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Whole-Fish Body Elemental Composition as Biomarker of Bacterial Infections in Wild *Gambusia holbrooki*

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Abstract

Infectious agents shape fish populations by inducing lethal and sublethal changes that alter nutrient metabolism and metal bioaccumulation. These shifts can manifest as changes in the ionome—the specific combination of essential and non-essential chemical elements defining the whole-body composition of an individual. Understanding how pathogens shape the fish ionome is critical for developing advanced monitoring tools and clarifying the ecological roles of hosts and their pathogens. This study reports the first documented outbreak of *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Pseudomonas mosselii*, and *Shewanella xiamenensis* bacterial infections in wild-caught eastern mosquitofish (*Gambusia holbrooki*) from three populations in Extremadura, southwestern Spain. Under laboratory-controlled conditions, we established associations between these bacterial outbreaks and the whole-fish body ionome of *G. holbrooki*. We compared 19 chemical elements and seven elemental ratios among diseased fish, healthy fish at the outbreak, and individuals fully recovered 100 days post-infection following antibiotic treatment. The fish ionome clearly discriminated between diseased and healthy states, and the response was consistent across all three populations. Our findings support the utility of whole-fish body elemental composition in *G. holbrooki* as a biomarker for environmental monitoring. Furthermore, as the bacterial infections were associated with the capture and transport-induced stress of wild individuals, this study provides critical data on the opportunistic pathogens that may be co-introduced into recipient ecosystems through the release of this widely distributed invasive fish species.

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Key Contribution: We found that *G. holbrooki* individuals naturally infected with *C. freundii*, *P. aeruginosa*, *P. mosselii*, or *S. xiamenensis* have a characteristic elemental signature, supporting the use of animal ionomes as biomarkers in ecological and ecotoxicological research.

1. Introduction

Infectious agents shape fish populations, inducing lethal and sublethal changes that alter nutrient metabolism and metal bioaccumulation [1,2]. These alterations can be quantified through biomarkers, which can be defined as any chemical and biological response capable of detecting sublethal effects of environmental stressors on individuals [1,3]. Determining how pathogens alter biomarker responses is critical for advancing disease ecology and refining the use of wild fish as bioindicators in environmental monitoring [2,3]. However, developing diagnostic tools requires establishing clear cause–effect relationships, which is often hindered in wild populations by the co-occurrence of environmental stressors and other confounding factors [3,4]. Consequently, studying wild fish populations under controlled laboratory conditions is essential for the identification of reliable biomarkers [4,5].

Many biomarkers, such as enzymatic and non-enzymatic antioxidants and blood cell profiles, are frequently used in fish studies [4,6,7]. However, a major caveat is their often-limited ecological relevance due to a lack of reference values for many wild species and the poor link between biomarker values and species' ecological performance in the ecosystem [4,8]. To address these limitations, the analysis of whole-body elemental composition—the ionome—is a promising tool [9,10]. The ionome reflects the systemic homeostasis of essential elements such as zinc (Zn), copper (Cu), iron (Fe), and the accumulation of non-essential toxic metal(oids)s such as mercury (Hg) and arsenic (As) [9,11]. Because all these elements are trophically transferred, infection-induced ionomic shifts can also provide critical insights into prey quality, nutrient cycling, and energy flow within aquatic ecosystems [9,10].

The usefulness of the ionome as a biomarker in disease ecology stems from the redistribution of minerals and trace elements between the host and pathogens [12,13], a phenomenon known as "nutritional immunity". For example, hosts can mobilize Zn for the synthesis of immune metalloenzymes, and bacterial pathogens often manipulate host mineral and trace element pools, such as sequestering Fe, to facilitate replication [13,14]. Infections also destroy tissues and alter fish osmoregulation [15], which may result in characteristic chemical signatures in hosts through altered ion uptake and excretion. However, our understanding of these shifts in chemical elements remains limited because studies typically measure a few elements in specific tissues [9,16]. Overlooking whole-body changes is a significant gap in fish ecology, as predators consume the entire organism [17], thereby ingesting the "total ionome" of the prey.

In this study, we address this question using the eastern mosquitofish (*Gambusia holbrooki* Girard, 1859) as a sentinel model, a widely distributed fish species [18]. This species is an ideal candidate due to its small size, extensive use in ecotoxicological and ecological research, and its role as a primary food source for piscivores [4,17,18]. Our study is also novel in that we report the first case of lethal bacterial infections in wild invasive populations of *G. holbrooki*, a notable occurrence given that invasive populations of this species are typically characterized by wide environmental tolerance and reduced

(nearly absent) parasite loads [19–22]. The aim of this study was to determine whether there are significant associations between the whole-body elemental composition and bacterial infections in *G. holbrooki* after fish transport under controlled laboratory conditions. Our hypothesis was that infection may significantly alter absolute chemical elemental concentrations and key physiological ratios (e.g., Fe:Zn) [12,14]. By comparing diseased and healthy fish at the outbreak with fish individuals that were fully recovered at 100 days post-infection following antibiotic treatment, we provide whole-body elemental biomarker data in *G. holbrooki* under variable health status conditions.

2. Materials and Methods

2.1. Host and Infection Origins

In September 2023, a total of 180 female eastern mosquitofish *G. holbrooki* (average length: 35.7 ± 3.1 mm) were caught using hand nets at three different locations in Extremadura, Spain (60 individuals per location): two agricultural ponds (Talayuela, 276,538.30 E–4,430,798.64 N; Valdeíñigos 256,252.68 E–4,427,890.19 N) and a polluted stream (Malpartida de Plasencia, 754,278.444 E–4,426,764.331 N). These sites are separated by a linear distance of 15–35 km and lack natural connectivity, which likely indicates isolated populations; we will therefore refer to them hereafter as Population 1 (Valdeíñigos), Population 2 (Malpartida de Plasencia), and Population 3 (Talayuela). The fish were transported by car to the aquatic facility of the University of Barcelona (UB) in plastic bags containing oversaturated oxygenated water and housed in isothermal EPS insulated boxes (Lab box®, Barcelona, Spain), following UB ethical standards for transport and fish keeping (License No. #11946). The transport took approximately 12 h for ~1000 km, with a mean velocity of 90–100 km/h. The vehicle was equipped with climate control (air conditioning) to maintain a stable internal temperature (25 °C) throughout the journey.

The three distinct fish populations were kept separate in three individually, fully equipped 500 L tanks, with 60 fish per tank. Fish were maintained in a mixture of 50% dechlorinated tap water and 50% distilled water so that the high conductivity of the tap water from Barcelona was reduced and the fish were kept in water with similar levels to the field (Tables S1 and S2). The aquatic facility was also at 25 ± 1 °C and 12 h light:12 h dark to mirror late summer temperature and daylight duration in their natural habitats (www.chtajo.es, accessed on 20 February 2026). Water quality was maintained through three biological filters and air-pumps (Eheim®, Deizisau, Germany), ensuring proper water oxygenation and safe water quality (Tables S1 and S2). Fish were fed daily with frozen chironomids Petra® (Prague, Czech Republic) and SERA VIPAN® (Heinsberg, Germany) fish flakes. To prevent cross-contamination, each tank utilized separate handling equipment.

During the second week of a planned 4-week acclimatization period for the original use of these fish (EU project INVASOMICS—101024805), an unplanned bacterial outbreak occurred, likely due to handling stress. Individuals from all three populations showed clinical signs of disease compatible with bacterial infection, including hemorrhagic ulcers, eroded fins, and petechiae [23]. Symptoms emerged simultaneously across all tanks by day 10 and the clinical signs of disease were visible in 5% of fish in Populations 1 and 2 and in 20% of the fish from Population 3. There was also an initial mortality of 2 fish in Population 3 on day 10, prompting the current study. Although the onset was concurrent, cumulative mortality at the end of the treatment varied by population, reaching 26.4% in Population 3, 6.4% in Population 2, and 8% in Population 1.

2.2. Experimental Design and Screening Procedure for Pathogens

All three fish populations were sampled at two distinct stages: at the peak of infection, including individuals with (D0) and without (H0) clinical signs of disease, and at 100 days

post-antibiotic treatment (H100). Tetracycline was added to the tanks (including D0 and H0 fishes) three days after the detection of infection at 100 mg/L for 10 days [23]. The fish were treated again on the third day after a 50% water change and the sequence was repeated until all fish were fully recovered. The tanks had diffuse light to reduce tetracycline photoinactivation, as recommended by Noga [23]. After the treatment was successful and no clinical signs of disease were detected, antibiotic residuals were removed by a 50% water exchange and the addition of activated charcoal to the filtration system [23]. To maintain a balanced experimental design and meet the sample size of the original project requirements, four size-matched females (average length: 32 ± 1 mm and wet weight: 0.4 ± 0.1 g) were sampled per tank for each group (D0, H0, and H100), resulting in 12 diseased fish (D0; 4 per stock tank), 12 healthy fish sampled during the outbreak (H0), and 12 healthy fish sampled 100 days post-treatment (H100). Healthy fish were identified based on their normal behavior and the lack of overt external signs of disease. The health status was confirmed during their dissection, as detailed below. We focused on females because *G. holbrooki* populations are strongly female-biased in summer [24].

Fish were euthanized with an overdose (250 mg/L) of buffered tricaine methane sulfonate (MS-222®, Sigma-Aldrich, Ferndale, WA, USA) [23,25]. All fish were processed for the analysis of bacteria, other parasites, and whole-fish body elemental composition. Systematic necropsy was conducted in sterile Petri dishes for bacteria and other parasites, as described below [20,26,27]. Firstly, external surfaces were inspected for parasites using a dissecting microscope with fiber-optic illumination (NexiusZoom 1903P, Euromex®, Duiven, The Netherlands). Plastic pipette tips were employed to manipulate the fish and thoroughly inspect all surfaces. Secondly, the body cavity was opened with a sterile scalpel, and a sterile swab was used to sample renal tissue, the liver, and the surface of all internal organs, except the gastrointestinal tract to prevent fecal contamination. To identify the bacterial pathogens, swab samples from all analyzed fish were sent in sterile 10 mL tubes with tryptic soya broth (TSB, Scharlab S.L., Barcelona, Spain) to the coauthor YS at the University of Santiago de Compostela, Galicia, Spain. Samples of broth cultures were seeded on plates of Brain Heart Infusion (BHI) agar and Trypticase Soy Agar (TSA) (Scharlab S.L., Barcelona, Spain) with 0.5% (*w*/*v*) NaCl (TSA-1 and BHI-1) and incubated for 24 to 72 h at 25 ± 1 °C. Bacterial cultures recovered from fish tissues were transferred onto TSA-1 and incubated at 25 ± 1 °C for 24 h for further characterization. Pure cultures of the isolates were stored in commercial Microbank storage medium (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at −30 °C until use. Initial characterization of the bacterial colony forming units was carried out using light microscopy for analyzing cellular morphology, motility, and Gram staining alongside the oxidase and Oxidative/Fermentative (O/F) test, following the guidelines of Austin and Austin [28]. Oxidase test sticks (Oxidase reagent, BioMerieux, Lyon, France) were used to determine oxidase activity.

Bacterial identification was confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [29]. The spectra were imported into BioTyper software (V. 3.1, Bruker Daltonics, Bremen, Germany) and analyzed by matching against standard patterns for bacterial identification, and the results were expressed using the criteria proposed by the manufacturer, e.g., [29–31]. The analysis had sterilized water as the negative control and *Aeromonas jandaei* CECT 4228 as the positive control. Finally, all internal organs (gut, liver, swim bladder, gonads, other organs, and tissues) were all individually screened for parasites. Muscle and skin were also crushed between two glass slides and examined by transparency.

2.3. Chemical Element Analysis

Whole-fish elemental composition was determined for all individuals subjected to necropsy (n = 36): 12 diseased fish (D0), 12 clinically healthy fish sampled during the outbreak (H0), and 12 healthy fish sampled 100 days post-treatment (H100). Individual fish were acid-digested HNO_3:H_2O_2 at 90 °C for 12 h using Instra-quality reagents (J. T. Baker®, Phillipsburg, NJ, USA). Elemental concentrations were determined using a Perkin-Elmer (Shelton, CT, USA) OPTIMA-3200RL Inductively Coupled Plasma Optical Spectrometer (ICP-OES) and a Perkin-Elmer ELAN-6000 Inductively Coupled Plasma Mass Spectrometer (ICP-MS), both at the University of Barcelona (UB) Technical Scientific Services, where analytical accuracy was ensured through calibration curves, blanks, and certified tissue standards (http://www.ccit.ub.edu/EN/tq01.html, accessed on 20 February 2026), e.g., [32,33].

We analyzed 13 essential elements involved in physiological processes and tissue structure: calcium (Ca), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), molybdenum (Mo), sodium (Na), phosphorus (P), sulfur (S), selenium (Se), and zinc (Zn). Additionally, we measured non-essential elements and potential pollutants, including arsenic (As), mercury (Hg), lithium (Li), and lead (Pb), as well as boron (B) [34,35]. Certain essential elements (e.g., Cu, Zn) were also monitored as potential environmental pollutants [34,35].

To further explore physiological shifts associated with health status, we also calculated the following chemical elemental ratios: Ca:P, Na:K, Zn:Cu, Se:Hg, Na:Mg, Ca:K, and Fe:Cu. While the fish-specific literature for some of these ratios is emerging, their clinical relevance in metabolic regulation, stress response, and toxicology is well-documented in other vertebrates [36–41]. For example, Fe:Cu and Cu:Zn (or Zn:Cu) ratios have been associated with infection susceptibility and immune alterations [37,38]. The Na:K ratio is regarded as a potential indicator of osmotic and immune-related responses [40]. The Se:Hg ratio is widely used to assess the antagonistic protective effect of selenium against mercury toxicity [42].

2.4. Statistical Analysis

All analyses were performed in the R software v. 4.5.1 [43] using the libraries *vegan* [44], *nlme* [45], and *car* [46]. To visualize primary patterns of variation in the 19 chemical elements and seven elemental ratios, a partial principal component analysis (pPCA) was conducted using the *rda* function. Tank identity was included as a "partial" effect to account for the nested structure of repeated samplings. The varimax rotation was applied to facilitate the interpretation of PC axes. Chemical elements or their ratios with loadings > |0.70| were identified as the primary drivers of the PCA ordination. To compare their values among fish groups (D0, H0, and H100), linear mixed models (LMMs) were implemented using the *lme* function. A separate model was constructed for the scores of each PC and the selected individual chemical elements or their ratios, with tank identity included as a random effect.

Statistical significance of fish group effects was determined using the R function *Anova*. Significant results were followed by pairwise post hoc comparisons at $p \leq 0.05$ using the R function *emmeans* [47]. Model assumptions were validated through visual inspection of residual diagnostic plots following the protocols of Zuur et al. [48].

3. Results

The preliminary characterization of the bacterial colony forming units isolated from fish revealed that all were Gram-negative rod-shaped bacteria. Isolates from Population 1 were oxidase-negative and fermentative, while bacterial isolates from Populations 2 and

3 included oxidative-positive bacteria with oxidative (Population 2) or fermentative and oxidative (Population 3) metabolism.

The analysis of the proteomic profiles obtained by MALDI-TOF MS identified bacterial pathogens characteristic of each fish population isolated in a separate tank. The outbreak in Population 1 was due to *Citrobacter freundii* (score value = 2.39 ± 0.026), in Population 2 it was due to *Pseudomonas aeruginosa* (score value = 2.26 ± 0.026) and *P. mosselii* (score value = 1.91 ± 0.023), and in Population 3 it was due to *Shewanella xiamenensis* (score value = 2.24 ± 0.131) and *P. aeruginosa* (score value = 2.10 ± 0.071). The strain used as a positive control for MALDI assays was correctly identified as *A. jandaei* CECT 4228 (score value = 1.90 ± 0.042). Since fish from each tank were processed in pools, it could not be determined if individual fish were co-infected. The bacteria-induced shifts in whole-fish elemental composition were not confounded by the presence of other parasites; only two colonies of the protozoan *Epistylis* sp. were observed on the caudal fins of a single *G. holbrooki* individual from Population 1.

Given that absolute concentrations of chemical elements or their ratios were all intercorrelated to some extent, PCA effectively summarized their variation into two principal components (Figure 1). PC1 explained 40% of the variance and represented the best indicator of infection status ($F_{2,33}$ = 128; $p < 0.001$) (Figure 1). Along this axis, diseased fish D0 were clearly distinct from both groups of healthy fish H0 and H100 ($p < 0.01$). PC1 loaded positively on Cu, Se, As, K, S, and the Na:Mg and Se:Hg ratios and negatively on Ca, Ca:P, Zn:Cu, and Ca:K (Table 1). PC2 explained 19% of the variance and was primarily defined by Mg, Fe, and P. While there were overall significant differences regarding fish infection status ($F_{2,33}$ = 9.85; $p < 0.001$), PC2 did not differentiate diseased fish D0 from the healthy fish group H100 (p = 0.92) (Figure 1).

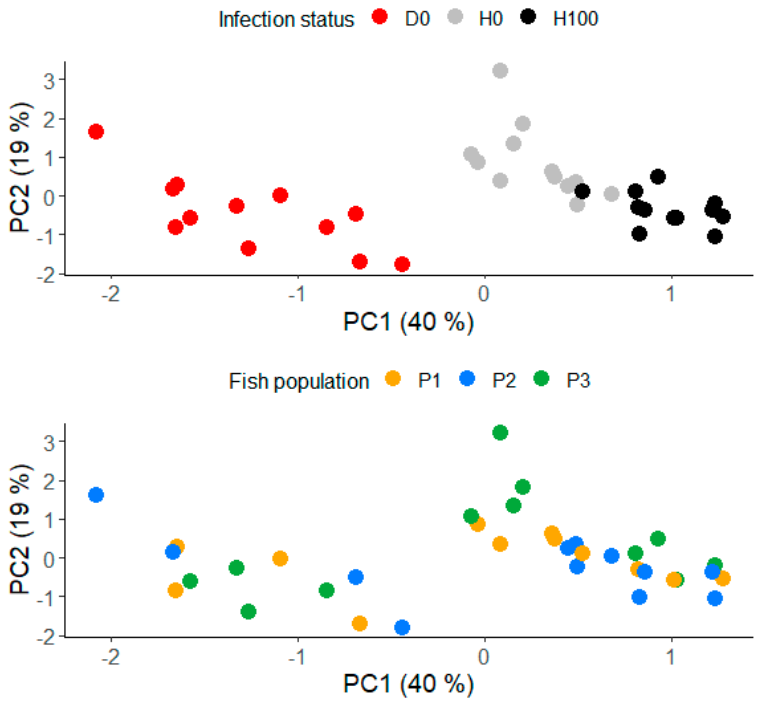

Figure 1. Principal component analysis ordination based on the whole-body chemical element concentrations in *Gambusia holbrooki*. The top plot shows differences in PC1 and PC2 scores among diseased (D0) and healthy (H0) fish during the bacterial outbreak, and healthy fish at 100 days post-outbreak (H100) to obtain baseline data. The bottom plot illustrates that the differences between healthy and diseased fish in PC1 and PC2 scores are not obscured by the effect of different fish populations (P1, P2, and P3). To assist in interpreting the PCA biplots, the strength and direction of associations of chemical elements with each PC axis are detailed in Table 1.

Table 1. Loadings of a principal component analysis (PCA) built based on the whole-body chemical element concentrations in *Gambusia holbrooki*. The elements or ratios that made the largest contribution (≥0.74) to the variation explained by PC1 and PC2 are highlighted in bold.

	PC1	PC2
Mn	−0.53	0.28
Cu	**0.75**	0.23
Se	**0.91**	
Cr	0.39	0.51
As	**0.75**	
B	−0.48	−0.19
Mo	0.48	
Li		
Co	0.66	
Pb	0.14	0.65
Hg	−0.22	0.59
Ca	**−0.74**	0.46
Mg		**0.91**
Na	0.63	0.61
Fe	−0.15	**0.81**
Zn	0.16	0.67
P	−0.43	**0.76**
K	**0.92**	0.13
S	**0.87**	0.35
Ca:P ratio	**−0.87**	
Na:K ratio	−0.61	0.41
Zn:Cu ratio	**−0.78**	
Se:Hg ratio	**0.85**	−0.18
Na:Mg ratio	**0.77**	0.34
Ca:K ratio	**−0.91**	
Fe:Cu ratio	−0.56	0.27

Separate linear mixed models confirmed the individual association of chemical elements with infection status, supporting all variables highlighted by the PCA except P (Table 2). At the time of the outbreak, healthy fish (H0) exhibited 1.2- to 2.3-fold higher concentrations of the elements Cu, Fe, Se, As, Mg, K, and S compared to diseased individuals (D0). These differences widened to 1.9–3.4-fold for all elements except Mg and Fe when compared to the healthy fish group at 100 days (H100) (Table 3). Conversely, diseased fish D0 were characterized by 1.3-fold higher levels of Ca than the healthy group at the time of infection outbreak (H0). Regarding elements with less influence on the PCA ordination, diseased fish D0 were significantly depleted in Na ($\chi^2 = 352$, $df = 2$, $p < 0.001$), Cr ($\chi^2 = 12.4$, $df = 2$, $p = 0.002$), and Zn ($\chi^2 = 10.5$, $df = 2$, $p = 0.005$), whereas they were enriched in B ($\chi^2 = 30.8$, $df = 2$, $p < 0.001$) (Table 3).

As with absolute elemental levels, the elemental ratios highlighted by the PCA also effectively discriminated between healthy and diseased fish (Table 2). Specifically, the ratios of Ca:P and Ca:K were significantly higher in diseased fish than in healthy fish at the time of infection, with values remaining stable in healthy fish until 100 days (H100) (Figure 2). In contrast, the Na:Mg ratio was markedly lower in the diseased group (Figure 2). Temporal shifts in healthy fish were most evident in the Se:Hg ratio, which increased steadily between H0 and H100 relative to the diseased group D0 (Figure 2). While the Zn:Cu ratio tended to be higher in diseased fish, it reached statistical significance only when compared against the healthy group at 100 days (H100) (Figure 2). Regarding the other two ratios less influential to PCA ordination, diseased and healthy fish significantly differed in both the Na:K ratio ($\chi^2 = 69.2$, $df = 2$, $p < 0.001$) and the Fe:Cu ratio ($\chi^2 = 72.6$, $df = 2$, $p < 0.001$). However, these

differences were not seen at the time of the outbreak (D0 vs. H0) but emerged at 100 days (D0 vs. H100) (all $p < 0.01$; Table S3).

Table 2. Results of linear mixed models comparing whole-body elemental concentrations and elemental ratios among fish groups D0, H0, and H100. The standard deviations for the random term population identity are also shown.

	χ^2	df	p Value	Random
Cu	57.5	2	<0.001	4.2×10^{-6}
Se	183.3	2	<0.001	0.03
As	149	2	<0.001	0.11
Ca	14.5	2	<0.001	3.4×10^{-6}
Mg	27	2	<0.001	1.2×10^{-6}
Fe	44.7	2	<0.001	0.009
P	3.3	2	0.19	7.3×10^{-7}
K	503.5	2	<0.001	1.5×10^{-6}
S	405.9	2	<0.001	2.5×10^{-6}
Ca:P ratio	76.2	2	<0.001	0.002
Zn:Cu ratio	40.4	2	<0.001	2.5×10^{-6}
Se:Hg ratio	91.8	2	<0.001	0.19
Na:Mg ratio	120.5	2	<0.001	0.04
Ca:K ratio	151.4	2	<0.001	3.8×10^{-6}

df, degrees of freedom.

Table 3. Whole-body chemical element concentrations and ratios (mean, standard deviation, minimum and maximum) in *Gambusia holbrooki* during a bacterial infection outbreak (diseased: D0, healthy: H0) and at 100 days post-antibiotic therapy and fish full recovery (H100). The table shows in the upper side the elements with |loadings| > 0.75 in the PCA and the rest of the elements in the bottom side.

	D0				H0				H100			
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**
As (µg/g)	0.43	0.33	0.11	0.96	0.72	0.13	0.60	0.98	1.22	0.22	0.91	1.51
Ca (mg/g)	41.45	12.41	22.45	64.98	31.65	7.09	21.39	47.54	28.07	6.48	20.54	36.97
Cu (µg/g)	4.64	1.96	2.02	8.15	8.03	2.84	4.41	14.96	11.30	1.90	9.11	15.91
Fe (mg/g)	0.09	0.03	0.04	0.15	0.15	0.05	0.08	0.25	0.06	0.03	0.00	0.11
K (mg/g)	2.99	0.41	2.40	3.74	6.96	1.06	5.30	9.11	8.25	0.54	7.67	9.59
Mg (mg/g)	1.37	0.22	0.98	1.74	1.64	0.10	1.53	1.93	1.40	0.06	1.31	1.55
P (mg/g)	23.49	5.70	14.48	34.29	23.94	3.12	20.07	30.66	21.19	2.35	18.16	25.21
S (mg/g)	5.25	0.86	3.67	6.42	11.06	0.63	10.00	12.03	10.44	0.66	9.52	11.56
Se (µg/g)	0.41	0.20	0.10	0.75	0.92	0.11	0.80	1.15	1.39	0.24	1.02	1.91
B (µg/g)	2.10	1.22	0.93	4.45	0.53	0.66	0.12	2.57	1.39	0.79	0.10	2.74
Co (µg/g)	0.03	0.02	0.00	0.06	0.04	0.02	0.01	0.07	0.08	0.04	0.04	0.16
Cr (µg/g)	0.06	0.06	0,00	0.16	0.16	0.13	0.04	0.49	0.15	0.05	0.05	0.21
Hg (µg/g)	0.20	0.07	0.12	0.33	0.21	0.09	0.14	0.47	0.18	0.04	0.14	0.25
Li (µg/g)	0.07	0.05	0.02	0.19	0.08	0.23	0.00	0.82	0.05	0.01	0.04	0.07
Mn (µg/g)	57.09	50.75	13.85	186.42	31.84	10.49	17.24	49.73	24.83	8.54	15.09	43.11
Mo (µg/g)	0.03	0.05	0.00	0.12	0.05	0.04	0.00	0.12	0.10	0.03	0.03	0.13
Na (mg/g)	2.88	0.47	2.13	3.96	6.21	0.92	4.47	7.44	4.82	0.46	4.25	5.77
Pb (µg/g)	0.04	0.03	0.00	0.11	0.07	0.08	0.00	0.25	0.07	0.05	0.02	0.20
Zn (mg/g)	0.15	0.06	0.08	0.29	0.20	0.02	0.16	0.25	0.18	0.02	0.16	0.22

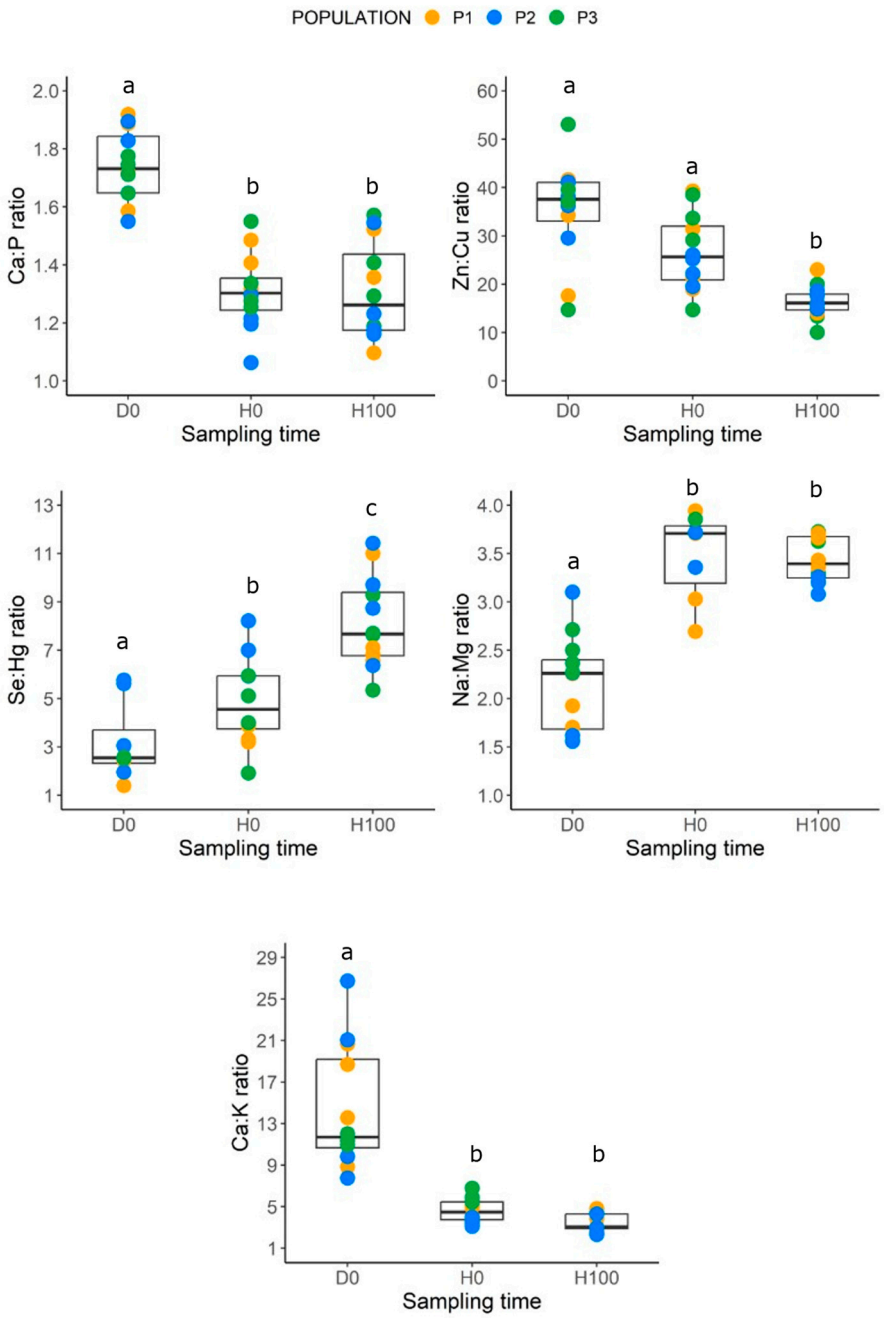

Figure 2. Boxplots of chemical elemental ratios (from Table 1) in diseased and healthy fish during the bacterial outbreak (D0 and H0, respectively) and in healthy fish at 100 days (H100) after full recovery to obtain baseline data in controlled laboratory conditions. The line inside the box indicates the median, the box itself shows the interquartile range IQR, and the whiskers the minimum and maximum data points. Dots represent individual observations, categorized by fish population (P1, P2, and P3). Different letters show significant differences among groups at $p \leq 0.05$.

4. Discussion

The present study reports for the first time an outbreak of *C. freundii*, *P. aeruginosa*, *P. mosselii*, and *S. xiamenensis* infections in wild-caught *G. holbrooki*. The clinical manifestations observed—including erythema, skin ulcers, and lethargy—align with the symptomatic profiles of these pathogens in other fish species [23,28,49]. Notably, the highest mortality was recorded in fish Population 3 (26.4%), where fish hosted potentially both *S. xiamenensis* and *P. aeruginosa*, suggesting that this specific pathogen combination or the presence of *S. xiamenensis* alone may pose a greater threat to *G. holbrooki*, as reported for

other fish species [50]. Despite this, the success of the tetracycline treatment is consistent with the known susceptibility of these bacteria to this broad-spectrum antibiotic, which inhibits protein synthesis [23,28]. In natural settings, moribund fish are typically removed rapidly by water currents or through predation; however, our laboratory observations provided a unique opportunity to quantify associations between bacterial infections and the whole-fish body elemental composition or "ionome" *sensu* Filipiak & Filipiak [9]. Capture and transport-induced stress probably catalyzed the bacterial infection outbreak in *G. holbrooki*, as overcrowding and the accumulation of metabolic waste in transport bags are well-established triggers for opportunistic pathogen replication in fish [51–53].

The variation in the elemental composition of the whole fish body was strongly associated with the presence of clinical signs at the time of infection (groups D0 and H0) and the subsequent 100-day recovery following antibiotic treatment. It is essential to highlight that the H100 fish group represents a physiological state influenced not only by the absence of infection but also by fish in captivity and the potential legacy effects of antibiotic treatment. Specifically, tetracycline, while effective in halting the outbreak, can affect the fish gut microbiota [54], with potential ramifications for nutrient assimilation and the overall fish physiology [55,56]. Interestingly, a low overall random effect variance for the term fish tank in the models (mean $\pm$ SD = 0.017 $\pm$ 0.036 in elements and 0.046 $\pm$ 0.082 in ratios) suggests a consistent pattern of elemental shifts across the three *G. holbrooki* populations. It is important to note that we confirmed that elemental shifts were not masked by other parasites; this is an aspect that is often overlooked in ecotoxicological studies, even though it is known to skew physiological data [1,2]. The negligible presence of *Epistylis* spp. on one fish fin was likely harmless, as these commensal ciliates only compromise fish health at much higher densities [23]. The elemental concentrations recorded here were consistent with previous data on metal bioaccumulation in *G. holbrooki*, even when studies focused on specific organs such as the liver [34,35]. In this regard, our whole-fish body measurements offer greater ecological relevance, as typical predators of *G. holbrooki*, including the Eurasian kingfisher *Alcedo atthis*, the squacco heron *Ardeola ralloides*, and the Eurasian bitten *Botaurus stellaris*, among many others, ingest the entire fish [17,57]. All elements regulated by EU legislation for fish consumption remained below safe limits (e.g., Pb = 0.3 mg/kg; Hg = 0.5 mg/kg; As = 0.1 mg/kg) [58,59].

The significant depletion of Cu, Se, Mg, and K in diseased fish likely reflects a disruption of physiological homeostasis, as the individuals sampled during the infection outbreak were moribund. The markedly higher levels of Cu and Se in healthy fish support their roles as essential cofactors for antioxidant enzymes, such as superoxide dismutase (Cu/Zn-SOD) and glutathione peroxidase (Se/GPx) [60]. Reactive oxygen species are typically produced during inflammatory and infection processes [60]; when the host's capacity to mitigate this oxidative stress is overwhelmed, widespread tissue damage and the loss of cellular homeostasis can occur [4]. Damage to internal tissues has also been associated with a significant reduction in Mg and K levels in diseased individuals due to passive efflux or alterations in Mg^{2+}-dependent and Na/K-dependent ATPases [39–41]. Furthermore, this tissue damage may be linked to the lower levels of arsenic (As), a non-essential metalloid, observed in diseased fish; this might suggest a functional failure of the tissues involved in its bioaccumulation, such as the gills and kidneys [35].

We acknowledge that, while it is tempting to view all these elemental shifts as a direct result of infection, the present study design can only support strong associations but not direct cause–effect relationships. While infection can impair mineral uptake through reduced feeding and compromised gastrointestinal function in many animals, including fish [4,61], it is also plausible that the fish had lower baseline levels of essential elements prior to the outbreak due to differences in microhabitat quality, which in turn may have

increased their susceptibility to opportunistic pathogens [11–14]. As this outbreak was unexpected, no baseline profiles were established upon arrival at the laboratory; however, given that the outbreak occurred immediately after arrival, it is possible that such profiles would also have reflected a poorer physical condition of the fish in the field or differences between individuals within the population. There may be differences in tolerance to changes in environmental conditions among individuals of the same population due to behavioral hierarchies, given that fish were in the breeding season [62], all of which can influence food access and body condition [61] and hence potentially the fish ionome. In this context, the depletion of Cu, Se, K, and S in diseased fish might indicate a pre-existing deficit in sulfur-containing amino acids and impaired protein metabolism [16,36]. Physiological impairments are expected in the field, as the sampled individuals were collected from sites surrounded by an extensive agricultural area, where the authors (OCR and AMV) saw the repeated use of pesticides and other chemicals. Nevertheless, the consistent changes found in elemental composition associated with infection across all populations suggest that differences in diet or in environmental conditions while the fish were in the field may have played a secondary role in the patterns we observed in captivity.

Beyond individual elements, our study supports the utility of elemental ratios as biomarkers of health status in *G. holbrooki*. The significantly higher Ca:P and Ca:K ratios in diseased fish were mainly associated with a peak in Ca concentration, as P levels remained stable across groups. Given the high mineral content of the water in the aquatic facility (Ca = 122 $\pm$ 1 mg/L), this increase was probably associated with an alteration in gill ion exchange caused by infection, as the gills are the main site of calcium entry and exit [41]. In part, the influx and efflux of Ca and other elements, such as B, were probably related to the water stock tanks having greater values of Ca and B than very soft waters at the fish collection sites in Extremadura (~10-fold). The observed Zn:Cu trends are concordant with the copper depletion often seen during active infections in mammals [12,38], suggesting a potential conserved physiological pattern. Conversely, the PCA outputs suggest that the Na:Mg and Ca:K ratios provided limited additional insight compared to individual elemental levels in this specific context, although these ratios have been associated with hormonal disorders in mammals [63]. Finally, the steady increase in the Se:Hg ratio over time in healthy fish (H0 and H100) may reflect the cumulative benefits of a balanced laboratory diet. While Se levels are known to provide protective antagonism against Hg toxicity [42], the very low Hg concentrations detected suggest that this shift was primarily associated with improved Se assimilation rather than a response to toxic stress in the studied *G. holbrooki* individuals.

5. Conclusions

Our study shows that *G. holbrooki* individuals naturally infected with *C. freundii*, *P. aeruginosa*, *P. mosselii*, or *S. xiamenensis* exhibit a characteristic elemental signature, supporting the use of animal ionomes as biomarkers in ecological and ecotoxicological research [9–11]. While the elemental composition observed after 100 days in the aquatic facility provides a tentative baseline for the 19 chemical elements and seven elemental ratios analyzed, we caution that these values may be influenced by the antibiotic treatment and the environmental conditions the fish experienced during acclimatization from the field to the laboratory, including changes in water chemistry and diet. Beyond physiological relevance, our findings underscore potentially biosecurity risks associated with the transport and release of *G. holbrooki*, a highly successful invasive species globally [18]. Future research should determine whether these ionomic signatures are pathogen-specific or represent a generalized stress response of *G. holbrooki* to infection and shifts in environ-

mental conditions, which would further refine the use of elemental profiling as a biomarker in environmental monitoring.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fishes11050257/s1, Table S1. Physicochemical characteristics of the water at the three fish collection points. Data represent the range of minimum–maximum values for all variables. The "Historic NO_3^- + NO_2^- (mg N/L)" column (green) is derived from the RECAREX and Confederación Hidrográfica del Tajo (www.chtajo.es) monitoring programs. For the Talayuela site, where historical records were unavailable, the maximum NO_3^- + NO_2^- values from the current study's four samplings were utilized as a reference. Table S2. Essential and non-essential elements measured in water from the three fish collection sites and from the three stocking tanks. Data represent the range of minimum–maximum values for all variables. Elements are classified based on the periodic table: non-metals in green, metalloids in yellow, alkali and alkaline earth metals in light gray, and metals in dark gray. Table S3. Whole-body chemical element ratios (mean, standard deviation, minimum, and maximum) in *Gambusia holbrooki* during a bacterial infection outbreak (diseased: D0, healthy: H0) and at 100 days after antibiotic treatment and fish full recovery (H100). The table reports the exploratory statistics of the five significant element ratios in Figure 2 and the remaining two ratios for completeness, despite the fact that these were not highlighted in the PCA.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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