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Experiments inducing prospective polar body nuclei to participate in embryogenesis of the sawfly *Athalia rosae* (Hymenoptera)*

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Summary. Mature eggs dissected from ovaries of unmated females of *Athalia rosae* (Hymenoptera: Tenthredinidae), if placed on a filter-paper soaked with distilled water, are activated and develop to haploid males. Occasionally, however, diploid females develop from these artificially activated eggs. Treatment of mature unfertilized eggs dissected from diploid females with ice-cold temperatures immediately before activation and with a high temperature (36° C) upon and immediately after activation resulted in the production of diploid males, diploid females, triploid females and gynandromorphs at high frequency. The same treatment of mature unfertilized eggs dissected from triploid females resulted in the production of only triploid survivors. These results, together with the results on the segregation of a marker mutation, yellow fatbody (*yfb*), appear to indicate that meiotic divisions were complete in the treated eggs, and that all four nuclei became potentially capable of participating in development with or without automictic fusion.

Key words: *Athalia rosae* – Hymenoptera – Thelytoky – Automixis – Gynandromorph

Introduction

Meiotic divisions in female animals ordinarily result in the production of one large egg cell and three small polar body cells. In insects, however, female meiosis is merely nuclear and not associated with cell divisions: the three nuclei corresponding to polar body nuclei usually degenerate within the cytoplasm of a single large cell, the egg (Counce 1961, 1973; Tremblay and Caltagirone 1973). There should be a mechanism then to allow only one of the meiotically produced nuclei to proceed to form a female pronucleus for fertilization. Alterations of this process caused by environmental factors or by mutations can easily occur. If there is subsequent fusion of nuclei, automictic parthenogenesis will follow, which is often observed in insects (White 1973; Suomalainen et al. 1987).

Hymenopterans are a unique group of insects in which parthenogenetic male production (arrhenotoky) is almost universal: fertilized eggs usually develop to diploid females and unfertilized eggs to haploid males (White 1973; Crozier 1975; Suomalainen et al. 1987). Gynogenesis, androgenesis, automictic parthenogenesis, and mosaics including haploid-diploid mosaics have been known in various species of this group (Crozier 1975; Rothenbuhler 1975; Cassidy 1975). At least in some species, sex is determined by a single-locus multiple-allele system: hemizygous and homozygous (homoallelic) individuals develop to males and heterozygous ones to females (White 1973; Crozier 1975). Since males do not show reduction in chromosome number in spermatogenesis even in diploid individuals, a cross between diploid males and normal diploid females results in the production of triploid individuals.

Artificial egg activation has been achieved in hymenopterans or in other insects in only a few cases (Went 1982; Sander 1985a, b, 1990; Vinson and Jang 1987; Saini et al. 1987). Sawflies of the family Tenthredinidae (Symphyta, Hymenoptera) are exceptional: mature unfertilized eggs dissected from the ovary initiate and complete embryonic development if placed on filter-paper wet with distilled water (Naito 1971). More than 200 species belonging to 44 genera of five subfamilies have been examined and shown to have the same characteristic (Naito 1982). We have chosen *Athalia rosae* (*A. rosae ruficornis* Jakovlev according to Abe 1988) as an experimental material (Sawa et al. 1989). This species has the single-locus multiple-allele sex determination system (Naito and Suzuki 1985). Mature unfertilized eggs dis-

* Studies on the sawfly, *Athalia rosae* (Insecta, Hymenoptera, Tenthredinidae), part V

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sected from unmated females can be activated to develop quite easily by various mechanical and non-mechanical means (Sawa and Oishi 1989a). These unfertilized eggs, if microinjected with genetically marked sperm, develop as heterozygous diploid females (Sawa and Oishi 1989b). Some aberrant adults observed in these studies led us to examine, employing mature unfertilized eggs dissected from the ovary, whether alterations can be induced in the developmental capability of meiotically produced nuclei. We report here that successive temperature shocks (cold and heat) given to unfertilized eggs from diploid females result in the production of diploid males, diploid females, triploid females and gynandromorphs, and that the same treatment to the eggs from triploid females results at high frequency in the production of triploids only. Together with the results on the segregation of a marker mutation, yellow fatbody (*yfb*), this leads us to conclude that at least in a considerable fraction of the cases meiotic divisions took place normally, and that some or all four nuclei resulting from meiosis became capable of participating in development with or without automictic fusion.

Materials and methods

A general description of *Athalia rosae* and methods of rearing and obtaining mature unfertilized eggs has been given previously (Sawa et al. 1989; Sawa and Oishi 1989a). The marker mutation was yellow fatbody (*yfb*), expressed in the pupal stage (Sawa and Oishi 1989b). It is a non-autonomous color mutation and hence haploid-haploid mosaic males, $+ \leftrightarrow yfb$, are normal haploid-size males but show a heterozygous $+/yfb$ color. Sex, since it is determined by the single-locus multiple-allele system in *A. rosae* as mentioned above, also serves as a marker.

Ploidy of adults was determined primarily on the basis of size. Diploid males are dinstinctly larger than haploid males. Triploid males, however, are about the same size as diploid males. Triploid females are larger than diploid females but the size distribution overlaps (Naito and Suzuki, unpublished). When size was ambiguous or further confirmation was desirable and necessary, progeny testing was carried out (see Results). Triploid males are sterile, although they show apparently normal spermatogenesis (Naito and Suzuki, unpublished). Triploid females are largely sterile apparently due to abnormal chromosome disjunction.

Chromosome preparations were made from 2-day-old embryos or from gonadal tissues of third-instar larvae and stained with Giemsa according to the methods described by Imai et al. (1988). Feulgen staining of eggs was done as previously described (Sawa and Oishi 1989a).

Results

Spontaneous development with more than one meiotic nucleus

In the course of our studies on the biology of *Athalia rosae* (Sawa et al. 1989; Sawa and Oishi 1989a, b), we have artificially activated mature unfertilized eggs and reared them to adulthood on many occasions. We have observed that apparently diploid females appear from these unfertilized eggs in a sporadic manner. To investigate this effect, heterozygous $+/yfb$ females were aged for 7 days or 10 days. They were given diluted honey but were not provided with *Raphanus* leaves on which they lay eggs. Under these conditions, females live up to 2 weeks and accumulate mature eggs in their ovaries. No apparent degradation or resorption of these eggs takes place (Sawa and Oishi 1989a). Eggs of females aged 7 days yielded, in addition to 103+ and 75 *yfb* haploid males, two $+ \leftrightarrow yfb$ haploid mosaic males and one wild-type $(+/+)$ diploid female. Out of 241 pupae from eggs of 10-day-old females, 116+ and 113 *yfb* haploid males, 3 $+ \leftrightarrow yfb$ haploid mosaic males, 2 $+/+$ diploid females, 4 $+/yfb$ diploid females, and 3 yfb/yfb diploid females were obtained.

The observed segregation of the *yfb* allele indicates that, although infrequent, spontaneous automictic parthenogenesis does take place in *A. rosae*, and that the rate increases as parental females age. Most probably, meiotic divisions took place normally. Haploid mosaic males were probably the product of two nuclei derived from meiotic divisions participating independently in development. The appearance of haploid $+ \leftrightarrow yfb$ mosaic males suggests that there were also $+ \leftrightarrow +$ and $yfb \leftrightarrow yfb$ haploid mosaic males, which went undetected. The possibility that more than two haploid nuclei participated in development, however, cannot be excluded. Diploid females must have resulted from fusion of two of the four haploid nuclei produced. Again a possible occurrence of diploid-diploid mosaics cannot be excluded. Since sex is determined by the single-locus multiple-allele system in *A. rosae* as mentioned above, if a large-scale experiment is done we may expect to obtain diploid males also (see below).

Artificial induction of development with several meiotic nuclei

The automictic parthenogenesis observed above, if consistently induced at high frequency by artificial means, would provide various possibilities for future developmental studies. We thus attempted to induce parthenogenetic production of diploid females or both males and females by artificial means employing mature eggs dissected from unmated 7-day-old diploid females.

First we examined the effect of temperature. Exposure of mature unfertilized eggs to temperatures at 38° C or over was quite deleterious even for a short period, and at 32° C or below was without effect in producing females. Temperature ranges 35°–37° C appeared most promising. Exposure to ice-cold temperatures was not effective, but at least it did not have a deleterious effect on development. We chose to expose the eggs first to ice-cold *Drosophila* Ringer solution for 60 min. *A. rosae* eggs were not activated in this solution and were still at the first meiotic metaphase following the completion of this treatment in Feulgen-stained preparations. The eggs were then exposed to warm distilled water (35°–37° C for 60 min). Eggs were activated by this treatment and began development. We, therefore, tried to shock the eggs with a large temperature difference. (Preliminary experiments appeared to indicate that the heat

Table 1. Effect of cold and heat shock on embryonic ploidy in eggs from diploid *Athalia rosae* females: results of chromosome examination on 2-day-old embryos

Treatment[a]	Parental genotype	No. of eggs activated	Normal embryos (%)	No. of embryos karyotyped	No. of embryos with chromosome number:					
					$1n$	$2n$	$n \leftrightarrow 2n$ mosaic	$3n$	$4n$	Others[b]
None	+/+	382	93.2	114	114	0	0	0	0	0
	yfb/yfb	399	83.2	102	100	1	0	0	0	1
1 h 35° C	+/+	524	50.2	116	82	19	2	9	0	4
	yfb/yfb	523	36.1	106	72	12	0	16	3	3
1 h 36° C	+/+	438	39.5	98	17	34	5	16	20	6
	yfb/yfb	727	18.0	102	31	35	5	16	12	3
1 h 37° C	+/+	650	6.3	42	1	14	0	3	12	12
	yfb/yfb	643	4.7	30	1	7	0	2	12	8

[a] After storage in ice-cold Ringer, see Materials and methods
[b] Mostly aneuploids such as $2n \pm 1$, $3n-1$ and $4n-1$

Table 2. Effect of cold and heat shock[a] on parthenogenetic genotypes in eggs from diploid (+/*yfb*) *Athalia rosae* females: results of rearing experiments

No. of eggs activated (%)	No. of larvae hatched (%)	No. pupated and examined (%)	Males						Females					Gynandromorph	Others[b]
			Haploid			Diploid			Diploid			Triploid			
			+	*yfb*	+↔*yfb* mosaic	+/+	+/*yfb*	*yfb/yfb*	+/+	+/*yfb*	*yfb/yfb*	+/+/+ +/+*yfb* +/*yfb/yfb*	*yfb/yfb/yfb*		
2045 (100)	307 (15.0)	186 (9.1)	38	28	3	0	5	2	10	43	7	27	0	5	18

[a] 1 h at 36° C after storage at 0° C in Ringer
[b] Includes 6 diploid-size females, 2 haploid-size males and 10 diploid or triploid-size males. One female did not eclose and 4 females died soon after eclosion. Eggs from the remaining single female were activated but they did not initiate development. Two haploid-size males, both heterozygous *yfb* in color, did not mate. One diploid or triploid-size male did not eclose. Eight diploid or triploid-size males either died soon after eclosion, or did not mate or did mate but were sterile. The remaining single diploid or triploid-size male, homozygous *yfb* in color, produced $2n$ and $3n$ progeny upon mating to normal diploid females (determined by cytological examination on 2-day-old embryos). He thus was a haploid male↔diploid male mosaic

treatment alone is less effective in producing diploids compared to the cold/heat treatment and is more deleterious to the survival of the eggs.) Treated eggs were transferred to distilled water at 25° C briefly, and then placed on filter-paper wet with distilled water and incubated at 25° C. From normally developing 2-day-old embryos, Giemsa-stained specimens were prepared for chromosome examinations. Table 1 shows the results.

As the heat treatment went up from 35° C to 37° C, the percentage of embryos developing normally on the second day decreased rather drastically, while the percentage of embryos with the chromosome number $2n$ or more increased sharply ($n=8$; Naito 1982). Individual examinations were made in batches of 16–67 eggs. The percentage of normally developing 2-day-old embryos in various batches fluctuated greatly (0%–82%), indicating that there are some presently uncontrollable parameters. In all the experiments that follow we similarly observed rather extensive fluctuations.

Individuals with chromosome numbers $2n$, $n \leftrightarrow 2n$, $3n$, $4n$ and others were observed at very high frequencies. That we did obtain $n \leftrightarrow 2n$ mosaics and $3n$ individuals would suggest that meiosis took place normally and that probably many embryos with chromosome numbers $2n$ or over were derived from the fusion of meiotically produced nuclei. Embryos from *yfb/yfb* mothers were consistently less viable than those from +/+ mothers. However, frequencies of individuals with chromosome numbers n, $2n$, etc. are not significantly different between the embryos from *yfb/yfb* and from +/+ mothers. Considering both the viability of treated eggs and the frequency of individuals with chromosome numbers $2n$ or over, we chose the 36° C treatment and examined the effect further.

Table 2 shows the results of rearing experiments with eggs taken from 7-day-old +/*yfb* females and given cold and heat (36° C) treatment. Out of 2045 eggs, 307 (15.0%) hatched and 186 (9.1% of the total, 60.6% of the hatched larvae) pupated and nearly all of them became adults. Among these we found 66 haploid males and 3 haploid ↔ haploid mosaic males, 7 diploid males, 60 diploid females, 27 triploid females, 5 gynandromorphs and 18 other aberrant individuals. Here ploidy was determined on two grounds: size and progeny test-

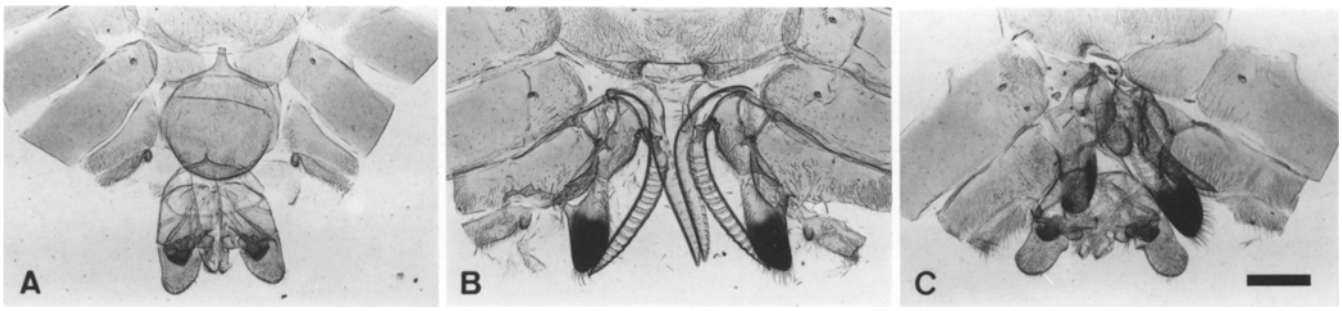

Fig. 1. Cuticular structures of the terminus of abdomen in **A** normal male, **B** normal female, and **C** a gynandromorph. *Bar* indicates 500 μm

ing. Diploid- or triploid-size males and haploid-size mosaic males were individually crossed to normal diploid females. Two-day-old embryos from these crosses were examined cytologically. If a male is diploid some of the progeny embryos (namely those fertilized) should show the triploid chromosome number, which was observed. Females obtained from shocked eggs were aged for a few days, and their mature unfertilized eggs activated artificially in individual batches. Many batches showed normal embryonic development. In this case, the female was concluded to be diploid. Twenty-seven batches showed abnormal development. The 2-day-old embryos were examined cytologically, and favorable preparations all showed aneuploid chromosome numbers between 9 ($=n+1$) and 15 ($=2n-1$). In these cases, the parental females were concluded to be triploids. Genotypes with respect to *yfb* are difficult to determine in triploids, especially when they appear sporadically and not all at once, hence the heading $+/+/+$ or $+/+/yfb$ or $+/yfb/yfb$, in Table 2.

Gynandromorphs were detected by the co-existence of partial or whole female and male external genitalia (Fig. 1). They were dissected and the internal reproductive organs were examined. Of the 5 gynandromorphs obtained, 2 had only male internal reproductive organs, while 3 had both male and female organs developed to various degrees. Three gynandromorphs had wings on one side of the body longer than those on the other. Most probably, then, at least these gynandromorphs were haploid male↔diploid female mosaics.

Tetraploids might have been expected from the results in Table 1. Their absence is probably due to death during development, as shown by the following experiment. A total of 500 eggs from $+/+$ and yfb/yfb females were given the same cold/heat treatment as above. Of these, 93 (18.6%) hatched and many developed further. A total of 46 third-instar larvae were dissected and their gonadal tissues were processed for cytological examinations. These included 8 haploids (all of them had testes), 22 diploids (16 had ovaries, 5 had testes, and 1 had an ovary and an ambiguous rudimentary structure), 5 $n\leftrightarrow 2n$ mosaics (4 had testes and 1 had an ovary and a testis), 9 triploids (all had ovaries) and 2 tetraploids (both had ambiguous rudimentary structures). Compared to the results in Table 2, these results indicate that tetraploid individuals, at least most of them, die before reaching the third-instar larval stage and probably none survive to adulthood.

Artificial induction of parthenogenesis in eggs from triploid females

Triploid females were obtained by crossing diploid females and diploid males, aged for 7 days, and mature unfertilized eggs were dissected. If these eggs are activated, simply by placing them on wet filter-paper, and incubated at 25° C, most of them develop abnormally and cytological examinations on 2-day-old embryos indicate that they are aneuploids with chromosome numbers ranging from $n+1$ to $2n-1$ as noted previously. A small number of normal embryos do develop, however, and some reach postembryonic stages. In one such experiment, 567 eggs were activated and of these 19 (3.4%) were developing normally on the second day. Of these, 8 developed further to become third-instar larvae. Gonadal tissues were examined morphologically and cytologically: 2 were haploid males, 3 were diploid females, and 2 were triploid females (the remaining 1 did not give a good specimen).

The frequency of normally developing 2-day-old embryos derived from triploid females greatly increased when the eggs were subjected to the cold/heat treatment. Out of 548 eggs treated, 131 (23.9%) were developing normally on the second day. A total of 129 embryos were successfully examined cytologically (in 2 others, we failed to prepare good specimens): 33 had the eu-triploid chromosome number, 34 were either $3n+1$ or $3n-1$, 9 were eu-hexaploids, 3 were aneuploids close to $6n$, and 50 were more extensive aneuploids. The prevalence of (near-)triploidy points to fusion of non-sister nuclei from the second meiotic division (see Discussion). In a separate experiment, 258 eggs from 7-day-old $+/+/yfb$ triploid females were given the cold/heat treatment. Of the 13 adults obtained, one was a sterile male of unknown ploidy and the 12 females produced abnormal offspring like triploid females originating from crosses. This indicates that these parental females were all triploids. Thus we did not obtain haploid and diploid individuals from eggs of triploid females given the cold/heat treatment. Also, hexaploid individuals, which might

have been expected from the above results of cytological examinations on 2-day-old embryos, apparently did not develop to adulthood.

Discussion

The present results have shown that in *A. rosae* parthenogenetic reproduction involving automixis occurs spontaneously, although infrequently. Automictic and mosaic reproduction can be induced in mature unfertilized eggs dissected from the ovary at high frequency by activating them with water at 36° C. We consider the possible mechanisms of this induced automixis.

Since mature unfertilized eggs dissected from the ovary employed in the present study are already at the first meiotic metaphase (Sawa and Oishi 1989a), we may disregard such mechanisms as premeiotic doubling and pachyreduplication (see Table 1 of Lamb and Willey 1987). One plausible explanation for the present results may be that meiosis still takes place normally producing four haploid nuclei, and that instead of producing one female pronucleus and three polar body nuclei these four nuclei all become potentially capable of initiating development with or without fusion among themselves. Thus, a developing embryo is either haploid, diploid, triploid, tetraploid, haploid↔haploid mosaic, or haploid↔diploid mosaic (Tables 1 and 2). Possible diploid↔diploid mosaics cannot be detected in the present system (note that *yfb* is a non-autonomous mutation). If this is correct, the fact that we obtained a relatively small number of diploid males but a relatively large number of diploid females may mean that the sex determination locus is close to the centromere and thus has a small probability of being recombined, and that fusion of the second-division non-sister nuclei rather than sister nuclei preferentially occurs when two nuclei fuse.

If the preferential fusion of the second-division non-sister nuclei is correct, we can similarly explain the results in triploids. Chromosome disjunction is apparently quite aberrant in triploids and the first meiotic division produces two nuclei differing widely in chromosome numbers and only rarely results in euploid chromosome sets. Following the second meiotic division, however, if fusion of non-sister nuclei occurs preferentially the resulting nucleus is expected to restore the triploid chromosome number.

It has been thought that the peripheral-most portion of egg cytoplasm furnishes a generally hostile environment to the nucleus during early stages of development and that the zygote nucleus suppresses further development of the polar body nuclei (Counce 1961, 1973). In *A. rosae* the first-division spindle is apparently parallel to the egg surface (Sawa and Oishi 1989a). Unfortunately further cytological analysis is difficult, at the moment, because of the technical problems. But suggestions of Tucker (1958) concerning the honeybee can provide a basis for explaining all the results presented here. In *Apis mellifera*, the first meiotic division spindle is formed parallel to the egg surface but it rotates and takes a perpendicular position before the completion of the first division. The second meiotic division produces four nuclei in a line at right angles to the egg surface. Tucker's (1958) explanation for automictic parthenogenesis is that occasionally the first-division spindle fails to rotate. The second-division spindles are then formed perpendicular to the egg surface, thus producing, at the completion of the second division, four nuclei in two parallel lines; the two inner nuclei then fuse resulting in the preferential fusion of the second-division non-sister nuclei, referred to as the central union.

In heat-treated *Athalia* eggs, the spindle rotation may frequently fail. In some eggs the first-division spindle might remain parallel to the surface but in others the spindle may slightly rotate and take an oblique position. Following the completion of the second division, which is perpendicular to the first, the former cases may result in fusion of the two inner nuclei and thus produce diploid individuals, while the latter cases might lead to fusion of two (producing diploids or haploid↔diploid mosaics) or three (producing triploids) inner nuclei. The occurrence of the first division at a position less close to the egg surface may result in the formation of tetraploids. On occasions one to four haploid nuclei may also begin development without fusion.

This abnormal spindle behavior may also, though infrequently, occur in untreated eggs especially in those long-stored in ovaries, thus providing an explanation for the spontaneous thelytokous reproduction observed.

Although the above explanation is simple and attractive since a single explanation applies to all the results, obviously it is not the only one possible, nor does it exclude other possible events from taking place in different eggs in the same batch. Other alternatives are possible but none explains the whole set of results. One possibility is that in a fraction of eggs, following normal crossing-over, the first division fails and forms a restitution nucleus, which then proceeds to the second division, one of its products initiating development alone or following fusion with the other nucleus. Although this explains only diploids and tetraploids from diploid eggs and triploids and hexaploids from triploid eggs, this may account for some of these classes. Another possibility is the occurrence of fusion of the second-division sister nuclei (terminal fusion). Since this would lead to a large number of homozygotes, both with respect to the *yfb* mutation and to the sex determination allele (hence males), if it occurs it could constitute only a minor fraction.

In any case, in principle we now have a means to obtain homozygous individuals from heterozygous females without going through the mating processes. Considering the rate of production of homozygous individuals achieved in the present study, this may not be useful for studying ordinary genes. For special classes of genes such as lethals or steriles, where hemizygous lethality or sterility may not necessarily mean homozygous lethality or sterility, it should provide a welcome means for developmental analysis. However, a significant fraction of deleterious mutations has been thought to be expressed in haplo-diploid insects only in the diploid and not in the haploid state (Bernstein et al. 1985). Thus,

it remains to be seen if the rate of automictic parthenogenesis can be improved.

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