

# New occurrences of the endangered *Notholebias minimus* (Cyprinodontiformes: Rivulidae) in coastal plains of the State of Rio de Janeiro, Brazil: populations features and conservation



Correspondence:  
Francisco Gerson Araújo  
gerson@ufrj.br

<sup>1</sup>Gustavo Henrique Soares Guedes<sup>1</sup>, <sup>1</sup>Carlos Henrique Pacheco da Luz<sup>1</sup>,  
<sup>2</sup>Rosana Mazzoni<sup>2</sup>, <sup>3</sup>Fábio Origuela de Lira<sup>3</sup> and  
<sup>1</sup>Francisco Gerson Araújo<sup>1</sup>

*Notholebias minimus* is an endangered annual killifish endemic to the coastal plains of the State of Rio de Janeiro, Brazil. This study aimed to present new occurrences in the Atlantic Forest biome, provide unprecedented population features (body and egg size, fecundity, sexual ratio, and length-weight relationship – LWR), and compare changes in land use and coverage between 1985 and 2021 in biotopes located inside and outside protected areas. Three new occurrence localities were found in shallow temporary wetlands with acidic pH ( $6.4 \pm 0.2$ ) and low concentrations of dissolved oxygen ( $2.0 \pm 0.9$  mg/L). Males and females total length ranged from 11.1 to 31 mm and 11 to 26 mm, respectively. Batch fecundity ranged from 18 to 40 oocytes ( $24.8 \pm 8.8$ ), corresponding to oocytes with sizes between 800–1,006  $\mu\text{m}$  ( $905 \pm 56$ ). Males were significantly larger than females ( $W = 2193.5$ ,  $p = 0.0067$ ), but both sexes occurred in similar proportions ( $p = 0.472$ ). LWR showed positive allometry ( $b = 3.18$ ). Biotopes located within protected areas exhibited higher conservation. Our discoveries expand the knowledge about habitat and population features of *N. minimus* and reinforce the importance of establishing protected areas for the conservation of annual fish biotopes.

**Keywords:** Annual fish, Atlantic Forest biome, Conservation units, Killifish, Threatened fauna.

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<sup>1</sup> Laboratório de Ecologia de Peixes, Departamento de Biologia Animal, Universidade Federal Rural do Rio de Janeiro, km 7, BR-465, 23890-000 Seropédica, RJ, Brazil. (GHSG) [gustavohsg@outlook.com](mailto:gustavohsg@outlook.com), (CHPL) [carloshenrique luz.100@gmail.com](mailto:carloshenrique luz.100@gmail.com), (FGA) [gerson@ufrj.br](mailto:gerson@ufrj.br) (corresponding author).

<sup>2</sup> Laboratório de Ecologia de Peixes, Departamento de Ecologia, Universidade do Estado do Rio de Janeiro, Av. São Francisco Xavier, 524 PHLC 220, 20550-013 Rio de Janeiro, RJ, Brazil. (RM) [mazzoni@uerj.br](mailto:mazzoni@uerj.br).

<sup>3</sup> Plano de Ação Nacional (PAN) – Rivulídeos, Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental – CEPTA, Rodovia SP-201, km 7.5, 13630-970 Pirassununga, SP, Brazil. (FOL) [fabiooriguela@hotmail.com](mailto:fabiooriguela@hotmail.com).

*Notholebias minimus* é um peixe anual ameaçado de extinção, endêmico das planícies costeiras do Estado do Rio de Janeiro, Brasil. Neste estudo, objetivamos apresentar novas ocorrências no bioma Mata Atlântica, fornecer características populacionais inéditas (tamanho do corpo e dos ovos, fecundidade, proporção sexual e relação peso-comprimento), e comparar mudanças no uso e cobertura do solo entre 1985 e 2021 em biótopos localizados dentro e fora de unidades de conservação. Registramos três novos locais em áreas úmidas temporárias rasas com pH ácido ( $6,4 \pm 0,2$ ) e baixas concentrações de oxigênio dissolvido ( $2,0 \pm 0,9$  mg/L). O comprimento total de machos e fêmeas variou de 11,1 a 31 mm e de 11 a 26 mm, respectivamente. A fecundidade do lote variou entre 18–40 oócitos ( $24,8 \pm 8,8$ ), correspondendo a diâmetros entre 800–1.006  $\mu\text{m}$  ( $905 \pm 56$ ). Os machos foram significativamente maiores que as fêmeas ( $W = 2193,5$ ;  $p = 0,0067$ ), mas ocorreram em proporções similares ( $p = 0,472$ ). A relação peso-comprimento detectou alometria positiva ( $b = 3,18$ ). Biótopos localizados dentro de áreas protegidas exibiram maior preservação ambiental. Nossas descobertas ampliam o conhecimento sobre as características do habitat e da população de *N. minimus* e reforçam a importância do estabelecimento de áreas protegidas para a conservação dos biótopos dos peixes anuais.

**Palavras-chave:** Bioma Mata Atlântica, Fauna ameaçada, Peixes anuais, Peixes das nuvens, Unidades de conservação.

## INTRODUCTION

Rivulidae (Cyprinodontiformes) is the ninth most speciose fish family in the world with about 473 valid species (Fricke *et al.*, 2023), occurring between southern Florida and southeast of the province of Buenos Aires (Costa, 2011; Calviño *et al.*, 2016; Loureiro *et al.*, 2018). Brazil is home to the largest component of this rich fish family, with at least 314 species distributed across all national biomes. This high richness is proportional to the anthropic threats. Rivulidae is the family with the highest number of endangered species among all vertebrates that occur in Brazil (ICMBio, 2022). Habitat loss and fragmentation are the main threats to rivulids (Costa, 2009). Wetlands have been drastically destroyed, both in agricultural areas and in areas undergoing urbanization, through deforestation, drainage, and landfills (Abrantes *et al.*, 2020; Castro, Polaz, 2020; Guedes *et al.*, 2020; Drawert, 2022). Despite this, research, funding agencies, policy, and freshwater conservation have historically neglected wetlands and focused on larger water bodies and flagship species (Guedes *et al.*, 2023).

Rivulidae is commonly subdivided into two major groups: annual/seasonal *vs.* non-annual/perennial, according to the presence or absence of resistant eggs capable of carrying out a complex process of embryonic diapause during the life cycle (Loureiro *et al.*, 2018). Embryonic diapause allows species to live in hydrologically ephemeral habitats, such as temporary wetlands, where eggs are able to remain buried in dry substrate for months waiting for environmental triggers for hatching (Furness, 2016; Ishimatsu *et al.*, 2018). This uniqueness makes annual species “invisible” during a considerable part of their life cycle, making it difficult to map species distribution areas.

The coastal plains of the State of Rio de Janeiro, located in south-eastern Brazil, are important hotspots of annual fish diversity (Costa, 2012). Among these endemic species, the genus *Notholebias* Costa, 2008 stands out including four valid species: *Notholebias minimus* (Myers, 1942), *N. cruzi* (Costa, 1988), *N. fractifasciatus* (Costa, 1988), and *N. vermiculatus* Costa & Amorim, 2013. All of these species are endemic to the Brazilian Atlantic Forest biome and are threatened with extinction (ICMBio, 2018, 2022). There are significant gaps in knowledge regarding the distribution, habitats, life history, and ecology of *Notholebias* species, as well as for most annual fish. These gaps are aggravated when considering the high number of endangered species, which should reflect a greater effort *in* and *ex situ* studies to support conservation strategies. To reduce these knowledge bottlenecks, this study has as main aims (i) to present new occurrence sites of *N. minimus* in the Brazilian Atlantic Forest biome, (ii) to provide unprecedented population features (individual size, fecundity and egg size, sex ratio, and length-weight ratio), and (iii) to compare anthropic impacts on land use and cover between 1985 and 2021 in temporary wetlands located inside and outside protected areas, which pose a threat to the conservation of this species.

## MATERIAL AND METHODS

**Sampling.** Fish samplings were conducted between February and December 2022 at 23 sites distributed in five localities in the coastal drainages of Sepetiba Bay and Lagoon System of Jacarepaguá (municipalities of Seropédica and Rio de Janeiro, State of Rio de Janeiro; Tab. 1). Three localities were visited for the first time during this study: Brisas APA (Área Proteção Ambiental das Brisas), UFRRJ (Universidade Federal Rural do Rio de Janeiro), and Chaperó (Chaperó solar power plant). Two other localities with previously known distribution of *N. minimus* were revisited: PMN Bosque da Barra (Parque Natural Municipal Bosque da Barra) and REBIO Guaratiba (Reserva Biológica Estadual de Guaratiba). The climate is seasonal tropical, with rainy summers and dry winters (Aw climate, according to the Köppen – Geiger classification). Fish were collected with immersion nets (hand nets with an oval shape, 50 x 40 cm, 1 mm of panel mesh size). After capture, they were anesthetized with hydrochloride benzocaine (50 mg/l), euthanized and fixed in 10% formalin *in situ*. In the laboratory, the fish were measured (precision 0.01 cm), weighed (precision 0.001 g), and after 48 h, preserved in 70% ethanol. Biometric analyses were conducted on the same day as the capture to avoid biases associated with specimen fixation/preservation. In order to reduce the impacts of sampling on fish populations, approximately 75% of specimens were returned alive to the pools after being counted (abundance). Fish were identified and sexed according to Costa (1988, 2008, 2009). Vouchers were deposited in the Ichthyological Collection of the Laboratório de Ecologia de Peixes of the Universidade Federal Rural do Rio de Janeiro (LEP–UFRRJ 2588–2593) and are available for online consultation via Global Biodiversity Information Facility – GBIF (Araújo *et al.*, 2023). Additional records were obtained from the bibliography (Costa, Amorim, 2013; Costa, 2016) and online fish collections database search at Sistema de Informação sobre a Biodiversidade Brasileira – SiBBR ([www.sibbr.gov.br](http://www.sibbr.gov.br)), SpeciesLink ([www.splink.org.br](http://www.splink.org.br)), and GBIF ([www.gbif.org](http://www.gbif.org)).

**TABLE 1** | Localities, number, and date (month/year) of samplings conducted in attempts to capture *Notholebias minimus* in coastal drainages of the State of Rio de Janeiro. Brisas APA = Área de Proteção Ambiental das Brisas; PNM Bosque da Barra = Parque Natural Municipal Bosque da Barra; REBIO Guaratiba = Reserva Biológica Estadual de Guaratiba; UFRRJ = Universidade Federal Rural do Rio de Janeiro.

Locality	Municipality	Area	Latitude, Longitude	Samplings	Date
UFRRJ	Seropédica	UN	-22.77734, -43.68428	2	Aug/22; Dec/22
			-22.772941, -43.68382	1	Aug/22
			-22.763572, -43.694206	1	Aug/22
			-22.771185, -43.679221	1	Dec/22
			-22.771145, -43.679254	1	Dec/22
			-22.782247, -43.706068	1	Nov/22
Chaperó	Seropédica	UN	-22.808623, -43.764165	2	June/22; Dec/22
			-22.807549, -43.765344	2	June/22; Dec/22
			-22.809576, -43.764480	2	June/22; Dec/22
			-22.806506, -43.767527	2	June/22; Dec/22
Brisas APA	Rio de Janeiro	AP	-22.991528, -43.6519	2	Oct/22; Nov/22
			-22.991543, -43.65200	2	Oct/22; Nov/22
			-22.989152, -43.656201	2	Oct/22; Nov/22
			-22.990631, -43.656565	2	Oct/22; Nov/22
			-22.994059, -43.653093	2	Oct/22; Nov/22
REBIO Guaratiba	Rio de Janeiro	AP	-22.983333, -43.566667	1	Mar/22
			-23.001301, -43.559622	1	Mar/22
			-22.999105, -43.573757	1	Feb/22
			-22.998559, -43.568107	1	Feb/22
PNM Bosque da Barra	Rio de Janeiro	AP	-22.997222, -43.37138	3	July/22; Aug/22; Sep/22
			-22.993886, -43.369539	1	Aug/22
			-22.993901, -43.375876	1	Aug/22
			-22.997522, -43.370991	1	Aug/22

To assess fecundity, ovaries from spawning females ( $N = 5$ ) were removed from the visceral cavity, weighted, and kept in Gilson's solution until a complete detachment of oocytes from epithelial and ovarian walls. Eggs were counted and measured (diameter, in  $\mu\text{m}$ ) in a microscope LEICA TL5000 Ergo. Microanatomy of the zona pellucida was examined under scanning electron microscopy Hitachi TM1000. The bath fecundity (BF), *i.e.*, the number of eggs produced in a single spawning batch, was established from the counting of vitellogenic oocytes (Rizzo, Bazzoli, 2020). The relative fecundity (RF) was determined by the number of vitellogenic oocytes per body size unit (1 cm).

Physical and chemical water characteristics such as temperature ( $^{\circ}\text{C}$ ), dissolved oxygen (mg/L), redox potential (mV), pH, electrical conductivity ( $\mu\text{S}/\text{cm}$ ), and turbidity (FTU) were measured using a multiprobe model Hanna HI9829. Depth (cm) was measured using centimeter rulers and a digital probe (SpeedTech SM-5) at the center of the temporary wetland (equidistant from opposite shores). Each environmental variable (physical, chemical, and depth) had the average value calculated from three replicates.

The measurements were taken at two sites belonging to the same sampling locality (Chaperó, codes 11–12; Tab. 2) during the dry (June) and rainy season (December) of 2022. Therefore, the environmental data presented here may not fully express the range of variability among different occurrence habitats of the species; however, they certainly provide useful evidence of the environmental characteristics to which annual fish are exposed.

**Land use and cover.** To assess changes in the landscape in the fish occurrence areas, buffers were established with a radius of 250 m from the centroids of the water body where fish were caught, totaling an analyzed area of  $\sim 0.1963$  km<sup>2</sup>. In these areas, land use and cover matrices for the years 1985 and 2021 were acquired through the Mapbiomas project (v. 7.0, <https://mapbiomas.org>). The classification was based on annual mosaics of Landsat satellite images, and the image classification process was carried out automatically through the use of decision tree algorithms of the Random Forest type (Souza *et al.*, 2020). The classification was carried out pixel by pixel, the minimum mapped unit was equivalent to 900 m<sup>2</sup> (30 x 30 m). A customized Spatial Reference System (SRS) was used to calculate the areas based on the Albers Projection, with parameters provided by the Instituto Brasileiro de Geografia e Estatística (IBGE). The different classes of land use and cover were grouped into two categories: natural (*e.g.*, Forest formation, Wetlands) and anthropic (*e.g.*, Urban Infrastructure, Pasture and Agriculture), and the rate (%) of progression or regression of anthropic cover (between 1985 and 2021) was compared between areas with different territorial policies (protected *vs.* unprotected areas). We included in our analyses 11 out of the 13 records (6 protected/conservation units; 5 unprotected areas) presented in Tab. 2. In two instances (codes: 10 and 13; 11 and 12; Tab. 2), the distance between the sites was less than 500 m, and to avoid buffer overlap and spatial redundancy in our analyses, we considered only one location. To address potential temporal biases of protected areas created after 1985, we observed if there were conspicuous changes in land use and cover between 1985 and the year of establishment of the protected area. We noticed that the land use and land cover matrices were similar between our lower limit (1985) and the date of creation of the conservation units. Therefore, we conducted our analyses by maintaining a standardized temporal scope of comparison of 36 years (1985–2021) for all 11 locations. All geoprocessing analyses, such as creating buffers, reprojections, transforming raster's into polygons, calculating areas of land use and cover classes, overlays, and layer sampling were performed using QGIS software v. 3.10 A Coruña (QGIS Development Team, 2022).

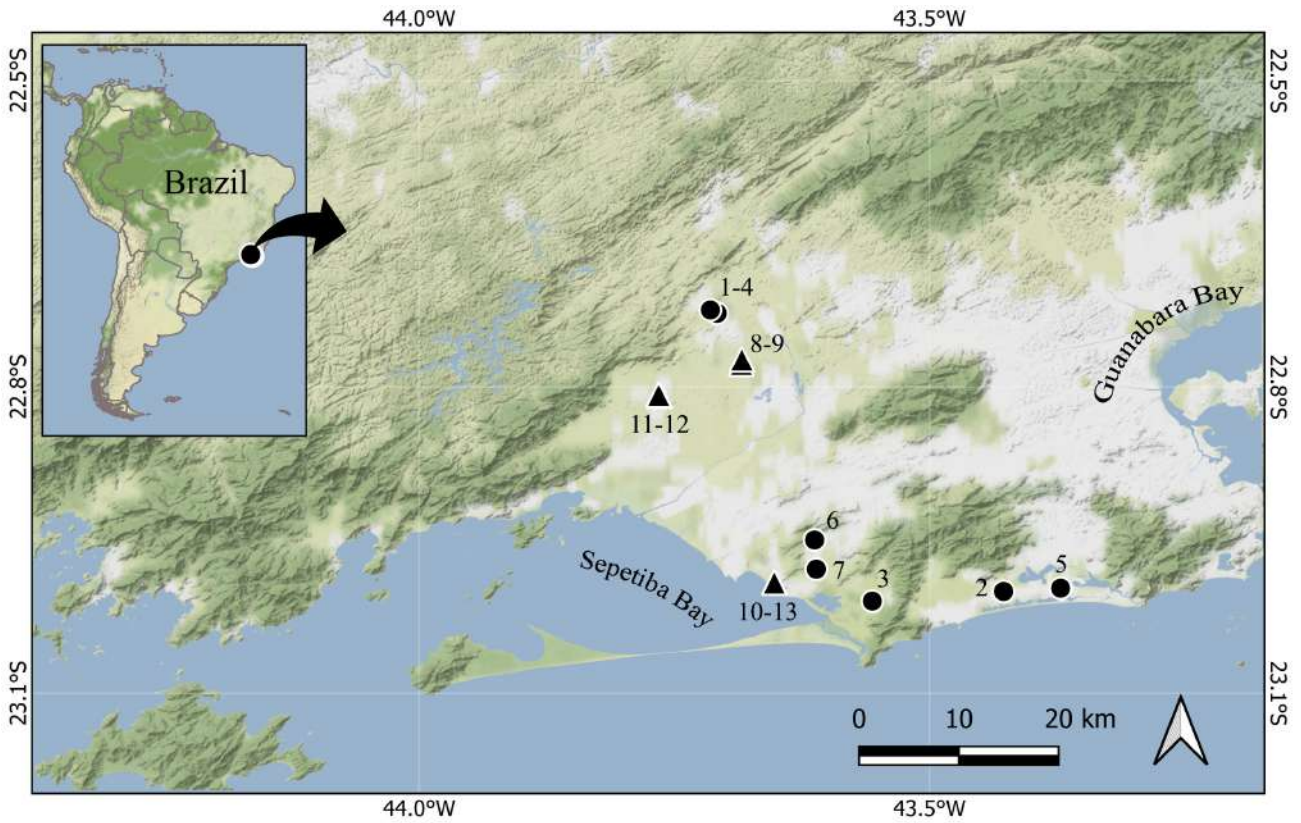
**Statistical analyses.** A Mann–Whitney–Wilcoxon test was performed to compare the differences in the total body length (TL) between males and females. A possible bias in the population sex ratio was assessed by comparing the expected rate of 1:1, and tested with a chi-square test ( $\chi^2$ ), with a 95% of the significance level. The length-weight ( $W = a \times TL^b$ ) relationships (LWR) based on measurements of 43 individuals (males + females) was estimated by linear regression on the transformed equation:  $\log(W) = \log(a) + b \log(TL)$  (Le Cren, 1951), where  $W$  is the body weight (g),  $TL$  is the total length (cm),  $a$  is the y-intercept, and  $b$  is the slope (Froese, 2006). All statistical analyses were conducted in an R environment (R Development Core Team, 2022).

## RESULTS

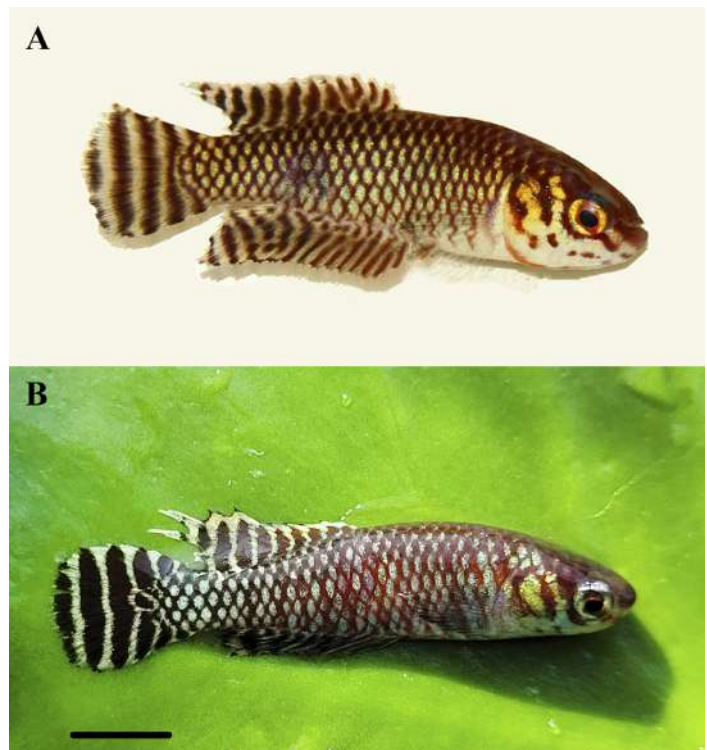
Three new localities of occurrence of *Notholebias minimus* were discovered in coastal plains draining into the Sepetiba Bay, State of the Rio de Janeiro (Tab. 2; Fig. 1). Two of the new records occurred in the Seropédica Municipality: (i) inside the campus of the UFRRJ (22°46'38.4"S 43°41'03.4"W; Tab. 2, cod. 8 and 9); and (ii) on land scheduled to receive the installation of the Chaperó solar power plant (22°48'31.0"S 43°45'51.0"W; Tab. 2, codes 11–12). The third new record occurred in the Rio de Janeiro Municipality, in the Brisas APA (22°59'29.5"S 43°39'06.8"W; Tab. 2, codes 10 and 13). In these localities, a total of 156 individuals of *N. minimus* (70 males, 84 females, and two juveniles with undefined sex; Fig. 2) were sampled. Two localities with the previously known distribution of the species were also revisited (code 3, REBIO de Guaratiba; code 13, PNM Bosque da Barra), however, the species was not recaptured there. Among the 23 sites inspected during the study period (Tab. 1), *N. minimus* was recorded in only six sites (Tab. 2). Other localities shown in Tab. 2 and Fig. 1, and not mentioned here, were not inspected.

**TABLE 2** | Records of *Notholebias minimus* in different areas (AP – protected/conservation units; UN – unprotected) in coastal drainages in the State of Rio de Janeiro. Year of establishment of the protect area also indicated. APA Tabebuias = Área de Proteção Ambiental das Tabebuias; Brisas APA = Área de Proteção Ambiental das Brisas; FLONA Mário Xavier = Floresta Nacional Mário Xavier; PNM Bosque da Barra = Parque Natural Municipal Bosque da Barra; REBIO Guaratiba = Reserva Biológica Estadual de Guaratiba. ZUEC-PIS, Coleção de Peixes do Museu de Zoologia of the Universidade Estadual de Campinas; MNRJ, Museu Nacional, Rio de Janeiro; UFRJ, Universidade Federal do Rio de Janeiro - Instituto de Biologia; LEP-UFRRJ, Coleção Ictiológica do Laboratório de Ecologia de Peixes of the Universidade Federal Rural do Rio de Janeiro. \*New records presented in this study.

Code	Municipality	Locality	Area	Latitude, Longitude	Voucher	Reference
1	Seropédica	FLONA Mário Xavier	AP 1986	-22.727872, -43.7081	ZUEC-PIS 2082	gbif.org/occurrence/2973819872
2	Rio de Janeiro	APA Tapeubias	AP 1999	-23.0004, -43.4273	MNRJ 25303	gbif.org/occurrence/1268715527
3	Rio de Janeiro	REBIO Guaratiba	AP 1974	-22.983333, -43.566667	MNRJ 26429	gbif.org/occurrence/1268716689
4	Seropédica	FLONA Mário Xavier	AP 1986	-22.724692, -43.71466	ICMBio 477424	sibbr.gov.br/55f2f6f9-599e-499c-96fd-74ab54f1ea12
5	Rio de Janeiro	PNM Bosque da Barra	AP 1983	-22.997222, -43.37138	MNRJ 25422	gbif.org/occurrence/1268715653
6	Rio de Janeiro	Campo Grande	UN	-22.9500, -43.6125	UFRJ 8270	Costa, Amorim (2013)
7	Rio de Janeiro	Guaratiba	UN	-22.978608, -43.61083	-	Costa (2016)
8*	Seropédica	UFRRJ	UN	-22.77734, -43.68428	LEP-UFRRJ 2590	gbif.org/occurrence/3988098303
9*	Seropédica	UFRRJ	UN	-22.772941, -43.68382	LEP-UFRRJ 2591	gbif.org/occurrence/3988098304
10*	Rio de Janeiro	Brisas APA	AP 1992	-22.991528, -43.6519	LEP-UFRRJ 2593	gbif.org/occurrence/3988098306
11*	Seropédica	Chaperó	UN	-22.808623, -43.764165	LEP-UFRRJ 2579	gbif.org/occurrence/3803056301
12*	Seropédica	Chaperó	UN	-22.807549, -43.765344	LEP-UFRRJ 2580	gbif.org/occurrence/3803056302
13*	Rio de Janeiro	Brisas APA	AP 1992	-22.991543, -43.652	LEP-UFRRJ 2592	gbif.org/occurrence/3988098305

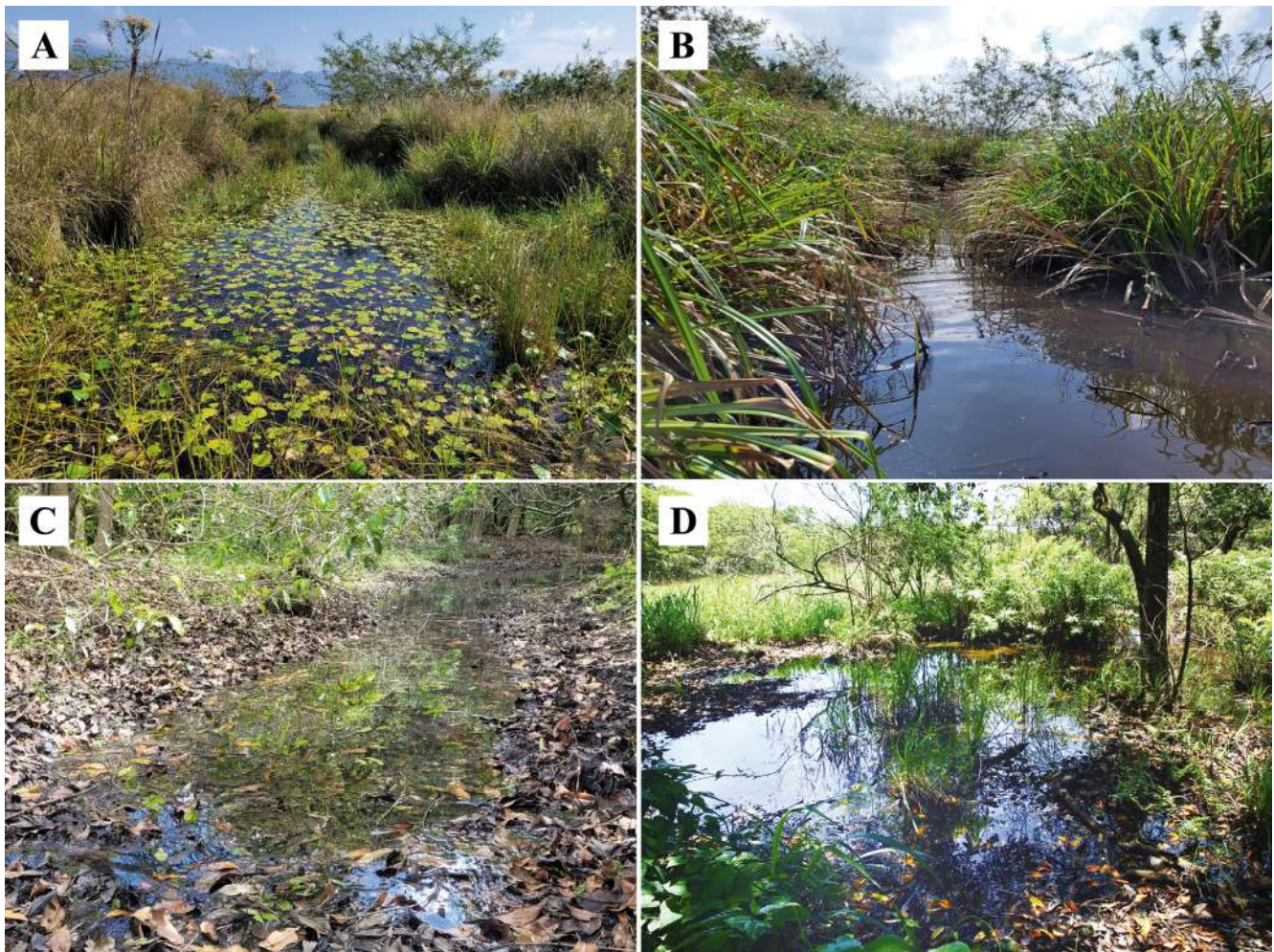


**FIGURE 1** | Map of occurrences of *Notholebias minimus* in coastal plains of the State of Rio de Janeiro, Brazil. Black triangles indicate the new records in this study. Black dots, records from previous studies (e.g., Costa, Amorim, 2013; Costa, 2016). Occurrence references (codes) are available in Tab. 2.



**FIGURE 2** | Males of *Notholebias minimus* captured in (A) Área de Proteção Ambiental das Brisas, Rio de Janeiro Municipality, and (B) in the campus of the Universidade Federal Rural do Rio de Janeiro – UFRRJ (Seropédica Municipality). Scale bar = 4 mm.

*Notholebias minimus* was recorded in temporary pools typical of annual killifishes, including unshaded (Figs. 3A–B) and shaded swamps in the interior/edges of small forest fragments. Floating macrophytes were present only in unshaded swamps (Fig. 3A). For the Chaperó locality, depth (cm) varied between the dry (average  $\pm$  s.d.,  $33 \pm 19$  cm) and wet ( $85 \pm 21$  cm) seasons, with swamps reaching up to 105 cm in depth (Tab. 3). Physical and chemical water characteristics indicate a pH with an acidity tendency (minimum–maximum, 6.25–6.76) and low oxygen concentrations (1.1–3.8 mg/ L; Tab. 3). Other non-annual fish species occurred in sympatry with *N. minimus*, such as *Trichopodus trichopterus* (Pallas, 1770) in the Brisas APA; *Phalloceros anisophallos* Lucinda, 2008, *Hyphessobrycon bifasciatus* Ellis, 1911, and *Deuterodon hastatus* (Myers, 1928) in the Seropédica Municipality (Chaperó and UFRRJ localities).



**FIGURE 3** | Temporary wetlands in the Guandu River Hydrographic Region (coastal drainages of the Sepetiba Bay, State of Rio de Janeiro, Brazil) with new occurrences of *Notholebias minimus*. **A–B.** Swamps of open vegetation in Chaperó locality, **C–D.** Swamps in forest fragments in the campus of the Universidade Federal Rural do Rio de Janeiro – UFRRJ, and in the Área de Proteção Ambiental das Brisas, respectively.

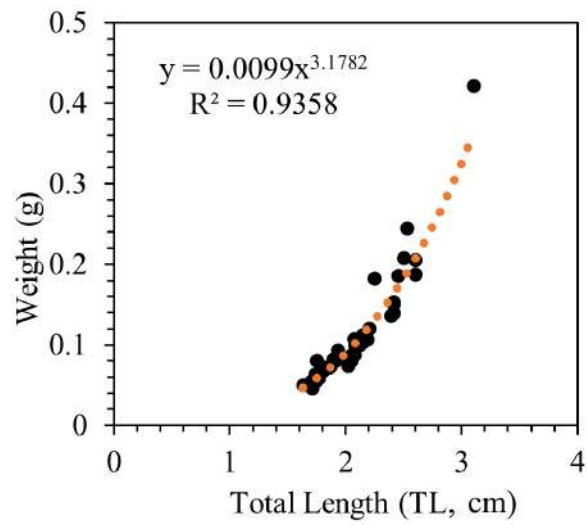


**TABLE 3 |** Physical and chemical water characteristics in the temporary wetlands associated with captures of *Notholebias minimus* in the Chaperó locality (codes 11-12; Tab. 2), during the dry (June) and wet (December) seasons of 2022. Minimum– maximum (mean  $\pm$  standard deviation).

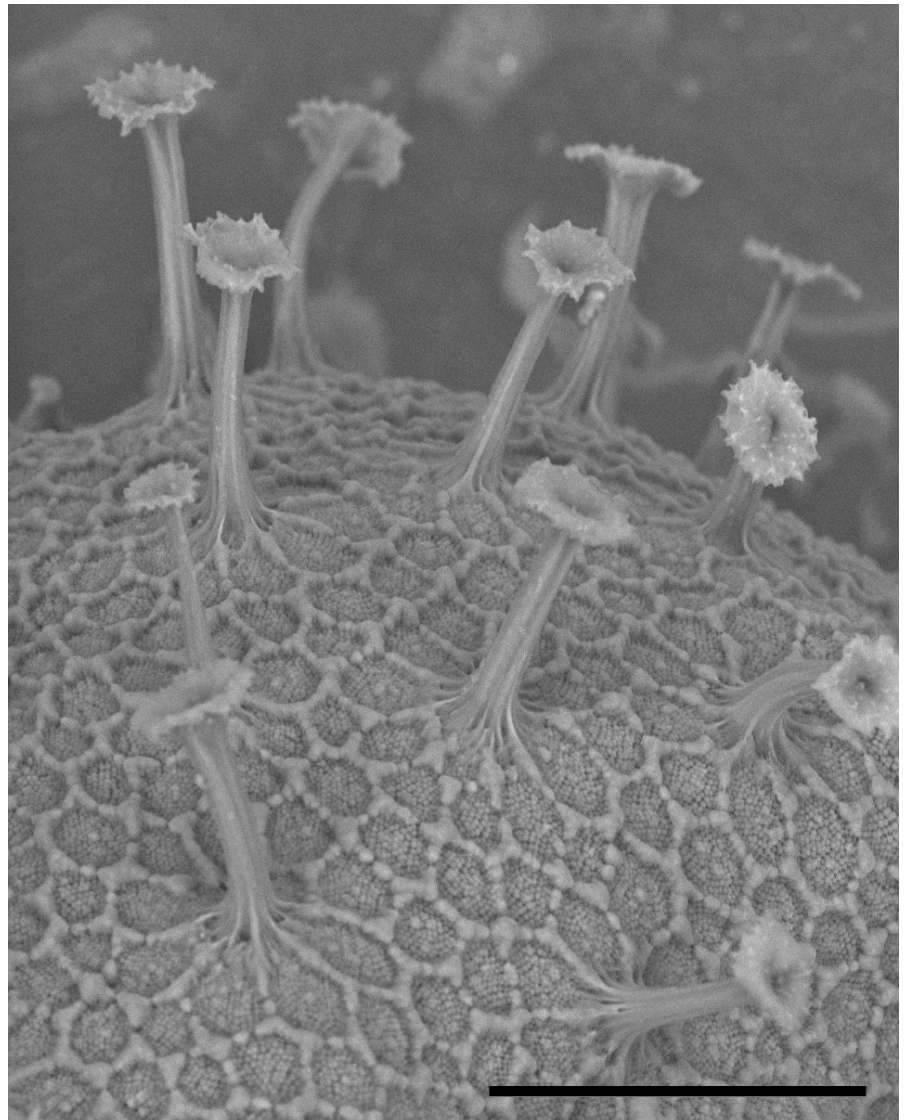
Variables	Dry	Wet
Depth (cm)	15–60 (33 $\pm$ 19)	55–105 (85 $\pm$ 21)
Temperature (°C)	21.2–25.7 (22.2 $\pm$ 1.4)	24.4–25.9 (25.3 $\pm$ 0.7)
pH	6.28–6.76 (6.5 $\pm$ 0.2)	6.25–6.42 (6.3 $\pm$ 0.1)
Dissolved oxygen (mg/L)	1.1–3.3 (1.8 $\pm$ 0.7)	1.1–3.8 (2.4 $\pm$ 1.2)
Oxidation–reduction potential (mV)	73.5–283 (175 $\pm$ 73)	166–281 (233 $\pm$ 43)
Conductivity ( $\mu$ S/cm)	77–190 (101 $\pm$ 29)	30–74 (65 $\pm$ 17)
Turbidity (FTU)	8.7–103 (42 $\pm$ 25)	57–294 (140 $\pm$ 118)

The chi-square test did not show significant differences in the sex ratio (1.1 female: 1 male), with both sexes being captured in similar proportions ( $\chi^2 = 0.516$ ,  $p = 0.472$ ). The body size ranged from 11.1 to 31 mm (mean  $\pm$  s.d., 19.1  $\pm$  3.9 mm TL) and 11 to 26 mm (17.5  $\pm$  3.0 mm TL), for males and females respectively. The mean body size of males was significantly larger than females ( $W = 2193.5$ ,  $p = 0.0067$ ). The length-weight relationship (LWR) with sexes pooled was determined by the following equation fitted to a potential curve:  $Wt = 0.0099 \times TL^{3.18}$  ( $N = 43$ ; Fig. 4). This equation corresponds to the logarithmic form,  $\ln W = 4.61 + 3.18 \times \ln L$  ( $R^2 = 0.92$ ). *Notholebias minimus* exhibits positive allometric growth with an exponent parameter ( $b$ ) equal to 3.18 (2.89–3.46; 95% confidence interval). The total number of oocytes present in the gonads (regardless of the stage of development) of females ranged from 35 to 63 (mean 50  $\pm$  12.3 s.d.). The bath fecundity (only vitellogenic oocytes) ranged from 18 to 40 (24.8  $\pm$  8.8), corresponding to oocytes diameter ranging from 800 to 1,006  $\mu$ m (905  $\pm$  56  $\mu$ m). Relative fecundity (eggs per body size unit – 1 cm) ranged from 8.1 to 16.6 (10.9  $\pm$  3.3). Oocytes in advanced stages of development have mushroom-like projections and polygonal grooves in the zona pellucida (Fig. 5).

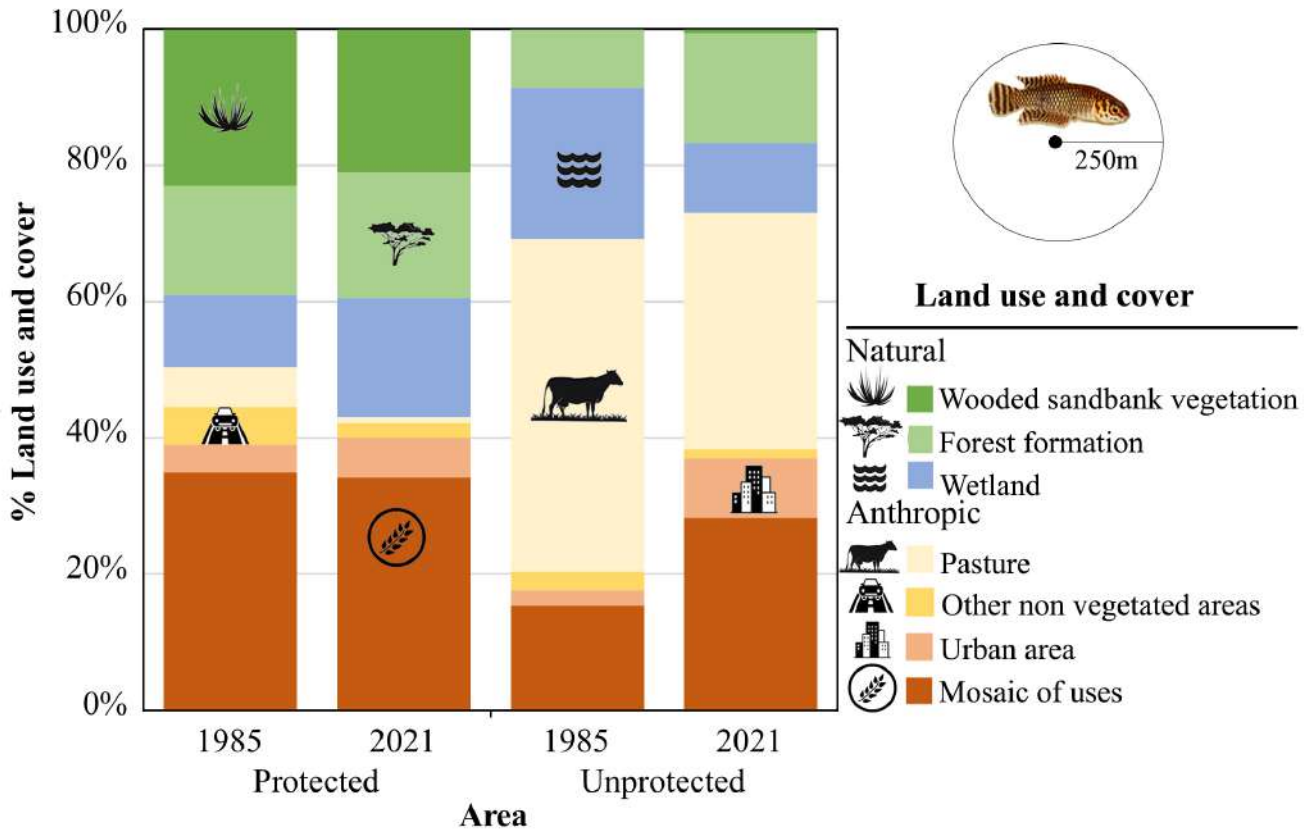
Seven different classes of land use and cover were mapped in adjacent areas (radius 250 m) of *N. minimus* occurrences (Fig. 6). The main impacts in the species occurrence areas were mosaic of land use (28.2%; areas of agricultural use where it was not possible to distinguish between pasture and agriculture), pasture (21.7%), urban area (4.8%) and other non-vegetated areas (3.2%; areas of non-permeable surfaces such as infrastructure or mining). The locations within conservation units exhibited greater relative coverage of natural matrices (total 48%; wooded sandbank vegetation 18.9%, forest formation 14.7%, and wetlands 14.2%) compared to unprotected sites (total 29.4%; wooded sandbank vegetation 0.26%, forest formation 11.7%, and wetlands 17.2%). Protected and unprotected areas also showed opposite temporal trends (1985–2021) of changes in the landscape, while unprotected areas showed an expansion of 4% of anthropic matrices, in protected areas there was a restoration of 7.3% of natural matrices (Fig. 6).



**FIGURE 4** | Length-weight relationship of *Notholebias minimus* (N = 43).



**FIGURE 5** | Unfertilized eggs of *Notholebias minimus*, evidencing mushroom-like projections and polygonal grooves in the zona pellucida. Scale bar = 100  $\mu$ m.



**FIGURE 6 |** Land use and cover (%) in 11 different localities (Protected/Conservation Units vs. Unprotected) and periods (1985–2021) at areas (buffer 250 m) of occurrence of *Notholebias minimus*.

## DISCUSSION

*Notholebias minimus* has a remarkably wide geographic distribution compared with other species of the genus *Notholebias*. Records of this species include the basins of the rivers Guandu, Guarda, Portinho, and drainages of the Lagoon System of Jacarepaguá (Costa, 1988; Costa, Amorim, 2013). This contrasts with the other species of the genus, which have lesser wide distribution and are restricted to the surroundings of the type localities (Costa, 1988; Costa, Amorim, 2013; ICMBio, 2018). There are alternative historical scenarios for the modern distribution patterns of Rivulidae (e.g., Garcia *et al.*, 2012; Costa *et al.*, 2017; Loureiro *et al.*, 2018), and at smaller spatial scales, there is evidence that some species could be dispersed by rearrangements of river drainages, large floods or even endozoochory (Costa, 2013; Silva *et al.*, 2019). Therefore, the explanation for the current distribution of *Notholebias* species is not trivial and deserves further specific studies, as they may encompass unique phylogeographic patterns.

The new biotopes were located inside shaded forest fragments and in swamps of open vegetation exposed to the sun, typical of *Notholebias* spp., which may still include sandy coastal areas covered by bush, grass and open woodland vegetation located up to 100 m from the sea (Costa, 1988). The water in temporary pools at Chaperó locality showed an acidity tendency and low oxygen concentrations, typical environmental

conditions of temporary wetlands (Bidwell, 2013). Overall, annual killifish have evolved to withstand significant daily and seasonal environmental changes, including variations in temperature, oxygen concentration, salinity, pH, and water availability, that approach the limits of vertebrate survival (Podrabsky *et al.*, 2016; Poláčik, Podrabsky, 2016; Ishimatsu *et al.*, 2018). The co-occurrence between *N. minimus* and other non-annual species (*T. trichopterus*, *P. anisophallos*, *H. bifasciatus*, *D. hastatus*) indicates a periodic connection of the temporary wetlands with adjacent perennial water bodies. Sympatry between *Notholebias* and other annual and non-annual species is common (Costa, 1988; ICMBio, 2018) and indicates that these species are able to complete their life cycle and maintain viable populations even under periodic competition or predation.

*Notholebias minimus* showed a positive allometric growth ( $b = 3.18$ ), with comparatively more gain in weight than in length (Froese, 2006). However, no previous references were found for the LWR of *N. minimus* and other species of *Notholebias*, what prevents comparisons of our results with other studies. Males of *N. minimus* are larger than females, corroborating the pattern of sexual dimorphism commonly observed in other species of Rivulidae (*e.g.*, Arenzon *et al.*, 2001; Lanés *et al.*, 2012; Guedes *et al.*, 2020). Preparation for reproduction can cause oxidative stress and affect maternal self-maintenance (Godoy *et al.*, 2020) and consequently the somatic growth of females. Differences in body size mediate the coexistence of annual fish in temporary pools by mitigating intra and interspecific competition (Arenzon *et al.*, 2001; Volcan *et al.*, 2019). Therefore, intraspecific differences observed in body size between males and females may be associated with different reproductive energy costs, in addition to playing an important role in population coexistence.

A reduced batch fecundity ( $24.8 \pm 8.8$  eggs) was found for *N. minimus*, as well as for other annual species such as *Cynopoecilus melanotaenia* (Regan, 1912) ( $19 \pm 26$  eggs; Gonçalves *et al.*, 2011), *Austrolebias nigrofasciatus* Costa & Cheffe, 2001 ( $21.5 \pm 12$  eggs; Volcan *et al.*, 2011), and *Leptopanchax opalescens* (Myers, 1942) ( $27 \pm 7.0$  eggs; Guedes *et al.*, 2023). However, the eggs are relatively large (maximum  $1.006 \mu\text{m}$ ) when weighted by the spatial limitations imposed by the coelomic cavity in this species of reduced body size ( $< 4$  cm). According to the optimal egg size theory, populations evolve a particular egg size that balances the tradeoff between egg size and fecundity to maximize reproductive yield (Smith, Fretwell, 1974). Therefore, larger eggs come at a cost of reducing the number of eggs, which is in accordance with the findings of this study. Annual species have smaller eggs when compared to non-annual species of the family Rivulidae (Guedes *et al.*, 2023). This may be associated with the extreme tolerance of embryos to hypoxia due to the process of embryonic diapause, which culminates in developmental arrest, metabolic depression, and G1 cell cycle arrest (Podrabsky *et al.*, 2016). For species without embryonic diapause, the optimal investment in offspring size increases as environmental quality decreases (Rollinson, Hutchings, 2013; Riesch *et al.*, 2014; Santi *et al.*, 2021). The zona pellucida of mature eggs of *N. minimus* featured mushroom-like projections similar to other species in the genera *Leptopanchax* and *Notholebias* (Costa, Leal, 2009; Thompson *et al.*, 2017). Wourms, Sheldon (1976) hypothesized that these projections are a chorionic respiratory system since there is a network of channels leading to hollow spikes that may function as egg-like aeropiles, similar to insect eggs. This may be an adaptation for annual fishes since a thick, hard, and consequently poorly oxygen-permeable zona pellucida may be necessary to prevent desiccation (Thompson *et al.*, 2017).

*Notholebias minimus* is currently found in five conservation units in the State of Rio de Janeiro, including the unpublished record in the Brisas APA presented here. However, other species such as *Notholebias vermiculatus* and *N. fractifasciatus* do not occur in protected areas (ICMBio, 2018). *Notholebias cruzi* whose type locality is outside a conservation unit, had its biotopes destroyed due to urban expansion and has not been found since 2002, and may be extinct (Costa, 2012; Lira, 2021). Biotopes of *N. minimus* located inside conservation units show great natural cover and environmental restoration trends between 1985 and 2021. On the other hand, locations without any protection show greater coverage of anthropic matrices (pasture, urban area) and a loss of temporary wetlands between 1985 and 2021. These results show the important role played by protected areas in the conservation of biotopes. However, even the protected areas showed high coverage (52%) of anthropic matrices, which may reflect the type of territorial policy, since part of these units are for sustainable use and consequently have fewer restrictions on land use (SNUC, 2000), and/or historical deforestation prior to 1985, since the Brazilian Atlantic Forest biome is historically impacted (Joly *et al.*, 2014; Egler *et al.*, 2020).

The wide geographic distribution of *N. minimus*, combined with records in conservation units, places this species in a more favorable conservation position when compared to other species of the genus *Notholebias*. Our findings reveal that biotopes located within protected areas show a trend of restoration between 1985–2021, with an advancement of natural matrices. Conversely, biotopes found in unprotected areas show an opposite trend, with an increase in anthropogenic impacts on land use and coverage. However, it is crucial to maintain continuous monitoring of the biotopes, both inside and outside protected areas, to ensure the successful preservation of these endangered fish. In conclusion, our findings expand the knowledge of the habitats and population structure of *N. minimus*, and reinforce the importance of establishing protected areas for the conservation and restoration of annual fish biotopes.

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#### AUTHORS' CONTRIBUTION

**Gustavo Henrique Soares Guedes:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing–original draft, Writing–review and editing.

**Carlos Henrique Pacheco da Luz:** Data curation, Formal analysis, Investigation, Methodology, Visualization.

**Rosana Mazzoni:** Formal analysis, Investigation, Methodology, Writing–review and editing.

**Fábio Origuela de Lira:** Data curation, Investigation, Methodology, Writing–review and editing.

**Francisco Gerson Araújo:** Conceptualization, Funding acquisition, Resources, Supervision, Writing–original draft, Writing–review and editing.

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The author declares no competing interests.

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