

## HELMINTHS PARASITES OF HARLEQUIN FROG (*Atelopus cruciger*) FROM VENEZUELA HELMINTOS PARÁSITOS DEL SAPITO RAYADO (*Atelopus cruciger*) DE VENEZUELA

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### ABSTRACT

The harlequin toad (*Atelopus cruciger*), endemic to Venezuela's Cordillera de la Costa, inhabits elevations from 30 to 2,000 m and persists in only two known populations. Despite the ecological importance of amphibians, helminth data for this species are scarce. This study examined 97 museum specimens collected across seven Venezuelan states between 1950 and 2009. A total of 422 helminths were identified, dominated by direct-life-cycle nematodes (*Aplectana*, *Cosmocerca*, *Oswaldocruzia*, *Rhabdias*) and the cestode *Cylindrotaenia americana*. Parasitization affected 80.41% of hosts, with nematodes comprising over 73% of the parasite burden. Aggregation coefficients and dispersion indices confirmed an aggregated distribution pattern. Prevalence and intensity varied across sex, size, and elevation, revealing ecological constraints shaping community structure. These findings expand the helminthological records for *A. cruciger* and highlight the need to integrate parasite ecology into conservation assessments of threatened amphibians.

### RESUMEN

El sapito arlequín (*Atelopus cruciger*), endémico de la cordillera de la Costa venezolana, habita entre los 30 y 2000 m de altitud y sobrevive en solo dos poblaciones conocidas. A pesar de la importancia ecológica de los anuros, la información helmintológica sobre esta especie es limitada. Este estudio examinó 97 especímenes de colección recolectados en siete estados venezolanos entre 1950 y 2009. Se identificaron 422 helmintos, dominados por nematodos de ciclo de vida directo (*Aplectana*, *Cosmocerca*, *Oswaldocruzia*, *Rhabdias*) y el cestodo *Cylindrotaenia americana*. El 80,41 % de los individuos mostró parasitación, con nematodos que representan más del 73 % de la carga parasitaria. Los coeficientes de agregación e índices de dispersión confirmaron un patrón de distribución agregado. La prevalencia e intensidad variaron según sexo, talla y altitud, revelando restricciones ecológicas que modelan la estructura de la comunidad. Estos hallazgos amplían el registro helmintológico de *A. cruciger* y destacan la necesidad de integrar la parasitología en la conservación de anfibios amenazados.

**Keywords:** *Atelopus*, ecology, helminths, prevalence, Venezuela.

**Palabras clave:** *Atelopus*, ecología, helmintos, prevalencia, Venezuela.

### 1. Introduction

Amphibians represent valuable biological models for investigating host–parasite dynamics due to their diverse life histories, permeable skin, biphasic life cycles, and their ecological linkage across aquatic and terrestrial habitats [1]. These attributes not only make them susceptible to a wide range of parasites but also position them as ecological indicators of environmental health. Among vertebrates, amphibians host an estimated 5.49% (n = 4,225) of known parasitic species, underscoring their significant role in parasite ecology and evolutionary interactions [2].

Parasitic infections in amphibians, particularly by helminths, often persist without overt symptoms under optimal physiological conditions. However, the presence of co-stress-

ors such as malnutrition, extreme environmental conditions, physical trauma, or senescence can undermine host immunity, increasing susceptibility to infection and pathology [3]. Particularly, certain trematodes such as *Ribeiroia ondatrae* (Looss, 1907) and *Echinostoma trivolvis* (Cort, 1914) Kanev, 1985; have garnered attention for their ability to induce severe developmental anomalies—including limb malformations and polymelia—in amphibian larvae, particularly during critical stages of growth [4]. These cases exemplify the broader ecological and evolutionary consequences that parasitic infections can impose on wild amphibian populations.

The structure of parasite communities in amphibians is shaped by a combination of host-intrinsic traits and extrinsic environmental factors. Host characteristics such as body size,

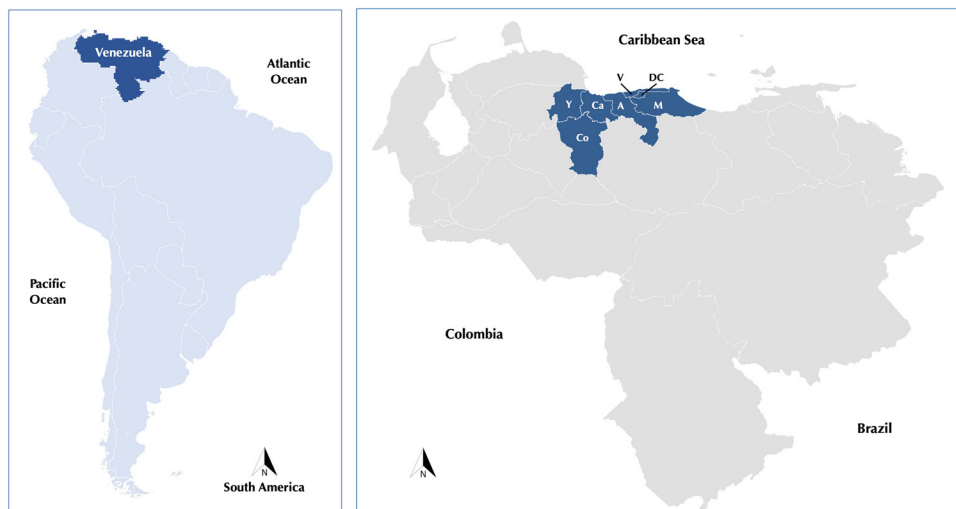
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sex, diet, behavioral ecology, and reproductive strategy all influence parasite richness, abundance, and composition [5-11]. For example, sexual dimorphism is common in anurans, with females typically exhibiting larger body sizes than males. This size disparity may provide a larger habitat or greater nutritional resources for parasites, potentially increasing parasite burden in females. However, empirical findings on this relationship remain mixed, suggesting the influence of additional ecological variables, such as parasite specificity and host immune defenses. Even under identical exposure conditions, differences in host resistance or parasite affinity may yield significantly divergent infection outcomes among individuals [12-16].

Habitat specificity and environmental context are equally critical in modulating parasite colonization success. In particular, the spatial and ecological overlap between host and parasite determines the likelihood of transmission. Elevational and climatic gradients further influence these dynamics. In temperate regions, seasonal temperature fluctuations constrain helminth transmission and development due to the ectothermic nature of amphibians, which limits their feeding activity and immune responses during colder months [1, 17, 18]. In contrast, tropical environments offer relatively stable thermal conditions that permit continuous host-parasite interactions and year-round parasite reproduction. Nevertheless, the extent to which tropical stability influences parasite community structure remains underexplored, necessitating studies that explicitly consider altitudinal variation as a proxy for ecological diversity.

Within Latin America, amphibian parasitology has expanded over the past two decades, revealing notable patterns in helminth diversity, host specificity, and geographic distribution [19-24]. Despite this progress, Venezuelan studies have been largely descriptive, with most data derived from isolated reports [25-29] or limited to two studies focused on helminth community structure in the Coastal Mountain Range [30] and the Eastern Plains [31]. These efforts provide a foundational baseline, yet they underscore the lack of systematic research on threatened and endemic species.

A case in point is the Harlequin Toad, *Atelopus cruciger* (Lichtenstein & Martens, 1856), a diurnal, primarily terres-



**Figure 1.** Relative position of Venezuela and the geographical distribution of *Atelopus cruciger*. The shaded area highlights the federal entities (states) where the species has historically been recorded, listed from west to east: Y, Yaracuy; Co, Cojedes; Ca, Carabobo; A, Aragua; V, Vargas; DC, Capital District; M, Miranda [33].

trial anuran endemic to Venezuela [32]. This species inhabits montane cloud forests, particularly rocky stream banks, from 30 to 2,000 m elevation along the Venezuelan Coastal Range [32, 33] (Figure 1). It is characterized by distinctive yellow coloration with black or brown dorsal markings—often forming an “X” on the head—smooth flanks, and long hind limbs. While males measure 28.2–34.6 mm in snout-vent length (SVL), females are considerably larger, ranging from 39.5–49.9 mm [34, 35]. Non-aggressive and territorial, *A. cruciger* occasionally perches up to 1.5 meters above ground level in nearby vegetation [32]. In recent decades, however, this species has experienced dramatic population declines attributed mainly to the emergence of chytridiomycosis, with habitat degradation playing a lesser role [36, 37].

Currently classified as Critically Endangered by the IUCN, *A. cruciger* has persisted in isolated populations within Henri Pittier National Park, prompting renewed interest in its conservation and biology [32, 38, 39]. Recent studies have focused on threats such as chytridiomycosis caused by *Batrachochytrium dendrobatidis* [40-44], but the parasitological component of its ecology remains virtually unknown. Given the susceptibility of amphibians to both environmental and parasitic stressors, understanding the parasite communities of endangered species like *A. cruciger* is critical for both conservation management and broader ecological insights.

This study seeks to address these knowledge gaps by: (a) characterizing the composition and structure of the helminth community in *A. cruciger*, (b) evaluating the relationships between parasitic burden, host body size, and sex, and (c) analyzing the distribution of helminth populations across its geographical distribution and altitudinal range.

## 2. Material and Methods

### 2.1 Specimen Collection

A total of 97 adult specimens of *Atelopus cruciger* were examined. These specimens were collected between 1950 and 2009 across seven states of Venezuela: Yaracuy, Cojedes, Carabobo, Aragua, Vargas, the Capital District, and Miranda. All specimens are deposited in the herpetological collections of the Rancho Grande Biological Station (EBRG), the La Salle Natural History Museum (MHNLS), and the Central University of Venezuela Museum of Biology (MBUCV). All examined material with information on the respective collection localities is listed in **Appendix I**.

#### 2.1.1 Sex determination

Sex determination was initially performed by examining external secondary sexual characteristics, such as the presence or not of vocal sacs, nuptial pads on the thumbs and the relative thickening of the forearms in males [34, 45] and confirmed by direct observation of the type and degree of development of the gonads.

#### 2.1.2 Morphometric Analysis

**Snout-vent length (SVL)**—defined as the linear distance from the tip of the snout to the posterior margin of the cloacal opening—was measured using stainless steel Vernier calipers with a precision of  $\pm 0.1$  mm. Each specimen was measured two to three times independently to assess measurement precision and reproducibility. The average value was used for statistical analyses.

### 2.2 Parasitological Examination

A full parasitological necropsy was conducted following the standardized protocol described by Díaz-Ungría [46]. This method involves dissection of the coelomic cavity and systematic inspection of the respiratory, digestive, excretory, and reproductive systems to recover endoparasitic helminths. Detailed dissection procedures are provided in **Appendix II**. All helminths were preserved in AFA (alcohol-formaldehyde-acetic acid) solution, cleared in lactophenol, and mounted in glycerin jelly for identification.

#### 2.2.1 Helminth Identification

Helminths were morphologically identified using standard taxonomic keys and descriptions [28, 47-54], complemented by comparative analysis of traits observed under light microscopy. Diagnostic criteria included external morphology (e.g., body shape, cuticular ornamentation), internal anatomy (e.g., esophageal length, genital structures), and sexual dimorphism, as detailed in **Appendix III**. When necessary, distinctions between closely related taxa were made based on

combinations of secondary characters such as vulvar position, papillae number, and shape of the caudal extremity.

Schematic drawings of selected helminth species were redrawn and adapted from published taxonomic keys [28, 47-54] to highlight diagnostic morphological features relevant to species identification. These illustrations were produced strictly for educational and scientific purposes, with no commercial intent, and fall under the principles of fair use.

Identification was restricted to genus or species level depending on specimen condition and diagnostic resolution. All nomenclature was validated against the Integrated Taxonomic Information System (ITIS) and the Global Biodiversity Information Facility (GBIF) taxonomic databases.

Helminth infections were categorized according to necropsy findings and taxonomic identification into distinct groups based on the type of parasite recovered: **nematode infections**, **cestode infections**, **trematode infections**, and/or **mixed infections**, the latter defined as the co-occurrence of helminths from different taxonomic groups within a single host.

### 2.3 Geographic and Altitudinal Variation in Parasite Composition

The **geographical variation** in helminth parasite composition was analyzed based on state-level identifiers, which correspond to specific collection sites detailed in **Appendix I**. Parasite composition was quantified using both absolute counts and proportional representations relative to the total number of parasites per collection site. It is important to note that, although political boundaries were used for classification, they may not accurately reflect underlying ecological or biogeographic zones, potentially influencing patterns of parasite distribution and abundance. Parasite distribution was evaluated across **altitudinal ranges** derived from host collection site elevations. Because exact altitudes were not available for all specimens, collection sites were grouped into three altitudinal intervals (AI) based on midpoints, covering the full elevation range sampled (250–2100 masl): **AI1: 250–750 masl**, **AI2: 750–1250 masl**, and **AI3: 1250–1750 masl**. These intervals were chosen to ensure adequate sample sizes per group while capturing ecologically meaningful gradients from lowland to montane environments.

### 2.4 Data Analysis

#### 2.4.1 Sex Determination and Morphometric Analysis

Sex assessments were confirmed through dissection and examination of the gonads: testes in males and vitellogenic oocytes in females. Differences in SVL between sexes were assessed using both parametric and non-parametric tests. Normality was evaluated using the Shapiro-Wilk test, and

homogeneity of variances with Levene's test. Depending on these assumptions, comparisons were made using Welch's *t*-test for unequal variances, and the Mann–Whitney *U* test was used as a non-parametric alternative [55].

#### 2.4.2 Qualitative analysis

The qualitative analysis involved synthesizing the taxonomic composition, ecological characteristics, and novelty of the helminth community in *A. cruciger*. This included assessing the diversity of helminth taxa, their life cycle types (direct vs. indirect), infection sites within the host, and the significance of new records for the species and Venezuela. Results of the qualitative synthesis are presented in a summary table listing identified helminth species and their taxonomic classification.

#### 2.4.3 Quantitative analysis

Quantitative parasitological descriptors encompassed both **parasite population parameters** and **community attributes**. Analyses were performed independently for male and female hosts, as well as for the combined population, whenever sample size and data distribution permitted. A significance level of  $\alpha = 0.05$  was used throughout. Population parameters included **helminth infection composition (HIC)**, **parasite burden (PB)**, **abundance (A)**, **relative abundance (RA)**, **prevalence (P)**, **mean intensity (MI)**, **mean abundance (MA)** [56], **the infection index (ii)** [57], **the Morisita dispersion index (Imor)** and **the aggregation coefficient (k)** [58, 59]. Detailed descriptions of the calculation methods and their interpretation are provided in **Appendix II**.

To analyze **parasite aggregation (k)** and **overall prevalence (P)** across body size gradients, hosts were grouped into SVL size classes using Scott's rule, based on the Central Limit Theorem [55]. Five SVL size classes were defined each spanning approximately 5 mm for the combined population, and parasite counts were categorized into ranges.

Parasite community attributes—including frequency, richness, abundance, diversity, and equity—were assessed using the **Shannon**, **Simpson**, **Pielou**, and **Bulla indices**, following established quantitative ecological methods [60, 61].

To evaluate whether parasitic infection was dependent on host sex and body size category, we applied the Chi-square test ( $\chi^2$ ) or Fisher's exact tests, depending on low counts. This test assesses whether infection status (infected/uninfected) is significantly associated with categorical variables such as sex or SVL class [58]. To examine whether the relationship between body size and parasite burden differed by sex, we conducted separate simple linear regressions for male and female hosts. In each model, snout–vent length (SVL) was used as the predictor variable and the number of helminths as the response

variable. Comparisons of slope and correlation strength allowed assessment of sex-specific patterns in parasite accumulation [58].

The total parasite variation per state were calculated as the ratio of cestodes or nematodes to the total parasites per state, while actual numbers reflected cumulative parasite burden. A Chi-Square Test of Independence was employed to test the hypothesis of no association between parasite type (cestodes vs. nematodes) and geographic origin. Within each altitudinal intervals, prevalence (P), mean abundance (MA), and mean intensity (MI) were calculated separately for male and female hosts and for the combined population. The Kruskal-Wallis test compared mean abundance and mean intensity across intervals, with post-hoc Dunn's test if significant. Prevalence was compared using Chi-square or Fisher's exact test [56, 58].

### 3. Results

#### 3.1 Sex Determination and Morphometric Analysis of *Atelopus cruciger*

Of the 97 individuals evaluated, 53 (54.64%) were males, 43 (44.33%) were females, and one could not be accurately sexed. Males had a mean SVL of  $30.63 \pm 3.02$  mm, while females were significantly larger, with a mean SVL of  $38.36 \pm 5.01$  mm, representing a size difference of approximately 25–29%.

The assumption of normality was satisfied for males ( $p = 0.093$ ) and marginally met for females ( $p = 0.049$ ), but the homogeneity of variances was violated ( $p = 0.018$ ). Consequently, both Welch's *t*-test and the Mann–Whitney *U* test were applied. Results from both tests confirmed a highly significant difference in body size between sexes (Welch's  $t = -8.66$ ,  $p < 0.0001$ ;  $U = 148.5$ ,  $p < 0.0001$ ), demonstrating robust evidence of sexual size dimorphism, with females consistently larger than males regardless of parametric assumptions.

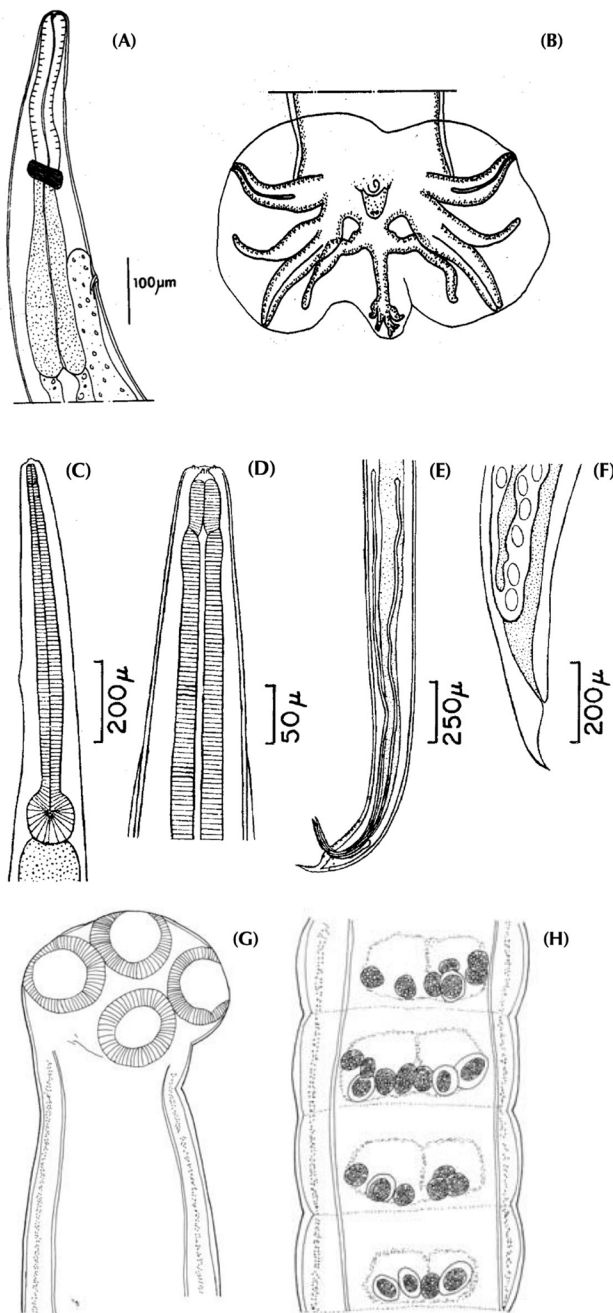
#### 3.2 Helminth Taxonomic Composition of *Atelopus cruciger*

The helminth fauna comprised five species spanning **four families** and **two phyla**. Identified families included Nematotaeniidae (1 species), Molineidae (1 species), Rhabdiasidae (1 species), and Cosmocercidae (2 species) (**Table 1**). All identified species represent novel helminthological records for *A. cruciger* and, in certain cases, also constitute new geographic records for Venezuela.

Schematic illustrations of three helminth species identified in *A. cruciger*—*Cylindrotaenia americana*, *Oswaldocruzia venezuelensis*, and *Aplectana* sp.—are presented to highlight diagnostic morphological features (**Figure 2**). These drawings depict key traits facilitating accurate identification and illustrating morphological diversity. For a comprehensive list of diagnostic characters, see **Appendix III**.

**Table 1.** Parasitic helminths identified in this work.

Phylum	Class	Order	Family	Specie
Platyhelminthes	Cestoda (van Beneden, 1849)	Cyclophillydea (van Beneden, 1850)	Nematotaeniidae (Lühe, 1910)	<i>Cylindrotaenia americana</i> Jewell, 1916 = <i>Cylindrotaenia quadrijugosa</i> Lawler, 1939
Nemathelminthes	Secernentea (Linstow, 1905)	Strongylida (Diesing, 1851)	Molineidae (Skrjabin & Schulz, 1937)	<i>Oswaldocruzia venezuelensis</i> Ben Slimane, Guerrero & Durette-Desset, 1996
			Rhabdiasidae (Railliet, 1915)	<i>Rhabdias pseudosphaerocephala</i> Kuzmin, Tkach & Brooks, 2007
		Ascaridida (Chabaud, 1965)	Cosmocercidae (Railliet, 1916)	<i>Cosmocerca podicipinus</i> Baker & Vaucher, 1984
				<i>Aplectana</i> sp. Railliet & Henry, 1916



The parasite species identified in this study display a variety of biological features, including differences in life cycle type, site of infection within the host, and transmission strategies [21, 50-52, 62]. Among the five species recovered, four (80%) exhibit a confirmed direct (monoxenous) life cycle. *Rhabdias pseudosphaerocephala* is a dioecious nematode, characterized by parasitic females residing in the **lungs** and free-living males in the environment. Transmission is achieved through skin penetration of infective larvae. *Oswaldocruzia venezuelensis* resides in the **pylorus** and **duodenum**, is dioecious, with parasitic males and females, with transmission via ingestion or active penetration of infective larvae. *Cosmocerca podicipinus* inhabits the **large intestine**, is dioecious, with parasitic males and females, with penetration-based transmission. *Aplectana* sp. occupies the **rectal ampoule**, is dioecious, with parasitic males and females, and is transmitted via ingestion of infective L3 larvae.

The cestode *Cylindrotaenia americana* inhabits the **pylorus** and **duodenum** of the host and is a hermaphroditic parasite, reproducing by releasing gravid proglottids that are expelled with host feces. Although tentatively classified as having a direct (monoxenous) life cycle, evidence from this and other studies suggests that an indirect (heteroxenous) transmission route involving one or more intermediate hosts may also be plausible. As discussed further in the *Discussion* section, the

**Figure 2.** Schematic Illustrations of Helminth Species Identified in *A. cruciger*. (A) Anterior region of male *O. venezuelensis*, showing buccal cavity and esophageal denticles. (B) Posterior end of male *O. venezuelensis*, depicting spicules and copulatory bursa. (C, D) Anterior region of *Aplectana* sp. in lateral (C) and dorsal (D) views, highlighting oral papillae and cuticular patterns. (E) Posterior end of male *Aplectana* sp., showing gubernaculum and spicules. (F) Posterior end of female *Aplectana* sp., illustrating vulva and tail morphology. (G) Scolex of *C. americana*, showing attachment structures. (H) Gravid proglottid of *C. americana*, depicting egg-filled uterus. Illustrations adapted from taxonomic keys [28, 47-54] under fair use for non-commercial scientific purposes.

**Table 2.** Abundance and Relative Abundance of helminth species and Fisher's Exact Test results in *A. cruciger*. n = 78. \*p < 0.05.

Species	Abundance Males (M) (n = 42)	Relative Abundance (M) %	Abundance Females (F) (n = 36)	Relative Abundance (F) %	p-value
<i>Aplectana</i> sp.	55	28.06%	81	35.84%	0.031*
<i>C. podicipinus</i>	72	36.73%	102	45.13%	0.042*
<i>C. americana</i>	57	29.08%	36	15.93%	0.066
<i>O. venezuelensis</i>	6	3.06%	3	1.33%	0.344
<i>R. pseudosphaerocephala</i>	6	3.06%	4	1.77%	0.767
<b>Total</b>	196	100%	226	100%	

\*p &lt; 0.05

complexity of its developmental stages and transmission pathways remains unresolved and warrants further investigation.

### 3.3 Parasite populations parameters

#### 3.3.1 Helminth Infection Composition

Among the 97 *A. cruciger* individuals examined, 78 (80.41%) were found to be parasitized, while 19 (19.59%) showed no evidence of parasitism. Males constituted 43.30% (42/53) and females 37.11% (36/43). Analysis of helminth infection types revealed three major categories: nematode infections, cestode infections, and mixed infections (co-infection with nematodes and cestodes), with no other helminth groups detected. The HIC values revealed that nematode-only infections were the most common ( $HIC_{nem} = 0.615$ ;  $n_t = 48$ ) affecting 79.25% of males (n = 42/53) and 83.72% of females (n = 36/43), followed by mixed infections ( $HIC_{mix} = 0.333$ ;  $n_t = 26$ ) occurred in 22.64% of males (n = 12/53), 32.56% of females (n = 14/43) and cestode-only infections ( $HIC_{ces} = 0.051$ ;  $n_t = 4$ ) observed in 1.89% of males (n = 1/53), 6.98% of females (n = 3/43). A Chi-square test of independence indicated **no significant association** evidence that the type of infection (nematodes, cestodes, or mixed) is associated with host sex in this sample of *A. cruciger* ( $\chi^2 = 2.79$ , df = 2,  $p = 0.248$ ).

#### 3.3.2 Parasite burden, Abundance, Relative abundance

A total of 422 helminths were recovered from all hosts examined, representing the parasite burden for the studied population of *A. cruciger*. The most abundant taxon was *C. podicipinus* with 174 individuals and a relative abundance of 41.23%, followed closely by *Aplectana* sp. (136 individuals, 32.23%). These two nematodes together accounted for over 73% of the parasite burden, indicating their numerical dominance within the intestinal helminth community. *C. americana* contributed 93 individuals (22.04%). In contrast, *R. pseudosphaerocephala* and *O. venezuelensis* were much less abundant, representing only 2.37% and 2.13% of the total burden, respectively.

The distribution of helminth species varied between male and female hosts in both absolute counts and relative representation, as assessed using Fisher's exact test (Table 2). In females, *Cosmocerca podicipinus* was the most abundant species, accounting for 45.13% of their parasite burden, followed by *Aplectana* sp. with 35.84%. Both species **showed statistically significant** sex-related differences: *Aplectana* sp. was more frequent in females (81 vs. 55;  $p = 0.031$ ), as was *C. podicipinus* (102 vs. 72;  $p = 0.042$ ). In males, these same two nematodes also predominated, but in lower proportions: *C. podicipinus* represented 36.73% and *Aplectana* sp. 28.06%. The cestode *Cylindrotaenia americana*, while slightly more frequent in males (57 vs. 36), did **not differ significantly** between sexes ( $p = 0.066$ ), though the trend suggests potential sex-biased exposure. Pulmonary and anterior intestinal nematodes, *Rhabdias pseudosphaerocephala* and *Oswaldocruzia venezuelensis*, remained rare in both sexes. Their frequencies did **not differ significantly** (6 vs. 4;  $p = 0.767$  and 6 vs. 3;  $p = 0.344$ , respectively), likely due to the low counts reducing statistical power. These results suggest sex-linked differences in susceptibility or exposure, particularly for dominant intestinal nematodes.

#### 3.3.3 Prevalence, Mean intensity, Mean abundance, Infection index

The parasitic helminths of *A. cruciger* exhibit significant variability in prevalence, infection intensity, and overall infection impact among the identified species. Prevalence (P%) ranged from 2.06% (*O. venezuelensis*) to 30.93% (*C. americana*). *C. americana* showed significantly higher prevalence in females (39.53%) than males (24.53%; Fisher's exact test, OR = 0.37,  $n=53\sigma$ ,  $43\phi$ ,  $p = 0.015$ ), while other species showed no differences (*Aplectana* sp.: OR = 0.93,  $p = 1.000$ ; *C. podicipinus*: OR = 1.81,  $p = 0.297$ ; *O. venezuelensis*: OR = 0.81,  $p = 1.000$ ; *R. pseudosphaerocephala*: OR = 1.65,  $p = 1.000$ ). Mean intensity (MI) was highest for *C. podicipinus* (1.36 males, 1.92 females),

**Table 3.** Prevalence (P%), Mean Intensity (MI), Mean Abundance (MA) and Infection Index (ii) of parasitic helminths identified in *A. cruciger*. M, males; F, females; C, combined population.

Species	Prevalence (P%)			Mean Abundance (MA)			Mean Intensity (MI)			Infection Index (ii)		
	M1	F2	C3	M1	F2	C3	M	F	C	M1	F2	C3
<i>Aplectana</i> sp.	26.4	27.91	26.80	1.31	2.25	1.74	1.04	1.88	1.40	0.274	0.526	0.376
<i>C. podicipinus</i>	22.64	11.63	17.53	1.71	2.83	2.23	1.36	2.37	1.79	0.308	0.276	0.314
<i>C. americana</i>	24.53	39.53	30.93	1.36	1.00	1.19	1.08	0.84	0.96	0.264	0.331	0.297
<i>O. venezuelensis</i>	1.89	2.33	2.06	0.14	0.08	0.12	0.11	0.07	0.09	0.002	0.002	0.002
<i>R. pseudosphaerocephala</i>	3.77	2.33	3.09	0.14	0.11	0.13	0.11	0.09	0.10	0.004	0.002	0.003

P %, MA, and ii were calculated using the total number of individuals examined: males (M, n = 53), females (F, n = 43), and combined (C, n = 97; includes M + F + 1 unsexed). MI was calculated using only infected hosts: males (M, n = 42), females (F, n = 36), and combined (C, n = 78).

with a significant female bias (Mann-Whitney U = 600,  $p = 0.030$ ) and borderline for *Aplectana* sp. (1.04 males, 1.53 females; U = 630,  $p = 0.050$ ). Mean abundance (MA) and infection index (ii) showed no significant sex differences (*Aplectana* MA: U = 1040,  $p = 0.070$ ; ii: U = 1030,  $p = 0.060$ ; others:  $p = 0.090$ – $1.000$ ), though *Aplectana* trended female-biased. Low prevalence (<4%) for *O. venezuelensis* and *R. pseudosphaerocephala* limited statistical power. The **Table 3** summarize prevalence, mean intensity, mean abundance, and infection index of helminth parasites in *A. cruciger*.

### 3.3.4 Morisita dispersion index, Aggregation coefficient

The calculated Morisita dispersion index for *A. cruciger* (**Imor = 1.875**) indicated aggregated distributions pattern for all species, common in many parasite-host systems, which fits a negative binomial distribution. The spatial distribution of helminths between male and female hosts exhibited **Imor values > 2**, indicating a strong pattern of **sexual aggregation** in parasite load. Specifically, *O. venezuelensis* showed the highest index (**Imor = 2.29**), followed by *C. americana* (**2.10**) and *R. pseudosphaerocephala* (**2.10**). *Aplectana* sp. and *C.a podicipinus* also demonstrated aggregation, with Imor values of **2.07** and

**2.06**, respectively. Such aggregation may reflect sex-specific differences in susceptibility, behavior, or exposure routes.

Parasite distribution was analyzed by SVL size class to assess aggregation patterns (**Table 4**). The aggregation coefficient (k) was calculated for each class to quantify clumping, assuming a negative binomial distribution. The parasite distribution shows low to moderate loads (1–6 parasites) dominating across most size classes, with high (>10) loads rare. Males (26–32 mm) have low-moderate loads; females (38–46 mm) show variable loads, including rare high loads, possibly due to larger size.

The values of the aggregation coefficient (k), calculated across five host size classes based on body length corroborate that the parasite load tends to be concentrated in a few individuals of the host population. The aggregation coefficient k calculated ranged from 0.94 to 2.53. Values of  $k > 1$  indicate an **aggregated distribution**, where parasites are disproportionately concentrated in certain hosts—a pattern observed in most classes. One class exhibited  $k \approx 1$ , suggesting a **random distribution**, while one class approached uniformity with  $k = 0.94$ . These findings confirm that the parasite population in *A. cruciger* tends to aggregate rather than distribute evenly among hosts, consistent with classical parasitological theory.

**Table 4.** Parasite Distribution and Aggregation Coefficient by Size Class in *A. cruciger* (n = 97).

SVL Class (mm)	n	Parasite Count					Mean	Variance	k
		0	1–3	4–6	7–10	>10			
I 23–28	17	5	5	5	2	0	3.06	6.76	2.53
II 28–34	39	7	15	7	4	6	4.23	17.10	1.39
III 34–39	27	2	10	7	3	3	4.85	29.98	0.94
IV 39–45	10	0	3	4	0	1	4.90	28.89	1.00
V 45–50	4	0	0	1	1	1	Not estimable		

n = sample size. No significant differences in parasite numbers by size class were found ( $\chi^2 = 2.59$ ,  $df = 4$ ,  $p > 0.05$ ). For Class V, k is reported as “not estimable” due to the small sample size (n = 4). Although some female hosts in this class harbored between 5 and 13 helminths, such low sample sizes can artificially inflate variance and yield misleading k values, making statistical interpretation unreliable.

### 3.3.5 Relationship between host body size and parasite burden

Following the description of parasite population metrics across host groups, the relationship between parasite burden and snout-vent length (SVL) was explored separately for males and females.

In males, the regression slope was 0.0322 (intercept = 36.98), with an  $R^2 = 0.0011$  and  $p < 1.84 \times 10^{-50}$ . Although the regression model detected statistical significance, the  $R^2$  value indicates that only 0.11% of the variation in parasite burden is explained by body size, meaning that SVL has virtually no predictive power for infection level. The data were widely dispersed (burdens 0–17.5), with no observable trend or clustering, suggesting that other unmeasured ecological or physiological variables dominate the variation.

In females, the slope was 0.1227 (intercept = 1.622), with an  $R^2 = 0.0121$  and  $p < 2.87 \times 10^{-37}$ . This also reflects a statistically significant but biologically weak association, where SVL explains only about 1.2% of the variance in parasite burden. Female data points exhibited broader dispersion, with a few individuals showing high parasite loads (up to 30) at larger SVL values ( $\approx 45$ –50 mm), but the overall relationship remained weak, consistent with a minimal size effect (Figure 3).

Both models yielded extremely low  $p$ -values, indicating statistically significant relationships between host size (SVL) and parasite burden, albeit very weak. In males, only **0.11%** of the variation in parasite load was explained by SVL, and in females, just **1.21%**, as reflected in the low coefficients of determination ( $R^2$ ). These low  $R^2$  values reflect the high dispersion of individual data points, which is expected in field-based parasitological studies where multiple uncontrolled

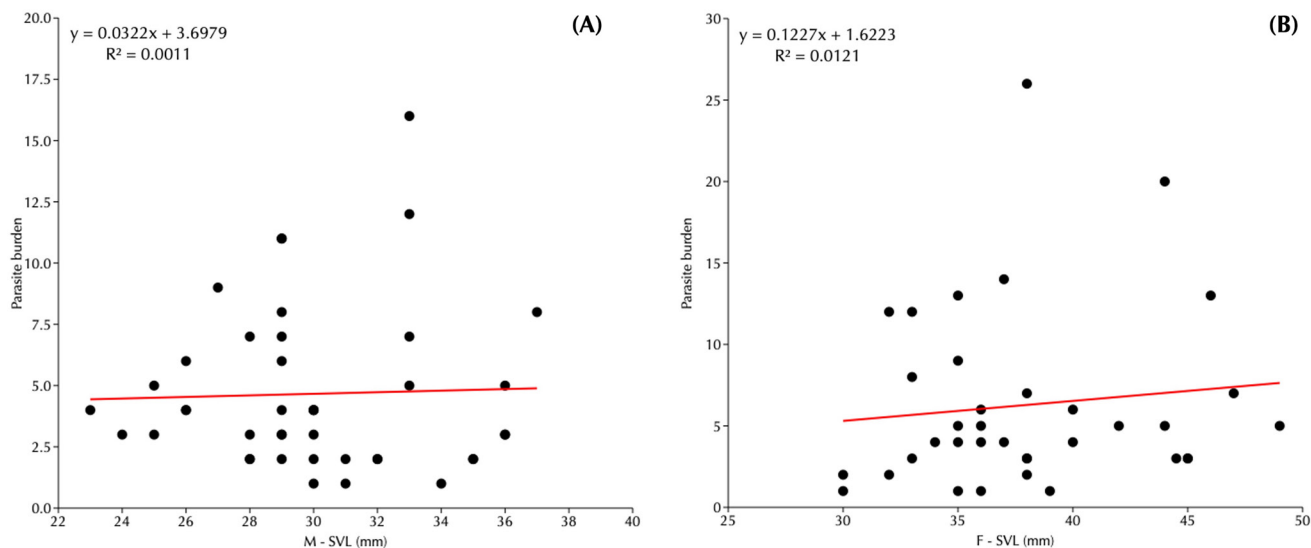
factors—such as duration of exposure, number of reinfection events, individual immune status, microhabitat use, and host behavior—contribute to variation in infection intensity.

While statistical significance is clear, it does not necessarily imply biological relevance. The results suggest that **SVL is a poor predictor of parasite burden** in this system, likely due to the overriding influence of other ecological or physiological factors. This interpretation is consistent with the strongly aggregated distribution of parasites observed across hosts.

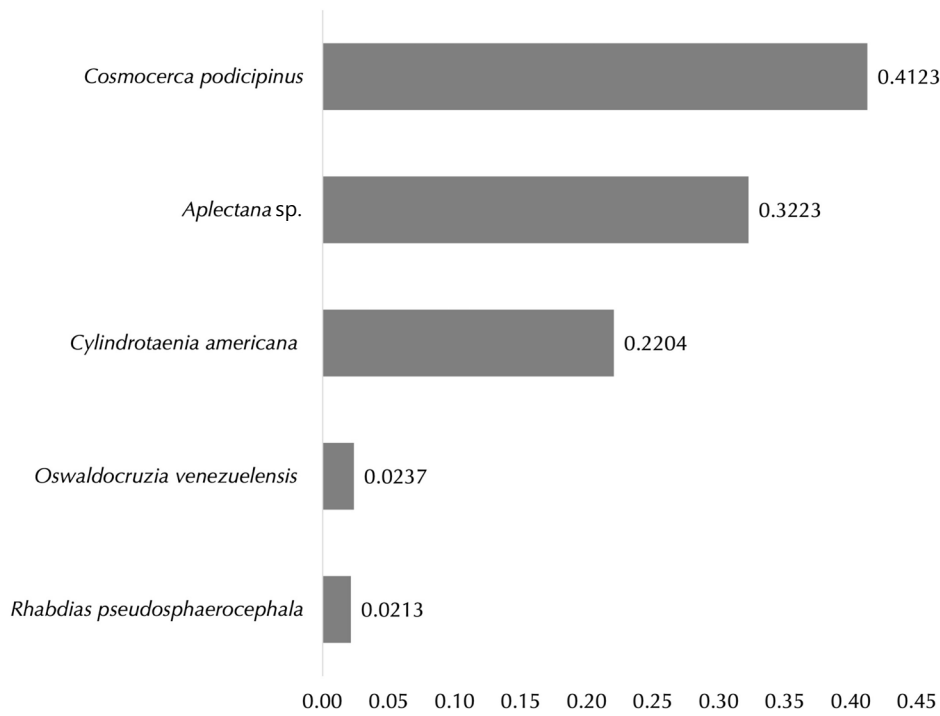
To further investigate the relationship between host size and parasite burden, particularly whether body size constrains the **maximum parasite load** a host can sustain rather than the mean, individuals were grouped into body size classes, and the maximum parasite count was extracted from each class. The regression analysis based on these maximum values revealed a **negative slope** (–17) with a higher coefficient of determination ( $R^2 = 0.1993$ ) compared to individual-level models. This result suggests that larger hosts do not necessarily carry the highest parasite burdens, and that intermediate-sized individuals may reach peak infection levels. Although the direction of the slope is contrary to expectations, it highlights the complexity of host–parasite interactions and supports the idea that **host size alone does not linearly predict upper limits of infection** in natural populations.

### 3.4 Parasites community parameters

A total of five helminth species were identified, comprising both nematodes and cestodes. The relative frequencies revealed a community dominated by intestinal nematodes. *C. podicipinus* was the most frequent species, representing 41.23% of all helminths recovered, followed by *Aplectana* sp.



**Figure 3.** Scatter plots of the parasite burden with the snout–vent length (SVL) by sex of *A. cruciger*. (A) M, males; (B) F, females. The coefficients of determination  $R^2$ , slope and intercept are reported.



**Figure 4.** Frequency distribution of parasites identified in *A. cruciger*.

(32.23%). Together, these two taxa accounted for over 73% of the total parasite burden.

The frequency distribution (see chart) highlights *C. podicipinus* and *Aplectana* sp. ( $F = 0.3223$ , 136 parasites) and as key parasites, with *C. americana* notable for high prevalence despite lower frequency (**Figure 4**).

This distribution indicates a **numerically skewed community**, characterized by a few dominant taxa and several low-frequency species—a typical pattern in amphibian helminth assemblages, where aggregated infections and host-parasite specificity influence community structure.

Diversity and evenness metrics indicated a moderately structured helminth community in *A. cruciger*. The Shannon index ( $H' = 1.234$ ) and Simpson index ( $D_{si} = 0.678$ ) suggest moderate to moderately high species diversity, dominated numerically by two species. The Shannon index indicates limited diversity within the community, consistent with Margalef's criterion, which considers values below 3 bits per individual as indicative of low diversity [63]. Pielou's evenness ( $J' = 0.767$ ) indicated a relatively balanced distribution of individuals among species, whereas Bulla's index ( $E = 0.306$ ) revealed a noticeable departure from perfect equitability, consistent with the presence of dominant taxa (*C. podicipinus* and *Aplectana* sp.) and rare species (*Rhabdias*, *Oswaldocruzia*). Overall, the parasite community exhibits low richness, moderate diversity, and partial equitability, characteristic of aggregated parasitic systems (**Table 5**).

### 3.5 Geographic Variation in Parasite Composition

The distribution of helminth parasites in *A. cruciger* exhibited significant variation across site of collection. Cestode counts were highest in Carabobo (48), followed by Aragua (22), Miranda (21), Distrito Capital (1), Cojedes (1), and absent in Yaracuy (0), with corresponding frequencies of 0.571, 0.173, 0.181, 0.033, 0.017, and 0.000, respectively. Nematodes predominated, with counts of 105 in Aragua, 95 in Miranda, 57 in Cojedes, 29 in Distrito Capital, 7 in Yaracuy, and 36 in Carabobo, yielding frequencies of 0.827, 0.819, 0.983, 0.967, 1.000, and 0.429, respectively. Total parasite numbers per

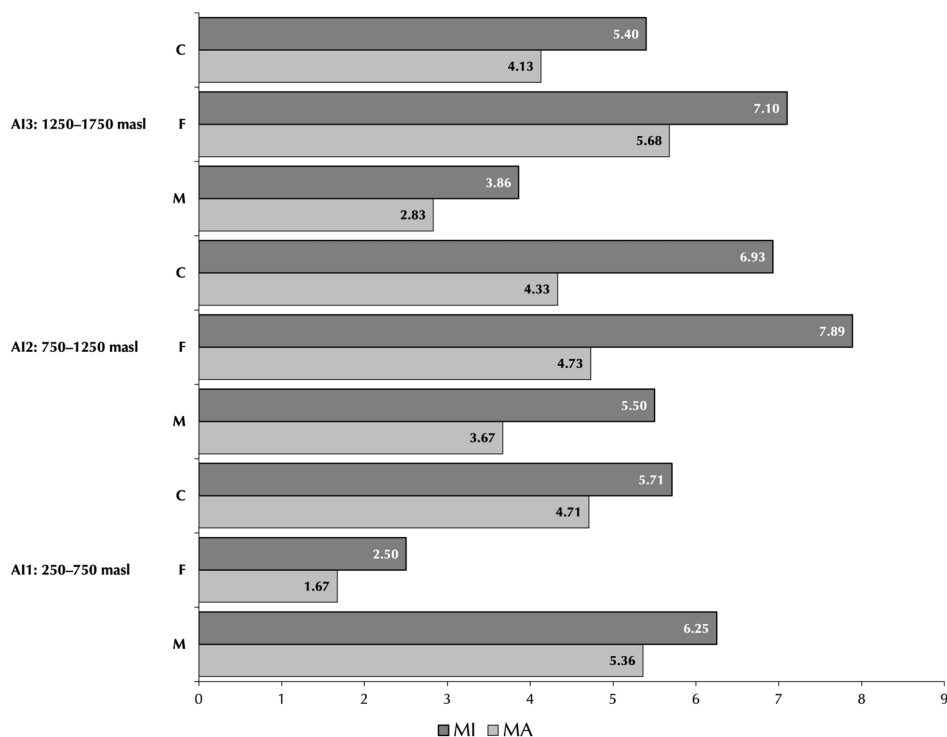
state were Aragua (127), Miranda (116), Cojedes (58), Carabobo (84), Distrito Capital (30), and Yaracuy (7), consistent with the overall sample. A Chi-Square Test of Independence suggested a significant association between parasite type and state ( $\chi^2 = 123.45$ ,  $df = 5$ ,  $p < 0.001$ ), with post-hoc analysis indicating Carabobo's higher cestode proportion and Yaracuy's exclusive nematodes as notable patterns. However, the broad political boundaries limited ecological inference, highlighting the need for habitat-specific data to refine these observations.

### 3.6 Altitudinal Distribution Analysis

Parasite population parameters varied across altitudinal ranges (**Figure 5**). Prevalence was highest at 250–750 m (82.4%), driven by male infections, while mean abundance peaked at 250–750 m (4.71) and 750–1250 m (4.33). Mean parasite abundance (MA) and mean intensity (MI) varied across altitudinal intervals and host sex. In males exhibited marked variation: MA was highest at low (AI1 = 5.36), but significantly reduced at high elevations (AI3 = 2.83). A similar pattern was observed in MI, which dropped from 6.25 in AI1 to 3.86 in AI3. Females showed higher intensity across all ranges, particularly at 750–1250 m (7.89), indicating more intense infections despite consistent parasite presence.

**Table 5.** Values of the Shannon, Simpson, Pielou and Bulla indices.

Shannon index ( $H'$ )	Pielou ( $J'$ )	Simpson index ( $DSi$ )	Bulla's index ( $E$ )
1.234	0.767	0.678	0.306



**Figure 5.** Mean parasite abundance (MA) and mean intensity (MI) in *A. cruciger* across three altitudinal intervals (AI). M, Males; F, Females; C, Combined. masl, meter above sea level.

Kruskal–Wallis tests confirmed that mean parasite abundance (MA) varied significantly across altitudinal ranges in both sexes. For males, the test yielded  $H = 12.30$  ( $p = 0.0021$ ), and for females,  $H = 12.57$  ( $p = 0.0019$ ). Kruskal–Wallis tests applied to mean intensity (MI) values across altitudinal bands revealed statistically significant differences for both males ( $H = 12.30$ ,  $p = 0.0021$ ) and females ( $H = 12.59$ ,  $p = 0.0018$ ). In males, intensity dropped markedly at mid elevations (AI3), whereas females exhibited a peak at AI2. These patterns suggest that the impact of elevation on helminth intensity may be sex-specific, possibly reflecting differences in behavior, physiology, or exposure dynamics.

#### 4. Discussion

This study represents the first comprehensive parasitological assessment of *Atelopus cruciger*. The dataset, comprising 97 individuals collected over nearly six decades from seven Venezuelan federal states, provides novel records for the species. These findings significantly enrich Venezuela’s biodiversity inventory and establish a crucial baseline for conservation-focused parasitological research.

The helminth community includes five species: four nematodes—*Aplectana* sp., *Cosmocerca podicipinus*, *Rhabdias pseudosphaerocephala*, and *Oswaldocruzia venezuelensis*—which together constitute 77.96% of the parasite assemblage, and one cestode, *Cylindrotaenia americana*, accounting for

22.04%. The species’ terrestrial habits, including diurnal activity and frequent soil contact, increase its exposure to nematodes with direct life cycles. This ecological trait aligns with the observed infection rate: 80.41% of individuals were parasitized. These results corroborate previous findings that terrestrial amphibians, especially bufonids, are predominantly infected by nematodes with direct transmission cycles [6, 10, 17, 20, 64–71].

The dominance of *C. podicipinus* can be attributed to its mode of transmission via skin penetration followed by pulmonary migration [52]. This nematode is known for its high prevalence in Neotropical anurans, particularly those inhabiting terrestrial and semi-terrestrial environments [8, 22, 23, 72–82].

Additionally, at least 23 species of *Aplectana* have been reported from a variety of Neotropical hosts [83, 84].

The cestode *Cylindrotaenia americana* has been reported in at least 45 anuran species across South America [8, 29–31, 85–91]. Although originally proposed to have a direct life cycle [92–95], more recent evidence—considering geographic variation and host dietary patterns—supports the likelihood of a heteroxenous life cycle involving arthropod intermediates [96–98]. In the present study, *C. americana* exhibited a strikingly uneven distribution, with a high prevalence in Carabobo (57.1%) and near absence in Yaracuy (0%), Distrito Capital (1%), and Cojedes (1%). Specifically, in the Cojedes population, where orthopterans dominate the anuran diet [98] *C. americana* represented only 1.7% of the total helminth community. This low prevalence suggests ecological or dietary constraints limiting transmission in that locality. Indeed, it is well established that various arthropod taxa, particularly mites, act as intermediate hosts in the life cycles of cyclophyllidean cestodes [46], which may be absent or scarce in these host diets.

The parasitological analysis of *A. cruciger* revealed a helminth community with characteristics typical of terrestrial anuran hosts—low species richness, strong dominance by a few taxa, and aggregated distributions across individuals. This high prevalence aligns with patterns observed in other tropical bufonid species that exhibit terrestrial lifestyles where exposure to infective stages via moist substrates facilitates infections by parasites with direct life cycles [1, 6, 10, 17, 20].

However, the lack of a clear sex-based differentiation in infection types (e.g., no significant association between sex and parasite species composition) diverges from findings in species like *Bufo americanus*, where male-biased parasitism has been linked to behavioral promiscuity [17]. This contrast suggests that *A. cruciger*'s ecology may mitigate sexual selection pressures on parasite acquisition, potentially due to its restricted habitat range or uniform exposure across sexes. The high representation of intestinal nematodes is comparable to that described for *Rhinella icterica* [99], reinforces the role of direct life cycles, yet the notable presence of a cestode challenges the typical nematode-centric description, inferring at a dietary or microhabitat influence like that observed by Goldberg *et al.* [64–68] in other anurans. The aggregated distribution of parasites, consistent with models of overdispersion, contrasts with studies like Iannacone [20], which found less clumping in *Atelopus bomolochos*, possibly reflecting differences in host population density or environmental stability.

The ecological niche of *Atelopus cruciger* as a diurnal, terrestrial anuran appears to shape a helminth community adapted to its lifestyle, with implications for both host fitness and parasite persistence. The prevalence of infections suggests a transmission environment robust enough to sustain a diverse parasite assemblage, likely supported by the species' frequent soil interaction, which favors nematodes with direct life cycles. The functional role of these parasites may extend beyond mere pathogenicity, potentially influencing host behavior or immunity, as intestinal nematodes could modulate digestion or nutrient absorption—a hypothesis supported by their numerical dominance. The presence of a cestode, reliant on intermediate hosts, implies a dietary component—possibly mites or small invertebrates—that introduces a secondary transmission pathway, enriching the community's ecological complexity. The observed sex-specific biases in parasite abundances (e.g., higher abundances of dominant intestinal nematodes in females) suggest functional differences in exposure dynamics between males and females. Although both sexes exhibit strong reproductive site fidelity, females spend considerably more time in the surrounding forest away from the stream, tend to be more sedentary, and move over smaller spatial scales than males [100]. These behavioral patterns may increase their cumulative contact with soil substrates and microhabitats harboring infective nematode larvae, thereby elevating exposure risk. Consequently, the higher abundances recorded for dominant intestinal nematodes in females are more plausibly linked to differential habitat use and reduced mobility rather than to body size or reproductive site fidelity alone. This interpretation aligns with the idea that parasite loads reflect not just exposure but also host life-history strategies, challenging the assumption that larger size or

sedentarism universally increases infection risk. The aggregated distribution further suggests that a subset of hosts acts as key reservoirs, possibly those in optimal microhabitats—a dynamic that could stabilize the parasite population amidst the species' endangered status. This ecological interplay underscores a community adapted to the host's terrestrial niche, where functional roles may enhance resilience against environmental stressors.

The findings in *Atelopus cruciger* engage directly with core principles of parasitological theory, particularly those concerning host-parasite co-evolution and community structure. The lack of a significant sex-infection type association challenges the hypothesis that sexual dimorphism drives differential parasitism, which expect sex-biased exposure based on behavior or immunity [101, 102]. Instead, the data support a model in which environmental transmission dominates, aligning with the theory on habitat as a primary determinant of parasite diversity [1, 14, 69]. The aggregated distribution, fitting a negative binomial model, reinforces argument that overdispersion reflects host heterogeneity rather than uniform susceptibility, suggesting that immune or behavioral variation among individuals' shapes parasite loads. The observed moderate diversity and uneven species composition align with Margalef's [63] criterion for low-diversity systems, where ecological constraints—such as habitat specificity or transmission limitations—favor the dominance of a few well-adapted taxa. In such communities, stability may be maintained by these dominant species; however, the presence of rare, low-frequency parasites introduces a potential vulnerability. This scenario supports the hypothesis that host population declines can lead to the local extinction of specialist or less abundant parasites, which are more sensitive to reductions in host density or environmental change [103]. Although most *A. cruciger* specimens included in this study were collected before the late-1980s chytridiomycosis-associated population collapse, the dataset also includes individuals from 2004, 2005, and 2009. Despite their limited number, these more recent hosts exhibited helminth assemblages consistent with historical patterns, with no evidence of community restructuring—apart from a single uninfected female collected in 2005. Because post-decline specimens mirror the parasitic composition of earlier decades, no formal temporal comparisons were performed, as the available data do not support a meaningful decade-level analysis. Nevertheless, additional sampling from extant remnant populations would be essential to determine whether subtle erosion of parasite diversity has occurred following the demographic bottleneck, **particularly considering potential synergies with chytrid infections** [104]. The absence of a size effect on parasite burden, consistent with Luque *et al.* [99], but contrasting with the patterns reported by Bolek and Coggins

[17, 18], further complicates size-based models of infection. These results suggest that physiological or microhabitat variables may override morphological predictors, a hypothesis that could be evaluated through immunological profiling or fine-scale spatial approaches.

The observed community aligns more closely with what Aho [1] termed “isolationist” parasitic assemblages, where few species dominate due to reduced colonization opportunities and low interspecific competition [101, 102]. This contrasts with the “interactive” communities seen in more gregarious or aquatic amphibians, which encounter a wider array of infective stages through multiple trophic pathways. Nevertheless, the dominance of *Cosmocerca podicipinus* and *Aplectana* sp. in *A. cruciger*—both nematodes with flexible, environmentally robust life cycles—suggests that even under isolationist constraints, selective pressures can drive intense colonization success for a few well-adapted taxa.

The analysis of parasite aggregation patterns supports the classical ecological expectation of overdispersed distributions. Most helminths were concentrated in a minority of hosts, while the majority harbored few or no parasites. This skewed distribution is common across vertebrates and reflects heterogeneity in susceptibility, exposure, and behavioral traits [1, 105–107]. The aggregation coefficient ( $k$ ) and Morisita index confirmed this pattern, reinforcing the notion that parasite transmission is not uniformly distributed but structured by complex host–parasite dynamics. Particularly striking is the finding that rare parasites such as *Oswaldocruzia venezuelensis* exhibited the highest levels of aggregation—underscoring the role of chance colonization and potentially strong individual-level variability in resistance or exposure.

Diversity indices further illuminate the structure of the helminth community. The moderate Shannon and Simpson values point to low richness but relatively balanced dominance between the two leading taxa. However, the low Bulla index and dominance by two nematodes suggest an unstable equilibrium, where any shift in host ecology or parasite transmission could restructure the community. These metrics, when viewed together, reflect a system where parasite colonization is tightly constrained, likely regulated by the host’s behavior, niche, and microenvironmental stability rather than by inter-specific interactions among parasites [1, 101, 108].

The composition and structure of the helminth community in *A. cruciger* reiterate broader ecological principles governing host–parasite systems: namely, that diversity is not synonymous with complexity, and that host biology often filters the potential richness of parasite assemblages. The dominance of generalist, direct-transmission nematodes, coupled with limited variation across host traits and geography, supports a

model of stability rooted in ecological constraint rather than dynamic turnover. However, the aggregated nature of infections and occasional sex-specific differences imply that individual-level traits and behaviors remain crucial determinants of infection risk.

Host size, measured via snout–vent length (SVL), did not emerge as a significant predictor of parasite burden in *Atelopus cruciger*, posing a nuanced challenge to established paradigms in amphibian parasitology. Although sexual dimorphism was confirmed—females (39.5–49.9 mm) being significantly larger than males (28.2–34.6 mm) [34, 35]—our findings contradict the widely held expectation that larger individuals harbor higher parasite loads [7, 10, 13, 16, 109, 110]. No significant associations were detected between SVL and parasite abundance, intensity, or species richness.

Regression analyses revealed negligible linear relationships between SVL and parasitological parameters for both sexes, with coefficients of determination ( $R^2$ ) of 0.001979 for males and 0.0121 for females, indicating minimal explanatory power. Moreover, no statistically significant differences in overall parasite burden were observed between males (range: 0–17.5 parasites; SVL: 22–40 mm) and females (range: 0–30 parasites; SVL: 30–50 mm), although certain species such as *Aplectana* sp. exhibited a mild female-biased prevalence. Regarding this sex bias, Joy and Bunten [111] and Bolek and Coggins [18] did not find a preference for cosmocercidae by a specific sex in their studies of anurans, as did Santos and Amato [112] with *C. parva* and *R. fernandezae*.

However, our results contrast with the common expectation that larger hosts would exhibit higher infection levels due to increased surface area for larval penetration or greater food intake. In *A. cruciger*, body size showed no significant relationship with parasite burden, as indicated by the scattered data distribution and the low explanatory power of regression models. Nevertheless, females tended to exhibit higher infection levels, a pattern that may be linked not to size but to behavioral differences. Female *A. cruciger* are generally more sedentary and exhibit stronger site fidelity during breeding [100], which could increase exposure to infective stages present in the substrate. Thus, although size did not predict infection intensity, behavioral ecology may explain the observed sex-related variation in parasitism.

This pattern aligns with several comparative studies, enhancing its robustness within the context of amphibian parasitology. For instance, Iannacone [20] reported no significant correlation between SVL and parasite prevalence, richness, or intensity in *Atelopus bomolochos*, despite analyzing a substantial dataset of 13,268 parasites across 33 individuals, while Luque *et al.* [99] observed similar results

in *Rhinella icterica*. These findings are echoed in studies on *Leptodactylus latinasus* and *Pseudopaludicola falcipes*, where González and Hamann [72, 78] and Hamann *et al.* [22, 23] suggesting that body size may be a weak predictor of parasitism in terrestrial amphibians.

In contrast, Bolek and Coggins [17, 18] documented a significant correlation between mean cosmoceroid intensity and host size in North American anurans, attributing this to an increased contact surface facilitating larval penetration—a mechanism potentially modulated by host behavior or integument thickness. The divergence between these findings and our results may reflect species-specific immunological responses or ecological adaptations in *A. cruciger*. For example, territorial males, exhibiting greater activity, might reduce exposure variability, while females' larger size could be offset by enhanced immune function, as suggested by Barton [113], who noted that infection levels in larger anurans may stabilize or decrease due to immune regulation or intraspecific competition. This hypothesis is supported by observations that larger males in some species exhibit higher parasite richness due to declining immune efficacy [102, 114]—a trend not replicated here, possibly due to *A. cruciger*'s unique behavioral or physiological profile.

The absence of a size-parasite burden relationship invites a deeper examination of alternative drivers, grounded in the ecological and physiological context of the species. Host immunity, potentially influenced by sex-specific hormonal profiles or life-history trade-offs, could play a pivotal role. The scattered distribution of parasite burdens across SVL ranges further supports the hypothesis that morphological traits alone are insufficient predictors, necessitating a multivariate approach that incorporates these factors. This perspective is reinforced by the moderate diversity and clumped distribution observed in the broader community, suggesting that parasite aggregation may be driven by host-specific microenvironments rather than size-related susceptibility.

Geographic variation in the helminth community of *A. cruciger* suggests that local environmental conditions, habitat structure, and host ecology jointly modulate the relative success of nematodes and cestodes across the species' range. The marked differences among states are consistent with patterns reported for other terrestrial anurans, where soil characteristics, forest integrity, and the availability of intermediate hosts strongly influence parasite assemblages [1, 69, 115]. Regions dominated by nematodes likely reflect environments where direct life cycle transmission is favored—such as moist substrates, high contact rates with the leaf litter, and limited trophic pathways. In contrast, areas where cestodes are proportionally more frequent may correspond to microhabitats

offering richer or more diverse intermediate host communities (e.g., mites, beetles), or dietary shifts in the local amphibian population.

These geographic differences may also mirror historical or ecological fragmentation within *A. cruciger* populations. Local declines, changes in prey availability, or microclimatic differences across cloud forest remnants could have altered transmission opportunities differently across sites. Such spatial heterogeneity aligns with theoretical expectations that helminth communities in declining or patchy amphibian populations become increasingly uneven, with some parasite taxa persisting only where environmental conditions remain favorable.

These findings reinforce the idea that parasite community composition in *A. cruciger* is not uniform across its range and instead reflects underlying variation in environmental conditions, host diet, microhabitat use, and potentially host population structure. However, an important caveat must be acknowledged: the use of broad geopolitical units (i.e., states) as proxies for ecological regions imposes limitations on the interpretation of distributional patterns. Political boundaries rarely align with ecological gradients, and habitat heterogeneity within a single state can be considerable. Therefore, while the Chi-square analysis offers robust statistical evidence of regional differences in parasite composition, ecological inferences should be tempered until more fine-scale data (e.g., vegetation type, soil structure, invertebrate community, and microclimate) are available.

Helminth infection parameters varied significantly across altitudinal range intervals, highlighting a complex interplay between elevation, host sex, and parasitic exposure. Rather than a uniform response to elevation, males and females appear to differ in how temperature, humidity, and microhabitat stability shape their exposure and susceptibility. At lower elevations—typically warmer and more humid—the ecological context likely enhances survival of free-living infective stages, particularly for nematodes with direct life cycles, increasing encounter rates for both sexes [1, 6, 17, 20]. Yet, the contrasting patterns between males and females along the gradient indicate that behavioral ecology modulates this environmental effect. Males, which tend to be more mobile and engage in calling activity, may experience fluctuating exposure as elevation changes, while females—generally more sedentary and exhibiting stronger site fidelity—may experience more consistent but environmentally mediated infection pressure. This altitudinal decline in parasitic metrics among males may reflect reduced activity levels, lower contact with infective substrates, or physiological constraints associated with cooler, more variable montane climates. These results align with established patterns in ectothermic hosts, where cooler environments limit parasite

recruitment and development due to reduced host metabolic and feeding rates [1, 17, 18].

Females, by contrast, displayed a different response: their infections intensified at mid-elevations rather than simply decreasing or increasing along the gradient. Such sex-specific divergence may stem from behavioral differences (e.g., differential substrate use, microhabitat preferences), reproductive status, or immune investment trade-offs [10, 13, 101, 114]. The elevated infection intensity observed in mid-elevation females may be linked to reproductive effort and hormonal cycles that transiently reduce immune efficacy, a phenomenon documented in amphibians and other vertebrates [116, 117]. During the reproductive season, elevated concentrations of estrogen and progesterone can modulate immune responses, particularly by downregulating cell-mediated immunity, thereby increasing susceptibility to parasitic infections [116]. In *Atelopus cruciger*, females exhibit strong site fidelity to breeding areas [100], which, combined with prolonged reproductive activity, may enhance repeated exposure to infective stages accumulated in these microhabitats.

Conversely, the decline in parasite intensity among males at higher elevations may be associated with environmental constraints that limit parasite viability and transmission. At cooler, high-altitude sites, the development and survival of free-living larval stages of nematodes are reduced [118, 119], resulting in fewer successful infections. Moreover, temperature-dependent metabolic suppression in ectothermic hosts can decrease feeding and movement rates, indirectly reducing exposure to infective stages [17, 18, 120]. Thus, the contrasting altitudinal trends between sexes likely reflect an interaction between host physiology, reproductive behavior, and environmental filtering of parasite life cycles.

Importantly, these results align with broader ecological principles. Altitude affects multiple environmental parameters—temperature, humidity, vegetation structure—that collectively influence the life cycles of parasites and the ecology of their hosts [12-16]. In tropical montane systems, such gradients are particularly steep, and even modest shifts in elevation can lead to pronounced ecological transitions. Given that most helminths identified in this study (e.g., *Cosmocerca podicipinus*, *Aplectana* sp.) rely on moist environments for larval development and transmission, altitudinal differences in microclimate likely play a key role [22, 52, 72].

In conclusion, the parasite burden in *A. cruciger* is strongly structured by altitude, but with sex-specific signatures that highlight the need to consider host biology in parasitological studies [1, 5-11]. The integration of microclimatic and behavioral data would offer further resolution to these patterns, potentially aiding in conservation efforts by identifying elevation zones with elevated parasitic risk. Future research

should explore whether these altitudinal patterns remain stable over time and how they interact with climatic changes, which are expected to shift the distribution and dynamics of parasite-host systems in tropical montane forests [1, 106, 107].

The helminth community of *A. cruciger* demonstrates that low richness does not equate to ecological insignificance. The dominance of a few well-adapted taxa, aggregated infections, and spatial or sex-specific patterns point to a system in equilibrium—one potentially vulnerable to disturbance. As an endangered species, *A. cruciger* faces threats from habitat loss, pollution, and disease. Parasites may exacerbate these pressures or serve as indicators of population health. Monitoring their presence, load, and distribution thus becomes critical—not merely for cataloging biodiversity, but for understanding and mitigating extinction risk.

Finally, the patterns observed in *A. cruciger* challenge traditional assumptions in parasitological theory. The absence of a clear size effect, the modest sex bias, and the prominence of environmental over morphological predictors all argue for a more nuanced, ecologically integrated approach. Rather than being defined solely by host traits, parasite community dynamics in this system appear to emerge from the intersection of host behavior, environmental context, and life-history constraints—a model that may apply broadly across threatened amphibians in tropical ecosystems.

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**Appendix I.** Specimens analyzed. P.N, National Park; n/a, No information; p/n = Pending number

**Museum of Biology of the Central University of Venezuela (MBUCV)**

<i>Catalog No.</i>	<i>Place of Collection</i>	<i>Date of Collection</i>
MBUCV 725a, MBUCV 725b, MBUCV 725c, MBUCV 725d	Curupao, Miranda	11.02.1966
MBUCV 726a, MBUCV 726b, MBUCV 726c, MBUCV 726d, MBUCV 726e, MBUCV 726f, MBUCV 726g, MBUCV 726h, MBUCV 726i, MBUCV 726j, MBUCV 726k	Quebrada Caurimare, Miranda	21.02.1968
MBUCV p/n a	Cata, Aragua	xx.08.2009
MBUCV p/n b, MBUCV p/n c, MBUCV p/n d	Cuyagua, Aragua	xx.12.2009

**Rancho Grande Biological Station Museum (MEBRG)**

<i>Catalog No.</i>	<i>Place of Collection</i>	<i>Date of Collection</i>
MEBRG 10	Rancho Grande, Aragua	25.08.1960
MEBRG 1208	Rancho Grande, Aragua	27.05.1980
MEBRG 1209, MEBRG 1211, MEBRG 1212	Rancho Grande, Aragua	08.04.1980
MEBRG 1062	Chivacoa, Yaracuy	12.05.1981
MEBRG 4020	Guatire, Miranda	13.06.1981
MEBRG 4021	Guatire, Miranda	17.06.1981
MEBRG 4033, MEBRG 4034	P.N Henri Pittier, Aragua	03.10.1981
MEBRG 5097	P.N Henri Pittier, Aragua	14.07.2004
MEBRG 5510	P.N Henri Pittier, Aragua	19.05.2005
MEBRG 89	n/a	n/a

**La Salle Natural History Museum (MHNLS)**

<i>Catalog No.</i>	<i>Place of Collection</i>	<i>Date of Collection</i>
MHNLS 176, MHNLS 177, MHNLS 178, MHNLS 182	El Hatillo, Miranda	19.02.1950
MHNLS 793	Chivacoa, Yaracuy	04.03.1953
MHNLS 1076	Rancho Grande, Aragua	17.04.1955
MHNLS 1077	Rancho Grande, Aragua	30.04.1955
MHNLS 1179	Rancho Grande, Aragua	10.09.1955
MHNLS 1306a	Sebucan, Distrito Capital	19.04.1956
MHNLS 1321, MHNLS 1322	P.N El Ávila, Distrito Capital	17.06.1956
MHNLS 1416a, MHNLS 1416b	Caraballeda, Vargas	14.12.1957
MHNLS 6828, MHNLS 6829	Las Cascadas, Distrito Capital	09.05.1962
MHNLS 1704, MHNLS 1705, MHNLS 1706	P.N El Ávila, Distrito Capital	01.04.1963
MHNLS 2691	Bárbula, Carabobo	10.02.1964
MHNLS 1968, MHNLS 1969	Las Cascadas, Distrito Capital	18.10.1964
MHNLS 4327, MHNLS 4328	Rancho Grande, Aragua	07.10.1965
MHNLS 1907, MHNLS 1909, MHNLS 1910, MHNLS 1922	P.N San Esteban, Carabobo	18.12.1965
MHNLS 2432	n/a	06.02.1967
MHNLS 4424	P.N El Ávila, Distrito Capital	15.04.1968
MHNLS 5542, MHNLS 5543	Rancho Grande, Aragua	16.05.1971
MHNLS 5744, MHNLS 5745, MHNLS 5747	Rancho Grande, Aragua	11.06.1972
MHNLS 6591, MHNLS 6594, MHNLS 6596, MHNLS 6597, MHNLS 6598, MHNLS 6599, MHNLS 6600, MHNLS 6603, MHNLS 6604, MHNLS 6606	Patanemo, Carabobo	01.09.1976
MHNLS 6864, MHNLS 6865	P.N Henri Pittier, Aragua	06.08.1977
MHNLS 7522, MHNLS 7525, MHNLS 7526, MHNLS 7528, MHNLS 7529, MHNLS 7540, MHNLS 7541, MHNLS 7542, MHNLS 7543, MHNLS 7549, MHNLS 7551, MHNLS 7553, MHNLS 7555	Cerro Azul, Cojedes	26.02.1979
MHNLS 11944	Cerro Azul, Cojedes	31.03.1980
MHNLS 8453, MHNLS 8454, MHNLS 8456, MHNLS 8458	Cerro Azul, Cojedes	03.04.1980

Appendix II. Key Parameters for Assessing Parasite Populations

a. Operational Protocol for Laboratory Procedures

Technique	Protocol
Parasitological necropsy	1. <b>Initial Incision and Examination:</b> Make a longitudinal incision along the midline of the animal's abdomen to access the coelomic cavity. Visually inspect for parasites in situ before removing any internal organs.
	2. <b>Organ Removal:</b> Clip at the base of the tongue and gently pull downward while carefully cutting with sharp scissors until reaching the rectal ampulla. In female specimens, first separate and remove the egg sacs to avoid damaging internal structures. Handle all tissues delicately to ensure a thorough examination and minimize tissue damage.
	3. <b>Tissue Preparation:</b> Place the extracted tissues in petri dishes containing 0.9% saline solution to maintain structural integrity and facilitate examination. Use stereoscopic magnifiers (Carl Zeiss, with 20x, 40x, and 80x magnifications) and dissection needles for: Extracting visible parasites from the body cavity or organs. Carefully opening and dissecting each organ to search for hidden parasites.
	4. <b>Parasitic Quantification:</b> Count all parasites in situ using a manual counter to ensure accurate enumeration.
Preservation and Identification of Helminths	1. <b>Helminth Preservation:</b> Initial Fixation: Immerse all helminths in 70% ethyl alcohol at boiling temperature for fixation. Storage Protocols: Cestodes: Preserve in an alcohol-formaldehyde-acetic acid (AFA) solution. Nematodes: Store in 70% ethyl alcohol.
	2. <b>Morphotaxonomic Identification:</b> Clarify the helminths in lactophenol to make internal structures visible. Prepare temporary wet mounts using glycerin for microscopic examination. Perform a detailed morphological assessment to ensure accurate taxonomic identification.

b. Parasite populations parameters

Parameter	Formula	Description	Interpretation
<b>Helminth Infection Composition index</b>	$HICt = nt/N$ $HICt = (nt/N) \times 100$	nt = number of hosts classified in infection type t, N = total number of hosts examined	Proportion—expressed as a fraction or percentage—of hosts assigned to each category of parasitic infection. Enables direct comparisons across host groups and environmental contexts.
<b>Parasite Burden</b>	$PB = \sum_{i=1}^n pi$	n = number of hosts in the group (all hosts, or by sex/locality), pi = number of parasites in host i	Total number of helminths recovered from a host population (or a subset, e.g., by sex or locality), regardless of parasite species
<b>Abundance</b>	$A = \sum_{i=1}^n pi$	n = total number of hosts examined, pi = number of parasites in host i	Total number of individuals of a particular parasite species recovered from all examined hosts (infected and uninfected)
<b>Relative Abundance</b>	$RAi = \frac{Ai}{\sum Aj} \times 100$	Ai = total number of parasites of species i, s = total number of parasite species, $\sum Aj$ = total abundance of all parasite species	Proportion (or percentage) of individuals of a parasite species relative to the total number of all parasites recovered from all hosts
<b>Prevalence</b>	$P = i/n \times 100$	i = number of positive hosts, n = number of hosts tested	Number of infected hosts
<b>Mean Intensity</b>	$MI = \sum pt/i$	$\sum pt$ = total number of parasites, i = number of positive hosts	Number of individuals of a parasite species present in the total number of infected hosts
<b>Mean Abundance</b>	$MA = \sum pt/n$	$\sum pt$ = total number of parasites, n = number of hosts examined	Average number of individuals of a given parasite species present in the total number of infected or uninfected hosts
<b>Infection index</b>	$ii = MA (P/100)$	MA = mean abundance, P = prevalence	Number of individuals of a parasite species present in a single infected host
<b>Aggregation coefficient</b>	$k = \bar{X}^2 / S^2 - \bar{X}$	$\bar{X}$ = arithmetic mean, S <sup>2</sup> =variance	<b>k ≈ 1:</b> Random distribution. Parasites are distributed evenly among hosts, with no tendency to clump together. <b>k &lt; 1:</b> Uniform or dispersed distribution. Parasites are distributed more evenly than randomly, meaning there is less variation in the number of parasites per host. <b>k &gt; 1:</b> Aggregate distribution. Parasites tend to cluster in certain hosts, while others have few or no parasites. This is common in many parasitic infections.
<b>Morisita dispersion index</b>	$Imor = n \frac{\sum pc^2 - \sum pc}{(\sum pc)^2 - \sum pc}$	n = number of hosts examined, $\sum pc$ = total number corresponding to each parasite	<b>Imor = 1:</b> Indicates a random distribution with no discernible pattern. <b>Imor &lt; 1:</b> Suggests a uniform distribution, where parasites are evenly spaced across hosts. <b>Imor &gt; 1:</b> Indicates an aggregated distribution, where parasites are concentrated in certain hosts rather than being evenly or randomly distributed.

**Appendix III.** Diagnostic Morphotaxonomic Characters of Helminths Recorded in *Atelopus cruciger*.

The following table summarizes the primary and secondary morphotaxonomic characters used to identify helminths of *Atelopus cruciger*. Primary characters (e.g., buccal capsule morphology, scolex structure, ovary arrangement) follow standard taxonomic keys [39,45–52], whereas secondary characters (e.g., cuticular spines, esophageal denticles, gubernaculum) derive from supplementary traits observed in our specimens. Characters are grouped by morphological category to facilitate species-level comparison and identification.

Morphological Category	Character	<i>Cylindrotaenia americana</i>	<i>Oswaldocruzia venezuelensis</i>	<i>Rhabdias pseudosphaerocephala</i>	<i>Cosmocerca podicipinus</i>	<i>Aplectana</i> sp.
<b>Dimension</b>	Body length	2.5–4.0 cm	males: 0.65 ± 0.07 cm females: 1.30 ± 0.25 cm	1.1–1.3 cm	males: 2.42 ± 0.42 cm females: 4.75 ± 1.43 cm	0.2–0.7 cm
<b>Head/Anterior Region</b>	Buccal capsule	-	Small, without evident lips	Without lips or pseudolips	Well-developed	Small, poorly developed
	Cephalic papillae	-	-	-	Four, prominent	Six, arranged in three pairs
	Scolex	Without rostellum or hooks; with discrete suckers	-	-	-	-
<b>Body/Cuticle</b>	Cuticle characteristics	Thin, fragile wall with transparent eggs	Fine striations; posterior end with cuticular spines; faint longitudinal bands	Smooth, without notable inflations	Evident transverse striations; posterior end with cuticular spines	Fine transverse striations; conical caudal end
	Body shape	-	Cylindrical with slight dorsal curvature	Elongated with slight mid-body widening	Cylindrical with slight dorsal curvature	-
	Cervical alae	-	-	-	Present in anterior region	Absent
	Lateral cords	-	Well-defined	-	-	-
<b>Digestive System</b>	Esophagus	-	Divided into muscular and glandular portions; esophageal denticles visible in fresh specimens	Muscular, without posterior bulb	Short and robust	Short and thick
	Digestive system	-	-	Straight, without marked internal differentiations	-	-
<b>Reproductive System</b>	Proglottids	Wider than long; mature with transparent genital organs; unilateral genital pores	-	-	-	-
	Uterus/Ovaries	Sacciform uterus without lateral branches	Opisthodelphic ovaries	Didelphic ovaries; ovoviviparous females with larvae in uterus; vulva displaced posteriorly	-	Short oviducts connected to uterus via narrow duct
	Vulva position	-	Displaced toward posterior region	Mid-body region	-	-
	Male structures	Multiple spherical testes per proglottid; posterior end expanded into lobes	Several caudal papillae	-	Curved, robust spicules; numerous precloacal papillae	Slender, elongated spicules; well-developed gubernaculum
	Sexual dimorphism	-	-	-	Females larger than males, with pronounced size difference	Females significantly longer than males
	Excretory glands	-	Large, branched	-	-	-
<b>Other Structures</b>	Sensory papillae	-	-	-	-	Reduced in number and size
	Nervous system	-	-	-	-	Distinct anterior ganglia