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Description of two threatened and polymorphic seasonal killifish species of the genus *Hypsolebias* (Cyprinodontiformes: Rivulidae) from the Piranhas-Açu River basin, in the Brazilian semiarid

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In this study we used integrative taxonomy approach to describe two new species of the *Hypsolebias antenori* species group found in the lower Piranhas-Açu River basin in Rio Grande do Norte State, northeastern Brazil. The new species differs from the other congeners primarily by a combination of morphological traits: color patterns of males and females, position of the dorsal-fin origin, number of filaments on anal and dorsal fins, number of scale series and cephalic neuromasts. Using sequences of the mitochondrial cytochrome c oxidase subunit I (cox1) gene, we reconstructed the phylogenetic relationships of the HAG through Bayesian inference with a molecular clock analysis. Our results suggest that the diversification events of the group occurred between the middle and end of the Pleistocene, a period marked by significant diversification among Caatinga's seasonal rivulids. We also identified color polymorphism in *H. guararug*, new species, and *H. negobispoi*, new species, and discussed its potential relationship with oil exploration activities in the region. Finally, we discuss the environmental impacts in the area of occurrence of the new species, suggesting the Endangered (EN) and Critically Endangered status (CR), respectively, based on IUCN criteria, as well as recommendations for their conservation in the semiarid temporary pools.

Keywords: Color polymorphism, Caatinga fishes, Conservation, Oil contamination, Temporary pools.

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Neste estudo utilizamos abordagem de taxonomia integrativa para descrever duas novas espécies do grupo *Hypsolebias antenori*, encontradas no baixo da bacia do rio Piranhas-Açu, no estado do Rio Grande do Norte, Nordeste do Brasil. As novas espécies diferem dos demais congêneres principalmente por uma combinação de características morfológicas: padrões de coloração de machos e fêmeas, origem da nadadeira dorsal, número de filamentos nas nadadeiras anal e dorsal, e série de escamas e neuromastos cefálicos. Com base em sequências do gene mitocondrial citocromo c subunidade I (cox1), reconstruímos as relações filogenéticas do HAG por meio de inferência bayesiana com relógio molecular. Nossos resultados sugerem que os eventos de diversificação do grupo ocorreram entre o meio e o final do Pleistoceno, um período de alta diversificação para os rivulídeos sazonais da Caatinga. Também identificamos a ocorrência de polimorfismo de cor em machos e fêmeas de *H. guararug*, nova espécie, e *H. negobispoi*, nova espécie, discutindo sua possível relação com a exploração de petróleo na região. Por fim, discutimos os impactos ambientais identificados na área de ocorrência das novas espécies, sugerindo o status de Em Perigo (EN) e Critimanete em Perigo (CR), respectivamente, base nos critérios da IUCN, além de recomendações para sua conservação nas poças temporárias do semiárido.

Palavras-chave: Contaminação de petróleo, Peixes da Caatinga, Conservação, Poças temporárias, Polimorfismo de cor.

INTRODUCTION

Rivulidae currently comprises 490 valid species, ranking among the ten most diverse fish families worldwide (Fricke *et al*., 2025). In addition to its high species richness, some members of this family exhibit unique reproductive strategies among vertebrates. For example, *Kryptolebias* (Costa, 2004) is known for self-fertilization, while many genera exhibit seasonal life cycle (Wourms, 1972; Berbel-Filho *et al*., 2022). These seasonal killifishes inhabit temporary aquatic environments and produce resistant eggs with thickened chorion, allowing the embryos to survive long dry periods through embryonic diapause (Furness *et al*., 2015; Thompson *et al*., 2024). These reproductive adaptations have likely enabled rivulid species to thrive in various habitats, ranging from mangrove swamps to ephemeral freshwater pools (Costa *et al*., 2010; Berois *et al*., 2016; Domínguez-Castanedo *et al*., 2022).

The genus *Hypsolebias* (Costa, 2006) comprises 55 species of seasonal killifishes that occur in the floodplains of the Cerrado and Caatinga in Brazil (Ramos *et al*., 2023; Fricke *et al*., 2025). Despite its widespread geographical distribution, *Hypsolebias* is the most threatened genus of freshwater fish in the Caatinga, with approximately 73% of the species on updated Brazilian red lists (ICMBio, 2018; MMA, 2022). Recent advances in integrative taxonomy have identified morphologically similar species, including several groups of *Hypsolebias* primarily found in the São Francisco River basin and in the coastal basins of the Brazilian semiarid (Costa, 2007; Costa *et al*., 2012; Britzke *et al*., 2016; Abrantes *et al*., 2023).

Among these groups, the *H. antenori* group (HAG) consists of three species distributed along the coastal basins of the Mid-Northeastern Caatinga ecoregion: *H. gongobira* Abrantes, Bento, Ramos & Lima, 2023 in the Pacoti River basin, *H. antenori* (Tulipano, 1973) in the Jaguaribe River basin, both from Ceará State, and *H. bonita* Abrantes, Bento, Ramos & Lima, 2023 in the Apodi-Mossoró River basin in Rio Grande do Norte State (Abrantes *et al*., 2023). The HAG is diagnosed by a subdistal orange-red band and a distal black band on anal fin, light blue or metallic band on caudal fin, and blue or white dots on body flank and fins of males. Additional features, such as the neuromast series, scale counts, and number of dorsal and anal-fin filaments, also distinguish the species within the group (Costa *et al*., 2014; Britzke *et al*., 2016; Abrantes *et al*., 2023).

Recent field trips in the lower portion of the Piranhas-Açu River basin, located east of the Apodi-Mossoró River, identified two new species of the HAG at three localities directly impacted by onshore oil activities. We conducted morphological and molecular analyses using cytochrome c oxidase subunit I (cox1) gene, including a time-calibrated Bayesian phylogeny, and discussed the diversification of the HAG species. Additionally, we report the remarkable color polymorphism in both sexes of the new species, and discuss its possible association with oil contamination, as detected through chemical analyses of total petroleum hydrocarbons in the water. The aim of this study is to provide the description of two threatened new species of the HAG from small wetland remnants directly impacted by multiple anthropogenic pressures in the Brazilian semiarid, such as oil extraction, wind farms and the São Francisco interbasin water transfer.

MATERIAL AND METHODS

Morphological analysis. Meristic and morphometric data were obtained using a stereomicroscope and a digital caliper following Costa (1995). Scale patterns were identified according to Hoedeman (1958). The nomenclature and counting of cephalic neuromasts followed Costa (2006). Body morphometric measurements are expressed in percentage of standard length (SL), and head measurements as percentage of head length (HL) (Tab. 1). A couple of each species were cleared and stained (c&s) according to Taylor, Van Dyke (1985). The description of the osteological characters followed Costa (2010). The description of coloration was based on specimens photographed in life. Both species present females with variable number of spots. In addition, some males showed typical female spots, indicating highly polymorphic species. To address this polymorphism, additional photos of preserved specimens were provided.

The specimens analyzed were deposited in the ichthyological collections of the Museu Nacional do Rio de Janeiro (MNRJ), Museu de Zoologia da Universidade de São Paulo (MZUSP), Universidade Federal da Paraíba (UFPB), and Universidade Federal do Rio Grande do Norte (UFRN).

TABLE 1 | Meristic and morphometric data of the holotypes and paratypes of *Hypsolebias guararug* and *H. negobispoi.* Numbers in parentheses represent number of males and females analyzed. Measurements of holotypes are not expressed as percentages.

	Holotype	*H. guararug*		Holotype	*H. negobispoi*	
		Paratype			Paratype	
	Male	Male (n = 41)	Female (n = 20)	Male	Male (n = 10)	Female (n = 9)
Standard length (mm)	30.8	21.0–31.1	20.3–26.4	33.2	22.2–34.4	22.6–39.4
Percentage of standard length						
Body depth	10.0	32.1–39.1	19.2–40.0	11.3	11.0–39.0	16.9–38.3
Caudal peduncle depth	4.3	13.6–16.7	12.5–16.2	5.0	9.6–15.5	8.1–15.2
Pre-dorsal length	14.8	44.5–54.7	55.2–65.8	16.4	37.2–50.4	45.0–60.1
Pre-pelvic length	12.6	40.7–48.6	41.4–52.9	15.8	32.1–45.0	35.1–48.2
Length of dorsal-fin base	10.3	30.0–40.8	17.4–32.1	10.5	19.3–36.0	13.5–23.6
Length of anal-fin base	11.0	32.1–49.6	21.9–29.4	12.1	27.9–39.4	19.0–29.5
Caudal-fin length	9.2	28.1–39.0	24.4–34.8	9.4	21.6–26.8	9.3–28.5
Pectoral-fin length	8.3	22.7–34.4	19.9–29.7	8.4	17.6–25.6	16.5–25.8
Pelvic-fin length	3.0	7.3–11.6	9.2–12.2	2.9	5.5–8.7	6.3–9.8
Head length	8.8	22.3–34.4	29.0–36.4	9.6	22.7–31.9	23.3–35.2
Percentage of head length						
Head depth	7.2	52.1–88.4	46.8–77.0	7.4	66.1–92.7	49.1–74.3
Head width	4.3	41.9 –68.9	41.7–57.1	5.4	45.2–56.2	41.0–56.1
Snout length	1.3	9.6–22.4	11.4–19.2	1.6	9.4–17.7	8.6–16.3
Lower jaw length	2.4	18.5–36.9	15.3–31.4	2.5	21.8–31.4	17.2–31.5
Eye diameter	3.0	27.9–41.3	29.4–39.7	2.8	28.2–43.5	24.7–42.1

Molecular analyses. The DNA extraction, amplification and sequencing procedures were performed following the methodology described in Abrantes *et al*. (2023). Analysis of the consensus sequences of the *cox1* mitochondrial gene of each specimen was carried out using the Geneious program (v. 10.11). Editing and alignment were executed in MEGA 11 (Tamura *et al*., 2021) using the Clustal W algorithm (Thompson *et al*., 1994). The final alignment included a 600 bp cox1 consensus sequence matrix for molecular analyses. Genetic distances were calculated in MEGA 11 using the K2P model (Tab. 2).

Phylogenetics analysis and haplotype network. We used a total of 37 cox1 sequences representing six species of *Hypsolebias*, including the two new species described herein. We analyzed 36 sequences from the *H. antenori* group and used a sequence of *H. lulai* Ramos, Nielsen, Abrantes, Lira & Lustosa-Costa, 2023, from the *H. flammeus* group (Ramos *et al*., 2023), as outgroup. Of the 37 sequences, 20 are original and 17 from Abrantes *et al*. (2023) (Tab. 3).

The best nucleotide substitution model (TN93) was selected a priori using the JModelTest v. 2.1.7 (Darriba *et al*., 2012). We reconstructed a time-calibrated tree with Bayesian inference (BI) in Beast 2 (Bouckaerte *et al*., 2014), using *cox1* sequences with relaxed substitution and a previously selected clock model. In this case, to estimate the divergence time we performed an analysis with uncorrelated lognormal relaxed molecular clock together with a mutation rate of 1.9% (substitution/site/million years), the mutation rate for cox1, in Actinopterygii fishes (May *et al*., 2020). The BI covered 20,000 MCMC generations with trees and parameters sampled every 1,000 interactions and 20% burn-in. The results of each run were visualized in Tracer v. 1.7 (Rambaut *et al*., 2018) to evaluate the parameters and stationarity of the MCMC chains. The posterior probabilities and the consensus tree were inferred using TreeAnnotator v. 1.5.4 (Bouckaerte *et al*., 2014). The tree was manipulated and edited using TreeViewer (Bianchini, Sánchez-Baracaldo, 2024). The haplotype network of the *H. antenori* group was inferred using the TCS method on PopART v. 4.8.4 (Leigh, Bryant, 2015) to visualize the geographic distribution and the number of mutation steps between the haplotypes.

TABLE 2 | Genetic distances (cox1, Kimura 2-parameter) among the species of the *H. antenori* group, including the two new species.

		1	2	3	4	5
1	***H. gongobira***					
2	***H. antenori***	0.026				
3	***H. bonita***	0.016	0.020			
4	***H. guararug***	0.015	0.020	0.014		
5	***H. negobispoi***	0.036	0.039	0.032	0.032	

TABLE 3 | List of *Hypsolebias* species used in the molecular analysis, with their respective coordinates of the collecting site, GenBank accession numbers for cox1 sequences and reference. Type localities are indicated by asterisk.

Species	Coordinates	Genbank number cox1	References
*H. gongobira**	03°53'52"S 38°24'13"W	OR899304, OR899305, OR899306	Abrantes *et al.* (2023)
H. antenori	04°57'39"S 37°54'26"W	OR896085, OR896086, OR896087, OR896088	Abrantes *et al.* (2023)
H. antenori	05°10'00"S 38°05'00"W	OR896089, OR896090, OR896091	Abrantes *et al.* (2023)
*H. bonita**	05°02'52"S 37°30'15"W	OR918882, OR918883, OR918884	Abrantes *et al.* (2023)
H. bonita	05°04'15"S 37°27'44"W	OR918885, OR918886, OR918887, OR918888	Abrantes *et al.* (2023)
*H. guararug**	05°15'26"S 36°38'58"W	PX434343, PX434344, PX434345, PX434346, PX434347, PX434348	This study
H. guararug	05°17'02"S 36°37'10"W	PX434349, PX434350, PX434351, PX434352, PX434353, PX434354, PX434355	This study
*H. negobispoi**	05°15'04"S 36°32'45"W	PX434356, PX434357, PX434358, PX434359, PX434360, PX434361	This study
*H. lulai**	06°10'33"S 35°31'47"W	PX43436280	This study

Chemical analysis of Total Petroleum Hydrocarbons. At the three localities in the Piranhas-Açu River basin, two replicate water samples were collected (on May 15, 2024) in 1L glass bottles and stored in a cooler with ice to preserve their chemical properties. The samples were sent for chemical analysis of total petroleum hydrocarbons (TPH) through the technical services of Laboratório de Análises Ambientais Processamento Primário e Biocombustíveis (LABPROBIO) at the Universidade Federal do Rio Grande do Norte (UFRN) (https://nupprar.ufrn.br/).

RESULTS

Hypsolebias guararug, new species

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(Figs. 1–3; Tab. 1)

Holotype. MNRJ 56075, 34.4 mm SL, male, Brazil, Rio Grande do Norte, Pendências Municipality, temporary pool near Piranhas-Açu River, Piranhas-Açu River basin, 05°15’26”S 36°38’58”W, 19 Jun 2023, R. Carvalho, Y. Abrantes & J. Soares.

Paratypes. All from Brazil, Rio Grande do Norte, Pendências Municipality, Piranhas-Açu River basin. MNRJ 56077, 2 males 26.7–27.7 mm SL, 2 females 21.4–28.7 SL, UFRN 5947, 4 males 22.0–29.8 mm SL, UFRN 5997, 7 males (1 c&s), 23.0–28.6 mm SL, 10 females, 21.7–26.4 mm SL (1 c&s), UFRN 6077, 4 males, 15.4–18.7 mm SL, collected with the holotype. UFRN 5598, 15 males, 20.5–31.1 mm SL, 8 females, 20.3–24.7 mm SL, 05°17’02”S 36°37’10”W. UFRN 6079, 5 males, 18.0–22.4 mm SL, 05°17’02”S 36°37’10”W, 15 May 2024, S. Lima, W. Berbel-Filho, Y. Abrantes & L. Torres.

Diagnosis. Differs from the three species of the *H. antenori* group (*H. gongobira*, *H. antenori*, and *H. bonita*) by having polymorphic males with 1–3 black spots distributed on anterocentral portion of flank and caudal peduncle (*vs*. absent in polymorphic males). It is distinguished from *H. gongobira* by number of pelvic fin rays in adult males (6 *vs*. 5); lower number scales on longitudinal series (27–28 *vs*. 33). Differs from *H. antenori* by lower number of scales on peduncle series (10–13 *vs*. 16); lower number of infraorbital neuromasts (15–16 *vs*. 20–24); and lower head depth in males (52.1–88.4% *vs*. 98.4–117.3% HL). Additionally, it is distinguished from *H. bonita* by position of dark grey bar in eye (vertical *vs*. oblique); higher number lateral mandibular neuromasts (10 *vs*. 4–8); lower number of vertebrae (27–28 *vs*. 30).

Description. Morphometric data in Tab. 1. Largest male examined 34.1 mm SL; largest female 26.4 mm SL. Body laterally compressed, relatively deep. Head narrow, elliptical in lateral plane view. Snout truncated. Eye positioned on lateral of head. Jaws short; conical teeth numerous, irregularly arranged; outer teeth large, inner teeth small and numerous. Vomerine teeth absent.

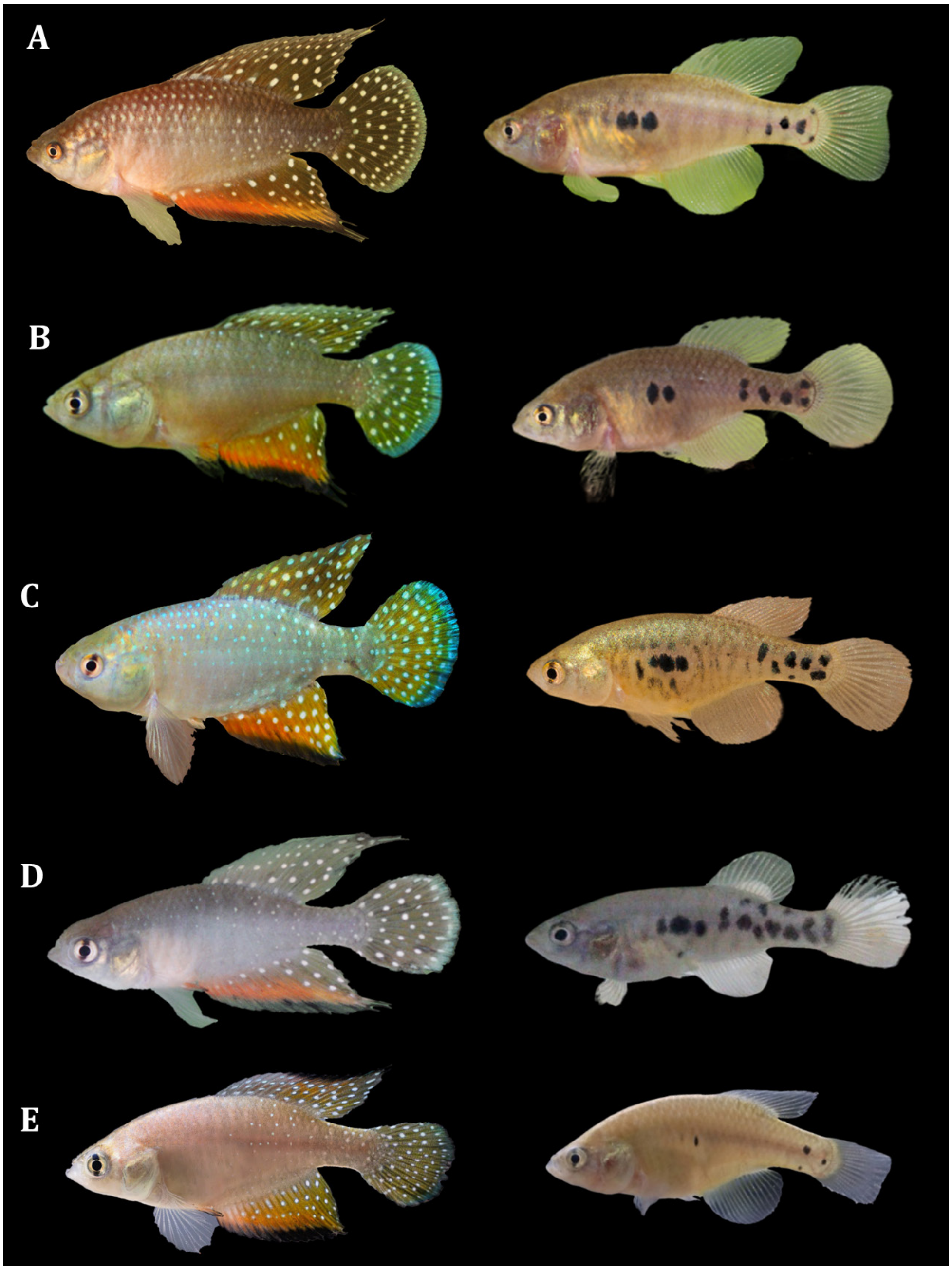

FIGURE 1 | Live coloration pattern of male and female specimens of the *Hypsolebias antenori* group. **A.** *H. gongobira* from Pacoti River, **B.** *H. antenori* from Jaguaribe River, **C.** *H. bonita* from Apodi-Mossoró River, **D.** *H. guararug* and **E.** *H. negobispoi*, both from the Piranhas-Açu River.

Dorsal and anal fins pointed in males and rounded in females. Anal fins with 1–3 filaments in males; filaments absent in females. Pectoral fins elliptical, posterior tip reaching vertical plane base, passing through7th and 8th anal-fin rays in males, and 4th and 5th in females. Pelvic fins pointed, tip reaching base of 2nd–3rd anal-fin rays in males, and 1st–2nd in females. Caudal fin subtruncate in males and rounded in females.

FIGURE 2 | *Hypsolebias guararug*, MNRJ 56075, holotype, male, 30.8 mm SL, Brazil, Rio Grande do Norte, Pendências, Piranhas-Açu River basin.

FIGURE 3 | *Hypsolebias guararug*, MNRJ 56077, paratype, female, 28.7 mm SL, Brazil, Rio Grande do Norte, Pendências, Piranhas-Açu River basin.

Dorsal-fin origin posterior to anal fin ray in males, at transversal plane passing through 3rd and 4th ray. In females, dorsal-fin origin posterior to anal-fin, at vertical plane through base of 4th anal-fin ray. Dorsal-fin rays 21–23 in males, 13–16 in females; anal-fin rays 20–24 in males, 19–21 in females; caudal-fin rays 20–23; pectoral-fin rays 13–14; pelvic-fin rays 6.

Dorsal profile in lateral view slightly concave from snout to end of dorsal-fin base; caudal peduncle slightly concave. Ventral profile convex from lower jaw to end of anal-fin base is straight along caudal peduncle.

Cephalic neuromasts: supraorbital 17–19, parietal 2, anterior rostral 1, posterior rostral 1, infraorbital 15–16, preorbital 4, otic 3–5, post-otic 3, supratemporal 1, median opercular 2, ventral opercular 3, preopercular 15, mandibular 15, lateral mandibular 10–13, and paramandibular 1. No contact organs on unpaired fins. One neuromast per scale of lateral line. Two neuromasts at base of caudal fin.

Frontal squamation follows E-pattern, with E-scales overlapping medially and no scale row anterior to H-scale. Longitudinal scale series 27–28; transverse scale series 10–12; scale rows around caudal peduncle 10–13. Total vertebrae 27–28 (2 c&s).

Coloration in alcohol. Males: flank brown, with 10–11 irregular pale yellow bars in vertical plane; 1–2 black spots on anterocentral portion of flanks and caudal peduncle. Venter pale yellow. Opercular region pale yellow. Iris white. Dorsal, anal and caudal fins dark, with white dots. Pelvic fin light brown. Pectoral fin hyaline with black distal margin. Female: Flank light grey, with dark brown on portion dorsal of flank; 1–3 black spots on anterocentral portion flank; 6–11 irregularly arranged black spots on caudal peduncle. Venter pale yellow. Opercular region silver golden. Infraorbital region light gray. Iris light yellow, with dark gray bar in vertical plane. Fins hyaline.

Coloration in life. Males: flank light grey on anterior portion grayish purple on posterior portion; bluish white iridiscent spots on latero-posterior portion of flank and caudal peduncle. Venter pale orange. Opercular region pale golden. Iris light yellow with dark brown bar in vertical plane. Dorsal fin yellowish-green with large white iridescent spots. One or three short black filaments on tips of dorsal and anal fins. Caudal fin greenish-yellow with greenish-blue distal margin. Anal fin yellowish-orange with white spots, subdistal area orange, and distal black stripe. Pectoral fin hyaline with distal black stripe. Pelvic fin orange with distal black stripe. Females: Sides of flanks light brown; 1–14 black spots on anterocentral portion flank (Figs. 1–9). Venter pale grey. Opercular region light yellow. Infraorbital region pale green. Iris light yellow. Paired fins hyaline.

Color polymorphism. In males, besides the typical male pattern described above, there are males (17 of 40; 42.5%) with 1–2 black spots on anterocentral portion of the flank and caudal peduncle, similar to females. Females highly variable (16 of 20; 80%), with 6–14 black spots on flank.

Geographical distribution. *Hypsolebias guararug* is only known from two localities in the lower portion of the Piranhas-Açu River basin, in the municipality of Pendências, Rio Grande do Norte, Brazil (Figs. 4, 8). The type locality is a long seasonal pool situated along the margin of road RN-18, and 18.6 km away from the Atlantic Ocean (Fig. 4). Four kilometers south, the second locality is a shallow and small pool in a private property. The area of occurrence of *H. guararug* is in an oil exploration field in the Potiguar basin (ANP, 2024), recognized as an economically important region for Brazil's petroleum industry and wind energy production in the semiarid Caatinga biome (https://atlaseolicosolarn.com.br) (Fig. 11).

Ecological notes. Both localities are in the semiarid coastal zone of Rio Grande do Norte. In the type locality, a pool with a total area of 11.368 m^2, at an altitude of 6 m above the sea level, containing dark water and depth ranging about 0.5 to 1 m, with sandy and rocky substrates. *Hypsolebias guararug* was found in syntopy with *Astyanax* aff. *bimaculatus* Linnaeus, 1758, *Poecilia vivipara* Bloch & Schneider, 1801, and the non-native poecilid *Xiphophurus maculatus* (Günther, 1866). At the second locality, at an altitude of 16 m above the sea level, the temporary pool had a total area of 487 m^2, with a depth ranging from 0.3 to 0.8 m, with muddy substrate, and dark water. In both localities, the water surface was covered by aquatic vegetation, predominantly *Echinodorus* sp. and *Nymphaea* sp., while the surrounding vegetation consisted of Carnaúba palms *Copernicia prunifera* (Mill.) H. E. Moore (Fig. 4).

Conservation status. *Hypsolebias guararug* is know from two sites comprising seasonal pools impacted by onshore oil exploration activities. Both sites have oil-contamined waters (see discussion). Five kilometers to the south, the advance of deforestation for the installation of wind farms may compromise the integrity of the forest wetlands where the new species occurs. Therefore, due to continue decline of habitat caused by impacts identified and restricted geographic distribution with an extent of occurrence (EOO) estimated at less than 5,000 km^2, an area of occupancy (AOO) below 10 km^2, this species should be classified as Endangered (EN), according to criteria B1ab(iii)+2ab(iii) of the International Union for Conservation of Nature (IUCN, 2024). The EOO and AOO were calculated on the GeoCAT online server; https://geocat.iucnredlist.org.

Etymology. The specific epithet *guararug* is derived from the Tupi-Guarani language, corresponding to the indigenous name of the Piranhas-Açu River, prior to the Portuguese colonization. The word "guararug" is translated as "river of birds" (Cascudo, 1968). The epithet is treated as a noun in apposition.

FIGURE 4 | Type locality of *Hypsolebias guararug*, Brazil, Rio Grande do Norte, Pendências, Piranhas-Açu River basin.

Hypsolebias negobispoi, new species

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(Figs. 1–5–6; Tab. 1)

Holotype. MNRJ 56076, 32.4 mm SL, male, Brazil, Rio Grande do Norte, Macau Municipality, temporary pool near Cabuji River, Piranhas-Açu River basin, *ca*. 05°15'S 36°32'W, 19 Jun 2023, R. Carvalho, Y. G. Abrantes & J. S. Soares.

Paratypes. All from Brazil, Rio Grande do Norte, Macau Municipality, Cabuji River, Piranhas-Açu River basin. MNRJ 56078, 2 males, 20.5–33.0 mm SL, 2 females, 26.4–34.5 mm SL, collected with the holotype. UFRN 5999, 4 males, 19.7–28.0 mm SL, 4 females, 22.6–24.4 mm SL, collected with the holotype, 19 Jun 2023, R. Carvalho, Y. G. Abrantes & J. S. Soares. UFRN 6080, 4 males, 33.3–37.7 mm SL (1 c&s), 3 females, 26.4–39.7 mm SL (1 c&s), 15 May 2024, S. Lima, W. Berbel-Filho, Y. Abrantes & L. Torres.

Diagnosis. *Hypsolebias negobispoi* differs from all species of the *H. antenori* group by presenting distal black strip in dorsal fin of adult males (*vs*. absence). Differs from *H. gongobira* by presenting higher number of scales on peduncle series (15–16 *vs*. 14). Dorsal-fin origin on transversal plane through bases of 3rd–4th anal-fin rays in males (*vs*. on transversal plane through base of 1st anal-fin ray); lower number of neuromasts on ventral opercular series (3 *vs*. 1). It is distinguished from *H. antenori* by lower number

FIGURE 5 | *Hypsolebias negobispoi*, MNRJ 56076, holotype, male, 32.4 mm SL, Brazil, Rio Grande do Norte, Pendências, Piranhas-Açu River basin. Photo: Diego Bento.

of dark filaments in dorsal and anal fins (1 *vs*. 2–3); lower number of neuromasts on infraorbital series (16–17 *vs*. 20–24); light orange flank color (*vs*. light gray). It also differs from *H. bonita* by presenting lower number neuromasts on preopercular series (12–13 *vs*. 16–17); lower length dorsal-fin base (19.3–36.0% *vs*. 37.8–43.5% SL). Additionally, the new species differs from *H. guararug* by presenting; lower number of ventral opercular neuromasts series (1 *vs*. 3), and preopercular (12–13 *vs*. 15); and by caudal-fin length (21.6–26.8% *vs*. 28.1–39.0% SL).

Description. Morphometric data in Tab. 1. Largest male examined 34.4 mm SL; largest female 39.4 mm SL. Body relatively deep, laterally compressed, greatest body depth at pelvic-fin base. Head narrow, elliptical in lateral plane view. Jaws long, teeth numerous, conical, irregularly arranged; outer teeth large, inner teeth small and numerous. Vomerine teeth absent. Snout truncated. Eye positioned on upper lateral of head. Urogenital papilla cylindrical and exposed in males; pocket-shaped urogenital opening in females.

Dorsal and anal fins pointed with filaments in males and pointed in females. Tip of dorsal and anal fins with one short filamentous rays, reaching vertical plane caudal-fin base in males; filaments absent in females. Dorsal profile slightly convex from snout to end of dorsal-fin base, slightly concave on caudal peduncle. Ventral profile convex, concave on caudal peduncle. Pectoral fin elliptical, posterior tip reaching transversal plane through bases of 6th and 7th anal-fin rays in males, reaching urogenital papilla in females. Pelvic fins pointed; tips reaching base of 1st–2nd anal-fin rays in males and urogenital papilla in females. Caudal fin rounded in males, subtruncate in females.

Dorsal-fin origin at transversal plane through anal fin in males; anal-fin origin at vertical plane through base of 2nd dorsal-fin ray in males. Dorsal-fin origin posterior to anal-fin origin in females, at transversal plane through base of 3rd anal-fin ray. Dorsal-fin rays 19–22 in males, 14–16 in females; anal-fin rays 22–24 in males, 20–23 in females; caudal-fin rays 21–22 in males, 17–20 in females; pectoral-fin rays 13; pelvic-fin rays 6.

Cephalic neuromasts: supraorbital 13–16, parietal 2, anterior rostral 1, posterior rostral with one lateral neuromast and one median neuromast, infraorbital 16, preorbital 3, otic 2, post-otic 3, supratemporal 1, median opercular 2, ventral opercular 2–3, preopercular 12, mandibular 17–18, lateral mandibular 11, and paramandibular 1. Contact organs absent in fins. One neuromast on each scale of lateral line. Two neuromasts at caudal-fin base. Frontal squamation E-patterned; E-scales overlapping medially; no scale row anterior to H-scale; longitudinal scale series 21–25; transverse scale series 10; scale rows around caudal peduncle 10. Total vertebrae 28–29 (2 c&s).

Coloration in alcohol. Males: Iris white. Flank brownish yellow. Opercular and venter region pale yellow. Pelvic and pectoral fin hyaline with black distal margin. Dorsal and caudal fins dark gray with white dots. Anal fin dark gray with white dots, subdistal stripe white and distal stripe dark. Female: Iris white. Flank pale yellow with one or two black spots on anterocentral portion of flank and 1–4 black spots on caudal peduncle. Venter and opercular region pale yellow. Fins hyaline.

Coloration in life. Males: flank light orange, with small bluish white dots. Venter light orange. Opercular region pale gray. Iris light yellow, with dark brown bar in vertical plane. Dorsal fin yellow-orange with bluish white dots. Short black filament is present at tips of the dorsal and anal fins. Caudal fin yellow-orange with bluish-white dots, distal margin with a metallic blue stripe. Anal fin yellow-orange with bluish white dots and a black distal stripe. Pectoral fin hyaline with a black distal stripe. Pelvic fin orange with black distal stripe (Figs. 1–5). Females: flank light gray, with one or two black spots on anterocentral portion of flank and 1–4 black spots on caudal peduncle (Figs. 1–6). Opercular region pale gray. Infraorbital region pale gray. Iris light yellow. Paired fins hyaline.

Color polymorphism. Males: As described for *H. guararug* (Fig. 9). There are 4/10 (40%) males with 1–2 black spots on the anterocentral portion of the flank and caudal peduncle, similar to females.

Geographical distribution. *Hypsolebias negobispoi* is only known from the type locality, a temporary pool near the Cabuji River, tributary of Piranhas-Açu River. The pond is located in an oil extraction unit on the margin of the road at 15 m altitude in the municipality of Macau, Rio Grande do Norte State (Figs. 7–8).

Ecological notes. The total area of the pool was 244 m^2, with turbid water, depth ranging about 0.5 to 1 m, and muddy substrate. During the different sampling efforts, we observed the foraging behavior of the great egret *Ardea alba* (Linnaeus, 1758) in the pool. Aquatic vegetation is composed of *Echinodorus* sp. and *Nymphaea* sp. on the water surface, while the marginal vegetation was composed of jurema-preta *Mimosa tenuiflora* (Willd.) Poir and faveleira *Cnidoscolus quercifolius* Pohl trees (Fig. 7).

FIGURE 6 | *Hypsolebias negobispoi*, MNRJ 56078, paratype, female, 34.5 mm SL, Brazil, Rio Grande do Norte, Pendências, Piranhas-Açu River basin.

Conservation status. *Hypsolebias negobispoi* is only known from its type locality, a single seasonal pool located in close proximity to onshore oil extraction infrastructure. An oil pumpjack operates less than 10 m from this pool, and the water is contaminated with petroleum residues (Fig. 7; see below). As in the case of *H. guararug*, deforestation associated with the installation of wind farms may further threatens the integrity of the surrounding wetlands. With an area of occupancy (AOO) < 10 km² (Calculated on the GeoCAT online server, https://geocat.iucnredlist.org), occurrence restricted to a single location, and evidence of ongoing habitat decline due to oil contamination and deforestation, this species meets the criteria for Critically endangered (CR) B2ab(iii) under the IUCN Red List Categories and Criteria (IUCN, 2024).

Etymology. The specific epithet honors Antônio Bispo dos Santos, known as Nêgo Bispo, a Quilombola teacher and philosopher from Brazil. Nêgo Bispo was born on December 12, 1959, in the municipality of Francinópolis in Piauí State, lived in the Saco-Curtume Quilombo, and died at the age of 63 on December 3, 2023. He fought against social inequalities and defended ancestral knowledge from the forest (Santos, 2023). A noun in apposition.

FIGURE 7 | Type locality of *Hypsolebias negobispoi*, impacted by oil exploration. Brazil, Rio Grande do Norte, Macau, Piranhas-Açu River basin.

Phylogenetic relationships, haplotype network, and genetic distances. The topology obtained through BI indicated a clear division of the *H. antenori* group into five distinct lineages. The specimens from the Piranhas–Açú River basin described here form two monophyletic, but not sister clades, suggesting a secondary contact in the basin. Although *H. guararug* is closely related to *H. bonita* and *H. gongobira*, and these are sister group of *H. antenori*, this relationship is weakly supported (PP = 0.5). On the other hand, *Hypsolebias negobispoi* samples formed a highly supported (PP = 1) sister clade of the species previously listed (Fig. 8).

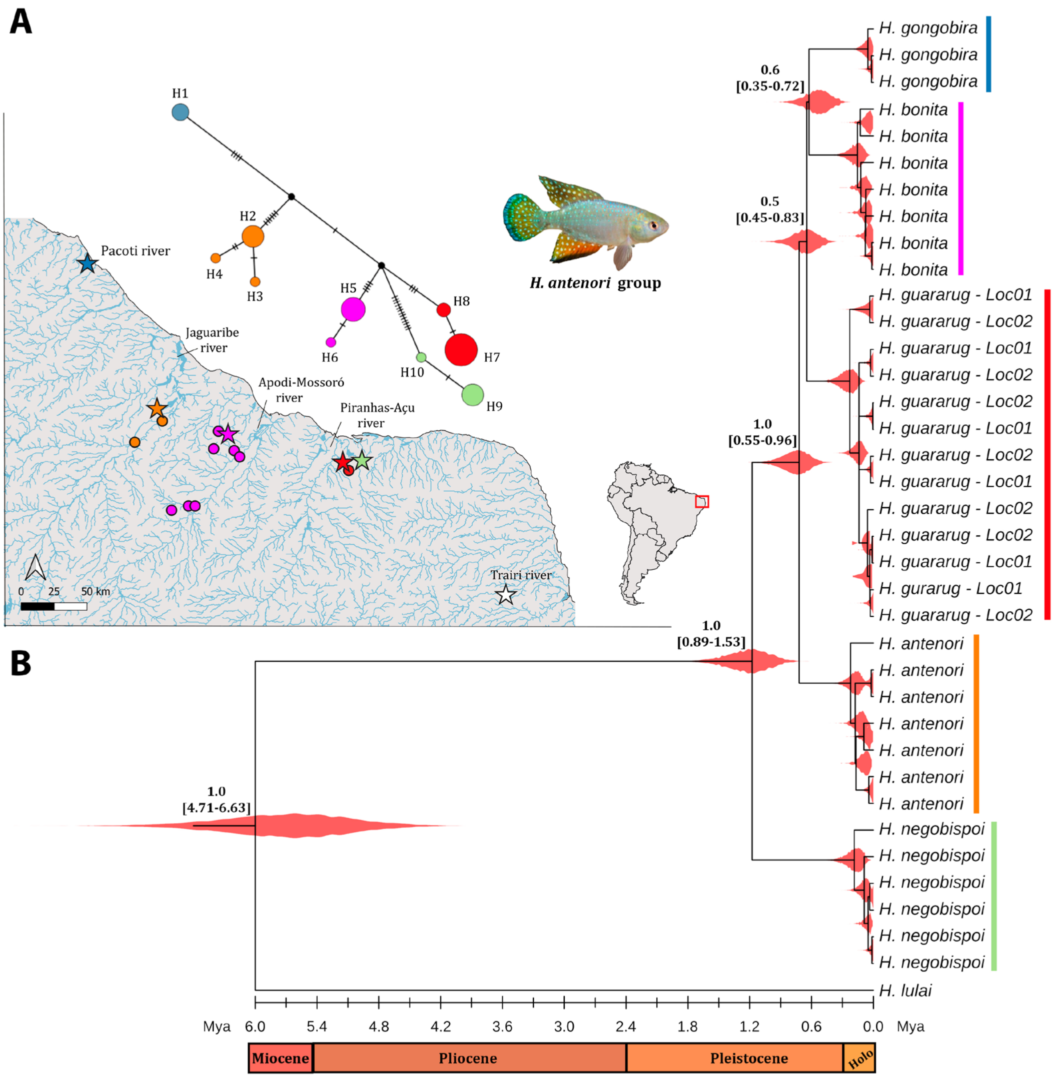

FIGURE 8 | **A.** Geographic distribution of *H. antenori* group in costal basins of the Caatinga. Stars represent type localities, and circles indicate sampled localities. *Hypsolebias gongobira* in dark blue, *H. antenori* in orange, *H. bonita* in purple, *H. guararug* in red, *H. negobispoi* in green, *H. lulai* in white. Light blue lines represent hydrographic costal basins of the Caatinga. TCS haplotype network from the mitochondrial *cox1* of *H. antenori* group haplotypes. Each circle represents a haplotype, lines between nodes represent single base changes, circle size indicates haplotype frequency, and black nodes represent hypothetical ancestral states. **B.** Phylogenetic relationships among the *H. antenori* species group. Bayesian inference tree based on *cox1* sequences. Values above the nodes represent support values, given by posterior probability values from the BI analysis. Values in brackets indicate divergence time estimates, with the 95% highest posterior density (HPD). Vertical bars represent the colors of the species on the geographical distribution map.

Hypsolebias negobispoi appears to have diverged from the other species of the HAG by approximately 1.2 Mya (95% HPD = 0.89–1.53 Mya), during the mid-Pleistocene. In contrast, *H. guararug* comprises a recent clade that diverged from *H. antenori* by about 0.7 Mya (95% HPD = 0.55–0.96 Mya), and from the sister clade composed of *H. bonita* and *H. gongobira*, about 0.6 Mya (95% HPD = 0.45–0.83 Mya), at the end of the Pleistocene (Fig. 8). We observed a total of 10 *cox1* haplotypes of the five HAG species distributed across four hydrographic basins. Our haplotype network also depicts the higher number of mutational steps separating *H. negobispoi* from the remaining species, including *H. guararug* also from the Piranhas-Açu River basin (Fig. 8A).

Genetic distances for *cox1* are presented in Tab. 2. The interspecific K2P distance among valid species ranged from 1.6% (between *H. gongobira* and *H. bonita*) to 2.6% (*H. gongobira* and *H. antenori*). Distances between the new species was 3.2% (*H. negobispoi* and *H. guararug*). The smallest distances were 1.4% (*H. guararug* and *H. bonita*) and 1.5% (*H. guararug* and *H. gongobira*), while the largest were 3.6% (*H. negobispoi* and *H. gongobira*) and 3.9% (*H. negobispoi* and *H. antenori*) (Tab. 2).

Concentrations of total petroleum hydrocarbons in water. The chemical analyses of the water indicated contamination by petroleum hydrocarbons in the pool of *H. guararug* with TPH concentration ranging from 284 µg/L (locality 01) to 447.88 µg/L (locality 02), and 291.73 µg/L in the single site of *H. negobispoi*

DISCUSSION

Phylogenetic relationships. Given the geographical proximity of only 10 km between the type localities of the new species in the Piranhas-Açu River basin, we initially expected them to be sister species, as for some congeners from the São Francisco River basin (Costa *et al*., 2012, 2018). However, our phylogenetic tree indicated that *H. guararug* is closely related with *H. gongobira* from the Pacoti River and *H. bonita* from the Apodi-Mossoró River. This close relationship is further supported by the lower genetic distances observed between these species (1.4 and 1.5%, respectively) (Tab. 2; Fig. 8). Similar genetic distances have been found between species of *H. igneus* group (Britzke *et al*., 2016; Abrantes *et al*., 2023). In contrast, *H. negobispoi* is sister clade of the remaining HAG species and exhibits the highest genetic distances (ranging from 3.2 to 3.9%). These differences are also observed number mutational steps in the haplotype network (Fig. 8). In addition to the molecular differences, *H. guararug* and *H. negobispoi* differ morphologically from each other and from other congeners of the clade, primarily in male and female color patterns, position of the dorsal fin origin, number of black filaments on anal and dorsal fins, scale series, cephalic neuromasts, and vertebral count (see diagnoses).

Our results indicate that the diversification of the HAG species occurred in the middle and late Pleistocene in the costal basins of Caatinga (Fig. 8). This is also consistent with the diversification of the *Hypsolebias flavicaudatus* group in the São Francisco River basin (Costa *et al*., 2018). This period was marked by glaciations with alternating cycles of dry and humid climates, which promoted contractions and expansions of Caatinga vegetation (Oliveira *et al*., 1999; Menezes *et al*., 2016), potentially influencing the speciation processes of the *Hypsolebias* lineages (Costa *et al*., 2017, 2018).

Water contamination by oil. The chemical analyses of the water revealed oil contamination in the habitats of *H. guararug* with total petroleum hydrocarbon (TPH) concentrations ranging from 284 μg/L at type locality, to 447.88 μg/L in the other locality. Similarly, TPH contamination was detected in the only locality of *H. negobispoi*, with TPH levels reaching 291.73 μg/L.

According to the Brazilian Environmental Council (Conselho Nacional do Meio Ambiente, CONAMA) resolution 357/05, which classifies the freshwater habitats, the seasonal killifishes habitats fall under class II and III, which are designated for the protection and natural equilibrium of aquatic communities (CONAMA, 2005). While this resolution sets permissible thresholds for toxic effluents in freshwater, it does not include petroleum hydrocarbons in its list of regulated substances. On the other hand, polychlorinated biphenyls (PCBs) have similar or greater toxic effects than hydrocarbons (Miller *et al*., 2024), are regulated with a maximum allowable concentration of 0.001 μg/L in freshwater (CONAMA, 2005). Despite the absence of specific regulation for petroleum hydrocarbons, the elevated concentrations identified in our analyses may adversely affect the physiology of seasonal killifishes, which are recognized for their rapid responses to environmental physical and chemical changes (Polačik *et al*., 2021).

Color polymorphism. Specimens of *H. gurarug* and *H. negobispoi* show a remarkable color polymorphism (Fig. 9), characterized by combining typical male (elongated, color dorsal and anal fins with filaments) and female traits (black spots on flank and caudal peduncle; Figs. 1–9). Molecular analyses based on *cox1* sequences indicated that these individuals share identical haplotypes of each species (Fig. 8), excluding the possibility of additional syntopic species. This color polymorphism has not been previously reported in *Hypsolebias*, including studies on the *H. antenori* group (Costa, 2002, 2007, 2010; Abrantes *et al*., 2023).

Due to their ephemeral life cycle, seasonal killifishes undergo a rapid senescence process (Thompson *et al*., 2024), which is often accompanied by quick changes in coloration during sexual development (Alonso *et al*., 2024). However, in *H. guararug* and *H. negobispoi* the atypical male phenotypes are observed consistently from juvenile to adult stages (Fig. 9), indicating that it is not result of different developmental stages (Fig. 9). The color polymorphism is generally understood as the coexistence of multiple color morphs in a single interbreeding population. Color polymorphism emerge and can be maintained by a complex interplay of evolutionary forces, including natural and sexual selection, genetic drift, gene flow, and ancestral polymorphism (Guerrero, Hahn, 2017; Rojas *et al*., 2020; Zerulla, Stoddard, 2021; Rodriguez-Silva *et al*., 2023).

Here we present the color polymorphism as a visible phenotypic variation in males and females of both new species, as observed in the North American killifish *Millerichthys robustus* (Miller & Hubbs, 1974) (Domínguez-Castanedo *et al*., 2021, 2022a,b). Once annual killifishes are highly sensitive to environmental changes (Polacik *et al*., 2021), the color polymorphism in both sexes of two *Hypsolebias* species from the Piranhas-Açu River basin may reflect physiological responses to local anthropogenic stressors (Mills, Chichester, 2005), particularly oil activities in the lower Piranhas-Açu River region (Figs. 10–11). This is particularly suggestive since it has not been observed in any other species of the genus, but is present in both species of the same basin, which are phylogenetically distant from each other and both impacted by oil activities.

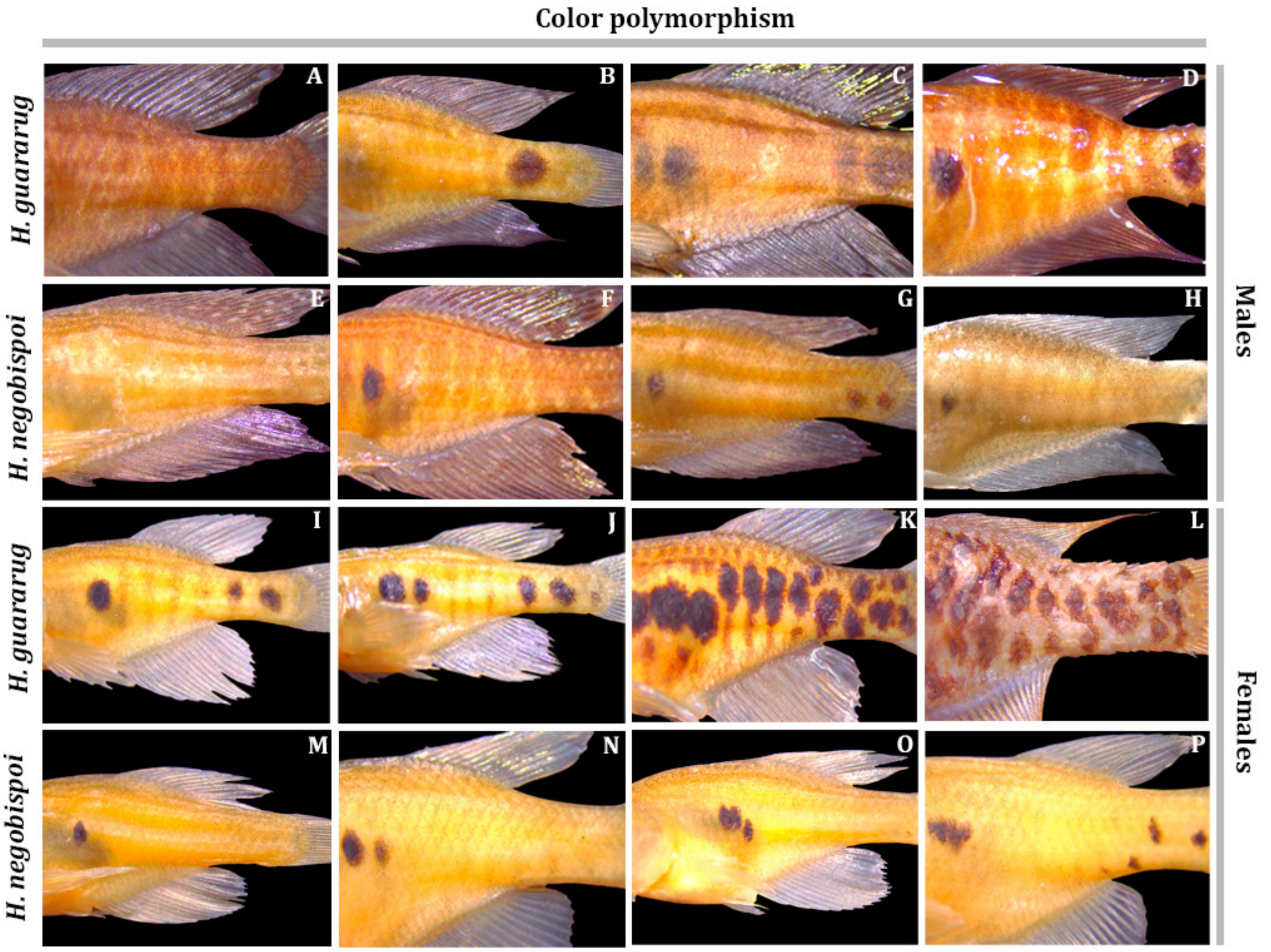

FIGURE 9 | Color polymorphism in alcohol preserved specimens of *Hypsolebias guararug* (males: **A**-**D**; females: **I**-**L**) and *H. negobispoi* (males: **E**-**H**; females: **M**-**P**).

Although we did not directly test the toxic effects of contamination, recent studies have shown that even low concentrations of TPH oil in water can be toxic, causing developmental deformities in embryos, immune system dysfunction, and endocrine disruption in fish (Miller *et al*., 2024; Kuhn *et al*., 2025; Park *et al*., 2025). It is also likely that other physical factors (*e.g*., water temperature, oxygenation, and pH) and ecological interactions (*e.g*., population density and sexual behavior) may influence the color polymorphism in the new species (Zerulla, Stoddard, 2021). Understanding the emergence of this color polymorphism and their impact on population dynamics particularly in the context of sexual and natural selection requires further investigation. Additionally, assessing how these biological factors interact with oil contamination will be critical for evaluating the long-term persistence of these species in the face of threatening environmental changes.

FIGURE 10 | Oil and wind energy impacts identified between the temporary pools of *Hypsolebias guararug* and *H. negobispoi*.

Recommendations for conservation. Since the 1970s, oil exploration in the lower Piranhas-Açu River (ANP, 2024) and the rapid development of wind farms over the last decade have emerged as major threats to the species described herein (Fig. 11). These projects often fail to evaluate their impacts on freshwater ecosystems and Caatinga's fish fauna, which are rarely considered in the environmental licensing processes. Consequently, wetland degradation, habitat loss, and water pollution have become significant factors contributing to the vulnerability of killifishes in Ceará State, including the Critically Endangered (CR) *Anablepsoides cearensis* (Costa & Vono, 2009) and the Vulnerable (VU) *H. longignatus* (Costa, 2008) (ICMBio, 2018; Abrantes *et al*., 2020; Gomes *et al*., 2025).

While *H. guararug* was recorded in two sites in the floodplains of the Piranhas-Açu River, *H. negobispoi* was found in a single pond in the tributary of the Cabuji River. Onshore oil exploration was observed to impact all three sampled habitats (Fig. 11). In addition to oil impacts, the rapid expansion of wind farms has been documented 5 km south of the localities where *H. guararug* and *H. negobispoi* were found. The installation of wind farms involves the suppression of Caatinga shrub vegetation, leading to the loss of riparian vegetation, a critical factor affecting freshwater fish habitats (Casatti, 2010).

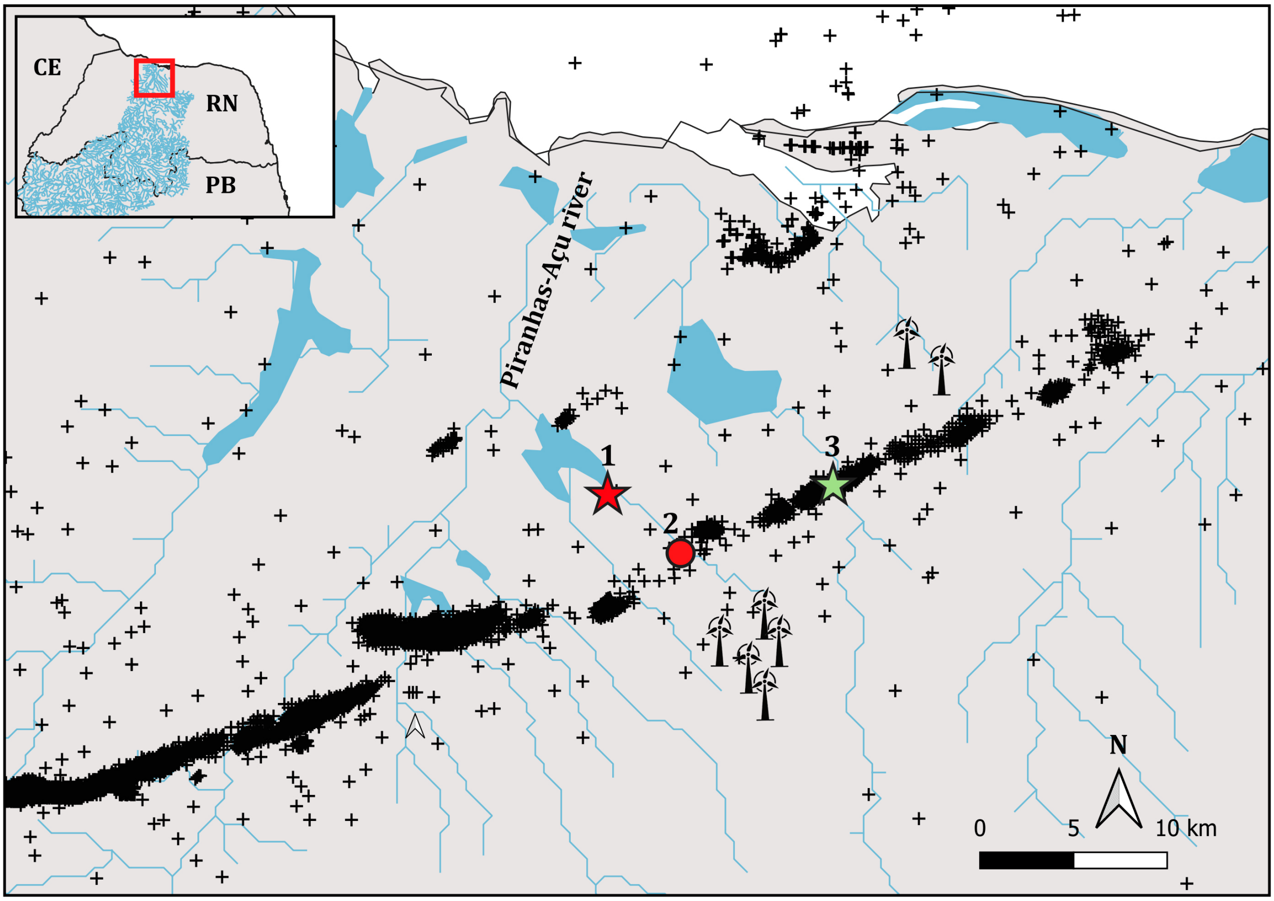

FIGURE 11 | Environmental impacts identified in the lower Piranhas-Açu River basin, Rio Grande do Norte State, Brazil, and the temporary pools of the new species, *Hypsolebias guararug* in red and *H. negobispoi* in green. Cross icons represent onshore oil wells indicated by Brazilian Agência Nacional de Petróleo (ANP, 2024). Wind turbines represent wind farm sites indicated by Plataforma de Energias do Rio Grande do Norte (https://atlaseolicosolarn.com.br/). Stars represent type localities, and circles indicate sampled localities. Light blue lines represent hydrography.

The new species have seasonal life cycle and are restricted to temporary habitats that are subject to continued decline due to environmental threats. With an area of occupancy (AOO) of less than 10 km^2, *H. guararug* can be classified as Endangered (EN) and *H. negobispoi* as Critically Endangered (CR) under the IUCN criteria (IUCN, 2024). Furthermore, the Piranhas-Açu River serves as a receptor basin for the São Francisco interbasin water transfer project, which poses additional risks by altering the hydrological characteristics of freshwater habitats and disrupting the life cycles of killifishes (Costa, 2002; Berbel-Filho *et al.*, 2016; ICMBio, 2018). These cumulative threats underscore the urgent need for conservation measures to protect these species and their fragile ecosystems.

Finally, we highlight the need of additional studies along the Piranhas-Açu River basin to monitor environmental impacts, identify additional localities, and further characterize life-history aspects of the species described here. We also suggest the development of *ex situ* studies as a conservation strategy to ensure the long-term preservation of the species and enable further research on their reproductive biology (da

Fonseca *et al*., 2018). We also emphasize the inclusion killifishes habitats as a priority in the environmental licensing policies following the recommendations proposed by the Plano de Ação Nacional para a Conservação dos Peixes Rivulídeos Ameaçados de Extinção (ICMBio, 2025). Such measures would enable actions to detect new species, expand the geographic distribution and improve the conservation status of known species, create or expand conservation units, plan strategies to protect riverine wetlands, and environmental education (Volcan, Lanés, 2018).

The discovery of two new species of the *H. antenori* group in the Piranhas-Açu River basin expands the group's known geographical distribution to the eastern coastal basins of the Caatinga. Developing technical and scientific studies, both *in situ* and *ex situ*, is crucial to ensuring the protection of these newly identified and threatened species. Additionally, the unprecedented occurrence of color polymorphism in these species encourages new research of evolutionary and ecological relevance. We hope that these perspectives will promote the persistence of these species in the Anthropocene and the implementation of mitigation measures to counteract the impacts on the wetlands in the Caatinga.

Comparative material examined. Brazil. *Hypsolebias antenori*: UFRN 5842, 4 males, 44.0–49.4 mm SL, 5 females, 33.8–39.7 mm SL, Ceará, Russas, rio Jaguaribe basin. UFPB 14900, 1 male, 34.7 mm SL, 12 females, 25.2–32.6 mm SL, Ceará, Limoeiro do Norte, rio Jaguaribe basin. *Hypsolebias bonita*: MNRJ 54899, holotype, male 38.8 mm SL, Rio Grande do Norte, Baraúna, córrego do Virgílio microbasin. UFRN 5226, paratypes, 5 males, 32.6–38.4 mm SL, 5 females, 22.6–36.2 mm SL, Rio Grande do Norte, Baraúna, córrego do Virgílio microbasin. UFRN 5228, paratypes, 2 males 35.1–38.2 mm SL, 5 females, 17.8–32.2 mm SL; Rio Grande do Norte, Felipe Guerra, rio Apodi-Mossoró basin. MZUSP 129608, paratypes, 5 males, 39.1–49.5 mm SL, 5 females, 30.4–37.8 mm SL, Rio Grande do Norte, Mossoró, rio Apodi-Mossoró basin. UFRN 5625, paratypes, 2 males, 34.0–35.8 mm SL, 5 females, 28.3–30.0 mm SL, Rio Grande do Norte, rio Apodi-Mossoró basin. UFRN 5626, paratype, 4 males, 41.5–45.5 mm SL, 4 females, 32.4–34.3 mm SL, Rio Grande do Norte, Mossoró, rio Apodi-Mossoró basin. *Hypsolebias gongobira*: MNRJ 54900, holotype, male, 44.8 mm SL, Ceará, Aquiraz, rio Pacoti basin. MZUSP 129607, paratype, 1 male, 25.0 mm SL, 3 females, 22.2–23.5 mm SL, Ceará, Aquiraz, rio Pacoti basin. UFRN 5847, paratype, 1 male, 34.5 mm SL, Ceará, Aquiraz, rio Pacoti basin.

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AUTHORS' CONTRIBUTION

Yuri Gomes Abrantes: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing-original draft, Writing-review and editing.
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Field activities were authorized by the Instituto Chico Mendes de Conservação da Biodiversidade/Sistema de Autorização e Informação da Biodiversidade (ICMBio/SISBIO), which issued the collection permits 30532–1/2011.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

AI STATEMENT

The authors used the free version of ChatGPT (GPT-5 series) exclusively for grammar review of the manuscript. No scientific content, data interpretation, or conclusions were generated or altered by the tool.

COMPETING INTERESTS

The authors declare no competing interests.

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