

Title: The evolution of insect germline specification strategies

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Highlights:

- Germline determination modes are evolutionarily labile.
- Cricket germline determination bears similarity to mammals
- *oskar* is the crucial molecule for organizing insect germ plasm
- *oskar* is heavily involved in transitions between germline determination modes

Abstract

The establishment of the germline is essential for sexually reproducing organisms. In animals, there are two major strategies to specify the germline: maternal provision and zygotic induction. The molecular basis of the maternal provision mode has been well characterized in several model organisms (fly, frog, fish, and nematode), while that of the zygotic induction mode has mainly been studied in mammalian models such as the mouse. Shifts in germline determination modes occur unexpectedly frequently and many such shifts have occurred several times among insects. Given their general tractability and rapidly increasing genomic and genetic tools applicable to many species, the insects present a uniquely powerful model system for understanding major transitions in reproductive strategies, and developmental processes in general.

Introduction

Two broad strategies for germline determination exist in animals. In maternal provision, a substance called germ plasm, which has the property of inducing naive cells to adopt the germline fate, is generated and localized during oogenesis, leading to the very early cloistering of the germline [1] (Fig. 1). In zygotic induction, the germline relies on the activation of the zygotic genome, and can occur at various time points in embryonic (and potentially post-embryonic) development [1] (Fig. 1).

Although most of the work on the mechanisms of germline specification has been done within model laboratory animals, understanding the evolution of germline determination mechanisms requires broad sampling of diversity, and thus the use of non-traditional model organisms. The insects are particularly well situated to provide important insights into how and why germline mechanisms change over time, as there has been an explosion of genomic information available, as well as a rapidly improving toolkit for functional analysis that is widely applicable to many species. In addition, the phenomenal species richness of insects makes it more likely that we will discover examples of independent, convergent evolution of developmental outcomes. These "natural experiments" can be leveraged to identify both essential properties of particular developmental modes, and the diverse palette of genomic raw material that evolution can draw from in order to solve developmental problems.

Here we review the recent literature, which is providing a deeper understanding of the biological processes underlying maternal provision and zygotic induction modes of germline determination, and will set the stage for understanding how and why germline determination modes change so often in evolution.

Evolution of Oskar, and the origins of maternal provision in insects

Classical experiments in the beetle *Leptinotarsa* and the fly *Drosophila* demonstrated that the cytologically conspicuous germ plasm is both necessary and sufficient to induce germ cell fate, proving that this substance is a germ cell determinant rather than just a germ cell marker [2,3]. *Drosophila* is the best characterized model system for the molecular mechanisms of germ plasm assembly. *Drosophila* germ plasm, in the form of polar granules, is assembled at the posterior pole of the oocyte in a process for which the gene *oskar* (*osk*) is both necessary and sufficient [4,5]. During early embryogenesis, when the syncytial nuclei reach the embryo cortex, the nuclei that associate with the polar granules cellularize precociously to form the pole cells, which are the primordial germ cells of *Drosophila* and many other holometabolous insects.

Osk is the central component of a regulatory network required to assemble germ plasm. Upstream components such as Stauf and Bruno ensure proper posterior localization of *osk* mRNA, and prevent ectopic *osk* translation prior to localization, respectively. The proteins Vasa and Tudor act downstream of Osk to assemble and

stabilize the germ plasm and to recruit other RNAs and proteins, such as *nanos* mRNA which are required for germ cell differentiation and development (reviewed in [6])

Given the essential role of *osk* and the evolutionary conservation of most other components of the *Drosophila* germ plasm assembly network, it was surprising that this gene seemed to be restricted to the Diptera [4,7,8] (Fig. 2). Eventually an *osk* ortholog was discovered in the wasp *Nasonia*, where it was also shown to be necessary for germ plasm assembly and primordial germ cell formation [9]. It was also shown that most of the regulatory interactions upstream and downstream of *Nasonia osk* are conserved with *Drosophila*, implying that these two networks are homologous and share a common evolutionary origin. Since the Hymenoptera and Diptera diverged at the base of the Holometabola, and neither *osk* genes, nor unambiguous maternal germ plasm had yet been found in any Hemimetabolous insects, it was proposed that *osk* was a novelty of the Holometabola, and its origin more or less correlated with the origin of maternal provision in this clade [9] (Fig. 2). A transcriptomic analysis of the beetle *Callosobruchus maculatus*, which has clear germ plasm and early pole cells [10], has identified an *osk* ortholog [11], further supporting the correlation between the presence of an *osk* gene and the presence of maternal provision of germ plasm.

Thus, it was a major surprise when an *osk* ortholog was discovered in the hemimetabolous cricket *Gryllus*, proving that this gene has a much longer evolutionary history than previously appreciated [12]. This discovery was particularly unexpected because *Gryllus* lacks any evidence of maternal germ plasm [13,14]. Indeed, strong knockdown of *Gryllus osk* had no effect on germ cell formation, development or viability of the crickets [12]. The only detected phenotype was sporadic defects in nervous system patterning [12]. Therefore, the evolutionary origin of *osk* appears to have predated its role in germ plasm assembly, and *osk* was apparently co-opted for a role in germline specification in a common ancestor of the Holometabola.

The origins and molecular functions of the *osk* gene are still somewhat mysterious, but progress has been made recently. While the origin of *osk* has been pushed back at least into more basally branching hemimetabolous insect lineages, it still appears to be a novelty of the insects as no *osk* genes have been found in non-insect arthropod genomes and transcriptomes [15-19]. The discovery of *Nasonia* and *Gryllus osk* has made it clear that there are at least two domains of Osk (that were only weakly detectable in *Drosophila* Osk), which are related to domains found in other molecules [9,12]. At the N-terminus is the LOTUS domain, which is also found at the N-termini of several Tudor-domain containing genes that are highly conserved among animals [20,21]. Toward the C-terminus there is a domain that bears some similarity to SGNH hydrolases and lipases, which was recently dubbed the OSK domain [9,12,22].

Two recent papers have shed light on the molecular function of Osk. Pull down experiments using embryonic extracts showed for the first time that Osk directly binds germline mRNAs *in vivo* [22]. *In vitro* analyses with isolated LOTUS and OSK domains showed that it is the OSK domain that binds RNA, while the LOTUS domain showed no such activity [22,23]. Instead the LOTUS domain forms dimers, which are predicted to block any nucleic acid binding potential [23], and mediates Osk interaction with the RNA helicase Vasa [22]. This is also important, as it demonstrates for the first time a molecular function for Osk's LOTUS domain in *Drosophila*, and provides a direct link to the downstream events of germ plasm assembly, which require Vasa protein localization [22].

Osk also has a more subtle role in the assembly of the germ plasm. The morphological characteristics of this substance vary among insects, from the uniform, stably anchored granules of flies, to the often large, spherical, spatially dynamic oosome of wasps [9,24]. It has been shown that even among species in the *Drosophila* genus, germ granule morphology and size is variable [25]. Later studies showed that the differences were due to species-specific properties of Osk proteins [26]. Changes in Osk protein can also lead more drastic changes in the protein's function. For example, while

Osk from *Drosophila immigrans* is able to induce functional germ plasm formation when expressed in the ovaries of *D. melanogaster*, Osk from the equally distantly related *D. virilis* cannot [27].

In order to gain an understanding of the above phenomena at a more detailed level, the evolutionary forces driving divergence of Osk protein sequences among *Drosophila* species were assessed. It was found that in general, *osk* loci are under purifying selection [28]. However when parts of the gene are examined in isolation, patterns of positive selection are observed for specific functional regions of the Osk protein, particularly those involved in interactions with the localization factor LASP, and the Long-OSK isoform specific sequence region [28]. It was proposed that selection on these regions might be responsible for the divergent morphology of the germ plasm granules in *D. immigrans*, and the inability of *D. virilis osk* to rescue germ plasm in *D. melanogaster* [28].

Mechanisms of Zygotic induction in Hemimetabolous Insects

Classical studies have strongly supported the idea that zygotic induction was the ancestral mode of insect germline determination, based on histological studies that consistently found a lack of maternal germ plasm, and relatively late appearance of primordial germ cells (e.g., [1,29]) This pattern has largely been confirmed in the molecular era (e.g., [30]). More recently, the absence of maternal provision was demonstrated in the milkweed bug *Oncopeltus fasciatus* (a hemipteran member of the Paraneoptera, the sister group to the Holometabola) [31] (Fig. 2). In this species, some germline markers are expressed maternally, but are not localized, and there is no *osk* ortholog. Instead, the first specific localization of germline genes such as *vasa*, *tudor*, *nanos*, and *piwi* occurs late in the cellular blastoderm stage at the posterior pole, just as the first signs of embryonic morphogenesis are taking place. So far the molecular basis of this zygotic induction of germ cell fate is not known [31].

Recently the zygotic induction mode of germline determination was characterized at the molecular level in the cricket *Gryllus bimaculatus*. The authors hypothesized that the role for BMP signaling in inducing germ cells might be a conserved mechanism used ancestrally by insects [32-37]. By both knocking down and overexpressing BMP signaling activity, they were able to show that indeed BMP has a crucial role in regulating the formation of primordial germ cells in the late stages of *Gryllus* embryogenesis [32]. It is not clear whether the conserved role of BMP signaling in *Gryllus* and mammals represents an ancient homology, present in their most recent common ancestor, or whether BMP was independently recruited for germline determination in these lineages.

Nakamura and Extavour recently demonstrated that the transcription factor Blimp-1 has a role in germline determination downstream of BMP signaling in *Gryllus* [38]. Since the Blimp-1 ortholog in mice has a similar role in specifying germline fate downstream of BMP signaling, the *Gryllus* result indicates that the gene regulatory networks for germline determination in crickets and mice share components and structure. This supports a common evolutionary origin of the zygotic induction mode in insects and vertebrates. Further analysis and comparison of the downstream gene regulatory network in currently established model species, as well as further sampling of zygotic induction mechanisms in additional basally branching insect lineages (such as cockroaches, dragonflies, mayflies and apterygote insect lineages), will hopefully give a clearer idea of what the ancestral mechanism of insect germline determination was. This can then be compared with additional data from other animal lineages to test the hypothesis of shared ancestry of BMP based germline determination.

Evolutionary Lability of Germline Determination Modes

Given that germ line cells are, from an evolutionary point of view, the only cells that matter in organisms that cannot reproduce by budding or fragmentation, it seems

counterintuitive that there would be much evolutionary change in their establishment. However, this is the pattern that is observed. In what we consider the current most parsimonious model of germline evolution in insects, there was at least one derivation of maternal provision at the base of the Holometabola, then at least three independent "reversions" to zygotic induction associated with loss of *osk* loci within the Holometabola: in the bees (*Apis*) [39,40], tenebrionid beetles (*Tribolium*) [8,41], possibly moths and butterflies (*Bombyx*, *Pararge*) [42-45], and likely more cases that have not been discovered due to the sparse sampling of insect embryonic developmental mechanisms relative to the massive diversity of Holometabola (Fig. 2).

Interestingly, these known "revertants" to zygotic induction have adopted distinct strategies for germline establishment. The honeybee germline is established very late in embryogenesis in association with the mesoderm [29,45,46], which is at least superficially reminiscent to the emergence of germ cells from among the mesoderm in *Gryllus*. In contrast, *Tribolium* germ cells form at the posterior pole at the onset of gastrulation and ingress to the interior of the embryo [41], in a manner very similar to *Oncopeltus* and other hemipterans ([31], and references therein).

Bombyx appears to be an outlier in that the first evidence of germ cells (based on high levels of Vasa protein) appears at the late blastoderm stage in the middle of the germband [43]. More recently it was shown that a *nanos* ortholog (*nanos-O*) is distributed in granules in the ventral region of early *Bombyx* embryos, and is also found in the early germ cells in the late blastoderm stage [47]. In addition a distinct cytoplasmic structure containing *nanos-O* RNA was found in the butterfly *Pararge* [48]. This may indicate that there is some type of maternal germ plasm in *Bombyx* and other Lepidoptera. However, given the lack of a strict spatial correlation between the *nanos-O* granules and the location of the first germ cells, and the late appearance of germ cells well after germband formation, it is premature to overturn the classical interpretations of higher Lepidopteran germ cell determination [44] without more direct functional evidence.

An example of the lability of germline determination modes moving in the opposite direction may be found in the aphid, *Acyrtosiphon pisum*, where a region of the early embryo accumulates the germline-associated gene *vasa* (mRNA and protein), and the cells deriving from this region appear to eventually become the primordial germ cells in both sexual and asexual forms [49,50]. Since the localization of germline markers occurs before the blastoderm forms, it is unlikely to be due to zygotic genome function, and thus could be considered a form of maternal provision that was independently derived in the aphid lineage.

This reinvention must use distinct molecular and cellular mechanisms from those known in Holometabola. First, like the other Paraneopterans so far sequenced [51,52], *A. pisum* lacks an *osk* ortholog [53] (Fig. 2). In addition, the mechanisms of localization must also be diverged, as the putative germ plasm only becomes localized after several syncytial divisions, appears as a ring at the cortex some distance away from the posterior pole, and is not cytologically distinguishable from the surrounding cytoplasm. Finally, aphid germ plasm does not induce precocious cellularization of the primordial germ cells [49].

Clearly caution is warranted in making strong statements about the evolutionary history of insect germline determination, as a full accounting of just how labile germline determination mechanisms are has not been achieved, and sampling of species relative to the total diversity of insects is almost vanishingly small. Looking at outgroups [54,55] is also not necessarily helpful. The myriapods [15,56] and chelicerates [17,57] examined thus far use zygotic induction and lack *oskar* (Fig. 2). However, representatives of closely related crustacean [58] and collembolan [59] clades apparently produce maternal germ plasm, and there is even some evidence for a maternal germline determinant in the apterygote insect *Thermobia* [60] (Fig. 2). Such evidence could be used to support the (unlikely, in our opinion) hypothesis that

maternal provision is ancestral to insects, and that some hemimetabolans simply lost this feature.

Another mystery is the role of *oskar* in the transitions between germline determination modes. Those species that lack clear maternal provision in the Holometabola also all lack *osk* orthologs in their genomes (Fig. 2). *osk* is also missing from several Paraneoptera, including true bugs and aphids, yet it persists in *Gryllus* [12]. Our current understanding indicates that *osk* arose early in insect evolution, where it initially took on a non-germline function, likely in the nervous system, and then was recruited for a germline function in a common ancestor of the Holometabola [12]. If the nervous system function is even weakly advantageous, *osk* should be maintained in the genome even if its germline function were lost. On the other hand, if its nervous system function is superfluous, it is hard to imagine how it persisted long enough to be repurposed as a germline determinant. In addition, it cannot be formally excluded that loss of maternal provision of germ plasm could be an indirect effect of selection favoring the loss of *osk* in another functional context. A better understanding of the non-germline functions of *osk* in the species where it has a germline function (such as *Drosophila*, *Callosobruchus*, and *Nasonia*) could provide insights into how this gene could have been lost in members of their respective Orders.

This question of *oskar's* evolutionary history leads to inquiries about the evolutionary forces driving transitions between germline modes. Are there any ecological and/or embryological conditions that favor one mode of germline determination over another? This question could be addressed by first gaining a higher resolution understanding of where in phylogeny transitions have occurred, which will, again, involve deeper sampling of insect diversity. Evans *et. al* found that in vertebrates maternal provision was correlated with significant increases in both species diversity and rates of molecular evolution in maternal provisioning clades relative to zygotic inducing ones [61]. This view was updated by a recently published hypothesis paper, which argued that the early germline specification during embryogenesis, rather than the way germ line is specified, that affects the evolvability of somatic development [62]. Whether such a pattern (and its reciprocal) is found in insects is an interesting open question, which may provide a partial explanation for the pattern of change in germline determination modes across insect phylogeny.

Conclusion

The lability of germline determination mode in the course of animal evolution continues to surprise, and simultaneously calls out for an explanation. The insects provide the best set of model systems to thoroughly characterize germline determination modes in diverse embryonic contexts, and to map and analyze multiple independent transitions between the two germline determination types. Thus, the insects will play a crucial role in understanding the evolution of this most essential of cell types.

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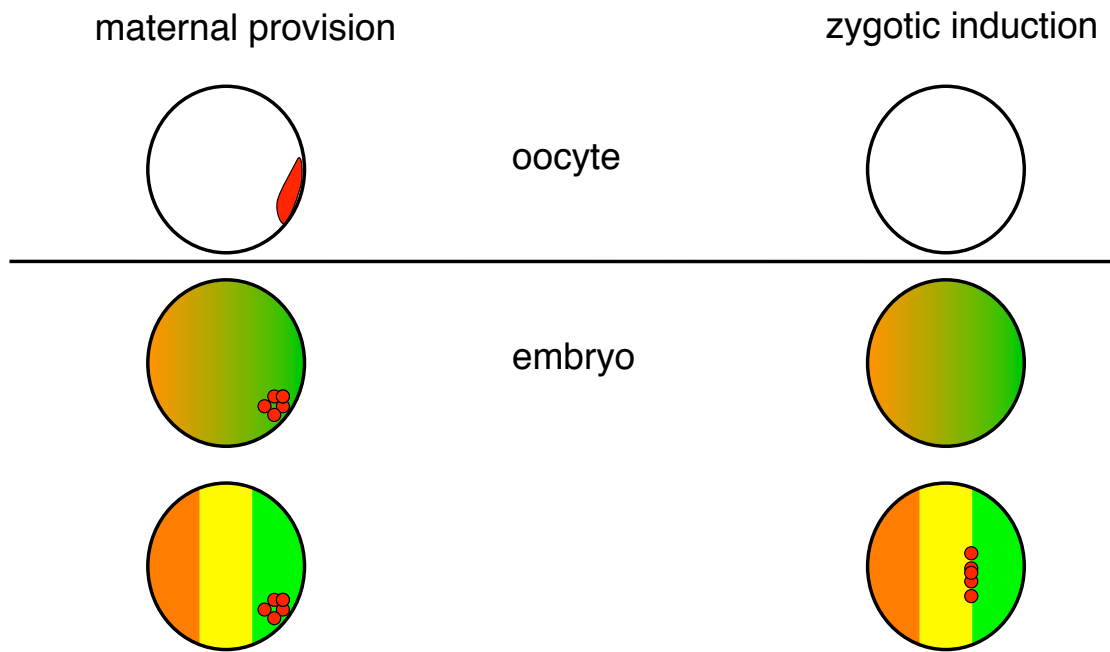


Figure 1. Modes of germline specification: maternal provision and zygotic induction. Red indicates germ plasm/germ cell markers. Gradient in middle two embryos indicates early stage of patterning with little differentiation of the soma. In maternal provision the germ cells are the first and only cells specified at this stage. The sharp borders in the bottom two embryos indicate that distinct tissues have been specified, and zygotic induction of germline fate can occur (right).

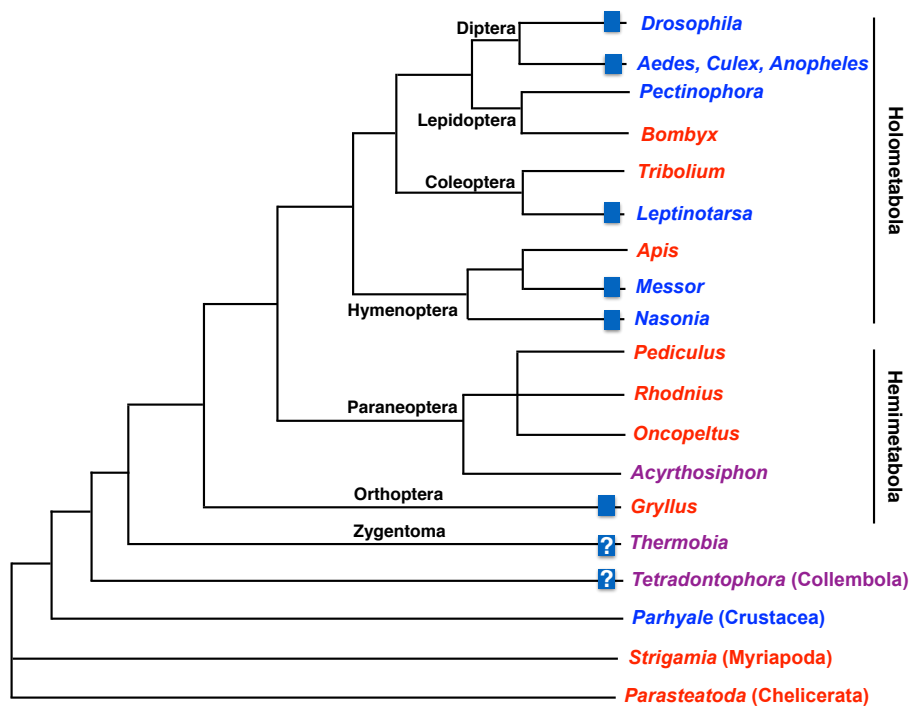


Figure 2. The distribution of germ cell specification modes and *oskar* orthologs. Blue boxes indicate *osk* ortholog in the genome [5,7,9,11,12], a box with a question mark indicates that no large scale genomic or transcriptomic data has been published to help determine whether *osk* is present, those without indications appear to lack *osk* based on its absence in large scale sequencing projects [8,39,51-53]. Genus names in blue are those in which maternal provision mode is used [3,7,9,58,63], while those in red use zygotic induction [13,31,40,41,56,57,64]. Those in purple indicate that the presence of maternal provision has been proposed and/or independently derived [49,59,60]. The topology of the phylogeny is based on [55] and [54].

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