Hold the germ cells, I'm on duty

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Summary
Germ cell segregation and gamete production are developmental problems that all sexually reproducing species must solve in order to survive. Many people are familiar with the complex social structures of some insect species, where specialised castes of adult insects perform specific tasks, one of which is usually to guard the sexually reproductive queen. The parasitic wasp *Copidosoma floridanum* adds another level of complexity to the caste system: a fertilised egg produces both sterile, short-lived ''soldier'' larvae and ''reproductive'' larvae that complete metamorphosis to produce sexually reproductive adults. How two morphologically and functionally distinct larval castes are produced by genetically identical groups of cells developing under the same environmental conditions is a baffling problem. A recent paper suggests that differential germ cell segregation during embryogenesis may be an event both necessary and sufficient for caste determination.[1] *BioEssays* 26:1263–1267, 2004. © 2004 Wiley Periodicals, Inc.

Introduction

The problem of caste, individuality and sociality

Genetic relatedness plays an important role in the behavioural and biological characteristics of social organisms, and was likely a crucial factor in the evolution of sociality and individuality.[2,3] Social insects often display polyphenism, which is polymorphism that occurs among genetically identical animals as a result of differing environmental cues.[4,5] For example, female honeybee larvae can develop into either queens or workers, and their developmental fate is initially controlled by the quantity and quality of the food they consume before metamorphosis.[4] Once a queen has differentiated and become sexually active, she produces a pheromone that inhibits oogenesis and queen-rearing behaviour in the surrounding female worker bees.[5]

Polyphenisms can also occur in the form of larval caste systems before metamorphosis.[6] Some parasitic wasps develop two types of larvae: ''soldier'' larvae that never undergo metamorphosis, and ''reproductive'' larvae that complete metamorphosis and form the next sexually reproductive generation.[7] Soldier larvae perform two functions. Since hosts are often parasitised by more than one species, soldier larvae protect their siblings by destroying competitor parasite species that may be present.[8] They also play a role in determining male-to-female ratios of the surviving reproductive larvae.[7,9] Soldier larvae have been reported to lack reproductive systems,[7,10] which begs the question of whether the two castes may regulate germ cell development differently during embryogenesis.

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Abbreviations: PGC, primordial germ cell, L1, L2 etc., first, second etc. host larval instar.

The problem of embryonic germ cell specification

In most animals, primordial germ cells (PGCs) are specified during embryogenesis and later incorporated into developing gonads by both morphogenetic movements and active migration.[11–13] PGCs may be induced to differentiate by signals from neighboring somatic cells, or specified cell-autonomously by inheritance of cytoplasmic germ cell determinants.[14] For example, in the fruit fly *Drosophila melanogaster*, maternal deposition of germ cell determinants occurs during oogenesis.[15] mRNA and protein products of genes necessary for PGC development are localised to the posterior of the ooplasm, and confer germ cell identity on cells forming at the posterior that inherit this special cytoplasm.[16] It seems straightforward: each embryo can produce its own complement of germ cells, ensuring that the resulting adult is capable of making gametes. However, almost everything that we know about insect germ cell development comes from studies of monoembryonic insects like fruit flies, where one egg gives rise to one individual. What about germ cell specification in insects where a single fertilised egg produces up to 2000 clonally related individuals?

Embryonic development in *Copidosoma floridanum*

As bizarre and unlikely as such a developmental strategy may seem, obligate polyembryony has evolved at least 15 times during metazoan evolution, in both invertebrates and vertebrates.[17] Among insects, polyembryony has been reported only in the Hymenoptera (bees, wasps, ants) and the Strepsiptera (twisted-wing flies).[6] Very little is known about strepsipteran development,[6,18] and most studies investigating the developmental mechanisms of polyembryony have focused on the Hymenoptera, specifically the parasitic wasp *C. floridanum*.[7,19–24] Within the parasitic wasps, polyembry-

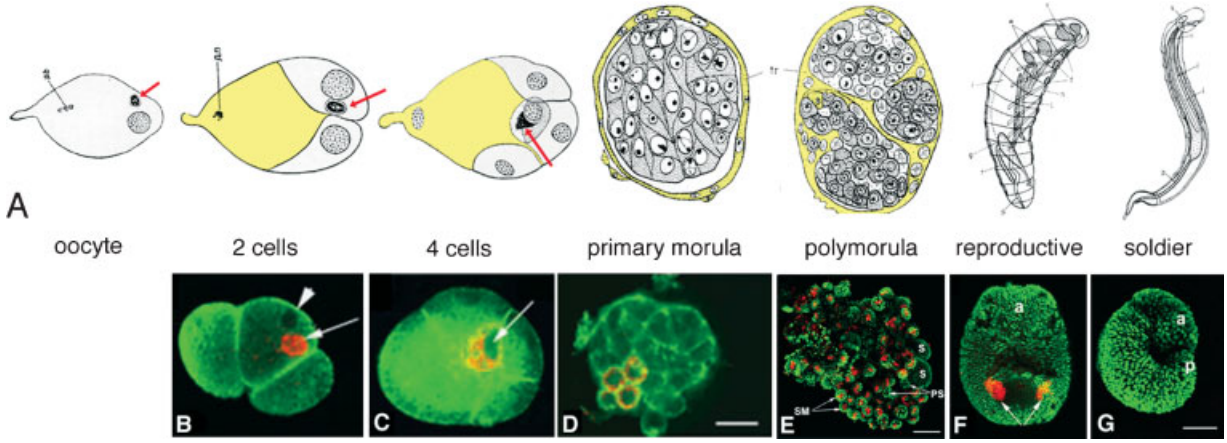

Figure 1. Embryonic development and Vasa protein distribution in *C. floridanum*. **A:** The progression through early holoblastic cleavage results in asymmetric inheritance of the oosome (red arrow) to the B_4 cell at the 4-cell stage. The polyploid trophamnion (yellow) grows to envelop the primary morula, then interdigitates to define secondary morulae at the polymorula stage. Morphological differences are very clear between reproductive and solider larvae. *pb*, polar bodies; *pn*, polar nuclei; *tr*, trophamnion. Adapted from Silvestri F. 1906. Ann. R. Scuola Sup. Agric. Portici 6:3–51. **B–G:**[1] Vasa protein distribution visualised by confocal microscopy. Red, Vasa protein; Green, filamentous actin (B–E) or nuclei (F–H). **B:** The oosome is inherited by only one cell at first cleavage. **C:** The oosome is inherited by B_4 at second cleavage, and subsequently breaks down into cytoplasmic particles. **D:** B_4 has four to eight descendants (putative PGCs) by the primary morula stage. **E:** Not all secondary morulae contain PGCs. SM, secondary morula; S, embryos undergoing morphogenesis into soldier larvae; PS, putative solider morulae. **F:** At the end of embryogenesis, reproductive larvae have two clusters of PGCs at the posterior. A, anterior. **G:** Soldier larvae do not possess PGCs. A, anterior; p, posterior.

ony has evolved convergently at least four times, correlated with the endoparasitic or viviparous life histories displayed by members of these families.[23,24]

In order to appreciate the complexity of caste determination and germ cell specification in this animal, we must first understand embryonic development in the context of the life cycles of both the parasite and the host. All stages described in the following section are shown in Fig. 1A.

C. floridanum adult females oviposit one or two eggs into the egg of the moth *Trichoplusia ni*. Embryogenesis takes 72 hours in *T. ni*, and up to 13 days in *C. floridanum*, so that embryogenesis of the wasp takes place during both embryonic and larval development of the host. *C. floridanum* cleavage is holoblastic (found in hexapods only among Collembola and parasitic Hymenoptera,[10,25–28]), unlike the syncytial cleavage characteristic of early embryogenesis in most insects. The first cleavage produces two diploid cells of roughly equal size, and the polar bodies, instead of disintegrating, become incorporated into a "polar cell". The second cleavage is equal in one cell (producing cells named B_1 and B_2), but unequal in the other, which produces one large (B_3) and one small cell (B_4). All cell proliferate until the embryo consists of roughly 200 cells, when it is called a primary morula. The polar cell nucleus divides syncytially, forming a multinucleate cell that encircles the primary morula, forming a syncitial extraembryonic membrane (trophamnion). When the host egg hatches into a larva, the trophamnion interdigitates between groups of proliferating cells, splitting the primary morula into a polymorula, now comprising up to 2000 secondary morulae of about 20 cells each. Each one of these clonally related secondary morulae proliferates and gives rise to one larva. The trophamnion does not contribute to any larval structures, but instead may protect the morulae from destruction by the host immune system.[29]

All wasp larvae hatch inside of, consume and eventually kill the host moth larva. However, not all larvae are created equal. Most larvae are reproductive larvae, with squat, overtly segmented bodies; they eat the host larva, pupate and metamorphose into sexually reproductive adult wasps. All embryos that form reproductive larvae undergo morphogenesis and hatch in the transition from fourth (L4) to fifth (L5) instar of the host larva, which is the final instar before pupation in uninfected moth larvae.[21] Some of the hatched larvae (4% to 24%[1,22]) are sterile soldier larvae, which have a vermiform morphology; these larvae never complete metamorphosis, instead dying after their reproductive siblings have consumed the host.

While male soldier larvae hatch at the same time as their reproductive siblings (host L4–L5), female soldier larvae begin hatching up to seven days earlier, between host instars L1 and L2.[21,30] Larval instar transitions and pupation are generally associated with changes in insect hormones titres.[4] However, heterochronic transplant and hormone treatment experiments have shown that *C. floridanum* caste determination is

not regulated by host hormonal factors.[21] If extrinsic factors do not control differences in morphology between genetically identical larval castes, then what does? The answer lies in intrinsic factors present in early embryogenesis, resulting in different embryonic cell compositions between the two larval morphs.

The intersection of germ cell biology and caste determination

In 1906 Filippo Silvestri reported a round granular structure in the oocytes of *C. floridanum* (at that time called *Litomastix truncatellus*) (Fig. 1A).[10] He referred to this structure as the ''nucleolo'' (oosome), and observed that it was inherited by the B_4 cell, and subsequently fragmented into cytoplasmic granules. Although Silvestri was only able to follow the granule-containing cells through to the primary morula stage, he proposed (1) that B_4 was the single PGC, and (2) that its descendants were distributed only to those proliferating morulae destined to become reproductive larvae, thus explaining the two larval castes. Contemporary workers on polyembryony dismissed this idea, proposing instead that spontaneous non-disjunction of the X chromosome or differences in egg fertilisation could explain the development of two larval morphs from a single egg.[6] However, Silvestri's first hypothesis was recently supported with molecular data: it was observed that a cross-reacting antibody against Vasa protein, which plays a role in germ cell identity and function in all metazoans studied to date, recognised a protein localised to the oosome in late oogenesis, one cell at the two-cell stage, B_4 at the four-cell stage, and four to six cells in the primary morula.[23,24] David Donnell and colleagues have now vindicated Silvestri's second conjecture by examining the endogenous *C. floridanum vasa* gene, *Cf-vas*.[1]

vasa gene products in *C. floridanum* persist only in reproductive larvae

A polyclonal antibody raised against Cf-Vas confirmed that the oosome contains Cf-Vas (Fig. 1B) and at the four-cell stage is segregated to B_4 (Fig. 1C,D), whose descendants (presumptive PGCs) number four to eight by the primary morula stage (Fig. 1E). However, at the polymorula stage, PGCs are not present in all proliferating morulae (Fig. 1F). Cf-Vas is absent from morulae undergoing morphogenesis into soldier larvae, and although the authors did not compare the percentage of Cf-Vas negative morulae with the percentage of soldier larvae known to hatch, it is likely that only those morulae destined to produce reproductive larvae inherit PGCs, while morulae that will produce soldier larvae do not. In later stages of morphogenesis, reproductive larvae possess PGCs at the sites of the presumptive gonads (Fig. 1G), while soldier larvae do not (Fig. 1H).

The data thus far support Silvestri's idea that receipt of PGCs during early embryogenesis correlates with the formation of reproductive larvae, and explains the previously reported absence of reproductive structures in soldier larvae. However, Donnell and colleagues were additionally able to establish an apparent causal relationship between germ cells and caste determination. Wild-type embryos almost always (91% of the time) give rise to broods containing both larval morphs. When the B_4 cell was ablated, however, 93% of ablated embryos gave rise to broods that were less numerous than controls, but were made up entirely of soldier larvae. When one of B_{1-3} were ablated, resulting broods were again smaller than controls, but 86% of ablated embryos contained both reproductive and soldier larvae. This result suggests a causal relationship between PGC inheritance and development of the reproductive larval morph.

Elaborate deduction or functional relationship?

When Silvestri suggested that PGCs might exert an organising influence over morulae containing them, these statements were considered ''purely speculative...elaborate deductions''.[26] Nevertheless, later workers, including Donnell's co-author Michael Strand, have not hesitated to put forward the possibility that PGCs might have wide-ranging organisational influence over developing morulae.[23] That would seem to be exactly what PGCs in *C. floridanum* are doing: somatic tissues of morulae that contain PGCs follow a different developmental program than morulae lacking PGCs. Although Donnell and colleagues comment that ''excluding gonads, soldier larvae seem to possess all major tissues and organ systems'',[1] data from other workers suggest that the somatic dimorphism involves all three germ layers.[6] Soldier larvae are reported to have differently structured musculature, lack functional excretory, circulative and respiratory systems, and show different cuticular and head morphologies as compared to reproductive morphs.[6,10,19,29] The results of Donnell and colleagues appear to reveal a novel developmental function of germ cells, which have not previously been observed to have dramatic effects on somatic tissues. Experiments performed on other systems indicate that, in the absence of PGCs, somatic development continues normally: even somatic gonads can assemble correctly in the absence of germ cells.[31–34]

In some animals, germ-line segregation and posterior patterning are coupled via genes like *nanos*, which play roles in both germ cell development and axial patterning. *C. floridanum* oocytes develop in a similar way to those of *D. melanogaster*, connected to nurse cells, which presumably provide maternal factors.[24] However, any potential polarity present in the egg at the time of fertilisation must be lost during the trophamniotic packaging and extensive proliferation of the morulae. Proliferating morulae in the polymorula are randomly oriented, and axial patterning of each larva is established de novo.[19,22] Germ cell specification is thus uncoupled from

posterior patterning in *C. floridanum*: the former is achieved by maternal oosome inheritance, while the latter must be determined autonomously in each morula.

Contrary to a previous speculation that partitioning of the germ cell lineage might provide axial polarity cues to individual morulae,[23] Donnell and colleagues argue that, since anteroposterior patterning genes are conserved in both morphs and soldier morulae have axial polarity but no PGCs, germ cells cannot contribute to axial patterning.[1] However, the study cited on *C. floridanum* axial patterning, by Grbic' and colleagues, did not deal with differences in embryonic patterning between the two larval castes.[22] Indeed, all of the markers used to asses axial polarity in that study were detectable only during late morphogenesis, at the end of the host L4 stage. The L4–L5 transition coincides with metamorphosis and hatching of reproductive larvae, but precocious larvae can metamorphose and hatch at earlier stages. Unless all the embryos examined by Grbic' and colleagues happened to be derived from unfertilised eggs (whose soldier larvae metamorphose and hatch at the same time as reproductive morphs, only at host L4–L5), something that was not specifically monitored in their paper,[22] if axial patterning mechanisms were conserved between the two larval morphs, then at least some of the conserved gene expression patterns should have been observed in morulae prior to host L4. It thus remains a formal possibility that morulae destined to make soldier larvae regulate axial polarity differently from reproductive morulae, and that the latter may somehow use PGCs to do so. This scenario, however, necessitates invoking either (1) differences in detectability of patterning gene products between larval and reproductive morphs, or (2) an anteroposterior specification pathway that does not terminate in segmentally repeated stripes of Engrailed, a situation without precedent among arthropods.[35] It also represents a departure from the quiescent nature of germ cells with respect to signaling. Although it is known that a small group of somatic cells can dictate axial patterning of the whole embryo in some arthropods,[36,37] once specified, embryonic PGCs are both transcriptionally quiescent and unresponsive to signaling cues present in the surrounding somatic tissues.[38]

How come I don't get some?

Regardless of the role of germ cells in somatic morula development, it is clear that a single PGC is established by oosome inheritance at the 4-cell stage, and that descendants of this single cell are distributed among most, but not all, morulae as they are sectioned off during the formation of the polymorula. Historical polemic aside, there is a temptation to despair at the biological problem this presents, and to share the sentiments of J. T. Patterson when he said, referring to Silvestri's hypothesis of predetermined germ cell distribution, "It is impossible to conceive of a mechanism which could operate in such a manner as to parcel out exactly predestined germ cells to the several hundred embryos".[26] An added complication to the problem of PGC distribution comes from the observation that the percentage of soldier larvae produced is highly variable, exhibiting plasticity in response to environmental changes associated with interspecific parasite competition.[8] If a host is parasitised by a single *C. floridanum* egg, the percentage of soldiers is 4%. If a *C. floridanum* egg and the egg of a competitor endoparasite doubly parasitise a host egg, the percentage of soldiers rises to between 7% and 24%. This might mean that about 24% of morulae do not receive PGCs initially, but could obtain them at a later point if there were no competitor parasite. However, this does not seem likely given the observations of Donnell and colleagues that very few morulae lack germ cells, and that the trophamnion has already parceled out individual morulae by the time that the generation of extra soldiers might be required.

Parceling of PGCs cannot be random, since the frequency of soldier larvae is responsive to environmental conditions. An alternative hypothesis is that the PGCs are normally programmed to populate every morula, but an environmentally determined frequency of failure causes absence of PGCs in a few morulae. However, morulae would then have to retain the potential to develop a soldier morph, even though the probability that they would have to carry out this developmental program could be low. A second possibility is that morulae that have received PGCs are not irreversibly committed to producing reproductive larvae, and are able to lose their PGCs and differentiate as soldiers in response to environmental cues. Could the initial localised pool of PGCs migrate singly or in small clusters to each morula, guided by external clues, possibly provided by the trophamnion? Many more experiments will be necessary to begin to answer some of these questions.

Concluding remarks

Although work on polyphenisms in other hymenopterans has demonstrated that caste determination can be genetically determined,[39] or result from differential changes in developmental gene expression,[40] this study by Donnell and colleagues[1] provides the first evidence for embryonic cell lineage involvement in caste determination. Like all good studies, this work makes a significant contribution to understanding the biological problem, and also raises numerous new questions and testable hypotheses about germ cell behaviour, embryonic development and the role of the germ line in polyembryonic animals.

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