



Lessons from the multitudes: insights from polyembryonic wasps for behavioral ecology

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Even for parasitic Hymenoptera, polyembryonic wasps are unusual creatures. Two features in particular, allow for novel exploration of major questions in behavioral ecology: the production of multiple offspring per egg and, in some species, the production of a soldier caste. Because final brood sizes of polyembryonic species are not constrained by trade-offs between current and future parental reproductive effort, we can clearly examine the selective forces at play that drive the balance between the number of offspring and their body size. Polyembryony also provides excellent opportunities to compare the performance of identical genotypes under different environmental conditions. Finally, polyembryonic species can provide unique tests of how genetic conflicts at multiple levels are resolved.

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Current Opinion in Insect Science 2018, 27:32–37

This review comes from a themed issue on **Behavioural ecology**

Edited by **Eric Wajnberg** and **Emmanuel Desouhant**

<https://doi.org/10.1016/j.cois.2018.02.001>

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Introduction

Polyembryony is a developmental mode whereby a single egg gives rise to multiple, genetically-identical offspring. Sporadic polyembryony is a very widespread phenomenon across all animal groups including humans. Obligate polyembryony is far more restricted in distribution; in the insects, it occurs only in two orders: the Hymenoptera (in four parasitoid families: Dryinidae, Encyrtidae, Braconidae, and Platygasteridae) and the parasitic Strepsiptera [1,2]. Polyembryony involves the production of yolk-poor eggs that undergo complex genetic [3,4], endocrine [1], and developmental [2,5,6] processes, many of which are unique to these species. With few exceptions (e.g. [7,8]),

nearly all developmental studies have involved the encyrtid wasp *Copidosoma floridanum*.

Polyembryony provides several selective advantages for parasitoids. First, polyembryonic development may provide a way for ovipositing females to overcome egg limitation. In other words, the cloning of embryos allows higher reproductive output without laying additional eggs. Second, it may alleviate conflict and aggression (which often lead to mortality) between genetically-identical larvae that develop within the same host. Finally, because all polyembryonic parasitoids are koinobionts, females may not be able to accurately predict the future quality of the host for their developing offspring at the time of oviposition. By dynamically adjusting the number of clonal divisions to the size of the growing host, brood size can be fitted to the carrying capacity of the host when that final carrying capacity cannot be foreseen by the parents [9,10].

The reasons for the rarity of polyembryony in parasitoids are far from clear. Craig *et al.* [9,11] suggested that polyembryony is costly because it clones an unproven genotype (different from that of the parent) at the expense of genetic diversity within a brood. However, this cost may be relatively small, because each mother produces several, genetically-distinct clones through regular sexual reproduction. Thus, polyembryony leads to a loss of within-clone genetic variation, but might not affect the overall population-level genetic diversity [12].

Two features of polyembryony permit unique tests of several important aspects of behavioral ecology [13]. First, as discussed below (see ‘Clutch versus brood-size’), polyembryony typically results in offspring brood sizes and sex ratios that are very different from the number and sex of eggs laid by the mother. This feature allows for unusually straightforward tests of the size–number trade-off problem as well as the role of sibling conflict in driving offspring sex ratio patterns. Second, some species within the Encyrtidae have evolved a larval caste system, where soldier larvae defend their clone-mates from intra-specific and inter-specific competitors (see ‘Soldiers, sex ratios, and sociality’, below). This raises the possibility of using these polyembryonic species to explore aspects of sociality as well as to study genetic conflict phenomena such as sibling rivalry and parent–offspring conflict. In addition, polyembryonic species can be excellent systems to differentiate between genetic and environmental effects on phenotype because it is easy to compare the effects of

different environments on different individuals of the same genotype [14,15*].

Clutch versus brood size

One of the most remarkable qualities of polyembryony is the apparent disconnect between maternal decisions — both clutch size (number of eggs laid per host) and sex allocation — and the resulting brood phenotype (total number of wasps emerging from a host and offspring sex ratios). While clutches often comprise only 1–2 eggs, several polyembryonic encyrtids produce broods that exceed 1000 offspring; in *C. floridanum* as many as 3400 offspring can arise from a single egg [2,16]. As a result, the investment in any one clutch is not expected to greatly constrain future reproductive effort, a confounding issue when trying to study clutch size decisions in monoembryonic species. When all brood-mates are genetically identical, parent–offspring and sibling conflict are also absent. These features permit exceptionally clean tests of classic questions in behavioral ecology including clutch size decisions and trade-offs between individual body size and the number of offspring per brood. As described below, such tests have received some attention to date in polyembryonic species.

Within the genus *Copidosoma*, ovipositing females typically lay clutches of either one or two eggs per host, which clonally divide to produce single-sex broods (all-male or all-female) or mixed-sex broods (originating from one male and one female egg). Whether one or more eggs are laid per host depends in part on host encounter rate, with low encounter rates resulting in a higher proportion of mixed-sex broods whereas high encounter rates result in a higher proportion of single-sex broods [17,18]. This maternal clutch size and sex allocation pattern has been interpreted in the context of mating opportunities for adult offspring (see ‘Soldiers, sex ratios, and sociality’ and ‘Future directions’ sections, below).

In species that produce broods of multiple offspring, there is a continuum of ways to partition limited resources among offspring ranging from producing few, relatively large body-sized progeny to producing many, relatively small body-sized progeny. The optimal balance point between individual body size and brood number is the one that maximizes the product of brood number and per capita offspring fitness [19–21]. Body size has been repeatedly shown to influence life history traits (i.e. survivorship, fecundity, and age at maturity) that are related to other traits linked to fitness such as dispersal, mating ability, and competitive ability [22,23], implying that selection acts on both number of individuals per brood and body size. Virtually all empirical studies of the size–number trade-off have shown that observed clutches comprise fewer individuals than the predicted optimum [20,21,24]. The most widely accepted explanation for this discrepancy is that other trade-offs such as those between

present and future reproductive effort [23,25,26], parent–offspring conflict, or sibling rivalry obscure the size–number trade-off [20,27]. These trade-offs are largely absent in polyembryonic species, permitting examination of the role of mating systems as selective forces on the body size–brood number trade-off. Furthermore, the influence of sibling rivalry on the size–number trade-off can be studied in the absence of present vs. future reproductive effort trade-offs. A second, less appreciated, reason for the discrepancy noted above is the fact that environmental conditions determining the optimal trade-off point often fluctuate spatially and temporally. Variation in such environmental conditions that affect reproductive success across generations can select for a phenomenon known as ‘bet-hedging’ [28]. Key to understanding how bet-hedging can be advantageous is recognizing that selection acts on the geometric mean rather than the arithmetic mean of a fitness related trait. Decreasing variance of fitness across generations can increase the geometric mean even if the arithmetic mean is reduced [28]. Therefore, a clutch size that is smaller than the predicted optimum (based on within-generation conditions) may be selected for if this reduces across generation variation in this trait. Thus, both bet-hedging and current vs. future reproductive effort trade-offs predict lower clutch sizes compared to the theoretical prediction; polyembryonic species permit the study of the role of bet-hedging in the absence of current vs. future reproductive effort trade-offs [29,30*].

A handful of studies of *Copidosoma* species suggest that all-male and all-female broods have different trade-off optima, possibly reflecting different selective pressures experienced by males and females as adults. In a study of *Copidosoma bakeri* that explicitly examined differences in the optima between all-male and all-female broods [31,32*], all-female broods comprised fewer, larger body-sized individuals compared to all-male broods. *Copidosoma sosares* [33,34] exhibits a similar pattern where all-female broods contain fewer, larger-bodied individuals. In the case of the univoltine *C. sosares*, females emerge, mate, and overwinter before they are able to mature eggs and locate hosts the following spring. Immediately after emerging, males (from both mixed-sex and single-sex broods) mate locally with females from nearby all-female and mixed-sex broods (multiple broods synchronously emerge within the same host plant). Unlike females, males die within a few days. Given these life history differences between the sexes, there is a premium placed on large body-sized females compared to males, as larger females are more likely to successfully overwinter and find hosts the following spring (at the cost of fewer females per brood) [33]. In contrast, all-male broods of *C. floridanum* and *Copidosoma koehleri* contain fewer, larger-bodied individuals compared to all-female broods [16,35–37]. It is tempting to infer that such differences between male and female broods in the trade-off optima

result from sex-differences in the relationship between body size and reproduction (e.g. finding and acquiring mates, dispersal, overwintering); however, little is known about mating and reproduction in the field in any of these species (see ‘Future directions’, below). Interestingly, males produced by mated females of *C. koehleri* form larger clones than sons of virgin females, suggesting that epigenetic effects of mating status affect offspring number and size [38].

Soldiers, sex ratios, and sociality

In several polyembryonic encyrtid wasps, a proportion of embryos (most commonly, females) develop into ‘precocious’ or ‘soldier’ larvae. Soldiers develop earlier and differ morphologically from their clone-mates that eventually develop into reproductively competent adults. Soldier larvae never become adults and die within the host prior to the emergence of their genetically identical siblings [39–42]. Two functions have been attributed to soldier larvae [43^{*}]: first, ‘sex ratio-conflict hypothesis’ — adjusting the secondary sex ratio (sex ratio of the brood of adult offspring) in mixed-sex broods, and second, ‘brood-benefit hypothesis’ — protecting their clone-mates from intra-specific or inter-specific competitors inside the host.

Tests of the sex ratio-conflict hypothesis

Whereas maternal clutch size and sex allocation decisions dictate the distribution of single-sex and mixed-sex broods (see above), asymmetries in proliferation and survival between male and female clones within mixed-sex broods throughout larval development can greatly modify adult offspring sex ratios. Even within a species, offspring sex ratios of mixed-sex broods can be incredibly variable; in *C. floridanum* for instance, sex ratios can range from more than 80% male to >99% female despite arising from a male and a female egg [16]. In several polyembryonic encyrtids, offspring sex ratios within mixed-sex broods are mediated in part by soldier larvae that kill reproductive larvae from competing clones [40]. Due to relatedness asymmetries arising from haplodiploidy (whereby diploid females develop from fertilized eggs and haploid males develop from unfertilized eggs; a characteristic of all Hymenoptera), sisters are less related to their brothers ($r = 0.25$) than are brothers to their sisters ($r = 0.5$). Within mixed-sex broods of *C. floridanum*, female clones produce soldiers earlier and in greater numbers than male clones [16,40], and male soldiers are less aggressive than female soldiers [44,45]. Consequently, female soldiers selectively kill male embryos resulting in a strongly female-biased sex ratio in the emerging brood. The significance of the soldier caste in sex ratio adjustments has also been supported by the results of a theoretical model based on the biology of *C. floridanum* [46]. In *C. koehleri*, soldiers are exclusively female and offspring ratios in mixed-sex broods are also female-biased, further supporting this hypothesis [47]. However, in a third species (*C. bakeri*), male and female

soldiers are equally abundant, and are mostly aggressive toward inter-specific competitors. Consequently, the secondary sex ratios of mixed-sex broods are ~ 0.5 [48]. In *C. sosares*, only a single soldier larva is produced regardless of sexual composition of the brood [49] and sex ratios of mixed-sex broods are relatively unbiased [33]. These variations may reflect differences among polyembryonic species in the intensity of the conflict over sex ratio.

Whether biased sex ratios represent a conflict of interest between brothers and sisters (i.e., sibling rivalry) depends on the mating structure of the population in which these mixed-sex broods exist. If brothers only have opportunities to mate with sisters that shared the same host, then both brothers and sisters should favor a strongly female-biased sex ratio and no genetic conflict of interest should exist. As mating opportunities for brothers away from the host increase, the intensity of conflict over within-brood sex allocation should increase. Resolution of this conflict is mediated in large part by the action of soldier larvae. Observations that both males and females of some species disperse immediately after emergence (e.g. *C. floridanum*; [16,40]) suggest that such conflicts do exist. However, a solid understanding of the mating structure of most field populations of polyembryonic parasitoids is lacking.

While interactions within the larval brood modify the secondary sex ratios, they can also exert potentially conflicting selective pressures on the primary sex ratios (clutch sex ratio of eggs laid by the adult females). In parasitoid species with unequal competitive ability of males and females, sexual selection is expected to favor increased production of the weaker competitors [50,51]. This should select for excess male production in polyembryonic species with female soldiers, while competition between brothers for mates could select for female-bias. A simulation model that incorporates these contradictory selective pressures, based on the life history of *C. koehleri*, predicts that a male-biased sex allocation would eventually evolve. The bias is expected to become more extreme as the probability of competition between clones increases. Both predictions have been confirmed experimentally [52].

Tests of the brood-benefit hypothesis and implications for sociality

As a non-reproductive caste, soldiers are widely appreciated to defend their clone mates against multi-parasitism (where individual hosts are parasitized by females of different species) and superparasitism (where individual hosts are parasitized by two or more females of the same species) [35,53,54]. Moreover, soldiers were directly observed to aggressively attack both inter-specific and intra-specific potential competitors, *in vitro* [39,42,45]; and the presence of a soldier larva was shown to reduce the survival of a competitor inside the host, *in vivo* [35,45,54]. Finally, in *C. floridanum*, soldier development

is plastic, with more soldiers being produced in the presence of competitors [41,55*]. The defensive soldier function is dependent on their ability to discriminate kin from non-kin. Soldiers were indeed shown to discriminate and adjust their aggression towards competitors according to their relatedness [35,42].

The sacrificial nature of the soldiers may be considered as either altruistic (for the benefit of their genetically identical clone-mates) or spiteful (against low-relatedness competitors), and is consistent with kin-selection theory [46]. Accordingly, the evolution of the soldier caste is often considered as equivalent to the evolution of non-reproductive castes in eusocial insects [39,42,46]. However, it should be cautioned that polyembryonic wasps do not share many of the basic characteristics that are thought of as pre-adaptations to eusociality such as parental care or cooperative breeding, suggesting a different evolutionary path.

Future directions

As discussed above, polyembryonic species provide rich opportunities for exceptionally clean tests of behavioral ecology theory. Largely through studies of a handful of *Copidosoma* species (*C. bakeri*, *C. floridanum*, *C. koehleri*, and *C. sosares*), our understanding of the behavioral ecology (esp., clutch size, sex ratios, caste systems and sociality) of this fascinating group of organisms has vastly improved. Similar sets of studies with non-encyrtid polyembryonic species with different life history traits and host use patterns will undoubtedly broaden and enrich our understanding of not only the behavioral ecology of polyembryonic species, but the field of behavioral ecology more generally.

At this point, one of the most pressing needs for future research is a better understanding of the fitness consequences of body size for males and females in the field. Also needed are better, direct field measurements of mating systems of polyembryonic species. This will provide desperately needed context for more fully understanding many aspects of the behavioral ecology of this group of organisms including the selective forces determining the outcome of the body size–clutch number trade-off and sibling conflict problems.

Finally, we need better phylogenies of polyembryonic species (e.g. [56,57]) and their monoembryonic relatives onto which we can map behavioral, ecological, developmental, and morphological traits to understand their evolution within these groups. For example, such a comparative phylogenetic approach is needed to decipher whether the evolutionary roots of polyembryony lie in solitary or gregarious parasitoid clades. With respect to behavioral ecology, detailed phylogenies would allow testing for evolutionary constraints that might shape salient traits, such as clone and body sizes, soldier

number, and function. A second challenge is to estimate the physiological cost of the proliferation process, as a potential barrier to the shift from monoembryony to polyembryony within evolutionary clades. An intriguing, but as yet untested, explanation for this cost is the long period of time spent as embryos in polyembryonic species. Whereas larvae may be able to defend themselves to some extent, embryos likely cannot; this would make polyembryony costly relative to monoembryonic species. The production of a soldier caste may be one way to reduce this cost. Indeed, clades of polyembryonic wasps that produce soldiers (i.e. the copidosomatine encyrtids) are more speciose than other clades of polyembryonic species.

Acknowledgements

We thank Éric Wajnberg and Emmanuel Desouhant for the invitation to write this review.

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Previous work by this group has shown that soldier larvae originate from embryos lacking primary germ cells. This study follows up on that work by showing that caste ratios ('soldier': 'reproductive' ratio) of female, but not male!, broods can rapidly shift in response to perceived competition (in this case, the chorion of *Microplitis demolitor* eggs) by increasing the proportion of embryos that lack primary germ cells.