

The Mesoamerican giant toad (*Rhinella horribilis*) as bioindicator of vegetation degradation in a tropical forest

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Identifying species that can serve as bioindicators of environmental quality is essential for monitoring the anthropogenic impact. Common and widespread species can be ideal bioindicators due to their abundance and easy monitoring, but a confirmation of their differential responses as a function of habitat perturbation is needed. Because amphibians are known as good bioindicators of environmental perturbation, we conducted this work to identify whether a common, generalist amphibian species, the Mesoamerican giant toad (*Rhinella horribilis*), could serve as a bioindicator of environmental degradation in a tropical forest. We sampled toads in two areas of tropical forest that differed in anthropogenic degradation (primary vs. secondary forest), establishing in each of these areas two sections of the same surface area but differing in substrate (grass vs. sand). We analyzed toad abundance, sex ratio, body length and condition, and the amount and distribution across the body of ectoparasites (ticks). We analyzed 59 toads that were infested with 503 ticks. Based on a multi-model inference approach, the results suggested that toads were more abundant and had lower body condition in the secondary than in the primary forest. In the secondary forest, females were proportionally less abundant than males. The tick loads responded to an interaction of the body area with either the forest type or the substrate, with increased occurrence of ticks in toads from secondary forests and from grass sections. The differences found between the primary and secondary forests in sex ratio, toad abundance, body condition, and tick load across body regions are consistent with previous studies in other less common species of amphibians and thus posit *R. horribilis* as a good bioindicator of anthropic disturbance in this tropical forest.

Key words: amphibian parasites; conservation; ecosystem degradation; ticks; tropical forests.

Deforestation of tropical forests is increasingly threatening biodiversity (GIBSON *et al.*, 2011). The ecological responses to deforestation may vary across taxonomic groups (e.g. ESTRADA-PEÑA &

VENZAL, 2006). Unfortunately, our knowledge of these responses is scarce and usually restricted to taxonomic groups with economic interest (DAVIC, 2003; GIBSON *et al.*, 2011). However, other organisms

have ecological relevance due to their important role in ecosystems, their cultural importance, or their intrinsic value for their uniqueness. In this sense, identifying the effects of anthropogenic disturbance in all taxonomic groups and the relationships between them is critical for anticipating possible cascading effects of anthropization on ecosystems (HERNÁNDEZ-ORDÓÑEZ *et al.*, 2019). As studying all groups is problematic because of the high costs and time resources needed, focusing efforts on potentially bioindicator species is a realistic alternative.

Candidate species to be good bioindicators should hold some characteristics, including sensitivity to environmental changes, abundance, and easiness to monitor (e.g. ZHOU & DING, 2010; MEGÍA-PALMA *et al.*, 2020). Furthermore, the protocols used for sampling potentially bioindicator species must be innocuous for both the species and the environment. In this sense, amphibians are one of the groups of terrestrial vertebrates most commonly used as bioindicators because of their inherent connection to both aquatic and terrestrial ecosystems, their sensitivity to environmental perturbations, and their limited dispersal capability (DAAM *et al.*, 2020; ZHANG *et al.*, 2020). The combination of these characteristics make amphibians excellent bioindicators of environmental degradation (ÁLVAREZ-GRZYBOWSKA *et al.*, 2020). Indeed, anthropogenic disturbances such as land use change and the associated habitat destruction are considered as one of the main threats to amphibian populations (LARA-TUFIÑO *et al.*, 2019).

Costa Rica is in one of the top biodiversity hot spots in the world. It has a wealth

of amphibian species, many of them endemic, and some threatened (LIPS *et al.*, 2005). However, there is scarce information about the impacts of human activities on amphibian biodiversity in the country, and even less about the parasites they have or the relationship between them. In this sense, the relationship of the species with its parasites can be very informative about the environment where it inhabits. Unlike the analyses of endoparasite abundance, ectoparasites can be studied with non-invasive techniques, according with the desirable characteristics of a good bioindicator described above. We postulate that ectoparasites can also serve as additional biomarkers of anthropogenic disturbance in tropical forests, because environmental degradation may influence the parasitic prevalence and the intensity of infections (MCKENZIE, 2007; MEGÍA-PALMA *et al.*, 2020). However, the relationship between habitat quality and parasite abundance can be ambiguous (MARCOGLIESE, 2005) and, hence, the use of more than one biomarker is recommended.

Hosts concentrate ectoparasites on certain areas of their body, which may be an adaptive anti-parasitic strategy that contributes to a better control of the infestation (ARNOLD, 1986; SALVADOR *et al.*, 1999; MEGÍA-PALMA *et al.*, 2018). We hypothesize that this antiparasitic strategy is impaired in disturbed habitats. In this sense, preferences of ticks for specific sites in the body of amphibians they parasitize have rarely been studied. When parasitizing toads, ticks of the genus *Amblyomma* may prefer attaching to the dorsal coelomic area (MENDOZA-ROLDAN *et al.*, 2020). However, there is no evidence of whether the distri-

bution of ticks on the body of amphibian hosts could vary in habitats with different degrees of anthropic disturbance. Should this occur, we expect to observe a differential distribution of ticks in toad bodies between habitats differing in degradation, likely with wider infestations across several body regions in toads occupying more degraded areas. This would suggest an impact of habitat degradation on this host-parasite relationship (e.g. LAZIĆ *et al.*, 2017; THAWLEY *et al.*, 2019).

Significant differences in amphibian traits are expected to arise between disturbed and well-preserved habitats, both at the population and the individual levels: (i) generalistic amphibian populations are expected to be more abundant, but with a lower proportion of females in degraded habitats (HUSTÉ *et al.*, 2006; KARRAKER *et al.*, 2018). The sex imbalance favoring males upon a scenario of presumed lower food resources is explained because males are smaller than females and thus generally have lower nutritional demands (HUSTÉ *et al.*, 2006; KARRAKER *et al.*, 2018). Moreover, male toads are typically more competitive and may have better survival prospects even in degraded habitats (ARNTZEN, 1999; KARRAKER *et al.*, 2018). (ii) Amphibians may have smaller bodies in poor quality environments, because growth rates may be reduced due to the lower availability of food resources and upon a putative higher competition context (ESTEBAN *et al.*, 1999, GONZÁLEZ-BERNAL *et al.*, 2016). This can lead to slower growth during development, resulting in smaller individuals. Moreover, the presence of stressors, such as pollutants or human activity, can lead to reduced lifespan in organisms, and hence

younger populations of individuals with smaller body size in species with indeterminate growth (PIKACHA *et al.*, 2015). (iii) Disturbed environments and parasites are also expected to impact amphibian body condition (BOSCH *et al.*, 2000, also see KARRAKER & WELSH, 2006). (iv) Disturbed habitats can influence the intensity and distribution of ectoparasites across the toads' body areas.

We compared several traits between individuals of a generalist amphibian species, the Mesoamerican giant toad, inhabiting forest areas with different degree of environmental degradation. Our aim was to determine habitat-related differences in these traits and contrast if the direction of those differences matched the above predictions with the purpose of confirming whether the Mesoamerican giant toad could serve in further studies as a bioindicator of ecosystem alteration in this tropical forest.

MATERIALS AND METHODS

Sampling

The study was performed between May 11th and 16th, 2019 in the Pacuare Matina Natural Reserve, Costa Rica. We studied the Mesoamerican giant toad, *Rhinella horribilis* (Anura: Bufonidae), in two sampling locations with different level of anthropogenic perturbation and connected by a matrix of forest. The approximate distance between the sampling locations was 6 km. The northern location (latitude 10.208°, longitude -83.265°) was surrounded by a primary forest, while the southern location (10.167°, -83.234°) was surrounded by a secondary forest. The anthropic pressure was more intense in the southern location

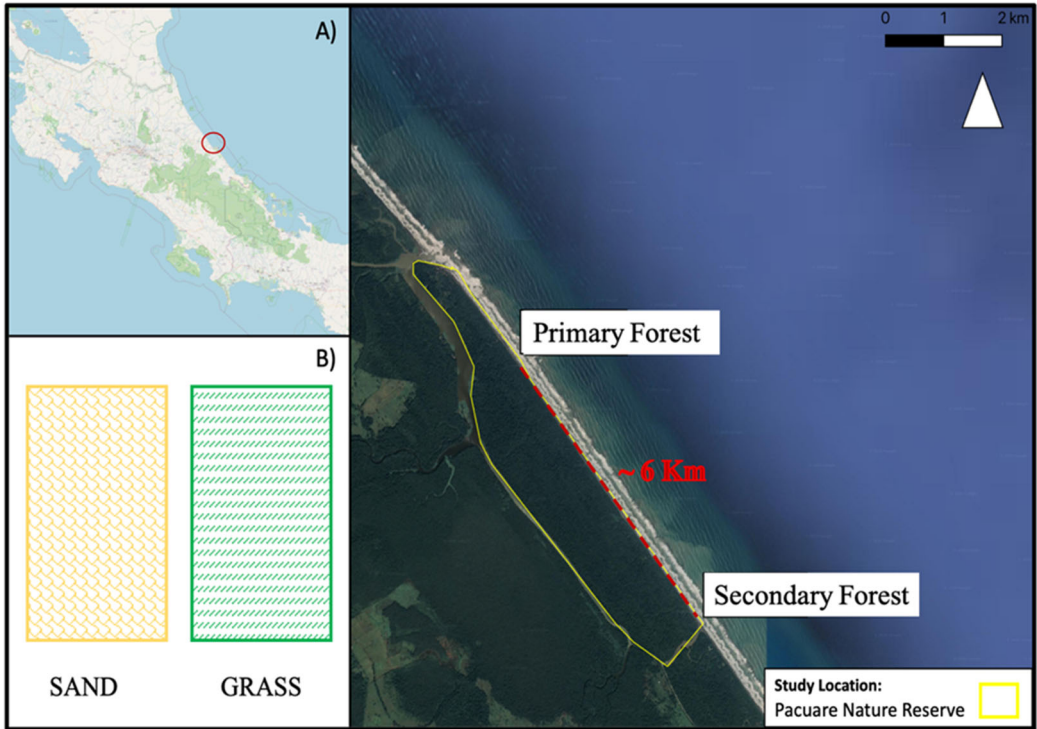


Figure 1: (A) Geographical location of the sampling site in the Pacuare Matina Nature Reserve of Costa Rica, which is delimited by the yellow line in the right picture. The white arrow indicates North. (B) Simplified graphical representation illustrating two distinct plots sampled within both primary and secondary forests, possessing identical area and shape but varying in substrate composition. Line drawings within boxes do not represent sampling itineraries. The distance between the two plots was 150 m in the secondary forest and 200 m in the primary forest.

due to the presence of farms and houses (Fig. 1a).

We standardized the sampling effort between sampling locations based on the prospected surface area and the invested sampling time. In each one of the two sampling locations, we defined two plots of 15 m x 50 m each (750 m²). One of the plots from each sampling locations (hereafter, grass) had gramineous plants as substrate and no tree cover, while the substrate in the other plot (hereafter, sand) was beach sand (Fig. 1b). Coconut trees (*Cocos nucifera*)

and beach almond trees (*Terminalia catappa*) overflow the sand plot at both sampling locations. The distance between the two plots was 150 m in the secondary forest and 200 m in the primary forest. This is consistent with the spatial ecology of *Rhinella* because toads usually re-use shelters and move on average less than 10-63 m between days, although some toads may move up to 200 m in one night before returning to their shelters (DEVORE *et al.*, 2021). All plots were sampled for three consecutive days. Plots were examined by

two people after the nightfall of each day between 19:30 and 20:00. To avoid pseudoreplication, the captured toads were held in captivity during the three days that the sampling was being performed. These toads were held at environmental temperature, with high humid conditions, and protected from direct sunlight. After that, the toads were released in the same spot where they had been collected.

Vegetation analysis

To investigate if the vegetation quality significantly varied between the primary and secondary forests, satellite radiation intensity data were obtained by remote sensing of electromagnetic bands that reflected the quantity, quality, and development of vegetation (HAYES & SADER, 2001; KIM *et al.*, 2007). This satellite data provided both the Normalized Difference Vegetation Index (NDVI) and the Enhanced Vegetation Index (EVI). The latter corrects the effect of some atmospheric components and is more sensitive than the former in areas with dense vegetation (Kim *et al.*, 2007). In both cases, we used satellite images as close to the dates where the sampling took place as possible, although avoiding satellite photos taken in cloudy days. The data on which NDVI was based were taken by the Moderate Resolution Image Spectroradiometer (MODIS) from May 16th to 25th, 2019 (EROS, 2019). The EVI was based on the multispectral data taken in this case by the Landsat 8 satellite (<https://eos.com/find-satellite/landsat-8/>) taken on May 8th, 2019. EVI was calculated using the formula applied to the data taken by the satellite as follows:

$$EVI = 2.5 * \left(\frac{(B5 - B4)}{(B5 + 6 * B4 - 7.5 * B2 + 1)} \right)$$

where B5 is the light band reflected in the near infrared wavelength, B4 the red spectrum, and B2 the visible spectrum.

We used the QGIS 3.10.0 free-code geographic information system (QGIS.org, QGIS Geographic Information System. QGIS Association. <http://www.qgis.org>) to process and analyze the satellite images. We sampled 3000 points at each sampling location, avoiding roofs and water bodies. Data from both indices did not follow a normal distribution and they could not be effectively transformed; therefore, we applied an unpaired Wilcoxon-Mann-Whitney non-parametric test with $\alpha = 0.05$ to compare the NDVI and EVI scores from both sampling locations.

Biomarkers

We estimated the abundance of toads by counting them in the four (two per forest type) sampled plots. As body length measure, we measured their snout-urostyle length using a caliper (± 1 mm) (MARAGNO & SOUZA, 2011; ESTEBAN *et al.*, 1999). All individuals included in this study measured more than 90 mm, a body length at which they are considered adults (HUDSON *et al.*, 2020). Thus, the adult toads captured were identified as males when they emitted vocalizations and / or had a nuptial callus on the first toe of the forelegs (BOWCOCK *et al.*, 2008; ARANTES *et al.*, 2015). We weighed toads with an analogical scale (± 0.1 g) and a body condition index was calculated taking the standardized residuals (mean = 0; standard deviation = 1) of the relationship between the

\log_{10} -transformed values of weight and body length (DUNLAP & MATHIES, 1993). Thus, individuals with positive mass residual values had better body condition than the average of the sample for a given body length, while negative mass residual values corresponded to individuals with worse body condition than the average.

Ticks were identified following BERMÚDEZ *et al.* (2018) and were quantified attending to their location on the toads' body regions. The division of body regions was made according to changes in skin thickness and contact with the ground. These were gular sac, ventral zone, dorsal coelomic area, cloaca, forelimbs, and rear limbs, following BURGON *et al.* (2012).

Statistical analyses of population traits and individual morphology

All the statistical analyses were performed in R v.3.6.2 (R CORE TEAM, 2019). A significance threshold of $\alpha = 0.05$ was assumed. We used a McNemar Chi-square

test to analyze if sex ratios differed between sampling localities. We used linear models (LM) to analyze the length and body condition of the toads. These traits were \log_{10} -transformed to improve model fit. Sex, number of ticks, and type of forest (primary vs. secondary) were included as predictors. We applied a model selection methodology implemented in the information theory (SAKAMOTO *et al.*, 1986), based on the Akaike Information Criterion corrected for small sample sizes (AICc) (AKPA & UNUABONAH, 2011). We also applied a model averaging method implemented with the R package MuMIn (BARTON, 2018). The latter method reduces the number of predictors initially included in the model (HEGYI & GARAMSZEGI, 2011; SYMONDS & MOUSSALLI, 2011). We considered all likely models with a difference of $AICc \leq 4$ (BURNHAM & ANDERSON, 2004). We also z-standardized the estimates to make comparable the magnitude of the effects.

Table 1: Results of model averaging for body length and body condition of *Rhinella horribilis*. The models with an AICc value within two units of the lowest AICc are considered. Significant model terms are shown in bold. Adj SE: adjusted standard error.

Variable	Source of variability	Importance	Estimate	Adj SE	z-value	P-value
Body length	(Intercept)		2.76	0.03	82.934	< 0.001
	Forest Type	1.00	-0.18	0.04	4.512	< 0.001
	Ticks	0.23	0.00	0.00	0.249	0.803
	Sex	0.19	-0.01	0.05	0.061	0.952
Body condition	(Intercept)		0.10	0.07	1.535	0.125
	Forest Type	0.90	-0.14	0.07	2.214	0.027
	Ticks	0.28	0.00	0.01	0.822	0.411
	Sex	0.23	0.03	0.16	0.412	0.681

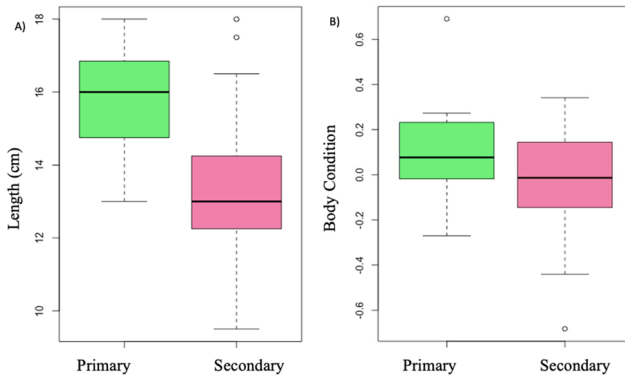


Figure 2: Differences between primary and secondary forest areas in (A) body length and (B) body condition of *Rhinella horribilis*. Dark lines represent the median value, boxes the interquartile range and whiskers indicate 25% of scores below and above the first and third quartiles, respectively.

Statistical analyses of ticks and type of forest and substrate

We used a McNemar Chi-square test to analyze differences in tick prevalence between sampling locations. We also compared the number of ticks in relation to the type of substrate and their distribution on the body of toad hosts in primary *versus* secondary forests. To do the latter analysis, we applied the same methodology as for the length and body condition described above, following the AICc and model averaging. Thus, we fitted a generalized mixed model (GLMM) with gamma residual distribution linked to an inverse function where the toad individual was the random factor. A random value of two units was added to every tick count and the new obtained values were \log_{10} -transformed to improve model fit (MEGÍA-PALMA *et al.*, 2023). The two-unit addition was done to avoid zeroes and hence \log_{10} -transformed negative values that are not allowed in Gamma distributions. Fixed predictors included the host's body area and a two-way interaction between substrate and forest type. We tested the parametric assumptions of the model using Shapiro-Wilks (normality), Ramsey Reset

(linearity), Ascombe (kurtosis) and Agostino (skewness) tests on the model residuals.

RESULTS

Quality of the vegetation across sampling locations

For the primary forest, the mean \pm standard error (SE) NDVI was 0.75 ± 0.001 , and the EVI was 0.7722 ± 0.003 . For the secondary forest, the NDVI was 0.70 ± 0.002 , and the EVI was 0.7693 ± 0.001 (NDVI: $W = 4.72 \times 10^6$, $P < 0.001$; EVI: $W = 7.02 \times 10^6$, $P < 0.001$). The spectral indices consistently supported a good discrimination of vegetation quality between forest areas, with a higher complexity of vegetation composition in the primary forest compared to the secondary one, in line with our initial expectations.

Toad abundance and sex ratio

We found 59 toads in the sampling plots of the two forest areas. In the primary forest, three toads were found in the sand and 13 in the grass, and in the secondary forest 13 toads were found in the sand and 30 in the grass. In the primary forest, the sex ratio was of 15 females per male, while in the secondary forest it was

Table 2: Results of the Chi-square test for tick loads on *Rhinella horribilis* after model selection. Significant model terms are shown in bold.

Source of variability	χ^2	df	P-value
Intercept	107.46	1	< 0.001
Body area	7.79	5	0.168
Forest	0.0027	1	0.959
Substrate	2.21	1	0.137
Body area: Forest	25.79	5	< 0.001
Body area: Substrate	12.95	5	0.024

of 4.38 females per male. This difference in sex ratios between the forest areas was significant ($\chi^2 = 30.25$, $P < 0.001$), with a lower proportion of females in the secondary forest.

Body length and condition in relation to forest type

Differences between forest areas in body length and body condition were significant. The toads sampled in the primary forest were longer (15.79 ± 0.37 cm vs. 13.32 ± 0.28 cm), heavier (290.31 ± 16.42 g vs. 168.48 ± 11.05 g) and had better body condition than in the secondary forest (Table 1, Fig. 2).

Prevalence and intensity of tick infestation in relation to forest type

Most of the observed ticks were at the nymphal stage and were identified as the iguana tick, *Amblyomma dissimile* (Acari: Ixodidae) BERMÚDEZ *et al.* (2018). This species commonly infests toads, lizards, and snakes in the Neotropical region (DUNN, 1918). We counted in total 503 ticks and 76.27% (45/59) of the toads were infested.

Infestation prevalence was 62.5% in the primary forest and 81.4% in the secondary forest ($\chi^2 = 0.056$, $P = 0.814$). The number of ticks on a single host varied from 0 to 13 in the primary forest and from 0 to 146 in the secondary forest. The dorsal coelomic area (with a total of 184 ticks) and gular sac (with 220 ticks) were the body areas with the highest tick loads. Interactions between body area and both forest and substrate

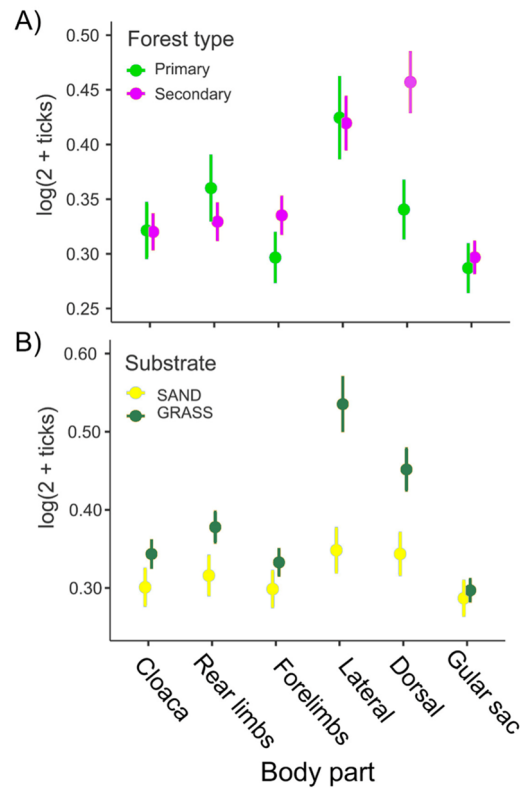


Figure 3: Mean \pm SE tick load values per body areas of *Rhinella horribilis* across types of (A) forest and (B) substrate. Tick infestation was higher on the dorsal area of the toads sampled in the secondary forest as well as in the dorsal and lateral areas of the body of those toads sampled on the grass substrate.

types had significant effects on tick load (Table 2). The dorsal coelomic area was more intensely parasitized in the secondary forest (Fig. 3a). Moreover, toads sampled on the grass in the secondary forest had more ticks on the cloaca, rear limbs, lateral, and dorsal coelomic areas of their bodies (Fig. 3b).

DISCUSSION

Toad abundance in the secondary forest was 2.7 times higher than in the primary one, which aligns with previous findings pointing that some generalist species, like *R. horribilis*, may thrive in degraded habitats by increasing their reproductive effort (GEUE & PARTECKE, 2008; LUCAS & FRENCH, 2012; KLAUS & NOSS, 2016; HERNÁNDEZ-ORDÓÑEZ *et al.*, 2019). Particularly, males were three times more abundant in the secondary forest, suggesting that they may cope with the putative lower food resources in this degraded habitat better than females, as the latter generally have higher nutritional demands (HUSTÉ *et al.*, 2006; KARRAKER *et al.*, 2018). Moreover, male toads are typically more competitive and may have better survival prospects than females even in degraded habitats (ARNTZEN, 1999; KARRAKER *et al.*, 2018). Nonetheless, the lower body length and condition of the toads in the secondary forest, for which the analysis of satellite data on vegetation confirmed a habitat degradation, consistently suggested a negative impact of the degraded habitat on the toads (ESTEBAN *et al.*, 1999; CAREY, 2005; JANIN *et al.*, 2011; Arantes *et al.*, 2015; Cayuela *et al.*, 2017; IGLESIAS-CARRASCO *et al.*, 2017; BIONDA *et al.*, 2018).

Toads from the secondary forest had

higher tick loads than those from the primary forest on the dorsum, but not in the rest of body regions. That higher tick load on the dorsum was also found in the toads sampled on the grass compared to those from the sands; toads from grass patches also had higher tick loads on the rear limbs and body lateral areas. Although grass and sand substrates were present in both forest areas, grass surfaces in tropical regions result directly from anthropogenic deforestation because vegetation competes vigorously for light, leading to rapid recolonization of tree clearings by new vegetation (SCHNITZER *et al.*, 2005). Since grassy substrates can provide microenvironments with humidity and temperature conditions that are favorable to ticks, deforestation, and particularly in habitats with degraded surrounding vegetation (ESTRADA-PEÑA, 2001), may increase parasite pressure on toads. This said, nymphs of the iguana tick, *A. dissimile*, can remain attached to their hosts for a period between 11 and 22 days (DUNN, 1918), hence the influence of substrate type on tick loads may be cautiously interpreted because, although normally toads would not move in one night a distance long enough to have occurred in both patches during our samplings (they do not move longer than 200 m on average), those individuals with worse body condition could move longer distances in the search for food (DEVORE *et al.*, 2021).

The degradation of natural habitats, driven by human activities such as deforestation, can lead to alterations in the ecosystem that favor the proliferation of parasites such as ticks, attributed to the presence of herbaceous substrates. These parasites can directly affect the health and fit-

ness of amphibians like toads, potentially impacting population demography (LAMPO & BAYLISS, 1996; BOWER *et al.*, 2019).

The traits measured in *R. horribilis* differed between the primary and secondary forest. This variation is consistent with previous studies that investigated amphibian species as bio-indicators of habitat degradation. Consequently, the traits analyzed in *R. horribilis* can serve as biomarkers of vegetation degradation in this tropical system. The relationships found between forest type and toad abundance, sex ratios, body length and condition, and parasite burden across body areas of toads, highlight the complex interactions between the environment, organisms' health, and population dynamics.

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