

Reproductive biology of the invasive bullfrog *Lithobates catesbeianus* in southern Brazil

Ígor Luís Kaefer^{1,*}, Ruben Alexandre Boelter² & Sonia Zanini Cechin¹

¹ *Setor de Zoologia, Departamento de Biologia, Universidade Federal de Santa Maria, Av. Roraima 1000, 97105-900, Santa Maria, Rio Grande do Sul, Brazil (*corresponding author's e-mail: igorkaefer@hotmail.com)*

² *Curso de Ciências Biológicas, Universidade da Região da Campanha, Campus São Borja, Av. Tancredo Neves 210, 97670-000, São Borja, Rio Grande do Sul, Brazil*

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We studied the reproductive biology of an invasive population of the bullfrog *Lithobates catesbeianus* by analyzing individuals captured in the central Rio Grande do Sul state, southern Brazil. Specimens were collected during 12 field trips held from May 2002 to June 2003. We analyzed gonad development stages in 216 individuals to evaluate the fecundity, reproductive effort, size–fecundity relationships, and seasonal distribution of individuals. Reproductive activity was potentially continuous, and was more intense during the spring and summer. The smallest mature male was 90.25 mm in snout–urostyle length (SUL); the SUL of the smallest mature female was 120.83 mm. The invasive potential of *L. catesbeianus* is probably associated with reproductive traits such as continuous gonad development, extended reproduction period, high fecundity, and early sexual maturity. The data presented here form a basis for future studies on the impacts and management of bullfrogs in invaded ecosystems.

Introduction

Lithobates catesbeianus (Ranidae) is an exotic invasive species widely distributed in southern Brazil. Its original range encompassed southern Quebec in Canada, the southern and eastern United States, and Veracruz in Mexico (www.globalamphibians.org). The species was introduced worldwide and has adapted to different environmental conditions. In places where it was introduced, the bullfrog influences the abundance of native anurans (Hecnar & M'Closkey 1997) and other aquatic species through predation and competition (Rosen & Schwalbe 1995,

Kiesecker & Blaustein 1998). In addition, bullfrogs are vectors of infectious diseases such as chytridiomycosis, one of the main causes of global amphibian declines (Berger *et al.* 1998, Longcore *et al.* 1999), as well as the cause of the extinction of at least one species (Daszak *et al.* 2003). This disease was identified in native amphibians in South America (Bonaccorso *et al.* 2003, Herrera *et al.* 2005), including Brazil (Carnaval *et al.* 2005, Carnaval *et al.* 2006), where the fungus was also recorded in *L. catesbeianus* (Toledo *et al.* 2006). Recently, another sanitary risk, *Ranavirus*, was detected in Uruguayan bullfrog farms (Galli *et al.* 2006). One of the most

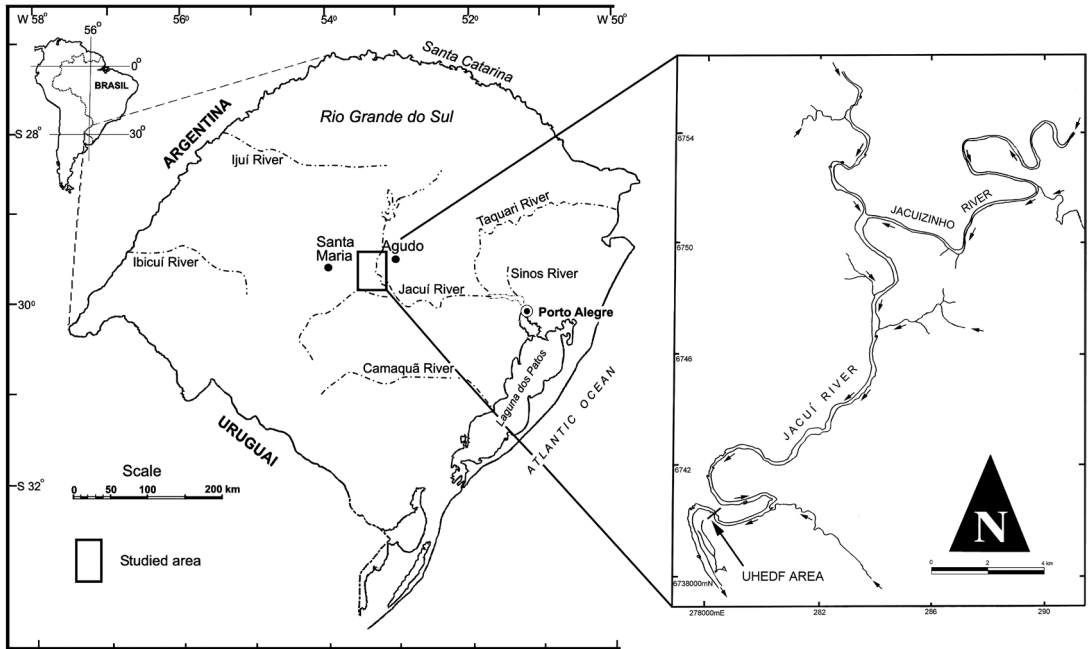


Fig. 1. Geographical position of the study area in the state of Rio Grande do Sul, Brazil.

important applications of modern ecology is to identify environmental, behavioral, and life-history attributes related to the impacts of such invasions (Parker *et al.* 1999, Mack *et al.* 2000).

Data on bullfrog reproductive traits usually pertain to populations in northern-hemisphere countries, where climatic conditions are markedly different from those in Brazil (Lima *et al.* 1998). In Brazil, reproductive data on *L. catesbeianus* are available for captive-bred populations in tropical areas; these regions differ markedly from our study area, classified by Maluf (2000) as subtropical. According to Bury and Whelan (1984), geographic location strongly influences body size and size at maturity in bullfrogs. Hence, analyses based on field data are of great importance to evaluate local impacts of biological invasions. In general, a minimum size must be reached before individuals become sexually mature, and local environmental factors determine the growth rate (Porter 1972). Anuran reproduction is influenced by various biotic and abiotic factors; in temperate regions, species with prolonged breeding periods, such as *L. catesbeianus*, must respond to a wider range of environmental variables than do species with short breeding seasons (Oseen & Wassersug 2002).

The present study aimed to characterize aspects of the reproductive biology of *L. catesbeianus* in southern Brazil and to relate these traits to the local frog fauna. We analyzed the male and female gonad development, fecundity, reproductive effort, size-fecundity relationships, seasonal distribution of individuals, and the presence of sexual size dimorphism. We also compared our results with data from the natural range of the species.

Material and methods

Fieldwork was conducted in 23 lentic waterbodies in the area of the Dona Francisca Hydroelectric Dam (UHEDF). The region lies in the final portion of the middle Jacuí River, between the cities of Agudo and Nova Palma, central Rio Grande do Sul state, Brazil ($29^{\circ}26'50''\text{S}$, $53^{\circ}16'50''\text{W}$) (Fig. 1). The mean annual temperature is 18°C , the mean annual rainfall is 2000 mm, and the mean annual air humidity is 87%, peaking in the winter months (Companhia Estadual de Energia Elétrica unpubl. data). Seasons are well defined, with low temperatures in the winter and high in the summer. The local

climate is classified as subtropical humid (Maluf 2000).

We captured *L. catesbeianus* individuals by hand and with the aid of fishing nets during 12 field trips from May 2002 to June 2003. Specimens were killed and subsequently deposited in the herpetology laboratory collection at the Universidade Federal de Santa Maria (ZUFMS).

We measured the snout–urostyle length (SUL, in millimeters) and mass (in grams) of each individual after preservation. We dissected specimens to remove and analyze gonads. In each animal, we measured the left testis length (with digital calipers to the nearest 0.01 mm) and mass (with a precision digital scale to the nearest 0.001 g).

To analyze the spermatogenic cycle, we embedded testes of 42 males in paraffin resin for later histological analyses. Sections of 5 μm were cut with microtomes, mounted on glass slides, and stained according to the standard hematoxylin-eosin techniques (Montero & Pisanó 1992). Males containing spermatozoa in seminiferous tubules were considered reproductive.

We weighed ovaries on a precision digital scale to the nearest 0.1 g. We analyzed oocyte maturation stages following the terminology proposed by Montero and Pisanó (1991), and classified them as follows: (1) pre-vitellogenic oocytes, (2) oocytes in primary vitellogenesis, (3) oocytes in late vitellogenesis, (4) oocytes in auxocytosis, (5) post-vitellogenic oocytes (hereafter PVOs), (6) atresic oocytes, and (7) pigment stain.

For ovarian maturation stages we followed the terminology proposed by Costa *et al.* (1998a). *Lithobates catesbeianus* showed five stages: (1) juvenile: ovaries thin, hyaline to whitish, and oocytes macroscopically indistinguishable; (2) beginning of maturation: whitish to yellowish ovaries; deeper invaginations in the ovaries, and oocytes milky white; (3) intermediate maturation: ovary grayish, with pigmented PVOs accumulated; (4) advanced maturation: maximum degree of ovarian development; high proportion of PVOs; (5) spent ovaries: flaccid, grayish ovaries with considerably reduced volume; high number of atresic oocytes. Juvenile females were excluded from the analysis.

We estimated the total number of PVOs by counting PVOs in 7%–100% of the weighed

ovaries (*see* Montero & Pisanó 1991, Díaz-Páez & Ortiz 2001). To facilitate counting, we separated the oocytes by immersing the ovary in a 10% sodium hypochlorite solution (Melchior *et al.* 2004). We measured the diameters of ten randomly selected oocytes with a digital caliper to the nearest 0.01 mm.

We estimated fecundity based on ovarian complement (number of PVOs in the ovary) and ovarian size factor (OSF; Duellman & Crump 1974), which relates the number and size of PVOs to the frog SUL according to the formula: $\text{OSF} = \text{OC} \times \text{OD}/\text{SUL}$, where OC is the mean number of PVOs, OD is the grand mean oocyte diameter, and SUL is the mean snout–urostyle length.

We determined the reproductive effort by calculating male and female gonadosomatic indexes (GSI) as follows: $\text{GSI} = \text{GM} \times 100/\text{BM}$, where GM and BM represent the gonad mass and body mass, respectively (Costa *et al.* 1998a). For males, we multiplied the value obtained for the left testicle by two. We evaluated GSI variation during the year with a Kruskal-Wallis test, using season as a factor (spring = September to December; summer = December to March; autumn = March to June; winter = June to September).

We evaluated the size–fecundity relationship with Pearson's (r) and Spearman's (r_s) correlation coefficients. The following variables were considered: Males: (1) SUL *vs.* testis length; (2) SUL *vs.* testis mass; (3) body mass *vs.* testis length; (4) body mass *vs.* testis mass. Females: (5) SUL *vs.* number of PVOs; (6) SUL *vs.* ovary mass; (7) body mass *vs.* number of PVOs; (8) body mass *vs.* ovary mass. We included only males containing spermatozoa in seminiferous tubules and females with PVOs in these analyses.

We estimated the recruitment period based on the proportion of juvenile individuals captured. We evaluated this aspect only for males, because immaturity can be precisely determined by the lack of spermatozoa in seminiferous tubules. We considered that mature males were those larger than the smallest reproductive individual found.

We evaluated sexual dimorphism in size of mature individuals using Mann-Whitney's U -test. We carried out all analyses using the program BioEstat 3.0 (Ayres *et al.* 2003).

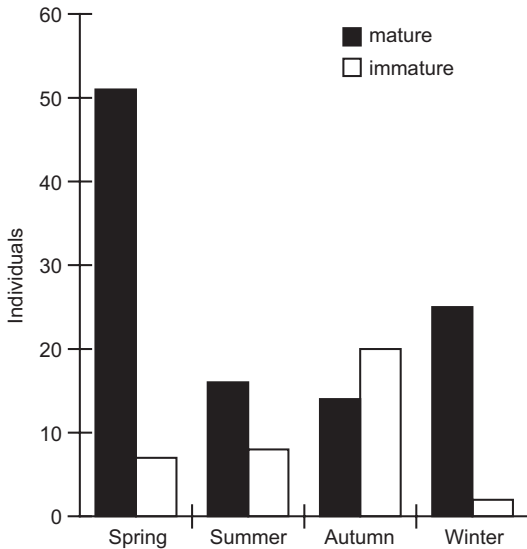


Fig. 2. Relationship between mature and immature male individuals of *Lithobates catesbeianus* in the central Rio Grande do Sul state, Brazil, during the seasons sampled.

Results

We analyzed 145 males and 71 females ($n = 216$ individuals). Mature male SUL ranged from 90.25 to 158.00 mm (mean \pm SD: 131.50 ± 17.42 mm; $n = 29$); mass ranged from 95 to 560 g (mean \pm SD: 311.60 ± 114.50 g; $n = 29$). Cells in different maturation stages occurred inside the testes, and germinative cells in more advanced stages usually occurred inside the lumen of tubules. Spermatozoa and all preceding spermatogenesis cell phases occurred in all four seasons of the year.

Male GSI values were higher in the spring and summer (Table 1), but this variation was not significant in all seasons (Kruskal-Wallis test: $H = 4.627$, $p = 0.2025$, $n = 29$). The SUL was

Table 1. Gonadosomatic indexes (GSI = Gonad Mass \times 100/Body Mass) of male *Lithobates catesbeianus* during the four seasons of the year in the central Rio Grande do Sul state, Brazil.

Groups (n)	GSI (mean \pm SD)
Spring ($n = 13$)	0.069 ± 0.020
Summer ($n = 4$)	0.060 ± 0.011
Autumn ($n = 4$)	0.049 ± 0.013
Winter ($n = 8$)	0.060 ± 0.030

correlated with the testis length ($r = 0.5529$, $p = 0.001$, $n = 29$) and the mass ($r = 0.6999$, $p < 0.001$, $n = 29$). Likewise, the individual mass was also correlated with the testis length ($r = 0.6186$, $p < 0.001$, $n = 29$) and the mass ($r = 0.7655$, $p < 0.001$, $n = 29$).

The seasonal proportion of mature and immature males is shown in Fig. 2. There was a higher juvenile recruitment in May (autumn). Still, juveniles occurred in all months of the year.

The SUL of females with post-vitellogenic oocytes (PVOs) ranged from 120.83 to 174.00 mm (mean \pm SD: 143.17 ± 11.78 mm, $n = 19$); mass ranged from 220 to 550 g (mean \pm SD: 311.6 ± 95.7 g, $n = 19$).

Depending on the growth or reproductive phase under consideration, it was possible to see oocytes in different degrees of development through the ovary wall. All ovarian developmental stages occurred in all four seasons of the year, except the advanced maturation stage, which was absent in the autumn, and the beginning maturation stage, which was absent in the summer (Fig. 3A).

Females harboring PVOs occurred in all seasons, but a sharp decrease was noticeable in colder seasons (Fig. 3B). For example, in April we found a single female with PVOs ($n = 7$), whereas in June ($n = 3$) and August ($n = 5$) we found no females with this ovarian maturation stage. The largest GSI value (9.84) occurred in a female collected in November. Females in advanced maturation stages (i.e., ready to spawn) occurred only from September to February. During the coldest months of the year (April to August) no females showed this gonad maturation stage. Morphometric measurements for different ovarian maturation stages are summarized in Table 2.

The OSF for the females analyzed was 74.8 ($n = 19$); the number of PVOs ranged from 61 in spent ovaries to 26 200 in ovaries in advanced maturation (mean \pm SD: 9225.8 ± 7045.4).

The number of PVOs was not significantly correlated with the SUL ($r = -0.0437$, $p = 0.858$, $n = 19$) or the body mass ($r = 0.0349$, $p = 0.887$, $n = 19$). Likewise, the ovary mass was not significantly correlated with the SUL ($r_s = 0.1333$, $p = 0.586$, $n = 19$) or the body mass ($r_s = 0.2847$, $p = 0.237$, $n = 19$).

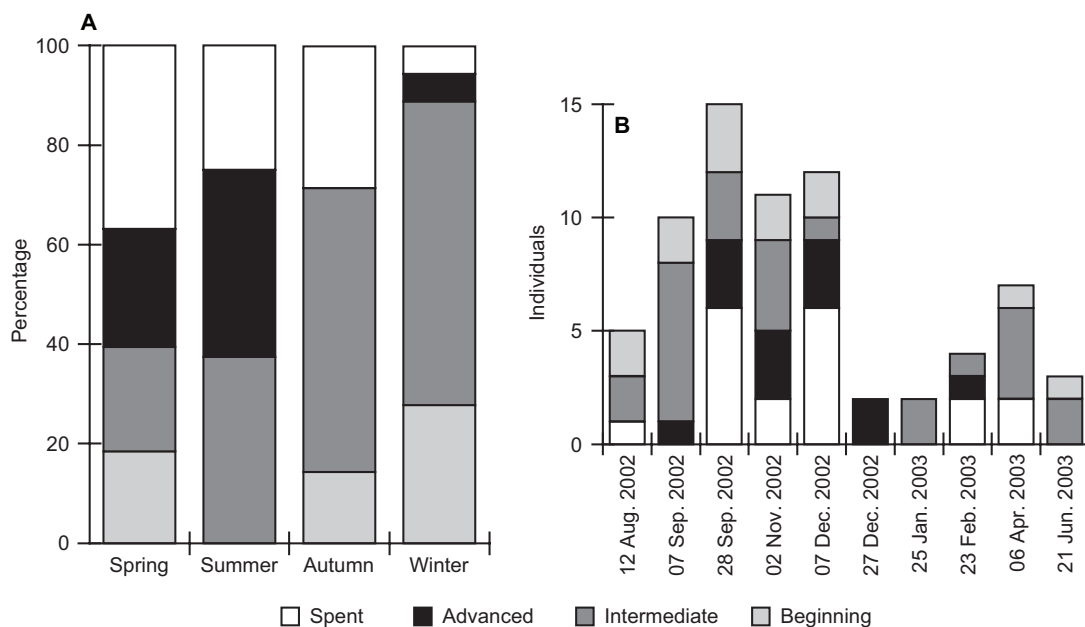


Fig. 3. Ovarian maturation stages during (A) the seasons of sampling and (B) field trips when females from the starting maturation stage were collected for female *Lithobates catesbeianus* in central Rio Grande do Sul state, Brazil. Spring $n = 38$, summer $n = 8$, autumn $n = 7$, winter $n = 18$, total = 71.

Adult females were significantly larger than adult males ($U = 165.00$, $p = 0.0198$). However, when we analyzed only the ten largest individuals per group, we found no significant differences in body size ($U = 46.00$, $p = 0.7624$). Morphometric data for both sexes appear in Table 3.

Discussion

Male reproductive characteristics

Our data indicate that this population has a continuous spermatogenic cycle, as reported for other subtropical frog species (Ceï 1949, Melchioris *et al.* 2004) and for captive-bred *L.*

catesbeianus in southeastern Brazil (Costa *et al.* 1998b, Sasso-Cerri *et al.* 2004). Amphibian species inhabiting cold regions have discontinuous spermatogenic cycles, whereas those in areas without remarkable annual temperature fluctuations have continuous or potentially continuous spermatogenic cycles (Lofts 1974).

Our results showed that male GSI did not vary significantly among seasons, as also reported for captive-bred populations of *L. catesbeianus* in southeastern Brazil (Sasso-Cerri *et al.* 2004). This result is in agreement with previously reported data on other animals that show continuous spermatogenic cycles (Rastogi *et al.* 1976, Delgado *et al.* 1989), including *L. catesbeianus* (Licht *et al.* 1983). The strong correlation

Table 2. Means \pm SD of snout–urostyle length (SUL), body mass, and gonadosomatic index (GSI) by stage of ovary development for *Lithobates catesbeianus* in the central Rio Grande do Sul state, Brazil.

Stage of ovary development	SUL (mm)	Body mass (g)	GSI	n
Spent	143.06 \pm 12.04	336.7 \pm 107.7	1.39 \pm 0.68	19
Advanced	140.08 \pm 9.47	352.7 \pm 76.8	4.74 \pm 2.34	13
Intermediate	137.67 \pm 12.43	328.3 \pm 90.8	2.03 \pm 1.34	26
Beginning	118.45 \pm 11.96	218.8 \pm 88.6	0.58 \pm 0.71	13

between the morphometric and testis measurements also suggests that testis weight remains steady in species that show this type of cycle. This characteristic may be related to compensatory differences in the testis volume and density during the year (Sasso-Cerri *et al.* 2004). These authors suggested that using testis weight only as a measurement of reproductive status in bullfrogs is inadequate. Their suggestion was corroborated by our data.

Female reproductive characteristics

Female bullfrogs can reproduce year-round, but final maturation and ovulation ultimately depend on environmental stimuli, such as rainfall and temperature rise after the winter (Agostinho *et al.* 2000). Even though females with post-vitellogenic oocytes occurred in all seasons, vitellogenic activity increased between August and April. Females in the advanced maturation stage (ready to spawn) occurred only from September to February, with maximum GSI in November, suggesting that oviposition may occur in the warmest months of the year.

Howard (1978) showed that older female bullfrogs in the US (larger than 130 mm) produce a second spawn with significantly fewer and smaller eggs than the first spawn. According to Vizotto (1984), two-year-old *L. catesbeianus* show two reproductive events in southeastern Brazil, first from September to January and the second from February to mid-April. These months include most females in advanced gonad maturation stage found in the present study.

However, the methods that we employed and the rapid rate of ovary recovery did not allow us to conclude that two reproductive events occur. In fact, only one female (139.20 mm, 410 g, February 2003) in advanced maturation stage was found during the second oviposition period indicated by Vizotto (1984).

As reported by Costa *et al.* (1998a), this species lacks an ovary “rest” stage, implying a continuous oogenesis in which ovaries reset to an intermediate condition after oviposition. This explains the high proportion of females found in an intermediate gonad maturation stage during the year, before and after the possible oviposition period (spring and summer). Monteiro and Pisanó (1991) noted that relatively constant numbers of pre-vitellogenic oocytes or oocytes in primary vitellogenesis are present during the entire cycle, regardless of the number of mature oocytes, indicating that these germinal cell lines represent a stock for subsequent maturation phases.

The present study showed the presence of oocytes in different levels of atresia, especially in females with spent ovaries, as also observed by Costa *et al.* (1998a). According to Saidapur (1978), the presence of atresic oocytes is common, and hormonal and environmental factors, such as temperature, food availability, and nutritional condition, regulate this process. Lofts (1974) reported the occurrence of atresic oocytes throughout the entire reproductive period in *Rana esculenta*. Horseman *et al.* (1978), investigating the influences of photoperiod on bullfrog gonad maturation in the laboratory, concluded that a 12/12 (L:D) photoperiod inhibits ovary

Table 3. Morphometric data (SD) for *Lithobates catesbeianus* in the central Rio Grande do Sul state, Brazil.

Characteristic	Male (<i>n</i> = 29)	Female (<i>n</i> = 19)
Mean body size (mm)	131.50 (17.42)	143.17 (11.78)
Smallest mature individual (mm)	90.25	120.83
Mean body mass (g)	311.6 (114.5)	365.5 (95.7)
Post-vitellogenic oocyte mean diameter (mm)	–	1.16 (0.10)
Post-vitellogenic oocyte mean number	–	9225.8 (7045.4)
Mean ovary mass (g)	–	13.74 (9.09)
Ovarian size factor	–	74.83
Mean testis mass (g)	0.100 (0.045)	–
Mean testis length (mm)	9.18 (1.80)	–
Gonadosomatic index (%)	0.062 (0.022)	3.84 (2.36)

regression.

Female GSI values for different ovarian maturation phases of bullfrogs in captivity (*see* Costa *et al.* 1998a) are clearly higher than those found in the present study, and maturation stages were reached at smaller body sizes. Like Costa *et al.* (1998a), we attribute these differences to the more favorable conditions in captivity than in natural environments.

The OSF (74.8) found for *L. catesbeianus* is very high as compared with that for other anuran species. Available values for frogs in the state of Rio Grande do Sul and Argentina indicate lower OSFs, such as 10.5 for *Pseudis minutus* (Melchior *et al.* 2004), 13.92 for *Leptodactylus latinasus*, and 17.57 for *Pseudopaludicola falcipes* (Basso 1990). Crump (1974) calculated the OSF for 66 species of a tropical anuran community, where only one, native species with high invasive potential, *Chaunus marinus*, had a higher OSF (97.26) than that calculated in the present study. Nevertheless, we must acknowledge that some non-invasive, but large-bodied anuran species can reach higher values of OSF, such as 167.4 (*Leptodactylus ocellatus*) and 162.5 (*Chaunus fernandezae*) (Basso 1990).

The mean number of PVOs recorded (9225.8) in *L. catesbeianus* is also high as compared with that in the most of the South American native frog species (*see* Crump 1974, Basso 1990), which provides evidence of the species's superior reproductive capacity. The maximum number of PVOs recorded in this study (26 200) is close to that reported by studies in the United States (> 25 000, Bury & Whelan 1984) and Canada (23 540, Bruneau & Magnin 1980). However, higher maximum numbers of PVOs were also reported for *L. catesbeianus* (40 000, Bury & Whelan 1984) and the invasive species *Chaunus marinus* (> 54 000, Seabrook 1993 as cited in Hagman & Shine 2006).

The wide variation in the estimated number of PVOs (61–26 200) is probably due to the dynamics of oviposition in the sampling period: females collected right after oviposition will naturally contain many fewer oocytes. Ontogenic changes occur in frogs in temperate regions (Camargo *et al.* 2005). Furthermore, Bonnet *et al.* (2003) observed that within a population, fecundity will be strongly correlated with the

body size only in analyses considering females with a high reproductive investment.

General reproductive characteristics

The reproductive pattern of this *L. catesbeianus* population is potentially continuous, where reproduction is most likely limited by low temperatures in the late autumn and early winter. Through the analysis of gonads and seasonal distribution of individuals, we observed an increase in reproductive activity during the spring and summer. This is evidenced by the high proportion of females with advanced gonad maturation stages and higher GSI values for both males and females during this period. The low number of females with PVOs from April to August further supports this hypothesis.

Frogs were collected year-round, and neither hibernation in the coldest months of the year (Bury & Whelan 1984) nor an arrest of gonad maturation were observed, corroborating previously reported data for captive-bred bullfrogs in southeastern Brazil (Costa *et al.* 1998a).

Our data indicated an absence of reproductive activity during the coldest months of the year, when no females ready to spawn were recorded. Furthermore, no males were heard calling from April to July (S. Z. Cechin pers. comm.). In southeastern Brazil, Sasso-Cerri *et al.* (2004) observed no signs of reproductive activity (calls or amplexing pairs) in bullfrogs collected in May and July. Also in Brazil, individuals of *L. catesbeianus* in the state of Paraná vocalized only in the warmest months of the year (Conte & Rossa-Feres 2006). In Canada, Oseen and Wassersug (2002) found that calling activity of bullfrogs is associated primarily with higher water temperature.

Our study also highlighted the importance of analyzing both sexes to correctly characterize the reproductive cycle. Because the presence of mature spermatozoa in testes throughout the year is a characteristic of continuous reproductive cycles (Lofts 1964), the description of only the male gametogenic cycle in bullfrogs would be misleading. For instance, Cei (1949) observed independent ovarian and testicular cycles in several frog species in the Chaco.

The mean sizes of both females and males, and the male minimum size at maturation are similar to those in a natural population in North America (see Howard 1981). Captive-bred frogs in Brazil (Lima *et al.* 1998) and elsewhere (Culley & Gravois 1971 as cited in Bury & Whelan 1984) mature earlier. Early male maturation also occurs in wild populations in North America (Howard 1981), where maturity was reached one year after metamorphosis in males, and two years after metamorphosis in females. Howard (1981) argued that, as found in the present study, the presence of females larger than males is a consequence of males reaching maturity at smaller sizes.

Cohen and Howard (1958) reported that the mean larval period in California was six months, and that most individuals of *L. catesbeianus* present at the end of summer in a temporary reservoir were juveniles of the current year. In the population that we studied, higher juvenile recruitment occurred in May, also at the end of the warm season. Although there are no data on the phenology of bullfrogs in southern Brazil, we suggest that oviposition, tadpole development, and metamorphosis can occur in the same warm period. This may contribute to the high reproductive potential of Brazilian bullfrog populations.

The reproductive traits described for this population have a strong association with the invasive potential of bullfrogs in southern Brazil. Continuous gonad development, extended reproduction period, high fecundity, and early sexual maturity are characteristics of *L. catesbeianus* shared with another exotic invasive species, the cane toad *Chaunus marinus* (see Phillips *et al.* 2007, Semeniuk *et al.* 2007). This toad has thrived in many countries where it was introduced, and, like bullfrogs, has significant negative effects on local faunas (see Semeniuk *et al.* 2007).

We expect that the information provided by this study will encourage further work on the impacts of *L. catesbeianus* in Brazilian ecosystems, related to its reproductive biology. Investigation on selection of breeding sites and adaptive changes in response to their new environments, such as those recently done on invasive cane toads in Australia (Phillips & Shine 2005, Hagman & Shine 2006, Phillips *et al.* 2006, Semeniuk *et al.* 2007), can act as tools to indi-

cate opportunities for bullfrog control, such as manipulation of waterbodies used for reproduction in order to reduce their suitability.

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