Veterinary Parasitology 187 (2012) 491–497

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Differences in helminth infections between captive and wild spur-thighed tortoises *Testudo graeca* in southern Spain: A potential risk of reintroductions of this species

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ARTICLE INFO

Article history:
Received 7 November 2011
Received in revised form 3 February 2012
Accepted 10 February 2012

Keywords:
Captive
Helminths
Angusticaecum
Tachygonetria
Medhiella
Thaparia
Alaeuris
Health hazard
Spur-thighed tortoise
Wild

ABSTRACT

Although the spur-thighed tortoise, *Testudo graeca*, is one of the most widely distributed species of tortoises, its natural populations are threatened through its whole range. Particularly at south-eastern Spain, the species is mainly threatened by habitat destruction and over-collection, given that this chelonian has been traditionally considered an appreciate pet. As south-eastern Spanish wildlife recovery centers shelter hundreds of captive animals mainly coming from illegal trade or captive-bred, there is a strong debate about what to do with these animals: maintaining them in captivity all along their lives or reintroducing them to wildlife. It is well known that the reintroduction of captive animals supposes a risk for the wild population due to the uncertainty of their genetic origin and to the possible spread of infectious diseases. However, despite the increasing evidence that infectious agents are a potential health hazard for wildlife, little is known about the risk that introduced parasites could suppose for the wild populations of spur-thighed tortoise.

The present study investigates for the first time the presence of helminth eggs and worms in faeces from 107 wild and captive individuals collected from mid-March to mid-June 2010, and relates the findings to different environmental and host variables. Sixteen oxyurid species and the ascarid *Angusticaecum holopterum* were identified. This last nematode and the oxyurid species *Tachygonetria palearticus* and *T. seurati* had not been reported in Spanish wild *T. graeca* previously. The prevalence of oxyurid eggs and worms were 94% and 70%, respectively; while, ascarid eggs and worms were found in 26% and 5% of tortoises, respectively. Ascarid infections affected mostly captive animals and were associated to caparace deformities and symptoms of upper respiratory tract disease ($p < 0.05$). Oxyurid infections were not associated to negative health traits and prevalence increased with age. In free-living tortoises, the distribution of pharingodonid genera also varied according to habitat; moreover, *T. longicollis*, *T. pusilla*, *T. conica*, *T. robusta* and *Mehdiella stylosa* where significantly more frequent in wild compared to captive tortoises ($p < 0.05$). Study results highlight important differences in the nematode fauna of captive and free-living tortoises and questions one more time if the reintroductions of captive animals suppose a risk for the wild population since the former ones can harbor and distribute among free populations pathogens like ascarid nematodes.

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0304-4017/$ – see front matter
doi:10.1016/j.vetpar.2012.02.007

1. Introduction

Habitat loss, trade, human consumption or the spread of diseases are some of the threats that many species of tortoises have to face (IUCN, 1989). Although the range of the spur-thighed tortoise (*Testudo graeca*) covers part of three continents (Europe, Africa and Asia) and represents the most widely distributed species of tortoises in the western Palearctic, its global population is declining through its whole range, being classified as vulnerable species at the Red List category (IUCN, 2010). At the Mediterranean Basin, spur-thighed tortoise presents most of its range in North Africa, and only occurs in a few small and isolated European populations in the Iberian Peninsula (in Doñana National Park and in south-eastern Spain) and in some islands (Mallorca, Sardinia and Sicily). Special characteristics like the small size of these animals or their tolerance to captive conditions promote its consideration of manageable and valuable pet, which is the main reason for the continuous removal of tortoises from their natural habitat and the illegal trade. Particularly, each summer hundreds of seized North African tortoises arrive to south-eastern Spanish wildlife recovery centers, admitted as confiscated specimens. Given their proximity to the wild population, these recovery centers as well receive wild damaged animals, captive animals collected from the field and maintained for a period, or captive-bred tortoises donated by its owners (Ferrández, 2011). There is a strong debate about how to manage and what conservation strategy must be implemented with captive population. Although south-eastern Spanish wild population is threatened by the destruction of its natural habitat due to urbanization and agricultural activity and by past over-collection, most of the recovery centers avoid reintroducing captive animals into the wild population, which was a traditional practice in the past but is still carried out by particular people. For scientists, administration and naturalists it is well known that these captive tortoises are not valid for reintroductions due to the uncertainty of their genetic origin and to the possible sanitary risk of introducing pathogen agents in its natural habitat that, subsequently, could provoke a disease outbreak (Pérez et al., 2010). However, little is known about the risk that introduced parasites could suppose for the wild population.

The role of parasites as limiting factor of tortoise populations is not well understood. With few exceptions, nematodes are the only helminths infecting terrestrial chelonians, and most of them belong to the orders Oxyurida and Ascaridida, which are considered to be transmitted by the oro-fecal route. There are isolated reports of mortality associated to large ascarid infestations (Rideout et al., 1987); in contrast, oxyurids can be very prevalent and are considered to have an almost commensal relationship with its host (Roca, 1999; Gagno, 2005). Most studies of helminths infections in tortoises have been carried out in captive populations kept in zoological parks and, on the best of our knowledge, there are not published epidemiological studies of helminths infections in *T. graeca* in its natural environment, where nematode transmission may differ significantly when compared to animals maintained in captivity. Moreover, nematode distribution and impact on the host may also vary depending on human intervention in the tortoise's natural habitat.

Therefore, the aims of this study are to describe and to compare the gastrointestinal helminth fauna in wild and captive spur-thighed tortoises. Additionally, we will evaluate the correlation between the prevalence and the infection intensity with host variables as age and sex, with indicators of tortoise's health conditions and, with the free-living or captive origin of tortoises. Finally, we will discuss the obtained pattern of parasitic infection and the potential risk of spreading parasites with tortoise's reintroductions.

2. Material and methods

2.1. Study area and population

Wild south-eastern Spanish population of *T. graeca* covers approximately 2600 km^2 of semiarid coastal mountains between Almeria and Murcia provinces (Fig. 1). We sampled 66 wild tortoises from areas with low, medium and high habitat fragmentation, and 41 from enclosures of the Wildlife Recovery center (WRC) of Santa Faz, in Alicante province (southeast of Spain). Samples were collected between mid march to mid June 2010 in both groups of tortoises (wild and captive animals). Study region lies between longitude N38° 23′ to N36° 20′ and latitude W0° 30′ to W2° 20′, and has a semi-arid climate with mild winters, hot summers and limited rain (200–350 mm/year) mostly during the cooler months of the year.

Tortoises were localized by sight during standardized transects. After sampling, specimens were marked and released in the capture sites.

The forty-one captive tortoises (that correspond to 20% of the individuals held in the WRC) were animals kept

Fig. 1. Distribution of *Testudo graeca* in the Mediterranean region (modified from Fritz et al., 2009); South-eastern Spanish population is included in the red grey square. Star represents the location of the Wildlife Recovery center of Santa Faz (Alicante, Spain). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Prevalence of faecal nematode egg shedding, median and interquartile (IQ) range eggs/g in a study in 107 *Testudo graeca* in southeast Spain, in 2010, according to independent variables.

Variable	Levels	No. Tortoises	All eggs		Oxiurid eggs		Ascarid eggs	
			% +ve	Median (IQ) eggs 10^3	% +ve	Median (IQ) eggs10^3	% +ve	Median (IQ) eggs 10^3
Age (yrs.)	≤1	7	43	6.9(3.2–17.8)	43	6.9 (3.2–17.5)	14	0.3 (0.3–0.3)
	2	3	67	1.9(1.8–2.1)	67	1.9 (1.8–2.1)	0	0
	3	4	75	6.5(0.9–6.7)	75	6.5 (0.9–6.7)	0	0
	≥4	93	100*	10.1(4.7–16.9)	99*	9.9 (4.6–17.6)	29	0.4 (0.2–1.5)
Sex	Male	29	100	6.8 (3.8–15.8)	100	6.8 (3.8–15.8)	67	0.3 (0.2–1.5)
	Female	37	100	10.3 (5.2–20.4)	97	10.5 (4.6–20.3)	92	0.6 (0.2–1.3)
Body weight (g)	12–168	36	83	9.6(5.2–15.7)	83	9.6 (5.2–15.1)	14	1.5 (0.2–4.8)
	169–450	36	100	7.8 (4.6–16.3)	100*	7.8 (4.1–16.3)	31	0.4 (0.2–1.0)
	451–1304	35	100*	10.3 (3.8–21.8)	97	10.5 (3.8–20.4)	34	0.4 (0.1–1.3)
Body condition	0.1429–0.1892	9	100	4.5 (1.2–5.3)	89	4.0 (1.5–9.4)	67	1.0 (0.6–3.1)
	0.1903–0.2298	44	98	10.7 (3.8–20.4)	98	10.7 (3.6–19.1)	80	0.3 (0.1–1.2)
	0.2305–0.4220	54	91	9.1 (5.5–15.1)	91	9.1 (5.5–15.1)	50	0.4 (0.3–1.9)
Symptoms of upper respiratory tract disease	Present	5	100	5.3 (4.7–6.1)	100	5.3 (4.7–6.1)	60	0.2 (0.1–0.2)
	Absent	102	94	9.6 (4.2–17.3)	93	10.1 (4.5–17.8)	25	0.5 (0.2–1.5)
Caparaces showing pyramidal growth	Present	8	100	4.9 (2.8–14)	100	4.9 (2.8–14)	63*	0.5 (0.2–1.0)
	Absent	99	94	9.1 (4.7–16.9)	93	9.6 (4.9–16.9)	23	0.4 (0.2–1.5)
Habitat	Captive	40	90	8.7 (3.6–22.2)	88	8.1 (3.5–20.3)	68*	0.3 (0.2–1.5)
	Wild	67	97	9.0 (5.2–13.6)	97	9.0 (5.2–13.6)	2	0.5 (0.5–0.5)

* Significant differences ($p < 0.05$).

sharing opened enclosures with scrubs as shelters (Table 1; Fig. 1). This group included one seized Algerian tortoise, while the remaining were mostly abandoned home-pet tortoises that had lived in the center for at least one year; they were specifically selected for the study to include 16 males (10 adults and 6 subadults) and 16 females (12 adults and 4 subadults), 1 subadult of undetermined gender and 7 immature individuals.

Tortoises are regularly feed with a mixture of fresh vegetables during the activity seasons. The WRC applies strict control measures for detecting and tackling the rhinitis disease, including quarantines for new arrived individuals, regularly disinfections of the enclosures and euthanasia of those animals with symptoms (Ferrández, 2011). However there is not applied any protocol for parasite's control.

2.2. Faecal sampling and tortoise data

Wild tortoises sighted were captured, and individual freshly voided faecal samples were obtained immediately following the handling of the animal or from the cloth bag where animals were placed until they defecated. After that, tortoises were returned where they had been found. Faecal samples from captive animals kept in the WRC were collected from individual cardboard boxes where the tortoises were hold until defecation occurred.

Individual characteristics recorded prior to the animals' release included sex, age estimated from the carapace growth ring (Lagarde et al., 2001; Hailey and Lambert, 2002; Bertolero et al., 2005), body weight and length, body condition score assessed by calculating Jacksons ratio dividing body weight by volume (Jackson, 1980), symptoms of upper respiratory tract disease (Muro et al., 1998) and pyramidal growth in the carapaces associated to captivity due to a protein based diet or a long lasting inadequate humidity (Wiesner and Iben, 2003).

2.3. Parasitological analysis

Faeces were maintained at 4 °C until processed within 48 h of collection. At first, saline solution was added to the faeces to obtain a 30% faecal solution to allow counting and collecting worms using an entomologic needle under a stereomicroscope.

Collected parasites were washed in distilled water and subsequently stored in 70% ethanol until morphometric identification. This was carried out in at least 100 females and males from each animal. Parasites were cleared in Amann lactophenol solution previously to be identified using the keys and descriptions of Petter (1966), (Bouamer and Morand, 2000, 2002, 2003a,b, 2005, 2006; Bouamer et al., 2001a,b, 2003) and Sprent (1980).

Faecal samples were then processed by the centrifugation-flotation and sedimentation techniques (MAFF, 1986) to identify helminths eggs, which were counted in a McMaster chamber. Oxyurid and ascarid eggs were classified to the family and species level, respectively (McArthur et al., 2004; Traversa et al., 2005). Prevalence and intensity of infection were calculated for adults, larvae and eggs.

2.4. Statistical analysis

EpiInfo 2002 (CDC, Atlanta) was used to investigate parasite distributions and their relationship with independent tortoise and environmental variables including age, sex, origin, body condition, presence of upper respiratory tract disease and carapace deformities. Yates corrected

Table 2
Prevalence of adult nematode species, median and interquartile (IQ) worms/g (wpg) in a coprological study in 107 *Testudo graeca* in southeast Spain, in 2010.

Species	All tortoises				Captive tortoises		Free-living tortoises	
	% +ve	Min. wpg	Median (IQ) wpg	Max. wpg	% +ve	Median (IQ) wpg	% +ve	Median (IQ) wpg
All species	70	<1	10 (3–39)	4128	53	10 (5–41)	81	9 (3–27)
Tachygonetria dentata	46	1	3 (2–9)	507	35	5(1–33)	52	3(2–8)
Tachygonetria longicollis	43	<1	5 (2–16)	1790	30	5(1–23)	51*	5(2–16)
Tachygonetria macrolaimus	22	1	2 (1–6)	331	15	6(1–37)	25	1(1–6)
Tachygonetria. conica	18	1	4 (1–29)	199	10	18(4–114)	24	2(1–20)
Tachygonetria pusilla	15	1	3 (1–15)	112	5	2(2–2)	21*	3(1–15)
Tachygonetria numidica	12	<1	3 (1–9)	56	13	1(1–4)	12	2(1–4)
Tachygonetria robusta	12	<1	2 (1–17)	57	5	1(1–1)	18*	5(1–17)
Tachygonetria setosa	5	2	9 (3–16)	29	3	29(29–29)	6	6(2–12)
Tachygonetria palearticus	5	1	3 (3–4)	6	8	4(3–6)	3	2(1–3)
Tachygonetria seurati	4	1	1 (1–17)	33	8	1(1–33)	2	1(1–1)
Alaeuris numidica	24	<1	3 (1–9)	56	18	5(1–10)	28	2(1–9)
Mehdiella stylosa	17	<1	3 (1–18)	43	8	5(3–8)	22*	3(1–8)
Mehdiella uncinata	15	<1	2 (1–6)	33	18	3(1–8)	13	1(1–5)
Mehdiella microstoma	8	1	2 (1–6)	299	5	151(2–299)	10	1(1–6)
Thaparia thapari	1	1	1 (1–1)	1	3	1(1–1)	0	0
Angusticaecum holopterum	5	<1	1 (1–1)	1	10*	1(1–1)	1	0(0–0)

+ve: Percentage of positive individuals.
* Significant differences ($p < 0.05$).

chi-squared test or, when appropriate, the Fisher exact test were used to compare proportions; the non-parametric Kruskal–Wallis test was used to compare means. Significance was taken for alpha <0.05 for a double test.

3. Results

3.1. Prevalence of infection

Coprological analysis revealed the presence of oxyurids and ascarids nematodes. The percentage of tortoises eliminating eggs, worms (both adults and larvae), exclusively adults and only larvae were 94%, 70%, 65% and 30%, respectively. Oxyurids were the most frequently encountered, and their eggs and specimens were present in 94% and 70% of animals, respectively. Instead, ascarid eggs and worms group were found in only 26% and 5% of tortoises, respectively. Samples containing worms always had eggs as well, and all ascarid-positive tortoises were oxyurid-positive except one captive tortoise which only had ascarid adults and eggs.

Sixteen nematode species with a direct life cycle were identified, including 15 species belonging to the oxyurid family Pharyngodonidae and one ascarid species identified as *Angusticaecum holopterum* (Table 2). Pharyngodonidae included *Tachygonetria longicollis*, *Ta. dentata*, *Ta. macrolaimus*, *Ta. conica*, *Ta. robusta*, *Ta. numidica*, *Ta. pusilla*, *Ta. setosa*, *Ta. seurati*, *Ta. palearticus y Ta. seurati*, *Mehdiella microstoma*, *M. uncinata*, *M. stylosa*, *Thaparia thapari*, and *Alaeuris numidica*. The most common species were *Ta. dentata* and *Ta. longicollis*, present in 46% and 43% of tortoises, respectively; the rarest included *Ta. seurati* and *Th. thapari*, found in four and one tortoises, respectively ($p < 0.05$; Table 2). The number of different worm species found in stool samples varied between one and ten species per tortoise: 21% of tortoises had only one species, 18% two species, 37% had three to five species and 25% had between six and ten different species.

3.2. Number of nematodes and intensity of infection

The estimated number of adult nematodes was 21,519, including 4651 males and 16,868 females. The number of larvae was 2152, while worms (adults and larvae per g of faeces -wpg-) ranged from 0 to 4128 wpg; their distribution was positively skewed since wpg were 0, 1–10, 12–68, 152–840 and 2719–4128 in 30%, 38%, 21%, 9%, 2% of tortoises, respectively. The mean, median and interquartile range of wpg among tortoises eliminating worms (worm elimination intensity -wei-) were 163, 10 and 3–39 worms, respectively (Table 2). Mean wei differed significantly between parasites and was greater for most oxyurid species when compared to the ascarid *A. holopterum* one ($p < 0.05$, Table 2). In contrast, the number of nematode eggs/g (epg) among parasitized tortoises (egg elimination intensity -eei-) ranged 89–38,063 epg and was more evenly distributed than worm counts. The mean of eei was 11,339, and the median and interquartile range were, 9009 and 4500–16,900, respectively. Egg counts differed significantly between nematode species ($p < 0.05$) (Table 2); in fact, eei median and interquartile range was 8702 (3872–16,337) epg for oxyurids, whereas it was 392 (171–1508) epg in ascarid species.

The prevalence of tortoises eliminating nematode eggs increased significantly with age; it was 43% among individuals of 1 year-old, 67% in 2 year-olds, 75% in 3 year-olds and 100% in older animals ($p < 0.05$). Moreover, prevalence of egg-positive tortoises was 100% in all the sexually-mature tortoises, and 83% among lightest (12–168 g) tortoises compared to 100% in heavier animals (169–1304 g) ($p < 0.05$); with regard to the body condition, prevalence was 100%, 98% and 91% among tortoises within ranges 0.142–0.189, 0.190–0.229 and 0.230–0.422 ($p > 0.05$), respectively. Prevalence of tortoises eliminating eggs was not associated with sex ($p > 0.05$), symptoms of upper respiratory tract disease (this variable was analyzed in captive tortoises since only one free-living specimen showed this symptoms), carapace deformities (pyramidal growth) or origin ($p > 0.05$), although prevalence was 90% and 97% among captive and wild tortoises, respectively ($p = 0.13$) (Table 1).

However, the relationship between parasite intensity and independent variables varied significantly between oxyurids and ascarids (Table 1). Given the overwhelming predominance of oxyurids, their associations with independent variables were similar to those described for nematodes as a whole. Instead, the percentage of ascarid egg positive tortoises was 68% among captive animals and 2% in free-living tortoises (only one wild individuals was positive; $p < 0.01$); with regard to the tortoise age, it was 14%, and 30% among 1 year-olds and ≥3 year-olds ($p = 0.08$). Moreover, ascarid egg positive prevalence was 60% and 25% in tortoises with and without evidence of upper respiratory tract disease ($p = 0.08$), and 63% and 23% in animals with and without carapace deformities ($p < 0.05$).

The greater oxyurid prevalence observed among free-living individuals was primarily due to a higher frequency of *Ta. longicollis*, *M. stylosa*, *Ta. pusilla*, *Ta. conica* and *Ta. robusta* in animals from this environment compared to those kept in captivity (Table 2). However, overall mixed infections were commonplace in both groups; in fact, the median and interquartile range of number of species were 3.5 (2–5) and 3 (2–6.5) species in captive and free-living tortoises, respectively. Differences in the number of species between individuals were not significantly related to age or the other independent variables ($p > 0.05$).

4. Discussion

This is the first large-scale coprological epidemiological study of helminths in *T. graeca* in their natural environment and, also, one of the few researches in which a captive population in open enclosures has been studied. Only oxyurid and ascarid nematodes were detected; a high prevalence of a large number of Pharyngodonidae oxyurids species was found in both wild and captive tortoises; instead, *A. holopterum* was the only ascarid species detected. Among sixteen nematode species identified, this last has not been previously described in free Spanish tortoises and neither the oxyurids *Ta. palearticus* and *Ta. seurati.* The ascarid species was almost exclusive of captive tortoises, except the finding of one free specimen probably recently re-introduced from captivity, given its carapace pyramidal growth, an external signal exclusively developed under captive conditions (Wiesner and Iben, 2003). It should be emphasized that animals which had a pyramidal growth showed a high ascarid eggs prevalence, being this percentage of infection higher also in tortoises with symptoms of upper respiratory tract disease. This result cannot be compared with other findings because we did not found any reference to these variables; so, in-depth studies on the association of ascarids, carapace pyramidal growth and respiratory tract disease are desirable.

The high prevalence and abundance of pharyngodonid nematodes in tortoises of all ages is well recognized, and suggests these parasites are well tolerated and have low pathogenicity. Dubinina (1949) estimated in up to 335,000 the number of nematodes in healthy *T. graeca*, and the number of adult worms counted in other similar studies in the same host showing no signs of infection were 5000–200,000 (Petter, 1966), 35,370 (Roca, 1999) and up to 37,000 (Gagno, 2005). Other authors suggest that these parasites may be beneficial to the tortoise by helping it break up faecal masses and preventing constipation (Telford, 1971), regulating the bacterial flora in caecum (Iverson, 1982) and making free amino acids available for the host (Rogers, 1969; Rothstein, 1970; Wright, 1975; Roca, 1999). Prevalence of Pharingodonidae egg shedding had not a statistic relationship with the body condition, going on these possible benefits and the low pathogenicity of the Pharyngondonidae family for tortoises.

Similarly, we have not found a statistic relationship between sex and nematode prevalence or intensity of infection, although female tortoises had marginally higher values for both parameters. Traversa et al. (2005) neither observed significant differences among the sex and the intensity of elimination of Pharyngodonidae eggs, and Al-Barwari and Saeed, 2007 did not found an association between this variable and the intensity of infection with *A. holopterum*.

A finding described in many of the previously cited studies and in the present one is that parasite infection in tortoises increases with age. So, animals below two years old have lower worm burdens (Petter, 1966) or even no worms at all (Traversa et al., 2005). The latter author, however, reported a significantly lower incidence of oxyurid faecal emission among tortoises over 5-years-old. The reasons behind differences in nematode intensity of infection according to age are poorly understood and may include the development of specific acquired immunity following repeated parasite exposures, preferential infection of young tortoises to prolific oxyurid species and differences in feeding habits, with younger animals being coprophagic and more carnivorous than older animals, which are essentially vegetarian (Gagno, 2005).

Increased contacts between tortoises following sexual maturity may explain the occurrence of the ascarid *Atractis dactyluris* in older tortoises but not in young ones (Petter, 1966). Atractids were not found in the present study but it is not possible to exclude that some of the larvae found may have belonged to this family. Competition between species could be the reason for the low numbers of some of them. In this sense, there is some evidence of this occurring between *A. dactyluris* and *M. uncinata* or *Ta. dentata* (Petter, 1966).

Translocations, breeding and re-introduction programs aim to prevent the extinction of threatened populations; nevertheless, the role that parasites play can be decisive in the success of such programs (Viggers et al., 1993; Cunningham, 1996; Woodroffe, 1999). Results here presented would reinforce the potential negative effect of parasites, particularly ascarids. The species composition in the present study was significantly associated to the tortoise's origin, wild or captive. The greater prevalence of ascarids in captive tortoises is likely to be related to higher environmental contamination as a result of greater animal density. The ability of ascarids to proliferate and cause disease in animals kept in confinement, such as *Toxocara canis* in dogs, is well documented (Gothe and Reichler, 1990; Overgaauw and Boersema, 1998; Claerebout et al., 2009). Heavy infection with *A. holopterum* was associated to gastrointestinal disorders (Stetter, 1934) and colon obstruction (Panikkar and Sproston, 1941), vomiting and anorexia (Holt et al., 1979); besides, larvae migration causes lesions in the lungs (Keymer, 1978) and aural abscesses (Cutler, 2004). Additionally, a large number of *A. holopterum* may limit the number of some oxyurid species. In this sense, loss of host-specific parasites from endangered species in captive conditions could suppose also a substantial risk for the wild population since the maintenance of the natural established host–parasite relations may be important for individual's immunity (Daszak et al., 2000).

Our results highlight that captivity conditions could be a major predisposing factor for parasitism and must be taken into account for breeding programs or when chelonians are assembled in recovery centers. Although these animals seem endure stress conditions when they live in captivity conditions, it is well documented that latent infections could be present threatening, not only the viability of the introduction, but also the survival of the wild population (Jacobson et al., 1995; Jacobson and Berry, 2009; Martel et al., 2009).

Acknowledgments

We are grateful to the Generalitat Valenciana for permission to obtain the samples and to Pedro María Mojica and Marcos Ferrández for their help with sampling.

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