

# Do fishes have nociceptors: evidence for the evolution of a vertebrate sensory system

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Nociception is the detection of a noxious, tissue damaging stimulus and is sometimes accompanied by a reflex response such as withdrawal. Pain perception, as distinct from nociception, has been demonstrated in birds and mammals but has not been systematically studied in lower vertebrates. We assessed whether a fish possessed cutaneous nociceptors capable of detecting noxious stimuli and if its behaviour was sufficiently adversely affected by administration of a noxious stimulus. Electrophysiological recordings from trigeminal nerves identified polymodal nociceptors on the head of the trout with physiological properties similar to those described in higher vertebrates. These receptors responded to mechanical pressure, temperatures in the noxious range (more than 40 °C) and 1% acetic acid, a noxious substance. In higher vertebrates nociceptive nerves are either A-delta or C fibres with C fibres being the predominating fibre type. However, in the rainbow trout A-delta fibres were most common, and this offers insights into the evolution of nociceptive systems. Administration of noxious substances to the lips of the trout affected both physiology and behaviour of the animal and resulted in a significant increase in opercular beat rate and the time taken to resume feeding, as well as anomalous behaviours. This study provides significant evidence of nociception in teleost fishes and furthermore demonstrates that behaviour and physiology are affected over a prolonged period of time suggesting discomfort.

**Keywords:** nociception; pain; rainbow trout; trigeminal

## 1. INTRODUCTION

Nociception, the detection of tissue damaging stimuli, is evident in a number of different phyla including birds and mammals (Walters 1996), but studies on lower vertebrates have suggested a lack of nociceptors and pain perception (e.g. Atlantic stingray (*Dasyatis sabina*), Coggeshall *et al.* (1977) and Leonard (1985); or long-tailed stingray (*Himantura fai*), Snow *et al.* (1993)). From the perspective of the evolution of sensory function in vertebrates, the study of sensory systems in lower vertebrates is of great interest. Olfactory, gustatory and chemosensory systems have been well described in fishes (Belousova *et al.* 1983; Kotschal 2000), but relatively little attention has been paid to nociception. The trigeminal nerve, the fifth cranial nerve, innervates the majority of sensory information from the head of vertebrates and as such conveys somatosensory information from potentially damaging stimuli to the brain. A study on the most primitive living vertebrate, the lamprey (*Petromyzon marinus*), suggested that there were trigeminal receptors that responded to burning of the skin (Matthews & Wickelgren 1978). The physiological responses of these receptors, however, were not well characterized and the responses recorded may have been a result of damage to the receptor field rather than the preferential sensitivity to a noxious temperature *per se*. Furthermore, the lamprey lacks myelination, and its closest evolutionary group, the elasmobranchs, are deficient

in unmyelinated fibres and no nociceptors have been identified (Leonard 1985; Snow *et al.* 1993). A recent study on the rainbow trout (*Oncorhynchus mykiss*) demonstrated that, although most primary afferent somatosensory fibres were A-delta fibres, unmyelinated C fibres were present in the trigeminal nerve (Sneddon 2002). Free nerve endings of A-delta and C fibres act as nociceptors in higher vertebrates and have been well characterized (Lynn 1994) and thus there is the potential for these neurons to act as nociceptors in the rainbow trout.

A number of different classes of nociceptors have been described in mammals but they are commonly slowly adapting mechanoreceptors that preferentially respond to noxious heat (greater than 40 °C) and are termed mechanothermal nociceptors (Lynn 1994). If these nociceptors also respond to noxious chemicals such as bee venom, acid, bradykinins, acetyl choline, then they are classified as polymodal nociceptors (Lynn 1994). Using electrophysiological techniques, nociceptors have been identified in amphibia (Spray 1976), birds (Gentle 1992, 1997; Gentle & Tilston 2000), mammals (Yeomans & Proudfit 1996) including primates (Kenshalo *et al.* 1989) and humans (Torebjörk & Hallin 1974; Hallin *et al.* 1981). Therefore, if we can demonstrate that the rainbow trout possesses the neural apparatus to detect noxious stimuli, then this confirms that the trout is capable of nociception, the simple detection and reflex response to a noxious stimulus (Kavaliers 1988; Bateson 1991). To suggest pain perception, it must be shown that any behavioural or physiological responses are not merely reflexive.

Pain in humans has been defined as an 'unpleasant sensory and emotional experience associated with actual or

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potential tissue damage' (IASP 1979●●5●●). It is impossible to truly know if an animal has an emotion because we cannot measure emotion directly. Therefore, emotion does not feature in the definition of pain in animals (Zimmerman 1986; Bateson 1991●●4●●). What an animal 'feels' is possibly nothing like the experience of humans with a more complex brain structure, however, the animal's experience may be unpleasant or cause suffering and their discomfort is no less important in terms of biology or ethics. To examine possible pain perception in an animal, indirect measurements of behavioural and physiological responses to a potentially painful event are made and then we decide upon the evidence collected from the data as is routinely done in welfare studies (Bateson 1991●●4●●; Broom 1991; Gentle 1992; Gonyou 1994; Bradshaw & Bateson 2000; Mason *et al.* 2001; Roughan & Flecknell 2001; Molony *et al.* 2002). If a noxious event has sufficiently adverse effects on behaviour and physiology in an animal and this experience is painful in humans, then it is likely to be painful in the animal.

To demonstrate that an animal is capable of pain perception, it must first perceive the adverse sensory stimulus and then react both physiologically (e.g. inflammation, cardiovascular changes) and behaviourally (move away from stimulus). However, to show that this is not simply a nociceptive reflex, it is necessary to show that the animal learns that the stimulus is associated with an unpleasant experience and avoids it. Certainly it has been demonstrated that fishes can learn to avoid an adverse stimulus such as electric shock (Ehrensing *et al.* 1982) and hooking during angling (Beukema 1970*a,b*). Additionally, suffering or discomfort is implicated if the animal's behaviour is adversely affected (Zimmerman 1986). These criteria have been demonstrated in mammals (Roughan & Flecknell 2001), birds (Gentle 1992) and amphibians (Stevens 1992) but not in teleost fishes.

The purpose of the present study was to determine if nociceptors were present in the trigeminal system on the head of the trout and to determine the physiological and behavioural consequences of prolonged noxious stimulation. Recordings were made from the trigeminal nerve to identify if nociceptors were present on the face and head of the trout. Behavioural and physiological responses of the fish to administration of acutely algogenic substances to the lips of the trout were assessed to examine if there was the potential for pain perception in this species. The criteria that must be met for animal pain are first, the demonstration of the sensory capability of detecting potentially painful stimuli, and second, the performance of adverse behavioural responses to a potentially painful event that are not simple reflexes.

## 2. METHODS

### (a) *Electrophysiological recordings from the trigeminal ganglion*

Rainbow trout ( $750 \pm 100$  g,  $n = 10$ ) were supplied by a commercial fish supplier. The fishes were maintained as described in a previous study (Sneddon 2002). Trout were caught individually by netting and initially anaesthetized by immersion in MS 222 ( $50 \text{ mg l}^{-1}$ ) to facilitate weighing and intraperitoneal

injection of Saffan ( $0.3 \text{ ml } 100 \text{ g}^{-1}$ ; Schering-Plough Animal Health, Welwyn Garden City, UK). Once deep anaesthesia was achieved, the fish was placed into a stainless steel cradle cushioned with wet paper towels and held in position with Velcro straps. The fishes had reached surgical, deep plane anaesthesia and were not conscious and had to be ventilated by flushing fresh water over the gills by means of a tube held in place by a specially constructed mouth piece. Skin and bone were removed above the brain and then the olfactory and optic lobes and cerebellum were removed via a suction tube connected to a vacuum pump. This procedure is known as decerebration and renders the animal insentient because it is only left with a brain stem. To prevent muscular twitching, Pavulon, a neuromuscular blocker (pancuronium bromide  $2 \text{ mg ml}^{-1}$ ), was injected intramuscularly ( $0.08 \text{ ml } 100 \text{ g}^{-1}$  fish weight). Bone was removed to expose the trigeminal ganglion and the ganglion was desheathed and covered in paraffin to prevent moisture loss. Glass insulated tungsten microelectrodes (tip diameter  $10 \mu\text{m}$ ) were used to record from afferent cell bodies. The extracellular action potentials were amplified using a NL100 head stage connected to a NL104 preamplifier (Neurolog System, Digitimer Ltd, UK). The signal was displayed on a storage oscilloscope (5113, Tektronix INC) and stored on a PC using a Micro 1401 interface and SPIKE 2 software (CED, UK).

Neural activity was recorded from single cells in the trigeminal ganglion following the application of stimuli to the head of the fish. A glass mechanical probe ( $0.1 \text{ mm}$  diameter) was lightly applied to the facial skin in order to locate a receptor field. Once located, the mechanical threshold of the receptor was determined by applying von Frey hairs ( $0.1\text{--}15.0 \text{ g}$  at  $0.1 \text{ g}$  intervals) to the receptor field. The diameter of the receptive field was measured to  $0.1 \text{ mm}$  using Vernier calipers. The receptor was then tested for thermal and chemical sensitivity. A thermal stimulator was placed  $1 \text{ mm}$  above the area of the receptor field so that it did not burn the skin and the stimulator raised the temperature to  $58 \text{ }^\circ\text{C}$ . Thermal sensitivity was determined by heating the skin at a rate of  $1 \text{ }^\circ\text{C s}^{-1}$  up to  $58 \text{ }^\circ\text{C}$  using a pre-focused quartz glass light bulb with built in reflector (A1231,  $12 \text{ V}$ ,  $100 \text{ W}$  Wotan) orientated vertical to the skin. If the receptor responded to the increase in temperature, the threshold was determined and the response had to be repeatable. Temperature was measured using a type K thermocouple placed in the centre of the bulb focus and was controlled by a feedback circuit. The skin temperature was held at  $58 \text{ }^\circ\text{C}$  for  $10 \text{ s}$  after which it rapidly returned to normal. The temperature increase of  $1 \text{ }^\circ\text{C s}^{-1}$  allowed the threshold to be determined. To ascertain chemosensitivity, a drop of  $1\%$  acetic acid was placed onto the receptor field. The first  $5 \text{ ms}$  after the addition of the drop was disregarded as this could be a response to the touch of the drop; a response to this noxious chemical stimulation was confirmed if the action potentials measured from mechanical and/or thermal stimulation of that receptor fired after this period. Again this response was repeatable. A drop of water was also placed onto the receptive field to act as a control stimulus. None of the receptors responded to this. Conduction velocities were obtained by placing silver wire stimulation electrodes onto the receptor field, and stimulating the receptor directly by an electrical pulse. This stimulated the fibre to produce an action potential and the conduction velocity was determined using the time that the action potential was recorded after the stimulus and the estimated distance travelled from the receptive field to the recording electrode in the trigeminal ganglion.

### (b) Behavioural responses to administration of algogenic substances

Twenty rainbow trout (30–100 g) were obtained from a commercial fish supplier, individually housed in rectangular tanks (45 cm × 25 cm × 35 cm) with a constant flow of water at  $11 \pm 1$  °C and a feeding ring (10 cm diameter) secured on the water surface at the same location in each tank. One half of the tank was covered by an opaque lid (22.5 cm × 25 cm) to provide an area of shelter, whereas the other half had a transparent lid and this was where the feeding ring was located. Each tank had a gravel substrate and was continuously aerated via an airstone and tubing connected to an air pump. Each fish was trained twice daily, am and pm, to come to the ring to receive food pellets (TROUW Aquaculture, UK) in response to a light cue above the tank (one test equals one trial; mean number of trials to learn,  $10 \pm 4$ ). Once the fishes had learned to feed at the ring by successfully performing six consecutive trials they received two weeks' further training to ensure that they were truly conditioned to the light stimulus (i.e. responded to light only before food presentation and they had to perform another 14 trials successfully to be included in the experiment). Fishes were then assigned to four treatment groups: (i) saline—0.1 ml sterile saline injected (25 g needle and 1 ml syringe) into frontal lips; (ii) venom—0.1 ml bee venom ( $1 \text{ mg ml}^{-1}$  sterile saline) injected into frontal lips; (iii) acid—0.1 ml acetic acid (0.1% in sterile saline) injected into frontal lips; and (iv) control—fish handled but received no injection.

Acetic acid and bee venom were chosen because the protons of the acid stimulate nociceptive nerves in mammals (Martinez *et al.* 1999) and frogs (Hamamoto *et al.* 2000), and the venom has an inflammatory effect in mammals (Lariviere & Melzack 1996) and both are known to be painful in humans. Before treatment the behaviour and opercular (gill) beat rate were measured continuously for 15 min. Behaviours recorded were their position in tank (under covered or exposed area) and swimming activity (direct movement of fishes more than one body length). Fishes were then individually anaesthetized using benzocaine ( $1.5 \text{ ml } (50 \text{ mg l}^{-1} \text{ ethanol}) \text{ l}^{-1}$ ) and were carefully injected with the appropriate substance into the upper and lower frontal lip or handled but not injected. The fishes were in medium to deep plane anaesthesia during this procedure and had lost all reflex activity and muscular control. Trout were placed back into their original tank and allowed 30 min to recover from the anaesthesia. Behaviour and opercular beat rate were recorded for 15 min and then the light was switched on and food subsequently introduced to the tank. If the fishes did not respond by swimming to the feeding ring to feed they were left for a further 30 min, then a further 15 min of observations were recorded and light cue and food given. This regime continued until the fishes resumed feeding. All fishes ingested food within *ca.* 4 h. The time to perform the feeding ring task and resume feeding for the four groups was compared using one-way ANOVA. The percentage of time spent in the covered area for each fish in all four groups was determined before and after the treatment and compared using Mann–Whitney *U*-tests. Frequency of swimming activity was calculated for each fish in the experimental groups and before and after the treatment, and also compared using Mann–Whitney *U*-tests.

In a second experiment, six rainbow trout were trained as described above, however, half of these were fed live red mosquito larvae instead of pellets to provide a softer foodstuff. All fishes were injected with bee venom and assessed for behaviour and opercular beat rate as already described. The time to resume

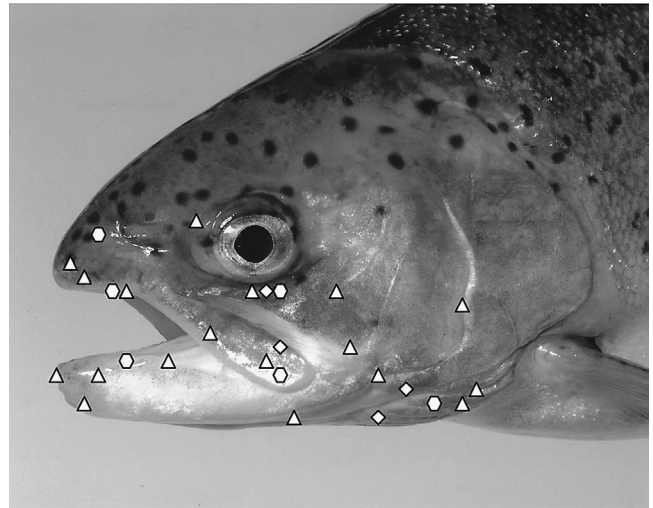


Figure 1. Position of polymodal mechanoreceptors or nociceptors, mechanothermal receptors and mechanochemical receptors on the head and face of the rainbow trout, *Oncorhynchus mykiss* (triangles, polymodal nociceptor; diamonds, mechanothermal nociceptor; hexagons, mechanochemical receptor).

feeding on the two different diets was compared using a Kruskal–Wallis test due to the low sample size, which was chosen for ethical reasons.

All the fishes used in both experiments were held for a further 3 days and trained in the conditioning task twice a day. All fishes continued to successfully perform the task and ingest food, therefore, there appeared to be no chronic effects on associative learning and appetite. At the end of the 3 days, the trout were individually killed by overdose in anaesthetic.

## 3. RESULTS

### (a) Characterization of nociceptors

Fifty-eight receptors were located on the face and head of the rainbow trout. Twenty-two of these receptors could be classified as nociceptors (figure 1) as they responded to mechanical pressure by a slowly adapting firing pattern and were also stimulated by noxious heat stimulation (more than 40 °C) and of these, 18 also responded to algogenic chemical stimulation (1% acetic acid; figure 2*a–c*). The response of the receptors to mechanical, noxious thermal and chemical stimulation clearly characterizes them as polymodal nociceptors (table 1). There were four receptors that did not respond to chemical stimulation and are classified as mechanothermal nociceptors. A third group of receptors ( $n = 6$ ) responded to only mechanical and chemical stimulation, but without a detailed investigation of their physiological characteristics they cannot be classified as nociceptors at present and are referred to as mechanochemical receptors. A further 16 receptors gave a slowly adapting response to mechanical stimulation and another 14 receptors gave a rapidly adapting response, but none of these responded to thermal or chemical stimulation and are possibly pressure and touch receptors, respectively (Sneddon 2003). The characteristics of the polymodal and mechanothermal nociceptors and the mechanochemical receptors are shown in table 1. Mechanical thresholds of the three types ranged between 0.1 and 7.1 g and conduction velocities were recorded

Table 1. Characteristics of the three types of receptor found on the head of the rainbow trout. Values shown are means  $\pm$  s.e.

	polymodal nociceptors ( <i>n</i> = 18)	mechanothermal nociceptors ( <i>n</i> = 4)	mechanochemical receptors ( <i>n</i> = 6)
diameter of receptor (mm)	3.20 $\pm$ 0.4	2.83 $\pm$ 1.0	2.52 $\pm$ 0.4
mechanical threshold (g)	0.83 $\pm$ 0.4	0.1 $\pm$ 0.0	0.78 $\pm$ 0.53
thermal threshold ( $^{\circ}$ C)	49.3 $\pm$ 1.4	46.2 $\pm$ 2.4	none
acid response	yes	none	yes
conduction velocity (m s $^{-1}$ )	3.96 $\pm$ 0.4	3.71 $\pm$ 0.5	4.28 $\pm$ 0.1

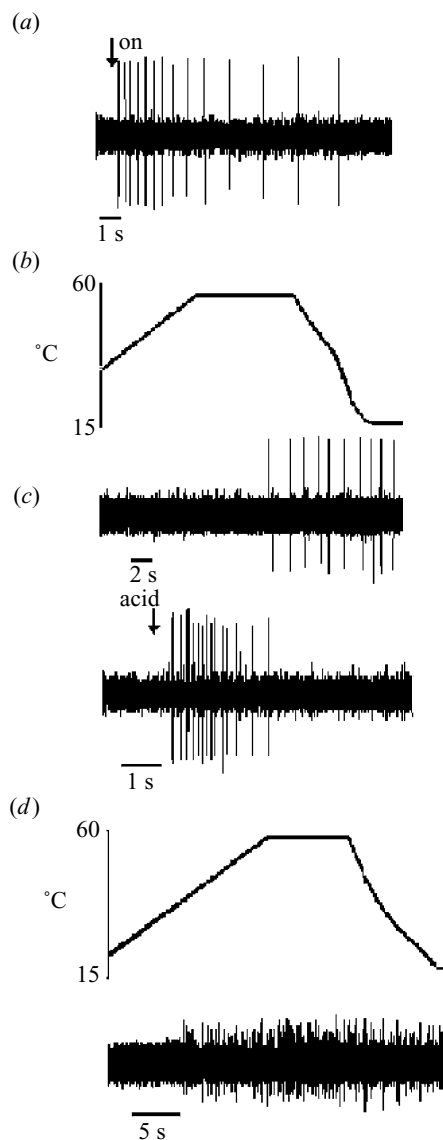


Figure 2. A polymodal nociceptor responding to (a) mechanical, (b) thermal and (c) chemical stimulation (1% acetic acid). The receptor is slowly adapting to mechanical stimulation (a) ('on' indicates application of stimulus), has a thermal threshold of 58  $^{\circ}$ C (b), and responds to application of a drop of acetic acid onto the receptive field (c). (d) A polymodal nociceptor with a thermal threshold of 42.3  $^{\circ}$ C.

between 0.97 and 8.5 m s $^{-1}$ . Out of all the polymodal nociceptors that were recorded from, only one was a unmyelinated C fibre and the rest were A-delta. Thermal responses were only seen above 40  $^{\circ}$ C and thresholds

ranged from 40  $^{\circ}$ C to 58  $^{\circ}$ C (figure 2*b,d*). The diameter of the receptor field ranged from 1.6 to 9  $\times$  1 mm. Interestingly, we found no thermal receptors that responded to temperature in the range of 20  $^{\circ}$ C to 40  $^{\circ}$ C.

#### (b) Behavioural and physiological responses to acute noxious stimulation

Significant increases in opercular beat rate were found in all four groups after the treatment (control and saline: *ca.* 52 beats min $^{-1}$  to 70 beats min $^{-1}$ ) although the venom and acid groups had greatly elevated rates after the treatment (*ca.* 52 beats min $^{-1}$  before to 93 beats min $^{-1}$  after treatment; figure 3*a*;  $F_{3,16} = 27.52$ ,  $p < 0.001$ ). This physiological effect was also coupled with profound effects on the fishes' behaviour. It took control and saline fishes *ca.* 80 min to begin ingesting food again whereas venom and acid fishes took *ca.* 170 min (figure 3*b*;  $F_{3,16} = 7.29$ ,  $p = 0.003$ ). In addition to this, we performed the second experiment that tested whether the fishes would resume feeding more quickly if fed on a softer foodstuff but there was no significant difference in the time to resume feeding ( $H = 0.05$ ,  $p = 0.827$ , d.f. = 1).

Activity levels were not affected by the treatment whether it was potentially painful ( $W = 130.5$ ,  $p = 0.057$ ) or not ( $W = 107.0$ ,  $p = 0.908$ ; median frequency before = 0.356 min $^{-1}$ ; after = 0.326 min $^{-1}$ ) although there was a trend for the venom and acid injected fishes to reduce the amount of swimming activity (median frequency before = 0.935 min $^{-1}$ ; median frequency after = 0.265 min $^{-1}$ ). Position in tank or use of the sheltered area was also not affected by the noxious injections ( $W = 103$ ,  $p = 0.910$ ; median percentage time spent under cover before = 53.3%; after = 55.8%) or the controls treatments ( $W = 106$ ;  $p = 0.970$ ; before = 53.9%; after = 63.0%). Observations following acid and venom injection found that the fishes performed anomalous behaviours after the treatment that were not seen in the control or saline groups; acid and venom fishes performed 'rocking' where the fishes moved from side to side balancing on either pectoral fin while resting on the gravel (mean frequency 0.37 min $^{-1}$  for venom group and 0.45 min $^{-1}$  for acid group). The acid group was also observed to rub their lips into the gravel and against the tank walls but the venom group did not perform this behaviour.

## 4. DISCUSSION

The polymodal nociceptors found here in the trout have similar properties to those found in amphibians (Stevens 1992), birds (Gentle 1992, 1997) and mammals

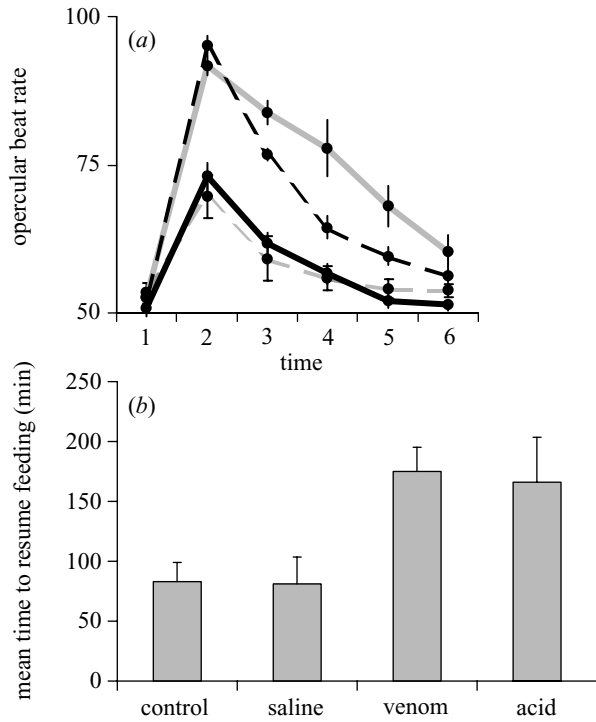


Figure 3. (a) Mean ( $\pm$  s.e.m.) opercular beat rate of each treatment group 20 min before treatment and at each observation afterwards (time 1 is 20 min before treatment; time 2 is 30 min after treatment and each time point after this is ca. 30 min apart). (Grey dashed line, control; black solid line, saline; grey solid line, venom; black dashed line, acid.) (b) The mean ( $\pm$  s.e.m.) time taken for each fish in each treatment group to resume ingesting food after the treatment.

(Handwerker *et al.* 1987) including humans (Lynn 1994). Nociceptors, by definition, preferentially respond to noxious, injurious stimuli and this demonstrates that the rainbow trout is capable of nociception (Kavaliers 1988; Bateson 1991●●4●●). Receptor diameter, thermal thresholds and mechanical responses are similar to those measured in higher vertebrate groups (Torebjörk & Hallin 1974; Spray 1976●●3●●; Hallin *et al.* 1981; Kenshalo *et al.* 1989; Yeomans & Proudfit 1996; Gentle & Tilston 2000). Mechanical thresholds were lower than those found in humans; at least 0.6 g is required for noxious stimulation in human skin (Lynn 1994) and many of the nociceptors on the fish skin were stimulated by 0.1 g. This may be due to the more easily damaged nature of the fish skin and as such the nociceptors have lower thresholds. Similar thresholds were found in mammalian eye nociceptors (Belmonte & Gallar 1996) and so the fish nociceptors have mechanical thresholds comparable with those in the cornea of the eye.

None of the trigeminal receptors in this study was stimulated by temperatures in the range of 30 °C to 40 °C. A number of studies have demonstrated a lack of thermal receptors in invertebrates and other lower vertebrates (Matthews & Wickelgren 1978; Leonard 1985; Walters 1996). This suggests that thermal receptors in the non-noxious range potentially evolved in vertebrate groups that lead a more terrestrial existence. These thermal receptors may have evolved in response to temperature fluctuations in the terrestrial environment. It is unlikely that the rain-

bow trout would come into contact with such high noxious heat as used in this study and this species inhabits waters below 25 °C. The nociceptors of this fish respond only above 40 °C and this is typical of nociceptors in higher vertebrates. This would suggest that either in the distant evolutionary past the animals encountered temperatures above 40 °C, or the response to such high temperatures may be a fundamental physiological mechanism or property of nociceptive nerve endings, as has been demonstrated in rat cultured dorsal root ganglion neurons (Lyfenko *et al.* 2002). These dorsal root neurons would also not come into direct contact with noxious temperatures, but they are responsive only to temperatures in the noxious range. It would be interesting from a comparative point of view to assess nociceptive responses in a tropical fish species because they would encounter higher temperatures. The mechanochemical receptors did not respond to thermal stimulation and cannot be classified as nociceptors. Further work is required to test these receptors with a variety of chemicals to ascertain if these are simply chemoreceptors, or if they are nociceptive, they only respond to noxious chemicals.

Assessing the subjective experiences of animals plays an increasingly large role in animal welfare (Broom 1991; Gentle 1992; Dawkins 1998; Bradshaw & Bateson 2000; Mason *et al.* 2001). To date, little attention has been paid to potential pain perception in fishes. In our behavioural experiments, we trained fishes to come to a feeding ring in response to a light cue and then assigned them to four treatment groups; three of these groups had either bee venom, acetic acid or saline injected into the lips and a fourth group was simply a handled control. After injection of algogenic substances, the resulting increase in opercular rate is similar to that recorded when trout are swimming at maximum speed (Altimiras & Larsen 2000) and much greater than the rate recorded after handling stress (increase to a maximum of 69 beats  $\text{min}^{-1}$  (Laitinen & Valtonen 1994)). The control and saline groups showed similar increases in opercular beat rate to stressed fishes (Laitinen & Valtonen 1994) and this is probably due to the handling and anaesthetic procedure. Respiratory changes have been demonstrated in mammals and humans enduring a nociceptive event (Kato *et al.* 2001) and so this dramatic rise in ventilation rate may be a physiological response to noxious stimulation in the rainbow trout.

The rainbow trout injected with acetic acid and bee venom performed anomalous behaviours that were not performed by the saline or control groups. Rocking behaviour was seen in both venom and acid treatment groups and this behaviour was performed only in the 1.5 h after injection. This is reminiscent of the stereotypical rocking behaviour of primates that is believed to be an indicator of poor welfare and thought to be performed as a comfort behaviour (Gonyou 1994). The performance of anomalous behaviours usually occurs within a short time period after the occurrence of a painful event when the pain is most intense (Molony *et al.* 2002). Only the acid group performed rubbing of the lips against the gravel and the sides of the tank. The act of rubbing an injured area to ameliorate the intensity of pain has been demonstrated in humans and in mammals (Roveroni *et al.* 2001). Overall, the administration of noxious substances had a negative

affect on the fishes' behaviour. To our knowledge, the performance of these behaviours has not been observed in fishes before. These behaviours may be indicative of discomfort and may have a potential use as indicators of pain or the occurrence of a noxious event in fishes. However, in humans and other animals pain is a specific experience and each different type of pain may have different behavioural responses and may also be species specific (Kavaliers 1988). Therefore, further studies should target noxious stimulation of other areas of the fish body to assess whether the behaviours seen in this study are universal.

The venom and acid injected fishes took *ca.* 3 h to begin ingesting food, whereas the saline and control groups took *ca.* 1 h. The venom and acid groups may be experiencing discomfort and so take longer to perform the task and resume feeding. This may be similar to guarding behaviour where an animal does not use a painful limb to prevent more pain and damage being caused to the affected area (Gentle 1992). Handling and anaesthesia are known to be stressful, causing an elevation in respiration rate (Laitinen & Valtonen 1994) and would account for the delay in the saline and control groups to perform the conditioning task. Giving the noxiously stimulated trout softer foodstuff did not affect the time to begin feeding again. Therefore, it appears as if the rainbow trout does not feed when affected by the administration of a noxious agent to the lips and only resumes feeding when the behavioural and physiological effects subside.

Our results demonstrate that the rainbow trout possesses nociceptors that detect noxious stimuli and that both the behaviour and physiology of the rainbow trout are adversely affected by stimuli known to be painful to humans. The behaviours shown by the trout after injection of a noxious stimulus are complex in nature and as such may not be simple reflexes. The performance of rocking behaviour and rubbing of the affected area, possible indicators of discomfort, suggests that higher processing is involved in the behavioural output and this is similar to some of the responses of higher vertebrates (Gonyou 1994; Roughan & Flecknell 2001) and man (Kato *et al.* 2001) to noxious stimuli. Other behavioural studies have shown that fishes learn to avoid aversive, noxious events such as electric shock but fishes that had morphine, an analgesic, administered failed to learn to avoid the electric shock (Ehrensing *et al.* 1982). Together, these electrophysiological and behavioural results show that the rainbow trout has a well developed nociceptive system. Previous anatomical studies have suggested marine elasmobranchs do not have nociceptors (Leonard 1985; Snow *et al.* 1993). This may represent an evolutionary divergence between the teleost and elasmobranch lineages.

Interestingly, there is a higher percentage of A-delta fibres (25%) in the trigeminal nerve compared with C fibres (4%; Sneddon 2002) and the majority of nociceptors were recorded from A-delta fibres. Only one of the 18 nociceptors we recorded from had a conduction velocity in the range of C fibre velocity ( $0.97 \text{ m s}^{-1}$ ) and the rest were A-delta fibres. Studies in mammals have stressed the importance of C fibres in prolonged nociceptive stimulation because they act as polymodal nociceptors with A-delta fibres, being mechanothermal nociceptors, participating only in acute short-term responses usually to alert

the nervous system to immediate injury (Matzner & Devor 1987; Lynn 1994; Gentle 1997). However, A-delta fibres predominate in the rainbow trout and the behavioural effects of a noxious stimulus, such as bee venom, were prolonged over *ca.* 3 h. Therefore, in teleosts, A-delta fibres potentially have a dual role in mediating reflex escape behaviour as well as prolonged noxious stimulation, whereas in higher vertebrates, C fibres may have evolved to become more numerous and have a more prominent function in prolonged noxious stimulation and inflammatory pain. More detailed electrophysiological recordings on A-delta fibres in the trout are necessary to confirm this hypothesis. Sneddon (2002) suggested that the higher proportion of C fibres in the higher vertebrates compared with the teleost was due to the advance onto land in evolution and the increased chance of injury due to gravity, extremes of temperature and noxious gases. The aquatic environment provides buoyancy, dilution of chemicals and a relatively stable thermal environment and so perhaps teleosts have not dedicated such a great amount of neural wiring to nociception as terrestrial vertebrates have.

The results of the present study demonstrate nociception and suggest that noxious stimulation in the rainbow trout has adverse behavioural and physiological effects. This fulfils the criteria for animal pain as stated in § 1. Future work should examine the cognitive aspects of noxious stimulation to assess how important enduring a noxious, potentially painful event is to the mental well-being of this species.

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## REFERENCES

- Altimiras, J. & Larsen, E. J. 2000 Non-invasive recording of heart rate and ventilation rate in rainbow trout during rest and swimming: fish go wireless! *J. Fish Biol.* **57**, 197–209.
- Bateson, P. 1992 Assessment of pain in animals. *Anim. Behav.* **42**, 827–839. ●●4●●.
- Belmonte, C. & Gallar, J. 1996 Corneal nociceptors. In *Neurobiology of nociceptors* (ed. C. Belmonte & F. Cervero), pp. 146–183. Oxford University Press.
- Belousova, T. A., Devitsina, G. V. & Malyukina, G. A. 1983 Functional peculiarities of fish trigeminal system. *Chem. Senses* **8**, 121–130.
- Beukema, J. J. 1970a Angling experiments with carp (*Cyprinus carpio* L.) II. Decreased catchability through one trial learning. *Neth. J. Zool.* **19**, 81–92.
- Beukema, J. J. 1970b Acquired hook avoidance in the pike *Esox lucius* L. fished with artificial and natural baits. *J. Fish Biol.* **2**, 155–160.
- Bradshaw, E. L. & Bateson, P. 2000 Welfare implications of culling red deer (*Cervus elaphus*). *Anim. Welfare* **9**, 3–24.
- Broom, D. M. 1991 Animal welfare: concepts and measurements. *J. Anim. Sci.* **69**, 4167–4175.
- Coggeshall, R. E., Leonard, R. B., Applebaum, M. L. & Willis, W. D. 1978 Organization of peripheral nerves and spinal roots of the Atlantic stingray, *Dasyatis sabina*. *J. Neurophysiol.* **41**, 97–107.
- Dawkins, M. S. 1998 Evolution and animal welfare. *Q. Rev. Biol.* **73**, 305–328.

- Ehrensing, R. H., Michell, G. F. & Kastin, A. J. 1982 Similar antagonism of morphine analgesia by MIF-1 and naxolone in *Carassius auratus*. *Pharm. Biochem. Behav.* **17**, 757–761.
- Gentle, M. J. 1992 Pain in birds. *Anim. Welfare* **1**, 235–247.
- Gentle, M. J. 1997 Sodium urate arthritis: effects on the sensory properties of articular afferents in the chicken. *Pain* **70**, 245–251.
- Gentle, M. J. & Tilston, V. L. 2000 Nociceptors in the legs of poultry: implications for potential pain in pre-slaughter shackling. *Anim. Welfare* **9**, 227–236.
- Gonyou, H. W. 1994 Why the study of animal behaviour is associated with the animal welfare issue. *J. Anim. Sci.* **72**, 2171–2177.
- Hallin, R. G., Torebjörk, H. E. & Wiesenfeld, Z. 1981 Nociceptors and warm receptors innervated by C fibres in human skin. *J. Neurol. Neurosurg. Psychiatry* **44**, 313–319.
- Hamamoto, D. T., Forkey, M. W., Davis, W. L., Kajander, K. C. & Simone, D. A. 2000 The role of pH and osmolarity in evoking the acetic acid-induced wiping response in a model of nociception in frogs. *Brain Res.* **862**, 217–229.
- Handwerker, H. O., Anton, F. & Reeh, P. W. 1987 Discharge patterns of different cutaneous nerve fibres from the rat's tail during prolonged noxious mechanical stimulation. *Exp. Brain Res.* **65**, 493–504.
- IASP (International Association for the Study of Pain) 1979 ●●6●●. *Pain* **6**, 249–252.
- Kato, Y., Kowalski, C. J. & Stohler, C. S. 2001 Habituation of the early pain-specific respiratory response in sustained pain. *Pain* **91**, 57–63.
- Kavaliers, M. 1988 Evolutionary and comparative aspects of nociception. *Brain Res. Bull.* **21**, 923–931.
- Kenshalo, D. R., Anton, F. & Dubner, R. 1989 The detection and perceived intensity of noxious thermal stimuli in monkeys and in humans. *J. Neurophysiol.* **62**, 429–436.
- Kotrschal, K. 2000 Taste(s) and olfaction(s) in fish: a review of specialized sub-systems and central integration. *Eur. J. Physiol.* **439**(Suppl.), R178–R180. ●●7●●.
- Laitinen, M. & Valtonen, T. 1994 Cardiovascular, ventilatory and total activity responses of brown trout to handling stress. *J. Fish Biol.* **45**, 933–942.
- Lariviere, W. R. & Melzack, R. 1996 The bee venom test: a new tonic-pain test. *Pain* **66**, 271–277.
- Leonard, R. B. 1985 Primary afferent receptive field properties and neurotransmitter candidates in a vertebrate lacking unmyelinated fibres. *Prog. Clin. Biol. Res.* **176**, 135–145.
- Lyfenko, A., Vlachová, V., Vyklícký, L., Dittert, I., Kress, M. & Reeh, P. W. 2002 The effects of excessive heat on heat-activated membrane currents in cultured dorsal root ganglia neurons from neonatal rat. *Pain* **95**, 207–214.
- Lynn, B. 1994 The fibre composition of cutaneous nerves and the classification and response properties of cutaneous afferents, with particular reference to nociception. *Pain Rev.* **1**, 172–183.
- Martinez, V., Thakur, S., Mogil, J. S., Tache, Y. & Mayer, E. A. 1999 Differential effects of chemical and mechanical colonic irritation on behavioural pain response to intraperitoneal acetic acid in mice. *Pain* **81**, 179–186.
- Mason, G. J., Cooper, J. & Clarebrough, C. 2001 Frustrations of fur-farmed mink: mink may thrive in captivity but they miss having water to romp about in. *Nature* **410**, 35–36.
- Matthews, G. & Wickelgren, W. O. 1978 Trigeminal sensory neurons of the sea lamprey. *J. Comp. Physiol. A* **123**, 329–333.
- Matzner, O. & Devor, M. 1987 Contrasting thermal sensitivity of spontaneously active A- and C fibres in experimental nerve end neuromas. *Pain* **30**, 373–384.
- Molony, V., Kent, J. E. & McKendrick, I. J. 2002 Validation of a method for assessment of acute pain in lambs. *Appl. Anim. Behav. Sci.* **76**, 215–238.
- Roughan, J. V. & Flecknell, P. A. 2001 Behavioural effects of laparotomy and analgesic effects of ketoprofen and carprofen in rats. *Pain* **90**, 65–74.
- Roveroni, R. C., Parada, C. A., Cecilia, M., Veiga, F. A. & Tambeli, C. H. 2001 Development of a behavioural model of TMJ pain in rats: the TMJ formalin test. *Pain* **94**, 185–191.
- Sneddon, L. U. 2002 Anatomical and electrophysiological analysis of the trigeminal nerve in the rainbow trout, *Oncorhynchus mykiss*. *Neurosci. Lett.* **319**, 167–171.
- Sneddon, L. U. 2003 Trigeminal somatosensory innervation of the head of a teleost fish. *Brain Res.* (In the press.) ●●8●●
- Snow, P. J., Plenderleith, M. B. & Wright, L. L. 1993 Quantitative study of primary sensory neurone populations of three species of elasmobranch fish. *J. Comp. Neurol.* **334**, 97–103.
- Stevens, C. W. 1992 Alternatives to the use of mammals for pain research. *Life Sci.* **50**, 901–912.
- Torebjörk, H. E. & Hallin, R. G. 1974 Identification of afferent C units in intact human skin nerves. *Brain Res.* **67**, 387–403.
- Walters, E. T. 1996 Comparative and evolutionary aspects of nociceptor function. In *Neurobiology of nociceptors* (ed. C. Belmonte & F. Cervero), pp. 92–116. Oxford University Press.
- Yeomans, D. C. & Proudfit, H. K. 1996 Nociceptive responses to high and low rates of noxious cutaneous heating are mediated by different nociceptors in the rat: electrophysiological evidence. *Pain* **68**, 141–150.
- Zimmerman, M. 1986 Physiological mechanisms of pain and its treatment. *Klinische Anästhesiol. Intensivtherapie* **32**, 1–19.

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