Coping styles and learning in fish: developing behavioural tools for welfare-friendly aquaculture

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Abstract

- Given the known stressful effects of many husbandry practices in carp aquaculture and the desirability of improving the welfare of farmed fish, the main aim of the study described in this thesis was to explore the possibility of developing a low-stress sorting system for common carp, based on a conditioned response to a visual cue signalling the presence of food.

- An additional aim was to investigate possible effects of individual stress coping strategy, which necessitated recording the behaviour of and keeping track of known individuals over periods of weeks to months. Photographic images of scales patterns in common carp can be used reliably for individual identification over periods of months. These individual identifiers, together with dye marks, were deemed sufficient for the purposes of the programme of research described in this thesis (Chapter 2).

- In general, rate of emergence from shelter into a potentially-dangerous novel environment containing food (a commonly-used method for screening fish for risk-taking) proved to be a consistent individual trait in common carp, even when fish were tested in different, randomly composed groups of fish on different occasions (Chapter 2).

- Consistent individual differences were also found in frequency of inspection of an unfamiliar object and in ability to gain access to a restricted food source. However, individual differences in performance in these 3 tests (novel environment, novel object and food competition) were unrelated when carp were tested in unfamiliar groups (Chapter 3).

- An examination was carried out on 5 data sets in which morphometric data were collected from common carp or goldfish assigned to a risk-taking phenotype on the basis of a novel environment test. Statistical differences were found in only 2 of these studies; both on common carp, with risk-takers in better condition than risk-avoiders. These support the “growth-mortality trade off” model (Chapter 6).

- Common carp classified as risk-taking, risk-avoiding and intermediate (on the basis of a series of novel environment tests) were given a simple conditioning treatment in which the presence of food in one of two potential feeding compartments was signalled by one of two movable coloured lights. Patterns of settlement (emergence from shelter

to explore the learning tank and time to feed) confirmed the original classification into risk-taking phenotype (Chapter 3).

- Over successive trials, the carp learned to forage fast and efficiently. 51.67% achieved this by using the coloured landmark; the remainder adopted a different strategy, swimming to one of the feeding compartments at random and switching immediately to the other compartment if no food was found. This was an efficient foraging strategy because of the close proximity of the two feeding chambers (Chapter 3).

- Once the criterion for learning had been reached, the fish that had learned to associate a particular visual cue were given a reversal learning test, in which food was associated with the previously un-rewarded colour. 83.33% of fish adjusted their behaviour (choosing LC/RS strategy), learning to swim to the previously un-rewarded colour within an average of 12 training sessions (Chapter 3).

- The colour red seems to be more efficient for training carp. In chapter 3, more fish learned to follow the red light compared to the yellow light and in chapter 4, fish trained with red light had a higher percentage of correct choices than fish trained with blue or green lights (Chapter 3 and 4).

- Some differences in behaviour between risk-taking categories were found during both the learning and the reversal learning phase. Risk-taking fish were faster to emerge and find food than risk-avoiders during the learning phase and tended to adopt the random switch strategy during the learning phase. Fish classified as risk-avoiders in terms tended to follow the cue (Chapter 3).

- Small groups comprising one risk-taking, one risk-avoiding and one intermediate carp (tentatively assigned on the basis of a series of novel environment tests) were exposed to a demand-feeding system in which pellets of food were delivered whenever a fish approached and/or touched a sensor identified by a coloured light of a specific colour (red, green or blue). 62% of the 18 groups (with a slight predominance of fish trained using a red light) tested were able to form this association and to feed efficiently under the demand regime. Within these groups, in general the individual that touched the sensor most gained most food. The behaviour of the groups that had failed to learn was unaffected by the addition of a trained “tutor” fish from one of the groups that had learned to touch the sensor for food (Chapter 4).

- For those groups that had learned to approach and touch the sensor, the fish were then exposed to three sensors located in different parts of the training tank signalled by different coloured lights, only one of which (that on which the fish had been trained) delivered food. The position of the sensors was changed between trials. In general, the fish tended to move towards and exploit the sensor signalled by the light colour on which they had originally been trained; this was particularly the case for fish trained on the red light. Carp classified as risk-avoiders made fewer correct choices early on in the three-light phase, but made predominantly correct choices in later trials (Chapter 4).

- Groups of 3 carp that had reached a criterion for having learned to approach a light of each of the three colours (i.e. one red-trained, one blue-trained and one green-trained) were then placed at the centre of a large tank with three lights, one of each colour, in the corner and the light approached by each fish recorded. In general, the fish were significantly more likely to approach the colour of light on which they had been trained, even though this meant separating from their companions. This effect was stronger for fish trained on the red light and disappeared after several (unrewarded) trials (Chapter 4). This result suggests that it might be possible to apply spatial separation of individuals within groups of carp on the basis of a learned association between the delivery of food and a light cue of a specific colour.

- During the course of this programme of work, the opportunity arose through the COST STSM programme to examine risk-taking phenotype, physiological stress response and brain structure in common carp of the 4 families reared either at high densities, in tanks under intensive farming condition or in natural ponds. A disease outbreak compromised the aims of this study, but significant family effects were found among both pond- and tank-reared fish for length, weight and condition factor as well as for emergence time in a novel environment test and approach to a novel object, indicating a heritable component to the variation in these traits. There was no relationship at the family level between emergence time and tendency to approach a novel object (Chapter 5)

- Fish from families that, on average, were heavier and longer took a long time to emerge from shelter, while those from families that were smaller and in poorer condition took more risks in this set up. Tank-reared fish were much slower to emerge than were pond-reared fish, possibly because the latter were in poorer condition (Chapter 5).

- Plasma cortisol levels were markedly higher in pond-reared fish compared to tank-reared fish of the same family, presumably due to the stressful experience of both harvesting and disease. In contrast, plasma glucose levels were lower in pond-reared fish, presumably due to their poor nutritional status (Chapter 5).

- The relationship between an estimate of forebrain size and overall brain size was different in pond and tank reared fish, with most pond reared families having a larger forebrain area than tank reared fish (Chapter 5).

- Also during the course of this programme of work, two related studies were carried out in collaboration with colleagues in the Division of Ecology & Evolutionary Biology. Together with Hussein Jen-Jan, we explored some hidden costs of an aggressive, proactive life style by examining respiratory function in relation to coping strategy in common carp (chapter 6).

- Morphometric analysis of the fine structure of the gills was used to estimate respiratory area and histological analysis of sections through the gill filaments was used to measure the extent to which the secondary lamellae were obscured by epithelial cells. There was a significant relationship between risk-taking phenotype and both the size of the respiratory surface and the extent to which this is exposed as opposed to covered with epithelial cells. Risk-taking fish had larger and more exposed respiratory surfaces than did risk-avoiding fish, with fish with intermediate risk-taking phenotype having intermediate scores. These differences are interpreted as an adaptation to the known high resting metabolic rate of risk-taking fish (Chapter 6).

- Together with Priyadarshini, we look at social interactions and growth in relation to risk-taking phenotype in goldfish. Within the social groups, though most goldfish showed no aggressive behaviour, some of the fish attacked their companions at least once per minute of observation and some individuals showed as many as 8 attacks per minute. These levels are surprisingly high for what is usually seen as a non-aggressive species. In groups comprising 3 goldfish of each risk-taking category, the risk-avoiding fish showed relatively little aggression. Overall, fish that showed any aggression within social groups gained preferential access to a restricted food supply (Chapter 6).

- There were no differences in weight, length or condition between risk-taking and risk-avoiding goldfish at the point of initial screening, but by the end of the experiment the risk-avoiding fish held in groups with other risk-avoiders had gained less weight and had strikingly lower condition factors compared to the other categories of fish (i.e. all risk-avoiders and risk-takers held in mixed groups). It is suggested that some sort of

social facilitation of fear keeps levels of stress high in groups composed entirely of risk-avoiding fish (Chapter 6).

- The implications of all these results are considered in a final general discussion (Chapter 7).

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Author’s declaration

I declare the work in this thesis is entirely my own, and of my own composition. No part of this work has been submitted for any other degree.

“It is not the strongest of the species that survives… it is the one that is the most adaptable to change (Charles Darwin)”

CHAPTER 1

General Introduction

1 Aquaculture and its importance

Fish are cultured for a variety of reasons, including for food, for the ornamental trade, for restocking and for conservation of threatened species (Flagg et al. 1995). Asia, the Indian Subcontinent and Southeast Asia dominate aquaculture production; however, Europe and the US are also substantial producers of aquaculture products. In the past decade, aquaculture has rapidly expanded, and is now recognized as a major food production industry (Figure 1.1).

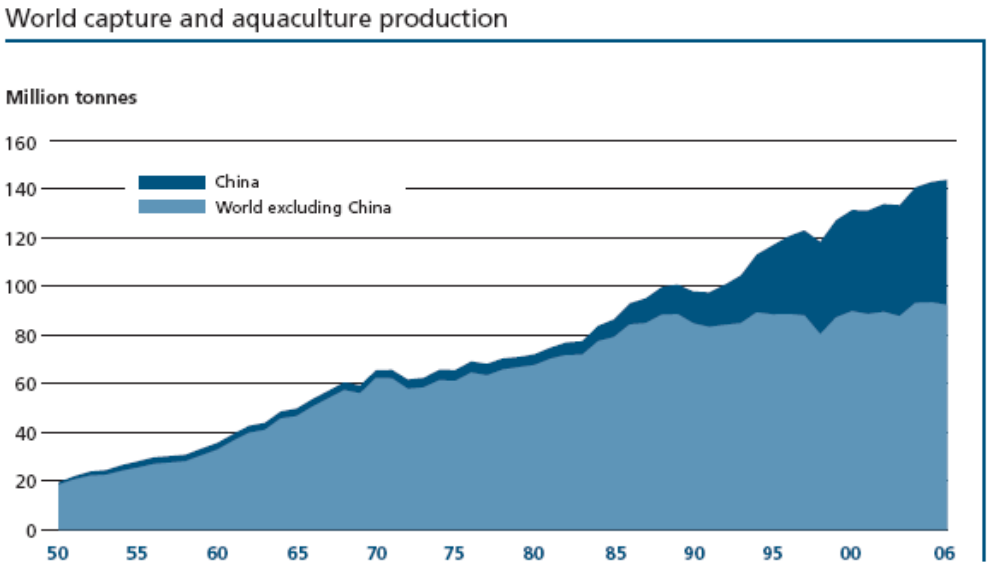


Figure 1.1 Growth of aquaculture on a worldwide basis by year (FAO - The state of world fisheries and aquaculture 2008).

Housing animals at high densities and with frequent husbandry interventions are common and necessary practices in intensive aquaculture. Even when in extensive systems (extensive aquaculture is more basic than intensive aquaculture in that less effort is put into the husbandry of the fish. Extensive aquaculture is done in the ocean, natural and man-made lakes, bays and rivers. Fish are contained within these habitats by multiple mesh enclosures which also function as trapping nets during harvest), fish culture inevitably introduces a number of stressors to the organism concerned. These may include poor water quality (for example, high levels of ammonia, unsuitable pH, high levels of carbon dioxide, low dissolved oxygen levels and inappropriate temperature), as well as handling, with resulting physical damage, disease treatments and incomplete nutrition. It is impossible to avoid many of the procedures known to induce stress in fish. Netting, grading and transport are integral components of the fish farming routine and all unavoidably induce stress responses in cultured fish (Pickering 1993).

2 Carp aquaculture

The family Cyprinidae is the most important in numbers of species of all freshwater teleosts. The Common Carp (*Cyprinus carpio*, Linnaeus 1758) is one of the most widespread members of the Cyprinid family. In Europe, the common carp is by far the carp species farmed in largest numbers. However, due to socio-economical changes in Central and Eastern Europe, the production of common carp has declined sharply, between 1990 and 2004. Carp production in Europe is currently of about 225,000 metric tons and 90% of this is produced by aquaculture.

Carp are omnivorous fish with a strong tendency to eat animal food. Carp occur naturally in summer-warm lakes and slowly flowing rivers. Carp are rarely found in clear, cool, swift-flowing streams. They prefer muddy areas where they search for food organisms. Carp can tolerate winter temperatures below 2°C. They can tolerate temperatures above 30°C for short periods. Three main production systems of common carp can be differentiated as: 1) monoculture of carp, 2) polyculture of carp, and 3) integrated carp culture with other agricultural activities. There are very few intensive systems in the region despite existing technology. There are numerous combinations of polyculture with common carp production and the species involved are all cyprinids and occupy only slightly different ecological niches in the pond system (EFSA 2009).

3 Stress responses in fish

Stress can be defined as any influence from the environment that disturbs an organism homeostasis. Fish respond to environmental challenges with a series of adaptive neuro-endocrine adjustments that are together named stress responses, manifested as the primary response, which is an endocrine response (for example, release of glucocorticoid hormones from the adrenal cortex or equivalent tissue), the secondary or metabolic stress response and the tertiary or behavioural stress response (Figure 1.2). These cause reversible metabolic and behavioural modifications that make fish more efficient at overcoming or avoiding the challenge and are doubtless beneficial, in the short-term at least (Barton 2002).

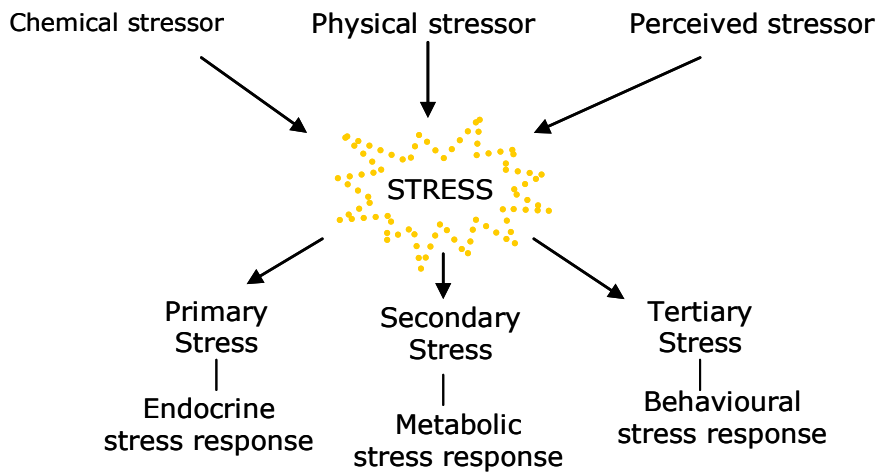


Figure 1.2 Causes of stress and responses to stressors (modified from Barton 2002).

In teleosts, cortisol is the main glucocorticoid released during stress and plasma cortisol concentrations can be used as an index of the stress response (Barton 2002). For example, basal levels of plasma cortisol in unstressed salmonid fish are normally in the range 0-5 ng ml^{-1}. An acute stress such as handling or confinement causes a temporary elevation of the plasma cortisol levels of both brown trout, *Salmo trutta* L., and rainbow trout, *Salmo gairdneri* Richardson, in the range 40-200 ng ml^{-1}, with a return to basal levels within 24-48 hours (Pickering & Pottinger 1989). Rainbow trout also showed increased cortisol levels when exposed to grading and transportation (Flos et al. 1988).

Behavioural responses are the first defence that an animal has against environmental changes, predators or social conflicts and are often caused by the same stimuli that elicit physiological responses to stress. Animals show different behavioural strategies when facing threatening situations and the type of behavioural and physiological response to stress is an individual characteristic called coping (Schjolden et. al 2005). Fish functions that are known to be affected by stressors include swimming performance, thermoregulation, orientation, avoidance, chemoreception, feeding, predator evasion and learning (Conte 2004). When attacked by a predator, fish may respond by shoaling, freezing, taking shelter, changing colour and also avoiding areas in which they have been attacked. Feeding behaviour may be suppressed following an encounter with a predator or inefficient feeding strategies may be adopted. Specific adaptive behaviour patterns are observed in response to parasitic disease and to tissue damage for example, carp that are hooked in the mouth show rapid darting, spitting and shaking of the head (reviewed by Huntingford et al. 2006).

4 Stress and welfare in fish culture

Research has identified adverse effects of various aspects of husbandry practice, including confinement, inappropriate densities, restricted feeding, handling, transportation and slaughter (Branson 2008). The extent of such effects depends on the purpose for which fish are being cultured. Fish that are farmed for the food market are mainly reared in intensive systems where the productivity in terms of growth rate and stocking density must be high to be economically viable (Brannas & Johnsson 2008). This kind of system is more stressful than an extensive system to the fish and raises more concern about their welfare. When carp are reared intensively at high stocking densities they show higher plasma levels of cortisol, glucose and free fatty acids which all are indicators of stress. They also are more sensitive to an additional acute stressor (netting) than carp reared under normal densities (Ruane et al. 2002). In a study with common carp, plasma cortisol levels were significantly increased after net confinement. Cortisol levels quickly returned to normal levels following release of the fish from the nets. Glucose levels were elevated during confinement; however the elevation of glucose in the plasma was more gradual and continued to increase for at least another 4 hs after the fish were returned to the tanks. Lactate levels were significantly increased during confinement and then returned to a normal level (Nematollahi et al. 2009).

Most sources of stress encountered, such as handling, sorting or transport, are part of routine fish culture operations and are generally inevitable. Even when carp, for example, are reared extensively, culture systems inevitably introduce a number of stressors to the organism. These may include poor water quality (for example, high levels of ammonia, unsuitable pH, high levels of carbon dioxide and low dissolved oxygen levels), inappropriate water temperature, handling, with resulting physical damage, disease treatments and incomplete nutrition. It is impossible to avoid many of the procedures known to induce stress in fish. Netting, grading and transport are integral components of the fish farming routine; all the fish farmer can do is try to minimize the effects of such stressors (Pickering 1993). Using impaired feed intake as an indirect index of stress in fish, Sørum & Damsgard (2003) showed that benzocaine anaesthesia, in Atlantic salmon, *Salmo salar* L, did affect on feed intake, but fish vaccinated with an oil adjuvant vaccine had a significantly reduced feed intake in a period of 12 days after vaccination.

Slaughter induced strong stress responses in fish. Slaughter methods include electrical stunning followed by decapitation, blunt trauma to the cranium, percussive stunning with a captive bolt, “cold stunning” and dewatering (Conte 2004). Slaughter methods in

which the fish are rapidly rendered and kept unconscious prior to killing are favoured. Of four methods of slaughter (exsanguination without prior stunning, carbon dioxide narcosis followed by exsanguination, percussive stunning and spiking the brain), only percussive stunning and spiking the brain resulted in no aversive reactions from the fish (Robb et al. 2000).

It is important to make husbandry practices (including separation, for example by size, age, reproductive status) less stressful. The impact of aversive stimuli or stressors is determined by the ability of the organism to cope with the situation. Whenever environmental stressors are too demanding and the individual cannot cope, its health is in danger. It is important to understand the mechanisms and factors underlying the individual's capacity to cope with environmental challenges.

4.1 Welfare in aquaculture

As is true for other agriculture sectors, aquaculture practices are now being examined to assess their impact on the environment and on animal welfare (Conte 2004). There is well-documented and legitimate concern about animal welfare in aquaculture. The fact that, even in extensive culture systems, carp and other fish are still exposed to a variety of stressors raises questions about their welfare.

4.1.1 Defining welfare

Animal welfare is a complex and controversial concept. Most definitions fall into one of three broad categories: (1) feeling-based definitions of welfare, in which the requirement for good welfare is that the animal should feel well, being free from negative experiences such as pain or fear and have access to positive experiences, such as companionship in the case of social species; (2) function-based definitions focussed on, an animal's ability to adapt to its present environment, here good welfare requires the animal be in good health with its biological systems functioning appropriately and not being forced to respond beyond their capacity and (3) nature-based definitions, in which each species of animal is seen as having inherent biological nature that it must express; good welfare requires that the animal is able to lead a natural life and express its natural behaviour (Huntingford et al. 2006). According to the feeling-based approach, for welfare to be a relevant concept for fish, they must have the necessary cognitive features of a sentient being, which is a controversial point.

4.1.2 Sentience, pain and welfare in fish

There is no doubt that practices in aquaculture, commercial and recreational fishing, and also scientific research do potentially represent painful and fearful situations, though there is controversy about the capacity of fish to feel pain and to suffer. Central to the discussion is the concept of sentience, or the capacity for basic consciousness, the ability to feel or perceive and respond to external stimuli, not necessarily including the faculty of self-awareness. Nociception is the detection of potentially harmful stimuli and is accompanied by a withdrawal response away from the noxious stimulus. Essential to survival in all animals, nociception can in some cases give rise to pain, which is defined as an unpleasant sensory and emotional experience associated with actual or possible tissue damage (Branson 2008). Although all animal groups are considered to have nociceptors this is relatively under-explored in fish.

In the book 'Do fish feel pain?' Braithwaite (2010) describes a series of studies with trout that yielded some very interesting results. At first, the study focussed on examining whether, like mammals, fish have the sort of receptors and fibre nerves that control nociception. Such receptors were indeed identified. The next step was to find nociceptors on the skin surface that when stimulated would transmit signals of connection to tissue damage. In that way the physiological part of pain and nociception in fish was proved.

The next experiment had the aim of examining the connection between the physiology of nociception and whether stimulating nociceptive receptors alters fish behaviour. To achieve this, fish were injected with one of two different noxious substances: bee venom or weakly acidic solution (vinegar) and had their reaction to a novel object after being injected was evaluated. The fish injected with the saline solution kept avoiding the novel object (Lego bricks tower) as trout normally do, but the fish injected with vinegar behaved oddly seeming much less fearful of the novel object. This result suggested that the vinegar solution impaired fish attention, as expected if the vinegar caused discomfort and pain for the animal. To test whether the distraction was pain-based the experiment was repeated but this time together with the vinegar or saline injection, fish received pain-relief (opiate morphine). Now there was no difference in the behaviour of fish treated with saline solution and those given vinegar, the levels of awareness and avoidance were similar.

Giving the fish an injection of a noxious substance distracted its attention, but when pain relief was administered the ability to focus increase again. For this to happen the

pain must have induced negative experiences, suggesting a degree of cognitive awareness (Braithwaite 2010). A study of pain perception using goldfish (*Carassius auratus*) and rainbow trout demonstrated that these two species have different responses to the same stimulus. Goldfish improved shock-avoidance learning and memory while the rainbow trout showed no significant stimulus discrimination and little information retention (Dunlop et al. 2006). All of these results suggest that fish, like other animal groups, have sufficiently complex mental processes for fish welfare to be a meaningful concept but there is variation between species and fish too.

4.1.3 Assessing welfare

Proper assessment and promotion of the welfare of farmed fish requires several aspects of their biology to be taken into account and species are likely to differ in their response to husbandry procedures. For example, many species of fish form schools in the wild and this is important when evaluating their adaptation to captivity. It is known that goldfish and common carp when kept in crowded spaces, liberates a hormone that inhibits growth and production and this will influence the welfare consequences of high stocking densities (Winfield and Nelson 1991). Yet, arctic charr (*Salvelinus alpinus*) eat more and grow faster at high (120 kg.mm^3) and or medium (60 kg.mm^3) densities than at low (15 kg.mm^3) densities (Joergensen et al. 1993).

On the subject of indicators by which welfare in farmed fish may be assessed, fish farmers use production variables such as growth rate and fish weight to assess the general status of their stock. In practice, good farmers also monitor behavioural indicators such as behaviour during feeding and visible indicators of health such as injuries and mortalities. In addition, easily measurable aspects of water quality such as temperature and oxygen (Stien et al. 2007) are also used to monitor the general well-being of fish in culture.

Other possible indicators of fish welfare include body condition, fin condition, colouration, swimming, behaviour during meals and food intake (Branson 2008). A study with Atlantic salmon used a multivariate analysis to combine four commonly used measures of fish welfare (condition of body and fins and plasma concentrations of glucose and cortisol) into a single welfare score. Using this multivariate index showed that stocking density can influence the welfare in production cages with higher densities having a lower welfare score, but only after a threshold density of ca 23 Kg/m^2 (Turnbull et al. 2005). Another study found a relationship between eye colour and status, showing that change in sclera colour in juvenile salmonids is a complex response

to local events which raises the possibilities of using colour patterns (Suter & Huntingford 2002). Rainbow trout with low cortisol-responsiveness were consistently more spotted than high-cortisol responsive fish (figure 1.3a) and, Atlantic salmon (figure 1.3b) individuals with more spots showed a reduced physiological and behavioural response to stress (Kittilsen et al. 2009).

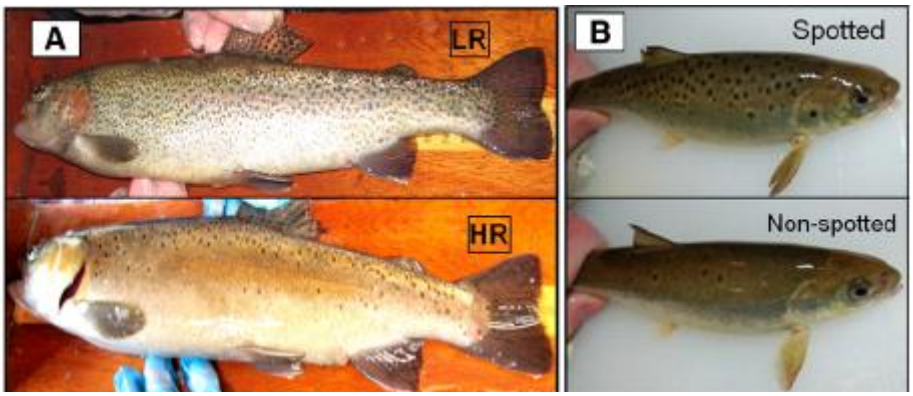


Figure 1.3 Examples of phenotypic variation in dermal melanin pigmentation in salmonid fish. a) Rainbow trout selected for low (LR, top) and high (HR, bottom) post-stress cortisol levels. b) Atlantic salmon defined as 'spotted' (top) and 'non-spotted' (bottom). Kittilsen et al. 2009.

Changes in behaviour was also related for Atlantic salmon in net pens where high rolling activity is an indication of recent acute stress, whereas high leaping activity indicates heavy louse infestation or present acute stress (Furevik et al. 1993).

5 Reducing the stressfulness of routine husbandry practices

5.1 Promoting welfare in cultured fish

Accepting that fish welfare is a meaningful concept and that various aspects of aquaculture practice can potentially compromise fish welfare, it is important to minimize stress and promote welfare among farmed fish. There are a number of strategies (not mutually exclusive) that could potentially be used to reduce the adverse effects of fish husbandry practices, including choosing to farm fish that adapt well to intensive rearing conditions, developing husbandry systems that minimise adverse effect on welfare and developing sensitive indicators that can be used on working farms to identify quickly and easily whether fish are in a state of good welfare.

In terms of selecting suitable species, stocks or individuals to farm, understanding the behavioural and physiological responses of farmed fish to aquaculture conditions is of major importance in improving animal welfare and consequently increasing production. The fact that individuals vary in stress responsiveness potentially has implications for the welfare of farmed fish, since individuals with different levels of responsiveness to risk are likely to be differentially affected by stressors met during intensive production (Pottinger & Carrick 1999). The fact that fish show specific behavioural responses to acute and chronic stress means that stress responses can be used to manipulate well-being. Section 5 presents more detailed information on behavioural responses to stress (coping strategies).

In terms of husbandry systems, simple and small changes can create significant improvements in the welfare of farmed fish. For example, simply changing the colour of tank in which Arctic charr are held (fish get darker when living on black background and this may suppress attack, fish interacting on a dark background showed less aggression than those interacting on a light one) (Höglund et al. 2002) or keeping salmon and Arctic charr in duoculture (Nortvedt & Holm 1991) can reduce levels of aggression and improve welfare (improve growth and reduce levels of aggression by effectively diluting the cues that elicit aggression). Improvements can involve slightly more complex technology. For example, comparisons were made between the behaviours of fish fed using a demand feeder and those of fish fed under the standard feeding practice of each farm. The results showed a decreased level of competition between the treatments with demand fed fish showing less scramble competition and fighting than those fed the same amount of food in meals (Andrew et al. 2002). An interesting illustration of a method to improve fish well-being is of a study where common carp submitted to a photoperiod of 12:12 and 30 minutes of classical music (1.5h intervals) showed similar growth to fish reared under darkness and no music. 0L:24D, therefore music transmission seems to reduce the negative effect that light brings to growth performance. Music could be regarded as a stress relief or inducing factor, possible using it as a growth and product quality promoter, as well as a means to ensure fish welfare under intensive fish farming (Papoutsoglou et al. 2007).

The development of methods for achieving less stressful farming making more use of the natural responses of fish could benefit both fish welfare and farm profitability (Lines & Frost 1999). To promote fish well-being and to help avoid stressful situations we can employ the fish's natural and/or learned preferences.

As an example of using the natural responses of fish to control their movement in culture systems, in Poland, medieval carp husbandry systems were constructed in such a

way that when draining the pond, the farmer used the fish natural attraction to water flow to gather the fish in one region of the pond and facilitate harvesting (Pilarczyk pers. communication). More recently, the use of natural responses, both in groups and individually, has proved to be promising for use in aquaculture systems. For example, the innate positive phototactic and rheotactic responses of guppies (*Poecilia reticulata*) were manipulated to stimulate fish to swim from one container to another, transferring them through pipes or narrow channels, allowing inspection by a computer vision system and enable sorting (Karplus et al. 2003, 2005). The natural attraction of fish to water flow is used to guide fish to a passage through the barrier of the Igarapava dam in Brazil in such a way that the fish swim close to a window. This window has a video camera to register the species using the ladder and also the size of fish that are able to exploit this type of aid to migration. The aim of this set up is to minimize the impact of the dam on the species that inhabit the river (Bizzotto et al. 2009).

Learning plays a major role in the behaviour of fish and may be useful as a means of controlling stress and promoting positive behaviour in aquaculture (Stien et al. 2007). Light has been used to facilitate and improve husbandry practices for a long time. Lekand & Færa (1993) demonstrated that small salmon and trout can be trained to associate light signals with feeding and so be collected or moved around a tank. An experiment by Lines & Frost (1997) showed that after training Atlantic salmon to associate a flashing light with food delivery, it was possible to selectively attract trained individuals to a feeding area by pointing a collimated beam of light to it; while such a system is probably unfeasible where fish are held at very high densities, it might well be used for managing high value cultured fish such as broodstocks. As a final example, tilapia held in groups learned readily to associate a visual cue (a blue light) with food, approaching the light to receive food; in contrast, groups of carp failed to do so, but when in mixed groups with tilapia the carp were able to learn the association (Karplus et al. 2007).

6 Learning in fish

The previous section shows that there are various ways in which learned responses to spatially-significant cues can be used to promote welfare in farmed fish. The overall aim of the present study is to develop such methods for common carp, which calls for a consideration of the process of learning in fish.

Learning is a process by which an animal benefits from experience, so that its behaviour is better suited to environmental conditions. The first experiment that analysed

learning capacity in fish was probably back on the late 1800s with the classic work on trial-and-error learning in pike (*Esox lucius*). The uniqueness of this study was that it involved experimentation to detect change in behaviour with experience (Kieffer & Colgan 1992a). More recently, the learning ability of fishes has been investigated in relation to several subjects, such as fish orientation (Warburton 1990; Braithwaite et al. 1996; Vargas et al. 2004) and foraging (Kieffer & Colgan 1992b).

Although some types of learning may occur with a single trial, most learning takes place gradually over several trials. Goldfish learn to distinguish between colour patterns in different orientation faster when given one training trial per day then when given 60 per day (Duecker 1982). Repetition can also lead to habituation, sometimes described as one of the simpler forms of learning. Habituation is a type of non-associative learning leading to a reduced behavioral response after repeated exposure to stimuli. It is a neutral form of learning in which a neutral stimulus is repeated many times. The first time it is applied it is novel and evokes a reaction, however, it evokes less and less response as it is repeated. Eventually the subject becomes habituated to the stimulus and fails to respond to it. When common carp is constant stimulated with cold shocks its cortisol response is lower than fish experiencing a single cold shock, indicating that habituation to this physical stressor occurred (Tanck et al. 2000). Rapid habituation is important in the aquaculture environment since it should reduce stress thus helping to adapt the fish better to farm daily procedures (Ferno et al. 2006).

6.1 Associative learning

Associative learning, also called conditioning, is a type of learning in which an association is made between a stimulus and a response. There are two types of conditioning; the first is called classical conditioning where an unconditioned stimulus (US) (to which an animal gives an inborn response that it does not have to learn) is associated with a second stimulus, one that does not initially elicit the response. Repeatedly presented before the US, after several pairings, the second stimulus is able to elicit the response. This new stimulus is now called the conditioned stimulus (CS). For example, carp can learn to associate a 400-Hz pure pulsed sound with food by classical conditioning, food being the US associating with a sound – second stimulus (Zion et al. 2007).

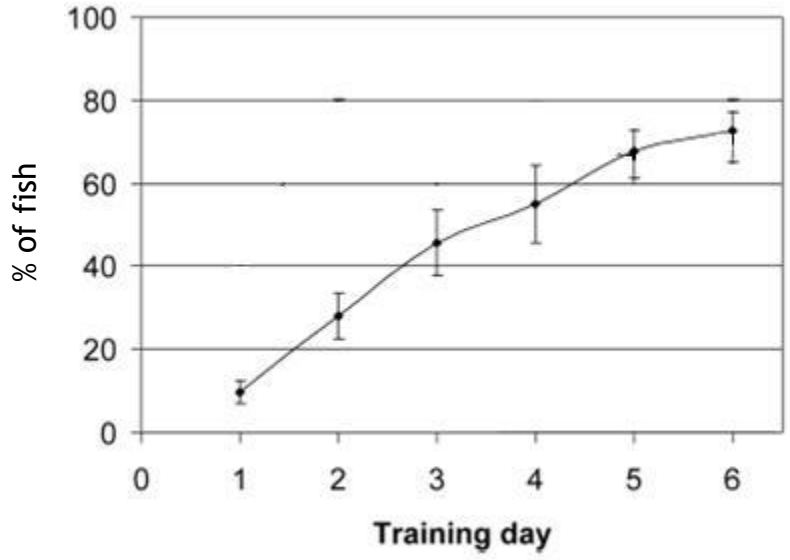


Figure 1.4 Proportion of naïve fish that were present at a feeding tray during an acoustic signal prior to the administration of food pellets (modified from Zion et al. 2007).

Another type of conditioning is operant conditioning, which happens when the consequence of performing an action (positive or negative) alters the probability of that action being performed. In the positive case if a behaviour has favourable consequences, animals learn to perform it in order to be rewarded. In this case though, the behaviour must be spontaneously emitted, not elicited by a stimulus and the favourable result, or positive reinforcement must follow it closely (Goodenough et al. 2001). For example, Atlantic cod *Gadus morhua* were trained to operate a trigger to receive food in an experiment using a self-feeding system (Nilsson & Torgersen 2010). In a recent study using a sound stimulus, common carp learned to discriminate between two stylistically different musical stimuli using positive reinforcement; while blues music was playing every response was reinforced with a food pellet, but during classical music no response was reinforced (Chase 2001). In the negative case, operant conditioning can take the form of aversive conditioning, in which instead of a reward, the animal receives a punishment so the rate of the response observed declines (Bolhuis & Giraldeau 2005). Goldfish reduced occupation of a specific zone at the tank after receiving electric shock, demonstrating spatially cued shock avoidance (Dunlop et al. 2006).

The process of learning can be affected by many different aspects of the situation in which learning occurs. These include strength and timing of the reinforcing stimulus in relation to performance of the relevant action. For example, delivery of a reinforcing stimulus immediately or shortly after a response results in faster learning than if the reinforcement is delayed. In goldfish the formation of learned associations between new

stimuli (e.g. visual cues) and rewards occurs more efficiently when the delay between the stimulus and the reward is short (Winfield & Nelson 1991).

Fish can also learn different types of tasks. Goldfish were able to locate a particular place in a tank that lacked relevant featural information by encoding geometrical properties of the experimental space (Vargas et al. 2004). Goldfish can also learn to locate a reward using featural information where geometrical landmarks (see below) are not available. A study using environmental enrichment to attract zebrafish (*Danio rerio*) to a specific place and then make them avoid it by putting an aversive stimulus (stroboscopic light) showed that fish can have a preferred spot in an environment and that its behaviour can change according to alterations at this environment (Mesquita et al. 2009). Using classical conditioning to teach hatchery-reared *Oreochromis niloticus* to avoid predators it was possible to show that fish can learn a new behaviour, swimming to the surface, to adapt to environment change (Mesquita & Young 2007).

Some organisms are able to learn from others, learning in this way is much more common in social species because they spend more time close to conspecifics. The adaptative value of social learning lies in saving some of the time and energy that might be wasted as an individual learned by trial and error. It is also generally assumed that social learning is beneficial, because naïve individuals can acquire adaptative behaviour quickly and efficiently from more knowledgeable individuals (Brown et al. 2006).

6.2 Learning about landmarks

In some of the examples given above, fish were relying on spatial learning, which often involves navigating in relation to specific localised features in the environment, or landmarks (Kieffer & Colgan 1992). Several studies have shown that both vertebrates and invertebrates are able to remember local features of the environment (visual, mechanical or olfactory) and use these to guide subsequent movement, on a variety of scales; in other words, they can remember and use landmarks.

Many studies have demonstrated the ability of fish to use visual landmarks. Warburton (1990) used plastic Lego columns to mark a food patch in a study with goldfish and the results showed that spatial learning was poor in the absence of clear local visual cues, the group trained with the Lego landmarks showed very high choice accuracy, less choice variability and a significant improvement with experience. Goldfish learned geometrical properties of the experimental space for locating food even in the absence of relevant featural information and vice-versa. And in the last experiment, using both

featural and geometrical information, fish were able to encode the information (Vargas et al. 2004). Juvenile Atlantic salmon were trained to find food in one of two potential feeding stations using visual landmarks. The fish were able to forage efficiently in this situation, though individuals varied in how they achieved this. Six out of 9 fish learned to use the coloured landmarks track the rewarded feeding site, with a mean performance of 75% The remaining 3 fish became site attached (to the left or right side of the tank (Braithwaite et al. 1996).

6.3 Spatial learning and brain

The hippocampus of birds and mammals has been discovered to be linked to spatial behaviour. Rats with lesions to the hippocampus lost their ability to navigate to a determine place using shape information provided by a solid-walled arena and an array of identical landmark information. This result is consistent with the theory that the hippocampus plays a key function in spatial learning (McGregor et al. 2004). The fish brain contains a structure that is homologous and functionally similar to the mammalian hippocampus within the telencephalon (specifically the lateral pallium). A recent study by Vargas et al. (2000) showed that after training goldfish in a spatial task, the brain presented a selective increase in protein synthesis in neurones located in this part of the telencephalon. Goldfish were trained in a radial arm-maze with numerous visual cues and then subjected to lesion in one of 4 different brain regions. Fish with ablation of the lateral pallium and telencephalon, but not of the other sites, lost their ability to navigate at the maze (Rodríguez et al. 2002). Vargas et al. (2006) found that goldfish with lateral pallium lesions learned a spatial learning task, where they have to escape from the enclosure to the open space of the aquarium using geometric information of the tank and position of striped panels on the walls, faster than control fish, but they were insensitive to geometric information, they only relied on feature information to locate food.

6.4 Variable responses to stress: coping strategies

Several studies have reported that animals that are similar in many ways (age, sex, size, maturity stage) may differ in learning ability (Iguchi et al. 2001). At the level of closely related species, pumpkinseed (*Lepomis gibbosus*) and bluegill sunfish (*Lepomis macrochirus*) behave differently in a task that involved learning to forage on a novel prey, whiteworms, with bluegill learning faster than pumpkinseed fish. In addition, individual fish within one species (pumpkinseed sunfish) exhibited individual variation in foraging efficiency such as capture rate and foraging success (measured by number of

captures attempts) (Kieffer & Colgan 1992). There are various possible explanations for such differences in learning ability, one being differences in what are called coping strategies.

As discussed above, an adequate ability to cope with stress is fundamental to fitness and quality of life. Behavioural and neuro-endocrine responses to stress are, however, characterized by large individual variation and understanding individual differences in stress coping ability has become a predominant task in biological and stress research (Koolhaas et al. 1999). The concept of individual stress coping strategy has been used to characterise this ability in a wide variety of animal species (Mouse: Benus et al. 1991, pig: Hessing et al. 1994, chicken: Blokhuis & Metz 1992, fish: Van Raaij et al. 1996). A coping style or strategy can be defined as a set of behavioural and physiological responses that is consistent over time and characteristic to a certain group of individuals. Two distinct stress response patterns exist reflected in both behavioural and neuro-endocrine processes: the proactive and the reactive stress coping styles (Pottinger & Carrick 1999; Frost et al. 2007. Table 1.1). Proactive animals are characterized behaviourally by a tendency to take risk in response to danger, by relatively high levels of aggression and by the tendency to form behavioural routines. In contrast, reactive animals avoid risk and aggressive conflict and are more flexible.

Table 1.1 Summary of the main differences between proactive and reactive coping styles (modified from Korte et al. 2005).

	Proactive	Reactive
Behavioural strategy	Fight-flight	Freeze-hide
Emotional state	Aggressive and risk-taker	Non-aggressive and timid
Exploration	Fast and superficial	Cautious and through
Flexibility	Rigid and routine-like	Flexible
Energy metabolism	High energy consume	Energy conservation
Physiological response	Adrenaline based	Cortisol based
Behavioural response	Proactive	Reactive

Such differences in “personality” may be reflected in several different contexts, including exploration of unfamiliar environments and objects, interactions with potential predators and encounters with conspecific rivals (Sih et. al 2004). The terminology used in the literature to describe differences in risk taking is controversial, since some of the words employed are those used to describe human feelings. The terms “bold” and “timid” or “shy” are a case in point. In this thesis wherever possible I use the terms “risk-takers” and “risk-avoiders” instead, unless referring to published studies in which the authors use the alternative terminology.

Behavioural and physiological variation in response to a stressor may be inherited and, in evolutionary terms, may be maintained by that spatial or temporal variation in selection regimes, the different behavioural phenotypes performing best in different conditions. Previous research has shown that rainbow trout (*Oncorhynchus mykiss*) segregated into high and low responding individuals represented inherited and environment-dependent cortisol levels (Pottinger & Carrick 1999).

Differences in behavioural flexibility are often associated with different coping strategies, with proactive animals tending to form rigid routines and reactive animals being flexible and highly sensitive to environmental change. This is likely to be reflected in differences in learning. For example, rainbow trout assessed as "bold" on the basis of time spent in an open area and level of activity learned a foraging task (approaching a specific area when a light was switched on to receive food pellets) faster than the fish assessed as "shy" in the same test (Sneddon 2003). This may be because the bold fish were less cautious and more willing to take more risks than shy fish and so may have experienced the association between light and food more frequently. In other cases, differences in learning between animals with different coping strategies seems to be a direct result of differences in general flexibility. For example, rainbow trout from lines selected for low cortisol responsiveness (arguably, proactive fish, see above) learned readily to feed in one of two feeding areas. However, when the food was moved to a different location, even to one in which it was clearly visible, these proactive fish failed to adapt. In contrast, in the same set up fish from a strain selected for high stress responsiveness (reactive fish, see above) were quick to adjust to the new feeding location. Thus it seems that the proactive, risk-taking fish produced by selection for low stress responsiveness are less flexible than their reactive, risk-avoiding counterparts from the high responsive strain and that this compromises their ability to learn about variable environmental features (Ruiz-Gomez et al. 2008).

6.5 Behavioural syndromes

There is an extensive body of literature on a topic related to that of coping strategies, describing the fact that individuals within a species often show consistent behaviour not just within a given context, but also among different contexts; in such circumstances, they are sometimes said to demonstrate a behavioural syndrome, with individuals having a specific status with respect to the syndrome (Sih et al. 2004a). Syndromes that have been described in the behavioural literature include functional categories such as feeding, antipredator response, exploration, competition and dispersal (Bell 2006). The existence of behavioural syndromes implies limited behavioural plasticity; this contrasts

with situations in which individuals have more plastic behaviour, can vary their behaviour in different functional contexts independently and so can exhibit the optimal response in all contexts. For this reason, behavioural syndromes are important because if they are genuinely fixed, then they limit behavioural plasticity, explain non-optimal behaviour and help to maintain individual variation (Sih et al. 2004b).

Many studies of behavioural syndromes have used vertebrates as subjects; for example, bluegill sunfish presented consistent behaviour in different contexts, individuals designated as bold being more active, more willing than those designated as shy to explore novel environment/object, to inspect a potential predator and to spend time in risky areas (Wilson & Godin 2009). The behaviour of invertebrates has also been investigated in this context. Cockroaches (*Blattella germanica*) reared in isolation showed stronger exploration-avoidance, reduced foraging activity, reduced willingness to interact socially and reduced ability to assess mating partner quality than conspecifics reared in groups. This study demonstrates the occurrence of a behavioural syndrome induced by social isolation, similar to syndromes described in vertebrates (Lihoreau et al. 2009). Another example of behavioural syndrome in invertebrates was demonstrated by Sinn et al. (2008) in dumpling squid (*Euprymna tasmanica*). Behaviour was measured in two different contexts, a threat and a feeding test. Across contexts, behaviour was not correlated at any age; while within context individual phenotypes were consistent both before and after sexual maturity. During sexual maturity, so-called shyer animals were more plastic in feeding tests, while so-called bolder animals were more plastic in threat ones.

Although syndromes, with behaviours that are significantly correlated across domains, have been described for many species, in other cases, individual variation is domain-specific. For example, Coleman and Wilson (1998) showed that individual differences do not correlate across contexts in pumpkinseed sunfish. In this case, fish that were considered intermediate in terms of their response to a novel object, allowing it to be moved close to them (not within 5cm) behaved boldly as foragers and in response to a predator. The same was established using rainbow trout in 5 different tests: 1) latency to consume food at the feeding apparatus, 2) latency to cross through a mesh partition to gain access to the feeder, 3) latency to cross through a mesh partition to gain access to the feeder under predation risk by a salmon, 4) latency to cross through a mesh partition to gain access to the feeder under predation risk by a aerial predator and 5) latency to cross a barrier in an artificial stream. The same individuals took or avoided risks (so were classified as “bold” or “timid”) in four different situations related to foraging, but behaved quite differently in a dissimilar context (explore the artificial stream) (Wilson & Stevens 2005).

Bell (2005) compared two populations of stickleback (*Gasterosteus aculeatus*) in three contexts: activity in an unfamiliar environment, aggression and boldness under risk. She showed that these behaviours were correlated in only one of the populations. The fact that the three behavioural variables were not positively related to each other in both populations allowed her to reject the hypothesis that behavioural syndromes inevitably act as an evolutionary constraint. Population differences in the existence of behavioural syndromes were also found in sticklebacks by Dingemanse et al. (2007). The often-documented syndrome between aggressiveness, activity and exploratory behaviour was found in large water bodies where vertebrate predators were present but not in smaller ponds with only invertebrate predators (figure 4.1). The lack of correlation between behaviours in predator-naïve populations did not arise because all individuals had the same behavioural type; individual fish did differ in all 3 behavioural tests, but covariance across contexts was weak or absent.

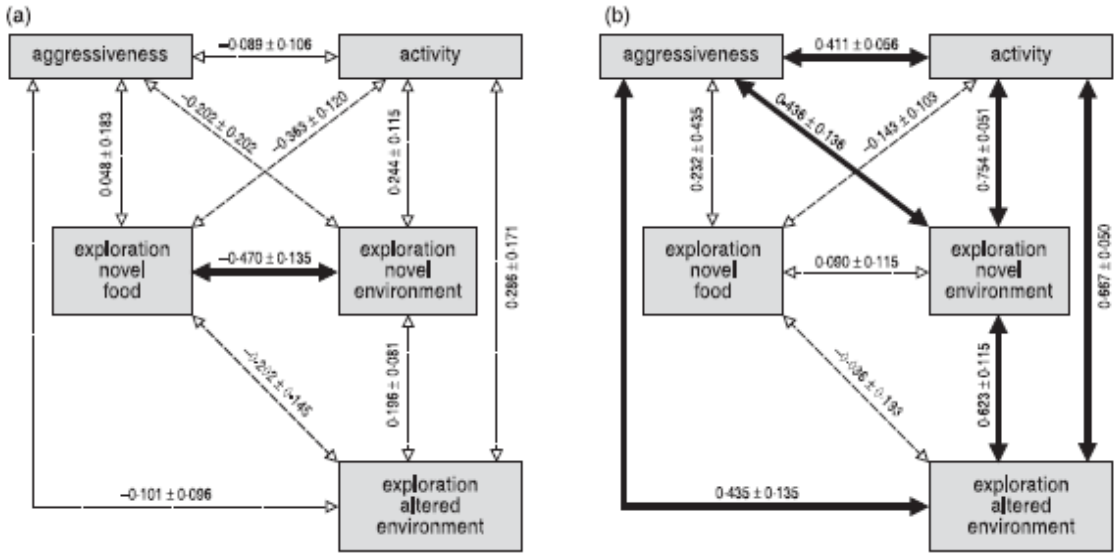


Figure 1.5. Behavioural syndromes for two populations: (a) predator-naive and (b) predator-sympatric. For each pair of behaviours, the average within-population r ± SE. Solid lines indicate significant correlations (p < 0.05). From Dingemanse et al. 2007.

For some of the well-studied vertebrate examples of behavioural syndromes, the underlying neuro-endocrine correlates are reasonably well documented and surprisingly conserved. For example, Huntingford et al. (2010) found that carp that took risks when exploring a novel environment showed low stress responsiveness, indicated both by lower plasma lactate and glucose levels and also by lower expression of cortisol receptor genes in the brain and head kidney.

6.6 Risk-taking and body condition

Where a correlation exists between risk-taking and aggression, labelling this a behavioural syndrome in some sense implies that the relationship is biologically significant and requires functional explanation. For example, in the case of the sticklebacks studied by Bell (2005) and Dingemanse et al. (2007), one might argue that in sites with piscivorous fish, individuals that are either risk-taking and aggressive or risk avoiding and non-aggressive do well, whereas those with the opposite combination of traits do poorly. According to a different approach put forward by Stamps (2007), risk-taking and aggression are independent manifestations of a life history decision for fast growth. It is not uncommon to find within the same population individuals that "opt" to grow fast and mature early and others that "opt" to grow more slowly and mature later. A study comparing similarly reared seventh-generation farm Atlantic salmon with wild salmon from the principal founder population of the farm strain showed that Atlantic salmon selected for fast growth show enhanced appetite, mediated in part at least by higher rates of production of growth hormone (Fleming et al. 2002).

Fast growing individuals are expected to show traits that make them more likely to gain food. For example, fast growing Atlantic salmon showed a marked increased appetite whereas the appetite of slow growing fish decreased (Metcalfe et al. 1986, Metcalfe et al. 1988). Among the behavioural traits that would be effective in individuals that have opted for fast growth are being ready to take risks in a potentially dangerous environment that contains food and competing aggressively when food is limited. Under such a scenario, individual differences in aggressiveness and risk-taking are independent, adaptive responses to a fast-growth developmental trajectory that involves a growth-mortality trade off. The often-observed correlation between these two aspects of behaviour is thus an incidental bi-product of a developmental switch to faster or slower growth.

If this view of co-varying risk-taking and aggression as a manifestation of a growth-mortality trade off is correct, then risk-taking, aggressive fish are expected to be the largest of their cohort and risk-avoiding, aggressive fish to be among the smallest. In a species in which both activity and boldness are positively related to food intake rates, individuals with consistently high growth rates should display high levels of activity and boldness (Biro & Stamps 2008). Several studies have found that bold, risk-taking individuals do indeed tend to be larger than shy individuals from the same population. In three-spined sticklebacks, fish that resumed foraging rapidly after a simulated predator attack (bold) have a higher growth rate than shy fish (Ward et al. 2004).

Dolomedes triton (fishing spiders) that showed more voracity in the prey foraging trials had higher feeding rates and consequently large adult size (Johnson & Sih 2005). Brown et al. (2007) showed in a study using the poeciliid *Brachyrhaphis episcopi* that bolder individuals screened using time to emerge from cover and response to a novel object had a greater body mass at a given standard length than shy fish (figure 1.6a).

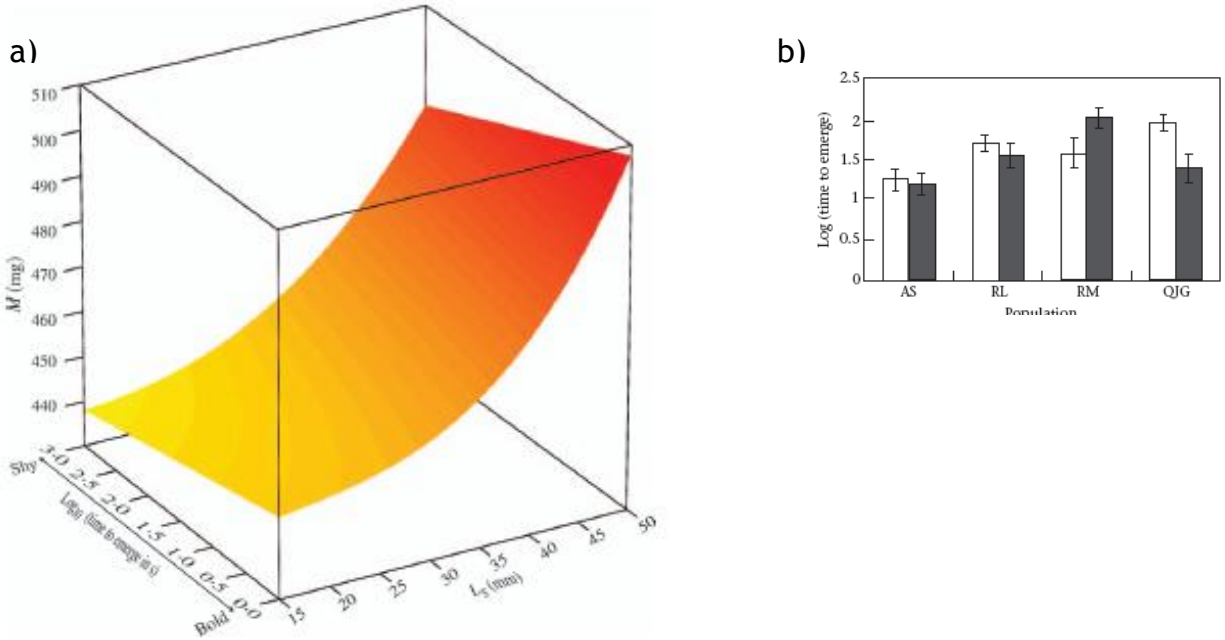


Figure 1.6 Variable relationship between body size and risk taking in the poeciliid fish. a) Relationship between boldness [log_{10}(time to emerge from shelter)], standard length (L_S) and body mass (M) showing the characteristics of a classic growth curve. (Brown et al. 2007). b) Mean (SEM) time to emerge from shelter adjusted for standard length for fish from downstream (white bars - high predation) and upstream (dark bars - low predation) sites in each of the four streams. (Brown & Braithwaite 2004).

However, other studies have found the opposite, namely that smaller individuals, and in particular those that have the lowest nutrient reserves, take risks; those with good reserves are more cautions, and can afford to be. This is sometimes described as the "asset protection" hypothesis. In 2004, Brown & Braithwaite found that in the same poeciliid *Brachyrhaphis episcopi* smaller fish emerged from shelter sooner than larger individuals, this was true only for populations that inhabited upstream sites which had low predation pressure (see figure 1.6b). These two frameworks are not mutually exclusive, since even if differences in risk-taking and aggression do reflect a growth-mortality trade off, in the short term even individuals on a slow growth trajectory will take risks to gain food if they are in very poor condition.

Another aspect that can influence the behaviour in relation to growth is the availability of food. In natural habitats, the variation of food availability causes many organisms to experience periods of low growth and in adaptation to that, many species compensate

this as soon as food is available growing faster than normal after this period of under nutrition. This 'compensatory growth' is observed in vertebrates and invertebrates. For example, Coho salmon (*Oncorhynchus kisutch*) changed their risk-taking behaviour when hungry, habituating faster after predator exposure and remaining in the risky areas; therefore they increased food intake and specific growth rate compensating their weight loss (Damsgard & Dill 1998). In another study, aggression was strongly connected to growth; fast-growing Atlantic salmon were more aggressive than slow-growing salmon (Nicieza & Metcalfe 1999).

6.7 Differences in behaviour, growth and mortality between wild and captive animals

In addition to long-term, inherited effects of domestication, as described for carp by Matsuzaki et al. (2009), animals of the same strain reared in captivity often show differences in behavioural and morphological traits when compared to their wild counterparts, arising from the fact that the environment experienced by cultured and wild animals is strikingly different. As an example of the effects of differential experience in wild and captive reared fish, the presence of predators in the wild stimulates the development of effective anti-predator responses in the cichlid Nile tilapia (*Oreochromis niloticus*). Lack of this experience makes tank-reared fish less prepared to react when subsequently confronting a predator (Mesquita & Young 2007).

Another process that can generate differences in behaviour between wild and captive-reared fish is differential mortality of individuals that behave in different ways. This can interact with internal differences in complex ways. Brown trout from four different families of wild parentage were reared in four tanks and fed high (100%) and low (25%) rations. Within each tank, highly significant differences in mortality were observed between families, but this was dependent on feeding treatment (Figure 1.7).

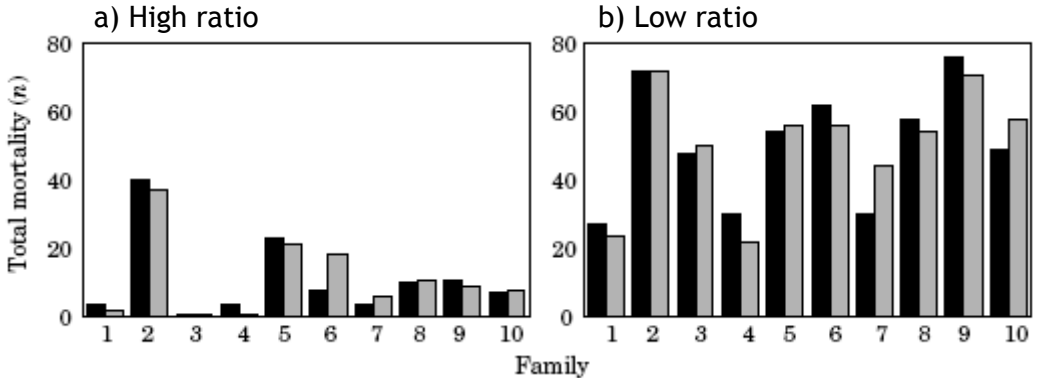


Figure 1.7. Overall family mortality during the 35 day start-feeding observation period for the (a) high [tank 1 - black bars, 2 - grey bars] and (b) low [tank 3 - black bars, 4 - grey bars] feeding regime. (Glover et al. 2004)

The family that experienced the lowest overall mortality in the high feeding treatment showed high mortality rates in the low feeding treatment. This difference in distribution of mortality among families observed between the low and high start-feeding treatments may be indicative of a genotype x environment interaction between feeding level and family survival (Glover et al. 2004).

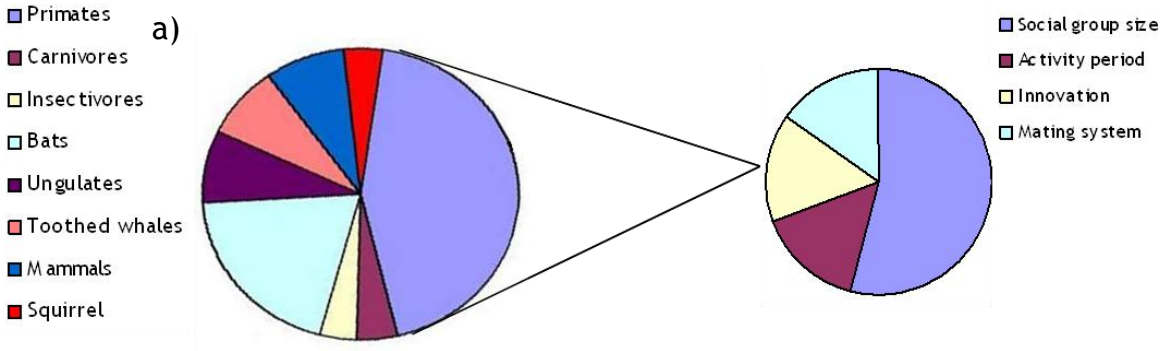
Domestication eventually selects out the reactive fish, but only after many have starved and/or died from the effects of chronic stress. In general, it is likely that captivity selects bold fish (Huntingford & Adams 2005). Less aggressive animals are more flexible in their responses and better at tasks that demand behavioural change (Koolhaas et. al 1999). Comparisons of wild and captive fish behaviour indicate that domestication selects aggressive and risk-taking behaviour. So, shy fish do not establish at regular conditions of production damaging its production and welfare (Huntingford & Adams 2005).

Differences in risk taking and in stress coping style have been reported for a number of fish species, including rainbow trout (Schjolden et al. 2005) and common carp (Huntingford et al. 2010). This has implications for aquaculture, since risk-taking, aggressive fish with low stress responsiveness (proactive copers) often do well and shy, non-aggressive fish with high stress responsiveness (reactive copers) often do poorly in husbandry practices. In common carp held at high densities under a variety of oxygen and temperature regimes, reactive fish tend to put on weight rather than length (and hence gain in condition), whereas proactive carp tend to grow in length (Pilarczyk et al in press). Proactive and reactive carp also show diametrically opposite responses at the level of changes in gene expression in the brain when given a simulated bacterial challenge (MacKenzie et al. 2009).

The best strategy will depend on the reasons concerning why fish are being reared. If this is to provide large numbers of fish for restocking purposes, then arguably farmed populations should include fish from across the whole spectrum of risk-taking. If, on the other hand, fish are being farmed for food so production and welfare are the main considerations, one approach might be to avoid placing shy fish in production systems, either by using domesticated strains (where these are available) or by pre-screening fish at the start of the production cycle. However, as described above, the performance of fish with different patterns of risk-taking is context-dependent, being influenced by many aspects of the competitive environment, particularly food distribution, environmental complexity and density (Huntingford 2004). So caution needs to be applied.

6.8 Brain structure and captive rearing

There is increasing evidence that both domestication and captive rearing have an effect on the brain structure of cultured animals, which in turn is likely to influence their behavioural capacities. On a broad taxonomic scale, variation in the relative size of the brain or of specific brain areas has been shown to correlate with some form of behavioural complexity. Figure 1.8 show the consensus from comparative studies published in the last 10 years that have looked for correlations between behavioural complexity and measures of brain size.



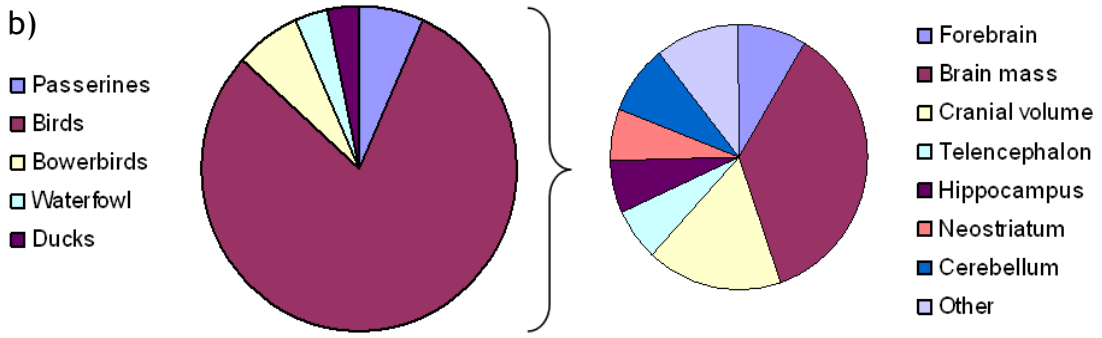


Figure 1.8 Summary of comparative studies published in the last 10 years that have looked for correlations between behavioural complexity and measures of brain size in (a) mammals classes and subjects of the comparative studies and (b) birds classes and brain areas studied. Figure based on data from Healy & Rowe 2007.

Clear results have been obtained when looking at the effects of domestication (with its known effects on behaviour) on brain size. Such differences can be caused by genetic differences consequent to domestication or to brain plasticity driven by the different environments in which wild and domesticated animals develop, or both. Ranched American mink were found to have, on average, smaller brain sizes than wild mink, independent of body size, sex and weight. Moreover, the captive mink had much more variable brain sizes and parts of brains than those of wild animals (Kruska 1996).

Several other studies that used different strains of animals have reported reduced brain sizes in captive-bred compared with wild individuals, including Mongolian gerbils *Meriones unguiculatus* forma domestica where they also showed differences in behaviour (Stuermer & Wetzel 2006), turkeys *Meleagris galopavo* (Ebinger & Rohrs 1995), pigs *Sus scrofa* (Plogmann & Kruska 1990).

Comparison between brain morphology (olfactory bulb, telencephalon, optic tectum and cerebellum) of hatchery and wild reared stocks of rainbow trout (2 hatchery-reared strains and 2 geographically distant populations of wild fish) showed that seven out of eight measures have smaller values in hatchery-reared fish than in wild fish and most strongly difference was found in the optic tectum and telencephalon. These areas of the brain that showed the greatest differences were those linked to aggression, feeding behaviour and reproduction, a finding that supported previous work that found that these were the areas in which captive-reared fish are deficient (Marchetti & Nevitt 2003).

7 Biology of common carp and carp aquaculture

The broad aim of this thesis is to look at various aspects of learning and coping strategies and their implications for the welfare of farmed fish, using the common carp (*Cyprinus carpio*) as a subject. The common carp is the oldest cultured fish in the world (Balon 2004). Culture of common carp has been performed from about the twelfth century in ponds and so comprehensive systems for its production under extensive conditions are well-established (Kocour et al. 2005).

Different species of carp are farmed in Europe for different purposes, some for food, sometimes for restocking, or else for angling or for sale as ornamental species (Table 1.2). The common carp, is the most widely cultured carp for all these purposes and, in terms both of the number of animals farmed and of economic value, the most important species.

Table 1.2 Some examples of carp species (Cyprinidae family) farmed for different purposes.

Species	Common name	Food	Restocking	Ornamental
Cyprinus carpio	Common carp	X	X	
Ctenopharyngodon idella	Grass carp	X	X	X
Hypothalmichthys molitrix	Silver carp	X		
Aristhichthys nobilis	Bighead carp	X		
Tinca tinca	Tench	X	X	X
Ciprinus carpio Koi	Koi carp			X
Carassius auratus	Goldfish			X
Abramis brama		X	X	
Carassius carassius			X	X

In the U.K, carp angling is now the largest and fastest growing sector of coarse fishing. Many fisheries are dedicated to this one species; they are highly managed and highly profitable (figure 1.9).

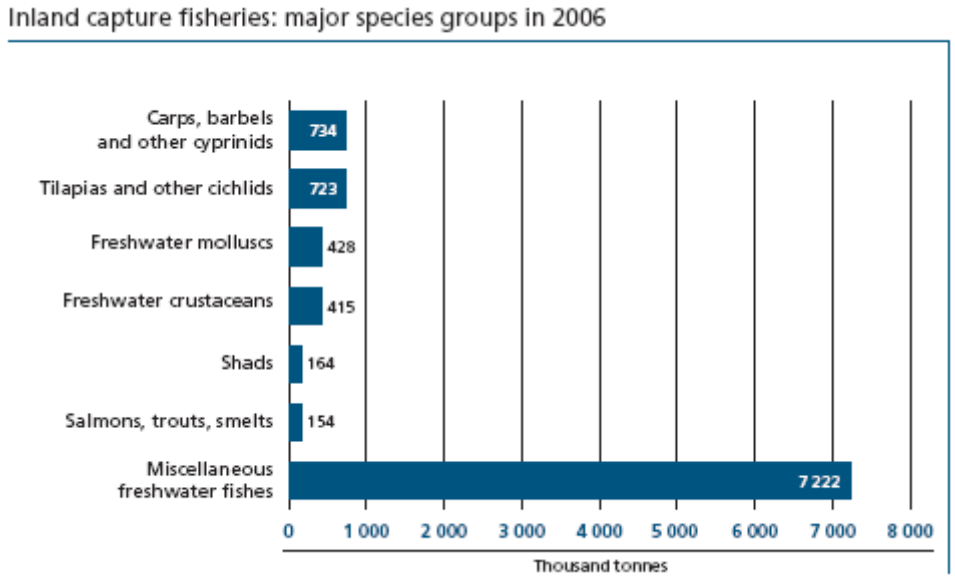


Figure 1.9 Amount of fish of different taxonomic groups produced by inland capture in 2006 (FAO - The state of world fisheries and aquaculture 2008).

Compared to intensively farmed species such as Atlantic salmon, pond-cultured carp are held in conditions that are similar in many respects to those experienced by wild fish. When carp are reared intensively at high stock density, they show higher plasma levels of cortisol, glucose and free fatty acids, which all are indicators of stress. They also were more sensitive to an additional acute stressor (netting) than are carp reared under normal densities (Ruane et al. 2002).

8 Aims of this thesis

With this background, the overall aim of the work described in this thesis was to explore the possibility of developing a low-stress sorting system for common carp, based on a conditioned response to a visual cue signalling the presence of food. A subsidiary aim was to explore sources of variability in pattern and speed of learning about spatially-relevant cues and, in particular, whether stress coping style was an influential factor. In order to achieve these aims, it was necessary to keep track of identified individuals over extended periods of time. Chapter 2 describes a study to validate the use of individual scale patterns in common carp for this purpose. It was also necessary to assign fish to coping strategy or risk-taking phenotype and to assess the consistency of individual differences in this context in fish assigned randomly to groups for testing. Chapter 2 also describes the novel environment test designed to make this assignment and how individual performance in a novel environment relates to risk-taking in other contexts (when the fish were faced with a novel object and when they were made to forage competitively for a localised and restricted food source). Having developed these

techniques, a large number of carp were individually identified, screened for risk-taking phenotype and then given the opportunity to learn to follow a visual cue to locate food when just one of two feeding sites was rewarded. The results of these learning trials, and of a subsequent reversal learning test, are described in Chapter 3. Having established that common carp can learn to follow a visual cue to find food, a separate group of fish were screened for coping strategy and trained to approach one of three feeding stations, signalled with different coloured lights, using a demand-feeding system for training. Groups of three carp trained to approach three different colours of light were then placed together in a tank offering all three light colours placed at a distance from each other and their movements observed to determine whether they separated on the basis of trained light cue. The results of this study are described in Chapter 4.

During the course of this programme of work, the opportunity arose for various additional studies. Firstly, an EC funded project allowed me to study the effects of captive rearing on behaviour and morphology in common carp. This was achieved by comparing risk-taking phenotype, morphology and brain structure in carp of 4 families reared from hatching either in natural ponds or in husbandry tanks in a research institute in Poland. The results (which were unfortunately compromised by a disease outbreak) are described in Chapter 5. I also took part in collaborative projects designed to explore the implications of risk-taking phenotype for performance in several different context. Firstly, a meta-analysis was carried out of a number of data sets of aspects of body size and condition in relation to risk-taking phenotype in common carp and a related species, the goldfish. Secondly, also using goldfish, I took part in a study of aggressive behaviour, access to food and patterns of growth in risk-taking and risk-avoiding fish held in small groups with different social composition. Thirdly, I took part in a study of gill structure in carp assigned to different risk-taking phenotypes, testing the hypothesis that, since risk-taking carp have a higher metabolic rate than do risk-avoiding fish, they will have a larger respiratory surface. The results are described in Chapter 6. Finally, Chapter 7 provides a general discussion of the results obtained from these various studies.

CHAPTER 2

General Methodology and Pilot Studies

This chapter describes methodologies that were used across other chapters of this thesis.

The programme of research described in this thesis depended on allocating fish to coping strategies and tracking their performance in a variety of contexts, which in turn depended on being able to track individual fish over time. This chapter looks at how fish identification was achieved in all the studies reported here and then describes the behavioural methods used to assign fish to coping strategies.

1 Identifying carp

When looking for individual differences in the following experiment it was necessary to identify the fish both within and across trials. This was achieved by a combination of dye marking with use of natural variation in the scales pattern.

1.1 Dye marking

Fish were lightly anesthetized using benzocaine (5ml of benzocaine per 1 L of water) until they ceased to respond to touch and were then weighed and measured for length (see figure 2.11). Using a Panjet inoculator (a pressure ink jet, see Hart & Pitcher 1969) filled with alcian blue dye, fish were marked in different body parts, including the dorsal, caudal and pectoral fin and the top of the head.

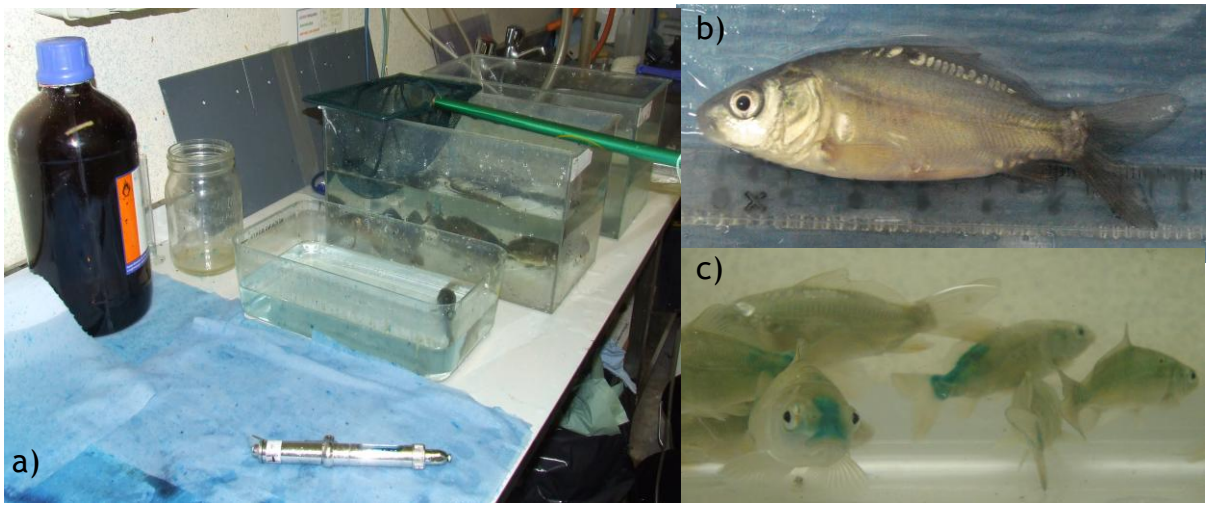


Figure 2.10 a) Marking procedure: A = marking equipment Panjet inoculator, B = anaesthetic bath, C = recovery tank, b) measuring the fish and c) examples of marked carp.

The durability of the marks depended on where they were made and how good they were. It was difficult to have fine control over the Panjet. Marks on the head and marks of the fins that entered the fin rays tended to last longer than others. Hence marks were combined with scale pattern to identify the fish in the experiments.

1.2 Using natural variation for identification

1.2.1 Introduction

Marking and tagging fish

Why do scientists mark fish?

An early and powerful motivation for marking individual animals was for the purpose of exploitation, for example the need to establish ownership, transport messages and determine movement patterns. One of the earliest references to the marking of fish appears in Izaak Walton's *The Compleat Angler,* first published in 1653. In this book, the author mentions experiments in which ribbons were attached to the tails of young salmon to evaluate movement patterns. In addition to such practical aims, the ability to identify individual animals is often critically important in zoological research, offering information on behaviour, distribution, habitat use, population structure and life-history traits.

There are many reasons why fish biologists and fisheries managers might need techniques for identifying particular individuals or categories on successive occasions during research into topics such as ecology and behaviour, and for management and conservation. Fish biologists have used identification methods for purposes ranging from studies of migration, monitoring population sizes and investigating behaviour. For example, McLean et al. (2005) studied the influence of social rank on the ability of Atlantic salmon to track profitable food patches using fish individually identified with PIT tag and alcian blue dye. Katano et al. (2006) used small cuts on the fins to analyse the effects of small-scale dams on fish communities, species diversity, population density, biomass, migration over dams and trophic relationships. Alcian blue marks were used to identify individual fish in a study of whether rainbow trout selected for high and low stress responsiveness differ in cognitive function (Moreira et al. 2004). In a study aimed at relating coping styles to social status of rainbow trout, fish were marked using small cut in the upper or lower part of the tail fin (Overli et al. 2004). As a final example, Suter & Huntingford (2002) examined the relationship between eye colour and social status in Atlantic salmon differentiated by using alcian blue dye. Fisheries

managers use various techniques for identifying and recognising fish including fin clipping, tagging and dye marking when monitoring stocks, making it easier to assess abundance, age structure and age- and year-specific survival and mortality.

What techniques are available for marking fish?

As the previous examples show, a number of methods have been developed for recognising particular fish on successive occasions, based on two main methods. Thus an animal may be individually distinctive to an observer either due to variation in phenotype acting as natural marks or by artificially modifying an animal's appearance (marking or tagging). Techniques for marking fish may include implanting structures in the fish's body, making external attachment of a physical object (including various kinds of numbered tags), marking external tissues with a dye or removal of external structures, often fin tissue. Most studies rely on physical capture of animals and placement of artificial tags, dye marks or other objects to allow following identification (Welch et al. 2007). Commonly used methods to individualize fishes artificially include alcian blue dye (Hart & Pitcher 1969; Adams et al. 1998), PIT (passive interactive transponder) tags (Armstrong et al. 1999), visible implant elastomer (Jensen et al. 2008) and calcein, a fluorochrome dye that exhibits a green fluorescence in fin rays and other calcified structures under specific optical conditions (Frenkel et al. 2002). The choice of mark will depend on fish size and aim of the project.

Identification requirements for different kinds of study

Different marking techniques are suitable for different kinds of study, which place different requirements on the identification system concerned. Relevant considerations include how long the identification must last, the number of categories that need to be recognised and whether identification must be from a distance or whether it can be "in hand".

Identification may simply require fish to be assigned to particular groups, in which case the relevant number of batch marks is all that is required; for example, fishes were fin clipped to distinguish population above and below dams (Katano et al. 2006). On the other hand, research aims may make it necessary to identify specific individuals, requiring a larger array of tags; for example, Japanese flounders *Paralichthys olivaceus* were given individual dye marks in a study of feeding patterns in hatchery-reared fish (Watanabe et al. 2006). The period for which fish need to be recognized varies between studies. For example, gobies (*Rhinogobius* sp) were individually identified with coloured implants for a single breeding season in a study of the determinants of male mating success (Ito & Yamagisawa 2006). In contrast, lemon sharks *Negaprion brevirostris* were

identified using PIT tags for up to 5 years in a study of early juvenile growth and population structure (Freitas et al. 2006). Another aspect that must be considered is the distance between the fish and the observer at the time of identification. Tagged fish are often captured and anaesthetized for repeat weighting, fish being identified "in hand", as in a study by Dumbrack et al. (2006) in which tags were used to measure growth rate in lungfish. In contrast, identification of free-swimming fish at a distance may be needed; for example, bennies (*Salaria fluviatilis*) were colour marked using pink fluorescent elastomer for visual identification in a field study of their reproduction, data being collected in the field by observation from a distance (Lengkeek & Didderen 2006). Finally, identification may take place in the laboratory or in the field. Many studies of fish in aquaria use PIT tags to identify individual common carp given endurance exercise in a study by Martin & Johnson (2006). Other studies use fish in the wild, for example, sea lampreys were marked with PIT tags and dorsal fin tags in a field study of the use of pheromones to control their movements (Wagner et al. 2006). Flat bed PIT detecting antennae were buried in the floor of a natural stream to monitor movement patterns of Atlantic salmon individually pit tagged (Armstrong et al. 1997).

Table 2.3 summarises the various marking requirements for different kinds of study, modified from Caro (1998).

Table 2.3 Techniques for identifying fish and the requirements they place on fish and researcher (adapted from Caro 1998).

Technique	Requirements	Example	Description
Numbered tags	Capture-recapture, handling anaesthesia, restrict to age, hand identification	Papoutsoglou & Lyndon 2006	Diet composition on individual performance
		Adkison et al. 1995	Visual recognition of fish underwater
Fin cuts	Capture-recapture, handling, anaesthesia, hand identification	Katano et al. 2006	Comparison of fish communities on dams
		Overli et al. 2004	Coping styles causes and consequences in social status
Radio transmitter	Capture, handling anaesthesia, restrict to age	Young 1994	Mobility of brown trout
		Keefer et al. 2006	Long-distance movements
Tattoos, dye injection	Capture-recapture, handling anaesthesia, restrict to age, hand identification occasionally	Hart & Pitcher 1969	Field trials using jet inoculator
		Louette & Declerck 2006	Evaluation of fyke nets as a sampling technique
Fin clipping	Capture-recapture, handling anaesthesia, restrict to age, hand identification	Wagner et al. 2006	Use of pheromones to control lamprey
		Blann & Healey 2006	Competitive ability of salmonids
VIE tags	Capture-recapture, handling anaesthesia, restrict to age, hand identification	Jensen et al. 2008	Marking of brown trout alevins
		Curtis & Vincent 2006	Survival, growth and movement patterns
PIT tags	Capture-recapture, handling anaesthesia, restrict to age	Armstrong et al. 1999	Individual space use strategies

	Imsland et al. 2006	Effect of temperature and fish size on growth and feed efficiency ratio

Some statistics on the use of tags in fish biology and fisheries research

Table 2.4 shows the results of an analysis of the use of different identification techniques in studies published in two key ichthyological journals (*Canadian Journal of Fisheries and Aquatic Sciences* and *Journal of Fish Biology*) for the period form 2000 to 2005. Clearly, a huge number of fish were marked using a variety of techniques. This is the case even though some studies did not mention the number of fish marked, so the figures are underestimates.

Table 2.4 Number of fish marked by different methods from 2000-2005 in studies published at Canadian Journal of Fisheries and Aquatic Sciences and Journal of Fish Biology.

Technique	Number of Fish
External tags (metal/plastic/ribbon)	102,922
Otholith marking	44,096
Radio/Acoustic transmitter	10,343
Dyes	9,150
PIT tag	8,617

Development of less invasive methods for identifying fish

Such invasive methodologies can be traumatic for the fish concerned and in the UK for example, much tagging is carried out under a Home Office license (Scientific Procedures Animals Act 1986). It is in the interest both of animal welfare and scientific quality to reduce the need for invasiveness of such procedures. No one is more aware of the necessity to reduce discomfort to an animal than the biologist who needs accurate scientific information. The prime requirement of a marking technique are that it should not modify the behaviour, mobility, dispersal, health, survival or any other aspect of the life history of the marked animal. Such practical considerations are additional but, related to, the biologist's ethical concern for the welfare of the animal. Conducted properly, tagging can afford a wealth of information including fish movements, fish populations, migration patterns, as well as statistics such as birth rates, mortality rates, and harvest levels. However, improperly conducted tagging programs can lead to collection of misleading information, due to increased mortality (Calvo & Furness 1992) and impaired growth (Cookingham & Ruetz 2008) in tagged fish. Hence the need for less invasive techniques.

Molecular biological techniques such as DNA profiling now allow variation in genotype to be used as a natural marker. Individual identification by DNA technology is increasingly

being used, though this requires collection of tissue and it is costly. Skin biopsy or sloughed skin samples from free-ranging humpback whales *Megaptera novaeangliae* were collected in a study to estimate abundance by mark-recapture (Palsbøll et al. 1997). Another, older, alternative to using invasive marks as tags involves visual identification of individuals using naturally occurring variation in phenotype. The ability to recognize individuals from natural differences in appearance has a number of advantages over invasive marking techniques. For example, if the features are sufficiently large to be seen from a distance, animals do not need to be physically captured. Some identifiable features persist over time, allowing individuals to be used in long term studies. For example, Tienhoven et al. (2007) showed that natural pigment marks of spotted raggedtooth shark (*Carcharias taurus)* are stable over time and can be used to track animals over several years. The use of natural marks is also preferable to artificial tagging, as it is relatively stress free, so the behaviour of the animal concerned is unlikely to be affected by the identifying trait. Finally, natural marks are cheaper than the artificial ones.

Natural body markings have been used successfully to identify individual animals in both terrestrial and aquatic environments for a variety of species, from Bewick's swan *Cygnus columbianus* (Scott 1978 using bill pattern) to cheetah *Acinonyx jubatus* (Caro & Durant 1991 using coat pattern). Table 2.5 summarises a number of such studies.

Table 2.5 Examples of the variety of naturally-varying morphological characteristics used to identify individual animals

Characteristics	Group	Example
Bill pattern	Bewick's swan	Scott 1978
Pelage pattern	Grey seal	Karlsson et al. 2005
Facial scale pattern	Sea turtle	Schofield et al. 2008
Pigmentation marks	Shark	Van Tienhoven et al. 2007
Dorsal pigmentation pattern	Salamander	Gamble et al. 2008
Melanophore pattern in eye and jaw	Atlantic Salmon	Leaniz et al. 1994
Dot pattern	Grayling	Persat 1982
Pigmentation on ventral side of fluke	Humpback Whale	Smith et al. 1999
Body pigmentation pattern	Brown Trout	Aparicio et al. 2005
Coat pattern	Cheetah	Kelly 2001
Parr marks	Japanese Charr	Yagyu et al. 2007
Scars	Dolphins	Lockyer and Morris 1990
	Mirror carp	Adamek et al. 2007
	Sea otter	Gilkinson et al. 2007

Photographic identification has proven to be a useful tool in long term monitoring of animal populations and is being used increasingly in studies of a wide range of animals. Here researchers photographically capture natural characteristics such as facial marks, scars, coloration patterns to identify and re-identify individuals. Thus Bradshaw et al. (2007) photographed whale shark (*Rhincodon typus*) for a capture-mark-recapture study

to estimate survival and capture probabilities. Photographs of the head of Loggerheads sea turtles (*Caretta caretta*) were used to explore the potential for both naïve and trained observers to use natural facial markings to identify individuals (Schofield et al. 2008). Photographs of natural spot patterns have been used to create a reference system catalogue in raggedtooth shark, in which species show that natural pigment marks have proved to be a reliable means of tracking individuals over several years (Van Tienhoven et al. 2007). Comparison of results from photographic and genetic identification based on microsatellite genetic markers in humpbacked whales confirmed that natural markings provide a reliable way of identifying individuals on a large scale (Stevick et al. 2001). Natural pigmentation patterns have been used to recognize various kinds of animals, including fish (Persat 1982; Bachmann 1984).

During routine procedures with Atlantic salmon and brown trout alevins in the field Garcia de Leaniz et al. (1994) noticed that melanophore spot patterns in the head region of these fish were highly variable. These subsequently proved to be unique even among closely related individuals and consistent over time and were used to tack growth and movement patterns on newly emerged wild fish. A subsequent study by Donaghy et al. (2005) showed that photographs of melanophore patterns in juvenile Atlantic salmon could be successfully and reliably matched over a period of 16 months.

Persat (1982) photographed more than a hundred grayling *Thymallus thymallus*, 40 of which were recaptured and correctly identified using photographic records even more than a year later. To identify the fish the number and position of black dots on the flanks were used; in some cases, when there were few dots or none at all, it was necessary the use of other features, such as the general disposition of the scales. In the grayling (Figure 2.12), the scales are mostly well ordered in parallel lines and the general arrangement of the scales is stable judging from photographs and can only be modified by wounds. In this case, therefore, the disposition of scales is a useful marker for individual identification.

Figure 2.11 Photographs of grayling (*Thymallus thymallus*) showing the disposition of black dots and the lines of scales. Source: http://eau.douce.free.fr/photos%20poissons/photos%20europe/thymallus%20thymallus.htm

As with other marking techniques, use of natural marks for recognising fish has some disadvantages. Thus, it is restricted to the variation that nature provides, for example with respect to the number of variants that can be identified and how easy these are to observe from a distance. In addition, it is necessary to establish how consistent natural variation is over time. The differences may be subtle, making them difficult and time consuming to use. It is possible that natural differences in coloration are associated with differences both in behaviour and habitat choice; where this is the case, their use may generate biased or partial information. A classical example is the peppered moth resting behaviour, peppered moths (*Biston betularia*) are cryptically camouflaged against their backgrounds, *typica* or white-bodied moths are camouflaged against lichens and *carbonaria* or black-bodied moths against plain bark (Steward 1977). Had this natural colour variation been used as a batch mark for studies of predation, for example, different results would have been obtained depending on the habitat in which the study was performed.

The common carp and its scale patterns

We report here on a study aimed at determining whether natural differences in scale patterns among common carp can be used for individual recognition.

The common carp is a cyprinid fish that originated in Western Asia and naturally dispersed to China, Siberia and the Danube basin. The carp was spread throughout Europe by monks between the 13th and 16th centuries as a food fish and has now been introduced to all continents. There are four main scale types displayed by carp (figure 2.13):

- Fully scaled carp (common or wild type, regular scales over the whole body)
- Mirror carp (small number of large, randomly clustered scales on body)
- Linear carp (usually a single row of large scales along lateral line)

- Leather carp (very few or no scales on body).

Figure 2.12 The 4 types of common carp using scale patterns. 1) fully scaled carp, 2) mirror carp, 3) linear carp and 4) leather carp.

Within each broad category, there is considerable variation in number, size, shape and position of scales that could potentially be used in individual identification.

Specific aims

With this background, the broad aim of the study described here was to test the effectiveness of a non-invasive method for identification of carp by eye from a distance, based on scale pattern. If such identification were possible, an additional aim was establish how difficult this is and whether the patterns remain sufficiently consistent to allow individual identification. Specific tasks involved were to:

- Determine whether individual carp within a population can be recognized by human observers on the basis of scale patterns alone, without reference to body size;
- Estimate the time required for such identification;
- Examine consistency in identification of fish by different observers;
- Establish whether naïve observers could easily and reliably learn to use photo-identification to recognise individual mirror carp;
- Determine whether scale patterns are sufficiently consistent to allow the same fish to be identified after a 3 months period.

1.1.2 Material & Methods

Subjects and husbandry

A group of 60 mirror carp were obtained from Barony College, Dumfries, UK. The carp were transferred to University of Glasgow and kept in 2 glass tanks (100 X 38 X 31.5 cm), one with 27 and the other with 33 carp. The temperature of the tanks was at 18ºC.

Collection of images

A month after the fish were established in their holding tank, 15 fish were chosen randomly from the group, lightly anaesthetized using benzocaine (HO licence nº 6003679) and photographed in air from the left side. Photographs were taken using a digital camera (Sony, model Cyber-shot 7.2 mega pixels (DSC-W70)). They were printed in black and white and laminated. A second set of photographs of the same fish (recognized by alcian blue dye marks not visible in the photographs) were taken in the same manner, 3 months later. The photographs were again printed in black and white (to preclude identification by any colour patterns); all images being printed at the same size (to avoid identification by size). The 15 photographs taken at the first session were given a number and those from the second session were assigned a letter, the number-letter pair being chosen randomly. Figure 2.14 shows two representative examples of these images.

Figure 2.13 Example of 2 of the photographs of carp used in the identification test.

Identification of images

12 volunteers (profiles given in table 2.6) were recruited from the Division of Ecology & Evolutionary Biology, University of Glasgow, to form an identification panel. All panel members were biologists and some were fish biologists, 6 being female and 6 male, ranging in age from 24 to 68 years old. Each panel member was given 15 individual cards (labelled from A to O), each with the LHS image of one fish taken at the second session.

S/he was also given a sheet with LHS photographs of all 15 fish (numbered from 1 to 15) taken at the first session. The volunteers were instructed simply to compare the photographs and match the pairs (number-letter) using whichever methodology they chose. The researchers recorded the time each volunteer took to complete matching of each image to the volunteer's satisfaction and whether each letter/number pair was correctly matched. After matching was completed, panel members answered the question: ''what strategy did you use to identify the carp?''.

Data analysis

From the records, the following variables were recorded: the time taken by each volunteer to identify each photograph, the total time taken by each volunteer to identify all fish, the order of identification and, for each image, whether identification was correct. The data analysis was made using Minitab series 15.

1.1.3 Results

Identification of images

Table 2.6 gives details of the mistakes made by each volunteer. From the 12 panel members, only 4 made mistakes matching the photos and all of these were made by males (Chi-square = 6.00, DF = 1, p = 0.014). Thus, in the vast majority of cases, the fish were correctly identified of pattern in the absence of additional information as size (162/180).

Table 2.6 Profile of the 12 volunteers in the study and the number of mistakenly identified images of 15.

Volunteer	Sex	Age range	Fish Biologist?	No of mistakes	Number of mistaken photos	Total time (s)
1	Female	26-30	no	0	-	720
2	Male	36-40	yes	3	9-11-12	1,596
3	Male	20-25	no	0	-	455
4	Female	26-30	yes	0	-	1,014
5	Female	26-30	yes	0	-	535
6	Male	61-70	no	2	2-8	1,169
7	Female	26-30	no	0	-	1,058
8	Female	20-25	no	0	-	1,470
9	Female	20-25	no	0	-	935
10	Male	31-35	no	2	2-3	698
11	Male	46-50	yes	0	-	409

Variability in time taken to identify images

Table 2.6 also shows the time taken to identify all 15 photos and figure 2.15 displays the mean completion time for male and female. The volunteers varied, with time to complete identification ranging from 409 to 1,596 seconds. There was no effect of gender on completion time; the median time for females was 955±131s and for males was 827±189s (Mann-Whitney test: W= 44.0, p = 0.4712). There was no effect of age on completion time (Kruskal-Wallis test: H_6 = 6.33, p = 0.388). Nor did the experience of the volunteer (whether or not they were a fish biologist) influence the time taken to match the images; thus the mean (SEM) for fish biologist was 889±269s and for not fish biologist was 892±117s (Mann-Whitney test: W = 54.0, p = 0.7989). The two males that made no mistakes were fastest to complete the task, so errors were not the results of over-hasty decisions.

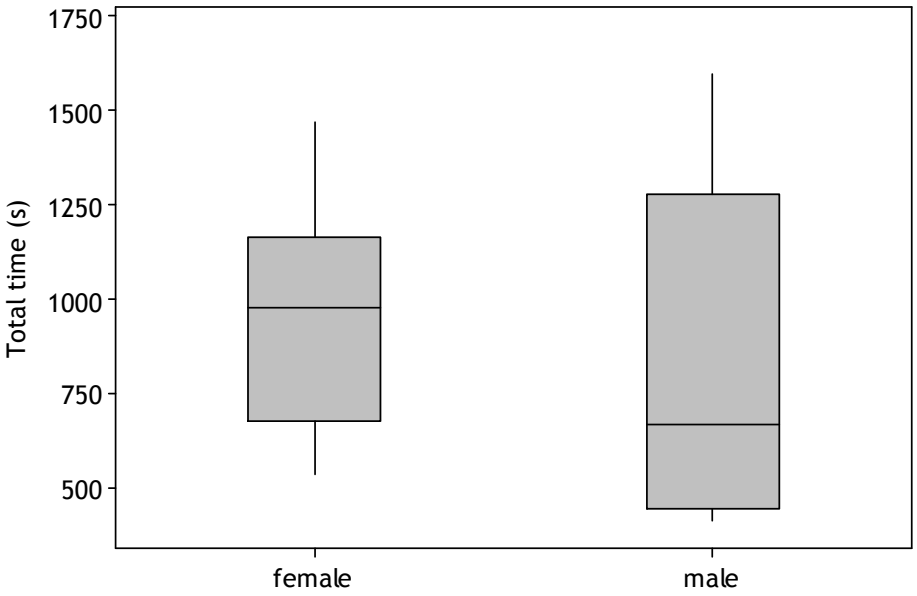


Figure 2.14 Median (IQR range) time (s) taken to complete the identification test by volunteer classified by gender.

Identification time and sequence for specific images

Table 2.7 shows the mean time taken by the 12 volunteers to identify each image and the mean (SEM) sequence in which images were identified. Kruskal-Wallis test shows no significant effect of image number of identification time (H_{11} = 11.0, p = 0.443), but a significant effect on identification sequence (H = 33.08, p = 0.003). Image number 2, 7 and 15 (Figure 2.16) tended to be identified earlier than the other images. These images are characterised by a relatively small number of large scutes in the midline.

Table 2.7 Mean (SEM) time taken by the 12 volunteers to identify each image and mean (SEM) sequence for each image.

Image	Mean (SEM) ID time	Mean (SEM) sequence
1	559.9 ± 313.5	7.64 ± 3.11
2	436.7 ± 348.5	5.11 ± 2.57
3	527.8 ± 365.7	8.60 ± 4.14
4	665.5 ± 396.7	9.91 ± 4.37
5	534.8 ± 365.8	7.00 ± 5.49
6	542.4 ± 314.7	7.09 ± 3.75
7	448.2 ± 353.9	5.54 ± 4.01
8	645.0 ± 402.4	10.70 ± 4.32
9	601.4 ± 449.1	8.60 ± 4.88
10	599.9 ± 304.4	8.45 ± 3.53
11	693.7 ± 332.6	10.70 ± 3.94
12	547.7 ± 342.0	8.00 ± 4.69
13	740.4 ± 312.9	10.91 ± 2.77
14	575.5 ± 311.5	7.45 ± 4.01
15	349.7 ± 187.5	4.18 ± 3.52

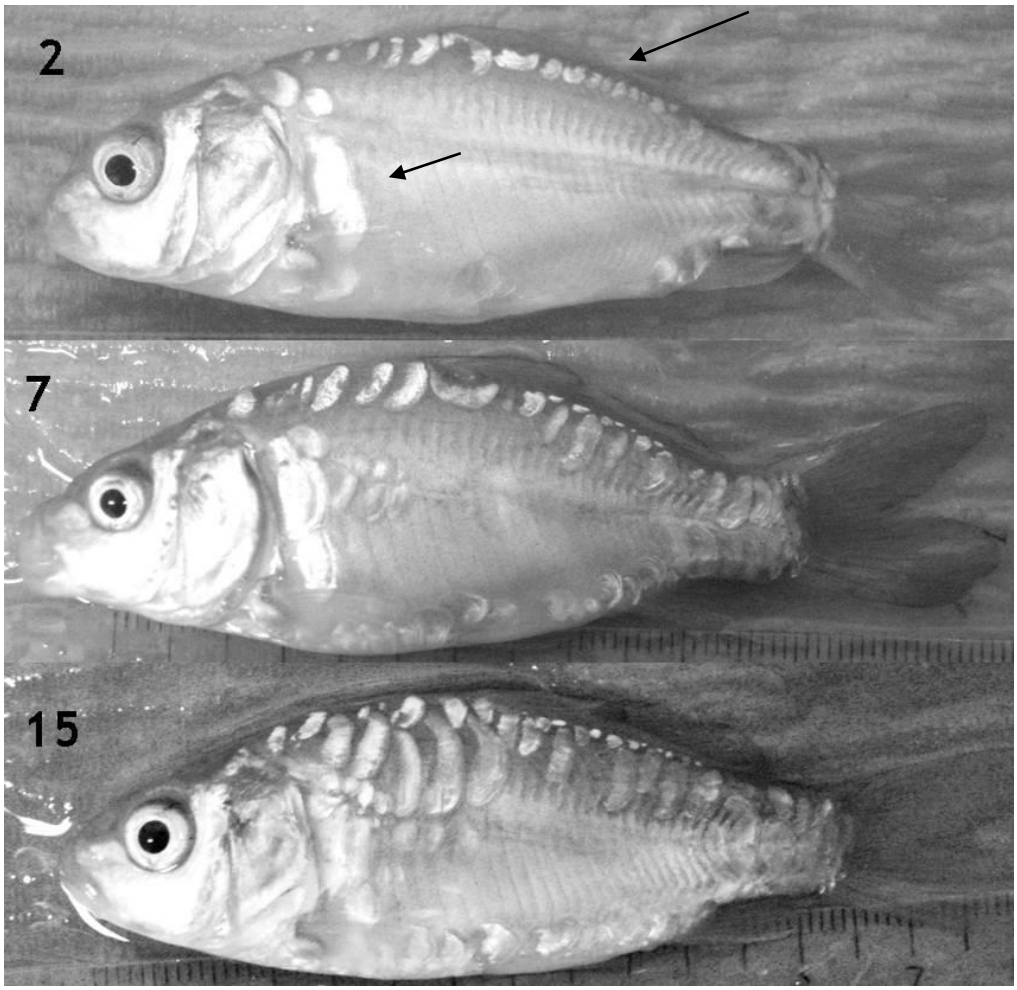


Figure 2.15 Image of the 3 carp identified early by volunteers. Arrows in first image shows features used by volunteers in the identification: scales near operculum and dorsal line of scales.

Figure 2.16 shows the cumulative time taken to identify successive images by 3 volunteers, selected to show different temporal patterns. Volunteer 6 made steady progress, taking similar amounts of time to identify successive images, while volunteer 8 was slow to identify the first few images, but then improved quickly. In contrast, volunteer 9 was slow at the start, then quickly recognized 9-10 images and took longer to identify the last 2.

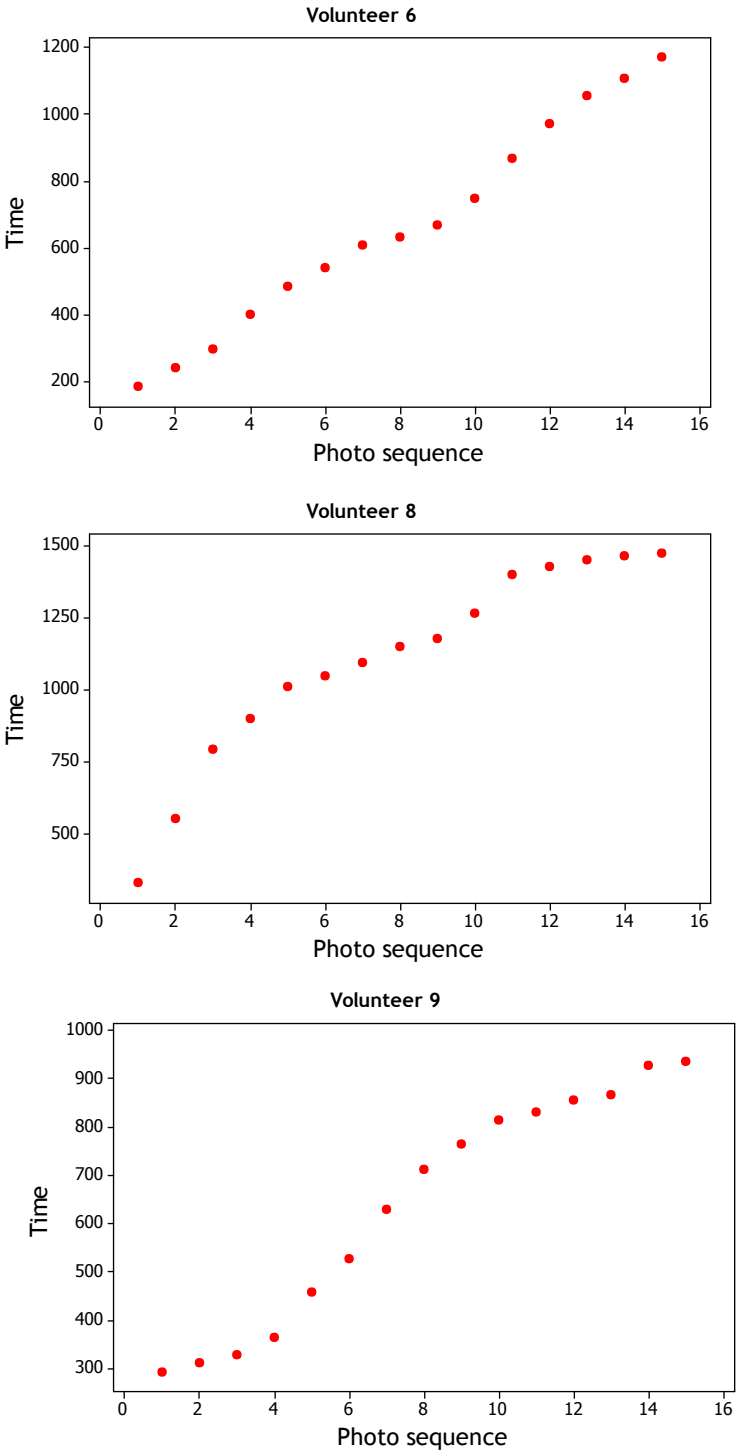


Figure 2.16 Example of three volunteers showing individual variability in temporal pattern and in identification sequence. The numbers in the graphs are the picture numbers.

Strategies used by volunteers

The volunteers were asked to comment on the strategy they used to identify the images and all reported that they used the pattern of scales at the fish's body. The majority used the dorsal line of scales to identify, comparing shape, size, position and regularity, but some also mention scales near the operculum as being particularly informative (see figure 2.16).

1.1.4 Discussion

The fact that our subjects mostly matched up the images correctly on the basis of visual features other than size, with just a few making (mostly a few) mistakes, shows that photographs taken at an interval of several months can be used to distinguish among individual common carp over relatively long periods. Most subjects used scales for matching images, particularly those along the back and behind the operculum. Thus natural characteristics can be used for accurate identification of mirror carp; once an image has been assigned to an individual, its marking can be used to match it to an existing catalogue of images from a group of fish. Photo-identification of natural features is increasingly being used to collect data on individual animals, for example Schofield et al. (2008, sea turtle), Gamble et al. (2008 marbled salamander, *Ambystoma opacum*), Yagyu et al. (2007 Japanese charr, *Salvelinus leucomaenis*) and Van Tienhoven et al. (2007 spotted raggedtooth shark). Obviously, the quality of photographs is important (Bateson 1976). Kelly (2001) showed that when low-quality photographs were excluded from the analysis the probability of matching Serengeti cheetahs using coat pattern increased from 59% to 80%.

Our group of subjects included several volunteer with experience of working with fish and one who had already worked with carp. Experience did not influence the outcome in terms of success rate for matching of images, which was high in most cases. Neither did it influence the speed of identification. This suggests that the method could be readily used by inexperienced observers, although with larger groups of fish experience might be more important. In a study with Bewick's swans based on individual identification from photographs, an experienced observer claimed (correctly) to be able to identify some 450 swans (Bateson 1976).

Our observers took between 6 and 27 minutes to identify all the images (mean = 579.31 ± 24.69). This compares with observers in the study of Garcia de Leaniz et al. (1994), who took between 1-5 minutes to correctly match 30 trout from close-up photographs

taken 4 weeks apart on the basis of patterns of melanophores on the jaw. Thus identification by natural marks identification can be time consuming and rather hard on the eyes. Computerized image analysis is often used to assist in the process. Examples include recognition based on coat pattern on cheetahs (Kelly 2001), shape and colour on mackerel (Strachan et al. 1993), wounds in mirror carp (Adamek et al. 2007), dorsal pigmentation patterns in salamanders (Gamble et al. 2008), pigmentation marks on sharks (Tienhoven et al. 2007) and dots patterns in grayling (Persat 1982). In computerized image analysis a reference system is created to enable comparison of the relevant markings, animals are mapped (based on an algorithm) and a score is calculated to indicate the quality of the match between the pictures.

For the future, we plan to use a kind of image analysis that highlights the scales and make them clear to see facilitating fish identification. Figure 2.17 shows an example of how this can be done using Metamorph software.

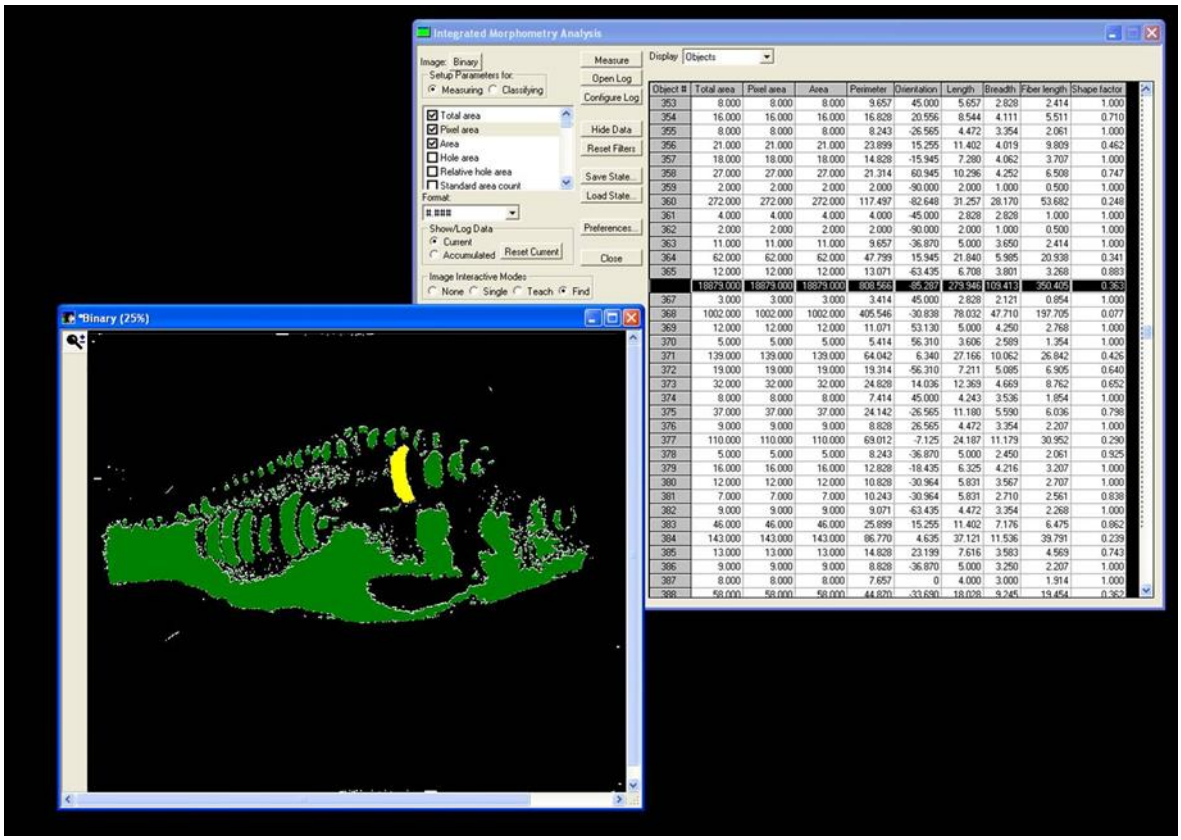


Figure 2.17 Example of image analysis that can be made to facilitate fish identification based on scales pattern.

As well as a high degree of stability in natural marks, a large number of variants is also desirable if they are to be used for identification. Persat (1982) calculated that the

probability of two individual grayling from the study river being identical in terms of dot pattern was of 1:1021. If the distribution of dots on the body was completely random, which is probably not the case; there would never have been two identical fish with thirteen dots since the species appeared. Thus, the probability of making a mistake when identifying the fish photographs was almost nil. Other morphological traits that might be used in addition to scales were identified, including features of the operculum. Aparicio et al. (2005) used five qualitative and seven quantitative variables to identify individual brown trout; these included the pattern of the stripes along the side of the body, black spot behind the eye, red spots on adipose fin, number of black spots on gill cover and number of black and red spots above the lateral line.

In conclusion, scale patterns in common carp can be used for reliable, non-invasive identification of individual fish. Combining scale patterns with other characteristics such as body shape, weight and size is likely to increase the accuracy and speed of matching and so could be used as a replacement for more invasive tagging techniques.

2 Screening for risk-taking

An important behavioural variable used throughout this thesis is “risk-taking”. This refers to an individual’s propensity to put itself in danger in a variety of situations. At one end of the spectrum of variability are risk-taking individuals (sometimes referred as “bold”) that ignore danger; at the other are risk-avoiders (sometimes called “shy”) that are sensitive to danger and avoid dangerous situations. Level of risk-taking can be evaluated using a number of different behavioural screening tests, including: foraging under predation risk (Bell 2005), resumption of foraging after predator attack (Ward et al. 2004), tendency to approach a predator (Dugatkin 1992), response to novel objects (Frost et al. 2007), exploration of novel environments (Huntingford et al. 2010), time to resume feeding in a novel environment (Ruiz-Gomez et al. 2008), escape behaviour (Korte et al. 1996) and behaviour in an open field (Sneddon 2003). More detailed information about risk-taking phenotypes can be found on chapter 1. In this study response to a novel environment and a novel object test were used to assess risk-taking phenotype, the aim being to develop methodologies, to check for individual consistency and to determine how many tests to perform.

2.1 Fish and general husbandry

6 carp were obtained from Barony College, Dumfriesshire, and kept at experimental aquaria at Graham Kerr Building, Glasgow University, at a temperature of 22^{o}C. Fish

were individually marked using alcian blue dye and tested in one group of six individuals (HO Project Licence number 60/3679).

2.2 Pilot screening for risk-taking: novel environment test

6 fish were screened for risk-taking using a variation of the well-established novel environment test (Yoshida et al. 2005). The screening tank was 100 cm by 38 cm by 31.5 cm, with a water depth of 30.5 cm (figure 2.19). Temperature was matched to that of the holding tanks (22°C). At one end of the screening tank there was a enclosed, darkened settling chamber (30 cm in length), from which a plastic tunnel (9.5 cm length and 9 cm diameter, with its base 5.5 cm from the bottom of the tank) formed an exit into the main section of the tank.

The opening to the tunnel was fitted with a removable plastic cover. The main section of the tank was covered with gravel and was illuminated from above representing a novel, potentially dangerous environment for exploration. Food was placed on a clear 3L container (14 cm in diameter and 30 cm in height) located in the centre of the main section, visible through the tunnel once the cover had been removed, fish did not have access to the food in the container.

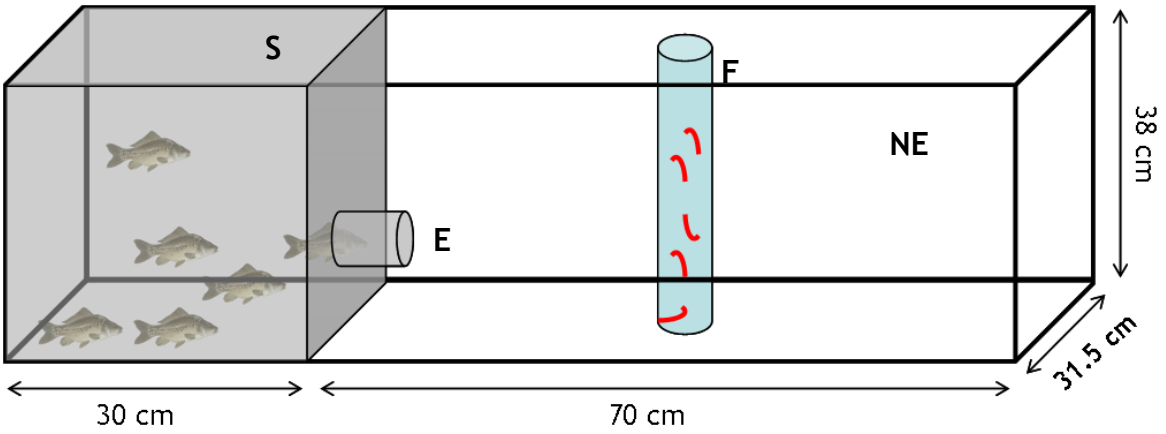


Figure 2.18 Schematic diagram of the tank used in the novel environment test. S = shelter, E = entrance to main section with removable cover, F = transparent container with food, NE = novel environment.

Prior to testing, defrosted bloodworm were placed in the transparent tube, with an airstone to create turbulence within the container and movement the bloodworms. Fish were deprived of food for at least 12hs prior to each trial (to standardize stomach

fullness) and the group of 6 was placed into the shelter to settle down for 15 minutes. Olfactory cues were introduced by adding a small amount of water in which bloodworm had been macerated to the tank. The time (in seconds) taken for each fish to leave the settling chamber was recorded, up to a maximum of 20 minutes; fish that failed to emerge were given a notional, high score of 2000 seconds. The test of the screening procedure was repeated 13 times with a interval of 24hs. All the fish were housed together between trials and were tested once a day. This screening potentially enables us to divide the group of fish into categories on the basis of time to emerge from shelter into the feeding chamber.

2.3 Pilot screening for risk-taking: novel object test

Another test to assess risk-taking behaviour is the novel object test (Frost et al. 2007). Individually identified common carp were placed in a group of 6 in a large glass tank (figure 2.20). After observing the fish for 5 minutes in aspects such as position and distance from where the object would be positioned, a novel object was dropped approximately 10 cm from the front of the fish. The subjects' behaviour was recorded for a further 10 minutes. Subjects were assessed for their location in the aquarium and their response to the object (see below). The novel object consisted of a Lego brick (approximately 6 cm in height) constructed of various colours and was either floating or sinking (5 times each).

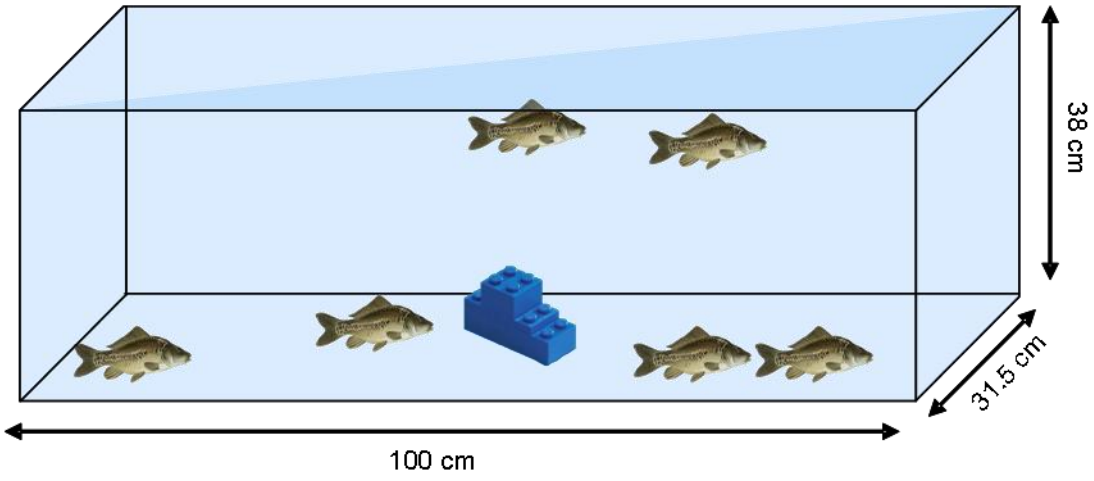


Figure 2.19 Schematic diagram of tank used in the novel object test.

2.4 Results and discussion

2.4.1 Novel environment test

The data were not normally distributed, so we used non-parametric statistics. Kruskal-Wallis showed highly significant decrease in emergence time with tests number, though all fish failed to emerge on test 6 (GLM Repeated Measures: $F_{10} = 70.03$, $p < 0.001$, test 1 ≠ from 5 on - $p < 0.001$), probably because this test occurred on a Monday, after a weekend, in which the fish were inadvertently fed (figure 2.21).

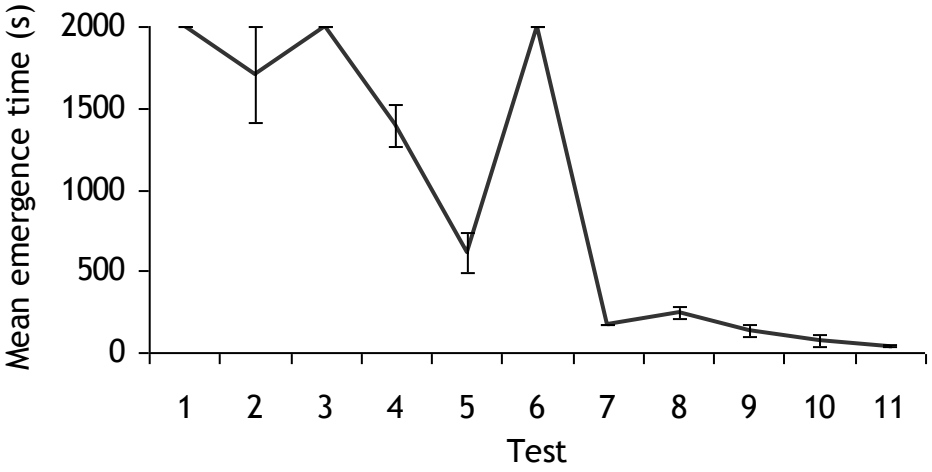


Figure 2.20 Mean (SEM) emergence time for all carp in the novel environment test. (N=6).

Almost no fish emerged in the first three tests, so these failed to provide any discrimination between the fish. In test 6, which followed a weekend, again no fish emerged. By tests 8-11, all fish were emerging fast, presumably having habituated to the test set up, so these too did no provide a useful discrimination. Omitting these non-informative tests, figure 2.22 shows that time to emerge clearly decrease with test.

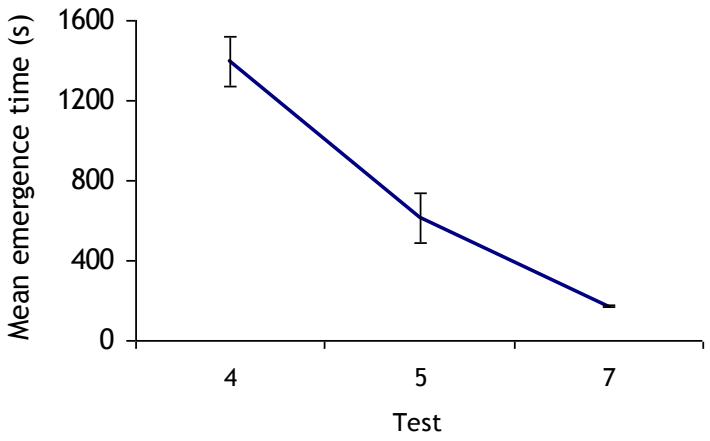


Figure 2.21 Mean (SEM) of emergence time on tests 4, 5 and 7 of the novel environment test.

Consistency in individual risk-taking was examined using Kruskal Wallis on the sequence in which fish emerged from the settling chamber. Kruskal-Wallis test showed no significant individual effect when comparing emergence time and fish number (H_5 = 0.61, p = 0.987). Using only tests 4, 5 and 7 and using the emergence rank (figure 2.23), Kendall's coefficient test was not significant (W = 0.618, p > 0.05). This low level of consistency may be because the number of trials run allowed the fish to habituate, obscuring individuals' differences. With the subsequent learning trials, the overall performance of individual fish in the risk-taking trials was summarised by the median of their emergence ranks across all tests (figure 2.23).

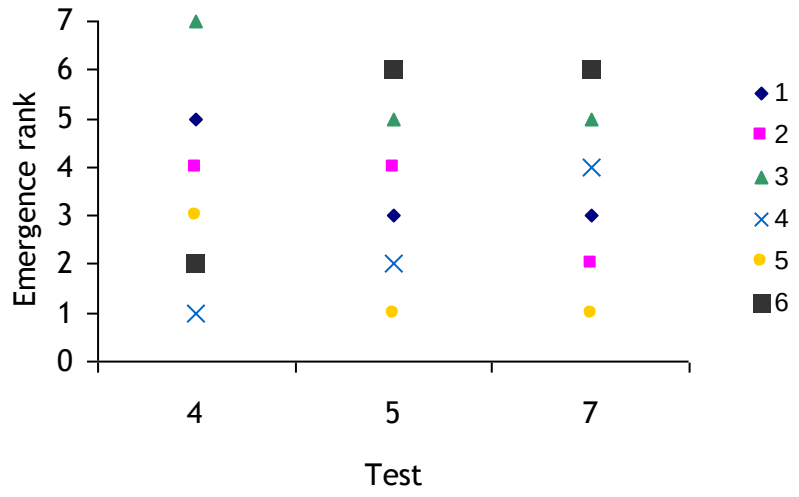


Figure 2.22 Emergence rank of the 6 carp tested on novel environment test considering tests 4, 5 and 7.

2.4.2 Novel object test

When the novel object was floating, carp approached it very fast and tried to bite it, potentially associating it with food. However, when it was weighted down, fish swim around it, inspecting it without approaching it, probably indicating they were afraid of it and avoid getting closer. This indicated that such a novel object could potentially be used as an independent test of risk-taking in carp, but that a smaller stimulus might elicit greater variability in behaviour. It was not possible to correlate this test with the novel environment test because the fish stayed all together far from it, and therefore it was not possible to evaluate individual responses.

After screening the fish to assess risk-taking, they were submitted to the experiment where the purpose was to train the fish to associate a source of light to food and therefore associate learning skills with risk-taking phenotype.

3 Pilot of training to associate light with food

3.1 Introduction

As discussed in chapter 1, there are many definitions for learning; one refers to a change in behaviour with experience, inferences about learning being based on examination of changes in the behaviour. For example, naïve Nile tilapia when trained to avoid predators changed their behaviour after several trials, switching to a different/new behaviour (Mesquita & Young 2007). Fish, like other animals, have the ability to learn; the experimental evidence for learning is widespread and dates back to the late 1800s. Several aspects of learning have been explored in fish, including foraging, migration, avoidance, social behaviour and spatial cognition (Kieffer & Colgan 1992).

Spatial learning happens when the animal learn to follow specific cues/landmarks to arrive at a certain place. Many species of fish have been trained to use landmarks as goal-directing cues (Odling-Smee & Braithwaite 2003). Individuals differ in rates of learning and differences in risk-taking may be responsible for some of this. For example, Sneddon (2003) found that bold rainbow trout (classified by the amount of time spent in an open area) learned to associate light with feed delivery in fewer trials that the shy fish, the task consisting of approaching a feeding ring when a light was switched on to receive food pellets. One aim of this thesis was to explore the link between risk-taking

phenotype and the capacity of fish on spatial learning and a pilot study was previously conducted to develop and test methodologies.

3.2 Methodology

The same group of 6 fish were divided into 3 pairs tentatively classified as risk-taking, intermediate and risk-avoiding according to the novel environment tests. The fish were tested in pairs due to its strong social behaviour as isolated carp show abnormal behaviour. Each pair was tested 13 times, the fish were separated in: one pair of risk-takers, two pairs of one intermediate and one risk-avoiding fish (see table 2.8).

At one end of the screening tank there was an enclosed, settling chamber (30 cm in length), from which a plastic tunnel formed an exit into the main section of the tank (figure 2.24). The opening to the tunnel was fitted with a removable plastic cover. At the other end, at the main section, the aquarium was divided into 2 sides were a Petri dish was put at each side. The fish could only see the Petri dish if it swam up to the entrance to the feeding compartment.

Outside the aquarium, two light sources with different colours (blue and yellow) were set up. These colours were selected because they are in the visual spectrum of carp, but differ clearly in wavelength. At the first 4 trials, both torches were on and there was food at both sides (pre-training sessions).

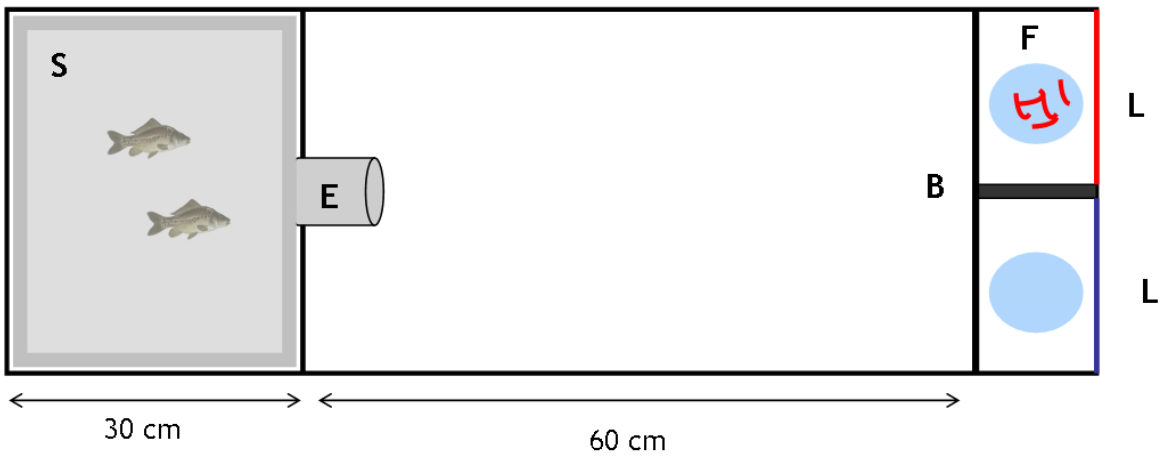


Figure 2.23 Schematic diagram of tank used to train a pair of common carp to approach a light to find food. S = shelter, E = entrance to main section, B = barrier that avoid carp do see the food from far, F = food in Petri dish, L = light (yellow on one side and blue on the other side).

From trial 5 onwards the food was given only at one of the sides (training sessions), for pair 1 the “correct” side was the one illuminated by the blue light and for pairs 2 and 3

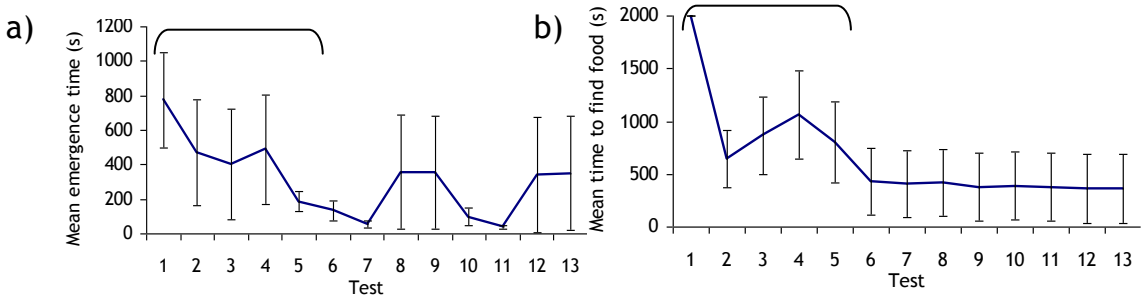
the “correct” side was the one illuminated by the yellow light. The lights remained on throughout the trial. The position of the light was changed at each 2 trials, thus in trials 4-5 the blue light was on the left side and yellow at the right side; trials 6-7 had the blue light on the right side and the yellow at the left side of the aquarium.

Table 2.8 Pairs of fish used in the learning sessions with risk-taking phenotype and colour of light used in the learning.

Pair	Fish	Risk-taking phenotype	Correct light
1	3	Risk-avoider	Blue
	4	Intermediate	Blue
2	2	Intermediate	Yellow
	6	Risk-avoider	Yellow
3	1	Risk-taker	Yellow
	5	Risk-taker	Yellow

3.3 Results and discussion

GLM test showed that the time the fish took to emerge from the settling chamber fell with test number ($F_{1,12}$ = 3.46, p = 0.001). Figure 2.24 shows time fish take to leave the box and to feed. Emergence time dropped in from test 1 to test 2 then continued to fall slightly. Time to eat fell with test number and was maintained until the end of the tests ($F_{1,12}$ = 7.15, p < 0.001). Time to eat continues to drop with test number, as expected if the fish are learning (though also if they were simply acclimatising the test set up). Search time (the time between leaving the shelter and finding food) fell rapidly between trials 1 and 2 and gradually thereafter. For all measures there was considerable variability between fish.



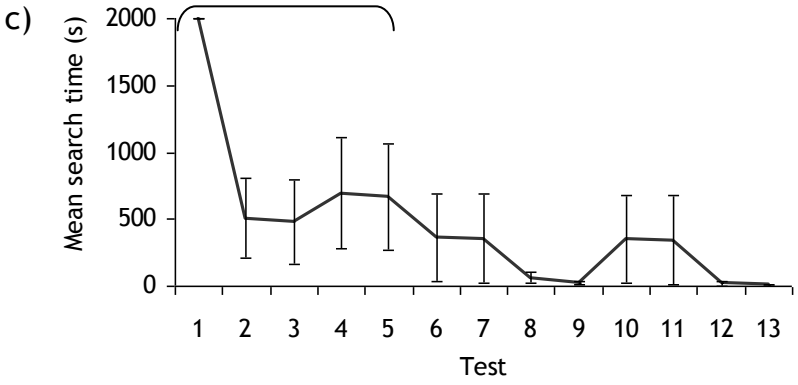
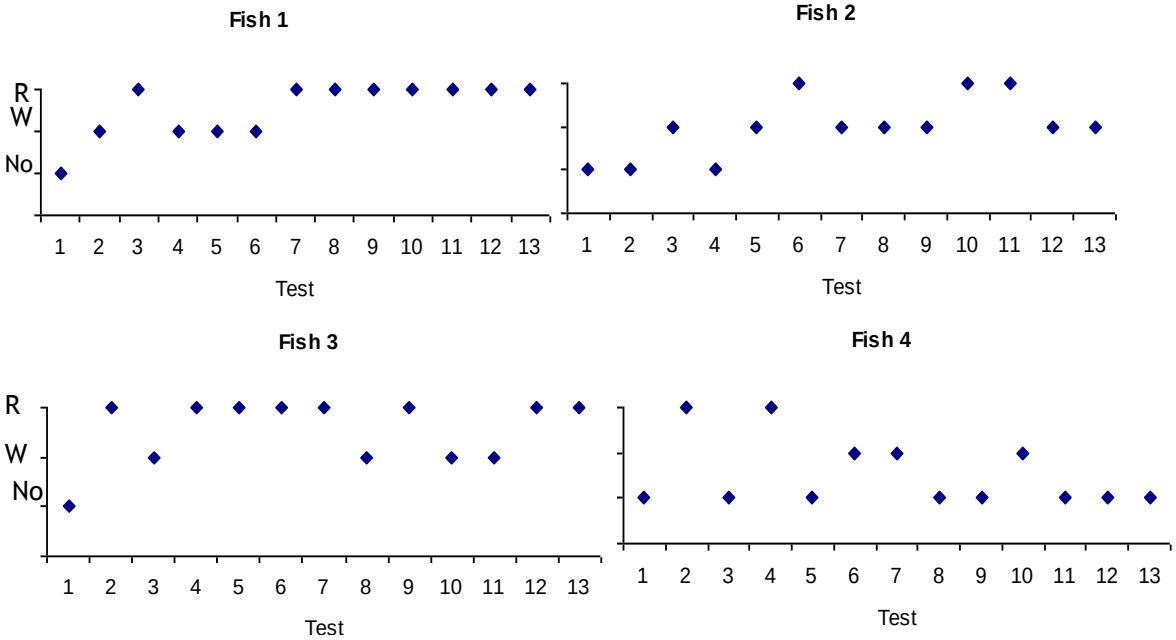


Figure 2.24 Mean (SEM) a) emergence times of carp in the learning trials, b) time fish take to find food, c) search time. Brackets represents settling trials.

Figure 2.25 shows the sequence of right and wrong responses shown by each fish and table 2.9 summarises these results. Some fish (fish 1 and 5) showed a strong tendency to approach the chamber with the rewarded light. Such fish seemed to have learned using landmarks, as they only went to the wrong side in the beginning of the tests, choosing the correct side in all subsequent tests, although fish 5 took longer to emerge and feed than fish 1. Other fish (fish 2 and 3) may have learnt that food will be available in one or other chamber, but not which. These fish would swim to either chamber at random and switch immediately to the alternative chamber if they found no food. Fish 4 failed to emerge in most of the trials and did not feed in the ones in which it did emerge.



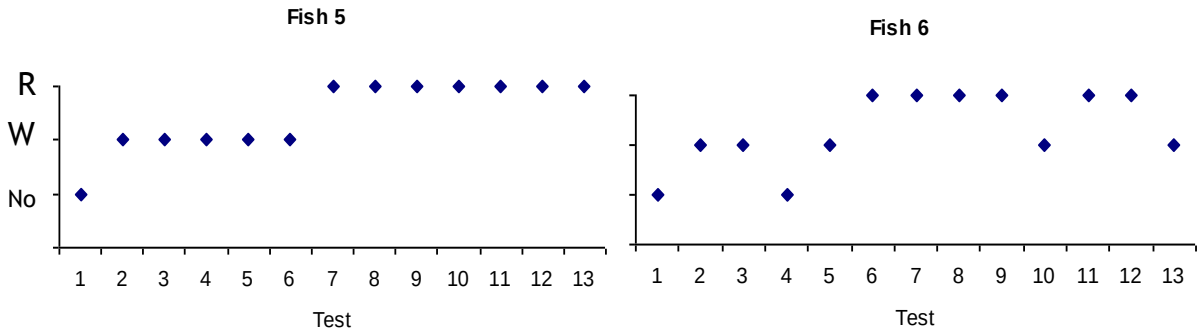


Figure 2.25 Sequence of side fish went to find food through tests. Y axis: R = correct side (rewarded side with food), W = wrong side (without food) and No = fish did not emerge from shelter.

Table 2.9 also allows the comparison of behaviour in the learning tests of fish tentatively assigned to different risk-taking phenotypes on the basis of the novel environment tests. Not surprisingly, since the initial allocations were tentative and sample sizes are small, there are few relationships. Although just one group of fish was tested, these preliminary results serve as a basis for designing the subsequent experiments. They were also valuable in developing my screening skills.

Table 2.9 Mean time to emerge, eat and number of "corrects" and "wrongs" for each fish on tests 5 to 13.

Fish	Risk-taking phenotype	Mean time to emerge	Mean time to eat	Right colour	Wrong colour	Strategy
1	Risk-taker	55.23	92.67	7	2	Learn cue
2	Intermediate	419.92	763.25	3	6	Random
3	Risk-avoider	70.38	114.6	6	3	Learn cue
4	Intermediate	918.38	2918.38	0	2	No
5	Risk-taker	167.46	254.9	7	2	Learn cue
6	Risk-avoider	262.77	365.32	6	3	Learn cue

CHAPTER 3

Association learning in carp with different risk-taking phenotype

The main aim of the work described in this chapter was to explore the possibility of training carp to approach a visual cue using food as a reward, with a view to using these responses to develop welfare-friendly husbandry practices in fish culture.

1 Introduction

1.1 Stress and welfare in aquaculture

As discussed in chapter 1, aquaculture (including carp aquaculture) is becoming increasingly important. Commercial pressure to improve production has meant that fish are often farmed in high intensity systems. This may include high stocking densities, predictable provision of formulated feed and frequent exposure to husbandry practices such as grading, disease treatment and high throughout slaughter processes. All these practices are known to be stressful to farmed fish.

Housing animals at high densities and frequent manipulation are common and necessary practices in intensive aquaculture. Even when carp are reared extensively, culture systems inevitably introduce a number of stressors to the organism. These may include poor water quality (for example, high levels of ammonia, unsuitable pH, high levels of carbon dioxide, low dissolved oxygen levels and inappropriate temperature), as well as handling, with resulting physical damage, disease treatments and incomplete nutrition. It is impossible to avoid many of the procedures known to induce stress in fish. Netting, grading and transport are integral components of the fish farming routine; all the fish farmer can do is to minimize the effects of such stressors (Pickering 1993).

Since chronic or repeated stress can compromise growth and health (Huntingford et al. 2006) this potentially impairs production. Relating stress to welfare in fish is complex (see chapter 1), but it is reasonable to assume that it also compromises welfare.

1.2 The potential for low stress husbandry practices

Increasing concern for efficient, welfare-friendly production in aquaculture puts pressure on the industry to develop low-stress husbandry techniques. The development of methods for achieving less stressful farming making more use of the natural responses of fish could benefit both fish welfare and farm profitability (Lines & Frost 1999). To promote fish well-being consequently avoiding stressful situations we can employ both natural and learned preferences. Attraction to food is an example of an innate response that could be exploited. Learning plays a major role in the behaviour of fish and may be useful as a means of controlling stress and promote positive behaviour in aquaculture (Stien et al. 2007). Aquaculture has long made use of light to facilitate and improve husbandry practices. An experiment by Lines & Frost (1997) showed that it is possible to selectively attract individuals of salmon that were trained to associate

food with a light signal to a feeding area. Lekand & Færa (1993) showed that small salmon and trout can be trained to associate light signals with feeding and so be collected or moved around a tank. The process of learning to associate a visual cue (blue light) with food was studied in groups of common carp and tilapia. Tilapia learned quickly to approach the light to receive food whereas carp failed to do so, although when in mixed groups carp were able to learn the association (Karplus et al. 2007).

1.3 Spatial learning in fish

Learning is a process by which an animal benefits from experience, so that its behaviour is better suited to environmental conditions. Associative learning, also called conditioning, is a type of learning in which an association is made between a stimulus and a response.

Fish have a well developed capacity for spatial learning, which involves adapting behaviour to spatially significant cues and often depends on the use of landmarks (Kieffer & Colgan 1992). Several studies have shown that vertebrates are able to remember local, visual and olfactory features of the environment and to use these to guide subsequent movement, on a variety of scales; in other words, they can remember and use landmarks. Many studies have demonstrated such ability in fish. Warburton (1990) used plastic Lego columns to mark a food patch in a study with goldfish and the results showed that spatial learning was poor in the absence of clear local visual cues, the group trained with the Lego landmarks showed very high choice accuracy, less choice variability and a significant improvement with experience. As another example, goldfish learned geometrical properties of the experimental space for locating food even in the absence of relevant featural information and vice-versa. Thus several studies have suggested ways in which learned responses to spatially-significant cues can be used to promote welfare in farmed fish. The overall aim of present study is to develop such methods for common carp.

1.4 Reversal learning

Reversal learning is defined as any situation where an animal is trained to respond differentially to two stimuli under reward (or punishment) and subsequently trained under reversed reward values. Goldfish trained to use visual cues (striped panel on the tank wall) to locate the exit decreased their performance at the reversal test (figure 3.26), but with training it learned the new set-up. Zebrafish trained to associate a colour (purple or green) of the arm of a T-maze for delivery of food also showed an

improvement in the performance across trials and learned to go to the reversed colour to gain food (Colwill et al. 2005).

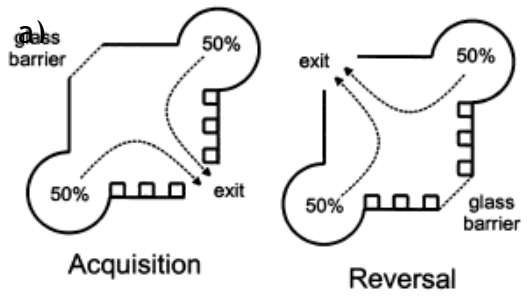


Figure 3.26 a) Schematic diagram of the directly cued test showing the location of the striped panels, the start compartment, the exit door and glass barrier during acquisition and reversal periods. The arrows shows the trajectory from the start to the exit. The percentage represent the usage of each compartment.

1.5 Differences in learning ability and a possible role for risk-taking phenotype

Several studies have reported that animals that are similar in many ways (age, sex, size, maturity stage) may differ in their behaviour patterns (Iguchi et al. 2001). At the level of closely related species, pumpkinseed and bluegill sunfish behave differently in a task that involved learning to forage on a novel prey item on whiteworms, with bluegill learning faster than pumpkinseed fish. In addition, individual fish within one species (pumpkinseed sunfish) exhibited individual variation in foraging efficiency such as capture rate and foraging success (measured by number of captures attempts) (Kieffer & Colgan 1992). Variable stress coping styles represents one possible source of such individual differences in learning.

As discussed in chapter 1, consistent individual differences in stress coping style have been described for a number of vertebrates and some invertebrates' species. Two distinct stress response patterns exist, reflected in both behavioural and neuro-endocrine processes: the proactive and the reactive coping styles (Pottinger & Carrick 1999; Frost et al. 2007). Proactive animals are characterized behaviourally by a tendency to take risk in response to danger, by relatively high levels of aggression and by the tendency to form behavioural routines. In contrast, reactive animals avoid risk and aggressive conflict and are more flexible.

Differences in risk-taking and in stress coping style have been reported for a number of fish species, including rainbow trout (Schjolden et al. 2005) and common carp

(Huntingford et al. 2010). This has implications for aquaculture, since risk-taking, aggressive fish with low stress responsiveness (proactive copers) often do well and shy, non-aggressive fish with high stress responsiveness (reactive copers) often do poorly in husbandry practices. In common carp held at high densities under a variety of oxygen and temperature regimes, reactive fish tend to put on weight rather than length (and hence gain in condition), whereas proactive carp tend to grow in length (Pilarczyk et al in press).

In behavioural terms, such differences are reflected in what is sometimes called the "shy-bold" continuum, with bold, risk-taking individuals at one extreme and shy, risk-avoiding individuals at the other. Such differences may be reflected in several different contexts, including exploration of unfamiliar environments and objects, interactions with potential predators and encounters with conspecific rivals (Sih et. al 2004). Such differences are relevant to the welfare of farmed fish, since reactive, risk-avoiding fish are likely to be more stressed by a variety of challenges. They are also likely to be relevant to the development and application of low stress husbandry systems based on learned responses. On the one hand, the risk-avoiding nature of some fish may compromise their ability to learn, perhaps by reducing their contact with the environment in which learning may occur. For example, rainbow trout assessed as "bold" on the basis of time spent in an open area and level of activity learned a foraging task (approaching a specific area when a light was switched on to receive food pellets) faster than the fish assessed as "shy" in the same test (Sneddon 2003). On the other hand, the tendency of proactive animals to form and stick to routines and the greater flexibility of reactive animals may mean that, once in contact with the opportunity for learning, risk-avoiding, reactive individuals may learn more readily. Rainbow trout from lines selected for low cortisol responsiveness (arguably, proactive fish) trained to feed in one of two feeding areas were slow to adjust when the food was moved to the previously un-rewarded locations. In contrast, fish from a strain selected for high stress responsiveness (reactive fish) were quick to adjust to the new feeding location. Thus it seems that the proactive, risk-taking fish produced by selection for low stress responsiveness are less flexible than their reactive, risk-avoiding counterparts from the high responsive strain (Ruiz-Gomez et al. submitted). The study described in this chapter examined learning ability in carp with different risk-taking phenotype. I looked first at the ability of fish to learn an association between a visual landmark and food. Once this was learned, I also looked at reversal learning, as an additional test of adaptative flexibility.

1.6 Aims of present study

With this background, the broad aim of the work described in this chapter is to characterize patterns of spatial learning in common carp and to determine whether carp with different risk-taking phenotypes differ in whether and how they learn to use visual landmarks to detect food. This was addressed through the following sub aims:

A. Examining patterns of learning

- To develop and deploy methods for examining and quantifying patterns of learning and reversal learning in carp.

B. Relating learning performance to risk-taking phenotype

- To characterize further the nature of variable risk-taking in carp,
- To compare behaviour when the fish are being familiarised with the experimental setup,
- To compare behaviour in learning period and in reversal learning period.

2 Material & Methods

2.1 Subjects and husbandry

60 mirror carp were obtained from Barony College, Dumfries, UK. All the fish were weighed (g) and measured (cm); total length ranged from 6.7cm to 10cm (mean 8.41cm) and weight ranged from 4.48g to 16.11g (mean 10.11g). The carp were transferred to the Experimental Aquaria, Division of Ecology and Evolutionary Biology, University of Glasgow, and kept in 2 glass tanks (100 X 38 X 31.5 cm), both with a re-circulating filter and airstones, one housing 27 and the other with 33 carp. The temperature of the tanks was at 18^{o}C. Carp were individually-marked using alcian blue dye (HO Licence number 60/3679) and photographed for future identification (see Chapter 2).

2.2 Pre-screening for risk-taking

2.2.1 Novel environment test

Fish were screened for risk-taking using a variation of the well-established novel environment test (Yoshida et al. 2005), details being based on pilot studies described in Chapter 2.

2.2.2 Novel object test

The same two sets of carp were screened using another commonly-used test of bold-shy behaviour, namely the novel object test (Frost et al. 2007), details being decided on the basis of pilot tests described in Chapter 2. In this period the fish were fed regularly once a day with frozen bloodworms. Again, fish were selected randomly from one of the holding tanks and tested in groups of 8 in a tank of the same dimensions as those used in the novel environment test and left to settle for 20 minutes.

After observing the fish for 5 minutes (to recognize them individually and notice their position in the tank), a novel object was dropped approximately 10 cm from the front of the fish. The subjects' behaviour was recorded for a further 10 minutes, recording for all fish their location on the aquarium, whether or not they approached the object and the order in which fish inspect it. The novel object consisted of a tower of Lego bricks approximately 5 cm in height constructed of various colours - red, blue, yellow, white, black and green (figure 3.27). Each fish was tested 3 times using a different object and in different, randomly assigned groups.

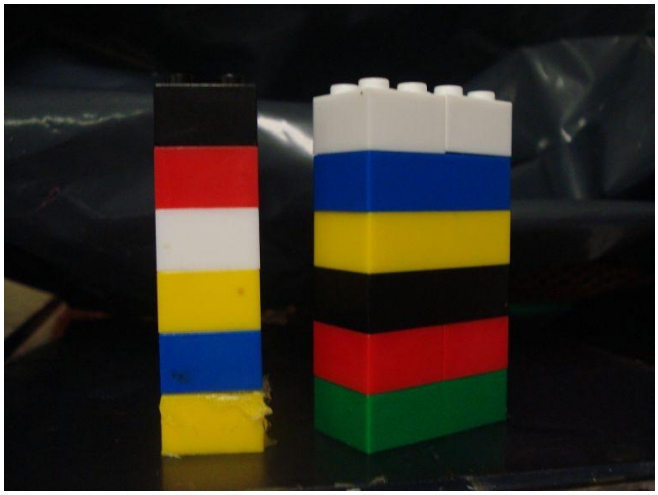


Figure 3.27 Examples of Lego towers used in the novel object test. Scale 1:1.

2.3 Screening for competitive performance

Previous observations show little direct aggression between the carp; instead competition for food usually took the form of jostling for favourable feeding positions. To quantify effectiveness in such competition, carp were deprived of food for at least 12h, which ensures an empty stomach and is not excessive for fish kept at this temperature. Fish were held for 5 minutes in groups of 6 in a holding tank of the same

dimensions as that used in the novel environment tests. A clear glass tube (5 cm in diameter and 42 cm in length) was placed vertically in centre of their holding tank, with one end in contact with the substratum and the other projecting above the water surface (figure 3.28). The tube had a semi-circular hole (2.3 cm high and 3.5 cm wide) cut into it at the base, of a size chosen to accommodate the snout of just one carp at a time. The hole was orientated towards the front of the tank, so as to be clearly visible to an observer.

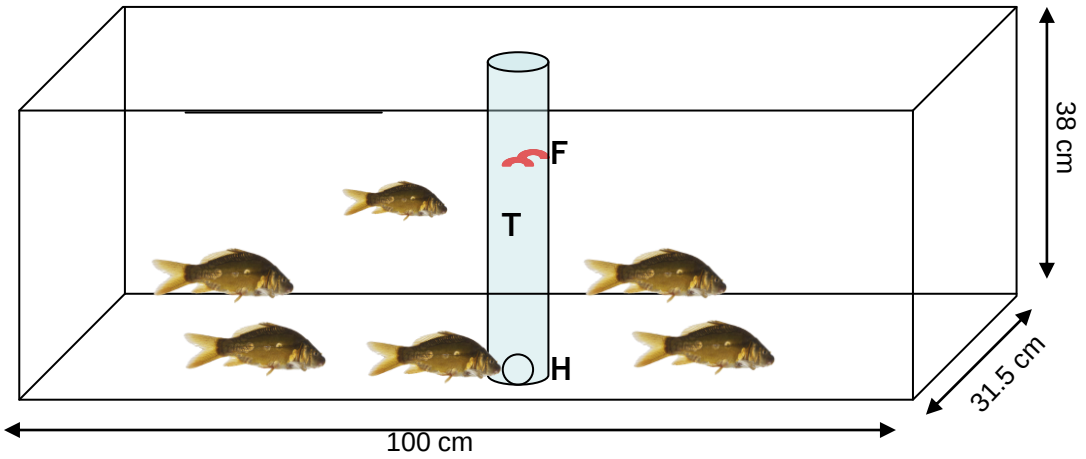


Figure 3.28 Schematic diagram of tank used to screen carp for competitive performance. F = food, T = clear plastic tube, H = small hole where fish could feed.

Defrosted chironomid larvae were introduced into the tube by pipette; these were clearly visible to the fish, which tracked the larvae as they sank to the bottom of the tube and jostled for a feeding position at the hole. This involved circling the base of the tube pushing for access to the feeding hole. The identity of the fish that took each one of the larvae was recorded, at each trial around 10 larvae were introduced in the tube. Each random group of 6 carp was tested 3 times.

2.4 Training carp to associate a light signal with food

The same fish classified previously as risk-takers, intermediate and risk-avoiders according to the pre-screening for risk-taking (see below for criteria) were formed into pairs with the same risk-taking phenotype; this was necessary as carp become very disturbed if held on their own. The behaviour of the fish was found to be independent of that of the other member of their pair at all stages in the trials on the basis of an absence of correlation between either time to emerge or time to find food in the learning set up.

Choice of colours for use with learning trials was based on consideration of the known spectral sensitivity of the carp eye and personal observations of the behaviour of the carp during routine handling. These suggested that carp may be frightened by blue objects such as nets and buckets while when in red buckets they behave normally. Carp eyes are sensitive to light with wavelength ranging from 490nm to 680 nm (Hanaoka & Fujimoto 1957) and a similar range has been shown for goldfish (Harosi & McNichol 1974). Tomita et al. (1967) identified three groups of cones in common carp, red cones (74%) with peak absorption at a wavelength of 611 ± 23 mμ, green cones (10%) absorbing at 529 ±14 mμ, and blue cones (16%) absorbing at 462 ± 15 mμ (figure 3.30). For these reasons, in this study red and yellow light sources were used.

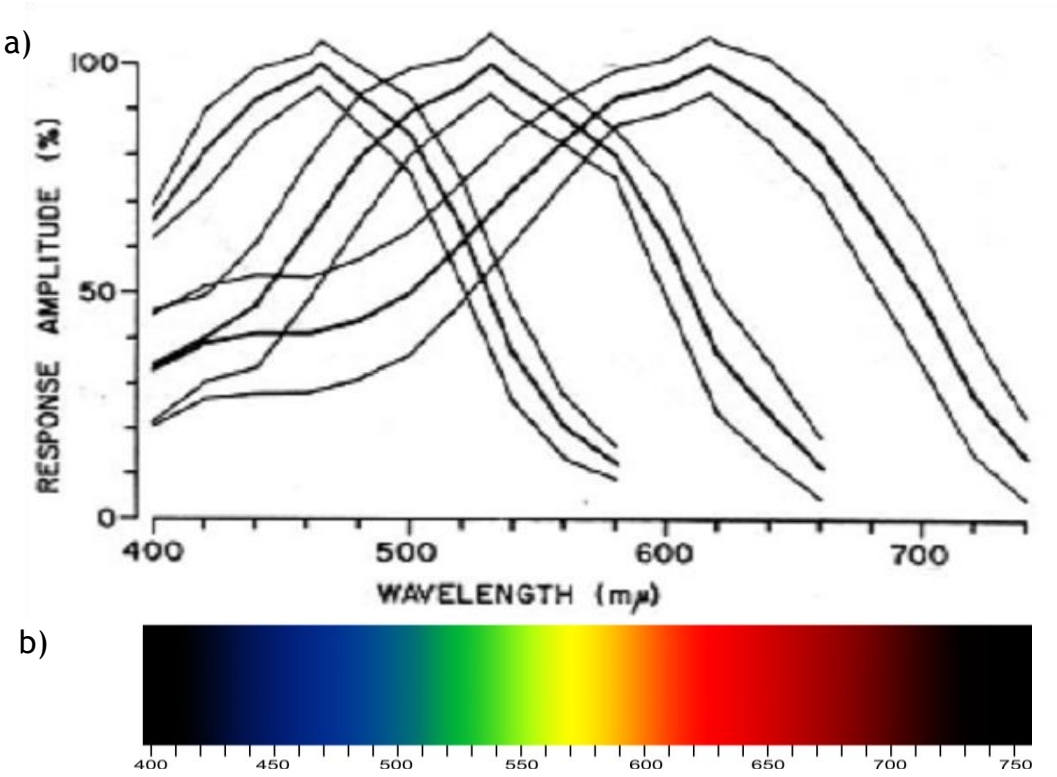


Figure 3.29 a) Averaged response and standard deviation curves of three types of cones of common carp (Tomita et al. 1967), b) the optical spectrum, its colours produced by visible light and their wavelength.

The experimental aquarium (figure 3.30) was a 1 meter tank, at one end of which the screening tank there was an enclosed, settling chamber (30 cm in length), with a plastic tunnel forming an exit into the main section of the tank. The opening to the tunnel was fitted with a removable plastic cover. At the other end, at the main section, the aquarium was divided into 2 sides in each of which a Petri dish was placed. The fish could only see the Petri dish if it swam up to the entrance to the feeding compartment.

Outside the aquarium, the same light source (a battery powered torch) with either red or yellow plastic placed in front of the light was set up. During an initial (pre-learning) period, both lights were on and food was placed in the Petri dish on both sides. A criterion for starting the learning trials was established, namely that at least one of the fish of the pair has to emerge 3 times in sequence in the pre-learning period. Based on pilot studies described in Chapter 2, the carp were given 20 minutes to settle before the door was opened in both pre-learning trials and learning trials.

After at least one fish of a pair had reached the criterion, the learning tests started; in these trials, the food was offered only on one side, the rewarded side, which was switched randomly between trials. Either red or yellow light signals were associated with the position of food, rewarded colour being assigned randomly to each pair.

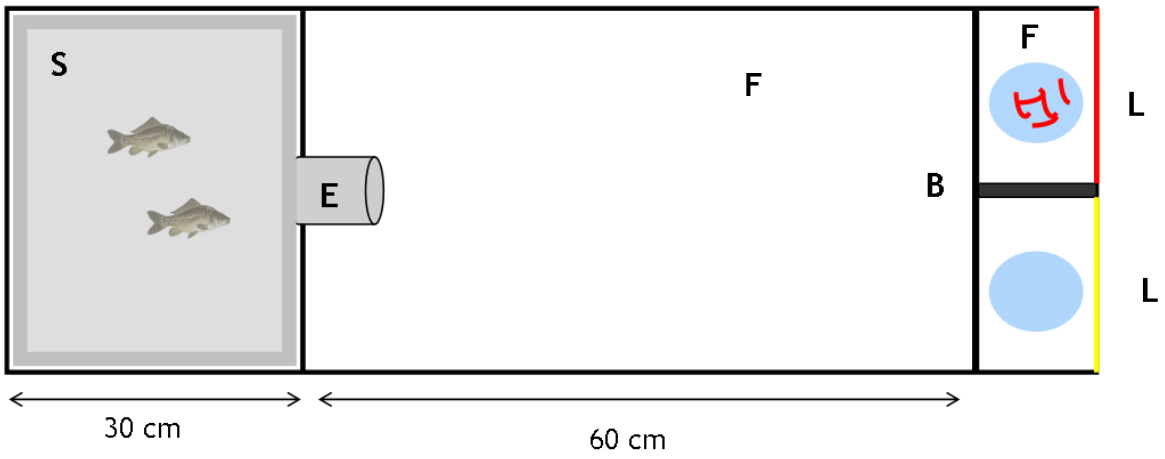


Figure 3.30 Schematic diagram of tank used in the learning trials. E = entrance to learning tank with removable lid, B = barrier to avoid fish seeing the food from far. F = food. L = torch outside the tank, one side red and the other yellow.

The criterion for the learning trials was that the fish had either to make 80% of corrects choices (going to the light that signalled food) or to go to the correct side 8 times in sequence. We used two criteria because some fish took longer to learn so they went through more tests than others and sometimes one fish of the pair took more time than the other. During pre-training and training the time taken to emerge from shelter and time to find food were recorded, search time was also calculated as the time from emergence to feeding. In the learning trials, the time taken by fish to go to the wrong side was also recorded (where relevant).

2.5 Reversal Learning

After the learning tests, fish that had reached the criterion for having learned to follow the light (see below) went through a period of reversal leaning. Fish that were first

trained with red light were rewarded at the yellow light during reversal learning and *vice-versa*.

3 Results

3.1 Pre-screening for risk-taking

3.1.1 Novel environment test

Overall distribution of emergence time: Figure 3.31 shows the distribution of emergence times for all fish in all tests. There is a great variability in response, with emergence times ranging from the fastest time of 54 seconds to a score of 2000, arbitrarily given to cases where the fish did not emerge during the screening period.

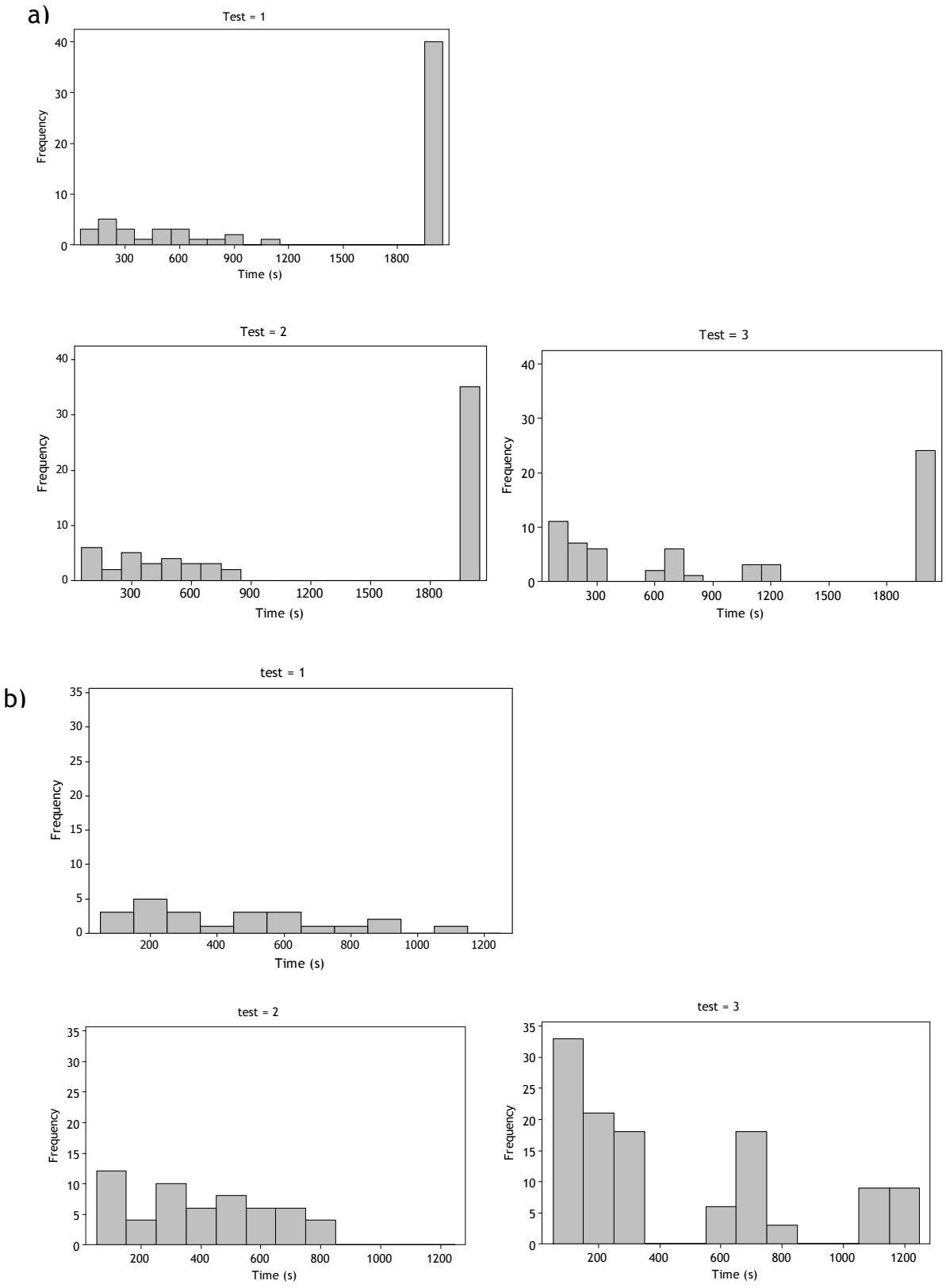


Figure 3.31 Frequency distribution of time to emerge from shelter in all fish by test. (a) Considering fish that never emerged represented by 2000 s (x-axis) which is a notional score for these fish. (b) Frequency distribution considering just the fish that emerged.

Effects of tank and test number: Figure 3.32 show the means (SEM) emergence time for all fish across the 3 tests for fish from holding tank 1 and 2. There was a marginally

significant tank effect, with mean emergence time for tank 1 being 1384.4 (median 2000) and tank 2 being 1158.5 (median 1175) (Mann Whitney test: W = 3.81, p = 0.051). Emergence time decreased marginally with test number for all fish together in tank 1 (GLM: F_1 = 3.11, p = 0.084) and decreased significantly for tank 2 (GLM: F_1 = 4.79, p < 0.001).

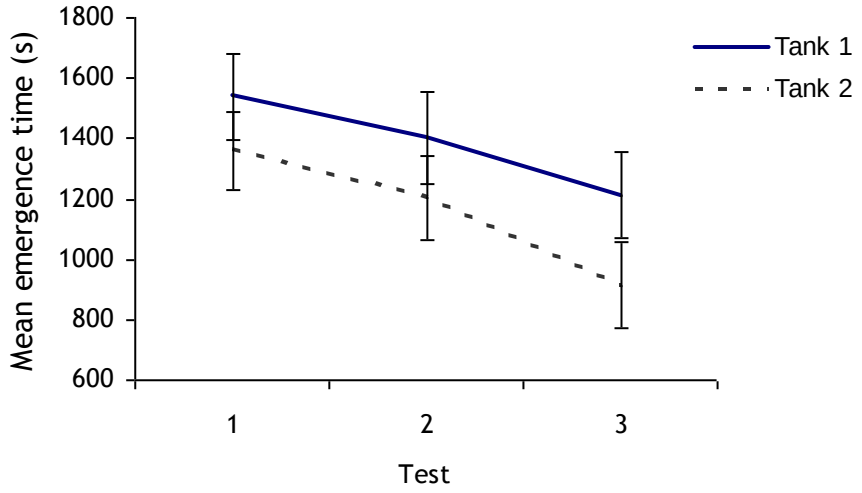


Figure 3.32 Mean (SEM) emergence time for all fish tested in the novel environment test by test and tank.

Classification of fish into risk-taking categories: Within tank 1 there were significant individual effects (GLM: F_{26} = 3.68, p < 0.001), but not in tank 2 (GLM: F_{36} = 1.17, p = 0.283). In both tanks, some fish consistently emerged within a short time while others did not emerge in any of the tests. Based on these differences in emergence time, the fish were divided into categories according to the mean time taken to emerge from shelter into the feeding chamber (figure 3.33 for the two tanks separately). The fish that emerged in a short time (in the lowest third of the mean emergence times - fish 1-5 in tank 1 and 1-10 in tank 2) were classified as risk-takers. Those that did not emerge or took a long time to do so (in the highest third of emergence tines – fish 20-27 in tank 1 and 28-36 in tank 2) were classified as risk-avoiders. Fish with intermediate emergence times (mean emergence times in the remaining third of the distribution) are designated “intermediate”, although scrutiny of their emergence times indicated that their behaviour was flexible (switching between fast and slow emergence) rather than having consistently moderate emergence times.

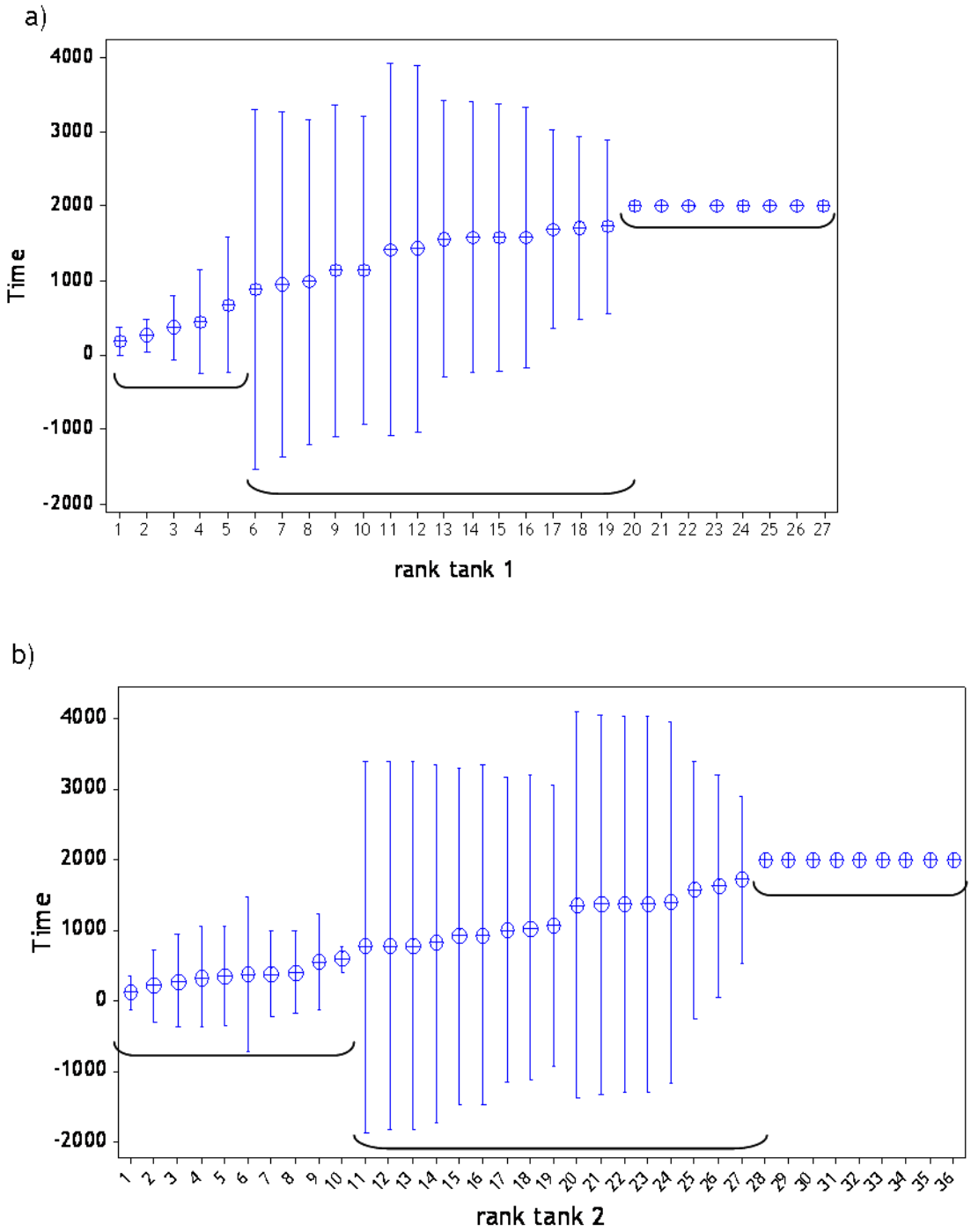


Figure 3.33 Mean (SEM) emergence times for all carp in the novel environment test in each tank. a) tank 1 and b) tank 2. Brackets sign the division between the risk-taking phenotypes.

Looking at the times to emerge from shelter in successive trials in fish with different risk-taking phenotypes (Figure 3.33), risk-avoider and risk-taker fish did not show a reduction in the emergence time with trial number, although risk-taking fish emerged faster than the others on the three trials (risk-avoider: Kruskal-Wallis test: $H_2 = 1.02$, $p = 0.601$; risk-taker: Kruskal-Wallis test: $H_2 = 4.36$, $p = 0.113$). Only intermediate fish showed a reduction in emergence time across the three tests (Kruskal-Wallis test: $H_2 = 11.57$, $p = 0.003$) with differences both between trials 1 and 3 (post-hoc < 0.05) and also between trials 2 and 3 (post-hoc < 0.05).

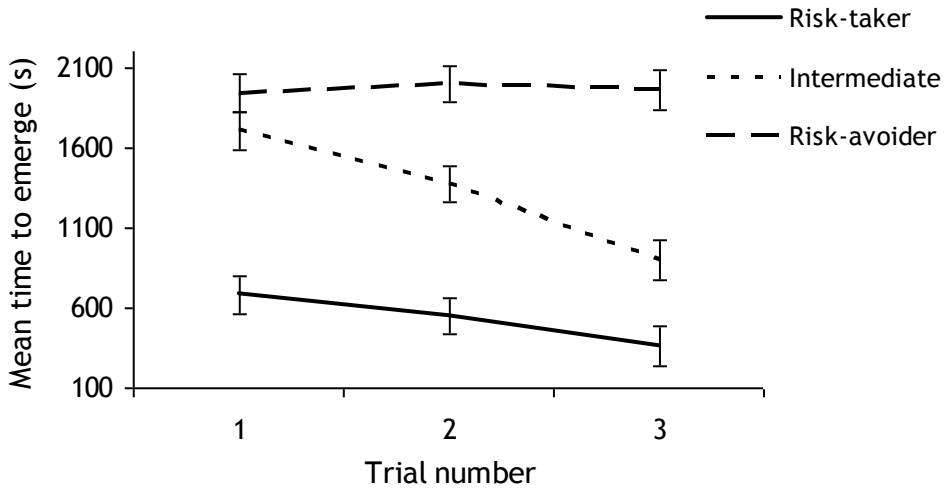


Figure 3.34 Mean (SEM) of emergence time in the three risk-taking phenotypes in successive trials.

Morphological status in relation to risk-taking phenotype: Figures for this section are shown in chapter 6. There was no significant relationship between weight and risk-taking phenotype (One-way ANOVA: $F_{2,56} = 0.80$, $p = 0.455$), although mean weight did increase in the sequence risk-takers (9.44 ± 0.75), intermediate (10.34 ± 0.63) and risk-avoiders (10.59 ± 0.60). Standard length was unrelated to risk-taking phenotype (One-way ANOVA: $F_{2,56} = 0.49$, $p = 0.613$). There was a marginally significant relationship between risk-taking category and condition factor, with risk-taking fish having slightly lower condition than intermediate and risk-avoiding fish (One-way ANOVA, $F_{2,56} = 3.13$, $p = 0.051$).

3.1.2 Novel object test

In this test, fish were classified by the number of inspections directed at the novel object in the 3 successive tests. 3 categories were recognized: (1) fish that did not inspect the object in any of the three tests; (2) fish that inspected the object once and (3) fish that inspected the object 2 or more times. Most fish (44.45%) fell into category 2 (category 1 = 22.22% and category 3 = 33.33%) and there was no significant tank effect on inspection categories (N = 133, DF = 62, Chi-Sq = 16.21, P = 1.00).

3.1.3 Competitive performance

Figure 3.35 shows the frequency distribution of the number of food items eaten by all fish in all 3 tests together.

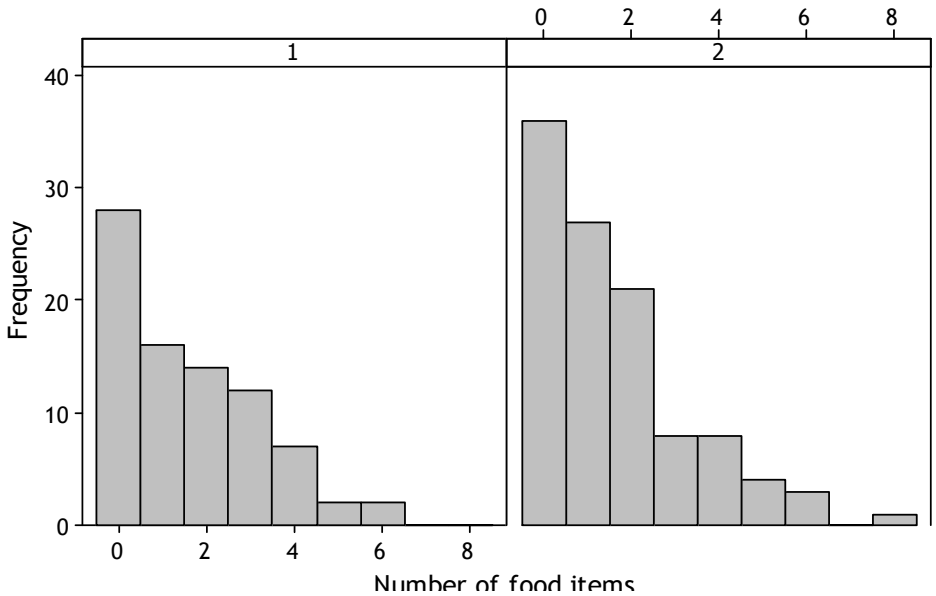


Figure 3.35 Frequency distribution of number of food items eaten by all carp in all the competition trials by tank.

The number of food items eaten did not differ with test number (test 1: 1.54 ± 0.211, test 2: 1.619 ± 0.213, test 3: 1.619 ± 0.207, Kruskal-Wallis test: $H_2 = 0.07$, $p = 0.964$). There was no significant tank effect on total food eaten (tank 1: 5.52 ± 0.735, tank 2: 4.22 ± 0.571; Mann-Whitney test: $W = 7789.0$, $p = 0.7957$). Again there was marked variability in both tanks, with some fish eating 8 worms and many eating none at all, giving a significant fish effect for number of worms eaten (Kruskal-Wallis test: $H_{62} = 101.62$, $p = 0.001$).

3.1.4 Relationship between the different pre-screening tests

There was a significant relationship between behaviour in the novel environment test and category from novel object test (Kruskal-Wallis test: $H_2 = 46.12$, $p = 0.000$). Thus fish that did not inspect the novel object (category 1) had longer emergence times than did fish that inspected the object relatively frequently, with category 2 fish coming in between. This is also reflected in the distribution of inspection categories in the 3 risk-taking phenotypes (Figure 3.37). There are more risk-avoiders in category 1, more intermediate fish in category 2 and more risk-takers on category 3.

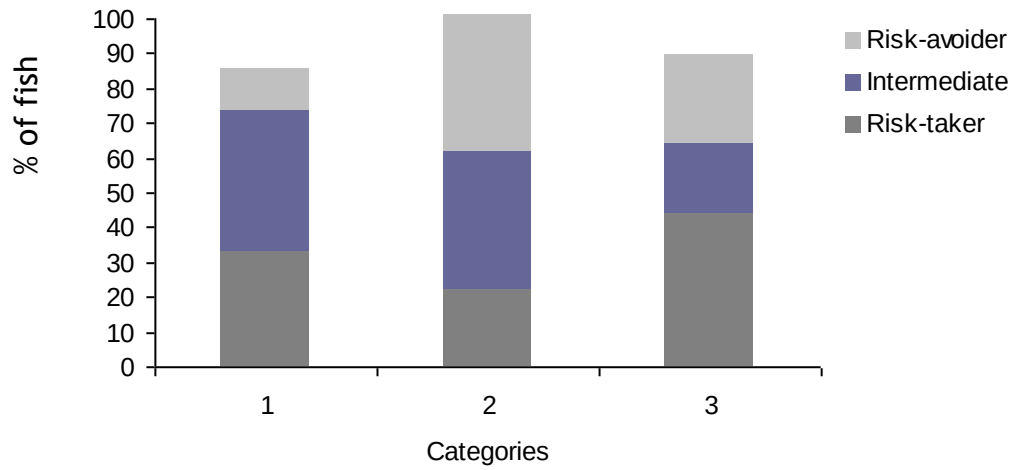


Figure 3.36 Comparison of classifications for the 2 tests - novel object and novel environment test.

Table 3.10 shows mean (SEM) number of food items eaten by fish in the competitive performance test according to risk-taking phenotype and inspection category for the novel object test. There was no significant relationship between risk-taking phenotype and competitive ability (Kruskal-Wallis test: H = 1.51, p = 0.469). Fish that did not inspect the novel object overall (category 1) on average ate more food than did fish in categories 2 and 3 (Kruskal-Wallis test: H = 6.24, p = 0.044).

Table 3.10 Mean (SEM) number of worms eaten by each risk-taking phenotype and inspection category from the novel object test.

Risk-taking phenotype	Mean (SEM) number of food
Risk-taker	1.433 ± 0.24
Intermediate	1.569 ± 0.25
Risk-avoider	1.789 ± 0.31
Inspection category	Mean (SEM) number of food
1	2.000 ± 0.38
2	1.429 ± 0.20
3	1.540 ± 0.27

3.2 Behaviour during the settling period (Pre-learning test)

In the settling period, the time taken for fish to emerge was very variable and showed significant difference between fish (repeated measures ANOVA by fish: $F_{1,168} = 5.78$, $p < 0.001$). Figure 3.37 shows mean (SEM) time to emerge and time to find food for carp in each risk-taking category, across successive trials in the pre-training period.

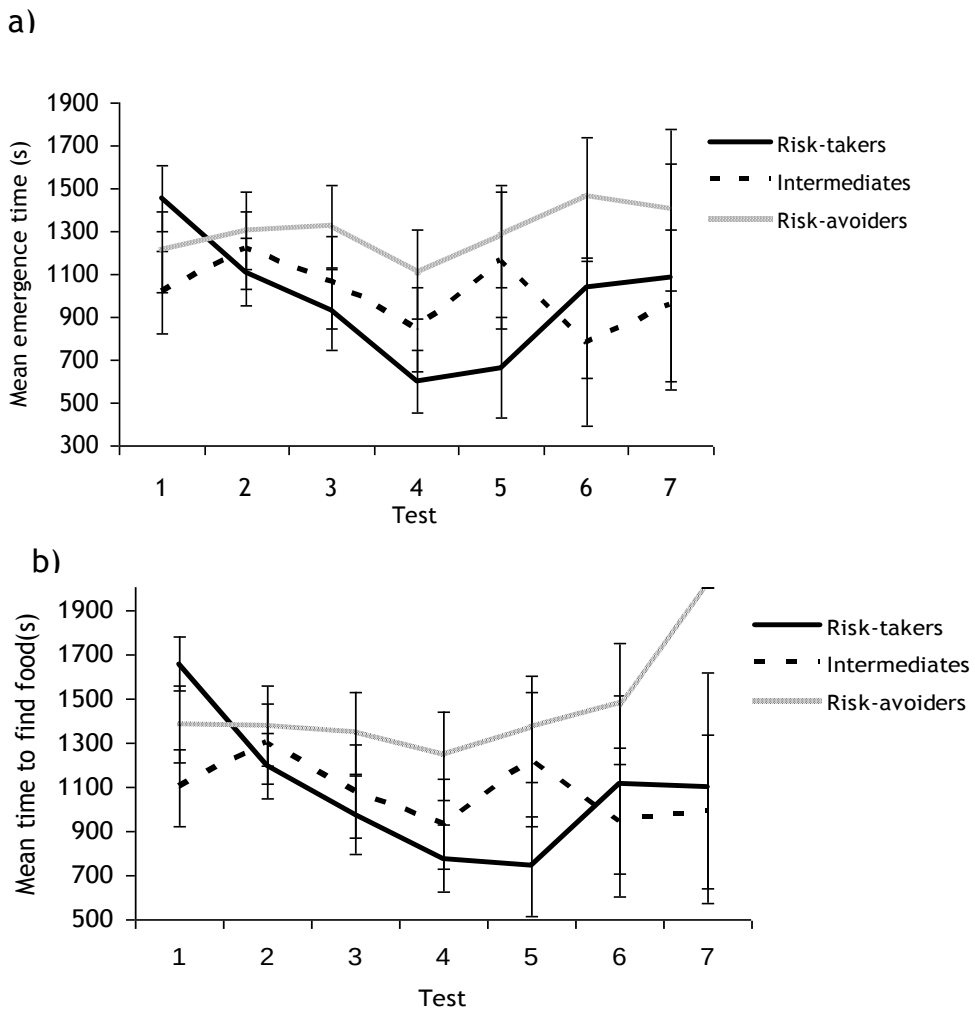


Figure 3.37 Mean (SEM) a) time to emerge from shelter and b) time to find food in successive trials for fish classified according to risk-taking phenotype.

In risk-taking fish, significant effects of test number effects were found for emergence time, time to find food and search time by test (repeated measures ANOVA: emergence time $F_{1,8}$ = 66.54, p <0.001; time to find food $F_{1,8}$ = 57.21, p < 0.001; search time $F_{1,8}$ = 60.96, p < 0.0001). The broad trend for these fish is for a decrease with trial number, although mean values started to rise in the later trials, as fish that had reached the criterion for settling were moved into the next phase. There was test effect for those same variables for intermediate fish (repeated measures ANOVA: emergence time $F_{1,7}$ = 27.94, p < 0.0001; time to find food $F_{1,7}$ = 33.59, p < 0.0001; search time $F_{1,7}$ = 41.70, p < 0.001) or for risk-avoiders (repeated measures ANOVA: emergence time - $F_{1,5}$ = 30.50, p < 0.0001; time to find food - $F_{1,5}$ = 27.07, p < 0.0001; search time - $F_{1,5}$ = 24.81, p < 0.0001). At least in the earlier trials, risk-taking fish were faster to emerge that the other two categories. Emergence and feed time remained high in risk-avoiders throughout the pre-learning period with intermediate fish falling in between risk-takers and risk-avoiders for both variables.

The numbers of tests taken for each fish to pass from pre-learning test to learning test was very variable (Figure 3.39); some fish took just 4 tests, while others took 20 tests to reach criterion. According to figure 3.39, most of risk-takers took fewer trials than intermediate fish and most of intermediate fish took fewer trials than risk-avoiders (Kruskal-Wallis test: $H_2 = 4.87$, $p = 0.088$).

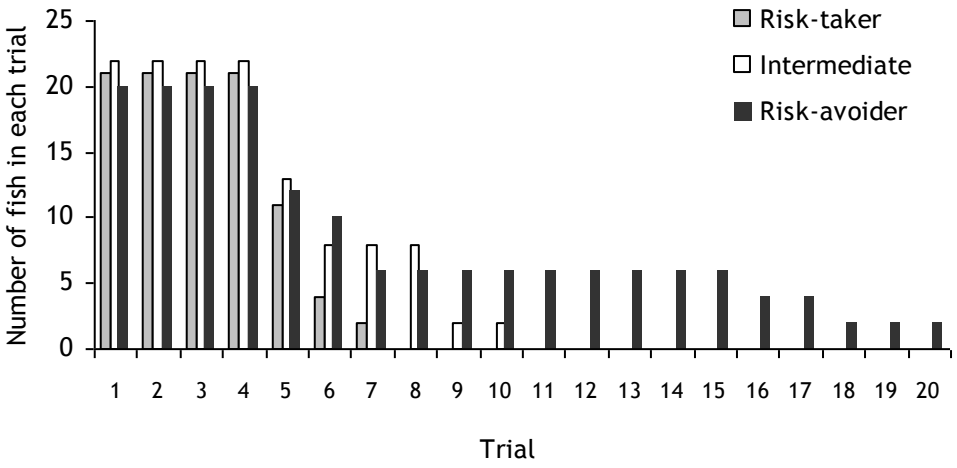


Figure 3.38 Frequency distribution of number of trials taken to reach criterion, separated by risk-taken phenotype.

Figure 3.38 shows the mean (SEM) emergence times and time to find food for the whole pre-settling period for risk-takers, risk-avoiders and intermediate fish (GLM repeated measures: emergence time – $F_{1,168} = 4.43$, $p = 0.037$, time to find food - $F_{1,168} = 1.71$, $p = 0.193$). The result showed difference only on emergence time.

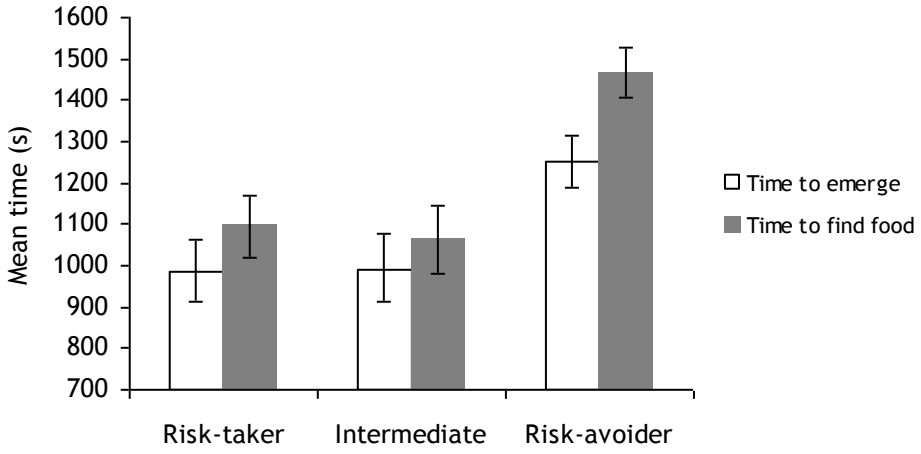


Figure 3.39 Total mean time (SEM) to emerge and find food for risk-taking phenotypes.

3.3 Learning test

3.3.1 Trial effects (effects of trial number on time to find food)

Figure 3.41 shows the distribution of time to emerge and time to find food across all learning trials; both were highly variable. For both variables there was a significant decrease with successive trials (Time to emerge: GLM, $F_{1,168}$ = 311.44, $p < 0.001$. Time to find food: GLM, $F_{1,168}$ =335.53, $p < 0.001$).

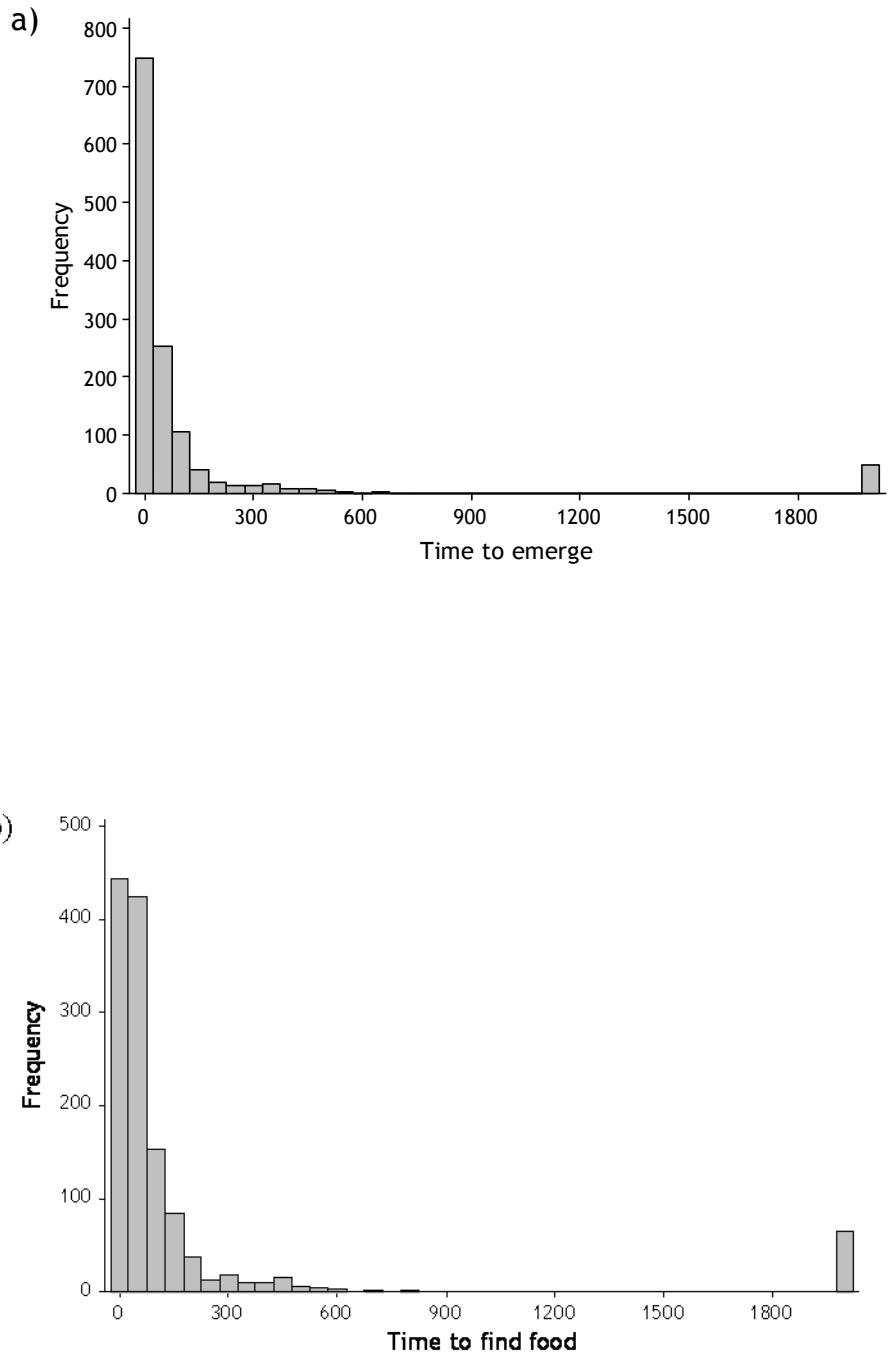


Figure 3.40 Frequency distribution of a) time to emerge and b) time to find food in all the learning tests.

The difference between the time to emerge and time to find food indicates how long the fish spent searching for food. Mean values for this score are shown in figure 3.42. Figure 3.42 shows search time in relation to trial number during the learning period. This falls significantly with trial number (GLM: $F_{1,168} = 237.30$, $p < 0.001$), such that by the end, all fish are finding food in less than 10 seconds. Together these figures indicate that the fish are adapting so as to forage efficiently in this set up.

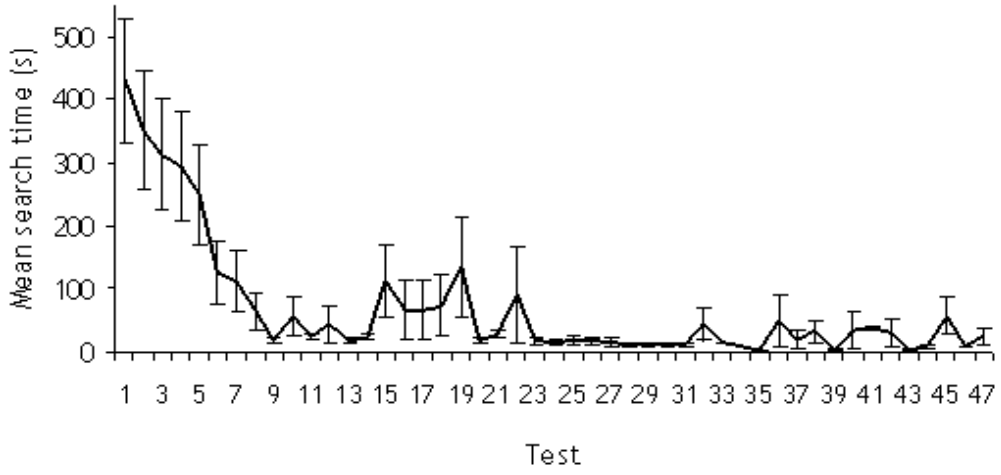


Figure 3.41 Mean (SEM) interval between emergence time and time to find food - search time by test.

3.3.2 Are the fish learning and if so, what are they learning?

Although by the end of the learning period most fish were finding food in less than 10 seconds, observation of the behaviour of individual fish suggested that they were achieving efficient feeding in different ways. Thus, some behaved as expected and seemed to have learned to associate food with one particular colour of light. Figure 3.43a shows a typical trace for such a fish.

a) b)

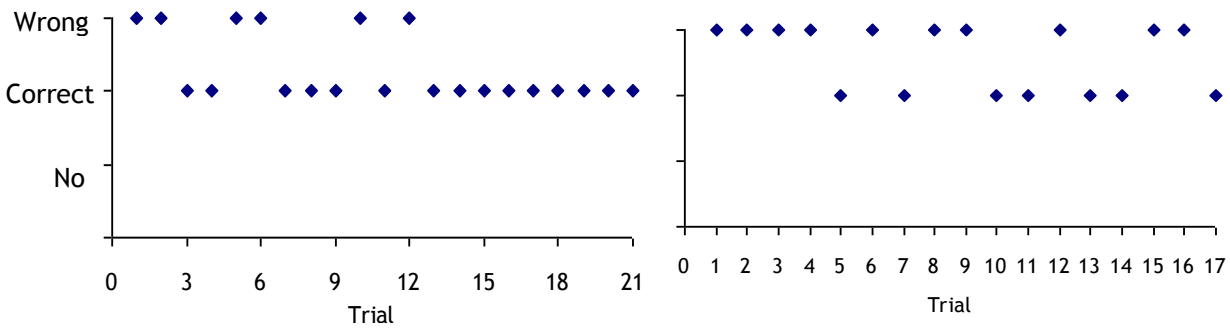


Figure 3.42 a) Example of a fish that learned to follow the light to find food; b) example of a fish went to one of the 2 sides randomly. No = did not emerge in the trial, correct = fish swam to the side with food, Wrong = fish swam to the side without food.

Other fish appeared to ignore the light, entering a compartment at random and, after no food was found, moving directly to the other compartment (figure 3.43b). To explore this further, binomial tests were carried out on number of times each fish went to the rewarded and unrewarded side, full results are shown on table 3.11.

All the significant results (binomial test) in table 3.11 are from LC (learn-cue) fish which has a bigger difference between number of correct and wrong choices. The LC fish that did not have a significant result was due to too few or too many tests. Figure 3.44 shows the percentage of correct choice by strategy. LC fish had a higher percentage of correct choice than RS (random-switch) fish in all trials.

Table 3.11 Number of right and wrong choices, % of correct responses overall tests, % of correct response on last 10 trials, results of a binomial test and assigned strategy. LC = fish that learned to follow the cue and RS = fish that enter a compartment at random.

Fish	N° of right/wrong	% right overall	% right last 10 trials	Binomial	Assigned strategy
1	9/2	81.82	90	0.033	LC
2	16/9	65.38	70	0.115	RS
3	16/10	61.54	60	*	RS
4	8/22	26.67	20	*	RS
5	10/1	90.91	90	0.006	LC
6	15/15	50	40	*	RS
7	11/12	47.83	60	0.500	RS
8	17/8	68	70	0.054	RS
9	22/9	70.97	80	*	LC
10	21/9	70	80	*	LC
11	10/3	76.92	80	0.046	LC
12	19/12	61.29	80	*	LC
13	11/2	84.61	90	0.011	LC
14	20/9	68.96	90	*	LC
16	15/6	71.43	90	0.039	LC
17	7/4	63.64	70	0.274	RS
18	9/9	50	40	0.593	RS
19	13/11	54.17	70	0.419	RS
20	23/8	74.19	80	*	LC
21	14/16	48.39	40	*	RS
22	13/17	43.33	60	*	RS
23	11/1	90.91	90	0.006	LC
24	8/3	72.73	80	0.113	LC
25	19/12	61.29	70	*	RS
26	13/10	56.52	40	*	RS
27	12/3	80	80	0.018	LC
28	7/4	63.64	60	0.274	RS
29	12/0	100	100	0.000	LC
30	8/3	72.73	70	0.113	LC
31	17/13	56.67	70	*	RS
33	7/2	77.77	77.77	0.090	LC
34	14/8	63.64	60	0.143	RS
36	24/22	52.17	50	*	RS
37	22/23	40	40	*	RS
38	2/7	25	25	0.090	RS
39	15/6	71.43	90	0.039	LC
40	7/1	85.71	85.71	0.035	LC
41	17/4	80.95	80	0.004	LC
42	12/8	60	60	0.252	RS
43	13/11	54.17	70	0.419	RS
44	13/5	72.22	70	0.048	LC
46	13/11	54.17	60	0.419	RS
47	14/15	48.27	50	*	RS
48	12/6	66.67	70	0.119	RS
49	20/2	90.91	80	0.000	LC
50	19/3	86.36	90	0.000	LC
51	9/7	56.25	60	0.402	RS
52	12/9	57.14	90	0.332	LC
53	14/4	77.77	80	0.015	LC
54	13/2	86.67	90	0.004	LC
55	5/2	71.43	71.43	0.227	LC
56	8/2	80	80	0.055	LC
57	16/10	61.54	80	*	LC
58	14/4	77.78	80	0.015	LC
59	10/5	66.67	70	0.151	RS
60	7/10	41.18	50	0.315	RS
61	14/5	73.68	90	0.032	LC
62	11/5	68.75	70	0.105	RS
63	12/3	80	90	0.018	LC
64	13/6	68.42	60	0.084	RS

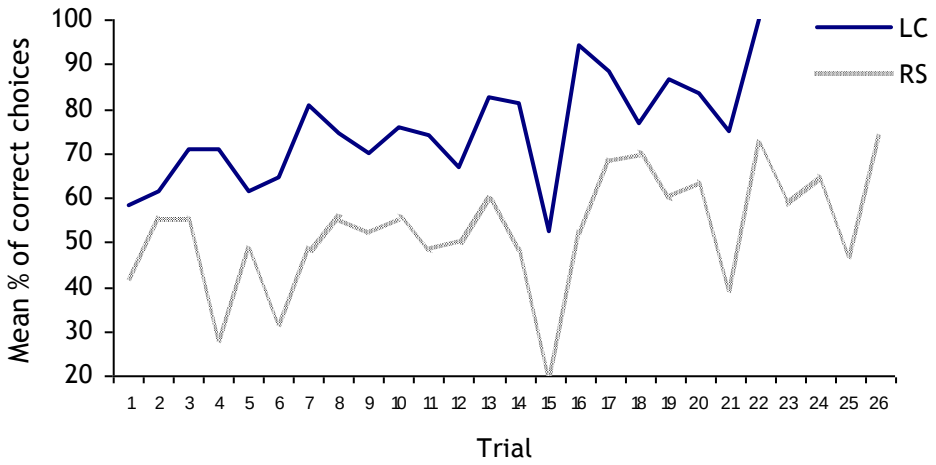
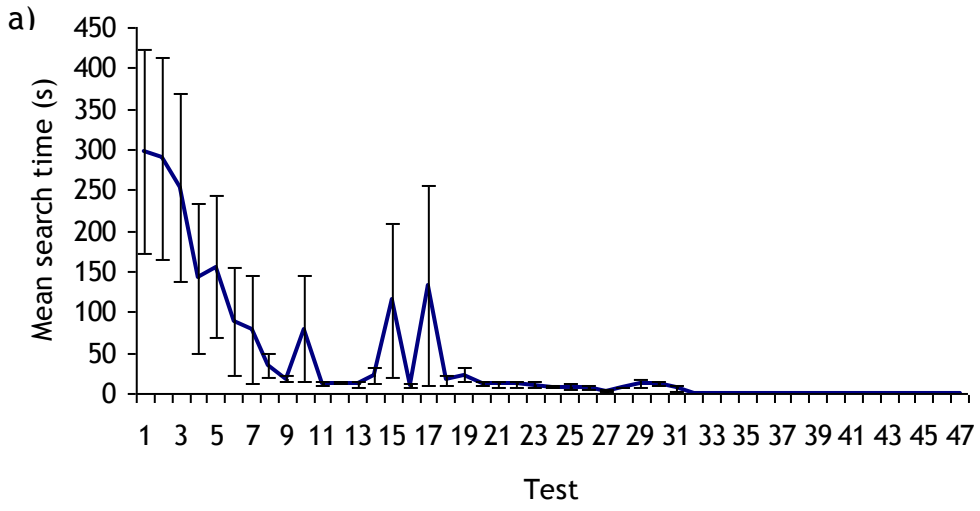


Figure 3.43 Mean percentage of correct choices in each trial separated by learning strategy.

Therefore the following criteria were used to classify the carp: 1) fish that made more than 70% correct choices in all tests were classified as having learned to follow the correct visual cue (LC) and those that made less than 70% were classified as random switch (RS); and 2) fish that made more than 80% on the last 10 tests were also classified as having learned to follow the correct visual cue. Figure 3.45 shows mean search time for fish adopting these two strategies. Both LC fish (Kruskal-Wallis test: H_{30} = 69.27, $p < 0.001$) and RS fish (Kruskal-Wallis test: H_{46} = 109.56, $p < 0.001$) showed significant differences by test in search time.



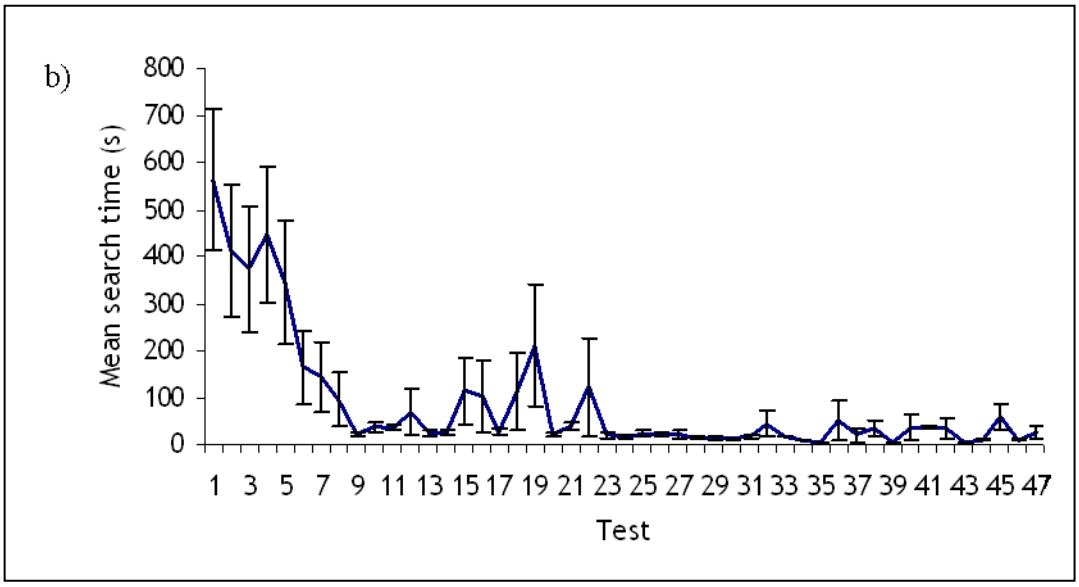


Figure 3.44 Mean (SEM) search time for a) LC fish and b) RS fish.

3.3.3 Effects of light colour

Figure 3.46 shows the mean (SEM) percentage of correct choices in fish trained to the red and the yellow light. Light colour had an effect on the percentage of correct choices, with fish that was trained with red light (median: 72.73, IQR: 18.23) having a higher percentage of correct choices (Mann-Whitney test, W = 1135.5, p = 0.0011) than the yellow light (median: 60.64, IQR: 21.09). There was no significant association between the learning strategy adopted by a given fish and the colour of light associated with food, although there was a marginally significant trend for more of the fish classified as LC being trained to follow the red light (Figure 3.46; Chi-square = 3.27, P = 0.071).

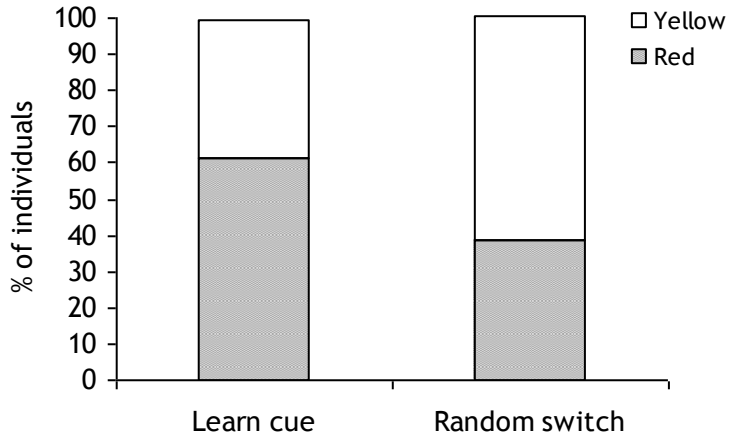
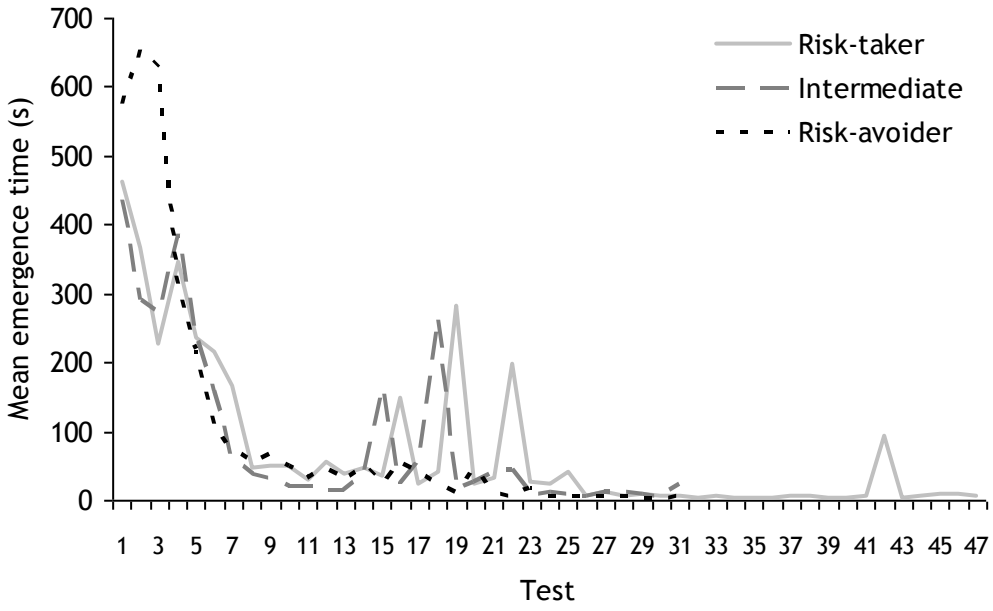


Figure 3.45 Percentage of carp adopting the random switch or learn cue strategy for which the location of food was signalled by red or yellow light.

3.3.4 Effect of risk-taking phenotype on learning

Overall the percentage of correct choice made during the learning trials were not related to risk-taking phenotype, with each of the 3 categories having similar mean percentage of correct choices (Kruskal-Wallis test, $H_2 = 3.97$, $p = 0.137$). Figure 3.47 shows time to emerge (GLM - Shy, $F_{1,7} = 108.55$, $p < 0.001$; Intermediate, $F_{1,7} = 151.02$, $p < 0.001$; Bold, $F_{1,10} = 104.67$, $p < 0.001$), time to find food (GLM – Shy, $F_{1,7} = 163.27$, $p < 0.001$; Intermediate, $F_{1,7} = 133.31$, $p < 0.001$; Bold, $F_{1,10} = 108.69$, $p < 0.001$) and search time (GLM – Shy, $F_{1,7} = 165.69$, $p < 0.001$; Intermediate, $F_{1,7} = 76.73$, $p < 0.001$; Bold, $F_{1,10} = 69.32$, $p < 0.001$) during successive trials for each risk-taking phenotype. It can be observed that by the end of the training period, all fish emerged quickly and found food on the learning tests but risk-takers took longer to learn.

a)



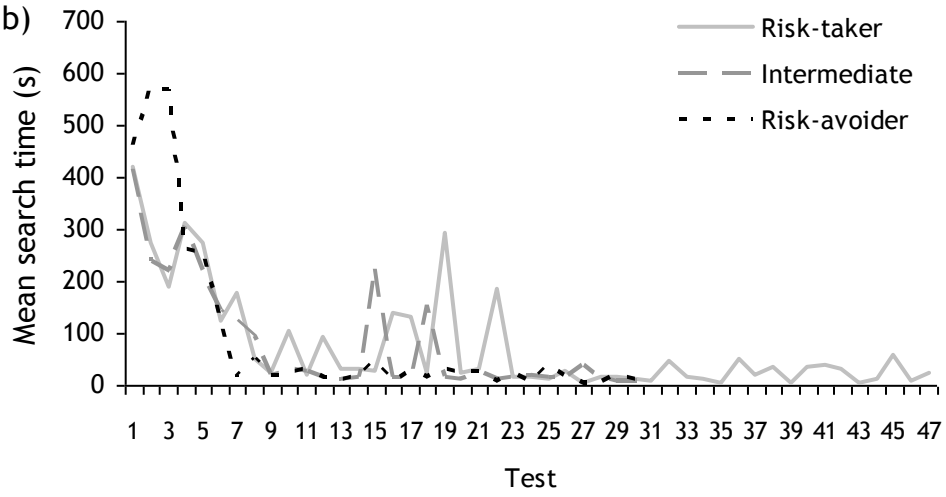


Figure 3.46 Mean a) emergence time in the learning tests for each one of the risk-taking phenotype; b) search time in the learning tests for each one of the risk-taking phenotype. In

Figure 3.48 shows the distribution of each risk-taking category in fish classified as having learned to follow the visual cue or as having adopted the random-switch strategy. Learning strategy was related to risk-taking phenotype (Chi-square = 7.116, p = 0.028), with risk-avoiders making up the largest group of fish that learned the cue and the smallest among the random-switch fish.

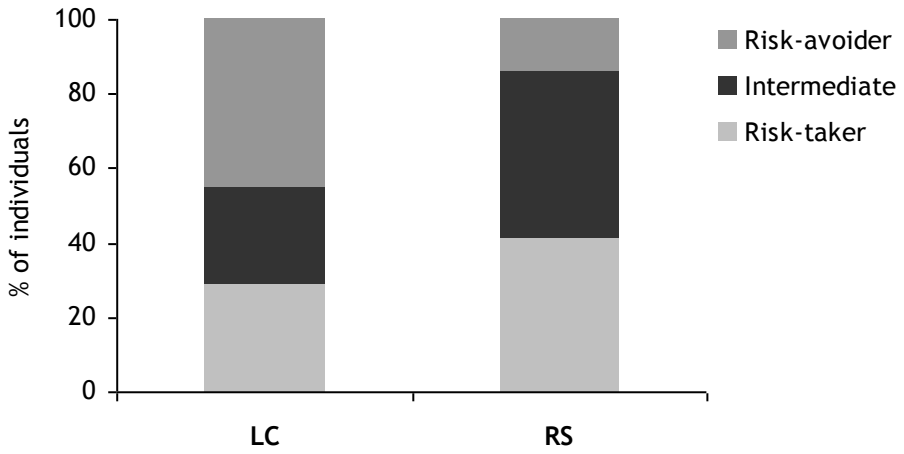


Figure 3.47 Distribution of risk-taking categories classified on the basis of the novel environment test.

There was significant effect of risk-taking phenotype on the number of tests that fish took to reach the criterion for learning (Kruskal-Wallis test: H_2 = 8.31, p = 0.016). Comparisons between the three categories demonstrated that the differences occurred between: risk-taker/risk-avoider (post-hoc $p < 0.05$) with risk-takers taking longer (post-

hoc $p < 0.05$) than risk-avoiders (post-hoc $p < 0.05$) to reach criteria; and risk-avoider/intermediate (post-hoc < 0.05) with risk-avoiders being faster to reach criteria than intermediate fish (post-hoc < 0.05). The difference between risk-takers and intermediate fish was not significant (post-hoc $p > 0.05$).

3.4 Reversal learning

Figure 3.49 shows time to emerge and find food in each test during the reversal period. Mean time to emerge (GLM repeated measures, $F_{1,7} = 94.54$, $p < 0.001$) and mean time to find food (GLM, $F_{1,7} = 91.38$, $p < 0.001$) both fell with test number as in the learning phase.

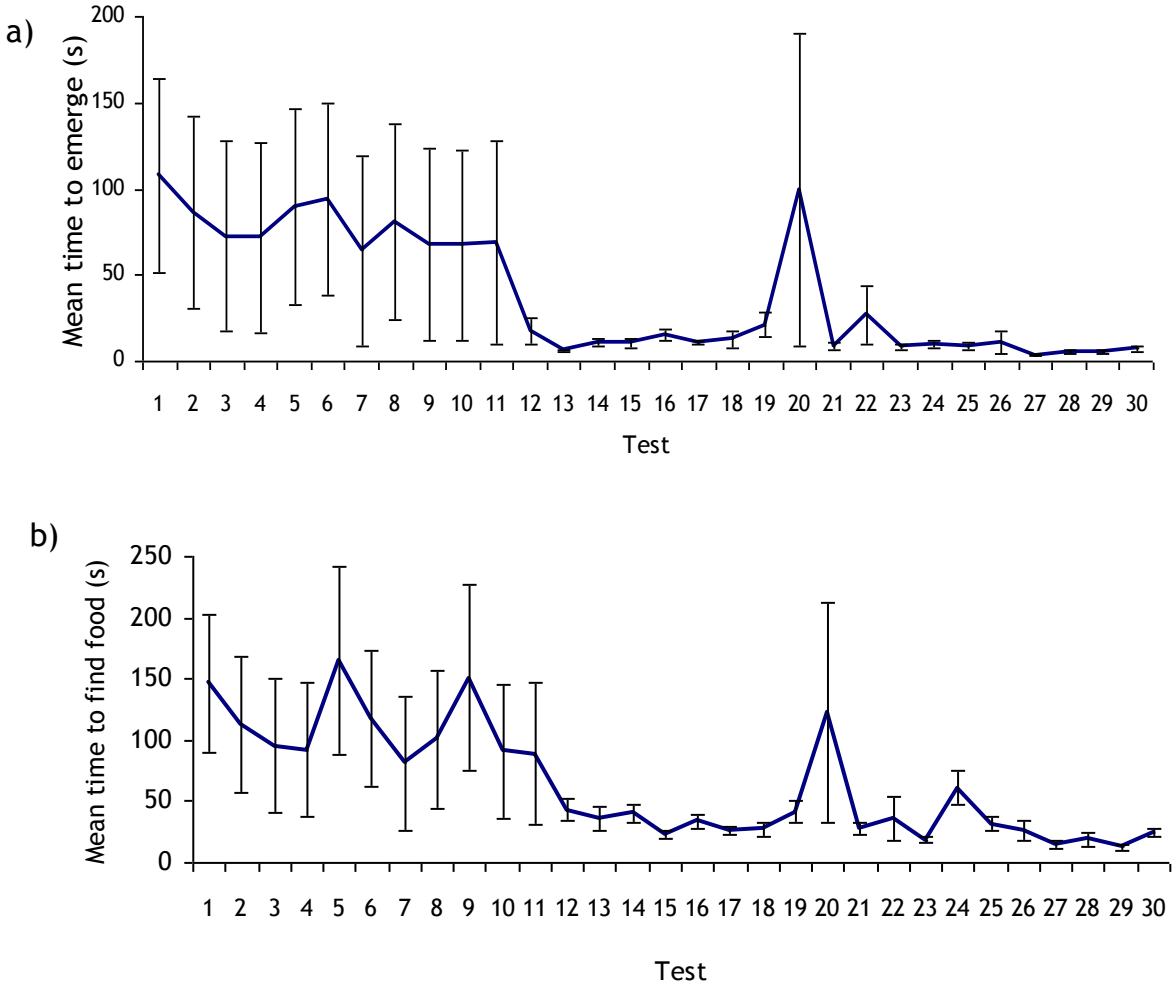


Figure 3.48 Mean (SEM) a) time to emerge and b) time find food in each test on reversal learning.

The same criterion to classify fish by learning category was used for the reversal learning tests: 1) fish that made more than 70% considering all tests were classified as

having learned to follow the cue (LC) and those that made less than 70% were classified as random switch (RS); and 2) fish that made more than 80% on the last 10 tests were classified as having learned to follow the cue. However a new category was added; fish classified as LC in the learning tests that continued to follow the colour from the learning phase (those that made less than 50% of correct choices) were classified as stick.

Figure 3.49 show the different strategies used by fish in the learning and reversallearning phases (Chi-square = 5.968, p = 0.051). Fish that learned to follow the cue in the learning phase showed a varied response on reversal learning. Fish that were considered random-switch in the learning phase also showed variable responses in the reversal phase. Since by definition they had not learned to follow the cue, the reversal learning test does not really make sense for these fish, which were put through the reversal tests to get further information on the effects of light colour on learning.

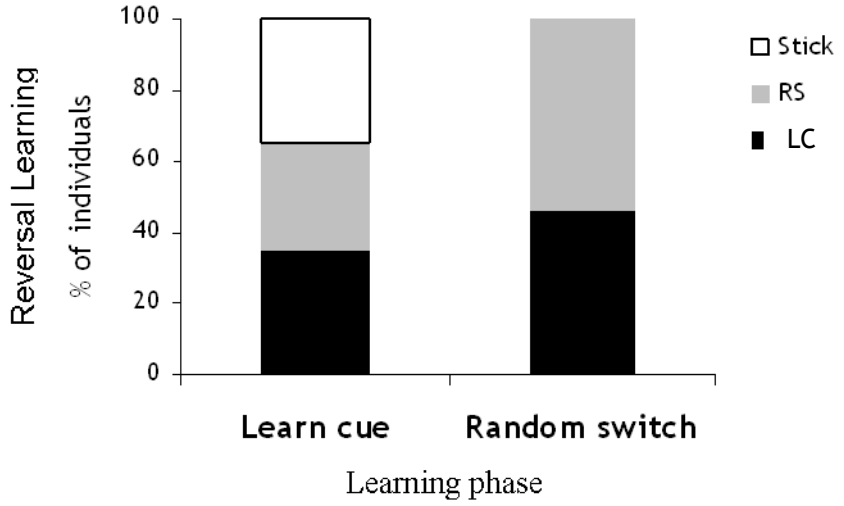


Figure 3.49 The percentage of fish in learning phase classified as learned to follow the cue or use RS strategy that either learned the cue, showed random switch or continued to follow the previous rewarded cue (stick).

Light colour had a strong influence on the strategy chosen in the reversal learning (Chi-square = 10.929, p = 0.004). In figure 3.51, we can see that all fish that stuck with the light colour on which they had been trained had been rewarded for the red light in the learning tests and so were now faced with the yellow light. Most of the fish that were trained with the yellow light chose the random-switch strategy and most fish that was trained with red light chose the LC strategy, as in the learning trials. All the fish that stuck with the previous colour were trained with yellow on reversal learning.

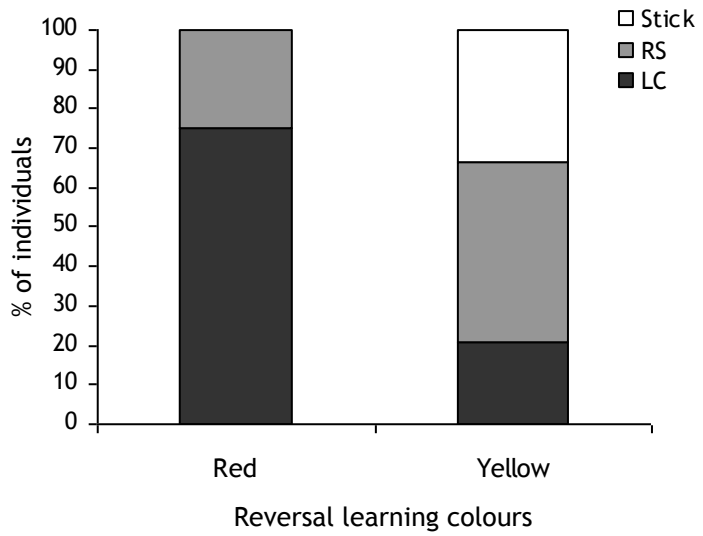


Figure 3.50 The percentage of fish in reversal learning phase classified as learned to follow the cue, use RS strategy or continued to follow the previous rewarded cue (stick).

Strategy in the reversal learning test was marginally significant related to risk-taking phenotype (Figure 3.52. Chi-square = 9.199, p = 0.056), with more risk-takers being classified as random-switch and more intermediate fish learning the cue.

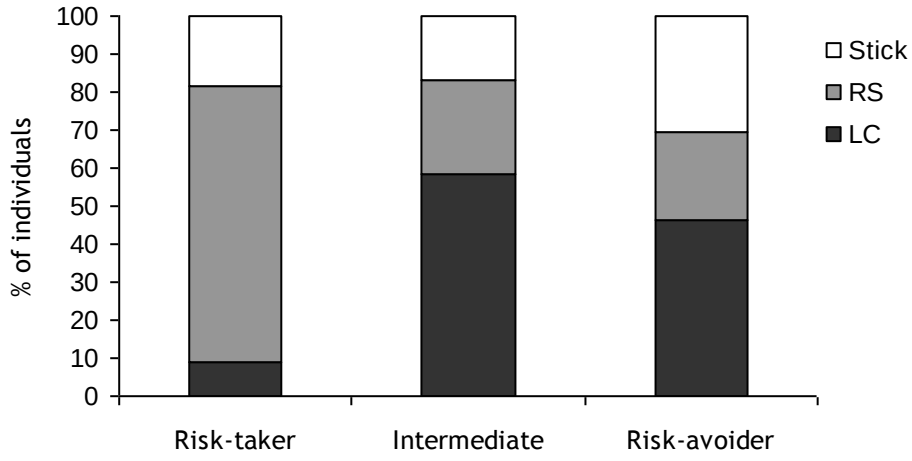


Figure 3.51 Distribution of the risk-taking phenotypes in relation to learning strategy on reversal learning phase.

The percentage of correct choices was not related to risk-taking phenotype (Kruskal-Wallis test: H_2 = 4.68, p = 0.096). Fish that learned to follow the light cue (LC) had a higher percentage of correct choices than for RS fish (post-hoc < 0.05) and than those that stick with colour from learning tests (post-hoc < 0.05). RS fish also showed higher percentage of correct choice than fish that stick with the learning test colour (post-hoc < 0.05). The red colour has a higher percentage (70%) of correct choices (Mann-Whitney test: W = 335.0, p = 0.0002) than the yellow (50%).

4 Discussion

Assignment of fish to risk-taking phenotype: The common carp used in this study showed great variability in response to the novel environment test. The fact that this variability was consistent at the individual level allowed them to be separated into three distinct categories: risk-takers, risk-avoiders and intermediate fish. It should be noted, however, that the fish classified as "intermediate" would perhaps be better characterised as "flexible", since instead of having an intermediate emergence time in all tests, they emerged quickly in some cases and slowly in others. This was associated with a marked fall in emergence time with successive trials, seen much more clearly in intermediate fish than in risk-takers or risk-avoiders. The classification into three risk-taking categories based on the novel environment test was validated by the differences in time to emerge in the pre-training period and by the related fact that fish classified as risk-takers took fewer trials than intermediate and then risk-avoiders to meet the criterion for moving on to the learning phase (emerge and feeding in 3 sequential trials).

The original allocation to risk-taking phenotype is also supported by the fact that there was an association between response to the novel object and risk-taking phenotype assigned on the basis of the novel environment test, with fish that did not inspect the object at all (category 1) having relatively long mean emergence times, more frequent inspectors (category 3) having relatively short mean emergence times and fish that inspected the object once (category 2) being intermediate. Studies with other species of fish show variable results with respect to consistency of individual differences across contexts. Thus, bluegill sunfish showed consistent behaviour when submitted to tests that involved aspects of activity, risk-taking and exploratory behaviour (Wilson & Godin 2009). In contrast, individual rainbow trout showed similar behaviour when the contexts concerned were related (i.e. involving foraging), but were ranked differently in a different context, i.e. when exploring a swim flume (Wilson & Stevens 2005).

In the present study, there was no relationship between competitive ability and behaviour in the other two screening tests. This is in contrasts to results obtained for common carp by Huntingford et al. (2010), namely that fish that emerged quickly into a novel environment were more likely than slow emerging fish to gain access to a restricted feeding location. It also contrasts with work on three-spined sticklebacks, in which "bold" individuals were competitively dominant (Ward et al. 2004). These differences may well have arisen because, in the present study, in contrast to that of

Huntingford et al. (2010) the fish were not housed in small groups with the same fish with which they were tested, in both the novel environment and the competition test.

Examining patterns of learning in carp: This study has shown that the common carp used in this study learned to forage fast and effectively when offered food in one of two feeding chambers, but that they used different strategies to do so. 51.67% of the fish (designated learned cue or LC fish) learned to follow the light cue to find food, swimming directly to whichever compartment was signalled by the appropriate cue. Criteria for having learned to follow the visual cue were of comparable stringency to those used in other studies. For example, Atlantic salmon were offered two feeding sites one of which (signalled by a visual cue) was rewarded; fish were deemed to have learned successfully to track the rewarded site using the visual landmarks if they were found in that location on 70% of occasions or more (Braithwaite et al. 1996). The same criterion (70% of correct choices) was used for goldfish in an experiment where it has to locate a place in the tank using geometric information (Vargas et al. 2004).

The remaining fish in the present study used a different strategy, swimming to one chamber at random and, if no food was found, switching quickly to the neighbouring chamber (designate random-switch or RS fish). Because the feeding chambers were close together and the initial visit to the non-rewarded site very short, this was an efficient way of locating food in the present set up. Short visits to non-rewarded sites were reported for goldfish trained to use landmarks to locate food (Warburton 1990).

An ability to use visual cues to improve the efficiency of foraging has been described for several species of fish. For example, goldfish trained to use a landmark to find food also showed increased speed and accuracy in finding food with successive trials. Spatial learning was poor in the absence of visual landmarks, but when food was signalled using Lego brick columns, fish showed high accuracy of choice, less variability and reduced time to locate food with experience (Warburton 1990). Goldfish also show an increase in effective use of space with successive trials when they have to escape from an enclosed space through an exit (goal) signalled by geometrical features (Vargas et al. 2004). Atlantic salmon learned to use shapes and colours of landmarks symbols to choose between one of two potential feeding locations only one of which produced food in each trial. The fish tended to be found waiting for food at the rewarded location, signalled by a visual cue, with a mean performance level of 75% at the rewarded location (Braithwaite et al. 1996). In contrast, common carp failed to learn to approach a flashing blue light to receive food, apparently because the carp were alarmed by this stimulus (Karplus et al. 2007).

The colour of the light used as cue in this experiment had an effect on learning, with fish trained with the red light having a higher percentage of correct choices than those trained with the yellow light. These two colours were chosen because carp are known to be sensitive to the wavelengths transmitted by both (Hanaoka & Fujimoto 1957) and because red and yellow were thought to be likely colours of the natural diet of these omnivorous fish. A number of studies have demonstrated clear colour preferences in fish in the context of feeding. For example, guppies (*Poecilia reticulata*) were reared on brown-coloured flake food from birth to maturity and then fed orangey-brown *Artemia nauplii* and Tetramin flake food (green, brown and red) for more than one week. They were then tested for attraction to simultaneously-presented coloured discs of 8 different colours (red, orange, yellow, green, black, white, blue and purple). Fish approached and bit at orange discs significantly more often than discs of any other colour, except red (Rodd et al. 2002). Attraction to red has also been described in three-spined and nine-spined sticklebacks (Smith et al. 2004). But red is a warning colour in other taxa.

Those fish that learned to follow a light cue to get food were subsequently tested in the reversal learning test. Not all fish made the transition, since some of them did not reach the criterion of this study. Even though random-switch fish never reach criterion, they were tested at the reversal learning to assess light influences on learning process, since some were in pairs with fish that had learned to follow the cue. Time to emerge and find food reduce with trial in the reversal learning as in the learning phase showing that the fish were also learning to emerge and feed in the reversal learning phase. Similar to the learning phase, in the reversal phase also the red colour was associated with a higher percentage of correct choices. Colour also had an influence on the strategy used by fish to find food in the learning tank, with more fish learning the cue when trained with the red light. Two effects might be involved. Firstly, fish trained to the red light in the reversal trials by definition had been trained to yellow in the original learning trials, so may not have learned so well, hence were quicker to learn the new rules. Goldfish in a reversal learning set-up showed a period of fixation to the old (now empty) patch (Warburton 1990) and the same was observed in the present study for some of the fish trained with the red light in the learning phase when altered to yellow light in the reversal learning (extinction process). Secondly, red colour might have a special salience as described above, promoting fast learning in the reversal phase as well as in the original learning phase.

Relating learning performance to risk-taking phenotype: During the learning phase, risk-takers were faster to emerge and find food than were risk-avoiders, with intermediate fish tending to fall in between. These results confirm what happened in

the pre-learning phase, where risk-takers are more willing to take risks and less cautious than risk-avoiders. These differences in behaviour during the learning trials could be a straightforward result of differences in response to risk, rather than to differences in the ability to form learned associations. This might be comparable to the faster learning behaviour of rainbow trout classified as bold on the basis of amount of time spent in an open area when required to approach a feeding area in response to a light. The trout classified as bold were fast, whereas those shown to be shy fish were cautious and slow (Sneddon 2003). In both this and the present study, it could be that bold, risk-taking individuals, being more willing to spend time in the open area have more opportunity to associate the visual cue with food.

However, during the learning phase risk-avoiders were marginally more likely than risk-takers to use the LC strategy. Thus more carp classified as risk-avoiders improved their speed of finding food by learning to follow the cue than did the risk-takers or intermediate fish. This suggests a difference in readiness to use visual landmarks to track a spatially-variable food source. This interpretation is complicated by the fact that during reversal learning risk-taking carp tended to adopt the random-switch strategy, whereas intermediate fish learned to follow the cue and risk-avoiders showed a mixture of the 3 strategies. This may represent a real difference arising from risk-taking phenotype, but may also be the result of the various complex processes that were going on in the reversal learning trials (see above).

A comment on intermediate fish: Fish classified as intermediate presented flexible behaviour in the initial novel environment tests, changing from slow to fast emergence over successive trials. In the learning test, intermediate fish did not show a preferred strategy, but emerged and found food quickly across the tests. They showed similar behaviour to risk-takers with respect to the number of tests to reach criterion, taking longer than risk-avoiders. In the reversal learning phase, intermediate fish learned to follow the cue to get food. These results suggest that fish classified as “intermediate” on the basis of a novel environment test (and often discarded in subsequent studied for experimental tractability) may have special behavioural features that warrant further study.

Conclusions, comments and future possibilities

The common carp used in this study were highly variable in the time taken to emerge from a shelter; these individual differences were consistent over successive trials, making it possible to classify the fish in three risk-taking categories, namely risk-takers (which consistently emerged quickly), risk-avoiders (which consistently emerged slowly

if at all) and intermediate fish (which were flexible in emergence time). When required to track a food source that was moved randomly between one of two locations signalled by a coloured light cue, over a variable number of trials (mean and range) the carp became fast and efficient at locating food. This was achieved in one of two ways. Some fish learned to follow the cue, consistently swimming directly to the feeding compartment signalled by the appropriate light cue. Others swam to one or other compartment at random, switching quickly to the other compartment if no food was found; there was no sign of side preference (left or right). Fish were more likely to follow the cue when this was red as opposed to yellow and more study is necessary to determine why this might be. Risk-taking phenotype influenced the behaviour of the fish in the learning trials. In some cases, as when risk-taking fish emerged from the start box more quickly during the learning trials, this seems to be a direct result of the initial behavioural differentiation. In others, as when risk-avoiding fish are somewhat more likely to follow the cue rather than to pick a compartment at random, this could reflect differences in flexibility between risk-taking categories, as have been well documented for mammals. Overall, the results of the study reported in this chapter suggest that a proportion of carp at least can learn to associate a light cue with the presence of food, suggesting that this might potentially be used as a tool for developing low stress husbandry systems.

The tank used at the learning trials were small and the alternative feeding sites close together, so fish did not have to incur a cost for going to the wrong light cue. It would be worthwhile making the switch more costly by placing the alternative feeding sites further apart and/or by adding a maze or making the fish swim longer distances. An expectation might be that elevating the cost of making a mistake would produce more LC fish and possibly accelerate the learning process.

CHAPTER 4

Sorting fish on the basis of a conditioned response

The main aim of this chapter was to examine the feasibility of using a conditioned response to a visual stimulus to control the movement of carp. A self-feeding set up was used for the conditioning and a secondary aim was therefore to examine the responses of carp to such a system.

1 Introduction

1.1 Aquaculture and welfare

Fish stocking is important for the fisheries industry and has recently been included as a tool for conservation of threatened species (Flagg et al. 1995). Also fish culture is essential for the food market. Asia, the Indian Subcontinent and Southeast Asia dominate aquaculture production; however, Europe and North America are also substantial producers of aquaculture products. In the past decade, aquaculture has expanded rapidly, and is now recognized as a major food production industry. As is true for other segments of agriculture, aquaculture practices are now being examined to assess their impact on the environment and on animal welfare (Conte, 2004). There is well-documented and legitimate concern about animal welfare in aquaculture. Welfare research has identified adverse effects of various aspects of husbandry practice, including confinement, inappropriate densities, restricted feeding, handling, transportation and slaughter (Branson, 2008).

Fish that are farmed for the food market are mainly farmed in intensive systems where the productivity in terms of growth rate and stocking density must be high to be economically viable (Brannas & Johnsson 2008). This kind of system is more stressful to the fish than extensive system and raises more concern about their welfare. When carp, for example, are reared intensively at high stocking densities they show higher plasma levels of cortisol, glucose and free fatty acids which all are indicators of stress. They also are more sensitive to an additional acute stressor (netting) than are carp reared at lower densities (Ruane et al. 2002).

Basal levels of plasma cortisol in unstressed salmonid fish are normally in the range 0-5 ng ml^{-1}. An acute stress such as handling or 1h confinement caused a temporary elevation of the plasma cortisol levels of both brown trout, and rainbow trout, in the range 40-200 ng ml^{-1}, with a return to basal levels within 24-48 hours (Pickering & Pottinger 1989). Rainbow trout also showed increased cortisol levels when exposed to grading and transportation (Flos et al. 1988).

Another criterion used to evaluate health and welfare in farmed fish is the feed intake. Sørum & Damsgard (2003) investigated the effects of anaesthesia and vaccination on feed intake and growth of Atlantic salmon. Benzocaine anaesthesia did not have a significant effect on feed intake, but fish vaccinated with an oil adjuvant vaccine had a significantly reduced feed intake in a period of 12 days after vaccination. The type of

slaughter used also affects fish stress responses. Slaughter methods include electrical stunning followed by decapitation, blunt trauma to the cranium, percussive stunning with a captive bolt, “cold stunning” and dewatering (Conte 2004). Methods that take into account welfare and quality are preferred in which the fish is kept unconscious until death without pain or suffering prior to killing. A study tested four types of slaughter: exsanguination without prior stunning, carbon dioxide narcosis followed by exsanguination, percussive stunning and spiking the brain. Only percussive stunning and spiking the brain resulted in no aversive reactions from the fish (Robb et al. 2000).

Most sources of stress encountered by cultured fish, such as handling, sorting or transport, are part of routine operations and are generally inevitable. Even when carp are reared extensively, culture systems inevitably introduce a number of stressors to the organism. These may include poor water quality (for example, high levels of ammonia, unsuitable pH, high levels of carbon dioxide and low dissolved oxygen levels), inappropriate water temperature, crowding, handling, with resulting physical damage, disease treatments and incomplete nutrition. It is impossible to avoid many of the procedures known to induce stress in fish. Netting, grading and transport are integral components of the fish farming routine; all the fish farmer can do is try to minimize the effects of such stressors (Pickering 1993).

1.2 Low stress husbandry techniques

Therefore it is important to make husbandry practices less stressful, including separation for example by size, age or reproductive status. In Poland, medieval carp husbandry systems were constructed in such a way that, when draining the pond, the farmer used the fish natural attraction to water flow to gather the fish in one region of the pond and facilitate harvesting (Pilarczyk pers. communication). More recently, natural or conditioned responses seem promising for use in aquaculture systems, both in groups and individually. For example, the innate positive phototactic and rheotactic responses of guppies were manipulated to stimulate fish to swim from one container to another, transferring them through pipes or narrow channels and allowing inspection and sorting using a computer vision system (Karplus et al. 2003, 2005). The natural attraction of fish to water flow is used to guide fish to a passage through the barrier of the Igarapava dam in Brazil in such a way that the fish swim close to a window. This window has a video camera to register the species using the ladder and also the size of fish that are able to exploit this type of aid to migration. The aim of this set up is to minimize the impact of the dam on the species that inhabit the river (Bizzotto et al. 2009). At the individual level, farmed Atlantic salmon can be trained to swim towards a

light to gain food; it is then possible by means of a focussed light beam directed at the eye of a particular fish to induce that fish alone to move away from the other fish and to the feeding point; such a system might be used for managing high value cultured fish such as broodstocks (Lines & Frost 1997).

The primary aim of the study described in this chapter was to examine the feasibility of using a conditioned response to a visual stimulus to control the movement of carp. A self feeding set up was used for the conditioning and a secondary aim was therefore to examine the responses of carp to such a system.

1.3 Self feeding systems for fish

Aquaculture is one of the fastest growing animal industries and its development depends on cost-effective feeds and feeding systems. Food costs are high in farming systems. Therefore the development of feed types, feed delivery systems and feeding routines that reduce feed losses and ensure effective consumption of nutrients is extremely important for the success of the practice (Le François et al. 2010).

In intensive fish culture, a common method of feed delivery is manual feeding. This may be more labour-intensive than the use of automatic feeding systems, but the cost is smaller. Hand feeding is often continued until the feeding activity of the fish is seen to decrease markedly or cease, so it offers the opportunity for a reasonable degree of matching of feed delivery to fish appetite. However, manual feeding is constrained by the regularity with which farm workers can gain access to the rearing units (e.g. ponds, tanks or cages) and the time it takes to distribute feed to each rearing unit. For this reason, feeding is sometimes carried out using either simple mechanical self-feeding devices which the fish trigger to release feed, or automated electric feeders (Houlihan et al. 2001; Le François et al. 2010). Farms using highly-capitalised, intensive culture systems usually employ automatic feeding.

1.3.1 Automatic feeding

In many cases farmed fish are fed by hand, but automatic feeders can be used to distribute food pellets to fish and there are many designs available such as conveyor belts feeders, feed hoppers or disc feeders. There are two main types of automatic feeders: timed release feeding systems and on-demand feeders; the latter can be subdivided in two categories, self-feeding and demand-feeding (with feedback mechanism).

Timed release feeders deliver portions of food pellets at pre-determined times that can be set up according to the age, species and size of the fish. For example, in the farming of Atlantic salmon fed using automated feeding systems, fish in the freshwater stage and in the period immediately after transfer to sea cages tend to be fed several meals each day; this is gradually reduced to 3 meals per day for larger, on-growing fish in summer and to one meal per day for large fish in winter (Le François et al. 2010).

Time-release feeding systems can save on labour costs, but may create problems for optimal feeding management. It is important that the timing matches the fishes natural patterns of feeding activity and, for example, the feed may be distributed when the fish are not hungry. Too little or too much food can be released resulting in poor growth and feed waste. The importance of giving fish the right rations is demonstrated by the fact that Atlantic salmon parr fed 100% ration grew in weight much more than those reared on 66% ration and into 33% ration (Figure 4.53 Berrill et al. 2006). Too much feed can also cause problems, such as decrease of water quality due to excessive dissolved and particulate waste.

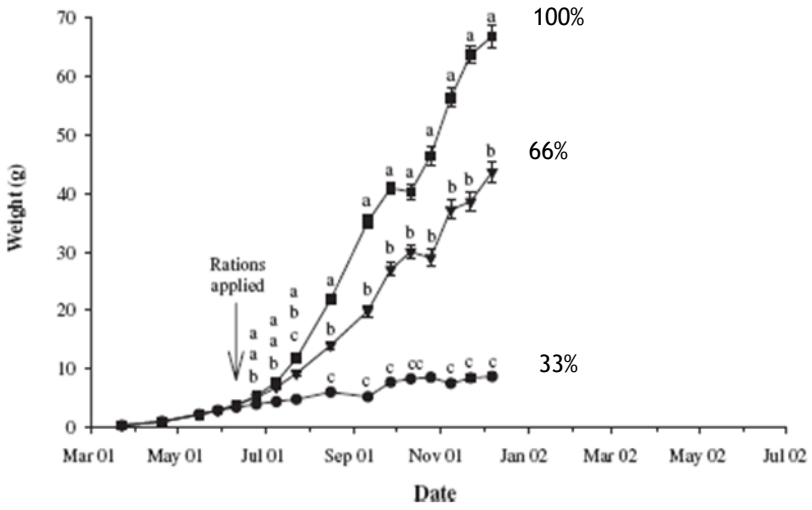


Figure 4.52 Changes in weight of Atlantic salmon parr fed different daily rations from early development. Figure legends denote the daily rations experienced in the respective experiments. Different lettering denotes statistical differences (P<0.05). When lettering has been stacked it is displayed in the same order as the graph lines (Berrill et al. 2006).

An alternative to carefully pre-programmed feed delivery by timed release is to use systems that adjust the frequency of feed delivery and the amount delivered according to appetite and/or feed wastage. For example, various kinds of demand feeding systems including automated feed delivery is matched to fish appetite. In feedback systems, feed delivery is controlled by some proxy for appetite, which may be detection of uneaten food (e.g the Aquasmart system, Blyth et al. 1993) or monitoring of fish movement patterns (Juell 1991). In self-feeding systems, the feeder is themselves

activated via a trigger that the fish activate, which directly controls the timing and amount of food delivered (François et al. 2010).

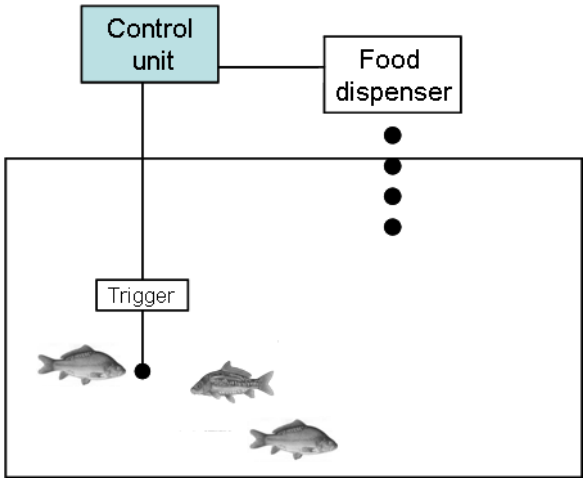


Figure 4.53 Schematic diagram of a self-feeder system. Actuation on the trigger by the fish send a sign to the control unit which activates the food dispenser allowing feed release.

In terms of production, such systems have the clear advantage of dispensing the pellets when the fish are most eager to feed and they also provide adequate amounts of food with little waste. Conversely, individual differences in behaviour can result in some fish being excluded from the feeding activity. In terms of monitoring and research, when all trigger activations are recorded, self feeders also provide an estimate of fish appetite and how this changes with, for example, time of day and environmental conditions.

1.3.2 Use of self-feeding system

Several studies have shown that fish can be trained to press a trigger to obtain food, including sea bass (Millot & Bégout 2009), thick lipped mullet (*Crenimugil labrosus*), common carp (Wright & Eastcott 1982) and ayu (*Plecoglossus altivelis altivelis*) (Amano et al. 2007). Self-feeding systems can also be used in aquaculture to evaluate growth, production and fin damage and also compare the efficiency of demand feeding with existing farm practices. Feeding fish using a demand feeder reduced the incidence of dorsal fin erosion (Noble et al. 2008). The fish learn to use the self-feeding system through exploration of the environment and formation of a learned association between an action (biting or touching a sensor) and a stimulus (food) through operant conditioning. Such associations are often formed inadvertently in aquaculture, as when fish learn to associate the footsteps of the farmer with the delivery of food (Ferno et al. 2006).

The majority of the published studies on the use of self-feeders in aquaculture focus on the feeding activity itself rather than on the learning process that underpin trigger activation, most just mentioning how long the fishes took to start touching the device. For example, barfin flounder *Verasper moseri* was able to learn to actuate the trigger within 2 days, but no more remarks on the learning itself were gained since the study was about feeding activity (Sunuma et al. 2009). The study described here was designed, among other things, to examine the initial behaviour shown by carp towards a self-feeding trigger and the time course of development of effective activation.

1.3.3 Differences in behaviour with self-feeding

Learning skills are extremely important in the aquaculture environment, since they help fish to adapt to the new environment and to cope with husbandry procedures. In the context of learning to use a self-feeding system, the behaviour of fish varies considerably in terms of, for example, the time to learn how to use the feeder and the relative frequency of trigger activation. Most studies using demand feeding demonstrate clear differences in trigger activation; for example, sea bass fed using a self-feeder could be divided according to their number of trigger actuations in three groups designated high-triggering fish, low-triggering fish and zero-triggering fish (figure 4.54 Millot & Bégout 2009).

Some of the variation in time taken to learn to use the feeder depends on the species concerned values ranging from 10 to 45 days being reported (Jobling et al. 2001). For example Juvenile sea bass (*Dicentrarchus labrax*) actuated the trigger for the first time 14 days after the beginning of the experiment (Di-Poi et al. 2008), although in another experiment with the same species, the first actuation occurred on average 10 days after the experiment started (Millot et al. 2008).

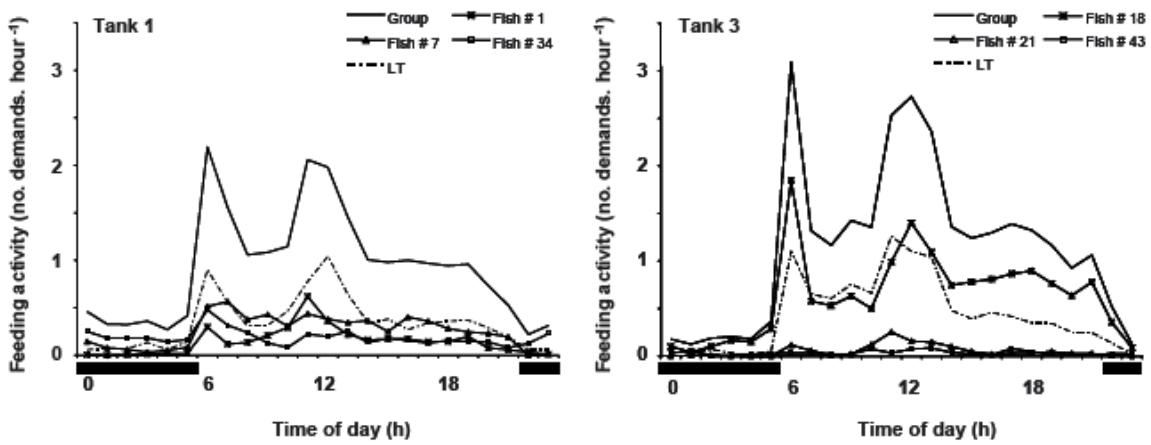


Figure 4.54 Mean hourly feed demand (sum of feed demand acts per every clock hour averaged over the entire experimental period) for the total group and for low-triggering (LT) and high-triggering fish in 2 experimental tanks. The *X*-axis represents time of the day (hours), and *Y*-axis represents mean clock hour feed demand acts number over 219 days. (Millot & Bégout 2009)

Goldfish learned how a self-feeder functioned and operated the trigger within few hours of its installation (Sanchez-Vazquez et al. 1996). A study using operant conditioning to train thick lipped mullet and common carp to use a self-feeder showed that groups of mullet (5, 10 or 20 individuals) were quicker to respond initially than groups of 5, 10 or 19 carp (Wright & Eastcott 1982). Surprisingly perhaps, the size of the fish may have little influence on the ability of fish to use self-feeders; in a study using groups of ayu even fish as small as 0.6g were able to use the self-feeder (Amano et al. 2009). However, the time taken to learn to use a self-feeder can vary with the number of fish in the group; for example, groups (of 8, 16 and 23 individuals) of rainbow trout learned to operate the trigger in 2 days while single rainbow trout needed 7 days (Landless 1976).

Many studies of the use of self-feeding systems have shown that most trigger activations are performed by a relatively small number of fish in the group. A study using cod *Gadus morhua* L. showed a bimodal distribution in trigger activation, with a peak on 0 and the other around 7 activations, although all the fish ate (Ablitt 2009). This means that some fish may be rewarded without performing the action of activating the trigger and at the same time the fish that carries out any given activation may not be rewarded, the learning situation and adjustment of reward level is complex.

Although most of the studies do not focus on learning and facts related to it, Nilsson & Torgersen (2010) present a conceptual model of the learning processes involved in demand feed triggering (figure 4.55). The actual results of their study were very similar to those predicted by the model. The model shows that the triggering rate of unrewarded fish reduces as a function of time (figure 4.55l). At first, a novel object

would attract the curiosity of the fish with a high triggering probability but then this curiosity would attenuate to an "acquainted" frequency (figure 4.55II).

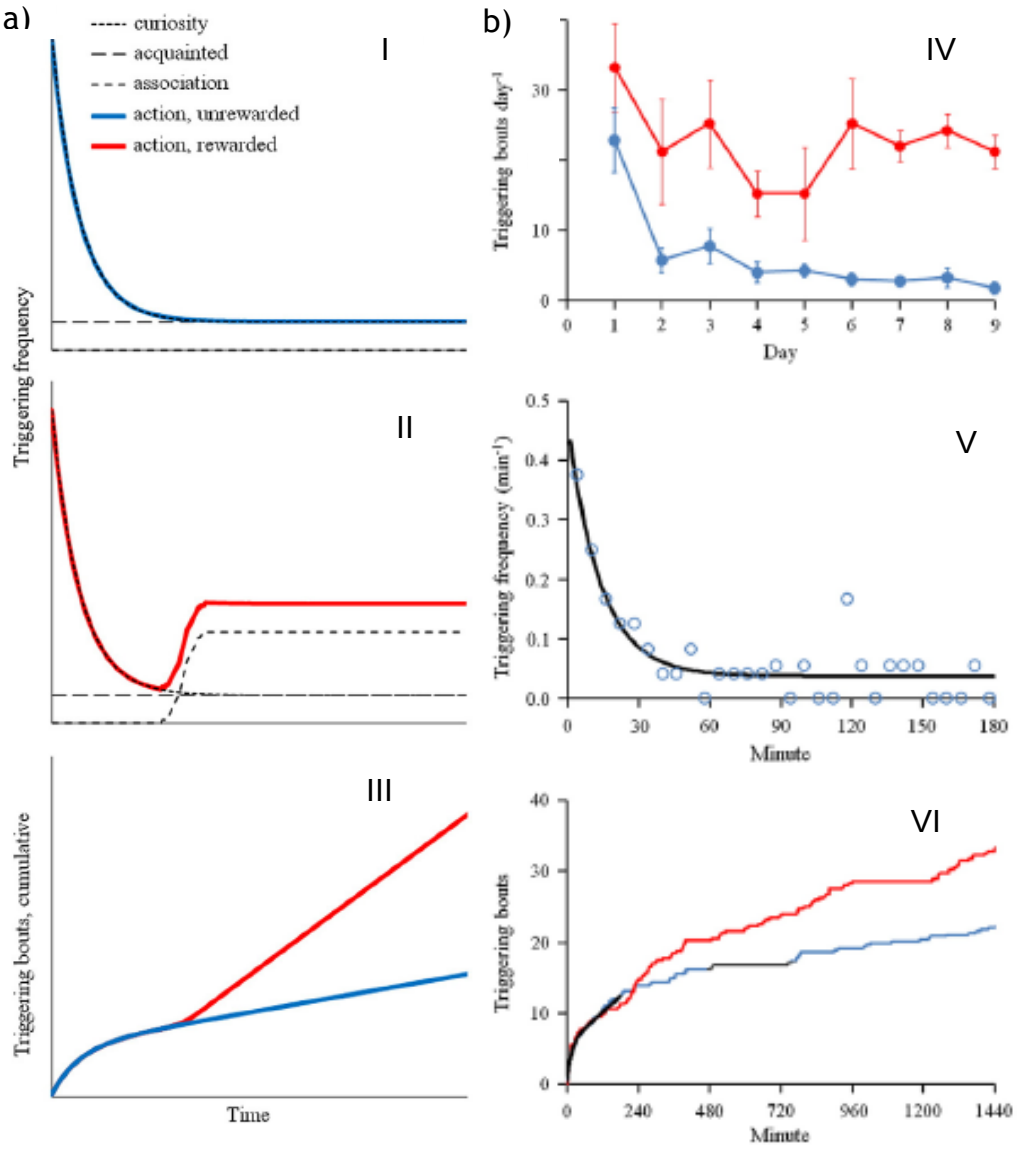


Figure 4.55 a) Outline of conceptual model proposed by Nilsson & Torgersen (*in press*). It is a model for triggering propensity as a function of motivation that can be due to curiosity towards novel objects or due to an established cognitive association between action and reward. I) Fish that are offered a novel bait but without reward. II) Fish that are offered a novel bait that provides reward. III) Cumulative triggering for rewarded (red line) and unrewarded (blue line) fish. b) Actual results of the study.

There are a number of possible explanations (not mutually exclusive) for variation in trigger activations within a group of fish, including social status, coping strategies and level of "curiosity". Individual differences become especially important when there is an increase level of intraspecific competition. The demand feeding behaviour of Arctic charr *Salvelinus alpinus* was affected by stocking density, the ability of the fish with high social status to monopolize the demand feeders was reduced at high densities but

when the individuals were grouped into quartiles based on their individual rank, charr ranked within the upper quartile accounted for the majority of actuations independent of stocking density (87% of the total). Instead of there being a single dominant individual, as was the case under low density conditions, a small group of individuals dominated the actuation of the trigger at high densities (Alanara & Brannas 1996). Social rank affected triggering activity in rainbow trout with dominant fish having a higher actuation level followed by sub-dominants; subordinates showed the lowest actuation level (Alanara & Brannas 1993).

Differences in risk-taking phenotype may also be important, since risk-taking fish and those with a tendency to approach a novel object may be more likely to approach and make contact with the trigger and hence more likely to learn the association between touching the trigger and obtaining food.

1.4 Coping strategies

As discussed in Chapter 1, coping style or strategy can be defined as a set of behavioural and physiological responses that is consistent over time and that is characteristic to a certain group of individuals. Although a continuum of responses is often observed, at the extremes two distinct categories of individuals are recognisable, namely proactive and reactive. Primarily, when exposed to a stressor, proactive individuals display a sympathetic activation (the fight/flight response), while reactive individuals respond with an parasympathetic activation (the conservation or withdrawal response). Consequently, reactive individuals respond to stressors with greater hypothalamic–pituitary–adrenocortical (HPA) axis reactivity, leading in a larger increase in plasma glucocorticoid levels compared to proactive animals (Schjolden et al. 2005).

Comparisons of wild and captive fish behaviour indicate that domestication selects aggressive and risk-taking behaviour. Risk-taking, aggressive fish with low stress responsiveness (proactive copers) do well and shy, non-aggressive fish with high stress responsiveness (reactive copers) do poorly in intensive husbandry conditions. Captive rearing and domestication eventually select out the reactive fish, but only after many have starved and/or died of stress, damaging both production and welfare (Huntingford & Adams 2005). On the other hand, less aggressive animals are more flexible in their responses, are better at tasks that demand behavioural change and flourish in more variable environments (Koolhaas et. al 1999). Such differences in what is sometimes called “personality” may be reflected in several different contexts, including interactions with potential predators, encounters with conspecific rivals and during

exploration of unfamiliar environments and objects (Sih et. al 2004). Such differences are likely to influence how fish respond on being exposed to a self-feeding system, a possibility that we examined in the study described in this chapter.

1.5 Colour preferences in fish

In the study described here, different groups of common carp were trained to approach a self-feeding trigger signalled by a light of a particular colour (red, blue or green). The wavelengths involved were known to be visible to the fish, but the possibility that preferences among detectable wavelengths might influence the process of learning to activate the trigger was also considered. A number of studies have demonstrated clear colour preferences in fish in the context of feeding. For example, guppies were reared on brown-coloured flake food from birth to maturity and then fed orangey-brown *Artemia nauplii* and Tetramin flake food (green, brown and red) for more than one week. They were then tested for attraction to simultaneously-presented coloured discs of 8 different colours (red, orange, yellow, green, black, white, blue and purple). Fish approached and bite at orange discs significantly more often than discs of any other colour, except red (Rodd et al. 2002).

Three-spined and nine-spined sticklebacks were tested for colour preference in a feeding trial having previously been fed exclusively on neutral coloured food (the chopped adductor muscles of *Mytilus edulis*) and subsequently exposed in small groups consecutively with each of nine differently coloured plastic strips (black, white, red, blue, green, orange, pink, purple and yellow). In males and females of both species (Figure 4.57), the highest rate of biting was directed at the red strips, the rank order of colour preference being red (most preferred), orange, pink, purple, yellow, white, blue, black, green (least preferred) (Smith et al. 2004).

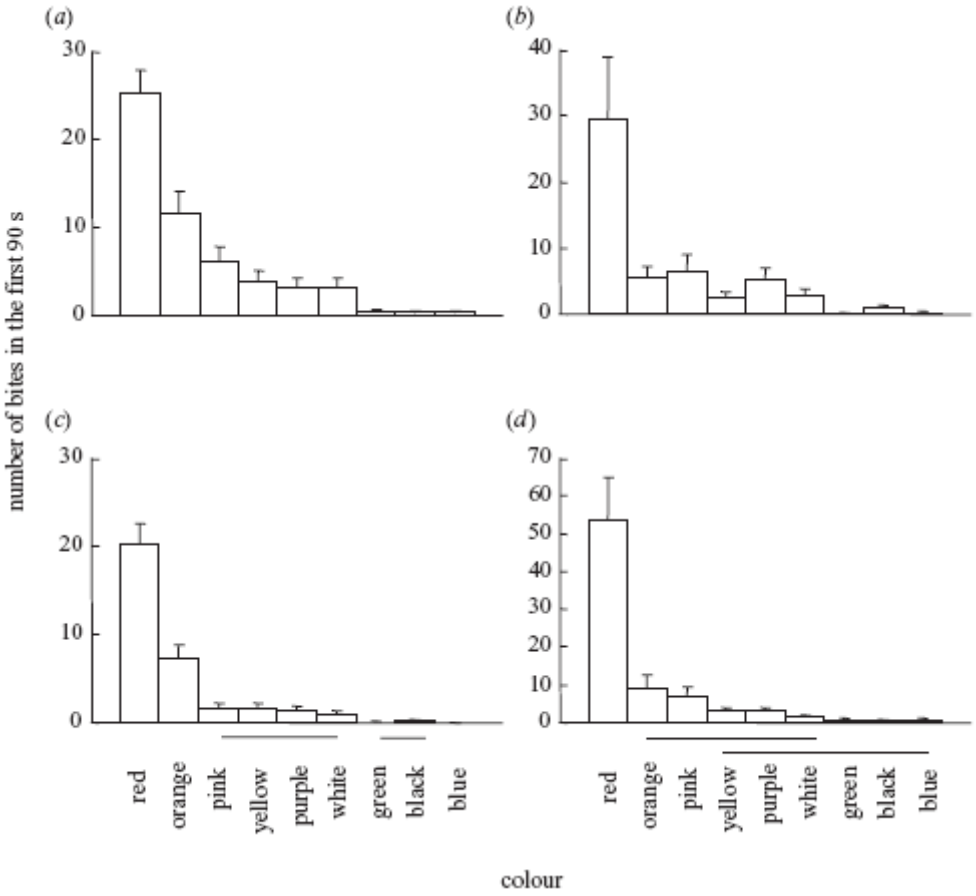


Figure 4.56 Mean responses to coloured plastic strips, measured as number of bites in the first 90 s for adult (*a*) male three-spined,(*b*) female three-spined, (*c*) male nine-spined and (*d*) female nine-spined sticklebacks.

On this basis, we chose three clearly distinct colours from within the range of the carp visual system, namely red, blue and green.

1.6 Aims of the present study

The main aim of the present study was to determine whether a conditioned response to a visual cue could be used to separate out the individual members of small groups in a strongly schooling species such as the common carp. Since the conditioning procedure was to be carried out using a self-feeding system, additional aims concerned the way in which individual carp learned to touch a trigger to receive food and the possible influence of coping strategy on this process. Thus the specific aims were to determine:

- Whether common carp can learn to approach and touch a trigger in response to food reward and if so, how quickly they learn this.
- The pattern of trigger activation, including which fish activate the trigger and which eat the food.
- Whether the colour of the light cue used to identify profitable feeders influences in rate of learning.

- Whether risk-taking phenotype influences rate of leaning and level of trigger activation.
- Whether carp can be trained to visit one of three feeders signalled by different coloured light to get food.
- If so, whether this learned response can be used to separate individual fish in small groups.

Since it turned out that some groups of fish failed to learn to touch the trigger in order to get food, a final, opportunistic, aim was to determine whether such carp could learn to use the trigger from association with a fish that had successfully learned this task (a "tutor"). Social learning in a foraging context has been demonstrated for several species of fish (Day et al. 2001, Pitcher & House 1987) so this was considered a possibility.

2 Material & Methods

2.1 Subjects and husbandry

54 mirror carp were obtained from VS Fisheries, Sparshold, Hampshire, UK (http://www.vsfisheries.co.uk). All the fish were weighed (g) and measured (cm); total length ranged from 7.4 cm to 10.6 cm (mean 8.98 cm) and weight ranged from 7.65 g to 19.82 g (mean 12.83 g). The carp were transferred to the Experimental Aquaria, Division of Ecology and Evolutionary Biology, University of Glasgow and kept in 9 glass tanks (100 X 38 X 31.5 cm), each tank with a re-circulating filter and airstones and housing 6 carp. The temperature of the tanks was at 12^{o}C. Carp were individually-marked using alcian blue dye (HO Licence number 60/3679) and photographed for future identification on the basis of scale pattern (see Chapter 2).

2.2 Pre-screening for risk-taking

2.2.1 Novel environment test

Fish were screened for risk taking using a variant of the well-established novel environment test (Yoshida et al. 2005), details being based on pilot studies described in Chapter 2. A group of 9 fish were tested at a time. This screening procedure was repeated 3 times for each fish, with a gap of at least 24 hours between trials. The fish were selected randomly for each trial from one of the holding tanks, to reduce the

possibility that social interactions within established groups might influence behaviour during screening.

2.2.2 Novel object test

At the end of the third novel environment test, the fish that did not emerge were netted to the same space as the fish that emerged and the groups of 9 carp were screened (at the same tank used in the novel environment test) using another commonly-used test of bold-shy behaviour, namely the novel object test (Frost et al. 2007), details being decided on the basis of pilot tests described in Chapter 2. Each fish was tested once in the same group as the last novel environment test.

2.3 The self-feeding system

The self-feeding system was composed by a platform over the tank, holding a sensor attached to a motor and feeder (figure 4.58a). The feeder was attached to a control box which in turn was attached to a computer. The platforms, sensor and motor were constructed by the Bioelectronics Department of the University of Glasgow. The feeders were made by Imetronic. They consisted of an internal disk with three holes that, when activated, rotated to allow a measured amount of around 0.17 grams of feed fell through a delivery tube and into the tank below.

The software designed to control the feeder (figure 4.58b) display allowed a bite limit (number of activations the sensor can have in each period before extracting the sensor) to be set. This could be different depending on fish behaviour and experimental setup. On the software, it was also possible to control the time when the system switched on and off and the accumulation time (the amount of time that the number of bites should be grouped together).

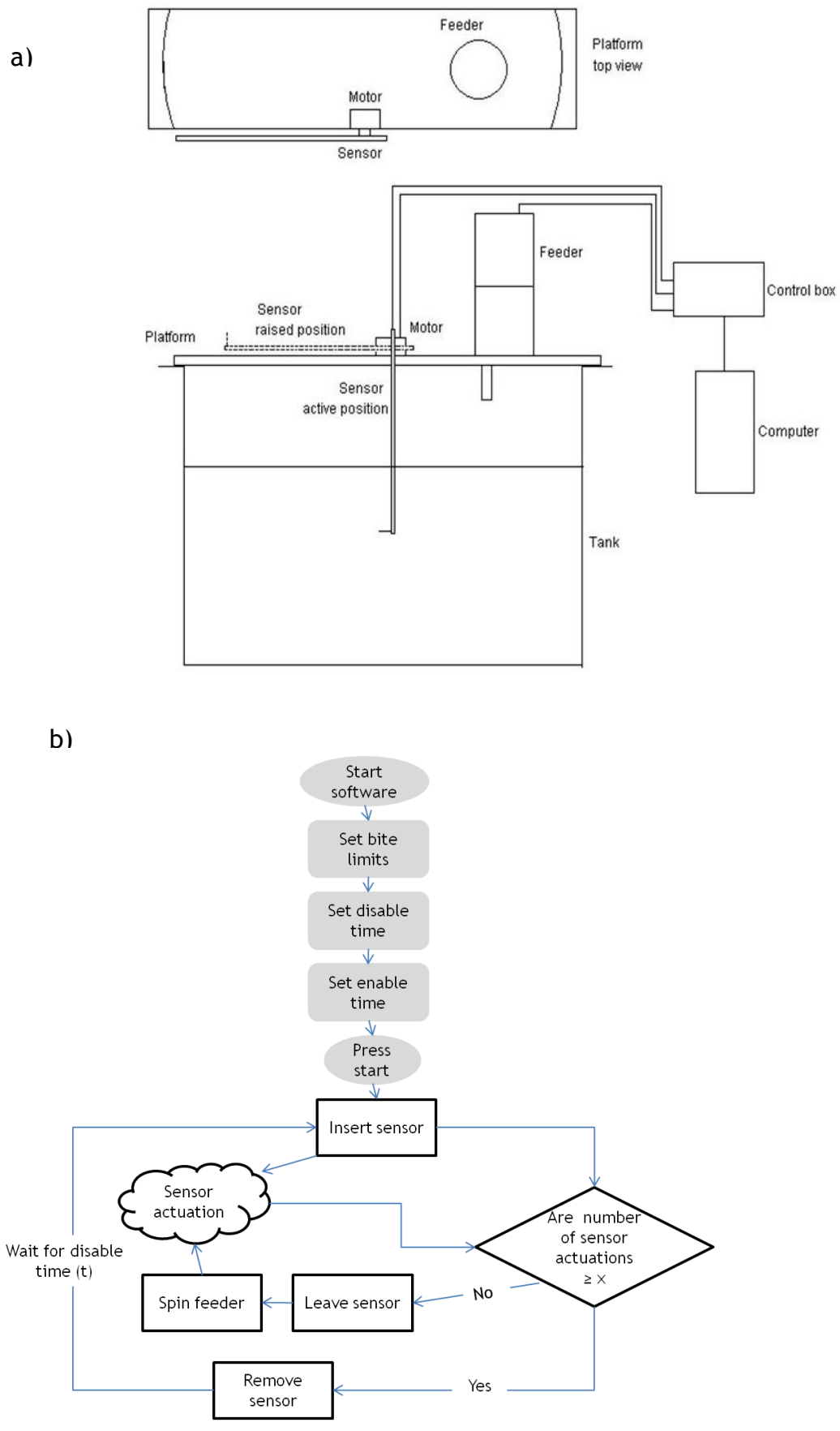


Figure 4.57 a) Schematic diagram of self-feeding system used in the tests, b) Flow diagram of the process involved in the running of the demand feeding system. The gray shapes represent stages where an operator input is required and the outlined shapes are those run by the processor.

Although this system had been used effectively in an earlier study (Ablitt 2009) a number of mechanical and software problems were experienced. Due to time constraints, it was necessary to improvise. The original sensor was utilized (with a small piece of red rubber on its tip), but this was not connected to the control box or to the computer), instead each time a fish actuated the sensor feed was delivery manually pellet by pellet by an assistant.

2.4 Initial observations of demand feeding

Groups of 3 carp were netted into the test tank and their reactions to the sensor were observed. A factor that influenced the behaviour of the fish was the presence or absence of pebbles on the bottom of the tank. In the first tests, there were a number of pebbles on the floor of the tank, which made it hard to visualize where the food pellets fell and whether they were eaten. The pebbles were therefore removed, but this appeared to frighten the fish. The pebbles were therefore replaced, but with a pebble-free "halo" around the lights so it was possible to see the food pellet and which fish ate it. Every time the fish come closer to the sensor (even without touching it) a food pellet was delivered. This approach is called shaping and is defined as "at first any coarse approximation of the desired outcome is reinforced, but reinforcement soon demands closer and closer matching to the required outcome to be effective" (Barnard 2004).

2.5 Demand feeding trials – one light

Carp were separated in 18 groups of 3 fish based on their response to the novel environment test and approximately matched for size. There were three different colours of light and 6 groups of carp were trained in each light. Fish were deprived of food and were only feed during the test.

The group of 3 individuals were netted to the release area (figure 4.59a) and then there was a 20 minutes observation period where the number of approaches to the feeding area (enter "halo"), number of touches to the sensor and the identity of these fish were observed as well as who ate the food pellets. To attract the fish to the area close to the demand feeder sensor each time they approach the light, a pellet was released in the water therefore every time a fish approach or touch the sensor a pellet was released into the water. This test was repeated 10 times with each group. The order of the tests was random meaning that at each day the groups were tested in a different order. After

10 trials, fish that had learned to approach (or touch) the light (sensor) were passed to the next stage of the experiment.

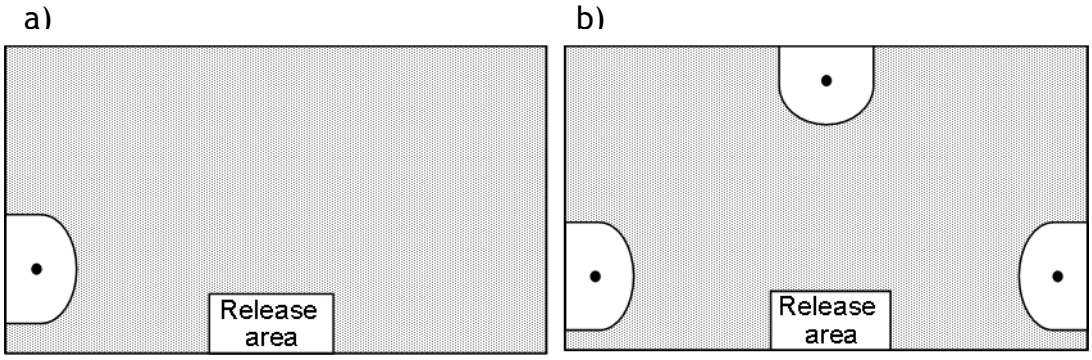


Figure 4.58 Schematic diagram of a) experimental tank used in the one light trials. Dark dot = position of the sensor (self-feeding system), white area = halo without pebbles around the feeding area, hashed area = area with pebbles and b) tank used on the three lights trials.

2.6 Demand feeding trials - three lights

The second phase of the experiment involved 3 colours of light in different positions in the tank (figure 4.59b). The groups of fish that learned the first phase of the experiment were now trained to approach the same colour of light on which they were initially trained, but now there were 3 colours of light in the tank, randomly moved between feeding locations at each trial.

One group of 3 individuals were netted at the release area and their behaviour was observed for 20 minutes. It was recorded: number of approaches to each light (enter "halo"), number of touches to the sensor (at correct and incorrect light), the identity of the fish that approach and/or touch the sensor and that ate each food pellet. At the end of the trial, the fish were netted back to their tank. The order of the tests was random, meaning that at each day the groups were tested in a different order.

2.7 Mixing groups trained on different colours

At the end of the three lights trials, 3 fish, one from each group trained on a different light colour, were placed in a group with 3 individuals, one trained with red light, one trained with green light and one trained with blue light. This group was placed inside a dark tube in the experimental tank (the same as was used in the three colours trials), the tube was removed and their behaviour was observed for 5 minutes. The following were recorded for each fish: the number of approaches to each light (enter in the "halo"), the number of touches to the sensor (at correct and incorrect light), the

identity of the fish that approached and/or touched the sensor and which fish ate each food pellet. This trial was run 3 times and no food was delivered during these trials.

2.8 Effects of addition of tutor fish

The groups of fish that did not learn the first phase of the experiment (one light) were placed in the training tank with a tutor from one of the groups that had learned, trained with the same colour as that of the group that had failed to learn (the not-learned group) forming a group of 4 individuals. This group was netted into the experimental tank (figure 4.59b) with the three colours of light and their behaviour was observed for 5 minutes. The following were recorded: the number of approaches to each light (enter in the "halo"), the number of touches to the sensor (at correct and incorrect light), the identity of the fish that approached and/or touched the sensor, which fish ate each food pellet and whether they follow the tutor or not.

2.9 Data analysis

All data were first tested for normality, which showed that they complied with the rules of non-parametric statistics. When the same fish were testes in successive trials, the data were analysed using repeated measures.The statistical tests used are shown in the results section. Analyses were performed using Minitab series 15 software.

3 Results

3.1.1 Pre-screening tests

The distribution of mean emergence time on the novel environment test can be seen on figure 4.60. Although the results for individual differences was not significant (Kruskal-Wallis test: $H_{53} = 56.74$, $p = 0.337$), the figure shows some variation with some fish emerging from the shelter faster than others. On this basis, fish were classified as risk-takers, risk-avoiders and intermediates as indicated in figure 4.60, but this classification was regarded as tentative.

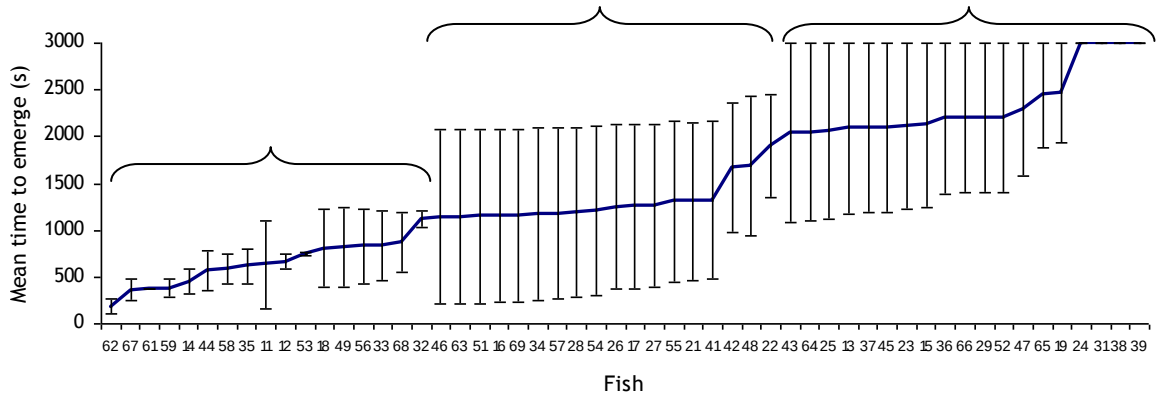


Figure 4.59 Distribution of mean emergence time (SEM) on the novel environment test for all the fish.

Figure 4.60 shows the frequency distribution of the number of approaches made by the carp to a novel object. There was a clear distinction between fish that approached the object and fish that did not.

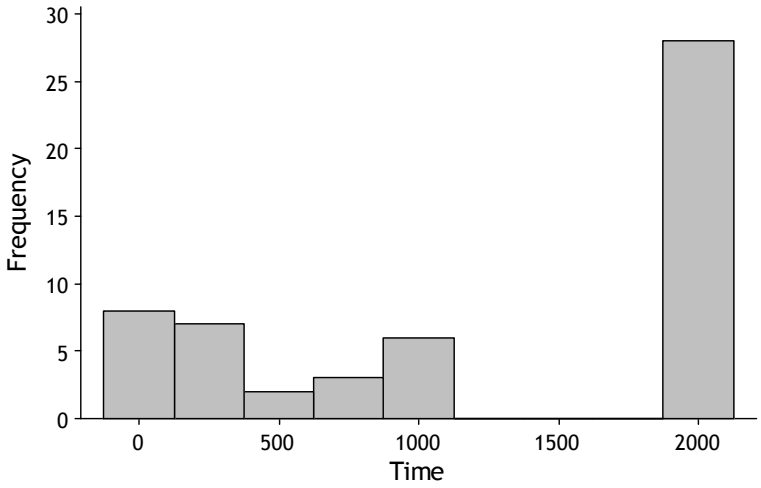


Figure 4.60 Frequency distribution of time to approach the novel object.

There was no difference in median emergence time between the fish that did or did not inspect the novel object (median for inspectors = 1227.2 seconds, median for non-inspectors = 1238.5 seconds, Mann-Whitney test: W = 774.0, p = 0.9517).

3.2 Self-feeding in the one light phase

3.2.1 Time course of learning

Figure 4.62 shows the mean (SEM) time taken by all groups of fish to touch the sensor for the first time in each test, for all tests and also only for those tests in which the sensor was touched at all. Considering all tests, touch latency fell (unevenly but significantly) with test number (dark line in figure 4.61 GLM repeated measures – $F_{1,25}$ = 6.10, p = 0.015). There was no significant effect of test number on time to touch the sensor just for the tests in which the sensor was touched (dotted line in Figure 4.62: Kruskal-Wallis test - H_9 = 11.44, p = 0.247). This result suggests that carp are capable of learning to use the demand feeding system and that once the sensor is touched at all, it is touched fast.

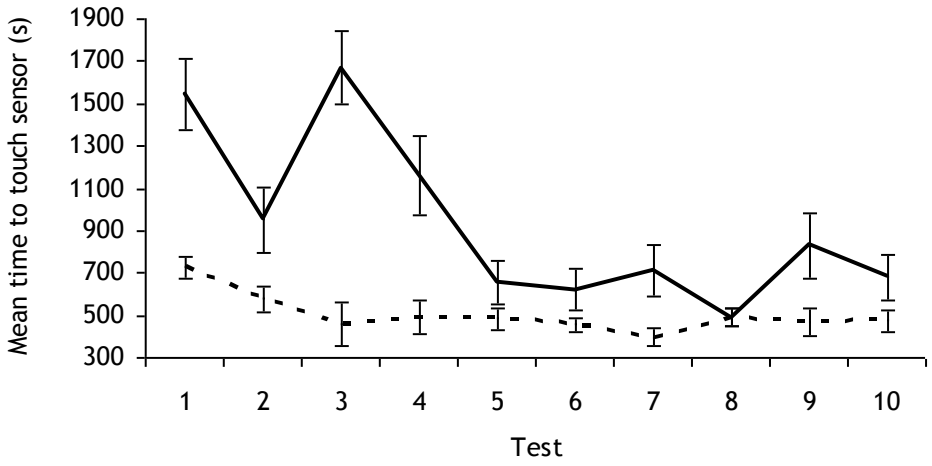


Figure 4.61 Mean (SEM) time to touch the sensor at each test. Dark line includes the fish that did not touch the sensor expressed by 2000 and the dotted line does not include these fish.

However, not all groups learned to use the demand feeding system. Of the 18 groups, 7 were considered not to have learned the task, since they approached the light and touched the sensor very few times. Since these fish rarely approached the light, they ate only a small quantity of food compared to the groups that learned to use the trigger. Figure 4.62 compares the number of touches and number of pellets eaten in each test for learners and non-learners.

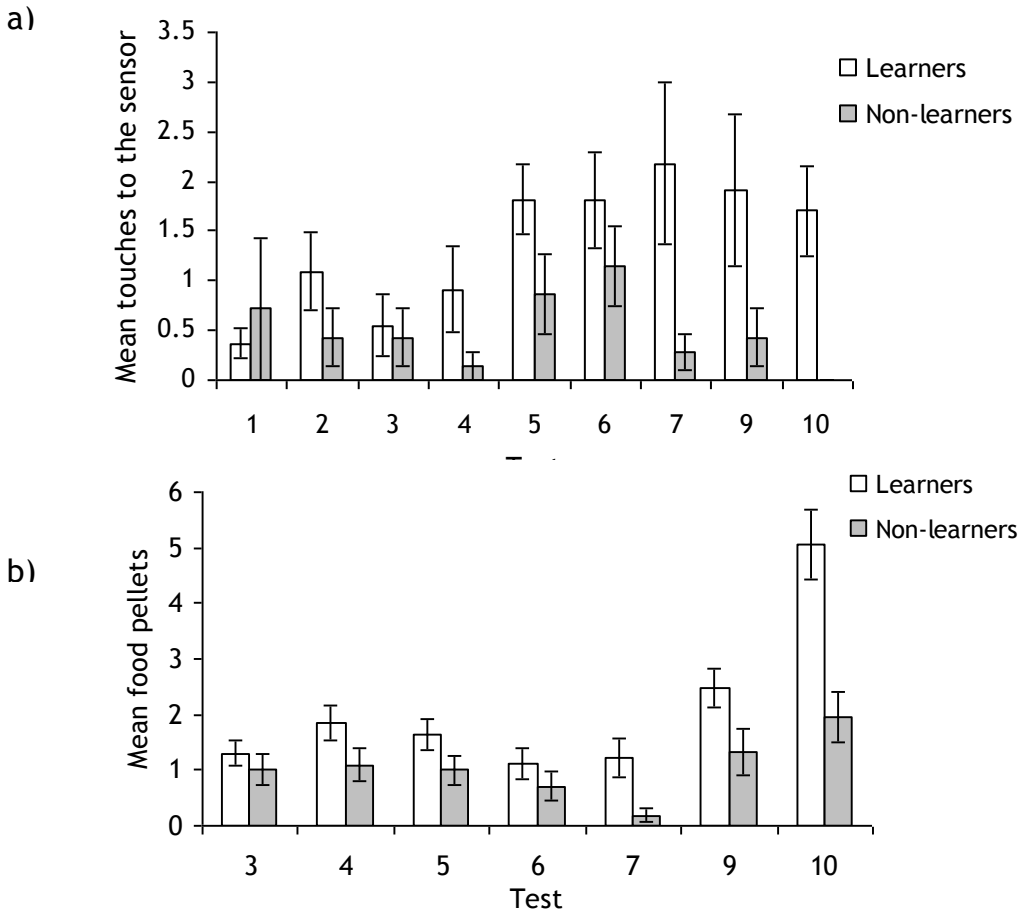


Figure 4.62 Mean (SEM) a) number of touches of the sensor and b) number of pellets eaten in groups designated learners and non-learners in the first phase of the experiment (with 1 light) in each successive test.

The group differences in learning were not due to an effect of light colour, because this had no influence of the time taken by the fish to touch the sensor (Figure 4.64). The groups of fish trained on the three different colours of light have similar median touch latencies (Median touch latency for blue = 548s, for green = 695s and for red = 614s; GLM repeated measures - $F_{1,27} = 1.35$, $p = 0.176$).

The incidence of learners and non-learners for each colour was: for the blue and green lights 3 groups that learned and 3 that did not, and for the red light had 5 groups that learned and 1 group that did not learn.

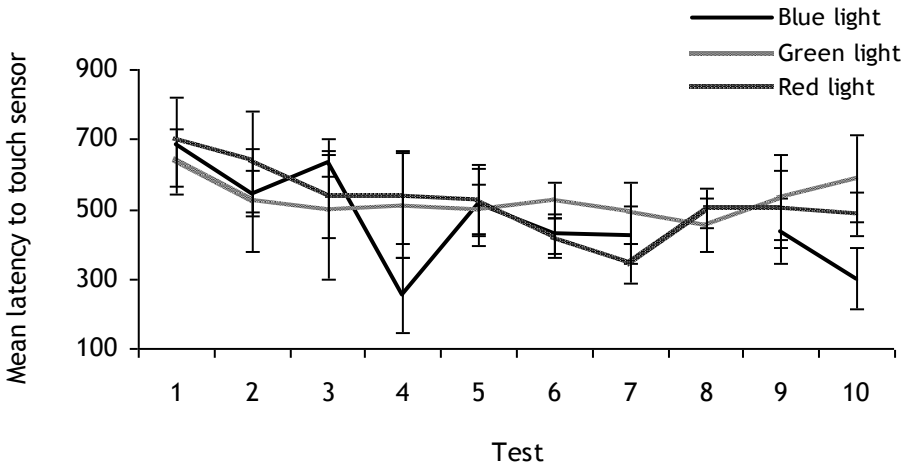


Figure 4.63 Mean (SEM) latency to touch the sensor for the three groups of fish coded by colour including both learners and non-learners.

3.2.2 Effects of risk-taking phenotype

The mean number of touches to the sensor made by each fish was unrelated to the number of times it approached the novel object (Pearson's correlation: R = -0.046, p = 0.743). The median number of sensor touches was also unrelated to risk-taking phenotype as determined by the novel environment test (risk-taker: 0.28, intermediate: 0.33, risk-avoider: 0.17. Kruskal-Wallis test: H_2 = 2.35, p = 0.308), as was the number of pellets eaten (risk-taker: 1.80, intermediate: 1.20, risk-avoider: 0.65. Kruskal-Wallis test: H_2 = 3.59, p = 0.166).

3.2.3 Tutoring the non-learners

The number of sensor touches in the groups that failed to learn the task did not increase when a tutor (a fish that had successfully learned to touch the sensor same with the rewarded colour to receive food) was introduced to the group. Thus the median number of touches in the test immediately before introduction of the tutor was 0.00 (mean 0.286) and the median number in the presence of a tutor was 0.00 (mean 0.143) (Wilcoxon matched pairs test: W = 6.0, N = 7, p = 0.181). While the fish that had failed to learn to demand feed were occasionally observed following the tutor, at other points the tutor was observed following them.

3.2.4 Individual differences within groups that learned the task

Figure 4.65 shows the relationship between the mean number of pellets eaten by each individual fish and the mean number of times the same fish touched the sensor, coded

by group, for all groups that learned the task. There is a significant positive relationship (Pearson's correlation = 0.609, $p < 0.001$), suggesting that, overall, the fish that touch the sensor tend to eat most of the resulting pellets. However, the pattern of sensor activation and food intake varied among groups. For example, the fish in group 11 (indicated by a cross in figure 4.65) showed relatively low levels of both sensor triggering and feed intake. In group 8 (indicated by a triangle pointing left in figure 4.65) a single fish (the "trigger" fish) made most of the sensor activations, but ate only a small proportion of the pellets. Finally, group 15 (indicated by the symbol x in figure 4.65) is a perfect example of a group in which one fish that triggers the sensor and ate most of the delivered feed.

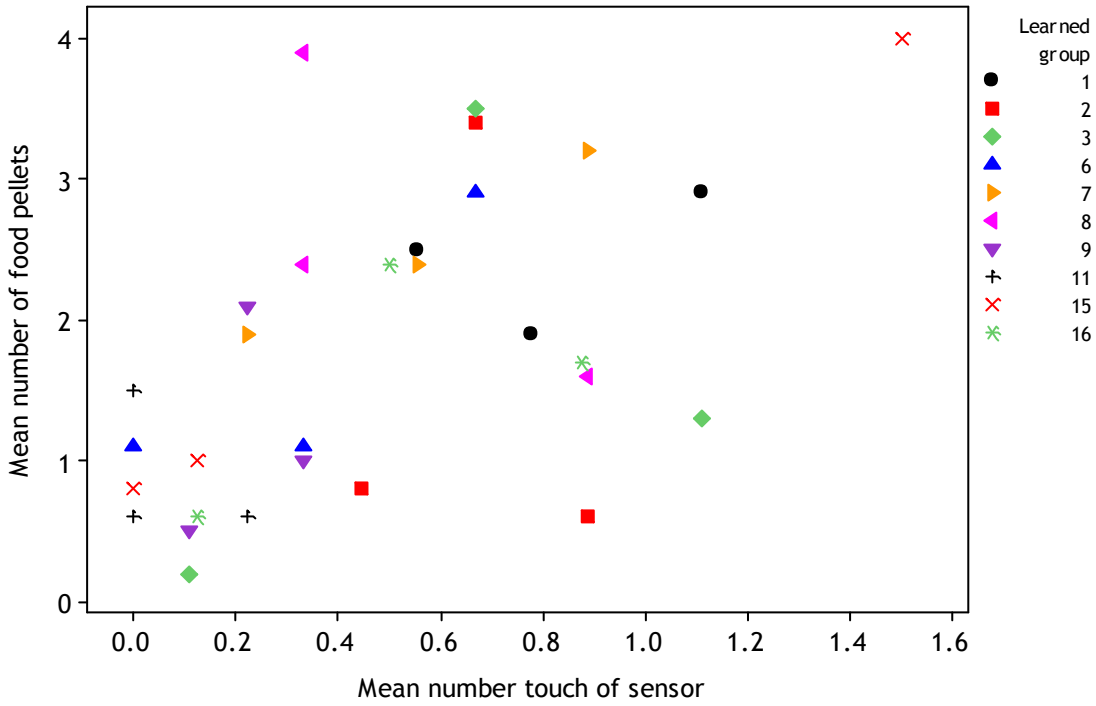


Figure 4.64 The mean number of food pellets eaten in relation to the mean number of times each fish touched the feeding system sensor, coded by group, for all groups that learned the task.

There was no correlation between the morphological variables (length, weight and condition factor) and the mean latency time of trigger fish (weight: R = -0.094, p = 0.824, length: R = -0.131, p = 0.757, condition factor: R = 0.078, p = 0.854).

Table 4.12 Correlations between morphological and behavioural variables

	Weight	Length	CF	Mean pellets	Mean bites	Mean latency
Weight	-	0.835*	0.108	-0.424	0.528	-0.094
Length	0.835*	-	-0.450	-0.338	0.493	-0.131
CF	0.108	-0.450	-	-0.107	0.001	0.078
Mean pellets	-0.424	-0.338	-0.107	-	0.422	0.288
Mean bites	0.528	0.493	0.001	0.422	-	-0.052

Mean latency	-0.094	-0.131	0.078	0.288	-0.052	-

* = p < 0.01

3.3 Three light phase

3.3.1 Incidence of correct choices

Figure 4.66 shows the percentage of times in which the groups that had learned the demand feeding task in the one-trial condition went first to the correct light colour (the one on which they had been trained) over all tests in the 3 light condition, coded for the colour on which they were trained. The percentage of correct choices varied between groups (Kruskal-Wallis test: H_{10} = 24.07, p = 0.007), with colour being a determining factor (red median: 65.4%, blue median: 52.6% and green median: 54.6%; Kruskal-Wallis test: H_2 = 13.99, p = 0.001). Pair-wise comparisons showed significant differences (post-hoc test: p < 0.001), with fish trained with the red light having a higher percentage of correct choices, followed by fish trained with green light and then fish trained with blue light.

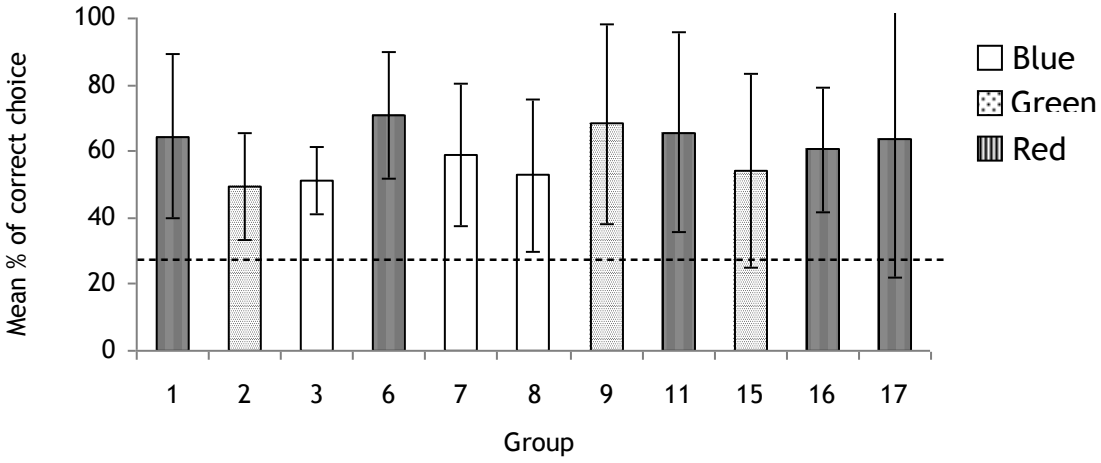
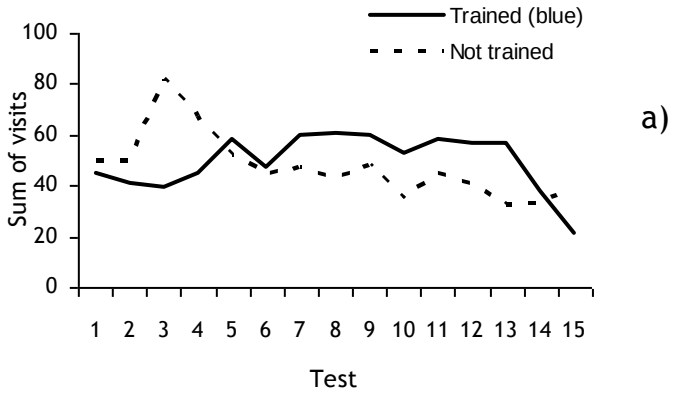


Figure 4.65 Mean (SEM) percentage of correct choices for each group coded by light colour they were trained. Dotted lined = expected % of fish chose colours randomly.

Figure 4.67 shows the sum of visits to each one of the colours used in the test as well as visits to the non-rewarded colours.



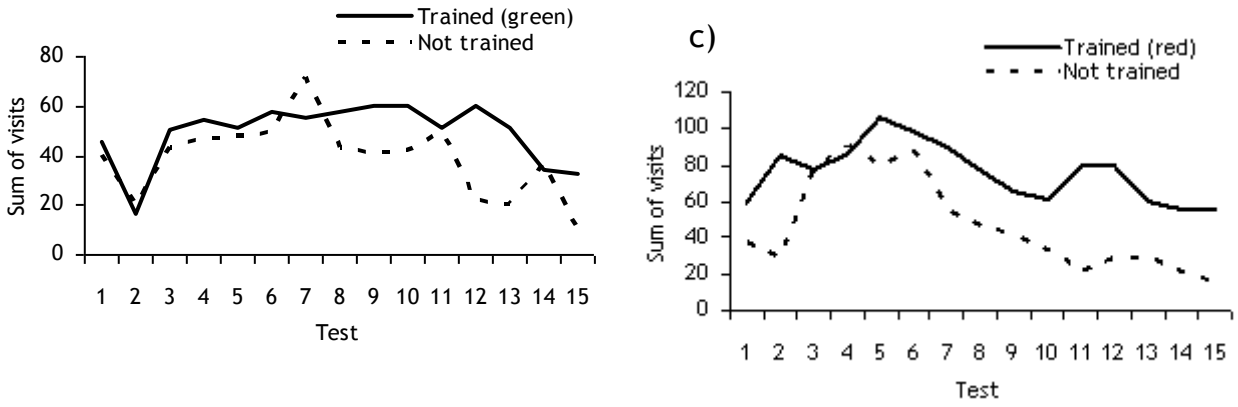


Figure 4.66 Sum of visits to trained and untrained light colours a) blue "rewarded" colour and not trained colours (green+red), b) green "rewarded" colour and not trained colours (blue+red), c) red "rewarded" colour and not trained colours (green+blue).

There was a statistically significant positive relationship between percentage of correct choices and test number (Regression: T = 2.65, p < 0.001). However, this effect was small (R2 = 3.8%), since the percentage of correct choices was quite high in all tests.

The number of touches to the demand feeder sensor was influenced by the colour of the light (Median – red: 60.63, blue: 50, green: 50. GLM: $F_{1,25} = 6.41$, p = 0.012). Pair wise comparisons between the colours showed that the fish trained with the red light made the highest number of touches (p < 0.001).

3.3.2 Effects of risk-taking phenotype

Figure 4.68 shows the median percentage of correct choices for each risk-taking phenotype. The three groups were significantly different (Kruskal-Wallis test: $H_2 = 8.13$, p = 0.017). Post-hoc comparisons results showed that risk-avoiding fish made fewer correct choices that did risk-taking or intermediate fish. Figure 4.69, which shows the pattern of correct responses across trials for the three categories, indicates that this difference is mainly seen in the earlier trials.

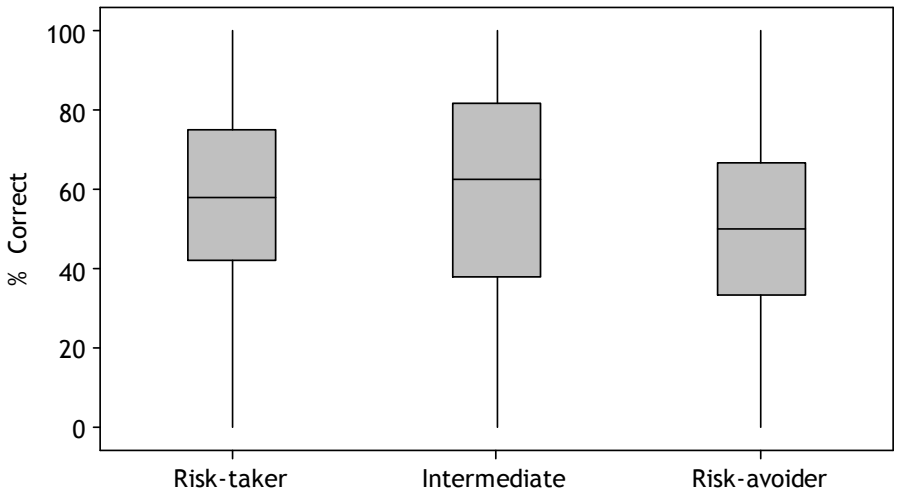


Figure 4.67 Comparison of the percentage of correct of each risk-taking phenotype.

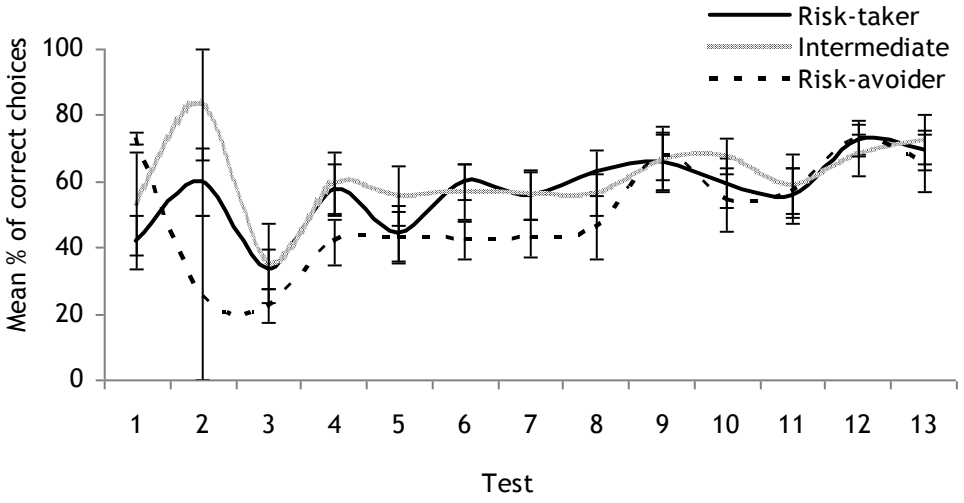


Figure 4.68 Mean (SEM) percentage of correct choices at each test for the 3 risk-taking phenotypes.

3.4 Separating carp in small mixed groups

Overall, the fish made the correct choice, in the sense of moving first towards the light of the colour on which they had been trained in 70.83% of trials. This is significantly different from random (48 out of 78 made the correct choice). The percentage of correct choices in tests 1, 2 and 3 decreases from 70.83%, to 62.96% in the second and 51.85% at the third test. There was no effect of training colour on the proportion of correct choices considering just the first test (red = 87.5%; blue = 50%; green = 75%. Pearson Chi-Square = 2.824, DF = 2, P = 0.244), nor did risk-taking phenotype have an effect (Risk-takers = 29.41%; intermediates = 29.41%; risk-avoiders = 41.18%. Pearson Chi-Square = 0.480, DF = 2, P = 0.787).

4 Discussion

The initial aim of this study was to determine whether common carp can learn to approach and touch a trigger in response to food reward and if so, how quickly they learn. The results show that carp can learn this task, since for some groups at least the time taken to touch the sensor fell significantly between trials. Thus, some groups learned the association between touching sensor and the delivery of food, approaching the sensor on the first test and learning to touch the sensor to receive food within about 3 tests. Other groups did not learn this association, showing very few touches to the sensor and consequently receiving few food pellets. This conforms with the results of numerous studies demonstrating both the ability of fish to learn to use a self-feeding system and difference between species in the rate at which this takes place. For example, juvenile sea bass first touched the trigger 14 days after the start of the experiment (Di-Poi et al. 2008) while goldfish actuate the trigger within a few hours after the installation of the demand feeder (Sanchez-Vazquez et al. 1996) and rainbow trout (Landless 1976) and barfin flounder *Verasper moseri* (Sunuma et al. 2009) took two days.

Several factors may have influenced the learning process in the present study; for example, for logistic reasons the interval between the triggering and delivery of food may have been too long, making it difficult for some groups of fish to relate the delivery of the food to the previous touch of sensor. There was insufficient time to extend the experiment and carrying out more tests, so it is not possible to determine whether, given more training, all individuals would eventually have learned to activate the trigger. A study using sea bass juveniles showed that, while some groups learned to activate the self-feeding system within 10 days of the start of the tests, another group showed a delay in the activation of the system starting only from the 50th day (Millot & Bégout 2009). In the present study, since the priority was to train the fish to approach a light of a given colour to obtain food, shaping (delivery of food when fish approached sensor area) was continued in all trials. As a consequence, the fish soon learned that simply approaching the feeding rather than specifically touching the trigger was sufficient to receive food pellets. However, this was not the reason why some groups did not learn the task, because not only did the fish in these groups fail to touch the sensor, they also failed to approach or to stay in the sensor area. Offering the groups of carp that had failed to learn to touch the trigger to get food the possibility of interacting with a trained tutor did not facilitate learning, since the tutor was just as likely to follow the untrained fish as the converse. Studies in which social learning about profitable feeding locations has been demonstrated tend to stage manage things so that

the fish first watch and are then given the opportunity to choose between alternatives on their own. This raises interesting questions about how important such effects might be in freely interacting fish.

The colour of the light did not influence on the time taken by the fish to learn to touch the sensor (in the 1 light phase) in those groups that learned the task. However, although sample sizes were small, light colour seemed to influence whether or not a given group learned to use the trigger, since most (5 in 6) of the groups trained with red light but only half of the remaining groups learned the task. In the 3 lights phase, the colour of the light was a determining factor, with fish trained with the red light making a higher percentage of correct choices than fish trained on the green and blue light.

Another aim of the present study was to examine individual variability in the pattern of trigger activation and in feed intake and to relate this to other aspects of the fish behaviour. The pattern of trigger activation varied among groups, but in all groups some individuals carried out the majority of activations. Overall, the frequency with which individual fish touched of the sensor predicted food intake, with the fish with more actuations being the ones with higher food intake. This agrees with studies of juvenile sea bass, in which fish could be classified as high triggering fish, responsible for most of the trigger actuations, as opposed to fish that showed little or no triggering fish (Covés et al. 2006, Di-Poi et al. 2008). In these studies, the high triggering fish did not have a higher growth rate or body mass, suggesting that a high rate of triggering does not necessarily result in fish eating more food (Di-Poi et al. 2008). Some studies with salmonids showed that fish with the highest triggering counts had the highest growth rate, indicating that the ability to release food is beneficial for food intake (*Salmo gairdneri*, Abbott & Dill 1989; rainbow trout, Brannas & Alanara 1993). In the present study, response to the sensor was unrelated to size and condition, as found by Millot et al. (2008) in sea bass.

The degree of polarisation of triggering and feed intake varied among groups of carp; in some cases the fish that made most trigger activations ate most of the delivered feed, but in others the high triggering fish ate little food. Differences in triggering behaviour between groups have been shown for the barfin flounder, with one fish doing all the actuations in half of the groups and no individual differences in activation in the other groups (Sunuma et al. 2009).

There were few relationships between risk-taking phenotype assessed by the novel environment and novel object tests and response to the self-seeding system. This may be because in this case the novel environment test failed to produce a clear

classification of carp according to consistent differences in emergence time. This is in contrast to the result found in chapter 3, where the novel environment test enable the classification of the carp in three distinct categories (risk-takers, intermediate and risk-avoiders). The differences from these carp to the one used in chapter 3 were that they were kept in the tanks in smaller groups (6 fish) while in the experiment of chapter 3, they were kept in 2 big groups (27 and 33 fish). Early on in the 3 lights phase, risk-avoiders made fewer correct choices, possibly because they were frightened or distracted by the new arrangement of lights. Ablitt (2009) found that the interactions with a novel object (swimming close to it and touching it) was correlated with trigger activation in cod, with fish that did most of the actuations being the ones that interact most with the object.

A further aim of the present study was to determine whether carp can be trained to visit one of three feeders signalled by different coloured light to get food and if so whether this learned response can be used to separate individual fish in small groups. All the groups that learned to activate the trigger in the 1 light phase went preferentially to their trained colour in the 3 light phase, though all showed some sampling of the potential feeding stations signalled by the other two colours. This preferential visiting was particularly the case for fish trained with the red light. Thus carp can learn to discriminate between one of 3 feeding points at the basis of colour of light. Rainbow trout can learn to discriminate between a trigger that provides food and one that does not, eventually activating the rewarded and unrewarded triggers in the ration of 1:20. The authors of this study speculates that the trout used aquarium characteristic (shape of tank, water inlet pipe and light position) as landmarks to identify the location of the profitable trigger (Adron et al. 1973). This same study also tested different trigger colours (red, green, blue and yellow) and showed that trout can differentiate between pairs of different colours, with no preference for particular colours.

The final test of the study described here showed that the individual fish in groups of 3 could be spatially separated even in a relatively small and visually simple space based on a learned association between the delivery of food and a light of a specific colour. This was most effective for fish trained to a red light and in the first of the three trials, probably due to the lack of a food reward during the trial itself and in spite of “retraining” between the trials. This was somewhat unexpected, since carp are a strongly schooling species and innate attraction to conspecifics might be expected to outweigh a learned attraction to a coloured light. Perhaps the trial tank was small enough for the fish to still assess themselves as being part of a shoal. It is therefore possible to control the movement of common carp using light cues; on the basis of the

present results, using fish trained to a red light, the success rate would be almost 90%. Several other studies have shown that fish can be separated on the basis of a learned response to light. For example, individual Atlantic salmon from groups that had been trained to associate a flashing light with food delivery were selectively drawn away from a group by using a beam of light focussed on their eye (Lines & Frost 1997). Currently any treatment or operation that need to be made with a subset of the fish stock requires captive and physical separating of the whole population. The development of low stress methods for sorting fish would therefore be of great use in aquaculture and fish welfare.

5 Conclusions

In spite of logistic problems with the self-feeding system, the study described in this chapter has shown that common carp held in small groups have the capacity to learn to approach and touch a trigger in order to receive a delivery of food. While the fish in a group activated the trigger to different extents, most got some food. This suggests that, as described in a handful of published studies, self-feeding may be an effective feeding strategy for this species, both in aquaculture and when held for scientific experiments. The fact that not all groups of fish learned this task during the timescale of the study suggests that larger groups and longer training may be needed for this to be fully effective. In carp, neither fish size, body condition nor risk-taking phenotype (assessed by a novel environment and a novel object test) predict the response of individual fish to the self-feeding system. Other factors must underlie the observed variability in performance and warrant further study.

All the groups that learned to use the self-feeding system in the one light situation (but particularly those trained on the red light) transferred this learned preference to the three-light phase, moving preferentially towards the light on which they had been trained. The learned preference was sufficiently strong to effect individual separation in groups composed of 3 fish trained on different lights, in spite of the fact that carp are a strongly schooling species and the learned preferences separated the fish from their companions. This raises the possibility of using a conditioned response to lights of different colours to separate carp into categories without the need for capture and manual sorting. This has implications for the welfare of fish held in captivity, both in the laboratory and in production systems.

CHAPTER 5

Effects of family and rearing condition on behaviour, morphology and brain development in common carp (*Cyprinus carpio*)

This chapter describes a study carried out in Poland in October/November 2008. The opportunity arose from a COST STSM programme to develop behaviour and morphological studies on common carp of 4 known families reared either in tanks or in semi-natural ponds. This material offered the potential for a study of the importance of differences in risk-taking and aggression and the relationship between them as well as the effects of captive rearing on behaviour and morphology.

1 Introduction

1.1 Behavioural syndromes and coping strategies

As described in chapter 1, the term behavioural syndrome is often applied to cases where individual animals vary consistently in how they respond in different contexts, with performance in different contexts being correlated. Many studies of behavioural syndromes have used vertebrates as subjects; for example, bluegill sunfish presented consistent behaviour in different contexts, individuals designated as bold being more active, more willing than those designated as shy to explore novel environment/object, to inspect a potential predator and to spend time in risky areas (Wilson & Godin 2009). However, the behaviour of invertebrates has also been investigated in this context.

Consistent behavioural variability is not always related across contexts. Another example in invertebrates, the dumpling squid (*Euprymna tasmanica*) behaviour was measured in two different contexts, a threat and a feeding test. Across contexts, behaviour was not correlated at any age, while within context individual phenotypes were consistent, both before and after sexual maturity. During sexual maturity, animals designated as shyer were more plastic in feeding tests, while so-called bolder animals were more plastic in the face of threat (Sinn et al. 2008). Rainbow trout were tested in 5 different tests: 1) latency to consume food at the feeding apparatus, 2) latency to cross through a mesh partition to gain access to the feeder, 3) latency to cross through a mesh partition to gain access to the feeder under predation risk by a salmon, 4) latency to cross through a mesh partition to gain access to the feeder under predation risk by a aerial predator and 5) latency to cross a barrier in an artificial stream. The same individuals took or avoided risks (so were classified as “bold” or “timid”) in four different situations related to foraging, but behaved quite differently in a dissimilar context (exploring the artificial stream) (Wilson & Stevens 2005).

For some of the well-studied vertebrate examples of behavioural syndromes, the underlying neuro-endocrine correlates are reasonable well documented and surprisingly conserved. For example, Huntingford et al. (2010) found that carp that took risks when exploring a novel environment show low stress responsiveness, indicated both by lower plasma lactate and glucose levels and also by lower expression of cortisol receptor genes in the brain and head kidney. In addition, it has been shown for some of these same vertebrate systems (e.g. great tits, rainbow trout) that the individual differences in behaviour reflected in particular behavioural syndromes are inherited.

1.2 Risk-taking and body condition

Where a correlation exists between risk-taking and aggression, labelling this a behavioural syndrome in some senses implies that the relationship is biologically significant and requires functional explanation. For example, in the case of the sticklebacks studied by Bell (2005) and Dingemanse et al. (2007), one might argue that in sites with piscivorous fish individuals that are either risk-taking and aggressive or risk-avoiding and non-aggressive do well, whereas those with the opposite combination of traits do poorly. According to a different approach put forward by Stamps (2007), risk-taking and aggression are independent manifestation of a life history decision for fast growth. It is not uncommon to find within the same population individuals that "opt" to grow fast and mature early and others that "opt" to grow more slowly and mature more slowly. A study comparing similarly reared seventh-generation farm Atlantic salmon with wild salmon from the principal founder population of the farm strain showed that Atlantic salmon selected for fast growth show enhanced appetite, mediated in part at least by higher rates of production of growth hormone (Fleming et al. 2002).

Fast growing individuals are expected to show traits that make them more likely to gain food. For example, fast growing Atlantic salmon showed a markedly increased appetite whereas the appetite of slow growing fish decreased (Metcalfe et al. 1986, Metcalfe et al. 1988). Among the behavioural traits that would be effective in individuals that have opted for fast growth are being ready to take risks in a potentially dangerous environment that contains food and competing aggressively when food is limited. On such a scenario, individual differences in aggressiveness and risk-taking are independent adaptive responses to a fast-growth developmental trajectory that involves a growth-mortality trade off. The often-observed correlation between these two aspects of behaviour is thus an incidental bi-product of a developmental switch to faster or slower growth.

If this view of co-varying risk-taking and aggression as a manifestation of a growth-mortality trade-off is correct, then risk-taking, aggressive fish are expected to be the largest of their cohort and risk-avoiding, non-aggressive fish to be among the smallest. In a species in which both activity and boldness are positively related to food intake rates, individuals with consistently high growth rates should display high levels of activity and boldness (Biro & Stamps 2008). Several studies have found that bold, risk-taking individuals do indeed tend to be larger than shy individuals from the same population. In three-spined sticklebacks, fish that resumed foraging rapidly after a

simulated predator attack (bold) had a higher growth rate than shy fish (Ward et al. 2004).

However, other studies have found the opposite, namely that smaller individuals, and in particular those that have the lowest nutrient reserves, take risks; those with good reserves are more cautions, and can afford to be. This is sometimes described as the "asset protection" hypothesis. In 2004, Brown & Braithwaite found that in the poeciliid *Brachyrhaphis episcopi* smaller fish emerged from shelter sooner than larger individuals; this was true only for populations that inhabited upstream sites with low predation pressure. These two frameworks are not mutually exclusive, since even if differences in risk-taking and aggression do reflect a growth-mortality trade-off, in the short term even individuals on a slow growth trajectory will take risks to gain food if they are in very poor condition.

1.3 Genetic effects

1.3.1 On risk-taking

Where such striking differences in behaviour and growth rate are found, it is of interest to determine the extent to which these are inherited, that is, they depend on genetic differences. Behavioural differences between pigs of Large White and Landrace breeds were evaluated using novel environment test, novel object test and tonic immobility test. The results presented significant differences in behaviour between large white and landrace pigs, with more large whites remaining immobile and that did not attempt to turn when held on their back. The immobility test proved better at predicting response in other behavioural tests for large white than for landrace pigs, large white spent less time exploring the pen in the novel object test. Moreover, some significant correlations were found between behaviour in the tonic immobility test and performance: pigs that remained immobile tended to grow more than did pigs that struggled and attempted to turn. The author suggested that breed should be considered when using tonic immobility test, since it is clearly an influential variable (de Sevilla et al. 2009).

As far as evidence for genetic effects and risk-taking in fish is concerned, inter-population experiments with first-generation offspring from wild zebrafish (*Danio rerio*) showed that four populations differ in response to a novel object. This experiment showed that the four populations have genetically based differences that affect their behavioural responses (Wright et al. 2003). Four different clonal lines of rainbow trout derived from four different sites were crossed with common eggs from two outbred

females; the progeny of this cross were tested in three different contexts: use of the water column, startle response and agonistic behaviour within and between clonal lines. Clonal lines differed significantly in behaviour in the three contexts. Lines derived from populations with at least 100 years of captive rearing (Arlee and Hot Creek line) swam at higher, more visible levels in the water column, fed more frequently and displayed shorter startle responses than did clonal lines derived from a more recently domesticated population (Swanson line) and a sea-ranched population (Clearwater River) (Lucas et al. 2004).

A study using common carp showed remarkable differences among one feral and two domesticated strains (D and O strains). Feral carp were more cautious, but quicker to attack prey than were those of the domesticated strains. Also feeding skill of the D strain was higher than the O strain. Moreover, depth selection, prey consumption rate and escape into shelter in response to predatory attack also differed between the two domesticated strains and between domesticated and feral fish. These behavioural differences were considered to be the result of genetic differences between strains, since the fish were reared from eggs under similar environmental conditions (Matsuzaki et al. 2009).

1.3.2 On patterns of growth

Three families of siblings of Donaldson strain juvenile Rainbow trout (BB, FF and BF) were evaluated with experiments on food competition, lure catching, fright recovery and the dominance and aggression experiment. In the food competition test, fish of one family out-competed fish from the two other families showed by the greater weight gain over 2 weeks. More BB fish returned to an open space after fright recovery and they also exhibited the highest frequency of aggressive behaviour (Azuma et al. 2005). Rainbow trout selected for a low or high cortisol response to confinement also presented differences in feeding efficiency (growth per unit feed consumed) with high responsive fish showing more variable size and lower growth rate than low responsive fish (Øverli et al. 2006) but cortisol suppresses appetite.

A relationship between behavioural response to challenge and growth rate, as described above for pigs, is of particular importance for species that are farmed for food. Genetic effects on patterns of growth have been widely studied in farmed fish species due to the increasing importance of quality traits in the aquaculture industry. Significant heritabilities have been reported for body weight and body length in rainbow trout (Gjerde & Schaeffer 1989) and for condition factor but not for weight and length in

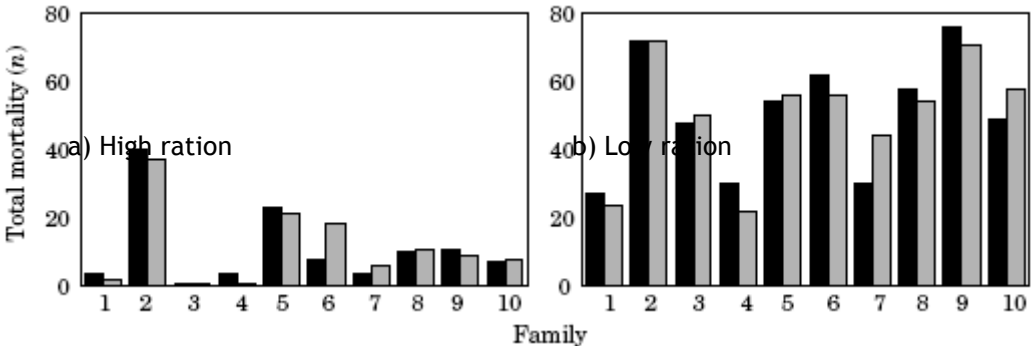
gilthead seabream (*Sparus auratus* L., Navarro et al. 2009). One-year-old wild-caught cod from 70°N were smaller, grew more slowly, weighed less and had a lower condition factor (CF) than southern cod from 60°N. In contrast, both a higher growth potential and an increase in CF were found in northern cod when offspring of northern and southern cod from the same area and of the same age as the wild cod were housed together in a 'common-garden' experiment. The rapid growth in northern cod was achieved by higher success in food competition when given a restricted amount of food (Salvanes et al. 2004).

1.4 Differences in behaviour, growth and mortality between wild and captive animals

In addition to long-term, inherited effects of domestication, as described for carp by Matsuzaki et al. (2009), animals of the same strain reared in captivity often show differences in behavioural and morphological traits when compared to their wild counterparts, since the environment experienced by cultured and wild animals is strikingly different. Differences in behaviour within one generation can be the result of differential experience (Huntingford 2004). As an example of the effects of differential experience in wild and captive reared fish, the presence of predators in the wild stimulates the development of effective anti-predator responses in cichlids Nile tilapia. Lack of this experience makes tank-reared fish less prepared to react when subsequently confronting a predator (Mesquita & Young 2007).

Like behaviour, growth rate can also be affected by origin of the animal. A study using offspring of farmed, wild and hybrid (cross farmed female x wild male) of Atlantic salmon reared under similar farming conditions, found that farmed salmon were over twice the size of wild salmon, whilst hybrids were intermediate and condition factor (K) was considerably higher in farmed compared to wild salmon, with hybrids intermediate values (Glover et al. 2009).

Another process that can generate differences in behaviour between wild and captive-reared fish is differential mortality of individuals that behave in different ways. This can interact with internal differences in complex ways. Brown trout from four different families of wild parentage were reared in four tanks and fed high (100%) and low (25%)



rations. Within each tank, highly significant differences in mortality were observed between families, but this was dependent on feeding treatment (Figure 5.69).

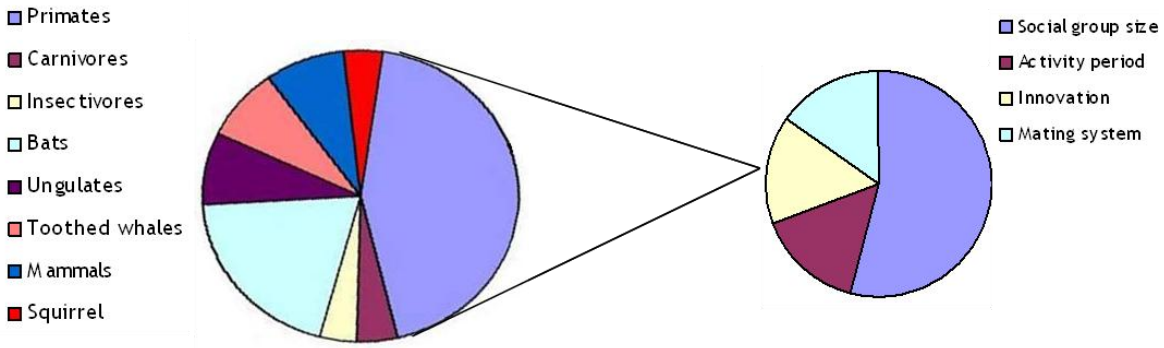
Figure 5.69 Overall family mortality during the 35 day start-feeding observation period for the (a) high [tank 1 - black bars, 2 - grey bars] and (b) low [tank 3 - black bars, 4 - grey bars] feeding regime. (Glover et al. 2004)

The family that experienced the lowest overall mortality in the high feeding treatment showed high mortality rates in the low feeding treatment. This difference in distribution of mortality among families observed between the low and high start-feeding treatments may be indicative of a genotype x environment interaction between feeding level and family survival (Glover et al. 2004).

1.5 Brain structure, behaviour and captive rearing

Attempts to relate differences in brain structure to differences in behaviour have a long (and not always honourable) history (Healy & Rowe 2010). On a broad taxonomic scale, variation in the relative size of the brain or of specific brain areas has been shown to correlate with some form of behavioural complexity (Figure 5.71).

a)



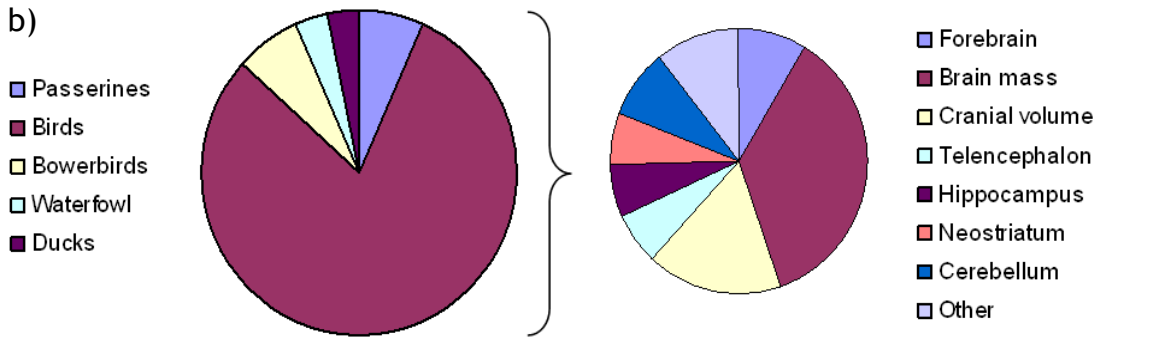


Figure 5.70 Summary of comparative studies published in the last 10 years have looked for correlations between behavioural complexity and measures of brain size in (a) mammals and (b) birds. Figure based on data from Healy & Rowe 2010.

Clear results have been obtained when looking at the effects of domestication (with its known effects on behaviour) on brain size. Such differences can be caused by genetic differences consequent to domestication or brain plasticity driven by the different environments in which wild and domesticated animals develop, or both. Ranched American mink were found to have, on average, smaller brain sizes than wild mink, independent of body size, sex and weight. Several other studies that used different strains of animals have reported reduced brain sizes in captive-bred compared with wild individuals, including Mongolian gerbils *Meriones unguiculatus* forma domestica where they also showed differences in behaviour (Stuermer & Wetzel 2006), turkeys *Meleagris galopavo* (Ebinger & Rohrs 1995) and pigs *Sus scrofa* (Plogmann & Kruska 1990).

Comparison between brain morphology (olfactory bulb, telencephalon, optic tectum and cerebellum) of hatchery and wild reared stocks of rainbow trout (2 hatchery reared strains and 2 geographically distant populations of wild fish) showed that seven out of eight measures have smaller values in hatchery reared fish than in wild fish and most strongly difference was found in the optic tectum and telencephalon. These areas that was selected as areas of the brain that showed the greatest differences were those linked to aggression, feeding behaviour and reproduction, a finding that supported previous work that found that these were the areas in which captive-reared fish are deficient (Marchetti & Nevitt 2003).

Kihslinger et al. (2006) examined brains of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) spawned from wild-caught adults and reared in two different environments: wild and hatchery-reared. They found that olfactory bulb and telencephalon volumes relative to body size were significantly larger in wild fish compared to hatchery-reared fish (figure 5.71). The same was found for guppies, where laboratory-reared fish when compared to wild-caught fish showed a considerable

reduction in both telencephalon and optic tectum (Burns et al. 2009). Juvenile steelhead salmon from the same strain also presented significant variation in brain growth between river and laboratory rearing environments. Fish reared in the river were larger and had larger total brain volumes than laboratory-reared fish (Kihslinger & Nevitt 2006).

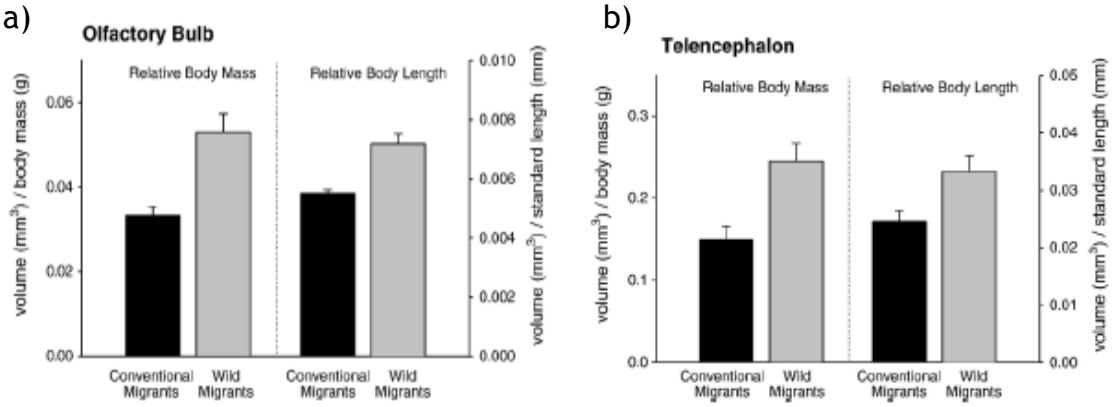


Figure 5.71 Relative volume of (a) the olfactory bulb and (b) the telencephalon shown normalized to body mass and standard length for migrating salmon reared in the wild and in conventional hatchery raceways. Value are plotted as mean (SEM). Black area = hatchery-reared salmon, gray area = wild salmon (Kihslinger & Nevitt 2006).

1.6 Aims of the present study

With this background and given the opportunity through the COST programme to study differentially reared common carp of the same genetic origin at the Institute of Ichtiobiology and Aquaculture in Poland (Gołysz), the aims of the study reported here were to:

- Monitor the response of individual fish both tank-reared and pond-reared carp of the same families to a novel object and to a novel environment both of which has been used successfully to discriminate coping strategy on other vertebrates.
- Compare the behaviour shown in these two tests among families and between rearing conditions.
- Compare plasma levels of glucose, lactate and cortisol in fish in different condition, family and risk-taking phenotype.
- Compare brains of tank and pond reared fish of different families.

Due to a disease outbreak it was not possible to achieve all those aims, specially related to comparison between pond and tank-reared fish and of tracking individual fish in different conditions.

2 Methodology

2.1 Fish provenance and rearing environments

The work described here was carried out in Poland (at the Institute of Ichthyobiology and Aquaculture, Gołysz 49,870300 N, 18,798637 E) during the period 16/10/2008 to 25/11/2008. The carp utilized in the study were progeny of two crosses: male of strain K with a female of strain 3 and, male of strain 3 and a female of strain K. The artificial reproduction was carried out on 15/05/2008 using Ovopel (Unitrade, Hungary) pellets containing GnRHa and dopamine antagonists to induce spawning.

The tank-reared carp were kept in tanks of 120 litres capacity, initially 300 fish per tank, but this changed during experiment accordingly to demand for separate groups. Temperature was 20°C. Oxygen concentrations were not monitored, but never dropped below 70% saturation. Fish were fed to satiation, initially with *Arthemia naupli* and later with AllerAqua classic 00 grade. Later grade 0 and 2mm were used. From 10 weeks old, fish were fed approximately 2.5% body weight/day. Illumination was by indoor lights, but also exposed to natural light conditions (windows of the building).

Pond-reared carp larvae were stocked at a density of 100.000 per hectare in pond of 670m^2, into outdoor ponds (3 ponds per family, 12 ponds in total - Figure 5.73). No supplementary feeding was given; so the fish relied on natural food only. Ambient temperature and light conditions prevailed (18-20°C). No predators were introduced into the pond, but fish were exposed to piscivorous birds.

a)

b)

Figure 5.72 a) Aerial image of the pond where carp were reared; b) photograph of one of the ponds used to rear carp.

On the 02/10/2008 and 03/10/2008 ponds were harvested and 250 fish from each pond were stocked in 120l glass tanks (12 tanks in total, in a recirculation system). Feeding, temperature, light and oxygen conditions were similar to those experienced by tank-reared fish. Prior to stocking in tanks, pond fish were bathed in 1.5% NaCl for 15 minutes and 20 minutes in 0.005% $KMnO_4$ to combat parasites. However, even with this treatment health problems arise and a disease outbreaks occurred in the pond reared fish. This posed a number of problems for the study. In the first place, it reduced the sample sizes available for pond fish. In the second place, many of the planned testing could not be carried out for quarantine reasons, since pond and tank fish could not be held together to avoid propagating diseases. Thirdly, to minimise stress fish were given batch marks only, rather than individual marks, which would have required more dye inoculation.

2.2 Response to a novel environment

All fish were batch-marked before testing using a Panjet marker (Hart & Pitcher 1969) and 2 types of dye: red tattoo pigment and alcian blue. Pond fish were marked with alcian blue and tank fish were marked in red. In both groups the marks were coded by family (4 different positions of mark); for example fish from family 3x3 were marked on the tail. Another feature used to identify the fish were their scale pattern, since this varied by family. Chapter 2 provides more specific information about marking and fish identification.

As in the work described in previous chapters, risk-taking was screened by monitoring emergence from shelter into a well lit, potentially dangerous novel environment with food stimuli. This is a commonly-used assay in the literature on risk-taking in animals,

including fish (Burns 2008). Since carp are a strongly schooling species and stressed by social isolation, the fish were tested in small groups and sequence of emergence used to classify them according to risk-taking phenotype. In carp time to emerge is repeatable and predictive of other aspects of behaviour (ability to compete for spatially-restricted food, which is greater in fast-emerging fish), metabolic physiology (resting metabolic rate is higher in fast-emerging fish) and stress and physiology (cortisol receptor expression is higher in risk-avoiding fish; Huntingford et al. 2010).

In the case of tank fish, groups of 12 tank-reared carp (3 from each family), individually identified by a combination of scale patterns and dye marking were screened for risk-taking, measured by time to emerge from shelter into a potentially dangerous environment. Fish (deprived of food for 12h which is not excessive for fish kept at this temperature) were placed in a darkened shelter with an opening into a well-lit tank (60x40x535 cm) filled with water to 20cm and left to settle for 20 minutes before the test. A few drops of food-flavoured water were tipped in front of the tube that leads outside the bucket to stimulate the fish to come out. For each fish we recorded time to emerge from shelter. This procedure was carried out on 16 groups of 12 fish, giving a total of 192 tank fish. After screening, the fish were put back in their holding tanks prior to being used in the novel object test.

For pond fish, groups of 8 pond carp, 2 from each family, individually identified by a combination of scale pattern and dye marking, were screened for risk-taking as explained above. For each fish we recorded its time to emerge from shelter. This procedure was repeated, giving a total of 63 (not a multiple of 8 due to mortality of fish during the experiment) fish screened for risk-taking. The number of fish used in this screening was smaller than the number of fish used for tank-fish due to a disease that affected pond-fish reducing the number of pond-fish available to testing.

2.3 Response to a novel object

To provide an additional indicator of risk-taking (Frost et al. 2007), the response of individual fish to a novel object was observed in tank fish only. 4 tank fish, one from each family, were placed in an empty aquarium (20x50x33 cm). Then a novel object (a small blue plastic clothes clip) was placed in the corner of the aquarium. The time taken by each fish to approach the object and the order in which fish approached it was recorded. The test was repeated 40 times, using the same fish as those in response to a novel environment. Because the fish were not individually identified (see above), it was

not possible to relate behaviour in the novel object and novel environment tests below the family level.

2.4 Stress physiology

After the behavioural screening was complete, 20 fish from each rearing condition (5 per family) were deeply anaesthetized and killed. They were measured and weighed. Immediately after sacrifice, blood samples were collected and assayed for plasma concentrations of lactate (Lactate Dry-Fast, Sentinel Diagnostics CH SpA, Via Robert Koch, 2-20152 Milano, Italy), glucose (HYDREX colorimetric end point enzymatic assay, ul. Zana 4, 04-313 Warszawa, Poland) and, cortisol (Cortisol determination Novatec kit for enzyme immunoassay).

2.5 Brain morphometrics

The brains were collected by opening the skull and preserved in buffered (0,1N phosphate, pH7) 4% formaldehyde solution. Risk-taking phenotype of sampled fish was unknown; due to constraints on marking we could not recognize fish individually. Within one week, the brains were then transferred to Bouin's for 12hrs, and embedded in paraffin. The sampling protocol was similar to the one used for Kihslinger et al. (2006) when examining Chinook salmon (*Oncorhynchus tshawytscha*) brain. Transverse sections (5 µm) of the forebrain area were mounted (see figure 5.74), and stained with Haematoxylin-eosin coloration. Cross-sectional areas of the forebrain were measured serially in every 8th section (at 40 µm intervals) and photographed using Zeiss AxioVision software. Areas were measured using Scion Image software (figure 5.75).

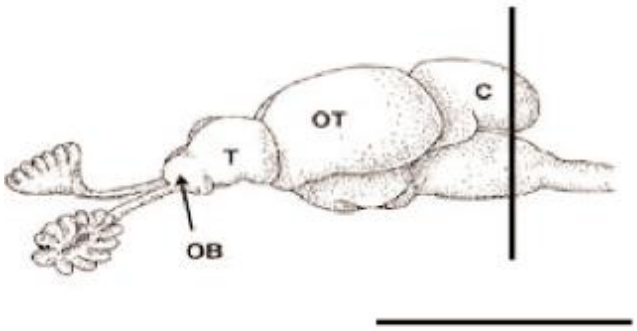


Figure 5.73 Direction of sectioning of the carp brain. OB = olfactory bulb, T = telencephalon, OT = optical tectum and C = cerebellum. Line shows the direction of section. Scale bar 1mm. (Image from Kihslinger & Nevitt 2006)

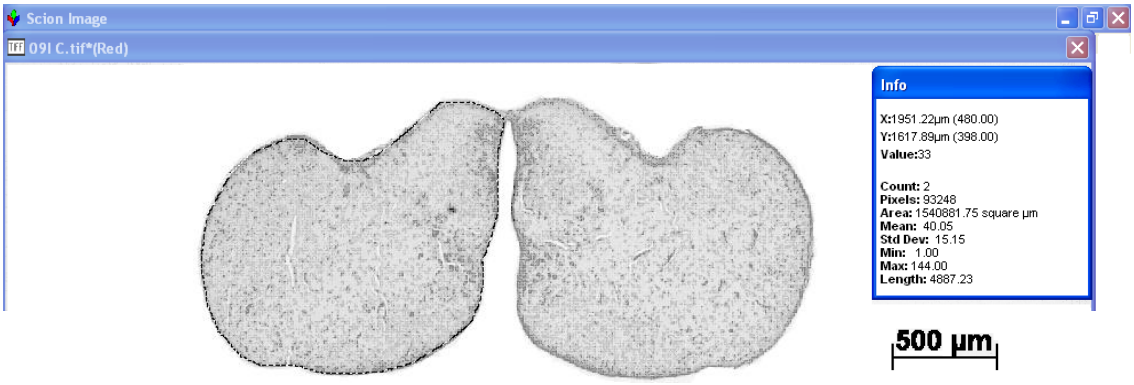


Figure 5.74 Example of image used to measure area of forebrain sections in carp using Scion Image software (area marked by dotted line, left side).

2.6 Statistical analysis

All the data were tested for normality. The different components of this study are sufficiently diverse to require different analysis test were carried out using Minitab series 15, which are therefore described in the relevant section.

3 Results

3.1 Morphological data

Table 5.13 shows the results of two-way ANOVA for length, weight and condition factor by family and rearing condition. Figure 5.76 shows means and standard errors for length, weight and condition factor (CF equation used: CF = Wx100/L3) for the 4 families in the two different rearing environments (pond and tank).

Table 5.13 Results of Two-way ANOVA for length, weight and condition factor by family, rearing condition and the interaction between them.

	Length		Weight		Condition factor	
	F_{DF}	p	F_{DF}	p	F_{DF}	p
Rearing condition	$7.53_{1,32}$	0.010	$0.20_{1,32}$	0.661	$34.27_{1,32}$	0.000
Family	$33.86_{3,32}$	0.000	$26.99_{3,32}$	0.000	$4.67_{3,32}$	0.008
Rearing condition x family	$3.81_{3,32}$	0.019	$3.32_{3,32}$	0.032	$5.06_{3,32}$	0.006

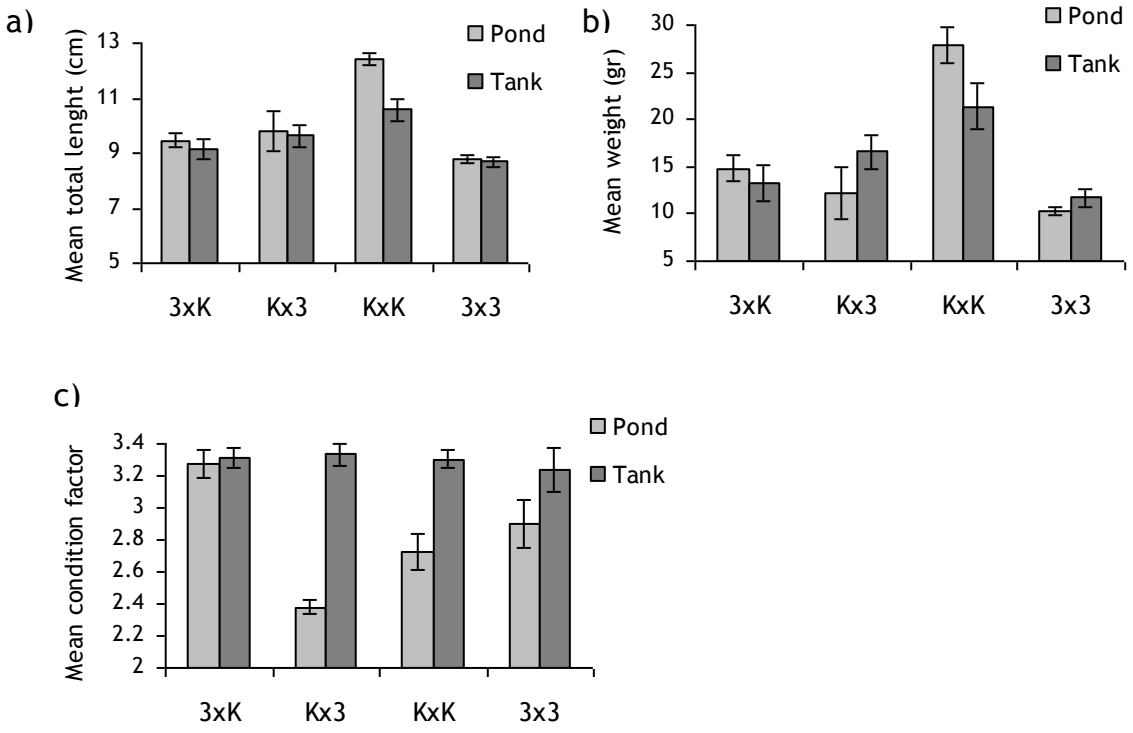


Figure 5.75 Mean (SEM) for each rearing condition (pond-tank) for each family for a) total length, (b) weight and (c) condition factor.

There were significant effects of both rearing condition and family on length, as well as a significant interaction between the two factors. Overall, pond fish were slightly longer than tank fish (Tukey test – T = -2.744, p = 0.01). There was a significant effect of family, but not rearing condition on weight. Overall, fish from family KxK were heavier than those for the other families (Tukey Test – KxK/3xK, T = 6.184 $p < 0.001$; KxK/Kx3, T = 4.849 $p < 0.001$; KxK/3x3, T = -8,00 $p > 0.001$). For condition factor, there were significant effects of rearing condition and family, as well as a significant interaction between these factors. Overall tank fish were in better condition (Tukey test – T = 5.854, $p < 0.001$) and condition was highest in family 3xK compared with family Kx3 (Tukey test – T = -3.471, p = 0.008).

3.2 Behavioural data

Figure 5.77 shows mean emergence times for pond and tank reared fish from each family in the novel environment test. Statistical results indicated a significant effect of rearing (Mann-Whitney test: W = 1281.0, $p < 0.001$), but not of family (Kruskal-Wallis test: $H_3 = 4.70$, p = 0.195). Emergence times were strikingly longer (approximately three times as long) in tank fish than in pond fish.

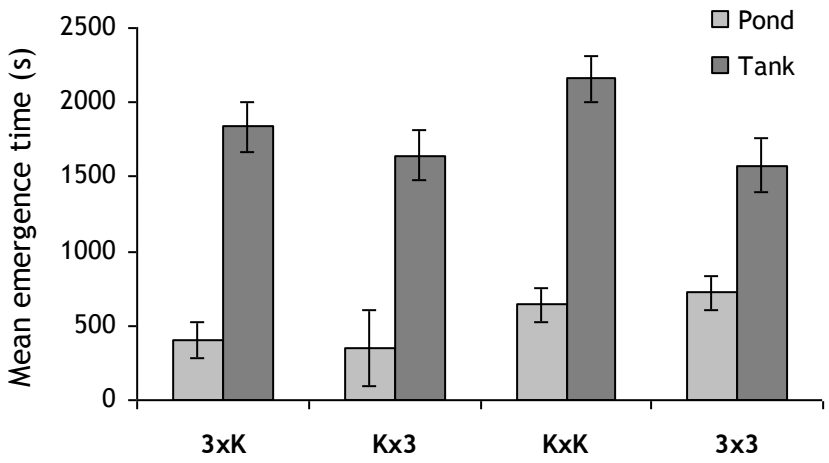


Figure 5.76 Mean (SEM) emergence times (s) for both rearing conditions in each family (3xK, Kx3, KxK and 3x3) in the novel environment test.

Because of the problems with disease on the pond reared fish and consequent quarantine constraints, it was only possible to carry out the novel object test with the tank reared group. Figure 5.76 shows the median time to approach the novel object in each family of the tank reared fish. There was a marginally significant effect of family, with family Kx3 taking the longest time to approach, family 3xK the shortest and KxK and 3x3 being intermediate (Kruskal-Wallis test: $H_3 = 7.50$, $p = 0.058$).

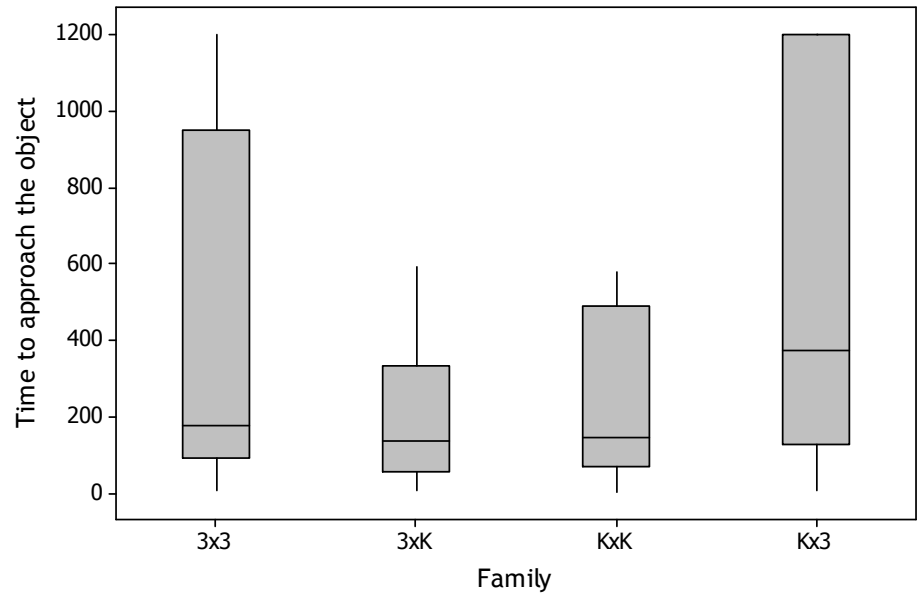


Figure 5.77 Median (IQR range) time to approach the novel object of each tank-reared family (3xK, Kx3, KxK and 3x3).

There was little association at the family level in tank fish between the time taken to enter the novel environment (rank by family = 3x3<Kx3<3xK<KxK) and the time taken to approach the novel object (rank by family = 3xK<KxK<3x3<Kx3). If anything, the

association is negative, with families 3x3 and Kx3 emerging quickly, but taking a relatively long time to inspect the novel object and families 3xK and KxK showing the opposite.

3.3 Physiological data

At the level of individual fish, there was a significant positive correlation between plasma levels of cortisol and lactate (R = 0.60, N = 40, p = 0.000) and a significant negative relationship between plasma glucose and plasma cortisol (R = -0.36, N = 40, p = 0.02). Lactate and glucose levels were uncorrelated (R = - 0.10, N = 40, p = 0.53).

Table 5.14 shows mean (±SEM) plasma concentrations of lactate, glucose, and cortisol for pond and tank-reared fish from each family, together with the results of Two-way ANOVA. Neither rearing condition nor family had an effect on plasma lactate levels, but there were significant rearing condition effects for the other 2 variables. Pond-reared fish showed a strikingly (4 times) higher level of plasma cortisol than tank-reared fish (pond: 120.2±137.2; tank: 27.5±35.0), and there were no family effects. In contrast, plasma glucose levels were higher in tank-reared than in pond-reared carp (pond: 17.73±4.56; tank: 130.32±11.53), but again there were no family effects.

Table 5.14 Mean (SEM) concentrations of lactate, cortisol and glucose for each family in the 2 rearing conditions.

Rearing condition	Family	Cortisol (ng/ml)	Lactate (mg/Dl)	Glucose (mg/Dl)
Pond	KxK	170.20 ± 72.00	351.96 ± 81.25	18.75 ± 4.07
	3x3	91.13 ± 35.52	284.83 ± 17.44	28.24 ± 7.41
	Kx3	242.39 ± 150. 74	416.77 ± 144.64	5.98 ± 5.45
	3xK	58.50 ± 39.16	227.84 ± 28.56	17.96 ± 3.15
Tank	KxK	17.01 ± 7.48	303.11 ± 14.85	98.91 ± 10.57
	3x3	31.67 ± 26.44	259.39 ± 9.11	129.14 ± 25.00
	Kx3	19.40 ± 5.79	263.86 ± 44.54	152.84 ± 12.93
	3xK	41.90 ± 16.59	289.79 ± 16.76	140.38 ± 30.95

Table 5.15 Two-way ANOVA results of cortisol, lactate and glucose by rearing condition, family and any interaction between them.

	Cortisol (ng/ml)		Lactate (mg/Dl)		Glucose (mg/Dl)	
	F_{DF}	p	F_{DF}	p	F_{DF}	p
Rearing condition	$11.88_{1,32}$	0.002	$1.49_{1,32}$	0.230	$90.70_{1,32}$	0.000
Family	$1.05_{3,32}$	0.385	$1.42_{3,32}$	0.255	$0.86_{3,32}$	0.474
Rearing condition x family	$1.84_{3,32}$	0.160	$1.49_{3,32}$	0.235	$1.34_{3.32}$	0.280

Table 5.16, figures 5.78 and 5.79 show the relationship between physiological and morphological variables, at the level of individual fish. In pond-reared fish, condition factor was negatively related to plasma levels of cortisol and lactate, but marginally

positively related to plasma glucose concentrations. In tank-reared fish there was no relation between the physiological and morphological variables.

Table 5.16 Correlation results for plasma concentration of cortisol, lactate, glucose and condition factor (CF) for a) pond reared fish and b) tank reared fish.

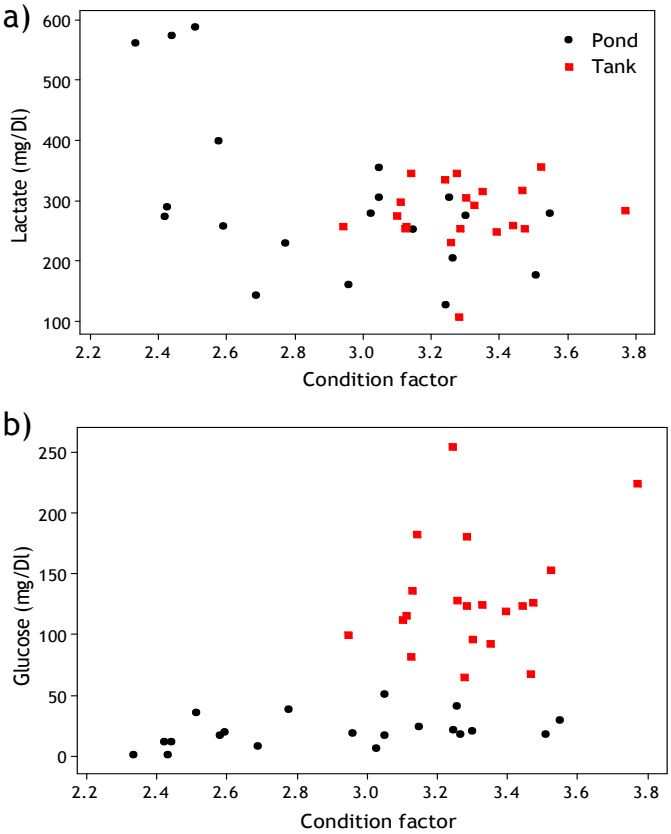
a) Pond

	Cortisol (ng/ml)	Lactate (mg/Dl)	Glucose (mg/Dl)
CF	-0.463, p = 0.040*	-0.552, p = 0.012**	0.389, p = 0.090
Cortisol		0.699, p = 0.001***	0.029, p = 0.905

b) Tank

	Cortisol (ng/ml)	Lactate (mg/Dl)	Glucose (mg/Dl)
CF	-0.304, p = 0.192	0.104, p = 0.661	0.259, p = 0.270
Cortisol		-0.124, p = 0.603	0.018, p = 0.939

Tank fish has consistently low levels of lactate (figure 5.78a), higher levels of glucose (figure 5.78b) and lower cortisol levels (figure 5.78c).



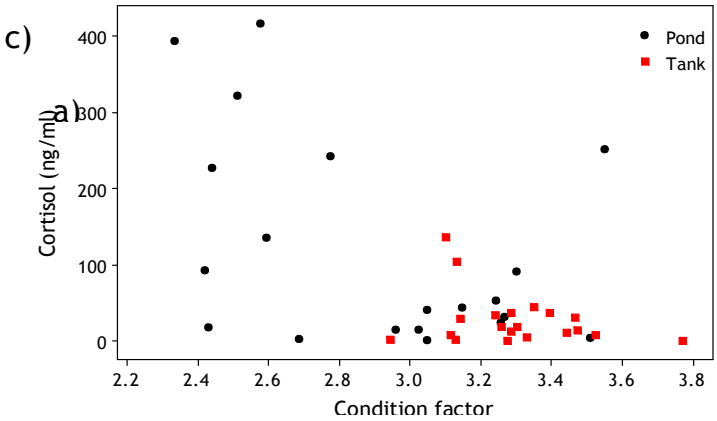


Figure 5.78 Relation between condition factor and physiological variables. a) condition factor x lactate, b) condition factor x glucose and c) condition factor x cortisol. Pond fish are represented by circles and tank fish with squares.

Figure 5.80 shows the relationship between cortisol and the other physiological measures, with rearing condition indicated. Glucose levels were lower in pond fish and cortisol levels were lower in tank fish (figure 5.80a), and there was a positive relationship between cortisol and lactate for the pond fish only (figure 5.80b).

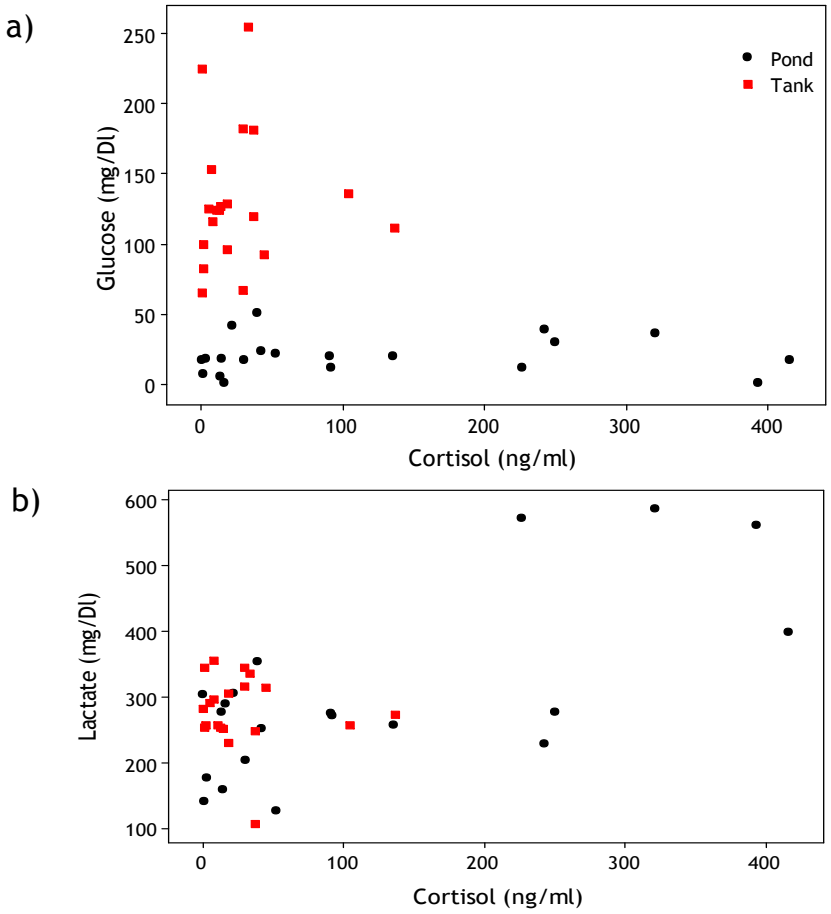


Figure 5.79 Relationship between physiological variables. a) glucose x cortisol and b) lactate x cortisol. Circles = pond fish and squares = tank fish.

3.4 Relative brain size

As expected, brain length was positively related to total length (Regression analysis: $F_{1,38}$ = 22.6, p = 0.000, RS = 39%), so for further comparison, residuals from this relationship were used to give length-corrected brain size. No relation was found between structured forebrain area and total length (Regression analysis: $F_{1,38}$ = 0.88, p = 0.353). Two-way ANOVA shows no significant effect of either family or rearing condition on length-corrected brain size (rearing condition: $F_{1,32}$ = 1.88, p = 0.180; family: $F_{1,32}$ = 0.42, p = 0.738); however there was a marginally significant interaction between rearing condition and family ($F_{1,32}$ = 2.74, p = 0.060).

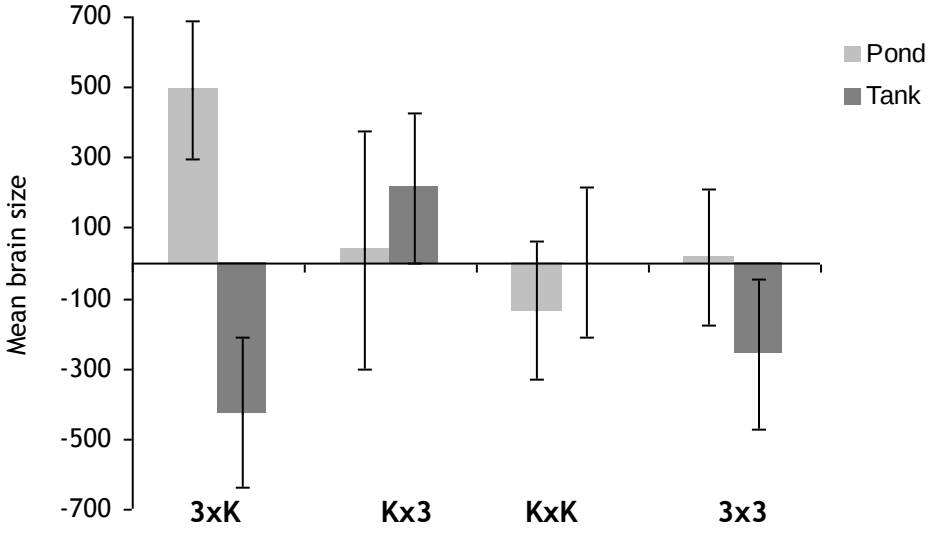


Figure 5.80 Mean (SEM) relative brain size of each family (3xK, Kx3, KxK and 3x3) for each rearing condition (pond and tank).

There was no significant effect of rearing condition on structured forebrain area (figure 5.81. One way ANOVA: $F_{1,32}$ = 2.67, p = 0.112). There was a significant family effect on forebrain area ($F_{3,32}$ = 4.37, p = 0.011), post hoc tests showed differences between families 3xK and Kx3 (Tukey test – T = 3.01, p = 0.025), 3xK and KxK (Tukey test – T = 3.03, p = 0,023).

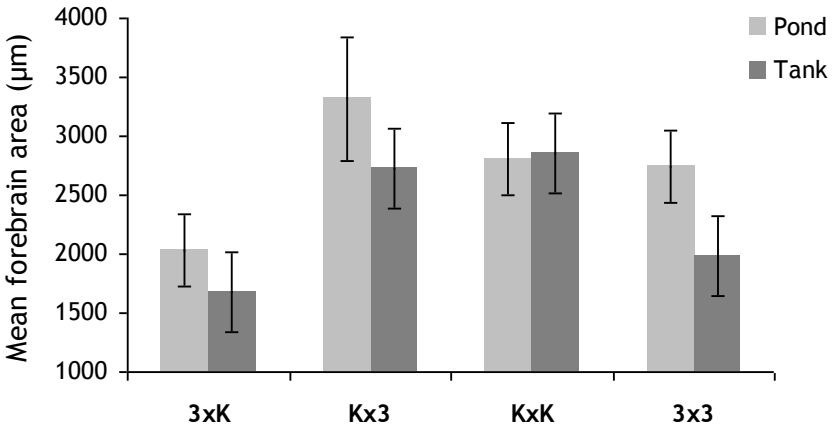


Figure 5.81 Mean (SEM) forebrain area (µm) for each family (3xK, Kx3, KxK and 3x3) on each rearing condition (pond and tank).

Figure 5.82 shows structured forebrain area and length-corrected brain size in pond and tank-reared fish. There was a significant relationship between these variables for tank reared fish (One way ANOVA: $F_{1,18} = 6.35$, $p = 0.021$), but not for pond reared fish ($F_{1,18} = 0.30$, $p = 0.592$). This is due to smaller pond fish having a relatively large forebrain area for their overall brain size.

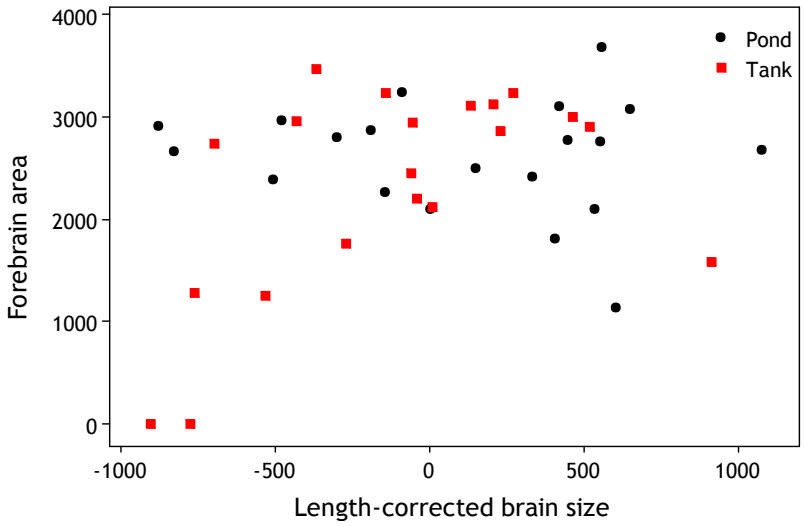


Figure 5.82. The relationship between forebrain area and length-corrected brain size in pond and tank-reared carp.

4 Discussion

The aims of this study were to compare morphology, risk-taking (using two different tests, the novel environment and the novel object tests), stress physiology and brain

size in identified individual common carp from 4 families reared either indoors in standard holding tanks or outside in earthen ponds.

A disease outbreak among the pond-reared fish compromised these aims by reducing the number of pond-reared fish available, by making it impossible to test tank and pond reared fish together as originally planned, for quarantine reasons, and by making it inadvisable to give fish more than batch marks. It was not possible to evaluate individual differences, because it was not possible to distinguish the same individual twice. Dye marks were only made to distinguish between families. The tables included in this section pull together the results by family and rearing condition.

Effects of family and rearing condition on morphmetrics: Briefly, to summarise the findings on each of these points, there was a significant family effect on length, weight and condition factor both for pond and tank-reared fish. Family KxK showed higher length and weight than family 3x3 in both rearing conditions, suggesting that fish with the KxK genotype have a tendency to grow faster. Also the two hybrid families were intermediate in length and weight. A similar result was found by Azuma et al. (2005) for Donaldson rainbow trout, in which fish from three families grew at different rates and hybrids between the fast and slow family showed intermediate growth rates.

Table 5.17 Summary of results of mean (±SEM) length, weight and condition factor for each family in the two rearing conditions.

Rearing condition	Family	Length (SEM)	Weight (SEM)	CF (SEM)
Pond	KxK	12.4±0.2	27.8±1.9	2.72±0.11
	3x3	8.8±0.2	10.3±0.3	2.90±0.15
	Kx3	9.8±0.7	12.2±2.7	2.38±0.04
	3xK	9.5±0.3	14.8±1.3	3.27±0.09
Tank	KxK	10.6±0.4	21.4±2.4	3.30±0.06
	3x3	8.7±0.2	11.7±0.9	3.24±0.14
	Kx3	9.6±0.4	16.6±1.7	3.33±0.07
	3xK	9.1±0.3	13.3±1.9	3.31±0.06

Tank-reared fish were in better condition that pond-reared fish. This could be because they had been reared with abundant food or because they had not experienced a disease outbreak. It is not possible to distinguish between these two possibilities, which are not mutually exclusive. While fish reared in captivity received a supply of nutritious pellets, wild fish and fish reared in outdoor ponds can be exposed to variation in the availability of food, predators and numerous diseases. Farmed fish tend to be in better condition than wild fish for other species, such as Atlantic salmon (Glover et al. 2009) and Masu salmon (Reinhardt et al. 2001).

Effects of family and rearing condition on behavioural traits

Emergence times were highly variably, some fish emerging within 16 seconds and others never emerging from shelter within the 30 minutes of observation. Some of this variability in both the pond and the tank reared fish depended on family. Among tanks, the family that took longest to emerge (KxK) was the one with the lowest levels of cortisol and in the pond fish the fastest family was the one with the highest levels of cortisol (table 5.18). There was a marginally significant effect of family on response to the novel object. Comparing the novel environment and novel objects tests, the ranking of family means were different. Thus tank fish behaved differently in the two tests.

Table 5.18 Summary of mean (±SEM) of time to emerge and cortisol.

Rearing condition	Family	Mean time to emerge (SEM)	Cortisol (SEM)
Pond	KxK	642.5 ± 116.5	170.2 ± 72.0
	3x3	719.3 ± 111.2	91.1 ± 35.5
	Kx3	348.7 ± 252.3	242.4 ± 150.7
	3xK	404.3 ± 119.7	58.5 ± 39.2
Tank	KxK	2157.8 ± 150.3	17.0 ± 7.5
	3x3	1576.5 ± 178.6	31.6 ± 26.4
	Kx3	1642.8 ± 168.5	19.4 ± 5.8
	3xK	1839.3 ± 168.7	41.9 ± 16.6

In the novel environment test, mean emergence time of the tank fish at the family level was related to morphological status, with heavier and longer families taking longer to emerge than lightest and smaller families. This result agrees with the “asset protection hypothesis”. According to this view, smaller individuals and in particular those that have the lowest nutrient reserves, tend to take risks and those with good reserves are more cautions, and can afford to be (Brown & Braithwaite 2004). For example, tank fish from family KxK were the slowest to emerge from shelter in the test and had higher weight and length than those of other families. The same was not true for pond-reared fish, in which family 3x3 being the slowest family to emerge and had lower weight and length than other families.

Table 5.19 Summary of ranks for emergence time in the novel environment test (1=faster, 4=slower), length, weight and rank (1= highest and 4=lowest) for all families in the two rearing conditions.

Rearing condition	Family	Emergence rank	Length rank	Weight rank	CF rank
Pond	KxK	3	1	1	3
	3x3	4	4	4	2
	Kx3	1	2	3	4
	3xK	2	3	2	1
Tank	KxK	4	1	1	2
	3x3	1	4	4	4
	Kx3	2	2	2	1
	3xK	3	3	3	2

Within populations, a growth-mortality approach predicts that consistent individual differences in growth rates will be accompanied by consistent individual differences in behavioural traits that contribute to growth-mortality tradeoffs (Stamps 2007). Correlation between boldness and body mass was shown by Brown et al. (2007) in poeciliid fish. The analysis in this chapter revealed a clear relationship between the boldness scores (time to emerge from shelter) and body mass, small fish tend to emerge from shelter sooner than large fish and they also show greater tendency to approach a novel object.

Correlations between risk-taking phenotype and body condition may depend upon the potential effects of those behaviour patterns on growth and mortality. Assuming that "bold" individuals would be more likely than "shy" individuals to forage under predator risk, inspect potential predators, and explore novel environments; it is important to note that these behaviours have different effects on growth and mortality rates. Foraging under predation risk provides resources, but increases the risk of mortality. On the other hand, exploratory behaviour may look like a waste of time that should be dedicated to growth-related activities, but it provides information that may increase growth and survival in the future (Stamps 2007).

Emergence time was significantly different in the pond reared and tank reared fish, with tank fish being much slower to emerge than pond fish. Although the two groups of fish had to be tested separately, for quarantine reasons, the tests were carried out at the same time and in the same way, so this probably represents a real difference between these groups of fish. There are various possible explanations for this difference and we are not in a position to distinguish between them:

1. It could be the results of differential mortality by behavioural phenotype in the ponds either during rearing or during the disease incident. Previously, in the same institute timid fish were found to be more susceptible to disease, than bold carp (Pilarczyk, personal communication). In Atlantic salmon, families that were characterised by high stress-responsiveness and high levels of activity when responding to acute stress showed increased susceptibility to infectious pancreas necrosis virus, but not to furunculosis (Kittilsen et al. 2009).

2. Some aspect of the pond environment may have modified the behaviour of the carp reared in this condition, making them less stressed by novelty. Results of a study with twelve populations of three-spined sticklebacks showed that fish predator-sympatric have a different behaviour from predator-naïve fish in novel environment and novel

object tests as well as in aggressiveness evaluation (Dingemanse et al. 2007). Matsuzaki et al. (2009) showed that feral carp (laboratory reared so there was a common garden experience) where more cautious of predator attacks and had a longer flight duration and a higher probability of escaping into the shelter than did domesticated strains. Pond carp emerged faster than tank carp, although they were exposed to a variable environment and occasional predation.

3. The differences in emergence time could have been the result of differences in body condition, resulting from the food regime in the ponds, the recent disease outbreak in the pond fish, or both. This seems to be the most likely explanation, although 1 and 2 could both also apply. If there is a growth-survival trade off, as suggested by (Stamps 2007), larger animals are likely to be those that consistently take risks, including while foraging in a potentially dangerous environment. Three-spined sticklebacks classified as bold measured by time to resume feeding following a simulated predator attack, position adopted in the shoal and shoaling tendency showed consistent behaviour in two different contexts (risk-taking and competitive ability) and moreover bold individuals had higher growth rates than shy individuals (Ward et al. 2004). The opposite is predicted if fish that have experienced poor feeding (and so are small and have few nutrient reserves) take more risks because they are more highly motivated to feed, as in the poeciliid *Brachyraphis episcopi* from upstream population (Brown & Braithwaite 2004).

Some studies show different behavioural responses between wild and captive reared animals. Johnsson et al. (2001) demonstrated that cultured Atlantic salmon have a reduced behavioural response toward predators than their wild counterparts. A similar study using a different behavioural test to evaluate risk-taking showed that rearing environment had a strong effect on salmon competitive ability (Metcalfe et al. 2003).

The novel object test presented a significant family effect, with family Kx3 taking longer to approach the novel object, family 3xK faster and KxK and 3x3 being intermediate. In rainbow trout, different families showed different response to fright recovery which consisted of time to return to open space or stay hiding after being chased by hand, more BB family returned to open space after being chased (Azuma et al. 2005). Zebrafish from four strains with a different history of domestication showed inherited differences in boldness measure by the total time spent close to the novel object inserted in the tank (Wright et al. 2003).

A recent study with common carp showed significant different and consistent individual differences in behaviour when exploring an unfamiliar environment and when competing

for a position in a feeding site. In addition, there was a significant correlation between individual performances in these two contexts with individuals that explore more quickly being more likely to gain access to the feeding place (Huntingford et al. 2010).

A number of studies in a variety of animals groups have shown behavioural syndromes with consistent individual differences being reflected in different contexts. For example, Ward et al. (2004) found that three-spined sticklebacks behaved consistently in three different contexts, fish which rapidly resumed feeding following a simulated predator attack also showed reduced shoaling tendency and a willingness to occupy front positions in a shoal. The same consistency was also encountered in bluegill sunfish with bolder individuals being more active, more willing to explore novel objects and environments and more disposed to inspect a potential predator and spend time in risky areas than shy individuals (Wilson & Godin 2009).

However, other studies have shown the opposite, namely that individual differences are context-specific. For example, rainbow trout behaved similarly when the context did not vary (foraging context) but it changed the behaviour when the context changed to exploration of a swim flume (Wilson & Stevens 2005). Also in a study with three-spined stickleback (Coleman & Wilson 1998) fish that was bold to approach a metrestick did not show a bold behaviour to approach a novel food source. More research is necessary to understand the circumstances in which novel environment does or does not affect behavioural syndromes.

Effects of family and rearing condition on physiology.

Plasma cortisol levels were strongly influenced by rearing conditions, pond-reared fish having 5 times higher levels than tank reared fish. It has to be considered that pond-reared fish had been brought into the laboratory to settle in tanks similar to those used for the tank-reared fish. The pond fish were harvested 14 days prior to testing. They therefore had a stressful experience (harvest and transfer) and had then been exposed to unfamiliar conditions previously. In tank-reared fish, there was no correlation between levels of cortisol and lactate, mainly because cortisol levels were all low; however, in pond reared fish, cortisol and lactate were positively related. Thus the high plasma cortisol levels in these fish and the associated high lactate levels, can readily be seen as the effect of recent stressful experiences.

Table 5.20 Summary of physiological results. Mean (±SEM) of plasma lactate, glucose and cortisol by rearing condition and family.

Rearing condition	Family	Lactate (SEM)	Glucose (SEM)	Cortisol (SEM)

Pond	KxK	351.9±81.2	18.7±4.1	170.2±72.0
	3x3	284.8±17.4	28.2±7.4	91.1±35.5
	Kx3	416.8±144.6	5.9±5.4	242.4±150.7
	3xK	227.8±28.6	17.9±3.1	58.5±39.2
Tank	KxK	303.1±14.8	98.9±10.6	17.0±7.5
	3x3	259.4±9.1	129.1±25.0	31.6±26.4
	Kx3	263.8±44.5	152.8±12.9	19.4±5.8
	3xK	289.8±16.8	140.4±30.9	41.9±16.6

The data presented here highlight a problem in using glucose as index of acute stress, as is commonly done (Huntingford et al. 2010, Tanck et al. 2001), because mobilisation of glycogen is an early component of the physiological stress response. This arises because of the additional relationship between plasma glucose and nutritional factors. In this study, plasma glucose levels were much higher in tank-reared than in pond-reared carp, which also had a higher condition factor, although the statistical results showed a marginally significant relationship between plasma glucose levels and condition factor.

Essentially, this looks like a negative relationship between levels of lactate and cortisol and pond fish condition factor, but uniformly low cortisol and lactate and high condition factor in tank fish. When comparing physiological and morphological data of pond-reared carp, condition factor was negatively related to plasma levels of cortisol and lactate, and cortisol had a positive relation with lactate.

Effects of family and rearing condition on gross brain morphology

Neither family nor rearing condition had any clear effect on length-corrected brain size. Estimate forebrain area was higher in pond reared fish in 3 out of the 4 families and, while estimated forebrain area was lower in fish with small overall brain size in pond reared fish, the area of this part of the brain tended to be large in tank reared fish regardless of overall brain size. Some studies affirm that rearing conditions impacts brain development and growth. Differences can be seen in cerebellar growth (Kihslinger & Nevitt 2006), telencephalon and olfactory bulb (Kihslinger et al. 2006), telencephalon and optic tectum (Burns et al. 2009). We could not observe these differences maybe because in our case these are not so extreme, or that such effects do not act in carp (although this seems unlikely), or that somehow the mass mortality have obscured the differences.

4.1 Conclusions

There were significant rearing conditions and genetic effects on the variables evaluated:

- Family effects on morphology with KxK family having a tendency to grow faster and hybrid families showing intermediate values;
- Tank-reared carp have a higher condition factor than pond-reared carp;
- Pond fish emerged faster from the novel environment test;
- The emergence time was related to weight and length in the tank fish with smallest and lightest fish emerging faster;
- There was no relationship between behaviour in the novel environment and the novel object test so the behaviour of the fish was context-specific;
- Pond fish have higher levels of cortisol than tank fish and their cortisol levels were positively related to lactate levels;
- Condition factor was related to the physiological variables. For tank fish, higher glucose levels were associated with higher condition factor. For pond fish, higher cortisol and lactate levels were related to lower condition factor.

CHAPTER 6

Collaborative projects

1 Overall introduction

During the present study, and in some related studies on the same or similar species, a number of data sets became available that allowed risk-taking to be related to aspects of body status. It seemed worthwhile to carry out a meta-analysis of these data sets to see if relationships were consistent and if not to seek possible explanations. The first section of this chapter relates this meta-analysis.

The other studies in this chapter include 2 collaborative projects to which I contributed behavioural expertise; I will be joint author on the two resulting publications, which are in preparation. The first of these two studies examined the implications of risk-taking phenotype for performance in a social context, by looking at social interactions and growth in relation to risk-taking phenotype in goldfish. This was carried out in collaboration within Priyadarshini Tamilselvan, MRes student at University of Glasgow. My role in the project was to train and supervise the MRes student and to discuss experimental design, data collection and interpretation of the results. The second study explored some hidden costs of an aggressive, proactive life style by examining respiratory function in relation to coping strategy in common carp. This was carried out jointly with Hussein Jenjan, Ph.D student at University of Glasgow. My role in this project was to carry out the behavioural screening for the fish used (which were the same as those in learning chapter) and to discuss data collection strategies, data analysis and data interpretation. These two studies are described in turn in this chapter.

2 Meta-analysis of morphological correlates of risk-taking

2.1 Introduction

The literature on body size and condition in relation to risk-taking phenotype is inconsistent, even within fish and the same species of fish. For example, three-spined sticklebacks classified as bold had higher growth rates than shy individuals (Ward et al. 2004). Brown & Braithwaite (2004) found that the relation between body size and time to emerge from a shelter was positive, with larger fish taking longer to emerge. However this relation differed between populations in the poeciliid *Brachyraphis episcopi*, being positive only in upstream population.

This suggests that some factor(s) that have not been taken into account are varying between studies. In the study of Brown & Braithwaite (2004), the results are explained by a metabolic hypothesis whereby juvenile fish in the upstream population were compelled to emerge earlier in order to resume feeding. The relationship did not occur in the other populations because fish from the site were more exposed to predation so all were more cautious and emerged later.

The general literature on coping strategies includes discussion of differences in energy metabolism, with proactive animals often adopting an energetically expensive strategy and reactive animals being energetically conservative (Korte et al., 2005). Stamps (2007) and Biro & Stamps (2008) argue that consistent individual differences in boldness and aggression as well as correlations between these traits may arise through a growth–mortality trade-off. According to this view, fast-growing individuals show both physiological and behavioural adaptations for efficient growth, including high metabolic rate and, in terms of behaviour, a tendency to take risks; those adopting a slow-growing trajectory will show the opposite traits. According to one model (the performance model), a positive relationship is predicted between resting metabolic rate and activity or aggressiveness, an active life style requiring well-developed machinery for acquiring and processing food, which will have higher than average maintenance costs (Daan et al., 1990). There have been no direct comparisons of metabolic rate in risk-taking and risk avoiding fishes, although differences in metabolic rate have been suggested as the reason for the observed association between risk taking and body size described in poeciliids (Brown & Braithwaite, 2004; Brown et al., 2007). Overall, however, there is relatively little information for fishes about the relationship between metabolic rate and risk taking or about physiological correlates of individual variability in risk taking.

2.2 Methodology

2.2.1 A. Common carp (pilot data)

6 mirror carp were screened for risk-taking phenotype using a novel environment test as described above and tentatively separated in risk-taker, intermediate and risk-avoider according to emergence rank (see Chapter 2, section 2.3).

2.2.2 B. Common carp (fish screened for the learning study).

62 mirror carp were screened for risk-taking phenotype using a novel environment test as described above and separated in risk-taker, intermediate and risk-avoider according to mean emergence time (see Chapter 3, section 3).

2.2.3 C. Common carp (fish screened for the demand feeding study)

54 mirror carp were screened for risk-taking phenotype using novel environment test as described above and tentatively separated in risk-taker, intermediate and risk-avoider according to mean emergence time (see Chapter 4, section 3).

2.2.4 D. Common carp (screened for Huntingford et al. 2010)

One-year old common carp (artificially reproduced crossbreeds of known production lines at the Polish Academy of Sciences' Institute of Ichthyobiology and Aquaculture, Zaborze, Poland) were held for one week in large groups at 20ºC. They were then deprived of food for at least 12 h and 10 randomly-selected fish were tipped gently into a small covered settling area at one end of a well lit tank (1.5 m x 1 m x 1 m), allowed to settle for 5 min and a door opened allowing access to the main compartment, into which food extract had been gently tipped. After the first 3 carp had emerged from the settling area or after a period of 10 minutes if fewer than three fish had emerged, the exit door was then closed and the fish that had emerged gently removed. These fish were classified as risk-takers. The door was then reopened a second recording period started, during which a further four fish were allowed to emerge and the door was closed again. These fish were classified as of intermediate risk-taking phenotype. The three remaining fish were classified as risk-avoiders. If fewer than four intermediate fish emerged during 15 min, all the remaining fish were classified as risk-avoiders. After screening, the intermediate fish were discarded and risk-taking and risk-avoiding fish were housed in separate 250 L holding tanks in a closed circulatory system and maintained at a temperature of 20 ± 0.5ºC prior to screening for resting metabolic rate.

2.2.5 E. Koi carp (screened by Huntingford et al., for a study of cortisol responsiveness)

As part of a study of stress responsiveness in risk-taking and risk-avoiding fish by Huntingford and colleagues (DD Delta Lts, Debrecen, Hungary), 69 Koi carp held in large groups at 18^{o}C were identified using natural pigmentation patterns. Groups of 8-9 fish were deprived of food for at least 12 hs and then placed in a small sheltered compartment in one corner of a well-lit 1m^2 tank and allowed to settle for 15 minutes. A moveable door was then raised and the sequence in which the fish emerged was recorded. Each group was tested 3 times and fish with mean emergence time in third highest and the third lowest of the distribution classified as risk-avoiders and risk-takers respectively.

2.2.6 F. Goldfish (screened for study of social interactions)

35 goldfish were screened for risk-taking by F. Mesquita and P. Tamilselvan as part of a study of performance in fish with different risk-taking phenotypes when held in small groups. Risk-taking phenotype was identified using a novel environment test as described above. Fish were separated in risk-takers, intermediate fish and risk-avoiders according to their mean emergence time (see Chapter 6, section 3).

2.3 Results

2.3.1 A. Common carp (pilot data)

A Kruskal-Wallis test showed that length was not significantly related to risk-taking phenotype (H_2 = 2.00, p = 0.368). In terms of means alone, figure 6.83 shows that risk-avoiders and intermediate fish were smaller than risk-takers. Lack of any significant effect may be due to very small sample size (N = 6) and the tentative nature of the allocation to risk-taking strategy.

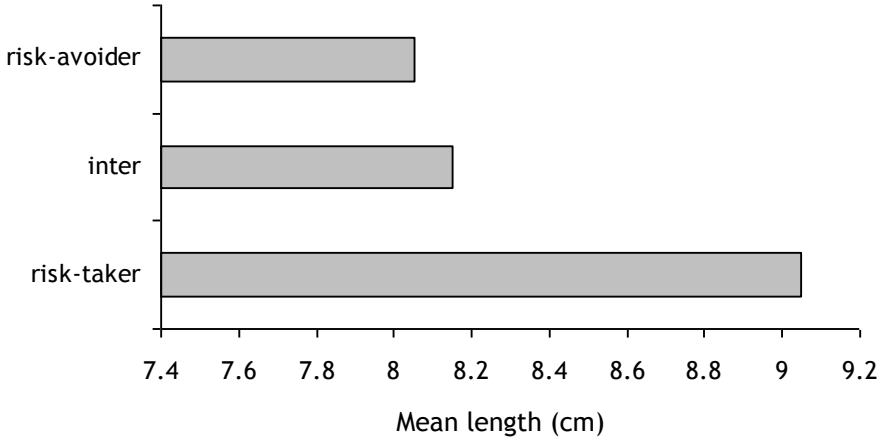


Figure 6.83 Mean length of fish classified using median emergence time based on 13 novel environment tests. SEM omitted due to small sample size.

2.3.2 B. Common carp (fish screened for the learning study)

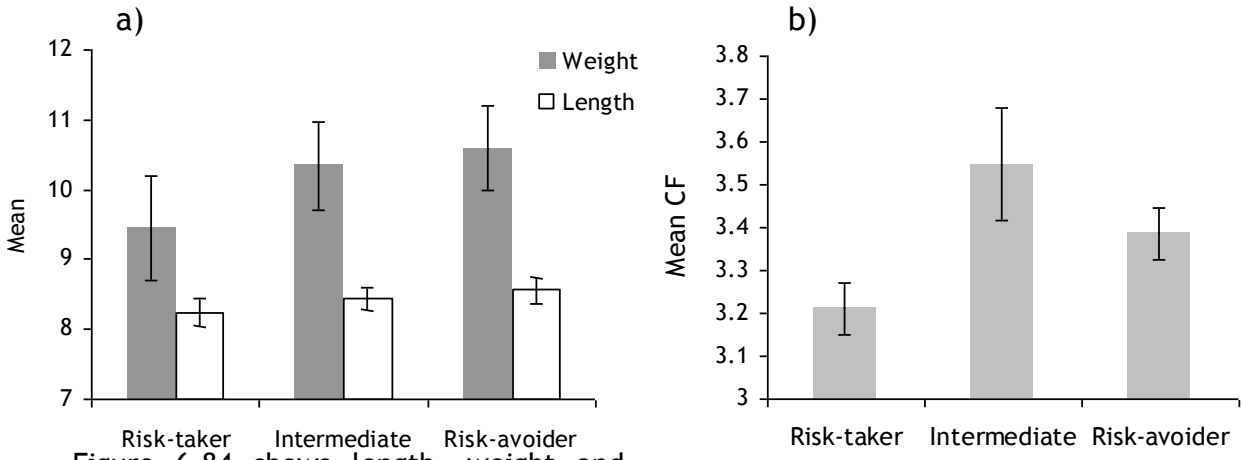


Figure 6.84 shows length, weight and condition factor in the three risk-taking phenotypes. Statistical results showed that there was no significant effect of risk-taking phenotype between on these variables (weight - $F_{2,56}$ = 0.80, p = 0.455; length - $F_{2,56}$ = 0.72, p = 0.493), although for condition factor the effect was marginally significant (One-way ANOVA: $F_{2,56}$ = 3.13, p = 0.051). Even though the statistics showed no significant relationship between length, weight and risk-taking phenotype, there was a sequential order in figure 6.84a, with intermediate fish having higher condition factor than risk-takers (Tukey test – T = 2.47, p = 0.0431).

Figure 6.84 Mean (SEM) of a) length (cm) and weight (g) by risk-taking phenotype, b) condition factor of carp by risk-taking phenotype.

2.3.3 C. Common carp (fish screened for demand feeding study)

Results for the demand feeding study did not show any significant relationship between tentatively assigned risk-taking phenotype and the morphological variables weight (One-way ANOVA: $F_{2,51} = 0.06$ p = 0.942), length (One-way ANOVA: $F_{2,51} = 0.18$, p = 0.835) and condition factor (One-way ANOVA: $F_{2,51} = 0.05$, p = 0.949). Looking at means alone, risk-takers were somewhat smaller and lighter although there was a lot of variation around the mean.

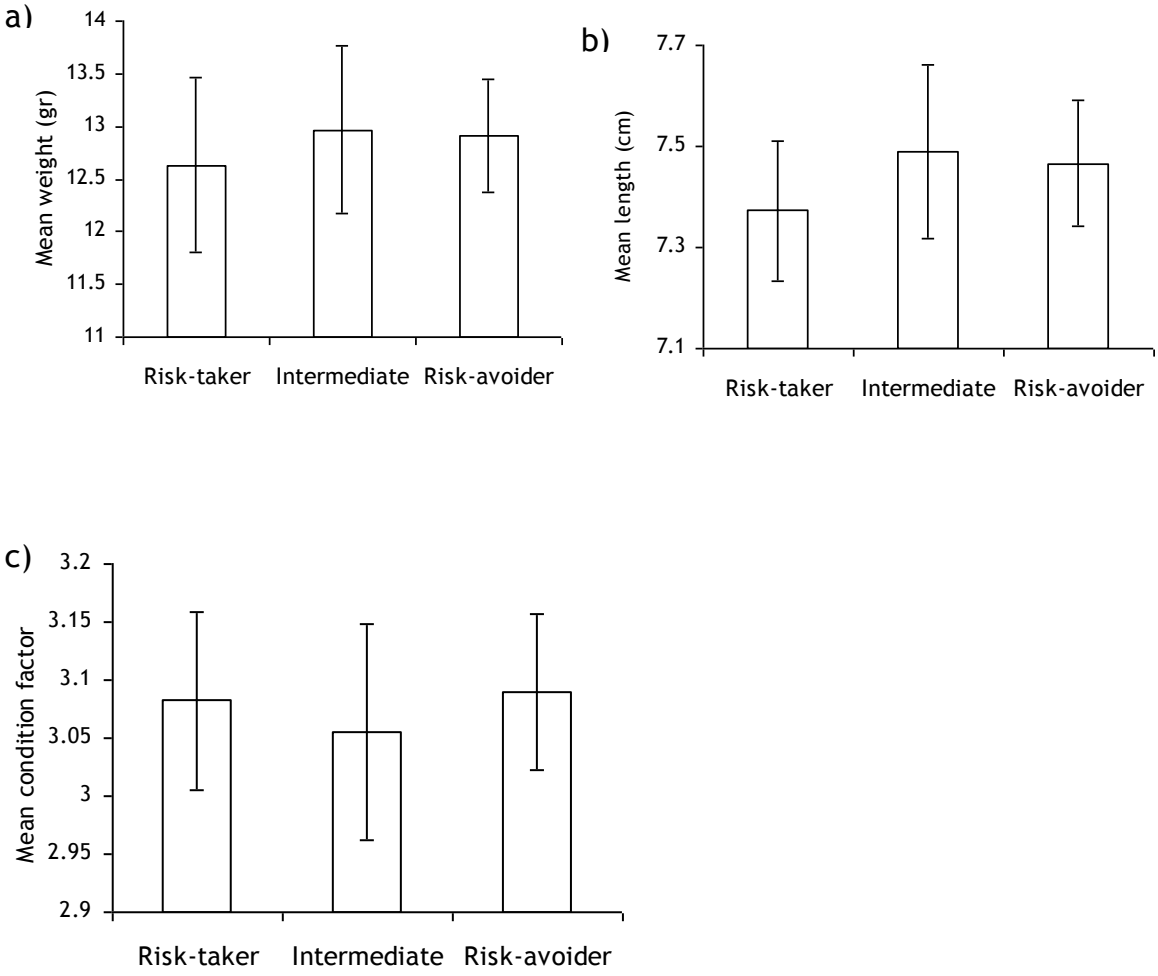


Figure 6.85 Mean (SEM) of a) weight and length, b) condition factor in carp assigned to risk-taker, risk-avoider and intermediate.

2.3.4 D. Common carp (screened for Huntingford et al. 2010)

These data showed significant differences when relating risk-taking phenotype to weight and condition factor (figure 6.86). Risk-takers were longer than risk-avoiders (One-way

ANOVA: $F_{1,358}$ = 30.36, p < 0.001), the same is observed for weight in figure 6.86b, although this was not a significant statistical result ($F_{1,358}$ = 0.21, p = 0.646). Figure 6.86c shows a difference in condition factor between risk-taker and risk-avoiding carp with risk-taking carp having a higher condition factor (One-way ANOVA: $F_{1,358}$ = 156.30, p < 0.001).

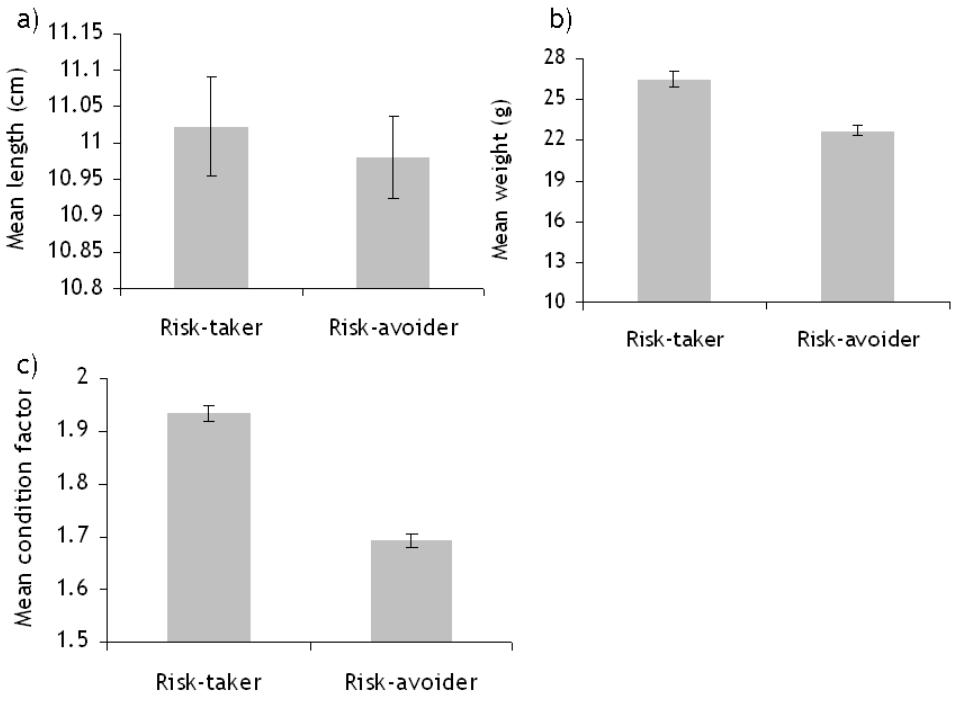


Figure 6.86 Mean (SEM) of a) length, b) weight and c) condition factor of carp by risk-taking phenotype.

2.3.5 E. Koi carp (fish screened by Huntingford et al., for a study of cortisol responsiveness)

Figure 6.88 shows the mean weight in Koi carp in relation to risk-taking strategy. For this data set fish weight was not significantly related to risk-taking phenotype (One-way ANOVA: $F_{2,67}$ = 1.19, p = 0.311). Taking mean values alone (although there was a lot of variation around the mean), risk-takers were on average lighter than the other 2 categories.

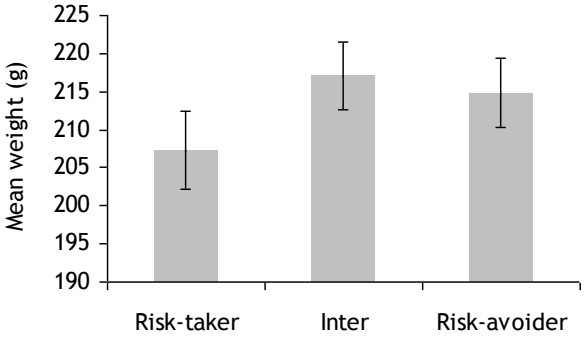


Figure 6.87 Mean (SEM) weight of carp by risk-taking phenotype.

2.3.6 F. Goldfish (screened for study of social interactions)

Results for the goldfish experiment (Table 6.21a) showed no significant relationship between risk-taking phenotype and the morphological variables length, weight and condition factor at the start of the experiment, although there was a trend for risk-taking goldfish to be longer and heavier than risk-avoiders.

Table 6.21a Mean (±S.D) length, weight and condition factor for the risk-taking phenotypes at the start of the experiment. B) Mean ± SD) rate of change in condition factor in risk taking and risk avoiding fish held in pure and mixed groups.

a)

Variable	Risk-takers	Risk-avoiders	Intermediates	F- ratio	P- value
Length	4.49±0.37	4.33±0.30	4.54±0.42	1.95	0.15
Weight	2.64±0.54	2.41±0.43	2.73±0.74	1.91	0.16
Condition factor	12.01±3.42	10.54±2.52	12.66±4.49	2.16	0.12

b)

Variable	Risk-takers Pure groups	Risk-takers Mixed groups	Risk-avoiders Pure groups	Risk-avoiders Mixed groups	F- ratio	P- value
Change in condition factor	14.70± 5.79	15.32±3.76	10.83±2.28	14.83±3.99	3.70	0.02

Different patterns of growth were subsequently shown, depending on risk-taking phenotype and social context. Risk-avoiding fish held for 8 weeks in groups of 6 fish, all of which were risk-avoiders, gained less weight than did risk-taking fish or risk-avoiders held in social groups with risk-taking fish and consequently had lower condition at the end of the study (Table 6.21b and see Chapter 6, section 3).

Although there were no significant differences, if we consider the means alone, risk-avoiders had a tendency to be shorter, lighter and in poorer condition than were fish in the other two risk-taking categories.

2.4 Discussion

For most of the data sets there was no significant relationship between risk-taking phenotype and weight, length or condition factor. However, as table 6.22 shows, based on common trends, 3 of the data sets (B, C and E) support the asset protection hypothesis (with risk-takers in poorer physical condition/nutritional status) whereas 3 data sets (A, D and F) support the growth-mortality model, in that risk-takers have better morphological status. In contrast, the latter group support the findings of Brown et al. (2007) that bolder poeciliid individuals (screened using time to emerge from cover and novel object test) were heavier at a given standard length than shy fish.

Table 6.22 Summary of results found (although some not statistically significant) for all data sets when comparing risk-taking phenotype with the three morphological variables. RT = risk-taker, I = intermediate fish, RA = risk-avoider, GM = growth mortality, AP = asset protection. Significant results are shown in bold.

Date	Weight	Length	Condition factor	Hypothesis
A. Common carp (Pilot)	-	RT>I=RA	-	GM
B. Common carp (Learning)	RT<I<RA	RT=I=RA	**I>RA>RT**	AP
C. Common carp (Demand)	RT<I+RA			AP
D. Common carp (Hunt.)	RT>RA	RT=RA	RT>RA	GM
E. Koi carp	RT<I=RA	-	-	AP
F. Goldfish	RT=I>RA	RT=I>RA	RT=I>RA	GM

The growth-mortality trade-off imply that risk-takers will have higher growth rate since they are more willing to take risks and at the same time this type of animal will increase its risk of mortality (Stamps 2007).

However, other studies have found the opposite, namely that smaller individuals, and in particular those that have the lowest nutrient reserves, take risks; those with good reserves are more cautions, and can afford to be. This is sometimes described as the “asset protection” hypothesis. In 2004, Brown & Braithwaite found that in the same poeciliid smaller fish emerged from shelter sooner than larger individuals, this was true only for populations that inhabited upstream sites which had low predation pressure.

3 Social interactions and growth in relation to risk-taking phenotype in goldfish

3.1 Introduction

As discussed in chapter 1, animals show different coping strategies. Two distinct stress response patterns exist reflected in both behavioural and neuro-endocrine processes: the proactive and the reactive stress coping styles (Pottinger & Carrick 1999). Proactive animals are characterized behaviourally by a tendency to take risk in response to danger, by relatively high levels of aggression and by the tendency to form behavioural routines. In contrast, reactive animals avoid risk and aggressive conflict and are more flexible. There is a considerable body of information about the physiological bases of differences in coping style and risk-taking and about their developmental origin (reviewed in Korte et al. 2005 and Koolhaas et al. 2007). Much less is known about the consequences for Darwinian fitness of adopting a particular coping strategy. In general, it is suggested that risk-taking, aggressive, proactive animals flourish at high population densities where resources are predictable, whereas risk-avoiding, non-aggressive animals flourish at low densities and when resources are unpredictable. In the case of mice, it seems that proactive animals do best during periods of build-up and at the peak of the population cycle, whereas reactive individuals do best after populations have crashed and when new sites for colonisation are needed (Korte 2005). Overwinter survival is higher for proactive than for reactive great tits (*Parus major*) in winters when beech trees produce many nuts and food is abundant and predictable; the converse is true for years with poor beech stands, when food is dispersed and unpredictable (Dingemanse et al. 2007).

It is also likely that there is a degree of frequency dependence in the fitness-consequences of adopting a particular coping/risk-taking strategy (Dall 2004), perhaps especially in highly social animals. The study described here addresses this possibility to some extent, by looking at food acquisition and growth in individuals of known risk-taking phenotype in social groups consisting of different mixtures of the same phenotypes, using goldfish as subjects. Goldfish are strongly schooling fish, whose behaviour within social groups might seem to be non-aggressive and somewhat uniform. However, individual differences in risk-taking have been described in this species when exploring of a novel environment (Yoshida et al. 2005). In addition, aggressive interactions have been reported within small groups of goldfish, in the context of competition for food (ref).

3.1.1 Aims of the study

With this background, the main goal of the study was to compare patterns of growth in risk-taking and risk-avoiding fish of the same size when held in small groups with different social composition with respect to coping strategy. The specific aims were:

- To screen individually identified goldfish for risk-taking phenotype.
- To compare social interactions in small groups of goldfish comprising either all risk-takers, all risk-avoiders or an equal mixture of both.
- To relate individual behaviour in small social groups of goldfish to previously-screened risk-taking category.
- To relate success in gaining access to restricted food to risk-taking category.
- To relate growth rates in social groups to risk-taking category.

3.2 Materials & Methods

3.2.1 Fish and husbandry

Goldfish of approximately 45mm in length were obtained from Murray Aquatics, Glasgow at the beginning of October 2009. They were kept in holding tanks (100 x 31.5 x 38cm) in experimental aquaria at the Graham Kerr Building, Glasgow University. The tanks were oxygenated and the average temperature of the water was 18^{o}C. Fish were allowed two weeks to adapt to the aquarium conditions, during which they were fed once a day to satiation on defrosted bloodworm, supplemented by flakes.

3.2.2 Screening for risk-taking phenotype and initial morphometrics

After the adaptation period, goldfish were screened for risk-taking phenotype using the novel environment test described in chapter 2. Screening was carried out in groups of 9 individuals; in total 54 goldfish were screened for risk-taking. After screening, fish were anaesthetized and weighed, measured (standard length) and individually identified by natural pigmentation supplemented by dye marking (HO License number 60/2930) using alcian blue applied by a Panjet inoculator, as described in chapter 2 (Pitcher & Hart 1969).

3.2.3 Experimental procedure

The fish were then established into groups of 6 fish matched as nearly as possible for length, in experimental tanks (100 x 31.5 x 38 cm) with a water temperature of 18°C. Groups consisted either of all risk-takers, or all risk-avoiders or of 3 fish of each category. Fish were held in these groups for 8 weeks, during which they were fed daily to satiation on frozen bloodworm dispersed through the tank, supplemented occasionally with pellet food. Both types of food mainly fell to the base of the tank, from where it was eaten. At the end of the study, the fish were killed by a Schedule I methods and again weighed and measured (standard length).

3.2.4 Behavioural screening

Behaviour of all fish was observed, with a total of 10 recordings and a minimum of 2 days between screenings. Focal animal sampling (Altman 1974) was used, with 1 a minute sample period during which the proximity of the focal fish to other fish (within 1 body length was considered shoaling) and the number of aggressive acts (biting and chasing) were recorded. At the end of each session, the fish were offered 10 clumped frozen bloodworms and the identity of fish that acquired food and the sequence in which they fed were recorded. Food was then added to excess.

The grouping behaviour and aggressive behaviour of the focal fish was scored for each 1 minute period as described in Table 6.23. The fish were given a food priority ranking based on the sequence in which they ate, gaining a rank of 0 if they ate no food and a rank of 6 if they were the first to eat. Thus fish that fed before their companions gained high scores.

Table 6.23 Description and scoring for the behavioural variables.

Behaviour	Score	Description
Grouping	4	In a group for the whole observation period
	3	In a group for more than half of the observation period
	2	In a group for less than half of the observation period
	1	Rarely or never in a group
Aggression	0	No aggression
	1	1 to 2 chases and bites during the observation period
	2	3 or more chases and bites during the observation period

3.2.5 Statistical analysis

Kruskal-Wallis test was used to test for significant individual effects on grouping, feeding and feed priority across all observation periods. The behaviour of each fish over

all observation periods was summarised by the mean score across tests. One-way ANOVA was then used to explore the combined effects of wave and risk-taking phenotype on the mean behavioural variables. Correlation analysis was used to examine the relationship between the different behavioural measures.

3.3 Results

3.3.1 Screening for risk-taking

There was considerable variation in the time taken by fish to emerge from shelter into the novel environment during the initial screening for risk-taking (Figure 6.89). Given this distribution, the cut-off time for allocation to each risk-taking category were as follows: fish that emerged in the first 15 minutes were classified as risk-takers, those that emerged between 15 minutes and 45 minutes were classified as intermediate fish and those that never emerged were classified as risk-avoiders.

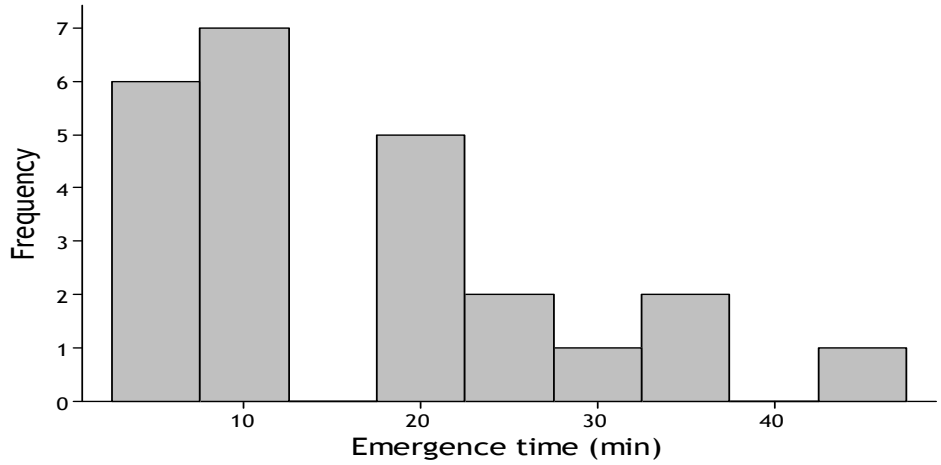


Figure 6.88 Frequency distribution of emergence time for goldfish at the novel environment test.

3.3.2 Behaviour in social groups

Individual goldfish differed in their behaviour in social groups. In terms of grouping, most fish fell into category 3, spending most but not all of the observation period within one body length of another group member.

As far as aggression was concerned, most fish showed no attacks during any given observation period, but 44% showed some aggression, the highest number of bites

observed per minute being 8. Comparing individuals across tests, significant individual effects were observed for aggression (Kruskal-Wallis test: H_{54} = 173.13, p < 0.001), grouping (Kruskal-Wallis test: H_{53} = 108.68, p < 0.001) and for feed priority (Kruskal-Wallis test: H_{53} = 177.13, p < 0.001).

Figure 6.89 shows the relationship between feed priority and aggression at the level of individual fish. Overall, there was a significant positive relationship between these 2 variables (Pearson's correlation = 0.31, P = 0.02). This is primarily the result of a lack of points in the bottom right hand corner of the figure; thus while the whole range of feed priority scores are found among fish that showed no aggression, all fish that showed attack rates of 0.5/min or higher gained relatively high feed priority scores.

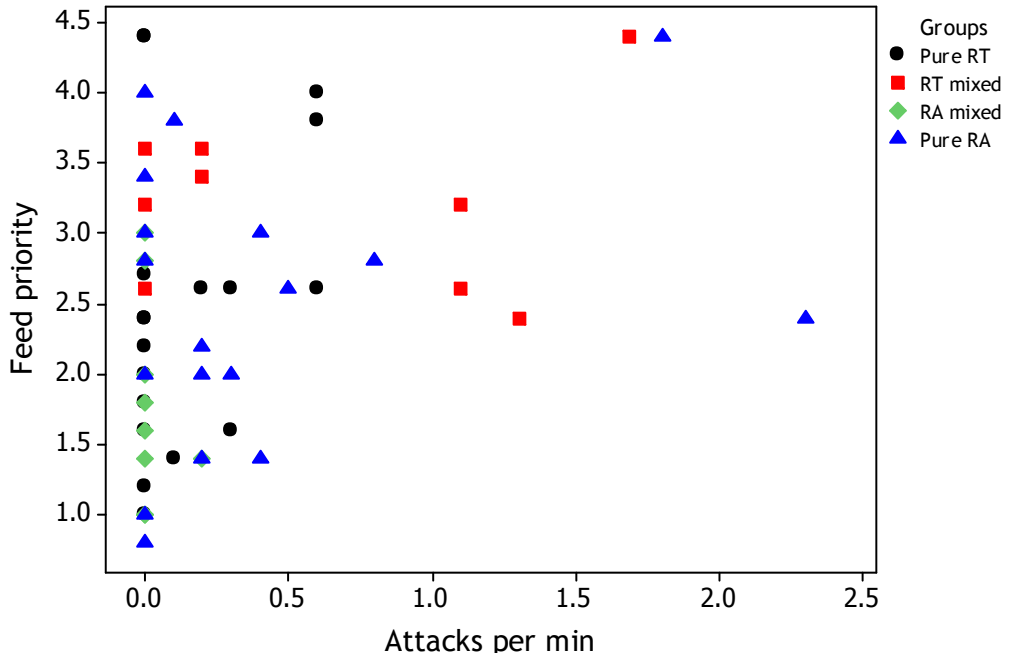


Figure 6.89 Comparison of mean feed priority with mean number attacks per minute for goldfish in groups of 6, coded by group category (pure risk-takers, pure risk-avoiders or mixed).

3.3.3 Behaviour in relation to group composition and risk-taking phenotype

Figure 6.90 shows mean (SEM) levels of grouping and aggression in the four categories of fish (risk-takers and risk-avoiders in pure groups and risk-takers and risk-avoiders in mixed groups. There were significant effects of fish category on grouping (Figure 6.91a. One-way ANOVA: $F_{3,50}$ = 14.35, p < 0.001), with risk-taking fish grouping less than groups

of only risk-avoiders in both pure and mixed groups. Risk-taking fish showed less grouping in mixed than in pure groups.

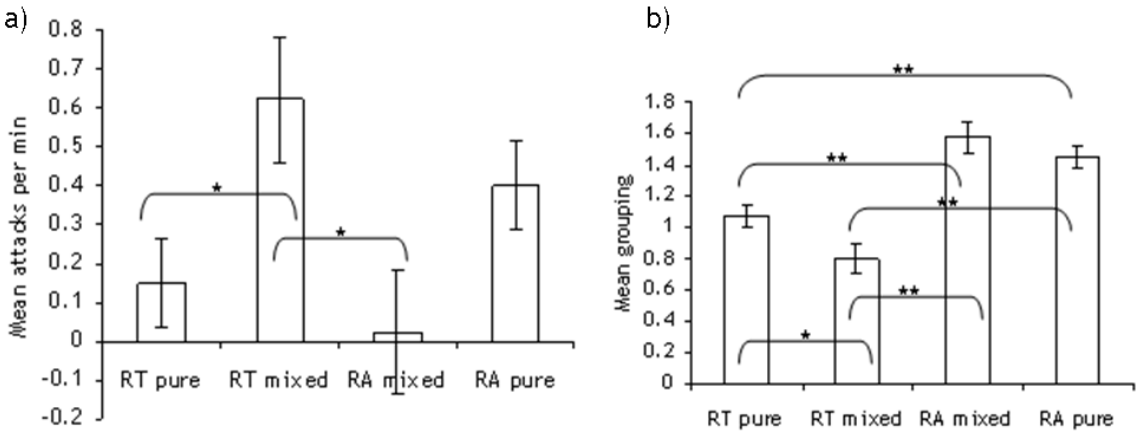


Figure 6.90 Mean (SEM) a) aggression and b) grouping for each category of fish. Brackets are showing the significant statistical difference between the groups based on Tukey comparisons. ** = statistically significant and * = marginally significant.

There were significant fish category effects for aggression (Figure 6.91a. One-way ANOVA: $F_{3,50} = 3.16$, $p = 0.032$). Levels of aggression were similar in risk-takers and risk-avoiders in pure groups. In mixed groups, risk-takers showed significantly higher levels of aggression than in pure groups, whereas the converse was the case for risk-avoiders. As a consequence, in mixed groups risk-taking fish were much more aggressive than were risk-avoiders.

Because of the way feed priority was defined, all groups had the same mean score, so the only useful comparison is between risk-takers and risk-avoiders in mixed groups. In this context, risk-taking fish had significantly higher feed priority scores than did risk-avoiders (RT = 3.22 ± 0.21; RA = 1.77 ± 0.24 - Two-sample T test: $T_{15} = 4.55$, $p < 0.001$).

3.3.4 Growth in relation to social context and risk-taking phenotype

There was no relation between growth in weight (One-way ANOVA: $F_{3,50}$ = 0.32, p = 0.808) and length (One-way ANOVA: $F_{3,50}$ = 0.70, p = 0.558) with risk-taking phenotype or social condition. Figure 6.92 shows a comparison of growth in condition factor between the social groups. Risk-avoiders in pure groups gain less in condition than the other groups (One-way ANOVA: $F_{3,50}$ = 3.70, p = 0.018).

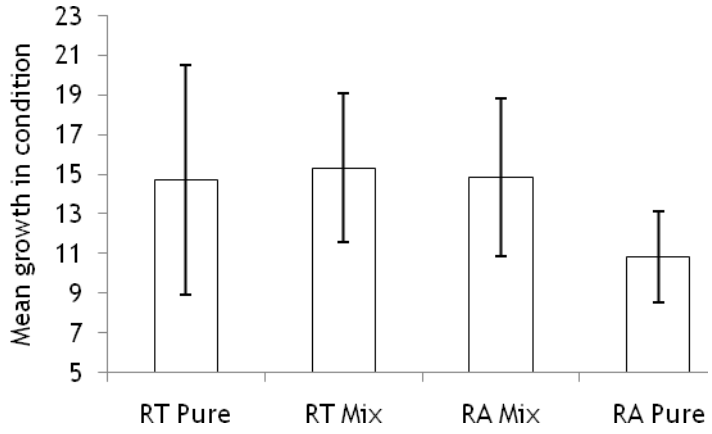


Figure 6.91 Mean (SD) growth in condition for each social group.

3.4 Discussion

Individual goldfish behaviour was variable in the time taken to emerge from shelter in the novel environment test. This agrees with the findings of Yoshida et al. (2005). For logistical reasons only a single screening was carried out, so it was not possible to assess the repeatability of these scores. However, risk-taking phenotype defined on the basis of a single screening predicted various aspects of behaviour in social groups over a subsequent 8 week period (see below). This does therefore seem to reflect at least semi-permanent differences in behaviour between fish; in other words, a bold-shy continuum may exist in goldfish, as it does for several other species of fish (Coleman & Wilson 1998).

When observed in small social groups, most of the fish spent much of their time within one body length of another fish, confirming the strongly schooling nature of this species. Propensity for shoaling was individual-specific and related to risk-taking

phenotype, with risk-avoiding fish being more likely to shoal than risk-taking fish, especially in mixed groups. The majority of fish did not attack their companions, but a non-trivial minority did do so. Aggressiveness was also an individual-specific trait and dependent on risk-taking phenotype, but in a complex way. Pure groups of risk-avoiders and risk-takers showed similar levels of aggression; however, in mixed groups risk-taking fish were more aggressive than risk-avoiding fish, attacking at approximately double the rate.

When feeding at a restricted food supply, consistent individual differences were observed in the sequence in which the fish fed and thus in the effectiveness with which the fish were able to scramble for this valuable resource. In mixed groups, risk-taking fish gained higher feed priority scores, consistently feeding earlier than their risk-avoiding companions. In these conditions at least, therefore, risk-taking phenotype predicts competitive ability; this is compatible with the existence of a loose risk-taking/aggression syndrome in goldfish, as described for several other species of fish (Sih et al. 2004).

Individual levels of aggression within groups predicted feed priority, those fish that showed moderate or high levels of aggression all gained above average feed priority scores; in contrast, while some non-aggressive fish gained early access to food, many others had low feeding priority. Thus, what goldfish in small groups gain from attacking their companions is reliable feeding when food is restricted. At a group level at least, this does not translate into higher growth rates, since rates of growth in weight and length are equivalent in the four categories of fish (risk-takers and risk-avoiders in pure and mixed groups), in spite of striking differences in aggression. This may be because aggression uses up nutrients that might otherwise have been used for growth and/or because (except during the feed priority tests) the fish were fed to excess. In combination with group composition, risk-taking phenotype does influence patterns of change in condition factor, since risk-avoiding fish gained in condition less than fish in the other three categories. It is known that the behaviour of perch (*Perca fluviatilis*) in small groups is influenced both by their own risk-taking phenotype and by that of their companions, with shy fish becoming bolder in the presence of bold fish (Magnhagen & Staffan 2005). It may be that the risk-avoiding fish in pure groups in the present study showed some sort of social facilitation of fear and so were particularly stressed, may have compromised their weight gain. Whatever the explanation, this result emphasises the fact that the fitness-related consequences of a particular level of risk-taking are variable and dependent on environmental conditions, in this case on the behavioural profiles of social companions.

4 Respiratory function in relation to coping strategy in common carp

4.1 Introduction

4.1.1 Coping strategies and respiratory physiology

According to the growth mortality trade-off view. One explanation of the existence of behavioural syndromes/coping strategies is that certain individuals within a species opt for a fast life history trajectory, which includes various adaptations for attaining fast growth, such as enhanced appetite, more active foraging and a greater propensity both to take risks to gain food and to fight with conspecifics over food. If individuals within a population pursue a range of growth rates, they will have associated with this a range of levels of risk-taking and a range of aggressiveness, hence the observed association between these traits in many cases (Stamps 2007, Biro & Stamps 2010). One implication of adopting a high-risk/high gain lifestyle is that this may require a higher metabolic rate in the individuals concerned in order to provide the necessary energy. In fish there are a number of examples of an association between high resting metabolic rate and an aggressive, dominant life style (examples in Huntingford et al. 2010). In addition, higher resting metabolic rates have been reported in risk-taking, proactive carp compared to risk-avoiding, reactive fish (Huntingford et al. 2010). This being the case, it might be expected that the respiratory surfaces of proactive individuals might be more extensive than those of size-matched reactive individuals. The purpose of the study described here was to determine whether this is the case, using common carp as subjects.

4.1.2 Gill structure and function

Fish gills are the main organ responsible for extracting oxygen from water. The respiratory structures are located on 4 paired gill arches lying within the buccal cavity and are composed of thin filaments covered with an epidermal membrane that is folded repeatedly to form the lamellae (figure 6.92). Fish constantly pump water through the mouth and over the gills arches. Each gill arch has two rows of filaments. Blood flowing through the capillaries within the lamellae picks up oxygen from the water (Hickman et al. 2001). The lamellar surface of fish gills also contain mucous cells; these that secrete the fluid that moistens the respiratory surface. Mucous cells proliferate in response to a variety of stressors, including poor water quality (Sollid et al. 2003), and so might be differentially developed in proactive and reactive fish. The extensive area exposed to

the water for the purpose of oxygen extraction also represents a surface across which dissolved ions can pass in or out of the body of the fish, depending on the osmotic pressure of the water in which the fish is living. It therefore poses osmoregulatory problems for the fish. A large respiratory surface also increases the ingress of dissolved contaminants. Thus larger gills, while beneficial in terms of respiratory efficiency, impose an extra cost for the fish and gill size reflects a balance between such costs and benefits.

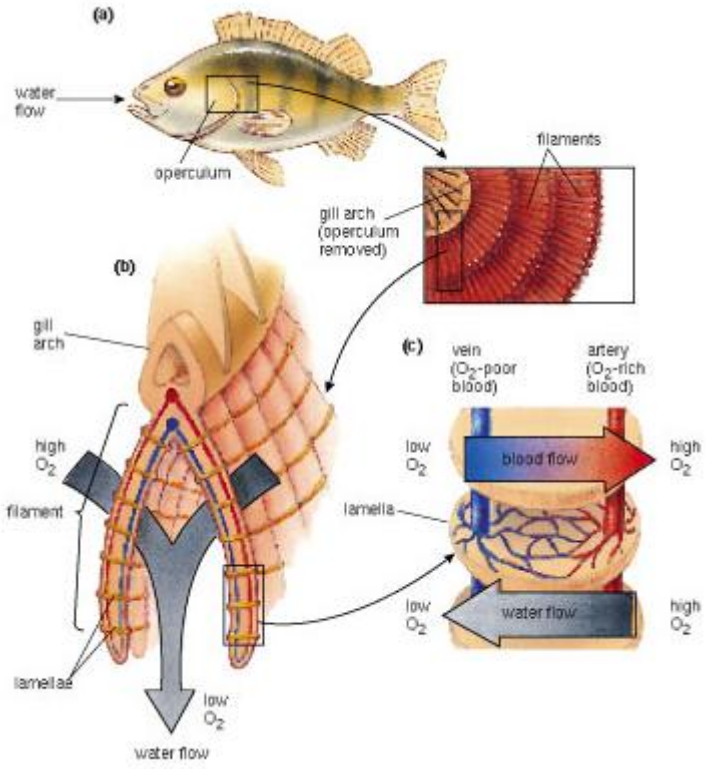


Figure 6.92 Gills of fish. a) Location of gills with and without operculum, revealing 4 gill arches on each side, b) a portion of the gill arch shows gill filaments that project to the bottom and gill rakers that projects to the top (responsible for feeding), also shows the lamellae and direction of O_2 flow, c) dissection of a filament showing the blood capillaries and the direction of blood and water flow. Figure from website: http://dobrinishte.org/fishes/index_files/Page512.htm

4.1.3 Adaptative variation in gill morphology

The extent of development of the gill filaments and secondary lamellae, and hence the area of the respiratory surface, varies strikingly both between and within species. This variation may relate to the quality of the water to which fish are exposed. For example, fish exposed to water with low levels of dissolved oxygen may develop larger respiratory surface. It may also relate to the life style of the fish concerned; in general, more

active fish with high oxygen requirements have longer gill filaments and more secondary lamellae than do slow-moving fish.

An optimal gill size is difficult to achieve, since fish live in a highly inconstant environment and moreover different life stages requires different rates of oxygen uptake. Therefore, in addition to longer term adaptive differences in the size of the respiratory structures, on a shorter time scale, fish are also able to modify the extent to which the respiratory surface is exposed to the water by altering the extent to which the secondary lamellae are covered by epithelial cells. For example, in crucian carp (*Carassius carassius*) at low temperatures (< 20°C) with well aerated water, the lamellae are largely covered by layers of epithelial cells (figure 6.94a). In carp held in hypoxic conditions or at an increased temperature (> 25°C), a reversible change occurs as the epithelial cells disappear, leaving the lamellae protruding into the water (figure 6.94b - Sollid et al. 2003 and 2005).

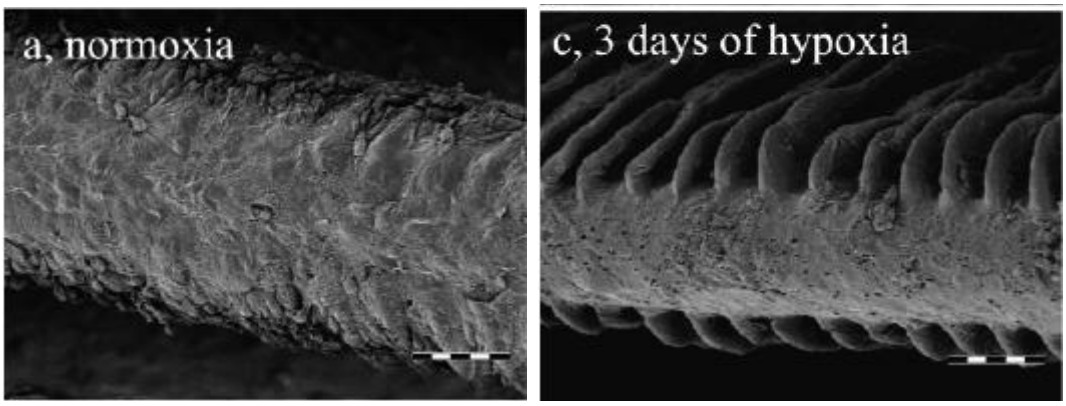


Figure 6.93 Scanning electron micrographs of gill filaments from crucian carp kept in a) normoxic water, b) 3 days of hypoxia (Sollid et al. 2003). Scale bar 50 µm

4.1.4 Aims

As stated above, the purpose of the study described in this section was to compare the development of the respiratory surface in common carp with different coping strategies. We used the fish from the study described in Chapter 3, testing the hypothesis that the higher resting metabolic rate of proactive fish may require a relatively large respiratory surface. The development of the respiratory surface was estimated from measurements of the gill filaments and secondary lamellae from all 4 gill arches. In addition, the percentage of this area that was exposed as opposed to occluded by epithelial cells was assessed from sections stained for light microscopy. The same sections were used to compare the abundance of mucous cells in carp with

different coping strategies, with a view to providing an additional insight into differences in stress responsiveness between them.

4.2 Materials & Methods

4.2.1 Screening for risk-taking

The carp were screened for risk-taking as described in chapter 3, the fish being used for the present study after the learning tests were complete.

4.2.2 Gill morphometrics

The carp were deeply anaesthetized, killed by a Schedule I methods and all four gill arches from both sides of each fish were dissected and placed in 10% normal saline. The gills from the two sides of the fish were removed, keeping the filaments intact. The gill arches were detached and various measurements (indicated in table 6.23) were taken for the left and the right sides of each arch. A binocular microscope at a magnification of 3x with an eyepiece micrometer was used (after Hughes 1984). To quantify individual status with respect to those variables, the mean value for the right and left sides were used.

Table 6.24. Measurements taken from the carp gills

Structure	Description
Filament number	Total number of gill filaments
Filament length	Length of every tenth filament
Secondary lamellae number	Number of secondary lamellae per mm on every tenth gill filament
Secondary lamellae length	Length of secondary lamellae at 3 points of every tenth gill filament
Distances between secondary lamellae	Distances between secondary lamellae at 3 points of every tenth gill filament

4.2.3 Light microscopy

The second gill arch from the left side was preserved for light microscopy. Tissues for histological analysis were placed into buffered formalin and embedded in wax following standard procedures. Wax embedded tissues were sectioned (thickness of section = 5 μm) and stained with haematoxylin and eosin, according to method outlined in Clark (1980). Figure 6.95 shows a typical section of a gill filament. The number of mucous cells (indicated in Figure 6.95) per millimetre of lamellar surface and the height of

interlamellar cells as a percentage of lamella height were quantified on different filament. The number of mucous cells, the height of the interlammellar epithelial cells and the height of the adjacent lamellae were measured for 9 lamellae per filament and mean values were calculated. From this a measure of percentage hyperplasia was calculated.

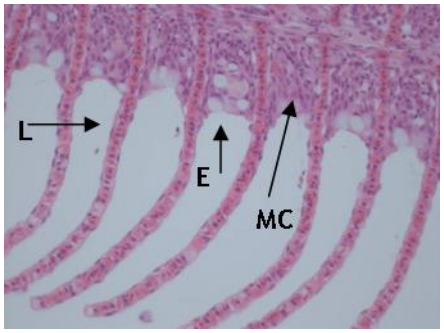


Figure 6.94 Typical section through the gill filament of common carp, with secondary lamellae (L) mucous cells (MC) and hyperplasia, which means the extent to which the space netween adjacent lamellae is filled with epithelial cells (E) indicated.

4.2.4 Statistical analysis

The following statistical procedures were carried out, using MINITAB series 15. First, the data were checked for normality and transformations performed where necessary. Relations among measured variables were studied by correlation analysis, followed by Principal Components Analyses (PCA), which was also used to generate compound scores where appropriate. For simplicity, separate PCAs were carried out for gill filament and secondary lamellae measures. Finally, ANOVA were used to compare gill morphology with risk-taking phenotype.

4.3 Results

4.3.1 Gill morphometrics

Table 6.25 shows the results of regression analysis of all measured variables against body length. Where the regression was significant, in other words for all variables except the distance between the secondary lamellae, length-corrected scores were derived from the residuals.

Table 6.25 Regression analyses of all variables measured from the gills of common carp, against body length with the results of statistical testing.

Variable	Arch	Regression equation	$F_{1,66}$	P
Filament length	1	Y= - 1.08 + 0.394 X	108.31	0.00
	2	Y= - 1.13 + 0.377 X	105.43	0.00
	3	Y= - 1.10 + 0.351 X	98.38	0.00
	4	Y= - 17.3 + 5.69 X	117.38	0.00
Filament number	1	Y= 28.3 + 3.28 X	59.09	0.00
	2	Y= 12.20 + 4.16 X	79.71	0.00
	3	Y= - 2.55 + 4.95 X	93.93	0.00
	4	Y= - 17.30 + 5.69 X	117.38	0.00
Secondary lamellae length	1	Y= - 0.162 + 0.031 X	183.70	0.00
	2	Y= - 0.091 + 0.023 X	154.17	0.00
	3	Y= - 0.094 + 0.023 X	149.60	0.00
	4	Y= - 0.108 + 0.024 X	168.16	0.00
Secondary lamellae number	1	Y= 46.00 + 2.72 X	21.41	0.00
	2	Y= 46.30 + 2.41 X	14.79	0.00
	3	Y= 46.60 + 2.14 X	12.71	0.00
	4	Y= 51.80 + 1.41 X	5.49	0.02
Distance between secondary lamellae	1	Y= 0.01 + 0.00 X	3.25	0.07
	2	Y= 0.02 + 0.00 X	0.88	0.35
	3	Y= 0.00 + 0.00 X	3.43	0.07
	4	Y= 0.01+ 0.00 X	3.16	0.08

Table 6.26a shows the matrix of correlations between gill filament number and length for all arches; lengths and numbers for the 4 arches were all strongly correlated. Table 6.26b shows the first two components from a principal components analysis for these variables. Together these 2 components explain 95% of variation in this data set. PC1, which accounts for 84% of the total variance, has positive loadings for all variables and so represents variability in filament size and number (and thus overall filament development) independent of body size. Fish with many, long filaments will gain high scores on this axis. PC2 accounts for 11% of the total variance and has negative loadings for filament length on all arches and positive loadings for filament number; fish with few, long filaments gain high scores in this axis.

Table 6.26 a) Correlation matrix for gill filament length and gill filament number for all gill arches. For each cell, the top figure is the person product moment correlation coefficient and the bottom row is the p value. b) Loading for all variables on the first two components in a PCA analysis of filament number and length for all arches.

a)	Length arch 1	Length arch 2	Length arch 3	Length arch 4	No. arch 1	No. arch 2	No. arch 3
Length 2	0.998						
	0.000						
Length 3	0.991	0.996					
	0.000	0.000					
Length 4	0.983	0.990	0.997				
	0.000	0.000	0.000				
Number 1	0.835	0.839	0.840	0.839			
	0.000	0.000	0.000	0.000			
Number 2	0.888	0.889	0.884	0.879	0.924		
	0.000	0.000	0.000	0.000	0.000		
Number 3	0.905	0..905	0.897	0.890	0.905	0.983	
	0.000	0.000	0.000	0.000	0.000	0.000	
Number 4	0.933	0.932	0.924	0.918	0.869	0.948	0.973

	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

b)	Length 1	No. 1	Length 2	No. 2	Length 3	No. 3	Length 4	No. 4
PC1	0.36	0.32	0.37	0.37	0.37	0.35	0.36	0.36
PC2	-0.32	0.43	-0.33	0.43	-0.35	0.39	-0.34	0.19

Table 6.27a shows the correlation matrix for secondary lamellae measures for all arches. Number and length of secondary lamellae are correlated across arches and with each other. Distances between lamellae are correlated across arches, but do not show any consistent pattern of correlation with the other scores. Table 6.27b shows the loadings for all lamellae variables on the first two components resulting from principal components analysis of the secondary lamellae, which together explain 73% of the variation in this data set. The first component accounts for 43% and has positive loadings for most variables, but negative for distance between secondary lamellae. It therefore represents an index overall development of the secondary respiratory surfaces, independent of body size. PC2 explains 30% of total variance, with high negative loadings for secondary lamellae length and distance between secondary lamellae, but positive for number of secondary lamellae.

Table 6.27 a) Correlation matrix for gill secondary lamellae length, number and spacing for all gill arches. For each cell, the top figure is the person product moment correlation coefficient and the bottom row is the p value. b) Loadings for all lamellae variables on the first two components resulting from PCA of the lamellae variables.

a)	Len 1	Len 2	Len 3	Len 4	No. 1	No. 2	No. 3	No. 4	Dist. 1	Dist. 2	Dist. 3
Length 2	0.991 0.000										
Length 3	0.991 0.000	0.988 0.000									
Length 4	0.989 0.000	0.989 0.000	0.994 0.000								
No.1	0.551 0.000	0.565 0.000	0.519 0.000	0.525 0.000							
No.2	0.493 0.000	0.504 0.000	0.461 0.000	0.464 0.000	0.968 0.000						
No.3	0.467 0.000	0.485 0.000	0.440 0.000	0.443 0.000	0.953 0.000	0.962 0.000					
No. 4^t	0.352 0.004	0.375 0.002	0.327 0.007	0.331 0.007	0.913 0.000	0.993 0.000	0.978 0.000				
Dist. 1	0.174 0.161	0.171 0.169	0.161 0.197	0.154 0.218	- 0.068 0.586	- 0.070 0.578	- 0.092 0.461	-0.14 0.240			
Dist. 2	0.100 0.424	0.103 0.412	0.090 0.471	0.092 0.463	0.059 0.638	0.065 0.606	0.055 0.659	0.030 0.800	0.795 0.000		
Dist. 3	0.162 0.195	0.154 0.218	0.154 0.216	0.156 0.211	- 0.094 0.451	- 0.104 0.404	- 0.121 0.333	- 0.160 0.180	0.880 0.000	0.841 0.000	
Dist. 4^t	0.184 0.139	0.174 0.162	0.181 0.146	0.176 0.158	- 0.150 0.228	- 0.163 0.192	- 0.176 0.157	- 0.220 0.070	0.918 0.000	0.812 0.000	0.961 0.000

b)

Arch		PC1	PC2
1st	Length	0.30	-0.31
	Number	0.36	0.04
	Distance	-0.20	-0.39
2nd	Length	0.35	-0.31
	Number	0.35	0.04
	Distance	-0.13	-0.37
3rd	Length	0.29	-0.31
	Number	0.35	0.05
	Distance	-0.22	-0.39
4th	Length	0.35	-0.31
	Number	0.41	0.06
	Distance	-0.22	-0.41

4.3.2 Gill microstructure

The extent of hyperplasia varied markedly among the carp, with a mean of 6.2%, a minimum of 0% and a maximum of 52.6%. Relatively few mucous cells were found and the mean number per lamellae was also variable, with a mean of 0.13, a minimum of 0 and a maximum of 1.

4.3.3 Gill structure in carp with different coping strategies

Table 6.28 shows means (± SEM) for the PCA-derived scores of gill filament and secondary lamellae dimensions, percentage hyperplasia and mucous cell number in risk-taking, intermediate risk-avoiding and intermediate carp, together with the results of one-way analyses of variance.

Table 6.28 Mean (±SEM) scores for PCA-derived gill dimensions fro gill filaments and secondary lamellae, mucous cell number and percentage hyperplasia in carp from the 3 risk taking phenotypes, together with the results of ANOVA.

Variable	Mean ± SEM			$F_{2,65}$	P
	Risk-takers	Intermediate	Risk-avoiders		
Filament PC1	1.19 ± 2.40	-0.36±2.36	- 0.62 ± 2.17	3.58	0.03
Filament PC2	0.78 ± 0.71	-0.13±0.73	-0.52 ± 3.17	2.43	0.09
Secondary lamellae PC1	1.58 ± 1.40	-0.18±1.87	-1.16 ± 2.54	9.63	0.00
Secondary lamellae PC2	0.11 ± 1.41	-0.18 ± 1.19	0.10 ± 2.79	0.17	0.85
Number of mucus cells	2.00 ± 0.47	1.65 ± 0.60	2.58 ± 0.22	5.13	0.03
Percentage hyperplasia	20 ± 10.9	30 ± 0.02	70 ± 0.02	10.9	0.002

Risk-taking fish had significantly higher scores than intermediate fish, which in turn had higher scores than did risk-avoiding fish for filament PC1 (a size-independent index of overall filament development) and secondary lamellae PC1 (a size independent index of overall lamellar development). *Post hoc* test showed risk-taking fish to be significantly different from intermediate and risk-avoiding fish, with risk-avoiders just different from

intermediate fish. In contrast, risk-taking carp had lower levels of hyperplasia and fewer mucous cells than did intermediate fish, which in turn had lower levels for both variables than did risk-taking fish.

4.4 Discussion

Using morphometric and histological techniques, this study has identified considerable variability in relative development of the respiratory surface among common carp of the same cohort that is independent of any differences in body size. This is reflected in the overall development of the gill filaments (represented by PC1 in our multi-variate analysis of filament dimensions), in the overall extent of the secondary lamellae (represented by PC1 in our multi-variate analysis of secondary lamellae dimensions) and in the extent to which the respiratory surface is obscured by epithelial cells. Together, these result show that the exposed respiratory surface is markedly larger in risk-taking than in risk-avoiding carp, with fish classified as intermediate in terms of their behaviour also being intermediate in terms of their gill development. Thus our initial hypothesis that the higher resting metabolic rate of proactive carp (Huntingford et al. 2010) may require a relatively large respiratory surface is supported. Our results agree with the general finding of better developed respiratory surfaces in fish with active life styles. It also agrees with the observation that under conditions of high oxygen demand, crucian carp show reduced levels of epithelial cover of their secondary lamellae (Sollid et al. 2003 and 2005). The fact that risk-avoiding fish have more mucous cells per secondary lamella (often taken as an index of stress) provides independent validation of our characterisation of the risk-avoiding fish as reactive.

Thus, the larger respiratory area of risk-taking fish can be seen as an adaptation to their higher metabolic rate and greater oxygen requirements. However, it also means that the fish have a greater surface area across which ions can be lost to the surrounding water and through which harmful substances can be absorbed. These therefore represent collateral costs of a proactive, aggressive lifestyle that, depending on environmental conditions and together with the direct costs of fighting, may counterbalance the advantages of gaining access to limiting resources through fighting.

CHAPTER 7

General Discussion

1 Introduction

The overall aim of my project was to investigate the extent to which it is possible to control the behaviour of a farmed fish species (the common carp) by using its ability to learn stimulus-response associations, with a view to developing welfare-friendly husbandry systems. In pursuit of this aim, the following questions were addressed:

1.1 Learning and variability in learning about visual landmarks

My results showed that carp were able learn to locate food by using light cues to direct them towards a profitable feeding site from a choice of 2. However, their behaviour was variable and 2 different strategies (both efficient) could be distinguished. Some fish learned to follow the cue to find food as expected, but an equal number went to one or other of the two potential feeding sites at random and, if they found no food, switched to the other site. Light colour appeared to have an effect on learning, with fish trained with a red light learning faster than fish trained with yellow light. This agrees with a number of studies showing that fish use various kinds of learning to forage efficiently in a variable environment (Warburton 2003). When 3 lights were used, some groups of fish also learned to forage efficiently and again the red light seemed to facilitate learning.

The influence of colour may be the result of the diet offered to the carp. While in their tanks before experiments, the fish were fed with frozen chironomid larvae (bloodworms) which have a dark red colour. Experience with red coloured food might explain the relative ease with which the fish formed an association between the red light and the presence of food. To the best of my knowledge, there are no conclusive published studies on colour preferences specifically in carp. A study with goldfish demonstrated faster learning with the blue when compared with green cues, but learned associations were formed between all the three colours (blue, green and red) and the presence of food (Muntz & Cronly-Dillon 1966). A non-specific bias for approaching short wavelength stimuli has been reported for some aquatic species, (Colwill et al. 2005). Muntz & Cronly-Dillon (1966) also reported variability in learning ability in goldfish, with some fish learning fast and making few mistakes and others never reaching the learning criterion.

1.2 Hands-off separation by learned association

For those carp that learned to approach a light of a particular colour to obtain food, it was possible to use these responses to draw specific individuals out of a school and to a specific location in the tank. The reliability with which this was achieved was moderate overall (though the effect was statistically significant), depending on the colour of the stimulus light and the fact that the response disappeared quickly if not rewarded. For fish trained on the red light the success rate was almost 90% in the first trial, which would be sufficient to effect reasonable separation. The results are similar to those found for Atlantic salmon previously trained to associate a light stimulus with delivery of food; these fish could be separated from the group by using a more selective light stimulus that could be focused in just one individual (Lines & Frost 1997). Learned responses to light cues could therefore be used to control the behaviour of carp. This might potentially promote efficient management (for example, selection of broodstock or disease treatment) of fish in semi-extensive aquaculture and also for developing welfare-friendly husbandry practices for intensive aquaculture.

1.3 Repeatability of risk-taking within randomly composed groups and risk-taking in different contexts

Some of the variability in performance of the carp used in the various learning trials may have arisen because they were not behaviourally equivalent, due to the existence of coping strategies. In the process of exploring the relevance of risk-taking phenotype for learning, I gathered various pieces of information of general relevance to behavioural syndromes and coping strategies.

On the subject of the repeatability of differences in emergence time, in most cases, even though fish were tested in randomly formed groups, the time taken by identified individual fish to emerge from shelter was consistent across tests. The exception was the study described in chapter 4, possibly because the groups of fish tested together were not being housed in the same tanks and also because they were kept in smaller groups (6 fish) while the others were kept in groups of more than 20 fish. This could arise because of social influences. The classification of fish in risk-takers, risk-avoiders and intermediate in chapter 3 was validated by differences in behaviour on both in the pre-learning settling period and in the learning period. It was also validated by the fact that risk-avoiding fish had more mucous cells in their gills than did risk-taking fish; mucous cells are produced in response to stress, suggesting that risk-taking fish had

higher stress responsiveness than the other categories. This would agree with the fact that they also have higher levels of expression of cortical receptor genes in their head kidney and brain (Huntingford et al. 2010) suggesting that hypothalamus-pituitary-interrenal axis is more active in risk-taking fish.

My results also throw light on the relationship between behaviour in the novel environment and other aspects of risk-taking. Thus my classification from novel environment test was also validated by the behaviour of the carp on the novel object test. On average, carp that approached the novel object more frequently had relatively short emergence times, and the converse (chapter 3). In contrast, in the study described in chapter 5 (the Poland study), at the family level the average behaviour in the novel environment test was not related to the average behaviour on the novel object test. Some studies showed that different contexts results in differences in behaviour (Wilson & Stevens 2005, Coleman & Wilson 1998), others points consistent behaviour across contexts; Dingemanse et al. (2007) found an association between risk-taking by three-spined sticklebacks in different contexts to be associated in populations coexisting with piscivorous fish, but not in other, predator-free sites. The behaviour shown by the carp in the present study in the competition tests was repeatable, but was unrelated to behaviour in both the novel object and the novel environment test. This is in contrast with the finding of Huntingford et al. (2010), in which fast-emergers were more likely than slow-emergers to gain access to limited food. This is presumably because carp in the present study were not held in small groups and not tested in the same groups, so no established social relationships could be formed. This is supported by the fact that in the goldfish used in the study described in chapter 6, risk-taking phenotype predicted aggressiveness in small established groups composed of risk-taking and risk-avoiding fish.

1.4 Risk-taking and learning

In chapter 3, fish of all risk-taking phenotypes learned quickly to find food and there was a difference in the learning strategy with more risk-avoiders learning to follow the light cue. This would seem to fit with the general finding of greater behavioural flexibility in reactive animals (Korte et al. 2005). In chapter 4, risk-taking phenotype did not influence rate of learning on the one-light phase, but on transfer to the 3 light phase there was a difference in the earlier trials, with risk-avoiders making more mistakes than risk-takers and intermediate fish. This may have occurred because risk-avoiding fish, were more frightened of the change to the new, 3-light set-up than were risk-taking and intermediate fish. This agrees with the findings of Sneddon (2003) that

rainbow trout classified as bold leaned faster than shy fish in a test that required them to leave shelter to obtain food in response to a light cue.

1.5 Risk-taking strategy and performance in other contexts

While the physiological mechanisms and developmental origins of differences in coping style have been extensively studies, there is relatively little information on their consequences for fitness. During the course of the work described in this thesis, various pieces of information relevant to this point were obtained. The meta-analysis of the relationship between risk-taking phenotype and morphological status (Chapter 6) was inconclusive. In terms of mean values, half of the data sets could be seen to support the growth mortality model, but half fitted better to the "asset protection" hypothesis. For example, in chapter 5, carp reared in ponds were in much poorer condition than those carp reared in tanks (which had been reared with higher food availability and had not suffered a recent disease outbreak) and emerged from shelter very much faster. The study of goldfish described in Chapter 6 throws some light on these findings, and the variable results described in the literature, by emphasising the complex nature of the relationship. Here, risk-avoiding goldfish had poorer condition than risk-taking fish, but only when held in groups consisting of all risk-avoiders; in mixed groups with risk-takers the same was not true. It is possible that some sort of social facilitation among risk-avoiding carp in pure groups leads to high levels of fear and stress and consequently to poor nutritional status.

In mixed groups, risk-taking goldfish gained more reliable access to restricted food than did risk-avoiding fish (Chapter 6), supporting the view that proactive animals gain fitness benefits through access to limited resources when these are clumped and predictable. On the other hand, carp classified as risk-taking had markedly larger exposed respiratory surfaces than did risk-avoiding carp, with intermediate fish being intermediate also being intermediate in terms of their gill development (Chapter 6). This result agrees with the hypothesis that the higher resting metabolic rate of proactive carp (Huntingford et al. 2010) requires a relatively large respiratory surface. Since a larger gill area presumably means greater loss of ions to the surrounding water in these freshwater fish as well as greater exposure to any damaging chemicals in the water, this adaptation could represent a hidden cost of a proactive/aggressive life style and may counterbalance the advantages of being aggressive and gaining access to food

1.6 Implications for Aquaculture

The results of the work described in this thesis potentially have a number of implications for carp aquaculture. In the first place, as described on chapter 1, husbandry practices are stressful to fish, so the possibility demonstrated here of developing low-stress methods for sorting fish could be of great value. In addition, the fact that some groups of carp at least learned to use a demand-feeding system (albeit one that functioned somewhat inefficiently) suggests that such systems could be deployed in carp aquaculture. The fact that risk-taking and risk-avoiding fish learned this equally well suggest that it might be possible to create culture systems in which risk-avoiders flourish by having several demand feeders, some delivering food at a lower rate (to deter risk-takers), but in covered areas (to attract risk-avoiders). That this might be necessary is suggested by the fact that when food is restricted, risk-taking goldfish gain preferential access to it. As a final point, the larger respiratory surface in risk-taking carp, which as pointed out above, can be seen as an adaptation to their high metabolic rate and greater oxygen requirements also means that fish may be more vulnerable to poor water quality in culture systems.

1.7 Problems, solutions, observations and thoughts

Some unexpected problems appeared in the course of the experiments. 2 batches of fish died, the first probably because the supplier did not send them in appropriate conditions (too many carp in just one plastic bag with very few water). The next batch, from a different supplier, came in good conditions, but most likely due to the stress of the journey from the farm to Glasgow, were more susceptible to disease, even after intensive treatment, they died.

At chapter 3, the finding of two different strategies was surprising and interesting as I thought fish would learn or not. In a next experiment, one should try to manipulate fish behaviour by making the switching from an unrewarded to a rewarded side more costly, for example testing fish in a bigger tank or putting some kind of obstacles. In that manner, it would possible to observe the possibility to “produce” fewer fish choosing the random-switch strategy.

Another problem we had was with the self-feeding system. It was previously used in other study but was left inside a room with salt water aquariums which caused corrosion of some parts of the equipment. This caused a malfunction and

it was not possible to use the whole equipment as previously thought. Because the self-feeding system was not working properly, we have to improvise therefore an assistant helped me delivering food to the fish which make the delivery of food not at the same time as the actuation of the trigger (although if the mechanical of the demand feeder was working it was also going to take some seconds to deliver the pellets). At the beginning of the trials, the pellets were not going to bottom of the tank (where generally carp forage) soon after the release into the water, so possibly this was delaying fish learning the association between touching the sensor and delivery of food. This problem had a simple solution: wet the pellets before throwing it in the tank. In the future, we have to more thoughtful and observe the time between the response and stimulus.

At chapter 4, carp showed some inconsistent behaviour on the novel environment test. This can be due to different factors, but one point I want to make is that fish were being kept in a room where not only me had access but other students too. That disturbs more and can affect fish behaviour. Another thing that happened in the last trials of the demand feeding experiment was a problem in the light system of the room, where part of it has illumination and the other did not. 3 of the tanks were in the darker area of the room and in the last trials (mixed groups trials and reinforcement tests between mixed groups' trials) and the fish from these tanks showed a different behaviour from the previous tests, decrease activity and swimming.

In Poland, we had a unique opportunity of exploring family differences and rearing conditions as well as interaction between these variables. However, because of disease, all fish from the pond transferred to tanks could not survive.

An interesting point to explore would be the fact that risk-taking fish showed larger gill surface area than risk-avoiding. For example, an experiment involving some kind of harmful substance in the water and observe the differences in behaviour and morphology of risk-takers and risk-avoiders.

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