Scientific Note

First laboratory culture of *Phortica variegata* (Diptera, Steganinae), a vector of *Thelazia callipaeda*

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The family Drosophilidae includes ~4,500 species in the two subfamilies Drosophilinae and Steganinae that are generally known as fruit flies or pomace flies because of their habit of feeding and growing on fermenting fruit. Within the Drosophilinae, *Drosophila* species are among the best characterized model organisms in genetic and genomic research. Conversely, knowledge of species ranked within the Steganinae is limited to a few morphological, taxonomic, and systematic investigations of selected members and information of the biology and ecology of most species is scarce. Over the past decade, fruit flies of the genus *Phortica* have been the subject of intense investigations aimed at identifying the arthropod vector and intermediate host of *Thelazia* spp. eyeworms (Otranto et al. 2003, 2005, 2006a, 2006b, Dorchies et al. 2007, Roggero et al. 2010). In particular, while *Phortica okadai* (Máca, 1977), *Phortica magna* (Okada 1968), and *Amiota nagatai* Okada, 1971 are vectors of *Thelazia* spp. in Asian countries (Kamakura et al. 1998), *Phortica variegata* (Fallén 1823) has been demonstrated, under both experimental (Otranto et al. 2005) and natural conditions (Otranto et al. 2006b), to harbor larvae of *Thelazia callipaeda* Railliet and Henry 1910 "eyeworm" nematodes in Europe. *Phortica* spp. differ remarkably from other fruit flies in that they are known to feed not only on decaying fruit and slime fluxes, but also on lachrymal secretions of animals and humans (Bächli et al. 2004). When feeding on the conjunctiva of vertebrates infested with *T. callipaeda*, *P. variegata* ingests the first-stage larvae released by adult female nematodes; within the body cavities of the arthropod vector, these larvae develop to second-stage larvae and, subsequently, to infective third-stage larvae which reach the proboscis of the fly and are invade the conjunctival sac of a new, receptive host (reviewed by Otranto et al. 2003). While both adult female and male *P. variegata* have been shown to harbour larvae of *T. callipaeda* under experimental settings (Otranto et al. 2005), only the latter gender has been demonstrated to act as a vector of this parasitic nematode under natural conditions (Otranto et al. 2006b, reviewed by Otranto et al. 2008a). The biological bases of this host-parasite relationship are still unclear.

Although the morphological features of adult *P. variegata* have been exhaustively described (Bächli et al. 2004) and their biology has been partially investigated (Otranto et al. 2006a), the morphology, ecology, and feeding habits of the pre-imaginal stages of this arthropod species remain unexplored (Okada 1968). In an effort to categorize and predict the geographical areas of Europe at risk of infection by *T. callipaeda*, Otranto et al. (2006a) integrated distributional data from ~250 sites in which *P. variegata* is known to occur in Europe, and identified large areas of central Europe that are likely to represent suitable habitats for the adult fruit flies. However, in order to improve the accuracy of these predictions, information of the biology and ecology of the pre-imaginal stages of *P. variegata* is necessary. Thus far, biological investigations of *P. variegata* larvae have been largely impaired by the absence of morphological descriptions of accurately determined species (cf. Okada 1968) which, in turn, have been made difficult by the unavailability of appropriate protocols for the rearing of *P. variegata* under laboratory conditions. In the present study, we address this gap in knowledge by describing the first protocol for the laboratory rearing of *P. variegata* as a basis for future investigations of the morphology, ecology, and biology of all developmental stages of this insect and its role as a vector of pathogens of humans and animals.

From April to November, 2005, a total 593 *Phortica* flies (418 males and 175 females) were collected around fruit-baited traps at monthly intervals in an area characterized by a high level of relative humidity (RH) and thick undergrowth (municipality of Oliveto Lucano, Potenza province, Italy, 16°15 E, 40°54 N), as described previously (Otranto et al. 2006a, 2006b). The area is characterized by a high prevalence of dogs infected by *T. callipaeda* (Otranto et al. 2003). On each day of collection, the temperature and RH of the site were recorded using a standard thermo-hygrometer. The flies were identified as *P. variegata* according to morphological keys of Máca (1977) and Bächli et al. (2004). The specimens collected were maintained in net-cages (26 x 21 x 21 cm, 1 mm diameter of mesh openings). The number of flies collected each month, as well as the male:female ratio and fly density in each cage were

registered. Three pieces of fresh fruit (i.e., apple, pear, banana) were placed on top of each cage (with their freshly cut surface facing the net) and replaced every 48 h as *pabulum* (substrate) for oviposition. Each cage was covered with humid clothes, to prevent dehydration of the fruit. Then, a black plastic bag was placed over each cage (for shadowing) and removed for 5 h each day from 8:00 to 13:00. Each cage was maintained at constant temperature and RH (21° C and 60%, respectively). The terminalia of all male specimens which died throughout the study were dissected and processed using a method previously described (Bächli et al. 2004). Each piece of fruit was observed daily (at 8:00) under the stereomicroscope for the presence of *P. variegata* eggs and when present, eggs were counted, transferred onto other pieces of freshly cut fruit, and placed in cages (20x16x16 cm) surrounded by a net to prevent contamination by other fruit fly species. Each cage containing the freshly laid eggs was then covered with a black plastic bag. Egg hatching was verified daily under a stereomicroscope and each larval stage was grossly identified according to the morphological keys proposed by Okada (1968). The timing of each larval molt was recorded. When observed, puparia were collected and transferred in separate cages and kept under the same laboratory conditions as the adult flies. The timing of emergence of adult flies from each puparium was recorded. In order to verify their fertility and fecundity, adult specimens reared under laboratory conditions (first generation = F1) were maintained until oviposition. The number of second-generation (F2) flies was recorded.

No specimens of *P. variegata* were collected in April (temperature: 16° C; RH: 70%) and November (temperature: 13° C; RH: 72%) (data not shown). *Phortica variegata* collected in July (n = 101 specimens; male:female ratio = 0.5; fly density = 8.8 flies/dm^3) laid the largest number of eggs observed throughout the experiment (n = 126, Table 1). Conversely, the smallest number of eggs (n =18) was obtained from flies (n = 35) collected in May (male:female ratio = 1.5, Table 1). While oviposition of specimens collected in July and August could be ascertained from one to four days following collection, that of flies collected in June started six days and terminated nine days following collection. First stage larvae (L1s) hatched from two to four days following oviposition in July and August, and from six to 12 days after oviposition in June (Table 1). Feeding activity of L1s on decaying fruit employed as substrate for the oviposition started immediately after hatching. The molt from L1 to L2 was observed three to six days following oviposition in July and August and from nine to 16 days following oviposition in June (Table 1). Third stage larvae were observed five to seven days following oviposition in August and from ten to 15 days following oviposition in June. Analogously, puparia were observed seven to ten and seven to eight days from oviposition in July and August respectively, and from 12 to 18 days from oviposition in June (Table 1). Adult flies (F1) emerged from nine to 13 and nine to 12 days following oviposition in July and August, respectively, and 15 to 18 days from oviposition in June (Table 1). Out of the 227 eggs laid throughout the experiment, 85 (37%) completed their development into adult flies (Table 1). Of these, 45 and 35 emerged in July and

Table 1. Number, sex, male:female ratio (M/F) of *Phortica variegata* collected each month, along with temperature (T expressed in °C) and relative humidity (RH expressed in percentage) registered at the site of collection. Number of specimens and timing of development (expressed as t minimum-t maximum) of life stages – 1st stage larvae- L1; 2nd stage larvae- L2; 3rd stage larvae- L3; pupae; adults – reared under laboratory conditions are also reported, together with survival rate (expressed in percentage) from egg to adult stage.

Month	T – r.h.	No. flies (M/F)	M/F ratio	No. eggs (t min/max)	No. L1 (t min- max)	No. L2 (t min- max)	No. L3 (t min- max)	No. pupae (t min- max)	No. adults (t min-max)	% of survival
May	22°C 45%	35 21M/14F	1.5	18 (+3/6d)	9 (+4/8d)	4 (+7/8d)	1 (+10d)	0	0	0%
June	14°C 80%	28 4M/24F	0.16	27 (+5/9d)	21 (+6/12d)	17 (+9/16d)	11 (+10/15d)	9 (+12/18d)	5 (+15/18d)	18.5%
July	23°C 75%	101 33M/68F	0.48	126 (+1/5d)	86 (+2/6d)	68 (+3/6d)	62 (+5/9d)	59 (+7/10d)	45 (+9/13d)	35.8%
August	28°C 68%	269 207M/62F	3.34	56 (+1/4d)	52 (+2/4d)	48 (+3/5d)	41 (+5/7d)	38 (+7/8d)	35 (+9/12d)	62.5%
September	23°C 75%	142 142M/0F	0	---	---	---	---	---	---	---
October	16°C 83%	18 11M/7F	1.58	---	---	---	---	---	---	

August, respectively (Table 1). The 45 F1 flies obtained in July (28 females and 17 males) laid a total number of 29 eggs from seven to 11 days (average eight days) from hatching. Out of these, nine (31%) completed their development into adult, F2 flies from 12 to 16 days (average 13 days) following oviposition. The 35 adult F1 specimens obtained in August (27 females and eight males) laid a total number of 17 eggs nine to 12 days (average ten days) from hatching, of which 12 (70.6%) completed their development into adult F2 flies from 11 to 14 days (average 13 days) following oviposition. The difference between the numbers of eggs laid by F1 females emerged in July (nine) and in August (12), respectively, that completed their development to adult flies, was statistically significant ($\chi^2 = 6.758$, df = 1, p = 0.009).

The present study describes, for the first time, a protocol for the laboratory rearing of *P. variegata* flies. The life cycle of *P. variegata* under laboratory conditions completed within a minimum of nine days (in July and August) to a maximum of 18 days (in June). In particular, eggs of *P. variegata* were laid approximately two days from collection, and egg hatching was observed two to 12 days following oviposition (Table 1). In contrast, eggs of *Drosophila melanogaster* are known to hatch and develop into a L1 within one day following oviposition, whereas pupae are found within seven days from oviposition (Demerec 1994). However, adult flies of *D. melanogaster* require approximately 5 days before emerging from their puparia (Bodenstein 1950), which contrasts with the two days required by adult *P. variegata* (Demerec 1994). In the present study, the shortest and the longest time spans for the completion of the life cycle of *P. variegata* under laboratory conditions corresponded to the highest (28° C) and lowest (14° C) temperatures recorded at the site of collection of wild specimens (Table 1), respectively. This finding is likely to be linked to the reduced fertility of wild flies collected in early spring and/or late autumn, due to the seasonal changes of climatic conditions observed at the site of collection, as previously suggested for *Drosophila* spp. (Muona and Lumme 1981, Chen and Walker 1994, Demerec 1994). Thus, the fact that no eggs were laid by *P. variegata* collected in October could be attributed to the occurrence of pre-hibernation physiological mechanisms (cf. Muona and Lumme 1981). The low survival rate from egg to adult fly observed in June (Table 1) is likely to be associated to the unusual low temperature and high RH observed at the site of collection (14° C and 80%, respectively), whereas high survival rates from egg to adult F1 flies were observed in July and August, in correspondence of the highest temperatures recorded throughout the study (23 and 28° C). This observation indicates that the fitness of wild flies collected under field conditions is essential for that of the offspring. In addition, a male:female ratio of 0.48 (in one cage) resulted in the largest number of F1 flies emerged (Table 1), thus suggesting that the reproductive capacity of the wild flies collected throughout the study was enhanced when the number of female specimens was twice that of males. This could be explained by the fact that under these experimental settings, wild flies were fed on freshly cut fruit which was used at the same time by adult females as substrate for oviposition. Where the number of male specimens was equal or larger than the number of females, intraspecific competition for the feeding substrate might have occurred, thus leading to the reduction in the number of eggs laid. At the same time, this would favor the development of a small number of hatched larvae and an improvement of the survival rate of the F1 specimens obtained (Table 1). However, the high density of flies in the rearing cage registered in August (23.4 flies/dm^3) could have been a limiting factor for the fertility of *Phortica* males. In the future, studies investigating the feeding preferences of both genders of *P. variegata* fruit flies (fruit and/or lachrymal secretions) could assist in addressing this point. In September, despite the relatively large number of male specimens of *P. variegata* collected, no females were captured. This could be linked to differences in behavior and/or mortality rates between male and female insects at the end of the reproductive period, as previously reported for some species of the order Hemiptera (Salomao and Vasconcellos-Neto 2010). For instance, it is possible that female *P. variegata* suffer high mortality as a consequence of their vulnerability to predators while ovipositing, and/or of the high energetic costs of the egg development and oviposition (Reguera and Gomendio 1999).

Wild flies collected in the present study were fed on fruit as the only substrate. Several protocols for the laboratory rearing of Drosophilidae involve various carbohydrate media with yeast (Ashburner et al. 2005). Previous studies had unsuccessfully attempted the use of such substrates for the rearing, under laboratory conditions, of fruit flies of the Steganinae subfamily (J. Máca, unpublished data). However, the small number of F1 and F2 specimens obtained using the protocol herein described suggests that the food sources utilized were suboptimal for reproductive fitness of wild specimens. Indeed, *P. variegata* have been reported as a nuisance in trying to enter the eyes and ears of human beings, suggesting that under natural conditions, this fly feeds on biological secretions as well as fresh and decaying fruit or slime fluxes (Bächli et al. 2004, Otranto et al. 2006a). This feeding habit represents the basis of the role that *P. variegata* plays as a vector and intermediate host of *T. callipaeda* (Otranto et al. 2006b). Whether infestations by *T. callipaeda* have, over the course of evolution, progressively led to a modification of the feeding habits of *P. variegata* flies, remains to be tested. Future studies could, for instance, compare the feeding habits of uninfested flies and flies experimentally infested with *T. callipaeda*, as well as explore feeding preferences of larvae of *P. variegata*.

The protocol for the rearing of *P. variegata* flies under laboratory conditions herein reported represents a useful tool for future studies of the morphology, taxonomy, systematics, ecology, and biology of selected members of the Steganinae subfamily. Based on the results of the present study, a male:female ratio of 2:1 in the rearing cage is advised for the maintenance of the *P. variegata* life cycle throughout the year. The ability to culture *P. variegata* in the laboratory will largely contribute to morphological and biological studies of eggs, L1s, L2s, and L3s. In addition, the availability of large numbers of specimens of *Phortica* spp. reared in the laboratory will assist molecular studies aimed at further elucidating the taxonomy

and systematics of the genus *Phortica* and other genera within the Steganinae subfamily (Otranto et al. 2008b, Cao et al. 2011). For instance, the standardization of a protocol for the rearing of *P. variegata* and other Steganinae species in the laboratory will provide the entomological community with a tool to rapidly access specimens (including pre-imaginal stages) for morphological and behavioral studies, as well as of both known and novel gene markers for phylogenomic and taxonomic investigations. Taken together, these advances will not only result in a substantial enhancement of current knowledge of the biology of this fruitfly species and other members of the Steganinae, but also assist the development of strategies for the treatment and control of thelaziosis in endemic areas.

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