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Behavioral Control of the Efficiency of Pharmacological Anesthesia in Fish

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Abstract—An original behavioral test was used to study the effect of opioid substances on the thresholds of nociceptive responses to pain stimuli—a series of electric impulses applied to nerve endings of the caudal fin—in the common carp (*Cyprinus carpio*). The substances tested included tramadol (μ-agonist of opioid receptors), DADLE (δ-agonist), and U-50488 (κ-agonist) injected intramuscularly in concentrations 10–100 nmol/g of body weight. Raised thresholds of sensitivity to the pain stimulus were observed in the studied fish 5 to 15 min after the injection. The degree of analgesia and the rate of its increase varied depending on the dose. The total duration of analgesia was 40 to 90 min and depended on the concentration of the injected substance. It was observed in some experiments that the analgesic effect of tramadol (the most efficient of the analgesics used) could last longer than 4 h. The analgesic effect of opioids was not detected in experiments where they were applied together with naloxone, an antagonist of opioids. Decreased motor response to pain stimuli after injections of analgesics was not caused by the immobilization of the animal, because the tested fish individuals released into an aquarium demonstrated normal swimming and their usual behavior. We concluded that the systems of opioid nociceptive regulation function similarly in fish and land vertebrates. This regulation can play an important role in defense behavior and in other behaviors in fish.

Keywords: fish, nociception, pain, analgesia, opioids, tramadol, behavioral test, *Cyprinus carpio*.

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INTRODUCTION

The idea of humane treatment of animals has gradually conquered over the minds of the scientific community in the last few decades. In particular, this trend manifested itself in the formulation of special requirements for methods of experiments with laboratory animals, mainly mammals. At the same time, the requirements regarding fish and other lower vertebrates, and invertebrates all the more, are currently either much looser or have never been formulated. Wishing to simplify organizational measures regarding fulfilling the conditions for experiments with animals, researchers are more and more often turning to experiments with fish and other lower vertebrates as model organisms. This, in turn, stimulated the study of pain and possibilities of relieving it in fish and other animals in which this sense was previously little known (Stevens, 2009).

At the current stage, the study of the biological foundations of humane treatment of fish as objects of fishing, economic usage, and scientific experimenting is a rapidly developing field of fish biology; it includes evolutionary, ecological, behavioral, biochemical, and physiological aspects (*VIIth Congress on Biology of Fish*, 2006). The number of studies aimed at investigating the reception of pain stimuli, the structure of nociceptors, and pharmacological methods of anesthesia has markedly increased (Chervova et al., 1992, 1994; Chervova, 1997; Chervova and Lapshin, 2000; Sneddon et al., 2003; Sneddon, 2004; Dunlop and Laming, 2005; Newby et al., 2006, 2008, 2009). However, the study of pain responses in fish is complicated by underdeveloped adequate methodological techniques allowing researchers to dose pain nociceptive stimuli and estimate quantitatively the degree of response to them.

The purposes of this study were to develop a method for pain stimulation of fish, recording their behavioral responses to near-threshold pain stimuli, and investigating the possibility of pharmacological regulation of their nociceptive sensitivity with analgesics of central action, agonists of opioid receptors.

MATERIAL AND METHODS

The experiments were performed with common carp (*Cyprinus carpio*) individuals 1–2 years old, 14–17 cm long, and weighing 50–100 g. A total of 47 individuals were used. Prior to the experiments, the fish were kept in a laboratory aquarium over at least three months and fed with living food (Chironomidae lar-

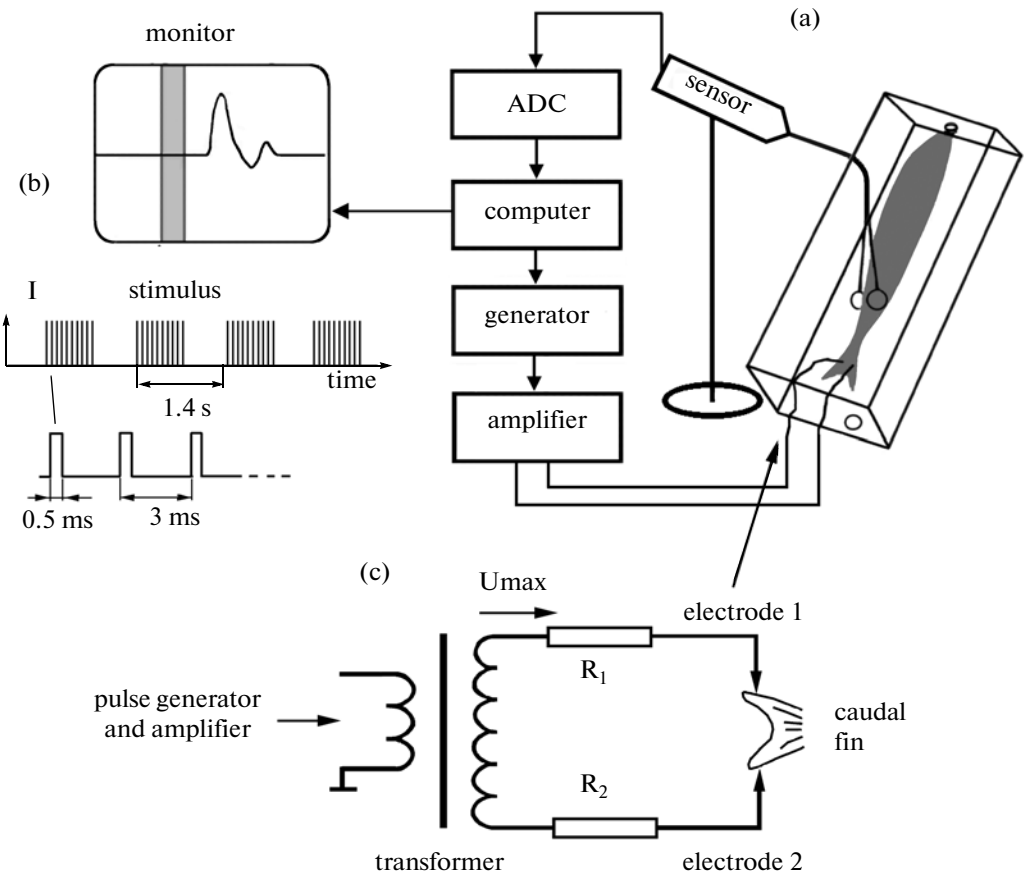

Fig. 1. Experimental setup: (a) diagram of the unit for recording motor responses of fish to nociceptive electric stimuli; (b) screen of the monitor shows an imaginary oscillogram of the recorded locomotor response of the fish to the nociceptive stimulus (shaded area), and the temporary structure of this stimulus is shown below; (c) electric circuit for attaching the stimulating electrodes to the caudal fin. See text for explanations.

vae). After the experiments, the fish were returned into the aquarium, where they continued normally swimming and feeding. No anesthetics or muscle relaxants were used in the experiments.

The tested fish was placed in a Plexiglas flowing water chamber of rectangular shape, 30 × 10 × 7 cm. Tap water from a tank where it had been left to settle over at least 24 hours was continuously flowing into the chamber in the course of the experiment via a pipe capped with a screwed connecting pipe of various size, depending on the size of the fish. When the fish was placed in the chamber, it tightly gripped the connecting pipe with its mouth, so that the pipe served as an additional fixation point. The area of pectoral fins was the principal area of fixation. This part of the body, including the fins, was wrapped in a moist cotton cloth and a foam rubber sleeve to protect the surface of the body from damage. The fish was then attached within the chamber to flat vertical holders by rubber bands, leaving the caudal peduncle free. The eyes of the fish were covered with moist cotton gauze to prevent any possible visual responses to movements of the experimenter. The water flowing into the mouth perfused the gills with the velocity of 150 ml/min. Water temperature was maintained within the range between 18 and 20°C (Fig. 1a).

Stimulating electrodes made of silver wire 0.3 mm in diameter were inserted into the tissue of the caudal fin, excluding the possibility of direct stimulation of muscle fibers. The nociceptive stimuli used were four successive series of brief electric impulses with a duration of 0.5 ms and a period of 3 ms. The amplitude of the impulses was measured in the course of the experiment within the range of 0.1–2.0 mA. The duration of each stimulating series was 0.7 s, with a period of the reretition the series of 1.4 s. The time pattern of the stimuli is shown in Fig. 1b.

The purpose of breaking the stimulus into four successive parts was to detect the possible motor responses caused by the direct effect of electric shock on muscles of the caudal part. Due to the small latent periods of muscle responses to electric stimulation, such contractions would take place four times, following each electric shock series. However, when the elec-

trodes were set correctly, no direct effects of electric shock on muscles were observed in our experiments.

Figure 1c shows the circuits of applying the stimulating electrodes to the caudal fin of the tested fish. The transformer provided galvanic isolation of the area of direct stimulation from the main body of the device. This isolation protected the fish from uncontrolled electric currents flowing through its body and the surrounding water. The choice of resistance values R_1 and R_2 was determined by the ratio $R_1 + R_2 > 10R_f$, where R_f is the resistance of the caudal fin in the tested fish over the area between stimulating electrodes 1 and 2.

The experimental device allowed recording the behavioral responses of the fish to the applied stimulus simultaneously with the process of nociceptive stimulation. The response was expressed in right or left deflections of the caudal peduncle (starting elements of the avoidance response). These movements deflected the wire "fork" gripping the caudal peduncle and attached to an optical-mechanical sensor (Fig. 1a). Information on the amplitude and duration of deflection, after converting the analog symbol into a digital code (ADC) proceeded to the memory of the computer and was visualized on the display. The transfer of commands into the hard part of the stimulator (the generator and amplifier of the stimulating signal) and the digital data were obtained from ACD by a double-byte input-output port controlled by our original program.

The initial testing was performed with a succession of stepwise ($\Delta I = 7.5$ μA) increasing values of stimulating current starting with clearly subthreshold values. The threshold was defined as the lowest value of impulse amplitude that produced small motor responses in the tested fish.

The threshold values were measured with intervals of 5 min over 1 h prior to the injection of the analgesic substance (agonist of opioid receptors) and over 1–2 h after the injection. The total duration of one experiment was 2–3 h and, in some cases when the pronounced effect of analgesia was prolonged, up to 4 h. The breathing frequency was simultaneously measured according to movements of the opercula.

Solutions of tramadol (μ-agonist of opioid receptors), DADLE (δ-agonist), and U-50488 (κ-agonist) were injected intramuscularly in 1 μl/g amount in concentrations 10, 30, 50, 80, and 100 nmol/g of body weight. The solvent and control solution used was 0.9% water solution of sodium chloride. In a series of six experiments, naloxone (universal antagonist of opioid receptors) was injected in the concentration of 100 nmol/g 1 h prior to the injection of the analgesic. In each experiment, the effect of only one analgesic was tested.

The degree of analgesia (A) was calculated as the decimal logarithm of the ratio between the nociceptive threshold values after the injection of the analgesic and prior to the injection: $A = \log(I_{th}/I_b)$, where I_{th} is the threshold value of stimulating current at successive moments after the injection of the analgesic and I_b is the average threshold value measured prior to the injection. Latent periods of nociceptive responses were calculated and the decimal logarithm of the ratio between the current latent period (L_i) and the background latent period (L_b) was calculated: $L = \log(L_i/L_b)$.

The significance of differences between recorded threshold values prior to the injection and after the injection was tested by the Wilcoxon nonparametric *U*-test.

RESULTS

Characteristics of the responses of fish to the pain stimuli. The behavior of fish in the experimental device usually remained stable over several hours. Rarely, the fish displayed spontaneous movements (one or two over 1 h): sometimes these were weak, low-amplitude undulations of the caudal peduncle; sometimes they were strong jerks, which faded over 1–5 s.

The nociceptive thresholds normally varied in particular individuals within a range of 10% ($p < 0.01$). The response usually started after the termination of the stimulating electric impulses, but sometimes it was observed simultaneously with the stimulus. The threshold stimulating value, latent period, and duration of responses to stimuli varied individually. Stronger stimulation led to increased amplitude and shorter latent periods of response (Figs. 2a, 2b).

Normally, the breathing frequency of the animals was 1.1 ± 0.1 s^{-1}. In response to threshold or suprathreshold stimuli, the frequency of opercular movements increased to 2.8 ± 0.7 s^{-1}. This increase lasted up to 4 s.

Injection with a solution of opioid receptor agonists tramadol, DADLE, or U-50488 in concentrations of 10, 30, 50, 80, and 100 nmol/g resulted in dose-dependent increases of the nociceptive threshold by a factor of 1.5–10.0, lasting over at least 1.5 h. The analgesic effect manifested itself in 5–15 min, depending on the dose (Fig. 2c). When the dose was increased, the rate of increase in the analgesia effect became higher. In graphs shown in Fig. 3, this dependence is visible in the increasing steepness of the growth trends during the period after the analgesic injection. The total analgesia duration is characterized by the length of the horizontal parts of the curves. In cases where tramadol was used in high concentrations (100 nmol/g), the total analgesia duration was up to 4 h.

In experiments where naloxone, an antagonist of opioid receptors, was injected prior to the opioid injection, the analgesic effect of tramadol was blocked or strongly reduced ($p < 0.05$). In control experiments, injections of pure solvent (0.9% NaCl) had no effect

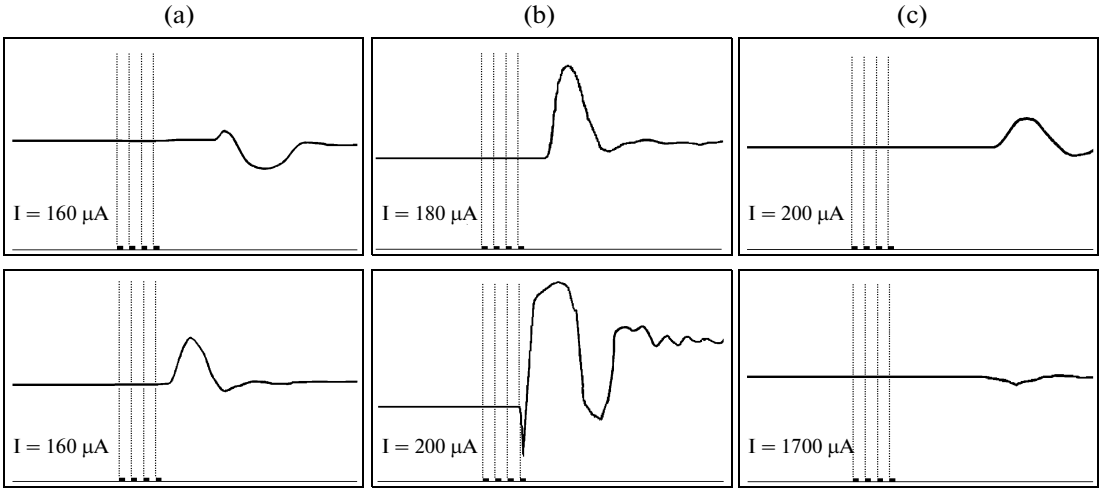

Fig. 2. Oscillograms of responses to nociceptive stimuli: (a) threshold response and (b) suprathreshold response under normal conditions (above and below); (c) response after injection of the analgesic tramadol in concentrations of 10 nmol/g (above) and 100 nmol/g (below). All data shown in this figure were obtained in the course of one experiment.

on response thresholds and characteristics of responses.

Latent periods of nociceptive responses after the injection of the opioid varied depending on the concentration and nature of the injected substance (Fig. 4). Tramadol and U-50488 led to a decrease in the duration of latent periods as early as 10 s after the injection. DADLE led to a decrease in the duration of latent periods during the first 10 s ($p < 0.05$) and an increase after 20 s ($p < 0.05$).

The tested fish individuals released in the aquarium after the experiments demonstrated normal swimming and usual behavior.

DISCUSSION

The last few years have been marked by considerably growing interest among specialists in the search for methods of studying pain responses in lower vertebrates, including in fish (Chervova et al., 1992, 1994; Chervova, 1997; Chervova and Lapshin, 2000; Sneddon, 2003a; Stevens, 2008; Roques et al., 2010; Correia et al., 2011). The principal difficulty of this search is developing adequate methodology that would allow dosing the pain stimulus and, thus, recording the response to changes in the degree of stimulus. The

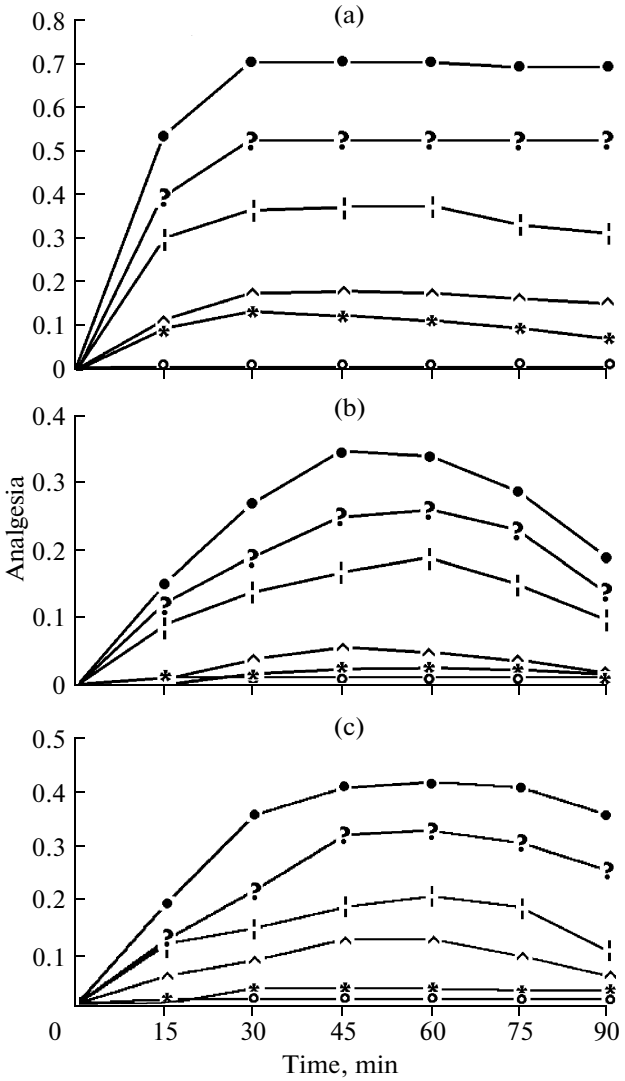

Fig. 3. Changes in pain sensitivity thresholds in the common carp (*Cyprinus carpio*) after injection of agonists of μ-, δ-, and κ-opioid receptors: (a) tramadol, (b) DADLE, (c) U-50488. Abscissa shows the time after injection (min); ordinate shows the degree of analgesia A = $\log(I_{th}/I_b)$. Concentrations of analgetics, nmol/g: (–•–) 100, (–?–) 80, (–¦–) 50, (–^–) 30, (–*–) 10, (–○–) control.

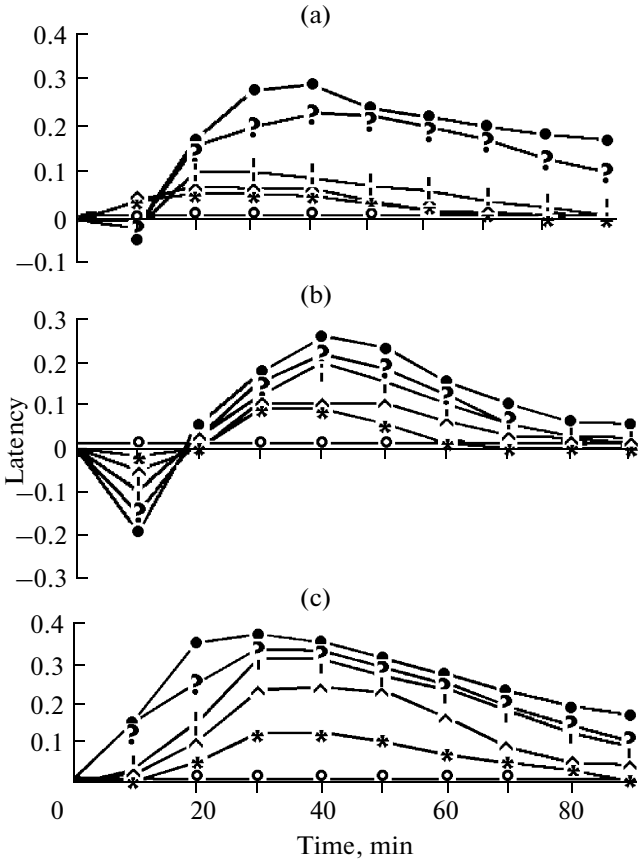

Fig. 4. Changes in the latent period of response to pain stimulation in the common carp (*Cyprinus carpio*) after injection of agonists of μ-, δ-, and κ-opioid receptors: (a) tramadol, (b) DADLE, (c) U-50488. Notation as in Fig. 3.

method proposed here is especially close to the solution of this problem.

We have earlier shown that fish are not only capable of sensing pain stimuli but display behavioral responses to pain similar to those displayed by mammals (Chervova et al., 1992, 1994; Chervova, 1997). In experimental studies of higher vertebrates, the estimated behavioral responses to nociceptive stimuli include alerting, starting, limb withdrawal, tail withdrawal, avoidance, attacking the source of pain, etc. These are components of more complex defensive and aggressive behaviors (Charpentier, 1968).

Swimming is the principal functional parameter of fish, and in many species it is realized mainly by lateral movements of the body, in which the caudal fin plays an important role. In our method, a discrete behavioral act was selected as the estimating measure: deflection of the caudal peduncle, the initial element of the avoidance response.

In our search for adequate methodology, we tested different ways of pain stimulation. In response to pricking with a steel needle of points above the upper lip fold, in periorbital and perinasal areas, where trigeminal nerve endings are present (Chervova, 1985), we observed undulations and sharp strokes of the caudal peduncle, as well as increasing breathing frequency (Chervova et al., 1994). However, these responses were not distinguished by stability. Repeated pricks, even stronger ones, sometimes caused no motor response. Difficulties with dosage of mechanical stimuli and low stability of behavioral responses to such stimuli in fish spurred us to developing a method of electric nociceptive stimulation of the nerve endings in the caudal fin. This approach allows regulation of the degree of the stimulus and, as our experiments have shown, obtaining stable responses.

The fish caudal fin has a great number of nerve endings. Sections of carp caudal fins revealed nerve tracts both within the lepidotrichia segment and in soft tissues (hypodermis) between the rays of the fin. Morphometric analysis revealed four categories of axons: three types of myelinated fibers and one type of nonmyelinated C fibers. The fibers were analyzed according to their diameters and identified as C, A-δ, A-β, and A-α types. C and A-δ comprised 38.7% of the fibers (Roques et al, 2010). These two fiber types are known in higher vertebrates as typical conductors of nociceptive signals (Lynn, 1994).

We did not use anesthetics in the course of our experiments for two reasons: first, they could distort the responses to the analgesics tested, and second, in our device, water was irrigating the gills at a rate of approximately 150 ml/min, creating favorable breathing conditions for the fish.

The rising thresholds of pain sensitivity caused by the tested opioid analgesics tramadol, DADLE, and U-50488 are probably determined by processes that take place in central structures. In mammals, responses to pain are regulated by the limbic system. In the diencephalon of zebrafish (*Brachydanio rerio*), diencephalic dopaminergic neurons, homologous to the mesolimbic dopaminergic system of mammals, mediated the effects of morphine. Morphine had a rewarding effect on zebrafish. This effect, as in mammals, was observed at early stages of development and could be lifted by naloxone, the nonspecific antagonist of opioid receptors. Furthermore, mutant zebrafish lacking diencephalic dopaminergic neurons were insensitive to the rewarding effect of morphine (Guo, 2004).

In our experiments, after the injection of the analgesic, shortening of the latent period over the first 5–15 min and hyperactivity of the tested fishes were observed. It has also been shown in experiments with land vertebrates that many narcotics can cause hyperactive motor response after acute injection. Similar hyperactive behavior is triggered by the dopamine system of the brain, which mediates the rewarding effect

of narcotics (Koob et al., 1998). The central action of the substances tested in our experiments is also indicated by the long (0.5 s and more) latent period of response to near-threshold stimuli.

The analgesic tramadol used in our experiments belongs to opioids, being a synthetic analog of codeine, an alkaloid of the phenanthrene group, but, unlike morphine and other opioids, it is widely used in current medical practice. Tramadol can provide an alternative to other analgesics that can be used for acute and chronic pain relief in animals. It is known that this drug can be used in veterinary practice with various mammals, birds, reptiles, and amphibians (Souza and Cox, 2011). We have shown that tramadol is also efficient as an analgesic for fish, as already reported earlier (Chervova and Lapshin, 2000). It can be recommended for usage in piscicultural and experimental practice.

The reported evidence that in fish, as in higher vertebrates, agonists of μ-opioid receptors also have a prolonged analgesic effect confirm the existence of a fish antinociceptive system, represented, as in land vertebrates, mainly by opioid receptors. The principal function of μ-opioid receptors in mammals is mediating analgesic effects. Our data show that these receptors play the same role in fish, raising the threshold of pain sensitivity. Thus, it can be assumed that the opioid antinociceptive system emerged at early stages of vertebrate evolution. In bony fish, it already has the same structural and functional features as in recent mammals.

The notions about pain perception in fish vary within a wide range of opinions, from complete denial to asserting the existence of conscious pain perception in fish (Rose, 2002; Sneddon, 2003b; Sneddon et al., 2003; Chandroo et al., 2004). The results of our study allow us to maintain that fish perceive pain stimuli. The development of efficient anesthetic measures for scientific experiments on fishes or industrial procedures, such as obtaining reproductive products, is quite an urgent challenge. The method of anesthesia and measures for controlling its efficiency proposed here contribute to solving this problem, in both its humanitarian and practical aspects.

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