

High prevalence of *Trypanosoma* infection in Iberian green frogs (*Pelophylax perezi*) and evidence of a negative relationship between blood parasites and two indices of frog body condition

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Trypanosoma commonly parasitizes anuran hosts but very few studies have investigated ecological relationships in multiparasitized amphibians. We analysed a sample of 29 adult Iberian green frogs (*Pelophylax perezi*) from a monitored population in central Spain and found that 28 of these individuals (96.5%) were infected with blood parasites. The protozoa genera *Lankesterella* (Apicomplexa: Eimeriorina) (72.4%) and *Trypanosoma* (Euglenozoa: Trypanosomatida) (69%) had the highest prevalence, followed by an intraerythrocytic bacteria of the genus *Aegyptianella* (Pseudomonadota: Rickettsiales) (31.0%). We also report an infection by hematic microfilariae (Nematoda: Spirurida) (6.8%), which to our knowledge represents the first documented case in Iberian amphibians. Infections with more than one parasite type occurred in 62.1% of the frogs. A multimodel inference approach indicated that the infection intensities of *Trypanosoma* and *Aegyptianella* were the most important predictors, both negatively affecting the body condition of the frogs. Furthermore, the number of leeches that frogs had when captured showed a strong positive association with *Trypanosoma* infection intensity. This suggests that leeches act as primary vectors for *Trypanosoma*. Our results revealed a high taxonomic diversity of blood parasites in green frogs, thus contributing to expand our knowledge of the biodiversity of Mediterranean wetlands and highlighted the potential negative effects of certain infections on the fitness of these amphibian hosts.

Key words: *Aegyptianella*; amphibian parasite diversity; *Lankesterella*; Mediterranean wetlands; microscopic diagnosis.

The immunological ability of hosts to respond to infections is influenced by the availability of energetic resources (MERINO *et al.*, 1996; MØLLER *et al.*, 1998; BLANCO *et al.*, 2001). Moreover, in ectotherms it is also conditioned by the thermal environment, because species differ in the thermal optimum at which their immune response most effectively operates (MEGÍA-PALMA *et al.*, 2020a; HERCZEG *et al.*, 2021). Amphibians, as other vertebrate hosts, usually harbour complex infracommunities of parasites that may undermine the overall energy available for the immune function, among other activities. Indeed, the ultimate outcomes of infections depend on the parasite taxa involved and the general health state of the host (JOHNSON & HOVERMAN, 2012). For example, the simultaneous occurrence of more than one parasite (i.e. co-infection) can produce strong (additive or even synergistic) effects on reducing fat storage and other reserves that otherwise could be allocated to key systemic functions (CATTADORI *et al.*, 2007; MABBOTT, 2018; HERCZEG *et al.*, 2021; but also see COMAS *et al.*, 2014). Parasitic infections can also favour or disfavour the invasion of subsequent parasites (HOVERMAN *et al.*, 2013). In this sense, a growing body of evidence supports the priority effect theory, where the parasite arrival order and timing (the simultaneous and/or sequential invasion of the host) strongly influence host-multiparasite interactions (CLAY *et al.*, 2019; HERCZEG *et al.*, 2021).

Assessing the health status of the host is pivotal to study the mechanisms and effects of parasitic infections, and thus body condition indices have been widely used in ecological studies as nutritional

status surrogates. In this regard, a meta-analysis investigated the negative effects of parasites across host taxa using different indices (SÁNCHEZ *et al.*, 2018). The use of these indices serves specifically to evaluate relationships between pre-existing infections and host nutritional status in amphibian populations when assessing the vulnerability of populations to emerging diseases (ROLLINS-SMITH, 2017). However, despite the potential negative impact of parasites on the fitness of host amphibians (e.g. HERNANDEZ-CABALLERO *et al.*, 2022), their pathogenic effects remain largely unknown (BARDSLEY & HARMSSEN, 1973; BOWER *et al.*, 2019).

A wide variety of hemoparasites have been reported in anuran hosts, such as intracellular haemogregarines (DAVIES & JOHNSTON, 2000; LEAL *et al.*, 2015; NETHERLANDS *et al.*, 2015, 2018), extracellular flagellates (FERREIRA *et al.*, 2015), and intracellular/extracellular coccidia (DUSZYNSKI *et al.*, 2007). In particular, European green frogs of the genus *Pelophylax* Fitzinger, 1843 (fam. Ranidae) are infected by blood protists of the genera *Trypanosoma* (Euglenozoa: Trypanosomatida), *Lankesterella* (Apicomplexa: Eimeriorina), *Dactylosoma* (Apicomplexa: Adeleorina), and *Hepatozoon* (Apicomplexa: Adeleorina), as well as microfilariae of the genus *Icosiella* (Nematoda: Spirurida) and intraerythrocytic bacteria of the genus *Aegyptianella* (Pseudomonadota: Rickettsiales) (WALTON, 1949; BARTA *et al.*, 1989; JIMÉNEZ-SÁNCHEZ, 1998; HERCZEG *et al.*, 2016; SPODAREVA *et al.*, 2018; PAVLÁKOVÁ *et al.*, 2024). However, the vectorial ecology of these genera of parasites is poorly known. Although *Trypanosoma* infections can be transmitted through

ectoparasitic leeches in the aquatic environment (O'DONOGHUE, 2017), adult frogs can also be infected by flying arthropods, whose role as vectors of these or other blood parasites remains understudied (BARTLETT-HEALY *et al.*, 2009; FERREIRA *et al.*, 2008; BERNAL & PINTO, 2016). Trypanosomiasis are important diseases in livestock and humans, e.g. sleeping sickness and Chagas disease (SWALLOW, 2000; BRUN *et al.*, 2010; RASSI & REZENDE, 2012). These infections cause immunosuppression and the eventual death of the host (GOODWIN *et al.*, 1972; ABRAHAMSOHN & COFFMAN, 1995). Furthermore, trypanosomiasis may impact male mate selection in birds, as evidenced by their association with variations in a secondary sexual character in female pied flycatchers, *Ficedula hypoleuca* (POTTI & MERINO, 1996).

The severity of trypanosomiasis may vary across anuran hosts and depends on the infecting *Trypanosoma* species (BARDSLEY & HARMSSEN, 1973, and references therein). There are more than 500 described species in the genus *Trypanosoma* and, even with the rapid progress of molecular taxonomic methods, phylogenetic relationships between clades of *Trypanosoma* that infect frogs are still unresolved (SPODAREVA *et al.*, 2018). Although negative effects on hosts were described for some *Trypanosoma*-hosts assemblages, these effects are not always obvious. For instance, no tissular damage produced by this parasite was found in infected rice frogs, *Hoplobatrachus rugulosus* (Wiegmann, 1834) from Thailand (SAILASUTA *et al.*, 2011). However, *Trypanosoma* infects the heart, kidneys, lungs, spleen, and peripheral blood of many frog

species, where parasites can replicate by a combination of both binary and multiple fissions, potentially leading to the death of the host (REICHENBACH-KLINKE & ELKAN, 1965; BARDSLEY & HARMSSEN, 1973). Given the global threat that invasive fungal and viral diseases pose for many amphibian populations (i.e. chytridiomycosis and ranavirosis, LESBARRÈRES *et al.*, 2012; SCHEELE *et al.*, 2019), there is an urgent need to further investigate the ecological relationships between amphibians and the autochthonous parasite communities with which they coexist (ROLLINS-SMITH *et al.*, 2017; HERCZEG *et al.*, 2021). In this context, it is especially relevant to study not only the potential negative effect of the parasites on their amphibian hosts, but also the interacting effects among different parasites (e.g. MEGÍA-PALMA *et al.*, 2022; HERCZEG *et al.*, 2021).

Co-infections may have a stronger impact on host health than infections by single parasites (BORDES *et al.*, 2011; KOTOB *et al.*, 2017; HERCZEG *et al.*, 2021 and references therein). In this study, we analysed how infection intensities (i.e. the number of parasites in a given host) of the different blood parasites found in adult Iberian green frogs, *Pelophylax perezi* (López-Seoane, 1885), are associated with their body condition. Several formulae describing the mass-body length relationship have been used in amphibians (BĂNCILĂ *et al.*, 2010; MACCRACKEN & STEBBINGS, 2012), of which an index inferred from the standardized residuals of the regression between body mass and body length has been shown to perform better than other body indices in tests to identify environmental effects on the health of individual

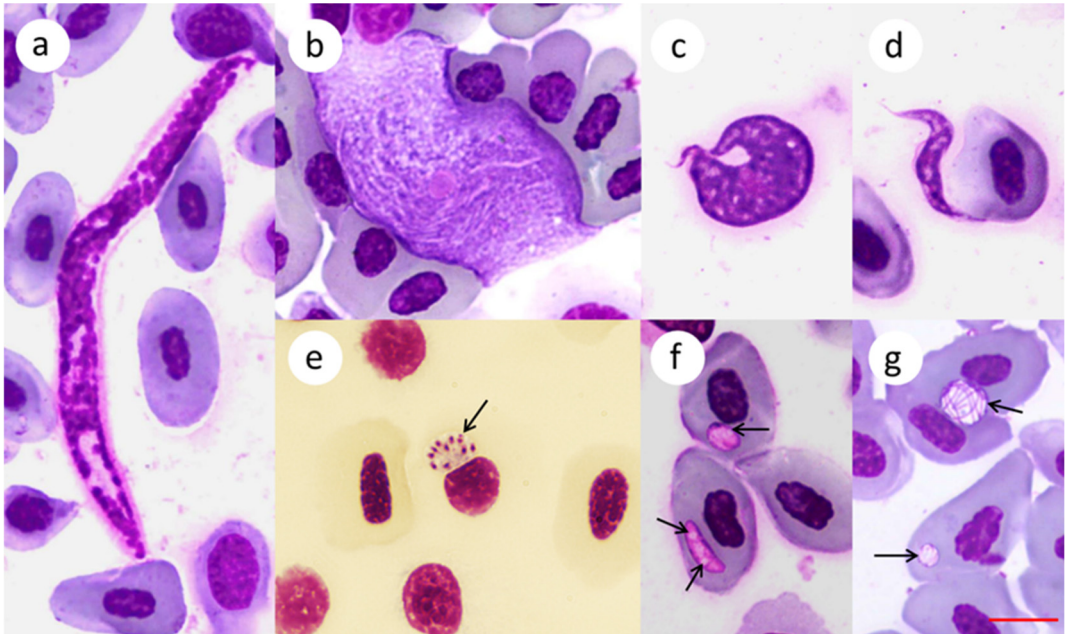


Figure 1: Microphotographs of blood parasites found in Iberian green frogs (*Pelophylax perezi*) in this study. (a) Unidentified microfilariae. (b-d) Genus *Trypanosoma*. (e) Meront of genus *Dactylosoma* in an hemolyzed erythrocyte. (f) Sporozoite of genus *Lankesterella*. The arrows indicate refractile bodies. (g) Intraerythrocytic bacteria of the genus *Aegyptianella* containing packed rickettsiae. Scale bar = 5 μm . All images were taken at the same scale.

amphibians (Băncilă *et al.*, 2010). In addition, the scaled mass index (PEIG & GREEN, 2009; 2010) can accurately reflect the content of protein and internal fat stores in small vertebrates, including amphibians (PEIG & GREEN, 2009; 2010; MACCRACKEN & STEBBINGS, 2012). We used these two metrics to assess consistency across body condition indices in the relationship between blood parasite infection and the health status of their Iberian green frog hosts.

MATERIALS AND METHODS

Sampling

We captured 12 female and 17 male Iberian green frogs (*P. perezi*) during the

2020 breeding season in a temporary pond in Central Spain (40.85°N, 3.65°W) that is the subject of a long-term population monitoring program (CAPELLÀ-MARZO *et al.*, 2020). All frogs were sexed based on sexually dimorphic characters (SALVADOR & GARCÍA-PARÍS, 2001), weighed to the nearest 0.1 gram (wet mass) and their body length was measured as the snout-to-vent length (SVL) to the nearest millimetre. We counted the number of leeches in each captured frog and collected blood samples (1–2 μL) from their brachial vein using insulin syringes (BD Micro-Fine, 0.5 ml, 30G). A drop of blood was smeared on a microscope slide and allowed to dry out, and later fixed with methanol 100%. We

stained the blood smears with a solution of Giemsa stain (1:10) for 40 minutes. The frogs were immediately released within a few minutes after data and sample collection, ensuring they behaved normally.

Quantification of blood parasites

A single researcher (RMP) analysed the blood smears. An area with a homogeneous distribution of blood cells at 100× magnification was screened in a light microscope (CX41, Olympus, Tokyo, Japan). In this area, intracellular and extracellular parasites were simultaneously counted at 1000× magnification in 40 microscope fields (~2000 cells). Thus, the intensity of infection is the number of parasites of each genus found in 2000 blood cells. To minimize observer bias, blind methods were used when we quantified parasite intensity because only a numerical code was associated with each blood smear. All the blood parasites found could be unambiguously identified based on morphological traits. Microfilariae are nematode worms with pluricellular hematic stages which are hence larger than other (unicellular) blood parasites (Fig. 1a). *Trypanosoma* has a central nucleus, a characteristic undulating membrane, and a flagellum (Figs. 1b-d). Intracellular parasites of the genus *Dactylosoma* (order Adeleorina) were identified due to the presence of meronts (Fig. 1e) whereas the presence of refractile bodies indicated the presence of genus *Lankasterella* (order Eimeriorina) (Fig. 1f) (MEGÍA-PALMA *et al.*, 2014). Intraerythrocytic bacteria of the genus *Aegyptianella* (order Rickettsiales) presented encapsulated rickettsiae (Fig. 1g).

Indices of body condition

We calculated two indices of body condition for the frogs. First, we calculated the standardized residuals (mean \pm SD = 0 ± 1) of the log-transformed values of body mass on body length (i.e. DUNLAP & MATHIES, 1993) (hereafter BCI) for males and females separately, because female frogs were larger and heavier (mean \pm SD = 81.42 ± 2.12 mm; 39.28 ± 3.50 g) than males (66.18 ± 1.19 mm; 21.07 ± 1.27 g) (ROLLINGS *et al.*, 2017). Thus, frogs with negative body mass residuals were lighter than the mean of the sample for a given SVL.

For the second index, we calculated the scaled mass index (SMI) of the frogs (PEIG & GREEN, 2009) as the reduced major-axis (LI, 2012) of the log-mass on log-body length for males and females separately using the rma.v1.21 software (BOHONAK, 2004; MEGÍA-PALMA *et al.*, 2020b). We calculated the exponential term of the formula proposed by PEIG & GREEN (2009) by dividing this reduced major-axis term by Pearson's correlation coefficient between log-mass and log-length. The resulting coefficients were z-standardized to partially remove the effects of sexual dimorphism in *P. perezi*.

Analysis of sexual differences in prevalence

Statistical analyses were run in R version 4.0.5 (R CORE TEAM, 2021). We analysed sexual differences in the one-by-one prevalence scores of the different blood parasites detected with separate Chi-square tests. In addition, we performed a Wilcoxon matched pair test to compare the overall prevalence of the different blood parasites, with sex as factor.

Analysis of frog body condition

We analysed the two indices of body condition with two independent general linear models. In each of the two models, either BCI or SMI was used as response variable, whereas the following set of variables were included as potential predictors in both models: the z-standardized date of capture of the frog, sex, body length, number of leeches, and the log-transformed infection intensities of each of the parasite genera found in the blood sample. In addition, we included the two-way interactions of the sex of the host with each of the intensities of the blood parasites and leeches. Microfilariae parasites and *Dactylosoma* were excluded from these analyses because they were only found in two frogs. In all the implemented models, we accounted for model collinearity by evaluation of variance inflation factor scores (all below 3.9 except factor sex, with a higher score of 6.65 because it was also added as a two-way interaction term with the different parasites) and confirmed assumptions of homoscedasticity, normality, and lack of both kurtosis and skewness of the distribution of model residuals using the libraries 'car' and 'moments' (KOMSTA & NOVOMESTKY, 2022; FOX & WEISBERG, 2019). We also performed an omnibus test on every model to confirm that the candidate models explained a significant portion of the variance as compared to a null random model.

We analysed the models by multimodel inference (BARTON, 2024) based on Akaike's criterion corrected for small sample size (AICc; BEDRICK & TSAI, 1994). The resulting candidate models were considered sufficiently informative and equally

likely when their $\Delta\text{AICc} \leq 2$ to the best model (i.e. the one with the lowest AICc coefficient) (BURNHAM & ANDERSON, 2004). Model averaging and model selection were used to obtain a final model and calculate the relative importance of each predictor (HEGYI & GARAMSZEGI, 2011). For this, only the models that included the effect (i.e. conditional average) were considered to estimate the magnitude of the effects of each predictor, which is based on a *P*-value ($\alpha < 0.05$) that derives from weighted averages across models within $\Delta\text{AICc} \leq 2$ with respect to the best model, their z-standardized β coefficient (estimate), and their adjusted standard error. For those predictors with effects estimated to be significant, we calculated the percentage of the variance explained by means of their sum of squares.

Predictors of the intensities of infection

As mentioned above, we calculated the intensity of infection of each parasite as the number of parasites found in 2000 blood cells. We analysed the intensities of infection of the different parasites in relation to the date of capture, sex, number of leeches found attached to the host, log-transformed intensity scores of the remaining blood parasites, and frog BCI and body length, the latter as a proxy to age (PATÓN *et al.*, 1991). We compared Gaussian linear models, where the intensity of infection of each parasite genus was log-transformed.

RESULTS

Blood parasites detected

The most prevalent blood parasite in the sample was *Lankesterella cf. minima* (Chaussat, 1850), occurring in 72.4% of the

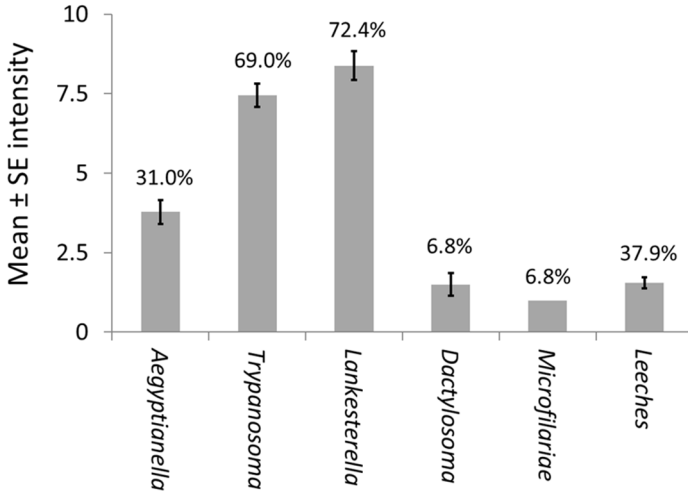


Figure 2: Mean ± SE intensity of infection of the parasites studied in the sample of infected adult Iberian green frogs (*Pelophylax perezi*). The prevalence (%) of each parasite is shown above the bars.

frogs, followed by extracellular parasites of the genus *Trypanosoma* (Euglenozoa: Trypanosomatida) in 69% of the specimens. We also found two female frogs (6.8%) infected by microfilariae nematodes. In addition, intracellular blood parasites *Dactylosoma* cf. *ranarum* (Lankester, 1871) infected 6.8% of the frogs, while intraerythrocytic bacteria *Aegyptianella* cf. *bacterifera* (Labbé, 1894) infected 31.0%. Co-infections by two or more genera of blood parasites occurred in 62.1% of the individuals. *Lankesterella* and *Trypanosoma* showed the highest mean intensities of infection

(Fig. 2). Ectoparasitic leeches were found in 37.9% (11/29) of the frogs.

Sexual differences in prevalence

We did not find significant differences between males and females in the prevalence of any of the infections or co-infections when performing one-by-one comparisons (Table 1). However, the pair test analysing the five blood parasites together indicated a significantly higher overall prevalence of blood parasites in female hosts ($Z = 2.02, P = 0.043$).

Table 1: Prevalence of blood parasites and ectoparasitic leeches in male and female Iberian green frogs (*Pelophylax perezi*). The term ‘co-infection’ refers to individuals infected simultaneously by two or more blood parasites. Partial chi-square tests revealed no significant differences between male and female frog hosts. However, females had an overall higher prevalence than males (see Results).

Parasite	Prevalence (%)			Contrast	
	Total	Males	Females	χ^2	P-value
<i>Lankesterella</i>	72.4	64.7	83.3	0.36	0.55
<i>Trypanosoma</i>	69.0	67	71	0.05	0.82
<i>Aegyptianella</i>	31.0	29.4	33.3	0.05	0.82
<i>Dactylosoma</i>	6.8	5.8	8.3	0.04	0.84
<i>Microfilaria</i>	6.8	0	16.7	3.04	0.08
Co-infections	62.1	52.9	75	1.45	0.23
Leeches	37.9	29	50	1.27	0.26

Table 2: Predictors of two indices of body condition (BCI and SMI) in Iberian green frogs (*Pelophylax perezi*), based on multimodel inference. The date of capture of the frog (Date) was z-standardized. The probability for the hypothesis contrast, $\Pr(>|z|)$, was based on the comparison with a random model assuming no deviation from the standardized mean (z) value; significant effects are highlighted in bold. Imp = importance; Exp. σ^2 = % of variance explained.

Index	Model terms	Imp	Estimate	Adj SE	z value	$\Pr(> z)$	Exp. σ^2
BCI	(Intercept)		0.023	0.024	0.946	0.344	-
	Date (stand)	0.21	-0.007	0.006	1.167	0.243	-
	Body length	0.06	0.000	0.001	0.057	0.955	-
	Sex	0.94	0.011	0.008	1.373	0.170	-
	log <i>Trypanosoma</i>	1.00	-0.033	0.013	2.616	0.009	13.62
	log <i>Aegyptianella</i>	1.00	-0.044	0.018	2.389	0.017	13.12
	log <i>Lankesterella</i>	0.06	-0.002	0.012	0.149	0.882	-
	Leeches	0.94	0.011	0.008	1.354	0.176	-
	Sex:log <i>Trypanosoma</i>	0.06	0.004	0.013	0.286	0.775	-
	Sex:log <i>Aegyptianella</i>	0.17	0.022	0.018	1.191	0.234	-
	Sex:log <i>Lankesterella</i>	0.00	0.014	0.011	1.294	0.196	-
	Sex:leeches	0.94	-0.022	0.007	3.241	0.001	24.24
SMI	(Intercept)		2.513	2.493	1.008	0.313	-
	Date (stand)	0.39	-0.267	0.182	1.468	0.142	-
	Body length	0.49	-0.049	0.032	1.568	0.117	-
	Sex	0.61	0.575	0.395	1.455	0.146	-
	log <i>Trypanosoma</i>	1.00	-1.004	0.390	2.576	0.010	13.63
	log <i>Aegyptianella</i>	0.90	-1.246	0.578	2.154	0.031	10.63
	log <i>Lankesterella</i>	0.04	-0.065	0.406	0.160	0.873	-
	Leeches	0.50	0.247	0.253	0.978	0.328	-
	Sex:log <i>Trypanosoma</i>	0.00	-0.093	0.431	0.216	0.829	-
	Sex:log <i>Aegyptianella</i>	0.06	0.577	0.595	0.969	0.332	-
	Sex:log <i>Lankesterella</i>	0.00	0.332	0.346	0.961	0.337	-
	Sex:leeches	0.46	-0.552	0.207	2.659	0.008	16.78

Infection intensity and body condition

The correlation coefficient between BCI and SMI was high ($r^2 = 0.81$). Three and four equally likely models explained the variance in BCI and SMI, respectively. Although the BCI model was better ranked than the SMI model ($\Delta\text{AICc} = -194$), they produced similar results (Table 2). The infection intensities of *Trypanosoma* and *Aegyptianella* were important predictors,

both with negative sign, of the body condition of the frogs in both models (Fig. 3). The interaction between the sex of the frogs and the number of leeches was significant in both models, although with lower importance than the blood parasites. Thus, female frogs with more leeches tended to have lower body condition (Pearson's $r = -0.55$), while the males showed the opposite trend (Pearson's $r = +0.37$).

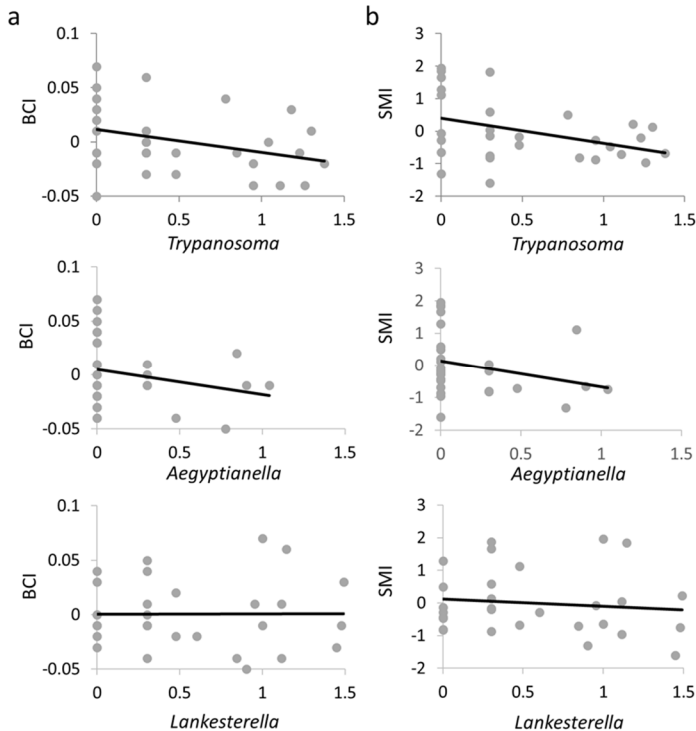


Figure 3: Relationships between the log-transformed intensity of the blood parasites with higher prevalence found in Iberian green frogs (*Pelophylax perezi*) and two body condition indices used to analyse their nutritional status. (a) Standardized residuals of body mass on body length (BCI), and (b) scaled mass index (SMI). The explanatory power (% of explained variance) of each parasite infection is shown in Table 2. The relationships with microfilariae and *Dactylosoma* are not shown because only two frogs were infected.

Predictors of infection intensity

The Gaussian models that used the log-transformed data of parasite intensity best fitted the statistical assumptions for parametric analyses for the three blood parasites analysed.

The intensity of infection by *Trypanosoma* was best explained by the number of leeches in frogs (estimate = 0.24), the body condition of the host (estimate = -7.30), and the intensity of *Aegyptianella* infection (estimate = -0.70). Thus, frogs with more leeches, lower body condition, and higher intensity of *Aegyptianella* infection had more *Trypanosoma* (Table 3).

The intensity of infection by *Aegyptianella* was best explained by the body condition of the host (estimate = -5.61), and the intensity of infection by *Trypanosoma*

(estimate = -0.39). Thus, lighter frogs with more severe infections of *Trypanosoma* had higher *Aegyptianella* infection intensities (Table 3).

None of the tested predictors explained a significant proportion of the variance in the infection intensity of *Lankesterella* (Table 3).

DISCUSSION

Our microscopy analyses displayed a relatively high diversity of blood parasites in Iberian green frogs in our study population from central Spain, which contrasts with the absence of blood parasites found in the same host species in southwestern Spain (PAREJO-PULIDO *et al.*, 2023). Environmental dissimilarities between sampling sites might explain such differences. For

Table 3: Predictors of the infection intensities of the genera *Trypanosoma*, *Aegyptianella*, and *Lankesterella* in Iberian green frogs (*Pelophylax perezi*), based on multimodel inference and model averaging. The date of capture of the frog (Date) was z-standardized. The probability for the hypothesis contrast, $\Pr(>|z|)$, was based on the comparison with a random model assuming no deviation from the standardized mean (z) value; significant effects are highlighted in bold. Imp = importance; Exp. σ^2 = % of variance explained.

Parasite	Model terms	Imp	Estimate	Adj SE	z value	$\Pr(> z)$	Exp. σ^2
<i>Trypanosoma</i>	(Intercept)		0.296	0.509	0.581	0.561	-
	Date (stand)	0.11	0.057	0.112	0.515	0.607	-
	Body length	0.11	0.008	0.014	0.550	0.582	-
	Body condition	0.84	-7.308	3.413	2.141	0.032	12.19
	Sex	0.05	-0.030	0.144	0.211	0.833	-
	<i>Aegyptianella</i>	0.76	-0.705	0.350	2.011	0.044	6.79
	<i>Lankesterella</i>	0.16	-0.175	0.243	0.719	0.472	-
	Leeches	0.81	0.240	0.118	2.027	0.043	12.00
<i>Aegyptianella</i>	(Intercept)		-0.241	0.582	0.413	0.680	-
	Date (stand)	0.13	0.077	0.092	0.844	0.399	-
	Body length	0.32	0.011	0.010	1.117	0.264	-
	Body condition	0.89	-5.613	2.768	2.028	0.043	14.04
	Sex	0.27	0.099	0.098	1.009	0.313	-
	<i>Trypanosoma</i>	0.84	-0.389	0.188	2.067	0.039	7.50
	<i>Lankesterella</i>	0.54	0.279	0.175	1.597	0.110	-
	Leeches	0.26	-0.118	0.110	1.069	0.285	-
<i>Lankesterella</i>	(Intercept)		-1.274	1.667	0.764	0.445	-
	Date (stand)	0.48	0.181	0.111	1.629	0.103	-
	Body length	0.74	0.034	0.019	1.748	0.081	-
	Body condition	0.07	1.000	3.514	0.284	0.776	-
	Sex	0.52	-0.281	0.183	1.538	0.124	-
	<i>Trypanosoma</i>	0.15	-0.177	0.249	0.713	0.476	-
	<i>Aegyptianella</i>	0.29	0.395	0.352	1.121	0.263	-
		Leeches	0.25	0.145	0.129	1.121	0.262

example, previous studies showed that frogs in water bodies with higher oxygen demand had higher diversity of blood parasites (RAHMAN & SHAKINAH, 2015). An alternative explanation is the inefficiency of the molecular primers used in detecting additional parasitic taxa (PAREJO-PULIDO *et al.*, 2023). Our results also contrast with the low prevalence of blood parasites ob-

served in *P. perezi* in Azores islands, where molecular primers targeting Nematoda and Apicomplexa detected a prevalence of 13% of spirurid nematodes and 7% of blood protists of the genus *Hepatoozon* in tissue samples from frog toeclips (HARRIS *et al.*, 2013). Dissimilarities in vector taxocenosis between the archipelago and the continent and/or the invasion history of *P.*

perezi and their parasites in the archipelago (e.g. missing-the-boat phenomenon; TOMÉ *et al.*, 2018) could explain the observed differences. Unfortunately, none of the two previous studies analysed blood smears, which precludes the detection of other parasites not targeted by the primers used (e.g. Euglenozoa). In this sense, a recent study that combined microscopic and molecular methods to examine frog samples of *Pelophylax esculentus* and *Pelophylax ridibundus* from Romania found a similarly high prevalence of *Trypanosoma* and a higher prevalence (84%) of *Hepatozoon*, a parasite not observed in our sample (PAVEÁKOVÁ *et al.*, 2024). Therefore, our findings are more in line with the high prevalence of *Trypanosoma* reported in green frogs from central Europe (SPODAREVA *et al.*, 2018; PAVEÁKOVÁ *et al.*, 2024).

The analyses of body condition supported independent negative relationships with the infection intensities of *Trypanosoma* and *Aegyptianella*, consistently estimated by two different indices. Furthermore, our results support previous studies suggesting that the residual body condition index (BCI) performs better than SMI when examining host-parasite interactions in *P. perezi* (BĂNCILĂ *et al.*, 2010). Our analyses suggest that blood parasites may draw resources from their hosts and limit their energy budget with a consistent effect on frogs of both sexes. However, with the present correlational data, we cannot determine whether the observed relationship results from parasite replication within the frogs or from increased host susceptibility to external reinfections, as a putative cost of the reproductive effort of the frogs dur-

ing the breeding season (NAVARRO & LLUCH, 2006; MCCALLUM & TRAUTH, 2007; DESPRAT *et al.*, 2015). The latter might be associated with the seasonal depletion of fat bodies in frogs (SAIDAPUR & HOQUE, 1996; GIRISH & SAIDAPUR, 2000; CAMPBELL *et al.*, 2019) which, if our hypothesis was validated, would suggest a trade-off in the allocation of energy between reproduction and anti-parasitic defences in Iberian green frogs, as shown in other amphibians (VENESKY *et al.*, 2012; CAMPBELL *et al.*, 2019).

Contrary to blood parasites, leech infestation was differentially related to body condition indices depending on sex, as evidenced by the opposing trends between the indices of body condition and leech load in females and males. Despite the larger body size of females, leech infestation might have a negative effect on their body condition; the latter being a determinant of reproductive investment and fitness in female anurans (HOWARD, 1978; CASTELLANO *et al.*, 2004; CADEDDU & CASTELLANO, 2012). Thus, females with more ectoparasites (specifically, leeches) and a lower nutritional status might represent a low-quality choice for males during the reproductive season. In contrast, although with a smaller effect, males with better body condition tended to have more leeches. Similar relationships between the same estimates of body condition and ectoparasites (ticks) have also been observed in males of other small ectotherm vertebrates (e.g. VÁCLAV *et al.*, 2007). If leeches, which can represent a selection force (e.g. HOWARD, 1978), reduce the body condition of frogs, those males with greater tolerance to leech infestation would be favoured (*sensu* ROY & KIRCHNER, 2000). In support of the

latter hypothesis, we found that the body condition of males infested by leeches (0.020 ± 0.019) was higher than the average BCI of males, while leech-free males had lower BCI than average (-0.006 ± 0.008). In anurans, the assessment of male quality by females takes place through the scrutiny of the males' acoustic signals (PLÉNET *et al.*, 2010), with males with larger body size producing calls more attractive to females (KLUMP & GERHARDT, 1987; ZIEGLER *et al.*, 2016). Thus, larger males might provide cues of individual quality to females in terms of tolerance to leech infestation. Males of *P. perezii* have a complex calling repertoire and we encourage future studies on this species investigating the females' mating preferences upon different males while also considering the relationships between male call characteristics and parasite load in the context of sexual selection mediated by parasites (HAMILTON & ZUK, 1982; HAUSFATER *et al.*, 1990; PRÖHL *et al.*, 2013).

We found similar prevalence and infection intensities of blood parasites in both sexes when we analysed each parasite genus separately, although the overall prevalence was significantly higher in the female frogs. This contrasts with a previous study about the intestinal infracommunity of helminths in *P. perezii* from southern Spain, where females had lower parasite abundance and richness (NAVARRO & LLUCH, 2006). It also differs from a previous study in túngara frogs, *Engystomops pustulosus* (Cope, 1864), from Panama, where *Trypanosoma* was found to infect male frogs almost exclusively (BERNAL & PINTO, 2016). These differences may be related to sexual dissimilarities in the diet composition in

the case of the intestinal parasites (NAVARRO & LLUCH, 2006), and in the transmission modes of different *Trypanosoma* species. For example, in the túngara frog, *Trypanosoma* is transmitted by a flying vector that may use acoustic cues (i.e. male calls) to find their hosts (BERNAL & PINTO, 2016; BOWER *et al.*, 2019) similarly as it has been recently described for another Onchocercidae nematode in South Africa (NETHERLANDS *et al.*, 2020). Interestingly, we found that the number of leeches positively correlated with the infection intensity of *Trypanosoma*. This supports the idea that aquatic leeches can transmit parasites of this genus to European frogs (O'DONOGHUE, 2017; SPODAREVA *et al.*, 2018). Since leeches rarely infest other co-existing amphibian species in the study population (authors' pers. obs.), we speculate that the leeches' host-specificity might limit the transmission of this parasite to other amphibian species, thus explaining the absence of this parasite in other amphibians in the local community, including *Pleurodeles waltli*, *Triturus marmoratus*, *Pelobates cultripes*, *Epidalea calamita*, and *Hyla molleri* (authors' pers. obs.). The exclusive presence of leeches on *P. perezii* could be associated with the differential behaviour of water frogs, which are strictly aquatic for almost the entire season, while most other species switch to terrestrial habitats right after the mating season.

The infection intensity of intraerythrocytic bacteria of the genus *Aegyptianella* and the body condition of individuals, both with negative sign, explained interindividual variation in the intensity of *Trypanosoma* infection. This suggests either that (i) frogs with better body condition might

be more successful in handling *Trypanosoma* infection, or that (ii) co-infected frogs with both *Aegyptianella* and *Trypanosoma* need to control at least one of the two infections. In this sense, we encourage future experimental studies analyzing the temporal dynamics (timing and order of arrival) of the infection with *Aegyptianella* and *Trypanosoma* in frogs and how that relates to their co-infection outcome in the context of the priority effect theory (HOVERMAN *et al.*, 2013). Moreover, the model of *Aegyptianella* intensity showed higher infection intensities in frogs with relatively lighter bodies. Thus, frogs with either low nutritional income or/and high allocation to body growth may increase their susceptibility to this bacteria (e.g., SOLER *et al.*, 2003). It is worth noting that this genus of bacteria was previously reported infecting the blood of water frogs, *P. esculentus*, in Corsica and in the Italic peninsula (BARTA *et al.*, 1989; GALUPPI *et al.*, 2012) but to our knowledge the current study represents the first report of its presence in Iberian water frogs.

Although our analyses indicated no effect of hematic coccidians of the genus *Lankesterella* on the body condition of Iberian green frogs, we recommend further research before ruling it out, because infection by this parasite was previously associated with detrimental effects in frogs (SAILASUTA *et al.*, 2011) as well as in birds and lizards (MARTÍNEZ *et al.*, 2018; DRECHSLER *et al.*, 2021; CHANG *et al.*, 2023; MEGÍA-PALMA *et al.*, 2024). The liver and spleen of infected frogs *Hoplobatrachus rugulosus* (fam. Dicroglossidae) showed inflammatory lesions with an accumulation of melanomacrophage centres surround-

ing the infective stages (meronts and merozoites) (SAILASUTA *et al.*, 2011). Nonetheless, the high prevalence and intensity of this parasite in the sample, with no evident effects on the hosts, suggest some degree of putative tolerance. This said, the potential role of *P. perezi* as a reservoir of *Lankesterella* from which it could infect other amphibian species in this community is unlikely, as for example parasites in this genus are highly host-specific in reptiles or bird hosts (MEGÍA-PALMA *et al.*, 2017; DRECHSLER *et al.*, 2021; VENKATACHALAM *et al.*, 2023). Moreover, *Lankesterella* is putatively transmitted by leeches (SANDERS, 1928), and in a sample of the other amphibian species from the same pond *Lankesterella* was only detected in a single *Hyla molleri* (representing a prevalence of 4.3% for this host species) (Megía-Palma, pers. obs.).

The molecular identification of the microfilariae observed in a single frog will be addressed in future studies. Candidate genera for this nematode include *Icosiella* and *Foleyella*, both known to infect frog hosts of the genus *Pelophylax* (WITENBERG & GERICHTER, 1944; MIKULÍČEK *et al.*, 2021; PAVLÁKOVÁ *et al.*, 2024). However, the microfilariae blood stage in species of both genera typically possesses a surrounding sheath, a diagnostic characteristic, which was absent in the microfilariae observed in this individual (WITENBERG & GERICHTER, 1944; GUPTA *et al.*, 2020).

In conclusion, the results of this study suggest the negative influence of *Trypanosoma* and the intraerythrocytic bacteria *Aegyptianella* on Iberian green frogs, as inferred by their negative relationship with two indices of body condition. While a co-

evolutionary equilibrium leading to asymptomatic effects of parasites is sometimes expected because of the ancient relationship with their hosts (PATERSON & BLOUIN-DEMERS, 2020), our results suggest that blood parasites can also have negative effects and call for additional efforts to (i) characterize their diversity and cumulative effects on amphibian population dynamics, and (ii) further analyse the effects of blood parasites of amphibians at a broader geographic level.

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