

# Ontogenetic morphological changes of the venom apparatus in 4 eupelmid egg parasitoids

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## Research Article

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### Abstract

Parasitoid wasps, notably egg parasitoids of the family Eupelmidae (Hymenoptera: Chalcidoidea), a key natural enemy of insect pests, offer a sustainable approach to pest management in agriculture. This study investigated the venom apparatus's developmental dynamics across 4 species of eupelmid egg parasitoids: *Anastatus japonicus*, *Anastatus fulloi*, *Mesocomys trabalae* and *Mesocomys albitarsis*. A comprehensive anatomical investigation revealed differences in the dimensions of the venom apparatus across different developmental stages in adult females. We found that the venom apparatus of these 4 studied species consists of a venom gland and a reservoir with an associated Dufour's gland. As the length of post-emergence increases, a significant enlargement in the venom apparatus is evident across all the studied parasitoid species. Notably, *M. albitarsis* consistently exhibits the shortest venom gland length, whereas that of *A. fulloi* is the longest among the observed species. At the high day age, the width of venom glands of the 2 *Mesocomys* species surpasses those of the *Anastatus* species; for the volume of the venom reservoir, there is a steady increase in all 4 species before the age of 6–7 days, with a decline on 8th day, especially for *A. japonicus*. This research provided new insights into the developmental trajectories of venom apparatus in eupelmid egg parasitoids and the potential impact of venom potency on their success.

## Introduction

Parasitoid wasps, a diverse group within the order Hymenoptera, exhibit a unique life cycle that involves parasitizing a host and usually resulting in its death (Godfray, 1994; Quicke, 1997). The adult female parasitoid typically oviposits its eggs within or on the immature stages of a host insect or other arthropod. During this oviposition process, various parasitoid-associated factors originating from the reproductive system, including venom, polydnviruses, virus-like particles, fibrous layer and ovarian fluids are injected (Beckage and Gelman, 2004; Asgari, 2006). These factors create a favourable environment for the parasitoid offspring, promoting their successful development by inducing host paralysis, hindering host development and hindering the host's immune response (Huw Davies and Vinson, 1986; Huang *et al.*, 2021). Venom systems are commonly found throughout Hymenoptera, and their general structure has been thoroughly investigated in various ichneumonid and braconid wasps (Edson and Vinson, 1979; Edson *et al.*, 1982; Quicke *et al.*, 1992, 1997; Zaldivar-Riverón *et al.*, 2004). The structural characteristics of the venom apparatus have also been extensively investigated across other Hymenopteran families, including social bees (Bridges and Owen, 1984; Roat *et al.*, 2006), wasps (Schoeters and Billen, 1995a; Gnatzy and Volkmandt, 2000), ants (Billen, 1990; Schoeters and Billen, 1995b; Billen and Al-Khalifa, 2018) and parasitic wasps (Edson *et al.*, 1982; Blass and Ruthmann, 1989; Wan *et al.*, 2006; Mao *et al.*, 2016) (Table 1).

Parasitoid wasps are classified based on the choice of host life stage, with primary categories including egg, larval, pupal and adult parasitoids (Pennacchio and Strand, 2006). Egg parasitoids are essential to study due to their role in biological control, aiding in sustainable agriculture by suppressing pest populations prior to the pest reaching the larval feeding stage (Vinson, 1998; Takada *et al.*, 2001). These wasps deposit their eggs within the host eggs. As their offspring grow, they consume the host eggs, effectively controlling pest damage at its earliest stages. This process frequently provides vital ecosystem services by regulating host populations and contributing to the preservation of ecological equilibrium (Bragança *et al.*, 1998). Research has been conducted on the venom apparatus of both larval and larval-pupal parasitoids (Zhu *et al.*, 2008; Mao *et al.*, 2016). However, there are limited studies on the composition of venom in egg parasitoids, and no other factors associated with these egg parasitoids have been identified (Zhao *et al.*, 2023). Although most Eupelmidae are larval and pupal parasitoids, members of some genera, such as *Anastatus* and *Mesocomys*, are egg parasitoids with significant implications in the field of biological control (Chen *et al.*, 2021). These wasps predominantly target larger eggs of insects, especially those belonging to the Lepidoptera (butterflies and moths) and Hemiptera (true bugs) (Li *et al.*, 2014; Chen *et al.*, 2021).

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**Table 1.** Distribution of character states from the venom apparatus characters

Parasitoids	Family	A	B	C	D	E	F	Reference
<i>Microctonus hyperodae</i>	Braconidae	1	1	0	1	1	0	Crawford et al. (2006)
<i>Bracon vulgaris</i>	Braconidae	22	1	1	1	1	0	Alves et al. (2015)
<i>Cotesia glomerata</i>	Braconidae	1	1	0	0	0	0	Arakawa et al. (2013)
<i>Yelicones delicatus</i>	Braconidae	1	1	0	1	1	1	Areekul et al. (2004)
<i>Trichomalopsis shirakii</i>	Pteromalidae	1	1	1	0	0	0	Mao et al. (2016)
<i>Pteromalus puparum</i>	Pteromalidae	1	1	1	0	0	0	Zhu et al. (2008)
<i>Ibalia rufipes</i>	Ibaliidae	1	1	0	0	0	0	Vårdal (2006)
10 species	Figitidae	1	1	0	0	0	0	
14 species	Cynipidae	1	1	0	0	0	0	

Note: A, number of venom gland; B, number of venom reservoir; C, number of Dufour's gland; D, the primary venom duct, 0 = no primary venom duct present, 1 = presence of primary venom duct; E, the secondary venom duct: 0 = no secondary venom duct present, 1 = presence of secondary venom duct without branching; F, the tertiary venom duct: 0 = no primary venom duct present, 1 = presence of primary venom duct.

More distribution of character states from the venom apparatus characters can be found in Fig. 1. Female adult of 4 egg parasitoids and venom apparatus. (A) *Anastatus japonicus*. (B) *Anastatus fulloi*. (C) *Mesocomys trabalae*. (D) *Mesocomys albitarsis*. (E) Venom apparatus of *Anastatus*. (F) Venom apparatus of *Mesocomys*. Vg, venom gland; Dg, Dufour's gland; Vr, venom reservoir.

In previous investigations, we identified the venom protein composition of 2 Eupelmid parasitoids, *Anastatus japonicus* and *Mesocomys trabalae* (Zhao et al., 2023). However, the development of their venom apparatus remained unobserved and unanalysed. In this study, we investigated the developmental dynamics of the venom apparatus, including the species *Anastatus fulloi* and *Mesocomys albitarsis*. A comprehensive anatomical exploration of the venom glands of females of the 4 eupelmid egg species was conducted. Hypothesizing that the developmental changes in parasitoids might influence their physiological functionalities, we carefully documented variations in the dimensions of the venom apparatus across different developmental stages. Specifically, the length and width of the venom glands were precisely measured at various age intervals, providing insights into the dynamic growth patterns and potential functional implications during their life cycle. This research sheds light on the link between venom gland development and potential variations in venom characteristics in Eupelmid egg parasitoids.

## Material and methods

### Insect rearing

The egg parasitoids, including *A. japonicus*, *A. fulloi*, *M. trabalae* and *M. albitarsis*, were initially collected from fields in the Kang County, Gansu Province (105–106° E, 32.9–33.7° N) in August 2018. They were bred at the Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University. For sustenance, the parasitoids were provided with the eggs of *Antheraea pernyi* as an alternative host. The incubation conditions were maintained in a temperature-controlled incubator set at 25 ± 5°C with a photoperiod of 14 h of light and 10 h of dark and a humidity level of 70 ± 5%. The duration of incubation lasted between 26 and 28 days. Post-emergence, adults were nourished with 30% honey water to promote ongoing growth and development.

### Venom glands dissection

After emergence, adult female parasitoids that had fully mated (paired and observed to ensure completion of mating behaviour) were allocated into 3 distinct replicates. The dissection of the venom glands was performed using ultra-fine tweezers, specifically by detaching them from the gaster under a dissection microscope (ZEISS, Oberkochen, Baden-Württemberg, Germany).

Once extracted, they were immediately immersed in a sterile 1 × Pringle's phosphate-buffered saline buffer (1 × PBS) (Biosharp, Hefei, China).

### Morphometric measurements and photographic documentation of the venom apparatus

Samples of parasitoids were collected post-eclosion in intervals of 0–1, 2–3, 4–5, 6–7 and 8–9 days. Each treatment included 20 venom apparatus. The described dissection method was employed for each sampling. Photographic documentation of the venom apparatus was executed using an ultra-depth field microscope. The built-in measurement tool of the microscope was then used to determine the dimensions of the venom glands.

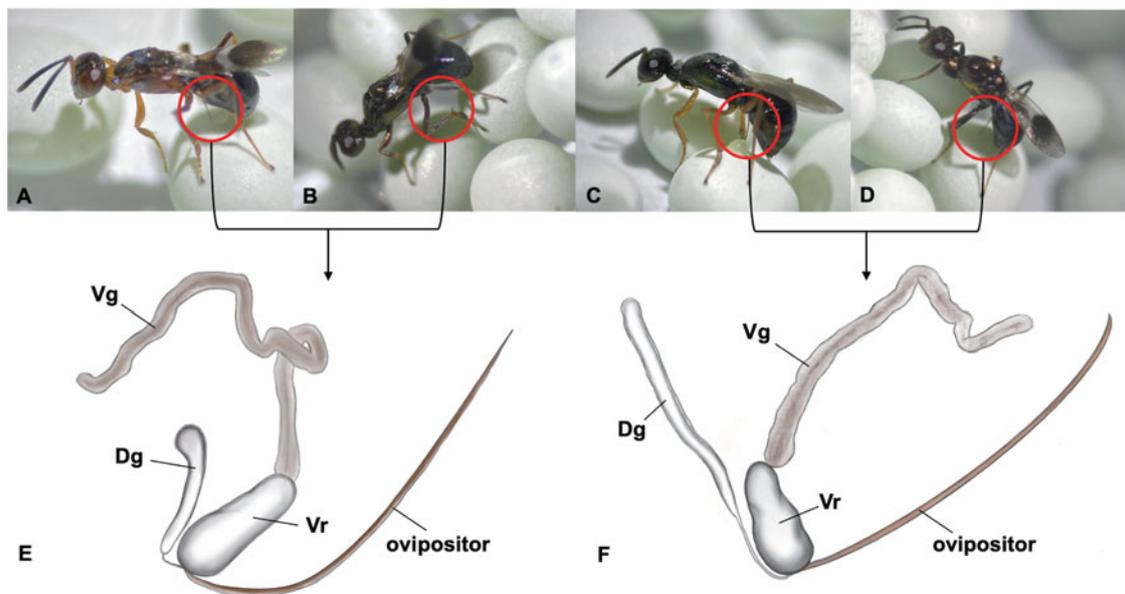
### Data compilation, statistical analysis and illustration

The measurements pertaining to the length and width of the venom glands and reservoir across different age intervals were collected. Data analysis was conducted using IBM SPSS v23.0 software. A 2-way analysis of variance (ANOVA) with corrections for multiple comparisons was used, employing Tukey's Honestly Significant Difference (HSD). Graphical representations were executed using GraphPad v9.0 software.

## Results

### Morphological comparison of venom apparatus

In this study, the venom apparatus of the 4 species of parasitoids of *Anastatus* and *Mesocomys* were found to be similar. The venom apparatus of the 4 studied species consists of a venom gland and a reservoir with an associated Dufour's gland (Fig. 1E and F). The venom reservoir, a nearly transparent cylindrical organ that tapers at the tip and widens at the base, is affixed to the base of the ovipositor, acting as a storage for the venom. Attached to the base of the venom sac is Dufour's gland, which exhibits morphological distinctions between the 2 genera. In *Anastatus*, the gland is noticeably curved, and it is slightly widened or expanded apically (Fig. 1E). Conversely, the Dufour's gland in *Mesocomys* is comparatively unexpanded apically and it has negligible curvature (Fig. 1F). The venom gland, serving as the site for venom synthesis, is attached to the end of the venom reservoir opposite that of the Dufour's gland, and is a non-branched tubular light-brown filament. The venom gland in *Anastatus* is slightly darker in



**Figure 1.** Female adult of 4 egg parasitoids and venom apparatus. (A) *Anastatus japonicus*. (B) *Anastatus fulloi*. (C) *Mesocomyx trabalae*. (D) *Mesocomyx albitarsis*. (E) Venom apparatus of *Anastatus*. (F) Venom apparatus of *Mesocomyx*. Vg, venom gland; Dg, Dufour's gland; Vr, venom reservoir.

colouration (Fig. 1E), whereas that of *Mesocomyx* is notably more transparent (Fig. 1F).

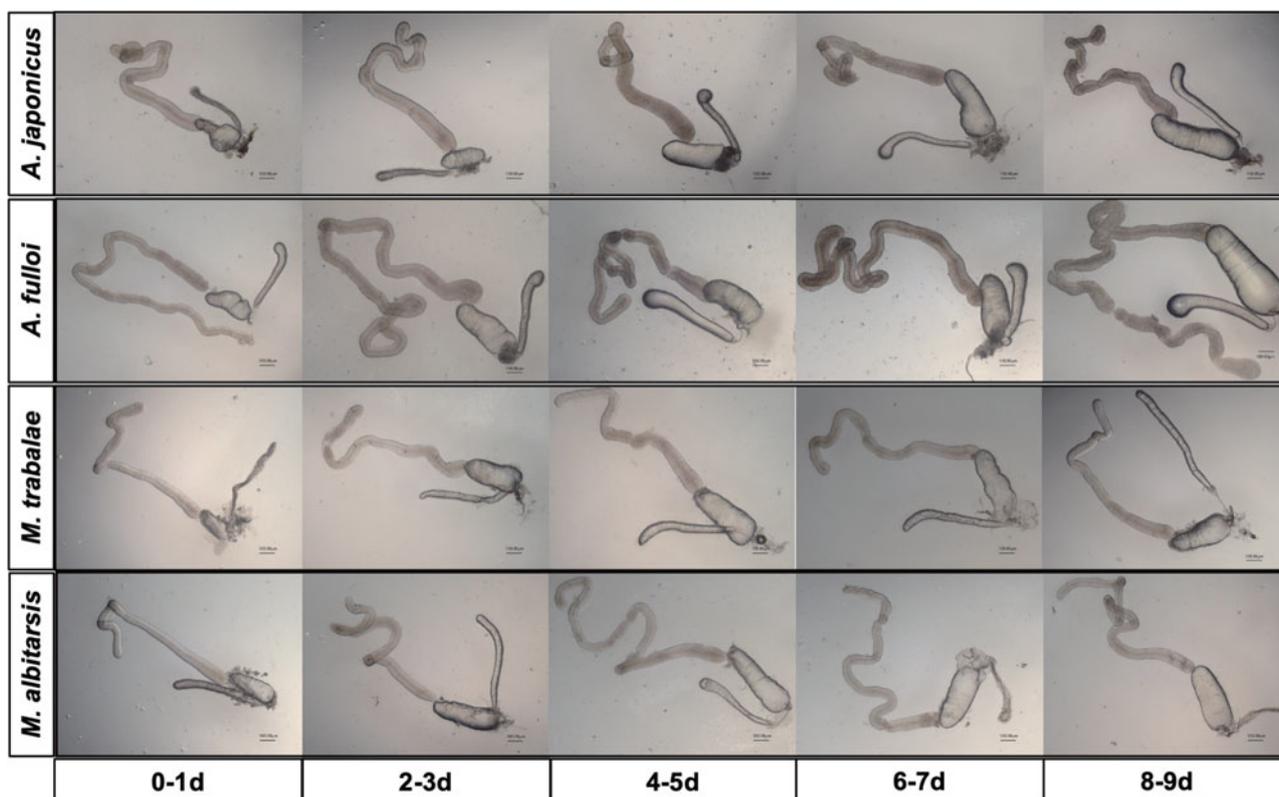
**Ontogenetic morphological variation of venom apparatus**

Morphological examination and comparison across various developmental stages were conducted to elucidate the developmental trajectory of the venom apparatus in the 4 species. Analysis of the imaging data revealed a significant enlargement of the venom apparatus in all 4 species as the length of the post-emergence increased. This growth was characterized by an elongation and

thickening of the venom gland, coupled with an expansion in the volume of both the venom reservoir and Dufour's gland (Fig. 2). In addition, differences were observed between the 2 genera. There was a significant enlargement of the venom reservoir as developmental time increased in *Anastatus*, whereas the increase was relatively modest in *Mesocomyx*.

**Temporal changes in venom apparatus dimensions**

Based on preliminary morphological assessments, it was evident that there was a temporal evolution in the dimensions of the



**Figure 2.** Ontogenetic morphological variation of venom apparatus after emergence in 4 egg parasitoids.

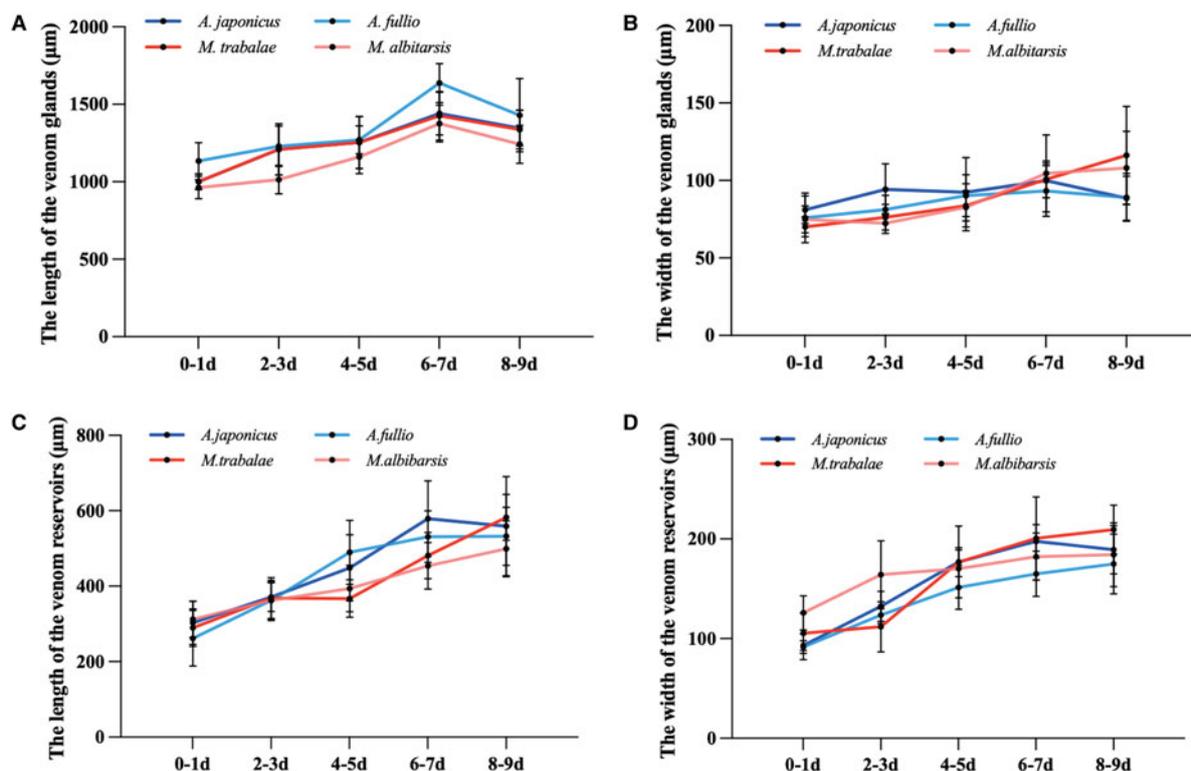
venom apparatus. To elucidate these complex morphological dynamics, we systematically quantified the ontogenetic changes in the venom reservoir and glandular structures (essential for venom biosynthesis and storage) across the 4 species. Upon post-emergence progression, there was a significant elongation in the venom glands within all 4 species (Fig. 3A). Specifically, during this 6–7 days interval, the venom gland of *A. fullio* was notably longer than for those of the other 3 species ( $P < 0.01$ ): *M. albitarsis* measured  $1376.05 \pm 309.98 \mu\text{m}$ , *M. trabala*  $1425.9 \pm 334.37 \mu\text{m}$ , *A. japonicus*  $1440.05 \pm 134.57 \mu\text{m}$  and *A. fullio*  $1636.7 \pm 364.35 \mu\text{m}$ . Throughout the developmental phase, the venom gland length of *M. albitarsis* consistently was the shortest, whereas that of *A. fullio* was the longest among the observed species (Figs 2 and 3A). Regarding the width of the venom gland, the 2 *Anastatus* species exhibited only negligible variation throughout the developmental duration. However, there was a consistent increase in the width of the venom glands in the 2 species of *Mesocomys*; notably, by the 8th–9th day, the venom glands of the 2 *Mesocomys* species surpassed those of the *Anastatus* species (Fig. 3B). With regard to the length of the venom reservoirs, during the initial 3 days post-emergence, 4 species are basically the same; while from 4 to 7 days, the 2 *Anastatus* species had greater lengths compared to the 2 *Mesocomys* species; on the 8–9 days, *M. trabala* arrived at the first place (Fig. 3C). However, starting from the 4th day onward, *Anastatus* had significantly longer venom reservoirs than for *Mesocomys*, surpassing them in length. For the venom width of the reservoir, all 4 species exhibited a progressive enlargement over time, with the most pronounced expansion occurring on the 8th–9th days post-emergence (Fig. 3D).

## Discussion

This research investigated the developmental dynamics of the venom apparatus in 4 species of Eupelmidae, *A. japonicus*, *A. fullo*, *M. trabala* and *M. albitarsis*. The venom apparatus of

these 4 parasitoid species all consists of a venom gland, a venom reservoir and a Dufour's gland. Their structure is similar to those of 2 species of Pteromalidae, the larval–pupal parasitoid *Trichomalopsis shirakii* and the pupal parasitoid *Pteromalus puparum*, with all having unbranched venom glands. In addition, all lack primary, secondary and tertiary venom ducts and the venom glands connect directly to the venom reservoir, which in turn connects directly to the ovipositor (Zhu et al., 2008; Mao et al., 2016). However, the venom reservoir of the eupelmid species does not have a distinct constriction at its centre (Fig. 2), unlike *T. shirakii* (Fig. 2 in Mao et al., 2016).

The venom gland, as the organ responsible for the synthesis and secretion of venom, plays a pivotal role in the evolution of venom in parasitic wasps. It also represents the most highly differentiated organ within the venom apparatus with different taxa. *Anastatus* the venom gland is slightly longer than in *Mesocomys*, though other morphological differences are minimal, with both being unbranched, tubular glands without any swelling at the tip. Differentiation of the venom gland is more pronounced in some other parasitic wasp families. For example, some parasitic wasps possess 2 or even more venom glands (Alves et al., 2015). Within Braconidae, some have only 1 venom gland, such as *Cotesia glomerata* and *Yelicones delicatus* (Areekul et al., 2004; Arakawa et al., 2013), whereas others, such as *Aptenobracon formicoides* and *Ecphyllus lychii*, have 2 venom glands. Each venom gland is connected to a primary duct, one end of this duct being attached to the venom sac and the other end to the ovipositor (Quicke et al., 1992). Further, some parasitic wasps have non-branching venom glands without an enlarged tip, which is characteristic of the 4 studied species as well as *C. glomerata* (Arakawa et al., 2013). Other species are known to possess branching venom glands without an enlarged apex and in certain cases the venom glands not only branch out but also have bifurcated tips (Quicke et al., 1992, 1997). Within various hymenopteran parasitoid families, analogous venom gland morphologies are evident,



**Figure 3.** Temporal changes in venom apparatus dimensions (length and width) across 4 egg parasitoids. (A) The length of the venom glands ( $\mu\text{m}$ ). (B) The width of the venom glands ( $\mu\text{m}$ ). (C) The length of the venom reservoirs ( $\mu\text{m}$ ). (D) The width of the venom reservoirs ( $\mu\text{m}$ ).

which implies potential convergent evolutionary trajectories for these structures. In the latest study by Guiguet *et al.* on the venom organs of Hymenoptera, it is true that venom organs differentiate among species with different phylogenetic relationships, and this differentiation is associated with evolution (Guiguet *et al.*, 2023).

Within parasitoids, the venom reservoir appears to be a highly conserved feature, invariably being a singular entity. However, there is variation in the configuration of the ducts connecting to the reservoir and the relative positioning vs the venom gland. In certain species, such as the 4 studied herein and in *T. shirakii* and *A. difficilis*, the venom gland attaches directly to the reservoir (Quicke *et al.*, 1997; Mao *et al.*, 2016), whereas, in some taxa both the venom reservoir and the gland converge *via* respective ducts, ultimately joining a primary duct linked to the ovipositor (Quicke *et al.*, 1997). There are also visible differences in the morphology of the venom reservoir among various parasitoid wasps. Reservoir shape ranges from droplet-like and circular, to ellipsoid and tubular, with some exhibiting central constrictions (Quicke *et al.*, 1992).

The Dufour's gland is commonly found in Hymenoptera, though in some instances it may be degenerate or absent (Robertson, 1968; Quicke, 1997). The Dufour's gland typically has a simple structure, although occasionally it appears bilobed; commonly it is connected to the ovipositor apparatus near the juncture between the reservoir and the ovipositor, but it may attach directly to the reservoir itself (Robertson, 1968). The Dufour's gland is likely instrumental in facilitating egg lubrication in the majority of symphytan species and parasitic wasps and in sting lubrication in most aculeate taxa (Bender, 1943; Bernard, 1951; Quicke, 1997). The gland has also been shown to produce alarm signals in ants and pheromones in certain ichneumonoid wasps (Guillot and Vinson, 1972; Syvertsen *et al.*, 1995). No evidence of the Dufour's gland, or any structure resembling this singular tubular gland, has been identified in any studied Cynipoidea (Vårdal, 2006). The specific function of Dufour's gland in parasitic wasps remains unclear. Consequently, its morphological development was not analysed in this study, but we intend to investigate its function in future research.

To successfully parasitize, parasitic wasps need venom to assist the normal development of their offspring, hence the venom apparatus plays a crucial role. This study also found that the venom apparatus itself requires development; in the early stages after eclosion, the size of the venom apparatus increases with age. However, upon reaching a certain equilibrium point, if the venom is not utilized, for instance, by injecting into a host to parasitize, the size of the venom apparatus will no longer increase and may even decrease. This could be because the parasitic wasp enters an energy-saving mode, as producing venom is also likely an energy-consuming process.

In conclusion, venom organ morphology and development in parasitic wasps appear to be highly conserved across species. However, our study of *Anastatus* and *Mesocomyus* reveals distinctive morphologic differences that distinguish them from many other parasitoids. These discoveries not only highlight the diversity within the parasitic wasps but also underscore the need for targeted research on specific wasp families to understand their unique behaviours and evolutionary trajectories.

**Data availability statement.** There is no other available data for this study.

**Author contributions.** L.-S. Z. conceived and designed the study. X. C. wrote the main manuscript text, carried out the experiments and prepared the figures. Q.-Y. Z. performed statistical analyses and carried out the sampling. Y.-M. C. and H. T. revised the manuscript. All authors reviewed the manuscript and approved the final version.

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**Competing interests.** None.

**Ethical standards.** Not applicable.

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