Parasitic behaviour and developmental morphology of *Anastatus japonicus* reared on the factitious host *Antheraea pernyi*

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Abstract

The egg parasitoid *Anastatus japonicus* is a key natural enemy in the biological control of various agricultural and forestry pests. It is particularly used against the brown marmorated stink bug *Halyomorpha halys* and the emerging defoliator pest *Caligula japonica* in East Asia. It has been proved that the eggs of *Antheraea pernyi* can be used as a factitious host for the mass production of *A. japonicus*. This study systematically documented the parasitic behaviour and developmental morphology exhibited by *A. japonicus* on the eggs of *A. pernyi*. The parasitic behaviour of *A. japonicus* encompassed ten steps including searching, antennation, locating, digging, probing, detecting, oviposition, host-feeding, grooming, and resting. Oviposition, in particular, was observed to occur in three stages, with the parasitoids releasing eggs during the second stage when the body remained relatively static. Among all the steps of parasitic behaviour, probing accounted for the longest time, constituting 33.1% of the whole time. It was followed by digging (19.3%), oviposition (18.5%), antennation (9.6%), detecting (7.4%), and the remaining steps, each occupying less than 5.0% of the total event time. The pre-emergence of adult *A. japonicus* involves four stages: egg (0 to 2nd day), larva (3rd to 9th day), pre-pupa (10th to 13th day), pupa (14th to 22nd day), and subsequent development into an adult. Typically, it takes 25.60 ± 0.30 days to develop from an egg to an adult at 25°C. This information increases the understanding of the biology of *A. japonicus* and may provide a reference for optimising reproductive devices.

Introduction

Anastatus japonicus Ashmead (Hymenoptera: Eupelmidae) is a key natural enemy insect in agroforestry ecosystems, which can control pests by parasitising their eggs (Cornelius *et al.*, 2016; Chen *et al.*, 2021; Zang *et al.*, 2023). This species is native to Asia and exhibits a wide host range and can parasitise the eggs of more than 15 species in two families of Hemiptera (Alydidae, Pentatomidae) and five families of Lepidoptera (Lasiocampidae, Lymantriidae, Notodontidae, Papilionidae, Saturniidae) (Noyes, 2019; Peng *et al.*, 2020). At present, it has been reported that this species is the dominant egg parasitoid for controlling the Japanese giant silkworm *Caligula japonica* Moore (Lepidoptera: Saturniidae) in East Asia, as well as a predominant egg parasitoid of the brown marmorated stink bug *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) (Jones *et al.*, 2014; Wei *et al.*, 2022).

Presently, due to the high control potential of *A. japonicus* as a parasitoid for diverse agricultural and forestry pests, it is crucial to advance the development of this parasitoid as a natural enemy product, aiming to substitute the use of chemical pesticides. In recent years, research on *A. japonicus* has gradually deepened, covering its biological characteristics, field population dynamics, and molecular biology (Meng *et al.*, 2012; Wang *et al.*, 2017; Stahl *et al.*, 2019; Zhao *et al.*, 2021). However, to develop it as a natural enemy product for pest control, it is pivotal to find alternative hosts for its mass reproduction. The research on the developmental period, host adaptability, reproductive attributes, and population structure of *A. japonicus* on the Chinese oak silkworm *Antheraea pernyi* Guérin-Méneville (Lepidoptera: Saturniidae) eggs showed that *A. japonicus* can be massively reared using *A. pernyi* eggs (Mi *et al.*, 2022; Chen *et al.*, 2022a; Mu *et al.*, 2023; Zang *et al.*, 2023). Particularly, *A. pernyi* eggs possess the characteristics of low production cost, being easy to rear, store, and transport, making them an optimal factitious host for the mass production of *A. japonicus* (Mu *et al.*, 2023).

In recent years, parasitoid products have been increasingly and widely used in agricultural and forestry production due to their high control efficiency and environmentally friendly properties (Aguirre *et al.*, 2019; Andorno *et al.*, 2022; Davidson *et al.*, 2023). For example,

the egg parasitoid *Anastatus fulloi* Sheng & Wang (Hymenoptera: Eupelmidae) has been used to control the litchi stink bug *Tessaratoma papillosa* Stål (Hemiptera: Tessaratomidae) (Li *et al.*, 2014), *Chouioia cunea* Yang (Hymenoptera: Eulophidae) has been used to control invasion pest *Hyphantria cunea* Dury (Lepidoptera: Arctiidae) (Yang *et al.*, 2006), and the egg parasitoid trichogrammatids have been used to control many species of Lepidopteran pests (Zang *et al.*, 2021). Therefore, large-scale artificial breeding techniques are needed to produce a greater quantity of high-quality natural enemy insects. Indeed, researchers have conducted more comprehensive investigations into the methodologies for large-scale artificial rearing of parasitoids (Mi *et al.*, 2022; Wei *et al.*, 2022; Chen *et al.*, 2022a). Observing the parasitic behaviour of wasps on the host could better evaluate their reproductive potential, improve their utilisation, and have vital reference value for the mass rearing of parasitoids (Strand, 1989; Wang and Zhou, 1996; Mehrnejad and Copland, 2006; Drost *et al.*, 2010). Up to now, there have been numerous reports on the observation of the parasitic behaviour of parasitoids, such as *Eupelmus messene* Walker (Hymenoptera: Eupelmidae), *E. microzonus* Frster (Hymenoptera: Eupelmidae), and *Mesocomys albitarsis* Ashmead (Hymenoptera: Eupelmidae) (Clausen, 1927; Gokhman and Nikelshparg, 2021). However, the parasitic behaviour of *A. japonicus* on *A. pernyi* eggs has not been documented. Furthermore, as the immature stage of *A. japonicus* within the host egg, there are also no detailed reports on its ontogenetic morphology. This limitation hinders a comprehensive understanding of the developmental biology of *A. japonicus*.

In the present study, the parasitic behaviour and the developmental morphology of *A. japonicus* on *A. pernyi* eggs were documented, to enhance the understanding of the biology of *A. japonicus* and the research results also provide information for developing devices with high reproductive efficiency.

Materials and methods

Parasitoid and host insects

Laboratory colonies of *A. japonicus* (GenBank Accession No. MK604240) were collected from the overwintering eggs of *C. japonica* in walnut orchards in Kang County (33°26′-33.81″N, 105°41′-52.10″E), Gansu Province, China in 2022 (Chen *et al.*, 2019, 2022b). The identification process involved a combination of both molecular (cytochrome oxidase subunit I gene, COI) and morphological characteristics (Chen *et al.*, 2019). This species was reared in the Key Laboratory of Green Pesticide and Agricultural Bioengineering of the Ministry of Education, Guizhou University, China.

The parasitoid was maintained on *A. pernyi* eggs. To obtain host eggs, *A. pernyi* cocoons were collected from fields in Yongji City, China, in the late fall of each year. The cocoons were stored at 4°C until late February and then were transferred to the emerging rooms under suitable conditions (25 ± 1°C, 60 ± 10% humidity, and photoperiod 14L: 10D) for adults' emergence (Chen *et al.*, 2022b). The host eggs were collected by squeezing the abdomen of newly emerged and virgin female moths (i.e., unfertilised eggs). Before use, the host eggs were cleaned with distilled water and air-dried (immature green eggs were removed). To raise the parasitoid colony, host eggs were exposed to individual females for 24 h in a cylindrical transparent plastic jar (diameter × height, 9.0 × 14.0 cm) covered with fine mesh. The parasitized eggs were incubated in a light and constant temperature incubator (MEMMERT HPP260) at 25 ± 5°C, 70 ± 5% humidity, and photoperiod 14L:10D, facilitating parasitoids development into the adult stage and subsequent emergence. Ten pairs of newly emerged (<6 h) wasps were introduced into each rearing jar to ensure their mating and were kept in the incubator as mentioned above. All the wasps were fed with 30% honey solution to ensure their continued growth and development after emergence.

Parasitoid species were reared for five generations on the *A. pernyi* eggs before they were used in this study. In all experiments, 3-day-old and mated female wasps were used.

Observation of parasitic behaviour of *A. japonicus* on *A. pernyi* egg

The parasitic behaviour of *A. japonicus* on host eggs was captured on camera using a video-recording device (TP-LINK-NVR6104C-B, China). A female adult *A. japonicus* was introduced into a transparent petri dish (3 cm in diameter), and an egg card with 8 *A. pernyi* eggs was arranged vertically and fixed at the centre of the petri dish with non-toxic glue (ChenGuang Liquid Glue, M&G Chenguang Stationery co., Ltd. Shanghai, China). The oviposition behaviour of *A. japonicus* was observed for two hours on *A. pernyi* eggs in each petri dish.

Parasitic behaviour was carefully observed and documented through continuous replay of recorded videos. Various parameters, such as the specific steps involved in parasitism, the distinct characteristics, and the duration of each step were meticulously recorded. We determined whether the wasp successfully parasitised the host eggs by observing the parasitic behaviour, and verified our judgment by dissecting host eggs after observing the parasitic behaviours of wasps. In total, 100 parasitic behaviour events were observed, which occurred in 38 parasitoids (no more than three parasitic behaviour events per parasitoid were observed).

We also recorded the sequence and frequency of each behaviour exhibited by the parasitoids after they encountered and mounted the egg card in every observation. The observation process was repeated for a total of 30 replicates (30 females were tested on 30 egg cards), and each record observed the continuous parasitic behaviour of the parasitoid on the first three eggs ($n = 90$).

Morphology of different developmental stages of *A. japonicus* inside the *A. pernyi* eggs

Ten pairs of newly emerged (<6 h) *A. japonicus* adults were randomly selected and were transferred into a rearing jar (diameter: 9.0 cm, height: 14.0 cm) for full mating. After three days of being fed with 30% honey solution, the parasitoids were provided with 300 eggs (i.e., in excess amount) that were extracted from unmated female moths of *A. pernyi* and were cleaned as mentioned above. After 4 h, the wasps were removed (to make the development status of the wasp as consistent as possible), and the parasitised eggs (the surface of host eggs parasitised by parasitoids usually has traces of egg fluid overflow, which can be used to screen for parasitised eggs) were kept in the environmental condition of 25 ± 1°C, 70 ± 5% RH, L14: D10 hours photoperiod. Finally, dissect 15 parasitised eggs under a stereomicroscope at the same time every day, and record the developmental time and status of various stages (i.e., egg stage, larval stage, pre-pupal stage, pupal stage) of *A. japonicus*, until adult emergence.

Data analysis

An ethogram was created to show the observed order and frequency of parasitic steps in *A. japonicus* parasitising *A. pernyi* eggs, and the transition percentage of each step that originates from the previous step is also displayed (Transition percentage = frequency of each step originating from the previous step/total frequency of previous step × 100%). In addition, Chi-square tests were used to further determine whether these behavioural transitions were random or statistically significant. Box plots of the durations of the three stages of the parasitoid oviposition step were generated using GraphPad Prism 9.3, and the Kruskal–Wallis test was used to compare the differences in the three stages. The mean time (±SE) spent by the parasitoids in each step of parasitism, the mean development time (±SE) of parasitoids at different stages, and the mean detection numbers (±SE) of parasitoids in parasitism were obtained by descriptive analysis. The duration difference between each parasitic step was compared using the Kruskal–Wallis one-way ANOVA on ranks. The difference between the time spent by the parasitoids in the first and secondary probing of the host egg was determined using a *t*-test. By analysing the average time allocated to each step of parasitism, we determined which step consumes the most time in the parasitic process. All data analyses were conducted using Office Excel 2019 and SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Parasitic behaviour of A. japonicus on A. pernyi egg

The entire process of parasitic behaviour of *A. japonicus* from host searching to oviposition completion was divided into the following ten steps. Because some of the steps of the parasitic behaviour cannot be recorded in a static image, fig. 1 shows only a part of those steps. Detailed parasitic actions are recorded as GIFs images, as shown in fig. S1.

(1) Searching. When the female parasitoid was introduced into the petri dish, the wasp walked and tapped the substrate with its antennae to search for host eggs (fig. S1A).
(2) Antennation. The female parasitoid climbed onto the host egg, crawled in the forwards direction, constantly moved the antennae up and down, palpated the host egg, and immediately turned and crawled forwards when touched the substrate, thus palpating most of the exposed surface of the host egg. (fig. 1a; fig. S1B).
(3) Locating. Antennation stopped; the female parasitoid lowered its abdomen and gently explored a small area on the surface of the host egg using the tip of its abdomen (i.e., the end of the acidotheca) to determine the oviposition site. After locating the oviposition site on the surface of the egg, the parasitoid slightly lifted the abdomen but kept the ovipositor in contact with the host eggshell, the ovipositor was withdrawn from its sheath, and then preceded to the digging step. (fig. 1b; fig. S1C).
(4) Digging. The end of the parasitoid's abdomen was gently shaken, and the tip of the ovipositor dug the size of the opening of the oviposition hole on the host surface (fig. S1D).
(5) Probing. The parasitoid's body trembled significantly, and the ovipositor was pressed down vertically to pierce the eggshell (successful probe when the body stopped trembling

significantly and the abdominal end began to sway left and right) (fig. 1c; fig. S1E).
(6) Detecting. The ovipositor penetrated the eggshell but was not fully inserted. The parasitoid twisted its abdomen to press down the ovipositor into the eggshell or inserted the end of the ovipositor into the eggshell, and paused for a moment, then pulled out the ovipositor (fig. S1F).
(7) Oviposition. Oviposition occurred in three stages (fig. S1G). (a) Abdominal torsion. The twisting of the abdomen presses all the ovipositor into the host egg, and the ovipositor then moves up and down or stirs in the host egg, sometimes even appearing to rotate their bodies, using the ovipositor as an axis (fig. S2a); (b) The body in a stationary state. The parasitoid stopped abdominal twisting, at this time the ovipositor was fully inserted into the host egg, occasionally with a small part exposed, and all parts of the wasp's body remained motionless (fig. 1d; fig. S2b); (c) Finishing stage of oviposition. Following the stationary state, the parasitoid pulled out most of its ovipositor without disengaging from the surface of the host egg. After lingering for a while, the ovipositor was completely pulled out from the host egg (fig. S2c).
(8) Host-feeding. After the parasitoids pulled the ovipositor out of the host egg, the host egg often spilled the egg fluid, and the parasitoids usually feed on the egg fluid of the host (fig. 1e, f; fig. S1H).
(9) Resting. When a suitable resting place was found, the parasitoids stopped moving and kept their bodies relatively stationary (fig. S1I).
(10) Grooming. Any actions involved in cleaning the body, e.g., the wasp brushed its antennae and face with the fore-legs, brushed its thorax with the mid-legs, brushed the abdomen, ovipositor, and wings with the hind-legs, or rubbed the legs together (fig. S1J).

After we observed and recorded the parasitic behaviour of *A. japonicus*, we immediately dissected the host egg to determine whether the parasitoid had laid eggs. Among the 100 observed parasitic behaviour events, after dissection, we found that parasitoids usually laid one egg (occurred 68 times, 68%) during a single oviposition process, but sometimes they also laid two (occurred 31 times, 31%) or even three (occurred 1 time, 1%) eggs. To determine in which step the parasitoids laid the egg, we performed another validation experiment by promptly removing the parasitoids from the host eggs using tweezers immediately after each step and dissecting the host eggs immediately to determine if they released the egg. Finally, we found that after the second stage (body stationery) of the oviposition step, dissecting the host egg can be observed in the presence of eggs of *A. japonicus*. In addition, we also found that when parasitoids lay two or three eggs, the stationary state during the oviposition process also appeared two or three times. Therefore, we determined that the parasitoid releases eggs during its stationary body state. By observing the parasitic behaviour of *A. japonicus* on host eggs and dissecting host eggs, it was determined that the accuracy rate of determining whether a wasp had parasitised a host egg based on the observed behaviour was 99%.

Ethogram construction

Female parasitoids started their behavioural pathway by searching (S), which could be interrupted by several events including

Figure 1. The image of part parasitic behaviour events in *Anastatus japonicus* parasitising *Antheraea pernyi* egg. (a) antennation, (b) locating, (c) probing, (d) oviposition, (e) start host-feeding, (f) end host-feeding. Scale bars: 1 mm.

grooming (G), resting (R), or antennation (A) (fig. 2). Among 117 searching events, 100 events were interrupted when the parasitoids found host eggs which led to antennation (A) (85.47%). Among 192 antennation events, 106 resulted in locating (L) (55.21%). Occasionally, the location would be unsuccessful. At this point, the parasitoid withdrew its acidotheca and ovipositor, turned around, tapped with its antennae, searched for another appropriate oviposition site on the surface of the host egg, and then located it again. This event occurred eight times in 258 locating events (3.10%). In 258 locating events, 94 progressed to the next step of parasitism, which was digging (D) (36.43%). However, digging occasionally failed. At this point, the parasitoid lowered its abdomen again, relocated the oviposition site with its acidotheca, and then proceeded with the digging step again. This situation occurred four times during the 94 digging processes (4.26%). Digging occurred only once in the process of parasitism, after digging the parasitoid started to probe (P) (95.74%). When the eggshell was penetrated, parasitoids sometimes immediately laid eggs, while in other cases they underwent multiple detection processes before laying eggs. Host feeding or antennation was also observed (fig. 2) immediately after probing rather than oviposition, which was in fact a detection process (to simplify the frequency diagram, the process of detection could be viewed as the process from locating to probing to host-feeding or antennation). Out of 246 probing events, 161 resulted in detection (65.45%).

After detecting the host egg, the parasitoid pulled out the ovipositor and usually fed (F) on the spilled egg fluid, which occurred 153 times out of 161 detection events (95.03%). Through detection, if the parasitoid wasp determined that the host egg was suitable for oviposition, the next oviposition step would be carried out. Among the 246 probing events, 85 (34.55%) resulted in oviposition (O). In most cases, host-feeding occurred after oviposition was completed. Of the 85 instances of oviposition, host-feeding occurred in 68 cases (80.00%). Generally, after successful parasitism of a host egg, the parasitoids departed and moved to the surrounding eggs to prepare for the next round of oviposition.

During the process of parasitism, parasitoids occasionally engaged in activities such as grooming and resting. These behaviours occurred both during the actual act of parasitism and after the parasitism process was completed. Among 117 searching events, 16 instances led to grooming (G) (13.68%). Among the 30 grooming events, seven events led to subsequent resting (R) (23.33%) (fig. 2).

Time budget construction

We defined the complete parasitic process as starting from the parasitoid searching for a host egg until it finished the oviposition and departed from the host egg. We observed and recorded the
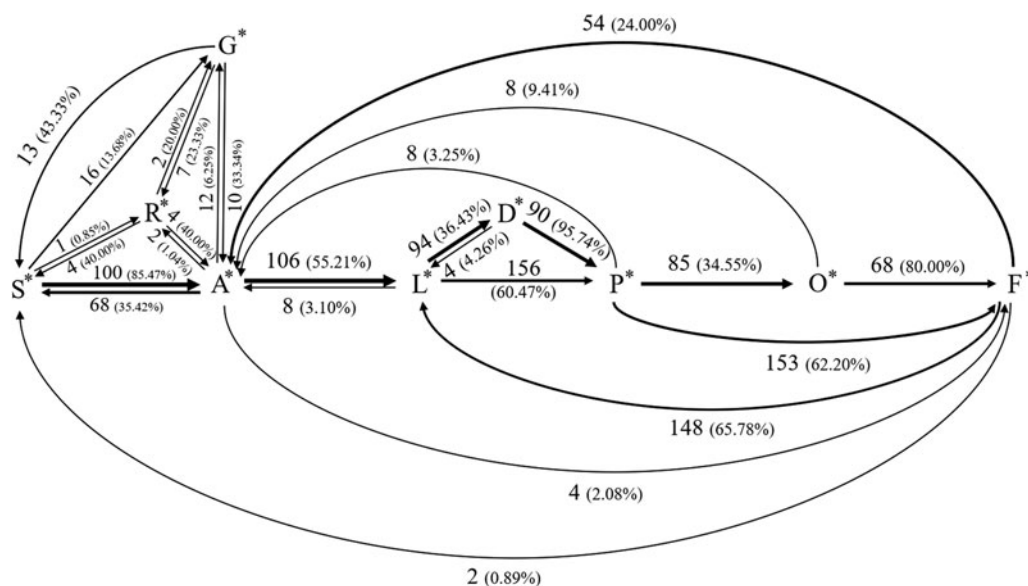
Figure 2. Ethogram of *Anastatus japonicus* parasitising *Antheraea pernyi* eggs (*n* = 90 parasitic events). Complete behavioural steps include searching (S), antennation (A), locating (L), digging (D), probing (P), oviposition (O), host-feeding (F), grooming (G), and resting (R). The arrow indicates the transition between two consecutive steps and the arrow's width is proportional to the transition frequency. The figures next to the arrow indicate the transition frequency for given steps, and the percentages indicate the transition percentage for given steps. Asterisks (*) indicate significant transitions (χ^2 tests, $P < 0.05$).

frequency and duration of each step of parasitic behaviour in a total of 68 instances (here, the parasitic behaviour of only laying one egg was used for data analysis).

Probing by *A. japonicus* occupied most of the time budget (33.08%), next was digging, oviposition, antennation, and detecting (accounted for 19.29, 18.50, 9.59, and 7.43% respectively), while host-feeding, locating, searching, resting, and grooming events, all occupied less than 5.0% each of the total time in events (4.52, 3.26, 2.09, 1.54, and 0.70%, respectively) (table 1).

The oviposition process was subdivided into three stages. During the stage of abdominal torsion, the ovipositor of the parasitoid was fully inserted into the host eggs and moved up and down. This movement was sometimes accompanied by body rotation. (fig. S2a). The average time spent on the abdominal torsion stage was 70.43 ± 11.35 seconds (*n* = 90), but due to individual differences in parasitoids, the time required to complete this stage varies, some parasitoids spent about 16 seconds which was enough, and some parasitoids needed to spend more than 400 seconds (fig. 3). Through dissection, it was confirmed that the parasitoid released the egg during the second stage of the oviposition step when the parasitoid's body was in a stationary state (fig. S2b). It took parasitoids 49.62 ± 2.23 seconds on average (*n* = 90) to release eggs. The shortest and longest durations recorded were approximately 18 and 110 s, respectively (fig. 3). The final stage of the oviposition step (fig. S2c) took time ranging from 23 to 210 s, with an average time of 96.93 ± 4.15 seconds (*n* = 90) (fig. 3).

Morphology of *A. japonicus* at different development stages in *A. pernyi* egg

The entire process of *A. japonicus* from egg laying to adult emergence occurs within the host egg. In an environment with a temperature of 25 ± 1°C, relative humidity of 70 ± 5%, and L14: D10 hours, its developmental period was 25.60 ± 0.30 days (*n* = 15). The embryonic development usually took about 2.85 ± 0.23 days

(*n* = 15), the larval stage 7.33 ± 0.11 days (*n* = 15), the pre-pupal stage about 4.82 ± 0.40 days (*n* = 15), and the pupal stage took about 10.39 ± 0.82 days (*n* = 15), and then developed into an adult and emerged.

Egg stage

The egg of the *A. japonicus* was long oval shaped and had a transparent egg membrane; the egg length was about 2.5–3 times as the egg width. One end of the egg had a solid conical protrusion, and the other end had a long tubular egg stalk, about 1/3 of the end of the egg stalk was noticeably thick (fig. 4, Day 0–2).

Larval stage

When the egg was laid about 3 days later, it developed into a larva. In the first two days of the larval stage, the larva had a 13-segmented spindle-shaped body, including three segments on the thorax and ten segments on the abdomen (including the anal segment), narrow and long, the front end was wide, and the rear end was pointed. The head was blunt and triangular, with mouth hooks, and the abdomen had a sharp and long fork at the end. The first segment of the larval thorax was smooth and hairless, and from the second segment of the larval thorax to the larval abdomen it was covered with long hairs. Actively, fed on the host ooplasm, and was moving freely within the host egg (fig. 4, Day 3–4).

When the larvae were on the third day of the larval stage, the first abdominal segment of the larvae became large, so that the middle part of the insect body was large, and the two ends were pointed. The 1st to 2nd thoracic segments of the larvae were smooth and hairless. From the third thoracic segment to the end of the abdomen, each segment still carried dense hairs, and there was still a tail fork at the end of the abdomen, but it was relatively thick and short. Larval activity slows down. (fig. 4, Day 5).

As the larvae developed into the fourth to seventh days of the larval stage, they had a robust body and were longer than the host

Table 1. The frequency and duration of all parasitic behaviour events of *Anastatus japonicus* parasitising *Antheraea pernyi* eggs (*N* = 68 parasitic events)

Behaviour	Frequency	Proportion of total events (%)	Total time in behaviour (s)	Mean duration ± SE (s)*	Proportion of total time (%)
Searching	64	6.50	1723	26.92 ± 4.21 de	2.09
Antennation	148	15.03	8043	54.34 ± 3.36 c	9.59
Locating	186	18.88	2703	14.53 ± 1.01 e	3.26
Digging	68	6.90	15,326	225.38 ± 9.27 a	19.29
Probing	172	17.46	28,542	165.94 ± 14.75 b	33.08
Detecting	102	10.36	7726	75.75 ± 4.41 bc	7.43
Oviposition	68	6.90	14,696	216.12 ± 12.64 a	18.50
Host-feeding	148	15.03	4495	30.37 ± 1.30 cd	4.52
Resting	7	0.71	1310	187.14 ± 71.82 ab	1.54
Grooming	22	2.23	575	26.14 ± 4.42 de	0.70

*Values represent the average time it takes to perform each behaviour once, while SE indicates the standard error of the data. Additionally, different lowercase letters following the values indicate significant differences in duration between each step (Kruskal–Wallis test, $H = 535.01$, $df = 9$, $P < 0.05$).

egg, so the larval body curved towards the abdomen along the curvature of the host egg. The head of the larva was small, slightly retracted into the thorax, circular, and smooth. The surface of each segment of the larva was hairless, and the end of the abdomen was blunt and round, without a tail fork. (fig. 4, Day 6–9).

Pre-pupal stage

After 7 days of larvae development, entered the pre-pupal stage. Its body shortens into a sac-shaped shape, with a large abdomen, and a sharp end. The insect's body was pale green, and then gradually became milky white. About 13 days after eggs were laid; insect organs begin to form gradually. First, the body wall appeared to have obvious depression, and then gradually formed

the foot bud, wing bud, external reproductive bud, antennal bud, and compound eye prototype (fig. 5, Day 10–13).

After the beginning of the pre-pupa stage, a new body wall formed under the larval body wall, and the original larval body wall became a semitransparent film and separated from the larval body to form a pre-pupa envelope.

Pupal stage

The *A. japonicus* pre-pupa entered the pupal stage about 14 days after the eggs were laid. In the early stage of pupa, the insect body gradually elongated, with clear boundaries between the head, thorax, and abdomen, as well as clear boundaries between the thoracic and abdominal internodes. As the pupa continued to develop, compound eyes formed and the colour gradually deepened from light brown, the antennae were segmented obviously, and the mouthparts of the adult insect began to form. The foot bud continued to expand, and the wing bud continued to develop in the wing envelope, folded up, and spread into a thin film at emergence. The abdominal ovipositor gradually formed and elongated, and the body colour of the pupa changed from brown-yellow to black (figs 5 and 6, Day 14–22).

Adult stage

About 23 days after eggs were laid; the mature pupa of *A. japonicus* was fully developed into a wasp-like shape (fig. 6, Day 23). Approximately one day later, the adult parasitoid emerged by chewing through the chorion of the host egg.

Discussion

This study provides a detailed observation and description of the ten steps involved in the parasitism behaviour of *A. japonicus* on the eggs of *A. pernyi*. The development of the wasp on the eggs of *A. pernyi* was also photographed at different stages. Since the parasitic behaviour of parasitoids is directly related to the implementation and effectiveness of key technologies such as indoor scale reproduction, natural population reproduction and dispersal in the field, studying the parasitic behaviour of *A. japonicus* on host eggs and understanding their developmental characteristics can provide valuable information for improving their reproduction and utilisation, and enhancing their control effect on hosts.
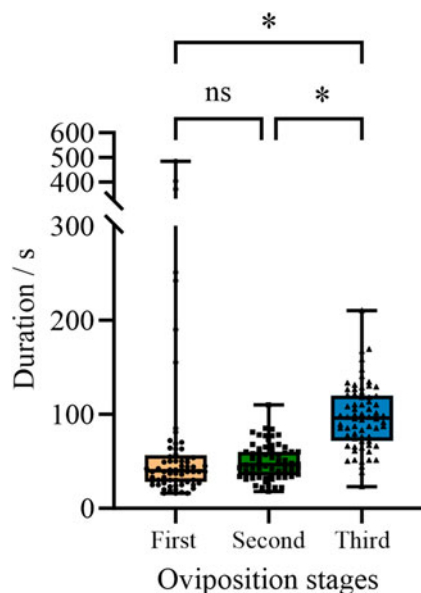

Figure 3. The duration of three stages of oviposition by *Anastatus japonicus*. First stage: abdominal torsion; Second stage: body stationary; Third stage: the finishing stage of oviposition (*n* = 68 ovipositional events). Boxplot depicts the median and upper and lower quartiles; dots represent individual data. Asterisks (*) indicate significant differences in durations between different stages of oviposition (Kruskal–Wallis test, $P < 0.05$).
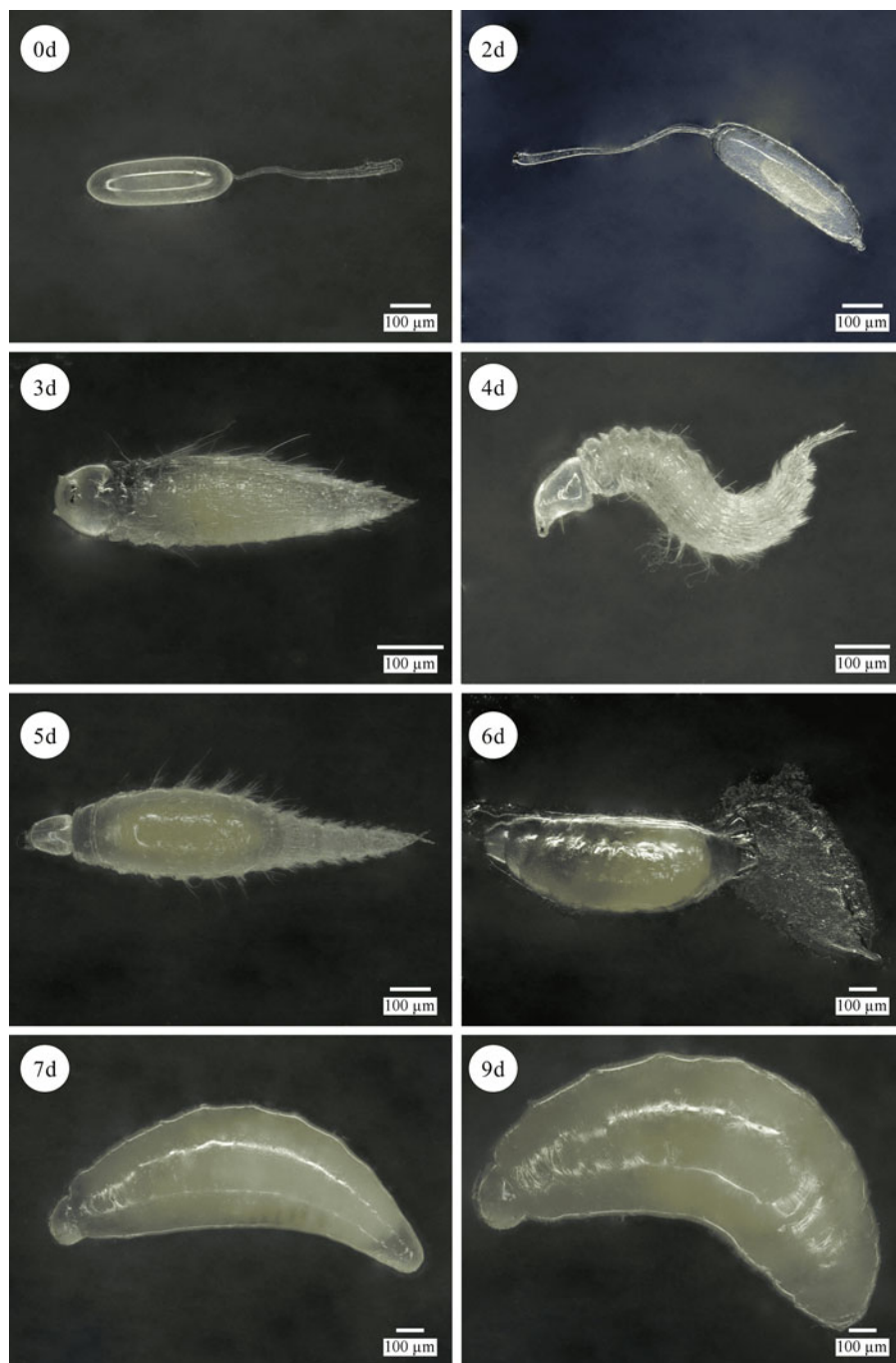
Figure 4. The developmental morphology of *A. japonicus* in egg stage and larval stage. Day 0–2, Egg stage; Day 3–9, Larval stage.

This is of great significance for the effective use of *A. japonicus* in biological control.

Although there is no report regarding the oviposition behaviour of *A. japonicus* on *A. pernyi* eggs, its oviposition characteristics on artificial eggs have been reported from an inside view (Xing and Li, 1989). Xing and Li (1989) reported that after random encounters with artificial eggs, *A. japonicus* found a suitable position to prick the ovipositor. Through the transparent artificial eggshell, it could be observed that first, it penetrated 1/3 of the ovipositor into the artificial egg, and then swayed the end of the abdomen along with the ovipositor to press the entire ovipositor. If the artificial egg was suitable, the female wasp laid eggs by first expelling the transparent end of the egg from the apex of the

ovipositor, then squeezing out the part of the egg containing the yellow protoplasm, and finally expelling the egg stalk. By observing the behavioural characteristics of parasitoids laying eggs outside and inside the host egg, we now have a more comprehensive and detailed understanding of the parasitic behaviour of *A. japonicus* on host eggs.

The oviposition behaviours of the egg parasitoid *M. albitarsis* on the eggs of *C. japonica* are similar to the wasp being studied. Both wasps follow a similar sequence of oviposition steps, including searching, antennation, locating the oviposition site, probing, detecting, oviposition, and host-feeding (Clausen, 1927). As the previous paper only provided a general description of the oviposition behaviour of *M. albitarsis*, it was not possible to make a
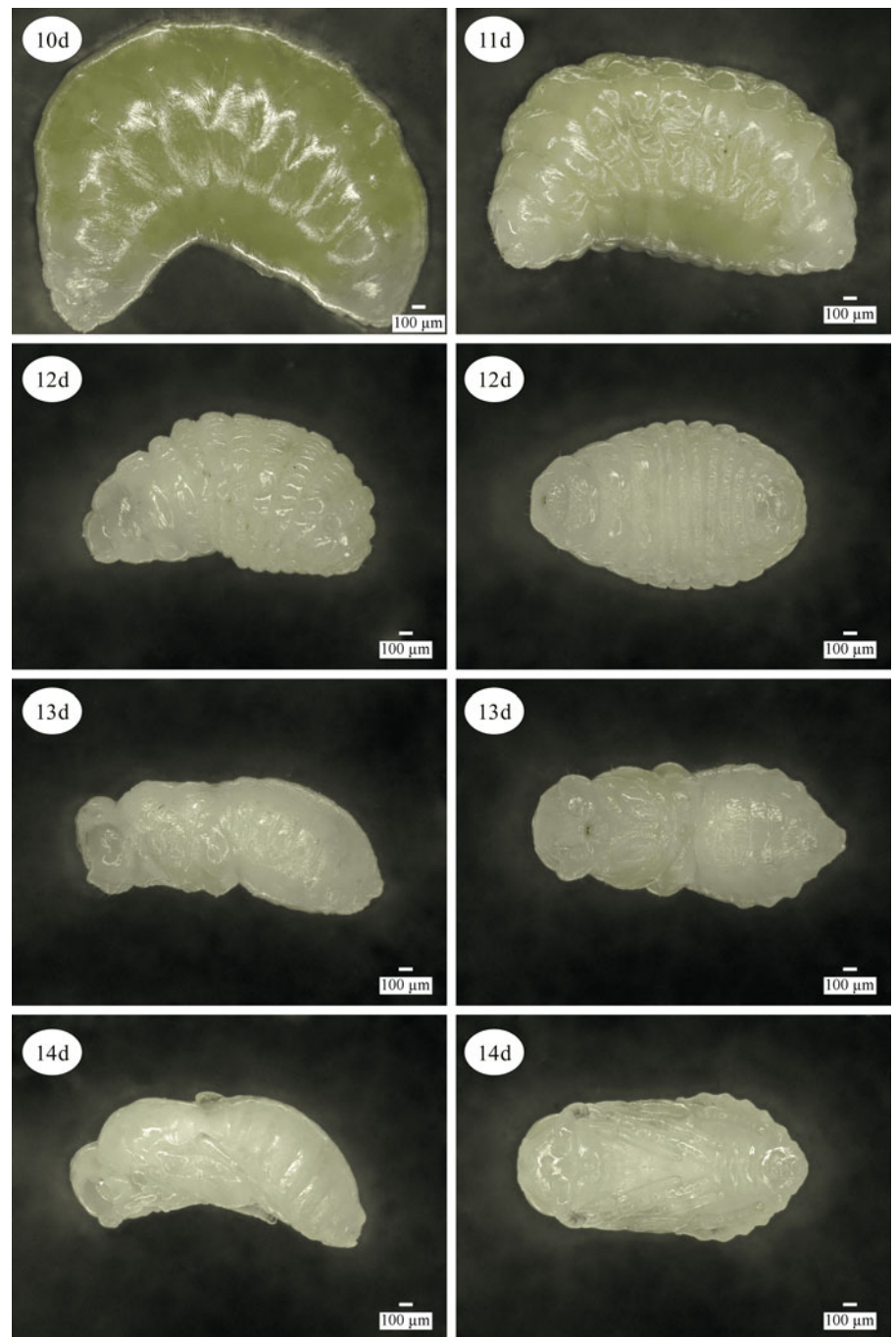
Figure 5. The developmental morphology of *A. japonicus* in the pre-pupal stage and pupal stage. Day 10–13, Pre-pupal stage; Day 14, Pupal stage.

detailed comparison of the behavioural characteristics. Therefore, this paper aims to provide a more comprehensive analysis of the behavioural details of *A. japonicus*.

During parasitism in *A. japonicus*, when the eggshell was penetrated (after the first probing), parasitoids sometimes immediately oviposited (48.00%), and sometimes went through multiple detection processes (at least two times, maximum nine times, with an average of 4.10 ± 0.27 times) before deposited eggs (52.00%). The time spent by *A. japonicus* in successful parasitism of the host egg was between 10 and 40 min, and the number of detections had the biggest impact on how long it took time. In addition, the detection step can only be performed after the ovipositor pierces the eggshell, with multiple probes accompanied

by multiple detections. In fact, the first probing took the longest time, with an average duration of 369.50 ± 19.14 s to puncture the shell. The subsequent probes occurred when the eggshell had already been pierced, and the time taken for probing was significantly reduced ($t = 21.34$; $df = 170$; $P < 0.001$), averaging 32.55 ± 22.63 s. The time parasitoids took to release eggs in the second stage of the oviposition step varied from a minimum of 18 s to a maximum of 110 s. This is related to individual differences in parasitoids, but may also be affected by the state of the parasitoids (e.g., nutritional state, oviposition posture, etc.), and the suitability of the host eggs. In addition, it has been reported that an increase in the temperature of the external environment can accelerate the laying of eggs by insects (Hans *et al.*, 2019).

Figure 6. The developmental morphology of *A. japonicus* in the pupal stage and adult stage. Day 14–22, Pupal stage; Day 23, Adult stage.

Nowadays, there have been reports on the developmental morphology of parasitoids in the genus *Mesocomys* and *Eupelmus* in the family Eupelmidae (Clausen, 1927; Berg, 1970; Gokhman and Nikelshparg, 2021; Nikelshparg *et al.*, 2023). The ontogeny of parasitoids in three genera of Eupelmidae is almost consistent, they all go through the egg stage, larval stage, prepupal stage, pupal stage, and adult stage. The egg morphology of the three genera parasitoids was long ovoid, with a conical protrusion at the back and an egg stalk at the front, but the length of the egg stalk varied (Berg, 1970; Lu and Yang, 1983; Gokhman and Nikelshparg, 2021). The egg stalk of the wasp of genus *Mesocomys* was short, accounting for about one-third to half of the egg length (Clausen, 1927, *cf.* fig. 2; Berg, 1970, *cf.* fig. 1; Berg, 1971). The egg stalk of the wasp of the genus *Eupelmus*

was slightly longer, almost equal to the egg length (Gokhman and Nikelshparg, 2021, *cf.* fig. 3a; Nikelshparg *et al.*, 2023, *cf.* fig. 1B). The egg stalk of the wasp of the genus *Anastatus* was the longest, about 1.5 times the egg length (Xing and Li, 1989). The larvae of all three genera of parasitoids have a 13-segmented spindle-shaped body, initially covered with long hairs; the body of the larva gradually bends and shortens into a sac-shaped as the larva grows larger by feeding on the egg fluid of the host. The antennal sheaths of *Mesocomys* parasitoids are prominent during the prepupal and pupal stages, but absent in the developmental stages of parasitoids from the genera *Anastatus* and *Eupelmus*. Apart from this difference, the morphological characteristics of the prepupal and pupal stages are essentially similar among the three genera. In pre-pupal and pupal stages, the bodies of the

parasitoid in three genera are stationary and deepening in colour, and all adult body organs are gradually formed. Eventually, the pupa of the parasitoids developed into an adult and emerged (Clausen, 1927; Lu and Yang, 1983; Gómez and Nieves-Aldrey, 2017; Gokhman and Nikelshparg, 2021).

In addition, our research may help improve rearing methods and even contribute to the development of efficient breeding equipment for *A. japonicus*. Through the observation of oviposition behaviour, we found that *A. japonicus* usually feeds on the host egg overflow to replenish its energy after the step of detecting and oviposition. Indeed, when breeding parasitoids, supplying 30% honey solution could help parasitoids replenish energy and promote egg-laying (Mu *et al.*, 2023). In the process of large-scale production of *A. japonicus*, egg cards are usually used to provide host eggs to wasps for parasitism. After the host egg is parasitised, screening equipment is used to identify and isolate the parasitised egg, which is then appropriately packaged as a natural enemy product and released in the field to control *C. japonica* (Wei *et al.*, 2022). Due to the limited upward mobility and phototropism of *A. japonicus* (unpublished data), they often remain on the surface of the host eggs after parasitism. This phenomenon makes the process of screening and separating parasitised eggs complex. Based on our current findings, however, the next step is to design a suitable breeding device for the large-scale production of *A. japonicus*. This device may include placing a layer of mesh gauze on the surface of the host egg on the egg card. The mesh size is smaller than the body of the *A. japonicus*, but larger than the eggs of *A. pernyi*, which will enable female wasps to evaluate and parasitise host eggs, and promote the separation of parasitoids and egg cards by simply removing the mesh containing wasps from the eggs.

In conclusion, the study of the parasitic behaviour and developmental morphology of *A. japonicus* on *A. pernyi* eggs can not only enhance our understanding of the biology of *A. japonicus*, but the research results also provide information for the development of high reproductive efficiency devices.

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