

## Field studies of ammonia excretion in *Aphanius iberus* (Pisces; Cyprinodontidae): body size and habitat effects

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### Summary

The effects of body size and habitat variability on ammonia excretion rates ( $R_{AMs}$ ) of *Aphanius iberus* were analyzed *in situ* for the first time. At hourly intervals during a 5-h field experiment, ammonia excretion was measured in 75 mature specimens from three sampling sites (small creek, marine salt-mine, and salt-marsh) established in a gradient of water salinity (0–5; 35–40; 65–70‰). Our results showed a specific size dependence pattern of  $R_{AMs}$  in the reproduction period, which might reflect an effect of the reproductive effort. In addition, the results point to a significant decrease in mean  $R_{AM}$  values of each population from freshwater aquatic systems ( $3.81 \pm 0.58 \mu\text{mol g}^{-1} \text{h}^{-1}$  in fish of  $2.8 \pm 0.3$  mm total length, TL) to salt aquatic systems with significantly higher alkalinity ( $2.52 \pm 0.35 \mu\text{mol g}^{-1} \text{h}^{-1}$  in fish of  $3.1 \pm 0.5$  mm TL in marine salt-mine;  $1.98 \pm 0.55 \mu\text{mol g}^{-1} \text{h}^{-1}$  in fish of  $3.1 \pm 0.4$  mm TL in salt-marsh). Due to the size-dependent pattern,  $R_{AM}$  in different habitats cannot be compared directly; ANCOVA, followed by residual compared analysis (regression-related techniques), is seen as a valid method for this purpose. This work presents the first field data on ammonia excretion in the *Aphanius* genus and the flexible physiologic response characteristic of Cyprinodontids has been demonstrated.

### Introduction

Fish from habitats characterized by a wide range of salinity fluctuations (estuaries, salt marshes, intertidal pools, etc.) show ecophysiological adaptations that enable them to tolerate and survive both extreme salinity levels and fluctuations (Nordlie and Haney, 1998; Plaut, 2000). Although most teleost fish are ammoniotelics (Wood, 1993), nitrogen metabolism and excretion are environmentally influenced (Hollingworth, 2002), and it is normal to find a relationship between the total ammonia proportion of their nitrogenous waste and the salinity of their habitat (Jobling, 2002). Very few studies have addressed the influence of water salinity on nitrogen excretion (Wright et al., 1995). Although laboratory studies have described this relationship (Sayer and Davenport, 1987, among others), little is known about the effect of salinity on excretion products via field studies. Moreover, ammonia excretion is strongly dependent on environmental pH (Danulat, 1995) and several authors have shown that fish in highly alkaline aquatic habitats reduce ammonia in their nitrogenous wastes (Wright et al., 1993; McGeer et al., 1994; Wilkie and Wood, 1996).

Cyprinodontids regularly inhabit freshwater and brackish waters (Parenti, 1981). *Aphanius iberus* (Valenciennes) is an

eurythermic and euryhaline fish mainly inhabiting brackish water of salt marshes, coastal lagoons, and river mouths (Moreno-Amich et al., 1999), although it also occurs in small creeks with little salinity. This ability to tolerate such a wide range of salinities makes it an excellent candidate for studying the effects of salinity on physiologic or ecologic traits. Moreover, this endemic Iberian species is catalogued as 'endangered' in the Red List of freshwater fish from Spain (Doadrio, 2002), and increasing our knowledge of the life history and ecophysiology traits of fish under threat is a necessary tool for management action and conservation programs (Wootton et al., 2000). A few studies describe some aspects of the *A. iberus* life history (Fernández-Delgado et al., 1988; García-Berthou and Moreno-Amich, 1992; Vargas and De Sostoa, 1997; Oltra and Todolí, 2000) or reasons for its decline (Rincón et al., 2002; Caiola and De Sostoa, 2005), but no study has been published on its ecophysiology. Several studies on Cyprinodontid species have examined the effects of salinity on physiologic functions (Nordlie et al., 1991; Nordlie and Haney, 1998; Plaut, 2000). However, to the authors' knowledge, no study has been published on field nitrogenous excretion in any species of the *Aphanius* genus.

The aim of the present work is to provide the first information about the field *in situ* ammonia excretion of *A. iberus* and to analyze its relationship with body size and habitat variations.

### Material and methods

#### Field collection and experimental procedure

Captured were 75 mature specimens from three sampling sites, two established in natural habitats (small creek and salt-marsh), and one on a semi-natural site (marine salt-mine; Table 1). All specimens were from wild populations from the same geographical and genetic group (Operational Conservation Unit *sensu* Doadrio et al., 1996). Legal limitations restricted sample sizes to a maximum of 25 individuals per collection. The experimental period was between 12 and 16 May 1999; details of the studied populations and sample sizes are listed in Table 1.

Because confinement stress could cause effects on nitrogen excretion and eliminate the influence of diet on excretion rates (Brett and Zala, 1975), the specimens at each sampling site were acclimated for 24 h by maintaining them individually in 600 ml vessels of filtered environmental water (Whatman GF/C filter, approximately  $1.2 \mu\text{m}$ ) in each of the studied habitats. The assays were performed *in situ* under natural temperature and photoperiod, but fish in the individual vessels ( $n = 25$  in

Table 1

Localities, sample size, total length of experimental individuals (TL), habitat data, and physicochemical parameters sampled each hour during 24-h acclimation (mean  $\pm$  SD; m = males; f = females)

Locality	Sample size (m/f)	TL (mm)	Habitat	Salinity (‰)	pH	Temperature <sup>a</sup> (°C)	Oxygen (mg L <sup>-1</sup> )
Chicamo	13/12	2.80 $\pm$ 0.32	Small creek	0–5	6.71 $\pm$ 1.60	19.9 $\pm$ 2.6	11.2 $\pm$ 2.6
Marchamalo	12/13	3.09 $\pm$ 0.45	Marine salt-mine	35–40	8.79 $\pm$ 0.30	19.8 $\pm$ 2.4	13.4 $\pm$ 3.0
Carmoli	12/13	3.07 $\pm$ 0.36	Salt-marsh	65–70	8.13 $\pm$ 0.43	25.3 $\pm$ 3.4	08.6 $\pm$ 2.2

each population) were protected from direct sunlight. After 24 h acclimation, the vessel water was replaced by filtered environmental water from the same locality; this procedure was carried out with care so as not to cause stress to the individuals.

After 2 h, in each procedure at hourly intervals (h1–h5) during the same 5 h (11.00–16.00 hours), 10 ml of chamber water of each specimen was transferred to a scintillation vial and immediately frozen on dry ice (volumes not replaced). Control vessels (no fish) were set up and sampled in an identical manner.

During acclimation and experimental periods a fixed structure to 25 experimentation vessels was placed in each locality and vessels were not moved. Furthermore, a control of physicochemical parameters (temperature, pH, and oxygen) was made at the individual level.

#### Laboratory analyses

Water samples were stored in the laboratory at  $-80^{\circ}\text{C}$  until analysis. All assays were performed in duplicate. Total ammonia ( $\text{NH}_3 + \text{NH}_4^+$  concentrations) was quantified using a micro-version (1 ml) according to the spectrophotometric method described by Ivancic and Degobbi (1984) and used in Walsh et al. (1990) and Danulat and Kempe (1992). In this method, total ammonia was measured directly in environmental water diluted with 10 mM HEPES, 3 mM EDTA, pH 6.5. A second aliquot was treated with a buffer containing urease, which specifically liberated ammonia from urea. Ammonia concentrations were read at 638 nm by a Fluostar-Galaxy<sup>®</sup> spectrophotometer.

#### Calculations and statistics

Weight-specific ammonia excretion rates ( $R_{\text{AM}}$ ) were calculated as follows:

$$R_{\text{AM}} = [(C_f - C_i) - (C_c - C_i)]VWt^{-1} = (C_f - C_c)VWt^{-1},$$

where  $C_f$  and  $C_i$  are final and initial ammonia concentrations ( $\mu\text{M}$ ),  $C_c$  is the ammonia concentration in the controls ( $\mu\text{M}$ ),  $V$  is volume of the system (L),  $t$  is the elapsed time (h), and  $W$  is the wet mass of the fish (g). Wet mass was estimated using mass-length relationships from each studied population obtained on the same date in specimens not exposed to the experiment ( $n_{\text{population}} > 50$ ), the measure of the goodness for estimated values was evaluated with the statistic  $R^2$ -adjusted ( $R^2_{\text{Chicamo}} = 0.958$ ;  $R^2_{\text{Marchamalo}} = 0.970$ ;  $R^2_{\text{Carmoli}} = 0.941$ ).

The statistical analysis used to compare fish excretion between sexes and among populations followed that proposed in García-Berthou and Moreno-Amich (1993), which is based on the application of univariate analysis of covariance (ANCOVA), using mean of excretion rate as the dependent variable and total length (TL) as the covariate. The relationships between TL and ammonia excretion rate were clearly

non-linear, although the log-transformation of the data appeared to linearize them. To avoid the use of negative values, we previously multiplied the variables by  $10^2$ . Ammonia excretion was compared by residuals from the relationship between TL and ammonia excretion rate (dependent variable) of the total specimens. Previously we tested the homogeneity of the regression coefficients (slopes) of the dependent-covariate relationship with an ANCOVA design that analyzed the pooled covariate-by-factor interaction. If the covariate-by-factor interaction (homogeneity of slopes) is not significant ( $P > 0.05$ ), this procedure removes the body length effects in the comparison (Sutton et al., 2000). To determine whether residual values differed significantly between populations an ANOVA test was carried out.

Statistical analyses were performed with SPSS<sup>®</sup> software package and accepted at a significance level of 0.05.

## Results

#### Data adjust

Prior to transferring the experimental individuals (zero time), total ammonia concentrations in the flask water were always below the detection limit of the assay. Control flasks (no fish) showed undetectable concentrations of ammonia (no toxic levels), which remained unchanged throughout the experiment.

Although the experiment did not show a totally homogeneous excretion pattern, ammonia was excreted at maximum rates during the first hour (Fig. 1). Two of the studied populations (Marchamalo and Carmoli in the case of males, Table 2) showed significant homogeneity of variance and no differences between mean  $R_{\text{AM}}$  values only in the last three experimental hours (h3–h5); hourly interval in Table 2 (see excretion data in Fig. 1). For this reason, weight-specific ammonia excretion rates were calculated using data only from the last three experimental hours when ammonia excretion rates were stabilized in variance and mean values.

#### Ammonia excretion: relation to body size and sex

There were significant differences in fish TL between populations [ANOVA,  $F(2,72) = 3.81$ ,  $P = 0.027$ ]; thus, we first compared the TL– $R_{\text{AM}}$  relationships between sexes in each population [ANCOVA test, sex as a factor: Chicamo  $F(1,21) = 0.81$ ,  $P = 0.380$  in the slope; Marchamalo  $F(1,24) = 0.14$ ,  $P = 0.715$  in the slope; Carmoli  $F(1,24) = 1.56$ ,  $P = 0.226$  in the slope] and between sampling sites for each sex [ANCOVA test, locality as a factor: males  $F(2,36) = 2.62$ ,  $P = 0.09$  in the slope; females  $F(2,35) = 0.19$ ,  $P = 0.826$  in the slope]. Secondly, we compared TL– $R_{\text{AM}}$  relationships between males and females in all specimens from the three sampling sites [ANCOVA test, sex as a factor:  $F(1,73) = 0.86$ ,  $P = 0.359$  in the slope]. Since no significant differences were obtained in any of these relationships, we could establish a specific relationship between TL

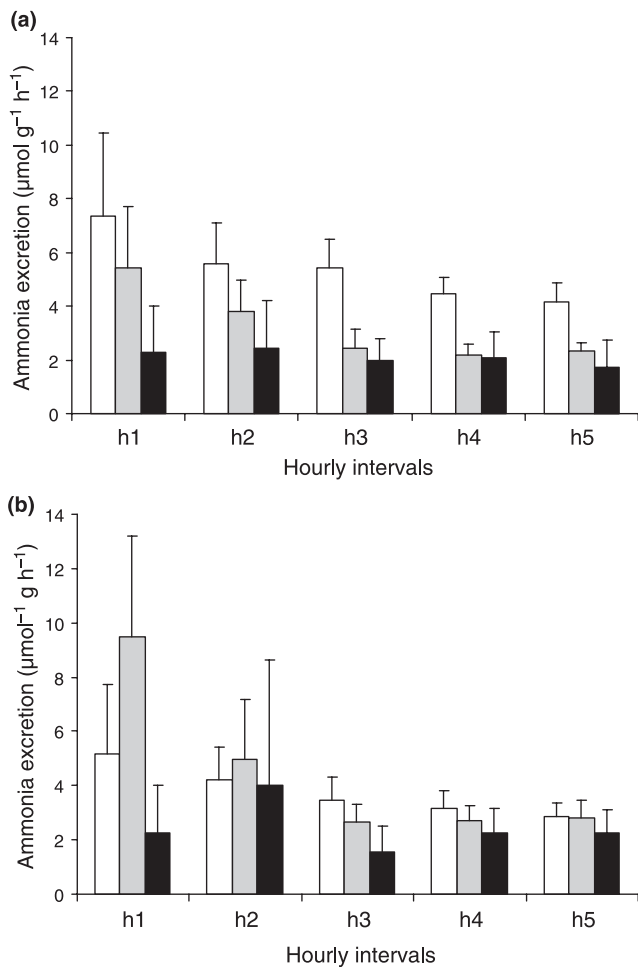


Fig. 1. Ammonia excretion ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ ) at hourly intervals (h1–h5; h1: first experimental hour, h2: second hour, etc.) in each flask containing one *Aphanius iberus* individual. Mean  $\pm$  CL (95%) for all studied females (a) and males (b) from each population (□ Chicamo; ■ Marchamalo; ■ Carmoli)

and  $R_{AM}$  for the studied populations. Weight-specific ammonia excretion rates decreased with increasing fish length and showed a length-scaling exponent of  $-1.80 \pm 0.29$  [log-transformation data; regression analysis:  $R^2 = 0.38$ ;  $F(1,73) = 41.22$ ;  $P < 0.0005$ ; Fig. 2].

There were significant differences in ammonia excretion rates between sexes because females showed significantly higher standard residual values than males [females:  $0.42 \pm 0.28$ ; males:  $-0.41 \pm 0.36$ ;  $F(1,73) = 14.77$ ;  $P < 0.0005$ ]. Significant differences were also obtained in Chicamo [females:  $1.16 \pm 0.23$ ; males:  $-0.13 \pm 0.24$ ;  $F(1,21) = 15.11$ ;  $P = 0.001$ ] and Carmoli [females:  $0.42 \pm 0.28$ ; males:  $-0.93 \pm 0.34$ ;  $F(1,24) = 6.14$ ;  $P = 0.021$ ], but not in Marchamalo [females:  $0.04 \pm 0.21$ ; males:  $-0.15 \pm 0.21$ ;  $F(1,24) = 2.36$ ;  $P = 0.138$ ]. Due to a size effect on samples, the differences between sexes were not confirmed by the analysis of the total experimental  $R_{AM}$  values (total females:  $3.06 \pm 0.51 \mu\text{mol g}^{-1} \text{h}^{-1}$ ; total males:  $3.11 \pm 0.48 \mu\text{mol g}^{-1} \text{h}^{-1}$ ; separated sampling sites in Table 3).

#### Ammonia excretion: relation to habitat

To compare weight-specific ammonia excretion rates among localities (Chicamo, Marchamalo, and Carmoli), we used the standardized residuals obtained from the specific relationship

for all studied populations. This procedure removed the body length effects due to differences in TL among populations. Standardized residuals showed significant differences between sampling localities [ANOVA,  $F(2,72) = 4.734$ ,  $P = 0.012$ ] and, although the number of compared localities was statistically low, they showed a significant decrease with water salinity [regression analysis:  $b = -0.014 \pm 0.005$ ,  $R^2 = 0.12$ ;  $F(1,73) = 9.13$ ;  $P = 0.004$ ; Table 3]. In absolute terms (mean  $R_{AM}$  for each population), there is a significant decrease (Chicamo > Marchamalo > Carmoli): Chicamo  $3.81 \pm 0.58 \mu\text{mol g}^{-1} \text{h}^{-1}$  (0.42 using standardized residuals); Marchamalo  $2.52 \pm 0.35 \mu\text{mol g}^{-1} \text{h}^{-1}$  (0.07 using standardized residuals); and Carmoli  $1.98 \pm 0.55 \mu\text{mol g}^{-1} \text{h}^{-1}$  (-0.43 using standardized residuals; see excretion data in Table 3).

#### Discussion

The high initial excretion rates detected in our field *in situ* procedure was thought likely due to 'handling stress' (Danulat and Kempe, 1992) caused by the transfer of the fish into the experimental vessels. However, our first step in the data analysis allowed us to eliminate this effect to calculate weight-specific ammonia excretion rates ( $R_{AMs}$ ).

Moreover, a critical factor when interpreting fish physiologic parameters in a way that may be both useful and applicable is to use the correct statistical methodologies when analyzing them. To investigate inter-population variations in relative size indices (ratio-related techniques) such as  $R_{AMs}$  in the present work, the use of ANCOVA before residual analysis (regression-related techniques) provided a valid method for this purpose (Sutton et al., 2000) because they allowed the adjustment of the size variation in individuals from different populations. In fact, adjustment of size variation in the data by residual values compared analysis has also been used in studies of ecophysiological traits (including nitrogenous excretion) where they provided valid results (Clarke et al., 1994; Boyce, 1999).

First, our results pointed to a significant size dependence of  $R_{AMs}$  in the studied *A. iberus* populations during the reproduction period. Data are expressed in logarithmic scale to achieve linearity (*sensu* Steinarsson and Moksness, 1996; Leung et al., 1999). This relationship has not been affected by differences between habitats, thus a specific-pattern that agrees in terms of negative slope with models shown by Jobling (1994, 2002) could be established. However, the establishment of the  $R_{AMs}$ -TL does not imply similarity of the mean  $R_{AM}$  value between populations in different habitats.

Because the studied period was included in the first phase of the reproductive period of the three studied populations (unpublished data) and because all experimental individuals were over the length of maturity, a related effect of the reproductive activity could be suggested. The biology of the species is characterized by early maturity, high reproductive effort, and high fecundity by multiple spawning (Vargas and De Sostoa, 1997). A high reproductive effort could lead to a considerable expenditure of energy and time, especially in larger sizes at the beginning of the reproductive period (Fernández-Delgado et al., 1988), which could coincide with a non-feeding period (Wootton, 1998). For example, adult males (studied specimens) spent their energy on defending their territory and on mating, and females of all sizes (especially the largest) showed the highest reproductive effort in terms of fecundity and ovule diameters in May (Vargas and De Sostoa, 1997; Oliva-Paterna et al., 2006).

Table 2  
Ammonia excretion rate ( $R_{AM}$ ) comparisons between experimental hours in each female and male population

Populations	Hourly intervals		
	h1-h5	h2-h5	h3-h5
Chicamo			
Females	$F_{Levene} = 2.940, P = 0.028^*$ $F_{ANOVA} = 2.937, P = 0.028^*$ d.f. (4,56)	$F_{Levene} = 1.371, P = 0.264$ $F_{ANOVA} = 2.405, P = 0.079$ d.f. (3,46)	$F_{Levene} = 0.536, P = 0.589$ $F_{ANOVA} = 3.156, P = 0.054$ d.f. (2,36)
Males	$F_{Levene} = 4.047, P = 0.006^*$ $F_{ANOVA} = 2.243, P = 0.075$ d.f. (4,57)	$F_{Levene} = 2.664, P = 0.060$ $F_{ANOVA} = 2.293, P = 0.091$ d.f. (3,45)	$F_{Levene} = 2.089, P = 0.140$ $F_{ANOVA} = 1.050, P = 0.361$ d.f. (2,35)
Marchamalo			
Females	$F_{Levene} = 16.80, P < 0.001^*$ $F_{ANOVA} = 6.499, P < 0.001^*$ d.f. (4,64)	$F_{Levene} = 6.348, P = 0.001^*$ $F_{ANOVA} = 5.089, P = 0.004^*$ d.f. (3,51)	$F_{Levene} = 1.930, P = 0.160$ $F_{ANOVA} = 0.303, P = 0.740$ d.f. (2,38)
Males	$F_{Levene} = 12.66, P < 0.001^*$ $F_{ANOVA} = 6.543, P < 0.001^*$ d.f. (4,57)	$F_{Levene} = 8.272, P < 0.001^*$ $F_{ANOVA} = 4.129, P = 0.012^*$ d.f. (3,47)	$F_{Levene} = 0.176, P = 0.839$ $F_{ANOVA} = 0.077, P = 0.962$ d.f. (2,35)
Carmoli			
Females	$F_{Levene} = 1.070, P = 0.380$ $F_{ANOVA} = 0.279, P = 0.890$ d.f. (4,59)	$F_{Levene} = 0.958, P = 0.420$ $F_{ANOVA} = 0.315, P = 0.814$ d.f. (3,48)	$F_{Levene} = 1.283, P = 0.292$ $F_{ANOVA} = 0.207, P = 0.814$ d.f. (2,36)
Males	$F_{Levene} = 11.15, P < 0.001^*$ $F_{ANOVA} = 1.494, P = 0.217$ d.f. (4,55)	$F_{Levene} = 13.77, P < 0.001^*$ $F_{ANOVA} = 1.855, P = 0.151$ d.f. (3,46)	$F_{Levene} = 0.535, P = 0.591$ $F_{ANOVA} = 0.746, P = 0.483$ d.f. (2,35)

h1-h5: 5 h; h2-h5: last 4 h; h3-h5: last 3 h.

\*Significant heterogeneity of variance using Levene's test.

\*\*Significant differences between mean  $R_{AM}$  values using ANOVA.

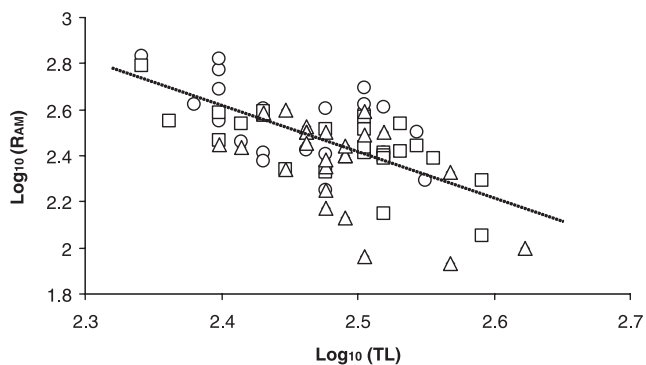


Fig. 2. Relationship between weight-specific ammonia excretion ( $R_{AM}$ ;  $\mu\text{mol g}^{-1} \text{h}^{-1}$ ) and total length (TL; mm) of experimental specimens from the three sampling sites ( $\circ$  Chicamo,  $n = 21$ ;  $\square$  Marchamalo,  $n = 25$ ;  $\triangle$  Carmoli,  $n = 25$ ; log-transformation data)

Moreover, adult individuals have been shown to have a loss of condition in May; this month was seen as a non-growing time (Vargas and De Sostoa, 1997; Fernández-Delgado et al., 1988). This all agrees with the supposition of a non-feeding time or a decrease in the feeding rate during reproduction, which was probably much more remarkable in larger sizes. Non-feeding or decrease in feeding rates are correlated with lower  $R_{AM}$ s in larger individuals (Preez and Cockcroft, 1988; Steinarsson and Moksness, 1996; Gelineau et al., 1998), as was seen in the present work.

The differences in ammonia excretion between sexes seem to be common and, in the present work, could also be related with the reproductive effort. However, we did not obtain significant differences in the Marchamalo sampling site, which is probably the habitat where the species is supporting the lower environmental stress (Oliva-Paterna et al., 2006).

Table 3  
Comparison in  $R_{AM}$  for each population between paired sampling localities (\*significant differences by ANOVA)

Paired comparisons	Total sampling				Males				Females			
	<i>F</i>	d.f.	<i>P</i>	Mean ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ )	<i>F</i>	d.f.	<i>P</i>	Mean ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ )	<i>F</i>	d.f.	<i>P</i>	Mean ( $\mu\text{mol g}^{-1} \text{h}^{-1}$ )
Chicamo (0-5‰)	1.81	1,45	0.185	21 3.81 $\pm$ 0.58	0.01	1,23	0.960	12 3.16 $\pm$ 0.37	8.48	1,21	0.009*	9 4.63 $\pm$ 0.41
Marchamalo (35-40‰)				25 2.52 $\pm$ 0.35				12 2.74 $\pm$ 0.30				13 2.32 $\pm$ 0.26
Chicamo (0-5‰)	7.60	1,45	0.008*	21 3.81 $\pm$ 0.58	3.73	1,23	0.066	12 3.16 $\pm$ 0.37	12.71	1,21	0.002*	9 4.63 $\pm$ 0.41
Carmoli (65-70‰)				25 1.98 $\pm$ 0.55				12 2.08 $\pm$ 0.48				13 1.92 $\pm$ 0.50
Marchamalo (35-40‰)	3.73	1,49	0.059	25 2.52 $\pm$ 0.35	3.78	1,23	0.065	12 2.32 $\pm$ 0.26	0.77	1,25	0.387	13 2.74 $\pm$ 0.30
Carmoli (65-70‰)				25 1.98 $\pm$ 0.55				12 2.08 $\pm$ 0.48				13 1.92 $\pm$ 0.50
Chicamo (0-5‰)	5.68	1,70	0.020*	21 3.81 $\pm$ 0.58	1.39	1,35	0.256	12 3.16 $\pm$ 0.37	13.12	1,34	0.001*	9 4.63 $\pm$ 0.41
Marchamalo and Carmoli (35-70‰)				50 2.28 $\pm$ 0.21				24 2.43 $\pm$ 0.38				26 2.14 $\pm$ 0.36

Shown are ranges of habitat salinity, statistics values using standardized residuals (*F*, d.f. and *P*) and original calculated data (mean values).

Our results showed a significant reduction in mean  $R_{AM}$  values of populations from freshwater aquatic systems (Chicamo) to salt aquatic systems with significantly higher alkalinity (Marchamalo and Carmoli; Table 3). Very few studies have focused on the influence of water salinity on nitrogen excretion in fish; however, several studies have shown a decrease in ammonia excretion with the increase in salinity (Gershanovich and Pototskij, 1995; Wright et al., 1995). On the other hand, several studies have analyzed the possible relationships between nitrogenous excretion in fish and alkalinity (revised in Danulat, 1995), showing different processes and products for excretion waste.

Since in the present study no attempt was made to differentiate between nitrogenous waste excretion products (ammonia-N vs urea-N), ammonia excretion in fish is strongly dependent on high water salinity and alkalinity; one possible way to maintain nitrogen excretion in these kinds of water would be to increase the proportion of nitrogenous waste excreted as urea (Randall et al., 1989; Wright et al., 1993). The Ornithine-urea cycle (OUC in Mommsen and Walsh, 1989) shown by some fishes is one possibility (Walsh et al., 1990). Moreover, most teleosts synthesize urea at relatively low rates through the metabolism of purines (uricolysis) or arginine (Mommsen and Walsh, 1991; Wilkie et al., 1994). However, ureotelism is not a prerequisite for fish living in saline or alkaline environments, and the ability of several species to excrete high levels of ammonia at high salinity or pH has been demonstrated (Danulat and Kempe, 1992). There are viable mechanisms of ammonia excretion existing in this kind of habitat (Wilkie, 1997). Clearly, the relationship between water salinity increases, and ureagenesis needs to be further analyzed in *A. iberus*.

The mean  $R_{AM}$  values of the populations in salt waters could be commensurate with a decrease in metabolic rates (Boyce and Clark, 1997; Boyce, 1999) due to increases in the water salinity (Swanson, 1998). In Cyprinodontids, the effect of salinity on metabolic rates has been shown in several studies (Nordlie et al., 1991; Jordan et al., 1993; Plaut, 2000).

Moreover, in the Carmoli salt-marsh (the habitat with the most significant differences, Table 3) the species can be locally abundant in small, isolated pools (surface < 5 m<sup>2</sup>; depth < 50 cm), and specimens are frequently altered by sudden changes in the environmental conditions; as a consequence, massive mortalities have been observed (unpublished data). In confined aquatic systems, the accumulation of excreted ammonia can lead to decreased growth, increased vulnerability to disease, and pathologic changes in gill structure, among others effects (Wilkie, 1997). It is possible that the low mean  $R_{AMs}$  values detected were an example of a flexible physiologic response characteristic of Cyprinodontids (Jordan et al., 1993).

In short, *A. iberus* is exposed to a wide range of habitat conditions (habitat-type, salinity, alkalinity, etc.) both within- and between-locations. The physiologic response in ammonia excretion showed a specific-pattern between  $R_{AMs}$  and fish size, which could be affected by the reproductive period of the species. In addition, we observed a significant decrease in mean  $R_{AM}$  values of populations in saline aquatic systems. As already mentioned, both may reflect the phenotypic plasticity that is characteristic of the family Cyprinodontidae.

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