

Effect of temperature on growth of the threatened annual fish *Austrolebias nigrofasciatus* Costa & Cheffe 2001

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VOLCAN, M.V., FONSECA, A.P., FIGUEIREDO, M.R.C., SAMPAIO, L.A. & ROBALDO, R.B. **Effect of temperature on growth of the threatened annual fish *Austrolebias nigrofasciatus* Costa & Cheffe 2001.** *Biota Neotrop.* 12(4): <http://www.biotaneotropica.org.br/v12n4/en/abstract?article+bn01712042012>

Abstract: This study evaluated the effect of temperature on growth of *Austrolebias nigrofasciatus*, an endemic and threatened annual killifish species of the Patos-Mirim lagoon system in Southern Brazil. In order to verify the effect of temperature on initial growth of *A. nigrofasciatus*, eggs stored in the laboratory were hatched and juveniles reared for eight weeks at 16 and 22 °C. The standard length of newly hatched fishes was 4.67 ± 0.25 mm and after eight weeks they reached 23.68 ± 3.73 and 22.68 ± 5.36 mm, respectively at 16 and 22 °C. However, initial growth of fish reared at 22 °C was faster and they reached sexual dimorphism at an earlier age compared to those reared at 16 °C. Final length of females reared at 22 °C was 23.00 ± 2.83 mm, they were significantly larger than those reared at 16 °C (17.91 ± 2.47 mm). Males were significantly larger than the females at 16 °C, but there was no difference for growth between sexes of fish reared at 22 °C. The sex ratios were 1:0.6 and 1:1.1 (M:F) at 16 °C and 22 °C, respectively, suggesting temperature determination of phenotypic sex. Considering the results, it appears that juveniles to be developed in captivity should be kept at 22 °C during the first six weeks of life, thus ensuring a higher growth rate until puberty.

Keywords: annual fish, laboratory conditions, Cyprinodontiformes, Rivulidae.

VOLCAN, M.V., FONSECA, A.P., FIGUEIREDO, M.R.C., SAMPAIO, L.A. & ROBALDO, R.B. **Efeito da temperatura no crescimento do peixe anual ameaçado *Austrolebias nigrofasciatus* Costa & Cheffe 2001.** *Biota Neotrop.* 12(4): <http://www.biotaneotropica.org.br/v12n4/pt/abstract?article+bn01712042012>

Resumo: Este estudo avaliou o efeito da temperatura no crescimento de *Austrolebias nigrofasciatus*, uma espécie de peixe anual endêmica e ameaçada do sistema lagunar Patos-Mirim no extremo sul do Brasil. Para avaliar o efeito da temperatura no crescimento inicial e na diferenciação sexual de *A. nigrofasciatus*, ovos mantidos em laboratório foram eclodidos e os peixes foram mantidos por oito semanas a temperaturas de 16 e 22 °C. O comprimento padrão dos peixes recém eclodidos foi de $4,67 \pm 0,25$ mm e ao final de oito semanas atingiram $23,68 \pm 3,73$ e $22,68 \pm 5,36$ mm, respectivamente para 16 e 22 °C. O crescimento inicial foi maior a 22 °C e os peixes apresentaram precocidade na maturação sexual quando comparados a 16 °C. As fêmeas criadas a 22 °C atingiram maior comprimento ($23,00 \pm 2,83$ mm) em relação aquelas mantidas em 16 °C ($17,91 \pm 2,47$ mm). Machos foram significativamente maiores que as fêmeas a 16 °C, mas não foi constatada diferença de comprimento entre os sexos dos peixes mantidos a 22 °C. A relação sexual foi de 1:0.6 e 1:1.1 (M:F) a 16 °C e 22 °C, respectivamente, sugerindo uma possível influência da temperatura na diferenciação sexual fenotípica. Considerado os resultados obtidos concluímos que juvenis de *A. nigrofasciatus* devem ser criados a 22 °C durante os primeiros meses de idade, garantindo assim uma maior taxa de crescimento até a puberdade.

Palavras-chave: peixe anual, condições de laboratório, Cyprinodontiformes, Rivulidae.

Introduction

Most species of Rivulidae are known as annual fish, they complete their life cycle in temporary ponds and as such their lifespan is short (Costa 2006). These fishes grow fast and reach sexual maturity within a short period, about two months after hatching (Walford & Liu 1965, Liu & Walford 1966, Arenzon et al. 1999, Errea & Danulat 2001). They leave their eggs buried in the substrate at depths of up to 15 cm (Vaz-Ferreira et al. 1966). Once these temporary ponds dry off, the entire adult population dies. However, the resting eggs remain on diapause, waiting the next rainy season, when they will hatch and start a new life cycle (Podrabsky & Hand 1999). Annual fish have been used as model organisms for laboratory (Arenzon et al. 2002a, 2003), bioindicators (Arezo et al. 2007), pest control (Fletcher et al. 1992, Frenkel & Goren 2000) and ornamental fish (Costa 2008).

Austrolebias are a genus of annual killifishes distributed in subtropical and temperate Argentina, Southern Brazil, Bolivia, Paraguay and Uruguay (Costa 2010). The most species inhabit seasonal ponds, where the rainy season coincides with winter months, therefore they are usually found in cold water (Costa 2006). *Austrolebias nigrofasciatus* Costa & Cheffe, 2001, are small fish, endemic of the Patos-Mirim lagoon system in Southern Rio Grande do Sul, Brazil. They present marked sexual dimorphism and are characterized by the color pattern of males, which consists of blue strips, parallel to the rays in basal half of dorsal and anal fins and a black spot bordering the posterior end of the dorsal and anal fins (Costa & Cheffe 2001).

This group of fishes requires several adjustments to assure their survival in highly variable environmental conditions such as drought stress, low levels of oxygen and wide fluctuations in temperature (Liu & Walford 1966, 1970, Errea & Danulat 2001). However, the same characteristics that make them able to live in such hostile environments make them dependent on the physical integrity of their biotope. In light of deleterious anthropomorphic activities in their habitat and restricted distribution area, *A. nigrofasciatus* is listed as a threatened species (Reis et al. 2003, Rosa & Lima, 2008, Volcan et al. 2009).

Temperature is considered one of the most crucial factors influencing the early development of fish (Brett & Groves 1979). Temperature has been shown to influence Rivulidae fish in terms of reproduction (Arenzon et al. 1999), time of the embryonic development (Arezo et al. 2007, Arenzon et al. 2002a), growth (Errea & Danulat 2001, Walford & Liu 1965, Liu & Walford 1970), and longevity (Walford & Liu 1965, Liu & Walford 1966, 1970).

The life cycle of *A. nigrofasciatus* is poorly studied. Considering the current threat of extinction for *Austrolebias* species in Brazil, it is important to understand the role of factors regulating its development and thus provide subsidies to improve the management of natural populations and to rear them in captivity (Reis et al. 2003, Rosa & Lima, 2008, Volcan et al. 2009, 2010a, b, 2011a, b, c). As such, this work aimed to study the effects of temperature on their survival, early growth and phenotypic sex differentiation of *A. nigrofasciatus* at laboratory conditions.

Materials and Methods

1. Production and hatching of eggs

Were captured 12 pairs of *A. nigrofasciatus* in a freshwater temporary pond located in Southern Brazil (31° 48' 25" S and 52° 25' 11" W) and maintained in laboratory for two months for obtained of fertilized eggs utilized in this study. Eggs were kept in opaque sealed bags buried in wet coconut fiber substrate at room temperature

(18-25 °C) for approximately six months, that is considered the approximate time that the biotopes of the species remain dry (M.V. Volcan, unpublished data).

For hatching, the eggs were deposited in Beaker glasses (1 L), buried to a depth of 10 cm in shredded coconut fiber, under 500 mL of water and kept at 18.0 ± 0.9 °C (SD) for 24 hours.

Broodstock fish used in the study were caught under license number 15108-1 issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA/ICMBio).

2. Laboratory tests

Fishes were randomly divided into two 30 L aquaria just after hatching. The stocking density was 1 juvenile/L and they were maintained for eight weeks at two constant temperatures: 16 and 22 °C. This range temperature was tested because it is considered within thermal variation recorded in the habitats of *Austrolebias* species (Walford & Liu 1965) and ideal for their maintenance in captivity (Calviño 2005).

Temperature was controlled by a submersible heater (20 W) coupled to a digital thermostat (Aquaterm®/FullGauge/0.1 °C). Water was kept under constant aeration and photoperiod of 12 hours of light per day. Fish were fed twice daily to satiation, at 12:00 PM and at 8:00 PM, with zooplankton, mainly native copepods and cladocerans, produced in mesocosm with organic fertilizer.

Fish were anesthetized in a benzocaine bath (50 mg/L) prior to standard length (SL) measurement. During the first week length was measured under a stereoscopic microscope equipped with micrometric ocular piece, thereafter a digital caliper was employed, all measurements were made to the nearest 0.01 mm. Fish were measured weekly, except for the second week of life, because a large mortality (25%) was found to be associated to the first biometry. No further mortalities were associated to length measurement. All fish in each tank were measured. The phenotypic sex differentiation and length of 50% individuals attained the sexual dimorphism was detected by development of secondary sexual characters of the species (color pattern and fin morphology) described by Costa & Cheffe (2001).

Temperature, dissolved oxygen, and pH were measured once a day with portable pHmeter Hanna HI9025 (0,01) and oximeter Quimis Q758P (00,1 mg/L). Water exchange was equal to approximately 20 %/day. Average temperature (±SD) was 16.1 ± 0.2 and 22.1 ± 0.2 °C. pH and dissolved oxygen concentration were equal to 7.45 ± 0.04 and 8.04 ± 0.29 mg O₂/L, and 7.52 ± 0.05 and 7.59 ± 0.32 mg O₂/L at 16 and 22 °C, respectively.

3. Statistical analysis

Length data and effects of temperature on standard length at the moment of 50% of sexual dimorphism were compared using the Student t-test. The possible bias of the sex ratio of the two temperatures from the expected 1:1 rate were determined by Chi-square test (χ^2). The assumptions of homogeneity of variance and normality distribution were analyzed by Cochran C and Kolmogorov-Smirnov tests, respectively. The significance level of all tests was 95% and they were performed using the software Statistica 8.0. The results are presented as mean ± standard deviation (SD).

Results

With the exception of the accidental mortality of one and five fishes at 16 °C and 22 °C, respectively, during the length measurement after hatching, no further mortalities were observed for both temperatures.

The standard length of newly hatched fishes was 4.67 ± 0.25 mm. Fish kept at 22 °C showed higher initial growth until six weeks of

life (t-test; $p < 0.05$). However, at the end of eighth week, there was no more significant difference in length (t-test; $p > 0.05$) of *A. nigrofasciatus* reached 23.68 ± 3.73 and 22.68 ± 5.36 mm, respectively at 16 and 22 °C (Figure 1).

The males responded similarly to temperature with average standard length at the end of eight weeks of 25.61 ± 4.46 and 24.67 ± 4.77 mm, under 16 and 22 °C, respectively (Figure 2). Females reared at 22 °C had significantly higher growth ($p < 0.05$) than those kept at 16 °C in all biometrics, with the average length for the lower and higher temperature of 17.91 ± 2.47 and 23.00 ± 2.83 mm, respectively (Figure 2).

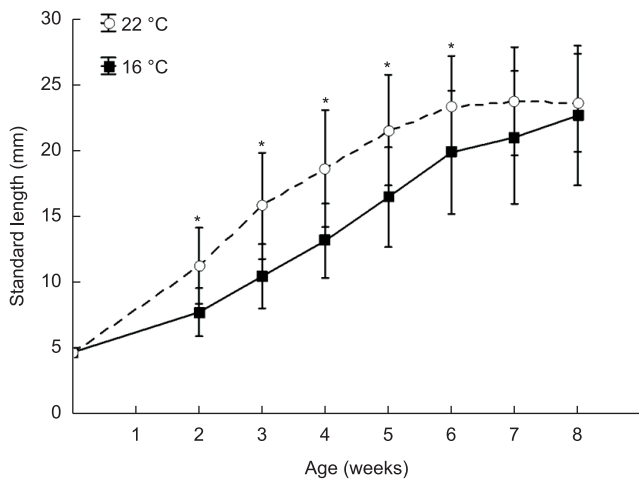


Figure 1. Standard length (mean \pm standard deviation) of *Austrolebias nigrofasciatus* kept in the laboratory at 16 and 22 °C during the first eight weeks of life. *Indicates significant difference in standard length between treatments during the first six weeks of study (t-test, $p < 0.05$).

Figura 1. Comprimento padrão (média \pm desvio padrão) de *Austrolebias nigrofasciatus* mantido em laboratório a 16 e 22 °C durante as primeiras oito semanas de vida. *Indica diferença significativa em comprimento padrão nas primeiras seis semanas de estudo (teste t, $p < 0.05$).

The males kept at 16 °C attained higher SL than females throughout the entire experimental period in contrast to similarity in size observed between the sexes to 22 °C (Figure 3). In the third week were registered sexual dimorphism in fishes at 22 °C, and the fourth week it was found the sexual maturity of females in this temperature, evidenced by reproductive behavior and egg laying, while at 16 °C the first male and females were identified only in the fifth and sixth weeks, respectively. Only from the eighth week was recorded egg laying for fish kept at 16 °C. In both treatments were recorded juveniles until the sixth week.

The age and standard length that more than 50% of specimens in each treatment reached the sexual dimorphism in females occurs in the seventh week and length of 17.08 ± 2.15 mm at 16 °C and in the fourth week and 21.38 ± 2.15 mm at 22 °C. For males, the dimorphism was reached in the fifth week at 16 °C with 20.45 ± 2.17 mm and the fourth week at 22 °C with 21.31 ± 4.33 mm (Figure 4). The coefficient of variation for male standard length ($17.89 \pm 2.30\%$) was significantly higher than females ($12.36 \pm 2.18\%$) only at 22 °C (t-test, $p < 0.0001$). The results show that fish kept at 22 °C reached maturity and sexual dimorphism earlier, and at a larger size than those kept at 16 °C.

The sex ratios were 1:0.6 and 1:1.1 (M:F) at 16 and 22 °C, respectively. Although the fish kept at 22 °C showed a tendency to a higher male proportion, there was no significant difference in sex ratio in the two treatments ($\chi^2 = 1.69$; $df = 1$; $p = 0.193$ for 16 °C; $\chi^2 = 0.04$; $df = 1$; $p = 0.841$ for 22 °C).

Discussion

In this study there was no mortality related to the rearing temperature, since the reported deaths were due to accidents during measurement of fish. The maximum standard length registered to *A. nigrofasciatus* is 42.7 mm (Costa & Cheffe 2001), considering it as a reference value, the species reaches about 60% of its maximum standard length within two months after hatching in the laboratory, proving the fast initial growth, typical to species of *Austrolebias* (Liu & Walford 1969, Errea & Danulat 2001).

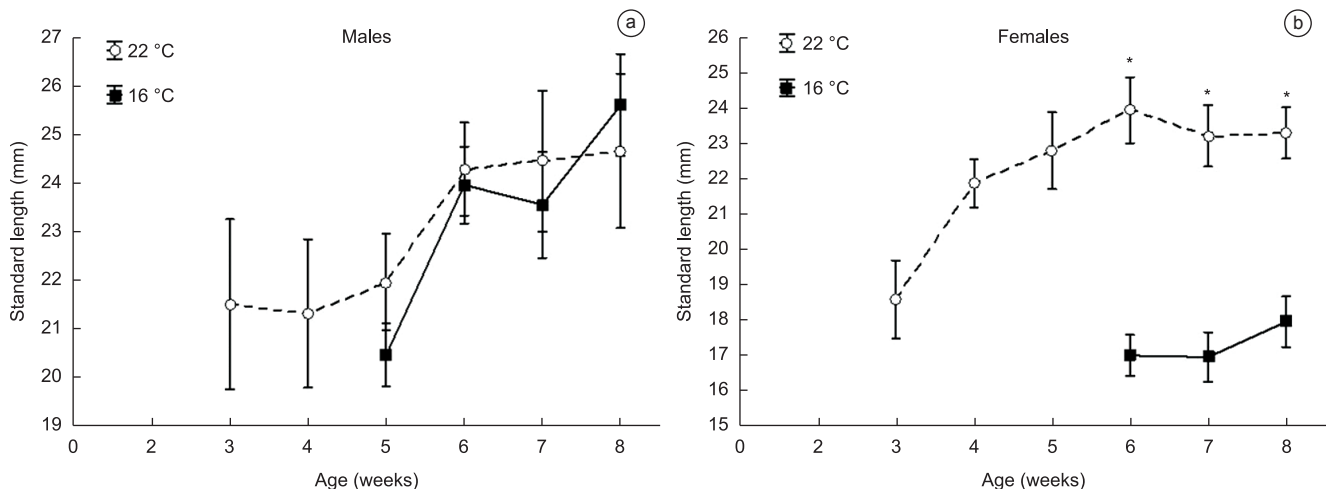


Figure 2. Growth of males (a) and females (b) of *Austrolebias nigrofasciatus* (mean \pm standard deviation of SL) reared in laboratory at 16 and 22 °C for eight weeks. Differences at the beginning of the growth curve occurred due to the time of sexual differentiation. In both treatments, females differed later than the males. *Indicates significant difference between the means (t-test, $p < 0.05$).

Figura 2. Crescimento de machos (a) e fêmeas (b) de *Austrolebias nigrofasciatus* (média \pm desvio-padrão de CP) mantidos em laboratório a 16 e 22 °C por oito semanas. Diferenças no início da curva de crescimento ocorreram devido ao tempo de diferenciação sexual. Em ambos os tratamentos as fêmeas diferenciaram mais tardiamente que os machos. *Indica diferença significativa entre as médias (teste t, $p < 0.05$).

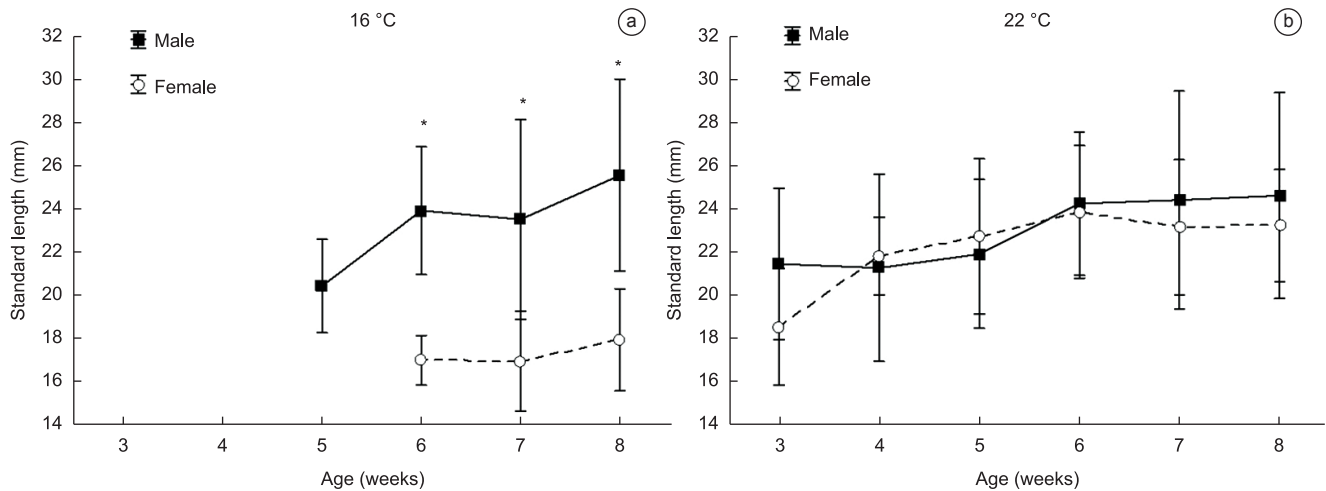
Growth of *Austrolebias nigrofasciatus*

Figure 3. Growth of *Austrolebias nigrofasciatus* in the laboratory at 16 (a) and 22 °C (b) (mean \pm standard deviation of SL) for eight weeks from hatching. Differences at the beginning of the growth curve occurred due to the time of sexual differentiation. Fishes of treatment of 16 °C differentiate later than those kept at 22 °C. *Indicates significant difference between the means (t-test, $p < 0.05$).

Figura 3. Crescimento de *Austrolebias nigrofasciatus* em laboratório a 16 (a) e 22 °C (b) (média \pm desvio padrão do CP) durante oito semanas após a eclosão. Diferenças no início da curva de crescimento ocorreram devido ao tempo de diferenciação sexual. Os peixes do tratamento de 16 °C diferenciaram mais tardiamente que os mantidos a 22 °C. *Indica diferença significativa entre as médias (teste t, $p < 0,05$).

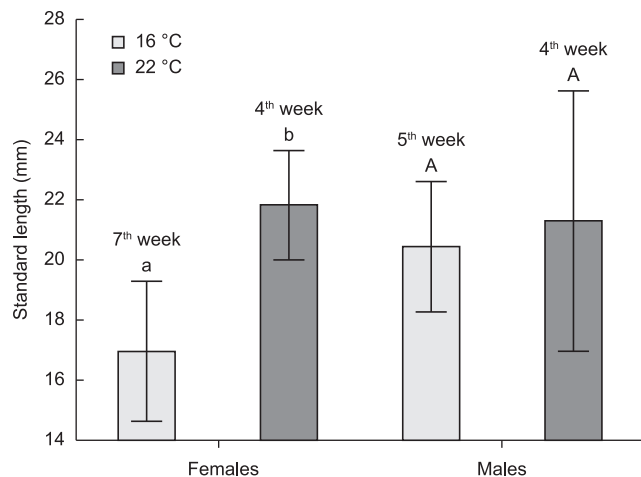


Figure 4. Effect of temperature on age and standard length at the moment of 50% of sexual dimorphism in *Austrolebias nigrofasciatus* (mean \pm SD). Different labels indicate significant difference for standard length to females (lowercase) and males (uppercase), respectively (t-test, $p < 0.05$).

Figura 4. Efeito da temperatura na idade e comprimento padrão no momento de 50% de dimorfismo sexual em *Austrolebias nigrofasciatus* (média \pm SD). Letras diferentes indicam diferença significativa entre o comprimento de fêmeas (minúscula) e machos (maiúsculas), respectivamente (teste t, $p < 0,05$).

The relationship of temperature with the growth of annual fish was also observed by Errea & Danulat (2001) that in the natural environment of *Austrolebias viarius*, reported the highest growth rates at the beginning and end of life, precisely in the months when the highest temperatures were recorded. These authors, in assessing the effect of temperature on growth of *A. viarius* in the laboratory, found that individuals maintained at 25 °C had higher growth and early sexual maturity when compared with those reared at 15 °C. The results of the present study showed higher initial growth for *A. nigrofasciatus* at 22 °C. This was a contrasting response in relation to sex, since males grow faster than females at 16 °C, but at 22 °C

there is no difference in growth rate related to sex. Independently of temperature effect, the higher length of males in rivulids was also observed for several species (e.g. Walford & Liu 1965, Liu et al. 1975, Laufer et al. 2009, Arenzon et al. 2001) and is considered a pattern of *Austrolebias* species (Costa 2006).

Annual fishes in general are exposed to marked fluctuations in abiotic conditions in their natural habitat (Errea & Danulat 2001, Arenzon et al. 2002b, Volcan et al. 2011c). Frenkel & Goren (2000) studying the effect of temperature on the growth of killifish, between 18 and 27 °C, showed higher growth of *Aphanius dispar* at 18 and 23 °C. Similarly, studies of effect of temperature on the life cycle of species of *Austrolebias* have shown that they have greater final length at temperatures between 15-16 °C when compared to 20-22 °C (Walford & Liu 1965, Liu & Walford 1970). The influence of temperature on initial growth of *A. nigrofasciatus* recorded in this study suggests that this group of fish present difference effect of the temperature associated with sex and initial growth.

As Liu & Walford (1969), for annual fish, higher temperatures stimulate the growth of the species only in the early stages, while throughout life lower temperatures favor the somatic growth by delaying the development of the gonads. Our study is in agreement with Liu & Walford (1969), where we also observed the effect of temperature in different stages of life. In the first six weeks we noticed a fast growth at 22 °C, however, after the fish reaching sexual maturity and dimorphism, the growth curve tended to stabilize at 22 °C and increased to 16 °C. Liu et al. (1975) reported that the transfer of adult fish from 22 °C to 16 °C results in increasing the growth of *Austrolebias bellotti*.

The early sexual maturity observed for *A. nigrofasciatus* was also recorded for *Austrolebias adloffii* (Walford & Liu 1965) and *Cynopoeilus melanotaenia* (Arenzon et al. 1999). These species reached sexual maturity after 6 (at 22 °C) and 8 (at 17-25 °C) weeks of life, respectively. The precocity of dimorphism in males was also observed for *Austrolebias toba*, the first secondary sexual characteristics were observed at 21 days after hatching, followed by courtship behavior, but still no spawning, since the females in this age were not mature (Calviño 2005).

During the study, territoriality and aggression were observed between males of *A. nigrofasciatus*, which has not occurred within females. According to Belote & Costa (2004) the general pattern of reproductive behavior of *Austrolebias* is similar to most other species of Rivulidae, except for the absence of fights between females. The higher dispersion of mean length of males when compared to females of *A. nigrofasciatus*, in both treatments, probably reflects a phenomenon of social pressure by size/hierarchy imposed over a longer period by the largest males.

Sexual differentiation of *Austrolebias* occurs still in the embryonic stage, as shown by histological analysis (Arezo et al. 2007). It was observed higher proportion of males of *A. nigrofasciatus* reared at 16 °C compared to those reared at 22 °C, suggesting possible role of temperature on the determination of phenotypic sex, a common phenomenon for many teleosts (Devlin & Nagahama 2002). In agreement with this hypothesis, there are 1% of males in natural populations of *Kryptolebias marmoratus* (Rivulidae) in Florida. However, rearing them at 19 °C (lower temperature than in the natural environment) resulted in a 100% male population (Harrington, 1967). Besides, this species presents a higher sex ratio when juveniles are kept at high temperature (30 °C). Harrington (1968) suggests a possible thermo labile sex determination in juveniles of *K. marmoratus* and denotes that the effect of temperature on induction of males differentiation may be a system of sex determination by environmental conditions.

In this work we observed a rapid initial growth of *A. nigrofasciatus*, which resulted in early maturation and sexual dimorphism in the temperature of 22 °C, compared to 16 °C. Despite the satisfactory results, the experimental design of this study did not have replicates for the treatments. An appropriate number of replicas and the exposure of fish to greater thermal amplitude could help to verify these results, mainly on the thermal influence on sex determination of killifishes.

We concluded that newly hatched fish to be developed in captivity should be kept at 22 °C during the first six weeks of life, in favor of more rapid growth until puberty while lower temperatures seems to improve growth after this period. Considering these results, future studies aiming to test the influence of temperature on the life cycle of Rivulidae are key to understanding the biology of these species and to contribute to future studies, mainly those aiming to manage populations in their natural habitat or at laboratory conditions.

Acknowledgements

The authors wish to express their gratitude to IBAMA/ICMBio for issuing the license for sampling (15108-1). M.V. Volcan and A.P. Fonseca thanks CAPES for the M.Sc. grant. L.A. Sampaio (301673/2009-3) and R.B. Robaldo (312035/2009-8) are research fellows of Brazilian Research Council (CNPq).

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Received 08/09/2011

Revised 10/07/2012

Accepted 07/11/2012