

Reproductive strategies of two Neotropical killifish, *Austrolebias vandenbergi* and *Neofundulus ornatipinnis* (Cyprinodontiformes: Rivulidae) in the Bolivian Gran Chaco

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Abstract: The dry Chaco, a semiarid thorn forest, is experiencing some of the highest deforestation rates globally, coupled with the fact that small-bodied fish are at the highest risk of extinction, the killifish inhabiting this region may be some of the most threatened taxa. Yet, aspects of ecology and life history for Neotropical killifishes in the Bolivian Gran Chaco region are completely lacking, and basic life-history data is of critical importance for the design and implementation of conservation measures. Collections were conducted during the early (January 2011) and late (March–April 2011) rainy season using an area-based sampler and dip net surveys. Fish standard length and body depth were measured as well as the number of oocytes per size class, mean oocyte diameter per size class, and total fecundity for the females of each species. A total of 490 specimens of rivulids were captured; *Austrolebias vandenbergi*: 85 females, 105 males and 39 juveniles, and *N. ornatipinnis*: 62 females, 113 males, 86 juveniles. Sexual size dimorphism, absolute fecundity, oocyte developmental stages, oocyte diameter, and population sex ratios were determined for each species. Both species exhibited sexual size dimorphism. Male *A. vandenbergi* exhibited longer standard length (mean±SD; males: 27.07±3.89mm, females: 23.6±2.02mm) and body depth (males: 8.9±1.7mm, females: 7.2±1.1mm) as compared to females. Male *N. ornatipinnis* had a similar pattern for both standard length (males: 26.0±7.1mm, females: 19.1±5.83mm) and body depth (males: 5.6±1.9mm, females: 4.7±1.0mm). *Austrolebias vandenbergi* had fewer and smaller oocytes per female (47±31.6) than *N. ornatipinnis* (206±131.2). There was a positive relationship between fecundity and female body size in both species. The presence of multiple developmental stages of oocytes (immature, maturing, and mature) suggest that both species of rivulids exhibit fractional spawning, a reproductive strategy that enhances reproductive success in these extreme habitats. *Neofundulus ornatipinnis* exhibited a higher mean oocyte diameter for all three developmental stages (immature, maturing, and mature) as compared to *A. vandenbergi*. *Austrolebias vandenbergi* exhibited an equal adult sex ratio (males:females, 1:1), but there was a slight female biased ratio for *N. ornatipinnis* (males:females, 1:1.8). These results provide fundamental and valuable information for ensuring rivulid conservation in tropical regions, and also improve the knowledge on the biology and ecology of these poorly known species. To our knowledge, this study represents the first contribution on the reproductive biology of two Neotropical annual rivulid fishes (*Austrolebias vandenbergi* and *Neofundulus ornatipinnis*) inhabiting semi-permanent and ephemeral ponds in the Gran Chaco of Southeastern Bolivia. Rev. Biol. Trop. 62 (1): 109-117. Epub 2014 March 01.

Key words: annual fish, ephemeral ponds, fecundity, life history, sexual dimorphism, tropical dry forest.

The family Rivulidae (Cyprinodontiformes) is a species-rich group of freshwater fishes with more than 350 valid species that extend from Southern Florida to Northeastern Argentina (Costa, 2011). Many of these species

are able to complete their life cycles in temporary aquatic habitats, and thus are often referred to as annual killifishes. These species possess a number of adaptations to deal with the constraints of ephemeral habitats. Populations can



persist in these habitats because their eggs are deposited in the substrate of the pond where they undergo diapause until the next rainy season (Wourms, 1972). Annual rivulids possess an opportunistic life-history strategy, characterized by having a small body and undergoing rapid maturation (Winemiller & Rose, 1992). Also characteristic of species possessing an opportunistic life-history strategy is sexual selection (Winemiller, 1992). Rivulids are often sexually dimorphic; males are brightly colored and conduct elaborated courtship displays (Belote & Costa, 2004; Garcia, Loureiro & Tassinio, 2008).

Globally, small-bodied freshwater fish are amongst the highest fish taxa at risk of extinction, which has been attributed to their vulnerability to habitat alterations and degradation (Olden, Hogan & Zanden, 2007). Killifish are no exception; they often possess a limited geographical range and are amongst some of the most threatened fish in Brazil (Rosa & Lima, 2008; Volcan, Lanés & Cheffe, 2010; Costa, 2012). Understanding life-history traits that are fundamental to population regulation, like a species reproductive biology, are essential in the design and implementation of conservation strategies (Winemiller, 2005).

While the phylogeny of rivulids has been the subject of much study (Costa, 1988, 1998, 2002, 2006), information on the ecology and life-history on a number of these species is lacking. The genus *Neofundulus* is comprised of five species, but two of the five species of *Neofundulus* are only known from their holotypes (Costa, 1998). There are 39 valid species recognized belonging to the genus *Austrolebias* (Costa, 2006; Loureiro, Duarte & Zarucki, 2011), and aspects of the ecology, behavior, and reproduction of some species have been the subject of study (Belote & Costa 2004; Calviño, 2005; Garcia et al., 2008; Volcan, Fonseca & Robaldo, 2011). In this study, we investigated the reproductive biology of two species of killifish that were recently found in the Bolivian Gran Chaco; Van den Berg's pearlfish (*Austrolebias vanderbergi*; Huber, 1995) and the ornate killifish (*Neofundulus*

ornatipinnis; Myers, 1935) (Montaña, Schalk, & Taphorn, 2012). Preliminary details on the reproductive biology and diet of *A. vanderbergi* from this site have been reported (Montaña et al., 2012), but nothing is known about *N. ornatipinnis* in the region. Here we present a more extensive analysis on the reproductive biology of both species of killifish from the Bolivian Gran Chaco.

MATERIAL AND METHODS

Study area: The Gran Chaco is a large xeric floodplain that contains vast expanses of semiarid thorn forest and palm savannahs distributed across the countries of Argentina, Brazil, Bolivia, and Paraguay. The Chaco has a warm, wet summer (November-March) and a cool, dry winter (April-October). In the study area in the Bolivian Chaco, average rainfall and temperature are 513mm and 24.6°C, respectively (Navarro & Maldonado, 2002). The surrounding forest is xerophytic thorn forest, with *Schinopsis lorentzii* (Engler, 1880) and *Aspidosperma quebracho-blanco* (Schlectendal, 1861) being common tree species and cacti (e.g. *Opuntia* sp., *Cleistocactus baumannii* [Lemaire, 1861], and *Eriocereus guelichii* [A. Berger, 1929]) and bromeliads constituting the common understory plants (Navarro & Maldonado, 2002). We sampled fish from both semi-permanent (N=1) and temporary (N=3) ponds around the Isoceño community of Kuaridenda, Provincia Cordillera, Departamento Santa Cruz, Bolivia (19°10'24" S -62°35'16" W and 19°10'34" S - 62°35'21" W).

Fish surveys: *Austrolebias vanderbergi* were collected only at the end of the rainy season (April 2011), whereas individuals of *N. ornatipinnis* were collected towards the beginning (January 2011) and the end (March-April 2011) of rainy season using a combination of methods including an area-based sampler (area: 1.3m²; height: 0.70m) that was cleared with a dipnet (mesh: 2mm) and dipnet surveys. In the instance when the depth of the pond exceeded the height of the area-based sampler,

we conducted five seine net sweeps (1m length x 1m height, 0.5mm mesh) (Werner, Skelly, Relyea & Yurewicz, 2007). The pipe-sampler was placed every three meters along a pond's longest axis in ponds <100m in length or every ten meters in ponds >100m in length. The abiotic characteristics of the ponds surveyed in this study are listed in Table 1. All individuals captured were immediately fixed in 10% formalin and later sorted and stored in 70% ethanol. Specimens collected and analyzed were deposited in the Biodiversity Research and Teaching Collections (TCWC 15182.01, 15183.01, 15184.01) at Texas A&M University (TAMU), College Station Texas, U.S.A. Additional specimens not examined in this study were deposited in the Museo Noel Kempff Mercado in Santa Cruz de la Sierra, Bolivia.

Fish measurements and reproductive analysis: Specimens were identified to species level using taxonomic keys (Costa, 1988; Huber, 1995a-b; Costa, 2006) and measured with a dial caliper (to nearest 0.01mm). Morphological measurements including standard length (SL) and body depth (BD) were measured for each individual. Standard length was used to classify the specimens into size groups. Specimens were identified as juveniles, males and females, and female gonadal state of maturation was determined following the methodology by Vazzoler (1996), which allows the inference of the degree of development of the ovaries. For each female ovary,

oocytes were separated and classified into three different levels of maturity using a dissecting microscope: immature (homogeneous and light colored oocytes and without follicle differentiation), maturing (oocytes in intermediate stages with yellow coloration and follicle in different stages), and mature (oocytes in final stage of maturation, totally developed ovaries with large follicle yellow to orange color). Fifteen oocytes were randomly chosen from each ovary and measured with an ocular micrometer in a dissecting microscope to estimate oocyte diameter (Gonçalves, Souza & Volcan, 2011). Absolute fecundity was estimated by counting the total eggs in the ovary of each female (Vazzoler, 1996). Finally, sex ratio was determined using the absolute frequencies of males and females collected during the sampling period. The Gonadosomatic Index (GSI) was calculated only for female *N. ornatipinnis*, for which there were samples collected during the early rainy season (only three mature females were examined because the majority of individuals were juveniles), March (11 females examined), end of rainy season (48 females examined). The GSI ($GSI = \frac{G_w}{W_t} * 100$) allows inferences to be made about species spawning periods. For example, an increase in the GSI suggests an approach to spawning season (Vazzoler, 1996).

The relationship between fecundity (F) and standard length was estimated for females of each species using the ordinary least squares regression model given by $F = a + b(x)$, where

TABLE 1
Average abiotic pond parameters of the ponds where *A. vanderbergi* and *N. ornatipinnis* were collected¹

Pond	Species	Average Pond Surface Area (m ²)	Average Depth (m)	Hydroperiod (%)	Canopy Cover (%)	pH
1	<i>N. ornatipinnis</i>	576	0.55	100	71	7.6
2	<i>N. ornatipinnis</i>	740	0.05	77.2	61	7.15
3	<i>A. vanderbergi</i>	777	0.21	77.2	70	7.27
4	<i>A. vanderbergi</i>	2487	0.28	77.2	83	7.12

1. Abiotic parameters for pond 1 were collected monthly from January - April 2011, whereas the parameters for ponds 2-4 were collected from February - April 2011. Hydroperiod is the percentage of days the ponds held water within the study period. Ponds were checked approximately every two weeks for the presence of water and were assumed to have dried in the midway point between surveys. Canopy cover was estimated using a spherical densitometer.

F represents fecundity (dependent variable), x represents length (independent variable), a is the numerical variable of intercept, and b is the slope. Length classes were determined to verify size differences between sexes (Sparre & Venema, 1997). Due to the variation in individual size classes and non-normality of the data, we used the Kolmogorov-Smirnov test (K-S), a non-parametric t-test, to test for differences in the standard lengths and body depths between species of killifish. A one-way analysis of variance (ANOVA) was performed to compare the means of the oocyte diameter among size classes between both species of killifish. Finally, chi-square (χ^2 , $p < 0.05$) was performed in the sexes of each species to determine whether or not they differ from the expectation (1:1). All analyses were conducted using the software JMP 7 (SAS Institute Inc. 2007).

RESULTS

A total of 490 specimens of rivulids were captured. Of those, 229 were *A. vanderbergi* (85 females, 105 males and 39 juveniles) and 261 were *N. ornatipinnis* (62 females, 113 males, 86 juveniles). *Austrolebias vanderbergi* were only collected from the two temporary ponds, whereas *N. ornatipinnis* were collected from both a temporary and the semi-permanent pond in the area. These species did not co-occur with one another (Table 1). Standard length varied between 19.0 and 24.9mm (23.6 mean \pm 2.02 standard deviation) for females and between 18.3 and 28.8mm (27.07 \pm 3.8) for males (Fig. 1A). Body depth was greater for males (8.9 \pm 1.7) than for females (7.2 \pm 1.1) (Fig. 1B). Standard length in *N. ornatipinnis* ranged from 10.6 to 34.7mm (19.1 \pm 5.8) for females and from 15.6 to 50.6mm (26.0 \pm 7.1) for males (Fig. 1A). Body depth was also greater for males (5.6 \pm 1.9) than for females (4.7 \pm 1.0) (Fig. 1B). The K-S test indicated significant differences in the standard length of males and females of *A. vanderbergi* ($D=0.51$, $p < 0.001$) and *N. ornatipinnis* ($D=0.66$, $p < 0.009$), with males showing larger sizes. Significant differences also were found in the

body depth of males and females of *A. vanderbergi* ($D=0.60$, $p < 0.008$) and *N. ornatipinnis* ($D=0.33$, $p < 0.0006$), respectively.

There were 85 ovaries of *A. vanderbergi* and 62 ovaries of *N. ornatipinnis* examined to determine absolute fecundity and ovarian developmental stages. The mean fecundity of *A. vanderbergi* (mean 47 oocytes) was lower than *N. ornatipinnis* (mean 206 oocytes) (Table 2). Examination of oocyte diameter revealed that both species of rivulids demonstrated fractional spawning, as determined by the presence of at least three oocyte developmental stages (Fig. 2, Table 2). *Neofundulus ornatipinnis* exhibited a higher mean oocyte diameter for all three developmental stages (immature, maturing, and mature) as compared to *A. vanderbergi* (Table 2). The ANOVA revealed significant

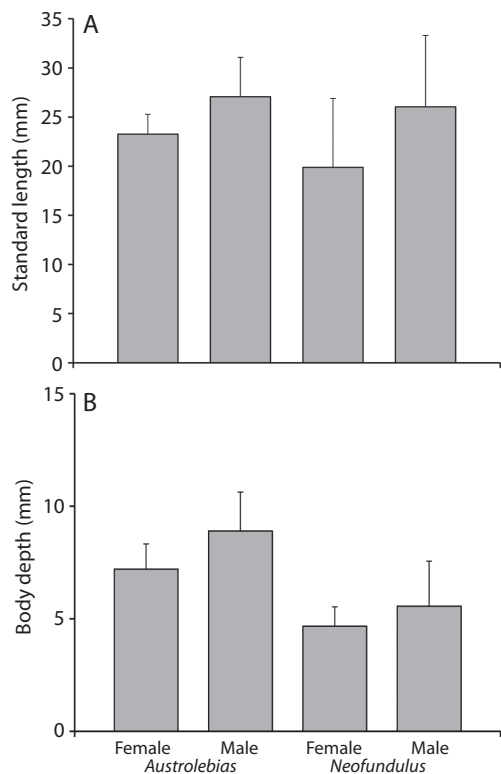


Fig. 1. Mean (+1 standard deviation) of (A) standard length and (B) body depth of male and female *A. vanderbergi* and *N. ornatipinnis*.

TABLE 2

Mean fecundity, range, and oocyte diameter developmental stages for female *A. vanderbergi* and *N. ornatipinnis*²

j	<i>A. vanderbergi</i> (N = 85)	<i>N. ornatipinnis</i> (N = 62)
Mean Fecundity (number of eggs)	47 (± 31.6)	206 (± 131.2)
Fecundity Range (number of eggs)	9 - 128	4 - 535
Mean Immature Oocyte Diameter (mm)	0.37 (±0.13)	0.38 (±0.18)
Mean Maturing Oocyte Diameter (mm)	0.85 (±0.19)	0.95 (±0.49)
Mean Mature Oocyte Diameter (mm)	1.49 (±0.21)	1.67 (±0.74)

2. Standard deviation values of the mean fecundity and oocyte diameters are given in parentheses. Oocyte developmental stages were determined from the criteria established by Vazzoler (1996).

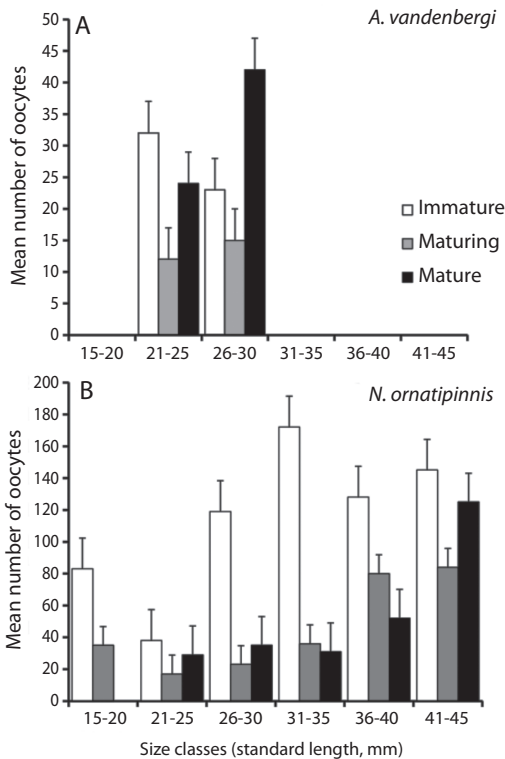


Fig. 2. Relationship between standard length size classes and the number of oocytes found within different developmental stages (immature [open bars], maturing [grey bars], and mature [black bars]) for both (A) *A. vanderbergi* and (B) *N. ornatipinnis*.

differences among oocytes diameter size for each development stage in both species, *A. vanderbergi* (df=82, F=61.7, p<0.02) and *N. ornatipinnis* (df=59, F=29.9, p<0.002). *Austrolebias*

vanderbergi exhibited an equal adult sex ratio (male:female 1:1; $\chi^2=45.7$, p=0.92), and there was a slight female bias was observed for *N. ornatipinnis* (1:1.8), but it was not statistically significant ($\chi^2=30.0$, p=0.20). The relationship between fecundity and standard length is represented by the equations: $y=-130.6 + 7.6x$ (r=0.48) for *A. vanderbergi* and $y=-208.3 + 13.6x$ (r=0.62) for *N. ornatipinnis*. There was a significant trend for increasing fecundity with increasing standard length in females for both *A. vanderbergi* (R²=0.24, p<0.0001) and *N. ornatipinnis* (R²=0.39, p<0.0008) (Fig. 3). The GSI estimates in females of *N. ornatipinnis* did not exhibit significant differences between early (January, GSI=7.24±0.42) and later (April, GIS=7.55±1.37) rainy season samples. However, there was a slight peak in March (GIS=8.08±1.44).

DISCUSSION

In the four ponds surveyed, *A. vanderbergi* and *N. ornatipinnis* did not co-occur together. *A.vanderbergi* were captured only in ephemeral ponds, whereas *N. ornatipinnis* were collected in both an ephemeral and a semi-permanent pond. All ponds had high canopy cover with a habitat structure in the form of leaf litter input and woody debris. Structural heterogeneous substrates in these ephemeral ponds may offer shelter to these annual fishes or egg deposition sites.

Most rivulids are sexually dimorphic, with males exhibiting bright coloration and

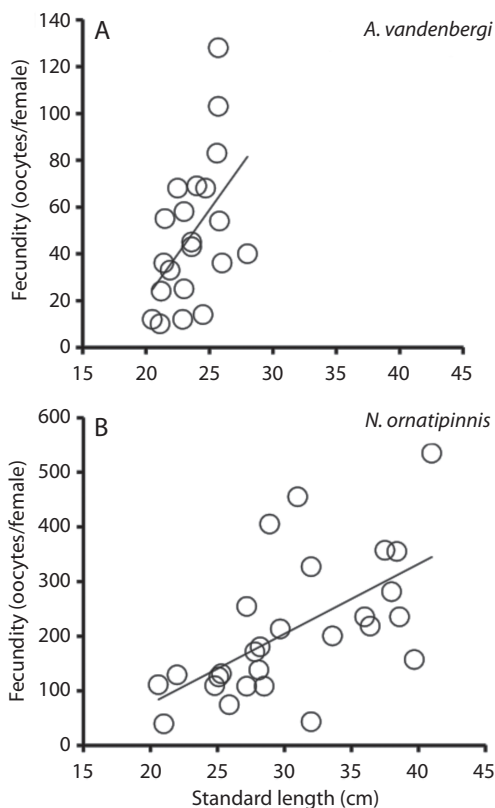


Fig. 3. Correlation between standard length and absolute fecundity in (A) *A. vanderbergi* and (B) *N. ornatipinnis*.

possessing relatively larger body sizes than females (Garcia et al., 2008; Gonçalves et al., 2011), and we observed a similar pattern in both species in this study. Overall, *A. vanderbergi* had shorter standard length but greater body depth than *N. ornatipinnis*, which reached a maximum standard length of 50.6mm. Males of both species exhibited a larger body size than females in regards to both standard length and body depth. This trend has been observed in other species of tropical annual rivulids including *Cynopoeilus melanotaenia* (Regan, 1912) and several species of *Austrolebias* (Laufer, Arim, Loureiro, Piñeiro-Guerra, Clavijo-Baquet & Fagúndez, 2009).

These differences in body size play an important role in sexual selection (Garcia et al., 2008). In fish, body size of females is favored

by increased fecundity, whereas in males, size is affected by sexual selection. Female mating preferences for large males can be attributed to superior genes inherited by offspring or a variety of direct benefits including increased sperm quality and better paternal care (Shine, 1989).

In this study, the *A. vanderbergi* had an equal adult sex ratio (i.e., 1:1), but there was a slight non significant female biased ratio for *N. ornatipinnis*. These values were similar to Laufer et al. (2009) who found a ratio of 1:1 (males:females) for *Austrolebias* spp. and *C. melanotaenia* in ephemeral ponds in Uruguay; but differed from Gonçalves et al. (2011) whom observed a ratio of 2:1 for *C. melanotaenia* in ephemeral ponds Southern Brazil, and from Calviño (2005) who reported a sex ratio of 1:4 for *N. ornatipinnis* from Argentina. Sex ratio data provide important information to understand the population dynamics in fish, but whether these sex ratios may be caused by physiological and/or environmental factors that affect sexes (Vazzoler, 1996) still remains unknown at this time.

According to Winemiller & Rose (1992), the opportunistic strategy of annual rivulids which is characterized by short life, with fast sexual maturation, and continual spawning, is related to an increase in offspring survivorship and fitness in environments subjected to frequent disturbance (in this system pond drying). In this study, most gonads examined did not show fat in the visceral cavity indicating that the short life cycle of these species may require the use of energy reserves for feeding and reproductive activities (Vazzoler, 1996). Results from this study showed that both species of rivulids are fractional spawners, continuously reproducing as evidence by the presence of three different oocyte stages (e.g., immature, maturing and mature). Females of both species appear to begin reproducing when reaching a size of 20mm SL. *Neofundulus ornatipinnis* has a significantly larger egg diameter than *A. vanderbergi*, but the egg diameters of both species in this study ranged within the sizes reported for *Austrolebias nigrofasciatus* Costa & Cheffe, 2001 (Volcan et al., 2011), *Leptolebias*

spp. (Costa & Leal, 2009), *Simpsonichthys* spp. (Fava & Toledo-Piza, 2007) and *C. melanotaenia* (Arenzon, Peret & Bohrer, 1999). Based on the oocyte diameter and developmental stages, both species in this study likely lay eggs over relatively short periods (e.g., days). Volcan et al. (2011) found that in Southern Brazil, *A. nigrofasciatus* spawned an average 21.5 eggs/female/week, and similar results have been reported for *Austrolebias belloti* (Steindachner, 1881) in Argentina (Calviño, 2005). No information is known on the spawning rate of the fishes within the genus *Neofundulus*, though we would expect it to be higher than that of *Austrolebias* due to the higher absolute fecundity of *N. ornatipinnis* as compared to *A. vanderbergi*. Fractional spawning is a common trait in fishes possessing an opportunistic life history (e.g., annual fishes), but also a consequence of physiological variation due to environmental alterations, and possibly the result of exposure to predation from snakes (Schalk & Montaña, 2012), macroinvertebrates (Schalk, 2010), or carnivorous tadpoles of *Ceratophrys cranwelli* (Anura: Ceratophryidae) (Barrio, 1980) (Schalk, unpublished data) in these shallow and temporal habitats.

Fecundity is an important variable in fish life history, because it is a major component in fitness (Endler, 1986). Fecundity may increase with increased body size or differ with species in a population and/or between populations of the same species or seasonally in a population (Wootton, 1990). Fecundity of *A. vanderbergi* and *N. ornatipinnis* was positively correlated with body length, though *N. ornatipinnis* had a higher absolute fecundity than *A. vanderbergi*. This relationship also has been observed in other annual fishes such as *Cynopeocilus melanotaenia* (Gonçalves et al., 2011), in live-bearing poeciliid fishes (Reznick & Miles, 1989), and in other freshwater fish (Lowe-McConnell, 1975).

Mean of gonadosomatic index (GSI) estimated in females of *N. ornatipinnis*, did not indicate significant variation in the gonadal status in relation to gonad weight at the beginning nor at the end of rainy season, probably

because of the continuously developmental stages observed in all females examined. However, higher values of GSI in March indicate a pronounced ovary weight towards the end of the rainy season. The GSI is an important indicator of the reproductive cycle and type of spawn of fishes. To better understand this index, more detailed studies are required to investigate the cues (e.g., feeding ecology and abiotic variables) that trigger these species to spawn and correlate them to GSI values across the entire rainy season.

The Gran Chaco is experiencing some of the highest deforestation rates compared with other ecoregions across the globe (Zak, Cabido & Hodgson, 2004). The restricted distribution of many species of rivulids in addition to their small body size and lack of information on their life history make these species particularly vulnerable to anthropogenic alterations to the landscape. *Austrolebias vanderbergi* and *N. ornatipinnis* are no exception; outside of the report by Montaña et al. (2012), virtually nothing is known about the ecology of either of these species. This study represents the first contribution of the reproductive biology of *A. vanderbergi* and *N. ornatipinnis*; and attempts to advance our understanding of the natural history of these species in extreme environments of the Bolivian Gran Chaco. In this region, there is a need for further studies in the biology and ecology of other species that inhabit these ponds (e.g. *Austrolebias monstrosus* Huber, 1995a; Osinaga, 2006). Our findings represent a basic tool for conservation of these species and their environments that are constantly threatened by anthropogenic activities.

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RESUMEN

Estrategias reproductivas de dos especies de rivulidos Neotropicales *Austrolebias vanderbergi* y *Neofundulus ornatipinnis* (Cyprinodontiformes: Rivulidae) en el Gran Chaco Boliviano: Información sobre la ecología e historia de vida de varias especies de rivulidos no ha sido estudiada, por lo tanto se analiza la biología reproductiva de dos especies neotropicales (*Austrolebias vanderbergi* y *Neofundulus ornatipinnis*) que habitan pozos semipermanentes y temporales en el Gran Chaco Boliviano. Las recolectas se realizaron al inicio y al final de la estación lluviosa. Dimorfismo sexual, fecundidad absoluta, diámetro de los ovocitos y proporción sexual fue determinada para cada especie de rivulido. Machos en las dos especies presentaron mayor tamaño de longitud estándar y altura del cuerpo cuando fue comparado con las hembras. *Austrolebias vanderbergi* tuvo menor fecundidad por hembra y huevos más pequeños que *N. ornatipinnis*, pero hembras en ambas especies presentaron una relación estrecha y positiva entre fecundidad y tamaño cuerpo. La presencia de varios estadios gonadales sugiere un desove fraccionado, lo cual es una estrategia reproductiva que promueve la viabilidad y sobrevivencia de estos peces en hábitats de extremas condiciones. La proporción sexual no varió entre machos y hembras de *A. vanderbergi* (1:1), pero en *N. ornatipinnis* la proporción sexual fue dominada por las hembras (1:1.8). Este estudio representa uno de los primeros que investiga aspectos de la biología reproductiva de *A. vanderbergi* y *N. ornatipinnis*, lo cual es importante para la conservación de rivulidos en regiones tropicales.

Palabras clave: bosque seco tropical, dimorfismo sexual, fecundidad, historia de vida, peces anuales, pozos efímeros.

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