($r_s = 0.632$, d.f = 2, $p = 0.368$, N = 4, Females A, C, D, E). The length of the aquatic period for the individual great crested newts captured in 2005 and 2006 was similar (Table 3.1). The recaptures in 2007 and 2008 showed a larger range in aquatic period. There was greater variation in arrival than

departure dates (Figure 3.4) so that earlier arrival was followed by earlier departure in some cases (D and E) but not others (K, L, N, O and P).

Table 3.1 Aquatic period of female great crested newts

The aquatic periods of nine female great crested newts in 2005-2008 are displayed. Aquatic periods were only calculated when newts were captured both arriving and departing at the Llysdinam Pond drift fence within a calendar year. The aquatic period of each was calculated by the date of departure capture minus date of arrival capture. The distinct great crested newt belly markings were used to determine when a newt was captured. In three cases (Newts C, D and E) the aquatic periods could be calculated for more than one year.

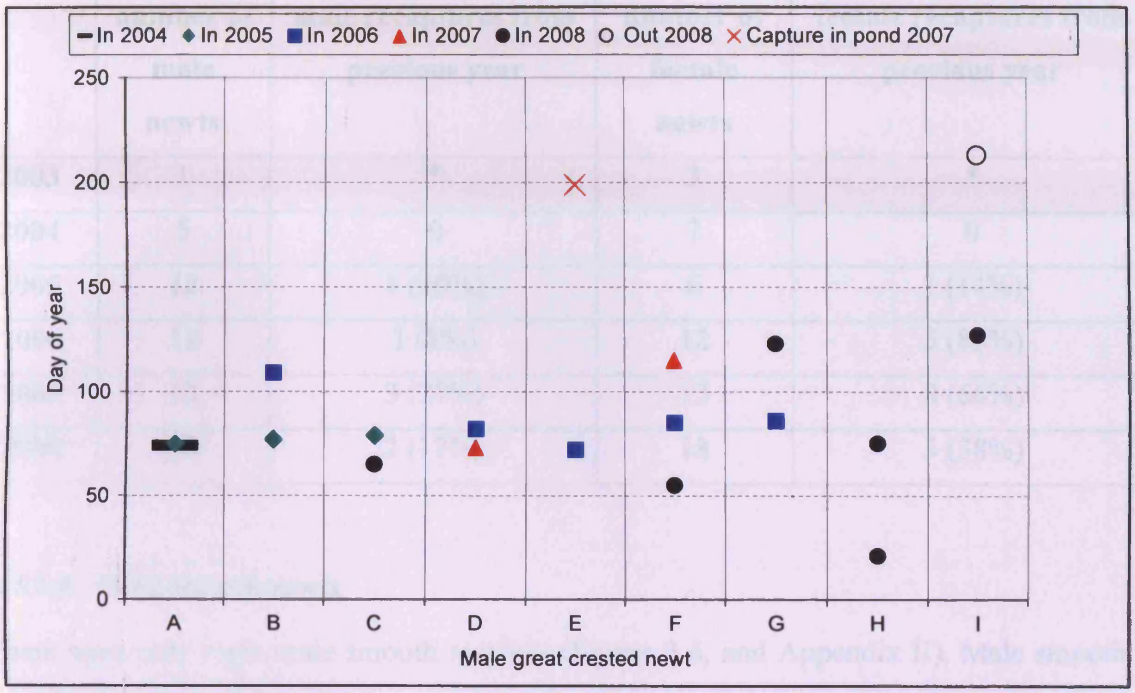
	Length of aquatic period in days (weeks in brackets)			
Female:	**2005**	**2006**	**2007**	**2008**
C	122 (17 weeks)	115 (16 weeks)		
D	137 (20 weeks)		198 (28 weeks)	
E	130 (19 weeks)		165 (24 weeks)	
F		127 (18 weeks)		
K			204 (29 weeks)	
M			123 (18 weeks)	
N				157 (22 weeks)
O				139 (20 weeks)
P				85 (12 weeks)

3.4.1.2 Male great crested newts

Only nine male great crested newts were captured twice or more. The nine male great crested recaptures were coded 'Male A-I'. There were some general patterns in captures dates (Figure 3.5). Male A showed high repeatability in captures, and was captured on Day 74 in 2004 and Day 75 in 2005. Male D was captured on Day 82 in 2006 and Day 73 in 2007. Only one male was recaptured exiting the pond suggesting the majority may exit via the water inlet, where a 1 m gap in the fence exists. Recaptured great crested newts males on average arrived three (2005) to 10 days earlier (2008) than females. No correlation between arrival dates was found (Males A-D, F and G, all years combined) with any combination of dates for Male F. Male H was captured three times at the pond fence with an interval of 19-54 days between recaptures but it was unknown if Male H remained terrestrial between recaptures or entered the pond. Male C was captured as a young juvenile in 2005 (10 cm in total length) then captured again in 2008 as a breeding adult. Only one male great crested was captured in the woodland (Day 249 in 2006, Fence 7) and he was not captured again.

Figure 3.5 Male great crested newt arrival and departure dates

The nine male great crested newts that were captured more than once from 2004-2008 are represented as letters A-I. Captures labelled as 'In' or 'Out' were made at the Llysdinam Pond drift fence. Arrival captures are labelled as 'In' and departure captures are labelled as 'Out'. The capture in the pond was made during netting surveys undertaken for Chapter 6. Each capture date at Llysdinam Pond drift fence or in the pond is represented with a different symbol.



3.4.1.3 Great crested newt recaptures

The great crested newt population increased in 2008 with 32 male and 18 female individuals. The increase in the number of great crested newts captured each year was probably because the population was recently introduced and therefore well below carrying capacity. Recapture rates for males varied between zero in 2004 to 27% in 2007. Female recapture rates varied between from zero in 2004 to 83% in 2006. Only one male (2% of 2006 captures, Male F) and two females (5% of 2006 captures, Females J and K) were captured annually between 2006 and 2008, and three females (6% of 2005 captures, Females C, D and E) were captured between 2005-2007 (Table 3.2 and Figures 3.4 and 3.5).

Table 3.2 Great crested newt numbers and recaptures at Llysdinam Pond
The annual number of male and female great crested newts captured between 2003 and 2008 is shown. The distinct great crested newt belly markings were used to determine when recaptures were made. All captures were made at the Llysdinam Pond drift fence and were a total of inward and outward bound captures. The number of recaptures as number of individual newts and percentage are displayed for each year.

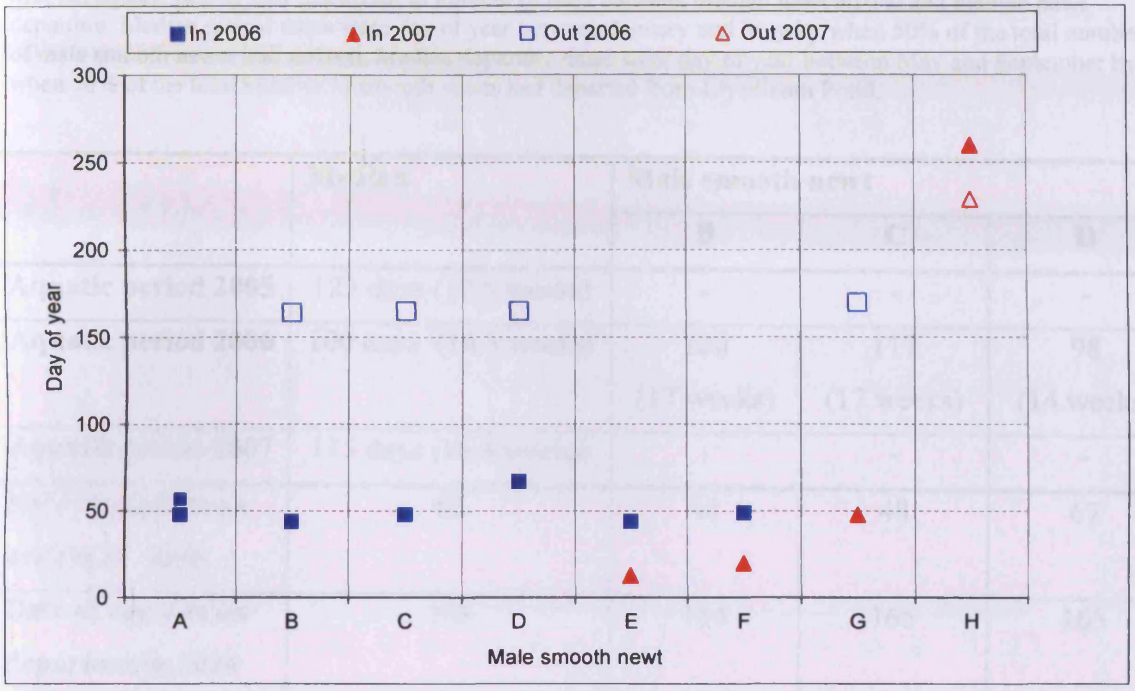
	Total number of male newts	Number and % of male recaptures from previous year	Total number of female newts	Number and % of female recaptures from previous year
2003	1	*	3	*
2004	5	0	7	0
2005	12	1 (20%)	6	1 (14%)
2006	11	1 (8%)	12	5 (83%)
2007	12	3 (27%)	13	8 (66%)
2008	32	2 (17%)	18	5 (38%)

3.4.1.4 Male smooth newts

There were only eight male smooth matches (Figure 3.6, and Appendix II). Male smooth recaptures were coded as 'Male smooth A-H'. Matching was more difficult because there were almost 700 records (mainly due to the large number of newt arrivals in 2006 when a total of 376 males were captured on arrival). Designation of photographs to categories reduced number of comparisons. Some male smooth newt belly markings appeared to fade during the aquatic period (Appendix II, Male smooth D) and some spots slightly changed in size (Appendix II, Male smooth F). Male smooth A was captured three times at Llysdinam Pond drift fence in 2006. Of the two between year recaptures, both individuals (Male smooth E and F) arrived earlier in 2007. Male smooth H was recaptured arriving at the pond drift fence 19 days after departing from the pond in 2007. (For dates of the individual male smooth captures see Appendix II).

Figure 3.6 Male smooth newt arrival and departure dates

The eight male smooth newts that were captured in pitfall traps more than once from 2004-2008 are represented as letters A-H. Captures labelled as 'In' or 'Out' were made at the Llysdinam Pond drift fence. Arrival captures are labelled as 'In' and departure captures are labelled as 'Out'. Each capture date at Llysdinam Pond drift fence or in the pond is represented with a different symbol.



The aquatic period for Male smooth B and C was three weeks longer than the average (Table 3.3). The median aquatic period has not significantly altered between 1981-1986 and 2000-2007 for male smooth newts ($T = 1.52$, $d.f = 9$, $p = 0.164$). Male smooth B and D gained body mass during the aquatic period (5% and 44% gain respectively) while C lost body mass (14% loss). The three newts recaptured in both 2006 and 2007 had gained weight (0.27-0.56 g, 15-23% gain).

Table 3.3 Aquatic period for male smooth newts

The aquatic periods of three male smooth newts in 2006 are displayed. Aquatic periods were only calculated when newts were captured both on arrival and departure at the Llysdinam Pond drift fence within a calendar year. The aquatic period of each newt was calculated by the date of departure capture minus the date of arrival capture. The distinct belly markings of male smooth newts were used to determine when recaptures were made. Median aquatic period of all male smooth newts are also displayed for 2005, 2006 and 2007. Median aquatic period was calculated as number of days between median newt arrival and median newt departure. Median arrival dates were day of year between January and May by when 50% of the total number of male smooth newts had arrived. Median departure dates were day of year between May and September by when 50% of the total number of smooth newts had departed from Llysdinam Pond.

	Median	Male smooth newt		
		B	C	D
Aquatic period 2005	123 days (17.6 weeks)	-	-	-
Aquatic period 2006	100 days (14.3 weeks)	120 (17 weeks)	117 (17 weeks)	98 (14 weeks)
Aquatic period 2007	115 days (16.4 weeks)	-	-	-
Date of capture on arrival in 2006	68	44	48	67
Date of capture on departure in 2006	168	164	165	165

3.4.2 Newt movements in woodland adjacent to Llysdinam Pond

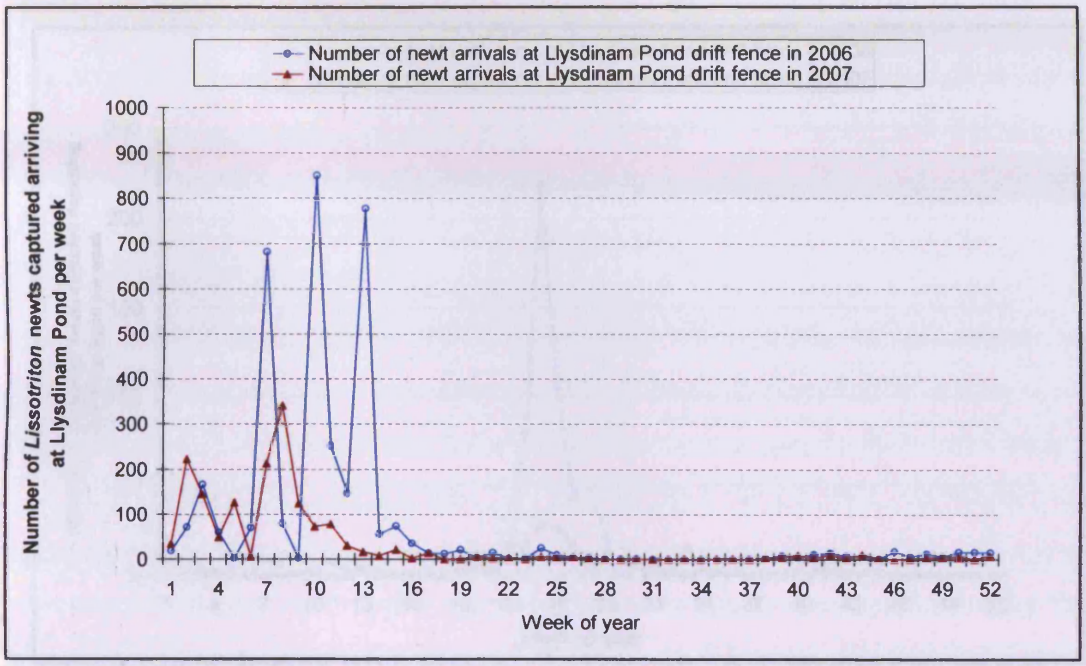
3.4.2.1 Adult newt captures

Peak newt captures in the woodland were mainly found on weeks with high captures at the pond drift fence (Figure 3.7 and Figure 3.8). Although around Week 37 (mid September) there were more newt movements in the woodland relative to the pond than during the rest of the year although still only in low numbers. This movement occurred in both directions so did not indicate a sub-migration towards the pond. In the woodland pitfalls, 35 and 18 adult *Lissotriton* newts were captured moving towards the pond in 2006 and 2007 respectively. This reflected the higher annual number of adults captured at the drift fence in 2006 (3614 adults) than 2007 (1617 adults). Newts moving away from the pond totalled 38 and 8 woodland pitfall captures in 2006 and 2007 respectively. This reflected the higher capture of newts departing from the pond in 2006 (620) than 2007 (238). Of these captures, only two male smooth and six female smooth newts were captured in the woodland (6% of total captures), this was a much lower proportion of smooth newt captures than at the pond drift fence (25 % of total captures in 2006 and 2007).

Figure 3.7 Adult *Lissotriton* newt arrival numbers at Llysdinam Pond and newt movement towards the pond in the woodland

(i) Number of adult *Lissotriton* newts captured arriving at Llysdinam Pond drift fence in 2006 and 2007 and (ii) number of adult *Lissotriton* newts captured moving towards the pond in woodland pitfall traps. Newt captures at the drift fence and woodland pitfalls are reflected in the two graphs. Newt movements in Weeks 37-43 occurred in both directions so did not indicate a sub-migration towards Llysdinam Pond.

(i) Number of newt arrivals at Llysdinam Pond drift fence



(ii) Woodland pitfall captures of newts moving towards Llysdinam Pond

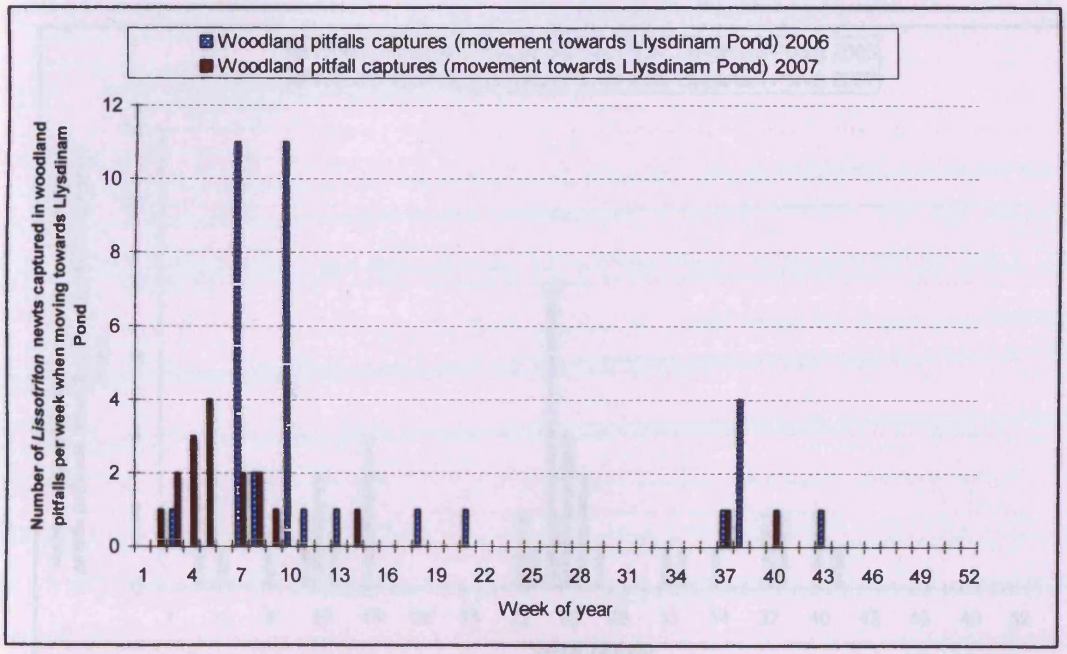
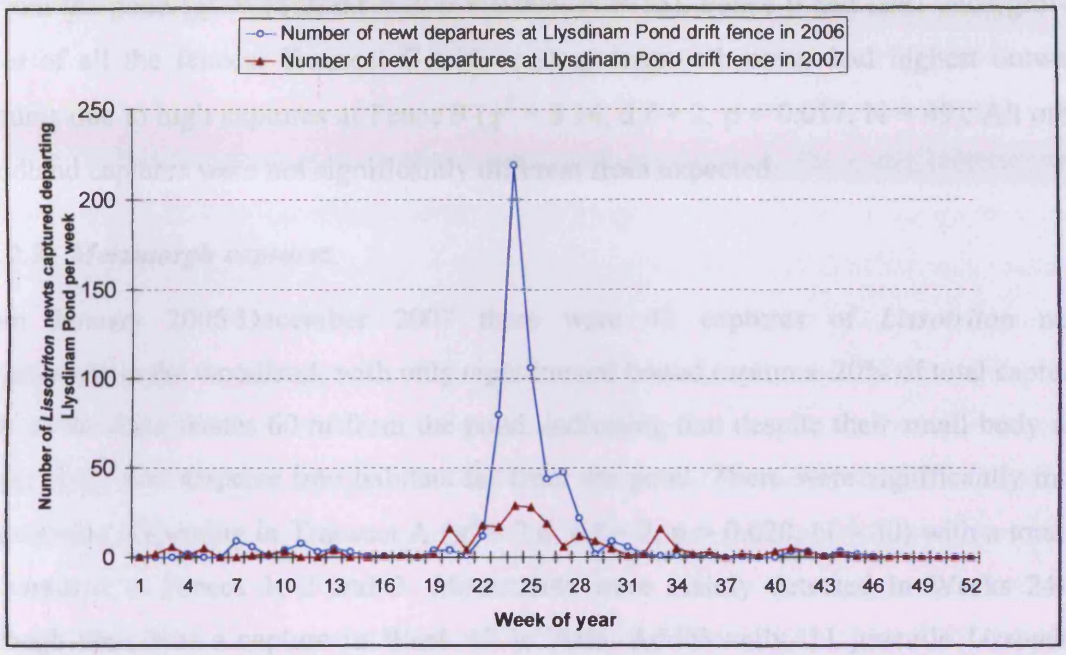


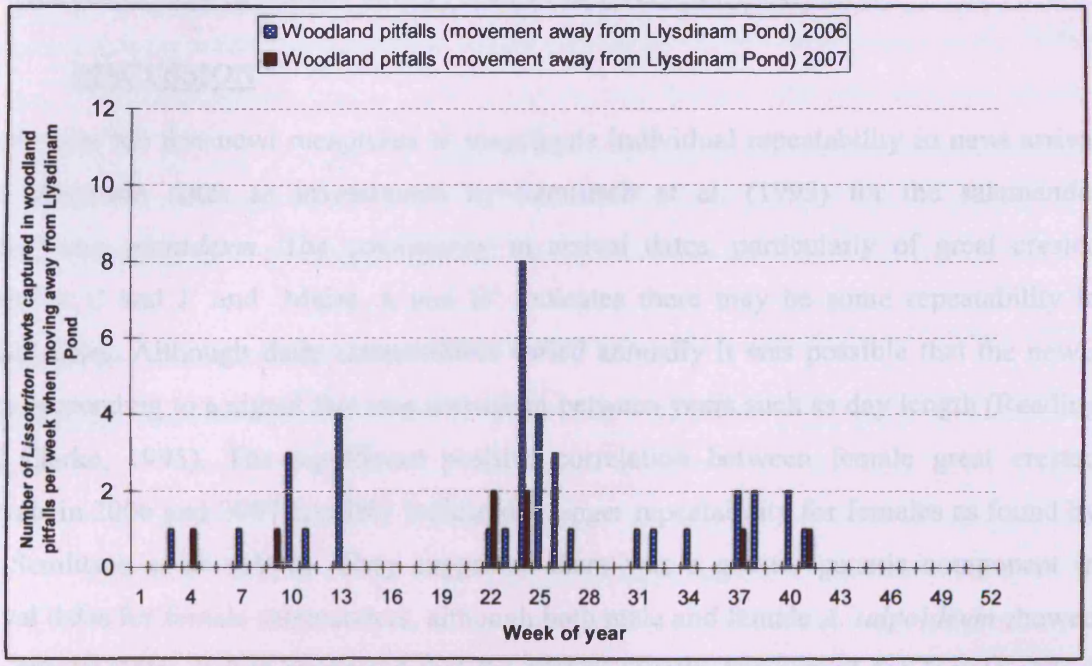
Figure 3.8 Adult *Lissotriton* newt departure numbers from Llysdinam Pond and newt movements away from the pond in the woodland

(i) Number of adult *Lissotriton* newts captured at the drift fence departing from Llysdinam Pond in 2006 and 2007 and (ii) number of adult *Lissotriton* newts captured moving away from the pond in woodland pitfall traps. Newt captures at the drift fence and woodland pitfalls are reflected in the two graphs. Newt movements in Weeks 37-43 occurred in both directions so did not indicate a sub-migration towards Llysdinam Pond.

(i) Number of newt departures at Llysdinam Pond drift fence



(ii) Woodland pitfall captures of newts moving away from Llysdinam Pond



20% of newt captures in the woodland were at the fences furthest (60 m) from the pond. 30% of inward migration was at Fence 10, significantly more than at the other fences (Fences 3, 6 and 9) that were located 20 m from pond (χ^2 = 16.82, d.f = 3, p = 0.001, N = 23) while metamorphs and departing adults showed no preference for that area of the woodland. Fence 9 had significantly more outward migration than the other two fences 20 m from the pond (χ^2 = 11.2, d.f = 2, p = 0.004, N = 15), Fence 9 had least undergrowth cover of all the fences. Transect C with least undergrowth cover had highest outward captures due to high captures at Fence 9 (χ^2 = 8.14, d.f = 2, p = 0.017, N = 49). All other woodland captures were not significantly different from expected.

3.4.2.2 Metamorph captures

From January 2006-December 2007 there were 42 captures of *Lissotriton* newt metamorphs in the woodland, with only eight inward bound captures. 20% of total captures were at the three fences 60 m from the pond, indicating that despite their small body size metamorphs will disperse into habitats far from the pond. There were significantly more metamorphs dispersing in Transect A (χ^2 = 7.8, d.f = 2, p = 0.020, N = 30) with a total of 17 captured at Fences 1, 2 and 3. Movements were mainly detected in Weeks 24-38 although there was a capture in Week 17 in 2006. Additionally, 11 juvenile *Lissotriton* newts that were not sexually mature were captured between May and September 2006 (nine of which were outward bound) and two male great crested juveniles were captured outward bound in the woodland in late August and early September 2006.

3.5 DISCUSSION

There were too few newt recaptures to investigate individual repeatability in newt arrival and departures dates as investigated by Semlitsch et al. (1993) for the salamander *Ambystoma talpoideum.* The consistency in arrival dates, particularly of great crested 'Females C and J' and 'Males A and D' indicates there may be some repeatability in arrival dates. Although daily temperatures varied annually it was possible that the newts were responding to a signal that was consistent between years such as day length (Reading and Clarke, 1995). The significant positive correlation between female great crested arrivals in 2006 and 2007 possibly indicated stronger repeatability for females as found by the Semlitsch et al. (1993). They suggested there was a greater genetic component in arrival dates for female salamanders, although both male and female *A. talpoideum* showed low repeatability. It was suggested that the higher genetic component for female arrival time may be because reproductive success was dependent only on the success of the eggs

she laid, while male reproductive success was divided between the numbers of females successfully mated. The low recapture rate of individual identified newts in this study meant that the first hypothesis that there was repeatability in arrival and departure dates between years could not be accepted.

Two female great crested newts, one male great crested and one male smooth were captured at Llysdinam Pond drift fence more than once annually, during inward breeding migrations. Griffiths (1984) found that some newts displayed a second return to water after being recaptured on land, and Gittins et al. (1980) have shown the same behaviour for common toads. It was likely that newts with a longer interval between captures entered Llysdinam Pond, while those caught within a day or so remained on land between captures.

Male great crested newts and male *Lissotriton* newts arrive slightly earlier than conspecific females (Arntzen, 2002; Chadwick et al., 2006). Males may have an earlier behavioural or hormonal response to migratory stimuli than females. It may be beneficial for males to arrive before females, in order to develop their secondary sexual characteristics (crest, webbed feet, or tail filament) in the aquatic environment. Factors influencing reproductive cycles of amphibians are complex and probably involve interactions between temperature, rainfall, photoperiod and endogenous circannual rhythms (Griffiths et al., 1986). Environmental changes may affect the timing of hormone production. Prolactin and sex steroids are known to play a vital role in a series of reproductive events including courtship in newts, and prolactin is believed to induce migration to the water (Iwata et al., 2000). Departure for female great crested individuals showed greater similarity than arrival dates (particularly in 2008) and may be a response to day length, so that earlier arrivals often but not always have a longer aquatic period. There were some indications that as predicted by the second hypothesis, early arrivals had a longer aquatic period but further evidence is required.

Only three identified individual newts (two female and one male great crested) were captured in the woodland so individual migration patterns in the woodland habitat could not be identified. The capture of a female great crested newt in the same woodland pitfall trap in concurrent years suggests that a winter refugia or the migration route that the female used was located there. Terrestrial refuge and site fidelity has been found for other amphibians (Jehle and Arntzen, 2000; Pittman et al., 2008). Newts use a range of orientation cues (Sinsch, 2006) including pond odours to find their way to ponds for

breeding (Joly and Miaud, 1993). By radio tracking great crested newts, Jehle (2000) showed that after breeding, newts would follow initial migration paths even if they did not return to the same refuge. In contrast, data from the woodland fences suggest that for *Lissotriton* newts there may be subtle differences in routes to and from the pond, since most inward bound captures were at Fence 10 and outward bound at Fence 9. Time of arrival may also be influenced by distance of emigration from the pond after the previous breeding season (Semlitsch et al. 1993), and if there is high fidelity to terrestrial sites then this could explain repeatability between years for some individuals.

The majority of *Lissotriton* and great crested newts were captured on the forested side of the pond drift fence to north-west (pers. obs). This contrasts with migratory patterns in the 1980s when more newts migrated from the east of the pond where there was deciduous woodland and hedgerows. Griffiths stated that the number of newts arriving from the small coniferous plantation were lower (Beebee and Griffiths, 2000). It may be that, as the coniferous woodland became more species rich and turned to a mixed (although predominantly coniferous) woodland, greater ground flora made it a more suitable habitat for newts. Preferred direction towards forest habitat has been found for great crested and smooth newts in other studies (Malmgren, 2002), with directionality greater for newts that had spent at least one year on land. The low ratio of smooth to palmate captures in the woodland, in comparison with captures at Llysdinam Pond drift fence, suggests that smooth newts move into different terrestrial habitat. It could be that the coniferous leaf litter creates acidic conditions to which palmate newts may show greater tolerance, since they tend to be more commonly found in more acidic ponds than smooth newts (Beebee and Griffiths, 2000). Fewer smooth newt captures in the woodland may be due to different catchability rates between the woodland traps and pond drift fence. There was a higher probability of catching newts at pond drift fence due to the strong breeding cues causing migration towards the pond. Newts climbing out of pitfall traps at the pond drift fence would be likely fall into another trap later that evening or the subsequent night, and sometimes newts were noticed in leaf litter by the drift fence on daily pitfall checks.

The woodland drift fences detected newt movement in both directions around Week 37 of 2006 and 2007 suggesting non-directional activity of newts prior to seeking refuge to hibernate for the colder months. There was no evidence for a large autumn sub-migration towards the pond, and therefore the third hypothesis was refuted. Sinsch (1988) found common toads migrated towards ponds in autumn, stopped before reaching the breeding

site and hid in holes and under the leaf litter. Unlike the study at Llysdinam Pond, Sinsch tracked toads at greater distance from the pond and all sub-migrations took place at 70-760 m from the pond. Denton and Beebee, (1993b) also found that some common toads made a substantial migration of over 500 m towards ponds in autumn before finding a hibernation site. 19% of adult *Lissotriton* newt captures were at the fences furthest from the pond indicating that some individuals move at least 60 m from the breeding pond. Using radio-tracking Jehle and Arntzen, (2000) demonstrated that great crested newts move large distances when they leave the pond but shorter distances on subsequent nights and could travel up to a 28 m per hour, and migrate as far 1.3 km (Kupfer, 1998). Jehle and Arntzen's work would indicate that if woodland fences had been located further than 60 m from Llysdinam Pond then a sub-migration may have been detected. However, they also found that 68% of all newt movement occurred within 20 m of the pond. Median distances of movement for great crested newts at three ponds during summer ranged from just 9-16 m (Jehle, 2000), and 96% of terrestrial smooth newts moved only 9 m in an urban environment (Griffiths, 1984).

Significantly more inward bound migratory behaviour was recorded at Fence 10 (the waterlogged site) which suggests that newts may follow water run off on breeding migrations possibly as a migratory cue or to minimise desiccation. Newt metamorphs and departing adults showed no preference for that area of the woodland. Unexpectedly, the fence with least vegetation around it (Fence 9) had significantly more outward migration suggesting that adult newts chose to move over relatively exposed areas on departure from the pond. Exposed ground would be advantageous in enabling faster movement than in undergrowth, but visibility and therefore predation risk would be greater. 20% of newt metamorphs heading away from the pond were captured at the three fences 60 m from the pond demonstrating that metamorphs can disperse considerable distances after departing from the pond. There were significantly more metamorphs dispersing in Transect A suggesting that metamorphs may migrate to different areas of surrounding habitat than adults which were mainly captured in Transect C. Transect A has greater vegetation cover than C so may be preferred by metamorphs whose small body size would make them more at risk of desiccation. The woodland pitfall captures provided evidence for directional movement by both *Lissotriton* adults and metamorphs so the fourth hypothesis was therefore accepted. Malmgren (2002) found that smooth metamorphs showed non-random multi-modal dispersal from a pond, while adult smooth newts had uni-modal dispersal, and there was significantly more migration of both species towards a forest habitat.

Furthermore, Rothermel (2004) found that juvenile spotted salamanders *Ambystoma maculatum* and American toads *Bufo americanus* migrated towards the nearest forested habitat in only one of 18 artificial pools. It was suggested that juveniles did not have an innate orientation system that improved their chances of find suitable terrestrial habitat after leaving ponds.

The low recapture rate identified by the photographic matching revealed very limited information about the male smooth newts. Individual changes in mass after the aquatic period and between years were identified for six male smooth newts. Information on changes in newt body mass has come from individual marking recognition in other studies (Andreone and Giacoma, 1989). Two of the three individual male smooth aquatic periods were three weeks longer than the median, demonstrating it can not be presumed that newts that arrive early also leave the pond early. The low male smooth newt recapture rate possibly indicated a large number of newts migrating via the water inlet, bypassing the drift fence, high annual mortality or little site fidelity. The low number of male smooth individual captured on departure from Llysdinam Pond suggests that high mortality was a strong possibility. Adult smooth newts often suffer a 50% annual mortality and few live to be older than six or seven years (Beebee and Griffiths, 2000). In contrast, great crested newts have a longer life span of around seven years (Beebee and Griffiths, 2000), and a female great crested returned six times to Llysdinam Pond in the 1980s recognised by photocopies taken on each visit (F.M. Slater, pers. comm.). Such longevity probably makes great crested newts a more suitable species for the study of repeatability in arrival dates.

It was possible there were more recaptures of male smooth newts than identified. Although the use of a Petri dish for recording belly patterns was relatively successful and has been used in at least one other study on salamanders (Nijhuis and Kaplan, 1998), the image quality was not consistent between the images over two years with some dark or out of focus images. Image quality has been identified as a problem in other image matching research (Bradfield, 2004), and in this study mainly varied with lighting conditions and the use of a lower quality macro camera for some of the study. Newts have flexible bodies and their shape and size can vary due to growth, feeding and hydration which may affect the appearance of the newt in successive photographs. Problems relating to shape and positioning of salamanders, and illumination of images were identified in work on the marbled salamander *Ambystoma opacum* (Gamble et al., 2008). Gamble et al. used a computer assisted method for matching photographs and had to factor out 'nuisance'

variables that would make the computer program less effective in identifying matches. After rescaling images, the computer assisted method successfully matched 447 images from 1008, these were 366 individuals captured between one-five times. Most recaptures were captured twice (345 specimens) during immigration and emigration.

Some male smooth belly markings altered between capture dates with a reduction in colouration or an enlargement of spot size. Despite the difference in orange colouration between male smooth newts, this was not used as a category since colouration altered through the breeding season. It has been suggested that colouration for identification should only be used as a secondary feature due to uncertainty of its stability over time (Bradfield, 2004). Assigning animals to subgroups for identification reduced the number of previous captures that an unidentified capture needed to be compared with. A problem with subgroups was that changes in markings over time may have caused images of the same individual to be assigned to different subgroups. This drawback occurred in the study by Bradfield (2004) when a discontinuous marking later became continuous in an Archey's frog. Such errors could lead to an increase in population estimates, which is particularly undesirable when dealing with an endangered species (Bradfield, 2004). Although some great crested newt belly patterns altered slightly between recaptures, the patterns were stabilised once the newt reached maturity as found by Arntzen and Teunis (1993).

Using subgroups has assisted photographic identification in other studies. Assignment of individual Archey's frog photographs to subgroups was based on the continuous and discontinuous nature of black markings and spots numbers on the anuran's dorsal surface, and made photo-matching 99.2% successful (Bradfield, 2004). Gill (1978) used the fact that the red-spotted newt *Notophthalmus viridescens* has a frequency of 0 to >25 spots on each side of its dorsal surface, thus a pair of numbers was used representing the left and right side. Gill (1978) found that old adults had significantly higher survival than first year adults and also that males in both age categories survived better than females, which was consistent with departure rates at Llysdinam Pond (Chapter 2). Mean life expectancy for male *N. viridescens* was 1.89 breeding seasons, and 1.31 for females, and 8500 individuals recorded at seven ponds over four years returned to the same pond annually.

Newts can sometimes be transient between populations, with juveniles and adults moving between ponds within a commutable distance which could explain the low recaptures in male smooth newts. Over 30% of alpine newts *M. alpestris* were found to be transient in

both a 100 year old established population and recently created ponds (Perret et al., 2003). At Llysdinam there were two ponds within migration distance, Buftons Pond 0.5 km away and Nina's Pond 100 m from the main study pond. Buftons had a low newt population as shown by torching surveys and egg searches conducted there during NARRS (National Amphibian and Reptile Recording Scheme) related work, while Nina's pond was small (approximately 5 x 8 m) so would not support a large breeding population. Some newts may remain within the confines of the drift fence in some years, reducing recapture rates. Toe-clipping of newts at Llysdinam in 1984, suggested that 12-30% of the actual breeding population of both species were captured at Llysdinam Pond drift fence (Griffiths et al., 1986). Since Griffiths never observed successful climbing of the fence it was suggested that many newts remained within 10 m of the pond. There was probably a greater trespass in the 1980s since the current 'newt guard' fence has less folds to assist climbing. During torching surveys in 2006 (Chapter 4), some newts (particularly male smooth newts) were observed climbing the fence but were not seen reaching the top.

Although a large range in the effectiveness of drift fences for the capture of great crested newts has been found (Arntzen et al., 1995; Ortmann et al., 2006), successful monitoring of their belly patterns has been used in previous studies to identify captures. Arntzen and Tenuis (1993) observed higher recapture rates than in this study, and for the three years 1979-1982, 25-35% of males and 23-45% of females returned on subsequent years. Hachtel et al. (2005) marked 101 female and 69 male great crested newts marked in 2001 and 2002. In 2001-2002 there were return rates of 25% for males and 18% for females returned and from 2002-2003 18% of males and 6% of females returned. For consecutive paired years from 2005-2008 at Llysdinam, male great crested return rates varied between 8-27% and female return rates varied between 14-83%. In the study by Hachtel et al. (2005) only 6% of males and 1% of females were captured in all three years which was similar to Llysdinam rates, but much lower than Arntzen and Tenuis (1993). Since the population of great crested newts at Llysdinam has increased with the largest number of individuals recorded in 2008, photographic records could be used to further investigate repeatability in future years.

Only a small number of newts were captured in the woodland pitfalls and the recapture rate of great crested and male smooth individuals was low, but there were indications of repeatability in captures, the use of a similar migration route for one female great crested newt and dispersal of adults and metamorphs into different areas of the woodland.

Different locations for woodland fences may have revealed greater information about newt migrations and movements, particularly before the main breeding migration. Photographic identification of naturally marked animals can be a 'powerful and non-intrusive technique for obtaining information on behaviour, population size and life history parameters in wild populations' (Kelly, 2001). Ventral markings were useful for great crested newt identification at Llysdinam Pond, but they were of limited use for gaining information on male smooth movements and aquatic period. Although this could be due to low return rates of male smooth newts, and not an intrinsic failure in the technique. Natural salamander markings have, however, been useful in mark recapture studies to identify critical habitat areas (Gamble et al., 2008), and could be of importance in the study of amphibian populations especially with the current concerns of amphibian declines.

Chapter 4: The breeding phenology of the three British newt species in Llysdinam Pond

4.1 SUMMARY

Lissotriton newts have advanced their migration dates to Llysdinam Pond, but it was not known if earlier arrival dates were reflected in earlier egg-laying. Surveys of egg-laying in Llysdinam Pond were conducted in 2006 and 2007, and were more successful for assessing breeding phenology than torchlight surveys for newt courtship behaviour conducted in 2006.

Lissotriton egg-laying phenology showed high variability between the two consecutive years of the study, with eggs detected 10 weeks earlier in 2007 (Week 2) compared with 2006 (Week 12), and over four times as many eggs were found in 2007 despite under half the number of *Lissotriton* newts arriving to breed. Great crested egg-laying dates and egg numbers showed less variability, but eggs were found four weeks earlier in 2007 than 2006, and oviposition continued for five weeks longer than in 2006. Factors contributing to the timing of egg-laying and number of eggs found probably included number of newt arrivals, vegetation availability, predation rates and temperature.

Molecular analysis of visually indistinguishable *Lissotriton* eggs in 2007 indicated that palmate newts have a protracted egg-laying season from January to June. In contrast, smooth newt eggs were found from January with a peak in April, but not afterwards. Palmate newts, which tend to be found more commonly in upland habitats than smooth and great crested newts, may have developed a longer period of egg-laying as a reproductive strategy to ensure survival of at least some offspring if unfavourable conditions occur during the breeding season. In contrast, since smooth newts were at the edge of their range at Llysdinam, which may have led to them laying fewer eggs, having a shorter oviposition period and investing time egg-laying in the middle of the breeding season. Female newts were opportunistic but selective in plant species used for oviposition with *Lissotriton* newts showing preference for *Callitriche stagnalis*, *Glyceria fluitans* and *Myosotis scorpioides,* and great crested newts laying the majority of eggs on *M. scorpioides*.

4.2 INTRODUCTION

Amphibian courtship and spawning behaviour can be used as a signal of phenological change. When the earliest dates of calling frogs were compared between the periods 1900-1912 and 1990-1999 in New York, it was found that four species were calling 10-13 days earlier, two were unchanged and none were calling later. It was suggested that climatic change had resulted in earlier breeding (Gibbs and Breisch, 2001). Although there has been research on anuran spawning phenology in ponds (Beattie, 1985; Tryjanowski et al., 2003), little research been conducted on the phenology of courtship and oviposition behaviour by European newts. Amphibians with protracted breeding seasons such as newts and the natterjack toad are more difficult to survey (Banks and Beebee, 1986). Although no research has taken place on the courtship phenology of European newts, amongst much laboratory work on courtship behaviour there has been research on the influence of temperature on alpine newt courtship (Denoël et al., 2005b). Similarly, oviposition phenology has been studied very little (Bell and Lawton, 1975), although selectivity of plants for oviposition has been studied in ponds (Miaud, 1995) and in the laboratory (Norris and Hosie, 2005b).

Only after entering the water do newts fully develop their secondary sexual characteristics (Griffiths and Mylotte, 1988). Early arrival can provide more time for the development of a dorsal crest by great crested and smooth newts, and webbed feet and a caudal filament by palmate newts. Newts show a crepuscular daily activity rhythm with peaks in activity around dawn and dusk, and higher activity levels at night than in daytime. Although palmate newts prefer well vegetated areas of water they move into open water at dusk (Beebee and Griffiths, 2000). Activity is flexible, but dawn and dusk peaks correspond with courtship while night time activity focused on feeding (Griffiths, 1985). *Lissotriton* newts do not defend territories during courtship whereas *Triturus* newts including the great crested establish transient territories in the pond, usually one evening at a time. Great crested territories are defended against other males, and females visit males at their territories (Zuiderwijk and Sparreboom, 1986). There are only subtle differences in the courtship behaviour of smooth and palmate newts (Halliday, 1977a) which can occasionally lead to interbreeding. Two adult male hybrid palmate-smooth newts have been found at Llysdinam Pond in April 1984 (Griffiths et al., 1987) and in March 2005 (pers. obs, Appendix IV).

Activities in the breeding season use a lot of energy and leave newts at an increased risk of predation which could account for the high mortality rates which occur during this time (Baker, 1992; Harrison et al., 1983). Newts obtain oxygen through the skin and lungs, and especially during courtship have to swim to the water surface periodically to breathe. When oxygen is abundant, those parts of the courtship that are under the male's control are much longer in duration than under conditions of oxygen depletion (Halliday, 1977b). The eggs of palmate and smooth newts are morphologically indistinguishable, both being buff coloured, 1.5-1.75 mm in diameter and up to 3 mm including the jelly coat. Great crested newt eggs are visibly very different, being cream-white, 2-2.75 mm in diameter and up to 5 mm including the jelly coat (Beebee and Griffiths, 2000; Green, 2001b). The protective jelly around a newt egg consists of several layers of transparent capsules. Soon after deposition the capsules swell by absorbing water. Gallien and Bidaud (1959) found that newt eggs undergo several developmental stages from segmentation (Stage 0-7) to gastrulation (Stage 8-13), neurulation (Stage 14-21) and then caudal budding (Stage 22, to before hatching. Oxygen can pass through the egg capsule, and the external gills may be used for respiration even before the embryo hatches (Griffiths, 1995). As the embryo develops it elongates, and it is possible to distinguish a rudimentary head and tail (Gallien and Bidaud, 1959).

Female *Lissotriton* and *Triturus* newts each lay between 200 and 300 eggs during the breeding season (Beebee and Griffiths, 2000). In common with many urodele amphibians, European newts show parental care of eggs (Potter and Hosie, 1998). After mating, females deposit and wrap their eggs individually in the submerged leaves of aquatic macrophytes. The wrapping of eggs laid over the season is a large parental investment (Norris and Hosie, 2005b). Female newts use their hind legs to wrap the eggs and an adhesive substance is used to stick the leaf together to enclose the egg. Newts may fold the leaf completely in half over the egg or lay many eggs on the same leaf, continually folding the leaf to produce a visible concertina effect on vegetation (Beebee and Griffiths, 2000). Wrapping eggs in vegetation may aid oxygenation of eggs (Wimpenny, 1951), prevent physical damage, reduce predation (Miaud, 1993) or protect from UV radiation (Marco et al., 2001). Wrapping will minimise desiccation if the plant substrate is exposed to air by a drop in water level for a short period of time (Green, 2001b). Females newts denied suitable oviposition sites, may lay several eggs, joined together as a string (Bell and Lawton, 1975).

Some species were more efficient at wrapping than others, palmate newts may leave up to 25% of eggs unwrapped (Miaud, 1993). Even wrapped eggs suffer high mortality with only about 12% of palmate eggs surviving to hatch (Miaud, 1993), but eggs laid nearer the middle of the breeding season usually survive better (Bell and Lawton, 1975). Caddisfly larvae, large snails such as *Planorbis corneus* and frog tadpoles are oophagous on newt eggs (Bell and Lawton, 1975; Green, 2001b). Adult great diving beetles *Dyticus marginalis* were found capable of taking wrapped and unwrapped eggs, while wrapping reduced oophagy by most predators (Miaud, 1993). Newts appear to select the vegetation on which they lay their eggs by sniffing potential leaves before selecting whether or not it is a suitable site for egg deposition (Norris and Hosie, 2005b). To facilitate egg-wrapping, newts prefer leaves which are flexible and wide enough to fully envelop eggs (Marco et al., 2001). The vegetation on which newts lay eggs may also affect the development stage at which the eggs hatch (Langdon et al., 2005). After hatching the larva is immobile and clings to the plant substrate subsisting on the remains of egg yolk until later morphological changes enable a full autonomous larval life (Bell and Lawton, 1975).

Time until hatching varies with environmental factors such as temperature and water quality, and can be from one week up to over a month (Griffiths and de Wijer, 1994). Great crested eggs take up to three weeks to hatch (Green, 2001b), and half of all great crested newt embryos abort at the tail-bud stage of development due to a chromosomal abnormality (Beebee and Griffiths, 2000). This developmental anomaly is found only in the marbled newt (*T. marmoratus*) and the four European crested newt species (Sessions et al., 1988).

4.2.1 Aims and hypotheses

Climate change has led to milder winters in Britain and this has enabled newts to migrate to ponds gradually over a longer period (Beebee and Griffiths, 2000). Although Chadwick et al. (2006) found *Lissotriton* newts, particularly male palmate newts, were arriving earlier at Llysdinam, it was not known whether arrival dates were reflected in breeding dates and investigation of courtship and oviposition phenology was suggested. There was not a large long term data set for great crested newts at Llysdinam since they were inadvertently introduced in the 1980s and only recently have annual captures reached over 20 individuals. Other research has found early arrival dates by newts but spawning phenology was only analysed for anurans (Beebee, 1995).

Egg-laying phenology has not been investigated in a pond where the three British newt species co-exist. This study aimed to investigate the breeding phenology of *Lissotriton* and great crested newts, and whether there is a delay between arrival and breeding activity (courtship and egg-laying). It was hypothesised that (i) there is a significant positive association between courtship behaviour and temperature, and (ii) there is a significant positive association between temperature and egg-laying activity.

Previously, molecular techniques have only been used to distinguish *Lissotriton* larvae (de Wijer, 1990), here molecular analysis of eggs was used to differentiate between the phenology of palmate and smooth newts, and it was predicted that (ii) earlier arriving species (palmate newts) begin egg-laying before smooth newts. The majority of newt selectivity for egg-laying substrates has been conducted in the laboratory (Norris and Hosie, 2005b). Therefore during egg searches to detect phenology of newts, a record of plant species used as egg-laying substrates were recorded and it was hypothesised that (iv) *Lissotriton* and great crested newts show selectivity for egg-laying substrates.

The courtship and egg-laying surveys conducted at Llysdinam Pond were in conjunction with the outdoor tank study (Chapter 5) which enabled investigation of the consequences of time of arrival on breeding phenology. The pond survey enabled a comparison in the wild.

4.3 METHODS

Surveys were conducted of newt courtship behaviour (2006) and egg-laying phenology (2005 – 2007). These were carried out in conjunction with surveys of newts in outdoor breeding tanks (Chapter 5). In 2007 a final year zoology student (Annabelle Philips) chose to undertake a Professional Training Year project on egg-laying phenology in conjunction with this research. Her project involved egg surveys in Llysdinam Pond and collection of *Lissotriton* eggs for molecular analysis.

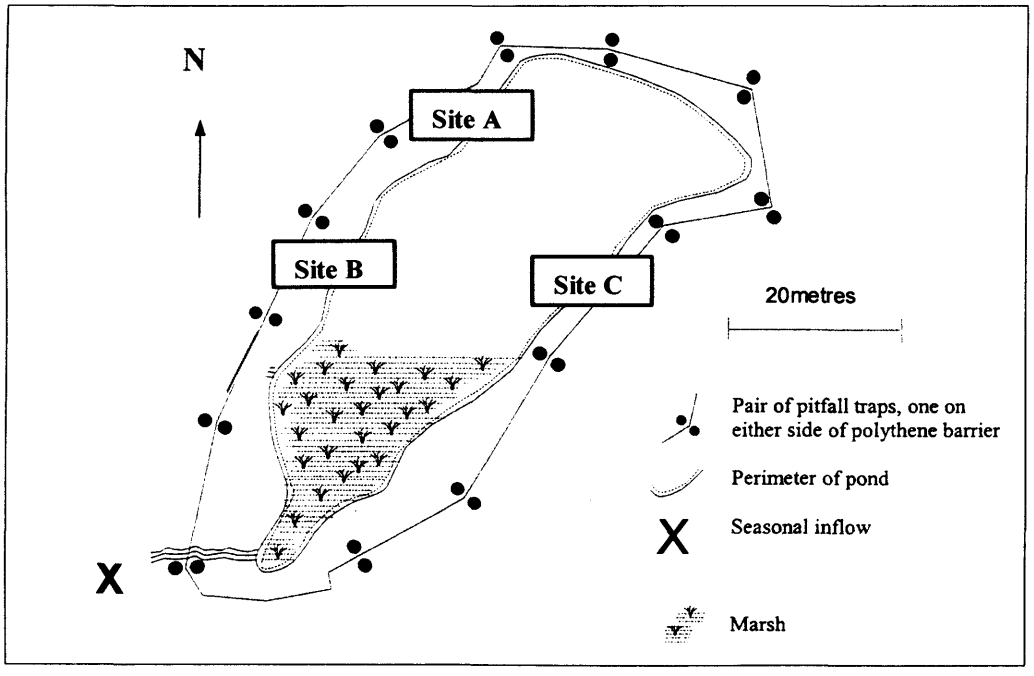
4.3.1 Surveying for newt activity and courtship

Courtship behaviour in the pond was surveyed on three evenings per week for 18 weeks from 19/1/2006 until 25/5/2006. Two people carried out the survey each time for safety reasons. Torchlight visual encounter surveys (VES) were used which entailed a systematic search for a prescribed period of time and was previously carried out by Griffiths (1984; 1985). Torches of 500 000 candlepower were used (Cluson Smartlite SM, 610) which

meets the recommendations for amphibian torch surveys by English Nature (2001). The minimum recommended was 50 000 candle power, and although 1000 000 candle power can increase detectability of newts, it also increases disturbance so was deemed unsuitable for surveys of courtship behaviour. Surveys were carried out one hour after sunset (taken from website http://www.timeanddate.com).

Three locations around the edge of Llysdinam Pond (Site A, B and C) were selected as observation points from which to monitor newt courtship in the pond. Llysdinam Pond was partially surrounded by trees, so these three sites were selected due to their good accessibility to the pond edge (Figure 4.1). A pair of surveyors conducted torchlight surveys at each of the three locations. Both surveyors scanned the water by torchlight at each site for a maximum of three minutes each, moving together in sequence from Site A-C. One of the surveyors also recorded any sightings. After this time the sighting of newt activity was unlikely due to the torchlight disruption. On nights when high numbers of newts were seen, surveyors left each site once it was likely that newts would be mistakenly re-counted. Recordings were made of the number of newts sighted within the water. The species and sex were identified where possible. Specific courtship interactions, for example newts following one another or tail fanning were recorded.

Figure 4.1 Llysdinam Pond showing courtship monitoring sites
Newt courtship behaviour and activity in the pond were recorded on three evenings per week from 19/1/2006-25/5/2006. Sites A, B and C were used as observation points from which to monitor the pond. Two surveyors conducted torchlight surveys from of the three sites. Both surveyors scanned the water by torchlight at each site for three minutes, and one of the surveyors also recorded any newt sightings. Diagram adapted from Harrison et al. (1983) and Chadwick (2003).



4.3.2 Egg-laying surveys

4.3.2.1 2005 egg survey: Egg-laying mops

In 2005 'egg-laying mops' resembling vegetation, were made from polythene strips to provide an artificial substrate for oviposition within the pond. Similar strips have been used in previous newt research (Arntzen and Hedlund, 1990; Miaud, 1993; 1994). Use of egg-laying mops was suggested as a means for great crested surveying by English Nature (2001). Green, black and transparent mops were made from double layered pieces of polythene approximately 20 cm wide by 22 cm length. Green and black egg-laying mops were made from refuse bags produced by the same manufacturer for consistency of colour and texture. Each transparent 'egg-laying mop' was made from one transparent bag (20 x 28 cm from Polybags Ltd). Each piece of polythene was cut into approximately 8 mm strips with a scalpel, and held together with duct tape at one end to make an egg-laying mop. A small stone was taped into the duct tape to weight it to the substrate, and string was used to tether the mop to a bamboo cane to assist relocation of each mop through the season.

20 egg-laying mops of each colour were positioned within the pond, three mops per bamboo cane. No eggs were found laid on the mops during the entire breeding season of 2005, although newts occasionally were found hidden within the black mops. Therefore an alternative method to study egg-laying phenology within the pond was used for the latter years of the study. In the outdoor tank study (Chapter 5) where plants were not available, female newts used egg-laying mops as a substrate on which to lay their eggs.

4.3.2.2 Egg survey of detritus

After failing to find newts eggs using egg-laying mops in 2005, two new methods were used in 2006. Egg surveys were conducted on vegetation growing within the pond (Section 4.3.2.3) and in detritus collected from the pond benthos.

Detritus was collected into tanks once every two weeks from 18.1.2006 to 19.4.2006. 20 detritus sampling sites (Sites 1-20) were dispersed evenly along the pond perimeter marked by bamboo canes. The marshy area was not used since the benthos there was composed of a mat of living vegetation rather than decaying plant material. From each detritus sampling session the decaying material was combined into five tanks with water (e.g. Sites 1-4 into one tank). The tanks of detritus were searched through for newt eggs in the laboratory. They were then left in the laboratory, and searched through again for any larval hatchlings

after three and six weeks. No eggs or newt larvae were found in the pond detritus. From Week 15 of the year, eggs began to be found on aquatic vegetation within the pond so detritus surveys were stopped and were not repeated in 2007.

4.3.2.3 Visual encounter survey for newt eggs on aquatic vegetation

A one man hour visual encounter survey (VES) (Crump and Scott, 1994) for newt eggs laid on aquatic vegetation was completed in the pond twice per week in 2006 and 2007 from January until end of August, when no more eggs were located. A one man hour VES was conducted by two people for 30 minutes each. The whole pond was sampled during the egg search, and each week one searched commenced from Site A and one from Site C (Figure 4.1). Caution was taken by each surveyor to sample different areas of the pond within a survey to avoid multiple encounters of the same individual eggs. The location of each egg was mapped and a note made recording (i) whether the egg was small (palmate or smooth) or large (great crested newt), (ii) the developmental stage, (iii) the plant species that the egg was laid on, and (iv) whether the egg was laid on the leaf surface or wrapped into the vegetation. For each search the total number of eggs of each developmental stage was summed. Newt eggs wrapped in aquatic plants were visible due to the distinctive folding of vegetation. When egg counts were high a third person acted as a scribe to record the data (Figure 4.2). Egg searches were not carried out more frequently than twice a week, due to concerns of disruption to the pond environment.

4.3.2.4 The 2007 breeding season

The results from 2006 led to a more detailed investigation of egg-laying phenology in 2007, with investigation of substrate choice for egg deposition, and the contribution of the two *Lissotriton* species to the two distinct peaks egg-laying periods that were found in 2006. The two peaks of egg-laying in 2006 occurred on Week 15 and Week 21. In 2007 the aim was to find out, using molecular methods, whether the first peak resulted from palmate newts and the second peak from egg-laying by both palmate and smooth newts.

4.3.2.5 VES for newt eggs on specified plant species

In 2007 additional 30 minute searches (two people for 15 minutes) were conducted on specific groups of plant species during the peak egg-laying season (11/01/2007 to 07/06/2007) to investigate egg-laying frequencies on specific plant groups. The five specific vegetation searches took place once every two weeks from January until June.

The searches included the following vegetation types:

1. Starworts, *Callitriche stagnalis* and *Callitriche intermedia*
2. Reed sweet-grass, *Glyceria maxima*
3. Water forget-me-not, *Myosotis scorpioides*
4. Floating sweet-grass, *Glyceria fluitans* and creeping bent *Agrostis stolonifera*
5. All other vegetation types included: water mint (*Mentha aquatica*), broadleaved pondweed (*Potamogeton natans*), soft rush (*Juncus effusus*), bottle sedge (*Carex rostrata*), bog-bean (*Menyanthes trifoliata*), duckweed (*Lemna minor*) and fallen dead tree leaves.

Figure 4.2 Egg surveys being conducted in Llysdinam Pond

In 2006 and 2007 a one man hour visual encounter survey (VES) for *Lissotriton* and great crested newt eggs laid on aquatic vegetation was completed in the pond twice per week (two people for 30 minutes from January until end of August, when no more eggs were found). Caution was taken by each surveyor within a survey to sample different sites in the pond to avoid multiple encounters of the same individual eggs. When egg counts were high a third person acted as a scribe to record the data.

(i) In May 2006 (with scribe) (ii) In February 2007 after snow

4.3.2.6 Quadrat survey of aquatic vegetation

Vegetation surveys within the pond were conducted in the middle of each month. 36 randomly placed quadrats were used to estimate the percentage cover of each plant species within the pond.

4.3.2.7 Temperature monitoring

Brannan minimum-maximum thermometers were placed in the pond at Sites A, B and C and weighted to be at a depth of 20 cm. These provided water temperatures for the evening courtship surveys and were checked twice weekly to compare with the outdoor breeding

tank temperatures (Chapter 5). Mean daily air temperatures (°C) were calculated from the average of the maximum and minimum temperature data collected from a standard meteorological weather station located 50 m south-west of the pond and accessed from the British Atmospheric data (BADC) website (UK Meteorological Office, 2008b). Three day rolling means and weekly means were also calculated.

4.3.3 Egg collection, development and preservation

Between 11/01/2007 and 16/06/2007 newly laid indistinguishable palmate and smooth eggs were collected every two weeks for an egg phenology and DNA analysis project in collaboration with a final year zoology student. The eggs were developed in the laboratory and preserved for identification at a later date using molecular methods.

4.3.3.1 Egg collection

The eggs were collected during egg searches on specified plant species (Section 4.3.2.5). A maximum of six eggs from each vegetation type and up to 25 eggs in total were collected every two weeks. It had been hoped to analyse the eggs collected every two weeks but due to large unforeseen losses of eggs due to infection (as discussed in more detail below) it was eventually only possible to analyse 120 eggs (20 eggs taken from the mid point of each month from January to June).

4.3.3.2 Egg development and preservation

Initially eggs were preserved immediately after collection in 70% ethanol and stored at -18°C. Molecular analysis trials proved unsuccessful possibly due to insufficient levels of DNA within the eggs or the jelly surrounding the egg (Section 4.3.4). Attempts at removing the jelly without disintegrating the eggs was difficult, therefore it was decided to let the eggs develop to a larger size before preservation.

After collection, eggs were placed in tanks of aerated pond water (Figure 4.3) to allow them to develop. Initially pond water was chosen as a suitable medium for simulating natural conditions. The tanks were positioned in an unheated room. When the eggs reached a developmental stage where the larval appearance could be distinguished, approximately Stage 33 to 37 according to Gallien and Bidaud (1959) (Appendix V), they were removed from their jelly, preserved in 70% ethanol, and stored at -18°C. In the early months, lower temperatures meant that the eggs took up to a month to reach a suitable size but later when temperatures had increased they could be preserved in under two weeks.

After two months problems began to occur with infection of eggs within the aerated pond water in the tanks, probably due to the fungus *Saprolegnia*. It may have been introduced in the pond water or on an individual egg, but infection rapidly spread through whole tanks of eggs. Regular cleaning of tanks did not prevent this and infected eggs could not be analysed successfully by molecular methods. To overcome the problem aerated tap water was used, which resulted in less infection but a significant number of eggs were still lost.

In an attempt to prevent or reduce further losses, collected eggs were placed individually in Petri dishes of tap water in an unheated room (Figure 4.4). This considerably reduced the spread of infection between eggs so only individual eggs were lost. Over 25% of the eggs collected were lost due to infection, most of which occurred before replacing the development tank with Petri dishes.

Figure 4.3 An egg development tank

Each development tank was compartmentalised with one egg in each section. The eggs were left attached to the plant substrate on which they were laid. The tank shown contains eggs laid on *Callitriche stagnalis*. (Photograph taken by Annabelle Phillips).

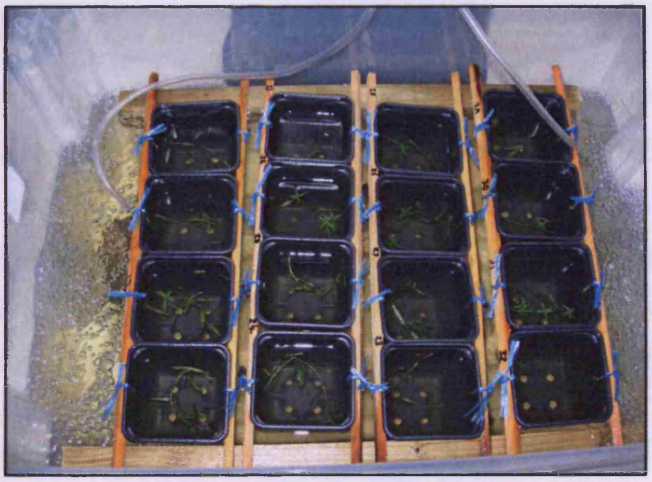
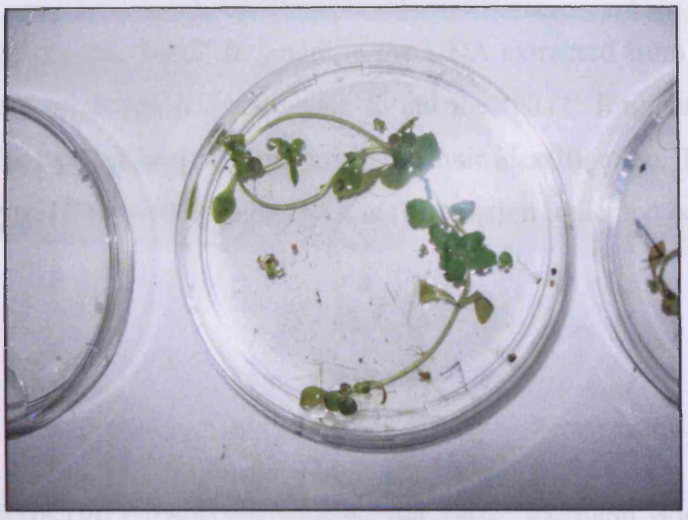


Figure 4.4 *Lissotriton* newt egg developing in a Petri dish

A Petri dish containing tap water in which a newt egg was placed to allow development to an adequate size for molecular analysis. Development of eggs in separate dishes instead of a communal tank, reduced fungal infection and reduced egg losses. The Petri dish contains an egg laid on *Callitriche stagnalis*. Photograph taken by Annabelle Phillips.



4.3.4 Identification of egg species using molecular methods

4.3.4.1 Extraction of newt DNA

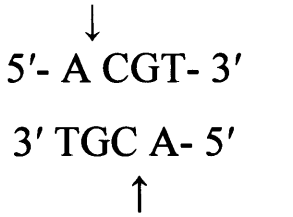
Extraction of DNA from all eggs to be analysed was carried out following the method taken from the DNeasy® Tissue Handbook (QIAGEN®, 2004) REF (Appendix VI). The DNeasy® method resulted in two stock solutions of DNA extract. One stock solution of 200 µl DNA extract was stored in a refrigerator (~ 4°C). The other stock solution of 100 µl DNA extract was stored in a freezer (~ -20°C) and kept as a reserve stock. All PCRs were performed in an Applied Biosystems® GeneAmp PCR System 9700. (See Appendix VII, Trials of molecular analysis of eggs).

4.3.4.2 Amplification of a mitochondrial DNA sequence

A protocol taken from Beebee et al. (1999) for differentiating between palmate and smooth adults was not successful for egg species identification. It was possible that by allowing the eggs to develop to a larger size and completely removing the jelly surrounding the eggs this method could have been used to distinguish between the palmate and smooth newt eggs. Nevertheless, it was felt that a different method should be attempted to successfully and reliably distinguish between the two species (pers. comm. G. Hobbs and A. King). Babik et al. (2005) amplified a c. 1016-bp fragment of the mitochondrial ND2 gene (which shall be referred to at 'ND2') in both palmate and smooth newts using the L3870 and H5018 primers. 10 µl and 30 µl reaction volumes were tried using DNA extracted from

known palmate and smooth newt adults. The individuals had died from a disease in 2005 and had been preserved at -18°C. CEFAS (Centre for Environment, Fisheries and Aquaculture Science) conducted tests and confirmed the disease was not a ranavirus.

By amplification of the 'ND2' fragment in the DNA extracted from the newt eggs, it was then planned to use a restriction enzyme to cut the 'ND2' fragment into different sized fragments in the two different species to enable their identification. The restriction enzyme chosen was HypCH4IV which splits DNA at the location indicated below by the arrows.



By using the HypCH4IV restriction enzyme the 'ND2' fragment would be split into a 587-bp and a 423-bp fragment in palmate newts, and a 260-bp and a 756-bp fragment in smooth newts. The method obtains two relatively equally sized fragments in palmate newts and two different sized fragments in smooth newts. Identification of each species should be possible by visualizing the different sized fragments on an agarose gel following electrophoresis.

The 'ND2' fragment was successfully amplified in smooth newt samples. Many unsuccessful attempts to amplify the 'ND2' fragment in palmate newt samples took place and, it was decided that it could not be achieved with the tissue samples being used. Babik et al. (2005) had successfully amplified this fragment in both palmate and smooth newts, but it is not uncommon for cross priming to be difficult in amphibians due to their large genome size (Garner, 2002). It was possible that the haplotype of the newts being analysed in this study had an effect on the ability to amplify this particular fragment (G. Hobbs, pers. comm.).

It would have been ideal to find a primer that would amplify a fragment in both palmate and smooth newts, but time and funding constraints prevented this. It was decided it would be acceptable to use the fact that the fragment could not be amplified in palmate newt eggs as a negative result, thus distinguishing from the positive amplification in smooth newt eggs (pers. comm. M. Bruford). When using a negative result it was necessary to always run a positive and a negative control. To ensure the 'ND2' fragment was amplified a

positive known smooth control was run along with a negative palmate newt control in addition to the H_2O control.

Table 4.1 Reagent concentration used for the PCR
The stock concentrations, reaction concentrations and reaction volumes of the reagents used for the PCR to amplify the 'ND2' fragment, using the primers L3870 and H5018, giving a total reaction volume of 10 µl.

Materials	Stock Concentration	Reaction Concentration	10 µl Reaction Volume
PCR buffer (20 mM Tris-HCL pH 8.3, 50 mM KCL)	10 x	1 x	1.0 µl
$MgCl_2$	50 mM	2.5 mM	0.5 µl
Taq DNA polymerase	5 units/µl	0.5 units	0.1 µl
dNTPs	2.5 mM	0.2 mM	0.8 µl
Primer L3870	10.0 mM	1.0 mM	1.0 µl
Primer H5018	10.0 mM	1.0 mM	1.0 µl
dH_2O	-----	-----	4.6 µl
DNA extract	------	-----	1.0 µl

20 eggs collected in the middle of January, February, March, April, May and June 2007 were analysed (120 eggs in total). DNA extracted from these eggs was assayed in 10 µl PCRs (Table 4.1 for concentrations and volumes of the reagents). The PCR cycling scheme was taken from Babik et al. (2005). Initial denaturation of 2 minutes at 94°C, 45 seconds at 56°C and 2 minutes at 72°C was followed by 35 annealing cycles, each with 30 seconds at 94°C, 45 seconds at 56°C, and 1.5 minutes at 72°C, followed by a final extension cycle at 72°C for 3 minutes. Following the PCR 7.8 µl of the PCR product, 1 µl dH_2O, 1 µl PCR buffer (10 x) and 0.2 µl of the restriction enzyme HypCH4IV were heated to 37°C for 1 hour. The 10 µl PCR product was mixed with 3 µl loading buffer and electrophoresis carried out through a 1.2% agarose gel containing 5.0 µl Gel Red. Gel electrophoresis enables separation of molecules through a gel by application of an electric field. Gel electrophoresis showed the 'ND2' fragment amplified in the smooth newt adult control and smooth newt eggs, but not in the palmate newt adult control or the palmate eggs (Section 4.4.3.3, Figure 4.13). Therefore DNA extracted from newt eggs that gave positive results were identified as a smooth newt eggs and DNA samples that led to no amplification of fragments were identified as palmate eggs.

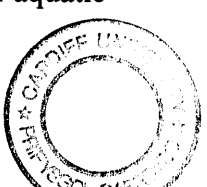
4.3.5 Data analysis

Average air temperatures from November 2005-January 2006 were lower than in November 2006-January 2007. Therefore differences in egg-laying phenology of *Lissotriton* and great crested newts in the breeding season of 2006 and 2007 were compared by Analysis of Covariance (ANCOVA) in Minitab 15. The slopes and intercept of the regression lines of number of eggs found in overall searches in 2006 and 2007 against week (Bowker and Randerson, 2008; Dytham, 2003) were compared. Analysis was conducted for *Lissotriton* eggs (palmate and smooth eggs were combined since they were morphologically indistinguishable) and great crested newt eggs. Week was the covariate as used in other ecological studies (Duffy et al., 2005; Heide-Jørgensen et al., 2007). Weeks 1-22 were analysed because by Week 22 peaks of egg-laying had occurred in both years for both *Lissotriton* and great crested newts. *Lissotriton* egg data were transformed using arcsinh calculation (in Minitab 15) while great crested egg data were log + 1 transformed.

Partial correlation analysis in SPSS assessed the association between mean weekly number of eggs found in overall searches (per one man hour search) for 2006 and 2007, and weekly mean and minimum temperatures. Partial correlation analysis enabled analysis of the association between temperature and egg-laying in Llysdinam Pond with the variable of female newt arrivals (cumulative number of females in the pond) held constant.

To determine whether the palmate and smooth eggs were in frequencies expected from the number of female newt arrivals, χ^2 tests were conducted (Bowker and Randerson, 2008). The number of palmate and smooth eggs identified from the 20 eggs, molecularly analysed from each month were the observed χ^2 values. The expected frequency was calculated from the number of adult female newts of each species entering the pond within a given time frame prior to the egg collection date: (i) the month prior to the egg collection date (ii) the two months prior to the egg collection date, and (iii) all months prior to the collection date.

χ^2 tests were also conducted on data from specific vegetation egg searches in 2007 to investigate whether newts were selective in plant substrate and whether this changed over the breeding season. Where more than 25% of cells contained expected values of fewer than five, some plant species were grouped to meet χ^2 assumptions. Observed values were number of eggs found per half man hour search in the middle of each month. Expected numbers of eggs on each plant were calculated from proportion of cover by each aquatic



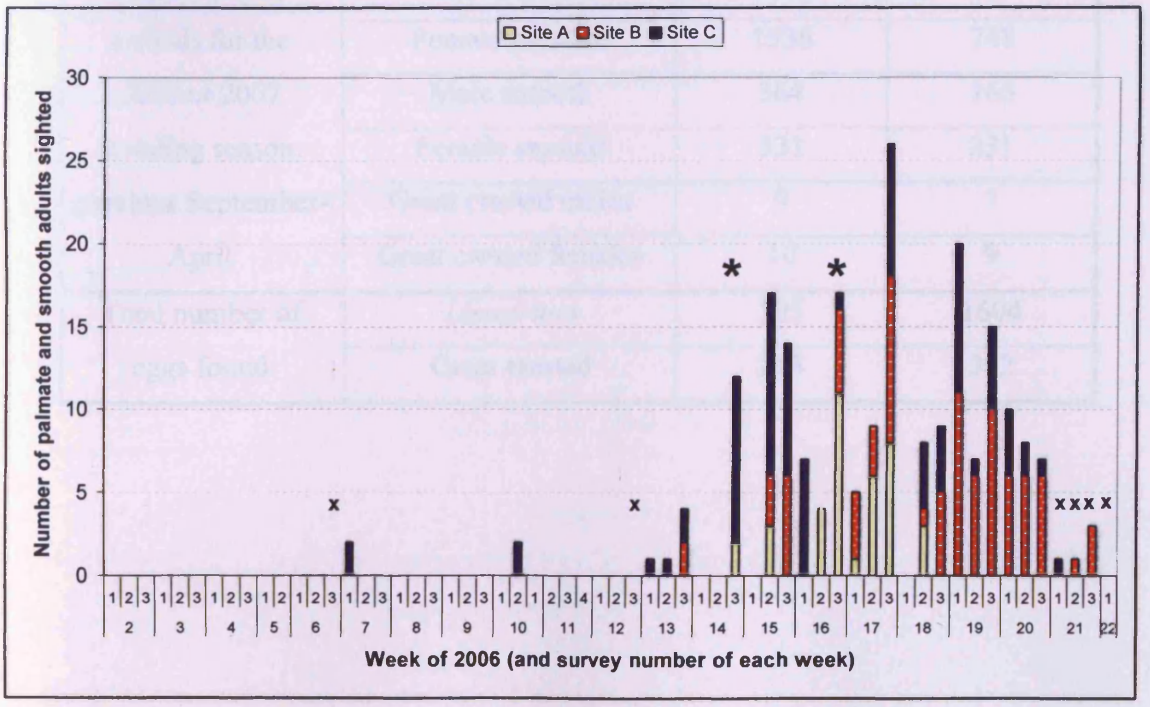
plant species in the pond. Due to the lower numbers of great crested eggs found, the χ^2 test was performed for the entire egg-laying period only (Bowker and Randerson, 2008). All analyses were done in Minitab 15.

4.4 RESULTS

4.4.1 Courtship survey

From September 2005 until Week 3 of 2006, 126 female palmate, seven female smooth, 247 male palmate and 21 smooth male newts migrated to the pond. Despite large numbers of newt arrivals, no newt activity was observed during evening torchlight surveys in the pond until Week 7 (Figure 4.5). Unfortunately specific courtship behaviours (tail fanning and other aspects of the courtship dance) were seen on only two surveys during Weeks 14 and 16. Observed newt activity levels peaked in Week 17. From Week 19 onwards duckweed growing on the pond surface started to obscure visibility (Section 4.4.3.4, Figure 4.17), eventually halting surveys during Week 22. Insufficient results were gained for statistical analysis. Courtship surveys were not pursued in 2007, since egg-laying surveys were much more successful for detecting differences in breeding phenology and activity over the season and between species (Section 4.4.3).

Figure 4.5 Newt activity in Llysdinam Pond on each survey night in 2006
Number of newts seen during torching surveys at Llysdinam Pond from Sites A-C located around the pond perimeter. Each survey week (Weeks 2-22) consisted of three survey nights (Survey 1-3) in 2006 indicated on the x axis. Surveys at each site lasted up to three minutes. Asterisks indicate evenings when courtship displays were observed. Crosses indicate evenings when visibility of pond benthos was impaired at all three sites due to prior rainfall or duckweed growth. Surveys were halted in Week 22 due to the amount of duckweed cover impairing visibility.



4.4.2 Egg survey using egg-laying mops and egg survey of in detritus

No newt eggs were found on the egg-laying mops placed in Llysdinam Pond during the amphibian breeding season of 2005. In 2006 no eggs were found in the pond detritus and no larvae were found in subsequent checks afterwards in the laboratory. From mid-April 2006 it was decided to stop monitoring detritus as an egg-laying substrate because by this time over 30 eggs per man hour egg search had been found on aquatic vegetation.

4.4.3 Results from egg surveys on aquatic vegetation in 2006 and 2007

4.4.3.1 Newt migrations and egg-laying

In 2007 only half the number of female and male *Lissotriton* newts migrated to the pond compared to 2006 but great crested newt numbers remained similar (Figure 4.6, 4.7 and Table 4.2). Surprisingly, the total number of eggs found from Weeks 2-34 was higher in 2007 (1604 eggs) than 2006 (395 eggs) which was not comparable with the arrival numbers. Great crested egg female arrivals and egg numbers were more closely matched in both years.

Table 4.2 Number of newts arrivals at drift fence and number of eggs found in egg searches in 2006 and 2007

The number of newt arrivals for the breeding season of 2006 and 2007 were calculated from the number of newts captured at the Llysdinam Pond drift fence from September of the previous year until April (eight months in total). Numbers of eggs were the total from all one man hour searches in Llysdinam Pond from Weeks 1-34 in 2006 or 2007. Two eggs surveys each lasting for one man-hour duration took place every week from January-August.

		2006	2007
Number of newt arrivals for the 2006 or 2007 breeding season: previous September-April	Male palmate	984	376
	Female palmate	1536	748
	Male smooth	364	165
	Female smooth	531	231
	Great crested males	9	7
	Great crested females	10	9
Total number of eggs found	*Lissotriton*	395	1604
	Great crested	215	362

Figure 4.6 Weekly number of female *Lissotriton* newt arrivals and eggs found

Weekly number of female *Lissotriton* newts that arrived at Llysdinam Pond from end of November to the end of August in 2005-2006 and 2006-2007 are represented by bars on the first y axis. All adult female newt captures were made at Llysdinam Pond drift fence. Mean weekly *Lissotriton* egg numbers found per one man hour search in 2006 and 2007 are represented by lines on the second y axis.

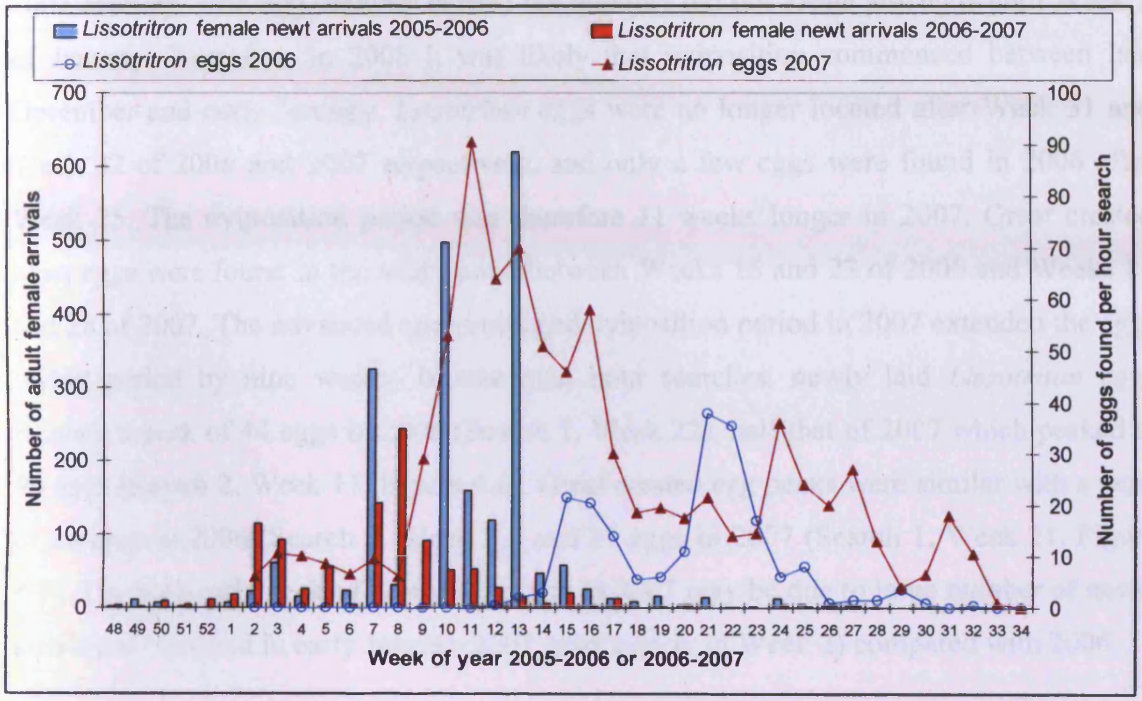
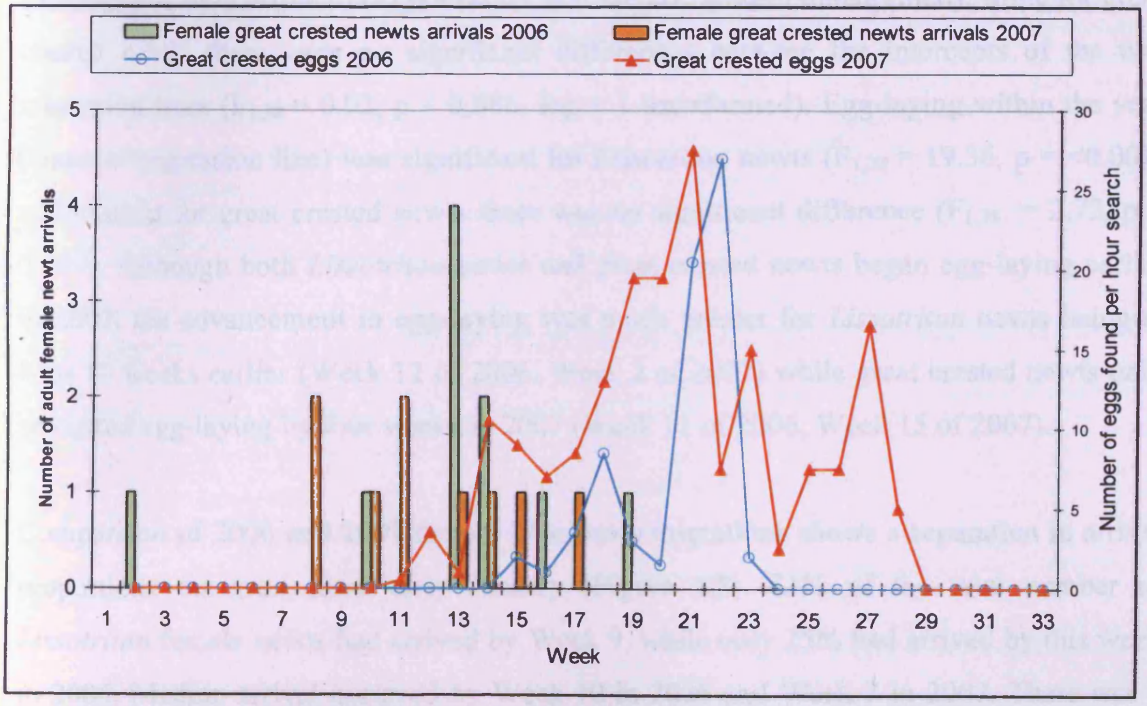


Figure 4.7 Weekly number of female great crested newt arrivals and eggs found

Weekly number of female great crested newts that arrived at Llysdinam Pond from January to the end August in 2006 and 2007 are represented by bars on the first y axis. All adult female newt captures were made at Llysdinam Pond drift fence. Mean weekly great crested newt egg numbers found per one man hour search in 2006 and 2007 are represented by lines on the second y axis.



In 2006 *Lissotriton* eggs were found from Week 12 in the pond. Therefore in 2007 it was thought appropriate to commence egg searches in Week 2, but eggs were found on the first search (Figure 4.6). Egg-laying was therefore at least 10 weeks earlier. From September 2007-January 2008 egg searches carried out monthly did not locate any eggs until Week 2 of January. Therefore in 2008 it was likely that oviposition commenced between late December and early January. *Lissotriton* eggs were no longer located after Week 31 and Week 32 of 2006 and 2007 respectively, and only a few eggs were found in 2006 after Week 25. The oviposition period was therefore 11 weeks longer in 2007. Great crested newt eggs were found in the study pond between Weeks 15 and 23 of 2006 and Weeks 11 and 28 of 2007. The advanced and prolonged oviposition period in 2007 extended the egg-laying period by nine weeks. In one man hour searches, newly laid *Lissotriton* eggs reached a peak of 44 eggs in 2006 (Search 1, Week 22), half that of 2007 which peaked at 94 eggs (Search 2, Week 11, Figure 4.6). Great crested egg peaks were similar with a peak of 28 eggs in 2006 (Search 2, Week 22) and 31 eggs in 2007 (Search 1, Week 21, Figure 4.7). The high early peak of *Lissotriton* eggs in 2007 may be due to large number of newts arriving at the pond in early January 2007 (particularly in Week 2) compared with 2006.

ANCOVA for Weeks 1-22 demonstrated a greater difference in egg-laying phenology between years for *Lissotriton* newts than great crested newts. For *Lissotriton* newts the start of egg-laying (intercept with the x axis) between years of the two regression lines were significantly different ($F_{1,38} = 65.03$, $p = <0.001$, arcsinh transformed), while for great crested newts there were no significant differences between the intercepts of the two regression lines ($F_{1,38} = 0.02$, $p = 0.886$, log + 1 transformed). Egg-laying within the year (slope of regression line) was significant for *Lissotriton* newts ($F_{1,38} = 19.36$, $p = <0.001$) while again for great crested newts there was no significant difference ($F_{1,38} = 2.72$, $p = 0.107$). Although both *Lissotriton* newts and great crested newts began egg-laying earlier in 2007, the advancement in egg-laying was much greater for *Lissotriton* newts being at least 10 weeks earlier (Week 12 of 2006, Week 2 of 2007) while great crested newts only advanced egg-laying by four weeks in 2007 (Week 11 of 2006, Week 15 of 2007).

Comparison of 2006 and 2007 female *Lissotriton* migrations shows a separation in arrival proportions occurred from early January (Figure 4.8). 81% of the total number of *Lissotriton* female newts had arrived by Week 9, while only 25% had arrived by this week in 2006. Median arrival occurred by Week 10 in 2006 and Week 7 in 2007. There was a large migration of 620 females in Week 13 of 2006.

Figure 4.8 Cumulative percentage of *Lissotriton* female newt arrivals

Female *Lissotriton* newt that arrived at Llysdinam Pond from September to the following May of 2005-2006 and 2006-2007 as a cumulative percentage of total female arrivals. The arrival proportions were calculated from Week 35 (September) to Week 20 (May). All adult female newt captures were made at Llysdinam Pond drift fence. Data displayed as a weekly proportion of total newt arrivals within each species and year.

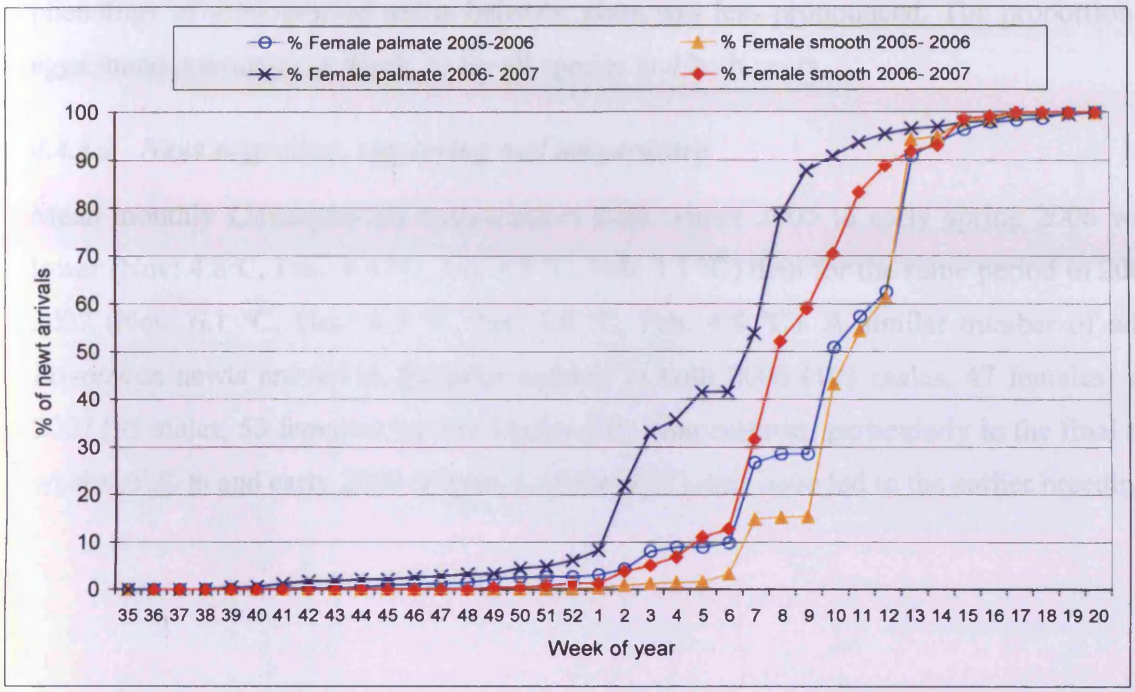
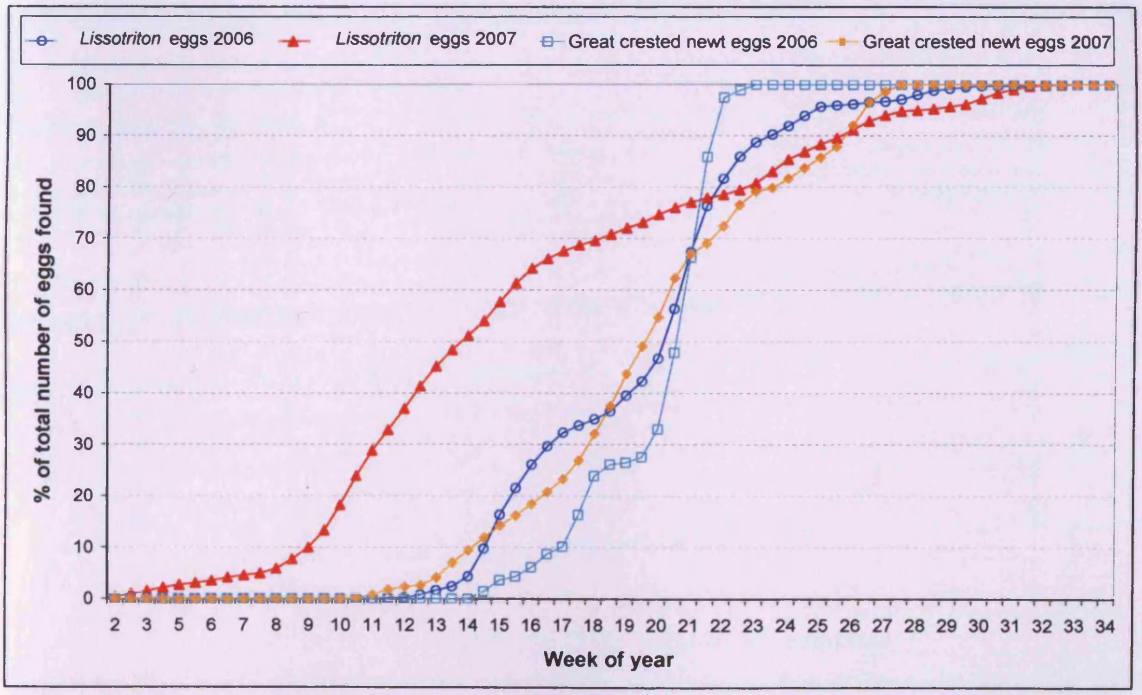


Figure 4.9 Cumulative percentage of newt eggs found over the breeding season

Lissotriton and great crested eggs as a cumulative percentage of egg found from January-August in 2006 and 2007. Egg numbers were totals from one man hour searches at Llysdinam Pond. Data displayed as weekly proportions of total eggs found. *Lissotriton* and great crested data are displayed individually for both years.



The early timing of egg-laying by *Lissotriton* newts in 2007 compared to great crested newts and *Lissotriton* newts in 2006 was apparent, with 50% of eggs found by Week 14 in 2007 compared to Weeks 20-21 in 2006 (Figure 4.9). The difference in egg-laying phenology of great crested newts between years was less pronounced. The proportion of eggs found converges at Week 20 for all species and both years.

4.4.3.2 Newt migration, egg-laying and temperature

Mean monthly Llysdinam air temperatures from winter 2005 to early spring 2006 were lower (Nov: 4.8°C, Dec: 4.3 °C, Jan: 3.9 °C, Feb: 3.1 °C) than for the same period in 2006-2007 (Nov: 6.1 °C, Dec: 4.9 °C, Jan: 5.8 °C, Feb: 4.8 °C). A similar number of adult *Lissotriton* newts arrived in the prior autumn in both 2006 (103 males, 47 females) and 2007 (95 males, 53 females) but the higher daily temperatures, particularly in the final two weeks of 2006 and early 2007 (Figure 4.10 and 4.11) may have led to the earlier breeding.

Figure 4.10 Daily female *Lissotriton* newt arrival numbers and mean air temperature at the start of the newt breeding season

Number of female *Lissotriton* newt arrivals at the start of the breeding season in 2006 and 2007 are represented by bars on the first y axis. All newts were captured on arrival at Llysdinam Pond drift fence. Data displayed from Day 350 (December 2005 or 2006) to Day 50 (February 2006 or 2007). Temperature (°C) is a three day rolling mean air temperature and represented by lines on the second y axis. Mean air temperature was calculated as an average of the daily minimum and daily maximum temperature recorded at the weather station located 50 m south-west of Llysdinam Pond.

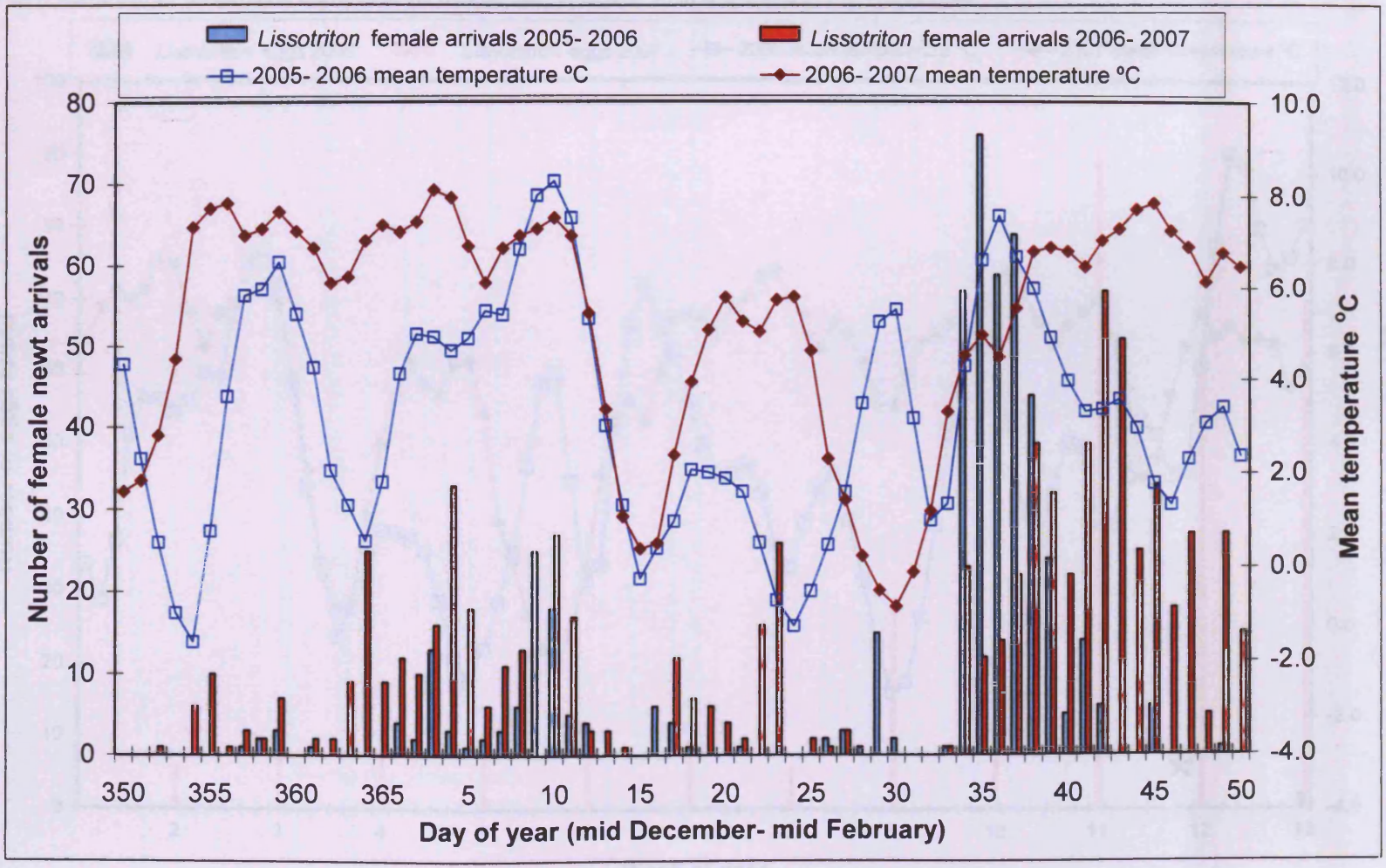


Figure 4.11 Number of *Lissotriton* newt *e*ggs detected in Llysdinam Pond and mean air temperature at the start of the newt breeding season

Number of *Lissotriton* eggs found in Llysdinam Pond compared with temperature at the start of the newt breeding season in 2006 and 2007. Egg numbers were weekly means from one man hour searches at Llysdinam Pond and are represented by bars on the first y axis. Asterisk indicates the week when the first egg was found in 2006. Temperature (°C) is a three day rolling mean of air temperature and represented by a line on the second y axis. Mean air temperature was calculated as an average of the daily minimum and daily maximum air temperature recorded at the weather station located 50 m south-west of Llysdinam Pond.

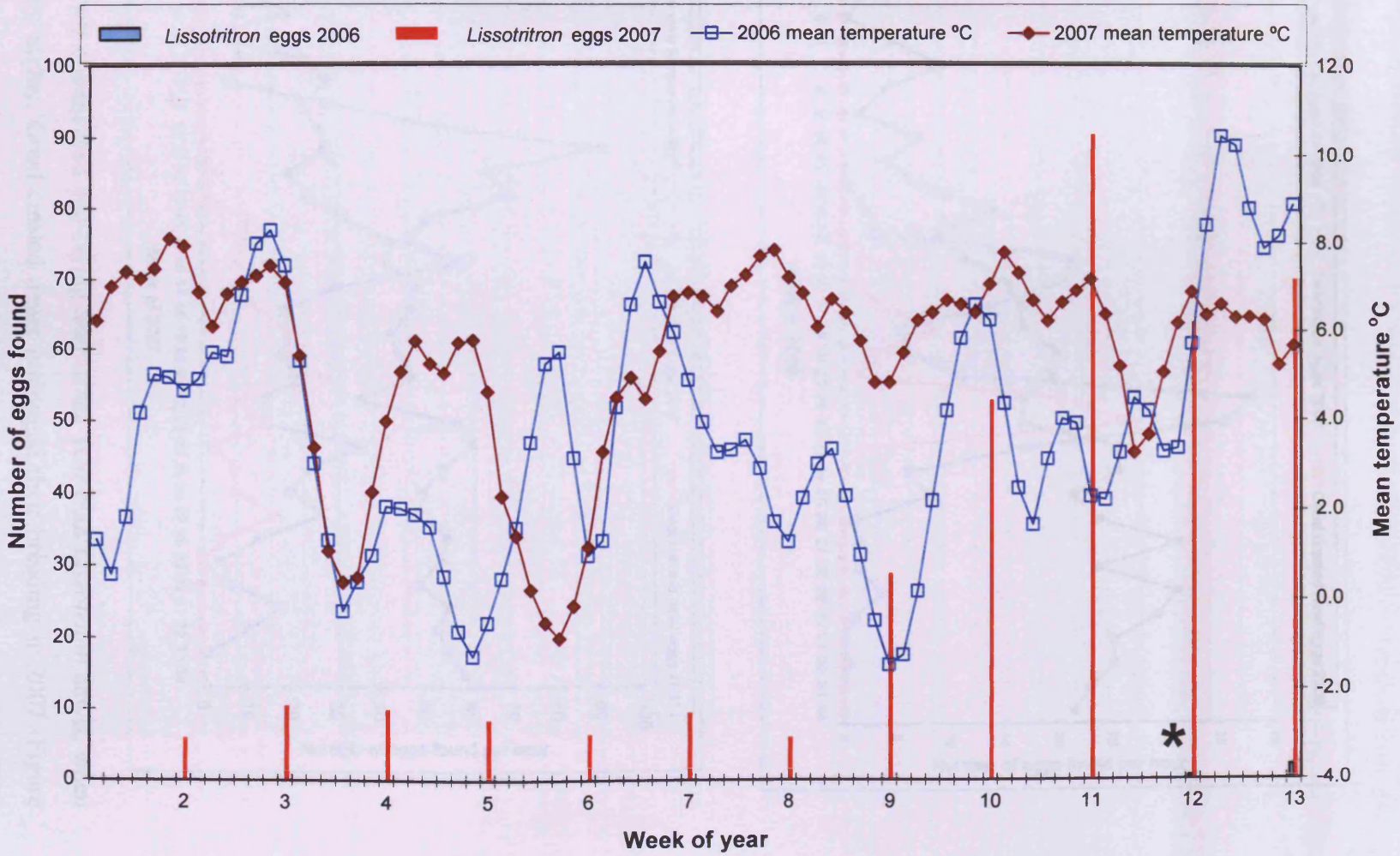
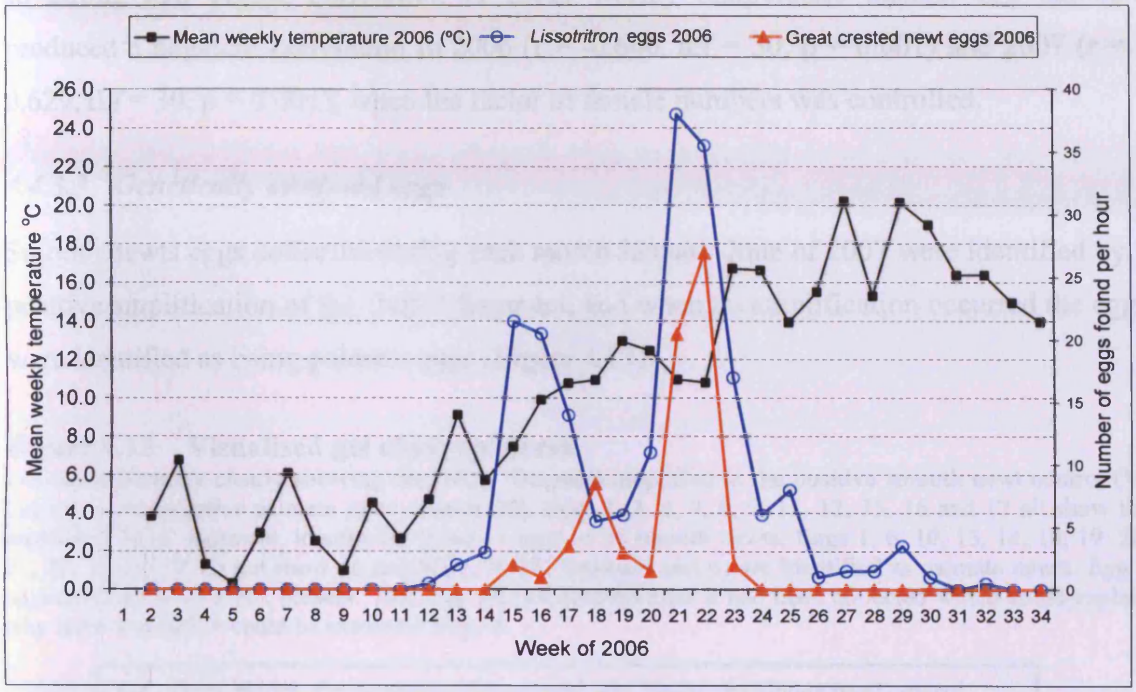


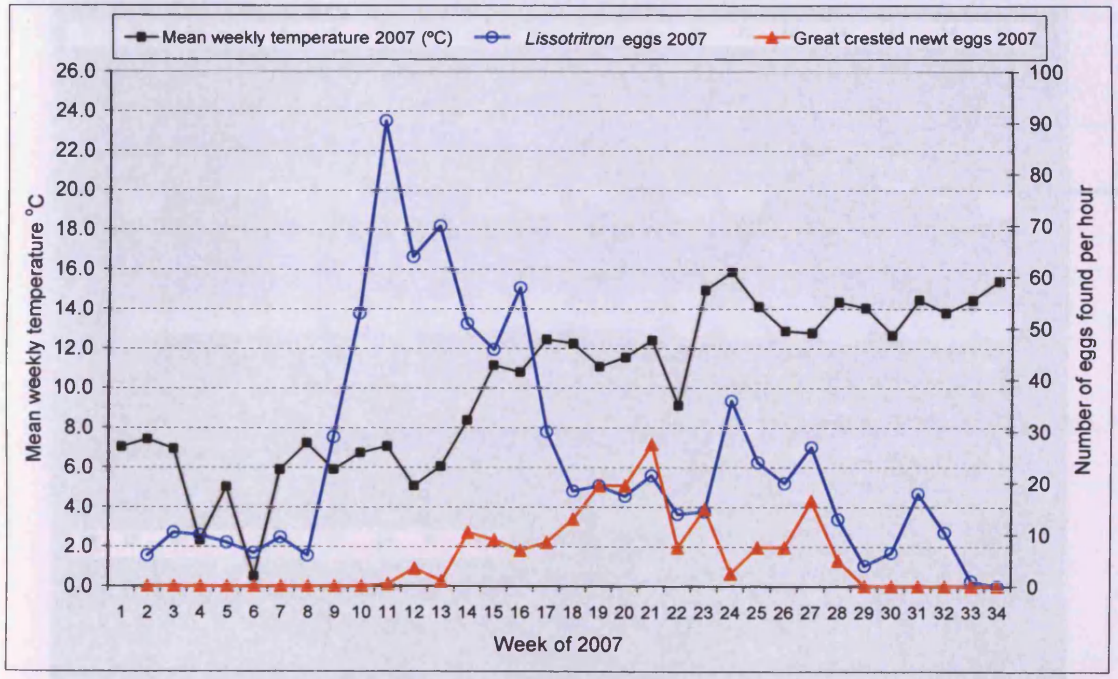
Figure 4.12 Newt egg-laying and mean weekly air temperature

Mean number of *Lissotriton* and great crested newt eggs found per man-hour at Llysdinam Pond in (i) 2006 and (ii) 2007 compared against mean weekly air temperature (°C). Mean weekly temperature was calculated from the data recorded daily at the weather station located 50 m south-west of the pond. Note difference in scale of second y axis between (i) 2006 and (ii) 2007 since more *Lissotriton* eggs were found in 2007.

(i) 2006



(ii) 2007



Great crested newts commenced egg-laying later in the year than *Lissotriton* newts, when temperatures were higher. Great crested newts prolonged their breeding in 2007 (Figure 4.12). The *Lissotriton* and great crested peaks of eggs were more synchronised in 2006

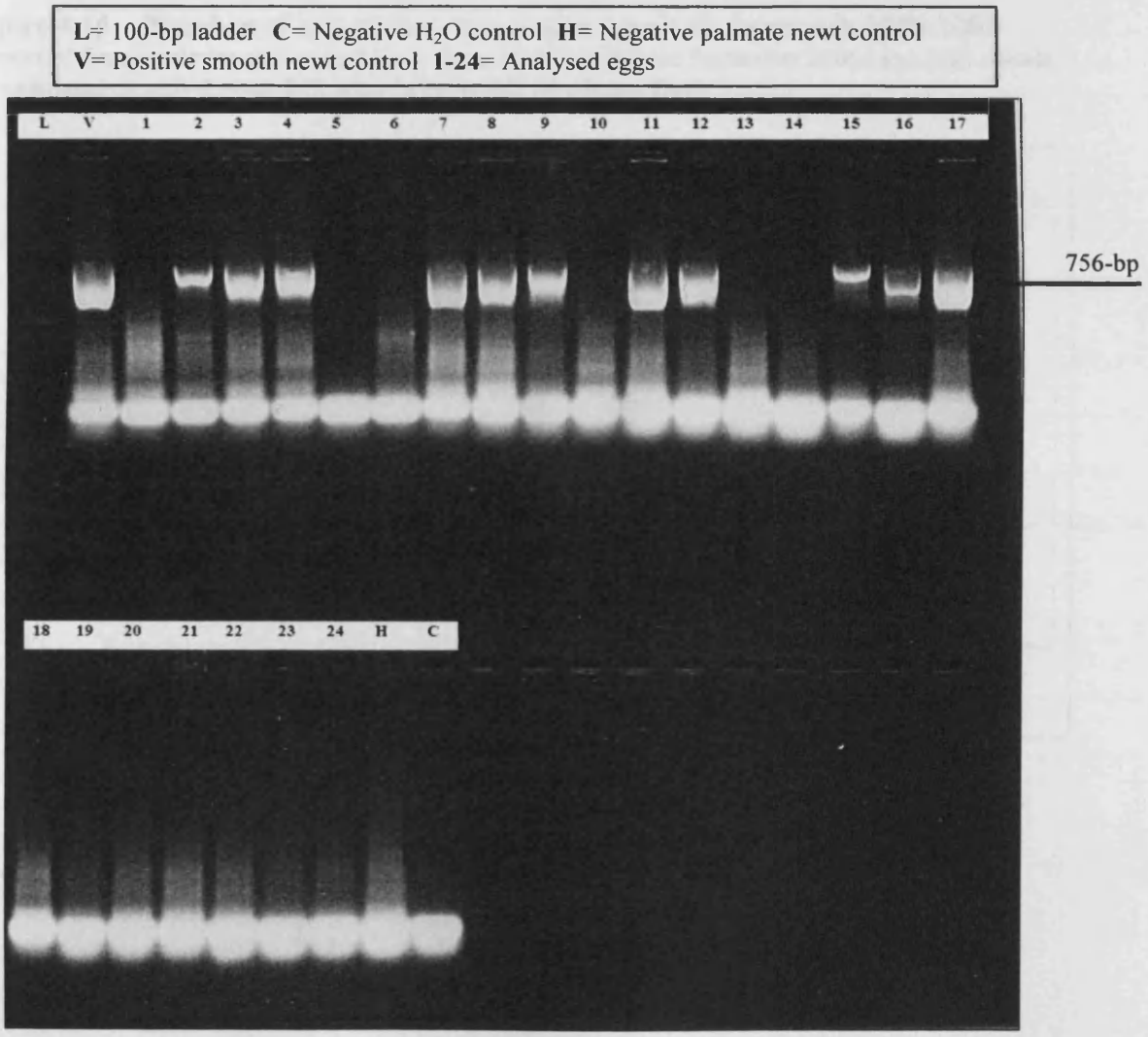
than 2007. There was a slight decline in number of *Lissotriton* eggs found in Week 6 of 2007 when there was high snow fall and a week of cold weather (Figure 4.12). Separating the factors of cumulative number of female newts and temperature was difficult. Partial correlation coefficients showed that the correlation between female arrivals and eggs was so strong that partial correlation of mean weekly temperature against egg numbers produced a negative correlation in 2006 ($r = -0.646$, $d.f = 30$, $p = 0.001$) and 2007 ($r = -0.629$, $d.f = 30$, $p = 0.001$), when the factor of female numbers was controlled.

4.4.3.3 Genetically analysed eggs

Smooth newts eggs collected during each month January-June of 2007 were identified by a positive amplification of the 'ND2' fragment, and when no amplification occurred the eggs were identified as being palmate eggs (Figure 4.13).

Figure 4.13 Visualised gel electrophoresis

Gel electrophoresis clearly showing the 'ND2' fragment amplified in the positive smooth newt control (V) but not in the negative palmate newt control (H). Eggs 2, 3, 4, 7, 8, 9, 11, 12, 15, 16 and 17 all show the amplified 'ND2' fragment, identifying these 11 eggs to be smooth newts. Eggs 1, 6, 10, 13, 14, 18, 19, 20, 21, 22, 23 and 24 do not show an amplified 'ND2' fragment and so are identified as palmate newts. Egg 5 appears to show no DNA present. This egg did not develop after it had been collected which could explain why little or no DNA could be extracted from it.



797 female palmate and 235 female smooth newts migrated to the pond for the 2007 breeding season giving a 3.5: 1 ratio (Figure 4.14). Of the 120 eggs molecularly analysed only 15 were identified to be smooth, the other 105 being palmate newt eggs giving a ratio of 7:1 palmate to smooth eggs. One of the collected smooth eggs was laid in January, one in February and two in March. The majority of smooth newt eggs were found in April (11 out of 20 eggs, Figure 4.15).

The egg species ratios for January-March were as expected from palmate and smooth arrival numbers (Table 4.3). The results were significantly different from expected in May and June so the palmate newts appear to have a longer egg-laying season than the smooth newts. Statistical significances did not differ when different time scales for female arrivals were used to generate expected values, except for eggs collected in April. Expected values generated from female arrivals in both February and March, or all months prior to collection, gave a significant result while expected values generated using only March arrivals gave a non significant result (Table 4.3). This indicated that individual female smooth newts may have a shorter egg-laying season than palmate newts.

Figure 4.14 Number of *Lissotriton* female newt arrivals by month 2006-2007
Number of female palmate and smooth newt arrivals per month from September 2006-June 2007. Newts were captured on arrival at the drift fence surrounding Llysdinam Pond.

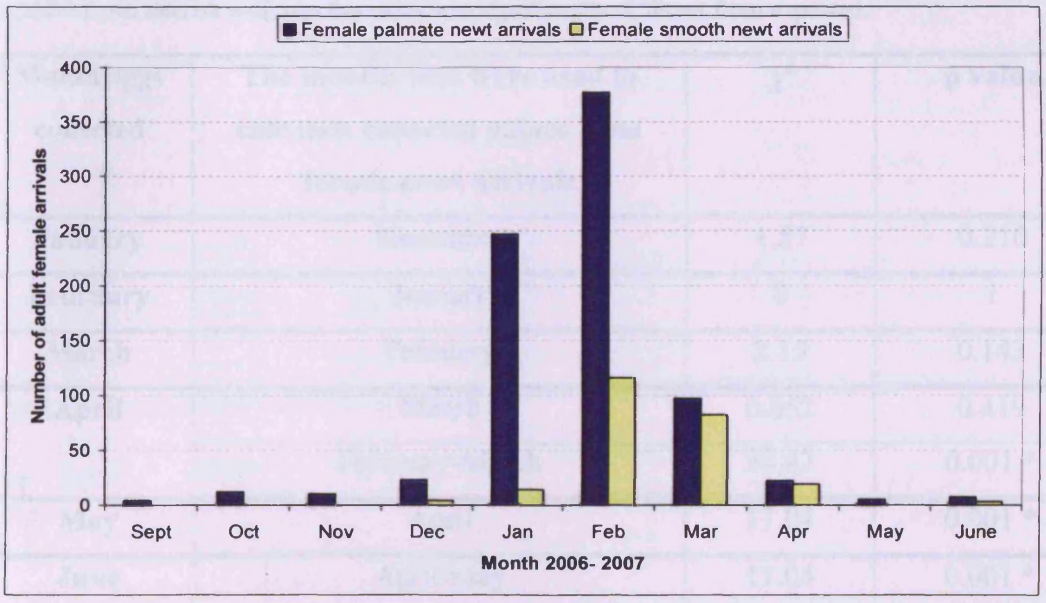


Figure 4.15 Smooth and palmate egg numbers determined by molecular analysis

Number of eggs identified as palmate or smooth by molecular analysis from January-June 2007. 20 eggs were analysed from eggs collected during specific vegetation surveys at Llysdinam Pond at the mid point of each month.

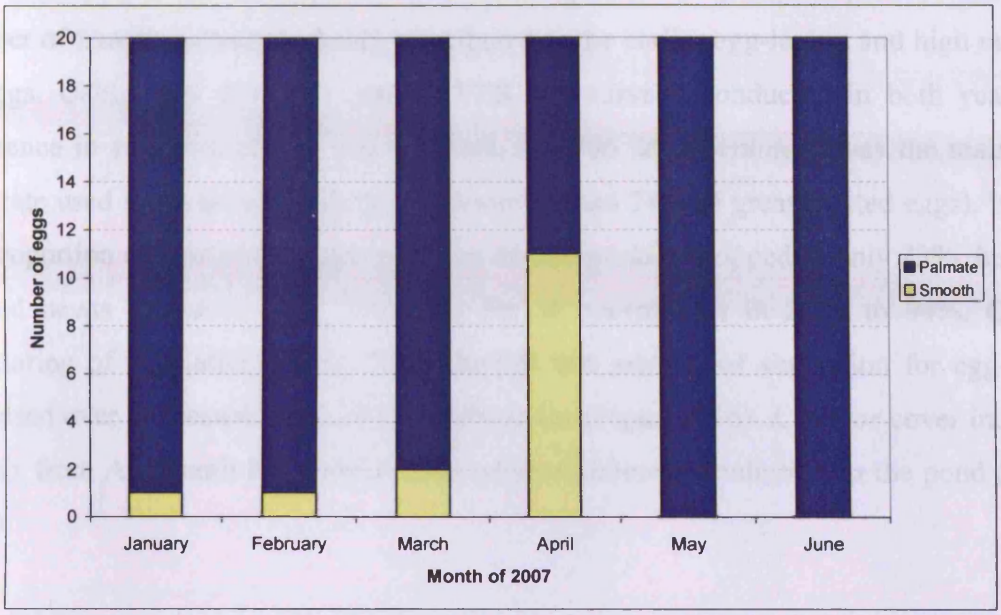


Table 4.3 χ^2 values from genetic analysis of palmate and smooth eggs against expected values

The species of newt eggs collected from the mid point of each month January-June 2007 were identified by molecular methods. Palmate and smooth eggs identified by genetic analysis of eggs provided the observed values for χ^2 analysis. Expected frequencies were calculated from number of adult females of each species arriving at Llysdinam Pond in a specific time period prior to the month of egg collection. N= 20 eggs analysed from those collected each month from Llysdinam Pond during egg searches on specified plant species, d.f = 1. An asterisk indicates the result was significantly different from expected.

Month eggs collected	The months that were used to calculate expected values from female newt arrivals	χ^2	p value
January	December	1.57	0.210
February	January	0	1
March	February	2.15	0.143
April	March	0.652	0.419
	February-March	20.47	0.001 *
May	April	17.04	0.001 *
June	April-May	17.04	0.001 *
All months combined	All previous months (September-May)	7.47	0.006 *

4.4.3.4 Vegetation

In January 2007 it was apparent that the pond vegetation was more abundant and diverse than in 2006 and this in conjunction with the higher early spring temperatures and high number of female arrivals probably contributed to the earlier egg-laying and high numbers of eggs. Using data from the general VES egg surveys conducted in both years, the difference in substrate choice was apparent. In 2006 *M. scorpioides* was the main plant substrate used for oviposition (81% of *Lissotriton* and 74% of great crested eggs). In 2007 the proportion of *Lissotriton* eggs found on *M. scorpioides* dropped to only 33%, but great crested newts increased their selectivity for *M. scorpioides* in 2007 to 94%. Quadrat monitoring of vegetation during 2007 showed that amount of vegetation for egg-laying increased over the season from 28% to 40% cover (Figure 4.16). *L. minor* cover increased rapidly from April until May which reduced penetration of sunlight into the pond (Figure 4.17).

Figure 4.16 Proportion of aquatic plant species through the season

Proportion of aquatic plant species found in quadrat surveys in Llysdinam Pond. 36 randomly placed quadrats were used on each survey. Surveys conducted in the middle of each month January-June 2007 showed how the percentage cover of each species of vegetation altered over the season. Cover of each species was a percentage of the whole pond. Open water accounted for 60% of the pond surface in June, when *L. minor* cover was excluded from the data.

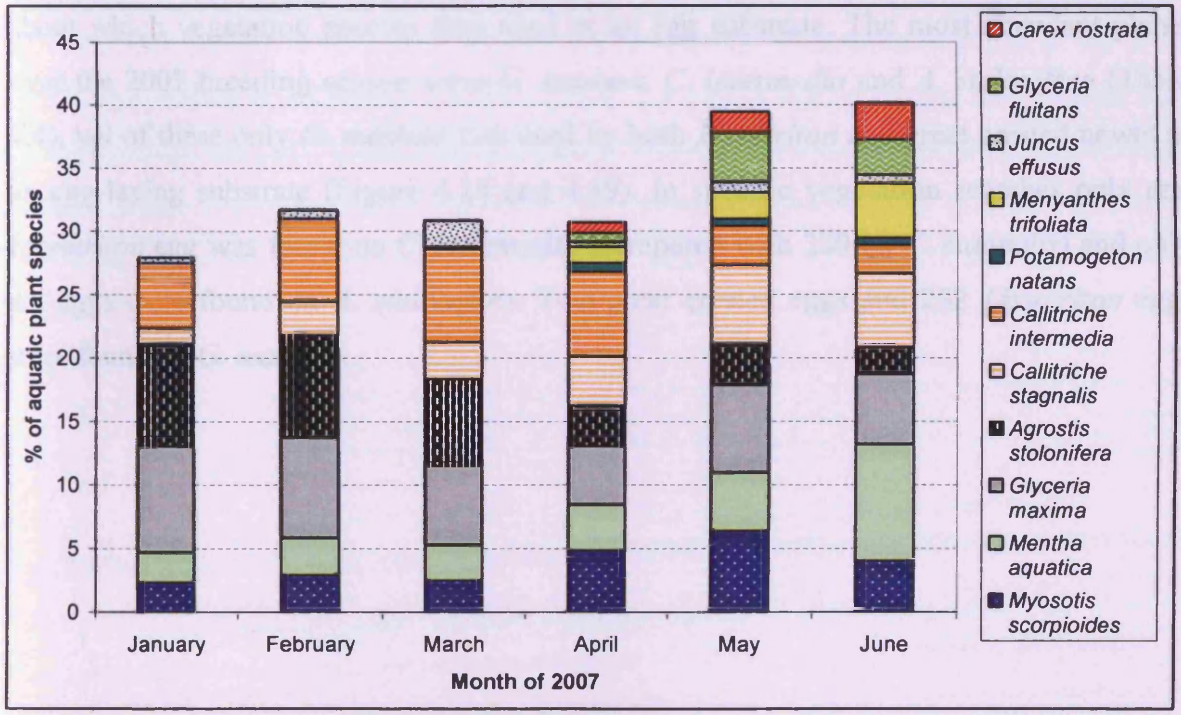
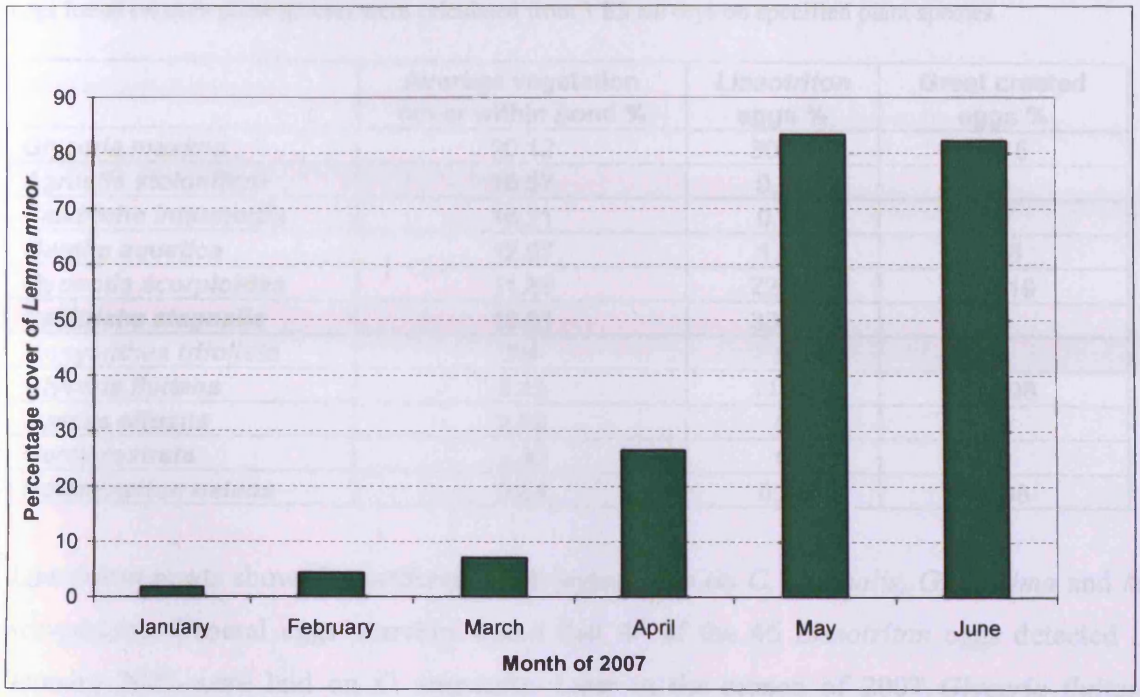


Figure 4.17 Percentage cover of duckweed (*Lemna minor*) on Llysdinam Pond

Percentage cover of duckweed over Llysdinam Pond estimated from quadrat surveys. 36 randomly placed quadrats were used on each survey. Surveys were conducted in the middle of each month January-June 2007 and showed how the percentage cover of duckweed varied across the newt breeding season.



Newts did not lay eggs on different vegetation species at random but instead were selective about which vegetation species they used as an egg substrate. The most abundant plants over the 2007 breeding season were *G. maxima*, *C. intermedia* and *A. stolonifera* (Table 4.4), yet of these only *G. maxima* was used by both *Lissotriton* and great crested newts as an egg-laying substrate (Figure 4.18 and 4.19). In specific vegetation searches only one *Lissotriton* egg was found on *C. intermedia* (compared with 280 on *C. stagnalis*) and only six eggs were found on *A. stolonifera*. Two great crested eggs and 252 *Lissotriton* eggs were found on *G. maxima*.

Table 4.4 Proportion of newt eggs and vegetation cover

Overall percentages (averages from January until June 2007) of aquatic plant species were calculated from quadrat surveys in Llysdinam Pond. Plant species are ordered from most abundant to least abundant. Quadrat surveys were conducted in the middle of each month. The percentage of *Lissotriton* eggs and great crested eggs found on each plant species were calculated from VES surveys on specified plant species.

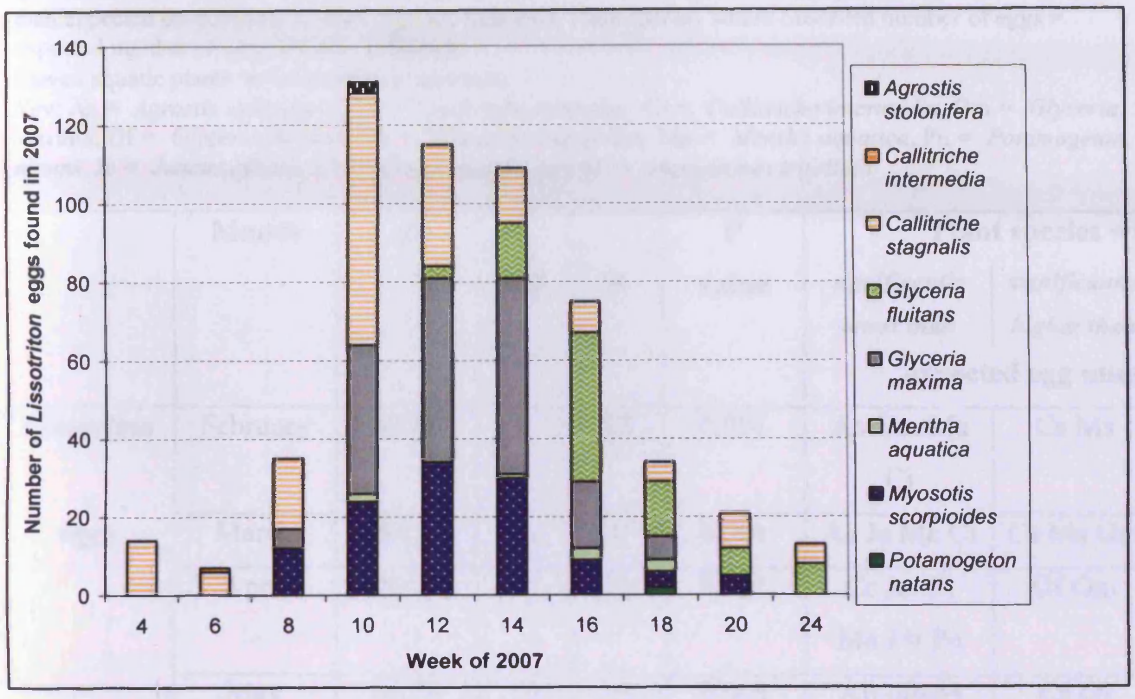
	Average vegetation cover within pond %	*Lissotriton* eggs %	Great crested eggs %
Glyceria maxima	20.12	30.14	2.15
Agrostis stolonifera	16.57	0.72	0
Callitriche intermedia	16.21	0.12	0
Mentha aquatica	12.07	1.56	8.6
Myosotis scorpioides	11.89	22.25	74.19
Callitriche stagnalis	10.81	33.49	0
Menyanthes trifoliata	3.4	0	0
Glyceria fluitans	3.15	11.48	13.98
Juncus effusus	2.59	0	0
Carex rostrata	2.35	0	0
Potamogeton natans	0.84	0.24	1.08

Lissotriton newts showed a preference for laying eggs on *C. stagnalis, G. maxima* and *M. scorpioides*. General eggs searches found that 44 of the 46 *Lissotriton* eggs detected in January 2007 were laid on *C. stagnalis*. Later in the season of 2007 *Glyceria fluitans* emerged in the pond and was selected for egg-laying by *Lissotriton* newts (Figure 4.18 (i) and Table 4.5). The high abundance of *C. stagnalis* in January and February when other aquatic plant species were in low abundance was likely to have contributed to the early egg-laying season of *Lissotriton* newts in 2007. In 2006 *Callitriche* was only present in a shallow area of the pond that dries out regularly and no newts eggs were found there. Great crested newts showed greater selectivity in substrate choice than *Lissotriton* newts, favouring *Myosotis scorpioides* over all other vegetation species (Figure 4.18 (ii) and Table 4.5).

Figure 4.18 Changes in plant selectivity for oviposition through the season by *Lissotriton* newts and great crested newts

Number of newt eggs found during egg searches on specified plant species in Llysdinam Pond during 2007. Each specified egg search was conducted by two people for 15 minutes. Data displayed for Weeks 4-24 for *Lissotriton* newts and Weeks 10-24 for great crested newts.

(i) *Lissotriton* newts



(ii) Great crested newts

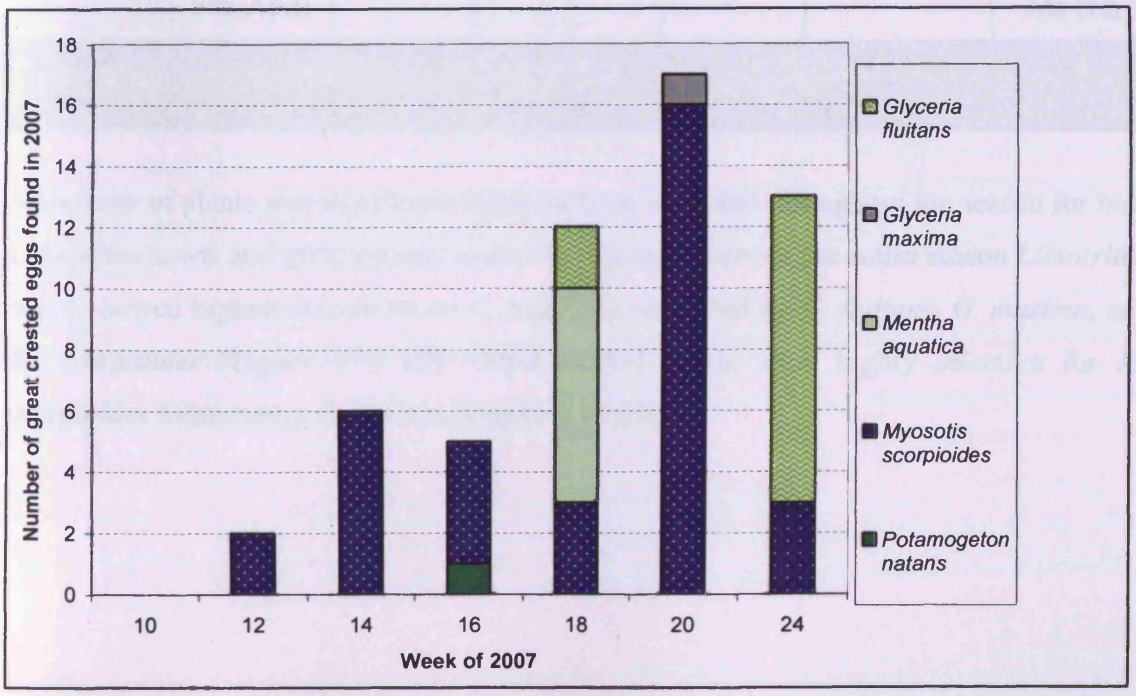


Table 4.5 χ^2 tests on selectivity of egg-laying substrates by *Lissotriton* and great crested newt eggs

Newt selectivity for plant species for egg-laying from January-June 2007 was analysed by χ^2 tests. Observed values were calculated from total number of eggs found in the mid point of each month during egg searches on specified plant species. Each egg search was conducted by two people for 15 minutes. Observed values overall from February-April were calculated from egg searches every two weeks, therefore two surveys from each month. Expected values were calculated from percentages of plants found in Llysdinam Pond during quadrat surveys conducted in the mid point of each month. Plant species with significantly lower or higher than expected proportions of newt eggs are indicated. Plant species where observed number of eggs = expected number of eggs are also indicated.

Eleven aquatic plants were tested in χ^2 analysis:

Key: As = *Agrostis stolonifera*, Cs = *Callitriche stagnalis*, Ci = *Callitriche intermedia*, Gm = *Glyceria maxima*, Gf = *Glyceria fluitans*, Ms = *Myosotis scorpioides*, Ma = *Mentha aquatica*, Pn = *Potamogeton natans*, Je = *Juncus effusus*, Cr = *Carex rostrata* and Mt = *Menyanthes trifoliata*

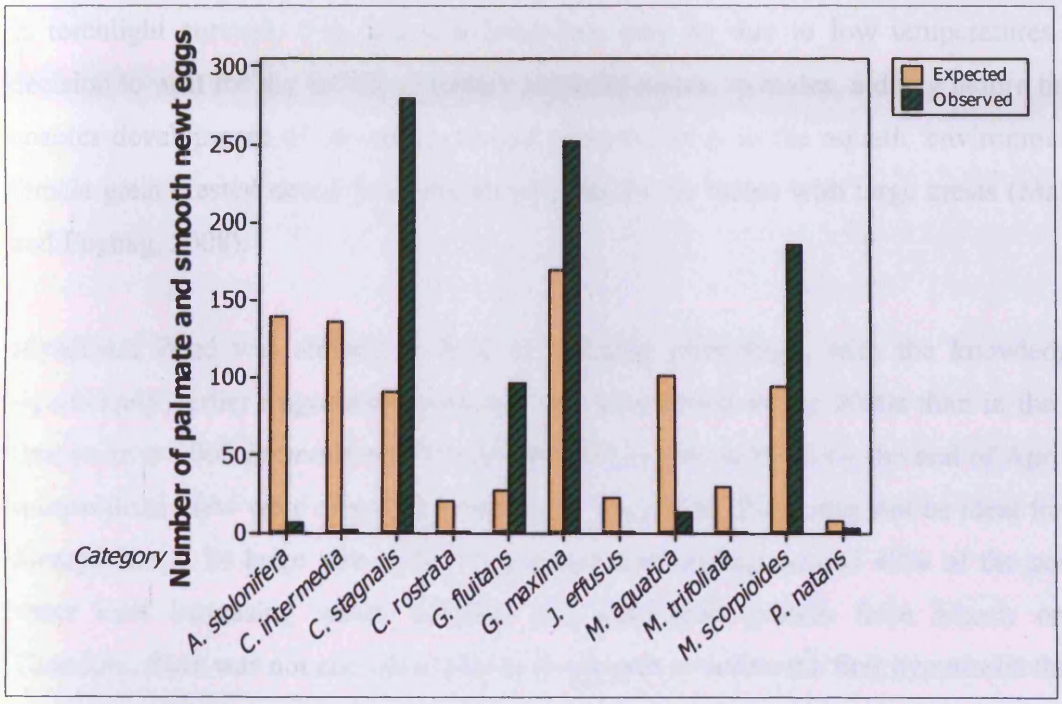
	Month	χ^2	**d.f**	**N**	**P value**	**Plant species with**		
						significantly lower than	***significantly higher than***	***Observed =***
						expected egg numbers		
Lissotriton	February	30.88	4	32	0.001	As Gm Ma Ci	Cs Ms	
eggs	March	194.41	7	117	0.001	As Je Ma Ci	Cs Ms Gm	Gf
	April	839.26	7	72	0.001	Cr As Ci Ma Mt Pn	Gf Gm	Cs Ms
	May	46.99	10	16	0.001	All others	Cs Gf	
	June	71.35	10	13	0.001	All others	Cs Gf	
	Overall Feb-April	1116.42	10	836	0.001	Ma, Ci, As	Gf Cs Ms Gm	

Selectivity of plants was significant different from expected throughout the season for both *Lissotriton* newts and great crested newts. Taking into account the entire season *Lissotriton* newts showed highest selectivity on *C. stagnalis*, followed by *G. fluitans*, *G. maxima,* and *M. scorpioides* (Figure 4.19 (i)). Great crested newts were highly selective for *M. scorpioides* followed by *G. fluitans* (Figure 4.19 (ii)).

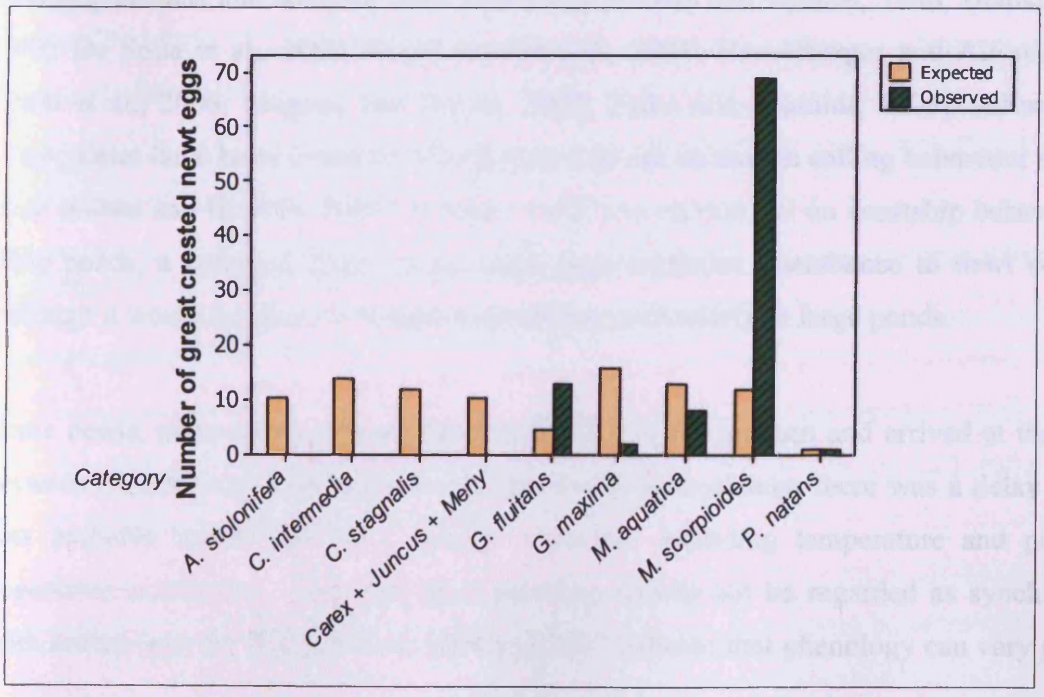
Figure 4.19 Newt selectivity for plants as egg-laying substrates

Expected and observed values for numbers of newt eggs found on specific aquatic vegetation in Llysdinam Pond. Numbers are totals from specified egg searches from January-June 2007. Expected egg numbers were calculated from percentages of plants found in Llysdinam Pond during quadrat surveys conducted in the mid point of each month. Observed values were calculated from total number of eggs per month found during egg searches every two weeks on specified plant species. Each specified egg search was conducted by two people for 15 minutes. χ^2 tests found that (i) *Lissotriton* females and (ii) great crested female newts showed significant selectivity in substrates chosen for egg-laying. For (ii) great crested newt *Carex* + *Juncus* + *Meny* = data merged from *C. rostrata, J. effuscus* and *M. trifoliata*.

(i) Female *Lissotriton* newt selectivity



(ii) Great crested newt selectivity



4.5 DISCUSSION

The surveys of egg-laying at Llysdinam Pond were more successful for studying newt breeding phenology than surveys of courtship behaviour. Courtship surveys to specifically assess the phenology of European newts have not been carried out before, and this may be due to difficulties in detecting newt interactions in ponds. The two surveys when specific courtship displays were observed occurred at a similar time to the first egg-laying peak. Earlier in the year newts were possibly seeking refuge in vegetation, and therefore not seen in torchlight surveys. This inactive behaviour may be due to low temperatures or the decision to wait for the arrival of further potential mates. In males, a delay before breeding enables development of secondary sexual characteristics in the aquatic environment, and female great crested newts have shown preferences for males with large crests (Malmgren and Enghag, 2008).

Llysdinam Pond was studied to look at breeding phenology, with the knowledge that significantly earlier migration phenology had been found in the 2000s than in the 1980s. Despite over 3000 *Lissotriton* newts arriving at Llysdinam Pond by the end of April 2006, comparatively few were observed in the pond. Llysdinam Pond may not be ideal for visual surveys due to its large size, dense vegetation surrounding around 40% of the pond, the water inlet increasing water turbidity and duckweed growth from March onwards. Therefore, there was not enough evidence to support or refute the first hypothesis that there was a significant positive association between courtship behaviour and temperature. In comparison, surveys for anuran calling behaviour in the wild have proved successful in detecting anurans and studying their phenology (Banks and Beebee, 1986; Blankenhorn, 1972; De Solla et al., 2006; Gibbs and Breisch, 2001; Hauselberger and Alford, 2005; Kirlin et al., 2006; Lingnau and Bastos, 2007; Pellet and Schmidt, 2005). Advances in calling dates have been found for a long term data set on anuran calling behaviour in New York (Gibbs and Breisch, 2001). If future work was carried out on courtship behaviour at other ponds, a coloured filter on the torch may minimise disturbance to newt activity, although it would be likely to reduce detectability particularly in large ponds.

Some newts, particularly palmate newts, migrated in the autumn and arrived at the pond between October and December but did not breed immediately, there was a delay which was probably determined by a variety of factors including temperature and possibly vegetation availability. Therefore newt breeding should not be regarded as synchronous with arrival, and the findings from 2006 and 2007 indicate that phenology can vary greatly

even between consecutive years. *Lissotriton* newts showed a greater plasticity in migration and breeding phenology than great crested newts. The delay between *Lissotriton* arrival and breeding was less in 2007 as egg-laying commenced by Week 2 while in 2006 it was at least 10 weeks later. The phenology of great crested egg-laying between years were more comparable, although great crested newts commenced breeding four weeks earlier in 2007 and prolonged breeding by five weeks. Beebee and Griffiths (2000) suggested the palmate and smooth egg-laying period extends from March to June but in 2007 eggs were found from the second week of January and it was likely egg-laying commenced before then. Newly laid eggs were found up until the second week of August indicating a longer period of egg-laying (at least 32 weeks) than previously suggested. A similar number of newts migrated to the pond in the autumn of 2005 and 2006 for the following years' breeding season, but there was an early peak of palmate newt arrivals in January 2007 that probably contributed to early abundance of eggs compared with 2006. The great crested newt has been found to lay eggs from late February if the winter has been mild, although it is more normal for egg-laying to begin in March (Beebee and Griffiths, 2000) which was the case for 2007. Great crested newt egg-laying can continue until July, with peak egg-laying from early-mid June (Green, 2001b) which was consistent with the peaks and finish of egg-laying at Llysdinam.

Two large distinct larval cohorts were identified in a study of the smooth newt in Oxford (Bell and Lawton, 1975) where vegetation was core sampled to detect eggs. Some of Bell and Lawton's results corroborate the 2006 data while other findings correspond better with the 2007 data. At Llysdinam two distinct cohorts were found in 2006 and three in 2007. Bell and Lawton (1975) found an early, main and late oviposition period. The early peak in Oxford contributed about 15% of total oviposition and was around Week 12, but at Llysdinam, eggs were only just detected at that time in 2006. In contrast in 2007 the early start to oviposition meant that by Week 12 the number of eggs found was declining, after a peak at Week 11. The sampling timing of Bell and Lawton did not start until early April, so they suggest that they may underestimate the importance of the early egg-laying. The peak of egg-laying in Oxford occurred when spring migrations had barely begun so must have principally involved the animals that migrated in the previous autumn. This was the case for 2007 at Llysdinam although in 2006 the egg-laying commenced during peak migration time. The main oviposition period peaked around Week 18 in the Oxford study similar to findings for 2006 in this study. In the late oviposition cohort, Bell and Lawton (1975) suggested the small proportion of eggs found from June could have been due to the

lower water levels, so that potentially, later oviposition could have added a more important component to the total. Two other factors that could lead to less eggs being oviposited later in the season are a certain amount of female mortality will have occurred by the end of the breeding season (Baker, 1992; Harrison et al., 1983), and many females would have depleted their store of fertilised eggs. Larvae that hatched from eggs laid late in the season would not have enough time to metamorphose, and would therefore need to overwinter in the pond (Chapter 6).

The eggs which were found during searches of the pond were only a very small proportion of total eggs laid within the pond as each female newt can lay up to 300 eggs (Beebee and Griffiths, 2000). A study conducted by Harrison (1985) on the Llysdinam newts found an average mean clutch size of 190 for palmate and smooth newts which was significantly lower then the 300 often suggested, but could be accounted for by the relatively small size of the newts at Llysdinam. It was apparent from estimations of clutch size that we only located a small proportion of eggs laid in Llysdinam Pond for *Lissotriton* newts, although great crested eggs appeared to be more detectable since 215 eggs and 362 eggs for nine and 10 female arrivals were located in 2006 and 2007, respectively. Following VES methodology, time constraints were placed on the egg search to make the sample times between weeks and years consistent and to reduce pond disturbance. It was difficult to monitor the delay between individual female newt arrival to the pond and egg-laying as newts lay their eggs over a long period of time. Although, outdoor tank based studies (Chapter 5) specifically investigated the delays between arrival and egg-laying. Smooth newts lay an average of 8.7 eggs per day but the rate can be as much as 54 eggs per day during peak egg-laying (dependent on temperature and body size). The egg-laying period can also be hugely variable, ranging from 11 to 74 days (Baker, 1992). This variability in egg-laying between individual newts due to factors such as female body size (Baker, 1992; Norris and Hosie, 2005a), makes it difficult to generalise about newts populations without data from direct observations of the population being studied.

Oophagy by predators in the pond could also influence the number of eggs found within the pond. One benefit of early egg-laying and presumably earlier hatching was low predation pressure since predator abundance in ponds peaked later in the season. Survival of eggs laid early would be particularly enhanced because survival increases with time from segmentation stages and gastrulation to late neural budding stages (Miaud, 1993). Also, near to hatching, a newt larva may escape predation, because if it is disturbed it

reacts to the potential predation risk by wriggling free through the degenerating egg capsule (pers. obs.). Newts minimise predation pressure by wrapping eggs in vegetation to reduce detectability. Amount of wrapping behaviour appears to vary between newt species. 100% of eggs laid by great crested eggs were wrapped (Miaud, 1994), while the figures have varied from 10% (Orizaola and Braña, 2003) to 25% wrapped eggs for palmate newts (Miaud, 1994). 12% of palmate newts eggs survived until hatching in a pond but 78% survived if protected by a mesh cage from predators (Miaud, 1993).

There is variability in the degree of oophagy by aquatic predators and it is likely that wildfowl consume newt eggs with aquatic vegetation. *Aeshna* dragonfly larvae predated half of unwrapped newt eggs but caused no noticeable mortality to wrapped eggs (Orizaola and Braña, 2003). It had been suggested that wrapping eggs reduces visibility and accessibility (Gabor, 1996), and Orizaola and Braña (2003a) observed that wrapping protected eggs from *Aeshna* dragonfly larvae, because *Aeshna* moved along leaves detecting eggs using their antennae. Wrapping behaviour almost guaranteed protection from dragonfly larvae while it minimised predation by male newts. Male newts predated 2% wrapped and 9% unwrapped, female newts predated 5% wrapped and 49% unwrapped, and the water beetle *Acilus sulcatus* predated 10% wrapped and 79% unwrapped. In contrast, adult great diving beetles (*Dytiscus marginalis*) were found to predate all wrapped eggs (Miaud, 1993).

Miaud (1993) found that in most cases the entire egg was consumed but in four incidents only the egg was taken and the jelly capsule was left intact, suggesting predation by an invertebrate by suction. The remains of the jelly capsule could explain the presence of transparent jellies found on plants during egg searches which were recorded in 2007, after being observed but not recorded in 2006. The jellies found may not have all been from newt eggs, but they were often visible on plants (particular *M. scorpioides*) with newt eggs laid on them. At least 10 jellies per man hour were found between Weeks 11 and 19 of 2007. The jellies peaked at almost 60 per man hour in 2007 at Week 14 and followed a similar trend line to the number of eggs located. Bell and Lawton (1975) found that dead eggs plotted as a proportion of total oviposition indicated that the average rate of mortality becomes less towards middle of main oviposition period due to predator satiation. They suggested that female newts may therefore increase oviposition rate towards the middle of the season to minimise risk of egg predation. In corroboration, at Llysdinam Pond the peak of jellies coincided with the peak in newly laid eggs and proportion of jellies to newly laid

eggs was highest after the peak in egg-laying had occurred. Nevertheless, further information on the jellies detected in Llysdinam Pond would be required before utilising their presence to infer predation rates.

Besides predation, another common threat to aquatic embryos is oomycetes. Although lower temperatures have not been found to affect embryo survival directly (Griffiths and de Wijer, 1994), *Saprolegnia* oomycetes has been found to be more prevalent at lower temperatures. In contrast, the incidence of fungal attack halting development in eggs to be used for molecular analysis occurred later in the year at higher temperatures. Fungal attacks often acts synergistically with other factors like UV radiation or unusually cold conditions (Kiesecker et al., 2001). Separating the newt eggs in Petri dishes reduced mortality, most likely by reducing spread of the fungal spores from infected eggs. In a similar way, communal eggs masses had higher mortality rates by fungal infection than eggs laid separately (Kiesecker and Blaustein, 1997). It is thought that communal egg-laying may have been the more primitive state in European newts before selection for individual oviposition due to mortality pressures. Susceptibility to oomycetes differs between species, partially due to their breeding phenology. Gomez-Mestre et al. (2006) studied three amphibians with different breeding phenology and oviposition habits. No infection of eggs of the earliest breeder, spotted salamanders (*Ambystoma maculatum*) occurred due to a jelly coat protection. Only wood frog (*Rana sylvatica*) eggs that were laid later in the year at warmer temperatures became infected with a water mould, while 60% of the latest breeder American toad (*Bufo americanus*) eggs became infected but plasticity in hatching enabled them to hatch 36% earlier in response to infection (Gomez-Mestre et al., 2006).

With only two years of data, causal affects between temperature and oviposition could not be identified. The influence of temperature on egg-laying was difficult to untangle from the other factors such as the number of females breeding and vegetation availability. Therefore the second hypothesis that there was a significant positive association between temperature and egg-laying activity could not be supported or rejected. Despite the lack of a relationship, it was likely the higher temperatures in late 2006 and early 2007 led to significantly earlier egg-laying by *Lissotriton* newts in 2007 breeding season. An advancement in *Lissotriton* newt arrival dates and their presence in ponds has been found in previous research (Beebee, 1995; Chadwick et al., 2006) but no work had taken place to investigate whether earlier arrival was reflected in earlier breeding. This study showed that

there was great variability in the commencement of oviposition but that mild winter temperatures, a high number of females arriving early and an abundance of vegetation for oviposition could cause a shorter delay between arrival and egg-laying. The egg searches from autumn 2007 until January 2008 indicate that the newts, mainly palmate newts, which underwent an autumnal migration and arrived at the pond between October and December, did not breed immediately. The length of the delay was probably determined by a combination of factors including mate availability, temperature and vegetation availability. Harrison et al. (1983) suggested that at temperatures below 0°C, palmate and smooth newts remain inactive. In a cold spell in early February 2007, there was a slight decline in egg-laying but since only 6-10 eggs per hour had been found up until then, the declines were not marked. Beattie (1985) suggested a different stimulus may control common frog spawning to emergence from hibernation since there were differences in the delay between arrival and spawning, even at ponds in close proximity. It has been suggested that great crested newts require higher temperatures (4°C to 5°C) in order to remain active (Arntzen and Hedlund, 1990; Verrell and Halliday, 1985). During this study it was observed that great crested newts did not arrive or begin egg-laying until temperatures have reached a higher threshold than those required by palmate and smooth newts.

The findings from 2006 and 2007 indicate that phenology can vary greatly even between years, so long term data sets such as the one at Llysdinam are very useful to phenological research. Long term data sets on anuran egg-laying are more common, probably due to anuran eggs being more detectable (Beebee 1995, Tryjanowski et al., 2003) and from these a 8-9 day shift towards earlier breeding was found over a 25 year period (Tryjanowski et al., 2003).

As far as I am aware, this was the first study to analyse *Lissotriton* newt eggs molecularly to investigate differences in egg-laying phenology within a community of both smooth and palmate newts. Although palmate newts began arriving at the breeding pond earlier than smooth newts, both species had commenced egg-laying by mid January. Beebee and Griffiths (2000) suggested palmate newts have a more distinct egg-laying peak occurring in May whereas smooth newts show more consistent egg-laying over the season. Molecular analysis showed that in 2007, palmate newts laid eggs more consistently over the entire breeding season with their eggs being found from January to June. It was the smooth newts that showed the most significant peak in egg-laying which occurred during April, possibly due to the accumulation of smooth females arriving in February and March.

The third hypothesis that earlier arriving species (palmate newts) begin egg-laying significantly earlier than late arriving species (smooth newts) was rejected since numbers of palmate and smooth eggs found in Llysdinam Pond were as expected from arrival numbers early in 2007. Instead molecular analysis revealed that smooth newt newts had a shorter egg-laying period that lasted from January-April, while palmate eggs were identified by molecular analysis from January-June.

The shorter oviposition period for smooth newts maybe due to them being at the edge of their range at Llysdinam and laying fewer eggs than palmate newts (egg-laying rates were lower for smooth newts, Chapter 5). Smooth newts may have invested time egg-laying in a more constricted period when overall newt egg numbers were in high abundance in the pond. Oviposition during times of peak egg abundance in ponds may increase chances of eggs surviving predation due to predator satiation (Ims, 1990). Also higher temperatures later in the season decrease hatching time and therefore reduce the period of vulnerability. Egg-laying later than this may not provide enough time for larvae to develop, metamorphose and leave the pond before winter. Eggs laid early were more likely to have developmental problems due to low temperatures and freezing, and longer developmental times increase predation risk. Eggs laid in summer have greater risk of desiccation and larvae may need to over winter in the pond due to lack of time for growth and development. Palmate newts, which tend to inhabit harsher environments than the smooth and great crested newts, may have developed a longer period of egg-laying as a reproductive strategy to ensure survival of at least some offspring if conditions were unfavourable at certain points during the egg-laying period (Denoël et al., 2005b; Harrison et al., 1983).

The use of a negative result in this study was believed to have provided accurate results in identifying smooth from palmate eggs (M. Bruford, pers. comm.). With more time and funding it would be preferable to develop a primer that would reliably amplify in both palmate and smooth newts. This would eliminate the possibility of unsuccessful DNA extraction or unsuccessful PCR going undetected. Also future studies should develop eggs separately to minimise oomycete infection. Development of eggs within Petri dishes was also successfully used by Bell and Lawton (1975) with an 87% survival rate.

Besides minimising predation risk, newts may also wrap eggs in vegetation to increase oxygen supply and therefore development rate of the larvae (Wimpenny, 1951). Preference

for living substrates rather than egg-laying mops, could explain why mops were not used by newts for oviposition in Llysdinam Pond in 2005. In contrast, Miaud (1995) found that equal numbers of eggs were laid by palmate newts on *G. fluitans* and a cotton support when offered the choice in tanks. The higher abundance of plant availability in 2007 (pers. obs.) was not the only factor that led to earlier breeding in 2007, since egg-laying by palmate and smooth newts commenced earlier in 2007 when oviposition substrate amount was controlled (Chapter 5). It was possible the egg-laying substrates at Llysdinam were reduced later in the season due to duckweed growth cutting off light levels and reducing the growth and condition of aquatic macrophytes.

Even when plants were in low abundance early in 2006, eggs were not found in the detritus of Llysdinam Pond. This may be due to reduced oxygen supply on the pond benthos due to standing water and decaying leaves (Miaud, 1995). Females may avoid egg-laying on the pond benthos since egg development would be slower due to low oxygen levels, and it would be a greater distance to swim to the surface to breathe. Egg frequency has been found to decrease as a function of depth between 0-40 cm with 60% of palmate newt eggs laid at 0-10 cm depth and 19% at 30-40 cm depth. Similarly great crested newts laid 52% of eggs at 0-10 cm depth and just 3.2% at 30-40 cm depth (Miaud, 1995).

Newts at Llysdinam were opportunistic but selective in their choice of substrate for oviposition, as found by Miaud (1995). Therefore the fourth hypothesis that (iv) *Lissotriton* and great crested newts show selectivity for egg-laying substrates was supported by this study. Green (2001b) proposed an order of vegetation species preference for great crested newts with which our findings largely agreed (taking account for the vegetation species present in the pond). However at Llysdinam, great crested newts selected *G. maxima* less and *M. aquatica* more than Green (2001) suggested. Our findings disagree with Green's statement that *Lissotriton* and great crested newts rarely lay eggs on the same vegetation species. There was large overlap in vegetation species preference at Llysdinam Pond, particularly with *M. scorpioides* and *G. fluitans*. Smooth and palmate newts laid eggs in large numbers on a variety of different vegetation species whereas the great crested newts showed a clear preference for one species, *M. scorpioides*. Selectivity of substrates by great crested females could partially explain the wider occupancy of ponds by smooth and palmate newts than the great crested newt as observed in ecological studies (Miaud, 1995).

Norris and Hosie (2005b) showed behavioural evidence for newt selectivity in the way newts 'sniffed' and 'flexed' leaves before deciding whether they were a suitable substrate for oviposition. The requirements of the vegetation for egg wrapping include leaf width and flexibility, since leaves need to be wide enough to completely encase the egg to provide adequate protection (Miaud, 1993), and need to be flexible enough to allow newts to easily bend the leaf around the egg (Miaud, 1995). Smaller newts require more flexible (Norris and Hosie, 2005b) and thinner supports (Miaud, 1995) which was demonstrated by female newts exploiting the abundance of *C. stagnalis* early in 2007 as an egg-laying substrate. The greater selectiveness of great crested newts for vegetation corroborates work by Miaud (1995) who found great crested newts laid almost all eggs on one plant species (Watercress, *Nasturtium officinale*) while palmate and alpine newt eggs were laid on four plant species. In both this and Miaud's study both newt species preferred linear plants such as the *Glyceria* species, the proportionately long leaves of *M. scorpioides* leaves and *C. stagnalis* than more oval shaped leaves such as *P. natans* and *M. aquatica*. The larger great crested newt might be better able to maintain a position for ovipositing on a more robust plant and leaf like *M. scorpioides* while *Lissotriton* newts could accomplish wrapping of eggs in small *C. stagnalis* leaves, although only one egg was found on the narrower leafed *C. intermedia*. *Lissotriton* females tended to fold eggs at the leaf tips of *M. scorpioides*, and in 2006 eggs were found that were folded on thinner flexible decaying yellow and black coloured leaves (20% of total eggs laid on *M. scorpioides*). In contrast, great crested eggs found on *M. scorpioides* tended to be enclosed in a larger fold at the leaf midpoint.

A limitation of a VES for newt egg detection was that not all microhabitats could be sampled with equal success (Crump and Scott, 1994), and so eggs laid on some plants species could have greater chance of being detected than others. The entire plant of *M. scorpioides*, *G. fluitans* and *Callitriche* were accessible but *G. maxima* grew mainly in three large clumps and it was difficult to sample deep in the clump. The characteristic folded leaves were easier to spot on plants such as *M. scorpioides*. Another assumption of VES is that detectability was equal between species but the proportion of great crested newts eggs found may have been higher due to their large size, larger plant folds and so great detectability. Eggs unsuccessfully folded or laid on the leaf surface might have been more difficult to spot, and since some species are less efficient at egg wrapping than others (Miaud, 1994), more of their eggs could go undetected as a result. Also factors affecting detectability over the season may include vegetation density, weather since water turbidity increased after rainfall, and predation of eggs. To overcome these problems, sections of

vegetation could be removed from the pond and studied in greater detail, but would result in greater disturbance to the pond.

Although there were no past data for amphibian egg-laying at Llysdinam, there were some limited data on past larval hatching phenology (Chapter 6). Further years of research, to a similar extent as the Llysdinam amphibian migration data set would be needed to draw conclusions on changes in breeding phenology. The results of this study demonstrate that the egg-laying phenology can show high variability even between consecutive years, and it was difficult to draw conclusions without long term data. Advancements in amphibian breeding phenology can be large and, the 2007 breeding season occurred up to two months earlier than had previously been suggested (Beebee and Griffiths, 2000). The timing of the start of egg-laying in previous years could possibly be predicted from the Llysdinam migration data sets and temperatures. Years with high late winter temperatures and early migration peaks, as occurred in 2007, would indicate possible early breeding, while lower temperatures and later peaks of arrivals as occurred in 2006 could indicate late breeding. Since newts have been arriving earlier in the 2000s than the 1980s (Chadwick et al., 2006), and higher winter and early spring temperatures occur more often, it is likely that earlier breeding also occurred more often.

As newts are poikilotherms, and much of their phenology is influenced by temperature, climatic warming is very likely to result in an earlier newt breeding season (Beebee, 1995; Blaustein et al., 2001). Changes in amphibian populations could affect other species within the pond community such as predators and prey, even if these species were not affected by global warming directly (Donnelly and Crump, 1998). Also a good ecological understanding of a species can assist conservation (Foster and Beebee, 2004), so phenological studies such as this may play an important role in guiding conservation efforts.

Chapter 5: Influence of arrival time on courtship behaviour and egg-laying phenology

5.1 SUMMARY

In 2006 and 2007 *Lissotriton* newts were sampled from arrival captures at the Llysdinam Pond drift fence to study courtship and egg-laying phenology in outdoor breeding tanks. Newts were sampled through the main migration season from January until April to study the effect of arrival time on the timing of the start of courtship and egg-laying. Palmate and smooth newts were studied separately with two males and two females assigned to each tank. In 2006 newts were assigned to two body size categories to investigate the effect of body size on egg phenology and numbers.

Each tank was monitored for several weeks using torchlight surveys for courtship behaviour three times a week, and a weekly check for eggs. Each tank contained egg-laying mops made of polythene strips to provide a controlled oviposition substrate over the study period. There was less delay between arrival and egg-laying by late arriving newts in both years, and newts arriving early in 2007 began breeding earlier in the year than newts that arrived early in 2006.

Palmate eggs were first found on Day 30 in 2007 compared to Day 57 in 2006. The first smooth eggs were also found on Day 30 in 2007 compared to Day 117 in 2006. In 2006 early palmate newt arrivals were in tanks on average 67 days before 20 eggs were detected but late arrivals only had an average delay of 17 days between arrival and egg-laying. For smooth newts there was a similar phenological trend with less delay between arrival and egg-laying for late arrivals, but smooth newts appear to have a higher temperature threshold for breeding. Mean daily air temperature remained above 0°C for 18 days before newts began egg-laying in 2006 and for seven days before egg-laying commenced in 2007. Mean weekly air temperature had remained over 2°C for at least three weeks before the first eggs were found in both 2006 and 2007. Therefore egg-laying in 2007 began earlier than in 2006 in both the wild and outdoor breeding tanks.

5.2 INTRODUCTION

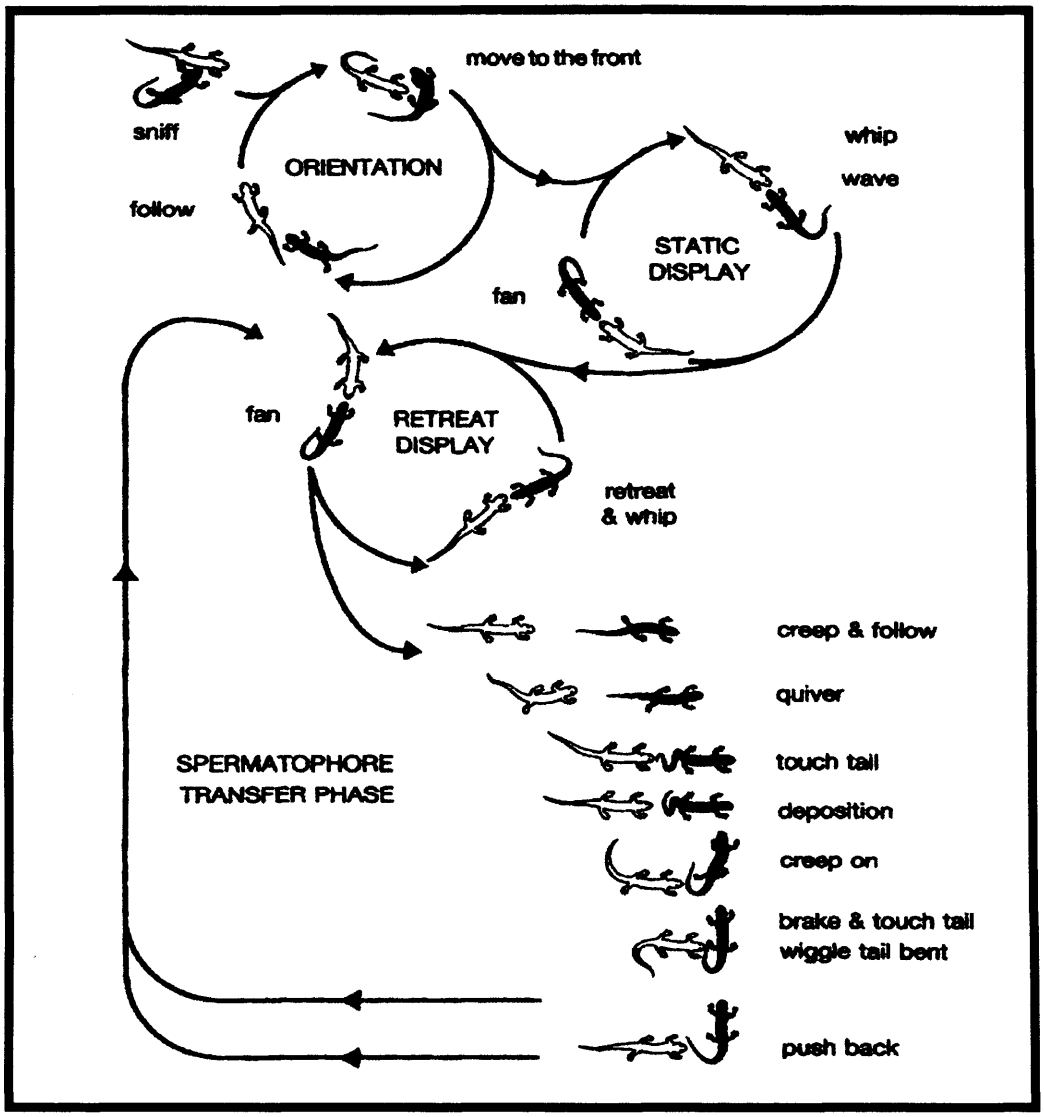
In the majority of salamanders and newts, fertilisation takes places in the female reproductive tract, after the male has manipulated the female to take up a spermatophore. In some species, including *Triturus* and *Lissotriton* newts, visual and olfactory cues are combined into an elaborate courtship display to enable the male newt to manipulate the female towards the spermatophore. Egg-laying then occurs later over a protracted period of weeks to a few months (Beebee and Griffiths, 2000; Griffiths, 1995).

Only after entering the water do *Lissotriton* and great crested newts fully develop their secondary sexual characteristics, which are thought to be advantageous to an aquatic lifestyle and to attract females during courtship displays. These characteristics include a dorsal crest in great crested and smooth newts, development of toe spurs by smooth newts, and webbing to the hind feet and growth of a tail filament in palmate newts. The courtship dance of the male newt enables display of secondary sexual characteristics which may provide clues to the female about his fitness. Larger crests may also aid oxygen absorption and allow the male to keep the attention of the female for longer periods without having to surface for air (Beebee and Griffiths, 2000).

The courtship behaviour seen in European newts is among the best described for all amphibian species (Arntzen and Sparreboom, 1989; Halliday, 1977a), and definitive stages of courtship have been outlined (Figure 5.1). In *Lissotriton* courtship, initially there is an 'orientation phase' where the male attempts to attract the attention of a female and takes up a position in front of her. If the female is interested then the 'display phase' takes place. Both male palmate and smooth newts use a combination of tail movements in displays, including fanning and waving as well as a violent whip of the tail against the body. Palmate newts whip their tail less violently than smooth newts, but fan about twice as much during the display (Arntzen and Sparreboom, 1989; Halliday, 1977a). In salamanders, chemical signals play an important role in species recognition and mate persuasion by increasing female receptivity (Houck and Reagan, 1990; Rollmann et al., 2003). Tail fanning by all *Lissotriton* newts sends male pheromones on a current of water towards the female (Halliday, 1975). The palmate newt (and Italian newt *L. italicus*) have ridges running along each side of the body which provide a concave shape when the fanning position is adopted, which may further assist with channelling the pheromones.

Figure 5.1 Smooth newt courtship sequence

Definitive stages of courtship behaviour by smooth newts (from Beebee and Griffiths, 2000; based on Halliday (1977a)). The male attempts to attract the female through a range of visual and olfactory cues. If courtship is successful a spermatophore is transferred to the female cloaca.



If the attention of the female has been maintained, then the 'retreat phase' occurs. The male turns away from the female and with the tail quivering, leads her away by creeping ahead of her. If the female touches the male's tail with her snout, the male releases a spermatophore onto the substrate and manipulates the female so that her cloaca picks up the spermatophore (Beebee and Griffiths, 2000; Griffiths, 1995). A male may deposit a spermatophore before the female has touched him, or may turn back and resume retreat display either before or after receiving the female's tail touch. Such variations appear to be related to the number of spermatophores that the male carries. Sometimes males will deposit a spermatophore without female responsiveness, and it has been suggested that this is to demonstrate male fertility to the female (Halliday, 1990).

Male interference may occur when a male 'pretends' to be a female by touching the courting male's tail during courtship. The misguided courting male then releases a spermatophore, while the interfering male leads the female away (Verrell, 1984). Despite this threat to courtship success, male alpine newts did not change their behaviours in the presence of other newts (Denoël et al., 2005a). When males outnumber females, sexual interference may be common but at Llysdinam Pond, pitfall trap captures indicated that females of both *Lissotriton* species were more abundant. Although it was possible that catchability of females was greater since males appear to be better climbers (pers. obs.). Female interference in courtship can also take place when a female 'steals' a spermatophore (Waights, 1996).

A female newt may mate with several males during the course of the breeding season and can pick up several spermatophores during one encounter (Pecio, 1992). Females have been found to be more selective in mate choice once they have mated (Gabor and Halliday, 1997; Hoeck and Garner, 2007). Teasing out the importance of each of the characters that may influence female choice has been difficult (Griffiths, 1995), but female palmate newts were found to prefer males with a long caudal filament (Haerty et al., 2007). Laboratory observations demonstrated that males preferred females that were larger and fatter, and therefore carrying more eggs (Verrell, 1986).

Amphibian courtship behaviour can be used as a signal of phenological change. There has, however, been debate over the sensitivity of amphibians with prolonged breeding to abiotic factors (Saenz et al., 2006), and the importance of month in analyses has been found to be important (Canavero et al., 2008). Several studies have focused on the effects of temperature and season on the acoustic displays in anurans (Banks and Beebee, 1986; Canavero et al., 2008; Canelas and Bertoluci, 2007; Hauselberger and Alford, 2005; Lingnau and Bastos, 2007; Oseen and Wassersug, 2002; Saenz et al., 2006). In contrast, fewer studies have taken place on the effects of temperature on visual displays in urodeles (Denoël et al., 2005b). Denoël et al. (2005b) found that at a lower temperature the male alpine newt fanned its tail for less beats but a longer duration, and Doherty (1982) showed similar findings for the courtship wave by fiddler crabs, *Uca minax* and *Uca pugnax*. Certain fin movements in the male guppy *Poecilia reticulata* were also directly temperature dependent (Laudien and Schlieker, 1981).

Female smooth and palmate newts each lay between 200 and 300 eggs during the breeding season. All *Triturus* and *Lissotriton* newts wrap individual eggs in the leaves of aquatic plants (Griffiths, 1995) which protects against predation (Miaud, 1993) and UV radiation (Marco et al., 2001). Differences in wrapping behaviour have been observed (Norris and Hosie, 2005b), and great crested newts were found to wrap 100% of eggs while palmate newts wrapped 75% of eggs (Miaud, 1994). Even wrapped eggs have only a 16% chance of survival to hatching (Miaud, 1993). To facilitate egg wrapping newts prefer leaves which are flexible and wide enough to fully envelop eggs (Miaud, 1995), and the vegetation on which newts lay eggs may also affect the development stage at which the eggs hatch (Langdon et al., 2005). Exposure of females during egg-laying increases the risk of predation which could account for the high mortality rates which occur during this time (Baker, 1992; Harrison et al., 1983).

Although Chadwick et al. (2006) found newts, particularly palmate newts, were arriving earlier at Llysdinam Pond, it was not known if earlier arrival was reflected by earlier courtship and egg-laying. Torchlight surveys and egg-laying surveys at Llysdinam Pond enabled courtship and egg-laying to be surveyed in the wild (Chapter 4). Research into newt breeding phenology in addition to arrival phenology is important since egg-laying phenology may have direct implications for number of eggs laid, predation risk to adults, eggs and larvae, and success of larvae and metamorphs.

5.2.1 Aims and hypotheses

No published data appears to have investigated the relationship between timing of arrival and breeding behaviour (courtship and oviposition) in an amphibian with a protracted breeding season. Assessment of amphibians with a protracted breeding season poses monitoring problems (Banks and Beebee, 1986) and relating the arrival dates to courting and egg-laying of newts in Llysdinam Pond was impossible. The main aim of this study was to overcome the problem of linking courtship and egg-laying phenology to arrival phenology. By using outdoor tanks, rather than a laboratory-based study it was hoped to simulate the pond environment more closely. Early in the migration season, larger newts made up a greater proportion of arrival numbers than later in the year (Chapter 2). Although there has been mixed findings about the influence of male body size on breeding success (Baker, 1990a; Griffiths, 1995; Haerty et al., 2007; Halliday, 1998), it has been found that larger females have greater breeding success (Baker, 1992; Nobili and Accordi, 1997; Semlitsch et al., 1993; Verrell, 1986). Therefore it was hypothesised (i) that larger

newts will have higher breeding success than small newts. Measures of breeding success used in this study were commencement egg-laying earlier in the season and having a higher egg-laying rate. The main aim of this study was to investigate differences in courtship behaviour and egg-laying phenology between early and late newt arrivals, and the influence of temperature. It was hypothesised that (ii) there is a significantly longer delay between arrival and courtship in *Lissotriton* newts that arrive early than late to Llysdinam Pond due to lower temperatures early in year. Thirdly it was hypothesised that (iii) there is a significantly longer delay between arrival and egg-laying in *Lissotriton* newts that arrive early than late to Llysdinam Pond. Finally it was hypothesised that (iv) frequency of courtship behaviour and egg-laying is positively associated with temperature.

5.3 METHODS

5.3.1 The outdoor breeding tanks

5.3.1.1 Location

The outdoor breeding tanks were set up in the woodland 50 m north-west from Llysdinam Pond. The site was chosen due to its accessibility from the field centre for evening torch surveys, and access to laboratory and pond. The location of the tanks under the tree canopy provided shelter which minimised freezing and overheating of the water in the tanks. The choice of location was made to avoid large temperature fluctuations since tanks had a low water volume relative to the pond. (For a diagram of the layout of the tanks in the woodland see Appendix VIII).

5.3.1.2 Outdoor breeding tank design

Due to limited availability, two different types of tanks were used for the study. Tanks used were either constructed from 10 large rectangular garden planters or were clear plastic storage boxes. The 10 rectangular garden planters (200 x 50 cm and a depth of 30 cm) were partitioned using plywood and building sealant, to make 20 replicate tanks (Figure 5.2.) The tanks were therefore 100 x 50 x 30 cm. A green plastic lip (made of newt guard) was added to the top of the tanks using duck tape to prevent newt escape. The storage boxes were 50 x 40 cm and a depth of 30 cm, with box lids which were used to prevent newt escape. The lids had several holes drilled in them to aid ventilation.

Figure 5.2 Newt outdoor breeding tanks

Outdoor breeding tanks were used to study courtship and egg-laying phenology of *Lissotriton* newts arriving at Llysdinam Pond in 2006 and 2007. Tanks were located in the mixed deciduous-coniferous woodland to the west of Llysdinam Pond. Two male and two female newts were allocated to each tank, and courtship behaviour and egg-laying was studied for several weeks. Palmate and smooth newt breeding was studied individually in separate tanks. Each outdoor breeding tank contained green and transparent egg-laying mops, and sweet chestnut leaves for benthic cover.

Each tank had fallen sweet chestnut leaves (20 leaves in large tanks, 10 in small) for benthic cover and newt refugia and a piece of wood 5 x 20 cm to enable newts to leave water. Water was filled to approximately 25 cm depth in tanks. To prevent the tank becoming stagnant, once every two weeks half the water in each tank was removed and the tanks topped up by hosepipe. Twice weekly Llysdinam and a nearby pond were sampled by a sweep net (30 cm diameter) to provide small invertebrates for newt prey. The netted invertebrates were divided evenly amongst the tanks. Newts are unselective in prey choice with a diet equivalent to the proportions of invertebrates found in the aquatic habitat (Griffiths, 1986). Large predatory invertebrates were removed from the sample. The small aquatic invertebrates provided were therefore equivalent to the prey available in the ponds at each sample session.

5.3.1.3 Temperature recording and tank location

Brannan maximum and minimum thermometers were placed in the outdoor breeding tanks to enable comparison of water temperatures between tanks and the pond. They were checked twice each week. Air temperatures from the weather station located 50 m south-

west of Llysdinam Pond were also obtained from the British Atmospheric Data Centre (BADC) website (UK Meteorological Office, 2008b).

5.3.1.4 Egg-laying mops

Egg-laying mops made from strips of polythene bags were provided in the tanks as an artificial substrate for egg deposition. They also provided refugia additional to the chestnut leaves. Similar strips of various colours have been used in previous newt research (Arntzen and Hedlund, 1990; Miaud, 1993; 1994), and were a suggested methodology for great crested surveying by English Nature (2001). Four green and four transparent egg-laying mops were provided in the large tanks and two of each type in the smaller tanks. Transparent egg-laying mops were used because eggs would be more easily detected on them. Green and transparent mops were made from a double layered piece of polythene approximately 20 cm wide by 22 cm length. Green egg-laying mops were made from garden refuse bags, bought from the same manufacturer for consistency of colour and texture. Transparent egg-laying mops were made from one transparent bag (20 x 28 cm from Polybags Ltd) and was cut into strips and constructed by the same method. Each piece was cut into approximately 8 mm strips and held together with duct tape at the one end to make an egg-laying mop. The mops were a suitable substrate for oviposition by *Lissotriton* newts that prefer to oviposit on flexible thin supports (Miaud, 1995). Each mop was weighted by a stone to the base of the tank, with the polythene strips floating freely in the water column.

5.3.2 Newt collection and allocation in study

From January-April in 2006 and 2007, palmate and smooth newts were collected for the study. When there were large migrations of newts towards the pond from January to April in each year, several newts were kept in order to monitor their courtship and egg-laying behaviour. In both years collection dates were separated by at least 17 days. Each collection was termed a batch (Table 5.1). No newts that were captured exiting the pond were used in the breeding study. Two male newts and two female newts of the same species were allocated to each tank. The number of batches that it was possible to set up varied according to migration peaks and the newt population size in each year. The number of newts taken for the study was minimised to reduce impact on the natural breeding of the population. In 2006 five palmate and four smooth newt batches were set up (of varying number of replicates). In 2007 only three batches of each species were used since only a small number of newts arrived to the pond in March.

Table 5.1 Newt collection dates in 2006 and 2007

From January-April in 2006 and 2007, sub-samples of palmate and smooth newts that arrived at the Llysdinam Pond drift fence were allocated to the outdoor breeding tank study. Each set of newts allocated to outdoor breeding tanks were named as a batch. Two male newts and two female newts of the same species were allocated to each tank. The number of batches that it was possible to set up varied according to migration peaks and the newt population size in each year. In 2006 five palmate and four smooth newt batches were set up (of varying number of replicates). In 2006 too few smooth newts arrived in April to enable a Batch 5 to be set up. In 2007 only three batches of each species were used since only a small number of newts arrived to the pond in March. Capture date = date of capture at Llysdinam Pond drift fence. Day of year into tanks = day that newts from each batch were allocated to outdoor breeding tanks.

Batch	Year	Capture date	Capture day	Day of year into tanks	Number of tanks set up (2 M and 2 F in each)	
					Palmate	Smooth
1	2006	19-20 January	19-20	20	10	3
2		13-14 February	44-45	46	10	6
3		9 March	68	72	10	6
4		28 March	87	88	10	6
5		13-17 April	104-107	108	7	0
1	2007	13-14 January	13-14	14	8	3
2		17-18 February	48-49	53	10	10
-		Low inward migration	-	-	-	-
-		Low inward migration	-	-	-	-
5		15 March-15 April	74-105	106	3	10

In 2006 newts were allocated to tanks according to their total length and body mass. When enough newts were captured at the drift fence to set up a batch for the study, the lengths and mass of each newt were measured (Chapter 2, Section 2.3.2). The newts were allocated into one out of three categories: small, medium or large size based on their mass and total length. Since mass and body condition were highly correlated (Chapter 2, Section 2.3.4.6) newts in the large category were generally of better body condition. There was a large range in newt size within the two categories dependent on the morphology of the newts arriving at the drift fence. Only newts in the small and large size category were randomly selected and allocated to tanks (Table 5.2). Newts categorised as medium sized were released into Llysdinam Pond.

Table 5.2 Total lengths of newts in the 2006 size categories

Range of newt sizes allocated to outdoor breeding tanks from January-April in 2006. On arrival newts were allocated into one out of three categories: small, medium and large total length. There was a large range in size within the categories dependent on the morphology of the newts arriving at the drift fence. Only newts within the small and large size category were randomly selected and allocated to tanks. All newts in the medium size category were released into Llysdinam Pond.

	Small newts (total length mm)	Large newts (total length mm)
Male palmate	57-65	67-77
Female palmate	66.5-74	78 -85
Male smooth	68-78	82-93
Female smooth	67-78	80-96

In 2007 newts were randomly assigned to tanks and not assigned to separate size categories. In 2006 palmate newts were studied in the large tanks and smooth newts in the small tanks. In 2007 the palmate and smooth newts were divided equally among the tank types. The newts were studied on a rotation system, as batches could not be studied simultaneously due to space constraints, and the length of time required for tank maintenance and surveys. Therefore early batches were released into holding tanks once later batches arrived. This was a precaution against affecting the study of egg-laying phenology in Llysdinam Pond (Chapter 4). Tanks were numbered in pairs and randomly assigned to the alternative batches (see Appendix VIII).

5.3.3 Recording courtship behaviour in tanks

Surveys for newt activity and courtship behaviour in the tanks were conducted by pairs of people on three evenings per week for 18 weeks from 19/1/2006 until 25/5/2006. Surveys were conducted approximately one hour after sunset (www.timeanddate.com). 500 000 candlepower torches (Cluson Smartlite SM, 610) were used.

Distances between each of the four newts in each tank were recorded on a grid that represented the tank (Figure 5.3). The distances between individual newts observed in the tanks were used as a measure of interaction and interest and named the 'inter-newt distance'. Frequencies of specific courtship display behaviours and females ovipositing on the egg-laying mops were annotated on the grids. Notes were also made of newts that looked like they were interacting but were not showing specific aspects of courtship (Table 5.1). During preliminary recordings, it was found that recording newt activity on a grid representing each tank was an efficient method. It enabled information on each newt position to be quickly recorded from multiple tanks. Speed of recording was important

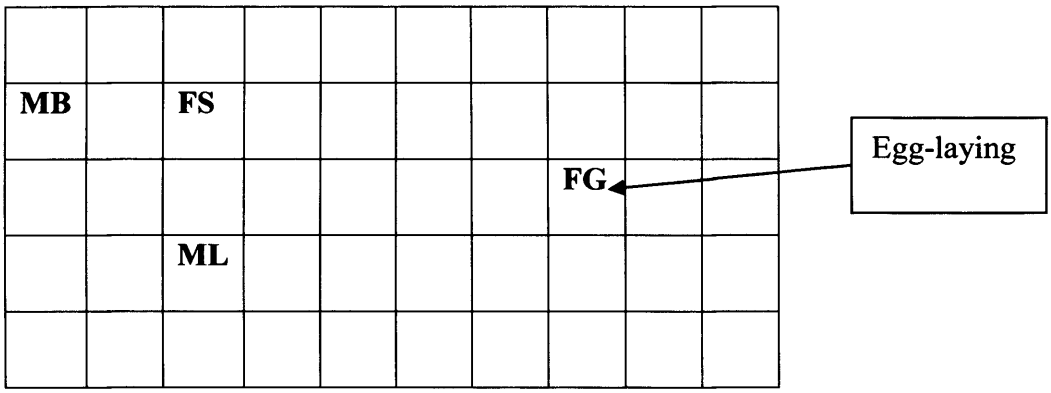
since newts often hid or changed position once torchlight was shone on the tanks. Distances between newt codes on the grid were used to calculate the 'inter-newt distance'.

Newt locations were recorded by a two or three letter codes representing sex and location.

- **Sex:** F = Female, M = Male
- **Location of newt:**

G = green egg-laying mop, T = transparent egg-laying mop, B = on tank bottom, L = in the leaves, S = surface of water, W = on the wood, Sw = swimming in mid water.

Figure 5.3 Example of a newt grid with data from one tank on one evening.
Positions of newts in each of the outdoor breeding tanks were recorded on a grid. Data were collected on evening torchlight surveys three times per week. A separate recording grid was used to record data for each tank. The positions of the newts within the tank were recorded by a code. M = Male, F = Female, B = on tank bottom, S = on surface of water, L = in the leaves, G = on the green egg-laying mop. Specific behaviour such as tail fanning by males or egg-laying by females was annotated on the grid.



Each 1 x 1 cm square within the grid represented a 10 x 10 cm square in the tank. Large rectangular garden planter tanks therefore had 5 x 10 squares and small storage box tanks had 4 x 5 squares. Courtship behaviour in the pond was surveyed on the same nights as the tanks using the 500 000 candlepower torches (Chapter 4, Section 4.3.1).

5.3.4 Recording oviposition in tanks

Surveys of the tanks for commencement of egg-laying and egg numbers took place approximately every seven days. When egg numbers were at a peak, the survey of all tanks would take up to 10 man hours to complete. Each egg-laying mop was removed from the water and checked on a plastic tray and the number of eggs recorded for each mop. Mops with eggs were removed from the tank and placed in developmental tanks (Chapter 6, Section 6.3.1). Mops which had not been used for egg-laying were left in the tank. Replacement mops were put in tanks to keep the number of mops per tank constant.

5.3.5 Data analysis

5.3.5.1 Temperature data

The relationship between temperatures recorded in Llysdinam Pond and air temperature from the Llysdinam weather station were assessed by correlation analysis. Friedman's tests were conducted to check that there were no significant differences in water temperatures of the outdoor tanks.

5.3.5.2 Courtship

Since palmate and smooth newts were held in two sizes of tank in 2006, courtship analysis was conducted separately for each species. Few observations of specific courtship behaviours were recorded, therefore statistical analyses of the timing of courtship behaviour and courtship frequency were not possible. Instead one-way ANOVA in Minitab 15 compared 'inter-newt distances' between batches after a duration of 1-6 weeks in tanks. Males and females on the courtship recording sheet were given codes M1, M2, F1 and F2 with M1 and F1 assigned to the newts that were the shortest distance from each other in the tank. All male-female distances (M1-F1, M1-F2, M2-F1 and M2-F2) were compared in the analysis. Kruskal-Wallis tests were used when data did not conform to parametric assumptions of normality or homogeneity of variance (Bowker and Randerson, 2008).

Repeated measures analysis with General Linear model in SPSS was used to compare 'inter-newt distances' for M1-F1. Batches held in tanks concurrently over time were compared, and within and between subject effects were detected. Comparisons were therefore conducted between Batch 1 and 2, Batch 2 and 3, Batch 3 and 4 and Batch 4 and 5. There were problems with missing values resulting from newts not being spotted and recorded on survey nights. Repeated measures GLM could not be performed on tanks with missing values, therefore nights with several missing M1-F1 values were removed before analysis (Dytham, 2003). Survey dates used for analysis contained at least three replicate tanks per batch. There were too many missing values to compare M1-F2 or M2 newts by Repeated measures analysis in GLM.

Correlation analysis in Minitab 15 was used to investigate relationships between the indicators of breeding phenology and success (egg numbers and male-female inter-newt distance). Data were checked for a normal distribution and Spearman's rank correlation

used where data did not meet the assumptions of Pearson's correlation (Bowker and Randerson, 2008).

5.3.5.3 Egg-laying

Tanks where no eggs were laid by the pair of females across the entire survey period were omitted from statistical analysis (Table 5.3). Comparisons of egg-laying phenology and number of eggs laid between body size categories for palmate and smooth newts were conducted by one-way ANOVA and Kruskal-Wallis. T-tests and Mann-Whitney tests were carried out to check there were no differences in egg-laying between the two tank sizes. Since no significant differences were found (Section 5.4.3.2 and 5.4.3.3) the data from all categories were analysed together.

Table 5.3 Number of replicates of outdoor breeding tanks used to investigate egg-laying in newts

In 2006 and 2007 samples of newts arriving at Llysdinam Pond were allocated to outdoor breeding tanks to study courtship and egg-laying phenology. Eggs were not laid by all female newts during each study period and these tanks were omitted from statistical analysis. Dates of newt allocations to tanks were for 2006: Batch 1 = 19-20 January, Batch 2 = 13-14 February, Batch 3 = 9 March, Batch 4 = 28 March and Batch 5 = 13-17 April. In 2006 too few smooth newts were captured in April to set up a Batch 5. In 2007 not enough newts arrived in March to set up a Batch 3 and 4. Newt allocations in 2007 were: Batch 1 = 13-14 January, Batch 2 = 17-18 February and Batch 5 = 15 March-15 April.
N = total number of tanks set up for study
N* = number of tanks in batch where no eggs were laid by the pair of females in the tank so tanks were omitted from statistical analysis.

	2006 Palmate		2006 Smooth		2007 Palmate		2007 Smooth	
	N	N*	N	N*	N	N*	N	N*
Batch 1	10	**4**	3	**3**	8	0	3	0
Batch 2	10	0	6	**6**	10	0	10	3
Batch 3	10	0	6	0	No batch set up		No batch set up	
Batch 4	10	0	6	0	No batch set up		No batch set up	
Batch 5	7	0	No batch set up		3	**1**	8	**5**

Four egg-laying criteria were used to compare egg-laying phenology and frequency between batches. The criteria were chosen partially because they enabled comparisons between tanks with few missing values to affect the analysis.

The criteria were:

- Daily egg-laying rates.
- Numbers of eggs laid by the two female newts within the first 30 days and first 50 days that newts were in the tanks.
- Number of days from arrival until a cumulative egg frequency of 10, 20 50 and 100 eggs had been reached by the two female newts. Therefore, the delay between arrival and egg-laying.
- Date (day of year) by when 10, 20, 50 and 100 eggs had been laid by the two female newts in each tank.

For egg numbers, batches with at least three replicate tanks were analysed for differences between batches by one-way ANOVA and Kruskal-Wallis in Minitab 15. For analysis of delay and date of egg-laying, batches with at least five replicate tanks were used. Post-hoc multiple comparisons by Tukey-Kramer tests after one-way ANOVA indicated which batches differed significantly. Pair wise comparisons by Mann-Whitney U tests in SPSS were conducted to indicate significances between batches after non parametric Kruskal-Wallis tests. The Bonferroni correction was applied to multiple comparisons.

5.4 RESULTS

5.4.1 Temperature

5.4.1.1 Comparison of tank temperatures with pond and weather station

Tank temperatures correlated strongly with pond temperature ($r = 0.878$ d.f = 398, $p = 0.02$) and with data from the weather station located 50 m south-west of Llysdinam Pond ($r = 0.861$, d.f = 398, $p = 0.013$). Since weekly means could be calculated from the weather station data, these were used for temperature analyses.

5.4.1.2 Comparison of water temperatures between tanks

There were some significant temperature differences between tanks (minimum temperature S = 58.22, d.f = 19, $p = <0.001$, maximum temperature S = 93.81, d.f = 19, $p = <0.001$, mean temperature S = 105.17, d.f =19, $p = <0.001$). Comparison of means and confidence intervals showed the significant results resulted from just six overall differences between tanks for minimum temperature, two differences for maximum temperature and eleven differences for mean temperature. The differences were distributed between batch categories. Tanks with significantly lower or higher temperatures were checked for

differences in egg-laying rates between them, and there were no significant difference for palmate newts (W = 576, d.f = 58, p = 0.616) or smooth newts (W = 581.5 d.f = 108, p = 0.70).

5.4.2 Courtship behaviour and interactions

Unfortunately, too few observations of courtship activity were made for statistical comparisons to be made, although statistical comparisons were made on the plotted inter-newt distances in tanks.

5.4.2.1 Observations of courtship behaviour and breeding activity

For palmate newts observations of courtship activity or male-female interactions were seen in all batches in 2006 (Table 5.4). For smooth newts, courtship display and observations of egg-laying were only seen in Batch 4 so comparisons of timing of behaviours could not be compared against arrival time (Table 5.5). In palmate newts earlier arrivals were seen interacting at an earlier date (Day 34 of year), but Batch 3 and 4 were in tanks the shortest duration of time (5 or 7 days respectively) when male-female interactions were first observed. Aspects of courtship behaviour and the obvious courtship tail fanning display by male palmate newts were not observed for Batch 1 and 2.

Table 5.4 Palmate newt courtship interactions observed in outdoor breeding tanks

Days when specific palmate newt courtship behaviours were observed in outdoor breeding tanks are indicated. Newts were allocated to outdoor breeding tanks soon after their capture day at Llysdinam Pond drift fence in 2006. Dates of newt allocations to tanks were for 2006: Batch 1 = Day 20, Batch 2 = Day 46, Batch 3 = Day 68, Batch 4 = Day 88 and Batch 5 = Day 108. Observations were made by torchlight surveys in outdoor breeding tanks. Numbers in bold = date (day) of 2006 when the observation was made. Italicised and bracketed numbers = the number of days that the newts had been in tanks when the observation was made. Frequencies of courtship and egg-laying are provided.

	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Day when male-female interaction first observed	**34** *(13 days)*	**60** *(16 days)*	**81** *(5 days)*	**94** *(7 days)*	**124** *(18 days)*
Day when male-female courtship behaviour first observed	-	-	**96** *(20 days)*	**116** *(29 days)*	**124** *(18 days)*
Day when tail fanning first observed	-	-	**99** *(23 days)*	**121** *(34 days)*	-
Number times courtship observed	-	-	2	4	1
Day when egg-laying first observed	-	-	**99** *(23 days)*	**96** *(7 days)*	**127** *(21 days)*
Number of times egg-laying observed	-	-	2	9	1

Table 5.5 Smooth newt courtship interactions observed in tanks for Batch 4

Days when specific smooth newt courtship behaviours were observed in outdoor breeding tanks for Batch 4 are indicated. Courtship behaviour in smooth newts was only observed for Batch 4. Newts allocated to Batch 4 arrived at Llysdinam Pond Day 87 of 2006 (28 March) and were allocated to tanks on Day 89. Observations made by torchlight surveys in outdoor breeding tanks. Numbers in bold = the date (day) of 2006 when the observation was made. Italicised and bracketed numbers = the number of days the newts had been in tanks when observation was made. Frequencies of courtship and egg-laying are provided.

	Batch 4
Day when M-F interaction first observed	**101** *(14 days)*
Day when M-F courtship behaviour first observed	**101***(14 days)*
Number times courtship observed	2
Day when tail fanning first observed	**127** *(40 days)*
Day when egg-laying first observed	**110** *(23 days)*
Number of times egg-laying observed	1

5.4.2.2 Differences in male and female inter-distances newt between batches

The inter-newt distances between males and female newts (M1-F1, M1-F2, M2-F1, M2-F2) in outdoor breeding tanks at 1-6 weeks were compared by Kruskal-Wallis tests. Repeated measures analysis on batches that were concurrently in tanks was used to compare between M1-F1 inter-newt distances between weeks. Both analyses demonstrated a large variance in values, but all differences were non significant for palmate newts and smooth newts once the Bonferroni correction was applied.

5.4.2.3 Relationship between newt interactions and egg-laying

Significant differences were found in egg-laying data between batches and it was apparent that arrival time had an affect on breeding phenology (Section 5.4.3). This suggested that the courtship survey technique was not a good indicator of breeding phenology. Correlation analysis between the two indicators of breeding used (M-F inter-newt distances and egg numbers) showed mostly highly non-significant correlations, and all correlations were non-significant once the Bonferroni correction was applied. There were no significant relationships between male and female palmate 'inter-newt distances' (at 7, 14, 21, 28 and 35 days in tanks) and number of eggs laid by Day 30 and 50. It was therefore concluded courtship was not an ideal methodology for monitoring breeding behaviour in tanks and was not repeated in 2007.

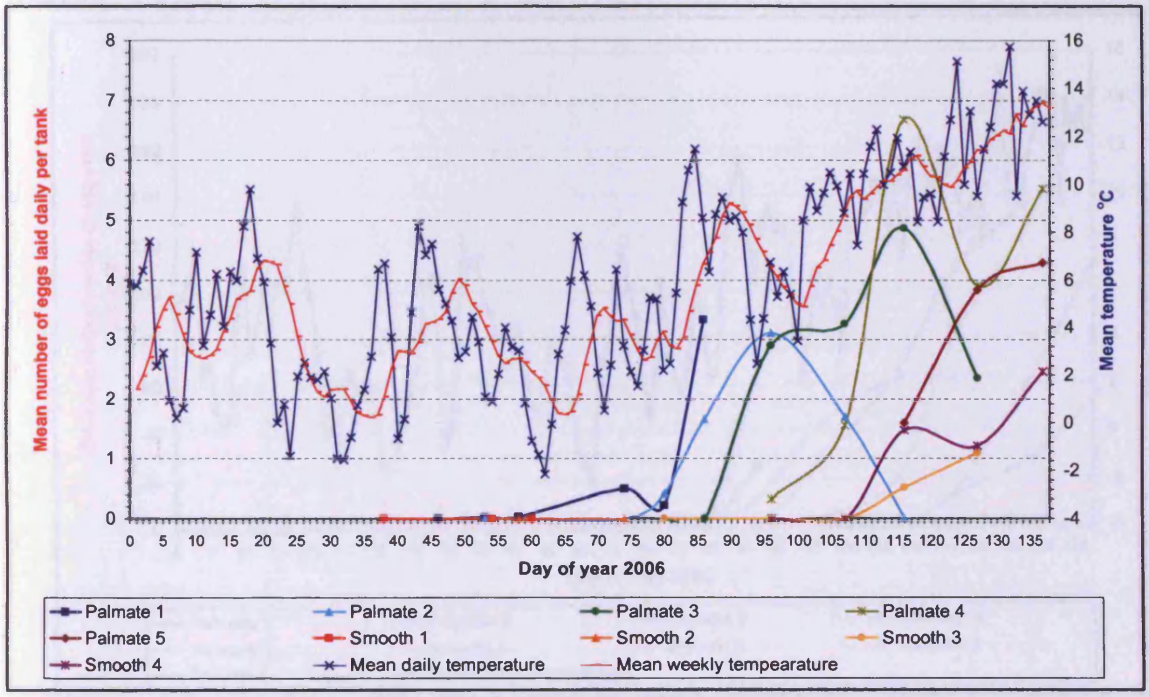
5.4.3 Egg-laying

The daily egg-laying rates per tank (by two females) were calculated by mean total number of eggs found per survey divided by the number of days between surveys (Figure 5.4, Figure 5.5 and Table 5.6).

Figure 5.4 Mean daily egg-laying rates per tank in 2006

Mean daily egg-laying rates for each batch were calculated from number of eggs found in each outdoor breeding tank divided by number of days between searches. Mean numbers of eggs laid by the two female *Lissotriton* newts allocated to each tank are indicated on the first y axis. Batches are represented in the key by species names (e.g. Palmate 1, Smooth 1 = Batch 1). (i) Dates of newt allocations to tanks in 2006 were: Batch 1 = Day 20, Batch 2 = Day 46, Batch 3 = Day 68, Batch 4 = Day 88 and Batch 5 = Day 108. No eggs were laid by smooth newts in Batch 1 and 2 of 2006. Due to the arrival of only a small number of smooth newts in April 2006 there was not a Smooth newt Batch 5. (ii) Dates of newt allocations to tanks in 2007 were: Batch 1 = Day 14, Batch 2 = Day 53 and Batch 5 = Day 106. Due to low numbers of newts that arrived in March 2007 there was not a Batch 3 and 4 in 2007 for either species. Mean weekly air temperatures were calculated as a running mean from mean daily air temperature (°C) and are indicated on the second y axis.

(i) 2006



(ii) 2007

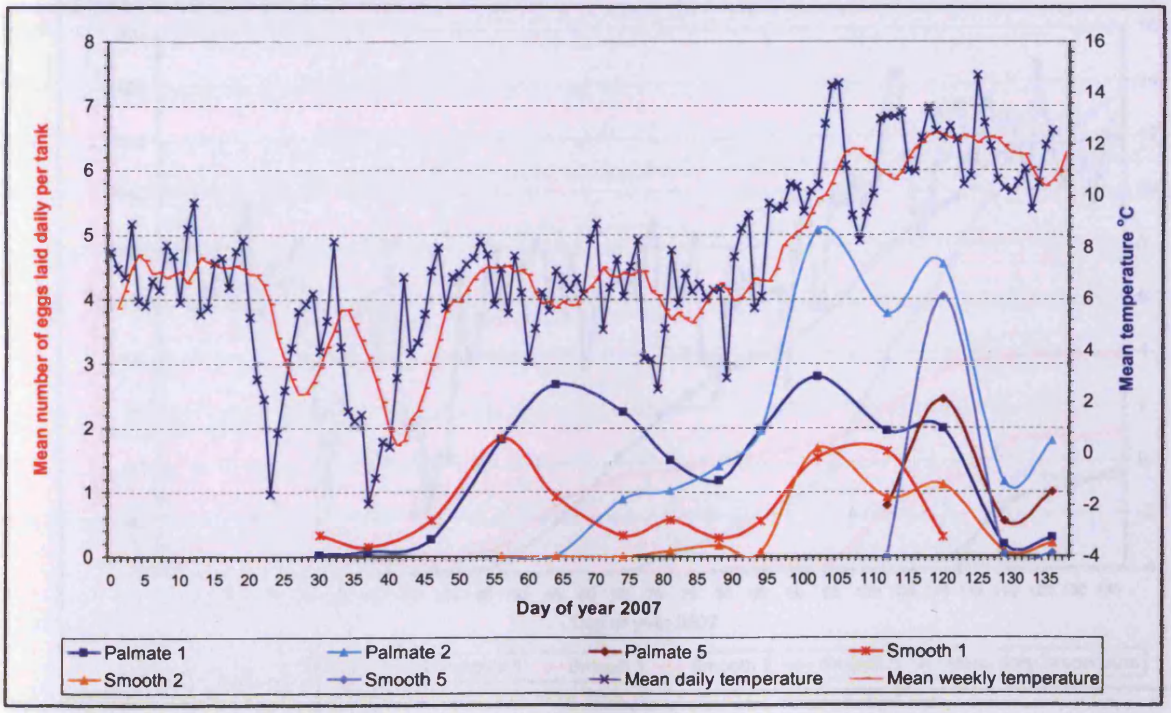
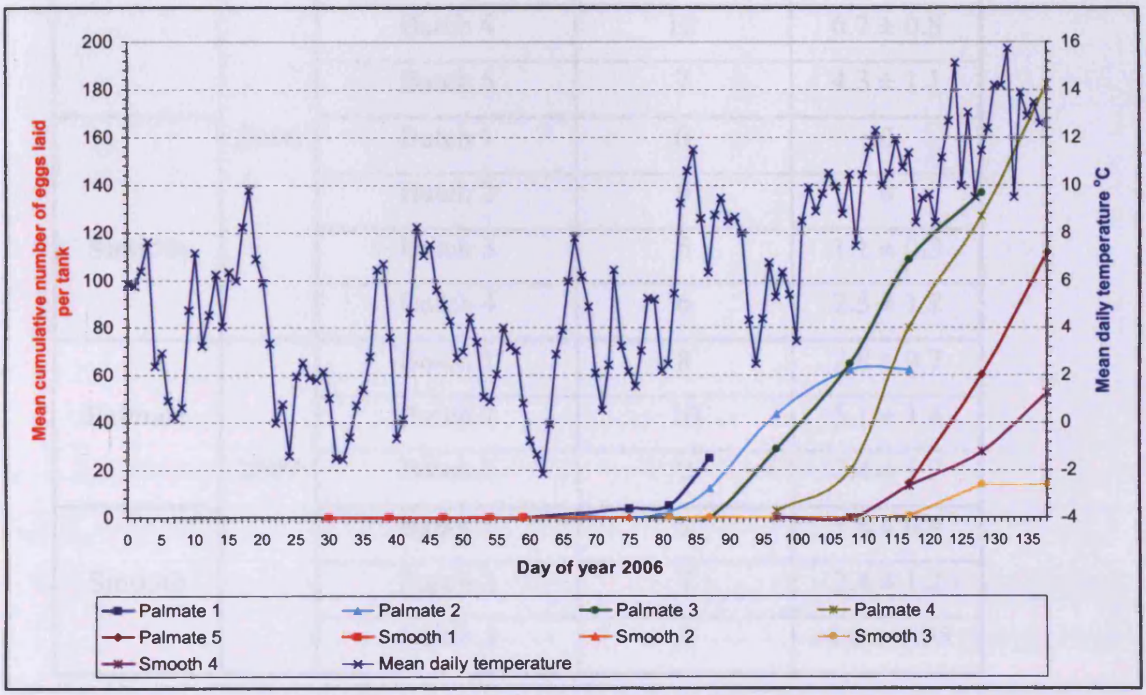


Figure 5.5 Mean cumulative number of eggs laid per tank in 2006

The mean number of eggs laid for each batch is shown as a cumulative frequency of the mean number of eggs found in each outdoor breeding tank. Mean numbers of eggs laid were for the two female *Lissotriton* newts allocated to each tank and are indicated on the first x axis. Batches are represented in the key by species names (e.g. Palmate 1, Smooth 1 = Batch 1). (i) Dates of newt allocations to tanks in 2006 were: Batch 1 = Day 20, Batch 2 = Day 46, Batch 3 = Day 68, Batch 4 = Day 88 and Batch 5 = Day 108. No eggs were laid by smooth newts in Batch 1 and 2 of 2006. Due to the arrival of only a small number of smooth newts in April 2006 there was not a Smooth Batch 5. (ii) Dates of newt allocations to tanks in 2007 were: Batch 1 = Day 14, Batch 2 = Day 53 and Batch 5 = Day 106. Due to low numbers of newts that arrived in March 2007 there was not a Batch 3 and 4 in 2007 for either species. Mean daily air temperatures (°C) were calculated as an average of the daily minimum and maximum temperature and are indicated on the second y axis.

(i) 2006



(ii) 2007

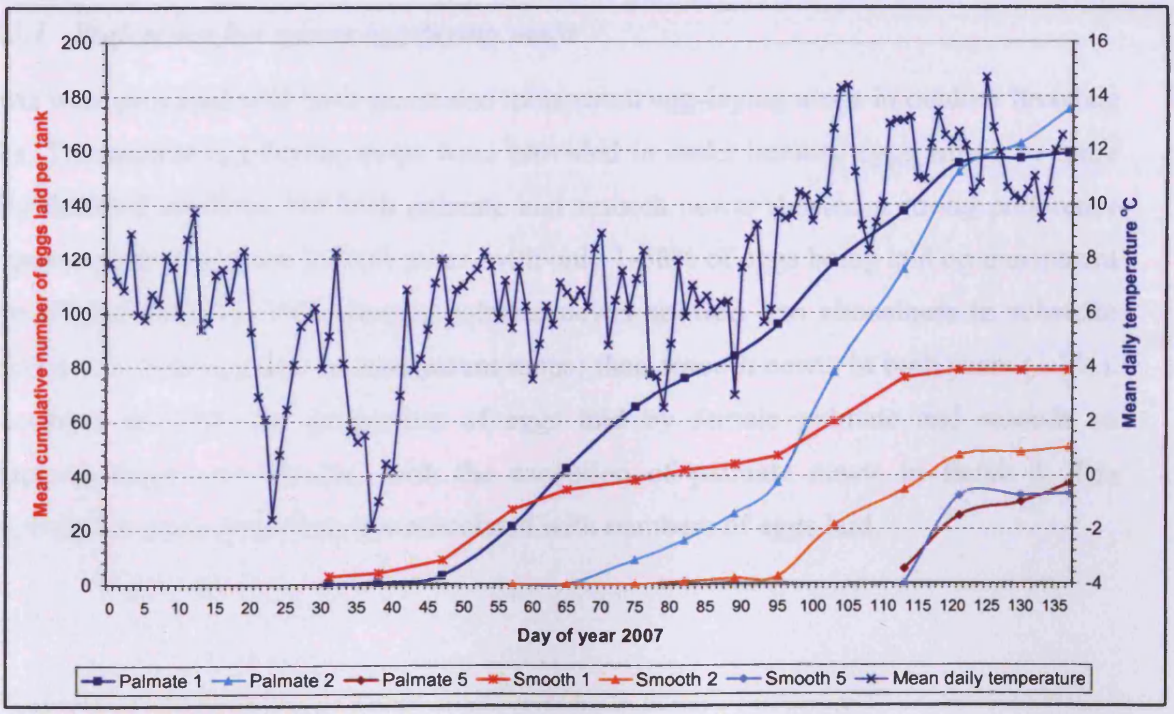


Table 5.6 Variability in egg-laying rates in outdoor tank study

Mean ± SE peak daily egg-laying rates are indicated for each batch of newts allocated to outdoor breeding tanks. N = number of tanks in which egg-laying was recorded. Number of eggs laid was for the two female *Lissotriton* newts allocated to each tank. Due to a small number of smooth newts that arrived in April 2006 there was not a Smooth newt Batch 5. Due to low numbers of newts arriving in February-March 2007 there were no Batch 3 and 4 in 2007. No eggs were laid by smooth newts in Batch 1 and 2 of 2006.

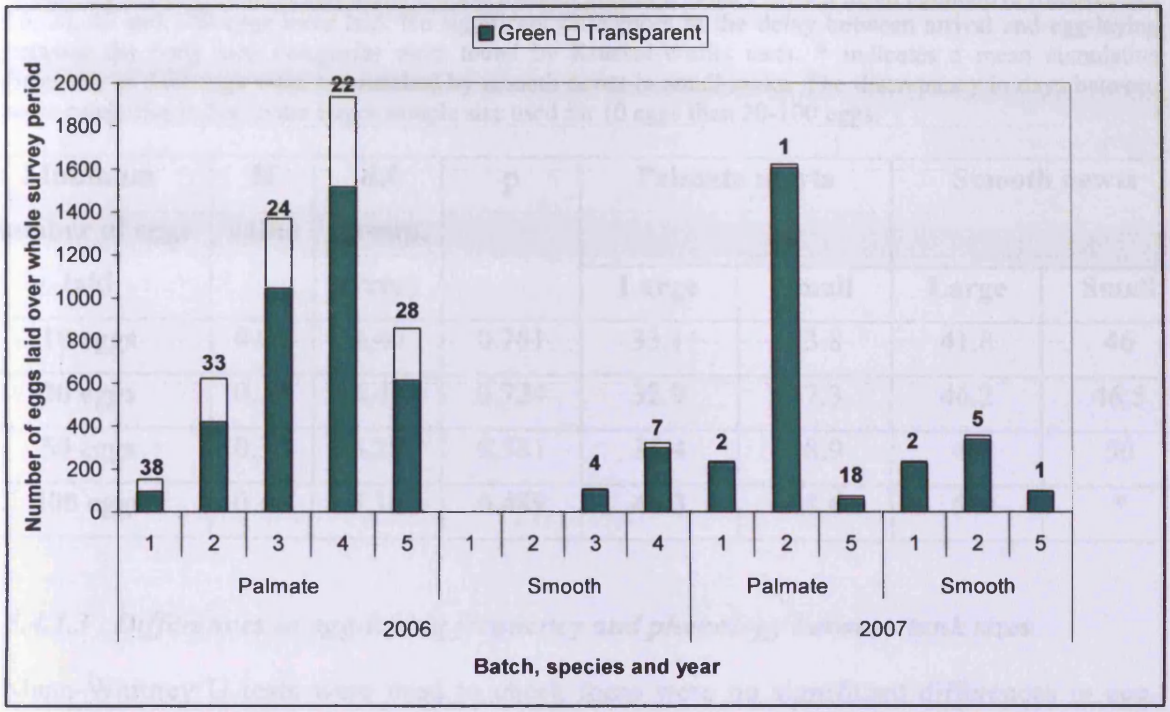
Species	Year	Batch name	N	Mean ± SE
Palmate	2006	Batch 1	8	2.5 ± 0.9
		Batch 2	10	3.1 ± 0.6
		Batch 3	10	4.9 ± 0.8
		Batch 4	10	6.7 ± 0.8
		Batch 5	7	4.3 ± 1.1
Smooth		Batch 1	0	0
		Batch 2	0	0
		Batch 3	6	1.1 ± 0.3
		Batch 4	6	2.5 ± 1.2
Palmate	2007	Batch 1	8	2.8 ± 0.7
		Batch 2	10	5.1 ± 1.4
		Batch 5	2	2.4 ± 1.7
Smooth		Batch 1	3	1.8 ± 0.9
		Batch 2	7	2.4 ± 1.2
		Batch 5	3	4.0 ± 1.95

5.4.3.1 Preference for 'green egg-laying mops'

Newts were provided with both green and transparent egg-laying mops in outdoor breeding tanks. Transparent egg-laying mops were provided in tanks because eggs would be more easily detected on them, but both palmate and smooth newts showed a strong preference for green egg-laying mops in both years, with only 1-38% of eggs being laid on transparent mops (Figure 5.6). In 2006, female palmate newts showed less choosiness in substrate selection (22-38% egg laid on transparent mops) than smooth newts in both years (1-7%). In contrast, in 2007 the proportion of eggs laid by female palmate and smooth on transparent mops was similar, with the exception of palmate newts in Batch 5. The selectivity for green mops was not associated with numbers of eggs laid.

Figure 5.6 Preference in egg-laying substrate in outdoor breeding tanks

Total number of eggs laid on green egg-laying mops and transparent egg-laying mops by newts in the outdoor breeding tank study. Numbers above the bar indicate the % of eggs laid on transparent mops.



5.4.3.2 Affect of newt size on frequency and phenology of egg-laying

On average large females laid more eggs than small females, but the results were non-significant. Smooth females oviposited under half the number of eggs of palmate females (Table 5.7). The mean delay between arrival and egg-laying was slightly less in large newts (for 10 eggs, a difference between the two size categories of 0.7 days for palmate newts and 4.2 days for smooth newts). The differences in delay between the two body size categories varied with numbers of eggs laid (Table 5.8)

Table 5.7 Differences in the number of eggs laid between newt body size categories

Mean ± SE number of eggs laid by small and large palmate and smooth newts after 30 and 50 days in outdoor breeding tanks. No significant differences in number of eggs laid between the two body size categories were found by Kruskal-Wallis tests.

Mean number of eggs laid within	H value	d.f (group, error	p	Palmate newts		Smooth newts	
				Large	Small	Large	Small
30 days	0.10	3,55	0.754	55.3 ± 14.6	40 ± 9.7	7.2 ± 4.7	6.2 ± 5.1
50 days	0.24	3,48	0.626	100.3 ± 22	81.2 ± 19	41.7 ± 22	30.8 ± 6.9

Table 5.8 Differences in the delay between arrival and egg-laying between newt body size categories

Mean ± SE number of days that newts were in outdoor breeding tanks before a mean cumulative frequency of 10, 20, 50 and 100 eggs were laid. No significant differences in the delay between arrival and egg-laying between the body size categories were found by Kruskal-Wallis tests. * indicates a mean cumulative frequency of 100 eggs were not reached by smooth newts in small tanks. The discrepancy in days between some categories is due to the larger sample size used for 10 eggs than 20-100 eggs.

Minimum number of eggs laid	H value	d.f (group, error)	p	Palmate newts		Smooth newts	
				Large	Small	Large	Small
10 eggs	0.09	3,49	0.761	33.1	33.8	41.8	46
20 eggs	0.12	3,43	0.724	32.9	37.3	46.2	46.5
50 eggs	0.30	3,29	0.581	36.4	38.9	45	50
100 eggs	0.48	3,18	0.489	40.3	38.6	50	*

5.4.3.3 Differences in egg-laying frequency and phenology between tank sizes

Mann-Whitney U tests were used to check there were no significant differences in egg-laying between the two sizes of outdoor breeding tanks used. For palmate newts although more eggs were laid in larger tanks (Table 5.9) and there was a greater delay before egg-laying in small tanks (Table 5.10), the difference was not significant between tank sizes. The opposite trend was found for egg-laying by smooth newts, as more eggs were laid in smaller tanks (Table 5.11) and there was a greater delay before egg-laying in larger tanks (Table 5.12). Again, the difference was non significant between tank size. Since data on egg-laying frequency and phenology did not differ significantly between either body size or tank size, the data were analysed together as one set.

Table 5.9 Influence of tank size on egg-laying in palmate newts

Mean ± SE number of eggs laid by palmate newts after 30 and 50 days in small and large tanks used in the outdoor breeding tank study. No significant differences in number of eggs laid between the two tank sizes were found by Mann-Whitney tests.

Mean number of eggs laid within	W value	d.f	p	Small tanks (50 cm length)	Large tanks (100 cm length)
30 days	34.5	8	0.171	7 ± 3.9	25 ± 11.6
50 days	34	8	0.210	49.4 ± 28.6	106.8 ± 37.7

Table 5.10 Differences in delay in egg-laying between tank size for palmate newts
Mean ± SE number of days that palmate newts were in outdoor breeding tanks before a mean cumulative frequency of 10, 20, 50 and 100 eggs were laid. No significant differences in the delay between arrival and egg-laying between the two tank sizes were found by T-tests.

Minimum number of eggs laid	T value	d.f	p	Mean number of days until eggs laid in small tanks	Mean number of days until eggs laid in large tanks
10 eggs	1.86	7	0.106	46.2 ± 6.4	30.4 ± 5.6
20 eggs	0.60	7	0.568	53.4 ± 6.7	47 ± 8.3
50 eggs	2.0	5	0.102	62.8 ± 7.2	46.8 ± 3.6
100 eggs	0.59	4	0.590	62 ± 6	57.3 ± 5.4

Table 5.11 Influence of tank size on egg-laying in smooth newts
Mean ± SE number of eggs laid by smooth newts in small and large tanks after 30 and 50 days. No significant differences in number of eggs laid between the two tank sizes were found. * indicates that there were not enough data at 30 days in tanks to conduct a Mann-Whitney test for differences in egg-laying between the two tank sizes.

Mean number of eggs laid within	W value	d.f	p	Small tanks (50 cm length)	Large tanks (100 cm length)
30 days	*	*	*	1.2	0
50 days	20.5	8	0.162	27 ± 16.4	4.2 ± 2.7

Table 5.12 Difference in delay in egg-laying between tank size for smooth newts
Mean ± SE number of days that smooth newts were in outdoor breeding tanks before a mean cumulative frequency of 10, 20, 50 and 100 eggs were eggs laid. T-tests found no significant differences in the delay between arrival and egg-laying were found between the two tank sizes.

Minimum number of eggs laid	T value	d.f	p	Small tanks (50 cm length)	Large tanks (100 cm length)
10 eggs	2.02	4	0.180	45.3 ± 8.1	67.3 ± 17
20 eggs	2.05	2	0.177	53 ± 5.8	64 ± 5.7

5.4.3.4 Egg-laying and temperature

Mean monthly temperatures in the period leading up to breeding were lower in 2006 than 2007 (Table 5.13).

Table 5.13 Mean monthly temperatures prior to and during the period when egg-laying commenced in 2006 and 2007

Mean ± SE monthly temperatures calculated from averages of the daily minimum and daily maximum temperatures. Temperature data obtained from Llysdinam weather station.

	November	December	January	February
2005-2006	4.8 ± 0.9	4.3 ± 0.5	3.9 ± 0.5	3.1 ± 0.4
2006-2007	6.1 ± 0.4	4.9 ± 0.6	5.8 ± 0.5	4.8 ± 0.6

Egg-laying in the earliest newt arrivals (Batch 1) began in both years after a period when mean daily air temperature had remained above 0°C for a period of 18 days in 2006 and 7 days in 2007 (Figure 5.4-5.5). Mean weekly air temperatures were above 2°C for three weeks prior to egg-laying in both years. Observation of scattergraphs of egg-laying rates plotted against temperature for each batch indicated that egg-laying rates were not strongly affected by temperature once egg-laying had commenced. There were no significant correlations between daily egg-laying rates and tank water temperatures or the weekly minimum and mean air temperature in the seven days prior to the egg surveys. Duration of time that the newts had been in the tanks had greater influence. A period of cold weather can cause a decline in egg-laying rates by female smooth newts as demonstrated by a cold period with snowfall from 3/2/2007-10/2/2007 (just after Batch 1 palmate and smooth newts had commenced egg-laying). The daily minimum air temperature remained below 0°C for eight consecutive nights and the daily mean air temperature was 2°C or less for seven consecutive days. The surface of the pond and the surface layer of water in the tanks froze. Mean daily egg-laying rates for smooth newts decreased from 0.33 eggs per day prior to the cold period to 0.14 eggs per day during the cold period, while palmate newts did not show a decline in egg-laying rates.

5.4.3.5 Egg-laying frequency

There was high variability in the numbers of eggs laid daily between batches and between replicate tanks (Figure 5.7). In 2006 no eggs were laid in the first 30 days by palmate newts in Batch 1 and 2, and smooth newts in Batch 3. No eggs were laid during the entire duration in tanks for smooth newts in Batches 1 and 2. In 2007 eggs were only found in two out of three palmate tanks that were set up for Batch 5 and there was a large variation

in the results (therefore the large confidence interval is not fully displayed in Figure 5.8). There was a significant difference in the number of eggs laid within the first 30 days in tanks ($H_{11,86} = 75.23$, $p = <0.001$, Figure 5.8) and within the first 50 days ($F_{9,73} = 14.32$, $p = <0.001$, square root transformed). Palmate newts in Batches 3, 4 and 5 laid significantly more eggs after 30 and 50 days than other batches (Table 5.14).

Figure 5.7 Variation in egg-laying rates per day over the time each batch were surveyed

Daily egg-laying rates for palmate and smooth newts in outdoor breeding tanks in 2006 and 2007. Daily egg-laying rates were calculated from the number of eggs laid by both of the two female newts allocated to each tank. Black circles represent means, diamonds represent medians, boxes represent middle half of data and vertical lines (whiskers) represent the lower and upper limits, while asterisks indicate outliers.

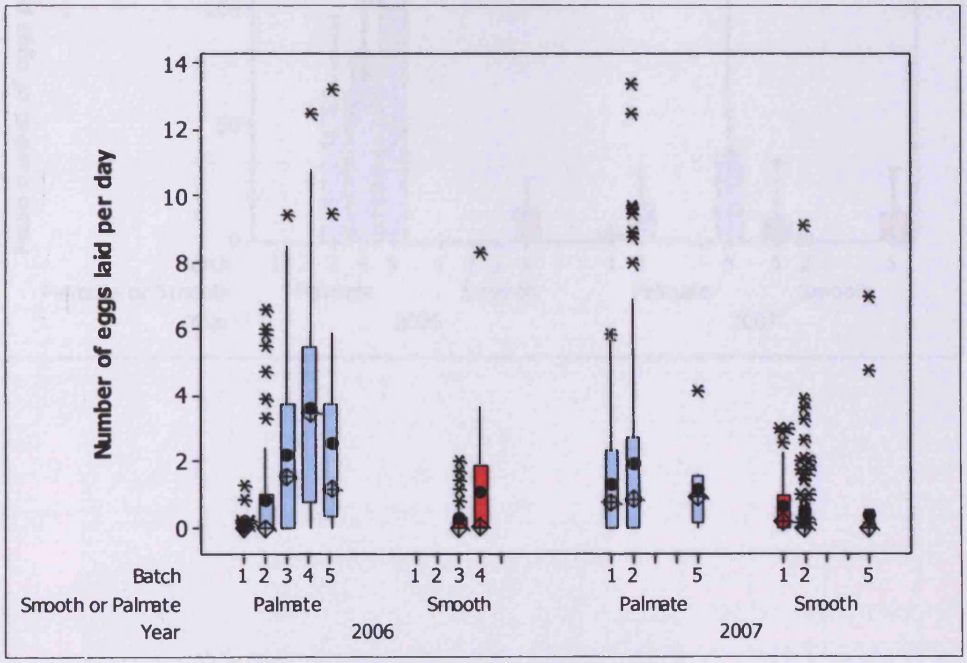


Figure 5.8 Number of eggs per tank laid within the first 30 days

Number of eggs laid by palmate and smooth newts during the first 30 days in tanks in 2006 and 2007. The number of eggs laid was for both of the two female newts allocated to each tank. Bars represent means, black diamonds represent medians and whiskers represent 95% confidence intervals. No eggs were laid during the entire duration in tanks for smooth newts in Batches 1 and 2 in 2006. In 2007 eggs were only found in two out of three palmate tanks that were set up for Batch 5 and there was a large variation in the results (therefore the large confidence interval is not fully displayed).

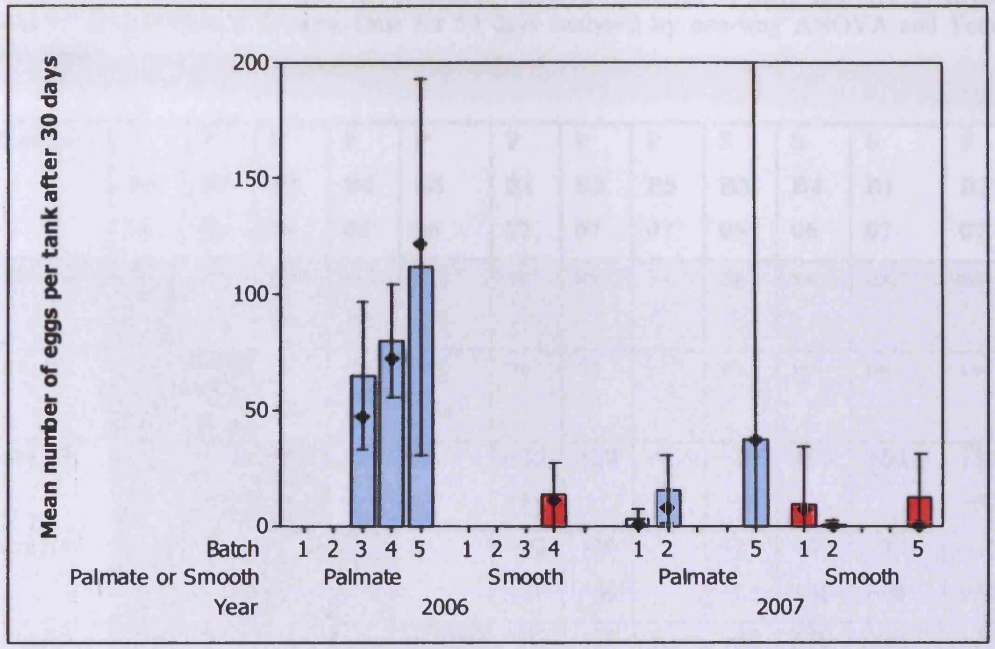


Table 5.14 Significant differences in egg numbers between batches

Significant differences in number of eggs laid between batches. There were a variable number of replicate tanks for each batch. Egg counts were for both of the two female newts allocated to each tank. A 30 in a cell indicates the cumulative number of eggs laid within the first 30 days in tanks differed significantly. A 50 in a cell indicates a significant difference in cumulative number of eggs laid after 50 days in tanks. A negative sign indicates the number of eggs laid was significantly lower for the batch represented in the row and a positive symbol indicates the number of eggs was higher. Egg were only found in two tanks for Batch 5 palmate newts in 2007, so data were not statistically testable. Data for 30 days analysed by Kruskal-Wallis followed by Mann-Whitney U tests. Data for 50 days analysed by one-way ANOVA and Tukey-Kramer post-hoc tests.

Batch name	P B1 06	P B2 06	P B3 06	P B4 06	P B5 06	P B1 07	P B2 07	P B5 07	S B3 06	S B4 06	S B1 07	S B2 07	S B5 07
Palmate B1 2006		ns	-30 -50	-30 -50	-30 -50	ns	ns	-	ns	ns	ns	ns	ns
Palmate B2 2006			-30 -50	-30 -50	-30 -50	ns	ns	-	ns	ns	ns	ns	ns
Palmate B3 2006				ns	ns	+30 +50	+30	-	+30 +50	+50	+50	+30 +50	+30 +50
Palmate B4 2006					ns	+30 +50	+30 +50	-	+30 +50	+30 +50	+30 +50	+30 +50	+30 +50
Palmate B5 2006						+30	+30	-	+30	+30	+30	+30	+30
Palmate B1 2007							ns	-	ns	ns	ns	ns	ns
Palmate B2 2007								-	ns	ns	ns	ns	ns
Palmate B5 2007	-	-	-	-	-	-	-		-	-	-	-	-
Smooth B3 2006								-		ns	ns	ns	ns
Smooth B4 2006								-			ns	ns	ns
Smooth B1								-				ns	ns
Smooth B2 2007								-					ns
Smooth B5								-					

5.4.3.6 Delay between arrival and egg-laying

Significant differences were found between batches in the delay in days that newts were in tanks before eggs were laid (for 10, 20, 50 and 100 eggs), 10 eggs ($H_{9,67}$ = 56.04, p = <0.001, Figure 5.9 and Table 5.15), 20 eggs ($H_{9,59}$ = 51.48, p = <0.001, Figure 5.10 and Table 5.15); 50 eggs ($F_{7,45}$ = 16.09, p = <0.001, square root transformed, Table 5.16) and 100 eggs (transformed by square root, $F_{7,28}$ = 22.06, p = <0.001). In 2006 there was a gradual decrease in the delay between arrival and egg-laying as the season progressed. In 2007 the data were limited due to less batches being set up. There was a high variability in the timing of egg-laying within batches (Figure 5.9). Smooth newts showed a longer delay between arrival and egg-laying than palmate newts in 2006. For palmate newts, Batch 1 had a significantly shorter mean delay between arrival and egg-laying in 2007 than in 2006. Batch 2 and 5 newts had a similar mean delay in egg-laying in both years. Earlier egg-laying phenology in 2007 for smooth newts was indicated by the delay for Batch 2 being similar to that for Batch 3 in 2006.

Figure 5.9 Variation in number of days newts spent in tanks before a minimum of 10 eggs were laid

Number of days that newts were in outdoor breeding tanks before a minimum of 10 eggs were laid in 2006 and 2007. No eggs were laid during the entire duration in tanks for smooth newts in Batches 1 and 2.
Black circles represents mean, diamonds represent median, boxes represent middle half of data and vertical lines (whiskers) represent the lower and upper limits, while asterisks indicate outliers.

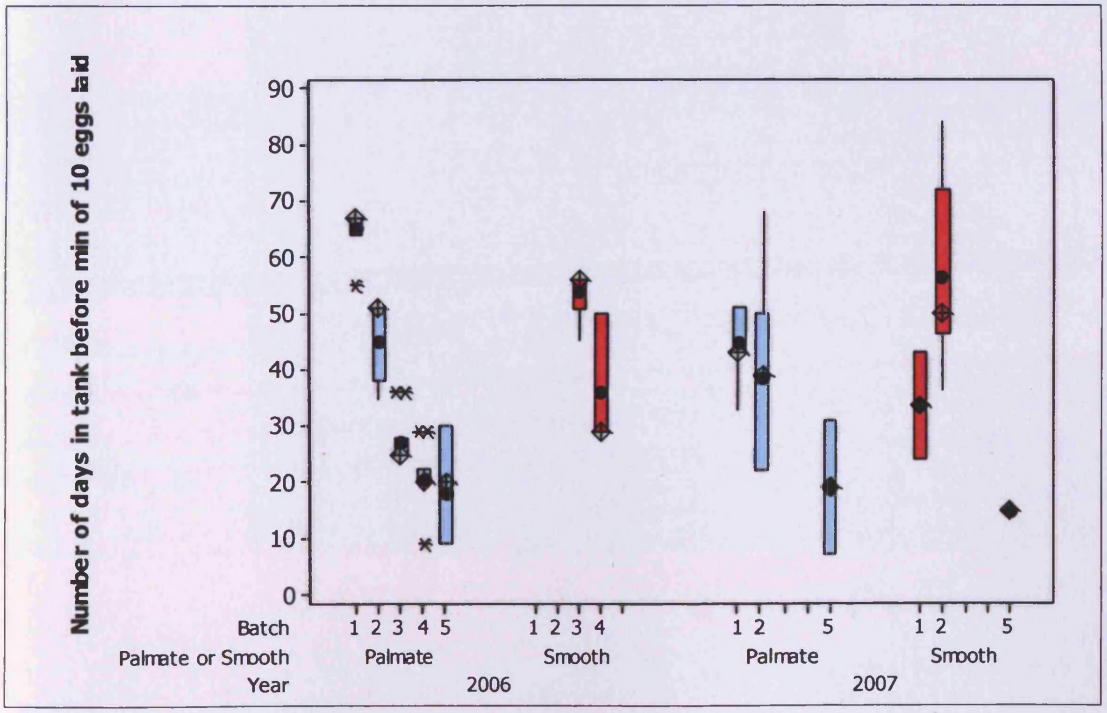


Figure 5.10 Number of days newts spent in tanks before a minimum of 20 eggs were laid

Number of days that newts were in outdoor breeding tanks before a minimum of 20 eggs were laid in 2006 and 2007. Bars represent mean. Black diamonds represent median and whiskers represent 95% confidence intervals. No eggs were laid during the entire duration in tanks for smooth newts in Batches 1 and 2.

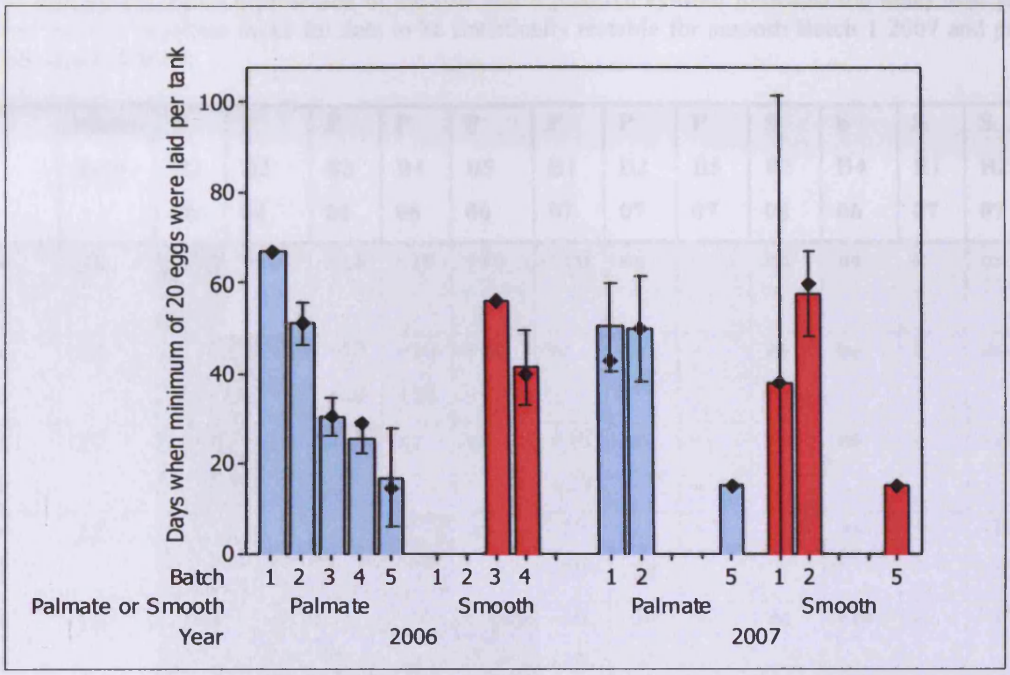


Table 5.15 Significant differences in egg-laying between batches

Significant differences in delay between arrival and egg-laying between batches of newts in 2006 and 2007. Data analysed by Kruskal-Wallis followed by Mann-Whitney U tests. Means displayed are for the number of days that newts were in tanks before a minimum of 20 eggs were laid. A 10 in a cell indicates a significant delay between arrival and a mean minimum of 10 eggs being laid per tank. A 20 in a cell indicates a significant delay between arrival and a mean minimum of 20 eggs laid per tank. A negative sign indicates the delay was less for the batch represented in the row and a positive symbol indicates the delay was greater. There were too few replicate tanks for data to be statistically testable for smooth Batch 1 2007 and palmate and smooth Batch 5 2007.

Batch name	Mean days	P B1 06	P B2 06	P B3 06	P B4 06	P B5 06	P B1 07	P B2 07	P B5 07	S B3 06	S B4 06	S B1 07	S B2 07	S B5 07
Palmate B1 2006	65		+10	+10	+10	+10	+10	ns	-	ns	ns	-	ns	-
Palmate B2 2006	45			+10 +20	+10 +20	+10 +20	ns	ns	-	ns	ns	-	ns	-
Palmate B3 2006	27				ns	ns	+10 +20	ns	-	-10	ns	-	-10	-
Palmate B4 2006	21					ns	-10 -20	-10 -20	-	-10	ns	-	-10 -20	-
Palmate B5 2006	18						-10 -20	-20	-	ns	-20	-	-10	-
Palmate B1 2007	45							ns	-	ns	ns	-	ns	-
Palmate B2 2007	38								-	ns	ns	-	ns	-
Palmate B5 2007	19	-	-	-	-	-	-	-		-	-	-	-	-
Smooth B3 2006	54										ns	-	ns	-
Smooth B4 2006	36											-	ns -20	-
Smooth B1 2007	33.5	-		-	-	-	-	-	-	-	-		-	-
Smooth B2 2007	56											-		-
Smooth B5 2007	15	-		-	-	-	-	-	-	-	-	-	-	

Table 5.16 Significant differences in delay for egg-laying 50 eggs between batches
Significant differences in mean number of days until a minimum of 50 eggs were laid by batches of palmate and smooth newts in 2006 and 2007. Data were transformed by square root and analysed by one-way ANOVA and Tukey-Kramer post-hoc tests. Batches are represented in the table by species names (e.g. Palmate B4 and Smooth B4 = Batch 4. Negative sign indicate the number of days in tanks before a minimum mean of 50 eggs were laid was significantly lower for the batch represented in the row and positive symbols indicate the delay was greater. P represents a palmate batch and S represents a smooth batch. Significances were determined by Tukey-Kramer post-hoc tests.

Batch	Mean number of days until minimum of 50 eggs were laid	Significant differences
Palmate B2 2006	55	+ P4 2006, + P5 2006,
Palmate B3 2006	43	+P4 2006, +P5 2006, +S4 2006, +S2 2007, - P1 2007
Palmate B4 2006	30	+S2 2007 -P2 2006, -P3 2006, -P1 2007, -P2 2007
Palmate B5 2006	22	-P2 2006, - P3 2006, -P1 2007, -P2 2007, - S4 2006, -S2 2007
Palmate B1 2007	62	+P3 2006, + P4 2006, +P5 2006,
Palmate B2 2007	56	+P4 2006, +P5 2006
Smooth B4 2006	47	+P3 2006, +P5 2006
Smooth B2 2007	63.5	+ P3 2006, + P4 2006, +P5 2006

5.4.3.7 Egg-laying dates

There was a significant difference in egg-laying dates between batches (Table 5.17). Significant differences were found for a minimum of 10 eggs, ($H_{9,67} = 61.85$, $p = <0.001$), 20 eggs ($H_{9,57} = 52.70$, $p = <0.001$); 50 eggs ($F_{7,145} = 17.82$, $p = <0.001$), 100 eggs ($H_{4,27} = 11.89$, $p = <0.001$). A prolonged delay between arrival and egg-laying by early newt arrivals sometimes resulted in them commencing egg-laying at similar dates to late arrivals.

Table 5.17 Significant differences in dates and delay in egg-laying between batches

Egg-laying dates and delay between arrival and egg-laying for newts in outdoor breeding tanks in 2006 and 2007. D = Mean number of days between arrival and when 10 eggs were detected for each batch. Y = Mean day of year by which 10 eggs had been detected for each batch. Y in the cell indicates there was a significant difference in the date (day of year) by when 10 eggs were laid and D indicates delay in egg-laying after arrival differed significantly. A negative sign indicates the date was earlier or the delay was less for the batch represented in the row and a positive symbol indicates the date was later or delay was greater. Not enough data were obtained to perform tests for smooth Batch 1 2007, and palmate and smooth Batch 5 2007. Significant differences determined by Kruskal-Wallis then Mann-Whitney U tests.

Batch name	D	Y	P B1 06	P B2 06	P B3 06	P B4 06	P B5 06	P B1 07	P B2 07	P B5 07	S B3 06	S B4 06	S B1 07	S B2 07	S B5 07
Palmate B1 2006	65	85		+D	+D -Y	+D -Y	+D -Y	+D +Y	ns	-	ns	+Y	-	+Y	-
Palmate B2 2006	45	91			+D	+D -Y	+D -Y	+Y	ns	-	ns	-Y	-	ns	-
Palmate B3 2006	27	99				ns	-Y	+D -Y	ns	-	-D -Y	-Y	-	-D	-
Palmate B4 2006	21	108					-Y	-D -Y	-D -Y	-	-D -Y	ns	-	-D	-
Palmate B5 2006	18	126						-D +Y	+Y	-	ns	ns	-	-D	-
Palmate B1 2007	45	59							-Y	-	-Y	-Y	-	-Y	-
Palmate B2 2007	38	91								-	-Y	ns	-	ns	-
Palmate B5 2007	19	125	-								ns	ns	-	ns	-
Smooth B3 2006	54	126								-		ns	-	ns	-
Smooth B4 2006	36	124								-			-	ns	-
Smooth B1 2007	34	48	-	-	-	-	-	-	-	-	-	-		-	-
Smooth B2 2007	56	129								-			-		-
Smooth B5 2007	15	101	-		-	-	-	-	-	-	-	-	-	-	

5.5 DISCUSSION

Although differences in egg-laying phenology were found between batches, this was not evident for courtship phenology using the 'inter-newt distance' recording method. Investigating male and female interactions by recording distances between newts was shown to be a poor indicator of breeding phenology and success. Few observations were made of specific courtship interactions, such a tail fanning so it was not possible to compare frequencies and duration of these activities between batches and over the season. Observations of courtship displays and females laying their eggs on egg-laying mops were only recorded for Batch 4 smooth newts, so phenology and frequency of smooth newt interactions could not be compared against arrival time. This finding does, however, indicate that smooth newts began both courtship activity and egg-laying later than palmate newts. The courtship study was not successful in supporting or refuting the first hypothesis that there was a significantly longer delay between arrival and courtship in *Lissotriton* newts that arrive early rather than late to Llysdinam Pond. An association between courtship and temperature was also not determined due to too few results achieved from the courtship surveys.

Interactions between palmate males and females were observed in all batches in 2006. Palmate newts arriving earlier in the year began interacting at an earlier date but in 2006 Batch 3 and 4 had the shortest duration of time between arrival and when male-female interactions was first observed. This indicates that newts that arrived earlier in the year spend longer in the water before courtship commenced and reached a high frequency of interactions. Later arrivals showed less delay before the commencement of courtship, but the delay was not reduced enough that commencement dates overlapped.

Research has shown that previous mating history of an individual female newt significantly affects its interest, with female attention and mating success decreasing once mating had occurred (Hoeck and Garner, 2007). Gabor and Halliday (1997) showed that until they had mated once, female smooth newts lacked discrimination for male traits indicating quality. This suggested choosiness for males increased once fertility had been assured. By mating with multiple males, females can compensate for any sterile males encountered. Sever (2002) found the structure of the female spermathecae enables female newts to store and use the most recently received spermatophore to fertilise eggs. Multi-seminated females produce on average 11.9% unfertilised eggs, while females that mated once laid 31.8% unfertilised eggs (Pecio, 1992). The fluctuations of female interest before and after mating

could account for the lack of relationship between male-female inter-newt distances and egg-laying. The finite time of the courtship observations by torch light provided just a 'snapshot' of the behaviour in the tanks, while egg surveys provide a measure of breeding behaviour over the length of time between each survey.

Male newt courtship displays need sustained energy levels to maintain female interest. Secondary sexual characteristics such as crests can increase oxygen absorption from the water and potentially decrease the need for males to surface for air during courtship and risk in losing their partner (Beebee and Griffiths, 2000; Griffiths, 1995). Males which can hold their breath longer maintain the interest of females (Halliday and Sweatman, 1976). Aspects of courtship displays in a range of animals have been linked directly to temperature, therefore displays may vary over the season due to changes in seasonal temperature. Temperature may affect courtship due to changes in body metabolism or as a result of the amount of dissolved oxygen in the water. Certain fin movements in the male guppy were directly temperature dependent (Laudien and Schlieker, 1981) and duration of advertisement calls in the torrent frog *Hylodes heyeri* showed a negative correlation with air temperature (Lingnau and Bastos, 2007).

Denoël et al. (2005b) investigated the effect of water temperature on alternative mating tactics used by alpine newt males, female responsiveness and the success of sperm transfer at 7°C and 13°C. There was no significant difference in number of encounters involving courtship at low and high temperatures or number of encounters involving sperm deposition. These findings may partially explain the lack of differences in recorded male-female inter-newt distance found between batches and over time. Denoël et al. (2005b) found no significant difference in the frequency of the commonly used courtship displays such as tail fanning, but at low temperatures fanning lasted for a significantly longer duration and the tail beat at a slower rate. Differences in tail fanning may alter the amount of pheromones received by the female newt. Also males deposited fewer spermatophores per encounter at low temperatures, indicating that frequency of courtship in this study may not be associated directly with breeding success. Denoël et al. (2005b) concluded that risk of sperm loss by reproducing in low water temperatures was not necessarily costly if males gained sufficient benefits. Males of lower fitness may benefit from this early breeding due to females becoming more choosy after mating (Gabor and Halliday, 1997; Hoeck and Garner, 2007). Twice the number of encounters included successful sperm transfer between male and female alpine newts at high temperatures (12 out of 40) than low

temperatures (6 out of 40), but the differences were not significant (Denoël et al., 2005b). This finding also indicates that observations of courtship behaviour suggest breeding interest but not necessarily breeding success (fertilisation). The success rate of spermatophore transfer in laboratory conditions was 25% for smooth newts, while rates are probably lower in the field (Halliday, 1990). There was a positive correlation between number of spermatophores transferred and number of eggs deposited by smooth newts (Pecio, 1992).

The courtship survey was not repeated in 2007 because it had been found that egg surveys in 2006 provided a better indicator of phenological change, and were shown to be more easily quantifiable. The methodology used for courtship surveys could be used in the future, with some potential improvements. A piece of coloured transparent material placed over the torch would act as a light filter, therefore potentially minimising newt disturbance and increase the likelihood of aspects of the courtship display being observed. Video cameras could be used to record courtship behaviour over a lengthened period of time in smaller tanks, as used by Denoël et al. (2005b). It would be beneficial to situate the tanks outside to enable natural conditions (photoperiod, temperature) but alternatively such variables could be controlled in a laboratory. Additionally leaves or other substrates provided for newt refuge should be fixed to the tank bottom because newt distances could be related to the dispersion of leaves within the tank.

Large females laid more eggs than small females and had a shorter delay between arrival and breeding, but the results were non-significant. Therefore the hypothesis that larger newts have significantly higher breeding success than small newts was not supported. Some previous studies on male newts also showed limited differences in breeding success between male body sizes. Male snout-vent length and male mass had no significant effect on number of spermatophores produced during laboratory trials where alpine newts deposited 0-6 spermatophores per courting trial (Hoeck and Garner, 2007), and Baker (1990a) found no relationship between the two variables in smooth newts. Larger males may, however, gain an advantage when in competition with males of smaller size. In the Mountain Dusky salamander, *Desmognathus ochrophaeus* (a species comparable in size to *Lissotriton* newts), large males chased away small males before courting with females and small males never deposited a spermatophore when a large male was present (Houck, 1988). The majority of studies on newts have found female body size and number of eggs (or ovarian oocytes) were positively related (Bell, 1977; Verrell, 1986; Verrell and

Francillon, 1986), although Hagström (1980) did not find any relationship between female body size and number of ovarian oocytes. Harrison (1985) found females moving away from Llysdinam Pond in July contained yolked oocytes, leading to debate about the ability to predict egg-laying frequency from oocytes numbers.

Weekly surveys on batches indicated a peak egg-laying rate of about 6.5 eggs per day by palmate newts and 4.5 eggs per day by smooth newts. This was less than the mean peak 32.3 eggs per day and mean oviposition rate of 8.7 eggs per day by smooth newts recorded in central England by Baker (1992). Baker found the total number of eggs deposited by female smooth newts ranged from 88-637, similar to clutch estimates in other studies (Bell, 1977; Verrell, 1986; Verrell and Francillon, 1986). Although the tank experiments were set up mainly to investigate the commencement of egg-laying, there were indications that egg-laying was declining to a finish in May 2007. In 2007 palmate newts in Batch 1 laid a mean of 80 eggs (range 24-178.5) and in Batch 2 laid a mean of 88 eggs (range 9.5-143). Egg-laying by smooth newts was lower in frequency, Batch 1 laid a mean of 39 eggs (range 2.5-59.5) and Batch 2 a mean of 25.5 (range of 3.5-59). Lower egg-laying rates were also found for smooth newts in less favourable environmental conditions in Romania (mean clutch sizes 51.2 and 74.6), where smooth females deposited eggs during short periods of less than 13 days (Cogalniceanu, 1999).

Harrison (1985) estimated mean clutch size (190 eggs) in 10 female palmate and 10 female smooth newts at Llysdinam Pond, by subtracting number of yolked ova in emigrating females from the number he had found in immigrating females. This corresponds to the maximum number of eggs laid by palmate females (Batch 1) in 2007. The estimated clutch size in mid-Wales (Harrison, 1985) was less than found by Baker (1992) but may be due to the cooler climate at Llysdinam. Lower ovarian counts were also found by Hagström (1980) in Sweden and may indicate a shorter foraging or breeding period. Smooth newts were at the edge of their range at Llysdinam which may account for the lower number of eggs laid than by palmate newts. Palmate newts were probably in their prime habitat at Llysdinam and the higher number of eggs laid by palmate females annually would help maintain the larger population.

Female palmate newts laid significantly more eggs than female smooth newts which contrasts with findings by Norris and Hosie (2005b). Norris and Hosie conducted a laboratory-based study at 18-20°C using the oviposition substrate of water cress, *Rorippa*

nasturtium-aquaticum. Possible reasons for the differences between our findings are that palmate newts have greater tolerance of cold water (de Wijer, 1990) and have greater substrate choosiness for egg oviposition. Smooth females may display more choosiness in egg-laying substrate, since they showed a strong bias for green egg-laying mops, and therefore may have higher oviposition rates on aquatic vegetation. In contrast, Norris and Hosie suggested that palmate females may be more selective than smooth females since they perform four or five more sniffs of the leaf for every successful deposition than female smooth newts. Female smooth newts are slightly larger in size and may have greater difficulty than female palmate newts in maintaining a steady position on egg-laying mops, although the sizes of the two species overlap so this seems unlikely.

Some salamanders, including the Ocoee salamander (*Desmognathus ocoee*) has been shown to store sperm for up to nine months (Adams et al., 2005) but no evidence of sperm storage from the previous year has been found in female smooth newts (Pecio, 1992). Therefore, it can be assumed the eggs laid were fertilised by one or both of the two males present in the tanks and not from any mating in the previous year. In April 2007, a small laboratory study was conducted in conjunction with the outdoor breeding tank study, with the intention of corroborating Pecio's findings. 10 female palmate newts and 10 female smooth newts were captured in pitfall traps migrating towards Llysdinam Pond. They were housed separately in tanks containing tap water and two egg-laying mops. All newts were captured in pitfall traps heading towards Llysdinam Pond and it was assumed they had not previously entered the pond to mate. None of the eggs that were laid developed into a bean shape structure (Stage 22 in development table: Gallien and Bidaud (1959) Appendix V). After two weeks the eggs became mouldy and it was assumed all eggs were unfertilised, since other newt eggs had been previously developed in the laboratory in 2005 and 2006 successfully. Results were therefore assumed to support lack of sperm storage between years in female *Lissotriton* species.

Palmate females laid a higher mean number of eggs within 30 days than smooth females in both years. Therefore later arrivals may show higher fecundity during the first month in the pond. In contrast, early arrivals have enhanced predation risk in the aquatic environment while not accruing benefits from egg-laying during the first month in water. In contrast to Pecio (1992) and Verrell and Halliday (1985), who found that female smooth newts begin egg-laying after 10 days in water, the number of days until egg-laying began varied over the season and between individuals. There was less delay from arrival to egg-laying for

newts that arrived later in the year, and the earliest newt arrivals showed less delay between arrival and egg-laying in mild springs and after mild winters, than after cold winters. Therefore the hypothesis that there was a significantly longer delay between arrival and egg-laying in *Lissotriton* newts that arrive early than late to Llysdinam Pond was supported.

Debate has taken place on whether prolonged breeders are as sensitive as explosive breeding anurans to weather related cues for the stimulation of breeding activities. Prolonged breeders might begin and end breeding in response to more generalised regional cues (Saenz et al., 2006). Prolonged breeders may be sensitive to abiotic factors in order to maintain energy reserves during the longer breeding season. In a study in Canada, prolonged breeding species responded differently to climatic variables through the breeding season with time of day and barometric pressure (predicting rainfall) becoming more important than water temperature later in the season (Oseen and Wassersug, 2002).

In years with low spring temperatures such as 2006, palmate newts began breeding earlier than smooth newts. This may be because their small body size meant they were able to be active at lower temperatures, and a higher surface area to volume ratio aids respiration through the skin. The greater resilience of palmate newts to cool temperatures corresponds to palmate newts being typically an upland species (Beebee and Griffiths, 2000) and showing greater tolerance of cooler ponds (de Wijer, 1990). For early newt arrivals, before egg-laying commenced, there appeared to be a period of three weeks with mild temperatures of over 2°C. Although the hypothesis that egg-laying and temperature were positively associated was not supported, there were indications that a certain threshold temperature was required before egg-laying commenced in each year. Reading (1998) found that common toads in southern England arrived after a threshold temperature of about 6°C was reached, and that early arrival occurred when the majority of the 40 days preceding main arrival were at or above 6°C.

Egg-laying rates of smooth newts in outdoor breeding tanks were affected by a cold spell when water froze and snow fell, whereas palmate newts did not decrease daily egg-laying rates. Banks and Beebee (1986) found the natterjack toad, a prolonged breeder, still called on nights when temperatures eventually dropped below 5°C and even called and spawned on one evening prior to a frost. Despite the risks of breeding early, earlier egg-laying may have been beneficial to both palmate and smooth newts in reducing the amount of UV

radiation that developing eggs were exposed to. Anuran eggs are more susceptible to UV radiation since wrapping eggs within a leaf has been shown to protect developing newt embryos from UV radiation (Marco et al., 2001). Miaud (1994) found that palmate newts left more eggs unwrapped (25.4% eggs unwrapped) than great crested and alpine newts which left zero and 10% of their eggs unwrapped respectively.

There was a greater delay from newt arrival to egg-laying in 2006, when air temperatures were cooler than in 2007. There were a number of variables that could cause changes in delay between and within years. Time needed for male newts to develop secondary sexual characteristics may be lengthened in cool water and could be subject to further study. Lower water temperatures can directly affect spermatophore transfer success and influence female receptivity (Denoël et al., 2005b). Female newts may delay the start of oviposition, until temperatures no longer fluctuate below freezing, to minimise egg mortality. Reproductive females have showed a greater preference for warmer water than non-reproductive females even during periods where they were not egg-laying. This could be due to thermal optima of some internal reproductive processes or a response to endocrine changes during reproduction (Gvoždí, 2005). In *Triturus carnifex* (that breeds in Italy in November) low temperature was found to regulate prolactin secretion showing a sex and season related control system (Mosconi et al., 2002).

No previous research has investigated newt breeding phenology in the quasi-natural environment of outdoors tank. The study of courtship and particularly egg-laying in outdoor breeding tanks led to important findings about breeding phenology. Suggestions for improvements include monitoring egg-laying rates on a more regular basis (every 2-3 days) as in laboratory-based studies (Baker, 1992; Pecio, 1992). Since this tank study was done concurrently with other data collection during the amphibian breeding season, more regular checks were not possible. Increasing the 'naturalness' of the outdoor tanks could increase validity of the results, but could impede data collection or increase time required for data collection. Suggestions for naturalising the tanks include gravel substrate and utilisation of one or more plant species found naturally in Llysdinam Pond as an egg-laying substrate. These suggestions could increase the probability that the behaviour was comparable between the pond and the outdoor breeding tanks. There are negative implications of using plants instead of inert polythene strips, because leaves would decrease in flexibility as they matured, and the plant coverage over the season would alter, leading to variability in egg-laying opportunities over time.

Allocating two newts of each sex per tank presented multiple breeding opportunities and simulated the breeding conditions of the pond where there would be the presence of and competition between other newts of both sexes. It might, however, be beneficial to conduct future studies of egg-laying phenology with one female per tank, since newts and in particular females are oophagous (Baker, 1992; Miaud, 1993). The incidence of egg wrapping was high (over 75%) in *Lissotriton* newts and predation by females on wrapped eggs was low (5% by females and 2% by males) which may have protected the majority of eggs in this study (Miaud, 1994). No oophagy was observed in the tanks on torching surveys and no jelly remains were found on the egg-laying mops (a possible indication of predation, Chapter 4, Section 4.5). In laboratory studies, female newts tended to avoid eating their own eggs and smooth newts have been shown to be reluctant to eat conspecific eggs (Gabor, 1996).

Outdoor tanks may have benefits over laboratory experiments in preventing an outbreak of disease, possibly due to lower ambient temperatures. A small scale version of this tank experiment set up in a laboratory in 2005 was halted after an outbreak of disease in adult newts caused high mortality. CEFAS recently completed testing of the newts that died and confirmed that ranavirus was not detected. A disease outbreak also occurred in a laboratory-based courtship study by Hoeck and Garner (2007).

To my knowledge, this was the first study of the relationship between arrival time and breeding phenology in *Lissotriton* newts. Although there were limited sightings of specific courtship behaviour and newt interactions, there was a greater delay between arrival and courtship for the earliest newt arrivals, but torching surveys only provided a 'snapshot' recording of the newt interactions taking place. The lack of relationship between courtship and egg-laying phenology was more likely due to inter-newt distance recording being a poor method of recording courtship phenology. This study demonstrated the greater delay between arrival and breeding for earliest newt arrivals. The delay between arrival and commencement of egg-laying was greater in years where the late winter and early spring mean temperatures were lower. This delay between arrival and egg-laying was particularly pronounced for smooth newts in 2006. Once egg-laying had commenced no relationship was found between temperature and egg-laying rates. Large females laid more eggs and showed slightly earlier egg-laying-phenology than small females but the results were not significant. The findings from this research warrant more investigation of *Lissotriton* newt

courtship and particularly egg-laying phenology in outdoor tanks, with some of the suggested improvements.

Chapter 6: *Lissotriton* larval and metamorph phenology

6.1 SUMMARY

Lissotriton newt hatching, larval and metamorph phenology were studied in tanks set up outdoors. With the effect of predation removed, hatching success did not differ over the breeding season, although eggs laid early took significantly longer to hatch. Hatchling size did not vary over the season, but larval growth was significantly faster later in the year. Faster larval growth may release larvae from predation risk by gape-limited or small aquatic predatory invertebrates such as damselflies. Nevertheless, most predatory invertebrates did not display size-selective predation of larval newt prey in a laboratory study.

Netting surveys for *Lissotriton* larvae and predatory aquatic invertebrates were conducted in 2007 to compare with a study at Llysdinam Pond in the early 1980s (Harrison, 1985). Larvae were detected in the pond significantly earlier in 2007 compared with the 1980s, with a four to five weeks advance in week of detection. Predatory aquatic invertebrates appear to have advanced their phenology to the same degree as *Lissotriton* newts. The peak of aquatic predatory invertebrates occurred at the time when *Lissotriton* larvae were first detected in both 1984 and 2007 and when *Lissotriton* eggs were at peak numbers. Anurans had not, however, advanced breeding phenology between the two years so there are still implications for asynchrony in interactions between species.

There were significantly fewer predators in Llysdinam Pond in 2007 compared to 1984. There was a significant difference in abundance of predatory beetle larvae detected over the newt breeding season with a peak at Week 16 of 2007. In the laboratory study, beetle larvae were shown to be voracious predators of newt larvae of all sizes. Although large newt larvae may be free from predation by gape-limited predators, predatory aquatic invertebrates are not gape-limited and most predated newt larvae in all size categories studied. Only predatory damselflies did not predate newt larvae over 20 mm.

Metamorphs emerging from Llysdinam Pond in 2007 were significantly larger than metamorphs captured in 1983-1984. This may be explained by the longer period for larval development in 2007 due to earlier egg-laying or due to the lower temperatures delaying metamorphosis and therefore prolonging the larval stage in 2007. Metamorphs emergence patterns were associated with rainfall during peak emergence months of July and August.

6.2 INTRODUCTION

Once a newt egg is laid on or wrapped within aquatic vegetation it develops at a rate dependent on environmental conditions, including temperature and water quality. Development of the eggs starts with initial cleaving, the cells continue to divide and differentiate so that a rudimentary head and tail are evident, then three pairs of external gills develop on the head (Gallien and Bidaud, 1959). Several layers of jelly surround the newt embryo to protect it from mechanical injury, desiccation, some types of predation (Stebbins and Cohen, 1995) and possibly even infection by aquatic phycomycetes (Gomez-Mestre et al., 2006). Nutrition during development is obtained from the yolk, and waste products are passed out into the chamber surrounding the embryo. Oxygen can pass through the egg capsule, and before the embryo hatches, the gills can be used for respiration (Griffiths, 1995).

The hatchling *Lissotriton* larva is approximately 7 mm long and the mouth is not fully functional, so the larva remains attached to an egg capsule or vegetation by prong-like organs on the head called 'balancers' while feeding on the remains of the yolk sac (Beebee and Griffiths, 2000; Griffiths, 1995). It then becomes a free-swimming 'gape-limited' predator that feeds on a wide variety of prey. Newt larvae are unselective in their prey and although they have small teeth they are only used to restrain the prey that is swallowed whole (Griffiths, 1995). At Llysdinam Pond, Harrison (1985) found larval diet was mostly comprised of small crustaceans, but diversified as the larvae became larger.

Great crested larvae are larger (approximately 12 mm in length on hatching), but they can be hard to distinguish from *Lissotriton* larvae early in development, although they soon develop dark spots (Griffiths, 1995). They will predate on the larvae of *Lissotriton* newts and cannibalise smaller conspecifics (Beebee and Griffiths, 2000; Griffiths, 1995). *Lissotriton* newt larvae hide in vegetation while great crested larvae swim freely in the water column which may reduce cannibalism by great crested adults which mainly dwell on the pond bottom (Beebee and Griffiths, 2000).

While anuran tadpoles undergo dramatic metamorphosis, newt larvae already have the basic adult body plan so metamorphosis is less extreme. The gills are reabsorbed and the young newts disperse onto land and are often known as 'efts'. Larvae that hatch later in the year may overwinter in the pond and emerge at a much larger size the following spring. Little is known about the 'eft' stage of the newt life cycle since they hide in inaccessible

hiding places and do not return to the pond until a few years later when ready to breed. The fact that newts breed over a protracted period and produce larvae that may or may not metamorphose the same year is an adaptive advantage to different pressures (Bell, 1977; Griffiths, 1995).

Although eggs that were laid earlier at lower temperatures seem to be particularly prone to fungal attack (Griffiths, 1995), a study on the direct influence of temperature (12 and 17°C) on egg development showed no differences in survival (Griffiths and de Wijer, 1994). Time to hatching decreased significantly at the higher temperature for palmate, smooth and great crested eggs (Griffiths and de Wijer, 1994), and for the Italian crested newt *Triturus carnifex* and alpine newts (D'Amen et al., 2007). *Lissotriton* size at hatching was unaffected by temperature but great crested larvae hatched at a smaller size at the higher temperature (Griffiths and de Wijer, 1994). Smooth newts at Llysdinam Pond have been shown to hatch at a smaller size than palmate newts but have faster growth rates to eventually emerge as the larger metamorphs (de Wijer, 1990; May, 1993).

Adult population sizes are maintained well below the potential carrying capacity of ponds by the controlling factors on larvae survival such as predation and desiccation (Beebee and Griffiths, 2000). Survival from egg to amphibian metamorph is low, Bell and Lawton (1975) estimated that 2.5% of eggs survived to hatching and that just 10% of the resulting larvae made it to metamorphosis. Harrison (1985) studied amphibian larvae at Llysdinam Pond in 1983 and 1984 to investigate the utilisation of resources and factors influencing survival. Survival of newt larvae was greater than anuran larvae at 1.6-2%. Newt larvae were exposed to lower levels of predation than anuran larvae but habitat desiccation was an important factor influencing survival.

Although research has taken place on predation of *Lissotriton* newt eggs (Miaud, 1993; Orizaola and Braña, 2003a), less has taken places on larval predation. Once great crested larvae reached approximately 27 mm (approximately Week 8 of development) they were found to begin predation on smaller *Lissotriton* larvae (Griffiths et al., 1994). In analysis of 205 specimens of *Lissotriton* newt larvae, Harrison (1985) found a small larva in the gut of a larger specimen, indicating cannibalism that had not been found in previous studies on newt diet in Britain. Fish, birds and amphibians are more likely to find amphibian prey unpalatable than predatory invertebrates (Gunzburger and Travis, 2005) because skin toxicity does not prove problematic for predatory invertebrates such as Dytiscid water

beetles, dragonfly larvae and greater water boatmen *Noctonecta glauca* which are not gape-limited (Harrison, 1985). *N. glauca* feeds by inserting its beak into prey, dragonfly larvae and adult dytiscid beetles feed by direct mastication (van Buskirk, 2001) while dytiscid larvae readily attack toad tadpoles and inject digestive enzymes and suck out the resulting fluid (Harrison, 1985).

The timing and success of metamorphosis and metamorph size depends on a number of factors including temperature (Alvarez and Nicieza, 2002; Baker, 1990b; Loman, 2002b), hydroperiod (Laurila and Kujasalo, 1999; Loman, 2002a; Paton and Crouch, 2002; Pechmann et al., 1989; Semlitsch and Gibbons, 1985; Werner, 1986), diet (Alvarez and Nicieza, 2002; Kupferberg, 1997) and predation (Laurila and Kujasalo, 1999; Loman, 2002a; Orizaola and Braña, 2005). In some amphibian species there is a crucial temperature threshold, such as the endangered green and golden bell frog (*Litoria aurea*) where juvenile length increases and male sexual maturation occurred only above 28°C (Browne and Edwards, 2003). Painted frogs *Discoglossus galgonoi* metamorphosed later and at a larger size at 17°C than at 22°C (Alvarez and Nicieza, 2002). Baker (1990b) found that smooth newt larvae raised at 20°C metamorphosed in 50 days in comparison to 275 days at 12°C, a six fold difference in growth rate. Size at metamorphosis also increased over the temperature range but decreased after 20°C (Baker, 1990b).

Although research has taken place on the direct effects of temperature on *Lissotriton* larval hatching times, survival and growth (Griffiths and de Wijer, 1994; May, 1993) and metamorphosis (Baker, 1990b), no work has taken place on the phenology and success of hatching through the year. Although arrival times of *Lissotriton* newts have advanced since the 1980s (Beebee, 1995; Chadwick et al., 2006) there appears to be little data on changes in newt breeding phenology. Anuran spawn and larvae are more conspicuous than urodele offspring and phenological records are more common (Beattie, 1985; Beebee, 1995; Scott et al., 2008; Tryjanowski et al., 2003), while newt phenological records mainly rely on arrival data. The outdoor tank breeding study (Chapter 5) was used to investigate changes in breeding phenology of newts arriving at Llysdinam Pond at different times through the year. To follow on from surveys of courtship and egg-laying phenology in this research, investigation of newt larval and metamorph phenology was required.

6.2.1 Aims and hypotheses

6.2.1.1 Differences in larval phenology between batches

Due to lower temperatures earlier in the breeding season it was predicted that (i) there would be less delay between egg-laying and hatching later in the year than at the start of the newt breeding season. With the effect of predation removed it was hypothesised that (ii) there is would be significantly greater hatching success for eggs laid late than those laid early. There would be higher prey supply and higher temperatures later in the breeding season so it was hypothesised that (iii) larvae hatching later in the year would grow at a faster rate than those that hatch early in the year. It was hypothesised that (iv) there would be a significant difference in hatching larval sizes and larval development between palmate and smooth eggs as found by May (1993). Smooth newts at Llysdinam Pond have been shown to hatch at a smaller size than palmate newts but have faster growth rates to emerge eventually as the larger metamorphs (May, 1993).

6.2.1.2 Larval phenology in Llysdinam Pond

Although limited, the data set on *Lissotriton* larval abundance from 1983-1984 (Harrison, 1985) and larval sizes (de Wijer, 1990) enabled some comparisons of the phenology of newt larvae. Data on larval phenology from the 1980s was compared with field data from 2007 and it was hypothesised that newt larvae hatch significantly earlier than 1980s (1983 and 1984).

6.2.1.3 Predatory aquatic invertebrates

Although there was only previous data from 1984 for predatory aquatic invertebrates at Llysdinam Pond, the aim was to collect data in 2007 to compare invertebrate phenology and abundance between the two years. Phenological records of predatory pond invertebrates have been infrequently recorded although Hassall et al. (2007) has analysed data on dragonfly phenology. Also laboratory-based experiments were set up to test the hypotheses that (i) there would be significantly more predations of small than large newt larvae by predatory aquatic invertebrates, and that (ii) there would be significant differences in predation rates between the invertebrate species.

6.2.1.4 Newt metamorphs

Using data on the emergence of newt metamorphs from Harrison (1985), the aim was to investigate the differences in phenology and size of metamorphs departing from Llysdinam Pond in 1983, 1984 and 2007. It was hypothesised that due to earlier breeding and more

time for development, newt metamorphs would be significantly larger on departure from Llysdinam Pond in 2007 than the 1980s. The distribution of metamorphs departures in relation to rainfall was also investigated.

6.3 METHODS

6.3.1 Monitoring hatching success of newt eggs and larval development

In 2006 larval hatching success and growth rates were monitored in baskets constructed from plastic mesh and a fine material mesh and submerged in Llysdinam Pond. The study was unsuccessful due to egg infections and larval escapes. Due to these problems and the concurrent disruption to the pond ecosystem in 2007 from egg searches (Chapter 4) and netting it was decided to attempt a different methodology in 2007 in both developmental and larvae growth tanks set up outdoors.

In 2007 a proportion of the nearly 4000 eggs laid by palmate and smooth newts in the outdoor breeding tanks (Chapter 5) were utilised for further research. Hatching success, hatching size and larval growth of palmate and smooth newts from Batch 1, 2 and 5 were studied. In the methodology of Chapter 6, two further tank types will be described more fully. To differentiate, the 'breeding tanks' were the large outdoor tanks in Chapter 5 where adult *Lissotriton* newt courtship and egg-laying were monitored. 'Developmental tanks' were tanks where eggs were placed to develop to monitor hatching success, time to hatching and larval hatching size. 'Larval growth tanks' were small tanks floating within an 817 litre water trough which provided a large water body simulating a pond.

Where numbers of eggs were high, up to 60 eggs per batch and species (from three replicate breeding tanks) were taken from the outdoor breeding tanks during weekly checks. Eggs were taken up to five times over the breeding season to monitor hatching success and hatchling size in developmental tanks. The eggs obtained were from one or more egg-laying mops, with 'mops' divided when necessary to provide not more than 20 eggs per replicate tank. Eggs were taken early in season (the first eggs laid by each batch), near the end of the outdoor tank study (May 2007) and at peak egg-laying times.

The developmental tanks were clear plastic storage boxes of 50 x 40 cm and 30 cm in depth, and filled to 25 cm water depth and water was changed approximately every two weeks using a hosepipe. Developmental tanks were checked every 3-4 days for infected eggs which were removed to prevent spread of infection, and tanks were checked more

regularly close to hatching. The date when 50% of the eggs in the tank had hatched was recorded. A median hatching date was used because the outdoor breeding tanks had only been checked for eggs every 7 days causing up to a 7 day range in laying date of the eggs collected. Once all larvae in a developmental tank had hatched they were removed via a large pipette to a sorting tray that had been filled with water. A laminated piece of graph paper taped to the bottom of the sorting tray, enabled snout-vent and total length of the larvae to be measured to the nearest 0.5 mm.

Larvae were then transferred to larval growth tanks (8-10 larvae were assigned to each tank, depending on original egg numbers and hatching success). Larval growth tanks were 21 x 12 cm and 11 cm in depth (available from www.Hagen.com) and floated in a large circular agricultural water trough (817 litres), to reduce temperature fluctuation and simulate a larger water body. The tanks were floated with use of two polystyrene floats attached to the either side of the tank by string and duck tape. Each tank had a lid to prevent rainfall causing overflow and larval escape. Each larval growth tank contained pond water from Llysdinam to provide a zooplankton diet. Diet therefore represented available small aquatic prey in Llysdinam Pond at the time. The egg-laying mops and a small section of *M. scorpioides* was used to provide larval refuge and reduce stress. The water was changed weekly and after four weeks the larvae were measured again to estimate growth before release. Individual increases were not recorded since larvae were not marked in any way. Due to the larger prey that larvae consume later in development (Harrison, 1985), and their more active hunting behaviour (Bell 1975), it was decided to release larvae after four weeks. Further length data would be unlikely to be representative of larval size in the pond environment. There were indications that larvae kept in indoor tanks in 2006 had reduced growth rates compared to those in the pond and other research supports this (Bonetti, 1996).

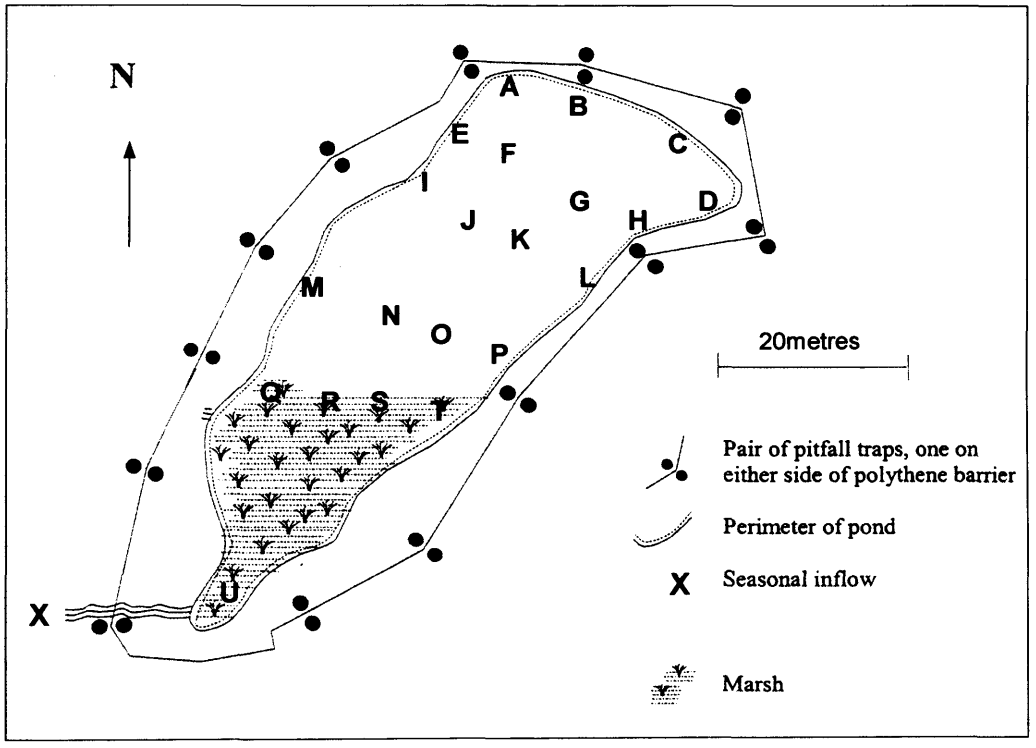
Three Brannan minimum-maximum thermometers in developmental tanks were used to record temperatures twice weekly. Two thermometers in the large agricultural tank containing the larval growth tanks were used to record temperatures twice weekly.

6.3.2 Larval newt and invertebrate surveys in Llysdinam Pond

Netting for the detection of newt larvae and aquatic predatory invertebrates was conducted in Llysdinam Pond from 28.2.2007-10.10.2007 in order to compare with data collected in 1983 and 1984 by Harrison (1985). 21 sites were chosen for the netting locations (Figure

6.1) to represent the pond microhabitats and include bank side locations and open water. The sites were located in five transect lines (e.g. A-D was Transect 1, E-H was Transect 2 etc) and the site 'U' was located in a small pool where the frogs spawned annually with low reproductive success since it dried up by April in most years. 11 of the sites (A, D, E, G, H, J, L, M, N, P and S) were at similar locations used by Harrison (1985). Although vegetation had altered at these sites over 23-24 year period, an abundance of *G. fluitans* remained at sites Q-T.

Figure 6.1 Netting sites used in 2007 at Llysdinam Pond
The location of the 21 netting sites (Site A-U) that were used for surveys of newt larvae and predatory invertebrates in 2007. Netting surveys were conducted between February and October 2007. Transect 1 = A-D, Transect 2 = E-H, Transect 3 = I-L, Transect 4 = M-P, Transect 5= Q-T. Site U was situated in a small pool which dried up annually. Map adapted from Harrison et al. (1983) and Chadwick (2003).



Sampling took place weekly from 28.2.2007-28.3.2007, then afterwards every 2-3 weeks due to concurrent field work commitments and the lengthened time required for checking each net sample. Netting surveys in 2007 followed the methodology of Harrison (1985) to enable comparison with the larval and invertebrate data sets from 1983-1984. Standard pond nets of approximately 30 cm diameter were used with a mesh size of approximately 1 mm. Three net sweeps were performed at each site with locations away from the bank accessed by wading. All newt larvae and aquatic predatory invertebrates captured and detected at each site were counted and measured to the nearest 0.5 mm.

The sampling was completed by a team of one to four people but sampling methods were consistent between team members. Newt larvae are reasonably fast swimmers so nets were not moved through the water slowly (Shaffer et al., 1994). The survey took 2-14 hours to complete dependent on the time of year. Surveys earlier in the year were quicker than later in the year, due to low numbers of invertebrates and newt larvae to measure. Additionally, surveys later in the year took longer due to the large amount of duckweed that needed to be sorted through. The sites were sampled in a random order to avoid affects of time of day between sites over the season. Bank side sites were always sampled before the nearby open water site to prevent disturbance of bank side sites by wading prior to netting.

Unless it was apparent that nothing had been captured in the net, the samples were sorted through in white sorting trays for newt larvae and aquatic predatory invertebrates on the bank side. Tap and rain water from large containers dispersed around the pond edge was added to the samples in the white trays to assist detection of larvae and invertebrates that were often inconspicuous when buried in vegetation. When large quantities of vegetation were netted and duckweed was prevalent, the samples were stored briefly in tanks on the bank side and sorted through in smaller amounts in the trays to enable detection of small invertebrates and larvae. Newt larvae and aquatic predatory invertebrates were tallied and total lengths measured to nearest 0.5 mm on a piece of laminated graph paper taped to the bottom of each sorting tray. Snout-vent lengths of newt larvae were also measured. The method enabled larval and invertebrate lengths to be recorded relatively easily once they remained motionless, regardless of their location within the tray.

The following aquatic predatory invertebrates were recorded and lengths measured:

- Predatory adult water beetles, Family *Dytiscidae*: mainly *Dytiscus marginalis* and *Agabus* species
- *Dyticius marginalis* larvae
- Small unidentified species of beetle larvae
- Greater water boatmen *Notonecta glauca*
- Dragonfly larvae (Anisoptera)
- Damselfly larvae (Zygoptera)

Although recordings were made of non-carnivorous lesser water boatmen *Corixa punctata*, mayflies (Ephemeroptera) and small carnivorous stoneflies (Plecoptera), these invertebrates were not used in analyses due to their diet or small size implying no

predation threat to newt larvae. Presence of common frog and common toad larvae in samples were also recorded.

6.3.3 Predation study

In May 2007, two laboratory studies on newt larvae predation by aquatic invertebrates were set up. Predators used in the study were captured during surveys in May at Llysdinam Pond and extra sampling at Buftons Pond (0.5 km north of Llysdinam). The predators were placed in tanks 34 x 18 cm and 18 cm in depth (available from www.Hagen.com), filled to 8 cm depth with tap water that had been left to stand for a day. A section of *M. scorpioides* was placed in each tank for a predator perch or refuge for newt larvae. The predators were left in the tanks for one day to reduce effects from satiation levels prior to the newt larvae being added. Newt larvae were then added to each tank in addition to pond water to make each tank of 16 cm water depth. Pond water provided small prey for the newt larvae.

Newt larval size categories selected for the predation study were dictated by the availability in the pond and from hatchlings from the outdoors breeding study. Newt larval sizes altered slightly for the two studies. The size and replicate numbers used for the seven predator categories were limited by the specimens captured at Llysdinam and Buftons Ponds.

Predator categories and size ranges used were:

Dragonfly larvae: large specimens (length 32-43 mm)
Dragonfly larvae: small specimens (length 18-24 mm)
Damselfly larvae (length 12-17 mm)
Water beetles *Agabus* species (length approximately 13 mm)
Greater water boatmen *Noctonecta glauca* (length 16-18 mm)
Large beetle larvae *Dytiscus marginalis* (larvae length 27-48 mm)
Beetle larvae: small specimens, unidentified species (16-20 mm)

6.3.3.1 Predation Study A

The first predator trial had nine newt larvae and one predatory aquatic invertebrate per tank. Each predator trial had three small recently hatched newt larvae measuring (6-9 mm), three medium sized larvae (12-14 mm) and three large larvae (20-24 mm). Predations were

recorded after one and two days. After the first day predated larvae were replaced to keep numbers consistent.

Controls that were run concurrently to the predation study showed that small newt larvae declined in numbers without presence of an predatory invertebrate so predation by the two other larval size classes must have been occurring. No predation of medium sized larvae by large larvae occurred.

6.3.3.2 Predation Study B

Subsequently another predation trial was set up with larger larvae separated from the smallest larvae to prevent conspecific predation. Tanks contained either: (i) six small larvae, 9-11 mm in length or (ii) six large larvae over 14 mm in length, (three 14-16 mm) and three 20-24 mm

Predators and prey from Study A were released and not reused for study. The number of larvae predated was recorded over three days. When larval numbers declined in a tank, new larvae were introduced into the tank on a daily basis to keep larval numbers in each size category consistent.

6.3.4 Metamorph phenology

The number of newt metamorphs captured daily in pitfall traps leaving Llysdinam Pond were recorded. In 2007 data were recorded on total length and snout-vent length (mm), mass to nearest 0.1 g and identification of the metamorph as a palmate or smooth using method from Roberts and Griffiths (1992). Roberts and Griffiths found that the dorsal strip (varying in colour from yellow to dark orange) on the back of newt metamorphs differed in length between the two *Lissotriton* species. In palmate metamorphs the dorsal stripe starts at neck and extends past the pelvic girdle and is uniformly pigmented. In smooth metamorphs the dorsal stripe runs from centre of head and terminates on the trunk near the pelvic girdle and is more pigmented at anterior. The accuracy of identification determined afterwards by gel electrophoresis was 93-100% for palmate metamorphs and 62%-100% for smooth metamorphs although only eight smooth metamorphs were used compared to 59 palmate metamorphs. Data on total length of metamorphs was compared with data by Harrison (1985) from 1983 and 1984. Caution must be drawn in data interpretation since Harrison used a different methodology (20 pieces of plastic tubing, 1 m in length as pitfall traps) to capture metamorphs.

6.3.5 Data from Harrison (1985) and de Wijer (1990)

Raw data from 1983 and 1984 were not available for comparison with the 2007 data. Only data for 1983 and 1984 presented in a graphical form in Harrison's thesis (1985) were available for analysis (Appendix IX, X, XI). These were converted back into values for analysis and comparison with 2007 data. Larval data were presented as mean catch per site (three sweeps of net) from May-November in 1983-1984 (Appendix IX). Predatory aquatic invertebrate data were presented as mean number per site (three sweeps of net) from March until September 1984 (Appendix X). Only a number of newt larvae captured were measured by Harrison (1985), and this was for a study on their prey size. However, boxplots for 1988 larval lengths (snout-vent length mm) in a report by de Wijer (1990) were used for comparison of larval snout-vent lengths by week for 1988 (Appendix XII) compared to 2007 (Appendix XIII). Metamorph data recorded by Harrison (1985) were numbers emerging from Llysdinam Pond through 1983 and 1984, and mean length at emergence (Appendix XI).

6.3.6 Data analysis

Kruskal-Wallis and Mann-Whitney U tests were conducted to test for differences in time to hatching, hatching success, larval hatching size, and larval growth between both species and time of year. None of the hatching and larval data could be transformed to meet assumptions of parametric tests (Bowker and Randerson, 2008). Previous work on larval growth had noted tail damage which made snout-vent lengths better for comparison (Bonetti, 1996). Since only a few larvae had been observed to have tail damage, and total length of larvae had been easier to measure accurately, data analysis was conducted on total larval lengths.

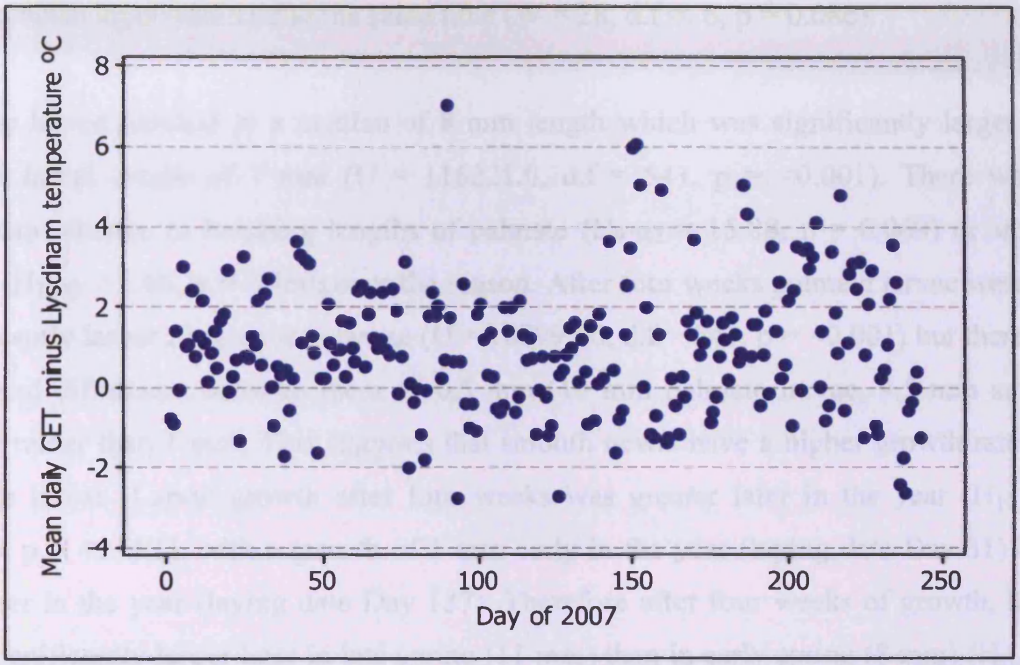
ANCOVA with week as a covariate was used to test for differences in the timing of the detection of *Lissotriton* newt larvae in Llysdinam Pond between 1983, 1984 and 2007 (Bowker and Randerson, 2008; Dytham, 2003). Weeks 13-22 were analysed, since after Week 22 in 2007 larval number began to decline from a peak of 18 per site. Comparisons of all three years and pair-wise comparisons were conducted. Data were transformed when assumptions of normality and homogeneity of variance were not met. Sites A-T were used to calculate larvae abundance in 2007 as no newt larvae were captured in Site U at any time. Patterns of mean abundance of newt larvae in 2007 were similar between the 11 sites

that corresponded to Harrison (1985) and the 20 sites A-T, confirming utilisation of data from all 20 sites was viable.

Two-way ANOVA was used to investigate the differences in aquatic predatory invertebrate abundance over the newt breeding season. The total number of beetle larvae captured per sampling session in 2007 (log+1 transformed) were analysed by week and by transect. In total 95 beetle larvae at greater than 15 mm length were captured during the entire netting study. Numbers of beetle larvae captured per individual site were too few for analysis. Too few other aquatic predatory invertebrate captures were made to warrant statistical analysis. For predation experiments in the laboratory, χ^2 analysis was performed on observed and expected values of predations by predatory aquatic invertebrates to investigate differences in predation on the three defined larval size categories.

Prior to 1988 local daily weather was not available. Daily Central England Temperatures (CETs) were applied for from the British Atmospheric Data (BADC) website (UK Meteorological Office, 2008a) to use as a measure of change between years. The CETs represent a roughly triangular area of the UK enclosed by Preston, London and Bristol. Although Llysdinam is outside the area the temperatures were highly correlated (Chadwick, 2003). Llysdinam 2007 temperatures were also obtained from the BADC website (UK Meteorological Office, 2008b) and CETs were highly correlated between January and August (mean temperatures 2007: $r_s = 0.911$, d.f = 241, p = <0.001 and minimum temperatures 2007: $r_s = 0.877$, d.f = 241, p = <0.001). Although the CETs were suitable as a measure of change between years, it should be noted that the CET mean daily temperatures were higher than at Llysdinam on 75% of days and CET mean was over 2°C higher on 23% of days (Figure 6.2). The CET minimum daily temperatures were higher than Llysdinam on 85% of days and 2°C higher in 43% of cases. All analyses were conducted in Minitab 15.

Figure 6.2 Difference in mean daily temperatures between CET and Llysdinam
Comparison of daily mean Central England Temperatures (CET) air temperatures and daily mean air temperatures calculated from Llysdinam weather station. A point at greater than 0 indicates that CET temperature was higher than at Llysdinam on that day. Llysdinam and CET mean temperatures were highly correlated but CETs were higher than Llysdinam on 75% of days. Data shown for January-August 2007 as that was the period when larval surveys were conducted.



6.4 RESULTS

6.4.1 Hatching phenology, success and larval growth

There was no significant difference in developmental and hatching success between palmate and smooth eggs (W = 373.0 d.f = 34, p = 0.269) with 92% of palmate and 95% of smooth eggs hatching. There was no significant difference in hatching success between time of year ($H_{10,25}$ = 14.70, p = 0.143) or between batches ($H_{5,30}$ = 9.60, p = 0.087) with a hatching rate usually within the range of 91-100%. However, the nine earliest laid smooth eggs (collected on Day 31) only had a 33% hatching success and the two palmate eggs collected on the same day did not develop successfully. Eggs collected on Day 38 had a poor hatching success (75% palmate and 50% smooth). Additionally late arriving palmate newts in Batch 3 laid few eggs and had only 62.5% hatching success. Some of the undeveloped eggs may have been unfertilized.

Time until hatching was significantly slower earlier in the year ($H_{10,26}$ = 34.65, p = <0.001) taking between a median of 60 days from end January (laying date Day 31) to median of 22 days by mid May (laying date Day 137). Although smooth newts hatched

approximately 1.5 days earlier on average this was non significant (W = 87, d.f = 15, p = 0.594) and could be due to egg-laying dates which could vary by as much as one week due to the intervals between egg checks. Differences between batches were due to egg-laying times and not inherent fitness, since there were no difference in hatching time between batches when eggs were laid at the same time (W = 28, d.f = 6, p = 0.086).

Palmate larvae hatched at a median of 8 mm length which was significantly larger than smooth larval length of 7 mm (U = 116221.0, d.f = 541, p = <0.001). There was no significant change in hatching lengths of palmate ($H_{9,342}$ = 15.88, p = 0.069) or smooth larvae ($H_{7,183}$ = 5.66, p = 0.560) over the season. After four weeks palmate larvae were still significantly larger than smooth larvae (U = 107992.0, d.f = 541, p = <0.001) but there was a reduced difference between them of 0.5 mm (10 mm palmate larvae, 9.5 mm smooth larvae) rather than 1 mm. This suggests that smooth newts have a higher growth rate than palmate larvae. Larval growth after four weeks was greater later in the year ($H_{10,532}$ = 399.96, p = <0.001), with a growth of 1 mm early in the year (laying date Day 31) but 3 mm later in the year (laying date Day 137). Therefore after four weeks of growth, larvae were significantly larger later in late spring (11 mm) than in early spring (8 mm) ($H_{10,521}$ = 181.18, p = <0.001).

There was no difference in larval survival between hatching date ($H_{10,25}$ = 9.43, p = 0.492) or between species (W = 392.5, d.f = 34, p = 0.648) and survival was approximately 85%. Larval hatching times and growth rates were reduced earlier in the year possible due to lower water temperatures and lower food availability. There was a negative correlation between number of days until hatching and mean water temperature (r_s = -0.973, d.f = 35, p = <0.001) and a positive correlation between water temperature and growth rates (r_s = 0.833, d.f = 543, p = <0.001).

6.4.2 Larval phenology in Llysdinam Pond

6.4.2.1 Comparison of larval phenology in 2007 with 1983-1984

Netting surveys have been conducted at Llysdinam in 1983, 1984 and 2007. Larvae were detectable earlier in the year in 2007 and latest in 1983 (Figure 6.3). Larvae were first found in Week 15 of 2007 and Weeks 21 and 20 of 1983 and 1984, respectively. Larval numbers peaked at Week 20 in 2007 and Week 28 in 1983 and 1984. The first larval detection was therefore advanced by at least five weeks in 2007 and the peak abundance

advanced by at least eight weeks. The 1984 and 2007 larval numbers had dropped to under one larvae per site by October (Weeks 40 and 41), but there were more larvae over wintering in 1983 with over two larvae per site detected in November (Week 44). The decline in larval numbers was very similar for 1984 and 2007 while in 1983 the later emergence of larvae was reflected in larval numbers still captured in autumn. Great crested newts did not occupy Llysdinam Pond in the early 1980s, and no great crested newt larvae were detected by netting in 2007. Mean cumulative captures of larvae per site indicated that there were more larvae in 1983 and 1984 than 2007 although this could be due to differences in netting techniques between Harrison (1985) and this study (Figure 6.4).

Figure 6.3 *Lissotriton* larval numbers in Llysdinam Pond

Mean number of *Lissotriton* newt larvae captured by netting surveys at Llysdinam Pond in 1983, 1984 and 2007. Mean numbers of newt larvae captured per sites are indicated. Newt larvae were captured at 20 of the netting sites dispersed across Llysdinam Pond. Each site was sampled by three sweeps of net. Surveys in 1983 and 1984 were conducted by Harrison (1985).

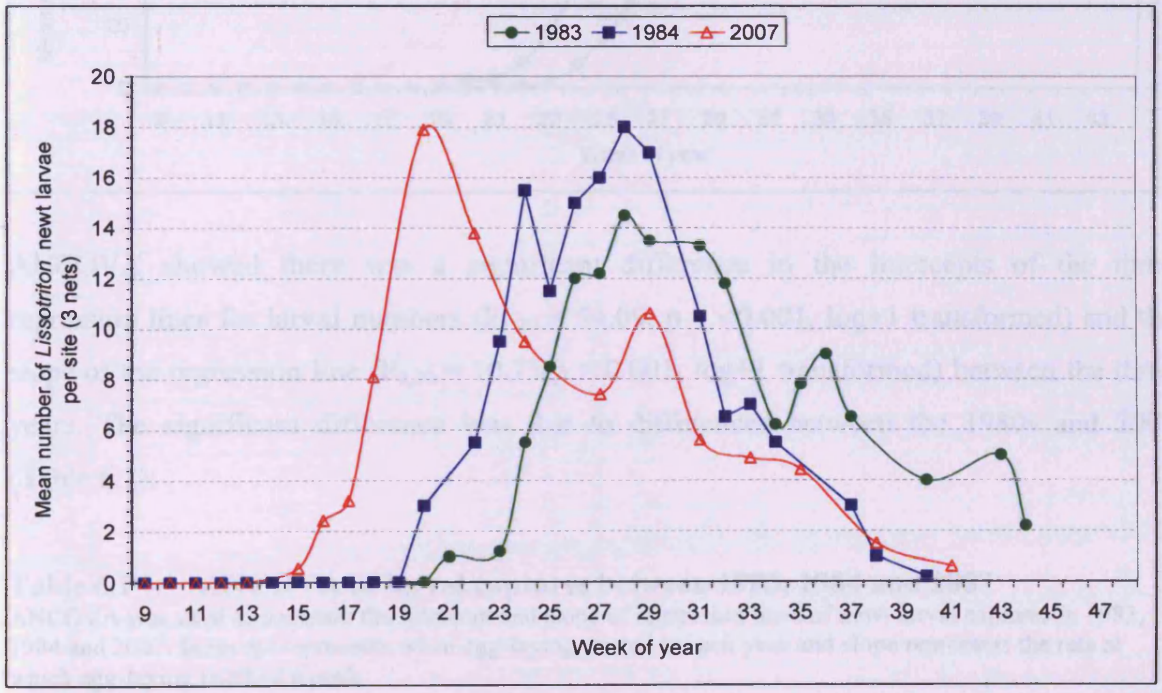
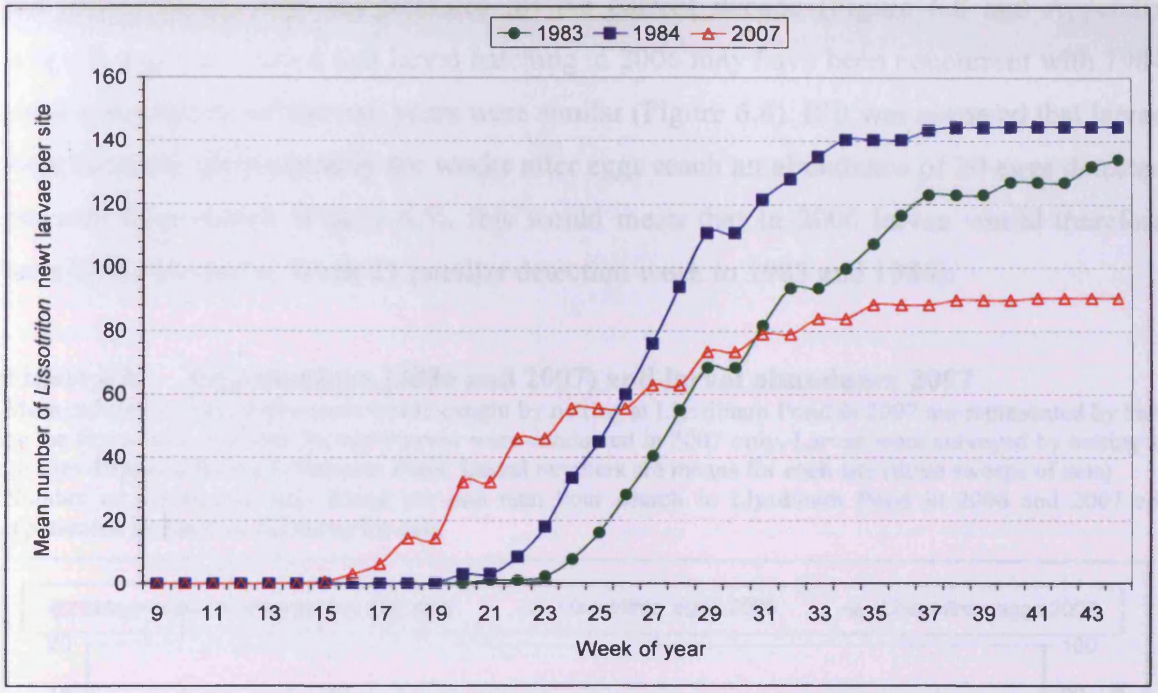


Figure 6.4 Cumulative *Lissotriton* captures in 1983, 1984 and 2007

Cumulative mean number of *Lissotriton* newt larvae captured by netting at Llysdinam Pond in 1983, 1984 and 1985. Cumulative mean numbers of newt larvae captured per site are indicated. The 20 netting sites where newt larvae were captured were dispersed across Llysdinam Pond. Each site was sampled by three sweeps of net. Surveys in 1983 and 1984 were conducted by Harrison (1985).



ANCOVA showed there was a significant difference in the intercepts of the three regression lines for larval numbers ($F_{1,20}$ = 54.09, p = <0.001, log+1 transformed) and the slope of the regression line ($F_{2,20}$ = 10.73 p = 0.001, log+1 transformed) between the three years. The significant difference was due to differences between the 1980s and 2007 (Table 6.1).

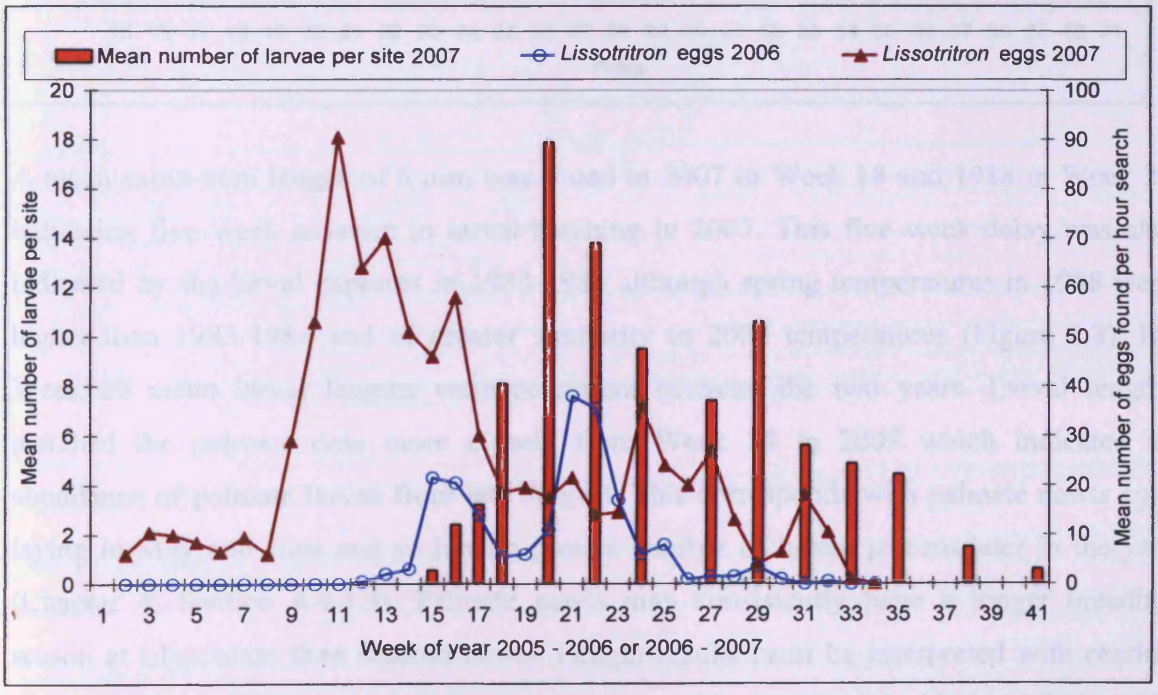
Table 6.1 ANCOVA of larval captures between 1983, 1984 and 2007

ANCOVA was used to compare the intercept and slope of regression lines of newt larval captures in 1983, 1984 and 2007. Intercept represents when egg-laying started in each year and slope represents the rate at which egg-laying reached a peak.

	F value (intercept)	p (intercept)	F value (slope)	p (slope)	d.f (group, error)	Transformation
1983-1984	0.28	0.607	6.72	0.021	1,13	
1983-2007	62.09	<0.001	36.79	<0.001	1,13	Log + 1
1984-2007	41.14	<0.001	14.61	0.002	1,13	

Although it was evident that larvae were detected earlier in 2007 this may not have been a consistent trend for previous years in the 2000s. Unlike larval abundance, egg-laying was surveyed in 2006 and 2007 and eggs were detected 10 weeks earlier in 2007 (Figure 6.5). Temperatures were higher in the spring of 2007 than in 1983-1984 (Figure 6.7) and 2007 had exceptionally high temperatures for the current decade (Figure 6.8 and Appendix XIV). It may be inferred that larval hatching in 2006 may have been concurrent with 1984 since temperatures of the two years were similar (Figure 6.8). If it was assumed that larvae were detected approximately six weeks after eggs reach an abundance of 20 eggs detected per man hour search (Figure 6.5), this would mean that in 2006 larvae would therefore have been detected at Week 21 (similar detection week to 1983 and 1984).

Figure 6.5 Egg numbers (2006 and 2007) and larval abundance 2007
Mean number of *Lissotriton* newt larvae caught by netting at Llysdinam Pond in 2007 are represented by bars on the first y axis. Surveys for newt larvae were conducted in 2007 only. Larvae were surveyed by netting at 20 sites dispersed across Llysdinam Pond. Larval numbers are means for each site (three sweeps of nets). Number of *Lissotriton* eggs found per one man hour search in Llysdinam Pond in 2006 and 2007 are represented by lines on the second y axis.

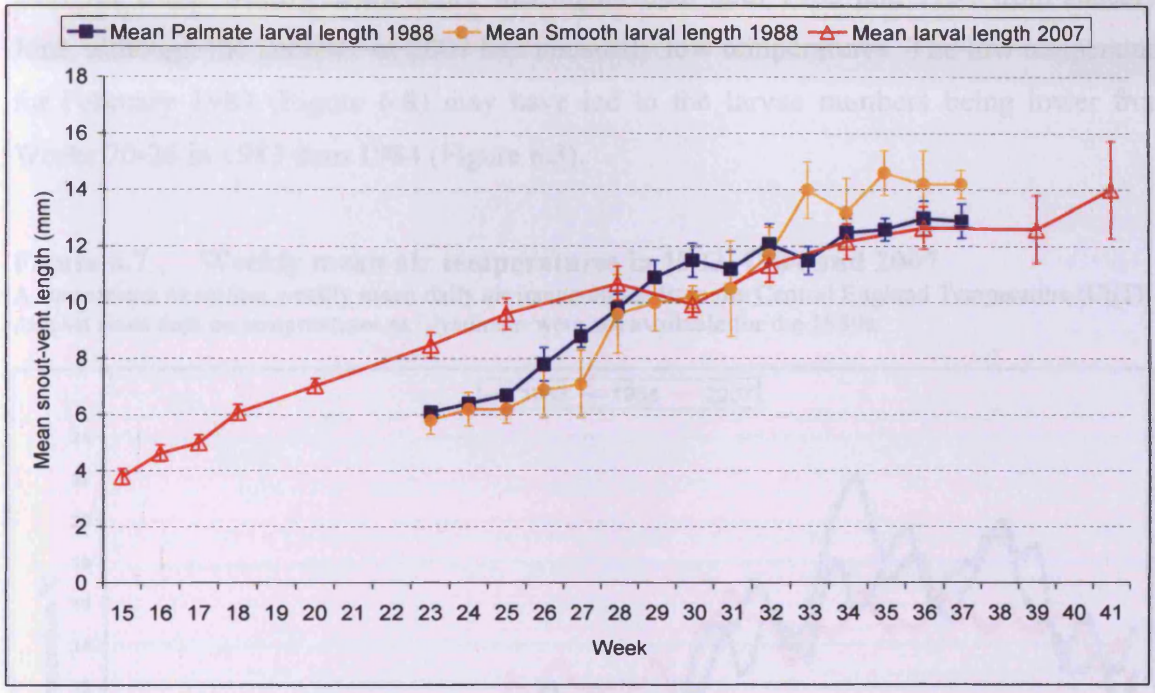


6.4.2.2 Comparison of larval lengths between 1988 and 2007

Data available from larval surveys at Llysdinam Pond in 1988 (de Wijer, 1990) enabled comparison of larval snout-vent lengths with 2007 captures (Figure 6.6). De Wijer only sampled newt larvae from Week 23 until Week 37 so presence of larvae and lengths prior to this were not known. De Wijer differentiated between palmate and smooth larval by removing a tail tip for electrophoresis. Species identification of palmate and smooth larvae did not take place in 2007. (Appendix XII and XIII for 1988 and 2007 larval newt lengths).

Figure 6.6 Comparison of larval snout-vent lengths in 1988 and 2007

Mean snout-vent lengths of *Lissotriton* newt larvae captured by netting at Llysdinam Pond in 2007, and by funnel traps in 1988 by de Wijer (1988). Palmate and smooth larvae were differentiated by de Wijer (1990) using electrophoresis of tail tips sampled from the larvae. *Lissotriton* species were not differentiated in 2007.

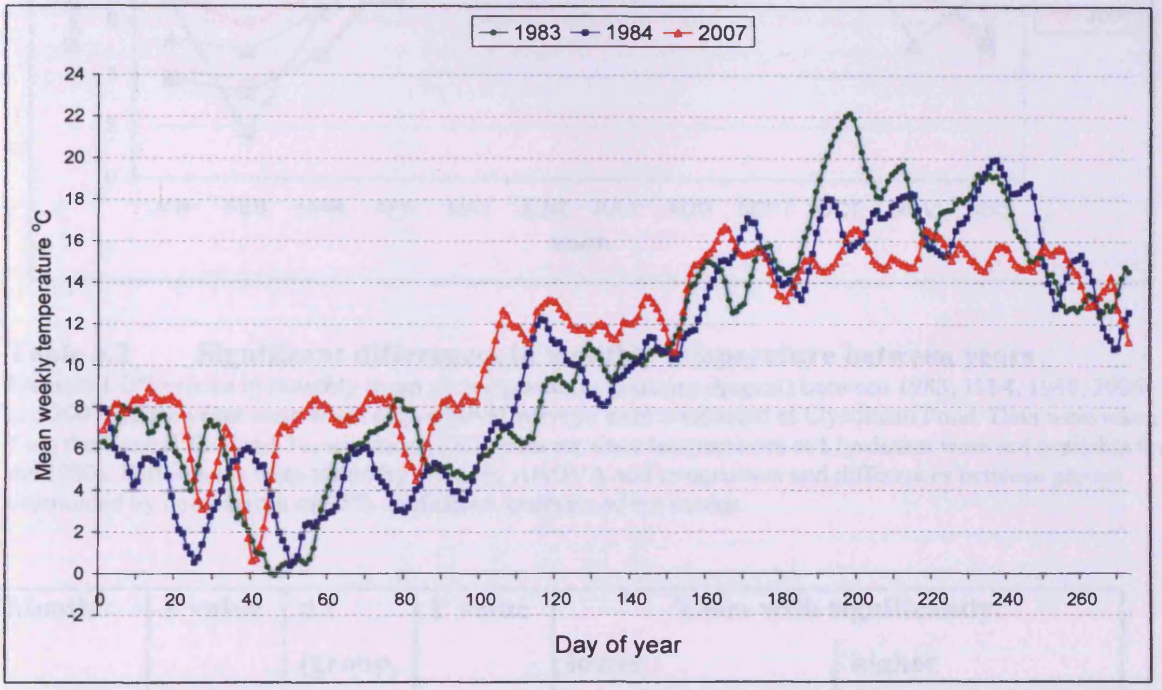


A mean snout-vent length of 6 mm was found in 2007 in Week 18 and 1988 in Week 23 indicating five week advance in larval hatching in 2007. This five week delay was also indicated by the larval captures in 1983-1984 although spring temperatures in 1988 were higher than 1983-1984 and of greater similarity to 2007 temperatures (Figure 6.8). By Week 29 mean larval lengths were consistent between the two years. Larval lengths matched the palmate data more closely from Week 34 in 2007 which indicated an abundance of palmate larvae from late August. This corresponds with palmate newts egg-laying in May and June and so having greater number of larvae present later in the year (Chapter 4, Section 4.4.3.3). Palmate newts may consistently have a longer breeding season at Llysdinam than smooth newts. Length results must be interpreted with caution since De Wijer (1990) used funnel traps (also named bottle traps) in 1988, while in 2007 larvae were captured using netting surveys. Funnels traps used by Fasola (1993) were only found effective in catching *Lissotriton* and *Triturus* larvae that were over 0.5 g while netting captured larvae over 0.1 g. This, however, would imply that de Wijer's data were biased towards larger larvae making the difference between 1988 and 2007 even greater.

6.4.3 Difference in weather between 1980s and 2007

Central England Temperature (CET) data were obtained to compare temperatures between years in which netting surveys were conducted (Figure 6.7). For the majority of days in 2007 the mean weekly temperature was higher than both 1983 and 1984 from January-June, although the summer of 2007 had unusually low temperatures. The low temperature for February 1983 (Figure 6.8) may have led to the larvae numbers being lower from Weeks 20-26 in 1983 than 1984 (Figure 6.3).

Figure 6.7 Weekly mean air temperatures in 1983, 1984 and 2007
A comparison of rolling weekly mean daily air temperatures from the Central England Temperature (CET) data set since data on temperatures at Llysdinam were not available for the 1980s.



Egg-laying was 10 weeks later in 2006 than 2007 which may have been the more common trend for the 1980s since 2006 temperatures were more similar to the 1980s (Figure 6.8). 2007 had significantly consistent higher temperatures earlier in the year (Table 6.2) while 1983 and 2006 had significantly higher summer temperatures. (Appendix XIV for graphs of monthly temperatures for the 1980s and 2000s).

Figure 6.8 Monthly mean temperature for 1983, 1984, 2006 and 2007

Temperature data at Llysdinam were not available for the 1980s. Therefore comparisons of monthly mean air temperatures were made from the Central England Temperature (CET) data set. The year represented were years when egg or larval surveys were conducted at Llysdinam Pond.

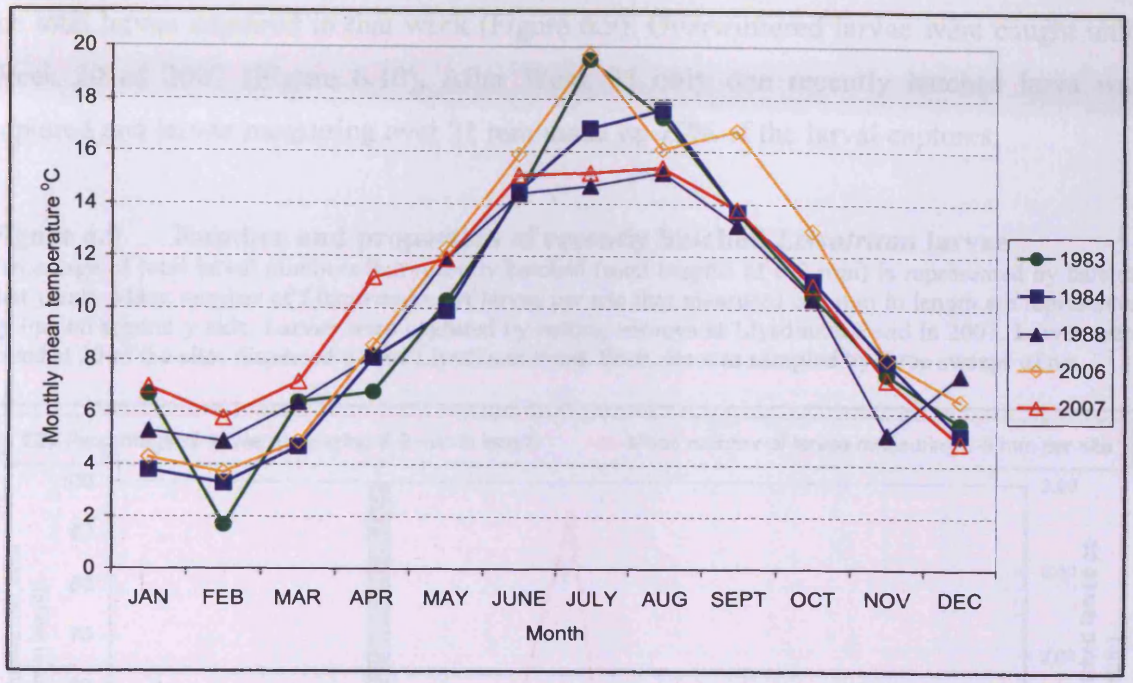


Table 6.2 Significant differences in monthly temperature between years

Statistical differences in monthly mean air temperatures (January-August) between 1983, 1984, 1988, 2006 and 2007. These years were when egg or larval surveys were conducted at Llysdinam Pond. Data were taken from the Central England Temperature (CET) data set since temperatures at Llysdinam were not available for the 1980s. Differences were tested by one-way ANOVA and comparison and differences between groups determined by comparison of 95% confidence intervals of the means.

Month	F value	d.f (group, error)	P value	Years with significantly:	
				lower temperatures	higher temperatures
January	11.6	4,150	<0.001	1984 2006	1983 2007
February	14.48	4,135	<0.001	1983	1988 2007
March	6.44	4,150	<0.001	1984 2006	2007
April	11.22	4,145	<0.001	1983	2007
May	10.33	4,150	<0.001	1983 1984	1988 2006 2007
June	3.0	4,145	0.021	1983 1984 1988	2006
July	47.83	4,150	<0.001	1988 2007	1983 2006
August	12.99	4,150	<0.001	1988 2007	1983 1984

6.4.4 Newt larval sizes in 2007

Larval size data were not available for 1983-1984. In 2007 recently hatched larvae (measuring 6-9 mm) were most abundant in Week 20, although they made up only 16% of the total larvae captured in that week (Figure 6.9). Overwintered larvae were caught until Week 20 of 2007 (Figure 6.10). After Week 31 only one recently hatched larva was captured and larvae measuring over 21 mm made up 75% of the larval captures.

Figure 6.9 Number and proportion of recently hatched *Lissotriton* larvae

Percentage of total larval numbers that recently hatched (total lengths of 6-9 mm) is represented by bars on first y axis. Mean number of *Lissotriton* newt larvae per site that measured 6-9 mm in length are represented by line on second y axis. Larvae were captured by netting surveys at Llysdinam Pond in 2007. Larvae were found at 20 of the sites dispersed across Llysdinam Pond. Each site was sampled by three sweeps of net.

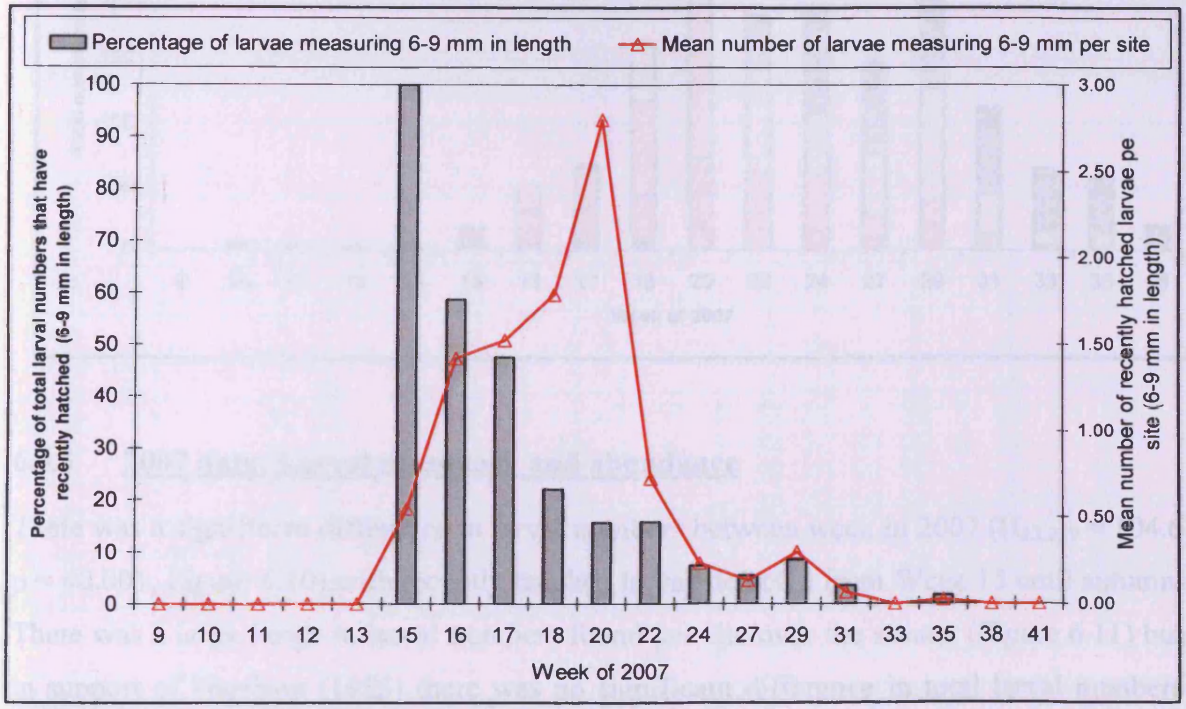
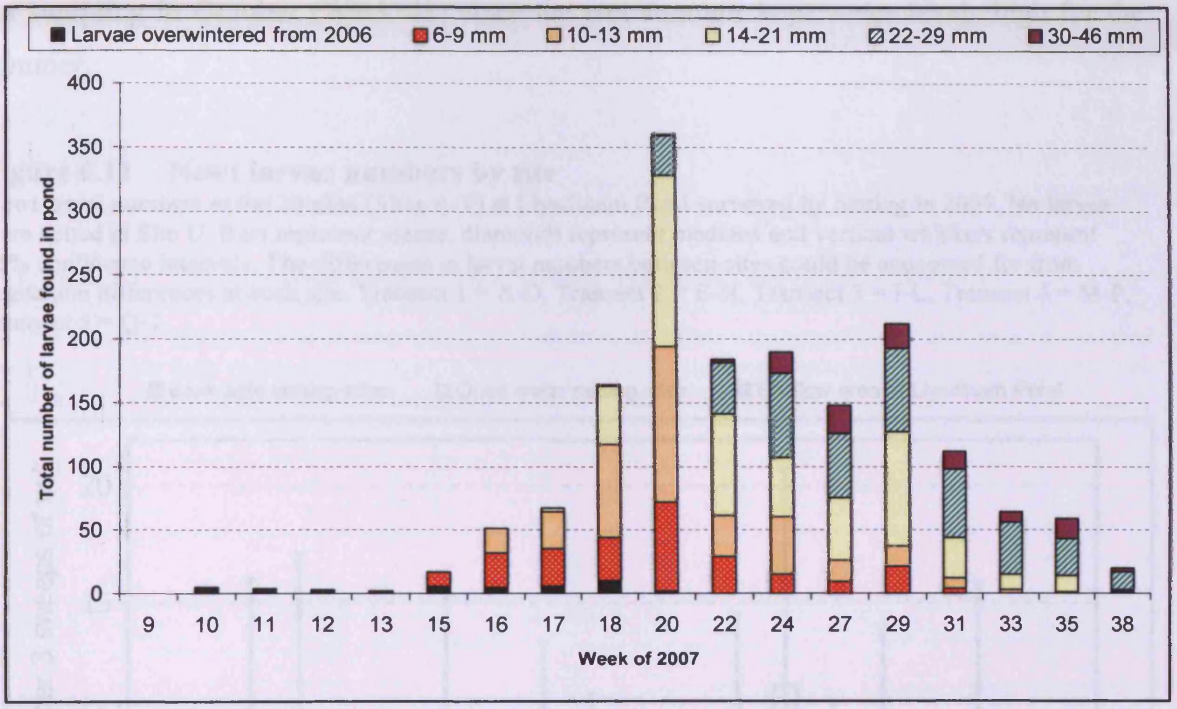


Figure 6.10 Abundance of newt larvae by size categories

Total numbers of newt larvae found in Llysdinam Pond per week for each of five size categories in the key. Overwintered captured between Weeks 10 and 20 were larvae that measured 30-40 mm total length. Larvae were captured by netting surveys at Llysdinam Pond in 2007. Total number of newt larvae captured in Llysdinam Pond from Weeks 9-38 of 2007 is indicated. Newt larvae were captured by netting at 20 sites dispersed across Llysdinam Pond. Each site was sampled by three sweeps of net.



6.4.5 2007 data: Larval phenology and abundance

There was a significant difference in larval numbers between week in 2007 ($H_{13,259} = 104.6$ $p = <0.001$, Figure 6.10) with recently hatched larvae detected from Week 15 until autumn. There was a large range in larval numbers found per site over the season (Figure 6.11) but in support of Harrison (1985) there was no significant difference in total larval numbers caught between sites ($H_{19,253} = 20.44$ $p = 0.369$). Sites with higher abundance reflected the distribution of aquatic vegetation used frequently as an egg-laying substrate.

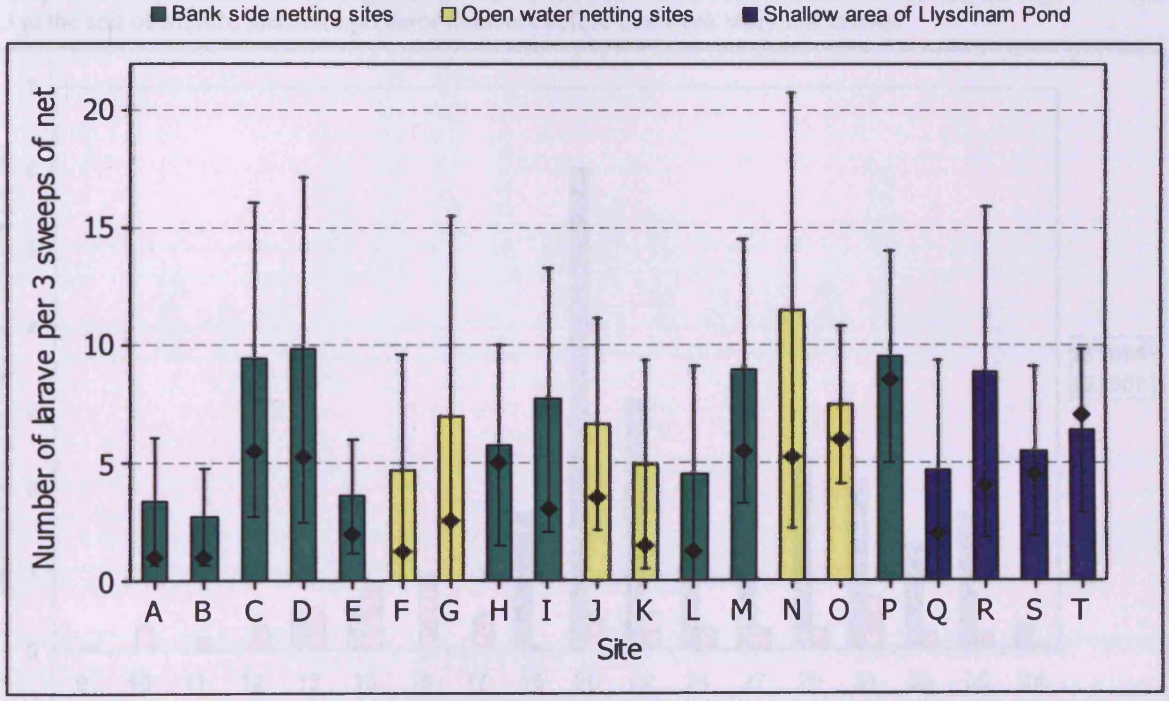
The larval abundance at sites in Llysdinam Pond could be accounted for by vegetation distribution:

- **Transect 1**: Site A and B had little vegetation, while *M. scorpioides* was present at Site C and D (and *G. maxima* at Site D)
- **Transect 2:** *G. maxima* was present at Site G.
- **Transect 3**: *M. scorpioides* was at Site I. Little vegetation at Site J-L.
- **Transect 4**: Abundance of *M. scorpioides*, *Callitriche*, *G. fluitans* and *G. maxima*

- **Transect 5**: *G. fluitans*, *M. scorpioides* were abundant in Q-T but *C. stagnalis*, the egg-laying substrate used in 30% of cases by *Lissotriton* newts in 2007 was not present in Site Q. Additionally Site Q was shallow and dried out, preventing sampling on four sessions (Weeks 16-18) when the peak larval numbers were captured. Sites R-T were only too dry for sampling in October (Week 41) since the wet summer kept water levels high for the summer.

Figure 6.11 Newt larvae numbers by site

Newt larval numbers at the 20 sites (Sites A-T) at Llysdinam Pond surveyed by netting in 2007. No larvae were netted in Site U. Bars represent means, diamonds represent medians and vertical whiskers represent 95% confidence intervals. The differences in larval numbers between sites could be accounted for from vegetation differences at each site. Transect 1 = A-D, Transect 2 = E-H, Transect 3 = I-L, Transect 4 = M-P, Transect 5 = Q-T.

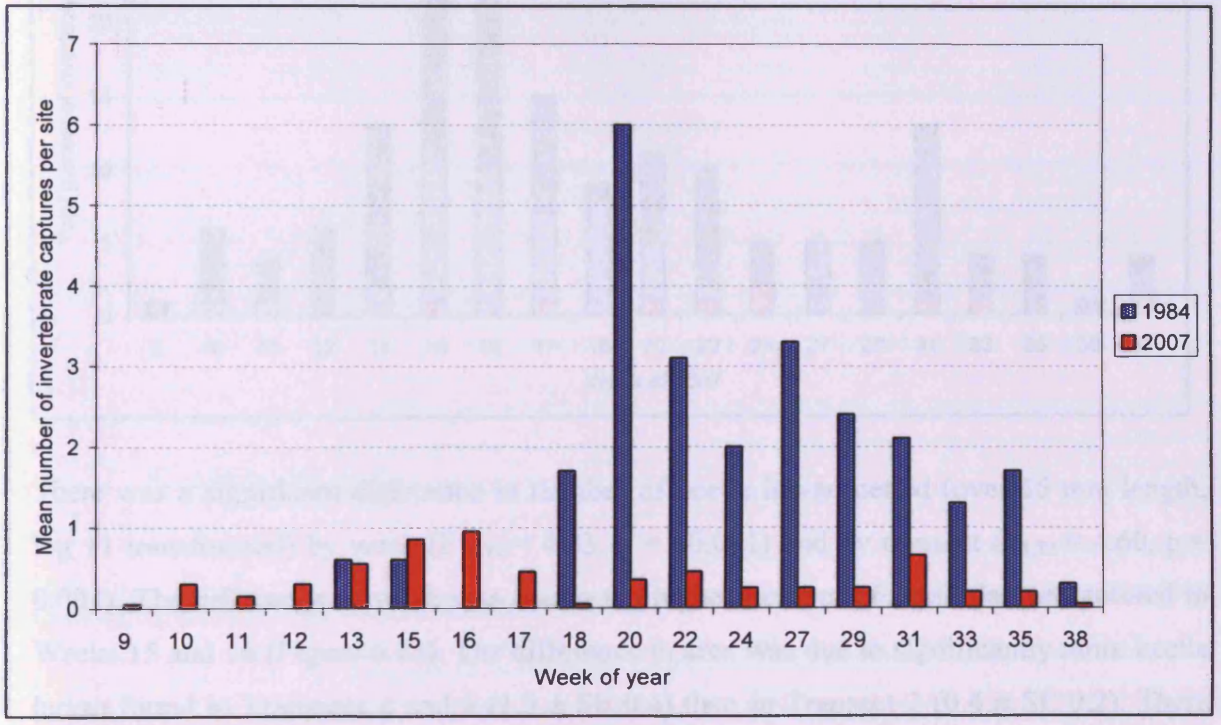


6.4.6 Invertebrate phenology in Llysdinam Pond

There were fewer predatory aquatic invertebrates in Llysdinam Pond in 2007 than 1980s (Figure 6.12 and Appendix X for full invertebrate data for 1984). This may be due to the large decline in toad numbers and therefore toad tadpoles from the mid-1980s since they were a major prey, particularly of beetle larvae (Harrison, 1985). Beetle larvae mainly contributed to the first peak in 1984 with approximately five larvae per site at Week 21, and large water boatman *N. glauca* contributed to the second predator peak at approximately two per site. In 2007 there were fewer aquatic predatory invertebrates, with less than one per site for all predatory groups recorded. In 2007 beetle larvae peaked at

Weeks 13 to 16, seven weeks earlier than the peak in 1984 (Figure 6.12). *N. glauca* fluctuated in numbers from Weeks 15-31 in 2007. Although there were similar fluctuations in *N. glauca* numbers in 1984, they were not detected until Week 18 and reached the highest abundance of two per site at Week 27. *N. glauca* was the main predator present during the larval stage of *Lissotriton* newts in 1984 but in 2007 the main predators were beetle larvae. There was a significant higher abundance of aquatic predatory invertebrate at Llysdinam Pond from Weeks 18-38 between 1984 and 2007 (W = 152.0 d.f = 18, p = 0.0004).

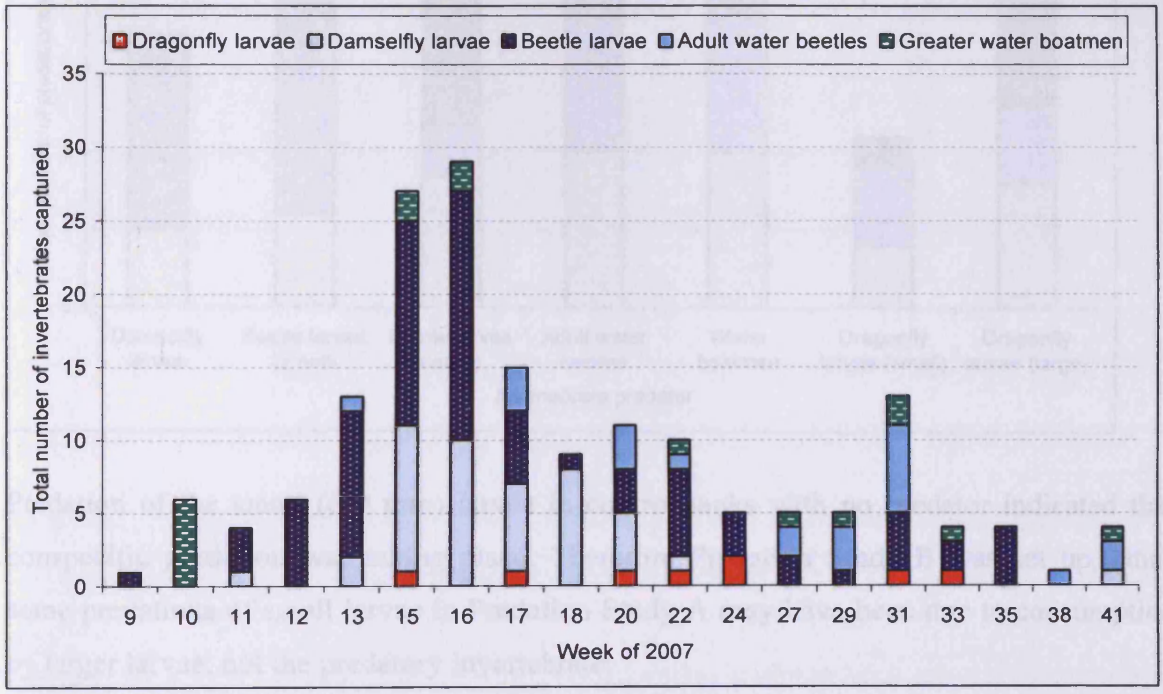
Figure 6.12 Aquatic predatory invertebrate captures in 1984 and 2007
Mean number of aquatic predatory invertebrates captures per site at Llysdinam Pond in 1984 and 2007. Mean numbers in 1984 calculated from three sweep nets at 11 sites. Mean numbers in 2007 calculated from three sweep nets from each of 20 netting sites (A-T). In 1984 Harrison only commenced netting surveys in Week 13 at the end of March, therefore predator numbers before this week were not known.



The predator peak in 2007 occurred at the same time as newt larvae were first detected in the pond (Weeks 15-16). Similarly in 1984 predators peaked on the same week as larvae were initially found (Week 20). In 2007 the predator peak coincided with peak newt egg-laying as newt eggs peaked in Week 11 of 2007 and remained in high numbers until Week 15 (Chapter 4, Figure 6.5). In 2007 toad spawn hatched in Weeks 15 and 16 and tadpoles were present in high numbers (of up to 30 per net) in certain areas of the pond until Week 18. Although frog tadpoles hatched on Week 12 of 2007 they were unlikely to be a major prey source since spawn was only deposited in Site U which dried up each year. Also only one predator was found in site U (a beetle larva 27 mm length), probably due to its

separation from the main water body. In 1984 toad spawn hatched in Week 16 and frog spawn hatched in Week 13 of 1984, which shows little change in spawning phenology between years (Harrison, 1985).

Figure 6.13 Total number of aquatic predatory invertebrates captured during 2007
Total numbers of aquatic predatory invertebrate captured in sweep netting surveys at all 21 surveys sites at Llysdinam Pond. Data displayed consists only of predatory invertebrates captured that were over 15 mm length (except great water boatmen (*N. glauca*) which are over 10 mm length).



There was a significant difference in number of beetle larvae netted (over 15 mm length, log +1 transformed) by week ($F_{18,72} = 4.33$, $p = <0.001$) and by transect ($F_{4,72} = 4.60$, $p = 0.002$). The difference in week was due to the higher number of beetle larvae captured in Weeks 15 and 16 (Figure 6.13). The difference in area was due to significantly more beetle larvae found in Transects 4 and 5 (1.3 ± SE 0.4) than in Transect 2 (0.4 ± SE 0.2). There were not enough captures of the other predatory invertebrates to warrant statistical analysis.

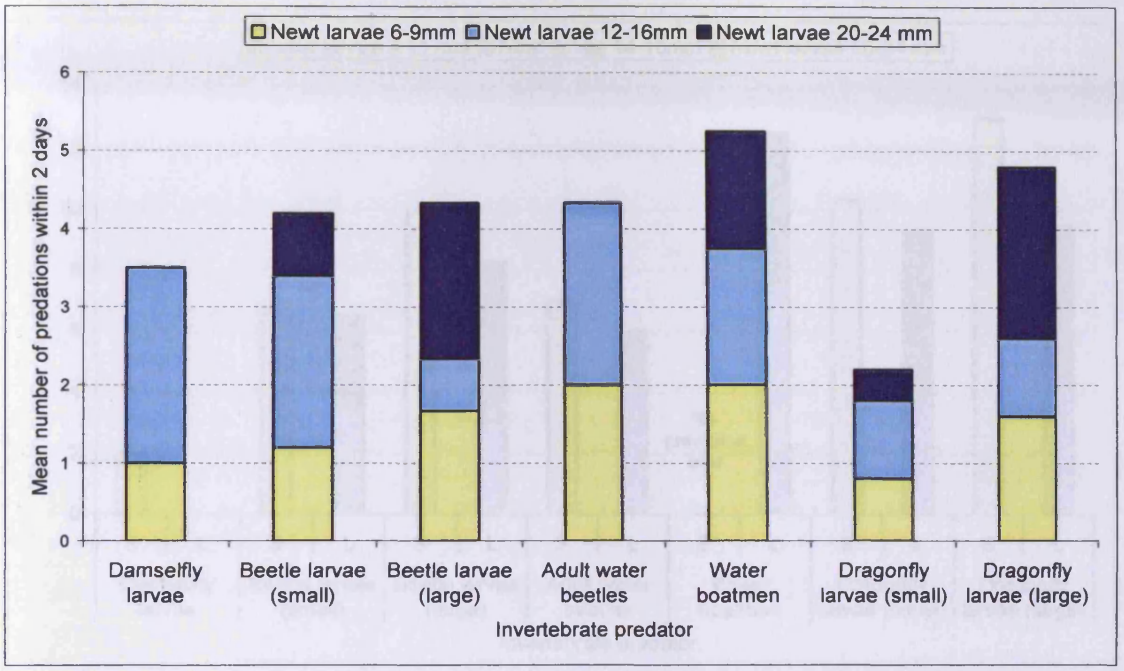
6.4.7 Predation study

6.4.7.1 Predation Study A

All invertebrates showed a sight preference for the median and large newt larval size categories (Figure 6.14). No large larvae were consumed by the damselflies although they showed a preference for medium sized larvae over small. Large dragonfly and beetle larvae showed a preference for the largest larvae.

Figure 6.14 Predation rates of newt larvae in Predation Study A

Mean number of newt larvae predated within two days by predatory aquatic invertebrates in a laboratory-based tank study. There were nine larvae per tank, three from each size category (6-9 mm, 12-16 mm and 20-24 mm).



Predation of the small (6-9 mm) larvae in control tanks with no predator indicated that conspecific predation was taking place. Therefore Predation Study B was set up, since some predations of small larvae in Predation Study A may have been due to consumption by larger larvae, not the predatory invertebrate.

6.4.7.2 Predation Study B

The proportion of larval predation between invertebrates differed significantly (Table 6.3). Damselflies, small beetle larvae and beetles predated fewer than expected larvae while more than expected larvae were predated by large beetle larvae and dragonflies. There was little difference in the most voracious predator groups between newt sizes. Too few *N. glauca* were captured to set up a small larval predation study. Unlike Predation Study A, small larvae were predated as readily as medium sized and larger larvae even though conspecific predation had been removed (Figure 6.15). There were no differences in number of small, medium and large larvae predated except damselflies only predated small larvae, and predatory adults beetles predated more than expected medium sized larvae (χ^2 = 3.9, d.f = 1, p = 0.048, N = 16). During predation studies observations were made that large dragonfly and beetle larvae were particularly voracious and stalked larvae as soon as the study was set up.

Figure 6.15 Predation rates of newt larvae in Predation Study B

Mean number of newt larvae predated within three days by predatory aquatic invertebrates in a laboratory-based tank study. There were six larvae per tank, coded 'S' for tanks with six small larvae (9-11 mm), and L for tanks with six large larvae (3 x 14-16 mm and 3 x 20-24 mm).

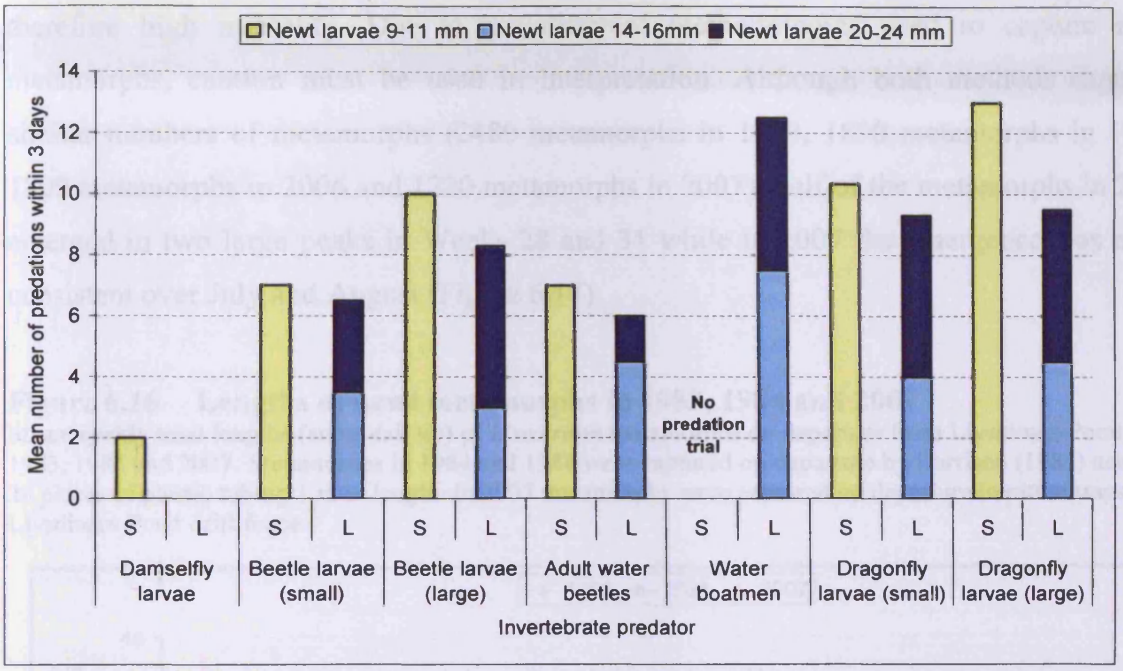


Table 6.3 Differences in predation rates of newt larvae in Predation Study B

Significant differences in predation levels of predatory aquatic invertebrates on the three different size categories of larvae in Predation Study B. Predation Study B took place in tanks in a laboratory. In the study there were six larvae per tank, either six small (9-11 mm) or six large larvae (3 x 14-16 mm and 3x 20-24 mm).

Larval size	χ^2	d.f	N	p	Invertebrates taking more than expected prey
9-11 mm	25.33	6	151	<0.001	Large dragonfly larvae, small dragonfly larvae, large beetle larvae
14-16 mm	32.75	7	135	<0.001	Large dragonfly larvae, small dragonfly larvae, adult water beetles
20-24 mm	18.68	7	66	0.009	Large beetle larvae, small dragonfly larvae, large dragonfly larvae, water boatmen, medium size beetle larvae

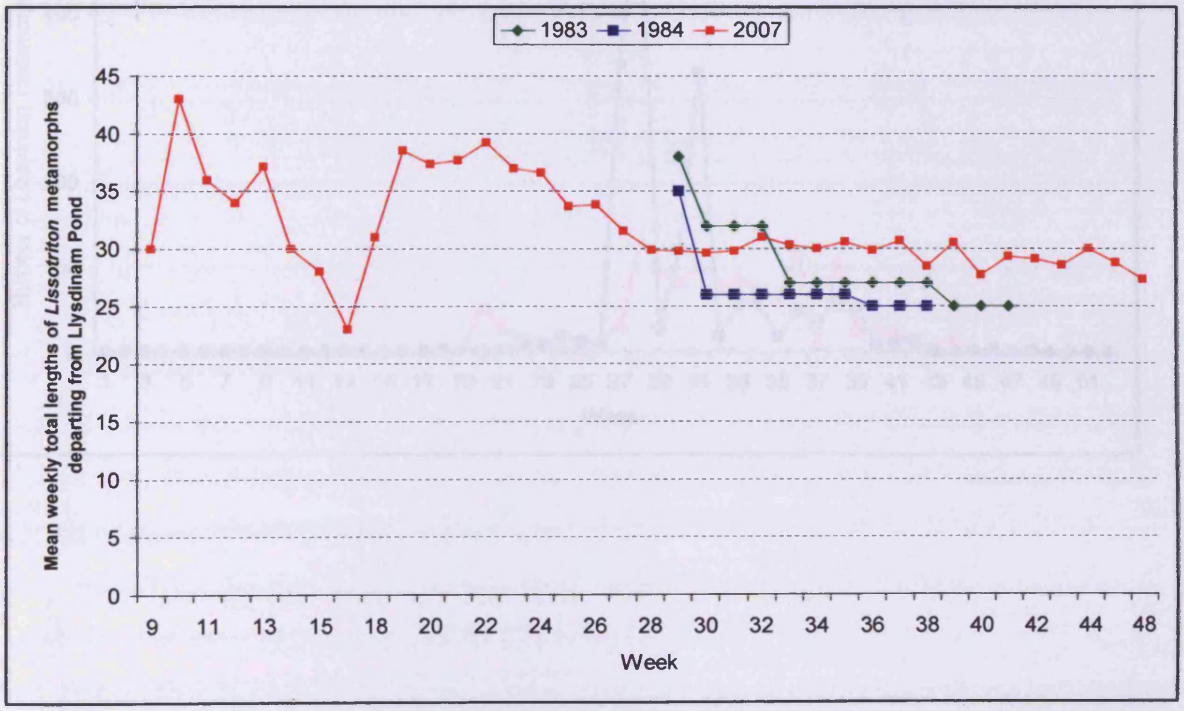
6.4.8 Metamorph phenology

6.4.8.1 Metamorph data from 1983, 1984 and 2007

Mean weekly newt metamorph lengths indicated that overwintered larvae emerged later in 1983 and 1984. Metamorphs that emerged from Llysdinam Pond during the main emergence period from Weeks 30-40 were also significantly larger in 2007 than in 1983 and 1984 ($H_{2,22} = 17.78$, $p = <0.001$, Figure 6.16).

Median metamorph emergence dates were similar in 1984 (12/8/1984) and 2007 (8/8/2007) although much later in 1983 (5/9/1983). Harrison (1985) stated that the median emergence date in 1984 was early and due to desiccation of the pond in the summer and therefore high mortality. Due to the different methodologies used to capture newt metamorphs, caution must be used in interpretation. Although both methods captured similar numbers of metamorphs (2480 metamorphs in 1983, 1890 metamorphs in 1984, 1809 metamorphs in 2006 and 1220 metamorphs in 2007), half of the metamorphs in 2006 emerged in two large peaks in Weeks 28 and 31 while in 2007 the emergence was more consistent over July and August (Figure 6.17).

Figure 6.16 Lengths of newt metamorphs in 1983, 1984 and 2007
Mean weekly total lengths (snout-tail tip) of *Lissotriton* metamorphs on departure from Llysdinam Pond in 1983, 1984 and 2007. Metamorphs in 1984 and 1984 were captured on departure by Harrison (1985) using 20 pieces of plastic tubing 1 m in length. In 2007 metamorphs were captured on departure in pitfall traps at Llysdinam Pond drift fence.



6.4.8.2 Metamorph emergence in 2006 and 2007

The higher rainfall in 2007 enabled a more continuous pattern of emergence by newt metamorphs (Figure 6.17), while in 2006 there were many weeks with low rainfall. In 2006 emergence of metamorphs occurred on weeks with at least one day of high rainfall (Figure 6.18). In Weeks 27, 28 and 31 there was one day with 26 mm, 8.2 mm and 11.8 mm rainfall respectively, leading to high captures of over 300 metamorphs that emerged within a few days.

In 2007 the method used to separate *Lissotriton* metamorphs by species indicated 765 palmate, 273 smooth and 181 unidentified metamorphs. Unknowns often lacked any dorsal stripe. Palmate metamorphs had a mean length of 29.8 mm and smooth metamorphs 32.1 mm. The median week of exit was 32 and 34 for palmate metamorphs and smooth metamorphs, respectively. Peak smooth metamorph emergence was over shorter period (Weeks 28-38) than for palmate metamorphs (Weeks 27-42).

Figure 6.17 Number of *Lissotriton* metamorphs emerging in 2006 and 2007
Weekly numbers of *Lissotriton* metamorphs captured departing from Llysdinam Pond in 2006 and 2007. All metamorphs were captured in pitfall traps at Llysdinam Pond drift fence.

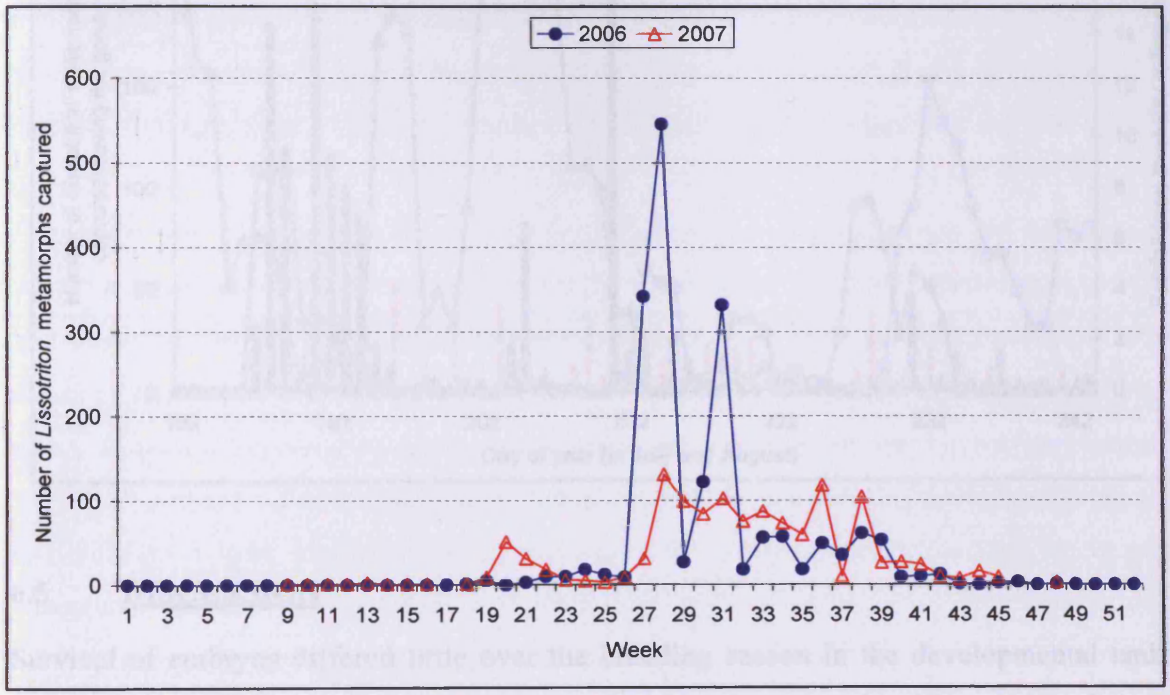
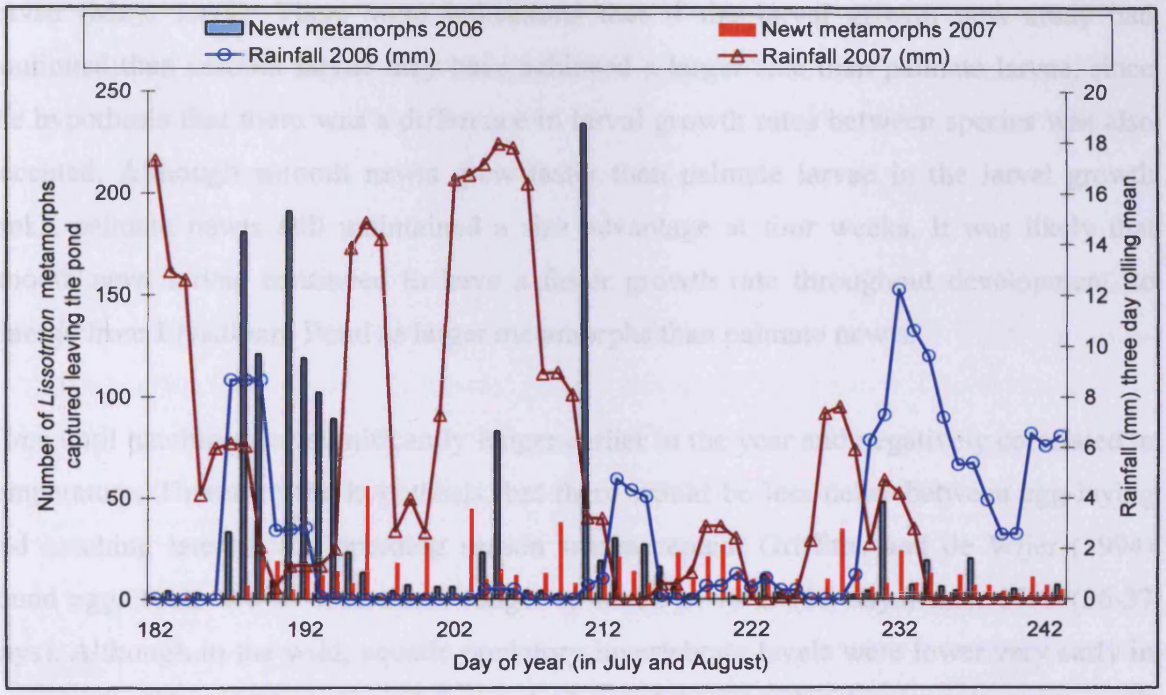


Figure 6.18 Association between the number of *Lissotriton* metamorphs emerging amount of rainfall

Daily numbers of *Lissotriton* metamorphs captured on departure from Llysdinam Pond in 2006 and 2007 are indicated by bars on the first y axis. All metamorphs were captured in pitfall traps at Llysdinam Pond drift fence. Data displayed from Day 182-242 (July and August). Rainfall is a three day rolling mean from rainfall measurements collected at Llysdinam weather station represented as a line on the second y axis. Metamorph departures in 2007 were more continuous with fewer large peaks due to the high rainfall throughout July-August 2007.



6.5 DISCUSSION

Survival of embryos differed little over the breeding season in the developmental tanks. Therefore the hypothesis that there would be significantly greater hatching success for eggs laid later in the newt breeding season was rejected. Griffiths and de Wijer (1994) also found no effect of temperature on egg survival with a high 85-95 % hatching success at 12 and 17°C. The newt egg-laying season began early in 2007 so the first eggs found in the outdoor breeding tanks may have had higher mortality due to a cold period that occurred a few days after they were laid. There were several days with a mean air temperature below 0°C and low temperatures can increase likelihood of fungal infection (Griffiths, 1995), but it was also possible that the eggs laid early were unfertilized. Although hatching lengths of palmate and smooth newts have been similar in many studies (Griffiths and de Wijer, 1994), palmate larvae at Llysdinam were on average 1 mm larger than smooth larvae. Therefore the hypothesis that there was a significant difference in palmate and smooth hatchling sizes was accepted. The reduced size of smooth larvae at hatching may be partially due to the slightly shorter egg stage of smooth newts although the differences

between palmate and smooth eggs were non-significant. Palmate newts were also found to hatch at a larger size in a study at nearby Cors-y-Llyn pond (2 km south of Llysdinam) by May (1993). Both species of *Lissotriton* larvae were larger in the study by May (1993) than at Llysdinam, but there was less difference between the palmate and smooth sizes at Cors-y-Lyn Pond. After 10 weeks smooth larvae had grown faster to be larger than palmate larvae (May, 1993). There were indications that if the larval growth tank study had continued then smooth larvae may have achieved a larger size than palmate larvae, since the hypothesis that there was a difference in larval growth rates between species was also accepted. Although smooth newts grew faster than palmate larvae in the larval growth tanks, palmate newts still maintained a size advantage at four weeks. It was likely that smooth newt larvae continued to have a faster growth rate throughout development, to emerge from Llysdinam Pond as larger metamorphs than palmate newts.

Time until hatching was significantly longer earlier in the year and negatively correlated to temperature. Therefore the hypothesis that there would be less delay between egg-laying and hatching later in the breeding season was accepted. Griffiths and de Wijer (1994) found eggs took two to four times longer to hatch at 12°C (16 days) than 17°C (36-37 days). Although in the wild, aquatic predatory invertebrate levels were lower very early in the year, by Week 15 larval numbers reached a peak but egg development times were still relatively low. Slow development increases time for predation when the developing newt was at an immobile defenseless stage, while later in the year the faster development rates would lead to a shorter period of vulnerability. No significant differences were found in the size of larvae hatching over the season which corroborates Griffiths and de Wijer's findings (1994). Unlike Griffiths and de Wijer, the developmental stages of the hatchling (Gallien and Bidaud, 1959) were not recorded in this study. Griffiths and de Wijer (1994) found that higher temperatures increased the hatching stage of palmate more than smooth larvae but not significantly (Appendix V for diagrams of *Lissotriton* hatching stages). The average developmental stage of hatchling palmate larvae was Stage 38 when the eggs developed at 12°C and Stage 39 at 17°C, while for smooth eggs most larvae hatched at stage 38 regardless of temperature during development.

Griffiths and de Wijer (1994) also studied great crested eggs experimentally and found that those raised at 17°C hatched at a smaller size but later stage so that there was a negative relationship between sizes and stage at hatching. Although no great crested larvae were captured in the pond during the netting surveys, the results for egg-laying indicate that they

have a later breeding phenology than *Lissotriton* newts. Research has shown that great crested eggs or larvae reared at low temperatures (13-16°C) produced more females and those raised at a high temperature (28°C) produced more males. Great crested newts may avoid egg-laying as early as *Lissotriton* newts to prevent a female biased population (Wallace and Wallace, 2000), due to different temperature thresholds (Arntzen and Hedlund, 1990; Verrell and Halliday, 1985) or different physiological mechanism controlling development (Griffiths and de Wijer, 1994). There was, however, no evidence that male and female *Lissotriton* newt ratios have changed significantly between the 1980s and recent years (Chadwick et al., 2006) due to climate change.

In addition to temperature increases, longer day length later in the year can stimulate growth (Boeuf and Le Bail, 1999) and development (Wright et al., 1988), which may have been a factor that led to an average larval growth increase of 3 mm over four weeks in May and June compared to 1 mm early in the year. The hypothesis that larval growth rates would be significantly greater later in the newt breeding season was therefore accepted. In addition to temperature differences, reduced food availability was likely to have stunted larval growth earlier in the year, since pond water provided in the larval growth tanks contained fewer prey. Heckel (1984) reared eastern red-spotted newt larvae *Notophthalmus viridescens* at 7, 21 or 25°C and fed them with 105, 150 or 195 brine shrimp per day. Higher food levels significantly increased growth but higher temperatures had no significant effect. In contrast, food levels had a greater effect on the passage through developmental stages than did higher temperatures. He concluded that higher food abundance mainly increased growth while temperature speeded up differentiation. If further work on larvae growth and development took place, two levels of food abundance could be provided to investigate whether the lower temperature or low food abundance affects growth in *Lissotriton* larvae.

Water temperatures in tanks were only recorded twice weekly and these small water volumes may have led to greater temperature fluctuations in ponds than in the wild. Therefore there may be some discrepancy between the hatching sizes and growth data recorded in developmental and larval growth tanks, and what would be recorded in the wild. Hatchling sizes in tanks were similar to the smallest larval captures in the pond, but larval growth may have been retarded in tanks due to space limitations and reduced food available. A laboratory-based tank study by Bonetti (1996) found very poor larvae growth rates for palmate newt larvae. Basket traps used to study newt larvae in Llysdinam Pond in

June 1995 (Bonetti, 1996) had slightly higher growth rates than the larval growth tanks (0.16 mm per day compared to 0.1 mm per day). Small size and slow growth after hatching may increase vulnerability to gape-limited predators (Griffiths and de Wijer, 1994). In Llysdinam Pond, adult newts and conspecific larvae would be examples of gape-limited predators, but most predatory aquatic invertebrates consume prey by direct mastication or injection of digestive juices (Harrison, 1985). Conspecific larvae have not been found to consume larvae in high numbers (Harrison, 1985). Predation Study A demonstrated that larger conspecific larvae would consume newly hatched larvae, but this may only have occurred because other large prey types were not available to the large newt larvae.

In Llysdinam Pond *Lissotriton* newt larvae were detected five or six weeks earlier in 2007 than 1983-1984. The hypothesis that larvae hatch significantly earlier in Llysdinam Pond in 2007 compared with the 1980s was therefore accepted. It was likely the high late winter and early spring temperatures in 2007 led to the earlier *Lissotriton* newt arrival and egg-laying and therefore earlier larval hatching and detection. Untangling the possible effects of weather, adult breeding numbers, vegetation abundance and predation numbers between the years would be difficult. The advancement in larval hatching was unlikely be a continual annual phenomenon in the current decade since in 2006 eggs were detected 10 weeks later than in 2007. Although in the 1990s and 2000s more *Lissotriton* newts have been migrating to Llysdinam Pond in autumn and winter (Chadwick et al., 2006), it may be that only when early spring temperatures were high enough did newts begin egg-laying in detectable numbers (Chapter 4), leading to higher numbers of larvae hatching earlier in the year. If temperatures are low in early spring, as in 2006, then there may be a longer delay between arrival and breeding. Factors affecting amphibian breeding timing can vary between pond habitats. In a five year study in the Mediterranean, breeding peaks were related to temperature in permanent ponds but reproductive success in temporary pond breeders was determined by rainfall pulses (Richter-Boix et al., 2006).

Two larval cohorts of an early and late hatchlings could be identified at Llysdinam Pond, particularly in 1983 and 2007 as found in previous research (Bell and Lawton, 1975). The two peak captures were also found for egg surveys (Chapter 4). In contrast, other research suggests a continuous egg-laying period and no distinct cohorts (de Wijer, 1990; May, 1993). The earlier hatching of larvae in 2007 led to a larger size cohort earlier in the year than in 1988, although later in each year the mean size of larvae in pond in 1988 and 2007 were similar.

Early breeding and larval hatching can have advantages over late hatching including lower predation, less competition and reduced exposure to UV radiation. Negative aspects of early hatching in the UK were likelihood of more exposure to days with low temperatures, lower food availability and reduced vegetation cover for refuge. Tadpole mortality has been positively correlated with number of cold days (Reading and Clarke, 1999), a direct temperature disadvantage of earlier breeding. Delayed breeding in the UK has the advantage of increased temperatures so that amphibian larvae can metamorphose faster, although often at smaller size in response to increased water temperature. *Bufo marinus* larvae showed direct preference for warmer temperatures (Floyd, 1984) although larval temperature preferences can vary with habitat (Freidenburg and Skelly, 2004). A shift to earlier breeding phenology can also limit exposure to UVB radiation (Cummins, 2003), although wrapping of newt eggs minimises exposure (Marco et al., 2001). The effects of UVB radiation have been shown to carry over from egg to larval stage in *Rana temporaria* with higher frequency of developmental abnormalities. Despite abnormalities being observed later, negative impacts of UVB radiation on hatching success and hatching size were not evident (Pahkala et al., 2000).

Environmental factors can affect feeding behaviour in newt larvae. Water acidity has been shown to affect snapping response to prey (Griffiths, 1993), so there are possibilities that other environmental factors, such as temperature may have implications for such behaviour. Amphibians adapt their breeding phenology according to the environmental conditions and larvae at higher latitudes may have higher genetic capacity for faster development (Laugen et al., 2003). Tadpoles from the north of the UK metamorphosed earlier and had higher growth rates than those from the south suggesting local adaptation to a shorter season and cooler temperatures (Merilä et al., 2000), and amphibians from high latitudes and altitudes were larger at all larval stages and had a longer larval period (Morrison and Hero, 2003).

Later in the 2007 newt breeding season, there were a high proportion of larger newt larvae which may predate on smaller larvae and compete directly for prey. The phenology of larval hatching has been shown to be of importance for competition and priority effects between and within species (Lawler and Morin, 1993). Even when adult *Triturus* newts used different resources, high aquatic niche overlap was found for larvae (Jehle et al., 2000). Many studies have taken place investigating priority effects in larvae (Alford and Wilbur, 1985; Morin, 1990; Ryan and Plague, 2004; Segev and Blaustein, 2007). Although

there were less food resources early in the season, early breeders reduced these before late breeders emerged. One disadvantage was that late hatchlings may have to compete against conspecific older larger hatchlings (Morin, 1987; Ryan and Plague, 2004). Survival of late hatching larvae was reduced to almost zero in one study on *Ambystoma taldpoideum* (Ryan and Plague, 2004). The protracted egg-laying season of *Lissotriton* newts meant that early hatchling larvae gained a size advantage by the time late larvae hatched but there was greater risk from low early spring temperatures.

Optimal hatching time will also depend on the other species present at the time of hatching and larval development. Newt larvae that hatched around Week 15 may have had greater predation risk due to the peak abundance of predatory aquatic invertebrates. The predators had alternative prey resources around this time, since there were numerous toad tadpoles which may have mitigated some of the predation risk. Since adults newts did not start to leave the pond in large numbers until June, the risk of predation by conspecific adults remained high until then. Many studies have investigated interspecific effects on larval development and success in amphibians. Early hatching *R. temporaria* larvae were eaten by smooth newts (Zahn, 1997) while *R. temporaria* inhibited the growth and reduce survival of natterjack toad *Bufo calamita* tadpoles (Griffiths et al., 1991). Great crested larvae had a size advantage over *Lissotriton* larvae by eight weeks, and were able to eliminate palmate newts and reduce smooth newts by predation (Griffiths et al., 1994). *Bufo americanus* larvae did better if they hatched before *Rana spenocephala* hatchlings while *R. spenocephala* did better when they hatched after *B. americanus* (Alford and Wilbur, 1985). Early breeding fire salamanders *Salamandra salamandra* reduced banded newt *Ommatotriton vittatus* by 90% and 70% in unvegetated and vegetated pools, respectively (Segev and Blaustein, 2007). Competition between amphibians and non-amphibian species has also been demonstrated (Mokany and Shine, 2003).

Large yearly fluctuations in amphibian populations are problematic in short term studies on amphibians. In 2007 there were half as many newt arrivals at Llysdinam Pond compared with 2006, but four times the number of eggs were detected, while metamorphs emergence in 2007 was two-thirds of that in 2006. Shoop (1974) also found that survival of *Ambystoma maculatum* varied each year, not dependent on number of eggs deposited but on the developmental rate of larvae, pond duration and climatic conditions at dispersal. Berven (1990) found large fluctuations in populations of the wood frog *Rana sylvatica*, where the adult population fluctuated by a factor of 10 and juvenile production by a factor

of 100. Long term data sets are required to explore amphibian populations and phenology thoroughly. It could be that despite the lower egg numbers in 2006, survival of eggs and larvae was greater than in 2007, leading to a greater number of newt metamorphs.

Netting has been demonstrated to be an effective method for capturing larvae (Willson and Dorcas, 2003) but dependent on larval size (Shaffer et al., 1994). Netting was more successful than funnel trapping for catching smaller specimens, since Fasola (1993) stated that only *Triturus* larvae over 0.5 g were captured in funnel traps. Netting can cause mechanical damage to the delicate larval tail and gills and it can be disruptive to the pond ecosystem (Shaffer et al., 1994). Presence and catchability of larvae depends on the habitat (Shaffer et al., 1994; Szetatecsny et al., 2004), and larvae were more abundant in well vegetated areas. Newt larvae were in high abundance in areas of the pond where there was an abundance of plants used for egg-laying such as *C. stagnalis, G. fluitans* or *M. scorpioides*. Although, as found by Harrison (1985), there was no significant difference in the distribution of *Lissotriton* larvae across the pond. Species bias in catchability between survey techniques was checked by de Wijer (1990) with a higher proportion of palmate larvae captured by netting than funnel trapping while the reverse was true for smooth larvae (de Wijer, 1990). Both the size and species differences between funnel trapping and netting noted above could have biased larval samples in 2007 to smaller sizes since smooth larvae attain a larger size. De Wijer found that palmate newt larvae size stopped increasing from Week 32 and that smooth newts were larger in size from Weeks 33-37. The size differences probably resulted from the number of recently hatched small palmate larvae contributing to the population later in the season (Chapter 4), that smooth larvae undergo faster growth (May, 1993) to emerge as larger metamorphs, and that palmate newts leave the pond earlier than smooth metamorphs (de Wijer, 1990). Differing growth strategies might influence susceptibility to predation in larvae, although risk of predation begins before the larval stage.

Oophagy by a range of predatory aquatic invertebrates has a significant impact on *Lissotriton* newt egg numbers, particularly when eggs were left unwrapped (Miaud, 1993; Orizaola and Braña, 2003a). Studies specifically on *Lissotriton* larval predation are more limited (Harrison, 1985), although a number of studies have looked at predator avoidance behaviour by amphibian larvae (Laurila and Kujasalo, 1999; Orizaola and Braña, 2003b; van Buskirk, 2001) and morphological changes of larvae in response to predator cues or presence (Kishida et al., 2006; Lardner, 2000; Mathis et al., 2003). Predator presence can

reduce larval growth and mass at metamorphosis (Lardner, 2000; Skelly, 1992) probably through reduced activity and foraging (Hokit and Blaustein, 1995; Kupferberg, 1997; Laurila, 2000; Skelly, 1992). Conversely, amphibians can increase growth rates when competitors are reduced (Morin, 1987; Wilbur, 1997) or prolong the larval period to gain body size (Laurila and Kujasalo, 1999). There are indications that developing embryos are sensitive to predator chemical cues before hatching and could then later elicit responses at the larval stage, even when no predation threat was present. For example, exposure of developing palmate eggs to predator cues resulted in higher larval growth rates and reduced time to and size at metamorphosis (Orizaola and Braña, 2005). This may have implications for those eggs laid at peak predator abundances in Llysdinam Pond.

In some studies (Loman, 2002a), but not others (Durward, 2005), a negative correlation has been found between predator numbers in ponds and *R. temporaria* tadpole and metamorph survival. There were fewer predators now than in the 1980s but there were fewer prey species too since common toad tadpoles were a major prey resource for beetle larvae in the 1980s but in 2007 were comparatively fewer (Harrison, 1985). Buftons Pond, located approximately 0.5 km north, sampled to provide some predators for the predation study, contained up to five dragonfly larvae, four water boatmen and three large beetle larvae per net, possibly due to the high availability of *R. temporaria* tadpoles for prey.

The peak of predators in 2007 occurred after newt larvae had been detected and were at a mean abundance of just two larvae per netting site. Similarly, in 1984, predatory aquatic invertebrates peaked at Week 20 when newt larvae were first detected by Harrison (1985). This suggests the predatory invertebrates and newt breeding showed similar synchrony between 1984 and 2007 but this would need further investigation as data were limited to just two years. The peaks of predator abundance and newt eggs coincided, newt eggs peaked at 92 eggs per man hour in Week 11 in 2007 and remained high at over 40 eggs per an hour until Week 15. Predatory invertebrate peaks also coincided with the hatching of toad spawn in 2007. Toad spawn hatched in Weeks 15 and 16 and tadpoles were present in high numbers (up to 30 per net) in certain areas of the pond until Week 18. Tail damage to toad tadpoles was noticed, possible evidence of predation attempts by aquatic invertebrates. Frog tadpoles hatched in Week 12 in Site U but were unlikely to be a main source of prey since there was high mortality due to desiccation. Toad spawn also hatched on Week 16 in 1984 (and frog spawn on Week 13) so the predatory aquatic invertebrates did not appear to show synchrony with anuran spawning dates. Despite the apparent

synchronous advance in phenology between newts and predatory invertebrates, there could be differences in predator-prey interactions since anuran hatching dates were apparently unchanged between the two study years.

From the limited two years of data, the phenology of *D. marginalis* larvae was earlier in 2007 than 1984, probably due to a change in optimal foraging time due to milder spring temperatures and earlier prey phenology. Harrison (1983) stated that *Dytiscid* larvae rapidly increased in abundance in May due to water temperatures since *Dytiscid* are inactive below 10°C but grow rapidly between 11-15°C. Optimal temperature may be around 14°C since at warmer temperatures surfacing frequency increased which may impact on dive time and successful predation (Calosi et al., 2007).

Harrison (1985) did a small tank study on predation with two replicates of *N. glauca*, *Aeshna* dragonfly larvae and *Agabus* predatory beetles and 10 newt larvae per tank. Daily predation rates of larvae were 0.8 for *Agabus* beetles, 1.7 per day for *D. marginalis* adults and 3.3 for *N. glauca*. *N. glauca* was the most prevalent predator at Llysdinam Pond in the 1980s at time of newt larval hatch. In Predation Studies A and B with greater replicates, dragonfly larvae, large beetle larvae and water boatman were the most voracious predators and predated significantly more larvae, therefore the hypothesis that rates of larval predation between predatory invertebrate species would differ was accepted. Little difference between the number of predations of small and larvae newt larvae was found. Therefore the hypothesis that there would be significantly more predations of small than large larvae by predatory aquatic invertebrates was rejected. However, there was a slight size advantage in being larger, since predation of the largest larval size class was lower for small beetle larvae, damselflies and adult water beetles. Beetle larvae were the most prevalent predator in Llysdinam Pond in 2007 so a reduction in predation risk by the most abundant predator would increase survival. Larger larvae may have less predation risk from adult newts (not studied as a predator) and conspecific larvae. Large aquatic predatory invertebrates might have found larger larvae easier to detect and grasp by mandibles (dragonflies and beetle larvae) or legs (*N. glauca*), but that small larvae were slower and once grasped, escaped less readily. Small frog tadpoles showed the strongest hiding response (95% hiding) to *N. glauca* and large tadpoles the strongest response (95%-100% hiding) to dragonfly larvae (van Buskirk, 2001). Predation results from laboratory studies need to be interpreted with caution when implying equivalence in predation pressures between predators (Loman, 2002a).

Although there were only two years of data on aquatic predatory invertebrate, results indicate that predators, although lower in abundance in 2007 have responded synchronously to prey presence. Although an advance in phenology has been shown for dragonflies (Hassall et al., 2007), there has been mixed evidence the consequences of climate change for freshwater macro-invertebrate abundance and therefore predator and prey abundance. Studies in microcosms indicated that the abundance of macro-invertebrates remained high and unaffected by warming (McKee et al., 2002; 2003) and invertebrate size was not affected (McKee and Atkinson, 2000). Weak affects of temperature on freshwater macro-invertebrate composition have been reported (Burgmer et al., 2007), and in ponds, annual variation in species distributions responded to long term climate trends and factors such as hydroperiod (Jeffries, 2005). Analysis of long term data from temperate headwaters in mid-Wales showed significant responses to climate change in macro invertebrates in a circum-neutral steam. It has been suggested that future climatic projections could cause considerable change with declines in abundance of 21% and although core species could persist, scarce taxa would risk extinction (Durance and Ormerod, 2007).

As found by Loman (2002b) for common frogs, newt metamorphosis was later in colder summers, although Loman could not establish a causal relationship. Median emergence dates varied between years and despite earlier egg-laying in 2007, median emergence dates of metamorphs were later in 2007. In July-August 2007 newt metamorph emergence from Llysdinam Pond was more dispersed than in 2006 due to the drier summer of 2006. Metamorphs often emerged on nights with rainfall which minimised desiccation and enabled higher activity levels (Rohr and Madison, 2003). Caution must be taken in comparisons with 1983-1984 metamorph emergence data since different methodologies were used to capture the metamorphs.

Climate change may alter pond hydroperiod and this may have a greater impact on amphibian breeding success than direct temperature effects, especially further south in Europe or in temporary ponds (Jakob et al., 2003). Although climate change has led to higher winter and early spring temperatures, it has not been confirmed whether summers will be significantly drier or wetter. In the summers of 2007 and 2008 the UK experienced higher than average rainfall and ephemeral ponds in mid-Wales did not dry up (F..M. Slater, pers. comm.) leading to extended hydroperiod for amphibians and possibly later

metamorphosis and increased size (Laurila and Kujasalo, 1999; Loman, 2002a; Semlitsch and Gibbons, 1985; Werner, 1986).

The newt metamorphs that emerged earlier were larger, as found for metamorphs of other amphibian species including *Ambystoma talpoideum* (Semlitsch et al., 1988). Durward (2005), however, found no differences in *R. temporaria* metamorph size between emergence time. Metamorphs with a greater mass to length ratio may have a slight advantage over metamorphs with a low weight to length ratio due to low desiccation and predation risk. Metamorphs were 3-5 mm larger in 2007, in comparison with 1983-1984 possibly due to earlier breeding or the cool summer increasing larval period. Therefore the hypothesis that newt metamorphs were larger on departure from Llysdinam Pond in 2007 than in the 1980s was accepted. Since *Lissotriton* metamorphs were not separated by species in 1983-1984, some of the variation could be due to a higher proportion of larger smooth newts in 2007 than the 1980s or possibly due to diet and predator abundance. Higher temperatures resulting from global warming have led to an increase in body size for common lizards over 19 years (Chamaille-Jammes et al., 2006). Body size has important effects for future life history. In common frogs, larger size at metamorphosis decreased predation risk (Werner, 1986), led to earlier maturation, larger adult size (Berven, 1990; Smith, 1987) and higher fecundity (Reading and Clarke, 1995).

Although there was no difference in hatching success and hatchling size over the season, longer hatching time and slower larval growth earlier in the season could increase mortality in the wild from predation. The limited data from 1983, 1984 and 2007 showed there were large inter-annual differences in breeding phenology of newts. There is probably a trend towards earlier larval hatching in years with mild early spring temperatures, which are predicted to occur more frequently in the future due to climate change. There were indications that aquatic predatory invertebrates have also synchronised their activity to peak earlier in the year. There was little selectivity in prey size by predators although larger larvae were released from predation pressure by the smaller predatory invertebrates. Median dates of metamorph emergence were highly variable but metamorphs may be emerging at a larger size now than in the 1980s. Although comparative data were limited, this research warrants further study of phenological change in larval amphibians and interactions with predatory invertebrates, particularly since the larval stage is of great importance to amphibian population regulation (Stebbins and Cohen, 1995).

Chapter 7: Discussion and Conclusions

Previous studies have taken place in Britain on anuran spawning phenology (Beebee, 1995; Reading and Clarke, 1999; Scott et al., 2008), but this was the first in depth study on newt breeding phenology in the UK. Previous research on *Lissotriton* newts had found an advancement in the phenology of newt arrival to breeding ponds (Beebee, 1995; Chadwick et al., 2006). There has been little research on the breeding phenology of European newts due to the cryptic nature of their courtship and egg-laying, which is more difficult to monitor than breeding in common toads and common frogs. The main aims of this site specific study were to investigate the differences in *Lissotriton* morphology between arrival times, determine whether early migration phenology leads to early breeding phenology, the consequences of arrival time for breeding success, and with the limited data available, determine whether *Lissotriton* larval and metamorph phenology had altered between the 1980s and present.

This study showed that newt breeding can not be assumed to be synchronous with adult arrival since there was a varying delay between years. The visual encounter survey (VES) for newt eggs and particularly the outdoor breeding tank study, demonstrated the variability in delay from arrival to egg-laying between years. *Lissotriton* arrival numbers were similar in the October-December period preceding the 2006 and 2007 breeding season, yet egg-laying commenced much earlier in 2007 than in 2006 (a 10 week advance in the pond and six weeks in the tanks). The advancement of breeding phenology in the pond in 2007 was also found in the outdoor breeding tanks, so it could not be due to higher egg detectability or vegetation abundance in Llysdinam Pond in 2007. Although egg-laying was earlier in both Llysdinam Pond and the outdoor breeding tanks in 2007, the time of the commencement of egg-laying differed between the two research methods. Eggs were detected three weeks later in the tanks than the pond in 2007 and five weeks earlier in 2006. If it had been possible to set up the outdoor tank breeding study in December before the 2007 breeding season, egg-laying may have been detected at a similar time within Llysdinam Pond.

Within years in the outdoor breeding tanks, smooth eggs were detected six weeks later than palmate eggs in 2006, while in 2007 egg-laying by both *Lissotriton* species commenced at the same time. Although both *Lissotriton* species began egg-laying at the same time in 2007, four weeks after egg-laying commenced, egg-laying rates were higher for palmate

than smooth newts. The greater delay in oviposition behaviour by smooth newts in 2006 was likely to be due to the cold early spring temperatures in 2006 compared to 2007. The outdoor breeding tank study demonstrated that for the earliest newt arrivals, air temperatures needed to reach weekly means of over 2°C. Further years of study would more clearly determine the effect of temperature on the commencement of egg-laying. There was a shorter delay between newt arrival and egg-laying for great crested newts and previous work has found that great crested newts have higher temperature thresholds to remain active (Arntzen and Hedlund, 1990). Therefore, in both years great crested newts possibly maximised breeding opportunities by arriving later than *Lissotriton* newts, although in 2007 eggs were detected four weeks earlier than in 2006.

Earlier arrival phenology could have direct affects on mortality due to temporal differences in competition, predation and temperature for the adults, eggs and larvae. The netting survey for aquatic predatory invertebrates showed predator abundance and species altered through the season as expected, and this corroborates the findings of Harrison (1985). Differences in breeding phenology exposed eggs and larvae to differing predation levels depending on the time of oviposition and hatching. There are different predation risks between the aquatic and terrestrial environment which could have impacts on adult newts, since they have a longer aquatic period now than previously (Chadwick et al., 2006). Additionally there are differing prey abundances and types between the aquatic and terrestrial environment, although only one study has looked at differences in foraging by newts between the two environments (Denoel, 2004).

There was higher than average seasonal mortality for the first laid eggs of both palmate and smooth newts in the outdoor tank study, before a short cold period in early February 2007. Reading and Clarke (1995) demonstrated tadpole mortality for common toads was higher when they were exposed to more cold days of under 1.5°C. If there had been a greater number or length of cold spells during 2007 a greater mortality of eggs may have occurred and the risks of breeding early would then have been apparent. Since average monthly temperatures vary greatly, even in consecutive years, further investigation would be needed to look into the consequences of early oviposition phenology between years. Although the hatching rate of eggs was consistently high in the developmental tanks, greater differences may occur over the season in the wild where eggs are exposed to predators. In 2007 the more gradual progression in egg-laying rates in the pond could have led to a higher proportion of egg predation due to lack of predator satiation (Ims, 1990).

Also the earlier extension of the breeding season may decrease the saturation level of eggs and larvae at peak egg-laying time which could again lead to increased predation of eggs and larvae. This could be further investigated in laboratory or in field studies.

Smooth newts generally begin breeding later in the season than palmate newts as found in outdoor breeding tanks in 2006 and by molecular analysis of eggs found during VES in 2007. There seems to be little difference between the British *Lissotriton* species in hatching success or hatchling size to indicate why smooth newts lay their eggs later. Although smooth eggs hatched at an average of 1 mm smaller in length than palmate eggs, this would be unlikely to affect predation rates between the species. In the absence of predation in the developmental tanks there was high hatching success of over 90% throughout the season for both species. There was a mild spring in 2007, but when prolonged cold periods in early spring occur as found in 2006, there may be greater mortality of smooth eggs or larvae which would be worth investigating further.

Lower larval growth rates in early hatchlings could be due to food availability as found by Heckel (1984) or a combination of factors including temperature and food which could be investigated experimentally. Higher larval growth rates lead to larvae spending less time at risk of predation by gape-limited predators such as conspecific larvae and adult newts. Conspecific larvae were found to predate recently hatched larvae in Predation Study A. Despite previous research finding that predation of anuran tadpoles decreased with increasing larval size (Travis et al., 1985), little difference in predation threat from dragonfly and beetle larvae between large and small larval sizes was found. There were only size advantages in that larger larvae were not predated by the smallest predatory invertebrates including damselflies. In the pond there may be greater differences in predation levels between larval sizes that were not found in the laboratory study. For example, in the tanks, larvae were highly visible, yet in the pond they may seek refuge in vegetation and remain inconspicuous. Future work could look at predation rates on larvae with the provision of refugia such as the leaf litter used by Van Buskirk (2001). There may be a differential hiding responses between larval sizes as found by Van Buskirk (2001) which may reduce predation levels. Also additional prey species could be provided for the predator in addition to larval newts to look at predator choice for species rather than just the affect of larval size on predation rates.

Netting detected much higher predator numbers in 1984 than 2007, but the proportion of metamorphs emerging taking into account the number of larvae captured by netting was similar between years. More eggs were detected in Llysdinam Pond in 2007 than 2006 despite the lower number of migrating adults captured at the pond drift fence, but fewer metamorphs emerged. There could be a number of reasons for the lack of association between the number of adult newts, eggs, metamorphs and predatory aquatic invertebrates. There may have been higher larval abundance in 2007 than 2006 resulting in greater competition and higher mortality and therefore fewer metamorphs. There may have been a greater number of predators in 2007 than 2006 resulting in higher egg and larval predation levels. There have been differing findings on whether predator abundance and anuran tadpole and metamorph survival are associated (Durward, 2005; Loman, 2002a). Conclusions about metamorph emergence in 1984 compared to 2007 must be made with caution since different methodologies were used. The limited data from 1984 and 2007 indicates that the phenology of predatory aquatic invertebrates was earlier in the 2000s probably due to temperature and prey availability (Formanowicz, 1982; Harrison, 1985). The jelly like substances found on aquatic vegetation in Llysdinam Pond while conducting egg surveys could be the remains of predated eggs as found by Miaud (1993), and is worth investigating.

Although the data on larval phenology were limited, larvae were detected significantly earlier in 2007 than in 1983-1984. Using temperature, egg-laying and larval data from 1983, 1984 and 2007 to predict when in 2006 newt larvae would have been at detectable levels, suggests that phenology in 2006 season may have been similar to 1983-1984. Reading (1998) found the toad breeding season did not show a significantly advancement in phenology but with warmer winter and late spring temperatures, more years with early egg-laying and hatching were likely to have occurred in the 2000s than the 1980s. For toad breeding he found that 44% of early years occurred in 1990s compared to just 10% in the 1980s. There have been concerns about a lack of synchrony between species within a community leading to changes in competition and predation between species (Visser and Holleman, 2001). The comparative data between 1984 and 2007 suggest that predatory invertebrate phenology has shifted to the same degree as newt egg-laying. In contrast, toad hatching occurred at the same time in 2007 as 1984 so there could be potential differences in predator-prey interactions. Although, using predictive work, Visser et al. (2006) showed that after an initial discrepancy between predator and prey, synchrony may later be restored. If synchrony is maintained between species in the freshwater environment, it

could be that other wildlife responses associated with climate change such as range shifts (Araújo et al., 2006), an increase in susceptibility to infectious diseases (Daszak et al., 1999; 2005a; 2003) and warmer weather leading to a shorter hydroperiod (Jakob et al., 2003; Laurila and Kujasalo, 1999; Loman, 2002a; Semlitsch and Gibbons, 1985; Werner, 1986) would have greater implications for conservation than changes in amphibian phenology.

Palmate newts seem to be adapted to breed at lower temperatures than smooth newts since they began egg-laying earlier in 2006, and did not reduce egg-laying rates unlike smooth newts during a cold spell in early 2007. Since palmate newts are typically thought to be an upland species (Beebee and Griffiths, 2000) they may have adapted to cooler conditions and opportunistically advanced breeding when early spring temperatures were mild. Fluctuations in adult population sizes may be a reflection of recruitment success due to levels of hatching, larval and metamorph survival (Griffiths, 1995). If the higher egg-laying frequency found in the outdoors breeding study was consistent with egg-laying in Llysdinam Pond, this may help maintain the higher palmate numbers returning to breed annually. Since *Lissotriton* eggs are visually indistinguishable there was no reason to believe they suffer different predation rates (Arntzen and Hedlund, 1990), although there are interspecific differences in wrapping behaviour (Miaud, 1994). The lower egg-laying rates by smooth newts may be because they were at the edge of their range in mid-Wales and phenological studies in other areas could reveal different findings. It is likely that egg-laying peaks vary between areas of the UK, but at Llysdinam Pond, in contrast to Beebee and Griffiths (2000) it was found that palmate showed a continuous egg-laying season between January-June in 2007 while smooth newt eggs peaked in numbers in April.

The study of *Lissotriton* courtship phenology was not successful, and observations of courtship behaviour in outdoor tanks did not show any consistent trends between batches unlike the research on oviposition behaviour. Anuran courtship is easier to study in the wild, and has been shown to have advanced with climate change (Gibbs and Breisch, 2001), while there has been less research on the association between newt courtship and temperature (Denoël et al., 2005b). It is possible that visible implant elastomers (VIE) could be used to investigate the effect of arrival time on courtship phenology, although such invasive marking practices can cause unknown stress to amphibians (Bradfield, 2004). Different coloured polymers could be inserted into the dorsal surface or tails of newts arriving at different periods through the breeding season. This could enable

recording of courtship behavior by torchlight surveys in relation to newt arrival time. Such a technique has been used by Woolley (1973) and marks were visible from 3-5 m. The same methodology could be used to monitor repeatability of arrival in both sexes of palmate and female smooth newts which do not have individual natural markings. VIEs can be visible for up to a year (Woolley, 1973), although such artificial markings may increase visibility to predators.

A higher proportion of the *Lissotriton* newts that arrived early were larger in mass and length than late arriving newts. This was particularly evident for palmate than for smooth newts. This contrasts with previous work at Llysdinam (Griffiths et al., 1986; Griffiths and Mylotte, 1988) which found no differences in size between arrival times. More data would need to be collected to find out whether temporal separation in arrival size was a new phenomenon associated with climate change. The milder winter conditions that have been more frequent in recent years may mean that large individuals migrate earlier because if they delay, the wait could mean a loss in reproductive time and a decline in body condition. Larger female palmate newts may have arrived earlier because they were in breeding condition earlier. Although male smooth newt body size has been associated with testis size (Verrell and Francillon, 1986) there has been less certainty over the effect of size on male fitness. The majority of studies have found a positive correlation between female body size and number of eggs (Bell, 1977; Verrell, 1986; Verrell and Francillon, 1986), although in outdoor breeding tanks there was no significant difference in egg-laying between small and large female newts. Few smooth newts arrived during the autumn which may explain the fewer significant differences in female smooth newt size between arrival times. In outdoor tanks in 2006, egg-laying by smooth newts was later than for palmate newts so it may not have been beneficial for female smooth newts to arrive early. Additionally, molecular analysis of eggs in 2007 demonstrated that smooth newt egg-laying did not peak until April in 2007.

There was no evidence of a sub-migration towards the pond as found by Sinsch (1988) for common toads. Although a much smaller number of newt captures were made at the woodland fences than at Llysdinam Pond drift fence, there were indications that a greater proportion of palmate than smooth newts were using the woodland. This could be because palmate newts having a greater tolerance for acidic soil created by fallen coniferous needles. The pond drift fence at Llysdinam may detect the earliest migration of newts but evidence of newt movements in other research suggests that alternative locations for the

woodland fences may have detected a greater amount of movement (Jehle and Arntzen, 2000). Photographic identification of belly markings indicated that great crested females may use similar migratory routes as found by (Jehle, 2000) and may show individual repeatability in arrival dates between years (Semlitsch et al., 1993).

The measurements of *Lissotriton* metamorphs from 2007 indicate that on average they were significantly larger than in 1983-1984 (Harrison, 1985). The earlier breeding in 2007 could have provided more time for feeding and growth before metamorphosis. The cool summer of 2007 may have increased time to metamorphosis and therefore metamorph size (Loman, 2002b). Lower temperatures have been shown to increase time to metamorphosis in other species (Alvarez and Nicieza, 2002). Anuran larvae reared at low temperatures (12°C) have been shown to accumulate higher lipid reserves than those raised at 17°C or 22°C, although there was no effect of this on body condition (Alvarez and Nicieza, 2002). Although findings in general for amphibians have disagreed over whether size at metamorphosis affects survival (Smith, 1987; Werner, 1986), larger body size at metamorphosis was often maintained at maturity (Berven, 1990; Smith, 1987) and larger body size in female newts was reflected in higher fecundity (Bell, 1977; Verrell, 1986; Verrell and Francillon, 1986).

This study showed that newt breeding phenology was earlier in years with high early spring temperatures and after mild winters. Newts have a protracted breeding season so an individual female may lay eggs over several weeks or months, which exposes each egg to differing levels of threat from abiotic and biotic factors. Predation risks, food abundance and competition between conspecific and other amphibian larvae therefore vary depending on laying date. In contrast, the explosive breeding behaviour of common frogs and common toads exposes the anuran larval population within a year to the similar risks.

There are limited published data on differences in egg-laying phenology but a large potential but non-scientific resource for such information are ecological consultancies since there has been an exponential increase in mitigation work on great crested newts in the UK (Edgar et al., 2005), and surveys to detect their presence will involve encounters of both *Lissotriton* and great crested eggs. Additionally, consultancies often survey for newt courtship behaviour providing another potential resource for phenological data (English Nature, 2001).

In comparison with other taxa, this study lends support to the growing amount of research showing that breeding, as well as migration has advanced with climate change. For newts, it appears that breeding only occurred earlier in the 2000s when winter and spring temperatures were high. Most breeding phenology research has taken place on birds, and advances in breeding dates of birds have often been found (Crick et al., 1997; Crick and Sparks, 1999; Møller et al., 2006). For plants advances in the pollen season have been reported (van Vliet et al., 2002), while phenological records for insects mainly still consists of sightings and flight dates (Hassall et al., 2007; Roy and Asher, 2000; Stefanescu et al., 2003) rather than egg-laying dates.

The limited data on predatory aquatic invertebrates and newt larvae indicated that both groups had advanced their phenology by a similar number of days in 2007 compared to 1984. Hassall et al. (2007) also documented an advance in the phenology of adult dragonflies. McKee and Atkinson (2000) found that although mayfly abundance and size was not significantly affected by an increase in temperature in microcosms, adults emerged up to two weeks earlier which could have implications for predator-prey interactions in the freshwater habitat. The spawning of anurans at Llysdinam Pond occurred at a similar time in 2007 as in 1984 so synchrony with predatory aquatic invertebrates was not maintained. Ecologists are concerned that changes in phenology may lead to an increasing lack of synchrony between species, and therefore alterations in interactions between predators and prey (Visser and Holleman, 2001). Species may start breeding in response to one or more of a range of cues such as photoperiod, ambient temperature, bud burst and abundance of food (Nilsson and Kallander, 2006). If plants, invertebrates and vertebrates differ in the cues they use then there is more likelihood of a mismatch in phenology. Warmer spring weather in Europe has disrupted the synchrony between winter moth hatching and oak bud burst, leading to a mismatch between the peak in caterpillar abundance and the peak food demands of great tit nestlings (Buse and Good, 1996; Visser and Holleman, 2001; Visser et al., 2006). Warmer springs are predicted to cause a mismatch between first-laying dates of golden plovers *Pluvialis apricaria* and Tipulidae prey (Pearce-Higgins and Yalden, 2005). It is hopeful that synchrony may be restored after an initial mismatch between the phenology of predator and prey (Visser et al., 2006). It is also possible that changes in phenology may lead to a greater synchrony between predators and prey, or larval emergence and the bud burst of host plants (Harrington et al., 1999).

Like newts, larger individuals of poikilotherms species, such as salmon have been found to arrive earlier than small individuals (Dickerson et al., 2002), and it has been suggested that larger males better able to overcome the costs of early arrival and reap the benefits (Verhulst et al., 1995). A trend in migrants being larger early in the breeding season has also been found in endothermic species such as redstarts (Lozano et al., 1996) nightingales (Kipper et al., 2006) and short tail barn swallows (Møller, 1994). Repeatability in arrival times between years has been studied more successfully in birds, with repeatability within individuals found to be typical for their reproductive events (Findlay and Cooke, 1982; van Noordwijk et al., 1981), although no repeatability in arrival times was found between pied flycatcher *Ficedula hypoleuca* individuals or between relatives (Potti, 1998),

In conclusion, although based at a single breeding pond this study indicated that despite the consistent earlier autumn arrival of adult *Lissotriton* newts to Llysdinam Pond, earlier breeding only followed if there had been high winter and early spring temperatures. There was less delay between great crested newt arrival and egg-laying within years although eggs were detected earlier in 2007 with higher spring temperatures. Due to human induced climate change, there were more years with high winter and early spring temperatures in the 2000s than in the 1980s, which may have led to a higher incidence of earlier breeding in newts. There were few differences in hatching success between early and late arrivals when the threat of predation was removed. However, larval growth was more rapid at the higher temperatures found later in the season which was advantageous by releasing larvae sooner from predation pressure by small predatory invertebrates and possibly gape-limited predators. Although predatory aquatic invertebrates and newts showed a similar advance in phenology, there could be difference in predator-prey interactions due to anuran spawning dates having remained unchanged in the 1980s and 2007.

Although newts were clearly sensitive to meteorological variables, further work to acquire long term data sets would assist with the study of newt breeding behaviour in relation to global warming. Further studies investigating egg-laying phenology, larval growth and synchrony with other amphibians, predatory invertebrates and invertebrate prey would be useful in understanding the implications of climate change and interactions between species.

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APPENDICES

Appendix I: Great crested newt photographs

Example photographs of the distinct belly patterns of great crested newts recaptured at Llysdinam Pond.

Male great crested newts

MALE D

MALE E

MALE F

Female great crested newts

FEMALE C

FEMALE D

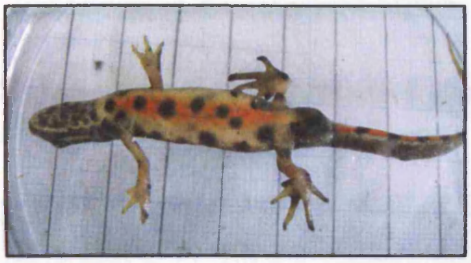
FEMALE E

Appendix III: Male smooth newt photograph matches

Photographs of the distinct belly patterns of the eight male smooth newts (Male Smooth A-H) recaptured at Llysdinam Pond. Captures dates and direction of movement are indicated. The 690 photographs of male smooth newts taken were categorised depending on the spot markings. The category each newt was put into is shown in brackets.

Male Smooth A (Line of spots):

17.2.06 in **25.2.06 out** **26.2.06 out**



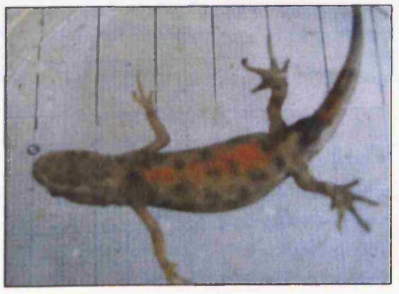
Male Smooth B (Small sized spots, few):

13.2.06 in **13.6.06 out**


MALE C (Line of spots): 17.2.06 in **14.6.06 out**


Male Smooth D (Medium sized spots few): Note how markings have faded during the aquatic period

8.3.06 in **14.5.06 out**

Male Smooth E (Line of spots):

13.2.06 in

13.1.07 in

Male Smooth F (Medium sized spots, few): Note how one spot has enlarged in second photograph

18.2.06 in

20.1.07 in

Male Smooth G (Small sized spots, few):

19.6.06 in

17.2.07 in

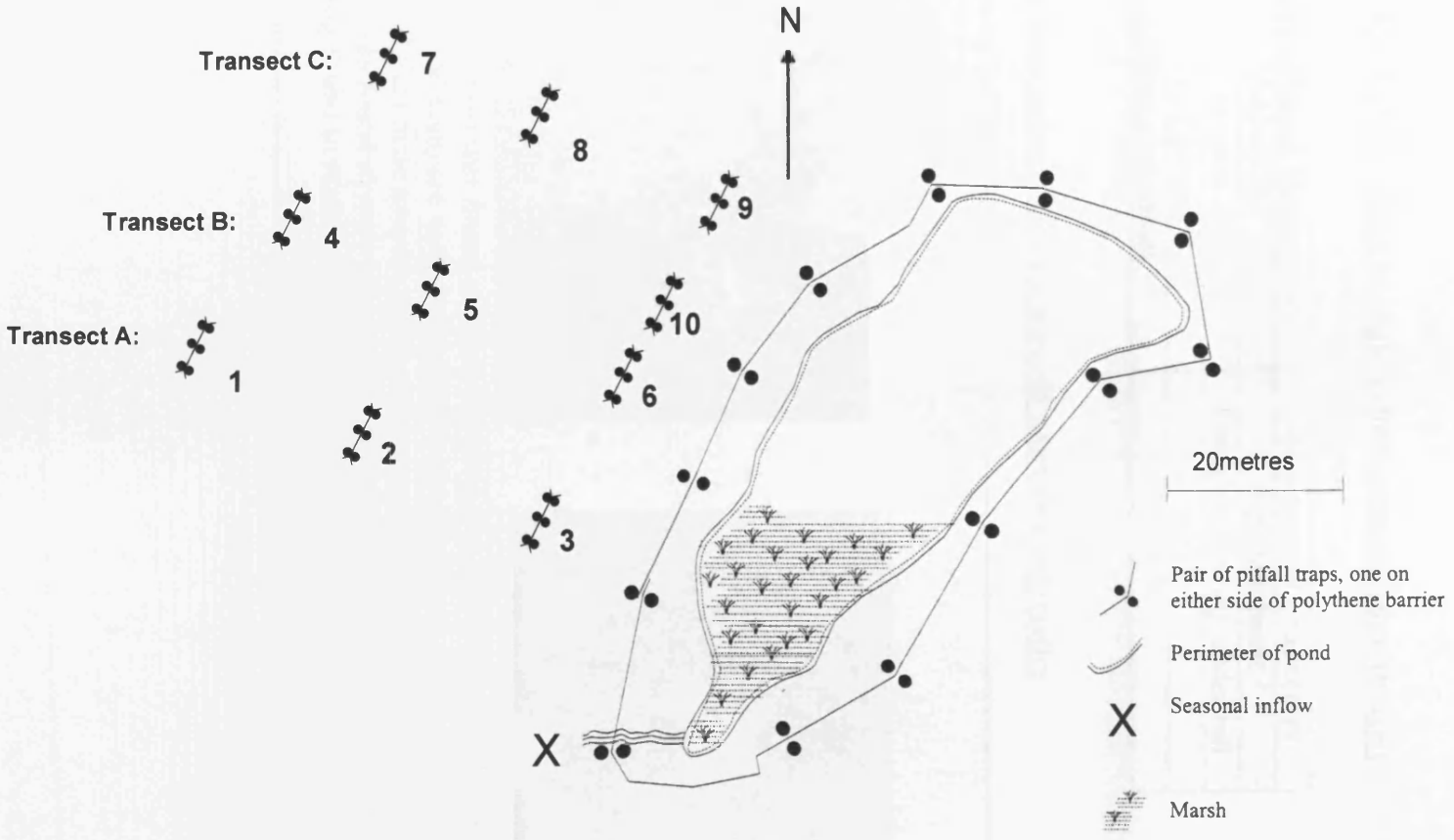
Male Smooth (Distinct markings):

17.8.07 out

17.9.07 in

Appendix III: Map of woodland fences and pitfall trap locations

10 drift fences of 5 m length each were constructed in the woodland to the north-west of Llysdinam Pond to enable patterns of newt movement to be monitored. Fences were numbered 1-10. There were three transects: Transect A = Fences 1-3, Transect B = Fences 4-6, Transect C = Fences 7-9. Fence 10 was an additional fence constructed within a boggy area of the woodland. Each fence had three pitfall traps (flower pots) on either side to capture amphibians.



Appendix IV: Male palmate-smooth hybrid newt

Characteristics and photographs of the male palmate-smooth hybrid captured at Llysdinam Pond on 19/3/2005 are shown.

Table of characteristics possessed by male hybrid palmate-smooth newt

Male palmate characters	Male smooth characters
Webbed feet	Spotted throat
Two lines of spots along tail	Orange colouring under tail
Tail filament	
Slightly pink throat	

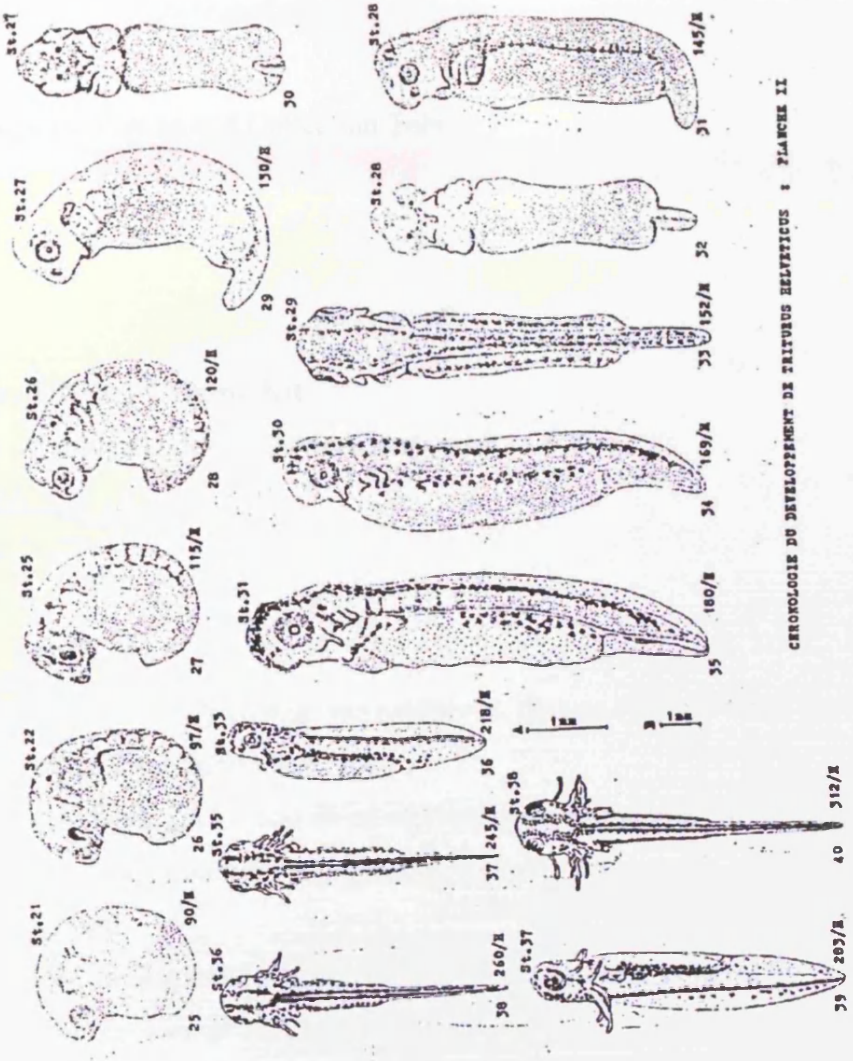
Photographs of male hybrid palmate-smooth newt characteristics

A male palmate- smooth hybrid was found entering the pond in a pitfall trap on 19/2/2005. Note characters listed in table and compare with males of both species below. Belly spots are less pronounced in the hybrid than male smooth newts. After several weeks in water the hybrid developed a slight crest (as found in male smooth newts) and very large webbed feet (from a combination of webbing found in male palmate newts and toe spurs found in male smooth newts).

Appendix V: Development stages of newt embryos

Developmental stages of smooth newts as defined by Gallien and Bidaud (1959).

Development stages of newt embryos (Gallien and Bidaud 1959).



Appendix VI: Method from the DNeasy® Tissue Handbook

DNA Extraction materials and method taken from the DNeasy® Tissue Handbook (QIAGEN®, 2004)

Materials

Provided by DNeasy® Tissue Kit

Buffer ATL

Proteinase K

Buffer AL

DNeasy® Mini Spin Columns and Collection Tubes

Buffer AW1

Buffer AW2

Buffer AE

Not Provided by DNeasy® Tissue Kit

Tissue Samples (in this case de-jellied newt embryos)

96-100% ethanol

Eppendorf tubes

Method

1. Blot away ethanol in which egg was preserved. Cut up egg into small pieces and place into a 2 ml Eppendorf tube.
2. Add 180 µl of Buffer ATL and 20 µl of Proteinase K and mix by vortexing.
3. Incubate at 55°C in a rocking incubator, vortexing occasionally, until tissue is completely lysed.
4. Vortex before adding 200 µl Buffer AL, immediately mix samples by vortexing and incubate at 70°C for 10 minutes
5. Add 200 µl ethanol (96-100%) and mix by vortexing.
6. Pipette mixture from step 5 into a Mini Spin column placed in a 2 ml collection tube.
7. Centrifuge at 8000rpm for 1 minute.
8. Discard flow through and collection tube. Place the Mini Spin column into a new 2 ml collection tube and add 500 µl Buffer AW1.
9. Centrifuge at 8000rpm for 1 minute.

10. Discard flow through and collection tube. Place the Mini Spin column into a new 2 ml collection tube and add 500 µl Buffer AW2.
11. Centrifuge at 14, 000rpm for 3 minutes.
12. Discard flow through and collection tube. Place the Mini Spin column into a clean 2 ml Eppendorf tube and add 200 µl Buffer AE.
13. Incubate at room temperature for 1 minute. Centrifuge at 8000rpm for 1 minute.
14. Store resulting 200 µl in fridge (c. 4°C).
15. Place the Mini Spin column into a clean 2 ml Eppendorf tube and add 100 µl Buffer AE.
16. Incubate at room temperature for 1 minute. Centrifuge at 8000rpm for 1 minute.
17. Discard Mini Spin column and store resulting 100 µl in freezer (c. -20°C).

Appendix VII: Trials of molecular analysis of eggs

RAPD Analysis

Preliminary trials used RAPD (random amplified polymorphic DNA) in an attempt to distinguish between palmate and smooth newt eggs. A protocol taken from Beebee et al. (1999) was followed in which they had successfully distinguished between palmate and smooth newt adults using nuclear primers to amplify a distinctive DNA fragment. The PCR stock concentrations, reaction concentrations and reaction volumes of the reagents used are listed the table below, giving a total reaction volume of 20 µl.

Table of the stock concentrations, reaction concentrations and reaction volumes

The reagents used for the PCR during RAPD analysis of the eggs, giving a total reaction volume of 20 µl.

Materials	Stock Concentration	Reaction Concentration	Reaction Volume
PCR buffer (20 mM Tris-HCL pH 8.3, 50 mM KCL)	10 x	1 x	2.0 µl
$MgCl_2$	50 mM	1 mM	0.4 µl
Taq DNA polymerase	5 units/µl	0.05 units	0.2 µl
dNTPs	2 mM	0.1 mM	1.0 µl
Nuclear RAPD primer 5′-CGGCCCCTGT-3′	10 mM	0.2 mM	0.4 µl
dH_2O	-----	-----	14.0 µl
DNA extract	------	-----	2.0 µl

The PCR cycling scheme was taken from Beebee et al. (1999) was: Initial denaturation of 4 minutes at 94°C was carried out before the PCR of 35 annealing cycles, each with 1 minute at 94°C, 1 minute at 40°C, and 2 minutes at 72°C, followed by a final extension cycle at 72°C for 5 minutes.

10 µl of the PCR product was mixed with 3 µl loading buffer. Electrophoresis was carried out through a 1.5% agarose gel containing 0.7 µl ethidium bromide. The results of the gel electrophoresis are shown in the figure below.

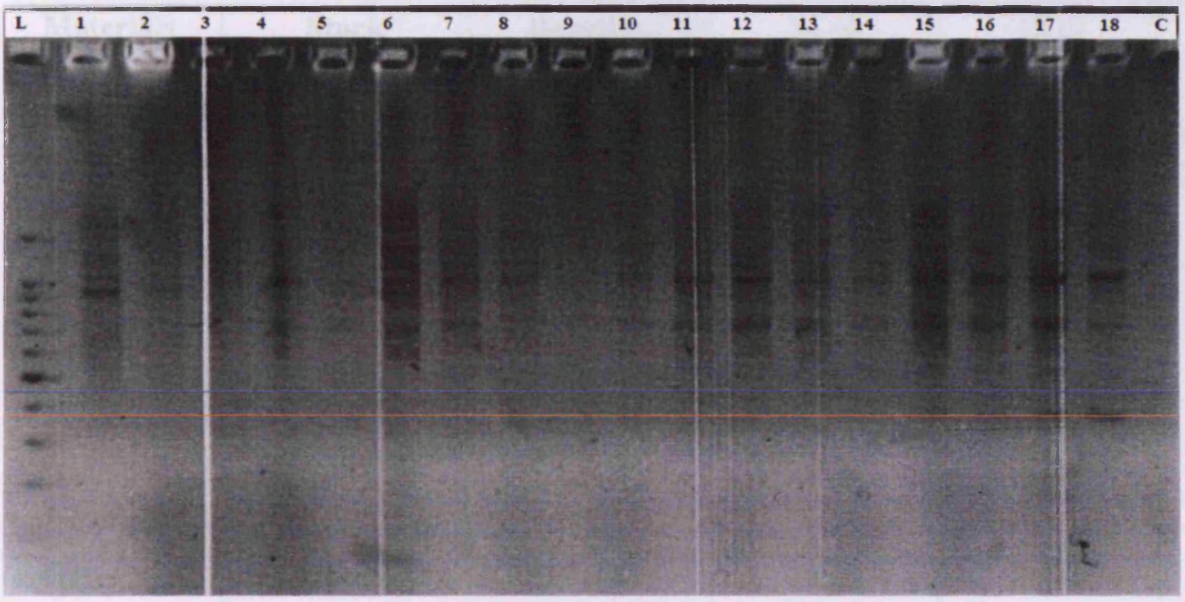
Gel electrophoresis photograph

Nuclear RAPD primer amplified using the protocol from Beebee et al. (1999).

L = 100bp ladder

C = Control

1-18 = Analysed eggs



The top line indicates the distance at which the distinctive smooth newt fragment band (>400-bp) should amplify and the bottom line indicates the distance at which the distinctive palmate newt fragment band (>300-bp) should amplify. The RAPD proved unsuccessful. Although DNA amplification proved successful, amplification of the distinctive bands required to distinguish between the species did not occur in any eggs except for a very faint band visible at >300-bp in eggs 15, 16, 17 and 18 which were shown to be palmate individuals. These four eggs had been completely de-jellied and allowed to develop to a slightly larger size whereas eggs 1-15 had been immediately preserved and their jelly had not been removed. It was likely that most eggs did not amplify due to DNA being present at only very low levels in the eggs or due to the presence of jelly affecting the success of DNA extraction or amplification.

Amplification of mitochondrial DNA sequences

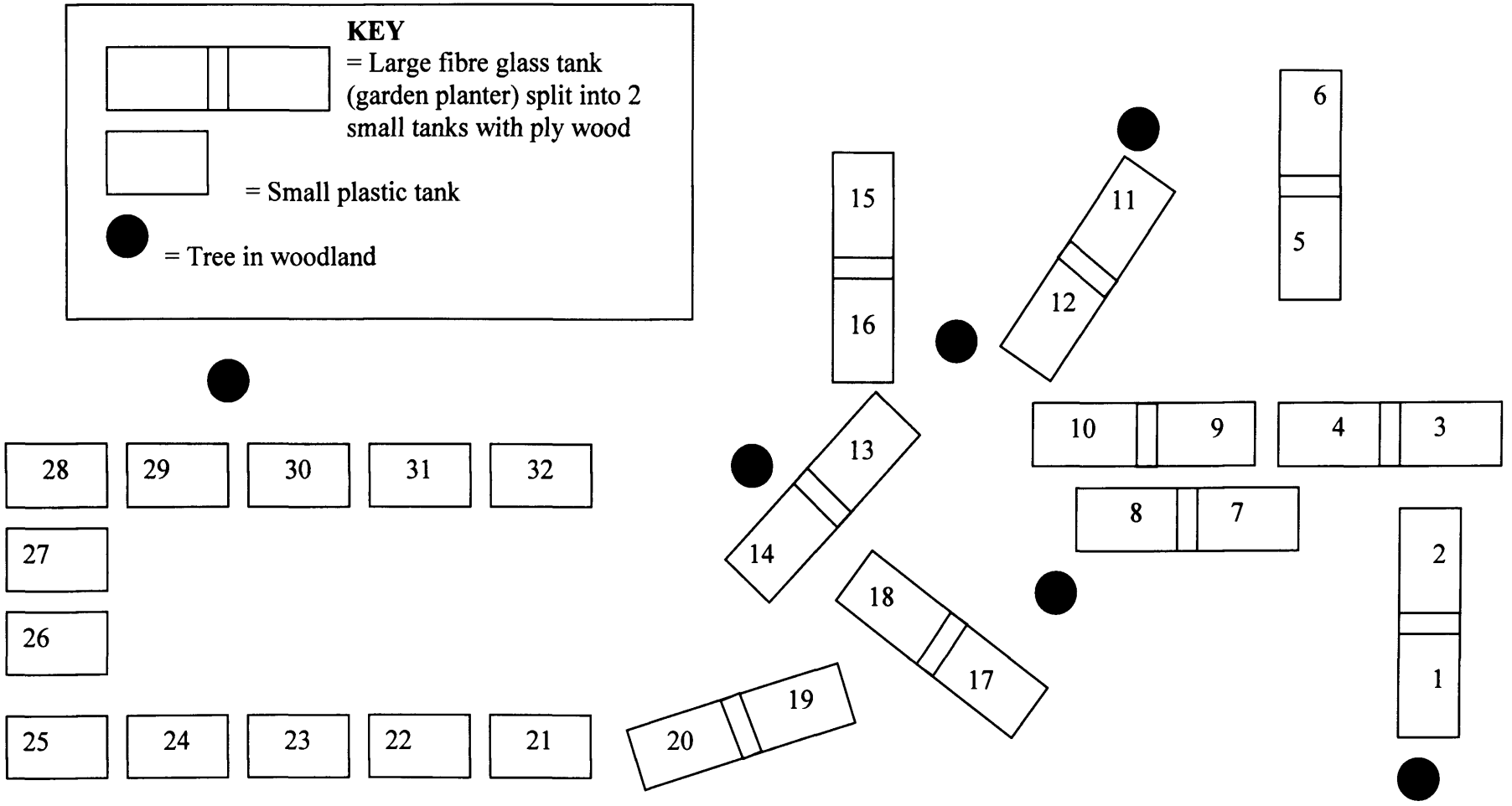
Babik et al. (2005) amplified a c. 1016-bp fragment of the mitochondrial ND2 gene (which shall be referred to at 'ND2') in both palmate and smooth newts using the L3870 and H5018 primers. 10 µl and 30 µl reaction volumes were trialed using DNA extracted from know palmate and smooth newt adult individuals. The concentrations and volumes of reagents used in this trial are listed below.

Table of the stock concentrations, reaction concentrations and reaction volumes

The reagents used for the PCR to amplify the 'ND2' fragment using the primers L3870 and H5018, giving a total reaction volume of 10 µl and 30 µl.

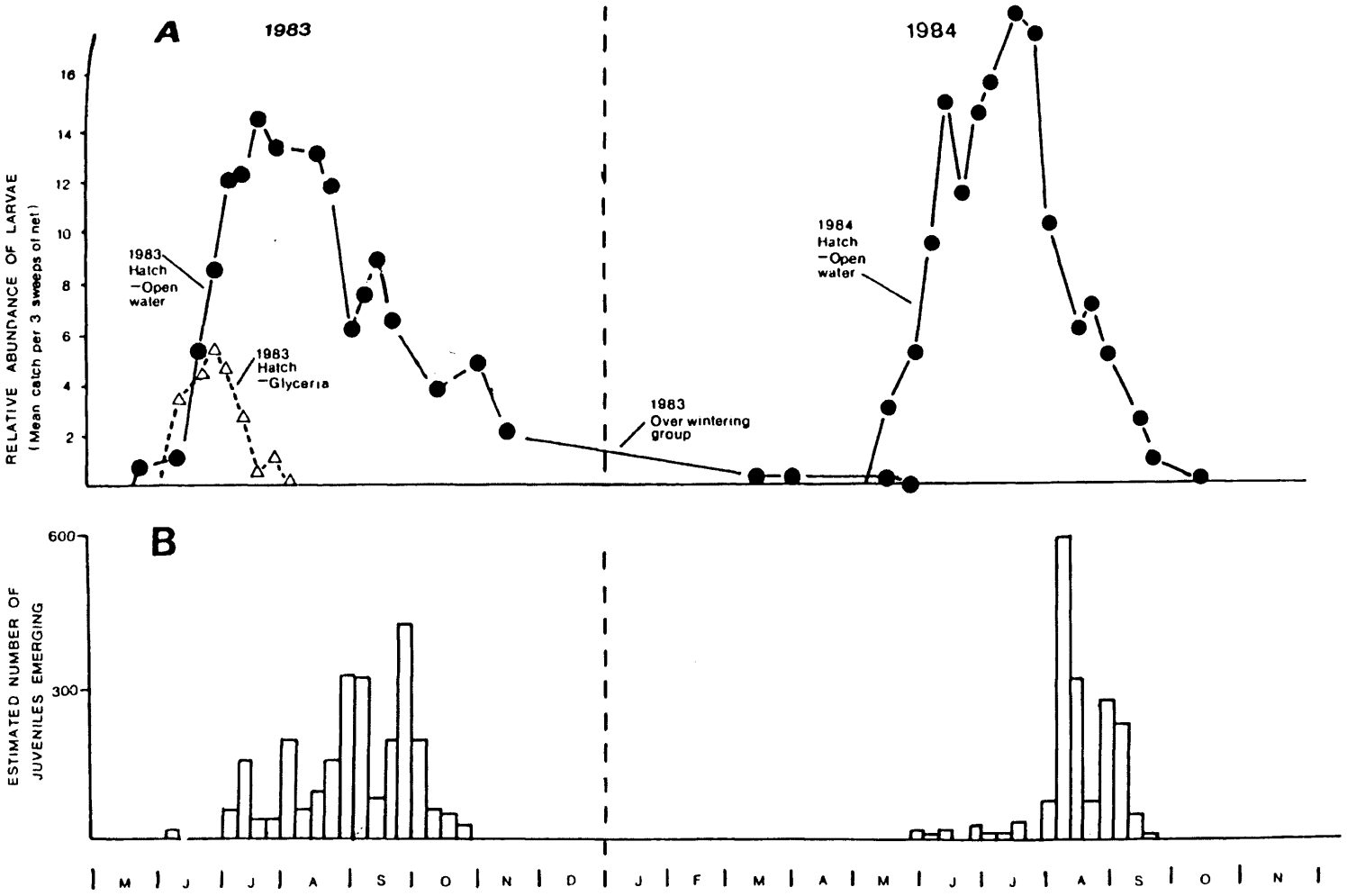
Materials	**Stock Concentration**	**Reaction Concentration**	**30 µl Reaction Volume**	**10 µl Reaction Volume**
PCR buffer (20 mM Tris-HCL pH 8.3, 50 mM KCL)	10 x	1x	3.0 µl	1.0 µl
$MgCl_2$	50 mM	2.5 mM	1.5 µl	0.5 µl
Taq DNA polymerase	5 units/µl	0.5 units	0.3 µl	0.1 µl
dNTPs	2.5 mM	0.2 mM	2.4 µl	0.8 µl
Primer L3870	10.0 mM	1.0 mM	3.0 µl	1.0 µl
Primer H5018	10.0 mM	1.0 mM	3.0 µl	1.0 µl
dH_2O	-----	-----	15.8 µl	4.6 µl
DNA extract	------	-----	1.0 µl	1.0 µl

Appendix VIII: Diagram of the layout of the outdoor breeding tanks



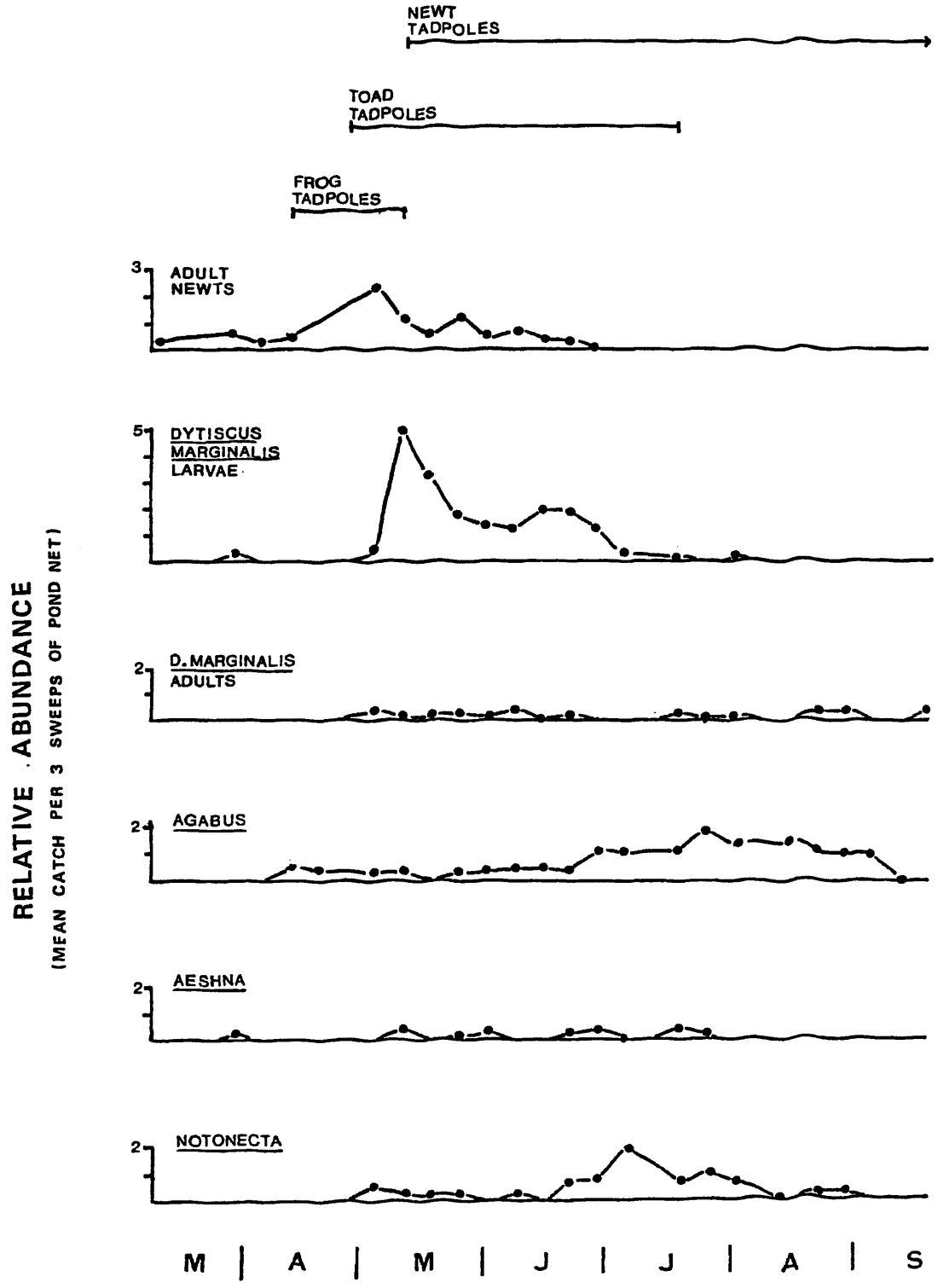
Appendix IX: Larval numbers 1983-1984 (Harrison, 1985)

Mean abundance of *Lissotriton* newt larvae captured by Harrison in 1983 and 1984. Means were calculated for each site from three sweeps of net. Months are represented on x axis by their initial letter. Estimated number of newt metamorphs captured departing from Llysdinam Pond are represented on the bottom graph. Estimates were made from 20 x 1 m funnel traps dug into the soil around the perimeter of Llysdinam Pond.



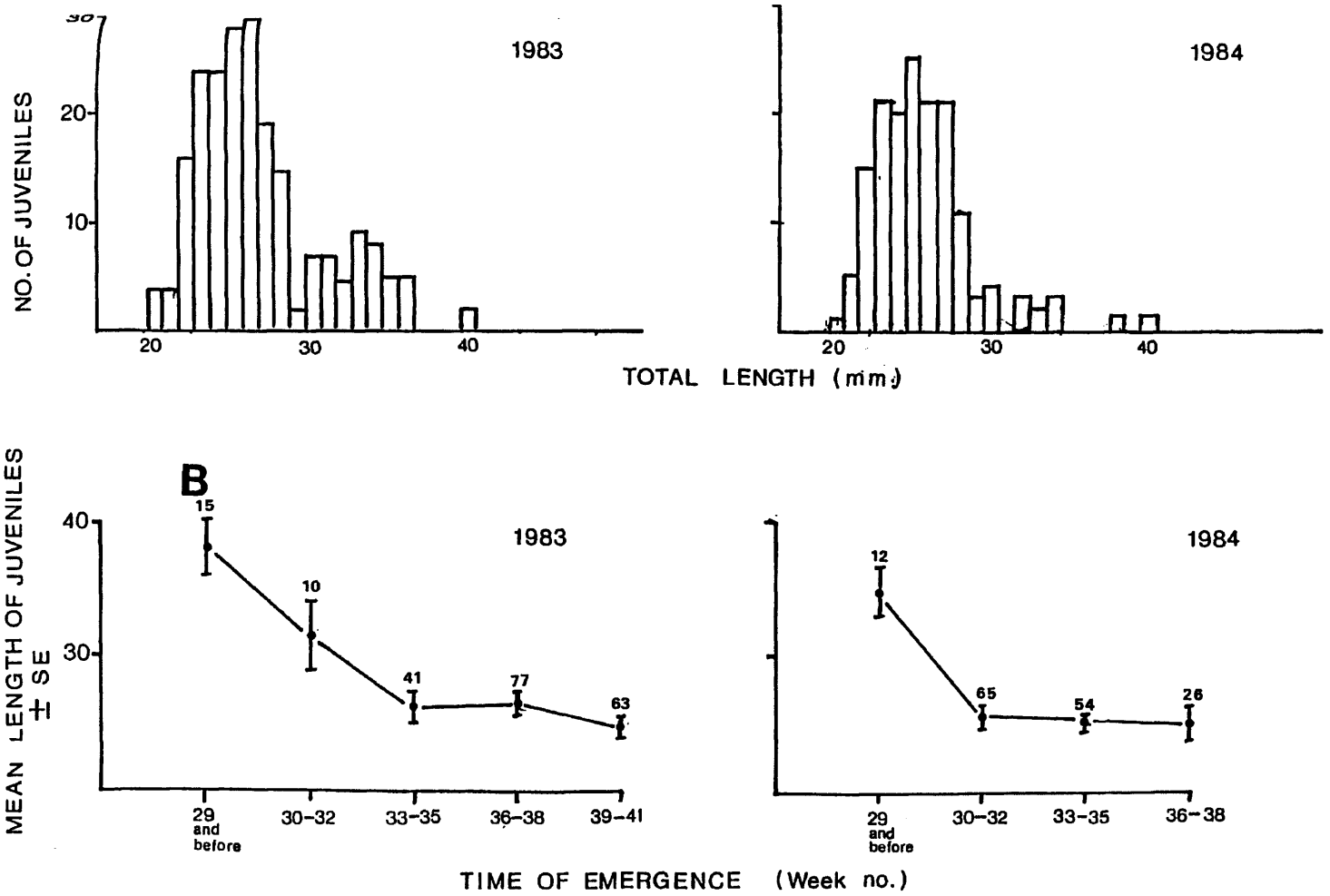
Appendix X: Invertebrate netting data 1984 (Harrison 1985)

Mean abundance (catch per three sweeps of net) of predatory aquatic invertebrates in Llysdinam Pond in 1988. Lines labeled newt, toad and frog tadpoles indicate the times when each larval type was present in Llysdinam Pond. Months on x axis are March to September represented by the initial letter of each month.



Appendix XI: Emerging metamorphs data 1983-1984 (Harrison 1985)

Top graph shows the number of newt metamorphs that emerged from Llysdinam Pond in 1983 and 1984 by total length (mm). Bottom graph shows the mean total length (mm) of *Lissotriton* metamorphs captured on emergence from Llysdinam Pond in 1983 and 1984. In 1983 and 1984 metamorphs were captured in 20 x 1 m pipes dug into the ground around the perimeter of Llysdinam Pond. Numbers above each data point = sample size.



Appendix XII: Larval sizes in 1988 (De Wijer, 1990)

Mean snout-vent lengths of palmate and smooth newt larvae captured in Llysdinam Pond in 1988 in funnel traps by De Wijer (1990). White box represent palmate larvae and black boxes represent smooth larvae. Species were identified by electrophoresis of larval tail tips. Means are represented by horizontal bars. 95% confidence limits are represented by bars. Ranges are represented by whiskers and sample size is indicated at the top of each whisker.

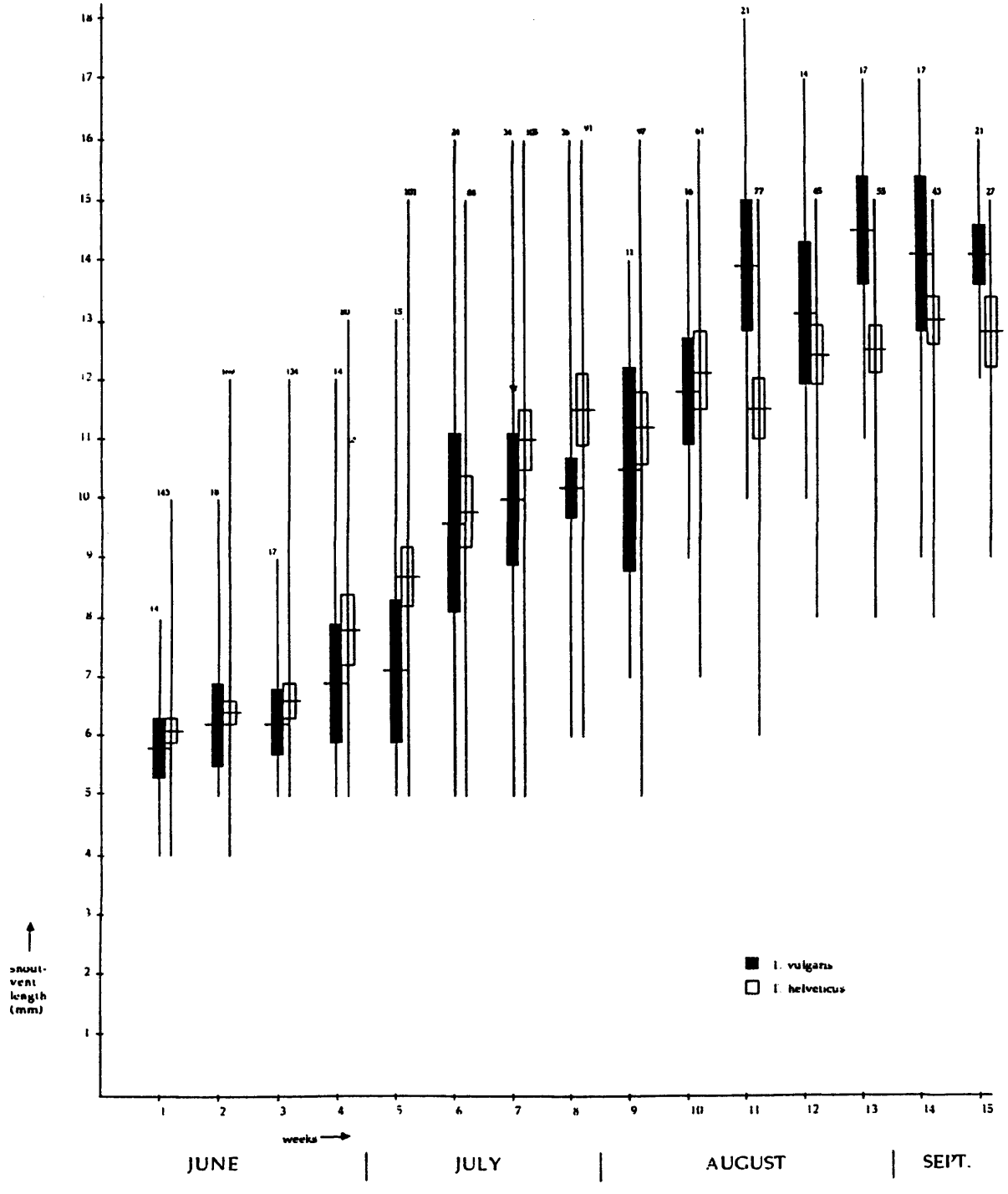
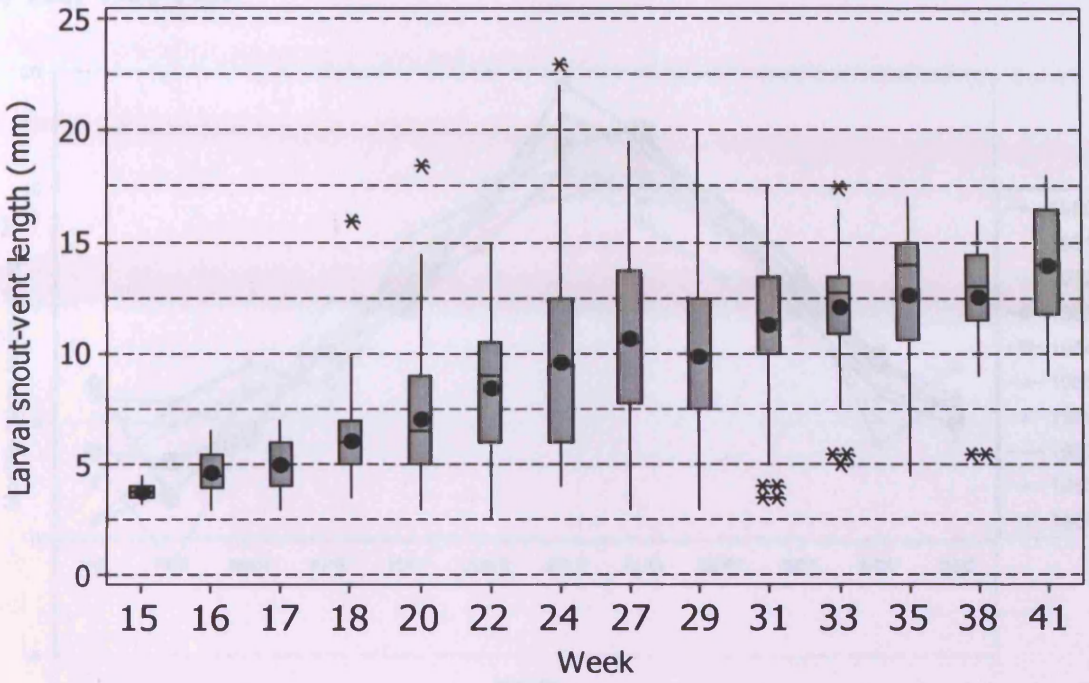


FIG. 9 Mean length of T. vulgaris and T. helveticus larvae per week. Shown are ± with 95 % confidence limits (boxed), range (bar) and sample size (at top of bar).

Appendix XIII: Larval sizes in 2007

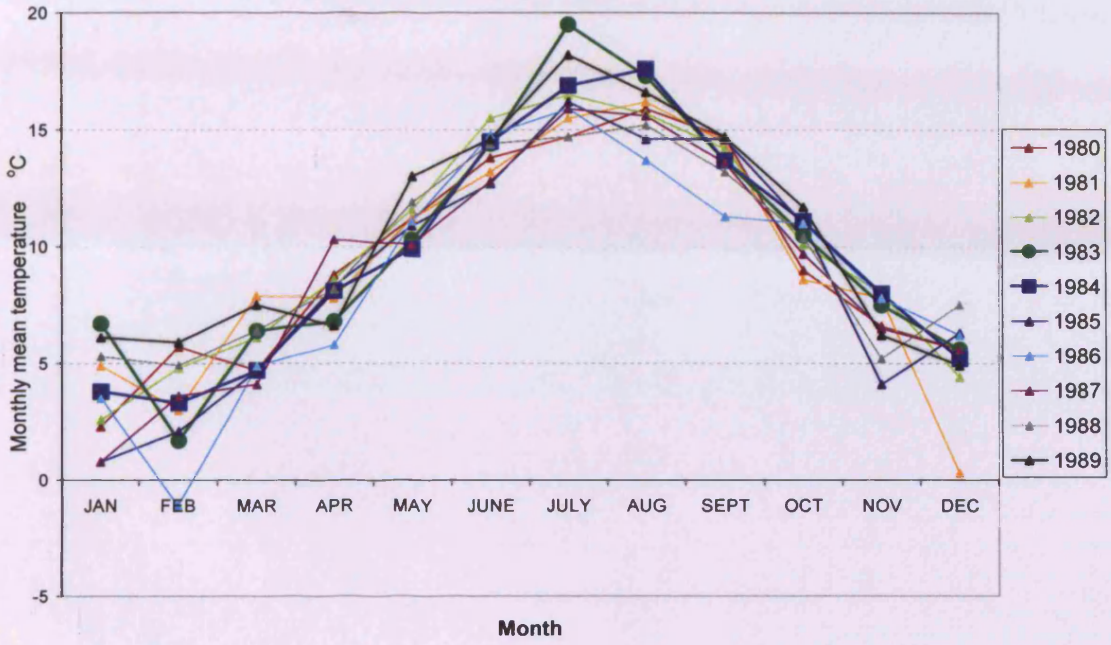
Mean snout-vent lengths of *Lissotriton* newt larvae captured in Llysdinam Pond in 2007 by netting surveys. Horizontal bars represent medians, black circles represent means, boxes represent middle half of data, and vertical lines (whiskers) represent the lower and upper limits, while asterisks represent outliers.



Appendix XIV: Monthly means for 1980s and 2000s

Mean monthly air temperature taken from Central England Temperatures (CETs) for (i) 1980-1989 and (ii) 2000-2007.

(i) Year 1980-1989



(ii) Year 2000-2007