[REVIEW]

Developmental Biology of the Sawfly, *Athalia rosae* (Hymenoptera)

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The order Hymenoptera is one of the most successful groups of insects and includes well over 100,000 species. So remarkable with this group is the fact that essentially all the members show haplo-diploidy: unfertilized eggs develop into haploid males (arrhenotoky: generative or haploid parthenogenesis) and fertilized eggs develop into diploid females (Crozier, 1975; Suomalainen *et al.*, 1987; Stouthamer *et al.*, 1990). In this group, indeed, egg activation and fertilization are separate events.

These characteristics in hymenopteran insects might provide a unique means of studying some of the important problems in developmental biology such as the mechanisms of egg activation and fertilization, the subjects which are little studied in insects but are being studied extensively in other animal groups, both invertebrates and vertebrates (Metz and Monroy, 1985; Dale, 1990). As a hymenopteran species with properties convenient as an experimental animal, we chose the sawfly, *Athalia rosae* (Tenthredinidae), a species belonging to the primitive suborder Symphyta.

In this review, we describe the results we obtained so far relating to the developmental processes of egg maturation, activation and fertilization. Since the sawfly *Athalia rosae* is probably not a familiar species even to the members of the Arthropodan Embryological Society of Japan and since our studies are mostly still in the preliminary stage, we will describe what can be done with this animal rather than present a critical review on the causes of developmental processes.

What is the sawfly, *Athalia rosae*?

First, we would like to introduce the reader to the sawfly, *Athalia rosae*. The order Hymenoptera is usually divided into two suborders, Apocrita (bees, wasps, ants, *etc.*) and Symphyta (sawflies, horntails, *etc.*). Sawfly larvae look much like those of Lepidoptera, and feed on leaves of various plants. *A. rosae* larvae feed on the family Cruciferae (Brassicaciae) and thus can be a serious agricultural pest. *A. rosae* is widely distributed in the Palearctic region and the form that occurs in the eastern half, including Japan, is a subspecies *A. rosae ruficornis*, while the one in the western half is a subspecies *A. rosae rosae*. *A. rosae* in Japan is a multivoltine species without estivation and produces 3-7 generations from late spring to late fall, depending on the local climate. During the winter they hibernate as prepupae (Abe, 1988).

A. rosae can be reared continuously in the laboratory, and the stocks can be maintained if kept at 25℃ under a 16 h light-8 h dark condition (Sawa *et al.*, 1989). Under these conditions one generation takes about 25 days. Egg shells are sufficiently transparent so that embryonic development (5 days) can be followed without any pretreatment (Fig. 1). Larval development takes 9 days in the female (6 instars) and 8 days in the

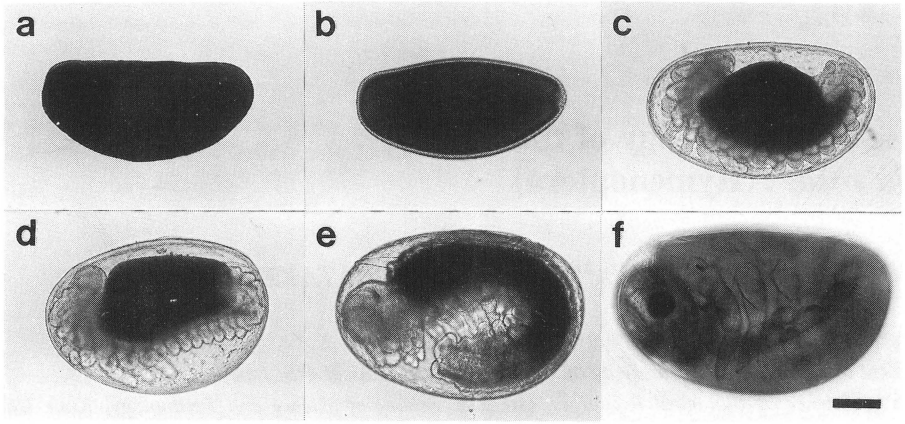

Fig. 1 Embryonic development in *Athalia rosae* as observed in fresh samples. Note the transparency of egg shell. The anterior end of the embryo is at the left and the dorsal side is at the top. a: 0 h, b: 15 h, c: 48 h, d: 55 h, e: 72 h, f: 114 h. Bar = 200 μm.

male (5 instars). Prepupal and pupal development takes 10 days in the female and 11 days in the male. Adults, both females and males, live about two weeks if fed on diluted honey.

Sex is determined by the single-locus multiple-allele system (Naito and Suzuki, 1991). Diploid males, homozygous for a sex determination allele and distinctly larger than haploid males in body size, can be obtained easily in the F_2 generation by making F_1 brother-sister matings. Both haploid and diploid males show non-reductional maturation division and produce haploid and diploid sperm, respectively. Triploid females, obtained by crossing diploid females and diploid males, do show normal maturation division and produce aneuploids hence mostly are sterile.

The adult ovary consists of about 14 meroistic and polytrophic ovarioles, each of which contains up to ten egg chambers (Fig. 2). The number of nurse cells per egg chamber shows considerable variation and does not

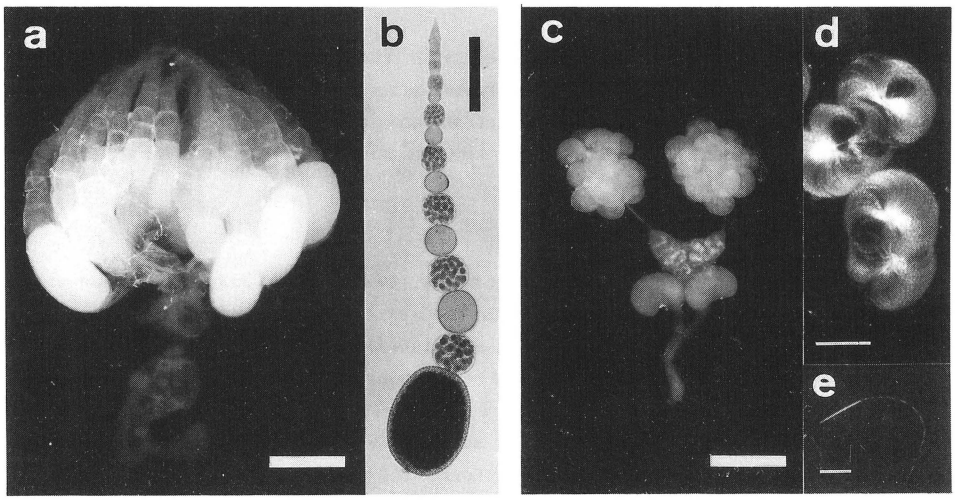

Fig. 2 A whole mount fresh preparation of internal reproductive organs from a young adult female (a), a single Feulgen-stained ovariole (b), a whole mount fresh preparation of internal reproductive organs from a young adult male (c), fresh sperm bundles (d), and a single sperm (e). Bars = a, c, 500 μm; b, 300 μm; d, 50 μm; e, 10 μm.

follow the 2^n-1 rule (Hatakeyama *et al.*, 1990a). Females store mature eggs in the ovarioles until they find fresh leaves of the Cruciferae plants to oviposit. Each female lays up to 100 eggs (Sawa *et al.*, 1989).

The adult testis consists of about 15 spheroidal bodies connected centripetally to the single sperm duct and eventually to the seminal vesicle (Fig. 2). Active spermatogenesis takes place in the spheroidal bodies during larval, prepupal and pupal development and up to a very young adult stage. Within a few days post-emergence, the spheroidal bodies become nearly empty. Sperm in the seminal vesicle are still in bundles, and the number of sperm per bundle varies considerably (Sawa *et al.*, unpublished; Iwaikawa, Y., personal communication). A mature sperm measures about 55 μm in length, of which 15 μm is the straight-shaped head and 40 μm is the curved tail with the appearance of a screw or spiral (Sawa *et al.*, 1989).

Unfertilized *Athalia rosae* eggs can be activated *in vitro* to develop into haploid males, and with a little trick into diploid females

Earlier we mentioned that unfertilized hymenopteran eggs (oocytes) develop into haploid males. This parthenogenetic egg activation occurs regularly in the laid eggs, but artificial activation in the unfertilized mature eggs explanted from the ovary has not been possible in most species (Sander, 1990). Tenthredinidae sawflies are exceptional: in more than 200 species examined the explanted eggs are activated to develop simply by immersing them in distilled water (Naito, 1982). Furthermore, the *A. rosae* mature eggs explanted can be activated to develop by various other means such as exposure to acidic pH, pricking with a needle, and brief desiccation (Sawa and Oishi, 1989a).

Generally, the mature insect eggs (oocytes) are arrested at the first meiotic metaphase and wait to be fertilized or activated. Subsequent to fertilization (or activation) meiosis resumes and as a rule results in the production of four nuclei, one of which becomes the egg pronucleus and three others form the polar body nuclei. Only the pronucleus participates in development and the polar body nuclei degenerate. How this is possible, that is, only one nucleus among four nuclei all in close proximity in the same cytoplasm participates in development, remains an important question to be solved.

In *A. rosae,* explanted eggs can be activated *in vitro* by immersing them in distilled water and they develop into haploid males as mentioned above. When the explanted eggs are first placed in ice-cold saline solution (in which eggs remain unactivated and in a healthy condition for up to 1 h) and then transferred to distilled water and activated, not at 25℃ as at an ordinary but at higher temperature (35-37℃), all four nuclei produced following the completion of meiosis are now capable of participating in development with or without fusion among themselves (Hatakeyama *et al.*, 1990b). As a result haploid-haploid male mosaics, haploid-diploid gynandromorphs (Fig. 3), diploid males, diploid and triploid females are produced (diploid females are most frequent). Clearly there exists a cytoplasmic factor(s) that determines the presumptive fate of the nuclei which is inactivated by treatment at 35-37℃.

The results also are of practical importance: *A. rosae* can be maintained in the absence of males. Effects of certain mutations (such as haploid male steriles and lethals) in homozygous diploid individuals can only be examined by this means.

Athalia rosae* eggs can be fertilized *in vitro

In vitro fertilization in insects has been attempted from time to time and success has been claimed in some cases (reviewed in Clarke, 1990 and in Sander, 1990). Unfortunately genetic markers were not employed in any cases other than *A. rosae* to be described below. Hence, the success is questioned since the possibility of rare parthenogenesis cannot be eliminated.

We attempted *in vitro* fertilization in *A. rosae* by a very simple means, but employing a genetic marker *yfb*, a non-autonomous mutation which alters the color of the fat body in late larvae, prepupae and pupae up to middle pupal stage from the dark blue of the wildtype to bright yellow (Sawa and Oishi, 1989b).

We took sperm bundles from the male (*yfb* or +) seminal vesicles, suspended in distilled water, dispersed by pipetting and injected about a dozen or more each into explanted mature eggs (+/+ or *yfb*/*yfb*). If fertilization takes place +/*yfb* diploid females will develop, and if not either + or *yfb* haploid males will de-

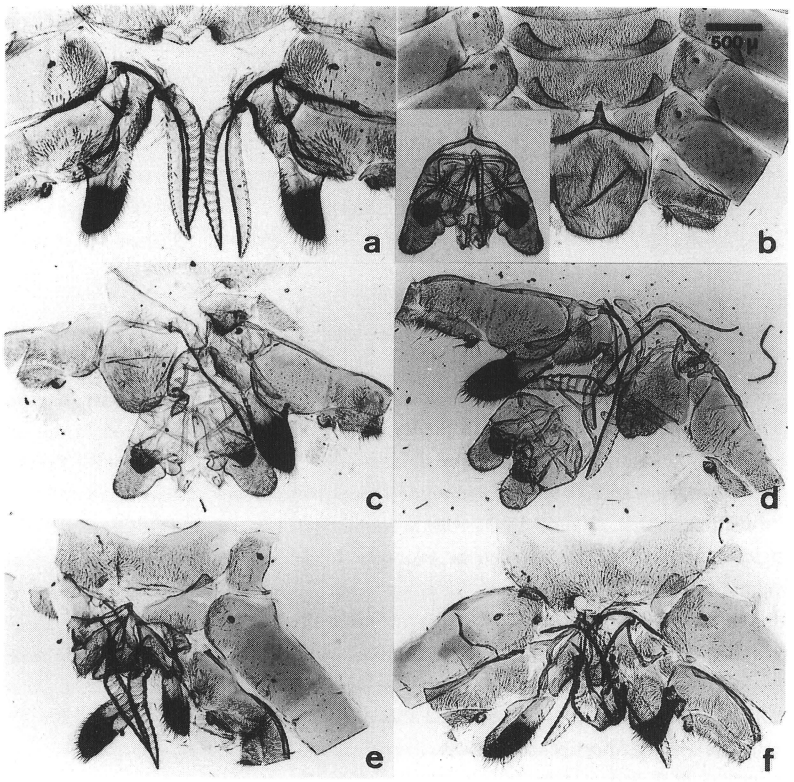

Fig. 3 Cuticular structures of the abdominal terminal in a normal female (a), a normal male (b), and gynandromorphs (c-f).

velop. About 10% of the injected eggs developed as diploid *+/yfb* females. These females were aged and eggs explanted and activated *in vitro*. In each and every case *+* and *yfb* haploid males appeared in a 1 : 1 ratio, confirming the diploid heterozygous nature of the mothers (Sawa and Oishi, 1989b).

The rate of *in vitro* fertilization can be increased greatly by injecting sperm into the eggs 20 min post-activation (corresponding to the anaphase-telophase of the first meiotic division). More than 20% of the eggs develop as fertilized diploid females. On the other hand, injection into the eggs 60 min post-activation results in the absence of fertilization (Sawa and Oishi, 1989c).

Since sperm were injected directly into the cytoplasm, the membrane had to be disrupted in some way for the sperm to be effective. This probably explains the effectiveness in the sperm suspended in distilled water. However, an important question remains to be answered. A dozen or more sperm were injected into each egg. When fertilization was successful, apparently the fusion of a single egg nucleus and a single sperm nucleus took place. What became of other sperm nuclei? More specifically, can the sperm nucleus participate in development independent of the egg nucleus? In the experiments mentioned above we injected sperm at the anterior pole of the eggs where sperm entry *in vivo* should take place. No sperm nuclei participated in development independently, or sperm nucleus participated in development only when it fused with the egg nucleus. When the sperm injection was performed at the posterior end of the eggs, however, sperm nucleus did participate in development independent of the egg nucleus in a small fraction (about 1%) of the cases, forming haploid-haploid chimeras (Hatakeyama *et al.*, 1994a). Progeny tests showed that both egg nucleus-derived nuclei and injected sperm-derived nuclei contributed in forming the germ cells of the chimeric males, indicating the occurrence of extensive mixing of cleavage nuclei during migration. Unfortunately it has not been possible to increase the fre-

quency of chimeric development.

As noted above sperm with some damage in membrane structure were effective in fertilizing the eggs *in vitro* by microinjection into the cytoplasm. This implies that abnormal non-motile sperm (*e.g.*, simple frozen sperm without cryoprotectant, and hence "dead") is capable of fertilization when micro-injected into the egg cytoplasm. Indeed this was shown to be the case. Sperm bundles taken from the male seminal vesicles were suspended in saline solution and directly placed into liquid nitrogen, and then kept in a deep-freezer at −80℃ for a month. The sperm were thawed in distilled water and injected as above into mature explanted eggs. Fertilization was observed although the rate was lower than that when fresh sperm were used (Hatakeyama *et al.*,1994b). The results indicate that the simply cryopreserved "dead" sperm can be regarded as a fully capable genetic reserve.

Sperm penetration in the normal pathway involves the acrosome reaction which activates the egg. Sperm injection into the cytoplasm of oocytes has been successfully employed to produce live offspring in the cow and in humans (reviewed in Goto, 1993). Additional stimulations such as electric pulse, however, had to be given to activate the eggs. In the *A. rosae* system, activation is induced in mature explanted eggs simply by immersing them in distilled water. Whether the injection of sperm into mature oocytes in insects other than Tenthredinidae hymenopterans, in which artificial activation has not been successful, results in fertilization and production of progeny remains to be seen.

***Athalia rosae* eggs can mature with heterospecific yolk protein**

In most insect species, yolk proteins are synthesized in the fat body as a precursor (vitellogenin, Vg), secreted into the hemolymph and then taken up by the developing oocyte and deposited (vitellin, Vn) (reviewed in Kanost *et al.*, 1990 and in Wyatt, 1991).

The *A. rosae* eggs contain two Vns, L (for large) with an apparent molecular mass of 180 kDa and S (for small) with that of 50 kDa on SDS-PAGE, and the female but not male hemolymph contains the corresponding Vgs, L-Vg and S-Vg (Hatakeyama *et al.*, 1990a; Kageyama *et al.*, 1994) (Fig. 4). We have cloned the cDNA for the *A. rosae* Vg and characterized the Vg gene expression (Kageyama *et al.*, 1994). The two Vgs are encoded by a single gene. The Vg gene is transcribed in the adult female fat body cells as a single 6.5 kb-long mRNA, which is translated into a single long polypeptide, >200 kDa in molecular mass. This long polypeptide is cleaved into two smaller polypeptides, pro-S-Vg from the N-terminal portion and pro-L-Vg from the C-terminal portion, which are then secreted into the hemolymph. Partial nucleotide sequencing show some interesting features. One is that the signal peptide contains no charged amino acid contrary to the situation in other invertebrate and vertebrate Vgs. The other is that the sequence in the S-L boundary region contains unusually long stretches of serine residues. These serine-rich sequences, similar to those of vertebrate phosvitins, were presumed to be vertebrate-specific (Nardelli *et al.*, 1987; Wahli, 1988). Recently, Chen *et al.* (1994) also observed the presence of serine-rich sequences in the mosquito (*Aedes aegypti*) Vg.

It is possible to induce the expression of the Vg gene in the adult male *A. rosae* fat body cells by a topical application of juvenile hormone III (Hatakeyama and Oishi, 1990). Immature previtellogenic ovaries mature when transplanted into the abdomen of adult males if they have received a topical application of juvenile hormone III. The eggs not only accumulate Vns but can complete development upon *in vitro* activation (Hatakeyama and Oishi, 1990). To what extent, then, are the yolk proteins of one species capable of supporting the development of another species?

The transplantation experiment was extended to see if *A. rosae* immature previtellogenic ovaries can accumulate heterospecific Vns in other host species. As a first step we chose *Athalia infumata.* This species lives sympatrically with *A. rosae.* There exists a strong premating isolation between the two species. Hybrids can be obtained by sperm injection, although they do not complete development (Sawa, 1991). *A. infumata* has two Vns and the corresponding Vgs which are indistinguishable from those of *A. rosae* on SDS-PAGE and on Western blot analysis by using the anti-*A. rosae* Vn antisera. However, the Northern blot analysis by using subclones of *A. rosae* Vg cDNA indicated that the *A. infumata* Vg mRNA is similar to but not identical with the *A. rosae* Vg mRNA (Hatakeyama *et al.*, 1995).

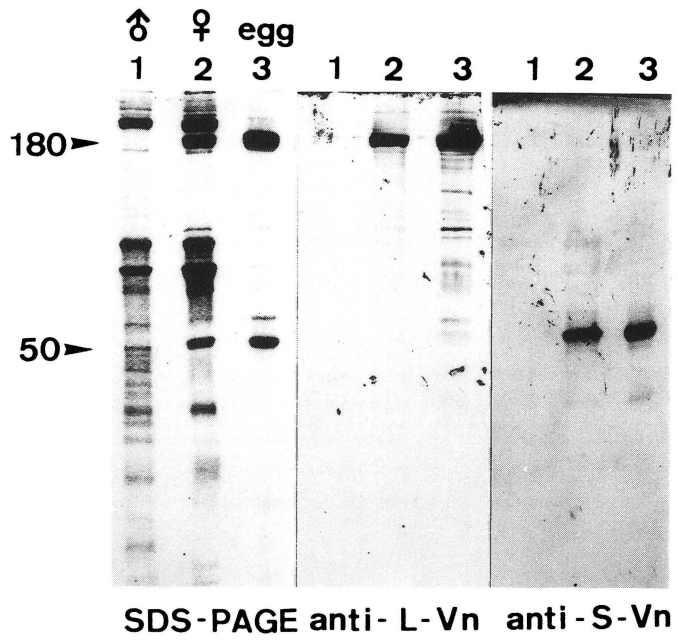

Fig. 4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of adult male and female hemolymphs, and of extracts from mature unfertilized eggs (Coomassie blue-stained) (left), and the results of Western blot analyses by using the anti-L-Vn and -S-Vn antisera, respectively (middle and right). Shown at the far left are molecular masses in kDa.

The transplanted previtellogenic *A. rosae* ovaries accumulated solely the heterospecific *A. infumata* Vns, and the eggs thus matured completed development upon *in vitro* activation. This was shown unequivocally by the Northern blot analysis. The Vg gene was induced to express in the host *A. infumata* male fat body cells upon topical application of juvenile hormone III, but was not expressed in the transplanted *A. rosae* ovaries (Hatakeyama *et al.*, 1995).

The results, together with the sperm cryopreservation mentioned earlier, bring about an interesting possibility. If previtellogenic ovaries were cryopreserved and then taken out and matured in transplanted heterospecific hosts, it may be possible to recover a species from these ovaries and cryopreserved sperm in the absence of the original species.

In the present review, we have concentrated on describing what can be done experimentally with *A. rosae,* and largely ignored the basic biological aspects such as descriptions of early embryonic development, and of spermatogenesis. Nor did we discuss the implications of the fact that non-reductional maturation division is sex (male)-specific and is independent of ploidy. We hope to have an opportunity to review these aspects in the future.

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