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EXPERIMENTAL INFECTION OF *RADIX NATALENSIS* AND *CULISETA LONGIAREOLATA* LARVAE WITH PLAGIORCHIID XIPHIDIOCERCARIAE IN EGYPT

By

YASSER DISOKY DAR* AND IMAN MOHAMAD EL-HUSSENY

Department of Zoology, Faculty of Science, Tanta University, Tanta, Egypt.
(*Correspondence: e-mails: yasser.dar@science.tanta.edu.eg, imanhusseny@yahoo.com)

Abstract

To clarify the life cycle of a plagiorchiid species which uses *Radix natalensis* as a first intermediate host for its larval development in Egypt, this study was carried out to investigate subsequent invertebrates where xiphidiocercariae of this digenean might encyst. Laboratory-bred *R. natalensis* and *Culiseta longiareolata* larvae (mosquito) were experimentally exposed to xiphidiocercariae under laboratory conditions. At 24h post-exposure, 70% of exposed juvenile *R. natalensis* were found harboring encysted cercariae in their tissues.

In addition, *C. longiareolata* larvae of each developmental stage were infected with these xiphidiocercariae. Compared to unexposed mosquito larvae, a significantly higher mortality rate in first- and second-stage (L1& L2) larvae was noted. Prevalence of infection was also significantly higher in these L1 & L2 (90% & 88%, respectively) than in third- and fourth-stage larvae. Mosquitoes emerging from exposed L1 & L2 were significantly less numerous when compared to corresponding control groups. Significant disturbance in male and female sex ratio was noted in L1- & L2-developing mosquitoes; it was equal in L1-emerging adults and slightly male biased for those coming from L2.

Key words: Egypt, *Culiseta longiareolata,* mosquito, *Radix natalensis*, snail, xiphidiocercaria**.**

Introduction

Xiphidiocercariae constitute a group of cercariae characterized by the presence of a stylet in the anterior margin of the oral sucker and a long unforked tail (Frandsen and Christensen, 1984). Three subtypes are recognized: ornatae (with a fin-fold tail), virgulate (with a virgula organ and a ventral sucker smaller than oral one), and armatae (without virgula organ but with oral and ventral suckers equal in size), each of which belonging to a particular parasite family. They may develop in sporocysts in a first intermediate host and encyst in a second intermediate host, or they may encyst in sporocysts according to the xiphidiocercaria subtype (Olsen, 1986).

The trematode species incorporating xiphidiocercariae in their life cycle such as Plagiorchiida families use two intermediate hosts, whatever cercarial subtype. The first is always a snail which sustains larval development and cercarial production, whereas the second, in which xiphidiocercariae encyst, may vary according to digenean species (Olsen, 1986). The arthropod aquatic larvae, other snail species, and tadpoles are most common examples as second intermediate hosts (Velasquez, 1964; Grabda-Kazubuska, 1969; Smyth, 1994). *Haematoloechus*, *Plagiorchis* and *Prosthogonimus* xiphidiocercariae could successfully encyst in mosquito larvae such as *Aedes aegypti*, *Ae albopictus*, *Anopheles quadrimaculatus* and *Culex quinquefasciatus*. These entomophilic cercariae could be also considered as an efficient mean of biological control for mosquito larvae (Rao *et al*, 1985; Webber *et al*, 1987; Dempster and Rau, 1991; Lacy and Orr, 1994; Carvalho *et al*, 2001). Infection by encysted xiphidiocercariae could have an influence on the usual larval development of these mosquitoes. Experimental infection of *Ae. aegypti* 4th stage larvae by *Plagiorchis noblei* metacercariae impaired survival and development of these mosquito larvae (Dempster *et al*, 1986). In addition, a delay in the life cycle, a high mortality rate and malformations of *Ae. albopictus* adults resulted from their infection with haematoloechid xiphidiocercariae in the laboratory (Carvalho *et al*, 2002). Exposure of *Ae. aegypti* to *P. elegans* xiphidioercariae can directly affect fitness parameters of mosquito larvae (Mitchell-Foster *et al*, 2012).

In Egypt, xiphidiocercariae of different types were detected in many freshwater snail species such as *Radix natalensis*, the principal intermediate host of giant liver fluke, *Fasciola gigantica* (Wanas *et al*, 1993; Dar *et al*, 2005). High prevalence of *R. natalensis* infection with xiphidiocercariae was reported in many Egyptian governorates (Helal *et al*, 2009), suggesting the role of this snail as a first intermediate host for cercarial development. Based on morphological and behavioural features of xiphidiocercariae obtained from *R. natalensis*, Dar (2009), using keys for cercarial identification (Frandsen and Christensen, 1984), identified these cercariae as the armatae subtype belonging to a plagiorchiid species of genus *Plagiorchis*. According to Dar (2009), these cercariae developed in sporocysts (with absence of the redial stage) and were shed from *R. natalensis* to encyst in another snail species, *Biomphalaria alexandrina.* This last snail could act as a second intermediate host in reason of natural and successful experimental infections with the encysted stage of these cercariae. Moreover, xiphidiocercariae were also capable of encysting in *Bulinus truncates* and *Physa acuta* under laboratory conditions.

Culiseta longiareolata (Diptera: Culicidae) is a common Mediterranean species found throughout North Africa. Its larvae were found in stagnant water bodies and the adult females affected birds and mammals, especially cattle,

but not humans (Kirkpatrick, 1925). In Egypt, an established colony of this mosquito was naturally recorded in the Beheira governorate (in an old well in the Wadi El-Natroun valley) and this colony was successfully maintained in the laboratory for experimental assays (Adham, 1982). *C. longiareolata* larvae showed a high predation capacity against larvae of other mosquito species and this species was thus considered as an alternative biological control agent (Shaalan and Canyon, 2008; Shaalan, 2012). However, the role of this mosquito species as a vector involved in the life cycle of digeneans in Egypt was not still reported.

The present study aimed to investigate subsequent invertebrate host species in which the xiphidiocercariae shed from naturally infected *R. natalensis* may encyst. The encystment of this cercarial type in different snail species (Dar, 2009) poses the question of whether *R. natalensis* itself may be a second intermediate host for cercarial encystment.

In addition, the role of mosquito larvae as a potential intermediate host for xiphidiocercariae was also examined. The *R. natalensis* were collected from several regions to obtain xiphidiocercariae for experimental assays and to investigate if they naturally harbour the encysted stage of these cercariae in tissues. Under the laboratory conditions, the laboratory-bred *R. natalensis* and *C. longiareolata* larvae of different developmental stages were experimentally exposed to the xiphidiocercariae.

Material and Methods

The xiphidiocercariae were obtained from naturally infected *R. natalensis* collected from an irrigation canal at Kafr-Hegazy Village, El-Mahala City, Gharbia Governorate, Egypt. This region was known for its high prevalence of infected snails with xiphidiocercariae throughout the year (Helal *et al*., 2009). Eighty snails (8-10 mm in height) were individually placed in 50-mm diameter Petri dishes with a water layer of 10 mm and were subjected to artificial light for 4 h to enhance cercarial emission. Petri dishes were examined under a light microscope to detect cercarial shedding. The number of cercariae per snail was daily calculated for three successive days. Non-shedding snails were dissected under a stereomicroscope to verify if they contained encysted stages of xiphidiocercariae. Surviving adult *R. natalensis* were then placed in a breeding aquarium and fed on lettuce ad libitum at room temperature (25°±1°C). Egg masses were collected from the aquarium using a fine needle and were incubated to allow egg development. Newly hatched snails were transferred to small aquaria containing dechlorinated water and finely powdered, dried lettuce. When snails attained 2 mm in height, they were exposed to xiphidiocercariae. Cercariae were fixed, either in the AFA (Alcohol-Formalin-Acetic acid) solution, or in formaldehyde-glutaraldehyde (4:1) for light or scanning electron microscopy (SEM), respectively.

Egg rafts of *C. longiareolata* were collected from a stagnant freshwater pool near the Faculty of Science, Tanta city, Egypt. They were placed in white enamel pans containing dechlorinated tap water and incubated at room temperature to obtain successive larval stages: first, second, third and fourth (L1, L2, L3 & L4). Larvae of each stage were placed in separated containers for experimental exposure to xiphidiocercariae. Other four groups of unexposed mosquito stages were used as controls. These larvae were fed on fish food (Tropical flakes-pets pacifical), sprinkled once daily over the water surface of breeding pans.

Fifty juvenile *R. natalensis* were individually exposed to infection by placing a Petri dish containing infected snails. One hundred larvae of each *C. longiareolata* developmental stage were exposed to xiphidiocercariae by putting three infected snails in their rearing pans. After 24h post-exposure (p.e.), the snails were dissected under a stereomicroscope to detect encysted stages of xiphidiocercariae in their tissues, whereas the mosquito larvae were carefully examined under a light microscope to observe the cysts in their see-through tissues before returning them to the pans where they were left until their adult stage. Survival and prevalence of infection were determined for each experimental or control group. Adult emergence and male/ female sex ratio from exposed and unexposed larval mosquito stages were determined. A Chi-square test and one-way analysis of variance (Stat-Itcf, 1998) were used to determine levels of significance.

Results

Snail-shed xiphidiocercariae showed vigorous movements in water via their tails and cercarial body contractions. By the presence of an oral and a ventral sucker of nearly equal sizes and an unforked single tail devoid of fins or any other structures. The tail arises from a deep pocket in the posterior end of the cercarial body. A distinctive stylet was observed at the anterior margin of the oral sucker and its tip protrudes at the anterior end of the cercarial body (Fig. 2). The mean number of shed cercariae varied from 142 to 228 per snail per day. Dissection of non-shedding snails showed the presence of encysted xiphidiocercariae inside the snail tissues with high density, especially in the mantle and the head-foot region (Fig. 3). However, these dissected specimens did not contain any other larval stages. Stylets detached from the cercarial bodies were observed inside cysts (Fig. 4). Of 50 juvenile *R. natalensis*, 35 snails (70%) were found harbouring the encysted stage of cercariae in their tissues. The mortality of juvenile snails subjected to experimental infection with xiphidiocercariae did not significantly differ from that of the unexposed control group.

At 24 h p.e., *C. longiareolata* larvae of each stage were found infected with the encysted stage of xiphidiocercariae. Metacercariae were detected in various regions of larvae, in their head, thorax, and abdomen (Fig. 5a, b). The infection intensity (mean number of cysts

per larva) in L1 (10 cysts) and L2 (12) was significantly greater ($F = 26.28$, $P < 0.05$) than that observed in L3 (5 cysts) and L4 (3). L1 and L2 mortality was significantly higher (L1: $\chi^2 = 19.7$, $P < 0.001$; L2: $\chi^2 = 51.9$, $P < 0.001$) than that of unexposed controls (Tab. 1). Compared to controls, no significant difference in L3 & L4 mortality was noted. Prevalence of experimental infections in L1 & L2 was significantly higher ($\chi^2 = 250.1$, $P < 0.001$) than that noted for L3 & L4. Contrary to adults emerging from exposed L3 & L4, the frequencies of adults developing from exposed L1 & L2 showed low significant values (L1: $\chi^2 = 116.6$, $P < 0.001$; L2: $\chi^2 = 90.1$, $P < 0.001$) when compared to corresponding controls (Tab. 2). Significant disturbances in sex ratios were noted in adults coming from exposed L1 ($\chi^2 = 73.7$, $P < 0.001$) and L2 ($\chi^2 = 66.5$, $P < 0.001$): the ratio was equal for L1-emerging adults and slightly male biased from those developing from L2. Adults originating from control L1 &L2 showed a female biased sex ratio. In exposed and control L3 & L4, adult sex ratios were unaffected.

Table 1: Mortality and infection rates of *Culiseta longiareolata* larvae exposed to xiphidiocercariae shed from *Radix natalensis**, 100 larvae per group at exposure.

Larval stages	No. of larvae (mortality %)		Overall prevalence (%)	Cysts per larva: M ± SD
	Exposed larvae*	Controls*		
L1	72 (28)	96 (4)	90	10 ± 0.8
L2	48 (52)	95 (5)	88	12 ± 1.6
L3	96 (4)	97 (3)	12	5 ± 1.2
L4	100 (0)	99 (1)	8	3 ± 0.8

Table 2: Percentages of emerging *Culiseta longiareolata* adults from larvae.

Larval stages	No. of emerging adults (%)		Adult male/female sex ratio	
	Exposed larvae	Controls	Exposed larvae	Controls
L1	12 (16.7)	94 (97.9)	1:1	1:1.6
L2	10 (20.8)	92 (96.8)	1:0.6	1:1.54
L3	90 (93.7)	94 (96.9)	1:1.2	1:1.47
L4	96 (96)	98 (99)	1:1.5	1:1.6

Discussion

The present study demonstrates the ability of xiphidiocercariae shed from naturally infected *R. natalensis* to encyst in other individuals of this species, either in the field, or under laboratory conditions. This result suggests the potential role of *R. natalensis* as a second intermediate host for xiphidiocercariae, in addition to its obligatory role as a first intermediate one. As this type of cercariae was previously thought to be produced by a plagiorchiid species of genus *Plagiorchis* (Frandsen and Christensen, 1984; Dar, 2009), this hypothesis was supported by the capacity of xiphidiocercariae produced by this genus to utilize lymnaeid species as second intermediate hosts for their encystment (Olsen, 1986). For example, *P. muris* xiphidiocercariae develop

in *Lymnaea emarginata*, the first intermediate host, and then leave it to penetrate other lymnaeid snails (*L. auricularia, L. peregra* and *L. stagnalis*) as subsequent hosts where they could encyst in their tissues. However, xiphidiocercarial encystment in the first intermediate host species was reported by Moema *et al*. (2008) who found that xiphidiocercariae, shed from *R. natalensis* originating from Pretoria in South Africa, encysted in the mantle of other individuals belonging to this snail species. In addition to *R. natalensis*, other common freshwater snails inhabiting Egyptian water bodies, *B. alexandrina*, *Bu. truncatus* and *P. acuta*, could also play the role of second intermediate hosts for xiphidiocercarial encystment (Dar, 2009).

Xiphidiocercariae showed also the aptitude to experimentally infected *C. longiareolata* larvae, as encysted metacercariae were detected in the tissues of the mosquito four larval stages, with high prevalence of infection in L1 and L2. As a result, these larvae could be considered an additional second intermediate host for this type of cercariae. This finding agrees with the fact that xiphidiocercariae of genus *Plagiorchis* could encyst in mosquito larvae. For example, cercariae of *P. noblei* develop in *L. reflexa* and encyst in *Ae. aegypti* larvae (Olsen, 1986; Dempster *et al*, 1986). Previous mosquito larvae could also be infected by *P. elegans* xiphidiocercariae emerging from *L. stagnalis* and *Stagnicola elodes* (Mitchell-Foster *et al*, 2012). Compared to L3 & L4, xiphidiocercarial encystment had an observable effect on larval mortality of exposed L1 and L2. Similar effect was noted in *Ae. aegypti* larvae infected by *P. noblei* metacercariae (Dempster *et al*, 1986). High mortality of *Ae. albopictus* larvae infected by haematoloechid xiphidiocercariae was also observed in the laboratory (Carvalho *et al*, 2002). Larval mortality may be due to increasing of parasite load that may eventually be fatal to the larvae (Mitchell-Foster *et al*, 2012). Besides, exposure of L1 & L2 to xiphidiocerariae obviously affects subsequent development of adults as well as male/ female sex ratio. This finding was reported in *Ae. aegypti* larvae exposed *P. noblei* xiphidiocercariae (Dempster *et al*, 1986), thus indicating that infection may interfere with adult emergence due to failure of pupae metamorphosis. Normal development of exposed L3 & L4 might be due to low infection intensity (number of cysts per larva), whereas a high number of cysts per larva in L1 & L2 might inhibit normal larval development (Carvalho *et al*, 2002; Mitchell-Foster *et al*, 2012).

Many authors have reported the importance of entomophilic xiphidiocercariae in biological control of mosquito larvae (Rao *et al*, 1985; Webber *et al*, 1987; Carvalho *et al*, 2002; Mitchell-Foster *et al*, 2012). Thus, the xiphidiocercariae developing in *R. natalensis* might be used as a biological agent to control *C. longiareolata* larvae in Egypt, as indicated in the present study. However, the question is still posed about the definitive host(s) in which encysted xiphidiocercariae develop into adult stage. As members of genus *Plagiorchis* have a wide range of

vertebrate definitive hosts (Olsen, 1986), other field and experimental studies should be conducted to determine the vertebrate hosts in the habitats where second intermediate hosts harboring encysted xiphidiocercariae exist.

Conclusion

The results showed that *Radix natalensis* could act as a second intermediate host for xiphidiocercariae, in addition of its obligatory role as a first intermediate one. The infection of *C. longiareolata* larvae with xiphidiocercariae underlines, not only the role of these larvae as an additional second intermediate host, but also their potential contributory function as a mosquito control measure.

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Explanation of Figures

Fig. 1: Ventral view of an unstained xiphidiocercaria with stylet at anterior margin of oral sucker.

Fig. 2: SEM of xiphidiocercaria anterior end with protruded tip of stylet.

Fig. 3: Encysted xiphidiocercariae in *Radix natalensis* tissues.

Fig. 4: Detached stylet within encysted xiphidiocercaria.

Fig. 5: Encysted xiphidiocercariae in head, thorax (a), and abdomen (b) of a second stage *Culiseta longiareolata* larva.

Figure 1

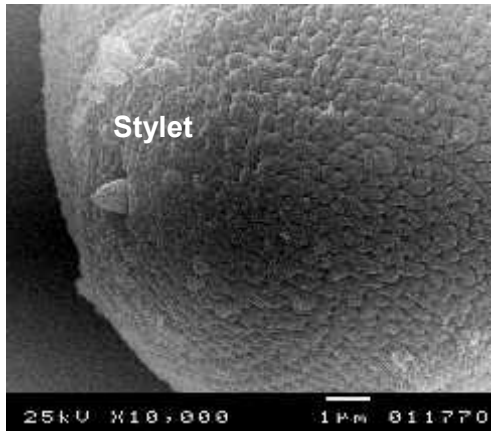

Figure 2

Figure 3

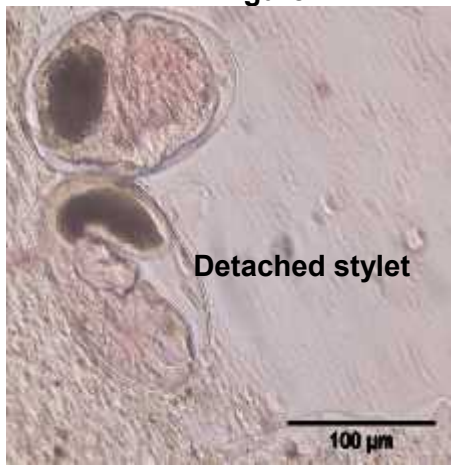

Figure 4

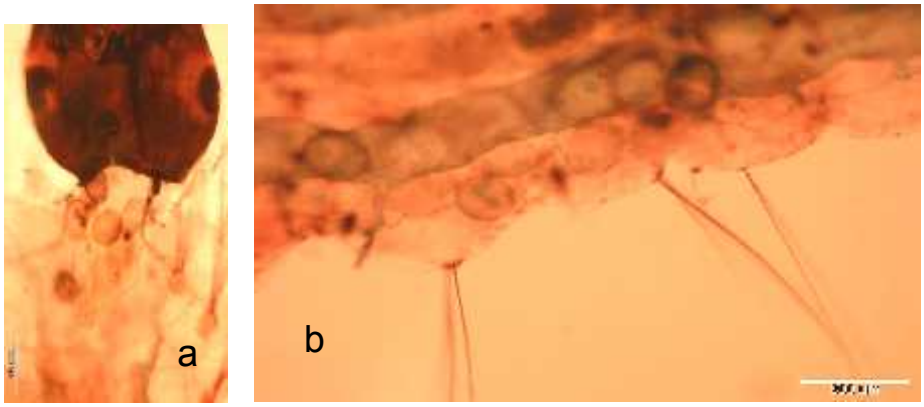

Figure 5