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Various Stages in the Life Cycle of Syrphid Flies (*Eristalis tenax*; Diptera: Syrphidae) as Potential Mechanical Vectors of Pathogens Causing Mycobacterial Infections in Pig Herds

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ABSTRACT. We defined the role of the syrphid fly *Eristalis tenax* in the survival and transmission of mycobacteria in pigs. The conditionally pathogenic mycobacterial (CPM) species *Mycobacterium chelonae* was isolated from 10 % of liquid dung samples, and both *M. chelonae* and another CPM species *M. fortuitum* were isolated from 7 (78 %) of the examined *E. tenax* larvae collected from the same location. Mycobacteriosis of the lymph nodes of pigs from 3 infected farms was caused by *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, and *M. fortuitum*. *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* of identical genotype and serotypes and *M. fortuitum* were isolated from 7 (1.9 %) larvae, 2 (7.4 %) puparia, and one (1.6 %) imago. The count of colony forming units isolated from larval skin covering (pouch) was higher ($p \leq 0.01$) than that isolated from the internal organs of larvae. These results showed the potential for *E. tenax* larvae to spread mycobacteria throughout pig herds and the surrounding environment.

Mammals and birds infected with mycobacteria shed the pathogen through their feces. We have demonstrated that ruminants can shed *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis*; Pavlík *et al*. 2000*a*,*b*; Ayele *et al*. 2001), whilst pigs can shed *M. avium* subsp. *hominissuis* (*M. a. hominissuis*; Mijs *et al*. 2002; Mátlová *et al.* 2003), and birds and other animals can shed *M. avium* subsp. *avium* (*M. a. avium*; Thorel *et al*. 1997; Fischer *et al*. 2000) in this way. Conditionally pathogenic mycobacteria (CPM) have also been demonstrated to be present in avian droppings (Beerweerth and Schürman 1969; Beerwerth and Kessel 1976*a*,*b*) and the feces of mammals including humans (Beerwerth and Schürmann 1969), as well as in feedstuffs, bedding materials, water, kaolin, and peat (Kazda 2000; Dvorská *et al.* 2002; Mátlová *et al*. 2003, 2004*a*,*b*, 2005; Trčková *et al.* 2004, 2005). Mycobacteria are quite resistant to environmental pressures (Jorgensen 1977; Richards 1981; Kazda 2000; Lloyd *et al.* 2001) and in particular can survive in liquid dung for months (Ayele *et al.* 2001; Whittington *et al*. 2004).

Syrphid flies are a common species of Diptera found in Czechia (Moucha and Štys 1956). Adult flies are active from March to December and hibernate over winter. Eggs are normally laid on surface water and larvae develop through three distinct larval stages in a still aquatic environment. The larvae are usually found in still or stagnant water such as water reservoirs or liquid dung. Just prior to pupation, the larvae leave their aquatic environment, searching for a dry and shaded place, sometimes traveling tens of meters (Reichholf-Riehmová 1997).

Pathogenic mycobacteria have been isolated from many invertebrates (Beerwerth *et al.* 1979; Fischer *et al*. 2001, 2004*a*). *M. a. paratuberculosis* has been isolated from blowflies *Calliphora vicina* and *Lucilia caesar* captured in slaughterhouses, where paratuberculosis-positive cattle were processed, and from dung flies (*Scatophaga* spp.) caught on pastures grazed by paratuberculosis-positive cattle (Fischer *et al*. 2001, 2004*a*). Fischer *et al*. (2003*a*) demonstrated the survival of *M. a. paratuberculosis* in one (3.1 %) earthworm sample from a cattle farm affected by paratuberculosis. *M. a. hominissuis* (Kunze *et al*. 1992; Ritacco *et al*. 1998) and *M. gastri* were isolated from three (6.4 %) earthworm samples collected from aviaries where avian tuberculosis had been previously diagnosed. *M. a. paratuberculosis* isolated from earthworms and their feces 1–2 d after contact with material contaminated with *M. a. paratuberculosis* was shown to be the same strain by RFLP typing (Fischer *et al*. 2003*a*). Larvae and adult blowflies of the species *Calliphora vicina* and *Lucilia sericata* may participate in the mechanical transmission of pathogenic mycobacteria from

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their abdomen, head, thorax, and wings (Fischer *et al.* 2004*a*). Viable and virulent mycobacteria can be harbored and shed by nymphs of the oriental cockroach (*Blatta orientalis*) and certain beetles (darkling beetles – *Tenebrio molitor* and *Zophobas atratus)* for up to 10 d after the initial contamination (Fischer *et al.* 2003*b*, 2004*b*). Previously we have detected the causative agent of paratuberculosis in *E. tenax* larvae caught at a site where a herd of cattle infected with paratuberculosis resided (Macháčková *et al.* 2004).

CPM species have also been isolated from invertebrates such as certain species of dragonflies (*Leucorrhinia rubicunda*) that survive in areas rich in peat, which often harbor populations of atypical mycobacteria (Kazda 2000). We have also shown that CPM can be isolated from certain dipterans species (*Drosophila* spp., *Musca* spp., *Stomoxys calcitrans* and *Scatophaga* spp.) captured at a pig farm infected with CPM (Fischer *et al.* 2001, 2004*a*). In recent years, *M. avium* complex (MAC) members *M. a. avium* and *M. a. hominissuis*, and CPM organisms, in particular *M. fortuitum* and *M. chelonae* (Actinomycetales: Mycobacteriaceae), have been frequently isolated from environmental samples (Dvorská *et al.* 1999; Pavlík *et al.* 2003). Further epidemiological studies revealed that MAC members and different species of CPM were frequently found in various constituents (including liquid dung) within domestic pig enclosures and the surrounding environment (Mátlová *et al*. 2003, 2004*a*,*b*, 2005).

The primary objective of this study was to investigate the participation of the syrphid fly *Eristalis tenax* (LINNAEUS 1758; Diptera: Syrphidae) at different developmental stages (larvae, imagoes *etc*.) in the transmission and spread of mycobacteria throughout pig herds. The secondary objective was to investigate the distribution of mycobacteria in the intestinal tract and internal organs compared to the skin covering (pouch) of naturally infected *E. tenax* larvae.

MATERIALS AND METHODS

Samples tested for the presence of mycobacteria included liquid dung ($n = 87$), water from the surface of stagnant reservoirs and ponds ($n = 15$), *E. tenax* syrphid flies at various developmental stages ($n = 503$), and their exuviae ($n = 12$). The samples were obtained from 6 locations in 4 districts of Czechia (*see* Table I). Locations *1* and *2* were natural forest-like environments. Location *3* was a pig farm where mycobacteriosis has been tested for but has not been diagnosed. Locations *4–6* were farms where pigs have been diagnosed with mycobacteriosis of the lymph nodes, caused by the following members MAC: *M. a. avium* of genotype IS*901*+ and IS*1245*+, serotype 2, and *M. a. hominissuis* of genotype IS*901*– and IS*1245*+, serotype 8. CPM (primarily *M. fortuitum*) have also been isolated from these sites (Mátlová *et al*. 2004*a*,*b*, 2005).

Sample collection and processing. Larvae (Fig. 1A, B) were captured from dung cesspools and stagnant water using a strainer attached to a 4-m long telescopic bar, or captured while migrating from their aquatic environment, mostly near sheds that housed pigs at locations *3–6*. Live samples were transported to the laboratory in a sealed sterile and humid bottle (high humidity prevented pupation). After identification (Doležil 1972) the larvae were rinsed and killed with 96 % ethanol. Randomly selected larvae ($n = 60$) were dissected as follows: Initially they were pinned to a paraffin board, the abdominal side was cut, and the digestive tract and internal organs were extracted under sterile conditions. The skins with the outer flesh and digestive tracts and/or internal organs were then cultured separately.

Puparia (Fig. 1C), imagoes (Fig. 2), and exuviae (Fig. 1D) were identified as *E. tenax* according to Doležil and Rozkošný (1997) and were examined for the presence of mycobacteria.

Isolation and identification of mycobacteria. Biological samples (one *E. tenax* individual, exuviae or 5 g of environmental material) were homogenized using a laboratory stomacher (*Kleinfeld Labortechnik*, Germany) in sterile polyethylene bags. The resulting suspensions were decontaminated according to Fischer *et al.* (2000, 2001). Samples were then cultured on Stonebrink's and Herrold's egg yolk medium without Mycobactin J, in duplicate (Fischer *et al.* 2003*a*). Colony forming units (CFU) were counted; averages of duplicates are presented.

Mycobacterial isolates were identified by the Ziehl–Neelsen staining technique. The presence of the *Mycobacterium*-specific gene *dnaJ* was demonstrated by PCR (Nagai *et al.* 1990). *M. a. avium* and *M. a. hominissuis* were identified by PCR, detecting specific insertion sequences IS*901* (Kunze *et al.* 1992; Švástová *et al*. 2002) and IS*1245* (Guerrero *et al.* 1995), and by serotyping (Wolinsky and Schaefer 1973). Isolates of other mycobacterial species were identified by biochemical methods (Wayne and Kubica 1986).

Statistical assessment. The χ^2-test (Stat Plus) was applied to the statistical evaluation of results (Matoušková *et al.* 1992).

Fig. 1. *Eristalis tenax*. Larvae in liquid dung on pig farm (**A**) and in Petri dishes during laboratory experiment (**B**); puparia near a reservoir of liquid dung on pig farm (**C**); exuviae during laboratory experiment (**D**); *bars* correspond to 10 mm; photo I. Pavlík (A, C) and O.A. Fischer (B, D).

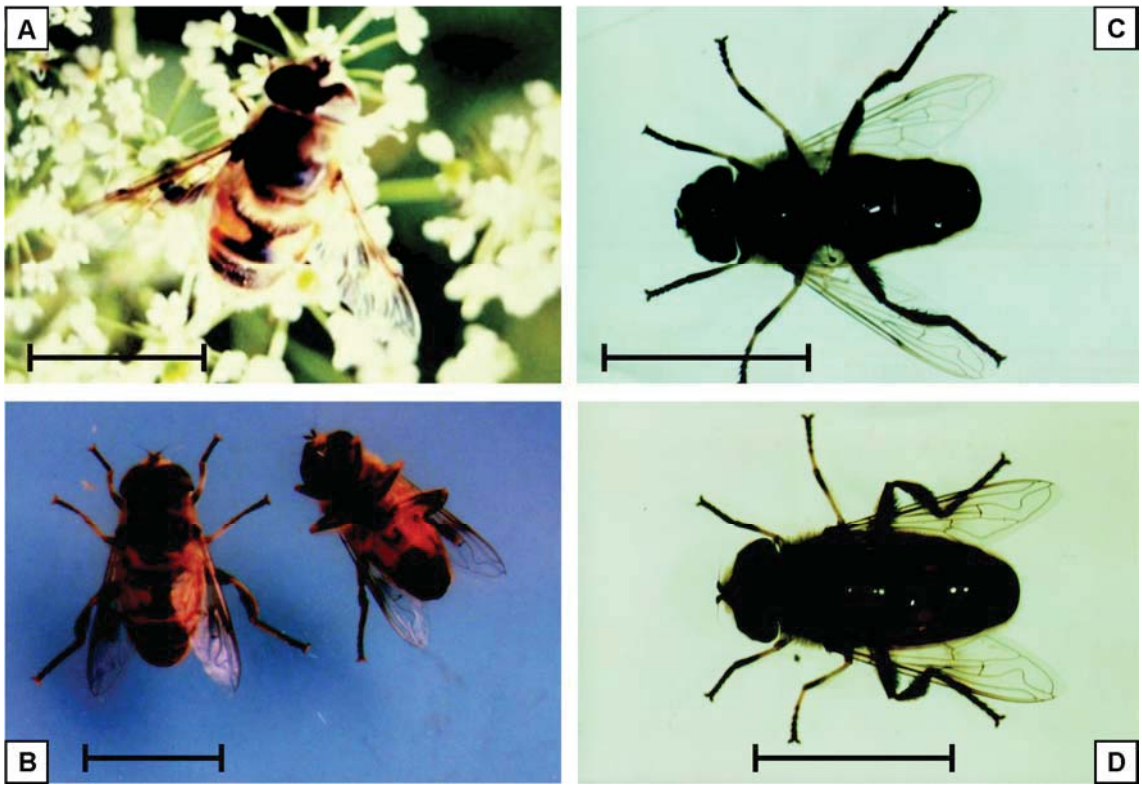

Fig. 2. Imago of *Eristalis tenax*. **A**: before the pig farm; **B**: found in the stable with pigs; received during laboratory experiment – ventral (**C**) and dorsal (**D**) view; *bars* correspond to 10 mm; photo O.A. Fischer.

Table II. Influence of submerged condition (SC; in BG 11 medium) and air-exposure (AE; on moist soil), agarized (2–8 %) solid BG 11 and NaCl-supplemented (0.2–0.6 mol/L) liquid BG 11 media, storage of dried vegetative cells and/or filaments air-exposed (SA) or in desiccators (SD; over fused calcium chloride for different time periods), different light intensity (0, 2 and 10 µmolm^{-2}s^{-1} for 12 h) on percentage survival of *S. millei*, *L. mesotricha* and *R. crassipellitum* (vegetative cells) and *P. bohneri*, *M. chthonoplastes* (filaments)[a]

Alga	Time months	SC	AE[b]	Agar, %			NaCl, mol/L					SA[c]	SD[c]	Light, µmolm^{-2}s^{-1}		
				2	4	8	0.2	0.3	0.4	0.5	0.6			0	2	10
S. millei	¼	91	96	–	–	–	93	89	64	26	0	–	–	–	–	–
	½	78	82	95	89	68	74	57	14	0	–	57	38	59	65	74
	1	61	65	82	67	33	51	23	0	–	–	25	10	50	51	56
	1½	29	31	44	36	10	20	0	–	–	–	5	2	14	20	20
	2	7.5	9	20	0	0	0	–	–	–	–	–	–	–	–	–
P. bohneri	¼	–	–	90	81	51	95	74	36	11	0	–	–	80	90	100
	½	100	80	88	69	30	89	31	10	0	–	38	20	56	69	73
	1	81	66	58	28	15	27	0	0	–	–	15	7	12	39	52
	1½	69	30	32	11	2	0	–	–	–	–	1	0	0	10	43
	2	52	8	6	2	0	–	–	–	–	–	–	–	–	–	–
	3	21	0	–	–	–	–	–	–	–	–	–	–	–	–	–
	4	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M. chthonoplastes	¼	86	90	87	71	68	92	69	42	15	0	51	21	70	81	86
	½	56	68	70	51	46	50	40	0	0	–	30	0	33	46	52
	1	16	22	43	18	8	18	7	–	–	–	3	–	4	11	12
	1½	0	6	10	0	0	0	0	–	–	–	–	–	0	0	0
	2	–	0	0	–	–	–	–	–	–	–	–	–	–	–	–
L. mesotricha	¼	77	79	100	79	60	71	42	2	0	0	50	35	57	68	72
	½	42	70	75	63	51	33	12	0	–	–	36	4	20	30	36
	1	16	50	50	29	15	0	0	–	–	–	4	0	0	4	13
	1½	0	31	20	6	0	–	–	–	–	–	0	–	–	0	0
	2	–	12	0	0	–	–	–	–	–	–	–	–	–	–	–
	3	–	0	–	–	–	–	–	–	–	–	–	–	–	–	–
R. crassipellitum	¼	93	94	95	66	59	85	79	61	40	21	46	25	66	80	88
	½	70	80	89	45	35	63	51	39	16	0	20	9	51	60	70
	1	47	59	69	10	0	31	20	8	0	–	0	0	15	23	30
	1½	23	10	36	0	–	6	0	0	–	–	–	–	0	0	8
	2	0	0	2	–	–	0	–	–	–	–	–	–	–	–	–

[a]Rounded mean of three replicates, from each of which ?6000–7000 vegetative cells or filaments were counted; algal materials maintained under a 12-h illumination (fluorescent light of 40 µmol m^{-2} s^{-1}) at 25 °C; mean relative humidity of culture chamber 47–55 %.

[b]Sterilized dry garden soil–sterilized tap water (1 : 1, *W/W*). [c]Survival of dried algal materials 3 d after inoculation in liquid BG 11 medium.

RESULTS

Forest environments and pig farm free of pathogenic mycobacteria. Mycobacteria were not isolated from any *E. tenax* or environmental samples from locations *1* and *2*. The CPM species *M. chelonae* was isolated from 9 (77.9 %) larvae of *E. tenax* and from 1 (10.0 %) sample of liquid dung from location *3* (Table I).

Pathogenic mycobacteria were isolated from *E. tenax* and liquid dung from pig farms with mycobacteriosis (locations *4–6*: farms that house herds of pigs affected by mycobacteriosis of the lymph nodes). Specifically *M. a. hominissuis*, *M. fortuitum*, and *Mycobacterium* sp. were isolated from liquid dung from locations *5* and *6*. *M. a. avium*, *M. a. hominissuis*, and *M. fortuitum* were isolated from different samples of *E. tenax* from locations *4* and *6* (Table I).

Isolation and identification of mycobacteria from E. tenax *found at sites of pig farming.* From a total of 490 samples of *E. tenax* at various developmental stages (larvae stages 1–3, puparia, imagoes, and exuviae) collected from locations *3–6*, sixteen (3.3 %) isolates of mycobacteria were obtained from locations *3*, *4*, and *6* (Table II).

A total of 60 larvae at developmental stage 3 of the *E. tenax* life cycle from sites, where mycobacteriosis has been diagnosed in pigs, were randomly selected for dissection. Five samples (8.5 %) yielded isolates of three mycobacterial species (Table II): *M. a. hominissuis* ($n = 3$), *M. a. avium* ($n = 1$) and *M. fortuitum* ($n = 1$). *M. a. hominissuis* (genotype IS*901*– and IS*1245*+, serotype 8) was isolated from the skin covering (pouch) of three larvae (14, 20, and 22 CFU). *M. a. avium* (genotype IS*901*+ and IS*1245*+, serotype 2) was isolated from the skin covering (pouch) of one larva (1 CFU); *M. fortuitum* was isolated from the intestinal tract and internal organs of one larva (1 CFU).

DISCUSSION

The syrphid fly, *E. tenax*, is a member of the order Diptera, suborder Brachycera. Dipteran insects undergo a complex metamorphosis. Larvae that hatch from eggs are classified to be at the second stage of development; after several molting stages they develop into pupas covered with puparium and remain dormant for a few days. The puparium is formed from the last larval skin (exuviae) that covers the coarctate pupa and provides protection. The imago that emerges from the pupa has the principal role of ensuring reproduction of the species (Fischer 1999). During the larval stage, bacteria can be ingested with feed and any that survive are concentrated into the anterior and posterior parts of the intestine, which are ectodermic and are therefore sloughed off together with skin (exuviae) each time molting occurs. This correlates with a decrease in the population of bacteria in the intestine of a larva immediately after molting (Povolný 1974).

Despite this decontamination mechanism, a small population of bacteria can survive in other parts of the intestine and remain viable throughout the rest of the life cycle of the organism (Povolný 1974). This study investigated at which stage of development the *E. tenax* was most commonly infected with mycobacteria. The lowest proportion of mycobacteria was found in exuviae (zero out of 12) and imagoes (mycobacteria were only isolated from one of 66 imagoes examined) compared to 1.9 % of larvae (7 out of 376) (Table I).

M. a. hominissuis was isolated from the skin covering (pouch) of 5 out of 60 larvae (Table II). This is an evidence that larvae living in an aquatic environment (*e.g.*, liquid pig dung) that harbors pathogenic mycobacteria could become contaminated with pathogenic mycobacteria. The *M. a. hominissuis* subspecies of mycobacteria is one of the predominant pathogens causing mycobacteriosis of lymph nodes in domestic pigs in Czechia (Dvorská *et al.* 1999; Pavlík *et al.* 2003). As such this subspecies is often detected at pig farms in Czechia (Mátlová *et al.* 2003). Thus larvae of *E. tenax* could contribute to the spreading and transmission of *M. a. hominissuis* and other pathogenic mycobacteria. Larvae migrating from a contaminated aquatic environment, looking for an adequate place for pupation, may in particular cause significant dispersal of pathogenic mycobacteria. This potential of mycobacteria transmission at locations where plague proportions of syrphid flies can develop would be particularly significant, especially in cases such as the reported incident from a cattle farm in the Mantua region in Italy, where masses of *E. tenax* larvae were seen to be leaving dung cesspools and the cavities under cattle grids searching for a sheltered, adequate place to transform into pupa, some were seen trying to get into the body cavities of cattle (Guizzardi *et al.* 1989). Such a scenario at a farm infected with pathogenic mycobacteria could lead to a widespread transmission of mycobacteria throughout the herd and surrounding environment.

Only one CFU of *M. fortuitum* was isolated from a sample of excised digestive tract and the internal organs from one larval sample. This result is not surprising as larvae are likely to ingest mycobacteria (if present) together with cell debris found in the liquid constituent of dung and mycobacteria have been pre-

viously shown to be able to survive in the intestinal tract or internal organs of both *E. tenax* and other invertebrates (Kazda 2000; Fischer *et al.* 2003*a*,*b*, 2004*a*,*b*).

Mycobacteria were isolated from 7.7 % of puparia samples and from only one imago (1.5 %) collected at pathogenic mycobacteria infected pig farms. The whole body of the imago was cultured, so it was impossible to determine specifically where on the imago the mycobacteria were located and the relative significance of body surface compared to internal contamination with respect to the spreading mycobacteria could not be assessed. Whatever the route of contamination, the imago was contaminated by mycobacteria, and could potentially help the spread and transmission of mycobacteria throughout pig herds (*cf.* Tables I and II).

Larvae of *E. tenax* are not the only larvae of the order Diptera or even the family Syrphidae that have been reported to develop in liquid dung. Doležil (1972) experimentally demonstrated that besides the larvae of *E. tenax* another 19 species from the family Eristaliini can develop in liquid dung. However, during this study, only the species *E. tenax* (Tables I and II) was found at pig farms examined.

Just prior to pupation, larvae leaving dung cesspools may be caught by predators such as birds. Consequently, mycobacteria may enter the food chain of other animals living in or around farms (Mátlová *et al.* 2003). It is clear from this study that larvae of *E. tenax* could be a potential vector of pathogenic mycobacteria. It is essential to know the morphological, physiological and developmental characteristics of these invertebrates, their occurrence and relation to the environment, and their involvement in the food chain to be able to assess potential spreading and transmission of mycobacteria by these organisms (Fischer 1999).

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