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Survey of *Phortica* drosophilid flies within and outside of a recently identified transmission area of the eye worm *Thelazia callipaeda* in Switzerland

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ABSTRACT

Phortica drosophilid flies are the intermediate hosts and vectors of the eye worm *Thelazia callipaeda*. This nematode originates from Asia and was first detected in southern Europe in 1989. The aim of the study was to assess the presence and the population dynamics of *Phortica* flies in a recently discovered new endemic area (Ticino, Southern Switzerland, south of the Alps) of *T. callipaeda* (site 1), at its border (site 2), at higher altitudes (beyond 1100 meters above sea level) within (site 3) or outside (site 4) the endemic area, and in a site north of the Alps (site 5). Flies were captured using two types of fruit-baited traps, the bait being changed once per week, and by netting around the eyes of a dog and human. A total of 1695 *Phortica* flies were collected. One of the fruit-baited traps, which can easily be assembled with cheap components, was found to be efficient for catching *Phortica* spp. At site 1, 644 such flies were collected with this trap during 34 weekly catches from April to October. The number of flies caught was highest at site 2 ($n=903$) and it was significantly lower ($n=36$) at site 5 north of the Alps. Virtually no *Phortica* at all were caught at higher altitudes (sites 3 and 4). Females were all in all predominant in the traps, accounting for 72.6% of *Phortica* flies (1150/1584), although males became dominant late in the season (male/female ratio 1.26 in October). In contrast, 80.2% of *Phortica* flies collected around the eyes of dog and human baits by netting ($n=111$) were males. No female at all was captured by netting until September. PCR for *T. callipaeda* was negative with all *Phortica* flies. Morphological examination of the 523 male flies based on features of the eye margin and the number of particular genital sensilla identified 89.1% *P. semivirgo*, 5.7% *P. variegata* but also 5.2% intermediate forms. Genetic analyses of partial mitochondrial *cox1* and rDNA internal transcribed spacer 1 sequences revealed that these three morphotypes were genetically not distinguishable. This study confirms the presence of *Phortica* spp. north to the Alps and therefore the potential risk of *T. callipaeda* infection outside the currently known endemic region, depending on local abundance and longevity of the drosophilid vectors.

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1. Introduction

Thelazia callipaeda, a nematode (Spirurida, Thelaziidae) infecting the eyes of mammals including humans, can cause mild to severe irritation leading to lacrimation, mucopurulent discharges, epiphora, conjunctivitis, keratitis and even to corneal ulcerations (Otranto and Traversa, 2005; Shen

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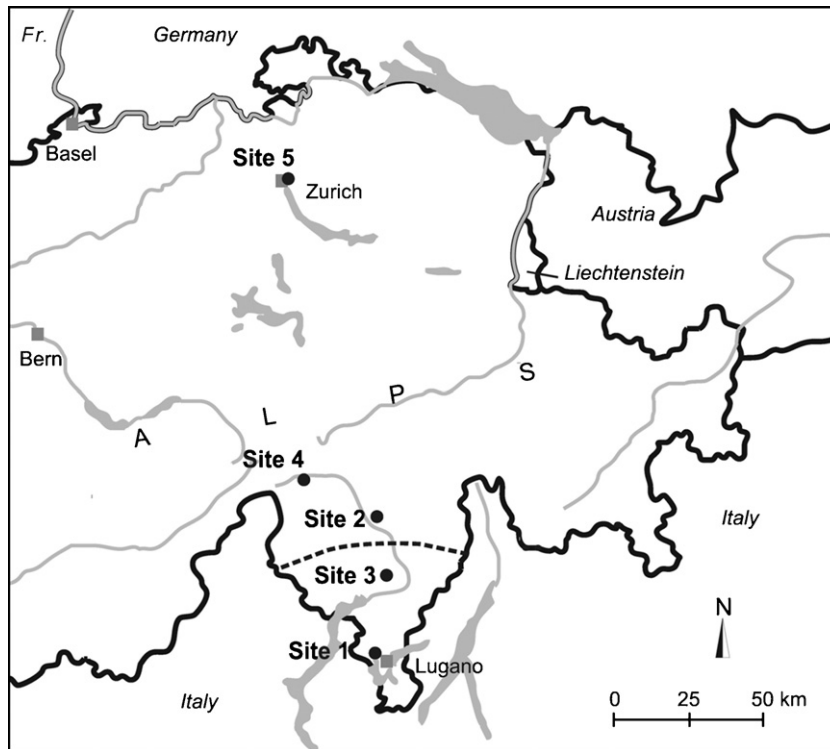


Fig. 1. Locations of the 5 trapping sites in Switzerland. Black dots: trapping sites; thick black line: country borders; grey areas: water bodies; grey squares: major Swiss cities. The approximate northern border where *Thelazia callipaeda* was previously found is depicted with the black dotted line (46°20'N latitude; longitude of the area is 8°57'E).

et al., 2006). This parasite was originally reported from Asian countries (Bhaibulaya et al., 1970; Shi et al., 1988), where human infections with this 'oriental eye worm' are considered to be emerging over the last two decades, particularly in poor rural communities (Shen et al., 2006). Further, the autochthonous presence of *T. callipaeda* has recently been reported from three European countries. Following the first report from dogs in northern Italy (Rossi and Bertaglia, 1989), autochthonous infections were subsequently recorded in dogs, cats and foxes from southern Italy (Otranto et al., 2003), Switzerland (Malacrida et al., 2008), from dogs in France (Dorchies et al., 2007) and very recently from one dog north of the Alps in Germany (Magnis et al., 2010). In addition, wolves, beech martens, brown hares and wild cats were identified as hosts in southern Italy (Otranto et al., 2009). Finally, *T. callipaeda* infections have recently been diagnosed in four human patients in northern Italy (Liguria) and neighbouring southern France (Otranto and Dutto, 2008).

Thelazia callipaeda requires a vector which also acts as intermediate host to accomplish its life cycle (Otranto et al., 2006b). Species of the dipteran family Drosophilidae (fruit flies, subfamily Steganinae) have been incriminated as vectors. *Amiota variegata*, recently taxonomically reclassified as *Phortica variegata* (Máca, 2003), was first identified as intermediate host and vector, but also *A. okadai* was considered to be a vector of this parasite in China (summarized in Otranto et al., 2006a). *Phortica* spp. and, to a lesser extent, *Amiota* spp. display a zoophilic behaviour, i.e. they feed

on ocular secretions of animals and humans in addition to feeding on fruits and on fermenting tree sap (Bächli et al., 2004). Interestingly only males of *P. variegata* were found to be infected with *T. callipaeda* under natural conditions in Italy (Otranto et al., 2006b), whereas also female flies were positive in dissection and/or molecular assays under experimental conditions (Otranto et al., 2005).

In Switzerland, *T. callipaeda* has been identified in vertebrate hosts in the southernmost part of the country (canton Ticino), neighbouring northern Italy, with prevalences up to 6.2% in dogs and 11.1% in foxes (Malacrida et al., 2008). The actual endemic area stretches to the latitude of approximately 46°20'N (Fig. 1). *Phortica* and *Amiota* spp. flies have previously been recorded from the canton Ticino but also from other regions in Switzerland north of the Alps (Bächli and Burla, 1985). Further, an ecological niche model suggested that large parts of Europe have a climate that could be suitable for *P. variegata* (Otranto et al., 2006a) and could potentially become endemic for *T. callipaeda*.

Phortica and *Amiota* spp. are usually caught by net sweeping around eyes of human or dog baits or around a fruit bait (Otranto et al., 2006b), which is time-consuming and poorly adapted to vector population dynamics study. The aims of this study were to establish an efficient method for trapping a high number of such drosophilid flies under natural conditions and to determine the presence and the population dynamics of *Phortica* and *Amiota* spp. in Switzerland in locations differing with regard to the present occurrence of *T. callipaeda* (endemic, border, non-

endemic regions) in order to assess the risk of spread and endemization.

2. Materials and methods

2.1. Collecting sites

Phortica spp. flies were trapped at 5 sites in Switzerland (Fig. 1). Four of them (site nos. 1, 2, 3, 4) were located in the canton Ticino, which is south of the Alps and where the climate is mainly influenced by the Mediterranean Sea (precipitation 1500–2000 mm/year). Sites 1 and 2 were selected in lowlands, where the annual mean temperature is 11.6 °C. Site 1 (locality Gentilino, altitude 469 m above sea level (a.s.l.), geographic parameters 45°59'25.01"N, 8°56'16.54"E) is situated in the southern part of Ticino where the highest prevalences of *T. callipaeda* in dogs in Switzerland was recorded (Malacrida et al., 2008). Site 2 (locality Bodio, altitude 320 m a.s.l., 46°22'40.20"N, 8°55'3.37"E) is located in central Ticino, just across the northern border of the known endemic area of *T. callipaeda*. Sites 3 and 4 were both selected at higher altitudes: Site 3 (locality Mornera, altitude 1400 m a.s.l., 46°12'26.34"N, 8°58'51.70"E) in central Ticino within the endemic area, and site 4 (locality Airole, altitude 1175 m a.s.l., 46°31'25.35"N, 8°36'31.08"E) in northern Ticino outside the endemic area. Site 5 was located north of the Alps, at the outskirts of the city of Zurich (47°23'59.44"N, 8°33'18.16"E, altitude 408 m a.s.l.), where the climate is cooler (Atlantic climate, precipitation 1000–1500 mm/year, annual mean temperature 2–3 °C lower than south of the Alps) and where no autochthonous cases of *T. callipaeda* in dogs have been reported so far.

All trapping sites were selected at the edge of a forest, and trapping was performed both in the woods and in the adjacent open land which in most places was grassland. Site 1 was located in an area where people frequently walk their dogs, and site 2 was adjacent to an orchard (apples, pears, figs, blueberry, raspberry, and vineyard). At both these sites, the vegetation is characterized by deciduous trees (chestnut and ash trees at both sites and oaks at site 1). At sites 3–5, the forest is characterized by conifers, increasing in numbers with increasing altitude. The grassland of sites 2–5 is temporarily being used as pasture for sheep and cattle during summer and early autumn, the one at site 1 is mainly used as sports field.

2.2. Fly collection

2.2.1. Bait trapping

Two trap types, A (adapted from Medeiros and Klaczko, 1999) and B (adapted from Toda, 1977) were used. The principle of both traps is based on the attraction of drosophilids by fruits positioned inside. Small apertures prevent larger insects to enter, and through a system of cones and dark components once captured insects are distracted from escaping the trap. Trap A is schematically represented in Fig. 2. For Trap B, a larger polyethylene terephthalate (PET) container was used, oriented horizontally, with a single circular aperture (radius of approximately 4.5 cm) covered by a net (mesh 0.4 mm) and narrowing through a funnel



Fig. 2. Schematic representation of trap A (courtesy of J. Peter). (a) Single components of the trap. (b) Assembled trap. Pieces of fruit are placed in the black-painted bottom of a 1.5 l polyethylene terephthalate (PET) bottle and covered by a net (stocking). Three small apertures of approximately 3 cm × 0.5 cm are cut in the dark end of another PET bottle and are covered by a net (mesh 4 mm). The conic system build with the second PET bottle that fits into the upper transparent part of the first bottle prevents captured insects to escape since insects tend to head towards the light. Paper-clips and cord fix the two dark bottle parts together.

towards the brighter side of the trap. During the first 8 weeks of collection, the efficiency of these trap types was compared. At sites 1, 2 and 5, pairs of both trap types were placed twice in a forest as well as in the adjacent open area, in a distance of about 20–50 m from each other (totally 8 traps per site). The two traps in the same habitat were installed close to each other (max. distance 40 cm) in a shaded place, with entrances at the same height (about 50 cm above ground). All traps were baited with sliced fresh apples and peeled bananas, the bait being changed once per week, after collection of the flies.

At sites 3 and 4, traps of type A were placed in the same way. Traps were run from the beginning (week 14, sites 1 and 2) or from the mid of April 2007 (week 15, site 5) until the third week of November 2007 (34/32 collection weeks,

respectively). At sites 3 and 4, which are characterized by a higher altitude and consequently by a cooler climate, traps were operated from mid-July (week 29) to the first week of October 2007 (12 weeks).

2.2.2. Netting

Flies were collected from around the eyes of a dog and a human bait using a net during 2 h at each site once per week (on the occasion of changing the fruit bait of the traps) as described (Otranto et al., 2006a).

2.2.3. Reference specimens

One male reference specimen each of *P. variegata* (collected near site 5, Zurich) and *P. semivirgo* (collected in central Ticino) from Switzerland was available, identified by expert taxonomists (J. Máca and G. Bächli).

2.3. Morphological identification of flies

The trapped flies were counted after sorting into 3 groups: *Phortica* spp., other Drosophilidae and non-Drosophilidae (which were not further considered). *Phortica* and *Amiota* specimens were examined under a stereoscopic microscope and morphologically identified at species level using the described diagnostic characters (Bächli et al., 2004). The sex of all captured *Phortica* flies was determined, and genitalia and eyes of all male specimens were further examined under a light microscope in order to distinguish the species *P. variegata* from *P. semivirgo* (Bächli et al., 2004). In particular, the number of sensilla localized on each medial and dorsal branch of the anterior parameres of the genitalia were determined (3 on each branch for *P. variegata*, 3–5 for *P. semivirgo*) and the pale ring around the eyes (pale yellow along the eye margin, but brownish along the lower half of the eye for *P. variegata*, pale yellow along the whole eye margin for *P. semivirgo*) was examined (Bächli et al., 2004).

2.4. Statistical analysis

Statistical tests were carried out using the software package SPSS 13.0 for Windows. A Wilcoxon signed ranks test (NPar Tests) was performed on the data, considering as significant a *p*-value <0.05.

2.5. DNA isolation, PCR and sequencing

All *Phortica* flies were individually homogenized for 2 min (Mixer Mill MM 300, Retsch, Haan, Germany) in Eppendorf tubes containing 200 µl of PBS and a single stainless steel bead of 2 mm diameter. After incubation at 95 °C for 5 min, the samples were supplemented with 50 µl of Chelex (50% (w/v); Biorad, Hercules, CA) and rotated during 10 min. The homogenate was centrifuged (13,000 rpm, 2 min) in a table centrifuge, the supernatant transferred to a new tube and stored at –20 °C. Genomic DNA was extracted from a pool of 10 µl homogenate from 20 flies using the QIAamp DNA mini kit (Qiagen, Hilden, Germany), following the blood protocol. DNA from *T. callipaeda* previously collected in the canton Ticino (Malacrida et al., 2008) was isolated using the same kit following the tissue protocol.

Table 1
 Features of PCR primers used in this study and reaction conditions.

Primer name (specificity)	Locus	Sequence (5'–3')	Fragment size (bp)	Annealing	Extension	No. of cycles	Reference
UEA7, UEA10 (insects)	Part of the mitochondrial <i>cox1</i>	TACAGTTCGAATAGACGTTGATAC	~700	58 °C, 60 s	72 °C, 60 s	40	Zhang and Hewitt (1996)
18S rDNA 5.8S rDNA (eukaryota)	Complete ITS-1 region of rDNA	TCCAATGCACATAATCTGCCAATATTA CGTAACAAGGTTTCGCTAGG	~600	50 °C, 60 s	72 °C, 60 s	35	Baffi and Ceron (2002)
rDNA-A G2 (<i>Thelazia callipaeda</i>)	Partial ITS-1	GCTCGGTTCTTCATCGAC AGGTGAACCTCGGAAGGA GACACCAACAGTGAACACCG	~500	58 °C, 45 s	72 °C, 45 s	40	Otranto et al. (2005), modified

Primers and PCR cycling conditions (all denaturation steps at 94 °C for 30 s) are listed in Table 1. Each 100 µl PCR mixture contained 10 or 20 µl of extracted DNA, buffer (50 mM KCl, 20 mM Tris–HCl pH 8.4, 2.5 mM MgCl₂, 0.5% Tween 20), dNTPs (0.2 mM; dUTP in place of dTTP), primers (Table 1) at concentration of 1 µM and 0.5 U UDG (uracil DNA glycosylase) to control for PCR carryover contamination (Longo et al., 1990). PCR was performed in an automatic thermal cycler (DNA engine, MJ Research, Waltham, MA). After a first step at 37 °C for 10 min and a second step at 94 °C for 12 min (heat inactivation of UDG), 2.5 U Taq DNA polymerase was added in a hot start (all reagents from Sigma–Aldrich, Buchs, Switzerland). A positive and a negative (no template) control were included in every run. When testing pools of DNA from *Phortica* flies for the presence of *T. callipaeda* by PCR, all samples were run in parallel spiked with DNA of *T. callipaeda* to check for the presence of PCR inhibition. PCR products were visualized under UV light after electrophoresis in 1.5% agarose gels stained with ethidium bromide.

DNA sequencing was done by a private company (SynGene Biotech, Schlieren, Switzerland) on the amplicons after direct purification from the reactions, after excision from agarose gels using the QIAquick PCR minielute purification kit (Qiagen, Hilden, Germany) or after re-PCR with dNTPs containing dTTP and cloning into the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Sequences were aligned using the Multalin program (Corpet, 1988) and manually edited. Midpoint rooted neighbour-joining phylogenetic trees were drawn using the software program package Treecon for Windows (Van de Peer et al., 1993). The statistical confidence of the branching patterns was evaluated by bootstrap analysis and expressed as the proportion of 100 replications at each node.

3. Results

3.1. Fly collection

During the first 8 trapping weeks at sites 1, 2 and 5, significantly ($p < 0.002$) more drosophilids were caught in the 12 traps of type A ($n = 26,548$) as compared to the 12 traps of type B ($n = 102$). *Phortica* specimens ($n = 263$) were only captured in traps of type A ($p < 0.003$). Trapping was therefore continued with trap type A only.

The number of total drosophilid and *Phortica* specimens trapped at weekly intervals at the 5 sites is depicted in Fig. 3. A total of 113,554 drosophilids were collected during trapping periods of 34 (sites 1 and 2), 32 (site 5) or 12 (sites 3 and 4) weeks: 34,611, 45,039, 5760, 2059 and 26,085 such insects were recorded at sites 1, 2, 3, 4 and 5, respectively.

A total of 1695 *Phortica* spp. specimens were collected during the entire season from April to November at the 5 collection sites with traps and by netting (Table 2), while 5 specimens of *Amiota* spp. were collected by netting during the same period at site 5. Among the *Phortica* flies, 93.5% were collected by bait trapping. The number of *Phortica* flies collected by netting for 2 h once per week was always significantly lower than the number caught at the same site

Table 2 Monthly number of *Phortica* spp. captured in southern (sites 1–4) and northern (site 5) Switzerland in 2007 by bait traps and netting. m, male; f, female; m/f ratio in square brackets. No *Phortica* spp. were caught at site 3 and in November at the sites listed.

Month	Site 1		Site 2		Site 4		Site 5		Total <i>Phortica</i> spp.	
	Trap (m/f)	Net (m/f)	Trap (m/f)	Net (m/f)	Trap (m/f)	Net (m/f)	Trap (m/f)	Net (m/f)	Trap (m/f)	Net (m/f)
April	51 (6/45) [0.13]	0	41 (12/29) [0.41]	1 (1/0)			2 (0/2) [0]	0	94 (18/76) [0.24]	1 (1/0)
May	103 (11/92) [0.12]	0	96 (10/86) [0.12]	2 (2/0)			3 (0/3) [0]	1 (1/0)	202 (21/181) [0.12]	3 (3/0)
June	47 (10/37) [0.27]	3 (3/0)	123 (29/94) [0.31]	0			10 (1/9) [0.11]	1 (1/0)	180 (40/140) [0.28]	4 (4/0)
July	215 (74/141) [0.52]	6 (6/0)	365 (113/252) [0.45]	22 (22/0)	1 (0/1)	0	19 (2/17) [0.12]	2 (2/0)	600 (189/411) [0.46]	30 (30/0)
August	150 (35/115) [0.30]	4 (4/0)	118 (13/105) [0.12]	18 (18/0)	0	0	2 (1/1) [1.0]	0	270 (49/221) [0.22]	22 (22/0)
September	59 (32/27) [1.18]	17 (10/7) [1.43]	136 (61/75) [0.81]	24 (12/12) [1.0]	0	0	0	0	195 (93/102) [0.91]	41 (22/19) [1.16]
October	19 (10/9) [1.11]	0	24 (14/10) [1.40]	10 (7/3) [2.33]			0	0	43 (24/19) [1.26]	10 (7/3) [2.34]
Total	644 (178/466) [0.38]	30 (23/7) [3.29]	903 (252/651) [0.39]	77 (62/15) [4.13]	1 (0/1)	0	36 (4/32) [0.12]	4 (4/0)	1584 (434/1150) [0.38]	111 (89/22) [4.05]

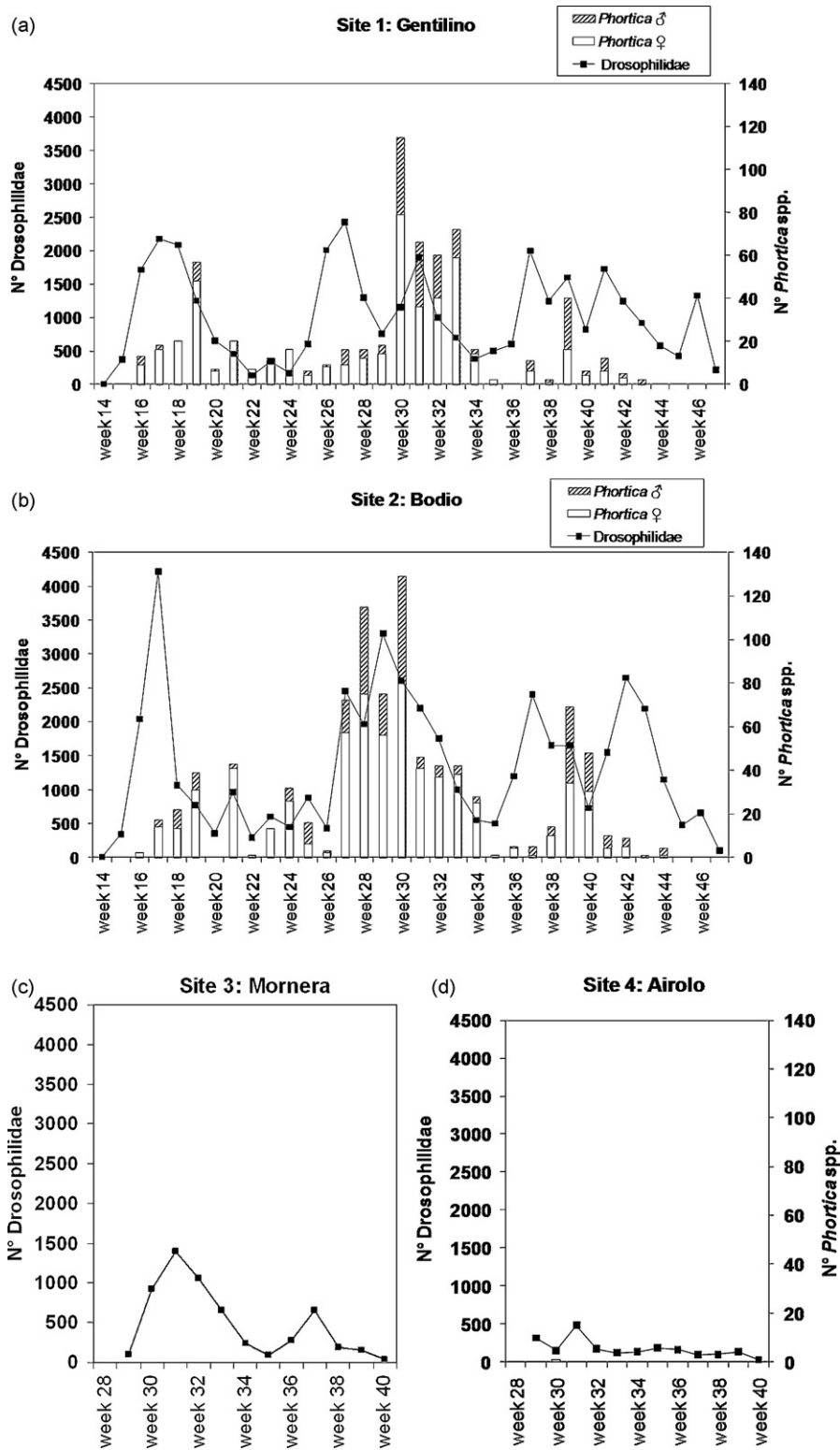


Fig. 3. Weekly captures by bait traps of *Phortica* spp. (bars) and other Drosophilidae (lines) at sites 1 (a, area endemic for *T. callipaeda*), 2 (b, just across the northern border of the known endemic area), 3 (c, at higher altitude within endemic area), 4 (d, outside endemic area south of the Alps, at higher altitude) and 5 (e, outside endemic area north of the Alps). Traps were run from the beginning (week 14) at sites 1 and 2 or from mid of April 2007 (week 15) at site 5 until the third week of November 2007 (34/32 collection weeks, respectively). At sites 3 and 4, traps were operated from week 29 to the first week of October 2007 (12 weeks).

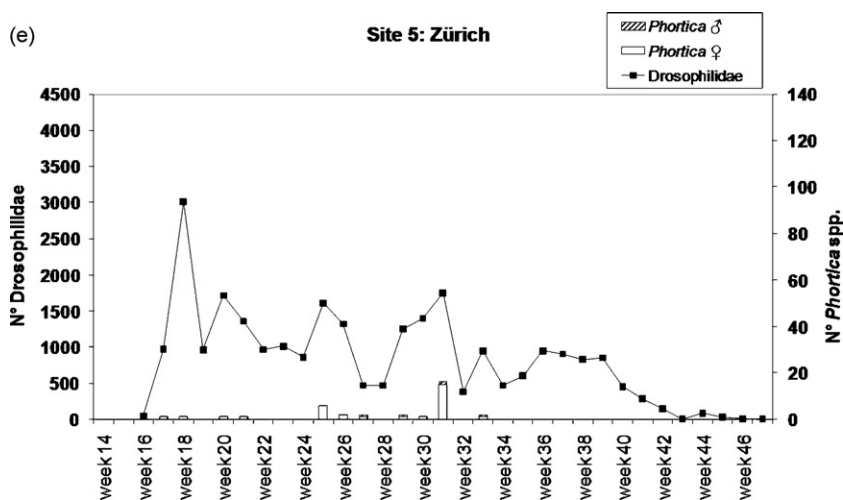


Fig. 3. (Continued)

with baited traps during 1 week (site 1: $p < 0.00001$; site 2: $p < 0.00003$; site 5: $p < 0.00677$).

At trapping site 1, placed in the endemic region of *T. callipaeda* in Ticino, a total of 674 *Phortica* specimens were caught starting from the third week of April to the end of October (Table 2). At trapping site 2, situated just across the northern border of the known endemic area of *T. callipaeda*, 910 *Phortica* specimens were collected during the same period, while at trapping site 5, the one north of the Alps in Zurich, only 40 were caught in a shorter season between the fourth week in April to the third week in August. The number of collected *Phortica* specimens was significantly higher ($p < 0.001$) at sites 1 and 2 (south of the Alps) as compared with site 5. At these 3 trapping sites, a peak in the number of captured specimens was observed in July.

Regarding sites 3 and 4, located in higher altitudes, one single *Phortica* specimen was collected at site 4 in July. From the *Phortica* specimens captured by traps ($n = 1584$, Table 2), 72.6% were females, with the males becoming dominant late in the season (male/female ratio 1.26 in October). In contrast, 80.2% of the *Phortica* flies collected around the eyes of dog and human baits ($n = 111$) were males. Interestingly, no female at all was captured by netting until September. Females were more abundant in the traps except at the end of the season (October, in addition also in September at site 1). In contrast, males were always predominant in catches with a net.

The overall number of *Phortica* spp. flies captured in the grassland traps (833/1583, 52.6%) and in the forest traps (750/1583, 47.4%) was similar (Table 3). However, at site 1, significantly more ($p < 0.006$) flies were trapped (434/644, 67.4%) in the forest, while 66.7% (602/903, $p < 0.008$) of the flies captured at site 2 were trapped in the grassland.

3.2. Identification of *Phortica* spp. by morphology

The male flies morphologically identified as belonging to the genus *Phortica* ($n = 523$) were further characterized. A pale ring around the eyes (indicative for *P. semivirgo*)

was observed in 466 males (89.1%). Of them, 415 had 4 or 5 sensilla confirming this species identification, while 51 specimens displayed 3 sensilla which is a shared feature of both *P. semivirgo* and *P. variegata*. Thirty males (5.7%) were identified as *P. variegata* by their shadowed ring around the eyes and the presence of 3 sensilla. Twenty-seven males (5.2%) could not unequivocally be assigned to one of the two species since they displayed a combination of a shadowed ring around the eyes (characteristic for *P. variegata*) and 4 or 5 sensilla (as *P. semivirgo*). These morphologically intermediate flies were captured by trapping or netting at all 3 sites.

3.3. Analysis of mitochondrial *cox1* and rDNA internal transcriber spacer-1 sequences of *Phortica* spp.

Part (700 bp) of the mitochondrial *cox1* gene was amplified and directly sequenced from 15 insects, and for 610 positions the nucleotides could be determined for all sequences. A neighbour-joining tree was constructed with these sequences, also including corresponding GenBank entries for *P. semivirgo*, *P. variegata* and *P. okadai* as out-group (Fig. 4).

Table 3

Monthly collection of *Phortica* spp. captured in traps placed in a distance of around 20–50 m in forest (F) or grassland (G) at sites 1 and 2 (south of the Alps) during 34 weeks and at site 5 (north of the Alps) during 32 weeks. No *Phortica* spp. were caught at any of these sites in the month of November.

Month	Site 1		Site 2		Site 5		Total	
	F	G	F	G	F	G	F	G
April	46	5	16	25	0	2	62	32
Mai	56	47	70	26	1	2	127	75
June	24	23	43	80	4	6	71	109
July	168	47	101	264	9	10	278	321
August	89	61	57	61	1	1	147	123
September	45	14	11	125	0	0	56	139
October	6	13	3	21	0	0	9	34
Total	434	210	301	602	15	21	750	833

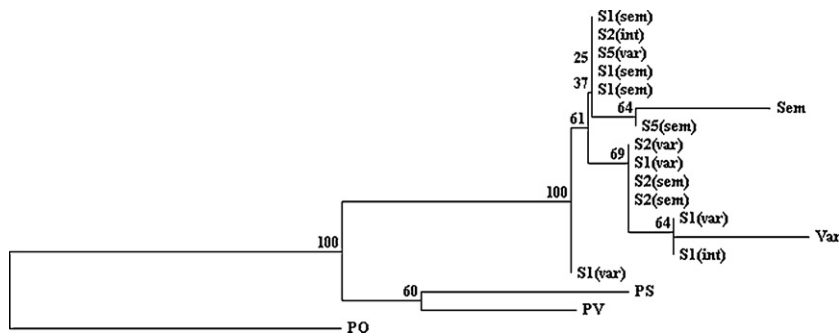


Fig. 4. Neighbour-joining tree reconstructed from partial mitochondrial *cox1* sequences of individual *Phortica* spp. Bootstrap scores are presented for each node. PV: *P. variegata* GenBank EF576934 (haplotype X; origin of insect: Italy); PS: *P. semivirgo*, GenBank EF576935 (haplotype III; origin of insect: Slovakia; Otranto et al., 2008); PO: *P. okadai* GenBank EF576924; S: site no. (1: in area endemic for *Thelazia callipaeda* in southern Switzerland, 2: just across the northern border of the known endemic area, 5: outside endemic area north of the Alps); Sem, Var: sequence of reference specimens of *P. semivirgo* (originating from central Ticino) or *P. variegata* (originating from vicinity of site 5); (var), (sem), (int): morphologically determination as *P. variagata*, *P. semivirgo* or as intermediate form based on eye ring features and the number of specific sensilla of the genitalia.

Neither the sequences of insects morphologically identified as *P. semivirgo*, *P. variegata* or as intermediate form did cluster separately nor did the sequences from individuals originating from areas south (sites 1 and 2) or north (site 5) of the Alps.

A second genetic locus, the rDNA internal transcriber spacer (ITS) 1, was amplified (586 bp) and cloned from the reference specimens (see Section 2) of *P. semivirgo* and *P. variegata*, and 4 clones each were sequenced. All sequences were identical except at position 283 where all sequences derived from *P. semivirgo* had an A, those of *P. variegata* a T. In additions, single polymorphisms in single sequences were present twice (position 407: T to C in 1 clone from *P. variegata*; position 419 G to T in one clone from *P. semivirgo*). No corresponding sequences are available in GenBank.

3.4. Infection of *Phortica* flies with *Thelazia callipaeda*

All the *Phortica* flies collected ($n = 1695$) were subjected to molecular analysis in pools of maximum 20 to detect larval stages of *T. callipaeda*. No inhibition of PCR was observed (as was obvious by the successful amplification of nematode DNA in spiked samples), and all the flies resulted PCR-negative (95% CI 0–0.18%).

4. Discussion

This study confirms the presence of *Phortica* flies in southern Ticino (Switzerland), within the recently described endemic area of *T. callipaeda* (site 1). No drosophilids of the genus *Amiota*, which are also incriminated as vectors, were collected in this area, neither by directly netting around the eyes of dogs nor by trapping, suggesting that *Phortica* flies are most likely the intermediate host of *T. callipaeda* in this area. However, the abundance of *Amiota* spp. might be underestimated in our study as these insects are less attracted by eyes and are more frequently caught with wine/beer baited traps placed as high as 5 m above ground in treetops (Bächli, 1996; Bächli et al., 2006). An even higher abundance of *Phortica* flies was recorded at site 2 just outside the endemic

area (Table 2) implying that the parasite may locally spread further northwards. At sites located at higher altitudes (sites 3 and 4, above 1200 m a.s.l.) within or close to the endemic area, virtually none of these flies were caught. At the site located north of the Alps (site 5, Zurich), *Phortica* flies were present although at lower abundance (less than one tenth) and during a shorter activity season (17 vs. 28 weeks) as compared with the sites in southern Ticino where the fly season is comparable to Southern Italy (Otranto et al., 2006a). This lower availability north of the Alps of *Phortica* flies, which genetically were not separated from those from Ticino (Fig. 4), maybe a limiting factor for the transmission and spread of *T. callipaeda*. However, with the large number of dogs travelling to and coming from endemic regions, local transmission of *T. callipaeda* cannot be excluded. Indeed, a first assumed such transmission of the eye worm to a dog has been reported from Southern Germany (Magnis et al., 2010).

PCR, which previously (Otranto et al., 2006b) was shown to be more sensitive than dissection for the detection of *T. callipaeda*, was negative with all our *Phortica* flies, including the specimens from the endemic region where the prevalences of the nematode infection are 5.3 and 11.1%, respectively, in dogs and foxes (Malacrida et al., 2008). However, our finding is not surprising, though, as even in hyperendemic (60.14%) areas of canine ocular thelaziosis the nematode prevalences in its incriminated zoophilic insect vector under natural conditions are as low as 1.34% (detected by PCR or dissection, Otranto et al., 2006b).

Phortica flies were collected by netting around the eyes of a dog for 2 h, and using traps loaded with sliced fruit and left for 1 week. Comparable traps have been used before for drosophilid collections, but to the authors' knowledge for the first time targeted for investigations on *Phortica* spp. Hence, 93.5% of all *Phortica* flies were captured by these traps, rendering this technique more efficient than the laborious netting around the eyes of dogs or around the cloth bags containing fermented fruits (Otranto et al., 2006a), for a similar amount of working time consumed on the field. Trapping is, in contrast to netting, a reasonably standardized technique, not depending on the skills of the investigator. Further, both traps can effortlessly be assem-

bled with cheap and easily available components. However, the traps are not specific, and huge numbers particularly of other drosophilids were captured, from which the *Phortica* need to be separated in the laboratory.

By netting around the eyes of dogs, 80.2% of all 111 collected *Phortica* were males, and females were not caught until late in the season. Hence, also females display a zoophilic behaviour, which is in contrast to a previous report (Otranto et al., 2006b) describing that exclusively males were caught throughout the season with this technique. In accordance with earlier observations (Otranto et al., 2006a) *Phortica* flies feed on ocular secretions mainly in the second half of the season (July–October, Table 2), maybe because of dietary needs or because of the higher abundance and activity of *Phortica* males. In the traps, both sexes were always present, with an overall dominance of females (72.2%) and the ratio male/female inverting over the season. Even though a lot of research about the vector competence of *Phortica* and *Amiota* flies for *T. callipaeda* has been done, also under controlled laboratory conditions (Otranto et al., 2005), the breeding of these drosophilids is a challenging task and there is still poor knowledge about their feeding requirements, their breeding substrate and the reasons for their changing population dynamics over the season.

At all sites, traps were placed in close vicinity in forests and grassland. The total number of *Phortica* flies captured at two similar sites (sites 1 and 2) was comparable. Catches within an orchard (at site 2) were the highest, followed by the ones of a trap in forest (at site, Table 3). Hence, the placement of the traps is not critical if the presence of *Phortica* is being investigated.

Several diagnostic morphological features have been described for differentiating *P. variegata* and *P. semivirgo* (Máca, 1977). Most of them are laborious to apply since they need further processing of the flies (i.e. mounting the genital apparatus on slides), and could not be used in this large study. We focused on commonly used characters such as the features of the eye margin and the number of particular male genital sensilla (Bächli and Burla, 1985; Bächli et al., 2004; Otranto et al., 2006a). Hence, the vast majority of males was attributed to *P. semivirgo* (89.1%) and 30 insects (5.7%) were identified as *P. variegata*. However, morphologically intermediate forms (5.2%) were identified, putatively related to different stages of maturation, variable conservation and preparation methods or other unknown reasons. Sequences of part of the mitochondrial *cox1* gene revealed that these three morphotypes were genetically not distinguishable (Fig. 4). This is in contrast to earlier findings of genotyping at the same locus with 20 specimens morphologically identified (by applying the same criteria) as *P. semivirgo* and 19 flies of *P. variegata* revealing 9 and 11, respectively, haplotypes, with an intraspecific variation of maximal 1%. A maximum likelihood tree placed these respective 9 and 11 haplotypes on separate branches, with 100% bootstrap values (Otranto et al., 2008), and a PCR-RFLP identification tool was developed on the basis of these differences (Cantacessi et al., 2008).

Analyses of another genetic locus, the rDNA ITS1, revealed nearly identical sequences of reference specimens of *P. semivirgo* and *P. variegata* (1 polymorphic site). This

locus is known to be variable, also between closely related species. For example, the corresponding sequences of the sibling species *Drosophila mulleri* and *D. arizonae*, of which interspecific crosses can be bred, differed by 100 bp in length (Baffi and Ceron, 2002).

Taken together, the presence of morphologically intermediate forms and the genetic analyses at two loci strongly suggest that the used morphological characters cannot be considered as diagnostic for differentiating *P. semivirgo* and *P. variegata*, and that all the investigated specimens collected in Switzerland including those identified by taxonomists are *P. variegata*. When comparing the populations from areas south and north of the Alps, we found no evidence of genetic differences. Thus, *Thelazia* transmission could also be ensured by *Phortica* populations located north to the Alps, depending on their local abundance and longevity.

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