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Genetics and biology of the sawfly, *Athalia rosae* (Hymenoptera)

Review

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Abstract

Hymenopteran insects are a unique group of animals in which arrhenotokous reproduction (haploid males develop from unfertilized eggs) is a rule. Males produce sperm through a non-reductional maturation division. A sawfly species, *Athalia rosae ruficornis* Jakovlev (Tenthredinidae, Symphyta, Hymenoptera), has been introduced as a new experimental material for studies on genetics and developmental biology. Basic features relating to the potential usefulness of the species in elucidating some of the important genetic and developmental biological problems are described.

Introduction

The insect order Hymenoptera is one of the largest orders in terms of the species included, and is divided into two suborders, Symphyta (sawflies, etc.) and Apocrita (wasps, ants, bees, etc.). Studies on the genetics and biology of hymenopteran insects have enjoyed attention from various sectors in the intellectual world. These come partly from the peculiar reproductive system (virtually all the species show arrhenotoky, namely unfertilized eggs develop parthenogenetically into haploid males while fertilized eggs develop to diploid females) and partly because the order includes groups with such unique and/or economically important features as sociality and parasitoidism. For these latter aspects the readers are referred to recent publications (e.g. Gauld & Bolton, 1988; Ross & Matthews, 1991).

While most studies, especially those concerned about genetics and developmental biology, have been carried out using the species in the advanced suborder Apocrita (DuPraw, 1967; Cassidy, 1975; Rothenbuhler, 1975), we believe that the lower suborder Symphyta provides better materials for studies on such subjects as egg maturation, activation, fertilization, and on meiotic divisions. For example, mature eggs explanted from adult females of the sawflies in the Family Tenthredinidae (Symphyta) can be activated *in vitro* simply by placing them in distilled water (Naito, 1982); in at least one species, *Athalia rosae*, diploid males can be easily obtained who show non-reductional maturation division and produce diploid sperm (Naito & Suzuki, 1991). Studies on the lower suborder should also provide a balanced understanding on the evolutionary aspects of the order. We thus began serious work using the sawflies in the hope that the results obtained in this insect system might be compared with those obtained in other groups of animals, both invertebrate and vertebrate.

General biology

We have chosen the sawfly, *Athalia rosae*, as an experimental animal because this species is easily maintained in the laboratory and also because the formal genetic mechanism of sex determination is known. Since, however, it is a newly-introduced experimental animal for genetic and developmental studies, a brief description of its general biology may be helpful. *A. rosae* is widely distributed in the Palearctic region and the form that occurs in the western half is a subspecies called *A. rosae rosae*

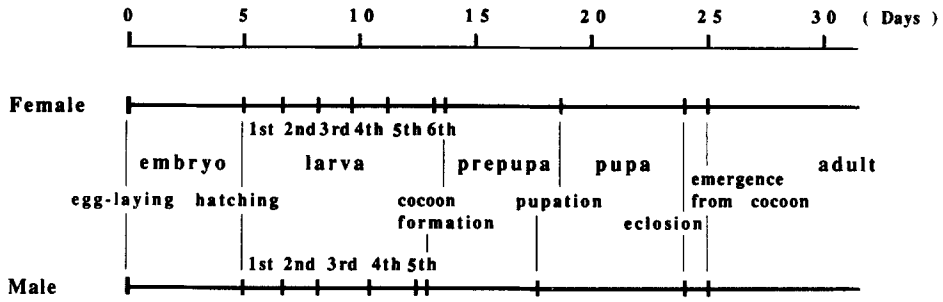

Fig. 1. Life cycle of *Athalia rosae* maintained at 25 °C under a 16 h light-8 h dark condition.

while the one in the eastern half, including Japan, is *A. rosae ruficornis* (Benson, 1962; Abe, 1988). Because the larvae feed on leaves of various species of the Family Cruciferae (Brassicaciae), they are known to be a serious agricultural pest (Saringer, 1976; Abe, 1988). In fact, the genus name itself suggests such damage: athal, Greek meaning not green, withered. Athalia, named in allusion to the devastation produced by its larvae (Jaeger, 1978). *A. rosae ruficornis* is a multivoltine species without aestivation and in Japan it produces 3-7 generations from the late spring to the late fall, depending on the local climate. During the winter they hibernate as prepupae.

A. rosae ruficornis can be reared continuously in the laboratory, and the stocks can be maintained if kept at 25 °C under a 16 hr light-8 hr dark condition (Sawa *et al.*, 1989). Under these conditions one generation takes about 25 days (Fig. 1). Mature eggs measure about 800μm in length and 350 μm in diameter. Egg shells are sufficiently transparent so that embryonic development can be followed easily with the help of the dissecting microscope. Larvae, caterpillar-like in morphology (resembling to those of lepidopteran insects), actively feed on leaves of cruciferous plants. The last instar larvae (6th instar in the female and 5th instar in the male) leave the plant, crawl around, dig into the soil, construct cocoons and become prepupae. Middle- to late-prepupae in the cocoons may be placed in a refrigerator and kept there for at least a month. Old prepupae and pupae may be removed from the cocoons so that the development to adulthood can be observed directly. Adults eclosed stay in the cocoon for a day and then come out through a hole they make at the anterior pole. Adults measure about 8 mm in length in the female and 6 mm in the male. Both females and males live, if fed on diluted honey, about 2 weeks, and each female lays up to 100 eggs.

Sex determination

Mechanism of sex determination in arrhenotokous hymenopteran insects has been studied using several symphytan as well as apocritan species (Crozier, 1975). At least in some species the sex is determined by the single-locus multiple-allele mechanism; hemizygous and homozygous (homoallelic) individuals develop into males, while heterozygous ones develop into females. *A. rosae ruficornis* conforms to this system as shown below (Naito & Suzuki, 1991).

Single-pair matings were made between virgin diploid females (collected in nature as larvae and reared to adults in the laboratory) and haploid males (obtained as above) derived from separate local populations. The F_1 progeny pupae were examined individually for the body weight and the head width, and a fraction of them were sacrificed to determine the ploidy cytologically (chromosome number, n = 8: Naito, 1982) by using the testis or the ovary. In all crosses only diploid females and haploid males were obtained; these females were distinctly heavier and larger than males. A number of single-pair sib matings were then made for each of the selected initial crosses and the F_2 progenies were examined similarly. In each case, about half the matings produced diploid females, haploid males and diploid males which weighed just in between diploid females and haploid males and

measured close to diploid females in head width. The remaining half of the matings produced only diploid females and haploid males. For those in which diploid males appeared, single-pair sib matings were made between diploid females and haploid males, and diploid females and diploid males. When the F_3 progenies were examined as above, the former crosses again produced diploid females, haploid males and diploid males. The latter crosses, however, produced triploid females (heavier and larger [in head width] than diploid females though distributions overlap) and, for the first time in hymenopteran insects, triploid males (measured both in weight and head width just as diploid males, thus positively identifiable only upon chromosome examinations), in addition to haploid males (apparently derived from unfertilized eggs).

These results can best be, and in fact can only be, explained by the single-locus multiple-allele system. Parental crosses were, say, $X^a/X^b \times X^c$, where X denotes the sex determination locus and the superscripts indicate various alleles. The F_1 progeny are X^a/X^c and X^b/X^c (both are diploid females derived from fertilized eggs), and X^a and X^b (both are haploid males derived from unfertilized eggs). By chance one half of the single-pair sib matings, $X^a/X^c \times X^a$ and $X^b/X^c \times X^b$, would produce diploid males, X^a/X^a and X^b/X^b, while in the remaining half of the crosses, $X^a/X^c \times X^b$ and $X^b/X^c \times X^a$, no diploid male progeny would appear. Single-pair sib matings, for example, between X^a/X^c diploid females and X^a/X^a diploid males would then produce $X^a/X^a/X^c$ triploid females and $X^a/X^a/X^a$ triploid males. That diploid males produce diploid sperm as a result of non-reductional maturation division has been shown cytologically (Naito & Suzuki, 1991). Triploid males similarly produce triploid sperm, but are not fertile when crossed to diploid females although the sperm are deposited in the female spermatheca (Naito & Suzuki, 1991). Triploid females are largely infertile apparently because of the abnormal chromosome disjunction (Hatakeyama *et al.*, 1990b; Naito & Suzuki, 1991).

Nothiger and Steinmann-Zwicky (1985), in their attempt to find a common principle for sex determination in insects, have proposed that the Sex-lethal (*Sxl*) gene (a pivotal gene for sex determination identified in *Drosophila melanogaster*) also plays an essential role in the Hymenoptera and that the gene product is only active when in heterozygous individuals. As the *Drosophila Sxl* gene has been cloned and is available as molecular probes (Keys *et al.*, 1992 and the references therein), the above proposal can and should be pursued.

Ovarian development and vitellogenesis

Ovarian development

The ovary of an adult female consists of about 14 meroistic and polytrophic ovarioles, each of which contains up to ten egg chambers. The posterior more mature egg chambers in the one-day-old (after emergence from the cocoon) adult females (see Fig. 1) tend to contain about 60 nurse cells but deviations, from 40 to 80, are frequently observed. In the anterior less mature egg chambers the number becomes smaller to about 30 again with frequent deviations (Hatakeyama *et al.*, 1990a).

The number of nurse cells per egg chamber is, for example, seven in most lepidopteran insects and 15 in *Drosophila*. A notable exception is seen in the honeybee, *Apis mellifera* (Apocrita, Hymenoptera), in which the number is about 48, clearly violating the 2^n-1 rule (cited and discussed in Telfer, 1975). Whether this violation of the rule is common in the Hymenoptera remains to be seen.

The ovaries in *A. rosae* become distinguishable from the testes in the third instar larvae. Differentiation of germ cells to the oocyte and nurse cells become apparent during the late-larval and prepupal stages. Vitellogenesis begins in the late pupal stage and continues to the adult life (Hatakeyama *et al.*, 1990a).

Vitellogenesis

In most insect species, yolk proteins (vitellins, Vns) are first synthesized as a precursor form (vitellogenins, Vgs) in the female (but not male) fat body, secreted into the hemolymph and then taken up by the developing oocytes (Postlethwait & Giorgi, 1985). In the Lepidoptera, another major yolk protein (called, for example, egg specific protein in the silk moth *Bombyx mori*) appears which is synthesized in the ovarian follicle cells and transported into the oocyte. In the higher Diptera such as *Drosophila*, the same vitellogenin genes are also expressed in the ovarian follicle cells.

Mature eggs of *A. rosae*, upon SDS-PAGE, are shown to contain two major yolk proteins L-Vn (with an apparent molecular mass of 180,000) and S-Vn (with an apparent molecular mass of 50,000) (Hatakeyama *et al.*, 1990a) (the earlier estimate for the L was in error and is to be corrected as mentioned here, Kageyama *et al.*, in preparation). Polyclonal antibodies raised against each of the L-Vn and the S-Vn were used in the Western blot analysis and demonstrated the presence of the corresponding L-Vg and S-Vg in the hemolymph of adult females but not of adult haploid or diploid males (Hatakeyama *et al.*, 1990a). Administration of juvenile hormone III to adult males induced appearance of hemolymph Vgs, but that of 20-hydroxyecdysone did not (Hatakeyama *et al.*, 1990a; Hatakeyama & Oishi, 1990).

Previtellogenic ovaries can be dissected out and they can be transplanted into the abdomen of other individuals in *A. rosae* with little difficulty. Previtellogenic ovaries accumulated yolk proteins in the abdomen of adult males when these males were treated with juvenile hormone III, and the eggs (some 10% of the mature-looking ones) upon artificial activation (see below) completed embryonic development (Hatakeyama & Oishi, 1990). A possibility remained, however, that the transplanted ovary itself provided at least a portion of yolk proteins as in the higher Diptera.

If it is shown that the yolk proteins are not supplied from the ovary itself in *A. rosae* (which is the case as shown in the next section), the system provides a means of studying to what extent the yolk proteins of one species can support the embryonic development of another species. Previtellogenic ovaries of *A. rosae* were transplanted into the abdomen of adult *Athalia infumata* males who were then treated with juvenile hormone III. The transplanted ovaries accumulated apparently only the host Vns and matured, and a fraction of the eggs (about 5%) upon artificial activation completed embryonic development (in fact they became perfectly normal haploid adult males) (Hatakeyama *et al.*, 1992b). Previous attempts to see the effect of interspecific ovarian transplantation could only detect the accumulation of yolk proteins (in *Drosophila*, though here the transplanted ovary itself should have made major contribution; Kambysellis, 1970; Srdic *et al.*, 1978; Lamnissou & Zouros, 1989).

Until recently, it was thought that yolk proteins are mere nutrition and hence the genes are subject to extensive accumulation of mutations (discussed in Postlethwait & Giorgi, 1985). This, however, is probably not so, since yolk proteins have to be digested in a well-regulated manner to support normal embryogenesis which should require a concerted expression of various digestive enzymes. Our preliminary examinations of major yolk proteins in various hymenopteran insects demonstrate (1) that species in the Symphyta have 1-2 large and 1-3 small polypeptides and in most species the large ones crossreact with the *A. rosae* anti-L-Vn antibody and the small ones with the anti-S-Vn antibody, and (2) that species in the Apocrita have mostly one large polypeptide and no small polypeptides in accordance with the previous results (Harnish & White, 1982; Wheeler & Kawooya, 1990; Martinetz & Wheeler, 1991). In many species the large one crossreacts, though only weakly, with both the *A. rosae* anti-L-Vn and S-Vn antibodies (Oishi *et al.*, 1992). The *A. rosae* system would hopefully provide some insight into the evolution of yolk proteins.

Cloning of cDNA for Vg and characterization of the Vg gene expression

A cDNA expression library was constructed from the poly(A)$^+$RNA prepared from the *A. rosae* adult female fat body cells, and screened with the anti-L-Vn and S-Vn antibodies, respectively. Most of the positive clones obtained with the anti-L-Vn antibody were short (<4.5 kb), while most of those with the anti-S-Vn antibody were long (4.5-6 kb). The restriction maps, however, agreed very well among them. In fact, rescreening of the positive clones with the other antibody showed that most of the L-positive clones are not S-positive but all of the S-positive clones are L-positive. The nucleotide sequence at the 5′-terminal in the longest clone showed the presence of ATG, 12 bp downstream from the linker site. The amino acid sequence was deduced from this site (other reading frames gave only short deducible sequences), and compared with the N-terminal amino acid sequence determined directly for the S-Vn. Up to 28 N-terminal amino acids (Asp-Gln-His-Ala-Trp-Lys-Ala-Gly-Gln-Glu-Tyr-Thr-Tyr-Gln-Val-Arg-Gly-Arg-Thr-Leu-Ala-Ala-Leu-His-Gln-Val-Ala-Asp) were determined and the sequence showed a perfect match

with the deduced amino acid sequence following the 16-residue long deduced putative signal peptide sequence (Met-Trp-Ser-Pro-Leu-Leu-Leu-Cys-Leu-Leu-Val-Gly-Ile-Ala-Ser-Ala) (Kageyama *et al.*, 1992). Northern blot analysis by using the subclones as probes demonstrates that the vitellogenin gene is expressed as a 6.5 kb mRNA in the adult female fat body but in neither the ovary nor in the adult male fat body (Kageyama *et al.*, 1992). Western blot analysis on the adult female fat body extract indicates the presence of a large polypeptide which reacts both with the anti-L-Vn and S-Vn antibodies in addition to the L-Vg and S-Vg (Kageyama *et al.*, in preparation). Thus, in *A. rosae* the vitellogenin gene is transcribed and translated as a single unit, and the initial large translation product is cleaved into the L-Vg and S-Vg and then secreted into the hemolymph. Now with the molecular probes available, we expect detailed studies be made on the Vg gene genomic structure and also be extended to other hymenopteran species.

Egg activation

Activation by artificial means

Although unfertilized eggs begin parthenogenetic development in the Hymenoptera, this generally applies only to the laid eggs and not to the unlaid mature eggs explanted from adult females in species of the higher suborder Apocrita (Sander, 1985, 1990). In contrast, in more than 200 species of sawflies of the Family Tenthredinidae (Symphyta) it has been shown that the unlaid mature eggs can be activated to develop simply by placing them in water (Naito, 1982). Egg activation here is such that the *in vitro* activated eggs show normal development, both embryonic and postembryonic, and become normal haploid males.

Conditions for the *in vitro* activation were further examined in *A. rosae* (Sawa & Oishi, 1989a). Eggs can be kept in 0.15M NaCl solution up to one hour without signs of activation and without any deleterious effect. Eggs were dissected out from adult females in the saline solution, transferred to distilled water for various periods of time and returned to the saline solution. If the eggs were kept for 20 min in distilled water, they were activated and continued development upon return to the saline solution. This 20 min period corresponds to the time required for the egg nucleus from the arrested first meiotic metaphase to proceed to telophase. These characteristics make *A. rosae* eggs especially suitable for biochemical and molecular studies, since a large number of exactly timed embryos and of particular sex (male) can be examined.

Various other stimulations, including those previously unknown in insects, are also effective but only to a lesser extent: brief desiccation, passage through narrow capillary tubings, and pricking (at any site). Changes in the ambient pH are, however, as effective as distilled water: at pH 5.0 in 0.15M citrate buffer more than 80% of the eggs are activated, while at pH 6.0 in the same buffer only some 30% and at pH 7.0 and 8.0 in 0.15M phosphate buffer 0% are activated.

Induced participation of prospective polar body nuclei in development

In insects mature eggs are arrested at metaphase of the first meiotic division and wait either for fertilization or for activation (as in the Hymenoptera) (Counce, 1972). When eggs are activated, meiosis resumes and results in the formation of one female pronucleus and three polar body nuclei. These polar body nuclei ordinarily do not participate in development.

Rare thelytokous reproduction (females develop parthenogenetically from unfertilized eggs) has been observed in *A. rosae*. One in 200-300 mature eggs activated *in vitro* develops to a diploid female (Sawa & Oishi, 1989b; Hatakeyama *et al.*, 1990b). Apparently the prospective polar body nuclei can in rare occasions participate in development. Furthermore, they can also do so independent of the female pronucleus as haploid male mosaics appear (Hatakeyama *et al.*, 1990b).

This can be shown decisively by using a non-autonomous fat body color mutation, yellow fat body (*yfb*). It is possible to distinguish wildtype females (*+/+*), mutant females (*yfb/yfb*), and heterozygous females (*+/yfb*), and wildtype males (*+*) and mutant males (*yfb*), by the fat body color which shows at mid-pupal stage through the still unpigmented integument. At this stage, *+/+* and *+* are green in color, *yfb/yfb* and *yfb* are bright yellow, and *+/yfb* is greenish yellow (Sawa & Oishi, 1989b) (also see below Fig. 4).

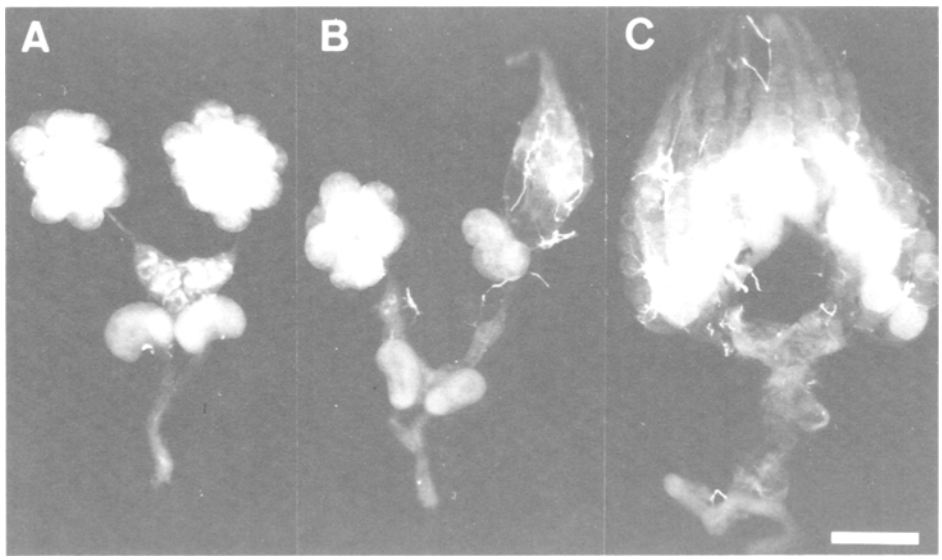

Fig. 2. Internal reproductive organs of a normal adult male (A), of a normal adult female (C), and of a gynandromorph (B). Scale indicates 500 μm.

The rate at which the prospective polar body nuclei participate in development can be greatly increased if the *in vitro* activation is done at an elevated temperature, 35-37 °C. Adults of diploid males, diploid females, triploid females and gynandromorphs (n-2n; Fig. 2) have been observed. Cytological examinations of the treated embryos even detect tetraploid individuals (Hatakeyama *et al.*, 1990b). Some cytoplasmic factors must be responsible for preventing the polar body nuclei from participating in development, and their functional abilities can be easily modified in *A. rosae*.

Fertilization and related problems

In vitro *fertilization*

Since the *A. rosae* mature unfertilized eggs can be activated *in vitro* and they develop to haploid male adults, it was thought possible to obtain *in vitro* fertilization by sperm injection. If fusion of the egg nucleus and the injected sperm nucleus takes place, the egg would develop to a diploid female (recognizable as *+/yfb* females), and if not to a haploid parthenogenetic male.

Sperm still in bundles were taken from the male spermatheca, dispersed by flushing them in and out of the microinjection pipet, and injected, up to two dozen each, into individual eggs, placed on a piece of double-stick tape attached to a microscopic slide, at the anterior pole. When unactivated eggs were injected some 10% of them developed as fertilized *+/yfb* females (Sawa & Oishi, 1989b). That the fusion of female and male nuclei did take place was confirmed by progeny testing; eggs from these females were activated *in vitro* and produced *+* and *yfb* haploid males in a ratio of 1:1.

Eggs at various times following the *in vitro* activation were next injected with sperm. Those at 20 min gave more than 20% fertilization, while those at 60 min gave none (Sawa & Oishi, 1989c). Most probably the sperm injected into the eggs 60 min post-activation did not become pronucleus. Changes in the egg cytoplasm or factors affecting the ability of injected sperm remain to be elucidated.

One application of the *in vitro* sperm injection is the attempt to obtain hybrids between species with strong premating isolation. Copulation rarely takes place between two sympatric species, *A. rosae* and *A. infumata*, and even in the rare cases when copulation takes place insemination occurs still more rarely. Taking advantage of the difference in karyotypes (*rosae*, K = 7M + 1A; *infumata*, K = 8M), interspecific sperm injections were made, and embryos examined cytologically (Sawa, 1991). In 4-5% of the embryos fertilization did take place

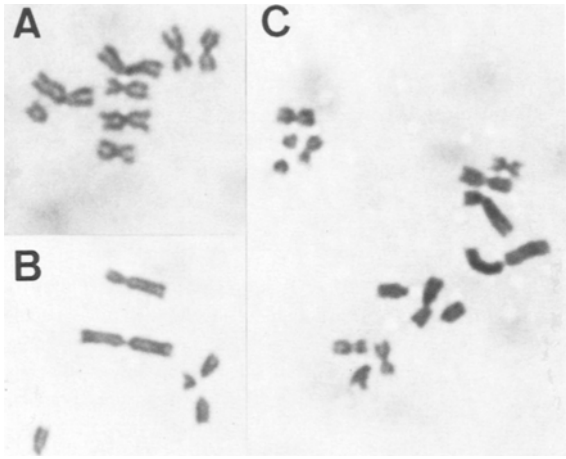

Fig. 3. Chromosomes of a haploid *Athalia rosae* male (A), of a haploid *A. kashmirensis* male (B), and of a hybrid produced by sperm injection (C).

which developed with the hybrid karyotype (remaining embryos were haploid). Similarly the hybrid between *A. rosae* and *A. kashmirensis* (K = 2M + 4A) of which larvae feed on different plant species have been obtained (Sawa, unpublished; Fig. 3). Limited attempts, however, have so far failed to produce adult hybrids.

Independent participation of injected sperm in development

In the initial attempt of *in vitro* injection, two apparently haploid chimeric (*+ − yfb*) males were obtained (Sawa & Oishi, 1989b). It later became apparent, when the embryos two days post-injection were examined for the presence of a microinjection pipet scar with respect to the orientation of the developing embryo, that in one in 200-300 injections mistakes occurred in the pole of injection. When this examination was included in the experiment the results were clear: the sperm injection at the anterior pole resulted in fertilization in a fraction of the eggs, while the sperm injection at the posterior pole never resulted in fertilization. Instead, a small fraction of the posteriorly injected eggs (about 1%) steadily produced haploid male chimeras, apparently derived from independent participation of the egg and sperm nuclei (Hatakeyama *et al.*, 1992a) (Fig. 4).

Five haploid chimeric males (*+* from sperm *−* *yfb* from egg) were crossed to *yfb/yfb* females and the phenotypes of progeny females were examined. In three crosses progeny females were all *+/yfb*. Two crosses, however, produced both *+/yfb* and *yfb/yfb* females, indicating that the anteriorly positioned egg nucleus can at least occasionally contribute to the formation of germ cells (Hatakeyama *et al.*, unpublished).

Prospects

We hope we have made it clear that the *A. rosae* system provides a means of studying such subjects as egg activation, fertilization, pronucleus formation, haploid-haploid chimeric and mosaic development, etc., which have been difficult if not impossible to study in other insects. In *Drosophila*, haploid embryos do not complete embryonic development, although when diploidization (either by central fusion or by gamete duplication) takes place these embryos become capable of completing development (Fuyama, 1984, 1986). Haploid development is a normal mode in the Hymenoptera and the underlying mechanism remains to be elucidated.

That we can make use of a large-scale exactly timed embryos of a particular sex (male) for biochemical and molecular studies may be emphasized. Hopefully the cytoplasmic factors responsible for the subjects mentioned above may be stud-

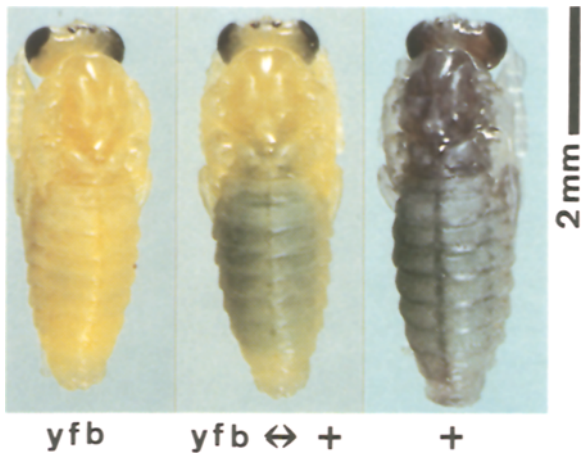

Fig. 4. A mutant haploid *yfb* male pupa (left), a wildtype haploid *+* male pupa (right), and a haploid (yfb *−* *+*) chimeric male pupa produced by sperm injection at the posterior end (center), all at the mid-pupal stage.

ied in detail. Functions of genes for sex determination in early embryos clearly are also an important subject. Another important subject we wish to pursue is the mechanism of meiosis. As mentioned earlier, diploid and triploid males can be obtained easily in *A. rosae*. These males, just like normal haploid males, produce their sperm by non-reductional maturation division (namely, diploid and triploid sperm, respectively) (Naito & Suzuki, 1991). Meiosis in *A. rosae* is, thus, ploidy-independent but sex-dependent.

One clear shortcoming in the *A. rosae* system is the lack of mutants (*yfb* is the only mutation available at the moment). This, however, should not be critical at least for the time being: it may be expected that genes involved in such important processes as meiosis and sex determination are evolutionally conserved. We should be able to make use of genes cloned in other species as probes and 'pick up' homologous genes in *A. rosae*. For other processes such as activation and fertilization, a shot-gun type examination of the cDNA library may prove to be useful.

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