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Is the morphology of *Culicoides* intersexes parasitized by mermithid nematodes a parasite adaptation? A morphometric approach to *Culicoides circumscriptus* (Diptera: Ceratopogonidae)

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ABSTRACT

Mermithidae is a family of endoparasitic nematodes known to cause intersexuality in arthropods. Intersexes of the genus *Culicoides* parasitized by mermithids have been the object of several studies aiming to describe their particular morphology. *Culicoides* intersexes are specimens with male genitalia and feminized sexually dimorphic structures, i.e. antennae, mouthparts and wings. To date, these specimens have only been described qualitatively and a quantitative approach supported by statistical analysis is lacking. Here we conduct morphometric analyses of sexually dimorphic structures in a sample of *Culicoides circumscriptus* that includes 34 intersexes with the aim of describing precisely the intersexual morphology. The morphology of antennae and the mouthparts was studied by multivariate statistical analysis of linear measures, and wing form by implementing geometric morphometrics techniques. While intersex wings proved to have a similar size to male wings, their shape was intermediate between males and females. However, when allometric shape variation was removed, the wing shape of intersexes was almost identical to that of females. The intersex antennae were morphometrically of the female type, especially when size variation was considered. In contrast, the measured mouthparts (the labrum and the third palpal segment) were halfway between males and females, even when body size was considered. Overall, the antennae and the wings showed a higher degree of feminization than the mouthparts. These findings indicate that the degree of feminization depends both on the morphological structure and on body size. Moreover, we propose that the feminization of the wings and antennae has an adaptive meaning for the parasite, which would favor female-like traits in order to access more easily its breeding sites, where the parasite has plenty of new hosts to infect. Female-like antennae would be beneficial to detect these sites, while having female-like wings would favor the host's capacity of dispersal to these sites.

1. Introduction

Parasites may affect their hosts in different ways. One of them is by altering their reproductive system (Hurd, 1993). Reproductive disturbances can range from a slight reduction in fecundity to a complete destruction of the reproductive tissues (castration), or even in some occasions to irreversible changes in the external appearance (Vance, 1996 and references therein). Mermithid nematodes are well known among insect parasites because they can produce stunning morphological changes in their hosts, making them adopt an intermediate external appearance between male and female sexes (Wülker, 1975). To date, two types of individuals simultaneously displaying both male and female characteristics have been described, gynandromorphs and intersexes (Narita et al., 2010; Tugletske and Stouthamer, 2012). Gynandromorphs are individuals composed of a mosaic of genetically male and female tissues with clear boundaries between them and with a sexual phenotype consistent with the genetic sex (Narita et al., 2010). In contrast, intersexes are composed of a single sexual genotype but exhibit both male and female sex characteristics, which often appear as a blend or intermediate of the two sex characters (Narita et al., 2010). Thus, intersex individuals may be masculinized females or feminized males (Vance, 1996). There are various possible origins of intersexuality and gynandromorphism, and the mechanisms of many are still not well understood. While

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gynandromorphism is thought to originate from developmental and fertilization errors, intersexuality likely originates from interference with one or more of the regulating factors involved in the sex determining process of the individual, often caused by mutations, polyploidy or parasitism (Tugletske and Stouthamer, 2012 and references therein).

In the genus *Culicoides* (Diptera: Ceratopogonidae), both gynandromorphs and intersexes have been described (Curtis, 1962; Navai, 1969; McKeever et al., 1997; Sarto i Monteys et al., 2003; Mullens et al., 2008). In accordance with general observations, while gynandromorphic specimens of *Culicoides* have not shown any sign of parasitism (Curtis, 1962; Navai, 1969) intersexes have often been associated to parasitization by mermithid nematodes (Sarto i Monteys et al., 2003; Mullens et al., 2008); though *Culicoides* intersexes with no signs of parasite infection have also been detected (Sánchez-Murillo et al., 2011). Most of the *Culicoides* intersexes parasitized by mermithid nematodes are specimens with male genitalia and secondary sexual traits modified toward the female condition (McKeever et al., 1997; Sarto i Monteys et al., 2003). These intersex specimens are assumed to be genetically male, with different degrees of feminization (McKeever et al., 1997). In contrast, parasitized genetic females undergo little or no modification (Smith and Perry, 1967; McKeever et al., 1997; Sarto i Monteys et al., 2003).

While changes in the morphology of the host can represent pathological by-products of infection, they can also represent adaptations, either of hosts or of parasites (Dingemanse et al., 2009). It has been recently suggested that differences between host sexes in many different traits, such as morphology, hormone levels, or behavior can impose divergent selection regimes on parasites, which can eventually lead to adaptations specific to the host sex (Duneau and Ebert, 2012). In the most extreme case, when the survival or the reproductive success of the parasite depends on the characteristics of one of the host sexes, the parasite can adapt in two different ways: i) evolving mechanisms for discriminating between the sexes, or ii) evolving mechanisms for manipulating the infected host so as to recover particular sex-specific traits necessary for parasite proliferation and/or transmission (Duneau and Ebert, 2012). Males and females of the genus *Culicoides* have marked morphological and behavioral differences (Kremer, 1965; Blackwell et al., 1992). Therefore, the divergent effect of mermithid nematodes on both sexes may respond to parasite adaptations to a specific host sex. Thus, the feminization of males could represent an adaptation of the nematode to recover particular female-specific traits necessary for parasite proliferation and/or transmission. The question is: what are these traits? Most of the studies reporting the presence of *Culicoides* intersexes produced by mermithid nematodes have noticed that intersexes are male-intended specimens that have the head, mouthparts, antennae and wings modified to resemble those of females (Sarto i Monteys et al., 2003). However, until now few studies have performed a detailed morphological analysis of these structures (but see McKeever et al., 1997). Actually, to our knowledge the wings have never been analyzed. Moreover, as far as we know, even in those structures that have been studied in depth a quantitative approach supported by statistical analysis is lacking.

Until now, *Culicoides circumscriptus* Kieffer, 1918, is the only species of the genus in which intersexes have been detected in Spain (Sarto i Monteys et al., 2003; Sánchez-Murillo et al., 2011). Specifically, among a group of ten specimens (two males and eight females) captured in northeast Spain, Sarto i Monteys et al. (2003) detected one intersex, with male genitalia and feminized antennae and mouthparts, that was parasitized by a nematode of the genus *Heleidomermis*. In a subsequent study, the nematode was described as a new species, which was named *Heleidomermis cataloniensis* (Poinar and Sarto i Monteys, 2008). With the aim of quantifying the degree of resemblance between *C. circumscriptus* intersexes and conspecific males and females, we performed a morphometric analysis of the main sexually dimorphic structures: the antennae, the mouthparts, and the wings. In order to gain insight into the evolutionary processes driving the feminization of parasitized males, the morphometric analyses were conducted separately in each of these structures. In particular, to analyze the morphological changes of the wing we applied geometric morphometrics techniques, which have proved to be very useful in describing the shape of *Culicoides* wings (Muñoz-Muñoz et al., 2011, 2014). The most well-known case of a parasite inducing the feminization of genetically male hosts is *Wolbachia*, an intracellular reproductive parasite of many species of arthropods and nematodes (Werren et al., 2008). Although *Wolbachia* has not been detected in non-filariid nematodes parasitizing arthropods (Duron and Gavotte, 2007), we considered the possibility that the feminization of *C. circumscriptus* intersexes could be caused by *Wolbachia* being transmitted by the parasitic nematodes. In order to further reject a possible role of *Wolbachia* in the feminization of *C. circumscriptus* we performed molecular analyses of the insect and the nematodes aiming to detect the bacteria.

2. Materials and methods

2.1. Sample composition and preparation

Eighty-two specimens of *C. circumscriptus* captured by means of light traps (Miniature blacklight trap model 912, John W. Hock Company, Gainesville, FL) at ten capture sites in Spain were used in this study (Table 1). Specimens were identified at the species level under the stereomicroscope according to their pattern of wing pigmentation (Kremer, 1965). Afterwards, they were dissected by using sterilized ultrafine tweezers for each individual (Muñoz-Muñoz et al., 2011), and the presence of adult nematodes was recorded. The head, the wings and the final part of the abdomen were mounted on glass microscope slides in Canada balsam for morphometric analyses. The remaining parts of the body and the nematodes of parasitized specimens were stored in absolute ethanol for molecular analyses.

2.2. Molecular analyses for Wolbachia detection

Individual nematodes and abdomens of parasitized specimens were homogenized in 200 μL PBS. DNA extraction was performed with homogenates using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Crawley, UK) following the manufacturer's instructions with a final elution volume of 100 ml and using positive and negative controls. Presence of *Wolbachia* sp. was assessed by means of specific single PCR in accordance with Duron et al. (2008). Briefly, the presence of *Wolbachia* was tested by amplification of a fragment of the wall surface protein (wsp) gene delimited by primers wsp81F and wsp691R.

2.3. Morphological data collection

Digital images of the antennae, the mouthparts, and the wings were obtained using a Nikon Eclipse 90i microscope equipped with a Nikon DXM 1200F camera (Tokyo, Japan). Only one of the sides of bilateral structures was photographed. The use of left and right structures was randomized as selection of one side may bias the results in the case of differential directional asymmetry between groups. Linear measurements from the antennae and the mouthparts were obtained from scaled digital images with the aid of the ImageJ software (Rasband, 1997–2014). Six linear measures were obtained from the antennae: the length of flagellomeres