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Stress-preventing effects of the anaesthetic agents 2-phenoxyethanol, MS-222, clove oil and metomidate in the Senegalese sole *Solea senegalensis*

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ABSTRACT

Objective: To determine the effectiveness of the anaesthetic agents 2-phenoxyethanol, MS-222, clove oil and metomidate in attenuating acute handling stress in juvenile specimens of *Solea senegalensis* subjected to two routine stressful events specific to aquaculture and/or fish research.

Methods: The stress-preventing effects of four anaesthetic agents (2-phenoxyethanol, 600 mg/L; metomidate, 5 mg/L; clove oil, 30 mg/L and MS-222, 75 mg/L) were evaluated in juvenile specimens of Senegalese sole (*Solea senegalensis*) subjected to two different types of acute (handling-related) stress: air exposure and net handling (chasing). To assess the stress-preventing effects of the four anaesthetic agents, diverse blood and plasma parameters (haematocrit, haemoglobin, glucose, lactate and cortisol levels) were determined as stress indicators. Fish were treated with the anaesthetic agents before being subjected to the different types of acute stress, and they were sacrificed 30 min, 2 and 24 h later. Control fish were processed in the same way without pretreatment with the anaesthetic agents.

Results: The net handling stress was of sufficient intensity to cause a significant increase in the levels of most of the stress indicators considered. By contrast, air exposure stress only induced significant increases in cortisol and haemoglobin levels.

Conclusions: The stress-preventing effects of the anaesthetic agents tested were ranked on the basis of their capacity to prevent increases in the haematocrit, haemoglobin, glucose, lactate and cortisol levels, as follows: metomidate (5 mg/L) > clove oil (30 mg/L) > MS-222 (75 mg/L) > 2-phenoxyethanol (600 mg/L).

1. Introduction

In commercial fish farming, the fish must be handled at various stages of culture (*e.g.* capture, classification, transport and vaccination). All of these procedures induce acute stress. When fish are exposed to a stressor, a series of adaptive responses are activated to enable the fish to deal with alterations in their internal balance and thus maintain or regain homeostasis. These physiological responses to stress occur sequentially and may be grouped broadly as primary and secondary responses. The primary physiological stress responses are mediated by activation of the sympathetic chromaffin cell axis, resulting in catecholamine release and activation of the hypothalamic-pituitary-interrenal (HPI) axis, leading to cortisol release[1,2]. Although cortisol and catecholamine levels can be used as indicators of stress, their measurement is almost wholly restricted to research on stress[3]. The secondary physiological responses result from the release of these hormones, which mainly induce mobilization of energy deposits and stimulation of the cardiovascular system; however, several plasma and tissue alterations are also detected, including changes in metabolite levels and haematological parameters[4,5]. Some of the parameters associated with secondary responses are easier to determine and are therefore of potential use in aquaculture. These

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include plasma glucose and lactate levels, as well as haematological indices such as haemoglobin and haematocrit levels[6-8].

Stress is a critical factor in fish health, but its harmful effects are not always immediately visible and may only appear after the medium or long period[9]. This may have adverse economic impacts on fish farming or could confound research findings[10]. Preventing or minimizing responses to stress during handling may reduce processing costs and thus increase profits for fish farmers[11].

It is generally accepted that anaesthesia is a useful way of minimizing stress in fish during routine handling in aquaculture[12], although it is also known that exposure to anaesthetic agents may itself cause stress[8,13-15]. Studies addressing the effectiveness of anaesthetic agents in reducing the stress associated with routine procedures in aquaculture are scarce. Most trials have addressed the effectiveness of anaesthetics in reducing stress induced by confinement or immobilization and fewer have considered handling stress[15-19].

Previous studies have shown that exposure to four commonly used anaesthetic agents [2-phenoxyethanol (2-PE), tricainemethanesulfonate (MS-222), clove oil (CLO) and metomidate (MET)] induced stress in Senegalense sole [*Solea senegalensis* (*S. senegalensis*)] to a greater or lesser degree[8]. However, the effectiveness of these anaesthetic agents in attenuating acute handling stress in flatfish has not previously been considered. The purpose of the present study was therefore to investigate the effectiveness of the anaesthetic agents 2-PE, MS-222, CLO and MET in attenuating acute handling stress in juvenile specimens of *S. senegalensis* subjected to two routine, stressful events specific to aquaculture and/or fish research (air exposure and net handling).

2. Materials and methods

2.1. Fish

Juvenile Senegalese soles (*S. senegalensis*) were obtained from the Instituto Español de Oceanografıa (Cabo Estai, Canido, 36200 Vigo, Spain). The fish were provided by Dr. Peleteiro JB and transported to the experimental aquarium (Faculty of Biology, University of Santiago de Compostela). The fish were allowed to acclimatize for at least 4 weeks in 300 L tanks (stocking density 2.6–2.8 kg fish/m^2) supplied with constantly running and aerated seawater from a recirculation system, under the following conditions: pH 7.3 ± 0.1; temperature (14 ± 1) °C; salinity 36 g/L; dissolved oxygen > 80% saturation level; and a 12 h light: 12 h dark photoperiod. The fish were then transferred to 100 L tanks, under the same environmental conditions, and acclimatized for further 10 days prior to the start of the experiments. The fish were fed to satiety once a day, at 17:00, with commercial dry pellets (Skretting LE-2), and excess food was removed daily. The fish were fasted for 24 h before the start of the experiments. The experiments complied with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were in accordance with Spanish national guidelines (decree 53/2013, BOE 34) for animal experimentation.

2.2. Anaesthetic agents

The anaesthetic agents 2-PE (PanreacQuímica SA, Barcelona, Spain), MET (Aquacalm; Syndell International Inc., Vancouver, Canada), CLO (Omya Peralta Gmbh, Hamburg, Germany) and MS-222 (Sigma Aldrich Co., St. Louis, USA) were used in the study. The doses of the anaesthetic agents used in all experiments were as follows: 2-PE, 600 mg/L; MET, 5 mg/L; CLO, 30 mg/L; and MS-222, 75 mg/L. Solutions of the anaesthetic agents were prepared a few minutes before use. CLO is poorly soluble in cold water, and it was therefore initially dissolved in 94% ethanol (ratio of CLO: ethanol, 1:9). Preliminary trials confirmed that the volume of ethanol used in each trial did not have any visible anaesthetic effect on fish during exposure for at least 15 min.

The doses of the anaesthetic agents were chosen on the basis of previous observations made in our laboratory[20]. The doses yielded induction and recovery times of around 3 and 4 min, respectively. An induction time of 3 min or less, with complete recovery in 5 min is considered acceptable for fish handling[20].

2.3. Experimental design

An experiment was carried out to determine reference levels, hereafter referred to as basal values, of the parameters analysed, which were subsequently used to determine the intensity of stress caused by air exposure and net handling. For this purpose, *S. senegalensis* specimens of (95 ± 3) g were distributed in 100 L tanks (8 fish/tank). All fish in each tank were captured and transferred to 50 L tanks (2 fish/tank) to which the anaesthetic agents had been added at the above-mentioned doses[8]. The fish were removed from the tanks after 5 min for blood sampling. This protocol was repeated 4 times. The values obtained for each anaesthetic agent were averaged to determine the basal values (n = 22–29).

The following experiments were designed to determine the effectiveness of the four anaesthetic agents (2-PE, MET, CLO and MS-222) to reduce or counteract the acute stress induced by air exposure or net handling (chasing). In the first set of experiments (air exposure stress), *S. senegalensis* specimens weighing (79 ± 2) g were used. For each anaesthetic agent, the fish were distributed in two 100 L tanks (12 fish/tank), which were lined with a rigid net. The fish were exposed to air by raising the rigid net out of the tank (non-anaesthetised, stressed fish). The fish were suspended in the air for 3 min and were then redistributed in 50 L tanks (two fish/tank). At intervals of 30 min, 2 and 24 h after air exposure, the fish were caught (in pairs, within 15 s) and finally placed in a 10 L tank containing the anaesthetic MET (10 mg/L) before blood sampling[21]. MET was used at this stage for the following reasons: 1) the dose used induces deep anaesthesia within 1.5 min[20], and 2) in *S. senegalensis* it produces a weaker stress response than other fish anaesthetics and it does not significantly affect cortisol levels 2 and 24 h after exposure[8]. This procedure was repeated with the fish from the other tank, although in this case, the fish were anaesthetised, directly in their tanks, with the corresponding test agent for 3 min before being subjected to the air exposure stress (anaesthetised, stressed fish). A total of 4–6 fish were tested for each anaesthetic agent and post stress sampling time.

In the second set of experiments (net handling stress), specimens of *S. senegalensis* weighing (92 ± 4) g were used. The procedure was similar to that indicated above. For each anaesthetic agent, the fish were distributed in two 100 L tanks (12 fish/tank). All fish in one of the tanks were chased with a net to simulate a capture procedure: 1) 1 min chase; 2) 2 min rest; and 3) 2 min chase. The fish were then placed in recovery tanks (50 L, two fish/tank). At intervals of 30 min, 2 and 24 h after the stressful event, the fish were caught (in pairs, within 15 s) and placed in a 10 L tank containing the anaesthetic MET (10 mg/L) before being removed for blood sampling. This procedure was repeated with the fish from the other tank, but in this case, the fish were first anaesthetised with the one of the test agents

for 3 min before net handling (anaesthetised, stressed fish). A total of 4–6 fish were used for each anaesthetic agent and post stress sampling time.

2.4. Tissue processing

At each sampling time, blood was collected in a heparinised syringe via the caudal vein. The haematocrit level was then determined, and 100 µL aliquots of blood were stored (at 4 ˚C) for haemoglobin analysis on the same day (≤ 4 h). The remaining blood was centrifuged (13 000 r/min for 5 min at 4 ˚C), and aliquots (50 µL) of the supernatant (not haemolyzed) were separated, frozen and stored at –80 ˚C for subsequent assay.

The haematocrit level was determined by the microhematocrit method with heparinised capillary tubes (75 mm × 1.5 mm diameter, Deltalab S. L., Barcelona, Spain). The haemoglobin in the blood was quantified by use of a commercially available kit based on the Drabkin method (Spinreact, S. A., Girona, Spain). Plasma levels of glucose and lactate were determined with commercial kits (Spinreact, S. A., Girona, Spain). Cortisol levels were also determined with a commercial kit (RADIM SpA, Rome, Italy) based on an enzyme immunoassay (EIA). Prior to its use, the kit was validated for use with *S. senagalensis* by tests of linearity and parallelism. The intra-assay (repeatability) and inter-assay (reproducibility) coefficients of variation were always below 7%. The mean recovery was 99.3% ± 6.7%[8].

2.5. Statistical analysis

The results were expressed as mean ± SEM. The data from experiments were analysed by One-way ANOVA followed by a Student–Newman–Keuls test for multiple comparisons or a Student's *t*-test. In all cases, differences were considered significant at $P < 0.05$. All analyses were performed using the SigmaStat 2.0 statistical software package.

3. Results

The data on the effects of air exposure and net handling relative to the global basal levels are summarised in Figure 1. Figure 1A shows the values of the parameters measured 30 min after the fish were subjected to the different types of stress. The haematocrit levels in fish exposed to air were significantly lower than basal levels ($P < 0.05$). The haemoglobin levels increased significantly ($P < 0.05$), relative to basal levels, in response to both types of stress. The levels of both glucose and lactate decreased significantly ($P < 0.05$) in fish subjected to air exposure stress. The opposite results occurred in fish subjected to net handling stress, in which the levels increased significantly ($P < 0.05$) relative to basal levels. The values determined 30 min after net handling were significantly higher ($P < 0.05$) than those determined at all times after air exposure. Figure 1B shows the cortisol levels 30 min and 2 h after the fish were subjected to the different types of stress. In all cases the values were significantly higher ($P < 0.05$) than basal levels. Cortisol levels in the fish 30 min after air exposure were higher than those measured 30 min after net handling. The opposite results occurred after 2 h, and at this time the cortisol levels associated with net handling were higher ($P < 0.05$) than those observed after air exposure.

The haematocrit, haemoglobin, glucose, lactate and cortisol levels obtained in the first experiment (air exposure stress) are shown in Table 1. The haematocrit levels were significantly higher ($P < 0.05$) only in the fish anaesthetized with CLO than those in the non-anaesthetised, stressed fish (control) 30 min and 2 h after the stressful event. In fish pretreated with MS-222, haemoglobin levels were significantly ($P < 0.05$) lower than those in the control fish after 30 min. The glucose levels were significantly lower ($P < 0.05$) only in the fish anaesthetised with MET than those in the control fish, after 2 h. However, significantly lower lactate levels ($P < 0.05$) were only observed 30 min after the stressful event in fish anaesthetised with 2-PE or MET, relative to the controls. In fish anaesthetised with CLO, MS-222 or MET, the cortisol levels were significantly lower ($P < 0.05$) than those in the control fish 30 min and 2 h after air exposure; this was also observed in fish anaesthetized with CLO 24 h after air exposure.

Table 1

Levels of plasma (glucose, lactate, cortisol) and blood (haematocrit, haemoglobin) parameters in non-anaesthetised fish (control) and anaesthetised fish (treated) 30 min, 2 and 24 h after being subjected to air exposure stress.

Parameters			2-PE	CLO	MS-222	MET
Haematocrit (%)	30 min	Control	11.20 ± 0.40	8.40 ± 1.10	12.40 ± 1.40	10.30 ± 0.60
		Treated	11.90 ± 1.80	12.80 ± 0.60*	13.00 ± 0.80	8.50 ± 0.70
	2 h	Control	8.40 ± 0.90	7.60 ± 1.90	9.80 ± 0.80	6.70 ± 1.00
		Treated	10.10 ± 0.60	10.20 ± 2.00*	9.70 ± 0.90	7.70 ± 0.80
	24 h	Control	7.70 ± 0.40	6.60 ± 0.60	6.70 ± 0.60	6.70 ± 0.90
		Treated	6.70 ± 1.50	6.80 ± 0.10	8.90 ± 0.80	7.00 ± 0.70
Haemoglobin (g/dL)	30 min	Control	2.50 ± 0.30	1.90 ± 0.30	2.05 ± 0.11	2.20 ± 0.06
		Treated	1.40 ± 0.30	2.20 ± 0.45	1.50 ± 0.04*	1.60 ± 0.22
	2 h	Control	2.40 ± 0.30	1.80 ± 0.30	1.80 ± 0.20	1.75 ± 0.16
		Treated	1.60 ± 0.20	1.90 ± 0.40	1.60 ± 0.20	1.50 ± 0.18
	24 h	Control	1.90 ± 0.10	1.65 ± 0.23	1.50 ± 0.10	1.40 ± 0.07
		Treated	1.70 ± 0.25	1.60 ± 0.30	1.30 ± 0.13	1.90 ± 0.19
Glucose (µmol/mL)	30 min	Control	1.60 ± 0.05	1.40 ± 0.20	1.80 ± 0.16	1.20 ± 0.08
		Treated	1.70 ± 0.30	1.20 ± 0.10	1.80 ± 0.11	1.04 ± 0.04
	2 h	Control	1.50 ± 0.10	1.50 ± 0.50	1.70 ± 0.11	1.34 ± 0.10
		Treated	1.80 ± 0.20	1.40 ± 0.40	1.70 ± 0.08	1.10 ± 0.08*
	24 h	Control	1.05 ± 0.04	1.00 ± 0.14	1.10 ± 0.16	1.03 ± 0.06
		Treated	0.80 ± 0.10	1.10 ± 0.20	1.20 ± 0.14	0.96 ± 0.04
Lactate (µmol/mL)	30 min	Control	0.22 ± 0.04	0.16 ± 0.02	0.34 ± 0.06	0.11 ± 0.01
		Treated	0.05 ± 0.03*	0.19 ± 0.11	0.30 ± 0.06	0.04 ± 0.01*
	2 h	Control	0.05 ± 0.01	0.13 ± 0.02	0.14 ± 0.03	0.02 ± 0.00
		Treated	0.08 ± 0.03	0.09 ± 0.05	0.07 ± 0.02	0.04 ± 0.01
	24 h	Control	0.04 ± 0.03	0.09 ± 0.07	0.07 ± 0.02	0.03 ± 0.01
		Treated	0.08 ± 0.04	0.08 ± 0.05	0.05 ± 0.02	0.02 ± 0.00
Cortisol (ng/mL)	30 min	Control	347 ± 161	353 ± 67	339 ± 75	353 ± 85
		Treated	202 ± 120	125 ± 43*	62 ± 14*	45 ± 49*
	2 h	Control	113 ± 46	158 ± 37	216 ± 65	172 ± 53
		Treated	77 ± 37	41 ± 30*	30 ± 19*	13 ± 5*
	24 h	Control	20 ± 12	23 ± 4	39 ± 17	16 ± 3
		Treated	6 ± 1	8 ± 1*	21 ± 5	11 ± 2

The values are expressed as mean ± SEM for 4–6 fish. *: $P < 0.05$ relative to the corresponding control.

The haematocrit, haemoglobin, glucose, lactate and cortisol levels obtained in the second experiment (net handling stress) are shown in Table 2. In this experiment, the haematocrit levels were significantly lower ($P < 0.05$) in the fish previously anaesthetised with CLO or MET than those in the non-anaesthetised, stressed (control) fish 30 min and 2 h after net handling stress. Haemoglobin and glucose levels were significantly lower ($P < 0.05$) than those in the respective controls 30 min and 2 h after net handling in fish anaesthetised with CLO or MET and 30 min after net handling in the fish anaesthetised with MS-222. The lactate levels measured 30 min after the stressful event were always significantly lower ($P < 0.05$) in fish treated with any of the four anaesthetic agents than those in the respective controls. The lactate levels measured 2 h after the stressful event in fish anaesthetised with CLO or MET were also significantly lower ($P < 0.05$) than those in the controls. Finally, the cortisol

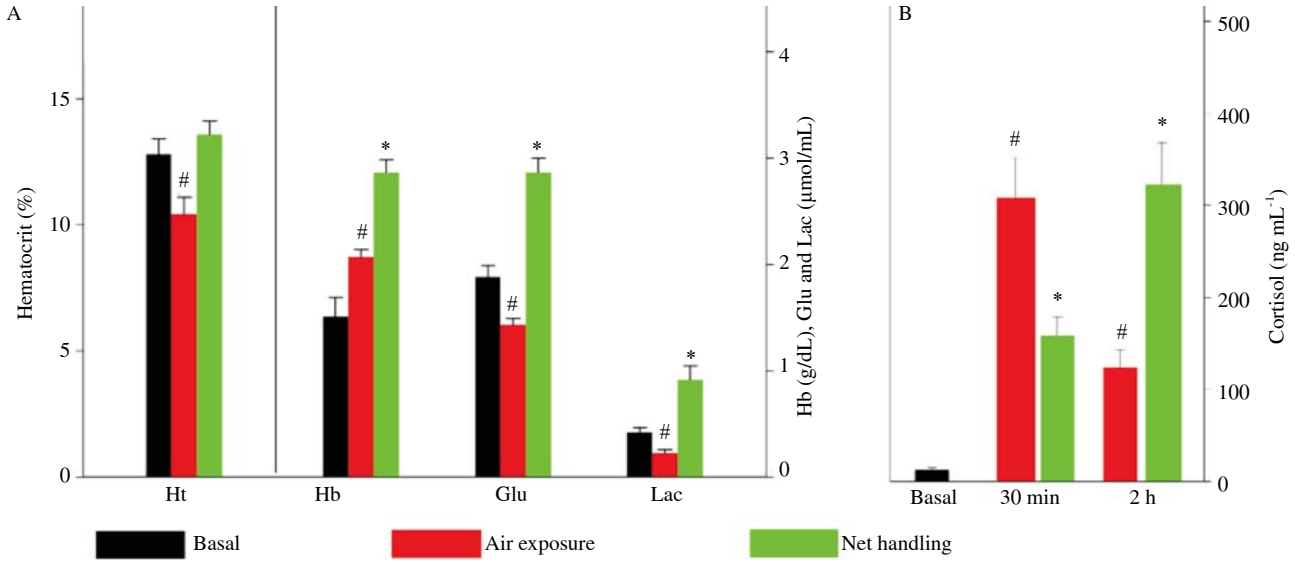

Figure 1. Haematocrit (Ht), haemoglobin (Hb), glucose (Glu) and lactate (Lac) levels in *S. senegalensis* 30 min after exposure to acute stress events (air exposure or net handling stress) (A); cortisol levels in the fish 30 min and 2 h after exposure to the different types of acute stress (B).
*: $P < 0.05$ in the comparison between levels after net handling stress, air exposure stress and basal levels. #: $P < 0.05$ in the comparison between levels after air exposure stress and basal levels. n = 22–29 for control fish and n = 16–24 for fish subjected to the stressful events.

levels measured 2 h after the stressful event in the fish anaesthetised with MS-222 were significantly lower ($P < 0.05$) than those in the control fish. In the fish anaesthetised with MET, cortisol levels were significantly lower ($P < 0.05$) than those in the control fish, at all times after net handling.

Table 2

Levels of plasma (glucose, lactate, cortisol) and blood (haematocrit, haemoglobin) parameters in non-anaesthetised fish (control) and anaesthetised fish (treated) 30 min, 2 and 24 h after net handling (chasing).

Parameters			2-PE	CLO	MS-222	MET
Haematocrit (%)	30 min	Control	11.4 ± 0.7	14.60 ± 1.01	13.50 ± 1.40	14.20 ± 0.80
		Treated	13.7 ± 0.6	8.80 ± 0.40*	13.00 ± 1.00	8.40 ± 0.70*
	2 h	Control	9.4 ± 1.2	13.40 ± 1.20	12.00 ± 1.90	12.60 ± 1.40
		Treated	12.0 ± 0.6	9.40 ± 0.80*	11.10 ± 1.30	6.60 ± 0.50*
	24 h	Control	9.2 ± 0.6	9.30 ± 1.00	11.50 ± 1.10	10.20 ± 1.20
		Treated	10.8 ± 0.8	8.70 ± 0.50	9.20 ± 1.05	7.40 ± 0.90
Haemoglobin (g/dL)	30 min	Control	2.8 ± 0.4	2.70 ± 0.20	3.10 ± 0.10	2.80 ± 0.40
		Treated	2.1 ± 0.2	1.40 ± 0.06*	2.10 ± 0.20*	1.60 ± 0.30*
	2 h	Control	2.1 ± 0.2	2.60 ± 0.20	2.40 ± 0.40	2.40 ± 0.07
		Treated	2.5 ± 0.2	1.80 ± 0.20*	2.30 ± 0.20	1.50 ± 0.10*
	24 h	Control	2.4 ± 0.2	1.80 ± 0.07	2.10 ± 0.20	1.70 ± 0.20
		Treated	2.4 ± 0.1	1.70 ± 0.20	2.30 ± 0.16	1.80 ± 0.10

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Table 2 (*continued*)

Parameters			2-PE	CLO	MS-222	MET
Glucose (µmol/mL)	30 min	Control	2.90 ± 0.40	3.30 ± 0.30	2.80 ± 0.30	2.40 ± 0.20
		Treated	3.00 ± 0.20	1.30 ± 0.15*	1.80 ± 0.15*	0.94 ± 0.03*
	2 h	Control	2.40 ± 0.60	2.80 ± 0.30	2.60 ± 0.40	2.70 ± 0.40
		Treated	2.70 ± 0.30	1.40 ± 0.10*	2.60 ± 0.50	1.00 ± 0.05*
	24 h	Control	1.70 ± 0.30	1.60 ± 0.40	1.20 ± 0.30	1.80 ± 0.22
		Treated	2.50 ± 0.50	0.90 ± 0.10	1.80 ± 0.30	1.30 ± 0.30
Lactate (µmol/mL)	30 min	Control	0.70 ± 0.20	0.90 ± 0.20	0.98 ± 0.20	1.10 ± 0.40
		Treated	0.30 ± 0.06*	0.10 ± 0.03*	0.18 ± 0.08*	0.04 ± 0.00*
	2 h	Control	0.40 ± 0.04	0.40 ± 0.20	0.50 ± 0.30	0.50 ± 0.20
		Treated	0.40 ± 0.06	0.10 ± 0.06*	0.27 ± 0.10	0.04 ± 0.02*
	24 h	Control	0.10 ± 0.06	0.11 ± 0.04	0.06 ± 0.02	0.09 ± 0.03
		Treated	0.03 ± 0.02	0.08 ± 0.02	0.12 ± 0.05	0.06 ± 0.02
Cortisol (ng/mL)	30 min	Control	203 ± 95	263 ± 35	135 ± 24	182 ± 41
		Treated	435 ± 92	136 ± 77	260 ± 45	5 ± 1*
	2 h	Control	386 ± 144	349 ± 112	466 ± 99	373 ± 196
		Treated	333 ± 139	108 ± 50	92 ± 33*	5 ± 2*
	24 h	Control	40 ± 3	36 ± 7	10 ± 3	26 ± 7
		Treated	59 ± 12	18 ± 4	22 ± 9	5 ± 2*

The values are expressed as mean ± SEM for 4–6 fish. *: $P < 0.05$ relative to the corresponding control.

An overview of the capacity of the anaesthetic agents under study to attenuate the stress induced in fish by air exposure or net handling is summarised in Table 3.

Table 3

Overview of the stress-preventing effects of the different anaesthetic agents studied in response to acute stress events (air exposure or net handling stress).

Stress		Ht			Hb			Glucose			Lactate			Cortisol		
		30 min	2h	24 h	30 min	2h	24 h	30 min	2h	24 h	30 min	2h	24 h	30 min	2h	24 h
Air exposure	2-PE	=	↑	↓	↓	↓	↓	=	↑	↓	⤓	↑	↑	↓	↓	↓
	CLO	⤒	⤒	=	↑	=	=	↓	=	=	↑	↓	↓	⤓	⤓	⤓
	MS-222	=	=	↑	⤓	↓	↓	=	=	↑	↓	↓	↓	⤓	⤓	↓
	MET	↓	↑	=	=	=	↑	↓	⤓	=	⤓	↑	↓	⤓	⤓	↓
Net handling	2-PE	↑	↑	↑	↓	↑	=	=	↑	↑	⤓	=	↓	↑	↓	↑
	CLO	⤓	⤓	=	⤓	⤓	=	⤓	⤓	↓	⤓	⤓	↓	↓	↓	↓
	MS-222	=	=	↓	⤓	=	=	⤓	=	↑	⤓	↓	↑	↑	⤓	↑
	MET	⤓	⤓	↓	⤓	⤓	=	⤓	⤓	↓	⤓	⤓	↓	⤓	⤓	⤓

Only the direction and intensity of effects of pretreatment with an anaesthetic agent relative to control are indicated in the table. ↓ or ↑: No significant decrease or increase above 10% relative to control; ⤓ or ⤒: Significant decrease or increase relative to control; =: Variations less than 10%. Ht: Haematocrit; Hb: Haemoglobin.

4. Discussion

Regarding the effects of air exposure and net handling relative to basal levels, in general, similar results were obtained in all cases: i) maximum values of the parameters were obtained 30 min after the fish were subjected to air exposure or net handling stress, except for the cortisol levels which reached maximal levels 2 h after net handling stress; and ii) the values of all parameters, except cortisol, measured 30 min after net handling, were much higher than those measured 30 min after air exposure. These findings indicate that the intensity of net handling stress was high enough to cause a significant increase in the levels of most of the stress indicators considered. By contrast, only the cortisol and haemoglobin levels increased in response to air exposure, and the levels of some parameters were even lower than basal levels in response to this type of stress. The intensity of the stimulus appeared to be lower for air exposure, allowing us to deduce that juvenile specimens of *S. senegalensis* are fairly insensitive to short periods of anoxia. The fish subjected to net handling stress showed a stronger response than those exposed to air exposure stress. Net handling therefore appears to be perceived by fish as highly stressful.

From a physiological perspective, the increase in cortisol levels, in response to both air exposure and net handling stress, was clearly a consequence of increased activity of the HPI axis, a typical primary response to acute stress. However, differences in the secondary responses (haematocrit, haemoglobin, glucose and lactate levels) were observed. These stress parameters are highly dependent on increased secretion of catecholamines from chromaffin tissue via activation of the sympathetic nervous system (SNS)[1,2,22]. Taking this into account, the results of the present study seem to indicate that exposure to air for a short time is not sufficiently stressful to activate the SNS. This is probably related to energetic adaptation in flatfish and the low sensitivity of these fish to hypoxia[23,24]. Moreover, the lack of an increase in lactate levels, even when cortisol levels are elevated, is attributed to the fact that flatfish have a lower capacity than round fish to produce and release lactate from muscles[25]. The slight decreases in haematocrit, glucose and lactate levels can probably be explained by natural fluctuations, as reported for other fish species[26]. However, the chasing (net handling) stress activated all responses mediated by the SNS-chromaffin cell and HPI cell axes, with clear signs of activation of glycogenolysis and gluconeogenesis.

The data obtained in the first experiment involving air exposure, which under the study conditions was perceived by *S. senegalensis* as a low intensity stressor, suggest that the stress-preventing capacity is difficult to detect. However, partial attenuation, mainly of the haemoglobin, lactate and cortisol levels, was observed. In the second experiment involving net handling which was perceived by *S. senegalensis* as intense stressor, it was easier to detect the stress-preventing capacity of the different anaesthetic agents, and except for 2-PE, the stress indices were often totally or partially attenuated by the anaesthetic agents. The stress-preventing effects thus appear to increase with the intensity of stress.

We found that, depending on the type of stress, MS-222 and CLO efficiently blocked, either totally or partially, activation of the HPI axis. These findings are consistent with the capacity of MS-222 to block or attenuate the release of cortisol in *Sciaenops ocellatus* 2 min after air exposure[19], and in *Puntius filamentosus* following transport stress[12]. They are also consistent with the capacity of CLO to do the same in *Ictalurus punctatus* and *Labeo rosita* after handling stress[10,27]. However, the potential stress-preventing capacity of the agents was not always fulfilled as in some cases, both MS-222 and CLO were incapable of attenuating the increase in levels of cortisol, *i.e*. after handling stress and crowding stress in *Pimephales promelas* pretreated with these agents[28], and following crowding stress in *Sparus aurata* treated with MS-222[29]. The findings of the present and other studies appear to suggest that the stress-preventing capacity of the anaesthetic agents may depend on the type of stress involved. However, other factors may also be involved, for example, species differences, type and duration of stress, differences in the doses applied and duration of exposure to the anaesthetic. The low stress-preventing capacity of 2-PE observed in the present study is consistent with the observations by Molinero and Gonzáles, and Toni *et al*. in *Sparus aurata* subjected to crowding stress[29,30]. We also found that MET was highly effective in counteracting the high levels of cortisol induced by both types of stress. These effects are consistent with those observed in other fish[10,18,19,31,32]. The ability of this anaesthetic to suppress the cortisol response is related to its capacity to inhibit the synthesis and release of cortisol[18,19,33].

Regarding the other parameters studied (haematocrit, haemoglobin, glucose and lactate levels), the stress-preventing capacity of the anaesthetic agents under study was most evident when the fish were subjected to net handling stress. As already mentioned, this is perceived as an intense stressor by *S. senegalensis*. In this case, CLO, MS-222 and MET were also able to totally or partially attenuate the increased levels of blood parameters such as haematocrit and haemoglobin. Release of catecholamines during periods of stress is known to cause the release of erythrocytes into circulation from the spleen, thus increasing haematocrit and haemoglobin levels[34,35]. The ability of these anaesthetic agents to block the increased levels of these parameters indicates that pretreatment with anaesthetic agents is effective in blocking activation of the SNS, the endpoint of which is the release of catecholamines into blood from chromaffin tissue. Likewise, attenuation of plasma glucose levels and lactate levels, a typical secondary stress response, also provides information about attenuation of primary stress responses by pretreatment with anaesthetic agents.

In summary, the study findings show that the stress-preventing efficacy of the anaesthetic agents studied can be ranked on the basis of the magnitude of their attenuating capacity, as follows: MET (5 mg/L) > CLO (30 mg/L) > MS-222 (75 mg/L) > 2-PE (600 mg/L).

Conflict of interest statement

We declare that we have no conflict of interest.

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