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Physiological parameters of Amur carps *Cyprinus rubrofuscus* (Lacépède 1803) under anesthesia following painful stimulus

Parámetros fisiológicos de carpas de Amur *Cyprinus rubrofuscus* (Lacépède 1803) bajo anestesia después de un estímulo doloroso

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ABSTRACT | The aim of this trial was to evaluate heart rate, opercular frequency, blood parameters and cortisol levels in Amur carps (*Cyprinus rubrofuscus*) anesthetized in benzocaine-based solutions at concentrations of 70, 100 and 130 mg/L (B70, B100 and B130) or eugenol-based solutions at concentrations of 20, 40 and 60 mg/L (E20, E40 and E60) following caudal fin pinching. A control group was handled without anesthesia. Bath anesthesia until the carps reached anesthetic plane was performed, followed by caudal fin pinching for one minute. It was recorded induction and recovery times, heart and opercular rates before and after fin pinching under anesthesia, and blood parameters, blood glucose and cortisol levels after fin pinching. E20 Amur carps only reached the sedation stage, with E40 and E60 groups showing motor response during caudal fin pinching, and higher blood glucose levels in E20 and E60 carps. Still, Amur carps anesthetized with benzocaine had no heart rate alteration after fin pinching, but in B130 higher glucose levels and elevated neutrophil counting in B70 carps were detected. Moreover, plasma cortisol levels were higher in B100 and E60 carps, with no difference between control and the experimental groups. In this assay, physiological parameters analyses suggest a more effective anesthesia when using 100 mg/L benzocaine with artificial ventilation. Eugenol should be avoided in Amur carps since it does not promote muscle relaxation.

Palabras clave
Anestesia
Pellizco de aletas
Parámetros fisiológicos
Cortisol

RESUMEN | El objetivo de este ensayo fue evaluar la frecuencia cardíaca, frecuencia opercular, parámetros sanguíneos y niveles de cortisol en carpas de Amur (*Cyprinus rubrofuscus*) anestesiadas en soluciones a base de benzocaína en concentraciones de 70, 100 y 130 mg/L (B70, B100 y B130) o soluciones a base de eugenol en concentraciones de 20, 40 y 60 mg/L (E20, E40 y E60) tras pellizco de la aleta caudal. El grupo control fue manejado sin anestesia. Se realizó anestesia en baño hasta que las carpas alcanzaron el plano anestésico, seguido de pellizco de la aleta caudal durante un minuto. Se registraron los tiempos de inducción y recuperación, frecuencia cardíaca y opercular antes y después del pellizco de las aletas bajo anestesia, y parámetros sanguíneos, niveles de glucosa y cortisol después del pellizco de las aletas. Las carpas del tratamiento E20 solo alcanzaron la etapa de sedación, y los grupos E40 y E60 mostraron una respuesta motora durante el pellizco de la aleta caudal, y niveles más altos de glucosa en sangre en las carpas E20 y E60. Aun así, las carpas de Amur anestesiadas con benzocaína no tuvieron alteración de la frecuencia cardíaca después del pellizco de las aletas, pero en las carpas B130 se detectaron niveles más altos de glucosa y un recuento elevado de neutrófilos en las carpas B70. Además, los niveles de cortisol en plasma fueron más altos en las carpas B100 y E60, sin diferencias entre los grupos de control y experimentales. En este ensayo, los análisis de parámetros fisiológicos sugieren una anestesia más eficaz cuando se utilizan 100 mg/L de benzocaína con ventilación artificial. Se debe evitar el eugenol ya que no promueve la relajación muscular.

INTRODUCTION

After more than 100 years of the Charles Scott Sherrington's publications about pain and nociception in humans (Sherrington 1900, 1906), studies into pain hampering and nociception comprehension are under continuous progress for small animals (Monteiro *et al*. 2023). The International Association for the Study of Pain describes pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (IASP 2011). The physiological component of pain is termed nociception, which consists of the processes of transduction, transmission, and modulation of neural signals generated in response to an external noxious stimulus (Lamont *et al*. 2000). On a complex level, this pathway

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involves a network of branches and communications with other sensory neurons and descending inhibitory neurons from the midbrain that modulate afferent transmission of painful stimuli (Lamont *et al*. 2000).

In turn, pain in fish is an up-to-date issue with conflicting hypotheses postulating that fish only react unconsciously against painful stimulus (Key 2015, Rose *et al*. 2014) but, in an opposite way of thinking, others argue that fish really feel pain and their responses are consciously orchestrated (Barbas *et al*. 2021, Braithwaite and Boulcott 2007, Chandroo *et al*. 2004, Sneddon *et al*. 2003 a,b, Yue *et al*. 2004, 2008). Even though nociception in fish is still in debate (Hart 2023), pain avoidance, stress and distress must be managed to improve fish recovery after medical procedures (Weber, 2011). Briefly, stress occurs when an animal's equilibrium is changed by either external or internal factors (Carroll 1999). Such environmental or biological imbalance may lead to distress, when an animal is unable to respond to stress (Carroll 1999). As in mammals, cortisol releasing is the first response to a stressful situation with positive and negative consequences along time (Tort 2011). Thus, monitoring of ordinary clinical signs of distress include subjective assessments of fish behavior (*e.g.* lethargy and feeding) and more accurate physiological/blood parameters (Weber 2011).

To avoid painful consequences in fishes under ordinary aquaculture practices, such as handling, transport and fishing, they are anesthetized with a range of natural and synthetic compounds in immersion bath anesthesia. The most common agents applied in Brazil are benzocaine and eugenol (Barbas *et al*. 2021, Jensch-Junior *et al*. 2005) to induce muscle relaxation, analgesia and a final unconscious stage (Martins *et al*. 2019, Zahl *et al*., 2012). However, data about physiological and molecular parameters in fish under anesthesia by both drugs at concentrations reported for aquaculture procedures and experiencing pain are lacking. Thus, to address fish pain physiological parameters and anesthesia, Amur carps *Cyprinus rubrofuscus* were anesthetized with different concentrations of benzocaine and eugenol, and plasma cortisol levels and blood parameters after painful stimulus were evaluated.

MATERIAL AND METHODS

Animal maintenance

Fifty-six juvenile female Amur carps (27.21 ± 2.95 g, $P = 0.12$) were purchased from Piscicultura Dinamarca (São Paulo, Brazil) and kept in a 500 L aquarium at the Aquatic Animals facility of the ICB/USP. Amur carps were fed omnivorous fish chow (360-AM, AMICIL S/A, Brazil) once a day until apparent satiety. During thirty-day acclimation, water physicochemical parameters were daily evaluated: temperature (19.79 ± 1.34 ºC), pH (7.47 ± 0.09), total and toxic ammonia (0.93 ± 1.10 mg/L and 0.0015 ± 0.0021 mg/L, respectively), nitrite (1.32 ± 0.75 mg/L) and dissolved oxygen concentrations (10.52 ± 1.10 mg/L) using rapid test kits (Alcon Pet®, Brazil).

Amur carps anesthesia and blood collection

For Amur carps anesthesia with benzocaine (Benzocaine, Sigma-Aldrich, United States), anesthetic solutions at concentrations of 70, 100 and 130 mg/L were prepared – groups B70, B100 and B130 (Antunes *et al*., 2008; Mohamed, 1999). Anesthesia with eugenol (Eugenol, Biodinâmica, Brazil) was performed with anesthetic baths at concentrations of 20, 40 and 60 mg/L – groups E20, E40 and E60 (Hikasa *et al*., 1986; Xu *et al*., 2023). All anesthetics were dissolved 1:5 (v/v) in 100% ethanol before their addition in water for fish anesthesia. A plastic box containing the anesthetic solution was kept with constant aeration and a stainless-steel grid lid (a rodent cage) was used. A second box with constant aeration was kept nearby to evaluate the recovery parameters after anesthesia. Amur carps of the control group were not anesthetized, but only handled to verify whether the consequences were due to stress or pain.

Each experimental group of Amur carps (n=8) was anesthetized in the respective bath kept at 19-20 ºC during all trial time, and the induction times of each carp was measured. Once the deep anesthesia plane was reached, characterized by total loss of muscle tone, total loss of balance and almost no ventilation (Stage II and Plane 2 anesthetic plane, as reported by Ross and Ross, 2008), each carp was removed from the anesthetic solution and placed on the grated lid. For continuous fish oxygenation and anesthesia, a hose connected to a submersible

aquarium pump into the anesthetic solution was inserted orally, with a flow rate of 300 L/h. Visual assessment of respiratory frequency was performed by counting the opercular movements in one minute (opercular frequency), and heart rate was performed with a vascular Doppler device (Doppler Vascular DV610, MedMega Indústria de Equipamentos Médicos, Brazil). To pinch the caudal fin, rat tooth forceps was set between the caudal fin and the anus. The forceps were pressed at maximum intensity with handgrip strength around 50-55 kg (Schlüssel *et al*., 2008) for one minute (Fig. 1a). Opercular frequency and heart rate (Fig. 1b) were measured again after the painful stimulus.

Dorsal vessels were punctured using a 13 x 0.38 mm (27.5G x 1.2") needles coupled to 1 mL syringes for blood collection (Fig. 1c). Previously, needles and syringes were internally coated with a 3% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, USA) dissolved in 0.65% NaCl saline solution to avoid blood clogging. A second syringe without anticoagulant was set for whole blood acquisition. Around 100 µL of blood were collected in each syringe per animal. Blood samples from the control group were collected without anesthesia.

Finally, each carp was weighed and relocated to a recovery box with constant water oxygenation and the recovery time was measured. Post-painful stimulus analgesia was performed with 0.15 mL lidocaine hydrochloride (Lidocaína 1%, Eurofarma, Brazil) IM administration at a concentration of 5 mg/mL (Chatigny *et al*., 2018). The Amur carps returned to the 500 L aquaria and were evaluated for 72 hours to observe post-painful signs and behavior (Harms *et al*., 2005).

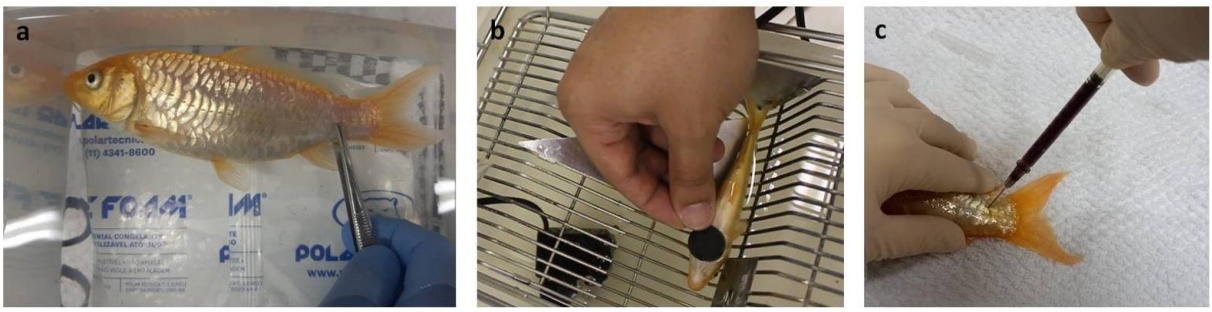

Figure 1. Experimental procedures undergone in anesthetized Amur carps. **a**, caudal fin pinching in an Amur carp *Cyprinus rubrofuscus* specimen after reaching the deep anesthetic plane; **b**, heart rate record using a vascular Doppler device; **c**, blood collection by dorsal vessels puncture.

Hematological analysis

Blood samples were used in the determination of blood glucose, hematocrit, blood smears preparation and samples for liquid nitrogen storage. Blood glucose was set using a portable blood glucose monitor kit with disposable strips (Accu-Chek Active, Roche, Germany) (Crosby *et al*. 2010). For hematocrit, microhematocrit glass capillary tubes were centrifuged at 700 x g for 1-2 min in a microhematocrit centrifuge (Microcentrífuga MH, SISLAB Ltda., Brazil). Blood droplets were used for blood smears preparation, which were stored until the stain.

Hemoglobin concentration was set using the cyanmethemoglobin method and each sample was read in a spectrophotometer (Biophotometer Eppendorf v.1.35, Eppendorf AG, Germany).

Two microliters of blood were further diluted in 398 µL of 0.65% NaCl saline solution for total blood cell count in a Neubauer's chamber at 400X magnification. Thus, the total number of erythrocytes was calculated by multiplying the total number of cells recorded using a Neubauer's chamber by 10,000 (number of cells/µL). Blood smears were stained using a Diff-Quick kit (Kit Panótico, Laborclin Produtos para Laboratórios, Brazil) and the total leukocyte number and differential leukocyte counting among 1000 erythrocytes was performed using an oil immersion light microscope objective at 1000X magnification. White blood cells were classified and

the actual concentration of each type of leukocyte was calculated as the proportion of the number of cells among 1000 erythrocytes and the erythrocyte concentration found in the Neubauer chamber (leucocyte type/µL).

Data on hematocrit, hemoglobin and number of erythrocytes were used to calculate the hematimetric values, such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) according to described by Tripathi *et al*. (2004).

Blood plasma cortisol analysis

Blood samples collected with anticoagulant were centrifuged at 700 x g for 3 minutes at 4 ºC (Universal 32R centrifuge, Andreas Hettich GmbH & Co KG, Germany) for blood plasma acquisition. After centrifugation, plasma from each sample was collected and packaged in microtubes, which were stored at -20 ºC for the determination of cortisol levels using ELISA tests.

Blood plasma cortisol levels were determined using a commercial microplate ELISA kit (Cortisol ELISA Kit # 500360, Cayman Chemical, USA), following the manufacturer's instructions. The microplate was read on a microplate spectrophotometer (SpectraMax 190, Molecular Devices, USA) under light wavelength of 405 nm after 120 min of development. Soft Max Pro 4.8 software (Molecular Devices, USA) was used to acquire the blood plasma cortisol levels expressed in ng/mL.

Statistical analysis

All data sets registered during Amur carps anesthesia were evaluated by normality test of Kolmogorov-Smirnov followed by parametric (ANOVA with Tukey's post-hoc test) and non-parametric tests of Kruskal-Wallis (*H* distribution) with Dunn's post-hoc test in GraphPad Prism 6 statistical packages (GraphPad Software Inc., USA). The results were expressed with a 95% confidence interval (α 0.05) as mean ± standard deviation.

Since blood plasma cortisol values did not show Gaussian distribution, the Grubb's test was used to remove outliers and data were evaluated by the Kruskal-Wallis non-parametric test (α 0.05). Blood plasma cortisol values are presented as mean ± mean standard error.

RESULTS

Physiological parameters related to anesthesia and fin pinching

Data on respiratory frequency, heart rate and blood glucose levels were measured during the carp anesthesia and are presented in Table 1.

During the anesthesia with eugenol solutions, Amur carps of the E20 group reached only the tranquilization stage after 15 minutes in the anesthetic solution and this group was excluded from induction and recovery time sets. Regarding induction times, they were affected by both the type of anesthetic and their respective concentrations ($H_{(4)}$ = 16.39, P<0.01). Groups B70, B100, B130 and E60 showed similar values, and E40 revealed the highest average value for induction time (465.12 ± 238.69 s; Dunn's test, B70 vs. E40 P<0.05; B100 vs. E40 P<0.01; B130 vs. E40 P<0.01; E40 vs. E60 P<0.05). Recovery times were longer ($H_{(4)}$ = 3.38, P = 0.5) showing a synergy among inhalation anesthetics and lidocaine hydrochloride via IM (Table 1).

It was also observed that E40 and E60 carps showed motor response during the painful stimulus application and increased heart rates after stimulus (Table 1). Amur carps anesthetized in benzocaine-based solutions did not change heart rate after caudal fin clamping, but Amur carp anesthetized in eugenol solutions showed significantly higher heart rate values (Tukey's test, P<0.0001). Respiratory rate values showed no significant differences among treatments (Tukey's test, P=0.23). The post-anesthesia evaluation revealed that all Amur carps treated with lidocaine hydrochloride showed appetite after the first 24 hours. In the control group, carps were lethargic at the bottom of the aquarium with no apparent satiety after 24 hours, but appetite returned after the first 48 hours.

Hematological analysis

During the differential leukocyte count, it was possible to distinguish eight cell types in carp blood: erythrocytes, erythroblasts, thrombocytes, lymphocytes, neutrophils, monocytes, eosinophils and basophils (Tripathi *et al*., 2004) (Fig. 2). Hematological results erythrocyte and leukocyte counts (excluding thrombocytes and erythroblasts) are shown in Table 2. The hematological analysis of the B70 group revealed a nonspecific response to an irritating agent detected as higher leukocyte counting, mainly neutrophils ($H_{(6)}$ = 17.96, P<0.01) (Table 2). Indeed, higher values for monocytes ($H_{(6)}$ = 42.83, P<0.001) and eosinophils ($H_{(6)}$ = 21.86, P<0.01) as seen among all groups contributed to leukocytosis in B70 carps as well.

Hematological parameters did not show significant differences among treatments except in MCHC values and blood glucose level. MCHC values in B100 treatment showed significantly lower values than the rest of the treatments ($H_{(6)}$ = 18.40, P<0.01) (Table 3). Anesthesia with benzocaine and eugenol led to altered blood glucose levels. In the case of benzocaine, B130 carps showed higher blood glucose levels compared to B70 and B100, with B100 significantly lower than control group (Dunn's test, P<0.05). However, Amur carps anesthetized with eugenol showed a distinct response, with mean glucose levels higher in E20 and E60 groups than that found in E40 carps when compared to control group (Tukey's test, P<0.05) (Table 3). As seen in carps anesthetized with the lower benzocaine concentration, E20 carps showed a similar response with higher red blood cell counting ($H_{(6)}$ = 9.68, P = 0.13) and higher monocyte counting among eugenol groups (Table 2).

Table 1. Physiological parameters measured before and after caudal fin pinching in Amur carps *Cyprinus rubrofuscus* anesthetized with benzocaine and eugenol-based solutions. Data are shown as mean±standard deviation (95% confidence interval).

Physiological parameter	Control+	B70	B100	B130	E20‡	E40	E60
Induction time (s)	---------	165.5±64.15a	157.37±60.51a	153.12±41.77a	----------	465.12±238.69b	162.62±48.37a
Recovery time (s)	---------	1961±1280a	2234.87±1000.36b	1574.37±566.87a	857.12±898.74c	1937.87±676.67d	1619.6212±737.86a
Heart rate before pinching (bpm)	104±23.49	94±21.49	91.5±25.52	73.75±34.89	99.50±6.21	104.5±9.66	108.5±11.40
Heart rate after pinching (bpm)	---------	84±21.80a	69.50±27.37a	63±14.77a	----------	102.5±11.69b	108.5±12.36c
Opercular frequency before pinching	104.5±11.2	96.5±25.24	103.25±23.32	101.75±26.26	77.5±21.58	99±12.96	118.75±9.96
Opercular frequency after pinching	---------	100.5±36.99	110.25±26.08	103±20.02	----------	72±14.81	91±19.47

OBS: In lines, different letters indicate statistical differences among groups by nonparametric ANOVA test (Kruskal-Wallis) with Dunn's post-hoc test, P<0.05.
Legends: + Control group was not anesthetized or undergoing fin pinching.
‡ E20 group only reached anesthetic tranquilization stage after 15 minutes and Amur carps were not pinched.

Table 2. Erythrogram and leukogram of Amur carps *Cyprinus rubrofuscus* after caudal fin pinching. Data are shown as mean±standard deviation.

Groups	Erythrocytes (10^6/µL)	Total Leukocytes (10^3/µL)	Lymphocytes (10^3/µL)	Neutrophils (10^3/µL)	Monocytes (10^3/µL)	Eosinophils (10^3/µL)	Basophils (10^3/µL)
Control	1.93±0.88a	52.7±19.28a	34.16±19.75a	17.54±8.14a	0.31±0.08a	0.56±0.97a	0.15±0.45a
B70	2.26±0.89a	70.78±46.28a	27.81±22.74a	31.76±22.81a	7.58±3.85b	2.9±1.35b	0.71±1.32a
B100	1.33±0.36a	34.1±21.1a	21.01±16.59a	8.08±5.87a	8.08±5.87a	1.27±0.35a	0.45±0.63a
B130	1.85±0.76a	52.95±58.39a	35.47±39.29a	12.77±20.88a	3.29±3.16a	1.17±0.78a	0.16±0.46a
E20	2.01±0.53a	44.91±17.37a	27.95±16.59a	12.39±4.64a	2.75±3.03a	2.05±1.5a	0.20±0.57a
E40	1.81±0.37a	42.25±12.56a	28.16±14.46a	10.60±3.75a	2.30±2.82a	1.8±0.36a	0.88±0.99a
E60	1.82±0.57a	43.73±21.19a	23.31±14.46a	18.28±21.1a	0.8±1.30a	1.31±0.98a	0.14±0.41a

OBS: In columns, different letters indicate statistical difference among groups analyzed by nonparametric ANOVA test (Kruskal-Wallis) with Dunn's post-hoc test, P<0.05.

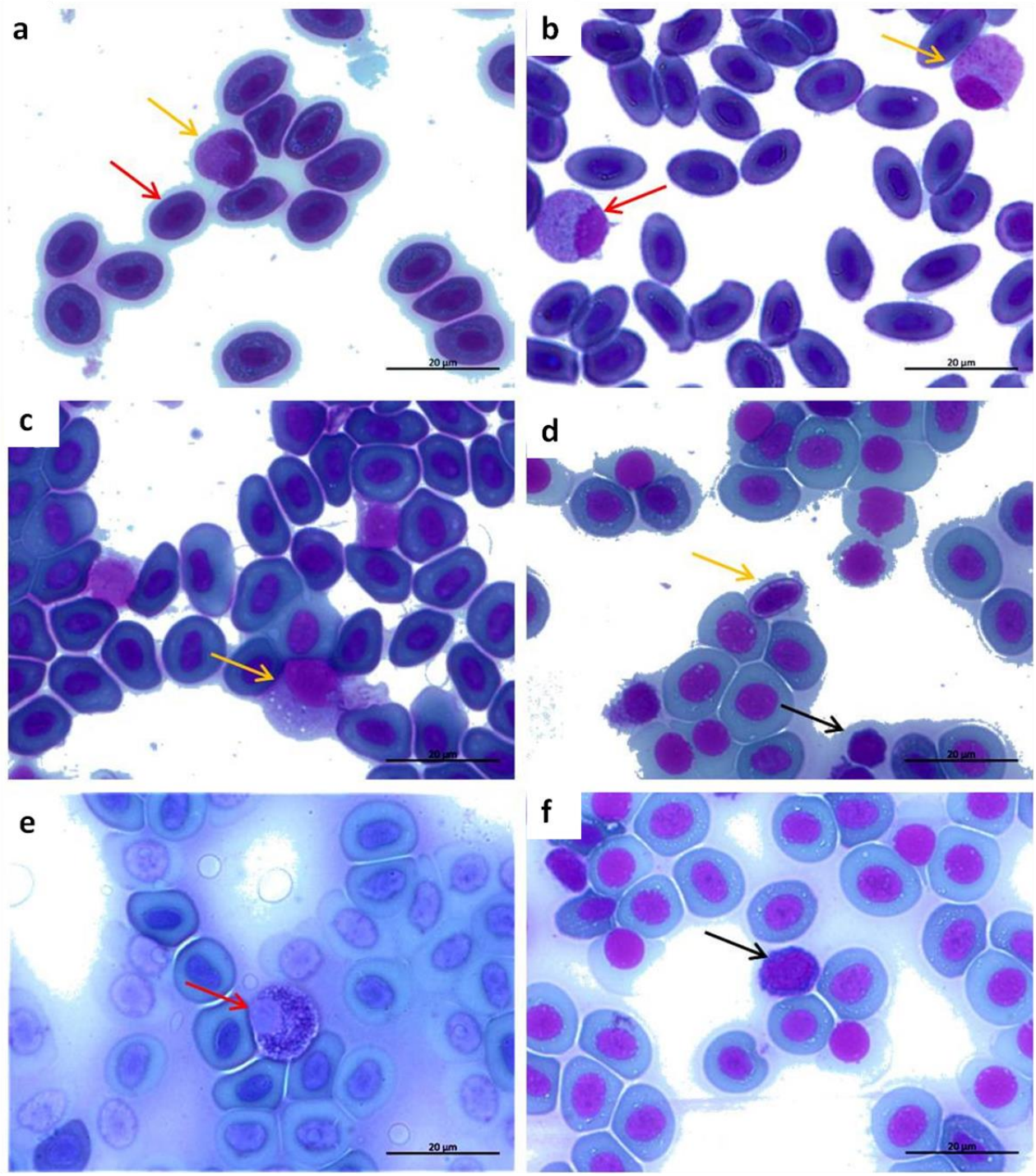

Figure 2. Blood cells of Amur carps *Cyprinus rubrofuscus*. **a**, bilobed neutrophil (orange arrow) and erythrocyte (red arrow). **b**, eosinophil (orange arrow) and neutrophil (red arrow). **c**, monocyte (orange arrow). Observe the presence of cytoplasmic vacuoles. **d**, thrombocyte (orange arrow) and lymphocyte (black arrow). **e**, basophil. **f,** erythroblast. Diff-quick dye technique. Immersion oil objective, 1000X magnification. Bars – 20 µm.

Table 3. Hematological parameters of Amur carps *Cyprinus rubrofuscus* after caudal fin pinching. Data are shown as mean±standard deviation.

Groups	Hematocrit (%)	Hemoglobin (g/dL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Blood glucose (mg/dL)
Control	24.62±7.78a	6.74±2.48a	141.64±57.79a	37.68±15.26a	27.29±7.67a	192.37±95.93a
B70	25.31±5.49a	6.25±1.94a	137.31±76.51a	30.26±9.18a	24.88±7.37a	84.12±51.61b
B100	33.0±11.67a	5.81±2.47a	251.51±91.83a	44.50±20.33a	17.66±4.19b	80.25±49.83b
B130	37.5±6.25a	7.0±1.07a	223.63±72.09a	41.92±13.35a	18.86±2.39a	158.37±65.99a
E20	28.9±10.02a	7.0±2.37a	149.64±66.55a	35.07±9.46a	24.64±5.18a	102.5±70.22a
E40	32.12±7.0a	8.48±0.61a	181.63±42.77a	48.26±9.86a	27.77±7.84a	79.5±26.26b
E60	30.93±9.38a	6.98±1.26a	186.48±75.99a	41.76±13.86a	23.51±3.93a	134.25±52.80a

OBS: In columns, different letters indicate statistical difference among groups analyzed by nonparametric ANOVA test (Kruskal-Wallis) with Dunn's post-hoc test, $P<0.05$.

Blood plasma cortisol after fin pinching

Blood plasma cortisol was not significantly affected by fin pinching in Amur carps under anesthesia ($H_{(6)}$ = 3.44, P = 0.75) (Fig. 3). Blood plasma cortisol levels had a tendency to an increase in B100 and E20 treatments when compared to the concentrations found in carps from the control group without prior anesthesia (87.57 ± 28.42 and 36.10 ± 9.13 ng/mL vs 68.68 ± 23.79 ng/mL, respectively). Lower values for cortisol levels were detected in B70 and E40 Amur carps (59.69 ± 20.39 and 25.83 ± 9.15 ng/mL, respectively).

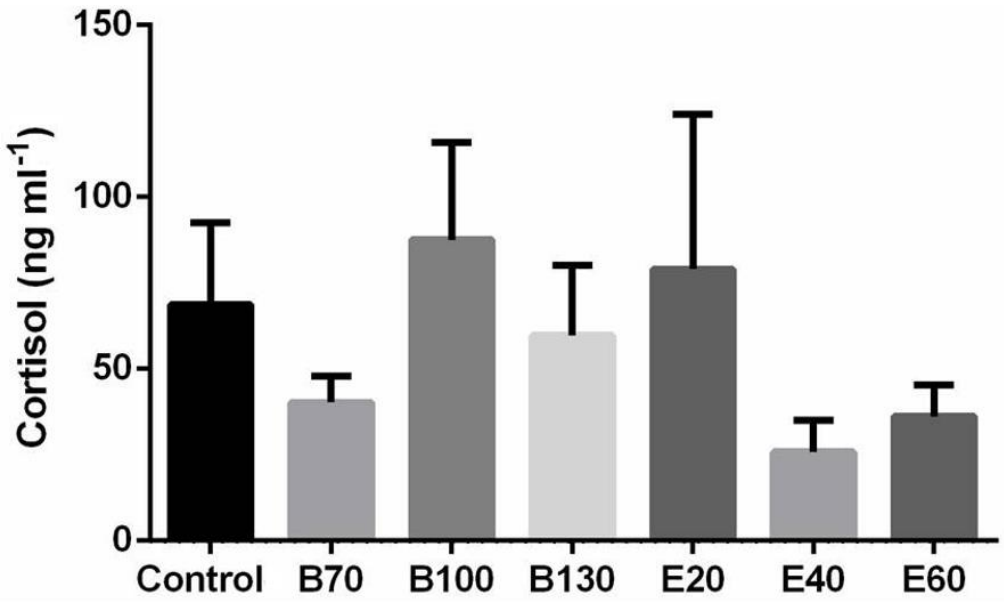

Figure 3. Plasma cortisol levels of Amur carps *Cyprinus rubrofuscus* in bath anesthesia using benzocaine and eugenol. There was no statistical difference among anesthetized groups and control non-anesthetized carps. Data shown as mean ± mean standard error.

DISCUSSION

Here we reported the physiological and biochemical parameters in Amur carps *Cyprinus rubrofuscus* anesthetized with benzocaine- and eugenol-based solutions following fin pinching.

Anesthesia with benzocaine and eugenol showed that intermediate concentration treatments of both anesthetics have increased the recovery times for Amur carps, corroborating with the results reported by Ferreira *et al*. (1984a) and Hikasa *et al*. (1986) for common carps *C. carpio*. The recovery time is directly influenced by physiological factors of fishes such as water temperature, gill perfusion, anesthetic absorption and intrinsic chemical factors related to anesthetic molecular characteristics (Ross and Ross 1999). However, higher concentrations may lead to consequences as seen in this trial, with lower recovery times than when the

recommended concentration is used for fish anesthesia. For benzocaine, higher concentrations can lead to gills' irritation due to their acid pattern (Ferreira *et al.* 1979), and rapid lipophilic eugenol absorption can lead to side effects as seen in *Anguilla anguilla* such as elevated red blood cell count and high hematocrit (Altun *et al*. 2006) as seen in this trial with Amur carps. It can be interpreted as indirect cortisol releasing consequences in fish (Zahl *et al*. 2012). Amur carps had longer recovery times than expected, suggesting a synergistic effect produced by the anesthesia and the post-analgesic treatment. The combination anesthesia improves the anesthetic effect as the drugs can act in different receptors or pathways and, in our case, it was detected with prolonged recovery times as reported by others (Zahl *et al.* 2012). Side effects were reported by other authors using analgesic agents after surgical procedures in fishes, such as nonsteroidal anti-inflamatories and opioids (Baker *et al*. 2013; Harms *et al*. 2005; Sneddon 2012). In our case, using lidocaine hydrochloride led to positive fish behavior, with appetite returning within 24 h. Corroborating with Weber (2011), pain medication in fish is a paramount action to avoid distress signals such as inappetence or anorexia in compromised fish. Stress and anorexia have straight relationship in fish (Bernier 2006) since distressed ones are more likely to illness (Roberts *et al*. 2009). Thereby, adjustments are necessary in lidocaine hydrochloride concentration and volume for future use in Amur carps.

Blood analysis showed variations in few hematological parameters, such as the increased leukocyte, lymphocyte, neutrophil and monocyte counting, increase in hematocrit and MCHC when compared to the control group. It can be seen as an irritation/acute inflammation response upon contact with xenobiotics. Adibi *et al*. (2024) showed that an environmental factor such as diluted ammonia levels is a bias for hematological parameters. In this trial, anesthesia followed by painful stimulus triggered a physiological response characterized as elevated lymphocyte, neutrophil and monocyte cell counting, which can be interpreted as leukocytosis when compared to the control group and with the reference intervals for common carps anesthetized with benzocaine (Ferreira *et al.* 1981) and clove oil (Velisek *et al*. 2005), and koi carps anesthetized with MS-222 (Tripathi *et al*. 2004). Yet, similar relationship between blood parameters higher values and low benzocaine and eugenol concentrations detected in Amur carps was observed in common carps anesthetized with benzocaine (Ferreira *et al*. 1981). However, Amur carps anesthetized with eugenol had less fluctuation on hematological parameters than carps anesthetized with benzocaine in relation to the control group, which suggests a greater fish tolerance possibly due to the former lipophilic profile and an irritating action of the latter on the gills (Ferreira *et al.* 1981). Those blood factors can be faced as side effects when using benzocaine and eugenol for fish anesthesia (Sneddon 2012) for routine procedures in fish farming.

Blood glucose levels also had a relationship to the concentrations of benzocaine and eugenol. Here, intermediate concentrations of both anesthetics led to the lowest blood glucose values as a consequence of cortisol release hampering. The intermediate concentrations used in this trial also had similar results in common carps anesthetized with benzocaine by Ferreira *et al*. (1980). In the case of anesthesia with benzocaine and fin pinching, B130 and E60 groups showed higher blood glucose compared to the other treatments, similar to reported by Heo and Shin (2010) for goldfish *Carassius auratus* anesthetized with benzocaine. Therefore, elevated hematocrit, erythrocyte count and blood glucose values may be indirectly interpreted as a consequence of the stress caused by the hypoxia of deep anesthesia plus the inducible fin pinching stressful condition (Kiessling *et al*. 2009; Wendelaar Bonga 1997).

Here, artificial ventilation was kept with a continuous oxygenation of the anesthetic solutions throughout the trial and its pumping to the gills using a cannula in the mouth and its contribution to the stress consequences cannot be ignored (Kiessling *et al.* 2009). Previous studies have reported that rapid increases of catecholamines, adrenaline and noradrenaline plasma levels occur primarily in fishes facing severe acute stress, particularly if it is accompanied by, or involves a significant reduction in blood oxygen concentration (Gesto *et al*. 2015). Using increased anesthetic concentrations lead to a reduction of pO_2 and an increase of pCO_2 in the bloodstream of common carp anesthetized with benzocaine (Ferreira *et al*. 1981) and in koi carps with MS-222 (Parker-Graham *et al*. 2020), which may trigger cortisol releasing by the cranial kidney tissue (Tort 2011).

Hence, hypoxia consequences cannot be discharged by hydrodynamic factors such as gill countercurrent exchange, heart rate and anesthetic flow through gill filaments. First fish contacts with anesthetic baths led to increased opercular frequency in all Amur carp groups with a second response profile characterized as a

decreased respiratory activities as reported by Ferreira *et al.* (1984b) in common carps. Benzocaine seems to be safer due to its gill absorption independent of the water flow through gill's lamellae as well as by the moderate benzocaine binding to blood plasma proteins (Hayton *et al*. 1996). On the other hand, E60 carps had higher mean opercular frequency values after reaching deep anesthesia plane before fin pinching, possibly a hypoxia side effect caused by high eugenol concentration (Hill and Forster 2004).

In juvenile cyprinid fishes, the relationship between anesthetic concentration and plasma cortisol levels is divergent. In our case, Amur carps anesthetized at 20 mg/L eugenol solution maintained increased motor responses, and elevated heart and respiratory rates with higher plasma blood cortisol levels, reflecting the absence of muscle relaxation. Even though intermediate eugenol concentration (40 mg/L) resulted in lower plasma cortisol levels in Amur carps as in other fish species (Corso *et al*. 2019; Filiciotto *et al.* 2012), it was not capable of hampering pain or nociception consequences. Yet, *Pimephales promelas* cyprinids anesthetized with eugenol at 30 mg/L does not alter plasma cortisol levels (Palić *et al.* 2006). Nonetheless, Le *et al*. (2019) observed that goldfish *Carassius auratus* anesthetized with eugenol solution at 20 mg/L had higher plasma cortisol levels when compared to the group anesthetized with MS-222 solution and by percussive stunning.

As seen in our trial, intermediary benzocaine and eugenol concentrations reported for aquaculture procedures in common and Amur carps - 100 mg/L and 40 mg/L, respectively - (Ferreira *et al.* 1984b; Hikasa *et al.* 1986; Mohamed 1999; Xu *et al.* 2023), should be used with caution in surgical cases, because they probably do not prevent pain from skin clamping with forceps or manual suture (Weber *et al*. 2009). Indeed, here the "optimal" benzocaine concentration was between 100 mg/L and 130 mg/L to reach the surgical anesthesia, in an optimistic way to avoid high cortisol peak and hence triggering high glucose levels. Besides, other aspect that must be considered in benzocaine concentration is the fish weight, as reported for common carps by Antunes *et al.* (2008). Using the reported weight-benzocaine concentration ratio equation reveals an "optimal" benzocaine concentration around 118-120 mg/L. So, future trials must evaluate whether those benzocaine concentrations may be useful for surgical anesthesia. Regarding to eugenol use, it is noteworthy that tambaquis fishes (*Colossoma macropomum*) keep brain activities and had an intense neuronal excitability when anesthetized in eugenol-based baths (Barbas *et al*. 2021). Considering that the morphological pathways between the central and peripheral nervous systems of fish have not yet been elucidated as in mammals (Chen 2011) and even though experiments showing that the potentials evoked after painful stimuli on the skin are processed and translated as pain by the fish encephalon are lacking (Dunlop and Laming 2005; Nordgreen *et al*. 2007; Sneddon *et al*. 2003a), given the physiological and molecular changes observed here, there is an ethical need to use anesthesia and analgesia protocols to guarantee the well-being of fish subjected to painful interventions (Chandroo *et al.* 2004; Livingston 2002). Indeed, the relationship between behavioral responses and drug concentration must be established for experimental fish species facing anesthesia (Readman *et al*. 2013). Therefore, safer anesthetic and analgesic drugs ensuring the well-being of fish undergoing painful are needed (Ross and Ross 2008). Studies have shown that anesthetic doses of benzocaine, MS-222, metomidate and 2-phenoxyethanol do not completely block responses to nociceptive stimuli in *Gadus morhua* cod (Zahl *et al*. 2009) and *Hippoglossus hippoglossus* (Zahl *et al*. 2011). Reflex reactions to caudal fin pinching and responsiveness to handling were most effectively reduced using two anesthetics, MS-222 and benzocaine. This is likely related to their mode of action, as these substances suppress signal transmission in both the central and peripheral nervous systems (Zahl *et al*. 2012).

CONCLUSIONS

Anesthesia plus fin pinching performed in Amur carps with benzocaine – based anesthetic solution at 100 mg/L had the best results when physiological parameters and cortisol levels were analyzed. Eugenol is not a safe anesthetic for painful procedures since it neither contributes to muscle relaxation nor prevents nociception when using the tested concentrations of this trial. Thus, eugenol must be employed only for routine aquaculture procedures.

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Conflict of interest

The authors declare no conflict of interest.

Ethical approval

The care and use of experimental animals complied with Brazilian animal welfare laws, guidelines and policies as approved by Ethics Committee on the Use of Animals of the Instituto de Ciências Biomédicas da Universidade de São Paulo (ICB/USP), São Paulo, Brazil # 5420020819.

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