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Differential sensitivity of offspring from four species of goodeine freshwater fish to acute exposure to nitrates

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Nitrate-nitrogen (NO_3-N) pollution related to anthropogenic activities is increasing in freshwater ecosystems. Knowledge about NO_3-N sensitivity in freshwater wild fish is needed to understand the differential tolerance between species. Goodeinae is a subfamily of 41 endemic fishes that inhabit central Mexico, with 33 species in the IUCN red list and three extinct. Distributional patterns suggest tolerant and sensitive goodeines related to the conservation gradient of freshwater ecosystems. Four species with a differential distribution and tolerance were selected to evaluate their physiological responses to NO_3-N. Fish were exposed to different NO_3-N concentrations for 96 h and the median lethal concentration (LC_{50}) was determined. Swimming disorders plus gill and liver histopathological indexes were estimated and incorporated into an Integrated Biomarker Response (IBR) for each species. *Skiffia lermae* (LC_{50} = 474.332 mg/L) and *Xenotoca variata* (LC_{50} = 520.273 mg/L) were more sensitive than *Goodea atripinnis* (LC_{50} = 953.049 mg/L) and *Alloophorus robustus* (LC_{50} = 1537.13 mg/L). The typical histological damage produced by $NaNO_3$-N exposure was fusion of secondary lamellae in gills. This was present in all species and cellular degeneration was observed at the highest concentrations. Secondary lamellae aneurysms were only observed in *G. atripinnis*. Liver alterations included vascular dilation in hepatic sinusoids, hyperemia and nuclear hypertrophy; higher concentrations produced hepatocyte cytoplasmic vacuolation and reduced frequency of cell nuclei. Behavioral and histopathological alterations could explain the differential species sensitivity. The results suggest that

species which preserve gill function and transfer the task of detoxification to the liver might have the best chance of surviving in polluted environments. Moreover, species previously considered as tolerant may be highly susceptible to $NaNO_3$-N exposure. Therefore, it is necessary to closely monitor $NaNO_3$-N concentrations in freshwater ecosystems and, if possible, reduce their levels to avoid the loss of wild populations.

KEYWORDS

ecotoxicology, LC50 96 h, histopathological changes, swimming behavior, gill, liver

Introduction

One of the most representative fish groups in central Mexico are the goodeines. The major diversification within this group occurs in the Río Lerma-Santiago basin, one of the most polluted basins in the world (Domínguez-Domínguez et al., 2006, 2008; Lyons et al., 2019). The Goodeinae subfamily is composed of 41 species from 19 genera (Domínguez-Domínguez et al., 2008; Lyons et al., 2019). This group presents unique reproductive characteristics such as internal fertilization, matrotrophy, and viviparity (Iida et al., 2019), and is considered one of the most at-risk groups in the world (Duncan and Lockwood, 2001). During the last decades, their populations have decreased related to pollution and other anthropogenic activities (Lyons et al., 2019). According to the IUCN (2022), 13 species are critically endangered, 14 endangered, six vulnerable, two extinct in the wild, and one extinct. Most Goodeinae species are endemic or micro-endemic to a single spring, portion of a river or drainage, whereas others are widely distributed in the region (Foster and Piller, 2018). These distributional patterns suggest tolerant and sensitive goodeines related to the conservation gradient of freshwater ecosystems (Rueda-Jasso et al., 2017; Lyons et al., 2019). For example: *Skiffia lermae* and *Alloophorus robustus* have been classified as endangered, sensitive, and vulnerable species, respectively; since they only are found in spring areas, lake shorelines and small tributaries (Miller et al., 2009; Lyons et al., 2019). *Xenotoca variata* and *Goodea atripinnis* are considered as least concern and tolerant species, because some areas they inhabit are classified as highly polluted aquatic environments (Domínguez-Domínguez et al., 2008; Rueda-Jasso et al., 2017; Lyons et al., 2019).

Nitrates (NO_3) constitute the most stable and abundant form of dissolved inorganic nitrogen in aquatic ecosystems (Galloway et al., 2004). Although nitrate-nitrogen (NO_3-N) may be naturally present, they accumulate in the ecosystem due to human activities such as livestock and agricultural use of inorganic nitrogenous fertilizers. Municipal and industrial wastewater effluents also increase nitrogenous compounds (Du et al., 2019; Goeller et al., 2019). Despite the abundance of NO_3-N, their impact on fish welfare has been underestimated since they are less toxic than other nitrogenous compounds (van Bussel et al., 2012; Kim et al., 2019; Presa et al., 2022). However, NO_3-N negatively impact fish health (Camargo et al., 2005; Guillette and Edwards, 2005; McGurk et al., 2006; Gomez-Isaza et al., 2018, 2020, 2021; Presa et al., 2022).

The standard sensitivity evaluation to acute toxicity is the median lethal concentration (LC_{50}). This index is usually complemented with other responses to increase the understanding of physiological trade-offs under acute exposure or predict the long-term permanence of fish in polluted environments (Kim et al., 2019; Dutra et al., 2020). Exposure bioassays to NO_3-N in fish revealed more sensitivity during early life stages (Rueda-Jasso et al., 2017), likely due to interference with biological processes such as body growth and reproductive development or behavior (Yu et al., 2021). The gills are the main route of NO_3-N absorption (Pereira et al., 2017). Physiological responses to pollutants aim to maintain gill activity preceding the loss of function related to the degree of damage (Emam et al., 2022). Inside the body, NO_3-N causes physiological and behavioral alterations (Torno et al., 2018; Yang et al., 2019; Yu et al., 2021). The liver is the detoxifying organ and its condition indicates environmental quality (Corredor-Santamaría et al., 2021; Tramunt et al., 2021; Santos et al., 2022). Toxic pollutants cause functional alterations of the gills and liver, such as gas exchange failure and metabolic damage. These changes are associated with altered swimming behavior, such as loss of balance, top or bottom position in the water column and lethargy (Rodrigues et al., 2011; Schram et al., 2012; Pereira et al., 2017; Yang et al., 2019).

Given the conservation relevance of goodeines and based on the premise that differential persistence in freshwater bodies could be related to differential sensibility to environmental pollutants, the main objective of this study was to evaluate NO_3-N sensitivity in offspring of *S. lermae*, *X. variata*, *G. atripinnis*, and *A. robustus*. Differential sensitivity was evaluated by LC_{50} values, histopathological indexes from the gills and liver, as well as swimming behavior alterations under acute exposure (96 h).

Integrated Biomarker Response (IBR) analysis was performed to contrast their sensitivity.

Materials and methods

Ethical statements

Sampling and laboratory fish handling protocols were reviewed and approved (SEMARNATSGPA/DGVS/00012/19, PPF/DGOPA-014/20). The protocols also followed the Guide for the Care and Use of Laboratory Animals (1996). Toxicity was evaluated with the Fish Acute Toxicity Test (OECD, 2019).

Field collection and fish maintenance

Gravid females of *S. lermae* (n = 25), *X. variata* (n = 20), *G. atripinnis* (n = 7), and *A. robustus* (n = 5) were collected from Zacapu Lake, central Mexico (19°49′26.88″N; 101°46′37.32″W), during years 2020 and 2021, using 2 mm aluminum mesh minnow traps (Gee-minnow-traps® G-40, USA). Gravid females were transported to the laboratory with water from the collection site. Females were acclimatized for 15 days in 120 L aquariums (loading capacity: 6 L per fish), with dechlorinated water and 7 mg/L of dissolved oxygen, at 22°C, under a natural light photoperiod. They were fed two times each day with *Artemia* sp. and commercial Wardley fish flakes (Rueda-Jasso et al., 2017). On post-natal days 1–7, offspring were separated from their mothers, fed and housed under the same conditions previously described. Offspring remain in these conditions until post-natal 21, when they were subjected to the experiment (see below).

Water quality monitoring and solutions

Temperature, dissolved oxygen, pH, salinity, total dissolved solids and electric conductivity were recorded every 24 h using a multiparameter probe (YSI EXO2. Ohio, USA). The toxicity assay solution was prepared with sodium nitrate ($NaNO_3$, 97%, Sigma-Aldrich) according to a stoichiometric calculation (Dutra et al., 2020). Concentrations of ammonium-nitrogen (NH_4-N), nitrite-nitrogen (NO_2-N), and NO_3-N were measured following the methodologies described in the American Public Health Association (American Public Health Association [APHA], 2017).

Toxicity test

Each fry was acclimatized for 7 days, weighted with an analytical precision balance (OHAUS™ Adventurer d = 0.0001 g, China), their length was measured using a digital Vernier caliper (Thermo Fisher Scientific™ S/N 1366162, USA) and then randomly assigned to a treatment. Three replicates were used per treatment, with 10 fish in 10 L tanks (loading capacity: 1 L per fish). A control group was included for each species.

During NO_3-N exposure, survival was monitored at 0.5, 1, 2, 3, 4, 6, 9, 12, 24, 36, 48, 72, and 96 h. Mortality was confirmed by the absence of response to stimulation. Swimming behavior (loss of balance and position in the bottom, middle, or surface of the water column) was recorded during 10 min by counting the animals that exhibited these actions at the same intervals mentioned above. Changes in swimming behavior were categorized as "low effect," indicating an alteration in 10–33% of individuals; "moderate effect" 34–63% and "high effect," 64–100%. Changes to swimming patterns were recorded only if they occurred in 10% of the fish within each aquarium (Ogueji et al., 2018). At the end of the experiment, surviving fish were anesthetized using clove oil (50 μl L-1) and processed for histology. Fish in the highest NO_3-N concentrations were removed from the aquariums immediately after they became immobile.

Histological evaluation

Three individuals from three treatments (control, that closest to the LC_{50} and the most lethal concentration, LC_{100}) per species were randomly selected and processed by routine histology. Gill and liver 5 μm transversal sections were obtained and stained with hematoxylin-eosin (Bancroft and Cook, 1994; Cano-Rocabayera et al., 2019). Slides were observed with a Leica microscope (DM3000, Germany), photo-documented using a camera (Leica DFC310 FX, Germany) and analyzed using NIH ImageJ software.

Gill and liver histopathological indexes

The histopathological condition index (HI) was calculated for each organism (Bernet et al., 1999; Antunes et al., 2017). Briefly, according to distinctive histological features, gill and liver alterations were classified into circulatory, progressive and regressive patterns. A pathological importance factor (w) was assigned for each type of damage (**Supplementary Table 1**). Similarly, a score value (a) was assigned for the occurrence of alterations from 0 to 5 (where 0 is no change; 1, changes in 1–20% of the sections; 2, 21–40%; 3, 41–60%; 4, 61–80%; 5, 81–100%, respectively). The HI for each rp was determined using the formula: $HI_{rp\ org} = \Sigma_{alt}\ (w \times a)$ where $HI_{rp\ org}$ is the reaction index of the organ for each reaction pattern (rp) and alt is the alteration (Antunes et al., 2017).

Liver damage tissue index

For the liver, tissue damage was assessed by nuclear density (nD = number of nuclei/mm^2) and nuclear area (nA = area

of hepatocyte nuclei in μm^2; Rodrigues et al., 2017). The average value of controls (nD_c and nA_c) was used as a reference to calculate a relative value for each feature. Since an increase in the nuclear area could indicate hypertrophy and a reduction could be related to pyknotic nuclei, the absolute value of the difference to 1 multiplied by 0.5 was used. Finally, the values were integrated into the following index: $LDTI = (nD^*1/nD_c) + [|1 - (nA^*1/nA_c)| \times 0.5]$, where values near 1 indicate a normal or reference condition and values closest to 0 indicate major tissue damage.

Statistical analysis and integrated biomarkers response

All analyses were performed with R software (R Core Team, 2022, version 4.1.3). Variables were tested for normality and homoscedasticity with Shapiro-Wilk and Levene's tests, respectively. The survival rate for each species was calculated by the Kaplan Meier method and compared with the log-rank test using the "survival" and "survminer" packages. Acute toxicity to $NaNO_3$-N was determined by the LC_{50} for each species (95% confidence intervals after 96 h) using the Spearman-Karber method and considered significantly different when not overlapping using the "tsk" package. Relationships between swimming disorders and concentrations at each exposure time were analyzed with Spearman correlations using the "stats" package; graphs were produced using the "ggpubr" package. Histomorphometric analyses were performed using the "car" and "pgirmess" packages. LDTI data distribution was analyzed by a Kruskal-Wallis test. Tukey multiple range *post-hoc* tests were used to discriminate means (Torno et al., 2018). The values obtained for each parameter were expressed as mean ± standard error of the mean. Survival rates, LC_{50}, swimming behavior, GHI, LHI, and LDTI were incorporated into an IBR to visualize and compare species-specific responses to acute $NaNO_3$-N exposure using the "IBRtools" package (Bertrand et al., 2016).

Results

$NaNO_3$-N exposure and water quality

Concentrations for acute toxicity assays were determined for each species. Thus, each species was exposed to slightly different ranges; *S. lermae*: 100, 200, 400, 800, and 1,200 mg NO_3-N/L; *X. variata:* 200, 400, 800, 1,200, and 1,600 mg NO_3-N/L; *G. atripinnis:* 400, 800, 1,200, 1,600, 2,000, and 2,400 mg NO_3-N/L; and *A. robustus:* 400, 800, 1,200, 1,600, 2,000, 2,400, and 2,800 mg NO_3-N/L. The body mass, length and total number of offspring for each species was: 0.028 ± 0.0004 g; 12.265 ± 0.058 mm; n = 180 for *S. lermae*, 0.072 ± 0.001 g; 15.656 ± 0.103 m; n = 180 for *X. variata*, 0.184 ± 0.001 g; 20.122 ± 0.047 mm; n = 210 for *G. atripinnis* and 0.182 ± 0.001 g; 21.382 ± 0.055 mm; n = 240 for *A. robustus*.

Water quality conditions (temperature: 22.059 ± 0.003°C; dissolved oxygen: 7.069 ± 0.001 mg/L; pH: 8.930 ± 0.004 log units; ammonium: 0.003 ± 4.88104E-06 mg/L and nitrite: 0.042 ± 0.002 mg/L) in the experimental tanks were similar for all groups and maintained in a range similar to the natural variations observed in Zacapu lake (Ramírez-García et al., 2022). Salinity (0.281 ± 0.001–12.041 ± 0.096 g/L), total dissolved solids (379.033 ± 1.913–13,017.266 ± 84.070 mg/L) and electric conductivity (551.290 ± 2.691–19,103.63 ± 141.191 µS/cm) increased as $NaNO_3$-N concentrations increased.

Survival rate and lethal concentration 50%

Survival rates for the four Goodeinae species decreased as $NaNO_3$-N concentrations increased and were related to exposure time. No mortality was observed at control concentrations (**Figure 1**). Survival rates for *S. lermae* and *X. variata* showed significant differences above 200 mg $NaNO_3$-N/L vs. control [*S. lermae*: $X^2_{(5)} = 306$; $p \leq 0.001$; *X. variata*: $X^2_{(5)} = 149$; $p \leq 0.001$]; no fish survived after 96 h at 1,200 mg $NaNO_3$-N/L for *S. lermae* and at 1,600 mg $NaNO_3$-N/L for *X. variata* (**Figures 1A,B**). Survival rates for *G. atripinnis* showed significant differences above 800 mg $NaNO_3$-N/L vs. control [$X^2_{(6)} = 131$; $p \leq 0.001$] and no fish survived at 2,400 mg $NaNO_3$-N/L (**Figure 1C**). For *A. robustus* significant differences were observed at 2,000 mg $NaNO_3$-N/L vs. control [$X^2_{(7)} = 940$; $p \leq 0.001$] and all fish died at 2,800 mg $NaNO_3$-N/L (**Figure 1D**).

The calculated LC_{50} values and corresponding 95% confidence intervals for each species followed the survival trend (**Figure 2**). *Skiffia lermae* was the most sensitive, LC_{50} = 474.332 (373.850–601.820) mg $NaNO_3$-N/L, followed by *X. variata* LC_{50} = 520.273 (380.390–711.594) mg $NaNO_3$-N/L, *G. atripinnis* LC_{50} = 953.049 (722.835–1256.584) mg $NaNO_3$-N/L, and *A. robustus* LC_{50} = 1537.130 (1203.223–1963.699) mg $NaNO_3$-N/L.

Swimming behavior

Swimming disorders were related to exposure time and concentration (**Tables 1**, **2** and **Supplementary Figure 1**). The first alteration in *S. lermae* offspring was that they remained on the bottom of the water column at 24 h of exposure to the lowest concentration (100 mg $NaNO_3$-N/L). The other three species showed surface permanence as the first alteration at 48 h of exposure to 200 mg $NaNO_3$-N/L in *X. variata*; while for *A. robustus* and *G. atripinnis* this occurred at 36 and 84 h of exposure to 400 mg/L, respectively (**Table 1**). The prevailing

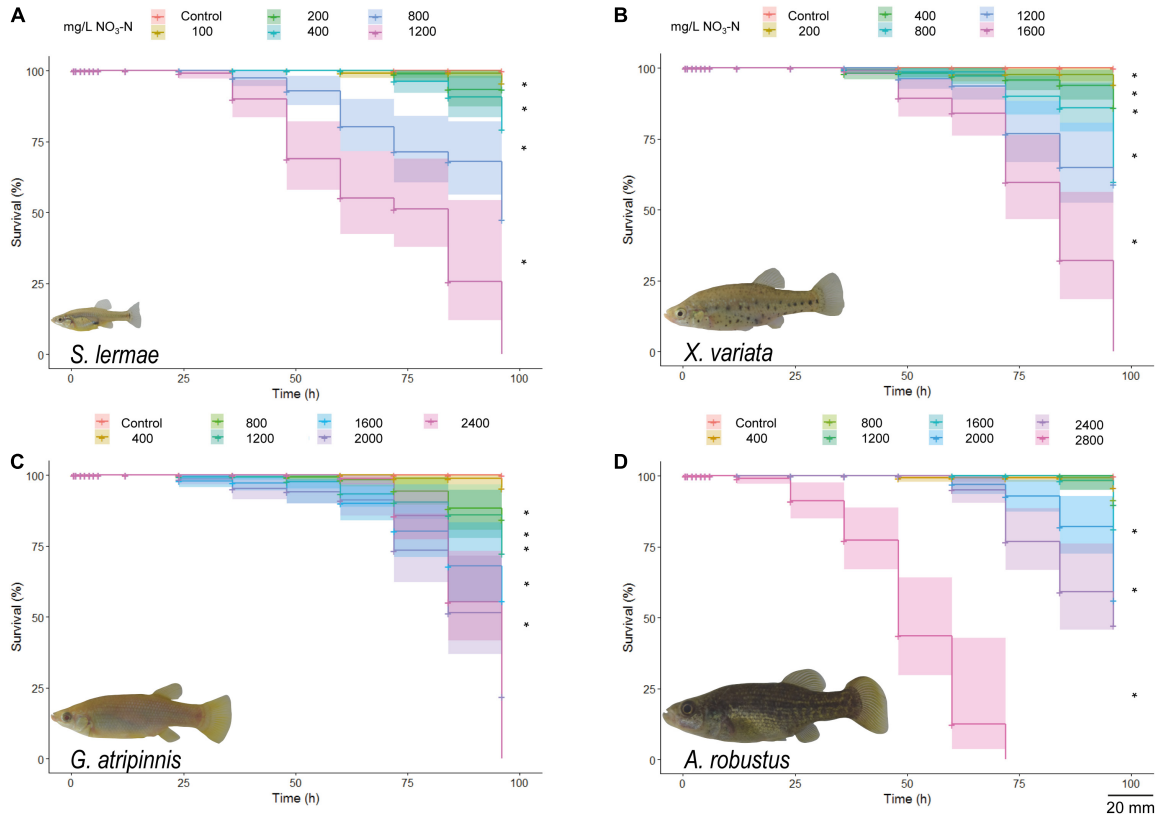

FIGURE 1
Kaplan-Meier survival curves for *S. lermae* **(A)**, *X. variata* **(B)**, *G. atripinnis* **(C)**, and *A. robustus* **(D)** exposed to $NaNO_3$-N. Log rank test indicated significant differences compared to the control groups (n = 30, *p < 0.001). Shading: 95% confidence intervals. Representative photograps of each species are shown (scale bar = 20mm).

behavior during acute exposure was permanence at the bottom without exploring the aquarium, but some fish also showed surface preference.

Loss of balance was related to exposure time and showed a similar trend, with *S. lermae* being more sensitive, followed by other species (Table 2 and Supplementary Figure 1). *Goodea atripinnis* did not show a loss of equilibrium at the lowest concentration.

Histological evaluation

Gill histopathological lesions integrated in the GHI showed a clear concentration-response pattern in all species (Table 3 and Figure 3). Circulatory index lesions were in a similar range; however, *G. atripinnis* was the only species that showed secondary lamellae aneurysms at the value closest to the LC_{50} (Figure 3). Lamellae aneurysms constitute the maximal circulatory damage associated with toxic exposure (w = 3 Supplementary Table 1). Secondary lamellae fusion was typical in *S. lermae* (Figure 3), where the progressive index reached the highest values (w = 3 Supplementary Table 1). Cellular degeneration was observed in the gills of all fish exposed to the lethal concentration. Thus, the regressive index showed the highest values at these concentrations for each species due to branchial degeneration resulting in death (w = 3 Supplementary Table 1).

In the liver, a concentration-response relationship for LHI also occurred (Table 3 and Figure 4). Vascular dilation in hepatic sinusoids occurred mainly in *S. lermae* (w = 1 Supplementary Table 1), while other species showed hyperemia as circulatory liver damage (Figure 4 and w = 3 Supplementary Table 1). Nuclear hypertrophy, as quantified in the progressive index (w = 1 Supplementary Table 1), was frequent at those concentrations close to the LC_{50} in all species. Hepatocyte cytoplasmic vacuolation and a relative reduction in nuclear frequency was observed in all species, especially in fish exposed to the lethal concentration, where the regressive index showed higher values than in other treatments (w = 3 Supplementary Table 1).

Quantitative hepatocyte density and nuclear area analysis were performed for the LTDI (values close to 0 indicate relevant tissue damage), which showed a concentration-response pattern

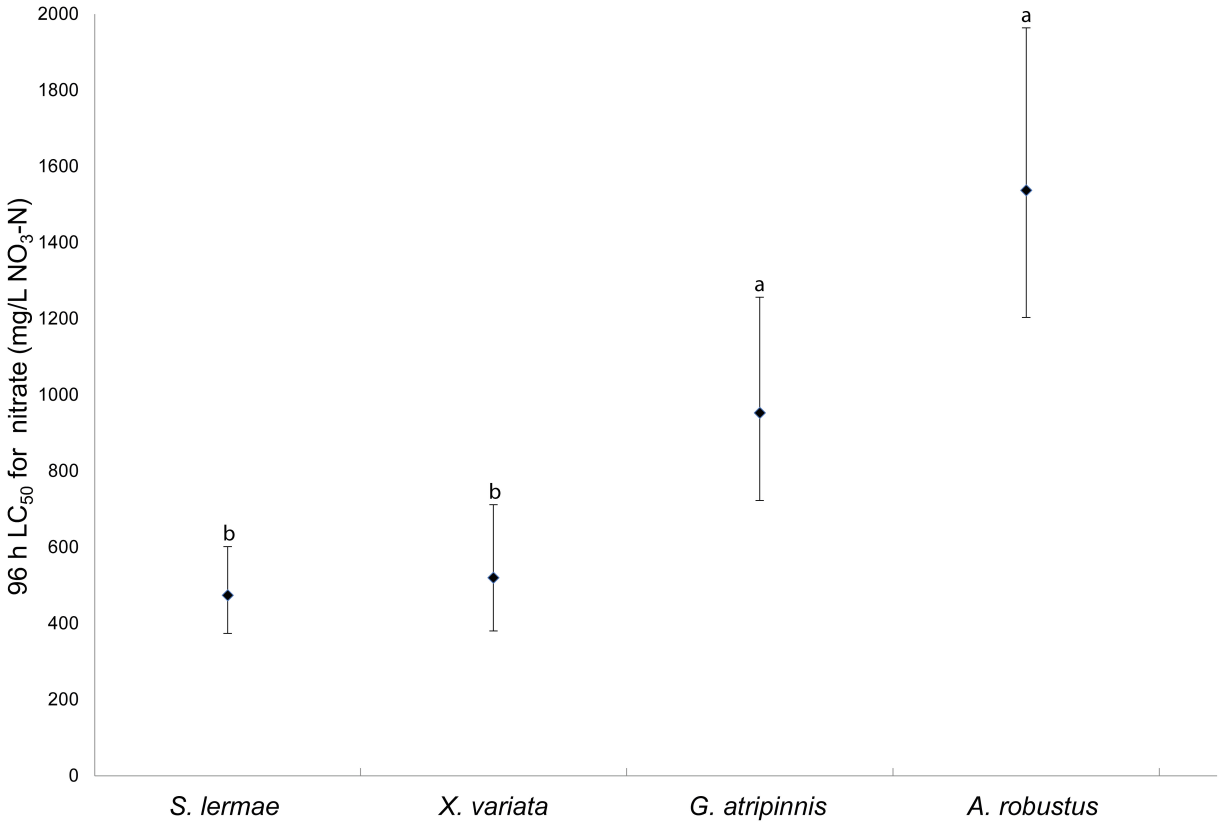

FIGURE 2
Lethal concentration 50% (LC_{50}) and 95% confidence intervals for four species of freshwater fish exposed to $NaNO_3$-N. LC_{50} were considered significantly different based on non-overlapping 95% confidence intervals. Different letters indicate significant differences (n = 30).

(Table 3). The lowest hepatocyte density was observed at the lethal concentration, while hepatocyte nuclear area was higher at concentration closest to the LC_{50} in all species. However, the nuclear area in *G. atripinnis* showed similar values to their respective control group (Table 3).

Integrated biomarker response

For comprehensive evaluation, all traits analyzed were incorporated into an IBR for each species (Figure 5 and Supplementary Table 2). IBR values for each species were well weighted; control fish showed lower values and the lethal concentration had the highest score in all cases. Intercomparisons at doses closest to the LC_{50} indicated that the most sensitive species was *S. lermae* (IBR = 2.208), with the highest value, followed by *X. variata* (IBR = 2.124) at 400 mg $NaNO_3$-N/L. *A. robustus* showed a higher IBR value than *G. atripinnis*; these were the most tolerant species to $NaNO_3$. However, they differed by twice the concentration, in *G. atripinnis* it was 800 mg $NaNO_3$-N/L (IBR = 0.977) and in *A. robustus* 1,600 mg $NaNO_3$-N/L (IBR = 1.560). An important factor for the IBR in tolerant species was the liver (LHI), contrary to the most sensitive species where the important factor was gill affectation (GHI); swimming behavior was a promising biomarker in all species.

Discussion

There was a clear concentration-response relationship, also dependent on time, in all exposed fish. This was evidenced by the Kaplan-Meier survival curves and their differential sensitivity. Low concentrations produced mortality in *S. lermae* and *X. variata,* while *G. atripinnis* and *A. robustus* were more tolerant to higher $NaNO_3$-N concentrations. The level of affectation, due to NO_3-N exposure, between goodeine species suggests differential tolerance. Reference parameters for $NaNO_3$-N acute toxicity in freshwater fish offspring are consistent with the results of *G. atripinnis* and *A. robustus* (Supplementary Table 3). However, the values for *S. lermae and X. variata* suggest they are the most sensitive species currently reported.

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TABLE 1 Aquarium distribution.

Species	Time (h)	Concentration (mg/L)								
S. lermae		0	100	200	400	800	1,200			
	3	–	–	–	–	/	+			
	4	–	–	–	–	/	+			
	5	–	–	–	–	/	+			
	6	–	–	–	–	/	+			
	12	–	–	–	–	/++	+			
	24	–	+	+	/++	/+++	+++			
	36	–	+	+	/++	+++	+++			
	48	–	/++	/++	+++	+++	+++			
	60	–	/++	/+++	+++	+++	+++			
	72	–	/+++	+++	+++	+++	+++			
	84	–	+++	+++	+++	+++	+++			
	96	–	+++	+++	+++	+++	x			
X. variata		0		200	400	800	1,200	1,600		
	12	–		–	–	–	–	+		
	24	–		–	+	/+	++	++		
	36	–		–	+	++	++	++		
	48	–		/	/++	++	++	+++		
	60	–		/++	/++	+++	+++	+++		
	72	–		/++	/++	+++	+++	+++		
	84	–		/+++	+++	+++	+++	x		
	96	–		/+++	+++	+++	+++	x		
G. atripinnis		0			400	800	1,200	1,600	2,000	2,400
	12	–			–	–	–	–	–	+
	24	–			–	/+	/+	/+++	/+++	+++
	36	–			–	/+	/++	/+++	/+++	+++
	48	–			–	/+	/+++	+++	+++	+++
	60	–			–	/+	/+++	+++	+++	+++
	72	–			–	/+	/+++	+++	+++	+++
	84	–			/	/++	/+++	+++	+++	+++
	96	–			/+	/++	+++	+++	+++	x
A. robustus		0			400	800	1,200	1,600	2,000	2,400	2,800
	5	–			–	–	–	–	–	–	/
	6	–			–	–	–	–	–	–	/+
	12	–			–	–	–	–	/	/	++
	24	–			–	–	–	/+	+++	+++	+++
	36	–			/	/	/+	/+	+++	+++	+++
	48	–			/	/++	/+++	/+++	+++	+++	+++
	60	–			/+	/++	/+++	/+++	+++	+++	+++
	72	–			/+++	/+++	+++	+++	+++	+++	x
	84	–			/+++	+++	+++	+++	+++	+++	
	96	–			/+++	+++	+++	+++	+++	+++	

Swimming behavior was recorded every hour during the six first hours; however, only the times when unusual behaviors were observed are shown, $n = 30$. Normal behavior in all fish (−); low affectation, unusual behavior was observed in 10–33% of the population (/top, + bottom); moderate affectation, 34–63% (//top, ++bottom); high affectation, 64–100% (///top, +++bottom); all individuals dead (x).

TABLE 2 Loss of equilibrium.

Species	Time (h)	Concentration (mg/L)								
S. lermae		0	100	200	400	800	1,200			
	24	–	–	–	–	++	++			
	36	–	–	–	+	++	+++			
	48	–	–	–	++	+++	+++			
	60	–	–	+	++	+++	+++			
	72	–	+	+	++	+++	+++			
	84	–	+	+	++	+++	+++			
	96	–	+	+	++	+++	x			
X. variata		0		200	400	800	1,200	1,600		
	24	–		–	–	+	++	++		
	36	–		–	–	++	++	+++		
	48	–			++	++	+++	+++		
	60	–		++	++	++	+++	+++		
	72	–		++	++	++	+++	+++		
	84	–		++	+++	+++	+++	+++		
	96	–		++	+++	+++	+++	x		
G. atripinnis		0			400	800	1,200	1,600	2,000	2,400
	24	–			–	++	+	++	++	++
	36	–			–	++	++	++	+++	+++
	48	–			–	++	++	+++	+++	+++
	60	–			–	++	+++	+++	+++	+++
	72	–			–	++	+++	+++	+++	+++
	84	–			–	++	+++	+++	+++	+++
	96	–			–	++	+++	+++	+++	x
A. robustus		0			400	800	1,200	1,600	2,000	2,400	2,800
	24	–			–	–	–	–	+	++	++
	36	–	–		–	–	–	–	++	++	++
	48	–			–	+	++	++	+++	+++	+++
	60	–			–	++	++	++	+++	+++	+++
	72	–			++	++	+++	+++	+++	+++	x
	84	–			++	+++	+++	+++	+++	+++	
	96	–			+++	+++	+++	+++	+++	+++	

Swimming behavior was recorded every hour during the six first hours; however, only the times when unusual behaviors were observed are shown, $n = 30$. Normal swimming in all fish (−); low affectation, loss of equilibrium was observed in 10–33% of the population (+); moderate affectation, 34–63% of fishes showed loss of equilibrium and some swam laterally (++); high affectation, 64–100% of fishes swam laterally (+++); all individuals dead (x).

Sensitivity, ontogenetic stage, and body size

Fish responses to NO_3-N depend on adsorption (accumulation time in plasma), distribution, metabolism, and excretion (Spurgeon et al., 2020). A relationship between sensitivity and ontogenetic stage has been found. Hamlin (2006) suggested that offspring were more tolerant to elevated NO_3-N than adults, contrary to other studies where earlier life stages were more sensitive (Adelman et al., 2009). Also, species with a bigger body size are less sensitive to NO_3-N (Camargo et al., 2005). In fact, a recent study in relatively larger species found that low NO_3-N concentrations did not alter growth and reproduction (Syed et al., 2022).

In this study, the four Goodeinae species had the same age during $NaNO_3$-N exposure but differed in body size and developmental stage. *Skiffia lermae* was the smallest species, followed by *X. variata*, while *G. atripinnis* and *A. robustus* were the largest species. Likewise, *S. lermae* have earlier maturation and begin their reproductive life approximately 3 months after birth (body length: 29.5 ± 5.7 mm in females and 25.2 ± 5.4 mm in males. Ramírez-García et al., 2021). In contrast, *G. atripinnis*

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TABLE 3 Gill (GHI) liver histopathological (LHI) and tissue damage indexes (LTDI) of fish exposed to $NaNO_3$-N (control, concentration closest to the LC_{50} and LC_{100}). $n = 3$.

Species	$NaNO_3$-N conc. (mg/L)	Circulatory index	Progressive index	Regressive index	GHI	Circulatory index	Progressive index	Regressive index	LHI	Nuclear area (μ m^2)	No. of cell nuclei/mm^2	LDTI
S. lermae	Control	2.400 ± 0.305	2.000 ± 0.721	0.000 ± 0.000	4.400	0.333 ± 0.066	0.000 ± 0.000	0.000 ± 0.000	0.333	13.454 ± 0.247	10319.783 ± 108.477	1.092^{a}
	400 mg/L	2.133 ± 0.290	13.266 ± 1.987	0.466 ± 0.133	15.866	3.133 ± 0.290	2.00 ± 0.230	2.866 ± 0.480	8.000	18.785 ± 0.295	6834.688 ± 63.488	0.864^{c}
	1,200 mg/L	1.200 ± 0.115	6.066 ± 1.576	11.200 ± 1.101	18.466	3.666 ± 0.133	0.333 ± 0.133	16.600 ± 0.901	20.600	8.919 ± 0.230	922.927 ± 22.645	0.272^{f}
X. variata	Control	3.333 ± 0.333	0.733 ± 0.176	0.000 ± 0.000	4.066	0.266 ± 0.066	0.000 ± 0.000	0.333 ± 0.066	0.600	13.179 ± 0.130	9975.610 ± 104.509	1.048^{ab}
	400 mg/L	4.200 ± 0.461	6.600 ± 0.808	1.066 ± 0.405	11.866	1.400 ± 0.115	0.733 ± 0.290	7.400 ± 0.611	9.533	16.724 ± 0.282	6325.203 ± 65.854	0.788^{d}
	1,600 mg/L	1.533 ± 0.240	4.133 ± 1.634	12.333 ± 0.940	18.000	2.333 ± 0.480	0.333 ± 0.066	14.266 ± 3.090	16.933	14.273 ± 0.308	2128.211 ± 123.568	0.316^{f}
G. atripinnis	Control	2.666 ± 0.437	1.200 ± 0.400	0.000 ± 0.000	3.866	0.466 ± 0.066	0.000 ± 0.000	0.000 ± 0.00	0.466	12.433 ± 0.170	11761.518 ± 97.938	1.067^{a}
	800 mg/L	5.333 ± 1.550	2.800 ± 0.808	1.133 ± 0.333	9.266	1.866 ± 0.240	2.266 ± 0.176	2.333 ± 0.176	6.466	13.398 ± 0.187	10883.469 ± 114.483	0.999^{b}
	2,400 mg/L	2.800 ± 0.721	10.133 ± 0.437	7.933 ± 1.87	20.866	2.600 ± 0.230	0.333 ± 0.133	8.866 ± 1.964	11.800	10.736 ± 0.275	3989.807 ± 290.373	0.458^{e}
A. robustus	Control	2.600 ± 0.305	0.533 ± 0.133	0.000 ± 0.000	3.133	0.266 ± 0.066	0.000 ± 0.000	0.400 ± 0.115	0.666	11.816 ± 0.159	8119.241 ± 117.643	1.064^{a}
	1,600 mg/L	2.533 ± 0.290	2.466 ± 0.896	1.533 ± 0.176	6.533	0.933 ± 0.133	1.466 ± 0.133	5.000 ± 1.514	7.400	16.470 ± 0.204	5349.593 ± 86.609	0.858^{c}
	2,800 mg/L	1.866 ± 0.240	3.933 ± 0.768	11.466 ± 0.835	17.266	1.466 ± 0.466	1.00 ± 0.230	9.000 ± 0.986	11.466	12.501 ± 0.205	3371.274 ± 36.225	0.501^{e}

Superscript letters indicate statistical differences (Kruskal-Wallis-test with Tukey's range *post-hoc* test, $p \leq 0.05$).

start its reproductive stage at 8 months (Silva-Santos et al., 2016), with a body length of 65.8 ± 17.6 mm in females and 65.6 ± 20.7 mm in males (Ramírez-García et al., 2021). Thus, the smallest and most precocious species were the most sensitive, while more tolerant responses were found in larger species.

Swimming behavior reflects differential sensitivity to nitrate-nitrogen

Swimming behavior alterations have been considered as an attempt to face unfavorable environmental conditions, from the regular swimming pattern that occurs in the middle of the water column (Gerhardt, 2007). The behavioral patterns observed in response to acute $NaNO_3$-N were: location on the surface or bottom of the water column, loss of balance and lethargy. *Skiffia lermae* showed swimming behavior alterations earlier and at lower $NaNO_3$-N concentrations, followed by *X. variata*, *A. robustus,* and *G. atripinnis*. Permanence in the surface of the water column is a compensatory response to inefficient oxygen uptake caused by damage to the gills or by conversion of oxygen-carrying pigments (i.e., hemoglobin) to forms that are incapable of carrying oxygen (i.e., methemoglobin Camargo and Alonso, 2006). Loss of balance and position at the bottom of the water column have been associated with a decreased capacity for respiratory gas exchange, as well as with the depletion of liver lipid reserves (Pereira et al., 2017). Other studies have reported a loss of balance and lethargy in fish exposed to NO_3-N (Shimura et al., 2004; Hamlin, 2006; Rodrigues et al., 2011; Pereira et al., 2017).

Histopathological alterations of gills and liver could reflect different sensitivity to nitrate-nitrogen in goodeine species

Remarkably, the LC_{50} value for *A. robustus* is similar to *C. clupeaformis* and *O. mykiss,* two euryhaline species having proficient ion exchange regulation to tolerate changes between freshwater and marine environments (Camargo et al., 2005; Takvam et al., 2021). This is interesting given that the gills are one of the main organs involved in ion regulation and ammonia excretion, where specific glycoproteins are involved (Hwang et al., 2011). At the histological level, *A. robustus* showed preserved gill structure after $NaNO_3$-N exposure. This suggests that gill function is maintained due to regressive, progressive and circulatory alterations restricted to the apical region. This was contrary to *S. lermae* which showed significant damage (highest GHI at minor concentrations), mainly lamellar fusion and cell hyperplasia; or *X. variata* and *G. atripinnis* which showed moderate gill

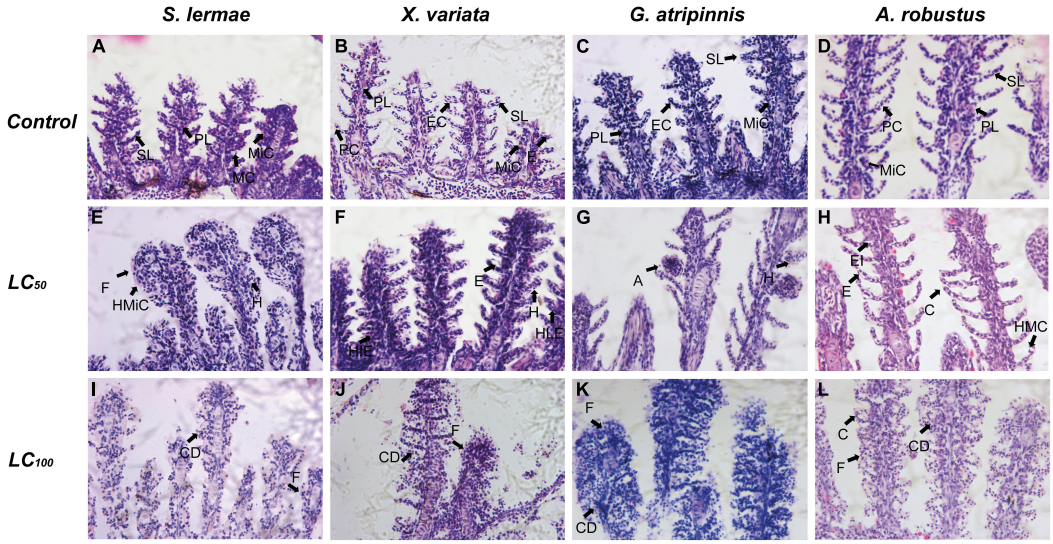

FIGURE 3
Effect of acute exposure to $NaNO_3$-N on gill histology of four goodeine species. Representative photomicrographs of gill sections stained with hematoxylin and eosin from control **(A–D)**, concentration closest to the LC_{50} **(E–H)** and LC_{100} **(I–L)** groups. A, Aneurysm of secondary lamellae; C, Change in curvature of secondary lamellae; CD, Cellular degeneration; E, Edema of secondary lamellae; EC, Epithelial cell; EI, Edema of interlamellar epithelium; F, Fusion of secondary lamellae; H, Hyperemia of secondary lamellae; HIE, Hyperplasia of interlamellar epithelium; HLE, Hyperplasia of lamellar epithelium; HMC, Mucus cell hyperplasia; HMiC, Mitochondria-rich cell hyperplasia; PL, Primary lamellae; SL, Secondary lamellae; MC, Mucus cell; MiC, Mitochondria-rich cell; PC, Pillar cell.

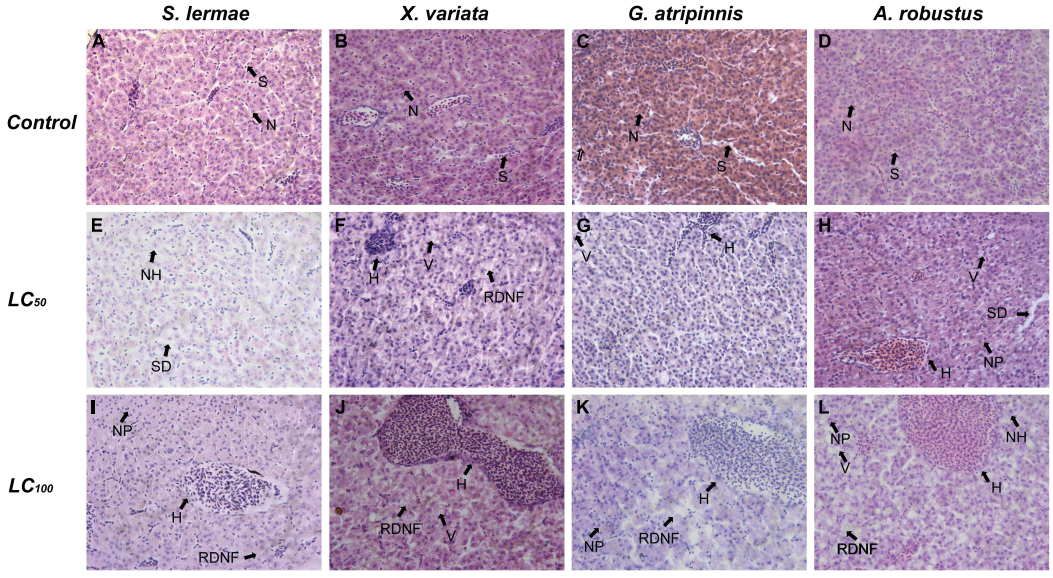

FIGURE 4
Effect of acute exposure to $NaNO_3$-N on liver histology of four goodeine species. Representative photomicrographs of liver sections stained with hematoxylin and eosin from control **(A–D)**, concentration closest to the LC_{50} **(E–H)** and LC_{100} **(I–L)** groups. H, Hyperemia; N, hepatocyte nucleus; NH, Nuclear hypertrophy; NP, nuclear pyknosis; RDNF, Relative decrease of nuclei frequency; S, sinusoid; SD, Blood sinusoid dilation; V, Vacuolation of the cytoplasm.

alterations, like edema and aneurysms. Gill histopathological alterations in response to water conditions and pollutants, including NO_2-N, have been described (Kroupova et al., 2005). The gills play a critical physiological role in nitrogenous waste detoxification; however, the response to NO_3-N has not been fully explained (Gomez-Isaza et al., 2020). The branchial epithelium responds to non-optimal environmental changes through circulatory and progressive alterations oriented

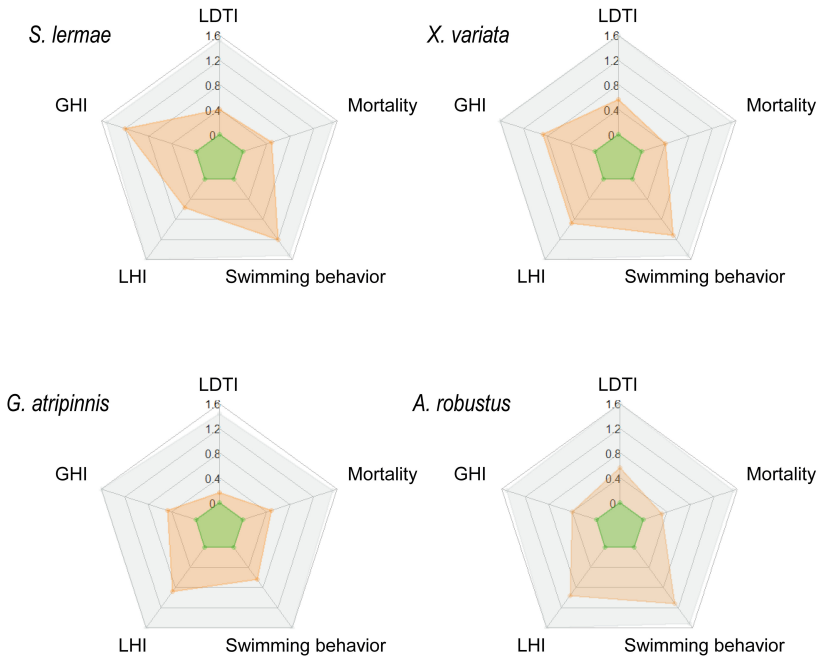

FIGURE 5
Integrated biomarker response (IBR) for four goodeine species exposed to $NaNO_3$-N. Radar graphs show the calculated IBR for *S. lermae*, *X. variata*, *G. atripinnis,* and *A. robustus* from the control condition (green), exposed to the concentration closest to the LC_{50} (orange) and LC_{100} (gray). The spokes of the radar indicate all traits evaluated: mortality and swimming behavior, as well as gill (GHI), liver histopathological (LHI), and tissue damage indexes (LTDI).

to preserve gill function. If ecological conditions remain unfavorable, degenerative mechanisms like lamellar fusion are triggered leading to loss of gill function (Antunes et al., 2017; Monsees et al., 2017). Thus, lamellar fusion observed in *S. lermae* fry could lead to ion-exchange failure and consequent fish anoxia. The different degrees of gill tissue damage observed in *X. variata*, *G. atripinnis,* and *A. robustus* suggest different mechanisms to face NO_3-N exposure.

Liver histopathology also confirmed differential tolerance due to variation in circulatory, progressive and regressive alterations. These constitute the LHI, which showed similar values between species. *Xenotoca variata* and *A. robustus* showed the highest regressive index associated with hepatocyte cytoplasmic vacuolation, pyknosis, and a relative decrease in nuclear density. Meanwhile, *S. lermae* and *G. atripinnis* showed circulatory and progressive alterations, respectively. The liver has an essential role in metabolic pathways and pollutant detoxification (Bruslé and Anadon, 1996). Exposure to environmental pollutants could result in erythrocyte accumulation in sinusoids (hyperemia), vascular dilation and hemorrhage, as well as hepatocyte hypertrophy. The degenerative indicators were hepatocyte cytoplasmic vacuolation, pyknosis, and decreased nuclear density. An increased size of hepatocyte nuclei (nuclear hypertrophy) reveals higher transcriptional and metabolic activity in response to pollutants. In contrast, a decreased cell density is related to liver degeneration (Strüssmann and Takashima, 1990). Here the hepatocyte nuclear area and number of cell nuclei were quantified and integrated into the LDTI. All species showed an increased cell nuclear area and a decreased number of cell nuclei. However, *X. variata* presented the highest liver damage, followed by *A. robustus* and *S. lermae*, whereas *G. atripinnis* showed less liver damage.

Combined histopathological evaluation suggested that gill and liver damage were associated with alteration of critical metabolic processes, such as inhibition of protein synthesis, depletion of glycogen reserves and reduced ability to detoxify environmental pollutants (Mohamed, 2009). In this regard, *S. lermae* showed the most severe gill damage and a relatively well-preserved liver structure. On the contrary, *X. variata* showed a high GHI and the highest LHI, whereas *G. atripinnis* and *A. robustus* showed lower GHI and LHI. Interestingly, *G. atripinnis* showed a higher GHI than *A. robustus* but a lower LHI. This supports the hypothesis that species that preserve gill structure and function, letting the liver carry out detoxification, likely have better survival opportunities. It has been demonstrated that lamellar fusion in response to acute NO_3-N exposure limits the absorption of toxic substances and inhibits ion exchange (Monsees et al., 2017).

The inclusion of all evaluated responses into IBR radar graphs allowed identifying the GHI, LHI, and swimming behavior as the main factors that contribute to explain the differential sensitivity of Goodeinae species to NO_3-N. Sensitive

species (*S. lermae* and *X. variata*) showed similar IBR values at the concentration closest to the LC_{50}, the first species showed the most severe gill damage. A certain tolerance level to NO_3-N exposure was found in *G. atripinnis and A. robustus* species which could be related to the activation of physiological detoxifying strategies for survival. In both tolerant species, gill damage was minor, suggesting that fish species showing gill NO_3-N tolerance have higher survival opportunities in polluted freshwater bodies.

The results show that NO_3-N are toxic since they produce behavioral, branchial, and hepatic alterations at a lower concentration in goodeines. Thus, it is recommended that their concentrations be monitored in freshwater bodies where goodeines inhabit. Their toxic effect may be increased by alterations in other water quality parameters like pH, hardness, and temperature. In this study, most water quality variables were close to Zacapu lake parameters; except salinity, TDS and conductivity, which are directly associated with the sodium ions in solution. Due to the large difference in progeny between species (*S. lermae* and *X. variata* females produce a mean of 12 and 38 hatchlings, respectively; whereas *G. atripinnis* and *A. robustus* females produce larger progeny of up to 150 offspring. Ramírez-García et al., 2021), this study evaluated offspring from a different number of gravid females. These differences in fecundity between goodeine species, in addition to differential sensitivity to $NaNO_3$-N, could increase the ecological risk for *S. lermae* and *X. variata* in wild freshwater ecosystems. However, population comparisons were outside the scope of this study.

Conclusion

Goodeinae species show a differential $NaNO_3$-N sensitivity. Since the most sensitive species showed higher gill damage, differential sensitivity could be related to different physiological mechanisms to face NO_3-N. Thus, species that preserve gill function and transfer the task of detoxification to the liver might have the best chance of surviving in polluted environments. Future studies should evaluate ecologically relevant NO_3-N concentrations to confidently predict goodeines persistence in polluted freshwater bodies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Mexican Ministry of Environmental and Natural Resources (SEMARNATSGPA/DGVS/00012/19) and the Secretariat of Agriculture and Rural Development (SAGARPA: PPF/DGOPA-014/20).

Author contributions

IV-V: investigation, methodology, formal analysis, and writing—original draft. BY-R: formal analysis and writing—review and editing. RR-J: conceptualization and methodology. MH-V and RH-M: methodology. OD-D: funding acquisition, conceptualization, and writing—review and editing. EM-H: funding acquisition, data curation, writing—original draft, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022.1014814/full#supplementary-material

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