Reservoir Role of Lizard *Psammodromus algirus* in Transmission Cycle of *Borrelia burgdorferi* Sensu Lato (Spirochaetaceae) in Tunisia

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ABSTRACT To investigate the reservoir role of the lizard *Psammodromus algirus* for the Lyme disease spirochete, 199 lizards were trapped from April to October 2003 in El Jouza, northwestern Tunisia. In this site, the infection rate of free-living *Ixodes ricinus* (L.) by *Borrelia* was evaluated by immunofluorescence as 34.6% for adult ticks and 12.5% for nymphs. Eighty percent of *P. algirus* (117/146) captured during this study were infested by *I. ricinus*, the predominant tick species collected from lizards. The intensity of tick infestation of this host by larvae and nymphs ranged from 0.14 to 7.07 and from 1.5 to 6.58, respectively. These immature stages of *I. ricinus* were found on lizards in spring and the beginning of summer, with a peak of intensity during June (10.16 immature ticks by lizard). Tissue cultures from lizards and xenodiagnosis with larval *I. ricinus* were used to assess the infection and the ability, respectively, of infected lizards to transmit *Borrelia* to naive ticks. Seventeen percent of xenodiagnostic ticks (40/229) acquired *B. lusitaniae* while feeding on *P. algirus*. Therefore, we demonstrated the ability of the lizards to sustain *Borrelia* infection and to infect attached ticks, and we proved that *P. algirus* is a reservoir host competent to transmit *B. lusitaniae*.

KEY WORDS *Ixodes ricinus*, *Psammodromus algirus*, *Borrelia lusitaniae*, xenodiagnosis, reservoir

Borrelia burgdorferi sensu lato (s.l.) is a bacterial species complex, at present comprising 12 delineated and named species. In Europe, six genospecies—*B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii* (Baranton et al. 1992, Canica et al. 1993), *B. valaisiana* (Wang et al. 1997), *B. lusitaniae* (Le Flèche et al. 1997), and *B. spielmani* (Richter et al. 2004)—are maintained in nature by complex zoonotic transmission cycles involving a variety of mammalian and avian hosts and the hard tick *Ixodes ricinus* (L.) as vector (Gern and Humair 2002). Globally, >300 vertebrate species have been identified as hosts for this tick species, but <50 of them have been reported as playing a role in the ecology of *B. burgdorferi* s.l. (Gern et al. 1998). Rodents have been identified as reservoirs for *B. afzelii*, *B. burgdorferi*, and OspA serotype 4 of *B. garinii*, whereas birds are reservoirs for *B. valaisiana* and *B. garinii* (Kurtenbach et al. 1995, Gern and Humair 2002). However, the reservoir hosts for *B. lusitaniae* still remain unknown.

In North Africa, more specifically in Tunisia and Morocco, *B. lusitaniae* has been reported as the predominant species infecting *I. ricinus* (Younsi et al. 2001, Sarih et al. 2003, Younsi et al. 2005). A *B. lusitaniae* strain has been isolated from a Portuguese patient (Collares-Pereira et al. 2004); thus, this species has pathogenic potential, even though there is, hitherto, no evidence of Lyme borreliosis being transmitted by this *Borrelia* species in North Africa. The present work was carried out in northwestern Tunisia, where *B. lusitaniae* is prevalent in *I. ricinus* (Younsi et al. 2001). It has been observed that the lizard *Psammodromus algirus* was the major host of *I. ricinus* immatures in this area (Bouattour et al. 1999). Therefore, the current study was undertaken to evaluate the role of *P. algirus* as host for *I. ricinus* immatures and its ability to transmit *B. burgdorferi* s.l. to feeding ticks. We demonstrated, by xenodiagnosis, that *P. algirus* has reservoir competence for *B. lusitaniae*.

Materials and Methods

Study Sites, Tick, and Lizard Collection. Ticks and lizards were collected in Jbel el Jouza in northwestern Tunisia (36° 58′, 9° 05′), a humid bioclimatic zone (rain fall ranges from 600 to 800 mm/yr) characterized by an oak formation (*Quercus faginea* Lam and *Quercus suber* L.). The under tree cover consists mainly of *Pteridium aquilinum* Kuhn and also *Erica arborea* L. and *Cytisus triflorus* Lam. In this biotope, cattle, sheep, and goats graze on the undergrowth and the wild fauna consists of wild boars, birds, and lizards.

Questing *I. ricinus* ticks were collected by blanket dragging the vegetation from March 2003 to February 2004 and examined for the presence of *B. burgdorferi* s.l.

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During this survey, lizards were hand captured in this site, mainly in the ferns, and brought to the laboratory for genus and species determination. Animals were aged, sexed, and examined for the presence of ticks. *P. algirus* having snout-to-vent lengths of 39 mm or less and those having snout-to-vent lengths equal to or >40 mm were classified as juveniles and adults, respectively (Blanc 1978). Ticks were removed from each lizard by using forceps and were identified according to Marquez et al. (1992). Prevalence of infestation (percentage of infested lizards) and intensity of infestation (total number of ticks attached to lizards divided by the number of infested lizards) were calculated (Kahl et al. 2002).

Ticks were removed from lizards with forceps immediately after capture. Because engorgement was not always completed, ticks have not been kept until molt, and therefore they were not analyzed for the presence of *Borrelia.*

***Borrelia* Infection in Field-Collected *I. ricinus*.** The prevalence of *B. burgdorferi* s.l. in field-collected *I. ricinus* was determined by direct fluorescent antibody assay (DFA), as described in Jouda et al. (2003). Briefly, ticks were surface sterilized by 70% ethanol and dissected to remove the gut. The gut was chilled into a drop of 1× phosphate-buffered saline (PBS) on glass slides, air-dried overnight at 37°C, and subsequently fixed in acetone for 10 min. Fluorescein isothiocyanate-conjugated antibodies prepared from a pool of Lyme borreliosis patient sera, which detect all *Borrelia* species, were used. Slides were incubated for 30 min in a humid chamber at 37°C and then rinsed with PBS and coverslipped after mounting in glycerol. They were examined for *Borrelia* by fluorescence microscopy.

***Borrelia* Infection in Lizards.** Lizards were anesthetized with ketamine (Imalgene) and disinfected. Samples of blood, skin, liver, and kidney were aseptically removed and then examined for *Borrelia* infection by culture. These samples were inoculated (n = 46 lizards) separately in 5 ml of BSK-H medium (Sigma, St. Louis, MO) supplemented with 6% rabbit serum, 7% gelatin, and 1% antibiotic mixture for *Borrelia* (Sigma). Tubes were incubated at 34°C and examined weekly by dark-field microscopy to detect spirochetes. After 2 mo of monitoring, DNA was extracted from centrifugation pellet of all positive and negative cultures by boiling at 100°C for 10 min. These bacterial thermolysates were used for polymerase chain reaction (PCR) amplification of the ribosomal *rrf-rrl* spacer as described previously (Postic et al. 1994). The positive amplicons, which produced a 250-bp DNA fragment, were submitted to enzymatic restriction by *Mse*I and *Dra*I to determine the *Borrelia* species (Postic et al. 1994).

Moreover, DNA was directly extracted from tissues (blood, skin, liver, and kidney) of 47 lizards by using the QIAamp tissue kit (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. DNA samples were used for *Borrelia* detection and identification by PCR-restriction fragment-length polymorphism as described previously (Postic et al. 1994).

Xenodiagnosis. To examine the reservoir status of *P. algirus*, xenodiagnosis was done. Approximately 100 uninfected larvae from a laboratory colony (Institute of Zoology, Neuchâtel, Switzerland) were placed on each anesthetized lizard. Lizards were then kept in a small container to increase tick–lizard contacts, for a better tick attachment rate. Then, lizards were placed in plastic cages (10 by 20 cm) over water to collect engorged larvae that were then kept at room temperature (≈25°C) and 85% RH until molting. Laboratory constraints did not allow us to keep lizards individually in plastic cages. Emerging nymphs were examined for *B. burgdorferi* infection by isolation in BSK-H medium. Individual ticks or pools of ticks were transferred into culture tubes. DNA was extracted from all culture tubes to perform PCR amplification for *Borrelia* detection and identification, as described above.

Statistical Analysis. Infestation prevalences of male and female lizards and of juvenile and adults were compared using chi-square test. A linear regression test was used to compare the monthly variations of the intensity of *P. algirus* infestation by *I. ricinus.*

Results and Discussion

Free-Living Ticks in Jbel el Jouza. During this study (from March 2003 to February 2004), 1,007 ticks were collected on the vegetation at Jbel el Jouza. The collected tick species were *I. ricinus* (609 adults, 132 nymphs, and 248 larvae) (Fig. 1), *Hemaphysalis sulcata* Canestrini and Fanzago (n = 9), *Hemaphysalis punctata* Canestrini and Fanzago (n = 6), and *Dermacentor marginatus* Sulzer (n = 3). *I. ricinus* was the dominant species (98%) in this site. The activity of adult ticks has been observed mainly between November and April, whereas that of larvae and nymphs has been observed from April to July (Fig. 1). These results agree with those reported previously (Bouattour et al. 1999, Younsi et al. 2001).

Tick Infestation of Lizards at Jbel el Jouza. From April to October 2003, 199 lizards, belonging to five species, were captured: 146 *P. algirus*, 47 *Podarcis hispanica*, four *Lacerta pater*, one *Tarentola mauritanica*, and one *Chalcides ocellatus. P. algirus* were present in the studied site every month from April to October, with a peak in May (Fig. 1). After this period, lizards enter in winter dormancy under rocks, broken trunks, and branches. This activity period overlapped that of immature *I. ricinus* ticks. Only *P. algirus*, *P. hispanica*, and *L. pater* were found infested by *I. ricinus* (Table 1).

One hundred seventeen of 146 *P. algirus* (80%) were infested by *I. ricinus* ticks. This prevalence was significantly higher than that observed in *P. hispanica* (55.3%) (Table 1). The infestation of *P. algirus* by immature ticks can be favored by the shape of the skin scales of this species, which are large, imbricated, keeled, and pointed and allow a solid attachment of ticks between scales. Moreover this lizard species is very active and ranges over a large area of fern veg-

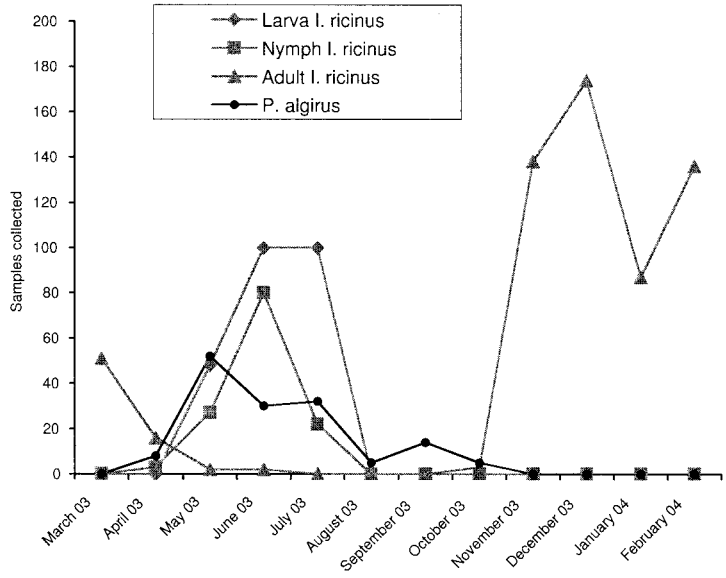

Fig. 1. Seasonal variations of *I. ricinus* and *P. algirus* numbers collected at Jbel EL Jouza (Tunisia) from March 2003 to February 2004.

etation, leading to a greater probability of tick encounter. In contrast, the skin of *P. hispanica* is smooth, and this species usually resides on tree trunks. *L. pater* seems to be favorable to the infestation by *Ixodes* immatures, but this species is very rare in this biotope, only four specimens were captured during the study.

The infestation prevalence of *P. algirus* males and females by *I. ricinus* immature stages was similar, 88.4% (61 /69) and 89.83% (53/59) respectively ($P = 0.79$). In contrast, only 16% (3/18) of juvenile lizards were infested by ticks, showing a very significant difference with adults ($P = 0.003$). The monthly variation of lizard infestation depends on the host breeding activity, particularly for males (Scali et al. 2001). However, monthly variation also may be recorded according to variable biotic and abiotic conditions (Eisen et al. 2001, Tälleklint-Eisen and Lane 2000).

From April to October 2003, 960 *I. ricinus* ticks were removed from 117 *P. algirus.* Therefore, a mean infestation intensity of *P. algirus* by *I. ricinus* larvae and nymphs was 8.20 ticks per lizard (Table 2), and the higher intensity was recorded in June, when the density of free-living immature ticks peaked. Twelve lizards were infested by larvae only, 10 were infested by nymphs only, and 95 were infested by both larvae and nymphs. The mean infestation intensity of lizards by larvae and nymphs was 3.30 (386 larvae per 117 infested lizards) and 4.90 (574 nymphs per 117 infested lizards), respectively. These results are within the range reported by Matuschka et al. (1991), who showed an intensity of infestation of *L. agilis* by *I. ricinus* larvae and nymphs of 2.45 and 4, respectively. In addition, the infestation intensity reached 8.38 (956 ticks per 114 adult lizards) in adult lizards, whereas it was only 1.33 (four ticks per three lizards) in juvenile lizards. This finding may be explained by the fact that lizards regulate their body temperature differently depending on the age. Particularly adult lizards are more able to maintain optimal temperatures and thus are more frequent in arboreous areas (Scali et al. 2001), a favorable biotope for *I. ricinus* ticks.

Table 1. Infestation prevalence of three lizard species captured at Jbel El Jouza (Tunisia) in 2003, by immature *I. ricinus* ticks

Mo	*P. algirus*	*P. hispanica*	*L. pater*
April	7/8 (87.5)	-	1/1 (100)
May	48/52 (92.3)	15/20 (75)	3/3 (100)
June	30/30 (100)	9/17 (52.9)	
July	30/32 (93.7)	2/2 (100)	
Aug.	2/5 (40)		
Sept.	0/14 (0)	0/7 (0)	
Oct.	0/5 (0)	0/1 (0)	
Total	117/146 (80)	26/47 (55.3)	4/4 (100)

Number of infested lizards/total number of captured lizards (%). *P. algirus* is significantly more infested than *P. hispanica* ($P = 0.0007$).

In our study, the tick infestation intensity was higher in males (9, 549 ticks per 61 males) than in females (7.68, 407 ticks per 53 females). This difference between sexes is in accordance with data reported by Lane and Loye (1989). Moreover, males are more active and have larger home ranges than females (Eisen et al. 2001). The higher infestation intensity observed in males also may be related to the difference in testosterone concentration. Indeed, Salvador et al. (1996) described that during the breeding season, *P. algirus* males that were implanted with testosterone carried a higher number of *I. ricinus* larvae and nymphs than control lizards.

Prevalence of *Borrelia* Infection in Field-Collected *I. ricinus*. *I. ricinus* samples, made up of 162 adults, 48 nymphs, and 32 larvae collected on vegetation at Jbel el Jouza, were analyzed for *B. burgdorferi* s.l. infection

Table 2. Intensity of infestation by *I. ricinus* larvae and nymphs (tick number/infested lizards) of *P. algirus* captured at Jbel El Jouza (Tunisia) in 2003

		April	May	June	July	Aug.
No. infested lizards		7	48	30	30	2
Infestation by larvae	No. of ticks removed from infested lizards	1	63	212	103	7
	Infestation intensity	0.14	1.31	7.07	3.43	3.5
Infestation by nymphs	No. of ticks removed from infested lizards	37	316	93	25	3
	Infestation intensity	5.29	6.58	6.43	0.83	1.5

No significant difference was observed along the year, either for infestation by larvae ($P = 0.36$) or by nymphs ($P = 0.06$).

by DFA. The prevalence of infection in adult *I. ricinus* was 34.56% (56/162). This rate is higher than prevalence in nymphs and larvae, which was 12.5 and 0%, respectively. The infection prevalences in adults and nymphs were lower than those previously observed in this biotope by Younsi et al. (2001) who reported, by using PCR technique, a rate of 55.2 and 36.6% for adults and nymphs, respectively. This variation could be explained by a higher sensitivity of PCR, annual variations in the level of tick infection, or both. However, our results confirm that prevalences in adults are higher than in immature stages. Indeed, adult *I. ricinus*, which have ingested a greater number of potentially infected meals than nymphs, usually show a higher prevalence of *B. burgdorferi* s.l. infection than immature stages (Aeschlimann et al. 1986, Strle et al. 1995, Younsi et al. 2001).

***Borrelia* Infection in Lizards.** The scarcity of *B. lusitaniae* reports over Europe may be the reason why its reservoir hosts have not yet been identified. In a first step, we studied the infection of lizards by *B. burgdorferi* s.l. by culturing various tissues (blood, skin, liver, and kidney) collected from a batch of 46 lizards (41 *P. algirus*, four *P. hispanica*, and one *T. mauritanica*) in BSK-H medium. One strain was obtained from the skin of one *P. algirus* and was identified as *B. lusitaniae* by PCR-restriction fragment-length polymorphism. Moreover, DNA of *B. lusitaniae* was detected by PCR in BSK-H medium containing the liver of two additional *P. algirus.*

In a second batch of 47 lizards (37 *P. algirus*, six *P. hispanica*, three *L. pater*, and one *T. mauritanica*), spirochetal DNA was extracted directly from tissues. From 14 of 37 *P. algirus* tested, PCR revealed *Borrelia* DNA in the skin ($n = 8$), the liver ($n = 5$), and the kidney ($n = 6$). The analysis of the restriction patterns of amplicons showed that all DNA samples belonged to *B. lusitaniae.* No *Borrelia* DNA was detected in samples from other lizard species.

Reservoir Role of Lizards. Detection of spirochetes in host tissues indicates that *P. algirus* is susceptible to infection but that it does not inform on its reservoir role, i.e., its ability to transmit the infection to ticks (Gern et al. 1998). Therefore, tick xenodiagnosis was performed on 45 *P. algirus.* In total, 628 larvae detached from them after a mean attachment duration of 10 d (range 6–15 d), with a peak at 7 d. This duration, ≈2 times longer than that observed with rodents, could be related to a slowing down of the bloodmeal uptake by ticks when the temperature of lizards decreases. Only 229/628 (36.5%) engorged larvae molted to the nymphal stage and this, between 28 and 39 d after drop-off. The molting success was higher in May (64.3%) when temperatures where more adequate for ticks than in July–August (33.7%) when most xenodiagnosis have been performed. This may explain the rather low molting success observed in the current study.

A total of 229 xenodiagnostic *I. ricinus* ticks fed on 45 lizards were analyzed for *Borrelia* infection after they molt as nymphs. Among these ticks, 183 nymphs were cultured in BSK-H medium either individually ($n = 44$ ticks) or as pools ($n = 139$ ticks, pools of three nymphs or fewer), resulting in 122 culture tubes. The presence of viable spirochetes was observed by dark-field microscopy examination in 11 culture tubes, and *Borrelia* DNA was detected by PCR in 25 additional tubes. In addition, the 46 remaining nymphs were analyzed individually by DNA extraction and PCR/restriction fragment-length polymorphism. *Borrelia* DNA was detected in 4/46 nymphs. Therefore, if we assume that PCR demonstrates actual infection, 40/229 (17%) xenodiagnostic ticks were infected by *B. lusitaniae.*

All DNAs amplified from nymphs issued from xenodiagnosis were identified by PCR-restriction fragment-length polymorphism as *B. lusitaniae.* One sample belonged to the genotype PotiB3, and another sample to the genotype TD12; these two genotypes were confirmed by sequencing (data not shown). One sample contained a mix of two genotypes PotiB2 and PotiB3. The remaining 37 DNAs belonged to the genotype PotiB2 of *B. lusitaniae* (randomly sequencing of two PCR products confirmed the identification), in agreement with the genetic homogeneity reported previously for this species in free-living *I. ricinus* ticks collected on the vegetation at Jbel el Jouza (Younsi et al. 2001, 2005).

These findings show that at Jbel el Jouza, the lizard *P. algirus* plays a reservoir role for *B. lusitaniae.* The role of lizards as reservoirs for *B. burgdorferi* s.l. was also demonstrated for *Eumeces inexpectatus* and *Anolis carolinensis* in eastern North America (Levin et al. 1996). Recently, Clark et al. (2005) reported evidence of DNA from three *B. burgdorferi* s.l. species among nine species of wild lizards in the southeastern United States, although they did not demonstrate their reservoir competence. In contrast, several other lizard species, such as the western fence lizard (*Sceloporus occidentalis*) (Lane and Loye 1989), the southern alligator lizard (*Elgaria multicarinata*) (Wright et al. 1998), the ground skink (*Scincella lateralis*), the broad-headed skink (*Eumeces laticeps*), and the east-

ern glass lizard (*Ophisaurus ventralis*) (Piesman 2002, Tälleklint-Eisen and Eisen 1999) serve as zooprophylactic hosts, meaning that they cannot sustain a *Borrelia* infection and that *Borrelia* is destroyed in infected feeding ticks. In Europe, *Lacerta viridis*, *Lacerta bilineata*, and *Lacerta agilis* were similarly reported to have a zooprophylactic effect (Matuschka et al. 1994, Scali et al. 2001). Lane and Quistad (1998) reported that the zooprophylactic effect of lizards may be because of borreliacidal factors present in the blood of various lizard species.

The infection rate of xenodiagnostic ticks fed on *P. algirus* is in the range reported in free-living *I. ricinus* nymphs. However, a cofeeding mechanism among infected nymphs and uninfected larvae feeding simultaneously on *P. algirus* (Gern and Rais 1996) also could be involved in the transmission of *Borrelia* between ticks in natural conditions. Hence, natural clustering of ticks on *P. algirus*, as observed in the current study, would plausibly facilitate the cofeeding transmission. The cofeeding transmission may greatly increase vector infection rates (Gern and Rais 1996, Hu et al. 2003). This phenomenon of cofeeding also was reported for some arboviruses (Randolph et al. 1999). Lizards seem to play an important role in the amplification of encephalitis arbovirus in ticks (Randolph et al. 1999). The contribution of lizards to the epidemiology of human Lyme borreliosis could be substantial if the pathogenicity of *B. lusitaniae* is confirmed, and this aspect deserves further study in different countries where *B. lusitaniae* is the main species infecting *I. ricinus.*

In conclusion, at Jbel el Jouza, *P. algirus* is abundant and its activity is temporally and geographically concurrent with that of immature *I. ricinus* ticks. In experimental conditions, we observed that *P. algirus* captured in this biotope transmitted *B. lusitaniae* to *I. ricinus* ticks. Therefore, *P. algirus* can be considered as a reservoir for *B. lusitaniae* in this site. However, the competence could be low as shown by a low rate of nymphs issued from xenodiagnostic larvae. Further studies are recommended to quantify the reservoir competence of *P. algirus* and to evaluate the reservoir status of other lizard species. In spite of our failure to detect spirochetes from the other lizard species, this cannot exclude the possibility of their implication in the transmission cycle of *Borrelia.* Similarly, the study of the reservoir competence of *P. algirus* for *B. lusitaniae* in other biotopes where these species are frequent, such as in Morocco, should be of highest interest.

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