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SHORT COMMUNICATION

Egg size, embryonic development time and ovoviviparity in *Drosophila* species

T. A. MARKOW, S. BEALL & L. M. MATZKIN

Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ, USA

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Abstract

Lengths, widths and volumes of eggs from 11 species of *Drosophila* whose genomes have been fully sequenced exhibit significant variation that is not explained by their phylogenetic relationships. Furthermore, egg size differences are unrelated to embryonic development time in these species. In addition, two of the species, *Drosophila sechellia* and, to a lesser degree, *D. yakuba*, both ecological specialists, exhibit ovoviviparity, suggesting that female control over oviposition in these species differs from what is observed in *D. melanogaster*. The interspecific differences in these reproductive characters, coupled with the availability of whole genome sequences for each, provide an unprecedented opportunity to examine their evolution.

Introduction

Drosophila gametes display surprising interspecific variability in size. Most astonishing is the variation in *Drosophila* sperm lengths (Pitnick *et al.*, 1995; Snook, 1997; Bjork & Pitnick, 2006). Although *Drosophila* egg size does not vary dramatically, significant species differences in size and shape have been observed (Starmer *et al.*, 2003; Lott *et al.*, 2007). Species' egg differences appear to have been shaped by evolution (Kambysellis & Heed, 1971; Montague, 1984) indicating a genetic basis to the observed variability. Yet, the relationship between an egg's dimensions and shape and its subsequent development, however, are largely unknown.

Are the ecologically driven changes in egg size and shape accompanied by modifications in early development? Recent studies in several species of Dipteran eggs suggest an influence of egg size and shape variability on embryonic gene expression (Lott *et al.*, 2007; Gregor *et al.*, 2008). Our ability to examine the developmental genetics of interspecific differences in egg size and morphology as well as early embryogenesis is enhanced tremendously by publication of the sequences of the genomes of 12 *Drosophila* species (Drosophila 12 Genomes Consortium, 2007). A critical first step in this process is to clearly measure eggs and their basic development. To this end, we report upon two egg characteristics in 11 of these 12 species. First, we determined the dimensions and volumes of the eggs. Second, for the same 11 species, we also recorded the times between oviposition and the hatching of first instar larvae. We then used these data to test the hypothesis that differences in egg size are related to differences in embryonic development time. We discovered, in addition to the independence of egg size and development time, that, in two species, facultative ovoviviparity is relatively common.

Correspondence: Therese Ann Markow, University of California, San Diego, Division of Biological Sciences, Section of Ecology, Behavior and Evolution, La Jolla, CA 92093 0116, USA.
Tel.: (858) 246 0095; fax: (858) 534 7108;
e-mail: tmarkow@ucsd.edu
Present address: Luciano M. Matzkin, University of California, San Diego, Division of Biological Sciences, Section of Ecology, Behavior and Evolution, Muir Biology, Rm. 2208, 9500 Gilman Drive 0116, La Jolla, CA 92093 0116, USA.

Materials and methods

Drosophila strains

Strains of the 11 species were those used in the genome sequencing projects (*Drosophila* 12 Genomes Consortium, 2007) and were obtained from the Tucson Drosophila Stock Center now located at the University of California at San Diego: *D. ananassae* (14024-0371.13), *D. erecta* (14021-0224.01), *D. melanogaster* (14021-0231.36), *D. mojavensis* (15081-1352.22), *D. persimilis* (14011-0111.49),

D. pseudoobscura (14011-0121.94), *D. sechellia* (14021-0248.25), *D. simulans* (14021-0251.195), *D. virilis* (15010-1051.87), *D. willistoni* (14030-0811.24), *D. yakuba* (14021-0261.01). All cultures were maintained at 24 °C on a 12-h/12-h light–dark cycle.

Collection of eggs and embryos

Mated females were placed on oviposition plates made of yeasted agar. In the case of *D. sechellia*, a drop of octanoic acid, the active component of their natural substrate, *Morinda citrifolia* was added to the medium. Plates were inspected every 15 min in order to obtain eggs as close to the time of oviposition as possible. All eggs were measured within 1 h of oviposition. For each species, the lengths and widths of 24 eggs were measured and their volumes were determined according to the formula $(1/6)\pi W^2 L$. The rest of the eggs were allowed to develop and were observed hourly to determine when first instar larvae hatched and embryonic development was complete. The minimum number of embryos observed was 44 for *D. pseudoobscura* and the maximum was 113 for *D. erecta*.

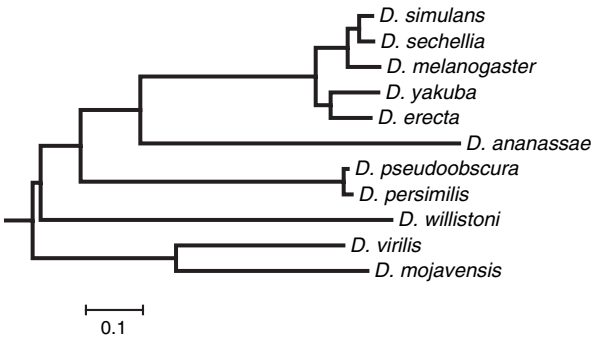

Fig. 1 Phylogenetic relationships among the 11 species studies. Tree is based on fourfold degenerate sites of the annotated genome sequences of the species (neighbour-joining method). Tree was modified form Drosophila 12 Genomes Consortium, 2007. Scale represents number of mutations per site.

Statistical analyses

Given the bias associated with the examination of phylogenetically related species, our analysis was based on phylogenetically independent contrasts of embryonic developmental time and egg volume. Phylogenetically independent contrasts were calculated using the relationship of *Drosophila* species shown in Fig. 1 (with equal branch lengths) and the CAIC version 2.6.9 software (Purvis & Rambaut, 1995). The relationships between the phylogenetically independent contrast of egg volume and embryonic developmental time were examined by performing correlations of 'positivized' contrasts through the origin as suggested by Garland *et al*. (1992). All statistical analyses (ANOVA, regression and correlations) were performed using the JMP version 5 software (SAS Institute, Cary, North Carolina).

Results

Egg size

Egg width (ANOVA, $F_{10,253} = 107.3$, $P < 0.001$), length (ANOVA, $F_{10,253} = 175.9$, $P < 0.001$) and volume (ANOVA, $F_{10,253} = 223.7$, $P < 0.001$) vary significantly among species (see Table 1). Egg widths and lengths both varied by approximately 23% across species. Widths ranged from 0.16 mm for *D. pseudoobscura* and *D. mojavensis* to 0.21 mm for *D. sechellia*. Lengths ranged from 0.57 mm in *D. sechellia* and 0.54 mm in *D virilis* to 0.44 mm in *D. pseudoobscura* and *D. mojavensis*. The eggs of *D. sechellia* were more than twice the volume of the smallest eggs produced by *D. pseudoobscura* and *D. mojavensis*.

Embryonic development time

Our initial goal was to determine the time from fertilization until hatching of first instar larvae. Because

Table 1 Egg widths, lengths and volumes, embryonic development (±SE), and ovariole numbers in 11 *Drosophila* species.

Species	Egg width (mm)	Egg length (mm)	Egg volume (mm^3) ($\times 10^{-3}$)	Embryonic development time hours (*n*)	Ovariole number*
D. simulans	0.18 ± 0.001	0.47 ± 0.003	8.58 ± 0.15	20.60 ± 0.18 (62)	40
D. sechellia	0.21 ± 0.001	0.57 ± 0.003	13.54 ± 0.17	1.68 ± 0.18 (56)	16
D. melanogaster	0.18 ± 0.001	0.51 ± 0.003	9.02 ± 0.14	22.24 ± 0.27 (53)	43
D. yakuba	0.18 ± 0.001	0.49 ± 0.004	8.52 ± 0.17	13.68 ± 0.80 (76)	28
D. erecta	0.18 ± 0.002	0.49 ± 0.002	8.05 ± 0.14	17.80 ± 0.44 (113)	27
D. ananassae	0.18 ± 0.002	0.49 ± 0.002	7.90 ± 0.17	19.05 ± 0.09 (93)	30
D. pseudoobscura	0.16 ± 0.001	0.44 ± 0.002	5.66 ± 0.10	23.39 ± 0.39 (44)	44
D. persimilis	0.17 ± 0.001	0.44 ± 0.002	6.80 ± 0.15	26.05 ± 0.15 (54)	36
D. willistoni	0.18 ± 0.001	0.49 ± 0.003	8.62 ± 0.14	23.52 ± 0.15 (53)	36
D. virilis	0.18 ± 0.001	0.54 ± 0.004	9.20 ± 0.12	31.90 ± 0.17 (51)	34
D. mojavensis	0.16 ± 0.001	0.44 ± 0.003	5.88 ± 0.06	28.59 ± 0.26 (68)	26

*From Markow & O'Grady, (2005).

fertilization occurs internally, the exact moment it happens cannot be directly measured. Females without attractive oviposition substrates may retain fertilized eggs for various periods. Retention of fertilized eggs, however, usually is preventable by frequently transferring females to fresh oviposition substrates suitable for the particular species (Nash *et al.*, 1973; Williamson *et al.*, 1978; Moreteau *et al.*, 1994) and this was the approach we employed in the current study. Thus, oviposition is assumed to occur within minutes of fertilization.

Embryonic development time was significantly different across the 11 species examined (ANOVA, $F_{10,716} = 363.2$, $P < 0.001$). Figure 2 presents the cumulative distributions of the times between oviposition and the hatching of first instar larvae. The striking variability among species, however, cannot entirely be attributable to embryonic development time. In *D. sechellia* and to a lesser extent in *D. yakuba*, females clearly retain fertilized eggs in their reproductive tracts for long periods. This pattern was unchanged by the addition of the active ingredient, octanoic acid, from *D. sechellia*'s native host, *M. citrifolia* (Legal *et al.*, 1994), by the density of females or by the presence of males. In *D. yakuba*, however, a small number of larvae did not hatch until approximately 20 h, indicating that retention is somewhat facultative.

A small degree of egg retention was observed in all species, but for the most part, all of embryonic development occurred after oviposition and required different periods of time in different species. With the exception of *D. sechellia* and *D. yakuba* the cumulative graphs in Fig. 2, along with the standard errors associated with the mean development times (Table 1), show clear interspecific differences in actual embryogenesis durations (ANOVA without *D. sechellia* and *D. yakuba*, $F_{8,586} = 224.3$, $P < 0.001$).

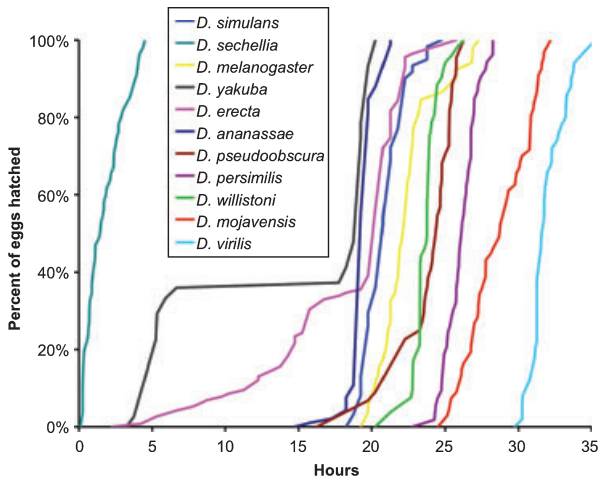

Fig. 2 Cumulative distribution of hatching times for all 11 species.

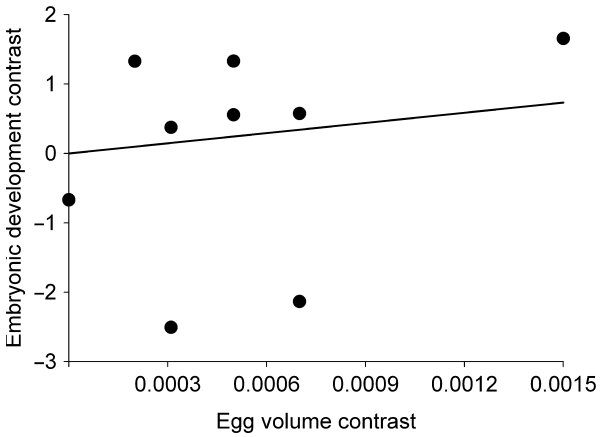

Fig. 3 Phylogenetically standardized contrasts for egg volume and embryonic developmental time. Correlation ($r^2 = 0.05$, $P = 0.56$) is shown.

Egg size and embryonic development duration

Testing for a relationship between egg size and embryonic development time must be performed in a phylogenetic context. Furthermore, our ability to make inferences using the species studied here is mitigated by the egg retention behaviour in *D. sechellia* and to a lesser extent in *D. yakuba*. For *D. yakuba*, the data were clearly bi-modal (see Fig. 2), therefore, for the independent contrast analysis we used only *D. yakuba* data from the greater mode and omitted *D. sechellia* data. Analysis based on the omission of both *D. yakuba* and *D. sechellia* produced identical results (not shown). Across the species analysed, there was no overall relationship between the size of the eggs and the embryonic developmental time (Fig. 3).

Discussion

Among the 11 *Drosophila* species whose genomes have been sequenced, there exists marked variability in egg size and embryonic development time. We found no support, however, for the hypothesis that egg volume is related to the length of the embryonic period. The lack of relationship between egg volume and embryonic development time (Fig. 3) suggests that, at least to some degree, these two traits are genetically independent.

With respect to embryonic gene expression, an obvious question is whether, in eggs that differ either in their size or embryogenesis duration, gene expression is scaled spatially and temporally? Lott *et al.* (2007) investigated spatial and temporal features of segmentation genes among strains of *D. melanogaster*, *D. simulans* and *D. sechellia*, as they had noted the substantial interspecific difference in egg size. Localization of gene expression relative to embryo length was found to differ significantly between all three species (Lott *et al.*, 2007). Similar

studies in additional species, such as the ones described here, for which full genome sequences are available, should reveal any consistent changes in expression associated with particular egg sizes, shapes or embryogenesis duration.

Surprisingly, two species, *D. sechellia* and to a lesser extent *D. yakuba,* exhibited striking retention of fertilized eggs in the uterus. *Drosophila* are considered to be oviparous, the type of reproduction in which fertilized eggs develop outside of the mother. At the other extreme are viviparous organisms in which embryonic development takes place inside the maternal reproductive tract, which transfers nutrients to the embryo and eliminates wastes. Ovoviviparity, thought to be an intermediate stage in the development of viviparity, occurs when fertilized eggs remain within the mother until they hatch or are about to hatch (Sellier, 1955). In ovoviviparous animals, all nutrition is derived from the egg's yolk rather than from the mother's body. Meier *et al*. (1999) suggest that viviparity has evolved at least 61 times within oviparous Diptera.

Most *Drosophila* researchers have observed that in many species, including *D. melanogaster*, females kept away from appropriate oviposition sites will retain fertilized eggs, hence exhibiting some degree of facultative ovoviviparity. In the present study, we handled *D. sechellia* females in a variety of ways that included different food types, substrate change frequency, crowding levels, presence of males and presence of their natural host *M. citrifolia*. On other occasions we have observed that an oviposited *D. sechellia* egg takes approximately 20 h before a larva hatched. Although these few examples were sporadic and observed to be outside the scope of the present study, they do demonstrate that egg retention, or ovoviviparity, in *D. sechellia* is at least somewhat facultative and strains may differ in the degree to which it occurs. The fact, however, that this phenomenon was so extreme in *D. sechellia* and to a lesser degree in *D. yakuba* leads us to speculate that these species represent an early step in the evolution of viviparity.

Ovoviviparity has been suggested to occur in nature in a number of *Drosophila* species, primarily flower breeders (Wheeler *et al*., 1962; Brncic, 1966; Pipkin, 1966; Throckmorton, 1966; Kambysellis & Heed, 1971; Hunter, 1979, 1988, 1992; Montague, 1984; Do Val & Marques, 1996). Both *D. sechellia* and *D. yakuba* are specialists, although not on flowers. However, perhaps specialization is a prerequisite for the development of facultative ovoviviparity. In other Diptera, ovoviviparity is associated with a reduction in ovariole number and an increase in egg size (Meier *et al*., 1999), a syndrome clearly observed in *D. sechellia*.

Of significance beyond the morphological changes accompanying shifts to ovoviviparity are the implications for male and female reproductive molecules controlling fertilization and oviposition in *Drosophila*. *Drosophila* male ejaculates contain at least 100 proteins, a number of which have been demonstrated to stimulate oviposition in the inseminated female (Wolfner, 2006). Obviously even in *D. melanogaster*, where females retain fertilized eggs in the absence of an oviposition site, some mechanism exists in the female to override the effects of the male-derived oviposition stimulants. In species with a stronger tendency for facultative ovoviviparity, such as *D. sechellia,* female control over oviposition must have developed to a greater degree. Although such control could exist at any of several levels, its identification and elucidation would provide important insights into the female side of reproductive control.

Eggs and pupae, being immobile, are life stages particularly vulnerable to environmental stressors such as heat, as well as to parasites and predators. For *D. melanogaster,* the embryonic period is 50% shorter than that of *D. virilis.* It's likely, however, that *Drosophila* embryogenesis can be shortened only thus far. Beyond that, should vulnerable embryos require further protection, the remaining option is larvaposition, or oviposition of an embryo that is about to hatch a first instar larva capable of burrowing into its substrate. In other Diptera, correlates of ovoviviparity are reduced ovariole number, large eggs and resource specialization (Meier *et al*., 1999). Indeed, resource specialization, on flowers, is characteristic of those Drosophilids mentioned above in which ovoviviparity was reported. In *D. sechellia,* not only have they specialized on *Morinda* fruit, but they have a vastly reduced ovariole number and a significantly larger egg relative to the other species (R'kha *et al*., 1997). The other fly exhibiting facultative ovoviviparity is *D. yakuba*, also a specialist, on *Pandanus*, although they exhibit no striking changes in ovariole number or egg size. These differences, especially in species so well characterized at the genome level, provide an unparalleled opportunity to examine the evolutionary genetic underpinnings of reproduction and development.

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