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ORIGINAL ARTICLE

Physicochemical parameters associated with the methods of application of salt baths and their field assessment of blood parameters of Atlantic salmon in water pre-smolt stage

Parámetros físico-químicos del agua asociados a los métodos de aplicación de baños de sal y su evaluación en terreno sobre parámetros sanguíneos de salmón del atlántico en etapa *pre-smolt*

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ABSTRACT. Salt baths in the production system of salmonids in fresh water, are important for the prevention and control of different diseases such as saprolegnia fungus. This standard procedure generates a rapid depletion of O_2 in the water, leading to a significant increase of CO_2, affecting blood parameters associated with animal welfare, such as Na^+, Cl^-, glucose and protracted hypercarbia. The aim of this study was to evaluate the response field of blood parameters related to fish welfare and the physical-chemical parameters of water to which they are subject during this period, comparing two sets of bath salt. One group of farmed Atlantic salmon (*Salmo salar*) in the pre-smolt stage was exposed in a salt bath system with the usual conditions and a second to a recirculation system with a waterfall for degasification, with two different exposure periods of one and two hours. The results of the concentration of CO_2 in the water show significant variations between the two systems without recirculation compared to the control condition and salt. Significant differences were observed between the recirculating system and the control in blood levels of pCO_2 and HCO_3, but not pH. Our field data also show that salt baths that are performed regularly at the fish farms generate significant effects on blood parameters associated with animal welfare specifically fish pCO_2 and HCO_3 causing minor hypercarbia without generating blood acidosis. The addition of water recirculation with a waterfall does not affect blood parameters.

Key words: salt bath, water chemistry, saprolegnia, animal welfare.

RESUMEN. Los baños de sal, dentro del sistema productivo de salmónidos en agua dulce, son un paso clave en la prevención y el control de diferentes enfermedades como la saprolegnia. Este procedimiento habitual genera un agotamiento rápido del O_2 en el agua, lo que genera un aumento significativo del CO_2 en el agua, afectando parámetros sanguíneos asociados a bienestar animal, como Na^+, Cl^-, glucosa y en situaciones prolongadas hipercapnia. Este trabajo tiene como objetivo evaluar en terreno la respuesta de parámetros sanguíneos asociados a bienestar de los peces y parámetros físico-químicos del agua a la que están sometidos en este periodo, comparando dos sistemas de baño de sal. Para ello se sometió a un grupo de *Salmo salar* en etapa *pre-smolt* a un baño de sal habitual, otro grupo a un sistema de recirculación con cascada de agua para su desgasificación y un grupo control sin sal, exponiéndolos a diferentes periodos de una y dos horas. Los resultados de la concentración de CO_2 en el agua muestran variaciones significativas entre ambos sistemas con y sin recirculación para la condición control y con sal. Los niveles sanguíneos evaluados en terreno de PCO_2 y HCO_3 presentaron diferencias significativas respecto del sistema con recirculación y al control, no así los niveles sanguíneos de pH. Nuestros datos de terreno demuestran que los baños de sal que se realizan con regularidad en los centros de cultivo generan efectos significativos sobre parámetros sanguíneos asociados a bienestar animal de los peces, específicamente PCO_2 y HCO_3, provocando hipercapnia leve sin generar acidosis sanguínea, al añadir recirculación del agua con una cascada a los baños de sal, no se ven afectados los parámetros sanguíneos.

Palabras clave: baño de sal, química del agua, saprolegnia, bienestar animal.

INTRODUCTION

In recent years, animal welfare has become an increasingly relevant subject for consumers and regulatory authorities in various countries, and the importance of animal welfare in the aquaculture industry has consequently grown (Hastein 2004). Animal welfare is particularly applicable to the aquaculture industry given the existing relationship between stressful situations and poor immune system responses in cultivated fish, which provokes increased rates of pathogenic infection (Dhabhar 2009, Tort 2011, Vargas-Chacoff *et al* 2014). There are a wide variety of stressful stimuli in salmonid farming, among which, animal management and confinement are the most significant (Kestin 1994). Confinement stress resulting from high stock density is produced due to a reduced water volume where fish are held, causing a social response and increased physiological activity (Thomas *et al* 1999). In turn, management procedures, such as vaccination, sanitary baths, transport, and taking biometric measurements,

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activate biochemical processes associated with the stress response (Gesto *et al* 2013).

In salmon farming, there are two types of stress responses. The first is an acute response to short-term events, such as fish capture and handling, biometric analyses, and transport. The second is a chronic response to persistent, long-term conditions such as high stock density, variations in water quality, exposure to new environments, social dominance, and exposure to certain diseases (Wendelaar Bonga 1997, Polakoff *et al* 2006, Tort 2011, Vargas-Chacoff *et al* 2014). Salt baths are one of the management procedures used by the aquaculture industry. These baths are generally administered in non-circulating tanks, generating rapid O_2 depletion in the water, a significant increase in CO_2 concentration, and a consequent pH decrease (Auro de Ocampo and Ocampo-Camberos 1999). In goldfish (*Carassius auratus*), salt baths can significantly affect blood parameters, such as Na, Cl, and glucose, depending on the salt concentration and exposure time (Burgdorf-Moisuk *et al* 2011).

In the culture of freshwater fish, salt baths are commonly used to treat pathogens such as Flavobacterium and Saprolegnia sp (Rasowo *et al* 2007, Mifsud and Rowland 2008, Wangen 2012). *Saprolegnia* sp are oomycete fungi more closely related to chromophyte algae than other fungi, meaning that the fungicides used for the control of this genus are ineffective (Avendaño 2012). *Saprolegnia* sp is both primary and secondary, or opportunistic, pathogens that can infect completely healthy salmon or dead tissue. Saprolegniosis was for a long time kept under control using green malachite. However, the use of this carcinogenic chemical compound is now prohibited (Mifsud and Rowland 2008, Avendaño 2012). Currently, salt is still used as an effective control method against saprolegniosis. Salt baths typically last one hour and employ concentrations between 1.5-3.0%, with oomycetes of *Saprolegnia* sp unable to proliferate at NaCl concentrations greater than 1.75% (Avendaño 2012, Wangen 2012). On the other hand, low concentration salt baths counterbalance the osmotic stress produced by cutaneous lesions by preventing electrolyte loss (Van West 2006, Wangen 2012).

The objectives of the present study were to evaluate the effects of salt baths in the field on the physicochemical parameters of the water and how these would impact blood parameters associated with the welfare of pre-smolt Atlantic salmon (*Salmo salar*). To achieve these aims, two salt bath systems for the control of *Saprolegnia sp.* were evaluated in the field using the i-STAT equipment to determine variations in the physicochemical properties of the water and in blood parameters (i.e. glucose, Na^+, K^+, Cl^-, pH, PCO_2, HCO_3).

MATERIAL AND METHODS

Pre-smolt *S. salar* salmon were acquired from a fish farm located in Hornopirén of the Lakes Region, Chile (Salmones FrioSur S.A.). The samples were translated to the bioassay laboratory of the same farm and were randomly distributed among eight tanks (0.9 m^3) at a density of 5.2 kg/m^3 (n = 350 fish weighing 110 ± 5 g per tank).

This study was performed in the field and within the framework of a Salmones FrioSur S.A. Salt Bath Improvement Program.

The fish were acclimated for ten days and were fed daily with a Golden Activia de Biomar diet in proportion to 1% corporal biomass. Prior to administering the treatments, the fish were maintained under fasting conditions for two days. The experimental treatments consisted in the following: i) a traditional salt bath with a closed water recirculation system (CS) and ii) a salt bath with an open water recirculation system (OS). The OS included a waterfall design to provide greater oxygenation and to extract CO_2 from the water. Both systems (CS and OS) without the addition of salt were used as controls. Each treatment and control were evaluated in duplicate.

Prior to salt bath administration, each treatment was saturated with oxygen (100%). Once the salt bath assay began, the following parameters were measured every 20 minutes in all groups: dissolved Oxygen (mg/L), measured with an oxygenometer (OxyGuard Handy Polaris); CO_2 (mg/L), determined with a portable device (OxyGuard CO_2), temperature (°C), conductivity (μS/cma), and salinity and pH, which were measured with a multiparameter meter (HANNA HI 9828). As a security measure, if the dissolved oxygen levels fell below 7.5 mg/L, O_2 was injected until reaching 10.0 mg/L.

For the analysis of blood parameters, six to eight fish were randomly selected at the end of each treatment, control, and corresponding replica. For sampling, the fish were anesthetised with Aqui-S. Then, blood was collected with 1 mL heparinized needles via caudal punctures. The blood samples were quickly placed in a CG8+ cartridge and analysed by the i-STAT equipment (Abbot) to quantify the blood levels of Na^+, K^+, Cl^-, pH, PCO_2, HCO_3, and glucose. The obtained measurements were corrected for temperature using previously described equations (Hosfeld *et al* 2008, Gallagher *et al* 2010, Merkin *et al* 2010).

STATISTICAL ANALYSIS

The physicochemical parameters of water are represented as the mean ± SD of 2-3 samples. Blood parameters are represented as the mean ± SE of 6-8 fish. The results were statistically evaluated using Statistica v7.0 for Windows. Prior to analyses, the data were evaluated for normality and homogeneity of variance. Then, a two-way analysis of variance was performed, where the independent variables were the control, the CS and OS conditions, and the exposure time to each treatment. In turn, the dependent variables were the salinity, pH oxygen and CO_2 concentrations in the water and the blood levels of glucose, Na^+, K^+, pH, Cl^-, PCO_2, and HCO_3. A posterior Tukey test was used to evaluate the differences between groups

RESULTS

Regarding the physicochemical properties of the water, no significant differences were observed in the O_2 levels for any of the treatments as compared to the controls ($P > 0.05$, table 1). In the case of the CS treatment group, 20 minutes after the start of the trial, O_2 was injected into the tank, but levels never surpassed 9.5-10.0 mg/L, maintaining the same conditions as the remaining groups. Significant differences ($P < 0.05$) were observed between the CO_2 levels of the control groups and treatment salt bath groups at both one and two hours. Specifically the traditional CS baths (figure 1A) evidenced a significant increase in CO_2 levels, surpassing the critical levels (10 mg/L) for salmonids (Tang *et al* 2009) in the two hour treatment group. In contrast, the CO_2 levels of the OS treatment groups (figure 1B) were slightly increased but remained well below critical levels for all treatment times.

The pH levels of the water did not show significant differences against the control ($P > 0.05$), with values between 6.6 and 6.1 in the CS treatment groups (table 1). Furthermore, no significant differences ($P > 0.05$) in water salinity were found between the CS and OS treatment groups (15.2 g/L; table 1). The control group, with no salt, presented a salinity of 0.06 g/L.

The plasmatic parameters of Na^+ did not present significant differences between the treatment and control groups (figure 2A). A similar result was found for the blood levels of Cl^-, which also showed no significant differences between groups (figure 2B). Additionally, the average levels of K^+ in the blood were 3.42 ± 1.36 mmol/L, with no significant differences between the treatment and control groups (figure 3). Blood pH did not significantly vary in relation to the control groups, presented average values of 7.16 ± 0.036. Glucose levels also did not significantly vary between the treatments and controls (figure 3).

Table 1. Average concentration of oxygen, pH and salinity in the water treatment ponds (± SD).
Concentraciones promedio de oxígeno, pH y salinidad en el agua de estanques en tratamiento (± DE).

	Closed recirculation						Open recirculation					
	Control			Treatment			Control			Treatment		
	Time (minutes)			Time (minutes)			Time (minutes)			Time (minutes)		
	0	60	120	0	60	120	0	60	120	0	60	120
Dissolved oxygen (mg/L)	8.2±0.8	8.3±1.0	8.7±0.9	11.3±1.6	9.9±2.2	9.4±1.7	9.5±0.4	9.1±0.4	9.3±0.4	10.0±0.9	9.5±0.8	9.1±0.8
pH	6.4±0.1	6.3±0.1	6.3±0.1	6.3±0.2	6.1±0.2	6.1±0.2	6.6±0.0	6.5±0.0	6.5±0.0	6.3±0.1	6.2±0.1	6.2±0.1
Salinity (g/L)	0.06±0.0	0.06±0.0	0.06±0.0	15.2±0.6	14.8±0.0	15.1±0.1	0.06±0	0.06±0.0	0.06±0.0	15.0±0.0	15.0±0.0	14.4±0.1

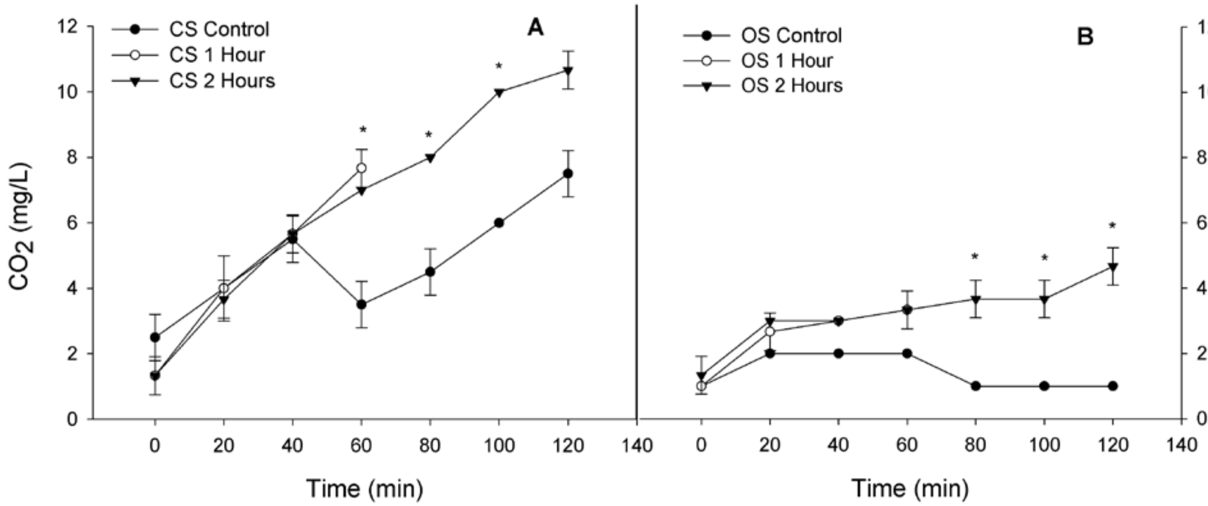

Figure 1. Water concentrations of CO_2 over the duration of the salt baths. A) Closed water recirculation system, CS. B) Open water recirculation system, OS. Data represent the mean ± S.E. * indicates significant differences ($P < 0.05$) compared to the control.

Concentraciones de CO_2 en agua durante el baño de sal. A) Sistema sin recirculación, CS. B) Sistema con recirculación OS. Los datos representan la media ± EE * significa diferencias significativas ($P < 0,05$) respecto del control.

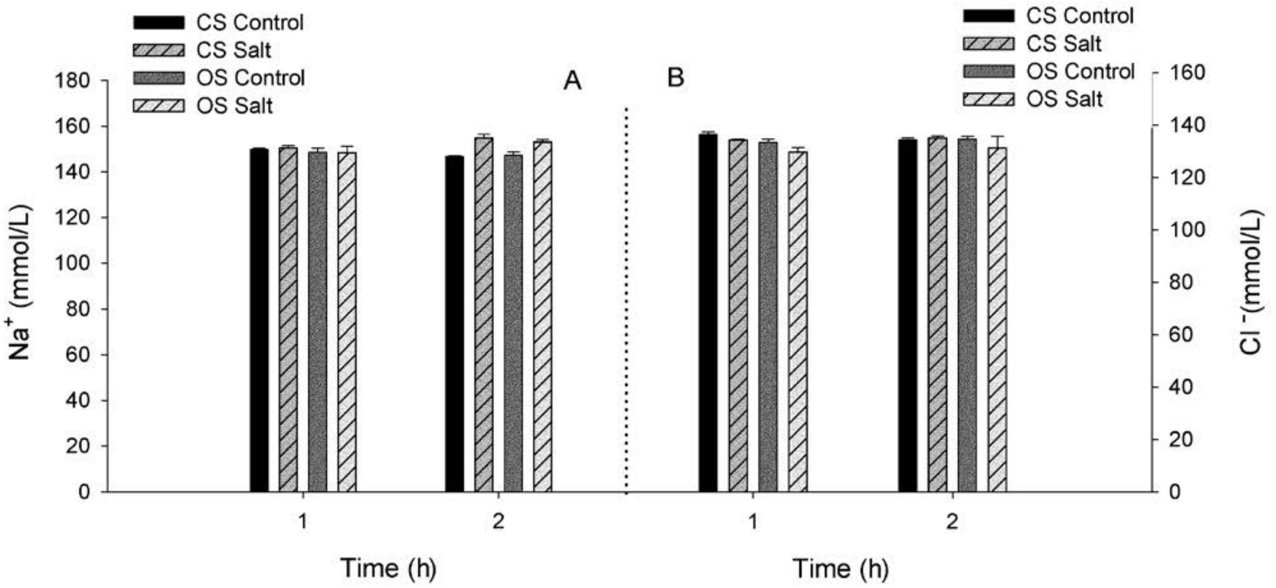

Figure 2. Changes in plasmatic A) Na^+ levels and B) Cl^- levels in fish from closed recirculation (CS) and open recirculation (OS) systems. Data are represented as the mean ± S.E. of 6-8 fish.

Concentración plasmática de sodio (Na^+) de peces *Salmo salar* en ambos sistemas sin recirculación (CS) y con recirculación (OS), los datos representan la media ± E.E.M. de 6 a 8 peces; Concentración plasmática de cloro (Cl^-) de peces *Salmo salar* en ambos sistemas, sin recirculación (CS) y con recirculación (OS). Los datos representan la media ± EE de 6 a 8 peces.

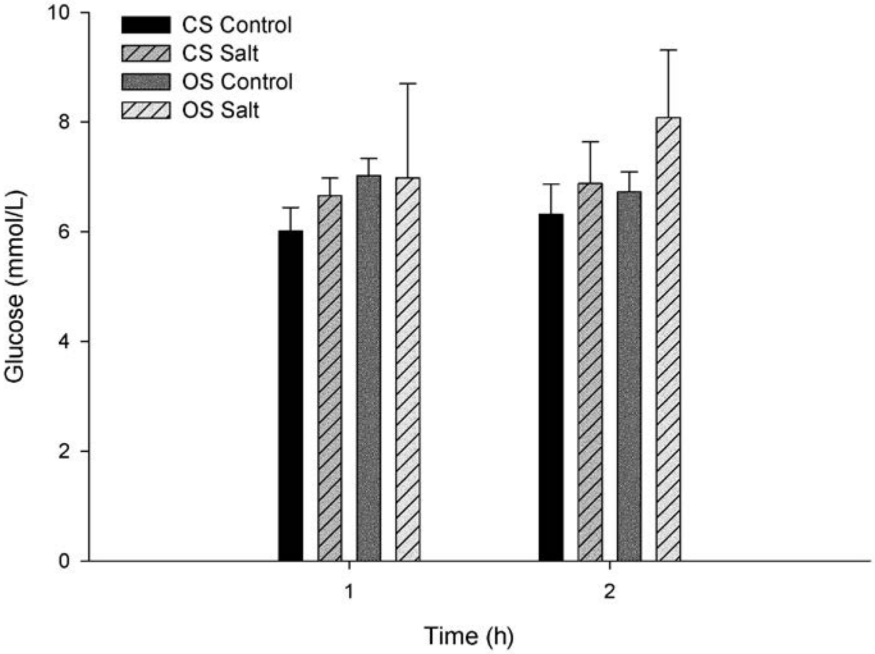

Figure 3. Plasma concentration of glucose fish in both systems; CS without recirculation with recirculation OS. The data represent the mean ± SE of 6-8 fish.

Concentración plasmática de glucosa de peces *Salmo salar* en ambos sistemas; Sin recirculación CS, con recirculación OS. Los datos representan la media ± EE de 6 a 8 peces.

Regarding the blood levels of gases, PCO_2 presented significant differences between OS and CS fish, where the PCO_2 levels of the two-hour CS treatment group were significantly greater ($P < 0.05$) than the one-hour CS control group (figure 4). Furthermore, both the treatment and control OS groups had lower PCO_2 levels at two hours than the CS groups, but these differences were not significant.

Fish exposed to a CS salt bath for two hours showed the highest blood levels of HCO_3, reaching levels significantly different than the control (figure 5).

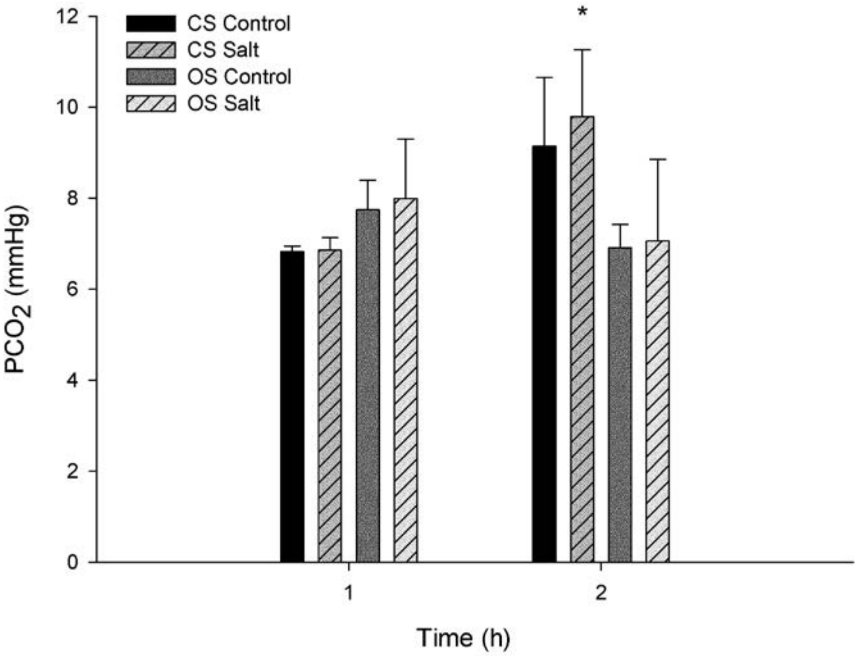

Figure 4. Plasma concentration of PCO_2 fish in both systems; CS without recirculation with recirculation OS. The data represent the mean ± SE of 6-8 fish. * Indicates significant differences ($P < 0.05$) compared to control.

Concentración plasmática de PCO_2 de peces *Salmo salar* en ambos sistemas; Sin recirculación CS, con recirculación OS. Los datos representan la media ± EE de 6 a 8 peces. * Indica diferencias significativas ($P < 0,05$) respecto del control.

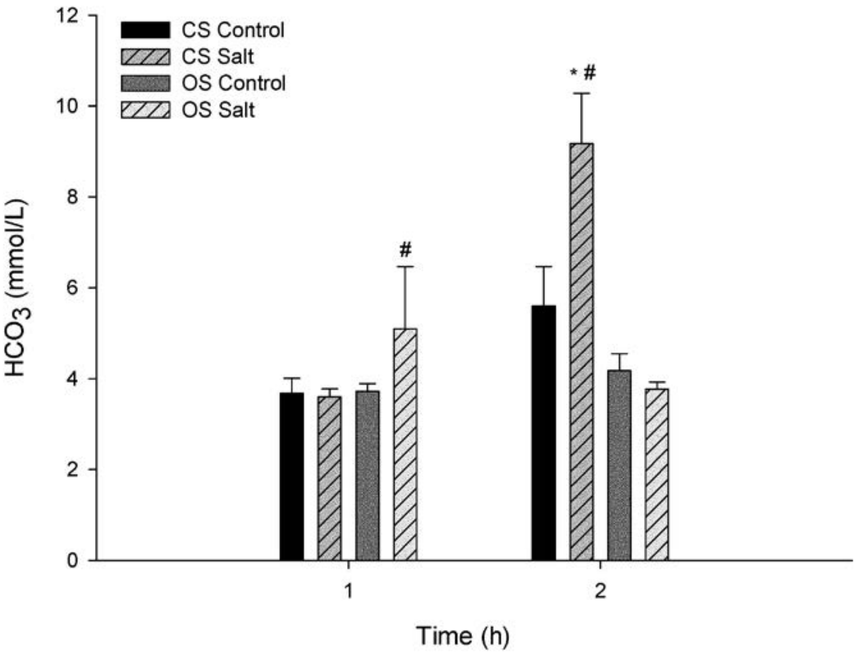

Figure 5. Plasmatic HCO_3 variations n fish from closed recirculation (CS) and open recirculation (OS) systems. Data are represented as the mean ± S.E. of 6-8 fish. * indicates significant differences ($P < 0.05$) compared to the control; # indicates significant differences ($P < 0.05$) between systems.

Variación de los niveles plasmáticos de HCO_3 de peces *Salmo salar* en ambos sistemas; Sin recirculación CS, con recirculación OS. Los datos representan la media ± E.E.M. de 6 a 8 peces. * significa diferencias significativas ($p < 0,05$) respecto del control, # significa diferencias significativas ($P < 0,05$) entre sistemas.

DISCUSSION

Salt baths alter physiological functions, such as metabolism and osmoregulation, in fish (Burgdorf-Moisuk *et al* 2011). These alterations are associated with increased respiration, as observed in the present study through the rapid uptake of O_2 and significant increase in CO_2 in the water of salt bath-treated groups (figure 1, table 1). Specifically, the CO_2 levels recorded for the water of the two-hour CS treatment group reached 12 mg/L, and CO_2 levels between 12 and 30 mg/L have been found to affect the growth, conversion rate, and immune status of *S. salar* (Smart *et al* 1979, Wedemeyer 1996, Fivelstad *et al* 1998). Wedemeyer (1996) suggested that salmonids can tolerate CO_2 levels up to 40 mg/L during transport or over short periods (Tang *et al* 2009). In contrast, the control OS and CS groups did not surpass CO_2 levels of 4 mg/L, which were nearly basal as compared to the CS treatment groups. Importantly, as individual factors, salt and a CS were able to significantly increase the CO_2 levels in water, as evidenced by the lower CO_2 levels in both the CS and OS control groups (figure 1). The increased CO_2 levels in the treatment groups likely represent a stress response due to increased salinity. This would be in agreement with that reported by Morgan and Iwama (1991), who found that a sudden increase in salinity is related to increased metabolic costs and, consequently, greater oxygen uptake.

The oxygen levels in water were variable for both the control and treatment groups. In the case of the CS groups, the oxygen levels decreased to 7.5 mg/L after 20 minutes, requiring the injection of oxygen to maintain levels near 10 mg/L. The OS treatment group permitted a greater exchange of gases between the water and air, thus effectively reducing CO_2 excess while naturally increasing the availability of oxygen. Ultimately, this would result in better respiration for the fish, thereby reducing physical stress and facilitating better adaptation to salt baths.

The levels of Na^+ and Cl^- in the plasma are relevant indicators of ion transfer through the gills, and changes in these levels represent the first response of a fish when the respiratory regulation of the acid-base balance becomes limited (Perry and Gilmour 2006). For example, rainbow trout (*Oncorhynchus mykiss*) evidence significantly increased plasma Na^+ levels when exposed to conditions of hypercapnia, or high levels of CO_2 (Larsen and Jensen 1997). The results of the present study did not reflect changes in plasmatic Na^+, which might be due to the short exposure period to hypercapnia conditions. Regarding Cl^-, plasmatic Cl^- levels are reduced as a result of an electro-neutral exchange of ions with HCO_3, resulting in a relationship between plasmatic Cl^- levels and HCO_3 synthesis (Lloyd and White 1967, Fivelstad *et al* 2003, Hosfeld *et al* 2008, Fivelstad *et al* 2015). Contrasting these prior studies, the present results did not evidence this positive relationship, which could, once again, be due to the short experimental period evaluated.

Likewise, blood glucose levels did not significantly vary between the currently assessed treatment and control groups. However, glucose levels in the CS treatment groups were slightly elevated as compared to the control groups for both evaluated time periods. In goldfish, one-hour salt baths at a concentration of 20 g/L modify plasmatic glucose levels (Burgdorf-Moisuk *et al* 2011). The present study was performed under field conditions and in water with controlled oxygen levels, which is noteworthy considering the possible relationship between blood glucose levels and plasmatic oxygen levels (Perry *et al* 1989).

It is very important to highlight that blood pH did not present significant variations between the assessed groups, suggesting an internal adaptation of fish to an acute period of stress. If plasmatic pH falls and respiratory acidosis occurs, rainbow trout are able to compensate for a period of 48-72 hours, while Atlantic salmon can compensate for 24-96 hours. One way in which salmonids adapt to pH variation is by increasing blood levels of HCO_3, which consequently promotes CO_2 transport in the blood (Foss *et al* 2007).

The recorded blood concentrations of HCO_3 and PCO_2 reflect the physicochemical parameters of the water, where a relationship was found between plasmatic HCO_3 and water CO_2 levels in both the OS and CS treatment conditions. This relationship is due to the high permeability of the gills to CO_2, and in conditions of hypercapnia, rapid internal equilibration occurs by elevating the levels of PCO_2 in the blood and tissues (Tang *et al* 2009). Once internalised, CO_2 is rapidly transformed by the enzyme carbonic anhydrase into carbonic acid (H_2CO_3), resulting in blood acidosis. Furthermore, studies have found that prolonged hypercapnia leads to increased blood levels of PCO_2, activating the primary stress response in fish (Perry *et al* 1989) and leading to changed pH levels (Eddy *et al* 1977, Thomas and Le Ruz 1982), increased respiratory volume and rate (Janssen and Randall, 1975, Thomas *et al* 1983, Fivelstad *et al* 1999)PWCO 2=5 TorrPWO 2=400\u2013450 TorrSalmo gairdneri R.9934\\uc0\\u8211{ }36, reduced O_2 levels, and variations in transport ability through the Bohr and Root effects (Eddy and Morgan 1969, Eddy *et al* 1977, Wedemeyer 1996).

Blood pH was not significantly affected, indicating that the assessed fish did not suffer prolonged hypercapnia. However, HCO_3 and PCO_2 levels were significantly affected, evidencing moderate hypercapnia, the effects of which were offset by the fish. Among the adaptation strategies of fish to hypercapnia exposure are increased ventilation and the use of chemosensors on the periphery of the gills that monitor environmental CO_2 levels (Burleson and Smatresk 2000, Reid *et al* 2000, Gilmour 2001, Perry and McKendry 2001). These chemosensors are only stimulated by changes in the water concentration of CO_2 and not by changes in pH (Perry and McKendry 2001).

Salt baths administered with an open recirculation system did not significantly affect the physicochemical parameters of the water as compared to the untreated control group. In contrast, salt baths administered with a closed recirculation system resulted in increased water CO_2 concentrations. Likewise, the two-hour, closed recirculation treatment group showed significantly affected blood parameters (i.e. PCO_2 and HCO_3) as compared to the respective control. These changes resulted in moderate hypercapnia, directly affecting animal welfare in culture conditions.

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