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Developmental patterns in the polyembryonic parasitoid wasp *Copidosoma koehleri*

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ABSTRACT

Polyembryony is a unique mode of development in which multiple genetically identical embryos develop from a single egg. In some polyembryonic species a proportion of the embryos develop into soldier larvae, which attack competitors in the host. We studied the development of the polyembryonic wasp *Copidosoma koehleri* in its host *Phthorimaea opercullela*. We dissected hosts parasitized by either virgin or mated female wasps at 2 day intervals from hatching to the final instars. We documented host mass and head width, the number and size of developing wasps and the presence of a soldier larva. Additionally, we kept a sample of parasitized hosts until emergence of wasps and measured the head width of emerging adults. We characterized wasp development in relation to host development. One half of the broods produced by mated wasps contained one soldier larva throughout development. This suggests that in *C. koehleri* each female brood produces a single soldier larva, and that the soldier probably survives and grows gradually during host development. Additionally, we found that female broods were larger than male broods during development and also upon emergence. Accordingly, body size was larger for males during development as well as upon emergence. These findings may extend the existing knowledge on polyembryonic development in general, and serve as a baseline for further experiments.

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1. Introduction

Polyembryony is a unique mode of development that involves the production of multiple genetically identical embryos from a single egg through clonal division. Polyembryony is relatively rare, but regularly occurs in several taxa such as bryozoans, oligochaetes, cestodes, trematodes, insects and mammals. Among insects, polyembryony is especially common in parasitoid wasps, in which it has evolved independently in four families: Braconidae, Platygasteridae, Encyrtidae and Drynidae. This suggests a high adaptive value for this developmental mode in the life history of parasitoids (reviewed by Godfray, 1994; Strand, 2003; Zhurov et al., 2007).

To date, the majority of data regarding polyembryonic development and life history originates from the study of one parasitoid species, *Copidosoma floridanum* (Hymenoptera: Encyrtidae). This wasp parasitizes the eggs of plusiine moths. After oviposition, the parasitoid egg goes through repeated mitotic divisions to produce a primary morula. The morula further proliferates to produce an aggregation of embryonic masses, which together form a polymorula (Grbic et al., 1998). The production of embryos continues as the host develops through its larval stages. Toward the end of host development, wasp larvae start feeding actively on host tissues until they fully consume the host. Wasp larvae then pupate inside the host cuticle, and eventually emerge as adults (Grbic et al., 1997; Strand, 2003). Brood size in this species is the largest known among parasitoids, and may reach 2000 offspring or more (Ode and Strand, 1995).

Another unique feature of polyembryonic encyrtids is the production of a soldier caste. During clonal division a proportion of embryos develop into sterile soldier larvae, which attack competitors in the host and never mature or emerge out of the host (Silvestri, 1937; Cruz et al., 1981, 1986a, 1990; Doutt, 1947, 1952). In *C. floridanum* it was shown that the caste of a larva (soldier or reproductive) is determined according to the distribution of germ cells between embryos at early developmental stages (Donnell et al., 2004; Zhurov et al., 2004). The number and development of soldiers varies among species, and may also depend on their sex. For example, *Copidosoma sosares* produces one soldier per host (Hardy, 1996) while *Copidosomopsis tanytmemus* produces about 10 (Cruz, 1986b; Cruz et al., 1990). In *C. floridanum*, male broods produce fewer soldiers ($\sim$10) than female broods ($\sim$50 soldiers, Grbic

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et al., 1992, 1997). In addition, male soldiers in this species develop more slowly, and are less aggressive, than female soldiers (Giron et al., 2007a).

Polyembryony probably resembles other modes of development in showing a great deal of variation based on a common developmental pattern. While some developmental features are probably shared by all polyembryonic parasitoids, other features likely vary among and within species. Focusing on a single species (such as *C. floridanum*) as a model of polyembryonic development limits one's ability to discriminate conserved developmental processes from species-specific developmental innovations. Conserved developmental processes can be identified by comparing developmental patterns within and among several polyembryonic species. We take a step in this direction by providing a description of developmental patterns in an additional Encyrtid, *Copidosoma koehleri*.

C. koehleri Blanchard (Hymenoptera: Encyrtidae) parasitizes the potato tuber moth *Phthorimaea opercullela* Zeller (Lepidoptera: Gelechiidae), and is a biological control agent of this pest (Horne, 1990; Kfir, 2003). Although the biology of *C. koehleri* has been previously described by Doutt (1947, 1952), many aspects of its development have not been quantified. The biology of *C. koehleri* differs from that of *C. floridanum* in several aspects: first, brood size is much smaller in this species, with an average of 40 wasps emerging from one host (Keasar et al., 2006; Kfir, 1981); second, in *C. koehleri* a female typically lays one egg per insertion of the ovipositor (Keasar et al., 2006), while in *C. floridanum* a female typically lays one female egg and one male egg in a single insertion (Strand, 1989a); and finally, in *C. koehleri* there is typically only one soldier within a host and male broods do not seem to produce soldiers at all (Doutt, 1952; Keasar et al., 2006). These differences make *C. koehleri* a promising candidate for the study of inter-specific variation in polyembryonic development. Additional attractive features of *C. koehleri* are that it is easy to rear and study under laboratory conditions. The small brood size allows easy determination of the number of wasps present per host during development and upon emergence.

In the current study we focus on the following aspects: (1) the timing of different developmental stages of *C. koehleri*, from oviposition to pupation, in relation to host development; (2) the number and size of wasps during development and at emergence; and (3) the presence of a soldier larvae and its development. To identify conserved developmental processes and species-specific developmental innovations, we compare our findings with existing knowledge, mostly collected from *C. floridanum*. To evaluate the extent of intra-specific variation in polyembryonic development, we describe the differences in development between male and female broods.

2. Methods

2.1. Animals

We used a laboratory stock of *C. koehleri* that originated from field-collected individuals from South Africa (courtesy of Dr. R. Kfir, Plant Protection Institute, Pretoria). Adult parasitoids were housed at 27 °C, natural daylight, and fed with honey. A laboratory stock of potato tuber moth (PTM) was housed at 27°, natural daylight, with adults fed with honey and water. PTM eggs were collected daily and were used within 12 h, since the eggs' age is known to influence the parasitoids' oviposition decisions (Ode and Strand, 1995).

2.2. Description of wasp development

A total of 54 virgin and 69 mated *C. koehleri* females, from 45 broods, were used in the experiment. Each female parasitized no more than six hosts. Virgin females were taken from all-female broods that had not been exposed to males. Mated females were taken from mixed broods (containing both males and females) at least 24 h after emergence, to allow mating. We allowed each parasitoid female to oviposit once inside each fresh host. This was done by placing one PTM egg at the center of a petri dish. We then introduced a female to the plate and directed her toward the host. Females that touched the host with their antennae normally started ovipositing. We removed the female from the petri dish after one insertion of the ovipositor into the host. This was done to prevent repeated ovipositions into the host, which are common in *C. koehleri* (Doutt, 1947; M. Segoli personal observations). We added a slice of potato to the petri dish and incubated it at 29 °C. We dissected samples of hosts during their larval development at 2-day intervals from days 4 to 14 after oviposition. During dissection, we measured the host's head width, counted wasp embryos and larvae inside the host and measured the maximal length of the largest developing wasp. We used maximal length since this measure can be used for any developmental stage, from embryos with no differentiated organs to feeding larvae. To avoid inaccuracy due to body twisting we took several pictures of each individual, and used the best available to measure length. We used the size of the largest developing wasp, rather than mean size, since it was often impossible to separate and measure all developing individuals. The presence of a soldier larva was noted, and its body length was measured. Additionally, we dissected a sample of host eggs immediately after oviposition to describe the wasp egg.

2.3. Morphometric analysis of adult wasps

An additional set of host eggs was parasitized by eight mated females originating from four broods as described above. These hosts were kept at room temperature until parasitoid emergence. The emerged wasps were counted and sexed. We measured head width for a sample of five wasps per brood.

We used image processing software (Analysis™) for all measurements of embryos and adults.

3. Results

3.1. Host development

Host eggs hatched after 3–5 days. We obtained head width measurements from 255 host larvae from different developmental stages. We were able to define four distinct size classes of host head width. Mean ± SD head width was 208 ± 12 μm ($n = 39$) for the first size class of hosts, 353 ± 21 μm ($n = 60$) for the second, 605 ± 56 μm ($n = 44$) for the third and 935 ± 59 μm ($n = 112$) for the fourth size class of hosts. Head width of moth larvae differed significantly between size classes (ANOVA, $n = 255$, $F_3 = 3291$, $P < 0.0001$). These size classes probably correspond to four host larval instars and thus were used as a surrogate of host instar. Accordingly, when dissecting the hosts we found that most hosts were at their first instar at days 4 or 6 after oviposition, second instar at day 8, third instar at day 10 and fourth instar at days 12 or 14 after oviposition (Table 1).

3.2. Parasitized vs. non-parasitized hosts

Out of 282 moth eggs that were handled by female wasps, 107 (38%) were apparently not parasitized (no developing wasps were found inside the host larva at dissection). We used GLM analysis to compare the mass of parasitized and apparently non-parasitized hosts during development. The model included days 8–14 since at earlier dissections most hosts were too small to be measured. Hosts that were too small to be measured at day 8 were also excluded

Table 1
The development of C. koehleri in relation to days from oviposition and the development of its host (P. opercullela) at 29 °C

Days	Host stage	Wasp stage
1–4	Egg	Formation of a primary morula
4–6	1st instar	Formation of a polymorula
		Emergence of a soldier larva
6–10	2nd instar	Proliferation continues
8–12	3rd instar	Morphogenesis of reproductive larvae
10–14	4th instar	Complete morphogenesis and host feeding by reproductive larvae

from the test. We found that host mass increased, especially between days 10 and 12, and that parasitized hosts were larger than non-parasitized hosts (Fig. 1A, GLM for days 8–14: days – $n = 151$, $F_1 = 139.7$, $P < 0.0001$, parasitism – $F_1 = 16.1$, $P < 0.0001$). Similarly, host head width was larger for parasitized than for non-parasitized hosts (Fig. 1B, GLM for days 8–14: days – $n = 199$, $F_1 = 559.2$, $P < 0.0001$, parasitism – $F_1 = 5.4$, $P = 0.021$).

3.3. Development of reproductive larvae

Five developmental stages of the wasps were apparent: (1) the egg is drop shaped. Following Doutt (1947) we identified a stalk and an ooplasm (Fig. 2A); (2) primary morula, which consists of an ovoid mass of cells (Fig. 2B); (3) polymorula, which consists of a cluster of morulae attached to each other arranged in several layers, the number of morulae increases from early (Fig. 2C) to later stages of development (Fig. 2D); (4) developing embryos at different stages of morphogenesis; at this stage the anterior–posterior axis of the embryos and segmentation are apparent; embryos gradually dissociate from each other (Fig. 2E); and (5) feeding larvae with mandibles often containing apparent host tissue in their gut (Fig. 2F). At emergence from the egg, most hosts contained a morula. Proliferation occurred mostly during the first and second instars of the host. Morphogenesis of reproductive larvae occurred mostly at the third instar of the host, and by the time hosts reached the fourth instar most of the larvae were already actively feeding. This sequence of development is summarized in Table 1.

### 3.4. Number and size of reproductive larvae during development

We characterized the effects of time since parasitism, and of parasitoid sex, on brood size and body size of wasps. To this end, we studied broods from hosts parasitized by virgin females (assumed to be male broods based on Hymenopteran sex determination). We compared them to soldier-containing broods from hosts parasitized by mated females. These were assumed to be female broods, according to Doutt (1952) and Keasar et al. (2006). We excluded broods without a soldier that were produced by mated females, since their sex could not be determined with high certainty. Female broods were significantly larger than male broods. Additionally, male brood size gradually increased during development whereas female brood size did not, resulting in a significant interaction between day and sex (Fig. 3A, GLM: $n = 84$, day – $F_1 = 0.277$, $P = 0.6$, sex – $F_1 = 16.0$, $P < 0.0001$, interaction – $F_1 = 6.3$, $P = 0.014$). The length of the largest wasp (embryo or larva) inside the host increased during development and was larger for males than for females (Fig. 3B, GLM: $n = 85$, day – $F_1 = 319.8$, $P < 0.0001$, sex – $F_1 = 5.25$, $P = 0.024$).

3.5. Presence of a soldier larva

The soldier was identified by its slender body and developed mandibles (Fig. 2F). None of the hosts that were parasitized by a virgin female contained a soldier larva. About one half of the hosts parasitized by a mated female contained a soldier larva, but no soldiers were observed in hosts dissected on day 14 after parasitism (Fig. 4A). Soldier size increased gradually during development (Fig. 4B, ANOVA, $n = 33$, $F_4 = 42.7$, $P < 0.0001$).
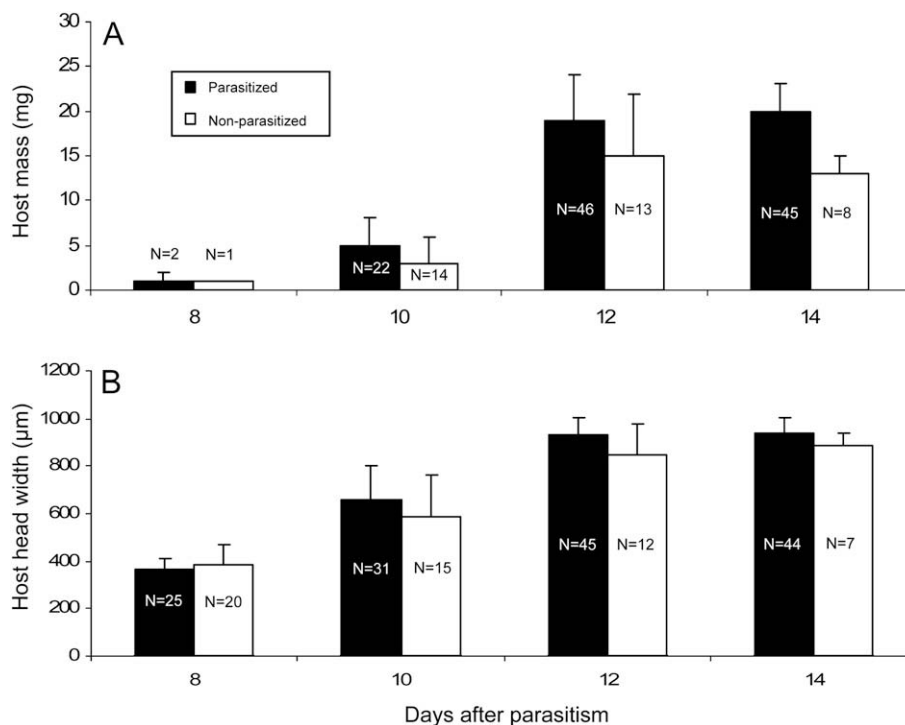
Fig. 1. A Mass (mg, mean and SD) of parasitized (black bars) and non-parasitized (white bars) hosts at different days after oviposition. **B** Head width (μm, mean and SD) of parasitized (black bars) and non-parasitized (white bars) hosts at different days after oviposition.
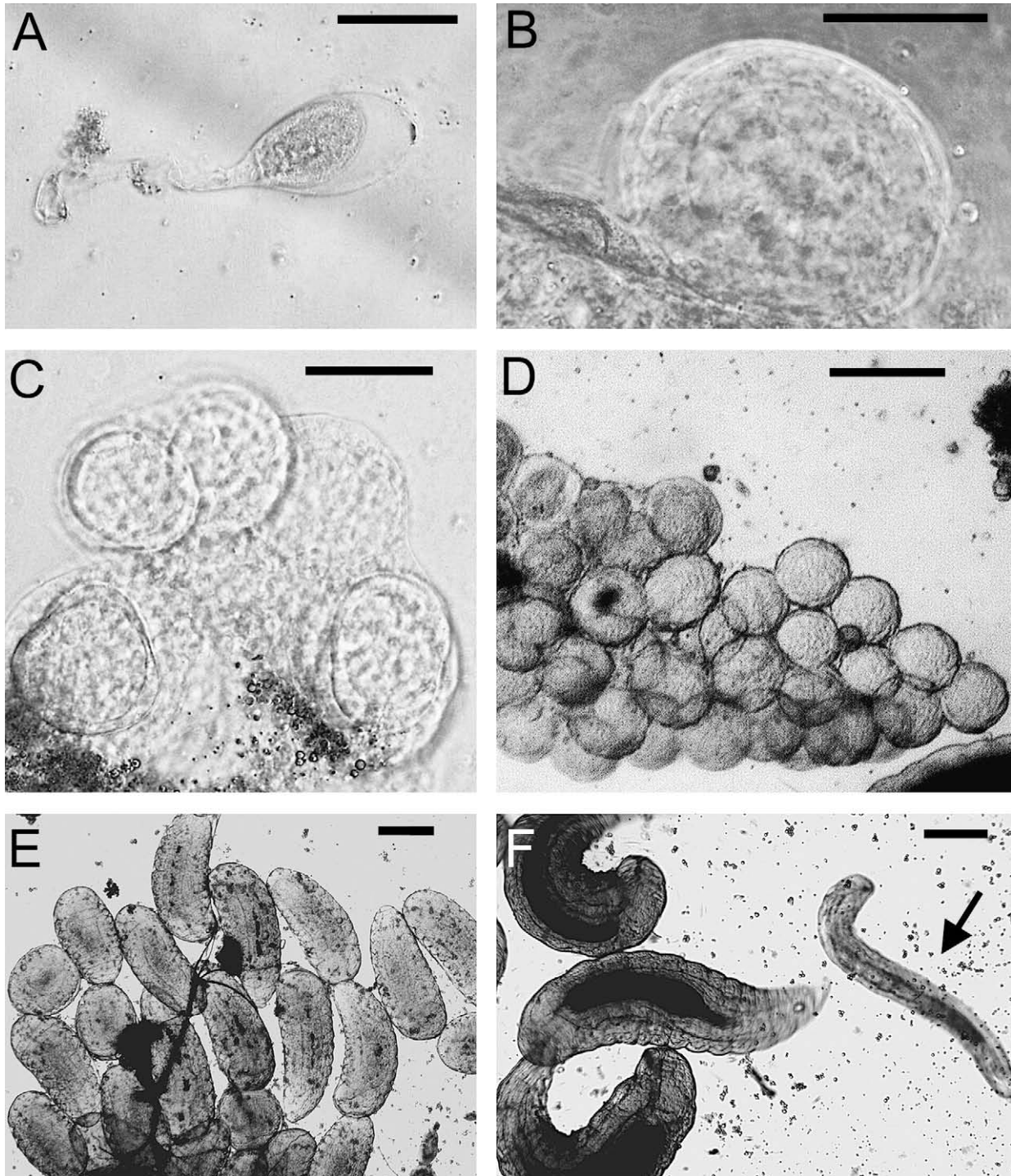
Fig. 2. Developmental stages of *C. koehleri* inside the host. **A** Egg (scale bar 50 μm). **B** Primary morula (scale bar 100 μm). **C** Early polymorula (scale bar 100 μm). **D** Late polymorula (scale bar 100 μm). **E** Reproductive larvae during morphogenesis (scale bar 200 μm). **F** Reproductive feeding larvae and a soldier (marked by an arrow) (scale bar 200 μm). Wasps were dissected out of the host and photographed under a dissecting microscope.

3.6. Wasps at emergence

Nine of the hosts observed parasitized by a mated female developed into a moth, nine contained a female brood and eight contained a male brood. The number of emerging wasps in female broods was significantly larger than in male broods (mean ± SD males – 34.1 ± 10.6 and females – 54.6 ± 9.5, ANOVA, $F_1 = 17.6$, $P = 0.001$). The average head width of emerging wasps was significantly larger for males than for females (mean ± SD males –

494 ± 40 μm and females 417 ± 23 μm, ANOVA, $F_1 = 24.3$, $P > 0.0001$). Head width was negatively related to the number of emerging wasps (linear regression, $R^2 = 0.47$, $P = 0.002$).

4. Discussion

This study was aimed to extend the existing knowledge on polyembryonic development by exploring the wasp *C. koehleri*. We distinguished five developmental stages of the host, and described
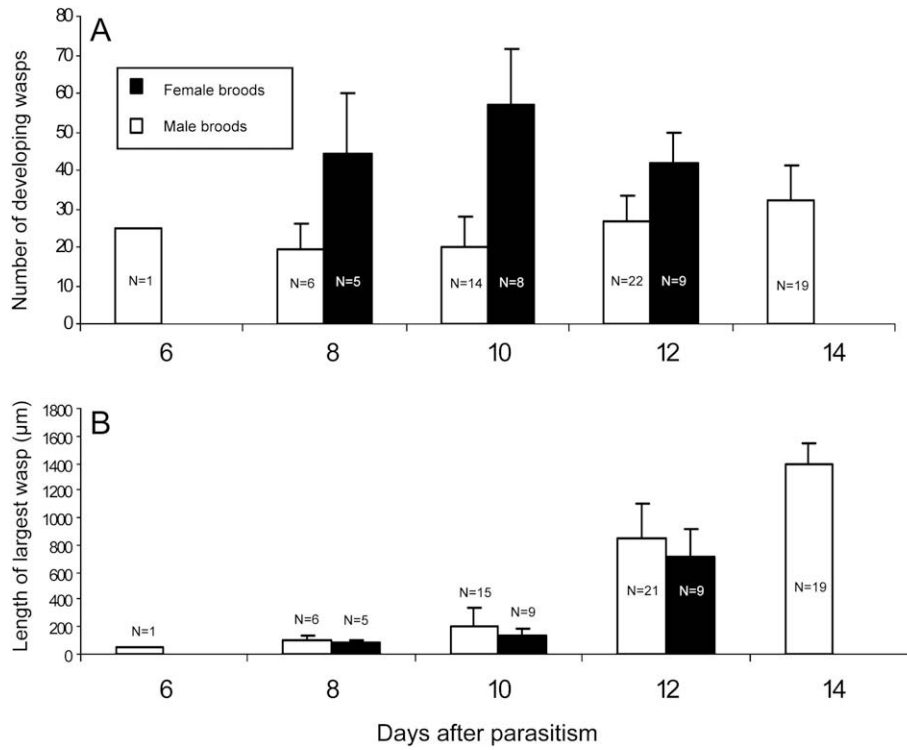
Fig. 3. A Wasp brood size (mean and SD) at different days after parasitism for male broods (produced by virgin females, white bars) or for female broods (produced by mated females and tentatively recognized by soldier's presence, black bars). **B** Length of the largest developing wasp found inside the host (μm, mean and SD) at different days after parasitism for male broods (white bars) and female broods (black bars). No female broods were recognized at day 14 since soldiers were probably already dead.

wasp development in relation to host development. Our results show that only broods produced by mated wasps contain a single soldier larva, thus these larvae are likely to be females. The results further suggest that the soldier increases in body length during development. Additionally we have demonstrated asymmetries in brood size and individual size between the sexes.

The developmental duration and head width of the different instars of the host *P. opercullela* was similar to those described in previous studies at similar temperatures (Broodryk, 1971; Flanders and Oatman, 1982). The development of *C. koehleri* was found to be highly synchronized with its host's development. Soon after host hatching the soldier was often already developed and
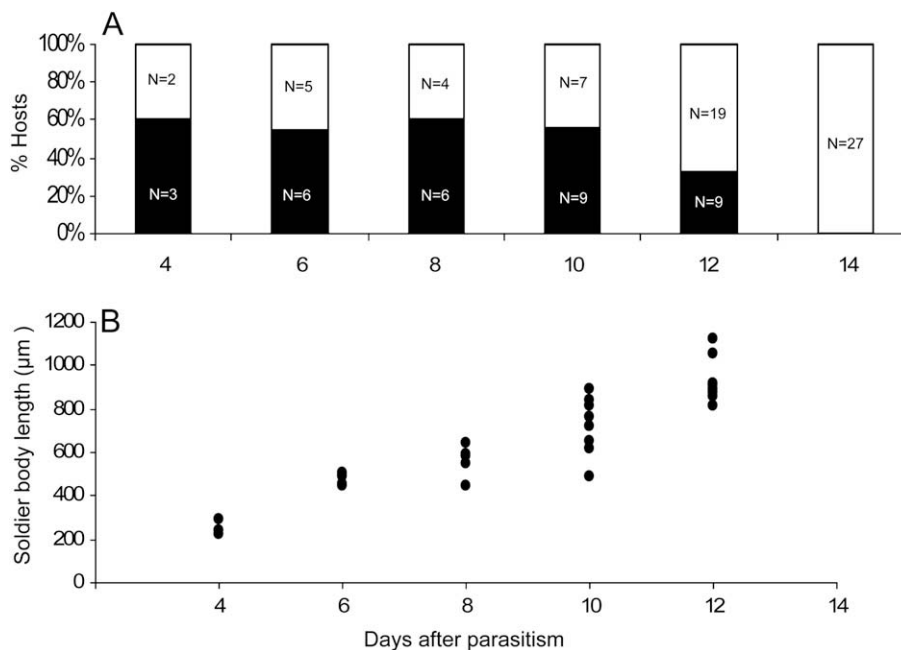
Fig. 4. A Percentage of hosts parasitized by a mated female that contained a single soldier larva (black) or no soldier larvae (white) at different days after parasitism. **B** Body length (μm, mean and SD) of soldier larvae at different days after parasitism.

potentially ready to defend its brood members from competitors. Proliferation occurred through the first and second instars of the hosts. Wasps increased in size during host growth and toward the fourth instar of the host, wasp embryos completed their development and became feeding larvae that consumed the host tissues before pupation. This developmental sequence is similar to that described for *C. floridanum* (Godfray, 1994; Grbic et al., 1992, 1998; Strand, 1989b, 2003, Zhurov et al., 2007) as well as for other polyembryonic Encyrtids (e.g. Cruz, 1986b; Silvestri, 1937; Wang and Laing, 1989).

Although we observed insertion of wasp ovipositor into the host egg in all cases, more than a third of the hosts were apparently non-parasitized. The number of non-parasitized hosts might have been overestimated especially during earlier developmental stages in which the morula might have been undetected (Cruz et al., 1990). Additional possibilities are that some wasps failed to parasitize the host, rejected the host after ovipositor insertion or that an egg was laid but was eliminated by the host's immune system. We found that *P. opercullela* larvae parasitized by *C. koehleri* increased in mass and size more rapidly than non-parasitized hosts. Increased growth by parasitized larvae is a general phenomenon in gregarious parasitoids (Slansky, 1986) and was reported for other *Copidosoma* species as well (e.g., *C. bakeri*, Byers et al., 1993; *C. floridanum*, Strand, 1989b).

In accordance with previous studies (Doutt, 1952; Keasar et al., 2006) we found that no soldiers were present in hosts parasitized by virgin females, suggesting that in *C. koehleri* male clones do not produce soldiers at all. In contrast, in *C. floridanum* male clones do produce soldiers, although fewer and later during development compared to female clones (Grbic et al., 1992, 1997). To our knowledge, no data on sex of soldiers from other species are available. About half of the hosts parasitized by a mated female contained a single soldier, except for hosts at late developmental stages, in which soldiers might have already died (Cruz, 1986b; Strand, 1989b). This proportion corresponded with that of male and female broods at adult emergence, suggesting that in *C. koehleri* each female clone produces a single soldier (Doutt, 1952; Keasar et al., 2006). Our indirect evidence regarding soldier sex awaits future confirmation through molecular markers or karyotyping. Although the number of soldiers is much smaller than in *C. floridanum*, the mean ratio between soldiers and reproductive larvae is similar ($\sim$1:40 compared with $\sim$50:2000 respectively). However, the number of soldiers in *C. koehleri* does not seem to be plastic, while in *C. floridanum* it is highly variable and was shown to respond to the induction of competition (Harvey et al., 2000).

If soldiers are indeed only females, this may explain the female-biased sex ratio ($\sim$ one third males) observed in mixed sex broods of *C. koehleri* (Keasar et al., 2006). Female-biased sex ratios also occur in other *Copidosoma* species (Patterson, 1919; Stoner and Weeks, 1975; Walter and Clarke, 1992). This bias is probably mediated by female soldiers attacking and killing male embryos and larvae (Grbic et al., 1992). A theoretical model further suggests that conflict over sex ratio may be sufficient to explain the evolution of the soldier caste (Gardner et al., 2007). Elimination of a large number of embryos by a soldier may be feasible especially if the soldier attacks the competing clone at relatively early stage of development. This is probably the case in *C. koehleri* (M. Segoli, unpublished data) as well as in *C. floridanum* (Giron et al., 2007b).

In several polyembryonic species, soldier larvae have been found to be polymorphic, with small soldiers present at the beginning of development, and large soldiers towards its end (Cruz et al., 1990; Giron et al., 2007b). In *C. floridanum*, the early-developing soldiers are specialized to combat intra-specific competitors and the later-developing soldiers are specialized for defense against inter-specific competitors (Giron et al., 2007b). In contrast, we found that in *C. koehleri* the size of the soldier increases gradually during development. This, along with the fact that we never found more than one soldier inside a host, suggests that there is probably only one type of soldier that grows and potentially defends its clone members throughout development.

In contrast to *C. floridanum*, where embryo production increases progressively during development (Grbic et al., 1997; Strand, 2003), in *C. koehleri* most of the proliferation is completed by the host's second instar. Possibly, in *C. floridanum* the proliferation phase is longer since the production of such a large number of embryos (up to 2000) is time consuming. This may imply that embryos of *C. floridanum* can adjust their numbers to the host carrying capacity by controlling the degree of proliferation more easily than can embryos of *C. koehleri*.

We found a difference in the development of male broods (produced by virgin females) and female broods (produced by mated females and tentatively recognized by the presence of a soldier). Female broods were larger than male broods both during development and upon emergence. Larger female broods at emergence were previously reported for *C. koehleri* (Doutt, 1947; Kfir, 1981), as well as for other Encyrtids (Cruz, 1986b; Ode and Strand 1995; Wang and Laing, 1989). The difference in brood size also during development, suggests that it probably results from sexual asymmetry in proliferation levels.

Most of the increase in wasp body size occurred between days 10 and 14, the same time at which wasp larvae started feeding actively on the host tissues. Body size of developing wasps was larger for males than for females. Accordingly, at emergence, the mean head width of males was larger than that of females, possibly due to the negative relationship between wasp number and size. The differences in size may be adaptive if optimal body size differs between males and females (Godfray, 1994). Alternatively, the haploid genome of males was suggested to constrain their ability to proliferate (Doutt, 1947), allowing each individual to attain a larger body size.

In summary, our study highlights some features of *C. koehleri*'s development that are shared with other polyembryonic species. These include the sequence of developmental stages, the synchronization with host development, and the presence of soldier larvae. We suggest that these features are conserved developmental motifs that are likely to be found in additional polyembryonic parasitoids. Other features, such as the number and size of reproductive larvae and the number, sex and size of soldiers, show both inter- and intra-specific variation. These traits are more likely to be developmental innovations. The identification of conserved vs. innovative traits in polyembryonic insects can serve as input for reconstructing the evolution of this developmental pathway.

Acknowledgments

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