Effects of acute changes in salinity and temperature on routine metabolism and nitrogen excretion in gambusia (Gambusia affinis) and zebrafish (Danio rerio)

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ABSTRACT

Acute stress may affect metabolism and nitrogen excretion as part of the adaptive response that allows animals to face adverse environmental changes. In the present paper the acute effects of different salinities and temperatures on routine metabolism, spontaneous activity and excretion of ammonia and urea were studied in two freshwater fish: gambusia, Gambusia affinis and zebrafish, Danio rerio, acclimated to 27 °C. The effects on gill morphology were also evaluated. Five salinities (0‰, 10‰, 20‰, 30‰ and 35‰) were tested in gambusia, while four salinities were used in zebrafish (0‰, 10‰, 20‰ and 25‰). Each salinity acute stress was tested alone or in combination with an acute temperature reduction to 20 °C. In gambusia, both salinity and temperature acute stress strongly stimulated urea excretion. Routine oxygen consumption was barely affected by acute salinity or temperature stress, and was reduced by the combined effects of temperature and high salinity. Gills maintained their structural integrity in all stressing conditions; hyperplasia and hypertrophy of mitochondria-rich cells were observed. In zebrafish, temperature and salinity acute changes, both alone and in combination, scarcely affected any parameter tested. The major effect observed was a reduction of nitrogen excretion at 20 °C–25‰; under these extreme conditions a significant structural disruption of gills was observed. These results confirm the high tolerance to acute salinity and temperature stress in gambusia, and demonstrate the involvement of urea excretion modulation in the stress response in this species.

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1. Introduction

In fish, the response to the osmoregulatory demand posed by salinity changes is species related. While most fish are stenohaline (Fiol and Kultz, 2007), there are a number of euryhaline fish species that tolerate great fluctuations in water salinity (Stickney, 1986; Plaut, 1999; Fiol and Kultz, 2007), including acute changes on a daily basis (Swanson, 1998; Scott et al., 2004). Moreover, salinity tolerance is affected by temperature (Moser and Hettler, 1989), as a consequence of its basic influences on metabolism and a number of related physiological processes (Crockett and Londraville, 2006).

Environmental stress, including temperature and salinity stress, affects metabolism and nitrogen excretion in fish, possibly as part of the adaptive response which allows survival under adverse conditions (Wright et al., 1995; Altinok and Grizzle, 2004; Wood et al., 1994; Polez et al., 2003; Loong et al., 2008). While both acute and chronic effects of salinity and/or temperature on oxygen consumption of freshwater and brackish teleost are well documented (Degani et al., 1989; Iwama et al., 1999; Dalla Via et al., 1998; Claireaux and Lagardère, 1999; De Boeck et al., 2000; Das et al., 2004; Das et al., 2005; Kim et al., 2005; Wuenhschel et al., 2005; Gracia-Lopez et al., 2006), the information on their effects on fish nitrogen excretion is scanty and somewhat contradictory. Following salinity increment (usually chronic), no change has been observed in Salmo trutta (Dodsat et al., 1997) and Allenbatrachus grunniens (Walsh et al., 2004). An increase in ammonia excretion with a decrease in urea excretion has been observed in Cyprinus carpio (De Boeck et al., 2000) and Rutilus marmoratus (Frick and Wright, 2002), while a decrease or constant ammonia excretion with an increase in urea excretion has been observed in the hybrid sturgeon (Gershonovich and Pototski, 1995) and Opsanus beta (Walsh et al., 2004). Gracia-Lopez et al. (2006) have reported that high salinity reduces ammonia excretion in Centropomus undecimalis, while Zheng et al. (2008) reported that ammonia excretion is affected by both salinity and temperature in Múchthys miuy. Changes in urea excretion in response to variable salinity have also been reported in catfish and goldfish (Altinok and Grizzle, 2004). It is of particular relevance that information on the acute effects on nitrogen excretion in fish is relatively scarce, as protocols investigating chronic stress may miss short term changes, which may be relevant both under natural and artificial conditions.

Physiological studies on Poeciliidae of the genera Gambusia and Limia, which include populations adapted to both freshwater and brackish environments, suggest that these animals may be very tolerant of temperature and salinity extremes (Otto, 1973; Meffe et al., 1995; Nordlie and Mirandi, 1996; Haney and Walsh, 2003). However, studies on their ability to face acute changes in these parameters and the...
involvement of nitrogen excretion in their short-term adaptive response are scanty. The main aim of the present study is to examine the physiological responses of gambusia (*Gambusia affinis*), in terms of oxygen consumption and excretion of ammonia and urea, to acute changes in temperature and salinity, and to compare these responses with those of zebrafish (*Danio rerio*), a phylogenetically distant stenohaline species adapted to live under very low salinity conditions (Boisen et al., 2003; Craig et al., 2007). Salinity stress in freshwater fish affects primarily gills, as the major organ involved both in osmoregulation and waste nitrogen excretion (Evans et al., 2005). The metabolic response to acute salinity and temperature stress in gambusia is likely to be correlated with changes at gill levels, and in particular in the mitochondria-rich cell population, as demonstrated in tilapia (Lin et al., 2004). Therefore, the effects of temperature and salinity acute treatments on gill morphology were also analysed.

2. Materials and methods

2.1. Experimental fish

Gambusia (*Gambusia affinis* Baird & Girard, 1853) were collected with nets from a pond near Cancello Arnone (Caserta, Italy). Identification of gambusia species was based on the number of dorsal and anal fin rays (Walters and Byron, 2000). Zebrafish (*Danio rerio* Hamilton, 1822) were obtained from a local pet supply store (CARMAR, Italy). Fish were kept in 2001 glass aquaria with dechlorinated, continuously filtered and aerated freshwater, with 10 h:14 h L:D photoperiod. Animals (100 per tank) were acclimated at 27±1°C for a minimum of 40 days prior to experiments. Acclimation temperature was set close to both the mean water temperature of the site were gambusia were collected and the optimal temperature for zebrafish (Lawrence, 2007). During acclimation, temperature, pH, conductivity, and the levels of ammonia, nitrite and nitrate were checked once a week. Fish were fed daily with commercial pelleted fish food (Tetramin, Tetra, Germany; 47% crude protein content, 6% humidity, 20.1 kJ/g dry mass). Twice a week Tetramin was replaced with *Chironomus* larvae (Eschematteo s.r.l., Italy. 7.01% crude protein content, 89% humidity, 21.9 kJ/g dry mass). Both species displayed a normal shoaling behaviour in the maintenance tanks. Acclimated fish were divided into experimental groups according to the experimental protocols described below. Each group was composed of 20 (gambusia) or 15 (zebrafish) individuals. The animals were selected randomly and isolated into appropriate net cages set in the same tank used for acclimation, and were fasted for 48 h before use (Rodela and Wright, 2006). All procedures were approved by the University Animal Care Review Board.

2.2. Experimental design

Preliminary tests demonstrated that gambusia can tolerate acute exposure to sea water, while zebrafish did not survive when exposed to salinity levels higher than 25‰. Therefore, the effects of acute changes of temperature and salinity on the two fish species were determined in a fully crossed, 2×5 factorial design with equal cell replication (temperature: 27°C and 20°C; salinity 0‰, 10‰, 20‰, 30‰ and 35‰) for gambusia (N = 200; mean mass 0.21 ± 0.01 g) and 2×4 factorial design with equal cell replication (temperature: 27°C and 20°C; salinity: 0‰, 10‰, 20‰ and 25‰) for zebrafish (N = 120; mean mass 0.52 ± 0.01 g). Water at different salinity was prepared fresh using sea salt *BiOMAR™ “C”* (CARMAR, Italy). Salinity was checked with a hand refractometer (S-10E, ATAGO, Japan). In this experimental design, the treatment with different salinities at 27°C (acclimation temperature) evaluated the effect of salinity acute stress, the treatment at 20°C and 0‰ salinity represented the acute temperature stress, and the treatment with different salinities at 20°C evaluated the effects of combined temperature and salinity stress. Five replicas were performed; each replica consisted of a single measurement on a pool of 4 gambusia or 3 zebrafish. In fact, preliminary results demonstrated that measurements on groups rather than on single individuals strongly reduced the adaptation time and minimized routine activity and metabolism both in gambusia (E. Uliano, unpublished) and zebrafish (Agnisola and Femiao, 2006).

A separate group of fish was used to check the effects of acute treatments on gill morphology. Animals were acutely treated at the following conditions: 27°C, 0‰ (control); 20°C, 0‰ (temperature stress); 27°C, 35‰ (gambusia) or 27°C, 25‰ (zebrafish) (salinity stress) and 20°C, 35‰ (gambusia) or 20°C, 25‰ (zebrafish) (temperature plus salinity stress). Animals, in groups of 4 (gambusia) or 3 (zebrafish) individuals, were exposed to the stressing conditions until distress appeared in at least 1 individual, as shown by hyperventilation, opercular congestion, reduced or altered motility, etc. At the end of treatment, individuals of each species and group were treated with excess MS-222, and gills were quickly removed, fixed in 10% formalin and dehydrated through a graded ethanol series. They were routinely embedded in paraffin wax for microscopy. Sections were cut at 5 μm and were dried over night at 37°C. They were stained with hematoxylin and eosin. Histopathological changes were examined under a light microscope.

2.3. Respirometry

Routine respiratory oxygen consumption (rMO2) was measured in a closed system. The respirometer consisted of a thermostatted plexiglass chamber (125 mL). An oxygen microelectrode (YSI 5357 Micro Probe, USA) was set through the chamber cover to continuously record the water oxygen content. The microelectrode was connected to an Oxygen Monitor System (YSI 5300 A), whose output signal was acquired via an analogical-digital interface (Pico Technology Ltd., UK) connected to a PC for automated data acquisition with a specific software (Picolog Pico Technology Ltd., UK). Water in the respirometer was fully aerated and continuously stirred to maintain uniform oxygen concentration. Before introducing fish into the respirometer, the oxygen sensor was calibrated at 100% oxygen saturated water. Animals were weighed, transferred into the respirometer chamber and left undisturbed to adapt to the respirometer. Animals were not disturbed by stirring; in fact, on a 10 min trial their spontaneous activity was not different with and without stirring. After adaptation (usually 10 min), aeration was set off, the chamber was closed and the decrease in oxygen partial pressure with time was recorded. The chamber was re-opened and aeration immediately re-started when the water oxygen saturation dropped to 85%, avoiding hypoxic stress. The decline in water oxygen concentration was linear and oxygen consumption rates were calculated by linear regression analyses. At the end of each rMO2 trial, samples of water were collected for successive evaluation of ammonia and urea excretion rate. Fish rMO2 was calculated as mg O2 kg⁻¹ h⁻¹. Atmospheric pressure during determination was measured and used to calculate PO2 according to the formula:

\[ pO_2 = (AP−SVP) \times 0.2096 \]

where AP was the atmospheric pressure and SVP the saturated vapour pressure (26.739 torr at 27°C and 17.535 torr at 20°C). Oxygen solubility was adjusted to temperature and salinity (Millero et al., 2002). The activity of each fish in the respirometer was evaluated as the number of turns per animal per minute (Lucas and Priede, 1992; Agnisola and Femiao, 2006), and was determined from video recordings taken during the period of oxygen measurements.

2.4. Waste nitrogen excretion: ammonia and urea determination

The concentration of ammonia was measured in water samples taken from the respirometric chamber at the end of each oxygen
consumption determination. Ammonia excretion (M<sub>amm</sub>) was determined with the Hach salicylate method (8155 Hach ammonium test kit, USA), with the DR/2400 Hach spectrophotometer (Hach, USA), and was expressed as mmol–N h<sup>−1</sup> kg<sup>−1</sup>. Urea levels in water samples were measured colorimetrically using a diacetyl–monoxime method (modified from Rahmatullah and Boyde, 1980) (after D. McKenzie, personal communication), and urea excretion (M<sub>urea</sub>) was expressed as mmol–N h<sup>−1</sup> kg<sup>−1</sup>. Urea levels in water were measured colorimetrically using a diacetyl–monoxime method (modified from Rahmatullah and Boyde, 1980) (after D. McKenzie, personal communication), and urea excretion (M<sub>urea</sub>) was expressed as mmol–N h<sup>−1</sup> kg<sup>−1</sup>.

**2.5. Statistical analyses**

Data are presented as means±SE. Two-way ANOVA was used to compare means (p<0.05), with Bonferroni post hoc test. The differences between the two species were evaluated at 0‰ only using the t-student test. Statistics was performed with GraphPad Prism 5.0 (Graphpad Software, San Diego, CA, USA).

**3. Results**

**3.1. Oxygen consumption, routine activity and nitrogen excretion of 27 °C acclimated animals**

In fish acclimated to freshwater at 27 °C, routine oxygen consumption (rMO<sub>2</sub>, Fig. 1, upper panels, 0‰ salinity) was significantly higher in zebrafish (~400 mg O<sub>2</sub> h<sup>−1</sup> kg<sup>−1</sup>) than in gambusia (~200 mg O<sub>2</sub> h<sup>−1</sup> kg<sup>−1</sup>), while routine activity (turns min<sup>−1</sup>, Fig. 1, lower panels, 0‰ salinity) was not different. Ammonia excretion rate (M<sub>amm</sub>, Fig. 2, upper panels, 0‰ salinity) was similar in both species (about 1 mmol–N h<sup>−1</sup> kg<sup>−1</sup>), while urea excretion rate (M<sub>urea</sub>, Fig. 2, lower panels, 0‰ salinity) was 10 times higher in zebrafish than in gambusia (2.68 ± 0.38 and 0.22 ± 0.05 mmol–N h<sup>−1</sup> kg<sup>−1</sup>, respectively). M<sub>urea</sub> represented the 14 ± 7% and 77 ± 7% of total nitrogen excretion for gambusia and zebrafish, respectively. O:N values were 6.58 ± 0.67 in gambusia and 5.74 ± 1.30 in zebrafish.

**3.2. Effect of acute temperature stress**

As shown in Fig. 1, when temperature was acutely decreased to 20 °C, rMO<sub>2</sub> did not change in gambusia (Fig. 1, upper-left panel) despite the significant increase in the activity (from ~3 to ~10 turns min<sup>−1</sup>, Fig. 1, lower-left panel), while it slightly decreased in zebrafish, although not significantly (Fig. 1, upper-right panel), in association with a reduced activity (~3 turns min<sup>−1</sup>, p<0.05, Fig. 1, lower-right panel).

Concerning nitrogen excretion, the acute exposure to 20 °C did not affect both M<sub>amm</sub> and M<sub>urea</sub> in zebrafish (Fig. 2, right panels, 0‰ salinity), but had significant effects in gambusia (Fig. 2, left panels, 0‰ salinity): while M<sub>amm</sub> was significantly reduced (becoming significantly lower than in zebrafish), M<sub>urea</sub> strongly increased. Interestingly, upon acute temperature reduction, M<sub>urea</sub> in gambusia represented the 80 ± 2% of total nitrogen excretion, which compares with the 66 ± 7% of zebrafish. O:N value for gambusia (5.19±0.55) was higher than in zebrafish (2.93±0.20).

**3.3. Effects of acute salinity stress**

As shown in Fig. 1, left panels, when gambusia acclimated to freshwater at 27 °C were acutely exposed to increased salinity, there was only a slight but significant increase in rMO<sub>2</sub> at 20‰; simultaneously, there was a significant increase in spontaneous activity with a maximum at 20‰ and 30‰. Routine activity decreased again at 35‰. In zebrafish (Fig. 1, right panels), there was no change in rMO<sub>2</sub>, while activity significantly increased at 10‰ and decreased at 25‰.

![Fig. 1](image_url)
As shown in Fig. 2, left panels, upon salinity stress gambusia \( M_{\text{ammon}} \) increased at 35\%, only, while \( M_{\text{urea}} \) increased significantly in hyperosmotic water (>10\%). A 40 times increase was observed at 35\% compared to freshwater. In zebrafish (Fig. 2, right panels), \( M_{\text{ammon}} \) increased significantly at 10\% and decreased at higher salinities, while \( M_{\text{urea}} \) remained similar to the control value. At the highest salinity tested, gambusia \( M_{\text{urea}} \) was 83 ± 1\% of total nitrogen excretion rate, a value similar to that of zebrafish (81 ± 4\%), while O:N was lower (1.81 ± 0.82) in gambusia than in zebrafish (5.55 ± 0.95).

3.4. Effects of simultaneous acute changes in salinity and temperature

In gambusia, the exposure to an increased salinity concurrent to a decrease in temperature to 20 °C induced a reduction of \( r\text{MO}_2 \) proportional to the value of salinity (Fig. 1, upper-left panel) becoming lower than the corresponding value at 27 °C at 35\% only. Spontaneous activity (Fig. 1, lower-left panel), increased significantly at 20\%, remaining higher than at 27 °C, and decreased at higher salinities. In zebrafish (Fig. 1, right panels), there were no significant changes in \( r\text{MO}_2 \) and routine activity, with the exception of an increased routine activity at 25\%. \( r\text{MO}_2 \) tended to be lower than at 27 °C, the difference being significant at 10\% and 25\%.

Concerning ammonia excretion rate, in gambusia (Fig. 2, upper-left panel) there was a significant increase at 35\%; \( M_{\text{ammon}} \) remained lower than at 27 °C at all salinities. \( M_{\text{urea}} \) (Fig. 2, lower-left panel) increased significantly at 30\% only, and was lower than at 27 °C only at the 35\% salinity. Interestingly, under simultaneous temperature and salinity acute stress conditions, urea excretion rate was always higher than ammonia excretion rate (80–90\% of total nitrogen excretion). In zebrafish (Fig. 2, right panels), \( M_{\text{ammon}} \) tended to decrease with salinity, being significant at 10\% and 25\%; also \( M_{\text{urea}} \) tended to decrease with salinity, being significant at 25\% only. Urea excretion rate was 60–70\% of total nitrogen excretion at all salinities. At the highest salinities tested, O:N ratio was 1.55 ± 0.60 and 6.13 ± 1.93 in gambusia and zebrafish, respectively.

3.5. Effects of acute changes in temperature and/or salinity on gill structure

As shown in Fig. 3, gills from gambusia submitted to acute salinity stress acutely displayed a slight hyperplasia and hyperthrophy of mitochondria-rich cells (Fig. 3B, arrow), when compared with control (Fig. 3A, thick arrow), while gills submitted to acute temperature stress showed only a mild hyperthrophy of these cells (Fig. 3C, arrow). In the animals treated with temperature plus salinity stress, the hyperplasia and hyperthrophy of mitochondria-rich cells was much more consistent (Fig. 3D, thick arrow), with an increase in the interlamellar tissue. In this last condition a certain degree of alteration of pavement epithelial cells was also observed (Fig. 3D, arrowhead). Gills structure of zebrafish (Fig. 4) was scarcely affected by the acute increase in salinity (Fig. 4B) or the temperature reduction (Fig. 4C). Vice versa, zebrafish acutely treated at 25\% and 20 °C (Fig. 4D) displayed a highly disrupted epithelium with a diffuse edema of both the primary and the secondary lamellae, with separation of epithelium from the underlying basement membrane.

4. Discussion

4.1. Salinity tolerance and effects of acute changes in temperature and/or salinity on routine metabolism and activity

Gambusia appear to tolerate higher salinity levels (up to 35\%) than zebrafish (max 25\%), confirming previous reports. Gambusia species are known to live at low salinities (<15\%). However, some populations living at salinities up to 25\% have been found (Renfro, 1960). Moreover, Gambusia affinis has been demonstrated to tolerate the acute transfer from fresh water to 19.5\% (Chervinski, 1983) and the chronic exposure (7 days) to salinity levels of 39\% and 58.5\% (with survival rate of 65% and 50%, respectively) (Al-Daham and Bhatti, 1977). Concerning Danio rerio, a species that is considered to be tolerant and well adapted to very low salinities (~ 35 μM NaCl)
Boisen et al., 2003; Craig et al., 2007), no data exist on adult response to salted water, although Sawant et al. (2001) reported that in zebra fish embryos the tolerance to 10‰ salinity increases with advancing developmental stage.

Fish behavioural characteristics must be considered when their basal metabolism, i.e. the minimum rate of energy consumption necessary to keep an organism alive (Brett and Groves, 1979), is measured. Since often fish display a basal spontaneous activity, which may influence metabolic rate, the concept of routine metabolism (rMO2) has been introduced, as the metabolism of a resting, unfed, but not starving fish which shows basal spontaneous activity (Fry, 1957). This implies that the routine metabolism measurement needs to be accompanied by a measure of activity (Fry, 1947), that in the present study was evaluated in terms of number of turns per unit time. Another behavioural feature which needs to be considered is the shoaling behaviour of animals. Both gambusia (Angelon and Petranka, 2002) and zebrafish (Spence et al., 2008) are shoaling species. Shoaling and schooling may have a calming effect within groups, with a reduction in spontaneous activity and metabolic rate (Parker, 1973; Klyashtorin and Salikyanov, 1981). Such calming effect of shoaling has been preliminary confirmed in our conditions, so that in both species measurements were made on groups rather than individuals.

The measured rMO2 values are similar to those reported by other authors for the same species, (Mitz and Newman, 1989; Akin and Neill, 2003; Lucas and Priede, 1992), and are relatively high, compared to other species, because of the small size and the high temperature adaptation of these animals (Gillooly et al., 2001; White et al., 2006).

Spontaneous activity was similar in both species. No data are available in literature on basal spontaneous activity in gambusia and zebrafish. Zebrafish spontaneous activity was in the lowest range (between 5 and 10 turns min⁻¹) of the activity levels reported on individuals by Lucas and Priede (1992) (5–60 turns min⁻¹).

Akin and Neill (2003) reported that routine metabolism of gambusia was lower in animals adapted at 10‰ salinity than at 0‰, in agreement with the hypothesis suggested by Stearns and Sage (1980) that they are not necessarily best adapted to the freshwater conditions in which they typically live. How important is this in the acute response to salinity changes is not clear, as we didn’t find great effects of salinity on rMO2. In fact, apart from the increase of rMO2 at 20‰, the main effect was a significant reduction following the 35‰ exposure combined with temperature reduction. This last result is in line with the typical metabolic response to a salinity increase over the tolerance limit found in all euryhaline fish, i.e. a decrease in the routine metabolic rate (Nordlie, 1987; Haney and Nordlie, 1997), with several physiological consequences, including possible reduction of swimming capacity and activity rate (Plaut, 2000). The effects on routine activity induced by acute salinity and temperature stress help to interpret routine activity results. They appear to be different in the two species. As expected, the acute reduction in temperature reduced both routine metabolism (although not significantly) and routine activity in zebrafish. However, in this species there is no apparent correlation between routine spontaneous activity and rMO2 under condition of increased salinity. This may suggest the occurrence of changes in the basal metabolic expenses, independent from

Fig. 3. Light microscope microphotographs of gill filaments of gambusia. Arrowhead: pavement epithelial cells; thick arrow: mitochondria-rich cells; thin arrow: pillar cells. (A) General view of the gills (control); (B) Gills acutely treated with 35‰ at 27 °C displayed hyperplasia and hyper trophy of mitochondria-rich cells. (C) Gills acutely treated with 0‰ at 20 °C showed a mild hypertrophy of mitochondria-rich cells. (D) Gills acutely treated with 35‰ at 20 °C showed alteration of gill pavement epithelial cells, hyperplasia and hypertrophy of mitochondria-rich cells with an increase in the interlamellar tissue.
spontaneous activity, possibly as a result of a stress response. On the contrary, routine metabolism in gambusia increased when temperature was reduced, because of a strong increase in routine activity. This response may reflect their tendency to escape the acute thermal stress, as gambusia in natural environments performs migrations in response to temperature changes, displaying preference for relatively high temperatures (Winkler, 1979). The gambusia routine spontaneous activity was also affected by the salinity changes, increasing at 20‰. Again, the increased spontaneous activity observed under these conditions may reflect an escape behaviour. This is in agreement with the observation made in our laboratory of the tendency of individuals of both sex of this species to avoid saline environments (unpublished), and the hypothesis that salinity limits gambusia invasive success (Alcaraz and Garcia-Berthou, 2007). On the other hand, in gambusia exposed to up to 25‰ salinity, behavioural changes and a reduced activity, in terms of aggression and food competition, have been reported (Alcaraz et al., 2008). This activity reduction (related with an oxygen consumption reduction) was induced in our experiments only at 35‰ combined with a temperature acute stress.

4.2. Effects of acute changes in temperature and/or salinity on nitrogen excretion

The excretion rates of ammonia and urea allow to evaluate the fish nitrogen balance and are useful tools to determine the effects of environmental and nutritional factors on protein metabolism (Rychly and Marina, 1977; Jobling, 1981; Perera et al., 1995; Frick and Wright, 2002; Fournier et al., 2003). Ammonia, the main end-product of protein metabolism, usually represents 70–90% of nitrogenous wastes in teleost (Mommsen and Walsh, 1992; Engin and Carter, 2001). A substantial proportion (10–30%) of nitrogenous wastes is also excreted as urea (Verbeeten et al., 1999; Engin and Carter, 2001; Kajimura et al., 2004; Merino et al., 2007), while other nitrogen compounds usually constitute a minor component (Kajimura et al., 2004). Ammonia is mainly produced in the liver through deamination of free amino acid, although the enzymes associated with this process may be present in other tissues including muscle, intestine, and kidney (Mommsen and Walsh, 1992). Urea production in fish is mainly a by-product of dietary arginine catabolism (argininolysis) and/or purine degradation (uricinolysis) (Terjensen, 2008). However, the ammonia detoxifying pathway, the ornithine–urea cycle (OUC), can be expressed in teleost, depending on species and life stages. Indeed, recent studies have expanded the list of teleost which excrete the bulk of their waste nitrogen as urea (ureotelic) (Anderson and Walsh, 1995; Saha and Ratha, 1998; Terjensen, 2008), and OUC enzymes have been localized in the liver as well as in other tissue, and in particular in the skeletal muscle (Terjensen, 2008).

Gambusia adapted to 27 °C appear to be ammoniotelic, with the rate of urea excretion as low as 14% of total nitrogen excretion. Vice versa, in zebrafish, under our acclimation conditions, the urea-N excretion is about 70% of total nitrogen excretion, suggesting that these animals can be facultative ureotelic fish (Evans et al., 2005). While no data exist on the nitrogen excretion rate in gambusia, Rodella and Wright (2006) reported that approximately 83% of total nitrogen in Danio rerio was excreted in the form of ammonia. The contrast with our results can be due to differences in adaptation conditions and maintenance, including feeding, water characteristics, animal density, etc. (Verbeeten et al., 1999; Walsh et al., 2004). On the other hand,
zebrafish embryos are known to use urea as nitrogen wastes and are considered ureotelic (Braun et al., 2009a). We also evaluated the ratios of oxygen consumption to ammonia-N excretion in atomic equivalents, that can provide information on changes in energy substrate utilization under various environmental regimes (Corner and Cowey, 1968). In particular, O2 (oxygen/nitrogen) values up to 16 mean a preferably protein oxidizable substrate; with a mixture of protein and lipid as substrates O2/N ranges between 16 and 60, while O2 above 60 represents the elevated energy demand from a predominant lipid substrate (Mayzaud and Conover, 1988). O2 values recorded under our conditions for both species suggest a predominant protein catabolism related to the high protein content of diet (47%, see M&M).

Water salinity affected differently ammonia and urea excretion in gambusia and zebrafish. While in zebrafish there was a significant increase in ammonia excretion at 10% only, i.e. in a close to isosmotic environment, in gambusia no significant change was observed apart from a strong increase at the highest salinity tested (35%). Vice versa, whilst zebrafish urea excretion was not affected, in gambusia there was a strong increase in the excretion of this compound, which was proportional to the environmental salinity. Clearly, gambusia (but not zebrafish) display the ability to shift from ammoniotelism to ureotelism upon salinity increase, associated with an increase in total nitrogen excretion. At the moment it is not possible to assess the meaning of such changes in energy substrate utilization under various environmental conditions as well as the role as osmolyte, as suggested by the fact that it increases at high temperature, brings to a hypertrophy and hyperplasia of mitochondria-rich cells are involved in the regulation of urea excretion. At the moment it is not possible to assess the meaning of such changes in energy substrate utilization under various environmental conditions as well as the role as osmolyte, as suggested by the fact that it increases at high temperature, brings to a hypertrophy and hyperplasia of mitochondria-rich cells are involved in the regulation of urea excretion.


