The Reproductive and Mating Strategies of the Twisted-winged Parasites (Insecta: Strepsiptera)

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Novel Insights in the Reproductive Biology of

an enigmatic Insect Order

Dissertation

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by

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"Quoi qu'il en soit,
cet insecte est un des plus singuliers et des plus inte'ressants que
puisse offrir la nature."

("This insect is one of the strangest and most interesting that nature can offer")

Luis Jurine – 1816 about *Xenos vesparum*

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1   Introduction

The overall aim of my dissertation is to make a significant contribution to the understanding of the poorly studied reproductive biology of the often-neglected insect order Strepsiptera. In my introduction, I aim to open up the hidden world of twisted-wing parasites and provide a basic context for the work presented here. I will start with a general overview of the insect order and proceed through a phylogenetic classification into morphological features of males and females. In doing so, I will focus on the peculiarities of sexual reproduction and interaction between the two sexes. In addition, I will present previous findings on the reproductive biology of Strepsiptera and explain important basic terminology. Finally, I will briefly outline the individual studies conducted with a clear objective, elaborate on the main questions of the dissertation and introduce the broad methodological approach to solving them.

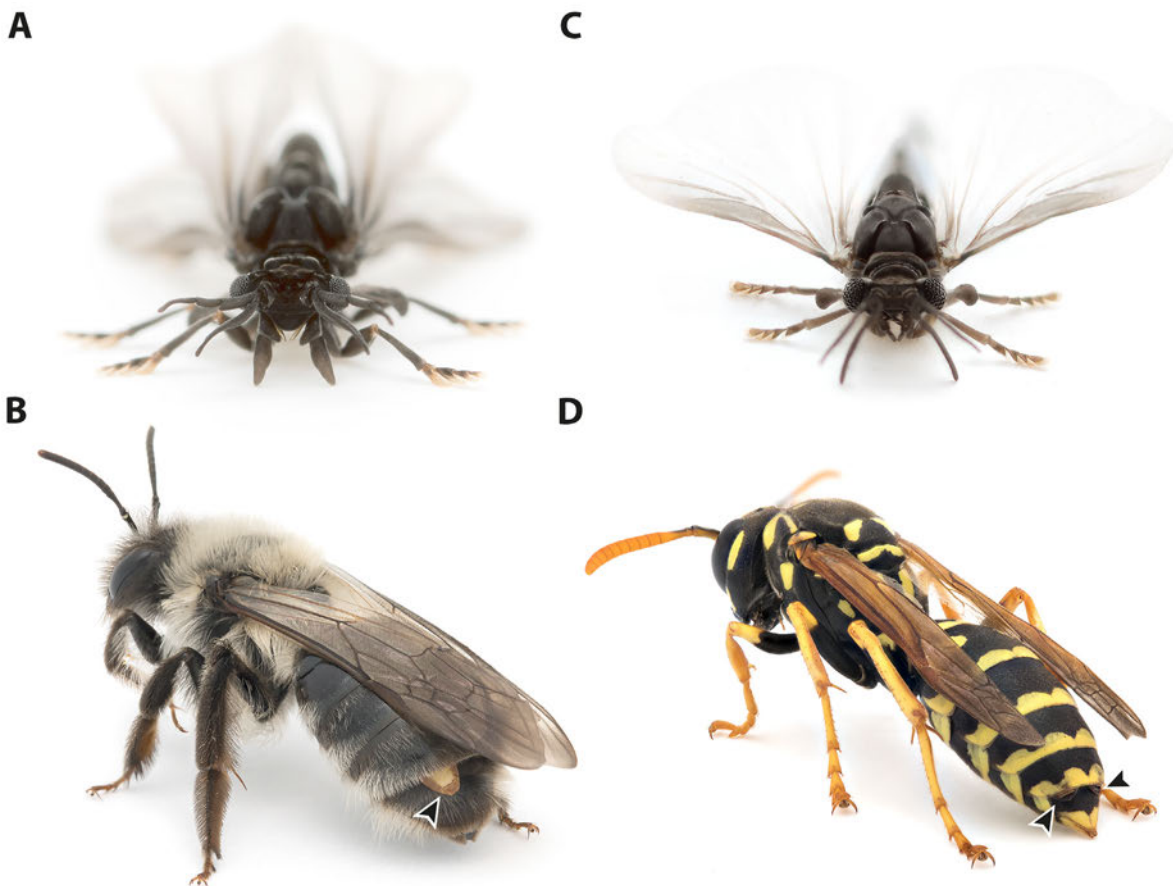Figure 1: Photographs of *Stylops ovinae* and *Xenos vesparum*. (A) Frontal view of an adult male *S. ovinae*. (B) One female of *S. ovinae* protrudes from its host's metasoma (*Andrena vaga*). (C) Frontal view of an adult male *X. vesparum*. (D) Two females of *X. vesparum* protruding from their host's metasoma (*Polistes dominula*). Arrowheads indicate strepsipteran females. Photos by Hans Pohl, from Jandausch et al. (2022).

1.1   A General Opening to the World of Strepsiptera

The twisted-winged parasites belong to the smaller orders of insects, with only about 600 described species (Pohl & Beutel, 2005; Pohl & Beutel, 2008). With an age of 100 million years from Burmese amber, †*Cretostylops* (Schawaroch, Kathirithamby & Grimaldi, 2005) is one of the oldest fossils in a growing pool of known fossil specimens, mostly represented by males (e.g., Grimaldi & Kathirithamby, 1993; Kinzelbach & Pohl, 1994; Pohl, 2009; Kogan & Poinar Jr, 2010; Pohl & Beutel, 2016; Pohl et al., 2018; Kogan & Poinar, 2019; Pohl et al., 2019; Pohl et al., 2021). Due to their predominantly endoparasitic lifestyle and mostly small size, they often elude even the most attentive observer and are therefore considered to be an enigmatic group of insects. The larvae are extremely miniaturised, sometimes as small as 70 µm (Pohl, 2000; Beutel, Pohl & Hünefeld, 2005; Pohl & Beutel, 2019), and possess the smallest known photoreceptors in insects (Fischer et al., 2021). While females, with the exception of two families – Bahiaxenidae and Mengenillidae – remain in their host for life, males are free-living after leaving the puparium within the host, albeit often living only a few hours (Figure 1). During their short life span, the
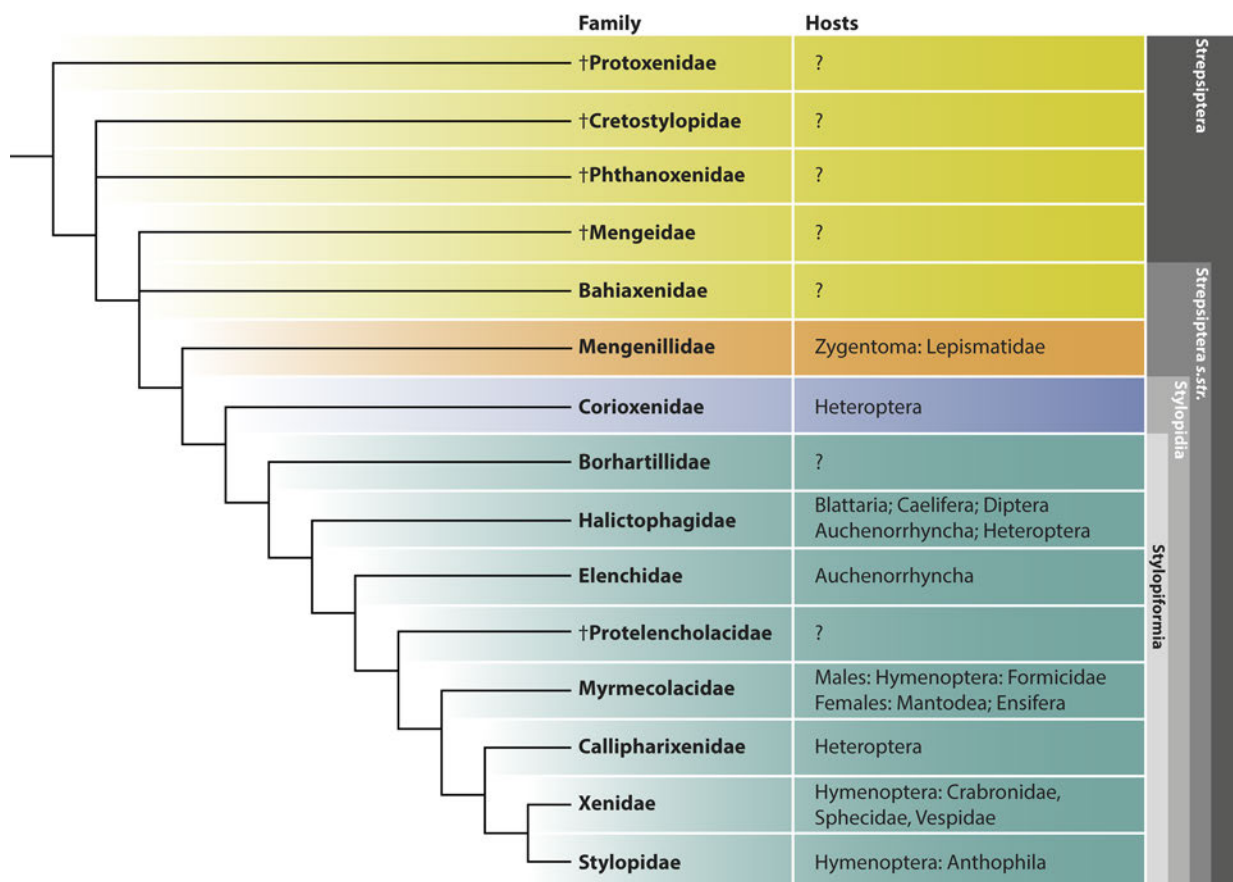

Figure 2: Phylogeny of Strepsiptera. Tree is based on the morphological phylogeny of Pohl et al. (2021). Information on associated hosts added according to Pohl & Beutel (2008).

main task of a male is limited to searching for females and mating. This distinct difference in adult lifestyle is also reflected in extreme sexual dimorphism. The host spectrum of these insects is extremely broad as they are associated with at least seven different insect orders (Pohl & Beutel, 2008; Kathirithamby, 2009) (Figure 2). While Mengenillidae are found in several species of silverfish (Zygentoma), all other families parasitise neopteran insects. Hosts include cicadas (Auchenorrhyncha), bugs (Heteroptera), flies (Diptera), grasshoppers (Orthoptera), ants (Formicidae), praying mantises (Mantodea), wasps (Vespidae), and bees (Anthophila). The degree of host-parasite specificity varies with taxonomic level. Some families are clearly associated with specific host groups (e.g., Mengenillidae with silverfish, Elenchidae with cicadas, and Stylopidae with bees). However, within families there are species that are restricted to a single host species (e.g., *Stylops ovinae*; Jůzová, Nakase & Straka (2015); Straka, Jůzová & Nakase (2015)) and others that parasitise several closely related host species (*Xenos vesparum*; Benda et al. (2020). A unique sex-specific parasite-host relationship is known in the Myrmecolacidae, where males infest ants and females parasitise praying mantises or crickets. How Strepsiptera overcome host immune responses is not well understood, but Manfredini et al. (2007) concluded that in *X. vesparum* the immune response is manipulated by the first instar exuvia. Even if clear mechanisms are not clear, the exuvia serves as a pseudo-target for immune response and the emerged secondary larva stays unaffected.

The life cycle of Strepsiptera is fascinating in its complexity. Studies dealing with embryonic development are scarce (e.g., Hoffmann, 1913; Hoffmann, 1914; Schrader, 1924; Noskiewicz & Poluszyński, 1928). However, the most recent work of Fraulob et al. (2015) on *Stylops ovinae* provides exceptional morphological details of the 18 differentiable embryonic stages. A few specific studies have been carried out on the postembryonic development of individual species such as *Caenocholax fenyesi* Pierce, 1990 (Myrmecolacidae) (Cook, Vinson & Gold, 1998), *Elenchus tenuicornis* Kirby 1815 (Elenchidae) (Baumert, 1958; Kathirithamby et al., 1984), and *Eoxenos laboulbenei* Peyerimhoff, 1919 (Mengenillidae) (Tröger et al., 2020; Weingardt, Beutel & Pohl, 2023). From these works, the major and minor life cycle differences between the families have been outlined (e.g., Kathirithamby, 1998; Beani et al., 2005; Tröger et al., 2020). The most striking discrepancy is probably between the Mengenillidae and the rest of the Strepsiptera. While the larvae of the former leave the host at some point (Kinzelbach, 1971; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Tröger et al., 2023), those of the latter remain inside the host and differentiate into adults (Kinzelbach, 1971; Kinzelbach, 1978; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Löwe, Beutel & Pohl, 2016; Richter et al., 2017).

The development of Strepsiptera can be roughly generalised as follows. The minute primary larvae are very mobile and are therefore considered to be the "infective" stage (Pohl, 2000; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Osswald, Pohl & Beutel, 2010; Pohl & Beutel, 2019; Tröger et al., 2020). The tasks of these larvae are to identify, reach, and infest the appropriate host organisms. After attaching to the exoskeleton of the host, the larva begins to penetrate the host with its mandibles, eventually reaching the inner sanctum of its new habitat (Kirkpatrick, 1937; Baumert, 1958; Pohl, 2000; Manfredini et al., 2007; Knauthe et al., 2016). After infestation the larva immediately molts into its secondary larval stage, which is very different in morphology. As this part of the life cycle is strictly endoparasitic, vital functions such as mobility and sensory organs are superfluous, and consequently legs and sensory organs are observably vestigial. Again, the Mengenillidae are an exception, as legs are developed in these stages (Tröger et al., 2020). Even if the number of clearly distinguishable endoparasitic larval instars differs between groups (e.g., two endoparasitic larval stages in Mengenillidae and one in Stylopiformia), they can be considered together as a trophic period, as only these instars feed on the haemolymph of their host (Strambi & Strambi, 1973; Giusti et al., 2007). During development, the endoparasitic instars grow in biomass and later start to differentiate internal tissues. At the end of endoparasitic development, the larva breaks through the host's cuticle at the membranous junctions of the segments, which are usually from the abdomen. Larvae of the Mengenillidae emerge completely from the host to pupate outside (Silvestri, 1943), while in other families only the cephalothorax protrudes between the sclerites (Kirkpatrick, 1937; Kinzelbach, 1971; Kinzelbach, 1978; Kathirithamby, 1983; Kathirithamby, 1989; Pohl & Beutel, 2005; Pohl & Beutel, 2008). In both cases, the cuticle of the final larval stage becomes a puparium in which the adults mature. Mature adults emerge from the puparium, except for the females of Stylopidia. Mating then occurs and, if fertilisation is successful, numerous viviparous larvae develop inside the mother – for example, ca. 1,400 in *Eoxenos laboulbenei* (Mengenillidae; Tröger et al. (2023)), about 29,000 in *S. ovinae* (unpublished data of H. Pohl & H. Stark), and up to 750,000 in *Stichotrema dallatorreanum* (Myrmecolacidae; O'Connor (1959)).

1.2   Strepsiptera Phylogeny and Morphology with Emphasis on Reproduction

Clarifying the relationships of Strepsiptera in the insect phylogenetic tree has been a long journey after the initial placement where *Xenos vesparum* (Rossi, 1793) was nested within the huge order of Hymenoptera close to Ichneumonidae (Rossi, 1793). Apart from the classifications into many different orders, including Ephemeroptera, Odonata,

Hymenoptera, Lepidoptera, or Siphonaptera, the Halteria hypothesis was probably the most prominent (see Pierce, 1909; Boussau et al., 2014). This hypothesis was based on the inference of a sister-group relationship with the Diptera, basis mainly on the presence of halteres and analysis of 18S and 28S ribosomal DNA sequences (e.g., Whiting et al., 1997; Wheeler et al., 2001). It should be noted that the halteres are located on different thoracic segments, being on the mesothorax in Strepsiptera and on the metathorax in Diptera. However, more recent phylogenetic analyses based on genomic data, and morphological data established a sister-group relationship with Coleoptera (Beutel et al., 2011; Niehuis et al., 2013; Boussau et al., 2014). The internal phylogeny of Strepsiptera resolved three major groups: Mengenillidae, the sister group to Stylopidia (all other Strepsiptera), and the division of this latter clade into Corioxenidae and Stylopiformia (Figure 2). This topology is strongly supported by morphological and genetic data (Kinzelbach, 1971; Pohl, 2000; Pohl & Beutel, 2005). The peculiarities of these three main groups will be presented in the following sections, with a focus reproductive aspect. Disentangling the relationships at the family and genus level has begun, although only a handful of datasets have been published (e.g., Jůzová et al., 2015; Straka et al., 2015; Benda et al., 2020; Benda et al., 2022).

1.2.1 Males

Unlike the evolutionary highly derived females, males of the order Strepsiptera can be clearly recognised as insects by their morphology. This is mainly due to the appearance of a distinct head, a three-segmented thorax with well-developed legs, and a distinct ten-segmented abdomen. The antler-like antennae are conspicuous and are formed by the scape, the pedicel, and up to 6 flabellate flagellomeres. The flagellomeres are equipped with densely packed chemoreceptors for long-range detection of females as potential mating partners (Kinzelbach, 1971; Kinzelbach, 1978; Pohl & Beutel, 2005; Pohl & Beutel, 2008). This is complemented at close range by the unique and diagnostic large raspberry-like eyes (Buschbeck, Ehmer & Hoy, 1999; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Fischer et al., 2021). The mouthparts, including the mandibles and maxillae, are present but highly simplified. The shape of the mandibles is specialised for opening the puparium and is therefore critical for male hatching. The mesothoracic wings are modified as halteres and serve to stabilise the insect in flight, which is propelled by the fan-shaped metathoracic wings (Pix, Nalbach & Zeil, 1993). Evolutionary derived modifications related to mating behaviour also occur on the legs of Stylopidia. Pohl & Beutel (2008) identified specialised adhesive structures on the tarsomeres and the complete

reduction of claws, among other features, as adaptations to endoparasitic females and their flying hosts (Pohl, Gorb & Gorb, 2020). As such adhesive pads are absent in male fossil Strepsiptera, this feature is used as evidence for the claim that the females of the extinct families †Protoxenidae, †Mengeidae, †Cretostylopidae and the extant family of Bahiaxenidae had free-living females (e.g., Pohl & Beutel, 2005; Pohl & Beutel, 2008). With respect to sexual reproduction, several features of the abdomen are striking. Strepsiptera have a simplified genital apparatus: parameres are absent except in †*Heterobathmilla* (Pohl et al., 2021), the sperm pump is unsclerotized, and pumping is likely generated by a thin muscular layer (Hünefeld & Beutel, 2005; Hünefeld et al., 2011). The penis can be short and slightly curved, elongated and straight or hooked. In general, however, the intromittent organ is always pointed.

1.2.2   Females

The morphology of female Strepsiptera is highly derived, as their endoparasitic lifestyle does not make them look like insects at the first sight, and anatomically, they are clearly distinguished from males (Kinzelbach, 1971; Kinzelbach, 1972; Kathirithamby, 1989; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Kathirithamby et al., 2015; Kathirithamby, 2018). The phylogenetically early branching family Mengenillidae is the only extant group where free-living adult females have been identified (Silvestri, 1933; Silvestri, 1941b; Silvestri, 1942; Silvestri, 1943; Kinzelbach, 1971; Kinzelbach, 1972; Kinzelbach, 1978; Tröger, Beutel & Pohl, 2019; Tröger et al., 2023). That at least females of the extinct †Mengeidae were also free-living is evident from the morphology of an immature free-living female of †*Mengea* described by (Pohl et al., 2019). Female Mengenillidae are characterised by a distinct head, a three-segmented thorax with one pair of legs each, and a ten-segmented abdomen. Antennas, mandibles, and maxillae are present, although the maxillae are small and greatly simplified. Inside the abdomen Mengenillidae carry an unpaired birth organ which is opens between the VII. and VIII. abdominal segments and where the primary larva are released (Tröger et al., 2019; Tröger et al., 2023). The most drastic morphological changes are observed with the switch of Stylopidia to winged hosts. This is accompanied by a change to a permanent endoparasitic lifestyle of the females. The most striking morphological changes are the restructuring of the body organisation a cephalothorax and abdomen, the loss of all extremities, and the formation of a functional unit with the exuvia of the previous larval stages (Pohl & Beutel, 2005; Pohl & Beutel, 2008). The latter novelty forms the brood canal, which is connected to the birth organs located in the abdomen and allows the primary larvae to hatch. While in the family Corioxeni-

dae the larvae still have to break through the outer larval skin, in all other Strepsiptera (= Stylopiformia) a birth opening is formed on the morphologically ventral (= physiologically dorsal) side of the cephalothorax at the transition from head to prothorax. As the entire animal in the host is oriented with the morphological ventral side outwards, this birth opening is exposed to the outside and is the site where the penis is introduced for copulation and where the primary larvae leave the brood canal. Another structure has been described for members of the Stylopidae, namely a paragenital organ (Peinert et al., 2016), an invagination of the integument in front of the birth opening. How widespread such a structure is in Strepsiptera remains unclear and will be answered in this dissertation.

1.3   Sexual Reproduction

1.3.1   Theoretical Background

Before discussing the current state of sexual reproduction in Strepsiptera, I will give a brief overview to traumatic insemination and polyandry, as these two biological phenomena play a crucial role in these insects.

1.3.1.1 Traumatic Insemination

Copulatory behaviour is referred to as traumatic insemination when direct sperm transfer involves injury to the female body. Although this definition seems straightforward, traumatic insemination is often confused with other copulatory behaviours. The most precise definition of traumatic insemination to date was provided by Lange et al. (2013), who introduced two additional categories for clarification. Figure 3 shows a four-step decision tree to identify traumatic insemination: (I) males of the species under study have a structure suitable for wounding females; (II) the male structure used in the mating behaviour is responsible for wounds occurring on the female; (III) males transfer a secretion into the female's body cavity that is nonequivalent to sperm; (IV) males transfer sperm into the female's body cavity. If I and II are fulfilled, the copulatory behaviour can be classified as traumatic mating. If, in addition, condition III is true, we speak of traumatic secretion transfer. Conversely, if IV is true, traumatic insemination has occured. If there is no secretion transfer at all, but there is wounding, the copulation is defined as traumatic penetration.

While traumatic penetration and traumatic secretion transfer are associated with effects on the physiology, fecundity, or fitness of females in a sexual conflict (e.g., Crudgington & Siva-Jothy, 2000; Lange et al., 2013; Matsumura et al., 2017; Tong et al., 2021)), traumatic insemination serves as a direct method to deposit spermatozoa near unfertilized female eggs (e.g., Stutt & Siva-Jothy, 2001; Brand, Harmon & Schaerer, 2022; Jandausch et al., 2023). Traumatic insemination is often viewed as a result of sperm competition, as it allows males to bypass female genital tract structures, such as a spermatheca, and gain an advantage in fertilising eggs (Parker, 1970; Tatarnic, Cassis & Siva-Jothy, 2014; Simmons & Wedell, 2020). In my dissertation, one of my main aims is to elucidate the copulation mode of the different families of Strepsiptera.
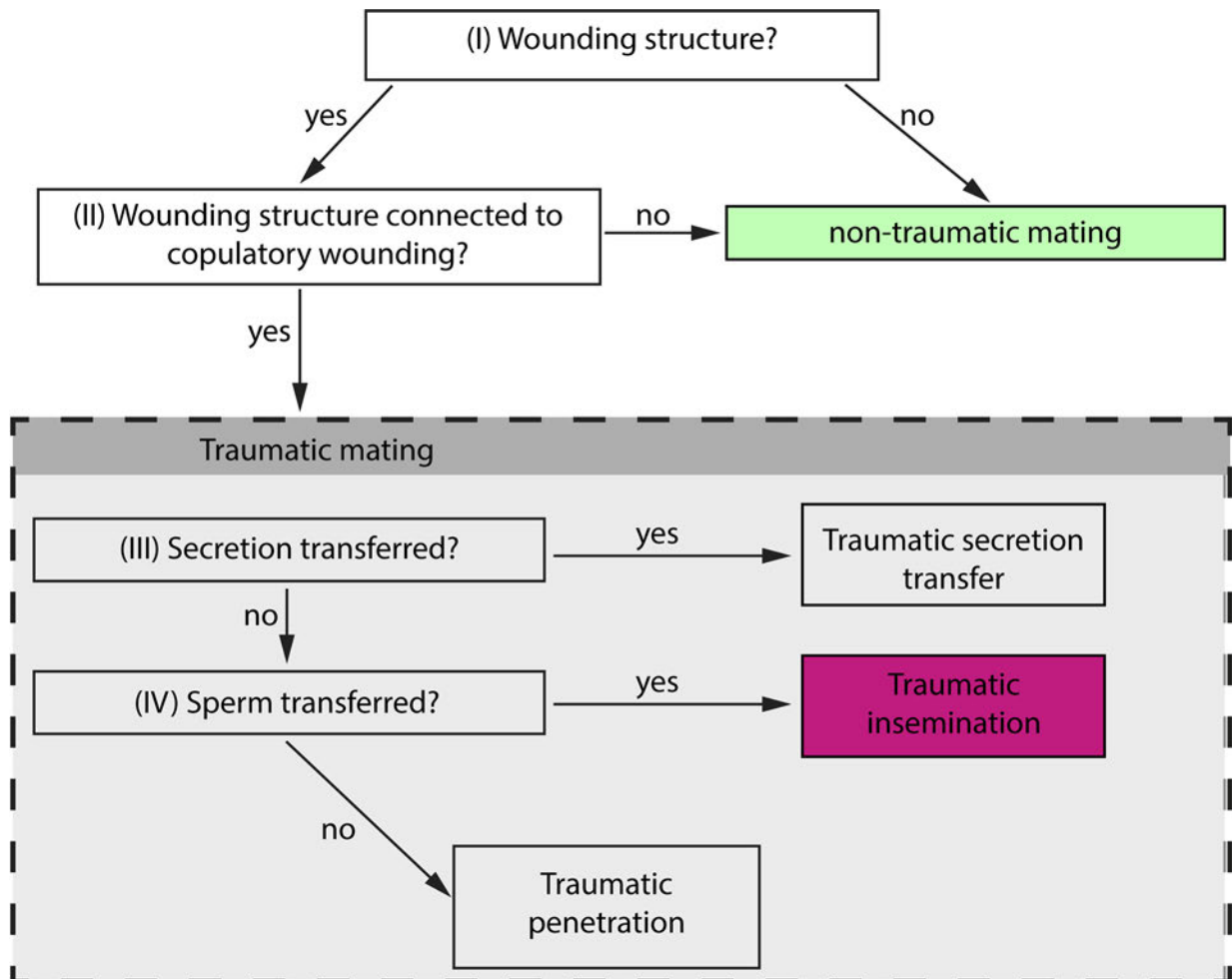
1.3.1.2 Polyandry

Figure 3: Schematic overview of traumatic insemination; modified from Lange et al. (2013).

Polyandry occurs when females mate with more than one mating partner (Arnqvist & Nilsson, 2000; Parker & Birkhead, 2013). This behaviour is known to have a strong influence on sexual conflict by setting up sperm competition in species. (Parker, 1970; Parker, 1990; Simmons, 2001; Wedell, Wiklund & Cook, 2002; Parker, 2006; Parker & Birkhead, 2013; Pizzari & Wedell, 2013; Parker, 2020; Simmons, Parker & Hosken, 2020). By mating with two or more males, sperm from different mates may overlap in time and location within a female and therefore compete for fertilisation. Such scenarios place high selective pressure on males to outcompete others and gain an advantage in fertilisation, triggering the evolution of beneficial mating strategies and traits. The most prominent of these appear to be in Odonata, where males have adapted their penis to the remove sperm from premating males (e.g., Waage, 1979). However, other strategies to reduce sperm competition are common in arthropods, such as mating plugs in Araneae (e.g., Uhl, Nessler & Schneider, 2010), Diptera (Polak et al., 2001), Hymenoptera (e.g., Duvoisin, Baer & Schmid-Hempel, 1999), and other groups that block the genital tract of females to precent the transfer of competing sperm. Fisher, Doff & Price (2013) described two types of polyandry. In addition to "true polyandry", they also describe "pseudopolyandry". The authors suggest that "true polyandry" refers to females mating with more than one male, after which sperm competition occurs. In contrast, "pseudopolyandry" refers to females that mate with more than one male without sperm competition.

1.3.2 State of the Art

When reviewing the literature on the reproductive biology of Strepsiptera, one inevitably comes across the term traumatic insemination. Some authors point to Strepsiptera as one of the best examples of this type of copulation (Tatarnic et al., 2014; Reinhardt, Anthes & Lange, 2015), while there is only one family for which there is actual scientific evidence. Even more surprisingly, not much is known about the sexual reproduction of Strepsiptera in general, as there are for too few studies that have actively observed the sperm transfer in Strepsiptera, including Mengenillidae.

The original hypothesis that Strepsiptera reproduce parthenogenetically was rejected with the first observations of sexual reproduction in *Xenos peckii* by Schrader (1924). It should be noted that parthenogenesis does occur in some species, such as *Stichotremma dallatorreanum* (e.g.,Kathirithamby, 2018) or *Halictoxenos spencei* (Perkins, 1918; Müller, 1944; Hofeneder, 1949; Kinzelbach, 1978), but is considered to be at least facultative. In the Mengenillidae, the question of the copulatory mechanism seems to be have been clarified already in the first half of the 20th century, since different authors have observed

without contradiction the penetration of the female body with the help of the penis (Parker & Smith, 1933; Silvestri, 1941b; Silvestri, 1942; Silvestri, 1943). Also, for Stylopidia, a small number of studies deal with the mode of copulation (e.g., Kirkpatrick, 1937; Grabert, 1953; Lauterbach, 1954; Baumert, 1958; Beani et al., 2005; Hrabar et al., 2014; Peinert et al., 2016). Although Lauterbach (1954) was not able to clarify the copulation mode of *S. ovinae* by direct evidence, he made an observation of inestimable importance. A few days after copulation, females showed a dark pigmented spot, which he interpreted as a melanised "mating sign" resulting from traumatic penetration. Another milestone was reached by Peinert et al. (2016), who showed that in *S. ovinae* the penis actually penetrates the integument to reach the female's hemocoel, thus providing the first real evidence for traumatic penetration in Strepsiptera. However, in contrast, Beani et al. (2005) already doubted that traumatic mating is the exclusive mode of copulation in *X. vesparum*. Using scanning electron microscopy and transmission electron microscopy they observed sperm in the haemocoel, the brood canal, and the genital organs, suggesting that the sperm may migrate through the brood canal and reach the haemocoel by passing through the birth organs. They therefore presented arguments against traumatic insemination, at least in Xenidae.

Strepsiptera are generally considered to be monandric (e.g., Kathirithamby et al. (2015)). This idea is based on several behavioural observations (Grabert, 1953; Linsley & MacSwain, 1957; Dallai et al., 2004; Hughes, Kathirithamby & Beani, 2004). Grabert (1953) observed males of *Stylops* copulating with different females — the species was probably *S. ovinae*, as the author collected them on the bee *Andrena vaga* in Germany. She describes that after a certain number of copulations with different females, a male lost interest in the female and eventually died. (Linsley & MacSwain, 1957) reported that if three males of *Stylops pacifica* found a conspecific female at the same time, two of them would fly away and only one would copulate with the female. *Xenos vesparum* males are not attracted to mated conspecific females and therefore make no effort to copulate with them (Dallai et al., 2004). A possible explanation for the latter behaviour was provided by (Tolasch, Kehl & Dötterl, 2012), who showed that females of *Stylops ovinae* (= *S. melittae* auct.) reduce, but do not completely stop, the production of a sex pheromone that attracts conspecific males. In *Xenos peckii*, mated females retreat into the body of their host after copulation, reducing the likelihood of further copulations (Hrabar et al., 2014). While these observations suggest monandrous reproduction in Strepsiptera, there are also anecdotal reports of polyandry in Strepsiptera that have never been validated (e.g., Kirkpatrick, 1937 on *Corioxenos antestiae* (Corioxenidae)).

The length of the receptivity period may allow for polyandry, simply because more males take advantage of the opportunity to copulate. Kirkpatrick (1937) reported serial mating of *Corioxenos antestiae* (Corioxenidae) females by several males. In one case described, the reported time between copulations by different males was remarkably long, suggesting a prolonged attractiveness of the female: "One male was observed to penetrate a female that had originally been fertilised some four months before, had been producing triungulins for two months, and of which the host died of old age 'a week later" (Kirkpatrick, 1937, p. 317). This case seems very unlikely, and its veracity is doubtful, as the females degenerate during the development of the larvae. Long-lasting attractiveness of mated females was also reported by Silvestri (1941a), who discussed the attractiveness of females of *Halictophagus tettigometrae* for up to three weeks after the extrusion of the cephalothorax. (Riek, 1970) also described that virgin females of *Halictophagus* sp. attracting males for more than four weeks. Both failed to correlate long-term attractiveness with potential polyandry and fertilisation success of specific fathers.

The most recent report of possible polyandry was given by Peinert et al. (2016), who showed that multiple matings occur in *Stylops ovinae* and that mated females remain attractive to males. The authors showed that the female attractiveness does not decrease until approximately 2 h, and that at least two males can mate with a female within this limited time period. As males of this species emerge synchronously and are characterised by a very short flight duration time (Grabert, 1953; Lauterbach, 1954; Tolasch et al., 2012; Lagoutte et al., 2013), multiple matings are likely to occur but still need to be documented in more detail. Mass male emergence is not unique to *Stylops ovinae*, as (Jandausch et al., 2022) were able to attract over 100 males of *X. vesparum* in one day with unmated females, with some males simultaneously approaching an air-permeable cage containing the females. The data reported by Peinert et al. (2016) on *Stylops ovinae* were obtained by studying Strepsiptera under laboratory conditions. Experiments demonstrating that multiple mating also occurs under natural conditions, resulting in the insemination of the eggs of a given female by different males, are completely lacking. We therefore decided to design experiments and establish genetic tools to assess the occurrence of polyandry in *S. ovinae* and *X. vesparum* under natural conditions. To clarify whether polyandry occurs in Strepsiptera, I will perform microsatellite based paternity tests on two species.

1.4   Aim of this Study

Strepsiptera are a group of insects whose phylogeny, lifestyle and reproduction have long remaind a mystery, and many questions remained unanswered. However, the first time you come into contact with Strepsiptera, you realise the fascination these insects can inspire. Their extensive adaptations to their parasitic lifestyle result in many impressive features, such as the absurd appearance of the female, which only remotely resembles an insect. As is so often the case, a fundamental question quickly arises: how do these organisms reproduce? While some well-founded observations by previous researchers provide clues and initial suggestions, many assumptions about copulation and reproduction are based on speculation or are conclusions drawn from data with few references. An example of this is the derivation of monandry over the period of pheromone release. It quickly becomes clear that with the wide range of modern methods available, there is an exciting field of entomology waiting to be explored. However, it is not only important to increase the knowledge in the field of Strepsiptera, but the very unusual aspects of reproduction in these animals and can also enrich research fields such as the mechanism of sperm competition, the evolution of traumatic insemination or mating behaviour. To achieve such enrichment, I used in collaboration with others a broad spectrum of methods including traditional histology, microphotography, scanning electron microscopy histology, microsatellite analysis, micro-computed tomography, 3D modelling, confocal laser scanning microscopy, and micro-indentation experiments. Using a combination of these techniques, this study aims to resolve fundamental puzzles about the reproductive system of Strepsiptera and to provide details in case studies that can be placed in a broader evolutionary context.

1.4.1   Study I

This study focuses on the distribution of traumatic insemination as a mode of copulation within the Strepsiptera. Understanding how sperm are transferred and which structures are involved is the essential for subsequent studies. The demonstration of traumatic insemination as a basic mode of copulation allows the detailed study and interpretation of mating behaviour and female adaptations to this bizarre mode of reproduction.

1.4.2 Study II

The second study is the first to describe the paragenital organ within the family Stylopidae. In addition to the distribution and the first descriptions in four genera other than *Stylops*, a characterisation of this exclusive structure is given. For the first time, the structure otherwise known as an invagination, is provided with an appropriate nomenclature for substructures. In addition, structural studies have allowed me to interpret the functionality of the organ.

1.4.3 Study III

Study III uses force measurements and confocal laser scanning to examine in detail the structural composition of the integument at the traumatically penetrated sites of two Strepsiptera species. Data from *Stylops ovinae* and *Xenos vesparum* will allow conclusions to be drawn about evolutionary adaptations of females to traumatic insemination and, in combination with shape analyses of male penises of the same species, will allow further discussion of potential functions of the paragenital organ.

1.4.4 Study IV

Study four aims to dispel the myth that the traumatically inseminating Strepsiptera are generally monandrous. Using microsatellite-based paternity testing, *Stylops ovinae* and *Xenos vesparum* will be used to show that a general assumption of monandrous behaviour is not tenable. Thus, the knowledge gained in the families Stylopidae and Xenidae contributes to our understanding of the reproductive behaviour of twisted-winged parasites and has implications for our understanding of polyandry in Strepsiptera and for the growing field of sperm competition.

2   Publications

Study I: Jandausch, K., van de Kamp, T., Beutel, R. G., Niehuis, O., & Pohl, H. (2023). "Stab, chase me, mate with me, seduce me." How widespread is traumatic insemination in Strepsiptera? Biological Journal of the Linnean Society, blad046.

Significance in the present thesis: First solid evidence of traumatic insemination in Strepsiptera, including penetration site, sperm transfer and phylogenetic implications. With the proof of the copulation mode of the investigated order, this research lays the cornerstone to all other studies.

Study II: Jandausch, K., Straka, J., van de Kamp, T., Stark, H., Beutel, R.G., Niehuis, O., Pohl, H. The paragenital organ of Stylopidae (Strepsiptera) and the functional incorporation of the secondary larval exuviae. In preparation

Significance in the present thesis: First detailed description of the paragenital organ and its distribution in the family Stylopidae including thickness measurements of the integument. The significance of the paragenital organ is discussed and a comparison with representatives of Strepsiptera without such a paragenital organ in terms of mating behavior is made.

Study III: Jandausch, K., Michels, J., Kovalev, A., Gorb, S., van de Kamp, T., Beutel, R. G., Niehuis, O., & Pohl, H. (2022). Have female twisted-wing parasites (Insecta: Strepsiptera) evolved tolerance traits as response to traumatic penetration? PeerJ, 10, e13655.

Significance in the present thesis: This study contains penetration force measurements on two species of Strepsiptera showing that female Strepsiptera tolerate the cost of traumatic insemination through a thickened integument. The significance of this morphological feature is illustrated with the help of penis shape comparisons and attraction experiments.

Study IV: Jandausch, K., Wanjura, N., Escalona, H., Sann, M., Beutel, R.G., Pohl, H., Niehuis, O. Polyandry and sperm competition in two traumatically inseminating species of Strepsiptera (Insecta). Submitted to *Scientific Reports*

Significance in the present thesis: First study showing polyandry in two species of Strepsiptera. The phenomenon was observed in laboratory experiments as well as in animals from wild populations. Our data provide further evidence for mate guarding in Stylops ovinae in implications on sperm competition in both investigated species.

FORM 1

2.1   Manuscript No. I

Manuscript title: 'Stab, chase me, mate with me, seduce me': how widespread is traumatic insemination in Strepsiptera?

Authors: Jandausch, K. , van de Kamp, T., Beutel, R.G., Niehuis, O., Pohl, H.

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Status: published

Authors' contributions (in %) to the given categories of the publication

Autor/-in	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Jandausch, K.	10 %	60%	40 %	50 %	10 %
van de Kamp, T.			20 %		
Beutel, R.G.	10 %			10 %	
Niehuis, O.	40 %	40 %	40 %	20 %	
Pohl, H.	40 %			20 %	80 %
Others					10 %
Summe:	100 %	100 %	100 %	100 %	100 %

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Signature candidate   Signature supervisor (member of the Faculty)

'Stab, chase me, mate with me, seduce me': how widespread is traumatic insemination in Strepsiptera?

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Traumatic insemination refers to mating in which males pierce the female's integument with his penis for insemination. Strepsiptera are often listed as an example for this mode of copulation. However, while traumatic insemination in Mengenillidae with free-living females is undisputed, its occurrence in Stylopidia with permanent endoparasitic females – 97% of the known species of Strepsiptera – has remained unclear. Rather, observations from a single study on *Xenos vesparum* (Xenidae) that questioned traumatic insemination in this species became generalized for Stylopidia. Here we show that integration of data from various imaging methods provides convincing evidence for traumatic insemination being phylogenetically widespread in Strepsiptera. Specifically, we provide the first evidence of injury wounds from traumatic insemination in species of Mengenillidae, Corioxenidae, Elenchidae, Halictophagidae and Xenidae. Using three-dimensional models of copulating pairs of *Stylops ovinae* (Stylopidae) and *X. vesparum*, we visualize the physical piercing of the female's integument by the male's penis. Finally, we show in species of Mengenillidae, Xenidae and Stylopidae that traumatic mating is associated with the injection of sperm in the female's haemocoel. Our results significantly alter the understanding of the reproductive biology of Strepsiptera and imply that traumatic insemination has been the ancestral mode of copulation and retained in most, if not all, extant families.

ADDITIONAL KEYWORDS: evolution – mating – SEM histology – Strepsiptera – traumatic insemination.

INTRODUCTION

Sexual reproduction is undisputedly one of the strongest forces driving the evolution of species (Williams, 1975; Crow, 1994). In groups with direct sperm transfer, such as pterygote insects, the sexual interaction in respect of mating behaviour, copulation mode and sperm transfer can vary strongly (Shuker & Simmons, 2014). An essential process for achieving egg fertilization is the efficient and successful transfer of sperm. Sperm transfer in hexapods was ancestrally indirect, and this strategy has been retained in all primary wingless lineages (e.g. Collembola; Schaller, 1971; Proctor, 1998). Winged insects evolved direct sperm transfer that relies on an intromittent organ, subsequently referred to as the penis (Hennig, 1969; Beutel *et al.*, 2017). While inserting a penis into a corresponding structure of the female genital tract is by far the most widely distributed sperm transfer strategy, some alternative strategies have evolved in pterygote insects, for instance attaching a spermatophore onto the external abdominal surface in a zorapteran species (Dallai *et al.*, 2013), a female penis in a species of Psocodea (Yoshizawa *et al.*, 2014), or just penetrating the body surface of the female and thus injuring it.

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Lange *et al.* (2013) defined copulation modes involving the injury of one sexual partner as traumatic mating. It represents an aberrant form of sexual reproduction, rare but relatively widespread in insects (Reinhardt *et al.*, 2015). Traumatic mating has been reported, for instance, in earwigs (Dermaptera), in some beetle species (Coleoptera) and in vinegar flies (Diptera: Drosophilidae) (Kamimura, 2007; Kamimura *et al.*, 2016; Dougherty & Simmons, 2017). The best-known examples for this mode of reproduction are bed bugs (Heteroptera: Cimicoidea) and twisted-winged parasites (Strepsiptera). However, only in the former group has traumatic mating been studied in detail. The citation in the title of the present paper is from the great comical series 'Seduce Me' episode 'Bed Bug' of Isabella Rossellini (Rossellini, 2010), in which she illustrates this peculiar mating strategy. Lange *et al.* (2013) distinguished three forms of traumatic mating, of which only one includes the direct transfer of sperm through the wound inflicted by the male's intromittent organ. This form of traumatic mating is referred to as traumatic insemination.

The endoparasitic insect order Strepsiptera comprises ~600 described species. It is characterized by numerous derived features in all life stages and in both sexes (Pohl & Beutel, 2005, 2008). All species of the order display extreme sexual dimorphism. The males are free-living; the only function of their extremely short life span of a few hours is to find females and to copulate with them. They have large antler-shaped, flabellate antennae with numerous dome-shaped chemoreceptors with which they sense the sex pheromones of the females (Cvačka *et al.*, 2012; Tolasch *et al.*, 2012). The pheromones are produced in glands called Nassonow's glands in Stylopidia females (Dallai *et al.*, 2004). Female Strepsiptera are usually obligatory endoparasites of other insects, in which they stay during most of their larval development and as adults. Only females of Mengenillidae are an exception, as they are free-living in the adult stage. However, some of the females remain inside their puparium. Female Strepsiptera are wingless and structurally strongly simplified compared to the males. Their genital apparatus is extremely reduced: ovaries, vagina, receptacula seminis, genital chamber, bursa copulatrix and accessory glands are missing; the eggs float freely in the female's haemolymph (Fig. 1). A single birth organ, which is probably derived from primary female genital ducts (Tröger *et al.*, 2019), is present in females of Mengenillidae, with the birth organ's opening situated in the region of sternite VII, where the minute (~200–250 µm) primary larvae are released. Members of Stylopidia utilize only pterygote insects as hosts, and the females of this group are obligatory endoparasites. Females of Stylopidia are characterized by secondary tagmosis: head, thorax and the anterior part of abdominal segment I form a compact cephalothorax, while antennae, compound eyes and legs are missing. The large sack-shaped posterior portion of the body remains within the host's abdomen, whereas the cephalothorax is exposed (Pohl & Beutel, 2005, 2008).

In Stylopiformia (Stylopidia excl. Corioxenidae), a secondary copulation opening, the birth opening, is located on the morphological ventral side of the exposed cephalothorax, between the head and prosternum. This is the external opening of the brood canal, which is connected with several birth organs in the abdomen. The birth opening enables the primary larvae to leave the female. Female Corioxenidae do not possess a birth opening. However, they possess very weakly sclerotized regions on their cephalothorax where penetration takes place. These regions are around the mouth and the membranous pleural areas of the cephalothorax (Kirkpatrick, 1937; Pohl & Beutel, 2005, 2008). The primary larvae leave the female at these regions.

The reproductive biology of Strepsiptera is only documented fragmentarily and with conflicting results and interpretations. It was initially proposed that Strepsiptera reproduce via parthenogenesis, as female genital organs are absent, and males appeared to be exceptionally rare. However, Schrader (1924) demonstrated sexual reproduction in Strepsiptera in the case of *Xenos peckii* (Xenidae). The mating behaviour of Mengenillidae, the only family with free-living females, is only documented in studies from the first half of the 20th century. Based on a single observation of the mating of *Eoxenos laboulbenei*, Parker & Smith (1934) assumed that the intromission of the penis takes place ventrally in the region of the postabdominal female birth opening. However, the authors were unable to observe the precise location of the penis intromission. Silvestri (1941a, 1943b) suggested that copulation in species of the mengenillid genera *Eoxenos* and *Mengenilla* does not involve the birth organ, but that males in these species penetrate the female's body wall with their penis at a random site on the thorax or abdomen, either dorsally or ventrally. Silvestri's statements were accepted by later authors (e.g. Kinzelbach, 1971; Kathirithamby *et al.*, 2015), even though they were preliminary notes without a precise documentation. Note in this context that Cook (2014) suggested in a recent review that copulation in Mengenillidae involves the birth opening on the ventral side of the female abdomen, citing Silvestri (1941a, 1943b) who actually postulated the opposite. There are few detailed studies on the copulation of Stylopidia (i.e. all Strepsiptera with endoparasitic females: Lauterbach, 1954; Beani *et al.*, 2005; Hrabar *et al.*, 2014; Peinert *et al.*, 2016). Lauterbach (1954) examined histological sections of freshly mated females of *Stylops ovinae*
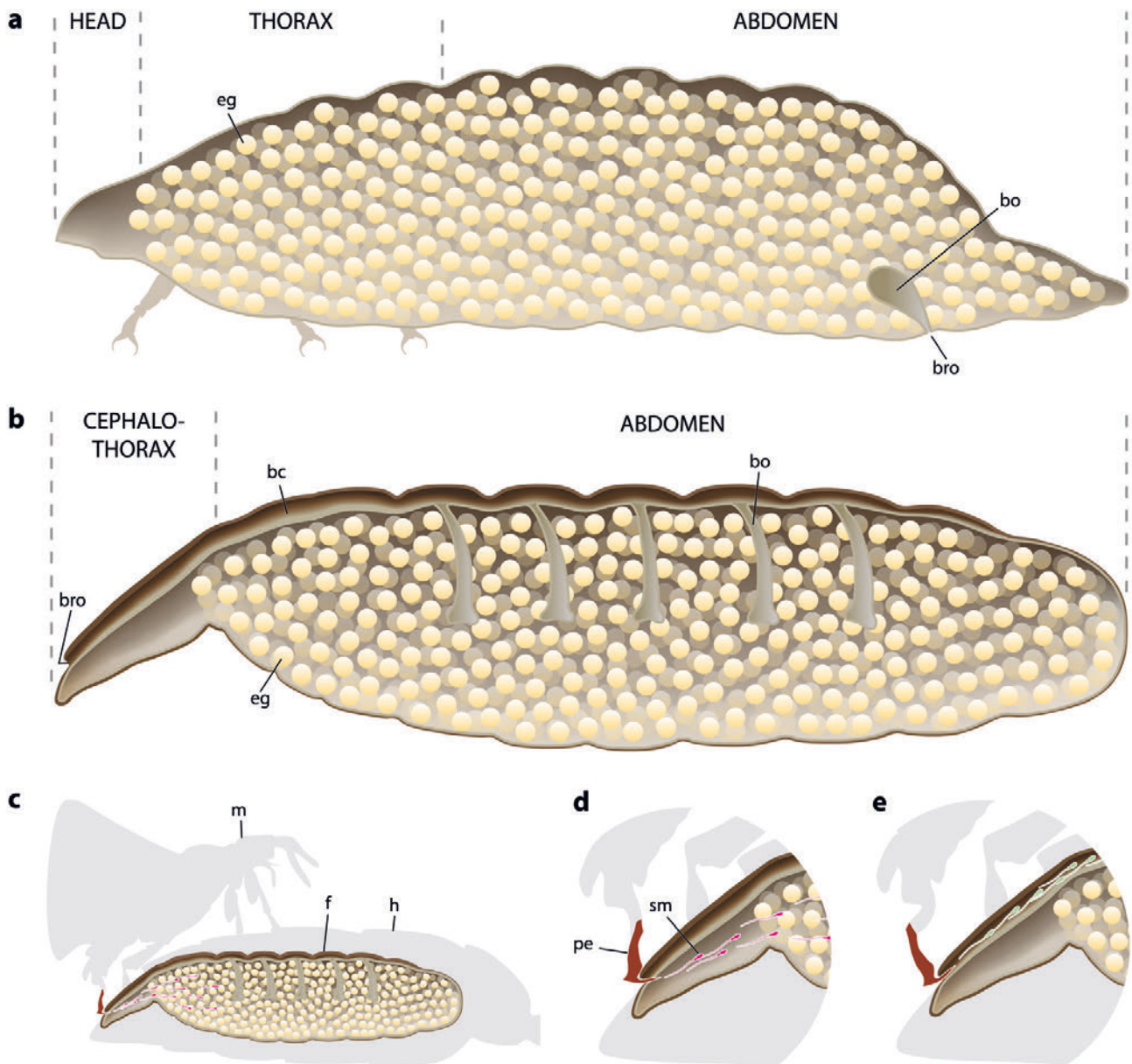
Figure 1. Schematized mediosagittal sections through females of Mengenillidae (A) and Stylopidia (B) with hypotheses on the copulation mode of Stylopidia (C–E). Only reproductive organs depicted; in B, the ventral side (physiological dorsal side) is facing upwards. C, D, traumatic insemination. E, copulation through the brood canal without injury of females. D and E are opposing hypotheses. Abbreviations: bc, brood canal; bo, birth organ; bro, birth opening; eg, egg cell; f, female; h, host; m, male; pe, penis; sm, sperm.

(Stylopidae) and found sperm neither in the brood canal nor in the birth organs. However, one or few days after the females copulated, he identified strongly pigmented areas in the wall of the females' brood canals, which he interpreted as mating scars and consequently as evidence for traumatic insemination. Peinert *et al.* (2016) observed traumatic insemination by using micro-computed tomography (μCT) imaging of *S. ovinae* in copula. They observed that the penis is inserted in an invagination of the female cephalothorax and perforates its cuticle (Fig. 1D). As Beani *et al.* (2005) found no mating scars in the wall of the brood canal, the authors assumed this was evidence of non-traumatic insemination via the birth opening (Fig. 1E). They also suggested that sperm can enter the female body cavity via the brood canal and the birth organs, in addition to traumatic insemination.

It was hitherto unclear whether traumatic insemination is a ground plan feature of the insect order Strepsiptera or a derived feature of Stylopidia. Kathirithamby *et al.* (2015) stated that the switch to an obligatory endoparasitic lifestyle of the females of Stylopidia resulted in a switch from traumatic to non-traumatic insemination (brood canal mating). To test this hypothesis, we applied a wide array of techniques to investigate and document structures that are relevant in the context of traumatic insemination. Penetration sites can be identified by melanized mating signs in the cuticle, comparable to those found, for instance, in *Drosophila* (Kamimura, 2007) and *Callosobruchus maculatus* (Coleoptera, Chrysomelidae, Bruchinae: Crudgington & Siva-Jothy, 2000). We used histological cross-sections and microphotography of cephalothoraces of females to identify penetration sites in members of different families of Strepsiptera. To document the mode of sexual interaction directly, we visualized copulating pairs of *S. ovinae* and *Xenos vesparum* via three-dimensional (3D) models based on μCT data. We assessed whether sperm was directly transferred into the female's haemolymph by using scanning electron microscopy histology (SEM histology). This technique allows us to locate sperm very shortly after sperm transfer without artificial effects often occurring in transmission electron microscopy preparations (e.g. dislocation of sperm). Interpreting the obtained data in a phylogenetic context, we finally address the question of which specific mode of sperm transfer evolved at which point in this highly specialized group of insects.

MATERIAL AND METHODS

A detailed description of the used Strepsiptera is provided in Supporting Information Table S1. This includes sex, location, date, collector, preservation and the applied method of all individuals used in this study.

PARTHENOGENESIS IN *EOXENOS LABOULBENEI*

To identify possible parthenogenesis in females of *Eoxenos laboulbenei* that have hatched from their puparia, we collected 14 female puparia of *Eoxenos laboulbenei* in Italy (Supplementary File). The puparia were kept at room temperature in small polystyrene containers with a plaster bottom (10 × 10 × 2 cm). The plaster was moistened to prevent desiccation. Eight females hatched and were controlled for the appearance of primary larva until death and remained in the polystyrene containers.

To detect parthenogenesis in females staying within their puparia, we collected 12 female puparia of *Eoxenos laboulbenei* in Croatia and Italy (Supplementary File). The puparia were kept as described above.

FIXATION OF COPULATING PAIRS

For gathering μCT data of copulating pairs of *X. vesparum* and *S. ovinae*, males of these species were exposed to conspecific females protruding from their host's metasoma in a small Petri dish. The metasoma of the parasitized host was removed from the rest of the host's body and attached to a lump of modelling clay with its anterior end on the bottom of the tray. Shortly after the males started to copulate, the pairs were fixed at –80 °C in pure ethanol and stored for 2 weeks in a refrigerator at the same temperature. This procedure secured the mating position by hardening the soft tissue. We used the same technique for one couple each of *Eoxenos laboulbenei*, *S. ovinae* and *X. vesparum* for SEM histology. In contrast to the procedure used to collect μCT data, ethanol was added 30 s after the start of the copulation to ensure that sperm transfer was accomplished. The copulation of *Eoxenos laboulbenei* was fixed with pure ethanol (10 °C) and was then stored in a refrigerator.

LOCATION OF PENETRATION SITE IN *EOXENOS LABOULBENEI*

To observe the location of the penetration site in *Eoxenos laboulbenei*, we initiated three copulae of male and female specimens collected in Makarska, Croatia. Freshly hatched males were exposed to females in a small Petri dish and were observed with a 10× magnification glass to document the penetration sites of the penis at the female's integument. After mating, the specimens were fixed with pure ethanol. To identify mating signs, a freshly hatched male was placed in a small Petri dish with a female as described above. One day after mating, the female was photographed to document mating signs (see below).

MICROPHOTOGRAPHY

Photographs for documenting mating signs and copulations were taken with a Canon EOS 6D or a Canon EOS 7D Mark II digital SLR camera equipped with either a Canon MP-E 65-mm macro lens (Canon, Krefeld, Germany), or a Nikon M Plan 20 ELWD microscopic lens (Nikon, Tokio, Japan), or a Mitutoyo M Plan Apo 10 microscopic lens (Mitutoyo, Kawasaki, Japan) fitted with a StackShot macrorail (Cognisys, Traverse City, MI, USA). The Canon lens was used to document the mating signs of *Eoxenos laboulbenei*. The Nikon lens was used to photograph *Elenchus tenuicornis*, *Halictophagus agalliae*, *Halictophagus silwoodensis* and *Tridactylophagus etoi*. Photographs of *Dundoxenos kinzelbachi*, *Malayaxenos trapezonoti*, *Paraxenos erberi*, *Stylops nevinsoni*, *S. ovinae* and *X. vesparum* were taken with the Mitutoyo microscopic

lens. All specimens were illuminated with two flashlights (Yongnuo Photographic Equipment, Shenzhen, China). Single stack shots with different focus were combined using Zerene Stacker (Zerene Systems LLC, Richland, WA, USA). Specimens were placed in a small Petri dish filled with hand disinfection gel (Septigel+, Laboratoires Prodene Klint, Marne-la-Vallée, France) and covered with a cover slip. *Tridactylophagus etoi* was dried at the critical point (see below) and photographed without any supporting medium. The studied specimen of *S. ovinae* was embedded in Euparal (Karl Roth GmbH, Karlsruhe Germany) before photographs were taken.

SEM HISTOLOGY

Females in copula of *Eoxenos laboulbenei*, *S. ovinae* and *X. vesparum* were embedded in methacrylate. The specimens were subsequently longitudinally sectioned (*S. ovinae*) or cross-sectioned (*Eoxenos laboulbenei* and *X. vesparum*) with an RM2265 rotation microtome (Leica, Wetzlar, Germany) equipped with a diamond knife. Sections were stained with toluidine blue and pyronin G and analysed under a microscope to determine the presence of sperm. This was necessary to ensure that the specimens were cut at suitable regions for the following steps. Xylol was used to dissolve the methacrylate and was then gradually replaced by acetone. The specimens were dried at the critical point with an Emitech K850 Critical Point Dryer using liquid CO_2 (Sample preparation division, Quorum Technologies Ltd, Ashford, UK), glued to a minute insect pin with cyano-acrylate glue (Ergo 5925 Elastomer, Kisling AG, Wetzikon, Switzerland), and mounted on a rotatable specimen holder (Pohl, 2010). Using an Emitech K500 (Sample preparation division, Quorum Technologies), specimens were sputter-coated with gold. Images were taken with a Philips ESEM XL30 (Philips, Amsterdam, Netherlands) and processed with the Scandium FIVE software (Olympus, Münster, Germany).

HISTOLOGICAL SECTIONS

Specimens of *T. etoi* and *Elenchus tenuicornis* were embedded in Araldite CY 212 (Agar Scientific, Stansted, UK). Serial cross-sections were made with a Microtome HM 360 (Microm, Walldorf, Germany) equipped with a diamond knife. The sections were stained with toluidine blue and pyronin G. The slides were digitized with an Olympus dotSlide microscope (BX51, software version 3.4, Olympus, Tokyo, Japan). Histological sections of *S. ovinae* were those previously used by Peinert *et al.* (2016), and sections of *X. vesparum* were produced during the SEM histology preparation (see above).

MICRO-COMPUTED TOMOGRAPHY AND 3D RECONSTRUCTION

One copulating pair of *X. vesparum* and one of *S. ovinae* were scanned in pure ethanol at the Imaging Cluster at the KIT Synchrotron Radiation Facility using a polychromatic X-ray beam produced by a 1.5-T bending magnet spectrally filtered by 0.5 mm Al. A fast indirect detector system was employed, consisting of a 13-μm LSO:Tb scintillator (Cecilia *et al.*, 2011), a diffraction-limited optical microscope (Optique Peter) (Douissard *et al.*, 2012), and a 12-bit pco.dimax high-speed camera with 2016 × 2016 pixels. Scans were done by taking 3000 projections at 70 f.p.s. over an angular range of 180°. An optical magnification of 10× resulted in an effective pixel size of 1.22 μm. The control system 'concert' (Vogelgesang *et al.*, 2016) was used for automated data acquisition and online reconstruction of tomographic slices for data quality assurance. Data processing included flat field correction and phase retrieval of the projections based on the transport-of-intensity equation (Paganin *et al.*, 2002). X-ray beam parameters for algorithms in the data processing pipeline were computed via *syris* (Faragó *et al.*, 2017). Execution of the pipelines, including tomographic reconstruction, was performed within the UFO framework (Vogelgesang *et al.*, 2012). We segmented tomographic data using Dragonfly 4.1 for Windows (Object Research Systems Inc., Montreal, Canada) and used VGStudiomax 2.0.5 (Volume Graphics, Heidelberg, Germany) for visualization and rendering.

IMAGE PROCESSING

All images were processed with Adobe Photoshop 21.2.1 (Adobe Systems Incorporated, San Jose, CA, USA) and arranged as plates with this software. Adobe Illustrator 24.2.1 (Adobe Systems) was used for labelling plates and drawings.

RESULTS

PARTHENOGENESIS AND MATING OF *EOXENOS LABOULBENEI* (MENGENILLIDAE)

Eight of a total of 14 collected females from 2021 hatched from their puparium while kept at room temperature. None of the hatched females gave birth to primary larvae before their death. All females which remained in their puparia produced primary larvae without being fertilized by a male.

In total, we observed three copulae of *Eoxenos laboulbenei* and documented the penetration site used by the male's penis. In the first copula, the male instantly began to mate with the female. Its penis was inserted dorsally between the third and fourth

abdominal segment. Copulation lasted for ~10 s. After copulation, the male was no longer interested in the female and did not attempt to mate a second time. In the second copulation experiment, the male mated with the female for a few seconds. Penetration took place dorsolaterally between abdominal segments III and IV (Fig. 2A). The female was walking inside the Petri dish during mating. Note that this copula was used for SEM histology, which is described in detail below. The male of the third mating experiment penetrated the female four times. The penis was inserted three times into the abdominal tergites and once ventrally in front of the birth opening. Each copulation lasted between 3 and 10 s. The female was walking during the mating process.

MATING SIGNS

Microphotography and histological sections revealed the presence of mating signs in species of the following Strepsiptera families: Mengenillidae, Corioxenidae, Halictophagidae, Elenchidae, Xenidae and Stylopidae.

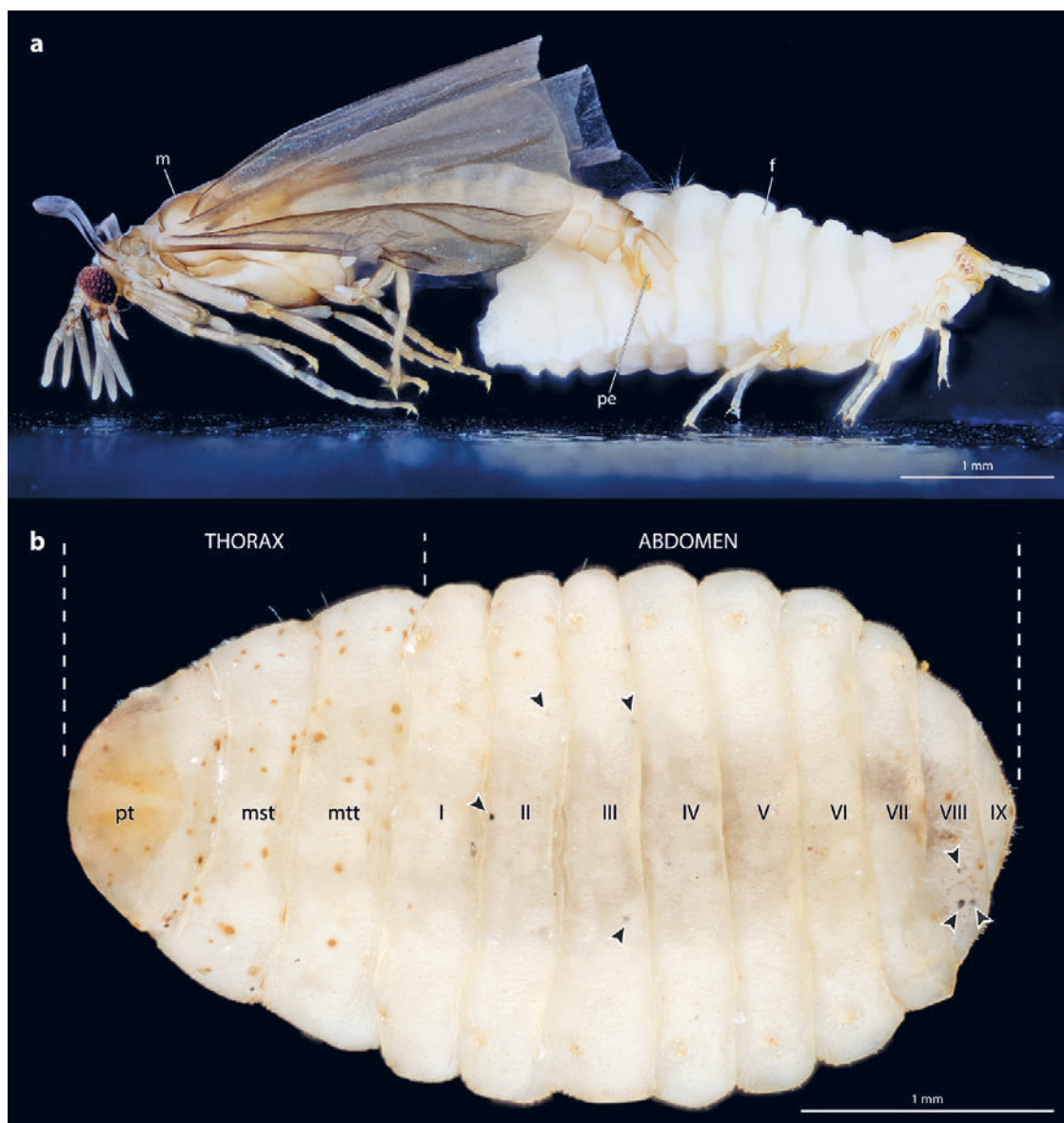**Figure 2.** Photographs of a copulating pair of *Eoxenos laboulbenei* fixed in absolute ethanol (A, lateral view) and female *E. laboubenei* with mating signs (B, dorsal view). Male turned backwards as female was not immobilized immediately by the absolute ethanol. Arrows indicate mating signs. Abbreviations: f, female; I–IX, abdominal segments I–IX; m, male; mst, mesothorax; mtt, metathorax; pe, penis; pt, prothorax.

We examined one species of Mengenillidae for the presence of mating signs resulting from wound healing (Lai-Fook, 1968). Small melanized mating signs were located on abdominal tergites II (number of mating signs: 2), III (2) and VIII (3) in the female of *Eoxenos laboulbenei* (Fig. 2B). We did not find any mating signs on the ventral side.

We observed dark pigmentation in females of two genera of Corioxenidae (Fig. 3A, B). The mating sign in the sample of *D. kinzelbachi* was located anteriorly, close to the mouth region, and was recognizable as a dark brown spot with blurry edges. A similar mating sign was visible in *M. trapezonoti*. The exact penetration site in *M. trapezonoti* remained undetermined, as species of this family can retract into their larval exuvia after copulation (Pohl & Beutel, 2005). As a consequence of the retraction, the female is detached from the cuticle of the puparium (2nd instar larva), and the mating sign therefore no longer corresponds to its original location beneath the larval exuvia.

We investigated three species representing two genera of Halictophagidae with respect to mating signs at the female cuticle. No external evidence was visible in *T. etoi* (Fig. 3C). The bean-shaped birth opening of *T. etoi* was located in the centre of the cephalothorax and was ~0.1 mm wide and 50 μm long. A second specimen of *T. etoi*, from which the cuticle of the 2nd instar was removed, revealed a melanized mating sign that was medially close to the posterior border of the birth opening (Fig. 3A). A second mating sign was located more posteriorly and slightly more to the right, not clearly separated from the anterior mating sign. Additionally, via histological cross-sections we identified mating signs as dark pigmented spots in the cuticle of *T. etoi* (Supporting Information, Fig. S1a). The pigmentation of the mating sign extended through the entire width of the cuticle and was located in the brood canal. In specimens of *Halictophagus silwoodensis* and *Halictophagus agalliae*, mating signs were visible through the cuticle of the 2nd instar larva (Fig. 3E, G). In *Halictophagus agalliae*, melanization was vaguely recognizable. After removing the larval cuticle of the specimen of *Halictophagus agalliae*, a mating sign was distinctly visible (Fig. 3F). As in *T. etoi*, the sign was located slightly posterior to the birth opening medially in the anterior brood canal. The mating sign in the specimen of *Halictophagus silwoodensis* was located on the left side of the cephalothorax and closer to the posterior end of the cephalothorax. In contrast to the narrow and linear birth opening of *Halictophagus agalliae*, the birth opening of *Halictophagus silwoodensis* was arched, with its tip pointing anterad, while the opening of *T. etoi* was broadly open and curved.

Elenchus tenuicornis (Elenchidae) is characterized by a wide orifice of the birth opening (Fig. 3H). Compared to *T. etoi*, the kidney-shaped birth opening of *Elenchus tenuicornis* occupies up to one-third of the whole cephalothorax, with a size of ~120 μm × 70 μm. It is located between the spiracles at the posterior end of the cephalothorax. Due to the wide birth opening, mating signs can be easily documented without removing the cuticle of the 2nd instar larvae, close to the right posterior border of the birth opening. A postcopulatory scar was also visible in sagittal sections of *Elenchus tenuicornis*, visible as a dark pigmented spot that extended through the cuticle at the same position as demonstrated with microphotography (Fig. 1B).

We found postcopulatory scars in females of two genera of Xenidae. Two very small and fine mating signs were found on a specimen of *Paraxenos erberi* (Fig. 4A). Both were located at the anteriormost part of the brood canal. The melanized spots were located at the lateral areas of the brood canal, one on the right side and one on the left side. A similar situation was observed in specimens of *X. vesparum*, where mating signs always appeared on the lateral parts of the anterior brood canal and never in the centre (Fig. 4B, C). This was also confirmed by cross-sections, as mating signs were visible along the lateral areas of the brood canal (Supporting Information, Fig. S2a). Specimens of *X. vesparum* also displayed several mating signs visible through the cuticle of the 2nd instar larva (Fig. 4B, C). In both species, we observed that the birth opening was located at the anterior third of the cephalothorax, being fissure-shaped and narrow.

We examined mating signs resulting from copulatory wounding in two species of Stylopidae. Peinert *et al.* (2016) described that copulation in *S. ovinae* involves a specialized paragenital organ. This organ is also present in other species of the genus (Jandausch *et al.*, 2022). In *S. nevinsoni* and *S. ovinae*, we found mating signs at the ventral integument of the paragenital organ (Fig. 4D–F). Even though the sign was located inside the female's body, it was clearly recognizable after removing the cuticle of the 2nd instar larva (the specimen of *S. ovinae* was embedded in Euparal for visualization of the mating sign). Cross-sections also showed that the mating sign of *S. ovinae* was visible from the inside, indicating that penetration had occurred at the ventral wall of the paragenital organ (Supporting Information, Fig. S2b; Peinert *et al.*, 2016). The birth opening of both investigated *Stylops* species was slightly curved posterad and almost extended over the full width of the anterior cephalothorax.

3D RECONSTRUCTIONS

μCT scans of pairs fixed in copula allowed us to confirm that penetration occurred traumatically in *S. ovinae* and *X. vesparum*. Based on these data and the observed mating signs, we can exclude the possibility of penis
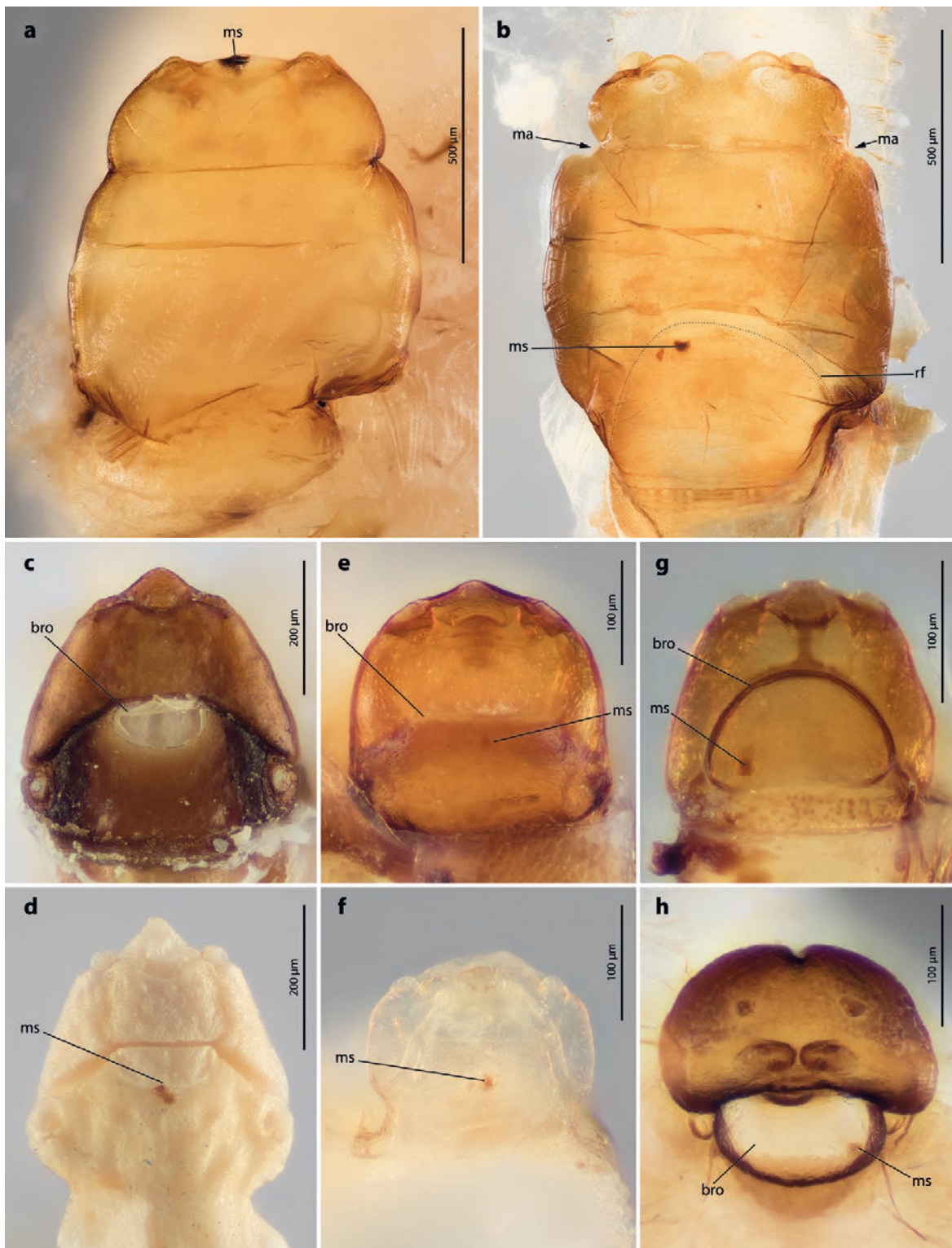
Figure 3. Photomicrographs of the cephalothoraces of different Strepsiptera females displaying mating signs as an indication of traumatic insemination. A, *Dundoxenos kinzelbachi*. B, *Malayaxenos trapezonoti*. C, *Tridactylophagus etoi*. D, *Tridactylophagus etoi* (larval exuvia removed). E, *Halictophagus agalliae*. F, *Halictophagus agalliae* (larval exuvia removed). G, *Halictophagus silwoodensis*. H, *Elenchus tenuicornis*. Abbreviations: bro, birth opening; ma, membranous area; ms, mating sign; rf, retracted female.
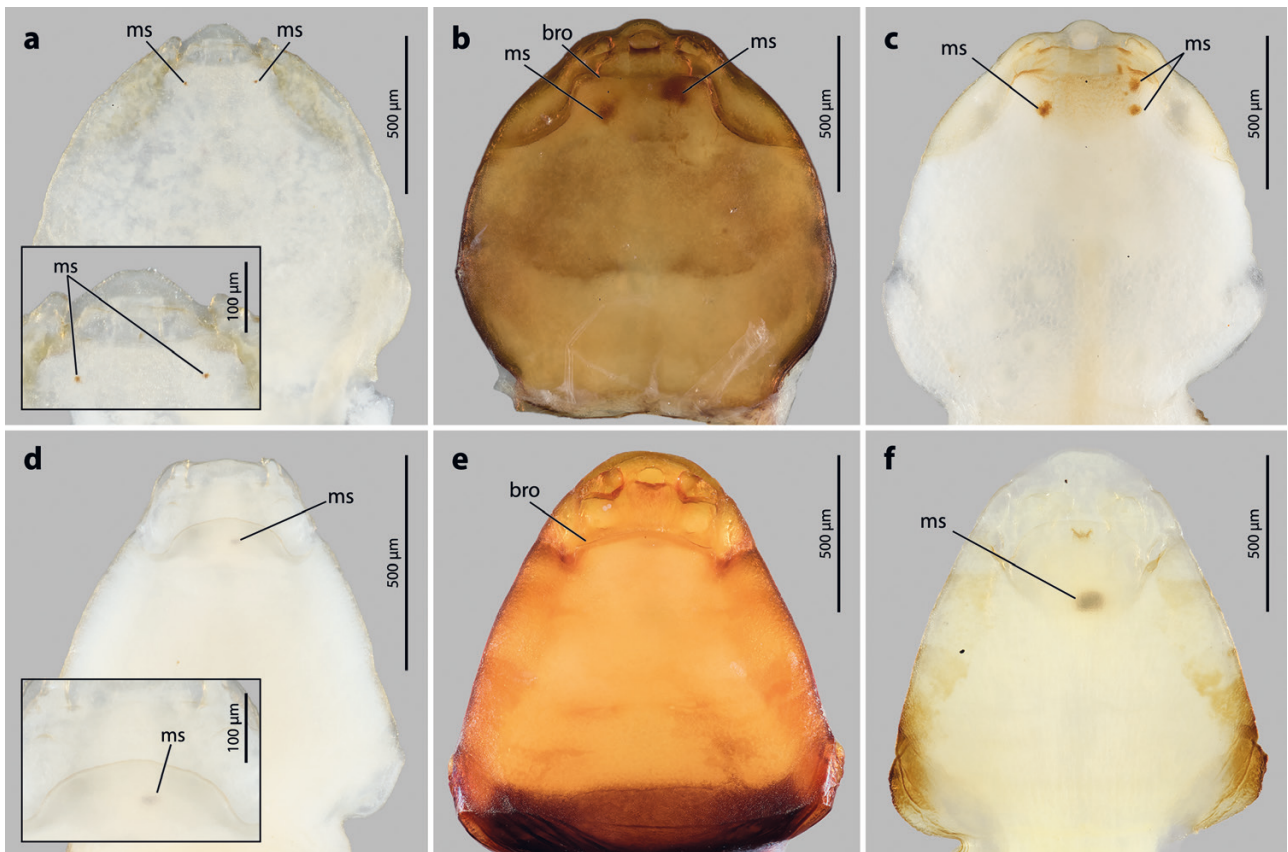
Figure 4. Photographs of the cephalothoraces of different Strepsiptera females displaying mating signs as indication of traumatic insemination. A, *Paraxenos erberi*. B, *Xenos vesparum*. C, *Xenos vesparum* (larval exuvia removed). D, *Stylops nevinsoni*. E, *Stylops ovinae*. F, *Stylops ovinae* (larval exuvia removed). Abbreviations: bro, birth opening; ms, mating sign.

insertion in the brood canal without penetration of the female's integument.

Stylops ovinae

(Fig. 5A, B). The penis of male *S. ovinae* was introduced into the female's paragenital organ by piercing through a thin membranous closure of the 2nd instar larva in front of the birth opening. Penetration took place on the morphological ventral surface and at the inner end of the paragenital organ. In this position, a spike at the base of the intromittent organ touched the outer cuticle of the female extruding from the host's metasoma. The acumen was tilted slightly upwards, which may facilitate penetration of the ventral wall of the paragenital organ.

Xenos vesparum

(Fig. 5C, D). In contrast to *S. ovinae*, the penis of male *X. vesparum* was inserted into the female birth opening through the cuticle of the 2nd instar larva. Penetration occurred at the lateral areas of the brood canal, which corresponds to the constant lateral position of mating signs. Because this species lacks

a paragenital organ, the acumen directly penetrated the anterior region of the brood canal. In contrast to *S. ovinae*, the intromittent organ was slightly tilted downwards to allow the acumen to reach the female's integument. A spike-like structure, as found in *S. ovinae*, is not present on the penis of *X. vesparum*.

SEM HISTOLOGY

Eoxenos laboulbenei

(Fig. 6A–C). Using histological cross-sections combined with SEM visualization, we found unambiguous evidence that sperm was injected into the female's body cavity. The spermatozoa were located dorsally in abdominal segments IV and V, corresponding to the penetration site of the specimen analysed. We found large sperm patches concentrated in the abdomen, extending between other structures such as muscles, eggs and trachea.

Xenos vesparum

(Fig. 6D–F). We found sperm in the haemocoel of the cephalothorax of the *X. vesparum* female. A large
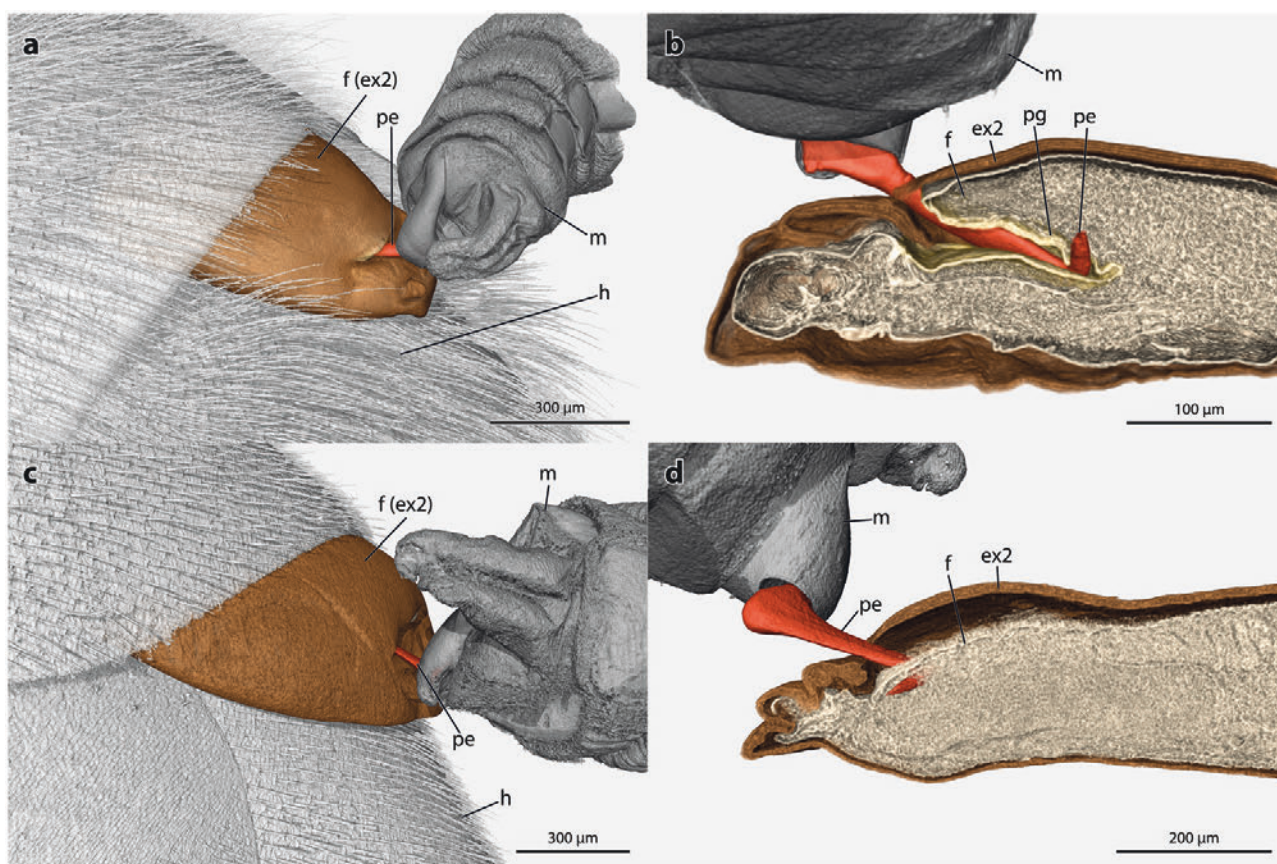
Figure 5. 3D reconstructions of copulating *Stylops ovinae* (A, B) and copulating *Xenos vesparum* (C, D). A, female *S. ovinae* protruding dorsally from the host's metasoma in copula with a male; B, sagittal view of a female *S. ovinae* (ochre) with the male's penis (red) penetrating the ventral wall of the paragenital organ; C, female *X. vesparum* protruding dorsally from the host's metasoma in copula with a male; D, sagittal view of a female *X. vesparum* (ochre) with the male's penis (red) penetrating the anterior part of the brood canal. Abbreviations: ex2, exuvia of the secondary larval stage; f, female; h, host; m, male; pe, penis; pg, paragenital organ.

portion of it was concentrated laterad the pharynx in the anterior region of the cephalothorax (Fig. 6D, E). The location corresponded to that of the observed mating signs and of the penetration sites in the 3D model presented in this study (Fig. 5C, D). We did not find sperm in the brood canal, neither in histological cross-sections (Supporting Information, Fig. S2a) nor in the SEM images shown in Figure 6D–F.

Stylops ovinae

(Fig. 6G–I). As in *X. vesparum*, sperm was exclusively found in the haemocoel of the cephalothorax and was completely absent in the brood canal (Fig. 6G). Sperm was located slightly posterior to the paragenital organ, where insemination typically takes place. Figure 6H shows parts of the male's penis located close to the thickened cuticle of the paragenital organ.

DISCUSSION

Our study revealed traumatic insemination in all species of Strepsiptera that we examined. Mating signs documented in females of Mengenillidae, Corioxenidae, Halictophagidae, Elenchidae, Xenidae and Stylopidae indicate that the penis penetrates the female's integument in these groups (Table 1). We also demonstrated, using µCT data and 3D modelling of copulating pairs, that the cuticle of females of *S. ovinae* and *X. vesparum* is penetrated by the males' penis. The same approach allowed us to correlate the location of mating signs with the penetration sites of the intromittent organ. In *Eoxenos laboulbenei* – a species with free-living females (Mengenillidae) – we confirmed earlier observations by Silvestri (1941a, 1943b), and by Parker & Smith (1934), namely that the males of this species do not insert their penis into the female's single birth opening, but that they transfer their sperm
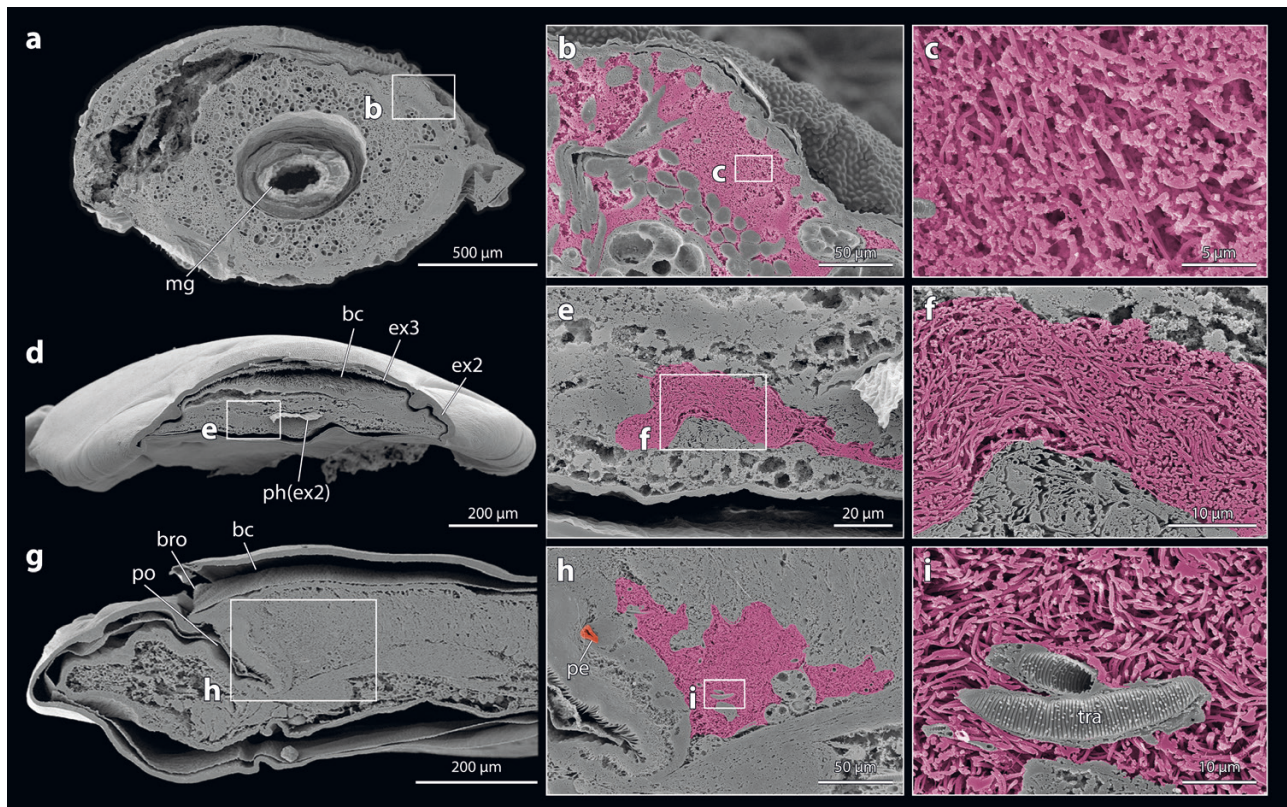
Figure 6. SEM histology of traumatically inseminated females of *Eoxenos laboulbenei* (A–C, caudal view), *Xenos vesparum* (D–F, frontal view) and *Stylops ovinae* (H, I, mediosagittal view). A, female *Eoxenos laboulbenei* sectioned at abdominal segment IV. B, detail showing the location of sperm (pink) in female *Eoxenos laboulbenei*. C, sperm at higher magnification in female *Eoxenos laboulbenei*. D, female *X. vesparum* sectioned at anterior region of cephalothorax (compare Fig. 5C, D). E, detail showing the area where sperm (pink) was found in female *X. vesparum*. F, sperm at higher magnification in female *X. vesparum*. G, cephalothorax of female *S. ovinae* sectioned along midline. H, detail showing the tip of the penis (red) and sperm (pink) in female *S. ovinae*. I, sperm at higher magnification in female *S. ovinae*. Abbreviations: bc, brood canal; bro, birth opening; ex2, exuvia of the secondary larval stage; ex3, exuvia of the tertiary larval stage; mg, midgut; pe, penis; ph(ex2) – pharynx; po, paragenital organ; tra, trachea.

directly into the female's body cavity during traumatic penetration. In two species with endoparasitic females (Stylopidia, i.e. *S. ovinae* and *X. vesparum*), we showed that sperm is transferred directly into the body cavity during the penetration process. This renders an alternative sperm route to the haemocoel of the female, the brood canal and birth organs as discussed by Beani *et al.* (2005), very unlikely. The widespread documented distribution of traumatic insemination in Strepsiptera suggests strongly that this specific trait belongs to the groundplan of the order. It also shows that this character has been retained probably in all subordinated lineages, from Mengenillidae with free-living females to the genera *Stylops* and *Xenos*, which are deeply nested in Stylopidia. While we cannot rule out reversal in some genera or families not covered by our sampling, the shape of the penises of species in these groups, indicative of traumatic insemination, renders this possibility very unlikely.

TRAUMATIC INSEMINATION IS A GROUNDPLAN FEATURE OF STREPSIPTERA

The confirmed traumatic insemination in Strepsiptera with free-living and endoparasitic females refutes the hypothesis raised by Kathirithamby *et al.* (2015) that the switch to an obligate endoparasitic lifestyle of females (Stylopidia) resulted in a switch to non-traumatic insemination (brood canal mating). Our current results, namely that penises with a sharp tip are used by males for traumatic insemination of females of the families Mengenillidae, Xenidae and Stylopidae, are supported by published research on Mengenillidae (Parker & Smith, 1934; Silvestri, 1941a, 1943b) Corioxenidae (Kirkpatrick, 1937), Halictophagidae (Silvestri, 1943a), Elenchidae (Baumert, 1958) and Myrmecolacidae (Luna de Carvalho, 1972). Sharp-tipped penises, which represent typical traumatic insemination genitalia (Lange *et al.*, 2013; Tatarnic *et al.*, 2014), have been

Table 1. Overview of evidence for traumatic insemination in studied representatives of Strepsiptera families from this study and the literature

Family	Species	Penis shape	Mating	Parthenogenesis	Reference
†Protoxenidae	†*Protoxenos janzeni*	Morphotype I	—	—	This study; Figure 7
†Phthanoxenidae	*Heterobathmilla kakopois*	Morphotype I + paramers	—	—	Pohl *et al.* (2021); Figure 7
†Cretostylopidae	†*Cretostylops engeli*	Morphotype I	—	—	This study; Figure 7
†Mengeidae	†*Mengea tertiaria*	Morphotype I	—	—	Kinzelbach (1971); Figure 7
Bahiaxenidae	*Bahiaxenos relictus*	Morphotype I	—	—	Bravo *et al.* (2009); Figure 7
Mengenillidae	*Mengenilla chobauti*	Morphotype I	Traumatic insemination on variable sites on the abdomen	No, in females leaving their puparium	Silvestri (1943b)
	Mengenilla chobauti	Morphotype I		Yes, in females overwintering in their puparium	Silvestri (1943b)
	Eoxenos laboulbenei	Morphotype I	Traumatic insemination on variable sites on the abdomen	No, in females leaving their puparium	This study; Figures 6A–C, 7
	Eoxenos laboulbenei	Morphotype I		Yes, in females overwintering in their puparium	This study; Parker & Smith (1934); Silvestri (1941a)
Corioxenidae	*Dundoxenos kinzelbachi*	Morphotype II	Traumatic penetration at the mouth region	—	This study; Figure 3A
	Corioxenos antestiae	Morphotype II	Traumatic penetration on the lateral site of the cephalothorax	No	Kirkpatrick (1937); Figure 7
	Malayaxenos trapezonoti	Morphotype II	Traumatic penetration, lateral site of the cephalothorax or at the mouth region	—	This study; Pohl & Melber (1996)
Bohartillidae	*Bohartilla megalognatha*	Morphotype III	—	—	Kinzelbach (1969); Figure 7
Halictophagidae	*Tridactylophagus etoi*	Morphotype III	Traumatic penetration, at the anterior brood canal	—	This study; Figure 3C, D; Supporting Information, Figure S1a
	Halictophagus tettigometrae	Morphotype III	Traumatic insemination, at the anterior brood canal	—	Silvestri (1943a)
	Halictophagus agalliae	Morphotype III	Traumatic penetration, at the anterior brood canal	—	This study; Abdul-Nour (1970); Figure 3E, F
	Halictophagus silwoodensis	Morphotype III	Traumatic penetration, at the anterior brood canal	—	This study; Waloff (1981); Figure 3G

Table 1. Continued

Family	Species	Penis shape	Mating	Parthenogenesis	Reference
Elenchidae	*Elenchus tenuicornis*	Morphotype III	Traumatic penetration, membranous region anterior to the brood canal	—	This study; Baumert (1958); Pohl (1991); Figures 3H, S1b
†Protelencholacidae	†*Cryptelencholax*	Morphotype III	—	—	Kogan & Poinar (2019)
Myrmecolacidae	*Caenocholax vilhenai*	Morphotype III	Traumatic penetration, membranous region anterior to the brood canal	—	Luna de Carvalho (1972)
	Stichotrema dallatoreanum	Morphotype III	—	Yes	Kinzelbach (1971); Kathirithamby (2000); Figure 7
Callipharixenidae	*Callipharixenos muiri*	—	—	—	Pierce (1918)
Xenidae	*Paraxenos erberi*	Morphotype III	Traumatic penetration, at the anterior brood canal	—	This study; Figure 4A
	Xenos vesparum	Morphotype III	Traumatic insemination, at the anterior brood canal	—	This study; Figures 4B, C, 5C, D, 6D–F, 7, S2a
	Pseudoxenos hookeri	Morphotype III	—	—	Kinzelbach (1971); Figure 7
Stylopidae	*Halictoxenos spencei*	Morphotype III	—	Yes	Kinzelbach (1971); Müller (1944)
	Stylops nevinsoni	Morphotype III	Traumatic penetration, at the ventral wall of the paragenital organ	—	This study; Figure 4D
	Stylops ovinae	Morphotype III	Traumatic insemination, at the ventral wall of the paragenital organ	—	This study; Peinert *et al.* (2016); Figures 4E, F, 5A, B, 6G–I, 7, S2b

A dash indicates unknown.

reported in all those species of Strepsiptera for which males have been recorded and morphologically studied, including representatives of the stem group (e.g. Fig. 7; Table 1; Kirkpatrick, 1937; Abdul-Nour, 1970; Kinzelbach, 1971; Pohl & Melber, 1996; Bravo *et al.*, 2009; Pohl *et al.*, 2021). Thus, a change in the mode of mating from non-traumatic in the stem group of Strepsiptera to traumatic insemination in the crown group seems very unlikely. We therefore assume that in the stem group of Strepsiptera, the penis was already used for traumatic penetration of the female's integument (Table 1).

EVOLUTIONARY TRENDS IN STREPSIPTERA WITH
REGARD TO TRAUMATIC INSEMINATION

The penises of Strepsiptera can be grouped into three morphotypes, each of which seems correlated with the body morphology and lifestyle of the conspecific females.

Penises of the first morphotype (morphotype I) are slightly curved and have a sharp tip. These simply structured penises are found in the stem group, i.e. in †Protoxenidae, †Cretostylopidae, †Phthanoxenidae and †Mengeidae, and also in the extant families Bahiaxenidae and Mengenillidae (Fig. 7, orange section). Additionally, only males that mate with permanently endoparasitic females seem to possess specialized adhesive hairs on their tarsomeres, probably to improve attachment on their pterygote hosts (Pohl & Beutel, 2005, 2008; Pohl *et al.*, 2020). Since the tarsi of male stem group representatives (including Bahiaxenidae) lack adhesive hairs on their tarsi, it is reasonable to assume that the females are free-living as in Mengenillidae. A remarkable

exception is †*Heterobathmilla kakopoios*, with a penis equipped with 'parameres' (Pohl *et al.*, 2021), paired structures commonly found in Coleoptera. Since †*Heterobathmilla kakopoios* is the only species of Strepsiptera with 'parameres' known so far, an interpretation of this feature is highly speculative at this point, especially as the morphology of the conspecific females is unknown. Assuming that females of †Protoxenidae, †Cretostylopidae, †Phthanoxenidae, †Mengeidae and Bahiaxenidae are free-living, we conclude that the simple shaped penis is used for penetration at variable sites of the abdomen

of the females and is therefore associated with this form of lifestyle. Furthermore, we demonstrate that those unmated females of *Eoxenos laboulbenei* that leave their puparium do not produce primary larvae, but that this occurs in females staying within the puparium. Since males of *Eoxenos laboulbenei* are apparently unable to penetrate the highly sclerotized integument of the female's puparium, we conclude that offspring of females remaining in the puparium are reproducing parthenogenetically. This result is consistent with the observations made by Silvestri (1941a, 1943b) on *Eoxenos* and *Mengenilla*.
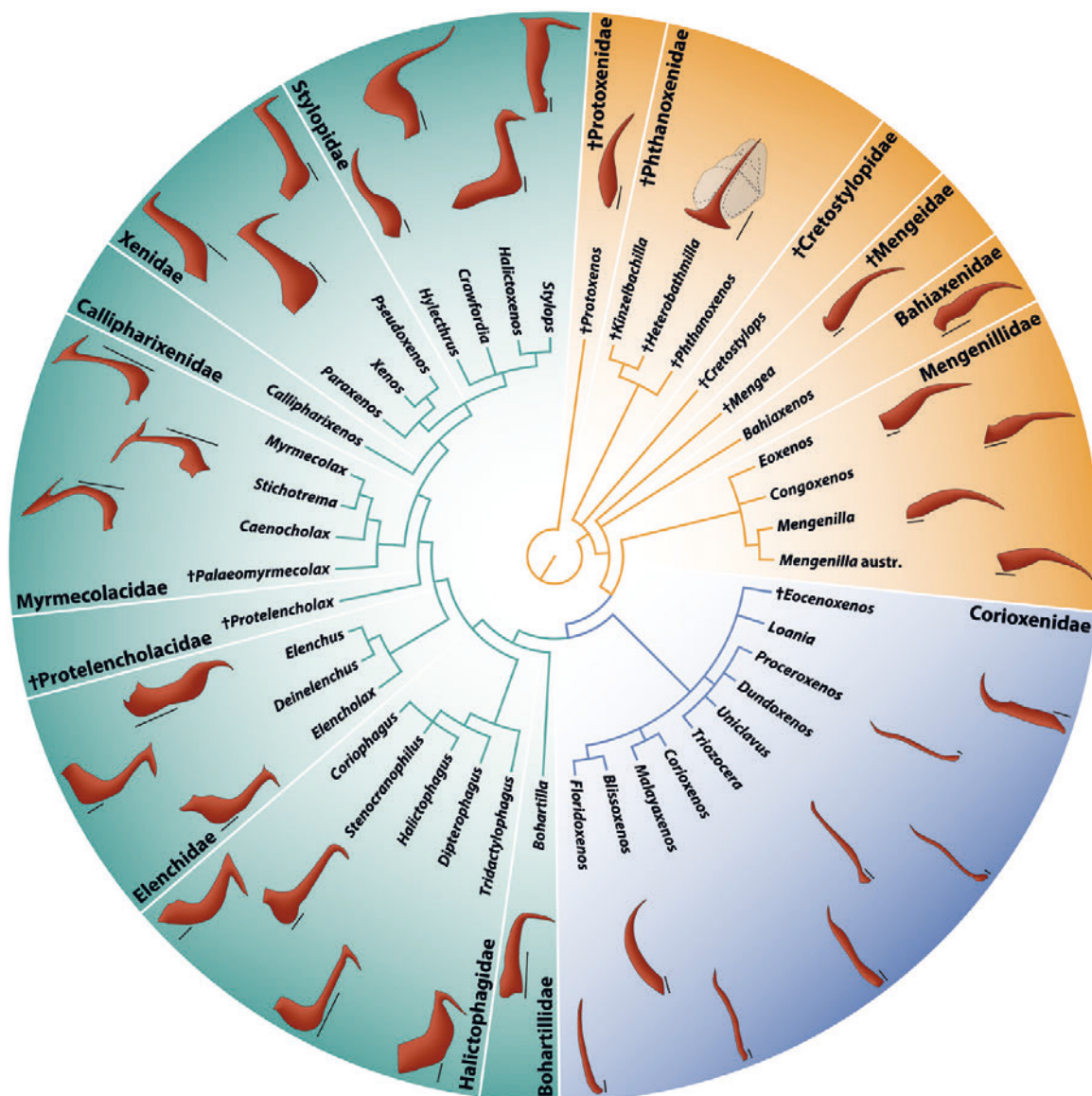
Figure 7. Phylogeny of Strepsiptera, taken and modified from Pohl *et al.* (2021). Genera and penises of a representative species of each genus, arranged clockwise, starting with †Protoxenidae. Penises are illustrated in lateral view (except for †*Heterobathmilla* in caudal view, including the paramers in grey). The references to the original descriptions of the penises are given in Supporting Information, Table S2. Scale bars: 50 μm.

The second morphotype (morphotype II) is restricted to Corioxenidae, the sister group of Stylopiformia (i.e. all remaining Stylopidia) (Fig. 7, blue section). The penises of Corioxenidae are conspicuously elongated (Pohl & Beutel, 2005, 2008). As endoparasitic females of Corioxenidae lack a birth opening (in contrast to females of Stylopiformia), mating cannot take place within the anterior brood canal. Penetration is achieved through the exuvia of the second larva at the weakly sclerotized mouth region or laterally on the cephalothorax (Fig. 3A, B). The cephalothoraces of female Corioxenidae are located below the hemelytra of their heteropteran host. The elongated penis is probably a modification that enables mating despite the concealed anterior female body region. Exceptions are the penises of *Loania* and *Blissoxenos*, which display a slightly curved acumen (Fig. 7).

The third morphotype (morphotype III) regarding penis shape is found in the males of species of Stylopiformia. Their intromittent organs are mostly characterized by a pronounced hook-shape (Fig. 7, green section). Particular features, such as distinct dorsal spines (e.g. found in *Stylops*, Stylopidae) or lateral spines (e.g. found in *Caenocholax*, Myrmecolacidae) are present. An important autapomorphy of females of Stylopiformia is the presence of a fissure-shaped birth opening where the primary larvae are released. It is located between the head and the prosternum. Penetration takes place by introducing the penis in the birth opening and by piercing the integument of the female at the anterior regions of the brood canal. A potential autapomorphy of *Stylops* is the presence of a paragenital organ in females (Schrader, 1924; Jandausch *et al.*, 2022). The paragenital organ is a pocket-like invagination located directly in front of the birth opening in which traumatic penetration takes place. We assume that the hook-shaped penis evolved to perform traumatic insemination inside the brood canal or the paragenital organ. Due to the spatial limitation inside the brood canal (or paragenital organ), penetration can probably be more easily achieved with an angled penis than with a straight one. The intromittent organs of *Elenchus tenuicornis* and *Hylecthrus rubi* are exceptions within the third morphotype, as the distal part is only weakly angled (Fig. 7). In *Elenchus tenuicornis*, such a penis is well suited to penetrating the exceptionally wide birth opening of conspecific females (compare Fig. 3H). However, such a wide opening is not present in females of *Hylecthrus rubi*.

In summary, the shape of the penis seems to correlate with the lifestyle of conspecific females, i.e. free living vs. endoparasitic. Strepsipteran males adapted to changes in the life history and morphology of the conspecific females, especially the enclosure in the secondary and tertiary larval exuviae. A slightly curved but simple penis is present in families of Strepsiptera with free-living females (or which are hypothesized to be free living, such as the stem group or Bahiaxenidae). Males of Corioxenidae have slender, straight and elongated penises, arguably an adaptation to female cephalothoraces hidden below the hemelytra of their hemipteran hosts. A hook-shaped penis evolved with the evolutionary novelty of the female birth opening in Stylopiformia. It probably facilitates traumatic insemination within the female's brood canal. A detailed discussion of evidence for traumatic insemination and parthenogenesis in each currently recognized family of Strepsiptera is given in the supporting information.

CONCLUSION

The evidence we present regarding the copulatory mode of Strepsiptera clearly refutes the hypothesis of a switch from traumatic to non-traumatic insemination in Strepsiptera (brood canal mating), corresponding to a switch from free-living to endoparasitic females. Looking at the different lifestyles and morphologies of females, we find different penis morphotypes independent of traumatic insemination. We hypothesize that traumatic insemination is the ancestral mode of copulation in Strepsiptera and consider alternative scenarios, such as multiple origins of traumatic insemination or a reversal to non-traumatic insemination, in Stylopidia unlikely. Although further research is needed to fill gaps in the knowledge of the morphology and life history of female Strepsiptera, especially in the families Bahiaxenidae and Bohartillidae, it appears reasonable to assume that traumatic insemination represents a groundplan feature of the insect order Strepsiptera, without reversal in Stylopidia.

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AUTHOR CONTRIBUTIONS

H.P., O.N. and R.G.B. conceived the project and designed the experiments. Microphotography was carried out by H.P. and K.J. T.vdK. performed X-ray computed tomography. Segmentation and 3D modeling of the µCT data was executed by K.J. SEM histology and cross-sectioning was carried out by H.P. and K.J. H.P. and K.J. prepared the figure plates and wrote the manuscript with helpful input from all other authors.

DATA AVAILABILTY

All data needed to evaluate the conclusions in the paper are present in the paper and its supplementary information.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article on the publisher's website.

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Supporting Information

'Stab, chase me, mate with me, seduce me': how widespread is traumatic insemination in Strepsiptera?

Kenny Jandausch, Thomas van de Kamp, Rolf G. Beutel, Oliver Niehuis and Hans Pohl

Table S1. Species identify, sex, location, date, collector, and preservation of Strepsiptera samples analysed.

Species	Family	Specimen	Fixation	Location and date	Method/detection of
Eoxenos laboulbenei Peyerimhoff, 1919	Mengenillidae	1 pair in copula	abs. EtOH	HR, Makarska, breeding ex. *Tricholepisma aurea* infected with primary larva, 08/2017, leg. H. Pohl	observation, SEM histology/ location of penetration, sperm
Eoxenos laboulbenei	Mengenillidae	8 ♀♀	abs. EtOH	IT, Tuscany, Val d'Orcia and southwest of Bagni San Filippo, 08/2021, leg. H, Pohl, K. Jandausch & D. Tröger	parthenogenesis of free-living females
Eoxenos laboulbenei	Mengenillidae	12 ♀♀	abs. EtOH	2 ♀♀, HR, Makarska, 03/2017, leg. H. Pohl 2 ♀♀, HR, Makarska, 04/2022, leg. H. Pohl & D. Tröger 2 ♀♀, IT, Tuscany, Tuoro sul Trasimeno, 09/2000, 09/2002, leg. H. Pohl 6 ♀♀, IT, Tuscany, Val d'Orcia and southwest of Bagni San Filippo, 08/2021, leg. H, Pohl, K. Jandausch & D. Tröger	parthenogenesis of females remaining in puparia
Eoxenos laboulbenei	Mengenillidae	1 ♀	abs. EtOH	HR, Makarska, breeding ex. *Tricholepisma aurea* infected with primary larva, 08/2022, leg. H. Pohl	microphotography/mating sign
Eoxenos laboulbenei	Mengenillidae	2 ♀♀ and 2 ♂♂	abs. EtOH	HR, Makarska, breeding ex. *Tricholepisma aurea* infected with primary larva, 08/2017, leg. H. Pohl	observation/location of penetration
Malayaxenos trapezonoti Pohl & Melber, 1996	Corioxenidae	1 ♀	70 % EtOH	DE, Niedersachsen, Oberhaverbeck, 06/1995, leg. A. Melber	microphotography/mating sign
Dundoxenos kinzelbachi Luna de Carvalho, 1985	Corioxenidae	1 ♀	70 % EtOH	YE, Al Kadan, 10/2001, leg. T. van Harten	microphotography/mating sign
Halictophagus silwoodensis Waloff, 1981	Halictophagidae	1 ♀	70 % EtOH	DE, Niedersachsen, Neustädter Moor, 2001, leg. A. Melber	microphotography/mating sign
Halictophagus agalliae Abdul-Nour, 1970	Halictophagidae	1 ♀	70 % EtOH	DE, Baden-Württemberg, Kaiserstuhl, Oberbergen, 08/1995, leg. C. Gack	microphotography/mating sign
Tridactylophagus etoi Nakase, 2013	Halictophagidae	1 ♀	70 % EtOH	JP, Tokyo, Machida City, 05/2016, leg. Y. Nakase	microphotography, histological section/mating sign
Elenchus tenuicornis (Kirby, 1815)	Elenchidae	1 ♀	70 % EtOH	DE, Thüringen, Rothenstein, Spitzenbergen, Schießplatz, 05/2018, leg. H. Pohl	microphotography/mating sign
Elenchus tenuicornis	Elenchidae	1 ♀	70 % EtOH	PL, Podlaskie, Biebrza National Park, Gugny, 09/2013, leg. H. Pohl	histological section/mating sign

Table S1. Continuation.

Species	Family	Specimen	Fixation	Location and date	Method/detection of
Xenos vesparum (Rossi, 1793)	Xenidae	1 ♀	70 % EtOH	DE, Rheinland-Pfalz, Mettenheim, 08/2014, leg. H. Pohl	microphotography/mating sign
Xenos vesparum	Xenidae	1 pair in copula	abs. EtOH	breeding ex. *Polistes nympha* (DE, Thüringen, Rothenstein, 06/2018) infected with primary larva, DE, Baden-Württemberg, Kaiserstuhl, Oberbergen, 07/2018, leg. H. Pohl	3D-reconstruction/sexual interaction
Xenos vesparum	Xenidae	1 pair in copula	abs. EtOH	breeding ex. *Polistes nympha* (DE, Thüringen, Rothenstein, 06/2018) infected with primary larva, DE, Baden-Württemberg, Kaiserstuhl, Oberbergen, 07/2018, leg. H. Pohl	SEM histology, histological section/sperm transfer, mating sign
Paraxenos erberi Saunders, 1872	Xenidae	1 ♀	70 % EtOH	IL, Qirayat Shemona, 05/1996, leg. C. Schmid-Egger	microphotography/mating sign
Stylops nevinsoni Perkins, 1918	Stylopidae	1 ♀	70 % EtOH	DE, Thüringen, Jena, surroundings, 02/2020, leg. H. Pohl	microphotography/mating sign
Stylops ovinae Noskiewicz & Poluszyński, 1928	Stylopidae	1 ♀	70 % EtOH	DE, Niedersachsen, Niedringhaussee, 02/2013, leg. H. Pohl	microphotography, histological section/mating sign
Stylops ovinae	Stylopidae	1 pair in copula	abs. EtOH	female: DE, Nordrhein-Westfalen, Langerwehe; male: DE, Thüringen, Jena, surroundings, 02/2020, leg. H. Pohl, K. Jandausch	3D-reconstruction/sexual interaction
Stylops ovinae	Stylopidae	1 pair in copula	abs. EtOH	DE, Niedersachsen, Niedringhaussee, 02/2013, leg. H. Pohl	SEM histology/sperm

Figure S1. Histological sections of the cephalothorax of *Tridactylophagus etoi* (a, cross-section) and *Elenchus tenuicornis* (b, sagittal section).
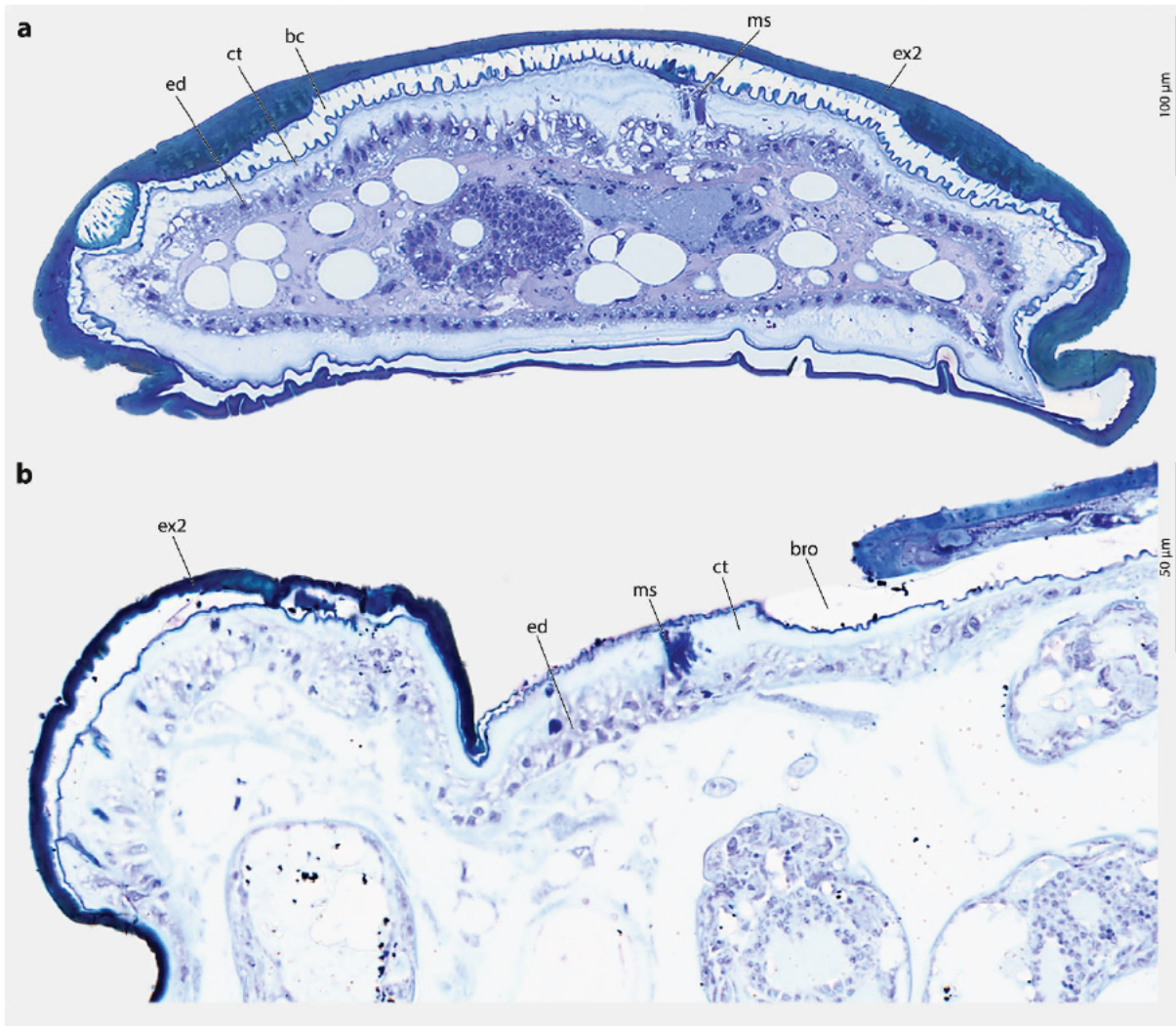
Abbreviations: bc – brood canal, bro – birth opening, ct – cuticle, ed – epidermis, ex2 – exuvia of the secondary larval stage, ms – mating sign.

Figure S2. Histological cross-sections of the cephalothorax of *Xenos vesparum* (a) and *Stylops ovinae* (b).
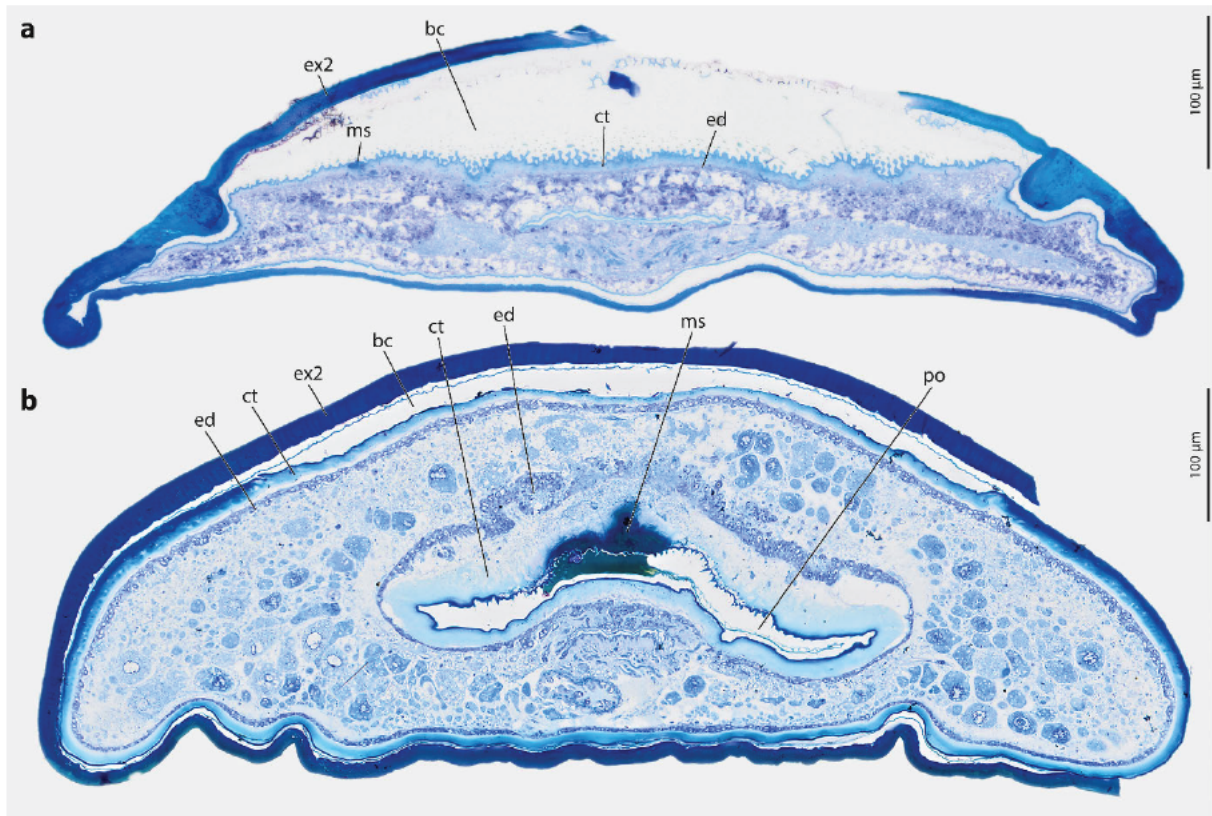
Abbreviations: bc – brood canal, ct – cuticle, ed – epidermis, ex2 – exuvia of the secondary larval stage, ms – mating sign, po – paragenital organ. **(b)** Modified from Peinert et al. (2016).

Table S2. References to the original descriptions of the penises illustrated in Figure 7.

Family	Species	Reference
†**Protoxenidae**	†*Protoxenos janzeni*	this study
†**Phthanoxenidae**	†*Heterobathmilla kakopoios*	modified from Pohl et al. (2021)
†**Mengeidae**	†*Mengea tertiaria*	modified from Kinzelbach (1971)
Bahiaxenidae	*Bahiaxenos relictus*	modified from Bravo et al. (2009)
Mengenillidae	*Eoxenos laboulbenei*	modified from Kinzelbach (1971)
	Mengenilla moldrzyki	modified from Pohl et al. (2012)
	Congoxenos stami	modified from Kinzelbach (1972)
	Mengenilla australiensis	modified from Kifune and Hirashima (1983)
Corioxenidae	*Loania canadensis*	modified from Kinzelbach (1971)
	Proceroxenos jordanicus	modified from Pohl et al. (1996)
	Dundoxenos breviphlebos	modified from Pohl et al. (1996)
	Uniclavus zambezenis	modified from Kathirithamby (1989)
	Triozocera mexicana	modified from Kinzelbach (1971)
	Corioxenos antestiae	modified from Cooper (1938)
	Malayaxenos trapezonoti	modified from Pohl and Melber (1996)
	Blissoxenos esakii	modified from Miyamoto and Kifune (1984)
	Floridoxenos monroensis	modified from Kathirithamby and Peck (1994)
Bohartillidae	*Bohartilla megalognatha*	modified from Kinzelbach (1969)
Halictophagidae	*Tridactylophagus coniferus*	modified from Kinzelbach (1971)
	Halictophagus agalliae	modified from Abdul-Nour (1970)
	Stenocranophilus anomalocerus	modified from Kinzelbach (1971)
	Coriophagus rieki	modified from Kinzelbach (1971)
Elenchidae	*Elencholax noonadanae*	modified from Kinzelbach (1971)
	Deinelenchus australensis	modified from Kinzelbach (1971)
	Elenchus tenuicornis	modified from Pohl (1991)
Myrmecolacidae	*Caenocholax fenyesi*	modified from Kinzelbach (1971)
	Stichotrema dallatorreanum	modified from Kinzelbach (1971)
	Myrmecholax furcatus	modified from Kinzelbach (1971)
Xenidae	*Paraxenos* sp.	modified from Kinzelbach (1971)
	Xenos vesparum	this study
Xenidae	*Pseudoxenos hookeri*	modified from Kinzelbach (1971)
Stylopidae	*Hylecthrus rubi*	modified from Kinzelbach (1971)
	Crawfordia pulvinipes	modified from Kinzelbach (1971)
	Halictoxenos ulrichi	modified from Kinzelbach (1971)
	Stylops ovinae	this study

**Detailed discussion of evidence for traumatic insemination and parthenogenesis
in each currently recognized family of Strepsiptera**

Stem group Strepsiptera
The mode of mating of extinct Strepsiptera is unknown, as the fossil record of adults of †Creto-
stylopidae, †Mengeidae, †Phthanoxenidae, and †Protoxenidae is restricted to males e.g., Engel et al.
(2016), Kathirithamby and Engel (2014) Pohl (2009), Pohl an Beutel (2016), Pohl et al. (2021).
However, the shape of the penis provides indirect evidence of the copulation mode of stem group
strepsipterans. In several genera (e.g., †*Protoxenos*, †*Heterobathmilla*, †*Cretostylops* and †*Mengea*),
the needle-like apex of the intromittent organ strongly suggests that the males were capable of traumatic
insemination (Fig. 7). It is in this context noteworthy that the intromittent organ of †*Heterobathmilla
kakopoios* was equipped with parameres, which are absent in all other extinct and extant strepsipterans
(Pohl et al., 2021). However, the exact function of these paired lateral elements of the copulatory
apparatus remains elusive. In any case, comparisons with penis shapes found in species of the family
Mengenillidae and others make it very likely that males of extinct groups were capable of perforating
the cuticle of their mating partner, even though virtually no information is available on adult females.
Only one single (immature) fossil female, from Eocene Baltic amber, is known, most likely belonging
to a free-living species of †*Mengea* (†Mengeidae) (Pohl et al., 2019). This immature female and the fact
that only males of Strepsiptera with permanent endoparasitic females (Stylopidia) have adhesive hairs
on their tarsi (Pohl & Beutel, 2005; Pohl & Beutel, 2008), strongly suggests that females of the stem
group were free living, like the females of Mengenillidae, with which they probably also shared a similar
lifestyle and reproductive biology. Based on these assumptions and considering the shape of the penises,
we hypothesize that stem group strepsipterans also performed traumatic insemination.

Bahiaxenidae
As only the male holotype of the single species *Bahiaxenos relictus* is known (Bravo et al., 2009),
nothing is known about the reproductive biology. The shape of the penis closely resembles the
intromittent organ of Mengenillidae, for instance of *E. laboulbenei*. The penis' sharp apex (Bravo et al.,
2009) (Fig. 7) makes it suitable for traumatic penetration and consequently traumatic insemination.

Mengenillidae
Earlier studies on *E. laboulbenei* and *Mengenilla chobauti* (Parker & Smith, 1934; Silvestri, 1941a,
1943b) suggested that copulation can take place at several sites of the female's abdomen, reaching from
lateral to ventral areas, including close proximity to the birth opening. Our observations on penetrations
of three copulae of *E. laboulbenei* (Fig. 2a) and observation of several mating signs on one female of *E.
laboulbenei* (Fig. 2b) strongly support the observations by Silvestri (1941a, 1943b) and Parker and
Smith (1934). Additionally, our SEM histology data revealed injection of sperm during the penetration
process. Our data thus do not support the idea of the birth opening serving as insertion site of the penis
in this species (suggested by Parker and Smith (1934)). While traumatic insemination was never really
questioned in Mengenillidae, Silvestri (1941a, 1943b) and Kinzelbach (1978) considered the occurrence
of parthenogenesis in *Eoxenos* and *Mengenilla*. It is conceivable that this phenomenon plays a role in
Mengenillidae, as some of the females remain inside their puparium and produce primary larvae

(Kinzelbach, 1978; Silvestri, 1941a; Tröger et al., 2019). These females clearly impede the penetration by a male. In fact, injection through the puparium is not documented and appears quite unlikely due to the strongly degree to which the cuticle of the puparium is sclerotized. In contrast, our experiments with unfertilized *Eoxenos* females that had left their puparium did not produce any primary larvae. This makes parthenogenesis in free-living females very unlikely.

Corioxenidae

There are very few studies on the reproductive biology and mating of Corioxenidae. Kirkpatrick (1937), who studied *Corioxenos antestiae* and its pentatomid host *Antestesia* sp. in detail, kept 200 females of the former species without granting them access to conspecific males, and he never observed hatching primary larvae. It is consequently unlikely that this species is able to reproduce via parthenogenesis. Kirkpatrick described the copulation as penetration of "a window" on the lateral side of the female's cephalothorax. In *Malayaxenos*, Pohl and Beutel (2008) described this "window" as a membranous area on the lateral cephalothorax as a zone of weakly sclerotized cuticle that possibly facilitates penetration (Pohl & Beutel, 2008). However, the same authors discussed copulation through the mouth opening as a possible option and as a groundplan feature of Corioxenidae. In the case of *Malayaxenos*, we verified traumatic penetration by a clearly visible mating sign on the cephalothorax. Due to the retracted position of the female within the larval exuvia, it was not possible to identify the precise region of the penetration. It remains consequently unclear whether the oral region or the pleural membranous window had been used. In the case of *Dundoxenos kinzelbachi*, microphotography of a protruding female clearly confirms traumatic penetration in the mouth region. The mating sign is distinctly visible in the anteriormost region of the cephalothorax in the area of the mouth opening. This is also the region where the primary larvae emerge. The outer cuticle appears slightly thinner at this area, which would make it more suitable for penetration by a penis compared to other regions of the exposed cephalothorax, with the noteworthy exception of the membranous pleural areas. Parthenogenesis was reported for the genus *Triozocera* (Esaki & Miyamoto, 1958), but Kinzelbach (1971) pointed out that this statement is not based on reliable data. Considering the presently available data, there is no conclusive evidence for parthenogenesis in Corioxenidae. In contrast, different pieces of evidence support traumatic mating in this family. Although insemination during penetration has not yet been confirmed, the elongated penises of the Corioxenidae have a needle-like, sharp tip that should facilitate traumatic penetration.

Bohartillidae

Like in Bahiaxenidae, only males of a single extant species, that of *Bohartilla megalognatha*, are known (Kinzelbach, 1969). Additionally, males of two species are known from Dominican amber, *Bohartilla kinzelbachi* (Grimaldi & Kathirithamby, 1993) and *Bohartilla joachimscheveni* (Kinzelbach & Pohl, 1994). Larval stages, females, and hosts are unknown (Pohl & Beutel, 2008). Information on the brood canal and possible penetration sites therefore remain obscure. No information on mating behaviour is available. Even though direct evidence is lacking, the hook-shaped penises of *B. joachimscheveni* and *B. megalognatha* suggest that traumatic penetration was used by species of this family.

Halictophagidae

The available information of the mating behaviour of Halictophagidae is sparse. Silvestri (1943a) observed extragenital traumatic mating in *Halictophagus tettigometrae* and found sperm in the hemocoel of female specimens. We provided evidence for traumatic penetration in three species of two genera by documenting strongly pigmented mating signs, comparable to those found in females of all other studied taxa in this study. In *Tridactylophagus*, the widely open brood canal can be easily reached by the penis. This feature of a wide opening is shared with species of Elenchidae (Fig. 3c, h) and parts of Myrmecolacidae. We documented a mating sign at the brood canal close to the posterior border of the birth opening in females of *T. etoi*. Males of this species are unknown so far, but a typical hook-shaped penis with a sharp acumen is present in closely related species, such as *T. coniferus*, *T. sinensis* and *T. similis*. *Halictophagus agalliae* and *H. silwoodensis* both display mating signs in the anterior region of the brood canal. The exact location differs slightly in both species, most likely due to the different shape of the birth openings. While the birth opening of *H. silwoodensis* is distinctly curved, it is straight in *H. agalliae*. The penises of species occurring in the genus *Halictophagus* are all hook-shaped e.g., Kinzelbach (1971) as in *H. agalliae* and *H. silwoodensis* Figure 7; (Abdul-Nour, 1970; Waloff, 1981). Males of other genera not included in this study also display penis shapes with a sharp tip, such as for instance those of males of *Dipterophagus* (Drew & Allwood, 1985), of *Stenocranophilus*, and of *Coriophagus* (Kinzelbach, 1971). Based on the available data on the Halictophagidae, we conclude that traumatic insemination is the common mode of copulation in this family.

Elenchidae

Baumert (1958) documented hypodermal injection in *Elenchus tenuicornis* in a single study of the mating behaviour of Elenchidae. He observed that males penetrate females at the ventral birth opening with the sharp acumen of their penis. Our observations suggest that traumatic penetration is the common mode of copulation in this family. Females of Elenchidae are characterized by an extraordinary wide birth opening directly exposed externally, providing easy access for the males (Fig. 3h). The penises are more or less sickle-shaped in the genera *Elenchus* and hook-shaped in *Deinelenchus* and *Elencholax* (Fig. 7). Despite of slight differences in shape, the tip of the penis always displays a sharp acumen, likely facilitating traumatic penetration (Baumert, 1958; Kinzelbach, 1971; Kogan & Poinar, 2019; Luna de Carvalho, 1985; Pohl, 1991). Additionally, piercing of the female at the birth opening is documented by mating signs at several locations in this region (e.g., Fig. 3h). This observation is consistent with the findings by Baumert (1958), who describes that males pierce the female at random locations in the region of the birth opening.

†Protelencholacidae

This family compromises only the genera †*Cryptelencholax* and †*Protelencholax* (Kogan & Poinar, 2019; Pohl & Beutel, 2008; Pohl et al., 2021). Like in Bahiaxenidae and Borhartillidae, the females of †Protelencholacidae are unknown. The only evidence for the possible mode of copulation is again the shape of the penis, which has been described in †*Cryptelencholax poinari* Kogan and Poinar (2019). The penis of †*C. poinari* is hook-shaped, with an angle of approximately 90° between stylet and acumen. The sharp tip appears suitable for penetration and suggests traumatic insemination.

Myrmecolacidae

The two sexes of species in the family Myrmecolacidae utilize different hosts – a unique feature of the family – males develop in ants, females in Mantodea and Ensifera (Orthoptera). Females of most Myrmecolacidae feature a wide birth opening (e.g., females of *Stichotrema dallatorreanum*), which is easily accessible to conspecific males (Hofeneder, 1910; Kathirithamby, 2000; Kinzelbach, 1971). In all genera, the males possess a penis that is hook-shaped and exhibit a distinct dorsal spine (Fig. 7). The penis of male *Caenocholax fenyesi* feature a very specific shape (Fig. 7): the entire distal portion is shield-like shaped with two lateral spines slightly posterior to the acumen. The birth opening of females of *C. fenyesi* is very wide, with a large portion of the integument exposed externally (Kathirithamby & Spencer Johnston, 2004). We did not study females of species of Myrmecolacidae, but mating signs were reported by Luna de Carvalho (1972). The author described two scars in the birth opening of female *Caenocholax vilhenai* induced by conspecific males during fertilization. Luna de Carvalho (1972) also mentioned that he repeatedly found in *S. dallatorreanum* the apex of the male genital left in the female after copulation. Considering the shape of the penis, the shape of the birth opening, and the appearance of mating signs in Myrmecolacidae, we consider traumatic penetration to be the likely mode of copulation in this family. Except for *Stichotrema dallatorreanum*, which seem to be able to reproduce parthenogenetically, there is no further evidence for parthenogenesis in this group.

Callipharixenidae

In contrast to other understudied groups of Strepsiptera, only the females of the species of this family are known, all of which were parasitizing Scutelleridae (Heteroptera) (Pohl & Beutel, 2008). Drawings of *Callipharixenos muiri* and *C. siamensis* (Pierce, 1918) show a narrow birth opening, similar to the condition we observed in Xenidae (Kinzelbach, 1971). Information on mating signs is not available. As males are unknown, the penis cannot be used as indirect evidence for traumatic insemination. As the morphology of females comparable with other families (e.g., the narrow birth opening), traumatic insemination as mode of copulation seems plausible. However, the absence of males is potential evidence that parthenogenesis occurs instead of bisexual reproduction.

Xenidae

The mode of copulation in Xenidae received more attention than that of other families of Strepsiptera. Schrader (1924) documented fertilization of eggs in *Xenos* and hence bisexual reproduction in this family. However, the study did not address the mode of sperm transmission. Beani et al. (2005) used transmission electron microscopy to determine the pathway of the sperm released through the penis into the birth opening of *Xenos vesparum*. The authors found spermatozoa in the hemocoel and (although in low quantities) in the brood canal. They consequently questioned transfer of sperm via traumatic insemination but could not excluding this mode of copulation entirely. An extensive behavioural study on *Xenos peckii* (Hrabar et al., 2014) showed that the penis of males of this species is inserted into the birth opening. However, it remained unclear how and where spermatozoa are released. Our µCT-data provide clear evidence for males of *Xenos vesparum* introducing their penis through the birth opening and piercing the female's body wall in the anterior region of the brood canal (Fig. 5c, d). The combined information about the penetration site gathered by our 3D data and the observed mating signs with microphotography in *X. vesparum* prove a direct connection of mating signs and penetration sites.

Furthermore, we were able to falsify the hypothesis that sperm passes through the brood canal using the birth organs to reach the free-floating eggs in the hemocoel e.g., Beani et al. (2005), Kathirithamby et al. (2015). Sperm found in the brood canal by Beani et al. (2005) can be explained in two different ways: 1) The observed spermatozoa in the brood canal are an artifact caused by cutting during the TEM processing. 2) A small amount of sperm was released by the male when the male removed its penis from the female's integument and pulled it out of the brood canal. SEM histology by us revealed that sperm is directly released into the hemocoel, and that traumatic insemination occurs in Xenidae. Considering that the penises of species in different genera of this family exhibit a high degree in morphological similarity, we consider it reasonable to assume that the mode of copulation does not differ between the species. We consequently assume traumatic insemination is the common mode of copulation in species of Xenidae.

Stylopidae

Parthenogenesis in Stylopidae was long discussed due to special behaviour of the polar bodies, which are repelled during the fertilization of the eggs (Brues, 1903; Smith & Hamm, 1914). This issue was evaluated by different authors investigating the embryonic development (Noskiewicz & Poluszyński, 1928; Richter, 1956). An exception marks *Halictoxenos spencei* as Müller (1944) found only female strepsipterans among 800 infested hosts. In general, there are only a few males described of *H. spencei* (Hofeneder, 1949; Perkins, 1918). From this sex ratio Kinzelbach (1978) deduces an at least facultative parthenogenesis in *Halictoxenos*. However, clear evidence for parthenogenesis has never gathered and seems overall unlikely. Lauterbach (1954) described the copulation of *Stylops* and the sperm's pathway in and trough the female in detail. He found that species of *Stylops* mate traumatically and that the sperm is directly released into the female's hemocoel after the integument of the female is pierced by the male's penis. Grabert (1953) described the insertion of the penis through the birth opening into the "Brutkanaltasche" (paragenital organ). The hooked-shaped penis pierces through several layers of cuticles before sperm is directly released into the female's hemocoel. The observations by Lauterbach and Grabert are verified by our data. 3D modeling of the μCT data revealed the precise position of the penis during copulation in *Stylops ovinae* and provided clear evidence for traumatic penetration. SEM histology additionally documented for the first time the injection of the sperm by traumatic insemination. That traumatic insemination is likely the common mode of copulation in this family can be inferred by the presence of a paragenital organ in females of all species studied so far. That the paragenital organ serves as penetration site was demonstrated by Peinert et al. (2016) and is confirmed in the present study by the location of mating signs in the organ. Whether this organ is phylogenetically widespread in the family Stylopidae or restricted to the genus *Stylops* is currently not known. In contrast to the paragenital organ of Stylopidae several penises of different genera are well described in this group. The intromittent organs of Stylopidae are in general hook shaped with an approximately 90-degree angled acumen. Most important, all penises have a sharp tip that appear suitable for traumatic penetration. The genus *Hylecthrus* marks an exception with a relatively straight penis, just slightly bend.

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FORM 1

2.2   Manuscript No. II

Manuscript title: The paragenital organ of Stylopidae (Strepsiptera) and the functional incorporation of the secondary larval exuviae.

Authors: Jandausch, K., Straka, J., van de Kamp, T., Stark, H.., Beutel, R.G., Niehuis, O., Pohl, H.

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The candidate is

☒ First author, ☐ Co-first author, ☒ Corresponding author, ☐ Co-author.

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Authors' contributions (in %) to the given categories of the publication

Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Jandausch, K.	15 %	80 %	50 %	60 %	10 %
Straka, J.					70 %
van de Kamp, T.			20 %		
Stark, H.		20 %			
Beutel, R.G.	5 %				
Niehuis, O.	40 %			20 %	
Pohl, H.	40 %		30 %	20 %	20 %
Total	100 %	100 %	100 %	100 %	100 %

_____   _____

Signature candidate   Signature supervisor (member of the Faculty)

The paragenital organ of Stylopidae (Insecta: Strepsiptera) and the functional incorporation of the secondary larval exuvia

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Abstract

Females of the insect order Strepsiptera are known to be traumatically inseminated. Traumatic insemination is the process of insemination by sperm transfer through a wound inflicted by the male in the female's integument, rather than by the male transferring sperm through the female's genital opening. Females fertilized by traumatic insemination are likely to exhibit morphological adaptations that help them to reduce the fitness costs associated with the integument wounding. One such adaptation is the presence of a paragential organ. It has been described in traumatically inseminated bugs of the superfamily Cimicoidea and in species of the Strepsiptera genus *Stylops*. Although the paragenital organ appears to play a critical role in the mating biology of *Stylops* species, its phylogenetic roots are unknown. Here we show that the paragenital organ in Strepsiptera may be an autapomorphy of the family Stylopidae, where we found it present in species of the genera we studied (i.e., *Eurystylops*, *Halictoxenos*, *Hylecthrus*, *Kinzelbachus*). Integument thickness measurements based on μCT data revealed that regardless of the presence of a paragenital organ in Strepsiptera, penetration sites in the female's integument are thickened relative to control sites. Our data refute the notion that the paragenital organ in Strepsiptera is exclusive to the genus *Stylops*. In addition, we found a functional interaction between the paragenital organ and the lateral processes of the secondary larval exuvia. Our study contributes to the basic understanding of the evolution and the function of the paragenital organ in Strepsiptera and suggests potentially important morphological characters for a species-level phylogeny of the Stylopidae.

Introduction

Species of the insect order Strepsiptera exhibit highly derived morphologies related to their endoparasitic lifestyle. Females of Strepsiptera are also known to be traumatically inseminated. In most Strepsiptera species, the female is pierced by the male's penis in close proximity to a structure known as the birth opening. In contrast, females of the genus *Stylops* are pierced by the male's penis in a secondary genital organ: the paragenital. It represents a cephalothoracic invagination and was first described by (Nassonov, 1893), who hypothesized that it morphologically corresponds to an internal structure of the insect head called the tentorium and serves as a shelter for the primary larvae to retreat in case of danger. That the structure instead actually represents a secondary genital organ was discovered six decades later by Lauterbach (1954). The functional morphology of the paragenital has recently been studied in detail in *Stylops ovinae* (Peinert et al. 2015).

However, the phylogenetic distribution of the organ in Strepsiptera, its functional interaction with the female's larval exuvia (retained throughout the female's life), and its functional interaction with the male's penis remain to be investigated and are the subject of the present study.

The insect order Strepsiptera comprises ca. 600 described species. Its species are characterised by numerous derived characters in all life stages and in both sexes (Pohl & Beutel, 2005; Pohl & Beutel, 2008; Straka, Jůzová & Batelka, 2014; Tröger, Beutel & Pohl, 2019; Tröger et al., 2020; Benda et al., 2022; Tröger et al., 2023; Weingardt, Beutel & Pohl, 2023). All Strepsiptera species show extreme sexual dimorphism. The males are free-living, and the only function of their short adult lifespan of a few hours is to find females and mate with them. The females are obligate endoparasites of other insects in which they spend most of their larval development and adult life. Species of the family Mengenillidae are an exception, as their females are free-living – a plesiomorphic character state. Species with permanently endoparasitic adult females form the monophyletic lineage Stylopidia, which comprises about 97% of all known Strepsiptera species (Pohl & Beutel, 2008). Due to their endoparasitic lifestyle, female Stylopidia are unable to shed their larval exuvia and form a functional unit with it. This unit provides the morphological basis for the formation of a morphological structure called the brood canal through which the primary larvae exit the maternal body cavity (Kinzelbach, 1971; Pohl & Beutel, 2005).

The body of the female of the free-living Mengenillidae reflects in its morphology the three insect-typical functional units: head, thorax, and abdomen. In contrast, the body of Stylopidia is sack-shaped and has been reduced to only two functional units: cephalothorax and abdomen. Specifically, the cephalothorax of the Stylopidia is composed of the head, the thorax, and the anterior part of the first abdominal segment, which bears the only functional spiracles. The abdomen consists of all remaining segments. The posterior part of the body of Stylopidia resides inside the host's abdomen, and only the cephalothorax is exposed to the host's external environment.

Stylopidia lack a primary genital opening through which the male could transfer his sperm into the female. Females of the lineage Stylopiformia (Stylopidia excl. Corioxenidae) are oriented with their ventral side facing away from the host. They have a secondary birth opening that connects the female's birth organs to the outside environment via the brood canal. The secondary orifice serves as an exit for the primary larvae from the female's body. However, in species of Stylopiformia, it also serves as the exclusive site where the male's penis penetrates the female's cuticle during traumatic insemination (Grabert, 1953; Lauterbach, 1954; Peinert et al., 2016; Jandausch et al., 2022; Jandausch et al., 2023). Species of the genus Stylops are a known exception in this regard, as they

possess a paragenital located in front of the birth opening, where penetration occurs instead. However, whether this paragenital is unique to the genus *Stylops* or also occurs in other lineages of Stylopiformia has not been investigated. While females of Stylopiformia are oriented with their ventral side facing away from the host, the anterior part of the body of females of Corioxenidae is oriented with its dorsal side facing away from the host's abdomen, but remains hidden under the hemelytra of the heteropteran host. Female Corioxenidae lack a copulatory opening on the exuvia of their secondary larvae. Male Corioxenidae therefore pierce the exuvia of the secondary larva with their penis during copulation, usually in the region of the female's mouth or on membranous pleural areas of the prothorax (Kirkpatrick, 1937; Pohl & Beutel, 2008).

Lauterbach (1954) discovered that the paragenital, which he named "Brutkanaltasche" and described as a pocket-shaped invagination of the integument at the anterior end of the brood canal, is the exclusive site of penetration and sperm transfer in *Stylops ovinae* (= *Stylops ater* sensu Straka, Jůzová & Nakase (2015)). Consequently, he considered the paragenital to be functionally equivalent to a bursa copulatrix. Löwe, Beutel & Pohl (2016) also concluded that the paragenital organ of *Stylops ovinae* represents a cephalothoracic invagination and reported that the ventral wall of the invagination cuticle is thickened and rests on a multilayered epidermis. The authors found the same character states (i.e., presence of a thickened cuticle, multilayered epidermis) in *Elenchus tenuicornis* (Elenchidae), *Tridacylophagus etoi* (Halictophagidae), and *Xenos vesparum* (Xenidae) (Peinert et al., 2016; Richter et al., 2017; Jandausch et al., 2022; Jandausch et al., 2023).

In their work on the copulatory behaviour of *S. ovinae*, Peinert et al. (2016) highlight the potential importance of the organ in the process of traumatic fertilisation. Specifically, the authors suggest that the structural properties of the paragenital organ could help reduce trauma caused by multiple copulations. Thus, the paragenital could be considered functionally analogous to the spermalege of bed bugs (Reinhardt, Naylor & Siva–Jothy, 2003; Michels, Gorb & Reinhardt, 2015). The idea that the paragenital is an adaptation that reduces wound-inflicted trauma in species with traumatically inseminated females was further supported by recent experiments analysing penetration forces and structural composition at penetration and control sites in *S. ovinae* and *X. vesparum* (Jandausch et al., 2022). The authors found that the cuticle of both species was consistently thickened at penetration sites compared to control sites. This feature is likely to aid wound closure and could thus reduce the negative effects of traumatic insemination on the female. The authors also reported the occurrence of a paragenital organ in two other Stylops species: *S. hammella* and *S. melittae*. The paragenital may also serve as a prezygotic mating barrier preventing heterospecific mating: using behavioural experiments and morphological covariation analyses, Jandausch et al. (2022) found that *S. ovinae* females are attractive

to males of several sympatric *Stylops* species, but only the conspecific males are able to successfully mate with these females.

Female Strepsiptera, with the exception of those in the family Mengenillidae, remain in their larval exuvia throughout their adult lives. Such long-term use (or re-use) of the larval exoskeleton after moulting by arthropods is not restricted to species of the order Strepsiptera. Some larvae of lacewings (Neuroptera: Chrysopidae) carry debris for camouflage, including parts of their larval skin (Tauber, Tauber & Albuquerque, 2014); turtle beetle larvae (Coleoptera: Chrysomelidae: Cassidinae) use their exuvia to shield their abdomen (Olmstead, 1994; Ramos, Boligon & Moreira, 2019); and some caddisflies (Trichoptera) that remain in their pupal case for an extended period as adults use parts of their exuvia to connect their claws inside the pupal case to the claws of the pupa via a tendon formed by the pupal integument (e.g., Betten, 1934; Hinton, 1946; Friedrich & Kubiak, 2018). While the functional link between an adult and its exuvia in (some) caddisflies is remarkable, the formation of a lifelong functional unit consisting of the adult female and the exuvia of the secondary larva remains a unique feature of Strepsiptera.

Some morphological particularities resulting from adult female Strepsiptera forming a lifelong functional unit with their secondary larval exuvia have been described in detail (e.g., Pohl & Beutel, 2005; Pohl & Beutel, 2008; Löwe et al., 2016; Richter et al., 2017). These include the brood canal of Stylopidia and the pores on the ventral side of the larval exuvia, which appear to function as openings for Nassonov's glands (Löwe et al., 2016). The function of another morphological particularity, the processes laterad of the paragenital organ in the second larval exuvia, interpreted by Kinzelbach (1971) as a tentorium, remains to be investigated.

In the present study, we (1) investigate the phylogenetic distribution of the paragenital organ in the Strepsiptera family Stylopidae; (2) present detailed μCT-based 3D reconstructions of the female cephalothorax and male penis in different species of Stylopidae; (3) assess whether a thickened cuticle on the ventral side of the paragenital organ correlates with the presence of processes laterad of the paragenital organ in the second larval exuvia; and we (4) discuss possible functions of these processes from analysis of 3D models of the female cephalothorax.

Table 1. Sex, species, family, locality, date, collector, and preservation of Strepsiptera specimens analysed. Abbreviations: EtOH, ethanol; abs., absolute

Species	Family	Locality	Date	Collector	Idenitifier	Preservation
		Female specimens				
Triozocera macroscyti Esaki & Hashimoto, 1958	Corioxenidae	Japan, Nagano campus area Shinshu University Faculty of Agriculture	05/2017	Y. Nakase	Y. Nakase	abs. EtOH
Paraxenos erberi Saunders, 1872	Xenidae	Slovakia, Virt env., sand dune pasture	06/2018	J. Straka	J. Straka	abs. EtOH
Pseudoxenos schaumii Saunders, 1872	Xenidae	Czechia, Hradec Králové, PP Na Plachtě	08/2007	P. Bogusch	D. Benda	abs. EtOH
Tuberoxenos sphecidarum (Siebold, 1839)	Xenidae	Mongolia, Arvaykheer, 137km NE, Övörkhangay prov.	07/2004	J. Straka	J. Straka	abs. EtOH
Xenos vesparum (Rossi, 1793)	Xenidae	Breeding ex. Polistes dominula (Germany, Thüringen, Rothenstein) infected with primary larva, DE Baden-Württemberg, Kaiserstuhl Oberbergen	06/2019	H. Pohl, F. Schweitzer	H. Pohl, F. Schweitzer	abs. EtOH
Crawfordia warnckei Kinzelbach, 1970	Stylopidae	Morocco, Idelsane env., Ouarzazate prov.	03/2019	D. Benda	D. Benda	abs. EtOH
Eurystylops sp. Bohart, 1943	Stylopidae	Türkiye, Budaklı env., Kahramanmaraş prov.	07/2011	J. Straka	-	abs. EtOH
Halictoxenos arnoldi Perkins, 1918	Stylopidae	Türkyie, Budaklı env., Kahramanmaraş prov.	07/2011	J. Straka	J. Straka	abs. EtOH
Halictoxenos simplicis Noskiewicz & Poluszynski, 1924	Stylopidae	Hungary, Dunatetétlen env., salt marshes	06/2013	D. Benda, P. Bogusch, J. Straka	J. Straka	abs. EtOH
Hylecthrus rubi Saunders, 1850	Stylopidae	Hungary, Bikolpuszta, loess steppe	08/2018	D. Benda	D. Benda	abs. EtOH
Kinzelbachus friesei (Hofeneder, 1949)	Stylopidae	Tajikistan, Firdavsi/Buragen env., 6km SW Shakriston	07/2019	J. Straka	J. Straka	abs. EtOH

Table 1.: Continuation

Species	Family	Location	Date			
Stylops hammella **Perkins, 1918**	Stylopidae	Czechia, Havraníky, Znojmo env.	05/2017	J. Straka	J. Straka	abs. EtOH
Stylops japonicus **Kifune & Hirashima, 1985**	Stylopidae	Poland, Gozdnica, sandpit	04/2015	K. Kodejš, J. Straka	J. Straka	abs. EtOH
Stylops melittae **Kirby, 1802**	Stylopidae	Czechia, Stroupeč env., PP Stroupeč	04/2016	P. Bogusch	P. Bogusch	abs. EtOH
Stylops nassonowi **Pierce, 1909**	Stylopidae	Czechia, Pavlov env., NPR Děvín-Kotel-Soutěska	04/2016	P. Bogusch	P. Bogusch	abs. EtOH
Stylops ovinae **Noskiewicz & Poluszyński, 1928**	Stylopidae	Czechia, Káraný nad Labem, V Dejmlovce	03/2008	J. Batelka	J. Straka	abs. EtOH
Stylops spreta **Perkins, 1918**	Stylopidae	Czechia, Káraný nad Labem, V Dejmlovce	03/2008	J. Batelka	J. Straka	
Male specimens						
Tuberoxenos sphecidarum **(Siebold, 1839)**	Xenidae	Mongolia, Arvaykheer, 137km NE, Övörkhangay prov.	07/2004	J. Straka	J. Straka	abs. EtOH
Xenos vesparum **(Rossi, 1793)**	Xenidae	Germany, Rheinland-Pfalz, Mettenheim	07/2020	H. Pohl, K. Jandausch	H. Pohl, K. Jandausch	abs. EtOH
Stylops hammella **Perkins, 1918**	Stylopidae	Czechia, Dolní Věstonice, Pálava	04/2014	J. Straka	J. Straka	abs. EtOH
Stylops japonicus **Kifune & Hirashima, 1985**	Stylopidae	Poland, Gozdnica, sandpit	04/2015	K. Kodejš, J. Straka	J. Straka	abs. EtOH
Stylops melittae **Kirby, 1802**	Stylopidae	Czechia, Písečný vrch, České středohoří	02/2008	J. Batelka	J. Straka	abs. EtOH
Stylops nassonowi **Pierce, 1909**	Stylopidae	Czechia, Dymokury env., pond bank	05/2012	J. Straka	J. Straka	abs. EtOH
Stylops ovinae **(*S. ater* sensu Straka et al. (2015))**	Stylopidae	Czechia, Hlásná Třebáň, štěrková cesta u zahr. Kol., Český Kras	03/2011	P. Špryňar	J. Straka	abs. EtOH
Stylops spreta **Perkins, 1918**	Stylopidae	Czechia, Kamýk, Praha-Modřany	04/2013	J. Straka	J. Straka	abs. EtOH

Micro-computed tomography and 3D reconstruction

With the exception of females of *Stylops melittae* and *Triozocera macroscyti*, all specimens were scanned in pure ethanol at the Imaging Cluster of the KIT Synchrotron Radiation Facility, using a polychromatic X-ray beam generated by a 1.5 T bending magnet and spectrally filtered by 0.5 mm Al. A fast indirect detector system consisting of a 13 µm LSO:Tb scintillator (Cecilia et al., 2011), a diffraction-limited optical microscope (Optique Peter) (Douissard et al., 2012), and a 12-bit pco.dimax high-speed camera with 2016 x 2016 pixels was used. Scans were derived from 3,000 projections at 70 fps over an angular range of 180°. An optical magnification of 10x resulted in an effective pixel size of 1.22 µm. The concert control system was used for automated data acquisition, and online reconstruction of tomographic slices was used for data quality assurance (Vogelgesang et al., 2016). Data processing included flat field correction and phase retrieval of the projections based on the intensity transport equation (Paganin et al., 2002). The X-ray beam parameters for the algorithms in the data processing pipeline were calculated using syris (Faragó et al., 2017). The execution of the pipelines, including tomographic reconstruction, was performed with the UFO framework (Vogelgesang et al., 2012).

To improve the contrast in some micro-CT scans, the female of *Stylops mellitae* was stained in an iodine solution (1% iodine in 100% ethanol) for 3 days. It was then transferred to pure ethanol and scanned in a SkyScan221 micro-CT (Max Planck Institute for the History of Mankind, Jena, Germany) with a beam strength of 40 kV and 300 mA. Images were taken every 0.2° in a 360° scan, with an exposure time of 5,800 ms. The resulting final pixel size was 1.22 µm.

The female of *Triozocera macroscyti* was dried at the critical point using an Emitech K850 Critical Point Dryer with liquid $CO2$ (Sample Preparation Division, Quorum Technologies, Ashford, England). The sample was scanned in a SkyScan221 micro-CT (Max Planck Institute for the History of Mankind, Jena, Germany) with a beam strength of 70 kV and 300 mA. Images were taken every 0.15° in a 360° scan, with an exposure time of 3,000 ms. The resulting final pixel size was 0.6 µm.

We pre-segmented tomographic data using Amira 6.0.1 and completed the segmentation semi-automatically using the online platform Biomedisa (Lösel et al., 2020). The segmented material was then exported from Amira as a tiff file stack using the "multiExport" plugin script (Engelkes et al., 2018), and NIFTI data were exported as meshes (.ply). VGStudiomax 2.0.5 (Volume Graphics, Heidelberg, Germany) was used to visualise and rende the data.

Thickness measurements

The thickness of the cuticle was measured using ImageXd 6.2.6 (Heiko Stark, Jena, Germany, URL: https://starkrats.de). We measured at the penetration sites and in the surrounding regions of the integument. Thickness measurements were made 1) using voxel-based data from rendering with VGStudiomax 2.0.5 (Volume Graphics, Heidelberg, Germany) and 2) using mesh data from 3D modelling uploaded to https://sketchfab.com/entomology_uni_jena/collections/thickness-measurements-strepsiptera-ca9eb803e04e-4512a42bbf144fb7a56d.
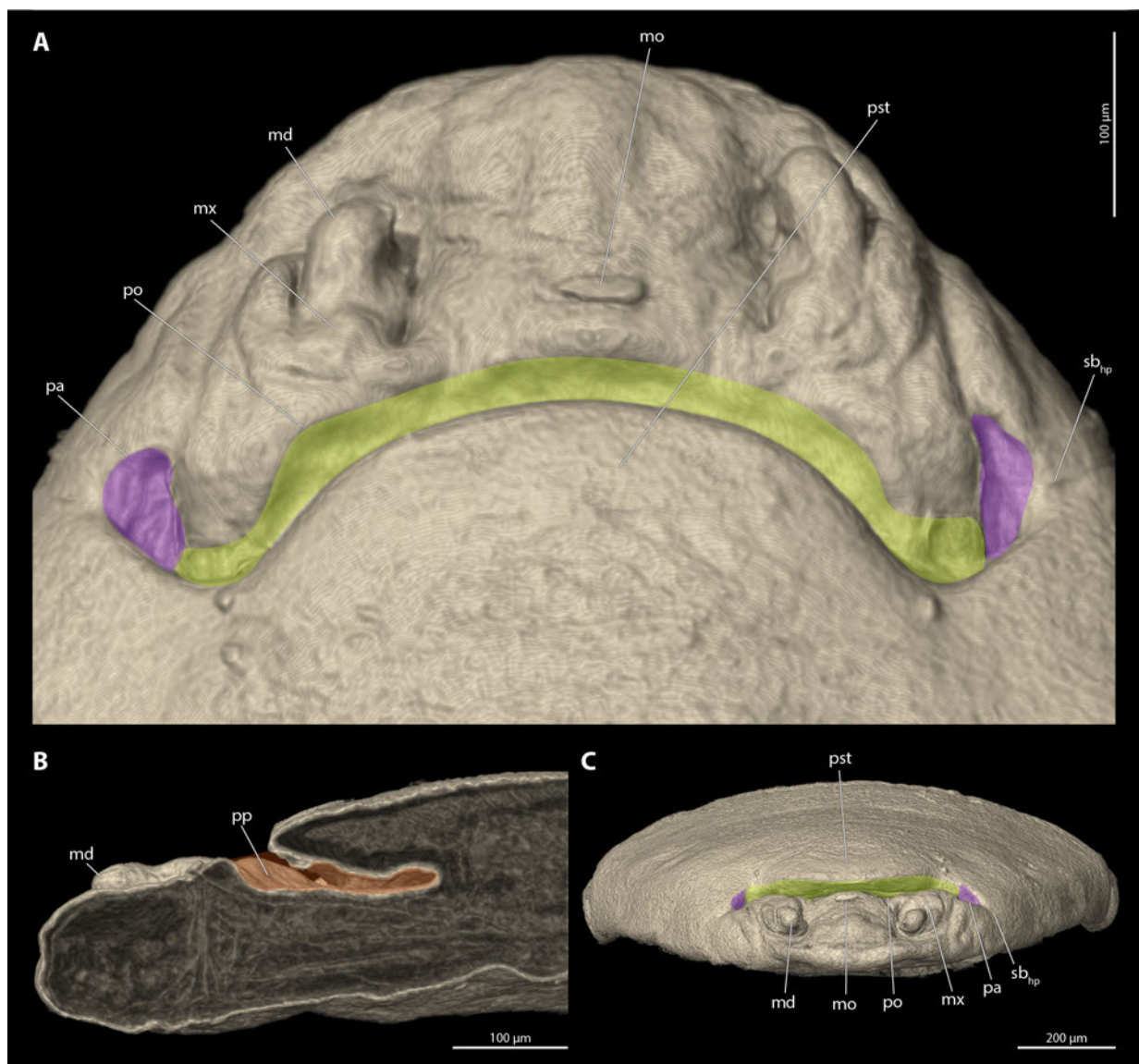
Figure 1. Cephalothorax (larval exuvia removed) of *S. mellittae* illustrating the general morphology of the paragenital organ. A: Ventral view. **B:** Medio-sagittal section. **C:** Frontal view. Shown are the opening of the paragenital organ (green), the paragenital auricles (pink), and the paragenital pouch (orange). Abbreviations: md – mandible, mo – mouth opening, mx – maxilla, pa – paragenital auricle, po – paragenital opening, pp – paragenital pouch, pst – prosternum, sbhp – segmental boundary between head and prothorax.

3D models

Analysed 3D models of penetration sites were imported into Blender 2.82.7 (Blender Foundation, Amsterdam, The Netherlands) and aligned with models of the corresponding female's cephalothorax. All models were uploaded to Sketchfab using the Sketchfab Blender Addon 1.5.0 (The Khronos Group Beaverton, USA).

Image processing. Adobe Photoshop 21.2.1 (Adobe Systems, San Jose, USA) was used to process Images and arrange them into plates. Adobe Illustrator 24.2.1 (Adobe Systems, San Jose, USA) was used for labelling plates and drawings.

Results

The results are presented in tabular form to provide a comparable overview of the species studied. A detailed description of each species in terms of paragenital organ and integument thickness can be found in the supplementary material.

General description of the paragenital organ (Fig. 1)

The paragenital organ is an invagination of the integument of the adult female, located on the ventral side between the head and the prothorax. Its opening is transverse and fissure-like, located on the ventral side, extending almost from one side to the other in most species studied. At each lateral end of the opening there are membranous folds (hereafter referred to as paragenital auricles), which in various species have strongly sclerotised cuticular projections of the secondary larval exuvia extending into their lumen (Fig. 2). These lateral exuvial projections are absent in *Halictoxenos*, weakly developed in *Kinzelbachus* and *Eurystylops*, and distinct in *Stylops* (Fig. 2). The paragenital opening is externally covered by a thin membrane of the secondary larval exuvia (e.g., approximately 2 μm thick in *S. ovinae*; (Löwe et al., 2016; Peinert et al., 2016; Richter et al., 2017). This membrane is ruptured when the penis enters the paragenital during copulation. The paragenital extends into the body lumen as a shallow pouch, reaching different depths in different species. In the case of deep invaginations (found in members of the genus *Stylops*), the pouch extends obliquely towards the dorsal side of the female body and ends in the prothorax.

We found that the cuticle of the ventral wall of the paragenital organ was always signifi-
cantly thickened compared to the dorsal wall and to other cuticular areas, such as the
anterior region of the brood canal. On each side of the paragenital opening, the process-
es of the secondary larval exuvia extend into the paragenital pouch to varying degrees.
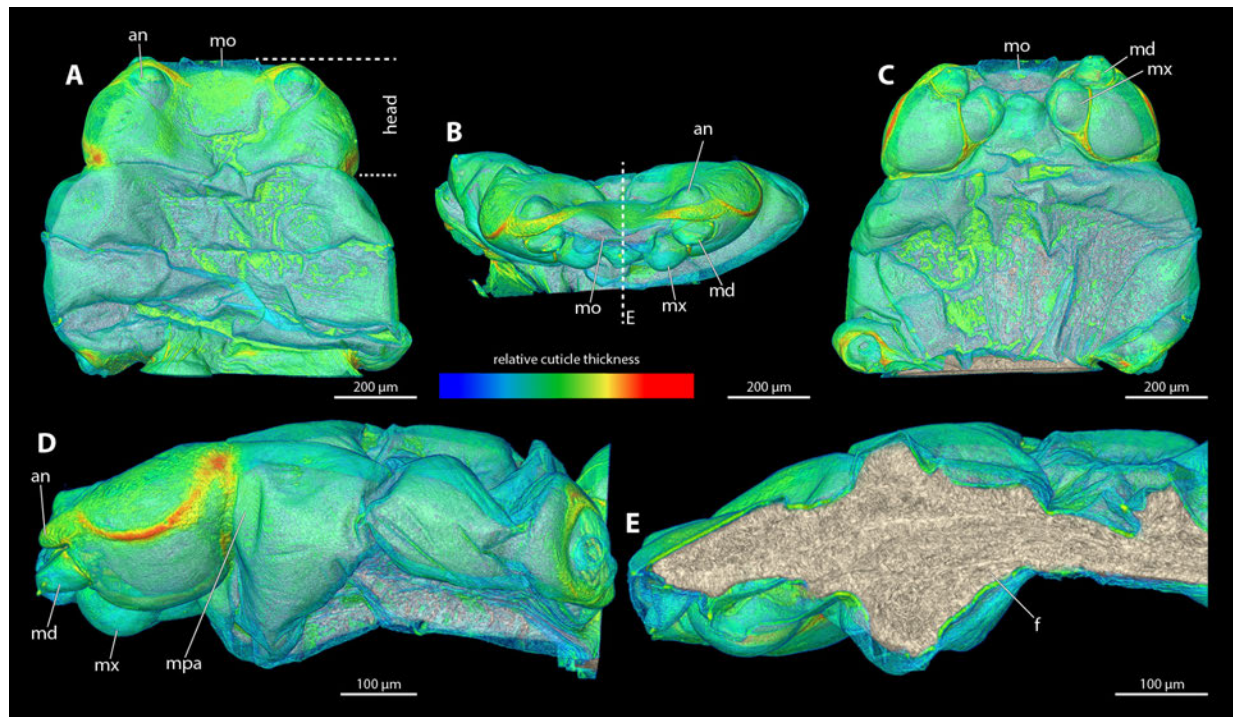
Figure 3. Thickness of the cephalothorax cuticle of *Triocozera macroscyti*. A: Exuvia of female sec-
ondary larva (dorsal view). **B:** Exuvia of female secondary larva (frontal view). **C:** Female cephalothorax
with exuvia of secondary larva (ventral view). **D:** Female cephalothorax with exuvia of secondary larva
(lateral view). **E:** Female cephalothorax (beige) within exuvia of secondary larva in colour correlated with
thickness of the exuvia (medio-sagittal section). Blue colour indicates a thin cuticle, red indicates a thick
cuticle. Abbreviations: an – antennal bud, f – female, md – mandible, mo – mouth opening, mpa – mem-
branous pleural area, mx – maxilla.

Table 2. Summary of the results of this study. A detailed description to each species studied in relation to the information in this table is given in the supplementary material. A "—" indicates missing information; CL – cephalothorax lenght.

Species	Paragenital organ	Shape of paragenital opening	Paragenital auricles	Paragenital pouch	Lateral exuvial process	Integument thickness	Fig.
Triozocera macroscyti	absent	absent	absent	absent	absent	thin area around the mouth	3
Crawfordia warnckei	absent	absent	absent	absent	absent	—	4
Eurystylops **sp.**	present	sinuate in ventral view, with a flat and elongated apex	absent	weakly pronounced, 12.9 % of CL	present	ventral wall of paragenital organ	2, 4
Halictoxenos arnoldi	present	flattened arch in ventral view	absent	weakly pronounced, 3.3 % of CL	absent	—	5
Halictoxenos simplicis	present	arcuate and very slit	absent	weakly pronounced, 11.4 % of CL	absent	ventral wall of paragenital organ, with a massive lobe	5
Hylecthrus rubi	present	very slightly curved and is bent posterad	absent	reaches deep into the body cavity, 25 % of CL	absent	ventral wall of paragenital organ, with a massive lobe	6
Kinzelbachus friesei	present	strongly arched, with a flat apex	weakly developed	does not reach deep into the body cavity, 6.3 % of CL	present	very slightly thickened	2, 6
Stylops hammella	present	slightly arched with a flattened apex	weakly developed	reaches deep into the body cavity, 20.6 % of CL	present	ventral and dorsal walls of the paragenital organ	2, 7

Species						
Stylops japonicus	present	arched with a flattened apex	well developed	reaches deep into the body cavity, 16.7 % of CL	present	ventral wall of the paragenital organ	2, 7
Stylops melittae	present	curved	well developed	reaches deep into the body cavity, 21 % of CL	present	ventral wall of the paragenital organ	2, 8
Stylops nassonowi	present	almost straight with only a slight curvature	well developed	reaches deep into the body cavity, 20 % of the CL	present	ventral wall of the paragenital organ	2, 8
Stylops ovinae	present	almost straight with only a slight curvature	well developed	reaches deep into the body cavity, 20.7 % of CL	present	ventral wall of the paragenital organ	2, 9
Stylops spreta	present	slightly concave at the apex and trapzoidal in appeaance	does not reach deep into the body cavity	does not reach deep into the body cavity, 12.1 % of CL	present	does not reach deep into the body cavity	2, 9
Paraxenos erberi	absent	absent	absent	absent	absent	—	10
Tuberoxenos sphecidarum	absent	absent	absent	absent	absent	—	10
Pseudoxenos schaumii	absent	absent	absent	absent	absent	—	10
Xenos vesparum	absent	absent	absent	absent	absent	anterior parts of the brood canal	11

Stylopidae
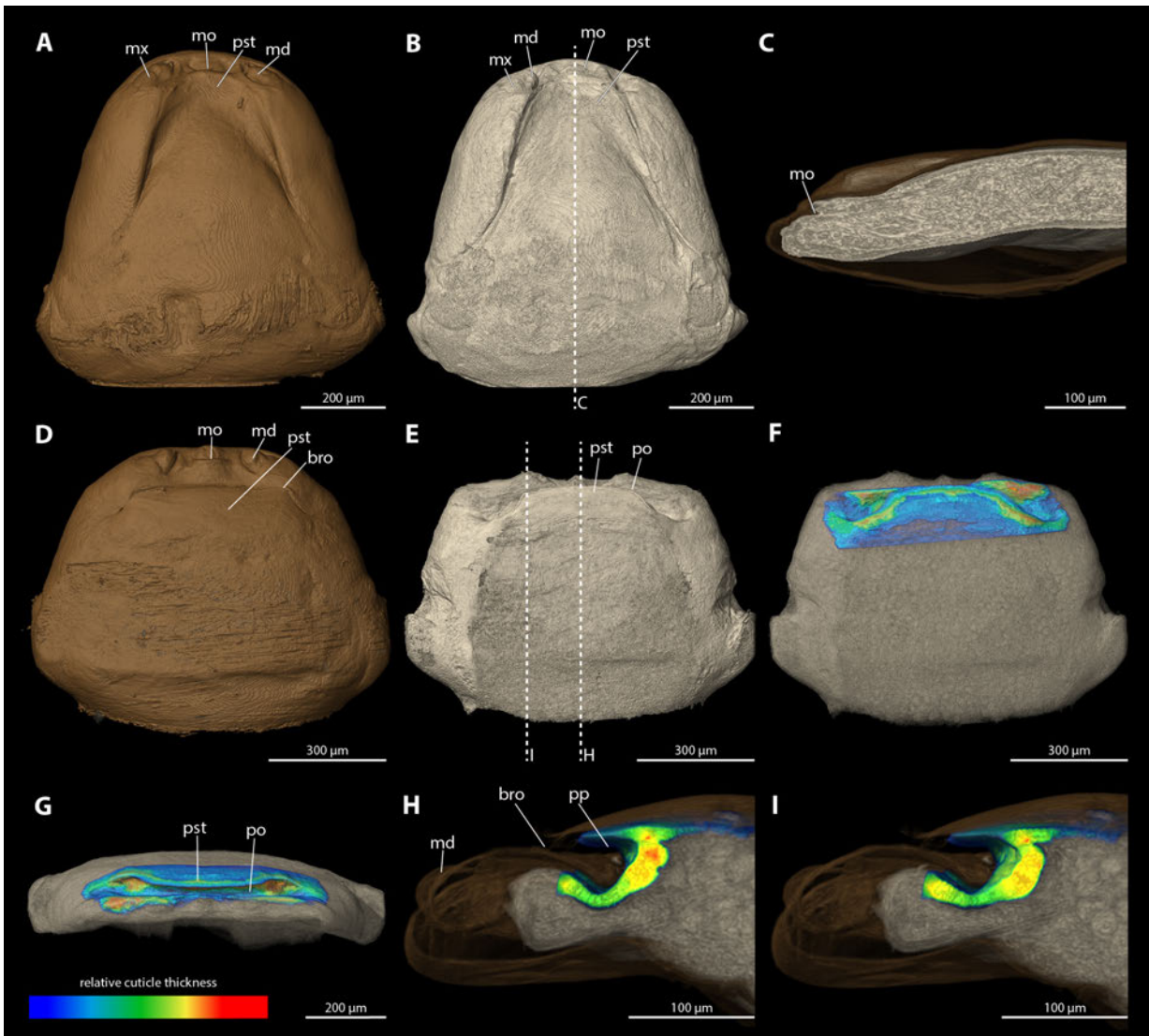
Figure 4. The female cephalothoraces of *Crawfordia warnckei* (A–C) and *Eurystylops* sp. (D–I) with the ventral side facing upwards. The thickness of the cuticle is indicated by colour. Blue areas represent thin cuticular regions, while red areas represent thick cuticular regions. The available μCT data did not allow measurements of the integument thickness of Crawfordia warnckei. **A, D:** Exuvia of the secondary larva. **B, E:** Female cephalothorax, exuvia of secondary larva removed. C: Female cephalothorax (medio-sagittal section). F: Female cephalothorax. **G:** Frontal view. **H:** Medio-sagittal section. **I:** Sagittal view. Abbreviations: bro – birth opening, md – mandible, mo – mouth opening, po – paragenital opening, pp – paragenital pouch, pst – prosternum.
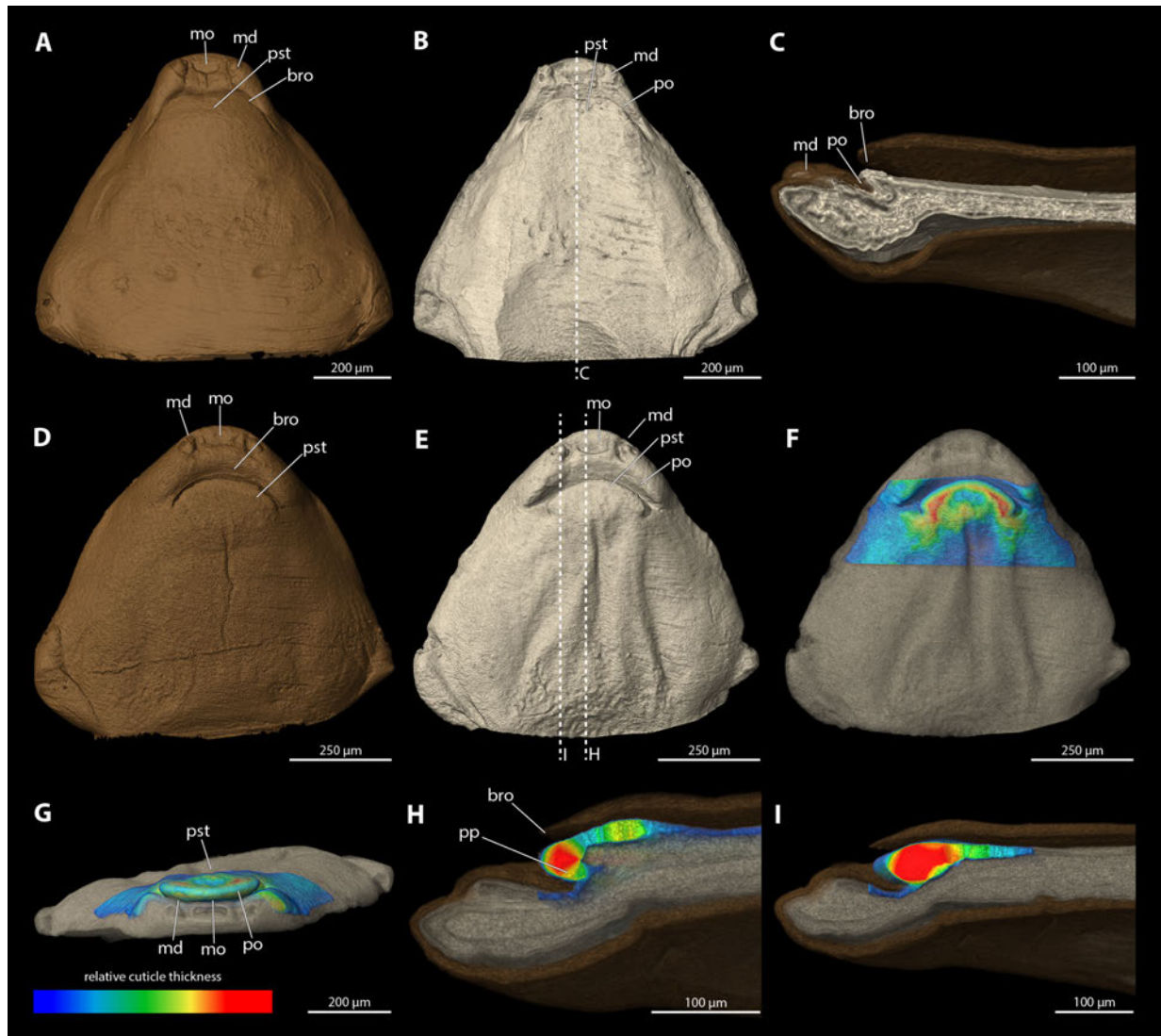
Figure 5. The female cephalothoraces of *Halictoxenos arnoldi* (A–C) and *Halictoxenos simplicis* (D–I), with the ventral side facing upwards. The thickness of the cuticle is indicated by colour. Blue areas represent thin cuticular regions, while red areas represent thick cuticular regions. The available μCT data did not allow measurements of the integument thickness of Halictoxenos arnoldi. **A, D:** Exuvia of the secondary larva. **B, E:** Female cephalothorax, exuvia of secondary larva removed. **C:** Female cephalothorax (medio-sagittal section). **F:** Female cephalothorax. **G:** Frontal view. **H:** Medio-sagittal section. **I:** Sagittal view. Abbreviations: bro – birth opening, md – mandible, mo – mouth opening, po – paragenital opening, pp – paragenital pouch, pst – prosternum.
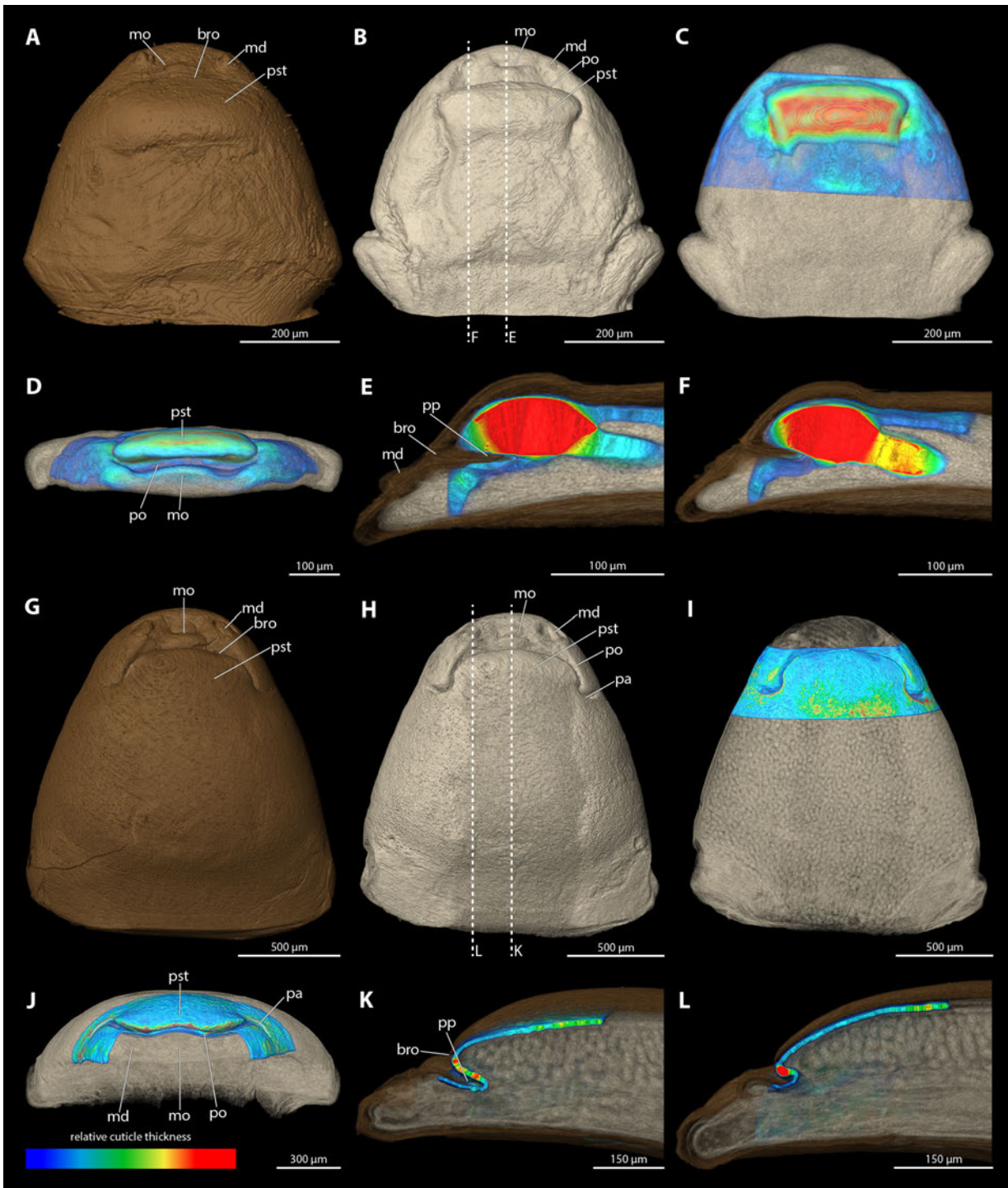
Figure 6. The female cephalothoraces of *Hylecthrus rubi* (A–F) and *Kinzelbachus friesei* (G–L), with the ventral side facing upwards. The thickness of the cuticle is indicated by colour. Blue areas represent thin cuticular regions, while red areas represent thick cuticular regions. **A, G:** Exuvia of the secondary larva. **B, H:** Female cephalothorax, exuvia of secondary larva removed. **C, I:** Female cephalothorax. **D, J:** Frontal view. **E, K:** Medio-sagittal section. **F, L:** Sagittal view. Abbreviations: bro – birth opening, md – mandible, mo – mouth opening, pa – paragenital auricles, po – paragenital opening, pp – paragenital pouch, pst – prosternum.
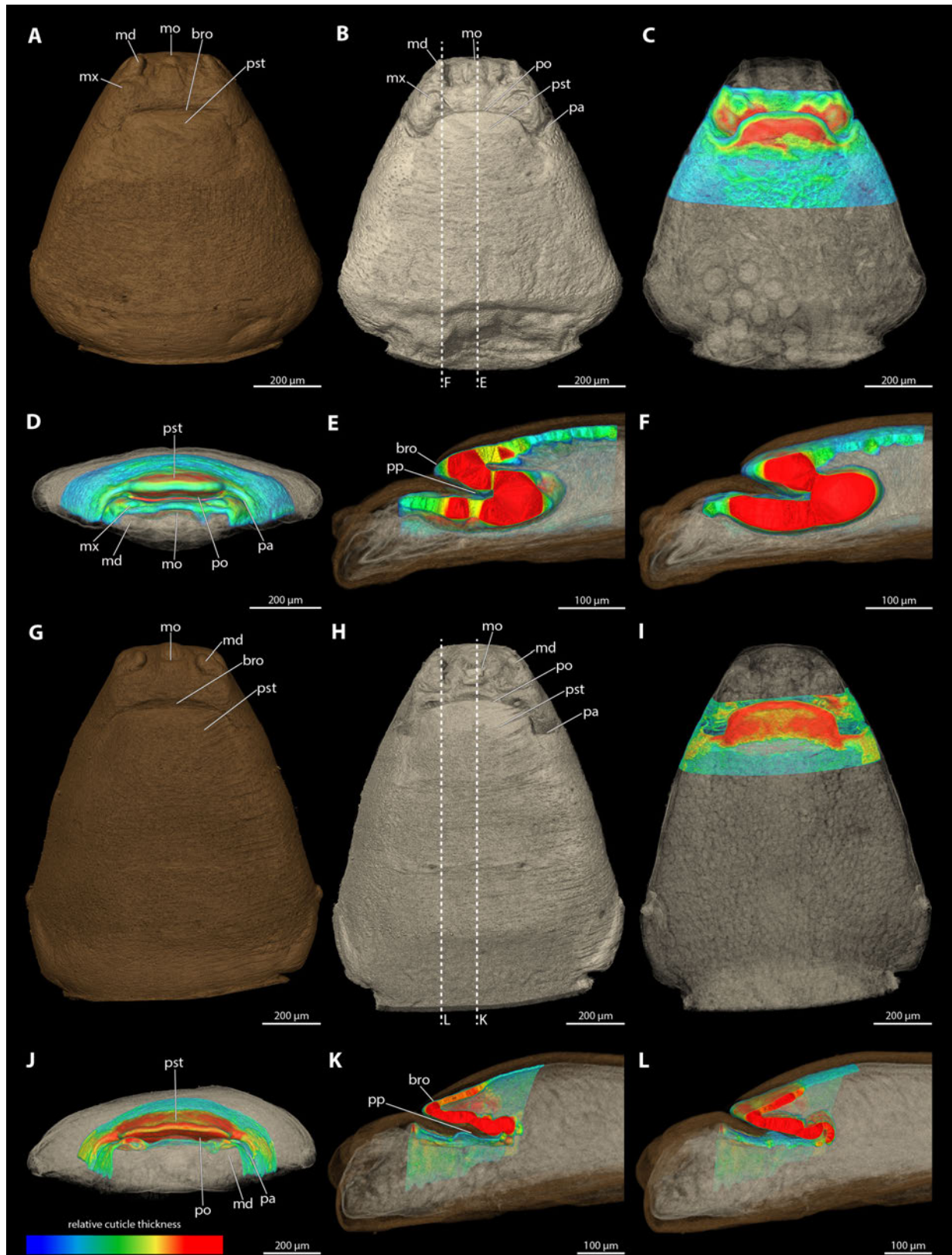
Figure 7. The female cephalothoraces of *Stylops hammella* **(A–F) and** *Stylops japonicus* **(G–L), with the ventral side facing upwards.** The thickness of the cutcile is indicated by colour. Blue areas represent thin cuticular regions, while red areas represent thick cuticular regions. **A, G:** Exuvia of the secondary larva. **B, H:** Female cephalothorax, exuvia of secondary larva removed. **C, I:** Female cephalothorax. **D, J:** Frontal view. **E, K:** Medio-sagittal section. **F, L:** Sagittal view. Abbreviations: bro – birth opening, md – mandible, mo – mouth opening, mx – maxilla, pa – paragenital auricles, po – paragenital opening, pp – paragenital pouch, pst – prosternum.
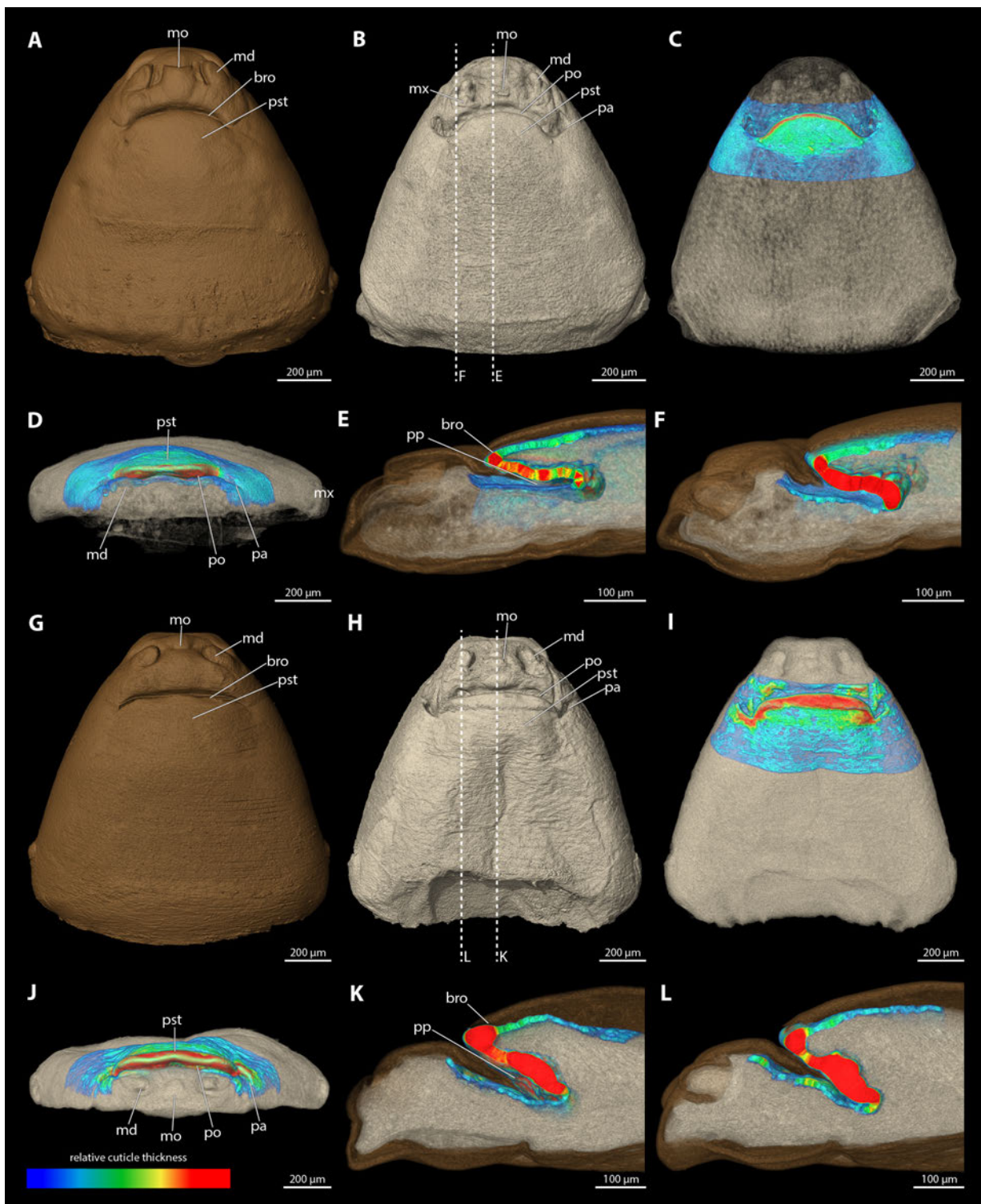
Figure 8. The female cephalothoraces of *Stylops melittae* (A–F) and *Stylops nassonowi* (G–L), with the ventral side facing upwards. The thickness of the cuticle is indicated by colour. Blue areas represent thin cuticular regions, while red areas represent thick cuticular regions. **A, G:** Exuvia of the secondary larva. **B, H:** Female cephalothorax, exuvia of secondary larva removed. **C, I:** Female cephalothorax. **D, J:** Frontal view. **E, K:** Medio-sagittal section. **F, L:** Sagittal view. Abbreviations: bro – birth opening, md – mandible, mo – mouth opening, mx – maxilla, pa – paragenital auricles, po – paragenital opening, pp – paragenital pouch, pst – prosternum.
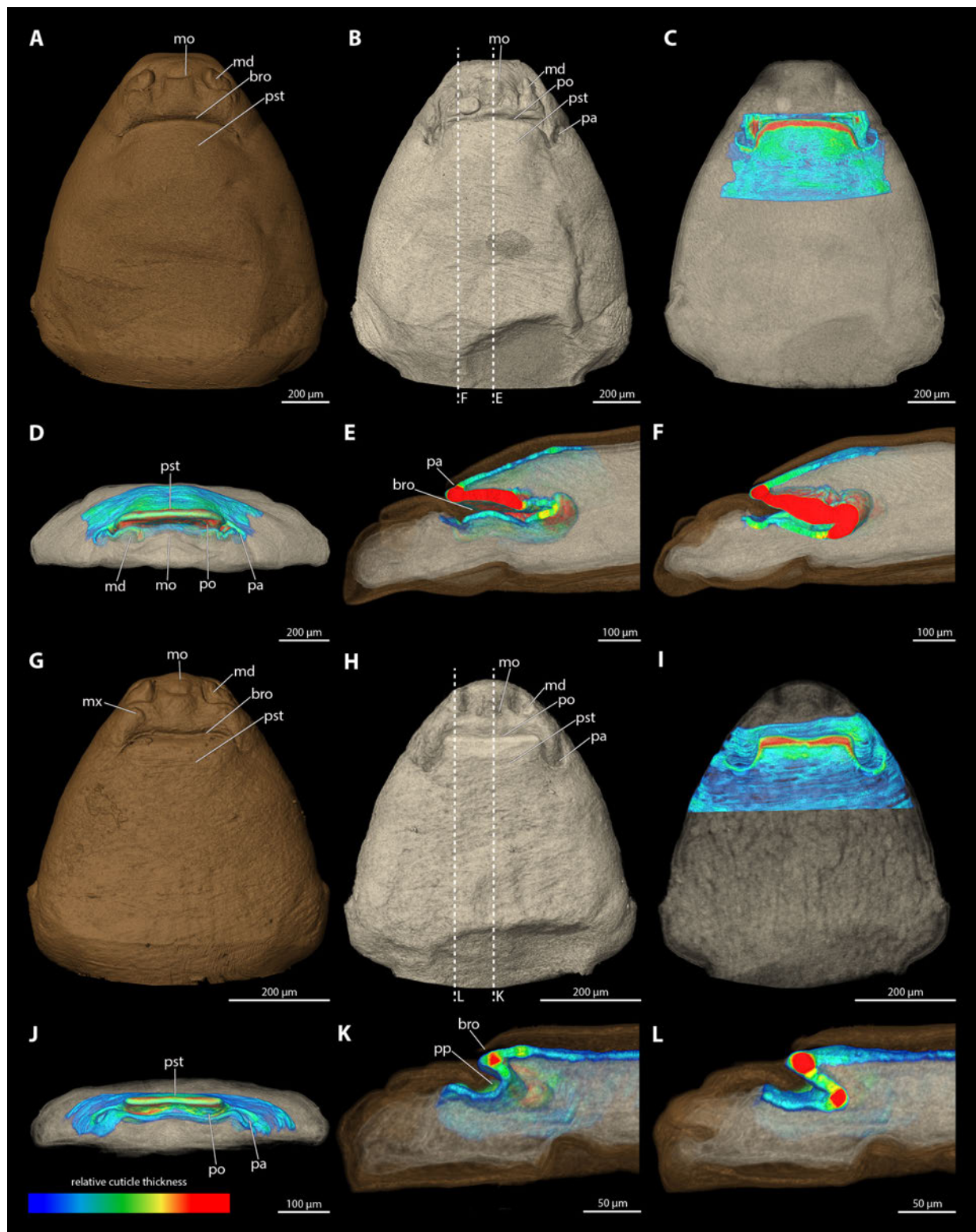
Figure 9. The female cephalothoraces of *Stylops ovinae* (A–F) and *Stylops spreta* (G–L), with the ventral side facing upwards. The thickness of the cuticle is indicated by colour. Blue areas represent thin cuticular regions, while red areas represent thick cuticular regions. **A, G:** Exuvia of the secondary larva. **B, H:** Female cephalothorax, exuvia of secondary larva removed. **C, I:** Female cephalothorax. **D, J:** Frontal view. **E, K:** Medio-sagittal section. **F, L:** Sagittal view. Abbreviations: bro – birth opening, md – mandible, mo – mouth opening, mx – maxilla, pa – paragenital auricles, po – paragenital opening, pp – paragenital pouch, pst – prosternum.
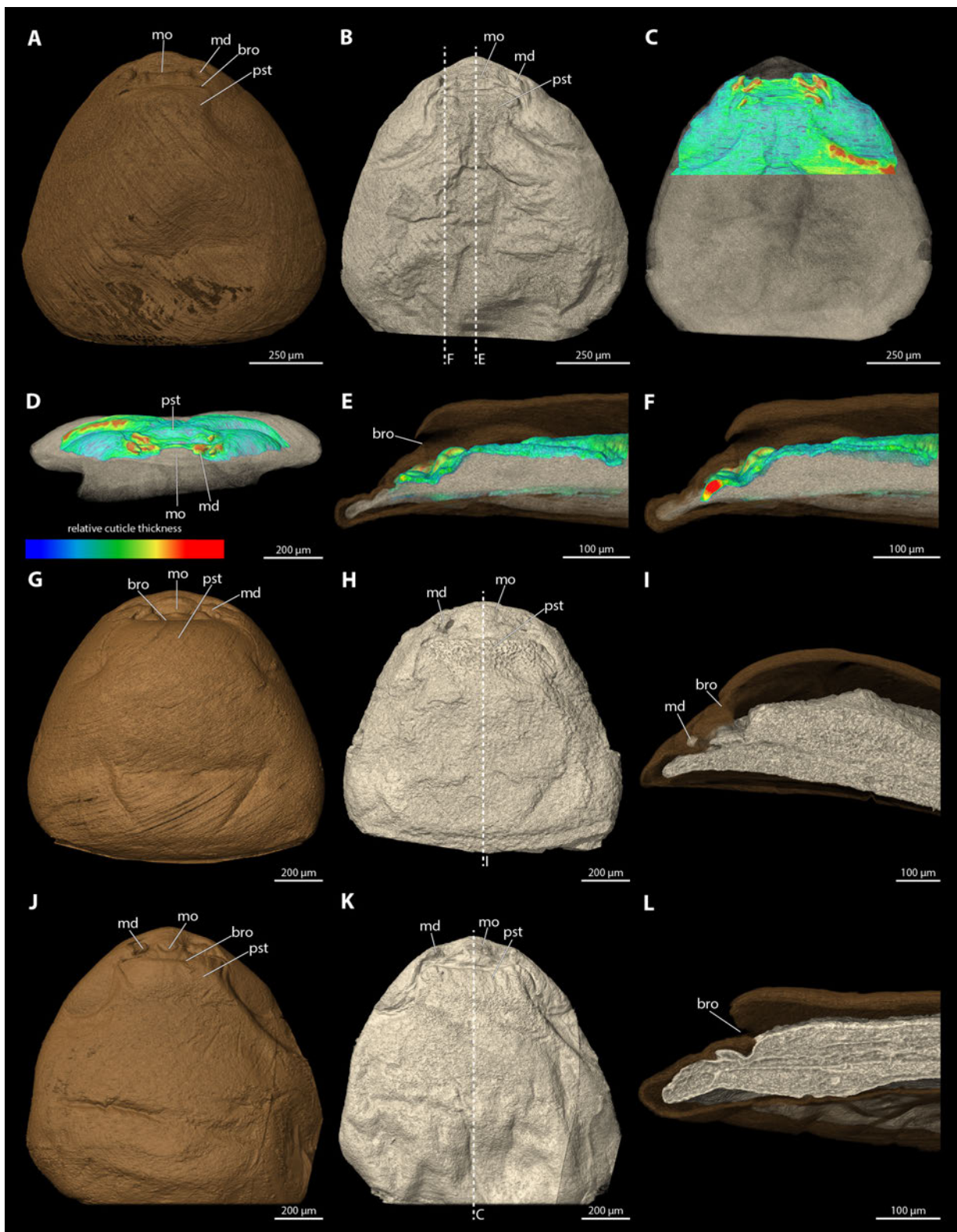
Figure 10. The female cephalothoraces of *Paraxenos erberi* (A–F), *Tuberoxenos sphecidarum* (G–I), and *Pseudoxenos schaumii* (J–L), with the ventral side facing upwards. The thickness of the cuticle is indicated by colour. Blue areas represent thin cuticular regions, while red areas represent thick cuticular regions. The available µCT data did not allow measurements of the integument thickness of Tuberoxenos sphecidarum and Pseudoxenos schaumii. **A, G, J:** Exuvia of the secondary larva. **B, H, K:** Female cephalothorax, exuvia of secondary larva removed. **C:** Female cephalothorax **D:** Frontal view. **E:** Medio-sagittal section. **F:** Sagittal view. **I, J:** Female cephalothorax (medio-sagittal section). Abbreviations: bro – birth opening, md – mandible, mo – mouth opening, pst – prosternum.
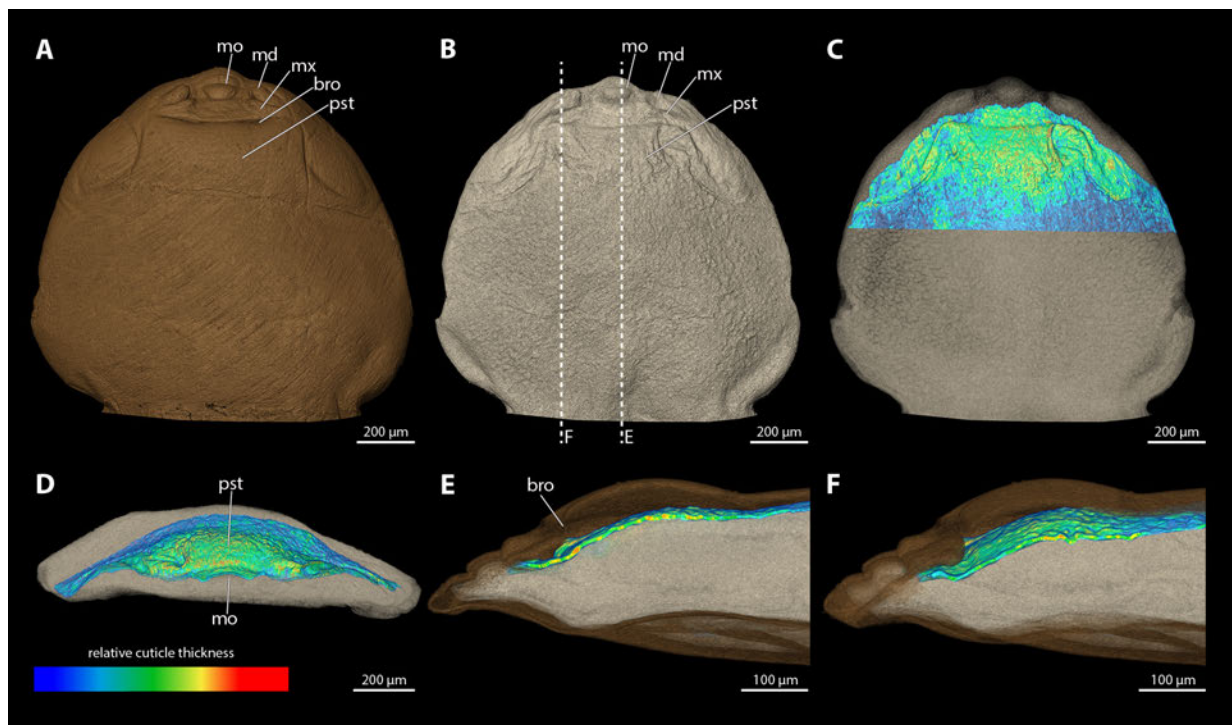
Figure 11. The female cephalothorax of *Xenos vesparum*, with the ventral side facing upwards. The thickness of the cuticle is indicated by colour. Blue areas represent thin cuticular regions, while red areas represent thick cuticular regions. **A:** Exuvia of the secondary larva. **B:** Female cephalothorax, exuvia of secondary larva removed. **C:** Female cephalothorax **D:** Frontal view. **E:** Medio-sagittal section. **F:** Sagittal view. Abbreviations: bro – birth opening, md – mandible, mo – mouth opening, mx – maxilla, pst – prosternum.

Discussion

As previously noted by various authors (Lauterbach, 1954; Kinzelbach, 1971; Kinzelbach, 1978; Löwe et al., 2016; Jandausch et al., 2022; Jandausch et al., 2023), a paragenital organ appears to be present in all species of the genus *Stylops*. The organ varies in its length, the depth of its pouch, and the shape of its opening. Characteristic of *Stylops* compared to other genera of Stylopidae are the distinct lateral auricles at the lateral ends of the paragenital opening.

In addition to the genus *Stylops*, we also detected the paragenital organ in selected species of the genera *Eurystylops*, *Halictoxenos*, *Hylecthrus*, and *Kinzelbachus*. Compared to the paragenital in the genus *Stylops*, the paragenital in these genera differs in two aspects; (I) the paragenital organ extends less deeply into the cephalothorax of the female than *Stylops* species, (II) the lateral auricles are completely absent in all genera except *Stylops*. The genus *Crawfordia* is an exception, as its species lack the paragenital organ.

Considering our results in the context of the current phylogeny of Strepsiptera (Jůzová, Nakase & Straka, 2015; Pohl et al., 2021; Benda et al., 2022; Jandausch et al., 2023), we interpret the paragenital organ as a potential autapomorphy of the Stylopidae. *Kinzelbachus*, which we found to possess a paragenital organ is considered as sistergroup of all remaining Stylopidae (Jůzová, Nakase & Straka, 2015). *Crawfordia*, which is the only genus of Stylopidae investigated in our study that lacks a paragenital organ, is nested inside Stylopidae. We therefore hypothesis that the paragenital organ is reduced in *Crawfordia*. Furthermore, our data suggest that the lateral auricle of the paragenital organ is an autapomorphy of the genus *Stylops*. Morphological studies on females of other families do not provide evidence for the occurrence of comparable structures outside the Stylopidae (e.g., Kirkpatrick, 1937; Silvestri, 1943; Baumert, 1958; Kinzelbach, 1971; Pohl, Katbeh-Bader & Schneider, 1996; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Pohl et al., 2012; Löwe et al., 2016; Tröger et al., 2023). We could not find a paragenital organ in Corioxenidae (*Triozocera*) and Xenidae (*Paraxenos*, *Pseudoxenos*, *Tuberoxenos* and *Xenos*).

The reproductive mode of traumatic insemination is costly for mated females (Morrow & Arnqvist, 2003; Lange et al., 2013; Tatarnic, Cassis & Siva-Jothy, 2014; Michels et al., 2015; Reinhardt, Anthes & Lange, 2015). Paragenital organs occur in females of many species of the Heteroptera superfamily Cimicoidea, and their occurrence has been found to be strongly associated with traumatic insemination (Tatarnic et al., 2014; Reinhardt et al., 2015; Jung et al., 2023). Since traumatic insemination is the general copulatory mode in Strepsiptera and the paragenital organ only occurs in Stylopidae, it is not a driving adaptation in cost reduction for females. In contrast, it is conceivable that cuticular thickening we have demonstrated in this order represents the cost-reducing adaptation, which

is consistent with the conclusions drawn from microindentation experiments carried out on *S. ovinae* and *X. vesparum* by Jandausch et al. (2022). They have shown that Strepsiptera females potentially reduce the costs of traumatic insemination by thickening the integument and thus tolerate the costs induced by copulation. Like other authors before (Richter et al., 2017; Jandausch et al., 2022; Jandausch et al., 2023), we found a thickening of the integument in the anterior brood canal of *X. vesparum* (Xenidae). However, a thickening of the integument at the penetration sites has also been observed in Elenchidae (Baumert, 1958; Jandausch et al., 2023) and Halictophagidae (Jandausch et al., 2023) and is therefore apparently functionally related to traumatic insemination in Strepsiptera.

The genus *Triozocera* must be considered with special attention, as Corioxenidae lack a birth opening, which is otherwise a groundplan condition of Stylopidia. The penis of the male Corioxenidae pierces the sclerotized exuvia of the female secondary larva before the enclosed female can be penetrated. It should be noted in this context that in other families of Strepsiptera whose species have a birth opening (e.g., Xenidae or Stylopidae), the penis also pierces the exuvia of the secondary larva, but at a region where the cuticle is very thin (Löwe et al., 2016; Peinert et al., 2016; Richter et al., 2017; Jandausch et al., 2023). Therefore, we expected the areas of Corioxenidae larval exuvia to be particularly thin at typical penetration sites, namely the oral region or the membranous pleural areas of the cephalothorax. Interestingly, a thin integument is only found in the mouth region, but not in the pleural areas. We consider two possible alternative reasons for this pattern: 1) mating in the genus *Triozocera* takes place exclusively in the oral region (Kirkpatrick, 1937; Pohl & Beutel, 2008), 2) the membranous pleural areas vary in material composition rather than in their thickness. Varying material composition is a known condition in the bed bug *Cimex lectularius* (Michels et al., 2015), whose integument of the spermalege (paragenital organ) is softened by resilin. Although further studies in more families and different genera of Strepsiptera are needed to evaluate these two alternative explanations, we hypothesize that the thickening of the resilin-rich integument in Strepsiptera is the primary cost-reducing adaptation to traumatic insemination. Even a thickened integument on the ventral wall of the paragenital organ may be a coincidence, since the paragenital organ serves as a penetration site.

The paragenital organ of the Stylopidae plays a crucial role as a penetration site during copulation, as convincingly demonstrated by Peinert et al. (2016). Jandausch et al. (2022) showed that females of *S. ovinae* attract heterospecific males via a pheromone signal (Cvačka et al., 2012; Tolasch, Kehl & Dötterl, 2012), but cannot mate with them. Consequently, Jandausch et al. (2022) hypothesised that the paragenital organ allows the prevention of heterospecific mating. Our data presented here provide further support for this interpretation. The variability of the paragenital organs and the variation

in penis shape of different *Stylops* species (paragenital organs: Figs. 7–9; penises: Fig. 12) suggest a precopulatory mating barrier. This may be achieved by a mechanical interaction between the paragenital pouch and the male's penis (lock and key). The length of the acumen penetrating the thickened integument of the paragenital organ may contribute to this barrier (Fig. 13). It is conceivable that all these factors have a cumulative effect. Whether mechanical barriers that prevent heterospecific mating also occur in other Strepsiptera genera should be addressed in future studies.
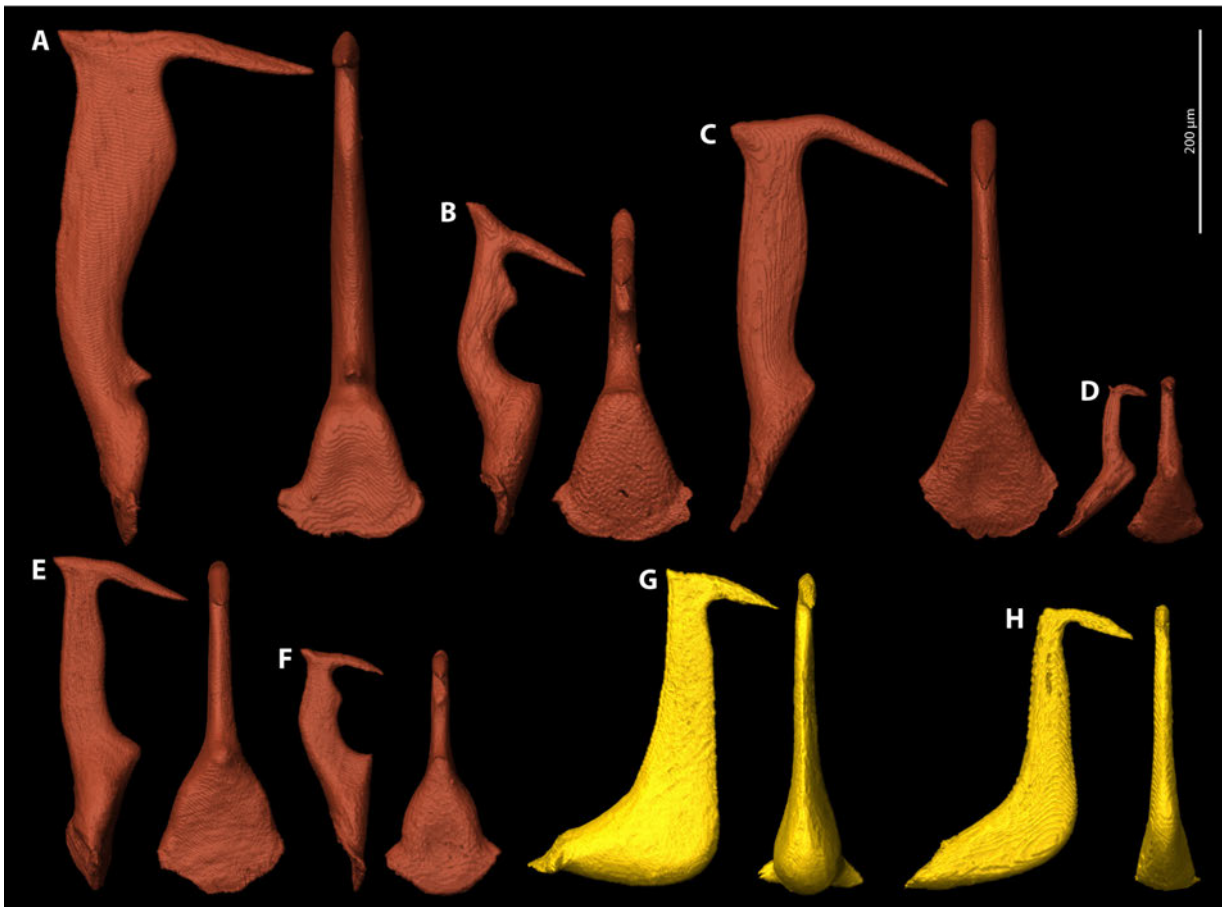
Figure 12. 3D reconstructions of penises of Stylopidae (red) and Xenidae (yellow) in lateral view (left) and frontal view (right). A: *Stylops ovinae* **B:** *Stylops melittae* **C:** *Stylops nassonowi* **D:** S*tylops spreta*m **E:** *Stylops japonicus* **F:** *Stylops hammella* **G:** *Xenos vesparum* **H:** *Tuberoxenos sphecidarum* An interactive version of these models is available in the HTML version of this article online and on Sketchfab at: https://sketchfab.com/entomology_uni_jena/collections/strepsiptera-penises-79603a06f3c74a2aacf76d98e875202e.
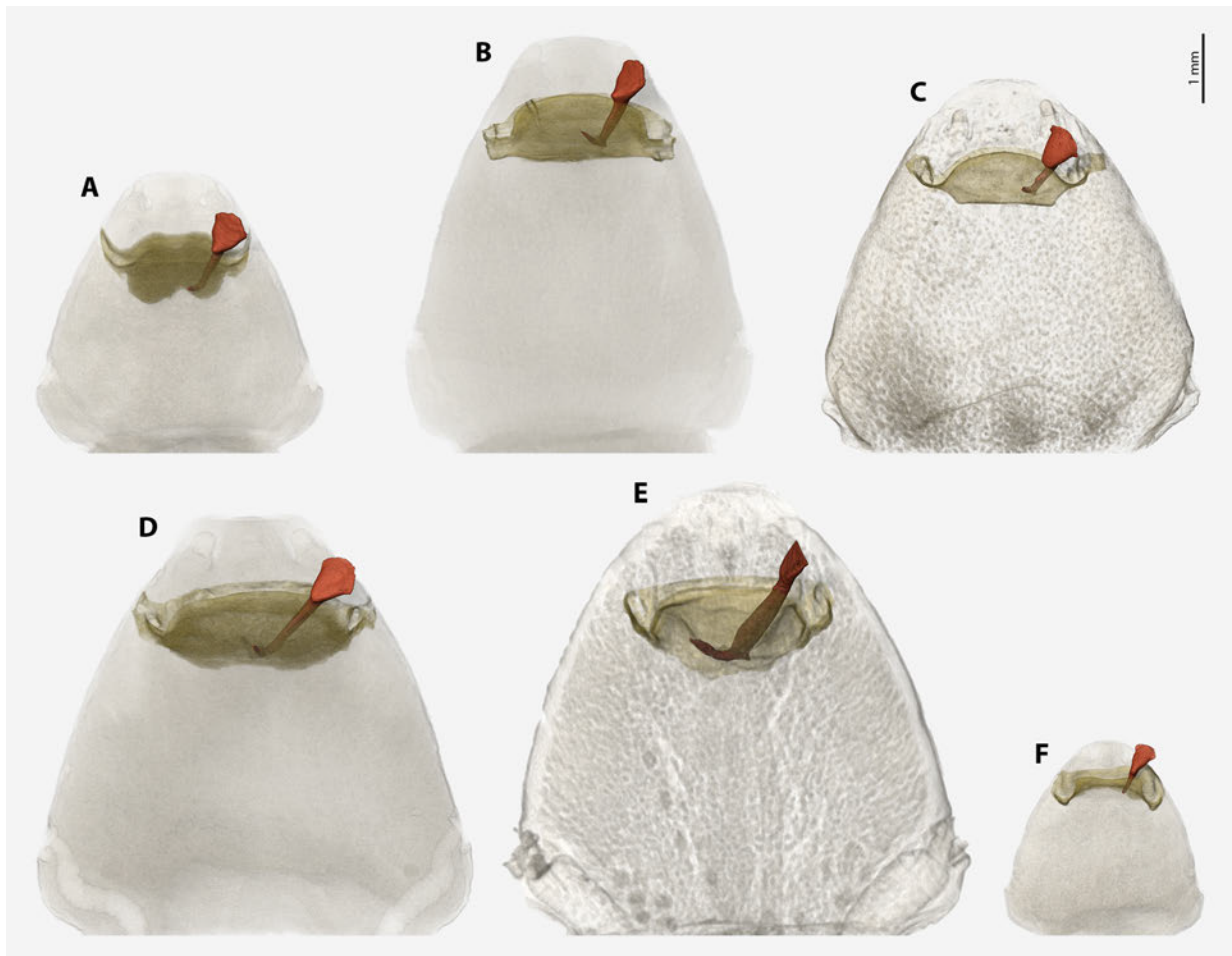
Figure 13. Pairs of female cephalothoraces (outer cuticle of the cephalothorax removed) and male penises. A: *Stylops hammella* **B:** *Stylops japonicus* **C:** *Stylops melittae* **D:** *Stylops nassonowi* **E:** *Stylops ovinae* **F:** *Stylops spreta* Paragenital organs highlighted in ochre, penises in red and orange. *S. ovinae* was scanned in copula (Peinert et al., 2016). The penises of *S. hammella*, *S. japonicus*, *S. melittae*, *S. nassonowi*, and *S. spreta* were virtually inserted into the corresponding female paragenital organs of conspecifics to assess the fit of these genital structures.

The lateral processes of the secondary larval exuvia, which were described by Kinzelbach (1971) as elements of the tentorium, are related to the paragenital organ. As these structures are not located in the head or cephalic region, we reject the homology statement of Kinzelbach (1971) and suggest a functional relationship between these structures and the paragenital organ. The lateral processes occur exclusively in females of Stylopidae with a paragenital organ. We found that the length and the thickness of these processes increase with increasing depth of the paragenital organ. Females of *Eurystylops*, for example, have only a very shallow paragenital pouch and weakly developed lateral processes of the larval exuvia. In contrast, in species of the genus *Stylops*, the paragenital pouch extends deeply into the cephalothoracic lumen, where also more prominent processes of the exuvia are present. It seems plausible that the lateral projection of

these structures into the paragenital organ stabilises the pouch and thus keeps it open for penetration. If the dorsal and ventral walls of the paragenital organ were to collapse due to a lack of stability, this could interfere with the copulation process by making penal penetration more difficult and thus interfering with fertilisation.

Conclusion

We propose that the presence of a female paragenital organ is a potential autapomorphy of the Stylopidae. In contrast, the paragenital organ is confirmed to be absent in the studied species of Xenidae, the sister group of Stylopidae, and is also absent in species of other families of Stylopidia. Compared to the surrounding cuticular regions, the paragenital organ of Stylopidae is characterised by structural modifications, in particular a very pronounced thickening of the integument. Despite the lack of a paragenital organ, a thickened cuticle at the penetration site is also observed in *Elenchus tenuicornis*, *Halictophagus*, *X. vesparum*. We also showed that the exuvia of the secondary larva acquired a new function in species with a paragenital organ, namely the stabilisation of the paragenital pouch. Finally, we found that the paragenital organ is likely to be stabilised by lateral exuvial processes that facilitate the intromission of the male penis into the paragenital pouch.

Acknowledgement

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Supplementary Information

The paragenital organ of Stylopidae (Insecta: Strepsiptera) and the functional incorporation of the exuvia of the secondary larva

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A detailed description to each species studied in relation to the information given in table 1.

Corioxenidae

Triozocera macroscyti **(Fig. 3)**

The paragenital organ is absent in *Triozocera macroscyti*. The exuvia of the secondary larvae lacks a brood canal opening. Since penetration in Corioxenidae occurs by piercing the female's exuvia of the secondary larva, we examined the thickness of the female's exuvia. We identified a weakly sclerotized area in the oral region and found that the integument in membranous pleural areas was not thickened in compared to surrounding regions.

Stylopidae

Crawfordia warnckei **(Fig. 4)**

The paragential organ is missing. The available µCT data did not allow measurements of the integument thickness.

Eurystylops **sp. (Fig. 4)**

The paragenital organ is present. The paragenital opening is sinuate in ventral view, with a flat and elongated apex. The shape of the overlying birth opening reflects the shape of the paragenital's opening, which is slightly more angular and thus appears trapezoidal. Lateral auricles of the paragenital organ are absent. The paragenital pouch is weakly developed (12.9 % of the cephalothorax length). The cuticle is strongly thickened along the entire paragenital organ, but especially on its ventral wall. The greatest thickness is found in the lateral areas of the ventral wall in the region of the lateral opening of the paragenital organ.

Halictoxenos arnoldi **(Fig. 5)**

The paragenital organ is present. The paragenital opening appears like a flattened arch in ventral view. The birth orifice mirrors the shape of the opening of the paragenital opening. The lateral auricles of the paragenital organ are absent. The paragenital pouch is weakly developed and does not extend far into the cephalothorax (3.3% of the cephalothorax length). The available µCT data did not allow to measure the thickness of the integument.

Halictoxenos simplicis **(Fig. 5)**

The paragenital organ is present. The paragenital opening is arched and strongly fissured. The birth opening above is much narrower but of the similar shape to the paragenital opening. The lateral auricles of the paragenital organ are absent. The pouch of the paragenital organ is shallow (11.4% of the length of the cephalothorax). The ventral wall of

the paragenital organ is strongly thickened and forms a massive mushroom-shaped lobe with the anteriormost parts of the brood canal. The ventral wall of the paragenital organ was found to be very thick. The thickness of the cuticle in the anterior parts of the brood canal decreases rapidly and continuously posteriorly. The dorsal wall of the paragenital organ is thin and completely covered by parts of the exuvia of the secondary larva.

Hylecthrus rubi (Fig. 6)

A paragenital organ is present. The opening is very slightly curved and is bent posterad. The birth opening above it is straight and extends transversely over the opening of the paragenital organ without lateral posterior extensions. The paragenital pouch extends deeply into the cephalothorax (25% of the length of the cephalothorax). The ventral wall of the paragenital organ is extremely thick and forms a massive trapezoidal lobe with the anteriormost portion of the brood canal. The ventral wall of the paragenital organ was found to be very thick throughout. The cuticular thickness of the anterior brood part of the canal decreases abruptly after about 100 μm posterior to the paragenital opening. The dorsal wall of the paragenital organ is thin and mostly covered by parts of the exuvia of the secondary larva.

Kinzelbachus friesei (Fig. 6)

The paragenital organ is present. Its opening is strongly arched, with a flat apex. The pouch of the paragenital organ is shallow and does not reach deep into the body cavity. The birth opening above it is very similar in shape to the paragenital opening. The lateral auricles of the paragenital organ are very weakly developed. The paragenital opening is very shallow (6.3% of the length of the cephalothorax). The thickness of the ventral wall of the paragenital organ varies only slightly, but significantly compared to the dorsal wall of the paragenital organ or the anterior parts of of the brood canal. The dorsal wall of the paragenital organ is largely covered by parts of the exuvia of the secondary larva.

Stylops hammella (Fig. 7)

The paragenital organ is present. The opening is slightly convex with a flattened apex. The birth opening above it is very similar in shape to the paragenital opening. The lateral auricles of the paragenital organ are weakly developed and covered by the exuvia of the secondary larva. The paragenital pouch extends deeply into the body (20.6% of cephalothorax length). Both the ventral and dorsal walls of the paragenital organ are extremely thick compared to the surrounding integument. Relative differences to the dorsal wall of the paragenital organ and the anterior parts of the brood canal are slight but clearly noticeable. The anterior part of the dorsal wall of the paragenital organ is covered by the exuvia of the secondary larva.

Stylops japonicus (Fig. 7)

The paragenital organ is present. The opening is arched with a flattened apex. The birth opening above it has exactly the same shape as the paragenital opening. The lateral auricles of the paragenital organ are well developed. The paragenital pouch extends deep into the body cavity (16.7 % of the length of the cephalothorax). The ventral wall of the paragenital organ is much thicker than the surrounding integument. The relative thickness differences to the dorsal wall of the paragenital organ or the anterior areas of the brood canal are very pronounced. The thicker anterior areas of the brood canal rapidly become thinner posteriorly. The dorsal wall of the paragenital organ is largely covered by the exuvia of the secondary larva.

Stylops melittae (Fig. 8)

The paragenital organ is present with an arched opening. The lateral auricles of the paragenital organ are well developed. The birth opening has the same shape as the paragenital opening, but completely covers the auricles. The paragenital pouch is very deep, extending well into the cephalothorax (21% of the length of the cephalothorax). The ventral wall of the paragenital organ is much thicker than the surrounding integument. The differences in thickness are very pronounced compared to the dorsal wall of the paragenital organ or anterior areas of the brood canal. Posterior to the anterior areas of the brood canal, the thickness decreases rapidly. The dorsal wall of the paragenital organ is largely covered by the exuvia of the secondary larva.

Stylops nassonowi (Fig. 8)

The paragenital organ is present. The opening is almost straight with only a slight curvature. The lateral auricles of the paragenital organ are well developed. The birth opening has the same shape as the paragenital opening but completely covers the auricles. The paragenital pouch is very prominent and extends well into the cephalothorax (20% of the length of the cephalothorax). The ventral wall of the paragenital organ is significantly thicker than the dorsal wall of the paragenital organ or the anterior areas of the brood canal. Posterior to the anterior areas of the brood canal, the thickness decreases rapidly but never reaches the extent of the dorsal cuticle of the paragenital organ. The dorsal wall of the paragenital organ is largely covered by the exuvia of the secondary larva.

Stylops ovinae (Fig. 9)

The paragenital organ is present. Its opening is almost straight, with only a slight curvature at the sides. The lateral auricles of the paragenital organ are well developed and follow the curvature of the opening. The pouch of the paragenital organ extends very deeply into the body lumen (20.7% of the length of the cephalothorax). The birth opening has the same shape as the paragenital opening, but it completely covers the auricles. The ventral wall of

the paragenital organ is strongly thickened compared to the dorsal wall of the paragenital organ or the anterior areas of the brood canal. The thickness of the cuticle is increased in the sagittal parts of the ventral wall of the paragenital organ compared to the medial parts. Compared to areas of the anterior regions of the brood canal, which are only slightly thickened, the cuticle becomes thinner posteriorly, but it is never as thick as the dorsal cuticle of the paragenital organ. The dorsal wall of the paragenital organ is almost as thick as the anterior regions of the brood canal, and only its anterior region is covered by the exuvia of the secondary larva.

Stylops spreta (Fig. 9)

The paragenital organ is present. The opening of the paragenital organ is slightly concave at the apex and appears trapezoidal throughout. The lateral auricles of the paragenital organ are well developed. The pouch of the paragenital organ extends only moderately inwards (12.1% of the length of the cephalothorax). The birth opening above it is rectilinear with a slight lateral curvature and completely covers the auricles. The ventral wall of the paragenital organ is thickened, and the thickness increases again in the sagittal parts of the ventral wall of the paragenital organ compared to the medial parts. Compared to the anterior regions of the brood canal, which are only slightly thickened, the thickness of the cuticle decreases posteriorly, but it never reaches the thickness of the dorsal cuticle of the paragenital organ. The dorsal wall of the paragenital organ is as thick as the cuticle of the anterior parts of the brood canal and is mostly covered by the exuvia of the secondary larva.

Xenidae

Paraxenos erberi (Fig. 10)

A paragenital organ is not present. We did not observe any thickening of the cuticle of the female at the anterior parts of the brood canal, where penetration takes place. A thickened integument only occurs in the region of the mouthparts and their articulations.

Tuberoxenos sphecidarum (Fig. 10)

A paragenital organ is not present. Thickness measurement could not be performed based on the available µCT data.

Pseudoxenos schaumii (Fig. 10)

Pseudoxenos schaumii does not have a paragential organ. The available µCT data did allow measurement of the thickness of the integument.

Xenos vesparum (Fig. 11)

Xenos vesparum lacks a paragenital organ. A thickened cuticle was observed in the anterior regions of the brood canal. The thickness of the cuticle decreases posterad, until it reaches the metathoracic region.

FORM 1

2.3 Manuscript No. III

Manuscript title: Have female twisted-wing parasites (Insecta: Strepsiptera) evolved tolerance traits as response to traumatic penetration?

Authors: Jandausch, K., Michels, K., Kovalev, A., Gorb, S.N., van de Kamp, T., Beutel, R.G., Niehuis, O., Pohl, H.

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The candidate is

☒ First author, □ Co-first author, ☒ Corresponding author, □ Co-author.

Status: published

Authors' contributions (in %) to the given categories of the publication

Author	Conceptual	Data analysis	Experimental	Writing the manuscript	Provision of material
Jandausch, K.		80 %	30 %	60 %	50 %
Michels, J.		10 %	15 %		
Kovalev, A.		10 %	15 %		
Gorb, S.N.	10 %				
van de Kamp, T.			10 %		
Beutel, R.G.	10 %				
Niehuis, O.	40 %			20 %	
Pohl, H.	40 %		30 %	20 %	50 %
Total:	100 %	100 %	100 %	100 %	100 %

_____ _____

Signature candidate Signature supervisor (member of the Faculty)

Have female twisted-wing parasites (Insecta: Strepsiptera) evolved tolerance traits as response to traumatic penetration?

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ABSTRACT

Traumatic insemination describes an unusual form of mating during which a male penetrates the body wall of its female partner to inject sperm. Females unable to prevent traumatic insemination have been predicted to develop either traits of tolerance or of resistance, both reducing the fitness costs associated with the male-inflicted injury. The evolution of tolerance traits has previously been suggested for the bed bug. Here we present data suggesting that tolerance traits also evolved in females of the twisted-wing parasite species *Stylops ovinae* and *Xenos vesparum*. Using micro-indentation experiments and confocal laser scanning microscopy, we found that females of both investigated species possess a uniform resilin-rich integument that is notably thicker at penetration sites than at control sites. As the thickened cuticle does not seem to hamper penetration by males, we hypothesise that thickening of the cuticle resulted in reduced penetration damage and loss of haemolymph and in improved wound sealing. To evaluate the evolutionary relevance of the *Stylops*-specific paragenital organ and penis shape variation in the context of inter- and intraspecific competition, we conducted attraction and interspecific mating experiments, as well as a geometric-morphometric analysis of *S. ovinae* and *X. vesparum* penises. We found that *S. ovinae* females indeed attract sympatrically distributed congeneric males. However, only conspecific males were able to mate. In contrast, we did not observe any heterospecific male attraction by *Xenos* females. We therefore hypothesise that the paragenital organ in the genus *Stylops* represents a prezygotic mating barrier that prevents heterospecific matings.

INTRODUCTION

Sexual reproduction and copulation occur in many different varieties across the animal kingdom. One of the most bizarre forms of sexual interaction is traumatic mating, which involves the injury of one sexual partner during copulation. In one particular form of traumatic mating, traumatic penetration (*Lange et al., 2013*), the female gets injured during the mating process, but receives no sperm. In contrast, traumatic mating resulting in the injection of sperm and the insemination of the female is referred to as traumatic insemination (*Lange et al., 2013*). Traumatic insemination occurs in different groups of animals, such as flatworms, snails and slugs, annelid worms, and arthropods (*Lange et al., 2013*; *Tatarnic, Cassis & Siva-Jothy, 2014*; *Brand, Harmon & Schaerer, 2022*). However, one of the best known and studied organisms in this context is the bed bug (*Cimex lectularius*) (*Michels, Gorb & Reinhardt, 2015*; *Reinhardt, Anthes & Lange, 2015*; *Brand, Harmon & Schaerer, 2022*), in which traumatic insemination occurs within a paragenital organ: the spermalege.

Traumatic insemination leads to injuries of females and is recognised as an example of sexual conflict (*Lange et al., 2013*; *Tatarnic, Cassis & Siva-Jothy, 2014*). Females unable to avoid unnecessary mating are predicted to likely evolve resistance traits or tolerance traits that reduce the fitness costs associated with sexual interaction with males. Resistance traits, which reduce the fitness of the copulating male(s), can result in a co-evolutionary arms race. Such arms races are generally thought to lead to accelerated trait exaggeration, such as the formation of species-specific differences in copulatory organs (*Arnqvist & Rowe, 2002*; *Parker, 2006*; *Lange et al., 2013*). *Michels, Gorb & Reinhardt (2015)* found evidence for female tolerance traits (*i.e.*, traits that reduce the fitness costs associated with a sexually conflicting interaction of the female without decreasing the fitness of the male) in the form of resilin in the spermalege in bed bugs. The elastomeric protein resilin seals the sexually imposed wounds and physically facilitates copulation by males (*Michels, Gorb & Reinhardt, 2015*, p. 6).

The endoparasitic insect order Strepsiptera comprises ca. 600 described species. It is characterised by numerous derived characters of all life stages and in both sexes (*Pohl & Beutel, 2008*). All species of the order display extreme sexual dimorphism. The males are free-living (Figs. 1A, 1C); the only function of their extremely short life span of a few hours is to find females and to mate. The females are usually obligatory endoparasites of other insects, in which they stay during most of their larval development and as adults (Figs. 1B, 1D). Only females of the family Mengenillidae are a notable exception, as they are free-living in the adult stage. Female Strepsiptera are wingless and structurally strongly simplified, as compared to the males. Their genital apparatus is extremely reduced: ovaries, vagina, receptacula seminis, genital chamber, bursa copulatrix, and accessory glands are missing, and the eggs float freely in the hemolymph. A single birth organ is present in females of Mengenillidae, with an opening in the region of sternite VII through which the minute (ca. 200–250 μm) primary larvae are released.

The genera *Stylops* and *Xenos* investigated in the present study belong to the clade Stylopidia, whose members utilise only pterygote insects as hosts and whose females are
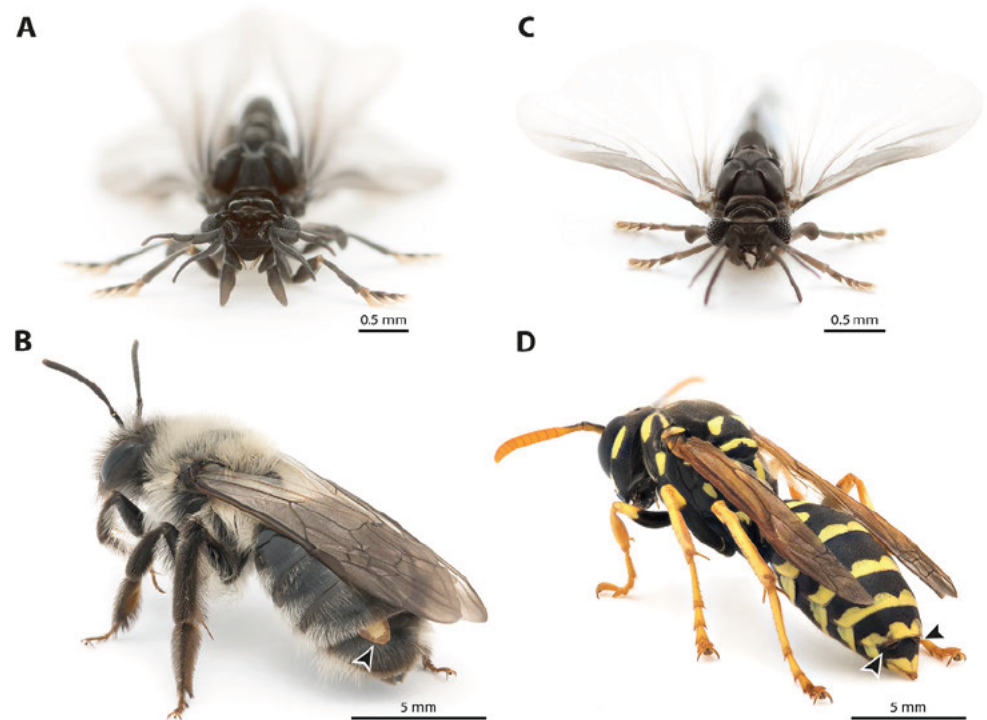
Figure 1 Photographs of *Stylops ovinae* and *Xenos vesparum*. (A) Frontal view of an adult male *S. ovinae*. (B) One female of *S. ovinae* protrudes from its host's metasoma (*Andrena vaga*). (C) Frontal view of an adult male *X. vesparum*. (D) Two females of *X. vesparum* protruding from their host's metasoma (*Polistes dominula*). Arrowheads indicate strepsipteran females.

Full-size ☐ DOI: 10.7717/peerj.13655/fig-1

obligatory endoparasites. Females of Stylopidia are characterised by secondary tagmosis: head, thorax, and the anterior part of abdominal segment I form a compact cephalothorax, while antennae, compound eyes, and legs are missing. The large sack-shaped posterior portion of the body remains within the host's abdomen, whereas the cephalothorax is exposed. A secondary copulation opening is located in the exposed cephalothorax. On the ventral side of the cephalothorax, between head and prosternum, a birth opening is present in the majority of species. This is the external opening of the brood canal, which is connected with the birth organs in the abdomen. The birth opening enables the primary larvae to leave the females and is used in most species for insemination (Stylopiformia). In Corioxenidae, insemination occurs either in the region of the mouth opening or in the membranised pleural region (*Pohl & Beutel, 2008*). The cuticle of the female is three-layered, as the female does not shed its larval exuviae. The cuticle layers are detached in the ventral area and form the brood canal. The exuvia of the second larval stage is strongly sclerotised in the cephalothoracic region, thinning only at the birth opening. The exuvia of the tertiary larva is extremely thin and weakly sclerotised (Figs. 2A, 2B).

The endoparasitic lifestyle of female Stylopidia has strongly influenced the mating strategy and the mating behaviour of its species, as only the female's cephalothorax protrudes from the host's abdomen (Figs. 1B, 1D). In *S. ovinae*, traumatic insemination
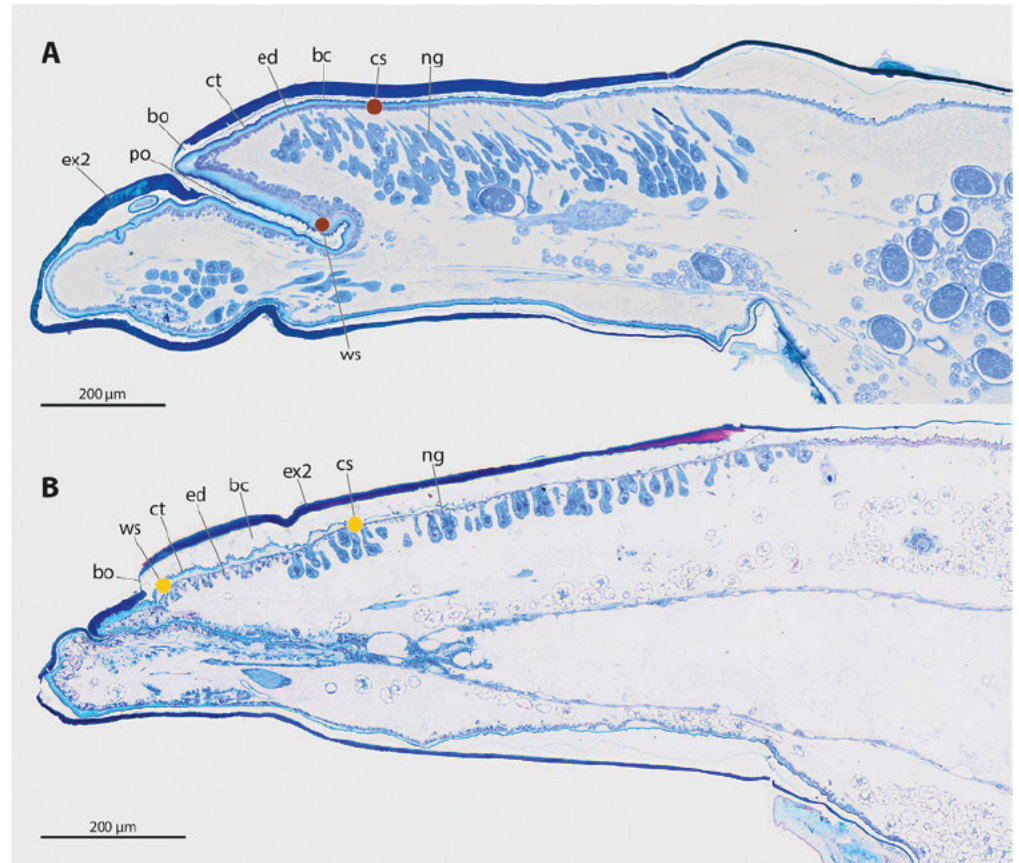
Figure 2 Sagittal sections of female cephalothoraces of *Stylops ovinae* (A) and *Xenos vesparum* (B). Abbreviations: bc, brood canal; bo, birth opening; ct, cuticle; ed, epidermis; ex2, exuvia of the secondary larval stage; ng, Nassonow's gland; po, paragenital organ; ws, wounding site; cs, control site. (A) Modified from *Peinert et al. (2016)*. (B) Modified from *Richter et al. (2017)*.

Full-size 🖼 DOI: 10.7717/peerj.13655/fig-2

takes place at a paragenital organ (po) located in front of the birth opening (*Peinert et al., 2016*) (Figs. 2A, 3A), while in *X. vesparum*, traumatic insemination occurs at the anterior part of the brood canal (bc) (Figs. 2B, 3B). However, *Beani et al. (2005)* discussed an alternative sperm route in *X. vesparum*, namely by release of sperm into the brood canal: the sperm could then reach the hemocoel of the female *via* the birth organs.

Previous studies have shown that the cuticle of the paragenital organ of *S. ovinae* and the cuticle of the anterior brood canal of *X. vesparum* is about three times thicker than the cuticle in spatial proximity (*Löwe, Beutel & Pohl, 2016*; *Peinert et al., 2016*; *Richter et al., 2017*). However, the material composition of the cuticle at specific penetration sites has not been studied and compared to that of the surrounding areas. This information could give additional clues whether Strepsiptera have evolved resistance traits or tolerance traits in response to traumatic penetration.

The penises of different species of *Stylops* vary greatly in shape and size, whereas those of males of *Xenos* differ almost exclusively in size (Fig. 4). Variation in penis shape, as reported for *Stylops*, has so far only been described from the genus *Caenocholax*
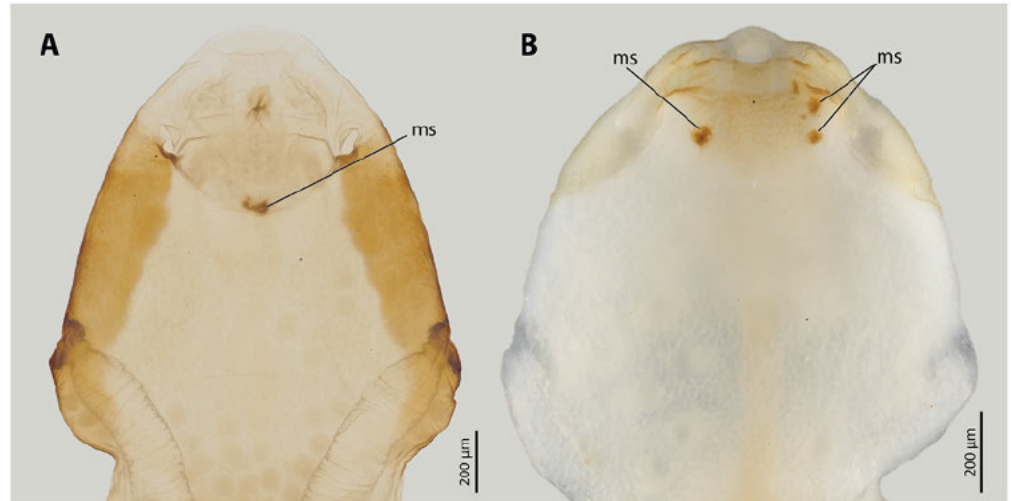
Figure 3 Female cephalothoraces of *Stylops ovinae* (A) and *Xenos vesparum* (B) with mating signs (outer cuticle of the cephalothorax removed). Abbreviation: ms, mating sign. (A) Modified from *Peinert et al. (2016)*.   Full-size ☒ DOI: 10.7717/peerj.13655/fig-3
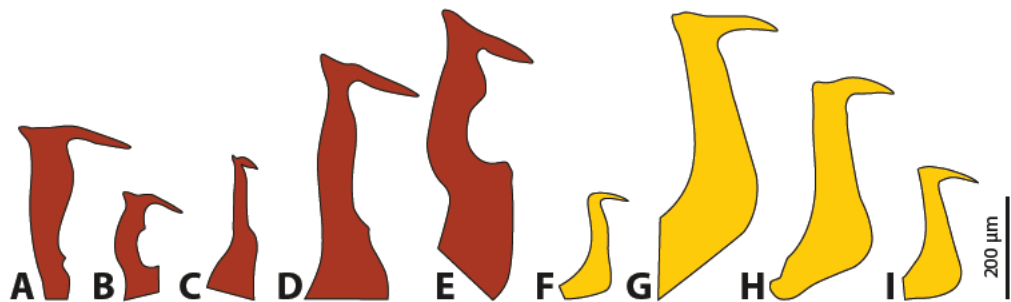
Figure 4 Penises of different species of *Stylops* (red) and *Xenos* (yellow). (A) *S. ovinae*. (B) *S. hammella*. (C) *S. liliputanus*. (D) *S. aterrimus*. (E) *S. melittae*. (F) *X. columbiensis*. (G) *X. moutoni*. (H) *X. oxydontes*. (I) *X. vesparum*. (B E) Modified from *Kinzelbach (1978)*, (F) modified from *Cook, Mayorga-Ch & Sarmiento (2020)*, (G) modified from *Kifune & Maeta (1985)*, (H) modified from *Nakase & Kato (2013)*.   Full-size ☒ DOI: 10.7717/peerj.13655/fig-4

(Myrmecolacidae) (*Kathirithamby et al., 2015*). The varying shape and size of the intromittent organs of different *Stylops* species could be either the result of intersexual co-evolution or avoidance of interbreeding between sympatric occurring congeners. Following *Eberhard (2004a)*, females encountering males may be passively protected *via* species-specific pheromones, for example by decreasing the chance of interspecific mating.

It is interesting to note that several of the 67 described *Stylops* species (*Straka, Jůzová & Nakase, 2015*) occur in sympatry and can be encountered in the same habitat at the same time of the year (*e.g.*, at least three different *Stylops* species have been recorded flying in March in the vicinity of Jena; H. Pohl, 2020, personal observations). In contrast, out of the 32 described *Xenos* species, only very few occur in sympatry (*e.g.*, only two species in the Western Palearctic, one likely restricted to Libya, while the other one is widespread). Furthermore, *Eberhard (2004b)* concluded that species-specific traits in males are missing

when females lack specific contact organs associated with copulation. We hypothesise that the paragenital organ of *Stylops* is such a specific contact organ, which suggests that the male genital armature will be more species-specific in this genus. To date it remains unclear whether heterospecific copulations or heterospecific attraction of Strepsiptera occurs in nature and if so, whether the species-specific morphology of the females' paragenital organs pose a significant prezygotic reproductive barrier that prevents penetration by heterospecific males.

We combined observational and experimental methods to address the question of whether *Stylops* and *Xenos* females evolved resistance traits or tolerance traits as counter-adaptations against traumatic wounding. We used confocal laser scanning microscopy to evaluate the material composition of the cuticle at the wounding sites of *S. ovinae* (paragenital organ) and *X. vesparum* (anterior brood canal) as well as at surrounding areas. We specifically assessed the presence of resilin, a soft and elastic protein found in large proportions in the cuticle of the spermalege of female bed bugs (*Michels, Gorb & Reinhardt, 2015*). We then measured the force required to penetrate the cuticle of the paragenital organ of *S. ovinae* and the anterior brood canal of *X. vesparum* using micro-indentation. We compared the results to the force necessary to penetrate the cuticle in control areas.

To clarify the question why the penises of different *Stylops* species strongly differ in size and shape, but not those of *Xenos*, we used field experiments to assess whether virgin females of both species attract heterospecific males. We subsequently used laboratory mating experiments to test whether heterospecific *Stylops* males are able to anchor and penetrate the female paragenital of *S. ovinae* with their penis. Additionally, we reconstructed the three-dimensional morphology of the penises and the female paragenital organ in *Stylops* using X-ray computed tomography to assess the morphological fit of the two organs. Positive correlation of shape and size of penises and paragenital organs of different *Stylops* species may indicate an interspecific copulatory barrier comparable to a lock-and-key mechanism. Finally, we used 3D models of penises of *S. ovinae* and *X. vesparum* to estimate the extent of intraspecific variation using geometric morphometrics. Comparing the magnitude of intraspecific penis variation of both investigated species could indicate whether this contributed to the higher diversification of penis morphology on the genus level.

MATERIAL AND METHODS

Studied insects

A total of 70 *Andrena vaga* (Hymenoptera, Andrenidae) parasitised by *S. ovinae* were collected in Langerwehe (North Rhine-Westphalia, Germany) (February 16, 2020, and March 11, 2021, by E. Holtappels, K. Jandausch, H. Pohl, and D. Tröger). During transport and preparation of the experiments, the bees were kept dark in glass vessels (0.5 L) half filled with moist sand at ~4 °C to prevent males of *S. ovinae* from hatching.

The hosts of *X. vesparum*, *Polistes dominula* (Hymenoptera, Vespidae), were collected in Mettenheim (Rhineland-Palatinate, Germany) (July 1, 2018; July 21, 2020, and August 11, 2021, by K. Jandausch, H. Pohl and D. Tröger) on a trumpet creeper bush (*Campsis*

radicans). Parasitised *P. dominula* are attracted to trumped creeper bushes and feed on extrafloral gland secretions (*Beani et al., 2018*). Wasps collected in 2018 and 2021 were used for the attraction experiments (see below). A total of 137 wasps were collected in 2020 for the micro-indentation experiments. In the laboratory, each wasp was assigned to one of four groups: wasps with extruded female *X. vesparum* ($n = 32$), wasps with extruded male puparia ($n = 28$), wasps with extruded females and male puparia ($n = 3$), and wasps without externally visible infestation ($n = 40$). Only one male puparium was empty, indicating that the majority of females were unfertilised. All parasitised wasps were kept in small groups ($n = 5$–8) in glass vessels (0.5 L) covered with gauze at room temperature and were fed *ad libitum* with water and diluted honey. Wasps without externally visible infestation were kept in an "aerarium" (40 cm × 40 cm × 60 cm) (Papa Papillon, Bern, Switzerland) and checked every day for freshly extruded *X. vesparum*.

One female specimen of *S. melittae* (ID: SFl87) and one of *S. hammella* (ID: SFc12) preserved in ethanol were provided by Jakub Straka.

Confocal laser scanning microscopy

Cephalothoraces of a virgin female *S. ovinae* and of a virgin female *X. vesparum* were dissected, transferred to glycerol (≥99.5%, free of water, two times distilled; Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and mounted in glycerol on object slides with high-performance cover slips (Carl Zeiss Microscopy GmbH, Jena, Germany) as described earlier (*Michels & Büntzow, 2010*; *Michels & Gorb, 2012*). Four autofluorescences exhibited by the cephalothoracic cuticle structures were visualised with a ZEISS LSM 700 confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using four solid-state lasers and bandpass and longpass emission filters as previously described (*Michels & Gorb, 2012*; *Michels, Gorb & Reinhardt, 2015*). The microscope system was controlled by the software ZEISS Efficient Navigation 2009 (ZEN 2009; Carl Zeiss Microscopy GmbH, Jena, Germany). A ZEISS Plan-Apochromat lens with a numerical aperture of 0.45 (Carl Zeiss Microscopy GmbH) was applied. Using ZEN 2009, a maximum intensity projection (MIP) was created from each of the obtained data sets. The four different autofluorescence visualisation results shown on the MIPs were colour-coded and overlaid as described earlier (*Michels & Gorb, 2012*). The resulting micrographs show differences in the autofluorescence composition of the analysed cuticle structures and indicate differences in the material composition of these structures. Red cuticle structures consist mainly of strongly sclerotised chitinous material, green ones are chitinous and are weakly sclerotised and membranous and either weakly sclerotised or non-sclerotised, and blue ones contain large proportions of resilin (*Michels & Gorb, 2012*).

Specimen preparation

Living females were extracted from their hosts with fine tweezers immediately before the micro-indentation experiments. The strongly sclerotised outer cuticle of the cephalothorax was removed to gain access to the penetration sites. The cephalothoraces were then fixed with micro-needles on a silicon block (Silicone HR-N; Reckli, Herne, Germany). Two types of fixations were used when handling *S. ovinae*: to make the ventral wall of the paragenital

organ (wounding site) accessible, the females were fixed with the morphological dorsal side directed upwards, and the head area of the female was folded back. To expose the anterior part of the brood canal (control site), females were fixed with the ventral side directed upwards. The brood canal was chosen as the control site for *S. ovinae*, as it resembles the location where traumatic penetration takes place in other families of Strepsiptera (*e.g.*, Xenidae). Females of *X. vesparum* were fixed exclusively with the ventral side directed upwards, as wounding and control sites are located on the same side. At the wounding sites, micro-indentation in *S. ovinae* and in *X. vesparum* was carried out in the areas where mating signs are located in mated females (Fig. 3). In *X. vesparum*, reference measurements at control sites were carried out at the posterior end of the brood canal where the cuticle is much thinner, representing the general state of the cuticle at the cephalothorax. All preparations were performed using a stereomicroscope (Olympus SZX12; Olympus, Tokyo, Japan).

Micro-indentation experiments

Micro-indentation experiments were carried out on living virgin females without mating signs. We studied 24 females of *S. ovinae* (wounding site: 14; control site: 10) and 12 females of *X. vesparum*. In *X. vesparum*, we performed both measurements on the same females. The force to penetrate the cuticle of the females was measured by inserting steel micro-needles with diameters of 5.6 μm when studying *S. ovinae* and 4.1 μm when studying *X. vesparum*. In comparison, the tips of the penises of *S. ovinae* measured on average 2.8 μm ($n = 3$, min. 2.6 μm, max. 3.0 μm), and those of *X. vesparum* measured on average 0.8 μm ($n = 3$, min. 0.7 μm, max. 0.9 μm). All tips were analysed using scanning electron micrographs of the penises and tips of the micro-needles. Each micro-needle was directly glued to a 10-g force transducer (World Precision Instruments, Sarasota, FL, USA) with cyano-acrylate glue (Ergo 5925 Elastomer; Kisling AG, Wetzikon, Switzerland). The force transducer was attached to a motorised micro-manipulator. To perform the indentation experiments, the force transducer was moved down with 200 μm/s velocity. During the experiments, the females were moistened with a drop of tap water to prevent drying artefacts. Each specimen was penetrated several times on slightly different positions. The whole system was connected to a computer running the software AcqKnowledge 3.7.3 (Biopac System Inc., Goleta, CA, USA) (Fig. S2). This software was used to record and process the measured force and time/travelled distance.

All measurements were controlled visually with a stereomicroscope (LEICA MZ 12.5, Wetzlar, Germany) to guarantee the penetration on the chosen location. Individual measurements were documented using a video camera (Basler piA1900-32g; Basler Vision Technologies, Ahrensburg, Germany) attached to the stereomicroscope. The software SteamPix5 (Norpix Inc., Montreal, QC, Canada) was used to record the videos.

Scanning electron microscopy

Scanning electron micrographs of the penises of *S. ovinae* and of *X. vesparum* were taken with a Philips ESEM XL30 (Philips, Amsterdam, Netherlands) (Fig. S3). The same equipment was used to obtain micrographs of the tips of the steel microneedles used in the

micro-indentation experiments. The penises were air-dried, glued to a microneedle, fixed on a rotatable specimen holder (*Pohl, 2010*), and sputter-coated with gold using an Emitech K500 (sample preparation division, Quorum Technologies Ltd., Ashford, England).

Attraction experiments

In 2020, four bees, and in 2021, eight bees from Langerwehe parasitised with *S. ovinae* females were kept in an "aerarium" (see above). The "aerarium" was placed in the field and orientated in the direction of the wind flow to increase the dispersion of female pheromones. The attraction experiments were conducted on March 18 and 19, 2020, and between March 24 and March 26 and on March 29, 2021, in the vicinity of Jena (Thuringia, Germany). Approaching males were collected from the gauze of the "aerarium" with an aspirator. In 2020, two, and in 2021, 40 of the attracted males were taken to the lab alive in snap-cap vials. To keep the males vital for the mating experiments, they were transported in a cold bag at ~5 °C. The remaining males were immediately fixed in 100% ethanol.

An identical setup was used to attract male *X. vesparum* in the backyard of the Phyletisches Museum Jena (Thuringia, Germany). On July 18, 2018, five, and on August 16, 2021, eight infected wasps from Mettenheim were placed in an "aerarium". *Beani et al. (2018)* carried out similar attraction experiments with parasitised wasps in small vials to attract males of *X. vesparum*. In contrast to *S. ovinae*, males of *X. vesparum* were fixed in 70% ethanol after collecting them from the gauze, as no further mating experiments were carried out with this species.

Species identification of the attracted males

To identify *Stylops* species, we analysed a 605-bp-long fragment of the mitochondrial gene COI in all males collected in 2020 and in the four males that we used in the mating experiment in 2021. The DNA was extracted from legs using the QIAGEN QIAamp DNA Micro, following the protocol of the manufacturer. PCR amplification was carried out with the oligonucleotide primers 5′-TCW ACA AAT CAT AAA ATA ATT GG-3′ (CO122For), 5′-TCC TCC TCC TAA AGG RTC RAA-3′ (CO16669Rev), 5′-TWT CWA CHA AYC ATA ARG ATA TTG G-3′ (Cox1LCO_DEG) and 5′-TCA ATT TCC AAA YCC YCC YAT-3′ (Cox1ALEX_DEG) published by *Folmer et al. (1994)*, *McMahon, Hayward & Kathirithamby (2009)*, and *Jůzová, Nakase & Straka (2015)*. PCRs were performed with the Invitrogen Taq DNA Polymerase for standard PCR (Thermo Fisher Scientific Inc., Waltham, MA, USA) including dNTPs, PCR buffer, $MgCl_2$, and *Taq* Polymerase. Applied primers were manufactured by Metabion GmbH (Munich, Germany). PCRs started with a 180 s initial phase at 94 °C, followed by 30 cycles of 45 s at 94 °C, 30 s at 50 °C and 90 s at 72 °C, and ended with one final extension at 72 °C for 10 min. Products were purified using ExoProStar 1-Step (Global Life Sciences Solutions USA LLC, Marlborough, MA, USA). For direct bidirectional Sanger sequencing, samples were sent to Macrogen (Amsterdam, Netherlands). After removing the primer binding sites, forward and reverse sequences of each specimen were aligned using Geneious prime 2021.0.3 and compared to reference sequences for the genus *Stylops* established by *Jůzová, Nakase & Straka (2015)*

(Fig. S4). All other males were determined by comparing their penis to the penises of the barcoded males.

The attracted *Xenos* males were identified with the key provided by *Kinzelbach (1978)*.

Mating experiments

In order to determine whether the attracted males are able to insert their penis into the paragenital organ and anchor themselves within virgin females of *S. ovinae*, we followed the method established by *Peinert et al. (2016)*. The mating experiments were carried out directly after collecting the specimens in the field and returning with them to the lab to ensure the vitality of the short-lived males. The copulations were initiated in transparent plastic trays (4 cm diameter, 1 cm high) at 21 ± 1 °C. The metasoma of each parasitised host was removed and was attached directly to modelling clay with its anterior end on the bottom of the trays. The males were placed one at a time in plastic dishes, and the dishes' opening were closed with a transparent lid to prevent the males from escaping. After about 2 min, when each male had mounted the metasoma of the host bee and attempted to mate with the female, the males were removed and fixed in 100% ethanol for later species identification unless otherwise stated. Note that we determined all males after the experiments, either by barcoding or by comparing the penises of the males with those of barcoded males. Mating attempts were recorded with a Canon EOS 7D digital SLR equipped with a Canon MP-E 65 mm macro lens (Canon, Krefeld, Germany) or an Apple iPhone SE (second generation) (Cupertino, CA, USA) through the eyepiece of a Leica MS 5 stereomicroscope (Leica, Wetzlar, Germany). We used a cold light source (KL 750; Schott, Mainz, Germany) as lighting.

In total, we introduced 18 of the attracted males to females of *S. ovinae* and tested whether they tried to mate or not. The following mating experiments were performed with virgin females of *S. ovinae* as described in the preceding paragraph: one male of *S. hammella* with two females (video recording), and one male of *S. ovinae* with two females. This latter male mated with one of the females and was fixed in copula with 100% ethanol cooled to −80 °C for a μCT scan. Eleven males of *S. melittae* were successively placed to one female (video recording). To detect possible injury to females by these heterospecific males during their mating attempts, four males of *S. melittae* were each placed to four infested host metasomas. Of these, two host metasomas were parasitised by one, one by two, and one by three virgin females. Since the penetration site is marked by a melanised spot on the cuticle 1 day after mating, we stored these females in their host metasoma for 48 h after the mating attempts in the refrigerator at 5 °C. To document the mating signs, we extracted the females from the host, removed the outer cuticle of the cephalothorax, dehydrated the specimens in an ascending ethanol series, and mounted the cephalothorax in Euparal (Carl Roth, Karlsruhe, Germany) on microscope slides. Photographs were taken with a Canon EOS 7D digital SLR equipped with a Mitutoyo M Plan Apo 10x lens (Mitutoyo, Kawasaki, Japan). The slides were illuminated with two flashlights (Yongnuo Photographic Equipment, Shenzhen, China).

X-ray computed tomography and 3D-reconstruction

One female and one male of *S. hammella*, one male of *S. melittae*, and one pair of *S. ovinae* fixed in copula (see mating experiments) were scanned in pure ethanol at the Imaging Cluster at the KIT Synchrotron Radiation Facility using a polychromatic X-ray beam produced by a 1.5 T bending magnet spectrally filtered by 0.5 mm Al. A fast indirect detector system was employed, consisting of a 13 μm LSO:Tb scintillator (*Cecilia et al., 2011*), a diffraction limited optical microscope (Optique Peter, Lentilly, France) (*Douissard et al., 2012*), and a 12-bit pco.dimax high speed camera with 2,016 × 2,016 pixels. Scans were done by taking 3,000 projections at 70 fps over an angular range of 180°. An optical magnification of 10× resulted in an effective pixel size of 1.22 μm. The control system concert (*Vogelgesang et al., 2016*) was employed for automated data acquisition and online reconstruction of tomographic slices for data quality assurance. Data processing included flat field correction and phase retrieval of the projections based on the transport of intensity equation (*Paganin et al., 2002*). X-ray beam parameters for algorithms in the data processing pipeline were computed by *syris* (*Faragó et al., 2017*). The execution of the pipelines, including tomographic reconstruction, was performed by the UFO framework (*Vogelgesang et al., 2012*). One female of *S. melittae* was scanned in a SkyScan221 micro-CT (FSU Jena) with beam strength of 40 kV and 300 μA. In a 360° scan, pictures were taken every 0.2° with an exposure time of 5,800 ms. A pixel size of 1.22 μm was achieved. We segmented tomographic data using Dragonfly 4.1 for Windows (Object Research Systems (ORS) Inc, Montreal, QC, Canada, 2019) and used VGStudiomax 2.0.5 (Volume Graphics, Heidelberg, Germany) for visualization and rendering.

Geometric morphometrics

To estimate the intraspecific variation, penises of 18 specimens of *S. ovinae* (Niedringhaussee, Lower Saxony, Germany) and of 17 specimens of *X. vesparum* (Jena, Thuringia, Germany) were carefully removed with fine tweezers and dried at the critical point with a Emitech K850 Critical Point Dryer (Sample preparation division, Quorum Technologies Ltd., Ashford, England). Penises were transferred into a plastic pipette tip and scanned in a SkyScan221 micro-CT (FSU Jena) with beam strength of 40 kV and 200 μA. In a 360° scan, pictures were taken every 0.2° with an exposure time of 2,300 ms. A pixel size of 0.7 μm was achieved. Segmentation was performed with Dragonfly 4.1 for Windows (Object Research Systems Inc, Montreal, QC, Canada, 2019). Exported *stl* files were smoothed and the polygons were reduced and rendered with Blender before exportation as *obj* files (Blender Foundation, Amsterdam, Netherlands). Landmarks and semilandmarks were placed with Stratovan Checkpoint (Stratovan Cooperation, Davis, CA, USA).

Landmarks

Nine landmarks and 126 semilandmarks on two curves were placed on the 3D objects to describe the overall size and outline shape of the penises. Landmark 1 describes the

proximal edge of the phallotreme. Landmark 2 was placed at the deepest point of the ventral angle of the acumen (Fig. S3). Landmark 3 represents the transition from the flat distal part of the penis to the broader base, and landmark 4 marks the posterior edge of the sclerotised penis base. Along these first four landmarks, 54 semilandmarks where projected to represent the posterior outline of the penis shape in detail. Landmark 5 was placed at the distal edge of the phallotreme, followed by landmark 6 at the tip of the acumen. Landmark 7 marks the maximum point of the dorsal curvature of the entire acumen. Landmark 8 represents the dorsal spine. Landmark 9 represents the anterior edge of the sclerotised penis base. A curve consisting of 72 semilandmarks was placed between landmark 5 to landmark 9 to describe the shape of the acumen and the anterior outline.

Statistical analysis

We conducted a total of 146 penetration force experiments (*S. ovinae* wounding site: 43; *S. ovinae* control site: 41; *X. vesparum* wounding site: 38; *X. vesparum* control site: 36). We analysed 30 measurements from the wounding site of *S. ovinae*, 40 measurements from the control site of *S. ovinae*, 38 measurements from the wounding site of *X. vesparum*, and 36 measurements from the control site of *X. vesparum*.

For statistical analysis and significance tests, we used the software RStudio (R Core Team, Auckland, New Zealand). Boxplots and raw diagrams were drawn with the same software. We applied the Shapiro-Wilk test for testing for normal distribution and F test for testing for equality of variances among independent datasets. The level of significance was checked with a Kruskal-Wallis test and with a Wilcoxon pairwise comparison (including Bonferroni-Holm correction). In case of the data of *X. vesparum*, we used Levene's test for assessing the equality of variances instead of applying an F test, because the data did not follow a normal distribution (Shapiro-Wilk test: $p = 0.03403307$ [wounding site] and $p = 0.01891762$ [control site]). The data of *S. ovinae*, however, were normally distributed (Shapiro-Wilk test: $p = 0.09288335$ [wounding site] *vs.* $p = 0.7888944$ [control site]).

Geometric morphometric analysis was carried out with RStudio (R Core Team, Auckland, New Zealand) and the geomorph package (*Adams et al., 2021*; *Baken et al., 2021*). General Procrustes analysis was performed using the gpagen function with semilandmarks allowed to slide between landmarks by minimizing bending energy (define. sliders). Resulting centroid size for each specimen was used for calculation of the coefficient of variance. PCA of the Procrustes shape variables was performed with the function gm.prcomp and the results were visualized with the packages pca3D and rgl to explore major aspects of the geometric variation.

Image processing

All images were processed with Adobe Photoshop Version 21.2.1 (Adobe Systems Incorporated, San Jose, CA, USA). We used Adobe Illustrator Version 24.2.1 (Adobe Systems Incorporated, San Jose, CA, USA) for labelling the plates and for drawings and processing diagrams.
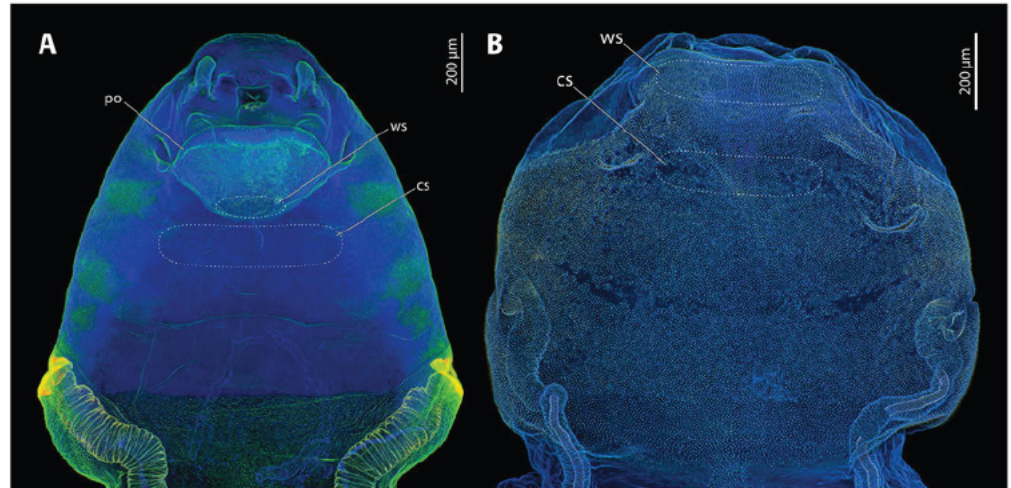
Figure 5 Confocal laser scanning micrographs (maximum intensity projections) showing the cuticle autofluorescence composition of female *Stylops ovinae* and *Xenos vesparum* cephalothoraces (outer cuticle of the cephalothorax removed). (A) Morphological ventral side of *S. ovinae*. (B) Morphological ventral side of *X. vesparum*. Blue structures contain large proportions of the elastomeric protein resilin. Green structures are chitinous and membranous and either weakly-sclerotised or non-sclerotised. Abbreviations: cs, control site; po, paragenital organ; ws, wounding site.

Full-size ⊞ DOI: 10.7717/peerj.13655/fig-5

RESULTS

Confocal laser scanning microscopy

Major parts of the cephalothoracic cuticle of both investigated species contain large proportions of resilin, including the areas where traumatic insemination takes place (Fig. 5). The composition of the cuticle in the control areas does not differ from sites where traumatic insemination takes place. In *S. ovinae*, the spiracles and the main tracheal stems of the cephalothorax consist of sclerotised chitinous material (Fig. 5A). In contrast, different areas on the ventral side of the cephalothorax are not or only weakly sclerotised. These include individual structures of the cephalic area, patches of the lateral pro-, meso-, and metathoracic regions, as well as microtrichia on the surface of abdominal segment I (Fig. 5A). The microtrichia covering almost the entire ventral cephalothoracic surface of *X. vesparum* are apparently slightly more sclerotised than those on the cephalothorax of *S. ovinae* (Fig. 5B). However, the spiracles, the main tracheal stems, and the lateral regions of the cephalothorax of *X. vesparum* are less sclerotised than corresponding areas in *S. ovinae*.

Micro-indentation experiments

The force required to penetrate the paragenital organ of *S. ovinae* (wounding site; see Fig. 5A) with a micro-needle was statistically significantly higher (mean: 9.732 mN) than the force required at a control site at the brood canal (see Fig. 5A) (mean: 4.578 mN) (Wilcoxon pairwise comparison; $p = 2.1e{-}08^{*}$; Tables 1 and 2). Likewise, the force required to penetrate the anterior brood canal (wounding site; see Fig. 5B) of *X. vesparum* was significantly higher (mean: 3.669 mN) than the force required at the posterior control site

Table 1 Means, standard deviations (SD) and *p* values from Shapiro-Wilk tests (SWT).

	Penetration force			Critical stress		
	Mean (mN)	SD	SWT	Mean (GPa)	SD	SWT
S. ovinae (wounding site) *n* = 30	9.723	3.498	0.09288335	0.402	0.138	0.09427896
S. ovinae (control site) *n* = 40	4.578	1.483	0.7888944	0.186	0.060	0.7888944
X. vesparum (wounding site) *n* = 38	3.669	1.011	0.03403307	0.273	0.075	0.03404492
X. vesparum (control site) *n* = 36	2.003	1.077	0.01891762	0.149	0.080	0.01892339

Note:
 mN, millinewton; GPa, gigapascal.

Table 2 Results of the Wilcoxon pairwise comparison of means for the penetration force and the critical stress of female *Stylops ovinae* and *Xenos vesparum*.

Penetration force	*S. ovinae* (wounding site)	*S. ovinae* (control site)	*X. vesparum* (wounding site)
S. ovinae (control site)	p = 2.1e−08*		
X. vesparum (wounding site)	p = 2.8e−11*	p = 0.00159*	
X. vesparum (control site)	p = 1.6e−13*	p = 5.5e−11*	p = 1.5e−08*
Critical stress			
S. ovinae (control site)	p = 7.5e−09*		
X. vesparum (wounding site)	p = 4.1e−05*	p = 1.2e−06*	
X. vesparum (control site)	p = 1.7e−09*	p = 0.0327*	p = 2.7e−08*

Notes:
 * Indicates significance.
 p, probability value.

in the brood canal (see Fig. 5B) (mean: 2.003 mN) (Wilcoxon pairwise comparison; p = 1.5e−08*; Tables 1 and 2). The absolute forces required to penetrate the cuticle were consistently higher in *S. ovinae* than in *X. vesparum*.

Critical stress (force per unit area) was calculated to compare the mechanical impact of the intromittent devices on female structures in both species, independent of differences in the diameter of the device. The highest mean value (0.402 GPa) was measured at the wounding sites of *S. ovinae* (Fig. 6, Table 1). The mean critical stress at the corresponding control sites (0.186 GPa) proved to be statistically significantly lower (Wilcoxon pairwise comparison; p = 7.5e−09*) (Tables 1 and 2). The critical stress value was significantly higher than critical stress values obtained for measurements at wounding and at control sites of *X. vesparum* (Fig. 6, Tables 1 and 2). The critical stress at the wounding site of *X. vesparum* (mean value of 0.273 GPa) was significantly higher than that at the control site (mean value of 0.149 GPa) (Wilcoxon pairwise comparison; p = 2.7e−08*) (Tables 1 and 2).
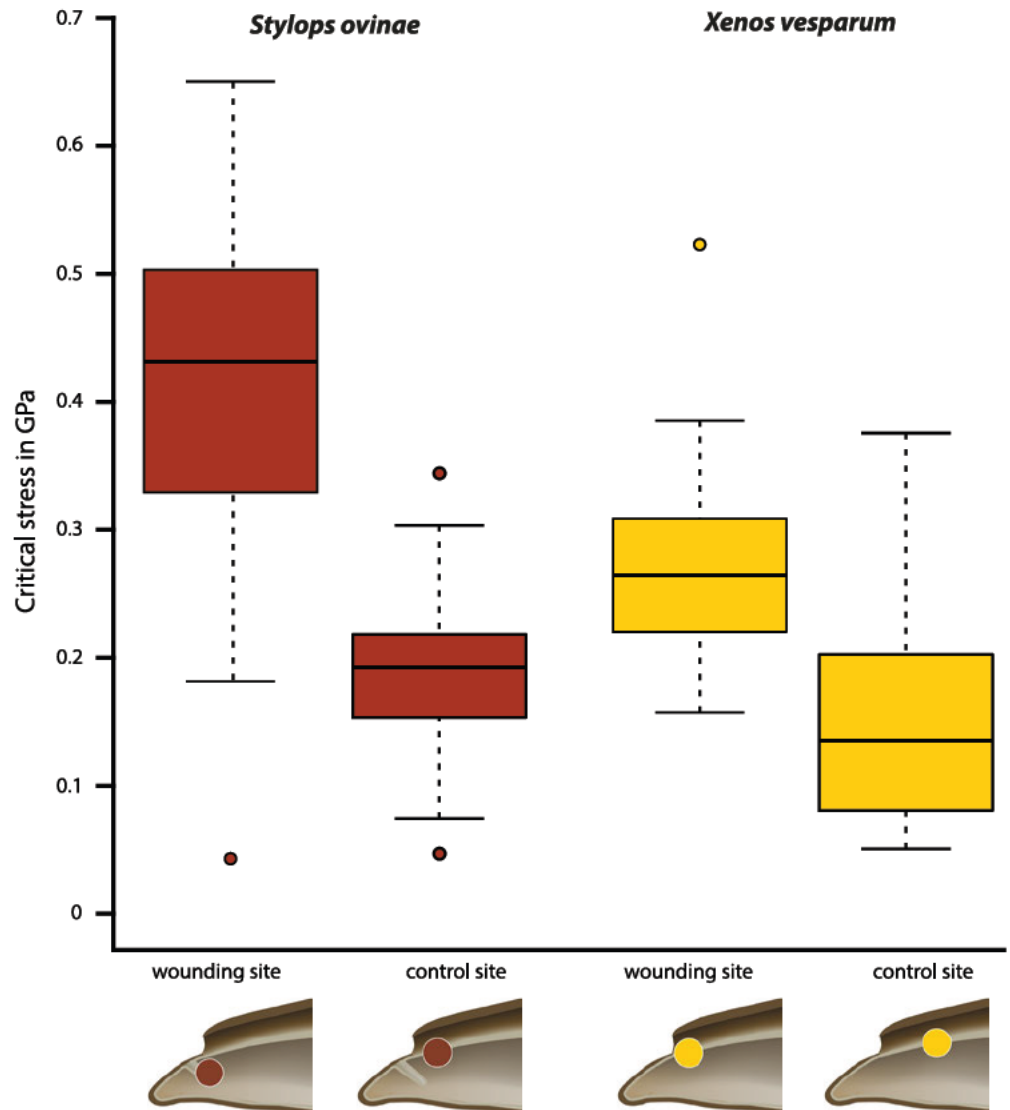
Figure 6 Critical stress values for wounding and control sites of *Stylops ovinae* (red) and *Xenos vesparum* (yellow). *Stylops ovinae*: wounding site ventral wall of the paragenital organ, control site anterior brood canal. *Xenos vesparum*: wounding site anterior brood canal; control site posterior brood canal. Boxes represent the interquartile range between first and third quartiles, and the line inside represents the median. Whiskers denote the lowest and highest values within 1.5× interquartile range from the first and third quartiles, respectively. Circles represent outliers beyond the whiskers.
Full-size 🖼 DOI: 10.7717/peerj.13655/fig-6

Attraction experiments

During two consecutive days in 2020, we were able to attract a total of 18 *Stylops* males in the field by exposing four *Andrena vaga* parasitised with females of *S. ovinae*. Using DNA barcoding and/or morphology (*i.e.*, the species-diagnostic shape of the penis), we identified the 18 captured *Stylops* males as *S. hammella* (two individuals), *S. melittae* (13 individuals) and *S. ovinae* (three individuals). During four consecutive days in 2021, we attracted an additional 94 *Stylops* males by exposing eight *A. vaga* parasitised with females of *S. ovinae*.
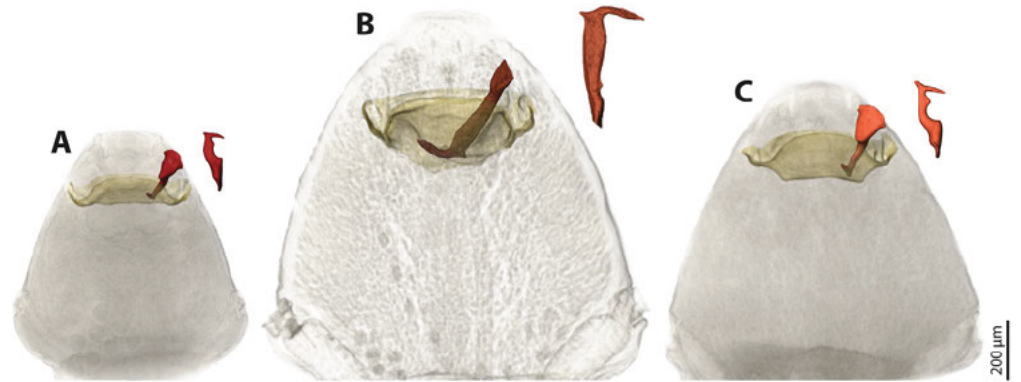
Figure 7 Pairs of female cephalothoraces (outer cuticle of the cephalothorax removed) and male penises of *Stylops hammella* (A), *Stylops ovinae* (B) and *Stylops melittae* (C). Paragenital organs highlighted in ochre, penises in red. *S. ovinae* was scanned in copula (*Peinert et al., 2016*). The penises of *S. hammella* and *S. melittae* were virtually inserted into the corresponding conspecific female paragenital organs to assess the fit of these genital structures.   Full-size 🖼 DOI: 10.7717/peerj.13655/fig-7

Of these, 91 were identified as *S. melittae* and one as *S. ovinae*. Two specimens were accidentally crushed during capture and remained unidentified. In 2018, we were able to collect 104 males of *X. vesparum* during a single day, attracted by a single *P. dominula* female parasitised by a single *X. vesparum* female. In 2021, we additionally collected 81 males of *X. vesparum* by exposing 10 *P. dominula*, each parasitised by *X. vesparum* females during a single day. Note that each of the above females of *S. ovinae* and *X. vesparum* attracted multiple males simultaneously.

Mating experiments

All *Stylops* males of the attraction experiments, irrespective of their species identity, mounted the parasitised host bee and searched for a suitable position on the bee's metasoma to mate with the *S. ovinae* females. The male of *S. hammella* was unable to insert its penis into the paragenital of two female *S. ovinae* and to anchor itself (Video S1). All *S. melittae* males were able to insert their penises into the paragenital organ, but they were unable to anchor (Video S1). In three out of six copulation attempts of *S. melittae* males with *S. ovinae* females, the female's cuticle was punctured. However, none of the resulting scars in the cuticle were found at the terminal end of the paragenital organs, the only location penetrated by conspecific males (H. Pohl and K. Jandausch, 2020, personal observations), but rather in the anterior region of the paragenital organ (Fig. S1). Only the conspecific male was able to perforate the terminal part of the female's paragenital organ (fixed in copula, µCT scan).

Co-adaptation of the penises with the females' paragenital organs

We used µCT scans of the penises and of the paragenital organs of *S. ovinae* (raw data taken from *Peinert et al. (2016)*), *S. hammella*, and *S. melittae*, and digitally inserted the penises into the paragenital organs with VGStudiomax 2.0.5 (Volume Graphics, Heidelberg, Germany). Doing so illustrated that the size and shape of a specific penis fits only with the paragenital organ of conspecific females (Fig. 7).
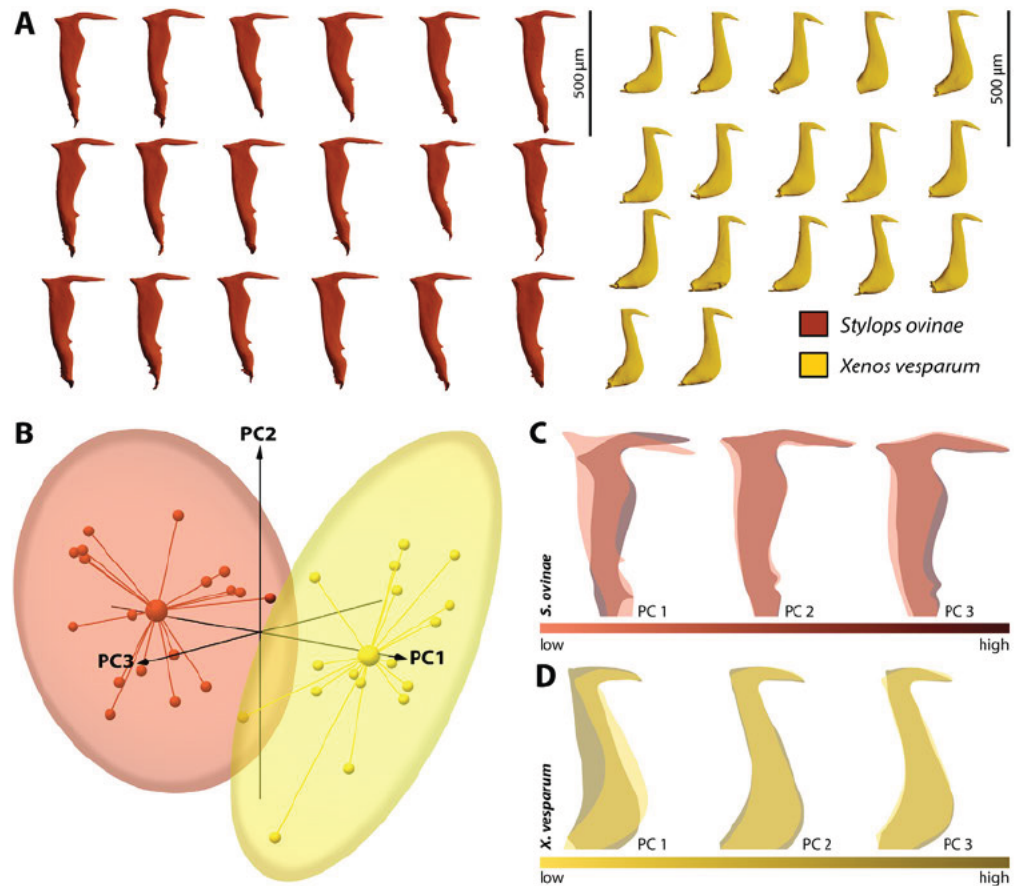
Figure 8 Geometric morphometric analysis of penises of *Stylops ovinae* (red) and *Xenos vesparum* (yellow). (A) Lateral view of all investigated penises of both species. (B) Shape spaces of penises of both species visualised with the three most representative principal components (see Supplemental for interactive 3D version). (C) and (D) Shape change along the PC axes of *S. ovinae* (C) and *X. vesparum* (D). Darker colours represent lower PC values, while brighter colours outline higher PC values.

Full-size 🖼 DOI: 10.7717/peerj.13655/fig-8

Geometric morphometrics

By performing Generalised Procrustes analysis of the morphometric data of *S. ovinae* penises, we calculated a mean centroid size of 1,829.35, with a standard deviation of 84.88. The coefficient of variation of 4.64% describes the extent of variation of *S. ovinae* penises with respect to centroid size. Based on a 95% coincidence interval, one outlier was found among the *S. ovinae* data (Fig. 8B).

The first three axes of the principal components analysis ordination plot explain 64.1% of the sample shape variation of *S. ovinae* penises (Fig. 8C). PC1 explains 32.1% of the shape variation. From negative to positive PCA values, the dorsal spine shifts towards the base of the penis, resulting in a slightly s-shaped or in a non-s-shaped back of the acumen's dorsal outline compared to penises related to lower values. Furthermore, the posterior bulge is shifted ventrad towards higher values. PC2 accounts for 20.4% of the sample shape variation. Moving from negative to positive PCA values, shape changes are expressed by a

slightly shorter acumen and a sharper ventral angle of the dorsal spine. PC3 describes 11.6% of the total sample shape variation of *S. ovinae* and relates to a more prominent posterior bulge and slightly elongated posterior spine for positive values.

Generalised Procrustes analysis of morphometric data of *X. vesparum* penises resulted in a mean centroid size of 1,243.18 with a standard deviation of 45.08. Regarding centroid size, the variation is described by coefficient of variation of 3.6%. As in *S. ovinae*, an outlier was identified among the data of *X. vesparum* based on a 95% confidence interval (Fig. 8B).

The first three principal components of the PCA explain 64.9% percent of the overall sample shape variation (Fig. 8D). With a proportion of 38.7% of sample shape variation, negative PC1 values associated with penises having a strongly curved posterior edge straightened towards positive values. The shape variation also influences the dorsal curvature of the acumen, being less curved with positive values than with negative ones. PC2 accounts for 14.1% of total sample shape variation. Compared to negative values, positive values are related to a slightly more downward tilted tip of the acumen for positive PCA values. PC3 describes 12.1% of the sample shape variation. Going from negative to positive, the dorsal spine shifts more dorsad. This appearance leads also to a more strongly bent dorsal curvature of the acumen in *X. vesparum*.

DISCUSSION

Increased thickness and sclerotization of the insect cuticle are common adaptations to reduce the risk of sexual wounding (*Merritt, 1989*; *Baer & Boomsma, 2006*; *Kamimura, 2007*; *Rönn, Katvala & Arnqvist, 2007*; *Lange et al., 2013*; *Dougherty & Simmons, 2017*; *Matsumura et al., 2017*). In *S. ovinae* and *X. vesparum*, the cuticle of females is weakly sclerotised and uniformly rich in resilin. Most notably, however, it is also three times thicker at the wounding site than at control sites in close spatial proximity. Note that the endoparasitic females are enclosed by the exuviae of the secondary and tertiary larval stage, and that the former is strongly sclerotised, thinning only at the birth opening. The males can only insert their penis at the strongly thinned exuvia of the birth opening.

The remaining exuvia of the secondary larva is not suitable for male penetration. *Michels, Gorb & Reinhardt (2015)* considered bed bugs as an example of a species in which the females have evolved tolerance against traumatic mating. This is due to higher proportions of resilin in the females' spermalege compared to surrounding areas of the abdomen. The flexibility and resilience of resilin allow efficient sealing of the cuticle after penetration and help reducing loss of haemolymph (*Michels, Gorb & Reinhardt, 2015*). At the same time, the forces required to pierce the cuticle of the spermalege are notably lower than those at control sites. The high proportion of resilin in the cuticle of the spermalege has been consequently interpreted as a response to traumatic mating, as it facilitates piercing by the male genitalia. The increased thickness of the integument in Strepsiptera requires a higher force to pierce the cuticle at the penetration sites (compared to control sites). This result could indicate that Strepsiptera have evolved resistance rather than tolerance against traumatic insemination. However, *Peinert et al. (2016)* found *S. ovinae* males to be consistently able to penetrate their female partners within seconds, indicating that the specific structure of the female cuticle does not seem to hamper penetration by males.

We consequently favour an alternative interpretation, namely that the thickened cuticle is also a tolerance trait. As the weakly sclerotised integument of female strepsipterans is already rich in resilin, further increasing the amount of this protein in the integument may not help to mitigate the male-inflicted trauma on the female's integument. However, increased thickness of the body wall could have such an effect. Potential positive effects of a thickened cuticle could be a reduced risk of integument rupture, improved sealing of the copulation wounds, and reduction of haemolymph loss.

We demonstrated that the sex pheromone emitted by *S. ovinae* females attracts not only conspecific males, but also males of two additional congeneric species. In contrast, females of *X. vesparum* attracted only conspecific males. The sympatric occurrence of congeneric species is much more common in *Stylops* than in *Xenos*, whose species do not possess a paragenital organ (*Kinzelbach, 1971*). In the Western Palearctic, only two species of *Xenos* are described (*X. vesparum* and *Xenos zavattarii* (Pierce, 1911) (*Benda et al., 2022*), the latter only documented from Tripoli, Libya). In contrast, 32 different species of *Stylops* are currently documented and are accepted as valid species in the Western Palaearctic, and many of them occur in sympatry (*Straka, Jůzová & Nakase, 2015*). Male *Stylops* are known to hatch in synchronised masses during a few days in late winter/early spring (*Grabert, 1953*; *Lauterbach, 1954*; *Tolasch, Kehl & Dötterl, 2012*; *Lagoutte et al., 2013*). This situation most likely leads to copulation of one female with multiple males and to increased interspecific competition. In contrast to males of *Stylops*, hatching of males of *Xenos* is not synchronised. They are released over a period from mid-July until mid-August (*Hughes et al., 2004*). Competition between males should consequently be comparatively low. However, our attraction experiments showed that the elongated flight period of *X. vesparum* does not mean that males of this species necessarily encounter females in lower frequency than in *S. ovinae*. In both species, we found multiple males having been attracted by females in short periods of time, even simultaneously. *Hughes et al. (2004)* and *Beani et al. (2018)* also observed that several males of *X. vesparum* were attracted to *X. vesparum* females at the same time. We consequently hypothesise that in *S. ovinae*, interspecific competition and intraspecific competition for females are relatively high, whereas in *X. vesparum*, intraspecific competition is prevalent across most of the distribution range.

Kathirithamby et al. (2015) speculated that females of Strepsiptera produce species-specific pheromones to entice conspecific and to exclude heterospecific males. The sex pheromones are produced by the Nassonow's glands, which open on the ventral surface of the brood canal in the female's cephalothorax (*Dallai et al., 2004*). As we found that the female sex pheromone of *S. ovinae* attracts heterospecific males, another mechanism is (or additional mechanisms are) likely in place to reduce the chance of heterospecific mating. In his review on the rapid divergent evolution of sexual morphology, *Eberhard (2004b)* stated that species-specific traits in males are typically present when specific contact organs exist in females. Males among different *Stylops* species differ strongly in the shape and the size of their intromittent organ (*Kinzelbach, 1971*; *Kinzelbach, 1978*) (Figs. 4A–4E). The size of their penises positively correlates with the size of the conspecific female paragenital organ, at least in all three investigated species.

In contrast, the penises in *Xenos* species differ only slightly in size and shape (*Kinzelbach, 1971*; *Kifune & Maeta, 1975*; *Kifune & Maeta, 1985*; *Kifune, 1986*; *Kathirithamby & Hughes, 2006*; *Nakase & Kato, 2013*; *Cook, Mayorga-Ch & Sarmiento, 2020*) (Figs. 4F–4I). We therefore hypothesise that the paragenital organ in *Stylops* evolved as a species-specific contact organ for copulation. The proximal part and the acumen (Fig. S3) of the penises vary in width and length among species (Fig. 4). Our mating experiments on *S. ovinae* showed that only conspecific males are able to mate successfully. Therefore, we conclude that interspecific mating is prevented by structural differences of the paragenital organ of *Stylops* females. Hence the paragenital organ, in combination with the penis shape, likely functions as a prezygotic barrier between different species. This barrier is apparently missing in the genus *Xenos*, possibly because heterospecific mating does not occur or is rare for other reasons.

It remains to be investigated whether intraspecific sexual conflict may further accelerate the evolution of the genital morphology within the two sexes of *Stylops*. Intersexual conflict should increase variation within a population. Therefore, we initially hypothesised that the variation of the penis morphology is larger in *S. ovinae* than in *X. vesparum*. However, we found no difference in the magnitude of interspecific variation between the two species, and the males' intromittent organs of *S. ovinae* and *X. vesparum* vary to a similar extent (approximately 4% in centroid size). Therefore, our results do not support this interpretation. However, they do not rule out intraspecific sexual conflict either, as our assumption of intraspecific sexual conflict restricted to species of *Stylops* could have been wrong.

CONCLUSIONS

The results of our study suggest that female strepsipterans of the genera *Stylops* and *Xenos* have likely evolved tolerance traits against traumatic insemination. This is achieved by thickening of the uniformly resilin-rich integument at the site of penetration. In contrast, female bed bugs achieved tolerance by incorporating the elastomeric protein resilin at the site of penetration only. This thickening in *Stylops* and *Xenos* does not seem to have any negative effect on the male partner, which appears to be always able to pierce the penetration site within seconds. Whether or not mating behaviour and morphological diversity of male penises and female paragenital organs are directly correlated to mating success of either sex is the subject of future research. However, we predict that tolerance traits in the context of traumatic insemination are more widespread in insects than currently assumed.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Grant Disclosures

Competing Interests

Stanislav Gorb is an Academic Editor for PeerJ.

Author Contributions

- Kenny Jandausch conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Jan Michels conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Alexander Kovalev conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Stanislav N. Gorb conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Thomas van de Kamp conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Rolf Georg Beutel conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Oliver Niehuis conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

● Hans Pohl conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw measurements of the micro-indentation experiments are available in the Supplemental Files.

MicroCT Data are available at Morphosource:

https://www.morphosource.org/projects/000430676

- Copulating pair of *Stylops ovinae* CT Image Series; Media 000431013; MorphoSource DOI 10.17602/M2/M431013.

- Female cephalothorax of *Stylops hammmella* CT Image Series; Media 000430994; MorphoSource DOI 10.17602/M2/M430994.

- Female cephalothorax of *Stylops melittae* CT Image Series; Media 000431002; MorphoSource DOI 10.17602/M2/M431002.

- Penis of *Stylops hammmella* CT Image Series; Media 000430950; MorphoSource DOI 10.17602/M2/M430950.

- Penis of *Stylops melittae* CT Image Series; Media 000430981; MorphoSource DOI 10.17602/M2/M430981

18 Penises of *Stylops ovinae* surface models:.

- pmj:Strep_42/01: Media 000432425; MorphoSource DOI 10.17602/M2/M432425.
- pmj:Strep_42/02: Media 000432431; MorphoSource DOI 10.17602/M2/M432431.
- pmj:Strep_42/03: Media 000432437; MorphoSource DOI 10.17602/M2/M432437.
- pmj:Strep_42/04: Media 000432442; MorphoSource DOI 10.17602/M2/M432442.
- pmj:Strep_42/05: Media 000432448; MorphoSource DOI 10.17602/M2/M432448.
- pmj:Strep_42/06: Media 000432454; MorphoSource DOI 10.17602/M2/M432454.
- pmj:Strep_42/08: Media 000432460; MorphoSource DOI 10.17602/M2/M432460.
- pmj:Strep_42/09: Media 000432466; MorphoSource DOI 10.17602/M2/M432466.
- pmj:Strep_42/10: Media 000432472; MorphoSource DOI 10.17602/M2/M432472.
- pmj:Strep_42/11: Media 000432478; MorphoSource DOI 10.17602/M2/M432478.
- pmj:Strep_42/12: Media 000432484; MorphoSource DOI 10.17602/M2/M432484.
- pmj:Strep_42/13: Media 000432489; MorphoSource DOI 10.17602/M2/M432489.
- pmj:Strep_42/14: Media 000432495; MorphoSource DOI 10.17602/M2/M432495.
- pmj:Strep_42/15: Media 000432501; MorphoSource DOI 10.17602/M2/M432501.
- pmj:Strep_42/16: Media 000432507; MorphoSource DOI 10.17602/M2/M432507.
- pmj:Strep_42/17: Media 000432512; MorphoSource DOI 10.17602/M2/M432512.
- pmj:Strep_42/19: Media 000432518; MorphoSource DOI 10.17602/M2/M432518.
- pmj:Strep_42/22: Media 000432529; MorphoSource DOI 10.17602/M2/M432529.

17 Penises of *Xenos vesparum* surface models:

- pmj:Strep_43/02: Media 000432535; MorphoSource DOI 10.17602/M2/M432535.
-pmj:Strep_43/03: Media 000432541; MorphoSource DOI 10.17602/M2/M432541.
-pmj:Strep_43/04: Media 000432547; MorphoSource DOI 10.17602/M2/M432547.

-pmj:Strep_43/05: Media 000432553; MorphoSource DOI 10.17602/M2/M432553.
-pmj:Strep_43/06: Media 000432559; MorphoSource DOI 10.17602/M2/M432559.
-pmj:Strep_43/07: Media 000432565; MorphoSource DOI 10.17602/M2/M432565.
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-pmj:Strep_43/10: Media 000432583; MorphoSource DOI 10.17602/M2/M432583.
-pmj:Strep_43/11: Media 000432589; MorphoSource DOI 10.17602/M2/M432589.
-pmj:Strep_43/12: Media 000432595; MorphoSource DOI 10.17602/M2/M432595.
-pmj:Strep_43/13: Media 000432601; MorphoSource DOI 10.17602/M2/M432601.
-pmj:Strep_43/14: Media 000432607; MorphoSource DOI 10.17602/M2/M432607.
-pmj:Strep_43/15: Media 000432613; MorphoSource DOI 10.17602/M2/M432613.
-pmj:Strep_43/16: Media 000432619; MorphoSource DOI 10.17602/M2/M432619.
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Have female twisted-wing parasites (Insecta: Strepsiptera) evolved tolerance traits as response to traumatic penetration?

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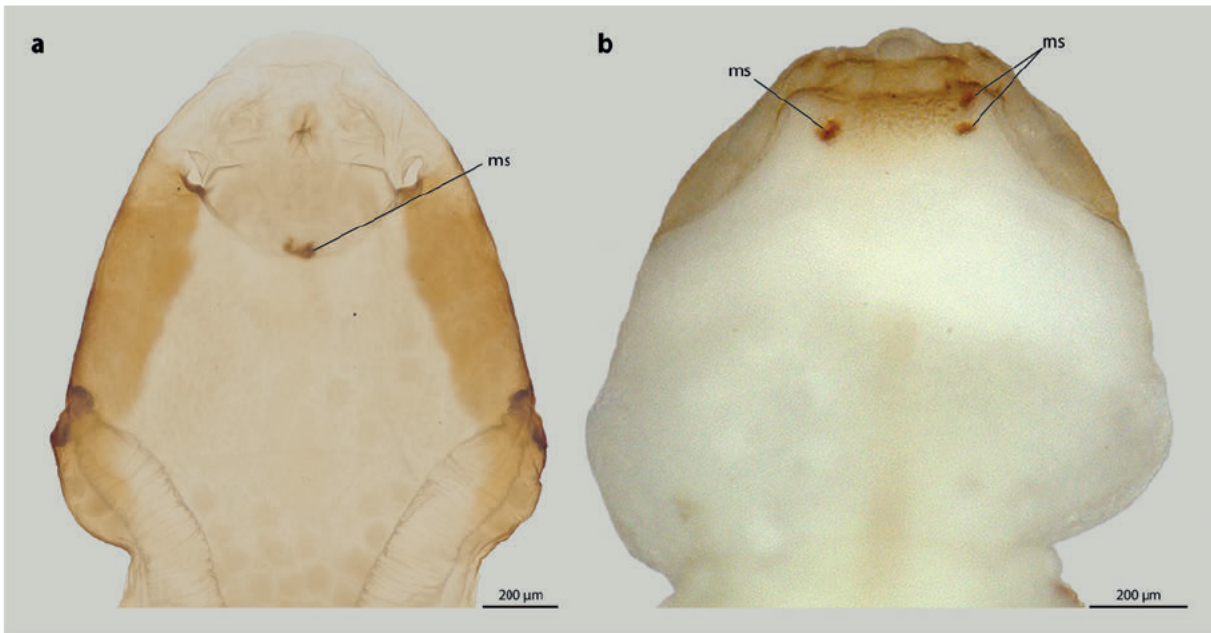
Supplementary Figure S1. Female cephalothoraces of *Stylops ovinae* (a) and *Xenos vesparum* (b) with mating signs (outer cuticle of the cephalothorax removed). a: modified from Peinert et al. (2016). Abbreviation: ms – mating sign.
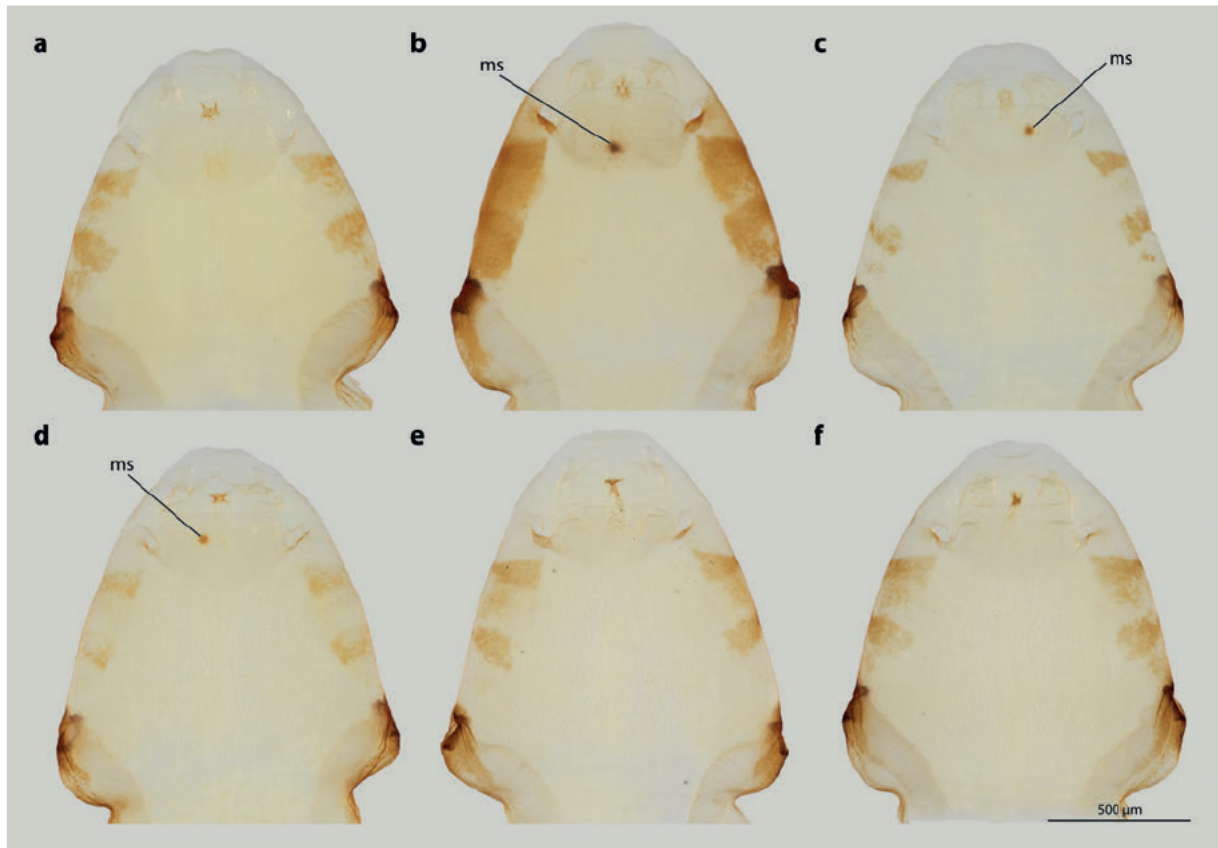
Supplementary Figure S2. Female cephalothoraces of *Stylops ovinae* from mating experiments with males of *Stylops melittae* described in this study. a, e, f: Female *Stylops ovinae* without mating sign after allospecific mating attempts. b, c, d: Female *Stylops ovinae* with injurys from allospecific mating attempts. Abbreviation: ms – mating sign.
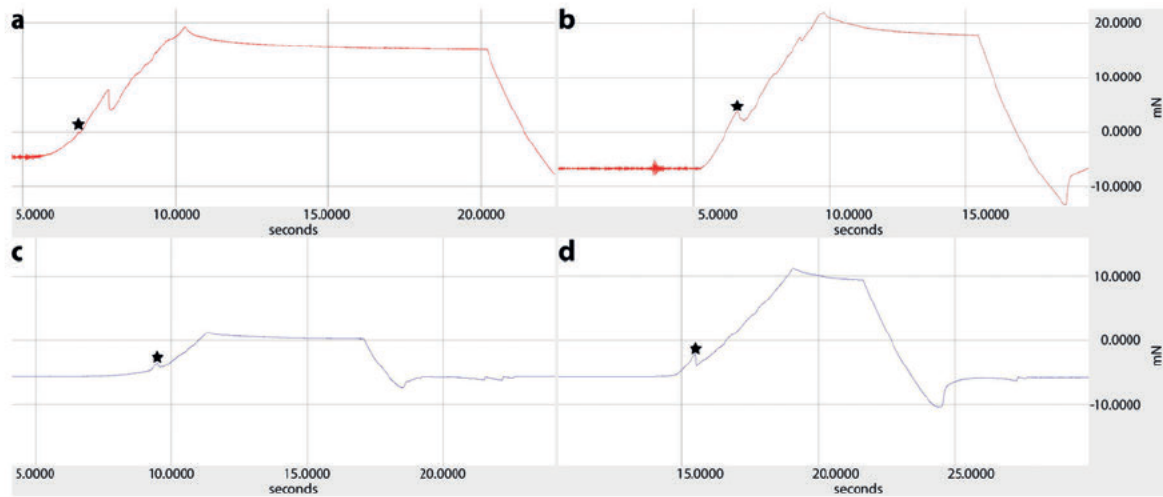
Supplementary Figure S3. Exemplary Penetration force curves for each of the four tested scenarios. a: Control site of *Sytlops ovinae.* b: Wounding site of *Stylops ovinae* c: Control site of *Xenos vesparum*. d: Wounding site of *Xenos vesparum*. Star indicates penetration of either control or wounding site.

Supplementary Figure S4. Scanning electron micrographs of the penises of *Stylops ovinae*, *Xenos vesparum* and the tip of the microneedle used in the penetration experiments. a: Penis of *Stylops ovinae*, lateral view. b: Penis of *Xenos vesparum*, lateral view. c: Tip of the microneedle.
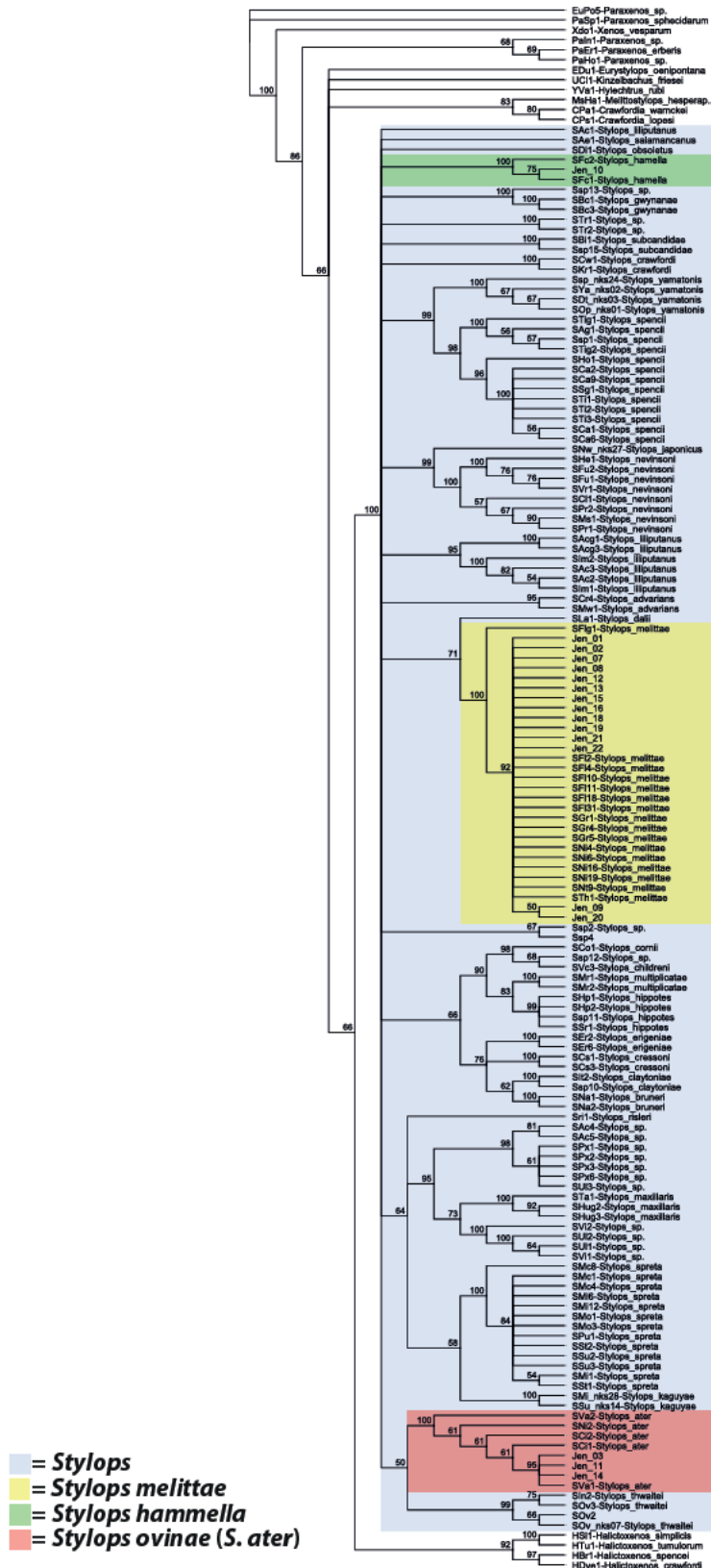
Supplementary Figure S5. Phylogenetic relationships of COI barcode nucleotide sequences from species of the genus *Stylops* and of closely related genera. COI nucleotide sequences published by Juzova et al. (2015) served as references. Sample IDs starting with Jen and followed by a number indicate male *Stylops* samples studied in the present investigation. The phylogeny was inferred with the neighbour-joining method and applying the Tamura-Nei substitution model. Values at branches indicate bootstrap support over 50 %.

FORM 1

2.4 Manuscript No. IV

Manuscript title: *Polyandry and sperm competition in two traumatically inseminating species of Strepsiptera (Insecta)*

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Authors' contributions (in %) to the given categories of the publication

Author	Conceptual	Data analsis	Experimental	Writing the manuscript	Provision of material
Jandausch, K.	10 %	45 %	60 %	60 %	40 %
Wanjura, N.	10 %	45 %	30 %		
Escalona, H.			5 %		
Sann, M.			5 %		
Beutel, R.G.	10 %			10 %	
Pohl, H.	35 %			15 %	60 %
Niehuis, O.	35 %	10 %		15 %	
Total:	100 %	100 %	100 %	100 %	100 %

_____   _____

Signature candidate   Signature supervisor (member of the Faculty)

Polyandry and sperm competition in two traumatically inseminating species of Strepsiptera (Insecta)

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1

Abstract

Polyandry, the practice of females mating with multiple males, is a strategy occurring in many groups of insects. Whether it increases the likelihood of receiving beneficial genes from male partners and other potential benefits for females is a contentious subject. Strepsiptera are generally considered monandrous, however, in a few species, females have been observed copulating serially with multiple males. Here we show that the offspring of a single female can have multiple fathers in two species of Strepsiptera: *Stylops ovinae* (Stylopidae) and *Xenos vesparum* (Xenidae). We studied female polyandry in natural populations of these two species by analysis of polymorphic microsatellite loci. Our results showed that several fathers can be involved in both species, in some cases up to four. Mating experiments with *S. ovinae*, revealed that the male that mates first with a given female contributes to a higher percentage of the offspring than subsequent males. In *X. vesparum*, however, we found no significant correlation between mating time and offspring contribution. The conspicuously prolonged copulation observed in *S. ovinae* has probably the advantage of reducing competition with sperm from other males. Our results show that monandry may not be the general pattern of reproduction in the insect order Strepsiptera.

Keywords: monandrous, microsatellites, mate guarding, paternity tests, *Stylops ovinae*, *Xenos vesparum,* sperm competition

Introduction

21   Copulation with multiple mating partners is a strategy that can provide fitness advantages over

22   intraspecific competitors [1]. This kind of promiscuous sexual reproduction has long been considered a

23   domain of males [2,3], which — according to the Bateman principle — have higher variance in their

24   reproductive success than females. However, not only males can exert multiple copulation as a mating

25   strategy. Polyandry, the mating of a female with multiple males, is widespread in the animal kingdom

26   e.g., [4,5-8]. The strategy provides females advantages and disadvantages. Parker and Birkhead [9] list as

27   advantages of this mating strategy among others an increased number of fertilised eggs, a higher genetic

28   diversity of the offspring, and the general permission for postcopulatory mate choice. The same authors

29   mention as disadvantage an increased risk of predation [9]. In his monograph on sperm competition, [10]

30   summarised five factors that could explain the evolution of polyandry: (i) sperm replenishment – females

31   remate to top up sperm stores depleted by previous ovipositions, to replace inviable sperm, or to ensure

32   fertility by other means; (ii) material benefits – females remate to acquire resources controlled by males,

33   such as nesting sites, food resources, or protection from conspecific and/or heterospecifics; (iii) genetic

34   benefits – females replace sperm of previous matings with sperm from a genetically superior mate,

35   encourage competition between sperm to ensure fertilization by sperm of high quality, or ensure genetic

36   diversity in offspring; (iv) convenience: females remate to minimize costs of harassment from males;

37   and (v) correlated evolution – females remate because of correlated response to sexual selection on

38   multiple mating males. However, the idea that females exerting polyandry benefit genetically has been

39   questioned by Slatyer, et al. [11], who found only weak support for this in a comprehensive meta-analysis

40   of experimental studies.

41   Polyandric behaviour generates a competitive environment for sperm of different males within the

42   body of the inseminated females after copulation [1,10,12,13]. Two key factors for generating this competitive

43   environment are currently considered: the temporal and the spatial simultaneous presence of sperm from

44   different males in the genital tract, or generally, inside the female's body [1,10]. This overlap can be

45   strongly influenced by the morphology of the female's reproductive apparatus. For instance, the

46   structure, function, and number of the spermathecae can strongly influence sperm competition [14]. Such

47   adaptations enable females to select preferred sperm for fertilization. Males may have evolved counter

48   strategies in their post-copulatory behaviour [1]: the removal of sperm from a previous mating partner

49   using specialised structures e.g., described in dragonflies; [15]; applying a mating plug to close the genital

50   opening of a female after having mated with it [16]; extending the copulation time mate guarding; [1,17].

51   Sperm competition can thus amplify sexual selection [1].

52   The endoparasitic holometabolous order Strepsiptera comprises ca. 600 described species and is

53   characterised by numerous derived features in both sexes throughout the life stages [18,19]. All species of

54   Strepsiptera show extreme sexual dimorphism. Their males are free-living; the only function of their

55   short adult life span of a few hours is to find females and to mate with them. The females are obligatory

56   endoparasites of other insects, in which they stay during most of their larval development and as adults,

57   except for species of Mengenillidae, whose adult females are free-living. Strepsiptera are well known

58   for their bizarre morphology and life history and serve as a prime example of an insect taxon that exhibits

59   traumatic insemination [18-21]. The fact that females cannot actively prevent conspecific males from

60   copulating increases the chances of multiple males contributing to the fertilization of a female's eggs

61   (i.e., polyandry) [20,22]. However, there is only anecdotal evidence for polyandry in Strepsiptera and it is

62   based on few random observations of multiple males serially copulating with the same female [23-25].

63   Whether these observations are representative for the behaviour of the investigated species or even the

64   entire insect order is unclear as is the question of whether multiple males also fertilize a given female's

65   eggs. We undertook field and controlled laboratory experiments in combination with DNA paternity

66   tests to provide answers to these questions. These answers could in turn help explaining the peculiar

67   reproductive behaviour that some Strepsiptera exhibit (e.g., prolonged copulation).

68   The Strepsiptera species whose mating behaviour we investigate are *Stylops ovinae* (Stylopidae) and

69   *Xenos vesparum* (Xenidae). Both belong to the clade Stylopidia, which are characterized by pterygote

70   insect hosts and adult females remaining within the host's body. The large sack-shaped posterior body

71   of the female resides within the host's abdomen, whereas their sclerotized cephalothorax is exposed

72   [18,19,26,27]. In females of Corioxenidae penetration takes place at a very weakly sclerotized region of the

73   cephalothorax. In the majority of species (Stylopiformia, Stylopidia excl. Corioxenidae), a birth opening

74   is present on the ventral side of the cephalothorax, between the head and prosternum. This external

75   opening of the brood canal, which is connected with the birth organs in the abdomen, serves as birth

4

76   opening. The birth opening enables the primary larvae to leave the females (Figure 1). In species of

77   Stylopiformia, the birth opening also serves as the site where the penis penetrates the female's cuticle

78   during traumatic insemination.

79   Kathirithamby, et al. [28] considered Strepsiptera as monandric. This idea is based on various

80   behavioural observations, particularly in species of the family Xenidae [29,30] and Stylopidae [31,32]: for

81   example, *S. ovinae* (= *S. melittae* auct.) females reduce the production of a sex pheromone, which attracts

82   conspecific males, immediately after the first copulation. However, females do not seem to stop

83   releasing their sex pheromone entirely [33]. At the same time, there are numerous scattered hints in the

84   literature that indicate the possible occurrence of polyandry in Strepsiptera. For example, Silvestri [25] and

85   Riek [24] reported females of species in the family Halictophagidae to have mated with multiple males

86   and that these females remained still attract other potential mating partners. Similar evidence for the

87   occurrence of polyandry was provided by Kirkpatrick [23] in one species of Corioxenidae. The most recent

88   and detailed evidence for the occurrence of polyandry in Strepsiptera has been presented by Peinert, et

89   al. [20], the authors conducted controlled laboratory experiments showing that female *S. ovinae* serially

90   mate with multiple males and that these remained attractive for an extended period of time (up to 2

91   hours) after the first copulation.

92   All species of Strepsiptera are thought to be traumatically inseminated [21]. Traumatic inseminating

93   (TI) is defined as a copulation method in which the copulatory organ is pierced directly through the body

94   integument of the female, allowing the male's sperm to enter directly the female's body cavity. This

95   bizarre mode of copulation in combination with the endoparasitic lifestyle of the female Strepsiptera,

96   which thus cannot prevent copulation with males and may not have any precopulatory influence on the

97   choice of mating partners, strongly suggests mating by several males (i.e., polyandry). The occurrence

98   of TI itself is probably triggered by sperm competition, as this allows males to bypass the female

99   genitalia and thereby also sperm previously deposited by other males [34-37]. Whether females of

100   Strepsiptera gain an advantage from polyandry is currently unknown. Given the large quantity of eggs

101   produced by females of Strepsiptera (e.g., *Eoxenos laboulbenei* (Mengenilldae): ca. 1,400, [38]; *S. ovinae*:

102   ca. 29,000, unpublished data by H. Pohl & H. Stark; *Stichotrema dallatorreanum* (Myrmecolacidae): up

103   to 750,000, [39]), multiple mating could help females receiving the necessary amount of sperm to have all

104   their eggs fertilized (sperm replenishment hypothesis). However, this interpretation remains speculative,

105   as collecting supportive evidence is difficult due to various challenges associated with handling and

106   rearing Strepsiptera.

107   Polyandry likely puts strong selective pressure on males to mate first with a female and to prevent or

108   delay fertilization of the eggs by other males. The absence of sperm-storing structures and ovaries in

109   adult females excludes post-copulatory mate choice, as sperm is directly released into the haemolymph

110   by males. Structures such as the spermalege described in bed bugs by Carayon [40] and which are suspected

111   to enable cryptic mate choice with the help of haemocytes [41,42] are not found in Strepsiptera either. One

112   way of males to reduce sperm competition is to extend the duration of their copulation and/or to guard

113   their mating partner. Both strategies would delay access by other males to the female. Consistent with

114   this idea, Peinert, et al. [20] found not only exceptionally long copulation times of a first mating male of

115   *S. ovinae* (up to 34 min), but also significantly shorter copulation times of any following mating male.

116   In comparison, the reported copulation time of *X. vesparum* is 5–15 s [43], that of *Elenchus tenuicornis*

117   (Elenchidae) 1–3 s [44], and that of *Corioxenos antestiae* (Corioxenidae) a few seconds to 1 min, seldom

118   5 min [23]. Based on these observations, Peinert, et al. [20] discussed the possibility that a first male that

119   mates with a given female in *S. ovinae* guards the female, thereby reducing sperm competition with

120   other males. An extended copulation duration could alternatively or additionally enable the male to

121   inject more sperm into the female. However, we consider this as unlikely. If the total amount of sperm

122   rather than the sequence of insemination would primarily determine how many eggs are fertilized by a

123   male ('fair raffle' scenario *sensu* Parker [45]), the copulation duration of subsequent males should not

124   drastically differ from that of the first male. We consider it more likely that *S. ovinae* exerts mate

125   guarding and that this strategy evolved in this species in correlation with the highly synchronized

126   hatching of its males. This creates a highly competitive environment in which (often) several males

127   compete for a given female (supplementary movie 2; Peinert, et al. [20]). If it is very likely that a male's

128   sperm compete with that of other males in a given female, and if injected sperm result in a timely

129   fertilization of this female's eggs, males should benefit from restricting access to this female. If the

130   chance of a given female to be mated by multiple males during the short lifespan of the first mating male

131   (males typically life less than six hours) is low, a male could benefit more from searching for additional

132   females than from guarding a female.

133   In the present investigation, we use experimental behavioural assays in combination with paternity

134   tests based of newly established microsatellite markers to assess whether multiple mating in Strepsiptera

135   indeed results in the fertilization of a female's eggs by multiple males. Having established all necessary

136   tools, we additionally address the question whether the extended copulation duration of the first

137   copulating male in *S. ovinae* reduces the chance of subsequently copulating males to inseminate the

138   same female's eggs, compared to species that do not exert mate guarding behaviour. We used *S. ovinae*

139   and *X. vesparum* to conduct our tests. While *S. ovinae* can be found exclusively in the grey-backed

140   mining bee (*Andrena vaga*, Andrenidae; Straka, et al. [46]), *X. vesparum* is found in various species of the

141   paper wasp genus *Polistes*, especially in the European *Polistes dominula* [47], but also in *Ropalidia*

142   (Vespidae). Males of *S. ovinae* hatch in synchronised masses in a time period of only a couple of days

143   in late winter/early spring [31,33,48,49]. In contrast, the hatching period of *X. vesparum* lasts at least a month

144   under natural conditions, from mid-July until mid-August [50], and hence can be considered asynchronous.

145   Mass emergence of males is obviously not unique to *S. ovinae*, but can potentially also occur in other

146   species under certain circumstances. Jandausch, et al. [22] attracted over 100 conspecific males with

147   unmated *X. vesparum* females during a single day, with some males even approaching the cage with the

148   stylopized paper wasps simultaneously. Mass occurrence of males was also observed in *Mengenilla*

149   *moldryzki* (Mengenillidae) in Tunisia [51].

150

Results

Polyandry in field-collected Strepsiptera

Stylops ovinae

154   Among the ten pools of larvae, seven exhibited more than four different alleles at least at one locus,

155   indicating the participation of more than one father in the generation of a female's offspring (Table 1)

156   (min = 1; max = 4; mean = 2.1; n = 10). The number of alleles (i.e., nine) in one specific pool of primary

157   larvae (S08) suggests a minimum of four fathers. Polyandry thus clearly occurs in *Stylops ovinae* and

158   seems to be common in the studied population.

159   *Xenos vesparum*

160   Among the ten pools of larvae, one (X07) exhibited more than four different alleles at one locus,

161   indicating the participation of more than one father in the generation of a female's offspring (Table 2)

162   (min = 1; max = 2; mean = 1.1; n = 10). Polyandry thus clearly occurs in *X. vesparum*, but does not seem

163   to be common in the studied population.

164

165   **Polyandry under laboratory conditions**

166   *Stylops ovinae*

167   We were able to infer the paternity of 397 of the 400 genotyped primary larvae (Figure 2, Table S8).

168   The electropherograms of the remaining three primary larvae lacked detectable signal. The offsprings'

169   genotypes indicate in nine of the ten experiments that both males contributed genetically. Only in one

170   mating experiment (So10; n =40) only a single male was involved, the one that mated first. In seven of

171   the ten mating experiments the genetic contribution by the two males is statistically biased, with the first

172   male being more frequently a father than expected by chance: So01 (n = 40), So02 (n =37), So05 (n =

173   40), So07 (n = 40), So08 (n = 40), So09 (n = 40), and So10 (n = 40) (χ2-test: χ2 = 10.0–40.0; df = 1; p

174   < 0.0016).

175

176   *Xenos vesparum*

177   We inferred the paternity of 354 of the 400 genotyped primary larvae (Figure 2, Table S9). The allelic

178   composition of the remaining 46 primary larvae did not allow identifying a father unambiguously. The

179   paternity data showed that both males contributed genetically to the offspring in all but one of the

180   experiments. Only in one mating experiment (Xv10, n = 39) a single male was identified as father, in

181   this case the one that mated second. The genetic contribution by the two males was statistically biased

182   in five of the ten mating experiments, with the first male being more frequently a father than expected

183   by chance: Xv02 (n = 39), Xv03 (n =31), Xv07 (n = 38), Xv08 (n = 40), and Xv09 (n = 31) (χ2-test: χ2

184   = 8.53–31.41; df = 1; p < 0.004). Only in mating experiment Xv10 (n = 39) the second male was more

185   often successful than expected by chance (χ2-test: χ2 = 38.011; df = 1; p = 7.034e-10). In contrast to

186   males of *S. ovinae*, males of *X. vesparum* frequently show multiple copulation attempts before

187   copulating for a longer duration with a given female. We assessed whether the number of copulation

188   attempts differed between the first and second mating male, but did not find a statistically significant

189   difference (Mann-Whitney U test: n = 18, W = 125.5, p-value = 0.2353).

190

191   **Effect of the first male's copulation duration on the paternity success by the two copulating males**

192   *Stylops ovinae*

193   We found a statistically significant positive correlation between the copulation duration of the first male

194   and the paternity success of the second male (generalized linear model: n = 10; p = 0.000061; Figure

195   3A). The positive correlation remained statistically significant after removing the data from experiment

196   So10, in which the second male did not contribute genetically to the offspring (generalized linear model:

197   n = 9; p = 0.00193). We found no impact of the copulation time of the second male on its paternity

198   success (generalizes linear model: n =10; p = 0.168).

199   *Xenos vesparum*

200   We found no statistically significant correlation between the copulation duration of the first male and

201   the paternity success of the second male (generalized linear model: n = 10; p = 0.774; Figure 3B). This

202   result was not affected after we removed the data from experiment Xv10, in which the first male

203   seemingly did not contribute genetically to the offspring (generalized linear model: n = 9; p = 0.456441).

204   However, we found a statistically significant correlation between the copulation duration of the second

205   male and the contribution of the first male to a female's offspring (generalized linear model: n = 10; p <

206   0.000487).

207

Discussion

Peinert, et al. [20] found that females of *S. ovinae* are sequentially mated by multiple males in laboratory experiments. However, it remained unclear whether subsequently copulating males also contribute genetically to a female's offspring. Our parental tests showed that the offspring of a female can indeed have multiple fathers. They also revealed that polyandry can be common in nature in Strepsiptera: ca. 70% in the studied population of *S. ovinae*. Peinert, et al. [20] pointed out that the first copulation in *S. ovinae* is significantly longer than subsequent copulations with other males and interpreted this conspicuous behaviour tentatively as mate guarding. The results of our paternity tests confirm this, as we found a statistically significant negative correlation between the copulation duration of the first mating male and the fertilisation success of a second male: the first mating male profits from extending its copulation with a given female in time. A positive correlation between the length of copulation and fertilisation success has also been described in other polyandric organisms, such as the scorpion fly *Panorpa cognata* [52], the nursery web spider *Pisaura mirabilis* [17,53], and the ensign fly *Sepsis cynipsea* [54]. Our interpretation of the results also appears plausible from a morphological point of view: Strepsipteran females lack structures to store sperm. Traumatic insemination results in an immediate contact of the sperm with the eggs that are freely floating in the haemolymph. As no mechanisms or morphological structures are known that would enable cryptic sperm choice of females, the first mating male gains a high level of control over how many eggs are fertilized by its own sperm. The second mating male cannot gain any advantage by mating with females that had been fertilized a longer time ago, because the egg production is completed with the end of the postembryonic development.

In contrast to the experiments of Dallai, et al. [29], which suggested that males of *X. vesparum* are not attracted to previously mated females, our experiments show that females of this species remain attractive to males for an extended period of time, even if these females had previously been fertilized by another male. In our laboratory experiments, males mated with females up to 20 min after the females had been mated by another male (Table S5, Xv01); and the paternity tests demonstrate that both males genetically contribute to the offspring (Figure 2). However, in nature, the fraction of females whose eggs are fertilized by multiple males is likely lower, 10% in the case of the population studied by us.

235 Males of *X. vesparum* hatch asynchronously over a period of ca. one month [50]. The chance of two males

236 competing for access to a given female simultaneously are thus likely distinctly lower in this species

237 than in *S. ovinae*. It is therefore unlikely that *X. vesparum* males benefit from mate guarding, and males

238 investing their short lifetime in search for a second mating partner could have an overall higher

239 reproductive success. Consistent with these considerations, we found no evidence for a prolonged

240 copulation duration of the first mating male compared to the second. We also found that the copulation

241 duration of the first male does not correlate with the male's relative genetic contribution to the female's

242 offspring, even if the female was subsequently mated by a second male. While we found that the

243 copulation duration of the second mating male negatively impacts the relative genetic contribution of

244 the first male to a female's offspring in laboratory mating experiments, we expect this pattern is largely

245 irrelevant under natural conditions. Laboratory experiments are likely affected by copulations of males

246 with a female in quick succession. It is unlikely that this happens in nature as *X. vesparum* males emerge

247 over a long period without a chance of noteworthy concentration. A possible explanation for the

248 copulation duration of the second mating male affecting the genetic contribution of the first male to the

249 offspring could be that sperm of both males directly compete with each other for a limited number of

250 eggs, with a longer copulation allowing a male to transfer more sperm ('fair raffle' scenario *sensu* Parker

251 [1]). However, we consider this scenario as unlikely for various reasons (see above), including the fact

252 that insemination starts right at the beginning of the copulation, and a brief mating duration is sufficient

253 for males to transfer enough sperm to fertilize all of the eggs [31,49].

254 We found no statistically significant difference in the duration of the first and the second copulation in

255 our experiments (Table S4), which were on average under 10 s in length. Therefore, we assume that *X.*

256 *vesparum* males are unable to assess whether or a female is already mated, in contrast to males of *S.*

257 *ovinae* [20]. We found that males of *X. vesparum* try to mate multiple times with a given female. While

258 we interpreted most of these copulations as failed attempts, they could possibly represent true matings

259 with transfer of sperm. Such a behaviour has been shown to allow males of some species to transfer

260 more sperm e.g., [55,56,57]. However, we expect this as unlikely in the case in *X. vesparum*, based on the

261 same arguments listed in the discussion on the possible impact of the mating duration on the fertilisation

262 success. Moreover, we found no statistically significant correlation between the number of mating

263   attempts of a male copulating with an already mated female and its reproductive contribution to the

264   offspring. An active or passive displacement of previously deposited sperm by subsequent males, as it

265   has been shown in dragonflies [58], also appears implausible, as strepsipteran females are traumatically

266   inseminated – with sperm directly injected into the female's haemolymph.

267   Hrabar, et al. [30] described the calling position of *X. peckii* and reported females leaving this posture

268   and retracting into the host after copulation. This behaviour was interpreted as a mechanism to prevent

269   serial mating by multiple males. We also observed distinct extrusion (hyperextrusion) of *X. vesparum*

270   females from their hosts in our experiments. However, we never observed females retracting their

271   cephalothorax directly after mating. Intriguingly, we even found males mating successfully with females

272   that had not been in calling position at all. Thus, while females may be able to reduce the number of

273   matings by retracting their cephalothorax into the host's body, it seemingly does not fully prevent being

274   mated multiple times and hence polyandry *per se*. In fact, females of the two species studied by us

275   probably mitigate possible negative consequences of traumatic insemination, such as wounding

276   [22,35,37,59,60], by morphological adaptations rather than by a reduced number of matings.

277   Strepsipteran females could benefit from mating with multiple males. Possible advantages are an

278   increased number of fertilized eggs, an increased genetic diversity of the offspring, and a reduced impact

279   of mating with a sterile male [9,11,61,62]. Given the present results, we assume that females either benefit

280   from copulations with multiple males or tolerate them as no negative costs are involved [22,59].

281   That the copulation duration of the second mating male of *S. ovinae* is on average shorter than that

282   of the first mating male suggests that the males are able to perceive a female's mating status. Potential

283   clues could be various substances such as the ejaculate of a previous male like in bed bugs [63],

284   haemolymph emerging from a previous mating wound, pheromones released by another male on the

285   host's abdomen, or specialized pheromones produced by mated females [20]. Another possible signal

286   perceived by the males could be the decrease of sex pheromone released by the females after copulation.

287   Tolasch, et al. [33]not only described the chemical structure of the sex pheromone compound (i.e.,

288   stylopsal; Cvačka, et al. [64]), but also monitored its release from a female. They found that the amount of

289   pheromone secreted by a female significantly decreases immediately after copulation. A perception of

290   the mating status with the copulatory organ was excluded by Peinert, et al. [20], as no sensilla are present

291   on the penis. The perception of the female's mating status is only one important aspect for the male to

292   be taken into account. When it comes to strategic ejaculation, as studied in detail by Kelly and Jennions

293   [65] in a meta-analysis, other factors can also play a role, like the number of rivals or the mating condition

294   of the female. Wedell and Cook [66] showed that the small white *Pieris rapae* (Lepidoptera: Pieridae) can

295   strategically determine the amount of ejaculated sperm depending on the probability of remating and

296   the degree of sperm competition. This is essential for provide enough sperm for multiple copulations in

297   regard to regeneration of sperm. Comparable data from Strepsiptera are not available, but strategic

298   ejaculation could on principle also play a role in the mating strategies of this taxon. However, as the

299   lifespan of male strepsipterans is too short for replacing ejaculated sperm, in contrast to Pieridae and

300   other groups, this appears very unlikely.

301

302   Conclusion

303   Our study revealed that, contrary to Kathirithamby, et al. [28], polyandry occurs not only in laboratory

304   experiments with strepsipterans[20], but also in natural populations. Considering the largely uniform

305   morphology and (as far as known) uniform behaviour of Strepsiptera, this tentatively suggests that

306   polyandry could be a ground plan feature of this insect order. Representatives of more families need to

307   be studied for an unambiguous conclusion. However, this hypothesis would be consistent with Kokko

308   and Mappes [67] who stated that polyandry rather than monandry should serve as a 'null hypothesis'. The

309   results of our experiments with *S. ovinae* are consistent with the mate guarding hypothesis of Peinert, et

310   al. [20], as the copulation duration of the first-mating male negatively impacts the fertilization success of

311   a second male. The prolonged copulation of the first male most likely prevents access of competing

312   males to the female, thus increasing the proportion of the first male's genetic contribution to the female's

313   offspring (Supplementary Video S2). In contrast, our data do not suggest mate guarding in *X. vesparum*.

314

315   **Material and Methods**

316   **Specimen collection, rearing, and mating experiments**

317   Field samples

318   To detect polyandry of *S. ovinae* and *X. vesparum* in nature, we collected fertilised females of the two

319   species and determined the number of alleles at different polymorphic loci in each female's offspring.

320   Specifically, to assess the extent of polyandry in *S. ovinae,* HP and KJ collected 50 host bees (*A. vaga*)

321   in the nature reserve Teverner Heide (North Rhine-Westphalia, Germany), parasitised by a total of 59

322   *S. ovinae* females. The bees were collected on a warm day (ca. 16 °C at noon) (February 28, 2022), with

323   male *S. ovinae* hatching in large numbers and mating with females. Given the short lifespan and flight

324   period of males, it is reasonable to assume that most (if not all) collected females had been fertilized by

325   at least one male. To rear primary larvae from the (likely) fertilized females collected in the field, we

326   kept the stylopised host bees under stable light in 0.5-l glass jars half filled with moist sand and sealed

327   with gaze in the laboratory for several weeks until the primary larvae started to hatch. The temperature

328   was regulated in a climate chamber as follows: 14 °C from 7 a. m. to 10 a. m., 19 °C from 10 a. m. to 5

329   p. m., 14 °C from 5 p. m. to 6 p. m., and 11 °C from 6 p. m. to 7 a. m. The light was turned on at 7 a. m.

330   and was turned off at 6 p. m., resulting in a regular photoperiod of 11 h. The host bees were supplied

331   with diluted honey and water *ad libidum*. The females were visually checked daily with a ten times

332   magnifying glass for the appearance of primary larvae, which was easily recognizable when the larvae

333   appeared at the birth opening. Hatching was accompanied by darkening of the female's cephalothorax,

334   caused by the primary larvae inside (Figure 1A). Females with offspring were removed from the host

335   bee with fine tweezers and stored in pure ethanol at -15 °C. Of the 59 collected *S. ovinae* females, 31

336   produced primary larvae. The larvae hatched between April 5 and April 20.

337   The females of *X. vesparum* were collected together with their host, *P. dominula*, on July 7, 2021, in

338   Mettenheim (Rhineland Palatinate, Germany) by HP and KJ. We collected 29 wasps with a total of 33

339   *X. vesparum* females, all of which had primary larvae at the birth opening (Figure 1D). The presence of

340   primary larvae made it unnecessary to keep *X. vesparum* females and their host for an extended period

341   alive in the laboratory. The females were removed from their hosts in the lab with fine tweezers and

342   stored in pure ethanol at -15 °C. As some hosts contained several females, special care was taken to

343   avoid cross contamination between tissues of the females and their primary larvae.

344   <u>Laboratory mating experiments</u>

345   To assess whether the first mating male fertilizes a higher number of offspring than subsequently mating

346   males, we conducted paternity tests on parents and offspring of *S. ovinae* and *X. vesparum* in controlled

347   laboratory mating experiments. In case of *S. ovinae*, the necessary experiments had already been

348   conducted by Peinert, et al. [20], whose samples were available to us and suitable for DNA genotyping.

349   The description of their mating experiments is given below. Note that Peinert, et al. [20] provided detailed

350   unpublished information on the parents of all offspring, the mating sequence, and the time and duration

351   of copulations.

352   To determine the duration and frequency of copulations, 68 living specimens of *A. vaga*, each parasitized

353   with a single virgin female of *S. ovinae*, were placed separately in glass vessels (0.5 l) with absorbent

354   paper (preventing males from getting stuck on excretions of the host bees) at 21 ± 1 °C. A cold light

355   source was used for illumination. A freshly hatched male of *S. ovinae* was placed in each glass vessel.

356   After the first copulation, the males were left in the vessel for ca. 10 min. Thereafter, the first male was

357   removed, and a second freshly hatched male was placed to 58 individuals of the females each. To assess

358   the duration of female attractiveness after copulation, a single newly hatched male was added to 17 *A.*

359   *vaga* individuals, each parasitized with a single *S. ovinae* female, from 50 min to 3 h 18 min after the

360   first copulation after [20]. In each experiment, the male's behaviour was recorded.

361   We genotyped the parents and offspring from a total of ten mating experiments. The males (fathers) had

362   been stored separately in pure ethanol. The bees containing the females (mothers) had been kept under

363   the following controlled conditions in a climate chamber till the emergence of the primary larvae: 14 °C

364   from 7 a. m. to 10 a. m., 18 °C from 10 a. m. to 5 p. m., 14 °C from 5 p. m. to 6 p. m., and 10 °C from

365   6 p. m. to 7 a. m. The light was turned on at 7 a. m. and was turned off at 6 p. m., resulting in a regular

366   photoperiod of 11 h. Bees were kept separately in 0.5-l glass jars half filled with moist sand and sealed

367   with gaze and provided with diluted honey and water *ad libidum*. After the emergence of the primary

368   larvae, we dissected the bees, removed the *S. ovinae* female(s), and stored them separately together with

369   the primary larvae in 100 % pure ethanol.

370   We set up 18 mating experiments with samples of *X. vesparum* similar in design to those conducted by

371   *Peinert, et al.* [20] with *S. ovinae*. However, Peinert, et al. [20] studied freshly hatched males in their

372   experiments, while we studied *X. vesparum* males that were attracted by unmated females in the field.

373   The experiments were performed in 2018, 2020, and 2021 using samples collected from localities listed

374   in Table S1. In each experiment, the host wasp with an unmated female of *X. vesparum* was fixed in a

375   plastic tube (20 mm long, 7 mm diameter) with a short thread attached to the wasp waist, leaving only

376   the metasoma protruding from the tube. To ensure that the strepsipteran male had only access to the

377   protruded wasp metasoma containing the strepsipteran female and to no other regions of the wasp, the

378   end of the tube with the wasp's head and the space between the wasp's metasoma and the tube were

379   filled with cotton to ensure that males did not get stuck in the tube. The tubes were then individually

380   placed and fixed with plasticine in containers (105 mm x 65 mm x 45 mm), in which the mating

381   experiments were carried out. We started each experiment by releasing a single *X. vesparum* male into

382   a container (Supplementary Video 1), where it remained until it had copulated with the female and

383   showed no further copulation attempts within 3 minutes. The male was subsequently removed from the

384   copulation vessel and fixed in pure ethanol. We then released a second male into the container and

385   repeated the procedure. After the second male had mated, each wasp was placed in individual 0.5-l glass

386   jars sealed with gaze, kept at room temperature (ca. 20 °C), and was fed *ad libidum* with diluted honey

387   and water for up to three months. We initiated artificial 12-week-long hibernation of the host wasps

388   following the procedure described by Gibo (1977). The hibernation was terminated by increasing the

389   ambient temperature to ca. 20 °C. We daily screened the wasps and their strepsipteran parasite(s) for

390   emergence of primary larvae. As soon as these appeared, the females were dissected from the host wasps

391   and stored in pure ethanol. In a few instances, the host wasps died before the release of primary larvae

392   from its parasite(s). We nonetheless included the offspring in our paternity tests, as these did not depend

393   on fully developed primary larvae. The duration of each copulation and the time between copulations

394   were documented and analysed as detailed above or described by Peinert, et al. [20](Table S2, S3, S4, S5).

395   Due to time and budget constraints, the fraction of offspring produced by the first and by the second

396   male was calculated from samples of ten of the 18 copulation experiments.

397

398   **Tissue dissection and DNA extraction**

399   All specimens were dissected under an Olympus SZ61 Zoom Stereo Microscope (Olympus, Shinyuku,

400   Japan). DNA was extracted from tissue of three legs and of pterothoracic muscles of males, from

401   cephalothoracic muscle tissue of females, and from entire primary larvae. To study the genetic

402   contribution of a given male to a female's offspring in the laboratory experiments, we extracted the DNA

403   from individual larvae (40 per female and species). The chosen sample size of 40 individual represented

404   a reasonable trade-off between available time and budget and the precision of the obtained results (i.e.,

405   is the second mating male able to fertilize a major fraction the female's large number of eggs; ca. 29,000

406   in *S. ovinae*, unpublished data H. Pohl, H. Stark). In contrast, we extracted DNA of batches of 50 primary

407   larvae to assess the occurrence and the extent of polyandry in fertilized females collected in the field

408   (one batch per female, ten batches per species). The DNA was extracted with the QIAamp DNA Micro

409   kit (Qiagen, Venlo, The Netherlands), following the manufacturer's protocol.

410

411   **Microsatellite design**

412   We screened unpublished shotgun genome sequence data of *Stylops ovinae* (obtained by curtesy of the

413   Leibniz-Institut für die Analyse des Biodiversitätswandels, Museum Koenig, Bonn, Germany) and

414   published shotgun genome sequence data of *Xenos vesparum* [68] for dinucleotide tandem repeats using

415   the software Microsatfinder [69]. We then used primer3 [70] to design oligonucleotide primers to PCR

416   amplify eight identified tandem repeats in each of the two investigated species (Tables S6 and S7). As

417   we were only interested in whether alleles specific for one (and only one) of the potential parents had

418   been transmitted to offspring, and we knew the allelic states of all possible parents, it was not necessary

419   to test for linkage disequilibrium and the occurrence of null alleles.

420

PCR

Oligonucleotide primers used for amplifying microsatellite loci in polymerase chain reactions (PCR) are given in Tables S6 and S7. PCRs were performed with the Invitrogen Taq DNA Polymerase standard PCR kit (Thermo Fisher Scientific, Waltham, USA), and oligonucleotide primers were manufactured by Metabion (Munich, Germany). The applied PCR temperature profile started with an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 20 s at 95 °C, 20 s at the annealing temperatures given in Tables S6 and S7, and 30 s at 72 °C. The profile ended with an extension step of 10 min at 72 °C. To reduce the number of PCRs required to assess the paternity of offspring, we first genotyped the parents of the samples collected in the lab experiments. We then selected a subset of up to four microsatellites to genotype the offspring based on the following criteria: 1. males in a given mating experiment did not share a common allele; 2. no uncertainty in the size of the microsatellite marker or only a minimum, for instance due to stutter bands.

Analysis of paternity

We used an ABI 3130xL Genetic Analyzer (Thermo Fisher Scientific, Waltham, USA) at the Department of Forensic Medicine at the University of Freiburg Medical Centre to determine the size of the PCR-amplified microsatellite loci. Analysed samples consisted of 2 µl PCR product, 10 µl Hi-Di™-Formamide (Thermo Fisher Scientific, Waltham, USA), and 0.5 µl of Red 500 DNA Size Standard 500bp (NimaGen, Nijmegen, Netherlands). The obtained electropherograms were evaluated with the Microsatellite plugin of the software Geneious Prime version 2021.0.3 (Biomatters, Auckland, New Zealand). The minimum number of fathers in pooled samples was inferred from the number of allele states at a given locus in the offspring. For example, five or more detected allele states indicated at least two fathers, as one female and one male can explain maximally four alleles in a diploid organism.

Statistics

445   Statistical analysis was done in RStudio version 2021.09.2 (R Core Team, Auckland, New Zealand).

446   We tested whether the distribution of the inferred proportions of paternity deviated from a normal

447   distribution with the Shapiro-Wilk test (*Stylops*: $W = 0.85401$, p-value $= 0.06483$; *Xenos*: $W = 0.85798$,

448   p-value $= 0.07224$). To show whether a given male contributed more to a female's offspring than another

449   male, we used a chi-squared goodness of fit test. The influence of the copulation time on the proportion

450   of offspring was assessed with a generalized linear model (function: glm) in RStudio (R Core Team,

451   Auckland, New Zealand). We used a Poisson distributed model to test whether the total copulation time

452   of a first copulating male affects the fertilization success (response variable) of a second male. The

453   copulation time of the second male was included as an additional parameter in the generalized linear

454   model.

455

456   **Imaging**

457   All images were edited with Adobe Photoshop Version 21.2.1 (Adobe Systems, San Jose, USA). We

458   used Adobe Illustrator Version 24.2.1 (Adobe Systems, San Jose, USA) for labelling plates and editing

459   diagrams.

460

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614   (Humana Press, 2000).

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627

628   Author Contributions

629   HP, ON and RB conceived the project and designed the experiments. Microphotography was carried

630   out by HP and KJ. HP and KJ executed the copulation experiments. HE, KJ, ON, MS, and NW

631   performed the laboratory and bioinformatic work on microsatellites. Statistical analyses were

632   performed by KJ and NW. HP, ON and KJ prepared the figure plates and wrote the manuscript with

633   helpful input from all authors.

634   **Data Availability**

635   All data needed to evaluate the conclusions in the paper are present in the paper and its supplementary

636   information.

637

638   **Competing Interest**

639   The authors declare no competing interests.

640

641

642   **Figures**

643
644   **Figure 1. *Stylops ovinae* and *Xenos vesparum*. A, B:** Cephalothorax and brood canal of females of *S.*
645   *ovinae* (A) and *X. vesparum* (B) filled with primary larvae, ventral view. **C:** The bee *Andrena vaga* with
646   primary larvae of *S. ovinae* hatch from the birth opening and disperse. **D:** The wasp *Polistes dominula*
647   with emerging larvae of *X. vesparum*. Abbreviations: bc, brood canal; bo, birth opening; cth,
648   cephalothorax.

649
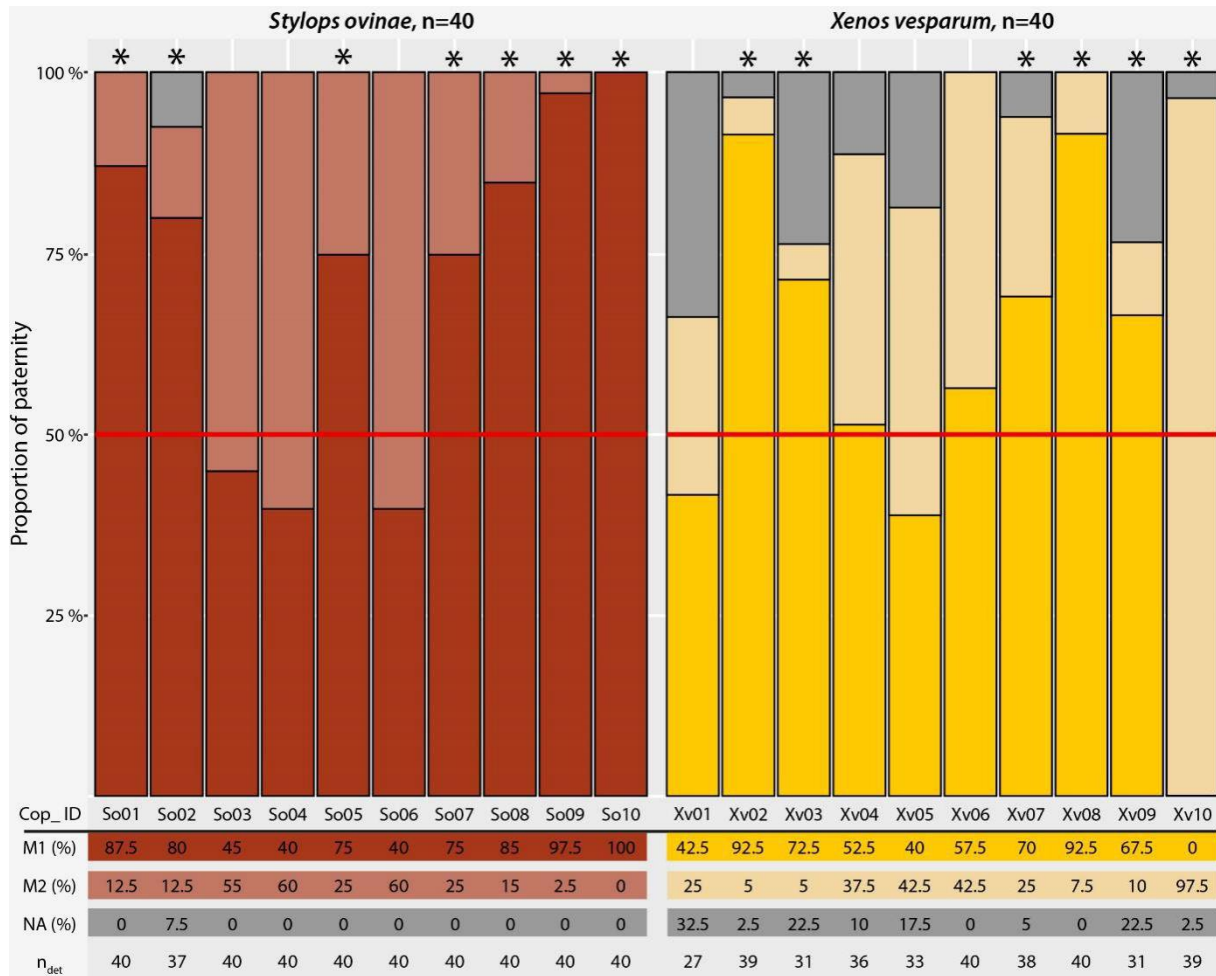
Figure 2: Genetic contribution of males that had subsequently mated with the same female to the female's offspring. Bar charts show the percentage of offspring in each sampled copulation experiment (ten per species). Proportions of *Stylops ovinae* are shown in red, those of *Xenos vesparum* in yellow. The red line marks the equal distribution of 50%. Copulation experiments marked with an asterisk show a statistically significant divergence from an equal contribution of the two fathers to the female's offspring ($\chi 2$-test: $\chi 2 = 8.53–38.011$; df = 1; p < 0.004). Abbreviations: Cop_ID = experimental ID of the copulation experiment; M1 = offspring proportion of the male that copulated first; M2 = offspring proportion of the male that copulated second, NA = not assignable; n = number of sampled larvae; n_{det} = number of larvae with known paternity.
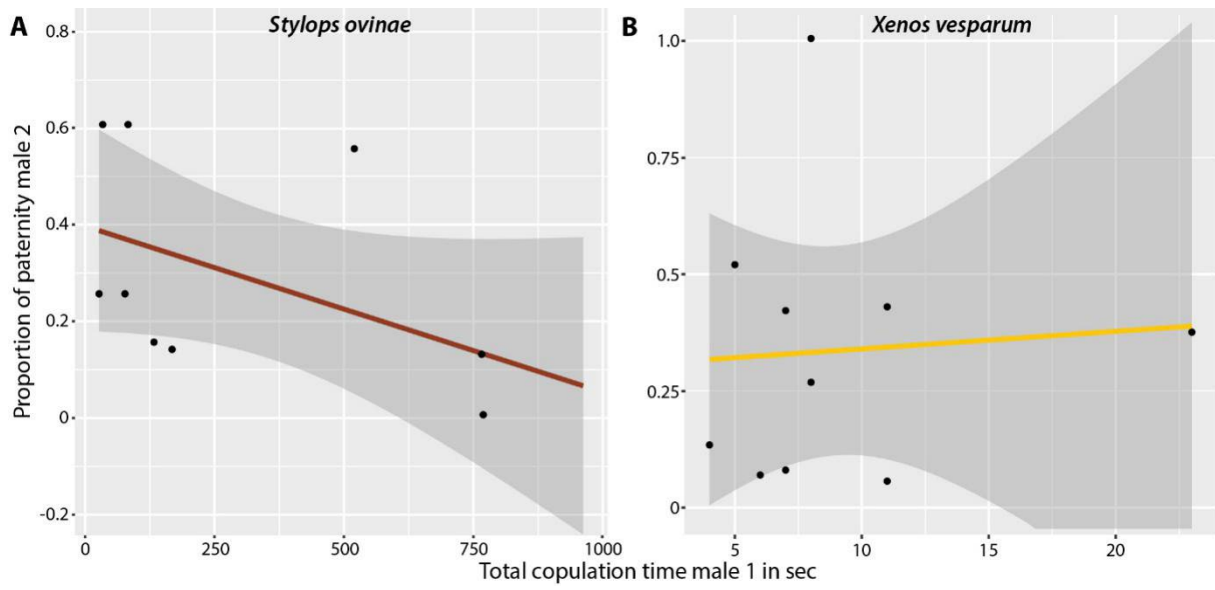
Figure 3: Correlation of the total copulation duration of the first male and the relative genetic contribution to the offspring of the second male in *Stylops ovinae* (A) and *Xenos vesparum* (B). Regression line in red (*S. ovinae*) and yellow (*X. vesparum*). Shape of confidence interval at 95% displayed in dark grey.

666   **Tables**

667   **Table 1. Number of alleles and estimated number of fathers of batches of 50 larvae produced by different**
668   **field-collected *Stylops ovinae* females.** Allele counts higher than four – indicative for polyandry – are highlighted
669   in green.

ID	Number of detected alleles per microsatellite locus				Minimum number of fathers
	So_A	**So_B**	**So_D**	**So_G**	
S01	4	1	4	3	1
S02	2	1	7	2	3
S03	3	2	6	3	2
S04	4	4	6	3	2
S05	3	2	4	3	1
S06	3	1	6	2	2
S07	2	0	4	3	1
S08	5	3	9	2	4
S09	3	4	6	4	2
S10	3	3	7	3	3

670

671   **Table 2. Number of alleles in and estimated number of fathers of batches of 50 larvae produced by different**
672   **field-collected *Xenos vesparum* females.** Allele counts higher than four – indicative for polyandry – are
673   highlighted in green.

ID	Number of detected alleles per microsatellite locus				Minimum number of fathers
	Xv_D	**Xv_E**	**Xv_F**	**Xv_H**	
X01	3	3	3	1	1
X02	4	2	3	2	1
X03	4	3	3	1	1
X04	3	3	3	2	1
X05	3	2	2	1	1
X06	3	1	3	3	1
X07	4	5	2	2	2
X08	3	3	3	2	1
X09	3	2	3	3	1
X10	3	2	4	2	1

674

675

Polyandry and sperm competition in two traumatically inseminating species of Strepsiptera (Insecta)

Kenny Jandausch, Nico Wanjura, Hermes Escalona, Manuela Sann, Rolf G. Beutel, Hans Pohl and Oliver Niehuis

Table S1. Mating experiments of *Stylops ovinae* under laboratory conditons.

ID from Peinert 2016	Lab ID	Sex of host	female *Stylops*	male *Stylops*	Copulation Duration								Copulation (Day)	Specimen Fixed
					male 1			male 2						
16	So01	female	1	0	12 min 46 s			5 min 8 s					28.01.2014	29.03.2014
17	So02	female	1	0	24 s	1 min 23 s	1 min 1 s	46 s	13 s	9 s			30.01.2014	29.03.2014
18	So03	female	1	0	8 min 40 s			1 min 56 s	8 s				30.01.2014	17.03.2014
22	So04	female	1	1	1 min 17 s			1 m 34 s	15 s	6 s	4 min 9 s	15 s	31.01.2014	26.03.2014
25	So05	female	1	0	34 s			13 m 33 s	50 s				01.02.2014	29.03.2014
29	So06	female	1	0	27 s			15 s					20.02.2014	29.03.2014
30	So07	female	1	0	2 min 13 s			6 min 28 s					20.02.2014	11.04.2014
33	So08	female	1	0	16 min 2 s			6 min 9 s	11 s				21.02.2014	11.04.2014
39	So09	female	1	0	33 s	39 s	11 s	1 min 15 s	15 s	23 s			23.02.2014	10.04.2014
52	So10	female	1	0	12 min 49 s			13 s					04.03.2014	11.04.2014

Table S2. Mating experiments of *Xenos vesparum* under laboratory conditons.

ID	Lab ID	Sex of host	female *Xenos*	Copulation duration										Copula (Day)	Begin of hibernation	End of hibernation	Specimen fixed
				male1			male2						male 3				
Pol_01		female	1											10.07.2018	no hibernation	–	not fixed
Pol_02		female	1											11.07.2018	no hibernation	–	not fixed
Pol_03	XL1	female	1	23 s			5 s	13 s						11.07.2018	06.11.2018	died during hibernation	15.11.2018
Pol_04		female	1	16 s			3 s	7 s						12.07.2018	06.11.2018	29.01.2019	18.03.2019
Pol_05		female	1	34 s			8 s	10 s	2 s					13.07.2018	06.11.2018	29.01.2019	17.03.2019
Pol_06		female	1	30 s			9 s	15 s	15 s	1 s				16.07.2018	06.11.2018	29.01.2019	31.01.2019
Pol_07		female	1	30 s			5 s						5 s	19.07.2020	no hibernation	–	19.07.2020
Pol_08		female	1	5 s	20 s	29 s	5 s	2 s	9 s	10 s				24.07.2020	07.10.2020	17.12.2020	03.01.2021
Pol_09	XL2	female	1	9 s	5 s	6 s	4 s	3 s						24.07.2020	died before hibernation	–	04.08.2020
Pol_10	XL3	female	1	3 s	1 s	2 s	2 s	2 s	3 s					27.07.2020	07.10.2020	17.12.2020	15.02.2021
Pol_11		female	1	3 s			5 s	2 s	several short attampts					05.08.2020	07.10.2020	died during hibernation	12.10.2020
Pol_12	XL4	female	1	4 s	3 s		7 s	2 s						30.07.2021	13.10.2021	died during hibernation	27.12.2021

Table S2 (cont.). Mating experiments of *Xenos vesparum* under laboratory conditons.

ID	Lab ID	Sex of host	female *Xenos*	Copulation duration male1	male2	male3	Copula (Day)	Begin of hibernation	End of hibernation	Specimen fixed
Pol_13	XL5	female	1	5 s	33 s		30.07.2021	13.10.2021	06.01.2022	09.02.2022
Pol_14	XL6	female	1	4 s, 2 s, 2 s, 3 s	15 s		30.07.2021	13.10.2021	06.01.2022	09.02.2021
Pol_15	XL7	female	1	6 s, 2 s	4 s, 2 s		30.07.2021	13.10.2021	06.01.2022	07.02.2022
Pol_16	XL8	female	1	5 s, 2 s, 2 s	1 s		03.08.2021	13.10.2021	died during hibernation	04.01.2022
Pol_17	XL9	female	1	4 s	6 s, 3 s, 3 s, 2 s, 2 s		05.08.2021	13.10.2021	died during hibernation	04.11.2021
Pol_18		female	1	14 s, 5 s, 3 s	15 s		06.08.2021	died before hibernation	–	21.09.2021
Pol_19	XL10	female	1	3 s, 5 s	4 s, 6 s, 5 s, 3 s		06.08.2021	13.10.2021	06.01.2022	11.02.2021
Pol_20		male	1	3 s, 4 s, 3 s	2 s, 3 s, 1 s		09.08.2021	died before hibernation	–	23.09.2021

Table S3: Duration and frequency of copulation in *X. vesparum*[1].

Copula		Virgin females (n = 18)			Mated females (n =18)		
		First n=18	Second n=10	Third n=6	First n=18	Second n=13	Third n=7
Duration of copula (s)	Average	11.2	5.9	7.5	7.4	5.4	5.4
	Min.	3	1	2	1	2	1
	Max	34	20	29	33	15	15
	median	5	3.5	3	5	3	3

[1] We recorded the duration and frequency of copulations in *Xenos vesparum* using a similar approach as Peinert et al. (2016) used when studying *Stylops ovinae*. In total, we observed 79 copulations and found the copulation time to not be normally distribution (Shapiro-Wilk test: W = 0.87311, p-value = 0.0007). In a single instance, a male copulated six times for a few seconds with a female that had previously copulated with another male. We observed no significant difference between the copulation duration of the first copulating male and the copulation duration of the second male (Mann-Whitney U test: W = 185.5, p-value = 0.4634), and no significant difference between the first copulation time and subsequent copulation times of a single male. This applies to copulations with virgin females (V = 39.5, p-value = 0.2393) and to previously mated females (V = 36.5, p-value = 0.8749).

Table S4. Documented copulation attempts, copulation duration of the first and of the second male, and time between the onset of the first male's copulation and the onset of the second male's copulation with a given female.

ID	Time between inseminations	Total copulation time M1	Copulation attempts M1	Total copulation time M2	Copulation attempts M2
So01	23 min 19 s	12 min 46 s	1	5 min 8 s	1
So02	13 min 21 s	2 min 48 s	3	1 min 8 s	3
So03	19 min 13 s	8 min 40 s	1	2 min 4 s	2
So04	11 min 56 s	1 min 23 s	3	1 min 53 s	3
So05	11 min 50 s	1 min 17 s	1	6 min 19 s	5
So06	11 min 7 s	0 min 34 s	1	14 min 23 s	2
So07	11 min	0 min 27 s	1	0 min 15 s	1
So08	12 min 46 s	2 min 13 s	1	6 min 28 s	1
So09	26 min 35 s	16 min 2 s	1	6 min 20 s	2
So10	90 min 22 s	12 min 49 s	1	0 min 13 s	1
Xv01	20 min 45 s	23 s	1	18 s	2
Xv02	8 min 34 s	11 s	3	6 s	2
Xv03	2 min 29 s	6 s	3	7 s	3
Xv04	1 min 15 s	7 s	2	9 s	2
Xv05	2 min 25 s	5 s	1	33 s	1
Xv06	3 min 47 s	11 s	4	15 s	1
Xv07	6 min 53 s	8 s	2	6 s	2
Xv08	7 min 33 s	7 s	2	1 s	1
Xv09	4 min 42 s	4 s	1	18 s	4
Xv10	4 min 28 s	8 s	2	18 s	4

Table S5. Allele states for tested larva and parents (green) in *Sytlops ovinae* (So_01-10).

So_01	Locus A.1	Locus A.2	Locus B.1	Locus B.2	Locus C.1	Locus C.2	Locus G.1	Locus G.2
Female	153	159	228	242	192	194	181	185
Male 1	151	151	242	242	186	186	185	187
Male 2	147	151	228	246	194	198	178	183
Larva 01	151	153	242	242	186	192	185	185
Larva 02	151	153	228	242	186	192	185	185
Larva 03	151	159	242	242	186	192	181	185
Larva 04	151	153	242	242	186	192	181	187
Larva 05	151	153	228	242	186	194	185	187
Larva 06	151	159	228	242	186	192	185	185
Larva 07	151	153	228	242	186	194	181	185
Larva 08	151	159	242	242	186	194	185	187
Larva 09	151	153	228	242	186	194	181	185
Larva 10	151	153	228	242	186	192	181	187
Larva 11	151	153	242	242	186	192	181	187
Larva 12	151	159	242	242	186	194	181	187
Larva 13	151	153	242	242	186	192	N.A.	N.A.
Larva 14	151	159	228	242	186	194	181	185
Larva 15	151	153	242	242	186	194	181	185
Larva 16	151	159	228	242	186	194	185	185
Larva 17	151	153	242	242	186	192	185	185
Larva 18	151	159	228	242	186	194	181	187
Larva 19	151	153	228	242	186	192	181	185
Larva 20	147	153	228	228	192	194	178	185
Larva 21	151	159	242	242	186	192	181	185
Larva 22	147	151	228	246	192	194	178	185
Larva 23	151	159	228	242	186	194	181	185
Larva 24	151	159	228	242	186	192	181	185
Larva 25	151	153	N.A.	N.A.	186	194	181	187
Larva 26	151	159	228	246	194	194	181	183
Larva 27	147	159	N.A.	N.A.	194	194	178	185
Larva 28	151	159	242	246	192	194	178	185
Larva 29	151	159	228	242	186	192	185	187
Larva 30	151	153	228	242	186	192	185	187
Larva 31	151	159	242	242	186	192	185	187
Larva 32	151	153	228	242	186	194	185	187
Larva 33	151	153	242	242	186	194	185	185
Larva 34	151	159	242	242	186	192	185	185
Larva 35	151	153	228	242	186	192	185	185
Larva 36	151	159	228	242	186	194	185	187
Larva 37	151	153	242	242	186	192	185	187
Larva 38	151	153	228	242	186	194	181	187
Larva 39	151	153	228	242	186	194	181	187
Larva 40	151	153	N.A.	N.A.	186	192	185	185

So_02	Locus A.1	Locus A.2	Locus B.1	Locus B.2	Locus C.1	Locus C.2	Locus D.1	Locus D.2
Female	149	151	228	230	187	187	152	156
Male 1	151	159	242	250	187	187	156	164
Male 2	145	151	236	250	187	193	140	156
Larva 01	149	159	230	242	187	187	152	164
Larva 02	149	151	230	242	187	187	152	156
Larva 03	149	151	230	242	187	187	156	164
Larva 04	151	151	230	250	N.A.	N.A.	156	164
Larva 05	151	151	228	242	N.A.	N.A.	152	164
Larva 06	151	159	228	242	N.A.	N.A.	156	156
Larva 07	149	151	228	250	187	187	152	156
Larva 08	151	159	230	242	N.A.	N.A.	156	164
Larva 09	149	151	228	236	187	193	156	156
Larva 10	151	159	230	242	N.A.	N.A.	152	164
Larva 11	149	151	228	250	N.A.	N.A.	156	164
Larva 12	149	151	N.A.	N.A.	187	193	152	156
Larva 13	151	159	228	250	N.A.	N.A.	152	164
Larva 14	149	151	230	236	187	187	140	156
Larva 15	151	159	228	242	N.A.	N.A.	156	164
Larva 16	149	159	228	250	N.A.	N.A.	156	164
Larva 17	149	151	230	242	N.A.	N.A.	156	156
Larva 18	149	159	230	242	N.A.	N.A.	152	164
Larva 19	151	159	230	250	N.A.	N.A.	156	156
Larva 20	151	151	230	242	N.A.	N.A.	156	156
Larva 21	149	151	230	250	N.A.	N.A.	152	164
Larva 22	151	151	228	242	N.A.	N.A.	152	164
Larva 23	151	159	230	242	N.A.	N.A.	152	164
Larva 24	151	151	228	250	187	187	156	156
Larva 25	151	151	230	242	N.A.	N.A.	156	164
Larva 26	149	159	230	242	N.A.	N.A.	156	164
Larva 27	151	151	228	242	N.A.	N.A.	156	156
Larva 28	149	159	230	250	N.A.	N.A.	152	156
Larva 29	151	151	228	242	N.A.	N.A.	152	156
Larva 30	151	151	228	250	N.A.	N.A.	156	164
Larva 31	151	151	230	250	187	193	152	156
Larva 32	145	151	230	236	187	187	140	152
Larva 33	149	159	230	242	N.A.	N.A.	152	156
Larva 34	149	151	N.A.	N.A.	N.A.	N.A.	156	164
Larva 35	151	151	228	242	N.A.	N.A.	152	156
Larva 36	149	151	230	242	N.A.	N.A.	152	164
Larva 37	151	151	228	242	N.A.	N.A.	156	156
Larva 38	151	151	228	250	N.A.	N.A.	156	156
Larva 39	149	151	228	250	187	187	152	156
Larva 40	149	159	230	242	N.A.	N.A.	152	164

So_03	Locus B.1	Locus B.2	Locus D.1	Locus D.2	Locus E.1	Locus E.2
Female	250	250	140	164	99	99
Male 1	228	242	154	156	99	105
Male 2	246	250	140	156	105	109
Larva 01	250	250	140	140	99	105
Larva 02	228	250	154	164	99	105
Larva 03	250	250	140	164	99	105
Larva 04	242	250	156	164	99	105
Larva 05	250	250	140	164	99	109
Larva 06	250	250	140	164	99	105
Larva 07	242	250	154	164	99	99
Larva 08	246	250	140	164	99	109
Larva 09	250	250	140	164	99	109
Larva 10	246	250	156	164	99	109
Larva 11	242	250	156	164	99	105
Larva 12	228	250	156	164	99	99
Larva 13	228	250	154	164	99	105
Larva 14	246	250	140	164	99	109
Larva 15	246	250	156	164	99	105
Larva 16	246	250	156	164	99	105
Larva 17	250	250	140	164	99	105
Larva 18	246	250	140	164	99	109
Larva 19	250	250	140	164	99	109
Larva 20	242	250	154	164	99	105
Larva 21	246	250	140	164	99	105
Larva 22	228	250	156	164	99	105
Larva 23	246	250	140	164	99	105
Larva 24	246	250	156	164	99	105
Larva 25	242	250	156	164	99	99
Larva 26	228	250	156	164	99	99
Larva 27	250	250	140	164	99	105
Larva 28	246	250	156	164	99	109
Larva 29	228	250	154	164	99	105
Larva 30	228	250	154	164	99	105
Larva 31	228	250	154	164	99	99
Larva 32	242	250	156	164	99	105
Larva 33	246	250	156	164	99	109
Larva 34	250	250	140	164	99	105
Larva 35	242	250	154	164	99	105
Larva 36	228	250	154	164	99	99
Larva 37	250	250	140	164	99	109
Larva 38	228	250	156	164	99	105
Larva 39	228	250	154	164	99	99
Larva 40	250	250	156	164	99	109

So_04	Locus B.1	Locus B.2	Locus D.1	Locus D.2	Locus G.1	Locus G.2
Female	228	242	150	154	179	185
Male 1	230	246	150	156	179	191
Male 2	214	214	150	154	191	193
Larva 01	214	228	154	154	185	191
Larva 02	214	242	150	154	N.A.	N.A.
Larva 03	214	228	154	154	185	191
Larva 04	214	228	154	154	179	193
Larva 05	214	228	150	154	185	193
Larva 06	242	246	150	150	179	179
Larva 07	214	228	154	154	185	191
Larva 08	214	228	150	154	179	193
Larva 09	228	246	154	156	185	191
Larva 10	242	246	154	156	185	191
Larva 11	242	246	150	156	179	185
Larva 12	214	242	154	154	179	191
Larva 13	214	242	150	154	179	191
Larva 14	214	228	154	154	179	191
Larva 15	214	228	150	150	185	191
Larva 16	230	242	154	156	179	191
Larva 17	230	242	150	150	185	191
Larva 18	230	242	154	156	179	191
Larva 19	242	246	154	156	179	179
Larva 20	228	246	154	156	179	191
Larva 21	214	242	150	150	185	193
Larva 22	214	242	150	154	185	193
Larva 23	228	246	150	154	179	185
Larva 24	228	230	150	156	185	191
Larva 25	228	246	150	154	179	191
Larva 26	214	228	150	150	185	193
Larva 27	214	242	150	154	179	191
Larva 28	214	242	150	150	185	191
Larva 29	242	246	154	156	179	191
Larva 30	228	246	150	150	179	185
Larva 31	214	242	150	154	185	191
Larva 32	214	242	150	154	179	193
Larva 33	214	228	150	154	185	193
Larva 34	214	242	150	150	179	191
Larva 35	214	228	150	154	179	193
Larva 36	214	228	154	154	179	193
Larva 37	214	242	154	154	179	193
Larva 38	214	228	150	154	179	191
Larva 39	228	246	150	154	179	185
Larva 40	228	230	150	154	179	187

So_05	Locus A.1	Locus A.2	Locus C.1	Locus C.2	Locus G.1	Locus G.2
Female	151	159	193	195	185	185
Male 1	149	159	187	195	185	185
Male 2	147	151	195	199	179	187
Larva 01	151	159	193	195	185	185
Larva 02	149	151	187	195	185	185
Larva 03	151	151	193	195	179	185
Larva 04	149	159	193	195	185	185
Larva 05	151	159	193	195	179	185
Larva 06	159	159	193	195	185	185
Larva 07	149	159	193	195	185	185
Larva 08	149	151	193	195	185	185
Larva 09	149	159	195	195	185	185
Larva 10	149	151	187	195	185	185
Larva 11	159	159	195	195	185	185
Larva 12	151	159	187	193	185	185
Larva 13	149	151	193	195	N.A.	N.A.
Larva 14	147	151	195	195	179	185
Larva 15	151	159	187	195	185	185
Larva 16	151	151	193	199	185	187
Larva 17	149	159	195	195	185	185
Larva 18	151	159	193	195	185	185
Larva 19	149	151	187	193	185	185
Larva 20	149	159	187	195	185	185
Larva 21	151	159	187	195	185	185
Larva 22	149	159	187	195	185	185
Larva 23	159	159	187	195	185	185
Larva 24	149	159	187	193	185	185
Larva 25	149	151	187	193	185	185
Larva 26	151	159	195	199	185	187
Larva 27	149	151	187	195	185	185
Larva 28	159	159	187	195	185	185
Larva 29	151	159	193	195	185	187
Larva 30	151	159	187	193	185	185
Larva 31	149	151	187	195	185	185
Larva 32	147	159	195	195	185	187
Larva 33	159	159	195	195	185	185
Larva 34	151	159	187	195	185	185
Larva 35	159	159	193	195	185	185
Larva 36	147	159	195	195	185	187
Larva 37	151	159	193	195	185	185
Larva 38	147	151	195	195	185	187
Larva 39	151	159	195	195	179	185
Larva 40	149	151	187	193	185	185

So_06	Locus A.1	Locus A.2	Locus B.1	Locus B.2
Female	147	147	230	242
Male 1	151	151	242	250
Male 2	147	159	214	242
Larva 01	147	159	214	242
Larva 02	147	151	N.A.	N.A.
Larva 03	147	151	242	242
Larva 04	147	159	230	242
Larva 05	147	147	214	230
Larva 06	147	159	214	242
Larva 07	147	159	214	230
Larva 08	147	151	242	242
Larva 09	147	147	214	230
Larva 10	147	151	230	242
Larva 11	147	147	214	230
Larva 12	147	159	214	230
Larva 13	147	159	242	242
Larva 14	147	151	230	242
Larva 15	147	151	230	242
Larva 16	147	151	230	242
Larva 17	147	151	242	242
Larva 18	147	147	214	230
Larva 19	147	147	214	230
Larva 20	147	151	230	242
Larva 21	147	159	214	230
Larva 22	147	151	242	242
Larva 23	147	159	230	242
Larva 24	147	159	214	242
Larva 25	147	147	214	242
Larva 26	147	151	242	242
Larva 27	147	159	214	242
Larva 28	147	151	242	242
Larva 29	147	147	214	230
Larva 30	147	151	230	242
Larva 31	147	159	214	242
Larva 32	147	159	214	242
Larva 33	147	151	230	242
Larva 34	147	147	214	230
Larva 35	147	151	242	242
Larva 36	147	159	214	230
Larva 37	147	147	214	230
Larva 38	147	151	242	242
Larva 39	147	147	214	230
Larva 40	147	147	214	230

So_07	Locus A.1	Locus A.2	Locus D.1	Locus D.2	Locus G.1	Locus G.2
Female	147	151	140	164	183	191
Male 1	151	151	150	156	183	185
Male 2	149	153	156	158	179	185
Larva 01	N.A.	N.A.	156	164	179	191
Larva 02	147	153	140	158	179	183
Larva 03	147	151	140	156	185	191
Larva 04	151	151	140	150	183	191
Larva 05	147	151	150	164	185	191
Larva 06	147	151	140	156	183	183
Larva 07	147	149	158	164	183	185
Larva 08	147	151	140	156	183	183
Larva 09	147	151	140	156	N.A.	N.A.
Larva 10	149	151	158	164	179	191
Larva 11	151	151	140	156	183	185
Larva 12	149	151	158	164	183	185
Larva 13	N.A.	N.A.	140	158	179	191
Larva 14	149	151	156	164	179	183
Larva 15	151	151	150	164	183	183
Larva 16	147	151	150	164	183	183
Larva 17	151	151	140	156	183	185
Larva 18	151	151	140	150	183	183
Larva 19	151	151	140	156	183	185
Larva 20	147	151	156	164	183	183
Larva 21	147	151	140	156	183	183
Larva 22	147	151	156	164	183	183
Larva 23	147	151	140	156	185	191
Larva 24	147	153	156	164	179	183
Larva 25	151	151	140	156	N.A.	N.A.
Larva 26	151	151	156	164	183	185
Larva 27	147	151	140	150	185	191
Larva 28	151	151	140	156	183	183
Larva 29	147	151	150	164	N.A.	N.A.
Larva 30	151	151	140	156	N.A.	N.A.
Larva 31	147	151	150	164	183	191
Larva 32	151	153	158	164	179	183
Larva 33	151	151	156	164	185	191
Larva 34	151	151	156	164	185	191
Larva 35	147	149	140	158	179	191
Larva 36	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Larva 37	151	151	140	150	183	183
Larva 38	151	151	N.A.	N.A.	183	183
Larva 39	151	151	150	164	185	191
Larva 40	147	151	150	164	183	191
Larva 41	147	151	N.A.	N.A.	N.A.	N.A.

So_08	Locus A.1	Locus A.2	Locus B.1	Locus B.2
Female	151	153	214	228
Male 1	151	159	244	246
Male 2	149	149	230	250
Larva 01	151	153	214	246
Larva 02	151	153	214	244
Larva 03	151	153	228	244
Larva 04	153	159	214	246
Larva 05	149	153	228	250
Larva 06	151	159	214	244
Larva 07	153	159	228	246
Larva 08	153	159	214	246
Larva 09	149	151	228	250
Larva 10	151	151	228	246
Larva 11	151	151	214	244
Larva 12	151	153	228	246
Larva 13	153	159	214	246
Larva 14	151	153	228	244
Larva 15	149	153	214	230
Larva 16	151	159	214	246
Larva 17	151	159	214	244
Larva 18	151	159	228	244
Larva 19	149	153	228	250
Larva 20	151	151	228	244
Larva 21	151	153	214	246
Larva 22	151	153	214	246
Larva 23	151	153	228	246
Larva 24	153	159	228	246
Larva 25	149	153	214	230
Larva 26	153	159	228	246
Larva 27	153	159	214	244
Larva 28	151	159	214	244
Larva 29	149	153	228	250
Larva 30	151	151	214	246
Larva 31	151	159	214	244
Larva 32	151	153	228	246
Larva 33	151	151	228	246
Larva 34	151	159	214	244
Larva 35	153	159	214	246
Larva 36	151	151	228	246
Larva 37	151	159	214	244
Larva 38	151	151	214	244
Larva 39	151	153	214	244
Larva 40	153	159	214	246

So_09	Locus B.1	Locus B.2	Locus C.1	Locus C.2	Locus D.1	Locus D.2	Locus G.1	Locus G.2
Female	242	250	187	193	140	150	185	187
Male 1	214	228	187	187	154	156	178	187
Male 2	242	246	197	199	140	156	183	185
Larva 01	228	242	187	187	140	156	185	187
Larva 02	228	242	187	193	140	154	178	187
Larva 03	228	242	187	187	140	154	187	187
Larva 04	228	250	187	187	150	156	185	187
Larva 05	228	250	187	193	140	156	178	185
Larva 06	228	250	N.A.	N.A.	150	154	178	187
Larva 07	214	250	187	193	140	156	178	185
Larva 08	214	250	187	193	150	154	178	187
Larva 09	228	250	187	187	N.A.	N.A.	178	185
Larva 10	214	250	187	187	140	156	178	187
Larva 11	228	250	187	193	150	154	185	187
Larva 12	228	242	187	193	N.A.	N.A.	N.A.	N.A.
Larva 13	214	242	187	187	140	156	185	187
Larva 14	214	250	187	187	140	156	178	187
Larva 15	228	242	187	187	150	156	178	187
Larva 16	214	250	187	193	140	156	187	187
Larva 17	N.A.	N.A.	187	187	140	156	178	187
Larva 18	N.A.	N.A.	187	187	140	156	178	185
Larva 19	N.A.	N.A.	N.A.	N.A.	140	154	178	185
Larva 20	N.A.	N.A.	187	187	150	154	178	185
Larva 21	N.A.	N.A.	N.A.	N.A.	140	150	185	187
Larva 22	N.A.	N.A.	187	187	140	156	178	185
Larva 23	N.A.	N.A.	N.A.	N.A.	140	154	178	187
Larva 24	N.A.	N.A.	187	193	140	154	187	187
Larva 25	N.A.	N.A.	187	187	140	154	187	187
Larva 26	N.A.	N.A.	187	193	140	156	N.A.	N.A.
Larva 27	N.A.	N.A.	187	187	150	154	N.A.	N.A.
Larva 28	N.A.	N.A.	187	193	140	156	N.A.	N.A.
Larva 29	N.A.	N.A.	187	193	140	156	N.A.	N.A.
Larva 30	N.A.	N.A.	187	187	140	154	N.A.	N.A.
Larva 31	N.A.	N.A.	187	193	150	154	187	187
Larva 32	N.A.	N.A.	187	187	140	156	178	187
Larva 33	N.A.	N.A.	187	187	140	154	178	187
Larva 34	N.A.	N.A.	187	193	150	154	178	187
Larva 35	N.A.	N.A.	187	193	140	156	178	187
Larva 36	N.A.	N.A.	187	187	150	156	185	187
Larva 37	N.A.	N.A.	187	187	150	156	185	187
Larva 38	N.A.	N.A.	187	193	140	154	185	187
Larva 39	N.A.	N.A.	187	193	150	156	178	187
Larva 40	N.A.	N.A.	187	187	150	156	187	187

So_10	Locus C.1	Locus C.2	Locus D.1	Locus D.2
Female	187	199	140	152
Male 1	195	197	140	164
Male 2	187	193	150	150
Larva 01	197	199	140	152
Larva 02	187	197	140	140
Larva 03	195	199	140	164
Larva 04	187	195	152	164
Larva 05	187	197	152	164
Larva 06	197	199	140	164
Larva 07	187	195	140	140
Larva 08	187	195	140	140
Larva 09	187	197	140	164
Larva 10	197	199	140	164
Larva 11	N.A.	N.A.	152	164
Larva 12	197	199	140	164
Larva 13	197	199	140	164
Larva 14	187	197	140	152
Larva 15	195	199	140	164
Larva 16	195	199	140	152
Larva 17	187	197	140	140
Larva 18	195	199	140	140
Larva 19	187	197	140	140
Larva 20	187	197	140	140
Larva 21	187	195	140	152
Larva 22	197	199	140	140
Larva 23	187	195	140	152
Larva 24	187	197	N.A.	N.A.
Larva 25	N.A.	N.A.	140	140
Larva 26	187	195	152	164
Larva 27	197	199	140	164
Larva 28	187	195	140	152
Larva 29	N.A.	N.A.	152	164
Larva 30	195	199	140	140
Larva 31	187	195	140	152
Larva 32	197	199	140	164
Larva 33	N.A.	N.A.	140	152
Larva 34	195	199	152	164
Larva 35	N.A.	N.A.	140	152
Larva 36	187	195	152	164
Larva 37	N.A.	N.A.	140	140
Larva 38	N.A.	N.A.	152	164
Larva 39	187	195	152	164
Larva 40	187	197	140	152

Table S6. Table S5. Allele states for tested larva and parents (green) in *Xenos vesparum* (XL_01-10).

XL_01	Locus F.1	Locus F.2
Female	227	231
Male 1	227	227
Male 2	231	233
Larva 01	227	227
Larva 02	231	231
Larva 03	227	227
Larva 04	227	231
Larva 05	227	227
Larva 06	231	231
Larva 07	227	231
Larva 08	227	231
Larva 09	227	227
Larva 10	227	227
Larva 11	227	227
Larva 12	227	227
Larva 13	227	227
Larva 14	227	227
Larva 15	227	227
Larva 16	227	227
Larva 17	227	231
Larva 18	231	231
Larva 19	231	231
Larva 20	227	231
Larva 21	231	231
Larva 22	227	231
Larva 23	227	231
Larva 24	231	231
Larva 25	227	227
Larva 26	227	227
Larva 27	227	231
Larva 28	227	231
Larva 29	231	233
Larva 30	227	227
Larva 31	231	231
Larva 32	227	231
Larva 33	227	227
Larva 34	227	231
Larva 35	227	231
Larva 36	227	227
Larva 37	227	233
Larva 38	231	231
Larva 39	227	231
Larva 40	227	227

XL_02	Locus C.1	LocusC.2	Locus E.1	Locus E.2	LocusF.1	Locus F.2
Female	184	188	164	176	227	243
Male 1	184	188	164	182	243	243
Male 2	182	188	164	170	233	233
Larva 01	184	188	164	176	227	243
Larva 02	184	188	164	176	227	243
Larva 03	184	188	164	176	227	243
Larva 04	184	188	164	176	227	243
Larva 05	182	188	164	176	N.A.	N.A.
Larva 06	184	188	164	176	243	243
Larva 07	184	188	176	182	227	243
Larva 08	184	188	164	176	227	243
Larva 09	N.A.	N.A.	164	176	N.A.	N.A.
Larva 10	184	188	164	176	227	243
Larva 11	184	188	164	176	227	243
Larva 12	184	188	164	176	227	243
Larva 13	184	188	164	176	227	243
Larva 14	184	188	164	176	227	243
Larva 15	184	188	164	176	227	243
Larva 16	184	188	164	176	227	243
Larva 17	184	188	164	176	227	243
Larva 18	184	188	164	176	227	243
Larva 19	184	188	164	176	227	243
Larva 20	184	188	164	176	227	243
Larva 21	184	188	164	176	227	243
Larva 22	184	188	164	176	227	243
Larva 23	184	188	164	176	227	243
Larva 24	182	188	164	176	N.A.	N.A.
Larva 25	184	188	164	176	227	243
Larva 26	184	188	164	176	227	243
Larva 27	184	188	164	176	227	243
Larva 28	184	188	164	176	227	243
Larva 29	184	188	164	176	227	243
Larva 30	184	188	164	176	227	243
Larva 31	184	188	164	176	227	243
Larva 32	184	188	164	176	227	243
Larva 33	184	188	164	176	227	243
Larva 34	184	188	164	176	227	243
Larva 35	184	188	164	176	227	243
Larva 36	184	188	164	176	227	243
Larva 37	184	188	164	176	227	243
Larva 38	184	188	164	176	227	243
Larva 39	184	188	164	176	227	243
Larva 40	184	188	164	176	227	243

XL_03	Locus C.1	LocusC.2	Locus E.1	Locus E.2	LocusF.1	Locus F.2
Female	184	188	164	176	233	243
Male 1	186	188	164	170	233	233
Male 2	188	188	164	176	243	243
Larva 01	186	188	164	170	233	233
Larva 02	184	186	164	170	233	243
Larva 03	184	188	164	164	243	243
Larva 04	186	188	164	170	233	233
Larva 05	184	188	164	170	233	243
Larva 06	184	186	164	176	243	243
Larva 07	184	188	170	176	243	243
Larva 08	188	188	170	176	243	243
Larva 09	188	188	170	176	233	233
Larva 10	184	188	164	164	243	243
Larva 11	184	188	170	176	243	243
Larva 12	184	188	164	164	233	233
Larva 13	N.A.	N.A.	164	164	233	233
Larva 14	184	188	164	164	233	233
Larva 15	186	188	164	170	233	233
Larva 16	184	186	170	176	243	243
Larva 17	184	186	164	170	233	233
Larva 18	186	188	164	170	233	243
Larva 19	184	186	164	164	233	243
Larva 20	186	188	164	176	233	233
Larva 21	188	188	170	176	233	233
Larva 22	186	188	170	176	233	233
Larva 23	186	188	170	170	233	233
Larva 24	184	188	170	176	233	233
Larva 25	188	188	164	164	233	243
Larva 26	184	186	164	170	243	243
Larva 27	184	188	164	164	233	233
Larva 28	184	188	164	176	233	233
Larva 29	184	186	164	170	243	243
Larva 30	184	186	164	170	243	243
Larva 31	184	186	164	170	233	233
Larva 32	186	188	170	176	233	243
Larva 33	184	186	164	176	233	243
Larva 34	188	188	164	170	233	233
Larva 35	184	188	164	176	233	233
Larva 36	188	188	164	176	233	243
Larva 37	184	186	170	176	233	233
Larva 38	N.A.	N.A.	164	164	233	233
Larva 39	186	188	164	176	233	233
Larva 40	184	188	164	176	233	233

XL_04	Locus C.1	LocusC.2	Locus E.1	Locus E.2
Female	182	190	164	182
Male 1	188	190	164	164
Male 2	184	186	164	164
Larva 01	182	186	164	164
Larva 02	188	190	164	164
Larva 03	184	190	164	182
Larva 04	N.A.	N.A.	N.A.	N.A.
Larva 05	182	188	164	182
Larva 06	190	190	164	182
Larva 07	182	184	164	164
Larva 08	182	188	164	164
Larva 09	N.A.	N.A.	164	182
Larva 10	184	190	164	164
Larva 11	182	186	164	164
Larva 12	N.A.	N.A.	164	164
Larva 13	186	190	164	182
Larva 14	188	190	164	182
Larva 15	188	190	164	182
Larva 16	186	190	164	164
Larva 17	182	186	164	182
Larva 18	182	190	164	164
Larva 19	184	190	164	182
Larva 20	182	190	164	182
Larva 21	188	190	164	182
Larva 22	182	186	164	164
Larva 23	190	190	164	182
Larva 24	182	188	164	164
Larva 25	184	190	164	164
Larva 26	188	190	164	182
Larva 27	188	190	164	164
Larva 28	184	190	164	182
Larva 29	190	190	164	182
Larva 30	182	188	164	182
Larva 31	190	190	164	182
Larva 32	188	190	164	164
Larva 33	182	184	164	182
Larva 34	188	190	164	182
Larva 35	186	190	164	164
Larva 36	182	188	164	182
Larva 37	186	190	164	182
Larva 38	182	184	164	164
Larva 39	188	190	164	182
Larva 40	N.A.	N.A.	164	182

XL_05	Locus C.1	LocusC.2	Locus F.1	Locus F.2	LocusG.1	Locus G.2
Female	186	188	233	243	123	133
Male 1	188	188	233	233	123	137
Male 2	186	188	243	243	123	123
Larva 01	186	188	243	243	N.A.	N.A.
Larva 02	186	188	243	243	N.A.	N.A.
Larva 03	188	188	233	233	N.A.	N.A.
Larva 04	186	188	233	233	N.A.	N.A.
Larva 05	188	188	233	233	N.A.	N.A.
Larva 06	186	188	233	243	N.A.	N.A.
Larva 07	186	188	233	233	N.A.	N.A.
Larva 08	186	188	N.A.	N.A.	N.A.	N.A.
Larva 09	186	188	243	243	133	137
Larva 10	186	188	233	233	N.A.	N.A.
Larva 11	186	188	233	243	N.A.	N.A.
Larva 12	188	188	233	233	N.A.	N.A.
Larva 13	188	188	233	233	123	137
Larva 14	188	188	243	243	N.A.	N.A.
Larva 15	186	188	243	243	N.A.	N.A.
Larva 16	186	188	233	243	N.A.	N.A.
Larva 17	186	188	243	243	N.A.	N.A.
Larva 18	186	188	233	233	133	137
Larva 19	186	188	233	233	N.A.	N.A.
Larva 20	186	186	243	243	N.A.	N.A.
Larva 21	188	188	233	233	N.A.	N.A.
Larva 22	186	188	233	233	133	137
Larva 23	186	188	233	243	N.A.	N.A.
Larva 24	186	188	233	233	N.A.	N.A.
Larva 25	N.A.	N.A.	243	243	N.A.	N.A.
Larva 26	188	188	233	233	123	137
Larva 27	186	188	233	243	N.A.	N.A.
Larva 28	186	186	243	243	N.A.	N.A.
Larva 29	188	188	243	243	N.A.	N.A.
Larva 30	186	188	243	243	N.A.	N.A.
Larva 31	186	188	243	243	N.A.	N.A.
Larva 32	188	188	233	233	N.A.	N.A.
Larva 33	188	188	243	243	N.A.	N.A.
Larva 34	188	188	233	233	N.A.	N.A.
Larva 35	186	188	243	243	N.A.	N.A.
Larva 36	188	188	233	243	N.A.	N.A.
Larva 37	188	188	243	243	N.A.	N.A.
Larva 38	188	188	243	243	N.A.	N.A.
Larva 39	188	188	243	243	N.A.	N.A.
Larva 40	188	188	233	233	N.A.	N.A.

XL_06	Locus C.1	LocusC.2	Locus E.1	Locus E.2	Locus G.1	Locus G.2
Female	188	190	176	182	133	133
Male 1	186	186	164	174	133	133
Male 2	184	188	174	176	133	135
Larva 01	188	190	176	N.A.	N.A.	N.A.
Larva 02	188	188	174	176	N.A.	N.A.
Larva 03	186	188	174	182	N.A.	N.A.
Larva 04	186	188	164	176	N.A.	N.A.
Larva 05	186	190	174	182	N.A.	N.A.
Larva 06	188	188	174	182	N.A.	N.A.
Larva 07	186	188	164	176	N.A.	N.A.
Larva 08	186	188	164	182	N.A.	N.A.
Larva 09	184	190	176	176	135	135
Larva 10	186	190	174	176	N.A.	N.A.
Larva 11	184	190	176	182	135	135
Larva 12	186	188	164	176	N.A.	N.A.
Larva 13	188	188	174	182	N.A.	N.A.
Larva 14	186	188	174	176	N.A.	N.A.
Larva 15	188	188	176	176	N.A.	N.A.
Larva 16	186	190	164	176	N.A.	N.A.
Larva 17	186	190	174	182	N.A.	N.A.
Larva 18	184	188	174	182	135	135
Larva 19	186	188	174	182	N.A.	N.A.
Larva 20	186	190	174	176	N.A.	N.A.
Larva 21	186	188	174	176	N.A.	N.A.
Larva 22	184	188	174	182	135	135
Larva 23	186	190	164	182	N.A.	N.A.
Larva 24	186	190	164	182	N.A.	N.A.
Larva 25	188	190	176	182	N.A.	N.A.
Larva 26	186	188	164	182	N.A.	N.A.
Larva 27	188	190	174	182	N.A.	N.A.
Larva 28	184	190	174	182	N.A.	N.A.
Larva 29	188	188	174	176	N.A.	N.A.
Larva 30	186	188	174	182	N.A.	N.A.
Larva 31	188	188	176	176	N.A.	N.A.
Larva 32	186	188	164	182	N.A.	N.A.
Larva 33	186	190	174	182	N.A.	N.A.
Larva 34	188	190	174	176	N.A.	N.A.
Larva 35	184	188	174	182	135	135
Larva 36	186	188	164	176	N.A.	N.A.
Larva 37	184	188	174	176	135	135
Larva 38	186	190	164	182	N.A.	N.A.
Larva 39	186	190	174	182	N.A.	N.A.
Larva 40	188	188	174	176	N.A.	N.A.

XL_07	Locus A.1	Locus A.2	Locus E.1	Locus E.2	Locus F.1	Locus F.2
Female	156	164	166	176	233	233
Male 1	164	168	174	174	233	233
Male 2	156	164	164	164	241	241
Larva 01	156	164	164	176	233	241
Larva 02	164	164	164	166	233	233
Larva 03	164	164	164	166	233	241
Larva 04	164	164	164	166	233	241
Larva 05	164	164	164	166	233	233
Larva 06	156	164	164	166	233	233
Larva 07	156	164	164	176	233	233
Larva 08	156	164	164	176	233	233
Larva 09	164	164	164	166	233	233
Larva 10	164	164	164	176	233	241
Larva 11	164	164	164	166	233	233
Larva 12	164	164	164	166	233	233
Larva 13	164	164	164	166	233	233
Larva 14	156	164	164	176	233	233
Larva 15	164	164	164	166	233	233
Larva 16	156	164	164	166	233	233
Larva 17	156	164	164	166	233	241
Larva 18	164	164	164	166	233	233
Larva 19	156	164	164	176	233	233
Larva 20	156	164	164	176	233	233
Larva 21	156	164	164	166	233	233
Larva 22	156	164	164	166	233	233
Larva 23	164	164	164	166	233	233
Larva 24	156	164	164	176	233	241
Larva 25	164	164	164	176	233	233
Larva 26	164	164	164	166	233	241
Larva 27	156	164	164	176	233	241
Larva 28	164	164	164	166	N.A.	N.A.
Larva 29	164	164	164	176	233	233
Larva 30	164	164	164	166	233	233
Larva 31	164	164	164	166	233	233
Larva 32	164	164	164	176	233	241
Larva 33	156	164	164	176	233	241
Larva 34	156	164	164	176	233	233
Larva 35	156	164	164	176	233	233
Larva 36	164	164	164	166	233	233
Larva 37	156	164	164	166	233	233
Larva 38	N.A.	N.A.	164	166	N.A.	N.A.
Larva 39	156	164	164	176	233	233
Larva 40	164	164	164	166	233	233

XL_08	Locus D.1	Locus D.2	Locus F.1	Locus F.2	Locus G.1	Locus G.2
Female	141	139	233	241	127	133
Male 1	131	149	233	233	123	127
Male 2	133	139	233	235	127	127
Larva 01	131	141	233	233	127	133
Larva 02	131	139	233	241	127	133
Larva 03	139	149	233	241	123	133
Larva 04	139	149	233	241	127	133
Larva 05	131	139	233	233	123	133
Larva 06	131	141	233	233	127	133
Larva 07	131	141	233	233	123	133
Larva 08	139	149	233	233	127	133
Larva 09	133	139	233	241	133	133
Larva 10	141	149	241	241	123	133
Larva 11	131	139	233	241	127	127
Larva 12	131	141	233	241	123	133
Larva 13	131	141	233	241	127	133
Larva 14	131	141	233	241	123	133
Larva 15	141	149	233	241	123	133
Larva 16	139	149	233	241	123	133
Larva 17	131	139	233	233	127	127
Larva 18	131	139	233	233	123	133
Larva 19	131	139	N.A.	N.A.	123	133
Larva 20	131	141	233	241	123	133
Larva 21	139	141	233	241	127	133
Larva 22	141	149	233	241	127	133
Larva 23	133	141	233	241	127	133
Larva 24	131	141	233	241	127	133
Larva 25	141	149	N.A.	N.A.	127	133
Larva 26	141	149	233	241	127	133
Larva 27	131	139	233	241	123	133
Larva 28	139	149	233	233	127	133
Larva 29	139	149	N.A.	N.A.	127	133
Larva 30	139	149	233	233	123	133
Larva 31	131	139	233	233	127	133
Larva 32	139	149	241	241	123	133
Larva 33	131	141	233	233	123	133
Larva 34	131	141	233	241	127	133
Larva 35	139	149	233	241	123	133
Larva 36	131	141	233	233	123	133
Larva 37	131	139	233	233	123	133
Larva 38	141	149	233	233	127	133
Larva 39	131	141	233	233	127	127
Larva 40	131	141	233	233	127	133

XL_09	Locus D.1	Locus D.2	Locus E.1	Locus E.2	Locus F.1	Locus F.2
Female	133	151	170	178	233	233
Male 1	139	151	164	164	233	233
Male 2	133	133	164	170	237	239
Larva 01	139	151	164	178	233	233
Larva 02	133	139	164	170	233	233
Larva 03	139	151	164	178	233	233
Larva 04	151	151	164	178	N.A.	N.A.
Larva 05	139	151	164	178	N.A.	N.A.
Larva 06	133	139	164	170	233	233
Larva 07	133	151	164	170	233	233
Larva 08	133	151	N.A.	N.A.	N.A.	N.A.
Larva 09	133	151	170	178	N.A.	N.A.
Larva 10	151	151	164	178	233	233
Larva 11	133	151	164	170	N.A.	N.A.
Larva 12	133	151	164	170	N.A.	N.A.
Larva 13	133	139	N.A.	N.A.	N.A.	N.A.
Larva 14	139	151	164	170	233	233
Larva 15	139	151	164	178	233	233
Larva 16	139	151	164	178	233	233
Larva 17	N.A.	N.A.	164	170	N.A.	N.A.
Larva 18	133	151	164	170	N.A.	N.A.
Larva 19	133	139	164	170	N.A.	N.A.
Larva 20	133	133	164	170	N.A.	N.A.
Larva 21	133	151	164	170	233	233
Larva 22	151	151	164	178	233	233
Larva 23	139	151	164	178	233	233
Larva 24	133	151	164	170	N.A.	N.A.
Larva 25	133	133	170	170	N.A.	N.A.
Larva 26	133	151	170	178	N.A.	N.A.
Larva 27	133	151	164	170	N.A.	N.A.
Larva 28	133	151	164	170	233	233
Larva 29	133	139	164	170	233	233
Larva 30	151	151	164	178	233	233
Larva 31	151	151	164	178	233	233
Larva 32	139	151	164	178	233	233
Larva 33	133	151	164	170	233	233
Larva 34	133	151	164	170	N.A.	N.A.
Larva 35	133	151	164	170	N.A.	N.A.
Larva 36	133	139	164	170	233	233
Larva 37	133	139	164	170	233	233
Larva 38	133	151	164	170	233	233
Larva 39	133	151	164	170	233	233
Larva 40	151	151	164	178	233	233

XL_10	Locus A.1	Locus A.2	Locus D.1	Locus D.2	Locus F.1	Locus F.2
Female	156	168	139	143	227	233
Male 1	164	168	131	139	243	243
Male 2	164	168	133	149	233	233
Larva 01	156	164	133	143	233	233
Larva 02	164	168	133	139	233	233
Larva 03	156	164	143	149	233	233
Larva 04	164	168	139	149	233	233
Larva 05	156	164	143	149	227	227
Larva 06	156	164	133	143	233	233
Larva 07	164	168	139	149	227	233
Larva 08	156	164	143	149	233	233
Larva 09	164	168	N.A.	N.A.	227	233
Larva 10	156	164	143	149	227	227
Larva 11	164	168	139	149	233	233
Larva 12	156	164	133	143	227	227
Larva 13	164	168	133	139	227	227
Larva 14	164	168	139	149	233	233
Larva 15	164	168	139	149	233	233
Larva 16	164	168	139	149	227	227
Larva 17	164	168	139	149	227	233
Larva 18	164	168	139	149	233	233
Larva 19	164	168	133	139	227	233
Larva 20	164	168	139	149	233	233
Larva 21	164	168	133	143	233	233
Larva 22	164	168	133	139	227	227
Larva 23	156	164	143	149	227	227
Larva 24	156	164	143	149	233	233
Larva 25	156	164	133	143	227	233
Larva 26	156	164	133	143	233	233
Larva 27	156	164	133	143	227	233
Larva 28	156	164	143	149	233	233
Larva 29	156	164	133	143	233	233
Larva 30	156	164	133	143	227	233
Larva 31	164	168	133	139	227	227
Larva 32	156	164	143	149	233	233
Larva 33	156	164	143	149	233	233
Larva 34	156	164	133	143	227	227
Larva 35	164	168	133	139	233	233
Larva 36	164	164	139	143	227	233
Larva 37	156	164	133	143	227	233
Larva 38	164	168	133	139	227	233
Larva 39	164	168	133	139	227	227
Larva 40	164	168	139	149	233	233

3 Discussion

As described in the introduction, the overall goal of this dissertation is to contribute to the reproductive biology of Strepsiptera. To achieve this goal and to resolve conflicting evidence regarding the general reproductive biology of Strepsiptera, the mechanism of penis insertion and sperm transfer and morphological reproductive traits of Strepsiptera will be elucidated. This overall objective is linked to clearly defined sub-objectives:

(I)   Describe the way of penis insertion and sperm transfer of different families of Strepsiptera;

(II)   Describe the morphological an anatomical adaptation of females to traumatic insemination;

(III)   Analyze morphological structures associated with reproduction to understand the function of morphological and anatomical adaptations to traumatic insemination;

(IV)   Assess the evidence for polyandry within Strepsiptera;

(V)   Contribute to the understanding of Strepsiptera phylogeny and the identification of phylogenetically informative characters;

(VI)   Make significant contributions to our understanding of the mechanisms of traumatic insemination and sperm competition by gaining knowledge in the poorly studied group of Strepsiptera.

In order to obtain high quality results on these sub-objectives from different research disciplines, it was necessary to apply a wide range of different methods from different research fields. In addition to a wide range of different morphological methods, biomechanical approaches and genetic techniques were included.

3.1   Summary of the Main Results

To understand the reproductive system of any species or group of organisms, basic knowledge of essential mechanisms such as sperm transfer and copulation mode is crucial. As the studies could only be realized as a team, I will speak in the "we" form. My own contributions to the respective studies can be found in the author contributions of the manuscripts as well as in the appendix of this thesis. Therefore, the aim of Study I was to elucidate how Strepsiptera achieve sperm transfer and to resolve the ongoing controversy over the copulation mode. We were able to collect clear evidence for trau-

matic insemination in twelve species from six different families distributed along the entire phylogenetic tree of Strepsiptera (Figure 2). In the three species *Eoxenos laboulbenei* (Mengenillidae), *Stylops ovinae* (Stylopidae), and *Xenos vesparum* (Xenidae), we were able to document the mode of sperm transfer and to provide reliable evidence for traumatic insemination. The results of this study were extended with additional evidence from the literature (Kinzelbach, 1971; Kinzelbach, 1978; Kinzelbach & Pohl, 1994; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Pohl et al., 2021), thus completing our understanding of traumatic insemination in a phylogenetic context. Since morphological features such as the shape of the penis or the structure of the female cephalothorax are also associated with traumatic insemination, Study I also provide insight to new identified phylogenetic characters (Question IV). As traumatic insemination (TI) must be the general copulatory mode in the entire order Strepsiptera based on the presented results, morphological characters also provide a general contribution to the understanding of the evolution of TI, as this is only well understood in a few groups (e.g., Řezáč, 2009; Tatarnic, Cassis & Siva-Jothy, 2014; Brand, Harmon & Schaerer, 2022). Traumatic insemination in Strepsiptera and the detailed results are discussed in chapter 3.4.

Using the information on the incidence of traumatic insemination obtained in Study I, it is possible to take a more focused look at the adaptations of females to this physically damaging form of sperm transfer. Several historical studies have pointed to a structure in females of the genus *Stylops* (Stylopidae) that has been suggested to play a role in copulation or the release of primary larvae (e.g., Nassonov, 1893; Lauterbach, 1954; Kinzelbach, 1971; Kinzelbach, 1978). Lauterbach (1954) already questioned whether this was an exclusive morphological feature of *Stylops*. Peinert et al. (2016) confirmed that the invagination of the integument in front of the birth opening indeed serves as a paragenital organ and is the cephalothoracic site where conspecific males introduce their penis and penetrate the cuticle. The new essential information on the reproductive biology of Strepsiptera raises several new questions. A) Is this an exclusive structure of a single species or more common in the genus *Stylops* or even widespread in the family Stylopidae? B) What is the functional role of this structure in relation to the paragenital organ in traumatic insemination? C) Are there specialized features of the integument compared to other parts of the cephalothorax? In study II, we show that the paragenital organ is not only present in *Stylops*, but also in *Eurystylops*, *Hylechtrus*, *Halictoxenos*, and *Kinzelbachus*. Therefore, it can be assumed that the paragenital organ is a typical feature of the Stylopidae, with the notable exception of *Crawfordia* and a possible synapomorphy. Studies II and III together provide the first insight into the material composition and integument thickness at the penetration site of seventeen Strepsiptera species and their paragenital organs. With these results, we conclude that the paragenital organ is

an evolutionary adaptation of females that allows them to tolerate the induced costs of traumatic insemination; in addition, it functions as a precopulatory mating barrier in the genus *Stylops*, preventing heterospecific copulation. Thereby, Study III is a prime example of interdisciplinary research, combining results from DNA barcoding, confocal laser scanning microscopy, principal component analysis, micro-computed tomography, 3D modeling, micro indentation measurements, force measurements, and statistics. The paragenital organ in general will be discussed and compared to other groups of organisms in a later chapter of this dissertation (Chapter 3.5).

Having gained insight into traumatic insemination as a general mode of copulation in Strepsiptera, and having illuminated the adaptations of females to TI, there remains a central question regarding the reproduction of Strepsiptera: Are these insects monandrous or polyandrous? The assumption of several authors that the group is generally monandrous (e.g., Linsley & MacSwain, 1957; Dallai et al., 2004; Beani et al., 2005; Kathirithamby et al., 2015) has been made without presenting substantial evidence; this assumption seems unrealistic because we observed that females are not able to actively prevent successive matings by different males due to the endoparasitic lifestyle and traumatic insemination. Therefore, it is not surprising that Study IV — which includes work from the BSc thesis of Nico Wanjura (University of Freiburg) — provides clear evidence for polyandry in the two species *Stylops ovinae* (Stylopidae) and *Xenos vesparum* (Xenidae) based on microsatellite analysis. Under laboratory conditions two males were able to mate with one female and contribute to the offspring. These results were confirmed for animals in the field, where we showed that several fathers, up to four in *Stylops ovinae*, share the contribution to the development of larvae in a single female. Based on these data ,we draw conclusions about sperm competition and provide further evidence for the hypothesis of mate guarding in *Stylops ovinae* proposed by Peinert et al. (2016). Therefore, Study IV demonstrates true polyandry in Strepsiptera with genetic evidence for the first time, and it is the first investigation of sperm competition in this cryptic insect order. In addition, this work is connected to Study I, as the discussion of sperm competition is only possible based on confirmed information on the mode of copulation.

Taken together, the four studies clarify fundamental issues of Strepsiptera reproductive biology. They provide the first clear evidence for the occurrence of traumatic insemination is provided and dispel the myth that Strepsiptera are generally monandrous. In the following, I will discuss individual aspects and alternative interpretations of these studies in detail and place them in a more general context.

3.2   Key Species in an Experimentally Challenging Insect Order

Working with Strepsiptera is generally considered to be as challenging as it gets for several reasons. The most obvious problems are the difficulty of finding Strepsiptera in the field, the challenge of maintaining them in culture, even if this is possible in principle (Baumert, 1958), and the minute size of the primary larvae, which are on average only 200 μm long (Beutel, Pohl & Hünefeld, 2005; Osswald, Pohl & Beutel, 2010; Knauthe et al., 2016; Pohl & Beutel, 2019). Due to the parasitic life cycle of Strepsiptera, it is often necessary to perform experiments with only a few specimens within a short period of time. Furthermore, it is almost impossible to breed Strepsiptera in successive generations, which means that they have to be collected every year and the breeding has to start from scratch every year. A particular challenge is the collection of enough virgin females, as the mating status of living individuals cannot be determined. This can only be done with special experience and timing. Although the selection of appropriate organisms is the basis of any experiment on organisms, the choice of Strepsiptera species should be made with an adequate phylogenetic background knowledge to allow a meaningful evolutionary conclusion. Therefore, I will briefly discuss the three key species treated in this thesis — *Eoxenos laboulbenei*, *Stylops ovinae*, and *Xenos vesparum* — with respect to their specific advantages and potential shortcomings.

3.2.1   *Eoxenos laboulbenei*

Eoxenos laboulbenei was chosen as the representative of the family Mengenillidae. In contrast to the closely related species of *Mengenilla*, its morphology and biology have been studied in great detail (Parker & Smith, 1933; Silvestri, 1933; Parker & Smith, 1934; Silvestri, 1941a; Müller, 2009; Marquart, 2010; Delgado et al., 2014; Tröger, Beutel & Pohl, 2019; Tröger et al., 2020; Tröger et al., 2023). This species is of particular interest in the context of the reproductive biology as it represents one of only a few lineages whose females are free-living, i.e. the extinct Mengeidae, the extant Mengenillidae, and probabily also the monospecific Bahiaxenidae (Parker & Smith, 1933; Silvestri, 1941a; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Pohl et al., 2019; Tröger et al., 2019; Tröger et al., 2023). This is in marked contrast to the obligate endoparasitism of females in all other Strepsiptera lineages. In addition, the phylogenetic position as one of the earliest branching extant families makes Mengenillidae a critically important candidate for identifying ground-plan features of the taxonomic order (Pohl & Beutel, 2005). *Eoxenos laboulbenei* develops in silverfish of the family Lepismatidae (Silvestri, 1941a; Delgado et al., 2014; Tröger et al.,

2020). This species was primarily studied by me in in Study I to elucidate the mode of copulation in an early branching extant family of Strepsiptera. *E. laboulbenei* occurs only in arid and semi-arid habitats with Mediterranean climate, where it is mainly found under stones. Material can only be obtained by collecting trips to Croatia and Italy, which makes collecting costly and time consuming. Breeding is even more difficult because the Zygentoma hosts are associated with ants in the wild. A method to keep the animals in small plastic containers is described by Tröger et al. (2020). In summary, the species is challenging to handle but essential for the study of Strepsiptera in an evolutionary context. It was originally planned to use this species also for paternity testing in Study IV, but due to the Corona pandemic it was not possible to collect a sufficient number of specimens. However, we were able to sequence the genome of one male and one female specimen from Croatia. This laid the foundation for further experiments with this species.

3.2.2 *Stylops ovinae*

The hosts of *S. ovinae* females are mining bees of the species *Andrena vaga* (Jůzová, Nakase & Straka, 2015; Straka, Jůzová & Nakase, 2015; Hoffmann et al., 2023), which live in colonies and usually emerge from their nests in spring (Brandenburg, 1953; Antoine & Forrest, 2021; Hoffmann et al., 2023). Compared to other Strepsiptera species, *S. ovinae* is relatively easy to collect. My supervisors, Prof Dr. Oliver Niehuis and PD Dr Hans Pohl, knew good collecting sites in the greater Aachen area, which were easily accessible. *Stylops* was important for our studies for several reasons. On the one hand, it represents the family of Stylopidae, one of the most speciose families of Stylopidia with approximately 160 known species and is characterized by a brood canal opening. On the other hand, *S. ovinae* has a paragenital organ, the origin and function of which was an overarching question in this study (Study II, chapter 3.5). Living specimens can be kept to a certain extent in small glass vials (Peinert et al., 2016). Furthermore, the species was of particular interest as a model organism for our study. Sufficient work on morphology, biology, embryology, and host interactions is available, allowing for interpretation and discussion in a broader context (e.g., Nassonov, 1893; Hoffmann, 1913; Hoffmann, 1914; Smith & Hamm, 1914; Noskiewicz & Poluszyński, 1928; Brandenburg, 1953; Grabert, 1953; Richter, 1956; Lagoutte et al., 2013; Fraulob et al., 2015; Jůzová et al., 2015; Löwe, Beutel & Pohl, 2016; Pohl, Gorb & Gorb, 2020; Nakase & Kato, 2021; Hoffmann et al., 2023).

3.2.3 *Xenos vesparum*

Like *S. ovinae*, *X. vesparum* has been intensively studied (Weingardt, Beutel & Pohl, 2023). Futhermore, it is the first described species of Strepsiptera and additionally one of the most abundant species with a Palearctic distribution (Rossi, 1793; Benda et al., 2020; Benda et al., 2022). The distribution of the endoparasitic species is closely linked to the distribution of its most common host, *Polistes dominula* (Höcherl & Tautz, 2015). The distribution of the host, among other factors, has greatly facilitated access to this species. Fresh males of *X. vesparum* could be attracted with pheromones, which call females in large numbers in the backyard of the Phyletic Museum in Jena. The preference of infested wasps to feed on trumpet creeper bushes (*Campsis radicans*) allows for highly effective collection in suitable locations (Beani et al., 2018). In addition, *X. vesparum* can be reared under laboratory conditions with certain limitations. *P. dominula*, a model organism for studies of eusociality, is well established in many laboratories, and methods with limited cost and effort are well established (e.g., Beani et al., 2005; Beani, 2006; Weingardt et al., 2023). A wealth of studies on morphology, anatomy, and behavior are also available, increasing the value of our new data in a broader context (e.g., Kifune & Maeta, 1985; Dallai et al., 2004; Beani et al., 2005; Kathirithamby & Hughes, 2006; Giusti et al., 2007; Manfredini et al., 2007; Hrabar et al., 2014; Beani et al., 2017; Richter et al., 2017; Weingardt et al., 2023). It is noteworthy that in studies on this species in different disciplines have produced numerous contrasting opinions on the reproduction of *X. vesparum* have arisen. This is another reason why *X. vesparum* was chosen as for this study. Details of the controversies are discussed in chapter 3.4.

3.3   Interdisciplinary Approach and Methodology

An interdisciplinary approach and several interrelated methods were used in this dissertation (Table 1). As mentioned in chapter 3.2, Strepsiptera are exceptionally difficult to handle, and almost all species can only be obtained in very limited numbers. Therefore, a careful choice of methods was essential to establish an efficient working pipeline and to obtain maximum information from limited samples. The focus was necessarily on feasibility, but also on transparency of applied methods, data and results. An example of how different methods from different disciplines are linked is given in Study III: DNA barcoding in experiments with males attracted by females of *S. ovinae* showed that the pheromone-driven attraction also involves heterospecific males. The attracted males were then analyzed using μ-CT scanning and 3D models, which allowed shape varia-

tion to be studied using geometric morphometrics and principal component analysis. In the following paragraphs I will outline some of the main methods used in my thesis and discuss and how their coordinated application can facilitate and enhance the study of Strepsiptera.

Modern morphological research relies heavily on micro-computed tomography, which has developed rapidly since its first application in insect research (Hörnschemeyer, Beutel & Pasop, 2002). The advantages are obvious: The generated data allow a detailed view of the internal morphology, in most cases without being invasive or destructive, which is an additional advantage when dealing with rare specimens, e.g., in historical collections. Histological section have long provided anatomical information. However, three-dimensional modeling based on μ-CT scans very clearly surpasses traditional methods. μCT data and 3D models greatly facilitate the understanding of complicated morphological configurations consisting of endo- and exoskeletal cuticular structures and different types of soft tissues (e.g., Jandausch, Beutel & Bellstedt, 2019; Jandausch et al., 2021; Aibekova et al., 2022). The morphological research in this dissertation is essentially based on a series of more than 30 μCT datasets obtained in different facilities in Germany, mainly Karlsruhe Institute of Technology [KIT], Deutsches Elektronen-Synchrotron [DESY], and Max-Planck-Institut für Menschheitsgeschichte, Jena. A network of collaborators provided an enormous amount of experience in the field of μCT scanning, which made it possible to optimize the acquisition of high-quality data and their subsequent processing. Very different requirements can play a role, e.g., high throughput of specimens (Study II), maximum resolution (Study I), or also special settings for amber fossils (e.g., Pohl et al., 2019; Boudinot et al., 2022; Richter et al., 2022). Micro-computed tomography has not only been used to obtain detailed information on three-dimensional anatomical configurations. It can also be used to generate detailed models that make morphology more accessible and can be used for further applications such as geometric morphometrics (Study III).

Geometric morphometrics was first applied to Strepsiptera in my thesis. While geometric morphometrics based on three-dimensional data is now common in vertebrates (e.g., Durrleman et al., 2012; Stoessel et al., 2016; Taszus et al., 2023), insect studies predominantly focus on two-dimensional data based on photographs (e.g., Sasakawa, 2016; Tatsuta, Takahashi & Sakamaki, 2018). The fact that 3D data have long been neglected is surprising, as geometric morphometrics in general is a powerful tool to address problems such as species delimitation or character variation (e.g., Mutanen & Pretorius, 2007; Friedrich et al., 2014; Braga et al., 2019). 3D data contain significantly more information than 2D data, which improves the results obtained with geometric morphometrics. This is also true for an intensively studied insect structure, the wings (Schubnel et al., 2023).

The established protocol of geometric morphometrics in Study III could be integrated into a Bachelor's thesis of Malte Lehmann (Morphometrics on *Polistes dominula* agg. utilizing synchrotron Micro-CT; University of Freiburg, Supervisor: Prof. Dr. Oliver Niehuis; C-supervisors: Fabian Schweitzer, Kenny Jandausch). In addition to DNA barcoding, the method was used to examine morphological characters for the delimitation of two paper wasp species. Based on the characters obtained, it was possible to unambiguously identify the more than 200-year-old syntypes of *P. dominula* from the Gerning collection in the Hessisches Landesmuseum für Kunst und Natur. This is of particular interest because *P. dominula* is widely distributed and has been used as a model organism in evolutionary biology and other disciplines (Turillazzi & West-Eberhard, 1996; Starks, Turillazzi & West-Eberhard, 2006), and is also the host of a key species of this thesis, *X. vesparum*. The results of this thesis confirm the usefulness and impact of 3D geometric morphometrics in insect research and fundamentally change certain perspectives. In addition, they demonstrate the value of morphology when molecular methods such as DNA barcoding reach their limits, as old museum specimens are often unsuitable for DNA extraction. Furthermore, the non-invasive nature of the techniques used has great potential for the study of very rare species or irreplaceable museum specimens.

A major result of the present work is the first genetic evidence for polyandry in Strepsiptera. Apart from the difficulty of handling and collecting the tiny primary larvae from controlled laboratory mating experiments (see section 3.2), it was very challenging to assess paternity for single, extremely small individuals by establishing polymorphic sets of microsatellites. Even a standard procedure like DNA extraction is extremely difficult with objects of about 200 μm length (e.g., Kinzelbach, 1971; Osswald et al., 2010; Knauthe et al., 2016; Pohl & Beutel, 2019). In the BSc project of N. Wanjura (included in Study IV) we established the laboratory procedure for handling the tiny larvae of *Stylops ovinae* ("Polyandry and mate guarding in *Stylops ovinae* (Strepsiptera, Insecta): Using paternity analyses to investigate the reproductive biology of Stylopidia"; University of Freiburg, Supervisor: Prof. Dr. Oliver Niehuis; Co-supervisor: Kenny Jandausch). Due to the viviparous development of Strepsiptera, it was necessary to isolate individual larvae under binocular control using a pipette. The exclusion of maternal tissue was essential for the accuracy of the microsatellite analysis. The transfer of the larvae had to be performed in a minimal amount of absolute ethanol to avoid problems with the static of the plastic tubes. Transferring larvae without liquid was not possible as the tiny individuals were passively dislodged and could not be safely transferred to the reaction tube. To maximize the yield of DNA from the tiny larvae, we found that it was necessary to crush them in liquid nitrogen with a mortar and pestle. From this point on, the handling of DNA samples could be handled using standard protocols for DNA extraction and

microsatellite analysis (see Study IV for details). The established methodological adaptations were successfully applied to adults and to all samples of the second studied species, *X. vesparum*. In addition, the protocols were also used for DNA extraction of pooled samples from the field. The standard genetic procedures, established for research on tiny larva of Strepsiptera, can be easily applied to other tiny organisms. In addition, pre-amplification protocols could be used to maximize the DNA yield when it is expected to be critically low for downstream applications (e.g., Spits et al., 2006; Niehuis, Judson & Gadau, 2008). For example, very low DNA yields can be expected from very small organisms, single insect eggs, or even single cells. In Study IV, we show that pre-amplification is not strictly necessary even in *X. vesparum*, where in addition to primary larvae, progeny at early embryonic stages were also analyzed. Such additional steps always increase the risk of contamination and translation errors. However, the need must be proven, and the benefits must be carefully weighed against the potential disadvantages. Since our DNA yield could not be improved using DNA amplification kits, we decided to exclude them from the experimental protocol, thus reducing the risk of translation errors and contamination. Overall, it was possible to perform reliable paternity tests with two Strepsiptera species and change the perspective of their mating behavior.

3.4 The Mating Mode of Strepsiptera

The data obtained in Study I increased the evidence for traumatic insemination in Strepsiptera. By documenting mating signs in species from six different taxonomic families, we show that traumatic insemination is the general mode of copulation in this group. Other applied methods, such as micro-computed tomography or scanning electron microscopy histology of specimens fixed in copula, allowed a precise documentation of penis insertion and sperm transfer in *E. laboulbenei* (Mengenillidae), *S. ovinae* (Stylopidae), and *X. vesparum* (Xenidae). Furthermore, by combining data obtained by different techniques, we can show that penetration is indeed the cause of melanized mating signs and can therefore be used as evidence for traumatic mating in Strepsiptera. In the following section I will focus specifically on *X. vesparum*, a species that has been discussed more controversially than others. The reproductive biology will be discussed, also with respect to insufficiently supported findings of other authors.

3.4.1 Brood Canal Mating

Tartanic et al. (2014) summarizes two generall hypothesis of the evolution of trauamtic insemiantion in Strepsiptera. First it could have evolved due to sexual selection and make males capable of circumventing mechanism of female cryptic choice. As to date such mechanism are unknown, Tartanic et al. (2014) put forth the hypothesis that traumatic insemination in Strepsiptera is the result of evolutionary constraints. The lack of developed female genitalia and the endoparasitic lifestyle makes only the cepahlothorax accesable to males and consequently could lead to traumatic mating as possible mechanism of sperm transfer. An alternative hypothesis is provided by Beani et al. (2005) that point at the possbility of brood canal mating.

Beani et al. (2005) provided the evidence tentatively contradicting traumatic insemination in *X. vesparum*. Although the authors do not completely rule out this traumatic mating, they clearly argue for brood canal mating. Using scanning electron microscopy and transmission electron microscopy, they show that spermatozoa can be found in the brood canal of females after mating, and that sperm do not appear in the cephalothorax until 1 or 2 hours after copulation. These results are in contrast to Study I of this dissertation, where we show that a) the penis is inserted into the brood canal but pierces the ventral integument of the female directly in the anterior part, b) mating signs result from this penetration at the observed insertion sites, and c) sperm is transferred directly into the cephalothorax without passing through the brood canal. We assume that the spermatozoa found in the brood canal in the study by Beani et al. (2005) were dislocated during the preparation of the female specimens for TEM. We addressed this problem of contamination by embedding females in methacrylate prior to sectioning. This ensured that spermatozoa were not dislocated from the hemocoel to the brood canal during sectioning. This technical problem was already pointed out by Silvestri (1941b) in a comment on observations by Schrader (1924) on Acroschismus wheeleri (junior synonym of *Xenos peckii*).

In addition to their findings of sperm in the brood canal, Beani et al. (2005) argued that the evolutionary advantages of traumatic insemination in Strepsiptera are difficult to demonstrate unequivocally. Hypotheses put forward for other taxa, such as male competition in cimicids (Lloyd, 1979), were refuted by Beani et al. (2005). They estimate male competition in Strepsiptera to be relatively low, arguing that adult males have a short lifespan of only a few hours and never occur in large numbers to potentially competing for females. The fact that these authors never observed clusters of flying males can be refuted, as Study III confirms that males are attracted to unmated females in large numbers (Jandausch et al., 2022). In 2018, we were able to attract over 104 males in

a single day, including several males clustering on the air-permeable cages containing the unmated females. We obtained comparable results for *Stylops* in 2020 (Jandausch et al., 2022), where we observed females in a similar cage attracting 18 males in 3 hours. Interestingly, several heterospecific males were also attracted, but the exposed females were still encountered by several conspecific males at the same time. These observations strongly suggest that there is indeed competition between males for females and that traumatic insemination is likely to have evolved in this context.

In addition, some authors have described hosts counteracting males on their abdomen and trying to get rid of them during their mating attempts. For example, this was described by Hubbard (1892) for *Polistes* and by Hassan (1939) for Delphacidae (Auchenorrhyncha). In our own experiments, we have observed wasps kicking to remove male Strepsipteran from their metasoma. Beani et al. (2005) noted that such host defensive behavior should favor rapid insemination to the exclusion of integument penetration. In contrast to this, we hypothesize that integumental penetration with an intromittent organ may produce a strong anchoring force that makes males more resistant to losing female contact duing mating, especially when the host is kicking.

This thesis combines several arguments against brood canal mating. The fact that we found sperm exclusively in the hemocoel, in close proximity to the undoubted site of penetration, and the evidence for male competition for females support the traumatic insemination hypothesis. Overall, brood canal mating in *Xenos vesparum* is highly unlikely, as the evidence presented for this interpretation is most likely an artifact, in addition to a misinterpretation of the reproductive behavior, i.e., competition. Consequently, the assumption is also untenable for all other groups of Strepsiptera. Traumatic insemination is vermosty likely the ancestral mode of copulation in Strepsiptera. The switch from traumatic insemination in Mengenillidae to brood canal mating in all other groups, as suggested by Kathirithamby et al. (2015), can be clearly refuted on the basis of the results presented here.

3.4.2   Traumatic Insemination in Other Terrestrial Arthropods

Traumatic insemination has evolved several times in the animal kingdom, but it is still something unusual, including terrestrial arthropods, where it is has been described in Cimicoidea (Heteroptera) and Strepsiptera (e.g., Tatarnic et al., 2014). It is therefore useful to compare different groups in terms of morphology and mechanisms of traumatic insemination. The most striking feature of female Strepsipteran is the absence of TI-associated structures in most families. Such structures, termed "paragenitalia",

have been described for several representatives of Heteroptera (Tatarnic et al., 2014). The most detailed studies have been outlined for the family Cimicidae, which includes the prominent example of TI, the bed bug (*Cimex lectularius*). Several studies on morphology, mating behavior, and material composition are available and contain most of the documented knowledge on traumatic insemination (e.g., Abraham, 1934; Carayon, 1966c; Carayon, 1966b; Carayon, 1966a; Stutt & Siva-Jothy, 2001; Morrow & Arnqvist, 2003; Reinhardt, Naylor & Siva-Jothy, 2003; Siva-Jothy & Stutt, 2003; Rossellini, 2010; Horton & Lewis, 2011; Benoit, Jajack & Yoder, 2012; Michels, Gorb & Reinhardt, 2015; Jung et al., 2023). However, other groups such as dysderid spiders or fruit flies have received more focused work than Strepsiptera with respect to traumatic insemination (e.g., Kamimura, 2007; Řezáč, 2009; Lange et al., 2013; Tatarnic et al., 2014; Reinhardt, Anthes & Lange, 2015; Ma et al., 2023).

3.4.2.1 Araneae

Although traumatic insemination is only known in one spider species, namely *Harpactea sadistica* (Dysderidae), it is an interesting example (Řezáč, 2009). Compared to other species of the genus, *H. sadistica* is the only spider that has a needle-like embolus, with which it repeatedly stings the female and transfers the sperm directly into the hemolymph in the opisthosoma. From there, the sperm bypass the uterus and fertilize the eggs directly in the ovaries. This means that parts of the spider's internal genitalia are still well developed, unlike the spermatheca that is usually present in Dysderidae (Austad, 1984). This is interesting because Beani et al. (2005) used the lack of sperm storage organs in *X. vesparum* as an argument against traumatic insemination in Strepsiptera, while the case of *H. sadistica* shows that the loss may be a consequence of traumatic insemination. This scenario provides excellent conditions to study the shift from genital mating to traumatic insemination and could reveal morphological consequences, although this opportunity has not yet been exploited. Since all extant Strepsiptera perform traumatic insemination, the details of such a switch are difficult to follow. Unfortunately, fossils of female Strepsiptera that provide details of the internal anatomy are currently lacking and are unlikely to appear.

3.4.2.2 Diptera

Traumatic insemination was described by Kamimura (2007) in the *Drosophila bipectinata* complex. He describes blind end pockets dorsad the genital opening of females that are pierced by basal processes of the male genitalia. Wounds in the genital tract of *D. pseudoananassea* are used for sperm transfer and were identified by melanized spots, which are comparable to mating signs in Strepsiptera and probably functionally equivalent. Interestingly, this diagnosis creates parallels with the insemination process of several Stylopidae species. In Study II, we show paragenital organs that are structurally similar to the blind end pockets described by Kamimura (2007). Similar to in the *D. bipectinata* complex, the intromittent organ is inserted into this paragenital organ and pierces the integument, leaving melanized scars after healing. These wounds are used for sperm transfer, which is another similarity. It is noteworthy that traumatic insemination in the *D. bipectinata* complex has been questioned by Polak & McEvey (2022), who questioned the validity of some criteria, such as the ability of the basal processes of the male genitalia to transfer sperm. The methodology used in this thesis could provide a definitive answer to this question. Scanning electron microscopy histology and micro computed tomography of specimens fixed in copula could shed light on the true position of the basal processes, the aedeagus, and the route of sperm. However, the route of sperm after insemination differs because Stylopidae lack a genital tract, while species of the *D. bipectinata* complex retain internal female genitalia.

3.4.2.3 Heteroptera

In several groups of Heteroptera, such as Anthocoridae, Cimicidae, and Miridae, a paragenital organ known as the "spermalege" is typical. An external part of this structure, the ectospermalege, can evolve as an invagination of the integument that guides the male aedeagus to a specific intromission site (Reinhardt et al., 2003). A counterpart to the guiding function of the ectospermalege for the male's penis in bed bugs may be superfluous in Strepsiptera. Options for penetration are severely limited by the endoparasitic lifestyle of most of the females, as only the cephalothorax exits the host. In addition, females of Stylopidia are enclosed by the exuvia of the secondary larva, which lies only anterior to the brood opening and is weakly sclerotized and penetrable by males. Interestingly, in females of some Lyctocoridae species lack a true ectospermalege and instead have a thickened endocuticle at the intromission sites on the abdomen (Carayon, 1977). A comparable situation is found in several species of Strepsiptera, where the integument is

thickened at the sites where males penetrate females for insemination (Study II; Peinert et al., 2016; Jandausch et al., 2022; Jandausch et al., 2023). This feature is most likely an adaptation to traumatic insemination to reduce the damage caused by stabbing (Jandausch et al., 2022). Associated with the ectospermalege is the mesospermalege, which is filled with phagocytic hemocytes, although they vary in shape and degree of membranization. These cells are capable of digesting both spermatozoa and seminal fluid and are therefore likely candidates for enabling female cryptic choice (Carayon, 1966a; Klein & Kallenborn, 2000; Reinhardt et al., 2003). Structures similar to the spermalege are completely lacking in Strepsiptera. Only the family Stylopidae has evolved a simple paragenital organ, which will be discusssed in chapter 3.5. However, because of the simplified female's genital tract, structures or mechanisms that could be used to select preferred sperm remain unknown. Tentative evidence for female cryptic choice by cell trapping was discussed by Beani et al., 2005. The authors found spermatozoa in adipocytes of newly mated females and suggested that sperm could be manipulated to lose sperm-egg recognition. At present, this remains speculative, and a possible underlying mechanism remains unstudied.

Although some similarities with other traumatically inseminating species can be outlined, the peculiar life history characteristics of Strepsiptera make it difficult to draw conclusions from comparisons. Endoparasitism and the complete reduction of the female genital tract make Strepsiptera isolated and highly specialized, making meaningful comparison with other groups. very difficult. However, this situation offers the opportunity to establish a new model system for traumatic insemination that may be useful to test hypotheses about this bizarre mode of copulation, which is currently interpreted mainly based on the study of bed bugs.

3.5 Evolution of the Paragenital and its Possible Function

Paragenitalia are interpreted as structures that evolve adin addition to, and often alongside, the primary genitalia and may take over some or all of their functions. As mentioned above, such structures often appear in correlation with extra-genital traumatic insemination and are well studied in several species of Heteroptera, especially Cimicoidea (e.g., Carayon, 1966a; Horton & Lewis, 2011; Lange et al., 2013; Tatarnic et al., 2014; Reinhardt et al., 2015; Jung et al., 2023).

In bed bugs, traumatic insemination has been shown to be costly for females due to the damage induced by the intromittent organ piericing for sperm transfer. Morrow & Arnqvist (2003) argued that female bed bugs are able to reduce this cost for life by evolv-

ing a paragenital organ as a potential counter-adaptation. This specialized abdominal region is rich in resilin compared to the highly sclerotized surrounding areas and is therefore much more efficient at closing wounds caused by penetration (Michels et al., 2015). In Study III, we looked for such a compositional gradient that might be part of similar mechanisms in female Strepsiptera. As female Strepsiptera are generally weakly sclerotized and rich in resilin throughout their integument, such a gradient was not identified. Rather, we found that the cuticle is thickened at the penetration sites, which potentially have the same wound-closing effect. This thickening of the integument has also been described in traumatically inseminating species of Lyctocoridae (Carayon, 1977). Whether traumatic insemination imposes costs on female Strepsiptera similar to some groups of true bugs remains unclear and should be the focus of future research.

The only paragenital structure known so far in Strepsiptera is that of Stylopidae. This invagination of the integument on the ventral side of the border between the fused head and the prothorax serves first as a blind end pocket into which the penis is inserted and second as a penetration site for traumatic insemination. Since the function of the paragenital organ can be seen as a kind of bursa copulatrix and is not homologous to any primary genitalia, the definition of this pouch as a paragenital system fits. Whether it has been evolved as a direct adaptation to traumatic insemination itself is not clear. Using mating experiments in Study III, we found that heterospecific males are attracted to the sexual pheromone of females but are unable to copulate with the females. We therefore hypothesize that the paragenital organ itself is not a direct adaptation to reduce the damage of traumatic insemination, but that it prevents the endoparasitic females from mating with heterospecific males. Such mismatches are highly costly for females, as they involve the cost of traumatic mating without any reproductive success, as discussed by Reinhardt & Siva-Jothy (2007) in bed bugs. In Study II, we provide additional evidence for this hypothesis, as penis shape and size correlate with the depth of the paragenital pocket (see also, Jandausch et al., 2022). Nevertheless, the penetration site on the ventral wall of the paragenital organ is thickened, as is the penetration site in females of other Strepsiptera families (e.g., Elenchidae, Halictophagidae, and Xenidae; (Jandausch et al., 2022). It is an open question why such structures have evolved in only one family of Strepsiptera. In the Western Palearctic, only two species of *Xenos* are described (*X. vesparum* and *Xenos zavattarii* (Pierce, 1911) (Benda et al., 2020; Benda et al., 2022), the latter only documented from Tripoli (Libya). In contrast, 32 different *Stylops* species are currently documented and are accepted as valid species in the Western Palearctic, and many of which occur in sympatry (Straka, Jůzová & Batelka, 2014; Jůzová et al., 2015; Straka et al., 2015). Since sympatry is apparently much more common in *Stylops*, we infer that this interspecific competition may have been a trigger for the formation of the paragenital organ.

For a more detailed discussion of the paragenital organ, see Study II of this dissertation. To draw more detailed conclusions about the evolution of the paragenital organ in Stylopidae, it is important to know more about the distribution of this structure based on my results. For this, it is necessary to study the paragenital organ in more species from different genera and to gain more information about the reproductive mechanisms in less studied genera such as for instance *Kinzelbachus*, *Halictoxenos*, or *Eurystylops*.

3.6   Polyandry and Sperm Competition in Strepsiptera

3.6.1   Polyandry

We show in individuals from wild populations of *S. ovinae* and *X. vesparum* that females produce offspring from at least two fathers. Thus, we refute the idea of strict monandry in Strepsiptera. By analyzing the allelic state of polymorphic microsatellites in both species, we show an allelic composition in the offspring that exceeds the potential of a single father in diploid organisms, which is a maximum of four different alleles. We validated these findings in laboratory mating experiments and were able to determine the paternity of single offspring individuals of double mated females for both species studied. With the data collected, we were able to test the mate guarding hypothesis proposed by Peinert et al. (2016) for *S. ovinae*.

The hypothesis of monandry in Strepsiptera was mainly supported by behavioral observations (e.g., Grabert, 1953; Linsley & MacSwain, 1957) or indirect evidence based on sexual pheromone emission (e.g., Tolasch, Kehl & Dötterl, 2012). At the same time, studies on the chemical stylopsal have impressively demonstrated the efficiency of the emitted pheromone by attracting multiple males (Cvačka et al., 2012; Lagoutte et al., 2013). For example, Lagoutte et al. (2013) were able to attract over 500 males of *S. muelleri* in three hours using synthetic stylopsal-laden traps. Comparable results are presented in Study III of this dissertation, where we used females of *S. ovinae* and *X. vesparum* located in their host to attract conspecific males (Jandausch et al., 2022). Surprisingly, females of *S. ovinae* also attracted males of two other species, providing evidence that the pheromone produced is not species-specific, as claimed by Kathirithamby et al. (2015), at least not in this species. However, the large number of attracted males indicates that intraspecific competition for mating is potentially high in Strepsiptera. Even though pheromone emission appears to decrease immediately after mating (Tolasch et al., 2012), it does not stop completely, allowing males to still detect potential mating partners. In the case of the synchronously hatching *S. ovinae*, polyandry was likely, in contrast to the results of the

above studies. Nevertheless, a decrease in pheromone emission after successful mating could be beneficial to females. In *S. ovinae*, no mechanism is described that would block males access to endoparasitic females. In contrast, a calling behavior has been described for *X. vesparum* by Hrabar et al. (2014): Females hyper-extrude their cephalothorax from the host metasoma, presumably to mate in this position and to re-ingress after mating. However, we also observed matings of females not in such a hyperextruded position, so it may not completely prevent multiple copulations. With respect to traumatic mating in Strepsiptera, increasing the number of copulations would impose additional costs on females through wounding (Morrow & Arnqvist, 2003; Morrow, Arnqvist & Pitnick, 2003). Therefore, a strategy of minimizing the number of penetrations to likely to be beneficial.

In gerenal, Strepsiptera females of thought to benefit from multiple mating because cryptic female choice is unknown, and they lack the morphological structure to enable such strategies (see chapter 3.5). Possible benefits include an increased number of fertilized eggs, an increased genetic diversity of the offspring, and a reduced effects of mating with a sterile male (e.g., Tregenza & Wedell, 1998; Arnqvist & Nilsson, 2000; Slatyer et al., 2012; Parker & Birkhead, 2013). However, whether multiple mating and egg fertilization by multiple males is common in Strepsiptera requires further evaluation in additional species. However, given the very similar lifestyles and mating strategies in different families, this seems very likely.

3.6.2   Sperm Competition

Polyandry results in male competition for fertilization, thus ultimately leading to sperm competition (Parker, 1970; Parker, 1990; Parker & Pizzari, 2010; Simmons & Wedell, 2020). A competitive environment can be created by the temporally or spatially simultaneous presence of the sperm in the female's genital tract. Because Strepsiptera females lack sperm storage organs and the seminal fluid is transferred directly into the hemolymph by traumatic mating, it is proposed that sperm are subject to high competition for fertilization when they overlap in time. To gain an advantage over competitors, a common strategy in such systems is to create a time-space in which only the sperm of the first mating male can fertilize eggs and competitors are excluded. This is achieved, for example, mechanically by a mating plug in spiders (Uhl, Nessler & Schneider, 2010), the removing competitor sperm in dragonflies (Córdoba-Aguilar, Uhía & Rivera, 2003), or by extending mating times to block access for other males (Matzke et al., 2022). Evidence for the latter option in Strepsiptera was provided by Peinert et al. (2016), who observed that S. ovinae males copulating with virgin females had significantly longer mating time

than successive competitors. However, as these authors did not examine the paternity of the offspring, they could not determine the effect of this behavior on the fertilization success of different potential fathers. Our data show a correlation between prolonged copulation time and fertilization success in S. ovinae. Therefore, we hypothesize that mate guarding exists in this species and occurs as an adaptation to the high mating competition and polyandry of the synchronously hatching males (often on only one day of the year). Interestingly, in *X. vesparum*, where males hatch over at least a month (Hughes, Kathirithamby & Beani, 2004), we found no such positive correlation between mating time and fertilization success.

An alternative hypothesis for *S. ovinae* males is that they do not simply block access to females by competitors, but that they use the prolonged copulation time to increase the amount of sperm transferred. If the fertilization of eggs in the hemolymph were random, this would result in a lottery in which each spermatozoon could be counted as a "ticket". Simply increasing the number of sperm increases the chances of a higher proportion of fertilization (Wedell & Cook, 1999; Parker & Pizzari, 2010; Abe & Kamimura, 2015; Ramm, 2020). This process has been described by Parker (1990) as a "fair raffle". To date, it remains unclear how much sperm is transferred during a single mating attempt, but my personal observations and those of H. Pohl suggest that the sperm transfer is completed after a few seconds, as the abdominal pumping responsible for sperm transfer during copulation stops well before the end of the process.

Further work is needed to assess the processes driven by polyandry and the resulting sperm competition in Strepsiptera. Factors such as the amount of sperm transferred, and mate status recognition mechanisms may shed new light on these issues. Regardless, the presented studies lay a foundation for basic strategies and possible adaptations to polyandry and sperm competition.

3.7   Phylogenetic Implications of Investigated Morphological Characters

The phylogenetic position of Strepsiptera as the sister group to Coleoptera is now well established, based on both morphological and genomic data (Beutel et al., 2011; Niehuis et al., 2013; Boussau et al., 2014). Furthermore, the internal relationships between the families are well understood (e.g., Kinzelbach, 1971; Pohl, 2000; Pohl & Beutel, 2005; Pohl & Beutel, 2008; Pohl et al., 2021). However, the internal relationships between genera and species are largely unresolved. There are only few pioneer works that include host specificity in the analyses (Jůzová et al., 2015; Benda et al., 2020; Benda et al., 2022; Hui, Mukherjee & Hazra, 2023).

Despite the studies on the two families Xenidae and Stylopidae, the internal phylogeny of Strepsiptera at the level of genera and species is far from being resolved.

In Study I, we showed that traumatic insemination is most likely the standard mode of copulation in Strepsiptera and belongs to the ordinal groundplan. In this context, penis morphology was interpreted and three general penis morphotypes were identified. Morphotype I is characterized as small and needle-like, correlating with free-living females in Mengenillidae, most likely in Bahiaxenidae, almost certainly also in stem group taxa such as †Protoxenidae. The elongated straight penises of morphotype II are restricted to Corioxenidae and may be related to the hidden position of females under the hemelytra of their heteropteran host. The most prominent feature of morphotype III is the distal hook of the penis, which probably evolved in direct correlation with the female birth opening. Morphotype III has the greatest potential for phylogenetic information content, as shape variation is highly variable not only among families, but also among species of individual genera (e.g., Kinzelbach, 1971; Jandausch et al., 2022). In Study III, we examined shape variation in the penises of X. vesparum and S. ovinae and variation in centroid size (a parameter of generalized shape), which was relatively small in both species, accounting only for about 4% of shape variation. The differences in the shape between congeneric species of S. ovinae demonstate the potential of penis shape analysis to effectively discriminate between different species (Figure 4). This should be tested in further research with a larger dataset of more species.

Studies II and III show that the morphology of the paragenital organ in Stylopidae is more complex than expected. Morphological characters described for the first time in these studies, such as the shape of the paragenital opening, the paragenital pocket, or the paragenital auricles, may elucidate phylogenetic relationships at the genus or species level.

Although the focus of this work was not on the phylogeny of Strepsiptera, some results are of potential interest for future phylogenetic analyses and may provide new character sets that can complement genomic approaches.
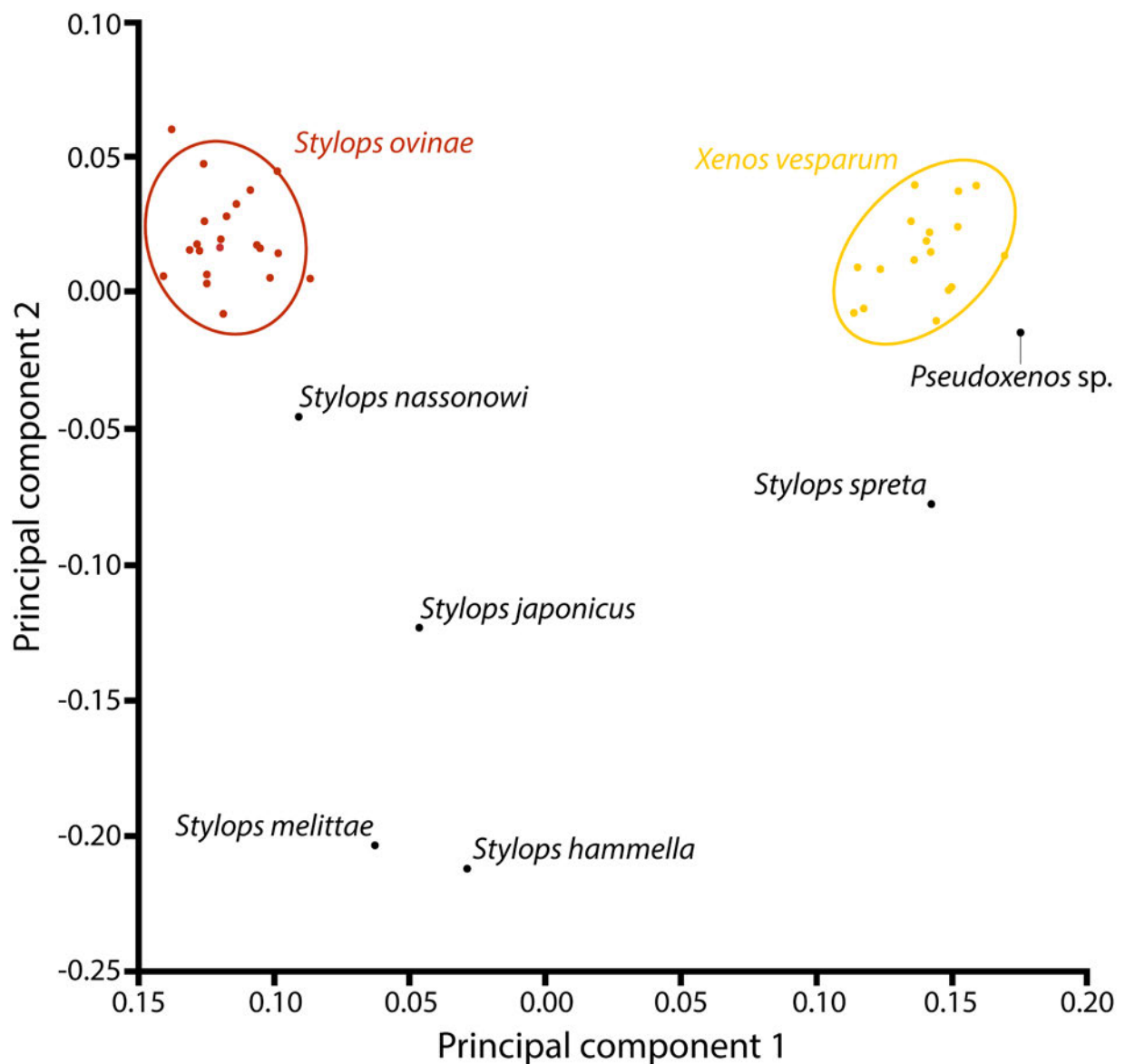
Figure 4: Principal component analysis of male penis shape. Shape space of penises of eight species of Xenidae and Stylopidae visualized with the two most representative principal components. PC1 explains 74 % and PC2 14 % of the total variation. Shape data from Study III were used and supplemented with data for *Pseudoxenos* sp., *Stylops hammella*, *Stylops japonicus*, *Stylops melittae*, *Stylops nassonowi*, and *Stylops spreta*. Generalized Procrustes fit and principal component analysis were performed using MorphoJ (Klingenberg, 2011). Circles represent the 95% confidence interval.

3.8   Conclusion

The present work provides a substantial contribution to the knowledge of the repro-
ductive biology of Strepsiptera. For the first time, conclusive evidence for traumatic in-
semination has been provided for species of seven taxonomic families. Using modern
morphological methods, the exact position of the penis, the exact site of penetration,
and the transmission route of the sperm were determined. This leads to the rejection of
the previous hypothesis that Strepsiptera have evolved brood canal mating secondarily
with the transition to an endoparasitic lifestyle. Traumatic insemination as the ancestral
mode of copulation in the order is now unambiguously confirmed. Based on these find-
ings, further detailed structural insights related to traumatic insemination have been
obtained. Adaptations such as the cuticle thickening and the evolution of a paragenital
organ are essential contributions to the biology of twisted winged parasites. However,
it will be necessary to document structures such as the paragenital organ in Stylopidae
in more detail. For the first time, Strepsiptera have been studied in an interdisciplinary
approach, e.g., combining force measurements and structural studies of the cuticle com-
position, both novel approaches in this group. It was shown that female twisted-winged
parasites are very likely to tolerate the costs associated with traumatic insemination
through adaptations in the cuticle thickness. The establishment of microsatellites in *S.
ovinae* and *X. vesparum* provided the first clear evidence for true polyandry in the group.
This first molecular evidence for polyandry in two species is a good starting point for fu-
ture studies on the related topic of sperm competition. With their endoparasitic lifestyle
and traumatic insemination, Strepsiptera can be used as a model system to study evolu-
tionary processes related to modified reproduction. This work has lied the groundwork
for the study of other evolutionary phenomena within Strepsiptera. But as Tatarnic not-
ed in his 2014 study of traumatic insemination in terrestrial arthropods: "There is much
to be learned of strepsipteran reproductive biology before we are able to confidently
ascertain the underlying forces behind TI in this system" (Tatarnic et al., 2014, pp. 256).

4   Summary

The reproductive biology of the various organisms on this planet has always been a highly attractive field of research and evoked broad interest among numerous scientists. Since knowledge about reproduction and mating behavior plays a central role in understanding our environment, this dissertation also deals with these areas. The small insect order Strepsiptera, which comprises only 600 species, has many unusual biological characteristics, such as the endoparasitic lifestyle of most females or an extremely pronounced sexual dimorphism. Probably the most bizarre and at the same time most controversial feature is traumatic insemination. Due to this, it holds great potential for generating new knowledge in areas such as reproduction, mating behavior or sexually associated morphological structures. This is also because twisted winged parasites are generally not in the focus of research, and knowledge about the reproductive biology of these animals is limited to a few, mostly old works. In addition, various studies repeatedly present unclear or even contradictory opinions. Probably the best example of these discrepancies is that 230 years after the discovery of the first twisted winged parasite, there is still no unambiguous knowledge about their mode of copulation. Besides questions like the occurrence of polyandry or adaptations of females to a possible traumatic insemination, elucidation of the strepsipteran copulation mode is one core questions of the present thesis.

In Study I, we demonstrate that the integration of data from various imaging techniques provides compelling evidence for the widespread occurrence of traumatic insemination across the phylogenetic tree of Strepsiptera. Especially in the families Mengenillidae, Corioxenidae, Elenchidae, Halictophagidae, and Xenidae, we present mating signs induced by traumatic mating for the first time. In the two species *Stylops ovinae* (Stylopidae) and *Xenos vesparum* (Xenidae) we were able to produce micro computed tomography data and transfer them into 3D models of the copulation act. Thereby the actual penetration site is identified corresponding to observed mating signs. Furthermore, we show for the first time that the penetration process is undeniably related to the process of sperm transfers. Therefore, we refute the hypothesis of a switch to brood canal mating in endoparasitic living females and argue that the traumatic insemination is the ancestral mode of copulation in Strepsiptera.

Having laid the groundwork for further in-depth work with the first study of this dissertation, in Study II we turn our attention to the adaptations of females to the bizarre form of copulation. To reduce the cost of copulation, females have evolved several strategies. In bugs of the superfamily Cimicoidea, traumatic fertilization is widespread and closely associated with the presence of a paragenital organ. Such a paragenital organ, al-

beit in a different form, has also been described in a single genus of Strepsiptera, *Stylops*. Although this structure plays a critical role in mating biology, studies of its phylogenetic distribution, morphology, and function are almost completely missing. In this study, we show that the paragenital organ is a possible autapomorphy of the Stylopidae, with the exception of the genus *Crawfordia*. Furthermore, using thickness measurements based on µCT data, we show that regardless of the presence of a paragenital organ, sites penetrated during mating exhibit a thickened integument. Our data provide the first evidence of paragenital organs in the genera *Eurystylops*, *Hylecthrus*, *Halictoxenos*, and *Kinzelbachus*, refuting the assumption that this structure is exclusive to the genus *Stylops*. In addition, we found a functional interaction between the paragenital organ and the processes of exuviation of the secondary larva. Our study contributes to the basic functional understanding of the paragenital organ of Strepsiptera and points to potentially important morphological features for a species-level phylogeny of Stylopidae.

The adaptation of tolerance behavior in response to traumatic insemination has been previously documented in the bed bug (*Cimex lectularius*). In Study III, we present data suggesting that female twisted winged parasites of the two species *Stylops ovinae* and *Xenos vesparum* have also evolved such adaptations. We found a uniform resilin rich integument that is significantly thickened at the sites of penetration by analyzing the cephalothorax with confocal laser scanning microscopy and micro indentation experiments. As this thickened cuticle does not seems to hamper males in their penetration process, we argue that it is an adaptation to reduce damage and hemolymph loss due to traumatic insemination. To better understand our results in an evolutionary context, we conducted attracting- and interspecific mating experiments and geometric morphometric analysis of the penis shape in *S. ovinae* and *X. vesparum*. We demonstrate that females of *S. ovinae* can attract heterospecific males occurring in sympatry. However, only conspecific males were able to successfully copulate. This phenomenon was not observed in *X. vesparum* at all, as no heterospecific males were attracted. Therefore, we conclude that the paragenital organ is a prezygotic mating barrier to exclude erroneous mating with heterospecifics.

Study IV addresses polyandry, the mating of females with multiple males. Although the positive effects to females are hotly debated, it is a strategy that occurs in many insect groups. Despite documentation of serially mating females, Strepsiptera are generally considered to be monandric. To assess paternity share in wild populations of *S. ovinae* and *X. vesparum* we designed specific microsatellite markers for both species and studied the allele composition of female offspring. We can assign both investigated species to being polyandric in the wild, with up to 4 fathers per female occurring in *S. ovinae*. We followed this up by conducting mating experiments in the laboratory to specifically as-

sign paternity to individual offspring to check the existing hypothesis of mate guarding in *S. ovinae* and if comparable strategies exist in *X. vesparum*. We can correlate a significantly increased copulation time of first mating males with their increased fertilization success in *S. ovinae*. We assume that this mechanism is beneficial for sperm competition with later mating males.

Through the work presented in this thesis, I was able to greatly change the perspective and state of knowledge regarding the reproductive biology of Strepsiptera. Fundamental findings such as the mechanism of traumatic insemination will allow future study of the costs and benefits of this copulatory mode on a new model system. Likewise, with the findings on polyandry, I was able to lay the groundwork to enable the study of this broad and evolutionarily complex phenomenon in fan-winged insects. Last but not least, the presented morphological results are also of phylogenetic importance. If these characteristics are considered in greater species diversity in the future, decisive conclusions may be drawn about relationships at the genus and species level.

5   Zusammenfassung

Die Reproduktionsbiologie der verschiedenen Organismen auf diesem Planeten ist seit jeher ein Forschungsgebiet mit hoher Anziehungskraft und rief breites Interesse bei zahlreichen Wissenschaftlern hervor. Da die Kenntnis über Vermehrung und Paarungsverhalten eine zentrale Rolle für das Verständnis unserer Umwelt spielt, beschäftigt sich auch die vorliegende Dissertation mit diesem Gebieten. Die nur 600 Arten umfassende und damit kleine Insektenordnung der Strepsiptera hat viele ungewöhnliche biologische Merkmale, wie die endoparasitische Lebensweiße der meisten Weibchen oder einen extrem ausgeprägten Sexualdimorphismus. Die wohl bizarrste und gleichzeitig umstrittenste Eigenschaft ist die traumatische Insemination. Sie birgt damit ein großes Potential für neue Erkenntnis in Gebieten wie Reproduktion, Paarungsveralten oder sexuell assoziierter morphologischer Strukturen. Dies begründet sich auch darin, dass Fächerflügler generell wenig im Fokus der Forschung stehen, und dass Wissen über die Fortpflanzungsbiologie dieser Tiere auf wenige, zumeist alte Arbeiten beschränkt ist. Zudem bilden diverse Studien immer wieder unklare oder gar Konträre Meinungen ab. Das wohl beste Beispiel für diese Diskrepanzen zeigt sich darin, dass 230 Jahre nach der Entdeckung des ersten Fächerflüglers noch immer keine einstimmige Erkenntnis über deren Art der Kopulation herrscht. Neben Fragestellungen wie dem Auftreten von Polyandrie oder Anpassungen der Weibchen an eine mögliche traumatische Insemination ist genau die Aufklärung dieses Kopulationsmodus Kernfrage der vorliegenden Schrift.

In Studie I zeigen wir, dass die Integration von Daten aus verschiedenen bildgebenden Verfahren überzeugende Argumente liefert, dass die traumatische Insemination bei Strepsiptera phylogenetisch weit verbreitet ist. Konkret liefern wir den ersten Beweis für Verletzungswunden durch traumatische Insemination bei Arten der Mengenillidae, Corioxenidae, Elenchidae, Halictophagidae und Xenidae. Anhand dreidimensionaler Modelle kopulierender Paare von *Stylops ovinae* (Stylopidae) und *Xenos vesparum* (Xenidae) visualisieren wir das physische Durchstechen des weiblichen Integuments durch den Penis des Männchens. Schließlich zeigen wir bei Arten der Mengenillidae, Xenidae und Stylopidae, dass die traumatische Paarung mit der Injektion von Spermien in das Hämocoel des Weibchens verbunden ist. Die Ergebnisse dieser Studie erweitern das Verständnis der Fortpflanzungsbiologie der Strepsiptera erheblich und deutet darauf hin, dass die traumatische Befruchtung die ursprüngliche Form der Kopulation ist und in den meisten, wenn nicht allen, Familien erhalten geblieben ist.

Nachdem mit der ersten Studie dieser Dissertation der Grundstein für fortlaufend vertiefende Arbeiten gelegt wurde, widmeten wir uns in Studie II den Anpassungen der Weibchen an die bizarre Form der Kopulation. Um die Kosten der Paarung zu senken,

haben Weibchen unterschiedlicher Organismen verschiedene Strategien entwickelt. Bei den Wanzen der Überfamilie Cimicoidea ist die traumatische Befruchtung weit verbreitet und eng mit dem Vorhandensein eines paragenitalen Organs verbunden. Ein solches paragenitales Organ, wenn auch in einer anderen Form, wurde auch bei einer einzigen Gattung der Strepsiptera, *Stylops*, beschrieben. Obwohl diese Struktur eine entscheidende Rolle in der Paarungsbiologie spielt, fehlen Studien über ihre phylogenetische Verbreitung, Morphologie und Funktion fast vollständig. In dieser Studie zeigen wir, dass das paragenitale Organ eine mögliche Autapomorphie der Stylopidae ist, mit Ausnahme der Gattung *Crawfordia*. Darüber hinaus zeigen wir anhand von Dickenmessungen basierend auf μCT-Daten, dass unabhängig vom Vorhandensein eines paragenitalen Organs die Stellen, die während der Paarung durchdrungen werden, ein verdicktes Integument aufweisen. Unsere Daten liefern den ersten Nachweis von Paragenitalorganen in den Gattungen *Eurystylops*, *Hylecthrus*, *Halictoxenos* und *Kinzelbachus* und widerlegen damit die Annahme, dass diese Struktur ausschließlich in der Gattung *Stylops* vorkommt. Darüber hinaus fanden wir eine funktionelle Interaktion zwischen dem paragenitalen Organ und inneren Fortsätzen der Exuvie der Sekundärlarve. Mit unserer Studie leisten wir einen Beitrag zum grundlegenden funktionellen Verständnis des paragenitalen Organs der Strepsiptera und weisen auf potenziell wichtige morphologische Merkmale für eine Phylogenie der Stylopidae auf Artniveau hin.

Die Entwicklung von Toleranzverhalten gegenüber traumatischer Insemination wurde bereits für die Bettwanze (*Cimex lectularius*) beschrieben. In Studie III präsentieren wir Daten, die darauf hindeuten, das weibliche Fächerflügler der beiden Arten *Stylops ovinae* und *Xenos vesparum* ebenfalls solche Anpassungen evolviert haben. Mithilfe von Mikroindentationsexperimenten und konfokaler Laser-Scanning-Mikroskopie fanden wir heraus, dass die Weibchen der beiden untersuchten Arten ein einheitliches, resilinreiches Integument haben, das an den Penetrationsstellen deutlich dicker ist als an den Kontrollstellen. Da die verdickte Kutikula die Penetration durch Männchen nicht zu behindern scheint, stellen wir die Hypothese auf, dass Verdickung der Kutikula zu einer Verringerung der Penetrationsschäden und des Verlusts von Hämolymphe und zu einer verbesserten Wundabdichtung führt. Zur Bewertung der evolutionären Bedeutung des Stylops-spezifischen paragenitalen Organs und der Penisformvariation im Kontext der inter- und intraspezifischen Konkurrenz, haben wir Lock- und interspezifische Paarungsexperimente sowie eine geometrisch-morphometrische Analyse der Penisform von *S. ovinae* und *X. vesparum* durchgeführt. Es zeigte sich, dass weibliche S. ovinae sympatrisch vorkommende heterogenerische Männchen anlocken. Allerdings waren nur artgleiche Männchen in der Lage, sich zu paaren. Im Gegensatz dazu konnten wir keine artübergreifende Anziehung von Männchen durch *Xenos*-Weibchen beobachten.

Wir stellen daher die Hypothese auf, dass das paragenitale Organ der Gattung *Stylops* eine präzygotische Paarungsbarriere darstellt, die heterospezifische Begattungen verhindert.

Studie IV befasst sich mit Polyandrie, der Paarung von Weibchen mit mehreren Männchen. Dies ist eine Strategie, die bei vielen Insektengruppen vorkommt. Es ist umstritten, ob dies die Wahrscheinlichkeit erhöht, von männlichen Partnern nützliche Gene zu erhalten, und ob die Weibchen dadurch andere potenzielle Vorteile erhalten. Strepsiptera gelten jedoch im Allgemeinen als monandrisch. Jedoch wurden bei einigen wenigen Arten Weibchen beobachtet, die seriell mit mehreren Männchen kopulieren. Mit der Analyse polymorpher Mikrosatelliten zeigen wir, dass die Nachkommen eines einzigen Weibchens bei *Stylops ovinae* (Stylopidae) und *Xenos vesparum* (Xenidae) mehrere Väter haben können: Wir untersuchten unter anderem Weibchen aus natürlichen Populationen auf Polyandrie. Unsere Ergebnisse zeigten, dass bei beiden Arten mehrere Väter beteiligt sein können, in einigen Fällen sogar bis zu vier. Paarungsexperimente mit *S. ovinae* ergaben, dass jenes Männchen, das sich zuerst mit einem bestimmten Weibchen paart, zu einem höheren Prozentsatz zur Nachkommenschaft beiträgt als nachfolgende Männchen. Bei *X. vesparum* hingegen fanden wir keine signifikante Korrelation zwischen Paarungszeit und Beitrag zur Nachkommenschaft. Die bei *S. ovinae* beobachtete auffallend lange Kopulation hat mit hoher Wahrscheinlichkeit den Vorteil, dass sie die Konkurrenz durch Spermien anderer Männchen verringert. Unsere Ergebnisse belegen Polyandrie in Strepsiptera und legen nahe, dass Monandrie möglicherweise nicht das allgemeine Fortpflanzungsmuster in dieser Insektenordnung ist.

Mit dieser Arbeit war es mir möglich die Perspektiven und den Wissensstand bezüglich der Reproduktionsbiologie der Strepsiptera stark zu erweitern. Grundlegende Erkenntnisse wie der Mechanismus der traumatischen Insemination werden zukünftige Studien zu Kosten und Nutzen dieses Kopulationsmodus an einem neuen Modelsystem ermöglichen. Ebenso war ich in der Lage mit den Befunden zur Polyandrie den Grundstein zu legen, um Forschung an diesem weitgreifenden und evolutiv komplexen Phänomen für Fächerflügler zu öffnen. Nicht zuletzt sind die präsentierten morphologischen Ergebnisse auch von phylogenetischer Bedeutung. Wenn diese Charakteristiken zukünftig in größerer Artenvielfalt betrachtet werden, könnten daraus entscheidenden Rückschlüsse auf Verwandtschaftsverhältnisse auf Gattungs- und Artniveau gezogen werden.

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8 Appendix

FORM 2

Manuscript No I

Short reference Jandausch et al. (2023), *Biol. J. Linn. Soc*.

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure(s) # 5	☒	Approximate contribution of the doctoral candidate to the figure: <u>80%</u> Brief description of the contribution: parts of data acquisition, data analysis and image composition

Figure(s) # 2, 3, 4	☒	Approximate contribution of the doctoral candidate to the figure: <u>60%</u> Brief description of the contribution: photographs and preperation was done with other co-authors, image composition

Figure(s) # 1, 6	☒	Approximate contribution of the doctoral candidate to the figure: <u>40%</u> Brief description of the contribution: based on data gatherd by co-authors, partially data acquistion, image composition

Figure(s) # 7, S1, S2	☒	Approximate contribution of the doctoral candidate to the figure: <u>20%</u> Brief description of the contribution: data of other studies (citied at given palces) and co-authors, image compostion

Manuscript No. II

Short reference Jandausch et al., Manuscript

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure(s) # 1	⊠	100 % (the data presented in this figure come entirely from experimental work carried out by the candidate)

Figure(s) # 12, 13	⊠	Approximate contribution of the doctoral candidate to the figure: <u>80</u>% Brief description of the contribution: parts of data acquisition, data analysis and image composition

Figure(s) # 2-11	⊠	Approximate contribution of the doctoral candidate to the figure: <u>60</u>% Brief description of the contribution: parts of data acquisition, data analysis and image composition

Manuscript No. III

Short reference Jandausch et al. (2022), *PeerJ*.

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure(s) # 8, S4	⊠	100 % (the data presented in this figure come entirely from experimental work carried out by the candidate)

Figure(s) # 7, S5	⊠	Approximate contribution of the doctoral candidate to the figure: <u>80</u>% Brief description of the contribution: data acquisition with other co-authors or studies (cited at given places, data analysis, image composition

Figure(s) # 3, 6, S1, S2, S3	⊠	Approximate contribution of the doctoral candidate to the figure: <u>60</u>% Brief description of the contribution: parts of data acquisition, parts of data analysis, image composition

Figure(s) # 1	⊠	Approximate contribution of the doctoral candidate to the figure: <u>40</u>% Brief description of the contribution: based on data gatherd by co-authors, partially data acquistion, image composition

Figure(s) # 2, 4, 5	⊠	Approximate contribution of the doctoral candidate to the figure: <u>20</u>% Brief description of the contribution: data of other studies (citied at given palces) and co-authors, image composition

Manuscript No. IV

Short reference Jandausch et al., submitted to *Scientific Reports*

Contribution of the doctoral candidate

Contribution of the doctoral candidate to figures reflecting experimental data (only for original articles):

Figure(s) # 2, 3	☒	Approximate contribution of the doctoral candidate to the figure: 80% Brief description of the contribution: parts of data acquisition, parts of data analysis, image composition

Figure(s) # 1	☒	Approximate contribution of the doctoral candidate to the figure: 60% Brief description of the contribution: parts of data acquisition, image composition

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Signature candidate   Signature supervisor (member of the Faculty)

9 Curriculum Vitae

Research interests

My research as an entomologist is in general focusing on the insect order Strepsiptera. More specific I am tackling several different aspects of their mating and reproductive biology. To deepen the understanding of this enigmatic insect order I am mainly engaged with morphology, systematics behavior, genomics and phylogeny.

Research Topics

- morphology of Strepsiptera genitalia and their male-female interaction
- Reproductive and behavioral biology of Strepsiptera
- Paternity tests and fertilization success
- larval morphology of Neuroptera and phylogenetic implications

Research Methods

- µCT scanning and 3D reconstruction, 3D- modelling
- Scanning electron microscopy
- Histology
- biomechanical force measurements
- Geometric morphometrics (principal component analysis)
- PCR and gel electrophoresis
- analyzing microsatellite markers via capillary sequencing

Education

04/2023 – present	Scientific assistant & lecturere, Universitätsklinikum Jena, Institut für Anatomie I
02/2020 – present	PhD student, Friedrich-Schiller-Universität Jena & Ludwig-Alberts-Universität Freiburg
	Project title: »The reproductive and mating strategies of the twisted-winged parasites (Insecta: Strepsiptera): novel insights in the reproductive biology of an enigmatic insect order.« Supervisors: PD Dr. Hans Pohl, Prof. Dr. Oliver Niehuis & Prof. Dr. Rolf Beutel
10/2015 – 04/2018	MSc in Evolution, Ecology and Systematics, Friedrich-Schiller-Universität Jena
10/2012 – 09/2015	BSc in Biology, Friedrich-Schiller-Universität Jena

Professionel experience

2015 – 2019	Teaching assistant, Friedrich-Schiller-Universität Jena, Courses in zoological Biodiversity, Morphology and Anatomie
2017	Technical assistant, Friedrich-Schiller-Universität Jena, Teaching, rebuilding the Mammut of Pfännerhall for the Landesmuseum Halle

Scholarships and awards

09/2019	Price of the German Zoological Association for the best zoological master thesis 2018 (100€)

Media

16.08.2022	»Twisted-wing parasites feel no pain—How females twisted-wing parasites endure the trauma of mating« Artcile in Lichtgedanken about Strepsiptera and traumatic insemination.
07.08.2020	»Wildbienen auf dem Rymelsberg in Langerwehe«Article in Biologie unserer Zeit about Stylops oviane and their host Andrena vaga.
19.04.2019	»10tons – Medusen – Ernst Haeckel« Article in Thüringer Museumshefte (1/2019) as a prequel to the Exhibition in the Phyletisches Museum

Publications

Jandausch, K., van de Kamp, T., Beutel, R. G., Niehuis, O., & Pohl, H. **2023**. "Stab, chase me, mate with me, seduce me." How widespread is traumatic insemination in Strepsiptera? Biological Journal of the Linnean Society, blad046.

Li, D., **Jandausch, K.**, Pohl, H., Yavorskaya, M. I., Liu, X., & Beutel, R. G. **2023**. Cephalic anatomy highlights morphological adaptation to underground habitats in a minute lacewing larva of *Dilar* (Dilaridae) and conflicting phylogenetic signal in Neuroptera. Insect Science, 0(1–19).

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Jandausch, K., Schwarz, D., Bock, B.L., Lukas, P., **2021**. A decharming metamorphosis: The larval and adult morphology of the common spadefoot toad, *Pelobates fuscus*. Zool. Anz. 296, 37–49.

Jandausch, K., Beutel, R., Bellstedt, R., **2019**. The larval morphology of the spongefly *Sisyra nigra* (Retzius, 1783)(Neuroptera: Sisyridae). J. Morphol. 280, 1742–1758.

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Book contributions

Beutel, R.G., **Jandausch, K.**, **2019**. 3.3.9. Digestive system. In: Beutel, R.G., Friedrich, F. (Eds.), Handbook of Zoology, Vol. IV Arthropoda: Insecta. Nannomecoptera and Neomecoptera. Walter De Gruyter, Berlin, New York.

Conference Abstracts

09/2022	Phylogenetic distribution of traumatic insemination in Strepsiptera (Insecta). 8 minutes talk at the annual meeting of the German Zoological Association.
11/2021	Evidence for female resistance traits in traumatically mating Strepsiptera (Insecta). 12 minutes talk at the annual meeting of the Entomological Society of America (online).
09/2021	Evidence for female resistance traits in traumatically mating Strepsiptera (Insecta). 8 minutes talk at the annual meeting of the German Zoological Association (online).
08/2021	Evidence for female resistance traits in traumatically mating Strepsiptera (Insecta). Poster at the annual meeting of the Royal Entomological Society.
09/2019	How to feed on sponges: The larval morphology of the spongefly Sisyra nigra (Neuroptera: Sisyridae). Poster at the annual meeting of the German Zoological Association in Jena.

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Signature candidate

10 Ehrenwörtliche Erklärung

Hiermit versichere ich das die hier vorliegende Abhandlung mit dem Titel „The Reproductive and Mating Strategies of the Twisted-winged Parasites (Insecta: Strepsiptera)." eigenständig angefertigt habe und keine anderen als die angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen benutzt habe. Die Stellen, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen wurden, habe ich in jedem einzelnen Fall durch die Angabe der Quelle, auch der benutzten Sekundärliteratur, als Entlehnung kenntlich gemacht. Zudem erkläre ich, dass mir die Promotionsordnung der Fakultät für Biowissenschaften bekannt ist. Alle Personen die bei der Auswahl und Analyse des Materials, sowie bei der Erstellung des Manuskriptes geholfen haben sind in der jeweiligen Publikation als Autor aufgeführt und deren Anteile sind in den entsprechenden Formblättern vermerkt.

Adrian Richter, Brendon Boudinot, Daniel Tröger, Oliver Niehuis und Hans Pohl haben zudem frühere Versionen von Einleitung und Diskussion dieser Dissertation gegengelesen und diskutiert. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen und es wurden keine Dritten weder mittelbar noch unmittelbar durch geldwerte Leistungen, die im Zusammenhang mit dem Inhalt dieser Dissertation stehen, entlohnt. Die Dissertation wurde nicht bereits zuvor als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.

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