Plasma Osmotic Regulation and Routine Metabolism in the Eustis Pupfish, *Cyprinodon variegatus hubbsi* (Teleostei: Cyprinodontidae)

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We assessed the influence of salinity on plasma osmotic regulation and routine metabolism in the freshwater Eustis pupfish *Cyprinodon variegatus hubbsi* and compared these results to published data for the widely distributed, euryhaline sheepshead minnow *C. v. variegatus*. The Eustis pupfish was able to tolerate salinities twice that of normal seawater. Plasma osmotic concentration increased with increasing salinity. In freshwater, Eustis pupfish maintained plasma osmotic concentrations lower than sheepshead minnow. However, this pattern reversed with increasing salinity. Routine metabolism did not differ between the Eustis pupfish and sheepshead minnow when the effect of body mass was statistically removed. The Eustis pupfish, although long isolated in fresh waters, has retained the physiological plasticity generally characteristic of the cyprinodontoids.

The Eustis pupfish *Cyprinodon variegatus hubbsi* is a small cyprinodontid endemic to the Eustis chain of lakes in the headwaters of the Oklawaha River, Florida (Guillory and Johnson, 1986). Carr (1936) first described the Eustis pupfish as *C. hubbsi*, and suggested that it was most closely related to Gulf of Mexico populations of the sheepshead minnow *C. v. variegatus*. Johnson (1975), who studied morphological and meristic variation in peninsular Florida populations of the sheepshead minnow, concluded that the Eustis pupfish was a subspecies of *C. variegatus* and erected *C. v. hubbsi*. Darling (1976), in a survey of allozymal variation in sheepshead minnow throughout its range, provided genetic evidence supporting Johnson's subspecific designation of the Eustis pupfish. Duggins et al. (1983) provided further molecular support for this designation. Currently, the Eustis pupfish is designated a Species of Special Concern in Florida (Florida Game and Fresh Water Fish Commission: Gilbert, 1992).

The sheepshead minnow is the most widely distributed member of the New World genus *Cyprinodon*, inhabiting coastal waters from the Yucatan Peninsula to Massachusetts (Darling, 1976; Johnson, 1980: Page and Burr, 1991). The species is exposed to a wide range of ambient salinities both within and between locations (Simpson and Gunter, 1956; Martin, 1968; Nordlie and Walsh, 1989). In addition to the Eustis pupfish, there are several southern Florida populations that have invaded freshwater habitats (Johnson, 1975). However, the Eustis
pupfish is unique in that it has been isolated in freshwater lakes since the recession of sea levels during the early Pleistocene (Guillory and Johnson, 1986; Gilbert, 1992). Correspondingly, the osmotic medium of the Eustis pupfish has been very stable and of low ionic concentration compared to the typical estuarine system occupied by the sheephead minnow.

The divergent habitats of Eustis pupfish and sheephead minnow, coupled with extended geographical isolation, could have led to divergence in their physiological responses to salinity. However, many freshwater cyprinodontid fishes (e.g., Fundulus spp., Griffith, 1974; Jordanella floridae, Nordlie and Walsh, 1989) have retained flexible physiological responses to variable osmotic conditions. In this paper, we compare physiological responses (plasma osmotic regulation and routine metabolism) of Eustis pupfish and sheephead minnow over a wide range of experimental salinities. We predicted that the Eustis pupfish has retained the ability to tolerate a wide range of salinities. Additionally, we predicted that the Eustis pupfish and sheephead minnow do not vary with respect to their osmoregulatory and routine metabolic responses to salinity. Eustis pupfish plasma osmotic regulation and routine metabolism data are reported for the first time herein, whereas sheephead minnow data were obtained in earlier studies (Nordlie, 1985, 1987; Nordlie et al., 1991).

**Material and Methods**

Eustis pupfish were seized from lakes Beauclair, Dora, Eustis, Griffin, and Harris (Lake and Orange counties, Florida) between June and Dec. 1990. Fish were held in water from the collection site (mean conductivity: 418 μmhos/cm) for 24–48 h to allow acclimation to laboratory temperature. Groups of 5–10 fish were then transferred into 38-liter aquaria containing well water (mean conductivity: 350 μmhos/cm). Experimental aquaria were aerated and equipped with undergravel filters covered with a layer of cochina sand. Fish were maintained in a constant environment room (20 ± 1 C, 12L:12D photoperiod) and were fed Tetramin® flake food at least once daily. Food was withheld from fish 24 h prior to metabolic and plasma osmotic measurements.

Fish were sequentially acclimated to experimental salinities ranging from freshwater to 70 ppt. Each acclimation step lasted at least 14 d, after which fish were either used for metabolic or plasma osmotic determinations, or were transferred into a tank 5 ppt higher in salinity. Desired experimental salinities were prepared by diluting filtered Atlantic Ocean seawater (34 ppt) with deionized water or by adding Instant Ocean® synthetic salt to seawater. Well water was used for the freshwater acclimations. Salinities were checked daily using an AO refractometer or YSI S-C-T meter and adjusted as needed.

Oxygen consumption (i.e., routine metabolism; Winberg, 1956; Fry, 1957) was measured on individual fish sequestered in sealed, opaque flasks. Prior to a trial, individual fish were held for 12–14 h in unsealed flasks containing oxygen-saturated water of their acclimation salinity and temperature. Experimental trials were carried out by sealing the flasks at approximately 0800 h and withdrawing a water sample hourly for determination of reduction in the PO₄ (partial pressure of oxygen). A full description of the experimental flasks and water sample collection routine is provided in Nordlie et al. (1991). A Radiometer oxygen electrode connected to a Radiometer PHM 71 Acid-Base Analyzer was used to make PO₄ determinations. Calculation of oxygen saturation values with respect to experimental conditions (i.e., salinity, barometric pressure, and humidity) were made using the equations of Truesdale et al. (1955), and mean routine metabolic rate (mg oxygen/h) was calculated for each fish. All trials were carried out between 0800 h and 1600 h, and at the end of a trial, fish were blotted dry and weighed individually to the nearest 0.01 g. Individual fish were used once during the metabolic trials.

For determination of plasma osmolality, fish were netted from their acclimation tanks between 0800 h and 1200 h and blotted dry. Blood was obtained by blind cardiac puncture using heparinized microhematocrit tubes drawn to a fine point, which were then centrifuged to separate plasma from formed elements. Plasma from one to three individuals was pooled in order to obtain the 5 μl's required for determination of osmolality (mOsm/kg) on a Wescor 5100B vapor pressure osmometer. Fish were used without regard to sex, and body size ranged between 24 mm and 60 mm standard length. Because of collection limitations associated with the conservation status of the Eustis pupfish, we measured plasma osmotic concentrations of some individuals from the respiratory studies. These fish were reacclimated to their experimental salinities for at least seven days before obtaining plasma measurements.

Plasma osmolality and routine metabolism data for Eustis pupfish and sheephead minnow acclimated to a range of experimental salinities
were statistically compared. Plasma data for sheephead minnow are from Nordlie (1985, 1987), and routine metabolism data are from Nordlie et al. (1991). These fish were collected from Gulf of Mexico salt marshes near Cedar Key, Florida. The same laboratory, acclimation routines, equipment, and techniques for measurement of physiological parameters were used for both the Eustis pupfish and sheephead minnow studies. Statistical procedures were performed as outlined in Winer et al. (1991) and Steel and Torrie (1980). Plasma osmolality (mOsm/kg) data were log_{10} transformed to meet analysis of variance (ANOVA) assumptions of homogeneity of variances ($P > 0.05$, $F_{max}$-test) and normality. Routine metabolic rates (mg oxygen/h) and weight (g) were log_{10} transformed to meet the ANOVA assumptions. All error terms shown are ±1 standard error.

RESULTS

Survivorship of Eustis pupfish across the range of experimental salinities was not quantitatively assessed. However, there were no obvious patterns of mortality up through 70 ppt, which was the highest salinity used in this study. The small sample size for plasma data at 70 ppt (see Fig. 1) was due to pooling of plasma from several individuals.

Sample sizes and mean plasma osmotic concentrations for the Eustis pupfish and sheephead minnow are shown in Figure 1. Mean plasma osmotic concentration increased with increasing experimental salinity in the Eustis pupfish, ranging from 316 ± 3.6 mOsm/kg in freshwater to 454 ± 9.8 mOsm/kg at 70 ppt. Mean plasma levels ranged from 334 ± 2.8 to 391 ± 4.7 mOsm/kg for the sheephead minnow. The plasma osmotic responses of the two populations to increasing salinity were different in magnitude. In general, Eustis pupfish maintained their plasma osmolality at levels higher than the sheephead minnow ($F_{1,115} = 40.79; P = 0.001$). However, there was a significant ($F_{2,115} = 11.85; P = 0.0001$) interaction between salinity and population (Fig. 1). The Eustis pupfish maintained plasma osmotic levels higher than the sheephead minnow in all experimental salinities except freshwater, wherein it had lower levels. Differences between populations were significant ($P < 0.01$; mean contrasts) for all experimental salinities except 35 ppt.

Body sizes used in the routine metabolism experiment ranged from 0.25–3.05 g in Eustis pupfish and from 0.20–8.50 g in the sheephead minnow. Weight (log_{10} g) was used as a covariate in an analysis of covariance (ANCOVA) model constructed to test for the effects of species and salinity on routine metabolism (log_{10} mg oxygen/h). This model was highly significant ($F_{10,115} = 37.21; P = 0.0001$) and explained 76% of the variation observed in routine metabolic rate. As suggested by Figure 2, weight was highly significant ($F_{1,115} = 528.57; P = 0.0001$) and accounted for 58% of the total variation explained by the model.

Table 1 provides the sample sizes, metabolic rates, and body masses for the routine metabolism experiment. The Eustis pupfish and sheephead minnow did not differ ($F_{1,115} = 0.35; P = 0.5534$) in their metabolic responses to salinity after factoring out the effect of weight. Experimental salinity significantly ($F_{7,115} = 4.09; P = 0.003$) influenced metabolic rate, but there was no consistent trend in ANCOVA adjusted
Table 1. Sample Size (n), Mean Metabolic Rate, and Mean Body Mass from Routine Metabolism Experiments. Sheepshead minnow data are from Nordlie et al. (1991).

<table>
<thead>
<tr>
<th>Species</th>
<th>Experimental salinity (ppt)</th>
<th>Sample size</th>
<th>Metabolic rate (mg oxygen/h)</th>
<th>Mean mass (g)</th>
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<tr>
<td>Eustis pupfish</td>
<td>0</td>
<td>8</td>
<td>0.39 ± 0.05</td>
<td>0.93 ± 0.24</td>
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<td></td>
<td>1</td>
<td>8</td>
<td>0.22 ± 0.02</td>
<td>0.68 ± 0.10</td>
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<td></td>
<td>5</td>
<td>8</td>
<td>0.29 ± 0.06</td>
<td>0.88 ± 0.15</td>
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<tr>
<td></td>
<td>10</td>
<td>8</td>
<td>0.38 ± 0.05</td>
<td>0.61 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>8</td>
<td>0.49 ± 0.06</td>
<td>1.44 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>8</td>
<td>0.38 ± 0.06</td>
<td>1.02 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>16</td>
<td>0.67 ± 0.04</td>
<td>1.75 ± 0.13</td>
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<tr>
<td></td>
<td>65</td>
<td>8</td>
<td>0.32 ± 0.03</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td>Sheepshead minnow</td>
<td>0</td>
<td>12</td>
<td>0.83 ± 0.10</td>
<td>3.49 ± 0.44</td>
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<tr>
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<tr>
<td></td>
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<td>3.26 ± 0.49</td>
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<tr>
<td></td>
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<td>2.49 ± 0.54</td>
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<tr>
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<tr>
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<td>3.50 ± 0.61</td>
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<tr>
<td></td>
<td>65</td>
<td>9</td>
<td>0.47 ± 0.10</td>
<td>1.52 ± 0.40</td>
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</table>

metabolic rates. Furthermore, salinity accounted for only 4% of the observed variation in routine metabolic rates. There was a significant ($F_{2,199} = 3.22; P = 0.0384$) interaction between population and salinity. Routine metabolic responses of the two populations were not different at 0, 1, 5, 20, 50, or 65 ppt ($P > 0.05$; mean contrasts). The Eustis pupfish had a higher mean metabolic rate at 10 ppt and the sheepshead minnow metabolic rate was higher at 35 ppt ($P < 0.05$), which accounted for the significant interaction.

**Discussion**

Eustis pupfish were acclimated to experimental salinities ranging from freshwater to twice that of normal seawater (70 ppt), which supports the hypothesis that the Eustis pupfish has retained its ability to tolerate a wide range of salinities. Furthermore, gravid females were observed at all salinities, which suggests that reproductive activity could occur throughout this range of salinities. Renfro (1960) and Martin (1972) also found that sheepshead minnow were capable of reproductive activity at elevated salinities.

The influence of salinity on Eustis pupfish plasma osmotic concentration was similar in form to other cyprinodontid species, including the sheepshead minnow. However, relative plasma osmotic responses to salinity varied significantly between the Eustis pupfish and sheepshead minnow. This result does not support the hypothesis of no physiological differentiation between the Eustis pupfish and sheepshead minnow. Plasma levels were generally higher for Eustis pupfish than for the sheepshead minnow. However, the Eustis pupfish had significantly lower plasma osmotic concentrations in freshwater. This pattern suggests that Eustis pupfish osmoregulate in response to salinity in a form similar to flagfish (*Jordanella floridae*), another freshwater cyprinodontid endemic to the Florida peninsula. Nordlie and Walsh (1989) found that, in salinities less than 10 ppt, flagfish maintained plasma osmotic concentrations lower than the sheepshead minnow, whereas they regulated at steeply higher levels in salinities greater than 50 ppt. Differences between plasma regulation in the Eustis pupfish and sheepshead minnow may reflect heritable physiological responses or nonheritable variation caused by different environmental conditions during development (Kinne, 1962; King et al., 1989; Laurent and Perry, 1991).

As shown previously for sheepshead minnow (Barton and Barton, 1987; Nordlie et al., 1991), metabolic rate was positively related to fish weight. Researchers working with other fish have demonstrated positive relationships between weight and respiration (e.g., Fry, 1957; Moser and Hetler, 1989). However, Peterson (1990) did not detect a relationship between fish weight and metabolic rate for sheepshead minnow under normoxic or hypoxic conditions. Differences in techniques used to determine metabolic rates may account for the contrasting results (Steffensen, 1989). Metabolic determinations in this study were made only when ex-
experimental flasks had pO₂'s above 80 torr, which is the critical pO₂ for Cinostomus variegatus at 100 ppt (D. C. Haney, unpubl. data).

In contrast to the plasma osmotic results, the results from the routine metabolism experiment support the hypothesis of no physiological differentiation between the Eustis pupfish and sheepshead minnow. However, the Eustis pupfish were less consistent in their responses to salinity (see Table 1). The routine metabolic rates of Eustis pupfish at the different experimental salinities fluctuated widely. The sheepshead minnow had much more stable metabolic rates with respect to the range of experimental salinities. This may partially reflect differences in sample sizes. Although there was a statistically significant salinity effect, there was no consistent trend in the relationship between salinity and routine metabolism. Eustis pupfish metabolic rate dropped beyond a salinity of 50 ppt, which was consistent with the sheepshead minnow response as described by Nordlie et al. (1991). Barton and Barton (1987) found a significant negative relationship between salinity and metabolic rate in the sheepshead minnow. However, Stuenkel and Hillyard (1981) found a positive relationship between salinity and metabolic rate in the Salt Creek pupfish (C. salinus), which inhabits saline (15–20 ppt) marshes around inland spring runs.

The two physiological responses that we measured appear to give contradictory results. There was population differentiation with respect to plasma regulation, but as already mentioned, this may reflect phenotypic plasticity (Kinne, 1962). On the other hand, metabolic responses to salinity did not vary between populations. Therefore, plasma regulation does not appear to have altered energetic costs as measured at the scale of the whole organism. The Eustis pupfish appears to have retained the physiological flexibility generally characteristic of the cyprinodontoids (Nordlie and Walsh, 1989).

Acknowledgments

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Literature Cited


