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Effect of noxious stimulation upon antipredator responses and dominance status in rainbow trout

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A potentially painful experience may modify normal behavioural responses. To gauge the importance of pain relative to predation or social status, we presented competing stimuli, a predator cue or an unfamiliar social group, to two groups of noxiously treated rainbow trout, *Oncorhynchus mykiss*. In the predator cue experiment, fish were classified as bold or shy. Noxiously stimulated fish did not show antipredator responses, suggesting that pain is the imperative. In the social status experiment, noxiously stimulated fish held individually and undisturbed showed an increase in respiration rate and plasma cortisol. As a comparison, we used the dominant or subordinate fish in a group as the noxiously stimulated fish. After the noxious treatment, we returned this test fish to a familiar or unfamiliar social group. Neither dominants nor subordinates showed a negative change in physiology compared to their controls. However, in a familiar group the dominant was much less aggressive, suggesting a behavioural impairment in response to noxious stimulation. In an unfamiliar group, no reduction of aggression was seen, suggesting that maintaining dominance status took priority over showing signs of pain. These findings may reflect an ability to prioritize motivational drivers in fish, and as such provides evidence for central processing of pain rather than merely showing a nociceptive reflex.

In animal models of pain, exposure to a new circumstance or a potentially fear-inducing or stressful situation reduces pain reactivity (e.g. Lester & Fanselow 1985; Harris & Westbrook 1994; Kavaliers & Colwell 1994; Gentle & Corr 1995; Gentle & Tilston 1999; Del Seppia et al. 2003; Nakama-Kitamura & Doe 2003; Smith et al. 2003). For example, chickens, *Gallus gallus domesticus*, with gouty arthritis exposed to a novel environment showed a reduction in their pain-related responses including the severity of inflammation in the affected joint (Gentle & Corr 1995; Gentle & Tilston 1999). Rodents exposed to predator odour also show reduced behavioural responses to pain, and anxiety tests have the same effect (Lester & Fanselow 1985; Kavaliers & Colwell 1991; Nakama-Kitamura & Doe 2003; Geerse et al. 2006). In humans, pain takes priority when it is chronic or particularly intense, and concurrent tasks are poorly performed (Kuhajda et al. 2002). Therefore, if pain is a high priority, it will affect behavioural responses to other stimuli (Eccleston 1995; Moseley & Arntz 2007).

These studies suggest that measuring the responses to competing stimuli during a painful event could be exploited as a tool to determine the relative importance of pain or nociception to an animal. Direct assessment of pain in animals is impossible owing to its subjective nature. However, many studies have identified behavioural and physiological responses to a potentially painful event in a variety of animals including fish (Sneddon et al. 2003a), amphibians (Willenbring & Stevens 1995), birds (Machin 2005) and mammals (Flecknell & Roughan 2004). Pain in animals is a contentious issue, especially for the cold-blooded vertebrates because of differences in their neurobiology from that of higher vertebrates (Chandroo et al. 2004). However, nociceptors have been identified in fish and these are similar to those found in mammals (Sneddon 2002, 2003a; Sneddon et al. 2003a; Ashley et al. 2006, 2007). Moreover, studies have shown that the brain of the fish is active during noxious stimulation and that this activity differs from the response to neutral stimuli (Dunlop & Laming 2005; Reilly et al. 2008a). Negative changes in behaviour and physiology have also been recorded (Sneddon et al. 2003a, b), suggesting an aversive affective state, and these are reduced by administering an analgesic (Sneddon 2003b). However, the real significance of this experience to the fish has not been explored. We tested this by providing noxiously stimulated rainbow trout, *Oncorhynchus mykiss*, with one of two different contexts to examine whether pain is the imperative. One context was the presentation of an antipredator cue to

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gauge responses to this threatening stimulus and the other was social novelty where fish were placed into an unfamiliar social situation.

Previous work demonstrated that noxiously stimulated fish do not show the classic neophobia when a fear-inducing stimulus, a novel object, is introduced (Sneddon et al. 2003b), indicating that pain dominates their attention in this context. However, it is possible that the novel objects used in that study were not sufficiently threatening to divert the fish's attention. In this study, we used more ecologically relevant and important distractors such as predation and social groupings to test the relative importance of noxious stimulation in these scenarios.

Avoiding being eaten by predators is a strong motivational drive for all prey animals and is, therefore, likely to be a strong stimulus to use as a competing stimulus. Fish are very sensitive to the presence of predators and predator-naïve fish show antipredator behaviours if they are given the odour of a predator in combination with the odour obtained from a damaged prey fish (Mirza & Chivers 2003; Zhao et al. 2006). This latter odour is called alarm substance or pheromone and is produced by damaged fish skin to elicit antipredator responses in conspecifics (Brown 2003; Scott et al. 2003). Antipredator behaviour can consist of freezing where the fish remains motionless, erratic escape swimming, increased refuge or cover use, sinking and spending more time in the bottom of the tank and a reduction in feeding attempts (Scheurer et al. 2007). Because these behaviours assist in avoiding detection, we used an antipredator cue to determine whether noxiously stimulated fish perform appropriate antipredator behaviour.

Rainbow trout are territorial and naturally form dominance hierarchies, usually including a bold individual that restricts the behaviour of subordinates (Sneddon et al. 2005). Individuals of lower status have higher stress levels as a result of losing contests with the dominant and have reduced access to food (Gilmour et al. 2005). Therefore, dominance status influences the probability of survival and overall fitness. As such, maintaining status is likely to be an important motivational driver in the behaviour of this species. We tested the behavioural and physiological responses of the top-ranked fish (dominant) and the lowest-ranked fish (subordinate) in a group to determine whether they show signs of pain or nociception when held in familiar groups where their dominance is already established. This was compared to returning the noxiously stimulated fish to unfamiliar groups where their dominance had not been established.

Behavioural analysis of large groups can be confounded by individual variation linked to boldness and this can affect responses to noxious stimulation (L. U. Sneddon, K. L. Edwards, S. Ringrose, L. J. Ashley & C. R. McCrohan, unpublished data). Rainbow trout can be either bold and aggressive or shy and timid, as reflected in their behaviour (Sneddon 2003c; Frost et al. 2007). Bold fish tend to take more risks, are more active, spend more time in open water, learn conditioning tasks faster and dominate shy fish (Sneddon 2003c; Frost et al. 2007). Therefore, we first determined the degree of boldness of our experimental subjects to investigate whether the response to noxious stimulation was affected by the 'personality' of the fish. We hypothesized that bold fish may be more likely to recover from a noxious event and respond to concurrent stimuli, such as exploring a new environment.

We also hypothesized that, if a potentially painful stimulus is important to the fish, noxiously stimulated fish will not respond in an appropriate manner to a predator cue. This may also be affected by the degree of boldness of the fish whereby bold fish may show more risk-prone behaviours during the presentation of the predator cue than shy fish. Finally, we tested the hypothesis that noxiously stimulated fish held in social groups may appear less affected by pain than individually held fish as they need to maintain their social status.

METHODS

Experiment 1: Predator Cue

Husbandry and set-up

Twenty-four juvenile rainbow trout (mean weight $\pm$ SE = 45 ± 2 g) were caught at random from a stock tank (2×2 m and 0.5 m high; $N = 100$ of original stock), transferred to individual experimental tanks (45×30 cm and 40 cm high) and left for 7 days to acclimate in a 12:12 h light:dark regime. Each tank had a constant flow of filtered freshwater at a temperature of 11 ± 1 °C, a gravel substrate and a refuge pipe (opaque plastic, 8 cm long $\times$ 8 cm in diameter). Half of the top of the tank had an opaque plastic cover (15 cm $\times$ 30 cm) to provide an area of shelter; the other half was left open. The cover was positioned on top of the tank (5 cm from the water surface as an overhang, so the fish could swim freely at the surface). The sides and backs of the tanks were covered in black plastic to isolate the fish visually and socially and to prevent any disturbance. A large screen with small openings for observation was placed in front of the tanks. The water was continuously aerated and fish were fed daily with their normal diet, commercial pellets (Skretting, Northwich, U.K.), at the recommended rate of 1% body weight per day. Prior to experiments the flow-through system was turned off so that water in each tank was contained. At the end of each experiment the tanks were thoroughly cleaned.

Experimental protocol

Fish were randomly assigned to one of four groups: control shy, control bold, acid treatment (noxious stimulation) shy and acid treatment bold. After acclimation to their new environment, the fish were tested for boldness using the novel object test (Wilson et al. 1993; Frost et al. 2007). Low-light-level cameras were set-up at the front and at the side of the tank to give 3D positioning, and rulers were positioned horizontally and vertically along the front and side of the tank to measure the distance from the object. We used a video recorder and monitor outside the experimental room including a video inset system which places one picture within another to allow both images to be viewed simultaneously. The behaviour was scored using custom-written behavioural analysis software on a PC. After the cameras were set-up, we left the fish overnight to recover from the disturbance. The next morning they were recorded for 10 min to obtain a baseline level of 'normal' behaviour, and then a novel object (constructed from a variety of white, black, yellow and green Lego bricks; dimensions $10 \times 15 \times 5$ cm) was added and left for a further 10 min. If a fish approached the novel object within 5 cm in the first 2 min it was deemed bold; if a fish did not approach the novel object within 10 min it was deemed shy. The experiment was conducted the following day.

The following procedures were conducted by experienced personnel (L.S. and P.A.) licensed by the U.K. Home Office. For the control groups, fish were individually removed from their tank and placed in a 25-litre bucket with 10 litres of anaesthetic-dosed water (1 ml/litre of a 300 mg benzocaine in 9 ml of ethanol). Once deep anaesthesia was reached (no reflex response to tail press), the fish was carefully removed from the bucket, placed on a wet paper towel and injected (25 g needle and 1 ml syringe) with 0.1 ml of sterile saline into the upper and lower frontal lips. This procedure took less than 2 min. The fish was then returned to its tank and allowed a 30 min recovery period from the anaesthesia and handling. The acid-treated fish were treated as above but were injected in the frontal lips with 0.1 ml of acetic acid (0.1% in sterile saline). Acetic acid has been shown to activate nociceptors (Hamamoto et al. 2000; Ashley et al. 2007).

Prior to injection, we observed the fish for 15 min to record normal behaviour for the entire period and respiration rate at