



Comparison of salinity tolerance and osmoregulation in two closely related species of blennies from different habitats

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Abstract

This study compares osmoregulatory capabilities of two closely related blennies inhabiting different habitats: *Salaria pavo* which inhabits marine rocky coasts in the Mediterranean and the eastern coasts of the Atlantic Ocean, and *Salaria fluviatilis* which inhabits freshwater habitats around the Mediterranean, both in rivers connected to the sea and in lakes that for a long time have been separated from the sea. Fishes for this study were collected in Lake Kinneret (Sea of Galilee) (*S. fluviatilis*) and Habonim beach, Israel, East Mediterranean coast (*S. pavo*). Both species tolerated salinities of 0, 40 and 100‰ seawater (0, 14.4 and 36 ppt sea salt respectively). Acclimation for a period of three months to freshwater (*S. pavo*) and seawater (*S. fluviatilis*) did not affect survival rates. After acclimation, total body water content of *S. fluviatilis* remained unaffected by experimental salinities but increased in *S. pavo* as salinity decreased. The hematocrit of both species increased as salinity increased. Plasma osmolality and Na⁺ concentrations were unaffected by experimental acclimation salinities in *S. fluviatilis*, but significantly decreased in *S. pavo* as salinity decreased. The results indicate that *S. fluviatilis* is able to osmoregulate both in fresh and seawater. *S. pavo*, living in seawater, while able to tolerate freshwater environment, fails to maintain constant extracellular fluid concentration. It is suggested that *S. pavo*, or its marine ancestor, has euryhaline capabilities that enabled it to invade freshwater habitats. *S. fluviatilis*, as the evolutionary result of this invasion, is thus better adapted to freshwater environment, but did not lose its ability to live in seawater.

Introduction

Marine fishes that invaded freshwater habitats during their evolution, had to reverse their patterns of osmoregulation. Instead of obtaining water by drinking and excreting excess salts, mostly through their gills, they have to absorb salts through the gills and intestines and excrete excess water, mostly through the kidney (Jobling 1995). Marine fishes that are already euryhaline in nature are preferred candidates for such invasion of freshwater habitats. For fishes that invaded freshwater habitats recently, one may expect that they will conserve their ability to tolerate and osmoregulate in seawater salinities.

Most members of the family Blenniidae are marine fishes. An exception is the freshwater blenny, *Salaria*

fluviatilis (Asso 1801) which inhabits freshwater habitats and is widely distributed round the northern and eastern parts of the Mediterranean (Steinitz 1954; Kosswig 1967). It is abundant in recent alluvial regions near sea level (e.g. rivers estuaries which are directly connected to the sea), lakes of postglacial age at higher altitudes and also in inner lakes that are separated from one another by great distances and lost their contact with the Mediterranean as long ago as the Pliocene (Zander 1973). The latter include Lake Kinneret (Sea of Galilee) and the Jordan Valley where specimens used in this study were collected.

S. fluviatilis is closely related to *Salaria pavo* (Risso 1810), a marine blenny that inhabits intertidal rocky coasts of the Mediterranean and European Atlantic coasts (Fishelson 1963; Zander 1973).

Kosswig (1967) considered *S. fluviatilis* to be a polytopic derivative of *S. pavo*, and Zander (1973) assumed an eurythermal and euryhaline ancestor for both species which tolerated not only the desalination of the Mediterranean Sea in the upper Pliocene period, but also adapted itself to the subsequent changing conditions.

Both species are considered to be euryhaline, and their habitats may overlap in estuaries, as in the harbour of Cagliari, Sardinia (Zander 1973). They are considered as being able to tolerate different levels of salinity (*S. pavo* down to 5 ppt and *S. fluviatilis* up to 15 ppt) but there are no published data to confirm this abilities (Zander 1973). Although considered euryhaline, most of the populations of both species live in relatively stable marine (*S. pavo*) or freshwater (*S. fluviatilis*) conditions (Fishelson 1963; Bernini et al. 1990; Freeman et al. 1990; Mir 1990; Zava and Violani 1991; Muller and Meng 1992; Oliveira et al. 1992; Bolik 1995; Changeux and Pont 1995).

Given its evolutionary and paleontologic history (Kosswig 1967), *S. fluviatilis* population in Lake Kinneret has been isolated from other *S. fluviatilis* or *S. pavo* populations for more than one million years. Except for a few short periods of partial salinization of the southern part of the lake by saline water from the Dead Sea (Gophen and Itzhaki 1992), they lived during this period in freshwater and for the last 30000 years, have not experienced salt concentrations above 2 ppt.

Fish osmoregulatory problems and adjustments associated with change in ambient salinity have been intensively studied in: (1) euryhaline fish species that encounter different levels of salinity in their habitat during lifetime (Evans 1993; Madsen et al. 1996), or in (2) fishes that move from one habitat to the other during their ontogeny, such as salmonid fishes (Hoar 1988; Boeuf 1993). The present study deals with fishes that may not have encountered substantial changes in habitat salinity for many generations, and examines the osmoregulation capabilities of *S. pavo* and *S. fluviatilis*. My working hypotheses were: (i) If *S. pavo* is the ancestor, or sister species, of the freshwater blenny, *S. fluviatilis*, it should have good osmoregulatory capabilities and tolerance to freshwater, since these are necessary conditions for invading freshwater habitats; (ii) *S. fluviatilis*, which recently invaded freshwater, should be adapted to freshwater, but may have excellent abilities to tolerate and osmoregulate in seawater as well, a remnant from the time it dwelled in the sea.

To examine these hypotheses, I acclimated fishes of both species to salinities of 0, 40 and 100% seawater, recorded their survival and examined their osmoregulatory response by measuring hematocrit, plasma osmolality, plasma Na⁺ concentration and total body water content.

Materials and methods

Fish collection, maintenance and acclimation

Specimens of *S. pavo* (92 males and 101 females) were collected in tide pools in the rocky beach of Habonim, Israel, in May and June 1996. Collections were performed on two different days during low tide when tide pools were disconnected from the sea. Quinaldin solution (10% Quinaldin in 90% ethanol) was poured into the pools in amounts sufficient to anesthetize the fish. Within 10–30 min all fishes in the pool were inactive. They were then collected into a seawater container and fully recovered within <5 min. Specimens of *S. pavo* were transferred into another 10 liter container with seawater and other fishes were released to the sea. Seawater temperature in the tide pools during the collection ranged between 23–28 °C. Specimens of *S. fluviatilis* (77 males and 89 females) were collected in shallow water at the northern beach of Lake Kinneret, Israel, in May 1996, by a snorkeling diver using a hand net. Water temperature at the collection site was 21.5 °C. All fishes were transported to the laboratory, within 2 h, in 10 l cans filled with water from the fish's natural habitat.

The fish were kept indoors in 20 l glass aquaria at temperature of 21 ± 1 °C with continuous biological filtration at 12:12 LD photoperiod. Dechlorinated tap water was used for *S. fluviatilis* and natural Mediterranean seawater for *S. pavo*. Fishes were fed twice a day with dry commercial fish food, live *Daphnia* spp. and *Tubifex* sp. Small stones and black PVC tubes were provided as shelters. They were kept under these condition for at least 3 weeks before acclimation began.

Acclimation and salinity tolerance experiments

Fish were acclimated to three different salinities: 0 (0 ppt sea salt), 40 (14.4 ppt) and 100% (36 ppt) seawater. From each species, three groups of 50 individuals were divided into five subgroups of 10 individuals. Each

subgroup was placed in a 20 l aquarium containing seawater (*S. pavo*) or dechlorinated tap water (*S. fluviatilis*). For each species, one group (5 subgroups, of 10 individuals each) was maintained in its natural salinity (100% seawater for *S. pavo* and 0% seawater for *S. fluviatilis*). The second group of each species was acclimated to 40% seawater (close to iso-osmotic concentration), and the third was acclimated to the opposite of its natural salinity (freshwater for *S. pavo* and 100% seawater for *S. fluviatilis*). Acclimation began 3 days after fishes were placed in the acclimation aquaria. By increasing (for *S. fluviatilis*) or reducing (for *S. pavo*) the salinity stepwise in 10% seawater increments per day by adding artificial aquarium sea salt (3.6 gr l^{-1}) or by diluting with dechlorinated tap water, until the desired salinities were obtained. Fish were maintained at the experimental salinities for 3 months. After this acclimation period, hematocrit, plasma osmolality, plasma Na^+ concentration and total body water content were measured. Deaths were recorded for 3 months after the experimental salinity for each group had been reached. First groups of each species (remained in its natural salinity) were used as control groups for the survival test.

Blood sampling, hematocrit, total body water content measurement, plasma osmolality and plasma Na^+ concentration

After the fish had been in the experimental salinities for three months, blood samples were collected and dry body mass was measured. Before taking blood samples, the fish were lightly anesthetized with 0.11 mg l^{-1} 2-phenoxyethanol (Summerfelt and Smith 1990). After losing balance, fishes were wiped dry with blotting paper, body live mass (M_b) was measured, the tail was removed and blood drained from vessels in the caudal peduncle into $75 \mu\text{l}$ lithium-heparinized capillary tubes. The capillary tubes were sealed and centrifuged for 10 min at 8000 RPM to determine hematocrit. The fish were then dried for 10 days at 55°C and dry body mass ($M_{b(d)}$) was determined. Relative total body water was calculated using the equation:

$$\text{Total body water content} = (M_b - M_{b(d)}) / M_b$$

Plasma osmolality was measured with Wescor 5500 osmometer and plasma Na^+ concentration was measured with Ciba-Corning 410C flame photometer. Because of the low volume of the blood samples,

plasma from different fishes were used for each of these two measurements.

Statistical analyses

Differences of each parameter between species and among the salinities within species were analyzed using one way ANOVA and Tukey HSD multiple comparisons. The tests were performed with SysTat 5.04 for Windows (Wilkinson 1990). Numbers are given as mean \pm SEM, statistical significance was declared at the $p \leq 0.05$ level.

Results

Salinity tolerance

During the three months of acclimation to the experimental salinities survival was high and not significantly different among salinities and among species (Table 1).

Total body water content

Acclimated to their natural habitat salinities (*S. pavo* to 100% seawater and *S. fluviatilis* to 0% seawater), total body water contents of the two species were not significantly different (Figure 1). However, when compared for the same salinities, *S. pavo* bodies contained significantly more water than those of *S. fluviatilis*. Total body water content of *S. pavo* acclimated to 0% seawater was $78.2 \pm 1.2\%$, significantly higher than those acclimated to 40 and 100% seawater (76.3 ± 1.1 and $76.2 \pm 1.1\%$ respectively). Between 40 and 100% seawater, the total water content of *S. pavo* was not significantly different. In *S. fluviatilis* total body water content was not significantly different among groups acclimated to 0, 40 and 100% seawater. ($74.8 \pm 2.2\%$, $74.0 \pm 1.5\%$ and $73.8 \pm 2.1\%$ respectively).

Hematocrit

When in their natural salinities, *S. pavo* had hematocrit values significantly higher than *S. fluviatilis* (Figure 2). For all the experimental salinities *S. pavo* had higher values of hematocrit than *S. fluviatilis*. *S. pavo* acclimated to 0% seawater had 0.34 ± 0.03 hematocrit value, significantly lower than hematocrit values for conspecifics acclimated to 40 and 100% seawater (0.39 ± 0.04 and 0.34 ± 0.03 respectively).

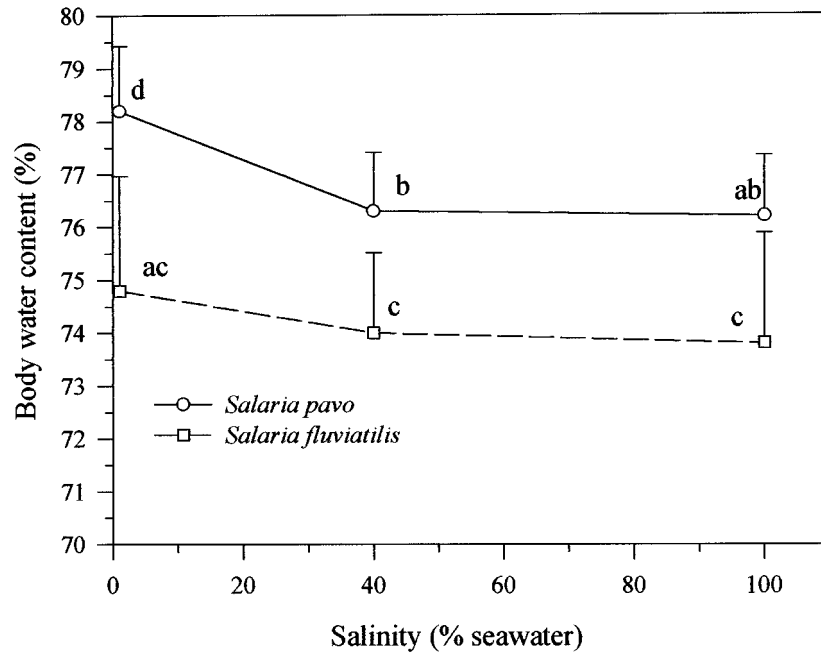


Figure 1. Total body water content of *Salaria fluviatilis* and *Salaria pavo* acclimated to salinities of 0, 40 and 100% (36 ppt) seawater. Data are shown as mean \pm SEM; points sharing the same letter are not significantly different ($p \leq 0.05$).

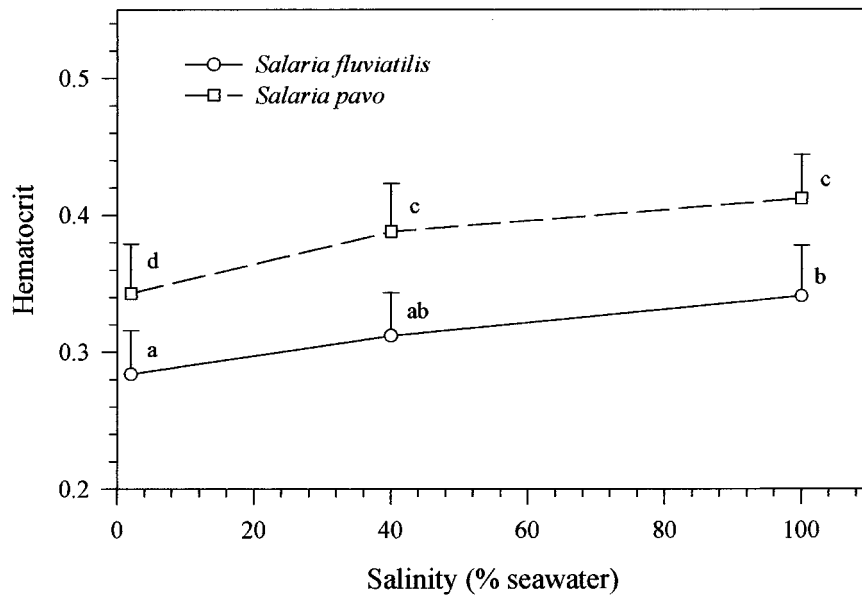


Figure 2. Hematocrit of *Salaria fluviatilis* and *Salaria pavo* acclimated to salinities of 0, 40 and 100% (36 ppt) seawater. Data are shown as mean \pm SEM; points sharing the same letter are not significantly different ($p \leq 0.05$).

Table 1. Survival of *Salaria pavo* and *Salaria fluviatilis* in 0, 40 and 100% seawater (0, 14.4 and 36 ppt salt) during three month of acclimation (mean \pm SEM)

	Survival rates			$F_{(2,12)}$	P
	0%	40%	100%		
<i>Salaria pavo</i>	0.94 \pm 0.02	0.96 \pm 0.01	0.92 \pm 0.02	0.333	0.72
<i>S. fluviatilis</i>	0.94 \pm 0.02	0.92 \pm 0.02	0.90 \pm 0.03	0.171	0.84

Hematocrit values in *S. fluviatilis* also increased as fish acclimated to higher salinities, although significant difference was detected only between acclimation to 0% seawater and to 100% seawater (0.28 ± 0.03 and 0.34 ± 0.04 respectively). Fish acclimated to 40% seawater had mean hematocrit value of 0.31 ± 0.03 , not significantly different from the other two salinities.

Plasma osmolality and Na^+ concentrations

Plasma osmolalities of *S. pavo* and *S. fluviatilis* (Figure 3), with each species acclimated to its natural salinity, were not significantly different (422 ± 22 mOsm and 359 ± 21 mOsm respectively, $p=0.318$). Within each salinity plasma osmolalities differed significantly only at freshwater.

Plasma osmolality in *S. pavo* was significantly lower as acclimation salinity reduced (422 ± 22 , 253 ± 21 and 161 ± 23 mOsm for 0, 40 and 100% seawater respectively). Plasma osmolality of *S. fluviatilis* was not affected by the different experimental salinities. (359 ± 21 , 316 ± 21 and 382 ± 20 mOsm for 0, 40 and 100% seawater respectively).

Plasma Na^+ concentration showed exactly the same pattern as plasma osmolality. In *S. pavo* it was not significantly different from that of *S. fluviatilis* when each species was acclimated to the salinity of its natural habitat (Figure 3). Within each salinity, *S. pavo* and *S. fluviatilis* significantly differed in their plasma Na^+ concentration only in 0% seawater, but not in 40 and 100%.

For *S. pavo*, plasma Na^+ concentrations were significantly lower as the salinity decreased (204 ± 11 , 120 ± 10 and 77 ± 11 mM for 100, 40 and 0% seawater respectively). Contrary to these, plasma Na^+ concentrations in *S. fluviatilis* were not significantly different among the experimental salinities. (173 ± 10 , 151 ± 10 and 181 ± 9 mM for 0, 40 and 100% seawater respectively).

Discussion

The results show that, although both species are euryhaline, *S. fluviatilis* osmoregulates better than *S. pavo*. In all the parameters tested here, *S. fluviatilis* maintained its water and ions balance more constantly than *S. pavo*.

Both species demonstrated similar tolerance to salinities between 0 to 100% seawater. Such wide-range salinity tolerance usually characterizes euryhaline fishes that encounter substantial salinity changes in their natural habitat, such as estuaries (House 1963; Hettler 1976; Chervinski 1977; Barton 1979; Davenport and Vahl 1979; Barton and Barton 1987; Plaza-Yglesias et al. 1989; Cataldi et al. 1995), inland saline ponds and lakes (Lotan 1969, 1971; Lui 1969; Stuenkel and Hillyard 1981), and in marshes (Peterson 1988; Susanto and Peterson 1996). Other fishes exposed to substantial salinity fluctuations are species that change their habitat during ontogeny (Evans 1993; Madsen et al. 1996). Both species studied here seem not to fall into any of these categories.

Hematocrit may usually reflect changes of water content in the blood. In both species hematocrit values increased with environmental salinity increase. This may indicate accumulation of excess water in the blood when fish are in hyposaline environment and/or loss of water from the blood when the fish are in hypersaline environment.

Nordlie (1985) stated that the more freshwater tolerant species generally show lower blood concentrations of low ambient salinities than the more marine/hypersaline forms. Such trend was observed in the blood of the species studied here, although the differences between them was not significant. However, total body water content of *S. pavo* was higher than that of *S. fluviatilis*.

Total body water content, plasma osmolality and Na^+ concentration show that *S. fluviatilis*, in which these parameters remained unaffected by the acclimation, is more successful in maintaining its body fluids

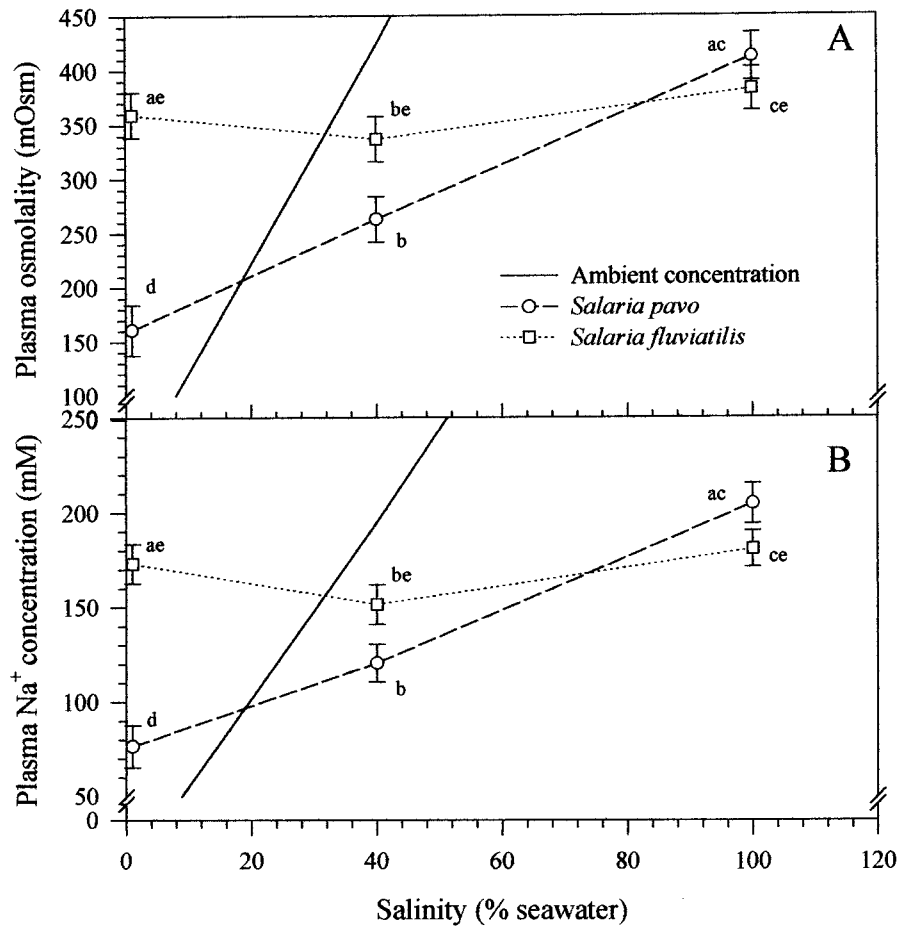


Figure 3. Plasma osmolality (A) and Na⁺ concentration (B) of *Salaria fluviatilis* and *Salaria pavo* acclimated to salinities of 0, 40 and 100% (36 ppt) seawater. Data are shown as mean \pm SEM; points sharing the same letter are not significantly different ($p \leq 0.05$).

and ions in homeostasis. In *S. pavo* these parameters were significantly affected as the ambient salinity decreased. For *S. pavo* osmolality and Na⁺ concentrations were lower than in any other cases studied (House 1963; Gordon, et al. 1965; Lotan 1971; Lee et al. 1987; Cataldi, et al. 1995; Madson, et al. 1996), but was similar to those of another blenny, *Blennius pholis*, acclimated to 10–20% seawater (House 1963; Evans 1969). These low values indicate that *S. pavo* is unable to actively absorb salts and get rid of excess water at a rate which will compensate for the loss to the environment due to osmotic gradient. The similarity with *B. pholis* (House 1963; Evans 1969) may suggest a general ability of blennies to survive in spite of severe hemodilution.

S. pavo in the eastern shore of the Mediterranean inhabits mainly intertidal rocky shores (Steinitz 1950, 1954; Fishelson 1963) and cannot be considered a typ-

ical estuaries fish. As an intertidal fish it is expected to be able to regulate its plasma osmolality extremely well against changing salinities (Bridges 1993). In its habitat it may encounter changes in water salinity when being trapped in tide pools, but considering low tidal amplitudes, this situation seems to be rare.

The ability of *S. pavo* (or its euryhaline ancestor) to tolerate wide range of salinities may be a result of evolutionary selective forces acting during several cycles of salinization and desalinization of the Mediterranean (Kosswig 1967; Zander 1973). This ability was the main feature that enabled it to invade freshwater habitats. While being trapped in bodies of water that gradually became freshwater lakes, selection favored individuals of *S. pavo* with better osmoregulatory capabilities. This is the probable scenario for the formation of the species *S. fluviatilis*.

S. fluviatilis inhabit fresh water habitats, including recent alluvial region near sea level, like rivers estuaries which are directly connected to the sea (Zander 1973), but the specimens used in this study were collected in Lake Kinneret, which has had no connection to the Mediterranean or any other sea for more than one million years. It follows that equally long, this population has not experienced salinity above 3 ppt. Thus, both species' euryhaline capabilities are probably a remnant of adaptation to previous environments.

The results of this study show that *S. fluviatilis* remained virtually unaffected by the acclimation to full strength seawater, and suggest that although *S. fluviatilis* gained the ability to invade and live in freshwater habitats, it did not lose its physiological ability to osmoregulate in seawater.

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