Leeches on Mudskippers: Host–Parasite Interaction at the Water's Edge

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ABSTRACT

A host–parasite relationship was observed, for the first time, between a piscicolid leech and a species of amphibious goby (*Scartelaos tenuis*) from an intertidal mud flat in southern Iran. Morphological and molecular investigations assign the leech to *Zeylanicobdella arugamensis*. Of the 3 endemic and sympatric mudskipper species living in the Persian Gulf (*S. tenuis*, *Boleophthalmus dussumieri*, and *Periophthalmus waltoni*), leeches were only found on *S. tenuis* (prevalence and mean intensity = 71.4% and 2.3 ± 2.5, respectively), which is also the most-aquatic mudskipper species. *Scartelaos tenuis* is not the largest species, but more leeches (≥ 4 leeches/host) were found on larger specimens (> 12 cm standard length [SL]). Nonetheless, in aquaria, leeches also attached on *P. waltoni*. This suggests either an ecological partitioning of host–parasite complexes, determined by host habitat selection, or leech limited resistance to air exposure, or both.

Mudskippers are peculiar, amphibious gobies (Gobiidae: Oxudercinae: Periophthalmini) that "are fully terrestrial for some portion of the daily cycle" (Murdy, 1989). They are distributed within tropical, subtropical, and temperate zones along the African Atlantic coasts and in the entire Indo-West-Pacific region, where they live in estuaries and intertidal areas such as mangrove ecosystems and tidal mud flats. Where sympatric species occur, they are typically differentially distributed along the intertidal gradient (Milward, 1974; Nursall, 1981). Different genera show different degrees of eco-physiological adaptations to terrestriality (Clayton, 1993). This is typically

paralleled by their habitat distribution (Takita et al., 1999). *Scartelaos tenuis* (Day, 1876), *Boleophthalmus dussumieri* Valenciénnes, 1837, and *Periophthalmus waltoni* Koumans, 1955, are present in an area comprising the Persian Gulf, the Gulf of Oman, Pakistan, and the west coast of northern India up to Mumbai (Murdy, 1989). Iranian mudskippers are differentially distributed from more-aquatic to more terrestrial habitats, respectively, from *S. tenuis* to *B. dussumieri* to *P. waltoni* (Clayton, 1985). Very few parasites have been reported in mudskippers, either in nature (Pearse, 1932; Das, 1934; Mhaisen and Al-Maliki, 1996; Bandyopadhyay and Dash, 2001) or in aquaculture (Zhang and Hong, 2003).

Here, we report the first record of leeches as ectoparasites on mudskippers (*S. tenuis*) from a tidal mud flat in southern Iran, together with laboratory observations in aquaria. Piscicolid leeches (Hirudinea: Piscicolidae) are widely distributed as fish ectoparasites in both fresh- and brackish water of all continents except Antarctica, and in all oceans; many species occur in estuaries. The Piscicolidae were recently revised based on both morphological and molecular characteristics (Williams and Burreson, 2006). The fish leeches of the Indian Ocean were reviewed by Sanjeeva Raj (1974), who reported only 3 species from teleost fishes, i.e., *Malmiana* (= *Ottoniobdella*) *stellata* Moore, 1958, *Piscicola olivacea* Harding, 1920, and *Zeylanicobdella arugamensis* De Silva, 1963. Examination of the holotype of *M. stellata* from the Natal Museum shows it to be conspecific with *Z. arugamensis* (E. Burreson, unpubl. obs.). *Piscicola olivacea* has not been reported since the original description (Harding, 1920), although Sawyer (1986) transferred the species to *Calliobdella*.

We document here the occurrence, in nature, of an aquatic parasite only on the most aquatic of 3 sympatric and related species of amphibious hosts; this suggests the presence of differential host selection with respect to hosts' habitat conditions. We examine this hypothesis in the present report.

Live mudskippers were collected, by hand, on the mudflats of Bandar Khamir (Hormozgan Province, Iran; 26°56'40"N, 55°35'55"E; **Fig. 1**) during 2 surveys in April and May, 2007 and 3 surveys in August, 2008. Surveys were made within ± 2 hr around the predicted spring low tide at the nearest reference tidal station (Bandar Abbas; Admiralty EasyTide© UKHO, data available online at: http://easytide.ukho.gov.uk/EasyTide/EasyTide/index.aspx [accessed: 16 August 2008]). Sampling was made along 3 linear transects at 3 different levels of the intertidal gradient, i.e., at increasing horizontal distances from the sand dune to the creek (transects A–C, **Table I**, **Fig. 1**). Individual fishes were placed into separate, transparent plastic bags and transported alive to the laboratory. Fish were killed by placing them at -25 C for <1 hr. Mudskipper specimens were morphologically identified to species level by means of the available morphological keys (Murdy, 1989), and leeches were counted on each host; mudskippers were then measured to the nearest 0.1 cm (standard length [SL]). Eight leech specimens were fixed and preserved in 96% ethanol for

molecular analyses, while other specimens were fixed in 5% formalin and preserved in 60% ethanol for morphological studies.

In the laboratory, several specimens of *S. tenuis*, *B. dussumieri*, and *P. waltoni* were observed for a few days in a 150 X 50 X 60-cm tank. The tank had been filled with a layer of mud and shallow water taken from a tidal mud flat, inhabited by mudskippers nearby the Hormozgan University, in Bandar Abbas, Iran. No filter system was used. They were maintained at room temperature and fed with algae and crab meat.

A sample of *S. tenuis* was deposited in the Genoa Museum of Natural History, Genoa, Italy (MSNG 54633), while a sample of the Iranian *Z. arugamensis* was deposited in the National Museum of Natural History of Washington, Washington D.C. (USNM 1114380) and in the Milan Civic Museum of Natural History, Milan, Italy (MSNM Ar4765). DNA leech haplotype sequences were submitted to the EMBL (codes: FM208109; FM208110; FM208111).

Two individual leeches, fixed in formalin, were examined using a dissecting microscope. One of them was embedded in paraffin and serial sectioned transversely at 5 μm through the clitellum and anterior urosome, following the methods in Burreson and Kalman (2006).

DNA from 8 leeches was extracted and purified by using the DNAeasy© Tissue kit (Qiagen Inc., Valencia, California) reagents. The concentration of the extracted solutions was adjusted to 3 ng/μl and was used directly for PCR amplification of an ~600 bp fragment of the COI region of mtDNA, using the primer LCO140 and HCO2198 (Folmer et al., 1994), and PCR reaction was performed as detailed in Williams and Burreson (2006). The templates obtained were purified, using a standard procedure, with NucleoSpin Extract (Macherey-Nagel, Düren, Germany). Direct sequencing was carried out using a 3730xl DNA analyzer (Applied Biosystems, Carlsbad, California) in forward and reverse directions.

Sequences were aligned by using the ClustalX 1.81 software (Thompson et al., 1997) and were adjusted by eye. The identification of haplotypes, polymorphisms, and parsimony informative sites were conducted with DnaSP 3.52 software (Rozas and Rozas, 1999). The species determination of the haplotypes was performed by aligning the obtained sequences with those found in the International Nucleotide Sequence Databases (INSD), by using the BLAST (Altschul et al., 1990) search engine. A set of closely related sequences from the international database was thus selected, i.e., 4 sequences of closely related taxa based on Williams and Burreson (2006) (DQ414344, *Z. arugamensis*; DQ414334, *Pterobdella amara*; DQ414305, *Aestabdella leiostomi*; and DQ414300, *Aestabdella abditovesiculata*) and the sequence of a species of Glossiphoniidae as an outgroup (DQ995310, *Helobdella robusta*).

These sequences were used to build a phylogenetic hypothesis. The best evolutionary model fitting the dataset was selected by Modeltest 3.06 (Posada and Crandall, 1998). Two different approaches were then employed. A maximum parsimony (MP) analysis was performed using PAUP 4.0b10 (Swofford, 2002), building a strict consensus of the most parsimonious trees by TBR

branch swapping. Bootstrap support values for nodes were computed (1,000 reps.). A Bayesian inference (BI) phylogenetic analysis was also performed (Huelsenbeck et al., 2001), using MrBayes (Huelsenbeck and Ronquist, 2001: 4 Markov chain, 300,000 generations; 100,000 generations were discarded as burn-in).
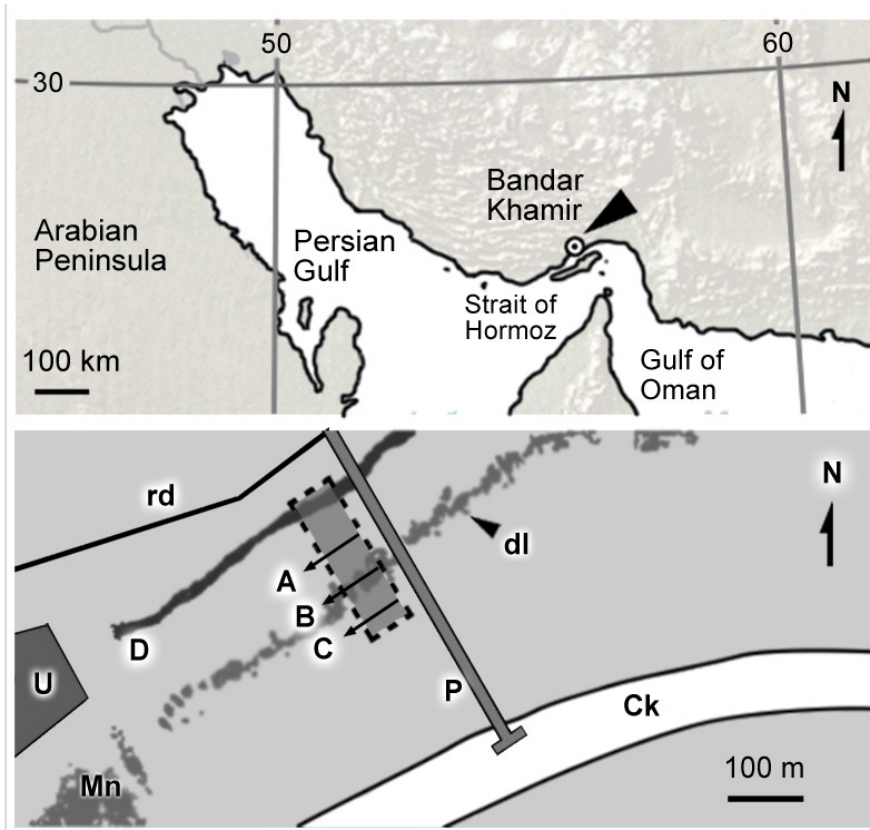
FIGURE 1. Above, Bandar Khamir (black arrow); below, area of study (shaded rectangle with a hatched contour). A, B, C: linear transects respectively made at 50, 100, 150 m from the sand dune, perpendicular to the water edge; Ck = creek; D = sand dune; dl = debris line deposited by the wave action during neap high waters; Mn = stands of stunted mangroves; P = pier; rd = road; U = urbanized area; maps drawn from satellite images (Google Earth Plus, v. 4.2, available online at: http://earth.google.co.uk/ [accessed: 14 June 2008]).

The marine leeches and their hosts were found at low tide on a tidal mud flat nearby Bandar Khamir, Hormozgan, Iran. The mudflat received freshwater input from the small town nearby and was separated from a salt flat by a sand dune, which was parallel to the creek (**Fig. 1**). Only a few, stunted mangrove trees (*Avicennia marina*) were present above, or at the same level of, a debris line, which indicates the approximate level of neap high tide (**Fig. 1**). Above this line, and nearby the sand dune (sand dune transect A, **Fig. 1**), the mud contained a sand fraction and was relatively

compact. In this zone, *P. waltoni* and *B. dussumieri* were present, together with numerous ocypodid crabs. A large majority of *P. waltoni*, with a few *B. dussumieri* specimens, were collected along transect A (**Table I**). Moving towards the sea (transects A–B, **Fig. 1**), the sediment progressively became finer, and the size and abundance of *B. dussumieri* increased while the abundance of *P. waltoni* decreased; some individuals of *S. tenuis* were present here (**Table I**). Below the debris line, at about 150 m from the sand dune (transect C, **Fig. 1**), *P. waltoni* and *B. dussumieri* rapidly declined in number as the mud became wetter. In this lower zone, the flat was covered by tide pools, and there were areas of thixotropic mud. Here, *S. tenuis* was dominant (**Table I**). Piscicolid leeches were found only on specimens of *S. tenuis* (n = 7), with up to 7 leeches per individual (n = 16; prevalence 71.4%; mean intensity 2.3 ± 2.5); they were attached on the dorsum of the head, opercula, throat (**Fig. 2a**), and nearby the attachment of the anal and dorsal fins. More leeches were found on the larger *S. tenuis*, and the highest number of parasites (n = 7) was found on the largest host (SL = 12.8 cm).

TABLE I. Mudskipper and leech sample size (n) and fish size (standard length, mean ±1 SD in cm) at each linear transect, A–C, as shown in **Figure 1**.

Species	A		B		C	
	Length	n	Lenght	n	Length	n
Zeilanicobdella arugamensis	-	0	-	0	-	16
Scartelaos tenuis	-	0	10.8	1	6.2-12.8 (9.7 ± 2.5)	6
Periophthalmus waltoni	2.8-7.4 (5.5 ± 1.9)	4	4.5-15.3 (8.7 ± 3.3)	18	-	0
Boleophthalmus dussumieri	3.9-11.2 (7.1 ± 1.7)	31	3.2-10.4 (7.8±2.3)	12	-	0

No leeches were found on *B. dussumieri* (n = 22) and *P. waltoni* (n = 43), even though their size was comparable to *S. tenuis*; the largest captured mudskipper was a *B. dussumieri* (**Table I**).

In aquaria, the leeches also attached on *P. waltoni* (**Fig. 2b**), although they were never observed on individuals completely out of water. No movements of leeches were observed while they were attached to their host; they remained tightly attached with their caudal sucker, even after death by freezing.
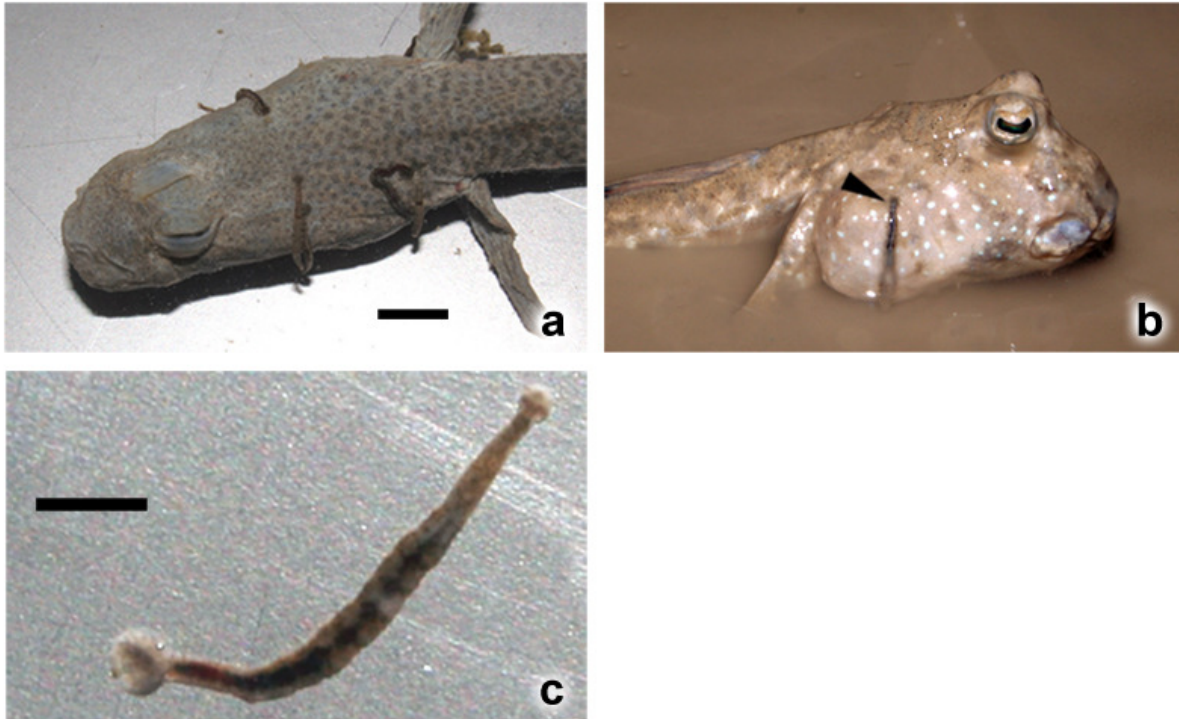
FIGURE 2. (a) Head of freshly dead *Scartelaos tenuis* with the piscicolid leech attached, dorsal view (Bar = 5 mm); (b) Live *P. waltoni* in aquarium with leeches attached; (c) The piscicolid leech (Bar = 2 mm). Photos by G. Polgar.

Both leeches examined morphologically were 15 mm in total length, including suckers. The body was smooth, lacking gills, pulsatile vesicles, or papillae. Any pigmentation, including eyespots and ocelli, had totally faded in the formalin. The caudal sucker was eccentrically attached and moderate in size. The oral sucker was small. Histological sections revealed 5 pairs of testisacs, 2 pairs of mycetomes, and an extensive, ramifying coelomic system. The sections confirmed the absence of pulsatile vesicles. The photographs (**Fig. 2c**) document dark brown or black pigmentation in the form of segmental transverse bands. There were also black pigment bands on the caudal sucker radiating from the sucker–urosome juncture to the margin of the sucker. The oral sucker had a transverse pigment band, and the crop of 1 leech contained blood from the host.

TABLE II. Matrix of p-distances (± 1 SD, when available) estimated among the piscicolid Iranian leech (Iran) and the most closely related sequences available in the INSD database, namely those of *Zeylanicobdella arugamensis* (DQ414344: arug), *Pterobdella amara* (DQ414334: amar), *Aestabdella leiostomi* (DQ414305: leio), and *Aestabdella abditovesiculata* (DQ414300: abdi). The intraspecific variability is reported only for the Iranian leeches.

	Iran	arug	amar	leio	abdi
Iran	0.16 ± 0.14				
arug	6.10 ± 0.10	-			
amar	8.48 ± 0.10	10.38	-		
leio	10.21 ± 0.10	10.80	9.29	-	
abdi	8.88 ± 0.07	8.42	9.72	10.15	-

Three haplotypes were identified (FM208109; FM208110; FM208111), among the 8 specimens examined, based on the alignment of 463 bp of the sequenced region. Variability among these sequences was relatively low (**Table II**), because only 3 polymorphic non-informative loci and 3 non-synonymous substitutions were detected. A BLAST search identified a COI sequence of *Z. arugamensis* (DQ414344) as the most similar one (94% identical), followed by those of *P. amara* (DQ414334, 91% identical), *A. abditovesiculata* (DQ414300, 91% identical), and *A. leiostomi* (DQ414305, 89% identical), respectively. These sequences were aligned with the new haplotypes and compared by means of p-distance (**Table II**). The new sequences showed a relevant divergence from the most similar taxon, namely *Z. arugamensis* (6.10 ± 0.10 SD). Nonetheless, the divergence between the Iranian leech and *Z. arugamensis* was determined by only 31 synonymous mutations, while the variability of all the piscicolid species was determined by 95 mutations, 11 of which were replacements.
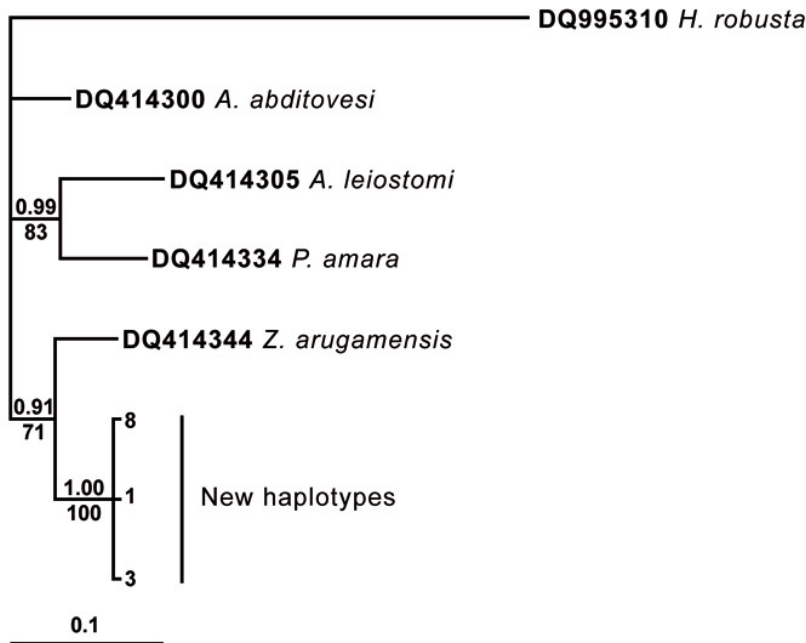
FIGURE 3. Phylogenetic relationships among the Iranian mtDNA COI haplotypes and other homologous sequences. A Bayesian inference tree (BI) is illustrated; the topology of a maximum parsimony analysis (MP) was identical. Numbers at each node indicate a-posteriori probabilities obtained from the BI analysis (above) and bootstrap percentages relative to the MP analysis (below). The glossiphonid leech *H. robusta* was used as outgroup. A. = *Aestabdella*; H. = *Helobdella*; P. = *Pterobdella*; Z. = *Zeylanicobdella*.

MP and BI phylogenetic hypotheses were highly congruent, and the trees had identical topologies (**Fig. 3**). Prior to analysis, Modeltest selected a GTR model (gamma correction = 0.3240). The new haplotypes formed a distinct clade, supported by a high bootstrap value and a-posteriori probability. Their affinity to *Zeylanicobdella* sp. was confirmed.

The morphology of the leech found on *S. tenuis*, i.e., 5 pair of testisacs, 2 pair of mycetomes, an extensive coelomic system, the shape of body and suckers, and black pigmentation in transverse bands, was consistent with *Z. arugamensis* De Silva, 1963. Previous molecular analyses demonstrated a monophyletic clade comprised of *Zeylanicobdella*, *Aestabdella*, and *Pterobdella* (Williams and Burreson, 2006); the present study shows that sequences from the Iranian leech are sister to *Z. arugamensis* (**Fig. 3**). Nonetheless, the bootstrap support value for the relationship between the Iranian leech and *Z. arugamensis* (71) is not as high as the support value for the relationship between A. leiostomi and P. amara (83), 2 clearly distinct species. This might suggest that the Iranian leech is a distinct species; however, the p-distances, although perhaps greater than expected, are less for the Iranian leech – *Z. arugamensis* relationship than between *A. leiostomi* and *P. amara* (**Table II**). *Zeylanicobdella arugamensis* is widely distributed in

the Indian Ocean and throughout Indonesia. Variability in pigmentation pattern has been documented (De Silva and Fernando, 1965; Sanjeeva Raj et al., 1977) and is consistent with our observations, but little is known of the molecular sequence variability across the broad range of this leech. The only deposited sequence data are from a specimen collected in Borneo (Williams and Burreson, 2006), at the opposite end of the range from Iran. Until more is known about the sequence variability of *Z. arugamensis* across its wide range, using multiple genes, we choose to identify the Iranian leech from *S. tenuis* as *Z. arugamensis*, based on morphology. The available molecular data are not inconsistent with this interpretation.

Zeylanicobdella arugamensis (= *Ottoniobdella stellata* Moore, 1958) was originally described from Sri Lanka, but is now known to be widely distributed throughout the Indian Ocean, Indonesia, the Philippines, and the Coral Sea, including the northern coast of Australia. It has been found as an ectoparasite on a wide range of demersal and benthic hosts from different fish families (Moore, 1958; De Silva, 1963; De Silva and Fernando, 1965; Jayadev Babu, 1967; Sanjeeva Raj et al., 1977; Hayward, 1997; Cruz-Lacierda et al., 2000). This leech, and its hosts, typically live in estuarine brackish waters; the only record on a coral-reef species (*Epinephelus coioides*) is a leech infestation that occurred after heavy rain in an aquaculture system (Cruz-Lacierda et al., 2000). This is consistent with our observations, as the tidal mudflat of Bandar Khamir receives continuous freshwater inputs from the nearby town (**Fig. 1**).

This is the first record of a leech parasitizing a mudskipper. Intertidal aquatic conditions, e.g., burrows and tide pools, are particularly harsh during low tide, including rapid and wide fluctuations of temperature (Tytler and Vaughan, 1983), salinity (Sasekumar, 1994), and oxygen levels (Ishimatsu et al., 2000). Therefore, mudskippers' ectoparasites should be specifically adapted to sub-aerial conditions and peculiar host behaviors, such as air gulping (Graham, 1997), air-phase maintenance in burrows (Ishimatsu et al., 1998; 2000), prolonged exposure to air (Sayer and Davenport, 1991), rapid and drastic temperature changes out of water (Tytler and Vaughan, 1983), and intense illumination. While piscicolid leeches were only found on *S. tenuis*, and in the most aquatic conditions in the field (transect C), they rapidly attached to other more-amphibious species in aquaria (*P. waltoni*). This suggests that these parasites are able to tolerate only a limited exposure to air, being present only in the most aquatic environments inhabited by mudskippers. In fact, *S. tenuis* is the most aquatic of the 3 Iranian mudskipper species; it mostly occurs in the lower intertidal zone (transect C: **Fig. 1**), is frequently found half immersed in shallow water, and often occurs in areas of thixotropic mud.

This scenario is compatible with a differential impact of parasites on hosts with different degrees of terrestriality. Host selection seems to be limited by hosts' habitat conditions.

Even though our sample of *S. tenuis* was relatively small, higher prevalence and mean intensity of parasites occurred during hotter months (April and June = 100%, 7.0 and 2.7, respectively; August = 33% and 0.3, respectively); *Z. arugamensis* presents demographic

explosions in small water basins where the salinity drops during periods of intense rainfall (Cruz-Lacierda et al., 2000). In southern Iran, the rainy season is also particularly intense, when ephemeral torrents are formed (wadis). Moreover, mudskippers are known to hibernate during winter in the Persian Gulf, when the temperature drops below 10° C (Tytler and Vaughan, 1983). Therefore, seasonal changes of this host–parasite relationship are highly probable.

A strikingly similar system was studied by Goater (2000) in North America. He examined the differential occurrence of glossiphoniid leeches on sympatric species of plethodontid salamanders with different degrees of terrestriality. In this case also, leeches parasitized more aquatic and larger hosts, and the system underwent drastic seasonal changes.

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