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High levels of prevalence related to age and body condition: host-parasite interactions in a water frog *Pelophylax* kl. *hispanicus*

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Abstract. Host traits can significantly influence patterns of infection and disease**.** Here, we studied the helminths parasitizing the Italian edible frog *Pelophylax* kl. *hispanicus,* giving special attention to the relationship between parasites and host traits such as sex, snout vent length, weight and body condition. The helminth community was composed of seven species: three trematode species (*Diplodiscus subclavatus, Gorgodera cygnoides, Pleurogenes claviger*), three nematode species (*Icosiella neglecta, Oswaldocruzia filiformis*, *Rhabdias* sp.) and one acanthocephalan species (*Pomphorhychus laevis*). We found that prevalence was positively correlated with snout-vent length and weight, but did not differ with body condition or sex. We found that prevalence and mean species richness increased with age. Our results show that abundance of *Icosiella neglecta* was positively correlated with higher values for host body condition. In fact, we found that high prevalence and mean species richness do not necessarily imply poorer body condition in the parasitized host. In conclusion, our results show that the helminth community in this taxon has great diversity, and this host-parasite system seems to be evolved to low levels of virulence, helminths maintaining a commensal relationship with this frog.

Keywords. Acantocephala, body condition, Italian edible frog, Nematoda, Trematoda.

INTRODUCTION

Wild amphibian populations are declining worldwide (Whiles et al., 2006; Wake, 2007) and factors such as global change and pollution may magnify the effect of pathogens and disease in this animal class (Harvell et al., 2002; Taylor et al., 2005; Paull and Johnson, 2011; Macnab and Barber, 2011; Tinsley et al., 2011). For example, higher water temperatures lead to a rise in the incidence of parasitic diseases due to increased pathogen development transmission and host susceptibility (Karvonen et al., 2010). Parasites can have a significant influence on the population dynamics of the host species (Longshaw et al., 2010) and understanding how host-parasite interactions will change in relation to host traits is one of the cornerstones of parasitology (McAlpine, 1997; Dobson, 2009; Lafferty, 2009).

The relationship between prevalence and factors such as age or sex is not clear. In some studies an age-related increase in prevalence in amphibians has been reported (McAlpine, 1997; Campiao et al., 2009). Nevertheless, some studies describe a decrease in prevalence (Ibrahim, 2008; Raffel et al., 2009; Tinsley et al., 2012), while others report no age-related patterns (Garvin et al., 2003; Hasselquist et al., 2007). Age-related changes in prevalence may be due to several causes. An increase in prevalence with age may be caused by prolonged exposure to parasite accumulation (Sanchis et al., 2000), while a decrease in prevalence with age may be linked to differential survival rates between individuals with and without parasites

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(parasitized individuals may be more likely to die and so over time there will be more non-infected individuals). A decrease in prevalence with age may also be influenced by changes in the immune response capacity of infected individuals. Levels of disease risk in hosts of different ages will vary due to differences in their susceptibility to infection and in their age-acquired immunity (Raffel et al., 2009; Tinsley et al., 2012). Nonetheless, prevalence may also decrease with time post-exposure due to parasite mortality, especially in the case of parasites that do not reproduce within a host (Telfer et al., 2008; Holland, 2009).

Relationships between sex and helminth prevalence show no clear patterns in amphibians. Some authors (González and Hamann, 2012) found significant differences in the intensity of infection between sexes, with females having higher mean intensities than males. Other authors found that levels of parasitism by sex depended on the studied species. For example, in wood frogs *Rana sylvatica* (LeConte, 1825) the parasite load in males was higher than in females (Dare and Forbes 2008), while in *Lithobates* frogs the parasite load was higher in females (Dare and Forbes 2009a). In the northern leopard frogs *Rana pipiens* (Schreber, 1782), prevalence and mean abundance were higher in breeding female frogs than in breeding males, even though no sex differences were observed in non-breeding adults (Dare and Forbes 2009b).

Host traits can significantly influence patterns of infection and disease (McAlpine, 1997; Dare and Forbes 2008; Raffel et al., 2009; Tinsley et al., 2012). In the present study, we analysed relationships between hosts and their parasites in the Italian edible frog *Pelophylax* kl. *hispanicus* and give special attention to host-parasite relationships and host traits such as host age, sex, snout-vent length, weight and body condition. We also examined the possible seasonality of the parasites.

The Italian edible frog is a hybridogenic species. These frogs are kleptospecies derived from *P. bergeri* and *P. ridibundus* or from *P.* kl. *esculentus*, which is itself of hybrid origin (Holsbeek and Jooris, 2009; Vorburger and Reyer, 2003). It is endemic to Italy, occurring on the Italian mainland south from Genoa to Rimini and Sicily. It is associated with a wide range of aquatic environments such as rivers, swamps and freshwater lakes and marshes, with either intermittent or permanent flow regimes. It is usually found in mixed populations with *Pelophylax bergeri* (Andreone et al., 2009) and normally occurs in sympatry with other amphibian species (Talarico et al., 2004; Sperone et al., 2009). As far as we know, there are no studies of the diet of the Italian edible frog, although the parental specie *P. ridibundus* (Pallas, 1771) has been reported to consume Coleoptera, Diptera, Hymenoptera and, less frequently, vertebrates such as amphibians (mainly tadpoles), reptiles and even mammals (Mollow et al., 2010). It has been shown that in other close related species of water frogs such as *P.* kl. *esculentus* and *P. lessonae* (Camerano, 1882) no significant differences in trophic niche exist between the hybrid and the parental species, which share the same habitats (Sas et al., 2007).

Herein, we addressed the following questions: 1) What is the helminth community of the Italian edible frogs? 2) What are prevalence, diversity, abundance and intensity of infection of helminths found in this host? 3) Is there a relationship between these parasitological parameters and host traits? 4) Does the prevalence of parasites vary seasonally?

MATERIAL AND METHODS

Study area and sampling

Our sampling site is located in the lower basin of a stream (Beltrame) in Calabria (southern Italy; 38°44’ N, 16°32’ E) at 10 m a.s.l.. This stream’s flow regime is characterized by sudden floods, alternating with long periods of drought, particularly in summer (Sabato and Tropeano, 2004). The climate is Mediterranean, with annual precipitation values of around 800–1000 mm (Talarico et al., 2004). Average temperatures range from 31 °C in the warmest to 13 °C in the coldest months. The vegetation is characterised by evergreen sclerophyllous formations (among which, *Quercus ilex* and *Q. suber)* and Mediterranean scrub and shrubs with species such as *Tamarix* sp. and *Nerium oleander*.

This study was carried out in autumn 2008 (81 individuals), in spring (17 individuals) and summer (19 individuals) 2009. In all, 117 frogs (54 males, 60 females and 3 unsexed) were netted along a 1-km transect along the lower course of the stream. Snout vent-length (SVL, nearest 0.1 mm) and body weight (nearest 0.1 g) were recorded for each individual. We measured the snout-vent length (SVL) from the tip of the snout to the rear border of the vent. Weight was measured using a precision balance. Body condition was calculated from estimated residuals of the relationship body weight-body length (log-transformed; Green, 2001). Hosts were grouped into three age classes on the basis of their SVL (juveniles: 35–60 mm; subadults: 61–70 mm; adults 71–110 mm) following Lanza (1983).

After collecting the biometric data, specimens were anaesthetized and killed with tricaine methane sulphonate, MS 222 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). All procedures were carried out following the recommendations of the Ethical Committee of the University of Calabria and under the supervision of authorized investigators. Then, gastrointestinal tract (oesophagus, stomach and small and large intestines), as well as lungs, urinary bladder, liver, heart and kidneys, were dissected and placed separately in Petri dishes containing 0.9% saline solution. The muscle (dewlap tissue) was also examined. Organs, muscle and gastrointestinal tract were then examined separately for helminths under a stereomicroscope.

Recovered helminths were placed in vials in 70% ethanol for subsequent examination. Nematodes and acantocephalans were examined on a temporary mounting in Amann lactophenol. Trematodes were stained in acetic carmine, with HCl at 5% to remove excess dye, dehydrated in series alcohol, cleared in xylene and mounted in Canada balsam for identification. Helminth were identified according to the literature (e.g., Anderson and Bain 1982; Brown 1987; Skryabin 1964; Slimane et al., 1993; Starzyska 1958). A subset of helminths recovered was deposited in the Museu de Zoologia de Barcelona (MZB), Catalonia, Spain, with accession numbers MZB 2013–3683- MZB 2013–3689.

Data analysis

We calculated prevalence (the percentage of hosts that are infected), mean abundance (total number of individuals of a particular parasite species found in the sample, divided by the total number of hosts examined) and mean intensity (average abundance of a particular parasite considering only the infected members of the host species) of helminth species following Bush et al. (1997). Mean species richness of helminths was calculated as the sum of helminth species per individual divided by the total sample size of host individuals (Bolek and Coggins, 2001). The Brillouin Index (HB) of diversity was calculated following the equation: $HB = (\ln N! - \sum \ln n_i!) / N$, where N is the total number of individuals and n_i is the number of individuals in the ith species (Magurran, 2004). Numeric dominance was determined using the Berger-Parker dominance index, following the equation $d = N_{max}/N$, where N_{max} is the number of individuals of the most abundant species and N is the total number of individuals in the sample.

To test the effect of sex, age and season on parasite prevalence a Maximum Likelihood Chi-square test was used. The effect of host sex on parasite abundance, taking into account body size, was tested using an ANCOVA. The effect of host sex on intensity was tested using a Mann-Whitney U test. Differences in snout-vent length (SVL) between males and females were tested with a t-student test. The difference in mean species richness among age classes was analysed using the Kruskal-Wallis test. We performed logistic regression analyses to test the relationships between snout-vent length (SVL), weight and body condition of the frogs with the presence/absence of parasites (i.e., prevalence). A one-way ANOVA was performed to test whether helminth mean species richness (introduced as categorical factor with values between 0 and 4 species) was related to (a) body size, (b) weight and (c) body condition. In order to test how prevalence for each species of parasite affects body condition, an ANOVA was performed. A multiple regression analysis was performed for each species of parasite in order to test how body condition changes with abundance.

The assumptions of normality and homoscedasticity were checked with the Shapiro-Wilk's test and Levene's tests, respectively. All statistical analyses were performed using STATISTICA 10.0.

RESULTS

The helminth community consisted of seven species: three trematodes, three nematodes and one acanthocephalan (Table 1). *Icosiella neglecta* had the highest prevalence (61.54%) and was the dominant species (d = 0.51), while *Pleurogenes claviger* was the rarest (0.85%). Three of the seven species were rare, with low prevalence (< 6%) and low mean abundances (< 0.16 parasites/host). Only 18 frogs were uninfected, while the remaining harboured 1–4 helminth species and 1–19 parasites.

We found sexual dimorphism ($t_{114} = 4.56$, $P < 0.0001$), females (75.13 ± 14.8 mm; mean ± SD) being larger than males (62.3 ± 13.6 mm). Our results showed a trend for higher prevalence in females than in males (77.7 % in males and 90 % in females; $\chi^2 = 3.23$, df = 1, P = 0.07). We also found that prevalence significantly increased depending on snout-vent length ($\chi^2 = 8.476$, df = 1, P = 0.0036) and weight ($\chi^2 = 10.176$, df = 1, P = 0.001), but not on body condition ($\chi^2 = 1.457$, df = 1, P = 0.227). Our results show that prevalence increased with age (Table 2). We did not find significant differences in the seasonal prevalence ($\chi^2 = 0.74$, df = 2, P = 0.69). Prevalence in autumn 2008 was 82.72%, in spring 2009 was 88.24% and in summer 2009 was 89.47%. Mean species richness did not differ between sexes (being 1.27 ± 0.9 in males and 1.55 ± 0.8 species/host in females; U = 1327, P = 0.097) and likewise, did not depend on either body size, weight or body condition (body size, $F_{4,117} = 0.8$, P = 0.53; weight, $F_{4,117} = 1.6$, P = 0.39; body condition, $F_{4,117} = 0.74$, P = 0.57). Nevertheless, mean species richness increased with age (Table 2). The Brillouin Index of diversity was 1.29.

For both *D. subclavatus* and *I. neglecta,* abundance significantly covaried with host body size but not with host

Table 1. Number of parasitized hosts and prevalence (%), mean intensity (MI ± SD), mean abundance (MA ± SD), number of helminth recovered and occupied microhabitat in the host *Pelophylax* kl. *hispanicus* from a stream (Beltrame) in Calabria, Italy (n = 117).

Helminth species	Number of individuals parasitized (prevalence, %)	MI ± SD (range)	MA ± SD	Number recovered	Microhabitat
Trematoda					
Diplodiscus subclavatus (Pallas, 1760)	52 (44.4)	3.08 ± 2.85 (1-16)	1.37 ± 2.85	160	Rectum
Gorgodera cygnoides (Zeder, 1800)	5 (4.27)	3.80 ± 3.35 (1-9)	0.16 ± 3.35	19	Urinary bladder
Pleurogenes claviger (Rudolphi, 1819)	1 (0.85)	7.0 (7-7)	0.06	7	Small intestine
Nematoda					
Rhabdias sp.	16 (13.68)	2.75 ± 2.74 (1-11)	0.38 ± 2.74	44	Lungs
Icosiella neglecta (Diesing, 1851)	72 (61.54)	4.25 ± 3.19 (1-14)	2.61 ± 3.19	306	Subcutaneous and intermuscular tissue
Oswaldocruzia filiformis (Goeze, 1782)	13 (11.11)	4.15 ± 4.18 (1-13)	0.46 ± 4.18	54	Small intestine
Acanthocephala					
Pomphorhychus laevis (Müller, 1776)	6 (5.13)	1.17 ± 0.41 (1-2)	0.06 ± 0.41	7	External wall Intestine/stomach
Total	99 (84.62)	6.03 ± 5.08	5.10 ± 5.08	597	

sex (Table 3) and there are not sex link patterns in intensity for any species of parasite (Table 4). Body condition did not covaried with prevalence in any helminth species (Table 5), although body condition was positively correlated with abundance of *Icosiella neglecta* (t_{108} = 2.28, P = 0.024, β = 0.22) and was almost significant for *Pomphorhychus laevis* (t_{108} = 1.96, P = 0.053, β = 0.18) (Table 6).

DISCUSSION

Our results show a significant increase in helminth prevalence related to snout vent length and weight in the Italian edible frog. Consequently, given that body size increases with age, older individuals are more parasitized. These imply that prolonged exposure to parasite accumulation is the most important factor explaining the high level of prevalence found in the studied population. As the time of exposure to parasites and vectors increase, parasite prevalence does too. This result is consistent with other studies that have found that prevalence increased with age (McAlpine, 1997; Sanchis et al., 2000; Abu-Madi et al., 2001; Treml et al., 2012; Haas et al., 2012). In other species of water frogs such as the levant water frog *P. bedriagae*, females live longer than males (Çiçek et al., 2011) and so, because we found higher levels of prevalence with age, we expected females to harbour higher parasite loads than males. Nevertheless, even though females were larger and older than males, our results showed no significant sex-linked patterns of prevalence (despite the fact that females tended to have higher parasite loads).

Moreover, we found no significant relationship between host body condition and prevalence or between host body condition and mean species richness, which may suggest that this population has a certain tolerance to these parasites. In fact, we found a positive correlation between host body condition and the abundance of two species of parasites, which was significant in *Icosiella neglecta* and almost significant in *Pomphorhychus laevis*. Hosts with the greatest abundances of these two parasites species were in better body condition. When the

Table 2. Prevalence (%), mean species richness and snout-vent length (SVL in mm) for each age class (n = sample size).

Age group	N	Prevalence (%)	Mean species richness ±SD (range)	SVL ± SD (range)
Juveniles	32	71.87%	1.06 ±0.94 (0-4)	52.06±6.86 (35-60)
Subadults	38	84.21%	1.29±0.8 (0-3)	65.84±2.69 (61-70)
Adults	47	93.61%	1.74±0.82 (0-3)	83.17 ± 10.21 (71-110)

Table 3. Abundance according to sex for each species of parasite (ANCOVA controlling for snout vent length (SVL)).

Helminth species	SVL $F_{(1, 111)}$	SVL p	Sex $F_{(1, 111)}$	**Sex p**
Diplodiscus subclavatus	10.44	0.001	0.064	0.799
Gorgodera cygnoides	1.887	0.172	0.000	0.976
Pleurogenes claviger	0.098	0.754	1.184	0.278
Rhabdias sp.	1.331	0.250	0.312	0.577
Icosiella neglecta	35.76	0.000	1.200	0.275
Oswaldocruzia filiformis	0.456	0.500	0.102	0.749
Pomphorhychus laevis	0.150	0.698	0.334	0.564

Table 4. Intensity according to sex for each species of parasite (results of Mann-Whitney U test).

Helminth species	U	Z	*p*
Diplodiscus subclavatus	251.500	0.570	0.568
Gorgodera cygnoides	2.500	0.288	0.772
Pleurogenes claviger	0.000	0.000	1.000
Rhabdias sp.	28.500	-0.317	0.750
Icosiella neglecta	465.500	1.738	0.082
Oswaldocruzia filiformis	15.000	0.731	0.464
Pomphorhychus laevis	3.000	-0.654	0.512

Table 5. Results of the ANOVA performed for each species of parasite in order to test how body condition changes with prevalence.

Helminth species	$\mathbf{F_{(1, 108)}}$	**p**
Diplodiscus subclavatus	0.363	0.547
Gorgodera cygnoides	0.165	0.685
Pleurogenes claviger	1.386	0.241
Rhabdias sp.	0.688	0.408
Icosiella neglecta	3.113	0.080
Oswaldocruzia filiformis	0.004	0.944
Pomphorhychus laevis	2.730	0.101

Table 6. Results of the multiple regression analysis performed for each species of parasite to test how body condition changes with abundance.

Helminth species	**Beta**	$\mathbf{t_{108}}$	**p**
Diplodiscus subclavatus	0.085	0.895	0.372
Gorgodera cygnoides	-0.034	-0.365	0.715
Pleurogenes claviger	-0.103	-1.144	0.255
Rhabdias sp.	0.149	1.576	0.117
Icosiella neglecta	0.220	2.279	0.024
Oswaldocruzia filiformis	0.023	0.236	0.813
Pomphorhychus laevis	0.178	1.955	0.053

host has evolved tolerance instead of resistance as a way of mitigating the harm caused by a parasite, a relationship may change from parasitic to commensal (Miller et al. 2006; Leung & Poulin, 2008). However, when the abundance of a parasite becomes too great for the host, host survival may fall. Consequently, we probably found high parasite loads in hosts that were able to tolerate this level of parasitism, that is, in those hosts that show higher body condition.

The prevalence (84.6%) and mean species richness (1.4) found in the present study are high, especially in comparison with frogs from temperate regions, where prevalence values that range 7.2 – 92% (Galeano et al., 1990; Yoder and Coggins, 2007; Comas and Ribas, 2014) and mean species richness of 0.8 (Aho, 1990; Muzzall et al., 2001) or even mean species richness as low as 0.072 (Comas and Ribas, 2014). Therefore, our findings show that the Italian edible frog in our study population harbours a community with high prevalence and mean helminth species richness.

In our study area, the Italian edible frog shares habitat with other amphibian species and also with a rich invertebrate community. A diverse community increases the likelihood of having more intermediate hosts and vectors available (Aho, 1990) and so living in a species-rich community may imply greater parasite loads. Moreover, during dry summer periods, host density dramatically increases and leads to greater parasitism (Arneberg et al., 1998; Krasnov et al., 2002; Harvell et al., 2002; Marina et al., 2005; Lindsey et al., 2009). However, we did not find any differences in prevalence among seasons, as also it occurs in other parasite studies where no seasonal patterns of occurrence were found (Sanchis et al., 2000).

In conclusion, we found that the Italian edible frog harbours seven different helminth species and infestation by these species increases with snout vent length, weight and consequently, age. Furthermore, we found that higher levels of infestation and parasite diversity do not seem to have any severe impact on host body condition.

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