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Efficacy of tricaine methanesulphonate, clove oil and medetomidine-ketamine and their side effects on the physiology of sturgeon hybrid *Acipenser naccarii* × *Acipenser baerii*

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Summary

Three anaesthetic agents were tested in sturgeon hybrid *Acipenser naccarii* female × *Acipenser baerii* male: clove oil at 100 mg L^{-1} (CLO); tricaine methanesulfonate at 150 mg L^{-1} (MS-222); combined medetomidine and ketamine hydrochloride (MK) administered by intravenous injection at 0.04 mg kg^{-1} + 4 mg kg^{-1}, respectively. Efficacy of the anaesthetics was evaluated as well as physiological effects on blood gas status, acid-base balance and stress response by measuring blood PCO_2, PO_2, pH, HCO_3^-, haematocrit and serum cortisol, glucose, NEFA, lactate, Na^+, K^+, Cl^- concentration. Anaesthetic dosages were safe and effective in order to rapidly anaesthetize the sturgeons: induction time was significantly shorter with MS-222 (3.2 min) compared with CLO (4.7 min) and MK (5.4 min). Recovery time was significantly longer in MK anaesthetized fish (16 min) in comparison with CLO (5.8 min) and MS-222 (3.8 min). Anaesthesia induced blood hypercapnia, respiratory acidosis and stress response, with differences among anaesthetics. Blood PCO_2 and cortisol levels were significantly higher in MS-222 (11 mmHg; 97.0 ng ml^{-1}) and CLO (9.6 mmHg; 65.3 ng ml^{-1}) anaesthetized groups compared to MK (7.8 mmHg; 39.6 ng ml^{-1}) and control (7.0 mmHg; 10 ng ml^{-1}). Overall results indicated that anaesthesia with MS-222 produced a greater physiological impact in sturgeons compared to the other anaesthetics. The effects were slightly attenuated in fish anaesthetized with clove oil, indicating that the use of this chemical was better than MS-222 in reducing handling stress. MK anaesthesia affected physiological parameters to a lesser extent. Intravascular administration of medetomidine at a dose of 0.04 mg kg^{-1} in combination with ketamine at a dose of 4 mg kg^{-1} proved to be a safe and effective anaesthetic protocol in this hybrid sturgeon, suitable for experimental studies and diagnostic procedures related to broodstock management and artificial reproduction.

Introduction

Sturgeon farming is a growing sector of the aquaculture industry in Europe (Bronzi et al., 2009). The application of anaesthesia is frequently needed to improve management practices for the handling, tagging, sexing, spawning, transportation and surgery of sturgeons. Very few studies on the comparative effects of anaesthetics in sturgeons are available (Di Marco et al., 1999; Fleming et al., 2003; Gomulka et al., 2008), despite their frequent use in different applications (Asadi et al., 2006; Flynn and Benfey, 2007; Askarian and Kousha, 2009). According to literature data, tricaine methanesulphonate (MS-222) is the compound most commonly used in sturgeons (Feist et al., 2004; Bronzi et al., 2006; Knowles et al., 2006). It is a water soluble benzocaine derivative generally administered by immersion. MS-222 is the only anaesthetic approved in the European Union with a compulsory 21-day withdrawal period prior to human consumption (Ross and Ross, 2008; EFSA, 2009; Reg (EC) No 470/2009).

Despite the widespread use of clove oil in several farmed fish (Mylonas et al., 2005; Park et al., 2009; Weber et al., 2009) this anaesthetic has not been extensively used in sturgeons and its effects have been poorly investigated (Jackson et al., 2006; Hurvitz et al., 2007; Gomulka et al., 2008; Feng et al., 2009). Clove oil or eugenol, as active ingredient, is considered an attractive anaesthetic in the aquaculture industry because it is effective, inexpensive and safe (Keene et al., 1998).

Medetomidine in combination with ketamine is extensively used for anaesthesia in mammals and produces good sedative and analgesic effects (Hall et al., 2001). Several applications of these injectable compounds have been investigated for anaesthesia in fish, including sturgeons (Fleming et al., 2003; Williams et al., 2004), providing interesting results on their efficacy and physiological effects. Knowledge of their properties such as efficacy, safety and side effects on fish physiology is a prerequisite for suitable anaesthetic application.

The hybrid sturgeon *Acipenser naccarii* × *Acipenser baerii* was obtained for the first time in 1993 from the cross breeding of the *A. naccarii* female and the *A. baerii* male in Northern Italy (Arlati and Bronzi, 1995; Arlati et al., 1999). This hybrid sturgeon, named AL in view of the geographical origin of the parental species, Adriatic Sea and Lena River, is currently intensively farmed in Italy for commercial purposes and has recently been exploited for caviar production (Vaccaro et al., 2005; Bronzi and Arlati, 2009; Pazzaglia and Giovannini, 2009).

The aim of the present study was to test the efficacy of clove oil, MS-222 and the combination of medetomidine-ketamine in the anaesthesia of the hybrid sturgeon and to investigate the effects of anaesthesia on blood gas status, acid-base balance and stress response. A multivariate statistical approach was followed in order to make a comprehensive evaluation of the physiological impact of chemical agents in anaesthetized fish vs control. The overall intent was to identify the most

appropriate chemical agent for anaesthetizing this hybrid sturgeon in order to reduce handling stress and improve welfare during aquaculture practices and in experimental studies.

Materials and methods

Origin and husbandry of fish

Six year old sturgeon hybrids (*A. naccarii* female × *A. baerii* male, n = 40; 12.4 ± 3.4 kg body weight; 120 ± 8.3 cm total length) were used for the experiment. Fish were reared from 2005 to 2009 in a small experimentally constructed wetland located inside a commercial fish farm (Ittica Spineto, Falerone, AP, Central Italy). Fish were kept in 600 m^3 concrete tanks supplied with freshwater pumped from the Tenna river (Gennaro et al., 2008; Tancioni et al., 2009). Fish rearing density was approx. 12 kg m^{-3}. A dry pellet diet (Ecolife 15, BIOMAR, Treviso, Italy) was given once per day, at a dose of 0.2–1.5% body weight, depending on the seasonal temperature and biomass.

Anaesthetics and sampling procedure

Three anaesthetics were tested: clove oil (85% eugenol, Sigma-Aldrich, Milano, Italy) at 100 mg L^{-1} (CLO); MS-222 Finquel® (95% tricaine methanesulfonate, Argent, Redmond, WA, USA) at 150 mg L^{-1} (MS-222); combined medetomidine (Domitor, Pfizer, Roma, Italy) and ketamine hydrochloride (Ketavet 100, Intervet , Shering-Plough, Latina, Italy) i.v. at a dose of 0.04 mg kg^{-1} + 4 mg kg^{-1} (MK). The anaesthetic dosages used in this study were determined in a pre-experimental study and according to literature data on anaesthesia in the parental species *A. naccarii* and *A. baerii* (Cataldi et al., 1998; Gomulka et al., 2008).

Before use, the clove oil was dissolved in 80% ethanol (1 : 4 v/v) and MS-222 in water to prepare a stock solution. The MK cocktail was prepared by diluting ketamine hydrochloride (100 mg ml^{-1}) with sterile saline solution to 10 mg L^{-1}, and then adding medetomidine hydrochloride (1 mg ml^{-1}). This preparation was then administered through the caudal vein with a 2.5 ml syringe using a 22 G × 1 1/2″ needle inserted behind the anal fin, modifying the anaesthetic protocols reported for a sturgeon species and two scombrid fish (Fleming et al., 2003; Williams et al., 2004).

The experiment was carried out in May 2009. Four groups of 10 fish each were tested. The fish were netted individually from the culture tanks and anaesthetized in a 150 L tank. Control fish were carefully immobilized, held down and immediately bled without anaesthesia.

The anaesthetic baths were prepared by collecting the water directly from the culture tanks in order to expose the fish to the same temperature, and were regularly renewed. Water temperature, dissolved oxygen concentration and pH were monitored and maintained at 18.6 ± 1.0°C, 10.4 ± 1.0 mg L^{-1} and 7.9 ± 0.2, respectively.

The different stages of anaesthesia were recorded for each fish following the criteria proposed by Mylonas et al. (2005). The end points monitored were the complete loss of balance in order to determine stage of sedation, and unresponsiveness to stimuli associated with slow opercular ventilation rate for the deep anaesthesia stage.

Blood sampling was performed between 9.00 and 12.00 a.m. Blood samples were withdrawn from the caudal vein and immediately used or centrifuged at 3000 ***g*** for 10 min. Serum aliquots were stored at −80°C for further analysis.

Sturgeons were then placed in 250 L well-aerated recovery tanks where time to regain equilibrium, regular opercular ventilation and responsiveness to visual stimuli were recorded.

Blood analysis

Haemogas analysis was performed on venal blood samples immediately after collection from 6 fish per group. Blood CO_2 and O_2 partial pressure (PCO_2, PO_2), pH and bicarbonate ion concentration (HCO_3^-), haematocrit (Hct) and electrolytes (Na^+, K^+, Cl^-) were determined by Voden Medical OPTI CCA blood gas analyser equipped with a temperature compensation device (Roche Diagnostics, Voden Medical Instruments, Milano, Italy) according to Marino et al. (2006).

Analysis of stress parameters was performed on serum samples collected from 10 fish per group. Cortisol concentration was measured by a solid-phase chemiluminescent enzyme immunoassay using an Immulite One analyzer (Siemens Medical Solution Diagnostic, Los Angeles, CA). Glucose, lactate and non esterified fatty acids (NEFA) were analysed by enzymatic colorimetric assays using commercial kits (BPC Biosed, Roma, Italy; WAKO Chemicals, Neuss, Germany; Greiner Diagnostic, Bahlingen, Germany) with a Cary 50 spectrophotometer (Varian Medical Systems, Milano, Italy).

Statistical analysis

Data were analysed using the SPSS 12.01 software statistical package (Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed in order to assess the effect of different anaesthetics on induction and recovery times, as well as on blood stress parameters. Post-hoc multiple comparisons were made using Student–Neuman–Keuls and Dunnett's T3 to assess inter-group differences in the mean values of the physiological parameters. Principal component analysis (PCA) and discriminant analysis on PCA factors were applied to the physiological data in order to assess discrimination among groups and the associated variables.

Results

The anaesthetic dosages were safe and no mortality occurred after anaesthesia up to 3 months after the experiment. Induction and recovery times differed significantly among anaesthetics (ANOVA $P < 0.005$). Induction times of anaesthesia were significantly faster with MS-222 (3.2 min) than with CLO (4.7 min) and MK (5.4 min), respectively (Fig. 1). Recovery times were significantly longer for sturgeons exposed to MK (16 min) compared to those exposed to CLO (5.8 min) and MS-222 (3.8 min).

Anaesthetics significantly affected blood gas status, acid-base balance and stress response (ANOVA $P < 0.05$). The mean level of O_2 partial pressure decreased in all groups and to a greater extent in MS-222 and CLO anaesthetized fish, compared to control (Fig. 2). However, differences among groups were not statistically significant. Blood PCO_2 level increased significantly in fish exposed to CLO and MS-222 compared to MK and control groups. Blood HCO_3^- concentration and pH significantly decreased comparably in all anaesthetized groups (Fig. 2).

Serum cortisol level increased significantly in all anaesthetized fish compared to control (Fig. 3), but to a lesser extent in the MK group (39.6 ng ml^{-1}) than in the CLO (65.3 ng ml^{-1}) and MS-222 (97.0 ng ml^{-1}) groups.

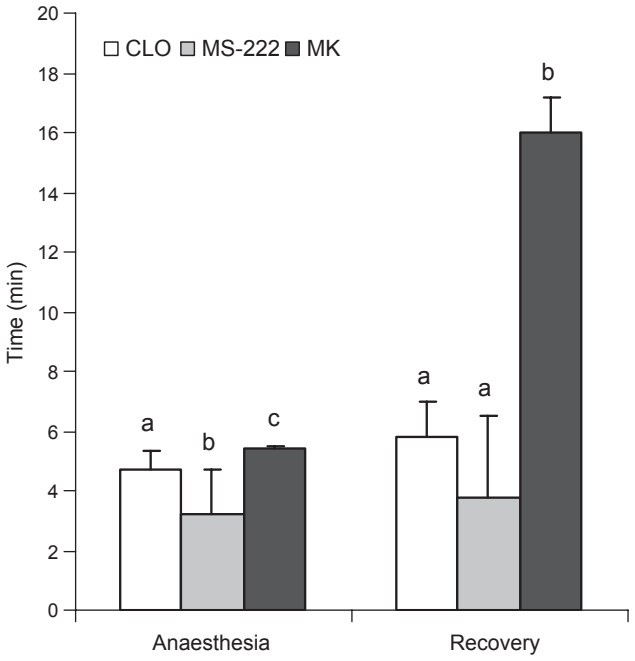

Fig. 1. Induction and recovery times in hybrid sturgeon *A. naccarii* female × *A. baerii* male anaesthetized with clove oil (CLO), tricaine methanesulphonate (MS-222) and combined medetomidine-ketamine (MK). Data are expressed as Mean ± SD; n = 10 fish per group. Different letters indicate differences among groups at $P < 0.05$

Serum glucose and lactate concentration increased significantly in all anaesthetized fish as a metabolic response to anaesthesia, whereas NEFA concentration did not display any well defined trend and no significant elevation was detected in control and anaesthetized fish.

Exposure to anaesthetics resulted in a significant increase of serum Na^+, K^+ and Cl^- concentration. Haematocrit did not change significantly in response to anaesthesia (ANOVA $P > 0.05$), although a slight increase was observed in sturgeons exposed to MK and MS-222.

Discriminant analysis applied to PCA factors as a function of anaesthetic treatment was significant (Monte Carlo test $P < 0.02$). The first two discriminant axes explained 75.2% of the variability of the data (38.1 and 37.1%, respectively). Plotting discriminant canonical scores grouped by confidence ellipsoids showed that anesthetized groups are well discriminated and significantly different from the control group depending on their physiological parameters (Fig. 4). Moreover, information from the first and third discriminant axis (38.1 and 24.8%, respectively) revealed that the MK group was displaced towards the control group, indicating a lesser physiological impact of MK anaesthesia with respect to CLO and MS-222 treatments.

Discussion

Anaesthetic agents are commonly used in aquaculture to reduce fish stress and physical injuries during husbandry practices (Davis and Griffin, 2004; Iversen et al., 2009). Culture and reproduction activities of sturgeons are currently increasing both for commercial and conservation purposes (Pikitch et al., 2005; Bronzi and Arlati, 2009; Bronzi et al., 2009), although knowledge of the comparative effects of different anaesthetics suitable for handling operations is still limited (Di Marco et al., 1999; Fleming et al., 2003; Gomulka et al., 2008).

Anaesthetic dosages used in this study were safe and effective in order to rapidly induce anaesthesia in the hybrid sturgeon *A. naccarii* × *A. baerii*. Fish were anaesthetized more rapidly

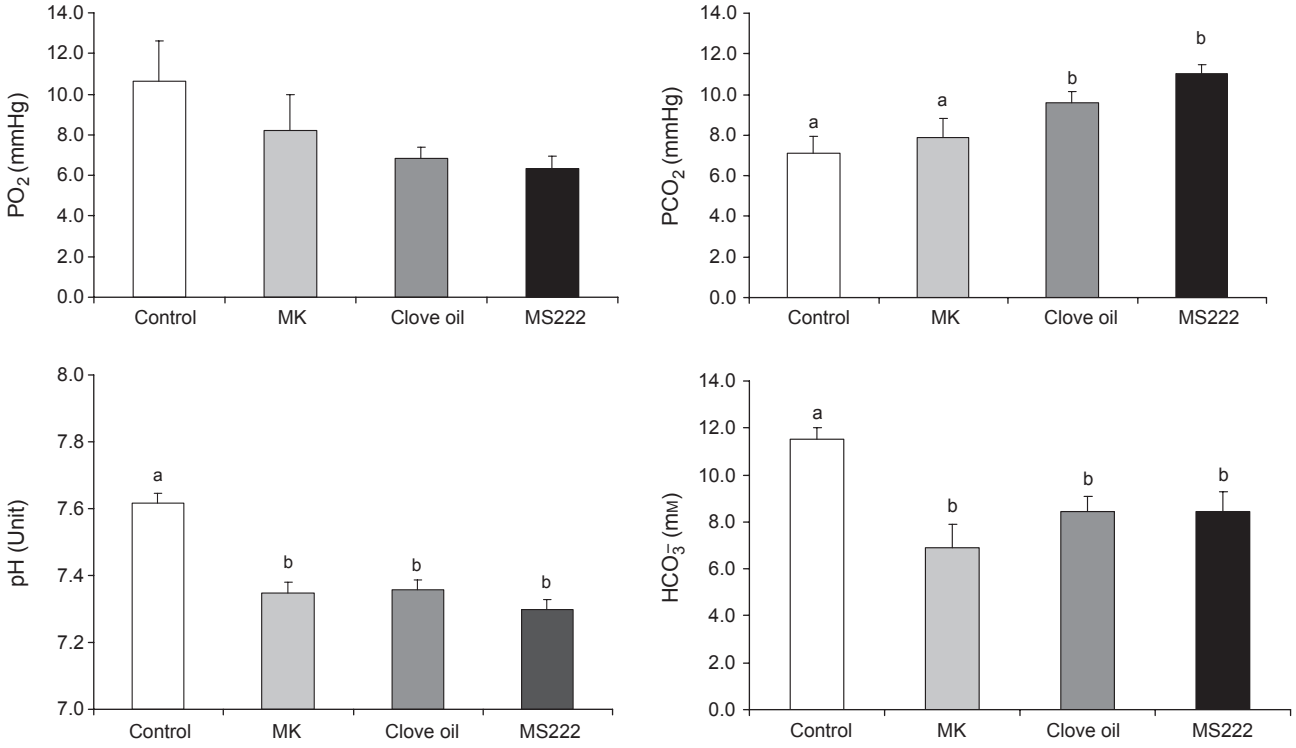

Fig. 2. Changes in blood gas pressure (PO_2 and PCO_2), pH and bicarbonate concentration (HCO_3^-) in control and anaesthetized hybrid sturgeon *A. naccarii* female × *A. baerii* male. Data are expressed as Mean ± SEM.; n = 10 fish per group. Different letters indicate differences among groups at $P < 0.05$

with MS-222 followed by CLO and MK. Induction time in sturgeon hybrids exposed to CLO was slightly longer compared to the time reported for Siberian sturgeon juveniles using 75 mg l^{-1} of eugenol and kept at 17°C (Gomulka et al., 2008), probably due to the large size of fish used in the present study.

The efficacy of the MS-222 at 150 mg L^{-1} dosage used was comparable with results reported on anaesthesia of the parental species *A. naccarii* and *A. baerii* using 140 and 125 mg l^{-1}, respectively (Di Marco et al., 1999; Gomulka et al., 2008).

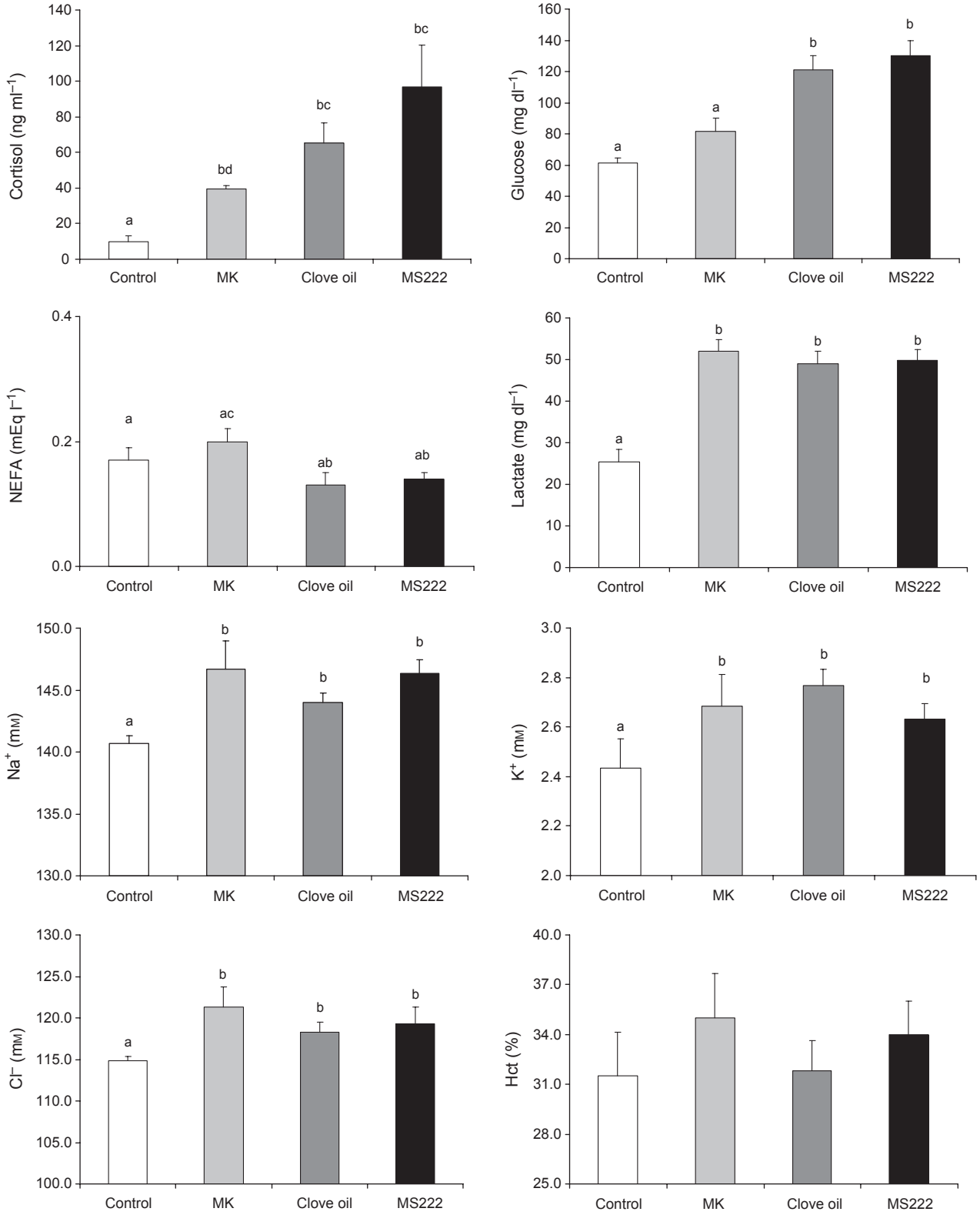

Fig. 3. Changes in serum cortisol, glucose, NEFA, lactate, Na^+, K^+, Cl^- concentration and Htc values in control and anaesthetized hybrid sturgeon *A. naccarii* female × *A. baerii* male. Data are expressed as Mean ± SEM; n = 10 fish per group. Different letters indicate differences among groups at $P < 0.05$

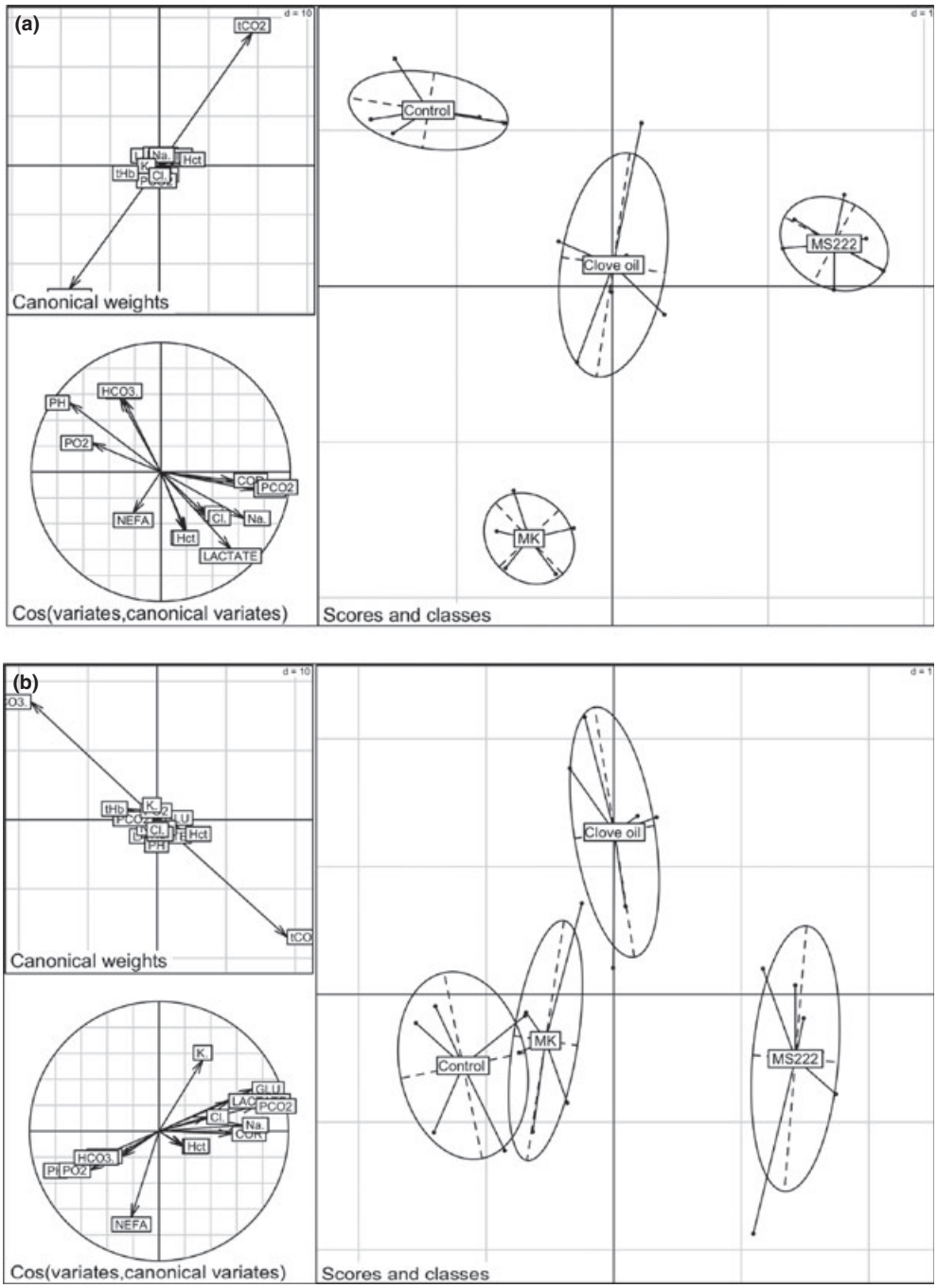

Fig. 4. Blood stress parameters in hybrid sturgeon *A. naccarii* female × *A. baerii* male analysed by multivariate analysis (n = 6 per group). The discriminant analysis was applied on PCA factors as a function of anaesthesia treatment (A = plotting of the first and second discriminant axis; B = plotting of the first and third discriminant axis). Monte Carlo test ($P < 0.02$)

Intravascular administration of MK at a dose of 0.04 mg kg^{-1} + 4 mg kg^{-1} was an effective method for reducing the induction time and chemical dosage (Graham and Iwama, 1990; Stoskopf and Posner, 2008) compared with intramuscular injection (Fleming et al., 2003; Williams et al., 2004).

Shorter recovery times were observed in MS-222 anaesthetized fish than in CLO treated fish, although without statistical difference. Conversely, recovery from the MK anaesthesia took longer and, in this regard, atipamezole administration should be tested to reverse anaesthetic effects of medetomidine and achieve good, more rapid recovery (Fleming et al., 2003; Williams et al., 2004).

Among the anaesthetics tested, the MS-222 at 150 mg L^{-1} seems to fit most closely the criteria for an ideal anaesthetic proposed by Marking and Meyer (1985) by inducing deep anaesthesia in about 3 min and recovery within 10 min. However, anaesthesia with MS-222 had significantly greater effects on physiological stress in sturgeon hybrids than CLO and MK.

All anaesthetic agents significantly affected blood gas status, acid-base balance and stress response, although with some differences among them. Blood PO_2 partial pressure decreased in all groups and to a greater extent in MS-222 and CLO anaesthetized fish, compared to control. Comparison among groups was not statistically significant, probably due to the higher data variability of this parameter with respect to the

other variables examined, as already reported in red pacu *Piaractus brachypomus* anaesthetized with clove oil and MS-222 (Sladky et al., 2001).

Blood PCO_2 level significantly increased in CLO and MS-222 anaesthetized fish compared to MK and control groups. Blood HCO_3^- concentration and pH decreased significantly in response to anaesthesia in accordance with the Henderson-Hasselbach equation (Evans et al., 2005), denoting an acid-base imbalance as reported in rainbow trout exposed to different anaesthetic agents (Iwama et al., 1989). Generally blood acidosis in fish is offset by a significant accumulation of bicarbonate ion in the blood. The extent and time of recovery of blood acidosis through HCO_3^- accumulation varies according to the species and it is more limited and slower in freshwater compared to marine fish, as reported in *Acipenser transmontanus* (Cech and Crocker, 2002).

Changes in these parameters are associated with the depression of respiratory activity due to anaesthesia, and differences among groups may be related to variable degrees of reduction in the ventilation rate. Anaesthesia with ketamine alone allows regular ventilation rhythm in fish (Graham and Iwama, 1990), but may decrease it when used in combination with an alpha-2 agonist such as metedomidine (Stoskopf and Posner, 2008). Anaesthesia with eugenol is reported to cause a 75% decrease in arterial PO_2 in fish and the risk of ventilatory failure increases with increasing chemical dose (Sladky et al., 2001; Hill and Foster, 2004).

Mean cortisol concentration measured in control fish was consistent with the resting values reported for unstressed *A. naccarii* (Di Marco et al., 1999). Lower cortisol increase was observed in MK anaesthetized group in comparison with CLO and MS-222 groups, suggesting that MK is the most effective in inhibiting the activation of HPI-axis and the cortisol release in response to sampling and anaesthesia stress. Very high cortisol levels measured in the MS-222 anaesthetized group indicate that this chemical exacerbates the cortisol stress response in sturgeon hybrids, as already reported in other species (Small, 2003; Davis and Griffin, 2004; King et al., 2005; Di Marco et al., 2007). Hyperglycemia and hyperlacticemia were observed in anaesthetized fish as metabolic effects induced by respiratory and muscular stress (Di Marco et al., 1999; Baker et al., 2008; McKenzie et al., 2007; Feng et al., 2009).

Although free fatty acids (NEFA) generally increased in stressed fish as a secondary stress response (Wendelaar Bonga, 1997), NEFA concentration did not differ significantly among groups. This result may be related to the short time of anaesthesia exposure and/or to fish nutritional status which may influence blood NEFA concentration.

A disturbance of electrolyte balance was observed following anaesthesia and resulted in higher concentrations of osmotically active ions in anaesthetized fish than in control. Osmolality and ions elevation was also reported in *A. naccarii* exposed to MS-222 (Cataldi et al., 1998; Di Marco et al., 1999) as in other species exposed to clove oil and medetomidine-ketamine (Sladky et al., 2001; Williams et al., 2004; Iversen et al., 2009).

Discriminant analysis confirmed a physiological impact of anaesthesia that was greater in MS-222 than in CLO and MK anesthetized fish. The effects were slightly attenuated in fish anaesthetized with clove oil, indicating that this chemical is better than MS-222 for reducing handling stress. Minor physiological changes were observed in MK anaesthetized fish. The MK dosage tested was effective in inducing anaesthesia in sturgeon hybrids within ~5 min. After drug administration the fish remained calm and unresponsive, had regular opercular movements and good muscle relaxation. Recovery occurred after about 15 min, but the use of atipamezole should further reduce recovery time.

In conclusion, intravascular administration of medetomidine at a dose of 0.04 mg kg^{-1} in combination with ketamine at a dose of 4 mg kg^{-1} is a safe and effective anaesthetic protocol in this hybrid sturgeon, suitable for experimental studies when the number of test animals is low, and for diagnostic procedures related to broodstock management and reproduction activities such as tagging, sampling for blood or gonadal biopsies, ultrasound analysis and surgery.

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