Conservation of the Native White-Clawed Crayfish, *Austropotamobius pallipes*, in the Uplands of Mid-Wales

by

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LIST OF ABBREVIATIONS

-	Negative
+	Positive
A	A statistic
AB	Aberedw
ABI	Applied Biosystems
AFLP	Amplified fragment length polymorphism
AI	Aire
ANOVA	Analysis of variance
AR	Army range
ASA	Noble crayfish, Astacus astacus
ATP	Adenosine triphosphate
AUP	Native white-clawed crayfish, Austropotamobius pallipes
AUT	Stone crayfish, Austropotamobius torrentium
B	Basket trap
BA	Banwy
BMWP	Biological Monitoring Working Party
BOD	Biological oxygen demand
bp	Base pairs
BU	Builth Road
CCW	Countryside Council for Wales
CL	Common land
cm	Centimetres
CPUE	Catch per unit effort
CR	Cregrina
CW	Cwmbach Llechryd
df	Degrees of freedom
DM	Dulas (Monnow)
DNA	Deoxyribonucleic acid
DS	Downstream
EA	Environment Agency
EN	English Nature
EQS	Environmental quality standards
ES	Escley
F	Flowerpot trap
F	F-value
F primer	Forward primer
F_{IS}	Inbreeding coefficient
FM	Nantyroffeiriad farm
FR	Franksbridge
ft	Feet
g	Grammes
G	G statistic

H	H statistic
ha	Hectares
H_E	Expected heterozygosity
H_o	Observed heterozygosity
HO	Honddu
ICONA	Institute of Conservation of Nature
IT	Itchen
kg	Kilogrammes
l	Litres
LC50	Lethal concentration of a chemical in water
LL	Llanbadarn y garreg
LU	Lugg
m	Metres
m^2	Metres squared
MEGA	Molecular Evolutionary Genetic Analysis
mgl^{-1}	Milligrammes per litre
ml	Microlitres
MLG	Multilocus genotype
mM	Millimetres
mM	Micrometres
mm	Millimetres
mtDNA	Mitochondrial DNA
Nall	Total number of alleles per locus
OP	Organophosphate sheep dip
p	P-value
PCA	Principal Components Analysis
PCR	Polymerase chain reaction
pmol	Picomoles
PP	Pontypool
R primer	Reverse primer
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RHS	River habitat survey
SAC	Special Area of Conservation
SE Coefficient	Standard Error coefficient
SG	Sgithwen
SP	Synthetic pyrethroid sheep dip
SSR	Simple sequence repeats
ST	Single trap
Ta	Annealing temperature
TDS	Total dissolved solids
UK	United Kingdom
US	Upstream

US	Usk
V	Volts
VMD	Veterinary Medicines Directorate
WHIP	Wye Habitat Improvement Project
z	Z-score
µg	Microgrammes
Ψ	Odds ratio

ABSTRACT

British native white-clawed crayfish, *Austropotamobius pallipes*, thrive in calcium rich freshwaters, but have declined dramatically in the last few decades due to competition with invasive species, disease, loss of habitat and pollution. This thesis examines the current status of Welsh *A. pallipes* populations, causes of decline, fine scale genetic structuring and rearing of *A. pallipes* in captivity for conservation purposes. A conservation action plan was also devised.

Distribution and abundance have continued to decline on tributaries from the 1990s into the 21st century. In remaining populations, numbers of females and juveniles were low, indicating further declines are likely. *A. pallipes* were no longer found on the main Wye River, were present in low numbers at only two sites on the main Usk River, while just a single individual was found on the main River Banwy of the Upper Severn Catchment. However, populations in the River Edw, a Wye tributary, had recovered somewhat by 2004.

Declines were attributed to siltation, from intensive livestock poaching and sheep dip pollution from the synthetic pyrethroid, cypermethrin. Both causes are exacerbated by steep landscape and heavy rains. The threat of crayfish plague and signal crayfish invasion remains in the Wye Catchment.

Genetic structuring of populations was found at three levels. Genetic structuring within a stream was observed in the River Edw, due to isolation by distance and human interference. Genetic variability differed between streams of the upper half of the Wye Catchment, where individuals were divided into two genetically dissimilar groups, those from the Upper Wye and those from the Lower Wye, possibly as a result of differing habitat characteristics. At the third level, genetic variability differed between catchments. For example, individuals of the Itchen Catchment in Hampshire, southern England, were genetically dissimilar from others surveyed such as those of the Wye catchment in Wales and those from the River Aire in West Yorkshire. This genetic structuring is important and should be considered when carrying out restocking programs.

Rearing *A. pallipes* in captivity was relatively successful due to a high protein, fresh diet of diatoms and zooplankton.

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General Introduction

1.0 INTRODUCTION

Crayfish play an important role in the world ecosystem as many other animals rely on them, particularly as converters of plant material (in the form of detritus) into meat, a high energy source. Native crayfish species are unfortunately declining across the world and as a direct consequence, other species which depend upon them will suffer. In Europe, the native white-clawed crayfish, *Austropotamobius pallipes* which provides a major food source for other animals, appear to be facing a similar fate (Gouin *et al.*, 2003).

This thesis aims to investigate the causes of problems facing *Austropotamobius pallipes* (Lereboullet) in the rivers of mid-Wales and methods of overcoming them.

The general introduction places the animal in its taxonomic, biological and ecological context, reviews its habitat requirements, describes its current and former distribution in Britain and Europe and briefly outlines the main evidence for and causes of decline. These factors are then discussed in relation to crayfish species in other parts of the world. Finally there is a brief introduction to legislation surrounding *A. pallipes* and molecular techniques to assess its population genetics with the view to conserving this species. These subjects are described in more detail in the introductions to specific chapters.

1.1 Crayfish Taxonomy and Distribution

Crayfish (Astacida) belong to the Order Decapoda, the largest group within the Phylum Crustacea (class Malacostraca). They are the only freshwater decapods to be classed as Reptantia Macrochelata (crawlers with large chela) (Holdich, 2002).

Crayfish can be split into two groups (super families), Astacoidea and Parastacoidea. Astacoidea can be further subdivided into two families, Astacidae and Cambaridae (both of which are found only in the Northern Hemisphere). Parastacoidea consists of only one family, Parastacidae, found in the Southern Hemisphere (Hobbs, 1988; Holdich, 2002) The distributions of these families are shown in Figure 1.1.

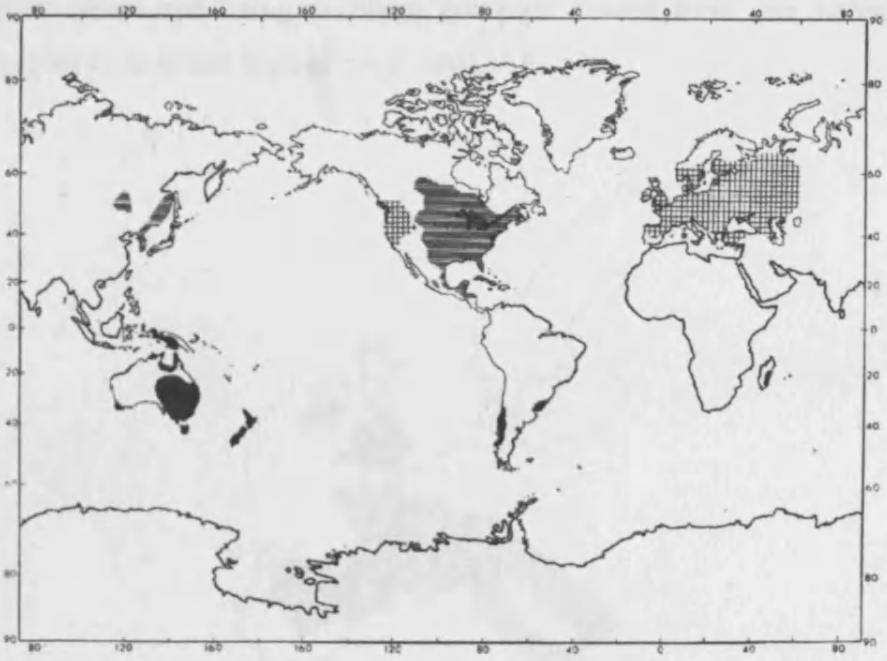


Figure 1.1 World map displaying distribution of the three crayfish families, Astacidae (crossed lines) and Cambaridae (horizontal lines) of the Northern hemisphere and Parastacidae (solid black) of the southern hemisphere. Adapted from Holdich & Lowery (1988).

The family Astacidae consists of three genera, *Austropotamobius, Astacus* and *Pacifastacus*. The species *Austropotamobius pallipes*, commonly known as the native white-clawed crayfish, is the only crayfish native to Britain and Ireland. However, it is thought to have originated in France and moved postglacially into Britain (Grandjean *et al.*, 1997; Gouin *et al.*, 2003) and by human translocation, possibly 12th century monastic orders, into Ireland (Reynolds, 1997). It is also widespread across Europe (Figure 1.2).

In Britain, six species of crayfish may currently be found, the native white-clawed crayfish, *A. pallipes*, the introduced North American Signal Crayfish, *Pacifastacus leniusculus*, narrow-clawed or Turkish crayfish, *Astacus leptodactylus*, noble crayfish, *Astacus astacus*, red swamp crayfish, *Procambarus clarkii* and the spiny-cheek or striped crayfish, *Orconectes limosus* (Holdich, 2003). However, in Wales, only two of these are usually found, *A. pallipes* possibly due to it being the only crayfish species "native" to Britain and *P. leniusculus* because it was introduced to a number of crayfish farms in

Wales in recent years and being excellent escapists moved from one watercourse to another with relative ease and high survival rates.

Figure 1.2 Diagram depicting the distribution of *Austropotamobius pallipes* in the British Isles and Europe (adapted from Holdich, 2002).

1.2 Anatomy and Identification

Crayfish are the "largest mobile freshwater invertebrates" (Holdich, 2002). Adult crayfish range in size from the very large such as *Astacopsis gouldi* from Tasmania which have been known to reach as much as 6 kg in weight (T. Walsh pers. comm.) to the very small, *Gramastacus* from the Grampian Mountains, Victoria, Australia (Holdich, 2002) which may be no larger than a fingernail. A generalised diagram of a crayfish is shown in Figure 1.3.

In general, *A. pallipes* tend to be smaller (12 cm maximum length) and paler than *P. leniusculus* (30 cm maximum length). *P. leniusculus* is usually black/brown with bright cream patches the junctions of the chelae, while their chelae are redder on the underside and large and broad, in relation to their body size. In comparison, *A. pallipes* is paler green/brown in colour and has a paler underside to the chelae although colour does vary somewhat with water chemistry (Figure 1.4). The rostrum of *A. pallipes* has smooth sides

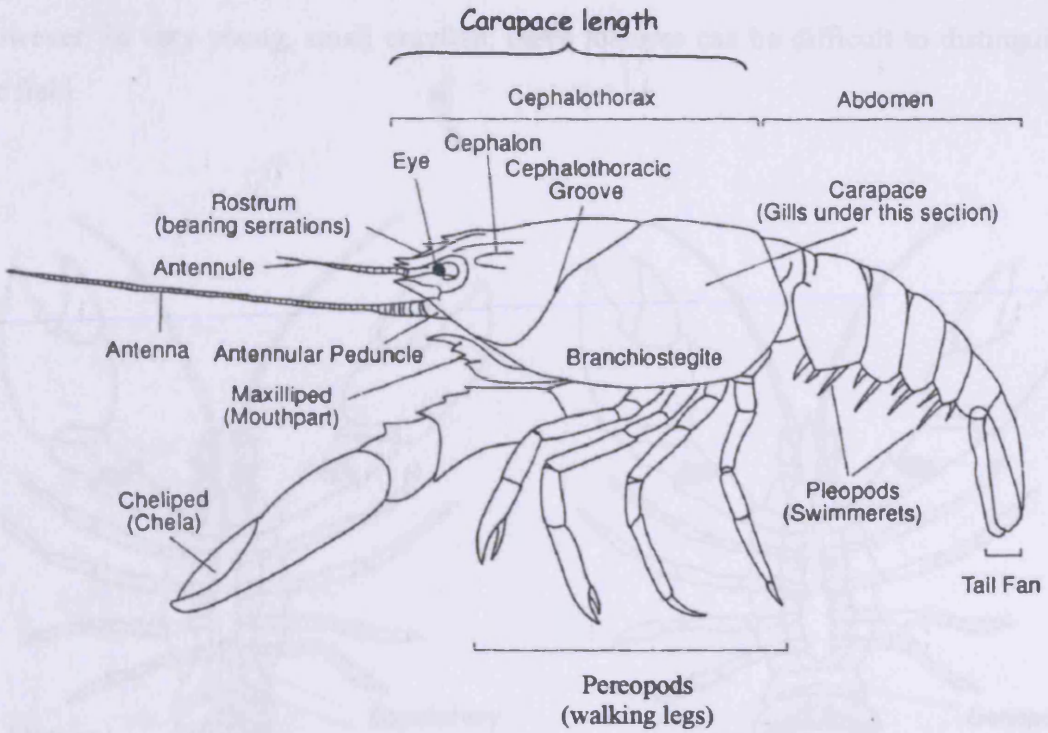


Figure 1.3 Diagram depicting the external anatomy of a generalized crayfish adapted from Merrick (1993).

which converge to the tip of a small triangular apex, while that of *P. leniusculus* has smooth sides which are parallel and have prominent shoulders with a narrow pointed apex (Environment Agency, 1997).

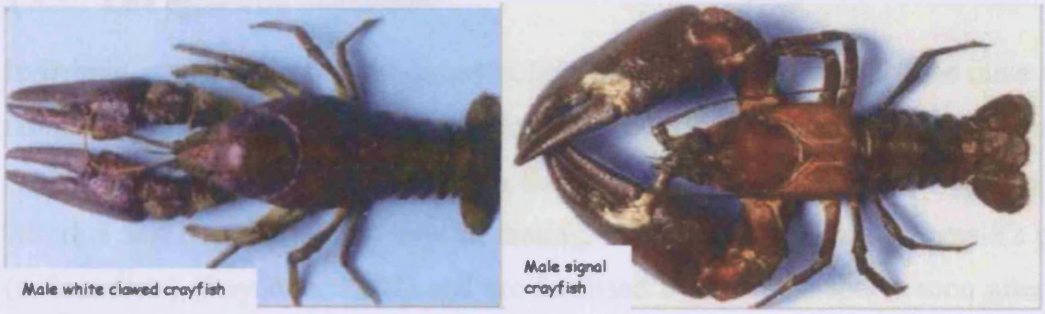


Figure 1.4 Photographs displaying a male white-clawed crayfish (*A. pallipes*) and a male north American signal crayfish (*P. leniusculus*) (not to scale). Adapted from the Environment Agency (1997).

In general, female *A. pallipes* have a wider abdomen than males. More reliably, in males the first pair of pleopods or swimmerets on the underside point forwards, toward the main body, away from the abdomen, and in females they are absent (Foster, 1996) (Figure 1.5).

However, in very young, small crayfish, these features can be difficult to distinguish in the field.

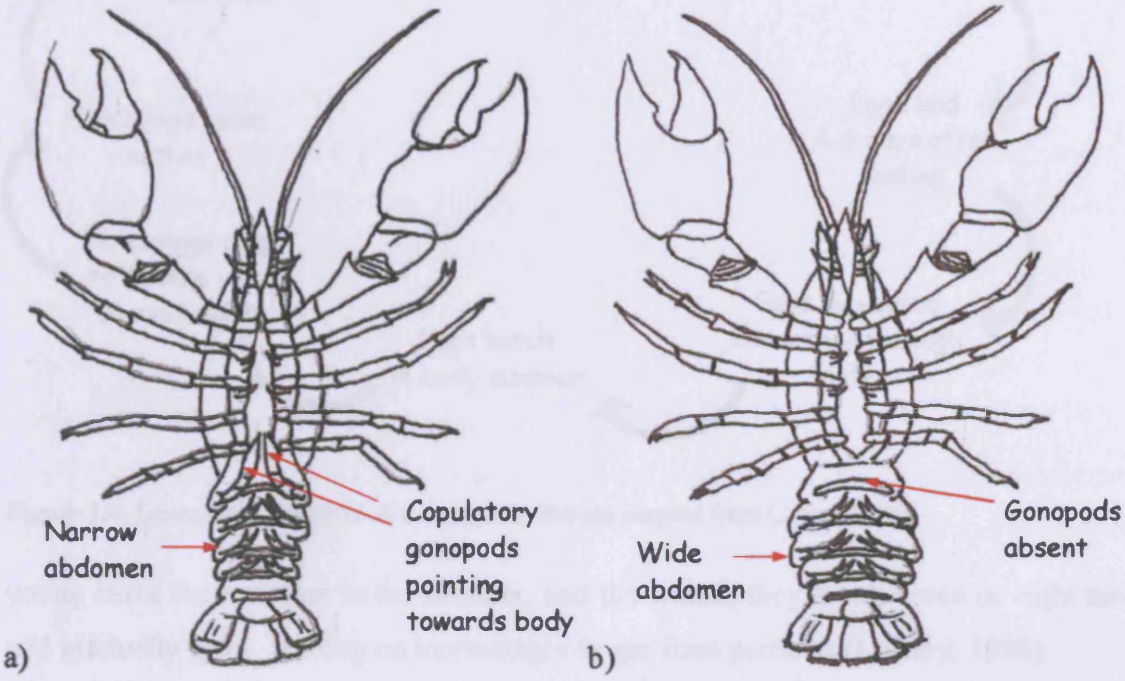


Figure 1.5 Ventral views of a) a male *A. pallipes* crayfish and b) a female *A. pallipes* crayfish.

1.3 Life cycle and behaviour

In Britain, crayfish copulation occurs during October and November. The male turns the female onto her back and secretes seminal fluid onto her abdomen after which the female retreats to her refuge e.g. a burrow in the river bank (Clegg, 1985). The eggs are laid within a few (usually 4 to 6) days of mating, become attached to the female's pleopods (swimmerets) (Reynolds, 2002) and are fertilised by the male sperm soon after (Clegg, 1985) (Figure 1.6). They remain attached to the underside of the abdomen where they are protected until they hatch in May or June. Clutch size tends to be correlated with female size. Older females are therefore likely to produce more young (Lowery, 1988).

When the eggs hatch, the juveniles cling to the underside of the abdomen until they reach a carapace length (Figure 1.3) of approximately 3.5 mm. During this time they undergo two moults and aquire food from the yolk mass (Lowery, 1988). Between the time the

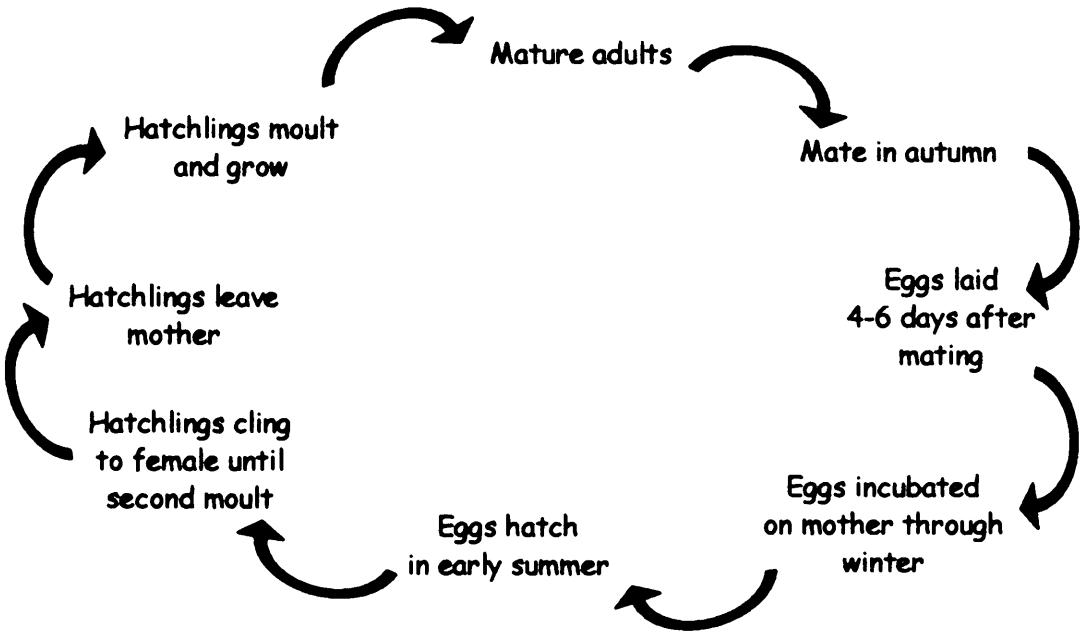


Figure 1.6. Generalised life cycle of a crayfish in Britain adapted from Coley (2000).

young leave their mother in the summer, and the winter, they moult seven or eight times and gradually grow, feeding on increasingly larger food particles (Lowery, 1988).

At 2 months of age, average crayfish length is 1.5 – 2 cm, while the average weight is 0.26 g. At 12 months of age, the average crayfish length is 4.5 – 5 cm and 2.7 g in weight (Cuellar & Coll, 1978). *A. pallipes* can live for a number of years, usually six or seven, with an average mature adult carapace length of 37 mm (Lowery, 1988).

A. pallipes is known to be particularly active during the night, preferring to take refuge during the day under boulders, tree roots, crevices or in burrows sometimes up to a depth of 8 ft (2.44 m) but usually around 3 ft (0.9 m) (Duffield, 1933; Lilley, 1977). This may be a way of avoiding predators that are active during the day (Lilley, 1977). Stone turning is the best day time method of searching for them, while torching enables them to be caught by night.

1.4 Ecology

The ecology of the white-clawed crayfish was described by Holdich (2003a) and is summarized in this section.

1.4.1 Habitat characteristics

A. pallipes tend to prefer fast flowing, highly oxygenated, shallow streams or rivers, although large populations of *A. pallipes* have also been found in reservoirs, lakes, ponds and canals (Lilley, 1977).

Steep channel banks, overhanging shrubbery, a tree canopy and roots extending from banks into the water are all habitat characteristics that positively influence crayfish population size, as these features provide "nursery" regions (Smith *et al.*, 1996). All of the above provide crayfish with food and shelter from predators and strong currents. Aquatic vegetation also provides refuge shelter and food by harbouring prey items such as invertebrates (Smith *et al.*, 1996). Overhanging boughs, boulders, tree shade and the number of riffles were also found to positively influence crayfish presence, while exposed tree roots, eroding cliffs, poached or reinforced banks, gravel/pebble/sand banks and cobble substrate have a negative effect on crayfish presence (Naura & Robinson, 1998). Bank "structure richness" in the form of sufficient bank shelter, i.e. burrows or hollows, overhanging roots and depth to the stream, has a positive association with crayfish density (Bohl, 1989; Kappus *et al.*, 1999). Alternating flat and steep banks are also a positive crayfish habitat feature (Kappus *et al.*, 1999), as this suggests habitat structure and diversity and consequently more crayfish refuges.

Preferred habitat characteristics vary diurnally, as during the day crayfish are found in crevices, among tree roots and under pebbles or boulders, while during the night they are mostly found moving around pebbles and boulders (Reyjol & Roquelpo, 2002).

1.4.2 Water Chemistry

Components of optimum water chemistry are described below:

1.4.2.1 Calcium concentration

Calcium, in the form of carbonates or bicarbonates, is vital to the formation and maintenance of the exoskeleton of the crayfish (Holdich, 2002) and is critical for crayfish presence. Crayfish are therefore often found in chalk or limestone areas.

1.4.2.2 pH

Some crayfish species can survive in environments of a more extreme pH than others, but water below pH 5.5 is detrimental to most (Nystrom, 2002). *Cambarus robustus* are found in lakes within a pH range of 4.7 – 5.6 (Berill *et al.*, 1985) while *Astacus astacus* is usually found in lakes above pH 6 (Svardson, 1974). Neutral or alkaline pH is preferred by most crayfish including *A. pallipes*, as this is likely to be richer in calcium ions (Ca^{2+}) (Foster, 1990).

1.4.2.3 Nitrogen and phosphorous levels

Increased nitrogen and phosphorous in the water cause increased vegetation growth and therefore increased oxygen. These can improve crayfish survival. If aquatic nutrient levels become too high, however, changes in biotic or abiotic factors can have a detrimental effect. For example, invertebrate diversity may decrease as a result of increasing organic matter mineralization, and toxic algal blooms may occur (Bronmark & Hansson, 1998), all of which could damage crayfish or their habitats.

1.4.2.4 Oxygen levels

The average optimum oxygen concentration in water for most species of crayfish is 6 mgl^{-1} (Nystrom, 2002). *Cambarus* is one of few crayfish species tolerant of oxygen concentrations below than 1.6 mg l^{-1}.

Some crayfish that are intolerant of low aquatic oxygen concentrations such as *Procambarus clarkii*, climb to the water's surface to get sufficient air. *A. pallipes* however, generally prefers highly oxygenated waters (Lilley, 1977).

1.4.3 Temperature

Although some crayfish species can survive large temperature ranges, there is a minimum water temperature below which they cannot grow. Each species has an optimum growth temperature, below which, activity and feeding decrease and growth rate is slowed (Holdich, 2002). Slower growth rates are reflected by a lower frequency of moults. For example, time between moults of *Paranephrops* sp. in New Zealand was shorter in pasture streams (16-23 °C) and longer in forest covered streams (12-15 °C) (Holdich, 2002).

1.5 Diet

A. pallipes are usually nocturnal feeders and use both mechanoreceptors and chemoreceptors to detect food, predators and conspecifics (Nystrom, 2002). They are able to detect substances produced by animals and plants (amino acids and carbohydrates respectively) and "hydrodynamic disturbances" caused by moving prey or predators (Breithaupt *et al.*, 1995). Once they have caught prey, crayfish hold it in their walking legs while feeding upon them with their mouthparts (Thomas, 1970; Nystrom, 2002).

Crayfish have been described as generalist feeders (Lorman & Magnuson, 1978; Smith *et al.*, 1996) and omnivores (Hill & Lodge, 1994; Foster, 1996). Foods include plant debris such as leaf litter from overhanging trees, aquatic and terrestrial macrophytes such as *Ranmunculus* spp. Animals make up a large proportion of their diet and include invertebrates such as insect larvae, tadpoles and gastropods (Lilley, 1977). Different authors suggest a number of foods which young crayfish prefer: detritus (Parkyn *et al.*, 1997); plants (Momot *et al.*, 1978) and crushed gastropod shells (Nystrom & Perez, 1998). By feeding on detritus, periphyton and macrophytes, crayfish play an important role in converting them into meat (Smith *et al.*, 1996). A crayfish may sometimes eat its own shed exoskeleton in order to retain calcium (Huxley, 1880) and may even resort to cannibalism, but only when other food is scarce (Lilley, 1977).

Diet is also important for crayfish growth. For example, *Paranephrops* sp. increased in size and moulted more frequently when feeding on invertebrates rather than detritus in both open (warmer) and shaded (colder) streams (Parkyn *et al.*, 1997).

1.6 *A. pallipes* decline and its causes.

1.6.1 Decline

A. pallipes distribution across Britain has altered dramatically over the past 30 years with a large decline in numbers and distribution in some regions (Holdich, 1993; Rogers & Holdich, 1995; Slater, 1998; Wilkins, 1999; Coley, 2000; Slater & House, 2001; Holdich, 2002; Sibley, 2003; Holdich *et al.*, 2004) (Figures 1.7 and 1.8).

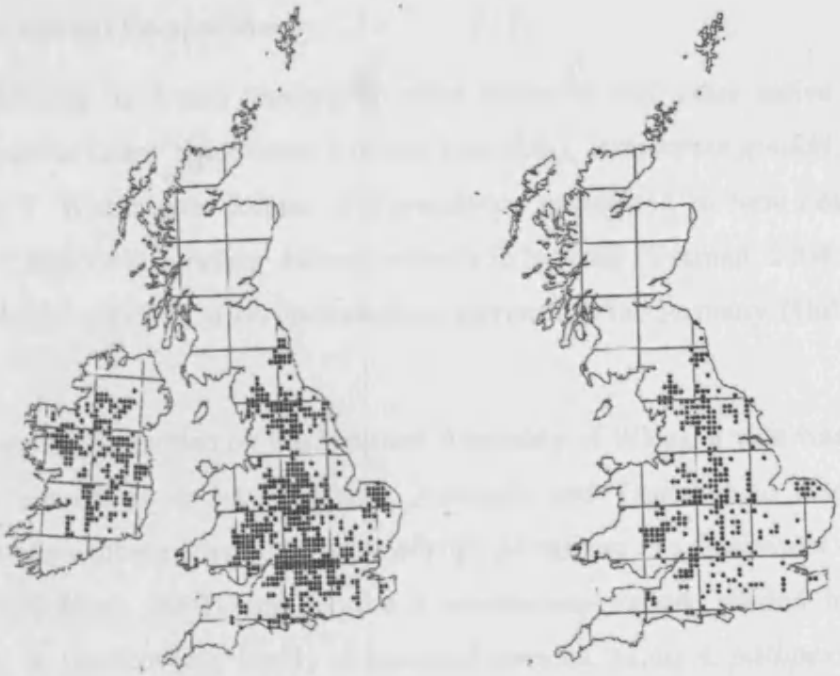


Figure 1.7 *A. pallipes* distribution 1970-90 and *A. pallipes* distribution 1990-96. Adapted from Sibley (2003).

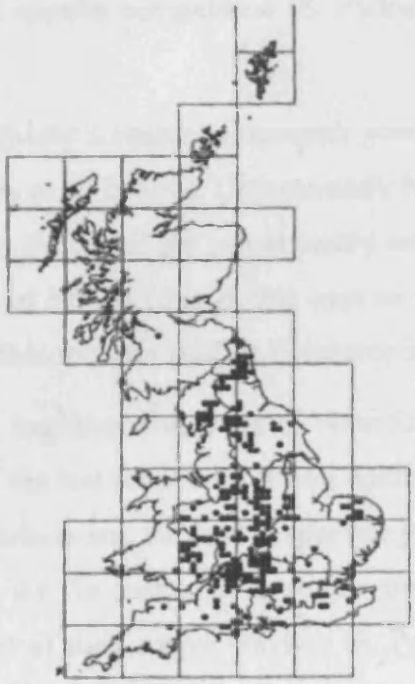


Figure 1.8 *A. pallipes* distribution 1997-2003. Adapted from Sibley (2003).

1.6.2 A Global Perspective

Similar declines have also occurred in other countries with other native species such as the Tasmanian Giant Freshwater Lobster (crayfish), *Astacopsis gouldii*, (Richardson *et al.*, 1999; T. Walsh pers. comm.); *Paranephrops planifrons*, in New Zealand (Parkyn *et al.*, 1997); the noble crayfish, *Astacus astacus* in Norway (Edsman, 2004; Taugbol, 2004) and the stone crayfish, *Austropotamobius torrentium* in Germany (Huber & Schubart, 2004).

In February 2002, funded by the National Assembly of Wales, a visit was made to fellow crayfish researchers in New Zealand, Australia and Tasmania to discuss declines in Southern Hemisphere crayfish. *Paranephrops planifrons* is a freshwater crayfish of New Zealand (Holdich, 2002) and require a structurally varied, shaded habitat of native scrubland in fast-flowing, highly oxygenated streams, as do *A. pallipes* in Britain. Both species, together with Australian freshwater crayfish, are in decline, apparently for similar reasons – agricultural, domestic and industrial pollution, structural habitat damage and loss and introduced alien species competition (S. Parkyn, pers. comm.; J. Merrick, pers. comm.)

In Tasmania, the Giant Freshwater Lobster, *Astacopsis gouldi*, actually a crayfish, can reach 6 kg in weight (T. Walsh, pers. comm). Unfortunately however, their size has made them a sought after trophy and food, and are consequently now severely declining. Other familiar reasons such as loss of habitat (due in this case to intensive logging practices) and pollution are also responsible for their decline (Richardson *et al.*, 1999)

New Zealand and Tasmania, together with parts of New South Wales in Australia are very similar to Wales, one of the last remaining strong holds of *A. pallipes* in Britain, in that the main land use is agriculture and the land is sparsely populated. Research suggests that toxicants such as sheep dip, in particular synthetic pyrethroids (SPs), are largely responsible for recent declines of their native crayfish (S. Parkyn, pers. comm; D. Jerry pers. comm.). It is therefore a reasonable suggestion that the *A. pallipes* decline in Wales is due largely to land use (agriculture) and toxicants (sheep dip), in areas where plague has been ruled out.

Crayfish decline in recent years is therefore clearly not just a national problem, but one of global importance. This was emphasised during the European CRAYNET Meeting in Halden, Norway in 2004. At a roundtable discussion, information on the importance of threats to native crayfish species across Europe was collated. No English representatives were present at the discussion hence the lack of results for this region. Schultz & Schultz (2004) summarised and discussed the findings, discovering that alien species, land use and toxicants were the first, second and third most important threats to native crayfish species in Europe. Fragmentation was also considered an important threat to native crayfish species of Europe (Table 1.1).

Threat	Alpine	Atlantic			Central		Eastern	Mediterranean		Scandi-navia
		France	Ireland	Wales	Germany	Polland	Estonia / Latvia	Italy	Portugal	
Crayfish Plague	3	3	1	2	2	1	2	1	3	3
Other diseases	?	?	1	1	?	1	?	?	?	?
Non indigenous species	3	3	1	2	3	3	3	3	3	3
Predators	1	1	1	2	2	2	2	1	2	1
Exploitation	1	1	1	1	2	1	3	2	2	1
Habitat alterations	2	3	1	2	3	2	2	2	1	1
Water level reductions	1	2	1	1	2	2	2	1	3	1
Eutrophication	2	1	1	1	2	3	2	1	1	2
Acidification	1	1	1	1	1	1	1	1	1	2
Toxicants	3	3	1	3	?	3	3	3	2	1
Land use	3	3	2	3	3	2	2	2	3	2
Fragmentation	3	3	1	1	3	1	2	2	1	2
Considered species	AUT, ASA, AUP	AUP	AUP	AUP	ASA	ASA	ASA	AUP	AUP	ASA

Table 1.1 Table displaying importance of various threats to indigenous crayfish species among different regions of Europe (where 1 = low importance, 2 = medium importance, 3 = high importance and ? = no information). The Considered species are those for which the information is most relevant (where AUP = *Austropotamobius pallipes*; AUT = *Austropotamobius torrentium* and ASA = *Astacus astacus*). Adapted from Schultz & Schultz, 2004).

1.6.3 Local Distribution

This study focuses on rivers in Wales. Up until the early 1990s, eastern Welsh river systems in Wales supported substantial *A. pallipes* populations (Holdich, 1993; Rogers & Holdich, 1995; Foster, 1996; Coley, 2000). In the early 1980s, the River Wye was known to support one of the largest populations in Powys and Herefordshire (Jay & Holdich, 1981).

In the late 1980s, a detailed survey of *A. pallipes* distribution was carried out in the River Wye. Although *A. pallipes* was still abundant and widespread, some decline in numbers had occurred (Foster, 1996).

Rivers of the Wye and/or the Usk, surveyed for *A. pallipes* in 1977, 1988, 1993, 1995 and 1998, were re-surveyed by Coley in 2000. *A. pallipes* were absent from 71 % of sites in the Wye catchment where they were previously present, and far fewer individuals were found where they remained. A similar trend occurred in the Usk Catchment, where 73 % of sites previously containing *A. pallipes* no longer supported any (Coley, 2000).

In 2001, a crayfish survey was carried out on the Edw, a tributary of the Wye. Although previously abundant on the Edw, of the 25 sites searched, only seven individuals were found, two of which were dead (Slater & House, 2001).

The results of these and other surveys provided much alarm for conservationists.

1.6.4 Why the decline?

A number of factors, listed by Smith *et al.* (1996), are thought to influence crayfish abundance and distribution. These include pollution, habitat loss or damage, disease, competition and predation.

1.6.4.1 Pollution

Pollution comes in many forms, through land and water and via the surrounding atmosphere. Most forms of pollution are the result of human activities for example mining, sewage and industrial effluents, fertilizers, sheep dips, biocides (Walker *et al.*, 1996). Each of the above affects crayfish directly, or indirectly by damaging habitat or food sources (Nystrom, 2002). In the Welsh uplands and borderlands, sheep dip has been one of the main sources of river pollution (Environment Agency, 1999) and is suspected

to have played a large part in reducing *A. pallipes* abundance.

There are two main types of insecticidal sheep dip: organophosphates (OPs) and synthetic pyrethroids (SPs). SP dips were licensed for use in 1999. They are thought to be 100 times more toxic to particular aquatic organisms than OPs (Coley, 2000), even though they are claimed to be safer for humans. One teaspoon of cypermethrin has been shown to kill the entire crayfish population of a 0.5 ha pond of the Western Yabby (*Cherax destructor*), Australia (D. Jerry, pers. comm.).

Chemical analysis of water and substrate is one method of detecting cypermethrin presence. However, SPs break down and get washed away very quickly making detection very difficult beyond 12 days after the pollution incident (Pesticide Action Network UK, 2000). Biological monitoring, for example measuring the Biological Monitoring Working Party (BMWP) Score (Mason, 1996), allows the severity of the effects of the pollution to be measured in terms of the abundance and diversity of aquatic and substrate dwelling organisms in that region. More recently, however, cypermethrin has been detected at reportable levels in moss samples, several months after sheep dipping occurred. A combination of moss analysis and biological monitoring is therefore recommended in assessing cypermethrin pollution in watercourses (Rutt, 2004).

The quantities of sheep dipping chemicals and their effects on aquatic organisms have been monitored in Wales since 1997 by the Environment Agency and results published in the Sheep Dip Reports of 1998, 1999, 2000 and 2001. The 1998 report showed sheep dip compounds to be present in almost half of all sampling sites, and at concentrations over those specified as acceptable by environmental quality standards (EQS).

In 1999, more sample sites were shown to be polluted by sheep dip, as three quarters of the sites tested positive, but only 29 % of all sites contained quantities of chemicals classed as "environmentally significant" i.e. above the EQS; while 9 % (1200 km) of the tested rivers were affected biologically i.e. aquatic life had suffered (Environment Agency, 1999). The survey also confirmed that since the OP ban in 1999, amounts of OPs being used by farmers were decreasing while SP usage was increasing. This increase continued to occur in 2000 and 2001 (Environment Agency, 2000 & 2001).

Slater & House (2001) pointed out that in 1997, BMWP (Biological Monitoring Working Party) scores dropped from 68 and 80 in the summer, to 30 and 46, respectively in one month in the autumn. It was thought that these declines might be attributed to sheep dip pollution. Closer investigation showed that a dead sheep was the immediate cause of the problem. Initially it was suggested that decomposition was causing organic pollution. The state of decay, however, was not sufficiently advanced for this to be the case. An alternative and more viable explanation was therefore that sheep dip had washed off the fleece of the sheep and into the water, thus affecting the invertebrate life for some distance down stream (Environment Agency, 1998).

In 2001, the arable form of the SP, cypermethrin, was found in a tributary of the Tywi in South West Wales (Environment Agency, 2000 & 2001). This chemical had been designed specifically for use on arable crops and it is illegal to use it as a sheep dip as it fails to bind properly to the wool (Rutt, 2004). Despite this its use as a sheep dip still occurs and therefore often results in pollution of nearby watercourses such as happened in the Tywi.

Despite the Groundwater Regulations introduced by the Environment Agency at the turn of the century, which enforce the safe use and disposal of sheep dip, more positive detections than ever of dip chemicals were found in rivers in 2000 and 2001 (92 % of 49 sites monitored) (Environment Agency 2000 & 2001) indicating that sheep dip pollution remained a serious problem which should be addressed.

The foot and mouth epidemic of 2001 restricted visits to farms for enforcement and pollution prevention. Also, more farmers had to dip their own animals as professional dippers' visits were also restricted, which probably resulted in more poorly applied dip entering rivers and causing more pollution incidents (Environment Agency, 2000 & 2001).

In an effort to reduce sheep dip pollution, other forms of sheep treatment are currenlty being considered such as injections, pour-ons and showers.

1.6.4.2 Habitat loss or damage

Physical habitat damage is a known cause of *A. pallipes* loss from an area (Nystrom,

2002). Habitat damage or loss can occur as a result of many situations, but these are usually through human interference.

Sediment movement can be the result of natural processes which depend upon river history and management (Naden *et al.*, 2002). Most rivers have some siltation, but excess siltation as a result of modern farming practices is a problem thought to cause a decline in crayfish numbers (Rogers & Holdich, 1995; Slater & House, 2001). It is caused mainly by overstocking of cattle and sheep in land adjacent to the rivers or streams. The banks are poached by stock each time they enter the watercourses, until vegetation is destroyed leaving banks exposed to erosion. The river bed becomes increasingly coated in silt, and in extreme cases becomes completely covered. Spaces under or between stones that are vital refuges for aquatic life become filled in, thus destroying the habitat for the dependent organisms such as *A. pallipes*.

Structural changes to rivers can also cause habitat degradation, for example, flood alleviation schemes, such as the one on the River Dulas of the Monnow Catchment of the Wye. At this site, the bed has been excavated and vertical, concrete flood defence walls have been built to replace the banks. All suitable crayfish habitat was destroyed as a result. Unsuitable management can also result in habitat degradation for example stream diversion or water extraction.

1.6.4.3 Disease

The two lethal crayfish diseases most important to British *A. pallipes* are crayfish plague, caused by the fungus *Aphanomyces astaci* (Oidtmann, 2000), and Thelohaniasis or Porcelain Disease caused by microsporidians of the genus *Thelohania* (Lowery, 1988).

Crayfish plague is carried by the North American signal crayfish, *P. leniusculus*. Although signals themselves are able to survive infection, they can easily transmit the disease to *A. pallipes*, which mostly die as a result (Lowery, 1988). Plague has already wiped out many populations of *A. pallipes*. Signal crayfish, however, usually only a vector of crayfish plague, can possibly die from this disease if stressed or suffering from some other sub-lethal infection although this is rare (Soderhall & Cerenius, 1999).

The spread of *P. leniusculus* and consequently crayfish plague across much of Britain and Europe is thought to be the main cause of *A. pallipes* decline (Figure 1.9). In Wales, particularly on the Upper Severn Catchment, signal crayfish farms were established. It is quite possible that the wild signal crayfish populations in Wales originated from these farms.

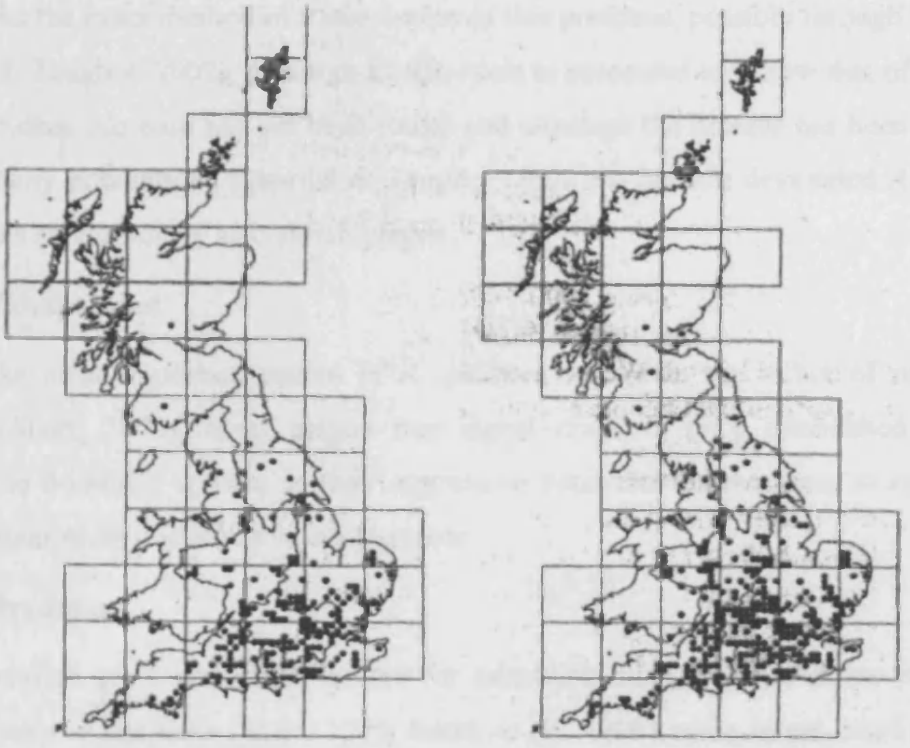


Figure 1.9 *P. leniusculus* distribution 1990-96 and *P. leniusculus* distribution for 1997-2003. Adapted from Sibley (2003).

Crayfish plague arrived in Italy in 1860, then spread through Europe. In 1898, Hofer in Germany originally thought plague was caused by a bacterium, *Bacillus pestiscictaci*. In 1906, Schikora identified crayfish plague as an infection by the fungus, *Aphanomyces astaci* (Oidtmann, 2000).

The sign of an infected signal crayfish is melanised spots in their cuticle. PCRs (polymerase chain reactions) have been used as a method of diagnosing crayfish diseases such as the crayfish plague (Oidtmann, 2000)

After the elimination of an *A. pallipes* population by an outbreak of plague it is possible for a region to become free of crayfish plague after a sufficient time has elapsed.

Restocking of the Sherston Avon and the Tetbury Avon in Wiltshire from an uninfected population proved successful after *A. pallipes* were originally wiped out from plague infection (Spink & Frayling, 2000).

Porcelain disease gets its name from the china white appearance of the muscle tissue on the underside of the abdomen of an infected crayfish (Hazard & Oldacre, 1975). Little is know about the exact method of transmission of this protozoa, possibly through ingestion (Skurdal & Taugbol, 2002), although its life cycle is suspected to follow that of a typical microsporidian. No cure has yet been found and although the disease has been found to exist in many populations (Skurdal & Taugbol, 2002), it has not devastated *A. pallipes* populations so vigorously as crayfish plague.

1.6.4.4 Competition

One of the most important causes of *A. pallipes* decline is the influx of non-native crayfish (Scott, 2000). Even plague free signal crayfish, once established, quickly become the dominant species as their aggressive behaviour allows them to easily out-compete their more docile native counterparts

1.6.4.5 Predation

Young crayfish are a good food source for salmonids such as trout, *Salmo trutta* and Chub, *Leuciscus cephalus* (Stein, 1977; Smith *et al.*, 1996) while larger crayfish are an important food source for otters, *Lutra lutra* (McFadden, & Fairley, 1984; Chanin, 1985; Slater & Rayner, 1993 and Smith *et al.*, 1996).

A number of animals such as herons, kingfishers, otters and fish (e.g. pike, perch, and bullheads) rely on *A. pallipes* as an important food source. If present in sufficient numbers, crayfish can be main food source of otters. In recent years, otter numbers have increased dramatically after their decline in the 70s (Foster & Slater, 1995). It is therefore possible that the current increase in otter numbers may be a contributory factor to the current *A. pallipes* decline. However, this is unlikely as otters are opportunistic feeders and eat whatever and whenever they can. They are therefore not considered a threat to *A. pallipes* in general (Slater & Rayner, 1993).

1.7 Legislation

A. pallipes in Britain has been given legal protection under Schedule 5 of the Wildlife and Countryside Act in 1988. This makes taking the native crayfish from the wild or selling it illegal, unless a license is obtained from the appropriate nature conservation agency, (Environment Agency, 1998). *A. pallipes* has also been listed under Appendix III of the Bern Convention and Annexes II and V of the European Union Habitats and Species Directive therefore requiring designation of protected areas such as Special Areas of Conservation (SACs) and regulation of exploitation (Rogers & Holdich, 1995). It is also classed as being globally threatened. Under the Habitats Directive, the UK Biodiversity Steering Group has established a Biodiversity Action Plan for the species described in the UK Biodiversity Steering Group report Volume 11, 1995.

Also, a number of "no go" areas have been established across England and Wales (Figure 1.9), making farming, movement or unlicensed keeping of introduced species in these regions illegal (Holdich *et al.*, 1995). Currently, the Severn Catchment remains the only area in Wales not designated as such due to the presence of *P. leniusculus*, however, recommendations have been made to include the Severn Catchment as a "no-go" area (Holdich, 1993).

1.8 Molecular Ecology

In order to tackle the problem of declining *A. pallipes* populations, it is initially important to assess the current extent of their distribution and abundance. Simply assessing abundance of crayfish, however, provides insufficient information with which to conserve the species as it tells us nothing of their genetic status and uniqueness or consequent capacity to survive. We must therefore thoroughly assess the genetic diversity of individuals from the populations in question, both on a large scale (between catchments) and on a small scale (within catchments). These results will subsequently allow the compilation of management plans based on the genetic resource. Information so gathered will enable breeding or restocking processes to take place knowing that the genetic integrity of populations will not be damaged.

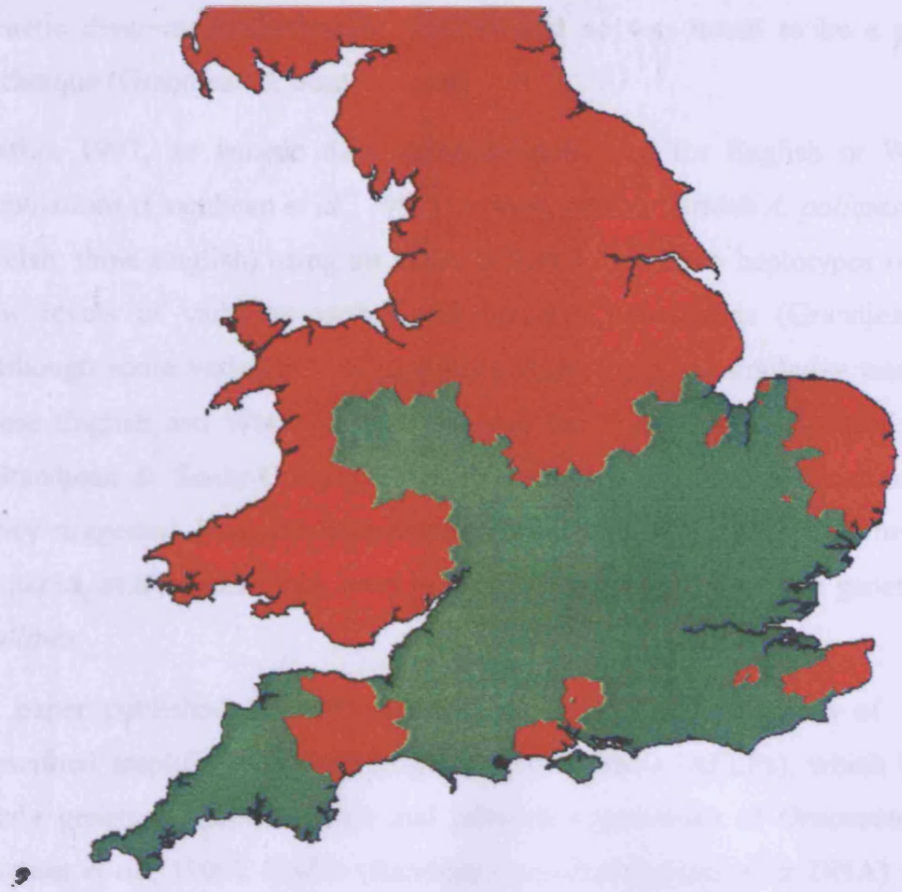


Figure 1.10 Map of Wales and England displaying "no go" areas (red). Adapted from Scott (2000).

1.8.1 Techniques to investigate genetic diversity

Most early genetic data specifically for *A. pallipes* was gathered by electrophoretic analyses (Grandjean *et al.*, 1997). Low levels of genetic diversity were found within and between populations using this technique (Albrecht & Von Hagen, 1981; Attard & Vianet, 1985).

Development of molecular analysis techniques, such as RAPDs, mitochondrial and nuclear sequence data, multi and single locus minisatellites have enabled improved detection of a high degree of genetic variation (Dawson *et al.*, 1997).

For example, an RFLP (restriction fragment length polymorphism) analysis of mtDNA (mitochondrial DNA) showed high levels of genetic diversity within and between French populations of the invasive North American signal crayfish. This technique revealed

genetic diversity in freshwater crayfish and so was noted to be a potentially useful technique (Grandjean & Souty-Grosset, 1997).

Before 1997, no genetic data had been published for English or Welsh *A. pallipes* populations (Grandjean *et al.*, 1997). A study on four British *A. pallipes* populations (one Welsh, three English) using mt DNA RFLPs found three haplotypes in total, indicating low levels of variation within and between populations (Grandjean *et al.*, 1997). Although some variation was present, a high degree of similarity was shared between these English and Welsh populations, and the French populations of a previous study (Grandjean & Souty-Grosset, 1996) indicating a common origin, possibly in France. They suggested, however, that despite these initial findings, further investigations were required, at a nuclear DNA level in order to thoroughly study the genetic diversity of *A. pallipes*.

A paper published in 1999 summarising molecular techniques of crayfish genetics described amplified fragment length polymorphisms (AFLPs), which had been used to study genetic variation within and between populations of *Orconectes luteus* (Souty-Grosset *et al.*, 1999). RAPD (Random Amplified Polymorphic DNA) was known to be another powerful molecular marker (Fritsch & Rieseberg, 1996) and was used to study genetic variability within and between populations of *A. pallipes* (Gouin *et al.*, 2001). The results of this study supported that of Grandjean *et al.* (1997) that no genetic differentiation was found between the British and French populations, indicating a common origin. Higher resolution markers such as microsatellites were therefore required. Microsatellites were thought to be the most suitable type of marker to investigate the "genetic and demographic structure of native crayfish populations" down to individual level (Souty-Grosset *et al.*, 1999).

Microsatellites or simple sequence repeats (SSRs) (Yu *et al.*, 1999) are "polymorphic tandem repeats of sequences which are two to five nucleotide pairs long." They are a subset of the satellite sequences present in highly repetitive eukaryotic DNA and so have been named "microsatellites" (Griffiths *et al.*, 1999). A microsatellite has also been described as a region within the genome where a single base pair or less than six base pairs is repeated multiple times (Rongwen *et al.*, 1995; Yu *et al.*, 1999). Microsatellites

can be three repeat types: perfect tandem repeats, imperfect (interrupted by several non-repeat nucleotides) or compound repeats (Herne *et al.*, 1992). The number of repeats can vary dramatically (Yu *et al.*, 1999) between individuals even of the same species (Griffiths *et al.*, 1999) and are known to be one of the most variable types of tandem repetitive DNA in both animals an plants (Edwards *et al.*, 1991), thus enabling scientists to distinguish between individuals.

Microsatellites are therefore one of the most useful types of nuclear genetic markers (Bruford & Wayne, 1993; Queller *et al.*, 1993). There are a number of advantages to using them, which aid in the study of many animal and plant species (Valsecchi & Amos, 1996). The main advantages are described below, taken from Yu *et al.*, (1999) and Valsecchi & Amos (1996).

1. Microsatellites are co-dominant and are therefore "more informative for linkage analysis than dominant markers."
2. They are PCR based enabling the process for their generation and analysis to be automated.
3. Identification of any genetic variability is easier as they are multiallelic and hypervariable.
4. Microsatellites are randomly and uniformly dispersed throughout eukaryotic genomes (Hamada *et al.*, 1982) and so can be relatively easily located.
5. The primer sequences are published making them easily available for use (Saghai-Maroof *et al.*, 1994).
6. Individual organisms can be identified using microsatellite markers. This is important in some species where natural phenotypic differences are difficult to spot or may change e.g. as with cetaceans.
7. Behaviour in some animals e.g. cetaceans can be difficult to observe. Microsatellite markers can therefore be used to recognise close relatives.
8. Even samples in poor condition due to damage or decomposition can be genetically typed using microsatellite markers.

9. Microsatellite markers can be used to match up a sample with its source. This technique is particularly useful for example, where commercial whaling is concerned (Conrad & Bjorndal, 1993; Valsecchi & Amos, 1996).

Numerous studies have shown that microsatellites are able to detect diversity in organisms, at a higher level of resolution than most other markers. Gouin *et al.* (2000) agrees, saying that microsatellite markers are more useful than allozymes or mtDNA in studying diversity of *A. pallipes*. This is also the case in other organisms. For example, in a study of brown trout (*Salmo trutta*), a higher level of polymorphism was found when using microsatellites than allozymes (Estoup *et al.*, 1998). Microsatellite and RAPD-PCR techniques were again found to be more sensitive than other markers in detecting genetic variability in crayfish (Grandjean *et al.*, 2001).

In order to further investigate the genetics of the declining *A. pallipes*, six microsatellite primers were specifically designed in France using DNA extracted from *A. pallipes* abdominal muscles (Gouin *et al.*, 2000). These primers, once developed, used genomic DNA as a substrate to amplify DNA. Primer pairs would amplify their "own repetitive tract and any size variants" of them in DNA from other individuals (Griffiths *et al.*, 1999). PCR primers would then show up marker alleles of different sized amplification products. Allelic diversity was found to be relatively low in *A. pallipes* in comparison with the other crayfish species, *A. italicus* (Gouin *et al.*, 2000). However, before we can begin to conserve *A. pallipes* through processes such as restocking, we must first undertake more detailed, small scale molecular investigations on remaining *A. pallipes* populations in order to establish whether localised variation is present which could dictate the success of future crayfish conservation programmes. We consequently decided that more microsatellite studies should be carried out using larger numbers of crayfish from a greater number of British sites enabling the genetics of each site to be studied in more detail.

It is believed that small-scale differences may be present and are therefore important in developing management plans specific to each unique group of crayfish.

Crayfish loss and decline in Britain does not simply represent the loss of yet another species in an isolated area, only detrimental to local biodiversity, but instead, illustrates

the loss of important indicator and keystone species from many countries of the world and a vital link in the global food chain.

The fragmentation and decline of native crayfish populations such as *A. pallipes* as a result of habitat loss and pollution is a situation replicated in many other species such as the red squirrel, *Sciurus vulgaris* in western Europe (Verboom & van Apeldoorn, 1990). If the problems of *A. pallipes* are studied in detail, there is a greater likelihood of survival of this species which could hopefully in turn be applied to other threatened species.
The aims of this thesis were therefore to:

- establish the current status of *A. pallipes* in Wales,
- investigate the suspected causes of their decline such as siltation and sheep dip pollution,
- study methods of overcoming them by searching for any fine scale genetic structuring and rearing hatchlings from eggs with a view to forming a conservation action plan for *A. pallipes* in Wales.

CHAPTER 2

Current status of *A. pallipes* in the mid-Wales uplands.

SUMMARY

The aim of this chapter was to establish the current status of the native white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet) in Wales and the Marches.

In Wales, *A. pallipes* distribution was originally mainly restricted to the eastern Wye, Usk and Upper Severn Catchments, these catchments supporting thriving populations. Despite the declines of English populations in the 1980s, surveys carried out in 1977 (Lilley, 1977) and 1988 (Foster, 1990) showed that populations in Wales were still thriving. However, by 1990, crayfish plague reached Welsh River catchments and surveys carried out in the late 90s revealed that *A. pallipes* populations had plummeted. A survey in 2000 revealed that in 72 % of sites previously holding crayfish in the Usk and Wye Catchments, *A. pallipes* could no longer be found. They were instead found to be restricted to remnant tributary stream populations with the exception of a few individuals in the main River Usk. Similar declines were noted in the Upper Severn Catchment.

In order to create management plans to conserve and increase remnant *A. pallipes* populations, the current status of *A. pallipes* in Wales was re-assessed in the present study using stone turning and trapping. Main rivers, tributary streams, canals and lakes in the Wye, Usk and Upper Severn Catchments were searched. For each site surveyed, total number of crayfish caught and catch per unit effort (CPUE) were established. Carapace length, sex and health status of each captured individual were measured on site before they were returned.

Further declines were found to have occurred in recent years in all three catchments, resulting in the extinction of some populations and an increased threat to the survival of this species in these catchments. A population recovery was noted in only one Wye tributary.

Habitat characteristics that positively influenced the presence of *A. pallipes* were boulder and cobble substrate, exposed boulders, bank structure diversity, overhanging boughs and vertical banks. Restoration of *A. pallipes* habitat should include the addition or enhancement of these characteristics.

2.1 AIMS

The aims of this chapter were:

- to establish current abundance and distribution of *A. pallipes* in the Wye, Usk and Upper Severn Catchments,
- to find out if the structure of *A. pallipes* populations in the Wye, Usk and Upper Severn Catchments are suffering,
- to determine which habitat characteristics, if any, influence the presence or absence of *A. pallipes* using river habitat surveys,
- to recommend habitat characteristics suitable for *A. pallipes* reintroduction.

2.2 INTRODUCTION

The native white-clawed crayfish, *Austropotamobius pallipes* is present in many watercourses throughout England and Wales (Lilley *et al.*, 1979; Alderman *et al.*, 1990; Holdich, 1993; Rogers & Holdich, 1995; Coley, 2000). *A. pallipes* distribution in Wales are mainly limited to the main eastern river catchments, the Wye, the Usk and the Severn (Foster, 1990) with the exception of some introduction sites in the Brecon Beacons and Pembrokeshire (Holdich, 1993; Howells, 2003; D. M. Holdich pers. comm.). The Wye, Usk and the Upper Severn Catchments once supported some of the best *A. pallipes* populations in Britain (Ratcliffe, 1977; Smith *et al.*, 1996).

The spread of the alien North American signal crayfish, *Pacifastacus leniusculus*, and crayfish plague caused by the fungus *Aphanomyces astaci*, for which signal crayfish are carriers, became an increasing problem by the 1980s and has been well documented (Holdich & Lowery, 1988; Holdich & Reeve, 1987; 1989; 1991; Foster, 1990; Alderman *et al.*, 1990; Alderman, 1993). Many *A. pallipes* populations across England were destroyed as a result and by 1990, crayfish plague had reached the Severn, Wye and Usk Catchments bordering into Wales (Alderman, 1993).

Despite this spread, however, it appears that the signal crayfish and plague outbreaks remained relatively contained and did not destroy all *A. pallipes* populations across

Wales (Holdich, 1993; Rogers & Holdich, 1995; Smith *et al.*, 1996; Wilkins, 1999; Coley, 2000; Slater & Howells, 2003c).

In the late 1980s, Foster surveyed the Wye and Upper Severn Catchments and found that *A. pallipes* were widespread (Foster, 1995). The distribution of *A. pallipes* was described as being present at altitudes of between 75 and 238 m by Foster (1995). At sites above 238 m he suggested that *A. pallipes* were absent for one or more of the following reasons:

i) ionic content of the water decreases above this altitude to levels which are detrimental to the osmoregulation and moulting success of crayfish,

ii) habitat and food supply deteriorate above this altitude

iii) *Austropotamobius pallipes* have never migrated to such altitudes probably due to physical and chemical barriers.

iv) growth is insufficient to maintain healthy populations above this altitude as a result of low temperatures. This has been disputed since *A. pallipes* have existed above this altitude at similar latitudes in Eire and on the Franco-Swiss border (Laurent, 1988).

Austropotamobius pallipes were absent from sites in Wales at altitudes below 75 m at this time due to pollution, channelisation, arable farming or habitat loss through urbanization (Foster, 1995).

Within the altitude range of 75 to 238 m, *A. pallipes* distribution also importantly depended upon the chemical characteristics of the water (Holdich, 1993), in particular calcium concentrations. *A. pallipes* are usually found in hard waters and are rarely found in Britain in water with calcium concentrations below 5 mgl^{-1} (Sourie & Chaisemartin, 1961; Jay & Holdich, 1981 as cited in Foster, 1995), which explains why they were present in base rich waters flowing over Ludlow strata and Old Red Sandstone e.g. in the Wye and Usk Catchments but absent from base poor waters flowing over Llandovery and Ashgill-Cardoc strata such as in West Wales (Foster, 1990; 1995). *A. pallipes* also require a pH of more than 6.8 (Jay & Holdich, 1977).

Within the Wye Catchment, *A. pallipes* were absent from tributaries upstream of the Dulas Brook at Builth Wells, the Ithon and Irfon Subcatchments, and the headwaters

upstream in the Wye (Lilley, 1977; Foster, 1990) although anecdotal evidence suggests they inhabited the former.

During 1990, signal crayfish and plague reached the Wye Catchment (Alderman, 1993). Concern was raised over the threat to the Welsh *A. pallipes* populations (Holdich, 1993; Slater, 1998; Wilkins, 1999; Coley, 2000). A number of surveys were consequently carried out in order to find out if declines had occurred (Moreley, 1997; Wilkins, 1999; Coley, 2000).

In 1988, 24 out of 26 sites surveyed in the Wye catchment contained *A. pallipes*, with a mean catch per man hour (CPUE) of 16.9 (Foster, 1990). Less than a decade later in 1997, *A. pallipes* were found in only five of these 24 sites and with a mean CPUE of 0.8 (Moreley, 1997).

It was suspected that these declines were mainly a result of sheep dip pollution incidents, particularly with the increase in use of cypermethrin, a synthetic pyrethroid 100 times more toxic to aquatic life than organophosphates, rather than plague or signal invasion (Wilkins, 1999; Coley, 2000; Slater & House, 2001).

Further surveys, for example by Coley (2000), established that between 1990 and 2000, *A. pallipes* had disappeared from 73 % of sites in the Usk Catchment and 71% of sites in the Wye Catchment while in 2001 a survey of the River Edw, a Wye tributary, once containing thriving *A. pallipes* populations, found only seven individuals, two of which were dead (Slater & House, 2001).

These findings suggested a general decline in numbers (measured by CPUE) and consequent distribution of *A. pallipes* in Wales and the Marches over the years. The suspected decline in *A. pallipes* numbers and distribution was therefore assessed within this chapter, by testing Hypothesis 1 that abundances and distribution of *A. pallipes* in Wales and the Marches have declined over the years, 1988, 1997, 1999 and 2002.

Also, by assessing the population structure such as female : male and adult : juvenile ratios of these potentially declining populations, it was hoped that the reasons for the decline may become clearer.

Previous studies have found female biased sex ratios in healthy crayfish populations (Svardson, 1949, Thomas & Ingle, 1971 and Lilley, 1977). It is therefore suspected that if a male biased sex ratio is present in a declining *A. pallipes* population, a lack of

females and consequently mature females with eggs may be occurring, which could be causing the decline as reduced numbers of young are being produced. Sex ratios were therefore assessed in this chapter by testing Hypothesis 2 that the sex ratios of surveyed *A. pallipes* populations are male biased.

A lack of females in a population could also be linked with a lack of juveniles as reduced numbers of juveniles are being recruited into the population. Also, reduced numbers of juveniles could be a sign of pollution as younger crayfish are known to be more susceptible to pollution than adult crayfish. Early American crayfish instars were found to be three times more sensitive to pollutants than juveniles, while juveniles were four times more sensitive than adults (Eversole & Seller, 1996). Adult : juvenile ratios were therefore also assessed in this chapter by testing Hypothesis 3 that adult : juvenile ratios of surveyed *A. pallipes* populations are adult biased.

A lack of juveniles in particular rivers could also be linked with differences in mean carapace lengths of crayfish between rivers, which could also indicate a problem in particular populations. For example, if the mean carapace length is particularly large in a river, this could indicate that the younger generations of crayfish had been destroyed for example due to pollution. A comparison of mean carapace lengths between rivers was therefore carried out in this chapter and tested Hypothesis 4 that mean carapace lengths of surveyed crayfish differed between rivers.

In the literature, crayfish were reported to be more common where particular habitat characteristics were present (Smith *et al.*, 1996). It is therefore likely that crayfish presence or absence in Welsh rivers is associated with certain habitat characteristics (Hypothesis 5). In order to investigate this, habitats of current crayfish populations and of regions where they were once present but are now thought to be absent were compared using a standard technique in order that features which positively and negatively influence crayfish presence or absence might be identified. It was thought that by identifying such features, suitable restocking sites could be found.

River Habitat Survey (RHS), the technique used to assess the habitat, is UK based and designed to assess the quality and structural variability of river habitats and adjacent land (Raven *et al.*, 1997, 1998) through examining their physical structure and noting the extent of any features present (Jeffers, 1998).

The 2003 RHS form was used to survey 53 sites across three catchments, the Wye, the Usk and the Upper Severn in Wales and the Marches. A PCA (principal components analysis) was used to reduce the number of environmental variables. The Scores produced were then subjected to an ordinal logistic regression to identify which, if any variables were significantly associated with the presence or absence of *A. pallipes*.

Hypotheses for this chapter are:

Hypothesis 1

Abundances and distribution of *A. pallipes* in Wales and the Marches have declined over the years, 1988, 1997, 1999 and 2002.

Hypothesis 2

Sex ratios of surveyed *A. pallipes* populations are male biased.

Hypothesis 3

Adult : juvenile ratios of surveyed *A. pallipes* populations are adult biased

Hypothesis 4

Mean carapace lengths of surveyed crayfish differed between rivers.

Hypothesis 5

Crayfish presence or absence in Welsh rivers is associated with certain habitat characteristics

2.3 METHODS

2.3.1 Location

This study focussed on rivers and streams of three catchments in Wales and the Marches: the Wye, the Usk and the Upper Severn (Figure 2.1). Appendix I presents a list of catchments, rivers and site names (with grid references) surveyed during this study.

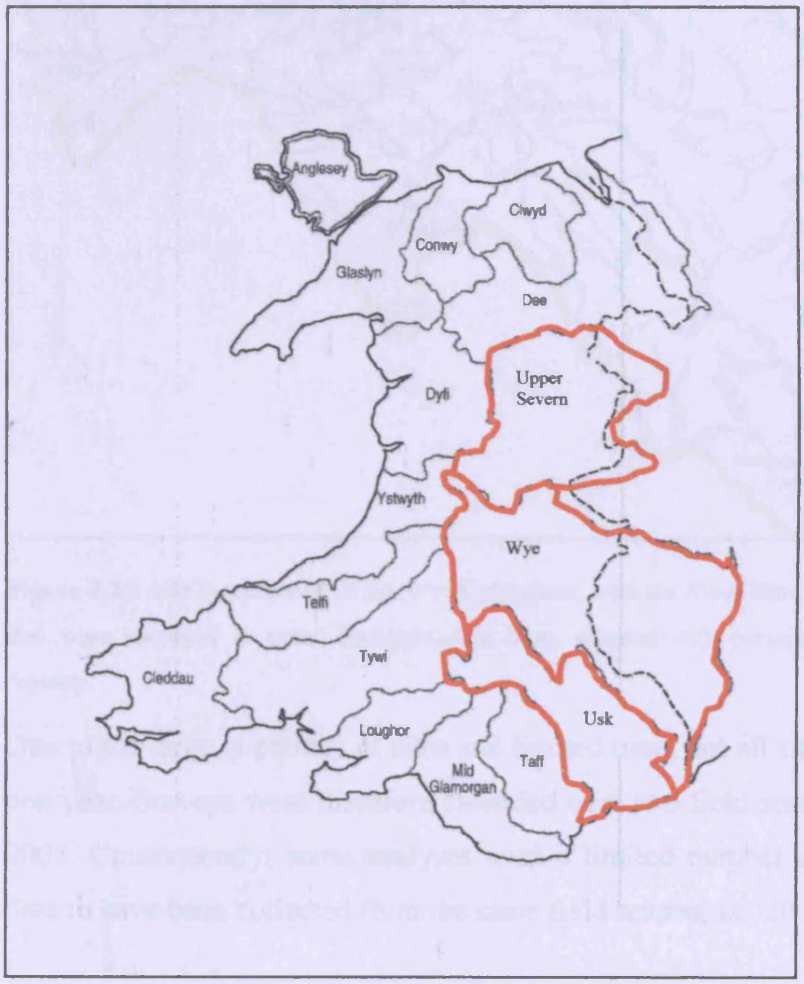


Figure 2.1 Map depicting main river catchments in Wales and the Marches. Sampling catchments, Upper Severn, Wye and Usk are highlighted in red, while the dashed line represents the Welsh-English border.

Particular attention was paid to the River Edw, a 16 km long tributary which enters the River Wye at Aberedw (grid reference SO 075 470), 5 km downstream of Builth Wells in Radnorshire, a district of Powys (Figure 2.2). All sub-tributaries and the main River Edw of the Edw sub-catchment were surveyed for crayfish.

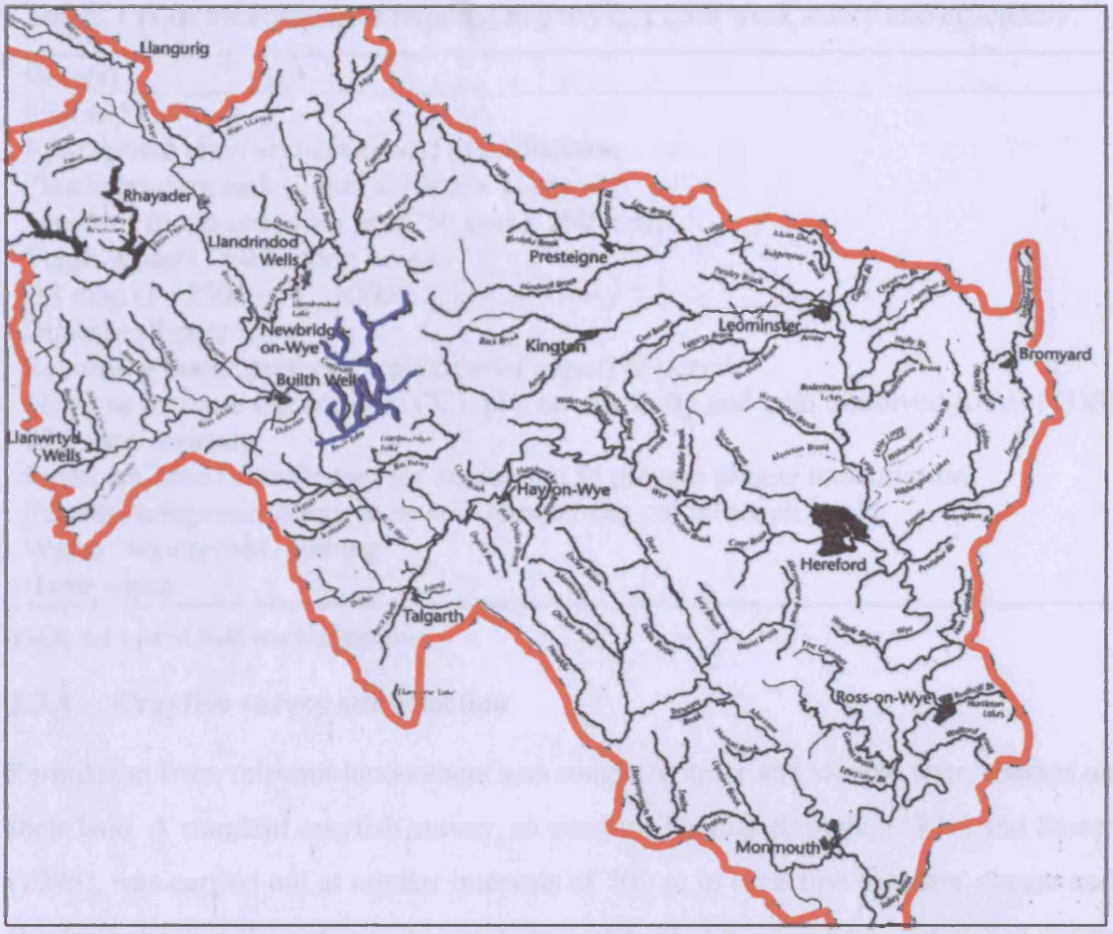


Figure 2.2 1:50000 scale map of the Wye Catchment, with the River Edw and its fine tributary streams that were surveyed in detail highlighted in blue, adapted with permission from the Environment Agency.

Due to the large quantities of sites and limited time, not all sites could be surveyed in one year. Surveys were therefore extended over two field seasons, Summer 2002 and 2003. Consequently, some analyses used a limited number of sites in order for the data to have been collected from the same field season, i.e. 2002 or 2003.

2.3.2 Safety

Health and Safety procedures were followed at all times. Route cards of exact locations with six figure grid references of fieldwork sites with estimated time at each site and return time were left with a responsible person. A first aid kit and mobile phone were carried at all times.

2.3.3 Equipment

Table 2.1 presents equipment required to carry out field work safely and efficiently.

Item(s)
First aid kit
Appropriate crayfish licence and identification
Plastic holding tank (20cm x 10cm x 10cm)
Standard frame sampling net (250 mm x 250 mm)
Thigh waders / wellington boots
OS map (1 : 2500 or 1 : 10000
Digital callipers
Recording book (preferably waterproof paper) & pencil
Meter to measure temperature (°C), pH, conductivity and total dissolved solids (TDS)
25m tape measure
Iodoform based disinfectant for equipment to prevent plague transmission
Rubber / neoprene gloves to protect hands from cut infection / cold
Warm / waterproof clothing
Hand wipes

Table 2.1 List of field work equipment.

2.3.4 Crayfish survey site selection

Permission from relevant landowners was sought to enter and sample watercourses on their land. A standard crayfish survey, as used by Thomas & Ingle, (1971) and Foster (1996), was carried out at regular intervals of 500 m in each fine tributary stream and the main river of the entire Edw Catchment and other rivers, canals, lakes and ponds of the Upper Severn, Wye and Usk Catchments. Any decreases or increases in abundance or distribution of *A. pallipes* populations were noted.

2.3.5 Crayfish search procedure

Crayfish searches were carried out using two methods: i) stone-turning (Thomas & Ingle, 1971) in shallow rivers and streams and ii) trapping as described by Foster (1996) in deeper or larger water bodies such as lakes, ponds or canals. These methods were used instead of the *A. pallipes* Monitoring Protocol designed by Peay (2003a), which enabled results to be comparable with those collected previously by Lilley (1977), Foster (1990), Holdich (1993), Rogers & Holdich (1995), Wilkins (1998), Coley, (2000) and Slater & House (2001). A detailed survey of crayfish in watercourses of Torfaen, in the lower Usk Catchment was conducted using the monitoring protocol of Peay (2002) as a contract for Torfaen County Council. For the purposes of the present study, however, catch per man-hour was also measured at these sites in order for results of this survey to be comparable with the present study.

2.3.5.1 Stone turning

In each stream, searches were carried out in an upstream direction. If a hand or fingers could fit underneath a stone, it was selected as being suitable for possible crayfish presence. The stone was turned after a standard kick sampling net (1 mm mesh size) was placed immediately downstream. Any mobile crayfish were captured in the net, while any sedentary crayfish were picked up and placed in a container with river water. The crayfish were then examined and the following data recorded:

1. date of capture
2. location
3. sex
4. carapace length (mm) (length from the posterior median edge of the thorax to the tip of the rostrum (Thomas & Ingle, 1971)
5. weight
6. number of missing appendages
7. disease status e.g. plague (by observation of lethargy and limb loss) or porcelain disease (by the white, china-like appearance of the underside of the abdomen)

Once measured, each individual was replaced in the exact location where it was captured in order to prevent migratory movements.

Catch Per Unit Effort (CPUE)

Stone turning was carried out at each site for one man-hour i.e. one person searched a site for 1 hour, two people for 30 minutes or three people for 20 minutes. This enabled the "*number of crayfish caught per man hour*" to be calculated for each site, which was expressed as "*catch per unit effort (CPUE)*" or "*catch per man-hour.*" This standard value was comparable with previous crayfish surveys. In order to ensure sufficient uniformity in survey efficiency during my surveys, each surveyor was trained and had attained a relatively efficient survey technique.

2.3.5.2 Trapping

There are several types of commercially available crayfish traps. Two types were used in this study, both of which were described by Holdich (2002). "Trappy" traps are 50

cm long and 20 cm in diameter with a mesh size of 2.5 cm x 2.5 cm and are suitable for capturing larger crayfish. The second trap type, supplied by EFE & GB Nets Ltd., has a smaller mesh size of 3 mm x 3 mm and is suitable for catching both juvenile and adult crayfish. Both trap types consist of a mesh cylinder with an inverted funnel at each end which allows crayfish to enter but not escape.

Traps were labelled as Cardiff University Property and were baited with tinned cat food and fish, replicating previous studies (Rogers & Holdich, 1995). Traps were completely submerged and secured tightly by strong cord to a sturdy object or the bank, left in place over a few consecutive nights and checked daily. Any captured crayfish were examined and measured then released in the same spot.

2.3.5.3 Crayfish sampling for DNA analysis

For genetic analysis in another part of the study (described in Chapter 4), one of the fourth pereopods (walking legs) was removed from each crayfish caught, providing the carapace length was over 20 mm. This method was thought to be the least invasive way of collecting samples.

2.3.6 River Habitat Surveys (RHS)

The 2003 version of the RHS form (Appendix II) was used to survey 53 sites across the Wye, Usk and Upper Severn Catchments of Wales and the Marches. Surveys were carried out between June and August of 2004. For replication, two or more sites were surveyed on each river.

Each RHS consisted of collecting data along a 500 m length of stream or river, and recording features of channel, banks and land use up to 50 m from the waterway at ten "spot checks" located every 50 m and a "sweep up" checklist which measured variables continuously between these spot checks along the 500 m stretch. Other features such as the shape of the valley and numbers of riffles were recorded (Jeffers, 1998).

Prior to commencing the surveys, an RHS accreditation course was completed. Ensuring surveyors across Britain have attended this course has enabled surveying errors to be minimised thus allowing compilation of a national RHS database by the Environment Agency (Fox *et al.*, 1998; Jeffers, 1998).

2.3.7 Data Analysis

Two-sample t-tests and one-way ANOVAs were used to compare past and present results of crayfish surveys.

River Habitat Survey (RHS) data was stored in an EXCEL spreadsheet. Data were split into three sections as they appeared on the survey form, "spot check," "sweep up" and "other variables." Data from left and right banks were summed. A Principal Components Analysis (PCA) was carried out to select the variables that account for the majority of data variation. This was followed by an Ordinal Logistic Regression (Vaughan & Ormerod, 2005) to identify the importance of these variables in accounting for the variation in crayfish presence/absence and abundance.

All statistical analyses were carried out using MINITAB 13.

2.4 RESULTS

2.4.1 Past and present *A. pallipes* abundance in terms of CPUE

2.4.1.1 Comparison of CPUE results between years in the Wye Catchment

Data were non-parametric even after transformation. A Kruskal-Wallis Test used to compare CPUEs of *A. pallipes* in the Wye Catchment between years showed that median CPUE values dropped significantly between 1988 (mean CPUE = 16.9) and 1997 (mean CPUE = 0.8) ($p = 0.000$, $df = 3$, $H = 45.98$). CPUE decreased again to 0.2 in 1999 but not significantly. However, CPUE marginally increased to 0.5 in 2002, although this recovery in no way reached pre-1990 levels (Figure 2.3 and Table 2.2).

In 1988, Foster surveyed 26 sites throughout the Wye Catchment and found 24 were occupied by *A. pallipes*. Nine years later, these sites were resurveyed and *A. pallipes* were only found in 5 of the 26 sites (Moreley, 1997). Their distribution had been dramatically reduced during this time. Two years later, *A. pallipes* were only found to be present in 4 of the 26 sites, 2 of which were sites where *A. pallipes* were not found 2 years earlier, an indication that these two sites were possibly recovering. In 2002, the present study found *A. pallipes* in only 4 of the original 26 sites. Also, they were not found in sites where they were present in 1999 (i.e. Sites 1, 3, 12 and 14 from Table 2.2). Instead, populations of four other sites (8, 9, 10 and 13 from Table 2.2) appeared to have made a slight recovery since 1999 as *A. pallipes* were found to be present here in 2002.

See Figure 2.4 for the mean abundance and distribution of *A. pallipes* across a river map of the Wye Catchment.

2.4.1.2 Comparison of CPUE results between years in the Edw Subcatchment

Data were non-parametric even after transformation. A Kruskal-Wallis Test was therefore used to compare CPUEs of *A. pallipes* in the River Edw between years. A marked decrease in CPUE was found between 1988 and 1995 (Figure 2.5).

There was no significant difference in the median CPUE values between 1988 and 2002 ($H = 6.22$, $df = 5$, $p = 0.286$). However, the mean CPUE was relatively high in 1988 (17) (Foster 1988), drops to 4.8 in 1995 (Rogers & Holdich, 1995), 2.2 in 1997 (Moreley, 1997), 0.5 in 1999 (Wilkins, 1999) and an all time low of 0.4 in 2001 (Slater & House, 2001) (Figure 2.5). By 2002, the CPUE had increased to 1.2

Site number	Site	Grid Ref.	CPUE (Foster, 1996)	CPUE (Moreley, 1997)	CPUE (Wilkins, 1999)	CPUE (Howells, 2002)
1	Dulas (A438)	SO 042552	26	2	1	0
2	Chwefru (Builth Wells)	SO 029513	12	0	0	0
3	Irfon (Builth Wells)	SO 032512	3	0	0	0
4	Duhonw (Mid)	SO 052495	19	7	0	0
5	Edw (Wern)	SO 117573	24	3	3	0
6	Edw (Hundred House)	SO 115545	0	0	0	0
7	Edw (Vron)	SO 124534	24	3	0	0
8	Edw (Cregina)	SO 124523	46.5	0	0	1
9	Edw (Common)	SO 100481	7.3	7	0	4
10	Edw Confluence	SO 077469	0	0	0	2
11	Lugg (Presteigne)	SO 316646	2	0	0	0
12	Hindwell (Combe)	SO 345634	10.8	0	1	0
13	Lugg (Mortimer's Cross)	SO 426637	21	0	0	6
14	Gilwern Brook	SO 287570	6.7	0	1	0
15	Arrow (Pembridge)	SO 391585	7.5	0	0	0
16	Llynfi (Llandefaelog)	SO 132304	45	0	0	0
17	Llynfi (u/s Enig)	SO 150344	20	0	0	0
18	Ennig (d/s Talgarth)	SO 152344	37.5	0	0	0
19	Trwffwrdd (Felin-Newydd)	SO 118358	12	0	0	0
20	Dulas (u/s Llynfi)	SO 148344	19.5	0	0	0
21	Llynfi (u/s Bronllys)	SO 163365	45	0	0	0
22	Velindre	SO 171374	15	0	0	0
23	Cilkenni	SO 183411	6	0	0	0
24	Dulas (Hay on Wye)	SO 231427	5	0	0	0
25	Hardwicke	SO 252444	18	0	0	0
26	Clyro	SO 229451	7.5	0	0	0
		Total	**440.3**	**22**	**6**	**13**
		Mean	**16.9**	**0.8**	**0.2**	**0.5**

Table 2.2 Site number, site, grid references and CPUEs for *A. pallipes* caught in the Wye Catchment by Foster (1996), Moreley (1997), Wilkins, (1999) and Howells (2002).

(Howells, 2002) and a total of 75 individuals were captured on the Edw between June and September in 2002 (Howells, 2002) as opposed to seven individuals, two of which were dead in 2001 (Slater & House, 2001).

2.4.1.3 Comparison of CPUE results between years in the Upper Severn Catchment

Data from Holdich (1993) and the present study (2002) were combined for the Upper Severn Catchment due to the lack of sites surveyed throughout these nine years. The resulting data was compared with that collected by Foster in 1988.

Despite transformations, data were non-parametric. A Mann-Whitney Test was

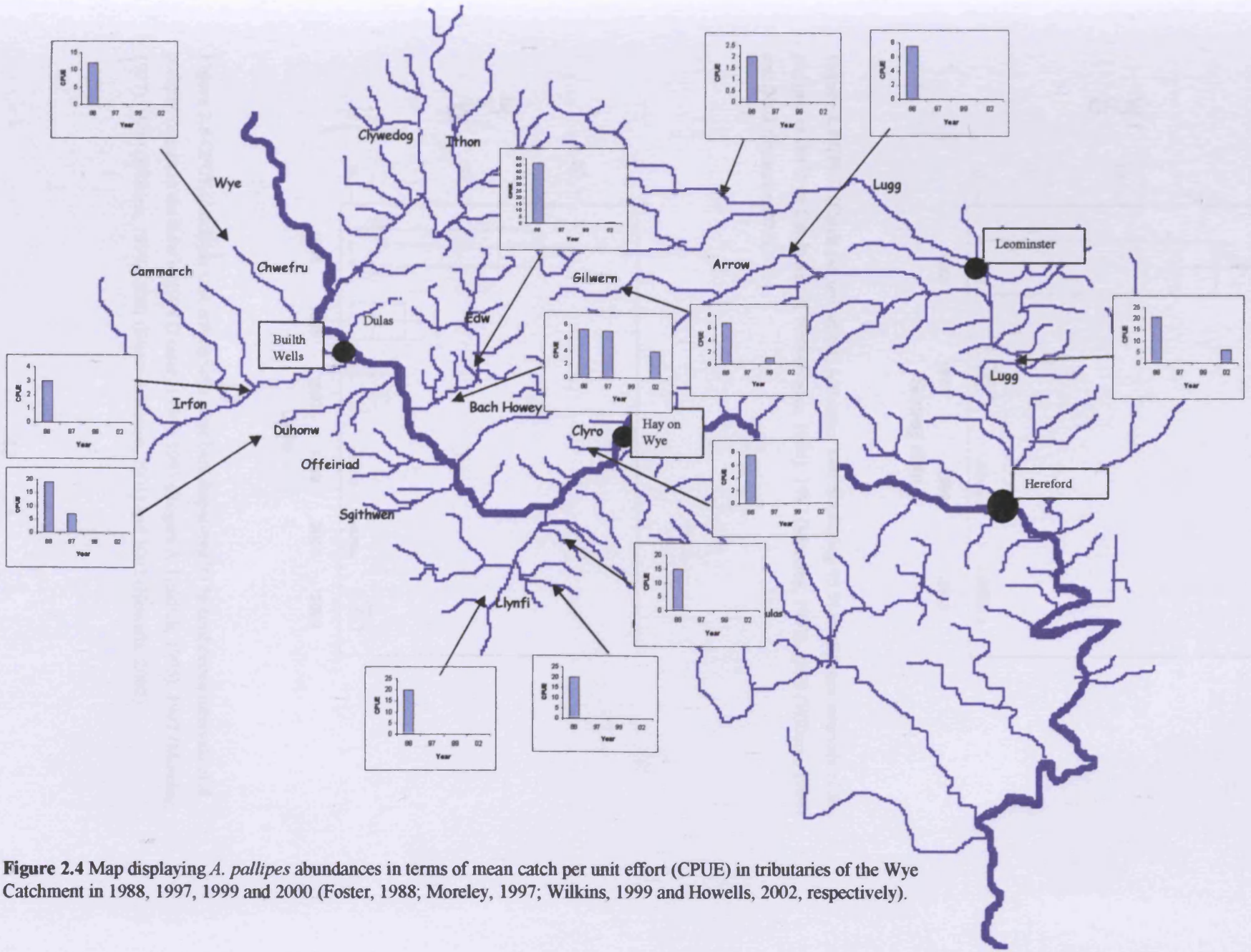


Figure 2.4 Map displaying *A. pallipes* abundances in terms of mean catch per unit effort (CPUE) in tributaries of the Wye Catchment in 1988, 1997, 1999 and 2000 (Foster, 1988; Moreley, 1997; Wilkins, 1999 and Howells, 2002, respectively).

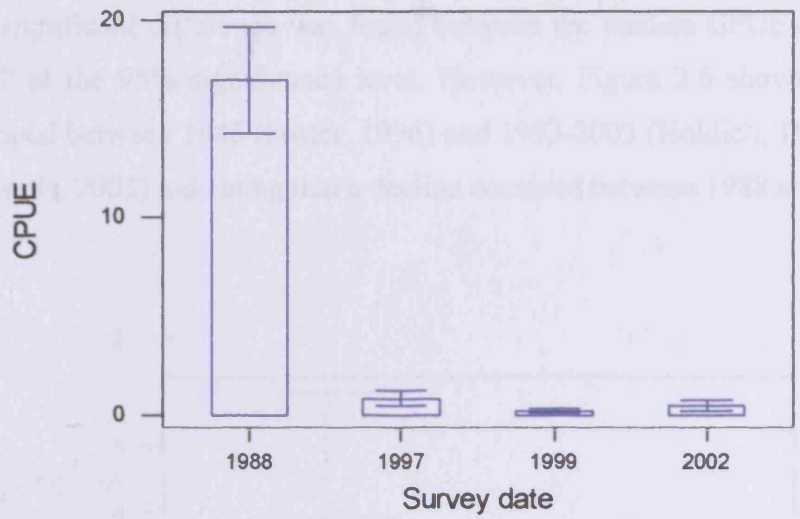


Figure 2.3 CPUE (Catch per unit effort) with error bars displaying 95 % confidence intervals of *A. pallipes* in the Wye Catchment in 1988 (Foster, 1996), 1997 (Moreley, 1997), 1999 (Wilkins, 1999) and 2002 (Howells, 2002).

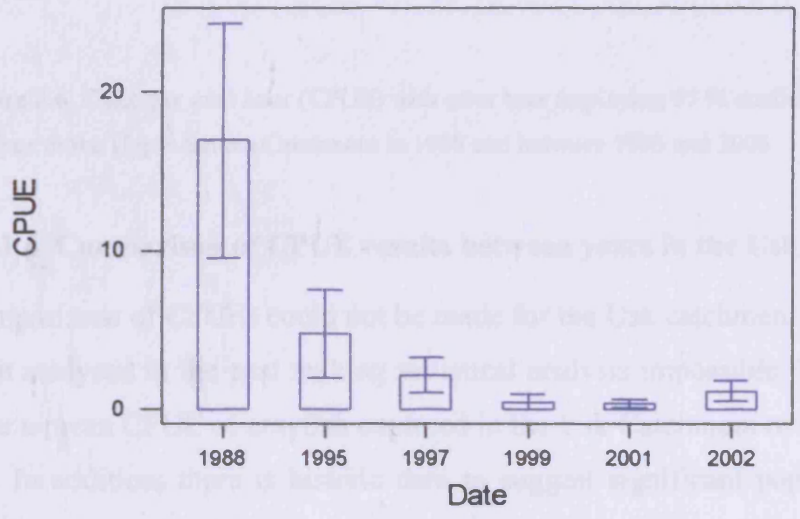


Figure 2.5 CPUE (catch per unit effort) with error bars displaying 95 % confidence intervals of *A. pallipes* caught in the Edw in 1988 (Foster, 1996), 1995 (Rogers & Holdich, 1995), 1997 (Moreley, 1997), 1999 (Wilkins, 1999), 2001 (Slater & House, 2001) and 2002 (Howells, 2002).

therefore used to compare CPUE values in the River Severn between the years 1988 and 1993 to 2000.

No significant difference was found between the median CPUEs of 1988 and 1993-2002 at the 95% significance level. However, Figure 2.6 shows that CPUE values dropped between 1988 (Foster, 1996) and 1993-2003 (Holdich, 1993; Moreley, 1997; Howells, 2003) indicating that a decline occurred between 1988 and 1993.

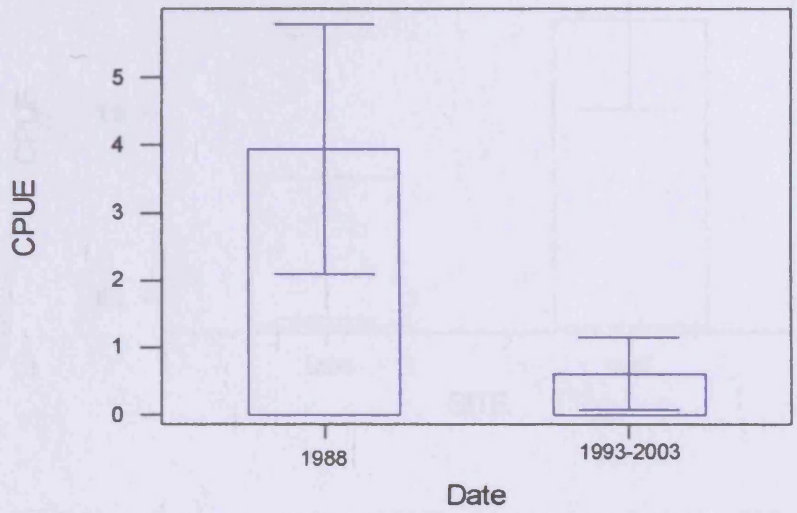


Figure 2.6. Catch per man hour (CPUE) with error bars displaying 95 % confidence intervals of *A. pallipes* in the Upper Severn Catchment in 1988 and between 1993 and 2003.

2.4.1.4 Comparison of CPUE results between years in the Usk Catchment

Comparisons of CPUEs could not be made for the Usk catchment as so few sites have been analysed in the past making statistical analysis impossible. However, this study gave a mean CPUE of crayfish captured in the Usk Catchment over 2002 and 2003 of 0.7. In addition, there is historic data to suggest significant populations in the past (Howells & Slater, 2004).

2.4.1.5 Comparison of CPUE results between catchments

A comparison of the Wye and Usk Catchment CPUEs could not be made with those of the Severn Catchment as too few values were available for the Severn catchment.

There was no significant difference between the median CPUEs of the Wye and the Usk Catchments. However, as illustrated in Figure 2.7, CPUEs were somewhat higher for the Wye than the Usk Catchment. Large amounts of variation, zero and missing values present in the data could account for the lack of significance.

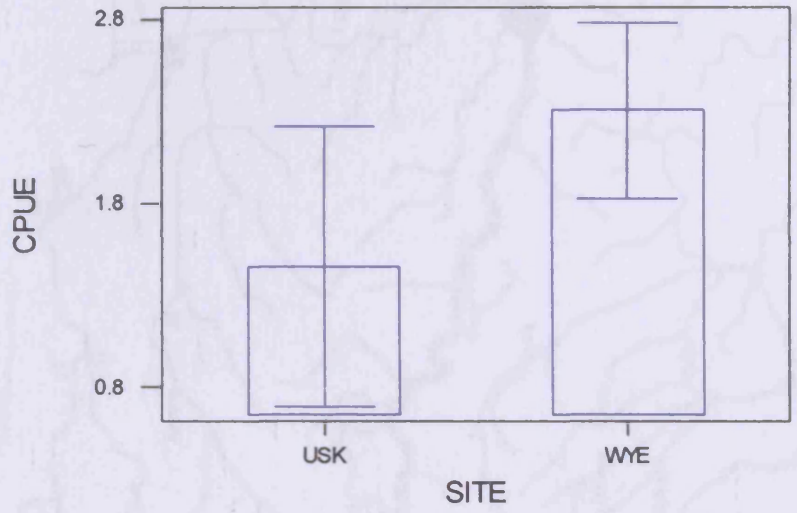


Figure 2.7. Mean Catch per man hour (CPUE) with error bars displaying 95 % confidence intervals of *A. pallipes* in the Usk and Wye Catchment over 2002 and 2003.

2.4.2 Past and present *A. pallipes* distribution

2.4.2.1 Wye Catchment

A comparison of presence/absence data from Lilley (1977) (Figure 2.8) and the present study in 2002 (Figure 2.9) showed that *A. pallipes* have either disappeared from sites where they were once present, or are have declined to the point where they are only present below detectable levels. For example, *A. pallipes* were abundant in the Llynfi Subcatchment and the Clyro Brook in 1977, but none were found here in 2002 although Rogers & Watson (2003) found low numbers of individuals in October 2003. They were also abundant in the Chwefru, Irfon, Duhonw, Hardwicke and Scallen Brooks in 1977 but were absent (or could not be found) at these sites in 2002.

2.4.2.2 Usk Catchment

Austropotamobius pallipes, once present in the Monmouthshire and Brecon Canal (Holdich, 1993) were no longer found here in a 2001 survey (Slater *et al.*, 2001) apart

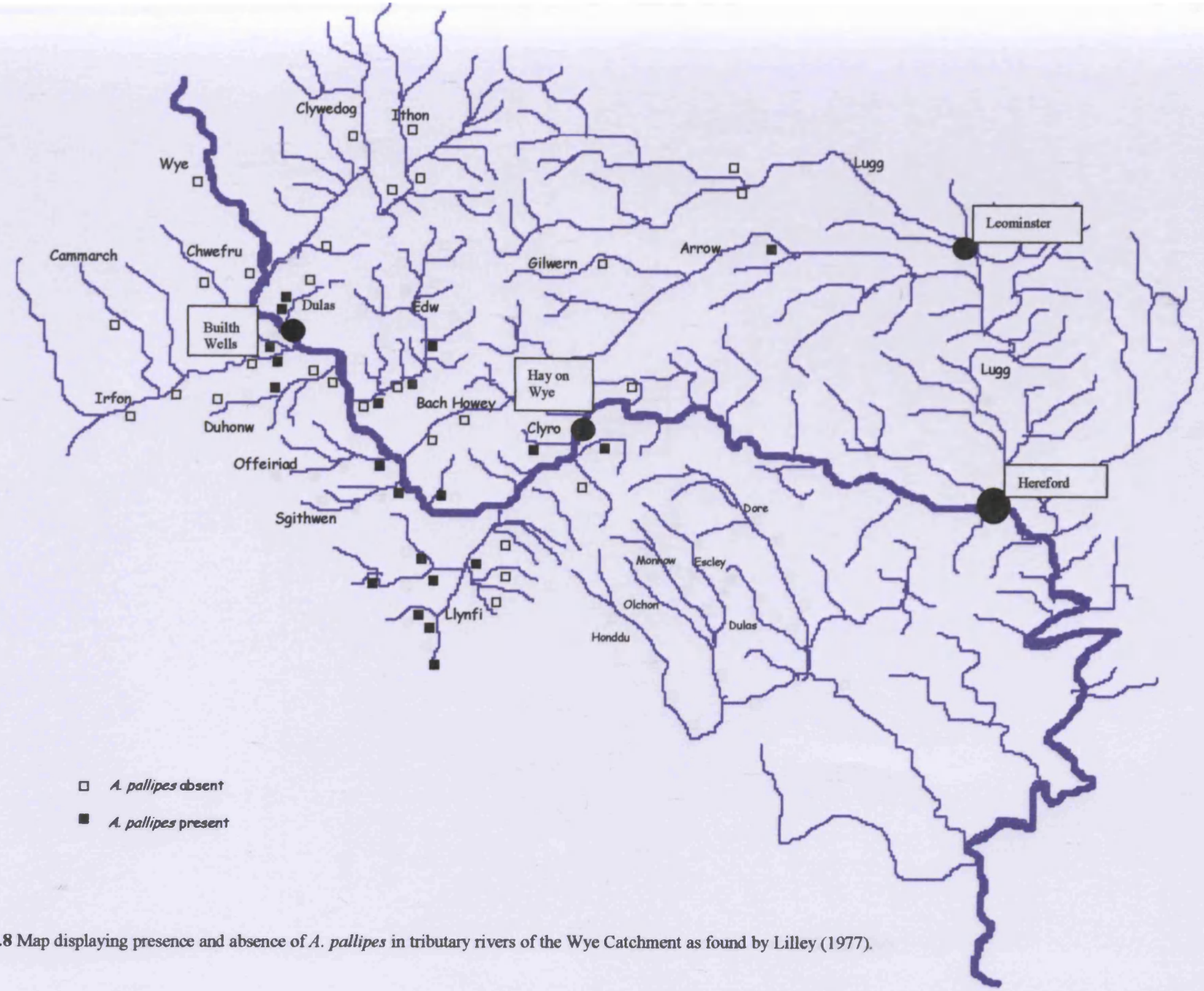


Figure 2.8 Map displaying presence and absence of *A. pallipes* in tributary rivers of the Wye Catchment as found by Lilley (1977).

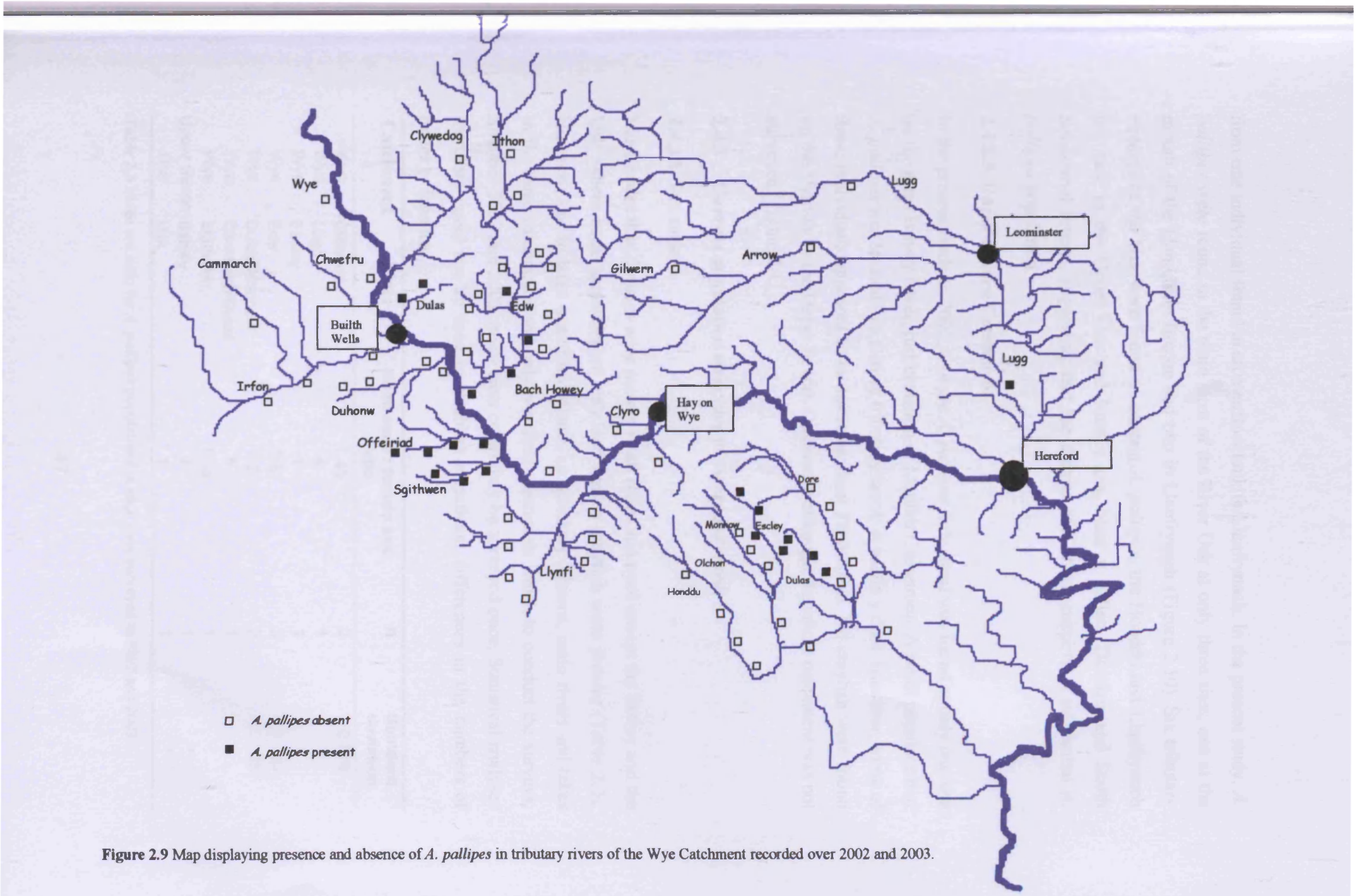


Figure 2.9 Map displaying presence and absence of *A. pallipes* in tributary rivers of the Wye Catchment recorded over 2002 and 2003.

from one individual found in an overflow tank in Llanfrynach. In the present study, *A. pallipes* were found in the main stem of the River Usk at only three sites, one at the mouth of the Honddu in Brecon and two in Llanfrynach (Figure 2.10). Six tributary streams of the Usk were found to contain *A. pallipes*, the Honddu and Llanfrynach tributary in the Upper Usk and Nant y pia, Nant y gollen, Dowlais and South Sebastapol brooks, suggesting that the district of Torfaen supports a substantial *A. pallipes* population.

2.4.2.3 Upper Severn Catchment

In the present study in 2002, a single *A. pallipes* individual was found at only one site on the main Banwy River, just upstream of Llanfair Caereinion. A small population of *A. pallipes* was located in a Banwy tributary brook at Melin y ddol. However, some of these individuals appeared to be suffering from *Thelohania*. No crayfish were found on the Vyrnwy in the Upper Severn Catchment although the whole catchment was not surveyed (Figure 2.11).

2.4.3 Current population structure of rivers surveyed

2.4.3.1 Sex ratio

More males than females were present in all rivers surveyed except the Escley and the Usk, where equal quantities of male and female crayfish were present (Table 2.3). However, due to large quantities of sites i.e. tributary streams, main rivers and lakes in the three catchments and only two field seasons in which to conduct the surveys, Summer 2002 and 2003, many sites could only be surveyed once. Statistical analyses therefore could not be used to establish significant differences in the numbers of males to females.

Catchment	River	Mean male : female sex ratio	n	Standard deviation
Wye	Offieriad	1.45	2	0.778
Wye	Lugg	4	1	*
Wye	Escley	1	1	*
Wye	Edw	2.8	3	2.800
Wye	Dulas Monnow	1.2	2	0.035
Wye	Cwmbach Dulas	5	1	*
Wye	Sgithwen	1.33	1	*
Upper Severn	Banwy	2	1	*
Usk	Usk	1	1	*

Table 2.3 Mean sex ratio for *A. pallipes* populations in each river surveyed in 2002 and 2003.

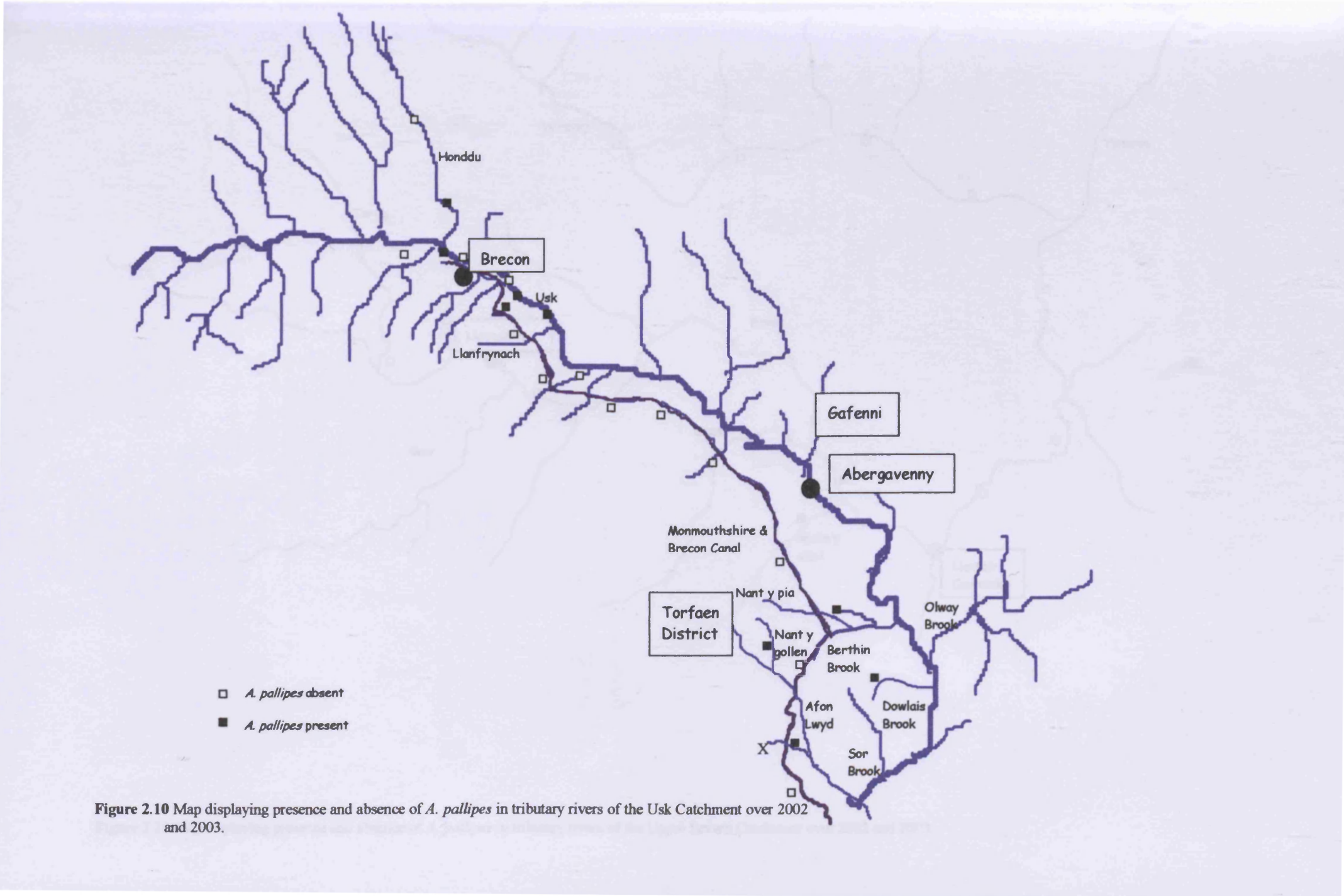


Figure 2.10 Map displaying presence and absence of *A. pallipes* in tributary rivers of the Usk Catchment over 2002 and 2003.

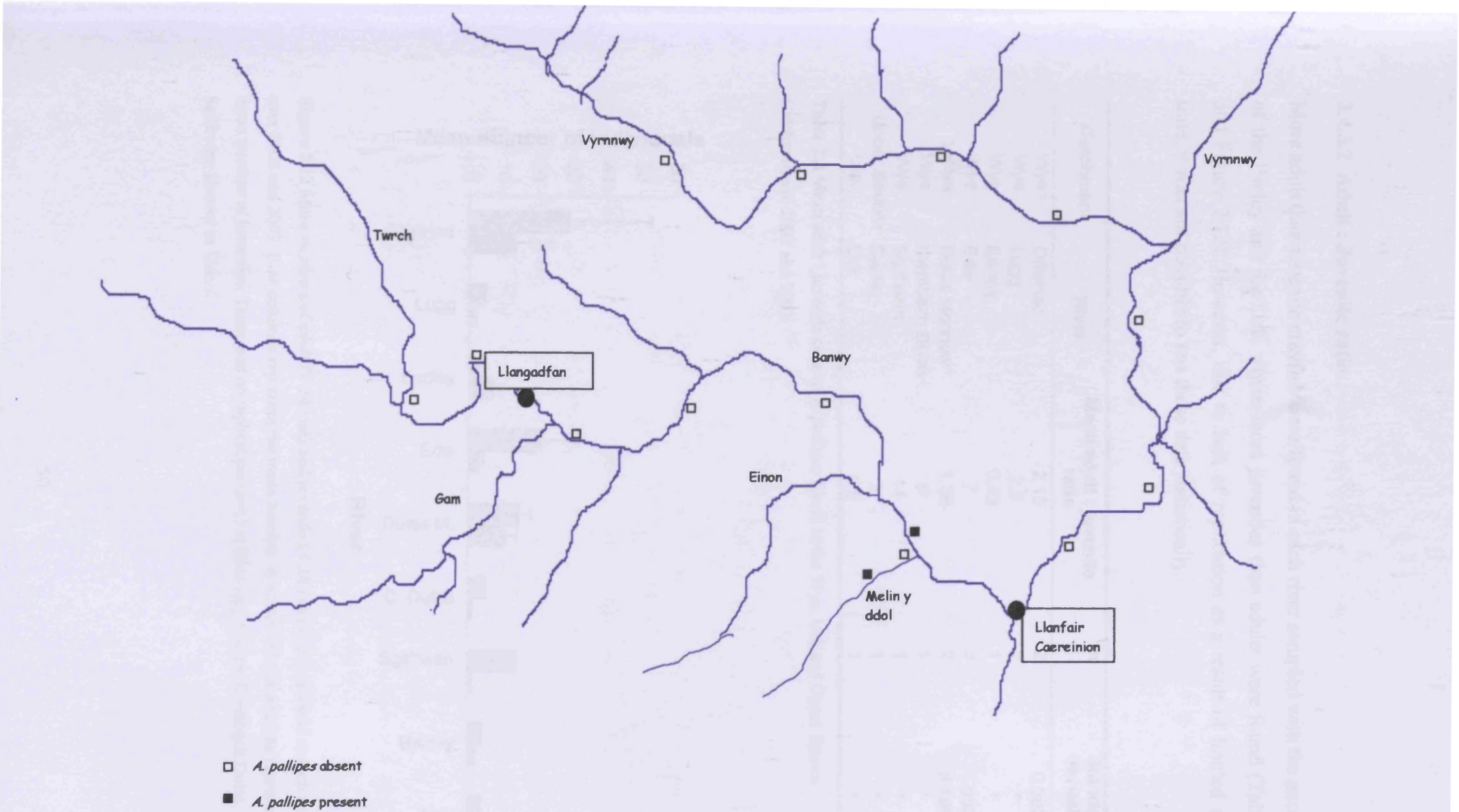


Figure 2.11 Map displaying presence and absence of *A. pallipes* in tributary rivers of the Upper Severn Catchment over 2002 and 2003.

2.4.3.2 Adult : Juvenile ratio

More adult than juvenile crayfish were found in each river sampled with the exception of the Escley and the Usk, where more juveniles than adults were found (Table 2.4 and Figure 2.12). However, due to lack of replication as a result of limited survey time, it was not possible to test these data statistically.

Catchment	River	Mean adult : juvenile ratio	n	Standard deviation
Wye	Offeiriad	2.15	2	0.382
Wye	Lugg	2.5	1	*
Wye	Escley	0.43	1	*
Wye	Edw	7	3	1.880
Wye	Dulas Monnow	1.38	2	0.198
Wye	Cwmbach Dulas	5	1	*
Wye	Sgithwen	14	1	*
Upper Severn	Banwy	2	1	*
Usk	Usk	0.5	1	*

Table 2.4 Mean adult : juvenile ratio of *A. pallipes* found in the Wye, Usk and Upper Severn Catchments in 2002 and 2003.

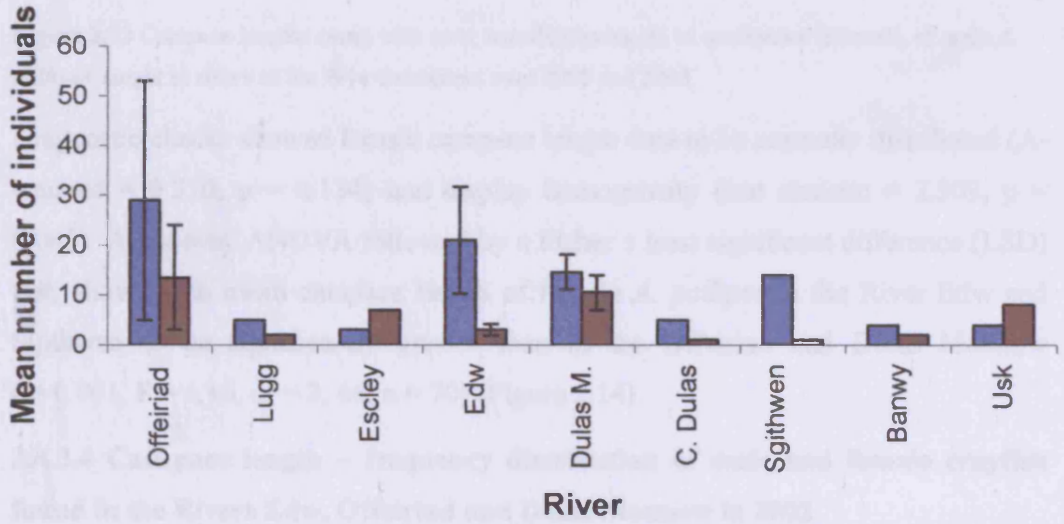


Figure 2.12 Mean numbers of adult (> 24 mm) and juvenile (< 24 mm) crayfish found at each river over 2002 and 2003. Blue columns represent the mean number of adults and red columns represent the mean number of juveniles. There was no replication (n=1) in the Lugg, Escley, Cwmbach Dulas, Sgithwen, Banwy or Usk.

2.4.3.3 Comparison of carapace length between rivers

Diagnostic checks showed male carapace length data were non parametric. A Kruskal-Wallis Test was therefore carried out and showed no significant difference between the median carapace lengths of male *A. pallipes* in each of five Wye tributary rivers in the Summer of 2002 (H = 1.91, df = 4, p = 0.752, n = 51) (Figure 2.13).

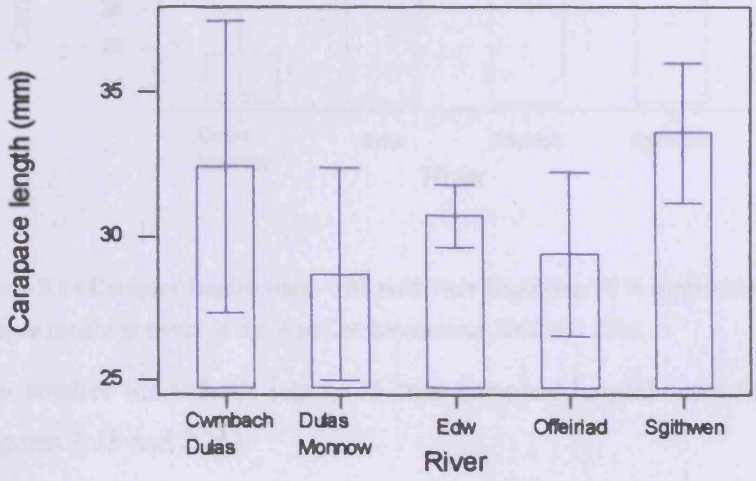


Figure 2.13 Carapace lengths (mm) with error bars displaying 95 % confidence intervals, of male *A. pallipes* caught in rivers of the Wye Catchment over 2002 and 2003.

Diagnostic checks showed female carapace length data to be normally distributed (A-squared = 0.570, p = 0.134) and display homogeneity (test statistic = 2.303, p = 0.085). A one-way ANOVA followed by a Fisher's least significant difference (LSD) test, showed the mean carapace length of female *A. pallipes* in the River Edw and Sgithwen to be significantly greater than in the Offeiriad and Dulas Monnow (p=0.001, F = 6.16, df = 3, 66, n = 70) (Figure 2.14).

2.4.3.4 Carapace length – frequency distribution of male and female crayfish found in the Rivers Edw, Offeiriad and Dulas Monnow in 2002

In 2002 on the River Edw, crayfish most frequently caught by stone turning were found to have carapace lengths of between 25 and 34.9 mm, while on the Offeiriad, the most commonly caught crayfish ranged between 20 and 24.9 mm carapace length.

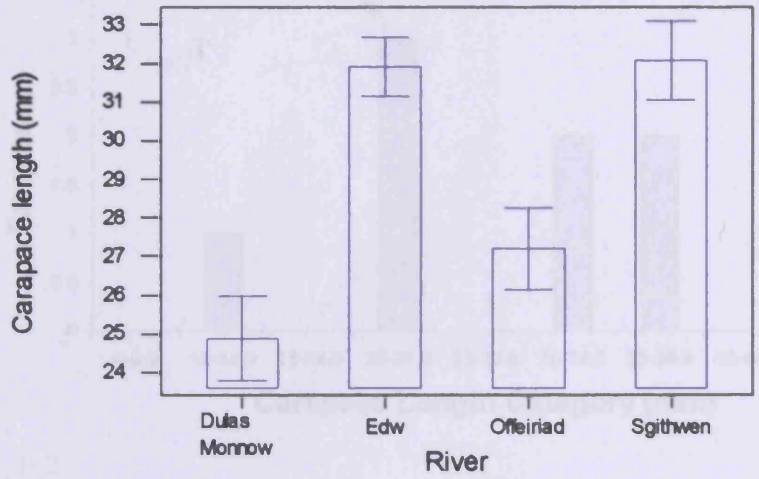


Figure 2.14 Carapace lengths (mm) with error bars displaying 95 % confidence intervals, of female *A. pallipes* caught in rivers of the Wye Catchment over 2002 and 2003.

Few smaller individuals (up to 15 mm carapace length) were found on these rivers (Figures 2.15 and 2.16).

Large crayfish with carapace lengths of between 40 and 44.9 mm were found in the Edw and Dulas Monnow Rivers (Figure 2.17). Only crayfish below this size were found in the Offeiriad in July, although in August, crayfish were found not only in the size category 40 to 44.9 mm, but also in the larger size category of 45 to 49.9 mm.

In the Dulas Monnow, crayfish of many sizes were frequently found in July 2002. Smaller individuals with carapace lengths of up to 10 mm were equally as common as larger individuals with carapace lengths of between 15 and 35 mm (Figure 2.17).

2.4.4 Using RHS to investigate crayfish presence/absence

Ten of 83 RHS variables originally measured at each site during field work in the present study were selected by the PCA and ordination analysis to be significantly positively associated with crayfish presence ($p = 0.011$; 0.002 and 0.022) (Table 2.5). The test on all spot check variables' coefficients was highly significant ($G = 31.067$, $df = 6$, $p = 0.001$) (Table 2.6). Tests on each individual coefficient, however, displayed different levels of significance, for example, spot check variables most positively associated with crayfish presence were bare bank face, uniform bank face

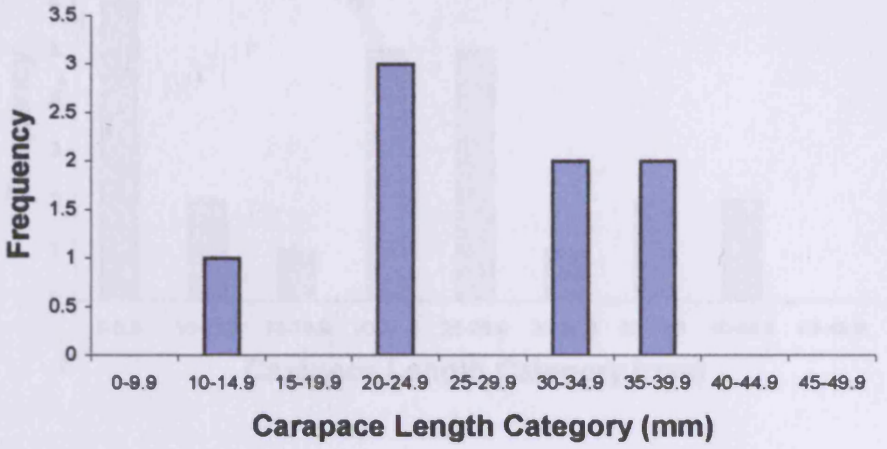


Figure 2.15 Carapace length – frequency distribution of *A. pallipes* in the Offeiriad River in July and August of 2002.

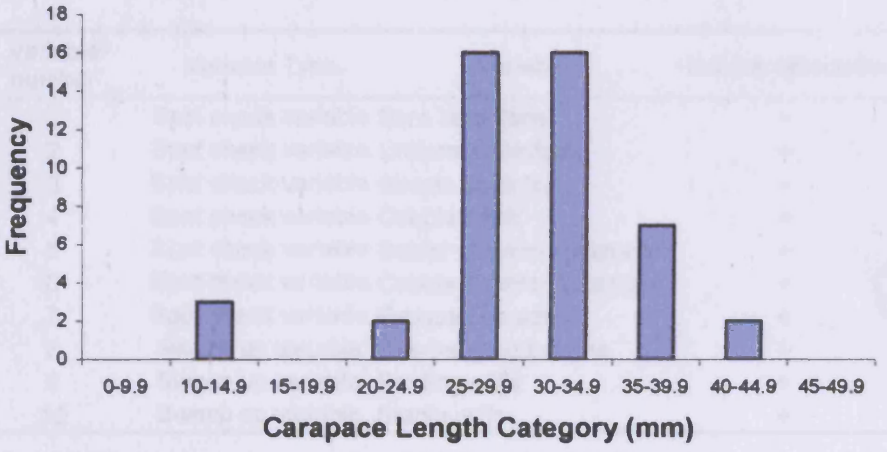


Figure 2.16 Carapace length – frequency distribution of *A. pallipes* in the River Edw in July, August and October in 2002.

displayed different levels of significance, for example, spot check variables most positively associated with crayfish presence were bare bank face, uniform bank face and simple bank face (Ψ = 2.28); cobble bank, boulder channel substrate, cobble channel substrate and exposed boulders (Ψ = 3.89).

The test on all sweep up variables' coefficients was not significant ($G = 8.725$, $df = 6$, $p = 0.190$), but tests on individual coefficients also displayed different levels of

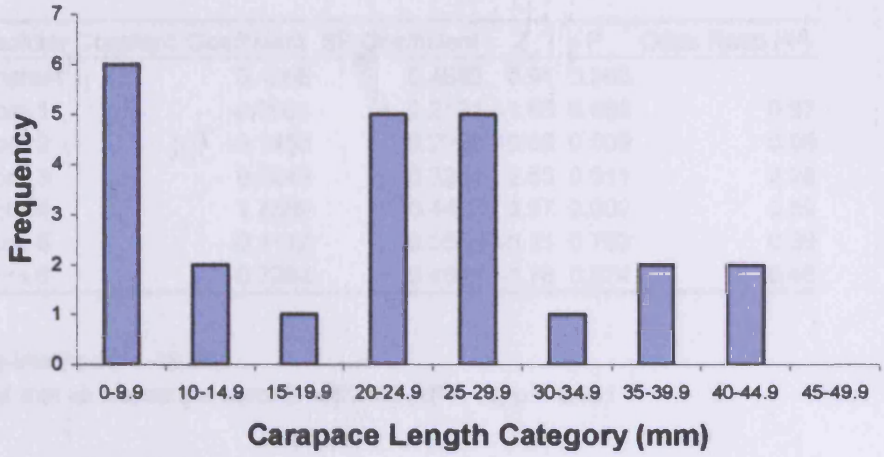


Figure 2.17. Carapace length – frequency distribution of *A. pallipes* in the Dulas Monnow in July and October in 2002.

significance (Table 2.7). For example, sweep up variables most positively associated with crayfish presence were overhanging boughs, eroding and stable cliffs ($\Psi = 1.52$).

Variable number	Variable Type	Variable	+ive/-ive association	p value
1	Spot check variable	Bare bank face	+	0.011
2	Spot check variable	Uniform bank face	+	0.011
3	Spot check variable	Simple bank face	+	0.011
4	Spot check variable	Cobble bank	+	0.002
5	Spot check variable	Bolder channel substrate	+	0.002
6	Spot check variable	Cobble channel substrate	+	0.002
7	Spot check variable	Exposed boulders	+	0.002
8	Sweep up variable	Overhanging boughs	+	0.022
9	Sweep up variable	Eroding cliffs	+	0.022
10	Sweep up variable	Stable cliffs	+	0.022

Table 2.5 RHS variables and p values from PCA Scores, and their association with crayfish presence.

The test on all "Other variables" coefficients were not significant ($G = 5.784$, $df = 6$, $p = 0.448$), neither were individual coefficients ($p > 0.05$). Goodness-of-fit tests with p values of between 0.519 and 0.703 indicated suitable fit of spot check data (Table 2.8).

In conclusion, cobble banks, boulder channel substrate, cobble channel substrate and exposed boulders, followed by bare, uniform or simple bank faces and finally overhanging boughs, eroding and stable cliffs were the variables most strongly associated with crayfish presence (Table 2.5).

Predictor Constant	Coefficient	SE Coefficient	Z	P	Odds Ratio (Ψ)
Constant	0.4256	0.4683	0.91	0.363	
Score 1	-0.3961	0.2131	-1.86	0.063	0.67
Score 2	-0.1458	0.2206	-0.66	0.509	0.86
Score 3	0.8245	0.3231	2.55	0.011	2.28
Score 4	1.3589	0.4427	3.07	0.002	3.89
Score 5	-0.1112	0.3536	-0.31	0.753	0.89
Score 6	-0.7264	0.4071	-1.78	0.074	0.48

Log-likelihood = -18.964
Test that all slopes are zero: G = 31.067, df = 10, p = 0.001

Goodness-of-fit Tests

Method	Chi-Square	DF	P
Pearson	33.863	39	0.703
Deviance	37.928	39	0.519
Hosmer-Lemeshow	6.800	8	0.558

Table 2.6 Logistic regression model using Scores from a PCA of selected Spot Check RHS variables.

Predictor Constant	Coefficient	SE Coefficient	Z	P	Odds Ratio (Ψ)
Constant	0.1791	0.3149	0.57	0.570	
Score 1	0.0490	0.1602	0.31	0.760	1.05
Score 2	-0.1413	0.1618	-0.87	0.383	0.87
Score 3	0.4220	0.1847	2.28	0.022	1.52
Score 4	0.0202	0.2102	0.10	0.923	1.02
Score 5	-0.1659	0.2174	-0.76	0.445	0.85
Score 6	0.2754	0.2383	1.16	0.248	1.32

Log-likelihood = -30.135
Test that all slopes are zero: G = 8.725, df = 6, p = 0.190

Goodness-of-fit Tests

Method	Chi-Square	DF	P
Pearson	51.240	43	0.182
Deviance	60.269	43	0.042
Hosmer-Lemeshow	6.533	8	0.588

Table 2.7 Logistic regression model using Scores from a PCA of selected Sweep up RHS variables.

Predictor Constant	Coefficient	SE Coefficient	Z	P	Odds Ratio (Ψ)
Constant	0.2501	0.3241	0.77	0.44	
Score 1	-0.2415	0.2257	-1.07	0.285	0.79
Score 2	-0.4147	0.2496	-1.66	0.097	0.66
Score 3	0.0451	0.2188	0.21	0.837	1.05
Score 4	-0.2413	0.2594	-0.93	0.352	0.79
Score 5	0.0568	0.3077	0.18	0.854	1.06
Score 6	0.0606	0.3067	0.2	0.843	1.06

Log-likelihood = -18.964
Test that all slopes are zero: G = 31.067, df = 10, p = 0.001

Goodness-of-fit Tests

Method	Chi-Square	DF	P
Pearson	33.863	39	0.703
Deviance	37.928	39	0.519
Hosmer-Lemeshow	6.800	8	0.558

Table 2.8 Logistic regression model using Scores from a PCA of selected Other RHS variables.

2.5 DISCUSSION

2.5.1 *A. pallipes* abundance and distribution in:

2.5.1.1 The Wye Catchment

The significant decrease in *A. pallipes* abundance and distribution that occurred between 1988 and 1997 in the Wye Catchment (Moreley, 1997) coincided with the increased usage of synthetic pyrethroid (SP) sheep dip – cypermethrin (Rutt, 2004) which is 100 times more toxic to aquatic life than organophosphates (Coley, 2000; Rutt, 2004; Howells, 2003).

A. pallipes numbers were dramatically depleted over the 1990s particularly in tributaries of the mid-Wye (Slater, 1998; Wilkins, 1999; Coley, 2000 and Howells, 2003). In 1990, a crayfish plague outbreak was thought to be present in the tributaries and main river in this region, which could account for these declines (Coley, 2000). Fifteen signal crayfish were recently caught in a fish farm at Painscastle although when they were introduced is not known. The Bach Howey, a mid-Wye tributary is located very close to this fishery. The presence of signal crayfish in the Bach Howey (probably originating in the fishery) has now been confirmed (Slater pers. comm.). This signal crayfish population poses yet another severe threat to the remaining *A. pallipes* populations of the Wye and may have already caused irreversible damage.

A crayfish plague outbreak was also confirmed on the River Arrow, a tributary of the Lugg (which joins the Wye in Hereford) and was strongly suspected in other Wye tributaries in Herefordshire in 1990 (Alderman, 1993).

The headwaters of the Bach Howey, and Arrow are within a few kilometres of those of the River Edw, a Wye tributary we have shown to originally support a substantial *A. pallipes* population (Foster, 1996). These distances on land could easily be covered by signal crayfish or other suggested vectors such as mink and otters (Foster & Slater, 1995). Plague and signal invasion are therefore very important threats to *A. pallipes* populations of the Edw and other Wye tributaries. It is, however, unlikely that plague was responsible for the dramatic declines in Edw *A. pallipes* numbers that were discovered in 2000 by Slater & House (2001) as no signal crayfish were found in the Edw or found there since this date. Also, the population has recovered somewhat since then (Howells, 2003), suggesting that other factors had caused that decline.

Sheep dip pollution and siltation are the more likely causes and are discussed in detail in Chapter 3 – "Causes of *A. pallipes* decline in Wales and the Marches."

Coley (2000) also warned of the potential threat of signal and plague invasion of the Lower Wye tributaries such as the Monnow sub-catchment from the Rivers Arrow and Lugg and the nearby headwaters of the River Gavenni (of the Usk Catchment) which are known to contain signal crayfish.

A. pallipes were found in the Monnow subcatchment in the Escley and Dulas (Monnow) Rivers, but none were found in the Honddu (Monnow), Olchon, Dore or Monnow Rivers (Slater & Howells, 2002a; 2002b; 2003c). By using electrofishing, the Monnow Habitat Improvement Project in 2003 found one dead individual on the River Monnow (SO 278361), one on Trenant Brook (SO 0348376) and six, four, eight and six individuals on the Slough Brook (at SO 350359, 349358, 345357, 343358 respectively). Trenant and Slough Brooks are both tributaries of the River Dore in the Monnow Sub-catchment. *A. pallipes* have therefore remained present in a number of tributaries in this sub-catchment although abundances are very low at some sites. In July 2004, a cypermethrin pollution incident occurred on a tributary of the Escley Brook of the Monnow Subcatchment at Cefn-Ceist Farm (SO 3143 3546). This was found to be one of the "best" sites for *A. pallipes* during our surveys and probably provided a good source of individuals to the Escley River itself. Unfortunately, a large number of dead crayfish were found along this tributary between the old school house and Firs Farm (Environment Agency, 2005). This incident demonstrated that a single pollution incident has the potential to wipe out the entire *A. pallipes* population of a stream.

A. pallipes were once present in the main stem of the River Wye, for example at the Dulas Brook Confluence at SO 021 513 (Lilley, 1977) and at Hay on Wye (Slater, 1998). However, during the present survey, no crayfish were found in the main stem. The reason for this could either be due to crayfish plague, pollution or predation.

With *A. pallipes* apparently absent from the main river stems of the Wye, only isolated tributary populations remain and even these appear to be under threat from pollution, signal and plague invasion and siltation as already discussed. Such factors, particularly habitat destruction and siltation are even threatening crayfish on the other

side of the world, such as the endangered giant freshwater crayfish, *Astacopsis gouldi* in Tasmania (T. Walsh pers. comm., Howells, 2002, Richardson *et al.*, 1999).

2.5.1.2 The Upper Severn Catchment

Median CPUEs from 1988 (Foster, 1990) and 1993-2002 (Holdich, 1993; Howells, 2003) in the Upper Severn Catchment were similar probably as a result of the lack of *A. pallipes* presence in many sites surveyed in both time periods. The mean CPUE of this catchment has however, actually decreased quite dramatically over time. *A. pallipes* were found in the main River Banwy in 1988 in two locations, Is y Coed and at the Cefnllwyd tributary confluence (Foster, 1990). In 97/98, a crayfish survey conducted in this region found a number of individuals in the main Banwy River at SJ 089075 (Slater *et al.*, 1998). Numbers dropped dramatically by 2002 as only one individual was found on the entire main Banwy River at this site in the present study. This decline was probably caused by pollution events. If plague had been the cause, then it would very likely have moved up into the nearby tributary at Melin y Ddol and destroyed any crayfish present. *Austropotamobius pallipes* were however, still present in this tributary in 2003 (Howells, 2003) and no signal crayfish were found here. Pollution and siltation are the most probable causes of decline at these sites (Foster, 1990). The small tributary stream at Melin y Ddol runs through some residents' gardens. In this instance the residents were aware of the crayfish presence and importance. The stream banks consisted of realigned stones, between which there were numerous gaps and a rocky substrate making ideal crayfish refuges. Downstream, however, near the confluence with the Banwy, no crayfish were found, possibly because improved grassland with livestock was present on one side of this section. Sheep dip pollution or excess siltation may therefore have wiped out crayfish from this section of stream.

A number of farmed signal crayfish populations were present in the Upper Severn Catchment, positioned close to or in contact with tributary streams (Holdich, 1993). *A. pallipes* were found in the headwaters of the Severn, the Vyrnwy and Camlad Rivers of the Upper Severn. Unfortunately, in 1990, the Camlad population was infected by crayfish plague (Alderman, 1993). It is therefore possible that the signals and plague could have spread to many other tributaries and the main river stem of the Upper Seven Catchment which could account for some of the decline observed in recent years.

2.5.1.3 The Usk Catchment

The Usk Catchment once supported substantial *A. pallipes* populations (Rogers & Holdich, 1995). However, between 1990 and 2000, *A. pallipes* disappeared from or were not found in 73 % of sites were they were originally present. Where they were present, their numbers were badly depleted (Coley, 2000).

In 2000, a substantial signal crayfish population was found on the River Gavenni, an Usk tributary at Abergavenny. This problem was considered a severe threat to *A. pallipes* of the Usk Catchment (Coley, 2000). Three years later, the present study found that *A. pallipes* were still remaining in tributary rivers downstream of this site in the Nant-y-pia, Nant-y-gollen and Dowlais Brooks and upstream of this site in the Honddu and at Llanfrynach. *A. pallipes* were also still found to exist in the main River Usk at the Mouth of the Honddu (SO 043286), Llanfrynach (SO 079273) and Llangattock (SO 215175), suggesting that crayfish plague had not yet spread into these upper reaches of the catchment. However, only one individual was found in the Honddu tributary in 2002 as opposed to the six caught in 1993 (Holdich, 1993) and 9 caught in the Honddu by Rogers & Holdich (1995) showing that numbers have probably declined since the early 90s. This decline is probably due to pollution and habitat loss through siltation as no signal crayfish were found here, so plague is less likely, though a potential threat to any crayfish remaining. The decline on this tributary and others is more likely to be a result of pesticide pollution and siltation, particularly as a substantial amount of adjacent land use was improved grassland and livestock farming. Poaching of banks, organic pollution and excess siltation were all observed at this site (Howells, 2003).

Anecdotal evidence suggests that *A. pallipes* were previously present in the Monmouthshire and Brecon Canal. Individuals were often spotted clinging to the canal sides in locks at Llanfrynach, downstream of Brecon. After the water level had dropped children found "crusty monsters" climbing the wall of the canal. However, a trapping survey carried out by Slater *et al.*, (2001) found none were present in the canal. This disappearance may also have been caused by pollution or possibly crayfish plague, as a signal population has been found near Abergavenny only a few 100 m from the canal (Coley, 2000).

One *A. pallipes* individual was, however, caught in a trap in a canal overflow tank in Llanfrynach. The water from this tank is regularly topped up from the canal, each time the lock gate is opened. When this happens, water overflows heavily into a small tributary stream of the main Usk River. However, despite this sporadic increase in flow rate in this tributary, it was found to contain a number of *A. pallipes* individuals. It is therefore most probable that the single individual captured in the tank came from this stream and not from the canal. Also, just at the mouth of this tributary, a number of juvenile crayfish were found actually in the main Usk River, together with some individuals downstream in the Usk, proving that they have not yet been entirely wiped out from all main stem Welsh Rivers.

A. pallipes were also found further upstream in the Usk, at the mouth of the Honddu, and in the Honddu itself, though they are thought to be absent from elsewhere in the upper reaches of this catchment.

A survey of the lower Usk Catchment showed *A. pallipes* to be present in three streams within a built up area (Figure 2.10). The presence of native crayfish in such an area may be surprising, although it is unlikely that such regions are polluted by cypermethrin as the land is not intensively farmed but rather industrialised and urban/suburban. Very few records of *A. pallipes* populations in this region appear in the literature. Perhaps they have been forgotten or simply not previously recorded.

The closest, most recent surveys previously carried out in this area were by Holdich (1993). He surveyed and found no crayfish in the Afon Lwyd, of which Nant y gollen is a tributary, and none in Sor Brook.

The Nant y gollen Brook runs through Pontypool Park and so is surrounded by parkland. It is shaded by overhanging trees and vegetation and has a boulder/cobble substrate with numerous bankside crevices – ideal crayfish habitat. The stream, not more than 2 m in width has at the centre of the park been partially diverted into two large consecutive ponds which appear to be highly anaerobic as they contain large quantities of silt, and water appears to be almost stagnant – hardly ideal for *A. pallipes*. No crayfish were found in these ponds, confirming their sub-optimal quality. Unfortunately, as a result of this partial diversion, the stream below this point dries up entirely during the warmer months of the year. Survival of *A. pallipes* downstream of this diversion is consequently highly unlikely, despite its excellent habitat. It is

recommended that less water should be diverted into these ponds as it would then allow *A. pallipes* to repopulate downstream and possibly move into other tributaries, thus making this population less isolated and less susceptible to stochastic events. Previous studies have confirmed that other crayfish can migrate in a downstream direction (Bohl, 1999; Schutze *et al.*, 1999). Alternatively, this link could have adverse affects on *A. pallipes* if signal crayfish moved into this region as they, and plague would be free to move upstream which is currently isolated by such threats. This, however, is a "no go" area, which could help to prevent signal crayfish from reaching this tributary. This disadvantage is, outweighed by the advantage of forming this link.

A. pallipes were also found in three other Usk tributaries, the Nant y pia, and Dowlais Brooks and an unnamed one, labelled X on Figure 2.10, another tributary of the Afon Lwyd, located in South Sebastapol.

The presence of *A. pallipes* in Tributary X was found during a contract survey carried out in 2003 (Slater & Howells, 2003a), at the edge of a site, South Sebastopol, which was to be developed. This stream passed through some residents' gardens. Unfortunately, one resident "cleared up" his section of stream and all of the boulders (potential *A. pallipes* refuges) were used to build his patio! Vegetation had also been cleared and only one boulder remained, under which a single crayfish was found. Improved public education in built up areas where *A. pallipes* are present is required in order to prevent such occurrences in the future.

Hypothesis 1 that abundances and distribution of *A. pallipes* in Wales and the Marches have declined over the years, 1988, 1997, 1999 and 2002, should therefore be accepted.

2.5.2 Current population structure of rivers surveyed

2.5.2.1 Sex ratio

In the Wye Catchment, eight out of ten rivers surveyed between June and October of 2002 had a male biased sex ratio of *A. pallipes*. Hypothesis 2 that the sex ratios of *A. pallipes* populations are male biased should therefore be accepted although levels of significance were not tested due to lack of repetition. Brooding females have been observed to moult once per year, just after releasing their young (Lilley, 1977). It is therefore likely that they will remain well hidden in burrows or bankside crevices

throughout brooding to protect their young, and during and after moulting to protect themselves from predators. Brooding females are therefore more difficult to detect than males using stone turning or kick sampling which could explain the apparent male biased sex ratio. Previous studies however, have produced female biased crayfish sex ratios at similar times of the year, for example, July to September in the Wye Catchment (Lilley, 1977), June to July in the River Darent (Thomas & Ingle, 1971) and in September in a Swedish lake (Svardson, 1949). Also, females carrying eggs and young were found during the present survey, proving that they could be caught while stone turning. These findings therefore suggest that there are currently fewer female than male crayfish present in Welsh rivers, a possible consequence of the decline of *A. pallipes* in this region, although establishing the sex ratio of a population accurately is difficult (Lilley, 1977). It is possible that breeding females may be older than their male mates. If this is the case then females take longer to mature than males, resulting in environmental pressures causing fewer females than males, thus indicating a population under pressure. The highest populated rivers in this study, the Offeiriad, Escley, Dulas Monnow and Sgithwen all had the most balanced male: female ratios.

2.5.2.2 Adult : juvenile ratio

A general adult (>24 mm carapace length as specified by Smith *et al.*, 1996) biased adult: juvenile ratio of *A. pallipes* was found in rivers surveyed. This finding could be due to sampling error where larger individuals tend to be caught by manual searching as smaller individuals are more difficult to see and catch (Lilley, 1977; Smith *et al.*, 1996) resulting in possible inaccurate estimations of population structure. Kick sampling should perhaps have been used to supplement stone turning in order to overcome this problem as it is 3.2 times more efficient at catching crayfish than stone turning (Smith *et al.*, 1996). However, kick sampling may damage some individuals (Howells, pers. obs.) and comparability of technique between sites was required. Stone turning was therefore used.

Another possible explanation for the lack of juveniles was that fewer were actually present due to reduced survival through habitat pollution and destruction. Young crayfish are particularly susceptible to pollution (Eversole & Seller, 1996).

One *A. pallipes* female with a carapace length of 32 mm produces approximately 60 eggs at a time (Foster, 1996). Low juvenile numbers could therefore be a symptom of the *A. pallipes* decline. CPUE and consequently has generally decreased since the 1980s (Howells, 2003) possibly due to lack of juvenile recruitment. Fewer juveniles may therefore be surviving into adulthood which should be of grave concern to conservationists. The low female to male numbers may also have a bearing on this. Hypothesis 3 that the adult: juvenile ratio of *A. pallipes* populations is adult biased could therefore be accepted although levels of significance were not calculated due to a lack of data replication.

2.5.2.3 Comparison of carapace lengths between rivers

Hypothesis 4, that mean carapace lengths of surveyed crayfish differed between rivers, was rejected for male crayfish, indicating that similar size ranges of male crayfish were present throughout these rivers.

However, Hypothesis 4 was accepted for female crayfish. The mean carapace length of female crayfish was greater in the Edw and Sgithwen than in the Offeiriad and Dulas Monnow, indicating that a greater proportion of smaller, younger individuals were present in the Offeiriad and Dulas Monnow than in the Edw and the Sgithwen. This could suggest that numbers in the Edw and Sgithwen are about to decrease again due to a lower recruitment of younger individuals to maintain the populations. In the Offeiriad and the Dulas Monnow however, the populations are more likely to remain stable or increase in coming years due to greater recruitment of younger individuals to reach maturity. Alternatively, a healthy population contains larger females as more eggs per female are produced. A lack of larger females, for example in the Offeiriad and Dulas Monnow, suggests there may have been environmental pressures on them, perhaps in earlier years, from which the younger population has recovered.

2.5.2.4 Carapace length-frequency distribution

Results suggested that stone turning allowed smaller crayfish i.e. <10mm in carapace length to be found, sometimes equally as frequently as other size categories e.g. 20-24.9 mm carapace length on the Dulas Monnow River, though not as frequently as larger crayfish in general i.e. those over 15 mm in carapace length. This contradicted results obtained by Smith *et al.* (1996). Although they found larger numbers of

smaller crayfish when they used kick sampling, they did not find any individuals with a carapace length less than 10 mm.

Results also showed that in other rivers, such as the Edw, no individuals with a carapace length smaller than 10 mm were found. This difference in success of stone turning between rivers may have been due to the differences in conditions of the rivers. For example, the Edw is wider with more broken water than the Offeiriad, making it more difficult to observe smaller crayfish through the water in the Edw than the Offeiriad. Although the differences between rivers were likely to be due to sampling errors, the possibility that fewer younger crayfish were present in some sites indicating a future decline in such rivers cannot be ignored. A similar situation could arise in other rivers for the same reasons (Holdich *et al.*, 2005).

In order to confirm these results, rivers should be resurveyed with the use of other techniques, e.g. kick sampling or electrofishing.

Individuals of all size categories between 1 and 50 mm in carapace length were only found in the Offeiriad. This indicated that the Offeiriad was able to support crayfish with the largest range of sizes (equivalent to ages) of all rivers sampled, suggesting that it was a healthy river whose habitat was of a high enough quality to support a healthy and sustainable *A. pallipes* population. In view of the small remaining numbers of such populations it is clear that this population should be protected.

The largest crayfish captured in the Offeiriad during the present study had a carapace length of 46.2 mm. The largest crayfish captured in the Wye Catchment in the 1980s came from the River Edw and had a carapace length of 52.1 mm (Slater & Foster, 1987). However, in the present study, the largest crayfish found in the River Edw was 44.2 mm. Throughout this entire study not a single crayfish caught had a carapace length of over 50 mm. This shows that perhaps the maximum crayfish size in the Wye Catchment has fallen slightly with the decline i.e. there are few very old crayfish. This suggests that very large, old crayfish may be susceptible to increased pollution, but also that perhaps crayfish had suffered from a pollution incident a number of years ago which resulted in the loss of what would now be classed as the older generation.

2.5.3 River habitat variables and crayfish presence

Ten River Habitat Survey (RHS) variables were positively associated with *A. pallipes* presence in this study. Hypothesis 5, that particular RHS variables were significantly

associated with crayfish presence, should therefore be accepted. The four variables with the greatest positive impact on *A. pallipes* presence were substrate features i.e. cobble bank, boulder and cobble substrates and exposed boulders. The presence of exposed boulders and cobble banks were found to be an important positive influence on crayfish abundance by Naura & Robinson (1998). They also suggested that where there is cobble channel substrate, crayfish are more likely to be found as boulders are usually present. Foster (1993) confirmed the importance of boulders as crayfish refuges.

Bare, uniform and simple bank faces were also found to have a positive impact on crayfish presence. The importance of all three reflects the importance of bank vegetation structural diversity to crayfish presence. Channel margins and consequently banks are important to crayfish presence, particularly as a nursery area to younger crayfish (Smith *et al.*, 1996). Where banks are uniform and simple, vegetation covers them suggesting that erosion, the products of which are thought to be detrimental to crayfish (Hogger, 1988; Summers, 1996; Slater & Howells, 2003c), is not occurring. The presence of these features are therefore not detrimental to crayfish, thus encouraging their presence. However, most rivers surveyed for the present study contained some bare bank faces usually as a result of livestock poaching. The rivers of the study are largely surrounded by upland livestock farms. It was therefore expected that even where crayfish were present, some livestock poaching occurred which led to some sections of bank being bare. This also explained why eroding cliffs appeared as a feature positively associated with crayfish presence. Although eroding cliffs may contain certain undercuts favoured by shade seeking *A. pallipes* (Smith *et al.*, 1996).

The presence of overhanging boughs had a positive influence on crayfish presence in the present study and in the study carried out by Naura & Robinson (1998). Overhanging boughs shade the river. Shade was positively associated with crayfish presence (Naura & Robinson, 1998) as it keeps the temperature of the water lower in warm summer months, a condition that appears to be favoured by crayfish in many parts of the world, e.g. the Tasmanian giant freshwater crayfish, *Astacopsis gouldi* (Richardson *et al.*, 1999) and Paranephrops of New Zealand (Parkyn *et al.*, 1997).

Overhanging boughs also signify the likelihood of the presence of exposed tree roots. A model produced by Naura & Robinson (1996) suggested that exposed tree roots

have a negative impact on crayfish presence. However, contrary to this, exposed tree roots were thought to have a positive impact on crayfish presence as they provide protection acting as a nursery area for young crayfish according to Smith *et al* (1996). Also, when a Mill race at Mortimer's Cross on the River Lugg, a Wye tributary was drained, large quantities of *A. pallipes* were found in crevices between the many exposed older tree roots by Foster (1996) and myself in 2003, proving their importance to adult as well as young crayfish.

Exposed tree roots also trap leaf litter, a primary *A. pallipes* food source (Smith *et al.*, 1996; Reynolds, 1979), while the trees drop leaves and canopy dwelling invertebrates into the water, a direct food source of crayfish and other species on which crayfish in turn feed (Naura & Robinson, 1998).

Stable cliffs are noted as having a positive impact on crayfish presence in the present study as are steep banks in the study by Smith *et al.* (1996). Stable cliffs indicate that erosion is not occurring. Increased livestock densities are likely to cause increased erosion, particularly of riverbanks (Howells, pers. obs.), which results in increased siltation in the vicinity (Coley, 2000), and organic excrement pollution as observed in the present study, which are both known to be detrimental to crayfish by eliminating and polluting the habitat (Hogger, 1988; Summers, 1996; Naura & Robinson, 1998; Slater & Howells, 2003b). The presence of stable cliffs therefore indicates that crayfish habitat is not in direct contact with sources of pollution.

2.5.4 General *A. pallipes* status in Wales and the Marches

A mean of 72 % of *A. pallipes* populations were lost from the Usk and the Wye Catchments during the period of 1990 and 2000 (Coley, 2000). Further declines have occurred since then with the only increase in *A. pallipes* abundances detected on the River Edw of the Wye Catchment. These declines have probably occurred as a result of sheep dip pollution but also partly due to signal crayfish, plague and predation as was suggested by Coley (2000). Even if pollution is curbed by such measures as the Ground Water Regulations introduced by the Environment Agency, further declines are inevitable if the spread of signal crayfish and plague continue. However, if "no go" areas work, perhaps the declines will cease or even reverse

2.6 CONCLUSIONS

Significant declines in *A. pallipes* abundances and distribution occurred between 1990 and 2000 in Wales and the Marches. Declines in distribution continued into the 21st century. By 2002 *A. pallipes* were only found in 15 % of 26 sites first surveyed in the Wye Catchment by Foster (1996) who originally found them to be present in 92 % of these sites. *A. pallipes* can no longer be found on the main Wye River. *A. pallipes* have declined in the Upper Severn Catchment and on the Usk catchment since 1988, although a few individuals could still be found in three sites on the main Usk River stem and in a few tributaries but in low numbers. Forgotten *A. pallipes* populations were discovered further downstream in tributary streams at Pontypool and Cwmbran.

Numbers of adult female and juvenile *A. pallipes* appear to have been reduced in Welsh Rivers. Further declines are consequently likely in many rivers of this region as a result although future investigations will prove this.

A. pallipes reintroduction programmes in Wales and the Marches should be directed to structurally diverse watercourses containing the following features: cobble plus boulder channel substrates and exposed boulders which provide important crayfish refuges, cobble banks, bare, uniform and simply vegetated banks, which are mostly stable thus reducing siltation and filling in of refuges, but some sections of which should be soft enough for crayfish burrowing and contain crevices or overhangs as refuges, overhanging boughs which provide shade, leaf litter and canopy invertebrates (that fall from the trees) on which *A. pallipes* can feed.

This chapter shows that *A. pallipes* have declined dramatically over the last decade. We must therefore next consider the causes of that decline. The general continuing declines are thought to be due to environmental pressures coupled with crayfish plague and competition with *P. leniusculus* and will be discussed in the next chapter entitled " Causes of *A. pallipes* decline in Wales and the Marches."

CHAPTER 3

Causes of *A. pallipes* decline in Welsh rivers.

SUMMARY

The decline of the once thriving Welsh *Austropotamobius pallipes* populations in the Upper Wye tributaries is thought to be attributable mainly to excess siltation, sheep dip pollution and other forms of habitat loss, rather than crayfish plague or competition from *P. leniusculus* as in many parts of England and Europe.

High densities of livestock, particularly sheep and increasing use of synthetic pyrethroid dip to control external parasites, combined with naturally heavy rainfall in a hilly landscape have inevitably led to sheep dip pollution and excess siltation in rivers from livestock poached banks.

Experiment 1 showed that *A. pallipes* preferred to live in regions such as dense aquatic vegetation or loose stones where refuges were present than where refuges were absent. These results strongly indicate that where excess siltation has coated the riverbed, *A. pallipes* is more likely to be absent.

In Experiment 2, siltation was measured in the Edw, a tributary of the River Wye and its effects on *A. pallipes* discussed. The use of basket and flowerpot traps enabled separate measurement of intrabed and surface siltation. Despite some riverside fencing erected by the Wye Foundation, siltation was still occurring confirming continuing bank erosion. Siltation was positively correlated with rainfall, so was found to be particularly high during winter months. During periods of low rainfall, more siltation and organic pollution was found directly downstream than upstream of controlled livestock entry points. Banks and riverbed at these access points should therefore be reinforced to prevent erosion.

Synthetic pyrethroid sheep dip, especially cypermethrin, has been found in many rivers across Wales. Its increased use and deadly effects on aquatic life have been reported, making it another of the main suspected causes of large-scale *A. pallipes* declines in Welsh Rivers.

Some rivers may be recovering as a result of new legislation, better management practices and farm visits. *A. pallipes* recovery is slow but occurring in some areas such as the River Edw.

3.1 AIMS

This investigation aims to establish:

- which type of substrate *A. pallipes* prefers in order to find out if excess siltation influences their presence,
- whether basket and flowerpot traps can be used to measure siltation in the River Edw, a Wye tributary,
- whether livestock access points contributed to siltation on the River Edw,
- whether siltation varied across the width of the River Edw,
- if sheep dip pollution is a likely cause of *A. pallipes* decline.

3.2 INTRODUCTION

The current nationwide decline in *A. pallipes*, the native white-clawed crayfish, is commonly attributed to the well-publicised encroachment of the North American signal crayfish, *Pacifastacus leniusculus* which can out compete *A. pallipes* and carry crayfish plague, caused by the fungus *Aphanomyces astaci* (Holdich & Rogers, 2000; Hiley, 2003; Sibley, 2003).

The non acidic upland rivers of rural mid-Wales once supported thriving populations of *Austropotamobius pallipes*, the native white-clawed crayfish (Foster, 1990; Holdich, 1993), until a dramatic decline began to occur around 1990 (Slater & House, 2001). Crayfish plague, a common cause of *A. pallipes* decline in England, had not been a major concern in Wales during this time. Extensive surveys carried out by the Environment Agency showed that although plague was probably present in a few locations within the Welsh region of the Wye Catchment before 1990, cases appeared to be isolated and did not spread (Coley, 2000; Holdich, 2003). Other factors must therefore be responsible for the decline that occurred here.

For the past 40 years, rural Wales has been subject to increasingly intensive farming practices mainly as a result of the Common Agricultural Policy, first established in 1962, where farmers were essentially paid for the quantity of their produce (NFU, 2005), which encouraged them to use more pesticides and other chemicals in order to increase their income. High densities of cattle and particularly sheep, the chemicals

used for exoparasite control combined with naturally heavy rainfall in this region (Environment Agency, 1999) inevitably led to sheep dip pollution and excess sediment deposition from livestock poached banks in many rivers. In recent years, flood alleviation schemes and other developments have been carried out on or near to rivers, also resulting in a reduction of habitat quality. Combinations of these factors are thought to be responsible for the continuing decline of *A. pallipes* (Slater & House, 2001) in Wales and the borderlands.

There are a number of reasons why sheep dip pollution is thought to be one of the main causes of *A. pallipes* decline despite specific legislation designed to protect watercourses. Farmers are reported to have allowed sheep to "drip dry" in fields near to watercourses. Sometimes, they were allowed to wander into rivers directly after dipping although these practices are against the law. Rain and river water can wash the excess chemicals from the sheep or dip soaked ground into the rivers (Environment Agency, 1999). To guard against this, the Groundwater Regulations provide a minimum distance between sheep dipping, drip-dry zones and any watercourses, but sometimes pollution incidents still occur.

Until the appearance of synthetic pyrethroids (SPs) as an alternative sheep dip chemical, organophosphates (OPs) such as Diazinon were the main pesticides used. Diazinon works by "inhibiting transfer of the acetyl cholinesterase enzyme" resulting in the neurotransmitter acetylcholine no longer being broken down which causes "uncontrolled firing of nerve impulses" (Croxford, 2005). However, Diazinon was found to produce harmful side effects in humans using them. Health concerns increased and more farmers began to use synthetic pyrethroids (SPs) such as cypermethrin. Unfortunately, although SPs appeared to be less harmful to humans, they are approximately 100 times more toxic to aquatic organisms than OPs (Coley, 2000) and works by inhibiting neural transmission in invertebrates (Fort Dodge, 2005). One teaspoon of cypermethrin will kill all crayfish in a 0.5ha pond (D. Jerry, *pers. comm.*) and is said to be able to destroy aquatic invertebrates hundreds of metres downstream of a pollution point. LC50 values are between 0.03 and 5 µg/l for many crustaceans, 2 µg/l for the water flea, *Daphnia*, (on which young crayfish feed) and as low as 0.009 µg/l for the freshwater shrimp, *Gammarus pulex*. Although many molluscs, amphibians and fish are much less sensitive to cypermethrin, some fish, particularly salmonids are sensitive and have an LC50 of 0.5 µg/l, while the olfactory

system of male North Atlantic Salmon was significantly affected resulting in decreased sperm production by 0.004 µg/l of cypermethrin and even by 0.4 µg/l of Diazinon. Cypermethrin works by "altering ion permeability of nerve membranes causing trains of nerve impulses that immobilise sensitive organisms and inhibiting ATPase enzymes, used to regulate oxygen exchange" (Croxford, 2005). *A. pallipes* individuals are very sensitive to such pollution while their slow growth rate makes them particularly vulnerable during their first few years of life (Foster, 1996).

With their ability to break down relatively quickly, SPs are very difficult to detect (Pesticide Action Network UK, 2000) unless affected organisms are tested immediately or riverside mosses are tested for chemical adsorption (Rutt, 2004). Biological assessment is another useful method of detecting water pollution as long lasting effects on biota can be measured indicating the degree of severity of the incident and recovery can be monitored. This method involves a three minute kick/sweep sample, using a standard pond net, in all major habitats present e.g. bottom substrata and vegetation. Each taxon, identified to family level is awarded a score of 1 to 10, 10 meaning it is highly susceptible to pollution. Scores of each family present in a sample are summed to give the Biological Monitoring Working Party (BMWP) Score (Mason, 1996). The higher the BMWP Score, the less polluted that area as the more pollutant sensitive taxa are present.

In the late 1990s, the Veterinary Medicines Directorate (VMD) introduced new usage procedures which reduced exposure to operators of sheep dipping (Croxford, 2005) making SPs the main sheep dip used. Although Groundwater Regulations to control safe use and disposal of sheep dip were introduced by the EA in 1999, both diffuse and point source pollution of waterways from sheep dip continued (Environment Agency, 2000), further exacerbated by frequent heavy rainfall and steep valleys that channel groundwater into tributaries and larger rivers. Sheep dip pollution is therefore thought to be a possible cause of *A. pallipes* decline in Wales and is discussed in detail in this chapter.

Anecdotal evidence suggests that felling and planting forestry operations and bank erosion through poaching by farm livestock has resulted in excess sediment deposition and organic pollution along many rural rivers. Excess sediment deposition within rivers is thought to be detrimental to crayfish as spaces between and underneath stones, vital *A. pallipes* refuges (Rogers & Holdich, 1995; Slater & House,

2001), become filled with this sediment. This could have helped accelerate the disappearances of *A. pallipes* and other river organisms such as salmonids from mid Wales rivers (Foster, 1990).

Figure 3.1. Photographs depicting cattle poached banks of a river in upland Wales.

Figure 3.2. Photograph depicting a silt coated river bed in upland Wales.

Suspected *A. pallipes* preference for unsilted refuges was consequently assessed in this chapter by testing Hypothesis 1 that *A. pallipes* prefer to reside on a substrate where refuges are present than where they are absent.

In an attempt to rectify the environmental problems faced by salmonid fish in tributary streams, the Wye Habitat Improvement Project (WHIP) was established (Wye Foundation, 1999). Fencing, thought to benefit fish, crayfish and other invertebrates by excluding stock (Environment Agency, 1997), and selective coppicing were carried out along stretches of several Wye tributaries including the Afon Edw. At specific points along each section of the river, livestock were allowed controlled access into the river to drink. It was, however, feared that even with such measures in place, large quantities of silt still entered the river at these access points as a result of livestock poaching and that deposition increased in a downstream

direction and filled in crayfish refuges, particularly as a result of heavy rainfall (typically experienced by upland Welsh rivers) washing silt into the river and downstream.

Following the methodologies of Naden *et al* (2002), specialised basket and flowerpot traps (designed to measure intrabed plus surface silt and surface silt respectively) were installed up and downstream of livestock access points, along the length of the River Edw. Silt captured within the traps was measured over a number of months to assess these suspicions by testing Hypothesis 2 that silt deposition increases in a downstream direction, Hypothesis 3 that silt deposition in a river increases with the quantity of rainfall and Hypothesis 4 that more silt will be deposited downstream than upstream of livestock access points.

The present study also attempted to find out whether the two trap types used (basket and flowerpot) measured both aspects of siltation i.e. intrabed and surface. Any organic pollution observed at livestock access points was also recorded.

There have been a number of publications dealing with the measurement of sediment deposition, often related to the siltation of salmonid spawning redds (Bunte & Abt, 2001; CEFAS, 2001; Fripp & Diplas, 1993; Lambert & Walling, 1988; Lisle & Eads, 1991; Wren *et al*, 2000).

The high loss rate of sediment traps in storm events and the known risk of large errors in suspended solids determination (Edwards & Glysson, 2000) make it difficult to achieve accuracy in such a study. The highly labour intensive installation and extraction of traps and extensive bedrock was such that only one trap of each type could be installed in as similar a position as possible at each sampling station. Although rates and directions of flow will vary across the width of a river, aligning traps at precisely the same distances from the banks at each sampling station was impossible due to bedrock and the difficulty in digging in some regions of the river bed. Variation in silt deposition across the width of the river was therefore also assessed within this chapter in order to minimise experimental error.

Hypotheses of this chapter are:

Hypothesis 1

Austropotamobius pallipes prefer to reside on a substrate where refuges are present than where refuges are absent.

Hypothesis 2

Silt deposition increases in a downstream direction.

Hypothesis 3

Silt deposition in a river increases with the quantity of rainfall.

Hypothesis 4

More silt will be deposited downstream than upstream of livestock entry points.

3.3 METHODS

3.3.1 Experiment 1 – Substrate peference

To determine if crayfish exhibited substrate preferences, the following apparatus was constructed. Two rectangular, glass fibre tanks measuring 185 x 46 x 40 cm filled to a depth of 25 cm were split into five sections with four airstones placed equidistant along one side, ensuring that oxygenation was evenly spread over the length of each tank. Each section consisted of two seed trays (37 cm x 23 cm x 7 cm), the first one (A) positioned furthest from the airstones and the second one (B) positioned closest to the airstones. Both trays were filled to the brim with one particular substrate type. Five different substrate types were present in each tank: aquatic vegetation, loose pebbles (fist size), gravel (16-64 mm in diameter) pebbles embedded in silt/sand and silt/sand (<2 mm in diameter). Substrate types were classified according to River Habitat Survey guidelines. In each of the tanks, the substrate types were arranged in random orders, selected using the statistical package, MINTAB 13 (Figures 3.3 and 3.4).

Prior to the experiment, crayfish were kept for 3 days in a nearby large holding tank in order for them to acclimatise to the water temperature. Two crayfish were placed carefully but randomly into each tank. Locations of the crayfish were recorded every hour between 7 am and 6 pm over two weeks.

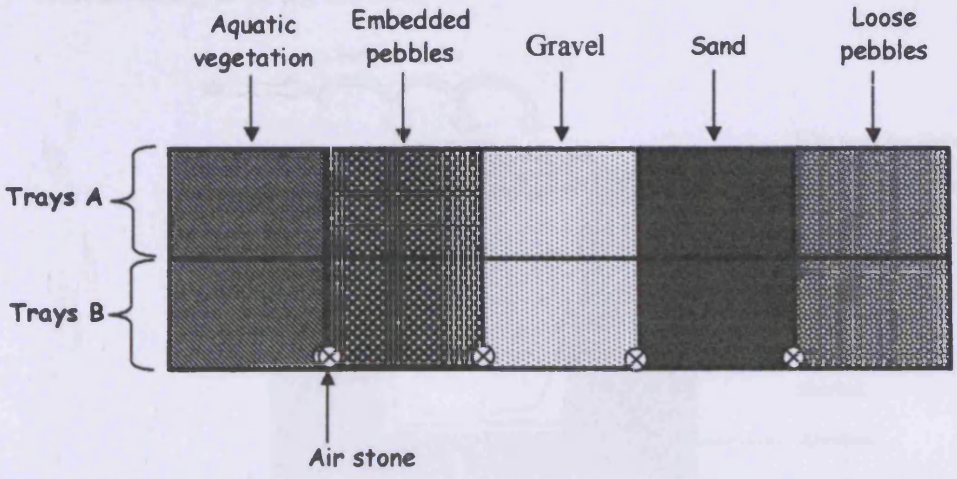


Figure 3.3 Layout of Tank 1 (view from above).

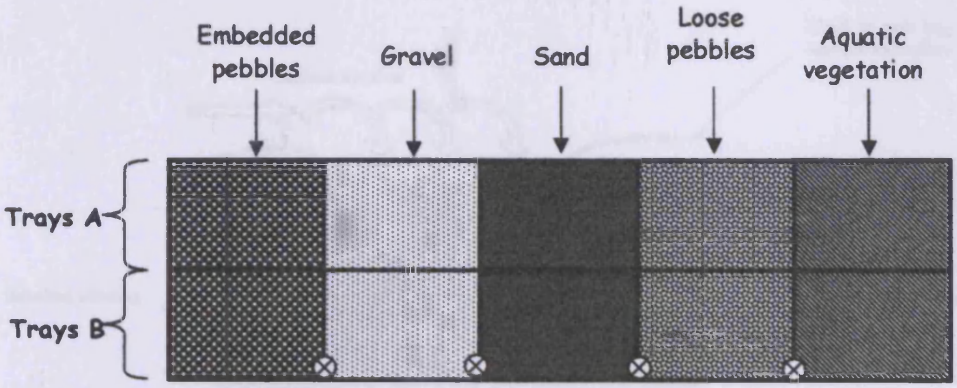


Figure 3.4 Layout of Tank 2 (view from above).

3.3.2 Experiment 2 - Sediment deposition

This experiment was carried out on the Afon Edw, a tributary of the River Wye in rural mid-Wales. Access permission was sought at each site visited.

Ten sampling stations were established in July 2002 along the length of the River Edw, from Franksbridge, the most upstream site, to its confluence with the Wye. Each station consisted of two silt traps, one a flowerpot trap designed to measure surface silt (Figure 3.5) and one a basket trap designed to measure surface plus intrabed silt (Figure 3.6). Silt traps were installed flush with the riverbed surface using pick axes. The basket traps were modified from the model designed by Naden *et al.*, (2002), by adding a slightly larger outer mesh cylinder, which maintained the hole in the riverbed during sample collection in order to allow easy replacement of the inner cylinder without having to re-dig the hole.

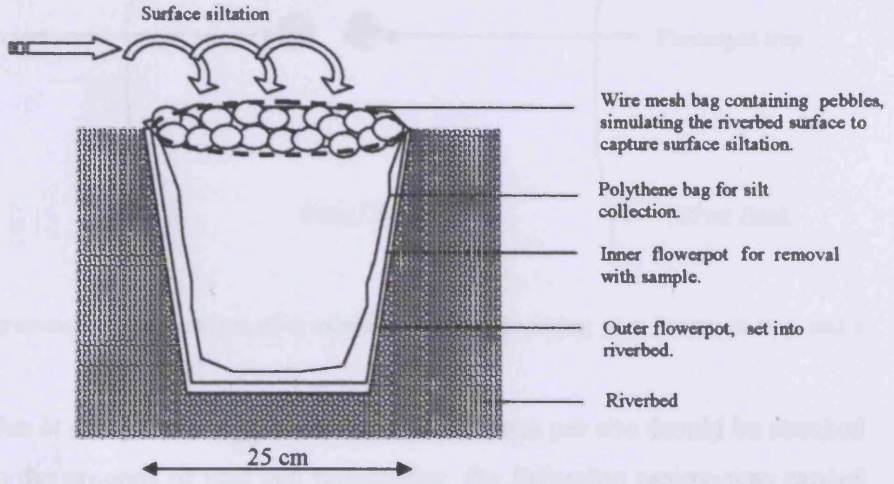


Figure 3.5 Diagrammatic representation of a flowerpot trap which captures riverbed surface silt, positioned within the riverbed.

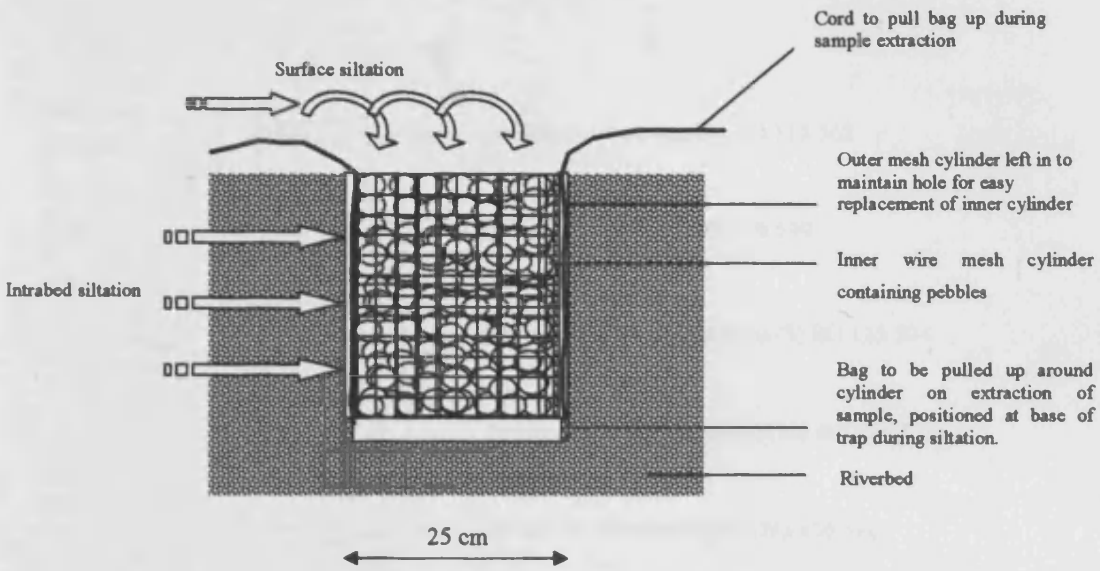


Figure 3.6 Diagrammatic representation of a basket trap which captures intra-bed plus surface silt, positioned within the riverbed.

In three regions of the river, sampling stations were located upstream and downstream of different river features, a tributary stream at Upper Cregrina, a sheep crossing at Llanbadarn y garreg and a cattle drinking area at Aberedw. The four remaining sampling stations were located at Franksbridge, Llanedw, Lower Cregrina and the Confluence with the Wye (Figures 3.7 and 3.8).

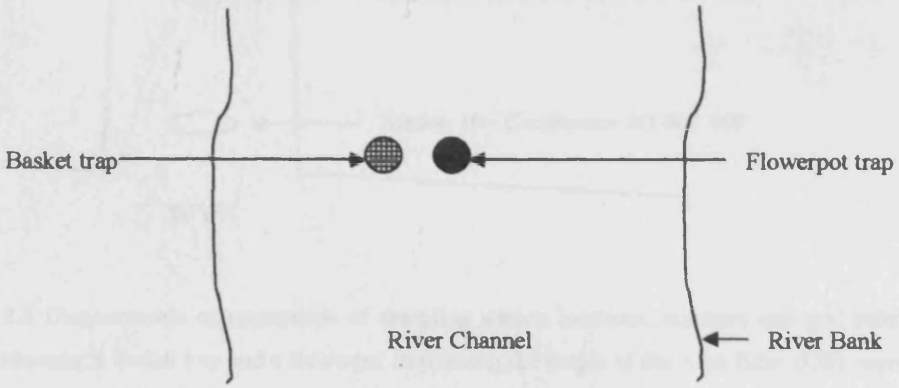


Figure 3.7 Diagrammatic representation of a sampling station consisting of a flowerpot trap and a basket trap.

Although Naden *et al.*, (2002) suggests 6-8 sample baskets per site should be checked fortnightly, on the grounds of cost and practicality, the following regime was carried out on the River Edw.

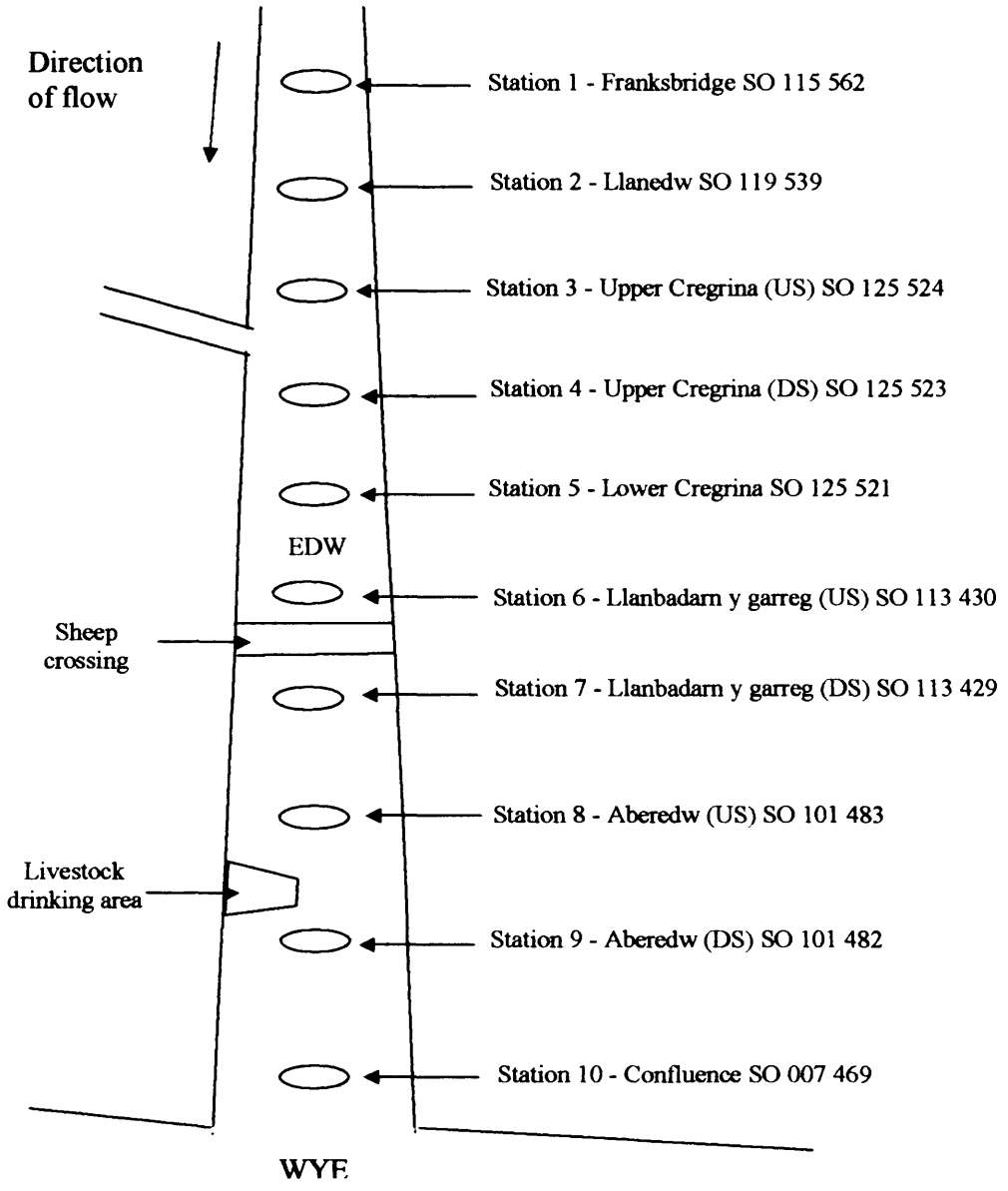


Figure 3.8 Diagrammatic representation of sampling station locations, numbers and grid references (each containing a basket trap and a flowerpot trap) along the length of the Afon Edw. (US) represents sites upstream and (DS) sites downstream of river features. (Not to scale).

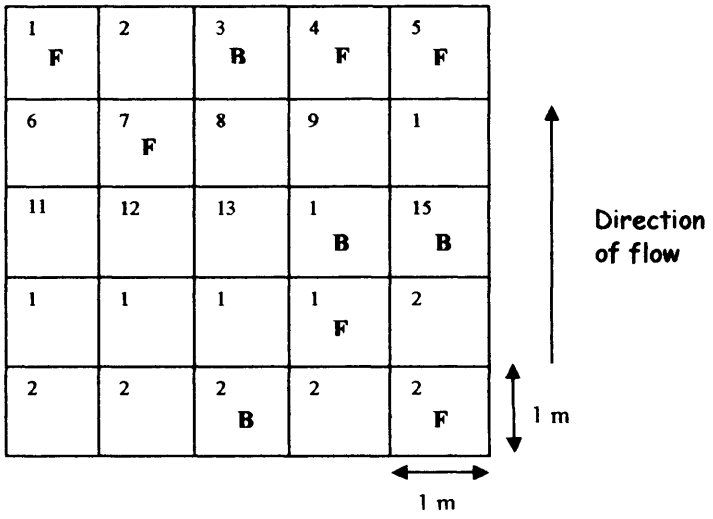
Samples were collected and transects observed two weeks after installation, in September 2002 and every two months thereafter.

Silt samples collected were sealed and labelled in the field. Once in the laboratory, bags were transferred to labelled, clean foil trays. Samples were oven dried for 24 hours at 105 °C. Weights of samples plus foil trays were measured and recorded. The

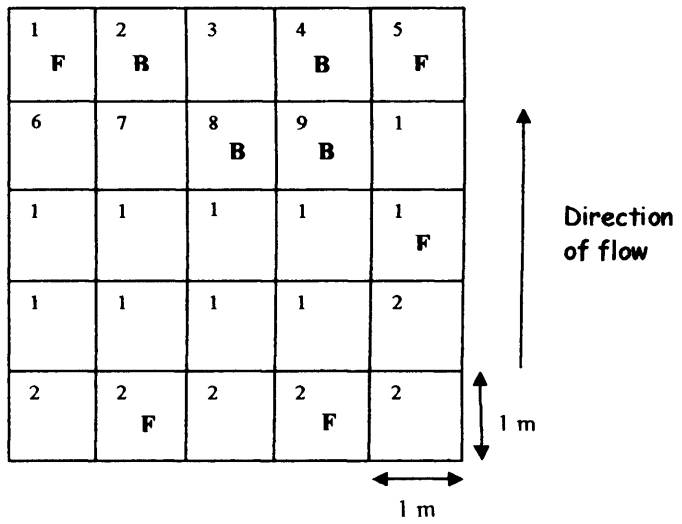
weight of a clean foil tray was measured and subtracted. Any trends in the data were observed.

In order to find out if the quantity of silt captured in a trap type varied depending upon the position of the trap across the width of the river, an extra study was carried out where all traps were removed and re-installed at two of the ten original sites in June 2004, Llanbadarn y garreg and Aberedw. Five flowerpot and five basket traps were installed in random positions at each site. A grid of twenty-five 1 m^2 squares was marked out across the river. Ten random squares were calculated for each site using MINITAB 13. A trap was installed in each of these randomly selected squares (Figure 3.9). However, if the bed at a square was too hard to dig, the trap was installed in the next square to the left. If this failed then it was moved to the next square above, then right, then below the original square.

Silt collection and measurement was carried out in August and December of 2004.



a)



b)

Figure 3.9 Diagrammatic representations of trap layouts at a) Aberedw and b) Llanbadarn y garreg. F represents a flowerpot trap and B represents a basket trap.

3.4 RESULTS

3.4.1 Experiment 1 - Substrate preference

A Kruskal-Wallis test carried out on the non-parametric data showed crayfish to be present significantly more often in sections containing loose pebbles and aquatic vegetation, both in Tank 1 ($p = 0.001$, $df = 4$) (Figure 3.10) and in Tank 2 ($p = 0.001$, $df = 4$) (Figure 3.11). Crayfish therefore appeared to prefer to live in regions containing aquatic vegetation or loose pebbles than gravel, embedded pebbles, silt/sand.

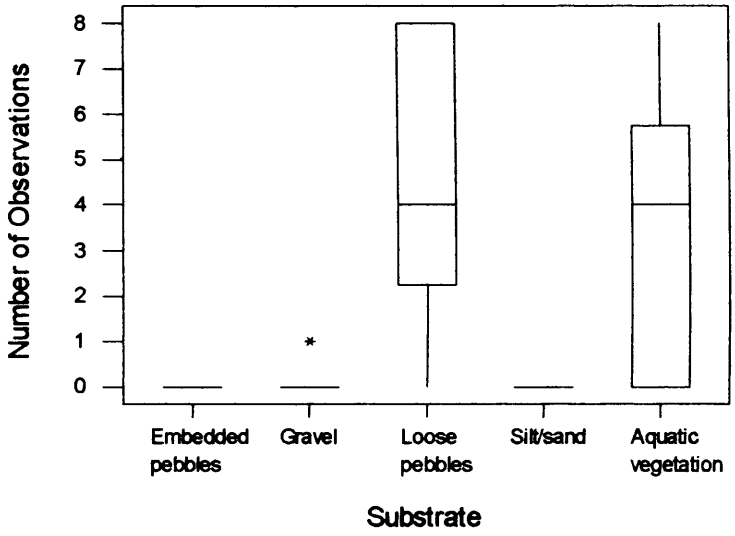


Figure 3.10 Boxplot displaying the number of times per day a crayfish was observed on each substrate type in Tank 1. Horizontal lines represent the medians, boxes represent the middle half of the data, vertical lines (whiskers) represent the lower and upper limits, while asterisks represent outliers.

3.4.2 Experiment 2 - Sediment deposition

More silt was collected from basket traps than the flowerpot traps indicating that the two trap types were measuring different components of siltation i.e. basket traps measure intrabed plus surface silt deposition while flowerpot traps measure only surface silt deposition.

The quantity of surface plus intrabed silt collected in basket traps was compared between sites using data collected from September 2002 to December 2003. Data transformed using the Log10 function were normally distributed and displayed

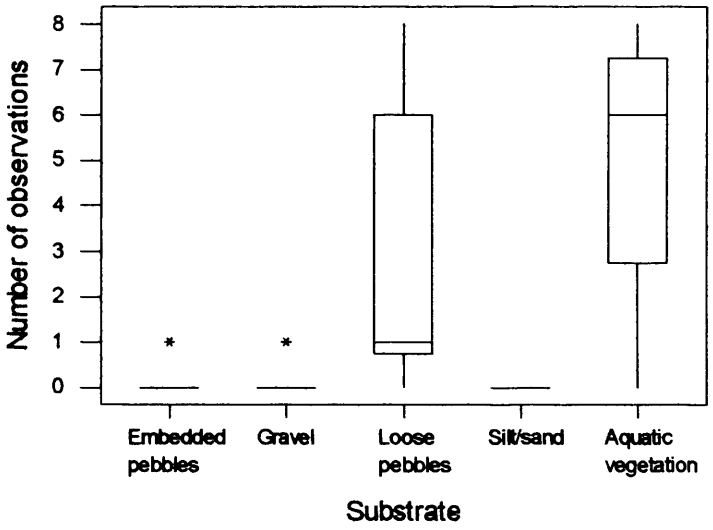


Figure 3.11. Boxplot displaying the number of times per day a crayfish was observed on each substrate type in Tank 2. Horizontal lines represent the medians, boxes represent the middle half of the data, vertical lines (whiskers) represent the lower and upper limits, while asterisks represent outliers.

equality of variances using Bartlett's Test (test statistic = 6.247, p = 0.620). The Anderson-Darling Test showed that residuals were normally distributed (test statistic = 0.644, p = 0.087).

A one-way ANOVA showed no significant differences between the mean weights of surface plus intrabed silt collected at each site (F = 0.69, p = 0.696, df = 9, 44) (Figure 3.12).

The quantity of surface silt collected in flowerpot traps was also compared between sites using data collected from September 2002 to December 2003. Data transformed using the Log10 function were normally distributed and displayed equality of variances using Bartlett's Test (test statistic = 4.042, P = 0.909). The Anderson-Darling Test showed residuals were normally distributed (test statistic = 0.435, p = 0.287). A one-way ANOVA showed no significant differences between the mean

weights of surface silt collected at each site (F = 0.31, P = 0.968, df = 9, 37) (Figure 3.13).

The mean quantity of surface plus intrabed silt deposited on and within a 0.049 m^2 river bed surface area, the equivalent of a basket trap's contents, in one month was

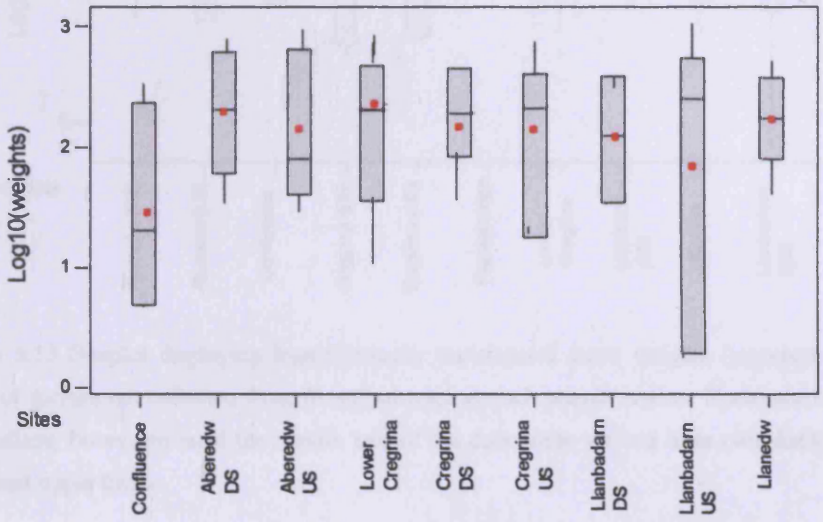


Figure 3.12 Boxplot displaying logarithmically transformed mean weights (represented by the red spots) of surface plus intrabed silt collected from basket traps at each sample station. Horizontal lines represent the medians, boxes represent the middle half of the data while vertical lines (whiskers) represent the lower and upper limits.

273.8 g. The calculated mean weight of surface plus intrabed silt deposited on 1m^2 riverbed surface area would therefore be 5587.8 g in one month.

The mean quantity of surface silt deposited on a 0.049 m^2 surface area of river bed was 263.2 g, while the calculated mean quantity of silt deposited per month on a 1m^2 riverbed surface area would be 5372.1 g.

The mean organic content of total silt collected in the Edw in one month was 22.6 % with a minimum of 4 % and a maximum of 29.5 %.

No significant differences in weights of surface plus intrabed or surface silt collected between sites ($p>0.05$) were found in the previous ANOVA comparison. Sites were therefore used as replicates when comparing the weights of silt collected between months.

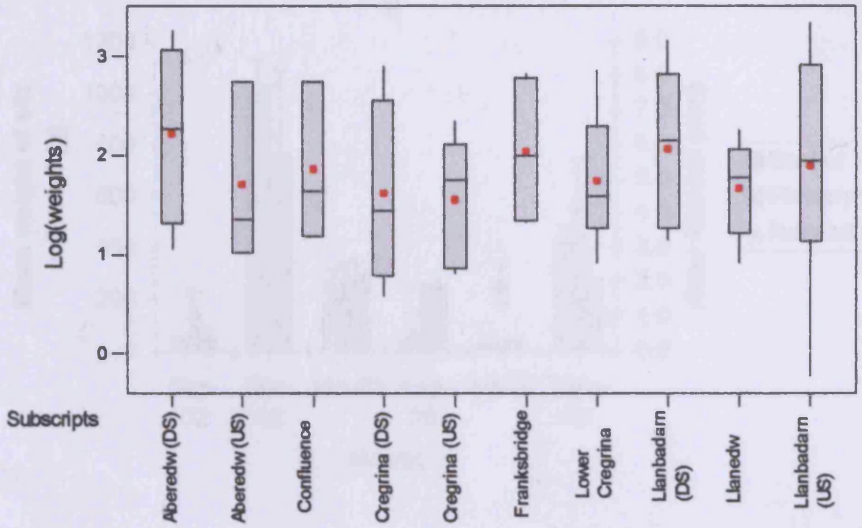


Figure 3.13 Boxplot displaying logarithmically transformed mean weights (represented by the red spots) of surface silt collected from flowerpot traps at each sample station. Horizontal lines represent the medians, boxes represent the middle half of the data while vertical lines (whiskers) represent the lower and upper limits.

Data were not normally distributed and variances were unequal despite transformation. A non-parametric Kruskal-Wallis Test consequently carried out showed significant differences in the median weights of surface plus intrabed silt and surface silt collected between each of six sampling months during September 2002 to December 2003 ($p = 0.00001$, $df = 5$).

Mean quantities of silt collected were greater in winter months, November 2002 and December 2003, than in summer and autumn months, September 2002, June 2003, August 2003 and October 2003 (Fig. 3.14).

A Pearson's Correlation Matrix showed that the mean monthly rainfall was significantly positively correlated with the mean quantities of surface plus intrabed and surface silt collected ($p = 0.003$ and 0.044 respectively at the 95% confidence level). The higher the mean quantity of rainfall in a month, the larger the quantities of silt deposited onto and passing through the river bed in that month. Also, the mean quantities of surface plus intrabed silt collected from basket traps and surface silt

collected from flowerpot traps were significantly correlated ($p = 0.004 < 0.05$) (Table 3.1).

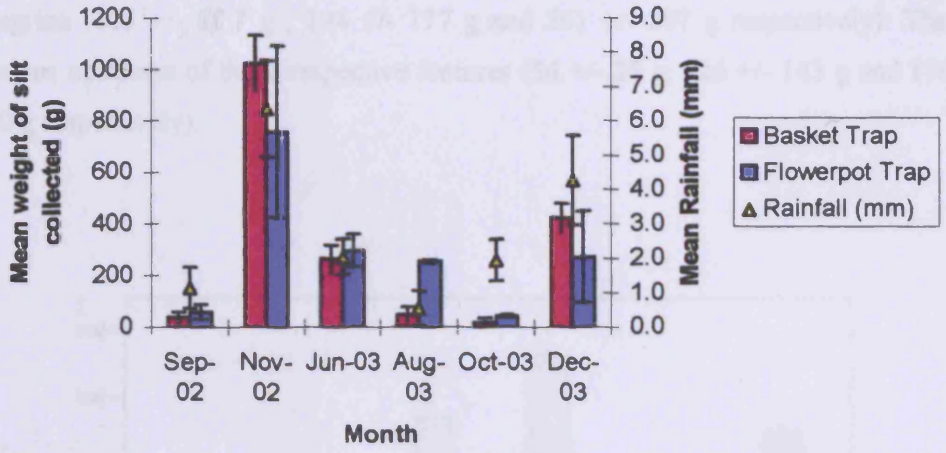


Figure 3.14 Plot to show mean silt weights (g) collected in basket and flowerpot traps and mean monthly rainfall (mm) during each of six sampling months with error bars of the standard errors of the mean for basket traps (n = 10), flowerpot traps (n = 10) and rainfall (n = 30).

	Surface plus intrabed	Surface
Surface	0.947, 0.004	
Rainfall	0.954, 0.033	0.823, 0.044

Table 3.1. Pearson's Correlation Matrix for rainfall (mm), quantity of surface plus intrabed silt (g) and quantity of surface silt (g) where cell contents = Pearson correlation, P-value.

Weights of silt collected upstream were compared with weights collected downstream of the cattle drinking area at Aberedw, the tributary stream entry at Cregrina and the sheep crossing at Llanbadarn y garreg and in order to find out if any differences were present during "summer" months (Figure 3.15).

A one-way ANOVA of basket trap data showed no significant differences in surface plus intra-bed silt weights between up and downstream sites during summer months ($F = 0.71$, $p = 0.626$, $df = 5, 16$). An Anderson-Darling test showed that residuals were normally distributed ($p = 0.351$) while Bartlett's Test showed data displayed equality of variance (test statistic = 6.377, $p = 0.271$).

However, although no significant differences were present, mean weights of surface plus intrabed silt were slightly greater at stations downstream of the cattle drinking area at Aberedw, sheep crossing at Llanbadarn and the tributary stream entry at Cregrina (115 +/- 85.7 g , 194 +/- 177 g and 261 +/- 207 g respectively). Than at stations upstream of these respective features (54 +/- 25 g, 126 +/- 143 g and 176 +/- 190 g respectively).

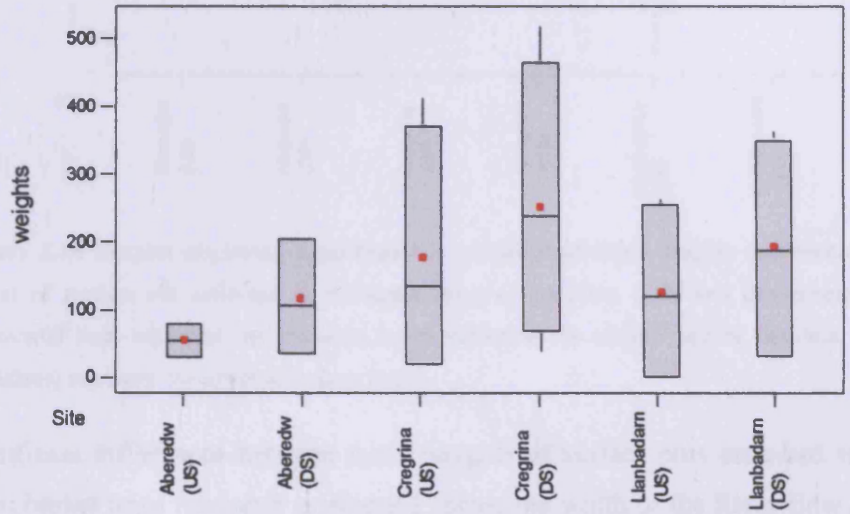


Figure 3.15 Boxplot displaying mean weights (represented by the red spots) of intrabed plus surface silt collected in basket traps at upstream (US) and downstream (DS) sites. Horizontal lines represent the medians, boxes represent the middle half of the data, vertical lines (whiskers) represent the lower and upper limits.

A one-way ANOVA of logarithmically transformed flowerpot trap data showed no significant differences in surface silt weights between up and downstream sites during summer months ($F = 0.27$, $p = 0.920$, $df = 5, 14$). An Anderson-Darling test showed that residuals were normally distributed ($p = 0.805$) while Bartlett's Test showed data displayed equality of variance (test statistic = 3.971, $p = 0.554$).

This part of the experiment was carried out to find out if the quantity of silt depositing onto or moving through the riverbed varied across the width of two sections of the River Edw at Aberedw and Llanbadarn y garreg.

A one-way ANOVA of logarithmically transformed basket trap data showed no

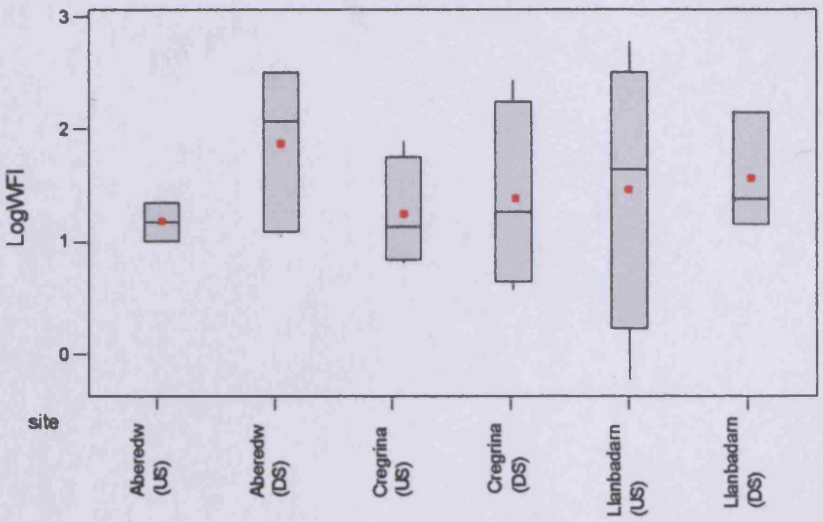


Figure 3.16 Boxplot displaying logarithmically transformed mean weights (represented by the red spots) of surface silt collected in flowerpot traps at upstream (US) and downstream (DS) sites. Horizontal lines represent the medians, boxes represent the middle half of the data, vertical lines (whiskers) represent the lower and upper limits.

significant differences between mean weights of surface plus intra-bed silt collected from basket traps randomly positioned across the width of the River Edw at Aberedw ($F = 0.65$, $p = 0.640$, $df = 4, 10$) (Figure 3.17). An Anderson-Darling test showed that residuals were normally distributed ($p = 0.304$) while Bartlett's Test showed data displayed equality of variance (test statistic = 4.142, $p = 0.387$).

A one-way ANOVA of logarithmically transformed flowerpot trap data showed no significant differences between mean weights of surface silt collected from flowerpot traps randomly positioned across the width of the River Edw at Aberedw ($F = 0.13$, $p = 0.969$, $df = 4, 10$) (Figure 3.18). An Anderson-Darling test showed that residuals were normally distributed ($p = 0.121$) while Bartlett's Test showed data displayed equality of variance (test statistic = 0.106, $p = 0.999$).

A one-way ANOVA of logarithmically transformed basket trap data showed no significant differences between mean weights of surface plus intra-bed silt collected from basket traps randomly positioned across the width of the River Edw at Llanbadarn y garreg ($F = 0.45$, $p = 0.723$, $df = 3, 11$) (Figure 3.19).

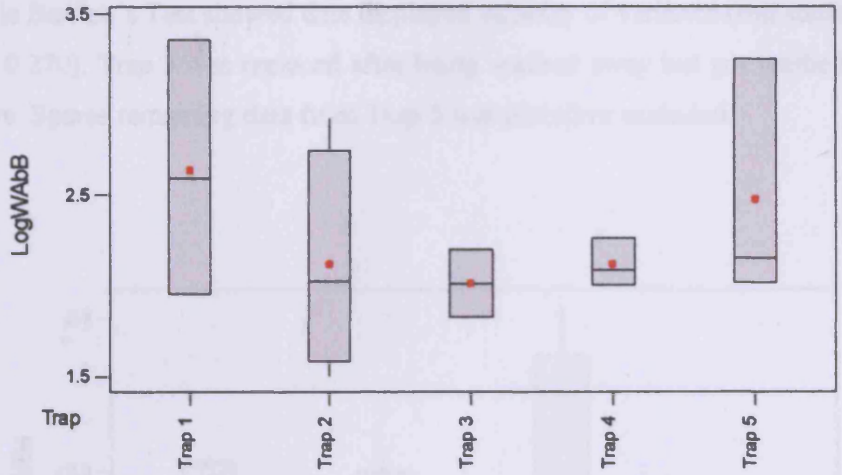


Figure 3.17 Boxplot displaying logarithmically transformed mean weights (represented by the red spots) of intrabed plus surface silt collected from basket traps at Aberedw. Horizontal lines represent the medians, boxes represent the middle half of the data, vertical lines (whiskers) represent the lower and upper limits.

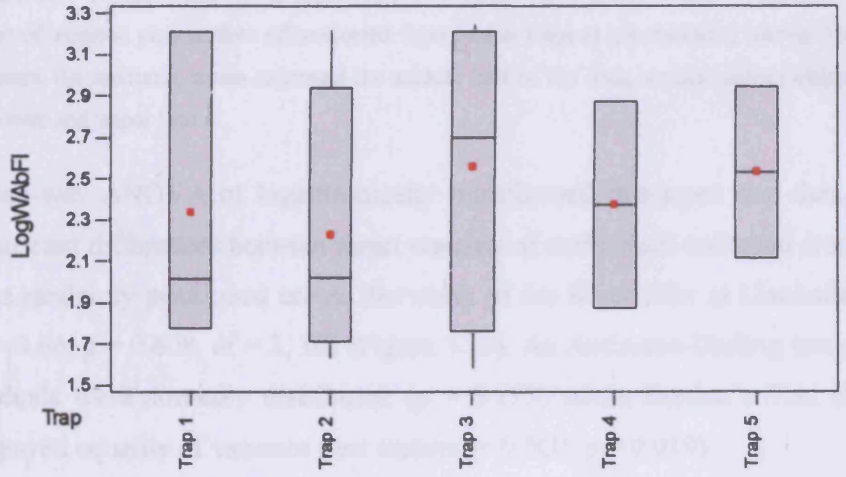


Figure 3.18. Boxplot displaying logarithmically transformed mean weights (represented by the red spots) of surface silt collected from flowerpot traps at Aberedw. Horizontal lines represent the medians, boxes represent the middle half of the data, vertical lines (whiskers) represent the lower and upper limits.

An Anderson-Darling test showed that residuals were normally distributed (p = 0.402) while Bartlett's Test showed data displayed equality of variance (test statistic = 3.919, p = 0.270). Trap 5 was replaced after being washed away but got washed away once more. Sparse remaining data from Trap 5 was therefore excluded.

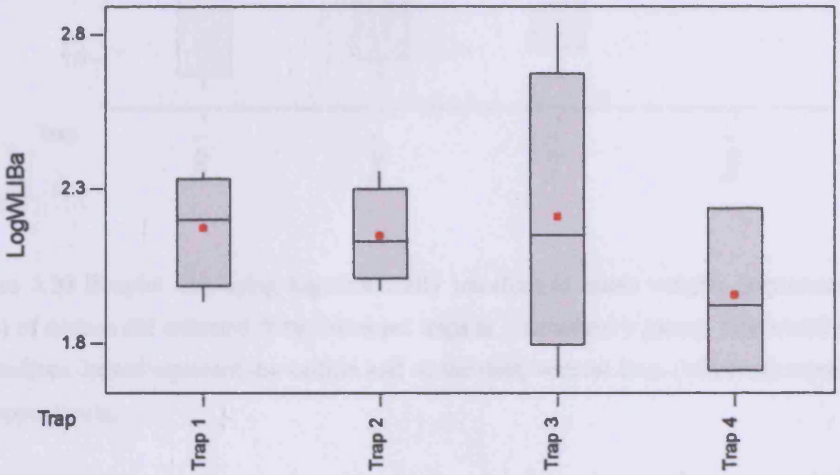


Figure 3.19 Boxplot displaying logarithmically transformed mean weights (represented by the red spots) of intrabed plus surface silt collected from basket traps at Llanbadarn y garreg. Horizontal lines represent the medians, boxes represent the middle half of the data, vertical lines (whiskers) represent the lower and upper limits.

A one-way ANOVA of logarithmically transformed flowerpot trap data showed no significant differences between mean weights of surface silt collected from flowerpot traps randomly positioned across the width of the River Edw at Llanbadarn y garreg (F = 0.64, p = 0.608, df = 3, 10) (Figure 3.20). An Anderson-Darling test showed that residuals were normally distributed (p = 0.193) while Bartlett's Test showed data displayed equality of variance (test statistic = 0.500, p = 0.919).

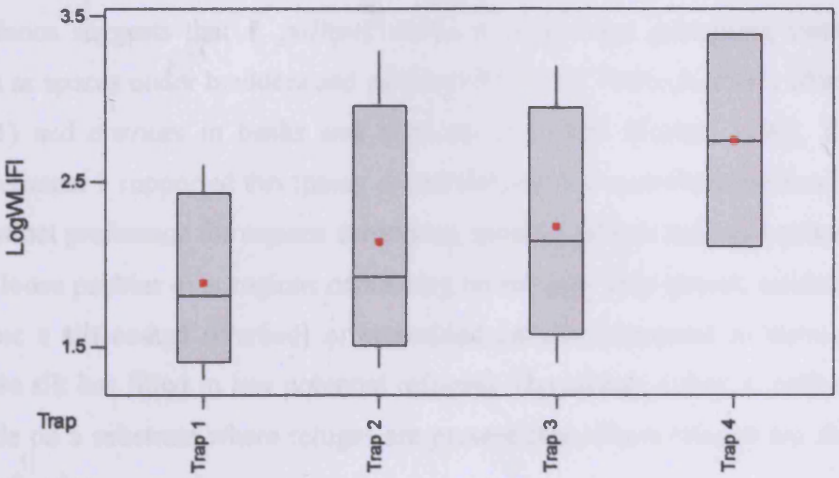


Figure 3.20 Boxplot displaying logarithmically transformed mean weights (represented by the red spots) of surface silt collected from flowerpot traps at Llanbadarn y garreg. Horizontal lines represent the medians, boxes represent the middle half of the data, vertical lines (whiskers) represent the lower and upper limits.

3.5 DISCUSSION

3.5.1 Experiment 1 – Substrate preference

Evidence suggests that *A. pallipes* reside in waterways containing suitable refuges such as spaces under boulders and pebbles (Rogers & Holdich, 1995; Slater & House, 2001) and crevices in banks and between tree roots (Foster, 1996). Results from Experiment 1 supported this theory as individuals in a controlled environment showed a distinct preference for regions containing suitable refuges such as aquatic vegetation and loose pebbles over regions containing no refuges, only gravel, sand/silt (meant to mimic a silt coated riverbed) or embedded pebbles (designed to mimic a riverbed where silt has filled in any potential refuges). Hypothesis 1 that *A. pallipes* prefer to reside on a substrate where refuges are present than where refuges are absent should therefore be accepted.

If a river bed becomes coated in silt and potential refuges get filled in, it is therefore likely that any *A. pallipes* individuals would leave that region to avoid vulnerability to predators. Where there are no refuges as a result of siltation *A. pallipes* are therefore less likely to be present (Foster, 1990). Also, if a river bed is less physically diverse e.g. due to excess siltation, less detritus accumulates (Lepori *et al.*, 2005). Detritus is a primary *A. pallipes* food source (Smith *et al.*, 1996; Reynolds, 1979) and also provides an indirect food sources as it harbours aquatic invertebrates on which *A. pallipes* also feed (Naura & Robinson, 1998).

Excess siltation could therefore undoubtedly have disastrous consequences for *A. pallipes* populations and other riverbed dwelling creatures such as bullheads (*Cottus gobio*) (Naden *et al.*, 2002) by filling in gaps underneath and between which they take refuge from the current and predators (Foster, 1996) and lay their eggs; salmonid fry by filling in gaps between stones where eggs are laid and newly hatched fry hide (Wheeler, 1991), and freshwater pearl mussels (*Margaritifera margaritifera*) as they require a sandy rather than fine silt substrate in which they bury themselves on the downstream side of rocks or boulders, and which also rely on salmonid fry to disperse young (Hastie *et al.*, 2000). Lampreys (*Petromyzon marinus*) are also threatened by excess siltation as it covers over the pebble river beds on which they spawn (Wheeler, 1991).

In conclusion, these experiments show that: siltation is still occurring in the river Edw and is likely to be a problem in other similar rivers, siltation is increased by livestock poaching of river banks and beds, increased siltation is likely to be a problem in rural regions where livestock is farmed, where heavy rainfall is common and where land is steep. A. *pallipes* prefers regions where refuges are present, whether they are spaces under stones e.g. the Dulas Brook, or dense aquatic vegetation e.g. the Offeiriad. Siltation and organic pollution are likely to be important contributing factors to the disappearance of *A. pallipes* in rural regions such as upland Wales.

3.5.2 Experiment 2 - Sediment deposition

Siltation is defined by Naden *et al.* (2002) as the deposition of fine sediment on the surface of and within the riverbed. We attempted to measure both aspects of siltation using two trap types. Basket traps allowed the measurement of intrabed plus surface silt, while flowerpot traps measured only surface silt.

The presence of significantly more silt collected in basket than flowerpot traps showed that the use of both trap types enabled both aspects of siltation to be measured separately, making them a suitable combination of apparatus to quantify aquatic siltation.

Summarising data collected during summer and winter months, mean quantities of surface and intrabed siltation were found to be similar along the length of the River Edw as no significant differences were found between sites. Hypothesis 2 that silt deposition increases in a downstream direction should therefore be rejected.

Silt must be continually entering the water column along the entire length of the Edw. This indicates that unstable, eroding banks must be present along the length of the river despite the presence, in parts, of WHIP bank reinforcement and fencing, suggesting that although these improvements may have reduced siltation, they are not preventing it.

This experiment showed that siltation continued to occur at an alarming rate three years after the WHIP fencing was erected in the Edw. Even after fencing, surface plus intra-bed siltation occurred at a mean rate of 5588 g m^{-2} per month, while surface siltation alone was detected to be occurring at a mean rate of 5372 g m^{-2} per month, showing that intrabed silt was very small component. Unfortunately, we have no previous records with which we can compare our findings, except that Mitchell

(1983) quotes the average sediment load of the River Wye to be 123824 tonnes per year. It is therefore impossible to know if siltation has been reduced since the fencing was erected. However, as livestock access and bank poaching has been prevented by large stretches of fencing along the Edw, it is likely that siltation in the Edw has been reduced. Despite this suspected reduction, current siltation rates remain relatively high and therefore pose a serious threat in many rivers to aquatic organisms such as *A. pallipes*, salmonid fry, freshwater pearl mussels (*Margaritifera margaritifera*), lampreys and bullheads (Naden *et al.*, 2002) as described in Experiment 1. Silt fills in gaps underneath and between which *A. pallipes* and bullheads (*Cottus gobio*) take refuge from the current and predators by day (Foster, 1996) and under which bullheads lay their eggs. Salmonid fry are affected by filling in gaps between stones where eggs are laid (Wheeler, 1991), while freshwater pearl mussels (*Margaritifera margaritifera*) require a sandy rather than fine silt substrate in which they bury themselves on the downstream side of rocks or boulders, and also rely on the declining populations of salmonid fry to disperse young (Hastie *et al.*, 2000). Lampreys (*Petromyzon marinus*) are also threatened by excess siltation as it covers over the pebble river beds on which they spawn (Wheeler, 1991).

Mean rates of siltation in the River Edw varied significantly between sampling months. Variation appeared to be seasonal as more sediment was deposited during the winter months (November 2002 and December 2003) than the summer months (September, 2002, June 2003, August 2003 and October 2003). Mean rainfall was also higher in winter than summer months. Siltation was found to be significantly positively correlated with mean monthly rainfall in the present study. Hypothesis 3 that silt deposition in a river increases with the quantity of rainfall should therefore be accepted.

Siltation is said to occur when the upward momentum transfer of the fluid eddies drops below the weight of the suspended solids (Naden *et al.*, 2002). If this is true then the amount of suspended solids being carried along in the river water must have been particularly high during periods of heavy rainfall when the Edw was flowing at a high rate. During such times, large quantities of silt must have entered the water column, possibly by erosion of river banks and steep surrounding landscape both previously heavily poached by intensively farmed livestock combined with frequent heavy rainfall. Although siltation occurs and varies naturally (Naden *et al.*, 2002), its

effects are exacerbated during periods of heavy rainfall in this instance by unstable, already eroding banks. Aquatic organisms such as *A. pallipes* would find it difficult to survive such times of heavy rainfall as so many refuges would be filled in by the large amounts of silt entering the River Edw and the increased risk of being washed away. Also, in spite of silt and erosion, in many areas there are no earth banks for *A. pallipes* to dig into as alternative refuges.

During periods of low rainfall, slightly more surface plus intrabed silt was found at sample stations positioned downstream than those upstream of livestock access sites in Llanbadarn y garreg and Aberedw. These differences, however, were not significant, possibly due to much variation and lack of replication within datasets. Hypothesis 4 that more silt will be deposited downstream than upstream of livestock entry points should therefore be rejected.

Silt must therefore be entering the river at the livestock entry points. Examination of habitat at these points in Aberedw and Llanbadarn y garreg revealed that the banks were poached bare and badly eroding. Also, on a number of occasions, livestock were observed entering the water at these sites, and each time, water immediately became opaque as large quantities of silt and organic excrement moved downstream, settling on the riverbed (pers. obs.) suggesting that this component of silt is likely to encourage anaerobic conditions, increasing the BOD which could be detrimental to *A. pallipes*. This organic matter can act as a substrate for microorganisms. When decomposition occurs, dissolved oxygen in the water may be used up more quickly than it can be replenished (Mason, 1996), which would have a deleterious effect on crayfish which usually require high dissolved oxygen levels (Foster, 1990). Organic matter also coats the riverbed preventing light important to bed dwelling organisms from getting through, and also contains ammonia which is toxic to crayfish and other aquatic organisms (Mason, 1996).

Despite livestock having limited access to the River Edw due to the WHIP improvements, siltation remains a problem, the cause of which should be treated. The bank and riverbed at controlled access points could be reinforced with hardcore or pebbles set in concrete, or a pump operated water point should be established on the field side of river fencing (Slater pers. comm.) (Figure 3.21). With these measures, livestock, while retaining access to the water, would not be continually eroding banks or riverbeds, thus reducing the input of loose silt to the river at these points.

Figure 3.21 Photograph displaying a livestock water dispenser positioned on the field side of a fenced river.

The cost and vast amounts of manual labour required for installing silt traps in the River Edw, meant that only one trap of each type could be used at each site, allowing very little replication. Also, positioning of traps at each trap station was not kept constant due to the difficulty in installing traps into a riverbed containing large amounts of near surface bedrock. Current, flow speed and direction are likely to vary across the width of rivers. However, results showed that this was not the case in this instance.

The absence of any significant amounts of variation in silt collected between traps of the same type at each site increases the validity of the results of the silt deposition experiment as it is unlikely that the position of the trap across the width of the river actually markedly influenced the quantity of silt collected in that trap. Other influencing factors such as heavy rainfall and positioning of fencing are more likely to be responsible for such differences.

3.5.3 Sheep dip pollution

Organophosphate (OP) and synthetic pyrethroid (SP) sheep dips have both caused river pollution in the past. Since the VMD introduced exposure reduction methods of OPs in the late 1990s (Croxford, 2005), however, increased use of SPs, particularly cypermethrin, has caused concern (Environment Agency, 2000; Rutt, 2004) as SPs are

100 times more toxic to aquatic life than OPs (Coley, 2000; Rutt, 2004; Howells, 2003).

The Welsh Assembly Government expressed the concern of the Environment Agency in 2003 over the illegal use of a form of cypermethrin, an arable crop pesticide, as a sheep dip chemical (Gwlad, 2003). It is cheaper than the one specifically designed for sheep dipping, which has tempted farmers to buy and use it (Rutt, 2004). The presence of agents to help this chemical bind to sheep fleece, the effects on sheep health and the concentrations at which it should be used as a dip are all unknown. The results of using this chemical, combined with its high toxicity to aquatic organisms is particularly threatening to the health of rivers, sheep, consumers, local trout and salmon fisheries (Gwlad, 2003) and *A. pallipes* populations. Younger crayfish are particularly susceptible as early instars are three times more sensitive to pollutants than juveniles, while juveniles are four times more sensitive than adults (Eversole & Seller, 1996).

A great number of sheep dip pollution incidents have been known to have occurred in Wales, some of which are listed in Table 3.3.

Site	Date	Pollutant	Detection method
Sgithwen (1 incident)	October 1996	Cypermethrin	BMWP scores & crayfish survey
Teifi & tributaries (8 incidents)	2003	Cypermethrin	BMWP scores
Ogmore Catchment (2 incidents)	2003	Cypermethrin	Moss analysis
River Loughour (1 incident)	2003	Cypermethrin	Moss analysis
River Tawe (2 incidents)	2003	Cypermethrin	Moss analysis
River Neath (1 incident)	2003	Cypermethrin	Moss analysis
River Cothi (1 incident)	2003	Cypermethrin	BMWP scores
Melindwr at Capel Bangor (1 incident)	2003	Cypermethrin	BMWP scores
Eastern Cleddau (1 incident)	2003	Illegal cypermethrin	Water analysis
Nant Paith – Ystwyth tributary (1 incident)	2003	Illegal cypermethrin	Water analysis
Nant Cwmdu – Llynfi tributary (1 incident)	2003	Illegal cypermethrin	Moss analysis

Table 3.3 Recent pollution incidents, pollutants and methods of detection in Welsh rivers from Wilkins (1998) and Rutt (2004).

Since August 2003, 57 dip pollution incidents were recorded over 29 catchments in Wales alone. It is therefore evident that despite the Groundwater Regulations and Best Practice Guidelines for the Management of Sheep Flocks, 2001, set out by the Environment Agency, both Diazinon and Cypermethrin continue to be detected in British waters and more disturbingly, at greatly underestimated levels (Croxford, 2005).

Proving sheep dip pollution in watercourses has been difficult as the chemicals get washed away and break down quickly. Cypermethrin has a half life of 7-12 days and so is much more difficult to detect than diazinon which has a half life of between 56 and 185 days (Pesticide Action Network UK, 2000). Cypermethrin has, however, been detected at reportable levels in moss samples, several months after sheep dipping (Rutt, 2004), when it has not been possible to detect in water samples and was only detected below reportable levels in sediment. For example, the illegal form of cypermethrin was found at reportable levels in moss samples from Nant Cwmdu, a Llynfi tributary (Rutt, 2004). Moss analysis and biological assessment are therefore recommended to detect sheep dip pollution incidents (Rutt, 2004), particularly if some time has elapsed since the incident.

Although rivers appear to have recovered somewhat after pollution incidents, *A. pallipes* recovery is very slow. For example, following a pollution incident on the Sgithwen Brook in 1996, BMWP Scores showed that by October 1997, macroinvertebrates appeared to be recovering well, with one exception, *A. pallipes* previously abundant, remained absent (Wilkins, 1998).

Sheep treatment was frequently found to be the cause of poor aquatic invertebrate fauna in some Welsh rivers, as acidity, heavy metal, nutrient and organic pollution were ruled out (Rutt, 2004). Numerous farm visits made by the Environment Agency often found poor use and storage methods of sheep dip pesticides (Rutt, 2004). This, combined with the many pollution incidents positively identified as having been caused by cypermethrin using chemical and biological assessment, strongly supports the theory that sheep dip has played a large part in the massive decline in *A. pallipes* numbers in Wales.

However, some signs of river improvement began in 1999 possibly due to the introduction of the Groundwater Regulations by the Environment Agency, better

farming practice and pollution prevention visits (Rutt, 2004). For example, *A. pallipes* numbers in the Edw and Cwmbach Dulas tributaries of the River Wye, have increased (Howells, 2003) since a survey carried out in 2000 by Slater & House (2001). It is possible that these recoveries have only recently become apparent. This timing is therefore more evidence that sheep dip pollution has helped contribute the Welsh *A. pallipes* decline. Another way of aiding river recovery would be to use less damaging alternatives to SPs. It was suggested in a paper presented to a recent Environment Agency Committee meeting (Croxford, 2005) that the Environment Agency should, with these alternatives in mind, aim for the withdrawal of SP dip licences in line with Recommendation 142 of the Salmon and Freshwater Fisheries Review. If such measures were taken, we would probably see a decline in damaging water pollution incidents in the Welsh Countryside and an increase in river quality, which could eventually result in the natural recovery of *A. pallipes* in many rivers. The alternative recovery strategy is to restock depleted rivers but, on conservation grounds, it is crucial that the genetic implications of such procedures are fully understood.

CHAPTER 4

Microsatellite analysis of British *A. pallipes* populations with special focus on Wales and the Marches.

SUMMARY

The decline of *Austropotamobius pallipes* populations in Welsh rivers dates from the early 1990s. Initially crayfish plague may have affected some rivers but subsequently the major decline in this area has been attributed to siltation and agricultural pesticides. The native white-clawed crayfish is an important component of the ecology of mid-Wales rivers. Restoration of lost or damaged populations is therefore desirable. If this has to be done by restocking, genetic structure of relevant populations should first be assessed, thus allowing management and maintenance of the natural diversity of the species.

This chapter describes the fine scale genetic variation of some remaining British populations using microsatellite markers at three levels, within stream, within catchment and between catchments and whether habitat variables can influence levels of genetic diversity in *A. pallipes* populations.

The present study found genetic variation at all three levels; suggests reasons for such variation and advocates genetic caution in future *A. pallipes* reintroductions, while particular river habitat features were found to be positively and negatively associated with *A. pallipes* genetic variability. The results of this study could assist with the selection of suitable donor populations and receptor sites for restocking.

4.1 AIMS

This chapter aims to investigate:

- the partitioning of genetic diversity in *A. pallipes* at three levels: within rivers, between rivers within a catchment and between catchments.
- whether RHS variables are positively or negatively associated with levels of genetic diversity in *A. pallipes* populations,
- whether possible suitable restocking sites can be identified using the results of this study.

4.2 INTRODUCTION

Native white-clawed crayfish (*Austropotamobius pallipes*) remain present in many parts of the UK and Europe despite the dramatic declines that have occurred in recent years (Holdich & Rogers, 1997, Slater, 1998; Wilkins, 1999; Holdich *et al.*, 1999; Coley, 2000; Sibley *et al.*, 2002; Holdich, 2003 and Sibley, 2003) as a result of habitat destruction, introduction of invasive species (particularly the North American signal crayfish, *Pacifastacus leniusculus)* together with crayfish plague (Alderman, 1993) and pollution (Holdich & Lowery, 1988 and Largiader *et al.*, 2000). A similar fate has also befallen other native crayfish across Europe such as *Astacus astacus* in Sweden (Edsman, 2004) and *Austropotamobius torrentium* in Germany (Huber & Schubart, 2004).

In 1990, crayfish plague, caused by the fungus *Aphanomyces astaci,* reached Welsh Rivers in Western Britain (Holdich, 2003) where it devastated once thriving *A. pallipes* populations of main river stems such as the Wye (Coley, 2000).

A. pallipes populations are now present at reduced densities and confined to headwaters and tributaries of main Welsh rivers such as the Wye (Holdich, 1993; Foster, 1995; Rogers & Holdich, 1995; Wilkins 1998; Slater, 1998; Slater & House, 2001 and Howells, 2003) while barely detectable numbers exist on the main river stems such as the Usk and Banwy (Howells, 2003). Loss of crayfish from main river stems has also occurred in France (Demers *et al.*, 2003) and with other native species in other European countries such as *Austropotamobius torrentium* in Germany (Huber & Schubart, 2004).

Isolated remnant populations have suffered further declines in rural upland areas of Wales, not from plague (Coley, 2000; Holdich, 2003; Howells & Slater, 2003), but probably as a result of sheep dip pollution (Wilkins, 1998; Environment Agency, 1998; 1999; 2000) and habitat degradation through excess siltation (Holdich, 2002; Howells & Slater, 2003).

Surveys for the present study (Chapter 2), however, showed that *A. pallipes* populations of some rivers were recovering, such as those of the Edw, a Wye tributary. This is therefore potentially an opportune time to begin restocking recovering watercourses.

It is now regarded as good practice that before restocking or translocation programmes for endangered species can commence, the genetic structure of relevant populations should first be assessed, thus allowing management and maintenance of the natural diversity of that species (Wayne *et al.*, 1991; Avise 1994; Waldman & Wirgin, 1994; Zaccara *et al.*, 2004). Effective management can prevent problems of genetic erosion through processes such as loss of diversity due to genetic drift or contamination (Moritz, 1994; Avise, 2000) or loss of local genetic integrity through hybridisation as occurred with native crayfish taxa in the Alpine Region (Largiader *et al.*, 2000). Genetic management can also prevent inbreeding and its associated depression in fitness which could result in an increased risk of extinction (Frankham *et al.*, 2002) as, for example, occurred in a small population of adders in Sweden (Madsen *et al.*, 1996).

Much of the literature on population genetics of crayfish has shown low levels of genetic diversity in Europe, using methods such as allozyme electrophoresis (Albrecht & Von Hagen, 1981; Attard & Vianet, 1985) and restriction fragment length polymorphism (RFLP) analyses of mitochondrial DNA (Grandjean & Souty-Grosset, 1996, 1997 and Grandjean *et al.*, 1997). For example, using allozymes, low levels of genetic variation were reported for *Procambarus clarkii* (Busack, 1988) and *A. pallipes*, which displayed heterozygosity values as low as 0.001 and 0.015 (Santucci *et al.*, 1997; Largiader *et al.*, 2000). Most of the earlier molecular studies of crayfish genetics and their techniques have been summarised by Souty-Grosset *et al.* (1999). More recently however, higher levels of native crayfish genetic diversity were found in some areas of Europe such as the Alpine Region using RFLP analyses of mtDNA and allozyme (nuclear) analyses (Largiader *et al.*, 2000) and in a French brook where

higher *A. pallipes* heterozygosity values of 0.394 were found using microsatellites (Gouin *et al.*, 2002) and 0.446 using RAPD markers (Gouin *et al.*, 2001).

Most studies previously conducted on crayfish have examined genetic variation at a large spatial scale such as those on *A. pallipes* (Fratini *et al.*, 2004) and *A. italicus* (Santucci *et al.*, 1997). Species management plans, however, should be established at the population level in order to minimise loss of local genetic integrity (Largiader *et al.*, 2000). Small scale genetic structuring within and between populations should therefore be investigated. Microsatellite markers are useful for investigating genetic diversity in species with low levels of allozyme variation (Bruford & Wayne, 1993; Choudhary *et al.*, 1993; Hughes & Queller, 1993 and Paetkau *et al.*, 1995) such as crayfish (Busack, 1988), as they are usually multiallelic and hypervariable making polymorphism identification much easier (Yu *et al.*, 1999). Microsatellites are therefore more likely to detect small scale genetic variability in *A. pallipes* populations (Gouin *et al.*, 2000; Grandjean *et al.*, 2001) than other molecular markers and were consequently used in the present study to investigate comparative, hierarchical, spatial differentiation of British *A. pallipes* populations at three levels, within a river, between rivers within a catchment and between catchments using microsatellite markers.

Small scale genetic differentiation within a river was previously investigated in a French brook, but no genetic structure was found, suggesting that *A. pallipes* individuals moved large distances in both upstream and downstream directions (Gouin *et al.*, 2002). Further, no genetic differentiation was found within and between *A. pallipes* populations in South Tyrol (Northern Italy) using mtDNA sequences suggesting that gene flow was occurring within a river (Baric *et al.*, 2004).

A study of rivers in eastern France, however, found for example, significant isolation by distance in brown trout, *Salmo trutta*, populations (Estoup *et al.*, 1998) and differentiation at a microgeographic scale using allozymes, showing that genetic drift occurred as a result of small population size and a limited interpopulation gene flow. Even when separated by waterway distances of only 1.5 to 2.5 km, populations were significantly differentiated. Another study, carried out in Quebec, Canada, have revealed fine-scale genetic population structure of brook charr, *Salvelinus fontinalis*, using microsatellites and found that the drainage pattern was the main factor influencing this genetic partitioning along with more minor environmental factors

(Angers & Bernatchez, 1998). It is therefore plausible that drainage pattern and in particular the direction of water flow may influence *A. pallipes* populations i.e. in a tributary river, genetic variability could be lowest at the most upstream site and greatest at the most downstream site. This theory should therefore be investigated.

Small differences have also been found at the population level in other crayfish species, *Cambarus* spp. (Brown, 1980); *Astacus* spp. (Fevolden & Hessen, 1989; Agerberg, 1990 as cited by Souty-Grosset *et al.*, 1999). It is therefore plausible to suggest that genetic differentiation of *A. pallipes* is present within a stream.

The presence of such small scale differences suggests that genetic differentiation may also be present between crayfish populations, between rivers of the same catchment and between catchments, as was discovered between French and British *A. pallipes* populations (Attard & Vianet, 1985), between Irish *A. pallipes* populations (Reynolds, 1997), between French *A. pallipes* populations (Souty-Grosset *et al.*, 1999) and between German *Astacus astacus* populations (Schultz & Spyke, 1999).

In order to investigate *A. pallipes* genetic differentiation on the three levels, populations were sampled from five river catchments in Britain: the Wye, the Usk and the Severn (located in Wales and the Marches), the Aire (West Yorkshire) and the Itchen (Hampshire). Four polymorphic microsatellite loci characterised by Gouin *et al.*, (2000) were used to assess genetic structure of *A. pallipes* populations within rivers, between rivers and between catchments.

If genetic differentiation was present within and between *A. pallipes* populations, it is possible that habitat variables could help to explain this variation. River Habitat Surveys (RHS) which assess the presence or absence of various habitat characteristics were consequently used in the present study to establish whether any genetic structuring was associated with habitat variables.

RHS is a UK based survey which allows us to picture the state of river habitats (Raven *et al.*, 1997, 1998) and assess their quality and variability through examining their physical structure and noting the extent of any features present (Jeffers, 1998). A study carried out in 1998 used RHS variables collected from across the UK to predict the environmental requirements of *A. pallipes*. Results were used to suggest suitable characteristics for *A. pallipes* reintroduction programmes (Naura & Robinson, 1998). This, however, was a nation-wide study which although very useful, covered many

different types of rivers. The present study therefore targeted the Wye, Usk and Upper Severn Catchments in an attempt to identify habitat characteristics that influenced genetic diversity of *A. pallipes* populations within a more localized area. If small scale genetic structuring was found to be present, and if this structuring was associated with particular habitat characteristics, more accurate and localized *A. pallipes* restocking and conservation programmes could be formed.

Hypotheses were:

Hypothesis 1

Levels of *A. pallipes* genetic diversity decrease in an upstream direction in a tributary river with increasing distance from the confluence with the Wye.

Hypothesis 2

Levels of *A. pallipes* genetic diversity differ between rivers within a Catchment.

Hypothesis 3

Levels of *A. pallipes* genetic diversity differ between Catchments.

Hypothesis 4

Particular habitat characteristics were significantly associated with differences in *A. pallipes* genetic diversity.

4.3 METHODS

4.3.1 Sample Collection

200 samples were collected from five river catchments (Table 4.1 and Figure 4.1) over three years, 2001, 2002 and 2003. Crayfish were captured using two techniques, stone turning (Thomas & Ingle, 1971) and trapping (described in Chapter 2). Once caught, samples were taken using the least invasive method available. A single fourth pereopod of each healthy crayfish with a carapace length of 25 mm or over was removed and placed in a labelled sampling tube containing 95 % laboratory grade ethanol. Tubes were sealed and stored at –20 °C pending further use.

Locations of sampling sites and rivers within the three main catchments where within stream and within catchment genetic differences were investigated are shown in Figure 4.2.

4.3.2 DNA Extraction

DNA was extracted from one half of each sampled pereopod using the Puregene Genomic DNA Isolation Kit for Cells and Tissues from Gentra Systems, Catalogue number D-5500A. The remaining half was stored in reserve. The protocol can be found on pages 41-43 of the Kit Instructions Manual and in Appendix IV.

The resulting DNA solution was electrophoresed on a 1.2-1.3 % agarose gel for 30 minutes at 100 V in order to ensure that the extraction procedure was successful (Figure 4.3). The second row of bright bands in Figure 4.3 show successfully extracted DNA.

Extracted DNA was stored at 4 °C. For long term storage, samples and extracted DNA were kept at –20 °C or –70 °C.

4.3.3 Polymerase Chain Reactions (PCR)

Microsatellite primers Ap1, Ap2, Ap3 and Ap6, previously characterised by Gouin *et al.* (2000) for *A. pallipes* were used in the present study (Table 4.2). GenBank accession numbers for these cloned sequences are respectively AF204815, AF204816, AF204817 and AF204820.

Although previously optimised by Gouin *et al.* (2000), the primers proved to be highly unstable in the present study and considerable time was spent re-optimising PCRs for successful reactions. The PCR mix consisted of the following quantities of

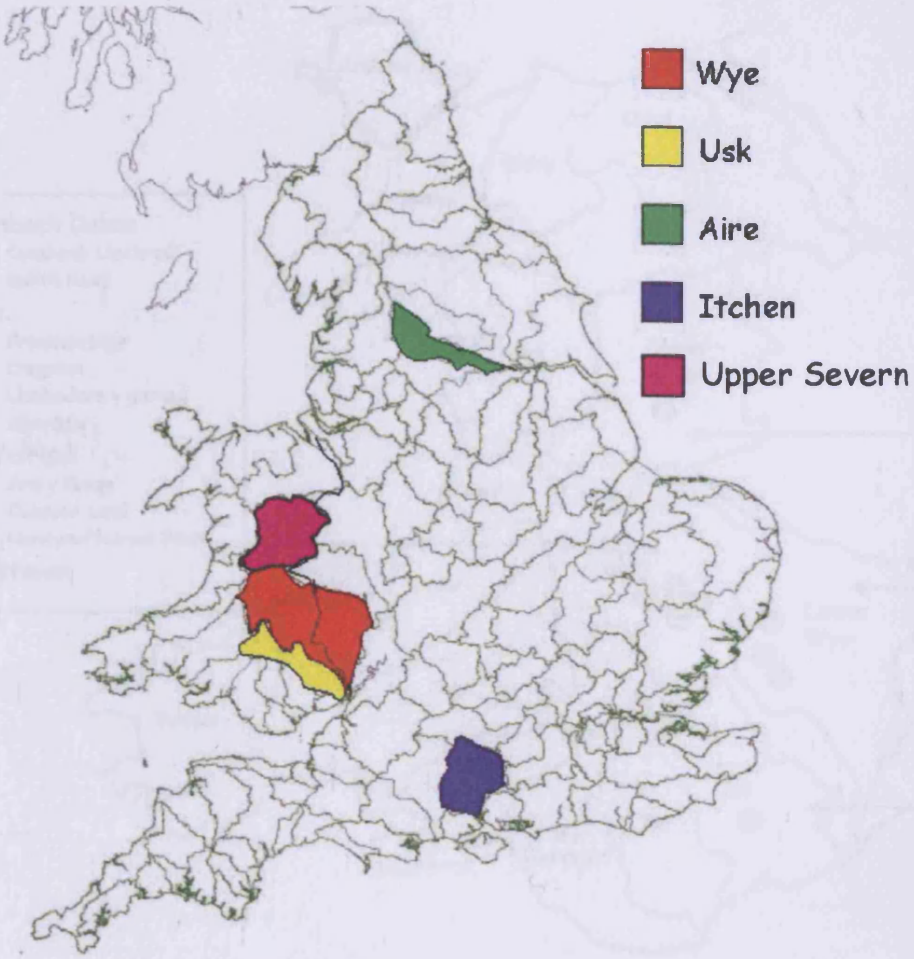


Figure 4.1 Map depicting catchments sampled between 2001 and 2003 during the present study.

reagents per sample with a total mix volume of 12.5 µl: 1.25 µl of 10 x PCR Buffer; 0.3 µl of 1.2 mM $MgCl_2$; 1.5 µl of 240 µM dNTPs; 1.25 µl of 0.5 pmol/µl F Primer; 1.25 µl of 0.5 pmol/µl R Primer; 0.25 µl of 0.5 units *Taq* polymerase; 1 µl of DNA and 5.7 µl of sterilised H_2O. The forward primer (5' to 3') of each pair was fluorescently labelled with one of the dyes, HEX, TET or 6-FAM.

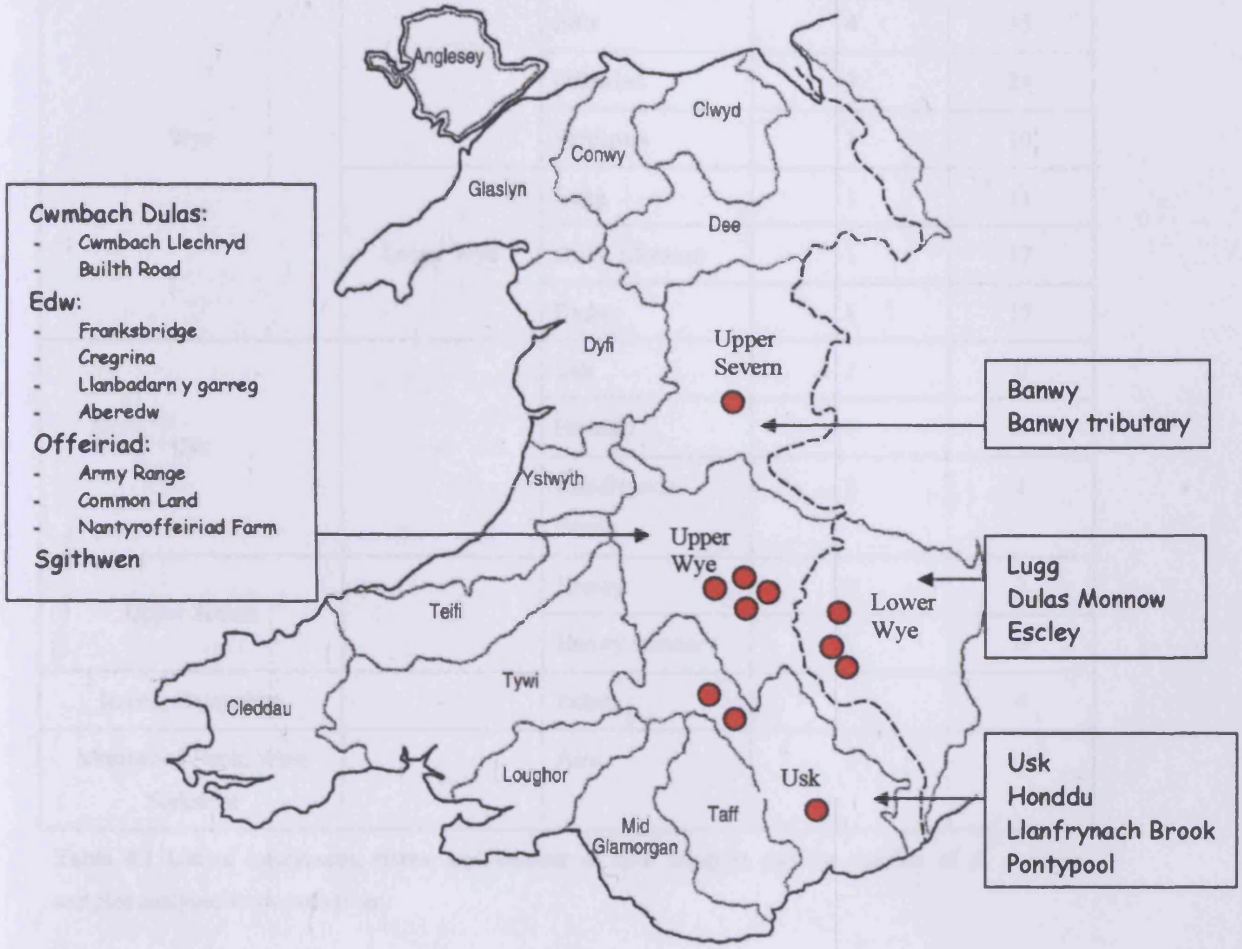


Figure 4.2 Map depicting rivers and sites sampled within the Upper Severn, Upper Wye, Lower Wye and Usk Catchments between 2001 and 2003.

Re-optimised PCR amplification conditions were as follows: 95°C for 3 minutes, 35 cycles of 95 °C for 30 seconds, specific annealing temperature of 60 °C for Ap1, 54°C for Ap2, 57 °C for Ap3 and 56 °C for Ap6 for 30 seconds, 72 °C for 45 seconds and a final extension of 72 °C for 30 minutes. 5 µl of PCR product with 1.5 µl of loading dye for each sample were electrophoresed on a 1.2-1.3 % agarose gel against a 500 bp ladder.

Catchment	Sub-catchment	Rivers sampled	Number of sites sampled	Sample size
Wye	Upper Wye	Cwmbach-Dulas	2	13
		Edw	4	35
		Offeiriad	3	24
		Sgithwen	1	10
	Lower Wye	Lugg	1	11
		Dulas Monnow	1	17
		Escley	1	17
Usk	-	Usk	1	0
		Honddu	1	2
		Llanfrynach Brook	1	1
Upper Severn	-	Banwy	1	2
		Banwy tributary	1	0
Itchen, Hampshire	-	Itchen	1	4
Meanwood Beck, West Yorkshire	-	Aire	1	10

Table 4.1 List of catchments, rivers, and number of sites sampled and the number of *A. pallipes* samples analysed from each river.

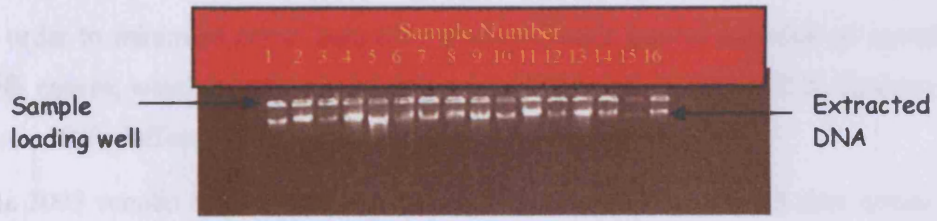


Figure 4.3 UV image displaying extracted DNA for 16 samples.

When optimised, 1 µl of PCR product was run with 10 µl of a solution made up of 0.5 µl of GeneScan-500 [ROX] size standard to 10 µl of Hi-di Formamide both from ABI (Applied Biosystems) per sample, on an ABI PRISM 3-100 sequencing machine at 60 °C for 2 hours and seven minutes using Pop 6 polymer and a 50 cm array, at 11 kV

Locus	Primer sequences (5'-3')	Repeat motif	Size range (bp)	Nall.	No. alleles (T_a)[H_O/H_E]
Ap1	F: TCTTGGGGATTGGCTAGTTG R: CCTGAACTAAAAGGTGCTTTGG*	$(CA)_{13}(CG)_2(CA)_6$	123-141	3	1 (60) [0 / 0]
Ap2	F: TTCGATATAACCGTTTGACCTG * R: TCAGACTTTGGCCATTGAAG	$(CA)_{31}$	151-191	7	3 (60) [0.35 / 0.5]
Ap3	F: CGCCTATCTAACCTTGGTTGTC * R: GGACTTGGGAAGCCTTGTG	$(CA)_{25}$	128-216	12	3 (60) [0.43 / 0.49]
Ap6	F: GCTGTGTGGGATGGAGGT * R: CACTAGCGTATTCAAGCAACT	$(TG)_7GGGT(TG)_8$ $GG(TG)_{40}TT(TG)_9$ $TT(TG)_7CA(TG)_3$	346-370	9	2 (56) [0.00 / 0.13]

Table 4.2 Characteristics of *A. pallipes* microsatellites where Nall. = total number of alleles per locus observed by Gouin; T_a = annealing temperature; H_O = observed heterozygosity; H_E = expected heterozygosity; * = 5' end-labelled primers (Gouin *et al.*, 2000).

and a mean current of 600 μA. Results were produced in the computerised form of electropherograms. Information was analysed in the package GENESCAN 3.7 (Applied Biosystems - ABI) and then exported to the package GENOTYPER 3.6NT (also from ABI) where results were further analysed.

4.3.4 River Habitat Surveys (RHS)

For each RHS, data were collected from a 500 m length of stream or river, by recording features of channel, banks and land up to 50 m from the waterway present at 10 "spot checks" every 50 m and between these spot checks using a "sweep up" checklist. Other features such as the shape of the valley and numbers of riffles are also recorded (Jeffers, 1998). This survey technique was described in p 36 of this thesis.

In order to minimize errors between surveyors, each person attended an accrediting RHS course, which has also enabled the compilation of a national RHS database (Fox *et al.*, 1998; Jeffers, 1998).

The 2003 version of the RHS form was therefore used to survey 53 sites across three catchments, the Wye, the Usk and the Upper Severn in Wales and the Marches.

4.3.5 Data Analysis

For analysis, the Wye Catchment was split into two halves, the Upper Wye, which includes the more upland rivers of the Welsh half, the Cwmbach Dulas, Edw,

Offeiriad and Sgithwen, and the Lower Wye, which includes more lowland rivers of the English half, the Lugg, Escley and Dulas Monnow (Figure 4.2).

Observed and expected heterozygosities, allele frequencies and F_{IS} statistics (Weir & Cockerham, 1984) were calculated using the analysis package GENEPOP ON THE WEB (Raymond & Rousset, 1995). Multi locus genotype frequencies (MLGs) and a pairwise allele sharing distance (ASD) (Bowcock *et al.*, 1994) had to be calculated by hand due to numerous gaps in the data. A pairwise ASD matrix was inserted into Molecular Evolutionary Genetic Analysis (MEGA) package Version 2.1 (Kumar *et al.*, 2001) and a Neighbour-Joining unrooted phenogram (Saitou & Nei, 1987) of Bowcock's individual pairwise genetic distances was produced as in Pryor *et al.*, (2001).

River Habitat Survey (RHS) data were stored in an EXCEL spreadsheet and split into three sections, "spot check," "sweep up" and "other variables." Data from left and right banks were summed. A Principal Components Analysis (PCA) was carried out to select the variables that account for the majority of data variation followed by an Ordinal Logistic Regression (Vaughan & Ormerod, 2005) in order to find out whether these variables account for the variation in allele frequencies of the 189 and 195 alleles at Locus Ap2.

4.4 RESULTS

4.4.1 Numbers of alleles

Seven of the primers designed by Gouin *et al.* (2000 and 2001) were originally used at the start of this study. Two of these, however, Ap5 and Ap7, both of which are polymorphic would not give results of a satisfactory quality, despite a prolonged effort. Ap5 and Ap7 were therefore discarded.

Ap1 and Ap4 were shown by Gouin *et al.* (2000) to be relatively monomorphic. These primers were therefore used on 20 samples in total taken from all five catchments. No variability was found as all results for these loci were monomorphic as suspected.

The remainder of the study was carried out with the three remaining polymorphic loci, Ap3, Ap2 and Ap6. Across the whole dataset at the three loci, a total of nine alleles were located: four, three and two alleles at the Ap3, Ap2 and Ap6 loci respectively. Population sample sizes ranged from one sample from Cregrina (of the Edw), the Honddu and Llanfrynach, to fifteen samples from the Dulas (Monnow). Due to the high degree of difficulty in obtaining quality results, however, there were a number of gaps in the dataset.

In the Ap3 locus, nine out of the 18 populations were polymorphic, the remainder were monomorphic. For the Ap2 locus, 14 populations were polymorphic, while at the Ap6 locus, only four populations were polymorphic.

4.4.3 Heterozygosity

Expected and observed heterozygosities calculated for the Ap2 locus were both relatively low with mean values across populations of 0.3 and 0.26 respectively.

Heterozygotes were found in 13 polymorphic populations at the Ap2 locus, in nine populations at the Ap3 locus and in four populations at the Ap6 locus. Heterozygotes for all three loci, heterozygotes were only found in two populations, the Dulas (Monnow), and the Lugg.

4.4.3.1 Within a river

Some differences in heterozygosity were apparent within some rivers. For example, in the Edw, an Upper Wye tributary, no heterozygotes were present in the most upstream site, Franksbridge at any of the three of the loci. At Llanbadarn-y-garreg further downstream in the Edw, observed heterozygosity was 0.25 at the Ap2 locus and 0.10

at the Ap3 locus. In Aberedw, the site nearest the confluence with the Wye, observed heterozygosity was again 0.25 at the Ap2 locus but it had also increased to 0.25 at the Ap3 locus. A similar downstream trend of increasing heterozygosity was also apparent at the Ap3 locus in the Offeiriad, another Upper Wye tributary. Here, observed heterozygosity increased from 0 at the most upstream site, the Army Range, to 0.14 in the mid stream site, Common Land, and 0.43 at the Nantyroffeiriad Farm Site farther downstream. However, the reverse occurred at the Ap2 locus where observed heterozygosity decreased from 0.40 to 0.20 to 0 at the same respective sites in a downstream direction.

In a third Upper Wye tributary, the Cwmbach Dulas, observed heterozygosity was slightly higher at the upstream site, Cwmbach Llechryd (0.25 at the Ap2 locus and 0.29 at the Ap3 locus) than at the downstream site, Builth Road (0.17 at the Ap2 locus and 0.25 at the Ap3 locus).

4.4.3.2 Between rivers

Using results of the Ap2 locus, the Anderson Darling Normality Test showed that observed heterozygosity values for the Upper Wye and Lower Wye followed a normal distribution ($p=0.266$ and 0.631 respectively). Bartlett's Test showed that observed heterozygosity values for the Upper and Lower Wye were homogeneous ($p=0.342$).

A 2-tailed t-test showed that the mean observed heterozygosity of the more upstream Upper Wye sites (0.24) was significantly lower than the mean of the downstream Lower Wye Sites (0.53) ($p=0.016$, $df=10$).

Statistical comparisons of observed heterozygosities between the entire Wye catchment and other sites were not possible due to the lack of quality results obtained in other catchments.

4.4.4 Allele frequencies at the Ap2 locus

The 187 allele at the Ap2 locus is present in three Upper Wye Sites, Llanbadarn y garreg, Aberedw (both of the River Edw) and the Sgithwen, but was absent from all Lower Wye sites (Figures 4.4 and 4.5).

The Anderson Darling Test for normality and Bartlett's Test for homogeneity were carried out and frequencies of allele 189 for both the Upper Wye sites and the Lower

Wye sites were found to be normally distributed (p = 0.126 and 0.126 respectively) and possess homogeneity or equality of variance (p = 0.762).

A 2-tailed t-test showed that the frequency of the 189 allele is close to significantly greater in the Upper Wye (mean frequency = 0.758) than in the Lower Wye (mean frequency = 0.416) (p = 0.058, df = 4).

The Anderson Darling Test for normality and Bartlett's Test for homogeneity carried out for frequencies of the 195 allele in both the Upper Wye sites and the Lower Wye sites showed data to be normally distributed (p = 0.126 and 0.126 respectively) and possess homogeneity or equality of variance (p = 0.762).

A two-tailed t-test showed that the mean frequency of the 195 allele was significantly lower in the Upper Wye (0.205) than in the Lower Wye Catchment (0.584) (p = 0.036, df = 4).

4.4.5 F_{IS} Values

Average inbreeding coefficient (F_{IS}) values were greater than zero in 12 of 17 populations sampled in the present study (Table 4.3).

The mean inbreeding coefficient was greater in the Upper Wye (0.306) than in the Lower Wye (-0.477), suggesting inbreeding had occurred in the Upper Wye, but that apparent outbreeding (represented by a negative F_{IS} value) occurred more commonly in the Lower Wye. Four sites, Nantyroffeiriad Farm (the most downstream Offeiriad Site), Franksbridge (the most upstream Edw site), both of the Upper Wye and the Honddu (an Usk tributary) and the Itchen (in Southern England), displayed F_{IS} values of 1 because only homozygotes were present at each of these sites.

In the Cwmbach Dulas, an Upper Wye tributary, apparent outbreeding occurred at both sites sampled. The more negative F_{IS} value at Cwmbach Llechryd, the most upstream site of this tributary indicated more apparent outbreeding occurring here than at the downstream site, Builth Road.

In the Edw (an Upper Wye tributary), F_{IS} was greatest (= 1) at Franksbridge, the most upstream site, while low levels of inbreeding occurred at the mid site, Llanbadarn y garreg (0.057) and relatively high levels of inbreeding from low numbers of heterozygotes occurring at the downstream site, Aberedw.

Although relatively high numbers of heterozygotes were present at the most upstream Offeiriad site (Army Range), inbreeding did occur here (F_{IS} =0.167). At the mid site, Common Land, some apparent outbreeding occurred (-0.111) while at the downstream site, Nantyroffeiriad Farm, an absence of heterozygotes found resulted in a high level of inbreeding (F_{IS} =1). Inbreeding also occurred on the Sgithwen (an Upper Wye tributary), Escley (a Lower Wye tributary), Banwy (an Upper Severn tributary), Honddu (an Usk tributary), Pontypool (an Usk tributary), Itchen (in Southern England) and Aire (in Northern England).

In the Lower Wye, both the Dulas Monnow and the Lugg display apparent outbreeding (-0.07 and –0.556 respectively) with high levels of heterozygosity. Apparent outbreeding appears to be particularly common in the Lugg at Mortimer's Cross.

Catchment	River	Site	n	H_E	H_o	F_{IS}
Upper Wye	Offeiriad	Army Range (upstream site)	5	0.480	0.400	0.167
Upper Wye	Offeiriad	Common Land (mid site)	5	0.180	0.200	-0.111
Upper Wye	Offeiriad	Nantyroffeiriad Farm (downstream site)	5	0.000	0.000	1.000
Upper Wye	Edw	Franksbridge (upstream site)	7	0.000	0.000	1.000
Upper Wye	Edw	Llanbadarn y garreg (mid site)	8	0.265	0.250	0.057
Upper Wye	Edw	Aberedw (downstream site)	8	0.508	0.250	0.508
Upper Wye	Cwmbach Dulas	Cwmbach Llechryd (upstream site)	4	0.219	0.250	-0.142
Upper Wye	Cwmbach Dulas	Builth Road (downstream site)	6	0.152	0.167	-0.099
Upper Wye	Sgithwen	Sgithwen	8	0.602	0.375	0.377
Lower Wye	Dulas (Monnow)	Dulas (Monnow)	15	0.498	0.533	-0.070
Lower Wye	Escley	Escley	14	0.336	0.286	0.149
Lower Wye	Lugg	Lugg	9	0.500	0.778	-0.556
Upper Severn	Banwy	Banwy	2	0.375	0.500	0.333
Usk	Honddu	Honddu	2	0.000	0.000	1.000
Usk	Pontypool	Pontypool	4	0.420	0.200	0.524
Itchen	Itchen	Itchen	4	0.000	0.000	1.000
Aire	Aire	Aire	10	0.495	0.300	0.394

Table 4.3. Number of samples (n), Expected heterozygosities (H_E), observed heterozygosities (H_o) and the average inbreeding coefficients (F_{IS}) for sites at the Ap2 locus.

4.4.6 Multi Locus Genotypes (MLGs)

Multi-locus diploid genotypes (MLGs, Pryor *et al.*, 2001) of the Upper Wye and Lower Wye *A. pallipes* populations were studied (Figures 4.4 and 4.5). In the River Edw, an Upper Wye tributary studied in detail, three MLGs (III, V and I) were present (Table 4.4, Figure 4.4). At Franksbridge (the most upstream site furthest from the confluence), only the homozygous MLG III was found, which consisted of the following alleles, 156, 189 and 360 at the Ap3, Ap2 and Ap6 loci respectively. At the

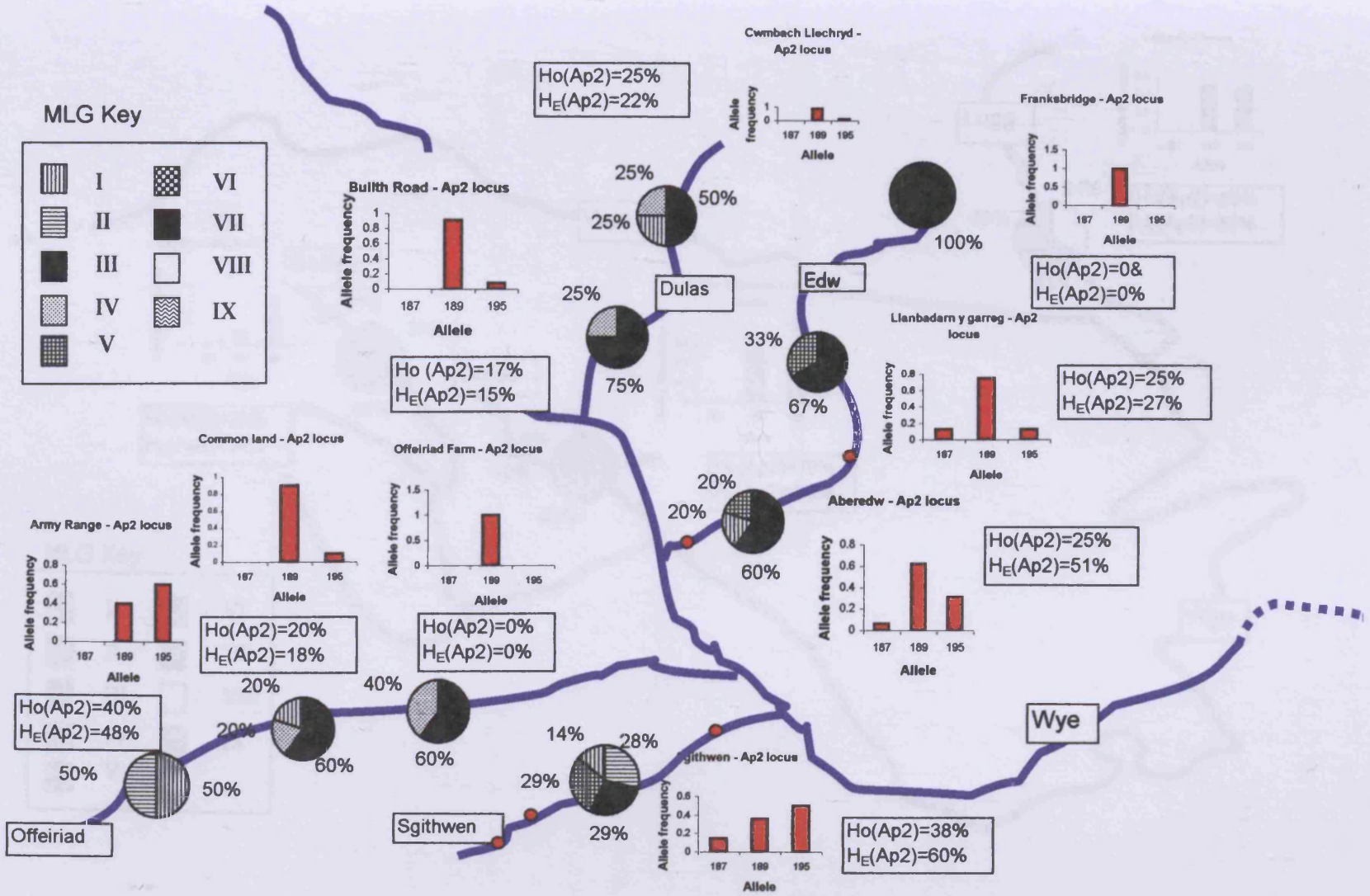


Figure 4.4 Map of Upper Wye Catchment rivers displaying plots of allele frequencies, observed and expected heterozygosities for the Ap2 locus and MLG pie charts. Red spots indicate other sites sampled.

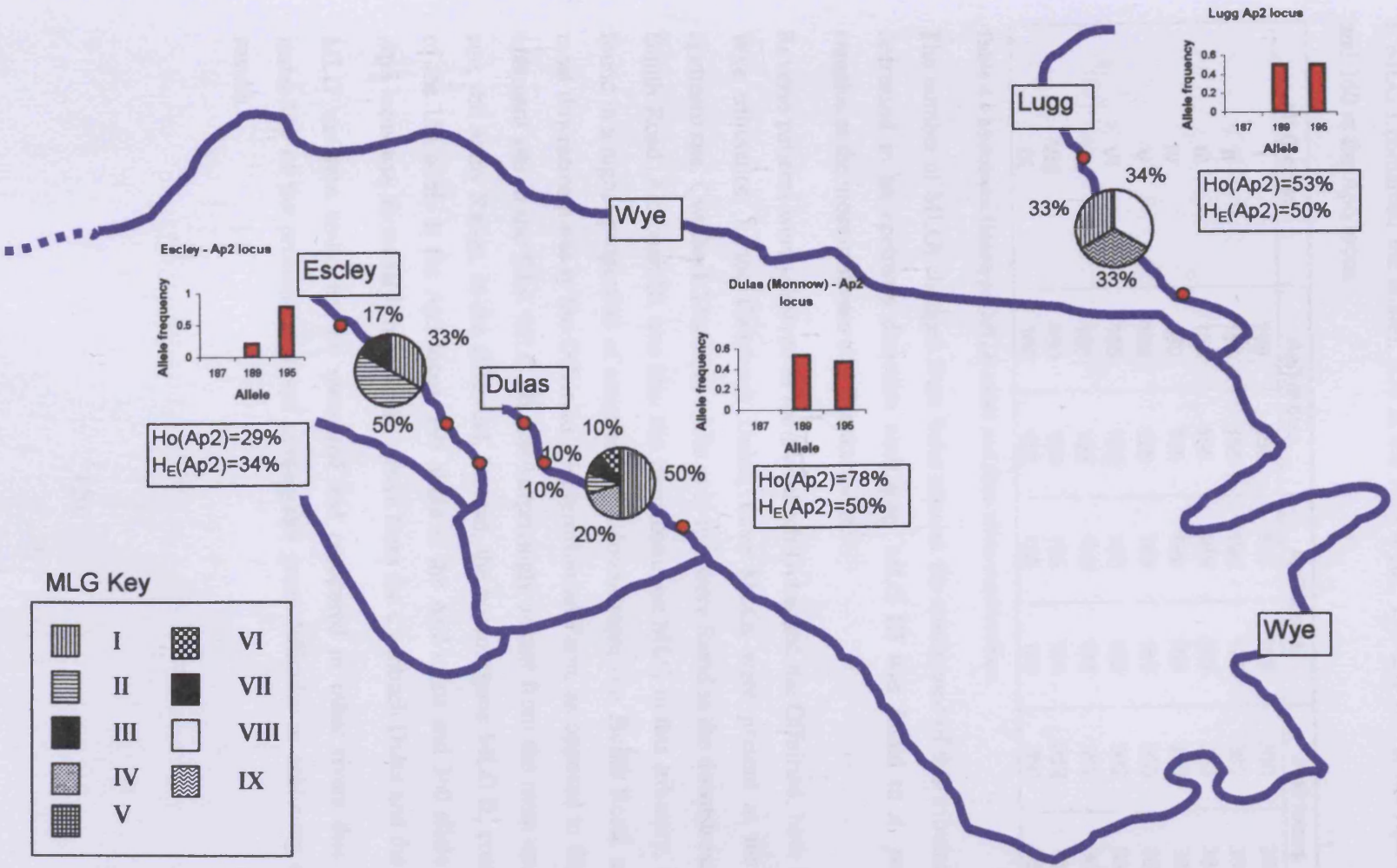


Figure 4.5 Map of Lower Wye Catchment rivers displaying plots of allele frequencies, observed and expected heterozygosities for the Ap2 locus and MLG (haplotype) pie charts. Red spots indicate other sites sampled.

mid site, Llanbadarn y garreg in the Edw, two MLGs were present, homozygous III being the most common and V, which contained alleles, 156 at the Ap3 locus, 189 and 187 at the Ap2 locus and 360 at the Ap6 locus. At Aberedw, the most downstream site of the Edw nearest the confluence with the Wye, three MLGs were found, with III again being the most common, followed in equal proportions by V and I. MLG I contained the alleles, 156 at the Ap3 locus, 195 and 189 at the Ap2 locus and 360 at the Ap6 locus.

MLG code	Ap3 locus		Ap2 locus		Ap6 locus	
I	156	156	195	189	360	360
II	156	156	195	195	360	360
III	156	156	189	189	360	360
IV	160	156	189	189	360	360
V	156	156	189	187	360	360
VI	156	156	195	189	362	360
VII	156	156	195	195	362	360
VIII	160	156	195	189	362	360
IX	160	156	195	189	360	360

Table 4.4 Multilocus Genotype (MLG) codes and their allele combinations.

The number of MLGs changed from three nearest the confluence of this tributary and decreased in an upstream direction until only MLG III was found in *A. pallipes* samples at the most upstream site, Franksbridge.

Reverse patterns were observed in the Cwmbach Dulas and the Offeiriad, both Upper Wye tributaries. In the Cwmbach Dulas, three MLGs were present at the most upstream site, Cwmbach Llechryd, while only two were found in the downstream site, Builth Road. Although III was also the most common MLG in this tributary, it was found in a higher proportion of samples in the downstream site, Builth Road, and the most downstream site in the Offeiriad, Nantyroffeiriad Farm, as opposed to the most upstream site in the Edw. MLG III was surprisingly absent from the most upstream site, the Army Range, in the Offeiriad. Instead, the homozygous MLG II, consisting of the 156 allele at the Ap3 locus, 195 allele at the Ap2 locus and 360 allele at the Ap6 locus was abundant here, but was absent from the Cwmbach Dulas and the Edw.

MLG variation could not be measured and compared in other rivers due to the instability of the primers used and consequent great difficulty in achieving quality results.

MLGs could not be established for samples from the River Itchen in Southern England or for the Banwy in the Upper Severn Catchment as results could only be obtained for two loci at these sites.

Homozygous MLG III was the most common in the Upper Wye, Aire and Usk Catchments (Figure 4.6) and was absent from only three of fourteen sites, the Army Range at the top of the Offeiriad, the Lugg and the Escley, all of which were located in the Wye Catchment.

The Anderson Darling Test for normality and Bartlett's Test for homogeneity were carried out and the proportion of samples being MLG III at each site in the Upper Wye and the Lower Wye were found to be normally distributed (where $p = 0.278$ and 0.057 respectively) and possess homogeneity ($p = 0.082$).

A two-tailed t-test showed that the mean proportion of samples being MLG III was significantly higher in the Upper Wye (0.557) than Lower Wye Catchment (0.033) ($p = 0.001$, $df = 9$).

4.4.7 Cluster Analysis based on Bowcock's Allele Sharing Distances (ASD)

4.4.7.1 Do individuals from the same river cluster together?

Samples from the most upstream site, Franksbridge (FR), of the River Edw, an Upper Wye tributary, cluster together in Group 1 of the dendrogram (Figure 4.7) indicating that these samples are genetically similar. Some samples from the mid-site, Llanbadarn y garreg (LL), of the Edw are also clustered in Group 1 indicating similarities with Franksbridge samples, while the remainder are found in Groups 3 and 4, thus displaying genetic differences. Samples of the most upstream site, Aberedw (AB), of the Edw nearest to the Wye confluence are, however, spread throughout the dendrogram and are present in Groups 1, 4, 8, 9, 11 and 12, indicating that many of these samples are genetically dissimilar from each other and from samples of other sites on the same river. The presence of a number different lineages (called the Wahlund effect), could explain the high F_{IS} value in this group of samples.

Similarly, samples from the most upstream site, Army Range (AR) on the Offeiriad River, another Upper Wye tributary, are spread throughout the Groups 2, 3, 8, 9, 10 and 12 on the dendrogram, indicating genetic diversity within this site. However, three samples from the mid site, Common Land (CL) and three from the most downstream site, Nantyroffeiriad farm, (FM) are clustered together in Group 1 of the

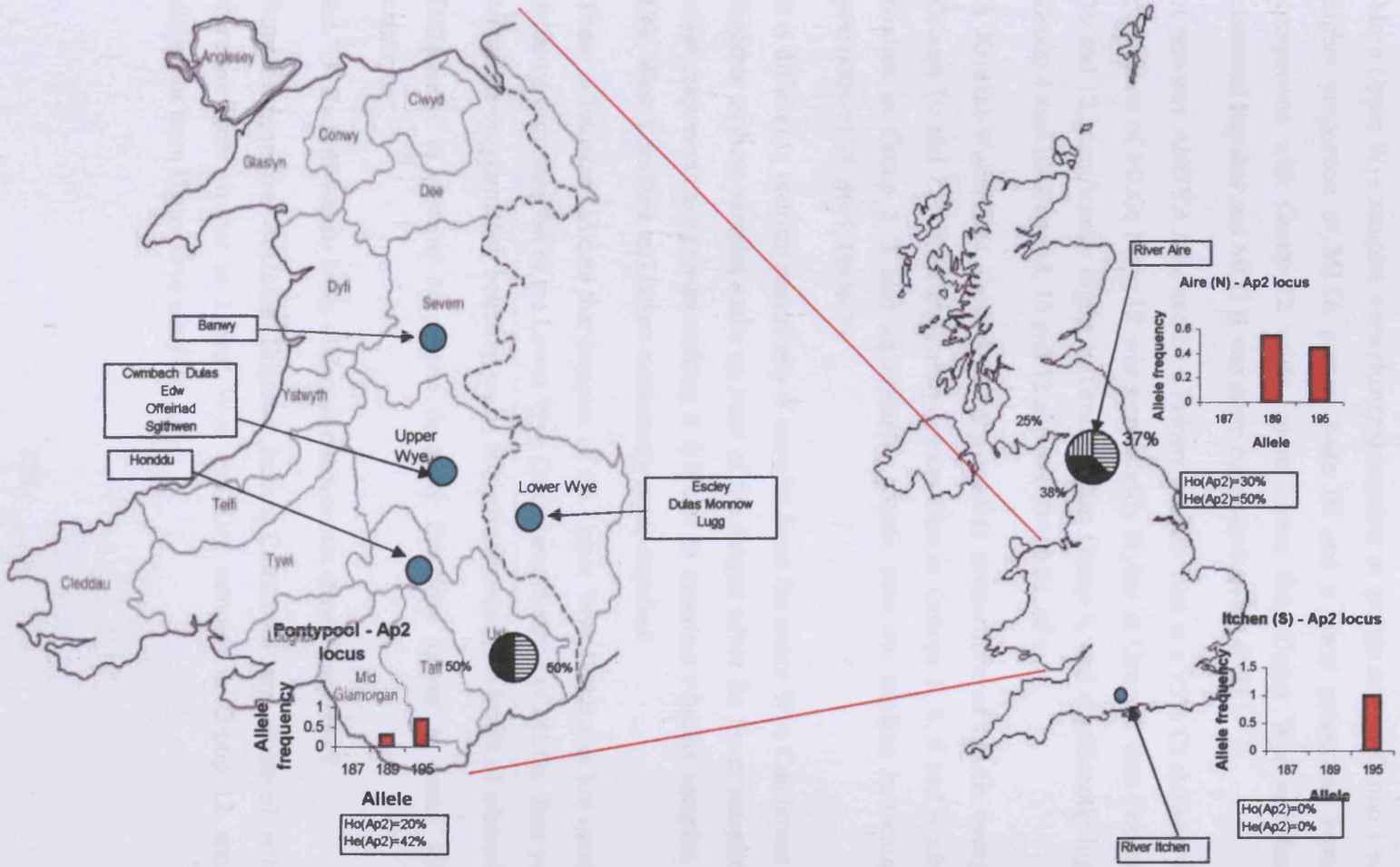


Figure 4.6 Maps showing locations of sampling sites with plots of allele frequencies, observed and expected heterozygosities for the Ap2 locus and MLG (haplotype) pie charts.

dendrogram indicating genetic similarities within and between these two sites and genetic dissimilarity between these samples and those of the most upstream site, AR. Results from both of these Upper Wye tributaries shown that genetic differences between sites occur within a river.

4.4.7.2 Do individuals from the same catchment cluster together?

More Upper Wye samples were clustered together in groups nearest Group 1 where a higher proportion of MLGs present were III and a lower proportion were II in comparison with Group 12 where, more Lower than Upper Wye samples were clustered together and MLG II was more common than MLG III.

A one-way ANOVA followed by a Fishers a priori Test at a 95% CI showed that the proportion of MLGs being III was significantly higher in Group 1 than Groups 6, 8, 10 and 12, significantly higher in Group 6 than Group 8, and significantly higher in Group 4 than in Groups 8, 10 and 12 at $p=0.000<<0.05$, $df = 5$.

A Kruskal-Wallis Test showed that the median proportions of MLGs being II in Groups 10 and 12 were significantly greater than in Groups 1, 4, 6 and 8 while the median in Group 8 is also significantly greater than the median in Group 1 at $p=0.000<<0.05$, $df=5$, $H=36.79$.

It is difficult to confirm statistically if samples from the entire Wye Catchment group together as these samples make up most of the dataset while far fewer samples from other catchments are present making it difficult to ascertain whether samples of the Usk, West Yorkshire and Itchen catchments group together.

These differences indicate that samples of the Upper Wye Catchment are genetically different from samples of the Lower Wye Catchment in terms of MLGs, their position on the dendrogram, their heterozygosity, allele frequencies and levels of inbreeding.

Differences in patterns of genetic diversity therefore appear to occur within a catchment.

4.4.7.3 Do individuals from different catchments cluster together?

Samples from the Southern England Itchen Catchment are clustered with and therefore most similar to Lower Wye and Usk samples in Group 12 and most dissimilar from Upper Wye samples.

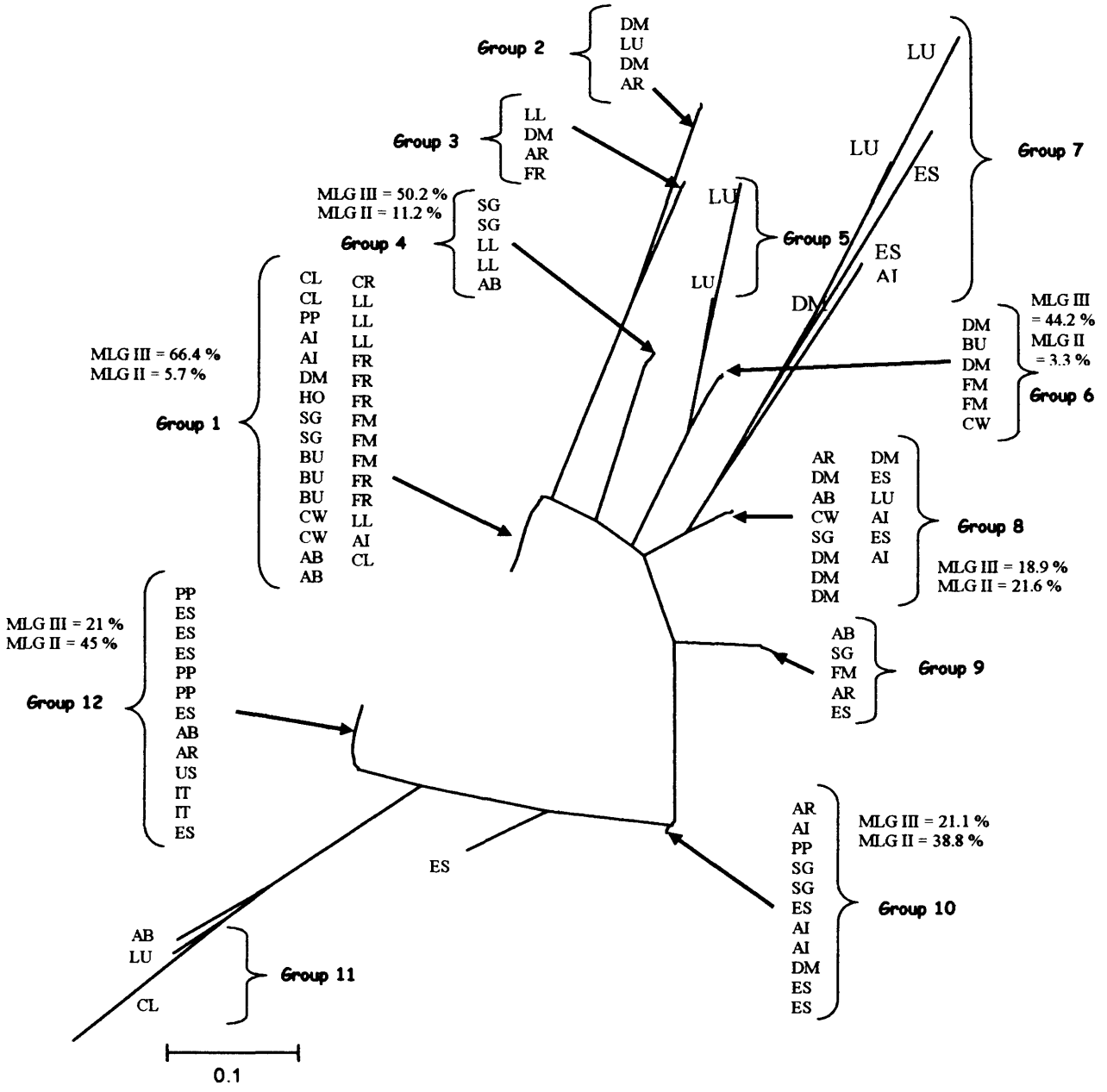


Figure 4.7 Neighbour-Joining Dendrogram based on Bowcock's allele sharing distances of sample sites and mean percentages of MLG II and III of samples clustered in Groups 1, 4, 6, 8, 10 and 12. In the Upper Wye: AR=Army range, Offeiriad (top site); CL=Common land, Offeiriad; FM=Nantyroffeiriad Farm, Offeiriad (lowest site); CW=Cwmbach Llechryd, Dulas (top site); BU=Builth Road, Dulas (lower site); FR=Franksbridge, Edw (top site); AB=Aberedw, Edw; CR=Cregrina, Edw; Ll=Llanbadarn, Edw (lower site); SG=Sgithwen. In the Lower Wye: LU=Lugg; DM=Dulas (Monnow); ES=Escley. In the Usk Catchment: PP=Pontypool; US=Usk; HO=Honddu. Also, IT=Itchen and AI=Aire.

However, only two Itchen samples are included in this part of the analysis. More replication is therefore required here to prevent misleading results.

Some samples from the Northern England Aire Catchment are clustered with and therefore genetically similar to Upper Wye samples of Group 1. However, other Aire samples are spread throughout the dendrogram in Groups 7, 8 and 10 indicating genetic diversity of *A. pallipes* within this catchment.

Aire samples also appear to be genetically dissimilar to Itchen samples as they are not clustered on the dendrogram.

These results therefore indicate differences in levels of genetic diversity exist between catchments.

4.4.8 River Habitat Survey (RHS) variables associated with 189 and 195 allele frequency

A PCA (Principal Components Analysis) followed by an ordinal logistic regression showed that particular RHS (River Habitat Survey) variables were significantly positively or negatively associated with the 189 allele frequency and 195 allele frequency both of the Ap2 locus. These variables were listed in Tables 4.5 and 4.6.

Habitat characteristics significantly positively associate with 189 allele frequency were earth bank, bedrock, broadleaf woodland (5 m land use) and bedrock substrate ($z = 2.04$, $p = 0.041$, odds ratio = 1.85). Habitat characteristics significantly negatively associated with 189 allele frequency were simple vegetated bank face, rip rap bank, cobble channel substrate, broken water flow type ($z = -2.25$, $p = 0.025$, odds ratio = 0.43), bare bank face, scrub and shrub (5 m land use) ($z = -2.95$, $p = 0.003$, odds ratio = 0.23), fringing reed beds, channel choked with vegetation, concave valley form, numbers of riffles and numbers of pools ($z = -2.13$, $p = 0.033$, odds ratio = 0.56).

Habitat characteristics significantly positively associated with 195 allele frequency were simple vegetated bank face, rip rap bank, cobble channel substrate, broken water flow type ($z = 2.03$, $p = 0.042$, odds ratio = 2.07), bare bank face, scrub and shrub (5 m land use) ($z = 2.86$, $p = 0.004$, odds ratio = 3.68), fringing reed beds, channel choked with vegetation, concave valley form, numbers of riffles and numbers of pools ($z = 2.13$, $p = 0.033$, odds ratio = 1.79). Characteristics significantly negatively associated with 195 allele frequency were broadleafed woodland (50 m land use), improved grassland and composite bank ($z = -2.06$, $p = 0.039$, odds ratio = 0.52).

Variable number	Variable type	Variable	+ive/-ive association	p-value
1	Spot check variable	Earth bank	+	0.041
2	Spot check variable	Bedrock bank	+	
3	Spot check variable	Broadleaf woodland 5m land use	+	
4	Spot check variable	Bedrock channel substrate	+	
5	Spot check variable	Simple vegetated bank face	-	0.025
6	Spot check variable	Rip rap bank	-	
7	Spot check variable	Cobble channel substrate	-	
8	Spot check variable	Broken water flow type	-	
9	Spot check variable	Bare bank face	-	0.003
10	Spot check variable	Scrub and shrub 5m land use	-	
11	Other variables	Fringing reed beds	-	0.033
12	Other variables	Channel choked with vegetation	-	
13	Other variables	Concave valley form	-	
14	Other variables	Number of riffles	-	
15	Other variables	Number of pools	-	

Table 4.5 Table displaying the positive (+) or negative (-) association of the 189 allele frequencies with RHS variables selected using a PCA (principal components analysis) followed by an ordinal logistic regression.

Variable number	Variable type	Variable	+ive/-ive association	p-value
5	Spot check variable	Simple vegetated bank face	+	0.042
6	Spot check variable	Rip rap bank	+	
7	Spot check variable	Cobble channel substrate	+	
8	Spot check variable	Broken water flow type	+	
9	Spot check variable	Bare bank face	+	0.004
10	Spot check variable	Scrub and shrub 5m land use	+	
11	Sweep up variable	Broad leaf woodland 50m land use	-	0.039
12	Sweep up variable	Improved grassland	-	
13	Sweep up variable	Composite bank	-	
14	Other variables	Fringing reed beds	+	0.033
15	Other variables	Channel choked with vegetation	+	
16	Other variables	Concave valley form	+	
17	Other variables	Number of riffles	+	
18	Other variables	Number of pools	+	

Table 4.6 Table displaying the positive (+) or negative (-) association of the 195 allele frequencies with RHS variables selected using a PCA (principal components analysis) followed by an ordinal logistic regression.

The 189 allele frequency is significantly higher in sites of the Upper Wye than the Lower Wye, while the 195 allele frequency is significantly higher in Lower than Upper Wye sites as previously described. RHS variables positively associated with

the 189 allele frequency are therefore more common in the Upper than Lower Wye, while those variables positively associated with the 195 allele frequency are more common in the Lower Wye than Upper Wye sites.

4.5 DISCUSSION

4.5.1 Within Rivers

Genetic diversity (in terms of observed and expected heterozygosity) decreased in a downstream direction in the Offeiriad and the Cwmbach Dulas, both of which are Wye tributaries. The most upstream site on the Offeiriad is located high in the uplands within a restricted army range (hence the site name, "Army Range") and is very near to the source. Although sheep are present here, the land consists of rough pasture with no improved grassland. This section of stream is therefore relatively unpolluted, and has suffered very little habitat degradation, destructive interference from humans or invasion of crayfish (due to its large distance from the confluence) and is unlikely to be contaminated with crayfish plague due to the highly restricted access and consequent prevention of humans transmitting the disease on boots or equipment or by introducing signal crayfish. A lack of these human induced pressures would allow the crayfish population at this site to flourish thus possibly explaining the relatively high levels of *A. pallipes* genetic diversity at this site. A small lake is situated directly adjacent to this section of stream in residential grounds. It is entirely possible that introductions made to this lake over time supplemented the stream's population, thus increasing genetic diversity.

The lower sites of the Offeiriad, "Common Land" and "Nantyroffeiriad Farm" are located downstream of a culvert under a road, through which the stream flows. Samples from sites downstream of the culvert contain MLG III and are clustered together on the Neighbour-Joining dendrogram thus displaying allele sharing through movement of individuals between sites. However, samples from these sites are not clustered with samples from the Army Range Site upstream of the culvert which also do not contain MLG III, suggesting that alleles are dissimilar upstream and downstream of the culvert indicating little movement of individuals through the culvert in an upstream direction.

Culverts, particularly those whose base is above the water level of the stream, by 5 cm in this instance, can restrict the upstream movement of some macroinvertebrate species as a result of increased water velocity due to channelisation of the water and the step up from the stream bed to the culvert base (Vaughan, 2002). It is therefore

likely that they also restrict the upstream movement of *A. pallipes* e.g. MLG III individuals.

Culverts also allow pollutants such as salt from the road, silt and soot to enter the stream (Vaughan, 2002). The culvert is at the bottom of a small valley down which the road passes. Salt containers are positioned at the roadside at the top of each valley side and are regularly used to prevent this high altitude road from freezing in the winter. Relatively large quantities of salt and road pollutants are therefore likely to be entering the stream.

Also, large quantities of logs were stored on open ground, directly adjacent to the stream at the Common Land Site. Some logs were carelessly dropped into the water and left for a number of days (investigated by Ray Woods, CCW). Logging vehicles moved regularly up and down the valley alongside the stream, causing large quantities of excess silt to enter the water and deposit out onto the stream bed a factor thought to be detrimental to *A. pallipes* populations by filling in their refuges as explained in Chapter 3. Phenolics seeping from the piled logs into the stream could have damaged aquatic life. Although sample collection for this chapter occurred prior to this incident, it is highly likely that similar logging practices have been previously carried out and could have contributed to pressures on crayfish genetic diversity.

This could explain the gradual reduction in genetic diversity of sites in a downstream direction in the Offeiriad Stream, particularly below the culvert.

A crayfish stocked lake was also within a small distance of the Cwmbach Dulas, another Wye tributary. Records have proved movement of *A. pallipes* down a small side tributary from the lake, thus boosting *A. pallipes* numbers and genetic diversity in the Dulas, particularly at downstream sites e.g. Builth Road. Increasing quantities of pollution and silt, due to improved grassland with livestock being the main land use along this river entering the river as it flows could have caused a greater genetic diversity in the upper site, "Cwmbach Llechryd" than in the more downstream site, "Builth Road."

In contrast, genetic diversity (in terms of heterozygosity and number of MLGs) decreased in an upstream direction in the Edw, a third Upper Wye tributary. This could have been a result of a greater movement of *A. pallipes* individuals in a

downstream than an upstream direction due generally to the relatively high flow rates of this rather wider tributary particularly during rainy periods and flooding events.

A. pallipes was once abundant in the main stem of the River Wye (Foster, 1996) and probably contained a natural source of genetically diverse individuals. It is therefore likely that due to greater difficulty in moving in an upstream direction against a current, fewer individuals would have reached the furthest, most upstream Edw site, Franksbridge, than Aberedw, the site nearest the confluence with the Wye. Individuals in Aberedw were therefore likely to be more genetically diverse than those located further from the confluence, a once replenishing source of genetic material from the main Wye (Hutchinson & Templeton, 1999).

Gouin *et al.* (2002) agree with Schutze *et al.* (1999) that crayfish are more likely to move upstream during the summer months when water levels and flow speed are lower, but also suggest that some crayfish may have a greater adaptive ability to move upstream and are therefore more likely to maintain their genes in the population. It is therefore possible that crayfish with a higher frequency of the 189 allele at the Ap2 locus are better at moving upstream than those with a high 195 allele frequency, thus explaining why those found at more upstream sites in the River Edw have a much higher 189 allele frequency. Microsatellites, however, are non-functional markers which do not usually confer adaptive value to the individuals who possess particular alleles (M. Bruford, pers. comm.). Although possible, it is statistically unlikely that microsatellites used in the present study may be linked to genes related to dispersal ability, particularly as only three microsatellites were used.

Data regarding actual tracking of *A. pallipes* individuals' movements within and between populations is limited and more is required (Gouin *et al.*, 2002) to confirm the reasons for any genetic variation within rivers.

A radio-tracking study in Germany found that the noble crayfish, *Astacus astacus*, moved both upstream and downstream, but if they were disturbed, they tended to migrate in a downstream direction, possibly hundreds of metres per night (Bohl, 1999). It is therefore likely that *A. pallipes* individuals in the Edw move in a downstream direction. This, together with the 100% homozygosity and high levels of inbreeding could explain why diversity is very low at the upstream site.

The increase in levels of genetic diversity in a downstream direction in the Edw was emphasised in the results of the Neighbour Joining Dendrogram.

Samples from Franksbridge were found to be genetically similar to each other and to some samples from the mid site, Llanbadarn, while other Llanbadarn samples were dissimilar. Samples from the most downstream Edw site, Aberedw, showed some similarities but also many dissimilarities from the upper sites. These findings indicate that *A. pallipes* individuals move between sites, but generally in a downstream direction in the Edw and that differences in genetic diversity were found between sites, within the Wye tributary Rivers Edw, Cwmbach Dulas and Offeiriad.

Hypothesis 1 that levels of genetic diversity of *A. pallipes* were similar throughout the length of a tributary river was therefore rejected.

In a radio-tracking study, in the River Sempt, Germany, restocked *Astacus astacus* moved in a downstream direction with no reasonable explanation other than the direction of current flow (Schutze *et al.*, 1999). However, other studies disputed this. For example, total and mean distances travelled by radio-tracked *A. pallipes* individuals did not differ between upstream and downstream directions in a North Yorkshire stream (Robinson *et al.*, 2000), while an equal distribution of upstream and downstream *A. pallipes* movements were found in a Tuscan stream (Gherardi *et al.*, 1998).

Environmental pressures, namely intensive livestock farming, heavy rainfall and steep landscape common to the area have resulted in excess siltation, sheep dip and organic pollution have probably helped to reduce genetic variation of *A. pallipes* in the upper reaches of the Edw.

Genetic structure therefore does occur within a river as was discovered in a French Brook by Neveu (2000). Neveu (2000) suggested that habitat structuring was thought to have controlled *A. pallipes* distribution and consequently caused genetic differences within the French Brook.

4.5.2 Within a Catchment

Genetic structuring was also evident on a higher level within the Wye Catchment which could be split into two genetically dissimilar sections, the Upper Wye, consisting of the more upstream tributaries, the Cwmbach Dulas, Edw, Offeiriad and Sgithwen of the Welsh half of the Wye, and the Lower Wye, consisting of the more

downstream tributaries, the Escley and Dulas of the Monnow sub-catchment and the Lugg, located in the Marches. The Upper Wye *A. pallipes* populations generally displayed a lower level of heterozygosity (0.24), a high 189 allele frequency, high proportions of the homozygous MLG III, a higher level of inbreeding and relatively low levels of genetic diversity, with samples more clustered near Group 1 of the Neighbour-Joining dendrogram, whereas, the populations of the lower Wye displayed significantly higher levels of heterozygosity (0.53), a higher frequency of the 195 than 189 allele, higher proportions of the heterozygous MLG I, than homozygous MLG III, lowest levels of inbreeding and more apparent outbreeding, with samples clustered nearer to Group 12, the opposite end of the Neighbour-Joining dendrogram. *A. pallipes* samples of the Upper Wye are therefore generally genetically dissimilar from samples of the Lower Wye.

Hypothesis 2 that levels of genetic diversity were similar throughout a catchment should therefore be rejected.

Results suggest that the Lower Wye Catchment supports relatively high levels of *A. pallipes* genetic diversity because of greater numbers of crayfish and reduced environmental pressures. *A. pallipes* populations of the Dulas and Escley Rivers in the Monnow sub-catchment and the River Lugg particularly at Mortimer's Cross (where most of the samples were collected) which make up the Lower Wye, are therefore very important in terms of genetic diversity and could provide donor individuals for restocking.

Although the Upper Wye supports relatively low levels of diversity, it remains an important region for *A. pallipes*, particularly as the Offeiriad's uppermost site, the "Army Range" and the Sgithwen River both support relatively high levels of genetic diversity, more consistent with the Lower Wye and therefore could also provide good donor populations. It is possible that these two sites together with those from the Lower Wye have retained high levels of genetic diversity due to good habitat quality and low pollution levels. A cypermethrin pollution incident struck the Sgithwen River in 1996 and destroyed many of its invertebrates including *A. pallipes* (Wilkins, 1998). This incident was however, a single event as the cypermethrin was being used to treat a pack of hunting dogs, and was not regularly used here as the river is mainly surrounded by woodland rather than farmland. Few pollution incidents meant that *A.*

pallipes numbers recovered and genetic diversity remained due probably to the survival of many upstream individuals.

Upper Wye populations, more isolated and fragmented than Lower Wye populations, were more susceptible to stochastic events and inbreeding leading to a loss of genetic diversity. Such a situation has also occurred with many other *A. pallipes* populations e.g. in Southern Italy (Paolucci *et al.*, 2002) and in Northern Italy (South Tyrol) (Baric *et al.*, 2004) and with many other species, for example, the red-cockaded woodpecker in Southeastern USA (Stangel *et al.*, 1992; Kulhavy *et al.*, 1995 and Daniels *et al.*, 2000)

In a study of brook charr (*Salvelinus fontinalis*) in Canadian Lakes, environmental variables partly accounted for genetic variation found among the lakes (Angers *et al.*, 1999). In the present study, environmental variables in the form of River Habitat Survey characteristics could offer some explanation for the genetic variation (in terms of 189 and 195 allele frequency variation) present between the Upper and Lower Wye Catchments, as they were used to explain the presence and absence of *A. pallipes* in a previous study by Naura & Robinson (1998). These findings could possibly indicate whether the 189 or the 195 allele would be the most common in an *A. pallipes* population using habitat characteristics. This is, however, difficult to confirm, as it may again be statistically unlikely that microsatellites used in the present study are linked to genes related to ability to survive in particular environmental conditions, as only one microsatellite was used in this part of the study.

Habitat characteristics positively associated with the 189 allele frequency i.e. earth and bedrock banks, broadleafed woodland and bedrock channel substrate, were typical of Upper Wye rivers together with characteristics negatively associated with the 195 allele frequency, i.e. broadleafed woodland (50 m land use), improved grassland and composite banks. Upper Wye land use is mainly livestock farming. In such regions, improved grassland is abundant and earthen banks are poached by livestock, resulting in excess siltation and a high incidence of sheep dip pollution. It is possible that *A. pallipes* individuals containing the 189 allele are more adaptable to these environmental pressures than those containing the 195 allele, which could explain why higher 189 allele frequencies were found in the Upper Wye than in the Lower Wye where these pressures are less pronounced.

Habitat characteristics positively associated with the 195 allele frequency i.e. simple vegetated bank face, rip rap, cobble channel substrate, broken water, scrub and shrub (5 m land use), fringing reed beds, concave valley form, numbers of riffles and pools were more typical of the Lower Wye, negatively associated with the 189 allele frequency and indicate good quality *A. pallipes* habitat. Cobble channel substrate, pools and riffles suggest that boulders suitable for crayfish refuges are present, while a simply vegetated bank face suggests a lack of erosion from a lack of livestock farming, resulting in less siltation and less sheep dip pollution. Scrub and shrubs, particularly alder (*Alnus glutinosa*), sometimes hazel (*Corylus avellana*) and willow (*Salix* spp.), amongst their exposed, underwater roots, provide protection from predators, being washed away and a nursery area for young crayfish (Smith *et al.*, 1996), together with detritus and invertebrates on which they feed (Naura & Robinson, 1998).

Results of the present study therefore show that within catchment variation in *A. pallipes* genetic diversity is present between rivers and suggest that these differences are influenced by habitat characteristics. Hypothesis 4 that particular habitat characteristics were significantly associated with differences in *A. pallipes* genetic diversity should consequently be accepted.

These results show that the Lower Wye sites, containing habitat characteristics more positively associated with the 195 allele, are more favourable to *A. pallipes* abundance and genetic diversity and would provide suitable donor populations in rearing, breeding and restocking programmes, which will be discussed in detail in Chapters 5 and 6.

4.5.3 Between Catchments

A. pallipes populations of the Aire Catchment in northern England were genetically similar to those of the Lower Wye, perhaps due to similar habitat characteristics, but genetically dissimilar from those of the more isolated, intensively farmed Upper Wye.

Also, the *A. pallipes* population of the River Itchen of the Itchen Catchment, Hampshire, southern England was genetically dissimilar from other populations sampled in this study, as all individuals were homozygous but for the 195 allele rather than the 189 allele at the Ap2 locus. Samples from the Itchen are therefore genetically

unique and show high levels of inbreeding. Hypothesis 3 that levels of *A. pallipes* genetic diversity differed between catchments was therefore accepted.

4.6 CONCLUSIONS

Genetic structuring of *A. pallipes* was present at three levels, within a river, within a catchment and between catchments.

Within stream genetic differentiation of *A. pallipes* was present in the River Edw, an Upper Wye tributary, as levels of genetic variation decreased in an upstream direction with increasing distance from the confluence. This also indicates that *A. pallipes* individuals moved in a general downstream direction.

The reverse of this trend in the Cwmbach Dulas and Offeiriad rivers can be attributed to human influence such as the stocking of adjacent lakes and the presence of a culvert which acts as a barrier to *A. pallipes* movement.

Crayfish in the Wye Catchment were split into two genetically different groups, the Upper Wye, consisting of the Cwmbach Dulas, Edw, Offeiriad and Sgithwen and the Lower Wye Consisting of the Escley and Dulas of the Monnow Sub-catchment and the Lugg, although the top site of the Offeiriad (Army Range) was genetically more similar to the Lower Wye than the Upper Wye in which it is located.

The Lower Wye plus Army Range Site is significantly more genetically diverse than the Upper Wye in terms of heterozygosity, 195 allele frequency and number of MLGs, possibly as a result of greater population isolation in the Upper Wye than Lower Wye and more livestock farming resulting in more sheep dip pollution and habitat damage such as excess siltation and bank erosion in the Upper Wye than in the Lower Wye.

Lower Wye sites, containing habitat characteristics more positively associated with the 195 allele, are more favourable to *A. pallipes* abundance and genetic diversity than Upper Wye sites and would provide suitable donor populations in rearing, breeding and restocking programmes.

A. pallipes populations were found to differ between catchments as individuals of the River Itchen of the Itchen Catchment, Hampshire, southern England were genetically dissimilar from all other catchments as they were much less genetically diverse and contained only a single genotype at the Ap2 locus.

Clearly there is genetic structuring at all scales within *A. pallipes* populations sampled. This has implications if sites are to be restocked. One option to retain

genetic structure would be to rear animals from residuals populations. In the next chapter I explore the feasibility of this option.

CHAPTER 5

Rearing *A. pallipes* in captivity for restocking purposes.

SUMMARY

Austropotamobius pallipes populations have declined dramatically in the past few decades as a result of plague, North American signal crayfish (*P. leniusculus*) invasion, sheep dip pollution and habitat destruction. Some rivers are now recovering as a result of legislation enforcement and habitat improvements. Restocking of *A. pallipes* populations in recovered rivers could help to conserve this declining species. Animals should be sourced and bred in captivity in order to provide the numbers required for restocking.

Relatively low success rates have previously been achieved in rearing *A. pallipes* in Britain, and mortality is particularly pronounced during the first stages of life. Crayfish rearing studies abroad have shown that a fresh, high protein diet positively influences hatchling survival. Crayfish were reared successfully in Australia using the algal food supplement, *Spirulina* (D. Jerry pers. comm.). A diet of fresh, natural food including diatoms with a supplement of *Spirulina* was therefore used in the present study to attempt to rear *A. pallipes* in captivity.

Although artificial incubation techniques used abroad have produced good survival rates, it was feared that the transfer of hatchlings from incubators into cooler British rivers would result in mass mortalities. This experiment was therefore carried out without incubation.

Most mortalities occurred before or during hatching of 657 eggs. 14.2 % of eggs hatched over a mean hatching period of 12 days. The majority of individuals that survived hatching, also survived to become summerlings, with 10.8 % of original egg stock progressing to summerling stage. These results strongly indicate that the abundant fresh food diet could account for the increased improvement in hatchling survival rate of this experiment when compared with previous attempts.

Growth and survival rates of *Spirulina* supplemented *A. pallipes* juveniles were found to be slightly greater than those not receiving the supplement, but not significantly so. If *Spirulina* had been provided in a more edible pellet form, this increase may have been more significant. Alternatively, sufficient protein may have already been provided in the main part of the diet without the *Spirulina* supplement.

5.1 AIMS

The aims of this chapter were:

- to investigate whether *A. pallipes* could be reared successfully from eggs in captivity without artificial incubation,
- to find out if *A. pallipes* could be reared using a combined diet of artificial and fresh food collected from local rivers,
- to establish the influence of a *Spirulina* supplement on crayfish hatchling growth rate and survival during rearing.

5.2 INTRODUCTION

The decline of *A. pallipes* populations in Wales has been well documented over the last decade (e.g. Holdich, 1993; Rogers & Holdich, 1995; Slater, 1998; Slater *et al.*, 1998; Wilkins, 1999; Slater, 2002). In 2000, *A. pallipes* were no longer found at 72 % of sites in the Usk and Wye Catchments where they were originally recorded (Coley, 2000). Although crayfish plague and invasion of the signal crayfish has been a problem in Wales, other factors, such as pollution from synthetic pyrethroid sheep dip, particularly cypermethrin and habitat degradation as a result of excess silt deposition (Slater, 2002) are thought to have largely contributed to the *A. pallipes* decline, (discussed in Chapter 3).

Organisations such as the Wye Foundation, Countryside Council for Wales (CCW) and the Environment Agency (EA) are currently working closely to clean up and improve affected rivers. For example, the Wye Habitat Improvement Project (WHIP), a river corridor fencing and coppicing scheme, was carried out in 1999 on the River Edw and other Wye tributaries, limiting livestock access to the water, to help prevent bank erosion and excess siltation (Wye Foundation, 1999). A similar scheme was also carried out on the Monnow Subcatchment, while more are planned at other sites. Together with the Groundwater Regulations implemented in 1999 to enforce safe sheep dip use and disposal (Environment Agency, 2000), it was hoped that these measures would improve the rivers. These improvements are thought to be responsible for the recovery of crayfish numbers on the River Edw (Howells, 2003). Perhaps, due to these mitigation measures, other rivers from which *A. pallipes* have

disappeared will soon be of a sufficient quality to support native crayfish once more, providing that the spread of signal crayfish and crayfish plague is controlled.

In order to facilitate the recovery of *A. pallipes* in improved rivers, restocking should be carried out (Kemp *et al.*, 2003). Animals should, however, first be sourced by assessing genetic variability of existing populations (discussed in Chapter 4). Once genetically robust source populations have been identified, a number of individuals should be taken from these sources, bred in captivity and offspring used to restock recovered rivers where *A. pallipes* are absent or where numbers are low. This would enable genetically diverse *A. pallipes* populations to be produced and maintained, thus promoting the survival of this currently declining species.

The life cycle of *A. pallipes* is such that it should, in theory, be relatively easy to breed. Mature males and females, which have a carapace length of >25 mm (Lowery, 1988), should be selected for breeding in late September or early October. Copulation occurs during October and November (Cuellar & Coll, 1978), when the female is turned onto her back by the male who secretes seminal fluid onto her abdomen where it stays, before she retreats to her refuge e.g. a burrow in the river bank (Clegg, 1985). The eggs are laid in this refuge 15 to 30 days later (Cuellar & Coll, 1978), become attached to her pleopods (swimmerets) and are fertilised by the stored male sperm soon after (Clegg, 1985).

After mating in the autumn, fertilised eggs remain attached to the underside of the female's abdomen over the winter months where they develop whilst being protected, until they hatch in May or June. The larger the crayfish, the larger the egg clutch size (Lowery, 1988). Older females are therefore more likely to produce more young and so could be more beneficial in a rearing and restocking programme.

When the young hatch, they cling to the underside of the abdomen until they reach a carapace length of approximately 3.5 mm. During this time they undergo two moults and are aquiring food from the yolk mass (Lowery, 1988).

Unfortunately, survival rates of eggs and hatchlings are particularly low during the first few phases of life when reared in captivity (Mason, 1977; Cuellar & Coll, 1978; Taugbol & Skurdal, 1989; Taugbol & Skurdal, 1992; Rhodes, 1981; Perez *et al.*, 1999 and Saez-Royuela *et al.*, 1995). The present study therefore focuses on this section of the life cycle, in an attempt to increase survival rates.

The use of artificial incubation techniques abroad have produced good survival rates (Mason, 1977; Cukerzis *et al.*, 1978; Rhodes, 1981; Carral *et al.*, 1992; Perez *et al.*, 1998; Celada *et al.*, 2001). It was, however, feared that the transfer of hatchlings from incubators into cooler British rivers may result in unacceptable mortalities.

Relatively low success rates have previously been achieved in rearing *A. pallipes* in Britain (Lebas & Rogers, 2000; F. Slater, pers. comm.). However, Cuellar & Coll (1978) successfully bred *A. pallipes* in Spain in association with the Institute of Conservation of Nature (ICONA) of the Ministry of Agriculture, in order to obtain young crayfish for restocking. They kept the adult crayfish in four ponds, each measuring 4 m width x 20 m length x 1.5 m depth, with limestone pebbles at the bottom. Before the eggs hatched, the females were placed on top of 1 cm wire mesh in the water of 6 basins measuring 2 m length x 1 m width x 0.6 m depth. When the young left the mothers, they dropped through the mesh, thus avoiding cannibalism by the parent (Cuellar & Coll, 1978). Mesh baskets were similarly used in the present study to separate independent hatchlings from the adult females. The young in the Spanish study were then reared in basins of 10 m length x 3 m width x 1 m depth, which contained shelters (refuges) and limestone pebbles. One fifth of the volume of water in each basin was renewed each day. A chemical analysis of the water showed: 8.7 mg l^{-2} of dissolved oxygen; 12.8 mg l^{-2} of CO_2; a pH of 8; 0 mg l^{-2} of Ca CO_3 (carbonates); 236 mg l^{-2} of $CaCO_3$ (bicarbonates); 0.02 mg l^{-2} of NH_3 (ammonical nitrogen); 42.5 mg l^{-2} of nitrates; while water temperature varied throughout the year (Cuellar & Coll, 1978).

Between the time young leave their mother and the winter, they develop setae on the mouthparts and moult, gradually growing, enabling them to feed on increasingly larger food particles (Lowery, 1988).

Rearing studies abroad have shown that a fresh, high protein diet consisting of small enough food particles, positively influences crayfish hatchling survival. For example, when fed on zooplankton such as *Cyclops* and *Daphnia*, with grated carrots and minced fish as a supplement, 74 % of *A. astacus* summerlings survived (Keller, 1988); a diet consisting of natural plankton from stagnant ponds such as *Daphnia*, *Tubifex* and young trout feed produced a 40-60 % survival rate of *A. pallipes* summerlings (Cuellar & Coll, 1978); 67% of *Astacus astacus* summerlings survived

when fed on natural food when reared in earthen ponds, while 58 % survived when fed on zooplankton, fish, vegetables and shrimp shell waste (Pursiainen, 1983).

A large part of the juvenile *A. pallipes* diet in the wild consists of microscopic algae, particularly diatoms (Clegg, 1985). Diatoms are yellow green algae that have patterned cell walls of silica (instead of cellulose). To reproduce, they split into two halves which then separate. Multiplication of diatoms in this way increases in the spring and early summer (Clegg, 1985), thus providing newly independent young *A. pallipes* with an excellent food source. It therefore follows that a good way of providing newly hatched *A. pallipes* with a natural food source in captivity, would be to feed them with diatoms. Diatoms can be found as a brown slimy film covering stones and aquatic plants (Clegg, 1985) in streams, rivers and ponds and were used as the main part of the diet in the present rearing experiment.

Fernandez *et al.* (1983) also attempted to rear *A. pallipes* in captivity, and found that when *A. pallipes* were fed a food supplement of fresh liver alongside an artificial diet, they survived. Those that were not given the fresh liver died due to the crayfish plague fungus, *Aphanomyces astaci* probably contracted from the *P. leniusculus* individuals also being kept in captivity during this experiment. Those that were fed the fresh liver appeared to be able to resist the fungus infection (Fernandez *et al.*, 1983).

A diet of fresh, natural food consisting of diatom packed algae collected locally from the River Wye and zooplankton such as *Daphnia* collected from local ponds, were therefore used in the present study to attempt to rear *A. pallipes* in captivity.

In Australian aquaculture, *Spirulina* supplements were recommended for rearing hatched crayfish (D. Jerry pers. comm.). *Spirulina* sp. is a type of blue-green algae and can be found in many lakes or ponds. It has a high protein content as over half of it is made up of amino acids, which are protein building blocks. *Spirulina* sp. is also rich in other nutrients such as B complex vitamins, beta-carotene, vitamin E, carotenoids, manganese, zinc, copper, iron, selenium and gamma linolenic acid (an essential fatty acid). It is also known to boost the immune system (Chamorro *et al.*, 1996). The present study therefore investigated whether *A. pallipes* hatchling survival and growth rates were influenced by the high protein *Spirulina* supplement.

After considering the above information, the present study involved, under licence, "borrowing" a number of egg laden *A. pallipes* females from local Welsh and borderland rivers in May 2003 in an attempt to successfully hatch and rear young crayfish for the purpose of restocking.

This experiment therefore investigated whether successful growth and survival rates of hatchlings could be achieved without artificial incubation, i.e. when left attached to the female, and at ambient temperature on the basis that the correct, high protein (*Spirulina* supplemented) diet and methodology would ensure survival. On this basis the following hypotheses were consequently formed:

Hypothesis 1

A. pallipes eggs left attached to the female can be reared successfully to summerlings.

Hypothesis 2

A. pallipes eggs can be reared successfully at ambient temperature in captivity.

Hypothesis 3

A. pallipes eggs can be reared to summerlings when fed a fresh algal diet containing diatoms.

Hypothesis 4

Spirulina supplement increases reared crayfish survival.

Hypothesis 5

Spirulina supplement increases crayfish hatchling growth rate.

5.3 METHODS

5.3.1 Experimental design

After obtaining appropriate licensing from the CCW allowing capture and rearing of *A. pallipes* in captivity, egg laden (berried) female crayfish were captured by stone turning (Thomas & Ingle, 1971) at a number of tributary rivers and streams throughout the Wye and Usk Catchments in May 2003. Once captured, the number of eggs/hatchlings attached to each female was counted. Each female was placed into a plastic basket, with a mesh size of 5mm, one of which would slide neatly into each crayfish rearing tank measuring 26 x 14 x 18 cm (Figure 5.1).

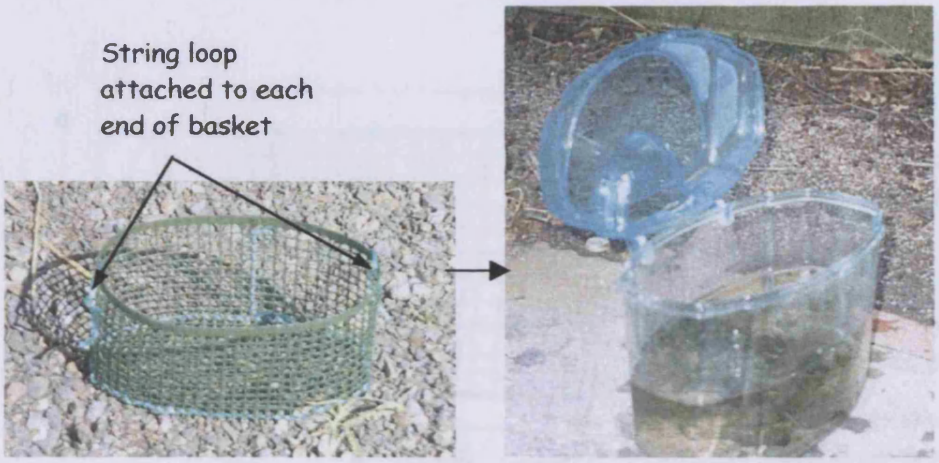


Figure 5.1 Photographs displaying an *A. pallipes* rearing basket and rearing tank, into which the rearing basket fits.

Loops of string attached to each end allowed the base of each basket to be permanently positioned 3 cm above the bottom of the tank. This enabling the basket to be easily removed (Figure 5.2). Water used in holding tanks came from a tap in the aquarium room with Ca^{2+} levels of approximately 80 mg l^{-2} and a temperature of 10 °C. All water was left to stand for over 48 hours in order to air out chlorine which is relatively unstable, but toxic to crayfish as it can pass into the bloodstream and destroy oxygen carrying cells.

A section of plastic pipe measuring 5 cm in diameter and 14 cm in length was used as a crayfish refuge in the water. Air pumps and long life air stones, obtained from Ultimate Discount Aquatics, were used to oxygenate the water.

Tanks were kept in a well ventilated, cool aquarium room and received artificial lighting for 12 hours per day, set on a timer. Adult female crayfish were regularly fed with mince morsels (a commercial, high protein dog food).

The young, once hatched, remained on the underside of the female's abdomen for a few days. On leaving the female, they dropped through the mesh basket into the 3 cm space at the bottom of the tank, thus preventing them from being damaged or eaten by the mother.

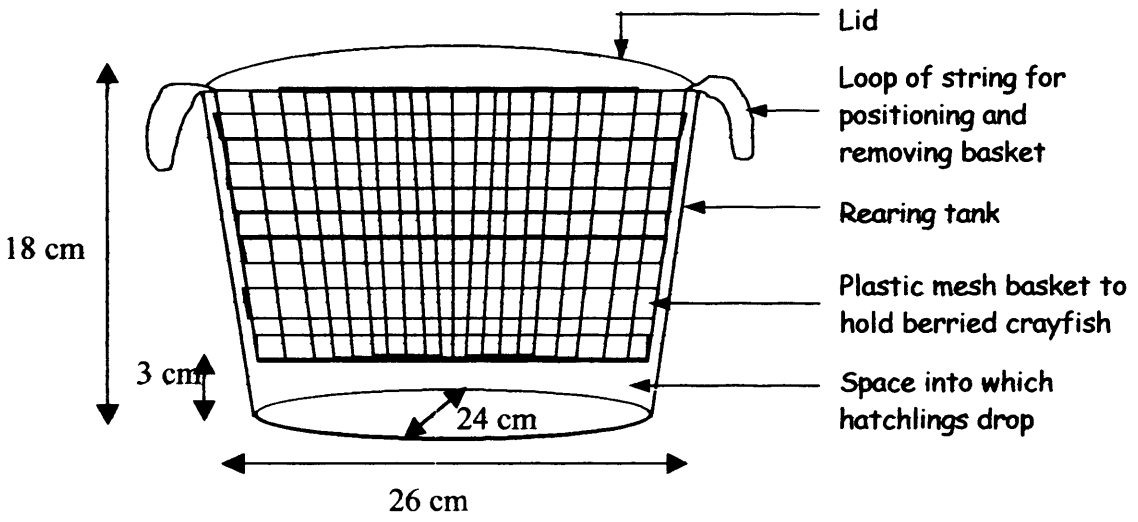


Figure 5.2 Diagrammatic representation of a rearing tank containing a basket.

5.3.2 Hatchling Feeding

Aquatic moss, pond weed, and small, algae covered pebbles were put into each tank in order to feed the hatchlings. The crayfish were fed regularly with mince morsels (a commercial high protein dog food), and provided with aquatic moss collected from exposed boulders in the river. The algae covered pebbles were collected from the River Wye and replaced each day, together with extra algae scraped off larger river stones. 10 g of the dried algal protein supplement *Spirulina* was added to each of 15 tanks labelled "A," as a food supplement every 5 days. 15 tanks not receiving the *Spirulina* supplement were labelled "B."

5.3.3 Monitoring

Tanks were checked twice daily (early morning and late afternoon). Any hatchlings that had dropped into the space beneath the baskets were removed using a custom

made net and separated into two tanks, A and B. Each tank contained sand as a substrate. Once all hatchlings had been separated from them, the adult crayfish were released at their original river location.

Hatchling condition was checked daily. Any dead ones were removed. Stages of development and lengths of all hatchings were recorded. They were measured by placing each individual in a clear petri dish over a sheet of laminated 1mm graph paper. The lid of the petri dish was inverted and used to lightly and carefully hold the hatchling in position in order to observe its length (mm). Care was taken not to damage hatchlings which were replaced quickly into the tanks in order to minimise stress.

At the end of the investigation, all hatchlings were returned to the locations from which their parents had been taken.

5.4 RESULTS

Fifteen berried female crayfish were captured in total in 2003 in preparation for the rearing experiment. One was caught in March, the other 14 were found between 8th May and 22nd June (Table 5.1).

Location	Date collected	Tank no.	Eggs/Hatchlings
Nantyroffeiriad (Army range)	15/03/03	1	E*
Nantyroffeiriad (Common land)	08/05/03	2	E*
Dulas (Builth Road)	14/05/03	3	E
Dulas (Monnow)	20/05/03	4	E
Dulas (Monnow)	20/05/03	5	E
Dulas (Monnow)	20/05/03	6	E
Sgithwen	14/06/03	7	H
Edw	14/06/03	8	H
Nantyroffeiriad Farm Brook	15/06/03	9	E
Nantyroffeiriad Farm	15/06/03	10	H
Dulas (Cwmbach Llechryd)	16/06/03	11	E
Dulas (Builth Road)	16/06/03	12	H
Dulas (Cwmbach Llechryd)	17/06/03	13	H*
Dulas (Builth Road)	17/06/03	14	E
Dulas (Cwmbach Llechryd)	22/06/03	15	H

Table 5.1 List of berried female crayfish original locations, date collected and maternity tank number (* = adult female died).

Of the 15 captured, three died, two before their eggs had hatched. The eggs of these two dead crayfish were carefully removed and placed in a tank of well oxygenated water but did not survive to hatching. One female died after the eggs had hatched while young were in the juvenile 1st stage and still attached to her abdomen. These hatchlings were carefully removed and also placed in a tank of well oxygenated water. Many of these hatchlings died while still in the 1st stage of development. However, some survived and moulted into the 2nd stage.

Berried crayfish were all found between 8th May and 22nd June with the exception of one being caught on 15th March, in 2003. Of the 15 crayfish, six collected in June possessed 1st stage hatchlings at the time of capture, while those found in May and three found in June possessed only unhatched eggs at the time of capture. Eggs of the females collected in May hatched between 17th and 19th June. Females were caught with hatchlings attached between 15th June in the Offeiriad and 22nd June in the Cwmbach Dulas, indicating that hatching in the wild occurs between 13th and 22nd June. Hatching period, the time taken for all eggs in a clutch to hatch, varied from 2 to

24 days with a mean of 12.2 days. By 1st July, all hatchlings had moulted to the 2nd stage with the exception of 1 egg on one female, which later failed to hatch. 14.2 % of all eggs hatched, 12.3 % of eggs reached the juvenile 1st stage and 10.8 % of eggs reached the 2nd stage (Figure 5.3).

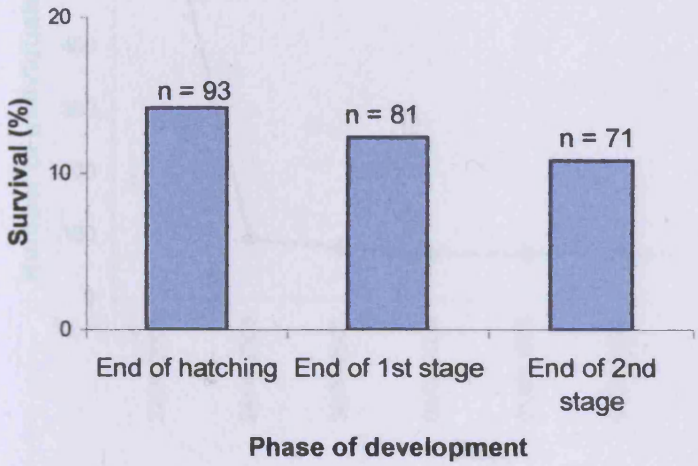


Figure 5.3 Percentage of *A. pallipes* hatchlings that survive the hatching process into Stage 1, that survive development from Stage 1 into Stage 2 and from Stage 2 into Stage 3.

12.1 % of hatchlings (40 individuals) supplemented with *Spirulina* and 9.4 % of those (31 individuals) not supplemented with *Spirulina* survived from eggs through to the juvenile 2nd stage and to become summerlings. A diet supplemented with *Spirulina* therefore slightly, but not significantly, increased hatchling survival. In all, 10.8 % of hatchlings (71 individuals) survived from eggs to become summerlings (Figure 5.4).

After measuring the total body lengths of the hatchlings on 1st September 2003, diagnostic checks carried out showed that the data were non-parametric. A Mann-Whitney Test was therefore carried out and showed no significant difference between the median lengths of hatchlings that received the *Spirulina* supplement and those that did not ($p = 0.1033$ or 0.0996 adjusted for ties) (Figure 5.5).

Anderson-Darling Tests showed hatchling lengths measured on 10th October 2003 to be normally distributed ($p = 0.076$, A-squared = 0.666 and $p = 0.343$, A-squared = 0.397 for with and without *Spirulina* supplement respectively) and an F-Test showed that variances were equal ($p = 0.494$, test statistic = 1.308). A 2-sample t-test showed there was no significant difference between mean body lengths of hatchlings

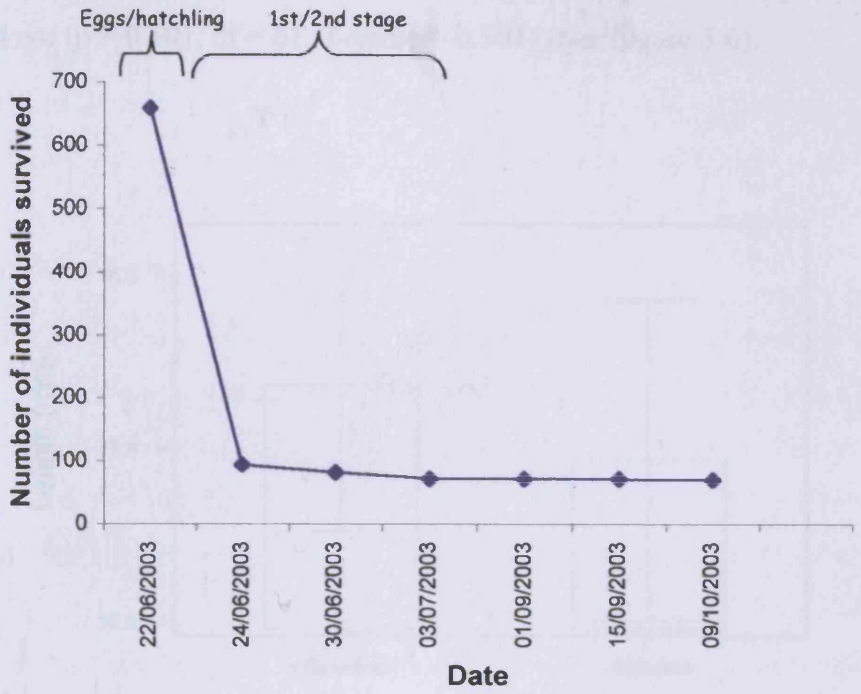


Figure 5.4 Number of individual eggs or juvenile crayfish that survived between 22nd June 2003 and 10th June 2004.

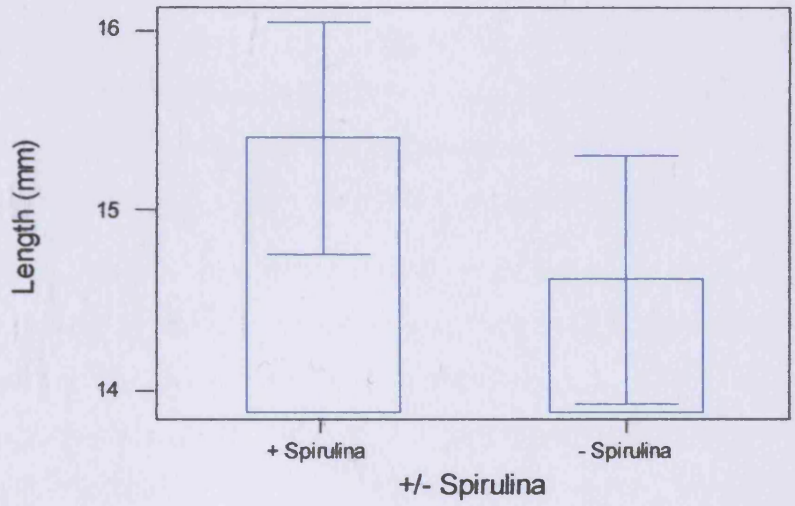


Figure 5.5 Mean body lengths (mm) with 95 % confidence intervals of 31 hatchlings supplemented with (+) *Spirulina* and 40 not supplemented with (-) *Spirulina* measured on 1st September 2003.

measured on 10th October 2003 that received the *Spirulina* supplement and those that did not (p = 0.501, df = 61, T-value = 0.501) (See Figure 5.6).

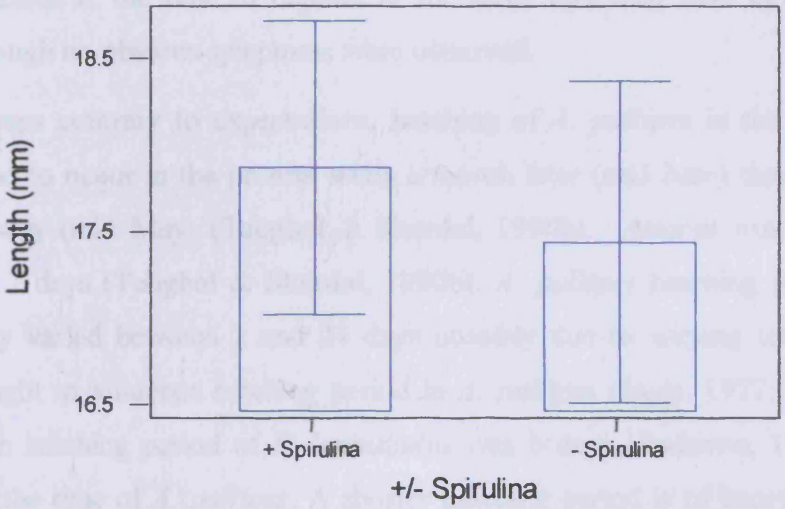


Figure 5.6 Mean body lengths (mm) with 95 % confidence intervals of 25 hatchlings supplemented with *Spirulina* and 38 not supplemented with *Spirulina* measured on 9th October 2003.

5.5 DISCUSSION

Three berried females died after capture. The three deaths were possibly due to stress, change in diet or water chemistry. However, the remaining twelve survived, suggesting other underlying causes may have been responsible, such as a lower state of health at the time of capture in the three that died than in those that survived, although no obvious symptoms were observed.

Perhaps contrary to expectations, hatching of *A. pallipes* in the wild in Britain was found to occur in the present study a month later (mid June) than *Astacus astacus* in Norway (mid May) (Taugbol & Skurdal, 1990b). *Astacus astacus* hatching period was 7 days (Taugbol & Skurdal, 1990b). *A. pallipes* hatching period in the present study varied between 2 and 24 days possibly due to varying temperatures, a factor thought to influence hatching period in *A. pallipes* (Ingle, 1977; Rhodes, 1981). The mean hatching period of *P. leniusculus* was 6 days (Andrews, 1907); approximately half the time of *A. pallipes*. A shorter hatching period is of benefit in such species as the hatchlings possess greater safety from predation when they hatch altogether, a phenomenon called breeding synchrony exhibited in other species from invertebrates to mammals e.g. wildebeest where 80 – 90 % of calves are born in a three week period which consequently restricts predator induced mortality (Berger & Cain, 1999). Some variation in the hatching period of a crayfish brood is however, thought to be due to the location of the egg within the brood cluster and consequent varying stages of embryonic development within the brood (Reynolds, 1989 as cited by Reynolds 2002) and could be beneficial to survival as this ensures that food supplies are not exhausted in a short period of time, but rather are allowed to be renewed.

Removal of dead fungus infected eggs and first stage juveniles every four days significantly improved survival during artificial incubation experiments in Spain (Carral *et al.*, 2002). Therefore, by checking eggs and young every 3-4 days in the present study, optimal survival was promoted through minimising disturbance and fungus transmission to surrounding healthy eggs. Despite this, most mortalities occurred during egg development or hatching in the present study, mostly due to fungal infection suggested by discolouration (orange rather than black) of the eggs, a classic symptom of *Saprolegnia* sp. (Reynolds, 2002).

Such results were somewhat expected as similar mortalities occurred in other studies (Carral *et al.*, 1988; Matthews & Reynolds, 1995; Perez *et al.*, 1999), while others found them to occur during the 1st juvenile stage and 1st moult (Mason, 1977; Rhodes, 1981 as cited by Perez *et al.*, 1999) or between hatching and 2nd stage (Carral *et al.*, 1992 as cited by Perez *et al.*, 1999). However, in the present study, only 3.4 % (23 individuals) of losses occurred after hatching. 76 % (71 individuals) of all eggs that hatched in the present study survived to become summerlings into October, a relatively high success rate in comparison with other rearing attempts; for example, the study by Lebas and Rogers (2000), when, by the end of September, only 1.3 % (5 individuals out of an initial 400) remained.

Reasons for the relatively high success rate of rearing *A. pallipes* hatchlings in this experiment in comparison with other studies e.g. that of Lebas & Rogers (2000), could probably be attributed to the provision of sufficient fresh, high protein, edible food i.e. diatoms and *Daphnia*, and lack of possible escape routes in the present study. It was suggested by Lebas & Rogers (2000) that the high losses of hatchlings could be due to their escape through the mesh holding cages, although it is debatable whether many hatchlings can escape through 2mm mesh size.

One of the aims of this study was to find out if *A. pallipes* rearing could be carried out successfully without artificial incubation. Incubation involves removing eggs from females at after a period of time and keeping the eggs at controlled temperatures (Carral *et al.*, 1992). In the present study, this was thought to be of particular importance in Britain as a drop in water temperature from incubator into the wild during reintroduction could shock and potentially kill many of the reared juveniles.

The relatively high success rate of the present study suggests Hypotheses 1 and 2 that *A. pallipes* eggs can be reared successfully when left attached to the adult female and at ambient temperature in captivity should be accepted.

Cannibalism of young by adult females could be responsible for some of the losses experienced during the present experiment before the young detached. The loss of 22 juveniles between hatching and the 2nd stage was probably also a result of cannibalism. Moulting occurs during the first few months after hatching. Exoskeletons are particularly soft for a period of time after a moult. Young are especially susceptible to cannibalism at this time which has, in previous studies,

partially accounted for the deaths of 40-60 % of young crayfish (Cuellar & Coll, 1978). Also, if the food provided was lacking in any way, this could have promoted cannibalism. Starvation was the most influential factor in promoting cannibalism when the noble crayfish, *Astacus astacus* was kept in captivity, particularly as the soft exoskeleton and mouth parts after moulting meant that feeding was difficult, thus increasing levels of hunger and provoking cannibalism once the exoskeleton had re-hardened (Gydemo & Westin, 1993). It was, however, thought that sufficient appropriate food of a high enough quality was provided during the present study to prevent this. It is therefore likely that some losses in the present study occurred as a result of fungal infections. However, tests were not carried out to confirm this.

Although hatchlings supplemented with *Spirulina* showed slightly increased growth and survival rates, increases were not significant. This could be due to the fact that *Spirulina* has little effect on *A. pallipes* growth at such a young age. Perhaps if the juveniles had been kept until they were older and continued to be supplemented with this alga then the effect would have been significant. Alternatively, the hatchlings may have received sufficient protein and nutrients from the fresh food and therefore did not need a supplement. Also, *Spirulina* was used in the present study as it is was reportedly effective on Australian species. However, it is possible that this supplement may have a less pronounced effect on other crayfish species such as *A. pallipes*.

Although Hypotheses 4 and 5, that *Spirulina* supplement increases reared crayfish survival and hatchling growth rate, cannot be accepted with regard to the present study, it is strongly suspected, because of the near significant results, that further, more advanced experiments would allow them to be accepted.

A. pallipes italicus reared in captivity in another study produced a higher survival rate of 40 % of juveniles by mid July. They were fed on vegetation and macroinvertebrates initially and 10 days after hatching, their diet was supplemented with pellets of 51 g 100 g^{-1} of proteins (DeLuise & Sabbadini, 1988). However, during the present study, the *Spirulina*, containing a higher proportion of protein (63 g 100 g^{-1}), but was suspended in the water. If it had been supplied in a different form e.g. mixed with other food into a pellet, perhaps this would have encouraged increased uptake by the hatchlings which could have increased hatchling growth and survival rates significantly.

Also, during the present study, one third of the water in each tank was changed every 3 to 4 days. A proportion of the suspended *Spirulina* was therefore unavoidably removed with each water change. This could have prevented sufficient nutrients from being taken up by hatchlings thus preventing a significant increase in hatchling growth and survival.

However, marron (*Cherax tenuimanus*) growth rate and size did not increase significantly when fed with the more nutrient rich, higher protein diet (43.6 g 100 g^{-1}) than when fed with the less nutrient rich, lower protein diet (23 g 100 g^{-1}) (Maguire *et al.*, 2002). In another study, marron growth rate was positively influenced by increased dietary protein (Morrisey *et al.*, 1995). However, Maguire *et al.* (2002) suggest that the difference was a result of the low protein diet in Morrisey's study (14 g 100 g^{-1}) being much lower than in their own study.

In yet another feeding study, 74 % of initial *Astacus astacus* juveniles reared in captivity survived to the end of the summer on a main diet of zooplankton e.g. *Cyclops* and *Daphnia*, artificial food and grated carrots fed every other day. From August, the diet was supplemented with minced fish (Keller, 1988), a good source of protein. It is thought that the extra protein supplement helped to increase survival rate in this instance.

Cuellar & Coll (1978) fed young *A. pallipes* hatchlings natural plankton from stagnant ponds and lakes and later supplemented the diet with ground bass, *Tubifex*, *Daphnia* and young trout feed. Only a 20 % egg loss was noted using this protein supplemented diet.

A fresh food supplement was noted as important to *A. pallipes* by Fernandez *et al.* (1983) when they found that *P. leniusculus* could survive with and without a fresh food supplement of liver, but *A. pallipes* could only survive if an artificial diet was supplemented with fresh food. They also observed that *A. pallipes* individuals not receiving the fresh liver supplement died from infection by the crayfish plague fungus *Aphanomyces astaci*, but those fed with it survived plague infection and appeared to develop a resistance to it. The state of health and level of immunity of individuals receiving the supplement were obviously much greater than those not receiving it.

These results were supported by another study where *Astacus astacus* juveniles reared in plastic tanks fed on zooplankton, fish, vegetables and shrimp shell waste at an

initial density of 100 individuals m^{-2} was 58 %, while juveniles reared in earthen ponds fed on natural food had a survival rate of 67 % (Pursiainen, 1983).

Results of the present study and other investigations therefore strongly indicate that a balanced, accessible, fresh, high protein diet increases growth and survival rates of crayfish hatchlings.

Hypothesis 3, that *A. pallipes* eggs can survive to summerlings when fed a fresh algal diet containing diatoms, should therefore be accepted.

Another way of improving egg survival may be to feed a protein supplemented diet to females before they produced eggs.

Stocking density of juvenile crayfish during rearing also appeared to greatly influence survival rates. For example, survival rates of 58% were observed in *Astacus astacus* juveniles when they were stocked at an initial density of 100 individuals m^{-2} in plastic tanks and 50 % at 300 individuals m^{-2}. Also, when kept in earthen tanks at an initial density of 100 individuals m^{-2} survival rate was 67%, but at 300 individuals m^{-2}, survival rate was only 32 % (Pursiainen, 1983). However, other literature showed that a high survival rate of 74 % of *Astacus astacus* juveniles could be reached with an initial stocking density of 400 individuals m^{-2} (Keller, 1988). Perhaps these differences were due to slightly different rearing conditions and feed types. Mean stocking density of juvenile *A. pallipes* in the present study was 117 individuals m^{-2} and the survival rate was much lower at 10.8 % for 2^{nd} stage juveniles from initial stock. Disease, lower water temperature and cannibalism, not stocking density were strongly suspected to be responsible for loss of eggs or hatchlings in this case.

This is supported by Cuellar & Coll (1978) who suggest that the loss of 40-60 % of all young *A. pallipes* during the first few months of their study were partially due to poor feeding and partially due to cannibalism (as previously mentioned).

5.6 CONCLUSIONS

Hatching occurred during mid June. Hatching period was on average 12.2 days, approximately twice that of *P. leniusculus*. Rearing was relatively more successful than previous studies in producing *A. pallipes* summerlings at lower temperatures as a result of providing fresh diatoms and zooplankton such as *Daphnia* every three days as a food source. The *Spirulina* supplement slightly increased growth and survival of hatchlings although it should be provided in a pellet form and its effects monitored further and for a longer period of time. However, it is also possible that the main diet in the present study was sufficiently rich in protein without the *Spirulina* supplement.

Survival rate was relatively low in comparison with studies where incubation had been used. Incubated rearing should probably therefore be used in order to boost survival rates. Investigations should then be carried out to acclimatise reared juveniles to the lower British water temperatures before reintroduction by gradually lowering water temperature in captivity while closely monitoring survival.

More replication should have been carried out, while survival rates based on different quantities of main food and *Spirulina* supplement could have been investigated in the present study, had time allowed. However, due to the limited number of berried females found, replication and further investigations would have been very difficult.
Contrary to the indications of previous studies, the present experiment showed that it is possible and practical to rear significant numbers of *A. pallipes* through to a size where they could be used for restocking. This finding has significance in formulating management plans for endangered native crayfish populations.

CHAPTER 6

General Discussion

and

A. pallipes Conservation Action Plan.

6.0 GENERAL DISCUSSION

6.1 Aims

The thesis aimed to assess the current status of *Austropotamobius pallipes* in the Wye, Usk and Upper Severn Catchments in Wales and the Marches, establish the causes of decline of the last 30 years, find out if genetic variation exists in populations on a local scale to determine whether genetics should be a consideration when restocking and finally, to successfully rear summerlings from eggs with a view to enable the breeding of *A. pallipes* in captivity in order to provide donor populations for restocking recovering watercourses.

6.2 Population structure, abundance and distribution

A survey carried out by Slater (1998) and repeated by Wilkins (1999) revealed dramatic declines during the 1990s in *A. pallipes* populations once abundant in Welsh Rivers (Lilley, *et al.*, 1979; Foster, 1990). Concern was intensified as later surveys discovered further declines in these regions. For example, by 2000, no crayfish were found in 72 % of sites in the Usk and Wye Catchments where *A. pallipes* were abundant ten years previously (Coley, 2000), while a detailed (25 man hour) survey of the River Edw, a Wye tributary thought to be an *A. pallipes* stronghold, found only seven individuals, two of which were dead (Slater & House, 2001).

Extensive surveys carried out for the present study across the Wye, Usk and Upper Severn revealed that the very existence of *A. pallipes* populations in these catchments was threatened.

In the Wye Catchment, eight out of ten rivers surveyed between June and October of 2002 had a male biased *A. pallipes* sex ratio. Brooding females have been observed to moult, just after releasing their young around June (Lilley, 1977) and are therefore more likely to take refuge in safer places such as crevices in riverbanks than males at this time in order to avoid predators. This could explain why more males than females were found during stone turning. Previous studies have, however, produced female biased crayfish sex ratios at similar times of the year (Lilley, 1977; Thomas & Ingle, 1971; Svardson, 1949). These findings therefore suggest that there are currently fewer female than male crayfish present in Welsh rivers, a possible consequence or cause of the decline of *A. pallipes* in this region. The highest populated rivers in this study, the

Offeiriad, Escley, Dulas Monnow and Sgithwen all had the most balanced male : female ratios, reinforcing this theory.

A general adult (>24 mm carapace length as specified by Smith *et al.*, 1996) biased adult:juvenile ratio of *A. pallipes* found in rivers surveyed in the present study could be the result of a sampling error known to occur using manual searching (Lilley, 1977; Smith *et al.*, 1996). Young crayfish are, however, particularly susceptible to pollution (Eversole & Seller, 1996), which could have caused the lack of juveniles by reduced survival through habitat pollution and destruction. The general *A. pallipes* decline since the late 1980s (Holdich & Rogers, 1997, Slater, 1998; Wilkins, 1999; Holdich *et al.*, 1999; Coley, 2000; Sibley *et al.*, 2002; Holdich, 2003 and Sibley, 2003) could therefore be due to lack of juvenile recruitment and survival into adulthood. The low female to male numbers may also influence this.

The mean carapace length of female crayfish was greater in the Edw and Sgithwen than in the Offeiriad and Dulas Monnow, indicating that a greater proportion of smaller, younger individuals were present in the Offeiriad and Dulas Monnow than in the Edw and the Sgithwen. This could suggest that numbers in the Edw and Sgithwen are about to decrease again due to a lower recruitment of younger individuals to maintain the populations.

Carapace lengths did not vary significantly over the summer into autumn showing that the populations remained relatively constant during the months July, August and October, perhaps because fewer deaths occurred due to warmer temperatures typical of this time of year

In order to reinforce these observations of *A. pallipes* population structure, the same rivers should be resurveyed with the use of other techniques such as kick sampling or electrofishing. However, limited time and man power prevented this from being carried out in the present study.

Offeiriad was able to support crayfish with the largest range of sizes (equivalent to ages) of all rivers sampled, suggesting that it was a healthy river whose habitat was of a high enough quality to support a healthy and sustainable *A. pallipes* population. In view of the small remaining numbers of such populations it is very important that this population should be protected.

A general pattern of decline emerged when site specific surveys were repeated over a number of years. A 1988 survey of 26 sites in the Wye Catchment showed that twenty four sites were occupied by *A. pallipes* (Foster, 1988). Nine years later, *A. pallipes* were found on only five of these sites (Moreley, 1997). Only four occupied sites were found two years later (Wilkins, 1999), while a further three years later, *A. pallipes* were still found at only four of the twenty six sites (Howells, 2002).

These declines have been attributed to crayfish plague and competition from *P. leniusculus* as in English populations, but also to sheep dip pollution, mainly by the synthetic pyrethroid, cypermethrin, combined with intensive livestock farming, steep, hilly landscape and consequent naturally high levels of rainfall, habitat degradation including bank erosion contributing to siltation resulting in loss of refuges and detritus, and habitat alteration for flood alleviation schemes such as near the confluence of the Dulas, a Monnow tributary.

Despite these declines, the continuing threat of plague and the increasing presence of the invasive signal crayfish, *P. leniusculus*, isolated *A. pallipes* populations were found during the present study at a number of locations in the Wye Catchment in the Offeiriad, Sgithwen, Cwmbach Dulas, Dulas Monnow and Escley. The populations of the Edw had even recovered as 75 individuals were captured on the Edw between June and September in 2002 (Howells, 2002) as opposed to seven individuals, two of which were dead in 2001 (Slater & House, 2001), although not to levels prior to the decline. Also, while electrofishing, the Game Conservancy Trust found a few individuals on the River Monnow in 2003, a population suspected to be almost extinct.

In the Usk Catchment, *A. pallipes* were still found, though in very low numbers on the main River Usk at only two sites, the mouth of the Honddu in Brecon and Llanfrynach, and on the Honddu and Llanfrynach tributaries, while previously unrecorded populations were discovered in Torfaen tributaries during a contract survey I carried out (Slater *et al.*, 2003). In the Upper Severn Catchment, only one individual was found on the main Banwy River at Melin y ddol and one other population was discovered on a small Banwy tributary also at Melin y ddol.

6. 3 River habitat variables and crayfish presence

Following river habitat surveys (RHS), some physical river habitat variables were found to be positively associated with crayfish presence i.e. cobble banks, boulder and cobble substrates and exposed boulders. This supported results of the national RHS study by Naura & Robinson (1998), showing that these features are particularly important to *A. pallipes* as they act as refuges (Foster, 1993) and increase detritus retention in the streams (Lepori *et al.*, 2005). Detritus retention is important as it provides refuge and is a primary food source of *A. pallipes* (Reynolds, 1979; Smith *et al.*, 1996) and of other invertebrates on which crayfish feed (Naura & Robinson, 1998).

Bank vegetation structural diversity was also found to have a positive influence on the presence of *A. pallipes* in this study. Where vegetation covers a bank it is usually not found to be eroding and it is therefore likely that siltation which can fill in and destroy crayfish refuges (Hoger, 1988; Summers, 1996; Slater & Howells, 2003b) is not such a problem, allowing crayfish to remain.

Overhanging boughs positively influenced crayfish presence in this study and that of Naura & Robinson (1998) probably because they provide shade which is also known to be favourable to crayfish (Naura & Robinson, 1998; Richardson *et al.*, 1999; Parkyn, 1997). Exposed tree roots, which provide crayfish nursery areas (Smith *et al.*, 1996) and refuges (Foster, 1996; Howells, 2003), are likely to be present below overhanging boughs. Exposed tree roots also trap leaf litter, a primary *A. pallipes* food source (Reynolds, 1979; Smith *et al.*, 1996), while trees drop leaves and canopy dwelling invertebrates into the water, providing a direct food source of crayfish and other species on which crayfish feed (Naura & Robinson, 1998).

The upland rivers of mid-Wales are largely surrounded by livestock farms. Consequently, some river banks have been poached and eroded because of high stocking densities. Despite this however, *A. pallipes* remain present in mid-Wales. This could explain why eroding cliffs surprisingly appeared as a feature positively associated with crayfish presence. Eroding cliffs may, however, contain undercuts favoured by shade seeking crayfish (Smith *et al.*, 1996) and indicate a bank material soft enough to allow crayfish to burrow, thus providing shelter (Gherardi *et al.*, 2000). The presence of some eroding cliffs therefore could somewhat benefit *A. pallipes*.

Stable cliffs are positively associated with crayfish presence in the present study. They indicate that extensive erosion and siltation are not occurring in these areas, which suggests that in such areas quality crayfish habitat still exists in rural Wales and the Marches. A combination of mostly stable and some eroding cliffs is therefore probably most beneficial to *A. pallipes*.

6.4 Causes of decline

6.4.1 Siltation

In the present study, it was attempted to quantify siltation by using basket and flowerpot type traps buried in the River Edw. Basket traps measured surface plus intrabed siltation while flowerpot traps measured only surface siltation. A new trap designed for but not tested in the present study could allow more accurate measurements to be made by solely quantifying intrabed siltation. This may be of use in future studies but should also be tested before using in experiments (Figure 6.1).

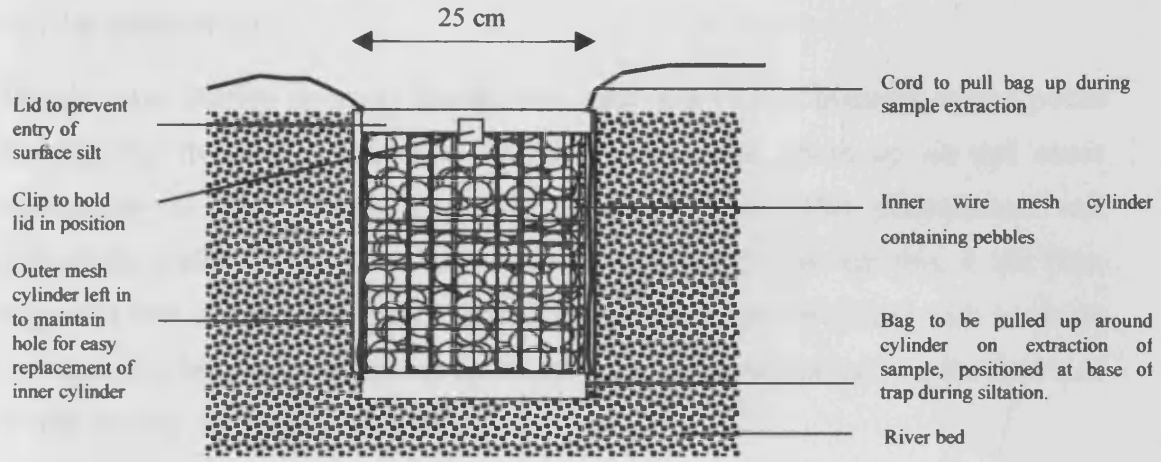


Figure 6.1 Diagrammatic representation of a trap designed to measure only intrabed siltation positioned in a river bed.

The results of the siltation experiment on the River Edw, showed mostly surface plus some intrabed siltation occurring at an alarming rate of 5587.8 gm^{-2} in one month along the entire length of the river, despite fencing, bank strengthening and coppicing improvements in place along parts of the river for the past three years as part of the Wye Habitat Improvement Project (WHIP).

Although siltation rates were not recorded prior to these improvements, it is likely that they have been reduced somewhat by preventing bank erosion and cattle poaching.

However, they still remain high enough to threaten many aquatic organisms. Siltation affects *A. pallipes* and bullheads (*Cottus gobio*) (Naden *et al.*, 2002) by filling in gaps underneath and between which they take refuge from the current and predators (Foster, 1996) and lay their eggs; salmonid fry by filling in gaps between stones where eggs are laid and newly hatched fry hide (Wheeler, 1991), and freshwater pearl mussels (*Margaritifera margaritifera*) as they require a sandy rather than fine silt substrate in which they bury themselves on the downstream side of rocks or boulders, and which also rely on salmonid fry to disperse young (Hastie *et al.*, 2000). Lampreys (*Petromyzon marinus*) are also threatened by excess siltation as it covers over the pebble river beds on which they spawn (Wheeler, 1991).

Although siltation rates vary naturally within seasons, they were significantly higher in winter months when rainfall was heaviest. Effects were probably exacerbated at these times by the heavy rains washing silt loosened from the steep landscape of often overgrazed pastures and felled forestry into the river. *A. pallipes* may therefore have been excluded as refuges were filled in or lost, increasing the risk of being washed away or predated upon.

Slightly more siltation occurred downstream than upstream of livestock access points showing that livestock continue to directly erode banks, churn up silt and cause siltation in the river despite the habitat improvements. This phenomenon was particularly evident at one location on the River Edw. To prevent this, it has been suggested that controlled livestock access points should be reinforced with hardcore or pebbles set in concrete or pump operated water points established on the field side of river fencing (F. Slater pers. comm).

The present study also showed that *A. pallipes* preferred to reside where refuges were present i.e. within aquatic vegetation or under loose pebbles rather than where refuges were absent i.e. gravel, sand/silt or embedded pebbles. Crayfish were found on a number of occasions during silt collection under stones closely surrounding embedded silt traps. These stones had more recently been disturbed than others in the river and therefore silt had been partially washed away and was looser. This suggested that if a watercourse becomes badly silted, *A. pallipes* seek less silted sites. Also, detritus, usually retained by a structurally diverse riverbed e.g. containing loose boulders and woody debris (Lepori *et al.*, 2005) provides an important food source and refuge for *A. pallipes* and invertebrates (Smith *et al.*, 1996) which will be washed away if the

river bed becomes structurally uniform and compacted by siltation or deliberate alteration. A loss of detritus would therefore have a detrimental effect on *A. pallipes*.

6.4.2 Sheep dip

Siltation is largely an effect of livestock farming as also is the chemical pollution caused by synthetic pyrethroid (SP) dips used in ectoparasite control of sheep.

Synthetic pyrethroids have increased in use since the VMD introduced exposure reduction methods of OPs in the late 1990s (Croxford, 2005). The SP, cypermethrin, has caused concern (Environment Agency, 2000; Rutt, 2004) as it is over 100 times more toxic to aquatic life than the OP, Diazinon (Coley, 2000; Howells, 2003; Rutt, 2004).

Twenty cypermethrin pollution incidents, three of which were caused by an illegal cypermethrin form were confirmed and listed by Wilkins (1998) and Rutt (2004) in Welsh rivers. Many other incidents were also confirmed and suspected by the Environment Agency and documented in their sheep dip monitoring reports of 1998, 1999 and 2000. Since August 2003, 57 dip pollution incidents were recorded by the Environment Agency across 29 catchments in Wales alone (Croxford, 2005).

The large numbers of proved and suspected cases of sheep dip pollution, particularly involving the synthetic pyrethroid, cypermethrin, has had and continues to have a detrimental affect on *A. pallipes* populations in Wales despite the Groundwater Regulations of 1999 and the Best Practice Guidelines for the Management of Sheep Flocks of 2001 set out by the Environment Agency. An example of how devastating a single pollution incident can be is that of a cypermethrin pollution incident in 2004 on the Escley Brook of the Monnow Subcatchment which resulted in the deaths of over 400 crayfish (Environment Agency, 2004).

Illegal use of the non fleece binding arable farming form of cypermethrin, again combined with heavy rainfall and poor control of sheep after dipping resulted in a number of pollution incidents on Welsh *A. pallipes* rivers (Gwlad, 2003). The younger the crayfish, the more susceptible they are to pollutants (Eversole & Seller, 1996). Younger generations on some rivers have therefore probably been destroyed, which could explain the lack of young crayfish found in many Welsh Rivers of the present study.

SPs were originally difficult to detect due to their short half-life of 7-12 days. They are now detectable in mosses within which they are retained for long periods of time after the pollution incident e.g. as occurred at Nant Cwmdu, a Llynfi tributary (Rutt, 2004). This now begs the question as to whether invertebrates eating these mosses are at risk.

Biological monitoring such as the Biological Monitoring Working Party (BMWP) Scores are useful methods of detecting changes in aquatic invertebrate fauna, many of which have characteristic responses to different types of pollution and can therefore be used as a tool for interpreting a pollution event.

This study therefore concludes that sheep dip pollution and habitat degradation through siltation are two of the main causes of *A. pallipes* decline in Welsh Rivers and are currently of more importance here than plague and *P. leniusculus* invasion.

Despite the factors exacerbating decline still being present, some rivers such as the Edw appear to be recovering perhaps as a result of the introduction of legislation which promoted the safe use and disposal of sheep dip. Once a river has recovered sufficiently, *A. pallipes* may return naturally although this process is likely to be slow. An introduction of one hundred *A. pallipes* individuals to the feeder stream of Llwyn Onn Reservoir in the Brecon Beacons in 1986 was not detectable after 2.5 or 9 years, but *A. pallipes* were found in the vicinity in 2002 i.e. after 16 years (F. Slater pers. comm). However, where natural recolonisation is difficult because of geographical isolation or obstacles, it has been recommended that restocking should be carried out (Lebas & Rogers, 2000) but approached with care.

6.5 Populations genetics of *A. pallipes*

Before restocking, genetic integrity of populations should be analysed (Wayne *et al.*, 1991; Avise 1994; Waldman & Wirgin, 1994; Zaccara *et al.*, 2004) in order for donor and receptor populations to be located.

The present study using microsatellite markers designed by Gouin *et al.* (2000) revealed that genetic differences were present in British *A. pallipes* populations from five catchments, the Wye, the Usk, the Upper Severn, the Aire and the Itchen, on three levels: within a river, within a catchment (between rivers) and between catchments. Also, samples analysed belong to one of three genetically dissimilar groups shown in Figure 6.2 and Table 6.1.

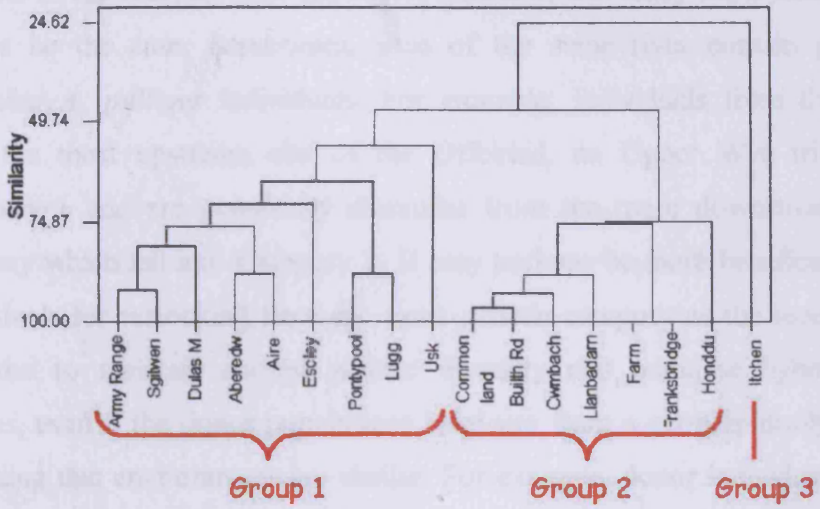


Figure 6.2 Cluster analysis based on allele frequency and heterozygosity values of *A. pallipes* populations sampled from sites of the Wye, Usk, Aire and Itchen catchments.

Group/category	Site/River	Catchment
1	Army range, top site - Offeiriad	Upper Wye
	Sgithwen	Upper Wye
	Aberedw, bottom site - Edw	Upper Wye
	Dulas Monnow	Lower Wye
	Escley	Lower Wye
	Lugg	Lower Wye
	Aire	Aire, N. England
	Nant-y-pia, Pontypool	Usk
	Usk	Usk
2	Common land, mid site - Offeiriad	Upper Wye
	Farm, lower site - Offeiriad	Upper Wye
	Builth Road, upper site - Cwmbach Dulas	Upper Wye
	Cwmbach, lower site - Cwmbach Dulas	Upper Wye
	Llanbadarn, mid site - Edw	Upper Wye
	Franksbridge, top site - Edw	Upper Wye
	Honddu, Usk	Usk
3	Itchen	Itchen, S. England

Table 6.1 Genetic groups/categories, their sites and their catchments derived from the Cluster Analysis.

Kemp & Hiley (2003) suggest that donor populations should be located as close as possible to the receptor site. However, results of this study show that this should not always be the case. Sometimes, sites of the same river contain genetically very dissimilar *A. pallipes* individuals. For example, individuals from the Army Range Site, the most upstream site of the Offeiriad, an Upper Wye tributary fell into Category 1 and are genetically dissimilar from the more downstream sites on this tributary which fell into Category 2. It may perhaps be more beneficial to take donor individuals for restocking from the same genetic category as the receptor population in order to maintain natural genetic diversity and minimise hybridisation of the species, even if the donor populations originate from a geographically distant region, providing that environments are similar. For example, donor individuals from the Aire Catchment in northern England could be used to restock the Escley, a Lower Wye tributary which suffered a massive crayfish loss in a recent sheep dip pollution incident, providing the river had recovered and the environments were sufficiently similar.

In the present study some *A. pallipes* populations displayed low levels of genetic diversity and high degrees of inbreeding e.g. Franksbridge, the most upstream site of the Edw, a Wye tributary, of Category 2 and the Itchen in southern England of Category 3. In order to boost levels of genetic diversity and reduce inbreeding and possible risk of extinction, donor populations should perhaps be taken from a genetically dissimilar site and category e.g. the Dulas Monnow of Category 1, or perhaps even from abroad i.e. Italy or France. However, this would depend on whether the stock would retain locally adaptive characters e.g. an ability to live in low calcium water or under a particular thermal regime.

Before such measures are taken, more replication and more sites across Britain should be sampled and analysed, possibly using more and new primers in order to confirm the presence of these and any other genetic categories and to further assess the risk of inbreeding.

This work would be specialised and time consuming and could comprise a new PhD study. Interpreting the genetic results in an ecological context would help in developing management plans for such populations.

Within the Edw, genetic diversity was greatest nearest the confluence with the Wye where heterozygosity was 0.25 and three haplotypes were present and lowest near the headwaters where heterozygosity was 0 and the only haplotype present was MLG III. These differences could be explained in two ways. First, the greater degree of genetic variation at the confluence end of the tributary has resulted from the closer geographical distance of the main River Wye acting as a physical conduit to other populations and where *A. pallipes* were found up until a decade ago. Movement of individuals between the Wye and Lower Edw could have maintained higher levels of genetic diversity in the lower Edw. Second, the few remaining genotypes are those which have been able to withstand the sheep dip pollution and siltation common to the region.

Other rivers such as the Cwmbach Dulas and the Offeiriad, both found in the Upper Wye, displayed a reversed trend. Lakes located near the upper sites of each of these rivers contained substantial *A. pallipes* populations, the origins of which are unknown. Imports to these lakes from elsewhere over time are highly likely. Movement of *A. pallipes* individuals into the rivers from these lakes has been recorded and may have maintained higher levels of genetic variation at these sites. Indeed, Foster (1996) recorded a dam burst at Pencerrig on the Dulas which resulted in thousands of crayfish being displaced downstream. The upper site of the Offeiriad is located in a restricted army range on unimproved grassland. Consequently, there is minimal pressure on *A. pallipes* genetic diversity at this site. The effects of livestock farming including sheep dip pollution and siltation are greater further downstream and could have added pressures on the genepools at these lower sites thus decreasing diversity and increasing the chances of inbreeding.

The greater genetic variability and more genotypes of *A. pallipes* in the Lower Wye than the Upper Wye may be explained by the fact that the Lower Wye sites are more lowland with less adjacent livestock farms, more arable farms and woodland and consequently experience less pressure from sheep dip pollution and siltation than the Upper Wye. Alternatively, it may be the case that those genotypes remaining in the Upper Wye are more able to withstand these pressures than those genotypes only found in the Lower Wye.

6.6 Rearing and Restocking

After considering the findings of the present study and other literature sources, I conclude that restocking is the most appropriate method of boosting *A. pallipes* numbers in recovering watercourses. A number of studies have shown this conservation method to be successful, particularly where *A. pallipes* has been wiped out as a result of crayfish plague. For example, a restocking programme carried out in the 1980s and early 1990s in the Sherston and Tetbury Avon, Wiltshire where crayfish plague had destroyed *A. pallipes* populations in the early 1980s, was successful as crayfish were restocked in the rivers a decade later (Spink & Frayling, 2000). Other successful *A. pallipes* restocking programmes included reintroductions, to a lake in Co. Westmeath and to Lough Lene in Ireland, from which *A. pallipes* had previously been wiped out by plague (Reynolds & Matthews, 1997; Reynolds *et al.*, 2000).

In the study of the Irish lake, a search confirmed the absence of crayfish. Individuals from a nearby healthy population that displayed relatively high levels of heterozygosity were introduced to the lake (Reynolds *et al.*, 2000). These introduced individuals were found in the lake three months later although numbers were depleted, perhaps due to predation or some avoiding recapture. Further introductions and regular monitoring were planned over a number of years in order to establish the level of success of this experiment.

A 1999 Polish study of *Astacus astacus* crayfish found them to be present in six out of nine restocked sites one year after reintroductions took place (Struzynski, 2002).

In Wales, few restockings have been carried out and those that have, appear to have been relatively unsuccessful in the long term. For example, a number of *A. pallipes* reintroductions were carried out using individuals from the Wye Catchment in 1988 and 1989 by John Foster and Fred Slater. Up to 115 individuals were stocked at 17 sites of suitable chemical and physical habitat quality (Foster, 1996). A resurvey of these sites in 1989 found no crayfish, which was attributed mainly to the fact that populations remained too small to be detected. In 1992, Foster (1996) reported that surveyors found no crayfish at any of the sites. Holdich (1993) found no crayfish at the restocked sites he surveyed, but in 1994, five years after restocking, a crayfish was detected at a reintroduction site in Solva, Pembrokeshire, suggesting that numbers

here had finally just reached detectable levels (Foster, 1996). In the present study, after resurveying these sites, no crayfish were found, indicating that numbers had again dropped below detectable levels, with the exception of some found in 2002 in the vicinity of the Llwyn Onn Reservoir in the Brecon Beacons (F. Slater, pers. comm.). A further search by the CCW also found that the Solva population had survived (M. Howe pers. comm.) Perhaps if larger numbers of crayfish had been used to restock these sites and supplemented with further introductions, greater success may have been achieved in the long term. However, as with many studies, restrictions of time and funding prevented this.

Before restocking is carried out in the future, results from the present study strongly suggest that genetically compatible crayfish should be found to act as donor populations for receptor sites. Unfortunately, Welsh *A. pallipes* abundance has become so low that breeding in captivity should, in my opinion, be used, although labour intensive and expensive to provide or at the very least to supplement donor populations in order to prevent too many crayfish from having to be removed from the wild. A cheaper alternative could be to use genetically appropriate stock from strong English populations.

Past breeding experiments in Britain (e.g. Lebas & Rogers (2000)) have found that *A. pallipes* breeding is possible but the rearing of summerlings from eggs has been extremely difficult, always resulting in many mortalities. The present study therefore tackled this problematic rearing stage with relatively successful results. A number of berried *A. pallipes* females were captured and their eggs reared to summerlings.

The provision of fresh diatoms every three days to hatchlings ensured a mean survival rate from eggs to summerlings of 10.8 % without incubation, while 76 % of all eggs that hatched in the present study survived to the 2nd stage, a relatively high success rate. The poor success of other non-incubation British *A. pallipes* rearing experiments may be due to a lack of fresh food, thought to be an important factor in promoting survival rates (Fernandez *et al.*, 1983).

Most mortalities in the present study occurred during egg development and hatching. A large quantity of eggs and some hatchlings became infected by the fungus *Saprolegnia* sp., recognisable by the orange/yellow discolouration of the usually dark brown/black eggs. This happened despite the checking for and removal of dead eggs

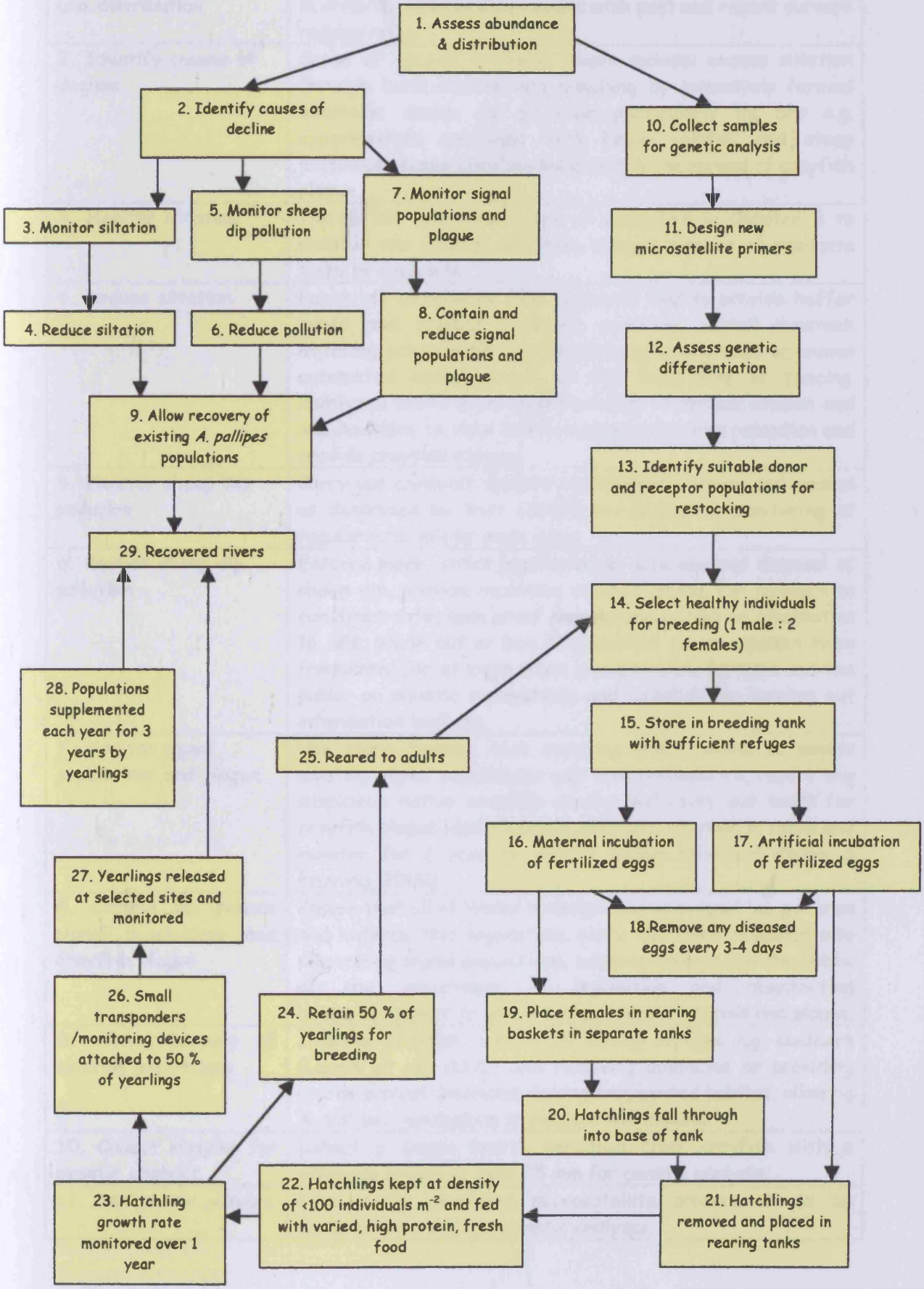
and young every 3-4 days to promote survival by minimizing disturbance and transmission to surrounding eggs as suggested by Carral *et al.* (2002). It is also likely that some mortalities occurred as a result of cannibalism.

A *Spirulina* supplement containing 63 g $100g^{-1}$ of protein, an important growth promoter used by aquaculture specialists in Australia, was used to try to increase growth and survival of half of the *A. pallipes* hatchlings in the present study. Those fed with the *Spirulina* supplement were found to have a slightly higher growth and survival rate than those not receiving *Spirulina*. This difference may have been greater if *Spirulina* had been provided in a more edible form such as a pellet rather than being suspended in water, and its effects monitored for a longer period of time. However, it was possible that the hatchlings were already receiving sufficient protein from their fresh food diet, resulting in the *Spirulina* making little difference.

Although artificial incubation techniques used abroad have produced good survival rates, it was feared that the transfer of hatchlings from incubators into cooler British rivers would result in mass mortalities. This experiment was therefore carried out without incubation. However, growth and survival rates of hatchlings in the present study would probably have been greater if incubation procedures had been used. I therefore recommend using incubation in future rearing studies, together with research into survival of juveniles during acclimatisation, by gradually lowering the water temperatures to those of the cooler British rivers while still in captivity before restocking begins. A lack of time prevented further progression of this study.

After the relative success of the present study in rearing *A. pallipes* from eggs, a captivity breeding programme using genetically selected individuals should be established. The individuals produced should then be used to restock recovering rivers and should be monitored after release perhaps by micro chipping or by radio tracking (Guan & Wiles, 1997; Armitage, 2000; Bubb *et al.*, 2002; 2004) although chips and transmitters used may be too large for very young individuals. However, time limitations did not allow this to be carried out during my PhD. Such a programme could comprise a new research project.

Conservation Action Plan for *A. pallipes* populations of upland Welsh rivers



Stage	Action
1. Assess abundance and distribution	Calculate CPUE and use the protocol designed by Peay (2002) in order to standardize results with past and recent surveys respectively.
2. Identify causes of decline	Cause of decline in Welsh rivers include: excess siltation through bank erosion and poaching by intensively farmed livestock; sheep dip pollution particularly by SPs e.g. cypermethrin combined with heavy rainfall and steep landscape; signal crayfish invasion and the spread of crayfish plague.
3. Monitor siltation	Install silt collecting traps as described in Chapter 3 to monitor the rate of siltation, using a number of replicate traps at each site.
4. Reduce siltation	Fence off riverbanks from adjacent land to provide buffer zones and prevent livestock poaching. Install livestock watering points with reinforced hardcore ground or animal automated water pumps on the field side of fencing. Reinforce banks with willow bundles to reduce erosion and add boulders to river bed to increase detritus retention and provide crayfish refuges.
5. Monitor sheep dip pollution	Carry out chemical analysis on substrate, water and mosses as described by Rutt (2004) and biological monitoring at regular intervals at more sites.
6. Reduce sheep dip pollution	Enforce more, strict legislation on safe use and disposal of sheep dip, provide incentive such as grants for farmers to construct safe, leak proof dipping areas, find an alternative to SPs, phase out or ban SPs, monitor water courses more frequently and at more sites, educate more farmers and the public on aquatic ecosystems and crayfish by handing out information leaflets.
7. Monitor signal populations and plague	Use stone turning, kick sampling and trapping to assess existing signal populations and their boundaries, report any suspicious native crayfish deaths and carry out tests for crayfish plague identification, and hold crayfish in tanks and monitor for 1 year to detect plague infection (Spink & Frayling, 2000)
8. Contain and reduce signal populations and crayfish plague	Ensure that all of Wales is designated as a signal "no-go" area and enforce this legislation, carry out more research into eliminating signal populations, educate anglers and the public of the importance of legislation and disinfecting boots/equipment to prevent the spread of signals and plague.
9. Allow recovery of existing populations	Improve crayfish habitat by adding refuges e.g. boulders (Lepori *et al.*, 2005) and removing obstacles or providing routes around obstacles, linking fragmented habitat, allowing A. pallipes populations to recover and expand.
10. Collect samples for genetic analysis	Collect a single fourth pereopod from crayfish with a carapace length of over 25 mm for genetic analysis.
11. Design new primers	New, easily amplified microsatellite primers should be designed to carry out genetic analyses.

12. Assess genetic differentiation	Genetic differentiation of *A. pallipes* populations as discovered in Chapter 4 of this thesis should be confirmed and any further groupings identified.
13. Identify suitable donor and receptor populations for restocking	Donor populations should come from habitats similar to those of the receptor populations, in order to maintain the natural genetic diversity of the area but also to prevent extinction through inbreeding. Introduction of individuals to Wales from Ireland, England, France or Italy to prevent inbreeding should also be considered.
14. Select healthy individuals for breeding (2 females : 1 male)	Healthy, disease free individuals should be selected for breeding programs. A few individuals in each population should be checked for diseases such as crayfish plague or porcelain disease. The sex ratio of breeding populations should be two females to 1 male (Struzynski, 2002).
15. Store in breeding tank with sufficient refuges	Crayfish should be stored in breeding tanks or ponds measuring 4 m width x 20 m length x 1.5 m depth, with weighted plastic tubing as refuges. Water temperature should be approximately 10 °C, 8.7 mg l^{-2} of dissolved oxygen, 12.8 mg l^{-2} of CO_2, a pH of 8, 0 mg l^{-2} of $Ca\,CO_3$ (carbonates), 236 mg l^{-2} of $CaCO_3$ (bicarbonates), 0.02 mg l^{-2} of NH_3 (ammonical nitrogen), 42.5 mg l^{-2} of nitrates (Cuellar & Coll, 1978) and adults should be fed on a varied, fresh, high protein diet supplemented by Spirulina.
16. Maternal incubation of fertilised eggs	If eggs are incubated maternally, females should be kept in separate holding tanks to prevent aggressive encounters (Ingle, 1977) throughout the wintering period until May.
17. Artificial incubation of fertilised eggs	Artificially incubated eggs should be kept at 10 °C until Phase XIII of embryonic development, then at 15 °C until the 2nd stage juveniles reached (Perez *et al.*, 1999).
18. Remove any diseased eggs every 3-4 days	In order to minimise disturbance and disease transmission, any diseased eggs (noted by their yellow/brown discolouration) should be checked and removed every 3-4 days (Carral *et al.*, 2002).
19. Place brooding females in rearing baskets in separate tanks.	Females with eggs should be held in rearing baskets immersed in separate tanks with a refuge pipe, as described in Chapter 5 at controlled temperatures and a regular supply of food.
20. Hatchlings fall through into base of tank	When hatchlings become independent of an adult female, they drop off the abdomen and fall thought he mesh basket into the base of the tank, thus preventing them from being cannibalised by the adult female.
21. Hatchlings removed and placed in rearing tanks	Tanks should be checked twice daily for any detached hatchlings, which are carefully removed and placed into separate tanks.
22. Hatchlings kept at density of <100 individuals m^{-2} and fed with varied, high protein, fresh food.	Hatchlings should be kept at density of no more than 100 individuals m^{-2}, less if possible, fed daily with a diet of fresh algae containing diatoms scraped from river bed rocks, vegetation, zooplankton e.g. Daphnia, fresh liver, Spirulina pellets and grated carrots (DeLuise & Sabbadini, 1988;

	Maguire *et al.*, 2002; Morrisey *et al.*, 1995; Keller, 1988; Cuellar & Coll 1978; Fernandez *et al.* 1983; Pursiainen, 1983).
23. Hatchling growth rate monitored over 1 year.	Hatchling length should be measured every 2-3 weeks using the petri dish and graph paper method described in Chapter 5, over a year.
24. Retain 50 % of yearlings for breeding.	50 % of yearlings should be retained for breeding.
25. Reared to adults.	These yearlings should be reared until they reach sexual maturity when they should be used to supplement the breeding program stock.
26. Small transponders/monitoring devices attached to 50 % of yearlings	The remaining 50 % of yearlings should be fitted with sufficiently small transponders/monitoring devices prior to release.
27. Yearlings released at selected sites and monitored	These yearlings should be released at the selected receptor sites at numbers of no less than 100 individuals per site (Spink & Frayling, 2000) and at a density of no more than 1 individual m^{-2} into stable refuges by night (Struzynski, 2002) and should be monitored at regular intervals of 2 weeks for the first 2 months, then every three months.
28. Populations supplemented each year for 3 years by yearlings	Introduced populations should be supplemented each year by yearlings for the next three years to produce a population structure with a good age range and to replace any individuals from previous years which may have died.
29. Recovered rivers	*A. pallipes* individuals should only be reintroduced to rivers of a high enough quality, which have recovered sufficiently from siltation or sheep dip pollution and which have had measures installed to prevent siltation or sheep dip pollution or plague from reoccurring.

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Appendix I

Crayfish Raw Data

Crayfish Samples - Dulas Monnow

Sample no.	Site	Catchment	Grid Reference	Date	Sex	Carapace length (mm)
1	Dulas (3)	Lower Wye	SO 387287	30/07/02	*	*
2	Dulas (3)	Lower Wye	SO 387287	30/07/02	M	10.9
3	Dulas (3)	Lower Wye	SO 387287	30/07/02	M	23.1
4	Dulas (3)	Lower Wye	SO 387287	30/07/02	M	25.4
5	Dulas (4)	Lower Wye	SO 386289	30/07/02	*	7
6	Dulas (5)	Lower Wye	SO 383292	30/07/02	*	*
7	Dulas (5)	Lower Wye	SO 383292	30/07/02	*	*
8	Dulas (5)	Lower Wye	SO 383292	30/07/02	*	*
9	Dulas (5)	Lower Wye	SO 383292	30/07/02	*	*
10	Dulas (5)	Lower Wye	SO 383292	30/07/02	*	*
11	Dulas (5)	Lower Wye	SO 383292	30/07/02	F	22.8
12	Dulas (5)	Lower Wye	SO 383292	30/07/02	F	21.2
13	Dulas (5)	Lower Wye	SO 383292	30/07/02	M	19.4
14	Dulas (6)	Lower Wye	SO 373295	30/07/02	M	13.5
15	Dulas (6)	Lower Wye	SO 373295	30/07/02	M	*
16	Dulas (6)	Lower Wye	SO 373295	30/07/02	F	21.7
17	Dulas (6)	Lower Wye	SO 373295	30/07/02	M	36.3
18	Dulas (7)	Lower Wye	SO 363302	30/07/02	F	26.8
19	Dulas (7)	Lower Wye	SO 363302	30/07/02	M	40.7
20	Dulas (7)	Lower Wye	SO 363302	30/07/02	M	34.7
21	Dulas (7)	Lower Wye	SO 363302	30/07/02	F	29.9
22	Dulas (7)	Lower Wye	SO 363302	30/07/02	M	39.9
23	Dulas (8)	Lower Wye	SO 361302	30/07/02	F	25.3
24	Dulas (8)	Lower Wye	SO 361302	30/07/02	M	42.4
25	Dulas (8)	Lower Wye	SO 361302	30/07/02	F	27.7
26	Dulas (8)	Lower Wye	SO 361302	30/07/02	F	23.4
27	Dulas (3)	Lower Wye	SO 387287	16/10/02	F	18.1
28	Dulas (4)	Lower Wye	SO 386289	16/10/02	M	19.7
29	Dulas (6)	Lower Wye	SO 373295	16/10/02	M	32.7
30	Dulas (7)	Lower Wye	SO 363302	16/10/02	M	30
31	Dulas (7)	Lower Wye	SO 363302	16/10/02	F	17.8
32	Dulas (6)	Lower Wye	SO 373295	16/10/02	*	30
33	Dulas (7)	Lower Wye	SO 363302	16/10/02	F	31.1
34	Dulas (7)	Lower Wye	SO 363302	16/10/02	M	33.3
35	Dulas (7)	Lower Wye	SO 363302	16/10/02	F	19.3
36	Dulas (7)	Lower Wye	SO 363302	16/10/02	M	17.5
37	Dulas (7)	Lower Wye	SO 363302	16/10/02	F	14.7
38	Dulas (7)	Lower Wye	SO 363302	16/10/02	M	20.4

Sample no.	Missing appendages	Disease status	Notes
1	0	Healthy	Escape (20-25mm)
2	0	Healthy	
3	0	Healthy	
4	0	Healthy	
5	0	Healthy	Juvenile
6	0	Healthy	Escape (10-15mm)
7	0	Healthy	Escape (10-15mm)
8	0	Healthy	Escape (15-20mm)
9	0	Healthy	Escape (15-20mm)
10	0	Healthy	Escape (15-20mm)
11	0	Healthy	
12	0	Healthy	
13	0	Healthy	
14	0	Healthy	
15	1 chela	Porcelain	White abdomen underside
16	0	Healthy	
17	0	Healthy	
18	0	Healthy	
19	0	Healthy	
20	0	Healthy	
21	0	Healthy	
22	0	Healthy	
23	0	Healthy	
24	0	Healthy	
25	0	Healthy	
26	0	Healthy	
27	0	Healthy	
28	0	Healthy	
29	0	Healthy	
30	0	Healthy	Left chela small
31	0	Healthy	
32	0	Healthy	Escape
33	0	Healthy	Recapture
34	0	Healthy	
35	0	Healthy	
36	0	Healthy	
37	0	Healthy	
38	0	Healthy	

Samples Summary – Dulas Monnow

Site	Catchment	Grid Reference	Date	Number of crayfish found
Dulas (3)	Lower Wye	SO 387287	30/07/02	4
Dulas (4)	Lower Wye	SO 386289	30/07/02	1
Dulas (5)	Lower Wye	SO 383292	30/07/02	8
Dulas (6)	Lower Wye	SO 373295	30/07/02	4
Dulas (7)	Lower Wye	SO 363302	30/07/02	5
Dulas (8)	Lower Wye	SO 361302	30/07/02	4
Dulas (3)	Lower Wye	SO 387287	16/10/02	1
Dulas (4)	Lower Wye	SO 386289	16/10/02	1
Dulas (6)	Lower Wye	SO 373295	16/10/02	2
Dulas (7)	Lower Wye	SO 363302	16/10/02	8
Ewyas Harold	Lower Wye	SO 392 282	25/09/03	3
Lower Maes Coed	Lower Wye	SO 355 310	13/08/03	8
Nr. Upper Grange	Lower Wye	SO 354 322	25/09/03	6
Gilfach	Lower Wye	SO 345 342	13/08/03	18

Crayfish Samples - Banwy

Grid Reference	Date	Sex	Carapace length (mm)	Missing appendages	Disease status	Notes
SJ 085 074	03/09/02	M	32.7	0	Healthy	
SJ 085 073	03/09/02	M	16	0	Healthy	Recently moulted
SJ 085 073	03/09/02	M	35.2	0	Porcelain	Recently moulted
SJ 085 073	03/09/02	F	29.9	0	Healthy	
SJ 085 073	03/09/02	M	12.7	0	Healthy	
SJ 085 073	03/09/02	F	32.5	0	Porcelain	
SJ 085 073	03/09/02	*	*	0	*	Escape
SJ 085 073	03/09/02	*	*	0	*	Escape

Samples Summary – Banwy

Site	Catchment	Grid Reference	Date	Number of crayfish found
Banwy	Upper Severn	SJ 085 074	03/09/02	1
Banwy tributary	Upper Severn	SJ 085 073	03/09/02	7
Llanfair Caereinion	Upper Severn	SJ 104 065	03/09/02	0
Llangadfan	Upper Severn	SJ 013 105	03/09/02	0
Railway bridge	Upper Severn	SJ 125 081	03/09/02	0

Crayfish Samples – Cwmbach Dulas

Site	Catchment	Grid Reference	Date	Sex	Carapace length (mm)
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	16/06/02	F	25
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	16/06/02	M	35
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	16/06/02	M	40
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	16/06/02	M	17
Builth Rd., Dulas	Upper Wye	SO 023534	16/06/02	M	45
Builth Rd., Dulas	Upper Wye	SO 023534	16/06/02	M	25
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	F	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	F	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	F	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	F	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	F	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	F	*
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	F	*
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	F	*
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	F	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	M	*
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	M	*
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	M	*
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	M	*
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	M	*
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	M	*
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	M	*

Sample no.	Missing appendages	Disease status	Notes
1	0	Healthy	
2	0	Healthy	
3	0	Healthy	
4	0	Healthy	
5	0	Healthy	
6	0	Healthy	
7	0	Healthy	Hatchlings attached
8	0	Healthy	
9	0	Healthy	
10	0	Healthy	
11	0	Healthy	
12	0	Dead	
13	0	Dead	
14	0	Dead	
15	0	Dead	
16	0	Healthy	
17	0	Healthy	
18	0	Healthy	
19	0	Healthy	
20	0	Healthy	Hatchlings attached
21	0	Healthy	Hatchlings attached
22	0	Healthy	Hatchlings attached
23	0	Healthy	Hatchlings attached
24	0	Healthy	Hatchlings attached
25	0	Healthy	
26	0	Healthy	
27	0	Healthy	
28	0	Healthy	
29	0	Dead	
30	0	Dead	
31	0	Dead	
32	0	Healthy	
33	0	Healthy	
34	0	Healthy	
35	0	Healthy	

Samples Summary – Cwmbach Dulas

Site	Catchment	Grid Reference	Date	Number of crayfish found
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	16/06/02	4
Builth Rd., Dulas	Upper Wye	SO 023534	16/06/02	3
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	15/06/03	16
Cwmbach Llechryd, Dulas	Upper Wye	SO 031541	22/06/03	5
Builth Rd., Dulas	Upper Wye	SO 023534	22/06/03	8

Crayfish Samples – Edw

Sample no.	Site name	Catchment	Grid Reference	Date	Sex
1	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	M
2	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	F
3	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	F
4	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	M
5	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	F
6	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	M
7	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	F
8	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	M
9	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	F
10	Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	F
11	Llanbadarn-y-Garreg	Upper Wye	SO 113488	10/07/02	M
12	Llanbadarn-y-Garreg	Upper Wye	SO 113488	10/07/02	F
13	Llanbadarn-y-Garreg	Upper Wye	SO 113488	10/07/02	M
14	Llanbadarn-y-Garreg	Upper Wye	SO 113488	11/07/02	M
15	Llanbadarn-y-Garreg	Upper Wye	SO 113488	11/07/02	M
16	Cregina	Upper Wye	SO 124522	11/07/02	F
17	Franksbridge	Upper Wye	SO 116561	15/07/02	M
18	Franksbridge	Upper Wye	SO 116561	15/07/02	M
19	Franksbridge	Upper Wye	SO 116561	15/07/02	M
20	Bettws Mill	Upper Wye	SO 116566	15/07/02	F
21	Bettws Mill	Upper Wye	SO 116566	15/07/02	M
22	Llanbadarn-y-Garreg	Upper Wye	SO 113488	15/07/02	F
23	Llanbadarn-y-Garreg	Upper Wye	SO 113488	15/07/02	M
24	Llanbadarn-y-Garreg	Upper Wye	SO 113488	15/07/02	F
25	Aberedw	Upper Wye	SO 098479	18/07/02	F
26	Aberedw	Upper Wye	SO 098479	18/07/02	M
27	Aberedw	Upper Wye	SO 098479	18/07/02	?
28	Aberedw	Upper Wye	SO 098479	18/07/02	M
29	Aberedw	Upper Wye	SO 098479	18/07/02	F
30	Aberedw	Upper Wye	SO 098479	18/07/02	M
31	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	F
32	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	F
33	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	M
34	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	M
35	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	M
36	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	?
37	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	F
38	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	M

39	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	F
40	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	F
41	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	F
42	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	?
43	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	M
44	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	F
45	Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	M
46	Cregina	Upper Wye	SO 124522	19/07/02	F
47	Aberedw	Upper Wye	SO 098479	08/08/02	F
48	Aberedw	Upper Wye	SO 098479	08/08/02	M
49	Aberedw	Upper Wye	SO 098479	08/08/02	M
50	Aberedw	Upper Wye	SO 098479	08/08/02	M
51	Aberedw	Upper Wye	SO 098479	08/08/02	M
52	Aberedw	Upper Wye	SO 098479	08/08/02	M
53	Aberedw	Upper Wye	SO 098479	13/08/02	?
54	Aberedw	Upper Wye	SO 098479	13/08/02	M
55	Aberedw	Upper Wye	SO 098479	03/09/02	F
56	Aberedw	Upper Wye	SO 098479	03/09/02	M
57	Aberedw	Upper Wye	SO 098479	03/09/02	M
58	Aberedw	Upper Wye	SO 098479	03/09/02	F
59	Aberedw	Upper Wye	SO 098479	03/09/02	M
60	Aberedw	Upper Wye	SO 098479	05/09/02	F
61	Aberedw	Upper Wye	SO 098479	05/09/02	M
62	Aberedw	Upper Wye	SO 098479	05/09/02	M
63	Aberedw	Upper Wye	SO 098479	05/09/02	F
64	Confluence	Upper Wye	SO 075470	17/09/02	M
65	Confluence	Upper Wye	SO 075470	17/09/02	*
66	Aberedw	Upper Wye	SO 098479	17/09/02	M
67	Aberedw	Upper Wye	SO 098479	17/09/02	M
68	Llanbadan-y-Garreg	Upper Wye	SO 113488	17/09/02	F
69	Llanbadan-y-Garreg	Upper Wye	SO 113488	17/09/02	M
70	Cregina	Upper Wye	SO 124522	17/09/02	F
71	Franksbridge	Upper Wye	SO 116561	17/09/02	F
72	Aberedw	Upper Wye	SO 098479	*	F
73	Aberedw	Upper Wye	SO 098479	*	F
74	Aberedw	Upper Wye	SO 098479	*	F
75	Aberedw	Upper Wye	SO 098479	*	M

Sample no.	Carapace length (mm)	Missing appendages	Disease status	Notes
1	21.1	0	Healthy	
2	30	0	Healthy	
3	33.4	0	Healthy	
4	31.2	0	Healthy	
5	30.4	0	Healthy	
6	30.7	0	Healthy	
7	29.2	0	Healthy	
8	30.3	0	Healthy	
9	29.7	0	Healthy	
10	29.4	0	Healthy	
11	27.8	0	Healthy	
12	37.4	0	Healthy	Two young on tail
13	30	0	Healthy	
14	27	0	Healthy	
15	32	0	Dead	
16	31	0	Healthy	
17	36.5	0	Healthy	
18	44.3	0	Healthy	
19	27	0	Healthy	
20	32.9	0	Healthy	
21	29	0	Healthy	
22	30.3	4th pereopod missing	Healthy	
23	29.5	0	Healthy	
24	27.3	0	Healthy	
25	38.9	0	Healthy	
26	27.8	0	Healthy	
27	11	0	Healthy	Juvenile
28	30.6	0	Healthy	
29	31.4	0	Healthy	
30	40.4	0	Healthy	
31	37.2	0	Healthy	
32	27.1	0	Healthy	
33	23.8	0	Healthy	
34	37.1	0	Healthy	
35	31.4	0.5 antennae	Healthy	
36	11.4	0	Healthy	Juvenile
37	35	2nd, 3rd, & 4th leg + 0.5 antenna	Healthy	
38	29.7	0	Healthy	
39	34.4	0.5 antenna	Healthy	

40	29.3	1 chela	Healthy	
41	31.7	0	Healthy	
42	18.5	0	Healthy	Juvenile
43	29.2	0	Healthy	
44	27.6	1st pereopod	Healthy	
45	28.4	0	Healthy	
46	35.8	0.5 antenna	Healthy	
47	32.1	0.5 antenna	Healthy	
48	30.9	4th right pereopod	Porcelain	
49	34	0	Healthy	
50	34.4	0	Healthy	
51	34	0	Healthy	
52	29.1	0	Healthy	
53	12.9	0	Healthy	Juvenile
54	35.9	1 chela & 0.5 antenna	Healthy	
55	30.7	0	Healthy	
56	25	4th pereopod	Healthy	Recapture
57	33.9	0	Healthy	
58	30.3	0	Healthy	1 small chela
59	33	0	Healthy	
60	42.5	1 Chela	Healthy	
61	33.2	4th pereopod	Healthy	Recapture
62	33.6	0	Porcelain	
63	35.1	4th pereopod	Healthy	Recapture
64	35.5	0	Healthy	
65	17.5	0	Healthy	Escape
66	18.1	0	Healthy	
67	32	0	Healthy	
68	42.6	1 chela	Healthy	
69	22.5	0	Healthy	
70	24.5	0	Healthy	
71	30.8	0	Healthy	
72	31	1chela	Healthy	
73	34.4	0	Healthy	Recently moulted
74	37.5	1 chela	Healthy	Recently moulted
75	23	0	Healthy	

Samples Summary – Edw

Site name	Catchment	Grid Reference	Date	Number of crayfish found
Llanbadarn-y-Garreg	Upper Wye	SO 113488	09/07/02	10
Llanbadarn-y-Garreg	Upper Wye	SO 113488	10/07/02	3
Llanbadarn-y-Garreg	Upper Wye	SO 113488	11/07/02	2
Cregina	Upper Wye	SO 124522	11/07/02	1
Franksbridge	Upper Wye	SO 116561	15/07/02	3
Bettws Mill	Upper Wye	SO 116566	15/07/02	2
Llanbadarn-y-Garreg	Upper Wye	SO 113488	15/07/02	3
Aberedw	Upper Wye	SO 098479	18/07/02	6
Llanbadan-y-Garreg	Upper Wye	SO 113488	18/07/02	15
Cregina	Upper Wye	SO 124522	19/07/02	1
Aberedw	Upper Wye	SO 098479	08/08/02	6
Aberedw	Upper Wye	SO 098479	13/08/02	2
Aberedw	Upper Wye	SO 098479	03/09/02	5
Aberedw	Upper Wye	SO 098479	05/09/02	4
Confluence	Upper Wye	SO 075470	17/09/02	2
Aberedw	Upper Wye	SO 098479	17/09/02	1
Llanbadan-y-Garreg	Upper Wye	SO 113488	17/09/02	2
Cregina	Upper Wye	SO 124522	17/09/02	1
Franksbridge	Upper Wye	SO 116561	17/09/02	1
Aberedw	Upper Wye	SO 098479	*	4

Crayfish Samples – Sgithwen

Sample no.	Site	Catchment	Grid Reference	Date	Sex	Carapace length (mm)
1	Sgithwen (1)	Upper Wye	SO 083401	22/08/02	F	33
2	Sgithwen (1)	Upper Wye	SO 083401	22/08/02	F	28.9
3	Sgithwen (1)	Upper Wye	SO 083401	22/08/02	M	30.9
4	Sgithwen (1)	Upper Wye	SO 083401	22/08/02	F	31
5	Sgithwen (1)	Upper Wye	SO 083401	22/08/02	M	28
6	Sgithwen (1)	Upper Wye	SO 083401	22/08/02	F	*
7	Sgithwen (1)	Upper Wye	SO 083401	22/08/02	M	41.4
8	Sgithwen (1)	Upper Wye	SO 083401	22/08/02	M	24
9	Sgithwen (2)	Upper Wye	SO 073394	18/09/02	F	36.1
10	Sgithwen (2)	Upper Wye	SO 073394	18/09/02	M	44
11	Sgithwen (2)	Upper Wye	SO 073394	18/09/02	F	33
12	Sgithwen (2)	Upper Wye	SO 073394	18/09/02	M	33.4
13	Sgithwen (2)	Upper Wye	SO 073394	18/09/02	M	37.2
14	Sgithwen (2)	Upper Wye	SO 073394	18/09/02	M	29.7
15	Sgithwen (2)	Upper Wye	SO 073394	18/09/02	F	30.3
1	Sgithwen (1)	Upper Wye	SO 083401	25/05/03	M	*
2	Sgithwen (1)	Upper Wye	SO 083401	25/05/03	M	*
3	Sgithwen (1)	Upper Wye	SO 083401	25/05/03	M	*
4	Sgithwen (1)	Upper Wye	SO 083401	25/05/03	M	*
5	Sgithwen (2)	Upper Wye	SO 073394	25/05/03	M	*
6	Sgithwen (2)	Upper Wye	SO 073394	25/05/03	M	*

Sample no.	Missing appendages	Disease status	Notes
1	0	Healthy	Cracked carapace
2	0	Healthy	
3	0	Porcelain	White abdomen
4	0	Healthy	Recently moulted
5	0	Healthy	
6	0	Healthy	
7	0	Healthy	
8	0	Healthy	
9	End of tail	Healthy	
10	0	Porcelain	White abdomen
11	0.5 both antennae	Healthy	Half of both antennae missing
12	0	Porcelain	Recapture. White abdomen
13	0	Healthy	Broken chela
14	0	Healthy	
15	0	Healthy	
1	0	Healthy	
2	0	Healthy	
3	0	Healthy	
4	0	Healthy	
5	0	Healthy	
6	0	Healthy	

Samples summary – Sgithwen

Site	Catchment	Grid Reference	Date	Number of crayfish found
Sgithwen (1)	Upper Wye	SO 083401	22/08/02	8
Sgithwen (2)	Upper Wye	SO 073394	18/09/02	7
Sgithwen (1)	Upper Wye	SO 083401	25/05/03	4
Sgithwen (2)	Upper Wye	SO 073394	25/05/03	2

Crayfish Samples – Offeiriad

Sample no.	Site	Catchment	Grid Reference	Date	Sex
1	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	M
2	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	M
3	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	F
4	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	M
5	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	M
6	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	*
7	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	F
8	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	M
9	Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	M
10	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
11	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
12	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
13	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
14	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
15	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
16	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
17	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
18	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
19	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	*
20	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
21	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
22	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	*
23	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	*
24	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	*
25	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
26	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
27	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
28	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
29	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
30	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
31	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
32	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	M
33	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
34	Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	F
35	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
36	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
37	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
38	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
39	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
40	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
41	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
42	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
43	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
44	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
45	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
46	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
47	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F

48	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
49	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
50	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
51	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
52	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
53	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
54	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
55	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
56	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
57	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	*
58	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
59	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
60	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
61	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
62	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
63	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
64	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
65	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
66	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
67	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
68	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
69	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
70	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
71	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
72	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
73	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
74	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	*
75	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	*
76	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
77	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	M
78	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
79	Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	F
80	Cletwr (Erwood Inn)	Upper Wye	SO 097431	30/08/02	F
81	Cletwr (Erwood Inn)	Upper Wye	SO 097431	30/08/02	M
82	Cletwr (Tircanvas)	Upper Wye		30/08/02	F

Sample no.	Carapace length (mm)	Missing appendages	Disease status	Notes
1	35.9	0	Porcelain	
2	24.6	3rd right pereopod	Healthy	
3	14.8	0	Healthy	Soft, just moulted
4	21.9	0	Healthy	
5	22.8	0	Healthy	
6	10.2	0	Healthy	
7	30.3	0	Healthy	Berried
8	36.8	0	Healthy	
9	34.1	0	Healthy	
10	33.8	0	Healthy	
11	46.2	0	Healthy	
12	26.7	0	Healthy	
13	25.9	0	Healthy	
14	24.7	0	Healthy	
15	25.9	0	Healthy	
16	23.4	0	Healthy	
17	15.3	0	Healthy	
18	28.8	0	Healthy	
19	7	0	Healthy	Juvenile
20	41.9	0	Healthy	
21	29	0	Healthy	Recently moulted
22	*	0	Healthy	Juvenile escape
23	*	0	Healthy	Juvenile escape
24	*	0	Healthy	Juvenile escape
25	28.9	0	Healthy	
26	22	0	Healthy	
27	28.6	0	Healthy	
28	19.3	0	Healthy	
29	30.8	0	Healthy	Recently moulted
30	33.5	0	Healthy	Recently moulted
31	36.6	0	Healthy	
32	27.9	0	Healthy	
33	32.5	0	Healthy	Recently moulted
34	27.9	0	Healthy	
35	31.9	0	Healthy	
36	29.1	0	Healthy	
37	39.3	0	Healthy	
38	25.4	0.5 antenna	Healthy	
39	29.9	0.5 antenna	Healthy	
40	32.9	0	Porcelain	White abdomen underside
41	34.6	0	Healthy	
42	29.7	0	Healthy	
43	21.5	0	Healthy	Too small to sample
44	30.7	0	Healthy	
45	266.5	0	Healthy	
46	32.5	0	Healthy	
47	30	0	Healthy	
48	27.7	1 chela missing	Healthy	
49	24.6	0.5 antenna missing	Healthy	

50	26.9	1 chela	Healthy	
51	27.8	0	Healthy	
52	37	1 chela	Healthy	
53	38.5	0	Healthy	
54	15.5	0	Healthy	Juvenile
55	11.1	0	Healthy	
56	36	4th pereopod	Healthy	
57	32.1	0	Healthy	Juvenile
58	14.8	0	Healthy	
59	24.4	0	Healthy	
60	33.3	0	Healthy	
61	30	White abdomen underside	Porcelain	
62	33	0	Healthy	
63	31.4	0.5 antenna	Healthy	
64	22.9	0	Healthy	
65	20.4	0	Healthy	
66	26.8	0	Healthy	
67	31.9	0	Healthy	
68	31.4	1 chela	Healthy	Soft exoskeleton
69	25.7	0	Healthy	
70	31.6	1 chela	Healthy	Soft exoskeleton
71	20	1 chela	Healthy	
72	17.8	0	Healthy	
73	30.4	0.5 antenna	Healthy	
74	10	0	Healthy	Juvenile
75	12	0	Healthy	Juvenile
76	16.8	0	Healthy	
77	31.4	0	Healthy	
78	29.8	0	Healthy	
79	18.8	0	Healthy	
80	32.6	0	Healthy	Carapace soft & cracked
81	41.3	0	Healthy	
82	35.3	0.5 antenna	Healthy	Half of antenna missing

Samples Summary – Offeiriad

Site	Catchment	Grid Reference	Date	Number of crayfish
Common Land, Offeiriad	Upper Wye	SO 023440	27/07/02	9
Army range, Offeiriad	Upper Wye	SO 015442	29/08/02	25
Nant yr Offeiriad Farm	Upper Wye	SO 035435	29/08/02	45
Cletwr (Erwood Inn)	Upper Wye	SO 097431	30/08/02	2
Cletwr (Tircanvas)	Upper Wye	*	30/08/02	1

Crayfish Samples – Escley

Sample no.	Site name	Grid Reference	Catchment	Date	Sex	Carapace length (mm)
1	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	F	19.4
2	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	M	19.8
3	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	F	24.2
4	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	M	37.9
5	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	*	*
6	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	*	*
7	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	*	*
8	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	*	*
9	Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	*	*

Sample no.	Missing appendages	Disease status	Notes
1	0	Healthy	
2	0	Healthy	
3	0	Healthy	
4	0	Healthy	
3	*	Healthy	Juvenile, escape
4	*	Healthy	Juvenile, escape
5	*	Healthy	Juvenile, escape
6	*	Healthy	Juvenile, escape
7	*	Healthy	Juvenile, escape

Samples Summary – Escley

Site name	Grid Reference	Catchment	Date	Number of crayfish found
Lower House Farm, Escley	SO 298369	Lower Wye	09/10/02	9
Michaelchurch Escley (PH)	SO 318 342	Lower Wye	18/09/03	2
Firs Farm	SO 315 352	Lower Wye	18/09/03	8
Lower House Farm, Escley	SO 308 362	Lower Wye	18/09/03	12
Escley Brook tributary	SO 310 354	Lower Wye	14/08/03	23

Crayfish Samples – Lugg

Sample no.	Site	Grid Reference	Catchment	Date	Sex	Carapace length (mm)
1	Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	20/09/02	M	*
2	Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	20/09/02	F	*
3	Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	20/09/02	*	*
4	Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	20/09/02	M	44.1
5	Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	20/09/02	*	*
6	Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	20/09/02	M	37.4
7	Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	23/09/02	M	*

Sample no.	Missing appendages	Disease status	Notes
1	0	Healthy	
2	0	Healthy	
3	1 pereopod	Healthy	
4	0	Healthy	Escape
5	0	Healthy	Recent moult
6	0	Healthy	Escape
7	0	Healthy	

Samples summary - Lugg

Site	Grid Reference	Catchment	Date	Number of crayfish found
Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	20/09/02	6
Mortimer's Cross (Mill), Lugg	SO 426637	Lugg	23/09/02	1

Crayfish Samples – Usk

Sample no.	Site	Catchment	Grid Reference	Date	Sex	Carapace length (mm)
1	Brecon bridge, Usk	Usk	SO 043286	12/09/02	M	33
2	Honddu	Usk	SO 050398	12/09/02	*	*
3	Llangattock	Usk	SO 215175	13/09/02	F	42.4
4	Llangattock	Usk	SO 215175	13/09/02	F	37.9
5	Llangattock	Usk	SO 215175	13/09/02	M	30.4
6	Llanfrynach	Usk	SO 079273	17/09/02	*	9.6
7	Llanfrynach	Usk	SO 079273	17/09/02	*	5.8
8	Llanfrynach	Usk	SO 079273	17/09/02	*	9.6
9	Llanfrynach	Usk	SO 079273	17/09/02	*	*
10	Llanfrynach	Usk	SO 079273	17/09/02	*	*
11	Llanfrynach	Usk	SO 079273	17/09/02	*	*
12	Llanfrynach	Usk	SO 079273	17/09/02	*	*
13	Llanfrynach	Usk	SO 079273	17/09/02	*	*
14	Dowlais Brook (3)		ST 184 944	*/09-10/03	F	25
15	Dowlais Brook (6)	Usk	ST 309 928	*/09-10/03	M	23.1
16	Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	M	21.4
17	Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	F	17.1
18	Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	M	19.1
19	Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	M	36
20	Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	M	39.2
21	Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	M	18.1
22	Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	M	17.7
23	Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	F	19.9
24	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	M	30
25	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	*	*
26	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	F	18
27	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	*	*
28	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	*	*
29	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	*	*
30	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	*	39.3
31	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	M	24.4
32	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	*	41.6
33	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	M	17.5
34	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	*	*
35	Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	*	*
36	Nant-y-Gollen (39)	Usk	ST 228 025	*/09-10/03	F	32.8
37	Nant-y-Gollen (39)	Usk	ST 228 025	*/09-10/03	*	*
38	Nant-y-Gollen (39)	Usk	ST 228 025	*/09-10/03	F	23.9

Sample no.	Missing appendages	Disease status	Notes
1	0	Healthy	
2	*	Healthy	
3	0	Healthy	
4	0	Healthy	Small right chela
5	0	Healthy	
6	0	Healthy	Juvenile
7	0	Healthy	Juvenile
8	0	Healthy	Juvenile
9	0	Healthy	Juvenile, escape
10	0	Healthy	Juvenile, escape
11	0	Healthy	Juvenile, escape
12	0	Healthy	Juvenile, escape
13	0	Healthy	Juvenile, escape
14	0	Healthy	
15	0	Healthy	
16	0	Healthy	
17	0	Healthy	
18	0	Healthy	
19	0	Healthy	1 small chela - left
20	0	Healthy	1 small chela - right
21	0	Healthy	
22	0	Healthy	
23	0	Healthy	
24	0	Healthy	
25	0	Healthy	Juvenile
26	0	Healthy	
27	0	Healthy	Juvenile
28	0	Healthy	Juvenile
29	0	Healthy	Escape
30	0	Dead	
31	0	Healthy	
32	0	Dead	
33	0	Healthy	
34	0	Healthy	Juvenile
35	0	Healthy	Juvenile
36	0	Porcelain	
37	0	Healthy	Juvenile, escape
38	0	Healthy	

Samples Summary - Usk

Site	Catchment	Grid Reference	Date	Number of crayfish found
Brecon bridge, Usk	Usk	SO 043286	12/09/02	1
Honddu	Usk	SO 050398	12/09/02	1
Llangattock	Usk	SO 215175	13/09/02	3
Llanfrynach	Usk	SO 079273	17/09/02	8
Stream 1	Usk	ST 286 974	*/08/03	0
Stream 2	Usk	ST 287 975	*/08/03	0
Stream 3	Usk	ST 287 978	*/08/03	0
Stream 4	Usk	ST 293 979	*/08/03	0
Stream 5	Usk	ST 292 974	*/08/03	0
Stream X (2)	Usk	ST 290 972	*/08/03	8
Stream X (1)	Usk	ST 293 970	*/08/03	1
Canal	Usk	ST 290 976	*/08/03	0
Dowlais Brook (1)	Usk	ST 273 952	*/09-10/03	0
Dowlais Brook (2)	Usk	ST 277 948	*/09-10/03	0
Dowlais Brook (3)	Usk	ST 184 944	*/09-10/03	1
Dowlais Brook (4)	Usk	ST 294 937	*/09-10/03	0
Dowlais Brook (5)	Usk	ST 300 934	*/09-10/03	0
Dowlais Brook (6)	Usk	ST 309 928	*/09-10/03	1
Dowlais Brook (7)	Usk	ST 317 926	*/09-10/03	0
Nant-y-Milwr (8)	Usk	ST 265 944	*/09-10/03	0
Nant-y-Milwr (9)	Usk	ST 268 940	*/09-10/03	0
Nant-y-Milwr (10)	Usk	ST 271 938	*/09-10/03	0
Nant-y-Milwr (11)	Usk	ST 275 936	*/09-10/03	0
Nant-y-Milwr (12)	Usk	ST 281 938	*/09-10/03	0
New House Farm (13)	Usk	ST 266 947	*/09-10/03	0
New House Farm (14)	Usk	ST 269 946	*/09-10/03	0
New House Farm (15)	Usk	ST 275 945	*/09-10/03	0
New House Farm (16)	Usk	ST 276 943	*/09-10/03	0
New House Farm (17)	Usk	ST 282 942	*/09-10/03	0
Bettws Brook (18)	Usk	ST 270 914	*/09-10/03	0
Bettws Brook (19)	Usk	ST 273 910	*/09-10/03	0
Nant-y-Pandy (20)	Usk	ST 261 934	*/09-10/03	0
Nant-y-Pandy (21)	Usk	ST 264 927	*/09-10/03	0
Nant-y-Pandy (22)	Usk	ST 266 920	*/09-10/03	0
Nant Henlys (23)	Usk	ST 271 926	*/09-10/03	0
Nant Henlys (24)	Usk	ST 270 920	*/09-10/03	0
Upper Cwmbran Brook (25)	Usk	ST 280 963	*/09-10/03	0
Upper Cwmbran Brook (26)	Usk	ST 285 958	*/09-10/03	0
Blaen Bran Brook (27)	Usk	ST 272 980	*/09-10/03	0
Blaen Bran Brook (28)	Usk	ST 285 969	*/09-10/03	0
Blaen Bran Brook (29)	Usk	ST 294 966	*/09-10/03	0
Candwr Brook (30)	Usk	ST 313 948	*/09-10/03	0
Candwr Brook (31)	Usk	ST 319 944	*/09-10/03	0
Berthin Brook (32)	Usk	ST 304 004	*/09-10/03	0
Berthin Brook (33)	Usk	ST 306 013	*/09-10/03	0
Berthin Brook (34)	Usk	ST 305 024	*/09-10/03	0

Berthin Brook (35)	Usk	ST 298 029	*/09-10/03	0
Berthin Brook (36)	Usk	ST 304 029	*/09-10/03	0
Nant-y-Pia (37)	Usk	ST 308 029	*/09-10/03	8
Nant-y-Pia (38)	Usk	ST 315 026	*/09-10/03	12
Nant-y-Gollen (39)	Usk	ST 228 025	*/09-10/03	3
Nant-y-Gollen (40)	Usk	ST 290 020	*/09-10/03	0
Nant-y-Gollen (41)	Usk	ST 290 011	*/09-10/03	0
Upper Nant Dar (42)	Usk	ST 279 997	*/09-10/03	0
Upper Nant Dar (43)	Usk	ST 276 994	*/09-10/03	0

Other Sites Surveyed

Site	Grid Ref.
Dulas (A438)	SO 042552
Chwefru (Builth Wells)	SO 029513
Irfon (Builth Wells)	SO 032512
Duhonw (Mid)	SO 052495
Edw (Wern)	SO 117573
Edw (Hundred House)	SO 115545
Edw (Vron)	SO 124534
Edw (Cregina)	SO 124523
Edw (Common)	SO 100481
Edw Confluence	SO 077469
Lugg (Presteigne)	SO 316646
Hindwell (Combe)	SO 345634
Lugg (Mortimer's Cross)	SO 426637
Gilwern Brook	SO 287570
Arrow (Pembridge)	SO 391585
Llynfi (Llandefaelog)	SO 132304
Llynfi (u/s Enig)	SO 150344
Ennig (d/s Talgarth)	SO 152344
Trwffwrdd (Felin-Newydd)	SO 118358
Dulas (u/s Llynfi)	SO 148344
Llynfi (u/s Bronllys)	SO 163365
Velindre	SO 171374
Cilkenni	SO 183411
Dulas (Hay on Wye)	SO 231427
Hardwicke	SO 252444
Clyro	SO 229451

Appendix II

River Habitat Survey Form 2003

RIVER HABITAT SURVEY 2003 VERSION: SITE HEALTH AND SAFETY ASSESSMENT			
Site Number[1]:	Site Ref:	River Name:	Date:
Grid References/Co-ordinates:	Spot 1[2]:	Mid-site:	End of site[2]:
Surveyor Name:		Accredited Surveyor Code:	
[1] Leave blank if new site.		[2] Optional	

Weather Conditions:
Flow Conditions:

Site details: (enter comments or circle if applicable and give details)	Risk Level (Low/Mod/High)
Access and Parking: (entry & exit)	
Conditions: comment on ground stability, footing, exposure/remoteness	
Obstacles/Hazards: fencing, stiles, dense vegetation, steep bank	
Occupied/Unoccupied: people, livestock, animals	
Activities/Land-use: agriculture, woodland, residential, industrial, construction, recreational	
Risk if lone-working	

IF THERE ARE ANY HIGH RISKS OR MORE THAN THREE MODERATE RISKS DO NOT CONTINUE WITH THE SURVEY.

Weil's Disease (*Leptospirosis*)

Instructions to card holders

1. As infection may enter through breaks in the skin, ensure that any cut, scratch or abrasion is thoroughly cleansed and covered with a waterproof plaster.
2. Avoid rubbing your eyes, nose and mouth during work.
3. Clean protective clothing, footwear and equipment etc. after use
4. After work, and particularly before taking food or drink, wash hands thoroughly.
5. Report all accidents and/or injuries, however slight.
6. Keep your card with you at all times.

Lyme Disease

1. Dress appropriately with skin covered up.
2. Regularly inspect for ticks when in the field.
3. Check for, and remove, any ticks as soon as possible after leaving the site.
4. Seek medical attention if bitten by a tick.

RIVER HABITAT SURVEY 2003 VERSION: SITE HEALTH AND SAFETY ASSESSMENT			
Site Number[1]:	Site Ref:	River Name:	Date:
Grid References/Co-ordinates:	Spot 1[2]:	Mid-site:	End of site[2]:
Surveyor Name:		Accredited Surveyor Code:	
[1] Leave blank if new site.		[2] Optional	

Weather Conditions:
Flow Conditions:

Site details: (enter comments or circle if applicable and give details)	Risk Level (Low/Mod/High)
Access and Parking: (entry & exit)	
Conditions: comment on ground stability, footing, exposure/remoteness	
Obstacles/Hazards: fencing, stiles, dense vegetation, steep bank	
Occupied/Unoccupied: people, livestock, animals	
Activities/Land-use: agriculture, woodland, residential, industrial, construction, recreational	
Risk if lone-working	

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RIVER HABITAT SURVEY 2003 VERSION: SPOT-CHECK KEY

PHYSICAL ATTRIBUTES (SECTION E)

BANKS

Predominant bank material

NV = not visible

BE = bedrock
BO = boulder
CO = cobble
GS = gravel/sand
EA = earth (crumbly)
PE = peat
CL = sticky clay

CC = concrete
SP = sheet piling
WP = wood piling
GA = gabion
BR = brick/laid stone
RR = rip-rap
TD = tipped debris
FA = fabric
BI = bio-engineering materials

Bank modifications

NK = not known
NO = none

RS = resectioned (reprofiled)
RI = reinforced
PC = poached
PC(B) = poached (bare)
BM = artificial berm
EM = embanked

Marginal and bank features

NV = not visible (e.g. far bank)
NO = none

EC = eroding cliff (**EC** if sandy substrate)
SC = stable cliff (**SC** if sandy substrate)

PB = unvegetated point bar
VP = vegetated point bar

SB = unvegetated side bar
VS = vegetated side bar

NB = natural berm

CHANNEL

Predominant substrate

NV = not visible

BE = bedrock
BO = boulder
CO = cobble
GP = gravel/pebble (**G** or **P** if predominant)
SA = sand
SI = silt
CL = clay
PE = peat
EA = earth
AR = artificial

Predominant flow-type

NV = not visible
FF = free fall
CH = chute
BW = broken standing waves (white water)
UW = unbroken standing waves
CF = chaotic flow
RP = rippled
UP = upwelling
SM = smooth
NP = no perceptible flow
DR = no flow (dry)

Channel modifications

NK = not known
NO = none

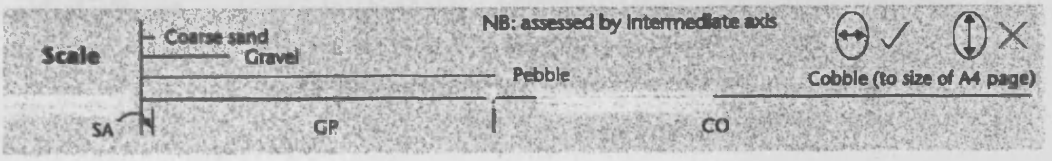
CV = culverted
RS = resectioned
RI = reinforced
DA = dam/weir/sluice
FO = ford (man-made)

Channel features

NV = not visible
NO = none

EB = exposed bedrock
EO = exposed boulders
VR = vegetated rock
MB = unvegetated mid-channel bar
VB = vegetated mid-channel bar
MI = mature island
TR = Trash (urban debris)

FLOW-TYPES	DESCRIPTION
FF: Free fall	clearly separates from back-wall of vertical feature – associated with waterfalls
CH: Chute	low curving fall in contact with substrate – often associated with cascades
BW: Broken standing waves	white-water tumbling waves must be present – mostly associated with rapids
UW: Unbroken standing waves	upstream facing wavelets which are not broken – mostly associated with riffles
CF: Chaotic flow	a chaotic mixture of three or more of the four fast flow-types with no predominant one obvious
RP: Rippled	no waves, but general flow direction is downstream with disturbed rippled surface – mostly associated with runs
UP: Upwelling	heaving water as upwellings break the surface – associated with boils.
SM: Smooth	perceptible downstream movement is smooth (no eddies) – mostly associated with glides
NP: No perceptible flow	no net downstream flow – associated with pools, ponded reaches and marginal deadwater
DR: No flow (dry)	dry river bed



RIVER HABITAT SURVEY: SPOT-CHECK KEY

LEFT **Banks are determined by looking downstream** **RIGHT**

CHANNEL MODIFICATION INDICATORS

One or more of the following may be indicative of resectioning:

1. Uniform bank profile
2. Straightened planform
3. Bankfull width/bankfull height ratio <4:1
4. Uniform/low energy flow-types
5. No trees/uniformly-aged trees along bank
6. Intensive/urban land-use

LAND USE WITHIN 5m OF BANKTOP (SECTION F) & 50m (SECTION H)

BL = Broadleaf/mixed woodland (semi-natural)
BP = Broadleaf/mixed plantation
CW = Coniferous woodland (semi-natural)
CP = Coniferous plantation
SH = Scrub & shrubs
OR = Orchard
WL = Wetland (e.g. bog, marsh, fen)
MH = Moorland/heath
AW = Artificial open water
OW = Natural open water
RP = Rough unimproved grassland/pasture
IG = Improved/semi-improved grassland
TH = Tall herb/rank vegetation
RD = Rock, scree or sand dunes
SU = Suburban/urban development
TL = Tilled land
IL = Irrigated land
PG = Parkland or gardens
NV = Not visible

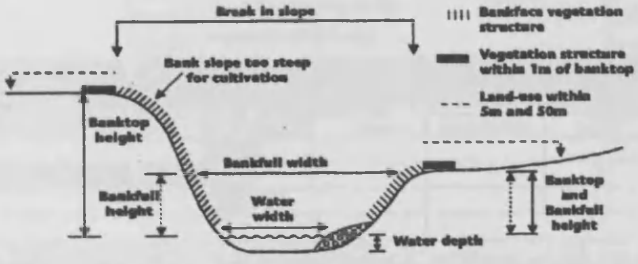
BANKTOP AND BANKFACE VEGETATION STRUCTURE To be assessed within a 10m wide transect (SECTION F)

bare	B	bare earth/rock etc.	vegetation types
uniform	U	predominantly one type (no scrub or trees)	bryophytes
simple	S	two or three vegetation types	short/creeping herbs or grasses
complex	C	four or more types	tall herbs/ grasses
			scrub or shrubs
			saplings and trees

Channel dimensions guidance (Section L)

- Select location on uniform section.
- If riffle is present, measure there. If not, measure at straightest and shallowest point.
- **Banktop** = first major break in slope above which cultivation or development is possible.
- **Bankfull** = point where river first spills on to floodplain.

Cross-section of channel showing definitions used to define where spot-check recording and channel dimensions measured



ENVIRONMENT AGENCY

EMERGENCY HOTLINE 0800 80 70 60

24 hour free emergency telephone line for reporting all environmental incidents relating to air, land and water.

RIVER HABITAT SURVEY 2003 Version Page 1 of 4

A FIELD SURVEY DETAILS

Site Number: (leave blank if new site)

Site Reference:

Spot-check 1 Grid Ref:

Spot-check 6 Grid Ref:

End of site Grid Ref:

Reach Reference:

River name:

Date / /20 Time:

Surveyor name:

Accredited Surveyor code:

Is the site part of a river or an artificial channel? River ☐ Artificial ☐

Are adverse conditions affecting survey? No ☐ Yes ☐

If yes, state .....

Is bed of river visible? barely or not ☐ partially ☐ ±entirely ☐

Is health and safety assessment form attached? Yes ☐ No ☐

Number of photographs taken: ☐

Photo references:

Site surveyed from: left bank ☐ right bank ☐ channel ☐

☐ When options shown with 'shadow boxes', tick one box only

LEFT banks determined by facing downstream RIGHT

B PREDOMINANT VALLEY FORM (within the horizon limit) (tick one box only)

(tick one box only)

☐ shallow vee

☐ deep vee

☐ gorge

☐ concave/bowl

☐ asymmetrical valley

☐ U-shape valley

☐ no obvious valley sides

Distinct flat valley bottom? No ☐ Yes ☐

Natural terraces? No ☐ Yes ☐

C NUMBER OF RIFFLES, POOLS AND POINT BARS (enter total number in boxes)

Riffle(s)		Unvegetated point bar(s)	
Pool(s)		Vegetated point bar(s)	

D ARTIFICIAL FEATURES (indicate total number of occurrences of each category within the 500m site)

If none, tick box ☐

	Major	Intermediate	Minor		Major	Intermediate	Minor
Weirs/sluices				Outfalls/ inlakes			
Culverts				Fords			
Bridges				Deflectors/ groynes/ croys			
Other - state							

Is channel obviously realigned? No ☐ Yes, <33% of site ☐ >33% of site ☐

Is channel obviously over-deepened? No ☐ Yes, <33% of site ☐ >33% of site ☐

Is water impounded by weir/dam? No ☐ Yes, <33% of site ☐ >33% of site ☐

SITE REF.	RIVER HABITAT SURVEY: TEN SPOT-CHECKS	Page 2 of 4

Spot-check 1 is at: upstream end ❑ downstream end ❑ of site (tick one box)

E PHYSICAL ATTRIBUTES (to be assessed across channel within 1m wide transect)

Where boxes 'banked', only one entry allowed	1 GPS	2	3	4	5	6 GPS	7	8	9	10	GPS
LEFT BANK	Ring EC or SC if composed of sandy substrate										
Material NV, BE, BO, CO, CB, GR, BA, PE, CL, CC, SP, WP, GA, BB, BR, TB, PA, EE											
Bank modification(s) NE, NO, ES, RI, PC(B), BM, EM											
Marginal & bank feature(s) NV, NO, EC, SC, PB, VP, SB, VS, NB											
CHANNEL	GP. Ring either G or P if predominant										
Channel substrate NV, BE, BO, CO, GP, SA, EE, FI, PE, BA, AR											
Flow-type NV, PF, CH, BW, UW, CF, RP, EP, SAR, NP, DR											
Channel modification(s) NE, NO, CV, RS, RI, BA, FO											
Channel feature(s) NV, NO, BB, BO, VB, MB, VB, MI, TR											
For braided rivers only: number of sub-channels											
RIGHT BANK	Ring EC or SC if composed of sandy substrate										
Material NV, BE, BO, CO, GR, BA, PE, CL, CC, SP, WP, GA, BB, BR, TB, PA, EE											
Bank modification(s) NE, NO, ES, RI, PC(B), BM, EM											
Marginal & bank feature(s) NV, NO, EC, SC, PB, VP, SB, VS, NB											

← Enter channel substrate(s) not occurring as predominant in spot-checks but present in >1% of whole site.

F BANKTOP LAND-USE AND VEGETATION STRUCTURE (to be assessed over a 10m wide transect)

Land-use: choose one from BL, BP, CW, CP, SH, OB, WL, NH, AW, OW, IP, IG, TH, RD, SU, TL, IL, PG, NV

LAND-USE WITHIN 5m OF LEFT BANKTOP											
LEFT BANKTOP (structure within 1m)	B/U/S/C/NV										
LEFT BANK-FACE (structure)	B/U/S/C/NV										
RIGHT BANK-FACE (structure)	B/U/S/C/NV										
RIGHT BANKTOP (structure within 1m)	B/U/S/C/NV										
LAND-USE WITHIN 5m OF RIGHT BANKTOP											

G CHANNEL VEGETATION TYPES (to be assessed over a 10m wide transect; use E if >1% area), ✓ (present) or NV (not visible)

None (✓) or Not Visible (NV)											
Liverworts/mosses/lichens											
Emergent broad-leaved herbs											
Emergent reeds/sedges/rushes/grasses/horsetails											
Floating-leaved (rooted)											
Free-floating											
Amphibious											
Submerged broad-leaved											
Submerged linear-leaved											
Submerged fine-leaved											
Filamentous algae											

Use end column for overall assessment over 500m, including types not occurring in spot-checks (use ✓, E or NV) →

SITE REF.	**RIVER HABITAT SURVEY : 500m SWEEP-UP**	Page 3 of 4

H LAND-USE WITHIN 50m OF BANKTOP Use ✓ (present) or E (≥33% banklength)

	L	R		L	R
Broadleaf/mixed woodland (semi-natural) (BL)			Natural open water (OW)		
Broadleaf/mixed plantation (BP)			Rough/unimproved grassland/pasture (RP)		
Coniferous woodland (semi-natural) (CW)			Improved/semi-improved grassland (IG)		
Coniferous plantation (CP)			Tall herb/rank vegetation (TH)		
Scrub & shrubs (SH)			Rock, scree or sand dunes (RD)		
Orchard (OR)			Suburban/urban development (SU)		
Wetland (e.g. bog, marsh, fen) (WL)			Tilled land (TL)		
Moorland/heath (MH)			Irrigated land (IL)		
Artificial open water (AW)			Parkland or gardens (PG)		
			Not visible (NV)		

I BANK PROFILES Use ✓ (present) or E (≥33% banklength)

Natural/semi-natural	L	R	Artificial/modified	L	R
Vertical/undercut			Resectioned (reprofiled)		
Vertical with toe			Reinforced - whole		
Steep (>45°)			Reinforced - top only		
Gentle			Reinforced - toe only		
Composite			Artificial two-stage		
Natural berm			Poached bank		
			Embanked		
			Set-back embankment		

J EXTENT OF TREES AND ASSOCIATED FEATURES *record even if >1%

TREES (tick one box per bank)

	Left	Right
None	☐	☐
Isolated/scattered	☐	☐
Regularly spaced, single	☐	☐
Occasional clumps	☐	☐
Semi-continuous	☐	☐
Continuous	☐	☐

ASSOCIATED FEATURES (tick one box per feature)

	None	Present	E (≥33%)
Shading of channel	☐	☐	☐
*Overhanging boughs	☐	☐	☐
*Exposed bankside roots	☐	☐	☐
*Underwater tree roots	☐	☐	☐
Fallen trees	☐	☐	☐
Large woody debris	☐	☐	☐

K EXTENT OF CHANNEL AND BANK FEATURES (tick one box for each feature) *record even if <1%

	None	Present	E(≥33%)		None	Present	E (≥33%)
*Free fall flow	☐	☐	☐	Exposed bedrock	☐	☐	☐
Chute flow	☐	☐	☐	Exposed boulders	☐	☐	☐
Broken standing waves	☐	☐	☐	Vegetated bedrock/boulders	☐	☐	☐
Unbroken standing waves	☐	☐	☐	Unvegetated mid-channel bar(s)	☐	☐	☐
Rippled flow	☐	☐	☐	Vegetated mid-channel bar(s)	☐	☐	☐
*Upwelling	☐	☐	☐	Mature island(s)	☐	☐	☐
Smooth flow	☐	☐	☐	Unvegetated side bar(s)	☐	☐	☐
No perceptible flow	☐	☐	☐	Vegetated side bar(s)	☐	☐	☐
No flow (dry)	☐	☐	☐	Unvegetated point bar(s)	☐	☐	☐
Marginal deadwater	☐	☐	☐	Vegetated point bar(s)	☐	☐	☐
Eroding cliff(s)	☐	☐	☐	*Unvegetated silt deposit(s)	☐	☐	☐
Stable cliff(s)	☐	☐	☐	*Discrete unvegetated sand deposit(s)	☐	☐	
				*Discrete unvegetated gravel deposit(s)	☐	☐	

SITE REF.	**RIVER HABITAT SURVEY : DIMENSIONS AND INFLUENCES**	Page 4 of 4

L CHANNEL DIMENSIONS (to be measured at one location on a straight uniform section, preferably across a riffle)

LEFT BANK		CHANNEL		RIGHT BANK	
Banktop height (m)		Bankfull width (m)		Banktop height (m)	
Is banktop height also bankfull height? (Y or N)		Water width (m)		Is banktop height also bankfull height? (Y or N)	
Embanked height (m)		Water depth (m)		Embanked height (m)	

If trashline lower than banktop, indicate: height above water (m) = width from bank to bank (m) =

Bed material at site is: consolidated ❑ unconsolidated (loose) ❑ unknown ❑

Location of measurements is: riffle ❑ other ❑ (state)

M FEATURES OF SPECIAL INTEREST Use C or E (≥ 33% length) *record even if <33%

None	❑	Very large boulders (>1m)	❑	Backwater(s)	❑	Marsh(es)	❑
Braided channels	❑	*Debris dam(s)	❑	Floodplain boulder deposits	❑	Flush(es)	❑
Side channel(s)	❑	*Leafy debris	❑	Water meadow(s)	❑	Natural open water	❑
*Natural waterfall(s) > 5m high	❑	Fringing reed-bank(s)	❑	Fen(s)	❑	Others (state)	❑
*Natural waterfall(s) < 5m high	❑	Quaking bank(s)	❑	Bog(s)	❑		
Natural cascade(s)	❑	*Sink hole(s)	❑	Wet woodland(s)	❑		

N CHOKED CHANNEL (tick one box)

Is 33% or more of the channel choked with vegetation? No ❑ Yes ❑

O NOTABLE NUISANCE PLANT SPECIES Use C or E (≥ 33% length) *record even if <1%

	bankface	banktop to 50m		bankface	banktop to 50m
None ❑ *Giant hogweed	❑	❑	*Himalayan balsam	❑	❑
*Japanese knotweed	❑	❑	*Other (state).....	❑	❑

P OVERALL CHARACTERISTICS (Circle appropriate words, add others as necessary)

Major impacts: landfill - tipping - litter - sewage - pollution - drought - abstraction - mill - dam - road - rail - industry - housing mining - quarrying - overdeepening - afforestation - fisheries management - silting - waterlogging - hydroelectric power

Evidence of recent management: dredging - bank mowing - weed cutting - enhancement - river rehabilitation - gravel extraction - other (please specify)

Animals: otter - mink - water vole - kingfisher - dipper - grey wagtail - sand martin - heron - dragonflies/damselflies

Other significant observations: If necessary use separate sheet to describe overall characteristics and relevant observations

Q ALDERS (tick one box in each of the two categories) *record even if <1%

*Alders? None	❑	Present	❑	Extensive	❑		
*Diseased Alders? None	❑	Present	❑	Extensive	❑		

R FIELD SURVEY QUALITY CONTROL (boxes to confirm checks)

Have you taken at least two photos that illustrate the general character of the site and additional photos of any weirs/ sluices and major/intermediate structures across the channel?	❑
Have you completed all ten spot-checks and made entries in all boxes in E & F on page 2?	❑
Have you completed column 11 of section G (and E if appropriate) on page 2?	❑
Have you recorded in section C the number of riffles, pools and point bars (even if 0) on page 1?	❑
Have you given an accurate (alphanumeric) grid reference for spot-checks 1, 6 and end of site (page 1)?	❑
Have you stated whether spot-check 1 is at the upstream or downstream end of the site (top of page 2)?	❑
Have you cross-checked your spot-check and sweep-up responses with the channel modification indicators given on page 2 of the spot-check key?	❑

Appendix III

Substrate Preference and Silt Data

Substrate Preference Data

Day	Tank	Crayfish	Embedded pebbles	Vegetation	Gravel	Sand/silt	Loose pebbles
1	1	1	0	3	1	0	4
		2	0	4	0	0	4
2	1	1	0	8	0	0	0
		2	0	4	0	0	4
3	1	1	0	0	0	0	8
		2	0	5	0	0	3
4	1	1	0	5	0	0	3
		2	0	0	0	0	8
5	1	1	0	8	0	0	0
		2	0	0	0	0	8
1	2	1	1	5	1	0	1
		2	0	7	0	0	1
2	2	1	0	7	0	0	1
		2	0	8	0	0	0
3	2	1	0	7	0	0	1
		2	0	8	0	0	0
4	2	1	0	2	0	0	6
		2	0	3	0	0	6
5	2	1	0	4	0	0	4
		2	0	0	0	0	8

Silt & Rainfall Data

Month	Basket Trap	Flowerpot Trap	Rainfall (mm)
Sep-02	38.3	59.9	1.1
Nov-02	1025	759.7	6.4
Jun-03	265.8	297.2	2.1
Aug-03	47.4	256.9	0.6
Oct-03	20.4	42.5	1.9
Dec-03	424.5	274.2	4.3

Silt Experiment Data – Part 1

Site	Sep-02	Nov-02	Jun-03	Aug-03	Oct-03	Dec-03
Aberedw US basket	54.1	928	79.4	*	29.3	452.4
Aberedw DS basket	205.2	780	106.6	*	34.5	490.8
Church US basket	2	1061	236.4	262.4	1.8	433.1
Church DS basket	34.4	34	330	362.8	47	466.6
Cregrina US basket	19.4	915	411.6	248.7	22.9	175.1
Cregrina DS basket	44.4	840	522.4	314.3	161.7	147.2
Cregrina ST basket	10.6	725.5	347.1	192.4	*	135.4
Franksbridge basket	21.9	1036.3	*	240.2	38.7	208.7
Llanedw basket	132.1	517.4	344.4	98.9	41.5	227.7
Confluence basket	75.5	*	*	335.1	5.4	4.8

Site	Sep-02	Nov-02	Jun-03	Aug-03	Oct-03	Dec-03
Aberedw US flowerpot	10.6	*	*	*	22.5	559.1
Aberedw DS flowerpot	292.2	*	117.6	*	11.5	1833.3
Church US flowerpot	0.6	2154	598.7	38.9	48.9	164.5
Church DS flowerpot	14.3	1439	141.8	*	24.4	312.1
Cregrina US flowerpot	8.1	*	225	77.6	6.6	57.3
Cregrina DS flowerpot	3.8	797	270.8	47.2	7.2	16.4
Cregrina ST flowerpot	8.4	735.2	125.3	44.4	33.2	23.7
Franksbridge flowerpot	21.9	*	465.2	*	21.7	655.3
Llanedw flowerpot	7.5	*	181.6	33.1	8.3	59.8
Confluence flowerpot	15.4	*	*	43.1	*	563.8

Silt experiment Part 2 Data

Site	Trap	Trap type	June-04	July-04	August-04	December-04
Aberedw	3	Basket	2253.6	*	90.7	388
Aberedw	4	Basket	824.8	32.1	171.4	65.7
Aberedw	14	Basket	158.7	67.1	*	*
Aberedw	15	Basket	182.1	100.7	123	*
Aberedw	23	Basket	1777.5	103.8	141.2	*
Aberedw	1	Flowerpot	*	60	103.4	1618.9
Aberedw	5	Flowerpot	127.8	43.4	87.6	1645.3
Aberedw	7	Flowerpot	1287.7	38.5	194.9	1735.7
Aberedw	19	Flowerpot	739.4	74.7	*	*
Aberedw	25	Flowerpot	881.8	130.4	*	*
Llanbadarn y garreg	2	Basket	226.1	86.4	187.6	132.7
Llanbadarn y garreg	4	Basket	133.2	94	226.7	135.4
Llanbadarn y garreg	8	Basket	127.8	49.3	155	684.7
Llanbadarn y garreg	9	Basket	172.6	51.4	84	*
Llanbadarn y garreg	1	Flowerpot	84.8	20.2	48.8	398.4
Llanbadarn y garreg	5	Flowerpot	76.7	23.8	91.6	1915.6
Llanbadarn y garreg	15	Flowerpot	157.3	25.5	120.9	1556.1
Llanbadarn y garreg	22	Flowerpot	*	*	*	*
Llanbadarn y garreg	24	Flowerpot	*	*	128.7	2405.3

Appendix IV

Genetics Data

Genetic Raw Data

Site name	Sample no.	Ap3		Ap2		Ap6	
		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
Army range	1	156	156	195	189	*	*
Army range	2	156	156	195	195	*	*
Army range	3	156	156	189	189	*	*
Army range	4	156	156	195	189	360	360
Army range	5	156	156	*	*	360	360
Army range	6	156	156	195	195	360	360
Army range	20	156	156	*	*	*	*
Army range	21	156	156	*	*	*	*
Army range	8	156	156	*	*	*	*
Common land	7	156	156	189	189	360	360
Common land	8	*	*	*	*	360	360
Common land	9	156	156	189	189	360	360
Common land	10	156	156	189	189	360	360
Common land	11	160	156	189	189	360	360
Common land	12	156	156	195	189	360	360
Common land	1	156	156	*	*	*	*
Common land	16	156	156	*	*	*	*
Farm	44	160	156	*	*	*	*
Farm	13	156	156	189	189	360	360
Farm	14	156	156	189	189	360	360
Farm	15	156	156	189	189	360	360
Farm	16	160	156	189	189	360	360
Farm	17	160	156	189	189	360	360
Farm	18	156	156	*	*	360	360
Franksbridge	11	156	156	*	*	*	*
Franksbridge	13	156	156	*	*	*	*
Franksbridge	6	156	156	*	*	*	*
Franksbridge	1	156	156	189	189	360	360
Franksbridge	2	156	156	189	189	360	360
Franksbridge	3	156	156	189	189	360	360
Franksbridge	4	156	156	189	189	360	360
Franksbridge	5	156	156	189	189	360	360
Franksbridge	11	156	156	189	189	*	*
Franksbridge	6	*	*	189	189	*	*
Llanbadarn y garreg	7	156	156	189	189	360	360
Llanbadarn y garreg	8	156	156	189	187	360	360
Llanbadarn y garreg	9	156	156	189	189	360	360
Llanbadarn y garreg	10	156	156	189	189	360	360
Llanbadarn y garreg	11	156	156	189	189	360	360
Llanbadarn y garreg	12	156	156	189	187	360	360
Llanbadarn y garreg	9B	156	156	189	189	*	*
Llanbadarn y garreg	13	156	156	*	*	*	*
Llanbadarn y garreg	37	161	156	*	*	*	*
Llanbadarn y garreg	19	156	156	*	*	*	*
Llanbadarn y garreg	13A	*	*	195	195	*	*

Cregrina	13	156	156	189	189	360	360
Aberedw	14	156	156	189	189	360	360
Aberedw	15	156	156	*	*	360	360
Aberedw	16	156	156	195	189	360	360
Aberedw	17	156	156	189	187	360	360
Aberedw	18	156	156	189	189	360	360
Aberedw	19	156	156	189	189	360	360
Aberedw	6	160	156	195	195	*	*
Aberedw	27	*	*	189	189	*	*
Aberedw	3	161	156	*	*	*	*
Aberedw	5	156	156	*	*	*	*
Aberedw		156	156	*	*	*	*
Aberedw	27	161	156	*	*	*	*
Aberedw	10	156	156	195	195	*	*
Cwmbach Llechryd	12	161	156	*	*	*	*
Cwmbach Llechryd	13	156	156	*	*	*	*
Cwmbach Llechryd	14	156	156	*	*	*	*
Cwmbach Llechryd	33	156	156	189	189	360	360
Cwmbach Llechryd	34	156	156	195	189	360	360
Cwmbach Llechryd	40	160	156	189	189	360	360
Cwmbach Llechryd	38	156	156	189	189	360	360
Builth Rd	35	156	156	189	189	360	360
Builth Rd	36	156	156	189	189	360	360
Builth Rd	37	160	156	189	189	360	360
Builth Rd	39	156	156	189	189	360	360
Builth Rd	11	*	*	189	189	*	*
Builth Rd	18	*	*	195	189	*	*
Sgithwen		156	156	195	195	360	360
Sgithwen	41	156	156	189	189	360	360
Sgithwen	42	156	156	189	187	360	360
Sgithwen	43	156	156	195	189	360	360
Sgithwen	45	156	156	189	187	360	360
Sgithwen	46	156	156	*	*	360	360
Sgithwen	47	156	156	189	189	360	360
Sgithwen	41	156	156	195	195	360	360
Sgithwen	3A	*	*	195	195	*	*
Sgithwen	7	156	156	*	*	*	*
Dulas	15	156	156	*	*	*	*
Dulas	25	156	156	195	189	360	360
Dulas	26	156	156	195	189	360	360
Dulas	27	156	156	195	189	360	360
Dulas	28	160	156	189	189	360	360
Dulas	29	156	156	195	189	360	360
Dulas	30	156	156	195	195	360	360
Dulas	31	160	156	189	189	360	360
Dulas	32	156	156	189	189	360	360
Dulas	19	156	156	189	189	*	*
Dulas	15	156	156	195	189	*	*
Dulas	19	156	156	195	189	360	360

Dulas	18D	156	156	195	189	*	*
Dulas	1	156	156	195	189	362	360
Dulas	5	156	156	*	*	*	*
Dulas	14D	*	*	195	195	*	*
Dulas	3D	*	*	195	195	*	*
Escley		156	156	*	*	*	*
Escley		156	156	195	189	360	360
Escley		156	156	195	195	360	360
Escley	11	156	156	195	195	*	*
Escley	71	156	156	195	189	360	360
Escley	29	156	156	195	195	360	360
Escley	81	156	156	195	195	*	*
Escley	74	156	156	195	195	*	*
Escley	73	156	156	195	195	*	*
Escley	77	156	156	195	195	*	*
Escley	3E	*	*	195	189	*	*
Escley	70	156	156	195	195	360	360
Escley	D1	156	156	195	195	362	360
Escley	E1	*	*	195	189	360	360
Escley	F1	*	*	*	*	*	*
Escley	G1	*	*	189	189	362	360
Escley	H1	156	156	*	*	360	360
Mortimers Cross	32	160	156	195	189	362	360
Mortimers Cross	20	160	156	195	189	360	360
Mortimers Cross	21	*	*	195	189	*	*
Mortimers Cross	22	156	156	195	189	360	360
Mortimers Cross	3	*	*	195	189	*	*
Mortimers Cross	23	160	156	*	*	360	360
Mortimers Cross	32	*	*	195	189	362	360
Mortimers Cross	1	156	156	195	189	*	*
Mortimers Cross	2	154	154	*	*	*	*
Mortimers Cross	3	161	156	195	195	*	*
Mortimers Cross	S1	*	*	189	189	*	*
Pontypool (308029)	76	156	156	195	195	*	*
Pontypool (308029)	89	156	156	*	*	*	*
Pontypool (308029)	80	156	156	*	*	*	*
Pontypool (308029)	A1	156	156	195	195	*	*
Pontypool (308029)	B1	156	156	195	195	360	360
Pontypool (308029)	C1	156	156	189	189	360	360
Pontypool (308029)	88	160	156	195	189	*	*
Llangattock (Usk trib)	18	156	156	195	195	*	*
Honddu	5	*	*	189	189	*	*
Honddu	44	156	156	189	189	360	360
Banwy	23	*	*	189	189	*	*
Banwy	9	*	*	195	189	*	*
Itchen (S)	D	*	*	195	195	*	*
Itchen (S)	E	156	156	195	195	*	*
Itchen (S)	F	156	156	195	195	*	*
Itchen (S)	G	*	*	195	195	*	*

Aire (N) 122	I1	156	156	195	195	360	360
Aire (N) 124	J1	156	156	189	189	360	360
Aire (N) 123	K1	156	156	195	189	360	360
Aire (N) 125	L1	156	156	189	189	360	360
Aire (N) 126	M1	156	156	189	189	360	360
Aire (N) 127	N1	156	156	195	195	360	360
Aire (N) 128	O1	156	156	195	195	360	360
Aire (N) 129	P1	156	156	195	189	360	360
Aire (N) 130	Q1	*	*	195	189	360	360
Aire (N) 131	R1	*	*	189	189	362	360

Allele Frequencies for the Ap2 Locus

Site	Ap2 locus		
	Allele 1 (187)	Allele 2 (189)	Allele 3 (195)
Army range	0.000	0.400	0.600
Common land	0.000	0.900	0.100
Farm	0.000	1.000	0.000
Franksbridge	0.000	1.000	0.000
Llanbadarn y garreg	0.125	0.750	0.125
Cregrina	0.000	1.000	0.000
Aberedw	0.062	0.625	0.312
Cwmbach Llechryd	0.000	0.875	0.125
Builth Road	0.000	0.917	0.083
Sgithwen	0.143	0.357	0.500
Banwy	0.000	0.750	0.250
Honddu	0.000	0.750	0.250
Dulas (Monnow)	0.000	0.533	0.467
Escley	0.000	0.214	0.786
Lugg	0.000	0.500	0.500
Pontypool	0.000	0.300	0.700
Itchen (S)	0.000	0.000	1.000
Aire (N)	0.000	0.550	0.450

DNA Isolation from 5-10 mg Fixed or Paraffin-Embedded Tissue

Fixed Tissue – Cell Lysis

1. Briefly blot excess fixative from tissue on clean absorbent paper and weigh 5-10 mg.
2. Place tissue into a 1.5 ml tube containing 300µl Cell Lysis Solution and heat for 15 minutes at 65 °C to soften tissue.
3. 3. Homogenize using 30-50 strokes with a microfuge tube pestle.
4. Either incubate lysate at 65 °C for 15-60 minutes, or, if maximum yield is required, add 3µl Proteinase K Solution (20mg/ml) to the lysate and mix by inverting 25 times; incubate at 55 °C for 3 hours to overnight, until tissue particulates have dissolved. If possible, invert tube periodically during the incubation.
5. Continue the isolation by beginning with Step 1 under RNase Treatment.

RNase Treatment

1. Add 1.5 µl RNase A Solution (4 mg/ml) to the cell lysate.
2. Mix the sample by inverting the tube 25 times and incubate at 37 °C for 15-60 minutes.

Protein Precipitation

1. Cool sample to room temperature.
2. Add 100 µl Protein Precipitation Solution to the cell lysate.
3. Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate.
4. Centrifuge at 13000-16000 x g for 5 minutes. The precipitated proteins will form a tight pellet. If the protein pellet is not tight, repeat Step 3 followed by incubation on ice for 5 minutes, then repeat Step 4.

DNA Precipitation

1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 1.5 ml microfuge tube containing 300 µl 100% Isopropanol (2-propanol).

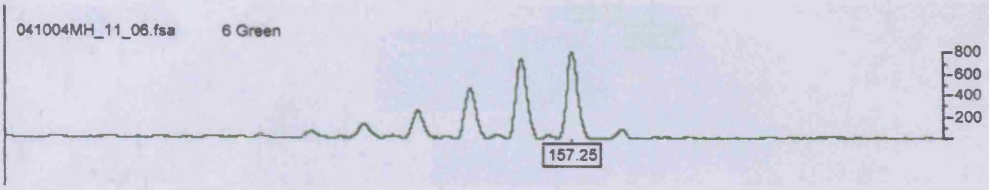
2. Mix by inverting gently 50 times.
3. Centrifuge at 13000-16000 x g for 8 minutes.
4. Pour off supernatant and drain tube on clean absorbent paper. Add 300 ul Ethanol and flick tube to wash the DNA pellet.
5. Centrifuge at 13000-16000 x g for 5 minutes. Carefully pour off the ethanol. Pellet may be loose so pour slowly and watch pellet.
6. Invert and drain the tube on clean absorbent paper and allow to dry in vacuum drier for 5-10 minutes.

DNA Hydration

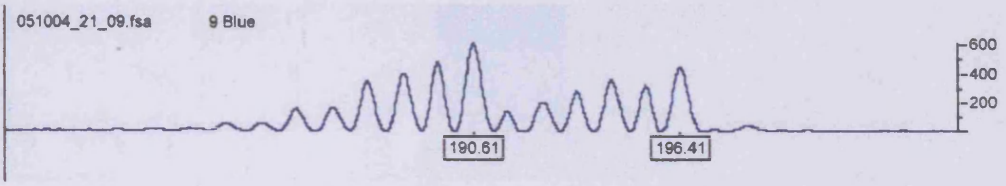
1. Add 50 µl DNA Hydration Solution (20 µl will give a concentration of 100 ng/µl if the total yield is 2 µg DNA).
2. 2. Rehydrate DNA by incubating sample for 1 hour at 56 °C and/or overnight at room temperature. Tap tube periodically to aid in dispersing the DNA.
3. Store DNA at 4 °C. For long term storage, place sample at –20 °C or –80 °C.

Examples of Electropherograms obtained for each Microsatellite Locus

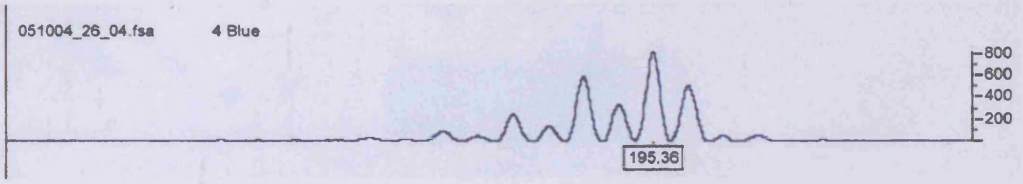
Locus Ap3, genotype 156/156



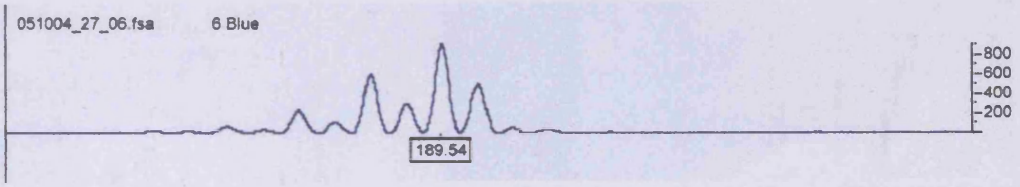
Locus Ap2, genotype 195/189



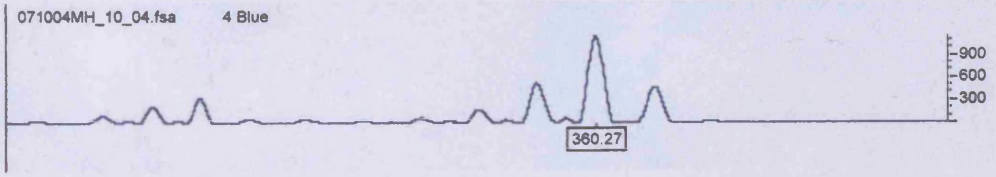
Locus Ap2, genotype 195/195



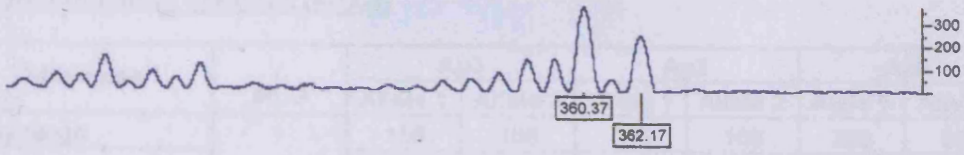
Locus Ap2, genotype 189/189



Locus Ap6, genotype 360/360



Locus Ap6, genotype 362/360



Multi-Locus Diploid Genotypes (MLGs)

Site name	MLG	Ap3		Ap2		Ap6		n
		Allele 1	Allele 2	Allele 1	Allele 2	Alele 1	Allele 2	
Army range	I	156	156	195	189	360	360	1
Army range	II	156	156	195	195	360	360	1
Common land	III	156	156	189	189	360	360	3
Common land	IV	160	156	189	189	360	360	1
Common land	I	156	156	195	189	360	360	1
Farm	III	156	156	189	189	360	360	3
Farm	IV	160	156	189	189	360	360	2
Franksbridge	III	156	156	189	189	360	360	5
Llanbadarn y garreg	III	156	156	189	189	360	360	4
Llanbadarn y garreg	V	156	156	189	187	360	360	2
Cregrina	III	156	156	189	189	360	360	1
Aberedw	III	156	156	189	189	360	360	3
Aberedw	I	156	156	195	189	360	360	1
Aberedw	V	156	156	189	187	360	360	1
Cwmbach Llechryd	III	156	156	189	189	360	360	2
Cwmbach Llechryd	I	156	156	195	189	360	360	1
Cwmbach Llechryd	IV	160	156	189	189	360	360	1
Builth Rd	III	156	156	189	189	360	360	3
Builth Rd	IV	160	156	189	189	360	360	1
Sgithwen	II	156	156	195	195	360	360	2
Sgithwen	III	156	156	189	189	360	360	2
Sgithwen	V	156	156	189	187	360	360	2
Sgithwen	I	156	156	195	189	360	360	1
Dulas	I	156	156	195	189	360	360	5
Dulas	IV	160	156	189	189	360	360	2
Dulas	II	156	156	195	195	360	360	1
Dulas	III	156	156	189	189	360	360	1
Dulas	VI	156	156	195	189	362	360	1
Escley	I	156	156	195	189	360	360	2
Escley	II	156	156	195	195	360	360	3
Escley	VII	156	156	195	195	362	360	1
Mortimers Cross	VIII	160	156	195	189	362	360	1
Mortimers Cross	IX	160	156	195	189	360	360	1
Mortimers Cross	I	156	156	195	189	360	360	1
Honddu	III	156	156	189	189	360	360	1
Nant y Pia, Pontypool	II	156	156	195	195	360	360	1
Stream 1, Cwmbran	III	156	156	189	189	360	360	1
Aire (N) 122	II	156	156	195	195	360	360	3
Aire (N) 124	III	156	156	189	189	360	360	3
Aire (N) 123	I	156	156	195	189	360	360	2

Appendix V

Rearing Data

Crayfish Hatchling Lengths with and without a *Spirulina* Supplement

Hatchling crayfish lengths (mm)			
1/9/03		9/10/03	
With *Spirulina*	Without *Spirulina*	With *Spirulina*	Without *Spirulina*
15	17	16	17
15	16	14	15
11	14	14	19
14	17	16	18
15	18	14	17
17	19	18	23
15	17	16	21
16	18	22	21
16	18	15	23
19	13	17	20
16	16	17	21
12	17	22	20
15	16	19	16
14	16	20	20
13	14	18	20
14	13	17	15
13	14	15	16
15	13	17	18
13	17	18	15
12	18	18	17
13	17	17	16
14	16	17	17
16	16	20	21
14	16	19	16
13	16	20	20
15	13		19
19	12		20
17	16		19
12	14		20
15	12		18
15	13		17
	13		18
	18		15
	19		14
	15		15
	15		14
	13		14
	14		14
	14		
	13		

Egg and Hatchling Survival Data

Date	Total no. juveniles/eggs alive
22/06/2003	659
24/06/2003	93
30/06/2003	81
03/07/2003	71
01/09/2003	71
15/09/2003	71
09/10/2003	70