Foraging, personality and parasites: investigations into the behavioural ecology of fishes

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Abstract

Individual behaviour varies considerably between and within species to balance the demands of resource acquisition in an ever-changing environment. This thesis investigated three aspects of behavioural variations: the occurrence of a conservative foraging strategy whereby predators avoid novel prey; the interaction between boldness (an aspect of personality) and behaviour; and the effect of boldness, host sex and social contact on parasite transmission. It was postulated that a temperate fish species, the three-spined stickleback (*Gasterosteus aculeatus*) was more dietarily conservative than poeciliid fish species, and that solitary fish exhibited a more conservative foraging strategy than fish in shoals. The thesis tested the hypotheses that shy fish shoaled more than bold counterparts, virgin females were bolder than mated ones, and shy fish had a greater chance of acquiring a parasite infection from an infected shoal member due to their closer proximity within shoals than bold fish. In addition, it was hypothesised that host contact was the main factor influencing parasite transmission within fish shoals.

Behavioural experiments, traditional parasitology and molecular techniques were used to test the hypotheses. Five fish species, and a directly-transmitted ectoparasite, were used as model organisms. Fish were tested individually and assessed within shoals for their avoidance of novel prey, shoaling behaviour and parasite loads. There was evidence for a conservative foraging strategy in the four tropical fish species (*Poecilia reticulata*, *P. sphenops*, *Xiphophorus maculates*, *X. hellerii*) and in a temperate species (*Gasterosteus aculeatus*), with the latter having greater dietary conservatism. Social context did not affect dietary conservatism, and there were with no significant differences in conservatism between isolated fish and shoals. Also, guppies showed reduced acceptance of novel, conspicuously-coloured prey. Using molecular scatology, both prey and host species-specific DNA were detected in fish faecal samples so this methodology can be used in the future to examine diet in the wild. Guppies from two wild populations differed significantly in their relative boldness, but individuals within a single population were similar in their relative boldness and this was consistent over time. Boldness of fish was affected by mating, with virgin females being bolder than their mated counterparts. Also, boldness impacted on shoaling behaviour, shy fish forming larger and tighter shoals than bold conspecifics. This had consequences for parasite transmission, with shy fish having higher parasite loads and a greater change in parasite load across an infection period than their bold counterparts. Host contact was the main factor influencing transmission of a directly-transmitted ectoparasite within a group-living host species. Significantly more parasites were transmitted between hosts when hosts were in more frequent and prolonged contact.

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Chapter 1: Introduction

The complex behaviour of fishes intimately reflects distinctive and effective solutions to problems raised by their three-dimensional environment. Behaviour is the interface between genetics and habitat pressures such as predators, finding suitable food, disease or parasite exposure and mortality risk. Fish constantly trade-off the conflicting demands of these factors, they show a range of flexible behavioural responses, allowing them to respond to many environmental factors at any one time. There can be considerable behavioural variation between and within members of a species with differences being caused by factors such as sex, body size, interactions with conspecifics, personality, food availability, predation pressure and parasitism. Of particular interest to this thesis are individual differences in foraging behaviour, personality and parasitic infection.

1.1: Shoaling behaviour

A well known aspect of animal behaviour is the formation of social groups (Bertram 1978, Pulliam and Caraco 1984). These are thought to arise from a continuous decision-making process, whereby individuals constantly assess the profitability of joining, staying with others or leaving a group, based on an ever changing trade-off between predation risk and the balance between foraging costs and benefits (Pitcher and Parrish 1993). Typically, competition for resources increases with increasing group size, whereas the risk of predation decreases (Alexander 1974). Costs associated with group-living also include kleptoparasitism, unproductive social interactions, and a higher likelihood of foraging on a recently depleted resource, leading to lower food intake (Beecham and Farnsworth 1999). However, the main advantage of living in a group is there are 'many eyes' for predator detection, so that increased group vigilance, together with dilution, confusion and selfish-herd mechanisms can reduce per capita predation pressure (Hamilton 1971, Pulliam 1973, Bertram 1978, Day et al. 2001). Another benefit comes from more individuals being able to detect food sources (Pulliam 1973, Bertram 1978, Lazarus 1979), leading to benefits for the group as a whole. Group-living may enable individuals to forage more effectively through information transfer (Ward and Zahavi 1973) and social learning (Zentall and Galef 1988, Heyes and Galef 1996).

Fish shoaling is a distinct, clearly measurable behaviour, ideal for studying the functions of group living (Godin 1986, Magurran 1990, reviewed by Pitcher and Parrish 1993). The term 'shoal' is commonly used to refer to any social aggregation of fish, whereas 'school' more specifically refers to synchronised groups of fish that show polarised swimming behaviour (Pitcher 1983). Amongst teleost fish, shoaling behaviour is very common in fry and juveniles, with an estimated 25% of species continuing to shoal as adults (Shaw 1978). Changes in both shoal size and composition can regularly occur (Pitcher et al. 1996, Mackinson et al. 1999, Krause et al. 2000, Svensson et al. 2000), providing individuals with the opportunity of using shoaling behaviour as a flexible response to changes in environmental conditions or internal state (Hoare et al. 2004).

In shoaling fish, as with other animals that form social groups, the behaviour of individuals is primarily influenced by the presence of predators and number of conspecifics present, with resulting feeding behaviour being a compromise between various costs and benefits (Hart 1993). A major benefit to shoaling is that individuals in shoals experience a lower predation risk compared to solitary individuals through anti-predator mechanisms, such as improved predator detection (Magurran et al. 1985, Godin et al. 1988), attack dilution (Foster and Treherne 1981, Morgan and Godin 1985), the confusion effect (Neill and Cullen 1974, Landeau and Terborgh 1986) and evasion tactics (Godin 1986, 1997, Magurran 1990, Pitcher and Parrish 1993). The impact of predation on shoaling behaviour can be seen in the responses of fish to changes in perceived predation risk. For example, European minnows (*Phoxinus phoxinus*) increase group size in response to a predator (Magurran and Pitcher 1987), shoaling decisions are made more quickly when a predator is present (Hager and Helfman 1991) and species from habitats with a high predation risk show a higher shoaling tendency than those from predator-free populations (Seghers 1974, Magurran and Pitcher 1987, Magurran et al. 1993, Brown and Warburton 1997). Shoaling also provides foraging benefits through faster location of food (Pitcher et al. 1982, Pitcher and Magurran 1983, Ranta and Juvonen 1993), more time for feeding (Magurran and Pitcher 1983), more effective sampling of feeding sites (Lester 1981, 1984, Pitcher and Magurran 1983), information transfer (see Pitcher and Parrish 1993) and learning from conspecifics (e.g. Pitcher and House 1987).

The primary cost of shoaling is that of increased competition for resources (Bertram 1978), and it is thought that this controls maximum group size (e.g. Pulliam and Caraco 1984). Shoal members respond to increased competition by varying their spatial position within the shoal (Krause 1993), or by altering food-handling times (Street et al. 1984) or foraging strategies (Milinski 1984a, Pitcher et al. 1986). Fish in shoals may also take more risks as a response to increased food competition (Grand and Dill 1999), and can trade-off energy gain with the risk of predation by changing their shoaling decisions to reflect their energy needs and environmental circumstances (Abrahams and Dill 1989, Ashley et al. 1993).

The foraging efficiency and predation risk of individual shoal members can be affected by a number of factors, for example, phenotypic variability. It has been shown that fish have a social preference for others of matching phenotype (Ranta and Lindstrom 1990, Ranta et al. 1992, Krause and Godin 1994, Krause et al. 1996a), and shoals can be assorted by size, body length, and parasite-infection status in the wild (Dugatkin et al. 1994, Krause et al. 1996b, Peuhkuri et al. 1997, Krause et al. 1998a, Hoare et al. 2000a, b). It is thought that this assortment arises from the 'oddity effect' (Landeau and Terborgh 1986, Theodorakis 1989), where individuals of a different phenotype to the majority of shoal members are at an increased risk of predation. There may also be foraging and growth-related benefits to assortative grouping, for example, if an individual's phenotype relates to its competitive ability (Ranta et al. 1993, Seppa et al. 1999). Another factor that may affect foraging behaviour and predation risk is the spatial position of an individual within a shoal. Individuals straggling from a shoal are preferentially attacked and have a much higher mortality rate than their more social counterparts (Morgan and Godin 1985, Magurran and Pitcher 1987, Parrish et al. 1989). Also, fish at the front of the shoal have a higher feeding rate than those at the rear of the shoal (DeBlois and Rose 1996, Krause et al. 1998a) but incur a higher predation risk as a result (Bumann et al. 1997, Krause et al. 1998b). Fish can also alter shoal cohesion in response to predator or food cues, reducing nearest neighbour distances to varying degrees in the presence of a predator as well as when hungry (Magurran and Pitcher 1987, Morgan 1988, Robinson and Pitcher 1989, Krause 1993, Sogard and Olla 1997). There may also be a genetic component to shoaling behaviour, specifically to improving shoaling responses to a predator (Seghers 1974, Giles and Huntingford 1984, Magurran and Pitcher 1987, Magurran 1989, Magurran and Seghers 1990), and shoaling behaviour can also be affected by kinship (e.g. Ferguson and Noakes 1981, Quinn and Busack 1985, van Havre and Fitzgerald 1988, Brown and Brown 1996) and familiarity with conspecifics (e.g. Brown and Colgan 1986, Dugatkin and Wilson 1992, Brown and Smith 1994, Magurran et al. 1994, Griffiths and Magurran 1997, 1999).

1.2: Foraging behaviour

Foraging is another very important aspect of an animal's behaviour. The foraging behaviour of a predator involves a set of hierarchical decisions, firstly that of deciding to feed or not. Once that decision has been made, an animal decides which search strategy to employ in order to find the food, and once found, which size, species and amount of prey it is appropriate to take. Together with this, a predator must adopt suitable prey handling tactics in order to physically ingest its meal. Fish have been an important study organism in the analysis of predator feeding decisions (Pitcher 1993, Godin 1997, Wootton 1998), with fish foraging behaviour being primarily influenced by the same hierarchical decisions made by other predators, but also by physiology and morphology (Brett 1979, Brett and Groves 1979, Bone and Marshall 1982), as well as by the presence of conspecifics and predators (Hart 1993). As a result, food gathering strategies are a compromise between the energy benefits derived from the food and the energy costs associated with the particular strategies. Optimal foraging theory (Stephens and Krebs 1986, Schoener 1987) assumes that the forager will adopt a strategy that will maximise its net energy gain, which is in turn correlated to its fitness (defined as the animals' lifetime reproductive success). There are two main ways an efficient foraging strategy can originate, firstly, the necessary skills can be acquired over evolutionary time through natural selection, or secondly, they can be learned during the lifetime of the fish.

A fish's decision when to feed is governed by its motivational state, which in turn is influenced by many intrinsic factors (physiological parameters) and extrinsic factors (e.g. presence of prey, species, presence of predators) (Warburton 2003). Hunger has a major influence on the motivation to feed, with hungry fish responding more quickly to the presence of prey as well as completing more prey capture attempts than a satiated counterpart (Tugendhat 1960, Beukema 1964, 1968, Clark 1986). A seasonal change can also affect feeding rate, with some river species feeding less in winter (Metcalfe et al. 1986). Feeding also takes second place to reproduction and vigilance in some circumstances (Heller and Milinski 1979, Milinski 1984a, b, Noakes 1986, Godin and Sproul 1988, Hart and Gill 1993), where fish will suppress feeding when the predation risk is greater than that of starvation, and during a fish's breeding season, it may preferentially search for mating opportunities, defend a territory or nest rather than feed.

Once a fish has decided to start feeding, it needs to find suitable food. Search behaviour of a foraging fish is constrained by habitat structure as this sets the context for a fish's feeding decisions, and can directly influence the rate at which predators detect and capture prey, affecting their foraging efficiency and ultimately their fitness (Webster et al. 2007). Some fish species show habitat-specific morphological adaptations in response to environmental pressures to maximise foraging efficiency (Bentzen and McPhail 1984, Ehlinger and Wilson 1988, McPhail 1993). Fish employ two main search tactics to encounter prey, either they sit and wait, or they cruise steadily through the water (O'Brien et al. 1990). The tactic a fish uses depends somewhat on the relative size and activity of both predator and prey; generally for prey that are relatively large, fish employ the 'sit and wait' tactic but for relatively small prey items, fish tend to spend more time moving through the water (O'Brien et al. 1990).

After encountering prey, a fish must decide what prey types to ingest and how much to eat. A fundamental aspect of these decisions is based on the size relationship between the predator and the prey, since many predatory fish eat their prey whole (Gill 2003). Some predators are limited by gape (jaw) size so cannot ingest prey that are too large for their jaw apparatus, and at the lower end of the scale are very small prey that are difficult to detect visually (Gill 2003). Some fish species have evolved prey manipulation tactics to overcome the limitation of gape size, such as nibbling, biting and rotational feeding (see Gill 2003). A general effect of prey size is that the time it takes to handle the prey increases exponentially with increasing prey size (Werner 1974, Kislalioglu and Gibson 1976, Hoyle and Keast 1986, Croy and Hughes 1991, Gill and Hart 1994), and the trade-off between keeping handling time and energy costs low and maximising energy intake affects many predator feeding strategies (Gill 2003). Other morphological features of fish affect their ability to exploit prey, such as mouth shape, position and general body form (Werner 1977, Webb 1982, Hart 1993). As regards the amount of prey ingested, stomach fullness is the main factor affecting this decision, as changes in satiation levels are linked to motivation (Strubbe and van Dijk 2002).

Two main factors affecting foraging behaviour are considered in this thesis, the first is that of dietary conservatism, the phenomenon whereby a predator avoids a fully palatable prey item specifically because of its novelty. The second factor is the capacity for learning.

1.3: Dietary Conservatism

Dietary conservatism (DC) is the lengthy avoidance of novel food, first described by Owen (1977), and defined by Marples and Kelly (1999). DC is distinguished from short-term neophobia (Barnett 1958) which is a short-lived, reversible aversion to novel objects (including food), lasting a few minutes at most (Brigham and Sibley 1999, Marples and Kelly 1999). In contrast, dietarily conservative foragers can overcome their neophobia to approach and touch the food, but nonetheless continue to avoid eating it for considerable periods of time (Marples & Kelly 1999). DC does not rapidly subside over repeated encounters with novel food, and some individual predators have shown an avoidance of novel prey for over two years (Marples and Kelly 1999, Kelly 2001). One reason for this difference could be that neophobia and DC are based on different learning processes (Marples and Kelly 1999).

The majority of studies to date on DC have used birds and they are often observed to be neophobic (e.g. Kelly and Marples 2004). Once neophobia has subsided, many birds accept the new food into their diets (termed 'adventurous consumers' AC), but a small proportion remain wary and do not regularly eat the novel food, exhibiting a DC foraging strategy. Four successive stages of DC have been described: i) visual inspection of the novel prey; ii) occasional sampling or acceptance of the novel prey but only in the absence of familiar food; iii) regular acceptance of the novel prey but only after any familiar food has been consumed; and iv) full acceptance as a familiar food (Marples and Kelly 1999).

The phenomenon of DC is present to some degree in all avian species tested to date (reviewed in Marples et al. 2005), including common quail (*Coturnix coturnix;* see Marples and Brakefield 1995), domestic chicks (*Gallus domesticus*; see Kelly 2001), European blackbirds (*Turdus merula*; see Marples et al. 1998), European robins (*Erithacus rubecula*; see Marples and Kelly 1999), zebra finches (*Taeniopygia guttata*; see Kelly and Marples 2004), red-winged blackbirds (*Agelaius phoeniceus*; see Neff and Meanly 1957) and snail kites (*Rostrhamus sociabilis*; see Beissinger et al. 1994). DC seems to be less pronounced in captive birds, but it is still present; in fact there are examples of DC being so strong in some captive individuals that they died of starvation rather than eat the palatable novel food available (domestic chicks, Rabinowitch 1965).

There is much variation in the occurrence of DC between individuals of a given species where some individuals are much less conservative and quickly start eating new prey. Some authors have proposed a genetic basis for individual variation in DC, through studies on common quail (*Coturnix coturnix*) (Marples and Brakefield 1995) and great tits (*Parus major*) (Carere et al. 2001, 2003). Another factor to consider is the behavioural plasticity of individuals, causing between-individual variation in the level of wariness towards novel foods, as this could be important for an animal's ability to exploit all resources in its particular habitat. A wary forager could miss out on a potential new food supply, leading to a net loss in resources (Greenberg 1990a, b). Behavioural plasticity with regard to novel food could also affect an individual's dispersal ability, with less wary individuals being able to colonise new areas as they can more readily utilise potentially novel food types compared to very wary individuals. This could also relate to migratory species, where migrants may be less wary than non-migrants (Mettke-Hoffman et al. 2002, 2005, Mettke-Hoffmann 2005). Innovation in foraging has been linked to range expansion and invasiveness in some birds (Sol et al. 2002). Furthermore, the social context in which an individual lives can also impact on its feeding behaviour, either with regard to solitary versus group-living (e.g. Magnhagen and Bunnefeld 2009), or the mixture of behavioural phenotypes within a group (e.g. Boogert et al. 2006, Dyer et al. 2009), and so this may also affect an individual's level of DC.

One well-studied potential effect of DC is as a mechanism for facilitating the evolution of warning colouration, because if predators avoid novel prey for sufficient time, novel colour morphs could invade a prey population and persist, rather than undergoing increased predation and rapid extinction (Coppinger 1969, 1970, Gotmark 1994, 1996, Marples et al. 1998). The assumption is often made that the foraging decisions of predators place conspicuous novel prey morphs at a selective disadvantage, i.e. predators are more likely to feed on conspicuous prey because of their conspicuous nature. However, studies showing clear evidence of predator avoidance of novel prey, i.e. a DC foraging strategy, call this assumption into question.

1.4: Learning

Another factor that affects a fish's foraging performance is learning, considered here briefly. Comparative psychology has revealed broad similarities in general laws of learning across invertebrate and vertebrate taxa (Logue 1988, Domjan 1998), so the general principles that apply to bees, pigeons and rats are also likely to apply to fish. A great number of studies have revealed that fish have a greater cognitive capacity than previously thought and it is now possible to compare the learning capabilities of fish with those of higher vertebrates (reviewed by Hart 1986, 1993, Hughes et al. 1992, Kieffer and Colgan 1992). Fish rely on a wide range of learning mechanisms that are vital to successful foraging, from the formation of foraging search images, to prey capture and handling.

A well-studied mechanism through which fish learn foraging skills is social learning, where socially transmitted information is used by conspecifics to locate food and assess changes in food patch profitability (Pitcher and House 1987, Ryer and Olla 1991). This has been seen for example in European seabass (*Dicentrarchus labrax*) that learned to press a lever to receive food after observation of trained demonstrator conspecifics (Anthouard 1987). Groups of fish find food more quickly than individuals because there are more individuals to be vigilant for food, as well as having more socially transmitted information available to them. This has been demonstrated in many species, e.g. goldfish (*Carassius auratus*), bluntnose minnows (*Pimephales notatus*), three-spined sticklebacks (*Gasterosteus aculeatus*) and guppies (*Poecilia reticulata*) (see Pitcher et al. 1982, Morgan 1988, Peuhkuri et al. 1995, Day et al. 2001). Fish can also learn foraging skills, such as prey manipulation, through observation of feeding conspecifics (e.g. Sundstrom and Johnsson 2001).

There are a number of other factors that affect the learning process, such as reinforcement, which influences what is learned rather than how, or how well, it is learned (Warburton 2003), and motivation, comprising two components, drive (deprivation) and stimulus attractiveness (incentive). When fish are hungry they are driven to search for food so are less distracted by other pressures (Milinski 1993) which enhances learning of foraging skills. Exploration and sampling are also factors integral to successful foraging. Fish find food through individual sampling, exploring their environment and paying attention to environmental stimuli (Pitcher and Magurran 1983). Fish use information about their habitat to deal with variability in that environment and it is thought this ability might explain observed fish behaviours such as spontaneous alternation, patrolling, effects of hunger, latent learning and behaviour following changes in food availability (Inglis et al. 2001). Another factor affecting the learning of foraging skills is cognitive processing which allows an individual to select from a wide range of pre-learned responses, not only innate ones, allowing flexibility of response. Goal-directed behaviour is the most basic behavioural marker of cognition. For example, fish can be trained to feed at a particular location and at a particular time of day by combining a stimulus (food) with an environmental cue (Reebs 2000). Individuals also remember their feeding success with particular conspecifics, using that information to shoal with, or remain separate from, those same conspecifics over several weeks (Dugatkin and Wilson 1992). These latter two studies show that fish use memory to adjust their behaviour; memory seems to be highly adapted to the specific requirements of each species, as in some circumstances forgetting may be as important as remembering (Warburton 2003).

1.5: Personality

Personality research in non-human animals has only become common in the scientific literature since the late 1990's, despite initial interest in the early 20th Century with Pavlov's series of studies on canines (e.g. Pavlov 1906, 1928), ape studies in the 1930's by Yerkes (Yerkes and Yerkes 1936, Yerkes 1939), followed by those of Hebb in the 1940's (Hebb 1946, 1949). Animal studies can inform human personality research in many ways, opening up new research opportunities and raising questions about the origins of personality traits. Specifically, the past decade of research (since 2000) has shown the viability of assessing elements of personality in animals, resulting in four main benefits to the field of psychology. Firstly, animals allow for greater experimental control as they permit a number of options for experimental manipulation that are not possible in humans. For example, controlling the environment of test subjects (e.g. De Jonge et al. 1996), conducting genetic manipulations (e.g. Chiavegatto et al. 2001), altering hormone levels (e.g. Briganti et al. 2003) and performing pharmacological tests (e.g. Ferris and Delville 1994). Animal studies also allow measurements of physiological parameters with greater ease than in humans. These include neurotransmitter and hormone concentrations, hormone receptor binding and subsequent genetic expression, since this data is normally collected through very intrusive access to cerebral spinal fluid, blood or specific brain areas (Mehta and Gosling 2006). Thirdly, animals can be observed for longer periods of time, in more detail and more contexts, and a greater variety of behaviours observed, relative to humans. Captive animals can potentially be observed for their entire lives from conception to death, but also observations on wild animals living in natural habitats can generate large amounts of behavioural and physiological data, and allow questions about how these parameters change in response to environmental cues, across seasons and over time (e.g. Virgin and Sapolsky 1997). The final main benefit of using animals in personality research is their accelerated lifespan, making it possible to conduct longitudinal studies across many generations of the same species, that provide relatively quick results and are much less costly than equivalent human studies (e.g. Mejia et al. 2002).

Defining the term 'personality' is difficult as there is not one specific definition of personality that would satisfy psychologists across all fields of study. However, Pervin and John's (1997) definition of 'those characteristics of individuals that describe and account for consistent patterns of feeling, thinking, and behaving', is a general enough description to cover most traits studied by psychologists. In animal personality research, terms such as 'temperament' and 'behavioural syndrome' have often been used instead of 'personality', with the latter term having become popular in the field of behavioural ecology since its definition (suites of correlated behaviours expressed either within a given behavioural context or across different contexts) by Sih et al. (2004), is very similar to the concept of personality in humans. Human-personality researchers focus on a wide range of constructs, including temperament and character traits, dispositions, goals, personal projects, abilities, moods, attitudes, physical health and life stories. The majority of animal personality studies consider just a subset of these constructs, called behavioural traits. A set of criteria are used to establish the existence of personality traits in animals: i) assessments by independent observers must agree, ii) assessments must predict behaviours and outcomes, iii) observer ratings must reflect genuine traits of the study individuals, not the observer's own theories or viewpoint. These criteria allow comparison of many individuals within and between species, as well as across different situations and through time, focussing on the consistency of behavioural patterns, rather than on any underlying emotional processes. This avoids criticisms about lack of objectivity and anthropomorphism (Weinstein et al. 2008), and further supports the viability of assessing personality in non-humans.

Gosling and John (1999) conducted one of the first cross-species reviews of animal personality traits, using the well-established human Five Factor Model (FFM; John 1990) as a framework for the findings. The FFM is a hierarchical model with five broad factors (Neuroticism, Agreeableness, Extraversion, Openness, Conscientiousness) representing personality in its broadest terms. They found the dimensions of Extraversion, Agreeableness and Neuroticism were present to some degree in all species (10 mammals, 1 fish, 1 mollusc) included in the review. Openness was found in 65% of the species tested whereas Conscientiousness was only identified in one species (Chimpanzees, *Pan troglodytes*). Dominance and Activity also emerged as clear separate factors in up to 35% of species. The most comprehensive review of recent animal personality studies was carried out by Gosling (2001), and identified 187 personality studies in 64 different species, providing much evidence for the wide distribution of personality traits across many animal taxa, such as numerous primates, birds, fish, reptiles, amphibians, arthropods and molluscs (e.g. Gosling 2001, Weiss et al. 2006, Cote and Clobert 2007, Kortet and Hedrick 2007, Kralj-Fiser et al. 2007, Magnhagen 2007, Sinn et al. 2008).

Within the field of behavioural ecology, and most pertinent to this thesis, three main personality traits have been well-studied: activity, aggressiveness and boldness. These traits are often interrelated (e.g. Huntingford 1976, Riechert and Hedrick 1993, Bell 2005, Johnson and Sih 2005) and all centre on a fundamental trade-off between gaining more resources but risking higher mortality (Stamps 2007). Firstly, when considering activity, the standard trade-off for prey animals is that, when predators are present, an increase in prey activity results in higher prey feeding rates but also a higher predation risk (Sih 1987, Werner and Anholt 1993, Lima 1998). Experimental studies have shown that average prey activity levels differ not only in the presence versus absence of predators, but also in different situations (e.g. Sih et al. 1988, Verbeek et al. 1994, 1996, Maurer and Sih 1996, Sih et al. 2003, Brodin and Johansson 2004). Across-situation correlations in activity are important to consider, since individuals that are more active than others in the absence of predators may be inappropriately active when predators are present, thus suffering a higher rate of predation (Sih et al. 2004). The second main ecologically important behavioural axis is aggressiveness, defined as the tendency to attack other individuals (Sih et al. 2004). More aggressive individuals attack both prey and territorial intruders (conspecifics and potential predators) more quickly than less aggressive counterparts (Riechert and Hedrick 1993). However, this can result in a higher risk of mortality, and, in some species, aggressiveness can incur fertility costs through precopulatory sexual cannibalism (e.g. Arnqvist and Henriksson 1997). Thirdly, the propensity of an animal to take risks, or its boldness, has been well documented (e.g. Wilson et al. 1994, Coleman and Wilson 1998, Wilson 1998), with bold and shy individuals differing in a variety of behaviours, such as predator inspection, acclimation and activity in a novel environment, foraging behaviour and refuge use, dispersal ability, reproductive success (Godin and Dugatkin 1996, Coleman and Wilson 1998, Reale et al. 2000, Fraser et al. 2001, Dingemanse et al. 2003, Reale and Festa-Bianchet 2003). Bold individuals trade off an increased level of risk across different situations against the chance of acquiring new and more resources.

1.6.1: Model species – the Trinidadian guppy

Guppies belong to the family of Poeciliidae, a group of fish characterised by internal fertilisation, viviparity and the male external sexual organ, the gonopodium (e.g. Rosen and Bailey 1963, Wourms 1981). The guppy, *Poecilia reticulata* Peters (1859) is a small (max standard length 45 mm) tropical fish with a short generation time, that gives birth to live young and is sexually dimorphic, with males being smaller and more colourful than females (Froese and Pauly 2008) (Figure 1.1).

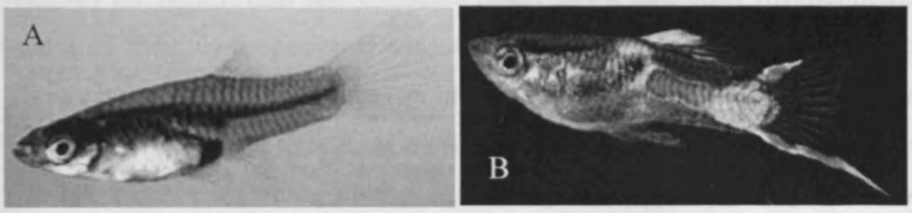

Figure 1.1: Wild strain (A) female (from www.thinkfish.co.uk) and
(B) male (from www.mysite.verizon.net) guppies.

In fact, guppies are notable amongst the poeciliids for having the greatest degree of sexual dimorphism and diversity of male colour patterns (Houde 1997). This, together with the adaptability of the guppy to live in a wide range of habitats under different environmental conditions, has meant that this species has been adopted as a model organism for testing key evolutionary theories (Amundsen 2003).

The natural range of the guppy is Trinidad, Venezuela, Guyana and Surinam (Farr 1975), although these fish now have a worldwide distribution due to their popularity as an aquarium fish, and use as a biological control agent for malaria mosquito larvae. Guppies tolerate a temperature range of 18-28 °C. The majority of fieldwork on this species has been conducted in the northern mountain range of Trinidad (Figure 1.2) and many laboratory studies have used guppies from this location also (Houde 1997). Guppies are usually found in freshwater, in clear, relatively fast-flowing mountain streams as well as larger and turbid lowland rivers (Kenny 1995, Houde 1997, Magurran and Philip 2001), but can also occur in small ponds and tolerate brackish water (Magurran 2005). This species can also be found in polluted water bodies, such as drainage ditches, as well as a natural pitch (tar) lake (Schelkle et al. in prep.).

Figure 1.2: Trinidad's northern range and location of key guppy populations (from Magurran 2005)

Guppies are omnivorous and consume insect larvae and other invertebrates, algae and detritus, and young of their own species (Dussault and Kramer 1981). Guppies are not only morphologically distinct but also display dimorphism in their feeding behaviour. Males ingest more algae per peck than females and spend less time foraging (Dussault and Kramer 1981, Magurran and Seghers 1994). Males also move between feeding sites more, and rely less on previous experience when choosing a foraging site than females (Abrahams 1989). These differences reflect the inherently different reproductive strategies of males and females, where males are continually search for mating opportunities, and females prioritise resource acquisition to cope with the energetic costs of reproduction (Houde 1997).

The configuration of river systems in Trinidad along with the physical distribution of fish species in them, including guppies, has resulted in numerous populations evolving rapidly and independently from each other. There is considerable variation between populations in predator pressure as well as other environmental factors that impact on a range of adaptive traits, leading to parallel but independent evolutionary changes between guppy populations (Houde 1997, Magurran 2005). Predation is a major factor influencing geographic variation in adaptive traits (reviewed by Endler 1995). The rivers on the southern slopes of the Northern Range in Trinidad contain guppies throughout almost their entire course, and these rivers are broken up by waterfalls that are sufficiently large to prevent upstream colonisation by larger fish predators (Magurran 2005). Therefore, upstream and downstream rivers differ in their predator fauna and hence predation risk, a major ecological factor. A number of traits are known to be affected by differences in predation pressure between populations, such as foraging and mating behaviours. Godin and Smith (1988) found that guppies that foraged on higher densities of prey had a higher risk of capture by a predatory cichlid. Also, guppies can continue to forage in to the night to avoid diurnal predators (Fraser et al. 2004). Many sexual selection studies have been performed using the guppy model system, assessing how the evolution of reproductive behaviour is affected by environmental variation, and to look at the joint evolution of male secondary sexual traits and female mate choice (Houde 1997).

Guppies exhibit varied mating strategies, such as male coercion, multiple mating, sperm competition, with the main one being female choice for male secondary sexual characteristics, namely colour pattern (e.g. Schmidt 1920, Bisazza 1993, Brooks and Endler 2001, Herdman et al. 2004). Male guppies produce sperm bundles and transfer these to the female using the gonopodium, a modified anal fin. Part of the anal fin is thickened and elongated to form the tube-like gonopodium structure (Clark and Aronson 1951, Constantz 1989). The logistics of copulation involve the male positioning himself so that he can swing the gonopodium forward and insert it into a female's genital opening, thus transferring sperm. Males usually employ two strategies to achieve copulation, either displaying in order to achieve a consensual mating with a receptive female, or engaging in 'sneaky' matings whereby males attempt to mate with unresponsive females (Baerends et al. 1955). Female guppies are typically more receptive to mating either as virgins or up to 2 days after giving birth (Liley 1966). Females can store sperm in their ovaries and gonoducts (Constanz 1989) and use it to fertilise ova for up to 8 months (Winge 1937), which means that a single pregnant female can potentially found a new population or colonise a new area (Houde 1997). A typical reproductive cycle lasts 25-30 days (Haskins et al. 1961) and female guppies can produce 20 or more young per litter per individual, largely depending on their body size (Reznick and Endler 1982, Reznick and Bryga 1987, Travis 1989, Houde 1997).

When born, guppy fry are well developed and do not need parental care. They can school immediately and display anti-predator behaviour (Magurran and Seghers 1990). Male and female guppies can be visibly distinguished from approximately 4 weeks old. Females begin to show a dark colouration around the anal region at this stage, whereas the anal fin in male guppies starts to thicken at 5 to 6 weeks of age and colour begins to appear on the tail (Houde 1997, Reznick et al. 2001). Males begin to exhibit sexual behaviour as the gonopodium develops and start to perform courtship displays as the colour patterns develop. Females may also mate before while their ova are still immature, and store the sperm until the ova mature (Houde 1997). Guppy females can continue to produce young until 20-34 months of age (Reznick et al. 2001a).

The other poeciliids used as study species in this thesis are mollies (*Poecilia sphenops*), platys (*Xiphophorus maculates*) and green swordtails (*X. hellerii*). They are small, live-bearing species that live in freshwater, and have a wide distribution throughout tropical and subtropical regions of the world. Mollies and swordtails both have a fairly diverse diet; mollies are mainly herbivorous and eat a great amount of detritus (Trujillo-Jimenez and Beto 2007) and swordtails are omnivorous, consuming aquatic plant tissue, algae and a range of invertebrates (Arthington 1989). Platys are more specialised feeders, primarily ingesting atyid shrimp (Arthington 1989).

1.6.2: Model species - three-spined stickleback

This fish belongs to the gasterosteid family that are restricted in their distribution to the temperate and sub-polar zones of the northern hemisphere, being found between 35°N and 70°N in Europe, parts of Asia and northern America. Three-spined sticklebacks, *Gasterosteus aculeatus* Linnaeus (1758) (Figure 1.3), are perhaps the best known of the species, and have attracted the most attention from biologists due to their hardy nature and adaptability to laboratory conditions.

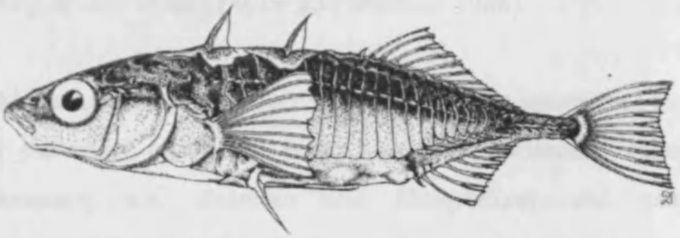

Figure 1.3: *Gasterosteus aculeatus* (from Wootton 1984)

This species is usually found in schools of closely-associated individuals. They can live in fresh, brackish and salt water, being most common in slow-flowing rivers, sheltered bays and harbours, but can also be found in lakes and ponds with submerged vegetation for cover. Adults are normally 35–80 mm total length and its common name, the three-spined stickleback, is taken from the three dorsal spines that precede the dorsal fin. These spines together with lateral body plates provide the fish with a flexible segmented armour that protects it from vertebrate predators (Hoogland et al. 1957, Bell and Foster 1994). The distribution and number of plates varies extensively within and among freshwater populations of three-spined sticklebacks (reviewed in Bell 1984, Wootton 1984), typically three morphs are recognised: complete, partial and low plated (Mattern 2007). This variability is one reason that this species has become a classic model for evolutionary and ecological studies (Bell and Foster 1994). Three-spined sticklebacks are also abundant and widespread, making them readily available for research, and within a relatively small area, populations may exhibit very different morphology and occupy diverse habitats. It is a small, common fish, so numerous individuals can be assessed over a small area in a short time in the wild, but also large sample sizes can be collected and maintained easily in the laboratory. This species also exhibits a range of conspicuous and ritualised behaviours, such as aggression, territoriality, courtship and male parental behaviours as well as foraging and anti-predator tactics, that have been studied to answer basic questions in behaviour, ecology and evolution (Bell and Foster 1994).

Specifically of interest in the context of this thesis is the foraging behaviour and diet of the three-spined stickleback. Survival, growth and reproduction are not possible without the resources that foraging provides. Sticklebacks are primarily visual predators (Beukema 1968, Wootton 1976), feeding only in the light (Wootton 1984), so cues such as size, movement, shape and colour contrast with the background, are important for prey detection. Habitat structure also affects foraging behaviour, and the differences in demands between habitats has caused sticklebacks to adapt specifically to them (Larson 1976, Bentzen and McPhail 1984, Lavin and McPhail 1986, Ibrahim and Huntingford 1988). This is most clearly seen when comparing sticklebacks from benthic and pelagic habitats, employing different search tactics to detect prey and being morphologically adapted to life in running or still water (Taylor and McPhail 1986).

Sticklebacks inhabit a variety of habitats but it seems that the same prey form the bulk of their diet, with the most prominent being the microcrustacean copepods, e.g. *Diaptomus* and *Epischura*, cladocerans, e.g. *Bosmina* and *Holopedium*, and ostracods. Chironomid (midge) larvae, ephemeropteran (mayfly) nymphs, oligochaetes, molluscs and algae are also ingested to varying extents (Wootton 1976, 1984). The diet of sticklebacks is partly affected by seasonal variations in the abundance and availability of prey, with the diet of *G. aculeatus* being dominated by copepods in spring and early summer and again in late summer and early autumn. Mayfly nymphs are the main prey eaten in mid-summer, algae ingested primarily in autumn and winter, whereas midge larvae are eaten all year round (Allen and Wootton 1984). Sticklebacks, especially males, vary the amount of prey ingested according to their reproductive behaviour. They eat more in the breeding season of spring and early summer when much energy is needed for nest building and courtship, whereas in mid-summer food intake drops, as they prioritise parental care.

1.6.3: Model species - Gyrodactylus

Parasites are known to be a major evolutionary driving force for many life history traits, such as sexual selection, reproductive behaviour, growth, immune defence and dispersal (Hamilton and Zuk 1982, May and Anderson 1983, Hamilton et al. 1990, Hochberg et al. 1992, Koskela 2002, Moller et al. 2004, Fredensborg and Poulin 2006). The monogeneans, *Gyrodactylus* spp. are ubiquitous and highly diverse ectoparasites of teleost fish. They reproduce viviparously, facilitating exponential population growth and making them one of the most invasive groups of fish parasite (Kennedy 1994). Particularly well studied are the species of gyrodactylids that are found on poeciliids and sticklebacks, namely *G. turnbulli* and *G. bullatarudis* that infect the guppy, and *G. gasterostei* that infects the three-spined stickleback. Adult gyrodactylids reproduce on the host epidermis, giving birth to offspring that contain developing embryos (reviewed by Cable and Harris 2002). Gyrodactylids can also survive for up to 32 hours off the host (pers. obs.). These parasites are economically important both in the farming and aquaculture industries (reviewed by Bakke et al. 2002, 2007), and are a conservation issue threatening endangered fish stocks (Leberg and Vrijenhoek 1994, Hedrick at al. 2001). Gyrodactylids were first used as model organisms mainly due to their unique reproduction (e.g. Braun 1966), with Scott and Anderson (1984) showing their use in an epidemiological model. Since their confirmation as important fish pathogens, resulting from the decimation of Atlantic salmon stocks in Norway following introduction of *G. salaris* (Heggberget and Johnsen 1982, Johnsen et al. 1999), the use of gyrodactylids as model organisms has grown rapidly, and they have recently been used as a model system to assess the impact of parasites on conservation practices (van Oosterhout et al. 2007, Faria et al. 2010).

The host-parasite model system used in this thesis is that of *G. turnbulli* infection of the guppy. These parasites have a prevalence of greater than 75% in some wild Trinidadian guppy populations (van Oosterhout et al. 2007). Parasite loads on wild fish can be as high as 100 parasites per individual guppy (van Oosterhout et al. 2006), with the average load on most hosts being <10 worms per individual (Harris and Lyles 1992). Infection reduces foraging rate (van Oosterhout et al. 2003) and the carotenoid content and resulting colour saturation of male guppy orange spots (Houde and Torio 1992), which is a primary target of female choice (Kodric-Brown 1989, Grether 2000). Infected males have been shown to be less attractive to females than uninfected males (Kennedy et al. 1987, Houde and Torio 1992, Houde 1997, Lopez 1998), indicating that gyrodactylids could be a strong selective force in wild guppy populations. Secondary pathology associated with heavy worm infection causes severe fin clamping (Cable et al. 2002) which may negatively affect swimming performance of these fish. Mortality rates of up to 50% have been seen in some experimental infections (Houde 1997).

Gyrodactylids are transmitted by direct contact between fish (Scott and Anderson 1984) making them highly contagious. Due to their direct life cycle it is thought that *Gyrodactylus* spp. are capable of continuous transmission, able to infect new hosts at any time of their life cycle (Boeger et al. 2005). There are four known transmission routes of gyrodactylids: i) direct transfer between live hosts; ii) contact between live and dead infected hosts; iii) transfer between fish and detached gyrodactylids on the surface water film; iv) transfer between fish and parasites on the substrate (Bakke et al. 1992, Soleng et al. 1999). El-Naggar et al. (2004) showed that *G. rysavyi*, that infects Nile catfish, is capable of unidirectional 'swimming' for up to 8 s if detached from the host. The particular transmission strategy used by gyrodactylids is possibly linked to host behaviour and ecology (Bakke et al. 2007). For example, *G. salaris*, infecting Atlantic salmon (*Salmo salar*), has been shown to remain attached to a dead host (Olstad et al. 2006), since this species of salmon is predominantly solitary, with individuals at all life stages preferring to be in close proximity to the substrate in shallow, fast flowing water (Bagliniere and Champigneulle 1986). In contrast, some species of gyrodactylid move off the dead host, e.g. *G. gasterostei* and *G. turnbulli* which infect the three-spined stickleback and guppy respectively (Cable et al. 2002a, b). These particular host species occur in shoals and so the detachment from a dead host, for these *Gyrodactylus* species, may be attributed to host shoaling behaviour as this would increase the likelihood of subsequent transmission to new hosts.

Gyrodactylids attach to their host using a posterior attachment organ called the opisthaptor. This consists of a central pair of larger hooks, the hamuli, surrounded by a ring of smaller marginal hooks (Figure 1.4), the exact arrangement of which varies hugely between species. The smaller hooks act as the primary mode of attachment while the hamuli prevent the parasite from being dislodged by sinking in to the host epidermis (Lester 1972). Marginal hook morphology has been widely used to identify gyrodactylids to species level, although there are a number of problems associated with this, partly due to the level of expertise required both for identification and sample preparation to avoid discrepancies in measurements of the hooks (e.g. Shinn et al. 2010). Furthermore, there is considerable morphological variation within species (Harris 1998) caused by factors such as temperature, water chemistry and salinity (reviewed in Bakke et al. 2007). To overcome such problems, new methods of identification have been developed, for example chaetotaxy (the use of sliver nitrate in mapping surface sensory structures) (Shinn et al. 1998a, 1998b), Scanning Electron Microscopy (Shinn et al. 2000), light microscopy (McHugh et al. 2000), as well as the development of molecular markers (e.g. Hansen et al. 2003).

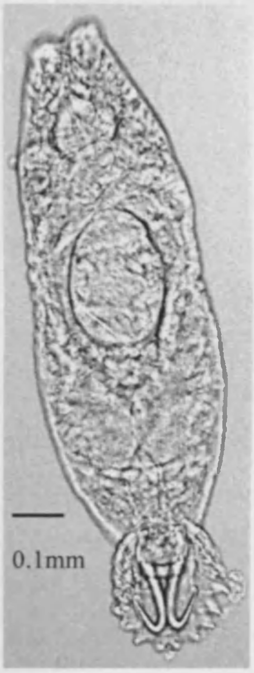

Figure 1.4: Light micrograph of *Gyrodactylus gasterostei* (from Dr. J Cable)

The success of gyrodactylids in colonising teleost hosts is due mainly to their viviparity. The parent individual contains an F1 embryo in its uterus, which in turn has a developing embryo (F2) within it, akin to ‘Russian dolls’. Therefore, under optimal conditions (25 °C), the parent gives birth to a fully formed offspring within 24-48 h of the parent’s own birth (e.g. Scott 1982). The new offspring attaches itself to the host next to the parent. This short generation time increases reproductive rate despite the low fecundity (typically 1-2 offspring per parent, see Scott 1982). Reproduction is primarily asexual (Harris 1993) but a small proportion of individuals (possibly as little as 1%) reproduce sexually (Harris 1993). The female reproductive system consists of an egg cell forming region (ECFR), uterus and vitelline cells (Cable et al. 1998), whereas the male system is comprised of a single testis, seminal vesicle and penis (Cable and Harris 2002). Reproduction in *Gyrodactylus* species follows a certain pattern with the first and successive offspring having different developmental origins (Cable and Harris 2002). The first born daughter develops as a ball of cells within the parent while second and subsequent offspring develop from oocytes (Cable and Harris 2002). Birth occurs very quickly when the anterior region of the daughter is freed from the parent, the offspring attaches to the host using its opisthaptor and pulls itself free from the mother. Temperature is the major factor affecting gyrodactylid population growth rate (e.g. Davidova et al. 2005), reproduction and survival (Scott and Anderson 1984, Jansen and Bakke 1991, Bakke et al. 2007). At higher temperatures (up to an upper limit of 30°C), population growth rate and reproductive rate increase, while survival is prolonged at lower temperatures (e.g. Harris 1982, Jansen and Bakke 1991, Scott and Anderson 1984, Scott and Nokes 1984). Temperature can also influence the abundance and transmission of gyrodactylids (Bakke et al. 2007).

1.7: Thesis aims and hypotheses

In this thesis, I investigate three main topics. First, I examine the occurrence of conservative foraging in four poeciliid fish species (Chapter 2), and whether such a strategy is affected by the social context of predators (Chapter 3). Dietarily conservative foraging occurs in many bird species and is known in one other fish species, but it is unknown to what extent it exists in others. In Chapter 2, I test the hypotheses that poeciliid fish species are less dietarily conservative than a temperate fish species, the three-spined stickleback (*Gasterosteus aculeatus*), and investigate the effects of sex, body size, foraging context (making the prey cryptic or conspicuous) and species differences between the poeciliids. Chapter 3 extends the work in Chapter 2 and tests the hypothesis that fish in shoals exhibit a less conservative foraging strategy than solitary fish.

Secondly, I consider the interplay between boldness (an aspect of personality) and fish behaviour, and the subsequent impact on parasite transmission. An animal's personality appears to affect how it interacts with its environment, for example, its reactions to predators, food sources, social and sexual encounters with conspecifics. However, there has been little work to date on how shoaling behaviour is affected by an individual's personality and specifically of interest in this thesis, how boldness is affected by social context and mating status. In Chapter 4, I investigate the occurrence of boldness in different populations of a fish species (the guppy, *Poecilia reticulata*), as well as the impact on behaviour of repeatedly testing for boldness. I test the hypotheses that shy fish shoal more than bold fish across the same time frame, and that shy and bold fish habituate to repeated boldness testing. In Chapter 5, I test the hypotheses that virgin female fish are bolder than their mated counterparts and that boldness decreases during gestation. Chapter 6 assesses the impact of boldness on parasite transmission as, to date, there is no empirical evidence linking the possible effect of host boldness on parasite transmission within social groups of animals. I specifically test the hypothesis that shy fish have a greater chance of acquiring a parasite infection from an infected shoal member because they shoal more than bold counterparts.

Thirdly, I examine the role of host sex and social contact in the transmission of directly-transmitted parasites. In Chapter 7, I investigate the role of sex specific differences in host grouping behaviour affecting transmission of an ectoparasite (*Gyrodactylus turnbulli*), hypothesising that greater parasite transmission occurs between female fish due to their higher shoaling tendency compared to male fish. Chapter 8 aims to determine more fully the effect of host contact on parasite transmission, hypothesising that those hosts in more prolonged and closer contact with each other experience a greater chance of acquiring a parasite infection from an infected shoal member.

The thesis is presented as 7 data chapters and each chapter is self-contained. As a result, there is some repetition between chapters, but each stands alone as a potentially publishable paper. Chapter 7 has been published in PLoS ONE. Chapters 2 and 3 have been submitted for publication. There are also two Appendices; Appendix I represents a preliminary study on the molecular scatology of fish faeces, detecting host and prey DNA, and Appendix II represents a collaborative study on the ability of guppies to forage on conspicuous and cryptic live prey, that will be submitted for publication with Dr Bart Adriaenssens as lead author.

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Chapter 2: Variation between and within fish species in the expression of dietary conservatism

2.1: Abstract

When animals encounter a new food, they must decide whether to eat it and risk being poisoned, or avoid it but risk losing out on a valuable food resource. Some individuals within a foraging population are 'adventurous consumers' (AC) and readily accept novel food items into their diets, while others display an active and long term avoidance of novel food, called 'dietary conservatism' (DC). Previous studies have shown the phenomenon of DC in many bird species and in a temperate fish, the three-spined stickleback (*Gasterosteus aculeatus*). However, it is unknown to what extent DC exists in other fish species, and so in the current study, we investigated the occurrence of DC in four species of tropical fish belonging to the poeciliid family (*Poecilia reticulata*, *P. sphenops*, *Xiphophorus maculates* and *X. hellerii*). Dietary conservatism strong enough to drive a novel prey morph from initial rarity to fixation in a prey population was detected in all four poeciliid species. The proportion of both prey types eaten by fish in one-off preference tests (to test for any pre-existing colour preference) varied between fish species and sizes, and was also affected by relative prey crypsis. Despite these underlying differences in pre-existing colour preferences, there was no significant difference between poecilid species, sexes or sizes in the likelihood of the novel morph reaching fixation; neither was there an effect of the relative conspicuousness or crypsis of the novel prey. Poeciliids were however less likely than sticklebacks to drive the novel prey morph to fixation. The current study provides strong evidence for the widespread existence of dietary conservatism in fish populations from a range of habitats, and the nature of the selection pressures exerted by DC foragers on prey populations.

2.2: Introduction

Animals are often faced with a choice between eating familiar food, or expanding their diet by consuming a novel item. It has long been recognised that animals hesitate to approach novel foods that they encounter (Barnett 1958, Brigham and Sibley 1999) and this initial fear of novelty (literally 'neophobia') lasts only a matter of minutes in most animals. In contrast, a second response to novel food has been identified in which the foragers overcome their neophobia to approach and touch the food, but nonetheless continue to avoid eating it for considerable periods of time (Marples and Kelly 1999). This much longer avoidance of novel food has been termed dietary conservatism (Marples et al. 1998) and, unlike neophobia, it does not rapidly subside over repeated encounters. For example, Kelly (2001) found that among wild birds, some individuals avoided novel food for more than 2 years and 200 exposures, even though the novel food was conspicuous and fully palatable. This surprising phenomenon of dietary conservatism has been described in a range of bird species, and recently, the first occurrence of this behaviour was recorded in a fish, the three-spined stickleback, *Gasterosteus aculeatus* (Thomas et al. 2010).

One remarkable observation about the phenomenon of dietary conservatism (DC) is that each of the forager populations examined so far have included some individuals which display DC and some individuals which do not. The individuals which display a DC foraging strategy exhibit the protracted avoidance of eating novel food described above. The other individuals have a much more adventurous foraging strategy and begin to eat the novel food either as soon as they encounter it, or after their neophobia has waned enough for them to approach it and touch it. Thomas et al. (2010) defined individuals following this second strategy as being 'adventurous consumers' (AC). It is possible that the balance of DC and AC foraging strategies in a population of predators is influenced by the predator's ecology. For example, DC may be favoured in habitats where prey are relatively cryptic, whereas AC may be favoured in environments with a high diversity but low abundance of any particular prey type.

Dietary conservatism could have facilitated the evolution of warning colouration, because if predators avoid novel prey for sufficient time, novel colour morphs could survive and spread in a population of familiar prey (Coppinger 1969, 1970, Gotmark 1994, 1996, Thomas et al. 2003, 2004). This idea of DC facilitating the survival and selective advantage of novel colour morphs has been tested using birds and fish as predators foraging on populations of artificial prey, into which novel colour morphs were introduced at a low initial frequency. The frequencies of the novel and familiar morphs were then allowed to change in response to the selection pressure imposed by the foragers, in an empirical simulation of the evolutionary process. These studies have demonstrated that DC can allow fully palatable novel colour morphs to survive and spread to fixation (i.e. the novel morph increases in frequency to reach 100% of the prey population) in small populations of otherwise familiar coloured prey (Thomas et al. 2003, 2004, 2010). The method used in these studies provides a very robust test of whether an individual forager shows DC, as only highly conservative foragers would exert sufficient selection pressure to cause an initially rare novel mutant to spread to fixation. Such a conservative forager would have to continue to avoid the novel colour morph even when it has become much more abundant than the originally common familiar morph. In the present study, we used the same method as Thomas et al. (2003, 2004, 2010) to address a series of related questions regarding the prevalence and ecological relevance of AC and DC foraging strategies in a range of fish species. We investigated the ratio of AC to DC foraging strategies in populations of four different species of tropical poeciliid, and compared the foraging strategies of these species with one temperate species, the three-spined stickleback, studied by Thomas et al. (2010).

We tested for differences in the frequency of strongly DC individuals between species, sexes, and fish of different sizes. In addition, it was important to consider the context in which fish encounter prey in the wild, where the colour of both the prey and the surrounding habitat could combine to make prey appear conspicuous or cryptic. Previous studies have shown an effect of relative prey crypsis on fish foraging ability (e.g. Gendron and Staddon 1983, Johnsson and Kjallman-Eriksson 2008). Therefore, we also tested whether the prevalence of DC was affected by the colour or the conspicuousness of the novel food. Finally, we investigated what happens if, after a novel morph has spread to fixation in a prey population, the originally familiar morph re-appears in the prey population, as might occur by immigration or mutation. We predicted that strongly DC fish would continue their avoidance of the originally novel morph and continue to place the originally familiar morph at a selective disadvantage, preventing its re-invasion of the prey population.

2.3: Materials and Methods

2.3.1: Experimental animals and prey origins

Four poeciliid species, namely Trinidadian guppy (*Poecilia reticulata*, n=121 individuals), mollie (*P. sphenops*, n=112), platy (*Xiphophorus maculates*, n=116) and green swordtail (*X. hellerii*, n=126), were purchased from a commercial supplier (J and K Aquatics, Taunton, U.K.). On arrival at Cardiff University, all fish were briefly anaesthetised in 0.02% MS222 (tricaine methane sulphonate) and externally screened for visible parasites under a stereo-microscope with fibre-optic illumination. Infected individuals were treated with 0.2% Levamisole and re-checked for parasites prior to use (see Schelkle et al. 2009). During this period, fish were habituated to laboratory conditions in glass aquaria with a 12 h light: 12 h dark lighting regime, and fed on a diet of food-flakes (Aquarian®), and frozen bloodworm (Chironomid larvae). Two days before the feeding trials, the fish were individually transferred to one-litre containers containing dechlorinated water at 25±0.5°C. The data for three-spined sticklebacks analysed in the current study were previously obtained by Thomas et al., using methods identical to those used here for the four poeciliid species, apart from the water temperatures in which the fish were kept (see Thomas et al. 2010 for full details).

For the feeding trials in the experiments described below, prey consisted of 1-2 mm diameter freeze-dried *Daphnia*, dyed in a 10 ml mixture of 'Supercook' food colouring (Dr. Oetker Ltd., Leeds, U.K.). Green *Daphnia* were dyed with 6ml green dye and 4ml water, and the brown *Daphnia* coloured with 0.5 ml red dye, 5.0 ml green dye and 4.5 ml water. The *Daphnia* were immersed in food dye solution for 10 min, removed with a spatula and rinsed twice in dechlorinated water before being used immediately in the feeding trials. The dried *Daphnia* retained the colour of the dye for over 2 h, which was sufficient time for each feeding trial to take place. The reflectance spectra of the food dyes used to colour the *Daphnia* were very different from each other (Thomas et al. 2003), allowing the fish to use colour to discriminate between the two prey types.

2.3.2: Experiment 1: Testing for pre-existing colour preferences

Fish may have pre-existing preferences for prey of, for example, a certain colour, shape or odour, based on previous foraging experiences or innate preference. To test for any such pre-existing colour preference for green or brown prey, fish with no prior exposure to artificially coloured prey were offered 10 green and 10 brown coloured *Daphnia*, on each background colour (white, green and brown). Thirty fish of each poeciliid species were tested with each background colour (i.e. n=90 individuals for each species), and the number of prey of each colour consumed was recorded. The fish used in the colour preference tests were not used in any further prey choice experiments, as they were no longer unfamiliar with the prey colours used.

2.3.3: Experiment 2: Testing for dietary conservatism where novel and familiar prey are equally conspicuous

The aim of this experiment was to investigate whether rare novel prey phenotypes can survive and increase to fixation in the face of predation by fish, when the novel prey are no more conspicuous than the familiar prey. The experiment therefore tests for the effects of novelty on the foraging decisions of predators, while excluding any differences in conspicuousness between the familiar and novel morphs. Fish were kept in one-litre containers that were covered on all sides with laminated white paper. This provided a homogeneous background against which all prey were equally conspicuous, and screened each fish from its neighbour (in order to avoid an individual fish being influenced by the foraging decisions of its neighbours). Sample sizes for each species were as follows: guppy n=36 individuals, molly n=24, platy n=20, swordtail n=36). Half of the individuals of each species were familiarised with foraging on either green or brown prey. Previous studies have shown that as birds and fish become familiar with a given colour of food, they acquire a preference for that food (Marples et al. 1998, Thomas et al. 2003, 2010).

In the present study, we made the assumption that fish fed a certain colour of *Daphnia* over a seven-day period would subsequently treat that colour of *Daphnia* as 'familiar' relative to *Daphnia* of colours they had not been fed. This familiarisation process involved providing the fish with 20 prey of the appropriate colour each day for 7 days, with half the fish receiving green prey and the other half brown prey. Non-responsive fish (i.e. individuals that failed to feed during the familiarisation period) and/or fish in poor health were excluded from the trials (n=10 individuals). We acknowledge that non-responsive fish could be failing to eat this food due to extreme DC, and by excluding these individuals we could actually be removing the most dietarily conservative proportion of the population. However, it was necessary to exclude such individuals in order to test the hypotheses of the current study, since we needed fish that made an active choice as to which prey to ingest, rather than making no choice at all.

On the 8th day of the experiment, each fish was offered 19 of its familiar prey and one of the novel colour morph. After 90 min of foraging, all remaining prey were removed and the numbers of each colour were counted. The next day, the fish was offered the two colours in proportion to the numbers of each colour left uneaten the day before, scaled up to make a total of 20 prey again. The formula employed for doing this was as follows:

$$\frac{\text{Number of novel prey remaining}}{\text{Total number of prey remaining}} \times 20 \quad = \quad \begin{array}{l}\text{Number of novel prey to be given the next day} \\ \text{(rounded to the nearest whole number)}\end{array}$$

Thus, a period of predation was followed by simulated 'breeding' in proportion to the survivors' colour morphs, and then another bout of predation the following day. This process continued day-by-day until one colour or the other reached fixation in the population, and the other had been driven to extinction.

2.3.4: Experiment 3: Testing for dietary conservatism where novel prey are more conspicuous than familiar prey

This experiment investigated whether rare novel mutants can increase to fixation in the face of predation, even when they are much more conspicuous than familiar prey (which is the expected situation for the initial appearance of a new warning colour signal). Methods were identical to those described for Experiment 2, except that the test pots that housed the fish (guppy n=30, molly n=31, platy n=34, swordtail n=34) were covered with laminated paper coloured (using the same food colouring solutions) to match the familiar prey for that individual fish, e.g. green containers for fish familiarised with green prey, and brown containers for those fish familiarised with brown prey. This caused the novel prey to be more conspicuous against the background than the familiar prey.

2.3.5: Experiment 4: Testing for dietary conservatism where novel prey are cryptic compared to familiar prey

The aim of this experiment was to investigate whether rare novel prey phenotypes can survive and increase to fixation in the face of predation by fish, when the novel prey are much less conspicuous than the familiar prey. Methods were identical to those described for Experiment 2, except that the test pots in which the fish (guppy n=21, molly n=25, platy n=29, swordtail n=25) were kept were covered with laminated paper coloured to match the novel prey for that fish, i.e. green containers for fish receiving green novel prey (familiarised on brown) and brown containers for those fish receiving brown novel prey (familiarised on green). In this case, the familiar prey would be more conspicuous against the background than the novel prey.

2.3.6: Experiment 5: Re-invasion of prey populations by previously familiar prey morphs

The subset of individual fish from Experiments 2-4 that drove the novel morph to fixation (n=10 guppies, n=4 mollies, n=12 platys, n=7 swordtails) were tested for their response to an immediate re-appearance in the prey population of the originally familiar colour morph. Re-invasion by the originally familiar morph was of interest since it seems reasonable to assume this would occur in a natural habitat in which dispersal from neighbouring prey populations was likely. These fish were tested in the same containers (i.e. with the same background colours) as in their original experiment, and given 20 of the originally novel morph on the day following fixation (referred to as day 2R, where day 1R represents the day of fixation). Then, on day 3R, they were offered one individual of the originally familiar colour, plus 19 of the originally novel prey, and another sequence of predation periods followed by 'breeding' of the prey was initiated, following the protocol described under Experiment 2. This continued until one colour or the other reached fixation in the population, and the other had been driven to extinction.

2.3.7: Statistical Analyses

A chi-square test was used to test for differences in the frequency of fixation events between the three-spined sticklebacks used previously by Thomas et al. (2010) and the poeciliid species in the current study. Following this, data for poeciliid species only was analysed in detail. Continuous data were natural log transformed where appropriate, to achieve normality. For all of the following multivariate analyses, a backwards stepwise approach was used to reach a final model. All analyses were performed in Minitab 15. We tested for pre-existing preferences for brown or green coloured prey, using a General Linear Model (GLM), with fish species ('Species') as a factor and fish standard length ('Size') as a covariate nested within species. The response variable 'Preference' was the difference between the number of brown and green prey eaten, expressed as a proportion of the total number of prey eaten. This was calculated as follows:

$$\text{Preference} = \frac{\text{Number of brown prey eaten} - \text{Number of green prey eaten}}{\text{Number of brown prey eaten} + \text{Number of green prey eaten}}$$

The effect of relative prey crypsis on any pre-existing prey preference was tested using a GLM, with background colour ('Background Colour') as a factor with fish standard length ('Size') nested within background, using the response variable 'Preference'.

The impact of species, sex, size, novel colour and relative novel crypsis on the likelihood of fixations of novel prey was assessed using a binary logistic regression, with fixation occurrence (0 = extinction, 1 = fixation) as a binary response variable, and with species ('Species'), sex ('Sex'), novel prey colour ('Novel') and novel prey crypsis ('Novel crypsis') as factors. Differences in the length of time taken by fish species to drive the novel morph to fixation were investigated using a GLM, with fish species ('Species'), sex ('Sex'), novel prey colour ('Novel') and relative novel crypsis, i.e. whether the novel is cryptic or conspicuous against the background colour ('Novel crypsis') as factors, with fish standard length ('Size') nested within species.

2.4: Results

2.4.1: Dietary conservatism in poeciliids and sticklebacks

Three-spined sticklebacks (data from Thomas et al. 2010) were compared with the four poeciliid species (pooled) in the current study for their overall level of DC, by comparing the total proportion of fixations occurring in both groups of fish. Thomas et al. (2010) showed that 33% of three-spined sticklebacks drove the novel morph to fixation, whereas the equivalent figure for the four poeciliid species in the current study was 11%. A chi-squared test revealed a significant difference between fixation rate observed among three-spined sticklebacks (from Thomas et al. 2010) and the four poeciliid species used in the current study ($\chi^2=32.89$, d.f.=1, $P<0.001$). Thus, though strong DC regularly occurs in each of these five fish species, it does so to different extents in sticklebacks and poeciliids. Since Thomas et al. (2010) previously investigated the extent of DC in three-spined sticklebacks, the remainder of the current study focuses on the foraging strategies of the four poeciliid species.

2.4.2: Experiment 1: Testing for pre-existing colour preferences

Within the poeciliids, the proportion of both brown and green prey types eaten when both prey types were equally conspicuous (white background) varied significantly between fish of different species (Nested ANOVA: $F_{20,133}=3.75$, $P<0.001$) and body size (nested within species: $F_{23,133}=3.96$, $P<0.001$) (Table 2.1A). The largest proportional difference between the number of both prey types eaten was seen in platys, with this fish species eating significantly more green coloured prey than brown (mean ±SE: brown 1.6±0.2, green 2.7±0.3). Furthermore, within fish species, there was a significant effect of fish size on the number of both prey types that were eaten, with the largest fish eating the most prey. Platys were the largest fish species with a mean length of 30.4±1.1 mm, followed by swordtails and mollies respectively (mean lengths 29.2±0.4 mm and 25±1.4 mm respectively), with guppies being the smallest species (mean length 15.4±0.2 mm).

Background colour significantly affected the pre-existing colour preference of poeciliids (Nested ANOVA: $F_{47,371}=1.78$, $P=0.002$), with fish eating the most brown and green prey when prey were presented against a brown background (Mean ±SE brown eaten 5.3±0.3, green eaten 5.8±0.3). Fish ate a lower number of both prey types when prey was presented against green and white backgrounds respectively (green background: mean ±SE brown eaten 4.8±0.2, mean green eaten 5.1±0.2; white background: mean brown eaten 4.7±0.3, mean green eaten 4.9±0.2). There was also a significant effect of fish body size (nested within species: $F_{27,371}=1.63$, P=0.028) (Table 2.1B).

2.4.3: Experiments 2, 3 and 4: Testing for dietary conservatism

After 7 days of the initial training with one colour of food, the fish were sufficiently familiarised with both the prey and its colour that they ate *Daphnia* readily. When a novel colour was introduced, individual fish showed one of two types of response; either rapid incorporation of the novel morph into the diet on day 1, or avoidance of eating the novel prey that lasted for a mean of 8 days (range 2-21 days) across all species and experiments. Across the four poeciliid species tested, 90% of individuals exhibited the first strategy (adventurous consumption, AC) and readily ate the novel prey, sending it to extinction, usually within 2 days. However, 10% of the fish displayed the alternative strategy (dietary conservatism, DC) and persistently avoided the novel colour, to the extent that the avoidance allowed the novel colour morph to increase in number and eventually reach fixation in the prey population. Table 2.2 shows the number of fixation and extinction events for the four different fish species across Experiments 2-4 (see also Figure 2.1 A-D).

(A)

Source	DF	Adj MS	F	P
Size	23	0.1680	3.96	<0.001
Species	20	0.1590	3.75	<0.001
Error	90	0.0424	*	*
Total	133			

(B)

Source	DF	Adj MS	F	P
Size	27	0.0973	1.63	0.028
Background colour	47	0.1064	1.78	0.002
Error	297	0.0597	*	*
Total	371			

Table 2.1: General Linear Model for the pre-existing colour preference for either green or brown prey, shown by the four poeciliid species. The response variable 'Preference' was the difference between the number of brown and green prey eaten, expressed as a proportion of the total number of prey eaten. The starting model contained the following independent variables: (A) fish species ('Species') as a factor and fish standard length ('Size') as a covariate nested within species, and (B) background colour ('Background colour') as a factor and ('Size') as a covariate nested within background colour. Only significant terms are reported.

Experiment number	Fish species				
	Fate of novel prey morph	Guppy	Mollie	Platy	Swordtail
2	Fixation	5	2	2	4
	Extinction	31	22	18	32
3	Fixation	2	1	3	2
	Extinction	28	30	31	32
4	Fixation	2	1	7	1
	Extinction	18	24	22	24
Total % fixation in experiments 2, 3 and 4		11.7%	5.3%	16.9%	8%

Table 2.2: Results for Experiments 2, 3 and 4 showing the number of individual fish that drove the novel coloured prey to fixation (100% remaining in prey population) or to extinction (0% in prey population), as well as the overall percentage of fixation events for each species that occurred across Experiments 2-4. Results are shown separately for the four poeciliid species, but pooled for sex and novel colour.

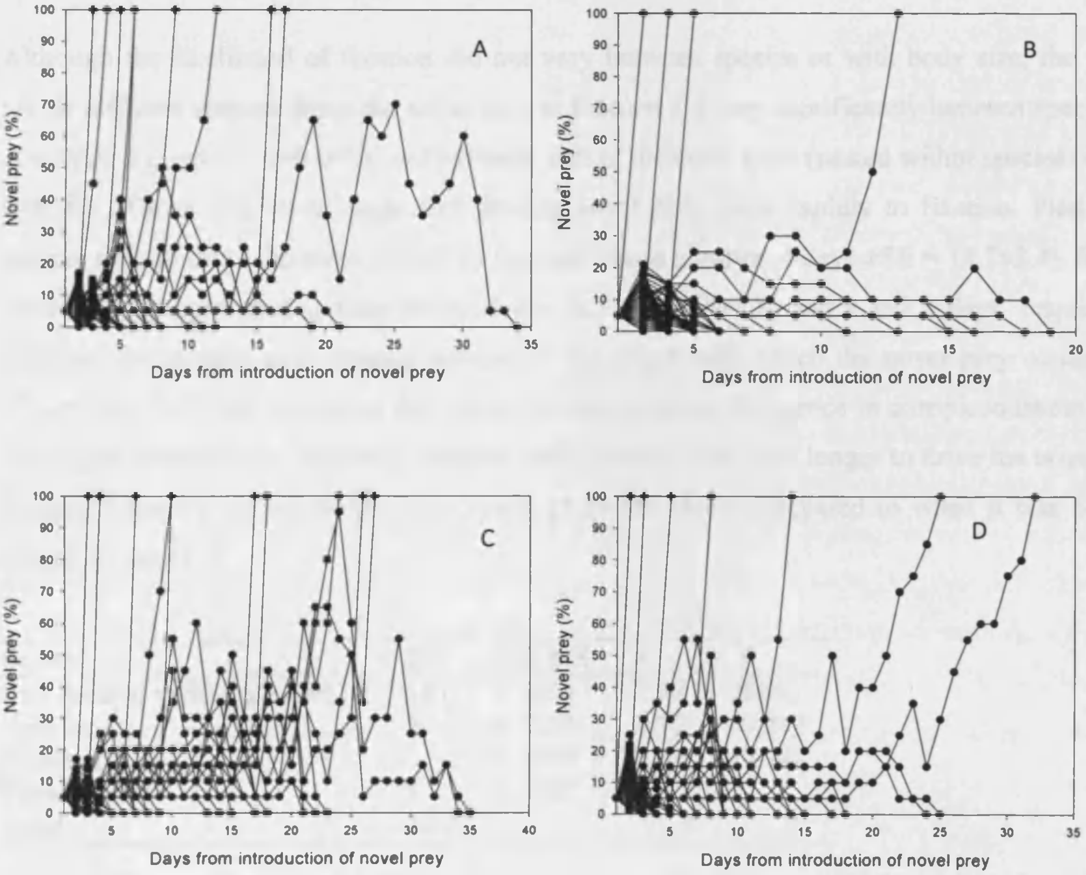

Figure 2.1: Frequencies of both green and brown (combined) novel morphs on successive days of Experiments 2-4 under predation by pre-trained individual poeciliids, showing that in some prey populations the proportion of the novel prey gradually increased to fixation, while in other populations the novel morph were driven to extinction. Results are presented for prey populations, under predation by (A) guppies, (B) mollies, (C) platys, (D) swordtails.

As expected, the probability of a fixation event occurring was not significantly associated with the sex (Logistic Regression: Z=-0.70, d.f.=5, P=0.481), or the size of the fish (Z=-0.57, d.f.=5, P=0.568). However, contrary to our expectation, neither was it associated with fish species (Z=0.30, d.f.=5, P=0.767), the colour of the novel prey (Z=-0.85, d.f.=5, P=0.394), or how cryptic the novel prey was against the background (Z=1.81, d.f.=5, P=0.070) (Table 2.3).

Predictor	Coef	SE Coef	Z	P	Odds Ratio	95% CI
Constant	-2.1104	1.2157	-1.74	0.083	*	*
Species	0.0578	0.1948	0.30	0.767	1.06	0.72 – 1.55
Sex	-0.2105	0.2986	-0.70	0.481	0.81	0.45 – 1.45
Novel	-0.3271	0.3841	-0.85	0.394	0.72	0.34 – 1.53
Novel crypsis	0.7281	0.4024	1.81	0.070	2.07	0.94 – 4.56
Size	-0.0191	0.0335	-0.57	0.568	0.98	0.92 – 1.05

Table 2.3: Binary logistic regression for the likelihood of a fixation or extinction event (0 = extinction, 1 = fixation) with species of fish ('Species'), sex ('Sex'), novel colour ('Novel') and relative novel crypsis ('Novel crypsis') as factors, and standard length ('Size') as covariate.

Although the likelihood of fixation did not vary between species or with body size, the speed with which different species drove the novel prey to fixation did vary significantly between species (Nested ANOVA: $F_{3,30}$=4.17, P=0.047), and between fish of different sizes (nested within species: $F_{18,30}$=7.92, P=0.003, Table 2.4), with larger fish driving novel prey more rapidly to fixation. Platys took the longest time to drive the novel morph to fixation (mean number of days ±SE = 14.7±3.4), followed by swordtails, guppies and mollies respectively (8.5±3.6, 7.6±1.8, and 4.3±2.3 days, respectively). In addition, the relative prey crypsis influenced the speed with which the novel prey reached fixation ($F_{1,30}$=5.44, P=0.048) indicating that there was indeed some difference in conspicuousness among the prey types presented on different coloured backgrounds. Fish took longer to drive the novel morph to fixation when the novel morph was cryptic (5.2±0.85 days) compared to when it was conspicuous (4.4±0.33 days).

Source	DF	Adj MS	F	P
Size (nested within species)	18	1.5864	7.92	0.003
Species	3	0.8353	4.17	0.047
Novel crypsis	1	1.0899	5.44	0.048
Error	8	0.2002		
Total	30			

Table 2.4: General Linear Model for the number of days taken by the four poeciliid species to drive the novel prey morph to fixation (100% remaining in prey population), with species of fish ('Species'), sex ('Sex'), novel colour ('Novel') and relative novel crypsis ('Novel crypsis') as factors, and standard length ('Size') nested within species. Only significant terms remaining in the final model are reported.

2.4.4: Experiment 5: Re-invasion of prey populations by previously familiar prey morphs

The novel morph was driven to fixation in 33 instances within Experiments 2, 3 and 4, and so these fish were tested for their response to the re-appearance of the originally familiar morph. In total, 32 out of 33 fish drove the originally familiar morph to extinction on day 3R (i.e. on the first day of reintroduction of the novel morph). However, one individual fish (of the species *P. sphenops*) drove the originally novel morph to extinction on day 3R, resulting in the re-invasion to fixation of the previously familiar prey morph. The likelihood of the originally familiar morph being driven to fixation was significantly lower than that of the originally novel morph (Fisher's Exact Test: $P<0.001$). This indicates that re-invasions can occur but they may be very rare. It also demonstrates that one day of being presented with nothing but the novel morph (day 2R) is not sufficient for the fish to accept that morph as a new familiar prey.

2.5: Discussion

It is clear from the current study that each of the four poeciliid species examined include individuals that exhibit strongly DC foraging strategies. Novel prey was consistently avoided by a small proportion of each poeciliid population, despite all prey being fully edible. The first evidence of DC in a fish species, the three-spined stickleback, was presented by Thomas et al. (2010). The present study provides the first evidence of DC in poeciliid species and confirms that, as in all other populations so far examined, DC and AC foraging strategies are both present in different individuals in the same population.

The colour preference tests (Experiment 1) revealed that platys have a pre-existing preference for green coloured prey compared to brown, since they ate significantly more green than brown prey when given both prey types simultaneously. The remaining three fish species tested did not show a colour preference. There is no obvious explanation for this difference in colour preference, since all fish in the current study were commercially bred strains, obtained from the same supplier and fed on the same diet while housed with the supplier, and when in the laboratory. However, we have no information as to the diet of these fish before they arrived with the commercial supplier. It may be that the platys previously received predominantly green coloured food which may have led to a pre-existing preference for that colour of food. Also, prey crypsis had an effect on prey choice, with fish choosing to eat both more brown and green prey when presented against a brown background, compared to either green or white backgrounds. It is assumed the brown prey would have appeared cryptic against a similar coloured background (since it is unknown exactly how fish would have viewed the prey in the different contexts provided by the differing background colours), and it could be suggested that fish may be less able to locate cryptic prey due to their cryptic nature. However, previous work has shown that fish can not only successfully locate cryptic prey (Johnsson and Kjallman-Eriksson 2008) but also eat more cryptic prey than conspicuous prey (Appendix II; Adraenssens et al. in prep).

The DC foraging strategies exhibited by some fish in Experiments 2, 3 and 4 was sufficiently strong that in some cases the novel prey reached fixation (100% novel prey remaining) in the simulated prey populations. This occurred despite the novel morphs being fully palatable, equally conspicuous (Experiment 2), more conspicuous (Experiment 3) or less conspicuous (Experiment 4) than the familiar prey. Fixations also occurred despite the novel morph being the more abundant prey available as it approached fixation. Murdoch (1969) and Murdoch et al. (1975) showed that predatory fish fed on equally familiar prey switched to feeding on the most abundant prey available. In our experiment, the effect of greater novelty of one prey compared to another overrode any such effect of relative abundance of the two prey morphs. Theoretic simulations by Thomas et al. (2010) have previous shown that such fixations cannot be explained by frequency-dependent selection. Therefore, we suggest it is highly likely that fish in the current study also avoid novel prey morphs based on their novelty rather than their rarity. Thus, DC seems to be a more powerful mechanism operating in the foraging decisions of these fish, than any underlying colour preferences or frequency-dependent foraging effects.

There is some suggestion of differences in the extent to which the four poeciliid species exhibit DC foraging tendencies (though not statistically significant in the current dataset), with guppies showing the highest proportion of fixation events at 11.7% (foraging against white backgrounds), and mollies the least at 5.3% (Table 2.2). Interestingly, the prevalence of DC was substantially (and significantly) lower among poeciliids (11%) than was previously found in three-spined sticklebacks (33%, Thomas et al. 2010). Differences between taxa in the relative proportions of DC and AC individuals may reflect gene frequency differences in different populations, (Marples and Brakefield 1995) due to differing selection pressures in different ecological situations. Owen (1977) suggested that birds in the tropics may be limited in their clutch size by the low abundance of any one prey type, and by the high diversity of toxic prey types. Similarly, the AC foraging strategy may be favoured over the DC strategy in habitats with high diversity but low abundance of palatable food types, and/or high abundance of toxic prey types. The lower frequency of the DC foraging strategy among poeciliids compared to sticklebacks may therefore have an ecological explanation. Alternatively, previous foraging experience may explain the differences, since it has been shown that a hatchery-rearing environment can have a strong effect on the subsequent ability of fish to forage successfully on prey of different types after release into the wild (Brown and Laland 2001, Sundstrom and Johnsson 2001). Similarly, Marples et al. (2007) showed that in domestic chicks, the expression of dietary conservatism can be modified by prior experience of novel food.

There was a significant difference between fish species in the length of time taken to drive the novel morph to fixation in the simulated prey populations. This could be due to inherent differences between the species in foraging behaviour or general foraging ecology. Mollies drove the novel morph to fixation or extinction in the shortest time compared to the other three fish species tested. Mollies have been observed to feed on a number of different food types in our laboratory (pers. obs.), and seemed to feed in a fairly unselective manner during Experiments 2-4 of the current study, showing relatively less aversion to the novel coloured prey than the other three fish species. Conversely, platys showed the most aversion to both approaching and ingesting novel prey (pers. obs.), and so it is not surprising that these fish took the longest time, of the species tested in the current study, to drive the novel morph to fixation or extinction. The diet of mollies in the wild is primarily herbivorous with a high intake of detritus (Alkahem et al. 2007, Trujillo-Jimenez and Beto 2007), suggesting this fish species may not be particularly conservative in its foraging decisions. Conversely, platys showed the most aversion to both approaching and ingesting novel prey (pers. obs.). Arthington (1989) showed that platys had the most restricted diet of four poeciliid species tested in a stream habitat in Australia. Therefore, it is not surprising that platys took the longest time, of the species tested in the current study, to drive the novel morph to fixation or extinction.

When considering the fate of the previously familiar morph upon its re-appearance into the simulated prey population following fixation of the novel morph, it was interesting to note that its survival probability was significantly less than that of the novel morph. This shows that within the proportion of the predator population that exhibits a DC foraging strategy, DC has a very strong and long-lasting impact, since it prevents an animal from foraging on a novel prey morph, even when that novel morph is the only prey type available to the predator. The enduring avoidance of novel prey has been seen previously in blackbirds (*Turdus merula*) that avoided novel prey for over two years (Kelly 2001). The consistent avoidance of novel prey by predators may impact indirectly on the predator's own survival, through a direct effect on prey population dynamics. A novel morph may reach such numbers in the prey population that it outcompetes the originally more abundant morph for resources, driving the latter morph to very low numbers in the prey population. This would subsequently have an effect on the DC proportion of the predator population, as there would be a greatly reduced amount of familiar prey available to these animals.

In the current study, novel morphs reached fixation a number of times despite being initially very rare in the artificial prey populations. One hypothesis to explain the survival of an initially rare prey morph is that predators avoid the rare form due to its rarity, rather than its novelty (Allen and Clarke 1968, Clarke 1969, Maskell et al. 1977). However, theoretic simulations carried out by Thomas et al. (2010) failed to support this explanation, and showed that three-spined sticklebacks foraging on artificial populations of dried *Daphnia* prey retained their bias against the novel prey throughout the experiment, even when it became common (as it approached fixation). Therefore, we suggest it is highly likely that fish in the current study also avoid novel prey morphs based on their novelty rather than their rarity.

Dietary conservatism is thought to occur in response to predominantly visual cues of novelty (Marples et al. 1998, Thomas et al. 2003, Thomas et al. 2004, Marples et al. 2007), but it may be possible that fish exhibiting a DC foraging strategy also use chemical cues to detect and avoid novel prey. It is doubtful that olfactory cues from prey in the current study were very different since both prey morphs were the same species and came from the same stock of dried food. It also unlikely that the food dyes used to colour the prey differed greatly in their chemical composition, being from the same manufacturer and containing the same base components. However, if we consider a wild fish foraging in a natural habitat encountering a variety of potentially novel prey, the question of olfactory prey detection becomes more pertinent. It is known that the detection of chemical cues underpins many aspects of fish behaviour, including prey location (Webster et al. 2007, Quesenberry et al. 2007), social organisation (Ward et al. 2004, 2005, 2007), mate choice (Milinski et al. 2005, Rafferty and Boughman 2006), kin recognition (Frommen and Bakker 2006) and predator detection (Magurran et al. 1996, Henderson et al. 1997). Novel prey morphs may exude different substances compared to more familiar prey, since novel morphs may be genetically dissimilar or use a chemical defence mechanism (aposematism). It has been shown that aposematic many insects give off a distinctive odour when attacked (Rothschild et al. 1984, Rothschild and Moore 1987, Moore et al. 1990). Furthermore, domestic chicks, *Gallus gallus domesticus*, (Marples and Roper 1996) and zebra finches, *Taeniopygia guttata*, (Kelly and Marples 2004) use odour cues in conjunction with visual cues to avoid consuming novel food. Differences in olfactory cues between prey may be used by predatory fish as part of their AC or DC foraging strategies.

We can also postulate that the mixture of AC and DC foraging strategies seen in the studies on DC to date (see Thomas et al. 2010) may be due to natural heterogeneity of response to prey found in populations of foragers. From previous studies on DC (see Thomas et al. 2010), it seems that a small proportion of a foraging population are conservative in their prey choices, while others are naturally much less wary and consume a greater variety of food. This mixture of behavioural phenotypes is also seen in the context of animal personality, where individuals within a population can be separated along a behavioural continuum in terms of boldness, aggression, activity levels or how proactive or reactive an animal is (see Sih et al. 2004).

The current study has implications for the rearing of fish within aquaculture as well as the foraging of wild fish in natural habitats. Fish raised in hatcheries for river stocking purposes are usually fed on a limited pellet diet and this may restrict their subsequent ability to feed on prey of different types (Brown and Laland 2001, Sundstrom and Johnsson 2001). Hatchery-reared fish suffer a substantial loss of mass following release and their mortality rates can be up to ten times greater than those of transplanted wild fish (Miller 1954). The highest mortality levels occur within the first few days after release (Howell 1994) and this is thought to be partly due to their lack of foraging experience with natural prey types, leading to starvation (Paszkowski and Olla 1985, Olla et al. 1998). If DC is twinned with a lack of foraging experience, it is highly likely that these individuals will be at a distinct disadvantage upon release in to the wild. When considering fish in natural habitats feeding on typical prey, the DC proportion of the fish population may be at a disadvantage in finding new food patches that could decrease the competition for resources within a habitat, or when moving into new territories that may contain previously unseen prey types.

The current study provides yet more evidence for the existence of a DC foraging strategy in previously untested populations of predators, specifically showing differences both between and within species in the occurrence of DC, an individuals' dietary conservative nature. It will be interesting to investigate the impact of other factors, such as energetic constraints and social context on the occurrence of DC in predator populations.

2.6: References

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Chapter 3: Does social context and prey conspicuousness influence the degree of dietary wariness exhibited by three-spined sticklebacks (Gasterosteus aculeatus)?

3.1: Abstract

Recent studies have shown an active and long-term avoidance of novel food by predators, known as 'dietary conservatism' (DC). Conservative prey choice may exert strong selective pressure on prey populations because novel coloured individuals will be protected from predation by their novelty. Recent work has provided the first evidence of DC in a fish species, where novel coloured morphs of dried prey (*Daphnia*) were under predation by individual three-spined sticklebacks (*Gasterosteus aculeatus*). However, in this earlier study sticklebacks were housed individually, whereas in the wild they are a strongly shoaling species. Shoals may provide foraging benefits, but as group size increases so does competition for food, and as a result shoal members may take more risks in their foraging decisions. Therefore, fish living in shoals might be less conservative in their prey choice than solitary fish. The current study tests the hypothesis that sticklebacks in shoals are less conservative in their choice of palatable live prey than individual fish, such that shoaling fish are expected to eat significantly more novel prey. DC strong enough to drive a novel prey morph from initial rarity to fixation in a prey population was detected when both novel and familiar prey types were equally conspicuous. DC was evident among both single sticklebacks and shoals, but we found no evidence that isolated fish and shoals are significantly different in the degree of dietary conservatism that they exhibit.

3.2: Introduction

'Dietary conservatism' (Marples et al. 1998) is the prolonged avoidance of novel food, and is distinguishable from 'neophobia' (Barnett 1958, Brigham and Sibley 1999) in that it is longer lasting and cannot be so easily reversed with repeated exposure to novel food (Marples and Kelly 1999, Marples et al. 2007). DC has previously been shown in a number of bird species (see Marples et al. 2005), but until recently it was unknown to what extent dietary conservatism existed in other taxa, or how it might vary between different foraging contexts. Recently, Thomas et al. (2010) provided the first evidence of DC in non-avian foragers, by showing that a novel colour morph arising in an artificial prey population can survive under the selective pressure imposed by a predatory fish species, the three-spined stickleback (*Gasterosteus aculeatus*). However, the study by Thomas et al. (2010) was conducted on isolated individual fish, whereas in the wild, sticklebacks are a highly social species. It is therefore important to discover whether the manifestation of DC in these fish is influenced by the social context (shoal or solitary) in which they forage.

Fish shoals are thought to arise as a result of trade-offs made by individuals between the costs and benefits of group membership (e.g. Pitcher and Parrish 1993), with individuals in shoals benefiting from reduced predation risk through improved predator detection, attack dilution, predator confusion and enhanced predator evasion (e.g. Magurran et al. 1985). Shoaling also provides foraging benefits, such as improved food location (e.g. Pitcher et al. 1982). Furthermore, individuals in shoals may be able to allocate more time to feeding (Magurran and Pitcher 1983) perhaps as a result of vigilance sharing between group members. However, as group size increases so too does competition for food within the group. A number of studies have documented increases in risk-taking behaviour with increasing shoal size (e.g. Magurran and Pitcher 1983). Therefore, shoals of fish may be predicted to be less dietarily conservative than solitary fish, because members of a shoal can spend more time investigating novel food types due to the decreased risk of predation, as well as being driven to exploit new resources due to the increased competition for food resources.

In the present study, we extend and develop the work of Thomas et al. (2010) by comparing the degree of DC exhibited by solitary and group-living three-spined sticklebacks. We experimentally simulated the appearance of a novel-coloured morph in artificial prey populations of *Daphnia* of two different colours. The colour morph frequencies in each successive simulated 'generation' of prey, were determined by the relative survival of the different morphs in the previous generation, under predation by isolated individuals or by shoals of fish.

3.3: Materials and Methods

The main aim of this experiment was to investigate whether rare, novel, prey phenotypes can survive and increase to fixation in the face of predation by individual and shoaling sticklebacks, when the novel prey are no more conspicuous than the familiar prey. The experiment tests for the effects of novelty alone on the foraging decisions of predators in different group sizes.

3.3.1: Experimental animals and prey origins

Wild-caught three-spined sticklebacks (n=463 individuals, mean standard length, i.e. from tip of snout to end of body excluding the tail = 22.1 mm, range 11-35 mm) were collected between May and August 2006 from Roath Park, Cardiff, Wales, UK (51°30'23"N, 3°10'30"W). On arrival at Cardiff University, all fish were briefly anaesthetised in 0.02% MS222 (tricaine methane sulphonate) and externally screened for visible parasites under a dissecting-microscope with fibre optic illumination. Infected individuals were treated (by manually removing ectoparasitic infections; see Schelkle et al. 2009) or excluded from the study (for digenean and cestode infections) and re-checked for parasites 1 week prior to use. Fish were captured and habituated to laboratory conditions over a period of 4 weeks, in small aquaria (30 x 30 x 60 cm) containing dechlorinated water at 14±0.5°C with a 12 h light: 12 h dark lighting regime, and fed on a diet of fish flakes (Aquarian®) and frozen bloodworm.

Two days before the experiments began sticklebacks were transferred to 14 litre plastic aquaria containing dechlorinated water, with some being housed individually, and others being housed in groups of three fish. Each 14 litre aquarium in which the fish were housed was covered on all six sides with white laminated paper, to provide a homogeneous background against which all prey were equally conspicuous. The tank covers also screened each tank from the neighbouring tank(s), as well as from disturbances by movements in the room.

Live *Daphnia magna* were purchased from a commercial supplier (Fish&Fins, UK) and upon arrival transferred to 20 litre plastic tanks containing dechlorinated water at 25±0.5°C. These cultures were fed by daily addition of 1 ml of a food solution. This solution consisted of 25 g of *Spirulina* powder (Holland & Barrett, UK) and 14 g of dried baker's yeast in 500 ml of dechlorinated water. A sample of the *Daphnia* culture was sieved (2 mm mesh) at the start of each experimental day to remove the largest individuals, so as to avoid predator feeding being limited by gape-size. The remaining prey were coloured by immersion in 50 ml of a brown- or green-coloured solution of edible food dye (5.0 g/l of dark brown and dark green food dust, www.squires-shop.com) for 20 min. Coloured *Daphnia* were netted, rinsed and released into dechlorinated water before the trial. *Daphnia* kept their colour for over 1 h, which was the maximum duration of each feeding trial.

3.3.2: Experiment 1: Testing for pre-existing colour preferences

It was important to test fish for any pre-existing prey colour preferences, as inherent preferences or previous experience with prey of certain types could influence the foraging decisions of fish in the current study. To test for any pre-existing colour preference for brown or green prey, 58 solitary fish, with no prior exposure to artificially coloured prey items, were fed 10 brown and 10 green coloured *Daphnia,* on white backgrounds. Forty shoals of 3 individuals were similarly tested, but with 30 brown and 30 green *Daphnia.* In each case, the number and colour of prey remaining was recorded after 1 hour. The fish tested in this way were not used in the main experiment (Experiment 2).

3.3.3: Experiment 2: Testing for dietary conservatism

A total of 60 individual sticklebacks and 61 shoals of 3 individuals were tested for their responses to novel prey. For logistical reasons, this experiment was conducted in three batches. Each batch comprised 20 individual sticklebacks and 20 (or 21) 3-fish shoals.

Fish were first familiarised with foraging on prey of a particular colour (either brown or green). Previous studies have shown that as birds and fish become familiar with a given colour of prey they acquire a preference for that food (Marples et al. 1998, Thomas et al. 2003, 2010). In the present study, we therefore expected that fish fed a particular colour of *Daphnia* over a seven-day period would subsequently treat that colour of *Daphnia* as 'familiar' relative to *Daphnia* of colours they had not been fed. For single fish, this familiarisation process involved providing the fish with 20 prey of the appropriate colour each day for 7 days. In order to test for the presence of DC, it was essential only to use fish that exhibited normal feeding behaviour during the training period. Any individual fish (or shoals) which did not feed for 5 or more days of the 7-day training period, and/or fish in poor health, were excluded from the experiments (n = 42 out of 285 individuals). We acknowledge that non-responsive fish could be failing to eat this food due to extreme DC, and by excluding these individuals we could actually be removing the most dietarily conservative proportion of the population. However, it was necessary to exclude such individuals in order to test the hypotheses of the current study, since we needed fish that made an active choice as to which prey to ingest, rather than making no choice at all. On the 8th day, each fish was offered 19 of its familiar prey and one of the novel colour morph. After 60 min of foraging, all remaining prey were removed and the numbers of each colour remaining were counted. The next day, the fish was offered the two colours in proportion to the numbers of each colour left uneaten the day before, scaled up to make a total of 20 prey again. For shoals of three fish, the familiarisation process involved providing the fishes with 60 prey (i.e. three times the number fed to individual fish) of the appropriate colour each day for 7 days. On the 8th day, the fish were offered 57 of the familiar prey and 3 of the novel colour morph. The remainder of the protocol is identical to that for single fish.

The formula employed for calculating the number of prey given each day was as follows:

$$\frac{\text{Number of novel prey remaining}}{\text{Total number of prey remaining}} \times N = \text{Number of novel prey to be given the next day}$$

where N is the total number of prey to be given, i.e. 20 for single fish and 60 for shoals. Fractions arising from the application of this formula were rounded up or down to the nearest whole prey item. Thus a period of predation was followed by simulated 'breeding' in proportion to the survivors' colour morphs, and then another bout of predation the following day. This process continued day-by-day until one colour or the other reached fixation in the population, and the other had been driven to extinction.

3.3.4: Prey reflectance spectra

Measurements of reflected light were measured with an Ocean Optics CHEM2000-UV-VIS fibre optic spectrophotometer, using an Avantes DH-2000 deuterium (215-400 nm) and halogen (360-1700 nm) light source calibrated against a WS-1 Spectralon diffuse reflectance standard, and viewed using SpectraWin. Due to the difficulty in measuring colour spectra of small live *Daphnia*, colour spectra for prey were measured from 6x6 cm pieces of very thin paper that mimicked the texture of *Daphnia* exoskeleton. Each piece of paper was coloured with the food dye and layered to produce the density of colour most similar to that of the coloured live prey (pers. obs.). The sample was illuminated and measured at an angle of 45° to the surface. Reflectance spectra of the prey colours differed substantially (Figure 3.1) and should have been readily distinguished by the visual sensitivity of the sticklebacks.

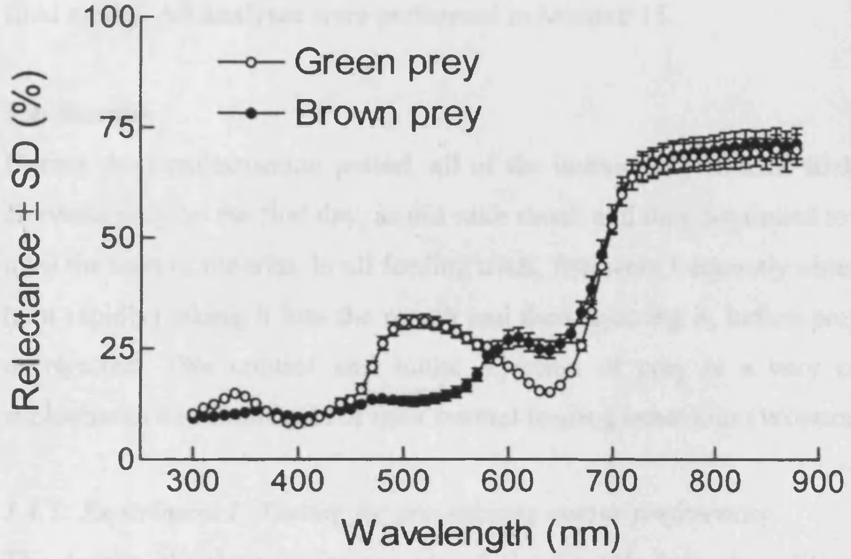

Figure 3.1: Reflectance spectra of the brown and green coloured prey. Measurements were taken from pieces of very thin paper that mimicked the texture of *Daphnia* exoskeleton, and layered to produce the density of colour most similar to that of the coloured live prey.

3.3.5: Statistical Analyses

Continuous variables were assessed for normality and natural log-transformed where necessary. For all analyses, the 'shoal' was treated as a single unit.

(i) Testing for pre-existing colour preferences

T-tests were used to investigate whether solitary fish or 3-fish shoals had a pre-existing preference for either prey colour, and whether the strength of any such preference differed between solitary fish and shoals. The response variable used in these tests was the difference between the number of brown and green prey eaten, expressed as a proportion of the total number of prey eaten (as calculated in Chapter 2).

(ii) Testing for dietary conservatism

Chi-square analysis was used to test for any difference in the number of novel prey extinction and fixation events between solitary fish and shoals. Variables influencing the likelihood of extinction and fixation events were analysed using a binary logistic regression with logit link function, where 0 = 'extinction' and 1 = 'fixation', using standard length of fish ('SL') as covariate, and social context ('Single/shoal'), novel prey colour ('Novel') and batch of experiment ('Batch') as factors in the starting model. The number of days taken for the novel morph to reach fixation or go extinct was investigated using a General Linear Model (GLM), with standard length ('SL') as covariate, and social context ('Single/shoal'), novel prey colour ('Novel') and batch of experiment ('Batch') as factors in the starting model. For all multivariate models, a backwards stepwise approach was used to reach a final model. All analyses were performed in Minitab 15.

3.4: Results

During the familiarisation period, all of the individually housed sticklebacks ate at least one of the *Daphnia* prey on the first day, as did each shoal, and they continued to eat the prey on most or all days until the start of the trial. In all feeding trials, fish were frequently observed to 'mouth' prey repeatedly (and rapidly) taking it into the mouth and then rejecting it, before prey were finally either swallowed or rejected. This contact and initial rejection of prey is a very common behaviour in foraging sticklebacks and forms part of their normal feeding behaviour (Wootton 1976).

3.4.1: Experiment 1: Testing for pre-existing colour preferences

The degree of colour preference varied significantly between solitary sticklebacks and 3-fish shoals (Two sample *t*-test: t=5.51, d.f.=70, P<0.001). Solitary sticklebacks had pre-existing preference for brown coloured prey, as they ate significantly more brown *Daphnia* (mean ±SE = 3.3±0.2) than green *Daphnia* (2.6±0.22; Matched-pairs t-test: t=3.31, d.f.=57, P=0.002). However, fish in shoals did not have an inherent preference for either prey colour, with fish in shoals consuming a mean (±SE) of 11.5±0.4 brown and 11.4±0.3 green *Daphnia* (Matched-pairs t-test: t=0.27, d.f.= 39, P=0.792).

3.4.2: Experiment 2: Testing for dietary conservatism

The novel morph was driven to fixation (i.e. 100% novel morph in prey population remaining at the end of a feeding trial) both by single fish (15/60 individuals; 25%) and by shoals (9/61 shoals; 15%) (Table 3.1; Figure 3.2 A-D). There was, however, no statistically significant difference in the likelihood of a fixation event between solitary fish and 3-fish shoals (χ^2=1.997, d.f.=1, P=0.158).

	Single fish		Shoals of 3 fish	
	Brown	Green	Brown	Green
Fixation	12	3	3	6
Extinction	18	27	27	25
% fixation of novel colour	40%	10%	10%	19%
Total % fixation	25%		15%	

Table 3.1: Summary of the number of fixation and extinction events of both brown and green novel prey for single and shoaling three-spined sticklebacks. Also shown are the percentage fixations for brown and green novel prey, and the total percentage fixations when brown and green fixations are combined.

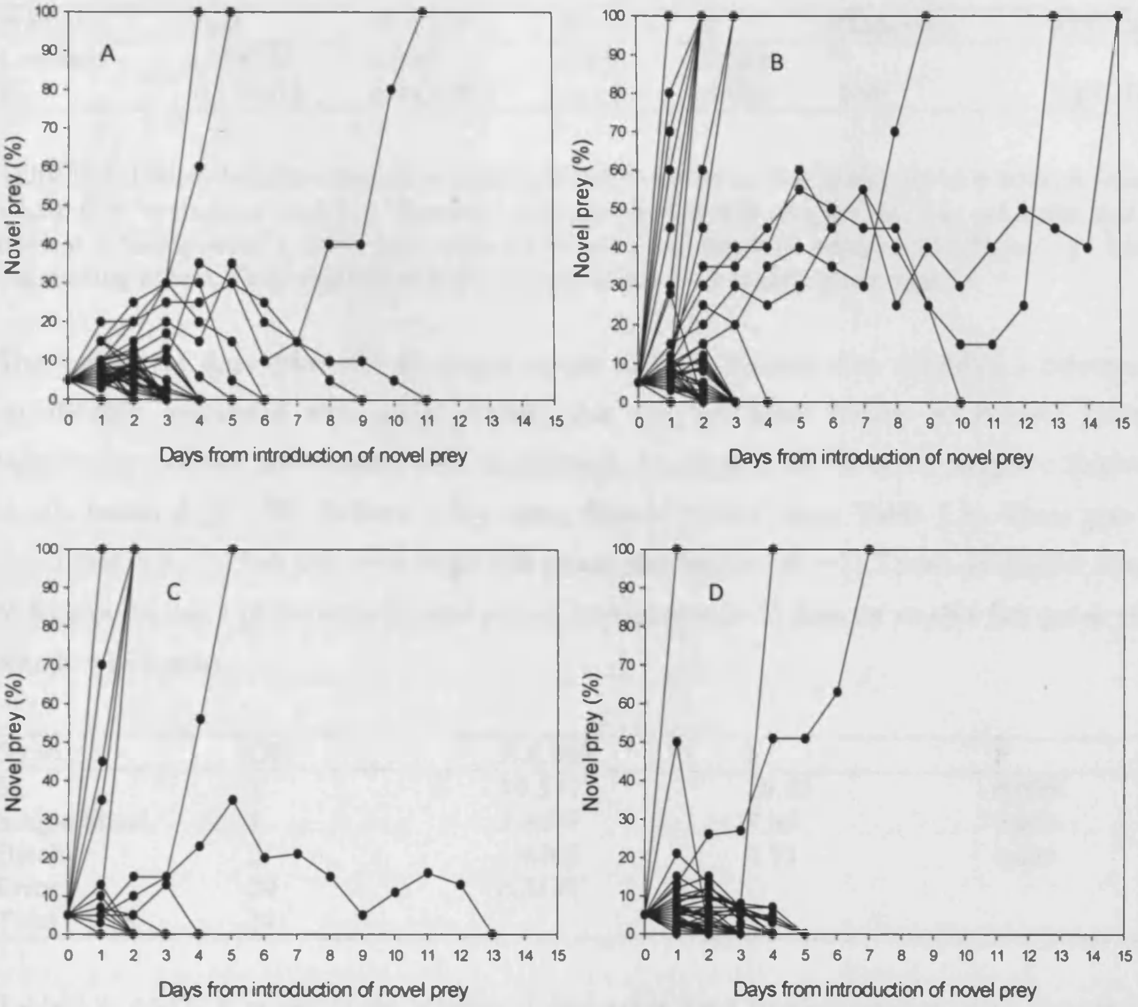

Figure 3.2: Frequencies of novel morphs on successive days of Experiment 2 under predation by pre-trained individual or shoals of three-spined sticklebacks, showing that in some prey populations the proportion of the novel prey gradually increased to fixation, while in other populations the novel morph were driven to extinction. Results are presented for prey populations, under predation by (A) and (B) individual fish, in which the novel prey were green or brown respectively, and under predation by (C) and (D) shoals of 3 fish, for the same novel colours respectively.

Using a binary logistic regression model to examine variables that may influence the likelihood of fixation of a novel morph, only fish size (‘SL’) had a significant effect on the probability of a fixation event occurring (Z=-2.41, P=0.016; Table 3.2). Specifically, smaller fish were more likely to drive the novel morph to fixation than larger fish.

Predictor	Coef	SE Coef	Z	P	Odds ratio	95% CI
Constant	0.568346	0.900713	0.63	0.528	*	*
SL	-0.104412	0.0433287	-2.41	0.016	0.90	0.83 - 0.98

Table 3.2: Binary logistic regression with logit link function on the likelihood of a fixation occurring, where 0 = 'extinction' and 1 = 'fixation', with standard length of fish ('SL') as covariate, and social context ('Single/shoal'), novel prey colour ('Novel') and batch of experiment ('Batch') as factors in the starting model. Only significant terms remain in the final model shown here.

The number of days taken for the novel morph to reach fixation (i.e. excluding extinctions) was significantly associated with social context, fish size and batch (GLM: all $P<0.05$, Table 3.3). Specifically, solitary sticklebacks took significantly longer to drive the novel morph to fixation than shoals (mean days ±SE: Solitary 3.3±1 days, Shoals 2.4±0.7 days; Table 3.3). There was also a significant effect of fish size, with larger fish (mean standard length = 21.7 mm) driving the novel prey to fixation on day 1 of the experimental period, compared to 2–13 days for smaller fish (mean standard length = 16.6 mm).

Source	DF	Adj MS	F	P
SL	1	10.5717	29.83	0.000
Single/Shoal	1	2.6077	7.36	0.013
Batch	2	1.6768	4.73	0.021
Error	20	0.3543		
Total	24			

Table 3.3: ANOVA model of the number of days taken for a fixation event to occur. For the starting model, standard length ('SL') of fish was treated as a covariate, with social context ('Single/shoal'), novel prey colour ('Novel') and batch of experiment ('Batch') as factors. Only significant terms remain in the final model shown here.

3.5: Discussion

Our study provides evidence of a dietarily conservative (DC) foraging strategy displayed by individual and shoals of foraging three-spined sticklebacks, since in both contexts there were repeated instances of fixation of the initially rare novel prey morphs. As in all previous studies of DC in vertebrates, only a small proportion of individuals exhibited a DC foraging strategy (25%); the remainder exhibited an adventurous consumer (AC) strategy, sending the novel morph to extinction. The current study extends the previous findings by Thomas et al. (2010) who found evidence of dietary conservatism in 33% of isolated three-spined sticklebacks by comparing the responses of individual foragers with the responses of small shoals.

The current study demonstrates that even shoals of sticklebacks can frequently (in 15% of cases) drive novel prey morphs to fixation. This is very surprising because one might expect that shoals containing one or more AC predators would drive the novel prey quickly to extinction, since the effect of one individual's level of wariness is likely to be diluted by being in a group of conspecifics of differing levels of wariness. In the current study, fish in shoals were not considered separately as individuals but as a single unit. The individual foraging decisions of shoal members were not measured; instead we considered the foraging outcome across all members of the shoal. Fish were randomly assigned to the single or shoal treatment groups, so shoals would be expected to contain a random selection of AC and DC individuals. As the number of single individuals displaying the DC foraging strategy in this experiment was 25% of all individuals in the population (Table 3.1), we would expect $((1 - 0.25^3) \times 100 =)$ 98% of randomly selected 3-fish shoals to contain at least one AC individual. Any shoal containing one or more AC individual is likely to eat the novel prey morph while it is still rare. Therefore, we might expect almost all of the shoals to send the novel prey extinct. Contrary to this expectation, we observed nearly 15% of the shoals sending the novel prey to fixation, which requires the active avoidance of novel morphs for an extended series of prey choices (see Thomas et al. 2010). This suggests that shoals tended to act more like DC than AC individuals, despite the likely presence in the shoal of AC individuals. This may indicate copying of DC behaviour from shoal-mates by individuals that would otherwise be AC when foraging alone.

A number of studies have shown that behaviour of animals at the group level is influenced by the decisions of individuals (e.g. Magurran 1986, Okubo 1986, Huth and Wissel 1994, Parrish & Turchin 1997, Beecham and Farnsworth 1999, Croft et al. 2003, Hoare et al. 2004), but much less is known about how individual decisions are influenced by collective patterns of behaviour (Ward et al. 2008). However, it is known that information can transfer between group members due to social learning (for review see Brown and Laland 2003). For example, Ward et al. (2008) showed that the swimming direction preferences of three-spined sticklebacks are influenced by the swimming directions of conspecifics. Although our data suggests that copying of the DC strategy may be occurring, our shoal-level data does not allow us to test directly for this possibility.

In addition to the above evidence that social context can affect the process by which fixations of novel prey can occur (via copying of DC behaviour), we also found that solitary sticklebacks took longer to drive the novel prey to fixation than shoals (Table 3.3). This may be due to greater foraging intensity by fish in shoals (i.e. more prey being eaten per fish) arising from increased competition between shoal members. Inter-individual competition for resources increases as a result of group-living (Krause and Ruxton 2002), and so members of a shoal may be driven to consume food items more readily. Indeed, fish in shoals have been found to eat more than solitary individuals due to increased competition for food between group members (Eggers 1976, James and Findlay 1989), and feeding rates of individual sticklebacks were found to be higher in shoals of five fish than for solitary individuals (Ranta and Lindstrom 1990, Ranta and Kaitala 1991). Three-spined sticklebacks were shown by Webster et al. (2007) to display higher activity levels in the presence of conspecifics than when tested alone. Also, in other species of fish (European perch *Perca fluviatilis*), and in birds (great tits *Parus major*), both the latency to feed and feeding rate of socially-foraging individuals, depended on the behavioural personality type of the other individuals present (Magnhagen and Staffan 2005, van Oers et al. 2005). Furthermore, Dyer et al. (2009) showed that the mix of personalities in shoals of guppies (*Poecilia reticulata*) influenced the foraging success of all shoal members.

All of the above influences make it more likely that shoals would cause the extinction of the novel morph. This is because fish which consume a greater proportion of the prey available would be left with a higher proportion of novel: familiar prey by the end of the foraging bout, and would have to select more actively in order to avoid eating the novel prey morph. Therefore, both increased foraging intensity and social group composition could be expected to reduce the likelihood of novel prey morphs reaching fixation in a population under predation by shoals compared to solitary fish. However, this expectation was not supported by our data, since shoals of fish displayed a higher tendency towards DC manner than would have been expected from their likely composition of AC and DC shoal members.

Fish body size was significantly associated with both the likelihood of a fixation occurring, and how long it took for the novel morph to reach fixation (Experiment 2, Tables 3.2 & 3.3). In other words, larger sticklebacks drove the novel prey to fixation more quickly than their smaller counterparts, as well as driving the novel morph to fixation more frequently than smaller fish. It is not surprising that larger fish drove the novel morph to fixation more quickly (since they consumed more food in each foraging bout), but it is surprising that they did so more frequently. Indeed, one might expect that larger fish might send the novel morph to fixation less often than smaller fish, since larger fish would consume a greater proportion of the prey, so for the novel morph to avoid extinction the larger fish would have to exert a stronger avoidance of the novel prey than smaller fish would.

In our study, novel morphs regularly reached fixation despite being initially rare (5%) in the prey populations. It could be argued that the novel morph is consistently avoided by foragers due to its initial rarity or subsequent abundance (i.e. due to apostatic or anti-apostatic selection), rather than due to its novelty (i.e. due to dietary conservatism). However, theoretic simulations performed by Thomas et al. (2010) showed that three-spined sticklebacks foraging on artificial populations of dried *Daphnia* prey retained their DC bias against the novel prey throughout the experiment, even when it became common (as it approached fixation). Therefore, it seems very likely that sticklebacks in the current study (which differed in its protocol for testing for DC only in the use of live *Daphnia* rather than dried prey) avoided novel prey morphs based on their novelty rather than their rarity.

Interestingly, the test for pre-existent colour preferences (Experiment 1) showed that solitary sticklebacks preferred brown coloured prey, since they consumed significantly more brown than green prey when presented with both colour morphs simultaneously, when they were both equally novel. However, this is not the case for shoals of sticklebacks, which exhibited no pre-existing colour preference. Increased competition for food in a group may prevent group members from being overly choosy when foraging (Krause and Ruxton 2002), resulting in the apparent absence of an inherent prey preference in shoaling individuals. Solitary fish may experience less competition for resources, which may allow them to be more selective in their choice of prey. It is possible that fish in the current study were already more familiar with brown coloured prey before being brought into the lab from the wild, and this may have influenced their responses during the experiment. Wild three-spined sticklebacks eat primarily copepods, ephemeropteran nymphs and chironomid larvae in the summer (Allen and Wootton 1984), which is the same time of year that fish in the current study were collected. These organisms tend to be primarily brown in colour, so it is possible that solitary fish in the current study show an inherent preference for brown coloured prey based on their immediate prior experience in the wild. Despite this inherent preference for brown prey, the brown novel morphs were driven to fixation on several occasions, by single sticklebacks as well as by shoals (Table 3.1), showing that fixations can occur even despite underlying inherent prey colour preferences.

The visual perception capability of fish is another factor that may influence the occurrence of a novel prey fixation or extinction event, since three-spined sticklebacks are visual predators (e.g. Wootton 1976, Hart and Gill 1994), and prey characteristics, such as size, shape, movement and colour contrast with the background, provide important visual cues for prey detection (Wootton 1984). Previous studies show the particular influence of movement on stickleback foraging motivation. For example, the fifteen-spined stickleback (*Spinachia spinachia*) preferred moving prey to stationary prey, with movement increasing the frequency at which the fish attempted or completed feeding (Kislalioglu and Gibson 1976). Furthermore, in the current study, fish were observed to attempt to feed much more readily on live *Daphnia* than the dried *Daphnia* used previously by Thomas et al. (2010) (pers. obs). If live prey promote a generally higher rate of feeding in sticklebacks compared to dried prey, then there may be a greater chance of the novel prey being eaten more often, and this would reduce the likelihood of the novel colour morph reaching fixation. Furthermore, there is evidence that visual acuity of fish correlates with body size (e.g. Hairston et al. 1982, Breck and Gitter 1983, Walton et al. 1992, 1994), so large fish may detect more prey from further away than smaller individuals in the same surroundings (Hairston et al. 1982). This could lead to an increased amount of food intake for larger fish (Mittelbach 1981). In the current study, it is possible that both increased visual perception in larger fish and inter-individual competition affected the likelihood of the novel prey reaching fixation or going extinct, although it is unknown to what extent such effects may be important within the current experimental design.

In conclusion, a proportion of the population of sticklebacks exhibited dietary conservatism which was strong enough to drive a novel prey morph to fixation despite it being (i) fully palatable live prey, (ii) just as conspicuous as the familiar prey and (iii) under predation pressure from a group of foraging predators, not only solitary individuals. This is particularly surprising since the majority of such shoals will have contained fish that would exhibit AC foraging strategies when tested in a solitary foraging context.

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Chapter 4: Boldness in guppy shoals: inter-population variations and the impact of habituation

4.1: Abstract

Boldness is an aspect of inter-individual variation in the behaviour of animals documented across a wide range of taxa. In the current study, we investigated differences in boldness between and within guppy populations (Experiment 1), and both the consistency of response to two boldness tests and the effect of social context on shoaling behaviour (Experiment 2). Female guppies were tested for boldness and placed into shoals of the same behavioural phenotype, consisting of either all bold or all shy individuals. Three parameters of shoaling behaviour were recorded and fish were tested for boldness a total of three times over the 19-day experimental period. We found inter-population differences between guppies from two different wild populations, but individuals within a single population were similar in their relative boldness. We also found shy fish formed larger and tighter shoals compared to bold fish. Furthermore, the boldness of female guppies correlated across contexts in the first round of testing, but their responses changed with time, where shy fish became bolder and bold fish became more shy. This study highlights the impact of habituation and familiarity on the relative shyness-boldness of individuals but also indicates an ability in guppies to exhibit a level of behavioural plasticity in response to stressful situations.

4.2: Introduction

Behavioural syndromes, otherwise referred to as personality, temperament or coping style (Dingemanse and Reale 2005), are suites of correlated behaviours reflecting between-individual consistency over time and/or across situations (Sih et al. 2004). Importantly, these individual differences persist after controlling for factors such as age, sex or reproductive state (Reale et al. 2007). Correlated behaviours respond to selection pressures concurrently, which may help explain how animals cope with fluctuating environments. This has important ecological and evolutionary consequences that may drive important life history trade-offs (e.g. Bell 2005, Sinn and Moltschaniwskyj 2005, Bell and Sih 2007, Duckworth and Badyaev 2007, Smith and Blumstein 2008). For example, exploratory individuals may gain access to important resources but this may be counter-balanced by an increased chance of predation (e.g. Biro et al. 2004, Sih et al. 2004, Wolf et al. 2007) or encountering parasites (Poulin et al. 1991, Wilson et al. 1993, Natoli et al. 2005, Easterbrook et al. 2007).

One commonly studied dimension of personality is boldness (e.g. Barnard 1984, Wilson et al. 1993, 1994, Budaev 1997, Reale et al. 2000, Rochette et al. 2001, Marchant-Forde 2002, Sneddon 2003, Brown et al. 2005, Natoli et al. 2005, Wilson and Stevens 2005, Bell 2007), defined as the propensity of an animal to take risks especially in novel environments (Wilson et al. 1994). Bold individuals tend to approach novel objects more readily, display increased activity levels and are more exploratory, whereas shy individuals retreat from novelty, reduce activity levels and are vigilant (symptoms akin to mild stress) (Brown et al. 2007). There is some debate as to whether shyness-boldness depends on context. In humans, relative shyness and boldness can be thought of as general personality traits that are expressed in many different situations, with an individual that is bold in one situation tending to be bold in others (Godin and Crossman 1994, Kagan et al. 1998, Segal and Macdonald 1998, Sih et al. 2004, Ward et al. 2004). However, the context-specific hypothesis (Wilson et al. 1994, Wilson 1998) predicts that an individual can be bold in one context, such as a social interaction, but shy in another, for example, when entering a new environment (Barnard 1984, Budaev 1997, Coleman and Wilson 1998, Reale et al. 2000, Wilson and Stevens 2005).

Behavioural phenotype appears to affect how an individual interacts with its environment, for example, its reactions to predators, food sources, social and sexual encounters with conspecifics (Reale et al. 2007). Increasing evidence suggests that boldness plays a role in group behaviour, influencing the degree of interactions within social networks (Pike et al. 2008), as well as affecting leadership potential (Leblond and Reebs 2006, Harcourt et al. 2009). Studies on fish have shown that individuals consider a number of factors when selecting to shoal with others. Fish tend to have a social preference for conspecifics of matching phenotype and assort strongly by species and body length (Hoare et al. 2000 and references therein, Rosenthal and Ryan 2005). They also prefer larger shoals that offer greater safety from predators (Krause et al. 1997, 1998), and are influenced by activity (Pritchard et al. 2001) and hunger levels (e.g. Krause et al. 1999). Boldness affects fitness characteristics such as exploration, body length and weight (Brown et al. 2005, 2007), as well as positioning within shoals (Ward et al. 2004). Previous studies have reported a greater shoaling tendency in shy compared to bold fish (Budaev 1997, Ward et al. 2004, Dyer et al. 2009), and differences in foraging behaviour depending on the mixture of behavioural phenotypes within a shoal (Magnhagen and Staffan 2005, Dyer et al. 2009). Magnhagen and Staffan (2005) was the first study to look at changes in individual behaviour in connection with placing subjects into groups of uniform behavioural phenotypes, and showed that the feeding behaviour and habitat use of both bold and shy fish was modified to some extent by the influence of other group members. However, Magnhagen and Staffan (2005) measured the responses of fish in groups of uniform behavioural phenotype after only one day of being placed in those groups, and did not measure any parameters of shoaling behaviour. Furthermore, it is important to consider the effect of habituation to novelty. Previous tests of boldness on mammals, birds and one other fish species revealed that habituation can cause both an increase or decrease in exploration tendency depending on the species (e.g. Martin and Reale 2008, Mettke-Hoffmann et al. 2006, Magnhagen and Staffan 2005).

In order to investigate the influence of behavioural phenotype on shoaling behaviour, and how habituation affects boldness, we used the guppy (*Poecilia reticulata*), a small, highly social species of tropical poeciliid that is one of the primary experimental and comparative model species in evolutionary biology (e.g. Reznick et al. 1996a, b, 2001). Guppies demonstrate pronounced between-population variation in many aspects of behaviour, morphology and genetics (e.g. Magurran and Seghers 1990, Carvalho et al. 1991, Shaw et al. 1991, Magurran et al. 1992, Magurran 1993), and have to cope with novel environments in the wild, due to translocation during flooding events and through escaping predators (Magurran 2005). Guppies have also been previously used in studies of temperament (Budaev 1997, Burns 2008) and to investigate the impact of boldness on foraging behaviour (Dyer et al. 2009).

In the current study, we quantified the proportion of shy and bold individuals in two different guppy populations (Experiment 1). Subsequently, using a single guppy population, this information was used to create shoals of uniform behavioural phenotype, containing fish that were either all bold or all shy. We assessed the relative boldness of fish independently three times, across a period of 19 days, and observed fish shoaling behaviour at intervals across the same time frame (Experiment 2). We predicted that shy fish would shoal more than bold fish across the experimental period. We also predicted that all fish would initially show consistency in their responses to two different boldness tests, but with time, there would be a degree of habituation to these tests. We predicted that habituation to the stress of the boldness tests would cause shy fish to become bolder, whereas habituation to the novelty of the tests would cause bold individuals to appear to become more shy.

4.3: Materials and methods

4.3.1: Experimental animal origins

Guppies originated from two different stocks, the Caura and the Tacarigua Rivers, both in the Caroni drainage basin in northern Trinidad. They were offspring of originally wild-caught fish maintained in the laboratory since 1997. All fish were routinely housed in aquaria (60 x 30 x 30 cm) in mixed sex groups (1:5 male to female ratio) with about 40 fish per 60 litres. Under normal laboratory conditions, and throughout the experimental period, guppies were maintained under a 12 h light: 12 h dark lighting regime at 25±0.5°C, and fed on a diet of flakes (Aquarian®), live *Artemia* (brine shrimp) and *Daphnia* (water flea).

4.3.2: Preliminary observations: assessment of bold-shy tests

In previous studies, six different tests each with between 1-4 modifications have been used to assess boldness in fish (Table 4.1). We discounted three of these tests because: fish in the current study were raised and maintained on a varied diet, possibly causing a reduced response towards novel food; recording the time spent shoaling with conspecifics was one of the shoaling behaviour parameters that we were measuring and therefore we wanted to avoid use the same measure for a boldness test; and finally, we preferred not to use 'time taken to leave a refuge to gain food' as a test of boldness, as we felt it was too similar to the 'time taken to emerge from shelter' boldness test. The three other methods were tested in a preliminary trial using 61 female guppies: (i) time taken to emerge from a shelter (Sundstrom et al. 2004, Brown et al. 2005), (ii) time spent investigating a novel object (Brown et al. 2005), and (iii) latency to feed after a startle (using the methods of Ward et al. 2004). Based on the results, we decided to abandon the 'latency to feed after a startle' test due to an inconsistent response by the guppies tested. Individuals reacted in one of two ways to the startle event, either they continued their normal behaviour without an apparent response, or individuals were greatly stressed by the startle and stayed immobile at the bottom of the test aquarium for the remainder of the test. These extreme responses did not sufficiently separate fish along a shy-bold axis. However, the time to emerge from shelter and the novel object tests clearly separated fish into more distinct shy and bold groups, and these were both used in the subsequent experiments.

4.3.3: Experiment 1: Testing for differences in boldness between populations

Female guppies from the Tacarigua (n=510) and Caura populations (n=360) were tested for boldness using the two independent boldness tests: i) time to emerge from shelter and ii) time spent investigating a novel object (Brown et al. 2007). Fish from the Tacarigua population were tested five months apart (due to being used by us in another study). A brief description of the boldness tests is necessary here since our methods differ slightly from those of Brown et al. (2007). We also had to adjust the timings of the tests to accommodate the innately more gregarious nature of guppies compared to previously used Panamanian bishops (*Brachyrhaphis episcopi*) that are comparatively more timid (Archard G., pers. comm.). Our test apparatus consisted of a clear plastic aquarium (30 x 20 x 20 cm) filled to a depth of 12 cm.

4.3.3 (i): Time to emerge from shelter

A small dark cylindrical container (8 x 8 cm) was positioned at one end of the test tank. Opening of the vertically lifting 'door' allowed access to the tank through a small opening (3 x 5 cm) at the front of the container. A rectangular white plastic sheet was placed underneath the container to provide a strong background contrast to the colour of the fish and the substrate. Fish were placed in the container and an opaque lid was placed on top. An individual was allowed to settle for 1 min before the trapdoor was opened. An observer sat motionless, 0.5 m away in front of the tank and recorded the time taken for the fish to emerge (defined as the individual's head crossing the line of the opening). If a fish had not emerged after 4 min, it was encouraged to do so by removal of the lid from the container (which reduced the value of the refuge). Any fish failing to emerge after 5 min was given a ceiling value of 300 s and the trial was terminated.

Test	Test method	Lab/Wild	Species	Reference
Novel Object	Bright pink and yellow rubber ball	Wild	*Brachyrhaphis episcopi*	Brown et al. 2007
	Cylindrical wire minnow traps	Wild	*Lepomis gibbosus*	Wilson et al. 1993
	Small teardrop-shaped lead weight	Lab	*Lepomis macrochirus*	Wilson and Godin 2009
Novel Food	Wide range of invertebrates	Wild	*Lepomis gibbosus*	Coleman and Wilson 1998
	Swimming through hole in yellow-coloured board to reach food	Lab	*Poecilia reticulata*	Dyer et al. 2009
Emerge from Shelter	Fish in opaque box. Time for fish head to emerge after trapdoor is opened	Wild	*Brachyrhaphis episcopi*	Brown et al. 2007
	Fish in opaque box. Time for fish head to emerge after trapdoor is opened	Lab	*Brachyrhaphis episcopi*	Brown and Braithwaite 2004
	Fish in opaque box. Time for fish snout to emerge from shelter and time taken to cross a mark 5 cm from opening after trapdoor	Wild	*Brachyrhaphis episcopi*	Brown et al. 2005
	Time to emerge after trapdoor is opened	Lab	*Lepomis macrochirus*	Wilson and Godin 2009
Threatening situation	Red-tipped metre stick	Wild	*Lepomis gibbosus*	Coleman and Wilson 1998
	Simulated aerial predation – two metal nuts dropped in tank. Time recorded for fish to resume movement	Lab	*Poecilia reticulata*	Dyer et al. 2009
	Simulated aerial predation – metal bolt dropped in tank. Time recorded for fish to resume movement	Lab	*Gasterosteus aculeatus*	Ward et al. 2004, Webster et al. 2007
	Predator model – time recorded for fish to leave shelter	Lab	*Lepomis macrochirus*	Wilson and Godin 2009
Other	Amount of time spent in proximity to a shoal	Lab	*Gasterosteus aculeatus*	Ward et al. 2004
	Latency of fish to leave a shaded refuge and cross an open, illuminated area to reach prey	Lab	*Gasterosteus aculeatus*	Webster et al. 2009

Table 4.1: Previous methods used to test relative boldness-shyness in fish species.

4.3.3 (ii): Time spent investigating a novel object

The test aquarium was divided along its length into 3 equal sections by drawing black lines on the back of the tank. A pair of stimulus fish (the 'shoal') taken from a different stock tank to the test fish were placed in a transparent cylinder (14 x 6 cm) at one end of the tank. (It was important that the stimulus fish were unfamiliar to the test fish to avoid any possible effect of familiarity (Griffiths and Magurran 1997) on the choice made by the test fish). A novel object consisting of a transparent cylinder (5 x 2 cm) containing brightly coloured gravel was placed at the other end of the aquarium. A single test fish was placed in a clear cylinder (12 x 8 cm) in the middle of the tank, equidistant from both the shoal and novel object, and left to settle for 1 min. After this time, the fish was gently released into the tank by removal of the container and allowed to swim freely for 5 min. After 5 min, the test fish was restrained in the clear cylinder and left to settle for 1 min, during which time the positions of the shoal and objects were swopped. The test was repeated for a further 5 min. An observer sitting motionless 0.5 m away from the front of the tank recorded the amount of time the test fish spent in the third of the aquarium closest to the novel object for the 10 min experimental period.

4.3.4: Experiment 2: Testing for the effect of social context and habituation on boldness

Fry were offspring of originally wild-caught fish from the Caura River, maintained in the laboratory since 1997, they were collected from the adult breeding tanks once a fortnight over 5 months and housed separately in smaller aquaria (30 x 20 x 20 cm). Upon reaching sexual maturity (6-8 weeks old) fish were transferred to larger aquaria (60 x 30 x 30 cm) where they were maintained in mixed-sex groups (1:5 male to female ratio) with about 40 fish per 60 litre aquaria. Fish were left for 6 months before the females were used in the study. We could more clearly test the proposed hypotheses using only female fish, because female guppies show an inherently greater propensity to shoal compared to males (Griffiths and Magurran 1998), whereas male behaviour is dominated by sexual display (Houde 1997). Also, it was important in this study to use fish of the same age and experience since it has been shown that shyness-boldness can vary with age (Brown et al. 2005) and rearing experience (e.g. Kelley et al. 2005). Furthermore, we had to control for reproductive status, as much as possible, since this can also impact on the relative boldness of an animal (Chapter 5). It was assumed that all female guppies used in the current study were previously mated since they had been housed with males since birth. A total of three fish (2%) were observed to give birth during the experimental period.

At the start of Experiment 2, female guppies (n=360) were individually assessed for boldness using two independent tests: (i) time to emerge from shelter and (ii) time spent investigating a novel object (Brown et al. 2007) (test round 1). The experiment was conducted in three separate batches to allow collection of sufficient data replicates. Females (n=120/batch) were randomly selected from each stock tank and assayed over 2 consecutive days for boldness (t = days 1-2). The boldness tests were conducted as in Experiment 1.

Following boldness measurements (t = days 1 and 2), we compared the responses of the fish in both tests and found a positive correlation between the time taken to emerge from the shelter and the time spent investigating a novel object. Therefore, we were justified in producing a composite rank of boldness across both tests, following the method of Ward et al. (2004). For the time to emerge from shelter test, the fish that emerged most quickly was given a rank of 1, and the fish that took the longest time to emerge was given a rank of 120. Similarly in the novel object test, the fish that spent the most time investigating the object was given a rank of 1, while the fish that spent the least time with the novel object was given a rank of 120. The two ranks for each fish were then summed and the combined score was itself ranked. The fish with the 36 lowest overall ranks were defined as being bold, relative to the others, and the fish ranked 84-120 were defined as being shy relative to the others. The fish ranked 37-85 were identified as intermediate and were removed from the experiment. To maintain a similar level of relative shyness-boldness of fish between the three batches of the experiment, it was pertinent to monitor the threshold levels in both boldness tests. For the time to emergence test, fish that emerged in less than 60 s were classed as bold, and those that emerged after 240 s were shy. In the novel object test, bold fish were defined as those that spent more than 60 s investigating the object, whereas shy fish were those that spent less than 20 s in close proximity to the object. Threshold levels were used to separate the fish into groups of bold and shy individuals.

After ranking was completed, standard length of all remaining bold or shy fish (n=72 per batch, after removal of intermediate fish) was recorded under anaesthetic (0.02% MS222) and all fish were injected with a Visible Implant Elastomer (VIE, Northwest Marine Technology, Inc.) dye, specifically designed for the purpose of marking fish, into the musculature (t = day 3). Fish were then allowed to recover in individual 1 litre pots for 1 h before being placed into groups (6 individuals per group) in test tanks (30 x 20 x 20 cm), and allowed to familiarise for a further 7 d (t = days 4-10). Standard length was controlled by size matching all individuals within a tank and by only using fish within a 18-26 mm size range. Each aquarium contained an air supply and a refuge (small plant pot). The location of tanks was randomised, and guppies in different aquaria were visually and physically isolated from one another. Shoals differed in their composition of bold and shy phenotypes: (i) Bold (6 bold fish) and (ii) Shy (6 shy fish), with 10 replicates of each.

On days 4 and 10 of the familiarisation period, the shoaling behaviour of each group was observed once daily. Three shoaling behaviour parameters were measured: i) shoal size, ii) nearest neighbour distances, iii) time spent shoaling. Observation periods lasted 1h 30 min per tank (5 min for each of the shoaling parameters which equalled 15 min per fish). During each observation period, 10 measurements of shoal size were recorded, by counting the number of fish in the largest shoal at the time of observation. A further 10 measurements of nearest neighbour distance were made for each fish. The time interval between each of these measures was 30 s, which was sufficient to make consecutive observations independent. Also the time spent shoaling by each fish was measured over 5 min. Horizontal and vertical lines drawn every 2 cm on three sides (back and two sides) of each test aquaria facilitated the estimation of between-individual distances, with all measurements being assessed in three-dimensional space (as previously performed in Chapter 7; Richards et al. in press). Shoal members were defined as fish within 4 body lengths of one another (Pitcher and Parrish 1993). After familiarisation, fish were kept in their groups for a further 7 days (t = days 11-17), during which time fish were tested for boldness (t = days 11-12) (test round 2) and observed for shoaling behaviour (t = day 17). At the end of the experimental period fish were re-tested for boldness on days 18 and 19 (test round 3).

4.3.5: Statistical analyses

Differences between guppy populations were examined using Kruskal-Wallis non-parametric tests, as the data was not normally distributed. All other data were log-transformed to achieve normality (assessed by Anderson-Darling tests). The responses to the boldness tests were analysed for consistency of response between the two different tests and between test rounds. Pearson correlations were used to test the consistency of responses to the boldness tests, with consistency between tests and between bold and shy fish being measured using Two-tailed T-tests. In total, 8% of fish tested failed to emerge from the shelter and 6% failed to leave the safety of the shoal and inspect the novel object and these individuals were excluded from further analysis. Repeated Measures ANOVAs were performed on the shoaling data with shyness-boldness behavioural phenotype ('Shy/Bold') as factor with standard length ('SL') and day of experiment ('Day') as covariates, and individual tanks ('Tank') and batches of the experiment ('Batch') as random factors. Variables that did not explain significant amounts of variation were excluded from the model using a backwards stepwise approach. In addition, Tukey post-hoc tests were performed where appropriate. All analyses were performed in Minitab 15.

4.4: Results

4.4.1: Experiment 1: Testing for differences in boldness between populations

There was no significant variation in boldness within the Tacarigua population, despite individuals being tested five months apart (Kruskal-Wallis: Time to emerge from shelter H=0.90, d.f.=1, P=0.344; Time spent investigating a novel object H=1.36, d.f.=1, P=0.243). However, the responses to both tests of boldness differed significantly between different guppy populations, with guppies from the Caura River being bolder than those from the Tacarigua River (Kruskal-Wallis: Time to emerge from shelter H=10.27, d.f.=2, P=0.006; Time spent investigating a novel object H=36.82, d.f.=2, P<0.001). Caura fish took the least time to emerge from shelter (mean ±SE 111.5±8.5 s) and spent the most time spent investigating a novel object (53.3±3.2 s) compared to Tacarigua fish (time to emerge from shelter: 149.2±11.3 s; time spent investigating a novel object: 36.6±3 s).

4.4.2: Experiment 2: Testing for the effect of social context and habituation on boldness

4.4.2 (i): Shoaling behaviour

Behavioural phenotype affected shoaling behaviour with shy fish forming significantly larger (Repeated Measures ANOVA: $F_{1,383}$=12.24, P=0.001) and more cohesive ($F_{1,383}$=5.42, P=0.020) shoals than bold fish. Shy fish also spent more time shoaling (mean ±SE 245.1±3 s) than bold fish (240.4±3 s) although this difference was not significant. Both bold and shy fish formed smaller and less cohesive shoals, and spent less time shoaling on days 7 and 14 of the experiment, compared to day 1 (all 3 shoaling parameters: P<0.01; Tukey post-hoc tests day 1 vs days 7 and 14: all P<0.001), indicating some habituation to the test environment and familiarity with other shoal members. The size of fish did not affect their shoaling behaviour (all 3 shoaling parameters: P>0.05).

4.4.2 (ii): Boldness tests

The responses of fish in the first round of boldness tests (used to identify individuals as initially either bold or shy) were significantly different in both tests (T-test: Time taken to emerge t=13.97, P<0.001; Time spent with novel object t=9.90, P<0.001), with 29% of fish tested being ranked as bold, and 32% ranked as shy. The responses of fish to both tests in the first round were correlated (Pearson correlations: r=0.472, P<0.001), so fish that were quickest to emerge from shelter also spent the most time with the novel object (Figure 4.1).

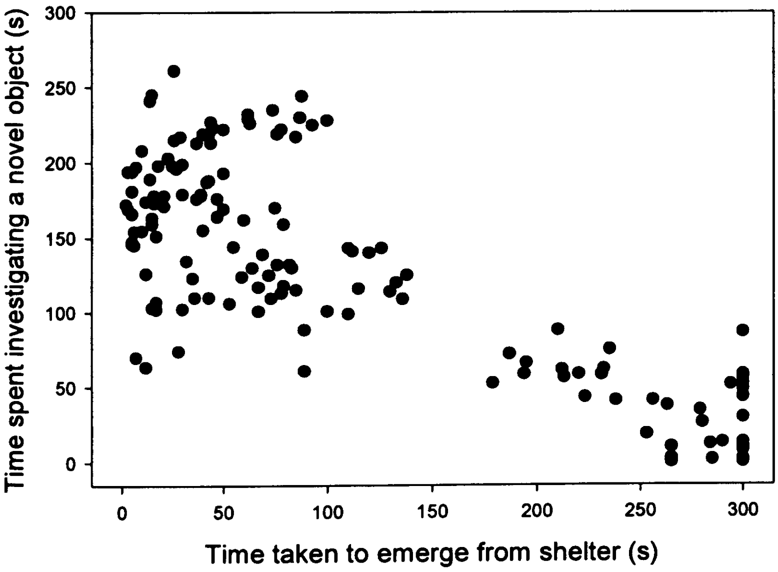

Figure 4.1: The relationship between the two measurements of boldness, for the first round of testing, time to emerge from shelter and time spent investigating a novel object, in Experiment 2.

However across the experimental period, shy fish became more bold, and bold fish became more shy (Figure 4.2). The responses of the originally bold and shy fish were no longer significantly different when comparing rounds 2 and 3 of the boldness tests (T-test: Time taken to emerge from shelter test P>0.40; Time spent investigating a novel object test P>0.05). Furthermore, responses to both the boldness tests in rounds 2 and 3 were not correlated for either bold or shy fish (Pearson correlations: Bold and shy fish test rounds 2 and 3 all P>0.09), which means fish that were bold in one test were not necessarily bold in the second.

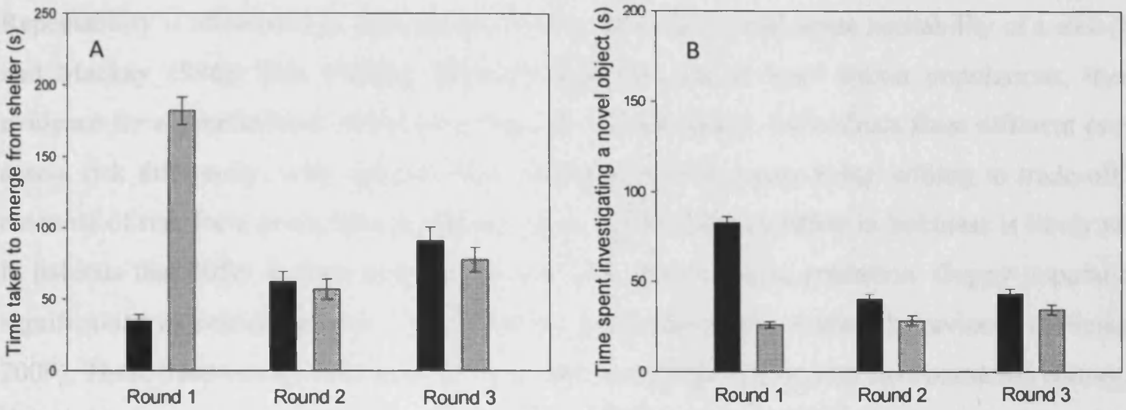

Figure 4.2: Mean ± S.E. of (A) time taken to emerge from shelter or (B) time spent investigating a novel object, for bold and shy fish across the 3 rounds of boldness tests in Experiment 2. Bold fish are shown in black and shy fish in grey.

Across all three boldness test repeats, there was no significant effect of fish size on either the time to emerge from shelter (One way ANOVA: F=1.02, d.f.=13, P=0.430) or the time spent investigating a novel object (F=0.36, d.f.=13, P=0.982).

4.5: Discussion

Guppies from two different populations differed significantly in their relative boldness, but when fish from one of these populations were re-tested five months later, there was no difference in the relative proportion of bold and shy fish in this population from the Tacarigua River (Experiment 1). In the Caura fish population (Experiment 2), as predicted, shy fish shoaled comparatively more than their bold counterparts. Also, boldness of both bold and shy individuals was affected to some degree by habituation to the test environment, reflected in a change in response to the boldness tests over the 14-day experimental period. The effect of habituation in the current study supports previous findings in European perch, where bold and shy individuals habituated to a test environment and modified their habitat use and feeding activity as a result (Magnhagen and Staffan 2005).

We found significant differences in boldness between fish populations from the Caura and Tacarigua Rivers (Experiment 1), the first study, to our knowledge, to compare relative boldness in these two populations. Guppies are one of the few species in which within-species, geographical and genotypic variation is known for many different traits (Endler 1995). Individual differences in personality traits can represent adaptive behaviour patterns that have arisen through natural selection in response to varying environmental conditions (Brown et al. 2007). However, we do not find evidence in this study to suggest that differences between populations are genetic. Although differences were detected, repeated observations of boldness on the same fish at different times were not always correlated. Repeatability is often used as an indicator for the maximum broad sense heritability of a trait (Falconer and Mackay 1996). This finding therefore suggests that at least within populations, there is no evidence for a genetic basis of boldness from the current study. Individuals from different populations assess risk differently, with animals from differing environments being willing to trade-off varying amounts of risk for a given benefit (Brown et al. 2005). This variation in boldness is likely to be seen in habitats that differ in their average level of risk, for example, predation. Guppy populations vary significantly in colour patterns, life histories, antipredator and mating behaviours (Huizinga et al. 2009). These traits covary with each other as well as with predation and environmental factors, such as light intensity, water temperature and food availability (Endler 1995). Therefore, it is not surprising that guppies from different populations vary in their relative proportions of bold and shy individuals.

In Experiment 2, as predicted, shy guppies shoaled significantly more than their bold counterparts, forming larger, more cohesive groups and tending to spend more time shoaling. This is in agreement with a number of other studies. For example, both Budaev (1997) and Ward et al. (2004) showed that shy European wrasse (*Symphodus ocellatus*) and three-spined sticklebacks (*Gasterosteus aculeatus*), respectively, have a higher tendency to shoal compared to bold fish. This behaviour is also seen in sheep (*Ovis aries*) where shy individuals form more cohesive groups than bold individuals (Michelena et al. 2008, Sibbald et al. 2009), and bold flocks tend to split into smaller sub-groups compared to shy flocks (Michelena et al. 2008). Many animals (including the guppy) form social groups to reduce predation risk (Krause and Ruxton 2002), but in doing so they incur the cost of increased competition (Pitcher 1986). Shy individuals tend to be risk-averse, accepting lower rewards in return for a lower degree of risk (Ward et al. 2004), so may prefer the perceived security of the shoal in the present study.

We also found that both bold and shy fish formed smaller and less cohesive groups, and spent less time shoaling on days 7 and 14 of the experimental period compared to day 1 (Experiment 2). This is most likely due to the development of familiarity between shoal members. Recognition of tank mates (i.e. familiarisation) develops over a period of 12 days in female guppies (Griffiths and Magurran 1997). In relation to the current study, shoaling behaviour observations were conducted when all fish were unfamiliar with each other (day 4), familiarity was developing between tank mates (day 10) and after familiarity had been established (day 14). Individual recognition has been shown to affect schooling behaviour in guppies (Magurran et al. 1994) and other fish species, e.g. the three-spined stickleback, *Gasterosteus aculeatus* (see van Havre and Fitzgerald 1988), fathead (*Pimephales promelas*) and European minnows (*Phoxinus phoxinus*) (see Brown and Smith 1994, Chivers et al 1995, Metcalfe and Thomson 1995) and bluegill sunfish, *Lepomis machrochirus* (see Dugatkin and Wilson 1992), with fish preferring to associate with familiar conspecifics rather than unfamiliar fish. However, to our knowledge, there are no animal studies that have investigated the possible interplay between temperament and familiarity, previously documented in humans, for example, Quilty et al. (2007) showed that adults with an inhibited (shy and introvert) personality showed stronger preferences for the familiar than more extrovert individuals.

Increased boldness of originally shy fish, as well as decreased boldness of originally bold fish, in Experiment 2 was most likely caused by some degree of habituation to the novelty of the boldness tests. Habituation to an experimental environment, or an aspect of that environment, has been seen previously: chipmunks (*Tamias striatus*) decreased their tendency to explore a novel environment over successive trials (Martin and Reale 2008), and marmosets (*Saguinus fuscicollis*), ravens (*Corvus corax*) and garden warblers (*Sylvia borin*) decreased approach latencies towards novel objects (Menzel and Menzel 1979, Heinrich et al. 1995, Mettke-Hoffmann et al. 2006, Stowe et al. 2006), whereas great tits (*Parus major*) and zebra finches (*Taeniopygia guttata*) increased their tendency to explore with successive trials (Dingemanse et al. 2002, Schuett and Dall 2009). Habituation can occur with repeated tests (Groves and Thompson 1970, Archer 1973, Wright et al. 2003, van Oers et al. 2005, Magnhagen and Staffan 2005), but it may be bypassed to some extent by exposing test subjects to different types of novel situations or objects (e.g. Verbeek et al. 1994), but this may itself lead to habituation if the animal can habituate to novelty itself, as opposed to specific properties of each of the novel objects (Reale et al. 2007). However, since all fish in this study experienced the stress of the boldness tests in the same form, between-individual differences in habituation should be minimal.

Female guppies in Experiment 2 may also be demonstrating a degree of behavioural plasticity, demonstrated in their responses to the boldness test environments. In the first round of boldness testing, there was a correlation between both tests, where individuals that emerged sooner from shelter also spent more time investigating a novel object. This is suggestive of a context-general response, where personality traits can be generalised across contexts (Reichert and Hedrick 1993, Benus and Rondigs 1996, Beauchamp 2000, Ward et al. 2004, Sih et al. 2004, Svartberg et al. 2005). However, in the second and third rounds of testing, the majority of bold and shy fish had altered their responses compared to the first round of tests, suggesting a context-specific response. The argument for and against both the context-general and –specific viewpoints has been much discussed (e.g. Sih et al.

2004, Reale et al. 2007, Webster et al. 2007), with no resolution to date. In fact, two very recent studies presented conflicting evidence for the existence of a behavioural syndrome in boldness in male and female guppies. Piyapong et al. (2009) showed responses to a simulated predator attack were correlated across two different social contexts for male, but not female, guppies. Another study found high repeatability for both male and female guppies in the time taken to emerge from shelter (Harris et al. 2010). In the current study, we did not find such sex differences in boldness as we only tested female guppies. Also, Piyapong et al. (2009) used two different boldness tests to those used in the current study and so this may account for the lack of a correlated response in female guppies in their study. There is much evidence to support the context-specific argument, where individuals adjust their behaviour to the context in which they find themselves, to avoid an inappropriate response that may, for example, increase their predation risk or decrease foraging or mating opportunities (Spoolder et al. 1996, Coleman and Wilson 1998, Reale et al. 2000, D'Eath and Burn 2002, Bell 2005, Wilson and Stevens 2005, Webster et al. 2007). Early emergence from shelter into a novel environment, or leaving the safety of a shoal and approaching a novel object, might be a high risk behaviour, especially in high-predation areas. However, it may be advantageous if there is significant competition for mates or food; in fact fish from high predation areas tend to be bolder than those from low-predation sites (Brown et al. 2007). It seems likely that an animal would benefit from displaying some degree of plasticity in response to its ever-changing environment, in order to avoid a maladapted response in a particular situation. For example, brown rockfish (*Sebastes auriculatus*) displayed behavioural syndromes over time in response to a predator, but individuals were inconsistent in their behaviour between test rounds (Lee and Bereijikian 2008). This complements earlier work on three-spined sticklebacks that found a stable behavioural syndrome across three ontogenetic stages (juvenile, subadult and adult), but inconsistent individual behaviour (Bell and Stamps 2004).

Furthermore, we must consider the impact of a change in social environment in the current study, since guppies were reared in shoals of mixed behavioural phenotypes but then placed into shoals of either all bold or all shy individuals (Experiment 2). The only other study, to our knowledge, that has transferred animals (in this case European perch) into groups of a single behavioural phenotype (Magnhagen and Staffan 2005) found that both originally bold and shy perch changed their behaviour after being re-grouped into shoals of either all bold or shy conspecifics. As in the current study, shy individuals became bolder, and bold fish seemingly became more shy. There is increasing evidence to suggest the social environment in which an animal lives affects individual behaviour (van Oers et al. 2005, Croft et al. 2006, Magnhagen 2007). For example, the relationship between risk-taking and exploration varies according to social context in great tits (*Parus major*), ravens (*Corvus corax*) and European perch (van Oers et al. 2005, Stowe et al. 2006, Stowe and Kotrschal 2007, Magnhagen 2007). Also, Dyer et al. (2009) showed that shy and bold guppies gain foraging benefits from associating with each other in mixed shoals. Admittedly, it is highly unlikely that groups of animals in the wild are composed of a single behavioural phenotype (e.g. Pike et al. 2008), but this sort of experimental methodology is necessary in order to elucidate the impact of social environment and how the mixture of behavioural phenotypes within a group impacts on individual behavioural variation.

In conclusion, shyness-boldness has a significant impact on behaviour with shy fish having a lower overall tendency to shoal compared to bolder fish. This study is the first to show the impact of habituation on boldness in a tropical fish species. The relative shyness-boldness of individuals significantly changes over time, indicating some degree of plasticity in behaviour, allowing individuals to adapt to ever-changing environments.

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Chapter 5: Personality studies on guppies (*Poecilia reticulata*): virgin females are less shy, and boldness increases during gestation.

5.1: Abstract

The relationship between animal personality, fitness and life history traits remains largely unexplored. An individual's personality has an important impact on its behaviour and fitness, and animals trade-off risks and benefits when it comes to the expression of boldness in their behaviour. In the current study we investigated the effect of mating and pregnancy on the boldness of female guppies (*Poecilia reticulata*). Groups of 6 virgin females were observed for shoaling behaviour before and after being housed with either two males ('mated' treatment group) or two unfamiliar females ('virgin' treatment group). Mated female guppies were subsequently monitored for signs of pregnancy and assessed for boldness. Virgin female guppies were bolder and formed significantly smaller, less cohesive shoals than mated females. Within the 'mated' treatment group, pregnant females were bolder than non-pregnant conspecifics, spending more time examining a novel object. This is the first study reporting a link between boldness and the prior sexual experience of an individual.

5.2: Introduction

Animal personality, broadly defined as consistency in an individual's behaviour over time and/or across situations (Reale et al. 2007), has been documented in a broad range of taxa including many mammals, birds, lizards, amphibians, fish, molluscs and arthropods (Gosling 2001). Personality impacts heavily on behaviour affecting, for example, intraspecific competition, dispersal behaviour and reaction to predators (Wilson et al. 1994, Wilson 1998, Fraser et al. 2001, Dingemanse et al. 2003, Dall et al. 2004, Sih et al. 2004, Reale et al. 2007, Stamps 2007, Wolf et al. 2007, Cote et al. 2010). One aspect of personality that has been relatively well-studied is boldness: the willingness of an animal to explore and take risks in many behavioural contexts (e.g. Wilson et al. 1994, Ward et al. 2004, Brown et al. 2005, 2007, Webster et al. 2007, Wilson and Godin 2009). Boldness is associated with a range of behaviours such as aggression (Johnson and Sih 2005), mate selection (Godin and Dugatkin 1996), general activity and exploration (Wilson and Godin 2009), dispersal (Fraser et al. 2001), invasiveness (Rehage and Sih 2004), foraging and anti-predator behaviour (Wilson and Stevens 2005). In light of this evidence, it seems very likely that there will be a relationship between boldness and reproductive status/traits.

Differences in animal temperament of mated and pregnant individuals were first recorded in monkeys. Captive female chimpanzees (*Pan troglodytes*) in the final months of pregnancy were observed to behave with extreme caution and avoided strenuous exercise in group activity (Yerkes 1943). This was well before investigations into human sexual behaviour began, since the first published studies on the development of human sexuality did not appear until 1955 (see Diamond 1965). Subsequently, Rowell

(1969) showed that pregnant female baboons (*Papio anubis*) reduced all types of social interaction (inter-female, male-female, friendly interactions), and pregnant female chimpanzees became less sociable as their pregnancies continued to full term (Wallis and Lemmon 1986). More recently, Maestripieri (1999) showed that female pig-tailed macaques (*Macaca nemestrina*) exhibited reduced grooming activity with other group members after the first month of pregnancy and reduced aggressiveness in the last month. In non-primates, temperament and sexual experience affect female sexual behaviour in sheep (*Ovis aries*). Bold ewes reproduced earlier, had a higher weaning success (Reale et al. 2000) and were significantly more receptive to initial contact with males (Gelez et al. 2003), compared to shy ewes. More recently, gravid female three-spined sticklebacks (*Gasterosteus aculeatus*) were found to be significantly bolder when inspecting a predator than non-gravid fish (Frommen et al. 2009), and fecundity and boldness were negatively correlated in the Eastern mosquitofish, *Gambusia holbrooki* (see Wilson et al. 2010). However, no studies to date have investigated the impact of mating on the boldness of virgin fish.

In the current study, we manipulated the degree of contact between male and virgin female guppies (*Poecilia reticulata*) to determine the effect of mating on the relative shyness-boldness of females. The Trinidadian guppy is an important model for studying the effects of natural and sexual selection (reviewed by Houde 1997) as well as the influence of both male and female choice on mating. Guppies naturally form groups and have a promiscuous mating system, where females invest heavily in reproduction but males spend a high proportion of time engaged in sexual behaviour (Endler 1987), contributing only sperm which can be stored by females for up to 8 months and used to fertilize multiple broods (Magurran 2005). Male guppies either court a female before attempting to mate, or they employ a sneaky mating strategy (Liley 1966). During courtship, males use a sigmoid display (Baerends et al. 1955, Liley 1966) forming an S-shape with their bodies they quiver in front of the female. This presumably allows females to select males based on their colour patterns and display behaviour (e.g. Houde 1997). Sneaky copulations occur when female guppies are unresponsive to a male's displays (Houde 1997). Most females actively avoid sneaky matings by darting away as soon as they detect the approach of a male (Magurran and Nowak 1991). Evans et al. (2003) showed 45% of females received sperm through sneaky matings, from eight localities in Trinidad, but the absolute success of sneaky mating behaviour remains unknown. Boldness has been well documented in fishes (e.g. Wilson et al. 1993, Coleman and Wilson 1998, Ward et al. 2004, Brown et al. 2005, 2007, Leblond and Reebs 2006, Wilson and Godin 2009). Guppies have been used previously in studies of temperament (Budaev 1997, Burns 2008), with boldness being specifically demonstrated in two different populations of laboratory-maintained strains (Chapter 4).

To our knowledge, this is the first study to examine the possible effect of mating status on relative shyness-boldness in fish. Firstly, we tested the hypothesis that virgin female guppies are more bold than their mated counterparts. Secondly, we examined the level of boldness of pregnant female guppies and predicted that boldness would decrease during gestation.

5.3: Materials and methods

5.3.1: Experimental animal origins

Guppies were offspring of a wild strain originally obtained from the Tacarigua River in the Caroni drainage basin of Northern Trinidad, maintained in the laboratory since 1997. Fry were collected from the adult breeding tanks once a fortnight over 5 months and housed separately in smaller aquaria (30 x 20 x 20 cm). Fish were monitored once every day for onset of sexual maturity (6-8 weeks old) which, in males, is characterised by a thickening of the anal fin (Houde 1997). Males were removed from the aquaria at this point and transferred to a larger stock tank (60 x 30 x 40 cm). Removal of males was necessary to prevent mating, since we required virgin females for this study. Females were maintained in single-sex groups, about 20 fish per 60 litre aquaria for one month before use, so fish used in this study were at least 12 weeks old. All fish were maintained under a 12 h light: 12 h dark lighting regime at 25±0.5°C, and fed on a diet of flakes (Aquarian®), *Artemia* (brine shrimp) and *Daphnia* (water flea).

5.3.2: Experimental design

Virgin female guppies (n=150) were individually assessed for boldness on days 1 and 2 of the experiment using two independent tests: (i) time to emerge from shelter and (ii) time spent investigating a novel object (Brown et al. 2005, 2007). Following these tests, all fish were ranked according to their performance in both tests (see Chapter 4 for exact details of the boldness tests and ranking procedure). The experiment was conducted in three separate batches to allow collection of sufficient data replicates (n=50 fish tested per batch).

On day 3, standard length of all fish was recorded under anaesthetic (0.02% MS222) and each fish was injected with a Visible Implant Elastomer (VIE) mark into the tail muscle. Fish were then allowed to recover in individual 1 litre pots for 1 h before being placed into groups (6 individuals per group) in test tanks (30 x 20 x 20 cm), and allowed to familiarise for a further 7 days (t = days 4-10). Standard length was controlled by size matching all individuals within a tank and by only using fish within 18-26 mm size range. Each aquarium contained an air supply and small flower pot for shelter. The location of guppy tanks was randomised, and guppies in different aquaria were visually and physically isolated from one another. Shoals were randomly divided between two treatment groups: 'mated' and 'virgin'. All shoals had the same relative composition of bold and shy phenotypes: 1 bold + 1 shy + 4 intermediate fish, with 10 replicates of each treatment.

After familiarisation, the shoaling behaviour of both the bold and shy fish in each group was observed using the methods of Chapter 7; Richards et al. (in press) once daily for 3 consecutive days (t = days 11-13). All fish were then tested for boldness a second time over two days, with each boldness test being performed on a separate day (t = days 14-15). Fish were given 24 h to settle after the boldness tests, to avoid possible fatigue affecting behaviour. On day 17, two adult male guppies were placed in with the 'mated' shoals, whereas 'virgin' shoals received two unfamiliar female guppies (of the same size or smaller to reduce interfering with dominance hierarchies within the familiar shoals). Pilot work for this study showed that adding only a single male guppy to shoals (n=5) of females resulted in a low level of pregnancy (4 out of 30 fish). Hence, we added two males to each 'mated' tank in order to increase the frequency of pregnancies. Consequently, in the current study, 66 females had the opportunity to mate and 30 of these became pregnant, giving birth to a (recorded) total of 61 offspring. (For details on assessment of pregnancy status, see below). Males and unfamiliar females were removed after 3 days (t = day 20), (based on findings by Houde 1997 that showed virgin female guppies become unresponsive to males after three days of continuous contact), after which shoaling behaviour of the 'virgin' and 'mated' fish was again recorded for 3 consecutive days (t = days 21-23). At the end of this time, all fish were tested for boldness a third time, with each boldness test being performed on a separate day (t = days 24 and 25).

Following this, 'virgin' treatment group fish were removed from the experiment and placed in large stock aquaria (60 x 30 x 40 cm). However, 'mated' treatment group fish remained in their shoals and were checked for signs of pregnancy by observing fish for an extended abdomen and/or darkening colour around anal opening, firstly 15 d post-mating, and then every day thereafter. Pregnant fish were tested for boldness at day 21 post-mating (a similar number of non-pregnant but mated females, from the 'mated' treatment group, were tested at this time for comparison). The gestation period in guppies is 3 to 4 weeks (Houde 1997), and females can abort embryos prematurely if stressed (pers. obs.). Testing fish at day 21 post-mating struck the balance between measuring boldness at a late stage of pregnancy, but not too late, so as to unduly stress the fish and cause premature birth/abortion. Following boldness tests, mated but non-pregnant fish were removed from the experiment and placed in large stock aquaria (60 x 30 x 40 cm). However, pregnant fish were housed separately in groups of two fish per tank (separated by a transparent divider). This reduced the stress of being housed individually and enabled an accurate count of offspring to be made for each pregnant female, providing a confirmation of pregnant status during the experiment. We assumed that only females that were observed with broods were pregnant. This method possibly underestimates the true number of pregnant females because (i) females without obvious signs of pregnancy in the first 21 days were disregarded, (ii) some pregnancies could have failed due to miscarriage, and (iii) some offspring may have been cannibalised before we had recorded them. The use of only confirmed pregnancies may have introduced additional error variance in the test if the 'mated non-pregnant' group also contained some (undetected) pregnant females. This error is conservative in that it only would have reduced the statistical power of our analysis.

5.3.3: Statistical analyses

All data were either log- or arcsine-transformed to reach normality, which was assessed using Anderson-Darling tests. Preliminary analysis revealed there were no significant differences in shoaling behaviour or the response to boldness tests between batches of the experiment. The data were analysed to see if shoaling behaviour was affected by standard length of fish, day of experiment, behavioural phenotype and mating status. Repeated Measures Analyses of variance (RM-ANOVAs) were performed on the shoaling data with shyness-boldness behavioural phenotype ('Shy/Bold') and mating status ('Virgin/Mated') as factors, with standard length ('SL') and day of experiment ('Day') as covariates. Variables that did not explain significant amounts of variation were excluded from the model using a backwards stepwise approach.

The responses to the boldness tests were compared between the two different tests and between the four test rounds. Two-tailed T-tests were used to examine the consistency of responses to the boldness tests across test rounds, with consistency between tests and between bold and shy fish being measured using Pearson correlations. In total, 4% of fish tested failed to emerge from the shelter and 6% failed to leave the safety of the shoal and inspect the novel object and these individuals were excluded from further analysis. All analyses were performed in Minitab 15.

5.4: Results

The relative boldness of virgin and mated female guppies was assessed using two independent tests, and as predicted, we found that virgin females were bolder than their mated counterparts. Furthermore, in the mated group, pregnant females are bolder than non-pregnant conspecifics.

5.4.1: Shoaling behaviour and effect of mating

Virgin female guppies form smaller (Repeated Measures ANOVA: $F_{1,239}=7.06$, $P=0.008$) and less cohesive ($F_{1,239}=60.68$, $P<0.001$) shoals than previously-mated females (Table 5.1A and B). However, there was no difference in the time spent shoaling by virgin and mated fish ($F_{1,239}=1.20$, $P=0.273$). Overall, this suggests that virgin female guppies are bolder than their mated counterparts. Fish were disturbed by the addition of males or unfamiliar females ('mated' and 'virgin' treatment groups, respectively), as indicated by the formation of tighter shoals ($F_{1,239}=11.64$, $P=0.001$) as well as longer time spent shoaling ($F_{1,239}=9.72$, $P=0.002$) on days 4-6 of the observation period (Table 5.1B and C). However, there was no effect of behavioural phenotype on the shoaling behaviour of female guppies in this study (RM ANOVA: all 3 shoaling parameters $P>0.1$), suggesting that bold and shy individuals do not group together more or less relative to each other. As expected, there was no effect of guppy size on shoaling behaviour (RM ANOVA: all 3 shoaling parameters P>0.05), since all fish were size-matched within and between shoals.

(A)

Source	DF	Adj MS	F	P
Virgin/Mated	1	2.1340	7.06	0.008
Error	234	0.3023	*	*
Total	239			

(B)

Source	DF	Adj MS	F	P
Day	1	1.3308	11.64	0.001
Virgin/Mated	1	6.9378	60.68	<0.001
Error	234	0.1143	*	*
Total	239			

(C)

Source	DF	Adj MS	F	P
Day	1	511.35	9.72	0.002
Error	234	52.62	*	*
Total	239			

Table 5.1: ANOVA model of (A) mean average shoal size (B) mean nearest neighbour distances, and (C) mean time spent shoaling for 'virgin' and 'mated' treatment group fish. For both starting models, standard length ('SL') of fish and day of experiment ('Day') were treated as covariates, with shyness-boldness behavioural phenotype ('Shy/Bold') and mating status ('Virgin/Mated') as factors. Only significant terms remain in the final model shown here.

5.4.2: Boldness tests

The behaviour of bold guppies differed significantly from that of shy fish in both trials conducted at day 11-13 and day 14-15 in round 1 (T-test: Time taken to emerge t=9.21, P<0.001; Time spent with novel object t=3.70, P=0.003). In these trials, 24% of fish were ranked as bold, 24% were ranked as shy and 52% ranked as intermediate. The responses of fish to both tests in the first round were strongly correlated (Pearson correlations: r=0.602, P<0.001, Figure 5.1), so fish that were quickest to emerge from shelter also spent the most time with the novel object. Across the experimental period, levels of boldness altered between days 1 and 14. Shy fish became bolder and bold fish seemingly became more shy. However, the responses of the originally bold and shy fish remaining significantly different in rounds 2, 3 and 4 of the boldness tests (T-test: Time taken to emerge from shelter test all P<0.003; Time spent investigating a novel object test all P<0.004). The responses to both the boldness tests in rounds 2 and 3 were, however, not correlated for either bold or shy fish (Pearson correlations: Bold and shy fish test rounds 2 and 3 all P>0.05, Figure 5.2), which means fish that were bold in one test were not necessarily bold in the other test.

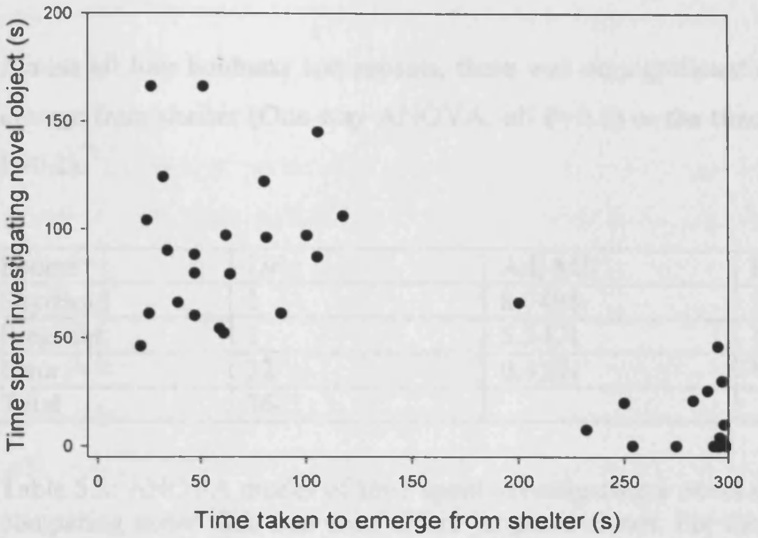

Figure 5.1: Responses of fish in the first round of boldness testing, showing a correlated response to both boldness tests. Fish that were quickest to emerge from shelter also spent most time with the novel object.

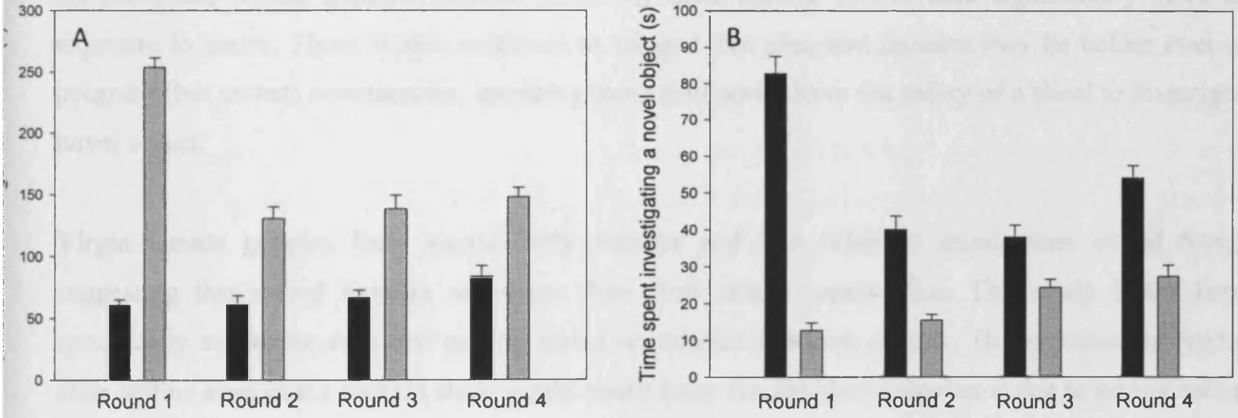

Figure 5.2: Responses of virgin and mated female guppies across the four boldness test rounds for (A) the time taken to emerge from shelter and (B) the time spent investigating a novel object. Mean ±SE shown for all. Bold fish are shown in black and shy fish in grey.

In round 4, when all fish were mated and some were pregnant, the responses of the shy mated fish to both boldness tests were correlated (Pearson correlations: r=0.586, P=0.011), but those of bold mated fish were not (r=0.223, P=0.359). Interestingly, pregnant fish spent more time investigating a novel object (Repeated Measures ANOVA: $F_{1,36}$=12.42, P=0.001, Table 5.2) than mated but non-pregnant conspecifics (Mean ±SE pregnant: 65.1±1.2 s, non-pregnant: 35.1±2.9 s), although no such difference was found for time taken to emerge from shelter (RM ANOVA: $F_{1,36}$=0.29, P=0.596). Pregnant females are thus bolder than non-pregnant females with regards to investigating novel objects but not for breaking shelter.

Across all four boldness test repeats, there was no significant effect of fish size on either the time to emerge from shelter (One way ANOVA: all $P>0.1$) or the time spent investigating a novel object (all $P>0.2$).

Source	DF	Adj MS	F	P
Shy/Bold	1	8.7498	20.34	<0.001
Pregnant	1	5.3421	12.42	0.001
Error	32	0.4301	*	*
Total	36			

Table 5.2: ANOVA model of time spent investigating a novel object for round 4 of the boldness tests, comparing mated fish that were either pregnant or not. For the starting model, standard length ('SL') of fish was treated as covariate, with shyness-boldness behavioural phenotype ('Shy/Bold'), mating status ('Virgin/Mated') and pregnancy status ('Pregnant') as factors. Only significant terms remain in the final model shown here.

5.5: Discussion

As predicted, female guppies became more shy after mating and shoaled significantly more after exposure to males. There is also evidence to suggest that pregnant females may be bolder than non-pregnant (but mated) counterparts, spending more time away from the safety of a shoal to investigate a novel object.

Virgin female guppies form significantly smaller and less cohesive shoals than mated females, suggesting that mated females are shyer than their virgin conspecifics. This study is the first to specifically assess the effect of mating status on relative boldness of fish. The reduction in boldness after mating seen in the current study could result from the females' experience due to non-consensual sneaky mating attempts, rather than from consensual mating through display and courtship. Male guppies can switch readily between both types of mating tactics (Magurran and Seghers 1990), but increase sneak mating attempts in the presence of rivals (Magurran 2005). Both in the wild and captivity, female guppies can receive up to one sneak mating attempt per minute (Magurran and Seghers 1994). Already mated female guppies may therefore be more inclined to avoid further (sneak) mating attempts, gathering in larger and more cohesive shoals than virgin females.

Our results furthermore suggest that pregnant female guppies are bolder than their mated but non-pregnant counterparts. Pregnant females left the relative safety of a shoal and spent significantly more time investigating a novel object. However, no such difference in behaviour between pregnant and non-pregnant fish was seen when emerging from a shelter. We predicted that pregnant female guppies would be less bold based on evidence from relevant studies in monkeys, which showed female baboons (*Papio anubis*), chimpanzees (*Pan troglodytes*) and pig-tailed macaques (*Macaca nemestrina*) all became less sociable and less aggressive when pregnant (Rowell 1969, Wallis and Lemmon 1986, Maestripieri 1999). The conflicting results from the present study may have partially been caused by differences in the boldness test environments. Pregnant fish may be more reluctant to leave a shelter that potentially provides a refuge from unwanted matings, and a place to give birth. However, this effect is unlikely to have occurred in the current study as we examined fish before the very final stages of pregnancy. Furthermore, the increase in size of a pregnant female, as the pregnancy continues to term, may impact on mobility. Ghalambor et al. (2004) showed that the fast-start swimming response of pregnant guppies is impaired as pregnancies continue to term. Gravid three-spined sticklebacks (*Gasterosteus aculeatus*) alter habitat use and escape behaviour compared with non-gravid females, to increase their probability of survival because they are more vulnerable to predation (Rodewald and Foster 1998). Furthermore, Brana (1993) showed that pregnant female lizards (*Podarcis muralis*) stayed closer to a refuge than males or non-pregnant females, and changed their predator-avoidance tactics from flight to crypsis. However, when faced with a novel object, perhaps pregnant females are driven to investigate it, for example, as a novel food source, due to the perceived energetic requirements of pregnancy (see Magurran 2005).

Responses of fish to both boldness tests in the first round were correlated, so that fish that were bold in one context were also bold in the other. We then observed a change in response in the subsequent three rounds of testing, most probably due to some degree of habituation to the novelty of the test environment, and/or the influence of other fish in the shoal. Similar results were shown in our earlier work (Chapter 4). However, in the present study, the degree of habituation seems lower because responses of bold and shy individuals remain significantly different from each other throughout the experimental period. This may be due to a difference in shoal composition between the current study and our previous work. The presence of intermediate conspecifics in the current study is likely to have influenced the behaviour of bold and shy shoal mates, maintaining the boldness differences between them. A few recent studies have shown the impact of the behavioural composition of a group on an individuals' boldness. Specifically, Magnhagen and Staffan (2005) and Dyer et al. (2009) showed for perch and guppies respectively, that shy fish become bolder in the presence of bold individuals, compared to when in groups of only shy conspecifics.

In summary, this study shows that mating status relates to boldness of female guppies, with virgin fish being more bold than mated counterparts. Furthermore, we provide evidence that pregnant females may also be bolder than non-pregnant (but mated) counterparts.

5.6: References

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Chapter 6: To be or not to be bold: host personality affects parasite transmission and infection status within fish shoals

6.1: Abstract

Boldness is a key element of behavioural variation in numerous animal species. Boldness influences many aspects of behaviour, but to our knowledge, the current study is the first to investigate the impact of boldness on parasite transmission and infection status within a group-living species. We screened individual guppies (*Poecilia reticulata*) for boldness using time to emerge from shelter and time spent investigating a novel object. We created artificial groups of one shy and five bold individuals (bold shoals) and one bold and five shy individuals (shy shoals). The single fish of a different behavioural phenotype in each shoal was designated the focal fish, and these individuals were infected with the ectoparasite, *Gyrodactylus turnbulli.* All fish were assessed for shoaling behaviour before and after infection of the focal individuals. As expected, we found that shy fish shoaled significantly more than bold fish. Interestingly, although shy and bold fish did not differ in the incidence of infection, shy focal fish had a significantly higher parasite loads than bold focals. A possible explanation for this is that shy focal fish had less chance of passing on their infection to bold conspecifics due to the reduced shoaling tendency of bold fish. Consequently, parasite population on these shy-focal fish continued to increase. This study highlights that besides playing a role in sexual selection, anti-predator behaviour and dispersal ability, boldness can have an important impact on parasite transmission within social, group-living species.

6.2: Introduction

One commonly considered dimension of personality is the shy-bold continuum (Wilson et al. 1993, 1994, Coleman and Wilson 1998, Gosling 2001, Bell 2007) with boldness being defined as the willingness to take risks, especially in novel situations (Wilson et al. 1994). Many species show considerable individual variation in boldness (Sih et al. 2004) and this has many possible fitness consequences, including influencing mate choice (Godin and Dugatkin 1996), lifetime reproductive success (Reale et al. 2000), anti-predator behaviour (Godin and Davies 1995) and dispersal ability (Fraser et al. 2001). More recently, it has been shown that individual personality may shape group behaviour by affecting leadership potential (Leblond and Reebs 2006, Harcourt et al. 2009), as well as influencing the degree of interactions within social networks (Pike et al. 2008). Shoaling behaviour of fish is a well known grouping behaviour, with shoals arising as a result of trade-offs made by individuals between the costs and benefits of group membership. Individuals benefit from reduced predation risk, improved food location and more time to feed (Hamilton 1971, Magurran and Pitcher 1987, Pitcher and Parrish 1993). However, as group size increases so too does competition for resources, and also the risk of acquiring parasites from infected group members (for directly transmitted parasites e.g. Alexander 1974, Cote and Poulin 1995, Chapter 7; Richards et al. in press).

Wilson et al. (1993) suggested that differences in parasite fauna of bold and shy individuals of pumpkinseed fish (*Lepomis gibbosus*) were related to how the fish interacted with their habitat that exposed them to different parasite species and loads. More recently, Natoli et al. (2005) found that a bold attitude in male urban domestic cats made them more likely to become infected with feline immunodeficiency virus (FIV), and hypothesised that natural selection favours proactive individuals despite the cost of increased disease risk, since proactive males even if FIV positive, reproduce significantly more than reactive individuals (at least before the last stage of infection). Furthermore, we have previously shown that the level of host contact is the main factor affecting ectoparasite transmission in single sex guppy (*Poecilia reticulata*) shoals (Chapter 7; Richards et al. in press). To date, there is no direct evidence showing the possible impact of host boldness on parasite transmission within social groups of animals. In particular, the transmission of ectoparasites is likely to be greatly affected by host personality, as transmission can occur directly between shoal members. Gyrodactylid monogeneans are a group of ubiquitous and highly contagious fish ectoparasites that are directly transmitted during host contact (reviewed in Bakke et al. 2007).

A particularly well studied gyrodactylid-fish system is *Gyrodactylus turnbulli* that infects the guppy. This small tropical fish is an important ecological and evolutionary model and has been widely used to explore host adaptations to natural and sexual selection pressures (e.g. Reznick et al. 1996a, b, 2001). Guppies are highly social with females displaying strong shoaling tendencies (Griffiths and Magurran 1998). In natural guppy populations, gyrodactylids are the most prevalent parasitic worms (Cable in press). They have a short generation time of 24-48h at 25°C (Scott 1982) resulting in rapid population growth. These parasites also give birth on their fish hosts where the offspring attaches alongside its parent and already contains a developing embryo (reviewed in Cable and Harris 2002).

In this study, we investigated whether boldness of guppies affected shoaling behaviour and as a result also affected gyrodactylid transmission. Previous studies (Budaev 1997, Ward et al. 2004, Dyer et al. 2009, Chapter 4) indicate that shy fish shoal more than bold individuals, and therefore as a result of this we predict shy fish would have a greater chance of acquiring a gyrodactylid infection from an infected shoal member.

6.3: Materials and methods

6.3.1: Host and parasite origins

Guppies were offspring of a wild strain originally obtained from the Tacarigua River in the Caroni drainage basin of Northern Trinidad, maintained in the laboratory since 1997. Fry were collected from the adult breeding tanks once a fortnight over 5 months and housed separately in smaller aquaria (30 x 20 x 20 cm). Upon reaching sexual maturity (6-8 weeks old) fish were transferred to larger aquaria (60 x 30 x 30 cm) where they were maintained in mixed-sex groups (1:5 male to female ratio) with about 40 fish per 60 litre aquaria, and fed on a diet of flakes (Aquarian®), *Artemia* (brine shrimp) and *Daphnia* (water flea). Fish were left for 6 months before the females were used in this study. We could more clearly test the proposed hypotheses using only female fish, because female guppies display a greater level of shoaling behaviour compared to males (Griffiths and Magurran 1998), whereas male behaviour is dominated by sexual display and the need to mate (Houde 1997). An isogenic strain of *Gyrodactylus turnbulli* (*Gt3*), originally isolated from petshop guppies in 1997, was used for all infections.

6.3.2: Experimental design

Throughout the experiment all fish were maintained under a 12 h light: 12 h dark lighting regime at 25±0.5°C. Standard tests for boldness (Brown et al. 2005, 2007) identified fish as relatively bold or shy, with shoals being composed of different ratios of bold and shy phenotypes, to more closely resemble the wild situation. Fish were infected with the parasite using a standard protocol (van Oosterhout et al. 2003). The infection period was of sufficient length to assess within-shoal parasite transmission but short enough to avoid high mortality in hosts, since gyrodactylids can cause up to 50% host mortality in laboratory infections (Houde 1997). The overall experimental design is shown in Figure 6.1. At the start of the experiment, female guppies (n=360) were individually assessed for boldness using two independent tests: (i) time to emerge from shelter and (ii) time spent investigating a novel object (Brown et al. 2007), conducted on two consecutive days (t = days 1 and 2). The experiment was conducted in three separate batches to allow collection of sufficient data replicates. Please see Chapter 4 for exact details of the boldness tests and ranking procedure.

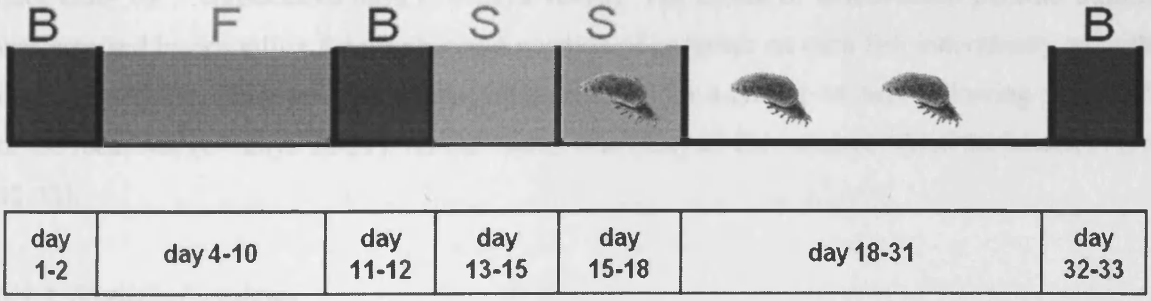

Figure 6.1: Experimental design summary. B = boldness tests; F = period of familiarisation; S = observations of shoaling behaviour; = infection of fish with *Gyrodactylus turnbulli*.

After shy-bold ranking was completed, standard length of all fish was recorded under anaesthetic (0.02% MS222) and all fish were injected with a Visible Implant Elastomer (VIE) mark into the tail muscle (t = day 3). Fish were then allowed to recover in individual 1 litre pots for 1 h before being placed into groups (6 individuals per group) in test tanks (30 x 20 x 20 cm), and allowed to acclimate for a further 7 d (t = days 4-10). Standard length was controlled by size matching all individuals within a tank and by only using fish within an 18-26 mm size range. Each aquarium contained an air supply and a small flower pot serving as a refuge. The location of guppy tanks was randomised, and guppies in different aquaria were visually and physically isolated from one another. Shoals differed in their relative composition of bold and shy phenotypes: (i) Bold (5 bold and 1 shy fish) and (ii) Shy (5 shy and 1 bold fish), with 10 replicates of each. The single guppy in each tank that had a different phenotype to its conspecifics was assigned as the focal fish. This particular shoal composition enabled us to specifically assess the impact of behavioural phenotype on parasite transmission, since we could carry out behavioural observations and parasite screening on the single bold or shy fish in each shoal, without its behaviour being affected by shoal mates with a similar behavioural phenotype. Note that this design does not allow for a direct comparison between the two types of focal fish (i.e. the shy and the bold guppies), because their biotic environment (shoal mates) co-varied. Rather, it allows us to evaluate the effects of being bold or shy when living in a group of individuals with an opposite behavioural phenotype.

After acclimation, all fish were re-tested for boldness as previously described (t = days 11-12). On days 13-15, the shoaling behaviour of each fish in each group was observed once daily for 3 consecutive days. At the end of day 15, all fish were removed from the test aquaria and kept individually in one litre containers (to prevent restructuring of social groups) while the focal fish from each test tank was infected with 4 individuals of *G. turnbulli*. Details of the infection procedure and shoaling observations are given in Chapter 7; Richards et al. (in press). Following infection, all fish were returned to their test tanks (t = day 16) and the shoaling behaviour of fish was again observed once daily for 3 consecutive days (t = days 16-18). The extent of within-shoal parasite transmission was assessed by recording the number and position of parasites on each fish individually anaesthetised in 0.02% MS222. This was performed every other day for a further 14 days following initial infection of the focal fish (t = days 18-31). At the end of this time, all fish were re-tested for boldness (t = days 32-33).

6.3.3: Statistical analyses

Preliminary analysis revealed that the shoaling data, parasite loads and boldness data were not normally distributed. The data was natural log- or square root-transformed to attain normality of residuals (assessed using Anderson-Darling tests). Furthermore, preliminary analysis showed there were no significant differences between different batches of the experiment in boldness, parasite growth and transmission, and in fish shoaling behaviour. Hence, the factor 'Batches' was dropped during the backwards elimination of non-significant factors in the multivariate analyses (see below). For the shoaling parameters, the data across all trials was pooled and analysed to test whether within-shoal parasite transmission was dependent on an individual's shy-bold phenotype or density of parasite load.

A Repeated Measures Analysis of Variance (RM ANOVA) with nesting was used to test for differences between bold and shy fish in their ability to spread infection to conspecifics. Day of experiment ('Day') and standard length of fish ('SL') were used as covariates, and infection status ('Parasitised') was crossed with shy-bold phenotype of fish ('SBF') as factors. In addition, 'SBF' was nested within shy-bold 'phenotype' of the tank ('SBT') as another factor. Differences in initial parasite loads on bold and shy fish were examined using Kruskal-Wallis non-parametric tests. A parasite load is defined as the total number of parasites per fish host. A binary logistic regression analysis (logit) was used with a dichotomous dependent variable, infected or not infected (coded as '1' and '0,' respectively), to test whether the infection status of fish at day 7 of the experimental period was associated with parasite population growth on focal fish ('Focal Growth') and shy-bold phenotype ('SB') of the guppy. The model uses 'SB' as a fixed factor crossed with 'Focal growth' as covariate.

The responses to the boldness tests were analysed for consistency of response between the two different tests and between the three test rounds. Pearson correlations were used to test for differences between the bold and shy individuals, and the consistency of responses to the boldness tests were expressed in the correlation coefficient 'r.' In total, 11% of fish tested failed to emerge from the shelter and 12% failed to leave the safety of the shoal and inspect the novel object and these individuals were excluded from further analysis. For all multivariate analyses, variables that did not explain significant variation were excluded from the models using a backwards stepwise elimination approach. All statistical analyses were performed using Minitab 15.

6.4: Results

Shoals were composed of a single focal fish with a diametrically opposite level of boldness to the other (non-focal) shoal members. As predicted, shy guppies shoaled significantly more than their bold counterparts, and this resulted in shy non-focal fish being significantly more likely to acquire a parasite infection from an infected tank mate compared to bold conspecifics. Also, shy focal fish carried higher parasite loads than bold focals. There was less chance of shy focals transferring parasites to bold tank mates due to the reduced shoaling behaviour of bold fish.

6.4.1: Shoaling behaviour and impact of parasitism

For focal fish, bold individuals seemed to be part of larger shoals (Repeated Measures nested ANOVA: $F_{2,851}=7.69$, $P=0.001$) than shy focals, but this was actually due to the higher shoaling tendency of shy non-focals associating with the single bold focal individual. Focal fish behaviour was unaffected by their infection status (all 3 shoaling parameters: $P>0.3$). When considering non-focal fish, shy individuals formed significantly larger (RM ANOVA: $F_{1,851}=16.34$, $P<0.001$) and more cohesive ($F_{1,851}=5.73$, $P=0.017$) shoals than bold non-focal fish. These results seem to apparently contradict the statistics on shoal size of focal fish. However, it is explained by the fact that the shy non-focal guppies were together in groups of 5 in single tanks. Hence, their shoaling aggregation was larger and significantly more cohesive than that observed in tanks containing 5 bold non-focal fish. Shy non-focals also spent more time shoaling ($F_{1,851}=8.06$, $P=0.005$) than their bold counterparts. There was also a significant effect of parasitism on shoaling behaviour of non-focals, with uninfected fish forming larger shoals than infected conspecifics ($F_{1,709}=8.38$, $P=0.004$).

6.4.2: Parasite transmission

There was no difference in initial parasite loads on bold or shy focal fish (mean number of parasites ±SE bold fish: 4.7±0.21; shy fish 4.6±0.29; Kruskal-Wallis: H=0.04, d.f.=1, P=0.841). In both bold and shy tanks, transmission of parasites to at least one other conspecific took place between days 3 and 7. Transmission occurred in 20 out of 23 tanks, with infection reaching epidemic levels (i.e. parasite loads >100 worms per individual) in 18 of these tanks. Out of the remaining 5 tanks, infection failed due to a combination of failed parasite population growth and little or no transmission to other shoal members. No fish presented clamped fins (secondary pathology characteristic of *G. turnbulli* infection) during the experiment but 6 fish deaths occurred. Focal fish were infected with an average of (mean ±SE) 17±3 worms across the 14-day infection period, with average parasite loads across all tanks peaking at day 10.

Parasite population growth on focal fish significantly affected the likelihood of non-focals acquiring an infection (Binary Logistic Regression: Z=2.65, P=0.008, Table 6.1). This means that a vigorous infection that resulted in a rapid increase in parasite numbers on the focal fish caused a faster outbreak of the infection with a higher proportion of non-focal fish becoming infected. There was, however, no significant difference between shy and bold fish in the incidence of infection.

Predictor	Coef	SE Coef	Z	P	OddsRatio	95% CI
Constant	0.2309	0.8978	0.26	0.797	*	*
Focal Growth	0.1207	0.0456	2.65	0.008	1.13	1.03-1.23

Table 6.1: Binary logistic regression for infection status (0 = not infected, 1 = infected), with shy-bold phenotype ('SB') of the host as factor crossed with parasite population growth on focal fish ('Focal Growth') as covariate. A vigorous infection that resulted in a rapid increase in parasite numbers on the focal fish caused a faster outbreak of the infection with a higher proportion of non-focal fish becoming infected. There was no significant difference between shy and bold fish in the incidence of infection. Only significant terms are reported.

By day 7 of the infection period, shy focals had a slightly higher mean (±SE) parasite load (27.8±3 worms) than bold focals (24±10 worms), and by day 14 this difference was much greater (shy focals: 33.4±8.7 worms, bold focals: 13.8±2.3 worms). This resulted in a significantly greater increase in parasite loads on both shy focal (RM nested ANOVA: $F_{2,75}$=4.52, P=0.014) and non-focal ($F_{1,75}$=4.60, P=0.035) fish across the 14-day infection period (Table 6.2, Figure 6.2). However, there was no difference in day to first infection between the shy and bold non-focals across all tanks (Kruskal-Wallis: H=0.28, d.f.=1, P=0.598).

Source	DF	Adj MS	F	P
SBF	1	5.106	4.60	0.035
SBT(SBF)	2	5.008	4.52	0.014
Error	71	1.325	*	*
Total	75			

Table 6.2: General Linear Model for the increase in parasite load on all fish (focal and non-focal) between day 0 and day 14 of the experimental period, using 'growth in parasite load' as response variable. The starting model contained shy/bold phenotype of fish ('SBF') and shy/bold 'phenotype' of the tank ('SBT') as factors, with 'SBF' being nested within 'SBT'. Fish size ('SL') was included as a covariate. Only significant terms are reported.

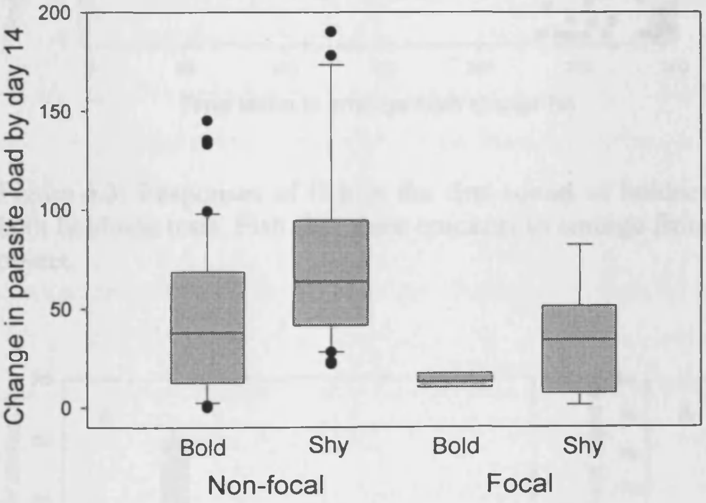

Figure 6.2: The increase in parasite loads on bold and shy fish, both non-focal and focal individuals, between day 0 and 14 of the experimental period. The dots represent outliers; the bars, the lower and upper limits; the box represents the first and third quartile value with the median.

6.4.3: Boldness tests

Fish were tested for boldness a total of 3 times during the 33-day experimental period (referred to here as rounds 1, 2 and 3). The first round of testing identified individuals as either bold or shy, since these fish had significantly different responses in both boldness tests (T-test: Time taken to emerge T=14.76, P<0.001; Time spent with novel object T=9.96, P<0.001), with 24% of fish tested being ranked as bold, and 24% ranked as shy. The responses of fish to both tests in the first round were correlated

(Pearson correlations: r=0.336, P<0.001), so fish that were quickest to emerge from shelter also spent the most time with the novel object (Figure 6.3). However, as previously observed, and discussed in Chapter 4, this changes across the observation period with shy fish becoming more bold, and bold fish becoming more shy (Figure 6.4), which means fish that were bold in one test were not necessarily bold in the second.

There was no effect of fish size on any of the response variables tested for shoaling behaviour, parasite population growth or transmission, or boldness tests (all P>0.1).

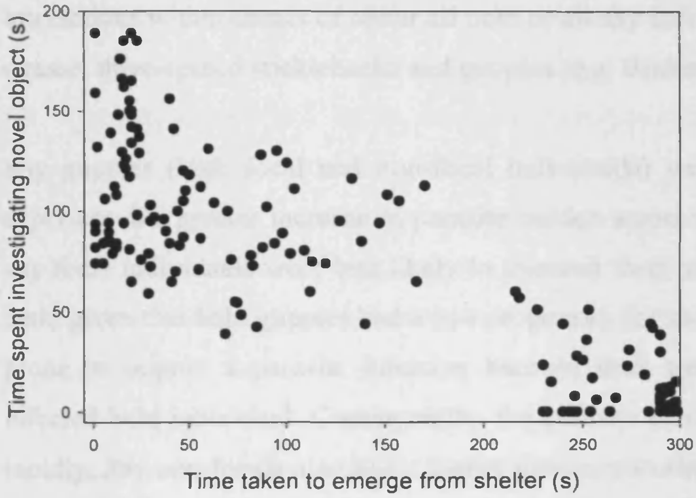

Figure 6.3: Responses of fish in the first round of boldness testing, showing a correlated response to both boldness tests. Fish that were quickest to emerge from shelter also spent most time with the novel object.

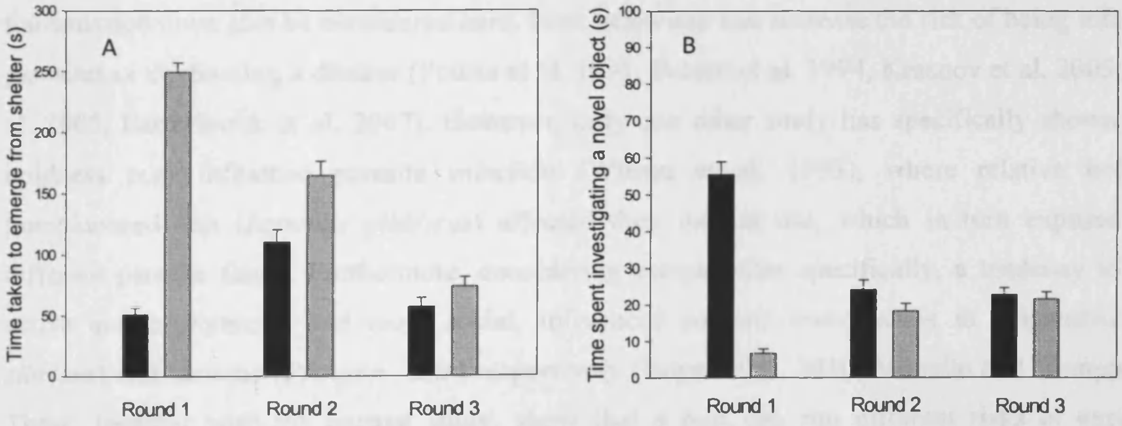

Figure 6.4: Responses of bold and shy fish across the three boldness test rounds for (A) the time taken to emerge from shelter and (B) the time spent investigating a novel object. Mean ±SE shown for all. Bold fish are shown in black and shy fish in grey.

6.5: Discussion

Behavioural phenotype significantly affected the shoaling behaviour of guppies, with shy fish shoaling significantly more than their bold counterparts, a finding consistent with previous studies (Chapter 4). As a consequence of the higher shoaling propensity, shy fish had a higher parasite load across the infection period compared to bold conspecifics. This is the first study to demonstrate the impact of boldness on parasite transmission within a group-living species.

Shy guppies shoaled significantly more than their bold counterparts, forming larger and more cohesive groups that spent more time shoaling. This agrees with our previous work (Chapter 4) on social interactions within shoals of either all bold or all shy individuals, as well as other studies on European wrasse, three-spined sticklebacks and guppies (e.g. Budaev 1997, Ward et al. 2004, Dyer et al. 2009).

Shy guppies (both focal and non-focal individuals) were infected with higher parasite loads, and experienced a greater increase in parasite burden across the infection period than bold fish. Possibly, shy focal individuals were less likely to transmit their parasite to the bold non-focal guppies in their tank, given that bold guppies had a low propensity for shoaling. The shy non-focal guppies were more prone to acquire a parasite infection because their tendency to shoal increased contact with the infected bold individual. Consequently, the parasite number of the bold focal fish decreased relative rapidly. Shy non-focals also had a higher tendency to shoal than their bold counterparts, so these fish may also transfer parasites to a greater extent between themselves compared to bold non-focals, after initial infection.

Host contact is a major factor in facilitating gyrodactylid transmission within shoals of female guppies (Chapter 7; Richards et al. in press). The interaction between social contact, personality and parasite transmission must also be considered here. Host behaviour can increase the risk of being infected with parasites or contracting a disease (Poulin et al. 1991, Brown et al. 1994, Krasnov et al. 2005, Natoli et al. 2005, Easterbrook et al. 2007). However, only one other study has specifically shown that host boldness may influence parasite infection (Wilson et al. 1993), where relative boldness in pumpkinseed fish (*Lepomis gibbosus*) affected their habitat use, which in turn exposed them to different parasite fauna. Furthermore, considering ectoparasites specifically, a tendency to be more active and exploratory, and more social, influenced parasite transmission in chipmunks (*Tamius sibricus*) and racoons (*Procyon lotor*) respectively (Boyer et al. 2010, Monello and Gompper 2010). These, together with the current study, show that a host can run different risks of encountering parasites according to its personality.

We observed that non-focal individuals altered their shoaling behaviour following introduction of the infected focal fish, forming smaller shoals. Other studies have shown aversion behaviour by fish in order to limit contact with parasites, for example, fish avoid joining shoals that contain parasitised members (e.g. Krause and Godin 1996, Barber et al. 1998). Fish also reject infected sexual partners (e.g. Milinski and Bakker 1990) and avoid particular habitats associated with infection risk (e.g. Poulin and Fitzgerald 1989). More recently, Tobler and Schlupp (2008) provided evidence that both parasitised and unparasitised cave mollies (*Gambusia affinis*) prefer to shoal with uninfected conspecifics. However, we have previously found that non-focal guppies did not alter their shoaling behaviour in the presence of an infected tank mate (Chapter 7; Richards et al. in press). Disparity between the current study and our previous work (Chapter 7; Richards et al. in press) could be due to the fact we used different guppy populations in both studies. Chapter 7; Richards et al. (in press) examined the shoaling behaviour in an ornamental (petshop) strain, while here, we examined the descendants of a wild population of the Tacarigua River that were maintained in a large stock tank in the laboratory since 1997. The ornamental strain has been inbred and shows reduced level of genetic variation (van Oosterhout et al. 2006). It may also have lost the appropriate aversion response to infected conspecifics common in many wild fish species (reviewed in Barber et al. 2000). This explanation is concordant with previous studies which reported that parasite-mediated selection and parasite resistance can be much reduced in captivity particularly in inbred populations (van Oosterhout et al. 2007).

In summary, we have shown the impact of individual variation in boldness on shoaling behaviour and its resultant effect on parasite transmission within a group-living host species. We show that shy fish have a higher tendency to shoal and as a result, carry greater parasite loads and are more at risk to contract novel infections than bold counterparts.

6.6: References

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Chapter 7: Sex-specific differences in shoaling affect parasite transmission in guppies

7.1: Abstract

Individuals have to trade-off the costs and benefits of group membership during shoaling behaviour. Shoaling can increase the risk of parasite transmission, but this cost has rarely been quantified experimentally. Guppies (*Poecilia reticulata*) are a model system for behavioural studies, and they are commonly infected by gyrodactylid parasites, notorious fish pathogens that are directly transmitted between guppy hosts. Parasite transmission in single sex shoals of male and female guppies were observed using an experimental infection of *Gyrodactylus turnbulli*. Parasite transmission was affected by sex-specific differences in host behaviour, and significantly more parasites were transmitted when fish had more frequent and more prolonged contact with each other. Females shoaled significantly more than males and had a four times higher risk to contract an infection. Intersexual differences in host behaviours such as shoaling are driven by differences in natural and sexual selection experienced by both sexes. Here we show that the potential benefits of an increased shoaling tendency are traded off against increased risks of contracting an infectious parasite in a group-living species.

7.2: Introduction

Social aggregation of fish, or shoaling behaviour, involves individuals trading-off the costs and benefits of group membership (Pitcher and Parrish 1993). Shoaling is thought to have evolved as an antipredator response (Helfman et al. 1997), but also provides foraging benefits as individuals in shoals can allocate more time to feeding (Magurran and Pitcher 1983) and profit from improved food location (Pitcher et al. 1982). Benefits are maximised with increasing shoal size through, for example, improved vigilance and attack dilution (e.g. Magurran et al. 1985), as well as increasing levels of coordination and phenotypic homogeneity within the group (Barber and Huntingford 1996). However, competition for resources also increases with group size (Krause and Ruxton 2002), and a number of studies have documented an increase in risk-taking behaviour with increased shoal size (e.g. Magurran and Pitcher 1983).

Parasitism may impose yet another cost of group living, since for uninfected individuals the probability of acquiring a directly transmitted parasite increases with the formation of a group (Dugatkin at al. 1994, Barber et al. 2000). Indeed, there is strong evidence of a positive correlation between host group size and parasite prevalence and load (Brown and Brown 1986, Côté and Poulin 1995). Many parasites have been assessed for their impact on fish behaviour (reviewed in Barber et al. 2000) but only a limited number of studies have considered the reverse situation, i.e. the direct impact of shoaling behaviour on parasite transmission (Poulin and Fitzgerald 1989a, Poulin 1991, Ranta

1992). This latter study (Ranta 1992) found that for some groups of parasites, shoaling host species harboured significantly greater parasite diversity than solitary host species, but this pattern did not hold for directly transmitted parasites. It is important to assess the impact of host shoaling behaviour on this class of parasite since they are likely to be greatly influenced by the shoaling behaviour of their hosts, as transmission can occur directly between shoal or group members. A ubiquitous and highly contagious group of fish parasites are the gyrodactylid monogeneans. They are directly transmitted but the possible influence of host shoaling behaviour on their transmission has never been examined.

A particularly well studied host-parasite system is the guppy (*Poecilia reticulata*) and its infectious parasite, *Gyrodactylus turnbulli*. Guppies are small tropical fish and an important ecological and evolutionary model. They have been widely used to explore host adaptations to natural and sexual selection pressures (e.g. Reznick et al. 1996a, b, 2001). Guppies are sexually dimorphic, with males being more colourful and smaller than females, and there are marked behavioural differences between the sexes (Houde 1997). They breed throughout the year and males spend a large proportion of time in courtship displays and sneaky mating attempts. Female guppies shoal more than males and show a greater preference for associating with their familiar shoal mates (Griffiths and Magurran 1998). Furthermore, guppies living in a high predation area shoal significantly more than fish in low predation streams and this may facilitate interhost transmission of ectoparasites (Endler 1995).

In natural guppy populations, gyrodactylids are the most prevalent parasitic worms (Cable in press). Amongst wild caught Trinidadian guppies, the parasite load of *Gyrodactylus* spp. is generally less than 10 worms/host (Harris and Lyles 1992), but can be as high as 100 parasites (van Oosterhout et al. 2006a). Larger guppies tend to harbour more parasites (Cable and van Oosterhout 2007a), and the maximum parasite load increases exponentially with increased host body size (van Oosterhout et al. 2008). These parasites give birth to a fully-grown offspring, which attaches to the host alongside its parent and already contains a developing embryo (reviewed in Cable and Harris 2002). They also have a short generation time of just 24h (at 25°C) (Scott 1982) resulting in rapid population growth. Gyrodactylids can be an important selective force in natural guppy populations (van Oosterhout et al. 2007a) and they adversely affect male colouration in guppies (Houde and Torio 1992). Infection also causes a number of behavioural changes in the host, such as a reduced feeding rate (van Oosterhout et al. 2003) and a reduced ability to compete for food with uninfected conspecifics (Kolluru et al. 2009). Also, fish with heavy infections can develop clamped fins (Cable et al. 2002), which presumably reduces swimming performance.

Here, we investigate whether the degree of host contact in single-sex shoals influences gyrodactylid transmission, and whether guppy behaviour is influenced by the presence of infected conspecifics.

7.3: Materials and Methods

7.3.1: Host and parasite origins

Ornamental (petshop) guppies (n=108) were purchased from a UK commercial supplier. On arrival at Cardiff University all fish were briefly anaesthetised in 0.02% MS222 and externally screened for visible parasites under a stereo-microscope with fibre optic illumination. All fish were infected with *Gyrodactylus* spp. but were subsequently treated with 0.2% levamisole (Norbrook, UK) and screened clear for visible parasites at least three times (see Schelkle et al. 2009) and then left for three to four months before use. The guppies were maintained under a 12 h light: 12 h dark lighting regime in mixed-sex groups (1:5 male to female ratio) with about 30 fish per aquaria (45 x 45 x 120 cm), and fed on a diet of flakes (Aquarian®) and frozen bloodworm. An isogenic strain of *Gyrodactylus turnbulli* (strain *Gt3*), originally isolated from ornamental guppies in 1997, was used for all infections. All experiments were conducted at 25±1°C.

7.3.2: Experimental design

Single sex groups of male or female guppies (6 individuals per group) were placed in test tanks (60 x 30 x 40 cm), and allowed to acclimate for 5 d. Standard length was controlled by size matching all individuals within a tank and by only using fish within a 20 - 30 mm size range. Male guppies did not have very large fan or forked tails. Each aquarium contained an air supply and water filter. The location of male and female guppy tanks was randomised, and guppies in different aquaria were visually and physically isolated from one another. A single guppy in each tank was randomly assigned as the focal fish. This focal guppy was recognisable by its colour pattern (in males) or pigment patch on the caudal fin (in females). The experiment was conducted in three separate batches for logistical reasons.

After acclimation, the shoaling behaviour of each group was observed once daily for 3 consecutive days (t = days 1-3). All observation periods lasted 15 min per group (5 min in total for each shoaling behaviour parameter). During each observation period, 10 measurements of nearest neighbour distance were made for each focal fish, and for one, randomly chosen, non-focal fish per tank. A further 10 measurements of shoal size were recorded, by counting the number of fish in the largest shoal at the time of observation. The time interval between each of these measures was 30 s, which was sufficient to make consecutive observations independent. Also the time spent shoaling by both focal and non-focal fish was measured over 5 min. Horizontal and vertical lines drawn every 2 cm on three sides (back and two sides) of each test aquaria facilitated the estimation of between-individual distances, as all shoaling behaviour measurements were evaluated in three-dimensional space. Shoal members were defined as fish within 4 body lengths of one another (Pitcher and Parrish 1993).

At the end of day 3, all fish were removed from the test aquaria and kept individually in one litre containers (to prevent restructuring of social groups) while the focal fish from each test tank was infected with *G. turnbulli*. Infection was achieved by anaesthetising each focal individual and allowing them to contact a euthanized heavily infected same-sex fish (donor) in a watch glass containing 0.02% MS222 on the stage of a stereo-microscope. The focal fish was removed once ca. 100 worms had transferred from the donor. Success of parasite transfer was estimated after 24 h by confining each focal fish in a crystallizing dish (5 cm diameter) containing dechlorinated water on the stage of a stereo-microscope and counting the number of parasites under fibre optic illumination. Non-focal fish were sham infected under anaesthetic using a similar procedure. Following infection, all fish were returned to their test tank (t = day 4). There was no evidence of secondary pathology (such as clamped fins or reduced mobility) among focal fish at this time.

Shoaling behaviour was again measured for 3 consecutive days following infection. On the first day post-infection (t = day 4), observations were made twice: once in the morning at 10:00 (1 h after the focal fish were returned to their home tank) and once at 14:00. During days 5-6, observations were made once daily. Trials (one male tank and one female tank) were repeated 9 times (18 tanks in total) and no fish was tested more than once. At the end of each trial the extent of within-shoal parasite transmission was assessed by recording the number and position of parasites on each individual fish anaesthetised in 0.02% MS222. No fish deaths occurred during the experiment and no fish presented with clamped fins (pathology characteristic of *G. turnbulli* infections) on day 6.

7.3.3: Statistical analyses

The data from all the trials was pooled and analysed to test whether within-shoal parasite transmission was dependent on an individual's behaviour, sex or density of parasite load. A preliminary analysis showed that the parasite loads and shoaling parameters were not normally distributed, and therefore the data was natural log-transformed. This resulted in normality of residuals, established using Anderson-Darling tests. Furthermore, preliminary analysis showed that there were no significant differences in fish shoaling behaviour, parasite growth or transmission between trials ('Tanks') and batches ('Batch') of the experiment. A Repeated Measures ANOVA was used to analyse whether differences in the three parameters of shoaling behaviour were explained by the day of the experiment, sex and infection status of the guppy. Day of experiment ('Day') was used as a covariate and infection status ('Parasitised') was crossed with sex ('Sex') as factors.

All guppies were assessed for parasite burdens at the end of the 3-day infection period. A parasite burden is defined as the total number of parasites per fish host. Differences in initial and final parasite burdens were assessed using Kruskal-Wallis tests. Comparisons between males and females in their ability to spread infection to conspecifics were tested using chi-square analysis, with the standard errors calculated using jackknife analysis. This was done by comparing the numbers of male and female non-focal fish that were carrying a naturally acquired gyrodactylid infection at the end of the experimental period, distinguishing four categories: (i) males that carried a parasite burden ('male infected'), (ii) uninfected males ('male clean'), (iii) females that carried a parasite burden ('female infected') and (iv) uninfected females ('female clean'). Focal fish were excluded from this analysis, as these fish were experimentally infected.

A binary logistic regression analysis (logit) was used with a dichotomous dependent variable, infected or not infected (coded as '1' and '0,' respectively), to test whether the infection status of fish at the end of the experimental period was associated with initial parasite load of focal fish ('Gyrostart') and sex ('Sex') of the guppy. The model uses 'Sex' as a fixed factor crossed with 'Gyrostart' as covariate. We used an iterative re-weighted least squares algorithm to obtain maximum-likelihood estimates of all parameters. The log-likelihood was used to test whether the coefficients of the predictors were significantly different from zero. A logit link function was employed to calculate the odds ratio and its 95% confidence interval (CI). The odds ratio represents the ratio in which an event occurs relative to a reference event. All statistical analyses were performed using Minitab 15.

7.4: Results

As predicted from Griffiths and Magurran (1998), female guppies shoaled significantly more than males. This resulted in focal females passing on their infection to non-focal conspecifics more readily than focal males in single sex shoals.

7.4.1: Shoaling behaviour

First we analysed the correlation between our three shoaling parameters (shoal size, average distance between nearest neighbours and the duration shoaling) separately for male and females guppies. These parameters are strongly correlated to one another for female fish but not for males (Pearson's correlation analyses: females all $r \geq 0.343$, all $P \leq 0.006$, males all $r \leq 0.152$, all $P \geq 0.235$, Figure 7.1 A-D).

Female guppies formed larger shoals than males with a significantly higher 'average shoal size' (Repeated measures ANOVA: $F_{1,121}=25.16$, $P<0.001$), and both focal and non-focal females formed significantly tighter shoals separated by shorter 'nearest neighbour distances' compared to males (Focal fish: $F_{1,103}=6.47$, $P=0.012$; Non-focal fish: $F_{1,121}=25.28$, $P<0.001$) (Figure 7.2A). Focal female guppies spent an average of 155.3±8.3 s per 5 min shoaling compared to 141.0±8.1 s for focal males, although this difference was not significant ($F_{1,121}=1.68$, $P=0.099$). Finally, non-focal females spent significantly longer shoaling with conspecifics than non-focal males ($F_{1,121}=4.38$, $P=0.038$) (Figure 7.2B).

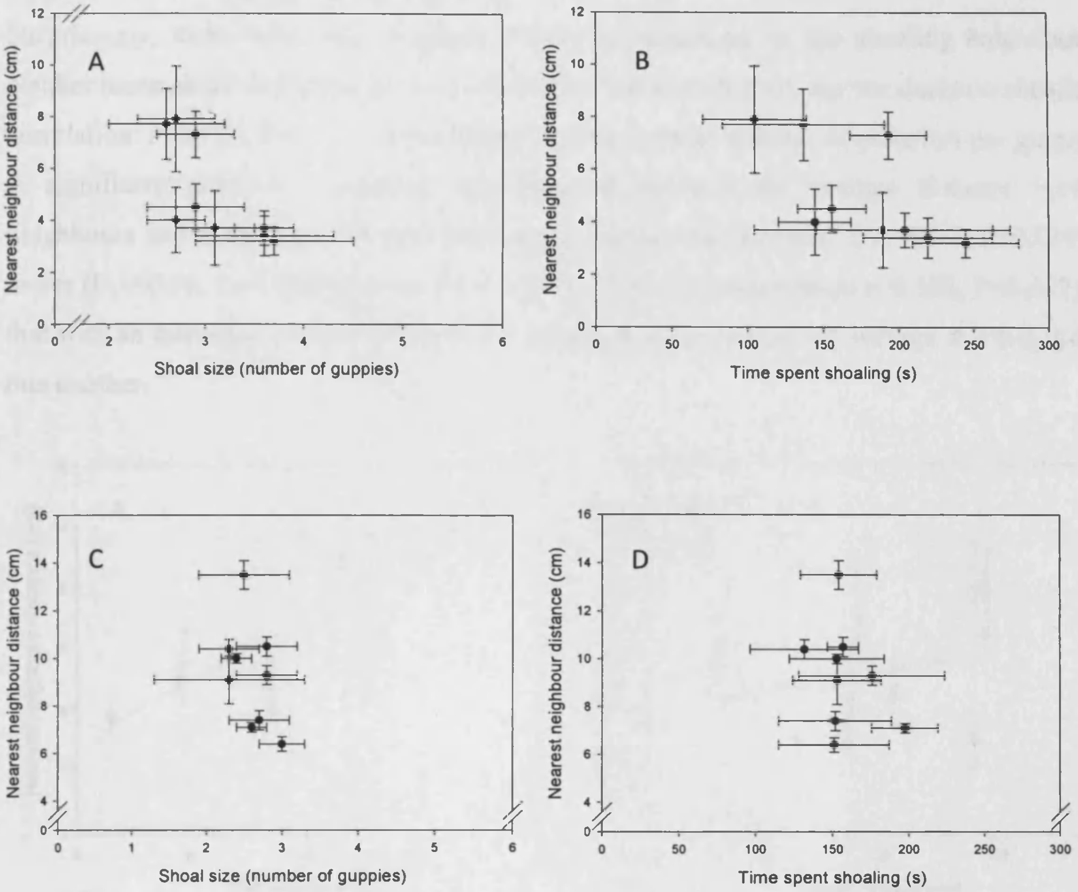

Figure 7.1: Correlation between the nearest neighbour distance and (A) the number of guppies per shoal, and (B) the time spent shoaling for female guppies. (C) and (D) represent the same correlations respectively for male guppies. Shown are the mean ±SE.

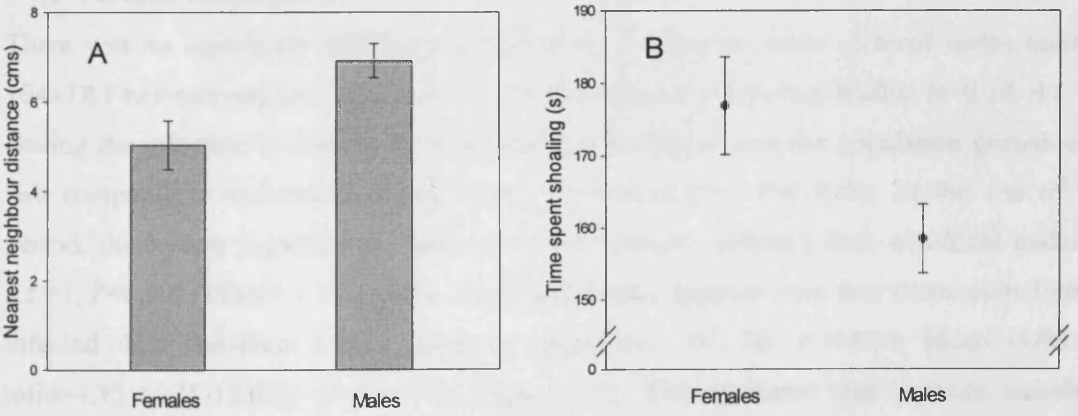

Figure 7.2: (A) Mean ±SE nearest neighbour distance of non-focal female and male guppies, pooled for Days 1-6. (B) Mean ±SE time spent shoaling by non-focal female and male guppies pooled for Days 1-6.

7.4.2: The effects of parasites on shoaling

Surprisingly, there were only marginal effects of parasitism on the shoaling behaviour in guppies. Neither mean shoal size (Pearson's correlation: r=0.190, P=0.450), nor the duration shoaling (Pearson's correlation: r=0.427, P=0.077) was affected by the average number of parasites per guppy in the tank. A significant positive correlation was detected between the average distance between nearest neighbours and mean parasite load for female guppies (Regression: $F_{1,7}$=9.27, P=0.019) but not for males ($F_{1,7}$=0.56, P=0.480) (Figure 7.3A and B) (Pearson's correlation: r=0.506, P=0.032). This shows that with an increased number of parasites, female guppies remain on average at a larger distance from one another.

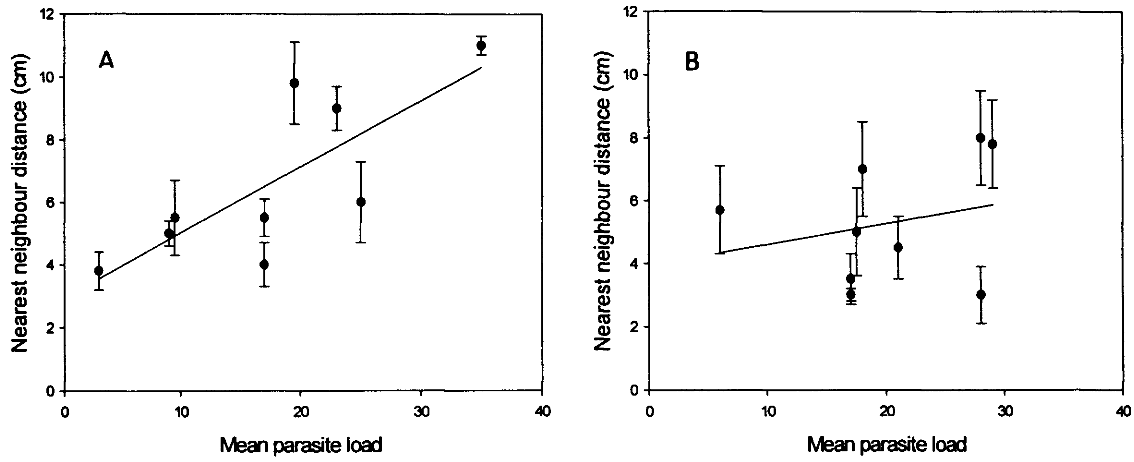

Figure 7.3. Nearest neighbour distance and parasite load for female (A) and male (B) guppies. Shown are the mean ±SE for both nearest neighbour distance and parasite load, averaged across the individuals within a tank.

7.4.3: Parasite transmission

There was no significant difference between initial parasite loads of focal males (mean load ±SE: 104±18.1 worms/fish) and females (117±16.1 worms/fish) (Kruskal-Wallis: H=0.34, d.f.=1, P=0.562). During the infection period, there was significantly higher parasite population growth on male focal fish compared to females (Kruskal-Wallis: H=5.48, d.f.=1, P=0.019). At the end of the infection period, there were significantly more non-focal females infected than non-focal males (χ^2=13.264, d.f.=1, P<0.001) (Table 7.1). Indeed, non-focal female guppies were four times more likely to become infected than non-focal males (Logistic regression: Z=2.46, P=0.014: Mean (5-95% CI) Odds ratio=4.33 (1.35-13.92)) (Table 7.2; Figure 7.4). This indicates that parasite transfer was more efficient between female guppies (female to female) than between males (male to male).

	Females	Males	Total
Clean	10	27	37
Infected	35	18	53
Total	45	45	90

Table 7.1: Contingency table with counts of clean (non-infected) and infected non-focal female and male guppies, at the end of the 3-day infection period.

Predictor	Coef	StDev	Z	P	OddsRatio	95% CI
Constant	-0.1220	0.4668	-0.26	0.794	*	*
Sex	1.4667	0.5954	2.46	0.014	4.33	1.35 - 13.92
GyroStart	0.0006	0.0019	0.32	0.747	1.00	1.00 - 1.00
Sex × GyroStart	0.0029	0.0043	0.70	0.486	1.00	0.99 - 1.01

Table 7.2: Binary logistic regression for infection status (0 - clean, 1 – infected) with sex of the host ('Sex') as a factor crossed with the initial tank burden ('GyroStart') as covariate.

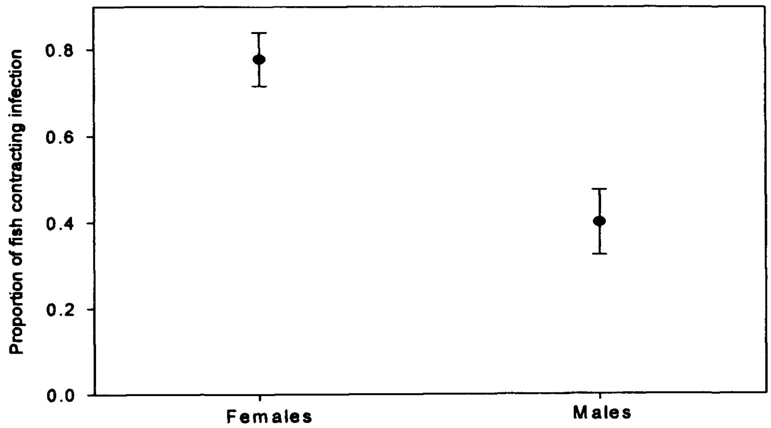

Figure 7.4: Proportion (mean ±SE) of non-focal male and female guppies contracting a *Gyrodactylus turnbulli* infection.

7.5: Discussion

There was a significant difference between the sexes in the degree of contact within single sex shoals of male and female guppies. Two of the three measures of shoaling behaviour (distance to nearest neighbour and time spent shoaling) used in the current study indicated that females shoaled significantly more than males. We believe that this sex-specific difference in shoaling behaviour explains why females were four times more likely to become infected with the ectoparasite, *Gyrodactylus turnbulli* than males. Males, on the other hand, sustained parasites with the fastest population growth rate, possibly because compared to females, they were less likely to lose parasites by transmission to conspecifics.

Contrary to our expectations, non-focal individuals did not alter their shoaling behaviour following introduction of the infected focal fish. Previous studies have shown that fish exhibit aversion behaviour to limit contact with parasites and avoid joining shoals that contain parasitised members (e.g. Krause and Godin 1996, Barber et al. 1998). Recently, Tobler and Schlupp (2008) provided evidence that both parasitised and unparasitised cave mollies (*Gambusia affinis*) prefer to shoal with uninfected conspecifics. Fish are also known to avoid particular types of habitat associated with infection risk (e.g. Poulin and Fitzgerald 1989b) as well as rejecting infected sexual partners (e.g. Milinski and Bakker 1990). Parasite-mediated selection can be much reduced in captivity (van Oosterhout et al. 2006b, 2007b), and hence, the ornamental (petshop) guppies used in the current study may have lost the appropriate aversion response to infected conspecifics common in many wild fish species (reviewed in Barber et al. 2000). The guppies used in our experiment may have been in captivity for as long as 300 generations (van Oosterhout et al. 2006b), and as a result, they could have lost the appropriate behavioural response to parasite infection. We believe that this crucial difference can explain the disparity between our results and previous studies on the effects of parasitism on shoaling.

The elevated rate of parasite transfer in females appeared to be due to increased host contact rather than faster parasite population growth rate (Cable and van Oosterhout 2007a, b). Sex-specific differences in shoaling (Griffiths and Magurran 1998) resulted in more host contact between females, which increased female-to-female parasite transmission. Of course, other factors influencing parasite transmission rate within fish shoals need to be considered. Fish populations may differ in predation risk, mating and/or foraging behaviour, which in turn can affect shoaling behaviour and thereby parasite transmission (e.g. Kolluru et al. 2005, 2009). For example, male guppies are known to have a lower propensity to shoal compared to females, instead preferring to move between shoals of female guppies searching for mating opportunities (Croft et al. 2003). Male behaviour could transfer parasites between shoals of females in a single pool or section of stream. Also, male behaviour may vary between different guppy populations, which could result in differences in parasite transmission between populations. Parasite transmission may also be linked with differences between wild and captive-bred fish, a point made previously but equally valid here. For example, van Oosterhout et al. (2007b) found dramatic differences in parasite load between wild and captive-bred fish when they occurred in the same (semi-natural) conditions in Trinidad, with 94% of captive-bred fish carrying an infection compared to only 40% of individuals in a wild population.

In summary, we have shown the impact of sex-specific differences in shoaling behaviour on parasite transmission within a group-living host species. We show that females have a higher tendency to shoal than males, and importantly, quantify a fitness-cost of shoaling. We demonstrate that guppies are exposed to a considerable risk of contracting a gyrodactylid infection by shoaling with parasitised conspecifics. We hope in future to more clearly separate the influence of sex and host behaviour on the spread of parasites within a host group, and explicitly test the relationship between shoaling and parasite transmission.

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Chapter 8: Transmission of the monogenean ectoparasite *Gyrodactylus turnbulli* is determined by the level of host social contact rather than a sex bias

8.1: Abstract

It has been shown previously that when female guppies shoaled more than males, there was greater transmission of the ectoparasite *Gyrodactylus turnbulli* between females, possibly indicating a host sex bias in transmission. In the current study, we conducted a similar experiment on single sex shoals of male and female guppies, observing host behaviour before and after the introduction of an infected shoal mate. This time, however, males aggregated more than females which resulted in highest parasite transmission occurring between male guppies. Together, these studies confirm that it is the extent of host contact that influences gyrodactylid transmission rather than a host sex bias, or differences in parasite population growth.

8.2: Introduction

Formation of social groups is a familiar aspect of animal behaviour (e.g. Bertram 1978) and is based on individuals evaluating the relative profitability of joining, leaving or staying with others due to constantly changing trade-offs between feeding opportunities and predation pressures (Pitcher and Parrish 1993). Parasitism has been implicated as a potential risk factor impinging on group formation and lab experiments indicate fish can use parasite-infection status as a cue in active shoal choice (Dugatkin et al. 1994, Krause and Godin 1996). Most previous studies have assessed the impact of indirectly transmitted endoparasites on fish shoaling behaviour and indicated that fish avoid infected conspecifics (review in Barber et al. 2000). We conducted similar experiments using a gyrodactylid-guppy system and predicted greater avoidance of infected conspecifics with this directly transmitted ectoparasite (Chapter 7; Richards et al. 2010). However this was not the case; there was no apparent change in the behaviour of guppies in single sex shoals after the introduction of a gyrodactylid infected host. However, because female-only shoals aggregated more than male shoals (as previously demonstrated by Griffiths and Magurran 1998), there was greater transmission between females than the equivalent groups of males (Chapter 7; Richards et al. 2010). The aim of the current study is to assess whether transmission of this pathogen is due to a host sex bias or influenced by the intensity of infection.

8.3: Materials and methods

8.3.1: Host and parasite origins

Ornamental guppies (n=120) were already infected with *Gyrodactylus* spp. when purchased from a UK commercial supplier, but were subsequently treated with 0.2% levamisole (see Schelkle et al. 2009) and then left to habituate in the aquarium for at least three months before use. The fish were maintained under a 12 h light: 12 h dark lighting regime in mixed-sex groups in 45 x 45 x 120 cm aquaria, and fed a diet of flakes (Aquarian®) and frozen bloodworm. An isogenic strain of *Gyrodactylus turnbulli* (strain *Gt3*), originally isolated from petshop guppies in 1997, was used for all infections. All experiments were conducted at 25±1°C between November 2007 - February 2008.

8.3.2: Experimental design

Experimental design was previously described in Chapter 7 (Chapter 7; Richards et al. 2010). The only difference between the current and previous study is that here we experimentally infected the focal fish with a lower initial parasite burden (mean±SE of 30±14 worms/fish cf. to mean 111±12; see Chapter 7; Richards et al. 2010). Briefly, single sex groups of male or female guppies (6 individuals per group with 10 replicate groups) were placed in test aquaria (40 x 60 x 30 cm), and allowed to acclimate for 5d. A single guppy in each tank, individually recognisable by its colour patterns, was randomly assigned as the focal fish. After acclimation, the shoaling behaviour of each group was observed once daily for 3 consecutive days (t = days 1-3). At the end of day 3, all fish were removed from the test aquaria and the focal fish was infected with *G. turnbulli*. Following infection, all fish were returned to their test tank (t = day 4). Different parameters of shoaling behaviour (shoal size, nearest neighbour distance, time spent shoaling) were measured for 3 consecutive days following infection; all shoaling behaviour measurements were evaluated in three-dimensional space. Shoal members were defined as fish within 4 body lengths of one another (Pitcher et al. 1993). At the end of each trial the extent of within-shoal parasite transmission was assessed by recording the number and position of parasites on each fish individually anaesthetised in 0.02% MS222. No fish deaths occurred during the experiment but 2 fish presented with clamped fins (pathology characteristic of *G. turnbulli* infections) on day 6.

8.3.3: Statistical analyses

Data was analysed as described in Chapter 7 (Richards et al. 2010). A brief description is included here for clarity. Data was natural log-transformed to achieve normality, established using Anderson-Darling tests. A Repeated Measures ANOVA was used to analyse whether differences in the three parameters of shoaling behaviour were explained by the day of the experiment, sex and infection status of the guppy. Day of experiment ('Day') was used as a covariate and infection status ('Parasitised') was crossed with sex ('Sex') as factors. All guppies were assessed for parasite burdens at the end of the 3-day infection period, with differences in initial and final parasite burdens assessed using Kruskal-Wallis tests, and comparisons between males and females in their ability to spread infection to conspecifics tested using chi-square analysis. A binary logistic regression analysis (logit) was used with a dichotomous dependent variable, infected or not infected (coded as '1' and '0,' respectively), to test whether the infection status of fish at the end of the experimental period was associated with initial parasite load of focal fish ('Gyrostart'), parasite population growth ('Gyrogrow') and sex ('Sex') of the guppy. The model uses 'Sex' as a fixed factor crossed with

'Gyrostart' or 'Gyrogrow' as covariate. For all multivariate analyses, a backwards stepwise approach was used to reach a final model. All analyses were performed in Minitab 15.

8.4: Results

8.4.1: Shoaling behaviour

Overall, the results with regard to shoaling behaviour were unexpected, since the males had more and prolonged contact with each other than female guppies. Male contact was characterized by typical courtship behaviour, with male guppies regularly performing 'sigmoid' displays (Baerends et al. 1955) directed to other males. Male guppies formed larger groups than females and showed a significantly larger 'average shoal size' (Repeated measures ANOVA: $F_{1,135}=15.65$, P=0.003). Focal and non-focal males were also closer together with a significantly shorter 'nearest neighbour distance' compared to female guppies (Focal males: $F_{1,135}=9.00$, P=0.015; Non-focal males: $F_{1,135}=11.64$, P=0.008). Furthermore, focal and non-focal male guppies spent a significantly longer time shoaling than their focal and non-focal female counterparts (Focal males: $F_{1,135}=35.65$, P<0.001; Non-focal males: $F_{1,135}=22.41$, P=0.001) (Figure 8.1 A-C).

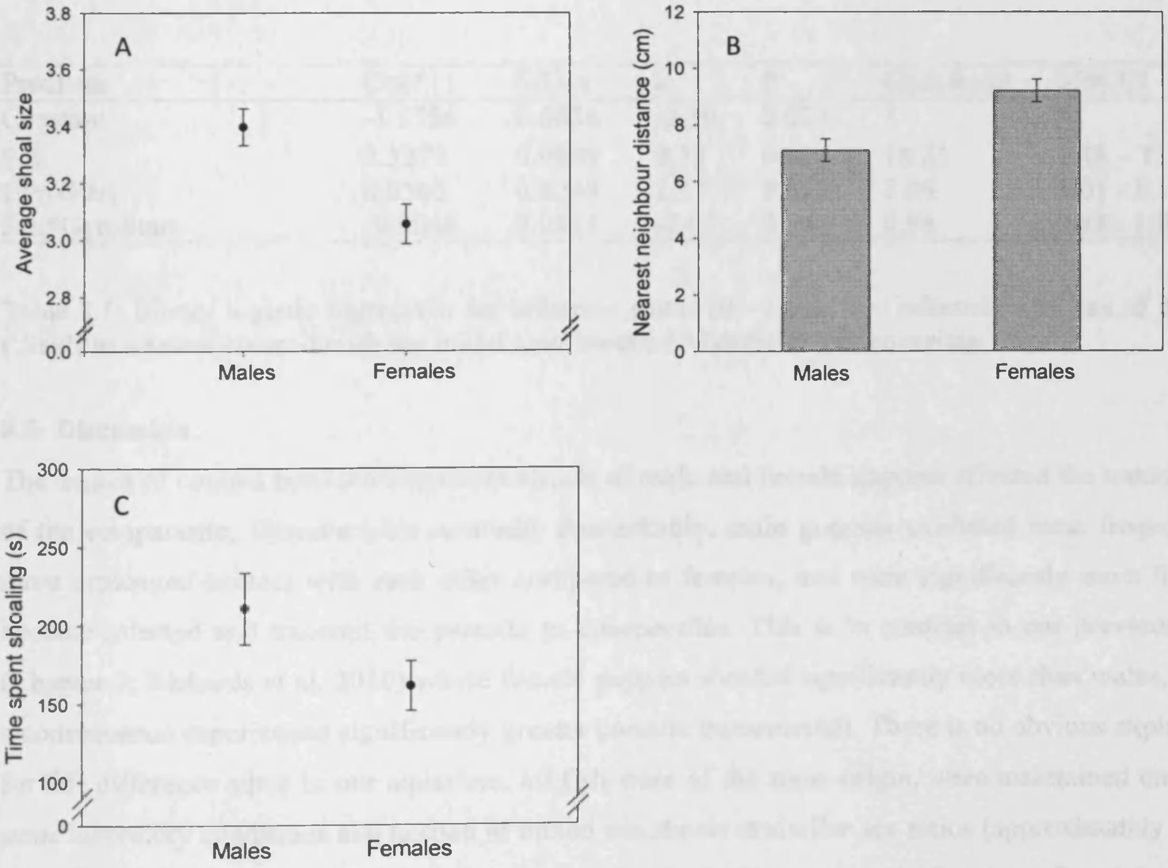

Figure 8.1: Mean ±SE (A) shoal size, (B) distance between nearest neighbours, (C) total time spent shoaling, for male and female guppies (focal and non-focal fish combined).

8.4.2: The effects of parasites on shoaling

There was a significant effect of parasitism on focal fish, both males and females. These fish shoaled significantly more than their uninfected counterparts, forming tighter shoals with a shorter 'nearest neighbour distance' ($F_{1,135}$=27.91, P=0.001). These parasitised fish also spent more time shoaling ($F_{1,135}$=5.81, P=0.039). For non-focal fish, surprisingly, there was no apparent effect of parasitism as they did not shoal more or less in the presence of an infected conspecific (all 3 measures of shoaling behaviour, Repeated measures ANOVA: P>0.1).

8.4.3: Parasite transmission

There was no difference between focal males (mean ±SE 34±4.6 worms/host) and females (mean 27±4.6) in their initial parasite loads (Kruskal-Wallis: H=1.23, d.f.=1, P=0.266). Over the 3-day infection period, focal males experienced significantly higher parasite population growth than focal females ($F_{1,135}$=4.51, P=0.049). Furthermore, focal males had a significantly greater chance of transferring parasites to conspecifics than females in this time (Logistic regression: Z=-2.05, P=0.040) (Table 8.1). In fact, non-focal male guppies were 10 times as likely to acquire an infection compared to females (Z=2.35, P=0.019).

Predictor	Coef	StDev	Z	P	OddsRatio	95% CI
Constant	-1.5754	0.6836	-2.30	0.021	*	*
Sex	2.3273	0.9889	2.35	0.019	10.25	1.48 – 71.21
GyroStart	0.0565	0.0249	2.27	0.023	1.06	1.01 - 1.11
Sex*GyroStart	-0.0646	0.0314	-2.05	0.040	0.94	0.88 - 1.00

Table 8.1: Binary logistic regression for infection status (0 - clean, 1 – infected) with sex of the host ('Sex') as a factor crossed with the initial tank burden ('GyroStart') as covariate.

8.5: Discussion

The degree of contact between single sex shoals of male and female guppies affected the transmission of the ectoparasite, *Gyrodactylus turnbulli.* Remarkably, male guppies exhibited most frequent and more prolonged contact with each other compared to females, and were significantly more likely to become infected and transmit the parasite to conspecifics. This is in contrast to our previous study (Chapter 7; Richards et al. 2010) where female guppies shoaled significantly more than males, and as a consequence experienced significantly greater parasite transmission. There is no obvious explanation for this difference since in our aquarium, all fish were of the same origin, were maintained under the same laboratory conditions and housed in mixed sex shoals at similar sex ratios (approximately 1 male to 5 females) prior to use. Also, both experiments were carried out at a similar time of year, under the same temperature, lighting and feeding regimes.

Focal fish in the current study altered their shoaling behaviour when infected. These fish formed tighter shoals and were more frequently observed shoaling compared to uninfected counterparts. This agrees with previous studies that have shown infected fish have an increased tendency to shoal (reviewed in Barber et al. 2000). For example, parasitised and unparasitised banded killifish (*Fundulus diaphanous*) and cave mollies (*Gambusia affinis*) prefer to shoal with uninfected conspecifics (Krause and Godin 1996, Tobler and Schlupp 2008). Contrary to our predictions, but following our previous findings (Chapter 7; Richards et al. 2010), non-focal individuals did not alter their shoaling behaviour following introduction of the infected focal fish. This was despite a small proportion of infected fish displaying clamped fins (indicative of secondary pathology, see Cable et al. 2002), causing these individuals to be phenotypically distinct from uninfected shoal mates. However, Scott (1985) stated that heavily infected guppies were inspected frequently by uninfected shoal members. These fish did not exhibit the expected avoidance behaviour, perhaps because the parasite itself is not conspicuous (Barber et al. 2000). Furthermore, healthy fish may approach individuals showing unusual behaviours to gain information on their condition and evaluate them as potential shoal mates (Barber et al. 2000).

The increased chance of parasite transfer seen in this study appeared to be due to increased host contact rather than faster parasite population growth rate or more efficient parasite transfer between focal and non-focal shoal members. There was no evidence to suggest a host sex bias in parasite transmission. In conjunction with our previous findings (Chapter 7; Richards et al. 2010) it demonstrates that the degree of host contact is a major factor influencing parasite transmission in shoals of guppies, and probably more generally in fish shoals. This agrees with the suggestion by Endler (1995) that a shorter nearest-neighbour distance of guppies would facilitate interhost transmission of gyrodactylids.

In summary, we have again shown the importance of host behaviour in controlling parasite transmission within a group-living host species, but this does not necessarily imply a host sex bias despite the distinct behaviours of male and female guppies.

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Chapter 9: Final Discussion

This thesis has focussed on the three research areas: fish feeding behaviour, the impact of fish personality (boldness) on shoaling behaviour and parasite transmission, and the effect of host sex and contact on transmission of a directly-transmitted fish parasite. Perhaps the most important findings to emerge are, firstly, conservative foraging strategies involving the avoidance of novel prey are more widespread in fish species than previously thought. Secondly, boldness affects shoaling behaviour with shy fish having a greater tendency to group together than bold fish, which in turn influences parasite transmission, resulting in shy fish having higher parasite loads and a greater change in parasite load across an infection period than bold counterparts. Thirdly, host contact was the main factor influencing transmission of a directly-transmitted ectoparasite between members of a group-living host species.

There is clear evidence that the phenomenon of dietary conservatism (DC), the active avoidance of novel palatable prey, is not limited to temperate species but is also found in tropical fish, as demonstrated in feeding trials on members of the poeciliid family (*Poecilia reticulata*, *P. sphenops*, *Xiphophorus maculates* and *X. hellerii*). However, poeciliids were more likely to drive the novel morph to extinction in simulated prey populations than a temperate species, the three-spined stickleback, *Gasterosteus aculeatus* (Chapter 2). Also, DC was evident among shoals of fish but not to a significantly different extent to that exhibited by isolated fish (Chapter 3). Furthermore, the role of learning in a fish's ability to feed on cryptic and conspicuous prey was investigated in Appendix II, and this has implications for studies on novel food wariness. Firstly, when testing fish for their response to novel food (Chapters 2 and 3) all species were given a period of training, during which fish became familiar with the particular colour prey presented. This period of familiarisation would also have allowed the fish to learn how to manipulate and consume the potentially novel dried and live *Daphnia* prey. Guppies learned to forage successfully on live novel prey over successive trials (Appendix II), as a significantly higher proportion of prey were consumed in the last trial than the first (when fish were naive). Many other studies in a variety of fish species have also shown an increase in foraging efficiency as the number of exposures to novel prey increased (e.g. Croy and Hughes 1991, Warburton and Thomson 2006). Therefore, it may be that both deficiencies in learning, as well as a high level of dietary wariness, are responsible for some fish not feeding sufficiently well at the end of the training period.

It is not always possible to identify food items consumed by an animal, sometimes due to difficulties in observing foraging behaviour, or prey are so digested that there are no identifiable remains in a predator's faeces. Molecular genetic techniques have been used to non-invasively assess the diets of a range of animal species (reviewed in Symondson 2002), now including fish, for the first time to our knowledge (Appendix I). When considering an animal following either an adventurous foraging strategy (AC) or a conservative one (DC), it may be possible to determine their feeding tendencies without the need for direct observation, since molecular techniques can quantitatively assess relative prey abundance (e.g. Matejuova et al. 2008). Furthermore, novel prey morphs may be morphologically distinct from more familiar prey species, but when consumed by a predator it may be impossible to visually distinguish them. However, genetic techniques can separate out prey species since it is expected that new morphs may arise in a population through small genetic changes over time. Molecular analysis of faeces can differentiate between closely related and congeneric species (e.g. see Parsons et al. 2005) and accurately distinguish between morphologically cryptic taxa (e.g. Springer et al. 1996) which are common in many animal groups (Knowlton 1993, Jarman and Elliott 2000). Upon completion of the molecular analysis of guppy faeces in Appendix I, it is hoped that two prey types will be identified along with their decay rates (the rate of decay of prey DNA in the gut of the guppy, *Poecilia reticulata*).

The avoidance of novel food, through short-term neophobia as well as the more prolonged process of DC, has ecological and commercial significance for individual animals. Many environments contain a variety of unfamiliar potential foods that are toxic and so DC may be an adaptation to such toxicity. However, it would be advantageous for foragers to sample new food sources and expand their diet, for example, due to seasonal fluctuations in food availability, in order to gain valuable resources. From an ecological perspective, individual variation in the degree of novel food wariness could affect an individual's dispersal ability, with less wary individuals being able to colonise new areas as they can more readily utilise potentially novel food types compared to very wary individuals. This may also relate to migratory species, where migrants may be less wary than non-migrants (Mettke-Hoffmann et al. 2002, 2005, Mettke-Hoffmann 2005). Innovation in foraging has been linked to range expansion and invasiveness in some birds (Sol et al. 2002). Furthermore, the responses of group-living species to novel foods may depend on the behaviour of conspecifics, with group members competing for new food sources but also providing social information on the value of new food patches (e.g. Barnard 1984, Wauters et al. 2002, Brown and Laland 2003, Boogert et al. 2006). Wariness of novel food also has implications for understanding the effects of human-induced environmental change, which itself has impacts for conservation. The introduction of invasive species, home range shifts due to climate change (Sanchez-Lafuente et al. 2001, Simmons et al. 2004, Suarez-Seoane et al. 2004, Chen and Kang 2005, Martin and Fitzgerald 2005), and modifications to farming practices all alter the foods available to many species (Fuhrer 2003, Logan et al. 2003, Travis 2003).

In a commercial context, novel food wariness has financial and welfare relevance for commercial poultry rearing and fish farming as well as the aquarium trade, for example, a reduction in food intake rate and growth of farmed birds is seen with each introduction of a new food during the rearing process (Cooper 1971, Murphy and Duncan 1977, Poole 1999). Also, recapture rates of hatchery-reared fish tend to be lower than those of wild fish, and this has been partly attributed to inefficient foraging due to a lack of previous feeding experience on various prey types, as fish in hatcheries are usually raised on dry pelletted feeds (see Stradmeyer and Thorpe 1987). Despite neophobia and DC now being well studied by psychologists and ecologists (Braveman 1976, 1978, Greenberg 1983, 1984, 1987, 1990, Jones 1986, 1987, Jones and Carmichael 1999, Mettke-Hoffmann 2005, Marples 2007), little is known of the factors affecting expression of novel food wariness in individual animals, both in natural habitats and under laboratory conditions (but see Barnett et al. 2007 for an exception). It is essential to explore the energetic and ecological controls of wariness to novel foods in order to predict optimal foraging responses, as well as further understand the broad ecological and social significance of novel food wariness.

The foraging behaviour of fish may also be affected by individual differences in personality traits such as sociability, activity and as studied in this thesis, boldness and shyness. Bold individuals are more likely to consume novel food items (Wilson et al. 1993, Magnhagen and Staffan 2003) compared to shy counterparts, and as a result, bold individuals may drive a novel prey morph to extinction in a prey population, both more quickly and more often, than shy individuals. Bold fish also spend more time in open habitats (Sneddon 2003, Westerberg et al. 2004, Magnhagen and Staffan 2005), and have a greater tendency to explore (Magnhagen 2007). It is reasonable to assume, therefore, that bold individuals are more likely to find new food patches and exploit this resource more readily than shy conspecifics. In natural shoals, it is likely that there is a mix of behavioural phenotypes and group composition can impact on the behaviour and fitness of individuals (Magnhagen and Staffan 2005, Sih and Watters 2005), with both bold and shy fish gaining foraging benefits from associating with one another in groups containing both behavioural phenotypes (Dyer et al. 2009).

There is clear evidence for the existence of individual differences in the personality trait, boldness, in guppies, both within and between populations (Chapters 4 - 6). Fish from two wild populations were found to differ significantly in their relative boldness, but the proportion of individuals within a single population showed similar levels of boldness over time (Chapter 4). However, the relative boldness of individual fish changed with time, mainly due to habituation to the boldness test environments, with shy fish becoming bolder and bold fish apparently becoming more shy (Chapter 4). Boldness of fish was affected by mating, with virgin females being bolder than their mated counterparts, forming smaller and less cohesive shoals. Also, pregnant females seemed to be bolder than non-pregnant conspecifics, spending more time in close proximity to a novel object (Chapter 5). Boldness also affected shoaling behaviour, as shy fish shoaled significantly more than bold conspecifics, and formed larger and tighter shoals (Chapters 4 - 6).

An animal's behaviour (Poulin et al. 1991, Brown et al. 1994, Krasnov et al. 2005, Natoli et al. 2005, Easterbrook et al. 2007), and more specifically to this thesis, an individual's boldness (Wilson et al. 1993) can increase the risk of infection by parasites. In this thesis, the impact of boldness on acquiring a parasite infection and subsequent transmission to other group members was shown in groups of guppies of mixed behavioural phenotype. Shy individuals tended to group with conspecifics more often and more tightly than bold individuals. This resulted in shy fish having higher parasite loads and a greater change in parasite load over an infection period, compared to their bold counterparts (Chapter 6), due to greater contact between shy fish. Host contact was shown to be the main factor influencing transmission of a directly-transmitted ectoparasite, where significantly more parasites were transmitted between hosts when hosts of both sexes had more frequent and more prolonged contact with each other (Chapters 7 and 8). When female fish shoaled significantly more than males, females had a four times higher risk of contracting an infection (Chapter 7), whereas when males aggregated more than females, this resulted in the highest parasite transmission occurring between males (Chapter 8). In the future, it will be interesting to assess parasite transmission in mixed sex shoals of wild-caught guppies, since only single sex shoals were used in this thesis. Mixed sex shoals are obviously more akin to the wild situation, and it would be exciting to explore what affect bold and shy males may have on female behaviour, and the possible resulting impact on parasite transmission.

The relationship between boldness and parasite transmission (Chapter 6) could also be examined in terms of social networks, which can be constructed from information on inter-individual interactions (reviewed by Newman 2003). Individuals are connected by their social exchanges, and networks have been shown to exist in guppies (Croft et al. 2004), as well as a range of mammal species (see Krause et al. 2007, 2009). Social network theory provides a framework for the study of social organisation in animal societies because social interactions rarely occur in isolation (Croft et al. 2005). For example, cooperation, information and parasite transmission in group-living species cannot be fully understood without considering the network of interactions through which such processes occur (Watts and Strogatz 1998, Moore and Newman 2000, Abramson and Kuperman 2001, Latora and Marchiori 2001). Recent work has attempted to directly map disease pathways between individuals, for example, Cross et al. (2004) and Corner et al. (2003) used a network approach to study the spread of bovine tuberculosis in African buffaloes (*Syncerus caffer*) and a possible vector species, the brushtail possum (*Trichosurus vulpecula*), respectively. Network theory may be able to help predict the speed with which a disease will spread through a population as well as the likely transmission pathway (Krause et al. 2007). In this way, 'super-spreaders' may be identified, i.e. individuals that are very highly connected and can potentially infect a large proportion of other individuals (Perkins et al. 2009). In terms of disease control, targeted vaccination of 'super-spreaders' could be an effective way of disrupting disease transmission despite only a small proportion of a population being vaccinated. Other potentially important contributions of network theory to disease control were recently seen in the foot-and-mouth outbreak in Britain and the SARS (severe acute respiratory syndrome) epidemic (Bowen and Laroe 2006), and also has present-day applications for modelling between-species interactions in badger-transmitted bovine tuberculosis.

Social network theory has also been applied to parasite transmission between groups of animals, for example, Godfrey et al. (2009) constructed a transmission network between lizards (*Egernia stokesii*) that shared the use of rock crevices, and showed that higher levels of network connectivity increased the risk of becoming infected with between one and four parasites (three blood-borne, one ectoparasite). Also, Monello and Gompper (2010) showed that host sociality impacted on ectoparasite load and transmission in free-ranging raccoons (*Procyon lotor*). Furthermore, behavioural phenotype of individuals has been shown to affect social interactions in animal networks. Bold and shy three-spined sticklebacks differed in the number and distribution of their interactions with conspecifics (Pike et al. 2008); shy guppies had more network connections that were on average stronger than those in bold fish (Croft et al. 2009). Furthermore, activity and exploration positively correlated with ectoparasite load in chipmunks, *Tamias sibiricus* (see Boyer et al. 2010).

Personality differences, such as relative boldness-shyness, between individuals have significant evolutionary and ecological consequences, in addition to those already discussed in this thesis (Chapters 4 - 6). For example, cooperation in animal groups may have evolved due to the maintenance of different personality types by natural selection (e.g. Crowley and Sargent 1996, Lotem et al. 1999, Fishman et al. 2001, McNamara et al. 2004). The presence of different personality types may affect how animals respond to change, facilitating speciation and adaptation (e.g.Wilson 1998), as well as coping with environmental change due to anthropogenic influences (Dall et al. 2004). Bold neophillic individuals may be able to locate new resources if familiar food types disappear, or aggressive individuals may be better at competing for resources if they become limited (see Sih et al. 2004). Personality traits have also been linked to growth-mortality trade-offs where faster-growing individuals may take more risks in foraging contexts than slower-growing individuals (see Stamps 2007), as well as variation in rates of energy metabolism in animals, where high activity, exploration, boldness and aggressiveness are energetically costly (see Careau et al. 2008).

An individual's behavioural phenotype is also related to its fitness, i.e. survival at different life stages, mating success, fecundity and reproductive success (see Dingemanse and Reale 2005, Reale et al. 2007, Bell 2007, Biro and Stamps 2008). The finding of this thesis that boldness is affected by the mating status of an individual (Chapter 5), with virgin and pregnant females being more bold than mated but non-pregnant counterparts, provides more evidence linking personality traits with fitness. A similar relationship was found in three-spined sticklebacks (*Gasterosteus aculeatus*) where gravid females were bolder when inspecting a predator than non-gravid fish (Frommen et al. 2009). A number of other studies have shown an association between aspects of fitness and behavioural phenotype, with boldness and fecundity being positively correlated in a range of animals, for example, chickens, *Gallus gallus*, (Barnett et al. 1992), Atlantic silverside, *Menidia menidia*, (Walsh et al. 2006), and domestic pigs, *Sus scrofa domestica* (Geverink et al. 2004). Previous studies have also examined the correlation between boldness and survival, finding a positive relationship in bighorn sheep during years of high predation (Reale and Festa-Bianchet 2003), but a negative correlation in swift foxes (*Vulpes velox*) where bold individuals had a lower survival rate after release from captivity than shy counterparts (Bremner-Harrison et al. 2004).

From personality studies conducted to date, there is clearly a huge number of further questions that can be addressed using animal models, for example, pertaining to central nervous system function, the role of genetics, the importance of prenatal experience, and the evolutionary origins of individual personality traits (Weinstein et al. 2008). There are also many practical real-world applications for personality research, such as predicting performance in working dogs (e.g. Svartberg 2005, Maejima et al. 2007), and for animal welfare and management (e.g. McDougall et al. 2006, Watters and Meehan 2007). Personality assessments of domestic animals have also been developed to help potential owners identify a suitable pet (Ledger and Baxter 1996, Coren 1998). It is essential that the various disciplines involved in personality research, such as Behavioural Ecology, Ethology, Developmental Psychobiology, Primatology, Zoo and Wildlife Management, Genetics and Comparative Psychology, continue to collaborate to understand individual behavioural differences, first proposed by one of the pioneers in behavioural trait research nearly a century ago (Pavlov 1928).

This thesis has addressed some specific questions regarding possible feeding preferences of fish, as well as relative boldness of individuals within shoals and the resulting impact on parasite transmission. In light of this work, many new questions have arisen, such as: how a dietarily conservative foraging strategy is affected by energetic constraints and social context, whether fish exhibiting such a foraging strategy are less likely to find new food patches or move into new habitats. Within shoals of fish, it would be interesting to assess individual foraging choices, learning mechanisms such as copying of conspecifics, and how dietary conservatism is affected by visual perception and competition.

With regard to the effect of boldness on behaviour, it would be exciting to examine if adult boldness predicted offspring boldness, and whether stress early in life (pre-natal to juvenile) affects the degree of boldness in adults. Also, how familiarity affects boldness, whether individuals are more or less bold in the presence of familiar conspecifics. The interplay between mating, gravidity and boldness could also be furthered examined.

In terms of parasite transmission within shoals, an essential next step would be to examine how this is influenced by interactions of bold and shy males and females in shoals of both mixed sex and behavioural phenotypes. Further to this, it would be interesting to specifically test if shy fish transfer parasites both more quickly and in greater numbers between each other than bold conspecifics, and specifically in guppies, how efficient males are at transmitting parasites between shoals of females. It would also be pertinent to compare aversion responses of both wild and ornamental strains to infected conspecifics. Furthermore, it would be interesting to incorporate social network theory (e.g. Krause et al. 2007) into further work on both shoaling interactions and parasite transmission in fish shoals, as it is a useful tool in elucidating animal social structure to bridge the gap between individual behaviour and population biology.

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Appendix I: Development of molecular scatology for the determination of the diet of the guppy (*Poecilia reticulata*)

Abstract

Assessment of predator-prey relationships often relies on analysis of the hard, undigested prey that remain in faecal (scat) samples. Although for many predator-prey interactions this can yield valuable information, for some species diagnostic characteristics are lost during the process of digestion, thus preventing morphological identification of prey species. Also for aquatic animals faecal collection can be difficult, and so previous studies using molecular scatology for marine species are rare, and there have been no previous attempts with freshwater species. In the current study, we tested the feasibility of using faecal DNA to detect the presence of a specific prey organism (*Eisenia vestiga*) in scat samples collected from a freshwater fish (*Poecilia reticulata*) fed on a monospecific diet. Novel earthworm mitochondrial DNA primers successfully amplified 230 bp region of the cytochrome oxidase 1 gene. This is the first time, to our knowledge, that molecular techniques have been used to identify prey DNA in scat samples from a fish species.

Introduction

A fundamental part of many ecological studies is to determine trophic relationships within an ecosystem (Trites 2003), and in order to do this, it is essential to reliably estimate the diet composition of the species involved. In interactions between specialist predators or parasitoids and their prey/hosts, there is little doubt about the identity of the prey, but identification and quantification of prey becomes more difficult for generalist predators that feed on a range of different species (Symondson 2002). The simplest method of visual inspection of gut contents has been used for a range of animal taxa (Pearson 1966, Pierce and Boyle 1991, Ewins et al. 1994, North 1996, Petty 1999, Elmhagen et al. 2000, Ingerson-Mahar 2002), and can provide quantitative information on prey that leave indigestible remains, such as arthropod cuticles, vertebrate bones, scales, feathers or fur. However, many invertebrate predators are fluid feeders and do not consume recognisable prey parts. Therefore, analyses based upon undigested remains are likely to provide biased results and will omit many trophic interactions (Dennison and Hodkinson 1983; Feller et al. 1985).

Faecal genetic analysis is one technique that has been used to overcome the problems of prey identification when prey do not leave identifiable remains. It is a powerful, non-lethal tool that can be used to obtain information on the identity and population genetics of predators, as well as information on prey and diet composition (see Parsons et al. 2005). Much of the focus has been on invertebrates (see Sheppard et al. 2005) but there is a growing vertebrate literature, using molecular scatology to determine diet, population structure and genetic variation of terrestrial mammals, e.g. baboons, *Papio cynocephalus ursinus*, (see Frantzen et al. 1998), seabirds (e.g. Walter and O'Neill 1986, Freeman and

Smith 1998), and marine mammals such as dolphins, *Tursiops truncatus*, seals, *Halichoerus grypus*, (e.g. Parsons et al. 1999, 2005), whale sharks, *Rhincodon typus*, (e.g. Jarman and Wilson 2004), despite obvious difficulties in collecting diffuse faecal plumes from aquatic species (Parsons et al. 1999).

To our knowledge, molecular techniques have never been applied to the study of fish diet to obtain information on species identification, population genetic structure or diet composition. Fish play an integral role in both freshwater and marine ecosystems; furthermore, for fish species that are used as evolutionary models, such as the Trinidadian guppy, *Poecilia reticulata* (see Magurran 2005), and the three-spined stickleback, *Gasterosteus aculeatus*, (see Bell and Foster 1994) it seems particularly important to investigate non-lethal methods of assessing population genetic structure. In this study, a polymerase chain reaction (PCR) technique was used to detect both prey and host species DNA extracted from guppy faecal samples.

Materials and methods

Faecal collection

Ornamental guppies (n=6) were housed individually in 1 litre containers (previously decontaminated using UV light) and starved for 24 h to allow previous gut contents to pass through before faeces collection. After this time, fish were fed a small amount (1 or 2 individual body segments) of fresh earthworm (*Eisenia vestiga*) twice daily for 2 days before faeces collection. This ensured when faeces were collected, it contained only earthworm prey DNA and no other prey DNA. Fish were observed to readily approach and ingest the earthworm prey throughout the period of faeces collection. Once fish had consumed all the earthworm prey, they were left to settle for 1 h before they were transferred to new aquaria to prevent contamination of the faeces by earthworm DNA present in the water after feeding. Fish were subsequently checked every 5 min for 2 h and faeces collected as soon as it had been passed by the fish. This limited the degradation of DNA in the water. Once collected, faeces were washed briefly in a 5% solution of disinfectant (Virkon) to remove any external contaminants, and then placed into ethanol and stored at -80°C.

Prey origins

Individual earthworms (n=8) were collected from a garden habitat and identified as *Eisenia vestiga* (A. King pers. comm.). Fresh worms were starved for 48 h to allow soil to pass through their guts before DNA extraction or being used as prey. Chironomid larvae, *Tubifex* sp. (common name 'bloodworm'), were purchased from a commercial supplier (J&K Aquatics, U.K.) and defrosted before use.

DNA extraction and PCR

The same DNA extraction method was applied to fresh earthworms (single specimen), guppy faeces (ca. 0.5mls) and defrosted chironomid larvae (five individuals). DNA was extracted by proteinase K digestion in a TE/Tween buffer followed by a Tris/Cl extraction and DNA precipitation (see Maniatis et al. 1982).

Universal invertebrate primers LCO1490 and HCO2198 (Folmer et al. 1994) and general earthworm primers EWF and EWR (see King et al. 2008) were used to amplify regions of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene, and guppy primers H16498 (Shields and Kocher 1991) and L15926 (Kocher et al. 1989) were used to amplify a 550 bp fragment of the mitochondrial control region gene (see Table 1).

Organism	Primer sequence (5'-3') and source	Amplicon size (bp)
General Invertebrates	LCO1490: GGTCAACAAATCATAAAGATATTGG HCO2198: TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al. 1994)	710
Earthworm (prey)	EWF: GTAGCTCGTYTAGTTTCGGG EWR: AGTGGGCCTAAAAGCAGCCA (See King et al. 2008)	230
Chironomid larvae (prey)	LCO1490: GGTCAACAAATCATAAAGATATTGG HCO2198: TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al. 1994)	223
Guppy (host)	H16498: CCTGAAGTAGGAACCAGATG (Shields and Kocher 1991) L15926: TCAAAGCTTACACCAGTCTTGTAAACC (Kocher et al. 1989)	550

Table 1: Primer sequences of the mtDNA prime pairs used in the current study and the predicted PCR fragment size.

PCRs were carried out in a total volume of 10 μl consisting of 1 x PCR buffer (Promega), 25 mM MgCl, 25 mM of each dNTP, 10 μM each forward and reverse primer, 5 U *Taq* DNA polymerase (Invitrogen) and 1 μl DNA (1:10 dilution of original extract). All reactions were performed in a GeneAmp 9700 (Perkin Elmer) thermal cycler. After an initial denaturing step at 94°C for 2 min 30 s, amplification proceeded for 35 cycles at 94°C for 30 s, 47°C for 30 s, 72°C for 45 s and a final extension at 72°C for 10 min. Positive (guppy and earthworm DNA) and negative (distilled water) controls were included in all PCR reactions to detect contamination. All PCR products were visualised under ultraviolet light using Geldoc-It imaging system software after electrophoresis at 110 V through a 1% agarose gel stained with ethidium bromide.

Results

From the guppy faecal samples, DNA from both earthworm and guppy was successfully amplified using the general earthworm CO1 mtDNA (230 bp fragment) and guppy mtDNA primers (550 bp). The universal Folmer et al. (1994) invertebrate primers failed to amplify either earthworm, chironomid larvae (710 bp predicted fragment size) or guppy DNA in guppy faecal samples. Only one of 20 PCR negative controls gave a false positive result, due mostly likely to contamination.

Discussion

This is the first study to use molecular scatology to detect both prey and host species-specific DNA from a fish faecal sample. The host DNA fragment from the guppy amplified by PCR in the current study was relatively large (> 400 bp) compared to fragments normally used in the analysis of faeces (Parsons et al. 2005). Smaller amplicons (< 400 bp) are usually targeted to maximise prey detectability among the multiple sources of degraded DNA present in faecal samples (Parsons et al. 2005), and such smaller sequences have been used previously in non-invasive faecal genetic studies (Kohn and Wayne 1997, Farrell et al. 2000) and to detect prey DNA in predator gut contents (Zaidi et al. 1999). Thus, is not surprising that the Folmer et al. (1994) primers, which successfully amplified DNA extracted directly from earthworms (one of the positive controls) failed to amplify DNA from predator faecal samples

Fortunately, the relatively cheap and rapid method of faecal DNA extraction (see Maniatis et al. 1982) used in the current study was suitable for subsequent PCR. Recovering DNA from faeces is often not straightforward since it can contain PCR inhibitors (Deuter et al. 1995, Kohn et al. 1995), and have a high bacterial content which may cause rapid degradation of any DNA present (Kohn et al. 1995). Nevertheless, the current study shows that faecal genetic analysis could be used as a viable method for looking at the diet of guppies, specifically wild fish foraging in a natural habitat. It is known that guppies have a fairly diverse diet, as they are omnivorous and opportunistic feeders, ingesting insect larvae, terrestrial and aquatic invertebrates, algae and detritus (Arthington 1989, Houde 1997). Guppies also cannibalise fry of their own species, and ingest the eggs and young of one of their fish predators, *Rivulus hartii* (see Houde 1997). In order to achieve application of faecal DNA analysis from wild populations, it is first necessary to successfully extract and detect more than one prey species, and it is hoped to achieve this in future studies.

In conclusion, the current study provides the first evidence of successful extraction and amplification of fish faecal DNA fragments from both a prey and predator species. At present, the use of DNA is an additional tool to augment faecal sample analyses, but using DNA techniques in conjunction with more traditional methods of prey identification, could enable non-invasive assessment of changes in prey consumption due to seasonal patterns, predation risk, dispersal events and anthropogenic influences, such as habitat destruction or climate change.

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This chapter is planned for submission to a behavioural journal with Dr. Bart Adraenssens as lead author.

Appendix II: Coping with contrast - foraging guppies (*Poecilia reticulata*) overcome neophobia by learning

Abstract

Prey colour and contrast can have profound effects on the outcome of an encounter with a naïve predator. At first, visual constraints can cause cryptic prey to be easily overlooked. Furthermore, once the prey is found, foraging biases may cause predators to avoid (neophobia) or prefer specific prey. Conspicuous colours may become associated with prey defence strategies causing predators to either be cautious (unprofitable prey hypothesis) or encourage attack if they signal particular nutritional value. The latter has been observed in several animal species that prefer red prey due to their high carotene content, a compound important in immune defence (carotenoid hypothesis*)*. Here, we studied the behaviour of guppies (*Poecilia reticulata*) foraging for live, artificially coloured water fleas (*Daphnia magna*, red or green) on either red or green backgrounds. Consistent with the unprofitable prey hypothesis, guppies were initially more cautious towards conspicuous prey. Furthermore, guppies consumed green prey faster than red prey, contrary to predictions of the carotenoid hypothesis. This suggests that naïve foraging was affected by prey acceptance mechanisms rather than visual constraints. Yet, foraging latency decreased with predator experience causing effects of prey colour and prey-background contrast to recede gradually. Finally, guppies initially trained on cryptic prey did not show reduced acceptance of conspicuous prey when they were later presented with both conspicuous and cryptic prey. This may suggest that positive experiences associated with stimuli such as shape and movement can outweigh initial neophobia against conspicuous prey.

Introduction

Recent research has revealed that prey characteristics can influence the foraging behaviour of predators in a number of ways. Firstly, cryptic colouration may influence a prey's risk of being detected (Ruxton et al. 2004). Cryptic colouration interferes with the visual skills of a predator such that cognitive constraints limit a predator's ability to detect small differences in the environment (Lythgoe 1979). As prey become more cryptic, the frequency of detection errors increases and more attention is required from foraging predators (Dukas and Ellner 1993, Dukas and Kamil 2000). When predators gain experience with cryptic prey they often enhance their prey detection success by processes such as increased search rate and/or search image formation (Reid and Shettleworth 1992, Plaisted and Mackintosh 1995).

Once prey is detected, a predator may trade off the prospect of a valuable food source against the risk of wasting energy on unprofitable or harmful prey. In such circumstances, fear of novelty (neophobia, Greenberg 1983) is commonly observed amongst foraging predators (Jones 1986, Marples et al. 1998, Sundström and Johnsson 2001). The duration of initial avoidance varies between predator species (Kieffer and Colgan 1992, Marples and Kelly 1999). Moreover, experiments on Japanese quail (*Coturnix coturnix japonicas*; see Marples and Brakefield 1995) and red-backed salamanders (*Plethodon cinereus*; see Gibbons et al. 2005) suggest a heritable component to the duration of neophobia. Prey characteristics can play a significant role in these acceptance decisions. In nature, profitable prey are often cryptic, while unprofitable prey species signal unprofitability with conspicuous warning colouration (i.e. aposematism, Wallace 1867). This may cause some avoidance of conspicuous prey by predators, also known as the unprofitable prey hypothesis (Cott and Benson 1970, Gotmark and Unger 1994). However, predator preferences for conspicuously coloured prey may evolve under certain conditions. As an example, some predators show an innate bias in favour of red prey that is suggested to represent an adaptive preference, as a mechanism of ingesting dietary antioxidants important for immune function and mate choice (carotenoid hypothesis, Rodd et al. 2002, Kolluru et al. 2006, Spence and Smith 2008, Sanchez et al. 2009).

The majority of studies investigating effects of prey colour and background contrast on predator behaviour have used motionless, artificial prey, thereby reducing the impact of prey movement and predator avoidance behaviour (e.g. Gotmark and Unger 1994, Marples and Kelly 1999, Dukas and Kamil 2000, Rodd et al. 2002). Furthermore, many studies used avian predators, whereas little is known about this phenomenon in aquatic predator-prey systems (but see Kerfoot 1982, Hargeby et al. 2004, Johnsson and Kjällman-Eriksson 2008, Thomas et al. 2010). Cryptic and warning coloured prey species are also common in these environments (Kerfoot 1982, Hargeby et al. 2004), and further work is needed to understand the relative benefits of different aquatic prey morphs and how they influence the feeding ecology of their predators.

In this study, we used guppies (*Poecilia reticulata*) and a small aquatic invertebrate crustacean (*Daphnia magna*) as model species. The guppy has been widely used to study adaptations to natural and sexual selection pressures (e.g. Reznick et al. 1996a, b, 2001). Guppies are visually hunting predators that primarily feed on benthic prey such as algae, diatoms and invertebrates (Dussault and Kramer 1981, Magurran 2005). The visual system of guppies has been well studied over the last two decades, and photoreceptor cells in their retina consist of cones with peak absorbances at 359 (UV) , 408 ('blue'), 464 ('green') and 533-572 nm ('red'), and rods at 502 nm (Archer et al. 1987, Archer and Lythgoe 1990, Endler et al. 2001). Guppies are known to use these different colour cues to make decisions during mating (Houde 1997) and in foraging contexts (Rodd et al. 2002). Even though *D.*

magna is not endemic to the native geographic range of guppies, captive guppies are known to readily take *Daphnia* as a prey (Dussault and Kramer 1981, White et al. 2005).

Our overall aim was to investigate how visual constraints and foraging biases influence the foraging behaviour of naïve predators on undefended prey. The behaviour of naïve male guppies foraging on live coloured *Daphnia* was assessed in four different contexts, using red and green *Daphnia* and red and green backgrounds, in all possible combinations. We examined four, not necessarily mutually exclusive, hypotheses (H) and predictions (P): (H1) Visual constraints reduce the foraging success of guppies on *Daphnia* and therefore cause (P1) guppies foraging on cryptic *Daphnia* to be less successful than those foraging on conspicuous *Daphnia.* (H2) The unprofitable prey hypothesis predicts that (P2) guppies will forage earlier on cryptic rather than conspicuous *Daphnia.* (H3) The carotenoid hypothesis predicts (P3) that guppies will forage more readily on red *Daphnia* than on green *Daphnia.* And finally, (H4) guppies are able to overcome visual constraints and/or foraging biases with learning thereby causing (P4) any initial differences to disappear with training on edible *Daphnia.*

Materials and methods

Experimental animals and prey origins

Domestic guppies (*Poecilia reticulata,* N = 80) were purchased from a commercial supplier (J&K Aquatics, Somerset, UK) where they were fed with commercial flake food (Tetramin). Upon arrival in the laboratory, fish were allowed to acclimate to their new environment for two months prior to the experiment. As feeding rates of guppies foraging on *Daphnia* differ substantially between the sexes and potentially also between females differing in reproductive status (Dussault and Kramer 1981), we used only male guppies in this experiment. Fish were maintained in mixed-sex groups in large aquaria (45 x 45 x 120 cm, 40 l) and fed on a diet of flakes (Aquarian®) and frozen bloodworm under a 12 h light: 12 h dark lighting regime, at 25 ± 0.5°C. Fish were not fed for 24 h before the start of the trials. Size did not differ between individuals in the different treatments (in length; One-Way ANOVA: $F_{3,76}$=0.87, P=0.46; mean ±SE standard length = 22.9±0.2mm). Approximately 12 h before the first trial, fish were randomly assigned to the start zone of an experimental tank for acclimation.

Live *Daphnia magna* were purchased from a commercial supplier (Fish&Fins, UK) and upon arrival transferred to 14l plastic tanks containing dechlorinated water at 25 ± 0.5°C. Cultures were maintained in the laboratory with regular doses of a solution of 25 g *Spirulina* powder (Holland & Barrett, UK) and 14 g dried bakers yeast in 500 ml dechlorinated water. A sample of the *Daphnia* culture was sieved (2 mm mesh) at the start of each experimental day to remove the largest individuals to avoid predator feeding being limited by gape-size. The remaining prey were coloured by immersion in 50 ml of a red- or green-coloured solution of edible food paint (red and dark green food dust, 5.0 g/l, www.squires-shop.com) for 20 min. Coloured *Daphnia* were netted, rinsed and suspended in dechlorinated water before the trial. New batches of *Daphnia* were immersed in the dye solutions approximately every 60 min to avoid differences in colour intensity.

Experimental tanks

We used 14l plastic aquaria (30 x 20 x 20 cm) containing dechlorinated water as experimental tanks for behavioural observations (Figure 1).

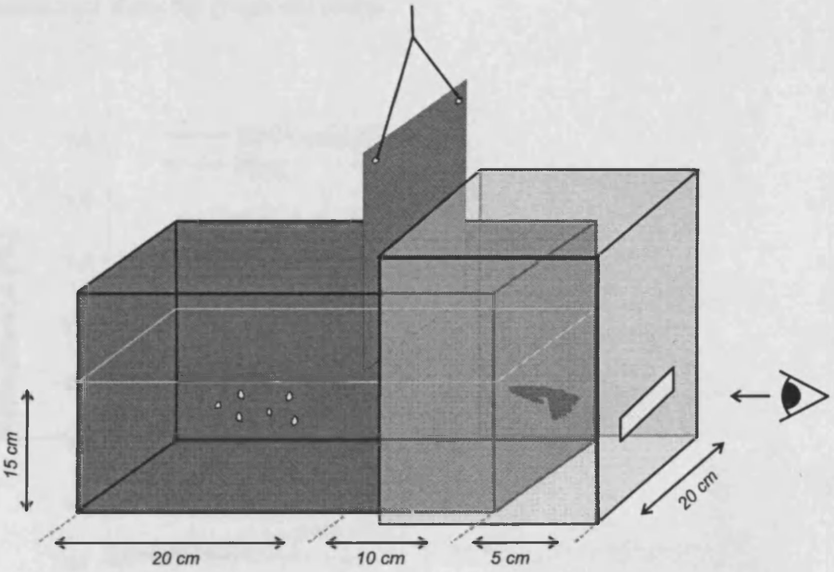

Figure 1: Experimental setup used for the foraging trials.

The tanks were covered on three sides and bottom with adhesive red- or green-coloured plastic sheets ('Fablon'). The fourth side of each tank was covered with a removable cardboard hide of comparable colour to the cover of the plastic aquarium, positioned 5 cm in front of the aquarium (Figure 1). A small viewing slot (10 x 3 cm) allowed observation without disturbance from movements outside the aquarium. The hide also encased the first third of the aquarium (*start zone*) and provided fish with a sheltered area during trials. The start zone was separated from the remainder of the tank (foraging zone) by a plastic divider of the same colour as the sides and hide. Dividers were operated from a distance with a simple counterweight system to avoid disturbance of the fish. All aquaria were lit from above by two fluorescent tubes 54W 'white light'. Luminance ranged from 4200 - 5600 Klux and did not differ between individuals from different treatments (Kruskal-Wallis test: $X^2_3 = 2.73$, $P = 0.44$). The location of test aquaria was randomised and guppies in different aquaria were visually and chemically isolated from one another.

Prey and background reflectance spectra

Measurements of reflected light with a USB2000 spectrometer system (Ocean Optics, Inc., Dunedin, USA) and C-spec software (Ancal, Inc., Las Vegas, USA) indicated that the colours used for the background and prey in the experiment were red and green and that spectra showed considerably more overlap in the cryptic treatment than in the conspicuous treatment within the wavelengths thought to be visible to guppies (Figure 2; Archer et al. 1987; Archer & Lythgoe 1990; Endler et al. 2001). Because red and green prey only differed in the food paint used for colouration, and due to the difficulty in measuring colour spectra of small submerged *Daphnia*, colour spectra for prey were measured directly from the paint.

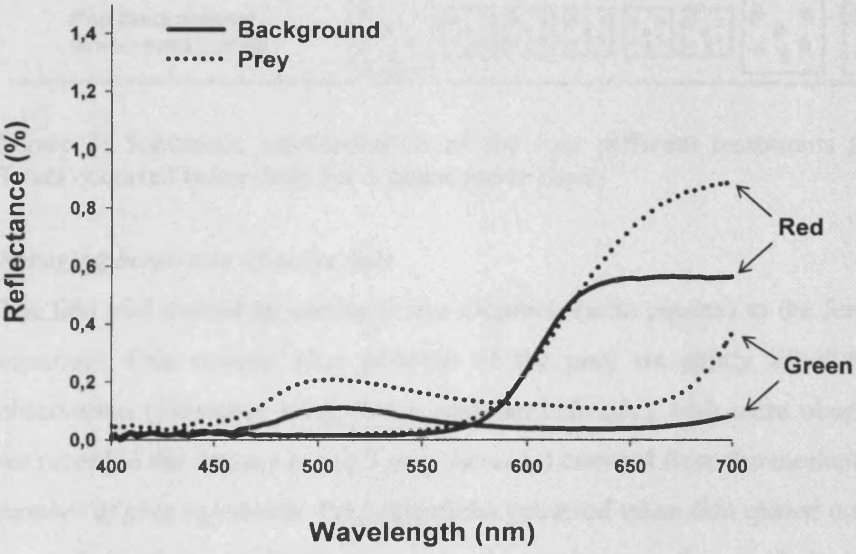

Figure 2: Reflectance spectra of red and green background and red and green dye used to colour the prey.

Experimental design

The behaviour of each guppy was observed for a total of 9 trials (Figure 3). Trials lasted 15 min each and took place twice daily for 5 consecutive days with a 5h interval between morning and afternoon trials. During trials 1-7 fish were trained to forage on cryptic or conspicuous *Daphnia*. Individuals tested with conspicuous prey received 6 green *Daphnia* on a red background or 6 red *Daphnia* on a green background, while in the cryptic prey treatments background colour matched prey colour (red or green). During training, we further distinguished foraging behaviour of naïve fish (trial 1) from foraging behaviour of experienced fish (trial 7). Fish received 3 red and 3 green coloured prey during trials 8 and 9 (further referred to as the mixed prey trials).

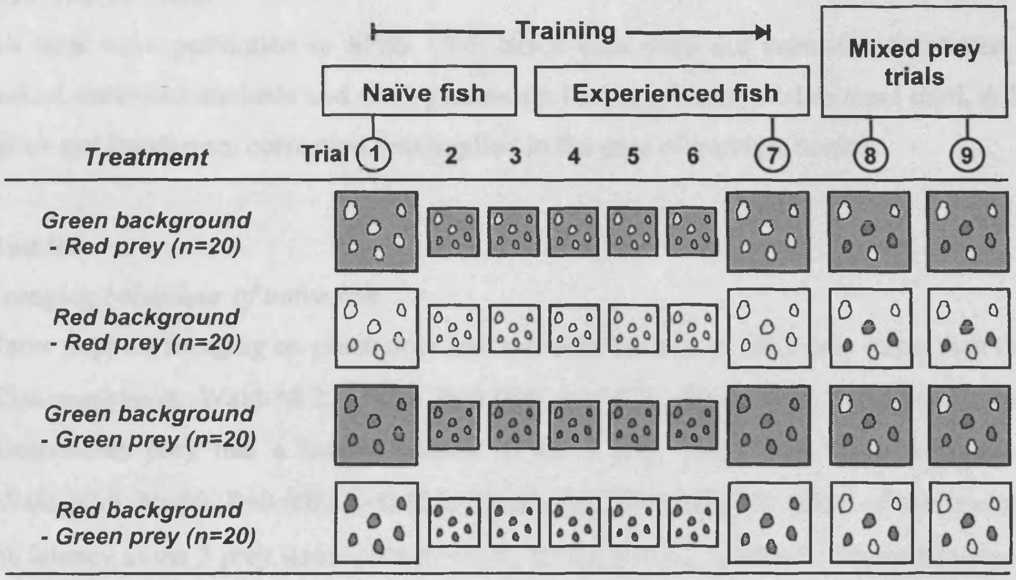

Figure 3: Schematic representation of the four different treatments throughout the different trials. Trials occurred twice daily for 5 consecutive days.

Foraging behaviour of naïve fish

The first trial started by adding 6 live *Daphnia* (with pipette) to the foraging area of the experimental aquarium. One minute after addition of the prey we gently lifted the plastic divider and started observation (JWatcher V1.0, www.jwatcher.ucla.edu). Fish were observed for 15 min during which we recorded the *latency to eat 3 prey items* (s) counted from the moment the divider was lifted and the *number of prey rejections*. Prey rejections occurred when fish spitted out an attacked prey. The latency to eat half of the available prey was chosen because this is likely to be least influenced by prey distribution or availability. After each trial, any remaining prey were removed, fish were moved back to the start zone (without netting) and the divider was lowered until the start of the next trial.

Effects of training

The procedure followed in the first trial was repeated for a total of 7 trials per individual to train each individual on its respective combination of prey and background colour. Data was analyzed for changes in behaviour over the 7 training trials and whether treatment affects foraging behaviour of experienced fish (trial 7).

Mixed prey trials

The same procedure followed in the first trial was repeated for trials 8 and 9, and here foraging was recorded as the latency to eat the first prey item. During these trials, the colour of the prey was recorded and when it was consumed. After trial 9, all fish were briefly anaesthetized, weighed and photographed to record standard length (from the tip of the snout to the base of the tail).

Statistical Analyses

All tests were performed in SPSS 15.0. Since data were not normally distributed, non-parametric ranked statistical methods and semi-parametric Cox regression models were used. All tests were two-tailed and Bonferroni correction was applied in the case of multiple testing.

Results

Foraging behaviour of naïve fish

Naïve guppies foraging on green prey had a shorter latency to eat 3 prey items than those fed red prey (Cox regression: $Wald_1=8.2$, N=80, P=0.004, $\alpha=0.025$, Figure 4a). Furthermore, naïve guppies fed conspicuous prey had a longer latency to eat 3 prey items than those foraging on cryptic prey ($Wald_1=7.8$, N=80, P=0.005, $\alpha=0.025$, Figure 4a). There was no effect of the background colour on the latency to eat 3 prey items ($Wald_1=2.26$, N=80, P=0.13, $\alpha=0.025$, Figure 4a). Prey rejections were numerous (median=7.5, interquartile range IQR: 1-15) but did not differ between treatments (Kruskal-Wallis: $\chi^2=1.62$, N=80, P=0.66) and were independent of fish length (Spearman rank correlation: *rho*=0.02, N=80, P=0.86).

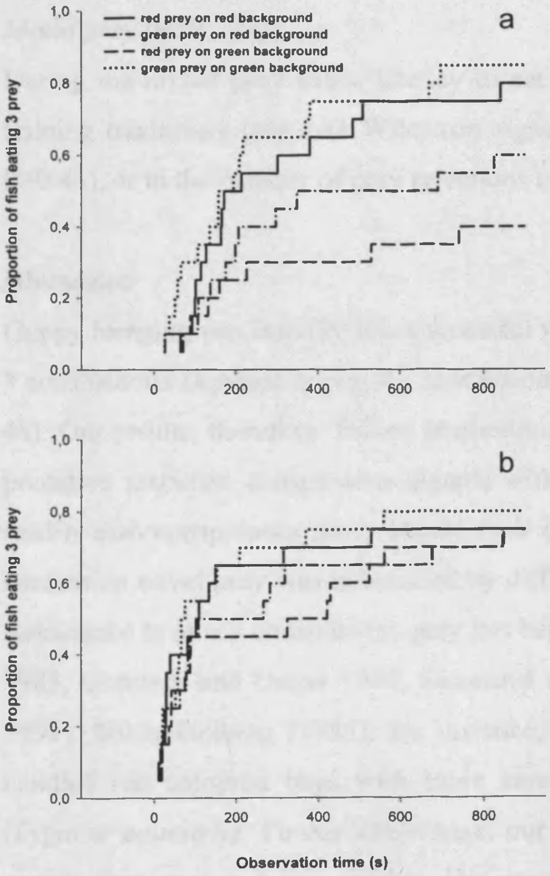

Figure 4: Proportion of fish eating at least 3 prey as a function of observation time and treatment for naïve fish (a) and experienced fish (b).

Effects of training

Guppies reduced their latency to eat 3 prey items during training across the first 7 trials (Table 1). This reduction was significant for all groups except guppies foraging on green cryptic prey (Table 1). Fish also reduced the number of prey rejections during training (Friedman ranked test; χ^2=29.87, N=80, P<0.001). Foraging behaviour of experienced fish was not influenced by prey colour, background colour or an interaction of these factors (Cox regression: N=80, d.f. =1, all P>0.4, α=0.025, Figure 4b).

Table 1. Results of different Friedman ranked tests to measure for changes in latency to eat 3 prey items in separate treatments and all fish during training

Background	Prey	X^2_6	*N*	*P*
red	red	15.75	20	0.02
red	green	17.35	20	0.01
green	red	15.38	20	0.02
green	green	12.06	20	0.06
all	all	45.85	80	<0.001

Mixed prey trials

During the mixed prey trials, latency to eat the first red or green prey did not differ in any of the training treatments (separate Wilcoxon signed ranks tests per treatment within each trial: N=20, all P>0.43), or in the number of prey rejections of red or green prey (N=20, all P>0.19).

Discussion

Guppy foraging was initially less successful when novel prey were conspicuous. Median latency to eat 3 conspicuous *Daphnia* during the first encounter was more than twice that of cryptic *Daphnia* (Figure 4a). Our results, therefore, follow predictions of the unprofitable-prey hypothesis that postulates that predators associate conspicuous signals with unprofitability and therefore attack cryptic prey more readily than conspicuous prey, despite their detection cost (Gotmark and Unger 1994). Thus foraging success on novel prey was influenced by differences in prey acceptance rather than visual constraints. Reluctance to attack conspicuous prey has been demonstrated in different bird species (Sillén-Tullberg 1985, Gotmark and Unger 1994, Exnerová et al. 2003, Halpin et al. 2008 but see Lindström et al. 1999). Sillén-Tullberg (1985), for instance, demonstrated that hand-raised great tits (*Parus major*) handled red coloured bugs with more care than the mutant cryptic bugs from the same species (*Lygaeus equestris*). To our knowledge, our study is the first to report reduced acceptance of novel conspicuous prey in a fish species. The occurrence of such a foraging bias has been suggested to be important for the evolution of warning colouration (Thomas et al. 2004, Marples et al. 2005) because it can help explain the, at first paradoxical, notion of the survival of a rare conspicuous morph in a population of cryptic prey. However, this explanation remains controversial due to the difficulty in determining whether foraging biases pre-existed or evolved as a consequence of the existence of defended prey with conspicuous colouration (Sherratt 2002).

Ioannou & Krause (2009) recently showed that a combination of cryptic colouration and immobility is a key for success in circumventing visually hunting predators. Under natural conditions, however, cryptic prey need to forage, forcing them to balance activity with predation risk (Werner and Anholt 1993, Vesakoski et al. 2008). Therefore, we speculate that increased prey activity may cause a shift from visually constrained predation towards predation dependent on prey preference mechanisms. As a consequence, the present dominance in the published literature of studies using immobile artificial prey (Ruxton et al. 2004) may have resulted in a bias when evaluating the relative importance of cognitive constraints and prey preference in shaping predator-prey interactions.

Guppies initially attacked green prey faster than red prey. This is surprising since Rodd et al. (2002) showed a preference to forage on orange and red over green artificial algae patches in guppies from wild populations. In female guppies, this preference is thought to have been a precursor to their preference for males with orange spots in a mating context (sensory bias hypothesis; Rodd et al. 2002). We wish to suggest two alternative mechanisms to explain this disparity. First, differences in either experience or selection pressures between captive-bred domestic and wild-caught guppies can cause their foraging behaviour to differ (Huntingford 2004, Brännäs and Johnsson 2008). Indeed, compared to natural prey, artificial flake food generally provided to domestic guppies leaves less, if any, associations between prey colour and their nutritional content. Studies of guppy mating behaviour show that female preference for male colour patterns is influenced by a complex interaction between experience and inheritance, and is influenced by local selection pressures (Endler and Houde 1995, Dugatkin 1996, Brooks 2002). It remains to be understood how colour preferences in a foraging context are affected by experience and relaxed selection pressures in domestic guppies. Alternatively, if our findings are representative for wild guppies, prey colour preferences in guppies may be context specific. Context-dependent prey colour preference has been demonstrated in birds when given the choice between red and green prey (Gamberale-Stille and Tullberg 2001, Gamberale-Stille et al. 2007). For example, domestic chicks (*Gallus gallus domesticus*) prefer to attack green over red prey if prey are insects, but show no colour preference with artificial pastry prey (Gamberale-Stille and Tullberg 2001). Further tests showed that preference for green prey remained when insects were motionless, suggesting that in this case prey colour preference was dependent on prey appearance, not movement (Gamberale-Stille and Tullberg 2001). Predators can, therefore, adjust prey colour preferences depending on other cues associated with the same prey. Similar differences may occur in guppies, but a test with multiple prey types should be performed to clarify this.

In nature, harmful prey often advertise their nature with conspicuous signals (Wallace 1867, Ruxton et al. 2004). Avoidance of brightly coloured or conspicuous novel prey may, therefore, be a strategy of guppies to avoid chemically defended prey (Sherratt 2002). Even though the effects of warning colouration and chemical defences in predator-prey relationships are less studied in aquatic than in terrestrial ecosystems, they may be more common than previously thought (e.g. Kerfoot 1982, Eisner and Aneshansley 2000, Härlin 2005, Walsh et al. 2006). Kerfoot (1982) suggested the role of warning colouration as an explanation for the bright red colouration of water mites, by demonstrating that guppies lose interest in artificial prey when this contained powder from different species of brightly coloured red water mites. Even though water mite fauna within the natural range of guppies remains poorly studied (T. Goldschmidt pers. comm.), water mites have a worldwide occurrence, and are likely to co-occur with wild guppies (Bader 1988, Rosso de Ferradas and Fernandes 2005, Goldschmidt 2007). Therefore, if wariness towards red or conspicuous prey in guppies evolved in response to co-occurrence with harmful prey with warning colouration, water mites represent likely candidates.

Experience improved guppy foraging success in three out of the four prey-background combinations (Table 1) and the foraging success of experienced fish was not affected by treatment. Guppies also reduced the number of prey rejections with experience. Initial prey rejection behaviour can indicate a lack of handling skills (Wainwright 1986) and/or taste-rejection behaviour scoring the edibility of the novel prey (Halpin et al. 2008). In the first case, however, rejections are expected to be fewer when predator size is small in relation to the prey size, a prediction which is not supported by our data. The alternative explanation of taste-rejection behaviour has been observed in different species, for example, in domestic chicks (Skelhorn and Rowe 2006), great tit, *Parus major*, (Sillén-Tullberg 1985) and guppies (Kerfoot 1982), and may be an efficient way of trying prey without taking the risk of ingestion. Studies on birds further demonstrated increased taste-rejection behaviour towards conspicuous prey during the first prey encounters (Sillén-Tullberg 1985, Halpin et al. 2008). However, no such effects were found in the present study.

The initial reduction in acceptance of conspicuous prey disappeared with training and did not occur during the mixed prey trials in guppies trained on cryptic prey. In fact, no signs were observed of preference for a specific prey colour during the mixed prey trials. Positive experience with the movement and form of the *Daphnia* during training may have reduced the novelty of the new colour morph during the mixed prey trials. As a consequence, reluctance for novel conspicuous prey in guppies can be reduced by relatively little experience with edible cryptic prey with similar form and behaviour. This observation is consistent with observations in domestic chicks, where experience with crumbs of one colour deactivates the reluctance to forage on similar food of all colours (Marples et al. 2007). Our results, therefore, suggest that guppies are to some extent capable of generalizing between similar prey types.

In conclusion, our results show that guppies are more reluctant to forage on novel conspicuous than novel cryptic prey. Background contrast may play an important role in modifying the motivation to feed on novel prey. Reluctance was, however, temporary and fish reduced their latency to eat 3 prey items with training, which resulted in similar foraging success for cryptic and conspicuous prey in experienced fish. Also, relatively little positive experience with similar, but cryptic, prey resulted in equal foraging success on the two prey types. Avoidance of conspicuous prey at first exposure was, therefore, not caused by contrast and prey colour alone, but may have been amplified by unfamiliarity with other stimuli such as the form and movement of the prey. Given that availability of different prey species within a predator's natural home range is subject to constant changes, the ability of a predator to rapidly adjust to these changes may be important for survival. Our results suggest that novel, undefended, conspicuous prey can initially survive predation if multiple encounters with the same predator are rare.

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