THE DOSE-RESPONSE RELATIONSHIP OF MORPHINE IN A ZEBRAFISH (*DANIO RERIO*) MODEL

BY

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ABSTRACT

Previous experiments in zebrafish (*Danio rerio*) have shown that a noxious ("painful") stimulus results in behavioural changes, namely a marked decrease in swimming activity. Analgesics may be effective at blocking this behavioural change but there is little evidence-based data regarding the efficacy of analgesics in fish. Before any analgesic can be used, a dose-response relationship must be demonstrated and first needs to be shown with morphine, the gold standard analgesic. I tested a model using subcutaneous acid injection as the noxious stimulus which has previously been shown to decrease swimming activity in zebrafish. Fish activity, before and after treatment, was recorded with a video camera and analyzed with Loligo® software. To alleviate this response, morphine was administered intraperitoneally at doses of 1,3,10,30, or 100 mg/kg in an attempt to show a dose-response relationship. Fish receiving doses of 1 or 100 mg/kg came from a different source and behaved so differently that their results could not be included in the statistical analysis. Acetic acid (5%) at both 5 and 10 µL significantly reduced activity in zebrafish in comparison with saline injected controls ($p<0.0001$), with the difference scaling with stimulus intensity. Morphine at doses of 10 and 30 mg/kg was effective at attenuating the decrease in activity associated with the noxious stimulus. The ED_{50} of morphine was 12.3 ± 1.2 mg/kg (90% C.I. 9.7-15.5). Activity of 10 mg/kg morphine/acid injected fish was not significantly different from control fish that did not receive the noxious acid injection at 60 and 90 min post injection ($p=0.39$). Activity of morphine-injected controls (no noxious

stimulus) did not differ significantly from saline control fish at 60 and 90 min post injection ($p=0.88$). Effective doses of morphine (10 and 30 mg/kg) were then injected in conjunction with naloxone, a known opioid antagonist. Naloxone, at both 10 and 30 mg/kg, was effective at attenuating the analgesic effect of 10 mg/kg morphine. These results show that morphine acts dose-dependently on opioid receptors to reverse behavioural changes associated with a noxious event in zebrafish. These results are consistent with other studies on zebrafish and confirm the robustness of the acetic acid-zebrafish model in testing analgesic drugs.

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LIST OF ABBREVIATIONS

ACC- Animal Care Committee

AVC- Atlantic Veterinary College

CCAC- Canadian Council on Animal Care

CNS- central nervous system

CO- cardiac output

DOR- delta opioid receptor

DR- dose-response

drDOR- *Danio rerio* delta opioid receptor homologue

drKOR- *Danio rerio* kappa opioid receptor homologue

drMOR- *Danio rerio* mu opioid receptor homologue

ED_{50}- The dosage that produces half maximal effect

ELISA- enzyme-linked immunosorbent assay

GPCR- G-protein coupled receptor

IASP- International Association for the Study of Pain

IP- intraperitoneally

IV- intravenous

KOR- kappa opioid receptor

LS Means- least squares means

MIF-1- melanocyte stimulating hormone release-inhibiting factor-1

MOR- Mu opioid receptor

PBS- phosphate buffered saline

SC- subcutaneous

SEM- standard error of the mean

1.0 INTRODUCTION

1.1 The Importance of Fish in Research

Over the past decade, fish have become one of the most widely used animal models in research. This is largely due to their low cost, portability, ease of laboratory culture, short reproductive cycle, ease of genetic study and their potential for *in situ* field monitoring. Various fish species, such as the rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*), have emerged as primary non-mammalian vertebrate research models in toxicological, genetic, and disease studies. As a result of these studies, fish are subjected to a variety of potentially "painful treatments," such as fin tagging, blood sampling, various surgeries and toxicology testing. This has led to the development of animal welfare regulations [Aquatic Animal Health (England and Wales) Regulations 2009; Aquatic Animal Health (Scotland) Regulations 2009; Canadian Council on Animal Care (CCAC) 2005] but a significant gap in our knowledge of fish pharmacology remains in regards to analgesic efficacy, pharmacokinetics, dose-response (DR) relationships, and side effects. Analgesics must be tested in fish prior to their use in research, teaching or testing. The main goal of my project was to test the usefulness of a zebrafish model to describe the DR relationship of an analgesic, morphine, in fish.

1.2 Nociception

In order to survive, animals, including humans, must react to potentially noxious stimuli and because of its obvious importance, nociception is expected to be widespread across vertebrate animal taxa. Nociceptors are free endings of cerebrospinal (in the body) or trigeminal (in the head) nerve fibres that react to damaging, or potentially damaging, stimuli. Many of these receptors display polymodal characteristics in that they respond to more than one noxious stimulus modality, e.g. heat, mechanical pressure, and/or noxious chemicals, particularly acids. Nociceptors have been characterized in both mammals and birds (Gentle, 1989; Meyer *et al.*, 1994), fish (Sneddon, 2003a) and even *Drosophila* larvae which show stereotypical rolling behaviour in response to a probe heated to 38°C (Tracey *et al.,* 2003). Using genetic screens and mutants, a *painless* gene has been identified in this larva that is hypothesized to be involved in nociceptive signalling.

Nociception is defined as "the neural processes of encoding and processing noxious stimuli" (International Association for the Study of Pain- IASP, 2008). The nociceptive fibres conduct action potentials to the dorsal horn of the spinal cord and synapse with secondary neurons that transmit the information to the brain. In mammals and birds, the axons from nociceptors are divided into two classes: small, faster conducting myelinated A-δ fibres and smaller, slower conducting unmyelinated C fibres. Previous studies have shown that fish have free nerve endings beneath their epidermis (Harder, 1975). Furthermore, Sneddon (2002; 2003a) demonstrated that the three branches of the trigeminal

nerve in the rainbow trout are all comprised of a range of fibre types including A-δ and C fibres. These fibres were first described based on the size range of the cell bodies and axon diameters. Electrophysiological recordings of evoked activity and conduction speed confirmed the presence of these fibre types with the proportion mirroring the proportion of fibre types in anatomical analyses (Sneddon, 2002).

Cutaneous A-δ fibres are involved in the immediate pain response, whereas C fibres are believed to be involved in the secondary pain or dull pain response, which plays a role in the development of chronic pain (Torebjork and Ochoa, 1990). In humans, C fibres can comprise 50% of the total fibre type (Young, 1977). In the rainbow trout, C fibres are found only in distinct bundles and comprise only 4% of the total while A-β fibres are the most common followed by A-δ fibres, then A-α fibres. Sneddon (2002) speculated that the difference in C fibre proportion may be related to the advancement onto land in vertebrate evolution as terrestrial animals are exposed to more dramatic changes in temperature and have an increased chance of mechanical injury due to gravity. However, this relative proportion of C fibres to A-δ fibres has been estimated for very few species of mammals or fishes making generalizations tenuous.

Sneddon (2003a), using microelectrodes placed in cell bodies of afferent neurons in the trigeminal ganglion, applied various stimuli to the head of the rainbow trout and measured evoked neural activity and located and measured the diameter of receptive fields. To ascertain chemosensitivity, a drop of 1%

acetic acid or water was placed on these receptive fields and, while none of the units responded to the droplet of water, six of 62 units responded to the acid stimulation with conduction velocities in the range of 2.58 to 5 m/s indicating that A-δ fibres were responsible for these responses. These general features of the peripheral nociceptive system are accepted by most scientists. However, some questions remain regarding the processes that take place after the signal reaches the spinal cord.

Rose (2002; 2007) suggested that nociceptive responses to noxious stimuli in fish are simply reflexive and do not involve higher brain areas, stopping at the spinal cord or hindbrain. However, electrical activity during noxious stimulation has been recorded in the forebrain and midbrain of rainbow trout, goldfish (*Carassius auratus*), and Atlantic salmon (*Salmo salar*) (Dunlop and Laming, 2005; Nordgreen *et al.*, 2007) and this electrical activity was shown to differ according to stimulus type and intensity. Thus, nociceptive activity has been detected in both the midbrain and forebrain in fish, but we cannot conclude from this observation that the response is not reflexive in nature.

1.3 Pain

Pain is a perception which requires interaction and processing of nociceptive input at the cortical level. Pain is more than an avoidance response; it is a protective reaction to stimuli that could lead to tissue damage. This response is an evolutionarily adaptive property that enhances awareness of

potentially damaging stimuli. In the absence of the avoidance response, the ability of animals to detect hazardous stimuli is lost and the probability of injury or death is increased. Pain in humans has been defined as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage" (IASP, 2008). The problem in applying this definition to animals is that we cannot directly measure 'emotional experience' in animals. To assess possible pain perception in animals, we can make indirect measurements using behavioural and/or physiological responses to a potentially painful event and then test to see if these changes can be reduced by the administration of an analgesic. There are many definitions of animal pain. For example, Zimmerman (1986) defined pain as "an adverse sensory experience caused by an actual or potential injury that elicits protective motor and vegetative reactions, results in learned avoidance and may modify specific species behaviour". When discussing pain in animals with definitions like this, it is possible that we are actually referring to nociception, rather than the subjective emotional characteristics of the pain experienced by humans.

Motivational affective states including conscious experiences, such as pain, fear, hunger, thirst and pleasure, play an important role in the causation of some types of behaviour (Fraser and Duncan, 1998). It is these motivational affective states that are often used in animal welfare evaluations, frequently relying on indirect evidence from behaviour, as a window to the subjective states of an animal (Duncan, 2002). IASP (2008) states that "the inability to communicate verbally does not negate the possibility that an individual is experiencing pain";

although this text refers to neonates, it also has been applied to animals. Thus we need to ensure that the definition of animal pain is based on what we observe and measure, not subjective states. The extent to which changes in behaviour actually reflect changes in 'conscious' experiences of pain, fear, hunger, thirst, pleasure and play is subjective and controversial. However, at the present time, it is the only approach tractable to experimental evaluation.

The issue of pain in fish has been a controversial one and has been a popular topic, especially in the grey literature. Victoria Braithwaite came into the spotlight in 2003 with the publication of the controversial paper by her student, Lynn Sneddon "*The evidence for pain in fish: the use of morphine as an analgesic*" (Sneddon 2003b). Braithwaite states that because the injection of a noxious substance acts to distract a fish's attention, thereby decreasing normal behaviour (novel object avoidance), and that an opioid is effective at returning normal behaviour, the fish must be cognitively aware and experiencing negative feelings associated with pain (Braithwaite, 2010). Rose (2002; 2007), on the other side of the debate argues that the action of analgesic drugs in fish is not evidence of pain perception because these compounds act at lower, sub-cortical levels of nociceptive processing. He also argues that fish cannot experience states associated with pain and suffering because they lack the specific brain structures required for conscious pain perception in humans. In humans, pain causes brain activity in areas associated with emotion, mainly the limbic system (Damasio 1998). Suffering is only possible if animals are conscious, as you

need a conscious brain to develop sentience- the ability to generate feelings that cause the mental experience of discomfort. In Braithwaite's book, *Do Fish Feel Pain,* she states that "fish, with their less complex brain structure, may not feel pain the same way humans do but this does not mean they will be completely devoid of emotion, or incapable of suffering" (Briathwaite, 2010). In contrast, Rose (2002; 2007) concludes that only primates can experience pain. This view is not held by most animal care organizations around the world. While the fish brain is much smaller and lacks the cerebral cortex present in the forebrain of mammals, it must be recognized that different brain structures may perform similar functions. For example, the dorsal telencephalon has been shown to be activated during nociception in fish, similar to what is observed in the limbic system in mammals (Nordgreen, 2007).

1.4 Analgesics: Opioids

Analgesics are a class of drugs used to relieve pain without producing loss of consciousness. There are five major classes of analgesic drugs used clinically; α_2-adrenergic agonists, dissociative anesthetics, local anesthetics, nonsteroidal anti-inflammatory drugs (NSAIDs), and opioids. Opioids can be classified via their binding to opioid receptors (μ, δ, κ) or based upon chemical structure. There are four classes of opioids: endogenous opioids (e.g., endorphins), opium alkaloids (e.g., morphine), semi-synthetic opioids (e.g., heroin), and fully synthetic opioids (e.g., fentanyl).

Opioids produce analgesia and reduce anesthetic requirements. Morphine, hydromorphone, and oxymorphone are potent analgesics, have a long duration of action (3-4 h), and are used to manage moderate to severe pain (Schug *et al.*, 1990). Morphine is the prototypical opioid receptor agonist derived from the opium poppy and is the standard against which all other opioids are compared. Morphine is a phenanthrene (polycyclic aromatic hydrocarbon composed of three fused benzene rings) opioid receptor agonist that binds to and activates μ-opioid receptors in areas including the mammalian central nervous system (CNS), with high densities in the posterior amygdala, hypothalamus, thalamus, nucleus caudatus, and certain cortical areas. Receptors are also found on the terminal axons of primary afferent neurons within laminae I and II (*substantia gelatinosa*) of the dorsal horn of the spinal cord and in the spinal nucleus of the trigeminal nerve. In mammals, activation of μ-opioid receptors (MOR) can result in analgesia, sedation, and respiratory depression, depending on dose and species. Morphine, albeit with less affinity, is also a κ-opioid (KOR) and δ-opioid (DOR) receptor agonist. κ-opioid activation is associated with spinal analgesia, miosis (pinpoint pupils) and psychotomimetic effects whereas δ-opioid activation is thought to play a role in analgesia (Kieffer, 1995).

The effects of morphine can be countered with opioid antagonists such as naloxone which is frequently used to manage the life-threatening CNS and respiratory depression associated with opioid overdose. Naloxone is a competitive antagonist with high affinity for μ-opioid receptors in the CNS, but

also shows affinity, albeit lower affinity, for κ- and δ-opioid receptors. The chemical structure of naloxone resembles that of oxymorphone, the only difference being the substitution of the *N*-methyl group with an allyl (prop-2-enyl) group.

1.5. Animal Models of Pain

There are many models that are used to measure and study pain in animals. These involve various types of stimuli (electrical, thermal, mechanical, and chemical) and subsequent monitoring of responses ranging from spinal reflexes to complex changes in behaviour and/or physiology. Electrical stimuli are frequently used but are ineffective in differentiating between fibre types which evoke a variety of sensations (Le Bars *et al.*, 2001). Also, an electrical stimulus is not one an animal would normally encounter. Thermal noxious stimuli are more selective in stimulating cutaneous receptors, but the speed of heating can be quite slow when using lamps or contact thermodes which prevent reflexive synchronous excitation of fibres. CO_2 laser stimulators are more effective at ensuring a more synchronous and selective activation of free nerve endings (Treede *et al.*, 1984), and can evoke motor and vocalization responses in rats (Danneman *et al.*, 1994; Bragard *et al.*, 1998). However, overall cost and technical complexity has limited the use of CO_2 lasers in the study of pain. Mechanical responses can be graded in relation to the intensity and/ or duration of the stimulus, but has the disadvantage of activating low

threshold mechanoreceptors as well, and can be difficult to apply in unrestrained animals.

The injections of chemical noxious stimuli have prolonged effects that become inescapable once applied. Synchronized activity in primary afferents and typical reflex responses are not produced; therefore, it is not the threshold, but rather, the behaviour that is measured. Behaviour after chemical injection is relatively consistent in rodents, and these models are the closest in nature to most clinical pain in humans (Le Bars *et al.*, 2001).

Nociceptor response to acetic acid application and injection has long been used as a standard noxious stimulus in studies in mammals and amphibians. For example, the application of acetic acid to the hind leg of a frog which is similar to the commonly used rat paw formalin test, will induce a spinally mediated wiping reflex at acetic acid concentrations above a certain threshold (Pezalla and Stevens, 1984).

Due to the subjective nature of pain in humans and presumably other mammals, difficulty lies in the inconsistency in response to standard stimuli. Responses vary from reflexive twitches and vigorous and repeated escape attempts, to licking, jumping, latency to feed, nonspecific flight responses, raised blood pressure and immobilization. Behavioural and physiological responses such as these can be looked for in recognizing pain in humans and other animals.

Rats and mice have commonly been used as subjects in a variety of pain models. The tail flick or tail withdrawal protocol assesses the spinal antinociceptive responses to noxious thermal stimuli as does the hotplate test. Paw pressure tests are also used to test the mechanical paw withdrawal threshold in response to the application of noxious pressure and the formalin test has been used in the assessment of persistent nociceptive pain responses.

Evidence for the capacity of non-mammalian species to experience pain is supported by the presence of: appropriate neurological components with the ability to elicit an action potential in response to a noxious stimulus, endogenous anti-nociceptive mechanisms to modulate the response, and the demonstrable modulation of nociceptive pathways and behavioural responses using pharmacological agents effective in other species (Spray, 1976).

Electrical and thermal stimulation have been used to evaluate responses to noxious stimuli in birds. Evaluation of withdrawal threshold and response to analgesics has been evaluated utilizing two different stimuli. The effect of electrical current and thermal gradients on nociceptive thresholds were evaluated in conscious parrots (Paul-Murphy *et al.*, 1999a), and the response to noxious stimuli was compared before and after administration of butorphanol or saline (Paul-Murphy *et al.*,1999b). In the African gray parrot, *(Psittacus erithacus*), 1.0 mg/kg butorphanol significantly decreased the response to a noxious electrical stimulus. Studies such as these have led to butorphanol tartrate being considered the analgesic drug of choice for the management of acute and chronic pain in birds at doses of 1-3 mg/kg (Curro *et al.*, 1994).

As in mammals, endogenous opioid peptides have been shown to modulate the central processing of noxious information in amphibians (Stevens, 1988). Spinal administration of the opioid peptides dynorphin, β-endorphin or met-enkephalin, resulted in a dose-dependent increase in nociceptive threshold in frogs (Pezall*a* and Stevens, 1984; Stevens *et al.*, 1987) which was then blocked with naloxone, an opioid receptor antagonist (Stevens *et al.*, 1995). The analgesic effect of morphine has been demonstrated in frogs which display a spinally mediated wiping reflex after acetic acid application to the hind leg. Acetic acid is used as the noxious stimulus in most pain experiments involving amphibians (Stevens, 1996).

1.5.1 Animal Models of Pain: Fish

Pain can be very difficult to assess in humans and other mammals. Veterinarians responsible for writing guidelines for assessing pain in animals have even greater difficulty when they get to fish, even though there is evidence which supports the conclusion that these complex animals possess mechanisms for avoiding noxious stimuli and responding to injury (Bateson, 1991). Acid sensitive nociceptors have been found on the head of the rainbow trout around the lips and operculum (Sneddon, 2002; Sneddon, 2003a). These nociceptors have similar properties to those found in mammals and, thus, the acetic acid pain test should be an effective noxious stimulus to use in a fish model.

The effect of acetic acid injection has been studied in rainbow trout, common carp (*Cyprinus carpio*), and zebrafish (Reilly *et al.*, 2008). Although no differences in behaviour were observed between noxiously stimulated carp and control fish, both the trout and zebrafish displayed a significant reduction in swimming frequency. This may prove to have a protective role in terms of energy expenditure, i.e. saving energy for recovery and prevention of further damage and pain (Sneddon *et al.*, 2003; Reilly *et al.*, 2008; Ashley *et al.*, 2009). Changes in activity are easily measured, but normal pre-treatment behaviour must be measured to establish baselines for each fish. Correia *et al.* (2011) also studied the behavioural effects of acid injection on zebrafish. They reported that the magnitude of effect on locomotor response scaled with acetic acid concentration as the response of fish injected with 5% acetic acid differed significantly from 10% acetic acid, and both treatments were statistically different from saline controls. Correia *et al.* (2011) used both an electric biosensor system that measured changes in water impedance as well as a more traditional video tracking system and concluded that both systems were effective and yielded similar results. These studies demonstrate the behavioural responses to noxious stimuli in zebrafish and support the utility of the zebrafish model in pharmacological and behavioural studies.

There are reports of acid injection altering other aspects of trout behaviour. Sneddon *et al.* (2003) reported a latency to feed in rainbow trout injected with acetic acid of 170 min compared with 80 min in fish injected with saline, as well as a marked increase in rocking and rubbing behaviour in the

acid-injected fish. These experiments were repeated by Newby *et al.* (2007), who, in contrast, saw no delay in feeding, rocking or rubbing behaviour. Both experiments were conducted in rainbow trout under similar conditions, but fish were un-anesthetised during the acid injection in the Newby study, which may explain the differences observed.

Anesthetic use for fish is common in commercial aquaculture, laboratory research and field studies to reduce damage and stress during a variety of procedures. The six stages of anesthesia are easily recognizable in fish, from stage 1 that involves slight loss of reactivity to tactile stimuli and a slight decrease in opercular beat rate to stage 6 where opercular movements cease (Cotter and Rodnick, 2006). Anesthetics are deemed efficacious if procedures can be carried out with minimal restraint. However, most "anesthetic" drugs used in fish simply produce immobilization and do not necessarily produce analgesia.

The molecular evolution of opioid receptors in fish has been examined by isolating cDNAs that encode six opioid receptor-like proteins from the white sucker, *Catostomus commersoni*. The identification of these six distinct opioid receptor-like proteins indicates that opioid receptors have been conserved over the course of vertebrate evolution (Darlison *et al.*, 1997).

Opioid receptors also have been studied in zebrafish. The expression patterns of the four opioid receptors has been analyzed in the zebrafish brain using immunohistochemistry (Porteros *et al.*, 1999; Alvarez *et al.*, 2006; Pinal-

Seoane *et al.*, 2006). The receptors showed a wide distribution in the brain with labelling intensity being greatest in areas involved in sensory information processing. This leads to the conclusion that the zebrafish brain is to some extent neuro-anatomically and functionally comparable with that of mammals (Guo, 2004).These opioid binding sites have also been located in the zebrafish brain in binding studies with relatively non-selective agents such as morphine or naloxone and endogenous opioid peptides. These exogenous and endogenous opioids show high affinity binding and displace the antagonist [^{3}H] – diprenorphine at two different sites in zebrafish brain membranes (Gonzalez-Nunez *et al.*, 2006). While the wide distribution of opioid receptors has been shown in the zebrafish brain using whole body autoradiography, no morphine binding activity was observed in the spinal cord or in transverse brain sections of Atlantic salmon after injection of morphine at a dose of 100 mg/kg (Nordgreen et al., 2009).

Four opioid receptors have been identified and cloned from zebrafish: The δ opioid receptor (drDOR), which shows a high degree of similarity to the mammalian DOR (Barrallo *et al.*, 1998a,b); drDOR2, a drDOR1 duplicate that shows high sequence similarity to other DORs (Pinal-Seoane *et al.*, 2006); the μ opioid receptor homologue drMOR; as well as drKOR, which is homologous to the mammalian κ opioid receptor (Alvaraz *et al.*, 2006), all of which are members of the G protein-coupled receptor (GPCR) superfamily.

Several pharmacological studies on these four receptors have been carried out. The overall conclusion is that non-selective opioid ligands bind to

zebrafish opioid receptors in similar fashion to the mammalian receptors; however, zebrafish receptors showed lower binding affinity with the highly selective opioids that show proven activity in mammalian counterparts. If this is examined from an evolutionary perspective, it suggests the binding pocket of opioid receptors is highly conserved throughout vertebrates, whereas extracellular domains that relay the sensitivity of ligands to receptors are less conserved (Gonzalez-Nunez *et al.*, 2007).

There is evidence that two rounds of whole genome duplication (2R) occurred in early vertebrate evolution but only the genome of teleost fish doubled again (3R) (Dreborg *et al.,* 2008). This would explain the fact that zebrafish appear to have two versions of proenkephalin, pro-opiomelanocortin, pronociceptin (Dreborg *et al.*, 2008) and the DOR (Gonzalez-Nunez and Rodriguez, 2009) whereas mammals do not.

The ability of analgesics to modulate behaviour associated with noxious stimuli is also indicative of pain perception. Pezalla and Stevens (1984) applied acetic acid to the hind legs of frogs (*Rana pipiens)* inducing a wiping reflex. Morphine sulphate, injected into the lumbar area of the spinal cord, was shown to act as a potent analgesic in preventing this behaviour in doses as little as 0.0316 µg/frog. There have been a few behavioural experiments using opiate analgesics in fish. Goldfish showed behavioural avoidance to a strong acoustic stimulus and this behaviour was modulated by opiate analgesics (Olson *et al.*, 1978). Morphine injected into the brain of paradise fish (*Macropodus opercularis*) at 2 mg/kg altered certain aspects of routine behaviour (Doka *et al.*,

1985). Goldfish have been shown to avoid an electric shock, but 30 mg/kg morphine applied directly to their brain resulted in an increase in threshold to the shock (Ehrensing *et al.*, 1982).

Recent studies on the nociceptive capabilities of the rainbow trout (Sneddon, 2002; Sneddon, 2003a) have led to questions regarding the effect of noxious stimulation on behaviour and physiology and what potential attenuation analgesics may provide. After acetic acid injection to their snout, rainbow trout were shown to perform anomalous behaviours such as rocking on their pectoral fins and rubbing their lips onto the gravel and sides of the tank. Opercular beat rate also increased significantly in comparison with controls that were injected with sterile saline. Morphine (300 mg/kg) was effective in significantly reducing these behaviours and appeared to be effective as an analgesic in the rainbow trout (Sneddon, 2003b). These trout showed no significant difference in the amount of swimming performed in any of the treatment groups. A single dose of morphine has been shown to have no effect on normal behaviour, feeding, and physiology in the rainbow trout (Sneddon *et al.*, 2003). But further studies must be undertaken to determine the effects of morphine at different doses in different species.

Jansen and Greene (1970) transferred goldfish to water containing 10 mg/L of morphine and reported that tissue concentration was equivalent to 9.7 mg morphine/kg fish after 15 min of exposure as that amount had dissipated from the water. They theorized that this equilibrium occurred after 15 min and no further uptake occurred over the next 3 h. Furthermore, when these fish were

transferred to a morphine-free container, the efflux of morphine from the fish to the water occurred at the same rate. Newby *et al.* (2009) attempted to replicate this work in goldfish and found that morphine uptake was extremely slow when administered via the water and when they went on to measure concentrations in the plasma they found concentrations <1 % of that of the water after 2 h. They were also interested in the effects of water chemistry on morphine uptake and found that altering water pH or hardness was effective at causing small changes in morphine uptake from the water, but plasma levels remained at <1% of the morphine concentration in the water throughout the experiment. Morphine does not seem to move rapidly across fish gills, implying that simply adding morphine to water is not an appropriate route of morphine administration in fish (Newby *et al.*, 2009). However, morphine at 48 mg/L in the water reduced the rubbing behaviour exhibited in goldfish after the injection of acetic acid in the cheek by approximately 95% (Newby *et al.*, 2009). Jansen and Greene (1970) also reported that morphine administered via the water acted as an analgesic by increasing the threshold to a noxious electrical stimulus. These results are not consistent with the slow rate of uptake of morphine from the water and Newby *et al.* (2009) presented three possible explanations for this observation: morphine was absorbed via the nasal capsules and bound to central receptors, morphine bound to peripheral morphine receptors, or morphine uptake, while extremely slow, was sufficient to bind to central receptors to exert an analgesic effect.

Newby *et al.* (2006) carried out the first pharmacokinetic studies for morphine in fish. Single intraperitoneal (IP) injections of morphine sulphate (40

mg/kg) were performed in winter flounder (*Pseudopleuronectes americanus*) and sea-water acclimated rainbow trout. They used repeated blood sampling to measure the plasma morphine concentration for 100 h post IP injection. Plasma morphine was found to reach maximum concentration within one hour post injection with a rapid distribution phase but extremely slow elimination phase. A bi-exponential decrease in concentration, similar to mammals, was seen in these two species. The half-life for distribution was less than 3 h for both species while the half-life for elimination was approximately 34 h for winter flounder and 14 h for rainbow trout (Newby *et al.*, 2006), compared to an elimination half-life of 2.9 ± 0.5 h in humans (Stanski *et al.*, 1978). Newby also reported the half-life for elimination of morphine in freshwater acclimated rainbow trout to be 18 h (Newby *et al.*, 2008) and in goldfish to be 37 h (Newby *et al.*, 2009). These results demonstrate that there are substantial differences between species in the elimination of morphine and that the disposition of morphine is about one order of magnitude slower in fish than mammals.

Potential side effects of morphine injection were also studied in winter flounder (Newby *et al.*, 2007). Morphine administration, both IV and IP, resulted in immediate bradycardia with duration of approximately 5 min, followed by a slow and sustained increase in heart rate and cardiac output (CO) to approximately 20% greater than baseline values for the duration of the 72 h experiment. Morphine injection only transiently affected respiratory rate. Application of a noxious stimulus (5% acetic acid to the cheek) resulted in a significant (10%) but transient (< 5 min duration) increase in CO, which was

completely blocked by prior administration of morphine at 40 mg/kg (Newby *et al.*, 2007). While morphine was shown to block the physiological reaction (i.e. increased heart rate and CO) to a noxious stimulus, potential cardiovascular side effects must be noted, especially in research where its use could be a confounding factor.

Naloxone has been used in non-mammalian species to counter the effects of opioids. Acetic acid application and resultant wiping reflex was shown to be mediated in frogs by the injection of morphine sulphate (Pezalla, 1983). This analgesic effect was then completely blocked by naloxone HCl at doses of 0.158 or 0.316 µg with animals receiving the naloxone alone appearing to have a slightly hyperalgesic response when compared with saline controls, but this effect was not significant (Pezalla,1983). Acute administration of naloxone has been shown to induce increased swimming behaviour in zebrafish (Stewart *et al.*, 2010). This suggests that opioid antagonists may have effects on swimming behaviour in zebrafish, possibly through the inhibition of endogenous opioid ligands. Morphine antagonism via naloxone and the hypothalamic peptide melanocyte stimulating hormone release-inhibiting factor-1(MIF-1) has been shown in goldfish. Morphine increased the threshold to an electric shock stimulus and this increase was effectively attenuated by naloxone and MIF1 and again a lower voltage was required to elicit a response (Ehrensing *et al.*, 1982). These studies scratch the surface of the various behavioural and physiological consequences of noxious, potentially painful events in a variety of teleost species. While morphine has shown promise in ameliorating these effects, the

dose-response relationships for morphine have not previously been studied in fish.

1.6 Zebrafish

Many animal models exist to study the mechanisms of pain and to aid in the development of pharmacological methods and substances for pain management. Recently, in addition to classical models such as the rat hind-paw test, fish have emerged as a potential model animal for the study of nociception, pain, and analgesic drugs. One species that has emerged as a widely used vertebrate research organism, primarily as a model for developmental genetics and increasingly for toxicological, human health, and behavioural studies, is the zebrafish (Trede *et al.*, 2004; Wright *et al.*, 2006; Beckman, 2007). Zebrafish models can be important from an evolutionary perspective allowing identification of common, conserved pathways involved in anxiety regulation and opioid effects. Similar to mammals, zebrafish possess a functional opioid system complete with opioid peptides and their receptors (Gonzalez-Nunez and Rodriguez, 2009; Stevens, 2009; Sundstrom *et al.*, 2010).

Zebrafish opioid receptors have been shown to be fundamentally similar in their molecular, pharmacological, and biochemical profiles to those of mammalian counterparts. In addition, zebrafish are sensitive to the anxiolytic action of morphine, similar to what has been reported in rodents (Kahveci *et al.*, 2006; Zhang and Schulteis, 2008) and have also been shown to be sensitive to the addictive properties of morphine (Lau *et al.*, 2006; Bretaud *et al.*, 2007). This

allows for extrapolation of results to higher vertebrates, warranting the utility of the zebrafish model in testing novel analgesic pharmaceuticals *in vivo*.

Zebrafish have a relatively high genetic similarity to humans (Lieschke and Currie, 2007) and provide many advantages in comparison with other vertebrates, such as low cost, easy handling, maintenance, fast reproduction, as well as access to all developmental stages as the optical clarity of embryos and larvae allows for real-time imaging of developing pathologies (Guo, 2004; Egan *et al.*, 2009). Thus, zebrafish models represent the possibility of a rapid and inexpensive screening system for a variety of novel compounds. I used zebrafish in my project in an effort to reveal the dose-response relationship to morphine in this widely used research organism.

1.7 Objectives and Hypothesis

The basic hypothesis is that morphine acts as an analgesic in fish (the null hypothesis is that morphine has no analgesic effect in fish). I tested this hypothesis by carrying out two critical tests, listed below. In addition there were three important control experiments that were required. Thus there were 5 separate experiments associated with testing the central hypothesis that morphine acts as an analgesic in fish. These experiments were:

1. To test if there is a relationship between the magnitude of the noxious stimulus and the magnitude of the behavioural response in zebrafish.
2. To estimate the dose(s) of morphine that will decrease or abolish the behavioural changes associated with a noxious stimulus (acetic acid injection) in zebrafish.
3. To measure the effect of various doses of morphine in and of itself on zebrafish swimming activity.
4. To test the ability of an opioid antagonist, naloxone, to block the analgesic effect of morphine in zebrafish.
5. To test the effect of an opioid antagonist, naloxone in and of itself, on zebrafish swimming activity.

In summary, the overall goal of my thesis is to develop a robust, reliable model that can be used to define the dose-response relationship of morphine in fish. This model can then be applied to test the effect of other drugs as well as the effect of various environmental factors on the dose-response relationship to morphine.

2. MATERIALS AND METHODS

2.1 Animals

Two hundred and forty zebrafish (*Danio rerio)* of AB strain were obtained from 2 commercial fish suppliers (Aquatron Laboratory, Dalhousie University, Halifax, NS and Pets Unlimited, Charlottetown PE). Fish were held in 34-L glass aquaria (50.1 x 25.3 x 30 cm), containing well water (pH 7.5) at a stocking density of 2 fish L^{-1}. The water temperature was maintained at 25 ± 1 ° C by submersible heaters and filtered through multi-stage external power filters with mechanical, chemical, and biological components. The water was returned to the aquaria via a waterfall system which ensured adequate aeration. Fish were kept under a10:14 h light : dark regime and fed flake food daily (NutraFin®), except on the experimental day. Mean fish mass was 0.37 ± 0.19 g. Fish were maintained and treated according to the ethical guidelines of the CCAC; this project was approved by the UPEI Animal Care Committee (Protocol Number: 09-004 -1003051).

2.2 Procedures Common to All Experiments

The change in distance moved over time was used as the basic metric of the response to the noxious stimulus. Real time position tracking software-LoliTrack® (Loligo Systems,Tjele, Denmark), was used and provided x-y coordinates of fish twice per second. These coordinates were exported to a Microsoft Excel® file and the Pythagorean Theorem was used to calculate distance between points at a rate of 2 Hz. Thus, at each time (pre, 30, 60, 90,

and 120 min post noxious stimulus) there was a 3 min record for 3 min x 60 sec/min x 2 Hz = 360 points per time period with a total of 1800 distance measurements per fish. The fish movements were also recorded using LoliTrack® and these video files were analyzed for anomalous behaviors such as rubbing the lips against the tank walls and loss of equilibrium.

During the test, fish were individually placed in 2 L containers (12 cm diameter) containing 0.5 L well water. The test arena was opaque on the sides but translucent on the bottom and was illuminated from below. After 1 h acclimatization, swimming activity was recorded for 3 min using a Panasonic PCTV video camera (model number WV-BP 144) and analyzed using LoliTrack® software recording at 30 f/s. This activity is referred to as pre-treatment activity. Then fish were individually anaesthetised in a container containing 0.5 L well water containing benzocaine (0.5 mL of 1 g/30 mL ethanol, Sigma-Aldrich; St. Louis, MO) and once the fish reached medium–to-deep plane anaesthesia they were weighed and then carefully injected with the appropriate amount of morphine/saline/naloxone IP (10 µl gastight syringe, 30 gauge needle; Hamilton; Reno, NV). Injections of saline or 5 or 10 µl of 5 % acetic acid (pain stimulus) to the snout of the fish were then performed using a 10 µl gastight Hamilton syringe with a 33 gauge needle. The equivalent amount of saline was injected into control fish. Fish were returned to their original containers and given 30 min to recover from the anesthetic before post-treatment recordings began. The experimenter was not visible to the fish during recordings. After the injections fish were recorded for 3 min every 30 min for 2 h. Fish were then euthanized via

anesthetic overdose (benzocaine 200 mg/L) and fixed in formalin. Four fish were tested concurrently and fish were tested during the same time frame each day (9:00-15:00 hrs) to avoid the confounding effects of circadian variation in locomotion and hormonal secretion (Cachat *et al.*, 2010; Grossman *et al.*, 2010; Wong *et al.*, 2010). I performed all of the procedures.

2.3 Treatments (for summary, see Table 1).

2.3.1 Control Fish

In this treatment, 5 µl of sterile saline was injected subcutaneously into the front of the head as a control for the nociceptive stimulus and 5 µl of sterile saline was injected IP as a control for the morphine injection (n=20).

2.3.2 Experiment 1 - Noxious Stimulus

In this treatment group, 5 µl or 10 µl of 5 % acetic acid was injected into the front of the head and 5 µl of saline was injected IP (n=12/group).

2.3.3 Experiment 2 - Morphine Analgesia

In this treatment 5 µl acetic acid was injected into the front of the head and various doses of morphine sulphate (1, 3, 10, 30, 100 mg/kg; 10 mg/mL, injection USP, Sabex, Boucherville, QC), were injected IP immediately after into individual fish (n=12/group).

Table 1. Summary of injections of acid, saline, morphine and naloxone for each treatment group used in all of the experiments.

Experimental Group	Injections IP	Injections IP	Injections Lip	N
Control Fish	10 µL Sterile Saline	-	5µL Sterile Saline	20
1. 5µL Acetic Acid	5 µL Sterile Saline	-	5µL 5% Acetic Acid	12
10µL Acetic Acid	5 µL Sterile Saline	-	10 µl 5% Acetic Acid	12
2 Morphine /Acetic Acid	1,3,10,30,100 mg/kg Morphine	-	5µL 5% Acetic Acid	12-15/group 63 total
3 Morphine only	1,3,10,30,100 mg/kg Morphine	-	-	12/group 48 total
4. Naloxone/Morphine /Acetic Acid	10, 30 mg/kg Morphine	10, 30 mg/kg Naloxone	5µL 5% Acetic Acid	12/group 48 total
5. Naloxone only		10,30 mg/kg Naloxone		12/group 24 total

2.3.4 Experiment 3 - Morphine Side Effects

In this treatment various doses of morphine sulphate (3, 10, 30, 100 mg/kg; 10 mg/mL) were injected IP, but nothing (i.e., neither acetic acid nor saline) was injected into the front of the head as a noxious stimulus (n=12/group).

2.3.5 Experiment 4 - Antagonist Experiment: Naloxone

Four treatment groups were administered the opiate antagonist naloxone hydrochloride (Tocris Bioscience, Ellisville, MO) as well as morphine at 10 and 30 mg/kg (n=12 per group) and 5 % acetic acid as the noxious stimulus. ((M10, N10; M10,N30; M30,N30; M30,N10 (mg/kg)). Injections of morphine and naloxone were performed IP concurrently using two different syringes and needles.

2.3.6 Experiment 5 - Antagonist side effects

Two treatment groups injected with naloxone at 10 and 30 mg/kg were used to control for any potential effects of naloxone on swimming behaviour but nothing (i.e. neither acid nor saline) was injected into the front of the head as a noxious stimulus.

2.4 Statistical Analysis and Data Reduction

Data for fish were discarded if they displayed immobility for the duration of a 3 min recording period and a new trial done so that each treatment had 12 replicates in all experiments. For saline controls there were 20 replicates. The raw data set for each treatment was large -- 3 min per time period * 2 Hz sample rate * 12 fish = 4320 samples per time period per treatment for each experiment, or 21600 for the complete trial of 5 time periods (pre-treatment, 30, 60, 90, 120 min). This large data set per treatment was reduced by averaging total movement for the 12 fish over 15 sec bins yielding an n of 12 for each treatment at each time period. These values at each time period after the treatment were normalized to the pretreatment value and these 12 normalized values at each post-treatment time (30, 60, 90, 120 min) were used in the statistical tests. Treatment effects, time effects, and their interaction were evaluated using a general linear model, and differences between treatments within an experiment were tested using Tukey's test. The procedure files and their detailed outputs are contained in the appendices. The level of statistical significance was set to $p < 0.05$ and all statistical analysis was performed using SAS Statistical software (SAS Institute Inc., Cary, NC). The average of the means associated with each time period (LS means) were used graphically to show the relationship between groups.

3. RESULTS

3.1 Objective 1: Reduced Activity Associated with a Noxious Stimulus

In general, zebrafish were active swimmers; the average distance moved by control fish during the 3 min pre-treatment period was 752 ± 139 cm. The saline control fish showed a slight decrease in activity after treatment (Figure 1A), this was not significant in comparison with the decreases exhibited after acid injection (Figure 1B). Acetic acid injection resulted in a significant decrease in swimming activity with both 5 and 10 µL acid ($p<0.001$) in comparison with saline controls at all time points post injection. The injection of 5µL acetic acid differed significantly from 10 µL ($p=0.0023$); difference scaled with stimulus intensity (Figure 2) and differed significantly with time ($p<0.001$). Also, there was a significant time effect ($p<0.0001$) observed in fish injected with 10 µL acetic acid (Figure 2) with the lowest activity observed 90 and 120 min post injection.

3.2 Objective 2: Morphine Analgesia

The decrease in fish activity associated with the noxious stimulus was attenuated by the administration of morphine at doses of 10 and 30 mg/kg with both treatments resulting in a significant increase in activity (Figure 3) when compared with no morphine fish ($p<0.001$). The effective dose of morphine (ED_{50}) calculated using data at 60 and 90 min post injection was 12.3 ± 1.2 mg/kg (90% C.I. 9.7-15.5)(Figure 3). Activity of fish injected with morphine at 10 mg/kg was not significantly different than control fish receiving saline instead of acid at 60 and 90 min post injection ($p=0.39$). Time periods 60 and 90 were

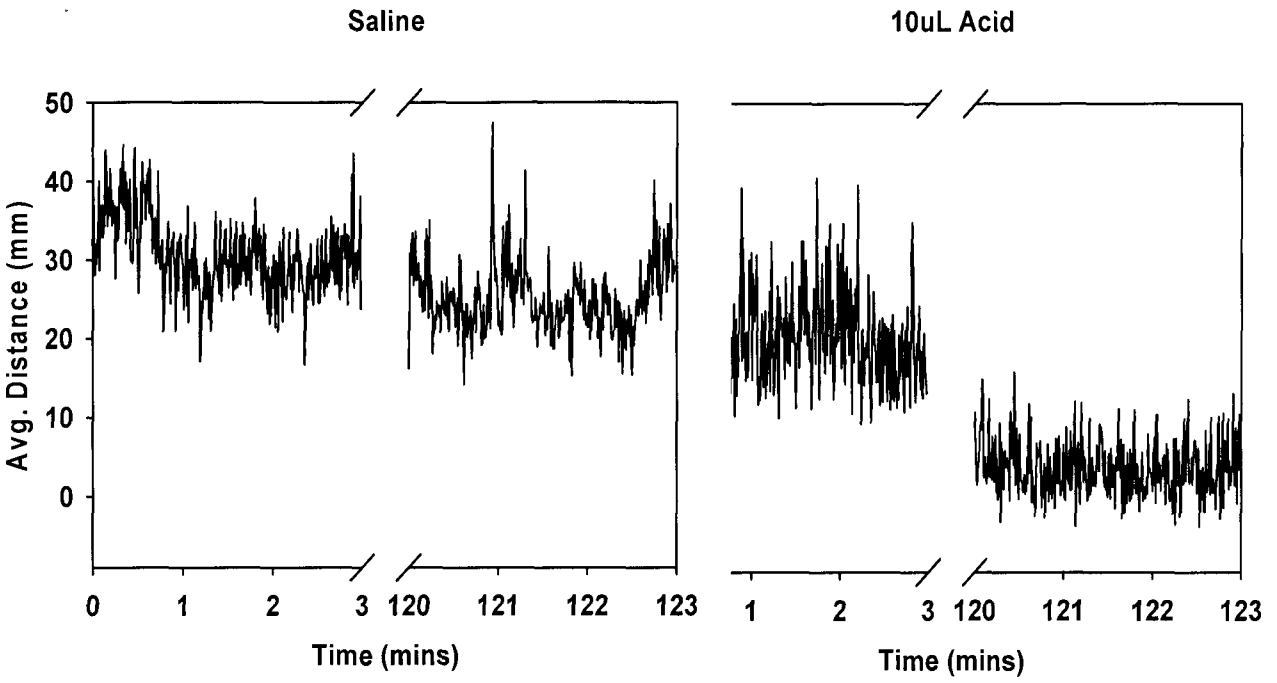

Figure 1. The effect of saline and acid injection: mean change in swimming distance over time in zebrafish injected with saline (A) or 10µL acetic acid (B) prior to and 120 min post injection. Values are means every 0.5 s.

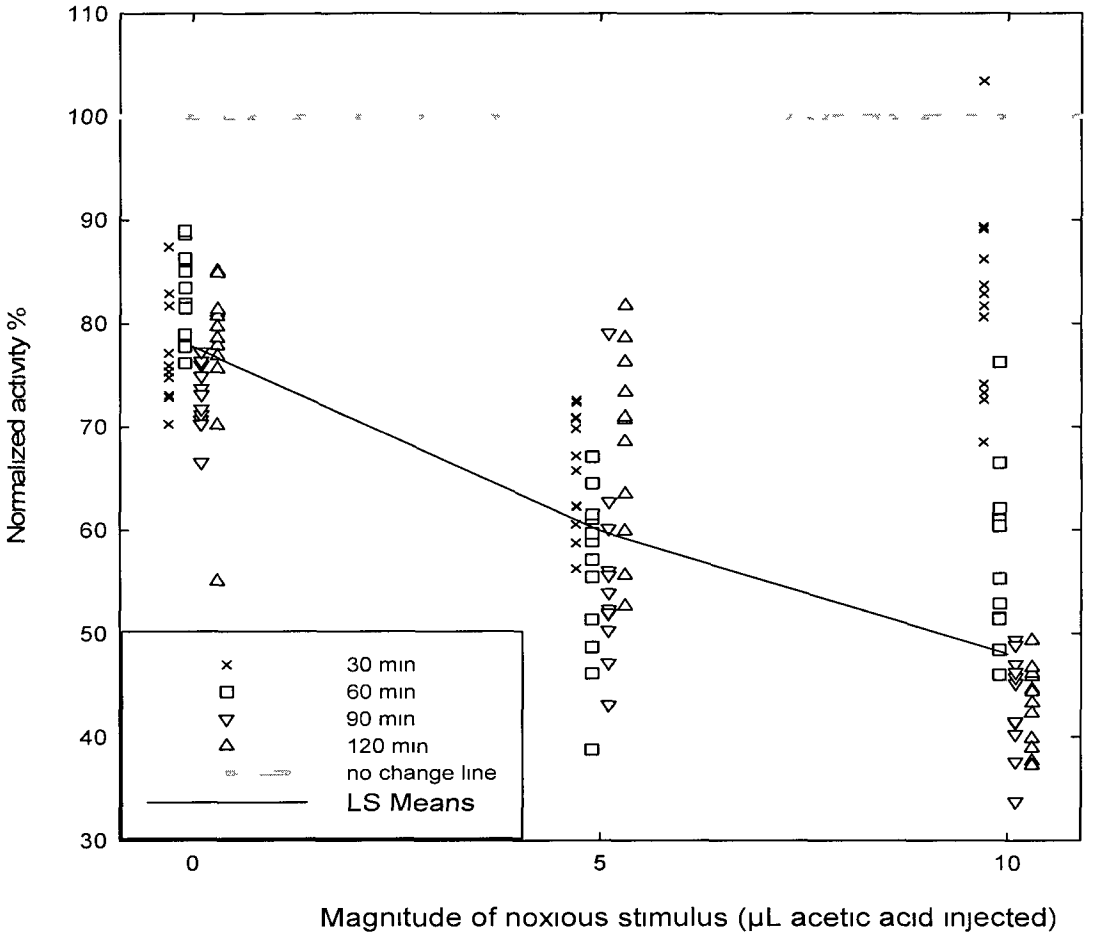

Figure 2. The effect of acetic acid (0, 5, 10 µL) injection on swimming activity (% normalized distance) of zebrafish (n=12 per group). Line joins LS means (Least squares means).

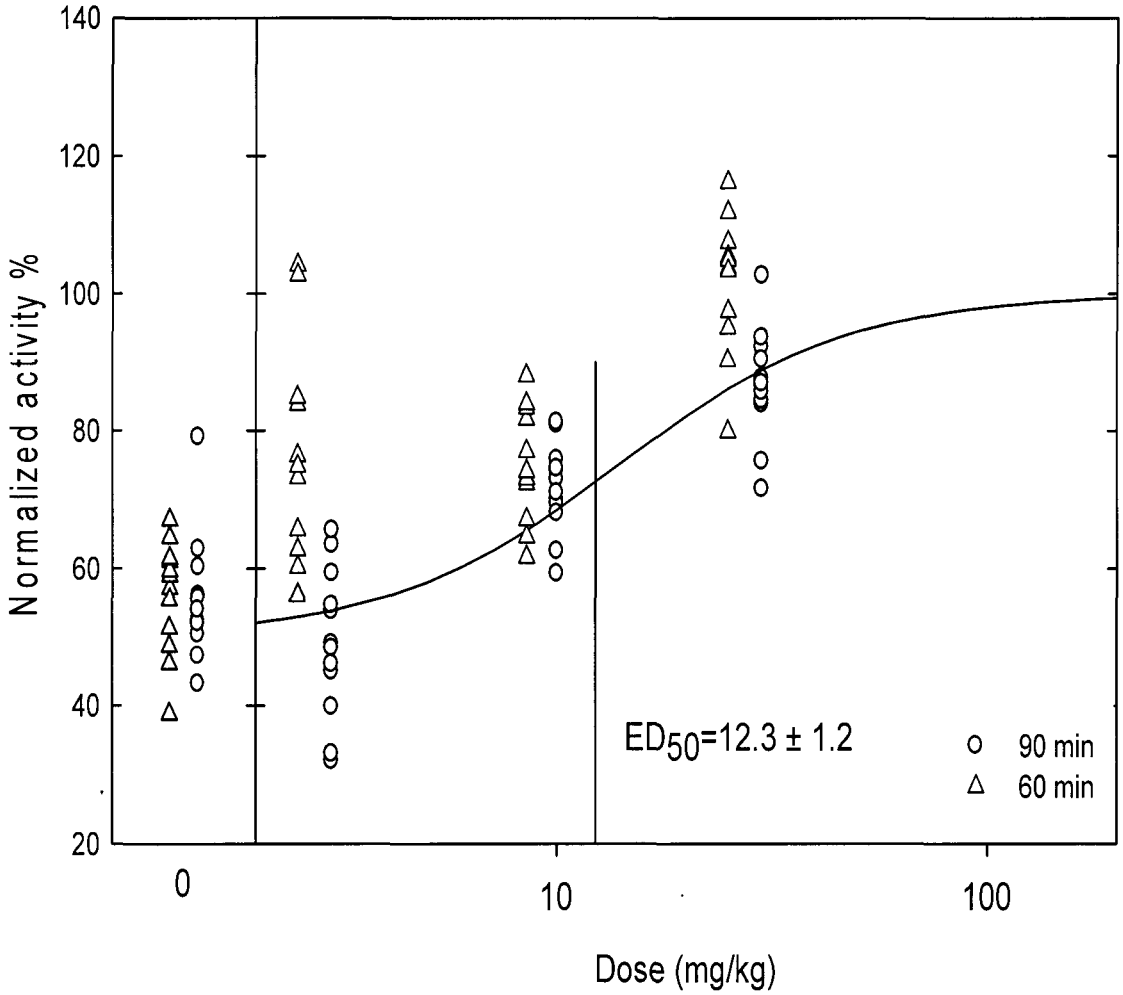

Figure 3. Normalized behavioural response of zebrafish injected with 5 µL of 5% acetic acid only (0) or acetic acid and 3, 10, or 30 mg/kg morphine at 60 (Δ) and 90 (o) min post injection; vertical line indicates the calculated ED_{50} at 12.3 ± 1.2 mg/kg (n=24/dose).

used as there was no significant difference in activity after 5 µL acetic acid injection at these times (LS means = 55.9 at 60 min; 55.4 at 90 min). Also, the time period after 30 min was not included as I wanted to mitigate any possible confounding effects of the anesthetic.

Fish treated with 3, 10, and 30 mg/kg morphine were all sourced from a local pet store. Fish treated with 1 and 100 mg/kg morphine were sourced from a breeding facility. Behaviour of fish from the breeding facility was different from fish from the pet store and showed excessive variance in activity (inactivity or extremely high activity) between fish and thus these data were not included in the analysis.

3.3 Objective 3: Morphine Side Effects

There was no significant difference between the normalized total distance of 3, 10, 30 mg/kg morphine in and of itself (no noxious stimulus) and saline injected controls at 60 and 90 min post IP injection ($p=0.88$) (Figure 4). Data were Winsorized to remove two extreme outliers (Wilcox and Keselman, 2003; Osborne, 2010).

3.4 Objective 4: Antagonist Experiment

Naloxone, at 30 mg/kg, was effective at blocking the analgesic effect of 10 and 30 mg/kg morphine (Figure 5). Naloxone at 10 and 30 mg/kg acted dose-dependently in reducing the analgesic effect of 10 mg/kg morphine (Figure 6). Naloxone treatment groups differed significantly from the saline control

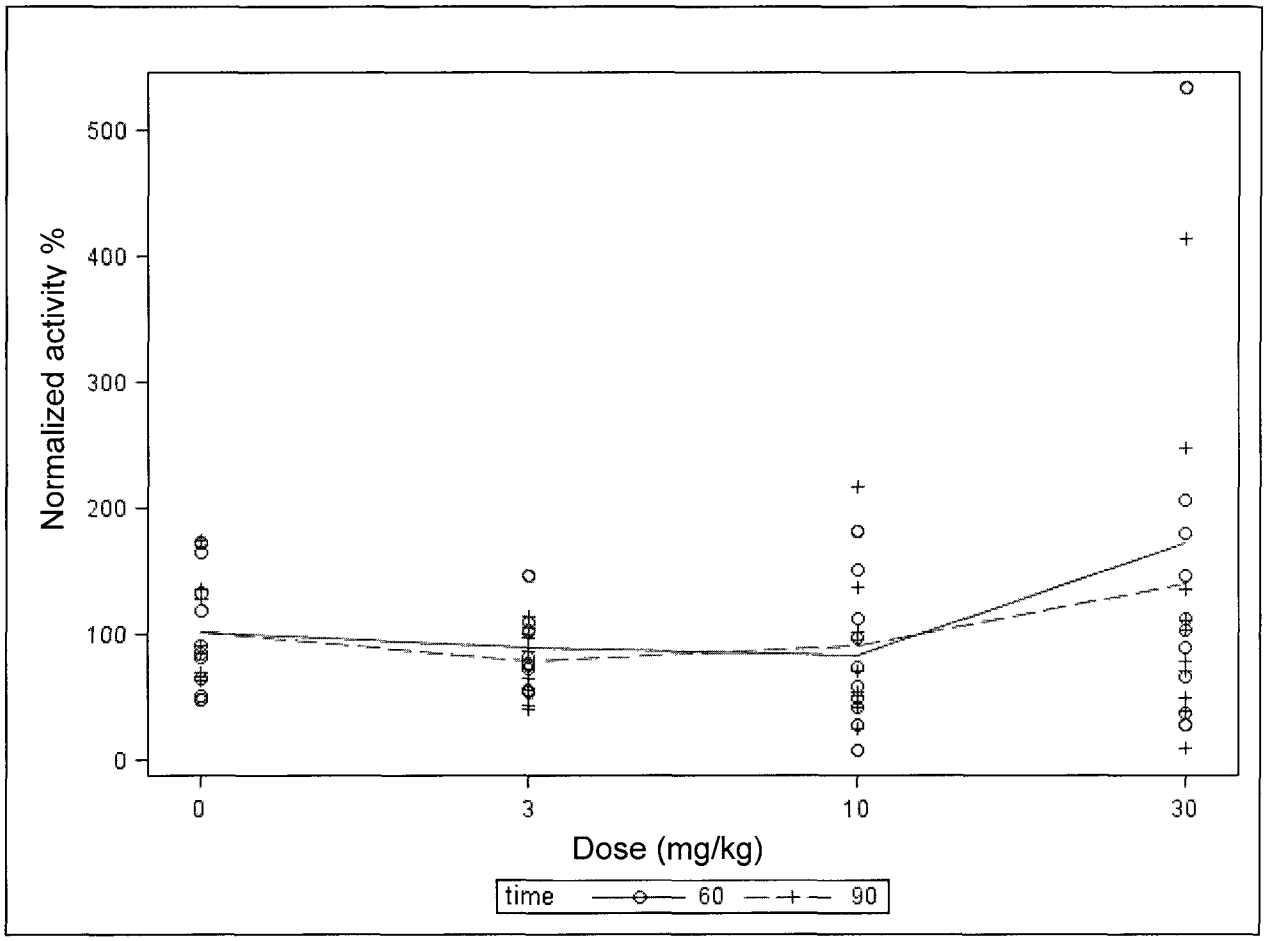

Figure 4. Interaction plot of the normalized activity of zebrafish 60 and 90 min post injection of saline or morphine at doses of 3, 10 and 30 mg/kg (no noxious stimulus). Lines join least squares means at each time period.

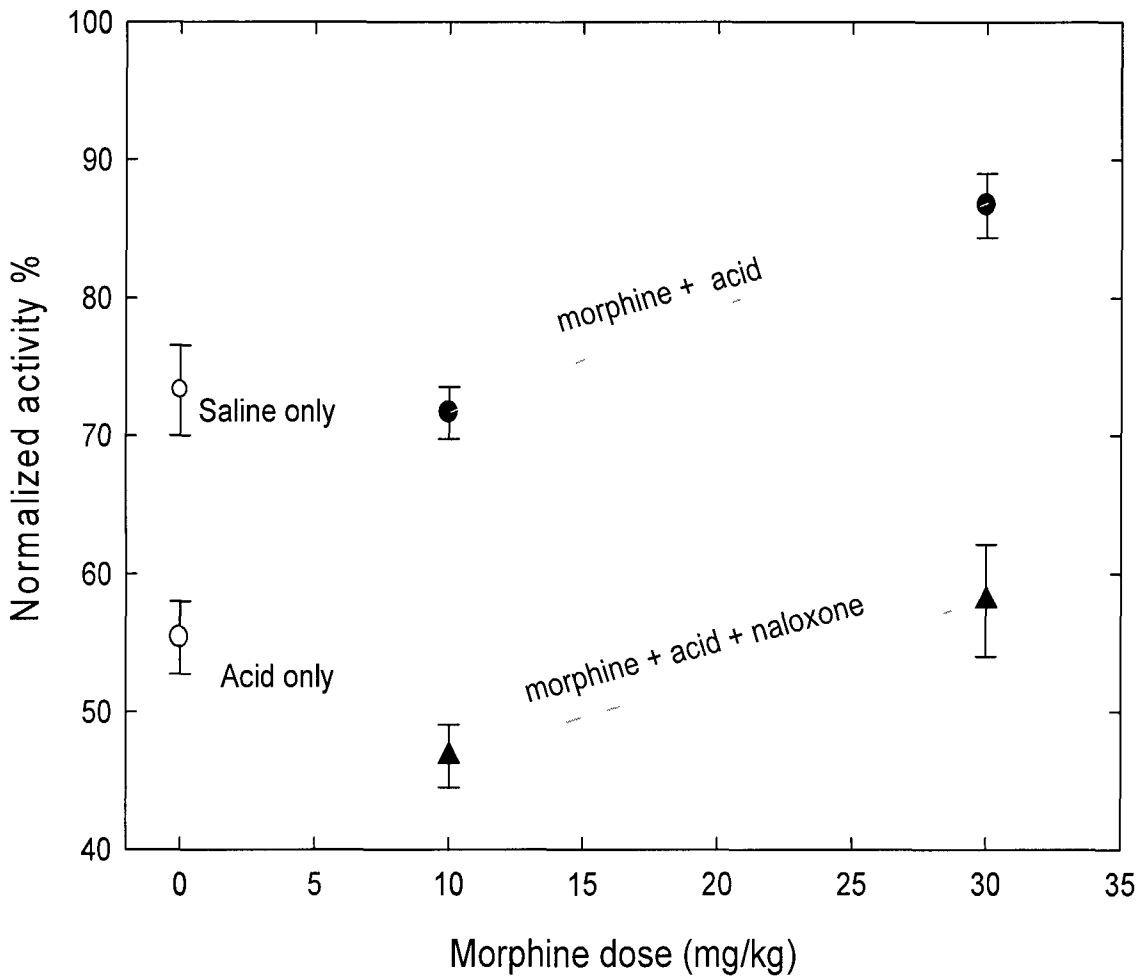

Figure 5. Blockade of the analgesic effect of morphine (●) in zebrafish 90 min post injection of naloxone (▲,30mg/kg) and 5 µL 5% acetic acid. Values are Mean ± SEM. (n=12/group, except n=20 for saline only). The dashed lines are not meant to imply a linear relationship; they simply connect similar treatment groups.

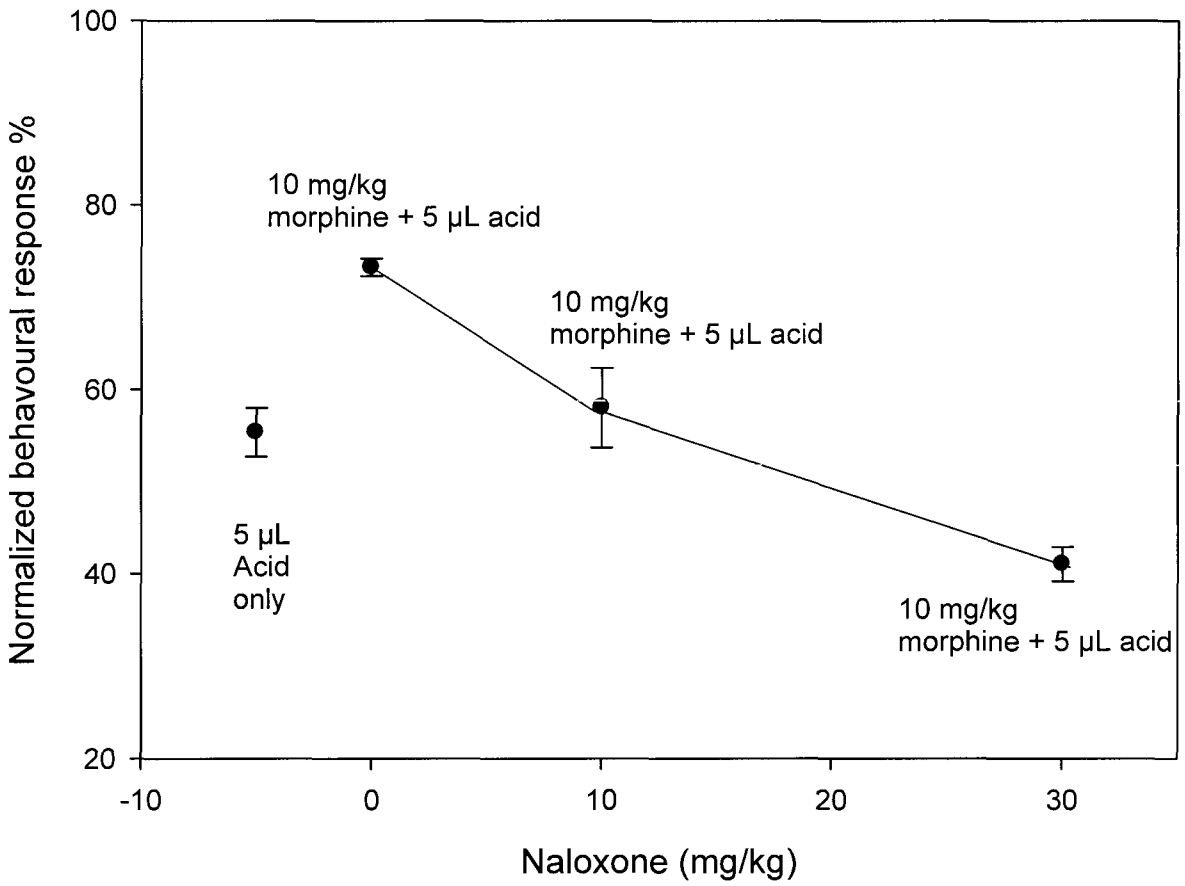

Figure 6. The dose-dependent decrease in activity in zebrafish 90 min post injection of naloxone at doses of 10 and 30 mg/kg. (Mean ± SEM, n=12/group).

treatment ($p<0.001$) at 60 and 90 minutes post injection. Naloxone and 10mg/kg morphine treated groups did not differ significantly from acid treated groups ($p= 0.275$ for 10mg/kg naloxone; $p= 0.391$ for 30 mg.kg naloxone) 90 min post injection (Figure 6).

3.5 Objective 5: Antagonist Side Effects

There was no significant difference between fish dosed with 10 mg/kg naloxone and control fish injected with sterile saline at 60, 90 and 120 min post injection (Figure 7) ($p=0.09$). However, naloxone at 30 mg/kg resulted in a significant increase in activity in comparison with control fish ($p<0.0001$).

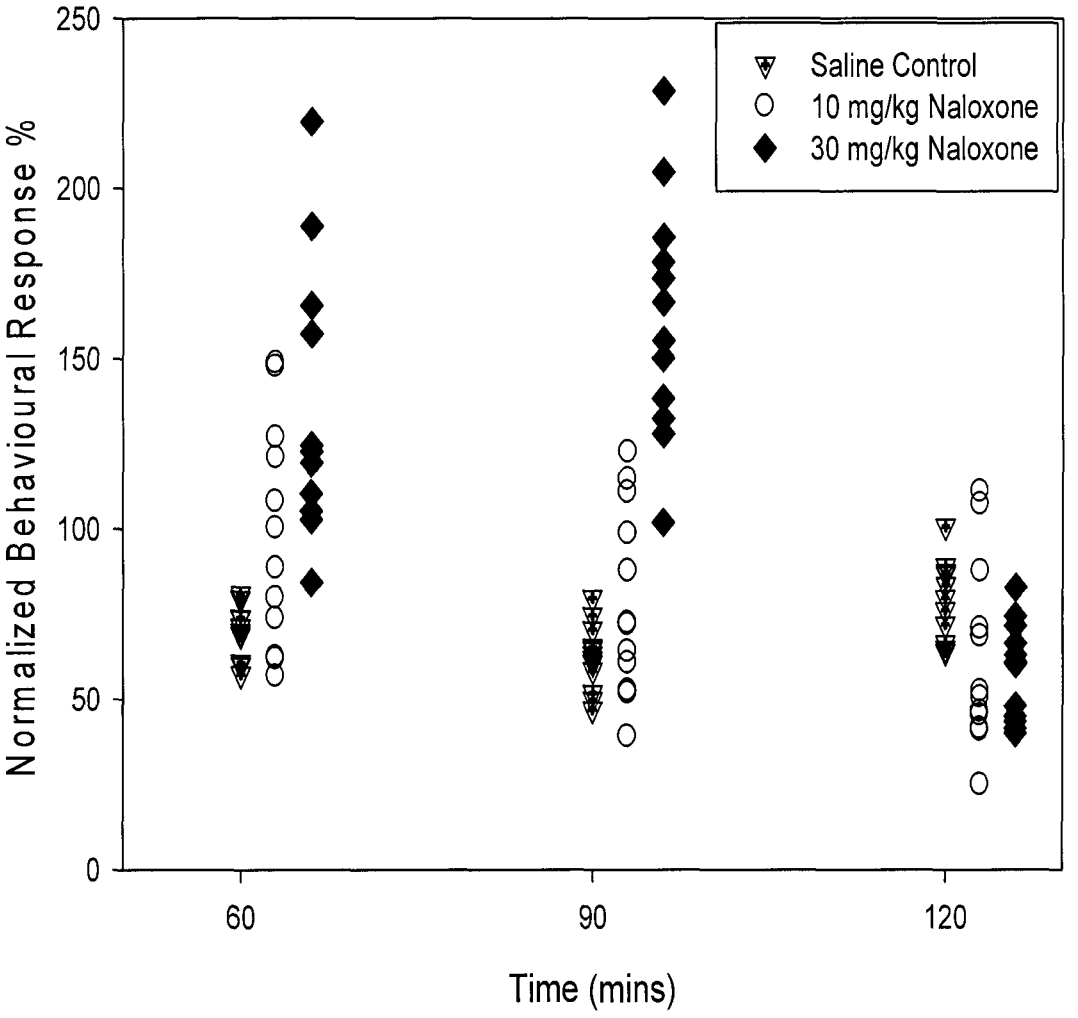

Figure 7. Normalized behavioural response of zebrafish 60, 90, and 120 min post injection of saline or naloxone at doses of 10 and 30 mg/kg. (n=12/group, except n=20 for saline control).

DISCUSSION

4.1 Behaviour as a Model to Measure Nociception in Zebrafish

My study looked at the behavioural response of zebrafish using a nociceptive stimulus that is commonly used in mammals. Acetic acid has previously been used as a noxious stimulus in fish (Sneddon, 2003b; Ashley *et al.*, 2007; Newby *et al.*, 2007; 2009), including zebrafish (Reilly *et al.*, 2008; Correia *et al.*, 2011). The experimental design of my zebrafish studies is based on Reilly *et al.* (2008). Their results showed a 68% decrease in swim rate (body lengths/min) post acetic acid injection (Reilly *et al.,* 2008). I began my study by using 5 µL of 5% acetic acid as this was what was used in the Reilly *et al.*2008 experiment but I was also interested in whether there would be a further decrease in activity if the dose was increased to 10 µL. While the increase in dose was effective at further decreasing activity throughout the duration of the experiment (120 min), I wanted to build upon the work done by Reilly *et al.* (2008) and have results comparable to that experiment. Therefore 5 µL of 5% acetic acid was used for the treatment groups requiring a noxious stimulus. Also, as these fish were tiny, it was easier to inject the smaller amount.

I was able to duplicate the results of the Reilly *et al.* 2008 experiment as well as show that the effect scaled with increasing amount of acid (5 µL, 10 µL). There was a significant time effect after the 10 µL injection where activity significantly decreased over time up to 90 min (Figure 2). The reason for the time effect using the higher concentration of irritant is unknown. Increasing the

concentration of acetic acid has also been shown to have an effect on zebrafish activity with the response scaling with stimulus intensity from 5 to 10% acetic acid (Correia *et al.*, 2011). These results are particularly important as they show the reproducibility of this model in that the results obtained are consistent in this species, even between laboratories.

Considerable variation was observed within and between groups in my experiments; although trends such as a decrease in activity after injection of the noxious stimulus were obvious and statistically significant. Increasing sample size, as observed in the saline control group (n=20) was effective at decreasing inter-group variability, although this was not a practical approach for all of the treatment groups in terms of time and animal care regulations. The sources of fish for the experiments also played a role in increasing variability. Fish in the 3, 10, and 30 mg/kg morphine treatment groups were sourced from a local pet store while fish in the 1 and 100 mg/kg morphine treatment groups were from the Dalhousie University breeding facility. There appeared to be excessive variability in the Dalhousie fish. In an attempt to explain this, a cortisol assay using fish from both suppliers was completed. While results from the assay were not sufficient to make any inference upon these differences I would recommend using only one supplier for experiments to prevent any possible increase in variation.

The extent of the decrease in activity associated with the noxious stimulus was only recorded for the duration of my experiment (120 min). It would be interesting to continue these recordings to assess the time period required

for normal behaviour to return. Acetic acid has been shown to produce prolonged behavioural and physiological responses over 3 to 6 h in rainbow trout (Sneddon, 2003b; Sneddon *et al.*, 2003; Reilly *et al.*, 2008; Ashley *et al.*, 2009; Mettam *et al.*, 2011)

4.2 Morphine Analgesia and Side Effects in Zebrafish

To assess possible pain perception in animals, we can make indirect measurements using behaviour and/or physiology in response to a potentially painful event and then test to see if these changes can be reduced by the administration of an analgesic. In this study, morphine was administered concurrently with the pain stimulus (5 µL 5% acetic acid) while the fish was anaesthetized. Concurrent administration was chosen because prior morphine administration would require further anesthetic use and further stress to the individual fish, both of which have the potential to be confounding factors in results. Correia *et al.* (2011) anaesthetised her fish twice, administering morphine post injection and then 30 min later during the injection of the noxious stimulus. This resulted in a more time consuming procedure, but, as the overall results were similar, this appeared to have no effect on swimming activity. However, the increased handling during the Correia procedure may have resulted in an increase in the stress hormone cortisol, as seen in previous work in zebrafish exposed to net handling stress (Ramsay *et al.*, 2009). As pain relief should not confer further stress on an organism, pre-emptive analgesia may not be ideal when analgesics are used in fish.

With a calculated ED_{50} of 12.3 mg/kg the effective dose of morphine is higher than recommended doses commonly used in mammals and birds. Morphine doses recommended for mammals range from 0.05- 0.1 mg/kg in cats to 2-5 mg/kg in rabbits, rats and mice (CCAC, 1993). In parrots (Psittacidae), a dose of 1 to 3 mg/kg of butorphanol, a κ-opioid receptor agonist, is recommended (Paul-Murphy *et al.*, 1999b).

Generally, dosage differences relate to slower metabolic and clearance rates in larger animals. Body surface area is frequently used to calculate the dose of various drugs but this may not be a viable method in predicting the metabolic rate of animals with variable body temperatures, such as fishes (Chappell, 1992). Reasons for the high dose of morphine required in fish may be a result of temperature. As most fish are ectothermic, lower temperatures can result in an overall lower rate of analgesic uptake with the converse true at higher temperatures (Gelwicks and Zafft, 1998; Peters *et al.*, 2001). Furthermore, Puig *et al.*, (1987) using guinea pig ileum preparations for ligand binding studies, demonstrated that the potency of morphine increased with temperature while the affinity of naloxone for opioid receptors was unaltered by temperature, and the affinity of morphine for μ receptors reached an optimal value within the range 30–37°C. The lower body temperature of fish may therefore impact the potency of morphine at μ receptors, preventing an overdose that would occur in most mammals at the doses frequently administered to fish. Also, the disposition of morphine has been shown to be one order of magnitude slower in fish than mammals (Newby *et al.*, 2007),

which may explain the overly high doses of morphine required for analgesia in fish in comparison with other vertebrates.

Morphine was injected IP because this is a common and simple procedure. Moreover, these fish were too small for IV injection and intramuscular injection can be less effective in administering the appropriate volume/dose into the dense muscle tissue.

4.3 Naloxone Antagonism: Dose and Side Effects

Concurrent treatment of morphine and naloxone effectively attenuated the anti-nociceptive behaviour associated with the morphine injection. Other studies in fish have demonstrated the effectiveness of naloxone at blocking the analgesic effect of opioids (Ehrensing *et al.*, 1982; Chervova and Lapshin, 2000; Correia *et al.*, 2011). It has been proposed that the mechanism of action involves competitive inhibition via naloxone binding to opioid receptors, similar to what is observed in amphibians (Stevens, 1996) and mammals (Akil *et al.*, 1976). The expression of opioid receptors in isolated zebrafish brain has been localized to limbic structures, as well as to other brain structures that have been implicated in pain mediation (Porteros *et al.*, 1999).

The high dose (30 mg/kg) of morphine and naloxone together resulted in a dramatic, almost four-fold increase in zebrafish activity. These data were removed from the graphical analyses of the antagonist effect. The effect we saw here could be due to hyperactivity, similar to that seen in neonatal rats injected

with κ- receptor agonists (Jackson and Kitchen, 1989), opioids in horses (Muir *et al.*, 1978), and mice following injections of either morphine or more selective opioid μ or δ receptor agonists (Mickley *et al.,* 1990). In the present study, a ratio of naloxone: morphine of 1:1 was effective at diminishing the analgesic effect of morphine. Work in frogs has shown that the analgesic effect of 0.316 μg of morphine was completely blocked by naloxone at 0.158 μg, half the analgesic dose (Stevens and Pezalla, 1983). It would be interesting to repeat the antagonist experiment testing a lesser dose of naloxone.

4.4 Conclusions

My study re-iterated the robustness of this model to test analgesic drugs in fishes. Acetic acid was effective at decreasing behaviour, morphine was effective at attenuating this decrease, and naloxone was effective at reversing the effect of morphine in adult zebrafish. Side effects of the injection of morphine or naloxone were minimal, except for the dramatic increase in activity observed after the highest dose of morphine and naloxone were injected concurrently. It must be stressed that while this model and analgesic dose was effective in zebrafish, given the anatomical, physiological, and behavioural variation among the over 30,000 species of fish, extrapolation of this data to other species should be approached with caution. Further work is necessary to assess the potential of this model and to test if a similar dose-response relationship exists in other fish species, especially those subject to invasive

experiments, as species-specific behavioural responses to noxious stimuli have been observed (Reilly *et al.*, 2008). In order to develop reliable analgesic protocols in fish, a model such as the acetic acid test must be employed under similar conditions for valid comparisons across species. The ED_{50} result in my study is lower than most analgesic doses that have been used in fish thus far with doses as high as 40 – 300 mg/kg not uncommon (Sneddon, 2003b, Newby et al., 2006; 2007; 2008; 2009). It seems obvious that due to a lack of data in the field of analgesia, fish may be receiving inappropriately high doses of analgesic drugs. Further experimentation and reporting on the ED_{50} values of analgesics in commonly used species of fish is required.

The ability of fish to centrally process pain is frequently contended. This study adds to the literature surrounding the fish pain issue and is the first to report ED_{50} values for an analgesic in a species of fish. Regardless of the debate, the evidence suggests that invasive procedures are capable of causing at least some degree of pain and appropriate steps should be followed to alleviate it.

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6. APPENDICES

APPENDIX A- Cortisol

Stress Induced Analgesia

It is important to appreciate that there are differences between the "stress response" and the "pain response." Cultured fish are routinely subjected to confinement, crowding, handling and transporting during husbandry (Klontz, 1995). The stress response associated with these procedures can elicit both physiological and behavioural responses (Davis, 2006) and usually involve a rapid increase in circulating catecholamines followed by a slower increase in cortisol and an even slower increase in blood glucose. However, the primary stress indicator in fish is the hormone cortisol, which typically increases in response to acute or chronic stressors (Ramsay *et al.*, 2006). After exposure to a stressor the hypothalamus secretes corticotropin-releasing hormone (CRH) which activates the pituitary to signal the release of adrenocorticotropic hormone (ACTH) which then stimulates the interrenal glands to synthesize glucocorticoid hormones from a cholesterol precursor. The glucocorticoid receptor (GR) regulates the transcription of targeted genes related to glucose metabolism, immune function, and behaviour (Mommsen *et al.*, 1999; Bury and Sturm, 2007). This signalling pathway resembles the human neuroendocrine system both in complexity and regarding cortisol utilization as opposed to corticosterone in rodents. This reinforces the utility of the zebrafish model in studies on the neurobiology of stress. The increase in glucocorticoid hormones

results in several metabolic effects including gluconeogenesis, immune system depression, as well as anti-inflammatory effects. The stress is alleviated through negative feedback to the hypothalamus and pituitary which suppresses CRH and ACTH release. This is an evolutionary conserved stress response that is observed in both fish and mammals.

In larger fish species it is common practice to use plasma cortisol as a stress indicator, whereas whole-body cortisol has been used in smaller fishes with an inadequate blood volume to measure plasma cortisol levels (Ramsay *et al.*, 2006). The nature of cortisol responses can be quite variable; in particular, handing stress has been described for a number of species. After a 30 s acute handling stress, salmonids were shown to have elevated plasma cortisol levels by 20 to 100 fold within 1 h (Barton and Iwama, 1991) but the magnitude of this response and recovery time to control levels varied greatly with species. A few studies have focused on stress in zebrafish (Ramsay *et al.*, 2006; Ramsay *et al.*, 2009) where each showed an increase in whole-body cortisol due to crowding and net-handling, respectively. Zebrafish have the potential to be developed as a model for stress in other aquaculture species and these results may be relevant to reared aquarium species (Dahm and Geisler, 2004). Zebrafish are a useful model for environmental and pharmacological manipulations as they show robust behavioural affects including a measurable cortisol response. As the complete genome has been sequenced, zebrafish genetic understanding is similar to what we know about fruit flies and mice (Sison *et al.*, 2006).

Preliminary experiments were not successful in estimating cortisol. Further work needs to be done in this area to evaluate any possible effect of stress on analgesia in fish.

APPENDIX B- Statistical Procedure and Data Used in Statistical Analysis

EXPERIMENT 1: EFFECT OF ACID

Input:

```
ods graphics on;
PROC GLM;
CLASS DOSE TIME ID;
MODEL ACTIV=DOSE TIME DOSE*TIME;
LSMEANS DOSE TIME DOSE *TIME;
LSMEANS DOSE / PDIFF=ALL ADJUST=TUKEY;
RUN;
ods graphics off;
```

The GLM Procedure

Class Level Information

Class	Levels	Values
dose	3	0 5 10 (Saline, 5µL acid, 10µL acid)
time	4	30 60 90 120
id	36	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 61 62 63 64 65 66 67 68 69 70 71 72

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	2	11555.31787	5777.65893	118.81	<.0001
time	3	5802.23234	1934.07745	39.77	<.0001
dose*time	6	8600.56555	1433.42759	29.48	<.0001

The GLM Procedure
Least Squares Means

dose	activ LSMEAN
0	77.4964258
5	61.4079700
10	56.5303221

time	activ LSMEAN
30	74.8726983
60	65.4597133
90	57.2936619
120	62.9535503

dose	time	activ LSMEAN
0	30	76.6866375

0	60	82.7809608
0	90	73.2757733
0	120	77.2423317
5	30	65.8063292
5	60	55.8621725
5	90	55.3744333
5	120	68.5889450
10	30	82.1251283
10	60	57.7360067
10	90	43.2307792
10	120	43.0293742

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

LSMEAN

dose	activ LSMEAN	Number
0	77.4964258	1
5	61.4079700	2
10	56.5303221	3

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: activ

i/j	SALINE	5µl	10µL
SALINE		<.0001	<.0001
5µL	<.0001		0.0023

Data used:

id	time	dose	activ
1	30	5	62.33819
2	30	5	70.91535
3	30	5	67.17222
4	30	5	56.27665
5	30	5	65.75771
6	30	5	60.55221
7	30	5	70.82413
8	30	5	72.59108
9	30	5	72.38401
10	30	5	69.84588
11	30	5	58.76011
12	30	5	62.25841
13	30	10	80.61222

14	30	10	89.35007
15	30	10	73.29854
16	30	10	89.11623
17	30	10	82.85344
18	30	10	81.66604
19	30	10	68.50556
20	30	10	72.60838
21	30	10	83.69423
22	30	10	86.21244
23	30	10	103.4582
24	30	10	74.12619
61	30	0	75.34292
62	30	0	72.90189
63	30	0	75.93213
64	30	0	87.4136
65	30	0	82.89867
66	30	0	74.77653
67	30	0	73.07882
68	30	0	77.13779
69	30	0	81.70273
70	30	0	75.91545
71	30	0	70.27916
72	30	0	72.85996
1	60	5	64.5186
2	60	5	51.35319
3	60	5	38.80165
4	60	5	46.14252
5	60	5	48.67999
6	60	5	57.12172
7	60	5	55.46604
8	60	5	61.0735
9	60	5	58.92363
10	60	5	59.67321
11	60	5	61.49533
12	60	5	67.09669
13	60	10	48.40612
14	60	10	61.10033
15	60	10	60.93841
16	60	10	66.51326
17	60	10	51.53462
18	60	10	62.09334
19	60	10	55.3129
20	60	10	60.37865

21	60	10	51.38634
22	60	10	52.88453
23	60	10	76.26247
24	60	10	46.02111
61	60	0	88.62867
62	60	0	88.97348
63	60	0	83.42844
64	60	0	86.08087
65	60	0	81.93699
66	60	0	78.57734
67	60	0	85.01857
68	60	0	76.19289
69	60	0	81.49679
70	60	0	78.96111
71	60	0	77.77822
72	60	0	86.29816
1	90	5	79.06807
2	90	5	51.90433
3	90	5	43.12128
4	90	5	47.1733
5	90	5	50.31523
6	90	5	52.34763
7	90	5	56.06858
8	90	5	55.62453
9	90	5	62.75734
10	90	5	51.99836
11	90	5	53.96595
12	90	5	60.1486
13	90	10	33.7602
14	90	10	41.42423
15	90	10	45.18962
16	90	10	45.80052
17	90	10	47.03626
18	90	10	49.4025
19	90	10	41.53534
20	90	10	48.90003
21	90	10	37.61878
22	90	10	41.51457
23	90	10	46.296
24	90	10	40.2913
61	90	0	66.57527
62	90	0	70.72342
63	90	0	74.91728

64	90	0	71.2263
65	90	0	71.76997
66	90	0	77.24743
67	90	0	76.01853
68	90	0	73.74053
69	90	0	76.37669
70	90	0	77.27398
71	90	0	73.14826
72	90	0	70.29162
1	120	5	52.66583
2	120	5	59.90207
3	120	5	63.4889
4	120	5	76.29334
5	120	5	73.39265
6	120	5	78.63888
7	120	5	70.97378
8	120	5	70.7377
9	120	5	55.65342
10	120	5	68.57882
11	120	5	70.98597
12	120	5	81.75598
13	120	10	43.31432
14	120	10	44.60317
15	120	10	42.29935
16	120	10	39.86946
17	120	10	45.83857
18	120	10	44.37692
19	120	10	37.65149
20	120	10	37.25567
21	120	10	38.95167
22	120	10	46.13699
23	120	10	46.71685
24	120	10	49.33803
61	120	0	70.17772
62	120	0	79.67135
63	120	0	77.87433
64	120	0	81.07839
65	120	0	78.57782
66	120	0	80.70024
67	120	0	75.6175
68	120	0	85.0817
69	120	0	84.86291
70	120	0	76.86341

71	120	0	81.34671
72	120	0	55.0559

EXPERIMENT 2: THE EFFECT OF MORPHINE

Input:

```
ods graphics on;
PROC GLM;
CLASS DOSE TIME ID;
MODEL ACTIV=DOSE TIME DOSE*TIME;
LSMEANS DOSE TIME DOSE *TIME;
LSMEANS DOSE / PDIFF=ALL ADJUST=TUKEY;
RUN;
ods graphics off;
```

Output:

The GLM Procedure

Class Level Information

Class	Levels	Values
dose	5	0 2 3 10 30 (Saline, 5µL acid, 3mgkg,10mgkg, 30mgkg morphine + acid)
time	2	60 90
id	60	1 2 3 4 5 6 7 8 9 10 11 12 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84

The GLM Procedure

Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	27532.15205	3059.12801	36.51	<.0001
Error	110	9216.12424	83.78295		
Corrected Total	119	36748.27629			

R-Square	Coeff Var	Root MSE	activ Mean
0.749209	12.59815	9.153303	72.65591

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	4	21514.92196	5378.73049	64.20	<.0001
time	1	3551.72502	3551.72502	42.39	<.0001
dose*time	4	2465.50507	616.37627	7.36	<.0001

The GLM Procedure

Least Squares Means

dose	activ LSMEAN
0	78.0283670
2	55.6183026

3	62.0834345
10	73.3277543
30	94.2216956

time	activ LSMEAN
60	78.0962884
90	67.2155332

dose	time	activ LSMEAN
0	60	82.780962
0	90	73.275772
2	60	55.862172
2	90	55.374433
3	60	75.065714
3	90	49.101155
10	60	75.023978
10	90	71.631530
30	60	101.748616
30	90	86.694775

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

LSMEAN

dose	activ LSMEAN	Number
0	78.0283670	1
2	55.6183026	2
3	62.0834345	3
10	73.3277543	4
30	94.2216956	5

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: activ

i/j	Saline	5µLacid	3mgkg	10mgkg	30mgkg
saline		<.0001	<.0001	0.3910	<.0001
5µLacid	<.0001		0.1108	<.0001	<.0001
3mgkg	<.0001	0.1108		0.0004	<.0001
10mgkg	0.3910	<.0001	0.0004		<.0001
30mgkg	<.0001	<.0001	<.0001	<.0001	

Data used for Exp.2

id	time	dose	activ
1	60	2	64.5185976
2	60	2	51.3531948
3	60	2	38.80165334
4	60	2	46.14251723
5	60	2	48.67999338
6	60	2	57.12171525
7	60	2	55.46603569
8	60	2	61.07350044
9	60	2	58.92362561
10	60	2	59.67321009
11	60	2	61.4953272
12	60	2	67.09669252
37	60	30	90.24947591
38	60	30	103.745621
39	60	30	116.2230002
40	60	30	105.2497117
41	60	30	111.8361626
42	60	30	107.5114408
43	60	30	105.4504197
44	60	30	79.94002891
45	60	30	104.925015
46	60	30	97.45760656
47	60	30	103.3743085
48	60	30	95.02059892
49	60	10	72.38290986

50	60	10	73.2058564
51	60	10	67.129379
52	60	10	77.05631538
53	60	10	73.05417198
54	60	10	81.7105542
55	60	10	88.01355638
56	60	10	64.62236757
57	60	10	74.14921137
58	60	10	61.67133289
59	60	10	83.33314194
60	60	10	83.95894406
61	60	3	65.62985779
62	60	3	73.20792896
63	60	3	60.18039981
64	60	3	56.2035168
65	60	3	56.04314384
66	60	3	83.8359472
67	60	3	76.41409006
68	60	3	104.1284474
69	60	3	62.74263389
70	60	3	74.86824632
71	60	3	84.85261497
72	60	3	102.6817449
73	60	0	88.62867412
74	60	0	88.97348223
75	60	0	83.42844109
76	60	0	86.08086856
77	60	0	81.93698874

78	60	0	78.57733839
79	60	0	85.01857412
80	60	0	76.19289449
81	60	0	81.49678559
82	60	0	78.96111369
83	60	0	77.77822123
84	60	0	86.2981575
1	90	2	79.06807223
2	90	2	51.90433115
3	90	2	43.12128016
4	90	2	47.1733009
5	90	2	50.3152309
6	90	2	52.34762679
7	90	2	56.06858256
8	90	2	55.62452719
9	90	2	62.75734153
10	90	2	51.99835692
11	90	2	53.96595046
12	90	2	60.14859957
37	90	30	83.82195382
38	90	30	92.12878208
39	90	30	102.594727
40	90	30	84.37995436
41	90	30	75.52652378
42	90	30	87.57737331
43	90	30	86.50466511
44	90	30	71.52698499
45	90	30	85.60997531

46	90	30	86.86523541
47	90	30	90.28182144
48	90	30	93.51930905
49	90	10	62.50501591
50	90	10	74.2000461
51	90	10	75.79176264
52	90	10	80.85860276
53	90	10	69.96945537
54	90	10	72.94012428
55	90	10	81.21087861
56	90	10	69.42341631
57	90	10	68.02703746
58	90	10	59.20137151
59	90	10	70.94264887
60	90	10	74.50800131
61	90	3	39.74796547
62	90	3	31.93811125
63	90	3	32.97589948
64	90	3	48.97198445
65	90	3	48.31069571
66	90	3	63.38338166
67	90	3	53.70276898
68	90	3	44.89388702
69	90	3	45.97369336
70	90	3	59.23509796
71	90	3	65.50576611
72	90	3	54.57460523
73	90	0	66.57527165

74	90	0	70.72341901
75	90	0	74.91727529
76	90	0	71.22629741
77	90	0	71.7699685
78	90	0	77.24742867
79	90	0	76.01853464
80	90	0	73.74053082
81	90	0	76.37668681
82	90	0	77.27397644
83	90	0	73.14825925
84	90	0	70.29162084

EXP 2- ED50

Pooled data- 60 and 90 min post injection

Input:

```
proc import datafile="C:\Documents and
Settings\default\Desktop\a_09\!_res_current\!_students_pei\!mystudents\Angela
_Douglas\SAS\expt2-60_90.csv" out=both60_90 dbms=csv
replace;getnames=yes;

*logD, activ60_90, activ60_90win1, activ60_90win2;
*output = both60_90;
run;
proc plot data=both60_90;
plot activ60_90*logD;
plot win1*logD;
plot win2*logD;
run;
proc print data=both60_90;
run;
proc means data=both60_90;
by logD;
run;
proc glm data=both60_90;
class logD;
model activ60_90=logD;
run;
proc glm data=both60_90;
class logD;
model win1=logD;
run;
proc glm data=both60_90;
class logD;
model win2=logD;
run;
proc nlin data=both60_90;
parameters
bottom=57
logEC50=1.09
Hillslope=1.9
;
predict=bottom + (100-bottom)/(1+10**((logEC50-logD)*Hillslope));
model activ60_90=predict;
run;
proc nlin data=both60_90;
```

```
parameters
bottom=57
logEC50=1.09
Hillslope=1.9
;
predict=bottom + (100-bottom)/(1+10**((logEC50-logD)*Hillslope));
model win1=predict;
run;
proc nlin data=both60_90;
parameters
bottom=56
logEC50=1.09
Hillslope=1.9
;
predict=bottom + (100-bottom)/(1+10**((logEC50-logD)*Hillslope));
model win2=predict;
run;
```

Output:

---------- logD=1E-6 ----------

The MEANS Procedure

Variable	N	Mean	Std Dev	Minimum	Maximum
activ60_90	24	55.6183026	8.4990152	38.8016533	79.0680722
win1	24	55.2994796	6.9600194	43.1212802	67.0966925
win2	24	55.3364081	6.1744528	46.1425172	64.5185976

---------- logD=0.477121255 ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
activ60_90	24	62.0834345	19.0051216	31.9381113	104.1284474
win1	24	62.0663964	18.7968750	32.9758995	102.6817449
win2	24	61.1449744	14.9622168	39.7479655	84.8526150

---------- logD=1 ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
activ60_90	24	73.3277543	7.4277294	59.2013715	88.0135564
win1	24	73.2617271	6.9234919	61.6713329	83.9589441
win2	24	73.2790505	6.7219204	62.5050159	83.3331419

---------- logD=1.477121255 ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
activ60_90	24	94.2216956	11.6667356	71.5269850	116.2230002
win1	24	94.2055582	11.0163078	75.5265238	111.8361626
win2	24	94.2129568	9.8529871	79.9400289	107.5114408

Class Level Information

Class Levels Values

logD 4 1 1E-6 0.477121255 1.477121255

Number of Observations Read 96

Number of Observations Used 96

Dependent Variable: **activ60_90**

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	20649.02339	6883.00780	44.07	<.0001
Error	92	14368.37115	156.17795		

Corrected Total 95 35017.39454

R-Square Coeff Var Root MSE activ60_90 Mean

0.589679 17.52437 12.49712 71.31280

Source DF Type I SS Mean Square F Value Pr > F

logD 3 20649.02339 6883.00780 **44.07 <.0001**

Source DF Type III SS Mean Square F Value Pr > F

logD 3 20649.02339 6883.00780 **44.07** <.0001

Dependent Variable: **win1**

Sum of

Source DF Squares Mean Square F Value Pr > F

Model 3 20874.12995 6958.04332 48.74 <.0001

Error 92 13134.33763 142.76454

Corrected Total 95 34008.46758

R-Square Coeff Var Root MSE win1 Mean

0.613792 16.77952 11.94841 71.20829

Source DF Type I SS Mean Square F Value Pr > F

logD 3 20874.12995 6958.04332 **48.74 <.0001**

Source DF Type III SS Mean Square F Value Pr > F

logD 3 20874.12995 6958.04332 48.74 <.0001

Dependent Variable: **win2**

Sum of

Source DF Squares Mean Square F Value Pr > F

Model 3 21276.11754 7092.03918 70.17 <.0001

Error 92 9297.91942 101.06434

Corrected Total 95 30574.03696

R-Square Coeff Var Root MSE win2 Mean

0.695888 14.16059 10.05308 70.99335

Source DF Type I SS Mean Square F Value Pr > F

logD 3 21276.11754 7092.03918 **70.17 <.0001**

Source DF Type III SS Mean Square F Value Pr > F

NOTE: An intercept was not specified for this model.

Sum of Mean Approx

Source DF Squares Square F Value Pr > F

Model 3 508612 169537 **1078.87 <.0001**

Error 93 14614.4 157.1

Uncorrected Total 96 523227

Approx

Parameter Estimate Std Error Approximate 95% Confidence Limits

bottom 56.9472 2.4537 52.0746 61.8198

logEC50 1.0888 0.0609 0.9678 1.2098

Hillslope 1.8870 0.4591 0.9754 2.7986

The NLIN Procedure Dependent Variable win1

Iterative Phase

Sum of

Iter bottom logEC50 Hillslope Squares

0 57.0000 1.0900 1.9000 13410.3

1 56.7169 1.0870 1.8738 13407.8

2 56.6800 1.0861 1.8658 13407.7

3 56.6690 1.0858 1.8633 13407.7

4 56.6657 1.0857 1.8626 13407.7

5 56.6647 1.0857 1.8623 13407.7

6 56.6644 1.0857 1.8623 13407.7

NOTE: Convergence criterion met.

Estimation Summary

Method Gauss-Newton

Iterations 6

R 5.895E-6

PPC(Hillslope) 0.000012

RPC(Hillslope) 0.000038

Object 4.82E-10

Objective 13407.72

Observations Read 96

Observations Used 96

Data used for ED50

Obs logD activ60_90 win1 win2

1 1E-6 38.80165334 43.12128016 46.14251723

2 1E-6 43.12128016 43.12128016 46.14251723

3 1E-6 46.14251723 46.14251723 46.14251723

4 1E-6 47.1733009 47.1733009 47.1733009

5 1E-6 48.67999338 48.67999338 48.67999338

6 1E-6 50.3152309 50.3152309 50.3152309

7 1E-6 51.3531948 51.3531948 51.3531948

8 1E-6 51.90433115 51.90433115 51.90433115

9 1E-6 51.99835692 51.99835692 51.99835692

10 1E-6 52.34762679 52.34762679 52.34762679

11 1E-6 53.96595046 53.96595046 53.96595046

12 1E-6 55.46603569 55.46603569 55.46603569

13 1E-6 55.62452719 55.62452719 55.62452719

14 1E-6 56.06858256 56.06858256 56.06858256

15 1E-6 57.12171525 57.12171525 57.12171525

16 1E-6 58.92362561 58.92362561 58.92362561

17 1E-6 59.67321009 59.67321009 59.67321009

18 1E-6 60.14859957 60.14859957 60.14859957

19 1E-6 61.07350044 61.07350044 61.07350044

20 1E-6 61.4953272 61.4953272 61.4953272

21 1E-6 62.75734153 62.75734153 62.75734153

22 1E-6 64.5185976 64.5185976 64.5185976

23 1E-6 67.09669252 67.09669252 64.5185976

24 1E-6 79.06807223 67.09669252 64.5185976

25 0.477121255 31.93811125 32.97589948 39.74796547

26 0.477121255 32.97589948 32.97589948 39.74796547

27 0.477121255 39.74796547 39.74796547 39.74796547

28 0.477121255 44.89388702 44.89388702 44.89388702

29 0.477121255 45.97369336 45.97369336 45.97369336

30 0.477121255 48.31069571 48.31069571 48.31069571

31 0.477121255 48.97198445 48.97198445 48.97198445

32 0.477121255 53.70276898 53.70276898 53.70276898

33 0.477121255 54.57460523 54.57460523 54.57460523

34 0 477121255 56 04314384 56 04314384 56 04314384

35 0 477121255 56 2035168 56 2035168 56 2035168

36 0 477121255 59 23509796 59 23509796 59 23509796

37 0 477121255 60 18039981 60 18039981 60 18039981

38 0 477121255 62 74263389 62 74263389 62 74263389

39 0 477121255 63 38338166 63 38338166 63 38338166

40 0 477121255 65 50576611 65 50576611 65 50576611

41 0 477121255 65 62985779 65 62985779 65 62985779

42 0 477121255 73 20792896 73 20792896 73 20792896

43 0 477121255 74 86824632 74 86824632 74 86824632

44 0 477121255 76 41409006 76 41409006 76 41409006

45 0 477121255 83 8359472 83 8359472 83 8359472

46 0 477121255 84 85261497 84 85261497 84 85261497

47 0 477121255 102 6817449 102 6817449 84 85261497

48 0 477121255 104 1284474 102 6817449 84 85261497

49 1 59 20137151 61 67133289 62 50501591

50 1 61 67133289 61 67133289 62 50501591

51 1 62 50501591 62 50501591 62 50501591

52 1 64 62236757 64 62236757 64 62236757

53 1 67 129379 67 129379 67 129379

54 1 68 02703746 68 02703746 68 02703746

55 1 69 42341631 69 42341631 69 42341631

56 1 69 96945537 69 96945537 69 96945537

57 1 70 94264887 70 94264887 70 94264887

58 1 72 38290986 72 38290986 72 38290986

59 1 72 94012428 72 94012428 72 94012428

60 1 73 05417198 73 05417198 73 05417198

61 1 73 2058564 73 2058564 73 2058564

62 1 74 14921137 74 14921137 74 14921137

63 1 74 2000461 74 2000461 74 2000461

64 1 74 50800131 74 50800131 74 50800131

65 1 75 79176264 75 79176264 75 79176264

66 1 77 05631538 77 05631538 77 05631538

67 1 80 85860276 80 85860276 80 85860276

68 1 81 21087861 81 21087861 81 21087861

69 1 81 7105542 81 7105542 81 7105542

70 1 83 33314194 83 33314194 83 33314194

71 1 83 95894406 83 95894406 83 33314194

72 1 88 01355638 83 95894406 83 33314194

73 1 477121255 71 52698499 75 52652378 79 94002891

74 1 477121255 75 52652378 75 52652378 79 94002891

75 1 477121255 79 94002891 79 94002891 79 94002891

76 1 477121255 83 82195382 83 82195382 83 82195382

77 1 477121255 84 37995436 84 37995436 84 37995436

78 1 477121255 85 60997531 85 60997531 85 60997531

79 1 477121255 86 50466511 86 50466511 86 50466511

80 1 477121255 86 86523541 86 86523541 86 86523541

81 1 477121255 87 57737331 87 57737331 87 57737331

82 1 477121255 90 24947591 90 24947591 90 24947591

83 1 477121255 90 28182144 90 28182144 90 28182144

84 1 477121255 92 12878208 92 12878208 92 12878208

85 1 477121255 93 51930905 93 51930905 93 51930905

86 1 477121255 95 02059892 95 02059892 95 02059892

87 1 477121255 97 45760656 97 45760656 97 45760656

88 1 477121255 102 594727 102 594727 102 594727

89 1 477121255 103 3743085 103 3743085 103 3743085

90 1 477121255 103 745621 103 745621 103 745621

91 1 477121255 104 925015 104 925015 104 925015

92 1 477121255 105 2497117 105 2497117 105 2497117

93 1 477121255 105 4504197 105 4504197 105 4504197

94 1 477121255 107 5114408 107 5114408 107 5114408

95 1 477121255 111 8361626 111 8361626 107 5114408

96 1 477121255 116 2230002 111 8361626 107 5114408

EXPERIMENT 3- Morphine in and of itself.

Input:

```
ods graphics on;

PROC GLM;
CLASS DOSE TIME ID;
MODEL ACTIV=DOSE TIME DOSE*TIME;
LSMEANS DOSE TIME DOSE *TIME;
LSMEANS DOSE / PDIFF=ALL ADJUST=TUKEY;
RUN;
ods graphics off;
```

Output:

The GLM Procedure: **TOT DIST MORPHINE AND SALINE:WINDSORIZED!!!**

Class Level Information

Class	Levels	Values
dose	4	0 3 10 30 (SALINE, 3MGKG,10MGKG,30MGKG; ALL WINDSORIZED).
time	2	60 90
id	48	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48

The GLM Procedure

Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	87432.9105	12490.4158	1.50	0.1776
Error	88	732651.8095	8325.5887		
Corrected Total	95	820084.7200			

R-Square	Coeff Var	Root MSE	activ Mean
0.106614	84.90966	91.24466	107.4609

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	3	80215.64736	26738.54912	3.21	0.0268
time	1	1791.34085	1791.34085	0.22	0.6439
dose*time	3	5425.92230	1808.64077	0.22	0.8842

dose	activ LSMEAN
0	102.407051
3	84.634684
10	86.708692
30	156.093060

time	activ LSMEAN
60	111.780571
90	103.141173

dose	time	activ LSMEAN
0	60	102.504101
0	90	102.310000
3	60	89.893878
3	90	79.375489
10	60	82.620661
10	90	90.796723
30	60	172.103643
30	90	140.082477

Least Squares Means
Adjustment for Multiple Comparisons: Tukey

dose	activ LSMEAN	LSMEAN Number
0	102.407051	1
3	84.634684	2
10	86.708692	3
30	156.093060	4

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: activ

i/j	Saline	3mgkg	10mgkg	30mgkg
Saline		0.9064	0.9330	0.1820
3mgkg	0.9064		0.9998	0.0394
10mgkg	0.9330	0.9998		0.0481
30mgkg	0.1820	0.0394	0.0481	

DATA USED FOR EXP 3 WINDSORIZED

ID	TIME	DOSE	ACTIV
97	60	30	174 8933
98	60	30	130 1377
99	60	30	108 4621
100	60	30	110 3294
101	60	30	117 2077
102	60	30	129 057
103	60	30	133 8937
104	60	30	111 7417
105	60	30	105 5695
106	60	30	107 9251
107	60	30	120 2761
108	60	30	113 1015
109	60	10	71 56696
110	60	10	57 06189
111	60	10	68 66449
112	60	10	62 70013
113	60	10	61 32089
114	60	10	69 70004
115	60	10	59 19176
116	60	10	76 51656
117	60	10	73 81867
118	60	10	73 80881
119	60	10	61 34631
120	60	10	77 05509
121	60	3	81 60945
122	60	3	76 76877
123	60	3	76 65713
124	60	3	71 8016
125	60	3	83 71276
126	60	3	98 96637
127	60	3	86 68019
128	60	3	90 73372
129	60	3	82 05245
130	60	3	86 84244
131	60	3	76 90237
132	60	3	72 27131
73	60	0	88 62867
74	60	0	88 97348

75	60	0	83.42844
76	60	0	86.08087
77	60	0	81.93699
78	60	0	78.57734
79	60	0	85.01857
80	60	0	76.19289
81	60	0	81.49679
82	60	0	78.96111
83	60	0	77.77822
84	60	0	86.29816
97	90	30	157.4771
98	90	30	140.9411
99	90	30	120.0304
100	90	30	120.2477
101	90	30	109.4901
102	90	30	117.3573
103	90	30	119.2451
104	90	30	102.7818
105	90	30	96.52761
106	90	30	100.9112
107	90	30	115.4639
108	90	30	94.60542
109	90	10	84.32382
110	90	10	71.43867
111	90	10	71.87608
112	90	10	68.99573
113	90	10	67.13561
114	90	10	73.31527
115	90	10	70.19053
116	90	10	61.76674
117	90	10	68.49081
118	90	10	72.37744
119	90	10	70.20819
120	90	10	85.24385
121	90	3	75.12647
122	90	3	85.15081
123	90	3	75.8439
124	90	3	69.24408
125	90	3	81.6805
126	90	3	95.7827
127	90	3	80.87515
128	90	3	77.50116
129	90	3	84.14205

130	90	3	71.69258
131	90	3	66.09552
132	90	3	55.83913
73	90	0	66.57527
74	90	0	70.72342
75	90	0	74.91728
76	90	0	71.2263
77	90	0	71.76997
78	90	0	77.24743
79	90	0	76.01853
80	90	0	73.74053
81	90	0	76.37669
82	90	0	77.27398
83	90	0	73.14826
84	90	0	70.29162

NON WINSORIZED= Large OUTLIERS
The GLM Procedure: MORPHINE ONLY NON-WINSORIZED

Input:
ods graphics on;
PROC GLM;
CLASS DOSE TIME ID;
MODEL ACTIV=DOSE TIME DOSE*TIME;
LSMEANS DOSE TIME DOSE *TIME;
LSMEANS DOSE / PDIFF=ALL ADJUST=TUKEY;
RUN;
ods graphics off;

Output:

Class Level Information

Class	Levels	Values
dose	4	0 3 10 30
time	2	60 90
id	48	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48

The SAS System 10:04 Tuesday, May 31, 2011 1150
The GLM Procedure
Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1204521.10	172074.44	0.62	0.7372
Error	88	24383726.89	277087.81		
Corrected Total	95	25588247.99			

R-Square	Coeff Var	Root MSE	activ Mean
0.047073	280.1605	526.3913	187.8892

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	3	1089195.111	363065.037	1.31	0.2762
time	1	8525.926	8525.926	0.03	0.8612
dose*time	3	106800.059	35600.020	0.13	0.9430

Least Squares Means

dose	activ LSMEAN
0	106.826750
3	98.204430
10	363.238759

30 183.286714

time activ LSMEAN
60 178.465166
90 197.313160

dose time activ LSMEAN
0 60 109.635730
0 90 104.017770
3 60 115.070842
3 90 81.338018
10 60 296.690518
10 90 429.787000
30 60 192.463573
30 90 174.109854

Least Squares Means
Adjustment for Multiple Comparisons: Tukey

dose	activ LSMEAN	LSMEAN Number
0	106.826750	1
3	98.204430	2
10	363.238759	3
30	183.286714	4

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: activ

i/j	1	2	3	4
1		0.9999	0.3364	0.9581
2	0.9999		0.3074	0.9436
3	0.3364	0.3074		0.6384
4	0.9581	0.9436	0.6384	

Data for exp 3-nonWinsorized

97	60	30	174.8932615
98	60	30	130.1377051
99	60	30	108.4621293
100	60	30	110.3294212
101	60	30	117.2077446

102	60	30	129.056968
103	60	30	133.8937237
104	60	30	111.7416747
105	60	30	105.5695484
106	60	30	107.925091
107	60	30	120.2761047
108	60	30	113.10149
109	60	10	71.56695511
110	60	10	57.06189143
111	60	10	68.66449164
112	60	10	62.70012841
113	60	10	61.32088991
114	60	10	69.70004406
115	60	10	59.19176224
116	60	10	76 51656165
117	60	10	73.81866516
118	60	10	73.80880913
119	60	10	61.34631444
120	60	10	77.05509377
121	60	3	81.60945133
122	60	3	76.7687668
123	60	3	76.65712868
124	60	3	71.80160106
125	60	3	83.71276305
126	60	3	98.96637077
127	60	3	86.68019201
128	60	3	90.73371819
129	60	3	82.05244981
130	60	3	86.84243822
131	60	3	76.90236577
132	60	3	72.27130747
73	60	0	88.62867412
74	60	0	88.97348223
75	60	0	83.42844109
76	60	0	86.08086856
77	60	0	81.93698874
78	60	0	78.57733839
79	60	0	85.01857412
80	60	0	76.19289449
81	60	0	81.49678559
82	60	0	78.96111369
83	60	0	77.77822123
84	60	0	86.2981575
97	90	30	157.4771123
98	90	30	140.9411437
99	90	30	120.0303733
100	90	30	120.2477287
101	90	30	109.4901077
102	90	30	117.3573406
103	90	30	119.2450791
104	90	30	102.7818331
105	90	30	96.52761409
106	90	30	100.9111892
107	90	30	115.46392
108	90	30	94.60541872
109	90	10	84.32382266

110	90	10	71 43867019
111	90	10	71 87608215
112	90	10	68 99573245
113	90	10	67 13561488
114	90	10	73 31526783
115	90	10	70 19052518
116	90	10	61 76673533
117	90	10	68 49081478
118	90	10	72 37743795
119	90	10	70 20818797
120	90	10	85 24385133
121	90	3	75 12647034
122	90	3	85 15080776
123	90	3	75 84390109
124	90	3	69 24408142
125	90	3	81 68049874
126	90	3	95 78270078
127	90	3	80 87514806
128	90	3	77 50116402
129	90	3	84 14205015
130	90	3	71 69257956
131	90	3	66 09551655
132	90	3	55 8391254
73	90	0	66 57527165
74	90	0	70 72341901
75	90	0	74 91727529
76	90	0	71 22629741
77	90	0	71 7699685
78	90	0	77 24742867
79	90	0	76 01853464
80	90	0	73 74053082
81	90	0	76 37668681
82	90	0	77 27397644
83	90	0	73 14825925
84	90	0	70 29162084

EXPERIMENT 4: NALOXONE

Output:
The GLM Procedure

Class Level Information

Class	Levels	Values
dose	5	0 1010 1030 3010 3030
time	2	60 90
id	60	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 61 62 63 64 65 66 67 68 69 70 71 72

Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	182420.9602	20268.9956	74.99	<.0001
Error	110	29732.9004	270.2991		
Corrected Total	119	212153.8606			

R-Square	Coeff Var	Root MSE	activ Mean
0.859852	19.99894	16.44078	82.20822

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	4	177087.5325	44271.8831	163.79	<.0001
time	1	2428.4883	2428.4883	8.98	0.0034
dose*time	4	2904.9394	726.2348	2.69	0.0350

Least Squares Means

dose	activ LSMEAN
0	78.028367
1010	65.670318
1030	54.072469
3010	157.145967
3030	56.123983

time	activ LSMEAN
60	86.7068208
90	77.7096207

dose	time	activ LSMEAN
0	60	82.780961
0	90	73.275773
1010	60	66.123108

1010	90	65.217528
1030	60	61.377835
1030	90	46.767103
3010	60	169.074057
3010	90	145.217877
3030	60	54.178144
3030	90	58.069821

Least Squares Means
Adjustment for Multiple Comparisons: Tukey

dose	activ LSMEAN	LSMEAN Number
0	78.028367	1
1010	65.670318	2
1030	54.072469	3
3010	157.145967	4
3030	56.123983	5

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: activ

i/j	1	2	3	4	5
1		0.0766	<.0001	<.0001	0.0001
2	0.0766		0.1116	<.0001	0.2674
3	<.0001	0.1116		<.0001	0.9926
4	<.0001	<.0001	<.0001		<.0001
5	0.0001	0.2674	0.9926	<.0001	

Removed 3010 doses
DATA USED:

1	60	1010	70.06015
2	60	1010	58.00864
3	60	1010	40.65573
4	60	1010	57.03531
5	60	1010	78.02433
6	60	1010	77.09567
7	60	1010	80.98928
8	60	1010	70.65634
9	60	1010	52.41337
10	60	1010	45.32695
11	60	1010	48.4902
12	60	1010	114.7213

13	60	3030	68.67075
14	60	3030	69.13252
15	60	3030	66.53557
16	60	3030	53.48252
17	60	3030	30.63021
18	60	3030	47.78299
19	60	3030	46.51258
20	60	3030	52.6347
21	60	3030	59.3647
22	60	3030	53.6673
23	60	3030	56.23512
24	60	3030	45.48876
25	60	3010	155.3129
26	60	3010	167.6289
27	60	3010	186.7479
28	60	3010	171.595
29	60	3010	205.4959
30	60	3010	169.4248
31	60	3010	175.4221
32	60	3010	200.7451
33	60	3010	188.5287
34	60	3010	154.0201
35	60	3010	138.8937
36	60	3010	115.0736
37	60	1030	64.7121
38	60	1030	57.46441
39	60	1030	66.91122
40	60	1030	60.74515
41	60	1030	80.41445
42	60	1030	73.78197
43	60	1030	52.09212
44	60	1030	42.87196
45	60	1030	76.25562
46	60	1030	50.80197
47	60	1030	46.42071
48	60	1030	64.06234
1	90	1010	78.17459
2	90	1010	76.60293
3	90	1010	49.47695
4	90	1010	36.71635
5	90	1010	50.09762
6	90	1010	54.73179
7	90	1010	74.20736

8	90	1010	52.58619
9	90	1010	73.70335
10	90	1010	51.85922
11	90	1010	81.39453
12	90	1010	103.0595
13	90	3030	75.5308
14	90	3030	67.75843
15	90	3030	71.16281
16	90	3030	77.43706
17	90	3030	26.97106
18	90	3030	44.08816
19	90	3030	54.19185
20	90	3030	56.79982
21	90	3030	58.33235
22	90	3030	57.32425
23	90	3030	51.0105
24	90	3030	56.23076
25	90	3010	172.4047
26	90	3010	154.0859
27	90	3010	152.9189
28	90	3010	148.5082
29	90	3010	154.2322
30	90	3010	134.2968
31	90	3010	147.3319
32	90	3010	178.4869
33	90	3010	172.2589
34	90	3010	127.994
35	90	3010	116.0769
36	90	3010	84.01912
37	90	1030	49.86514
38	90	1030	50.17209
39	90	1030	47.7457
40	90	1030	51.52729
41	90	1030	57.72192
42	90	1030	58.31856
43	90	1030	44.80385
44	90	1030	33.86113
45	90	1030	51.28597
46	90	1030	37.24759
47	90	1030	39.6318
48	90	1030	39.02422
61	60	0	88.62867
62	60	0	88.97348

63	60	0	83.42844
64	60	0	86.08087
65	60	0	81.93699
66	60	0	78.57734
67	60	0	85.01857
68	60	0	76.19289
69	60	0	81.49679
70	60	0	78.96111
71	60	0	77.77822
72	60	0	86.29816
61	90	0	66.57527
62	90	0	70.72342
63	90	0	74.91728
64	90	0	71.2263
65	90	0	71.76997
66	90	0	77.24743
67	90	0	76.01853
68	90	0	73.74053
69	90	0	76.37669
70	90	0	77.27398
71	90	0	73.14826
72	90	0	70.29162

Experiment 4: Naloxone

Class Levels Values
dose 4 0 1010 1030 3030 **(REMOVED 3010)!!!**
time 2 60 90
id 48 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 37 38 39 40
41 42 43 44 45 46 47 48 61 62 63 64 65 66 67 68 69 70 71 72

The GLM Procedure
Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	10536.28259	1505.18323	9.07	<.0001
Error	88	14598.61132	165.89331		
Corrected Total	95	25134.89392			

R-Square	Coeff Var	Root MSE	activ Mean
0.419189	20.29178	12.87996	63.47378

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	3	8617.558803	2872.519601	17.32	<.0001
time	1	669.703997	669.703997	4.04	0.0476
dose*time	3	1249.019794	416.339931	2.51	0.0639

Least Squares Means

dose	activ LSMEAN
0	78.0283671
1010	65.6703180
1030	54.0724693
3030	56.1239825

time	activ LSMEAN
60	66.1150118
90	60.8325566

dose	time	activ LSMEAN
0	60	82.7809608
0	90	73.2757733
1010	60	66.1231077
1010	90	65.2175282
1030	60	61.3778351
1030	90	46.7671035
3030	60	54.1781436
3030	90	58.0698214

Least Squares Means
Adjustment for Multiple Comparisons: Tukey

dose	activ LSMEAN	LSMEAN Number

0	78.0283671	1
1010	65.6703180	2
1030	54.0724693	3
3030	56.1239825	4

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: activ

i/j	1	2	3	4
1		0.0070	<.0001	<.0001
2	0.0070		0.0129	0.0568
3	<.0001	0.0129		0.9458
4	<.0001	0.0568	0.9458	

DATA USED:

ID	TIME	DOSE	ACTIV
1	60	1010	70.06015345
2	60	1010	58.00864142
3	60	1010	40.6557345
4	60	1010	57.03531492
5	60	1010	78.02433233
6	60	1010	77.09566697
7	60	1010	80.98928354
8	60	1010	70.65633691
9	60	1010	52.41336682
10	60	1010	45.32695019
11	60	1010	48.49020162
12	60	1010	114.7213101
13	60	3030	68.67075063

14	60	3030	69.13252016
15	60	3030	66.5355691
16	60	3030	53.48251643
17	60	3030	30.63021447
18	60	3030	47.78298694
19	60	3030	46.51258419
20	60	3030	52.63470092
21	60	3030	59.3646977
22	60	3030	53.66729986
23	60	3030	56.23512323
24	60	3030	45.48875991
37	60	1030	64.71210299
38	60	1030	57.46441272
39	60	1030	66.9112167
40	60	1030	60.74514532
41	60	1030	80.41445213
42	60	1030	73.78197397
43	60	1030	52.09212159
44	60	1030	42.87196414
45	60	1030	76.25561921
46	60	1030	50.80196695
47	60	1030	46.42070773
48	60	1030	64.06233789
1	90	1010	78.17458644
2	90	1010	76.60293351
3	90	1010	49.47695039
4	90	1010	36.71635086
5	90	1010	50.09761667

6	90	1010	54.73178858
7	90	1010	74.20736236
8	90	1010	52.58618884
9	90	1010	73.70334747
10	90	1010	51.85921728
11	90	1010	81.39453484
12	90	1010	103.0594609
13	90	3030	75.53080294
14	90	3030	67.75843452
15	90	3030	71.16280819
16	90	3030	77.4370614
17	90	3030	26.97106425
18	90	3030	44.08815928
19	90	3030	54.19184693
20	90	3030	56.79981703
21	90	3030	58.33235264
22	90	3030	57.32424596
23	90	3030	51.01050463
24	90	3030	56.23075914
37	90	1030	49.8651377
38	90	1030	50.17208599
39	90	1030	47.74569601
40	90	1030	51.52729306
41	90	1030	57.72191893
42	90	1030	58.3185595
43	90	1030	44.80384904
44	90	1030	33.861127
45	90	1030	51.28596539

46	90	1030	37.24759042
47	90	1030	39.63179728
48	90	1030	39.02422144
61	60	0	88.62867
62	60	0	88.97348
63	60	0	83.42844
64	60	0	86.08087
65	60	0	81.93699
66	60	0	78.57734
67	60	0	85.01857
68	60	0	76.19289
69	60	0	81.49679
70	60	0	78.96111
71	60	0	77.77822
72	60	0	86.29816
61	90	0	66.57527
62	90	0	70.72342
63	90	0	74.91728
64	90	0	71.2263
65	90	0	71.76997
66	90	0	77.24743
67	90	0	76.01853
68	90	0	73.74053
69	90	0	76.37669
70	90	0	77.27398
71	90	0	73.14826
72	90	0	70.2916

Experiment 4: Naloxone

The GLM Procedure

Class Level Information

Class	Levels	Values
dose	4	5 1010 1030 3030 (acid vs naloxone groups)
time	2	60 90
id	48	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	3379.29743	482.75678	2.67	0.0150
Error	88	15935.65753	181.08702		
Corrected Total	95	19314.95496			

R-Square	Coeff Var	Root MSE	activ Mean
0.174958	23.25309	13.45686	57.87127

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	3	2001.237840	667.079280	3.68	0.0150
time	1	220.064354	220.064354	1.22	0.2733
dose*time	3	1157.995238	385.998413	2.13	0.1019

The GLM Procedure

Least Squares Means

dose	activ LSMEAN
5	55.6183029
1010	65.6703180
1030	54.0724693
3030	56.1239825

time	activ LSMEAN
60	59.3853147
90	56.3572216

dose	time	activ LSMEAN
5	60	55.8621725
5	90	55.3744333
1010	60	66.1231077
1010	90	65.2175282
1030	60	61.3778351
1030	90	46.7671035
3030	60	54.1781436
3030	90	58.0698214

Least Squares Means
Adjustment for Multiple Comparisons: Tukey

dose	activ LSMEAN	LSMEAN Number
5	55.6183029	1
1010	65.6703180	2
1030	54.0724693	3
3030	56.1239825	4

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: activ

i/j	1	2	3	4
1		0.0540	0.9785	0.9992
2	0.0540		0.0189	0.0740
3	0.9785	0.0189		0.9521
4	0.9992	0.0740	0.9521	

Data Used:

ID	TIME	DOSE	ACTIV
1	60	1010	70.06015345
2	60	1010	58.00864142
3	60	1010	40.6557345
4	60	1010	57.03531492
5	60	1010	78.02433233
6	60	1010	77.09566697
7	60	1010	80.98928354
8	60	1010	70.65633691
9	60	1010	52.41336682
10	60	1010	45.32695019
11	60	1010	48.49020162
12	60	1010	114.7213101
13	60	3030	68.67075063

14	60	3030	69.13252016
15	60	3030	66.5355691
16	60	3030	53.48251643
17	60	3030	30.63021447
18	60	3030	47.78298694
19	60	3030	46.51258419
20	60	3030	52.63470092
21	60	3030	59.3646977
22	60	3030	53.66729986
23	60	3030	56.23512323
24	60	3030	45.48875991
37	60	1030	64.71210299
38	60	1030	57.46441272
39	60	1030	66.9112167
40	60	1030	60.74514532
41	60	1030	80.41445213
42	60	1030	73.78197397
43	60	1030	52.09212159
44	60	1030	42.87196414
45	60	1030	76.25561921
46	60	1030	50.80196695
47	60	1030	46.42070773
48	60	1030	64.06233789
1	90	1010	78.17458644
2	90	1010	76.60293351
3	90	1010	49.47695039
4	90	1010	36.71635086
5	90	1010	50.09761667

6	90	1010	54.73178858
7	90	1010	74.20736236
8	90	1010	52.58618884
9	90	1010	73.70334747
10	90	1010	51.85921728
11	90	1010	81.39453484
12	90	1010	103.0594609
13	90	3030	75.53080294
14	90	3030	67.75843452
15	90	3030	71.16280819
16	90	3030	77.4370614
17	90	3030	26.97106425
18	90	3030	44.08815928
19	90	3030	54.19184693
20	90	3030	56.79981703
21	90	3030	58.33235264
22	90	3030	57.32424596
23	90	3030	51.01050463
24	90	3030	56.23075914
37	90	1030	49.8651377
38	90	1030	50.17208599
39	90	1030	47.74569601
40	90	1030	51.52729306
41	90	1030	57.72191893
42	90	1030	58.3185595
43	90	1030	44.80384904
44	90	1030	33.861127
45	90	1030	51.28596539

46	90	1030	37.24759042
47	90	1030	39.63179728
48	90	1030	39.02422144
49	60	5	64.5186
50	60	5	51.35319
51	60	5	38.80165
52	60	5	46.14252
53	60	5	48.67999
54	60	5	57.12172
55	60	5	55.46604
56	60	5	61.0735
57	60	5	58.92363
58	60	5	59.67321
59	60	5	61.49533
60	60	5	67.09669
49	90	5	79.06807
50	90	5	51.90433
51	90	5	43.12128
52	90	5	47.1733
53	90	5	50.31523
54	90	5	52.34763
55	90	5	56.06858
56	90	5	55.62453
57	90	5	62.75734
58	90	5	51.99836
59	90	5	53.96595
60	90	5	60.1486

EXPERIMENT 4 – NALOXONE ONLY AT 90 MIN

The GLM Procedure

Class Level Information

Class	Levels	Values
dose	4	5 1010 1030 3030
time	1	90
id	48	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Number of Observations Read 48
Number of Observations Used 48

The GLM Procedure

Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2092.491229	697.497076	4.00	0.0132
Error	44	7666.459577	174.237718		
Corrected Total	47	9758.950807			

R-Square	Coeff Var	Root MSE	activ Mean
0.214418	23.42187	13.19991	56.35722

Source	DF	Type I SS	Mean Square	F Value	Pr > F
dose	3	2092.491229	697.497076	4.00	0.0132
time	0	0.000000	.	.	.
dose*time	0	0.000000	.	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	3	2092.491229	697.497076	4.00	0.0132
time	0	0.000000	.	.	.
dose*time	0	0.000000	.	.	.

The GLM Procedure
Least Squares Means

dose	activ LSMEAN
5	55.3744333
1010	65.2175282
1030	46.7671035
3030	58.0698214

time	activ LSMEAN
90	56.3572216

dose	time	activ LSMEAN
5	90	55.3744333
1010	90	65.2175282
1030	90	46.7671035
3030	90	58.0698214

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

dose	activ LSMEAN	LSMEAN Number
5	55.3744333	1
1010	65.2175282	2
1030	46.7671035	3
3030	58.0698214	4

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: activ

i/j	1	2	3	4
1		0.2748	0.3907	0.9586
2	0.2748		0.0071	0.5514
3	0.3907	0.0071		0.1699
4	0.9586	0.5514	0.1699	

Data used:

1	90	1010	78.17458644
2	90	1010	76.60293351
3	90	1010	49.47695039
4	90	1010	36.71635086
5	90	1010	50.09761667
6	90	1010	54.73178858
7	90	1010	74.20736236
8	90	1010	52.58618884
9	90	1010	73.70334747
10	90	1010	51.85921728
11	90	1010	81.39453484
12	90	1010	103.0594609
13	90	3030	75.53080294
14	90	3030	67.75843452
15	90	3030	71.16280819
16	90	3030	77.4370614
17	90	3030	26.97106425
18	90	3030	44.08815928
19	90	3030	54.19184693
20	90	3030	56.79981703
21	90	3030	58.33235264
22	90	3030	57.32424596
23	90	3030	51.01050463
24	90	3030	56.23075914
37	90	1030	49.8651377
38	90	1030	50.17208599
39	90	1030	47.74569601
40	90	1030	51.52729306
41	90	1030	57.72191893
42	90	1030	58.3185595
43	90	1030	44.80384904
44	90	1030	33.861127
45	90	1030	51.28596539
46	90	1030	37.24759042
47	90	1030	39.63179728
48	90	1030	39.02422144
49	90	5	79.06807
50	90	5	51.90433
51	90	5	43.12128
52	90	5	47.1733
53	90	5	50.31523
54	90	5	52.34763
55	90	5	56.06858

56	90	5	55.62453
57	90	5	62.75734
58	90	5	51.99836
59	90	5	53.96595
60	90	5	60.1486

Experiment 5- Naloxone only

The GLM Procedure

Class Level Information

Class	Levels	Values
dose	3	0 10 30 saline, 10gmkg naloxone, 30mgkg naloxone
time	3	60 90 120
id	36	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

Number of Observations Read 109
Number of Observations Used 108

The SAS System 17:24 Tuesday, June 14, 2011 2

The GLM Procedure

Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	123497.4238	15437.1780	23.35	<.0001
Error	99	65450.2182	661.1133		
Corrected Total	107	188947.6419			

R-Square	Coeff Var	Root MSE	activ Mean
0.653607	28.74654	25.71212	89.44424

Source	DF	Type I SS	Mean Square	F Value	Pr > F
dose	2	44999.06325	22499.53162	34.03	<.0001
time	2	28127.76173	14063.88087	21.27	<.0001
dose*time	4	50370.59879	12592.64970	19.05	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	2	44999.06325	22499.53162	34.03	<.0001
time	2	28127.76173	14063.88087	21.27	<.0001
dose*time	4	50370.59879	12592.64970	19.05	<.0001

The GLM Procedure
Least Squares Means

dose	activ LSMEAN
0	70.857759
10	79.609121
30	117.865837

time	activ LSMEAN
60	100.692356
90	101.018240
120	66.622121

dose	time	activ LSMEAN
0	60	70.686379
0	90	62.328608
0	120	79.558291
10	60	97.875235
10	90	78.785582
10	120	62.166547
30	60	133.515455

30	90	161.940529
30	120	58.141525

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

dose	activ LSMEAN	LSMEAN Number
0	70.857759	1
10	79.609121	2
30	117.865837	3

Least Squares Means for effect dose
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: activ

i/j	1	2	3
1		0.3225	<.0001
2	0.3225		<.0001
3	<.0001	<.0001	

Data used:

id	time	dose	activ
1	60	0	60.81828053
2	60	0	60.00835525
3	60	0	57.29658992
4	60	0	69.8086766
5	60	0	71.20017982
6	60	0	80.86286239
7	60	0	74.04614189

8	60	0	73.69100331
9	60	0	80.81032663
10	60	0	79.46676942
11	60	0	71.31436242
12	60	0	68.91300234
13	60	10	148.7014403
14	60	10	79.6843878
15	60	10	107.9125447
16	60	10	126.7159509
17	60	10	100.1643173
18	60	10	88.45817401
19	60	10	56.69580883
20	60	10	62.34393702
21	60	10	73 65024299
22	60	10	61.83434561
23	60	10	120.732703
24	60	10	147.6089623
25	60	30	105.1523317
26	60	30	102.6345733
27	60	30	165.5468871
28	60	30	219.5010366
29	60	30	124.3326644
30	60	30	84.27869255
31	60	30	122.5175628
32	60	30	119.2662399
33	60	30	110.2460814
34	60	30	102.6261669
35	60	30	157.2231032

36	60	30	188.8601258
1	90	0	49.92260868
2	90	0	46.95703548
3	90	0	51.91219363
4	90	0	58.55354833
5	90	0	64.58871961
6	90	0	63.20700943
7	90	0	61.78046811
8	90	0	70.59712131
9	90	0	74.65955469
10	90	0	79.7546755
11	90	0	65.3758008
12	90	0	60.63456074
13	90	10	114.4081393
14	90	10	71.8445091
15	90	10	98.50733694
16	90	10	110.5998149
17	90	10	87.58746878
18	90	10	63.97143288
19	90	10	51.74447656
20	90	10	60.59067698
21	90	10	52.52480367
22	90	10	38.93142415
23	90	10	72.35412265
24	90	10	122.3627786
25	90	30	132.2596024
26	90	30	204.7817855
27	90	30	173.5895854

28	90	30	228.5075159
29	90	30	155.3421342
30	90	30	101.8575496
31	90	30	178.4289404
32	90	30	150.195109
33	90	30	138.2292692
34	90	30	127.8415915
35	90	30	166.6325645
36	90	30	185.6207035
1	120	0	79.77217425
2	120	0	66.54310206
3	120	0	64.05718457
4	120	0	86.74783493
5	120	0	100.7219196
6	120	0	89.06668677
7	120	0	76.24623833
8	120	0	83.48395594
9	120	0	83.64264344
10	120	0	72.07308604
11	120	0	87.35963159
12	120	0	64.98502937
13	120	10	70.71789163
14	120	10	68.46230616
15	120	10	87.53085913
16	120	10	110.8711499
17	120	10	107.1326305
18	120	10	46.29787022
19	120	10	40.73840306

20	120	10	50.54571505
21	120	10	41.29401345
22	120	10	24.82544573
23	120	10	45.28347147
24	120	10	52.29880863
25	120	30	71.58033119
26	120	30	66.4132975
27	120	30	74.41932825
28	120	30	82.80087175
29	120	30	43.44814512
30	120	30	41.48988637
31	120	30	60.42832066
32	120	30	60.99252483
33	120	30	48.04514747
34	120	30	45.00687835
35	120	30	63.01737557
36	120	30	40.05619287

NALOXONE ONLY 0(SALINE) VS (10MG/KG

The GLM Procedure

Class Level Information

Class	Levels	Values
dose	2	0 10
time	3	60 90 120
id	24	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

GLM Procedure
Dependent Variable: activ

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	10823.12457	2164.62491	4.55	0.0013
Error	66	31411.15541	475.92660		
Corrected Total	71	42234.27998			

R-Square	Coeff Var	Root MSE	activ Mean
0.256264	28.99740	21.81574	75.23344

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dose	1	1378.554050	1378.554050	2.90	0.0935
time	2	2947.893022	1473.946511	3.10	0.0518
dose*time	2	6496.677496	3248.338748	6.83	0.0020

Least Squares Means

dose	activ LSMEAN
0	70.8577593
10	79.6091212

time	activ LSMEAN
60	84.2808069
90	70.5570950
120	70.8624188

dose	time	activ LSMEAN
0	60	70.6863792
0	90	62.3286080
0	120	79.5582906
10	60	97.8752346

10	90	78.7855820
10	120	62.1665471

Adjustment for Multiple Comparisons: Tukey

dose	activ	LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
0		70.8577593	0.0935
10		79.6091212	

Data Used:

id	time	dose	activ
1	60	0	60.81828053
2	60	0	60.00835525
3	60	0	57.29658992
4	60	0	69.8086766
5	60	0	71.20017982
6	60	0	80.86286239
7	60	0	74.04614189
8	60	0	73.69100331
9	60	0	80.81032663
10	60	0	79.46676942
11	60	0	71.31436242
12	60	0	68.91300234
13	60	10	148.7014403
14	60	10	79.6843878
15	60	10	107.9125447
16	60	10	126.7159509
17	60	10	100.1643173
18	60	10	88.45817401

19	60	10	56.69580883
20	60	10	62.34393702
21	60	10	73.65024299
22	60	10	61.83434561
23	60	10	120.732703
24	60	10	147.6089623
1	90	0	49.92260868
2	90	0	46.95703548
3	90	0	51.91219363
4	90	0	58.55354833
5	90	0	64.58871961
6	90	0	63.20700943
7	90	0	61.78046811
8	90	0	70.59712131
9	90	0	74.65955469
10	90	0	79.7546755
11	90	0	65.3758008
12	90	0	60.63456074
13	90	10	114.4081393
14	90	10	71.8445091
15	90	10	98.50733694
16	90	10	110.5998149
17	90	10	87.58746878
18	90	10	63.97143288
19	90	10	51.74447656
20	90	10	60.59067698
21	90	10	52.52480367
22	90	10	38.93142415

23	90	10	72.35412265
24	90	10	122.3627786
1	120	0	79.77217425
2	120	0	66.54310206
3	120	0	64.05718457
4	120	0	86.74783493
5	120	0	100.7219196
6	120	0	89.06668677
7	120	0	76.24623833
8	120	0	83.48395594
9	120	0	83.64264344
10	120	0	72.07308604
11	120	0	87.35963159
12	120	0	64.98502937
13	120	10	70.71789163
14	120	10	68.46230616
15	120	10	87.53085913
16	120	10	110.8711499
17	120	10	107.1326305
18	120	10	46.29787022
19	120	10	40.73840306
20	120	10	50.54571505
21	120	10	41.29401345
22	120	10	24.82544573
23	120	10	45.28347147
24	120	10	52.29880863