Cognition in freshwater fish: effects of the environment

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Abstract

For animals that live in a reasonably variable environment the capacity for learning and memory allow them to adapt to the changes they experience. Ecological factors that vary between habitats can affect a range of learning behaviours. Less attention has been directed at how this variation may affect memory processes, or how different ecological variables might interact when shaping cognition and behaviour. Therefore one aim of this thesis was to investigate how different ecological variables shape memory abilities and to test whether those same variables affect other related behaviours such as learning. In order to test this, I selected natural populations of a temperate freshwater fish, the three-spined stickleback (*Gasterosteus aculeatus*) from pond and river habitats that were proposed to differ in predation pressure, and assayed their learning, memory and other behavioural traits. Pond and river populations differed in their memory and orientation behaviour. An interaction between pond/river habitat and predation pressure affected learning rate, and a similar interaction affected temperament behaviours.

Two further studies were conducted to address how captive rearing environments and typical handling procedures affect behaviour in different species. Rearing environment affected memory, but not learning or temperament behaviours in three-spined sticklebacks. Handling caused stress responses in three-spined sticklebacks, Panamanian bishops (*Brachyraphis episcopi*) and Rainbow trout (*Oncorhynchus mykiss*), but handling with a water filled scoop compared to a traditional dip-net decreased these responses in three-spined sticklebacks and Panamanian bishops, and also affected behaviour in Panamanian bishops.

The results presented in this thesis suggest that ecological variables play a substantial role in shaping learning, memory and other behavioural traits in fish, and highlight the utility of behavioural assays in answering welfare-based questions.

Contents

Declaration

I declare that this thesis has been composed by me and is the result of my own work, except for the collaborations mentioned below. It does not exceed 70,000 words, and has not been submitted to any other university in application for a higher degree.

Chapters 2 & 3. A summer research student (Robert Heathcote) helped to collect the morphometric data used to classify populations as high or low predation (section 2.3.2.). He also helped with the field data work presented in appendix 1. Dr Nick Colegrave helped with the statistical analysis in Chapter 3.

Chapter 4. A summer student, Andrew Holmes, helped to run the behavioural trials in this chapter.

Chapter 6. The cortisol assay procedure was conducted by Dr Tim Ellis, Cefas Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB. The trout (*Oncorhynchus mykiss*) behavioural data in this chapter were collected by Dr Phil Boulcott, Division of Animal Production and Public Health, Faculty of Veterinary Medicine, Jarrett Building, University of Glasgow, Garscube Estate, 464 Bearsden Road, Glasgow G61 1QH, Scotland, UK.

The following papers have been submitted from the work described in this thesis:

N.M.Brydges, R.J.P.Heathcote & V.A. Braithwaite. Habitat stability and predation pressure influence learning and memory in populations of three-spined sticklebacks. *Animal Behaviour (in press).*

N.M.Brydges, N.Colegrave, R.J.P.Heathcote & V.A. Braithwaite. Habitat stability and predation pressure affect temperament behaviours in populations of three-spined sticklebacks. *Journal of Animal Ecology (in press).*

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Chapter 1. General Introduction

1.1. Learning and memory

1.1.1. Learning and memory – why?

In reasonably variable environments, learning and memory allow animals to adjust their behaviour in a flexible manner that more genetically fixed patterns of behaviour do not (Shettleworth 1998). It is easy to imagine how learning and remembering about certain aspects of the environment could enhance fitness. For example, those animals with a good learning and memory capacity for profitable feeding patches or refuges are more likely to obtain the best food or avoid predation. There are many examples of how learning can be beneficial in a wide variety of contexts and species. In a foraging context, shore crabs (*Carcinus maenas*) improve their ability to open hard-shelled prey such as clams and snails through experience (Cunningham & Hughes 1984), and are able to transfer these learned skills to novel prey that require similar handling (Hughes & O'Brien 2001). Similarly, both hatchery and wild caught salmon (*Salmo salar*) increase their foraging efficiency on different types of prey with experience (Reiriz *et al.* 1998, see Warburton 2003 for a review on learning in fish). Learning in this way allows animals to efficiently exploit whatever type of prey is currently available in the environment, and this will be beneficial if the availability of prey types differs over time. Learning also affects mate choice. Female zebra finches (*Taeniopygia guttata*) prefer males that display at a higher rate if they have previously seen males displaying at a high rate, but are not choosy if they have only seen males with a low display rate (Collins 1995). Similar results are found even in short-lived invertebrates: female fruit flies

(*Drosophila melanogaster*) that have been courted by small males accept small and large males for mating, whereas those only courted by large males only accept larger males (Dukas 2005). As males that display at a higher rate (zebra finches) or larger males (*Drosophila melanogaster*) tend to have greater mating success and are considered higher quality, this demonstrates how females can adjust their mate preference thresholds based on their own experience of what is currently available.

1.1.2. Learning and memory - when?

Learning and memory are proposed to be costly processes. For example, costs may be incurred through making mistakes, and the physical cost of producing and maintaining neurological machinery (Dukas 1999, Laughlin 2001). Empirical studies on *Drosophila melanogaster* support this; populations bred for enhanced learning ability have decreased productivity and the competitive ability of their larvae is reduced (Mery & Kawecki 2003, 2004). Further support comes from studies on divided attention. Silver perch (*Bidyanus bidyanus*) offered a single prey type reach maximal intake rates in only 5 learning trials, whereas those presented with two prey types take 12-20 trials to converge on the most profitable of the two prey types (Warburton & Thomson 2006). When presented with one prey type only, the fish can focus their attention on learning about it, but when two types are present, it is proposed that cognitive constraints on the amount of information able to be processed impairs efficiency (see Dukas 2004 for a review on limited attention). A further suggestion that learning and memory are costly comes from food storing in birds. Several species of bird store food, and retrieve it days to months later, using spatial memory to relocate their caches. A within-species

comparison revealed that a population of black-capped chickadees (*Poecile atricapilla*) inhabiting a harsher terrain have a greater learning and memory capacity for cache storage and recovery, and a larger hippocampus (a structure known to be important in spatial memory) than a population living in milder habitat, suggesting that the benefits outweigh the costs of investing in greater learning and memory in the harsher terrain (Pravosudov & Clayton 2002). Furthermore, food storing is seasonal in some species, including black-capped chickadees. Just before and during the storing season, the size of the hippocampus (Smulders *et al.* 1995) and recruitment into the neuron population (Barnea & Nottebohm 1994, Sumlders *et al.* 2000) increases, presumably to cope with increased spatial demand. The fact that hippocampal size and neuron population change seasonally suggests that they are costly to maintain.

So when should an animal invest in learning and memory, and how long should they remember for? This has been investigated in models that weigh up the proposed costs and benefits of learning and memory (e.g. Papaj & Prokopy 1989, Anderson 1991, Dukas 1999), and a key factor appears to be the stability of the environment (e.g. Stephens 1991, Kerr & Feldman 2003). If the environment was stable, and never changed, then the same behaviour would be appropriate time and time again, and we would expect such behaviour to become genetically controlled. Indeed, in environments experiencing little or no change animals often display no or reduced learning and memory (Potting *et al.* 1997). Conversely, if the environment was unpredictably variable, then nothing of any value could ever be learned and remembered (Shettleworth 1998), and a more appropriate strategy may be to base behaviour on environmental averages.

In terms of foraging behaviour, these ideas have been developed further, particularly with regard to how long learned information should be remembered for. Between the two extremes of a never changing environment and unpredictable environmental variation, it is expected that long-term memory will be advantageous in environments where food patches are relatively stable and predictable (Hirvonen *et al.* 1999, Fortin 2002). However, when the environment is changing more rapidly, the value of more recent information increases, and this should favour short-term memory (Cowie 1977, Eliassen PhD thesis 2006, also see Fig. 1.1).

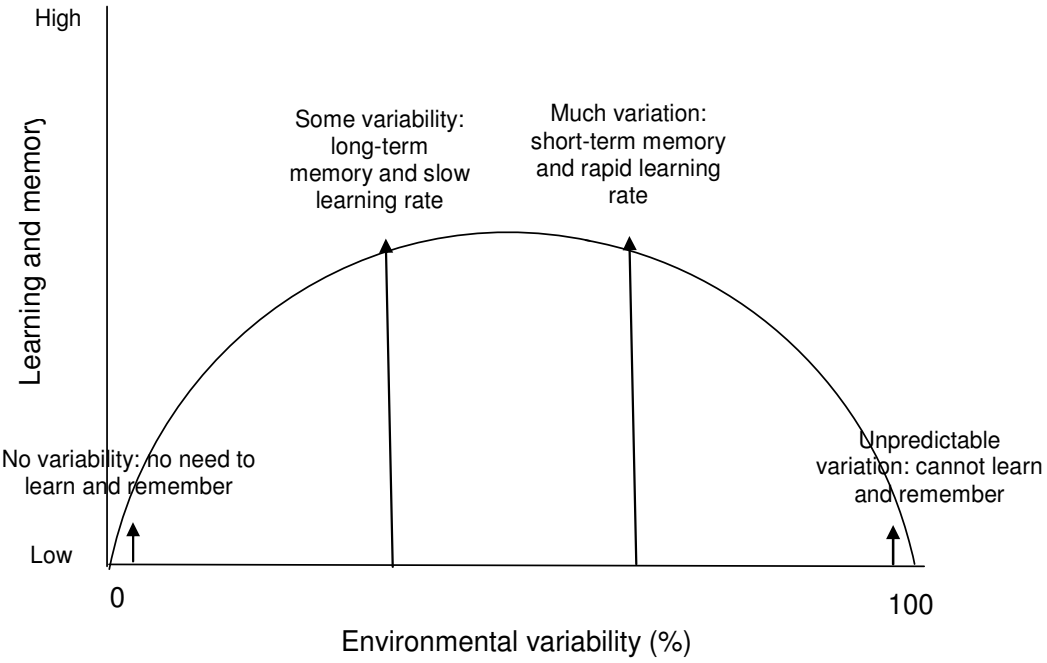

Figure 1.1. Predicted learning and memory of animals as a function of environmental variability.

Experiments with pigeons support this, as in a less predictable environment the birds place less emphasis on past experience and more on rapid adjustment to present circumstances (Shettleworth & Plowright 1992, Schofield & Davidson 1997, Bell & Baum 2002). In terms of memory duration, similar evidence comes from a study on prey handling skills. Three-spined sticklebacks (*Gasterosteus aculeatus*) living in a pond habitat with a relatively consistent prey fauna over time remember how to handle specific prey types for a longer amount of time than 15 spined sticklebacks (*Spinachia spinachia*) originating from marine habitats where prey type varies more frequently (Mackney & Hughes 1995). In contrast to the three-spined sticklebacks it is advantageous for the 15-spined sticklebacks to have shorter memory duration for prey handling skills so that when the relative abundance of prey types changes, they quickly learn to handle and exploit whatever is available. However, as only one population of each species was used in this study, it is difficult to be sure that prey stability is causing these differences, and not some other ecological variable or phylogenetic constraint.

1.1.3. Learning and memory – a role for temperament behaviours?

The temperament of an animal may have an effect on its learning and memory abilities. Measures of temperament include, for example, boldness, neophobia, activity and aggression, and are often based around the five axes of personality developed for humans (see Gosling & John 1999). Sometimes these behaviours are correlated with learning and memory abilities. A number of studies have found that bolder individuals learn simple conditioning tasks faster than less bold conspecifics, for example, trout, *Oncorhynchus mykiss* (Sneddon 2003) and guppies, *Poecilia reticulata* (Dugatkin &

Alfieri 2003)). There are a number of possible explanations for these results, for example, bolder individuals may explore their environment more or be less averse to novelty, which may increase their encounter rate with various environmental stimuli such as food patches, enhancing their learning rate.

Animals can also show consistency over time in their temperament behaviours, and different temperament behaviours can be correlated (often termed a 'behavioural syndrome'). For example, an animal that is bolder may also be more aggressive (Bell 2005). Consistencies in and correlations between temperament behaviours are not easy to explain, because animals are generally expected to be flexible in their behaviour, allowing them to cope with changing circumstances. For example, we can imagine a scenario where an animal that is always bold and always aggressive fares poorly, perhaps when faced with a predator. A recent model based on life-history strategies has begun to explore adaptive explanations for the persistence of animal personalities (Wolf *et al.* 2007). This model suggests that if there is a trade-off between, for example, current and future reproduction, then two different strategies may evolve: reproduce now or save resources and reproduce later. Wolf and colleagues (2007) suggest that the costs and benefits of certain levels of temperament behaviours such as boldness and activity will differ depending on the reproductive strategy chosen. If an animal chooses to wait to reproduce, then it should be consistently less bold in many circumstances, because it has to live to realise that reproductive benefit. Conversely, animals reproducing now do not have to be so cautious, and can perhaps benefit from being consistently bold. Considering animal personalities from a life-history point of view indicates how they

might be adaptive, and this type of approach will benefit from the development of more comprehensive models in the future.

In terms of correlations between behaviours, there are currently two competing hypotheses, the 'Constraints' and the 'Adaptationist' hypothesis (Bell 2005). The 'Constraints' hypothesis postulates that when behaviours are correlated, it is due to an underlying constraint, for example the pleiotropic effect of genes or proximal links. On other hand, the 'Adaptationist' hypothesis argues that when behaviours are correlated it is because they are adaptive. One way to untangle these two hypotheses is to compare correlations between behaviours in different populations of the same species. If the 'Constraints' hypothesis holds true, then if those behaviours are correlated in one population they must necessarily be correlated in all others. Using this approach, Bell (2005) recently found support for the 'Adaptationist' hypothesis, as boldness and aggression were positively correlated in a high predation population of three-spined sticklebacks but this was not the case in a low predation population (Bell 2005). This suggests that predation pressure may play a role in causing correlations between temperament behaviours. However, as this study only compared two populations, it remains unclear what ecological variables might be important in causing correlations between temperament behaviours.

1.2. The role of ecology

Ecological variables appear to play a role in shaping certain behaviours. Indeed, we might expect behaviours such as learning, memory and temperament to be fine-tuned within a population to suit specific environmental requirements. Comparing (i) between

closely related populations of animals inhabiting diverse ecological habitats or (ii) between distantly related species experiencing similar ecological selection pressures can test theoretical models and provide insights into what natural variables are important in shaping such behaviours (Sherry 2006). Traditionally, these comparisons have been made between species. For example, food-storing birds tend to have a better spatial memory and a larger hippocampus than species that do not store (e.g. Krebs *et al.* 1989). This provides support for the hypothesis that the ecological demand of needing to store food selects for a greater spatial memory capacity and a larger hippocampus. Within species, spatial habitat stability is hypothesised to affect the cues used in orientation by three-spined stickleback fish. Fish from pond habitats use visual landmarks whereas those from river environments use the turn direction of their own body when navigating to a food reward in a maze (Girvan & Braithwaite 1998, Braithwaite & Girvan 2003). Ponds are hypothesised to be more spatially stable environments, so here it is thought that visual landmarks will be reliable navigation cues, whereas in a river, where flow and flooding can move landmarks around, turn direction may be more reliable.

A compelling example of how different ecological demands can affect learning and memory comes from studies on sex differences in spatial behaviour in mammals and birds. Males of the polygynous meadow vole (*Microtus pennsylvanicus*) compete for females over a large home range, where a good spatial ability is advantageous (Spritzer *et al.* 2005). Females do not have such a demand on their spatial ability, and in spatial laboratory tests males perform better (Gaulin & Fitzgerald 1989), and they also have a larger hippocampus than females (Jacobs *et al.* 1990). In contrast, males and females of the monogamous pine vole (*Microtus pinetorum*) have similar home ranges, spatial

ability (Gaulin & Fitzgerald 1989) and hippocampal sizes (Jacobs *et al.* 1990). The opposite pattern is found in cowbirds. Female brown-headed (*Molothrus ater*) and shiny (*Molothrus bonariensis*) cowbirds lay their eggs in the nests of other species, where a good spatial ability is proposed to be advantageous. Males do not participate in selecting host nests, and females have a larger hippocampus than the males (Sherry *et al.* 1993, Reboreda *et al.* 1996). Importantly, no sex differences are found in two closely related species, the screaming cowbird (*Molothrus rufoaxillaris*), where both males and females search for suitable nests, and bay-winged cowbirds (*Molothrus badius*), which are not parasitic (Reboreda *et al.* 1996).

1.3. Cues used in orientation

There would be little point to an animal learning and remembering specific spatial details of their environment, such as the location of nest sites or foraging patches, if they did not have reliable cues to guide them back there. Animals have many techniques and tools that they use to guide their movements (reviewed in Healy 1998, Bingman & Cheng 2005). For example, they can follow compass directions or keep track of their own movements using path integration. Others are capable of learning to use cues and landmarks in their environments to generate maps. The cues used to create such maps can have several different modalities, for example, they may be based on visual landmarks (e.g. Collett & Collett 2002), smells (e.g. Papi 1990, Papi 2006) or even sounds (e.g. Payne & Webb 1971, Walraff 2003, Jensen *et al.* 2003). The use of geometric cues, defined as distances, angles and directions, has been particularly well studied in a variety of species (reviewed in Cheng & Newcombe 2005, Cheng 2005),

and was first investigated in the rat. Rats were trained to find food in a rectangular environment, and made systematic rotational errors between geometrically equivalent corners (Cheng 1986, Fig. 1.2.).

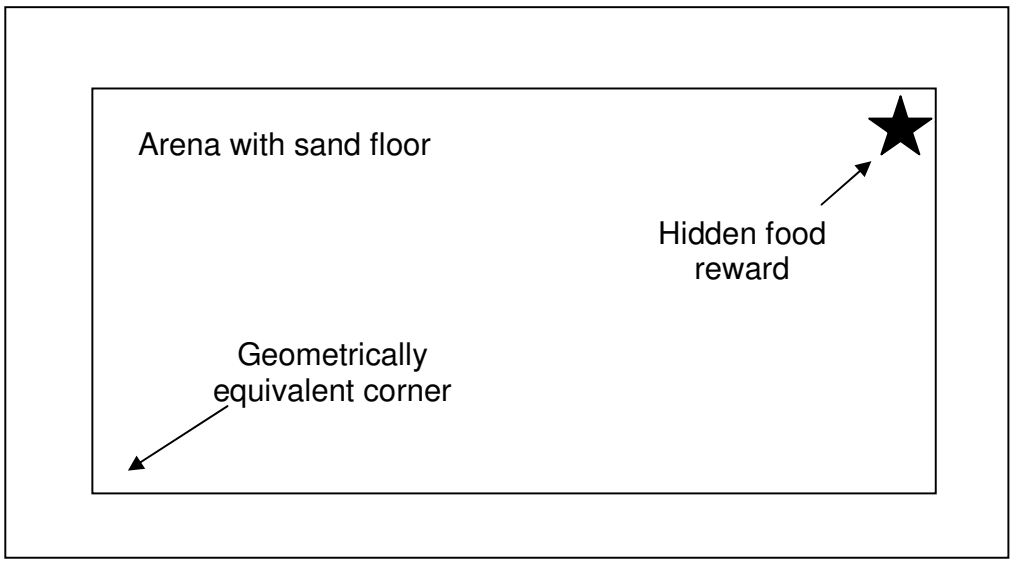

Figure 1.2. Rectangular arena used to train rats. Rats were trained to find food in one corner (represented by the star). The walls are identical in every way except for length. Using geometry alone rats could select the correct corner and its geometric equivalent (i.e. both the target location and its geometric equivalent have a long wall on the left and a short wall on the right).

Surprisingly, the rats ignore reliable non-geometric landmarks such as the colour of a wall and odours. This was subsequently found to be the case in human infants, who ignore a blue wall and solid landmark cue, and only use geometry when attempting to locate a hidden object (Hermer & Spelke 1994). This is not found in human adults (Hermer & Spelke 1994) or even species of fish, such as the red-tailed splitfin (*Xenotoca eiseni*) (Sovrano *et al.* 2002, 2003: see Vallortigara *et al.* 2005, Cheng & Newcombe

2005 for reviews). Humans and red-tailed splitfins can combine geometric information with non-geometric features in an environment to successfully locate targets. There is no consensus as to why some groups combine these types of information whereas others do not, but Sovrano *et al.* 2007 have suggested that ecological adaptations may be the reason. A within species comparison between populations inhabiting contrasting habitats could provide a good test of this hypothesis.

1.4. Study system

Three-spined sticklebacks are a suitable species to investigate questions of how ecological variables might shape behaviours such as learning, memory and temperament. After the retreat of the last ice age (around 10,000-15,000 years ago), three-spined stickleback populations colonised a wide variety of marine, brackish and freshwater habitats throughout the Northern hemisphere (Bell & Foster 1994). Consequently, they have experienced an equally wide variety of ecological circumstances, which have caused a divergence in numerous morphological and behavioural traits, probably aided by the fact that the majority of populations are reproductively isolated (Bell & Foster 1994). This system therefore could provide an opportunity to study how ecological variables can influence behaviour. Perhaps because of this, the ease of maintaining and breeding them in a laboratory environment, and their widespread occurrence, there exists a great wealth of information on many aspects of three-spined stickleback biology. For example, they have been used in studies of foraging (Schluter 1995, Coolen *et al.* 2003, Webster & Hart 2006, Quesenberry *et al.* 2007), courtship (Ishikawa *et al.* 2006, Shaw *et al.* 2007), parental care (Lachance &

Fitzgerald 1992), mate choice (Bakker & Mundwiler 1994, Barber *et al.* 2001, Aeschlimann *et al.* 2003, Smith *et al.* 2004) inbreeding avoidance (Frommen *et al.* 2006), shoaling (Peuhkuri 1997) environmental studies (e.g. Ernst *et al.* 1991, Katsiadaki *et al.* 2002, Gravenmier *et al.* 2005, Sanchez *et al.* 2005), phylogeny (e.g. Takamura & Mori 2005, Raeymaekers *et al.* 2005, Malhi *et al.* 2006), genetics (Peichel *et al.* 2001), morphology (Bell *et al.* 2004, Vamosi & Schluter 2004, Zimmerman 2007) social behaviour (Ward *et al.* 2005, Sneddon *et al.* 2006), vision (Boulcottt *et al*. 2005) parasite effects on behaviour (Dugatkin *et al.* 1994, Barber *et al.* 2004), anti-predator behaviour (Wright & Huntingford 1993) temperament (Huntingford 1976, Bell 2005), learning (Losey & Sevenster 1995, Girvan & Braithwaite 1998, Braithwaite & Girvan 2003, Odling-Smee & Braithwaite 2003) and memory (Milinski 1994, Mackney & Hughes 1995). A recent review highlights the utility of the three-spined stickleback as a model organism, particularly in answering developmental questions (Kiefer 2006). So popular is this organism as a model for a range of biological questions that entire books, '*The Evolutionary Biology of the Threespine Stickleback'* (Bell & Foster 1994) and '*Biology of the Three-Spined Stickleback'* (Ostlund-Nilsson *et al*., 2007) are devoted to it, and a small conference series has been established to bring together researchers working with sticklebacks (see Bell 1995, Braithwaite & Odling-Smee 1999).

1.5. Integrating behaviour and welfare

As indicated in the previous section, three-spined sticklebacks are widely used as a model system in biology, particularly in studies of behaviour. Although they are relatively easy to maintain and rear in the laboratory, little consideration has been given

as to how the laboratory environment itself affects their behaviour and physiology. If housing conditions and routine husbandry practices such as handling affect behaviour and physiology, this could have implications not only for the welfare of fish, but also for the validity of experimental data, areas that have recently received much attention in other species (see Balcombe 2004, Morgan & Tromborg 2007 for reviews). It has also been proposed that fish have sufficient cognitive capacity to suffer and experience pain, however this a debated topic (see Rose 2002, Braithwaite & Huntingford 2004, Chandroo *et al.* 2004a, Chandroo *et al.* 2004b, Dunlop & Laming 2005, Braithwaite & Boulcott 2007, Rose 2007 for reviews). Given the vast number of studies that use fish such as the three-spined stickleback in the laboratory (see e.g. Bell & Foster 1994), it would seem timely that we determine how housing conditions and routine husbandry practices affect them. There are a number of different approaches that can be taken to answer such questions. The effects of different housing conditions and husbandry practices on physiology can be used to compare how stressful different methods are (e.g. Laitinen & Valtonen 1994). Tests of cognitive behaviour can also be used to infer how these practices affect the animals' psychological state, a promising technique that has recently received attention (Paul *et al.* 2005).

1.5.1. Integrating behaviour and welfare – housing conditions

Housing conditions will be a primary concern in any study of captive animal welfare. These conditions are what the animals must live in day to day, and for the majority of their lives in many cases. It is well known, particularly from the rodent literature, that the nature of housing conditions can have dramatic effects on behaviour and physiology

(see Balcombe 2006 for a recent review). Traditionally, animals were housed in plain, barren environments, in an attempt to maintain good physical health and standardize behaviour between different groups (Olsson & Dahlborn 2002). However, barren housing conditions are thought to have detrimental effects on welfare (Dawkins 1988, 1998), and can potentially decrease the validity of experimental data, for example, through producing abnormal behaviour and physiology (Würbel 2001, Reinhardt 2004). Enriching the environment, for example through providing social stimulation or structural complexity, has a number of often beneficial effects on the animals. It can decrease stress responses (Fox *et al.* 2006), decrease stereotypies (Mason *et al.* 2007), equip commercial fish with better behavioural skills (Brown *et al.* 2003, Braithwaite & Salvanes 2005), and enhance learning and memory (Paylor *et al.* 1992, de Jong *et al.* 2000, Woodcock & Richardson 2000, Leggio *et al.* 2005). Enrichment is believed to be valuable to animals, because if given the opportunity, they will work to access it (Olsson & Dahlborn 2002). The majority of enrichment studies have focussed on rodents (reviewed in Balcombe 2006), animals housed in zoos (reviewed in Mason *et al.* 2007), and a few commercial fish species. To date, there has been no such investigation in a commonly used, laboratory fish species. Such a study would give an insight into how housing conditions might affect welfare and accuracy and consistency in experimental data, particularly if abnormal behaviours are produced in some environments (Würbel 2001, Reinhardt 2004). Furthermore, this type of study would give an insight into the mechanisms that underlie plasticity of behaviour.

1.5.2. Integrating behaviour and welfare – handling methods

Another potential welfare issue facing many captive animals is the method by which they are handled. Animals may be moved from place to place for a number of reasons, including routine cleaning or movement from the home environment to experimental or slaughter environments. Handling is thought to be a significant source of stress for many animals (Grandin 1997, Dwyer & Bornett 2004, Balcombe *et al.* 2004, von Borell & Schaffer 2005, Waiblinger *et al.* 2006, Muller *et al.* 2006, Portz *et al.* 2006), and can produce a variety of physiological and behavioural responses, for example, increased heart rate and blood pressure in rodents and increased corticosterone/cortisol (generally accepted physiological measures of stress) in rodents, birds and fish (reviews in Barton & Iwama 1991, Barton 2002, Balcombe *et al.* 2004, Portz *et al.* 2006), disrupted behaviour in rodents (Burman & Mendl 2004) behavioural and immunological effects in laying hens (Barnett *et al.* 1994) and fish (e.g. Frisch & Anderson 2000) and reduced growth rate in fish (Hoskonen & Pirhonen 2006). Reducing and or refining handling methods can improve animal welfare and productivity. For example, reducing handling stress increases productivity and welfare in farm animals (reviewed in Grandin 1998, Rushen *et al.* 1999). The nature of the handling technique used can also alter the responses of the animals. For example, Holstein-Fresian heifers that were 'positively' handled by encouraging them to move along using pats, strokes, and slow deliberate movements approached a stimulus person faster, had a shorter flight distance and lower cortisol levels than their 'negatively' handled counterparts, which were moved along using hits, slaps, prods with hard plastic tubing and quick movements (Breuer *et al.* 2003). Similarly, regular handling (or even visual contact with humans) of chickens in

some cases reduces adverse behavioural, physiological and immunological responses to humans and increases egg production (Barnett 1994, Zulkifli *et al.* 2002 but see Leonard & Fairfull 1992) and increases their food conversion efficiency (Jones & Waddington 1992, 1993), presumably through habituation to humans. These examples demonstrate that refining the method of handling is likely to be as useful as finding ways of reducing it. From an experimental point of view, stress may increase the variability of experimental data, particularly if some animals are more stressed than others by handling, so reducing it also helps to ensure consistency and validity in experimental data (Balcombe *et al.* 2004).

Stress should not automatically be considered detrimental, as it is essentially an adaptive mechanism allowing an animal to cope and maintain homeostatsis in the face of environmental challenges (Barton 2002, Davis 2006). The problem occurs when a stressor is prolonged or extreme (Barton & Iwama 1991, Wendelaar Bonga 1997, defined as *distress* by Balcombe *et al.* 2004) as this can have many deleterious effects on both the behaviour and physiology of captive animals (see Barton 2002, Portz *et al.* 2006, Morgan & Tromborg 2007 for reviews). This is potentially the case with handling in laboratory fish, where individuals may be handled repeatedly day after day during experimental trials. Presently, most laboratory and young commercial fish are handled with dip nets and spend some time out of the water. This may potentially have detrimental consequences, for example, by disrupting mucous coating and scales leading to pathogenic and parasitic attack, increasing oxygen demand and elevating stress levels (FSBI 2002, Conte 2004). Studies using commercial fish demonstrate how detrimental handling can be: juvenile rainbow trout that were repeatedly handled put on less weight

and had a reduced feed intake (Hoskonen & Pirhonen 2006), and it took 2 weeks for brown trout (*Salmo trutta*) to recover completely from just 2 minutes of handling (Pickering 1982). Similarly, coral trout (*Plectropomus leopardus*) that were captured, handled and transported displayed lower levels of cellular based immunity (Frisch & Anderson 2000). Adverse effects of handling have been well established in commercial species of fish (e.g. Baron & Iwama 1991, Barton 2002), but little information exists on how it may affect commonly used laboratory fish such as guppies and sticklebacks.

1.6. Aims of thesis

Investigations that compare different populations of the same species can provide valuable insights into how certain ecological variables shape and influence animal behaviour, and this type of approach allows us to study the adaptive variation of those behaviours. This kind of comparative method is widely used and there are numerous theoretical models suggesting which variables should be important in shaping certain behaviours. Despite the many models and hypotheses, however, in many cases there is still a paucity of empirical data that test these models. This is particularly true for behaviours relating to animal cognition, especially with regards to memory processes. Furthermore, empirical investigations typically only consider one ecological variable at a time, yet different variables in reality are likely to interact with each other when shaping behaviour. Therefore, one aim of my thesis is to investigate how multiple natural environmental variables shape behaviour across different populations. I primarily do this using a small freshwater fish, the three-spined stickleback, because different populations of this species are readily found in contrasting types of environment. As a

primary concern of anyone working with animals should be their welfare, the behavioural assays used to study questions about fish cognition are also developed to address questions associated with the best practice for handling fish for behavioural work within a laboratory setting, and this is the second aim of my thesis.

1.7. Structure of thesis

My thesis consists of 5 data chapters. Their contents are briefly described in the following section. I have opted to write the thesis up as a series of independent manuscripts, and each chapter has now been submitted for review to different journals (see declaration for further details). Taking this approach has led to a certain level of repetition between the Methods sections in some of the chapters.

In Chapter 2, I test the hypothesis proposed by several models that in a relatively stable environment, long-term memory will be advantageous (Hirvonen *et al.* 1999, Fortin 2002). In contrast, in a more rapidly changing environment, the value of more recent information should increase, and this should favour short-term memory (Cowie 1977, Eliassen, PhD thesis 2006). These ideas are supported empirically in a study of prey handling skills (Mackney & Hughes 1995), but have never been tested in a spatial context or with more than two populations. I use populations of three-spined sticklebacks from ponds and rivers, which are hypothesised to differ in their spatial stability (spatially stable ponds versus less spatially stable rivers), and compare their learning and memory ability for foraging patches. As predation pressure varies between the sites sampled, and this variable is known to affect learning (e.g. Brown & Braithwaite 2004), this is also quantified for each of the sites.

The third chapter extends these ideas and investigates which ecological variables might be important in shaping temperament behaviours. In particular, predation pressure is hypothesised to be a major variable determining whether certain temperament behaviours become correlated within populations (e.g. Bell 2005, Bell & Sih 2007). However, as this has never been tested in more than two populations, I investigate how predation pressure and pond/river environments affect these behaviours both within and between 8 populations of three-spined sticklebacks. As the individual fish used in this chapter are the same as those used in Chapter 2, the effects of temperament behaviours on learning and memory are also considered, as temperament behaviours such as boldness have previously been found to affect learning (e.g. Marchetti & Drent 2000, Sneddon 2003, Dugatkin & Alfieri 2003, Korte *et al.* 2005, Brown *et al.* 2005).

Chapter 4 examines how ecological variation affects cues used during orientation. Recent years have seen a growing body of work considering how information from more than one source might be combined during orientation tasks. To date, however, comparative studies on what factors might determine which of the available cues animals combine during orientation have been made exclusively between species. Here, I investigate the ability of populations of pond and river three-spined sticklebacks to use geometrical cues (the ability to use geometry has not thus far been tested in three-spined sticklebacks) and combine this geometry with other, non-geometric cues during an orientation task.

In Chapter 5 the effects of different rearing environments on learning, memory and temperament behaviours are considered. By comparing the behaviour of fish from

the wild with others reared in the laboratory in either enriched or plain tanks I investigate the mechanisms that underlie the plasticity of behaviour, and the implications that housing fish in different types of environments might have for welfare and the validity of experimental data. Environmental enrichment studies have previously focussed on rodents, animals housed in zoos (primarily mammals) and commercial fish species, where dramatic effects on behaviour, welfare and physiology have been found. This experiment is the first test of how enrichment might affect a non-commercial, commonly used laboratory fish species, the three-spined stickleback.

Chapter 6 uses the temperament assays developed in Chapter 3, alongside physiological measures of opercula beat rate and cortisol levels to investigate the effects of handling methods on behaviour and stress in three species of fish. Stress induced by handling is proposed to be detrimental to the welfare of fish for a number of reasons (FSBI 2002, Conte 2004). Allowing fish to remain submerged in water during handling by using a water filled scoop could potentially mediate some of these effects. In this chapter, I compare the effects of net versus scoop handling in three species of fish: three-spined sticklebacks, Panamanian bishops (*Brachyraphis episcopi*) and rainbow trout. I decided to compare responses in three species because there is a growing body of work that demonstrates how different species can differ in their reaction to the application of identical stressors (e.g. Barton 2002, Jentoft *et al.* 2005). This variation is not surprising, considering that fish are specious and phylogenetically diverse (Borski & Hodson 2003).

Chapter 2. Habitat stability and predation pressure affect learning and memory in populations of three-spined sticklebacks

2.1. Summary

Learning and memory enable animals to adjust their behaviour in variable environments. Not all habitats vary to the same extent, and thus different environments may affect learning and memory in different ways. Habitat stability is one of numerous environmental variables proposed to influence what animals learn, but it is unlikely to act alone. To investigate how multiple variables affect learning and memory behaviour, I compared spatial learning and memory in three-spined sticklebacks from four ponds (hypothesised to be stable habitats) and four rivers (hypothesised to be unstable habitats) thought to vary in their predation pressure. Contrary to initial predictions, river fish had longer memory duration (> week) than pond fish (<week). Learning rate was affected by an interaction between pond/river habitat and predation pressure, with low predation river populations learning faster than high predation river populations. These results demonstrate that learning and memory differ between populations, possibly as a result of contrasting ecological factors.

2.2. Introduction

2.2.1. Learning and memory

Learning and memory allow animals to adjust their behaviour to adapt to changeable environments and thus cope with a degree of unpredictability (Shettleworth 1998). In such environments, animals that use learning and memory to hone their behaviour will perform tasks better than other more behaviourally fixed individuals. For example, parasitoid wasps that select host substrate based on experience can parasitize a larger number of host eggs and produce more offspring than those forced to select at random (Dukas & Duan 2000). However, in environments where there is little or no change we find that animals sometimes show reduced, or even no learning and memory skills (Potting *et al.*1997). This suggests that there are costs associated with learning and memory; for example, it is speculated that there is a physical cost to producing and maintaining the required neurological machinery, and also there is the cost of making mistakes (e.g. Dukas 1999, Laughlin 2001). Surprisingly, there are only a few direct demonstrations of the costs associated with learning. In *Drosophila melanogaster*, populations selectively bred for enhanced learning ability had decreased productivity, and the competitive ability of larvae was reduced (Mery & Kawecki 2003, 2004). There are numerous theoretical models that consider the costs and benefits of learning and memory (e.g. Papaj & Prokopy 1989, Dukas 1999). Several of these models predict circumstances under which the benefits of learning and memory are greater than the costs and a key factor affecting this appears to be the degree of environmental variability (e.g. Stephens 1991, Kerr & Feldman 2003).

2.2.2. The role of ecology

Environments inhabited by different populations are likely to differ from one another in many aspects. As such, we might expect learning and memory processes to be fine-tuned within a population to suit specific environmental requirements that the animals encounter. A few avian studies have investigated this, both between and within species (e.g. Brodin 2005, Sherry 2006). For example, Pravosudov & Clayton (2002) found a population of black-capped chickadees inhabiting a less favourable habitat had a better learning and memory capacity for cache storage and recovery, and a larger hippocampus (a structure known to be important in spatial memory) than a population living in a more favourable environment. This suggests the benefits outweigh the costs of investing in enhanced learning and memory ability in the harsher terrain. Learning behaviour in fishes also appears to be fine-tuned to the local environment. Populations of Panamanian bishops originating from low predation sites solved a spatial task almost twice as quickly as those from high predation locations (Brown & Braithwaite 2004). Similarly, pond and river three-spined sticklebacks pay attention to different cues when learning the location of a food reward in a maze: pond fish prefer to use visual landmarks, whereas river fish prefer to use the turn direction of their own body (Girvan & Braithwaite 1998, Braithwaite & Girvan 2003). The stability of a landmark is known to affect its use as a spatial cue; the more unreliable the landmark, the less likely an animal will use it to guide it to a goal (Biegler & Morris 1996). Ponds are hypothesised to be spatially stable environments, for example in terms of foraging patch and landmark cue location, which would make visual cues reliable indicators of location in a pond. Rivers, however, are hypothesised to be less spatially stable due to flow and flooding causing, for example, a

greater turn over of foraging patches and moving landmarks around. In this situation, landmark cues would be less reliable than in ponds (Braithwaite & Girvan 1998, Odling-Smee & Braithwaite 2003). However, pond/river differences in spatially habitat stability have not been tested experimentally. Evidence to support this hypothesis could be obtained by, for example, taking invertebrate samples from patches of each habitat over time to determine if they are more variable in space and time in river compared to pond habitats, or by placing landmarks (such as small painted rocks) in each habitat and determining their spatial stability over time. Aside from the hypothesised differences in habitat stability, there are other systematic ways that ponds and rivers may differ. For example, ponds are enclosed environments, rivers open, which may have an impact on spatial memory. There is also the potential for fish to migrate in river habitats, whereas this is not possible in pond environments.

2.2.3. Memory

Although numerous studies have investigated learning (e.g. Moore 2003), less attention has been directed at memory. Learning and memory are linked, however, the processes have differences. Learning is essentially the acquisition of memory, whereas memory has other composites, such as retention and the potential for interference. Work directed at quantifying memory duration, how rates of forgetting progress, or what factors cause variation in forgetting rates is far less common than studies investigating the acquisition of information (Shettleworth 1998).

Traditionally, forgetting was considered a failing of memory, but over the past two decades we have moved towards the idea that forgetting may be advantageous

(Kraemer & Golding 1997). For example, forgetting the locations of previously rich but now poor feeding sites will benefit individuals. As such, forgetting is increasingly considered an adaptive trait rather than a flaw associated with failed memory processes (Kraemer & Golding 1997). For example, foraging nine-spined sticklebacks (*Pungitius pungitius*) use recently acquired private information about food patch profitability when choosing where to feed, but their tendency to use this information decreases over time and instead they begin to rely more on what other fish are doing, so called public information (van Bergen *et al.* 2004). This may demonstrate flexible memory use depending on the perceived reliability of available information, and shows how forgetting can be adaptive in certain circumstances. However, an alternative explanation for this observation is that after 7 days a fish may have forgotten its own experience, and so must rely on publicly acquired information.

2.2.4. Aim

In terms of explaining population differences in behaviour, typically only one ecological variable is considered at a time. However, habitats are likely to differ in many aspects, and variables may interact when shaping behaviour. Hence, studying them in isolation may be misleading. To date, few studies have investigated the influence of multiple ecological variables on learning and memory, and how these variables might interact. Thus here, I investigate how learning and memory varies across a range of pond and river three-spined stickleback populations originating from habitats that are proposed to differ in their levels of predation pressure.

I use a simple spatial task to investigate individual learning and memory ability in annual populations of pond and river fish sampled from sites proposed to differ their spatial stability and level of predation pressure. Both of these variables have previously been thought to affect learning behaviour (habitat stability: three-spined sticklebacks (Braithwaite & Girvan 1998, Odling-Smee & Braithwaite 2003), predation pressure: Panamanian bishops (Brown & Braithwaite 2004)). Working on the hypothesis that rivers are less spatially stable habitats than ponds, I hypothesised that fish from rivers would update their foraging information sooner, and hence be less likely to return to a previously rewarded patch than fish from ponds. I also hypothesised that fish from low predation sites would learn the task faster because they may not have to expend so much attention on predator vigilance, potentially allowing them to learn faster. This pattern was found in populations of Panamanian bishops, where fish originating from low predation populations learned a spatial foraging task significantly faster than those from high predation sites (Brown & Braithwaite 2004). It has previously been revealed that simultaneously focussing attention on two tasks can impair an animals' ability to perform either task in isolation. For example, it takes silver perch 5 trials to reach maximum intake rates when offered a single prey type, but they take 12-20 trials to converge on the most profitable prey type when offered two simultaneously. Similarly, blue jays (*Cyanocitta cristata*) showed a decreased response to peripheral targets (representing predators) when their attention was focussed on a foraging task (Dukas & Kamil 2000).

2.3. Materials and methods

2.3.1. Subjects and housing

Three-spined sticklebacks were collected from 4 ponds and 4 rivers in Central and Southern Scotland, U.K: Ponds - Beecraig Pond ($3^0$47'W,$55^0$57N), Craiglockhart Pond ($3^0$14'W, $55^0$55N), North Belton Pond ($2^0$35'W, $55^0$59N) and Balmaha Pond ($4^0$31.5'W, $56^0$05N), Rivers - Water of Leith ($3^0$14'W, $55^0$57N), River Biel ($2^0$35'W, $55^0$59N), River Endrick ($4^0$24'W, $56^0$02N) and River Esk ($3^0$10'W,$55^0$51N). These sites were presumed to represent independent samples that originated 11,000-10,000 years ago when, following the retreat of the Loch Lomond stadial, glaciers began to retreat (Sissons 1979). The four rivers used in the study are not directly linked to one another, and it would not be possible for fish to migrate between these rivers. There is at least one weir between each river sampled and the sea, preventing mixing of marine or estuarine fish with the sampled populations. All of the ponds are unconnected to other waterways, and coupled with the fact that three-spined stickleback populations are believed to have the ability to differentiate in morphology and behaviour in very few generations (for example, nearly 100% of a marine three-spined stickleback population that invaded an Alaskan lake had the full compliment of lateral defensive plates in 1990, but 12 years later, in 2001, only 11% of this same population had the full compliment, and low plated morphs (usually the monomorph observed in local freshwater populations) were dominant (Bell et al. 2004). See Kristjansson et al. 2002 for a similar example), I considered these populations to represent independent samples that had been subjected to specific selection regimes that may be expected to cause adaptive responses. It would have been ideal to collect genetic data, for example microsatellite or

mitochondrial DNA to determine the true phylogeny of these populations, however this was not possible. A one year survey of these sites indicated that they did not appear to differ in many factors which may be expected to influence the potential value of visual stimuli, for example turbidity and vegetation structure (see Appendix 1). Fish were collected in November 2004 with minnow traps and large nets. I found similar densities of fish in traps in all habitats, indicating similar school sizes. A total of 66 fish were tested (10 from River Biel and 8 from all other sites). Populations were housed separately in holding tanks (76cm long x 30cm wide x 38cm high) furnished with plastic plants, a gravel substrate, biofilters and refuges and fed on a diet of frozen blood-worm. Laboratory temperature was maintained on a day:night cycle at 14:9.5^{0}C, and light:dark cycle of 10:14 h for the duration of the experiment. Fish were collected outside of their breeding season, and as males and females are morphologically identical at this time, populations were assumed to be mixed sex, and outside of the breeding season male and female sticklebacks do not differ in their behaviour (Bell & Foster 1994). All populations were of a similar mean body length (ANOVA: $F_{7,57}$=1.4, P=0.2, mean=3.7cm ± 0.6se).

2.3.2. Quantifying predation pressure

Predation pressure was measured using 2 methods. The first involved taking morphometric measurements of defensive amour from three-spined sticklebacks from each of the eight populations. Previous studies have revealed a strong positive correlation between degree of defensive armour (number of lateral plates, pelvic and dorsal spine length) and predation pressure, which is thought to reflect evolutionary

responses to predation pressure from all predators (e.g. piscivorous, aerial and mammalian) over time (e.g. Reimchen 1994, Bell 2001, Vamosi & Schluter 2004, Bell et al. 2004). The sharp pointed spines, when locked erect, increase the diameter of the fish, making it harder for gape-limited predators such as trout to handle and consume them (Reimchen 1991), and the bony plates protect the epidermis from damage by toothed predators (Reimchen 1992). The second method involved collecting field data on piscivorous predators as a measure of current predation pressure. Although it would have been ideal to make many observations over several months to gain information not only over time but also on aerial and mammalian species such as herons, kingfishers and loons, which are also know to predate three-spined sticklebacks, this was not possible. All of the sites sampled were located either in national park land or on public walkways. As such, I would consider present predation threat from piscivorous predators to be greater than that from aerial or mammalian predators, which are likely to be disturbed by visitors to these sites.

Field Observations

Field observations of predation pressure were made in the summer of 2006. A 50m stretch of each river or the entirety of each pond was electrofished. All captured fish were allowed to recover fully in buckets before being replaced, and no adverse effects were observed on the resident wildlife. The number, relative size and species of piscivorous fish species were recorded.

Morphometric Measurements

I used 52 preserved (old) and 79 fresh (new) caught specimens (euthanased in MS222) to compare the morphology of three-spined sticklebacks from the eight populations. The data from preserved (old) and fresh (new) specimens was compared to ensure that the preservation process had not affected morphology (there was no effect, see Results).

Measurements and analysis of defensive armour traits were based on Vamosi & Schluter (2004), and were as follows: 8 external traits were measured on the left side of each fish: body length, body depth, gape width, first and second dorsal spine length, pelvic spine length, pelvic girdle length and lateral plate number. The first three traits were used to correct for body size. In order to count plate number, dead fish were stained with alazarin dye using the following protocol: fish were transferred from 70% ethanol into 50% ethanol 50% (3.5%) NaCl for 24 hours. They were then moved into 25% ethanol 75% (3.5%) NaCl for a further 24 hours, then into 100% (3.5%) NaCl for 24 hours. Finally fish were placed into alazarin solution (0.04g/l) for 24 hours. They were then transferred into 100% (3.5%) NaCl solution to rinse off excess dye for 24 hours. They were then placed directly into 70% ethanol, and stored until needed.

2.3.3. Learning and memory assay

During the experiment, fish were individually housed in tanks (35cm long x 20cm wide x 24.5cm high) with a water depth of 15cm, 1cm of gravel substrate and an individual bio-filter. Housing fish individually in this way eliminates the need for handling and transport between trials, which the fish find stressful (see Chapter 6). Tanks were placed next to one another in a row, so although they were physically separated, fish had visual

contact with neighbours to reduce isolation stress in this naturally shoaling species. Tanks were divided into a home chamber and two 'foraging patches' using plastic dividers (see Fig. 2.1.). The patches were accessible at all times (except when a patch

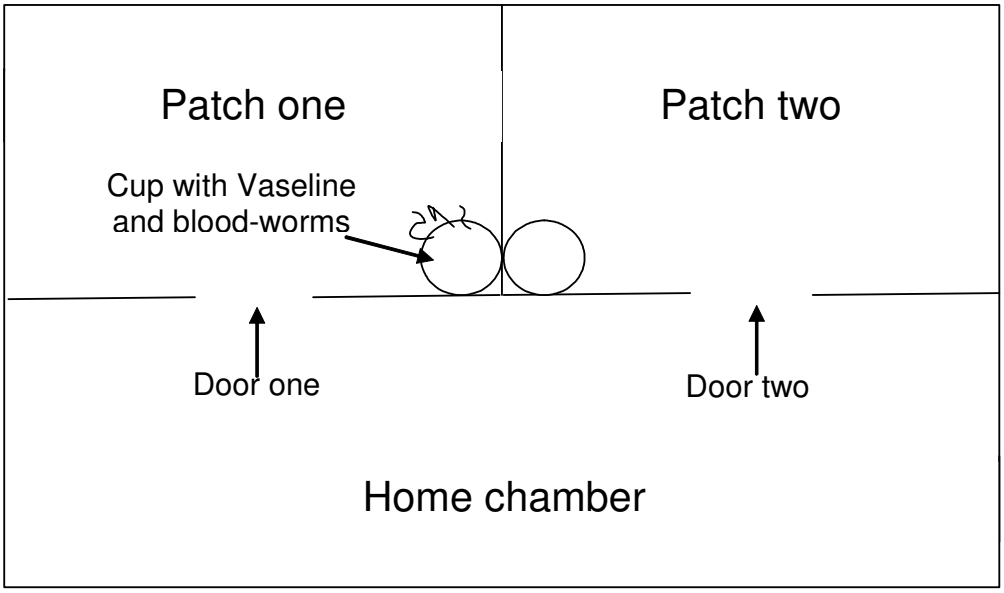

Figure 2.1. Schematic view of a tank used to house fish individually during the experiment.

was being baited) via doors cut into the dividing wall (measuring 4.5cm high x 2.5cm wide). Each door was surrounded by coloured white or yellow PVC to provide a conspicuous visual cue for each patch. Half of each population had the yellow door on the left, white on the right, and vice versa for the other half. This controlled for the possibility that associations may be more readily formed with certain colours. A small, weighted plastic cup (3cm diameter) filled with Vaseline was placed in each foraging patch. During a trial, opaque between-tank plastic partitions were placed down both sides of the tank so that a fish could not watch and learn the task from its neighbour. At

the start of a trial, an opaque plastic barrier was also placed in front of the doors and the plastic cups were removed from both patches to ensure fish were not following which compartment the feeder was placed into but were using spatial memory to locate the food rewards. Three blood-worms were placed into one of these plastic cups. Although recent studies indicate the importance of olfaction to three-spined sticklebacks, particularly in a social context (e.g. Ward et al. 2005, 2007), previous work has shown that three-spined sticklebacks cannot locate these worms by smell (Girvan & Braithwaite 1998), and the development of the olfactory epithelium compared to the development of the retina suggests that they are predominantly visual predators (Honkanen & Ekstrom 1992). Furthermore, filters in the tanks and regular cleaning prevented the build up of any potential olfactory cues in rewarded compartments. If fish were locating these worms by smell, I would expect performance to be above random chance at the beginning of the trials as fish directly located the worms, and this was not the case. Cups were then placed back into the compartments, always the left cup followed by the right, and a curtain was placed in front of the tank to ensure minimum disturbance to the fish during a trial. Fish were given two minutes to settle, then the barrier was gently removed remotely via a piece of string looped over a plastic rod suspended above the tank. Fish were observed over the top of the tank, with the observer standing 1m away from the tank, and remaining motionless. Pilot trials showed that fish did not alter their behaviour in response to the presence of an observer as long as the observer remained still during the observations. Door entered first (right or left), and the latency to move into the food patch and begin feeding was recorded. If it was an incorrect choice the fish was observed until it either entered the correct side, or until 15 minutes had elapsed. The

experiment was divided into three phases:

Phase One –Acquisition

Fish were given two trials a day, with the food in the same patch each time, until they selected the correct patch first in 9/10 trials, indicating they had learned the task, or until 45 trials had elapsed, at which point it was assumed the fish was incapable of learning the task.

Phase Two – Acquisition

When criterion performance was reached in phase one, fish were fed in the opposite patch until they reached the same criterion level of 9/10 correct choices.

Phase Three – Return to previously rewarded patch

During this phase, the plastic dividers that created the foraging patches were removed from the tank. Half of each population were left for an interval of 7 days, the other half 21 days. Fish were fed six blood-worms a day via a pipette at the front centre of their tanks for the duration of this phase. After the appropriate interval the apparatus was reinserted into each tank, and a trial was performed to determine if the fish returned to the last rewarded side (phase two rewarded side).

As a maximum of 18 fish could be tested at any one time, experiments were conducted in four blocks, using two fish from each population per replicate, except in the second replicate where four fish were used from River Biel. All fish were humanely euthanased using over-anesthesia with MS222 at the end of the experiment. To minimize

spreading infections between fish, I do not release them back into the wild after they have been maintained in the laboratory.

2.3.4. Data analysis

All data were checked for normality and homogeneity of variance, and were transformed to normality when assumptions were not met.

Predation Pressure

A principal components analysis (PCA) was run on body size traits to obtain a single 'body size' variable (PC1). All traits contributed equally, and significantly, to PC1: body length (component coefficient = 0.62), body depth (0.59) and gape width (0.51). The first principal component accounted for 78% of the variance among individuals. To correct for body size variation among individuals, each armour trait was then regressed against PC1 for all individuals from all populations. The remaining variation (residuals) was saved for each trait. Number of plates was uncorrelated with size, and so was not adjusted.

A PCA was then performed on the regressed values for first and second dorsal spine length, pelvic spine length and pelvic girdle length, to give an overall 'armour' variable. This resulted in a clustering of fish with long spines and pelvic girdles at one end, and fish with short spines and pelvic girdles at the other. PC1 accounted for 64% of the variation in the data. Length of the first dorsal spine had the highest loading coefficient (0.58), followed by the pelvic spine (0.57), the second dorsal spine (0.56), and finally the pelvic girdle (0.16). PC1 (overall armour variable) was analysed using an

analysis of variance (ANOVA), with old versus new samples and population as factors. Non-significant terms were removed to leave the minimal model.

As plate number data were not normally distributed, and could not be transformed to normality, a Kruskal-Wallis test was used to analyse the effect of population on plate number.

Populations were also categorized as high or low predation based on the field data. All three categories (spine measurements, plate number and field data) were considered when devising the final predation category for each population.

Learning and Memory Assay

One fish from North Belton was excluded from the analyses as it did not reach the criterion level of performance even after 45 trials. The number of trials taken to reach criteria in phases one and two (Box-Cox transformed, raw data can be found in table 2, appendix 2 (A.2.1.)). were analysed using general linear models. Maximal models, including habitat type (river or pond), predation pressure, population (a random factor nested within predation pressure and habitat type), habitat type*predation pressure interaction, length, replicate and tank number as factors were initially used. Non-significant terms were removed to create minimal models. Chi-square tests were used to determine if pond and river fish and high and low predation fish could remember the task after 7 and 21 days.

2.4. Results

2.4.1. Quantifying predation pressure

There was no effect of old versus new samples ($F_{1,123}$=0.0006, P=0.98) on PC1 (overall armour variable), so this term was removed from the model. There was a significant main effect of population on PC1 (overall armour variable) ($F_{7,124}$=6.1, P<0.0001). A post-hoc Tukey test revealed that River Esk, Water of Leith, Craiglockhart Pond and River Biel had significantly more armour than North Belton Pond, River Endrick and Balmaha Pond. Consequently, River Esk, Water of Leith, Craiglockhart Pond and River Biel were classified as high predation, and North Belton Pond, River Endrick and Balmaha Pond as low predation. Beecraigs Pond fell in the middle, but had a negative score that was closer to the low predation sites, so was classified as low predation (see Table 2.1). Raw data values can be found in table 1, appendix 2 (A.2.1.).

Table 2.1. Categorization of field sites as either high (H) or low (L) predation in all three predation categories, and the overall category.

Site	Morphometric data (PCA)	Plate number	Field data	Overall
Beecraig Pond	L	L	H	L
Craiglockhart Pond	H	L	H	H
North Belton Pond	L	H	L	L
Balmaha Pond	L	L	L	L
Water of Leith	H	L	H	H
River Biel	H	H	H	H
River Endrick	L	L	L	L
River Esk	H	L	L	L

There was a significant effect of population on plate number (Kruskal-Wallis: H_7=24.4, P=0.001). A post-hoc comparison of means (Games-Howell (Zar 1996)) revealed that North Belton fish were significantly more plated than all other populations except for River Biel and these fish were significantly more plated than Craiglockhart Pond and Balmaha Pond fish. There were no differences between any of the other populations. As such, North Belton and River Biel fish were classified as high predation, all other sites as low predation. When North Belton and River Biel were removed from the analysis, there appeared to be a significant effect of population on plate number (Kruskal-Wallis: H_7=16.4, P=0.006), but controlling for multiple comparisons (Games-Howell post-hoc test) revealed that there were no significant differences in plate number between the remaining populations.

Based on field observations, River Esk, River Endrick, North Belton Pond and Balmaha Pond were classified as low predation, as no or few small piscivores were caught at these sites. Water of Leith, Craiglockhart Pond, Beecraig Pond and River Biel were classified as high predation as many large piscivores were caught at these sites (Table 2.2).

Taking the majority of all three predation categories therefore classified River Esk, Beecraigs Pond, North Belton Pond, River Endrick and Balmaha Pond as low predation sites, Water of Leith, Craiglockhart Pond and River Biel as high predation sites (Table 2.1).

Table 2.2. Type of predators caught at each site. Numbers of each species caught are shown in brackets. The entirety of each pond and the entire width of a 50m stretch of each river was electrofished to sample the piscivorous predators present. The area of river sampled was that which the three-spined sticklebacks had previously been sampled from.

Site	Predator species	High/Low predation
Beecraig Pond	Brown trout (5), perch (20) (*Perca fluviatilis*)	High
Craiglockhart Pond	Perch (25)	High
North Belton Pond	None	Low
Balmaha Pond	None	Low
Water of Leith	Large brown trout (5), bullhead spp., (*Petromyzon fluviatilis*) (10), rainbow trout (6), salmon (3), sea trout (4)	High
River Biel	Large brown trout (20)	High
River Endrick	Small brown trout (5)	Low
River Esk	Small brown trout (3)	Low

2.4.2. Learning and memory assay

Phase One – Acquisition

Length, replicate and tank number had no effect on number of trials to learn phase one, and so were removed to leave the minimal model. There was no effect of habitat type ($F_{1,57}$=9.47, P=0.45), but there was an almost significant effect of predation pressure ($F_{1,57}$=3.76, P=0.06) and a significant interaction between habitat type and predation pressure on the number of trials to learn phase one ($F_{1,57}$=7.61, P=0.01). A post-hoc Tukey test revealed that this interaction occurred because low predation river fish learned significantly faster than high predation river fish (Fig. 2.2.). There was also an effect of population (nested within habitat type and predation pressure) ($F_{4,57}$=3.43,

P=0.01). A post-hoc Tukey HSD test revealed a similar trend to the habitat type*predation pressure interaction, with low predation river fish learning significantly faster than high predation river fish.

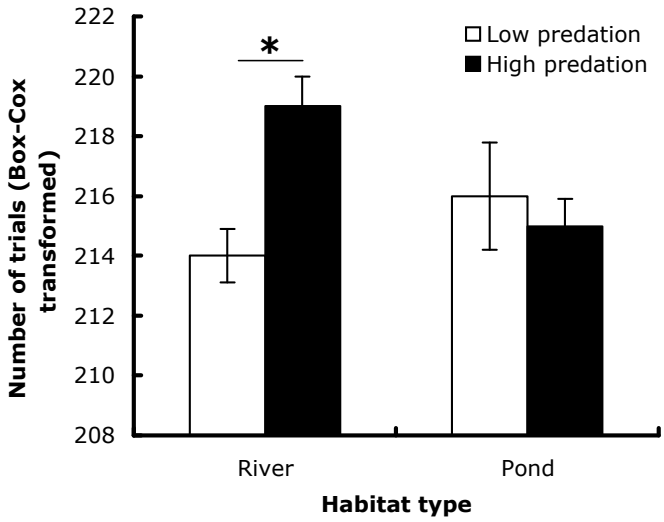

Figure 2.2. Mean number of trials to reach criterion performance (correct patch selection in 9/10 trials) in pond and river fish from habitats of differing predation pressure in phase one. Bars connected by an asterisk are significantly different from one another (P<0.05). Error bars represent one S.E.

Phase Two – Acquisition

There was no effect of habitat type ($F_{1,57}$=0.45, *P*=0.50), predation pressure ($F_{1,57}$=0.28, *P*=0.60) or population (nested within habitat type and predation pressure) ($F_{1,57}$=0.52, *P*=0.72) on the number of trials taken to learn phase two. However, the interaction between habitat type and predation pressure showed a trend in the same direction as learning in phase one, but this was not significant ($F_{1,57}$=3.03, *P*=0.09) (Fig. 2.3.).

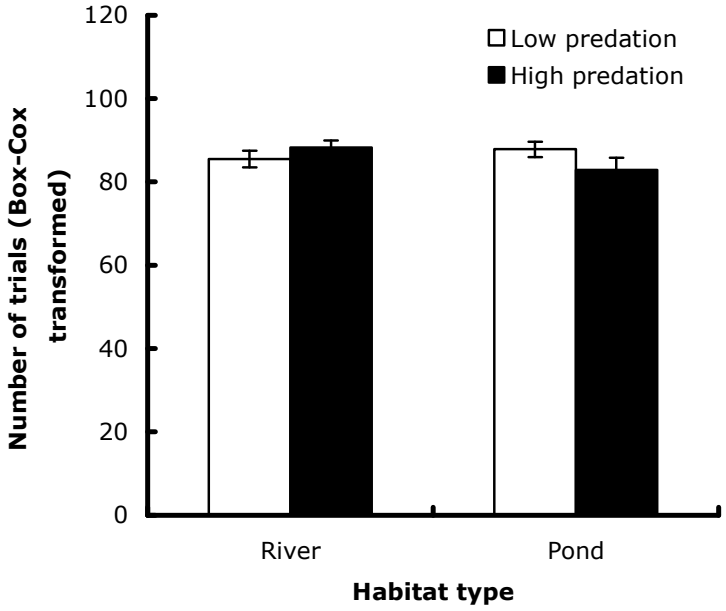

Figure 2.3. Mean number of trials to reach criterion performance (correct patch selection in 9/10 trials) in pond and river fish from habitats of differing predation pressure in phase two. Error bars represent one S.E.

Phase Three – Return to previously rewarded patch

The ability of pond versus river, and high versus low predation fish to return to the food patch that had most recently been rewarded in their last training phase was compared after 7 and 21 days. After 7 days, river (d.f.=1, Chi-square=13.2, $P<0.01$) but not pond (d.f.=1, Chi-square=2.25, $P>0.05$) fish performed significantly above chance levels, indicating river fish remembered the task (Fig. 2.4.a). Additionally, although not significant, there was a greater tendency for river fish to return to the previously

rewarded patch than pond fish after 7 days (Contingency table analysis: d.f.=1, Chi-square=3.57, *P*=0.059). After 21 days, neither river (d.f.=1, Chi-square=0.53, *P*>0.05) or pond (d.f.=1, Chi-square=0.5, *P*>0.05) fish performed above chance levels (Fig. 2.4.b.).

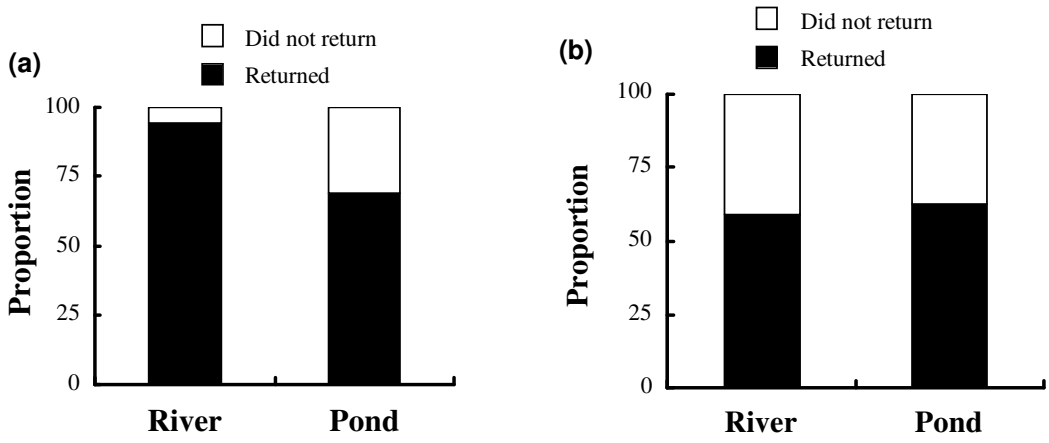

Figure 2.4.a. Proportion of pond and river fish returning to the last rewarded patch after 7 days.
Figure 2.4.b. Proportion of pond and river fish returning to the last rewarded patch after 21 days.

This indicates that river fish have a memory for this task that lasts at least 7, but not longer than 21 days, whereas pond fish have a memory of less than 7, but at least 1 day as they remembered the task from day to day during the acquisition phase. After 7 days, high (d.f.=1, Chi-square=5.4, *P*<0.05) and low predation (d.f.=1, Chi-square=4.3, *P*<0.05) fish performed significantly above chance levels, indicating they remembered the task (Fig. 2.5.a). After 21 days, neither high (d.f.=1. Chi-square=0.04, *P*>0.05) or low predation (d.f.=1, Chi-square=3.2, *P*>0.05) fish performed above chance levels (Fig.

2.5.b). This indicates that both high and low predation fish could remember the task after 7 days, but neither could remember after 21 days, demonstrating that predation pressure is not impacting on memory retention of this foraging task.

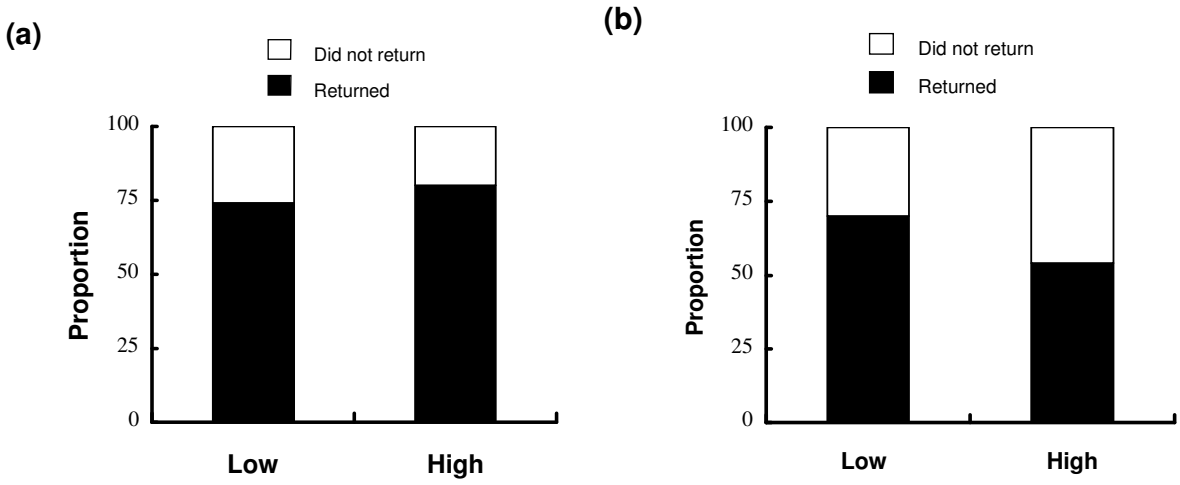

Figure 2.5.a. Proportion of high and low predation fish returning to the last rewarded patch after 7 days.
Figure 2.5.b. Proportion of high and low predation fish returning to the last rewarded patch after 21 days.

2.5. Discussion

2.5.1. Memory

River three-spined sticklebacks were less likely to update their foraging information than fish sampled from ponds. River fish returned to a previously rewarded foraging patch after 7 days, but did not show a preference to return to it after 21 days. This result suggests that fish originating from different habitats differ in the way they update their long-term memory. Surprisingly, pond fish showed no tendency to return to the foraging patch after only 7 days of the memory retention test. Contrary to my original habitat

stability hypothesis, which predicted fish originating from pond environments (hypothesised to be more spatially stable) would be less likely to update their memory and hence have a longer memory duration than those from rivers (hypothesised to be less spatially stable), I found the reverse to be true.

This differs to the results obtained by Mackney & Hughes (1995), who found that sticklebacks originating from habitat hypothesised to be more temporally stable with respect to prey availability had longer memory duration for prey handling skills compared to those from more changeable environments. In a more temporally stable habitat, longer memory duration for particular prey handling would be advantageous. Fish from the marine environment, which is hypothesised to have greater spatial and environmentally variability, are likely to encounter a greater diversity of prey over time, favouring shorter memory duration and an ability to learn how to exploit the prey type that is most locally available. If habitat stability does differ between pond and river habitats in the hypothesised direction, then my results would indicate that spatial memory duration is affected in a different way to memory for prey handling.

Memory is thought to be divided into discrete systems or cognitive modules, each with separate underlying neurology and physiology (e.g. Klein *et al.* 2002, Squire 2004). It has been suggested that different memory systems may be adapted in different ways to the environment, and have different rules of operation (Sherry & Schacter 1987, Shettleworth 1998). Hence, the factors that shape memory for prey handling skills may not be the same as those that shape memory for spatial locations. Compared to Mackney & Hughes (1995), my data would seem to support this hypothesis.

In terms of spatial memory, some factor other than spatial stability may be driving differences between river and pond populations. In a river habitat, fish have a greater chance of being relocated to new areas due to either the flow of the river or exploration. In this situation, having a good and extensive spatial memory may be beneficial, as it will allow fish to relocate shelter or feeding sites rapidly if they return to areas visited in the recent past. However, for pond fish living in a more enclosed environment, the same spatial memory capacity may not be as important; if food is plentiful then it may not be necessary to remember the positions of specific food patches. Habitat stability may still be an important factor in determining memory duration in three-spined sticklebacks, but comparing between ponds and rivers may not provide a sufficient test of this hypothesis. Ponds and rivers differ from one another in overall structure, ponds are enclosed, rivers open. This may affect memory duration in ways that obscures the true potential effects of habitat stability alone. Comparing between rivers that, for example, differ markedly in flow rate (and so would be hypothesised to differ in habitat stability), or habitats where stability had been quantified would provide a more robust test of the hypothesis that long-term memory is advantageous in comparatively more stable habitats, short-term memory in more variable habitats.

2.5.2. Learning phases

In contrast to memory retention, there were no clear pond/river habitat differences in the ability to learn phases one and two of a spatial foraging task. This is in agreement with earlier observations of spatial learning in pond and river three-spined sticklebacks

(Odling-Smee & Braithwaite 2003). However, adding predation pressure into the model reveals that learning was affected by an interaction between pond/river habitat and predation pressure. Low predation river fish learned phase one significantly faster than high predation river fish, but this was not seen within pond populations. There is a similar non-significant trend apparent for learning in phase two. This result mirrors what has previously been found in tropical rivers where predation pressure varies between different populations of Panamanian bishops. Here, populations from low predation sites learned a spatial foraging task almost twice as quickly as those from high predation sites (Brown & Braithwaite 2004).

A possible explanation for these observed differences in learning rate is divided attention. Animals continually receive information about their environment, and must filter this information in order to focus on those aspects most important to survival (Dukas 2002). The ability of an animal to successfully perform a given task can be affected by the amount of attention being focused simultaneously on other activities (see Dukas 2002 for a review on limited attention). For example, three-spined sticklebacks (Milinski 1984) and guppies (Krause & Godin 1995), engaged in more complex foraging tasks are more vulnerable to predation, and are preferred targets for predators (Krause & Godin 1995), presumably because their attention is divided between foraging and predator vigilance. Similarly, denser swarms of *Daphnia* decrease foraging efficiency of three-spined sticklebacks due to the confusion effect, whereby predators find in harder to target any one individual the denser a swarm of prey becomes (Ohguchi 1981). Furthermore, a recent study found that fish selectively bred to have a lateralized brain (i.e. they used different halves of the brain to process particular tasks) had a foraging

advantage over non-lateralized individuals when a predator was present, and this was attributed to lateralized fish being better able to process multiple sources of information, processing each task with one brain hemisphere (Dadda & Bissaza 2006).

In my system, it may be expected that high predation river fish have several activities to divide their attention amongst: they must be vigilant for predators and pay attention to their spatial location to avoid becoming moved to unfavourable areas by water currents or exploration. This would leave less attention for locating profitable feeding sites, and may explain why high predation river fish take longer to learn the spatial foraging task presented here. It could also partly explain why the trend is non-significant by phase two: having been in the maze for several days they may have learned it is a safe, predator-free environment. Fish are also more familiar with the task by phase two, which may increase their learning rate. Low predation river fish may not have to expend the same amount of attention on predator detection, enabling them to devote more attention to other tasks, such as locating feeding sites, possibly translating to faster learning rate in the present experiment. In contrast to this in pond environments, fish may not have so many tasks to divide their attention between. They will not be relocated to unfavourable areas by current or exploration, and it is expected that they have stable local landmark cues to aid navigation. Thus, high predation pond fish may not learn more slowly than low predation pond fish because they do not have so many variables to pay attention to, allowing them to learn this relatively simple spatial task at equal rates.

In conclusion, I have found the learning and memory ability of populations of three-spined sticklebacks differs. It appears that differences between ponds and rivers

create differences in long-term memory between pond and river populations, whereas an interaction between pond/river habitats and predation pressure influences learning rate. This suggests that although they are linked, learning and memory have differences, and may not necessarily be shaped in the same way by the same ecological factors. It also highlights the complex nature of natural habitats, and shows how multiple ecological factors can interact to affect behaviour.

Chapter 3. Habitat stability and predation pressure affect temperament behaviours in populations of three-spined sticklebacks

3.1. Summary

There is growing interest in the causes and consequences of animal temperaments. Temperament behaviours often have heritable components, but ecological variables, such as predation pressure, can also affect them. Numerous variables are likely to differ between habitats, and these may interact to influence temperament behaviours. Furthermore, temperament behaviours may be correlated within populations (behavioural syndromes), although the underlying causes of such correlations are currently unclear. I analysed three different temperament behaviours and learning ability in three-spined sticklebacks to determine how different ecological variables influence behaviour both within and between populations. I selected populations from four ponds and four rivers proposed to differ in their exposure to predators. High predation river populations were significantly less bold than a high predation pond and low predation river populations, and low predation pond populations were significantly less bold than a high predation pond population. Within populations, temperament behaviours were correlated in one high predation river population only. These results suggest that multiple ecological factors can interact to affect temperament behaviours between populations, and also correlations in those behaviours within populations.

3.2. Introduction

3.2.1. Temperament behaviours

Intraspecific differences in temperament behaviours were, until recently, considered to be non-adaptive variation surrounding an adaptive optimum. This view was generally accepted because of concerns over anthropomorphizing with respect to animal behaviour. Recently, however, we have seen a move away from this notion towards the view that such variation may be adaptive (e.g. Wilson 1998, Dall *et al.* 2004, Wolf *et al.* 2007). In particular, it has been proposed that animals exhibit specific temperament behaviours (sometimes referred to as 'personality traits' or 'personality behaviours') that are similar to the personality behaviours used to describe human behaviour. Psychologists working on human personality types have described five axes of personality (referred to as the human five-factor model - see Gosling & John 1999). Borrowing from these ideas research has begun to address whether animals express similar types of temperament (see Gosling 2001 for a review). This work has revealed that temperament behaviours generally have a heritable component (e.g. Bouchard & Loehlin 2001, Dingemanse *et al.* 2002), although this is relatively low in some populations: aggressiveness, boldness and activity were only weakly heritable in two populations of three-spined sticklebacks (Bell 2005). There are many potential ways to define and measure certain temperament behaviours. For example, boldness can be defined as foraging under the threat of predation, or boldness towards conspecifics. Boldness has also been measured using various methods. For example, previous studies have measured boldness as method used to capture food (Sneddon *et al.* 2003), predator inspection behaviour (Dugatkin & Alfieri 2003), time to emerge from a refuge (Brown

et al. 2005) and time to resume foraging after a predator attack (Coleman & Wilson 1998). In contrast, temperament behaviours such as activity and neophobia are fairly consistently defined as the amount of distance an animal covers in a given time (activity, e.g. Marchetti & Drent 2000, Bell 2005) and time to approach or time spent near a novel object (neophobia, e.g. Mettke-Hoffman *et al.* 2002). The particular experimental protocol used should be considered when interpreting the results of temperament behaviour studies.

3.2.2. The role of ecology

The environment experienced during development can play a role in shaping temperament behaviours. This is seen in captive reared species of fish (Huntingford & Adams 2005). For example, enhancing the spatial complexity of the rearing environment alters behaviour towards prey, exploratory and stress recovery behaviours in hatchery reared cod, *Gadus morhua* (Braithwaite & Salvanes 2005). Similarly, hatchery reared brown trout are bolder than their wild counterparts (Sundstrom *et al.* 2004). Less attention has been directed at the role that natural environmental variables play in shaping temperament behaviours. Comparing populations of the same species living in different natural ecological habitats may provide valuable insights into the environmental factors that affect temperament behaviours in animals. A recent study using this approach investigated boldness in natural populations of Panamanian bishops, and found that fish originating from high predation river sites were bolder than those from low predation river sites (Brown *et al.* 2005).

3.2.3. Temperament behaviours within populations – two hypotheses

Alongside differences between populations, different temperament behaviours (e.g. aggression and boldness), or the same temperament behaviour in different functional contexts (e.g. boldness towards a predator and boldness towards a competitor), can be correlated within populations, and this is known as a behavioural syndrome (e.g. Gosling 2001, see Sih *et al.* 2004a, 2004b, Bell 2007 for reviews on behavioural syndromes). For instance, positive correlations between anti-predator behaviours and activity levels have recently been reported in the chaffinch, *Fringilla coelebs* (Quinn & Cresswell 2005). There are two hypotheses for the existence of behavioural syndromes. The 'Constraints' hypothesis states that when correlations exist between behaviours it is because of underlying constraints that are difficult to break apart and so necessarily couple those behaviours together. For example, behaviours may be proximally linked or due to the pleiotropic effects of genes, so that selection on one behaviour necessarily causes correlated changes in other behaviours (Bell 2005). This hypothesis has been used to explain why some behaviours may appear maladaptive when considered in one functional context only. For example, populations of a desert spider (*Agelenopsis aperta*) living in food limited environments are more likely to attack prey and also kill more prey than they can consume, and this apparently energetically wasteful behaviour has been explained as a consequence of selection for general aggressiveness towards prey in food limited environments (Maupin & Riechert 2001). The second hypothesis, the 'Adaptive' hypothesis, proposes that when correlations between behaviours exist it is because they are adaptive (Wilson 1998, Bell 2005). In the spider example given above, this hypothesis would suggest that spiders living in food-limited environments show a

greater tendency to attack prey and participate in superfluous killing because both behaviours are beneficial in this environment. However, at present, it is difficult to imagine how superfluous killing could be adaptive in this system (Maupin & Riechert 2001). A way to disentangle these two hypotheses is to investigate the presence/absence of behavioural syndromes within populations of the same species. If the 'Constraints' hypothesis is true, when certain behaviours are correlated within one population, then due to underlying constraints they must necessarily be correlated within all others. A recent study on two populations of three-spined sticklebacks revealed that this was not the case for this species, as there were positive genetic and phenotypic correlations of activity, aggression and boldness in one high predation population only (Bell 2005). Reasons why these behaviours were correlated within a high but not a low predation population are unclear.

3.2.4. Aim

Studies of behaviour typically only consider the effects of one ecological variable at a time. This may be misleading, as numerous ecological variables are likely to differ between habitats, and these may interact to influence temperament behaviours. To date, no study has investigated the effects of multiple ecological variables on temperament behaviours, or how these variables may interact. Hence, I designed an experiment to investigate how two natural variables affect temperament and learning behaviours. Using three-spined sticklebacks from ponds and rivers that were proposed to differ in predation pressure, I quantified three temperament behaviours: boldness, neophobia and activity in an unfamiliar environment in the presence of a novel object. I also

investigated learning rate in a simple foraging task. I chose to measure temperament behaviours which I considered may affect foraging performance, in order to determine if there were any correlations between temperament behaviours and learning of a foraging task. I measured boldness as time to emerge from a refuge and time to begin a foraging trial, activity as general activity in a novel environment, and neophobia as time taken to approach and time spent near a novel object. All of these traits may be expected to affect an animal's foraging performance. Learning rate could feasibly be affected either way by temperament behaviours: 1) Bolder, less neophobic, more active fish may learn a spatial foraging task faster because they explore their environment and have a higher chance of encountering food items. This appears to be the case with guppies and Rainbow trout, where bolder individuals learn foraging tasks faster (Dugatkin & Alfieri 2003, Sneddon 2003). 2) Less bold, less active and more neophobic individuals may learn faster if they are more careful, and pay greater attention to their environment, as is the case with great tits, *Parus major* (e.g. Marchetti & Drent 2000, see Korte *et al.* 2005 for a review) and populations of Panamanian bishops (Brown *et al.* 2005, Brown & Braithwaite 2004). Between populations, I predicted that high predation site fish would be less bold, more neophobic and have lower activity levels in order to decrease the chances of being detected by a predator. I had no specific hypothesis for how pond and river fish might differ in their temperament behaviours, however, because previous studies found that pond and river three-spined sticklebacks differed in their learning behaviour (Girvan & Braithwaite 1998, Braithwaite & Girvan 2003, chapter 2), I considered it might also have an effect on temperament behaviours.

3.3. Materials and methods

3.3.1. Subjects and housing

Minnow traps and large nets were used to collect three-spined sticklebacks in November 2004 from 4 ponds and 4 rivers in Central and Southern Scotland, U.K: Ponds - Beecraig Pond ($3^0$47'W,$55^0$57N), Craiglockhart Pond ($3^0$14'W, $55^0$55N), North Belton Pond ($2^0$35'W, $55^0$59N) and Balmaha Pond ($4^0$31.5'W, $56^0$05N), Rivers - Water of Leith ($3^0$14'W, $55^0$57N), River Biel ($2^0$35'W, $55^0$59N), River Endrick ($4^0$24'W, $56^0$02N) and River Esk ($3^0$10'W,$55^0$51N). A one year survey of these sites indicated that they did not differ in many factors aside from predation pressure and habitat stability (see Appendix 1). A total of 66 fish were tested (10 from River Biel and 8 from all other sites). Populations were housed separately in holding tanks (76cm long x 30cm wide x 38cm high) furnished with plastic plants, a gravel substrate, biofilters and refuges. Fish were fed on a diet of blood-worm. Laboratory temperature was maintained on a day:night cycle at 14:9.5 ^{0}C, and light:dark cycle of 10:14 h for the duration of the experiment. Fish were collected outside their breeding season, and as males and females are morphologically identical at this time and school together, populations were assumed to be mixed sex. All populations were of a similar mean body length (ANOVA: $F_{7,57}$=1.4, P=0.2, mean=3.7cm ± 4.6 s.d.).

3.3.2. Quantifying predation pressure

Using a combination of direct field measurements as well as morphometrics quantifying the body armour of the fish I classified fish as coming from either high or low predation

sites (Table 2.1.). Details of the analyses and methods used to assign populations to high or low predation categories are given in Chapter 2 (see sections 2.3.2. & 2.4.1.).

3.3.3. Quantifying temperament behaviours

Boldness assay one

Boldness was quantified using two methods. The first was derived from the learning and memory assay presented in Chapter 2 (section 2.3.3.), and involved determining the average time taken for a fish to begin a foraging trial. I chose this as my first measure of boldness because I expected that all fish would be highly motivated to forage as they were maintained on a rationed diet of 3 blood-worms a day during the experiment, so the only factor preventing them from foraging should be their willingness to swim across the home chamber and enter a foraging compartment. Briefly, fish were individually housed in tanks (35cm long x 20cm wide x 24.5cm) with a water depth of 15cm, 1cm of gravel substrate and an individual biofilter. To allow fish visual access to one another and reduce isolation stress in this naturally shoaling species, tanks were placed next to one another in a row. The tanks were divided into three sections, a home chamber and two foraging patches using plastic dividers (see Chapter 2, Fig. 2.1.). A small, weighted plastic food cup was placed into each of the foraging compartments. Fish were trained to find food (blood-worms) in one of the two compartments, and were given two trials a day. During a trial, plastic dividers were placed down the sides of the tank to prevent fish from watching and learning the task from its neighbour. Food was placed into one of the food cups, and latency to enter a compartment was recorded. Fish were trained in this way until they entered the baited patch first in 9/10 trials (phase one). When fish had

attained this criterion, they were trained with food in the opposite compartment, again until they had entered the baited patch first in 9/10 trials (phase two). Half of the fish from each population were trained on the left side in phase one then the right side in phase two, and vice versa for the other half. The average latency over the first 10 trials of phase one and phase two comprised the first boldness score. Fish that entered a compartment sooner were defined as being bolder. A maximum of 18 fish could be tested at any one time, so the experiments were conducted in four blocks, using two fish from each population per replicate, except in the second replicate where four fish were used from River Biel.

Boldness assay two

One week after the end of boldness assay one, fish participated in boldness assay two. The second assay was based on the method employed by Brown *et al.* 2005, and involved timing fish to emerge from a darkened, enclosed box (refuge). This is a commonly used assay of 'boldness' or 'fearfulness' (e.g. Jones & Waddington 1992, Brown *et al.* 2005), and I considered this to be a suitable measure of boldness as fish had to emerge from this dark box into a brightly lit, novel tank environment. Fish were netted individually from their holding tanks and placed into a rectangular test tank (44.5cm long x 24.5cm wide x 21.5cm high) covered with black plastic to reduce outside disturbances. The fish were put into a darkened, enclosed box (refuge) (10.5cm long x 11cm wide x 21.5cm high) that was located in the test tank and had a removable lid. A door was cut into the front of the box (6cm wide x 9cm high), and this could be open or

closed with a sliding door (11cm wide x 24cm high). This box was positioned at one end of the rectangular tank on a white plastic semi-circle, which gave the fish a bright surface to cross upon leaving the refuge (see Fig. 3.1). Fish were left to

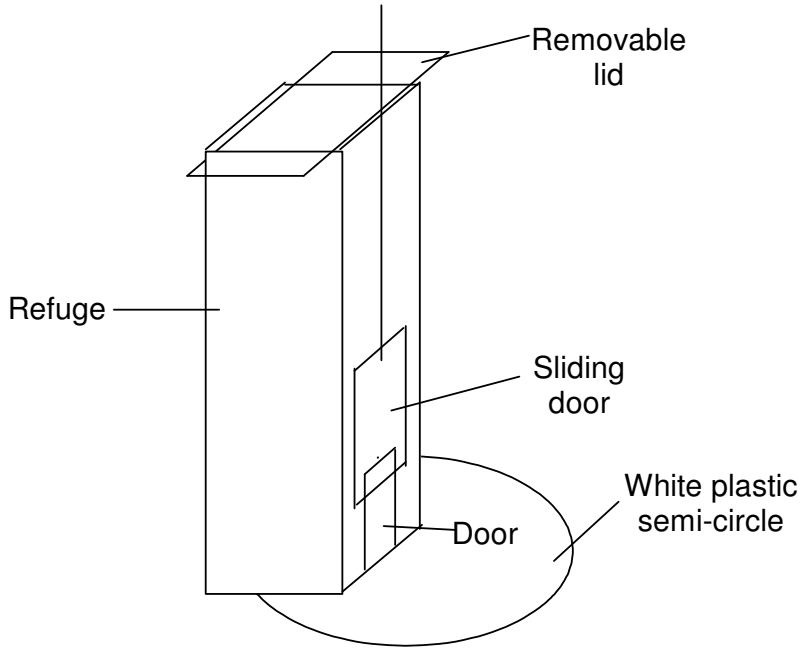

Figure 3.1. Diagram of boldness box used in boldness assay two.

settle for 2 minutes before the door was raised remotely via a length of monofilament, and to reduce disturbance to the fish all observations were made via a video camera positioned above the tank. Time taken for the fish to emerge fully from the box was recorded. Fish were given a maximum of 15 minutes to emerge, after which time they

were assigned a maximum score of 900 seconds. Fish that emerged sooner were assumed to be bolder. Observations of the fish during these trials support the notion that they were using this box as a refuge. Fish would typically emerge very slowly from the box, often emerging a small amount (front of the head protruding only), sometimes several times, before rapidly swimming across the white plastic semi-circle when they had decided to emerge fully.

Neophobia

Neophobia was quantified using two methods. The day after boldness assay two, fish underwent neophobia trials. Fish were individually netted from their home tanks into a test tank (44.5cm long x 24.5cm wide x 21.5cm high) containing a novel object (this was a brightly coloured red and blue plastic toy in the shape of a fish, measuring 6cm long x 6cm wide x 1cm high). It was assumed that all fish would be able to see this object, as there is behavioural and electrophysiological evidence that three-spined sticklebacks have good visual sensitivity in both the blue and red regions of the visible spectrum (Boulcott 2003). Furthermore, this object was placed about 15cm away from the start position of the fish and fish typically orientated towards the object before the start cylinder was removed, indicating that they had seen the object and it was within a visible distance. Animals generally find novel objects aversive, and will typically display a fear response to them. The novel object test is a widely used method of measuring neophobia in animals (e.g. Jones & Waddington 1992, Sneddon *et al.* 2003a). The object presented to the fish was novel for all fish, so I considered this assay an

appropriate measure of neophobia. The test tank was divided into three equal sections by marks along the edge of the tank, and the novel object was placed in the left section for half of each population of fish, the right for the other half. Fish were initially placed into a clear plastic cylinder (diameter 5cm x height 8cm) located in the middle section of the tank to standardise start location. They were given two minutes to settle, then the cylinder was gently raised remotely via a fine monofilament. Observations were made remotely via a video camera, filming from above, and the tank was covered in black plastic to minimise external disturbances to the fish. Fish were filmed for 15 minutes. Video replay was used to determine the time fish spent in the near, middle and far sections of the tank relative to the novel object. Fish that spent a larger proportion of time near the novel object were considered to be less neophobic, and this was the first measure of neophobia. Time taken for fish to approach the novel object was also recorded as a second measure.

Activity in a novel environment

Activity in a novel environment was determined during the neophobia trial. This tank was a novel environment for all fish. Over the 15 minutes, the number of times a fish crossed between the near, middle and far sections was recorded to give an 'activity' score for each fish.

Learning rate

The number of trials taken for a fish to learn the foraging task presented in phases one and two of boldness assay one was determined. More details of how the learning trials were set up can be found in Chapter 2 (section 2.3.3.).

3.3.4. Data analysis

All data were checked for normality and homogeneity of variance, and were transformed to normality when assumptions were not met.

One fish from North Belton was excluded from the analyses as it did not successfully complete the learning task presented in boldness assay one. Temperament behaviours were measured in three different contexts, boldness, neophobia and activity. There was only one measure for activity, but two measures each for boldness and neophobia. In order to obtain single measures of boldness and neophobia, principal components analyses (PCA) were run on the behaviours in each context (raw data can be found in table 3, appendix 2 (A.2.2.)). This simplifies the analysis, and reduces the problem of multiple comparisons. For boldness, PC1 accounted for 74% of the variation in the data, with loading coefficients of 0.71 for average time to begin a foraging trial (boldness assay one) and 0.71 for time to emerge from a box (boldness assay two). The more positive the value, the longer a fish took to emerge from the box and begin the foraging trial (i.e. less bold fish). For neophobia, PC1 accounted for 73% of the variation in the data, with loading coefficients of –0.7 for time to approach the novel object, and 0.7 for time spent near the novel object. The more positive the value, the longer a fish

took to approach the novel object, and the less time it spent near it (i.e. more neophobic fish).

To investigate the effects of temperament behaviours between populations, separate general linear models were run with activity, PC1 of boldness and PC1 of neophobia as dependent variables using fish length, replicate, population (a random factor nested within predation pressure and habitat type), habitat type, predation pressure and habitat type*predation pressure in the models. Non-significant terms were removed in a step-wise fashion to leave minimal models.

I used general linear models to investigate the relationship between temperament behaviours within populations. Here, the four dependent variables were the number of trials taken to learn the task in boldness assay one, activity, PC1 boldness and PC1 neophobia, and these investigated the effects of fish length, replicate, population, number of trials to learn the task presented in boldness assay one, activity, PC1 boldness and PC1 neophobia (with the dependent variable affecting which of these factors were included in each analysis). All two-way interactions were tested for, and non-significant terms were removed in a step-wise fashion to leave minimal models.

3.4. Results

3.4.1. Effects of temperament behaviours between populations

There was a significant main effect of population (nested within habitat type and predation pressure) ($F_{4,57}$=19, P<0.001), and a significant predation*habitat type interaction ($F_{1,57}$=19, P<0.001), but no overall effects of predation ($F_{1,57}$=0.028, P=0.86) or habitat type ($F_{1,57}$=1.43, P=0.24) on boldness. A post-hoc Tukey test revealed that the

predation pressure*habitat interaction arose because low predation river fish and high predation pond fish were significantly bolder than high predation river fish, whereas low predation pond fish were more timid than high predation pond fish (Fig. 3.2.).

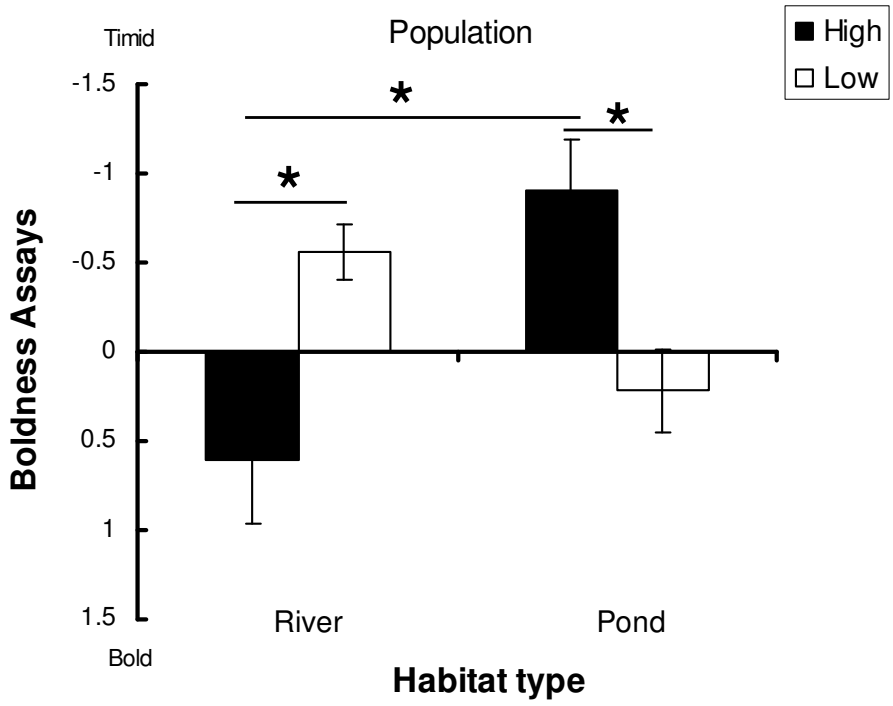

Figure 3.2. Principal component scores of boldness behaviours for pond and river fish from habitats of high and low predation pressure. Bars connected by an asterisk are significantly different to one another. Error bars represent one S.E.

A post-hoc Tukey test on population revealed a similar pattern to the predation pressure*habitat interaction, with low predation river fish and high predation pond fish being more bold than high predation river fish, and low predation pond fish more timid

than high predation pond fish. There were no overall effects of predation ($F_{1,57}$=0.90, P=0.35) or habitat ($F_{1,57}$=0.04, P=0.83) on activity in a novel environment, but there was a significant predation*habitat type interaction ($F_{1,57}$=6.27, P=0.02). There was also a significant effect of population ($F_{1,57}$=4.89, P=0.002). The means of the groups (Fig. 3.3) suggest a similar pattern to the boldness result, with low predation river fish and high predation pond fish being more active than high predation river fish, and low predation pond less active than high predation pond fish. However, a post-hoc Tukey test on the predation pressure*habitat interaction revealed that although predation pressure appears to affect activity in different ways in river and pond habitats, none of the means of the groups were significantly different to one another (Fig. 3.3.).

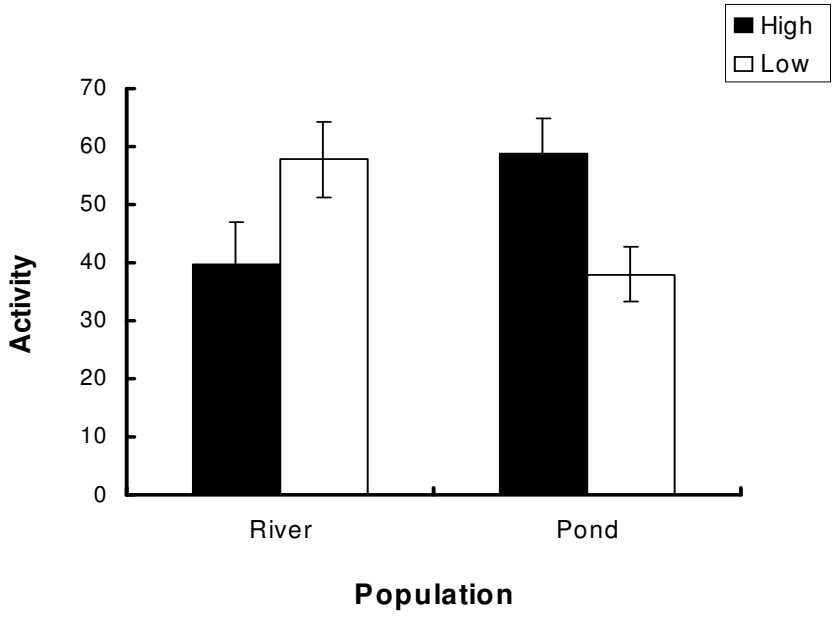

Figure 3.3. Activity scores for pond and river fish from habitats of high and low predation pressure. Error bars represent one S.E.

A post hoc Tukey test on population revealed that River Biel fish were significantly less active than Water of Leith and Craiglockhart Pond fish. There were no significant effects on neophobia ($F_{11,53}$=15.86, P=0.13, Fig. 3.4.).

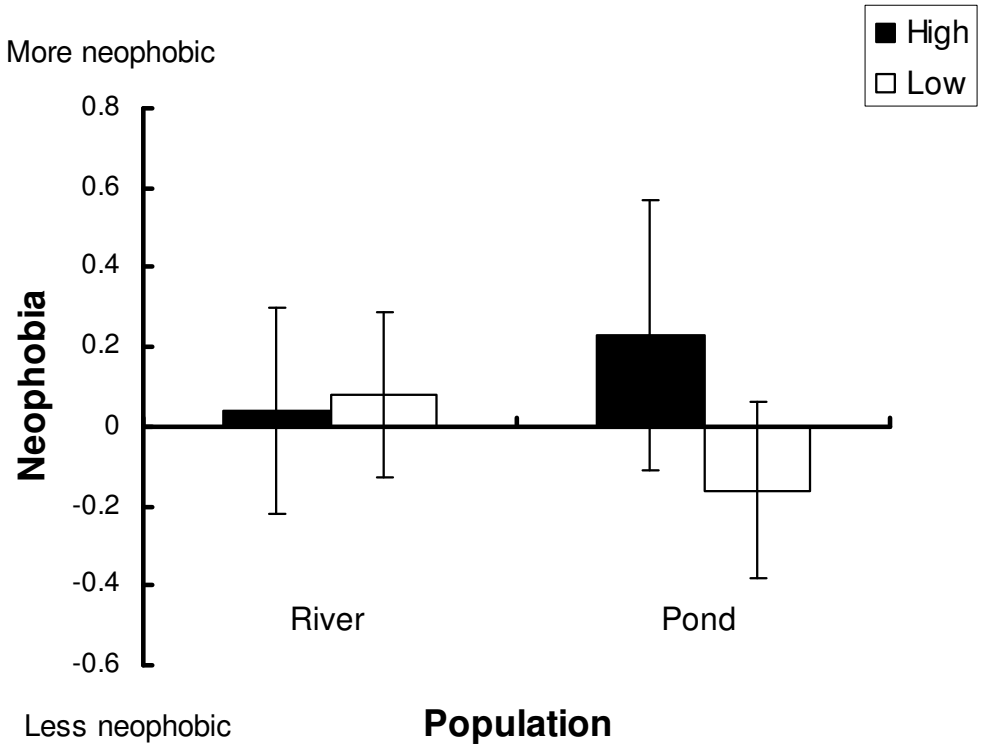

Figure 3.4. Principal component score of neophobia behaviours for pond and river fish from habitats of high and low predation pressure. Error bars represent one S.E.

3.4.2. Effects of temperament behaviour within populations

The relationship between boldness and activity differed among the populations (population*activity interaction $F_{7,49}$=3.28, P=0.006) with a negative relationship between boldness and activity in the River Biel population (t=-4.17, P=0.0001, Fig. 3.5),

but not in any other populations. Boldness and activity were not related to any other measures within populations. Similarly, there was no relationship between either neophobia or the number of trials taken to learn the task in boldness assay 1 and any of the other measures.

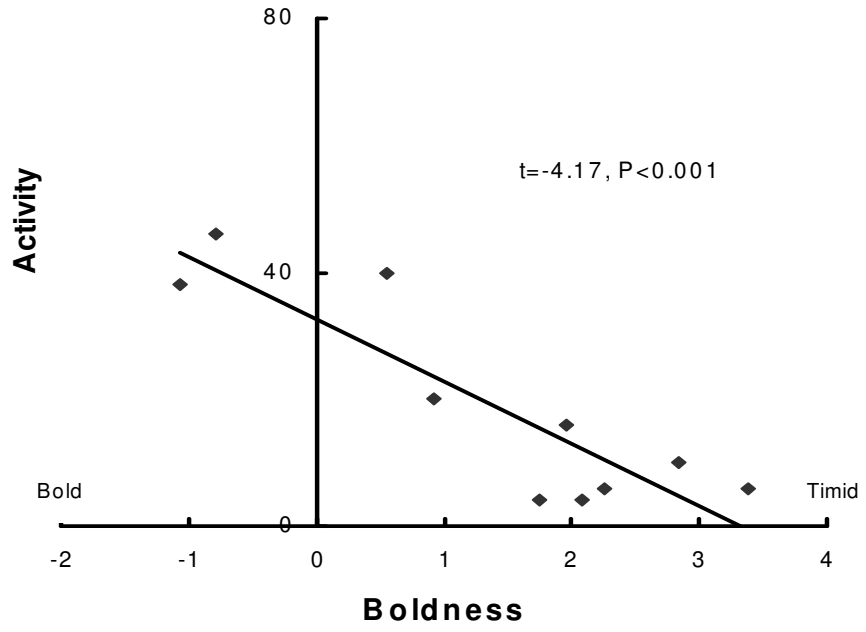

Figure 3.5. Correlation between activity and boldness for high predation river fish

3.5. Discussion

3.5.1. Temperament behaviours between populations

High predation river fish were significantly less bold and tended to be less active than high predation pond fish and low predation river fish. Although it can be difficult to determine precisely what factors are driving these differences, these results suggest that

predation pressure can interact with pond/river habitat to influence temperament behaviours in three-spined sticklebacks. High predation river populations were significantly less bold than a high predation pond population (Craiglockhart). Habitat stability has previously been hypothesised to alter behaviour in three-spined sticklebacks. Maze experiments revealed pond fish preferred to use visual landmarks to orientate to a food reward, whereas river fish preferred to use the turn direction of their own body (Girvan & Braithwaite 1998, Braithwaite & Girvan 2003). Ponds are hypotehsised to be more spatially stable environments, rivers less so as they are subject to flow and more general disturbance, so cues that might be used as landmarks in a pond environment may be less reliable in a river environment. Similarly, a habitat stability hypothesis may explain why high predation pond fish are bolder than high predation river fish. Prey in refuges are safe from predation, but there is a trade-off with other activities, such as foraging (Sih 1997). In pond environments, refuges and landmarks indicating their position are hypothesised to be more stable over time, which would allow these fish to rapidly find shelter if threatened by a predator. Furthermore, it may be predicted that the predation regime in a pond is more stable over time than in a river, where predators such as salmon and sea trout migrate through areas (see e.g. Moore 1998a,b). Hence, whilst high predation pond fish are likely to be relatively well informed about the presence and abundance of predators, river fish could face a greater degree of uncertainty. Indeed, models predict that prey with lower quality information about the presence of predators should remain in refuges for longer periods of time (Sih 1992). Additionally, river fish may be relocated to unfavourable areas by water currents or exploratory behaviours. Thus, they may need to devote more of their attention

towards their spatial location, which would give them less time for predator vigilance. In contrast, in a pond environment, there may be decreased benefit of remaining in a refuge for prolonged periods of time and this may lead to the loss of potentially valuable foraging opportunities. So even in the face of high predation in a pond environment, fish could afford to be relatively bold. In a river environment where there is hypothesised to be less stable local landmarks, refuge locations and predator populations, the alternative strategy of staying hidden for longer and being less active may therefore be more adaptive. However in the present study, caution must be applied. Only one high predation pond population was sampled, so it is somewhat difficult to be certain that differences in spatial habitat stability, rather than unique features of Craiglockhart Pond, are driving this difference between high predation populations. Sampling and testing fish from other high predation pond sites would be desirable to more vigorously test these explanations.

In agreement with my original hypothesis, low predation river fish were bolder and tended to be more active than high predation river fish. This agrees with a previous study on three-spined sticklebacks, where a low predation river population was found to be more active than a high predation population (Bell 2005). Predators have long been known to influence the behaviour of their prey. For example, fish sampled from high predation sites often display greater anti-predator behaviours (e.g. three-spined sticklebacks: Giles & Huntingford 1984, guppies: Seghers 1974) than those from low predation sites. Indeed, longer emergence times and lower activity levels will decrease the chances of meeting a predator in a high predation environment. However, the opposite pattern was revealed in pond habitats, as here high predation pond fish were

significantly bolder than low predation pond fish. It is currently unclear why this should be the case in pond habitats.

3.5.2. Temperament behaviours within populations

Temperament behaviours were correlated within one population but not the others. In one of the high predation river populations, River Biel, boldness and activity were correlated, with bolder fish having higher activity levels. There were no such correlations for any other populations. Similarly, a recent study found genetic and phenotypic correlations of activity, aggressiveness and boldness in one but not another population of river three-spined sticklebacks (Bell 2005). Interestingly, in this study the population displaying the correlation was also thought to be high predation. The 'Constraints' hypothesis for the existence of behavioural syndromes predicts that if suites of behaviours are correlated within one population, then owing to underlying constraints, they must necessarily be correlated in all other populations of that species. In conjunction with the results presented by Bell (2005), this study does not support that hypothesis. This suggests that when correlations do exist between behaviours it is because they are beneficial rather than due to underlying constraints.

In the present study, one population of river fish thought to be experiencing high predation either emerged quickly and were active, or emerged slowly and were less active. The same correlation was also found in another high predation river population in a recent study (Bell 2005). This suggests that the high predation river environment may be selecting for these two behaviours to become correlated, and may reflect two different strategies, similar to those found in other species, for example, great tits. Two

different temperament types have been described for great tits: proactive (e.g. fast explorers of novel environments, aggressive and bold) and reactive (e.g. slow explorers, passive and shy) (Verbeek *et al.* 1996). Different temperament types have greater survival depending on the particular selection regime in a give year, and this seems to underlie the co-existence of these two temperament types (Dingemanse *et al.* 2004). The same may be true for high predation river fish. If a fish emerges from a refuge quickly and is active, it risks greater predation, but also stands to gain, for example, from greater foraging rewards. This may be a preferential strategy at a time of year or in a particular year with lower predation pressure. Alternatively, a fish can emerge more slowly and be less active, which would appear to be a more adaptive strategy when predation pressure is higher. Predation pressure is likely to be less stable over time in a river compared to a pond, as certain types of predators such as salmon and trout move and migrate through areas in rivers (Moore *et al.* 1998a,b). In a pond environment with more stable and consistent predator populations, or in a river environment with consistently low levels of predation, fish might not experience this fluctuating exposure to predation events, resulting in uncorrelated behaviours. Indeed, a recent model predicts that behavioural syndromes should arise in environments where information is 'noisy', and animals are less well informed about environmental variables, such as the presence or absence of predators (Mcelreath & Strimling 2006). However, this correlation was not unveiled in one other high predation river population in the present study, the River Endrick. Overall, the River Biel was thought to be the highest predation habitat (see Chapter 2, sections 2.3.2. & 2.4.1.). This is also reflected in the fact that the River Biel fish are the least active, and most timid population overall. Indeed, I would expect fish living in high

predation environments to be less bold and display lower levels of activity, in order to decrease the chances of being detected by a predator. The reason we do not see the same correlation between boldness and activity within the River Endrick population may be due to slightly lower levels of predation in this habitat compared to the River Biel. However, it may be due to other factors more specific to the River Biel site. It would be interesting to investigate possible correlations in other river habitats with predation pressure comparable to that of the River Biel.

3.5.3. Temperament behaviours and learning

I also predicted that there might be a correlation between temperament behaviours and learning rate. Within populations however there were no correlations between any of my measured temperament behaviours and the rate at which fish learned boldness assay one, suggesting that in contrast to other species (e.g. trout (Sneddon 2003), guppies (Dugatkin & Alfieri 2003) and great tits (Marchetti & Drent 2000)) boldness, neophobia and activity do not impact upon learning in three-spined sticklebacks. However, the nature of the learning task presented and the methods used to quantify temperament behaviours need to be taken into account. For example, there are many potential ways to define boldness, e.g. boldness in the face of a predator versus time taken to emerge from a refuge. There are also numerous ways to measure it. In the present study, I defined boldness as time taken to emerge from a refuge. In previous studies, where correlations were found between boldness and learning, boldness was measured as method used to capture food (Sneddon *et al.* 2003), and predator inspection behaviour (Dugatkin & Alfieri 2003). Furthermore the task presented in these studies differ from that in the

present study: these studies simply involve fish learning to associate food with a food ring placed on the surface of the water. Bold fish that are not afraid of approaching a novel food ring may have a distinct advantage in learning such a task. In contrast in the present study, fish had the more complicated task of encoding spatial location in order to find food patches, and here boldness may not have such an impact on learning rate. This highlights the fact that the nature of the learning experiment and methods used to quantify temperament behaviours need to be considered when interpreting the results of such studies.

Although I did not find a correlation between boldness and learning rate within populations, between populations river fish from habitats thought to be experiencing low predation were not only bolder than river fish from habitats thought to be experiencing high predation, but have also previously been found to learn faster (see Chapter 2). Several studies (reviewed in Sih *et al.* 2004) have found correlations between behaviours at the population level (an average behavioural phenotype for that population). For example parrot species that explored more either lived in low predation habitats, fed on complex foods or lived in complex habitats (Mettke-Hoffman *et al.* 2002). These associations were proposed to occur because it was either more beneficial or less costly to explore in certain environments. Similarly in the present study, it may be less costly for low predation river fish to be bolder, allowing them to learn faster.

In conclusion, my results suggest that ecological variables can play a role in shaping temperament behaviours between populations, and that multiple variables might interact when fine-tuning behaviour. Although the underlying reasons are currently not clear, I have also shown that certain temperament behaviours are correlated within some

populations but not others, providing further evidence for the ‘Adaptationist’ hypothesis for the existence of behavioural syndromes. My results demonstrate the importance of considering multiple ecological variables when investigating the role of the environment in shaping an animals’ behaviour.

Chapter 4. Differences in geometric and non-geometric information use by pond and river three-spined sticklebacks

4.1. Summary

A number of animals can use large-scale features in their environment such as the geometry or shape of an area to guide their movements. There is evidence that human adults, rhesus monkeys and some species of fish and birds can combine this geometric information with non-geometric cues such as discrete landmarks to aid orientation. Other studies, however, have shown that human infants and rats do not integrate these types of cue and instead rely solely on geometry. To date, comparisons on the use of geometrical cues have been made exclusively between species. To investigate how ecological factors may influence the use of geometric and non-geometric cues at the level of the population, I compared orientation behaviours in different populations of pond and river three-spined sticklebacks. Populations from both types of habitat were able to use geometric cues for orientation, but contrary to initial predictions, only river populations were able to combine geometric information with a non-geometric cue to locate an exit. This suggests that even within a species, populations may learn about different cues when orientating.

4.2. Introduction

4.2.1. Spatial orientation

The ways animals use information to navigate and move around their environment has been extensively studied (Healy 1998). Until recently, however, experiments typically tested how animals use one type of spatial cue in isolation. For example, the use of the geometric features of an environment in orientation has been particularly well studied in a variety of species (see Cheng & Newcombe 2005 for a review). In the complex natural world, however, animals are likely to have multiple spatial cues available to them, and recent studies have begun to investigate how information from more than one source may be used and whether different types of spatial cue can be combined (see Cheng & Newcombe 2005). Two major sources of information available to animals for orientation are the shape of the environment (or its geometry), and more local cues or landmarks, such as discrete objects. For instance, in an environment with distinctive geometry, human infants (Hermer & Spelke 1994) and rats (Cheng 1986) use this geometry to find their way around, surprisingly ignoring other reliable landmarks (i.e. non-geometric cues) such as the colour of a wall. In contrast, human adults combine information from both geometric and landmark cues (Hermer & Spelke 1994), and until the past two decades we were thought to be the only species with such ability. Evidence is now accumulating, however, to show that certain bird, fish and mammal species also share this ability (see Sovrano *et al.* 2005, Vallortigara *et al.* 2005, Cheng & Newcombe 2005 for reviews). For example, work with young domestic chicks, *Gallus gallus*, demonstrates that they can encode and conjoin both geometric and non-geometric features in an environment (Vallortigara *et al.* 1990), and they use different hemispheres

of the brain to process these different types of information (Vallortigara *et al.* 2004, Chiesa *et al.* 2006).

Why do some animals combine this information and others not? Sovrano *et al.* (2002) have speculated that ecological adaptations may be at the root of why such radically different species share this ability. To date, such comparisons have been made exclusively between species. For example, differences were found in the relative importance that redtail splitfin fish and domestic chicks gave to geometric versus landmark information, and this was suggested to be due to general differences in ecology between birds and fish (Sovrano *et al.* 2007). However, several phylogenetic factors other than ecology are likely to contribute to differences between species. Within species comparisons would therefore be a more direct way to investigate the effects of ecological factors on the types of spatial cues used.

4.2.2. Aim

Populations of three-spined sticklebacks are good for this type of investigation because they inhabit ecologically diverse habitats - from marine environments to freshwater ponds and rivers. Furthermore, pond and river fish are already thought to differ in the types of spatial information they use, and this difference is related back to habitat; local visual landmarks such as small plants or rocks are used by pond fish but ignored by river fish when navigating to a food reward in a maze (Girvan & Braithwaite 1998, Odling-Smee & Braithwaite 2003). It has been suggested that the value of different types of landmarks (e.g. global versus local) will be affected by their uniqueness and stability in time and space (Vlasak 2006, Biegler & Morris 1996). River environments are

hypothesised to be less spatially stable than pond environments, because flow and flooding are expected to move landmark cues such as small plants and rocks around, making them unreliable navigational cues in these habitats (Odling-Smee & Braithwaite 2003). To date, however, whether sticklebacks are capable of using geometry has not been studied. I therefore devised an experiment to test whether pond and river three-spined sticklebacks are able to use and combine information from geometric and non-geometric sources. Large-scale geometric features are likely to be stable in both ponds and rivers, but smaller scale local landmark cues are only likely to be stable in ponds. As such, I predicted that pond fish would combine geometric with landmark information when orientating, but river fish would rely more on large-scale geometric features of the environment, and be less likely to combine the two cues.

4.3. Materials and methods

4.3.1. Subjects and housing

Three-spined sticklebacks were collected from 2 ponds and 2 rivers in Central and Southern Scotland, U.K: Ponds – Craiglockhart Pond ($3^0$14'W, $55^0$55N) and Balmaha Pond ($4^0$31.5'W, $56^0$05N), Rivers – River Esk ($3^0$10'W, $55^0$51N) and Water of Leith ($3^0$14'W, $55^0$57N). A one year survey of these sites indicated that they did not differ in many factors which may be expected to influence the potential value of visual stimuli, for example turbidity and vegetation structure (see Appendix 1). Fish were collected in March 2006 with minnow traps and large nets. A total of 64 fish were tested, 16 from each site. Populations were housed separately in tanks (76cm long x 30cm wide x 38cm high) furnished with plastic plants, a gravel substrate, biofilters and refuges and fed on a

diet of frozen blood-worm. Laboratory temperature was maintained on a day:night cycle at 14:9.5^{0}C, and light:dark cycle of 10:14h for the duration of the experiment. Fish were collected outside of their breeding season, and as males and females are morphologically identical at this time, populations were assumed to be mixed sex. All fish measured 3.5-4.5cm in length.

4.3.2. Experiment 1 – geometric cues

Eight fish from each population were tested (i.e. a total of 32 fish). The maze design was similar to that used by Sovrano *et al.* (2002). It consisted of a rectangular arena (28cm long x 8cm wide x 20cm high) with an opening at each corner (7cm high x 3cm wide). These openings led to small tunnels that ended in doors (9cm high x 6.5cm wide), which could be open or blocked (Fig. 4.1.). I incorporated tunnels into the design so that dead-ends could not be detected by fish until they reached the end of the tunnel, and hence their only method of locating the correct door was by using the features in the maze environment. The rectangular shape of the maze provided different types of geometric cues (for example a fish might learn that the correct corner was located where there was a long wall on the left and a short wall on the right, or vice versa). The maze was set-up within a larger opaque arena (50cm long x 35cm wide x 30cm high). This created an annular region with gravel, vegetation, food and conspecifics (one in each corner). The conspecific stimulus fish were restrained in containers (6cm long x 6cm wide x 12cm high): these fish were never tested and were changed at regular intervals. During trials, only one door in the maze was open, the others were blocked by clear plastic doors.

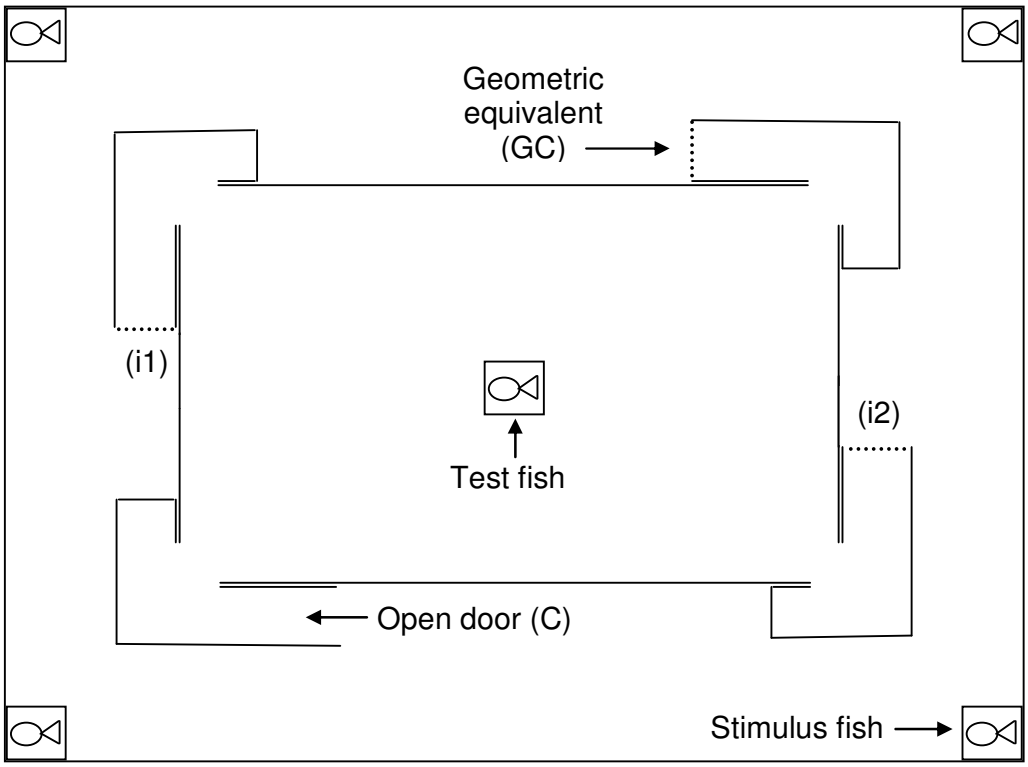

Figure 4.1. Schematic view of the experimental apparatus. Test fish could escape from the central arena by swimming down the small tunnel and through the open door into the annular region. In experiment 1, the walls were identical in every way except for length, and only one door was open (blocked doors are represented with the dotted lines). Using geometry alone fish could select the correct corner and its geometric equivalent. In experiment 2, one short wall was painted bright blue, and a cross shape was left unpainted in the middle of the wall to provide a conspicuous non-geometric cue. Again only one door was open, and fish could select the open door through combining geometry with the non-geometric information provided. (c)=correct corner, (gc)=geometrically correct corner, (i1)=incorrect corner 1, (i2)-incorrect corner 2.

Different fish were given a different open door position, and this was balanced across populations. To motivate the fish to leave the maze, I provided a food reward (a single blood-worm secured in a small Vaseline filled dish) and visual access to conspecifics. To eliminate the use of extra-maze cues a circular curtain of black plastic suspended from the ceiling surrounded the entire apparatus. A centrally positioned lamp and

camera provided light over the tank and the ability to film the movements of the fish so that their behaviour could be monitored remotely.

At the start of a trial, a fish was placed into a clear plastic cylindrical container (5cm diameter x 8cm high) in the middle of the test tank, and allowed to settle for one minute. After this time, the container was gently raised using a pulley made of monofilament.

A video monitor was used to observe the number of escape attempts from each corner until the fish exited the maze and entered the annular region, or until 10 minutes had elapsed. If a fish had not left the maze after this time, it was gently ushered towards and out of the correct door with a dip-net. There was an inter-trial interval of 10 minutes, during which the fish was allowed to remain in the annular region (reinforcement time). The maze was then rotated through 90^0, the fish was disorientated by rotating in an opaque cup, and then it was tested again. This was to ensure that the only reliable cue between trials was the geometry of the apparatus. Fish were given 5 trials a day for 5 days.

4.3.3. Experiment 2 – geometric and landmark cues

Eight new fish from each population were tested (n=32). The same apparatus and experimental procedure were used as in experiment 1, but one short wall was now painted bright blue, with the shape of a cross left unpainted in the middle of this wall to provide a conspicuous non-geometric cue. Again only one door was open, the others were blocked with clear plastic doors. Different doors were open for different fish, and again this was balanced across the different populations. Fish were given 5 trials a day

for 5 days, and were monitored via a video camera. The number of escape attempts from each door was recorded.

4.4. Results

All data were checked for normality and homogeneity of variance, and were transformed to normality when assumptions were not met.

4.4.1. Experiment 1 – geometric cues

Frequencies of escape attempts (Ln+1 transformed) were analyzed using a repeated measures analysis of variance (ANOVA) with habitat (river versus pond) as a between subject factor, and corner and day as within subject factors. All interactions were tested for. Data were Ln transformed to conform to the assumptions of normality, and statistical values were adjusted accordingly if sphericity (tested using the Mauchly criterion (Mauchly 1940)) was violated. The ANOVA revealed a significant main effect of corner ($F_{3,90}$=0.42, P=0.003), but no other significant effects (see Table 4.1.) on frequency of escape attempts. The lack of habitat*corner interaction demonstrates that there was no difference in the number of escape attempts that river and pond populations directed at each of the four corners. Examining the significant main effect with a post-hoc Tukey HSD test revealed that fish predominantly directed their escape attempts at two of the corners in particular: these were the correct and geometrically correct corners, and there was no significant difference in the frequency of escape attempts i) between the correct versus the geometrically correct corner, or ii) between incorrect corner 1 versus incorrect corner 2 (Fig. 4.2.a,b.). Thus the fish chose to use the correct and

geometrically correct corners more often than the two incorrect corners, showing that they could use the geometry of the test arena.

Table 4.1. Terms with non-significant effects on frequency of escape attempts in experiments 1 and 2.

Term	Experiment 1: all fish	Experiment 2: all fish	Experiment 2: pond fish	Experiment 2: river fish
Habitat/population	$F_{1,30}$=0.68, P=0.42	$F_{1,30}$=0.009, P=0.93	$F_{1,14}$=4, P=0.07	$F_{1,14}$=0.78, P=0.39
Corner	Significant	Significant	$F_{3,42}$=1.8, P=0.17	Significant
Day	$F_{4,120}$=2.10 P=0.09	$F_{4,120}$=1.79, P=0.114	$F_{4,56}$=1.7, P=0.16	$F_{4,56}$=1.10, P=0.35
Habitat/population *corner	$F_{3,90}$=0.42, P=0.74	Almost significant	$F_{3,42}$=0.90, P=0.43	$F_{3,42}$=1.97, P=0.13
Day*corner	$F_{12,360}$=0.95, P=0.50	$F_{12,360}$=0.76, P=0.69	$F_{12,168}$=0.60, P=0.84	$F_{12,168}$=0.87, P=0.58
Habitat/population *day*corner	$F_{12,330}$=0.61, P=0.83	$F_{12,360}$=0.74, P=0.71	$F_{12,168}$=0.25, P=1.00	$F_{12,168}$=1.00, P=0.40
Habitat/population *day	$F_{1,30}$=1.36, P=0.25	$F_{4,120}$=0.90, P=0.46	$F_{4,56}$=1.86, P=0.13	$F_{4,56}$=1.46, P=0.23

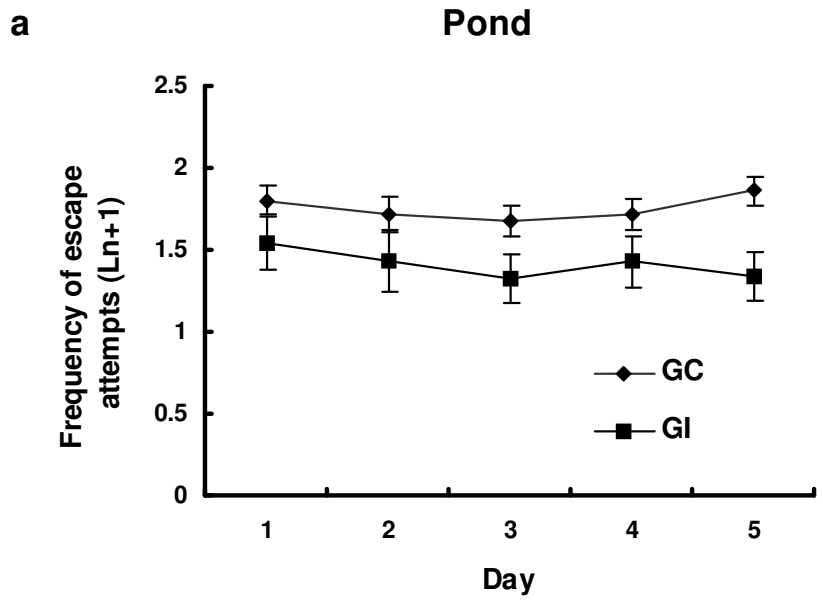

Figure 4.2.a. Frequency of escape attempts from correct + geometrically correct corners (gc) versus two incorrect corners (gi) for pond populations. Error bars represent one S.E.

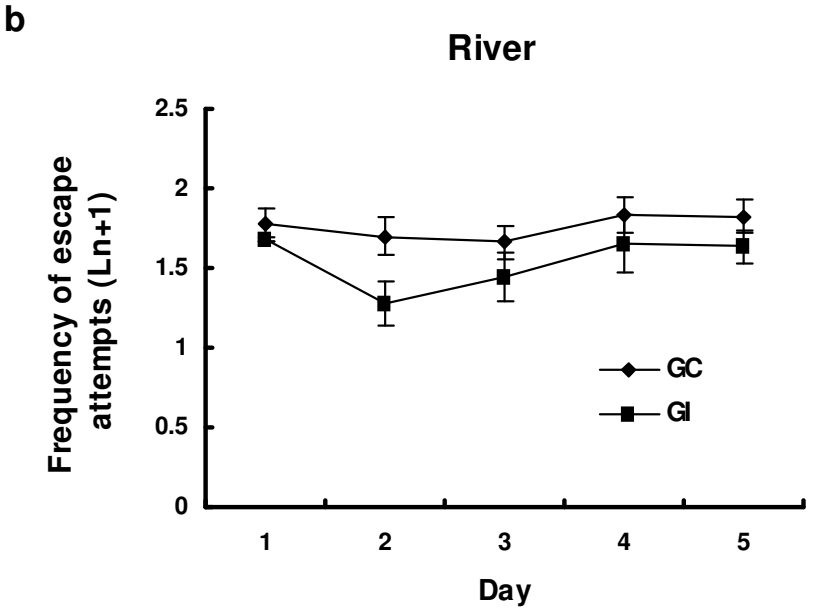

Figure 4.2.b. Frequency of escape attempts from correct + geometrically correct corners (gc) versus two incorrect corners (gi) for river populations. Error bars represent one S.E.

4.4.2. Experiment 2 – geometric and landmark cues

Frequencies of escape attempts (Ln+1 transformed) were analyzed by repeated measure analysis of variance (ANOVA) with habitat as a between-subject factor, and corner and day as within subject factors. All interactions were tested for. Data were Ln transformed to conform to the assumptions of normality, and statistical values were adjusted accordingly if sphericity was violated. There was a significant main effect of corner ($F_{3,90}$=3.75, P=0.01) and a non significant trend for the corner*habitat interaction ($F_{9,90}$=2.3, P=0.08). None of the other effects were significant (See Table 4.1.). The trend shown by the corner*habitat interaction suggests that pond and river populations had a tendency to attempt to escape from different corners at different frequencies from one another, with river fish directing more escape attempts at the correct corner than the three incorrect corners compared to pond fish (Fig. 4.3a,b.).

I investigated this further by analysing the frequencies of escape attempts of pond and river fish in separate ANOVA's, with population as a between-subject factor, and corner and day as within subject factors: **Pond fish**: there were no significant effects (see Table 4.1., Fig. 4.3.a.) **River fish:** there was a significant main effect of corner (ANOVA: $F_{3,42}$=4.3, P=0.01), but no other significant effects (see Table 4.1., Fig. 4.3.b.). A post-hoc Tukey HSD test on corner revealed that river fish directed significantly more escape attempts at the correct corner than the other three corners, and there were no differences between the number of escape attempts directed at the incorrect corners.

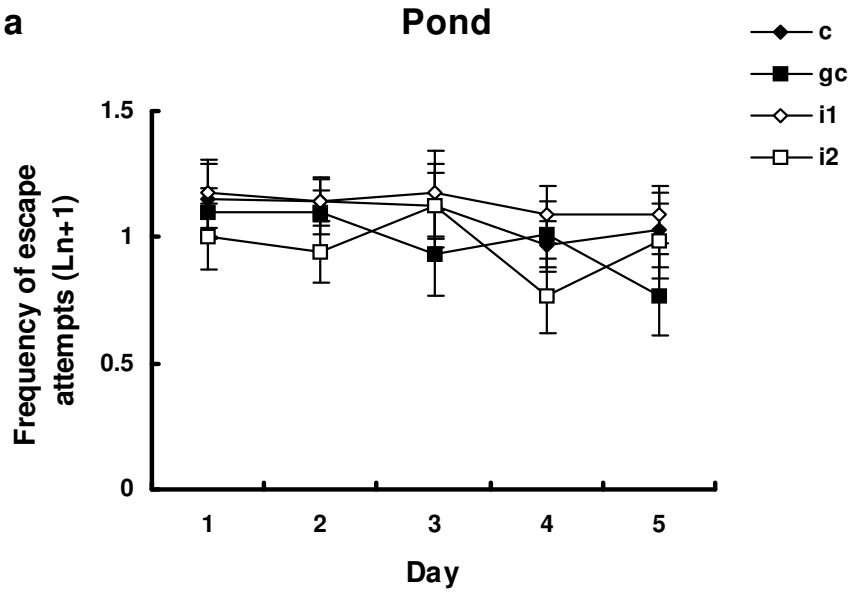

Figure 4.3.a. Frequency of escape attempts from all four corners (correct (c), geometrically correct (gc), and two incorrect (i1 and i2)) for pond populations. Error bars represent one S.E.

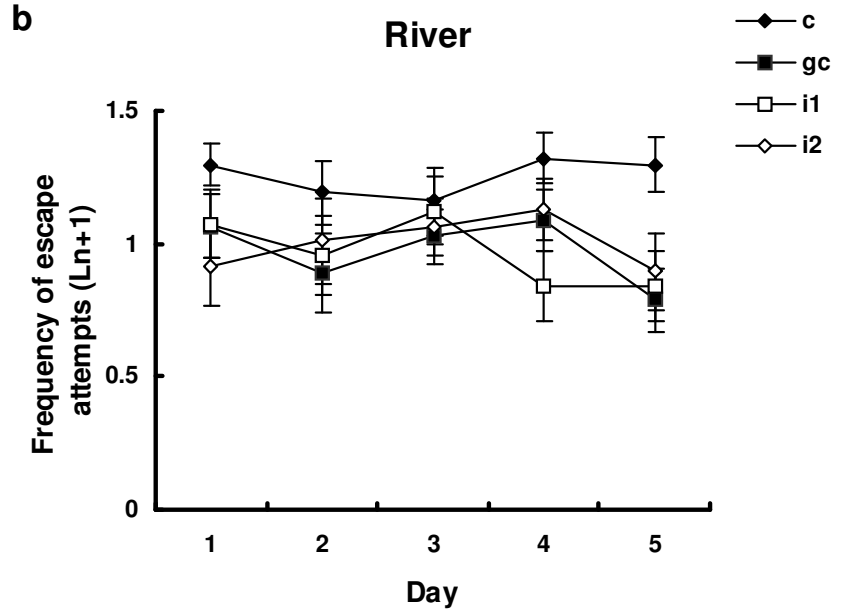

Figure 4.3.b. Frequency of escape attempts from all four corners (correct (c), geometrically correct (gc), and two incorrect (i1 and i2)) for river populations. Error bars represent one S.E.

4.5. Discussion

Both river and pond three-spined sticklebacks were able to use geometric information for orientation. This was demonstrated in experiment 1, where all fish predominantly directed their escape attempts at the open door corner (c) and its geometric equivalent (gc) over the other two, geometrically incorrect corners (i1 and i2, see Fig. 4.2a,b.). This result is consistent with findings in numerous other species, for example, fish (e.g. redtail splitfins (Sovrano *et al.* 2003)), mammals (e.g. rats (Cheng 1986)) and birds (e.g. domestic chicks (Vallortigara *et al* .2005)), (reviewed in Cheng & Newcombe 2005). It is not surprising that many species have the ability to use geometry for reorientation. Large-scale features of an environment are likely to remain quite stable over time and throughout seasons, and hence provide a reliable, basic cue for at least initial orientation processes (Sovrano *et al.* 2002, Cheng 2005). Local cues such as discrete landmarks may then be used to more precisely specify a particular location.

It is interesting that certain groups of animals such as human adults (Hermer & Spelke 1994), rhesus monkeys (Gouteux *et al.* 2001) and redtail splitfins (Sovrano *et al.* 2003) can combine geometric cues with non-geometric cues when orientating, using the geometry of the area and integrating it with local landmark information. There are, however, exceptions to this ability with human infants (Hermer & Spelke 1994) and rats (Cheng 1986, Jonasson 2005) that are unable to combine these two types of information. In the second experiment described here, river fish were able to combine geometric and non-geometric information to locate the exit of the maze, but pond fish were not. This result is interesting for several reasons. First it shows that even across populations within a species there are differences in ability to combine different sources of spatial

information. Second, this result is opposite to my original predictions; owing to the hypothesised relative spatial stability of landmark cues in pond habitats and previous work demonstrating that pond fish can use small discrete landmarks to help them find their way around a maze (Girvan & Braithwaite 1998, Odling-Smee & Braithwaite 2003), I predicted that pond fish would be able to combine geometry with a non-geometric landmark cue (represented by the blue wall with a cross in the middle) to locate the open exit. In contrast, I expected that river fish would ignore this non-geometric cue and be unable to successfully complete the task, as previous work has shown they tend to ignore local landmarks in other maze tasks (e.g. Girvan & Braithwaite 1998, Braithwaite & Girvan 2003).

It is possible that fish collected from pond environments have a different visual ability to those from river environments, and this difference in the visual sensory system precluded the pond fish from using the blue wall and cross shape as a spatial cue. I do not believe this is likely as previously no differences in visual colour discrimination were found between pond and river fish (Girvan & Braithwaite 1998), and there is behavioural and electrophysiological evidence that pond fish have good visual sensitivity in the blue region of the visible spectrum, around 400nm wavelength (Boulcott 2003).

A different explanation for my observations is related to the nature of the non-geometric cue used in the experiment. The landmark cue was a blue wall with a cross in the middle, whereas in previous experiments the landmarks have been small discrete objects placed next to places of interest, for example a plant or a rock next to an open door in a maze. These two types of landmark may represent two different categories of

non-geometric cue. A blue wall with a cross on it may be used as a global cue (i.e. a more distant source of reference, typically defined as features of a room, or in this case a maze), whereas a small discrete object closer to the target location may be used as a local cue (i.e. a cue relatively close to a target location, such as a small plant next to an open door). Previous studies have found that animals sometimes preferentially pay attention to certain categories of cues over others. For example, honeybees, pigeons and European jays (*Garrulus glandarius*) place greater importance on near landmarks rather than more distant cues when trying to locate hidden food rewards (Cheng *et al.* 1987, Cheng 1989, Bennett 1993). Near landmarks are proposed to provide a more accurate means for identifying a location compared to cues positioned further away. Weber's law illustrates this fact: as the magnitude of a measure increases so too does the uncertainty in estimating that measure (Cheng 1990). The proximity of a local cue to a target location can also affect how spatial information is used. Clark's nutcrackers (*Nucifraga columbiana*) trained with local cues in close proximity to a target location pay more attention to local cues, whereas those trained with local cues further away rely more heavily on global cues to orientate (Gould-Beierle & Kamil 1999).

It has been suggested that the value of different types of landmarks (e.g. global versus local) will depend not only on their proximity to a goal, but also on their uniqueness and stability in time and space (Vlasak 2006, Biegler & Morris 1996). In three-spined sticklebacks, it may be expected that river fish would pay more attention to global over local cues. We already know that they do not place much importance on local cues when those local cues are discrete objects such as small plants (Girvan & Braithwaite 1998, Braithwaite & Girvan 2003, Odling-Smee & Braithwaite 2003), as

these are hypothesised to be spatially unstable in a river habitat. Global cues, however, such as characteristics of the river bank (e.g. rock texture, colour or shape) may be relatively stable and reliable over time. Thus in experiment 2, if the blue wall and cross used are categorized as a global landmark, this might explain why river fish were able to combine the non-geometric with the geometric cue to locate the correct corner and exit the maze. It may be argued that such global cues are also stable in pond habitats, however, it is possible that pond fish place greater reliance on more local landmarks rather than global cues. Interestingly, in the ponds sampled for this study, the fish were rarely found close to the edges of the pond, rather they were caught in patches of vegetation that tended to be some distance from the edges. Hence, global cues such as the characteristics of the bank may have little relevance to orientation in natural pond environments, and may be the reason why pond fish did not combine the non-geometric cue with geometry in experiment 2. Indeed, the addition of this cue appeared, if anything, to confuse the pond fish because unlike in experiment 1 where they were able to distinguish between the geometrically correct and geometrically incorrect corners, in the second experiment they were no longer able to use geometry, demonstrated by them attempting to escape from all four corners with equal frequency. This apparent confusion also indicates that the pond fish did see the blue wall and cross, which also refutes the alternative explanation that pond and river fish have different visual capacities.

Although statistically the fish used in this study were able to use geometry and pond fish were able to combine this with non-geometric information to orientate, there were still a high proportion of 'incorrect' choices in both experiments one and two, even after 5 days of testing. This was not attributable to particular individuals, but was

distributed across all individuals. Training fish for a greater number of days may have decreased the number of incorrect responses, indeed, fish did appear to be increasing their frequency of correct choices over time.

In conclusion, these two experiments demonstrate that three-spined stickleback fish living in different types of habitat vary in their ability to integrate spatial information. The idea that the ability to learn and use geometrical cues is widespread is supported, as all four populations could use geometry. Furthermore, the fact that river and pond populations differed in their ability to combine geometry with a non-geometric cue supports the notion that the local environment is important in determining the cues that populations pay attention to during orientation and navigation. As previous experiments have demonstrated that pond fish use more local landmarks (for example, small plants) to navigate in a maze (e.g. Girvan & Braithwaite 1998, Odling-Smee & Braithwaite 2003, Braithwaite & Girvan 2003), it would be interesting to compare the ability of pond and river fish to combine local landmarks with geometry during orientation. We now need to determine how different types of cues are categorised (e.g. global or local) and used by pond and river fish.

Chapter 5. Environmental enrichment: implications for learning, memory and temperament behaviours in three-spined sticklebacks

5.1. Summary

Housing conditions can have significant effects on the behaviour and physiology of captive animals. In particular, barren environments can have detrimental effects on welfare. Enriching barren environments, for example through adding structural complexity or providing companions can decrease the occurrence of abnormal behaviours and physiology, improving both welfare and repeatability of scientific results. Many studies have investigated the effects of environmental enrichment on laboratory rodents, and although investigated in some commercial fish species, little consideration has been given to commonly used laboratory fish species. Hence, I designed an experiment to investigate the effects of environmental enrichment on learning, memory and temperament behaviours in three-spined sticklebacks. Fish were either caught in a natural environment, or reared in plain and enriched tank environments. I found no overall effect of rearing environment on learning or temperament behaviours, but there was a significant effect of replicate. Fish from replicate one learnt the initial phase of a foraging task more slowly, a subsequent phase faster and were bolder than fish from replicate two, suggesting either that boldness may affect learning, or that learning and temperament behaviours (e.g. boldness) are very

sensitive to environmental variation and differed between replicates due to an unidentified variable.

Rearing environment had a significant effect on memory: enriched and non-enriched fish were able to return to a previously rewarded location after 3 days, whereas wild fish did not. These results indicate that the rearing environment affects certain behaviours but not others in three-spined sticklebacks.

5.2. Introduction

5.2.1. Effects of environmental enrichment

Standard laboratory housing conditions consist of plain impoverished environments, often designed to standardize behaviour between different experimental groups and maintain good physical health (Olsson & Dahlborn 2002). However, such environments can severely restrict the natural behavioural repertoire of animals, and hence may compromise their welfare if the animal is highly motivated to carry out particular behaviours (Dawkins 1988, 1998). Furthermore, they can alter behaviour and physiology, and thus compromise the validity of research data (Würbel 2001, Reinhardt 2004). Enriching the environment can allow expression of certain behaviours, improving both welfare and research validity. Environmental enrichment refers to an environment that is 'enriched' compared to standard laboratory housing conditions (van Praag *et al.* 2000), and can take many forms, from social enhancement (i.e. by providing companions) through to structural complexity (e.g. providing toys for mice). Environmental enrichment studies have a long history, and there is currently much interest in its effects on a variety of captive animals, from those housed in the laboratory

(e.g. see Balcombe 2006 for a review in rodents) to those in zoo environments (e.g. see Mason *et al.* 2007 for a review).

Enriching the environment can have numerous effects on behaviour and physiology, and is often thought to be beneficial to the animals (see Balcombe 2005 for a review in rodents). It can decrease fear and aggression responses (see Reinhardt 2004 for a review) and stress responses to past, present and future stressors (see Fox *et al.* 2006 for a review). For example, female group housed rats were less stressed than those housed in isolation (Sharp *et al.* 2003). It can also decrease stereotypic behaviour (reviewed in Mason *et al.* 2007). Sterotypies are defined as apparently functionless, repetitive behaviours that are widespread in captive animals and are thought to be indicators of poor welfare. Mason *et al.* (2007) indicate that some 10, 000 captive wild animals are thought to be affected worldwide. Work with commercial fish species has shown that enhancing the complexity of the rearing environment can equip them with better behavioural skills. For example, structural enrichment and feeding with live prey increases foraging performance on novel live prey in Atlantic salmon (Brown *et al.* 2003b). Similarly, environmental enrichment alters behaviour towards prey, exploratory and stress recovery behaviours in hatchery reared cod (Braithwaite & Salvanes 2005), ultimately equipping them with enhanced behavioural skills compared to their non-enriched counterparts. This has great commercial importance because currently fewer than 5% of many millions of hatchery reared fish released into the wild survive to adulthood (McNeil 1991).

Enrichment can also enhance learning and memory. Rats exposed to a wide range of sensory stimuli demonstrate better learning and memory for a simple

conditioning task (Woodcock & Richardson 2000), and rats raised with conspecifics in more complex environments perform better on the radial maze task (Leggio *et al.* 2005). Even brief exposure (12 days) to enrichment is enough to improve performance on the Morris water maze test in rats (Paylor *et al.* 1992). Similar results have been found in pigs, as those raised in enriched environments have a better long-term memory capacity (de Jong *et al.* 2000). There is also evidence that animals prefer enriched environments because they will work for the opportunity to gain access to enrichment such as nesting material, shelter and raised platforms (reviewed in Olsson & Dahlborn 2002).

5.2.2. Laboratory fish and enrichment

The majority of laboratory studies investigating environmental enrichment have focussed on rodents, and little attention has been paid to other commonly used laboratory species, such as fish. There are several reasons why investigating housing conditions for laboratory fish may be important: (i) Recent studies suggest that fish demonstrate complex cognitive capacities, and may possibly have sufficient cognitive capacity to suffer from the experience of pain, although this remains a debated topic (see Rose 2002, Braithwaite & Huntingford 2004, Chandroo *et al.* 2004a, Chandroo *et al.* 2004b, Dunlop & Laming 2005, Rose 2007 for reviews). It has therefore been suggested that fish should be afforded a welfare status similar to other vertebrates (Chandroo *et al.* 2004a, Huntingford *et al.* 2006), and this will necessarily include a consideration of the conditions in which they are housed. (ii) The rearing environment may influence the validity of experimental data, if, for example, rearing or housing animals in unsuitable environments produces abnormal behaviours (reviewed in Sherwin 2004, Reinhardt

2004). (iii) The effect of housing conditions on behaviour and physiology can give insights into the mechanisms that underlie behavioural plasticity. For example, it is well known that domesticated animals tend to have smaller brains than their wild counter parts (see Kruska 2005 for a review), and this has generally been attributed to selection on genes over many generations. However, it has recently been shown that differences in behaviour and physiology can be observed within just one generation: juvenile rainbow trout raised in enriched tanks have larger cerebella and different locomotor behaviours to genetically similar individuals raised in conventional tanks (Kihslinger & Nevitt 2006). The cerebella of these enriched fish was also more similar in size to wild river reared individuals.

5.2.3. Aims

To date, there have been no studies investigating the effects of enrichment in non-commercial, commonly used laboratory fish species. Thus here, I investigate how environmental enrichment affects learning, memory and temperament behaviours in three spined sticklebacks reared in enriched, non-enriched and natural environments. Based on previous findings that exposure to more complex or naturalistic environments can promote brain growth and enhance learning and memory (e.g. de Jong *et al.* 2000, Woodcock & Richardson 2000, Leggio *et al.* 2005), I hypothesized that fish from the natural environment (wild fish) would learn fastest and have the greatest memory capacity, followed by enriched fish and finally non-enriched fish. I expected that wild fish would be the least bold, least active and most neophobic, as they will have experienced or witnessed natural predators and so should be more cautious. Animals

experiencing higher levels of predation often display enhanced anti-predator behaviour and morphology (e.g. sticklebacks, (Giles & Huntingford 1984; Bell 2005), guppies, (Seghers 1974; O'Steen, Cullum & Bennett 2002), Daphnia *spp*., (Fisk *et al.* 2007), larval anuran *spp*. (Relyea 2001) and Seychelles warblers, *Acrocephalus sechellensis* (Veen *et al.* 2000)). I also hypothesised that enriched fish would be bolder, more active and less neophobic than non-enriched fish as the majority of previous studies have found that enrichment tends to enhance these types of behaviour (e.g. greater activity in enriched fish (Braithwaite & Salvanes 2005), lower anxiety and fear in enriched rodents (reviewed in Sherwin 2004), enhanced exploration of mazes and novel objects (reviewed in Fox *et al.* 2006), increased activity and quicker emergence times in mice (Olsson & Dahlborn 2002) greater activity and lower response distances in enriched spiders (Carducci & Jakob 2000), although enrichment had the opposite or no effect on exploration of a novel environment in pigs (de Jong *et al.* 2000)).

5.3. Materials and methods

5.3.1. Subjects and housing

48 three-spined stickleback fry were collected from Craiglockhart Pond in Edinburgh, Scotland ($3^0$14'W, $55^0$55N) in June 2005 with large nets. These fish were naturally spawned, and caught after experiencing only 2-3 weeks of life in the pond. These 48 fry were split into four groups of 12, and housed in four holding tanks (76cm long x 30cm wide x 38cm high). Two of these tanks were furnished with four plastic plants, a gravel substrate, biofilters and four refuges (which were upturned plant pots), and these were the enriched environments. The other two tanks were unfurnished with biofilters and

gravel, but otherwise were non-enriched environments. All tanks were cleaned every two weeks, and the position of plants and refuges were altered at random in the enriched tanks. All fish were fed on a diet of frozen blood-worm, delivered via a pipette onto the surface of the water. Enriched fish were fed at variable places in the tank, non-enriched fish were always fed at the front left corner of the tank. These fish were reared to adulthood in the laboratory over the course of 10 months. In April 2006, 24 adult three-spined sticklebacks were collected from Craiglockhart Pond in Edinburgh, Scotland ($3^0$14'W, $55^0$55N) with large nets, and were from the same generation as the juveniles caught the previous year in this annual population of sticklebacks. They were split into two groups of 12, and housed in holding tanks (76cm long x 30cm wide x 38cm high) furnished with one plastic plant, a gravel substrate, biofilters and one refuge, a typical housing situation for fish kept in the laboratory. They were fed at the front centre of their tanks, again standard practice in the laboratory. These were the wild groups. All fish were fed on a diet of frozen blood-worm. Laboratory temperature was maintained on a day:night cycle at 14:9.5^{0}C, and light:dark cycle of 10:14h for the duration of the experiment. All populations were of a similar mean body length (4.75cm) at the time of testing (ANOVA: $F_{2,27}$=0.72, P=0.50).

5.3.2. Learning and memory assay

The learning and memory assay was based on the method used in Chapter 2 (see section 2.3.3.). Briefly, fish were housed individually in tanks (35cm long x 20cm wide x 24.5cm high) with a water depth of 15cm, 1cm of gravel substrate and an individual bio-filter for the duration of the experiment. These tanks were divided into three

compartments: a home chamber and two 'foraging patches' (see Chapter 2, Fig. 2.1.). The patches were accessible at all times (except when a patch was being baited) via doors cut into the dividing wall (measuring 4.5cm high x 2.5cm wide). A small, weighted plastic cup (3cm diameter) filled with Vaseline was placed in each foraging patch.

During a trial, an opaque plastic barrier was placed in front of the doors and the plastic cups were removed. Three blood-worms were placed into one of these plastic cups, both cups were then placed back into the compartments. Fish were given two minutes to settle, then the barrier was gently removed remotely via a piece of string looped over a plastic rod suspended above the tank. Fish were observed over the top of the tank, with the observer standing 1m away from the tank, and remaining still. Door entered first (right or left), and the latency to move into the food patch and begin feeding was recorded. If it was an incorrect choice the fish was observed until it either entered the correct side, or until 15 minutes had elapsed. The experiment was divided into five phases:

Phase One – Acquisition: learning one compartment is rewarded

Fish were given two trials a day, with the food in the same patch each time, until they selected the correct patch first in 9/10 trials, indicating they had learned the task, or until 45 trials had elapsed, at which point it was assumed the fish was incapable of learning the task.

Phase Two - Acquisition: learning a different compartment is rewarded

When criterion performance was reached in phase one, fish were fed in the opposite patch until they reached the same criterion level of 9/10 correct choices.

Phase Three – Memory retention: do the fish relocate the last rewarded compartment?

During this phase, the plastic dividers that created the foraging patches were removed from the tank, and all fish were left for a retention interval of 3 days. Fish were fed six blood-worms a day via a pipette at the front center of their tanks for the duration of this phase. After 3 days the apparatus was reinserted into the tank, and a trial was performed to determine if the fish could return to the last rewarded side (phase two rewarded side).

Phase Four – Acquisition: relearning a compartment is rewarded

The trial in phase three (return to previously rewarded patch) comprised the first trial of this phase. Fish were again trained to locate food in one patch – the same patch that they were trained to in phase two - until they reached the same criterion level of 9/10 correct choices.

Phase Five - Memory retention: do the fish relocate the last rewarded compartment?

During this phase, the plastic dividers that created the foraging patches were removed from the tank, and all fish were left for a retention interval of 7 days. Fish were fed six blood-worms a day via a pipette at the front center of their tanks for the duration of this phase. After 7 days the apparatus was reinserted into the tank, and a trial was performed to determine if the fish could return to the last rewarded side (phase four rewarded side).

Experiments were conducted in two blocks, using 5 fish from each treatment group (enriched, non-enriched and wild) per replicate.

5.3.3. Temperament assays

Temperament assays were based on methods used in Chapter 3 (see section 3.3.3. for extended details).

Boldness assay one

Boldness was quantified using two methods. The first involved determining the average time taken for a fish to begin a foraging trial (determined as entry into a foraging patch) in the first 10 trials of phases one, two and four of the learning and memory assay presented above, generating a mean value for each fish. Fish that entered a compartment sooner were assumed to be bolder.

Boldness assay two

The second assay was based on the method employed by Brown *et al.* 2005, and involved transferring an individual fish from its holding tank and placing it into a darkened, enclosed start box located in a rectangular test tank. The box had a door cut into it that closed with a sliding door (see Chapter 3, Fig.3.1.). Fish were left to settle for 2 minutes before the door was raised remotely via a length of monofilament, and to reduce disturbance to the fish all observations were made via a video camera positioned above the tank. Time taken for the fish to emerge fully from the box was recorded. Fish

were given a maximum of 15 minutes to emerge, after which time they were assigned a maximum score of 900 seconds. Fish that emerged sooner were assumed to be bolder.

Neophobia

Neophobia was quantified using two methods. The day after boldness assay two, fish underwent neophobia trials. Fish were individually netted from their home tanks into a test tank that was divided into three equal sections, and contained a novel object at one end. Fish were initially placed into a clear plastic cylinder located in the middle section of the tank to standardise start location. They were given two minutes to settle, the cylinder was then gently raised remotely via a fine monofilament. Observations were made via a video camera, filming from above, and the tank was covered in black plastic to minimise external disturbances to the fish. Fish were filmed for 15 minutes. Videos were replayed in order to determine the time fish spent in the near, middle and far sections of the tank relative to the novel object. Fish that spent a larger proportion of time near the novel object were considered to be less neophobic, and this was the first measure of neophobia. Time taken for fish to approach the novel object was also recorded as a second measure.

Activity in a novel environment

Activity in a novel environment was determined during the neophobia trial. This tank was a novel environment for all fish. Over the 15 minutes, the number of times a fish crossed between the near, middle and far sections was recorded to give an 'activity' score for each fish.

5.3.4. Data analysis

All data were tested for normality and heterogeneity of variance, and were transformed to normality when assumptions were not met.

Learning and memory assay

One fish from the enriched group (replicate two) was excluded from the analyses, as it did not reach the criterion level of performance even after 45 trials. The number of trials taken to reach criteria in phases one (Box-Cox transformed) and two (Box-Cox transformed) were analysed using general linear models (Raw data values can be found in table 4, appendix 2 (A.2.3.)). The number of trials taken to reach criterion in phase four were not normal and could not be transformed to normality, so were analysed using non-parametric Kruskal-Wallis tests. Maximal models, including rearing environment (enriched, non-enriched and wild), replicate, length, and tank number as factors were used. All interactions were tested for, and non-significant terms were removed in a step-wise manner to leave minimal models. Chi-square tests were used to determine if enriched, non-enriched and wild fish could return to the previously rewarded side after 3 and 7 days.

Temperament assays

One fish from the enriched group (replicate two) was excluded from the analyses, as it did not successfully complete the learning and memory assay. Temperament behaviours were measured in three different contexts, boldness, neophobia and activity. As there were two measures each for boldness and neophobia, principal components analyses (PCA) were run on the behaviours in each context (Raw data values can be found in table 4, appendix 2 (A.2.3.)). This resulted in a single measure for each behaviour, simplifying the analysis, and reducing the problem of multiple comparisons. For boldness, PC1 accounted for 67% of the variation in the data, with loading coefficients of 0.71 for average time to begin a foraging trial (boldness assay one) and 0.71 for time to emerge from a box (boldness assay two). The more positive the value, the longer a fish took to emerge from the box and begin the foraging trial (i.e. less bold fish). For neophobia, PC1 accounted for 77% of the variation in the data, with loading coefficients of –0.7 for time spent near the novel object, and 0.7 for time to approach the novel object. The more positive the value, the longer a fish took to approach the novel object, and the less time it spent near it (i.e. more neophobic fish). Separate general linear models were then run to determine the effect of rearing environment (enriched, non-enriched and wild), replicate, length, and tank number on activity, PC1 of boldness and PC1 of neophobia. All interactions were tested for, and non-significant terms were removed to leave minimal models.

5.4. Results

5.4.1. Learning and memory assay

Phase One – Acquisition: learning one compartment is rewarded

There was no effect of rearing environment (Fig.5.1.) but there was a significant main effect of replicate ($F_{1,27}$=14.80, P<0.001), on number of trials to learn phase one, with fish in replicate two learning significantly faster than those in replicate one (Figs.5.1., 5.2.).

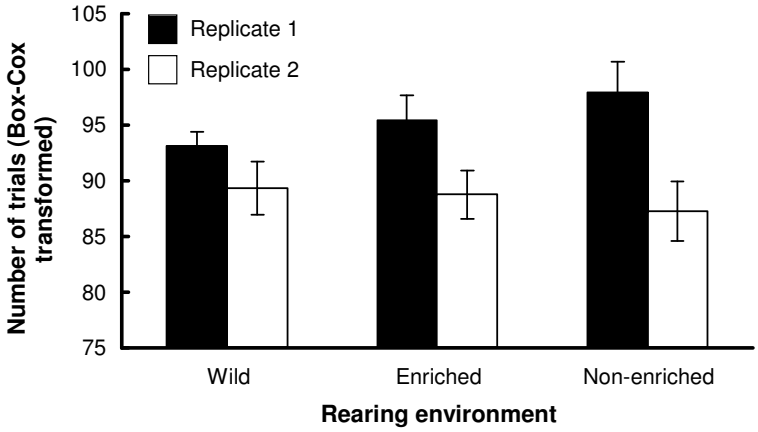

Figure 5.1. Mean number of trials to reach criterion performance (correct patch selection in 9/10 trials) in fish from different rearing environments in the two replicates of phase one. Error bars represent one S.E.

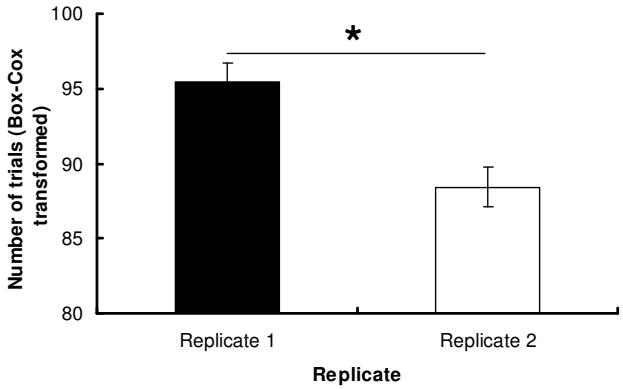

Figure 5.2. Mean number of trials to reach criterion performance (correct patch selection in 9/10 trials) in fish from replicates one and two. Bars connected by an asterisk are significantly different from one another ($P<0.05$). Error bars represent one S.E.

Phase Two – Acquisition: learning a different compartment is rewarded

Data were Box-Cox transformed to conform to meet the assumptions of normality.

There was no effect of rearing environment (Fig.5.3.) but there was a significant main effect of replicate ($F_{1,27}$=13.02, P=0.001) on number of trials to learn phase two, with fish from replicate one learning significantly faster than those from replicate two (Figs. 5.3., 5.4.).

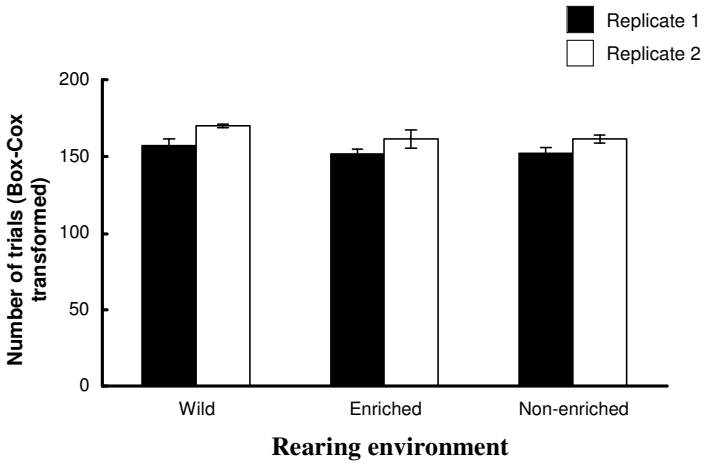

Figure 5.3. Mean number of trials to reach criterion performance (correct patch selection in 9/10 trials) in fish from different rearing environments in the two replicates of phase two. Error bars represent one S.E.

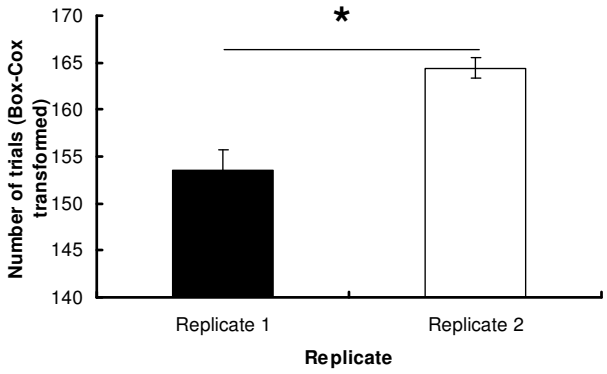

Figure 5.4. Mean number of trials to reach criterion performance (correct patch selection in 9/10 trials) in fish from replicates one and two. Bars connected by an asterisk are significantly different from one another ($P<0.05$). Error bars represent one S.E.

Phase Three – Memory retention: do the fish relocate the last rewarded compartment?

The ability of enriched, non-enriched and wild fish to return to the patch that had most recently been rewarded was compared after 3 days. Enriched (d.f.=1, Chi-square=9,

P<0.01) and non-enriched (d.f.=1, Chi-square=6.4, *P*<0.05), but not wild caught (d.f.=1, Chi-square=0.4, *P*>0.05) fish performed significantly above chance levels, indicating that enriched and non-enriched fish could return to a previously rewarded location after 3 days (Fig. 5.5.). Laboratory reared fish also performed significantly better than wild caught fish (Contingency table analysis: d.f.=1, Chi-square=5.54, *P*<0.01).

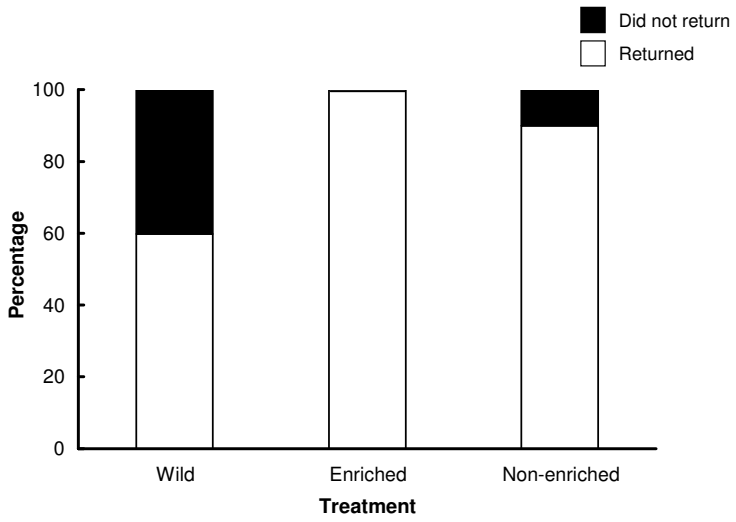

Figure 5.5. Proportion of wild, enriched and non-enriched fish returning to the patch rewarded in phase two after 3 days.

Phase Four – Acquisition: relearning a compartment is rewarded

There was no effect of rearing environment (Kruskal-Wallis d.f.=2, Chi-square=2.13, *P*=0.344) or replicate (Kruskal-Wallis d.f.=1, Chi-square=0.30, *P*=0.58) on number of trials to learn phase four.

Phase Five – Memory retention: do the fish relocate the last rewarded compartment?

The ability of enriched, non-enriched and wild fish to return to the patch that had most recently been rewarded was compared after 7 days. No treatment group performed above chance levels: enriched (d.f.=1, Chi-square=2.8, P>0.05), non-enriched (d.f.=1, Chi-square=1.6, P>0.05) and wild caught fish (d.f.=1, Chi-square=3.6, P>0.05).

5.4.2. Temperament assays

There was no significant effect of rearing environment (Fig. 5.6.), but there was a significant main effect of replicate on PC1 of boldness ($F_{1,27}$=4.42, P=0.045, Figs. 5.6., 5.7.), with fish in replicate one being significantly bolder. There were no significant effects on PC1 of neophobia ($F_{11,17}$=1.75, P=0.144) or activity in a novel environment ($F_{11,17}$=1.39, P=0.260) (Box-Cox transformed).

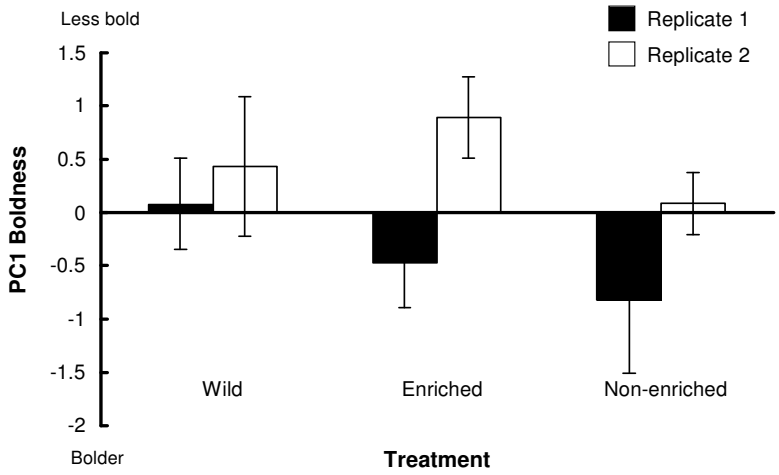

Figure 5.6. Principal component score of boldness behaviours in fish from different rearing environments in different replicates. Error bars represent one S.E.

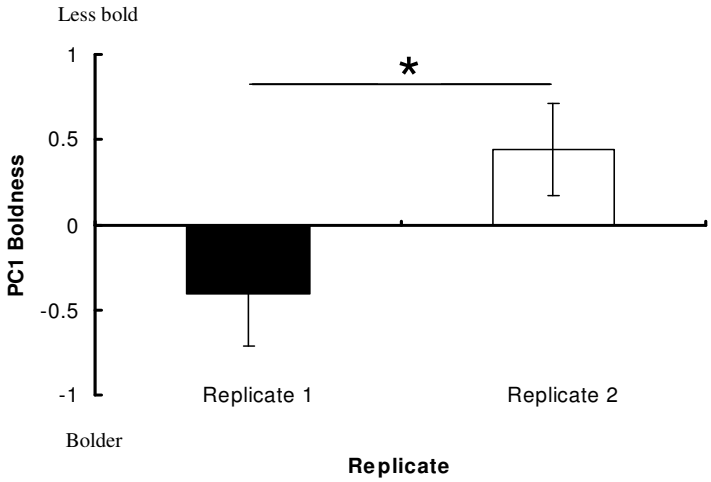

Figure 5.7. Principal component score of boldness behaviours in fish from replicates one and two. Error bars represent one S.E. Bars connected by an asterisk are significantly different from one another ($P<0.05$).

5.5. Discussion

5.5.1. Learning and temperament behaviours

There were no significant effects of rearing environment on learning ability. This is contrary to my initial hypothesis, which predicted that fish reared in wild and enriched environments would demonstrate an enhanced learning ability compared to those reared in plain environments. Numerous studies in other species have found that enriching the environment enhances neural growth and learning and memory ability (e.g. Woodcock & Richardson 2000, Leggio *et al.* 2005, but see de Jong *et al.* 2000). This is mainly thought be a product of enrichment stimulating cells and neurons to fire at one another more often, which in turn makes them more effective at firing, enhancing processes such

as learning and memory (van Pragg *et al.* 2000). There are several possible reasons to explain why there were no effects in the present study. Firstly, perhaps the enrichments provided were not suitable to stimulate the brain and enhance learning behaviour in the three-spined stickleback. My populations are of wild origin, and have spent only one generation in the laboratory, whereas the majority of rodent species tested originate from laboratory strains that have spent many generations in the laboratory, perhaps making them more sensitive to changes in the captive environment. A second possibility is that learning behaviours may have a stronger genetic influence in three-spined sticklebacks than in rodents, rendering them less sensitive to environmental variation. Although we may expect most behaviours to be the product of an interaction between genetics and environment (e.g. Girvan & Braithwaite 2000), certain behaviours do appear to have a stronger genetic component. For example, the migratory activity of bird species such as blackcaps (*Sylvia atricapilla*) is largely under genetic control (Berthold & Querner 1982), and anti-predator responses can also be largely genetically determined (e.g. Miklosi *et al.*1995, Veen *et al.* 2000).

Although there was no overall effect of rearing environment, there was an effect of replicate on learning rate in phases one and two of the learning and memory assay, with fish from replicate one learning phase one slower and phase two faster than fish from replicate two. Fish from replicate one were also bolder than those from replicate two, indicating that bolder fish learned phase one more slowly, and phase two faster. Various studies have found the opposite pattern to that found in phase one, with bolder individuals learning simple conditioning tasks faster than less bold conspecifics (e.g. trout (Sneddon 2003a) and guppies (Dugatkin & Alfieri 2003)). In these studies, fish

simply had to learn to associate food with a food ring placed on the surface of the water. Bold fish that are not afraid of approaching a novel food ring may have a distinct advantage in learning such a task. In contrast in the present study, fish had the more complicated task of encoding spatial location in order to find food patches. Perhaps in this situation, fish that are less bold, spend longer observing their environment and take a longer amount of time to make a choice have a learning advantage. This pattern is found in great tits, where less bold, more careful reactive individuals learn faster (Marchetti & Drent 2000), and also in Panamanian bishops solving a spatial foraging task, where less bold populations (Brown *et al*. 2005) learn faster than bolder populations (Brown & Braithwaite 2004). In the present study, this pattern is reversed in phase two, with the bolder fish learning faster. Perhaps by phase two, the bolder fish are paying more attention to their environment, and coupled with their boldness, this allows them to learn phase two faster than the less bold fish. Alternatively, learning and boldness may not be causally linked, and may both differ between replicate due to another, unidentified factor. This could include, for example, some unidentified effect of the laboratory environment that differed between the times of testing. If this is the case, it would suggest that learning and boldness behaviours are very sensitive to environmental variation in this species.

Similarly to learning, there were no effects of rearing environment on any temperament behaviour, with all three groups demonstrating similar levels of boldness, neophobia and activity. I expected that fish from the natural environment would exhibit the lowest boldness and activity levels and highest neophobia, as they had been reared with predators, in a relatively high predation environment (see Chapter 2, section 2.3.2

& 2.4.1.), where such behaviour would be adaptive in reducing the chances of being detected by a predator. Indeed, in natural systems, fish sampled from high predation sites often display greater anti-predator behaviours (e.g. three-spined sticklebacks: Giles & Huntingford 1984, guppies: Seghers 1974) than those from low predation sites. I also expected that enriched fish would demonstrate higher levels of boldness and activity, and lower levels of neophobia than non-enriched fish, as previous studies have found that enrichment tends to enhance these types of behaviours (e.g. Sherwin 2004, Braithwaite & Salvanes 2005, Fox *et al.* 2006). This is thought to be because increased exposure to novelty in more enriched environments (e.g. through the introduction of novel objects, novel arrangements of objects in the environment) should habituate animals to novelty, causing them to exhibit greater levels of behaviours such as boldness, activity and neophilia (Zimmermann *et al.* 2001). Similar explanations to those given above for learning may explain why fish from the different rearing environments do not differ in their temperament behaviours in the present study: in contrast to the majority of rodent studies, my populations have spent only one generation in the laboratory, and either temperament behaviours may have a stronger genetic influence in three-spined sticklebacks than in previously tested species, or they may be so sensitive to environmental variation that an unidentified third variable affected these behaviours between replicates. A way to test the influence of genetics versus environment would be to rear fry from different habitats (e.g. river and pond environments) to adulthood in the same conditions in the laboratory, and then test their behaviour.

5.5.2. Memory retention

There was a significant effect of rearing environment on ability to return to a previously rewarded location after 3 days (phase three of the learning and memory assay), with laboratory reared (enriched and non-enriched) but not wild fish returning to the patch. No group returned to a previously rewarded patch after 7 days (phase five of the learning and memory assay). Previous studies have found enrichment enhances memory (e.g. Paylor *et al.* 1992, de Jong 2000 *et al*.), hence I predicted that compared to non-enriched fish, those reared in wild and enriched conditions would exhibit a greater ability to return to a previously rewarded location. However, it appears that the laboratory environment promotes a greater propensity to return to a previously rewarded location, as laboratory reared fish were able to relocate a food patch after 3 days, whereas wild caught fish were not. Even in enriched tanks, the laboratory environment is likely to be less changeable than the natural environment. Non-enriched fish were used to being fed in the same location every day, and experienced minimal structural complexity, and even in enriched tanks where feeding location was varied, there were a limit of places that fish could be fed, and the structural complexity provided is unlikely to match that found in nature. Furthermore, the tanks in which the populations were housed in the laboratory were certainly smaller than the natural pond environment. Hence, fish in laboratory tanks are likely to possess a very accurate representation of their relatively small spatial environment. Perhaps the enhanced stability and predictability of laboratory life, with very little to learn about and remember, is the reason laboratory fish returned to the previously rewarded patch after 3 days, whereas the wild fish did not. Indeed, in the natural pond, these fish have to learn about many aspects of their

environment, for example predators, as the pond sampled is thought to be a high predation site (Chapter 2, sections 2.3.2 & 2.4.1.). The fact that no group returned to the rewarded patch after 7 days agrees with what has previously been found for pond three-spined sticklebacks (see Chapter 2, section 2.4.1.).

Unfortunately, due to small sample sizes, it was not possible to test for an effect of replicate on the ability of fish to return to a previously rewarded location. It seems unlikely that replicate is having an effect here because of the high proportion of fish returning to the previously rewarded location after 3 days in the enriched (100%) and non-enriched (90%) groups, and the low proportion returning (20-30% in all groups) after 7 days. Furthermore, where an effect of replicate was revealed, there was never an effect of rearing environment.

5.5.3. General discussion

Although the majority of previous studies have revealed that enrichment enhances learning, memory and temperament behaviours, I did not find this to be the case with three-spined sticklebacks. There were no effects of housing conditions on either learning or temperament behaviours, suggesting that in the three-spined stickleback, these behaviours may have a strong genetic influence. The rearing environment did, however, enhance the ability of laboratory reared individuals to return to a previously rewarded location, perhaps due to the enhanced predictability of laboratory life. This suggests that in contrast to learning and temperament behaviours, memory is more sensitive to environmental change. Previous studies have found that the environment can have marked effects on behaviour after just one generation, for example, locomotor

behaviours differed between juvenile salmonids raised in enriched versus plain tanks (Kihslinger & Nevitt 2006).

Perhaps the most striking result is the difference in learning and temperament behaviours between the two replicates. This could be due to an effect of temperament behaviours on learning, as bolder fish also learned phase one of the learning and memory assay slower, and phase two faster. This raises the question of what might maintain such variation in temperament behaviours in nature (see Wolf 2007 for a recent discussion), or if it is simply a product of laboratory rearing in the present experiment. Alternatively, it may be a product of a third, unidentified variable that differed between the two replicates and caused the differences in learning and boldness behaviours. This would suggest that learning and boldness behaviours are extremely sensitive to environmental variation. Further testing would be required to distinguish between these possibilities. It would also be interesting to determine how these same rearing environments (enriched, unenriched and natural) affect behaviour in three-spined sticklebacks originating from rivers, as chapter 2 revealed that memory differs between natural populations of three-spined sticklebacks originating from pond and river environments. Indeed, river fish returned to a previously rewarded patch after 7 days whereas pond fish did not, so perhaps the rearing environment may have a greater effect on memory in river fish. In terms of welfare requirements, the present study suggests that three-spined sticklebacks are at least sensitive to changes in their rearing environment (as their memory ability differed), and future studies should aim to determine how these changes may affect behaviours more directly related to welfare, for example stress responses.

Chapter 6. Variation in handling induced stress responses in three species of fish

6.1. Summary

A growing body of literature suggests that fish have sufficient cognitive capacity to experience pain and suffer. Our use of various fish species is extensive and increasing, and while considerable attention has been given to determining how our interactions with fish may impair welfare in aquaculture, little work has addressed the welfare of fish we maintain in research facilities. Stress induced by handling is likely to affect both behaviour and physiology in captive fish; hence I investigated the effects of two handling techniques on stress responses. Given that different species are likely to differ in their stress responses, I compared different handling methods across three species. Handling caused stress responses in three spined sticklebacks, Panamanian bishops and Rainbow trout, although handling with a scoop (a modified net which allowed fish to remain submerged in water) compared to a traditional dip-net significantly reduced these responses in three-spined sticklebacks and Panamanian bishops. Motivation and avoidance responses also differed between Panamanian bishops handled with nets and scoops. These results suggest that keeping fish in water in a scoop whilst transferring them between tanks can decrease the impact on stress responses in some fish species. These results show that handling techniques can affect stress, behaviour and laboratory performance in fish, and illustrate that these responses vary across different species.

6.2. Introduction

6.2.1. Fish welfare

Although a contentious issue, (e.g. Rose 2002, Braithwaite & Huntingford 2004, Chandroo *et al.* 2004a, Braithwaite & Boulcott 2007, Rose 2007) it has been argued that anatomical, pharmacological and behavioural data suggest that fish have sufficient cognitive capacity to experience pain and potentially suffer (e.g. Dunlop & Laming 2005, see Braithwaite & Huntingford 2004, Chandroo *et al.* 2004a,b for reviews). For example, research by Sneddon *et al.* (2003a), showed trout possess specialised receptors capable of detecting noxious stimuli, and that the administration of noxious chemicals affects trout behaviour and physiology in a manner consistent with the fish experiencing pain and discomfort. This type of empirical approach indicates that the experience of aversive or noxious stimulation in fish generates a complex suite of behaviours that are more than just associatively learned avoidance (see also Dunlop *et al.* 2005). Although it is misleading to equate such processes to the pain and suffering experienced by humans (see Boissy 1995, Griffin & Speck 2004, Paul *et al.* 2005 for reviews), current evidence seems to suggest that fish have a capacity for fear and suffering. It has therefore been suggested that fish should be afforded a welfare status similar to other vertebrates (Chandroo *et al.* 2004a, Huntingford *et al.* 2006).

Our use of fish has seen a dramatic increase in recent years, for example fish use in aquaculture has more than doubled over the past decade (FAO 2000). Multiple fish species are also used in scientific studies, kept as pets, or fished for sport. With such an extensive and increasing use of fish, it would seem timely that we determine what welfare requirements they have. Recent years have seen a growing interest in this area

(e.g. DEFRA 2002), and some guidelines do exist (e.g. DeTolla *et al.* 2001, Erickson, 2003, Nickum *et al.* 2004, CCAC 2005), but to date these are based either on mammalian guidelines (Borski & Hodson 2003) or on one or two commercially used species (e.g. Atlantic salmon). Fish are clearly very different from terrestrial vertebrates, and their welfare requirements are likely to differ considerably. Therefore the use of guidelines developed for terrestrial vertebrates will need modification before they can be usefully applied to fish (Huntingford *et al.* 2006). Further to this, fish are the most diverse group in the vertebrate phylum (Borski & Hodson 2003), and it is likely that requirements of different species will also vary (reviewed in Johansen *et al.* 2006). Thus, knowledge of species specific requirements would be useful not only in terms of welfare, but also in terms of productivity (commercial operations), and performance (scientific experiments). This is particularly apparent when considering the vast number of behavioural studies that are conducted on laboratory fish (Johansen *et al.* 2006). If natural behaviour of fish is under investigation, the most accurate results will be obtained if animals are living in conditions that promote natural behaviour.

6.2.2. Handling stress

A potential welfare issue for all captive fish is stress induced by handling. Previous studies have found that handling does appear to be stressful for a number of commercial fish species (e.g. increased cortisol levels in greenback flounder (*Rhombosolea tapirina*) (Barnett & Pankhurst 1998), coral trout (Frisch & Anderson 2000) and brown trout (Pickering 1982), see Portz *et al.* 2006 for a review)). At present, most laboratory and young commercial fish are caught by hand nets and experience some time out of the

water during handling. This method may have detrimental effects on the welfare of the fish in terms of elevated stress levels, oxygen deprivation and disruption to mucous coating and scales, potentially increasing susceptibility to parasitic and pathogenic attack (FSBI 2002, Conte 2004). A method of handling that allows the fish to remain submerged in water may mediate some of these problems and keep stress levels to a minimum. Hence, the aim of the present study was to investigate the effects of two handling techniques on stress and behavioural responses.

6.2.3. Aims

Experiment 1 investigates the hypothesis that handling with a darkened scoop (a net modified to hold water so the fish never leaves the water) will cause a lower stress response (measured by opercula beat rate) than handling with a traditional dip-net in three-spined sticklebacks, Rainbow trout and Panamanian bishops. These three species were chosen as they are phylogenetically diverse, and differ in numerous ways, from their habitats (cold, freshwater–Rainbow trout and three-spined sticklebacks; tropical-Panamanian bishops), to their human utility (commercial farming–Rainbow trout; scientific investigation–three-spined sticklebacks and Panamanian bishops). This allows an additional comparison into species differences in response to the same stressors.

In order to obtain an additional physiological measure of stress, experiment 2 was set up to compare plasma cortisol levels in net versus scoop handled three-spined sticklebacks.

As experiment 1 revealed an effect of handling method on the stress responses of three-spined sticklebacks and Panamanian bishops, experiment 3 was set up to

investigate how these two handling techniques affect behaviour in these species. Behavioural assays can test whether stress responses are purely physiological (i.e. cognitive performance is not affected) or if there is an indication of an additional psychological component to the stressor (i.e. higher order brain states are affected by the application of a particular stressor). The use of cognitive assays to gain an insight into animal psychological state has recently received attention, and promises to be a useful technique in assessing how animal welfare is impaired (Paul *et al.* 2005). In the present experiment, fish were screened to quantify their willingness to leave a start-box (which is a procedure typical for a number of behavioural assays of boldness, maze or foraging trials (e.g. Odling-Smee & Braithwaite 2003, Brown & Braithwaite 2004)), and their responses to a novel object after handling with a net and handling with a scoop.

6.3. Materials and methods

6.3.1. Experiment 1 – opercula beat rate increase and recovery time

6.3.2. Subjects and housing

Three species of fish were compared in this study: 23 mixed sex domesticated Rainbow trout, 23 mixed sex wild-caught three-spined sticklebacks and 23 female Panamanian bishops.

Rainbow trout were reared at the Niall Bromage Freshwater Research Facility, Stirlingshire, Scotland before their transferral to experimental apparatus in August 2005. During the course of the experiment, fish were housed in a flow through tank (2m long x 2m wide x 1m high) lined with a gravel substrate, and fed on a diet of fish food pellets for the duration of the experiment. The laboratory was maintained at ambient

temperature and light levels, and experiments were conducted in October 2005. Length ranged from 8 to 10cm. Adult three-spined sticklebacks were collected with minnow traps and long handled dip-nets from Craiglockhart Pond, Edinburgh ($3^0$14'W, $55^0$55N) in October 2005. They were housed in aquaria (76cm long x 30cm wide x 38cm high), furnished with plastic plants, a gravel substrate, biofilters and small upturned pots as refuges and fed on a diet of defrosted blood-worm for a three week settling period. Laboratory temperature was maintained on a day:night cycle at 14:9.5^{0}C, and light:dark cycle of 10:14h for the duration of the experiment. Length ranged from 2.9 to 5.7cm. Panamanian bishops were reared at the Kings Buildings, Institute of Evolutionary Biology, University of Edinburgh. These fish were the offspring of parents originating from four different populations in Panama: (i) River Limbo (RL) upstream, (ii) RL downstream, (iii) Quebrada Juan Grande river (QJG) upstream, and (iv) QJG downstream. Upstream populations were located above waterfalls, downstream below. Populations were housed in separate aquaria (92cm long x 29cm wide x 30cm high) furnished with plastic plants, a gravel substrate, biofilters and small upturned pots as refuges and fed on a diet of tropical flake fish food. Laboratory temperature was maintained at 32^{0}C, on a light:dark cycle of 12:12h for the duration of the experiment in October 2005. Length ranged from 2.9 to 4.3cm.

6.3.3. Apparatus

Trout and three-spined sticklebacks

A series of individual observation tanks (45cm long x 24cm wide x 26cm high (trout) and 20cm long x 35cm wide x 24.5cm high (three-spined sticklebacks)) were screened off from potential visual disturbance by opaque plastic sheeting, and continuously aerated by an airstone. Illumination was provided by a 60W tungsten lamp positioned behind the tank. A series of observation windows were cut into the plastic sheeting, allowing an observer to record the behaviour of the subject fish without disturbance.

Panamanian bishops

Due to the smaller size of this species, a slightly different protocol was followed. It was not possible to measure opercula beat rate by eye, so fish were isolated overnight in tanks (44.5cm long x 24.5cm wide x 26cm high), and then transported to a smaller holding container (6cm long x 6cm wide x 6cm high) via a net or scoop for filming from above. Both holding tanks and filming containers were covered with black plastic to avoid external disturbances to the fish.

6.3.4. Procedure

Trout and three-spined sticklebacks

Individual fish were moved to an observation tank one day prior to testing to allow them to settle in this novel environment. To assess responses to handling, opercula beat rate (OBR) was recorded. OBR is a commonly used measure to quantify stress levels in fish (e.g. Laitinen & Valtonen 1994, Sneddon *et al.* 2003b, Artigas *et al.* 2005) and is an easy, unobtrusive measurement to make without disturbing the fish (it is simply measured by counting the frequency of beats of the operculum). Basal OBR was

established by recording opercula beats for 1 minute (bpm), every 5 minutes, over a 30 minute period for each individual fish. Once basal OBR was recorded, one of two designated handling regimes was performed: *net handling*, where fish were lifted from the tank using a dip-net (12cm x 10cm) for a 5 second interval; or *scoop handling*, where fish were lifted from the tank using a net lined with opaque plastic (12cm x 10cm), which allowed them to remain in water during the 5 second interval. To ensure that the method of capture was comparable between the two experiments (and in all subsequent experiments), all fish were initially trapped in a large net before being quickly raised out of the water by one of the two handling treatments. Fish were allowed to return to individual basal OBR before the second treatment was applied. All fish received both treatments but the order was pseudorandomised, with half experiencing the net first, the other the scoop.

Following their return to the observation tank, fish were given an initial 2 minute period to settle. Subjects typically underwent a short period of strong swimming, precluding the accurate assessment of OBR immediately after returning to their tank. Thus, I used a 2 minute settling time before measuring OBR. Although the strong swimming would be expected to elevate OBR further, it was regarded as a response to the handling regime. Following the 2 minute settling period, OBR was recorded for 1 minute every five minutes until it dropped back to individual basal level. In addition to the recording of OBR, the time taken, to the nearest five minutes, for subjects to return to basal levels was recorded.

Panamanian bishops

Prior to the test, individual fish were allowed to settle in a tank (44.5cm long x 24.5cm wide x 26cm high) overnight. The next day they were either netted/scooped (pseudorandomised for each fish) into a smaller container (6cm long x 6cm wide x 6cm high), after being lifted outside the tank for 5 seconds. Fish were then recorded on a video camera for an hour, released back into the larger tank for another night, then transported to the filming container via the alternative method (net/scoop). Opercula beat rate was recorded from the video recording for 1 minute, every five minutes, for 1 hour. All fish received both treatments but the order was pseudorandomised, with half experiencing the net first, the other the scoop.

6.3.5. Experiment 2 – plasma cortisol levels

6.3.6. Subjects and housing

30 mixed-sex three-spined sticklebacks were collected from Craiglockhart Pond ($3^0$14'W, $55^0$55N) in February 2006 using minnow traps and long handled dip-nets nets. Fish were housed in aquaria (76cm long x 30cm wide x 38cm high) lined with gravel and furnished with plastic plants, refuges and a bio filter. Length ranged from 3.2 to 6cm.

6.3.7. Procedure – handling and sample collection

A series of tanks were set up (20cm long x 35cm wide x 24.5cm high). Fish were moved individually into these tanks ten days prior to testing to allow them to settle in this novel environment and to allow their cortisol levels to return to basal levels, as cortisol levels can remain elevated for 1-2 weeks after handling (e.g. brown trout: Pickering 1982,

Laitinen & Valtonen 1994; coral trout: Frisch & Anderson 2004). To assess response to handling method, plasma cortisol levels were assayed. Cortisol is a common measure of stress, with higher levels believed to indicate increased stress (e.g. Barton & Iwama 1991, Rotllant & Tort 1997, Wendelaar Bonga 1997, review in Barton 2002). Fish were assigned at random to one of three categories *Control*: fish were not handled. *Net handling*: fish were removed from the water for 5 seconds in a dip-net (12cm x 10cm) before being returned to the water. *Scoop handling*: fish were removed from the water for 5 seconds in a scoop (12cm x 10cm), then returned to the water. Fish were left for 90 minutes before being transferred quickly by net to a jar of MS222 where they were anaesthetized before being decapitated. Blood was then collected using 20 microlitre capillary tubes, and stored in eppendorf tubes. These were centrifuged at 13,000 rpm for 5 minutes at 4^0C, and the supernatant plasma was removed, placed in Eppendorfs and stored at -20^0C until the assay. Fish were left for 90 minutes before sampling because although plasma cortisol levels are believed to increase several minutes after a stressful event (e.g. Barnett & Pankhurst 1998, see Portz *et al.* 2006), levels often continue to increase for 1-2 hours, peaking at about 90 minutes in three-spined sticklebacks (Sebire *et al.* 2007).

6.3.8. Procedure – cortisol assay

Plasma samples were quantified by Tim Ellis, Cefas Marine Laboratories, Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB. Plasma cortisol concentrations were measured blind (without knowledge of treatment). Cortisol was extracted from the plasma samples using ethyl acetate, as described by Sebire *et al.*

(2007). Aliquots (5uL) of thawed plasma were transferred to 1.5mL Eppendorfs, and 100µl of distilled water and 1ml ethyl acetate were added. The liquids were vortex mixed (10s) and then centrifuged (13,000rpm for 3min). The aqueous phase was frozen by briefly placing the base of the Eppendorf in liquid nitrogen, and the ethyl acetate was separated by decanting. A further 1mL of ethyl acetate was added to the remaining aqueous fraction, and the mixing and separation repeated. The combined ethyl acetate extracts (2mL) were dried down under nitrogen at 45°C. The residue was re-dissolved in 500µL of buffer, and 100uL aliquots were assayed for cortisol using the radioimmunoassay described by Ellis *et al.* (2004).

6.3.9. Experiment 3 – motivation to emerge from start box and neophobia

This experiment investigated two behaviours often used to assess temperament behaviours: motivation to leave a shelter (commonly used as a measure of boldness) and neophobia. Methods were based on the temperament assays used in Chapter 3 (see section 3.3.3. for extended details of these assays). These assays were performed with three-spined sticklebacks and Panamanian bishops after handling with scoops and handling with nets. All fish received both treatments but the order was pseudorandomised, with half experiencing the net first, the other the scoop. Methods used for each species were identical. This experiment was not conducted with Rainbow trout because they appeared highly stressed by both net and scoop handling and reacted with equal increases in OBR to both methods. I therefore thought it unlikely that their behaviour would differ between the two methods of handling.

6.3.10. Subjects and housing

20 mixed-sex three-spined sticklebacks were collected from Craiglockhart Pond ($3^0$14'W, $55^0$55N) in February 2006 using minnow traps and long handled dip-nets nets. Fish were housed in aquaria (76cm long x 30cm wide x 38cm high) lined with gravel and furnished with plastic plants, refuges and a bio filter. Fish were fed on a diet of defrosted blood-worm for a three-week setting period before the experiment began. For Panamanian bishop housing refer to *subjects and housing* for experiment 1 (section 6.3.2.).

6.3.11. Motivation to leave shelter

Motivation to leave a shelter was assessed by timing how long it took a fish to emerge from an enclosed, darkened start box. Fish were isolated in individual tanks (44.5cm long x 24.5cm wide x 21.5cm high) overnight to settle before each experiment began. During an experiment, fish were transported in either a net or a scoop to the start box (10.5cm long x 11cm wide x 21.5cm high) that was located in a test tank (44.5cm long x 24.5cm wide x 21.5cm high) covered with black plastic to reduce outside disturbances. Fish were lifted from the water in either a net or a scoop for 10 seconds, used to reflect a typical handling time for such an experiment. An individual fish was then placed into the start box and left for a 2 minute settling period (a standard length of time in such experiments). After settling, a door (11cm wide x 24cm high) positioned in the centre of one wall of the box was raised remotely using a fine monofilament to leave an open doorway (6cm wide x 9cm high), and fish were timed until they emerged fully from the

box. All observations were made remotely via a video camera positioned above the tank to reduce disturbance to the fish. If a fish had not emerged after 10 minutes, it was given a maximum score of 600 seconds. Fish were then replaced into their individual tanks, left to settle for another night, and then given another trial after handling with the alternative method. All fish received both treatments (trial one and trial two) but the order was pseudorandomised, with half experiencing the net first, the other the scoop.

6.3.12. Neophobia

Neophobia was assessed by determining the amount of time a fish spent near a novel object. The day after boldness trials, fish began neophobia trials. After settling in individual tanks overnight (44.5cm long x 24.5cm wide x 21.5cm high), fish were lifted from their tanks for 10 seconds in either a net or a scoop and taken to a test tank (44.5cm long x 24.5cm wide x 21.5cm high) containing a novel object (this was a brightly coloured red and blue plastic toy fish, measuring 6cm long x 6cm wide x 1cm high). The test tank was divided into three equal sections by the use of marks on the edge of the tank, and the novel object was placed in the left section for half the fish, the right for the other half. Fish were initially placed into a clear plastic container located in the middle section of the tank to standardise the start location of each fish. This container was immediately gently removed remotely via a fine monofilament. Observations were made remotely via a video camera, filming from above, and the tank was covered in black plastic to avoid external disturbances to the fish. With the aid of marks along the bottom and up the side of the tank, the distance of the fish from the novel object was recorded every 20 seconds for 10 minutes, giving a total of 30 observations. Fish that spent a

larger proportion of time near the novel object were considered to be less neophobic. After the trial, fish were individually isolated overnight and given another trial after handling with the alternative method. All fish received both treatments (trial one and trial two) but the order was pseudorandomised, with half experiencing the net first, the other the scoop.

6.3.13. Data analysis

Data were tested for normality and heterogeneity of variance. Data were transformed to normality when assumptions were not met.

For experiment 1, results were analysed using repeated measures ANOVA, with treatment as a within subject factor, population (Panamanian bishops only) and trial order (i.e. net or scoop first) as between subject factors and length fitted as a covariate. Non-significant terms were removed in a step-wise manner to leave minimal models. Results from experiment 2 were analysed using an ANOVA to compare plasma cortisol levels (Box-Cox transformed, raw data values can be found in table 5, appendix 2 (A.2.4.)) in control, net and scoop handled fish, with treatment and length as explanatory variables. Results from experiment 3 were analysed using repeated measure ANOVA's, with length, population (Panamanian bishops only), and trial order (i.e. net or scoop first) as explanatory variables. Time to emerge from a refuge was transformed (Ln+1) in three-spined sticklebacks and Panamanian bishops (raw data values can be found in table 6, appendix 2 (A.2.4.)). Non-significant terms were removed in a step-wise manner to leave minimal models.

6.4. Results

6.4.1. Experiment 1 – OBR increase and recovery time

Trout

In trout, both handling methods induced a highly elevated OBR (97% net versus 94% scoop increase above basal level). There was no difference between net and scoop handling treatments in increase above basal OBR over time (RMANOVA: $F_{1,22}$=1.06, p=0.31) and no interaction between handling method and time, indicating that recovery was similar with both treatments (RMANOVA: $F_{13,10}$=1, p=0.53). OBR decreased significantly over time (RMANOVA: $F_{13,10}$=41.26, p<0.0001) (Fig.6.1.).

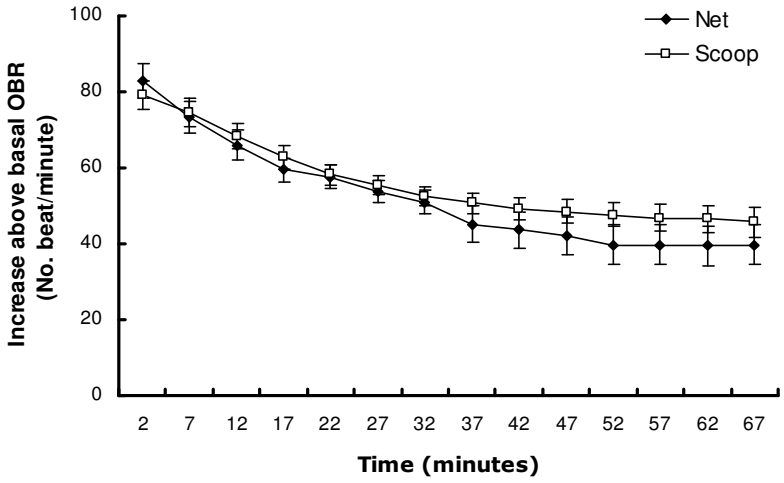

Figure 6.1. Increase above basal OBR after net and scoop handling, and subsequent decrease over time in trout. Error bas represent one S.E.

Three-spined sticklebacks

There was a tendency for net handling to cause a greater increase above basal OBR than scoop handling over time (RMANOVA: $F_{1,22}$= 3.22, P=0.08), and a separate RMANOVA revealed that maximum increase in OBR (OBR after 2 minutes) was significantly greater in net compared to scoop handled fish (65% net versus 52% scoop, RMANOVA: $F_{1,22}$=12.67, P<0.001). OBR also decreased significantly over time (RMANOVA: $F_{2.59,57.07}$=2.59, P<0.0001). Nevertheless, there was no difference in time taken to return to basal OBR between the two treatments, signified by the lack of treatment*time interaction (RMANOVA: $F_{3.6,79.3}$=1.62, P=0.18). P=0.74, Fig. 6.2.).

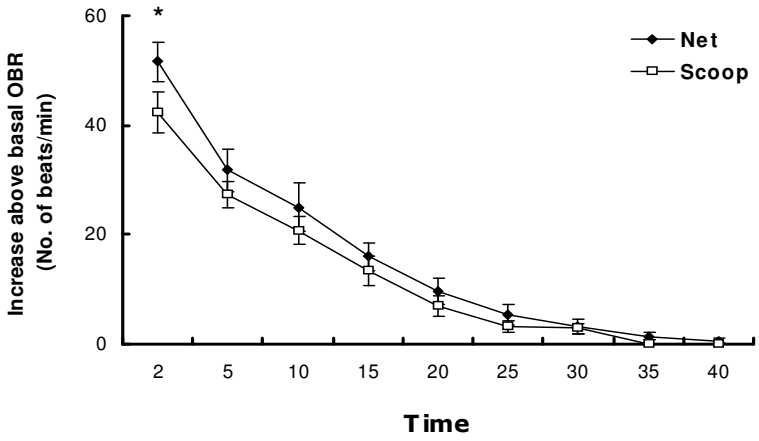

Figure 6.2. Increase above basal OBR after net and scoop handling, and subsequent decrease over time in three-spined sticklebacks. Error bars represent one SE. Bars connected by an asterisk are significantly different to one another.

Panamanian bishop

Net handling caused a significantly greater increase in OBR than scoop handling over time (RMANOVA: $F_{1,19}$=60.9, P=0.032). OBR also decreased significantly over time (RMANOVA: $F_{4.87,92.5}$=34.9, P<0.0001). Nevertheless, there was no difference in time taken to return to basal OBR between the two treatments, signified by the lack of treatment*time interaction (RMANOVA: $F_{3.35,63.3}$=1.64, P=0.18, Fig. 6.3.).

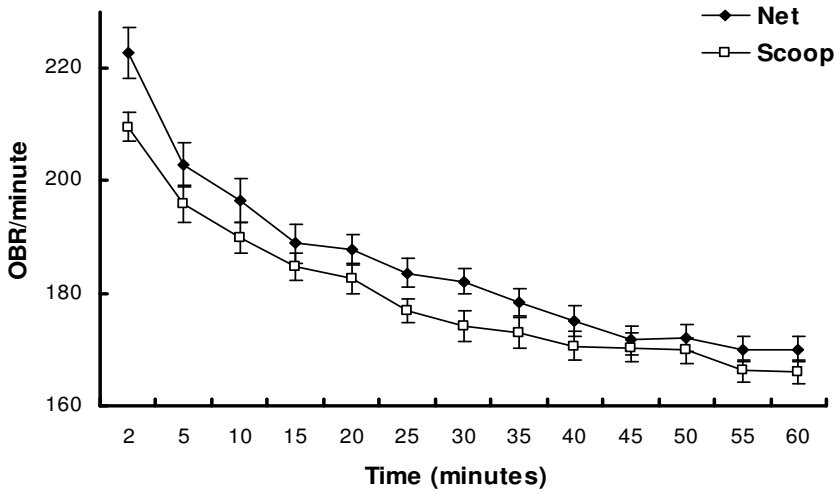

Figure 6.3. OBR in Panamanian bishops after net and scoop handling and subsequent decrease over time. Error bas represent one SE.

6.4.2. Experiment 2 – cortisol assay

Three-spined sticklebacks

There was a significant effect of handling on plasma cortisol levels (Box-Cox transformed, $F_{2,26}$=6.42, P<0.01). A post-hoc Tukey test revealed that plasma cortisol

levels were significantly higher in handled compared to control fish. Although there was no significant difference in plasma cortisol levels between scoop and net handled fish, average levels were higher in net handled fish (Fig. 6.4.).

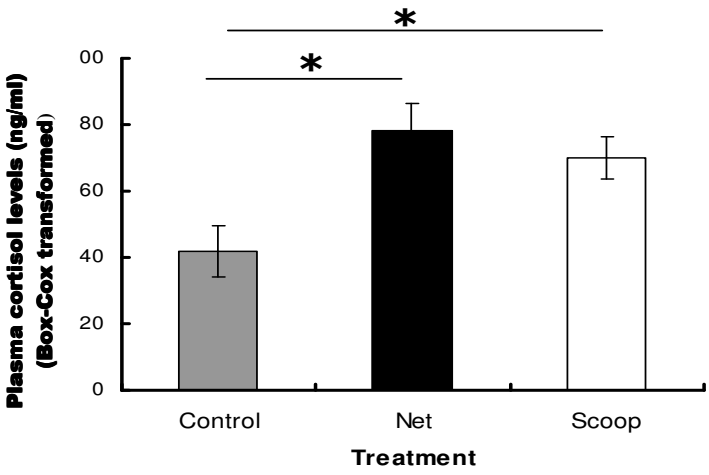

Figure 6.4. Box-Cox transformed plasma cortiosol levels (ng/ml) in three-spined sticklebacks handled with net, scoops and unhandled (control). Bars that are significantly different to one another are connected by an asterisk (P<0.05). Error bars represent one S.E.

6.4.3. Experiment 3 – motivation to emergence from start box and neophobia

Three-spined sticklebacks

There was no effect of handling method on Ln time to emerge from a start box ($F_{1,18}$=2.29, P=0.15, Fig. 6.5.a.). However, smaller fish emerged significantly sooner than larger fish ($F_{1,18}$=7.63. P=0.01). There were no significant effects on neophobia ($F_{2,17}$=0.95. P=.0.40, Fig. 6.5.b.).

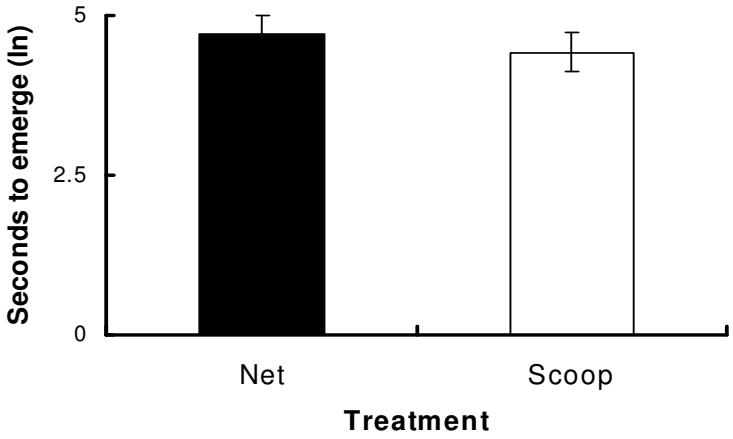

Figure 6.5.a. Seconds taken for three-spined sticklebacks to emerge from a start box after net and scoop handling. Error bars represent one S.E.

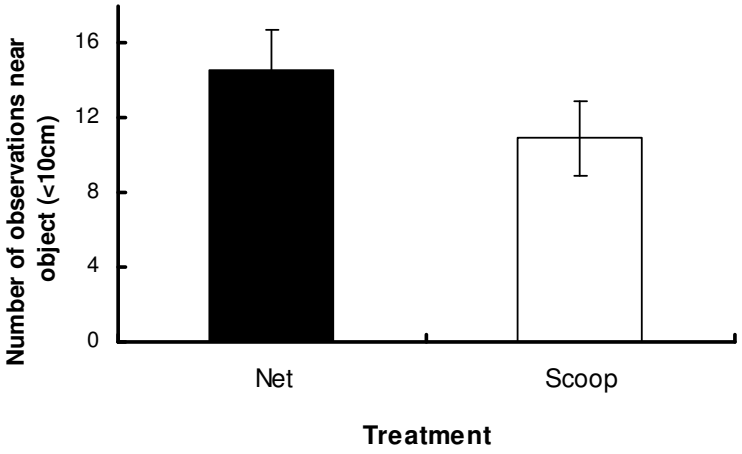

Figure 6.5.b. Number of times (/30) sticklebacks were observed <10cm away from a novel object after net and scoop handling. Error bars represent one S.E.

Panamanian bishops

As there was a significant effect of trial order (i.e. trial one affected trial two) on emergence times, only the first treatment for each fish was used in the following ANOVA. There was a significant main effect of handling method on Ln emergence time from a start box, with net handled fish emerging sooner than those handled with scoops ($F_{1,14}$=7.81, P=0.01, Fig. 6.6.a.). There was also an effect of length on emergence time ($F_{1,14}$=4.77, P=0.04) with smaller fish emerging sooner, and an effect population ($F_{3,14}$=4.09, P=0.02). A post-hoc Tukey HSD test revealed that fish from River Limbo downstream emerged significantly sooner than fish from River Limbo upstream.

As there was a significant effect of trial order on neophobia, only the first treatment for each fish was used in the following ANOVA. There was a significant main effect of population on neophobia ($F_{3,14}$=4.62, P=0.02). A post-hoc Tukey HSD test revealed that fish from River Limbo downstream spent a greater proportion of time near the novel object than fish from River Limbo upstream. There was also an effect of treatment ($F_{1,14}$=5.13, P=0.04) and length ($F_{1,14}$=4.70, P=0.048), with net handled fish and larger fish spending a greater proportion of time near the novel object (Fig. 6.6.b.).

(a)

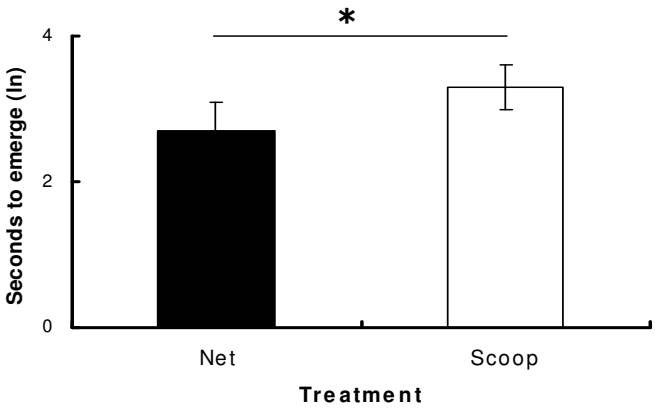

Figure 6.6.a. Seconds taken for Panamanian bishops to emerge from a start box after net and scoop handling. Error bars represent one S.E. Bars connected by an asterisk are significantly different to one another (P<0.05).

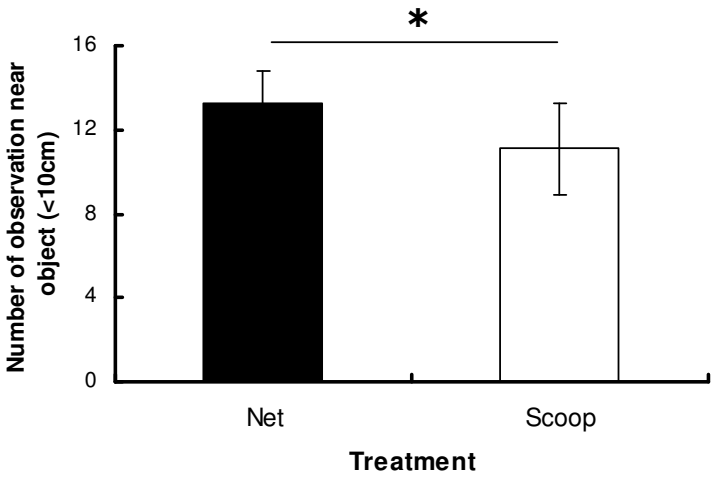

Figure 6.6.b. Number of times (/30) Panamanian bishops were observed <10cm away from a novel object after net and scoop handling. Error bars represent one S.E. Bars connected by an asterisk are significantly different to one another (P<0.05).

6.5. Discussion

Both handling techniques (scoop and net) caused an elevation of OBR in all three species (average increases: Rainbow trout: 97% net versus 94% scoop, three-spined sticklebacks: 65% net versus 52% scoop). Furthermore, plasma cortisol levels were significantly elevated in handled (net and scoop) three-spined sticklebacks compared to unhandled controls. This suggests that any form of handling causes an increase in stress levels (as measured by respiratory rate and cortisol levels). However, scoop handling resulted in significantly lower OBR elevation in sticklebacks and Panamanian bishops, lower OBR after 60 minutes recovery time in Panamanian bishops, and, although not significant, lower average cortisol levels in three-spined sticklebacks. Using OBR and cortisol levels as proxy measures of stress, there are several possible explanations for these results. Higher OBR and cortisol levels may be observed after netting due to purely physiological reasons - the removal of fish from water in a net probably causes elevated oxygen deprivation, which would increase OBR, and the release of corticosteroid stress hormones, such as cortisol. Increased swimming activity after handling may also add to the increase in OBR. It would therefore have been useful to include a treatment where the effects of increased swimming activity on OBR without handling were assessed. There may also be psychological (fear related) components to the elevated stress response – fish may find removal from water in a net more distressing than removal in a water filled scoop, which could again increase OBR and the release of stress hormones. The use of behavioural assays such as those used in experiment 3 can help to disentangle these two possibilities.

In experiment 3, Panamanian bishops, a typical tropical poecillid fish, emerged from a start box sooner and were less neophobic when handled with a net compared to a scoop. This suggests that the increased stress associated with netting is distracting the attention of the fish away from the threat of a novel environment and a novel object. This is similar to the results of a study on pain perception in trout (Sneddon *et al.* 2003a), which found that fish experiencing a painful stimulus show a less neophobic response to a novel object, presumably because their attention is diverted towards coping with the pain. In support of this conclusion, it was shown that fish experiencing pain showed increased neophobia again after a pain reliever was administered (Sneddon *et al.* 2003a). It is impossible to be certain that these are true psychological reactions, as it is not presently possible to directly determine or measure conscious experience in animals. However, the use of cognitive assays can give indirect measures, and the emotional state being experienced by an animal can be inferred (see Paul *et al.* 2005 for a review).

There was also an effect of length on emergence time in Panamanian bishops and three-spined sticklebacks, with larger fish taking longer to emerge. This relationship has been previously revealed in Panamanian bishops and is explained by a metabolic hypothesis, where smaller fish need to leave shelter earlier in order to feed (Brown & Braithwaite 2004). An effect of population was also found in experiment 3 in Panamanian bishops, with fish from River Limbo downstream (a high predation site) emerging from a refuge sooner spending and greater proportion of time near a novel object than fish from River Limbo upstream (a low predation site). This difference is consistent with other observations in these populations, which have also revealed that Panamanian bishops from high predation downstream sites emerge from a refuge sooner

than those from low predation upstream sites (Brown & Braithwaite 2004). Although this result seems to conflict with the hypothesis that high predation populations should be more cautious in order to avoid predators (e.g. Seghers 1974, Pitcher & Parish 1993), it has been explained as a result of high predation pressure forcing fish to behave relatively boldly in order to carry out activities such as foraging and reproduction (Brown & Braithwaite 2004).

Few studies have investigated stress responses to handling in laboratory fish (e.g. Artigas 2005), although handling has been found to induce stress related reactions in many commercial species of fish. For example, higher levels of cortisol are found in handled chinook salmon (*Oncorhynchus tshawytscha*) (Barton *et al.* 1986), coral trout (Frisch & Anderson 2000) and brown trout (Pickering 1982) (see Barton 2002, Portz *et al.* 2006 for reviews). Stress is generally thought to be detrimental to animals, but this may not always be the case. Stress can be an adaptive mechanism that allows animals to cope and maintain homeostasis (Barton 2002), and may even be beneficial (see Davis 2006 for a review). The problem occurs when stressors are prolonged or extreme, preventing the animal from coping and maintaining homeostasis (Barton & Iwama 1991, see Wendelaar Bonga 1997 for a review of stress in fish). Such stressors can have a variety of detrimental effects, including reductions in growth rate, disease resistance, reproductive capacity, normal behaviour and survival (see Barton 2002, Portz *et al.* 2006 for reviews). The stress experienced by fish during handling is a potentially serious issue, as it is a stressor that can be applied repeatedly. This may have serious welfare implications. For example, a recent study by Hoskonen & Pirhonen (2006) found juvenile Rainbow trout that were repeatedly handled had significantly reduced feed

intake and weight gain compared to an unhandled control. Similarly, coral trout exposed to capture, handling and transport stress had lower levels of cellular based immunity (Frisch & Anderson 2000). It is therefore important to identify sources of stress and determine methods of reducing them in order to safeguard fish welfare and, in the case of commercial operations, productivity. The results of experiments 1 and 2 demonstrate that net and scoop handling cause greatly elevated stress responses in three species of fish, but that the use of a scoop reduces the severity of this response in two species.

The results of experiments 1 and 3 also demonstrate how species can differ in their reactions to the same stressors. In experiment 1, in contrast to three-spined sticklebacks and Panamanian bishops, the difference in net/scoop response was not seen in Rainbow trout, with maximum OBR responses reaching equally high levels after net and scoop handling. A possible reason for this is that trout are easily stressed and regardless of handling method may always experience equally high levels of OBR. Indeed, brown trout can show signs of stress for several days after handling (e.g. Laitinen & Valtonen 1994), and a recovery period of 2 weeks has been suggested for complete recovery from 2 minutes of handling (Pickering 1982). Similarly, in experiment 3, handling method affected emergence times and neophobia in Panamanian bishops, but not three-spined sticklebacks. This suggests either that the elevated stress responses in three-spined sticklebacks handled with nets has a stronger physiological rather than psychological component, or that this species is better able to cope with elevated psychological stress.

These results contribute to a growing body of literature that illustrates species differences in response to identical stressors. For example, Jentoft *et al.* (2005) recently

found stressed Eurasian perch (*Perca fluviatilis)* experienced a greater loss in body growth than stressed Rainbow trout. Barton (2002) reports that species differ by more than two orders of magnitude in their corticosteroid responses (a measure of stress) after the application of an identical stressor. Recent years have seen a growing interest in the welfare requirements of fish, and have led to the development of some guidelines (e.g. DEFRA 2002, CCAC 2005). However, these guidelines do not always take into account species differences. The growing number of studies demonstrating that species differ in their stress responses suggests that existing welfare guidelines are likely to need modification, taking into account species specific requirements, before they can be appropriately applied to different fish species (Huntingford *et al.* 2006).

The results of experiment 1 show a handling method that allows three-spined sticklebacks and Panamanian bishops to remain submerged in water (scoop) does not generate the same elevated levels of OBR as net handling. Further to this, scoop versus net handling has a significant impact on the laboratory performance and behaviour of Panamanian bishops. Thus, handling Panamanian bishops and three-spined sticklebacks with water filled scoops will reduce stress-related reactions, and reducing these effects is likely to improve consistency and quality of behavioural observations (Artigas 2005). This has particular application in ensuring consistency of results between experiments, experimenters and laboratories. This is an area that has received considerable attention in the mouse literature, and the importance of standardizing procedures in order to obtain comparable results across laboratories has been emphasized (reviewed in Wahlsten *et al.* 2003).

In conclusion, the results of this study highlight the fact that issues such as routine handling techniques need to be reconsidered for laboratory fish, and future experiments should aim to determine if similar responses to handling techniques are observed in other fish species, particularly those used in scientific experiments, where there is a great paucity of information on welfare.

Chapter 7. General Discussion

7.1. Conclusions

The experiments in this thesis have revealed that ecological variables can affect learning, memory and orientation behaviours in three-spined sticklebacks, and that different ecological variables appear to interact when shaping such behaviours (Chapters 2-4). The behavioural assays from Chapters 2-4 were used to investigate how routine laboratory procedures affect fish behaviour and physiology in Chapters 5 and 6. The results of these experiments demonstrate that handling method and housing conditions can affect behaviour and stress in fish, and highlight the fact that different species can differ in their responses to the application of identical stressors.

7.1.1. Ecology, learning and memory

Several models propose that in a relatively stable environment, long-term memory will be advantageous (Hirvonen *et al.* 1999, Fortin 2002), whereas in a more rapidly changing environment, the value of more recent information should increase, favouring short-term memory (Cowie 1977, Eliassen, PhD thesis 2006). If the hypothesis that rivers and ponds differ in their spatially stability is true, then the results of Chapter 2 do not support this hypothesis, because fish from 4 river populations (thought to be less spatially stable environments) were able to return to a previously rewarded foraging patch after 7 days, whereas fish from 4 pond populations (thought to be more spatially stable environments) were not. This result contrasts a study on prey handling skills in

three and nine-spined sticklebacks, where fish originating from marine habitats where prey fauna was temporarily variable had a shorter memory duration for prey handling skills than fish from a pond environment where prey fauna was more thought to be more stable (Mackney & Hughes 1995). Compared to the results presented in Chapter 2, this indicates that spatial memory is affected in a different way to memory for prey handling, supporting the hypothesis that different memory systems may have different rules of operation, and be shaped in different ways by the environment (Sherry & Schacter 1987, Shettleworth 1998).

A suggested reason for the difference in spatial memory between the pond and river populations tested in Chapter 2 is that the river habitat may place greater demands on spatial ability in general. In a river habitat, there is a greater chance of fish being relocated to unfamiliar or unfavourable areas due to either the flow of the river or exploration. Here, a good and extensive spatial memory may be advantageous in relocating familiar or preferred areas. The same may not be true for pond fish living in a more enclosed environment. In particular, if food is plentiful, it may not be necessary to remember the positions of specific food patches. Animals that are assumed to have greater demands on their spatial ability in nature often demonstrate enhanced performance in laboratory tests. For example, males of the polygynous meadow vole compete for females over a large home range, where a good spatial ability is thought to be advantageous (Spritzer *et al.* 2005). These demands are not so great for females, and in spatial laboratory tests males perform better (Gaulin & Fitzgerald 1989).

In terms of determining how habitat stability *per se* affects memory duration, it would be interesting to try and sample populations from either pond or river habitats that

differ in their spatial stability. In particular, river habitats that differ markedly in their flow rate throughout the year are likely to differ in spatial stability and hence may provide a more valid test of the hypothesis that long-term memory will be advantageous in a more stable environment (Hirvonen *et al.* 1999, Fortin 2002), and short-term memory in a more changeable one (Cowie 1977, Eliassen, PhD thesis 2006). Comparing between pond and river habitats appears to be confounded by the general differences in structure between ponds and rivers – ponds are enclosed, rivers open. This may impact on memory duration in ways that obscures the true potential effects of habitat stability alone. Alternatively, fish could be artificially reared in environments of differing spatial stability, although it may be difficult to create substantially variable environments in the laboratory.

Consistent with previous observations, there was no difference in the rate at which pond and river populations learned the task presented in Chapter 2. However, there was an interaction with predation pressure: two river populations thought to be experiencing low levels of predation learned the task significantly faster than two river populations thought to be experiencing high predation. This may be explained by a divided attention hypothesis (e.g. Dukas 2002). This hypothesis supposes that animals must filter the continuous amounts of information they receive about their environment so that they can focus on those aspects most important to survival, and that dividing attention between numerous tasks will decrease the efficiency with which any one of those tasks can be performed (Dukas 2002). In a high predation river environment, fish may have many variables to pay attention to – predators and their own spatial location, for example. This may leave less attention to be directed towards learning about other

aspects of the environment, for example, foraging patches. Low predation river fish may not have to devote so much attention to predator vigilance, and pond fish do not have to pay so much attention to their spatial location, potentially allowing them to learn faster. Previous studies have found that an animal's ability to perform a task can be impaired when attention is simultaneously being focussed on other activities (see Dukas 2002 for a review on limited attention). For example, silver perch take only 5 trials to acquire maximum intake rates when offered a single prey type, but require 12-20 trials to converge on the most profitable prey type when offered two prey types (Warburton & Thomson 2005). Similarly, when participating in complex foraging tasks three-spined sticklebacks (Milinski 1984) and guppies (Krause & Godin 1996) are more vulnerable to predation, and are selectively predated. This may be due to their attention being divided between predator vigilance and foraging. A further example comes from a laboratory study with blue jays. Birds were less responsive to peripheral targets (which could be said to represent predators) when their attention was focussed on a difficult central task (supposed to represent foraging) (Dukas & Kamil 2000).

7.1.2. Ecology and temperament

Predation pressure and pond/river habitat appear to interact to shape temperament behaviours. Chapter 3 revealed that river populations thought to be experiencing high predation were less bold and less active than a pond population thought to be experiencing high predation. Previous studies have found that orientation behaviour differs between pond and river three-spined sticklebacks, and this is hypothesised to be because of differences in spatial stability between ponds and rivers. A similar habitat

stability hypothesis may explain why high predation pond populations are bolder than high predation river populations in Chapter 3. Hiding in a refuge may protect prey from predators, but there is a trade-off with other activities, such as foraging (Sih 1997). Compared to a river, in more spatially stable pond environments, refuges and landmarks indicating their location are likely to be more stable over time, so they can be rapidly relocated by fish. Furthermore, in a pond, prey might have a greater knowledge of the predator population, as it is likely to be more consistent over time than in a river, where predators can migrate through areas (see e.g. Moore 1998a,b). It is theoretically predicted that prey with poorer information about the local predation regime should remain in refuges for longer amounts of time (Sih 1992). River fish may also need to devote attention towards their spatial location, as there is a risk that they will become relocated to unfamiliar or unfavourable areas by exploratory behaviours or water currents. This may leave less attention to be devoted towards predator vigilance. So it is potentially less risky for a fish in a high predation pond to emerge from a refuge (i.e. be bolder) and resume other activities, such as foraging, than for a fish living in a high predation river environment.

River populations thought to be experiencing low predation were bolder and more active than river populations thought to be experiencing high predation. This makes sense in terms of avoiding predators in a high predation environment, as longer emergence times and lower activity levels will decrease the chances of meeting a predator in a high predation environment. Animals experiencing higher levels of predation often display enhanced anti-predator behaviour and morphology (e.g. three-spined sticklebacks, (Giles & Huntingford 1984, Bell 2005), guppies, (Seghers 1974,

O'Steen *et al.* 2002), Daphnia spp., (Fisk *et al.* 2007), larval anuran spp. (Relyea 2001) and Seychelles warblers, *Acrocephalus sechellensis* (Veen *et al.* 2000)).

In Chapter 3, I showed that temperament behavious were correlated within one high predation river population only (the River Biel). This correlation (both phenotypically and genotypically) was also found in another high predation but not a low predation river population of three-spined sticklebacks in a recent study (Bell 2005). According to the 'Constraints' hypothesis for the existence of behavioural syndromes, if traits are correlated within one population of a species then they must be correlated in all others, due to underlying constraints coupling those traits together. In contrast, the 'Adaptationist' hypothesis suggests that when correlations between traits occur it is because they are adaptive. Coupled with the results of Bell (2005), the results from Chapter 3 provide support for the 'Adaptationist' hypothesis. What factors might be important in causing traits to become correlated? The results of Chapter 3 and Bell (2005) both suggest that a high predation river environment may select for certain behaviours to become correlated. Further evidence that predation pressure may be important in causing correlations between behaviours comes from a recent laboratory study using three-spined sticklebacks originating from a low predation environment. Initially, there was no correlation between boldness and aggression in these fish, but exposure to and predation by an introduced trout induced such a correlation in the remaining fish (Bell & Sih 2007). However, in Chapter 3, no correlation was found in another high predation river population. Although this population did not appear to experience as greater predation pressure as the River Biel, it suggests that further work is

required before we can conclude that predation pressure is an important selective factor in causing behavioural correlations.

As correlations between temperament behaviours were found in one high predation river population only, it would be interesting to look for behavioural correlations in a wider range of populations, experiencing a greater diversity of predation pressure. Models concerning the evolution of behavioural syndromes are beginning to emerge (e.g. Wolf *et al.* 2007), and this field will benefit greatly from the development of more comprehensive models predicting when and why behavioural syndromes should be expected to occur.

7.1.3. Ecology and cue use

Many different animals are able to use geometry for orientation purposes, and it appears to be a basic, widespread ability. In particular, it has been well documented in birds and mammals (see Cheng & Newcombe 2005 for a review). There is now growing evidence that fish can also use geometry for orientation purposes (e.g. Sovrano *et al.* 2002, 2003, 2007). More recently, studies have begun to investigate how cues from multiple sources (e.g. geometry and landmark information) might be combined during orientation (Cheng & Newcombe 2005). Species sometimes differ in their ability to combine certain categories of cue, and it has been suggested that this is due to differences in ecology between those species (Sovrano *et al.* 2002). A more convincing test of this ecological hypothesis is gained by comparing populations of the same species living in contrasting habitats, and this was the aim of Chapter 4.

The results of Chapter 4 demonstrate that two populations of pond and two populations of river fish were able to use the geometry of a maze to locate an exit (Chapter 4, experiment 1). However, only the two river populations combined this information with non-geometric information to locate an exit (Chapter 4, experiment 2). The results of experiment 2 contrasted with initial predictions. As landmark cues are hypothesised to be relatively spatially stable in ponds, and based on previous work that demonstrates pond fish can use small discrete landmarks to navigate around a maze (Girvan & Braithwaite 1998, Odling-Smee & Braithwaite 2003), I predicted that pond fish would be able to combine geometry with a non-geometric landmark cue. In contrast, due to the hypothesised instability of landmark cues in river environments, and the fact that these fish largely ignore such cues in maze experiments, I predicted that river fish would be unable to combine these two cues. Why was the opposite result obtained? A suggested reason is the nature of the non-geometric cue used. The non-geometric cue presented in experiment 2, Chapter 4, was a blue wall with a cross shape left unpainted in the middle. This might be considered to be more of a global cue, for example, similar to the characteristics of a river bank (e.g. rock texture, colour or shape). This type of global cue is likely to be relatively stable and reliable over time, and may explain why river fish were able to use this cue to exit the maze. Global cues are also likely to be stable in pond habitats. However, pond fish may pay greater attention to more local landmarks (e.g. small plants and rocks) rather than global cues. In the ponds sampled for this study, it was rare to find fish close to the edges of the pond; rather they were caught in patches of vegetation some distance from the edges. In this situation, global cues such as the characteristics of the bank may have little relevance to orientation, and may be the

reason why pond fish could not combine the non-geometric cue with geometry. Previous studies have shown that the type of cue presented and the situation it is presented in can affect how much attention an animal pays to it. When trained with cues close to a target location, Clark's nutcrackers pay more attention to local cues, but when they are trained with cues that are further away they rely more heavily on global cues (Gould-Beierle & Kamil 1999). Also, when European jays relocate stored food items they prefer to use near landmarks compared to more distant cues (Bennett 1993).

My experiments showed that river three-spined sticklebacks were able to combine geometry with a global non-geometric cue to locate an exit in a maze, but pond fish were not. It has been shown in previous experiments that pond fish are able to use more local landmarks (for example, small plants) to navigate in a maze (e.g. Girvan & Braithwaite 1998, Odling-Smee & Braithwaite 2003, Braithwaite & Girvan 2003). It would be interesting then to compare the ability of pond and river fish to combine local landmarks with geometry during orientation. The results in this chapter also indicate that we now need to determine how different types of cues are categorised (e.g. global or local) and used by pond and river fish.

7.1.4. Environmental enrichment: learning, memory and temperament behaviours

Chapter 5 revealed that the rearing environment can affect behaviour. Three-spined sticklebacks reared in enriched and non-enriched environments were able to return to a previously rewarded location after 3 days, whereas wild caught pond fish were not. This contrasts with my initial hypothesis, which was that enriched and wild reared individuals

would exhibit a greater ability to return to a previously rewarded location. Numerous studies have revealed that memory is enhanced by environmental enrichment (e.g. Paylor *et al.* 1992, de Jong 2000 *et al.*). The reason that laboratory reared fish in Chapter 5 were better at returning to a previously rewarded location than the wild fish may be due to the small scale and enhanced predictability of laboratory life compared to the natural environment.

In contrast to the ability to return to a previously rewarded location, there was no effect of rearing environment on learning or temperament behaviours. Again, this contrasts my initial predictions, that fish reared in enriched and wild environments should exhibit greater learning ability, as has been found in rodents (e.g. Woodcock & Richardson 2000, Leggio *et al.* 2005), although not in pigs (de Jong *et al.* 2000). I also expected that wild reared fish would exhibit greater levels of neophobia, lower activity and lower boldness levels, because of being exposed to predators, and that enriched fish would demonstrate lower neophobia, greater activity and greater boldness than unenriched fish, as previous work has shown enrichment tends to enhance these types of behaviours (e.g. Sherwin 2004, Braithwaite & Salvanes 2005, Fox *et al.* 2006). There are several possible reasons why the results of Chapter 5 contrast with previous work (the majority of which has been with rodents). The three-spined sticklebacks used in Chapter 5 have spent only one generation in the laboratory. In contrast, rodents used in such studies have often been there for many generations. Spending many generations in the laboratory may increase sensitivity to changes in the laboratory environment. A second reason is that learning and temperament behaviours may be under a greater genetic influence than memory in the three-spined stickleback. Although we may expect

most behaviours to be the product of an interaction between genetic and environmental components (e.g. Girvan & Braithwaite 2000), some behaviours do have a strong genetic component (e.g. migratory activity in blackcaps (Berthold & Querner 1982) and some antipredator behaviours (Miklosi *et al.* 1995, Veen *et al.* 2000).

There was an effect of replicate on behaviour, with fish from replicate one learning the initial phase of a foraging task more slowly, a subsequent phase faster and also being bolder than fish from replicate two. This suggests that boldness may affect learning. Previous studies have found that bolder fish learn simple conditioning tasks faster (e.g. trout (Sneddon 2003) and guppies (Dugatkin & Alfieri 2003)). This is opposite to the pattern revealed in phase 1 of the learning task in Chapter 5. However studies by Sneddon *et al.* (2003) and Dugatkin & Alferi (2003) simply required fish to make an association between food and a food ring. In this situation, bold fish that are not afraid to approach and explore the ring may gain a learning advantage. In contrast, the fish in Chapter 5 had the more complicated task of encoding spatial information in order to locate a foraging patch. Perhaps in this situation, less bold fish learn faster initially because they pay more attention to their environment. This is also found in populations of great tits and Panamanian bishops (Brown & Braithwaite 2004, Brown *et al.* 2005) where more careful, reactive individuals learn faster (Marchetti & Drent 2000). This pattern is reversed in phase two, with bolder fish learning faster. It is possible that by phase two, bolder fish had learned to pay attention to the task, and coupled with their boldness, this allowed them to learn phase two faster than the less bold fish. Alternatively, boldness and learning may not be causally linked, and may both differ between replicates due to an unidentified third environmental variable. If this is the case,

it would actually suggest that learning and boldness are very plastic behaviours that are extremely sensitive to environmental variation.

The rearing environment then can affect fish behaviour, but it may also have substantial effects on stress. It would be interesting to investigate this by comparing, for example, the basal opercula beat rate and cortisol levels of fish reared in different environments.

7.1.5. Handling stress

The first experiment in Chapter 6 revealed that handling is highly stressful for three species of fish (three-spined sticklebacks, Panamanian bishops and Rainbow trout), as measured by opercula beat rate and cortisol (in three-spined sticklebacks). However, handling with a darkened, water filled scoop is less stressful (as measured by opercula beat rate and indicated by cortisol level in three-spined sticklebacks) than handling with a traditional dip-net for two species of fish, the three-spined stickleback and the Panamanian Bishop. These differences may be purely physiological, for example due to oxygen deprivation caused by removal from the water. There may however be a psychological component to the elevated stress responses, with fish finding net removal more distressing. The use of behavioural assays in experiment 3, Chapter 6 suggests that the response may be partly psychological in Panamanian bishops, as they were faster to leave a shelter and less neophobic when handled with a net compared to a scoop. This suggests that the attention of the fish is diverted away from the threat of a novel object and novel environment and towards coping with elevated stress levels after net compared to scoop handling. A similar observation was made with trout experiencing a

noxious stimulus, as these fish also showed a decreased response to a novel object (Sneddon *et al.* 2003a). Although it is presently impossible to directly measure psychological state in any animal, the use of cognitive assays such as neophobic response can give indirect measures (see Paul *et al.* 2005 for a review).

The results of Chapter 6 also highlight the fact that species of fish differ from one another in their stress responses. Net handling caused a significantly greater opercula beat rate in three-spined sticklebacks and Panamanian bishops, but not in Rainbow trout, and net handling affected behaviour in Panamanian bishops but not three-spined sticklebacks. This is in agreement with a growing body of work demonstrating that different species of fish display different responses to the application of identical stressors, and highlights the fact that fish requirements are likely to differ from species to species (reviewed in Barton 2002, Jentoft *et al.* 2005, Huntingford *et al.* 2006).

7.2. Concluding remarks

The results presented in this thesis demonstrate that cognitive behaviours differ between populations of three-spined sticklebacks. The behavioural plasticity exhibited by this species is undoubtedly one of the reasons they have expanded so successfully since the retreat of the last ice age to occupy a vast diversity of habitats throughout the Northern hemisphere. This thesis also demonstrates the utility of cognitive behavioural assays in determining the effects of routine laboratory procedures on behaviour and stress, in particular highlighting the fact that different species of fish can differ in their responses to identical stressors. This is not surprising, as species of fish differ from one another in

numerous ways, but these differences are often overlooked. However, they need to be considered when devising guidelines and legislation for fish welfare as species will differ in their requirements. There are still many unanswered questions about how the environment and routine laboratory procedures can affect behaviour and stress. The three-spined stickleback remains a useful system in which to investigate these types of questions.

Appendices

A.1. A one-year survey of ecological parameters of the three-spined stickleback habitats used in this thesis

A.1.1. Water chemistry

Table 1. Water chemistry of three-spined stickleback habitats used in this thesis. Temperature, ammonia, nitrite and nitrate were measured on site. Water clarity was determined using a secchi disk.

Site	Season	Temperature (^{0}C)	Water Clarity	Ammonia	Nitrite	Nitrate
Craiglockhart Pond	Spring	6.9	100%	0	0	0
	Winter	2	50%	0	0	0
	Summer	15.6	100%	0	0	0
Balmaha Pond	Spring	6	100%	0	0	0
	Winter	1	100%	0	0	0
	Summer	11	100%	0	0	0
Beecraigs Pond	Spring	6.4	100%	0	0	0
	Winter	3	95%	0	0	0
	Summer	17	95%	0	0	0
North Belton Pond	Spring	7.6	100%	0	0	0
	Winter	2	0%	0	0	0
	Summer	19	95%	0	0	0
River Biel	Spring	7.6	100%	0	0	0
	Winter	1	100%	0	0	0
	Summer	12.5	100%	0	0	0
River Esk	Spring	7.4	100%	0	0	0
	Winter	1.5	75%	0	0	0
	Summer	11.7	100%	0	0	0
River Water of Leith	Spring	6.8	100%	0	0	0
	Winter	2.5	50%	0	0	0
	Summer	18.8	100%	0	0	0
River Endrick	Spring	7.3	100%	0	0	0
	Winter	1.5	100%	0	0	0
	Summer	14.6	100%	0	0	0

A.1.2. Physical parameters

Table 2. Physical parameters of three-spined stickleback habitats used in this thesis. Flow was measured on a comparative scale (1-5), with 1 representing slow flowing, 5 fast flowing. Flow values shown are averages of summer, spring and winter measurements. Electrical conductivity (μS/cm) is a measure of water purity, with lower values indicating purer water.

Site	Flow	Electrical conductivity (μS/cm)	Perimeter/width (metres)
Craiglockhart Pond	0	476	220
Balmaha Pond	0	112	150
Beecraigs Pond	0	359	163
North Belton Pond	0	345	306
River Biel	2	345	7
River Esk	1.5	228	10
River Water of Leith	3	261	25
River Endrick	1.5	187	17

A.1.3. Substrate

Ponds: The substrate of Craiglockhart Pond is boggy and peaty, with the presence of a few large rocks (>30 cm diameter) and a few small stones (<30 cm) diameter. Balmaha Pond has lots of small stones (<30 cm) and much spongy vegetation. North Belton Pond is very peaty and boggy, with a few large (>30 cm) rocks. Beecraigs Pond is fairly boggy and peaty, with a few large rocks (>30 cm). These are likely to be spatially stable habitats as there is no flow to move substrates around.

Rivers: The River Esk is fairly sandy and muddy, with a few large logs, many small (<30 cm) stones and much gravel. Similarly, the River Biel has many small stones (<30 cm) and gravel. The River Endrick has some small (<30 cm) and some larger (>30 cm) rocks, whereas the River Water of Leith has fewer small stones (<30 cm) and more large ones (>30 cm). The presence of smaller stones and in particular gravel substrate of Rivers Esk and Biel suggests that these two habitats are likely to be less spatially stable

than ponds as flow can easily move this substrate downstream. Rivers Endrick and Water of Leith may be more stable as their substratum is comprised of larger stones, but the smaller stones are still likely to be moved by current making these habitats comparatively less stable than ponds. Flow is similar in all river habitats.

A.1.4. Vegetation

Ponds: Craiglockhart Pond is full of dense aquatic vegetation during the spring and summer, including water lilies and reeds. Much vegetation remains during the winter, although it is considerably less than in the spring and summer. There is always plenty of shelter for fish and fry in this pond. Balmaha Pond is full of pond weed, particularly during the spring and summer. Many trees and plants surrounded this pond around 75% of its perimeter. North Belton Pond has lots of areas of dense pond weed, particularly in the spring and summer. This pond is surrounded by grass and trees around its entire perimeter. Beecraig Pond is full of leaves, plus grass and a few large logs.

Rivers: The River Esk has little vegetation in the water, aside from small amounts of grass and decomposing leaves in autumn. There are many large logs in this river. The surrounding bank is full of overhanging trees and shrubs, plus flowers and grass, which are denser in the spring and summer. The River Biel has little vegetation in the water, apart from small patches of grass and weeds, which expand in the spring and summer. The surrounding bank contains grass and small flowers on one side, and overhanging trees, shrubs and plants on the opposite side, denser in the spring and summer. The River Endrick has a small amount of grass in the water, and dead leaves in autumn. Otherwise, vegetation within the water is sparse here. The banks of this river

are flat, containing grass, small flowers and the occasional tree. The River Water of Leith has small amounts of grass in the water by the bank. Vegetation is dense on the banks, including much grass, plants, overhanging trees and shrubs.

A.2. Raw data values

A.2.1. Chapter 2

Table 1. Morphometric measurements in 8 populations of three-spined sticklebacks.

Population	Old/New Samples	Body Length (mm)	Gape Width (mm)	Body Depth (mm)	1st Dorsal Spine (cm)	2nd Dorsal Spine (cm)	Lateral Plate Number	Pelvic Spine Length (cm)	Pelvic Girdle Length (cm)
Balmaha Pond	New	42.9	3.1	10.1	2.6	3.1	3.1	4.7	9.9
Balmaha Pond	New	50	3.4	10.8	2.8	3.3	3.3	5.4	10
Balmaha Pond	New	39.8	3.2	8.6	2.7	2.9	2.9	4.4	8.6
Balmaha Pond	New	43.4	2.8	10.5	2.9	3.4	3.4	5	10.7
Balmaha Pond	New	43	2.6	9.6	2.8	3.3	3.3	5	10.1
Balmaha Pond	New	38.8	2.6	7.7	2.3	2.9	2.9	4	7.2
Balmaha Pond	New	41	2.7	8.4	2.5	3	3	4.5	8.8
Balmaha Pond	New	39.7	2	8.6	3	3.2	3.2	4.5	8
Balmaha Pond	New	35.7	2.4	7.9	2.1	2.5	2.5	4.3	7
Balmaha Pond	Old	44.2	3.6	9.6	2.6	3	3	3.8	9
Balmaha Pond	Old	42	3.6	9.9	2.2	2.5	2.5	4.4	9.1
Balmaha Pond	Old	42	2.9	9.9	2.7	3	3	5.1	8
Balmaha Pond	Old	46.1	3.9	11	2.1	2	2	3.4	8.4
Balmaha Pond	Old	44.7	3.4	9.9	2.2	2.7	2.7	4.1	9.7
Balmaha Pond	Old	50.4	3.1	12.1	2.6	2.9	2.9	4.5	11.4
Balmaha Pond	Old	43.6	3	11.1	2.5	2.9	2.9	4.5	10.5
Beecraig Pond	New	34.8	2.5	9.4	2.7	2.8	2.8	4.6	7.6
Beecraig Pond	New	37.8	3.5	9.7	2.8	3.5	3.5	5.6	8.2
Beecraig Pond	New	38.5	3	9.1	2.5	3.2	3.2	4.8	7.8
Beecraig Pond	New	33.8	2.5	2.9	2.1	2.6	2.6	4.1	6.9
Beecraig Pond	New	36.3	2.3	9.1	2.5	2.9	2.9	4.6	7.6
Beecraig	New	38	2.4	9.6	3.5	3.7	3.7	5.7	8.9

Pond									
Beecraig Pond	New	40.3	2.6	10.3	2.8	3.4	3.4	5.1	10
Beecraig Pond	New	48	3.3	11.2	3.4	4	4	6.3	10.3
Beecraig Pond	New	39.5	2.8	9.5	2.4	2.9	2.9	4.4	9.1
Beecraig Pond	New	35.9	2.3	8.6	1.9	2.2	2.2	4.1	7.6
Beecraig Pond	New	40.7	2.9	9.9	2.5	3.3	3.3	4.6	9.3
Beecraig Pond	Old	36.5	2.4	8.8	2.9	3.1	3.1	5.6	8.4
Beecraig Pond	Old	40.8	1.8	9.2	3.5	4.3	4.3	5.6	8.8
Beecraig Pond	Old	39.7	2.6	8.9	2.5	2.8	2.8	4.7	8.7
Beecraig Pond	Old	41.7	3.2	10.4	2.4	2.7	2.7	4.9	8.7
Beecraig Pond	Old	55.4	4.2	14.7	4	2.9	2.9	6.6	14.4
Beecraig Pond	Old	41.8	3	10.1	2.4	3.2	3.2	5.1	9.7
Beecraig Pond	Old	39.1	3.5	9.7	2.5	3	3	5	8.5
Beecraig Pond	Old	40.8	2.9	9.8	2.8	3.4	3.4	5.1	9.9
Beecraig Pond	Old	43.2	3	10.6	3.2	3.8	3.8	6	9.8
Beecraig Pond	Old	43	3.6	11.8	2.4	1.6	1.6	4.6	9
River Biel	New	41	3	10.4	2.8	3.5	3.5	5.7	9.3
River Biel	New	37	3	9.5	2.6	3	3	4.7	7.6
River Biel	New	37.5	2.3	9.5	2.5	2.7	2.7	4.3	7.7
River Biel	New	37	2.2	9.5	2.4	2.8	2.8	4.3	8.2
River Biel	New	40.4	2.6	10.5	2.8	3.4	3.4	5	8.5
River Biel	New	39.1	2.4	9.6	2.7	3.4	3.4	5.6	8.4
River Biel	New	36.6	2.1	9.3	2.4	2.7	2.7	4.5	7.9
River Biel	New	40.4	2.2	10.2	2.4	3	3	5.1	9
River Biel	New	41.6	2.7	10.9	3	3.4	3.4	5.7	9.1
River Biel	Old	43.8	2.2	11.1	3.5	3 .6	3.6	5.6	10.6
River Biel	Old	32.3	2.6	7.9	2.7	3.2	3.2	5	8
River Biel	Old	46.4	2.7	10	2.6	3.1	3.1	5.2	9.7
River Biel	Old	34.4	2.4	8.3	1.8	2.1	2.1	4.2	7.8
River Biel	Old	34.2	1.7	8.1	2.5	3	3	4.7	7.6
River Biel	Old	36.5	2.1	8.6	2.6	3	3	5.5	8
River Biel	Old	32.3	1.7	7.6	2.3	2.8	2.8	4.6	7.7
River Biel	Old	46.5	3.1	11.4	3.2	3.4	3.4	5.7	10.2
Craiglockhart Pond	New	47	2.8	10.7	2.3	3.3	3.3	5	10.7
Craiglockhart Pond	New	55	3.2	13.9	3.7	4.1	4.1	6.9	3.5

Craiglockhart Pond	New	48.4	3.1	13.4	3.9	4.5	4.5	6.1	11.7
Craiglockhart Pond	New	48.3	2.9	12.9	3.7	4.4	4.4	6.6	11.5
Craiglockhart Pond	New	47.6	2.6	12.8	2.5	3.9	3.9	6.1	10.9
Craiglockhart Pond	New	60.6	4.3	15.7	2.8	3.4	3.4	5.4	13.9
Craiglockhart Pond	New	49	3.3	12.5	2.8	3.5	3.5	5.3	10.5
Craiglockhart Pond	New	46	2.9	11.9	2.5	3.2	3.2	4.9	10.4
Craiglockhart Pond	New	43.3	2.6	11.8	2.6	3	3	5	10
Craiglockhart Pond	Old	44.8	3.5	10.9	2.8	2.3	2.3	6	11.2
Craiglockhart Pond	Old	48.8	3.7	11.3	3	3.5	3.5	5.4	9.8
Craiglockhart Pond	Old	53	3.3	13.2	3.4	4.2	4.2	6	13.2
Craiglockhart Pond	Old	53.6	3.2	13.4	3.8	4.3	4.3	6.2	12.5
Craiglockhart Pond	Old	49.7	3	11.8	3.2	3.3	3.3	5.5	10.9
Craiglockhart Pond	Old	50	3	12.9	3.4	3.9	3.9	5.6	11.7
River Endrick	New	31.2	2.9	7.9	1.8	2.1	2.1	3.4	6.7
River Endrick	New	37.6	2.4	9.4	2.6	2.7	2.7	4	9.1
River Endrick	New	36.5	2.3	9.4	2.4	2.4	2.4	4.6	7.9
River Endrick	New	37	2.5	9.5	2.5	2.7	2.7	4.7	7.7
River Endrick	New	35.8	2.2	8.4	2.2	2.5	2.5	4.3	7.6
River Endrick	New	38.4	2.4	9.7	2.8	3.3	3.3	4.8	8.4
River Endrick	New	35.8	2.7	9.4	2.5	2.7	2.7	4.5	7.8
River Endrick	New	40.1	2.2	9.9	2.2	2.8	2.8	4.4	9.4
River Endrick	New	35.9	3.4	9.3	2.5	2.7	2.7	4.5	7.9
River Endrick	Old	46.1	3.6	10.8	2.9	2.2	2.2	4.7	9.4
River Endrick	Old	37.6	1.7	8.2	2.9	3.8	3.8	5.4	7.8
River Endrick	Old	49.4	3	13.6	2.9	3.7	3.7	5	3.4
River Endrick	Old	46	2.5	13.7	3.7	4.6	4.6	5.7	11.5

North Belton Pond	New	43.1	2.8	10.8	2.5	3.3	3.3	4.7	8.6
North Belton Pond	New	46.3	3.2	12.2	2.9	3.3	3.3	4.7	10.4
North Belton Pond	New	41.5	3	9.8	2.8	3.3	3.3	5.2	9.3
North Belton Pond	New	39.5	2.9	9.5	2.9	3.4	3.4	4.5	7.8
North Belton Pond	New	41.9	2.7	9.2	3.1	3.7	3.7	5.3	8.6
North Belton Pond	New	37.1	2.6	8.8	2.5	2.8	2.8	4.1	7.2
North Belton Pond	New	51	2.9	12.6	3	3.5	3.5	5.9	11.6
North Belton Pond	New	41.7	2.6	10	3	3.5	3.5	5.4	9.4
North Belton Pond	Old	50.5	2.8	13.6	3.4	3.8	3.8	6.2	12.6
North Belton Pond	Old	44.2	3.4	10.7	2.5	3	3	4.5	7.6
North Belton Pond	Old	48.8	3.4	12.8	3	3.4	3.4	5.5	12
North Belton Pond	Old	37	2.7	9.3	1.9	2.5	2.5	4.5	8.5
North Belton Pond	Old	49	3.6	13.2	3.2	3.9	3.9	6.4	12.3
North Belton Pond	Old	39.2	1.8	9.9	2.7	2.3	2.3	5.4	8.7
North Belton Pond	Old	39.4	3	9.1	2.2	2.8	2.8	4.5	8.9
River Esk	New	45.3	3.1	11.3	3.7	3.8	3.8	6.7	9.3
River Esk	New	43.7	2.5	10.9	2.8	3.2	3.2	5	10
River Esk	New	44.3	2.6	11.6	3.8	3.8	3.8	6.3	9.3
River Esk	New	45.8	2.2	10.8	3.2	3.3	3.3	5	7.8
River Esk	New	42.7	2.9	10.5	3.3	3.6	3.6	5.3	9
River Esk	New	46.8	3.4	12.7	3.4	4	4	5.5	11.4
River Esk	New	42.9	2.7	10.5	2.9	3.3	3.3	4.7	9.5
River Esk	New	45.2	3	10.9	3.4	3.8	3.8	6	9.3
River Esk	New	47.7	4.1	12.3	3.4	3.7	3.7	6.2	9.9
River Esk	New	39.5	2.7	10.1	3.4	3.7	3.7	5.5	8.7
River Esk	Old	40.6	2.3	9.5	3.5	3.9	3.9	5.6	9
River Esk	Old	45.5	3	12	3.8	4.2	4.2	6.2	9.5
River Esk	Old	44.5	3.5	11.6	3	3.2	3.2	5.5	8.3
River Esk	Old	41	2	10.4	3.5	3.6	3.6	5.4	9.3
River Esk	Old	44.5	2.7	10	3	3.5	3.5	5.3	9.9
River Esk	Old	43	2.3	11.8	3	3.5	3.5	6.3	10.1
River Esk	Old	40.1	2.3	10.4	2.9	3.6	3.6	5.2	9.1
River Esk	Old	42.6	3	11.1	3.3	3.5	3.5	5.6	9.3
River Esk	Old	39.2	2.3	9.5	3.3	3.6	3.6	6.2	9.5
River Water of Leith	New	42.1	2	10.5	3.5	3.7	3.7	6	9.3
River Water	New	40.9	2.7	10.4	3.3	3.5	3.5	5.4	8.5

of Leith									
River Water of Leith	New	43.1	3.2	10.4	3.1	3.8	3.8	5.7	8.6
River Water of Leith	New	43.9	3	12.3	2.4	3.1	3.1	4.9	9.8
River Water of Leith	New	43.6	3.6	11.4	3.6	4	4	6.5	9.8
River Water of Leith	New	45.9	3.3	11.7	2.5	3.2	3.2	4.7	9.8
River Water of Leith	New	42.9	3.2	10.4	3.4	4	4	5.5	8.7
River Water of Leith	New	40.4	2.3	9.9	2.5	3.1	3.1	5.1	8.4
River Water of Leith	New	41.2	3.2	11.2	3.5	3.7	3.7	5.7	8.5
River Water of Leith	New	40.8	2.8	9.3	2.7	3	3	4.8	7.6
River Water of Leith	Old	46.4	3.1	12.1	3.5	4	4	6.3	9.6
River Water of Leith	Old	32	2.1	7.8	2.3	3	3	4.5	6.9
River Water of Leith	Old	41	3.1	10.7	3.1	3.1	3.1	4.9	8.8
River Water of Leith	Old	44.2	3.6	10.5	3.2	3.8	3.8	5.6	9.2
River Water of Leith	Old	44.7	3.4	11.8	3.1	3.4	3.4	6	9.3
River Water of Leith	Old	49.4	3.7	13.3	3.8	4	4	6.3	12.6

Table 2. Number of trials taken by 8 populations of three-spined sticklebacks to learn phases one and two of the learning and memory task

Population	Trials to learn phase one	Trials to learn phase two
Balmaha Pond	19	17
Balmaha Pond	15	18
Balmaha Pond	22	52
Balmaha Pond	35	24
Balmaha Pond	15	16
Balmaha Pond	17	11
Balmaha Pond	10	12
Balmaha Pond	17	34
Beecraig Pond	12	43
Beecraig Pond	13	30
Beecraig Pond	11	21
Beecraig Pond	14	15
Beecraig Pond	11	13
Beecraig Pond	16	17
Beecraig Pond	10	27
Beecraig Pond	10	35
North Belton Pond	24	22

North Belton Pond	15	21
North Belton Pond	11	29
North Belton Pond	19	13
North Belton Pond	20	19
North Belton Pond	14	28
North Belton Pond	14	36
River Biel	17	17
River Biel	26	29
River Biel	13	26
River Biel	22	30
River Biel	19	25
River Biel	11	31
River Biel	36	15
River Biel	23	25
River Biel	27	18
River Biel	20	30
Craiglockhart Pond	15	11
Craiglockhart Pond	12	30
Craiglockhart Pond	31	24
Craiglockhart Pond	14	14
Craiglockhart Pond	16	27
Craiglockhart Pond	14	13
Craiglockhart Pond	10	14
Craiglockhart Pond	10	15
River Endrick	11	17
River Endrick	17	17
River Endrick	11	33
River Endrick	14	18
River Endrick	13	21
River Endrick	11	21
River Endrick	10	16
River Endrick	10	33
River Esk	12	14
River Esk	12	26
River Esk	16	43
River Esk	14	20
River Esk	18	15
River Esk	20	11
River Esk	10	11
River Esk	13	21
River Water of Leith	14	18
River Water of Leith	20	36
River Water of Leith	20	13
River Water of Leith	18	28
River Water of Leith	15	26
River Water of Leith	20	19
River Water of Leith	13	12
River Water of Leith	13	23

A.2.2. Chapter 3

Table 3. Temperament trait values for three-spined sticklebacks from 8 populations.

Population	Activity (number of times a section was crossed in 15 minutes)	Time to approach a novel object (seconds)	Time spent near novel object over 15 minutes (seconds)	Time to emerge from a darkened box (seconds)	Average time to begin a foraging trial (seconds)
Balmaha Pond	78	74	400	10	158.15
Balmaha Pond	104	70	393	25	191.1
Balmaha Pond	17	141	668	35	326.25
Balmaha Pond	20	345	73	258	469.3
Balmaha Pond	30	150	472	264	266.45
Balmaha Pond	13	205	528	900	113.75
Balmaha Pond	8	22	12	236	57.55
Balmaha Pond	32	29	484	325	415.2
Beecraig Pond	53	80	293	177	40.95
Beecraig Pond	43	2	605	145	184.95
Beecraig Pond	40	487	114	153	113.4
Beecraig Pond	48	3	680	131	95.05
Beecraig Pond	44	380	292	8	89.4
Beecraig Pond	46	23	206	110	122.95
Beecraig Pond	39	12	470	49	41.2
Beecraig Pond	43	1	210	28	22.45
North Belton Pond	24	503	200	23	265.1
North Belton Pond	58	196	150	300	318.55
North Belton Pond	4	1	321	688	212.2
North Belton Pond	35	134	195	57	217.4
North Belton Pond	26	436	148	305	285.5
North Belton Pond	42	140	307	362	618.9
North Belton Pond	26	379	128	376	498.15
River Biel	46	211	274	17	206.7
River Biel	4	780	56	900	420.6
River Biel	20	59	309	900	145
River Biel	10	1	414	724	629.1
River Biel	16	188	434	736	416.5
River Biel	40	271	234	113	303.25
River Biel	38	1	364	14	161.6
River Biel	6	537	274	900	463.5
River Biel	4	105	795	481	419.05
River Biel	6	691	8	900	731.3
Craiglockhart Pond	79	81	275	132	16.8
Craiglockhart Pond	70	20	251	91	19.7
Craiglockhart Pond	40	237	269	3	16.7
Craiglockhart Pond	29	1	725	213	25.3
Craiglockhart Pond	66	90	306	331	49.8
Craiglockhart Pond	67	8	351	10	43.6
Craiglockhart Pond	65	21	429	131	69.5
Craiglockhart Pond	55	66	215	27	65.95
River Endrick	54	18	553	136	115.75
River Endrick	87	121	227	64	258.7

River Endrick	70	11	360	29	127.55
River Endrick	19	14	146	14	128.4
River Endrick	76	86	314	19	54.9
River Endrick	34	455	80	32	118.7
River Endrick	50	121	436	262	70.7
River Endrick	31	179	186	160	75.45
River Esk	70	95	352	89	133.45
River Esk	77	110	541	4	26.1
River Esk	16	121	641	343	49
River Esk	30	39	268	366	91.65
River Esk	56	11	600	57	83.15
River Esk	35	305	329	264	100.75
River Esk	99	87	397	37	131.1
River Esk	24	1	463	34	153.6
River Water of Leith	57	27	375	42	40
River Water of Leith	60	8	293	233	353.85
River Water of Leith	72	154	330	297	112.55
River Water of Leith	118	42	440	29	58.05
River Water of Leith	32	1	442	21	96.8
River Water of Leith	60	17	364	34	403.55
River Water of Leith	36	1	480	91	91.25
River Water of Leith	88	42	362	42	125.65

A.2.3. Chapter 5

Table 4. Number of trials taken by three-spined sticklebacks reared in enriched, unenriched and wild environments to learn phases one, two and three of the learning and memory task, and their temperament trait values

Rearing environment	Replicate	Trials to learn phase one	Trials to learn phase two	Trials to learn phase four	Activity (number of times a section was crossed in 15 minutes)	Time to approach a novel object (seconds)	Time spent near novel object over 15 minutes (seconds)	Time to emerge from a darkened box (seconds)	Average time to begin a foraging trial (seconds)
Enriched	1	20	11	10	17	1	549	302	194.05
Unenriched	1	24	28	10	36	1	563	719	466.1
Unenriched	1	18	12	10	29	85	583	163	30.95
Enriched	1	18	12	10	62	51	415	240	91.35
Wild	1	18	40	14	46	1	469	290	249.4
Enriched	1	30	22	17	12	456	223	72	182.1
Wild	1	18	12	10	44	249	271	172	79.2
Wid	1	21	14	10	46	43	425	391	430.55
Unenriched	1	16	11	13	36	1	532	48	44.8
Enriched	1	27	10	10	55	66	385	54	154.35
Wild	1	20	12	10	66	118	230	26	280.1
Unenriched	1	33	11	16	0	7	0	98	32.65
Unenriched	1	42	12	17	40	1	319	93	32.35
Wild	1	14	22	10	16	476	16	67	83.4
Enriched	1	14	15	10	10	1	176	3	84.05
Unenriched	2	12	29	10	7	640	232	900	166.1

Wild	2	19	32	10	2	2	898	497	498.6
Enriched	2	11	14	12	24	141	305	827	192.2
Unenriched	2	10	16	10	32	1	828	229	69.4
Unenriched	2	19	30	10	8	900	0	366	62.45
Unenriched	2	10	19	10	34	1	330	84	216.55
Enriched	2	14	36	27	2	900	0	900	280.75
Wild	2	17	54	23	35	1	448	97	364.65
Enriched	2	14	44	17	58	3	200	28	335.4
Wild	2	10	35	11	56	87	255	45	102.4
Wild	2	18	30	10	4	431	469	900	478.35
Enriched	2	19	12	15	10	396	495	208	342.1
Unenriched	2	18	16	10	11	421	133	315	92.45
Wild	2	12	32	10	74	1	213	8	175.85

A.2.4. Chapter 6

Table 5. Plasma cortisol levels in three-spined sticklebacks handled with net, scoops and unhandled (control)

Handling method	Plasma cortisol (ng/ml)
Scoop	52
Net	64
Scoop	24
Net	29
Control	56
Net	48
Conrol	6
Net	34
Scoop	52
Control	6
Control	6
Net	9
Control	3
Scoop	23
Scoop	18
Net	20
Control	26
Scoop	61
Control	19
Net	93
Net	27
Scoop	41
Net	178
Scoop	95
Control	23
Net	118
Control	3
Scoop	7

Table 6. Temperament traits in three-spined sticklebacks and Panamanian bishops handled with nets and scoops

Species	Fish number	Handling method	Time to emerge from a darkened box (seconds)	Time spent near novel object over 15 minutes (seconds)
Three-spined stickleback	1	Net	91	9
Three-spined stickleback	2	Net	868	17
Three-spined stickleback	3	Net	484	14
Three-spined stickleback	4	Net	22	2
Three-spined stickleback	5	Net	473	17
Three-spined stickleback	6	Net	64	30
Three-spined stickleback	7	Net	709	0
Three-spined stickleback	8	Net	557	30
Three-spined stickleback	9	Net	353	19
Three-spined stickleback	10	Net	130	9
Three-spined stickleback	11	Net	240	5
Three-spined stickleback	12	Net	55	17
Three-spined stickleback	13	Net	24	30
Three-spined stickleback	14	Net	12	12
Three-spined stickleback	15	Net	8	14
Three-spined stickleback	16	Net	24	17
Three-spined stickleback	17	Net	40	30
Three-spined stickleback	18	Net	116	10
Three-spined stickleback	19	Net	80	9
Three-spined stickleback	20	Net	654	0
Three-spined stickleback	1	Scoop	43	18
Three-spined stickleback	2	Scoop	232	17
Three-spined stickleback	3	Scoop	74	12
Three-spined stickleback	4	Scoop	202	22
Three-spined stickleback	5	Scoop	30	14
Three-spined stickleback	6	Scoop	89	5
Three-spined stickleback	7	Scoop	480	0
Three-spined stickleback	8	Scoop	900	24
Three-spined stickleback	9	Scoop	65	7
Three-spined 11stickleback	10	Scoop	109	11
Three-spined stickleback	11	Scoop	36	0
Three-spined stickleback	12	Scoop	525	0
Three-spined stickleback	13	Scoop	2	1
Three-spined stickleback	14	Scoop	123	8
Three-spined stickleback	15	Scoop	7	6
Three-spined stickleback	16	Scoop	22	18
Three-spined stickleback	17	Scoop	153	19
Three-spined stickleback	18	Scoop	240	0
Three-spined stickleback	19	Scoop	111	7
Three-spined stickleback	20	Scoop	75	30
Panamanian bishop	1	Net	2	14
Panamanian bishop	2	Scoop	10	13
Panamanian bishop	3	Net	12	22

Panamanian bishop	4	Scoop	39	3
Panamanian bishop	5	Net	33	9
Panamanian bishop	6	Scoop	26	18
Panamanian bishop	7	Net	28	15
Panamanian bishop	8	Net	58	12
Panamanian bishop	9	Scoop	53	2
Panamanian bishop	10	Scoop	183	0
Panamanian bishop	11	Net	15	6
Panamanian bishop	12	Scoop	33	9
Panamanian bishop	13	Net	53	11
Panamanian bishop	14	Scoop	22	15
Panamanian bishop	15	Net	3	18
Panamanian bishop	16	Scoop	17	18
Panamanian bishop	17	Net	58	10
Panamanian bishop	18	Scoop	10	16
Panamanian bishop	19	Net	3	16
Panamanian bishop	20	Scoop	31	17

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