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Nociception or pain in a decapod crustacean?

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Nociception is the ability to perceive a noxious stimulus and react in a reflexive manner and occurs across a wide range of taxa. However, the ability to experience the associated aversive sensation and feeling, known as pain, is not widely accepted to occur in nonvertebrates. We examined the responses of a decapod crustacean, the prawn, *Palaemon elegans*, to different noxious stimuli applied to one antenna to assess reflex responses (nociception) and longer-term, specifically directed behavioural responses that might indicate pain. We also examined the effects of benzocaine, a local anaesthetic, on these responses. Noxious stimuli elicited an immediate reflex tail flick response, followed by two prolonged activities, grooming of the antenna and rubbing of the antenna against the side of the tank, with both activities directed specifically at the treated antenna. These responses were inhibited by benzocaine; however, benzocaine did not alter general swimming activity and thus the decline in grooming and rubbing is not due to general anaesthesia. Mechanical stimulation by pinching also resulted in prolonged rubbing, but this was not inhibited by benzocaine. These results indicate an awareness of the location of the noxious stimuli, and the prolonged complex responses indicate a central involvement in their organization. The inhibition by a local anaesthetic is similar to observations on vertebrates and is consistent with the idea that these crustaceans can experience pain.

Keywords: benzocaine; decapod; invertebrate; nociception; pain; *Palaemon elegans*; welfare

Nociception is the physiological detection of noxious, tissue-damaging stimuli (Sneddon et al. 2003a), allowing for swift reflexes that assist in withdrawal from those stimuli. However, whereas virtually all animal taxa seem capable of nociception (Sneddon 2004), it is not widely accepted that they all experience pain. Animal pain is defined as 'an adverse sensory experience of a stimulus that can or potentially could cause tissue damage; and this experience should elicit protective motor and vegetative reactions, having an adverse effect on the animal's general behaviour' (Zimmerman 1986) and as 'an aversive sensation and feeling associated with actual or potential tissue damage' (Broom 2001). Pain may provide an awareness of the stimulus and, as the stimulus is 'unpleasant' (Iggo 1984) or provides an 'aversive sensation' (Broom 2001), the animal may learn to avoid subsequent situations associated with that experience (Molony 1997; Broom 2001). For example, fish learn to avoid a particular area of a tank in which electric shock has been given and higher stimulus intensity enhances learning and memory (Dunlop & Laming 2005). Presumably, pain enhances fitness by increasing the likelihood that the animal will stay alive long enough to produce offspring (Bateson 1991; Rutherford 2002). Because pain perception is beneficial, we expect it to be widespread among animal taxa but possibly with different degrees of development. Among the vertebrates, the capacity to experience pain has been suggested to increase through Pisces, Amphibia, Reptilia, Aves to Mammalia (Stevens 1992).

In contrast to vertebrates, there have been few studies of pain in invertebrates (Sherwin 2001) and there is little evidence that invertebrates can experience something akin to mammalian pain (Fiorito 1986). Nevertheless, avoidance learning of electric shock has been shown in molluscs and insects (Carew & Sahley 1986) and annelids (Sahley 1995), and avoidance learning has been demonstrated in decapod crustaceans (Fernandez-Duque et al. 1992). Furthermore, bee venom, which typically induces pain responses in vertebrates (Sneddon et al. 2003a), when injected into the leg of a spider may cause autotomy of that leg, whereas saline injection does not (Eisner & Camazine 1983). Also, various taxa including decapods (Lüschen et al. 1991) have opioid binding sites (Sherwin 2001), suggesting an endogenous analgesic system like

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that found in vertebrates. These findings are congruent with the idea of pain. Because various invertebrate taxa have had long separate lineages, it is quite possible that the ability to experience pain has evolved independently in different groups.

Evidence that an animal can experience pain must show that the animal can perceive an adverse stimulus and that it reacts both physiologically and behaviourally by way of nonreflex responses (Sneddon et al. 2003a). Following an adverse stimulus, pain-specific behaviours, such as guarding or rubbing the affected area, may be observed (Stasiak et al. 2003). The alleviation of these relatively long-term responses by the application of an analgesic or local anaesthetic provides further evidence for pain. For example, fish injected in the lip with acetic acid show a distinct rocking behaviour and rubbing of the lip against the gravel in the tank, but this was reduced by analgesics (Sneddon 2003). A general behavioural change, such as rocking, may be interpreted as suggesting pain, but long-term rubbing of specifically affected sites denotes an awareness of the location of the potential tissue damage (Weary et al. 2006). In vertebrates, this awareness and subsequent rubbing activates spinal mechanoreceptor Aβ fibres that inhibit nociceptor afferents as part of an endogenous analgesic system (Melzack & Wall 1965). Other approaches to pain assessment include recording the decline in the frequency or magnitude of certain activities or evaluating how animals perceive the relative value of different treatments by choice and preference tests (Weary et al. 2006) or how pain responses alter attention processes to other tasks (Sneddon et al. 2003b). Furthermore, studies have stressed the possession of particular types of neurons (Sneddon et al. 2003a), sensory structures or areas of the brain necessary for pain. For example, it has been suggested that the possession of a well-developed cerebral cortex is a requirement and thus only the advanced mammals are regarded capable of experiencing pain (Rose 2002), although this stance has been questioned (Sneddon et al. 2003a, b).

We attempted to apply some of the criteria noted above to the question of potential pain experience in decapod crustacea, using the glass prawn, *Palaemon elegans*, as a model. We examined the occurrence of specific activities in response to noxious chemical and mechanical stimulation of the antennae. If the animal is capable only of nociception then there should be no significant change in behaviour, other than a reflex response such as the tail flip movement. However, if pain is experienced then a longer-term change in the animal's behaviour that is directed specifically at the site of the noxious stimulus may occur, for example grooming or rubbing the treated area. Furthermore, we used a local anaesthetic to determine whether the expected pain-relieving properties alter responses to noxious chemical and mechanical stimulation.

METHODS

Animal Collection and Husbandry

Palaemon elegans were collected in hand nets from rock pools during low tide from the shore at Kilclief Bay, Co Down, Northern Ireland (5°34′W; 54°19′N), between April and July 2006. They were immediately transported to Queen's University Belfast, housed in aerated seawater and, maintained between 11 and 13°C on a 12:12 h light: dark photoperiod with seaweed (*Fucus serratus*) present in the tanks. Before each treatment, a prawn was removed using a small net, placed in a glass dish containing seawater, covered with a paper towel to prevent the animal escaping and transferred to an adjacent observation room.

Experimental Treatments

Prawns were assigned randomly (by drawing tokens from a bag) to one of eight experimental groups ($N = 18$ per group), each involving two treatments.

First treatment

The animal was placed into a clean treatment dish containing a paper towel dampened with seawater. The first treatment, water or 2% benzocaine, was applied to a randomly (coin toss) chosen antenna using a small brush. One treatment was applied from halfway along the antenna to the tip and a separate brush was used for each treatment and for each prawn. Immediate reaction to the treatment by performing a tail flick reflex was noted. The prawn was then placed in the observation tank (19.5 × 9 × 9 cm) containing fresh seawater (11–13°C) to a depth of 3 cm. The observation tank was housed in an observation chamber behind a one-way mirror and behaviour recorded for 5 min.

We recorded general movement as the number of times the prawn crossed a marked line which divided the tank in half, the time taken to first cross the line, the number of tail flick movements, the number of times the animal groomed its treated or untreated antennae with its mouthparts or chelipeds, and the number of times the animal rubbed its treated or untreated antennae against the tank wall.

Second treatment

The prawn was removed from the observation tank for the second treatment and placed into a new clean treatment dish. The same, previously treated antenna was then treated either with seawater, 10% NaOH or 10% acetic acid or by pinching with forceps. The second treatment was applied following a procedure similar to that used before but to the distal quarter of the antenna. This ensured that the second treatment was applied to an area of the antenna covered by the first treatment. In the case of mechanical treatment, the antenna was gently pinched with wide-end forceps (width 3 mm), again on the distal quarter. Tail flick following treatment application was again noted before the prawn was placed into the observation tank for another 5 min, recording the same activities as before. The animal was then removed from the observation tank and euthanized immediately in liquid nitrogen.

Statistical Methods

First treatment

The occurrence of tail flick immediately following first treatment was compared using chi-square (χ^2) tests.

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General activity, as indicated by the time taken to cross the line and the number of line crosses, was compared using unpaired t tests. The occurrence of tail flick in the observation tank was compared using χ^2 tests. Grooming and rubbing activity were analysed by means of two-factor ANOVA (factor 1: seawater or anaesthetic; factor 2: treated or untreated antenna), with data transformed to $\log(x+1)$ and treated and untreated antennae as repeated measures.

Second treatment

The occurrence of tail flick immediately following second treatment was compared between treatments using Fisher's exact tests on collapsed data when there were small expected values. General activity, as indicated by the time taken to cross the line and the number of line crosses, was compared using two-factor ANOVA (factor 1: seawater or anaesthetic; factor 2: NaOH, acetic acid, seawater, or pinch). The occurrence of tail flick in the observation tank was determined using χ^2 tests. Grooming and rubbing of antennae against the tank were analysed using three-factor ANOVA (factor 1: seawater or anaesthetic; factor 2: NaOH, acetic acid, seawater, or pinch; factor 3: treated or untreated antenna), with data transformed $\log(x+1)$ and activity directed at the treated and untreated antennae as repeated measures. Each second treatment was then analysed separately by two-factor ANOVA (factor 1: seawater or anaesthetic; factor 2: treated or untreated antenna), with treated and untreated antennae as repeated measures.

RESULTS

First Treatment: Effects of Seawater or Anaesthetic

Significantly more animals flicked their tails upon application of anaesthetic compared to seawater (37/72 versus 0/72; $\chi^2_1 = 47.14$, $P < 0.001$). However, the time taken to first cross the line during the 5-min observation did not differ significantly between treatments ($t_{142} = 1.3$, $P = 0.196$) and there was no significant difference in general activity as indicated by the number of line crossings ($t_{142} = 0.98$, $P = 0.329$). There was no significant difference in the occurrence of tail flicking during the 5-min observation in animals treated with seawater or anaesthetic (17/72 versus 21/72; $\chi^2_1 = 0.57$, $P = 0.45$).

Overall, grooming of the antennae occurred significantly more following anaesthetic compared to seawater treatment ($F_{1,142} = 23.05$, $P < 0.001$; Fig. 1) and the treated antenna was groomed significantly more than the untreated antenna ($F_{1,142} = 16.87$, $P < 0.001$; Fig. 1). There was a significant interaction effect ($F_{1,142} = 22.2$, $P < 0.001$; Fig. 1) due to the high level of grooming directed at the treated antenna. There was no significant difference in rubbing antennae against the tank between treatments (seawater versus anaesthetic) ($F_{1,142} = 0.11$, $P = 0.74$) or between the treated antenna and the untreated antenna ($F_{1,142} = 0.01$, $P = 0.92$). There was no interaction effect ($F_{1,142} = 1.19$, $P = 0.28$).

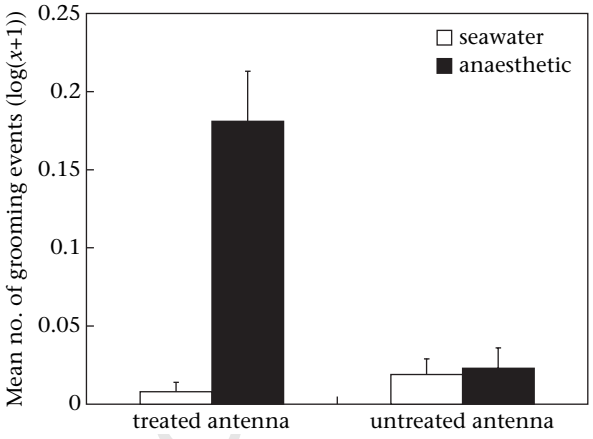

Figure 1. Mean ± SE ($\log(x+1)$) of grooming of treated and untreated antennae following application of seawater or anaesthetic in the first observation.

Second Treatment: Effects of Seawater, NaOH, Acetic acid and Pinching Following First Treatment

When the first treatment was seawater, significantly more animals tail flicked upon second treatment compared to when the first treatment was anaesthetic (9/72 versus 0/72; Fisher's exact test: $P = 0.003$). Testing for differences among the four groups that had not received benzocaine (Table 1) was precluded due to the small expected values, but collapsing the data into nonnoxious treatment (seawater) and noxious treatment (sodium hydroxide, acetic acid and pinch) indicated that significantly more animals tail flicked when given a noxious treatment (Fisher's exact test: $P = 0.0027$).

There was no significant effect on the time taken to first cross the line in either the first treatment ($F_{1,136} = 1.64$, $P = 0.202$) or the second treatment ($F_{3,136} = 1.61$, $P = 0.190$) and there was no interaction ($F_{3,136} = 0.74$, $P = 0.526$). Furthermore, the number of line crosses during the second treatment was not significantly affected by either the first treatment ($F_{1,136} = 0.002$, $P = 0.963$) or the second treatment ($F_{3,136} = 0.86$, $P = 0.856$) and there was no interaction ($F_{3,136} = 0.47$, $P = 0.706$). There was no significant difference in the occurrence of tail flicking in the observation tank in animals first treated with seawater or anaesthetic (14/72 versus 16/72; $\chi^2_1 = 0.17$, $P = 0.68$). Tail flicking did not differ depending on the nature of the second treatment ($\chi^2_3 = 1.52$, $P = 0.68$).

There was no main effect of first treatment on the number of antennal grooms during the second treatment

Table 1. Effect of the second treatment on the occurrence of a tail flick following the first treatment with seawater

Second treatment	Tail flick: No	Tail flick: Yes
Seawater	18	0
Sodium hydroxide	12	6
Acetic acid	16	2
Pinch	17	1

($F_{1,136} = 0.75$, $P = 0.386$); however, grooming differed among the second treatments ($F_{3,136} = 6.17$, $P < 0.001$), with most grooming after sodium hydroxide and least after seawater. The treated antenna was groomed more than the untreated antenna ($F_{1,136} = 20.08$, $P < 0.0001$) and there was a significant interaction between the first treatment and the antenna that was groomed ($F_{1,136} = 4.38$, $P = 0.038$) due to high levels of grooming directed at the treated antenna when the first treatment was seawater. The nature of the second treatment also had a substantial effect on which antenna was groomed ($F_{3,136} = 4.54$, $P = 0.0046$). The two-way interaction between first and second treatments, however, was not significant ($F_{3,136} = 0.90$, $P = 0.44$). Importantly, there was a three-way interaction between first and second treatments and the antenna that was groomed ($F_{3,136} = 3.75$; $P = 0.013$; Fig. 2). Grooming was directed most at the treated antenna when the treatments were seawater followed by NaOH or acetic acid.

For grooming of antennae, separate two-way ANOVAs were conducted for each second treatment but, because of the similarity in responses to the two chemical treatments, these data were combined. When treated with chemicals, there was no effect of the first treatment with anaesthetic or seawater ($F_{1,70} = 2.115$, $P = 0.15$); however, there was more grooming of the treated antenna ($F_{1,70} = 22.4$, $P < 0.0001$) but, more importantly, there was a significant interaction ($F_{1,70} = 9.76$, $P = 0.0026$) because grooming occurred much more when the first treatment was seawater and the antenna groomed was the treated antenna. When animals had their antenna pinched, there was no effect of anaesthetic on the amount of grooming ($F_{1,34} = 0.094$, $P = 0.76$) or on which antenna was groomed ($F_{1,34} = 0.85$, $P = 0.36$), and there was no interaction ($F_{1,34} = 0.734$, $P = 0.398$). Similarly, when the second treatment was seawater, there was no effect of first treatment with anaesthetic or seawater on the amount of grooming ($F_{1,34} = 1.03$, $P = 0.32$) or on which antenna was groomed ($F_{1,34} = 0.001$, $P = 0.99$), and there was no interaction ($F_{1,34} = 1.009$, $P = 0.32$).

There was more antennal rubbing against the tank when the first treatment was seawater than when it was local anaesthetic ($F_{1,136} = 8.94$, $P = 0.003$), but there was no main effect of second treatment ($F_{3,136} = 0.19$, $P = 0.903$). The treated antenna was rubbed more than the untreated antenna ($F_{1,136} = 20.12$, $P < 0.0001$), and there was a significant interaction between the first treatment and the antenna that was rubbed ($F_{1,136} = 9.57$, $P = 0.002$) due to high levels of rubbing when the first treatment was seawater and the rubbed antenna was the treated antenna. There was also a significant interaction between the second treatment and which antenna was rubbed with more rubbing of the treated antenna after chemical treatment ($F_{3,136} = 3.07$, $P = 0.03$). The interaction between first and second treatment, however, was not significant ($F_{3,136} = 1.04$, $P = 0.38$). The three-way interaction between first and second treatments and which antenna was rubbed against the tank was significant ($F_{3,136} = 3.21$, $P = 0.0253$; Fig. 3). The treated antenna was rubbed most when the treatments were seawater followed by NaOH or acetic acid.

For rubbing of antennae, separate two-way ANOVAs were conducted for each second treatment with the data for the two chemical treatments combined. When treated with chemicals, there was an effect of the first treatment ($F_{1,70} = 11.06$, $P = 0.0014$), with high levels of rubbing when seawater was the first treatment. There was more rubbing of the treated antenna ($F_{1,70} = 14.24$, $P = 0.0003$) but, more importantly, there was a significant interaction ($F_{1,70} = 22.34$, $P < 0.0001$) because rubbing occurred much more when the first treatment was seawater and the antenna rubbed against the tank was the treated antenna. When animals had their antenna pinched, there was no effect of the first treatment ($F_{1,34} = 0.192$, $P = 0.664$), but there was an effect of which antenna was rubbed ($F_{1,34} = 14.37$, $P = 0.0006$), with the pinched antenna being rubbed more than the untreated antenna. There was no interaction ($F_{1,34} = 0.37$, $P = 0.544$). When treated with seawater, there was no effect of the first

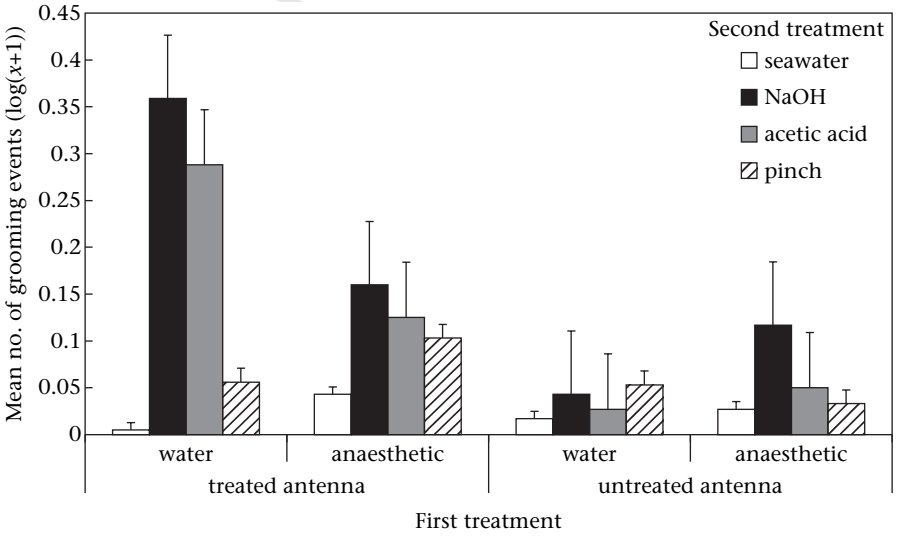

Figure 2. Mean ± SE (log($x + 1$)) of grooming of treated and untreated antennae in the second observation.

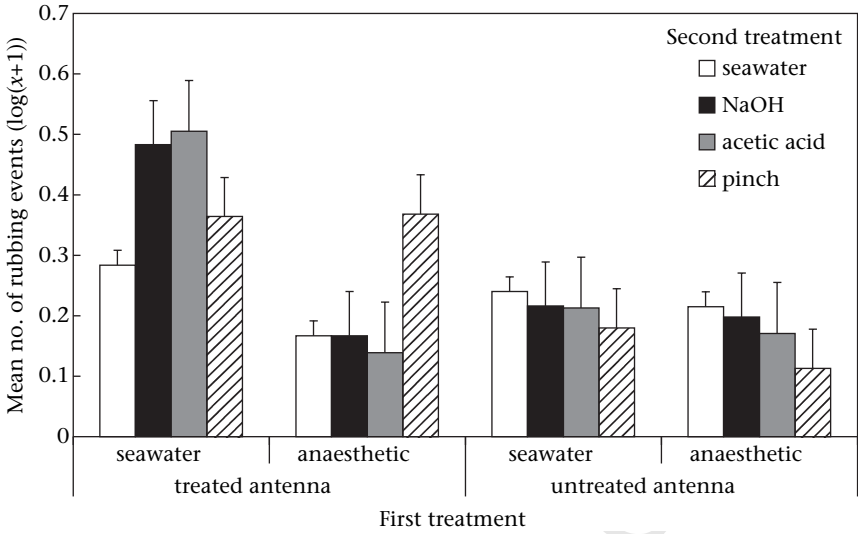

Figure 3. Mean ± SE (log(x + 1)) of rubbing activity of the treated and untreated antennae in the second observation.

treatment with anaesthetic ($F_{1,34} = 0.71$; $P = 0.407$), no effect on which antenna was rubbed ($F_{1,34} = 0.001$, $P = 0.975$) and no interaction ($F_{1,34} = 0.71$, $P = 0.41$).

DISCUSSION

Application of benzocaine to one antenna was more likely than seawater to produce an immediate reflex reaction as shown by the tail flick escape response. However, benzocaine did not act as a general anaesthetic (or stimulant) because the general level of activity was not altered. This is important, as general anaesthesia can occur if doses are sufficient and benzocaine is used to immobilize fish for capture and surgical procedures (Munday & Wilson 1997). Despite general activity not being affected, subsequent grooming of the treated antenna was greater with anaesthetic than with seawater and there was more grooming of the treated antenna than of the untreated antenna. That is, the effect was specific with respect to type and site of treatment. This suggests that benzocaine is an aversive stimulus. However, there were no effects of benzocaine on rubbing antennae against the glass sides of the tank. The increased grooming in the 5 min after application of benzocaine was not apparent during the second observation following subsequent treatment with seawater. Furthermore, there was no effect of benzocaine on rubbing the antennae against the side of the tank after subsequent treatment with seawater, indicating that the aversive effect of benzocaine was short-lived.

No animal tail flicked on the second treatment when the first treatment was anaesthetic or when the second treatment was seawater, but fail flicking occurred in animals that had seawater as the first treatment and a noxious second treatment, suggesting that benzocaine was effective in preventing the perception of the noxious treatment. The tail flick movement has been described as an escape response in many decapod crustaceans, involving medial giant interneurons, lateral giant interneurons and fast flexor motor giant neurons (Faulkes 2004). These escape responses elicited by the giant neurons are rapid bends of the abdomen that thrust the animal away from the origin of sufficiently abrupt disturbance (Edwards et al. 1999). Therefore, it is not possible to infer any more than nociception from this response.

The second treatments had considerable effects on both grooming the antennae and rubbing the antennae against the side of the tank. Both activities occurred more with the treated antenna than with the untreated antenna and so were specific to location. Both also occurred more with the treated antenna when the first treatment was seawater and the second treatment was sodium hydroxide or acetic acid than when other treatments were applied. That is, chemical treatment resulted in a prolonged location-specific change in behaviour that was not merely a reflex.

Grooming is well developed in many decapod crustaceans, although the nature of stimuli promoting grooming has not been elucidated (Bauer 1989). In this experiment, it is plausible to suggest that the animal is attempting to ameliorate the painful effect of the stimulus by grooming or rubbing the affected area. This difference between general grooming and focused grooming, provoked by local irritation or noxious stimulation, has been witnessed in rats (Roveroni et al. 2001). Grooming by rats in relation to a noxious stimulus has been found to have an organization different from those related to maintenance of the pelage, thermoregulation, social signalling or arousal reduction (Vos et al. 1998). In these cases, grooming actions appear to be aimed at removing the cause of pain (Vos et al. 1998). In mammals, selective activation of large-diameter afferent axons through gentle mechanical stimuli inhibits the pain response by closing the gate in the dorsal horn of the spinal cord, which is believed to mediate pain (Melzack & Wall 1965). Although the mechanisms in mammals and crustaceans may not be the same, it could be that an analogous system may achieve a similar pain-relieving effect by different means. The prolonged, specifically directed rubbing and grooming seen here are consistent with the idea of pain. Similar responses are seen in fish (Sneddon 2003) and amphibians (Stevens 1992).

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These responses to chemical treatment were both significantly lower when the first treatment had been a local anaesthetic. This effect is similar to that of xylocaine anaesthetic, which has been shown to alleviate stress due to eyestalk ablation in female *Litopenaeus vannamei* shrimp, as indicated by swimming and feeding behaviour (Taylor et al. 2004). There is also similarity with the study by Sneddon (2003), which showed that behavioural and physiological responses to a noxious stimulus in rainbow trout, *Oncorhynchus mykiss*, were reduced by morphine analgesia. Opioids, such as morphine, produce analgesia by acting on the central nervous system (Sawyer 1998). In comparison, local anaesthetics function by blocking sodium channels that prevent the generation and conduction of impulses from nociceptors (Machin 2005). The nerve-blocking properties of xylocaine and procaine anaesthetics occur in crayfish, *Procambarus clarkii*, by this mode of action (Leech & Rechnitz 1993). In the present study, benzocaine eliminated nociception, which otherwise would lead to the prolonged grooming/rubbing behaviour, indicative of a pain response. Furthermore, these results were not due to an overall change in activity as evidenced by the lack of treatment effects on latency to move and amount of swimming. This is important because any decline in specifically directed activities after benzocaine treatment was not due to general anaesthesia.

Mechanical pinching of an antenna did not markedly increase grooming but did increase rubbing; however, anaesthetic did not alter the responses to this treatment. Thus pinching had a prolonged effect on specifically directed behaviour. The lack of an effect of benzocaine, however, may be due to different receptors of the two types of stimuli that have different sensitivities to benzocaine. Other decapod species have up to 10 morphologically different receptor types on the antennae, some of which are bimodal (chemomechanoreceptive) sensilla (Cate & Derby 2001), and mechanoreceptors are modulated by various monoamines (Pasztor & Bush 2000).

We conclude that noxious stimuli on the antennae are detected by prawns resulting in an immediate escape tail flick consistent with the concept of nociception and reflex response. The prolonged grooming after chemical stimulation and rubbing after chemical and mechanical activities shown by the prawn after exposure to noxious chemicals, however, is more complex than a reflex action. Both activities are directed specifically at the site of stimulation, indicating an awareness of the location and suggesting that higher processing is involved in the behavioural output, similar in many respects to responses of fish (Sneddon et al. 2003a) and higher vertebrates (Gonyou 1994; Roughan & Flecknell 2001). However, the responses to chemical stimulation are reduced by benzocaine local anaesthetic. These observations are similar to observations on pain responses in vertebrates (Sherwin 2001). Coupled with observations of opioid receptors (Lüschen et al. 1991) and avoidance learning (Fernandez-Duque et al. 1992), they fit criteria for pain (Sneddon et al. 2003a). The prolonged, specifically directed grooming may be a pain-specific behaviour or a pain-coping strategy in this decapod crustacean.

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