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Thermonociception in fish: Effects of two different doses of morphine on thermal threshold and post-test behaviour in goldfish (*Carassius auratus*)

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ABSTRACT

The question of whether fish can perceive pain is controversial, and pain is a potentially grave threat to fish welfare. To be able to study pain in a species, knowledge of its nociceptive system is necessary. There is therefore a need for standardised, repeatable and quantifiable measures of nociception and pain in fish. Sensitivity to noxious heat is readily quantifiable. We developed an apparatus to expose goldfish to controlled, localised heat stimulation, and tested the hypothesis that goldfish perceive heat as aversive. We predicted that they would respond to increasing heat with an escape response, that morphine would decrease their heat sensitivity and that the heat stimulation would affect post-test behaviour. A safety cut-off temperature of 50 °C was built into the test apparatus. All 16 fish responded to the heat with an escape response, with a mean baseline of 38 °C. However, morphine at 40 and 50 mg kg^{-1} could not be demonstrated to have a biologically relevant analgesic effect, but did significantly decrease the impact of heat stimulation on behaviour in the home tank. To our knowledge, this study is the first to systematically investigate thermonociception in unanaesthetised fish.

1. Introduction

Both as production and laboratory animals, fish are subjected to noxious, potentially painful treatments such as blood sampling, fin tagging, toxicology testing and different surgical procedures. Our knowledge of fish physiology and the efficacy of different analgesics does not match the severity of the procedures to which fish are exposed (Zottoli and Freemer, 2003) and there is a need for research aimed at developing good analgesic protocols for fish.

Pain in humans is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage," and further "activity induced in the nociceptors and nociceptive pathways by a noxious stimulus is not pain, which is always a psychological state." There are considerable challenges in designing experiments that specifically address pain in fish; the focus of research has therefore been on describing the nociceptive system. Galvanic stimulation has been employed to investigate nociception in fish (Dunlop et al., 2006; Nordgreen et al., 2007), and fish clearly react aversively to this stimulation. However, an electric current will not activate nociceptive afferents specifically, and is therefore less well suited for testing of analgesics. Injection of acid does stimulate nociceptors specifically, and has been used to show an analgesic effect of morphine in trout (Sneddon, 2003) and

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winter flounder (Newby et al., 2007). However, acid can denature tissue, and affects behaviour in rainbow trout for up to 4 h (Sneddon, 2003; Sneddon et al., 2003a). Repeated testing within relatively short time spans is therefore not possible. Furthermore, handling of the fish, often combined with anaesthesia is necessary in order to inject the acid subcutaneously.

In rodents, cats and poultry, tests of heat nociception have been used successfully to assess the efficacy of different analgesics, including opioids (Hughes, 1990; Dixon et al., 2002; Robertson et al., 2003; Morgan et al., 2006; Taylor et al., 2007). Heat is a well-suited stimulus modality for experiments on nociception because it stimulates heat sensitive nociceptors selectively, does not damage the tissue if proper safety cut-offs are used, and readily gives a quantifiable measure of nociception and analgesia in the form of a thermal threshold (TT); the temperature at which the subject shows an escape response from a thermal stimulus, or latency to withdraw. Goldfish can sense temperature changes of as little as 2 °C by point stimulation, and 0.1 °C by whole-body stimulation (Bardach, 1956). However, noxious heat stimulation has not previously been used to investigate the efficacy of analgesics in fish. To the author's knowledge, the only report of thermonociception in conscious fish is Bardach (1956) who, in his paper on maximum sensitivity of thermoreceptors in goldfish also reported that point stimulation of 38–45 °C elicited an escape-response. However, the temperature was increased at a rate that in mammals will elicit an A-delta fibre mediated response (Yeomans and Proudfit, 1996), whereas a slower heating rate, stimulating C fibre nociceptors, would be better suited for testing the analgesic effect of μ receptor agonists such as morphine (Yeomans et al., 1996). Morphine has strong analgesic properties in mammals and is the standard against which other opioid analgesics are assessed (Rang et al., 1999). Even though it is known that morphine has analgesic effects in rainbow trout (Sneddon, 2003), the very high doses used makes it clear that more knowledge about fish analgesia and the fish opioid system is needed.

The main aim of the current experiment was to develop an apparatus to expose goldfish to controlled, localised heat stimulation and use it to test the hypothesis that increasing heat activates the nociceptive system and that goldfish perceive heat as aversive. On the basis of this hypothesis we predicted that (1) goldfish would respond to increasing heat applied to the trunk with an escape response and that (2) morphine would have an analgesic effect, seen as an elevation of the TT. The last aim was to study the behaviour in the home tank before and after the TT test to reveal sub-acute behavioural effects of the test itself and the drug.

2. Methods

2.1. Animals and housing

For each of the experiments 1 and 2, $N = 8$. Thus, 16 common goldfish (*Carassius auratus*) were used in total, and they were purchased from Ozark fisheries (1100 Ozark Fisheries Rd. Stoutland, MO, USA). They were fed the same diet as used at Ozark twice a day, and the mean body weight of the fish was 30.1 g (range 19.8–39.0) The lighting regime was 12:12 h L:D. The water was declorinated prior to being led into the tanks and the tanks were cleaned twice a week. Water temperature was kept at 22 ± 1 °C. The tanks were kept on a part flow-through part recirculation system and the total water volume of all tanks was renewed approximately every 18 h. Each tank was aerated by an air pump. All fish were brought into the lab at least one week prior to the start of the experiment. Two days before the first behavioural observation, they were put into separate tanks. Both the holding and the experimental tanks measured 122 cm × 32 cm × 53 cm (W × D × H) and contained gravel and plastic aquarium plants.

2.2. Overview of the experimental design

In experiment 1, the fish were injected with 40 mg morphine kg^{-1} or a similar volume of saline, and in experiment 2, the fish were injected with 50 mg morphine kg^{-1} or a similar volume of saline. Other than the dose, the experiments were similar (Table 1). On days 1 (24 h before injection), 2 (2 h after injection, i.e., 30 min after the last TT test) and 3 (24 h after injection), behaviour was observed in the home tank for 30 min per day using sampling of the focal animal for 1 s every 15 s. The behaviours were: Activity (constituted of swimming, i.e., movement of more than one head-length, and picking of gravel) and hovering (holding the same position in the water) in the lower half of the tank. Other behaviours were scored as "other". On day 2, the baseline TT for each fish was measured before it was injected with either morphine or saline and the TT measured 30, 60 and 90 min after injection. On days 9–11, the observations and TT tests were repeated, but the fish that had received saline on day 2, received morphine on day 10, and vice versa. Half of the fish received saline as their first injection, and the other half received morphine. The wash out period (the period between the administration of morphine and saline) was eight days.

2.3. Heat stimulator apparatus

We designed a custom made heat stimulator (HS), which consisted of a kapton thermofoil heater (1.27 cm × 1.27 cm HK5572, Minco, Minneapolis, USA)

Table 1
An overview of the experiment for one fish. The timeline is general and shows the flow of experiment 1 as well as 2.

Day	Procedure
1	Observation of behaviour (24 h prior to injection)
2	Thermal threshold testing Observation of behaviour (2 h post injection/30 min after the last TT test)
3	Observation of behaviour (24 h post injection)
2–10	Wash-out period
9	Observation of behaviour (24 h prior to injection)
10	Thermal threshold testing Observation of behaviour (2 h post injection/30 min after the last TT test)
11	Observation of behaviour (24 h post injection)

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and a thermal ribbon RTD sensor (0.76 cm × 0.76 cm S651, Minco) glued together with a thin layer of epoxy. This assembly was approximately 0.79 mm thick, and moderately flexible. A belt made of chamois leather and vet-wrap with a small patch of gauze held the heater and sensor in contact with the fish skin. The heater and sensor were connected to a CT325 temperature controller (Minco). The CT325 converted the sensor reading to a calibrated voltage signal which was passed through a second amplifier (which was in turn, calibrated by the CT325's reference voltage signal) to a Phidgets voltage sensor (1117 voltage sensor, Phidgets, Alberta, Canada) and Phidgets interface card (1018 Phidgets Interface Kit 8/8/8) to the computer. The interface also controlled the CT325, allowing us to control the heater directly from the computer. In addition, the CT325 provided a safety cut-off that prevented the heater exceeding 50 °C in the event of an interface or computer failure. The safety limit of 50 °C was determined in a pilot study on anaesthetised fish. During a trial, custom-written software controlled the heater, displayed the readings on the computer screen and databased every temperature reading and heater control signal 32 times per second. A web-camera (QuickCam Communicate STX, Logitech, CA, USA) was mounted on the test chamber and the image displayed on a monitor in real-time. An LED attached to the tank within the view of the camera was connected to the heat stimulator. It lit up at the start of the trial and was turned off when it was terminated. The database saved, and co-ordinated this video with the temperature readings for further analysis.

2.4. Thermal threshold testing

On the day of the test, the fish was anaesthetised in metacaine (0.15 g l^{-1}) (Finquel, Argent Laboratories, Redmond WA, USA). Following cessation of voluntary swimming movements it was weighed, placed on moist chamoise leather and connected to a recirculation system by means of a mouthpiece. Viscotears® (Novartis Healthcare A/S, Copenhagen, Denmark) was applied to the eyes to prevent drying out of and damage to the cornea. Anaesthetic solution (metacaine 0.1 g l^{-1}) was pumped across the gills while the belt holding the stimulator and sensor in place, was fitted. The fish was then placed in the test tank, which measured19 cm × 12 cm × 14 cm (W × D × H) and was placed in a larger aquarium. The test chamber had a plastic wall that could be adjusted to the size of the individual fish, and the mean size of this restricting crate within the test tank was 8.5 cm × 3 cm × 14 cm (W × D × H). The adjustable wall was placed so that the fish was limited in its movement but not immobilised, to allow fish to show escape behaviours. Water was aerated and pumped through the test cage in the nose-tail direction for the fish. Thirty minutes after the fish regained consciousness (judged by voluntary breathing and/or swimming movements), it was tested twice with an interstimulus interval of 15 min to find the baseline thermal threshold (TT B1 and TT B2). The heater was stable at the temperature of the fish skin between each test. For all tests, the temperature was increased at a mean rate of 0.9 °C s^{-1}. This level of temperature increase was chosen as it predominantly stimulates C-nociceptive fibres (Yarnitsky et al., 1992; Yeomans et al., 1996), which in mammals are the nociceptive fibres most responsive to morphine (Kellstein et al., 1990; Taddese et al., 1995). The presence of nerve fibres responding to noxious input with a conduction velocity similar to that of mammalian C fibres has been confirmed for goldfish (Dunlop and Laming, 2005). Following the last baseline stimulation, the fish was transferred to a small box with metacaine (0.15 g l^{-1}), sedated and injected with morphine (Morphine sulphate, 15 mg ml^{-1}, Baxter Healthcare Corporation, Deerfield, IL, USA) or 0.9 % saline. The injection was given intramuscularly (IM) in the dorsal epaxial musculature. Thereafter the fish was again transferred to the experimental tank and the TT recorded at 30, 60 and 90 min after injection. When the fish showed an escape response (see definition below); the heat was immediately turned off by the observer and the trial was terminated. Categorisation of the escape behaviours and determination of the exact TT were made from the video recordings. An escape response was defined as a response that would have propelled the fish away from the source of stimulation had its movement not been limited. The exact start of the trial and response was determined to the closest 0.01 s. The escape responses were classified as either C-starts (movement of the head and tail towards the same side of the body forming a "C"), swimming (movement of the body to form an S-shape) or tail-flicking (flicking the tail without sideways movements of the head or trunk region). After the last test, the fish was lightly sedated, the belt removed and the fish placed in its home tank.

2.5. Data processing

2.5.1. Thermal threshold

The thermal threshold was calculated as the linearly interpolated temperature between the two temperature readings closest to the timestamp of the onset of the avoidance response. Two trials had to be discarded, leaving a total of ten TT-results for 14 fish and nine for two fish.

The 95 % confidence interval (CI) for the difference in baseline responses (within and between test days) for each fish contained the value zero, and the mean baseline for each fish and day was calculated and used in analysis. Two readings from two fish in experiment 2 had to be discarded from the computations, as they were below 30 °C, most likely due to a strong learning effect. Similar effects of retesting have been found in mice (Plone et al., 1996). The total number of each of the escape responses recorded (experiment 1 and 2 together), was calculated and presented as the percentage of the total number of escape responses scored.

2.5.2. Behavioural observations in the home tanks

The behaviours were presented as the percentage of the total number of observations in which the behaviours occurred.

2.6. Statistical methods

Analyses of the effect of morphine on TT and behaviour in the home tank were done by repeated measures analysis of variance (ANOVA), using the statistical software JMP for

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Windows, version 7.0.1 (SAS Institute Inc., Cary NC, USA). The mean TT baselines were normally distributed and computation of means and confidence intervals were done using MINITAB (Minitab Inc., PA, USA). The criteria for using the general linear model were met for all variables, and analyses were therefore done using untransformed data. We analysed the TT from experiment 1 and 2 separately, using a multifactorial model with interactions. Unless otherwise mentioned, all models were significant. The factors were fish (as a random effect, nested in crossover-order), treatment (saline or morphine), time (mean baseline before injection and 30, 60 and 90 min after injection) and crossover-order (whether the fish received saline or morphine first). To test whether treatment had any effect on behaviour in the home tank, we used a multifactorial model with interactions. The dependent variables were hover low and activity. The factors were fish (as a random effect nested in crossover-order), treatment (saline or morphine), time (24 h before, 2 and 24 h after injection), and crossover-order. For testing whether morphine had an effect on TT and whether the test affected home tank behaviour, the most important variable was the interaction of treatment and time and the variable time, respectively. All post hoc tests were conducted with the Tukey HSD test in JMP, and the values presented are least square (LS) means. Results were accepted as significant if $p < 0.05$.

3. Results

3.1. Baseline thermal threshold

The 95% confidence interval (CI) for the median baseline TT in experiment 1 and 2 overlapped, and the 16 mean baseline TTs could therefore be pooled. The resulting dataset was normally distributed, with a mean of 38.0 °C and 95 % CI of 37.4–38.6 °C.

3.2. Escape behaviour

Three of the 16 fish showed only C-starts and swimming behaviour, but the remaining fish performed all three behaviours. Out of the total 155 responses categorised (three responses could not be placed in any of the three categories and were excluded), 45.8% were C-starts, and tail flick was the least frequent behaviour seen (15.5 %). In addition, fish were seen to display exaggerated (i.e., more than was needed for respiration) mouth opening and movement of the opercular lids just before, during or just after the escape response in 61 of the 155 trials.

3.3. The effect of 40 mg kg^{-1} morphine

3.3.1. Thermal threshold

The effect of time × treatment ($F_{(3,18)} = 3.84$; $p < 0.028$) on TT was significant. Thus morphine and saline affected the TT differently at different time points of testing. The post hoc Tukey HSD test showed that the TT 30 min after injection was significantly higher when the fish received morphine than when they received saline injection (41.4 °C ± 0.63 and 36.8 °C ± 0.63, respectively). However, neither the TT30 for saline nor morphine differed significantly from their respective baselines (baseline morphine: 39.6 °C ± 0.63, baseline saline: 38.2 °C ± 0.63). At 60 and 90 min after injection, there were no treatment differences.

Furthermore, the interaction between treatment and fish ($F_{(6,18)} = 5.13$; $p < 0.0031$) was significant. This indicates that there were significant individual differences in how the fish responded to the two treatments.

3.3.2. Behaviour in the home tank

There was a significant effect of time ($F_{(2,12)} = 26.09$; $p < 0.0001$) on the dependent variable hover low. The post hoc test showed that there was significantly more hovering 2 h after injection (LS mean ± sem, 33.5 % ± 2.5) than 24 h before and after (10.3 and 12.8 % ± 2.5, respectively). Thus, the test affected home tank behaviour.

Interestingly, there were also significant effects of the interactions treatment × time ($F_{(2,12)} = 5.98$; $p < 0.016$) and treatment × time × crossover-order ($F_{(2,12)} = 4.86$; $p < 0.028$). The treatment × time interaction showed that the post-test increase in hovering happened only 2 h after saline injection (42.1 % ± 3.3), whereas the level of hovering 2 h after morphine injection was not significantly different from baseline (25 % and 12.4% ± 3.3, respectively) (Fig. 1a). Furthermore, post hoc analysis of the treatment × time × crossover-order interaction showed that the increase in hovering 2 h after saline injection was found only in the fish that had been injected with saline on the first day of the experiment (57.3 % ± 4.6). In fish that received saline on the second injection day, the level of hovering did not increase significantly (26.9 ± 4.6 % of time spent hovering).

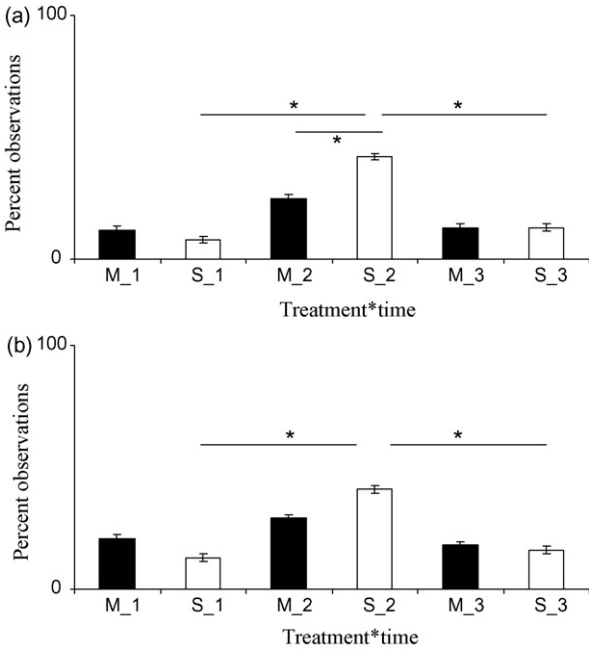

Fig. 1. Percentage of observations in which the fish hovered in the lower half of the tank. M = morphine, S = saline, time 1 = 24 h before injection, 2 = 2 h after injection and 3 = 24 h after injection. (a) Experiment 1, 40 mg morphine kg^{-1}; (b) experiment 2, 50 mg morphine kg^{-1}. Columns are LS means ± standard error of the mean. Horizontal upper lines mark significant differences within treatment and the lower lines mark significant differences between treatments. ($\alpha = 0.05$).

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As for hovering, there was a significant effect of time ($F_{(2,12)} = 14.51$; $p < 0.0006$) on activity. The post hoc test revealed that there was a significant drop in activity at 2 h after injection (from 78.5 to 59.2 ± 2.7 %). Thus, the test affected also this aspect of home tank behaviour.

Furthermore, there was a tendency towards effects of treatment × time ($F_{(2,12)} = 3.14$; $p < 0.08$) and treatment × time × crossover-order ($F_{(2,12)} = 3.56$; $p < 0.06$). The treatment × time and treatment × time × crossover-order interactions indicated that the drop in activity seen 2 h after injection was significant only in the fish who received saline as their first injection.

In conclusion, the TT test did induce changes in behaviour 30 min but not 24 hrs after testing, and morphine seemed to alleviate some of the effects the test had on the amount of time spent hovering in the lower part of the tank, whereas the results for activity showed only tendencies in the same direction.

3.4. The effect of 50 mg kg^{-1} morphine on the thermal threshold of goldfish

3.4.1. Thermal threshold

The treatment × fish interaction had a highly significant ($F_{(6,17)} = 14.84$; $p < 0.0001$) effect on TT, indicating interindividual variation in how the treatments affected the fish. However, by excluding two of the fish in experiment 2 that had shown an extreme drop in TT from testing day 1 to 2, none of the interactions or factors had a significant effect on TT.

3.4.2. Behaviour in the home tank

For hover low there was a significant effect of time ($F_{(2,12.2)} = 4.86$; $p < 0.028$) which was caused by an increase in hovering at 2 h after injection (LS mean ± sem, sem, 35.2 ± 4.7 %) compared to 24 h before injection (16.9 ± 4.7%). Thus, as for the 40 mg kg^{-1} study, the test altered home tank behaviour in the short term.

Furthermore, there was a significant effect of the interaction between treatment and time ($F_{(2,11)} = 5.07$; $p < 0.028$) which revealed that the increase in hovering was seen only after saline injection (13.2, 41.4 and 16.4 ± 3.2% hovering at 24 h before, two and 24 h after) (Fig. 1b). The model failed to reach significance for activity.

4. Discussion

The main findings in these experiments were that goldfish showed an escape response to increasing heat, that morphine at the doses used could not be demonstrated to have an analgesic effect, but, interestingly, did counteract the effect of the test on some aspects of behaviour in the home tank. Goldfish can perceive temperature increments of 2 °C above the water temperature when the area stimulated is as small as 2 mm^2 (Bardach, 1956). In the current experiment, the thermal threshold was demonstrated to be as much as 16 °C above ambient temperature, and remarkably uniform within and between individuals. Thus, the findings supported the current hypothesis, and the earlier observations by Bardach (1956), that goldfish, like birds, reptiles and mammals, perceive heat as noxious, and that the avoidance responses observed were caused by activation of heat sensitive nociceptors, and not by thermal receptors. The presence of heat sensitive nociceptors in fish is slightly puzzling. Unlike terrestrial organisms, fish do not get exposed to tissue-injuring heat sources such as fire. However, heat sensitive nociceptors may serve an important evolutionary role in fish as they respond to temperatures within the lethal range of the species. Rainbow trout nociceptors respond to heat in a range of 28–33 °C (Ashley et al., 2007). The upper lethal temperature for this species lies between 25 and 31 °C depending on exposure time (Hokanson et al., 1977; Ineno et al., 2005). The goldfish baseline TT, 38.0 °C, was higher than that reported for rainbow trout nociceptors and lower than those reported for mammals and reptiles (Yeomans and Proudfit, 1994; Sladky et al., 2007; Taylor et al., 2007). However, it is in agreement with the critical maximum temperature for goldfish of 37.5–38.3 °C, as calculated from the formula of Ford and Beitinger (2005). Thus, thermonociception may have evolutionary significance in that it allows fish to avoid damaging or lethal temperatures, but experiments on more species will be needed in order to test this hypothesis.

To test the prediction that morphine would increase the thermal threshold, i.e., have an analgesic effect, we chose the doses 40 and 50 mg kg^{-1}. The choice of dosages was based on Newby et al. (2006,2007,2008) who described the pharmacokinetics of morphine in rainbow trout and winter flounder after an intraperitoneal (IP) injection of 40 mg kg^{-1} and demonstrated an analgesic effect of 40 mg kg^{-1} in winter flounder 50 min after IP injection. In experiment 1, we found that morphine at a dose of 40 mg kg^{-1} resulted in a higher TT than saline injected at a similar volume at 30 min after injection. However, the increase was not significant when compared to the baseline TT, and probably too small to be biologically significant or clinically useful. Morphine given at 50 mg kg^{-1} did not increase the thermal threshold. This lack of effect contrasts to the finding in Winter Flounder, and may have several explanations. Firstly, the pharmacokinetics of morphine differs between fish species (Newby et al., 2006), and the pharmacodymanics may differ as well. Secondly, we may have allowed too little time for morphine to diffuse from the injection site to the blood stream. However, this is not likely as experiments carried out in our laboratory (Nordgreen et al. *in prep*) show that the maximum plasma concentration of morphine is reached after 30 min following intramuscular injection in goldfish. Thirdly, the same experiment has shown that the plasma concentration of morphine following intramuscular injection in goldfish shows a high degree of individual variation. This individual variation is reflected in the highly significant fish × treatment interaction in the current experiments, and may have contributed to the lack of an observable effect. The rate at which the temperature was raised, 0.9 °C sec^{-1}, was in accordance with heating rates that predominantly activates C fibres in mammals (Yarnitsky and Ochoa, 1990; Yarnitsky et al., 1992; Yeomans and Proudfit, 1996), and was chosen because morphine is most effective against C

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fibre mediated nociception (Kellstein et al., 1990; Taddese et al., 1995). However, the optimal increase rate for activation of C fibres has not been determined in goldfish, and may differ from that of mammals, something that could diminish the antinociceptive effect of morphine. Lastly, even though afferents with C fibre-like conduction velocity seem to dominate the response to noxious stimuli in goldfish when applied just caudal to the operculum (Dunlop and Laming, 2005), this may not be the case for the trunk, which was the area stimulated in the current experiment.

The TT test led to changes in behaviour 2 h after injection. Interestingly, morphine did significantly counteract some of these changes. Several explanations for this observation can be offered: (1) Morphine did have an analgesic effect, but the TT test was not sensitive enough to detect it, whereas the behavioural observations revealed the effect. (2) Exposure to noxious heat might have led to a longer-lasting irritation at the stimulation site, and a possible delayed analgesic effect of morphine might have alleviated that at 2 h after injection. (3) It is well documented that uncontrollable exposure to novelty and involuntary, uncontrollable restraint induces fear in animals (Boissy, 1995; Jones, 1997; Forkman et al., 2007). Morphine reduced neophobic responses in rainbow trout (Sneddon et al., 2003b). The behavioural changes following the TT test may therefore have been caused by fear in the goldfish, which was reduced by morphine treatment. However, the present study cannot be used to differentiate between the three possible explanations. Further experiments would be necessary to clarify whether the effect is one of analgesia or fear-reduction.

5. Conclusion

The results presented in this paper support the hypothesis that goldfish perceive increasing heat as aversive, as other vertebrates are known to do (Hughes and Sufka, 1989; Dixon et al., 2002). Even though morphine had no analgesic effect in the thermal threshold test, it did attenuate effects of testing on post-test behaviour. This indicates that there is a need for further study of the opioid system of teleosts, and the thermal threshold model presented in this paper is a promising model for studies on nociception and the effects of different analgesics in fish.

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