Aversive responses by shore crabs to acetic acid but not to capsaicin

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Abstract

Nociception is the ability to encode and perceive harmful stimuli and allows for a rapid reflexive withdrawal. In some species, nociception might be accompanied by a pain experience, which is a negative feeling that allows for longer-term changes in behaviour. Different types of stimuli may affect nociceptors, but in crustaceans there is conflicting evidence about the ability to respond to chemical stimuli. This study attempts to resolve this situation by testing behavioural responses of the common shore crab, *Carcinus maenas,* to two chemical irritants frequently used in vertebrate pain studies (acetic acid and capsaicin). In our first experiment acetic acid, water, capsaicin or mineral oil were applied by brush to the mouth, and in a second experiment treatments were applied to the eyes. Application of acetic acid had a marked effect on behaviour that included vigorous movement of mouth parts, scratching at the mouth with the claws and attempts to escape from the enclosure. Acetic acid also caused holding down of the acid-treated eye in the socket. By contrast, capsaicin had no effect and was no different to the control treatment of mineral oil and water. These results demonstrate responsiveness to acetic acid and thus nociceptive capacity for at least some chemicals. Further, the responses that persist after application were consistent with the idea of pain, however, proof of pain is not possible in any animal.

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1 Introduction

Nociception is the ability to detect and respond to noxious stimuli and nociceptors are “sensory systems that respond to noxious stimuli and mediate protective reflexes” (Sherrington 1906, Sneddon et al. 2014). There is no suggestion of any awareness by the animal about the stimulus or response or of any long-term motivational change in behaviour. By contrast, pain in animals is “an aversive sensory experience caused by actual or potential injury that elicits protective motor and vegetative reactions, results in learned avoidance and may modify species-specific behaviour, including social behaviour” (Zimmerman 1986). Pain can result from nociceptive input but nociception does not always result in pain. Indeed, it is possible that many taxa have nociception without the ability to experience pain (Elwood 2011). Nociceptive reflexes enable the animal to withdrawal from tissue-damaging stimuli and thus protect the animal from harm. The adaptive value of nociception is obvious and nociception had an early appearance during evolution and is thus widespread in the animal kingdom (Elwood et al. 2009, Crook et al. 2014). Presumably pain offers further benefits and it appears that the negative emotional component causes a long-lasting motivational change that enables the animal to avoid situations that gave rise to the original pain experience (Bateson 1991). Pain provides information that nociception alone cannot and thus increases the likelihood that the animal will survive long enough to produce offspring.

The ability to experience pain presumably requires a neural network enabling complex processing by a large number of neurons (Crook & Walter 2011). For this reason pain or pain-like experience in invertebrates has been considered unlikely (Rose et al 2014; but also see Klein & Barron 2016). Several studies, however, suggest that decapod responses to noxious stimuli are more than reflexes (Barr et al. 2008; Appel & Elwood 2009a,b; Elwood & Appel 2009; Magee & Elwood 2013, 2016a,b; Fossat et al 2015; Dyuizen et al. 2012). One of these examined responses of glass prawns, *Palaemon elegans*, to acetic acid, sodium hydroxide or seawater controls being brushed onto a single antenna (Barr et al 2008). Prawns treated with the noxious chemicals showed an increased grooming of that specific antenna and also of rubbing that antenna against the sides of the tank compared to controls. This appeared too complex to be merely nociceptive reflexes and was interpreted as being consistent with the idea of pain. Further, when terrestrial hermit crabs compete with ants for access to carrion, the ants spray the crabs with formic acid and drive the crabs away from the food resource (McNatty et al. 2009). This indicates that the acid is aversive and causing the retreat from an important resource is consistent with the idea of pain (Elwood & Appel 2009). Further, the crabs keep away from areas with large numbers of ants suggesting avoidance learning (Elwood & Appel 2009).These studies and interpretations, however, were put into doubt when three other species of decapod showed no responses to either hydrochloric acid or sodium hydroxide (Puri & Faulkes 2010). This latter study questioned whether crustaceans had nociceptors for noxious chemicals and suggested the evidence for such receptors was weak. Recently, the same authors found no aversion by crayfish (species) to eating chillies or wasabi, which contain capsaicin and isothiocyanate, and those substances rubbed on the antennae failed to cause grooming (Puri & Faulkes 2015). Nevertheless, crayfish did respond with vigorous escape responses when touched with a hot soldering iron. Thus studies on decapods show they respond to heat but the evidence for extreme pH nociceptors is contradictory (Barr et al. 2008; Puri & Faulkes 2015).

Capsaicin is a powerful chemical irritant for most mammals, including humans, and has been found to be noxious to other invertebrates such as the nematode, *Caenorhabditis elegans*, (Wittenburg & Baumeister 1999) and the leech, *Hirudo medicinalis* (Pastor et al. 1996). However, capsaicin has no effect on fruit flies, *Drosophila melanogaster* (Al-Anzi et al. 2006). Acetic acid has long been used in pain studies of vertebrates such as fish (Sneddon et al. 2003) and mammals (Pavao-de-Souza et al. 2012). One mammal, the African naked mole-rat, *Heterocephalus glaber,* shows a lack of responses to both capsaicin and acid (Park et al. 2008) and birds are unresponsive to capsaicin (Jordt & Julius 2002). These studies indicate marked variation in nociceptive ability within particular broad taxa.

Elucidating nociceptive capability has significant implications on the welfare of the species. Evaluating the capacity for nociception in different invertebrate species aids in understanding the evolution of that sensory modality and thus bears on the potential for suffering in these animals. Such research also has the potential to create new models for human pain (Puri & Faulkes 2010). Evidence for pain-like states in crustaceans is growing (Denti et al. 1998; Kawai et al. 2004; Patterson et al. 2007; Barr et al. 2008; Elwood and Appel 2009; Appel and Elwood 2009a, b; Puri &

Faulkes 2015). However, we need to resolve which, if any, chemical stimuli induce nociception to enable advances in potential pain research in this taxon. For this reason we investigated the nociceptive abilities in the common shore crab, *Carcinus maenas.* We conducted two experiments in which capsaicin and acetic acid or controls were brushed on either the mouth parts or on the eyes.

2. Methods

Shore crabs, *Carcinus maenas,* were collected using baited pots from Barr Hall Bay, Strangford Lough, Co. Down, UK (OS; J 617464) between May and June 2014. The crabs were transported to Queen's University, Belfast and housed about 25 per plastic tank (76cm x 38cm x 17cm), filled with aerated seawater to a depth of 5cm, and seaweed (*Ascophyllum nodosum)* was included for shelter. The crabs were maintained in a cold room at a temperature of 11-13°C with a 12 hour light/dark schedule for a maximum of 10 days prior to the experiments. Crabs were fed with Tetra Pond Floating Food Pellets (Melle, Germany) and the water changed every 3 days.

Each crab was each brought from the cold room to an adjacent observation room at about 20°C where it was immediately tested singly in a glass tank (62cm x 25cm x 25cm). The tank contained gravel, rocks and seawater (just enough to moisten the gravel). The area was lit by a 100 W bulb (2060 Lux) suspended over the tank and 2 minute recording made using a hand-held Sony Handycam (HDRCX240EB) HD camcorder, which was moved if the crab moved, to facilitate recording. The same person (ND) did all manipulations and recording and thus was not blind to treatments.

2.1 Experiment one: application to mouth

Crabs (N=60) were randomly assigned (dice) to one of four experimental treatments, 10% acetic acid, distilled water (control), capsaicin (0.018g per 10ml mineral oil, which is approximately the capsaicin concentration of a scotch bonnet chilli), mineral oil (control) but with equal numbers per group. The crab was held in one hand and the treatment was then brushed onto the mouth after gently prying open the maxillipeds (2 brush strokes per treatment). Small individually coloured brushes were used to ensure that each brush was used for one treatment only.

Seven activities were recorded to measure potential responses to treatment: threat display (holding the claws upright at either side of the carapace); claws scratching at mouth (moving claws against the mouth parts in a scraping motion); escape (crawling up the side of the tank and scrabbling at the glass with limbs); mouth parts up and down (third maxillipeds moving in unison up and down); mouth parts side to side (moving third maxillipeds independently of each other, left and right) ; inside mouth moving (movement of first and second maxillipeds) and the mouth parts held out (third maxillipeds held away from the main body).

The 2 minute recordings of each crab were divided into 5 second parts and each behaviour was recorded as occurring or not in each period (maximum score for each activity was 24). To reduce the number of statistical tests, however, the last four activities that involved the mouth parts where combined to a single score for "mouth part movements" with a maximum score of 96. Statview (Version 5, SAS Institute, Cary, California, NC, USA) was used to calculate one way ANOVAs with alpha set at 0.05. Power analyses provided by Statview are presented for significant results.

2.2 Experiment two: application to eyes

Each crab had both eyes treated but the treatment for each eye was different. (N=48). The choice and order of treatment was fully randomised. One eye received either capsaicin or mineral oil control and the alternative eye received either acetic acid or water, applied by a gentle stroke of a brush. This gives four experimental groups (N=12 each), denoted by the treatment of each eye, in a 2 x 2 design (1. capsaicin and water, 2. capsaicin and acid, 3) oil and water and 4) oil and acid). The crab was then filmed for 2 minutes. The same activities were recorded as in experiment 1. In addition we recorded the duration in seconds of how long each eye remained down in the socket.

The scores for each activity and the time of holding down the eye were analysed using a two-factor ANOVA (factor 1: acetic acid or water applied to one eye; factor 2: capsaicin or mineral oil to the other eye). For the time that the eyes were held down we conducted two analyses, one for each eye. First we focus on the specific eye that had acid or water. Factor 1 is acid or water, whereas factor 2 is the oil or capsaicin applied to the alternative eye. Next we focus on the eye that had capsaicin or oil. Factor 1 is capsaicin or oil to that eye and factor 2 is the effect of acid or water to the alternative eye. This design allowed for us to examine if one eye is only responsive to treatments to that specific eye or if it responds to treatments given to the other eye. Again alpha was set at $P<0.05$ and power tests presented for significant results using Statview.

2.3 Ethics

No licence is required to on crustaceans in the United Kingdom. Nevertheless sample sizes were kept to a minimum as judged from other studies. The 2 x 2 design for experiment 2 was chosen as it required fewer subjects than experiment 1. All crabs appeared to recover after treatment without intervention by the experimenters, and were provided with suitable housing conditions similar to that used prior to tests, and returned to shore within 10 days.

3. Results

3.1 Experiment one: application to the mouth

Several activities differed significantly between treatment groups. These were mouth part movements, ($F_{3,56}$=19.26, $P<0.0001$, Power = 1.0)(figure 1), claws scratching at mouth ($F_{3,56}$=14.24, $P<0.0001$, Power = 1.0)(figure 2) and escape ($F_{3,56}$=6.49, P= 0.0008, Power = 0.97)(figure 3). In each case animals treated with acetic acid had the highest scores and post hoc tests (Tukey's) showed that in all cases the acetic acid group was significantly different from each of the other three groups ($P<0.01$ all cases) whereas the other three groups did not differ from each other. Threat displays, however, did not differ significantly across the groups ($F_{3,56}$= 0.84, P=0.47)

3.2 Experiment two: application to eyes

Acetic acid applied to an eye caused crabs to move their mouth parts more than those receiving water ($F_{1,44}$ = 9.78, p =0.003, Power = .88), however, there was no effect of capsaicin ($F_{1,44}$ = 2.8, p =0.1) and no interaction between acetic acid and capsaicin ($F_{1,44}$ = 2.8, p =0.1) (figure 4). Crabs also scratched at the their mouth using their claws more after acetic acid application compared to those with water ($F_{1,44}$= 12.16, p = <0.001, Power = 0.90) but capsaicin had no effect for this behaviour ($F_{1,44}$= 1.05, p = 0.31), and there was no interaction effect ($F_{1,44}$= 1.05, p = 0.31) (figure 5). Acid also caused more escape behaviour ($F_{1,44}$= 21.72, p = <0.001, Power = 0.99) but capsaicin had no effect ($F_{1,44}$= 1.47, p = 0.23), and there was no interaction effect ($F_{1,44}$= 2.49, p = 0.12) (figure 6). For threat displays, however, there was no effect of acetic acid ($F_{1,44}$= 1.45, p = 0.24), or capsaicin ($F_{1,44}$= 0.0, p =1.0) and there was no interaction effect ($F_{1,44}$= 1,44, p = 0.48) .

We describe the responses of each eye to treatment to that specific eye and treatment to the alternative eye. Acetic acid rather than water on an eye (the acid/water eye) significantly increased the duration for which that specific eye was held down in the socket ($F_{1,44}$= 4.76, p = 0.034, Power = 0.56), but there was no effect on the acid/water eye of capsaicin applied to the alternative eye ($F_{1,44}$= 0.12, p= 0.74), and there was no interaction between acetic acid on one eye and capsaicin on the other eye ($F_{1,44}$= 0.26, p = 0.61) (Figure 7). By contrast, application of capsaicin rather than mineral oil to an eye (capsaicin/mineral oil eye) had no effect of withdrawal of that specific eye ($F_{1,44}$= 0.52, p = 0.48). Application of acetic acid rather than water to the alternate eye had no effect on the capsaicin/mineral oil eye ($F_{1,44}$= 0.68, p = 0.42), and there was no interaction effect ($F_{1,44}$= 1.78, p = 0.18). That is, acetic acid only affected the eye to which it was applied and did not affect the alternate eye, whereas capsaicin did not affect the eye to which it was applied or the alternative eye.

4 Discussion

Crabs with capsaicin applied to either the mouth or an eye did not differ in their responses from those treated with mineral oil control. This is in marked contrast to some other taxa such as the nematode, *Caenorhabditis elegans*, (Wittenburg & Baumeister 1999) and the leech, *Hirudo medicinalis* (Pastor et al. 1996), which responded to capsaicin. It agrees, however, with a recent study that found no effect of capsaicin applied to the antennae of crayfish on the behaviour (Puri & Faulkes 2015). Further, crayfish did not avoid foods containing capsaicin and capsaicin did not affect firing of sensory neurons (Puri & Faulkes 2015). Thus neither of the two

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species of decapod with capsaicin applied to different body regions are responsive to that substance. In the vertebrates there is variation in responsiveness to capsaicin with some behavioural modification in fish (Eckroth et al. 2014), major effects in humans with reports of painful burning (Caterina et al 1997) but no effects in birds (Jordt & Julius 2002).

By contrast, acetic acid applied to the mouth of shore crabs resulted in high levels of movement of the small appendages that make up decapod mouth parts. These were unlike normal feeding movements and involved flaring and rubbing movements of the various mouth parts. In addition, the claws were used to scratch and scrape at the mouth parts. These activities demonstrate that acetic acid is detected and that it appears to be aversive because the crabs appear to be attempting to rid the mouth area of the substance.

When acetic acid was applied to an eye, the mouth parts were moved and the claws scratched at the mouth, similar to experiment 1. One reason for this is that the eyes are situated just dorsal to the mouth and a small grove near to the base of each antenna might allow some of the acetic acid to trickle down to the mouth area. There is no reason, however, to suggest that this might be as large a volume compared to that when the acetic acid was applied directly to the mouth. Indeed, whilst the activities involved were the same as in experiment one, moving of mouth parts was not as active in the eye experiment, although the amount of scratching with the claws was similar in the two experiments.

When an eye was brushed, the crabs typically withdrew that eye into the eye socket indicting sensitivity to touch. In most cases, the eye was swiftly brought out again enabling normal vision but this did not occur if the eye was brushed with acetic acid. That eye was then held down for significantly longer than when brushed with water, however, we note that the power of the test is weaker than other significant results. The effect of the acid was specific to the eye to which application was made and did not affect withdrawal of the alternative eye. That is the crabs ‘blinked’ with one eye only, keeping the unaffected eye to receive visual information indicating independent control of each eye (Crothers 1968). Crabs in the first experiment were not seen to withdraw their eyes into the socket so this was a specific response to acetic acid on the eye.

We had predicted that an aversive stimulus would result in the defensive threat display shown by crabs when they hold out their claws towards a potential predator. However, this was seen in very few crabs and was not affected by chemical application in either experiment. It appears that the stimulation from the acetic acid was not perceived as a potential predatory threat from which a threat display in return might benefit the crab. In both experiments, however, there was a significant increase in escape responses after acetic acid application, which involved relatively prolonged scrabbling and attempting to climb the walls of the container. This indicates that the crabs found the substance highly aversive and is similar to the escape by hermit crabs when attacked by ants spraying formic acid (McNatty et al. 2009). It does not appear to be a mere reflex, rather it is more like a complex goal directed activity to escape the vicinity of the stimulus.

In general, the present data agree with those of Barr et al. (2008) when glass prawns with acetic acid applied to one antenna groomed that antenna with their pincers and rubbed that antenna against the side of the tank. Barr (2009) also noted complex grooming responses towards an eye of the prawn that was treated with acetic acid. The complexity was noted because the prawns used pincers on both their first walking legs to reach to the eye but this could only be achieved by markedly different postures of the front legs to both reach the one eye. Shore crabs cannot reach the eye with their claws and thus this type of grooming was not seen in the present study. In the study of Barr et al. (2008) the prawns were treated out of water but then immersed for the observations, whereas in the present study crab treatment was out of water and then the observations occurred without immersion. Thus the acetic acid used on the prawns may have largely washed off but that would not be possible in the present study. That both studies report prolonged grooming and rubbing indicates that acid might have effects that last after it washes off.

These observations of Barr et al. (2008) and Barr (2009) and those of the present study are in marked contrast to those of Puri and Faulkes (2010) in which three species of decapods (Louisiana red swamp crayfish, *Procambarus clarkii*, white shrimp, *Litopenaeus setiferus*, and grass shrimp, *Palaemonetes paludosus*) showed no response to hydrochloric acid applied to the antennae. Thus, whilst hydrochloric acid had no effect on those three species, acetic acid has considerable effect when applied to antennae, eyes and mouth of other two other decapods (Barr et al. 2008). It seems unlikely that this is a species effect and it might be that different acids act differently on nociceptors. The responses, however, do not appear to be specific to one acid because formic acid also appears to evoke marked responses and avoidance in hermit crabs (McNatty et al. 2009). Dyuizen et al. (2012) also reported abnormal behaviour after a cheliped of crabs (*Hemigrapsus sanguineus*) was injected in the distal joint of the cheliped with 1% formalin. This involved flexion, extension and shaking of the claw. These crabs also showed rubbing of the injected claw and 20% autotomized the appendage. These activities were not seen in saline injected controls. Further, sodium hydroxide applied to an antenna of glass prawns caused rubbing and grooming (Barr et al. 2008) but not in tests on three other species by Puri and Faulkes (2010). However, those authors did report strong responses of crayfish, including tail flip escape responses, when touched with a hot soldering iron. Electric shock also causes marked behavioural change, including giving up valuable resources to escape from the shock (Magee & Elwood 20013, 2016; Appel & Elwood 2009b). Thus, decapods show marked changes in behaviour after treatment with various stimuli that are noxious to vertebrates and, presumably, these changes are mediated by nociceptors. However, capsaicin does not appear to stimulate decapod nociceptors and it is also not effective in birds (Jordt & Julius 2002).

4.1 Conclusions

The responses to acetic acid are consistent with the idea that they are mediated by nociceptors. They demonstrate immediate responses, some of which are likely to be a reflex e.g. withdrawal of the eye. The movement of mouth parts and the scratching/scrapping of the mouth parts with the claws are prolonged and complex and less likely to be merely reflex. Further, the escape attempts involve various activities, including unsuccessful attempts to climb up the walls of the tank and indicate that the crabs find the acetic acid aversive. These complex responses are consistent with the idea of pain (Sneddon et al. 2014).

Acetic acid has been used to induce pain-like behaviour in vertebrates, for example in trout (Sneddon et al. 2003) and mice (Gawade 2012). Trout injected with acetic acid into the lip showed rubbing of that area against the gravel bottom of the tank and against the glass wall. They also showed a rocking movement. These anomalous activities declined with analgesic treatment. Mice injected with acetic acid show writhing of the body and this is reduced by analgesic administration and has been used to test analgesic efficiency in pain tests (Gawade 2012). We did not attempt to determine if the responses of crabs to acetic acid were reduced by application of local anaesthetics because that had been shown with glass prawns (Barr et al. 2008). Formalin has been used to assess pain in rats by injection into a paw (Abbott et al. 1995) and results in lifting, licking and shaking of the specific paw. That is the behaviour of vertebrates are remarkably similar to decapods when similarly treated with noxious chemicals. Thus, using the argument by analogy (Sherwin 2003), the responses of the decapods are consistent with the idea of pain. We stress, however, that total proof of pain is not possible in any animal (Elwood 2011, Stamp Dawkins 2012). Pain is often presumed in vertebrates but, with invertebrates, that possibility is often rejected, even when the evidence is similar (Sherwin 2003).

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Figure legends:

Fig 1. Experiment 1. Mean (+/- SE) of mouth part movements, recorded as the number of 5 second periods the activity was shown per 2 minutes.

Fig 2. Experiment 1. Mean (+/- SE) of scratching at their mouth with their claws, recorded as the number of 5 second periods the activity was shown per 2 minutes.

Fig 3. Experiment 1. Mean (+/- SE) of escape activities, recorded as the number of 5 second periods the activity was shown per 2 minutes.

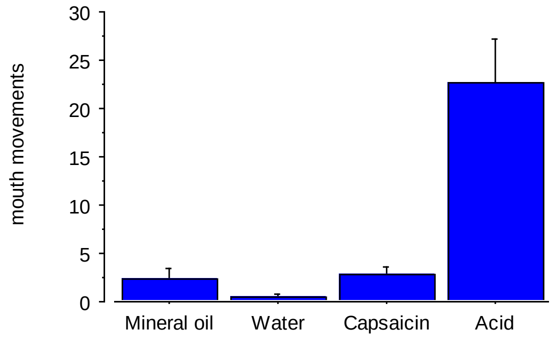
Fig 4. Experiment 2. Mean (+/- SE) of mouth part movements, recorded as the number of 5 second periods the activity was shown per 2 minutes. One treatment of acid or water was given to one eye and oil or capsaicin to the other eye.

Fig 5. Experiment 2. Mean (+/- SE) of scratching at their mouth with their claws, recorded as the number of 5 second periods the activity was shown per 2 minutes. One treatment of acid or water was given to one eye and oil or capsaicin to the other eye.

Fig 6. Experiment 2. Mean (+/- SE) of escape activities, recorded as the number of 5 second periods the activity was shown per 2 minutes. One treatment of acid or water was given to one eye and oil or capsaicin to the other eye.

Fig 7. Experiment 2. Mean (+/- SE) duration (sec) for which the eye receiving either acetic acid or water was held down in the eye socket. The alternative eye received either capsaicin or mineral oil.

Fig 1



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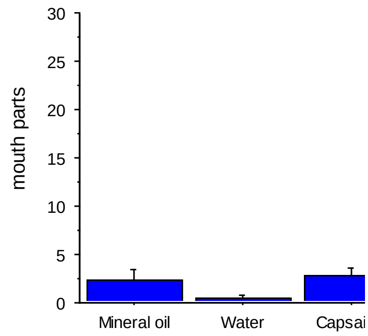
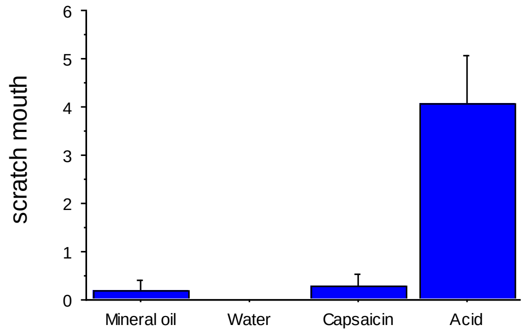


Fig 2



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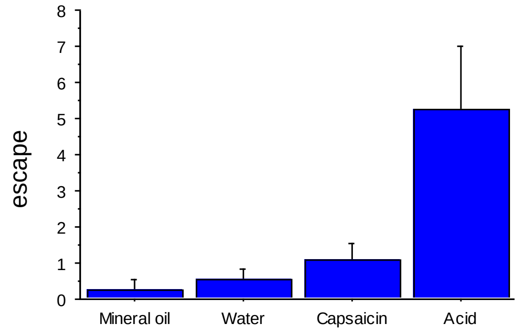

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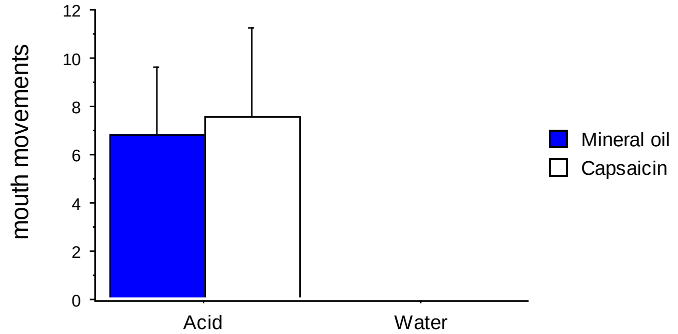

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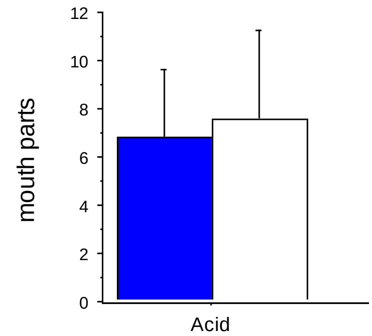

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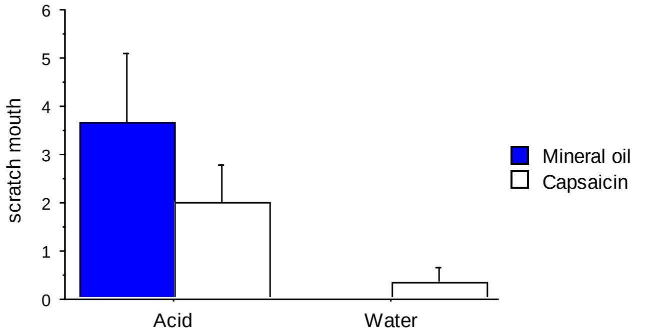
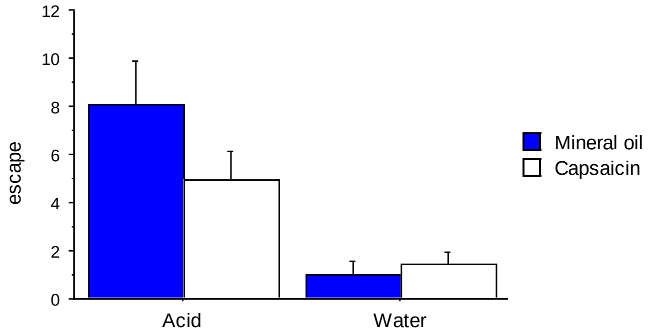


scratch mouth
0
1
2
3
4
5
6
Acid
Water
Mineral oil
Capsaicin

Fig 6



escape
0
2
4
6
8
10
12
Acid
Water
Mineral oil
Capsaicin

Fig 7