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ORIGINAL RESEARCH ARTICLE

Acanthocephalus ranae (Acanthocephala: Echinorhynchidae) from amphibians in Turkey, with special reference to new morphological features revealed by SEM, and histopathology

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Abstract. Specimens of *Acanthocephalus ranae* (Schrank, 1788) Lühe, 1911 were collected from 6 species of amphibians in 3 families: *Bufo bufo* (Linnaeus, 1758) (Bufonidae), *Hyla arborea* (Linnaeus, 1758) (Hylidae), *Rana dalmatina* Bonaparte, 1838, *Rana macrocnemis* Boulenger, 1885, *Rana ridibunda* Pallas 1771, and *Rana tavasensis* Baran and Atatür, 1986 (Ranidae), from various locations in Turkey between 2000 and 2009. The record from the latter species is a new host record. Compared to the original and subsequent descriptions of *A. ranae*, specimens from Turkey had consistently smaller trunks, lemnisci, testes, and eggs, but measurements of the proboscis, proboscis receptacle, and proboscis hooks were comparable or greater. Our SEM study of morphology revealed new features that were not possible to observe using light microscopy, such as the proboscis/neck area, epidermal micropores, gonopores, and special features of the bursa and its sensory papillae. Histopathological observations and notes on hosts and distribution are also presented.

Keywords: *Acanthocephalus ranae;* Acanthocephala; Amphibians; Turkey; Description; Histopathology; SEM; *Asellus aquaticus.*

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Introduction

There are 31 species of amphibians in 7 families known from Turkey, including 16 species of anurans in 6 families (Baran and Atatur, 1998). The Turkish parasitological literature includes references to *Acanthocephalus ranae* (Schrank, 1788) Lühe, 1911 from 16 species of amphibians including *Mertensiella caucasica* Waga, 1876 (see

Yildirimhan et al., 2005a), *Bombina bombina* (Linnaeus, 1761) (see Yildirimhan et al., 2001), *Bufo bufo* (Linnaeus, 1758) (see Yildirimhan and Karadeniz, 2007), *Bufo viridis* Laurenti, 1768 (see Yildirimhan, 1999), *Hyla arborea* (Linnaeus, 1758) (see Düşen and Öz, 2004), *Rana dalmatina* Bonaparte (1838) (see Düşen et al., 2009), *Rana macrocnemis* Boulenger, 1885 (see Yildirimhan et al., 2006a, Düşen, 2007), *Rana camerani* Boulenger, 1886 (see Yildirimhan et al., 2006b), *Rana ridibunda* Pallas, 1771 (see Oğuz et al., 1994; Yildirimhan et al., 1996; Kir et al., 2001; Yildirimhan et al., 2005b; Düşen and Öz, 2006; Sağlam and Arikan, 2006). Of the 6 species of Amphibia sampled in our study: *B. bufo*, *H. arborea*, *R. dalmatina*, *R. macrocnemis*, *R. ridibunda*, and *Rana tavasensis* Baran and Atatür, 1986, from various locations in Turkey between 2000 and 2009, only the latter species represents a new host record in Turkey.

Morphometric data is provided and SEM images further enhance our knowledge of *A. ranae*, especially regarding anatomical features not previously reported. Because of the wide distribution of *A. ranae* in European amphibians, this report will be limited to Turkish materials only. Many species of trematodes, nematodes, and cestodes have also been collected from the same amphibian host species, but these will not be covered here.

Materials and methods

The present study was undertaken between May 2000 and July 2009 in Denizli: Çivril Işikli Gölü (38°19'19N, 29°51'12E), Çakýroluk (Çakiroluk) (37°41'40N, 29°02'55E), Başkarci (37°45'38 N, 28°59'120E), Antalya: Taşkesiği (37°13'14N, 30°04'05E), Bucak-Burdur: Kestel Lake (37°24'01N, 30°27'59E), Sakarya: (Akyazi) (40°41'N, 30°41'E), and Bursa Uludağ (40°04'N, 29°13'E). These collection sites do not appear to be connected by water. All collections were made in the spring or summer except for 7 specimens of *R. ridibunda* that were examined in Antalya in September, 2001 (3 frogs), and in November, 2007 (4 frogs).

Frogs were examined within 48 hours of capture. Frogs were anaesthetized in ether filled glass containers. Subsequently the body cavity was dissected and the intestinal tract was excised. The opened intestinal tract and other organ systems were dissected out and bathed in 0.5% saline solution, and examined. Acanthocephalans were relaxed in saline and heat-fixed under slight cover slip pressure in warm alcohol-formalin-acetic acid (AFA) then transferred to 70% ethanol before staining. They were stained with acetocarmine, dehydrated in ethanol series, cleared in cedar oil, and whole-mounted in Entellan® or Canada Balsam. Measurements were made using ocular micrometer.

Voucher specimens of the parasites were deposited in the Ege University, Museum of Zoology, Izmir, Turkey (ZDEU HEL-7-15/2003, ZDEU HEL-2/2006, ZDEU HEL-25-28/2007, ZDEU HEL-16-18/2008, ZDEU HEL-16-19/2009). Host specimens were deposited in the Pamukkale University, Department of Biology, Denizli Turkey (PAU-1/2000, PAU-1-5/2001, PAU-1-2/2003, PAU-1-2/2006, PAU-1-3/2007, PAU-1-3/2008, PAU-1-4/2009).

For SEM, samples of *A. ranae* that had been AFA-fixed and stored in 70% ethanol were processed following standard methods (Lee, 1992) that included critical point drying (CPD) in sample baskets and mounted on SEM sample mounts using conductive double sided carbon tape. Samples were then gold coated for 3 minutes using a Polaron E3500 sputter coater establishing an approximate thickness of 20nm. Samples were then placed in a FEI XL30 ESEM FEG under low vacuum conditions. Samples were imaged using 10 KV, spot size 3 at 0.7 torr using the GSE detector. Permanent records were obtained with a digital camera at various magnifications.

For histopathological sections, standard methods (Galigher and Kozloff, 1971; Keinan, 2002) were employed for the examination of the infected host intestinal tissue. Samples of *A. ranae* embedded in host tissue that have been AFA-fixed and stored in 70% ethanol were transferred to 10% buffered formalin (v/v). The infected host tissue was dehydrated and blocked in paraffin. The blocks were sectioned at 4-6 micrometers (μ), placed on glass slides and stained with Harris hematoxylin and eosin (HE), Mallory's trichrome stain, and then

viewed with an LSM laser (Carl Zeiss, Thornwoood, New York) equipped compound light microscope. Representative pictures were taken with an attached digital camera at various magnifications and stored in a memory disk for future reference. HE is a standard stain for viewing pathological tissue while Mallory's trichrome is used for viewing specific cells characteristic of tissue inflammation.

Results and discussion

Geographical distribution

The orientation of the western Anatolian rivers to the west appeared in early Pleistocene when there was a brackish water lake in the Denizli region (Demirsoy, 1999). The Çakiroluk area has meadow vegetation (Çakiroluk Spring). The existing little stream suddenly disappears in the soil (Baran, 1969; Düşen, 2009). The Işikli Lake (Çivril) is connected to the Büyük Menderes River. (www.dsi.gov.tr/bolge/dsi21/denizli.htm). The water of Karci stream (Başkarci) is formed and maintained by the Karci Mountain (2308 m) (www.dsi.gov.tr/bolge/dsi21/denizli.htm). In Taşkesiği (Korkuteli-Antalya), samples were collected in irrigating ponds. The Tethys Sea regressed in Mid Miocene from Western Taurus Mountains and formed the "Lakes District" (Göller Yöresi) (Demirsoy, 1999). In Kestel Lake (Bucak-Burdur), in Sakarya Akyazi region, and in Bursa Uludağ, samples were collected in small ponds and streams. At 2543 m high, Mount Uludağ is the highest point of the city. Where the major river is Nilüfer Brook, which is a branch of Susurluk River. There are many large and fertile plains within the borders of Bursa (mmfwww.uludag.edu.tr/about_bursa.pdf).

Acanthocephalus ranae (Schrank, 1788) Lühe, 1911

Acanthocephalus ranae is one of the most widely distributed species of acanthocephalans in Europe. Among the 68 frogs examined, 35 were found infected with 156 *A. ranae* (table 1). The record from *R. tavasensis* is a new host record. Its amphibian hosts have been frequently reported in Turkey and in most of Europe. Of the 31 species of amphibians in 7 families known from Turkey including 16 species of anurans in 6 families (Baran and Atatur, 1986; 1998), 16 species are known hosts of *A. ranae* (see Introduction). Its versatile crustacean intermediate host, *Asellus aquaticus* Linnaeus, 1758 (Asellidae) (Crompton, 1970; Canning, 1973; Kirbanov, 1978 in Schmidt, 1985) is also widely distributed in European waterways. *Asellus aquaticus* is also an intermediate host for other species of acanthocephalans in Europe including *Acanthocephalus anguillae* (Müller, 1780) Lühe, 1911 (see Dezfuli et al., 1994) and *Acanthocephalus lucii* (Müller, 1780) Lühe, 1911 (see Hasu et al., 2007). Infections with *A. ranae* might be expected to show a seasonal pattern since even the arboreal species, e.g., *H. arborea*, will only be exposed to infection during spawning. Crompton (1970), however, noted that *A. ranae* can persist in frogs that have not fed for 4 months. Accordingly, infections could have possibly been acquired while the frogs or toads were in the water, and then persisted (Canning, 1973). All collections reported in this study were made in the spring or summer from noted locations (table 1) with the exception of only 7 specimens of *R. ridibunda.* The 7 specimens were collected in Antalya (3) and Lake Denizli (4) during September and November, respectively, and all were infected with *A. ranae*.

Description of the Turkish material

Measurements of specimens from various geographical locations (table 2) were similar and were combined because of the small sample size from each host species in each location. Compared to the description of *A. ranae* based on Meyer (1932), Petrochenko (1956) and Golvan (1969) (their measurements in parentheses), our specimens from Turkey (table 2) had consistently smaller trunk (males 5.0-12.0 X 1.0 mm, females 20.0-60.0 X 2.0 mm), lemnisci (0.6-1.4 mm long), testes (0.97 X 0.34 mm) and eggs (0.110 mm long). Measurements of the proboscis (0.42-0.47 X 0.31-0.34 mm), proboscis receptacle (0.97-1.02 X 0.31-0.34 mm), and anterior, middle, and basal proboscis hooks (0.064-0.068 mm, 0.070-0.071 mm, and 0.045 mm long, respectively) were comparable or greater in the Turkish specimens.

Table 1. Infection rates of *Acanthocephalus ranae* in amphibians collected between May 2000 and July 2009 from different localities in Turkey

Locality		Amphibian species	Amphibians examined	Amphibians infected	Parasites collected	Prevalence (%)	Mean intensity
Denizli	Işikli	*Rana ridibunda*	54	21	75	39	3.6
	Çakiroluk	*Rana tavasensis*	2	2	14	100	7.0
	Çakiroluk	*Hyla arborea*	1	1	2	100	2.0
	Başkarci	*Bufo bufo*	1	1	5	100	5.0
Antalya	Taşkesiği	*Rana macrocnemis*	2	2	13	100	6.5
	Taşkesiği	*Rana ridibunda*	2	2	20	100	10.0
	Taşkesiği	*Hyla arborea*	1	1	1	100	1.0
Bucak-Burdur	Kestel Lake	*Rana ridibunda*	2	2	16	100	8.0
Bursa	Uludağ	*Rana dalmatina*	1	1	5	100	5.0
Sakarya	Akyazi	*Rana dalmatina*	2	2	5	100	2.5
Total			**68**	**35**	**156**	**51**	**4.5**

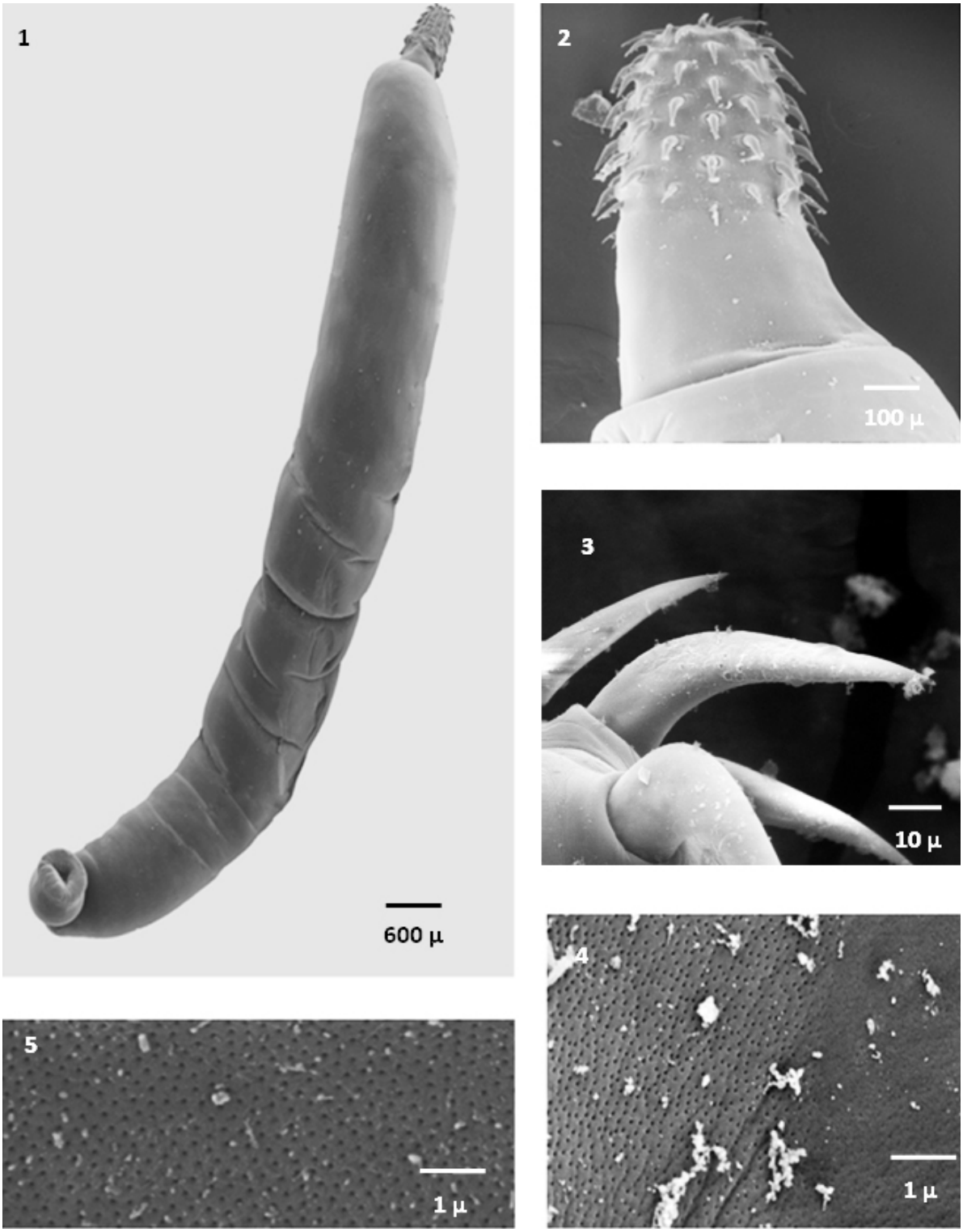
The number of proboscis hook rows of 12-18 and hooks per row of 4-6 fell within the reported range for the species (12-20 rows of 4-6 hooks each) (table 2).

Within the Turkish collection (table 2), the size of specimens appeared to vary in different geographical populations rather than by host species. For instance, specimens from *R. ridibunda* from Bucak-Burder were consistently smaller than specimens from the same host species and others elsewhere in all characters. There was no homogeneity in the size of all structures in the same location. For instance, the largest trunk was observed in specimens from Denzili, largest proboscis in specimens from Sakarya-Bursa, largest proboscis receptacle in specimens from Antalya, and longest proboscis hooks in specimens from Denzili and Antalya. The size of certain anatomical structures has been demonstrated to vary in different geographical populations, e.g., in *Acanthocephalus dirus* (Van Cleave, 1931) in North America (Amin, 1984) and in *Mediorhynchus papillosus* Van Cleave, 1916 (Gigantorhynchidae) in North America, Taiwan, and Eurasia (Amin and Dailey, 1998), among other species.

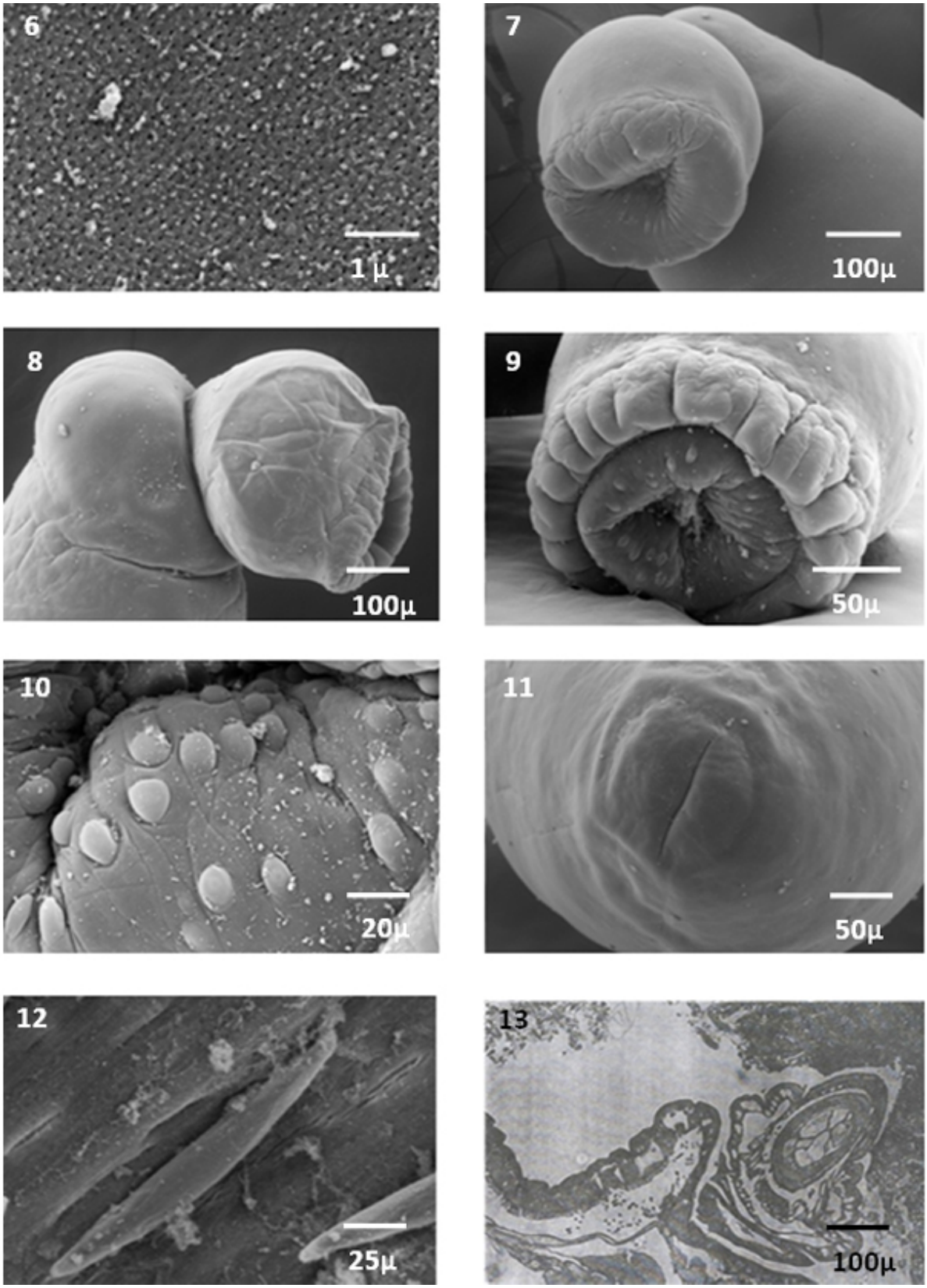
Morphologically, our SEM images have revealed new features that were not possible to observe using conventional microscopy (figures 1-12). The cylindrical male is shown in figure 1. The large neck almost as long as and wider than the proboscis (figure 2) is in contrast to the neck described by Petrochenko (1956) as "very short and conical". Apical hooks were seen as acutely curved (figure 3) compared to other hooks on the proboscis (figure 2).

The micropores in the epidermis appeared dense in the mid-trunk (figure 4), most dense in the anterior trunk (figure 5, left) where they come to an abrupt end at the junction with the neck which has no micropores (figure 5, right), and least dense in the posterior trunk (figure 6). This is the first time such a gradient in micropore density has ever been reported in the Acanthocephala. The reports that these peripheral canals are continuous with canalicular crypts that constitute a huge increase in the external surface area implicated in nutrient uptake, e.g., Whitfield (1979), might suggest that different regions of the epidermis of *A. ranae* are not equally involved in nutrient uptake and absorption.

The bursa is lateral and ventral against a dorsal bump at the dorso-terminal end of the trunk (figures 7, 8), and muscular with a pronounced rim and many elongate discoid sensory papillae (figures 9, 10). The female gonopore is slit-like and terminal with 2 muscular lips (figure 11). Eggs are fusiform and elongate with pointed ends (figure 12).



Figures 1-5. SEM of specimens of *Acanthocephalus ranae* from *Rana ridibunda*. 1. A male specimen. 2. The proboscis of the same specimen in Fig. 1, note the large neck. 3. Apical proboscis hooks with typical acute curvature. 4. Epidermal micropores in anterior trunk (left) absent from the neck (right). 5. Epidermal micropores in mid-region of trunk.



Figures 6-13. SEM of specimens of *Acanthocephalus ranae* from *Rana ridibunda* (Figs. 6-12), and histopathological section in the intestine of *R. ridibunda* (Fig. 13). 6. Epidermal micropores in posterior trunk. 7**.** Face view of a bursa. 8. lateral view of a bursa showing its lateral orientations and the dorso-posterior swelling in the trunk. 9. A higher magnification of the muscular rim of the bursa showing the location and shape of the sensory discs. 10. A close up of the sensory discs showing their shape. 11. Female gonopore. 12. An egg. 13. Section of host intestine infected with *A. ranae*. Note the obstruction of the lumen with concomitant hemorrhaging in the invaded area.

Table 2. Measurements of *A. ranae* from Denizli, Antalya, Bucak-Burdur, and Sakarya-Bursa in Turkey

	Rana ridibunda, Rana tavasensis Hyla arborea, Bufo bufo **Denizli**		***Rana ridibunda, Hyla arborea Rana macrocnemis*** **Antalya**		***Rana ridibunda*** **Bucak-Burdur**		***Rana dalmatina*** **Sakarya-Bursa**	
	(n=32) ♂♂	**(n=19)** ♀♀	**(n=14)** ♂♂	**(n=13)** ♀♀	**(n=5)** ♂♂	**(n=2)** ♀♀	**(n=6)** ♂♂	**(n=5)** ♀♀
TL*	9.42±4.03 (6.34-20.21)**	19.24±8.33 (9.18-33.00)	8.80±1.14 (6.93-9.93)	13.87±6.08 (7.90-25.27)	4.05±0.55 (3.23-4.55)	8.38±1.39 (7.39-9.36)	9.95±2.20 (6.93-11.92)	12.62±2.79 (8.83-16.43)
TW	1.07±0.49 (0.51-2.46)	1.19±0.52 (0.65-2.86)	0.949±0.17 (0.75-1.32)	1.21±0.32 (0.55-1.69)	0.48±0.09 (0.35-0.59)	0.45±0.01 (0.44-0.45)	1.04±0.23 (0.75-1.34)	1.38±0.41 (0.91-1.89)
PL	0.52±0.10 (0.32-0.69)	0.66±0.12 (0.51-0.85)	0.54±0.12 (0.35-0.69)	0.59±0.19 (0.35-0.96)	0.40±0.11 (0.30-0.49)	0.46±0.04 (0.43-0.49)	0.65±0.07 (0.53-0.71)	0.73±0.15 (0.57-0.91)
PW	0.26±0.07 (0.18-0.51)	0.31±0.07 (0.22-0.47)	0.316±0.04 (0.26-0.41)	0.39±0.08 (0.16-0.47)	0.18±0.01 (0.16-0.18)	0.22	0.31±0.06 (0.24-0.39)	0.40±0.06 (0.32-0.47)
PRL	0.78±0.43 (0.45-2.03)	0.82±0.31 (0.41-1.63)	0.92±0.30 (0.49-1.36)	1.06±0.40 (0.53-1.63)	0.40±0.09 (0.32-0.57)	0.43±0.03 (0.41-0.45)	0.83±0.36 (0.49-1.40)	1.43±0.47 (0.63-1.75)
PRW	0.25±0.07 (0.16-0.47)	0.32±0.09 (0.20-0.49)	0.32±0.07 (0.18-0.45)	0.34±0.08 (0.18-0.51)	0.18±0.03 (0.14-0.20)	0.18±0.03 (0.16-0.20)	0.27±0.03 (0.24-0.32)	0.41±0.08 (0.32-0.47)
L	0.88±0.25 (0.47-1.32)	0.97±0.43 (0.61-1.58)	0.80±0.11 (0.67-0.91)	0.97±0.21 (0.71-1.22)	0.45±0.14 (0.28-0.61)	0.54±0.13 (0.45-0.63)	0.97±0.21 (0.75-1.22)	0.86±0.19 (0.71-1.12)
AHL	59±18 (43-102)	79±26 (52-134)	78±16 (56-100)	78±19 (48-104)	51±2 (48-52)	58±3 (56-60)	67±12 (52-84)	64±23 (48-88)
MHL	72±10 (55-89)	90±19 (69-126)	88±13 (64-104)	95±15 (76-120)	68±3 (64-72)	68±6 (64-72)	77±10 (64-92)	99±28 (68-136)
BHL	54±17 (35-93)	61±26 (36-106)	75±20 (40-100)	69±22 (40-100)	47±2 (44-48)	46±3 (44-48)	60±17 (40-80)	54±31 (32-88)
HR	12-16	12-15	13-18	12-17	13-18	13-14	13-17	12-17
H/R	5-6	4-6	4-5	4-6	4-5	4-5	4-6	5-6
ATL	0.84±0.34 (0.51-1.69)	____***	0.83±0.16 (0.53-1.08)	——	0.42±0.03 (0.39-0.45)	——	0.82±0.17 (0.65-1.06)	——
ATW	0.51±0.29 (0.22-1.22)	____	0.44±0.10 (0.32-0.69)	——	0.23±0.05 (0.16-0.28)	——	0.61±0.08 (0.53-0.71)	____
PTL	0.85±0.37 (0.59-1.87)	——	0.80±0.15 (0.53-1.02)	——	0.46±0.05 (0.41-0.53)	——	0.81±0.22 (0.61-1.12)	——
PTW	0.50±0.29 (0.20-1.18)	____	0.44±0.11 (0.30-0.69)	——	0.27±0.05 (0.20-0.30)	——	0.59±0.08 (0.47-0.67)	——
BL	0.33±0.08 (0.22-0.43)	____	0.40±0.16 (0.20-0.61)	——	0.16	——	0.55	——
BW	0.31±0.04 (0.26-0.39)	____	0.48±0.11 (0.39-0.65)	——	0.14	——	0.51	——
EL	____	95±20 (52-112)	——	97±35 (56-140)	——	Immature	——	85±20 (64-104)
EW	____	13±2 (10-16)	——	16±3 (12-20)	——	Immature	——	20±4 (16-24)

* AHL: Apical hook langth. ATL: Anterior testis length. ATW: Anterior testis width. BHL: Basal hook length. BL: Bursa length. BW: Bursa width. EL: Egg length. EW: Egg width.
L: Lemniscus length. HR: No. of proboscis hook rows. H/R: No. of hooks per row. MHL: Median hook length. PL: Proboscis length. PRL: Proboscis receptacle length.
PRW: Proboscis receptacle width. PTL: Posterior testis length. PTW: Posterior testis width. PW: Proboscis width. TL: Trunk length. TW: Trunk width.
** Mean± standard deviation (range). All measurements are in mm except those of proboscis hooks and eggs (in μ).
*** Information not available.

Histopathology

Sections of fish intestine infected with *A. ranae* (figure 13) show obstruction to the lumen of the tract with necrotic tissue in the area. The proboscis is seen attached to the inner layer of the intestine. Normal host tissue with visible villi is seen to the left of the organism (figure 13) but extensive hemorrhaging with numerous nucleated red blood cells free in the lumen space appears on the right side. Granulocytes are visible in high concentrations around the anterior end of the worm typical of parasite invasion into soft host tissue. Columnar cells, typical of host villi, are absent in the area of the parasite but visible to the left

side. Blockage of the intestinal lumen was prominent in this (figure 13) and other sections examined.

The hosts

The Turkish, as well as the European, parasitological literature includes ample references to *A. ranae* from many species of amphibians. In the present treatment, we report on the distribution and diet of the six host species of amphibians in order to better understand the ecology of their infection with *A. ranae* in Turkey. No matter the nature of the habitats and reported diet of each of these six species of amphibians, because they are infected with *A. ranae* they must access the intermediate host, *A. aquaticus*, in its waterways during their breeding season.

Bufo bufo lives in damp, rocky or pebbly areas with sparse vegetation or in forest floor. It is fossorial and forages nocturnally, preying on insects, earthworms and some mollusks. It is found in Western Turkish Thrace, Middle, Western, and Northeasthern Anatolia (Yildirimhan and Karadeniz, 2007).

Hyla arborea is a small nocturnal arboreal species that shelters under leaves in the daytime. It goes to water only in the breeding season, preferring clean, deep, and heavily vegetated water. It feeds on various insects and spiders. In Turkey, it is known in Northwestern and Southwestern Anatolia (Düşen and Öz, 2004).

Rana dalmatina is a medium sized nocturnal agile strictly terrestrial anuran species that lives in deciduous forests and damp grasslands with dense vegetation usually far away from water bodies at up to 1500m elevation. Its diet consists of various insects. It is found in the Turkish Thrace region and the northern parts of Anatolia (Düşen et al., 2009).

Rana macrocnemis, the Uludağ frog, usually lives near small streams in open fields or forested areas as well as wet grasslands and other places in close proximity to water bodies. Its diet consists of 68% insects (primarily Coleoptera) and other prey items including Oligochaeta, Arachnida, Diplopoda, Chilopoda and anuran larvae (Uğurtaş et al., 2004). In Turkey it is found in west, south and north Anatolia. It is a typical montane form with vertical distribution between 1000-2300 m.

Rana ridibunda, the diurnal marsh frog, is a medium-sized aquatic anuran species that inhabits lakes, pools, or slowly flowing streams with much vegetation. It stays in proximity to water and prefers low plains or marshes (Baran and Atatür, 1998). It is an opportunistic feeder that also forages also at night (Atatür et al., 1993; Çicek and Mermer, 2006; 2007; Ferenti et al., 2009). Its main diet is comprised of Arthropoda (Insecta, Arachnida, Crustacea, Chilopoda, Diplopoda), Gastropoda, Annelida, and Pisces (Cyprinidae) (Atatür et al., 1993; Colak-Yilmaz and Kutrup, 2006; Çicek and Mermer, 2006; 2007), as well as some small size mammals (Atatür et al., 1993; Çicek and Mermer, 2006,; 2007; Ferenti et al., 2009). It also exhibits cannibalistic behavior (Ruchin and Ryzhov, 2002; Cicek and Mermer, 2006; 2007; Colak Yilmaz and Kutrup, 2006; Ferenti et al., 2009). In Turkey, it is the most widely distributed frog in all suitable habitats except for a portion of the Lakes District.

Rana tavasensis was originally described from the area of Akdag-Cakiroluk district, near Kizilcabölük-Tavas in Denizli province in Turkey (Baran and Atatür, 1986). It usually inhabits areas with small slowly flowing streams in open fields and wet grasses in forested areas (Budak and Göçmen, 2008). Its diet consists of various insects.

The wide diversity of the habitats of the anuran hosts noted above is clearly associated with the marked versatility of the intermediate host, *A. aquaticus*, in the same habitats making larval acanthocephalans accessible to the definitive hosts at such high prevalence rates (table 1).

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