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Pain in Aquatic Animals

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KEYWORDS

animal pain, crustaceans, experimental ethics, fish, molluscs, neurobiology, nociceptors

ABSTRACT

*Recent developments in the study of pain in animals have demonstrated the potential for pain perception in a variety of wholly aquatic species such as molluscs, crustaceans and fish. This allows us to gain insight into how the ecological pressures and differential life history of living in a watery medium can yield novel data that inform the comparative physiology and evolution of pain. Nociception is the simple detection of potentially painful stimuli usually accompanied by a reflex withdrawal response, and nociceptors have been found in aquatic invertebrates such as the sea slug *Aplysia*. It would seem adaptive to have a warning system that allows animals to avoid life-threatening injury, yet debate does still continue over the capacity for non-mammalian species to experience the discomfort or suffering that is a key component of pain rather than a nociceptive reflex. Contemporary studies over the last 10 years have demonstrated that bony fish possess nociceptors that are similar to those in mammals; that they demonstrate pain-related changes in physiology and behaviour that are reduced by painkillers; that they exhibit higher brain activity when painfully stimulated; and that pain is more important than showing fear or anti-predator behaviour in bony fish. The neurophysiological basis of nociception or pain in fish is demonstrably similar to that in mammals. Pain perception in invertebrates is more controversial as they lack the vertebrate brain, yet recent research evidence confirms that there are behavioural changes in response to potentially painful events. This review will assess the field of pain perception in aquatic species, focusing on fish and selected invertebrate groups to interpret how research findings can inform our understanding of the physiology and evolution of pain. Further, if we accept these animals may be capable of experiencing the negative experience of pain, then the wider implications of human use of these animals should be considered.*

Introduction: The occurrence of nociception and pain

The ability to detect dangerous, damaging stimuli is adaptive in terms of survival, and thus the evolution of an early warning system in animals seems intuitive. Indeed, the nociceptive system, which detects noxious, harmful, injury-causing stimuli such as extremes of temperature, high mechanical pressure and irritant chemicals, has been identified in invertebrates (e.g. *Drosophila* and *Caenorhabditis elegans*; Tobin and Bargmann, 2004; Neely et al., 2010; Im and Galko, 2012) through to humans (reviewed in Sneddon et al., 2014). Nociception is the simple perception of a noxious event and is typically accompanied by a reflex withdrawal response away from the source of damage. In humans, negative ‘feelings’ of discomfort or suffering are experienced alongside the injury and this is termed pain. The concept of pain occurring in animals has been extensively debated, with some authors suggesting only primates and humans can

experience the adverse affective component as they possess a human (or similar in primates) neocortex (Rose, 2002; Rose et al., 2014). Opposing this opinion, scientists suggest that the negative experience that accompanies tissue damage is crucial in altering an animal's subsequent behaviour to perform protective and guarding reactions, enabling the animal to avoid such stimuli in future, and for avoidance learning to occur (Sneddon et al., 2014). This implies that the unpleasant internal state of experiencing pain goes hand in hand with its perception as it has to be such a strong aversive stimulus to ensure animals will alter future behaviour and learn from the event. If this were not the case, animals would continue to damage themselves repeatedly, resulting in disease, loss of limbs and even mortality. It is unlikely that animals living in very different environments will have developed the same nociceptive or pain-detecting neural machinery as humans. Evolution and life history place very diverse pressures on different animal groups as well as exposing them to differing types of nociceptive stimuli. For example, fish living in an aquatic world can maintain buoyancy, so the risk of collision due to gravity is likely to be rare compared with a terrestrial vertebrate (Sneddon, 2004). Therefore, evolution, ecology and life history may have shaped nociceptive and pain systems in aquatic animals to meet the demands of their environment in quite a dissimilar way to terrestrial animals (Broom, 2001; Rutherford, 2002).

Studies on the bird brain have challenged old dogma on brain evolution and have shown that the theory of linear and progressive evolution, where the cerebral cortex was believed to have evolved from lower to higher vertebrates, with birds at an intermediate stage, are incorrect (see Jarvis et al., 2005 for review). The avian cerebrum was proposed to be relatively simple in structure and controlled primitive behaviours (Ariëns Kappers et al., 1936). Modern experimentation demonstrates that the avian cortex developed from pallial (or cortical-like), striatal and pallidal regions from a common avian/reptilian ancestor. The avian pallial region is differentially organized compared with the mammalian pallium or cortex in that the avian region is nuclear and the mammalian cortex is layered. However, the pallia of the two groups perform similar functions and have comparable connectivity. Thus, the avian cortical regions are different from the mammalian or human cortex but have evolved analogous roles. Thus, we should expect that other non-mammalian vertebrate taxa may have brain structures that differ from the mammalian cortex anatomically, but that may have evolved to perform similar functions. This example demonstrates how the study of disparate animal groups can enlighten us about the evolution of the nervous system. In this review, the evidence for pain and nociception in wholly aquatic animals will be considered by discussing the differences with terrestrial groups to ascertain how the watery environment may have resulted in anatomical, neurobiological and functional differences. Unfortunately, there are negligible data on aquatic mammals and aquatic birds, and very few data published on aquatic forms of amphibians (see Guenette et al., 2013); therefore, this review will focus upon the aquatic animal groups where there is significant empirical evidence, specifically fish, cephalopods and crustaceans.

Definitions of animal pain have proposed that there are two key criteria animals must fulfil to be considered capable of experiencing pain (Table 1; Sneddon et al., 2014). The first is that whole-animal responses to a noxious, potentially painful event differ from those to innocuous stimulation. More specifically, it is suggested that: animals must have the neural apparatus to perceive damaging stimuli with nociceptors, often free nerve endings that specifically detect harmful stimuli; the information is conveyed to the central nervous system (CNS) from the periphery; central processing occurs involving brain areas that innervate motivational and emotional behaviour and learning; physiological responses are altered that may be linked to a stress response; behavioural alterations are not simple reflexes, with long-term responses including protective behaviours and avoidance as part of the response; and finally all of these reactions should be reduced by the use of analgesics or painkillers (although note that these may only be effective in mammals and specific compounds may not necessarily work in invertebrates with different receptors, etc.; e.g. Barr and Elwood, 2011). The first invertebrate species in which nociceptors were identified was the leech (*Hirudo medicinalis*), an aquatic annelid inhabiting freshwater (Nicholls and

Baylor, 1968). These nociceptors have similar properties to mammalian nociceptors including responding to multiple types of noxious, damaging stimuli, classifying them as polymodal (Pastor et al., 1996). Further studies have demonstrated that analgesic compounds and stimulation of adjacent touch receptors can reduce nociceptor activity (Higgins et al., 2013; Yuan and Burrell, 2013), the latter being comparable with gate control in mammals whereby touch modulates pain transmission (Melzack and Wall, 1965). Therefore, the physiological and molecular mechanisms may be highly conserved between aquatic invertebrates, vertebrates and terrestrial vertebrate groups (Sneddon et al., 2014).

The second criterion is that the pain experience should result in the animal basing future behavioural decisions upon this negative event such that the animal's motivation is altered. For example, the animal may seek analgesia or pay a cost to access analgesia to reduce its pain, or may incur a cost to avoid the noxious stimulus and learn to avoid subsequent encounters; it may also perform less well on competing tasks, or act inappropriately when in pain such that pain is an attention-dominating state. Sneddon et al. (2014) propose that these criteria should not be considered in isolation but that specific animal groups must fulfil both sets to be considered capable of experiencing pain (Table 1). Thus, this provides a framework to identify how far aquatic animals meet these criteria, and where future studies are needed if evidence is currently lacking. This review will consider the evidence in fishes and selected invertebrate groups (crustaceans and molluscs), where there are substantial data from studies exploring nociception and pain, to discuss why differences occur between these animal groups and terrestrial animals that may inform our understanding of the evolution and comparative neurobiology and physiology of this important survival system (Table 2).

Pain in fish

Prior to 2002 it was believed that fish could not perceive pain because nociceptors, receptors that preferentially detect potentially painful stimuli, had not been identified (Rose, 2002). However, using electrophysiology and neuroanatomical approaches, nociceptors were subsequently identified in a teleost or bony fish, the rainbow trout (*Oncorhynchus mykiss*), for the first time (Fig. 1A; Sneddon, 2002; Sneddon, 2003a). Previous research found receptors responsive to damaging stimuli in a jawless fish, the lamprey (Matthews and Wickelgren, 1978), but other studies have failed to find nociceptors in elasmobranchs using neuroanatomy, where there is an absence of unmyelinated C fibres, which act as one type of nociceptor in mammals (e.g. Snow et al., 1996). The trout nociceptors were of two fibre types, C fibres and small diameter myelinated A-delta fibres, with three classes of nociceptor identified, including polymodal (responsive to mechanical, thermal and chemical stimuli), mechanothermal (no response to chemicals) and mechanochemical (no response to temperature) (Sneddon, 2003a; Ashley et al., 2006, 2007; Mettam et al., 2012).

The electrophysiological properties of the trout nociceptors are comparable to those found in mammalian models (Fig. 1B; Sneddon, 2004, 2012). Differences do occur in that trout nociceptors are not responsive to cold temperatures below 4°C (Ashley et al., 2007); this species can encounter very low temperatures and thus, in evolutionary terms, it would be adaptive for the fish nociceptors to be unresponsive. It would be interesting to test a tropical species of fish, as they would not have evolved to tolerate such extremes of cold. Anatomical and electrophysiological studies have found that a small proportion of fish nociceptors are innervated by C fibres (4–5%; Sneddon, 2002; Roques et al., 2010), in contrast to terrestrial vertebrates where some 50% of nociceptors are C fibres (Young, 1977), although reptiles have much fewer C fibres (Terashima and Liang, 1994). C fibres in mammals contribute to dull, 'thudding' pain whereas the faster conducting A-delta fibres are believed to signal 'first' pain to the CNS. Sceptics have suggested that the small number of C fibres means fish do not experience pain (Rose et al., 2014). Given the differences in lifestyle, morphology and so on, fish neuroanatomy is not identical to the human system; however, A-delta fibres conduct at a faster speed, so perhaps the fish system is more rapid and

efficient. When considering the ecology, life history and evolutionary pressures, fish live in an aqueous world and therefore there will be a difference in how damage occurs to fish compared with terrestrial animals. Buoyancy of fish in water means less damage due to gravity (falling), noxious chemicals may be more diluted in aquatic water bodies and changes in temperature are less dramatic compared with terrestrial environments; thus, pain from gravity, extremes of temperature and noxious chemicals may be experienced to a greater degree by terrestrial animals. This is just a hypothesis, but irrespective of this the trout electrophysiological studies show clearly that trout A-delta fibres act in the same way as mammalian C fibres, reacting to a variety of noxious stimuli, and many are polymodal nociceptors (Sneddon, 2002, 2003a,b; Ashley et al., 2006, 2007; Roques et al., 2010; Mettam et al., 2012).

Table 1. The two key principles and detailed criteria for pain in animals

Whole-animal responses to potentially painful events differ from innocuous stimulation	Change in motivational behaviours after a potentially painful event
<ul style="list-style-type: none"> • Possession of nociceptors, pathways to CNS, evidence of central processing involving areas that regulate motivated behaviour (including learning and fear) • Nociceptive action responsive to endogenous modulators (e.g. opioids in vertebrates; FMRFamide in <i>Aplysia</i>) • Nociception activates physiological responses linked to stress or an elevated state over and above stress (one or a combination of the following alterations: respiration, heart rate or hormonal levels (e.g. cortisol in some vertebrates)) • Evidence that responses are not just a nociceptive withdrawal reflex • Alterations in behaviour over longer term that reduce future encounters with the harmful stimulus • Protective behaviour such as wound guarding, limping, rubbing, licking or excessive grooming • All of the above reduced by analgesia or local anaesthetics 	<ul style="list-style-type: none"> • Self-administration of analgesia • Pay a cost to accessing analgesia • Selective attentional mechanisms whereby the response to the noxious stimulus has high priority over other stimuli; the animal does not respond appropriately to competing events (e.g. presentation of predator; reduced performance in learning and memory tasks) • Altered behaviour after noxious stimulation where changes can be observed in conditioned place avoidance and avoidance-learning paradigms • Relief learning • Long-lasting change in memory and behaviour especially those relating to avoidance of repeat noxious stimulation • Avoidance of the noxious stimulus modified by other motivational requirements as in trade-offs, e.g. hungry animal will return to area where pain was given to seek food after a relevant period of time • Evidence of paying a cost to avoid the noxious stimulus
<p><i>These criteria must be fulfilled in their totality for an animal to be considered capable of pain. Adapted from Sneddon et al. (2014) with kind permission from Elsevier.</i></p>	

Thus far, most anatomical and electrophysiological studies have been conducted on teleost or bony fish with relatively few on elasmobranch (cartilaginous) fish. The very few published findings tend to be lacking in experimental detail (Leonard, 1985); however, a more recent study in the long-tailed stingray (*Himantura fai*) has confirmed some of the previous experiments in that there is a lack of unmyelinated C fibres, but small myelinated fibres are in abundance and could potentially be A-delta fibres (Kitchener et al., 2010). However, electrophysiological studies are needed to determine whether nociceptors occur in this group. It may be that as many sharks, skates and rays engage in biting behaviour, causing often extensive injuries during breeding and courtship, this group has lost the capacity, or has a limited capacity, for pain (e.g. Kajiura et al., 2000; Porcher, 2005); this, combined with evidence that healing is relatively slow (Heupel et al., 1998; Ashhurst, 2004), means tissue damage may be less problematic than for other species. However, Porcher (2005) reported that courtship bite wounds in the blackfin reef shark, *Carcharhinus melanopterus*, healed within 10 days. These findings in elasmobranchs are especially interesting because the evolutionary predecessor of both teleosts and elasmobranchs, the agnathan sea lamprey, has nociceptors (Matthews and Wickelgren, 1978). Caution should be applied to ruling out nociception in sharks, skates and rays given the very meagre number of published studies.

Table 2. The criteria for animal pain and whether these criteria are shown by terrestrial vertebrates or aquatic animals including teleost fish, cephalopods and decapod crustaceans

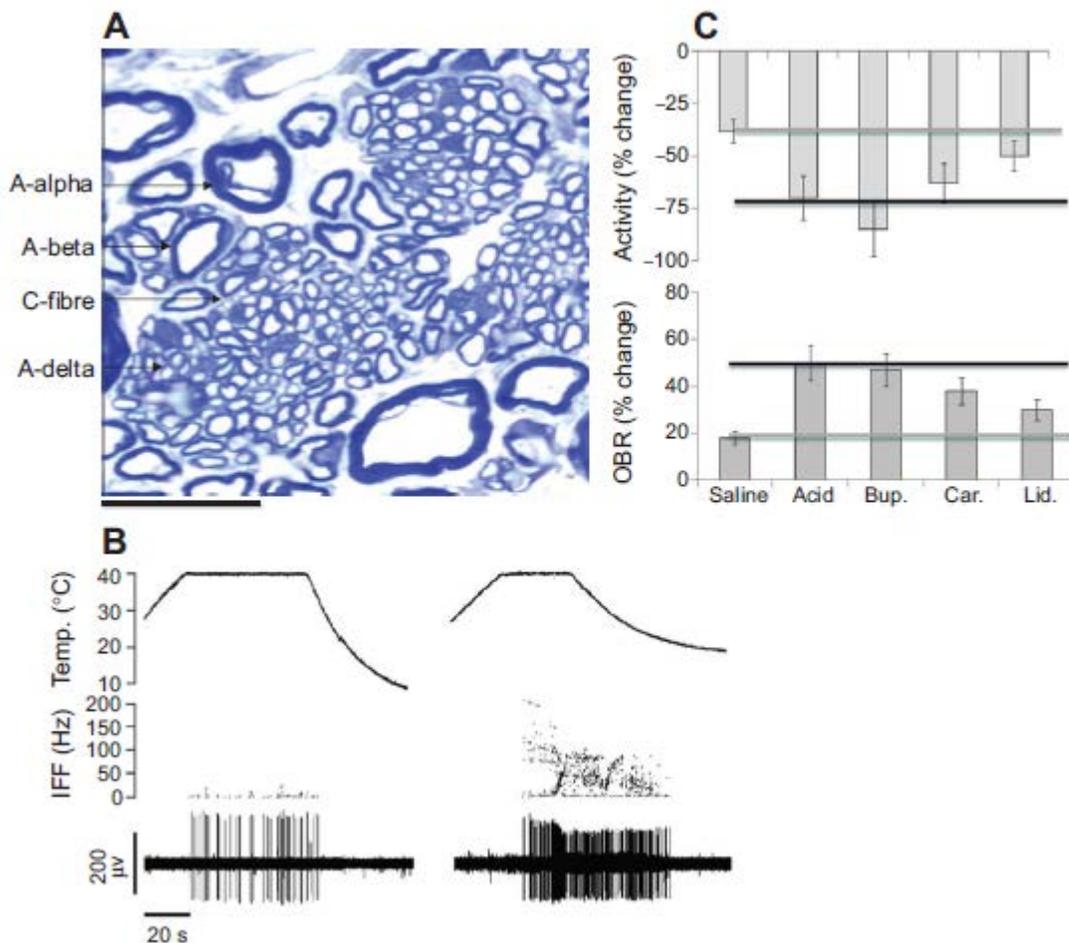
	Terrestrial vertebrates (mammals, birds, reptiles and amphibians)	Fish (teleosts)	Molluscs (cephalopods)	Crustaceans (decapods)
Nociceptors	Yes	Yes	Yes	Yes
Pathways to CNS	Yes	Yes	Yes	Yes
Central processing in CNS	Yes	Yes	Yes	Yes
Receptors for analgesic drugs	Yes	Yes	Yes	Yes
Physiological responses	Yes	Yes	Yes	Yes
Movement away from noxious stimuli	Yes	Yes	Yes	Yes
Abnormal behavioural changes	Yes	Yes	Yes	Yes
Protective behaviour	Yes	Yes	Yes	Yes
Responses reduced by analgesic drugs	Yes	Yes	Yes	Yes
Self-administration of analgesia	Yes ¹	Yes	Not yet	Not yet
Responses with high priority over other stimuli	Yes ²	Yes	Yes	Yes
Paying a cost to access analgesia	Yes ³	Yes	Not yet	Not yet
Altered behavioural choices/preferences	Yes	Yes	Yes	Yes
Rubbing, limping or guarding	Yes ¹	Yes	Yes	Yes
Paying a cost to avoid stimulus	Yes ¹	Yes	Not yet	Yes
Trade-offs with other requirements	Yes ¹	Yes	Not yet	Yes

'Yes' indicates the criterion is shown; 'Not yet' signifies a lack of empirical evidence for the criterion. Adapted from Sneddon et al. (2014) with kind permission from Elsevier. ¹ Not yet shown for amphibians and reptiles. ² Some studies show pain is imperative whereas others demonstrate reduced pain behaviour when birds are hungry or placed in novel circumstances. ³ Not yet shown for amphibians, birds or reptiles

Several tract tracing studies have demonstrated that the fish neuroanatomical pathways from peripheral areas to the brain are highly conserved. In the long-tailed stingray, dorsal horn distinguishable layers are apparent as seen in the equivalent of the spinal cord of mammals, and the synaptic ultrastructure is broadly similar to that of the dorsal horn of rodents and other mammals (Kitchener et al., 2010). The main tracts, including the spinothalamic and trigeminal, which convey pain from the body and face, respectively, are similarly organized (review in Sneddon, 2004), and within the teleost brain there are various connections to the thalamus and cortical areas (Rink and Wullimann, 2004) that innervate pain processing in mammals. Furthermore, multiple brain areas are active during noxious stimulation [e.g. studies of gene expression in the forebrain, midbrain and hindbrain of common carp, *Cyprinus carpio*, and rainbow trout (Reilly et al., 2008a); electrical activity in all brain areas in Atlantic salmon, *Salmo salar* (Nordgreen et al., 2007), goldfish (*Carassius auratus*) and rainbow trout (Dunlop and Laming, 2005); activity using functional magnetic resonance imaging (fMRI) in common carp (Sneddon, 2011)]; thus, activity differs from that in response to innocuous stimuli and is not limited to the hindbrain and spinal cord nociceptive reflex centres (Rose, 2002). More complicated responses occur in the whole brain, possibly innervating the prolonged behavioural responses described below. Finally, a range of analgesic drugs have been shown to be effective in ameliorating the pain-related changes in behaviour and physiology seen in fish when painfully stimulated (Fig. 1C; Sneddon, 2003b; Sneddon et al., 2003a; Mettam et al., 2011; reviewed in Sneddon, 2012). Opioid receptors as well as the action of non-steroidal anti-inflammatory drugs (NSAIDs) on cyclo-oxygenase (COX2) enzyme are also highly conserved

between fish and mammals (reviewed in Malafoglia et al., 2013). Thus, the pain neural apparatus in fish is directly comparable with the mammalian system and operates similarly.

Fig. 1. Anatomical, electrophysiological and behavioural evidence of pain in rainbow trout. (A) Section of the maxillary branch of the trigeminal nerve of the rainbow trout showing the presence of A-delta and C fibres that may act as nociceptors ($\times 1000$; scale bar, $2\ \mu\text{m}$). Adapted from Sneddon (2002) with kind permission from Elsevier. **(B)** Electrophysiological recordings from a nociceptive receptive field on the trout face showing responses of nociceptors to heat stimulation. This illustrates sensitization of a mechanothermal receptor to heat following noxious chemical stimulation. The firing response to ramp and hold heat stimulation is shown before (left) and 9 min after (right) subcutaneous injection of 1% formalin, $<1\ \text{mm}$ from the receptive field. The upper trace shows the heat stimulus, the middle panel plots the instantaneous firing frequency (IFF) as scatter graphs and the lower trace shows an extracellular single unit recording from the trigeminal ganglion. Thermal threshold remains the same but firing frequency is greatly increased following formalin injection. Adapted from Ashley et al. (2007) with kind permission from Elsevier. **(C)** The percentage change in activity (top) and opercular beat rate (OBR; bottom) in rainbow trout 30 min after they were injected subcutaneously with saline or a noxious substance (0.1% acetic acid), or acid combined with intramuscular injection of the opioid buprenorphine ($0.1\ \text{mg}\ \text{kg}^{-1}$; Bup.) or the non-steroidal anti-inflammatory drug (NSAID) carprofen ($5\ \text{mg}\ \text{kg}^{-1}$; Car.), or injected at the same site as the acid with the analgesic lidocaine ($1\ \text{mg}$; Lid.). The grey line represents the impact of saline (control) treatment whereas the black line represents the impact of pain (acid injection). Adapted from Mettam et al. (2011) with kind permission from Elsevier.



When exploring whole-animal behavioural and physiological responses to potentially painful stimuli, there is much evidence for fish withdrawing from a noxious event. Common carp (*C. carpio*) withdrew from electrical stimulation with reduced responses after anaesthetic was administered, yet normal motor activity was unaffected (Chervova and Lapshin, 2011). Fish learn to avoid electric shocks usually in one or a few trials (e.g. Yoshida and Hirano, 2010). This avoidance behaviour persists for up to 3 days (Dunlop et al., 2006), but after 3 days of food deprivation fish will risk entering the shock zone to obtain food (Millsopp and Laming, 2008). This demonstrates that teleost fish find electric shocks so aversive they alter their subsequent behaviour. Among the sharks and rays, the ampullae of Lorenzini detect low-frequency (i.e. 0.5 Hz) electric fields (Murray, 1962; Kalmijn, 1971; von der Emde, 1998). Elasmobranchs possess an acute sensitivity to electric fields, facilitating the development of shark repulsion devices (SRDs) that create an electrical field around the wearer to prevent large sharks attacking them. Exposure to SRDs results in muscle spasms and motivates sharks to leave the area (www.sharkshield.com; Broad et al., 2010). Whether this is nociceptive or not is unknown.

In vivo administration of potentially painful stimuli results in prolonged, complicated responses (reviewed in Sneddon, 2009). Opercular beat rate (ventilation of the gills) is dramatically increased more than in a stress response in rainbow trout and zebrafish (*Danio rerio*) when they are injected with noxious chemicals. Additionally, an increase in plasma cortisol has been recorded in rainbow trout (Sneddon, 2003b; Ashley et al., 2009) and Mozambique tilapia (*Oreochromis niloticus*; Roques et al., 2012). Behavioural responses are also affected; for example, decreased swimming observed after painful treatment (Sneddon, 2003b; Reilly et al., 2008b; Correia et al., 2011; Roques et al., 2012). Guarding behaviour (such as avoiding using an area in which a painful stimulus has been administered) has been recorded in trout, who avoid eating after a painful injection to the lips for up to 3 h (Sneddon, 2003b); sham-handled (anaesthetized only) and saline-injected controls resume feeding after 80 min as do acid-injected fish when treated with a painkiller.

Mammals show very different behavioural responses to pain between species (Flecknell et al., 2007) and these species-specific responses have been shown in fish. For example, piauçu (*Leporinus macrocephalus*) injected with formalin and Nile tilapia that had had the tail fin severed increased swimming (Roques et al., 2010; Alves et al., 2013). In contrast, Mozambique tilapia subjected to electric shock and Atlantic salmon experiencing abdominal peritonitis due to vaccination decreased swimming (Bjørge et al., 2011; Roques et al., 2012). Therefore, these disparate responses highlight that pain indicators will have to be quantified on a species-by-species basis and to different modes of pain in fish. Pain-related changes in behaviour last from 3 h up to 2 days and are not simple instantaneous nociceptive reflexes (Sneddon, 2003b; Bjørge et al., 2011). In rainbow trout, acetic acid is used below 2% when injected subcutaneously, as concentrations above this destroy nociceptor activity (Ashley et al., 2007; Mettam et al., 2012). However, acid concentrations above 5% are needed to elicit pain-related behavioural responses in common carp (Reilly et al., 2008b). It would appear that the cyprinids are more robust and possibly have a higher pain threshold, again demonstrating species-specific differences.

When potentially painful events are applied to fish, anomalous, novel behaviours, such as tail beating in zebrafish, are elicited. When zebrafish were injected with acid near the tail fin, they performed vigorous tail fin wafting, yet were not swimming and activity was reduced (Maximino, 2011). Other examples of anomalous behaviours only observed in response to noxious chemical injection are rocking to and fro on the substrate by trout and carp, and rubbing of the injection site by trout and goldfish (Sneddon, 2003a,b; Sneddon et al., 2003b; Reilly et al., 2008b; Newby et al., 2009). These responses have only been reported in fish given a potentially painful treatment and were not observed in sham-handled controls and saline-treated fish, nor were they reported in toxicological studies. This suggests these anomalous

behaviours are a direct result of the painful treatment, and studies have shown they are reduced by painkillers (Sneddon, 2003a; Mettam et al., 2011).

Because fish do not feed when in pain (Sneddon, 2009), it is difficult to attempt the type of analgesic self-administration paradigms where food or water is laced with a painkiller and animals can self-select the drugged water or food to reduce their pain (e.g. Pham et al., 2010). However, will fish pay a cost to accessing analgesia? If the internal experience of pain is aversive then they should sacrifice either effort or access to a resource or favourable area to obtain pain relief. Zebrafish, given the choice between a bare, brightly lit chamber or a less brightly lit, enriched chamber with a stimulus shoal and enrichment of gravel and plants, chose the enriched chamber and spent most of their time there on consecutive occasions (Sneddon, 2012). When these fish were given either acid or saline as an innocuous treatment, they still chose their preferred enriched chamber. However, zebrafish that were painfully treated lost their preference for the preferred area and spent most of their time in the unfavourable, bare chamber if an analgesic, lidocaine, was dissolved in the water of this chamber. Controls that were not noxiously stimulated but were given access to the lidocaine-dosed barren chamber did not lose their preference and spent most of their time in the enriched chamber, demonstrating that it is neither an addictive effect nor a sedative effect of lidocaine that resulted in painfully treated animals spending most of their time in an unfavourable area. This suggests that zebrafish seek to reduce their pain by forgoing the opportunity to be in a preferred area and spending time in a non-preferred chamber to access analgesia.

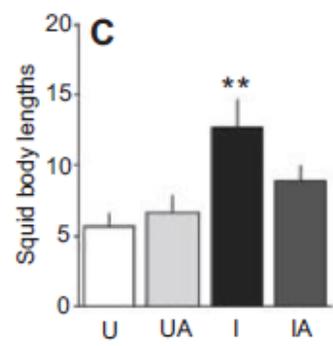
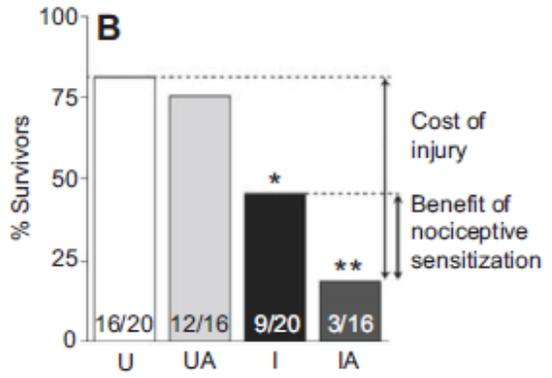
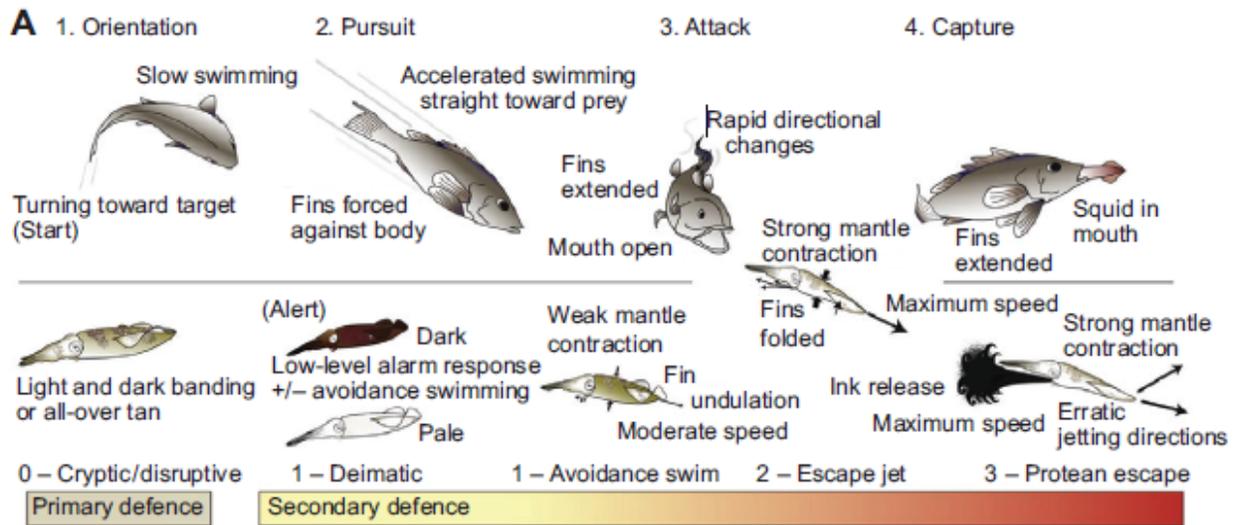
If pain is important to fish then they should perform other competing tasks less well or ignore them. Trout will ignore novel objects rather than show avoidance when in pain; however, avoidance is shown if morphine is administered to the fish (Sneddon et al., 2003a). Anti-predator behaviour such as seeking cover and escape behaviour are reduced when trout are noxiously stimulated (Ashley et al., 2009). Socially subordinate trout with high plasma cortisol concentrations exhibit almost no signs of pain, possibly because of endogenous analgesia (Ashley et al., 2009). These studies combined show that pain takes precedence over competing stimuli and that central mechanisms may be activated to ameliorate pain. When considering all of the empirical evidence together, these studies show teleost fish do fulfil the criteria for animal pain as proposed by Sneddon et al. (2014; Table 1).

Molluscs

Molluscs and other invertebrates have a substantially different CNS from that of vertebrates; thus, pain in invertebrates is subject to debate (Crook and Walters, 2011). Rather than cover the wider issues concerning the existence of pain and suffering, which are comprehensively addressed by other authors (e.g. Crook and Walters, 2011; Elwood, 2011), this review will focus on the empirical evidence. Molluscs are a highly diverse group with substantial divergence in body plan, life history and ecology, living in both terrestrial and aquatic habitats. Evidence for nociception does exist in aquatic bivalves, gastropods and cephalopods, with nociceptors identified in many species (reviewed by Crook and Walters, 2011). *Aplysia californica* is one of the leading models for the study of nociception with electrophysiological properties highly conserved between molluscs and mammals. It is interesting to note that whilst *Aplysia* is an established model for nociceptor activity and nociceptive behavioural responses, the same argument is not accepted for a pain experience in that these nociceptive responses are consistent with a negative affective component linked to suffering and discomfort. *Aplysia* has nine central ganglia innervating some 10,000 neurons with cell bodies in peripheral nerve nets (Cash and Carew, 1989), so has a relatively simple CNS. The nociceptors found in *Aplysia* exhibit comparable electrophysiological properties to those of mammals (Illich and Walters, 1997; Walters and Moroz, 2009), and noxious stimulations results in reflex withdrawal of the gill, siphon, tail and head, and ink ejection (Kandel, 2001; Crook and Walters, 2011). Indeed, damage to tissues and subsequent stimulation result in sensitization of *Aplysia* nociceptors and enhanced withdrawal reflex responses. *Aplysia* also show non-reflexive responses such as escape

movements and protective behaviours as well as suspension of feeding (Walters et al., 1981; Kandel, 2001). Most investigations have focused on the plasticity of primary nociceptors and their central synapses to defensive motor neurons, so little is known about other aspects of nociceptive processing. Opioids inhibit nociceptive transmission in vertebrates and although there is some pharmacological evidence for their action in invertebrates, there are currently no direct data on molecular signalling of opioid receptors in nociception (Dores et al., 2002). However, the peptide FMRFamide does produce inhibitory effects on *Aplysia* nociceptor activity via reduced excitation and synaptic transmission, resulting in decreased withdrawal reflexes; thus, the CNS does have internal mechanisms for reducing the perception of damage (Small et al., 1992).

Fig. 2. Impact of injury on anti-predator flight behaviour and survival in squid. (A) The predator-prey sequence of seabass feeding upon squid prey. (B) Injured squid lacking nociceptive sensitization had the lowest odds of survival at the conclusion of a 30 min trial with free interaction of squid and seabass. Squid in the injured (I) and injured treated with anaesthetic to prevent nociceptor sensitization (IA) groups had lower overall survival, showing that predators target injured animals compared with the uninjured (U) and uninjured with anaesthetic (UA) group. IA squid were most likely to be killed. The difference in survival between the U and the IA group can be considered the cost of being injured, while the difference in survival percentage between the IA and I groups ($P=0.05$) reveals the benefit that nociceptive sensitization provides to injured animals. Odds ratios are given in the bars: $*P\leq 0.05$, $**P<0.01$. (C) Flight initiation distance. Injured squid fled from predators at a greater distance and thus were more sensitive to predator threat than the other groups ($**P<0.01$). Adapted from Crook et al. (2014) with kind permission from Elsevier.



Nociceptors have only recently been identified in cephalopods (e.g. Crook et al., 2013; Alupay et al., 2014). Cephalopods (which comprise cuttlefish, nautilus, octopus and squids) are considered to have the most highly developed CNS of all invertebrates, and this is reflected in their complex behavioural repertoire and cognitive ability (Broom, 2007; Crook et al., 2011). They possess brains that are divided into specialized areas or lobes, which process incoming stimuli integrating somatosensory, visual and chemosensory information. In contrast to the relatively small number of neurons in *Aplysia*, cephalopods possess some 500 million cells in the CNS and a complex arrangement of cell bodies innervating the periphery (Young, 1963). Noxious stimuli such as mechanical damage do elicit withdrawal responses in cephalopods, and the squid *Doryteuthis pealeii* is responsive to mechanical damage but not to heat (Crook et al., 2013). It is unlikely that in the marine environment that this squid inhabits it will come into contact with noxious heat (>30°C), so it is improbable that the nociceptor system will have evolved to detect such high temperatures. Thermal stimulation greater than 30°C does excite nociceptors in rainbow trout (Sneddon, 2002, 2003a,b; Ashley et al., 2007) and elicits behavioural responses in goldfish (Nordgreen et al., 2009). Of course, these are freshwater species and they live in relatively small water bodies in contrast to the oceans; these aquatic environments are known to increase in temperature to over 30°C during heatwaves or in desert climates (e.g. Carveth et al., 2006); thus, it would be important for these animals to avoid these lethal temperatures. Neural and behavioural sensitization have been recorded in *D. pealeii* for up to 48 h after damage. However, enhanced activity 1 day after injury was seen in sensory neurons on both sides of the body rather than at the site of injury, suggesting that long-lasting nociceptive information is not location specific as seen in vertebrates (Crook et al., 2013).

Does tissue damage in molluscs alter their motivational state such that their subsequent behavioural decisions are based upon noxious stimulation? In *Aplysia*, experiments have shown that these animals can learn to associate a neutral stimulus, the smell of shrimp, with electric shock. Indeed, when the smell was presented alone, *Aplysia* froze in what could be considered a motivated fear response, and when innocuous light touches were applied, these animals responded in an overt manner with escape movements, ink directed at the location of the stimulus and withdrawal (Walters et al., 1981). Therefore, previous experience with a noxious event was remembered and the animal's motivational responses were altered. In the octopus *Abdopus aculeatus*, animals exhibited hypersensitivity to touch and wound-directed behaviours, whereby other arms would curl around the injured arm; this protective behaviour persisted for 24 h (Alupay et al., 2014). Crook et al. (2014) investigated whether increased nociceptive sensitivity affected anti-predator behaviour by examining whether injured *D. pealeii* altered their escape strategy (Fig. 2). Injured squid reacted to predators at a greater distance than non-injured animals; thus, sensitization has evolved as a survival tactic, demonstrating that nociception motivates protective behaviours to reduce predation risk. Indeed, in animals that were anaesthetized during injury, sensitization did not occur, making these individuals more vulnerable to predation.

Crustaceans

Crustaceans are a diverse group comprising crabs, lobsters, crayfish, shrimps, krill and barnacles with a hard exoskeleton. As with cephalopods, crustaceans can autotomize limbs and regenerate them. Nociceptors with a precise receptive field have not been identified in crustaceans yet. Indeed, Puri and Faulkes (2010) applied noxious chemicals to the antennae of Louisiana red swamp crayfish (*Procambarus clarkii*), white shrimp (*Litopenaeus setiferus*) and grass shrimp (*Palaemonetes* sp.) and found no specific neuronal activity during application of acid and saw no evidence of increased grooming of antennae. In contrast, when acid was applied to the antenna of the glass prawn (*Palaemon elegans*), there was an increase in grooming of the treated antenna for up to 5 min (Barr et al., 2008). Dyuzen et al. (2012) injected formalin into a cheliped of the crab *Hemigrapsus sanguineus* and observed shaking and rubbing of the appendage, as well as reduced use, which could be considered as a protective response

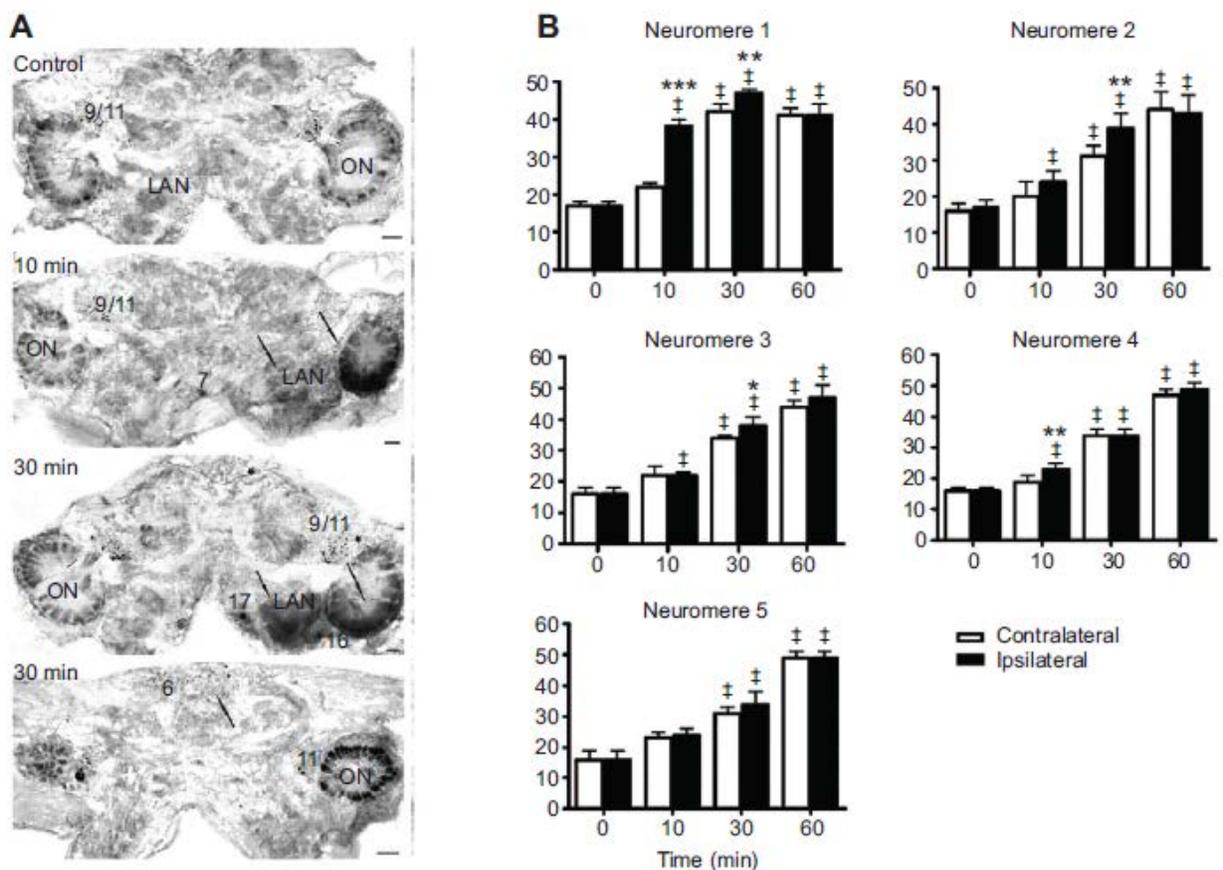
(Dyuzen et al., 2012). Concurrently, neurons expressed the enzyme nitric oxide synthase on the ipsilateral side initially; it was then recorded contralaterally in the CNS (Fig. 3). These responses were primarily detected in nerve fibres in the thoracic sensory neuropils and were associated with neurons that modulate cheliped action. The behavioural responses were observed over a 60 min period, and could be considered as a motivational change after noxious stimulation that elicits defensive reactions where cheliped use is suspended during the event. Hermit crabs (*Pagurus bernhardus*) leave their shells when given an electric shock and subsequently perform prolonged abdominal grooming (Appel and Elwood, 2009a). Hermit crabs subjected to increasing electric shocks left their shell at a reduced shock intensity when the shell was from a less preferred species than did those in shells of a more desirable species (Appel and Elwood, 2009b). This demonstrates that the soft-bodied hermit crab is willing to risk predator attack by evacuating its shell to avoid a noxious stimulus and that this is dependent upon how valuable the shell is. Shore crabs (*Carcinus maenas*) do show avoidance learning to electric shock. When these crabs were given the choice of two shelters, they switched their initial preference if they were given an electric shock in the first choice shelter (Magee and Elwood, 2013). These studies suggest that there is behavioural evidence of nociception and that the experience alters subsequent behavioural decisions; however, very little is known regarding the neurobiology of nociception in crustaceans.

Exploring motivational change in the crayfish (*Procambarus clarkii*), Fossat et al. (2014) tested the animals in a fear paradigm, the elevated plus maze, where animals chose to walk on a cross where two arms were lit and two were dark. Crayfish subjected to electric shock displayed enhanced fearfulness or anxiety and chose the dark arms more than the light. The shocked crayfish had relatively higher brain serotonin concentrations coupled with elevated blood glucose, which suggests a stress response (Patterson et al., 2007). The anti-anxiety drug chlordiazepoxide, a benzodiazepine, resulted in reduced turnover of serotonin in mammals (Antkiewicz-Michaluk et al., 1975). Fossat et al. (2014) found that this drug reduced fearfulness in the crayfish, showing that these reactions may be mediated by the serotonergic system as demonstrated in mammals; however, further investigation is required to confirm this. The behavioural responses may be comparable with vertebrate anxiety and indicate the ability of crayfish to exhibit a state similar to mammalian fear.

Implications for the use of aquatic animals

We have a complicated relationship with many aquatic animals where we use them as an important foodstuff, an experimental model, essential species within our conservation efforts and a source of entertainment in angling, public exhibits and scuba diving or for companionship as pets (Sneddon, 2013). If we adopt the precautionary principle where we give animals the benefit of the doubt as to their internal experience of negative states such as pain, then we should apply ethical thinking to how we treat all animals. This does not necessarily mean that we should avoid using such animals but that we should do so as humanely as possible in order to improve their welfare whilst under our care. If animals fulfil all criteria as proposed by Sneddon et al. (2014) then they should be considered as capable of experiencing pain. The evidence from teleost fish is compelling; therefore, this should be considered when making decisions regarding the ethics of their use and welfare. However, there is much evidence yet to be gathered for other aquatic invertebrates and it is essential that we do not rule out pain in these animals where gaps in our knowledge exist. Instead, future studies should explore whether aquatic invertebrates do meet pain criteria (or not, as the case may be). It is important to avoid placing human needs or biology on to an animal that has a completely different life history, experiences different environmental demands, and has been subject to entirely distinct evolutionary pressures that have shaped its nervous system. Thus, clever experimentation should consider these factors when designing approaches to make them relevant to the test species.

Fig. 3. Dynamics of NADPH-d/iNOS activity in the brain of *Hemigrapsus sanguineus* after nociceptive stimulation. (A) The distribution of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), a histochemical marker for inducible nitric oxide synthase (iNOS), in the olfactory lobe (ON), the lateral antenna 1 neuropils (LAN), antenna II neuropils (AnN) and cluster 9/11 is shown in control animals. Ten minutes after nociceptive injury, NADPH-d staining increased in the ON, LAN (arrows) and cluster 17. Thirty minutes after injury, NADPH-d activity maximally increased in the ON and LAN (arrows), and solitary NADPH-d-positive neurons appeared in clusters 9/11 and 6 (arrowheads). The bottom panel shows NADPH-d-positive neurons that appeared in cluster 6. Scale bars, 100 μ m. (B) The temporal dynamics of NADPH-d activity (optical density) in leg neuromeres 1–5 of *H. sanguineus* induced by nociceptive stimulation. Data are shown for the side contralateral to injury and ipsilateral to injury. In the graphs, double daggers represent $P < 0.05$ when compared with intact (and control) animals whose activity was unchanged during the observation period; asterisks indicate the difference obtained when contralateral and ipsilateral sides of the same neuromere were compared ($*P < 0.05$, $P < 0.01$, $***P < 0.001$). Results represent the mean \pm s.e.m. from five different sections of five animals. Adapted from Duyizen et al. (2012).**



By accepting that animals experience pain, we must consider the manner in which they are treated by refining existing procedures in aquaculture, fisheries, angling, experimentation, public exhibits and the ornamental animal industry. There are many examples of consumers paying more for better welfare in terrestrial farm animals; for example, eggs from free-range chickens. This also applies to fish, where consumers are now demanding to know where the fish and shellfish are caught and the method used. Lack of consideration for fisheries has led to unsustainable fishing practices and population crashes of important species (Sneddon and Wolfenden, 2012) as well as the death of non-target animals such as

birds, cetaceans, turtles and so on in fishing apparatus. Surely it is better to be proactive and ensure the wellbeing of fish and other aquatic animals for improved economic return in aquaculture, fisheries and the ornamental fish trade.

Recently, the European Directive (Directive 2010/63/EU) included all cephalopod species as protected animals in experimentation in Europe. Fish as vertebrates are already included in the regulations of many countries. However, there is very little known regarding the welfare of aquatic animals in comparison with terrestrial vertebrate models. More specific information on appropriate analgesic protocols for fish and cephalopods is lacking. If experimental procedures cause tissue damage but the study of pain or nociception is not the goal of the research, appropriate analgesia should be applied to minimize the impact on animals, so further research is required. Improved welfare can also result in better quality outputs from experimental studies. Research in rodents has shown that animals with better welfare kept in improved conditions yield higher quality data with reduced intraspecific variation (Singhal et al., 2014).

Conclusions

The study of a diverse range of animal taxa can yield interesting insights into the evolution and comparative biology of nociception and pain. Generally, the electrophysiological properties of nociceptors are conserved from molluscs through to teleost fish and mammals. Common behavioural responses such as withdrawal, suspension of normal behaviour and performance of behaviours that promote protection and healing are seen in crustaceans, molluscs and fish. After a potentially painful event, there are also examples of modifications in motivation that demonstrate the significance of a damaging event in terms of learning, avoidance and altered behavioural decisions based upon the experience associated with tissue injury. Differences in nociception and pain between terrestrial animals and aquatic animals are apparent and thus life history, ecology and evolutionary history need to be factored into experimental design. The ethical treatment of animals has many benefits economically and scientifically; thus, the treatment of animals should be informed by empirical evidence with regard to the capacity of animals to experience negative affective states.

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Competing interests

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