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Short communication

Gasterophilus intestinalis (Diptera: Oestridae) in the diaphragmatic muscle: An unusual finding

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ABSTRACT

Larval forms of the bot-fly *Gasterophilus* are obligate parasites commonly found in the gastrointestinal tract of equids, causing intestinal myiasis. Five species are reported so far in Italy, mostly observed during necroscopy, located in different portion of gastrointestinal tract of equids: *G. intestinalis*, *G. nasalis*, *G. inermis*, *G. pecorum* and *G. haemorrhoidalis*. An unusual finding of larval *Gasterophilus intestinalis* deeply inserted into the diaphragmatic muscle is here reported. Due to the uncommon localization, to the absence of clinical signs related to myiasis and subsequent uncertainty of species identity, identification was performed using an integrative taxonomical approach combining morphology with molecular tools for confirmatory reasons. This finding adds information on migration patterns of erratic larval forms in *G. intestinalis*, a feature of interest as gasterophiliasis is among the less studied intestinal myiasis of horses.

1. Introduction

Gasterophilus (horse bot-fly) are parasites of equids including horses, donkeys and zebras, throughout the world. They occasionally affect also humans in close contact with horses (Zumpt, 1965; Francesconi and Lupi, 2012). The larval forms are obligate parasites, commonly found in the gastrointestinal tract, causing intestinal myiasis and consequent considerable economic loss due to swallowing, gastro and intestinal ulcerations, gut obstructions, rectal prolapses, anemia, diarrhea, and digestive disorders (Zumpt, 1965; Sequeira et al., 2001; Gao et al., 2016). Six of the eight species so far described (*Gasterophilus nasalis, Gasterophilus haemorrhoidalis, Gasterophilus intestinalis, Gasterophilus pecorum, Gasterophilus nigricornis, Gasterophilus inermis; Gasterophilus lativentris, Gasterophilus meridionalis)* typically infect horses and three of them show a worldwide distribution: *G. nasalis, G. haemorrhoidalis* and *G. intestinalis*.

Following eggs deposition by a bot-fly female on a horse, the host becomes infected by ingesting the larvae hatched from eggs. After ingestion, first instar larvae (L1) pass through the digestive tract where they remain attached to the gastric mucosa up to 10–12 months moulting to the third instar (L3) (Zumpt, 1965). The localization of larvae in the gastric mucosa is species-specific, as well as pathogenic lesions they may induce.

For instance, *G. nasalis* (Linnaeus, 1758) is recovered prevalently in the pyloric mucosa and in the first tract of duodenum, while *G. intestinalis* (De Geer, 1776) occurs in the *pars oesophagea* of the stomach (Urquhart et al., 1998). The precise larval migration pattern from ingestion to the arrival in the gastro-intestinal trait of the host remains obscure, causing mild-to severe "tunnel-like" lesions mostly on tongue, gums and cheeks often related to secondary infections and abscesses. Due to the difficulties in estimating pathogenic damages, gasterophiliasis is often underestimated and less studied than other myiases of livestock (Otranto et al., 2005).

In Italy, five species have been reported (Principato, 1989): *G. intestinalis*, *G. nasalis*, *G. inermis* (Brauer, 1858), *G. pecorum* (Fabricius, 1794) and *G. haemorrhoidalis* (Linnaeus, 1758), mostly recovered following necropsy and *post-mortem* inspection at the slaughterhouse, where larval forms are discovered in association with different portions of gastrointestinal tract of equids.

This contribution reports the use of an integrative taxonomical approach combining the microscopy and molecular analysis to

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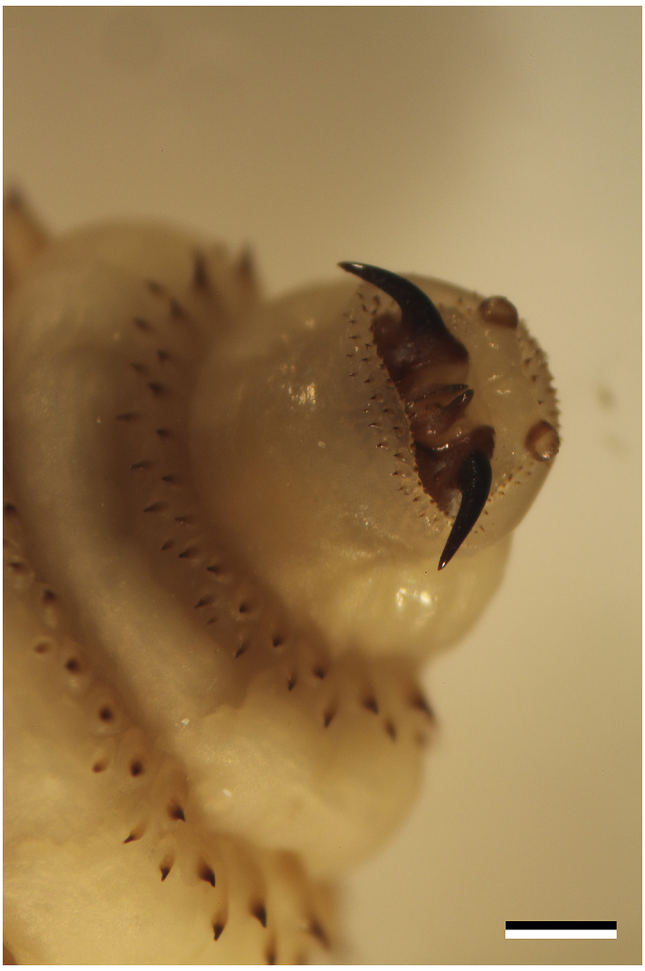

Fig. 1. Detail of the morphology of the cephalic and first thoracic segments of one of the two *Gasterophilus intestinalis* specimens collected. Bar = 0.5 mm.

characterize larvae recovered as an unusual evidence of diaphragmatic myiasis in a horse.

2. Material and methods

During a *post-mortem* inspection on a horse born in Italy and slaughtered in April 2016 according to European Regulation (CE) n. 854/2004 in a public slaughterhouse (Meat Centre of Rome), two larvae were detected as actively moving excrescences inside the diaphragm. The animal did not show any *"ante mortem"* apparent clinical sign or visible lesion associable to previous or ongoing disease, and other larvae were not detected in the gastrointestinal tract. The specimens were removed from the diaphragmatic muscle, washed in physiological saline solution and then stored in ethanol 70% for subsequent morphological and molecular analyses.

2.1. Morphological analysis

Larval specimens were measured *in toto* and morphologically identified according to Zumpt (1965) and Colwell et al. (2007) after cutting of the terminal segment (i.e. spiracular plate). A stereomicroscope (Nikon SMZ1000) coupled with a Canon EOS 5D Mark II camera has been used for observations, species identification and image acquiring. A magnification from 10× to 80× has been used to detect morphological characters useful for species identification. In particular, morphological features of cephalic and first thoracic segments, thoracic and abdominal spines, and spiracular plates of terminal segment have been examined to identify the species of the myiasis-causing fly.

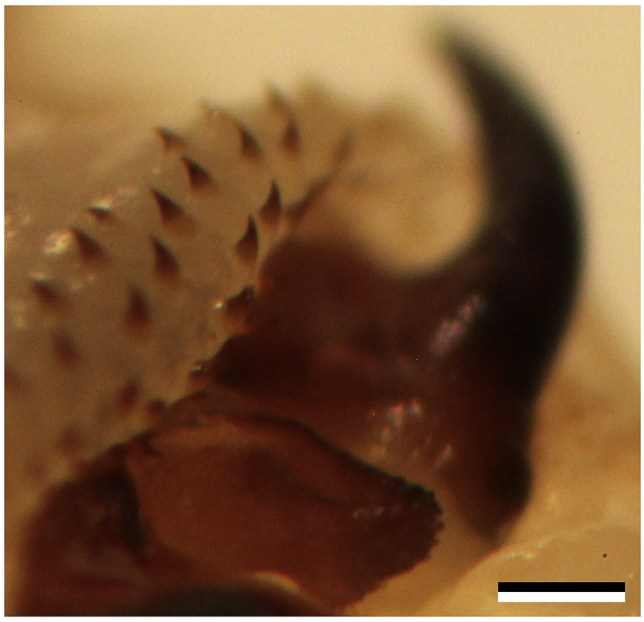

Fig. 2. Detail of the mandibles of one of the two *Gasterophilus intestinalis* specimens collected showing an extended dorsal lobe with regular notches on the edges. Bar = 0.1 mm.

2.2. Molecular analysis

Partial mitochondrial gene encoding for the subunit 1 of the cytochrome oxidase (*cox*1) was analysed at sequence level, since it has proven to be informative as molecular marker for taxonomic differentiation and systematics of insects, including members of the Oestridae family (Otranto et al., 2000, 2003).

Total genomic DNA was obtained from the larval middle-part (abdominal segments) using the Wizard Genomic DNA Purification kit (Promega, USA), according to manufacturer's instructions. A partial region of mitochondrial *cox*1 gene was amplified using a modified protocol from Otranto et al. (2003) combining the most external primers (Zhang and Hewitt, 1997). PCR amplification was performed in a final volume of 50 μL under the following conditions: 10 × buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1U of DNA polymerase (BIOTAQ^{TM} DNA Polymerase, Bioline, UK), 3 μL of genomic DNA and primers 50 pmol/μl. After an initial denaturation (95 °C for 3 min), reactions were subjected to 39 cycles of 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and72 °C for 30 s (elongation), followed by a final extension at 72 °C for 5 min.

PCR runs included positive and negative control to each run: DNA from *Oestrus ovis* previously characterized by morphological and molecular approaches (Zammarchi et al., 2014) was used as positive control. Positive amplicons were purified using Sure Clean (Bioline, UK) and shipped to external service for sequencing (Eurofins Genomics, DE). Sequences obtained were manually checked and reconstructed consensus sequences were compared to GenBank retrieved sequences.

3. Results and discussion

We here describe the unusual finding of third instar *Gasterophilus intestinalis* in the diaphragmatic muscle in a horse. Larval identification was performed using combined morphological and molecular analyses.