

## Physiological changes of sturgeon *Acipenser naccarii* caused by increasing environmental salinity

R. M. Martínez-Álvarez<sup>1,2</sup>, M. C. Hidalgo<sup>1</sup>, A. Domezain<sup>2</sup>, A. E. Morales<sup>1</sup>, M. García-Gallego<sup>1</sup>  
and A. Sanz<sup>1,\*</sup>

<sup>1</sup>Dept Biología Animal y Ecología, Univ. de Granada, 18071 Granada, Spain and <sup>2</sup>Dept I+D, Piscifactoría 'Sierra Nevada', 18313 Riofrío, Granada, Spain

\*Author for correspondence (e-mail: anasanz@ugr.es)

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### Summary

The possible repercussions of osmoregulatory processes on some indicators of classical and oxidative stress were examined during gradual acclimation of sturgeons (*Acipenser naccarii*) to full seawater (35‰ salinity) and after a period of 20-days at this salinity. Erythrocyte constants and levels of cortisol, protein and glucose in the plasma were determined. In addition, plasma osmolality and muscle-hydration values, as well as liver and heart protein, were determined. Catalase, glutathione peroxidase and superoxide dismutase activities and lipid-peroxidation levels were measured in blood (plasma and red blood cells) and tissue (liver and heart). A number of physiological responses, such as disturbance in body fluid, activation of osmoregulatory mechanisms, augmented antioxidant defences in blood and alteration of energy

metabolites, were detected with increasing environmental salinity. After 20 days at 35‰ salinity, plasma osmolality, erythrocyte constants and muscle water content all returned to values usual for low environmental salinity, indicating that osmoregulatory processes have achieved their objective. However, cortisol values, antioxidant enzyme activities in the blood (plasma and red blood cells), lipid peroxidation in plasma, and hepatic proteins did not return to initial values, showing that osmoregulatory processes cause major physiological changes in the fish.

Key words: *Acipenser naccarii*, osmoregulation, water salinity, oxidative stress, physiology.

### Introduction

Many species of sturgeons are anadromous, as they spawn in rivers and, after a variable length of time, migrate downstream to brackish areas or even the open sea, where they mature until they return to spawn upstream. *Acipenser naccarii* is usually included as one of these euryhaline species because it has been captured in the Adriatic sea, although it breeds in freshwater. Nowadays, this sturgeon species and/or its hybrids are being raised in freshwater fish farms in Italy and, notably, in Spain. Some recent studies (Garrido-Ramos et al., 1997; Hernando et al., 1999) support the possibility that the historical distribution of this species included certain Spanish rivers.

Cataldi et al. (1995) pointed out that, if the euryhaline character of *A. naccarii* is verified, new perspectives for widening culture areas to littoral zones would become of great interest. Anadromous fish must develop complex osmoregulatory mechanisms to survive successfully both in hypoosmotic environments (e.g. rivers) and hyperosmotic environments (e.g. estuaries and open sea). Some aspects of these osmoregulatory processes have been previously studied in several sturgeon species (Natochin et al., 1985; McEnroe and Cech, 1985; Krayushkina, 1998), including *A. naccarii* (Cataldi et al., 1995, 1999; McKenzie et al., 1999). Age

(maturation stage) and body size (ratio of gill surface area:body surface area) have been postulated as determining factors of the salinity tolerance of the fish (McEnroe and Cech, 1985; García-Gallego et al., 1998).

Any environmental disturbance can be considered a potential source of stress, as it prompts a number of responses in the animal to deal with the physiological changes triggered by exterior changes. In theory, these responses can be detected in fish and in other vertebrates in the form of changes in hormonal or substrate concentrations in the plasma or alterations in erythrocyte parameters, such as cell volume or enzyme activities (Donaldson, 1981). If the internal perturbation of the fish, either directly or as a result of alterations of the environment, overwhelms the physiological mechanisms of the animal for response and adaptation to new conditions, survival can be threatened and death can result.

In addition, as a consequence of metabolic activity, reactive oxygen species (ROS) are continuously produced and act as strong oxidants. As a defence mechanism, a large repertory of antioxidant enzymes, in addition to small antioxidant molecules, are produced by the cell. Superoxide dismutase (SOD), which hastens the dismutation of O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub>, catalase

(CAT), and glutathione peroxidase (GPX), which converts  $H_2O_2$  to  $H_2O$ , are the most important antioxidant enzymes found in all vertebrates. Their activities differ among the organs and tissues of freshwater and marine fish (Wdzieczak et al., 1982), depending upon feeding behaviour (Radi and Matkovics, 1988), environmental factors and other ecological conditions (Winston and Di Giulio, 1991; Roche and Bogé, 1996). When the antioxidant defences are inadequate to combat the action of the ROS, the result is oxidative stress. The formation of ROS can be increased in response to different variations in the internal or external medium, whereupon oxidative alterations occur in the cellular constituents. One of the alterations is increased lipid peroxidation as a consequence of the oxidation of the lipid constituents of cell membranes.

One circumstance involving great metabolic changes in fish is their acclimation and survival at different degrees of salinity. Thus, Roche and Bogé (1996) reported changes in antioxidant enzymes in the red blood cells of sea bass (*Dicentrarchus labrax*) subjected to hypoosmotic shock. Osmoregulation undoubtedly implies, among other circumstances, a greater energy expense, which is caused in turn by a metabolic activation and, consequently, an increase in ROS formation. In fish, a direct relationship has been found between metabolic intensity and activation of oxidative enzymes (Wilhelm Filho et al., 1993).

The present study examines the possible repercussions that osmoregulatory processes can induce on the classical indicators of stress (e.g. cortisol, glycaemia and haemoglobin) in *A. naccarii* subjected to gradually increasing environmental salinity and maintained for 20 days at a constant salt concentration of 35‰. The activities of SOD, CAT and GPX, as well as lipid-peroxidation levels in blood (plasma and red blood cells), liver and heart, were studied.

## Materials and methods

### *Animals and maintenance*

The saltwater-acclimation experiment was conducted with Adriatic sturgeons *Acipenser naccarii* (Bonaparte 1836) of >2 years of age (mean age 31 months; mean initial mass 1500 g) that had been previously reared in a freshwater (0‰ salinity;  $Na^+$  0.41 mmol l<sup>-1</sup>,  $Cl^-$  0.5 mmol l<sup>-1</sup>,  $K^+$  0.01 mmol l<sup>-1</sup>,  $Ca^{2+}$  1.45 mmol l<sup>-1</sup>,  $Mg^{2+}$  0.53 mmol l<sup>-1</sup>) facility (Piscifactoría ‘Sierra Nevada’, Riofrío, Granada, Spain) at 14°C. During the 67 days of the experiment, sturgeons were kept in a closed circuit, in which the water was recirculated through mechanical and biological filters, at 10–12°C, and salinity was progressively increased by controlled addition of highly saturated saline water according to the following temporal pattern: fish were transferred directly from 0‰ to 4–5‰ salinity; this initial increase was followed by a daily increase of 2‰ up to 13‰ salinity, 1‰ up to 15‰ salinity and 0.5‰ up to the final salinity (35‰). Sturgeons were kept at a constant salinity of 35‰ for a further 20 days. Over the entire experimental period, temperature, dissolved oxygen (6–7 p.p.m.) and pH (8–9) were controlled daily using a

GRANT-YSI 3800 data-logger (Grant Instrument Ltd, Cambridge, UK) and ammonia and nitrite concentration (never reaching toxic levels) was controlled by colorimetric tests (Merck, Darmstadt, Germany).

The experimental fish were subdivided into two groups: one group was left undisturbed throughout the experimental period except for salinity changes and daily measurements of water-quality parameters (‘undisturbed’ fish), while the other group was sampled at pre-determined intervals, after anaesthetization to allow blood and tissue samplings. The survival index in the undisturbed sturgeons was 96.4%.

### *Sampling*

Fish were sampled at salinities of 0‰, 15‰, 22‰, 29‰ and 35‰ and after the period at constant 35‰ salinity ( $N=5$  fish at each concentration). At each sampling, blood, muscle, liver and heart were taken from killed fish after anaesthetization by immersion, until sedated, in an ethylene-glycol-mono-phenyl-ether (Merck) 1:2000 (v:v) solution. For 0‰ salinity samples, only blood and muscle were taken to determine plasma osmolality and muscle moisture, respectively. The rest of the determinations were made at  $\geq 15$ ‰ salinity.

Blood was drawn from the caudal vein and collected into heparinized syringes. Haematocrit (Hct), total haemoglobin (Hb) concentration and red-blood-cell count (RBCC) were immediately determined. Plasma was separated by centrifugation (650 g, 15 min), and the haemolysate supernatant from red blood cells was obtained according to Marcon and Wilhelm Filho (1999). Both plasma and haemolysate were stored at –80°C until analysis.

Muscle samples were taken from the anterior dorsal area, weighed and placed in an oven to determine tissue moisture. Samples of heart and liver were submerged in liquid nitrogen and then stored at –80°C until analysis.

### *Analytical methods*

The RBCC was performed after blood dilution (1:100) with Hendrick’s reagent (made with reagents from Sigma, Akobendas, Spain) by using a Neubauer chamber (Afora, Sevilla, Spain) and light microscope (magnification  $\times 100$ ). Hct was determined by blood centrifugation (2600 g, 5 min) using a special microfuge. The Hb concentration in the samples was determined by colorimetry (540 nm), after mixing blood with Drabkin’s solution (Van Kampen and Zijlstra, 1961).

Plasma cortisol and glucose were assayed by immunoassay (Immulite®, Diagnostic Products Corporation, Los Angeles, USA) and the glucose oxydase method (Hugget and Nixon, 1957), respectively; plasma osmolality was evaluated using an osmometer (Osmostat OM620, Daiichi Kagaku Co Ltd, Kyoto, Japan). The protein contents of plasma, liver and heart were measured according to Bradford (1976). For moisture-level measurements, muscle samples were weighed before and after drying in an oven (Heraeus, Madrid, Spain) at 105°C until they reached constant weight.

For the determination of the parameters indicating oxidative stress (CAT, GPX, SOD and lipid-peroxidation levels),

samples of plasma, haemolysate, liver and heart were used. The tissue extracts were prepared by homogenization in nine volumes of extraction buffer (100 mmol<sup>-1</sup> Tris HCl, 0.1 mmol<sup>-1</sup> EDTA, 0.1% (v/v) Triton X-100, pH 8). These homogenates were centrifuged at 27 170 g for 30 min at 4°C. The supernatants were divided into different aliquot samples and stored at -80°C until analysis.

CAT (EC 1.11.1.6) activity was determined by measuring the decrease of hydrogen peroxide concentration at 240 nm. The decline in absorbance was registered in 12 s intervals for the first 2 min in a cuvette containing 50 mmol<sup>-1</sup> potassium phosphate buffer (pH 7) and 10.6 mmol<sup>-1</sup> hydrogen peroxide freshly added, according to Aebi (1984).

GPX (EC 1.11.1.9) activity was measured following the method of Flohé and Günzler (1984), where freshly prepared glutathione reductase solution (2.4 U ml<sup>-1</sup> in 0.1 mol<sup>-1</sup> potassium phosphate buffer, pH 7) was added to a cuvette containing 50 mmol<sup>-1</sup> potassium phosphate buffer (pH 7), 0.5 mmol<sup>-1</sup> EDTA, 1 mmol<sup>-1</sup> sodium azide, 0.15 mmol<sup>-1</sup> NADPH and 0.15 mmol<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>. The NADPH-consumption rate was monitored for 2 min. After the addition of 1 mmol<sup>-1</sup> GSH (reduced glutathione), the decrease in absorption at 340 nm at intervals of 12 s for the first 2 min was measured.

SOD (EC 1.15.1.1) activity was measured spectrophotometrically by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals. One unit of activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome *c* reduction rate (McCord and Fridovich, 1969).

Lipid-peroxidation levels were determined by quantifying the concentration of thiobarbituric-acid-reacting substances (TBARS), expressed as the malondialdehyde (MDA) concentration, according to Buege and Aust (1978).

#### Statistical analysis

The differences in parameters were tested for significance using a one-way analysis of variance (ANOVA) and the LSD (least-significant difference) test ( $P < 0.05$ ).

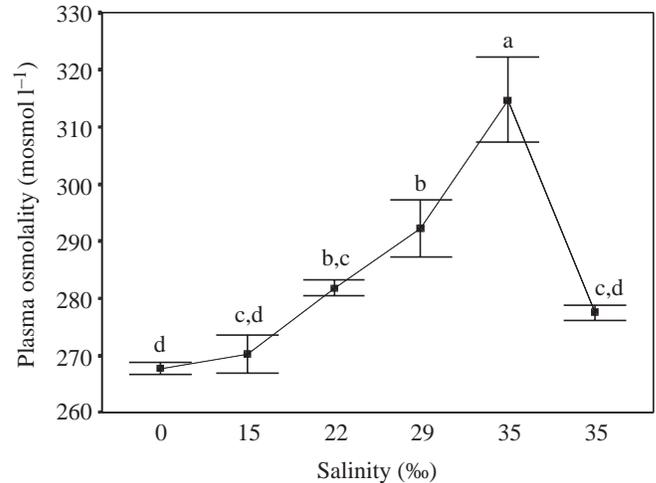


Fig. 1. Plasma osmolality values of sturgeon *Acipenser naccarii* during acclimation to seawater (35‰) and after a period of 20 days at this salinity. Values are means  $\pm$  S.E.M. Data values with different letters are significantly different (one-way analysis of variance,  $P < 0.05$ ,  $N = 5$  fish at each salinity).

#### Results

Fig. 1 presents the plasma osmolality over the experimental period. The increase was gradual and significant during acclimation to the water with salinity rising from 0‰ to 35‰. After the fish remained for 20 days in water with 35‰ salinity, osmolality values fell to levels similar to those of fish in freshwater (0‰).

The results presented in Table 1 show an increase in plasma cortisol level with salinity, although the difference was not significant. No significant changes were found in glycemia values. The total plasma proteins showed a statistically significant decline with increasing salinity (from 15‰ to 29‰), remaining stable from 29‰ to 35‰ salinity. Hepatic protein increased significantly during acclimation to salinity, while the protein level in the heart did not significantly change during this process.

Table 1. Values of plasma parameters and heart and liver protein of the sturgeon *Acipenser naccarii* during acclimation to seawater (35‰) and after a period of 20 days at this salinity

	Salinity (‰)				
	15	22	29	35	35*
<b>Plasma</b>					
Cortisol ( $\mu\text{g dl}^{-1}$ )	0.9 $\pm$ 0.2	0.8 $\pm$ 0.4	1.1 $\pm$ 0.4	1.2 $\pm$ 0.4	1.2 $\pm$ 0.6
Glucose ( $\text{mg dl}^{-1}$ )	47.6 $\pm$ 2.1	47.2 $\pm$ 2.2	47.8 $\pm$ 3.7	49.0 $\pm$ 4.6	44.8 $\pm$ 1.8
Protein ( $\text{mg ml}^{-1}$ )	48.5 $\pm$ 2.3 <sup>a</sup>	38.8 $\pm$ 4.2 <sup>a,b</sup>	28.2 $\pm$ 2.6 <sup>c</sup>	27.4 $\pm$ 2.6 <sup>c</sup>	34.3 $\pm$ 4.2 <sup>b,c</sup>
<b>Tissue protein</b>					
Liver ( $\text{mg g wet tissue}^{-1}$ )	59.1 $\pm$ 0.2 <sup>c</sup>	74.0 $\pm$ 0.4 <sup>b,c</sup>	84.0 $\pm$ 0.5 <sup>a,b</sup>	74.0 $\pm$ 1.3 <sup>b,c</sup>	95.0 $\pm$ 0.2 <sup>a</sup>
Heart ( $\text{mg g wet tissue}^{-1}$ )	76.8 $\pm$ 0.5	81.2 $\pm$ 0.7	82.3 $\pm$ 0.2	93.1 $\pm$ 0.5	90.0 $\pm$ 0.7

Values are means  $\pm$  S.E.M. Values with different superscript letters within each row are significantly different (analysis of variance,  $P < 0.05$ ,  $N = 5$  fish at each salinity).

\*After a period of 20 days at 35‰.

Table 2. Values of some haematological parameters of the sturgeon *Acipenser naccarii* during acclimation to seawater (35‰) and after a period of 20 days at this salinity

Parameter	Salinity (‰)				
	15	22	29	35	35*
Hct (%)	20.2±0.5 <sup>b,c</sup>	24.0±0.3 <sup>a</sup>	17.2±1.2 <sup>c</sup>	19.0±1.8 <sup>b,c</sup>	21.4±1.1 <sup>a,b</sup>
Hb (g 100 cm <sup>-3</sup> )	8.5±0.3 <sup>b</sup>	10.9±0.6 <sup>a</sup>	6.5±0.4 <sup>c</sup>	5.8±0.4 <sup>c</sup>	10.0±1.2 <sup>a,b</sup>
RBCC (10 <sup>3</sup> cells µl <sup>-1</sup> )	822.0±53.4 <sup>a</sup>	894.0±45.9 <sup>a</sup>	764.0±63.0 <sup>a</sup>	560.0±52.3 <sup>b</sup>	773.3±105.0 <sup>a</sup>

Values are means ± s.e.m. Values with different superscript letters within each row are significantly different (analysis of variance,  $P < 0.05$ ,  $N = 5$  fish at each salinity).  
\*After a period of 20 days at 35‰.

Table 2 shows that Hct, Hb concentration and RBCC showed similar responses to increasing salinity. Values during the first phase (0–29‰) increased as salinity rose, only to decline when the salinity level reached 29‰ and then increase again during the 20 days at a constant salinity of 35‰.

The water content of the muscle fell significantly as the animals acclimated to the saline water, but values returned to normal at the end of the period of constant 35‰ salinity (Fig. 2).

Enzyme activities and lipid-peroxidation levels in plasma and red blood cells are shown in Fig. 3 and Fig. 4, respectively. High levels of SOD activity were found in red blood cells, compared with levels found in plasma; SOD activity in plasma increased significantly when the salinity level reached 22‰, remaining high at higher salinities. SOD activity in red blood cells increased with increasing salinity, but this trend was only significant after 20 days at 35‰ salinity. The CAT activity in red blood cells was higher than in plasma and increased significantly with increasing salinity in both samples (red blood cells and plasma). No appreciable responses in GPX activity were found in plasma at higher

salinity, although red blood cells showed an increase in GPX as salinity increased.

Major changes in MDA concentration were found in red blood cells at differing salinities; levels were high at 15‰ salinity but fell when salinity reached 22‰. Subsequent increases in salinity (22–35‰) did not alter these low values but, after 20 days at 35‰ salinity, a significant increase was found, although values did not reach those previously recorded at 15‰ salinity. In addition, although lipid-peroxidation levels in plasma were lower than those found in the erythrocytes, they rose in relation to increased salinity.

Table 3 shows the parameters of oxidative stress in the liver and heart. First, it should be emphasized that the CAT and GPX activities in the liver were far greater than those detected in the heart. Levels of CAT and SOD in the liver significantly declined as salinity increased, while no significant change was detected in the heart with increasing salinity. Lipid-peroxidation levels showed no significant change in the two tissues with increasing salinity.

#### Discussion

The effect of cortisol on osmoregulatory parameters has been amply studied in teleosts (Maetz, 1969; Epstein et al., 1971; Kamiya, 1972; Scheer and Langford, 1976; Assem and Hanke, 1979; Hegab and Henque, 1984). Its primary action seems to be the stimulation of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. In the present study, the trend of increasing levels of cortisol at higher salinity in *A. naccarii* (Table 1) indicates that, for this chondrosteian, the role of cortisol must be similar to that in teleosts. Besides, the increase of serum cortisol levels is considered to be a primary indicator of stress response (McDonald and Milligan, 1992; Cataldi et al., 1998). In a previous test with animals at the same salt concentrations but at a higher environmental temperature (21°C; Martínez-Álvarez et al., manuscript submitted), the values for plasma cortisol were higher than in the present study, and, in this case, the increase at higher salinity proved significant. The cortisol levels found in our tests were comparable with those reported by Cataldi et al. (1998) for the same fish species. These also show that, after acclimation to low temperatures, the cortisol concentration diminished significantly. According to McKenzie et al. (1999), although these sturgeons showed no fluctuations in plasma cortisol levels at different salinities (0‰,

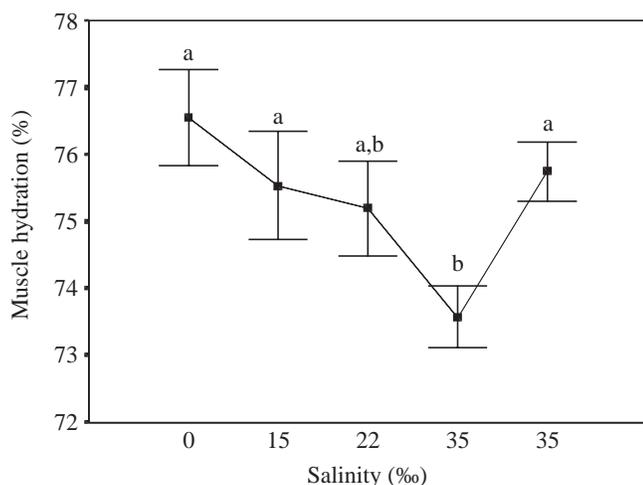


Fig. 2. Muscle hydration levels of sturgeon *Acipenser naccarii* during acclimation to seawater (35‰) and after a period of 20 days at this salinity. Values are means ± s.e.m. Data values with different letters are significantly different (one-way analysis of variance,  $P < 0.05$ ,  $N = 5$  fish at each salinity).

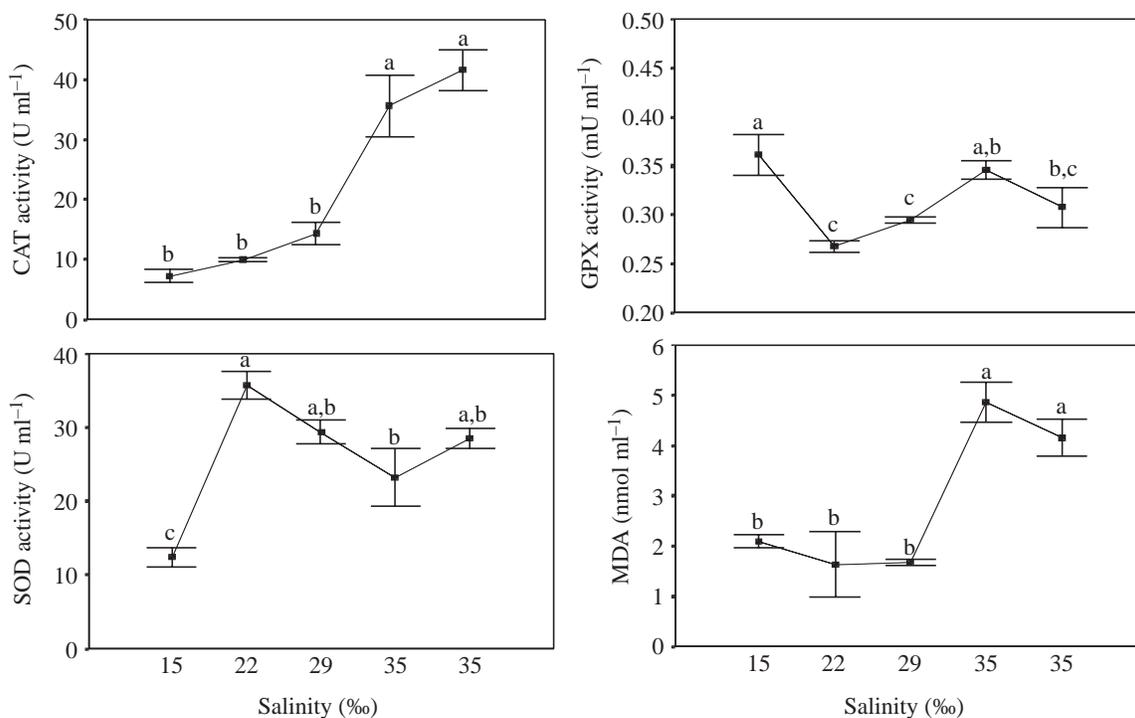


Fig. 3. Antioxidant enzyme activities and lipid-peroxidation levels in the plasma of sturgeon *Acipenser naccarii* during acclimation to seawater (35‰) and after a period of 20 days at this salinity. Values are means  $\pm$  S.E.M. Data values with different letters are significantly different (one-way analysis of variance,  $P < 0.05$ ,  $N = 5$  fish at each salinity). Catalase (CAT) and glutathione peroxidase (GPX) activities, 1 unit (U) = 1 mmol min<sup>-1</sup>; superoxide dismutase (SOD) activity, 1 U = 50% inhibition of cytochrome *c* reduction min<sup>-1</sup>; lipid-peroxidation levels are expressed as malondialdehyde (MDA) concentration (nmol MDA ml<sup>-1</sup>).

Table 3. Antioxidant enzyme activities and lipid-peroxidation levels in liver and heart of the sturgeon *Acipenser naccarii* during acclimation to seawater (35‰) and after a period of 20 days at this salinity

	Salinity (‰)				
	15	22	29	35	35*
<b>Liver</b>					
CAT (U mg protein <sup>-1</sup> )	487.8 $\pm$ 44.7 <sup>a</sup>	324.2 $\pm$ 12.5 <sup>b</sup>	302.8 $\pm$ 13.9 <sup>b,c</sup>	318.8 $\pm$ 17.7 <sup>b</sup>	235.6 $\pm$ 21.6 <sup>c</sup>
GPX (U mg protein <sup>-1</sup> )	30.0 $\pm$ 4.0	30.0 $\pm$ 4.0	20.0 $\pm$ 3.0	30.0 $\pm$ 4.0	22.0 $\pm$ 2.0
SOD (U mg protein <sup>-1</sup> )	12.7 $\pm$ 0.8 <sup>a</sup>	9.8 $\pm$ 0.4 <sup>b</sup>	9.3 $\pm$ 0.9 <sup>b</sup>	10.2 $\pm$ 0.9 <sup>b</sup>	7.0 $\pm$ 0.1 <sup>c</sup>
MDA (nmol ml <sup>-1</sup> )	2.1 $\pm$ 0.2	1.2 $\pm$ 0.2	1.3 $\pm$ 0.2	1.4 $\pm$ 0.1	1.4 $\pm$ 0.2
<b>Heart</b>					
CAT (U mg protein <sup>-1</sup> )	11.6 $\pm$ 0.5	11.2 $\pm$ 0.6	9.2 $\pm$ 0.2	9.1 $\pm$ 0.9	12.8 $\pm$ 1.6
GPX (U mg protein <sup>-1</sup> )	7.8 $\pm$ 0.8	7.2 $\pm$ 0.8	8.0 $\pm$ 0.1	6.0 $\pm$ 0.6	7.0 $\pm$ 0.3
SOD (U mg protein <sup>-1</sup> )	11.1 $\pm$ 0.5	9.6 $\pm$ 0.8	9.5 $\pm$ 0.8	8.5 $\pm$ 0.5	10.2 $\pm$ 0.7
MDA (nmol ml <sup>-1</sup> )	1.0 $\pm$ 0.2	0.9 $\pm$ 0.2	0.6 $\pm$ 0.2	0.4 $\pm$ 0.1	1.2 $\pm$ 0.2

Values are means  $\pm$  S.E.M. Values with different superscript letters within each row are significantly different (analysis of variance,  $P < 0.05$ ,  $N = 5$  fish at each salinity).

\*After a period of 20 days at 35‰.

Catalase (CAT) activity, 1 unit (U) = 1 mmol min<sup>-1</sup>; glutathione peroxidase (GPX) activity, 1 unit = 1 nmol min<sup>-1</sup>; superoxide dismutase (SOD) activity, 1 unit = 50% inhibition of cytochrome *c* reduction min<sup>-1</sup>; lipid peroxidation levels are expressed as malondialdehyde (MDA) concentration (nmol MDA ml<sup>-1</sup>).

11‰ and 23‰), poor growth and high mortality following disturbances in animals in water at 20‰ salinity indicate only a partial tolerance to brackish water (20‰ salinity). These circumstances, among others, induced these authors to

conclude that a salinity of 20‰ represents the upper tolerance limit in *A. naccarii* (Cataldi et al., 1995, 1999). Our animals, on the other hand, remained for 20 days at a salinity of 35‰, with only 4% mortality. In addition, some specimens were

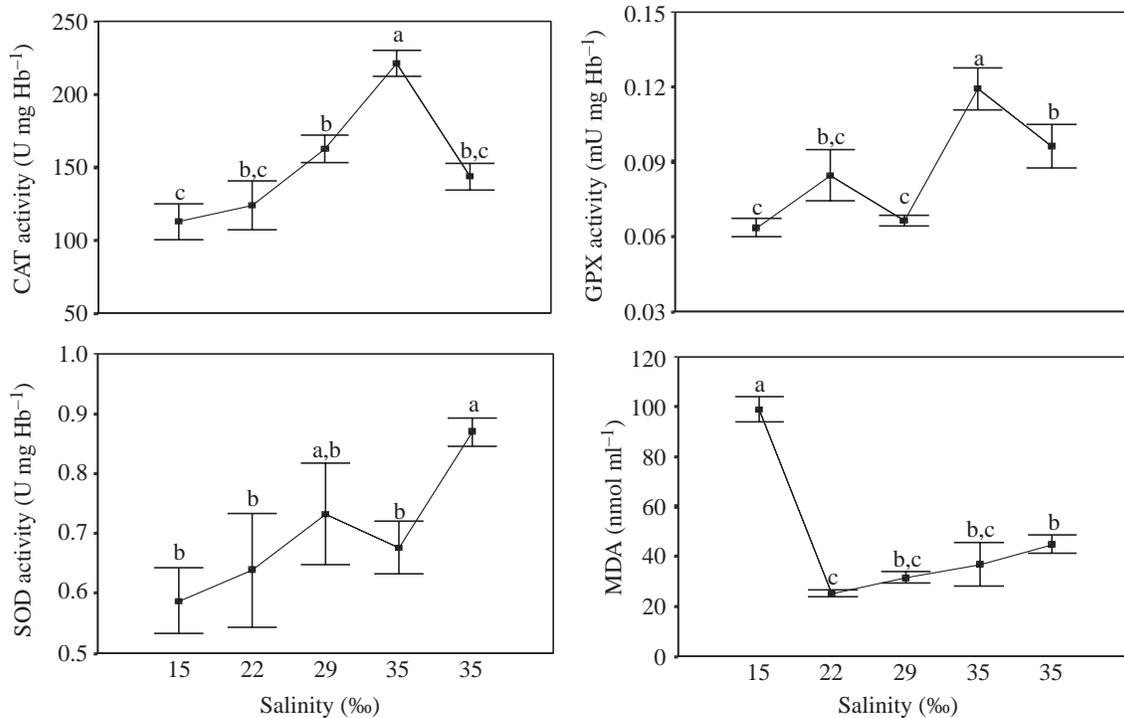


Fig. 4. Antioxidant enzyme activities and lipid-peroxidation levels in red blood cells of sturgeon *Acipenser naccarii* during acclimation to seawater (35 ppt) and after a period of 20 days at this salinity. Values are means  $\pm$  S.E.M. Data values with different letters are significantly different (one-way analysis of variance,  $P < 0.05$ ,  $N = 5$  fish at each salinity). Catalase (CAT) and glutathione peroxidase (GPX) activities, 1 unit (U) = 1 mmol min<sup>-1</sup>; superoxide dismutase (SOD) activity, 1 U = 50% inhibition of cytochrome *c* reduction min<sup>-1</sup>; lipid-peroxidation levels are expressed as malondialdehyde (MDA) concentration (nmol MDA ml<sup>-1</sup>).

transferred afterwards to an exhibition marine aquarium in northwestern Spain, where they survived for several months, although monitoring was no longer possible. In addition, the results presented in the present and other studies of different experiments involving acclimation to rising salinity indicate a compensatory response that enables survival at an environmental salinity of 35‰.

Thus, during a number of saltwater-acclimation experiments with sturgeons of >2 years of age (mean initial mass 1500 g), we have found an increase in chloride cells (Carmona et al., manuscript submitted) and branchial Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (Morales et al., 2001), changes in fatty-acid composition of branchial-cell membranes (R. M. Martínez-Álvarez, unpublished data) and rising levels of plasma urea (Martínez-Álvarez et al., manuscript submitted; in this study we also used fish of  $\geq 1$  year and a mean initial mass of 750 g). All these responses encourage osmolality (Fig. 1) and the electrolyte levels in blood (Martínez-Álvarez et al., manuscript submitted) to stabilize in a hyperosmotic medium. The different age/mass of the sturgeons used in our studies (14 months, 932 g) and in those of Cataldi et al. (1995) (20 months, 1900 g), Cataldi et al. (1999) (0–150 days, 0.015–35.9 g) and McKenzie et al. (1999) (5 months, 56 g) and/or the differences in acclimation times and rhythms could explain the differences in the results found in these studies compared with the present study.

The trend for the cortisol level to rise in response to growing

environmental salinity should, like a hyperglycemia-causing hormone, raise the plasma glucose level. However, we found no such rise (Table 1). Previous studies of this issue are contradictory, showing both a rise (Assem and Hanke, 1979; Bashamohideen and Parvatheswararao, 1972) and a fall (Krumnschnabel and Lackner, 1993; Soengas et al., 1991) in glucose during seawater adaptation. There appears to be a high glucose demand in order to supply the energy by osmoregulatory mechanisms (Krumnschnabel and Lackner, 1993; Plaut, 1998), whereupon glyconeogenesis even increases (Jürss and Bittorf, 1990). The greater use of glucose could mask the plasma glucose increase prompted by the cortisol.

The decline found in total plasma proteins (Table 1) during increasing salinity could also be accounted for by the high osmoregulatory energy demand. This, together with a reduced appetite of the animals at higher salinity (Usher et al., 1991; Plaut, 1998), would account for this reduction. The latter would also contribute to the failure to find a rise in blood glucose.

During the first stage of acclimation, the Hct, Hb concentration and RBCC increased (Table 2); they subsequently decreased as the salinity rose further and returned to initial values when the fish had remained for 20 days at a constant salinity of 35‰. These changes can be attributed to changes in the water content in the blood, caused by the change in environmental salinity (Plaut, 1998). Thus, at the beginning

of exposure to a hyperosmotic environment, the fish would lose water passively, and thereby undergo increases in the concentrations of blood-cell elements. Afterwards, the compensatory increase in water ingestion would provide a transitory dilution of the blood parameters. Finally, these would return to initial values as a result of the rest of the osmoregulatory mechanisms, which act to re-establish the extracellular volume. This hypothesis is reinforced by the results found for the muscle water content (Fig. 2), which, on the one hand, reflect a certain dehydration of the muscle during acclimation to the saline water and, on the other hand, reflect the return to normal values after 20 days at 35‰.

Our results indicate that, in *A. naccarii*, blood (plasma and red blood cells) antioxidant defences (SOD, CAT and GPX activities; Figs 3, 4) have the ability to strengthen under increasing environmental salinity. The increase of these enzyme activities in red blood cells could explain the decline in lipid-peroxidation values and the stability of these values despite changing salinity. Nevertheless, it seems that the activity of these enzymes in the plasma is not sufficient to avoid a certain degree of lipid oxidation at 35‰ salinity (Fig. 3). However, this fact did not apply to red blood cells, where lipid oxidation remained low at all times (Fig. 4).

It should be emphasized that the CAT and GPX activities in the liver (Table 3) were far greater than those detected in the heart, a circumstance that has also been noted by Wilhelm Filho et al. (1993) in other fish species. In addition, SOD and CAT decreased significantly in the liver as environmental salinity rose. This trend, when not accompanied by lipid peroxidation, would indicate a certain alteration of the cell metabolism rather than a state of oxidative stress. It should be borne in mind that enzymatic activities are expressed as a function of the protein content; the relative protein content of the liver increased with rising environmental salinity. This increase may result from a decrease in fatty content in response to a deficit in energy intake, as discussed above, or to reduced body-water content. Thus, the increase in hepatic protein is a possible reason for the depressed SOD and CAT activities. In the heart, the invariability in the protein content would promote constancy in enzyme activities during acclimation to environmental salinity.

It could be stated that there were no alterations in oxidative state during acclimation to salinity in the sturgeon, either in the liver or in the heart, during the osmoregulatory process, with lipid-peroxidation levels remaining low at all times. The fact that we did note alterations in the antioxidant enzymatic activities in the blood could indicate that the blood was more affected by being more directly involved in osmoregulatory activities.

There are studies demonstrating that tissues differ in their responses to oxidative stress induced by the same circumstance. However, we have not found any studies that determine the parameters of oxidative stress under conditions of hyperosmotic stress; only one study refers to the changes in enzyme activities (SOD, CAT and peroxidase) in *D. labrax* during replacement of seawater (37‰) with freshwater (5‰),

reporting a stimulation of SOD and CAT activities by hypoosmotic shock (Roche and Bogé, 1996).

In conclusion, the sturgeon *A. naccarii*, when subjected to growing environmental salinity up to 35‰, revealed a number of physiological responses, such as disturbance in body fluid (detected by increased plasma osmolality, altered number of red blood cells and decreased levels of muscle hydration), activation of osmoregulatory mechanisms (increased cortisol levels) and antioxidant defences (augmented antioxidant enzyme activities in the blood), and alteration of energetic metabolites (changes in protein concentration in the plasma and liver), indicating that the acclimation of sturgeons to increased salinities involves osmotic stress counteracted by osmoregulation. In fact, after the period of acclimation to growing salinity (up to 35‰) and after the fish remained for 20 days at this salinity, the plasma osmolality values, the concentration of blood parameters, and the water content of the muscle all returned to the values obtained for water with low salinity, showing that osmoregulatory processes have achieved their objective.

By contrast, levels of cortisol, antioxidant enzymes in the blood (plasma and red blood cells), lipid peroxidation in plasma, and hepatic proteins did not return to initial values. This indicates that osmoregulatory processes cause major physiological changes in the fish.

Further study on osmoregulatory physiology is needed to determine the point at which maintenance of an environmental salinity level enables growth and utilization of food compatible with the possibility of raising this fish species in brackish or seawater.

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