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Toward a Novel Model of Pain in Zebrafish: Exposure to Water Containing Dilute Concentrations of Acetic Acid

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Abstract

Existing models of pain in zebrafish (*Danio rerio*) require injection of acetic acid into localized areas including the lips. We are currently developing an alternative assay of pain in zebrafish that involves immersion in dilute concentrations of acetic acid. This assay involves placing subjects in a 120 mL beaker containing 100 mL of water taken from the subject's original tank. After a 20-minute acclimation period, the experimental substances are added, if applicable. Subjects are exposed to the experimental substances for 30 minutes (unless otherwise specified), after which they are returned to their original tanks. A series of studies was conducted to determine the optimal concentration of acetic acid to be used in this model, to determine any changes in behavioral response over two hours of exposure, and to investigate the effect of concomitant exposure to morphine sulfate (MS) on top-dwelling behavior. A significant increase in top-dwelling behavior was observed upon exposure to 0.03% acetic acid. This response remained relatively constant over the two-hour time course analysis. These results demonstrate a significant, replicable increase in top-dwelling behavior upon exposure to 0.03% acetic acid. The three concentrations of MS tested herein did not significantly affect top-dwelling behavior in the presence of acetic acid, so the underlying state motivating this behavior is unclear. These results could suggest that a pain state is not motivating the top-dwelling behavior. Alternatively, it is possible that the doses of MS used in the current study are sub-threshold. After further investigation, this paradigm could serve as a model for use in future pain research.

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Note to Readers

This thesis is the product of the exciting and engaging work that I have completed over the past two and a half years with zebrafish at Macalester College. The idea for this project began as a research proposal for my Research in Psychology II course, and following this idea to its current state has been a challenging but very rewarding experience. Due to the extended duration of this project, readers should be aware that I have written about portions of this study in the past. Sections of this paper include updated versions of text that have appeared in my Directed Research paper and in scientific abstracts that have been (or will be) presented at national and international conferences. However, I felt it necessary to include all phases of this project in the current paper in order to provide a more holistic view of this idea and how it has evolved into its current state.

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Chapter 1: Introduction

Toward a novel model of pain in zebrafish: Exposure to water containing dilute concentrations of acetic acid

According to the International Association for the Study of Pain, pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 2012). Acute and chronic pain conditions can severely limit quality of life in humans, and the same is presumably true for non-human animals. Due to the subjective nature of painful experiences, however, knowledge about, and treatment of, pain is limited. Consequently, more research on pain and its treatment is warranted.

As it is currently defined, the perception of pain requires the sensation of a stimulus with the potential to cause tissue damage, followed by a negative sensory and emotional experience. In order to produce pain, a noxious stimulus first must activate nociceptors. This activation must then be relayed to the central nervous system through fiber tracts for further processing. These two steps constitute nociception, or "the neural process of encoding noxious stimuli" (IASP, 2012). It should be noted that nociception can be reflexive, as is the case for spinal reflexes, and pain perception is not implied in the term "nociception". In order to experience pain, an animal must experience a negative sensory and emotional state. This second component of pain is difficult to measure empirically and is the source of much scientific debate in the field of animal research. Nevertheless, animal models are integral components of pain research.

Anatomy of a pain assay

Although animals are unable to communicate a negative sensory and emotional experience verbally, this negative experience can often be inferred through studies of their physiology and behavior (Bateson, 1991; Smith & Boyd, 1991). Smith & Boyd (1991) and Bateson (1991) propose that the physiological structures necessary for the perception of pain include:

1. Nociceptors
2. A cerebral cortex
3. Fiber tracts connecting nociceptors to the cerebral cortex, and
4. An endogenous opiate system (Bateson, 1991; Smith & Boyd, 1991).

Animals must possess the sensory apparatus for the sensation of noxious stimuli and the transfer of these stimuli to structures in the CNS including the cerebral cortex. In addition, these authors require that an animal capable of perceiving pain must possess an endogenous opiate system. Although the significance of this criterion might not appear to be intuitively obvious, it is actually very important in demonstrating the ability of an animal to perceive pain. One of the central purposes of the endogenous opiate system is to suppress pain during times in which the perception of pain is not evolutionarily advantageous. In the case of a soldier who has been wounded in combat, the endogenous opiate system allows the soldier to continue to fight, or at least to find safety, before immobilizing pain sets in. An animal that is able to perceive pain must therefore have an endogenous opiate system in order to suppress the perception of pain at times when a pain response is not evolutionarily advantageous. Similarly, there would be little need for an endogenous opiate system in an animal that was not capable of perceiving pain. An extraneous system such as this would likely be eliminated eventually through the process

of natural selection. Possession of an endogenous opiate system is therefore an integral criterion in determining the pain-sensing capabilities of animals.

In addition to these physiological criteria for pain perception, Bateson (1991) and Smith and Boyd (1991) have defined a set of behavioral criteria for pain perception. Behaviorally, these authors suggest that animals capable of perceiving pain should:

1. Respond to painful stimuli in ways that minimize tissue damage
2. That these behaviors should be antagonized by known analgesics, and that
3. Animals should be capable of learning to avoid painful stimuli (Bateson, 1991; Smith & Boyd, 1991).

Animals capable of perceiving pain should therefore behave in ways that indicate that experiencing a pain state is a biologically significant state for the individual.

In addition to these criteria for determining an animal's pain-perceiving capabilities, a set of criteria that should be present in a particular model of pain have been developed. Dubner (1994) proposes a set of criteria that should be included in an animal assay of pain:

1. It should distinguish between responses to innocuous and noxious stimuli.
2. The behavioral response or responses to be measured should vary in magnitude with changes in stimulus intensity over a range from threshold to tolerance.
3. Multiple threshold and suprathreshold behavioral measures should be used to infer pain.
4. The model should be susceptible to behavioral and pharmacological manipulations that alter the perceived intensity of noxious stimuli.
5. The modification of behavioral responses by nonsensory variables such as attention, motivation, and motoric ability should be distinguishable from effects on sensory capacities.
6. There should be little or no tissue damage with repetitive stimulation. (p. 294)

This definition is very inclusive and can be applied to a variety of assays. These criteria allow for assays including both reflexive and more complex behaviors. Furthermore, this

definition allows for pain elicited by thermal, electrical, mechanical, or chemical stimuli (Dubner, 1994; LeBars, Gozariu, & Cadden, 2001).

Current animal models of pain were reviewed by Mogil (2009). The author reports that the vast majority of pain research utilizes assays based in rats and mice, but other common assays are based in the dog, the cat, and the rabbit. Although Mogil acknowledges that these models have facilitated valuable advances in the study of pain, he encourages the development of novel assays to address the weaknesses of current models. He suggests that new assays based on spontaneous and non-reflexive behaviors could provide more clinically relevant results.

The use of chemical stimuli in novel pain assays could address these weaknesses. Le Bars, Gozariu, and Cadden (2001) review the advantages of using chemical stimuli in behavioral assays of pain in non-human animals. The authors propose that chemical pain best approximates acute pain in humans because it is inescapable, leads to stereotyped changes in behavior within a species, and lasts longer than other classes of painful stimuli. These factors are advantageous for a model of acute pain because they decrease the likelihood of purely reflexive behaviors and increase the likelihood of behaviors representative of tonic pain (LeBars et al.).

The emergence of fishes as subjects in pain assays: Physiological evidence

The development of pain assays in fishes began with the characterization of their physiological capabilities for nociception. First, fish were discovered to possess a cerebral cortex (Northcutt, 1981; Ito & Yamamoto, 2009). Next, nociceptive fibers

(Sneddon, 2002) and receptors (Sneddon, 2003b, Mettam, McCrohan, & Sneddon, 2011) were identified. Lynne Sneddon has been at the forefront this research, and the work from her lab has provided a foundation on which most subsequent pain research in fishes has been built. Finally, the endogenous opiate system in zebrafish was identified (Alvarez et al., 2006; Barrallo, G-Malvar, Gonzalez, Rodríguez, & Traynor, 1998; Barrallo, Gonzalez-Sarmiento, Alvar, & Rodríguez, 2000; Gonzalez-Nuñez & Rodríguez, 2009; Pinal-Seoane et al., 2006).

A foundational study by Sneddon (2003b) investigated whether rainbow trout (*Oncorhynchus mykiss*) possess nociceptors sensitive to mechanical, thermal, and chemical stimuli. Throughout the procedure, microelectrodes were used to record afferent neural activity in the trigeminal ganglion. Because all receptors associated with A-delta and C fibers are sensitive to mechanical stimulation, a glass probe was first applied to the skin in order to map the receptive fields of each receptor. After receptors were identified, Sneddon applied mechanical, thermal, and chemical stimuli to the receptive fields of these cells in order to determine the sensitivity of the receptor. In order to determine chemosensitivity, she applied one drop of 1% acetic acid to each receptive field and observed the resulting neural activity. Fast-adapting mechanoreceptors, slow-adapting mechanoreceptors, polymodal nociceptors, mechanothermal nociceptors, and mechanochemical receptors were identified. With regard to chemical receptors, she identified one class of polymodal nociceptors that respond to mechanical, thermal, and chemical noxious stimulation. This receptor class responded selectively to noxious thermal stimulation, so it was considered a nociceptor. She also identified a class of

mechanochemical receptors, though this experiment did not investigate whether these receptors responded to all mechanical and chemical stimuli or the noxious stimuli exclusively. Both the polymodal nociceptors and the mechanochemical receptors appeared to be associated with A-delta fibers.

These mechanochemical receptors were further characterized by Mettam, McCrohan, and Sneddon (2011). The authors investigated a variety of noxious and non-noxious chemical irritants in rainbow trout to determine whether the mechanochemical receptors identified by Sneddon (2003b) were true nociceptors. The noxious chemicals tested were acetic acid, carbon dioxide, low pH, citric acid, citric acid phosphate buffer, and sodium chloride. Non-noxious stimuli included ammonium chloride, bile, sodium bicarbonate, and alarm pheromone. Similar to the Sneddon (2003b) study, electrodes were first placed in the trigeminal ganglion in order to record activity, and receptive fields were mapped using mechanical stimulation from a glass electrode. Experimenters applied a 15-µL drop of the chemicals of interest directly to the area of the receptive field using a pipette, then observed electrophysiological responses in the trigeminal ganglion. It was found that both the polymodal and the mechanochemical receptors responded exclusively to noxious substances, suggesting that they are nociceptors.

The results of the studies reported by Sneddon (2003b) and Mettam, McCrohan, and Sneddon (2011) suggest that fishes possess nociceptors. These results therefore fulfill the first criterion for the ability to perceive pain proposed by Bateson (1991) and Smith & Boyd (1991).

The cortical structures of teleost fishes have also been investigated. Fishes are some of the most evolutionarily distant vertebrates from humans, and as such, their brain structures differ considerably from mammalian and human brain structures. Many mammals, including humans, have a six-layered neocortex, and evidence suggests that this neocortex allows them to process and perceive painful stimuli (Rose, 2002). While fishes do not possess a neocortex, research suggests that they possess a rudimentary cortex surrounding the surface of the cerebrum (Northcutt, 1981; Ito & Yamamoto, 2009).This cortex appears to receive sensory information from a variety of sensory modalities, and this tissue appears to have numerous connections with other structures including the thalamus and the inferior lobe (Northcutt, 1981). It is possible that some of these connections could contribute to basic processing mechanisms that are functionally equivalent to those found in the neocortex of higher vertebrates (Ito & Yamamoto, 2009), meeting the second physiological criterion proposed by Bateson (1991) and Smith and Boyd (1991).

Sneddon investigated the third criterion for pain-sensing capabilities of teleost fish, which requires that animals possess fibers connecting nociceptors to the cerebral cortex, by studying the trigeminal nerve in rainbow trout, *Oncorhynchus mykiss* (2002). In mammals and birds, the trigeminal nerve is used to relay afferent signals from the head, including nociceptive signals, to the CNS (Dong, Chudler, Sugiyama, Roberts, & Hayashi, 1994). In mammals and birds, nociceptive signals are generally carried by bundles of small, myelinated A-delta fibers and by small, unmyelinated C fibers. In this study, Sneddon (2002) aimed to determine whether fishes possess these fiber types as a

first step in determining their pain-sensing capabilities. She used histological techniques paired with image analysis software to determine the size, and therefore the classification, of each fiber type. The trigeminal ganglion was composed of approximately 53% A-beta fibers, 33% A-delta fibers, 9% A-alpha fibers, and 4% C fibers. Furthermore, she used electrophysiological recording to determine the speed at which these fibers conducted information in order to verify that the fibers she identified were A-delta and C fibers. Although the relative percent of C fibers was found to be lower in rainbow trout compared to humans or other mammals, this study establishes that fishes possess fiber tracts linking nociceptors to the central nervous system, fulfilling the third criterion for pain perception identified by Bateson (1991) and Smith and Boyd (1991).

Numerous studies also provide evidence that fishes possess an endogenous opiate system. Gonzalez-Nuñez and Rodríguez (2009) reviewed existing evidence of an endogenous opiate system in zebrafish. The three major classes of opioid receptors present in humans have also been identified in zebrafish: δ (Barrallo, G-Malvar, Gonzalez, Rodríguez, & Traynor, 1998; Pinal-Seoane et al., 2006), μ (Barrallo, Gonzalez-Sarmiento, Alvar, & Rodríguez, 2000), and κ (Alvarez et al., 2006; reviewed by Gonzalez-Nuñez & Rodríguez, 2009). Furthermore, Gonzalez-Nuñez, Barrallo, Traynor, & Rodríguez, (2006) conducted a competitive binding assay on zebrafish brain homogenates and found that morphine displaced radiolabeled [^{3}H]diprenorphine ligands that were bound to endogenous opiate receptors. This evidence suggests zebrafish possess a variety of opioid binding sites and that morphine binds to these sites in the brains of zebrafish. The authors therefore conclude that zebrafish possess an endogenous opioid

system that is similar to the endogenous opioid system of mammals (Gonzalez-Nuñez & Rodríguez, 2009). This evidence suggests that zebrafish fulfill the fourth physiological criterion necessary for the perception of pain defined by Bateson (1991) and Smith and Boyd (1991).

Taken together, the evidence from these studies of teleost physiology meets all of the physiological criteria for pain perception proposed by Bateson (1991) and Smith and Boyd (1991).

The emergence of fishes as subjects in pain assays: Behavioral evidence

In addition to establishing the physiological criteria for pain perception in fishes, researchers have constructed a body of behavioral evidence suggesting that fishes are capable of perceiving pain. Sneddon and colleagues have conducted extensive research into the behavioral responses of fishes to potentially painful stimuli, including identification of a behavioral change in response to a putative nociceptive stimulus (Sneddon, Braithwaite, & Gentle, 2003) and antagonism of this behavior by an analgesic (Sneddon, 2003a). Furthermore, reports demonstrate that teleost fishes are capable of learning to avoid painful electric shocks (Donlop, Millsopp, & Laming, 2006), meeting the last of Bateson's (1991) and Smith's and Boyd's (1991) criteria necessary for the perception of pain. This evidence therefore suggests that it is very possible that teleost fishes are capable of perceiving pain.

After Sneddon (2003b) demonstrated that rainbow trout possess nociceptors, Sneddon, Braithwaite, and Gentle (2003) went on to investigate the behavioral response

of these fish to injections of noxious stimuli. After anesthetizing the fish, researchers injected saline (control), 0.1 % acetic acid, or bee venom into the lips (Sneddon, Braithwaite, & Gentle, 2003). Opercular (gill) beat rate, quantitative behavioral measures, and qualitative behavioral observations were recorded. It was found that opercular beat rate increased significantly after treatment in all groups but that the groups that received injections of acetic acid or bee venom demonstrated the greatest increase in opercular beat rate. Furthermore, it was found that the fish injected with acetic acid or bee venom took significantly longer to resume feeding compared to fish in the control group. There was not a significant difference in quantitative behavioral measures including swimming activity (defined as "direct movement of fishes for more than one body length" (p. 1117)) or use of cover in any of the groups. However, the authors noted some qualitative behavioral changes. Subjects that received injections of acetic acid exhibited complex behaviors such as rocking motions (defined as "moving from side to side balancing on either pectoral fin while resting on the gravel" (p. 1118)) and rubbing their lips against gravel. The authors concluded that these behaviors were complex and could not be attributed to simple reflexive responses, suggesting that they could be indicative of a pain response.

With respect to Bateson's (1991) and Smith's and Boyd's (1991) criteria for pain perception, these results fulfill the first behavioral criterion, which requires that animals should respond to painful stimuli in ways that minimize tissue damage. It was found that subjects that received an injection of noxious chemical irritants into the lips took significantly longer to resume feeding compared to fish in the control group. This

behavior helped to minimize damage to the injected tissue by minimizing its use, protecting it, and allowing it time to heal (Sneddon, 2009). It should be noted that the authors did not determine whether these behaviors were antagonized by the concomitant administration of an analgesic, so the underlying state motivating this change in behavior cannot be ascertained from this study.

In order to determine the underlying state motivating this change in behavior, Sneddon (2003a) investigated whether the prototypical analgesic morphine reduces the behaviors reported by Sneddon et al. (2003). After subjects were anesthetized, they received an injection of saline into the lips, an injection of 0.1% acetic acid into the lips, an intramuscular injection of 0.3 mg/mL morphine, or an injection of 0.1% acetic acid into the lips with an intramuscular injection of 0.3 mg/mL morphine. Opercular beat rate, quantitative behavioral measures, and qualitative behavioral measures were recorded after subjects recovered from the anesthesia. The behavioral changes in the acetic acid group were consistent with those reported by Sneddon et al. (2003); opercular beat rate, rocking behaviors, and rubbing the lips against the gravel increased significantly. The same results were found for the group that received morphine with acetic acid. However, further analysis revealed that subjects that received the injections of morphine and acetic acid displayed these behaviors significantly less compared to subjects that received the acetic acid injection alone. Sneddon therefore concluded that morphine reduced the pain-associated behaviors that the trout displayed after injection with 0.1% acetic acid. This evidence suggests that the behavioral response caused by the injection of the 0.1% acetic acid represents a pain response.

This evidence therefore meets the second behavioral criterion proposed by Bateson (1991) and Smith and Boyd (1991); the previously-observed change in behavior is antagonized by concomitant injections of morphine, a prototypical analgesic. Because the behavior was antagonized by an analgesic, it is likely that a pain state was motivating the increased opercular beat rate, rocking motions, and lip rubbing behavior identified by Sneddon, Braithwaite, and Gentle (2003).

Finally, learned avoidance has been demonstrated in rainbow trout and in goldfish, suggesting that teleost fish are capable of learning to avoid a noxious stimulus (Donlop, Millsopp, & Laming, 2006). In this experiment, subjects were placed in a tank and were given shocks of particular intensities depending on the quadrant of the tank in which they were swimming. When shocks were administered in certain quadrants, the fish spent less time in these quadrants, suggesting that subjects had learned to avoid them.

This evidence therefore meets the final criterion proposed by Bateson (1991) and Smith and Boyd (1991). Dunlop, Millsopp, and Laming demonstrated that teleost fish are capable of learning to avoid areas where they receive electric shocks. Taken together, these foundational studies suggest that teleost fishes are capable of perceiving pain. Consequently, behavioral assays of pain in fish have begun to emerge in the literature recently. I will now focus on the only existing model of pain in zebrafish, which was based on the model Sneddon and colleagues have developed in rainbow trout.

The lip injection model of pain in zebrafish

Soon after the lip injection model of pain was established in rainbow trout, researchers began to investigate this model in other species of fishes. This lip injection model has now been validated in a variety of species, including zebrafish (*Danio rerio*).

Reilly, Quinn, Cossins, and Sneddon (2008) first tested this assay in zebrafish. It is known that mammals exhibit species-specific differences in nociceptive behavior, so this group investigated the pain responses of three species of fish in order to determine whether behavioral responses to noxious stimuli varied between species. Zebrafish, common carp (*Cyprinus carpio*), and rainbow trout were investigated. Subjects were first anesthetized, and then they were injected with acetic acid or saline into the lips. Zebrafish received 0.05 mL injections of 5% acetic acid, rainbow trout received injections of 0.1 mL 0.1 % acetic acid, and common carp received 0.1 mL injections of either 5% or 10% acetic acid. The authors recorded behavioral measures including "swim rate" (defined as "a direct movement more than one body length" (251)), "use of cover" (defined as "percentage of time spent under cover" (251), opercular beat rate, and anomalous behaviors. Opercular beat rate increased significantly in zebrafish and in rainbow trout after injection of the acetic acid, but opercular beat rate in common carp remained constant. Swim rate decreased significantly in zebrafish and in rainbow trout after injection of the acetic acid, but no difference was observed between conditions in carp. Rainbow trout and common carp displayed anomalous behaviors including rubbing their lips against the gravel, but no anomalous behaviors were observed in zebrafish. From this data, the authors concluded that different species of fish exhibit different behavioral

responses to nociceptive stimuli. Specifically, the indicators of pain observed in zebrafish were increased ventilation rate and decreased swimming frequency in this assay.

Correia, Cunha, Scholze, and Stevens (2011) recently developed a model of acute pain in zebrafish. There were three specific aims to this series of experiments: to identify an effective dose of acetic acid to induce a behavioral response, to validate their model by reducing pain using morphine, and to test whether the analgesic effect could be reversed by the addition of naloxone. In the first study, subjects were anesthetized and injected in the lips with 5% acetic acid, 10% acetic acid, or saline. The number of tail flips the fish made per minute was recorded using Marine On-line Biomonitor System (MOBS), an electronic biosensor device which records behavioral activities of fishes by comparing low-power electrical signals emitted from one side of the tank to those received on the other side (Cunha, Goncalves, Silva, & Correia, 2008). It was found that subjects that received an injection of 5% acetic acid demonstrated significantly less tail flipping behavior compared to subjects that received a saline injection. Furthermore, it was found that subjects that received an injection of 10% acetic acid demonstrated significantly less tail flipping behavior compared to subjects in the 5% acetic acid group and the control group. The authors did not observe a greater loss of fish in the 10% acetic acid group compared to the other two groups, so they used this concentration in all of their subsequent studies. In the second study, subjects were anesthetized and received intramuscular injections of 3 mg/kg morphine, 6 mg/kg morphine, or saline. Then, subjects received injections of 10% acetic acid or saline in the lips. It was found that subjects that received an injection of morphine displayed significantly more tail flipping

behavior compared to fish that received an injection of acetic acid. The authors therefore concluded that morphine antagonized the decreased tail-flipping behavior that is observed after injection of 10% acetic acid. The final study investigated the effect of naloxone on the antinociceptive effect of morphine. Subjects were anesthetized and received intramuscular injections of 6 mg/kg morphine with saline or 6 mg/kg morphine with 6 mg/kg naloxone. Then, subjects received injections of 15% acetic acid into the lips. It was found that subjects that received naloxone exhibited significantly less tail-flipping behavior compared to subjects that did not receive naloxone, suggesting that the naloxone attenuated the analgesic effect of the morphine. The authors therefore concluded that injection of acetic acid into the lips of zebrafish is an effective model for testing pain in zebrafish because their behavioral response scales with stimulus intensity, is attenuated by morphine, and that this attenuation is reversed by the addition of naloxone.

Although there are many advantageous attributes of this lip injection model, some key features limit its applicability. One limitation is the necessity of anesthetizing each fish. A model that does not involve anesthesia would reduce the cost and the time necessary to complete the assay. Secondly, this model requires the delivery of precise injections into the lips of zebrafish. These injections take time and require great precision, further limiting the application of this model on a larger scale.

Toward a novel model of pain in zebrafish: The current study

Although the zebrafish's behavioral responses to the injection of acetic acid in the lips have been established, little is known about their behavioral responses to immersion

in water containing noxious chemicals. Mettam, McCrohan, and Sneddon (2011) explored the response of the rainbow trout's trigeminal ganglion to the topical application of noxious chemicals by placing drops of acetic acid on the receptive field of chemoreceptors, suggesting that teleost fish possess cutaneous nociceptors capable of sensing acetic acid. Furthermore, the lip injection model proposed by Correia, Cunha, Scholze, and Stevens (2011) demonstrated that zebrafish show reduced tail-flicking behaviors after injection of the lips with 10% acetic acid. However, it is possible that zebrafish behave differently in response to the presence of dilute concentrations of acetic acid administered through immersion in the water. It is important to establish this basic behavioral response in order expand the current understanding of pain in zebrafish and to better understand their use as a behavioral model of pain.

The current program of investigation aims to develop a behavioral model of pain in zebrafish. Specifically, we aim first to identify a significant behavioral response after immersing zebrafish in dilute concentrations of acetic acid. Secondly, we aim to demonstrate that this behavioral response is motivated by a pain state by showing that exposure to morphine attenuates this behavioral change. The specific hypotheses for each of the six studies contained in this paper will be presented briefly before the methods section of the appropriate study.

Zebrafish were selected as the animal to be used in this model for a number of reasons. First, zebrafish were chosen because a considerable amount of background research has already established that these animals are capable of perceiving noxious chemical stimuli, but their responses to immersion in noxious chemicals is not known.

Although a lip injection assay of pain has been established in zebrafish, their small size and the necessity of anesthetizing each fish are limitations of the current model. Secondly, zebrafish are small, and many fish can be housed in a single tank. Therefore, the amount of space needed to house these animals is less than the amount of space required for larger fishes or for rodents. Thirdly, the use of mammals is ethically questionable because their relatively large cerebral cortices increase the likelihood that they are capable of experiencing the negative affective component of pain. On the other hand, fishes have relatively smaller and less-developed cerebral cortices, and it could be argued that they are less sentient than mammals. Finally, the use of zebrafish in studies of behavioral neuroscience is increasing rapidly, and this study of pain-related behaviors in zebrafish could contribute to animal husbandry procedures. This characterization of nociceptive responses to immersion in water containing a noxious substance could aid in the identification of sub-optimal water conditions for these animals. This is an important step in maximizing the welfare of zebrafish in a laboratory setting.

In addition to contributing to knowledge of zebrafish welfare, the results of this study may contribute to the study of pain in humans by offering another model of nociception in fish. The current model offers an improvement on the existing model because it eliminates the necessity of anesthetizing and specifically injecting each fish. It therefore facilitates high-throughput phenotyping, allowing more animals to be tested in a shorter period of time. This type of model could lay the foundation for future studies of the tolerability and efficacy of novel analgesics before they are tested in rodents and

humans. Therefore, the current research could eventually contribute to increased quality of life in fishes and in humans.

Chapter 2: Methods, Results, and Discussion of Each Study

A series of six studies was conducted in order to characterize the current experimental model. First, the pH of a series of solutions of acetic acid was determined. Then, subjects were exposed to these concentrations in order to observe the behavioral response of the fish to immersion in dilute concentrations of acetic acid and in order to determine the concentration which elicited the greatest behavioral response in the absence of adverse health effects. After a significant behavioral change was observed, a two-hour time course analysis was conducted in order to identify any trends in the behavioral response over time. In order to determine whether the observed behavioral change was a response to pain, an analgesia study was conducted with morphine sulfate (MS). Another pH study was conducted to determine the pH of a series of solutions containing MS and acetic acid. A tolerability study was then conducted in order to identify any potentially toxic effects of exposure to water containing dilute concentrations of MS. No negative health effects were identified upon exposure to MS, so a larger study was conducted to determine the effect of three concentrations of MS on the observed behavioral response to acetic acid.

Study 1: Determination of pH change upon addition of acetic acid to water sample from fish tanks

Before any subjects were exposed to water containing acetic acid, a study was conducted to determine the effect of 0.01%, 0.03%, and 0.05% acetic acid on the pH of a sample of water taken from the zebrafish colony. Previous reports have suggested that a

pH of approximately 4 is tolerable to fish in the acute phase (Branson, 1993). Therefore, the pH of these three concentrations of acetic acid was measured.

Method

Apparatus

One 120 mL beaker was used to test a sample of water taken from the zebrafish colony. A Symphony VWR SB21 pH meter with a Corning G-P combo w RJ probe was used to take pH measurements.

Procedure

A time-course analysis was first conducted in order to determine whether the pH of a solution of 0.03% acetic acid (Pharmco-Aaper, Brookfield, CT) changed over 30 minutes. A 120 mL beaker was filled with 100 mL of water taken from one of the tanks in the zebrafish colony. The appropriate amount of glacial acetic acid was added to the beaker using a micropipette, and the pH of the solution was measured immediately thereafter. Measurements of pH were then taken every five minutes for the duration of the testing period.

Next, the pH of solutions of 0.01%, 0.03%, and 0.05% acetic acid were tested. A beaker was filled with 100 mL of water taken from one the tanks in the zebrafish colony, and the initial pH of the water sample was measured. The appropriate amount of glacial acetic acid was then added to the beaker using a micropipette, and the resulting pH was measured immediately. Three trials were conducted for each concentration of acetic acid. The probe was rinsed with deionized water between trials in order to prevent contamination of the tank water samples with acetic acid.

The pH of 0.01%, 0.03%, and 0.05% acetic acid were measured again on another day in order to account for daily variations in pH.

Results and Discussion

The time-course analysis of the 0.03% acetic acid solution revealed that the pH did not change greatly over the 30-minute testing period. The initial pH of the solution was 4.0, and the pH increased to 4.1 at 5 minutes. The pH stayed constant for the remainder of the 30-minute testing period. This analysis revealed that the pH of a 0.03% acetic acid solution prepared with water from the zebrafish colony remains relatively static over 30 minutes of testing.

On the first day of testing, the average initial pH of water taken from tanks in the zebrafish colony was 7.2. The average pH of the 0.01% acetic acid solution was 4.7. The 0.03% acetic acid solution had an average pH of 4.1, and the 0.05% acetic acid solution had a pH of 3.8. All results obtained on the second day of pH testing were identical to the results on the first day of testing.

Study 2: Observation of behavioral responses of zebrafish upon exposure to acetic acid and determination of acetic acid concentration necessary to cause a change in behavior

Although the behavioral response of zebrafish to injections of acetic acid into localized areas has been established (Sneddon, Braithwaite, & Gentle, 2003; Correia, Cunha, Scholze, & Stevens, 2011), the behavioral response of zebrafish to immersion in dilute concentrations of acetic acid is not known. Because injections of acetic acid appear

to cause a pain response in zebrafish (Sneddon, Braithwaite, & Gentle, 2003), and prior research has demonstrated that fish have cutaneous nociceptors that respond to acetic acid (Sneddon, 2003b; Mettam, McCrohan, & Sneddon, 2011), it was hypothesized that immersion in dilute concentrations of acetic acid would result in a behavioral change indicative of a pain response. Furthermore, it was hypothesized that the fish exposed to higher concentrations of acetic acid would exhibit a greater behavioral response. Because the behavioral response of zebrafish to exposure to water containing acetic acid is not known, one of the goals of this study was to observe this behavioral response of zebrafish to water containing 0.01%, 0.03%, and 0.05% acetic acid. Another goal of this study was to determine the lowest concentration of acetic acid that elicits a behavioral response in the absence of adverse health effects.

Method

Animals

Adult zebrafish (*Danio rerio*) obtained from a commercial supplier were housed in 10-gallon freshwater tanks. The water was maintained at 22°C, with a pH of between 6.8 and 7.4. Fish were fed *ad libitum* with Tetrafin flakes and frozen Mysis shrimp. A 20%-30% water change was performed 3-5 times weekly to maintain stable water conditions and water quality. All procedures were conducted in accord with protocols approved by the Macalester College Institutional Animal Care and Use Committee.

Apparatus

A novel apparatus was designed for these procedures. Four 120 mL beakers were used as testing chambers in this study to facilitate behavioral observation (Figure 1).

These beakers were placed side-by-side on a table and were surrounded by a barrier designed to prevent the fish from seeing the researcher. A small hole was cut into the barrier in order to permit video recording of the fish.

A Panasonic SD 2.3 megapixel camcorder was used to record the behavior of the fish. Data was recorded onto mini DV cassette tapes and was later imported to a computer using iMovie.

Research Design

This procedure was designed to characterize the behavioral response of zebrafish to water containing acetic acid and to identify the optimal concentration of acetic acid to be used to model pain. Fish were exposed to water containing 0.01% acetic acid, 0.03% acetic acid, 0.05% acetic acid, or a sample of water from the original tank (control) for a total of 31 minutes. Ten fish were exposed to each condition.

Procedure

Prior to exposure to experimental conditions, fish were selected to participate in a non-systematic manner and were transferred from their original tank into a holding tank with similar dimensions and environmental conditions. Each holding tank was assigned to undergo a particular experimental condition. The animals were allowed at least 24 hours to acclimate to the new tank conditions before testing.

Each of the four beakers was filled with 100 mL water from the fish's holding tank. Four fish were then transferred to these beakers (1 fish per beaker), beginning the experimental period. Generally, four fish that were assigned to the same condition were run concurrently. In some cases, however, two fish from one condition and two fish from

another condition were run concurrently, due to the number of subjects in each condition. The fish underwent a 20-minute acclimation period in the beaker to allow them to acclimate to the new environment. Twenty minutes after the beginning of this acclimation period, the ten-minute baseline period began. A single camera was used to record all four subjects simultaneously. Recording began during the baseline period and continued for the duration of exposure to experimental conditions.

After 10 minutes of baseline recording, the appropriate amount of acetic acid was added to the beaker using a micropipette, if applicable. In the control condition, nothing was added to the beaker. Fish were exposed to experimental substances for thirty-one minutes. During the entire experimental period, fish were observed for signs of considerable distress, such as attempts to jump from the water or overt signs of lethargy such as decreased ventilation rate and swimming upside-down or sideways. If a fish showed signs of considerable distress, it was removed from the experimental conditions and transferred back to the holding tank. Thirty-one minutes after addition of the acetic acid, a net was used to transfer the fish from the testing chamber to the appropriate section of the holding tank to minimize the amount of acetic acid transferred into the experimental tank. Tank dividers, constructed from a plexiglass frame and a plastic mesh barrier to allow adequate circulation of water, were used to separate pre-test fish from post-test fish, ensuring that a fish was not tested more than once.

Behavioral Analysis

After all recording was complete, the video tapes of the data were reviewed for behavioral analysis. The primary behavioral measure assessed in this study was the

amount of time spent top-dwelling, operationalized as the amount of time the fish spent with its head above the permanent 60 mL mark on the beaker. Please see Figure 1 for an example of top-dwelling and bottom-dwelling behavior.

Baseline behavioral observations were made for one minute at the following time points: ten minutes prior to the addition of the acetic acid and five minutes prior to the addition of the acetic acid. One-minute experimental observations were made immediately following addition of the acetic acid and after 5, 10, 15, 20, 25, and 30 minutes of exposure.

Results

Twenty-nine fish completed this study. All fish exposed to the control and 0.01% acetic acid conditions completed the study; however, nine fish completed the 0.03% acetic acid condition, and no fish completed the 0.05% acetic acid condition. One of the fish in the 0.03% acetic acid condition and the first two fish in the 0.05% acetic acid condition met the pre-determined criteria for signs of considerable distress before behavioral data could be recorded. Consequently, testing of the 0.05% acetic acid condition was abandoned, and no data were collected for any subjects that did not complete the procedure.

Qualitative Behavioral Observations

Note: The following observations do not apply to all fish tested. Rather, they are a summary of commonly-observed behaviors designed to characterize the general response of the fish to the experimental substances.

During the baseline period, fish demonstrated moderate levels of activity and tended to stay in the bottom half of the beaker. Most fish displayed short intervals of inactivity, in which they would appear to float at or near the bottom of the beaker. Some fish displayed this behavior throughout the baseline period. Although most subjects spent the majority of the baseline period in the bottom half of the beaker, subjects would commonly cross between the top and bottom halves of the beaker. When subjects were observed in the top half of the beaker during the baseline period, their behavior appeared to be qualitatively different than the characteristic top-dwelling behavior observed in the presence of acetic acid (described below). During the baseline period, the fish observed in the top half of the beaker would commonly touch their mouths or noses to the meniscus of the water for a second or two at a time, but they rarely displayed this behavior for more extended periods of time. When fish spent extended periods of time in the top half of the beaker during baseline, they would often float a few millimeters below the meniscus of the water. Some fish were also observed to swim against the edges of the beaker for a few seconds at a time. This behavior occurred in both halves of the beaker.

The behavior of fish in the control conditions generally continued as described above for the remainder of testing. Some changes in the relative frequencies of these behaviors were observed over time, however. The frequency and intensity of swimming against the sides of the beaker tended to decrease as time progressed, while the floating behavior tended to increase. Generally, these changes are consistent with a decrease in activity over time and could demonstrate habituation of the fish to the novel environment.

In contrast, fish exposed to acetic acid displayed distinct changes in behavior during the testing period. In the seconds following addition of the 0.01% acetic acid, a slight increase in swimming against the glass and diving behavior was observed, and more rapid turning was observed compared to baseline observations.

In the seconds following the addition of the 0.03% acetic acid, the fish tended to swim to the bottom of the beaker, make a lot of fast movements, turn around rapidly, and swim against the edges of the beaker. The fish also exhibited a repetitive and rapid diving behavior. Although some of these behaviors were observed after addition of 0.01% acetic acid, the magnitude and frequency of these behaviors was greater following addition of the 0.03% acetic acid. These initial responses appeared to be ones of discomfort, as if the fish were trying to escape from the newly added acid. It should also be noted that the time points immediately following addition of the acetic acid were marked by less top-dwelling behavior compared to the rest of the time points after the addition of the acetic acid. These initial responses were observed upon addition of the lower concentrations of acetic acid, as well.

After approximately one to two minutes of exposure to 0.03% acetic acid, many fish began to display the characteristic top-dwelling behavior. Fish that displayed this behavior tended to swim at the top of the beaker with their mouths or heads touching the surface of the water. These fish tended to open and close their mouths while swimming at the top of the beaker. During the first ten minutes following addition of the acetic acid, the top-dwelling behavior was characterized by rapid tail beating and swimming against the sides of the beaker. As time progressed, the size and frequency of tail beats decreased,

and fish appeared to float at the top of the beaker. This top-dwelling behavior was often punctuated by short periods of swimming at the bottom of the beaker.

Although this top-dwelling behavior was the most commonly-observed response to 0.03% acetic acid, another behavior was commonly observed, especially among the fish that did not display the top-dwelling behavior. Many fish seemed to float in the beaker, with their heads closer to the surface of the water and their tails closer to the bottom of the beaker. This floating behavior was marked by small, infrequent beats of the caudal and pectoral fins. Fish commonly displayed this behavior near the bottom of the beaker, but it was occasionally observed in the top half of the beaker.

Quantitative Analysis of the Effective Concentration of Acetic Acid

All data analysis was performed using SPSS Statistics Processor (International Business Machines Corp., Armonk, NY, USA). First, randomization effects were tested using a one-way ANOVA. No significant difference was found between any of the conditions at baseline ($F(2, 26) = 2.186$, $p = 0.133$).

Within-subjects differences were then assessed. Mauchly's Test of Sphericity revealed that the assumption of sphericity was violated (*Mauchly's W*= 0.007, $p < 0.001$). A repeated measures ANOVA with time point as a within-subjects factor and treatment as a between-subjects factor was run. A Greenhouse-Geisser correction revealed a main effect for seconds spent top-dwelling over time, such that fish spent more time top-dwelling after addition of the acetic acid than during baseline ($F(3.87, 100.59) = 4.91$, $p = 0.032$). A significant between-subjects effect was found for condition ($F(2, 26) = 11.72$, $p < 0.001$), revealing a difference in top-dwelling behavior between experimental

groups. A Games-Howell post-hoc test revealed that fish in the 0.01% acetic acid condition and the control condition did not spend significantly different amounts of time top-dwelling at any time points. Fish in the 0.03% acetic acid condition spent significantly more time top-dwelling than fish in the 0.01% condition at 0, 10, 15, 20, and 25 minutes after addition of the acetic acid. Fish in the 0.03% acetic acid condition spent significantly more time top-dwelling than fish in the control condition at 5, 10, 20, and 25 minutes after addition of the acetic acid (see Figure 2).

Discussion

The increased time the zebrafish spent top-dwelling in the 0.03% acetic acid condition demonstrates a significant behavioral response to exposure to acetic acid. The time spent top-dwelling was significantly higher in the 0.03% condition compared to the control condition at 5, 10, 20, and 25 minutes after addition of the acetic acid. The fish in this condition demonstrated a slightly delayed response to the addition of the acetic acid; they did not exhibit significant differences in time spent top-dwelling until 5 minutes after the addition of the acetic acid. Furthermore, the greatest amount of time spent top-dwelling occurred at 5 minutes after addition of the acetic acid and did not change appreciably for the remainder of the testing period (See Figure 1).

Although we expected time spent top-dwelling to increase as the concentration of acetic acid increased, the results of this study did not support this expectation. There was not a significant difference in time spent top-dwelling between the control and 0.01% acetic acid conditions. As expected, the difference in top-dwelling between the control group and the 0.03% condition was significant. It was also expected that the behavioral

response of the fish to the acetic acid would be modest at 0.03% and slightly higher at 0.05%. However, subjects in this study did not tolerate exposure to 0.05% acetic acid for more than five minutes. The 0.03% acetic acid therefore appears to be the most effective concentration to elicit a behavioral response without any major acute health effects.

Study 3: Time-course analysis

A significant increase in top-dwelling behavior was observed in subjects following exposure to 0.03% acetic acid. Following a sharp preliminary increase in time spent top-dwelling at the first experimental time point, the fish demonstrated possible signs of habituation, generally spending fewer seconds top-dwelling at each successive time point. Therefore, the purpose of this study was to observe the behavioral response of the zebrafish to immersion in water containing 0.03% acetic acid over two hours of exposure and to validate the behavioral effect observed in the previous study. It was hypothesized that subjects would habituate to the presence of the acetic acid, as demonstrated by a decrease in time spent top-dwelling at each successive time point.

Methods

The methods of this study were similar to the methods reported in Study 2, with the following exceptions:

Research Design

This procedure was designed to characterize the behavioral response of zebrafish to water containing 0.03% acetic acid over an exposure period of two hours. Subjects

were exposed to 0.03% acetic acid or to a sample of water from the fish's original tank (control). Ten fish were exposed to each condition.

Procedure

The duration of exposure to acetic acid was lengthened from 31 minutes to two hours and one minute.

Behavioral Analysis

The frequency of behavioral observations was also changed. In study 2, one-minute behavioral observations occurred every five minutes for the duration of exposure to experimental substances. In contrast, for this time-course analysis, one-minute behavioral observations occurred every 15 minutes, for the duration of exposure to experimental substances.

Results and Discussion

Mauchly's Test of Sphericity revealed that the assumption of sphericity was violated (*Mauchly's W*< 0.001, $p < 0.001$). A repeated measures ANOVA with time point as a within-subjects factor and treatment as a between-subjects factor was run. A Greenhouse-Geisser correction revealed a main effect for seconds spent top-dwelling over time, such that fish spent more time top-dwelling after addition of the acetic acid than during baseline ($F(3.68, 66.31) = 3.84$, $p = 0.009$). Furthermore, a significant time point * condition interaction effect ($F(3.68, 66.31) = 3.92$, $p = 0.008$) revealed that conditions were not different at baseline but diverged after addition of the experimental substances. However, a high degree of variability in the data prevented post-hoc analyses

from determining which time points were driving this difference between groups (Figure 3).

Although the results of Study 2 suggested that subjects displayed a downward trend in top-dwelling after 5 minutes of exposure to acetic acid, the results of the current study suggest that this trend can be attributed to normal variation in top-dwelling behavior. These results do not support the hypothesis for this study. Rather, they suggest that subjects do not habituate to 0.03% acetic acid over two hours of exposure.

Although no significant difference was found within conditions after addition of the acetic acid, the significant interaction effect validates the results reported in Study 2. Two studies, one with nine fish and one with ten fish, have demonstrated a significant increase in top-dwelling behavior following addition of 0.03% acetic acid. This result suggests that the top-dwelling behavior reported in Study 2 is relatively reliable in this particular paradigm.

Study 4: Determination of pH change upon addition of morphine sulfate and morphine sulfate + acetic acid to water sample from fish tanks

Before any subjects were exposed to water MS, a study was conducted to determine the effect of 0.5 mg/L MS, 1.5 mg/L , and 3.0 mg/L MS alone and in combination with 0.03% acetic acid on the pH of a sample of water taken from the zebrafish colony.

Methods

Apparatus

One 120 mL beaker was used to test a sample of water taken from the zebrafish colony. A Symphony VWR SB21 pH meter with a Corning G-P combo w RJ probe was used to take pH measurements.

Procedure

Morphine sulfate was obtained from Mallinckrodt (Hazelwood, MO, USA). First, a 5 mg/mL stock solution of MS was prepared by mixing solid MS with sterile water. This solution was refrigerated when not in use.

The pH of solutions of 0.5 mg/L MS, 1.5 mg/L MS, 3.0 mg/L MS, 0.5 mg/L MS with 0.03% acetic acid, 1.5 mg/L MS with 0.03% acetic acid, and 3.0 mg/L MS with 0.03% acetic acid were tested. A beaker was filled with 100 mL of water taken from one the tanks in the zebrafish colony, and the initial pH of the water sample was measured. The appropriate amount of glacial acetic acid was added first, if applicable, using a clean micropipette, and the resulting change in pH was measured immediately. The appropriate amount of MS was then added using a clean micropipette. The resulting pH of the solution was measured immediately after addition of the MS. Three trials were conducted for each solution. The probe was rinsed with deionized water between trials in order to prevent contamination of the tank water samples with acetic acid and/or MS. All pH measurements were taken again on a second day in order to account for daily variations in pH.

Results

On the first day of testing, the average pH of the water from tanks in the zebrafish colony was 6.7. The average pH of the 0.5 mg/L MS and 1.5 mg/L MS solutions was 6.9. The average pH of the 3.0 mg/L MS solution was 7.0.

On the first day of testing, the average pH of the water from tanks in the zebrafish colony after addition of 0.03% acetic acid was 4.0. The average pH of the 0.5 mg/L MS with 0.03% acetic acid, 1.5 mg/L MS with 0.03% acetic acid, and 3.0 mg/L MS with acetic acid solutions was 3.9.

On the second day of testing, the average pH of the water from tanks in the zebrafish colony was 6.9. The average pH of all other solutions containing water and MS was identical.

On the second day of testing, the average pH of the water from tanks in the zebrafish colony after addition of 0.03% acetic acid was 3.9. The average pH of the 0.5 mg/L MS with 0.03% acetic acid, 1.5 mg/L MS with 0.03% acetic acid, and 3.0 mg/L MS with acetic acid solutions was 3.8.

Discussion

The results of this study suggest that these three concentrations of MS do not have a great effect on the pH of these solutions. The pH measurements of all of the morphine-only solutions were within 0.1 pH unit of each other. The same was found for the solutions of MS and 0.03% acetic acid. Although all pH measurements were shifted down due to the presence of the acid, all pH measurements fell within 0.1 pH unit of each other.

When the procedure was replicated on the second day of testing, it was found that the average pH of water taken from the zebrafish colony was 0.2 pH units higher than the average pH measured on the first day. Despite this difference in starting pH, the average pH of the solutions of 0.5 mg/L MS, 1.5 mg/L MS, and 3.0 mg/L MS were identical on both days of testing. Furthermore, the average pH of the 0.5 mg/L MS with 0.03% acetic acid, 1.5 mg/L MS with 0.03% acetic acid, and 3.0 mg/L MS with acetic acid solutions was only 0.1 pH unit lower on the second day of testing. These results suggest that the final pH of all of the solutions tested is relatively resistant to minor variations in the pH of the sample of water taken from the tanks in the zebrafish colony.

Study 5: Tolerability of exposure to morphine sulfate

This procedure was designed to determine the behavioral response of zebrafish to water containing dilute amounts of morphine sulfate. Authors have previously exposed zebrafish to dilute concentrations of MS for studies of conditioned place preference and chronic opiate addiction (Khor et al., 2011). This study suggested that zebrafish exhibit a behavioral response to exposure to 0.5 mg/L MS, 1.5 mg/L MS, and 3.0 mg/L MS. Therefore, in the current study, subjects were exposed to 0.5 mg/L MS, 1.5 mg/L MS, 3 mg/L MS, or water from the original tank (control). Five fish were tested in each condition. Because authors have previously reported that zebrafish tolerate these three concentrations of MS for at least 30 minutes, it was hypothesized that subjects would tolerate these concentrations in the current model.

Method

The methods of this study were similar to the methods reported in Study 2, with the following exceptions:

Research Design

This procedure was designed to determine the tolerability of three solutions of MS in zebrafish. Fish were exposed to a control sample of water from the fish's original tank or to water containing 0.5 mg/L MS, 1.5 mg/L MS, or 3.0 mg/L MS. Ten fish were tested in each condition.

Procedure

The appropriate amount of MS was added to the beaker using a micropipette, if applicable.

Results

Qualitative Behavioral Observations

As with the addition of acetic acid, addition of the MS led to an immediate decrease in top-dwelling behavior. The behavioral response to the addition of MS was similar to the behavior observed in the presence of acetic acid in many aspects, with a few notable exceptions. Immediately following addition of the MS, the fish often swam against the sides of the beaker and exhibited a repetitive diving behavior. Aside from the diving behavior, which occurred in both halves of the beaker, most of this behavior was observed in the bottom half of the beaker. In contrast to the behavior observed after the addition of acetic acid, however, less rapid movement and turning were observed after addition of MS. This behavior is difficult to measure quantitatively. It was therefore not measured in the current studies.

Approximately one to two minutes after addition of MS, this acute response subsided. At this point, behavior appeared to return to baseline levels. Over time, the fish exposed to MS exhibited behavior similar to that described for the control condition.

Quantitative Analysis of the Effect of MS on Behavior

A repeated measures ANOVA with time point as a within-subjects factor and treatment as a between-subjects factor was run. A significant main effect of time ($F(8, 128) = 3.42$, $p = 0.001$) revealed a change over time in top-dwelling behavior across conditions. An insignificant time point * condition interaction effect ($F(24, 128) = 1.07$, $p = 0.392$) revealed that top-dwelling behavior did not change following addition of the MS (Figure 4).

Discussion

The results of the current study suggest that a 30-minute exposure to solutions of 0.5 mg/L MS, 1.5 mg/L MS, and 3.0 mg/L MS is tolerable to zebrafish in the current model. No overt signs of distress were observed in the fish at any time during testing, and no long-term health effects were observed in the weeks following testing. These results therefore support the hypothesis for this study. Furthermore, a statistical analysis revealed that addition of the MS did not significantly affect the top-dwelling behavior of the fish.

It should be noted, however, that a decrease in top-dwelling behavior was observed during the first time point after addition of the MS. It is therefore possible that MS causes a reduction in top-dwelling behavior, but that this effect subsides after five minutes of exposure. However, a decrease in top-dwelling behavior immediately following the addition of another experimental substance was observed in Study 2. In

Study 2, qualitative behavioral observations revealed an immediate decrease in top-dwelling behavior in the 15-20 seconds after addition of 0.03% acetic acid. Although top-dwelling behavior increased from baseline levels in the minute following addition of acetic acid, top-dwelling behavior at this time point was lower than that observed at most successive time points. It is therefore possible that an initial bottom-dwelling response to addition of the acetic acid was attenuating the top-dwelling behavior in the minute after addition of the acetic acid. The combined results of Studies 2 and 5 suggest that zebrafish might demonstrate a period of bottom-dwelling behavior after addition of any liquid to the beaker. Further research could investigate whether this behavioral response is observed in response to addition of water to the beaker.

Study 6: Effect of an analgesic on top-dwelling behavior

The outcome of the MS tolerability study did not reveal any signs of overt distress in subjects upon exposure to 0.5 mg/L MS, 1.5 mg/L MS, or 3.0 mg/L MS. Consequently, a follow-up study was conducted to observe the effect of MS on the behavioral response of the subjects to water containing 0.03% acetic acid. Because the top-dwelling behavior was believed to be a pain response, it was hypothesized that addition of the three concentrations of MS to solutions containing 0.03% acetic acid would cause the top-dwelling behavior to decrease. Furthermore, a dose-response relationship was expected, such that subjects exposed to the higher concentrations of MS with 0.03% acetic acid would spend less time top-dwelling than those exposed to lower concentrations of MS with 0.03% acetic acid.

Method

The methods of this study were similar to the methods reported in Study 2, with the following exceptions:

Research Design

This procedure was designed to determine the effect of three concentrations of an analgesic on top-dwelling behavior in zebrafish exposed to 0.03% acetic acid. Fish were exposed to a control sample of water from the fish's original tank or to water containing 0.5 mg/L MS, 1.5 mg/L MS, 3.0 mg/L MS, 0.03% acetic acid alone, 0.5 mg/L MS with 0.03% acetic acid, 1.5 mg/L MS with 0.03% acetic acid, or 3.0 mg/L MS with 0.03% acetic acid. Ten fish were tested in each condition.

When trials involved exposure to both acetic acid and MS, acetic acid was added first. For these conditions, timing began immediately after addition of MS. Generally, timing began approximately one minute after addition of the acetic acid. Timing for the condition exposed to 0.03% acetic acid therefore began one minute after addition of the acetic acid in order to account for the immediate bottom-dwelling response to acetic acid described in Study 2.

Procedure

The appropriate amount of acetic acid and/or MS was added to the beaker using a micropipette, if applicable. Acetic acid was added first, followed by the appropriate amount of MS. Approximately one minute elapsed between the addition of the 0.03% acetic acid and the addition of the MS.

Results

Mauchly's Test of Sphericity revealed that the assumption of sphericity was violated (*Mauchly's W*= 0.098, $p < 0.001$). A repeated measures ANOVA with time point as a within-subjects factor and treatment as a between-subjects factor was run. A Greenhouse-Geisser correction revealed a significant main effect of time ($F(4.62, 333.13) = 11.20$, $p < 0.001$), demonstrating an increase in time spent top-dwelling following the addition of the experimental substances. A significant time point*condition interaction effect ($F(32.39, 333.13) = 3.64$, $p < 0.001$) revealed that conditions were not different at baseline but diverged after the addition of the experimental substances. Finally, a significant between-subjects effect ($F(7, 72) = 10.45$, $p < 0.001$) revealed a difference in top-dwelling behavior between conditions. A Tukey post-hoc test revealed that groups exposed to the 0.03% acetic acid spent significantly more time top-dwelling than those exposed to morphine alone. No significant difference was observed between any of the groups exposed to morphine + 0.03% acetic acid and the group exposed to 0.03% acetic acid alone (Figure 5).

Discussion

The three concentrations of MS tested in the current investigation did not significantly affect top-dwelling behavior in the presence of acetic acid. These results do not support any aspect of the hypothesis for the current study. It is possible that this result demonstrates that a pain state is not motivating the observed top-dwelling behavior. On the other hand, it is possible that the doses of MS used in the current study are sub-threshold.

No literature was available regarding the exposure of zebrafish to MS for analgesic purposes. Reports of exposing zebrafish to MS for other purposes have been described, and these reports were used to select the MS concentrations in the current study. Khor et al. (2011) exposed zebrafish to 0.5 mg/L, 1.5 mg/L, and 3.0 mg/L MS in a conditioned place preference paradigm, and they found significant behavioral responses to 1.5 mg/L and 3.0 mg/L MS. Furthermore, they used these results to conduct a study of the behavioral effects of chronic morphine withdrawal in zebrafish. In order to create this morphine addiction, subjects were exposed to 1.5 mg/L MS for two weeks (Khor et al., 2011). These results therefore suggest that zebrafish are capable of sensing morphine at the concentrations tested in the current study. However, it is possible that the concentration of MS needed to produce acute analgesia is greater than the concentrations used for these conditioned place preference and chronic morphine addiction studies. Research is ongoing to investigate the effect of higher doses of MS on the top-dwelling behavior of zebrafish exposed to 0.03% acetic acid.

Although the MS concentrations in the current study were not effective in reducing top-dwelling behavior in the presence of 0.03% acetic acid, the significant time point * condition interaction effect provides additional support for the top-dwelling response to 0.03% acetic acid described in Study 2. In the current study, we observed a significant increase in top-dwelling behavior in the 40 subjects exposed to 0.03% acetic acid. This data demonstrates that the observed top-dwelling response to 0.03% acetic acid is reliable and can be replicated in large numbers of subjects.

Chapter 3: General Discussion

As a whole, the results of these studies suggest that exposing zebrafish to water containing 0.03% acetic acid causes a significant increase in top-dwelling behavior and that this behavior might be motivated by a pain state.

Top-dwelling behavior: Possible explanations

The current studies revealed that exposing zebrafish to acetic acid leads to a significant increase in top-dwelling behavior. However, it is unclear why subjects spent more time top-dwelling as a result of exposure to water containing acetic acid. Although fishes have vastly different behavioral repertoires compared to humans and other mammals, this top-dwelling behavior does not seem to have any parallels to known nociceptive behaviors in mammals.

Hypoxia

It is possible that the observed top-dwelling behavior represents an attempt to increase oxygen intake. Qualitative observation of subjects revealed that they would often swim near the top of the water, with their mouths or heads touching the surface of the water. Furthermore, some subjects tended to open and close their mouths while swimming at the top of the water, as if to increase the amount of oxygen reaching their gills.

The literature contains information that could support this explanation. Branson (1992) suggested that the acetic acid damages the gills of the fish, impairing their oxygen uptake. Furthermore, Randall and Brauner (1991) suggested that hematological changes in response to acidosis following exercise may impair the delivery of oxygen to tissues in

fishes, and a similar mechanism could explain the observations in the current study. Similar to humans, fishes possess hemoglobin proteins that carry oxygen in their red blood cells. The oxygen-carrying capacity of hemoglobin depends upon the pH of the red blood cells according to the following equation: $HHb + O_2 \leftrightarrow HbO_2 + H^+$ (Hb= hemoglobin; Randall & Brauner, 1991). An increase in H^+concentration (or an increase in acidity, or a decrease in pH) leads to the formation of HHb and the dissociation of the HbO_2complex. The oxygen carrying capacity of hemoglobin therefore decreases under acidic conditions. Under normal physiological conditions in the blood stream, the red blood cell is at neutral pH, so oxygen is bound to hemoglobin. When the red blood cell reaches the swim bladder, the acidic environment of the swim bladder leads to the dissociation of the oxygen from the hemoglobin so that the oxygen can be used for cellular respiration in the necessary tissues (Randall & Brauner, 1991). However, it is possible that the fish exposed to acetic acid in this experiment could have absorbed some of the acid through diffusion, causing acidosis of the red blood cells in the blood stream. This could cause a decrease in the oxygen carrying capacity of the red blood cells in the blood stream, leading the fish to search for more oxygen-rich water to compensate for the reduced amount of oxygen reaching the swim bladder (or, to shift the above equation to the right). It is therefore possible that the zebrafish spent more time at the top of the tank in an effort to increase oxygen intake.

If the top-dwelling behavior does represent a response to hypoxia, this leaves two possibilities for the nociceptive significance of this behavior. First, it is possible that the hypoxia does not cause a negative sensory and emotional experience, and therefore does

not cause pain. In this case, the behavior could be viewed as a reflex intended to increase oxygen intake. If this hypoxic situation is not painful for the fish, exposure to MS would not be expected to have an effect on top-dwelling behavior. On the other hand, it is possible that the oxygen deprivation is painful for the fish. In this case, the top-dwelling behavior could be motivated by a desire to escape a painful, hypoxic situation. If this is the case, concomitant exposure to MS would be expected to reduce pain-related behaviors.

Finally, it is possible that the increase in top-dwelling behavior is not related to oxygen uptake at all. The top-dwelling behavior could represent an innate avoidance mechanism or some other nociceptive behavior that does not have a correlate in mammals.

Future research could investigate these explanations by sampling the blood of fish exposed to 0.03% acetic acid and measuring the O_2saturation and the blood pH. Furthermore, the existing video tapes could be reviewed for other behavioral indicators of pain, including swimming activity, number of tail flips per minute, top-bottom transitions, swimming against the sides of the beaker, swim velocity, number of turns, or opercular beat rate. Top-dwelling was chosen as the primary outcome measure of the current study because this behavior is clearly defined, easily verified, and can be identified without the use of sophisticated bio-sensing programs. If more standardized methods of identifying the aforementioned behaviors are articulated, or if a more sophisticated bio-sensing software becomes available, these behaviors could provide a more complete account of the behavioral changes of subjects exposed to this assay.

Changes in pH

Acetic acid was chosen as the noxious stimulus in this study based on past research reporting that injection and topical application of acetic acid elicits a change in behavior indicative of pain in fishes (Sneddon, 2002; Sneddon, 2003a; Reilly, Quinn, Cossins, & Sneddon, 2008). The mechanism by which the acid stimulates the nociceptors is not clear, however. Ashley, Sneddon, and McCrohan (2007) hypothesized that this response could be due to the protons in the acetic acid dissociating from the acid and stimulating nociceptors, and Mettam, McCrohan, and Sneddon (2011) have published evidence supporting this hypothesis. They reported that topical application of acetic acid and low pH (which was tested using citric acid phosphate buffer) to the receptive fields of chemosensitive receptors (mechanochemical and polymodal) on the head of rainbow trout lead to similar activation in the trigeminal ganglion. Protons are a component common to both substances, so it is possible that the protons, and not the acetate anion, are responsible for the pain response. It is therefore possible that any acidic substance that loses a proton in water could elicit a similar pain response in fishes. This hypothesis warrants further investigation, but careful *a priori* research must be conducted to ensure that the substance and its anion are not toxic to the fish.

Alternative explanations

Researchers testing the effects of anxiolytic and antidepressant drugs on zebrafish have reported results similar to the findings of this study. Previous research suggests that top-dwelling increased on an acute basis in *Danio rerio* in response to antidepressants and anxiolytics, including Citalopram, Desipramine (Sackerman et al., 2010), lysergic

acid diethylamide (Grossman et al., 2010), and olanzapine (Selbit et al., 2010), so it is possible that the acetic acid has an anxiolytic effect. On the other hand, it is possible that the antidepressants and anxiolytics stimulated nociceptors (Sackerman et al., 2010), or that the responses in these two situations are not related. Nicotine has also been reported to cause increased time spent top-dwelling in zebrafish on an acute basis (Sackerman et al., 2010), complicating the issue and suggesting a need for further research.

Considering the evidence that increased time spent top-dwelling could be a response to drugs which are not likely to cause pain in zebrafish, it is necessary to investigate additional behavioral indicators of pain before this paradigm can be proposed as a model of human pain. Previous studies of pain in zebrafish have shown that the number of tail beats per minute decreases in response to potentially painful stimuli (Correia, Cunha, Scholze, & Stevens, 2011), so future research could investigate these behavioral measures. Changes in the fish's physiological processes could also be strong indicators of a pain response. Multiple studies of pain in fishes have reported that opercular beat rate increases significantly in response to injection of acetic acid and other potentially noxious chemicals (Sneddon, 2003a; Reilly, Quinn, Cossins, & Sneddon, 2008).

The current model as a pain assay

When the current model is compared to Dubner's (1994) criteria, it is clear that further research is necessary to develop this model as a pain assay. The current model will be discussed in terms of each criterion in turn.

First, Dubner (1994) requires that a pain assay "should distinguish between responses to innocuous and noxious stimuli" (p. 294). The current study begins to address this criterion, but additional research could contribute to the distinction between responses to innocuous and noxious stimuli. In the minutes following addition of the experimental substances, qualitative behavioral observations of subjects exposed to 0.03% acetic acid in the current model revealed different behaviors compared to subjects that were exposed to morphine or to subjects in the control condition. When the acetic acid was initially added to the water, subjects exhibited a repetitive and rapid diving behavior and tended to turn around rapidly and swim against the edges of the beaker. These behaviors were not observed in the control condition, but swimming against the sides of the beaker and rapid diving behaviors were also observed after addition of morphine. Therefore, in the acute phase, rapid turning and diving movements seem to distinguish between noxious and innocuous stimuli. Furthermore, after a few minutes of exposure to experimental conditions, top-dwelling behavior also came to distinguish between subjects exposed to noxious and innocuous substances. As mentioned previously, a significant increase in top-dwelling behavior was observed after addition of 0.03% acetic acid, but this change was not observed in the groups exposed to morphine or the control conditions. These behaviors therefore seem to distinguish between noxious and innocuous stimuli. It should be noted, however, that an increase in top-dwelling behavior has also been observed in zebrafish exposed to antidepressants and anxiolytics (Grossman et al., 2010; Sackerman et al., 2010; Selbit et al., 2010). The findings of these studies could suggest either that exposure to these drugs is painful for subjects or that

top-dwelling is not a pain-related behavior. Further research could identify additional behavioral differences in subjects exposed to 0.03% acetic acid, including tail flipping behavior and opercular beat rate, which are not observed in putative non-noxious situations.

Dubner's second criterion requires that "the behavioral response or responses to be measured should vary in magnitude with changes in stimulus intensity over a range from threshold to tolerance" (1994, p. 294) in a behavioral assay of pain. The current studies were designed to demonstrate a change in magnitude with changes in stimulus intensity, but the results do not meet this criterion. In Study 2, subjects were exposed to 0.01%, 0.03%, or 0.05% acetic acid, but a significant behavioral difference was identified only in the 0.03% acetic acid condition. We did not observe a difference between subjects in the 0.01% acetic acid and control conditions, and exposure to 0.05% acetic acid was not tolerated, so testing at this concentration was abandoned. However, it is possible that a dose-response relationship would be identified if concentrations closer to 0.03% acetic acid were tested. A future study of zebrafish exposed to water containing concentrations of acetic acid very close to 0.03% could yield valuable information regarding how the behavioral response changes over a range of stimuli from threshold to tolerance.

Third, Dubner requires that "multiple threshold and suprathreshold behavioral measures should be used to infer pain" (p. 294). The current model does not meet this criterion, but further research could identify additional behavioral measures indicative of pain. Although top-dwelling behavior increased significantly after exposure to 0.03% acetic acid, this was the only behavioral measure investigated in this study. Future studies

could review the videos collected for this study in order to identify an additional behavioral indicator of pain. This approach is favorable because it reduces the number of animals that are exposed to this potentially noxious stimulus. On the other hand, one of the weaknesses of the video footage collected in this study is its poor quality. The original study design included the collection of opercular beat rate as an indicator of pain, but initial review of the videos revealed that the video quality was not sufficient for these measurements. Future studies could therefore use more powerful cameras, test fewer fish at once, or devise another method to measure opercular beat rate.

Dubner's fourth criterion requires that a pain assay "should be susceptible to behavioral and pharmacological manipulations that alter the perceived intensity of noxious stimuli" (1994, p. 294). Again, the current studies were designed to manipulate concentrations of morphine in order to alter the magnitude of the behavioral response, but the results did not meet this criterion. The introduction of the prototypical analgesic morphine was intended to attenuate the observed top-dwelling behavior, but a change in top-dwelling behavior was not observed after addition of morphine. It is possible that this lack of effect demonstrates that top-dwelling behavior is not indicative of a pain state in zebrafish. On the other hand, it is possible that the doses of morphine used in the current study were sub-threshold. Previous work by Newby, Wilkie, and Stevens (2009) provides support for this explanation. These authors exposed goldfish (*Carassius auratus*) to morphine concentrations of 0, 0.12, 0.48, 2.4, 12, and 48 mg/L for fifteen minutes, and then they administered injections of 0.7% acetic acid into the cheek of each subject. Rubbing behavior was found to decrease significantly after exposure to 12 and 48 mg/L

morphine, but the lower concentrations had no effect on behavior. An ongoing study is investigating the effect of two higher concentrations of morphine on top-dwelling behavior in zebrafish exposed to 0.03% acetic acid.

Dubner's fifth criterion for a pain assay specifies that "the modification of behavioral responses by nonsensory variables such as attention, motivation, and motoric ability should be distinguishable from effects on sensory capacities" (1994, p. 294).The current studies were designed to begin to address this criterion, but further research is necessary to fulfill it completely. In order to fulfill this criterion completely, it would be necessary to demonstrate that the top-dwelling behavior is primarily a pain response, rather than a natural variation in behavior or a decrease in an anxiety- or depression-like state in the fish. The current study included a control group in order to demonstrate that the observed change in behavior is not observed in control subjects in this paradigm. The possibility remains that the top-dwelling response is primarily driven by a reduced anxiety- or depression-like state. If future research testing higher concentrations of MS reveals that this analgesic reverses the observed change in behavior, this could provide compelling evidence that the top-dwelling behavior observed in this model is indicative of a pain state.

Finally, Dubner proposes that a pain model should involve "little or no tissue damage with repetitive stimulation" (1994, p. 294). The current studies began to investigate the tissue damage aspect of this criterion, but the effect of repetitive stimulation is unknown. During the period of acute exposure to acetic acid, subjects in the 0.03% condition demonstrated a favorable survival rate, and no outward signs of

tissue damage were observed. Furthermore, subjects were maintained in the zebrafish colony at Macalester College after they underwent testing, and no long-term health effects have been observed. This evidence therefore suggests that the current model does not cause tissue damage. It should be noted, however, that the effects of repeated stimulation were not assessed in the current studies. In these studies, animals were exposed to acetic acid only once in order to avoid the confounding effects of learning on changes in the subject's behavior and in order to minimize tissue damage to these animals. Painful events often relay information of significant biological importance, so learning about the cues that signal these events or the behaviors that can be used to escape them is an important function of the nervous system. Therefore, animals were not exposed to the current model more than once in an effort to minimize the effects of learning on the experimental results. Future studies could investigate the effect of repeated exposure to 0.03% acetic acid on tissue damage in zebrafish, but incorporating such a design into future iterations of this model for pain research is not recommended.

Strengths and weaknesses of the current model

If future research reveals that most of these criteria for a pain assay are met, the strengths and weakness of this assay should still be considered before selecting this model for use in future studies. One of the strengths of this assay is its low cost. Compared to the costs of purchasing and maintaining rodents, zebrafish are relatively inexpensive.

Secondly, this model is amenable to high-throughput phenotyping. Although the limiting factor in the current model is the amount of time needed to review the behavioral recordings, application of a biosensing device similar to the MOBS developed by Cunha et al. (2008) could significantly reduce the amount of time needed to conduct a study using this assay.

Thirdly, subjects exposed to the current model exhibited a favorable survival rate; only one of the sixty fish exposed to 0.03% acetic acid did not survive the procedure.

Finally, acute exposure to 0.03% acetic acid does not appear to have any long-term effects on the fish. All subjects used in the current studies continue to be maintained and monitored in the zebrafish colony at Macalester College. Over two years have passed since the first subjects were exposed to acetic acid, and no adverse health effects have been identified by the researchers or by animal care workers.

Although the current assay has numerous strengths, its weaknesses should also be noted. First, a large degree of variability was observed in top-dwelling behavior, both within and between subjects. With regard to the between-subjects variability, qualitative observation revealed that some of the subjects tended to spend the majority of the time either at the bottom or at the top of the beaker, regardless of the presence of experimental substances. Furthermore, subjects exposed to acetic acid would commonly spend long intervals of time at the top of the beaker, punctuated with short intervals of bottom-dwelling. The reverse was also seen in subjects that were not exposed to acetic acid; they would often spend long intervals at the bottom of the beaker, punctuated with short intervals of top-dwelling.

A second weakness in the current assay is that it restricts the kinds of substances that can be tested for their analgesic effects. If analgesics are administered by immersion, they must be non-toxic to the fish. An important factor to be considered when determining the toxicity of potential substances includes its effect on the pH of the water used in the assay.

Finally, another weakness in this assay is the inescapable nature of immersion in a chemical irritant. Many of the established models of pain in rodents, including the tail flick test and the hot plate test, are ethically favorable because they involve termination of the noxious stimulus after the animal exhibits a response, allowing the animal to escape from the painful situation. Unfortunately, if subjects exposed to this assay experience pain, they are unable to escape from the irritant until they are removed from the beaker after testing has concluded. Although the inescapable nature of this assay has some ethical limitations, it also has some scientific advantages. While the previously mentioned assays are often used to measure pain threshold, the current assay could be used to measure the animal's response to supra-threshold stimuli. This aspect of the model could be viewed as advantageous for a model of pain because it meets Dubner's fourth criterion for an animal assay of pain (1994).

Pain and suffering in fishes

Although a fish's subjective state cannot be directly assessed, many researchers accept changes in behavior in response to a potentially noxious stimulus and amelioration of these behaviors in the presence of analgesics as evidence that the stimulus causes a

pain response (Smith & Boyd, 1991; Sneddon, 2003). Although no single criterion has been developed to prove or disprove the possibility that fishes can feel pain, many researchers suggest that a holistic approach be taken to the study of pain in animals, including physical health, physiological markers, and behavioral changes (Dawkins, 2008; Smith & Boyd, 1991).

There has been considerable debate over whether fishes can experience pain. Nearly all researchers agree that fishes are capable of nociception (Sneddon, 2009). Whether they are capable of feeling pain is an issue of debate, since some researchers argue that pain perception requires conscious awareness of a negative affective state. Rose (2002) suggested that fishes are not able to feel pain because they do not possess the necessary brain structures. He began his argument by noting that there are considerable differences in cerebral hemisphere development across species, with fishes having poorly-developed cerebral cortices and humans having well-developed, six-layered cerebral cortices. He then proposed that consciousness in humans depends on the function of this highly developed neocortex. He supported this claim with case studies demonstrating that people with damage to their neocortices appear to be unconscious and that they show reflexive, but not negative affective, responses to painful stimuli. Without the neocortex, he argued, humans are unconscious and are merely capable of reflexive responses, so any animals without a neocortex must not be conscious. Anatomical evidence has suggested that fishes do not possess a neocortex. Rose thus concludes that fishes are not conscious and are incapable of experiencing pain as humans experience it.

Due to the subjective nature of painful experiences, however, it may never be possible to know how fishes, or even other humans, experience pain. It is possible that fishes have developed neural structures and connections that, although they differ from pain circuitry in humans, allow them to experience pain in a way that is similar to the way that humans experience pain. Furthermore, even if humans and fishes do not experience pain in similar ways, it is possible that fishes' experiences of pain have equal biological relevance and unpleasantness to humans' experiences of pain. Because it is impossible to measure this subjective experience, internal states must be inferred from observable behaviors. The fish in the current studies demonstrated significant, observable changes in behavior that could be indicative of a pain response.

Although it may be impossible to determine fishes' subjective experiences, it is better to take pain-relieving measures now and learn that fishes might not feel pain later than to fail to minimize pain now and learn that fishes have been suffering later. The welfare of these animals should therefore be a priority during interactions between humans and fishes.

Conclusions

Based on the data collected in the current studies, it can be concluded that exposing zebrafish to 0.03% acetic acid causes a significant, reliable increase in top-dwelling behavior. Further research is ongoing to determine whether this behavior is a nociceptive response and is indicative of a pain state. After further investigation, this paradigm could serve as a model for use in future pain research.

Chapter 4: Figures

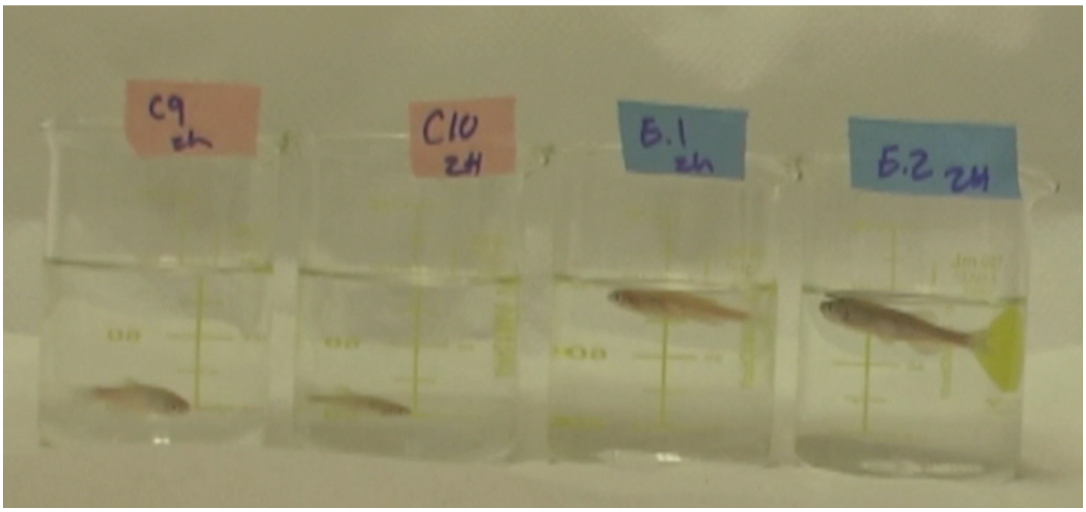

Figure 1. Experimental setup and examples of top-dwelling and bottom-dwelling behavior. The two subjects on the left were not exposed to acetic acid and are not exhibiting the top-dwelling behavior. The two subjects on the right were exposed to 0.03% acetic acid and are displaying the characteristic top-dwelling behavior.

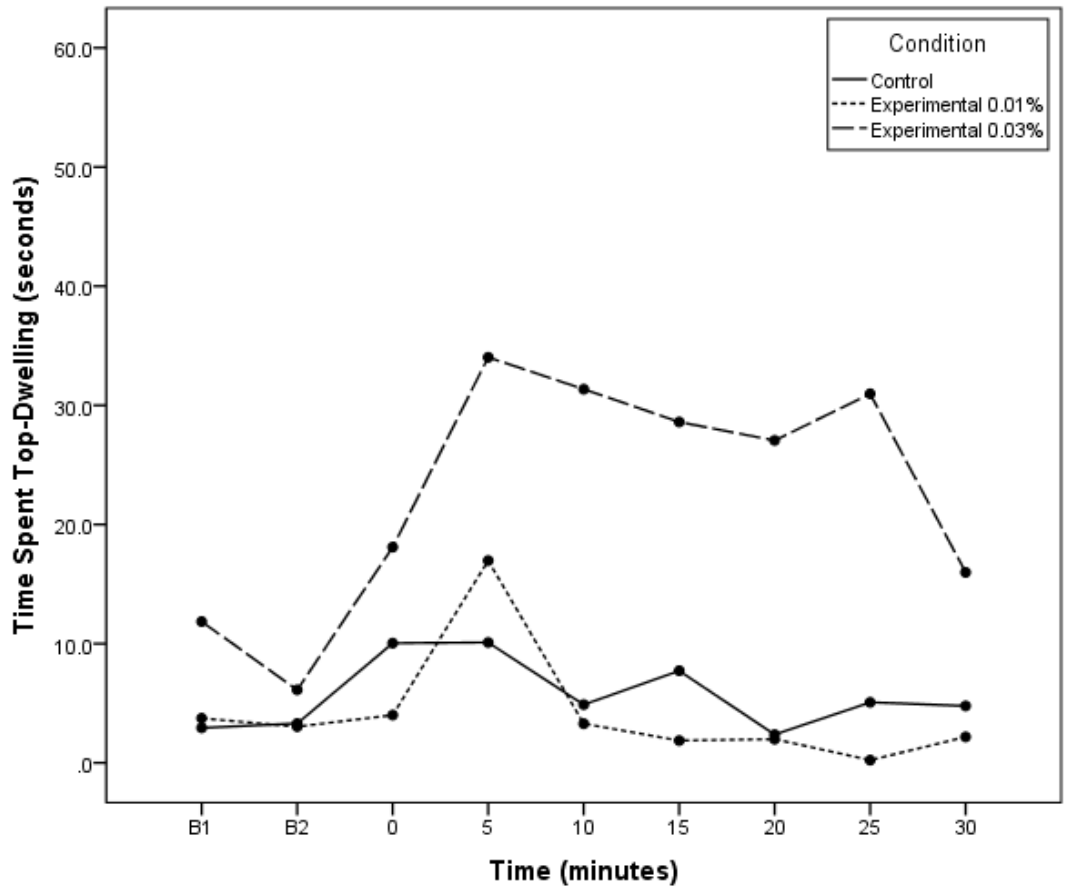

Figure 2. Mean time zebrafish spent top-dwelling following exposure to different concentrations of acetic acid. Two baseline measurements were collected, and acetic acid was added at t = 0.

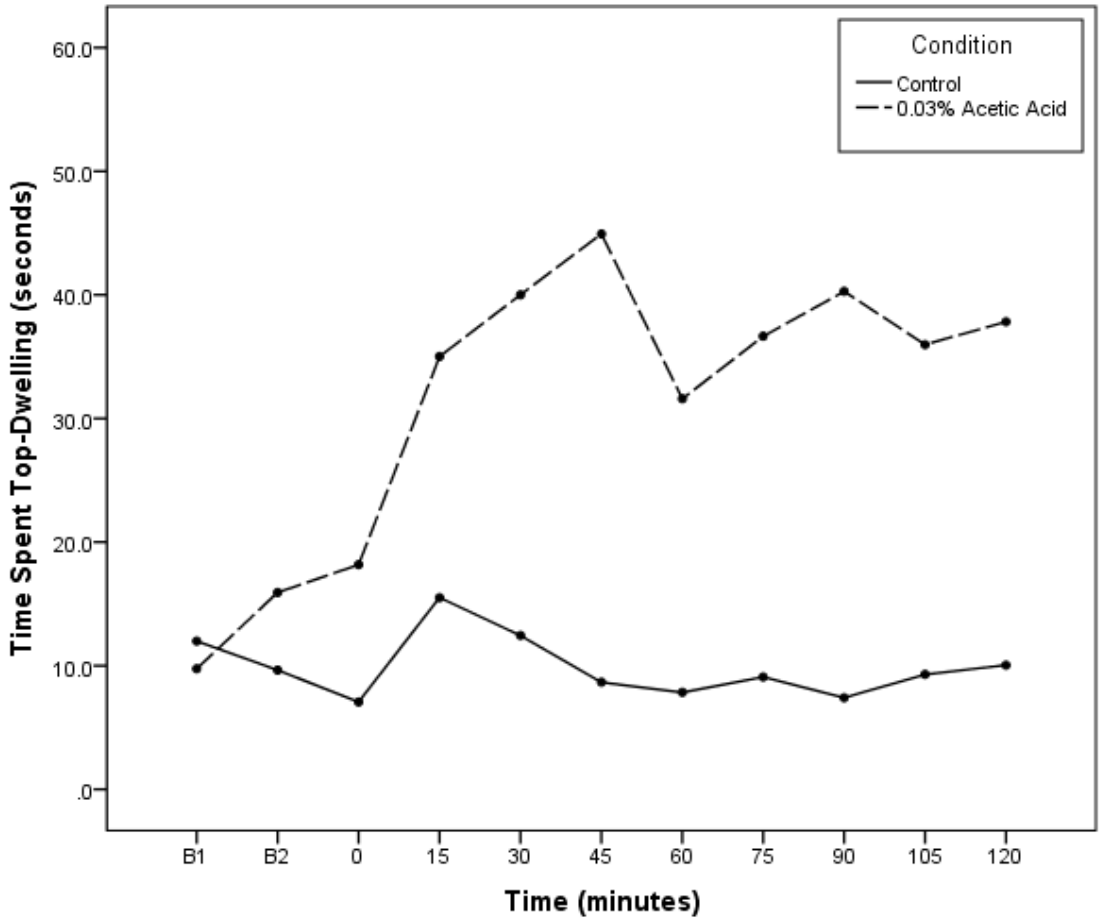

Figure 3. Change in top-dwelling behavior over two hours of exposure to 0.03% acetic acid. Two baseline measurements were collected, and acetic acid was added at t = 0.

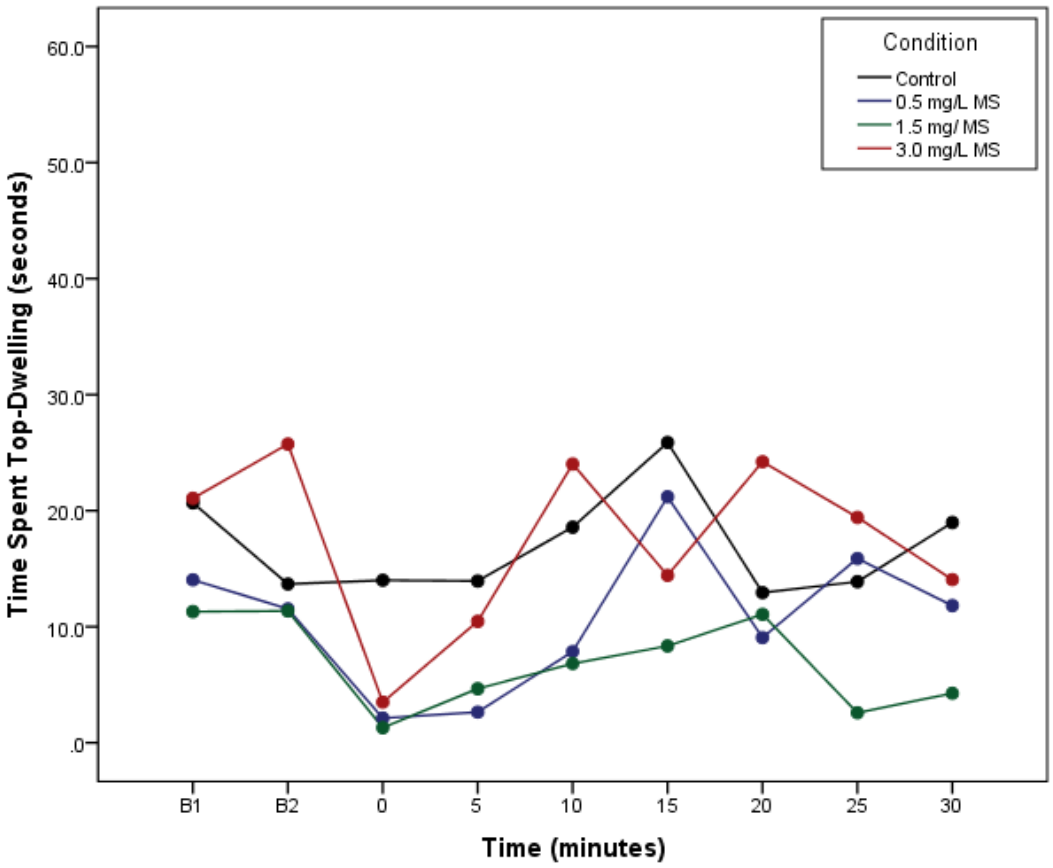

Figure 4. Effect of three concentrations of morphine sulfate on top-dwelling behavior in zebrafish. Two baseline measurements were collected, and morphine sulfate was added at t = 0.

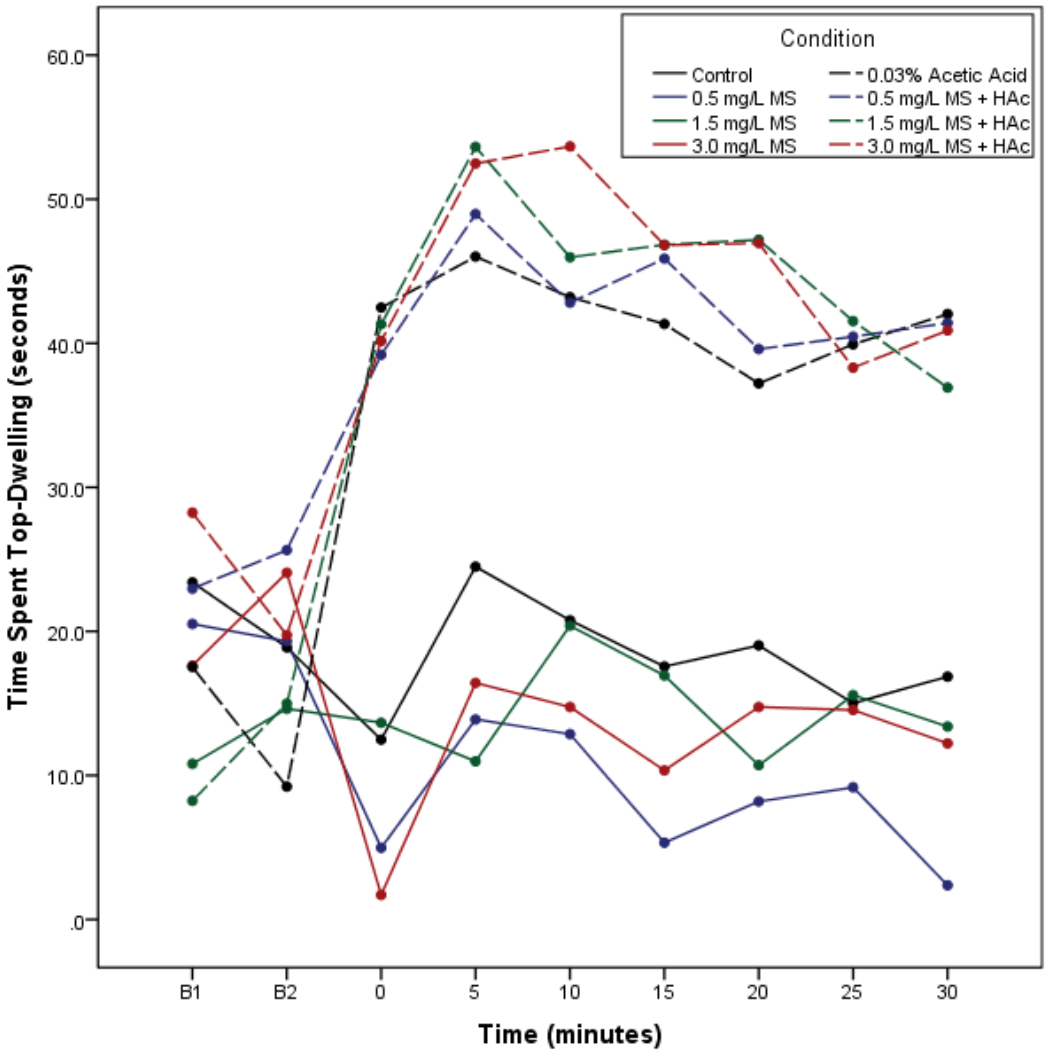

Figure 5. Effect of morphine sulfate on top-dwelling behavior in zebrafish exposed to 0.03% acetic acid. Acetic acid was added approximately one minute before t = 0. Morphine sulfate was then added (if applicable), and behavioral observation began thereafter.

References

Alvarez, F. A., Rodríguez-Martin, I., Gonzalez-Nuñez, V., Marrón Fernández de Velasco, E., Sarmiento, R. G., & Rodríguez, R. E. (2006). New kappa opioid receptor from zebrafish *Danio rerio*. *Neuroscience Letters, 405*, 94-99.

I used this reference to demonstrate that zebrafish possess κ opioid receptors.

Ashley, P. J., Sneddon, L. U., & McCrohan, C. R. (2007). Nociception in fish: stimulus-response properties of receptors on the head of trout *Oncorhynchus mykiss*. *Brain Research, 1166*, 47-54.

This article investigated the effect of stimulus intensity on the firing rate of nociceptors in rainbow trout. The authors used acetic acid to stimulate cutaneous nociceptors, and in the discussion, they suggest that the nociceptors could be sensitive to all kinds of acids, rather than responding to acetic acid specifically. I use this idea in my discussion section to suggest that the top-dwelling response identified in these studies could be a general response to changes in pH, rather than to a specific property of acetic acid.

Barrallo, A., G-Malvar, F., Gonzalez, R., Rodríguez, R. E., & Traynor, J. R. (1998). Cloning and characterization of a δ opioid receptor from zebrafish. *Biochemical Society Transactions, 26*, S360.

I used this reference to demonstrate that zebrafish possess δ opioid receptors.

Barrallo, A., Gonzalez-Sarmiento, R., Alvar, F., & Rodríguez, R. E. (2000). ZFOR2, a new opioid receptor-like gene from the teleost zebrafish (*Danio rerio*). *Molecular Brain Research, 84*, 1-6.

I used this reference to demonstrate that zebrafish possess μ opioid receptors.

Branson, E. (1992). Environmental Aspects of Aquaculture. In L. Brown (Ed.), *Aquaculture for Veterinarians: Fish Husbandry and Medicine* (pp.57-67). New York: Pergamon Press.

In this book chapter, the author discusses water quality and its effect on fish. Branson notes that exposure to low pH can cause gill damage, and I used this reference to support my suggestion that an oxygen deficiency could explain the results of these investigations.

Bateson, P. (1991). Assessment of pain in animals. *Animal Behaviour, 42*, 827-839.

This article provides a review of pain-sensing capabilities in animals. It also provides a checklist of important aspects for a pain assay, which I refer to in my discussion of the pain-sensing capabilities of fishes.

Correia, A. D., Cunha, S. R., Scholze, M., & Stevens, E. D. (2011). A novel behavioral fish model of nociception for testing analgesics. *Pharmaceuticals, 4*, 665-680.

This article validated the lip injection model of pain in zebrafish. The authors foudn that the behavioral response of zebrafish to injections of 10% acetic acid into the lips scales with stimulus intensity, is attenuated by intramuscular injections of morphine, and that this attenuation is reversed by intramuscular injection of naloxone.

Cunha, S. R., Goncalves, R., Silva, S. R., & Correia, A. D. (2008). An automated marine biomonitoring system for assessing water quality in real-time. *Ecotoxicology, 17*(6), 558-564.

I cited this reference because it provides more information on the automated biomonitoring system used by Correia et al. (2011) to collect behavioral data.

Dong, W. K., Chudler, E. H., Sugiyama, K., Roberts, V. J., & Hayashi, T. (1994). Somatosensory, multisensory, and task-related neurons in cortical area 7B (PF) of unanesthetised monkeys. *Journal of Neurophysiology, 72*, 542-564.

I use this article to support my claim that the trigeminal nerve relays afferent signals from peripheral nociceptors to the central nervous system.

Dubner, R. (1994). Methods of assessing pain in animals. In P. D. Wall & R. Melzak (Eds.), *Textbook of Pain* (pp. 293-302). Edinburgh: Churchill Livingstone.

I used this book chapter to establish the necessary components of a pain assay in animals.

Dunlop, R., Millsopp, S., & Laming, P. (2006). Avoidance learning in goldfish (*Carassius auratus*) and trout (*Oncorhunchus mykiss*) and implications for pain perception. *Applied Animal and Behaviour Science, 97*, 255-271.

I used this reference to support my claim that teleost fish meet the behavioral criteria proposed by Dubner (1991) for a pain assay. Specifically, this article demonstrates that two species of teleost fish are capable of learned avoidance of a painful shock.

Ehrensing, R. H., Mitchell, G. F., & Kastin, A. J. (1981). Similar antagonism of morphine analgesia by MIF-1 and naloxone in *Carassius auratus*. *Pharmacology, Biochemistry, and Behavior, 17*, 757-761.

This article presents some of the first evidence that teleost fishes are capable of learning to avoid noxious stimuli. I use this article to support my claim that teleost fishes fulfill the third behavioral criterion for pain perception proposed by Bateson (1991) and Smith and Boyd (1991).

Gonzalez-Nuñez, V., Barrallo, A., Traynor, J. R., & Rodríguez, R. E. (2006). Characterization of opioid-binding sites in zebrafish brain. *The Journal of Pharmacology and Experimental Therapeutics, 316*(2), 900-904.

This article reports the results of a competitive binding assay on zebrafish brain homogenates. It was found that morphine displaced radiolabeled diprenorphine, suggesting that morphine binds competitively to receptors in the central nervous system of zebrafish. I use this article to support my claim that zebrafish possess an endogenous opioid system and therefore meet the fourth physiological criterion for pain perception proposed by Bateson (1991) and Smith and Boyd (1991).

Gonzalez-Nuñez, V., & Rodríguez, R. E. (2009). The zebrafish: A model to study the endogenous mechanisms of pain. *Institute for Laboratory Animal Research Journal, 50*(4), 373-386.

I used this review to support my contention that fishes, and specifically zebrafish, possess an endogenous opiate system. This review summarizes the fundamental studies that identified endogenous opiate receptors and opioid ligand binding sites in zebrafish. I used this article to identify primary literature and to gain a clearer understanding of how these primary sources contribute to our knowledge of the endogenous opiate system in zebrafish.

Grossman, L., Utterback, E., Stewart, A., Gakiwad, S., Chung, K., Suciu, C., Wong, K., Elegante, M., Elkhayat, S., Tan, J., Gilder, T., Wu, N., DiLeo, J., Cachat, J., & Kalueff, A. V. (2010). Chatacterization of behavioral and endocrine effects of LSD on zebrafish. *Behavioral Brain Research, 214*(2), 277-284.

I used this reference as an example of top-dwelling behavior that has been identified previously in zebrafish. Because LSD increased top-dwelling behavior, the authors suggest that the top-dwelling behavior is motivated by a state of reduced anxiety.

International Association for the Study of Pain. (2012). IASP taxonomy. Retrieved September 23, 2012, from http://www.iasp-pain.org.

Pain can be a difficult concept to define. I cited this reference in order to provide a widely-accepted definition of pain in a research context. I also used this reference to clarify what I mean when I refer to the concept of "pain" throughout this paper.

Ito, H., & Yamamoto, N. (2009). Non-laminar cerebral cortex in teleost fishes? *Biology Letters, 5*, 117-121.

I used this article to support my claim that teleost fishes possess a cerebral cortex. In this article, the authors acknowledge numerous differences between the mammalian neocortex and the pallium of teleost fishes, but they argue that the two tissues might share some functional equivalents.

Le Bars, D., Gozariu, M., & Cadden, S. W. (2001). Animal models of nociception. *Pharmacological Reviews, 53*, 597-652.

This article provides a review of animal models of human pain, with a focus on rodent models. The authors discuss 4 main types of nociceptive stimuli: electrical,

thermal, mechanical, and chemical. Furthermore, the authors suggest that chemical pain is the closest approximation to acute pain in humans.

Mettam, J. J., McCrohan, C. R., & Sneddon, L. U. (2011). Characterisation of chemosensory trigeminal receptors in the rainbow trout, *Oncorhynchus mykiss*: responses to chemical irritants and carbon dioxide. *The Journal of Experimental Biology, 215*, 685-693.

The authors of this article tested the polymodal and mechanochemical receptors identified by Sneddon (2003b) in order to determine whether these responded exclusively to noxious stimuli and therefore could be considered true nociceptors. The authors placed a variety of noxious and non-noxious chemicals on the receptive fields and recorded activity in the trigeminal ganglion. They found that polymodal and mechanochemical receptors responded exclusively to noxious chemicals, so they concluded that those receptors are true nociceptors.

Mogil, J. S. (2009). Animal models of pain: Progress and challenges. *Nature Reviews Neuroscience, 10*, 283-294.

This relatively recent review discusses the challenges of translating discoveries related pain in animal models to therapies with clinical safety and efficacy in humans. I use this review to support my contention that a model of pain in zebrafish could be valuable to the field of pain research. The author also classifies pain assays into four different categories: acute, inflammatory, neuropathic, and painful disease.

Newby, N. C., Wilkie, M. P., & Stevens, E. D. (2009). Morphine uptake, disposition, and analgesic efficacy in the common goldfish (*Carassius auratus*). *Canadian Journal of Zoology, 87*, 388-399.

This article investigates the rate of morphine uptake by a goldfish when the drug is administered via the water. The authors found that morphine uptake from the water was slow compared to intraperitoneal injections (less than 1% of the morphine in the water was taken up into the plasma after 2 hours). However, the authors did note an analgesic effect when subjects were exposed to concentrations of 12 mg/L and 48 mg/L morphine. I use this information to justify my claim that the morphine concentrations tested in the current studies are likely sub-threshold, and that higher concentrations could provide an analgesic effect.

Northcutt, R. G. (1981). Evolution of the telencephalon in nonmammals. *Annual Review of Neuroscience, 4*, 301-350.

This article provides a review of the structure and evolution of the telencephalon in non-mammals. Northcutt suggests that fishes possess an outer layer of tissue called the pallium that is dedicated to the processing of multiple sensory modalities (not just olfactory input, as was previously believed). I use this article to support my claim that fishes possess a cerebral cortex.

Pinal-Seoane, N., Martin, I. R., Gonzalez-Nuñez, V., Marron, E., Alvarez, F. A., Sarmiento, R. G., & Rodríguez, R. E. (2006). Characterization of a new duplicate δ-opioid receptor from zebrafish. *Journal of Molecular Endocrinology, 37*, 391-403.

I used this reference to demonstrate that zebrafish possess δ opioid receptors.

Randall, D., & Brauner, C. (1991). Effects of environmental factors on exercise in fish. *Journal of Experimental Biology, 160,* 113-126.

I used this article to support my claim that hematological changes in response to acidosis could explain the increased top-dwelling behavior observed in the current study. These authors propose a mechanism by which acidic conditions could cause to a deficiency in oxygen, motivating the top-dwelling behavior.

Reilly, S. C., Quinn, J. P., Cosins, A. R., & Sneddon, L. U. (2008). Behavioural analysis of a nociceptive event in fish: Comparisons between three species demonstrate specific responses. *Applied Animal Behaviour Science, 114,* 248-259.

This is the first report of the lip injection model in zebrafish. I briefly review this article in order to highlight the behavioral changes that the authors observed in zebrafish as a result of this nociceptive event.

Rose, J. D. (2002). The neurobehavioral nature of fishes and the question of awareness and pain. *Reviews in Fisheries Science, 10*, 1-38.

Rose is one of the key figures in the scientific literature who firmly argues that fishes are incapable of feeling pain. I cited this article because I thought it was important to present the opinions of people who think that fishes cannot feel pain. Although much of the literature is in favor of fish being able to feel pain, this side of the argument should be represented. Rose's argument hinges on the fact that fishes do not possess a neocortex, which he claims is a structure that is necessary for consciousness. He also argues that the emotional aspects of pain cannot be

perceived without consciousness, leading him to conclude that fishes are incapable of feeling pain.

Sackerman, J., Donegan, J. J., Cunningham, C. S., Nguyen, N. N., Lawless, K., Long, A., Benno, R. H., & Gould, G. G. (2010). Zebrafish behavior in novel environments: Effects of acute exposure to anxiolytic compounds and choice of *Danio rerio* line. *International Journal of Comparative Psychology, 23*(2), 43-61.

This reference includes a report of increased top-dwelling behavior in zebrafish. The authors report a variety of drugs that were found to increase top-dwelling behavior, suggesting that the increases in top-dwelling behavior could be a response to anxiolytics or antidepressants.

Selbit, K. J., Oliveira, L., Zimmermann, F. F., Capiotti, K. M., Bogo, M. R., Ghisleni, G., & Bonan, C. D. (2010). Antipsychotic drugs prevent the motor hyperactivity induced by psychotomimetic MK-801 in zebrafish (*Danio rerio*). *Behavioral Brain Research 214*(2), 417-422.

This reference presents another example of top-dwelling behavior that has been identified previously in zebrafish. Olanzapine was found to increase top-dwelling behavior, so the authors suggest that the top-dwelling behavior is motivated by a state of reduced anxiety.

Smith, J., & Boyd, K. (1991). Pain, Stress, and Anxiety in Animals. *Lives in the Balance: The Ethics of Using Animals in Biomedical Research.* New York: Oxford University Press.

In this book chapter, the authors present a list of criteria that can be used to determine the pain-sensing capabilities of animals. Furthermore, they review the scientific literature in order to determine whether a variety of vertebrates, including fishes, meet these criteria. I use these criteria as a framework for discussing the components that are necessary for the perception of pain in animals. I also use these criteria to prime my discussion of existing knowledge of the pain-sensing capabilities of fishes, in order to suggest that fishes are capable of feeling pain.

Sneddon, L. U. (2002). Anatomical and electrophysiological analysis of the trigeminal nerve in a telelost fish, *Oncorhynchus mykiss*. *Neuroscience Letters, 319*, 167-171.

In this report, Sneddon describes the fiber types found in the trigeminal ganglion of a rainbow trout. She reports that A-beta fibers are the most prevalent fiber type in the trigeminal ganglion, followed by A-delta, A-beta, and C fibers. This study is important because it is the first report of the presence of A-delta and C fibers in teleost fish. This information was foundational in establishing the nociceptive capabilities of teleost fish.

Sneddon, L. U. (2003a). The evidence for pain in fish: the use of morphine as an analgesic. *Applied Animal Behavior Science, 83*, 153-162.

I use this article to support my claim that fishes exhibit pain-related behaviors that are antagonized by known analgesics. In this follow-up to work done by Sneddon, Braithwaite, & Gentle (2003), Sneddon demonstrates that the observed change in behavior can be antagonized by morphine. Sneddon observed a significant

reduction in behavior after administration of the morphine, suggesting that the earlier response was motivated by a pain state.

Sneddon, L. U. (2003b). Trigeminal somatosensory innervations of the head of a teleost fish with particular reference to nociception. *Brain Research, 972*, 44-52.

This is the first characterization of somatosensory receptors in teleost fish. It provides evidence for the presence of fast-adapting mechanoreceptors; slow-adapting mechanoreceptors; polymodal receptors sensitive to mechanical, thermal, and chemical stimuli; mechanothermal receptors, and mechanochemical receptors. I use this article to demonstrate that teleost fish are capable of sensing chemical stimuli. It should be noted, however, that this report does not demonstrate that these chemoreceptors respond exclusively to noxious chemical stimulation, so this article does not demonstrate the presence of chemical nociceptors.

Sneddon, L. U. (2009). Pain perception in fish: Indicators and endpoints. *Institute for Laboratory Animal Research, 50*(4), 338-342.

This review provided a helpful summary of the research on pain perception in fishes, and it also provided some novel interpretations of previously published data. In this review, Sneddon proposes that the increased time to resume feeding in fish following injection of noxious substances could be a protective response intended to promote healing of the affected tissue. I used this article to demonstrate that the first criterion proposed by Bateson (1991) and Smith and Boyd (1991) is met in teleost fish.

Sneddon, L. U., Braithwaite, V. A., & Gentle, M. J. (2003). Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. *Proceedings of the Royal Society of London B, 270*, 1115-1122.

In this study, the authors injected the lips of rainbow trout with acetic acid and observed the resulting physiological and behavioral changes. The authors found that injections of acetic acid led to increased opercular beat rate and complex behavioral changes including rocking motions and rubbing their lips against the gravel. I used this study to demonstrate that teleost fish exhibit a significant behavioral response to injections of 0.1% acetic acid. This fulfills the first behavioral criterion for pain perception that was proposed by Bateson (1991) and Smith and Boyd (1991). Although the authors reported a significant behavioral response in this study, it is unclear if this is a pain-related response.