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Investigating fear in domestic rainbow trout, *Oncorhynchus mykiss*, using an avoidance learning task

S. Yue*, R.D. Moccia, I.J.H. Duncan

Department of Animal and Poultry Science, University of Guelph, Guelph, Ont., Canada N1G 2W1

Abstract

The capacity of rainbow trout to experience fear was assessed using an avoidance learning task. Each of 13 fish was placed individually into a two-chambered shuttle tank where it could be subjected to the putative frightening stimulus of a plunging dip net in either chamber. The fish could escape from the stimulus by swimming through a doorway to the other chamber. The fish escaped from the plunging net by swimming through the doorway, some on the first occasion and all after a few exposures. Each fish was then presented with a neutral stimulus of a light that went on 10 s before the net plunged into the water. Over a 5-day period, all fish learned to avoid the plunging net by swimming through the doorway when the light was illuminated. All fish showed evidence of longer-term memory by showing the learned avoidance response on the first occasion they were tested after 7 days of no testing. Whereas the escape responses to the plunging net were immediate and reflexive-like, the avoidance responses to the light going on were delayed a few seconds and more deliberate in nature. This evidence suggests that trout can experience fear and that they can learn to avoid frightening stimuli. It implies that they are sentient animals, more complex than previously thought.
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Keywords: Fish; Fear; Avoidance; Learning; Memory; Animal welfare

1. Introduction

Fear is a primitive negative emotion resulting in physiological and behavioural changes and is caused by the perception of danger (Drever, 1981; Hurnik et al., 1995). It is an

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* Corresponding author. Tel.: +1-519-824-4120x6964; fax: +1-519-767-0573.
E-mail address: syue@uoguelph.ca (S. Yue).

extremely difficult state to assess, primarily because like any other feeling, it is a private, subjective experience. However, a possible method to investigate fear is to study avoidance behaviour. Avoidance of aversive stimulation by means of escape, non-approach or other appropriate responses may provide a window into an animal's mind revealing how negative it considers the stimulation to be. The principle of avoidance learning uses a combination of both classical and operant conditioning to determine what an animal finds aversive. The basic procedure first consists of allowing an animal to escape from, or terminate, aversive stimulation—a response that usually results from trial and error learning (operant component). The next step consists of signalling the onset of the aversive stimulus with a neutral cue immediately before each occurrence of the aversive stimulus. Prior to the pairing of the cue and noxious stimulus, the cue should not elicit any particular response from the animal. However, as a result of this association (classical component), the cue when presented alone, should bring about avoidance of the aversive stimulus. A good example of avoidance learning was demonstrated by Gentry (1934). White rats learned to avoid an electric shock delivered by the grid under their feet by running into an adjacent unelectrified compartment of a 'shuttle-box'. A light stimulus was then presented before each delivery of electric shock. The rats eventually learned that the onset of the light signalled the forthcoming electric shock; therefore, rats 'shuttled' from one side of the box to the other in order to successfully avoid receiving an electric shock. This concept has been widely employed and shuttle avoidance (using different noxious stimuli ranging from electric shocks to inflating balloons) has been demonstrated in a variety of species such as dogs (Solomon and Wynne, 1953), domestic pigs (Karas et al., 1962), pigeons (Hineline and Rachlin, 1969), guinea pigs (Rabedeau, 1970), and chickens (Duncan and Hughes, 1988; Rutter and Duncan, 1991). Interestingly, behavioural results are remarkably similar across different species.

Although the term 'fear' is used in everyday vernacular to describe the negative affect that most animals are assumed to feel during, or in anticipation of, some frightening stimulus, this term is more cautiously used today when referring to fish. This is partly due to the disbelief, by some, that fish have the capability to experience conscious feelings. Rose (2002) believes that conscious experiences like fear and pain are neurological impossibilities, due to the lack of a neocortex in fish—the presumed place where consciousness dwells in higher vertebrates. He therefore proposed that behavioural responses to noxious stimuli are separate from psychological experiences (of fear for example)—behavioural responses to frightening or aversive stimuli are merely reflexive responses and are not accompanied by a negative feeling. Nonetheless, the term 'fear' has been widely used to describe fish behaviour for some time (Pinckney, 1967; Gallon, 1972; Huntingford, 1990; Ledoux, 1990; Noakes and Baylis, 1990). Others have put forth the idea that fish derive conscious experiences through some mechanism other than the neocortically based consciousness of humans and other highly evolved mammals (Verheijen and Flight, 1997). Recent anatomical, physiological, neuropharmacological and behavioural data suggest that fish are likely to feel subjective experiences, like fear, in much the same manner as tetrapods. A full review of this evidence is beyond the scope of this paper, but briefly, the major argument lies in the fact that the neuroanatomical structure and function between fish and higher vertebrates are more similar than previously thought (Rakic and Kornack, 2001; Chandroo et al., 2004).

Oidtmann and Hoffman (2001) strongly believe that fish are indeed able to suffer (from pain and fear) because they satisfy the important criteria of anatomical and physiological similarities between mammals and fish in addition to showing similar avoidance behaviour to noxious stimuli. The idea of fish being able to derive conscious subjective feelings that lead to suffering is not a new idea, however, the paucity of information regarding fish suffering makes it a hotly debated topic.

Due to societal concern about farm animal welfare, researchers have already begun to investigate practices that may cause undue suffering to intensively cultured fish (Schreck et al., 1995; Ross and Watten, 1998; Skjervold et al., 2001; Robb et al., 2002). However, this presumes that fish do 'suffer' in the accepted context, which is still in question. Since animal welfare is to do with how animals 'feel' (Duncan, 1993, 1996) the question of fish being able to suffer psychologically needs to be addressed.

The objective of this study, therefore, is to evaluate avoidance and fear responses of domestic rainbow trout by use of an avoidance paradigm. Studies have shown that when an animal perceives a frightening stimulus, it can learn to avoid this stimulus, and the animal's degree of responsiveness can be measured to give insight as to how frightening or aversive it finds it. For example, Rushen (1986) ran sheep through a raceway at the end of which he administered either electro-immobilisation or physical restraint. Results showed that sheep found both of these procedures aversive because 'transit time' (time taken to run through the raceway) and 'push up' time (time experimenter spent moving toward or pushing sheep) both significantly increased; in addition, sheep also showed that electro-immobilisation was perceived to be more aversive than physical restraint, again through a longer 'transit' and 'push up' time of the former compared to the latter. Although we cannot know for sure that an animal's feelings are similar to human feelings, this is not essential when assessing welfare. What we need to know is whether the animal is in a state of negative or positive affect and how negative or positive the associated feelings are. We can do this by making use of the cognitive process of learning (Duncan and Petherick, 1991). Aversion learning will be used in the present study to better elucidate the nature of responses demonstrated by fish to a sudden stimulus—that is whether fish show evidence of true fear (a negative psychological experience) or merely reflexive responses.

Fear is an important area of research in animal agriculture, since this stressful state can seriously harm an animal's welfare, performance and profitability; it is thus important to alleviate fear from the viewpoint of both the public and producer (Jones, 1997).

2. Materials and methods

2.1. Animals and housing

Eighteen mixed-sex, domesticated Ontario rainbow trout (*Oncorhynchus mykiss*) were used in this study. They were hatched and reared at the University of Guelph's Alma Aquaculture Research Station (AARS). Six fish were kept in a group in 1125 l tanks with continuous flowing water (flow rate of 18 l/min) at 8.5 °C. The animals were 22 months of age (641 ± 37 g, mean weight ± S.E.) at the beginning of the experiment and were kept under a 12L:12D photoperiod. They were fed, daily, standard commercial trout pellets at

a rate of 0.9% body weight as recommended by the standard operating procedures at the AARS.

2.2. Experimental tank

The experimental tank was modelled after a modified shuttle box and was kept in the same room as the holding tanks. The testing tank was 80 cm wide × 190 cm long × 37 cm high and was divided into two equal chambers by a heavy opaque partition containing a doorway 18 cm wide × 21 cm high. The water level was filled to a depth of 27 cm. A 150 W blue light bulb was placed at each end of the tank just above the water surface. The perimeter of the test tank was surrounded by a black plastic sheet that was hung from the ceiling in order to prevent fish from seeing the observer and the surrounding room. A mirror was fixed at a 45° angle and hung from the ceiling above the test tank to allow the observer to watch the fish from outside the sheeting. On the outside of the tank, two dip nets were mounted on the side walls, one at each end, in front of the light fixtures. These nets could be plunged into the water of the tank by passing through a slit in the black plastic and could be withdrawn by the use of an attached string (Fig. 1). It was thought that the net plunging into the water would have the properties of a frightening stimulus, that is, it would be sudden and intense. Sudden and intense stimuli generally elicit fear in mammals and birds (Gray, 1971).
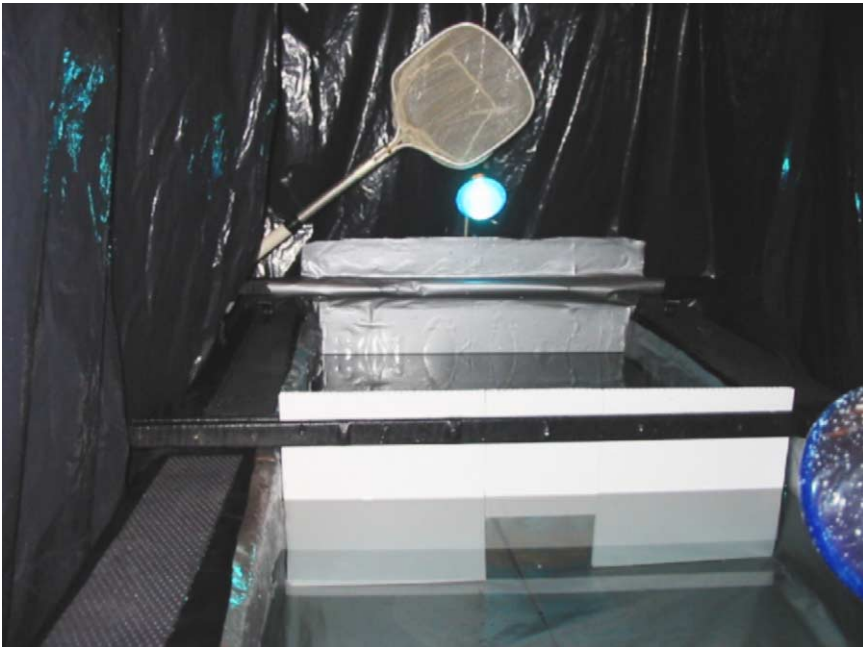

Fig. 1. View of the inside of the shuttle tank. A partition separated the tank into two identical chambers; the door was fully submerged under water. Two blue lights rested on each end of the tank. Automated dip nets also rested on each end of the tank directly in front of the lights. Automated dip nets (originating from the outside of the tank) were plunged into the water through slits in the tarp.

2.3. Procedure

Experimental procedures were approved by the University of Guelph Animal Care Committee, complying with the requirements of the Canadian Council on Animal Care.

Prior to the experiment, fish were anaesthetised in 70 mg/l MS-222 (tricaine methanesulfonate) then tagged for individual identification. Fish were allowed to recover, then left in their holding tanks undisturbed for 2 weeks before the experiment began.

Since there was time to test only six fish a day, six fish were trained and tested at a time and the whole procedure was repeated three times. All fish received a pre-exposure period in which they were allowed to explore and habituate to the test tank before the start of training (Phase 1). Random groups of three fish were placed into the test tank for 2 h a day for three consecutive days. Fish were placed into the tank as a small group during this phase of the experiment in order to encourage exploratory behaviour and facilitate adaptation to the tank. All other phases were carried out on individual fish, alone in the test tank.

Phase 2 consisted of training the fish to swim from one chamber of the tank through the door into the adjacent chamber upon being presented with the automated dip net. The net was plunged into the water on the side in which the fish rested. This was first achieved by using a manual dip net to gently guide the fish toward the door if it did not correctly swim directly through the door by itself when the net was plunged into the water. Within 2 days, most fish swam directly for the door (instead of fleeing into the corners of the tank or frantically swimming around haphazardly, for example) when the automated net was plunged into the water. Each fish received 10 training trials with an inter-trial interval of 2 min on each of five consecutive days.

Phase 3 consisted of establishing net avoidance behaviour. The fish received 20 trials in which they were presented with the automated net that plunged into the water. Fish were expected to swim through the door within 10 s to score a correct response. Five fish did not reach an 80% criterion (at least 16 successful avoidance responses out of the possible 20 trials); they were considered to have failed to demonstrate net avoidance behaviour reliably and were eliminated from the study. The remaining 13 fish continued on to Phase 4.

In Phase 4, each fish was placed into the test tank individually in a random order. The light at the end of the tank in which the fish happened to occupy was illuminated for 10 s and then the net was plunged into the water at that end. Each fish received 10 trials of light-net pairings each day for five consecutive days. Throughout this period, the behaviour of each fish was scored according to whether or not it swam through the doorway when the light was illuminated. Also recorded, was the latency; latency was defined as the time between the onset of the stimulus and the moment the fish completely passed through the doorway.

Phase 5 involved testing short-term memory and took place 1 day after completion of Phase 4. Each fish was placed into the test tank and the light at the end of the tank in which the fish happened to be was illuminated for 10 s. In this phase, the net was not plunged into the water. Each fish received 20 trials of light-only stimulation. The behaviour of each fish was scored according to whether or not it swam though the doorway when the light was illuminated. Latency was also recorded.

Phase 6 involved testing longer-term memory and consisted of repeating Phase 5 after an interval of 7 days.
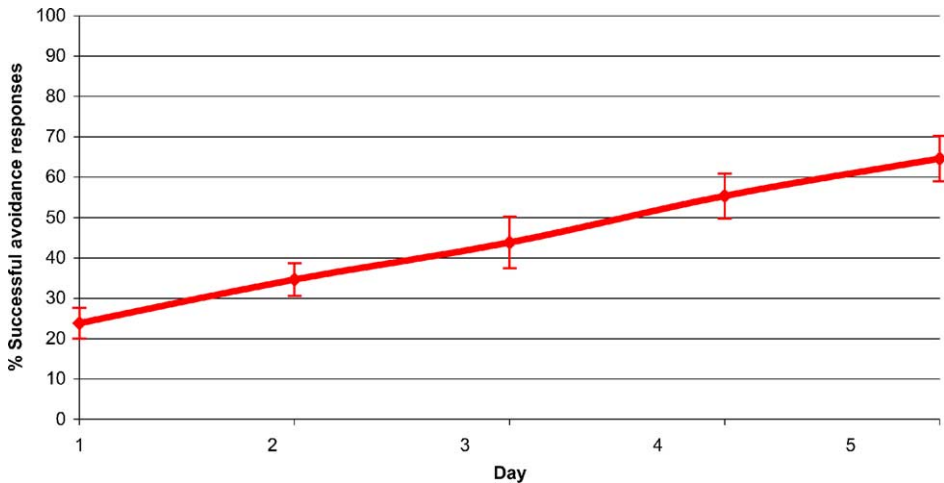
Fig. 2. Mean proportion (±S.E.) of successful responses over time during Phase 4. Fish ($n = 13$) showed a significant trend in learning the task of associating the illumination of a light (conditioned stimulus) with an aversive plunge of a dip net (unconditioned stimulus); the slope of the line is 1.023. Fish learned to anticipate the presentation of the net and thus swam through the door into the adjacent room of the shuttle tank within 10 s of the onset of the light in order to avoid receiving the noxious unconditioned stimulus.

2.4. Statistical analyses

Our binomial data (number of successful responses for 10 trials) could not be analysed with the GLM procedure in SAS. Therefore, the data were transformed from probability values (between 0 and 1) to values covering the normal distribution. Hence, using SAS statistical software, analyses were performed on logit transformed data. The number of successful avoidance responses due to the light-net association in Phase 4 were examined using a repeated measures analysis. The possible effect of sex was also examined using the same analysis. Furthermore, linear and quadratic contrasts were used to investigate the rate of learning over the 5 days comprising Phase 4. A t-test was used to reveal whether memory of the light-net association deteriorated between Phase 5 and Phase 6; correlations between successful avoidance responses to the light-only on Phase 5 and Phase 6 were computed using the Pearson's correlation coefficient. In addition, successful avoidance responses were regressed over the 20-trial, non-reinforced test of memory for all fish over Phase 5 and Phase 6, to investigate whether the number of responses declined over the course of the trials. Finally, a t-test and Pearson's correlation test were performed on the difference in time between net-stimulus onset and swimming through the door and light-stimulus onset and swimming through the door.

3. Results

The mean proportion of successful avoidances during Phase 4 are shown in Fig. 2. The trout showed an average response rate of 25% on the first day of Phase 4 and this had risen

to about 65% on the last day of this phase. A repeated measure ANOVA showed that this increase in successful responses was statistically significant ($F = 29.12$; d.f. $= 4, 36$; $P < 0.0001$). The linear component of the polynomial contrast analysis confirmed that the avoidance response varied linearly with increasing number of days over the course of Phase 4 ($F = 50.17$; d.f. $= 1, 9$; $P < 0.0001$). Therefore, the slope of the learning line rises in a significantly positive manner (Fig. 2). A quadratic contrast showed that there was no indication that the slope of the line was beginning to level off within the 5-day range in Phase 4 ($F = 0.01$; d.f. $= 1, 9$; $P > 0.05$). This indicates that the fish were continuing to learn throughout this period and their performance would probably have improved even further had the trials continued. Also, the sex of the fish had no significant effect ($F = 0.06$; d.f. $= 1, 9$; $P > 0.05$) on the acquisition of the light-net association.

Fish averaged 8 successful avoidance responses out of the possible 20 trials in Phase 5, and 7 successful avoidance responses out of 20 trials in Phase 6. However, the fish's memory of the light-net association did not deteriorate between Phase 5 and Phase 6 ($t = -1.72$; $P > 0.05$) as the number of avoidance responses remained at a constant level. Also, the correlation analysis ($r = 0.64$; $n = 13$; $P < 0.05$) showed that fish that consistently fled through the door at the onset of the light (even without negative reinforcement) also demonstrated a high response rate when tested 7 days later during Phase 6, demonstrating strong individual behavioural consistency. However, fish were showing a decline in the number of avoidance responses near the end of the 20 non-reinforced consecutive trials in both Phase 5 and Phase 6 ($r^2 = 0.12$; $n = 520$; $P < 0.001$; slope of regression line is -0.03).

Lastly, the mean latency to swim through the door when presented with the plunging net (in Phase 3) was 3.2 ± 0.1 s, whereas the mean time it took to swim through the door upon the onset of the light (in Phase 4) was 6.6 ± 0.2 s. This difference in response time was highly significant ($t = -18.49$; $P < 0.0001$). Pearson's correlation procedure was also significant ($r = 0.57$; $n = 13$; $P < 0.05$), showing that fish that reacted slower to the net, tended also to react slower to the light.

4. Discussion

In order to confirm that trout are capable of feeling fear, it must be shown that the behavioural responses to the noxious stimulus are not merely reflexive. Although it is impossible to measure emotions directly, we can measure them indirectly, through the responses that the animal shows. Because emotions can provide the motivation to perform certain behaviours, it is important to distinguish between non-reflexive, voluntary behavioural responses and unconscious reflexive motor patterns (e.g. knee jerk reaction). It is important that the fish not only immediately moves away from the aversive stimulus, but also learns to avoid this stimulus in the future. The cognitive ability of recognising that the performance of certain behaviours will lead to some desired effect (i.e. avoidance or prevention of an aversive situation) is evidence that the displayed behaviour may not be an automatic unconscious manifestation. Although some behavioural responses are "innate", associative learning can modify the behaviour, resulting in flexible usage. For example, Cantalupo et al. (1995) presented a 'predator' to mosquitofish, only when they swam across a certain

boundary of its test tank. The investigators found that not only did the fish perform an escape response by rapidly swimming away from the 'predator', but with repeated exposure, the fish began to avoid that particular part of the tank where the stimulus appeared and interestingly, began to perform an erratic zig-zagging behaviour when they happened to be within this 'danger zone'. It is probable that fear motivated this learned association and therefore the fish were able to develop a strategy to deal with the 'frightening' stimulus. A purely reflexive system would have simply led to the repetition of the same avoidance response (burst swimming away from the predator) over and over again, without the possibility of behaviour modification (for the development of a 'strategy' or escape tactic, for example).

It is difficult to discuss animal welfare without addressing the issue of cognitive ability. If fish show characteristics of having both 'basic' and more advanced, 'flexible' cognitive abilities then it supports the notion that these animals have more complex life-styles (i.e. foraging strategies, anti-predator behaviour, social learning, etc.) than traditionally thought—making them more comparable to other 'higher' vertebrate models. In the extensive literature that deals with animal learning, it has been shown that fish compare well to other species traditionally used in experimental psychology such as rats, pigeons and dogs.

In this study, fish learned that the illumination of a light signalled that a net was going to be plunged into the water. They revealed that they had learned this association by shuttling from one side of the tank to the other depending on where the light went on. Other studies have used a stricter criterion of learning than was used in the present study. For example, Piront and Schmidt (1988), investigating avoidance of shock in goldfish, used a criterion of 80%, i.e. the fish avoided the shock 8 times out of 10. In an experiment investigating memory in zebrafish, Williams et al. (2002), used a criterion of 75% and Peretti and Nowak (1974) working with maze learning in goldfish used a criterion of 90%. In the present study, the trout avoided successfully on 25% of occasions on Day 1 in Phase 4, but this rose in a straight line to 65% on Day 5. There was no sign of this trend diminishing and we maintain that it would have reached similar levels to those in previous studies had we continued with more trials.

A feature of this experiment that requires explanation is the fact that 5 of the 18 fish failed to demonstrate the operant shuttling response to the net stimulus. However, many previous investigators employing fear and escape paradigms have also reported similar results—subjects showed poor learning or failure to learn the desired response (McAllister and McAllister, 1971). An explanation may be that many species suppress active behaviour and adopt an immobile or freezing response when they perceive a threat or a frightening stimulus (Huntingford, 1990; Korte, 2001), and the freezing response may be incompatible with the performance of other instrumental responses (Pinckney, 1967; McAllister and McAllister, 1971). In the present study, the fish that failed to demonstrate a reliable avoidance response to the illumination of the light were frequently observed to sink to the bottom of the tank and remain motionless and were behaviourally unresponsive to the light. The fish that failed to avoid may have found the stimulus to be very frightening—so frightening that the appropriate avoidance response was inhibited—these fish were therefore eliminated from the study.

The trout in this experiment demonstrated the ability for both short-term and long-term memory. In a 20-trial test of memory, the illumination of the light was never reinforced with the plunging of the net. They scored an average of 8 successful avoidance responses

in Phase 5, and 7 in Phase 6, out of a possible 20. Interestingly, most of the responses usually happened in the first half of the test; fish showed a decline in responses towards the end of the trials. This is almost certainly evidence of habituation rather then a loss of memory part way through each day's testing. Habituation refers to "the relatively persistent waning of a response as a result of repeated stimulation which is not followed by any kind of reinforcement" (Hinde, 1970) and fear, as a classically conditioned response, would be expected to habituate in the same manner as other responses (McAllister and McAllister, 1971).

The present finding, that trout have the ability to remember an avoidance response in the short-term and longer-term, is in accordance with results obtained from other species of fish. Gobies have been found to remember the location of tide pools for up to 40 days (Aronson, 1971), Atlantic salmon fry have retained colour conditioning for up to 21 h (Clarke and Sutterlin, 1985), conditioned avoidance responses have been retained up to 47 days by goldfish (Manteifel and Karelina, 1996) and zebrafish have shown memory of an alternation task for a period of at least 10 days (Williams et al., 2002). Trout in the present experiment demonstrated memory retention of at least 7 days. The variation in duration of memory retention in other studies may reflect differences among species and in underlying motivation. Miklósi et al. (1992) suggested that the duration of memory traces depends on the relationship between the fish and whatever stimulus it encounters. They found that with paradise fish, the memory of an encounter with another paradise fish was forgotten within 7 days, whereas an encounter with a fish of another species was remembered for 3 months. They explained that there are few opportunities for paradise fish under natural circumstances to learn about engagements with a goldfish, for example, which could be a predator. It would therefore be adaptive to retain this information for a long time. However, within an ever-changing group of conspecifics there are dynamic connections between group members; it is therefore more adaptive to remember conspecifics for a short time only (Miklósi et al., 1992). Following this hypothesis, the trout in the current study, having been exposed to the potential danger of a plunging net, may have retained a memory of it for an even longer time period than was tested.

An interesting finding from the present study is the difference in avoidance responding to the net and the light. The latency of responding to the light was approximately double that to the net. This is evidence that the fish were demonstrating a more conscious voluntary response to the light rather than the reflexive, stereotypic response that they showed to the net. Rapid, explosive burst swimming commonly seen in fish in response to an abrupt and unexpected stimulus is known as the 'fast-start' response. A fast-start consists of an initial preparatory stroke followed by a propulsive stroke ending with a variable stage, involving continuous swimming (Domenici and Blake, 1997; Hale, 1999). However, without high speed cinematography, this sequence appears to be one quick fluid motion to the naked eye. Fast-start locomotor behaviour is especially important for escaping from predators and capturing prey (Webb, 1986). It seemed that the trout in this experiment were responding reflexively with fast-starts when they quickly swam into the adjacent chamber when presented with the net stimulus. The fact that the response latency to the light stimulus was much longer, makes it much less likely that reflexive fast-starts were involved. The increase in response latency suggests that the fish were able to have some mental representation of events not present in their immediate environment and then make a decision on how to act

on it—a mental process that takes longer than a reflexive response. Duncan and Hughes (1988) carried out a similar type of shuttle avoidance learning task with domestic hens. The frightening stimulus was a rapidly inflating balloon and the warning signal was a light that was illuminated for 20 s before the balloon was inflated. The hens not only learned to avoid the inflating balloon but their fear level decreased as they learned the task as evidenced by them alarm calling for a shorter and shorter duration. Other studies with a variety of species have also shown that during the acquisition of avoidance, the intensity of fear decreases as the animal masters the correct response (Solomon and Wynne, 1953; Kamin et al., 1963; Starr and Mineka, 1977). This is probably due to the animal having some sense of control over the situation.

The results of this experiment suggest that when trout are subjected to a threatening stimulus, they react in a reflexive way with fast-start and rapid swimming away from the stimulus. The evidence also suggests that the fish feel frightened at the time. These feelings of fear are averse and, if given the opportunity, trout will learn to avoid the threatening stimulus and so be less frightened. Having learned the shuttle avoidance task, trout can remember it for up to 7 days.

These results show that fish can learn, have memory and make conscious decisions to avoid being exposed to a sudden and intense stimulus when given a cue that such a stimulus is going to occur. In other words, the results suggest that fish can experience fear. Fear is a state of suffering that often reduces the welfare of animals being managed by human beings (Duncan, 1996). In addition, fear is a powerful stressor and has been shown to reduce production performance in some farm animals (Hughes et al., 1986; Grandin, 1991). Therefore, the capacity of fish to experience fear will have important economic, legal and ethical implications to the fish farming industry. Taken together with the recent evidence that fish can suffer in other ways, e.g. by experiencing pain (Sneddon, 2003; Sneddon et al., 2003), the results suggest that fish may be worthy of more moral consideration than they have had in the past.

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