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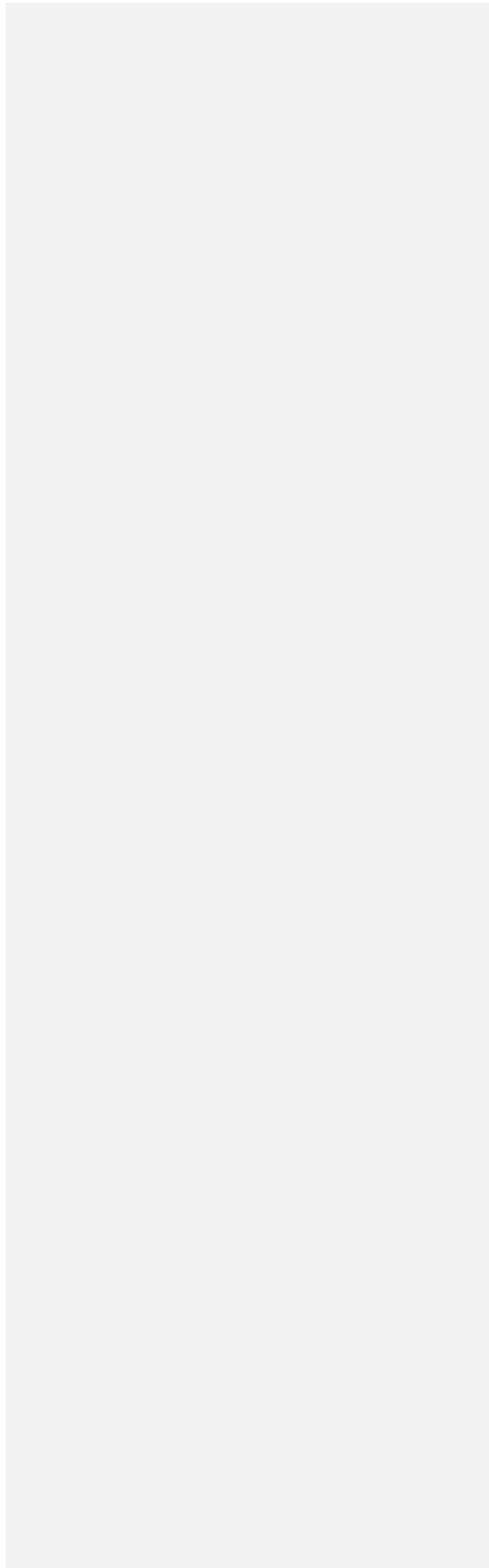
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Aversive responses by shore crabs to acetic acid but not to capsaicin

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31

32 **Abstract**

33 Nociception is the ability to encode and perceive harmful stimuli and allows for a
34 rapid reflexive withdrawal. In some species, nociception might be accompanied by a
35 pain experience, which is a negative feeling that allows for longer-term changes in
36 behaviour. Different types of stimuli may affect nociceptors, but in crustaceans there
37 is conflicting evidence about the ability to respond to chemical stimuli. This study
38 attempts to resolve this situation by testing behavioural responses of the common
39 shore crab, *Carcinus maenas*, to two chemical irritants frequently used in vertebrate
40 pain studies (acetic acid and capsaicin). In our first experiment acetic acid, water,
41 capsaicin or mineral oil were applied by brush to the mouth, and in a second
42 experiment treatments were applied to the eyes. Application of acetic acid had a
43 marked effect on behaviour that included vigorous movement of mouth parts,
44 scratching at the mouth with the claws and attempts to escape from the enclosure.

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45 Acetic acid also caused holding down of the acid-treated eye in the socket. By
46 contrast, capsaicin had no effect and was no different to the control treatment of
47 mineral oil and water. These results demonstrate responsiveness to acetic acid and
48 thus nociceptive capacity for at least some chemicals. Further, the responses that
49 persist after application were consistent with the idea of pain, however, proof of pain
50 is not possible in any animal.

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54 Key words: acetic acid; capsaicin; decapod; nociception; pain

57 **1 Introduction**

58 Nociception is the ability to detect and respond to noxious stimuli and nociceptors
59 are “sensory systems that respond to noxious stimuli and mediate protective
60 reflexes” (Sherrington 1906, Sneddon et al. 2014). There is no suggestion of any
61 awareness by the animal about the stimulus or response or of any long-term
62 motivational change in behaviour. By contrast, pain in animals is “an aversive
63 sensory experience caused by actual or potential injury that elicits protective motor
64 and vegetative reactions, results in learned avoidance and may modify species-
65 specific behaviour, including social behaviour” (Zimmerman 1986). Pain can result
66 from nociceptive input but nociception does not always result in pain. Indeed, it is
67 possible that many taxa have nociception without the ability to experience pain
68 (Elwood 2011). Nociceptive reflexes enable the animal to withdrawal from tissue-
69 damaging stimuli and thus protect the animal from harm. The adaptive value of
70 nociception is obvious and nociception had an early appearance during evolution
71 and is thus widespread in the animal kingdom (Elwood et al. 2009, Crook et al.
72 2014). Presumably pain offers further benefits and it appears that the negative
73 emotional component causes a long-lasting motivational change that enables the
74 animal to avoid situations that gave rise to the original pain experience (Bateson
75 1991). Pain provides information that nociception alone cannot and thus increases
76 the likelihood that the animal will survive long enough to produce offspring.

77 The ability to experience pain presumably requires a neural network enabling
78 complex processing by a large number of neurons (Crook & Walter 2011). For this
79 reason pain or pain-like experience in invertebrates has been considered unlikely
80 (Rose et al 2014; but also see Klein & Barron 2016). Several studies, however,
81 suggest that decapod responses to noxious stimuli are more than reflexes (Barr et
82 al. 2008; Appel & Elwood 2009a,b; Elwood & Appel 2009; Magee & Elwood 2013,
83 2016a,b; Fossat et al 2015; Dyuizen et al. 2012). One of these examined responses
84 of glass prawns, *Palaemon elegans*, to acetic acid, sodium hydroxide or seawater
85 controls being brushed onto a single antenna (Barr et al 2008). Prawns treated with
86 the noxious chemicals showed an increased grooming of that specific antenna and
87 also of rubbing that antenna against the sides of the tank compared to controls. This
88 appeared too complex to be merely nociceptive reflexes and was interpreted as
89 being consistent with the idea of pain. Further, when terrestrial hermit crabs compete

90 with ants for access to carrion, the ants spray the crabs with formic acid and drive
91 the crabs away from the food resource (McNatty et al. 2009). This indicates that the
92 acid is aversive and causing the retreat from an important resource is consistent with
93 the idea of pain (Elwood & Appel 2009). Further, the crabs keep away from areas
94 with large numbers of ants suggesting avoidance learning (Elwood & Appel
95 2009). These studies and interpretations, however, were put into doubt when three
96 other species of decapod showed no responses to either hydrochloric acid or sodium
97 hydroxide (Puri & Faulkes 2010). This latter study questioned whether crustaceans
98 had nociceptors for noxious chemicals and suggested the evidence for such
99 receptors was weak. Recently, the same authors found no aversion by crayfish
100 (species) to eating chillies or wasabi, which contain capsaicin and isothiocyanate,
101 and those substances rubbed on the antennae failed to cause grooming (Puri &
102 Faulkes 2015). Nevertheless, crayfish did respond with vigorous escape responses
103 when touched with a hot soldering iron. Thus studies on decapods show they
104 respond to heat but the evidence for extreme pH nociceptors is contradictory (Barr et
105 al. 2008; Puri & Faulkes 2015).

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

116 Elucidating nociceptive capability has significant implications on the welfare of
117 the species. Evaluating the capacity for nociception in different invertebrate species
118 aids in understanding the evolution of that sensory modality and thus bears on the
119 potential for suffering in these animals. Such research also has the potential to
120 create new models for human pain (Puri & Faulkes 2010). Evidence for pain-like
121 states in crustaceans is growing (Denti et al. 1998; Kawai et al. 2004; Patterson et al.
122 2007; Barr et al. 2008; Elwood and Appel 2009; Appel and Elwood 2009a, b; Puri &

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

128

129 **2. Methods**

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

139 Each crab was each brought from the cold room to an adjacent observation
140 room at about 20°C where it was immediately tested singly in a glass tank (62cm x
141 25cm x 25cm). The tank contained gravel, rocks and seawater (just enough to
142 moisten the gravel). The area was lit by a 100 W bulb (2060 Lux) suspended over
143 the tank and 2 minute recording made using a hand-held Sony Handycam
144 (HDRXCX240EB) HD camcorder, which was moved if the crab moved, to facilitate
145 recording. The same person (ND) did all manipulations and recording and thus was
146 not blind to treatments.

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149 2.1 Experiment one: application to mouth

150

151 Crabs (N=60) were randomly assigned (dice) to one of four experimental treatments,
152 10% acetic acid, distilled water (control), capsaicin (0.018g per 10ml mineral oil,
153 which is approximately the capsaicin concentration of a scotch bonnet chilli), mineral
154 oil (control) but with equal numbers per group. The crab was held in one hand and
155 the treatment was then brushed onto the mouth after gently prying open the

156 maxillipeds (2 brush strokes per treatment). Small individually coloured brushes were
157 used to ensure that each brush was used for one treatment only.

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

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

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176 2.2 Experiment two: application to eyes

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

186 The scores for each activity and the time of holding down the eye were
187 analysed using a two-factor ANOVA (factor 1: acetic acid or water applied to one
188 eye; factor 2: capsaicin or mineral oil to the other eye). For the time that the eyes

189 were held down we conducted two analyses, one for each eye. First we focus on the
190 specific eye that had acid or water. Factor 1 is acid or water, whereas factor 2 is the
191 oil or capsaicin applied to the alternative eye. Next we focus on the eye that had
192 capsaicin or oil. Factor 1 is capsaicin or oil to that eye and factor 2 is the effect of
193 acid or water to the alternative eye. This design allowed for us to examine if one eye
194 is only responsive to treatments to that specific eye or if it responds to treatments
195 given to the other eye. Again alpha was set at $P < 0.05$ and power tests presented for
196 significant results [using Statview](#).

198 2.3 Ethics

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

206 3. Results

207 3.1 Experiment one: application to the mouth

208 Several activities differed significantly between treatment groups. These were mouth
209 part movements, ($F_{3,56}=19.26$, $P < 0.0001$, Power = 1.0)(figure 1), claws scratching at
210 mouth ($F_{3,56}=14.24$, $P < 0.0001$, Power = 1.0)(figure 2) and escape ($F_{3,56}=6.49$, $P =$
211 0.0008 , Power = 0.97)(figure 3). In each case animals treated with acetic acid had
212 the highest scores and post hoc tests (Tukey's) showed that in all cases the acetic
213 acid group was significantly different from each of the other three groups ($P < 0.01$ all
214 cases) whereas the other three groups did not differ from each other. Threat
215 displays, however, did not differ significantly across the groups ($F_{3,56}= 0.84$, $P=0.47$)

219 3.2 Experiment two: application to eyes

220 Acetic acid applied to an eye caused crabs to move their mouth parts more than
221 those receiving water ($F_{1,44} = 9.78$, $p = 0.003$, Power = .88), however, there was no
222 effect of capsaicin ($F_{1,44} = 2.8$, $p = 0.1$) and no interaction between acetic acid and

223 capsaicin ($F_{1,44} = 2.8$, $p = 0.1$) (figure 4). Crabs also scratched at the their mouth
 224 using their claws more after acetic acid application compared to those with water
 225 ($F_{1,44} = 12.16$, $p = <0.001$, Power = 0.90) but capsaicin had no effect for this
 226 behaviour ($F_{1,44} = 1.05$, $p = 0.31$), and there was no interaction effect ($F_{1,44} = 1.05$, $p =$
 227 0.31) (figure 5). Acid also caused more escape behaviour ($F_{1,44} = 21.72$, $p = <0.001$,
 228 Power = 0.99) but capsaicin had no effect ($F_{1,44} = 1.47$, $p = 0.23$), and there was no
 229 interaction effect ($F_{1,44} = 2.49$, $p = 0.12$) (figure 6). For threat displays, however, there
 230 was no effect of acetic acid ($F_{1,44} = 1.45$, $p = 0.24$), or capsaicin ($F_{1,44} = 0.0$, $p = 1.0$)
 231 and there was no interaction effect ($F_{1,44} = 1.44$, $p = 0.48$).

232 We describe the responses of each eye to treatment to that specific eye and
 233 treatment to the alternative eye. Acetic acid rather than water on an eye (the
 234 acid/water eye) significantly increased the duration for which that specific eye was
 235 held down in the socket ($F_{1,44} = 4.76$, $p = 0.034$, Power = 0.56), but there was no
 236 effect on the acid/water eye of capsaicin applied to the alternative eye ($F_{1,44} = 0.12$,
 237 $p = 0.74$), and there was no interaction between acetic acid on one eye and capsaicin
 238 on the other eye ($F_{1,44} = 0.26$, $p = 0.61$) (Figure 7). By contrast, application of
 239 capsaicin rather than mineral oil to an eye (capsaicin/mineral oil eye) had no effect of
 240 withdrawal of that specific eye ($F_{1,44} = 0.52$, $p = 0.48$). Application of acetic acid rather
 241 than water to the alternate eye had no effect on the capsaicin/mineral oil eye ($F_{1,44} =$
 242 0.68 , $p = 0.42$), and there was no interaction effect ($F_{1,44} = 1.78$, $p = 0.18$). That is,
 243 acetic acid only affected the eye to which it was applied and did not affect the
 244 alternate eye, whereas capsaicin did not affect the eye to which it was applied or the
 245 alternative eye.

246

247 4 Discussion

248 Crabs with capsaicin applied to either the mouth or an eye did not differ in their
 249 responses from those treated with mineral oil control. This is in marked contrast to
 250 some other taxa such as the nematode, *Caenorhabditis elegans*, (Wittenburg &
 251 Baumeister 1999) and the leech, *Hirudo medicinalis* (Pastor et al. 1996), which
 252 responded to capsaicin. It agrees, however, with a recent study that found no effect
 253 of capsaicin applied to the antennae of crayfish on the behaviour (Puri & Faulkes
 254 2015). Further, crayfish did not avoid foods containing capsaicin and capsaicin did
 255 not affect firing of sensory neurons (Puri & Faulkes 2015). Thus neither of the two

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

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

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

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

289 We had predicted that an aversive stimulus would result in the defensive
290 threat display shown by crabs when they hold out their claws towards a potential
291 predator. However, this was seen in very few crabs and was not affected by
292 chemical application in either experiment. It appears that the stimulation from the
293 acetic acid was not perceived as a potential predatory threat from which a threat
294 display in return might benefit the crab. In both experiments, however, there was a
295 significant increase in escape responses after acetic acid application, which involved
296 relatively prolonged scrabbling and attempting to climb the walls of the container.
297 This indicates that the crabs found the substance highly aversive and is similar to the
298 escape by hermit crabs when attacked by ants spraying formic acid (McNatty et al.
299 2009). It does not appear to be a mere reflex, rather it is more like a complex goal
300 directed activity to escape the vicinity of the stimulus.

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

315 These observations of Barr et al. (2008) and Barr (2009) and those of the
316 present study are in marked contrast to those of Puri and Faulkes (2010) in which
317 three species of decapods (Louisiana red swamp crayfish, *Procambarus clarkii*,
318 white shrimp, *Litopenaeus setiferus*, and grass shrimp, *Palaemonetes paludosus*)
319 showed no response to hydrochloric acid applied to the antennae. Thus, whilst
320 hydrochloric acid had no effect on those three species, acetic acid has considerable
321 effect when applied to antennae, eyes and mouth of other two other decapods (Barr

322 et al. 2008). It seems unlikely that this is a species effect and it might be that
323 different acids act differently on nociceptors. The responses, however, do not appear
324 to be specific to one acid because formic acid also appears to evoke marked
325 responses and avoidance in hermit crabs (McNatty et al. 2009). Dyuizen et al. (2012)
326 also reported abnormal behaviour after a cheliped of crabs (*Hemigrapsus*
327 *sanguineus*) was injected in the distal joint of the cheliped with 1% formalin. This
328 involved flexion, extension and shaking of the claw. These crabs also showed
329 rubbing of the injected claw and 20% autotomized the appendage. These activities
330 were not seen in saline injected controls. Further, sodium hydroxide applied to an
331 antenna of glass prawns caused rubbing and grooming (Barr et al. 2008) but not in
332 tests on three other species by Puri and Faulkes (2010). However, those authors did
333 report strong responses of crayfish, including tail flip escape responses, when
334 touched with a hot soldering iron. Electric shock also causes marked behavioural
335 change, including giving up valuable resources to escape from the shock (Magee &
336 Elwood 20013, 2016; Appel & Elwood 2009b). Thus, decapods show marked
337 changes in behaviour after treatment with various stimuli that are noxious to
338 vertebrates and, presumably, these changes are mediated by nociceptors. However,
339 capsaicin does not appear to stimulate decapod nociceptors and it is also not
340 effective in birds (Jordt & Julius 2002).

341

342 4.1 Conclusions

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

351 Acetic acid has been used to induce pain-like behaviour in vertebrates, for
352 example in trout (Sneddon et al. 2003) and mice (Gawade 2012). Trout injected with
353 acetic acid into the lip showed rubbing of that area against the gravel bottom of the
354 tank and against the glass wall. They also showed a rocking movement. These

355 anomalous activities declined with analgesic treatment. Mice injected with acetic acid
 356 show writhing of the body and this is reduced by analgesic administration and has
 357 been used to test analgesic efficiency in pain tests (Gawade 2012). We did not
 358 attempt to determine if the responses of crabs to acetic acid were reduced by
 359 application of local anaesthetics because that had been shown with glass prawns
 360 (Barr et al. 2008). Formalin has been used to assess pain in rats by injection into a
 361 paw (Abbott et al. 1995) and results in lifting, licking and shaking of the specific paw.
 362 That is the behaviour of vertebrates are remarkably similar to decapods when
 363 similarly treated with noxious chemicals. Thus, using the argument by analogy
 364 (Sherwin 2003), the responses of the decapods are consistent with the idea of pain.
 365 We stress, however, that total proof of pain is not possible in any animal (Elwood
 366 2011, Stamp Dawkins 2012). Pain is often presumed in vertebrates but, with
 367 invertebrates, that possibility is often rejected, even when the evidence is similar
 368 (Sherwin 2003).

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374

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377

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512 **Figure legends:**

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

517

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

520

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

523

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

527

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

530 One treatment of acid or water was given to one eye and oil or capsaicin to the other
531 eye.

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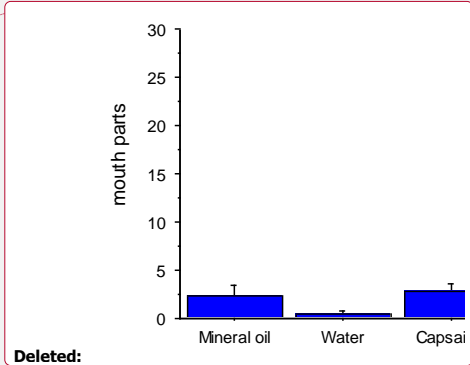
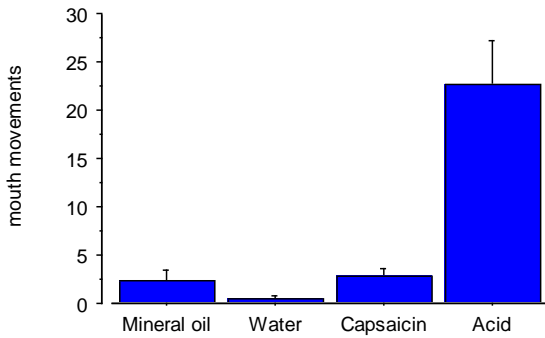
533 Fig 6. Experiment 2. Mean (\pm SE) of escape activities, recorded as the number of 5
534 second periods the activity was shown per 2 minutes. One treatment of acid or
535 water was given to one eye and oil or capsaicin to the other eye.

536
537 Fig 7. Experiment 2. Mean (\pm SE) duration (sec) for which the eye receiving either
538 acetic acid or water was held down in the eye socket. The alternative eye received
539 either capsaicin or mineral oil.

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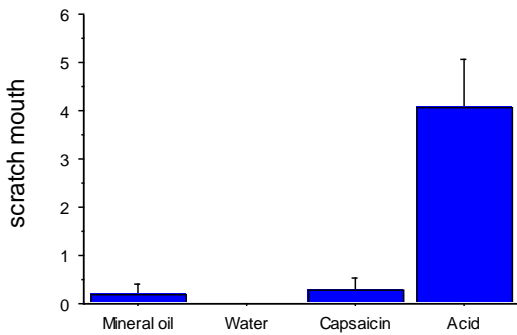
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Fig 1



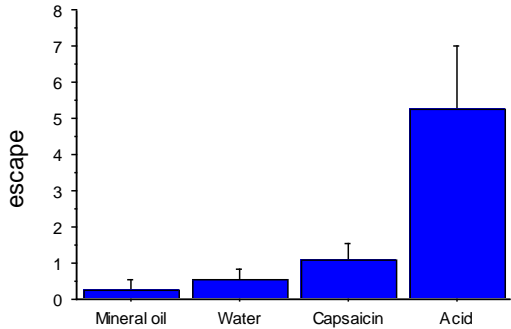
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Fig 2

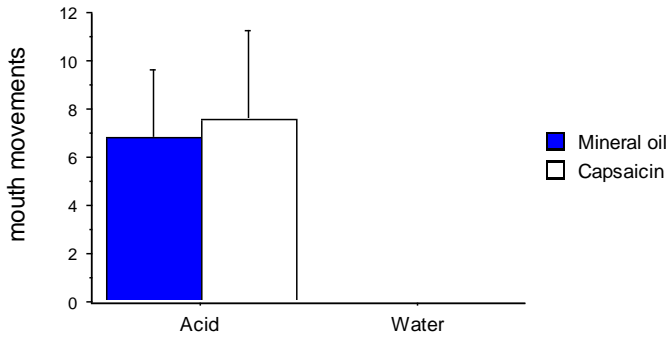


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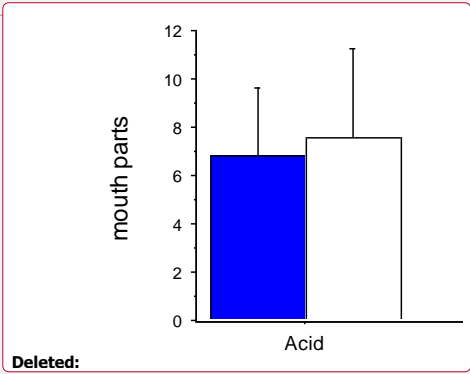
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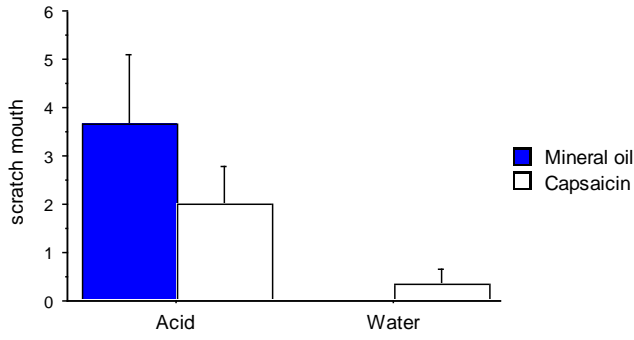
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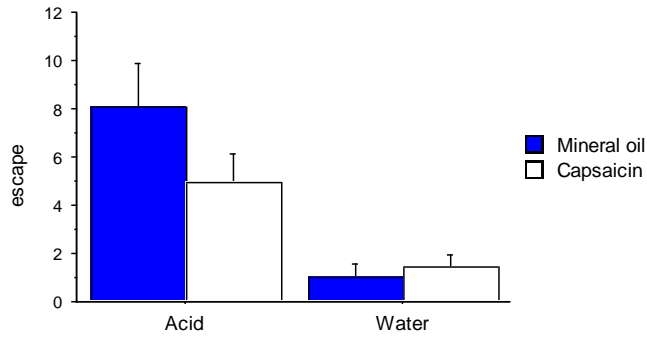
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Fig 5



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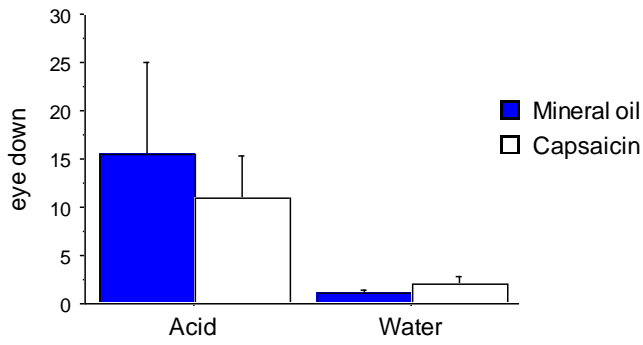
Fig 6



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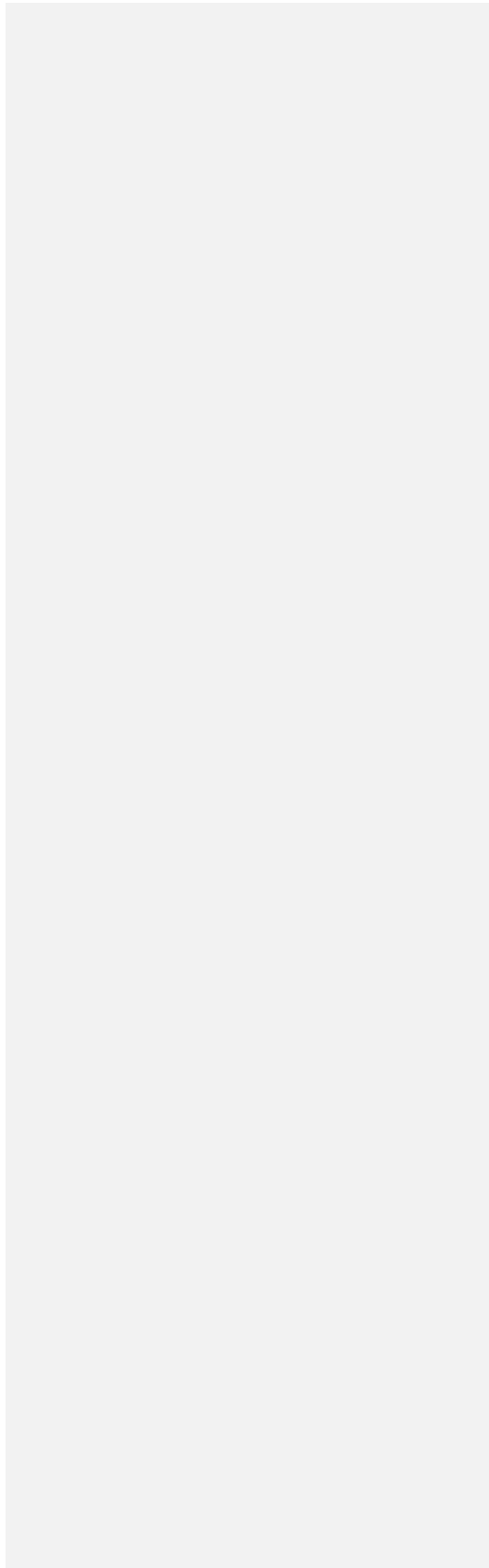
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Fig 7



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