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Effects of the sap of the common oleander *Nerium indicum* (Apocyanaceae) on male fertility and spermatogenesis in the oriental tobacco budworm *Helicoverpa assulta* (Lepidoptera, Noctuidae)

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Summary

We investigated the effects of sap of the common oleander *Nerium indicum* (Apocyanaceae) on male fertility and spermatogenesis in the oriental tobacco budworm *Helicoverpa assulta*. We found that continuous feeding of oleander sap during the larval period significantly affects fertility in males but not in females. This effect was also induced by direct injection of oleander sap into the hemocoel of 2-day-old pupae. Histological analyses of developing testes following oleander injection revealed a developmental delay and progressively more severe morphological abnormalities in the later stages of development. The effects of oleander sap on spermatogenesis in *H. assulta* were associated with greatly reduced levels of the two major polyamines, spermidine and spermine, in testis compared with saline-injected controls. In contrast, levels of putrescine, which is a precursor of both spermidine and spermine, and the activities of the enzymes ornithine decarboxylase and arginine decarboxylase, which are involved in the biosynthesis of putrescine, were initially elevated following oleander injection, but subsequently failed to undergo the induction that normally occurs during late pupal development. The effects of oleander sap on spermidine and spermine levels could be the result of direct inhibition by chemical constituents of the oleander sap of one of the enzymes used in common in the conversions of putrescine to spermidine and spermidine to spermine; alternatively, these effects on polyamine metabolism could be secondary to the disruption of a more fundamental process in the developmental program guiding spermatogenesis in *H. assulta*.

Key words: oleander, *Nerium indicum*, Apocyanaceae, oriental tobacco budworm, *Helicoverpa assulta*, Lepidoptera, spermatogenesis, polyamine, putrescine, spermidine, spermine, ornithine decarboxylase, arginine decarboxylase.

Introduction

The fertility of male lepidopteran insects is affected by prolonged exposure to elevated sublethal temperatures during the pupal period (Bodnaryk and Gerber, 1988; Gerber et al., 1991; Yoo et al., 1998). Heat-induced male sterility in the bertha armyworm *Mamestra configurata* has been shown to be caused by inhibition of spermatogenesis, resulting in greatly reduced production of spermatozoa (Bodnaryk and Gerber, 1988; Gerber et al., 1991). Similar heat-induced effects on male fertility and spermatogenesis in the oriental tobacco budworm *Helicoverpa assulta* have been shown to be accompanied by significantly reduced levels of testicular polyamine (Yoo et al., 1998). The latter effects were also produced by injecting *H. assulta* pupae with α-difluoromethylornithine (DFMO) and α-difluoromethylargine (DFMA) (Jeong and Kown, 1996). These compounds are known inhibitors of ornithine decarboxylase and arginine decarboxylase, respectively, enzymes that mediate distinct biosynthetic routes to putrescine, which is the precursor of both spermidine and spermine (Smith, 1985; Kaur-sawhney et al., 1989). These essential, ubiquitously occurring, polyamines are intracellular organic cations that play important roles in nucleic acid and protein synthesis (Abraham and Pihl, 1981; Tabor and Tabor, 1984) and in cell growth and differentiation in animals and plants (Pegg and McCann, 1988; Cayre et al., 1997; Mitsuhashi, 1998). Since high levels of spermidine and spermine are present in insect testes during the period of rapid cell division and differentiation in pupal development (Hamana et al., 1989; Jeong and Kown, 1996), it has been postulated that depressed polyamine metabolism could lead to incomplete or defective spermatogenesis in testes (Yoo et al., 1998).

Many groups of plants contain secondary metabolites that produce a variety of physiological and behavioral effects on insect herbivores (Berenbaum, 1986). Well-characterized non-toxic effects of plant phytochemicals on herbivorous insects include those influencing feeding behavior, for example as

gustatory repellents, feeding deterrents or antifeedants (Schoonhoven, 1982), and reproductive function (Saxena et al., 1977; Bodhada and Borle, 1985; Raju et al., 1990). Plants of the Family Apocyanaceae are widely recognized for containing compounds that are very toxic to mammals (Langford and Boor, 1996; Bose et al., 1997; Monzani et al., 1997; Al-Yahya et al., 2000; Oji and Okafor, 2000), and some species are also known to contain compounds producing major effects on insect behavior and physiology, including insecticidal activity in *Thevetia thevetiodes* (McLaughlin et al., 1980), insecticidal and repellent activity in *Nerium oleander* (El-Lakwah et al., 1996) and sterilant activity in *Thevetia neriifolia* (Raju et al., 1990). We were interested in investigating whether the common oleander *Nerium indicum*, which is well known in Korea for having no insect herbivores other than scale insects, possesses insect-sterilizing activity, as has been described for *T. neriifolia*. To this end, we performed feeding and injection experiments using *H. assulta*, which has a well-characterized reproductive biology and is easy to maintain in captivity. In the present paper, we describe initial investigations of the effects of the milky sap of the common oleander *N. indicum* on male fertility, spermatogenesis and polyamine metabolism in this lepidopteran species.

Materials and methods

Insects and plant sap

Larvae of *Helicoverpa assulta* were reared on an artificial diet (6.8 % soybean flour, 6.4 % corn flour, 2.4 % yeast extract, 1.5 % agar, 0.6 % skimmed milk, 0.43 % multi-vitamins, 0.41 % ascorbic acid, 0.24 % methyl-*p*-hydroxy benzoate, 0.22 % sorbic acid and 0.04 % formalin) at 25±1 °C and 60±5 % relative humidity under an 18 h:6 h light:dark photoregime. In early May 1998, sap was collected from a common oleander tree approximately 150 cm tall that had been transplanted from the subtropical Cheju Island, Korea, into a greenhouse at Hannam University, Taejon, Korea, in October 1996. Several stems were cut, and the exuded white viscous sap was collected and stored at −20 °C until experiments were performed.

Administration of oleander sap to insects

For feeding experiments, crude sap was diluted 10-, 20- and 40-fold with sterile distilled water, and one part of each dilution was added to 199 parts of molten diet at approximately 40 °C. After an immediate mix by rapid shaking by hand, 10 ml of the diet was poured into each rearing cup and allowed to congeal at room temperature. Single first-instar larvae were placed in individual rearing cups, where they fed during the entire larval period (15±1 days).

For injection experiments, a fine glass capillary needle, drawn using a microelectrode puller (Harvard), and a micrometer syringe pump (Harvard) were used. Because of its high viscosity, the sap had to be diluted at least 20-fold in sterile insect saline (Jungreis et al., 1973) to permit it to flow through the device. Healthy 2-day-old male pupae weighing 500±50 mg were selected, and 5 µl of each dilution was injected into the hemocoel through the third abdominal ventral fold.

Determination of fertility

Individual adult moths that had been fed or injected with oleander sap were placed with non-treated moths of the opposite sex in a transparent plastic cage (20 cm×20 cm×20 cm) and transferred to the controlled environmental chamber described above for mating and egg laying. The moths were supplied with a 10 % sucrose solution, and the tops of the cages were covered with a piece of gauze to provide oviposition sites. The gauze was removed from the cages daily until egg laying ceased. The eggs were counted and then incubated under controlled environmental conditions to permit embryonic development. The fertility of each treatment group, which consisted consisted of 20 pairs, was determined by counting the number of eggs that remained unhatched after 7 days. The data were analyzed using one-way analysis of variance (ANOVA), *F*-tests and least significant difference) (LSD) tests (SAS 8.0, SAS Institute Inc., Cary, North Carolina, USA).

Dissection of testes

Male pupae and adult moths were dissected under cold insect saline, and the testes were removed through the dorsal wall of the fourth abdominal segment using fine forceps. Dissection of pupal testes was carried out by the ninth day of the pupal period, which normally lasts 10–11 days. The extirpated testes were blotted onto filter paper to remove most of the hemolymph clinging to them, and then stored at −20 °C until they were used in experiments.

Polyamine analysis

Analysis of polyamine levels and enzyme assays of testicular extracts were performed at the 1-, 3-, 5-, 7- and 9-day-old pupal stages and the 1-day-old adult stage.

For polyamine extraction, the pooled testes were homogenized in a microfuge tube in 100 µl of cold 5 % perchloric acid, and the homogenate was centrifuged at 10 000 ***g*** at 4 °C for 10 min. The supernatant was applied to a Bio-Rad AG 50W-X4 cation-exchange resin (H^+ form, 200–400 mesh in a 0.7 mm×2.3 cm column), and a polyamine-containing fraction was eluted through the following steps; 2.8 ml of 0.7 mol l^{-1} NaCl in 0.1 mol l^{-1} sodium phosphate (pH 8.0), 2 ml of deionized distilled water, 3 ml of 10.1 mol l^{-1} HCl and finally 2.4 ml of 60.1 mol l^{-1} HCl. The final eluate was dried in a rotary vacuum evaporator at 40 °C and then dissolved in 1 ml of distilled water for analysis by high-performance liquid chromatography (HPLC). *o*-Phthalaldehyde-thiol (OPT) reagent was prepared by dissolving 10 mg of *o*-phthalaldehyde and 10 µl of 2-mercaptoethanol in 200 µl of ethanol and diluting this with 5 ml of 0.5 mol l^{-1} sodium borate buffer (pH 10.3). Polyamine solution (200 µl) was mixed with 25 µl of OPT reagent, stored overnight under argon to reduce background fluorescence, and reacted for 60 s before injection

onto an HPLC column. HPLC was carried out using a Waters system consisting of a 600E multisolvent delivery pump, a U6K injector and a 474 scanning fluorescence detector (338 nm excitation/400 nm emission). OPT-derivatized polyamine solution (25 μl) was injected onto a Beckman Ultrasphere ODS column (5 μm, 4.6 mm×15 cm) and eluted according to the method of Corbin et al. (Corbin et al., 1989).

Enzyme assays

The assays for ornithine decarboxylase and arginine decarboxylase measure $^{14}CO_2$ released from [^{14}C]ornithine and [^{14}C]arginine, respectively. The pooled testes were homogenized in 100 μl of 50 mmol l^{-1} Tris-HCl buffer (pH 7.4) containing 100 μmol l^{-1} EDTA and 1 mmol l^{-1} dithiothreitol, and the homogenate was centrifuged at 10 000 ***g*** at 4 °C for 10 min. A sample (50 μl) of the supernatant was mixed with a substrate mixture: 145 μl of 500 mmol l^{-1} L-ornithine or L-arginine plus 5 μl (0.5 μCi) of L-[^{14}C]ornithine or L-[^{14}C]arginine. Reactions were carried out for 30 min at 37 °C in test tubes (1 cm×9.5 cm) capped with serum stoppers carrying a small microfuge tube filled with 200 μl of 1 mol l^{-1} hyamine hydroxide (NEN) as a CO_2-trapping agent. Reactions were stopped by injecting 500 μl of 25 % trichloroacetic acid, after which an additional incubation for 30 min at 37 °C was carried out for additional CO_2 trapping. The radioactivity of $^{14}CO_2$ was determined by scintillation spectrophotometry using a Beckman LS 6000LL counter. Enzyme activity was expressed as nmol of $^{14}CO_2$ liberated per hour per testis. Each of the polyamine analyses and enzymes assays was carried with seven pooled testes, and all the experiments were performed three times to arrive at mean values from 21 testes.

Microscopy of cellular differentiation in testes

Testes were removed as described above for histological analysis from 5- and 9-day-old pupae and from 1-day-old adults that had been injected with saline or oleander sap at the 2-day pupal stage and from 1-day-old pupae prior to injection. Each extirpated testis was fixed with 2.5 % glutaraldehyde in 1 mol l^{-1} phosphate buffer (pH 7.4) for 24 h at room temperature. The testes were fixed again with 2 % osmium tetroxide, in the same buffer, and dehydrated through a graded acetone series. The dehydrated specimens were embedded in Epon/Araldite mixture, cut into sections 4–7 μm thick, using an ultramicrotome, and applied to glass slides using albumin solution. After removal of the Epon/Araldite embedding medium, the preparations were stained with hematoxylin and eosin, and examined by light microscopy under bright-field conditions (OPTIPHOT-2, Nikon).

Results

Effects of feeding oleander sap on male fertility

In preliminary studies, we evaluated the effects of oleander on the development of *H. assulta* by continuous feeding of crude sap mixed with diet throughout the larval period. We found that feeding 50 μl or more of undiluted sap significantly delayed the onset of pupal development (20–25 days compared with approximately 15 days in the untreated group) and caused a 5–10 % increase in the frequency of pupal failure relative to untreated controls; these effects were minimal at 10-fold lower dosages (data not shown). We then determined the effects of oleander sap on *H. assulta* fertility by feeding 10-, 20- and 40-fold dilutions of the sap to larvae, allowing them to develop to adults, and determining the percentage of eggs hatching from matings of treated moths with untreated moths of the opposite sex (Table 1). We found that males fed 50 μl of 10- and 20-fold dilutions of the sap as larvae had greatly reduced fertility (Table 1; treatment group I) with only approximately 35 % as many eggs hatching compared with the control group in which untreated males were mated with untreated females (Table 1; treatment group III). Feeding on a 40-fold dilution of the sap produced a less severe effect on male fertility, with approximately 80 % as many eggs hatching compared with the control group. In the reciprocal experiment, in which female larvae were fed oleander sap over the same dilution series, we found no significant effect on female fertility (Table 1; treatment group II), either as a function of the total number of

Table 1. *Effects of feeding oleander sap on male and female fertility in* Helicoverpa assulta

	Treatment group						
	I			II			III
Oleander sap dilution	10^{-1}	5×10^{-2}	2.5×10^{-2}	10^{-1}	5×10^{-2}	2.5×10^{-2}	–
Number of eggs laid per female*	599±237[a]	523±287[a]	619±338[a]	501±238[a]	660±467[a]	471±167[a]	549±216[a]
Number of hatched eggs*	151±59[b]	130±63[b]	351±116[c]	361±141[c]	420±287[c]	330±102[c]	393±187[c]
% Hatched eggs	25.2	24.9	56.7	72.1	63.6	70.1	71.6

Group I, mating between non-treated females and oleander-sap-fed males; group II, mating between oleander-sap-fed females and non-treated males; group III, mating between non-treated females and non-treated males.

Values are means ± S.D. (N=20).

*Means followed by the same letter were not significantly different (LSD test, α=0.05).

There was no significant difference in the number of eggs laid between the treatment groups (F=0.54; d.f.=6,63; P=0.7754); however, the numbers of hatched eggs from the 10- and 20-fold dilution subgroups of group I were significantly different from those of the other treatment groups (F=5.62; d.f.=6,63; P=0.0001), and this statistical difference was also confirmed by the LSD test (α=0.05).

Table 2. *Effects of injecting oleander sap on male fertility in* Helicoverpa assulta

	Treatment group		
	I		II
Oleander sap dilution	5×10^{-2}	2.5×10^{-2}	–
Number of eggs laid per female*	614±273[a]	560±315[a]	578±339[a]
Number of hatched eggs*	121±108[b]	209±162[b]	403±177[c]
% hatched eggs	19.7	37.3	69.7

Group I, mating between non-treated females and oleander-sap-injected males; group II, mating between non-treated females and saline-injected males.

Values are means ± S.D. (N=20).

*Means followed by the same letter were not significantly different (LSD test, α=0.05).

There was no significant difference in the number of eggs laid between the oleander-sap-injected and saline-injected groups (F=0.079; d.f.=2,27; P=0.9247); these numbers were also not significantly different from those of the non-treated control groups in Table 1 (F=0.015; d.f.=1,18; P=0.9036). However, the numbers of hatched eggs were significantly different from each other (F=9.02; d.f.=2,27; P=0.0009), and this difference was also confirmed by LSD test (α=0.05).

eggs laid or as a function of the percentage of eggs hatching relative to the control group.

Effects of injecting oleander sap on male fertility and spermatogenesis

We found that direct injection of oleander sap into the hemocoel of 2-day-old male pupae also strongly affected adult male fertility (Table 2). Since the oleander sap is too viscous to inject easily through a thin glass capillary, it was necessary to dilute it at least 20-fold. Males injected with 5 µl of 20- and 40-fold dilutions of the sap had greatly reduced fertility (Table 2; treatment group I), with only approximately 28 % and 54 % of the eggs hatching, respectively, compared with the control group, in which saline-injected males were mated with untreated females (Table 2; treatment group II). The latter treatment group did not differ significantly from uninjected controls (Table 1; treatment group III).

We next made histological preparations of testes removed from 5- and 9-day-old male pupae and 1-day-old adults after injection with oleander sap and compared them with control preparations from saline-injected males (Fig. 1). The testes of untreated 1-day-old pupae exhibited various stages of differentiating spermatocysts emanating from germaria located at the edge of each follicle (Fig. 1; P1-I and P1-II). In the testes of 5-day-old pupae (P5) of the saline-injected group, distinct eupyrene and apyrene spermatogenesis was well advanced and germaria were no longer present. In the testes of 9-day-old control pupae, dense well-developed cysts filled with spermatozoa were evident (P9). By the adult stage, the testes had become more compact as a result of massive water efflux and were filled with mature follicles and dense spermatozoan cysts (A1). In contrast to the well-ordered structures in the testes of the saline-injected controls describe above, spermatogenesis in the testes of the oleander-sap-injected treatment group was developmentally delayed and exhibited major abnormalities that were particularly evident in the later stages. In the testes of 5-day pupae of the sap-injected group, germaria were still quite prominent and follicle envelopes were not present (Fig. 1; OP5). In the testes of 9-day-old pupae of the sap-injected group, spermatozoan cysts were irregularly arranged because of the absence of any follicle boundaries and were diffuse in appearance (OP9). This disorganized pattern remained in the adult-stage testes in the oleander-injected group, with the spermatozoan cysts failing to condense and remaining very diffuse in appearance (OA1) compared with the testes of the saline-injected treatment group (A1).

We also found that the wet mass of the testes of the oleander sap-injected treatment group was significantly lower throughout pupal–adult development compared with that of the saline-injected control group (Fig. 2).

Effects of injecting oleander sap on polyamine metabolism

We found that injection of oleander sap into the hemocoel of 2-day-old male pupae altered the normal developmental profiles of putrescine, spermidine and spermine in *H. assulta* testes (Fig. 2). After injection of oleander sap into 2-day-old pupae, putrescene levels initially increased relative to the saline-injected control group, but subsequently declined and failed to undergo the large (fivefold) induction that normally occurs during late pupal development. In contrast, spermidine levels decreased immediately following injection of oleander sap and remained at levels significantly below those of the control treatment group for the remainder of pupal development and at day 1 of the adult stage. Similarly, spermine levels decreased immediately following injection of oleander sap and remained significantly lower than those of the control treatment group, which increased steadily throughout pupal development and at day 1 of the adult stage.

We also found that injection of oleander sap into the hemocoel of 2-day-old male pupae altered the normal developmental profiles of ornithine decarboxylase and arginine decarboxylase activities (Fig. 2) in *H. assulta* testis in a way that is similar to the effect of oleander sap on putrescine levels. Whereas the activities of both enzymes increased immediately after injection of oleander sap compared with the saline-injected control group, they remained unchanged during the second half of pupal development compared with significant induction of activity during this period in the saline-injected controls.

Discussion

The present study examined the effects of the milky sap of the common oleander *Nerium indicum* on the fertility, spermatogenesis and polyamine metabolism of the oriental tobacco budworm *Helicoverpa assulta*. The results from feeding experiments show conclusively that oleander feeding

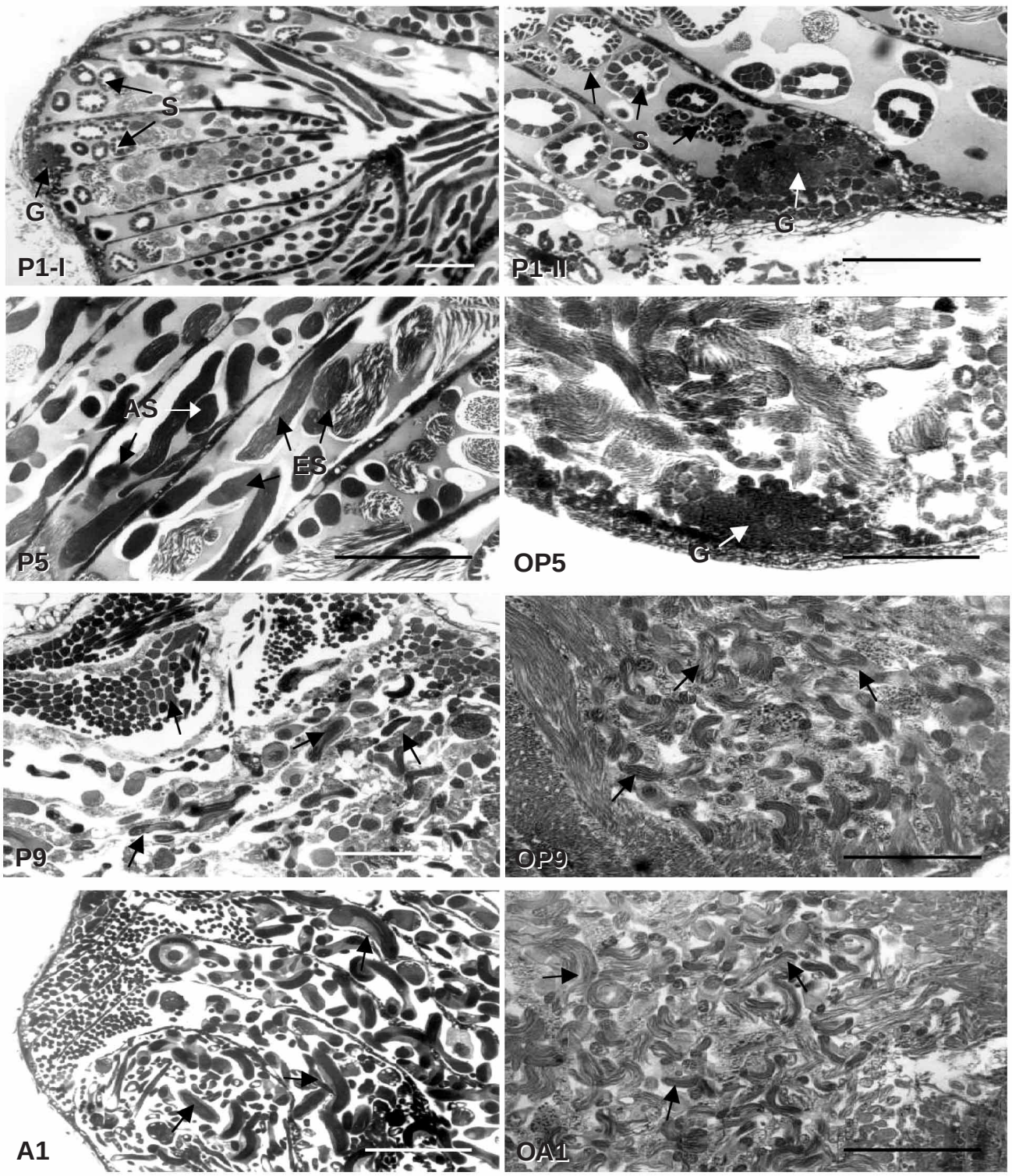

Fig. 1. Longitudinal sections of testes during pupal–adult development of *Helicoverpa assulta* following injection with saline or oleander sap. P1-I and P1-II, from a 1-day-old pupa before the injections, showing a germarium (G) and various stages of developing spermatocysts (S); P5, from a 5-day-old pupa following injection with saline, showing various stages of differentiating eupyrene (ES) and apyrene (AS) spermatozoan cysts; P9, from a 9-day-old pupa following injection with saline, showing well-developed dense cysts (arrows) filled with spermatozoa; A1, from a 1-day-old adult injected with saline, showing mature follicles and spermatozoan cysts (arrows); OP5, from a 5-day-old pupa injected with oleander sap, showing a persisting germarium (G) and the absence of follicle envelopes; OP9 and OA1, from a 9-day-old pupa and a 1-day-old adult, respectively, both injected with oleander sap, showing abnormally developing, irregularly arranged, cysts (arrows) and the absence of follicle envelopes. Scale bars, 100 μm.

by *H. assulta* larvae over the dosage range tested profoundly affects fertility in adult males but not in adult females (Table 1). This effect on male fertility was also produced when oleander sap was injected directly into the hemocoel of 2-day-old pupae (Table 2). Since the injection volume was only one-tenth of the feeding volume (5 μl compared with 50 μl), we conclude that the effects of injection are greater than those produced *via* the oral administration route. However, this observation is not surprising since the acute route of administration of the sap *via* injection offers much less time for metabolic inactivation of the component(s) of the oleander sap causing the male-specific fertility effects compared with administration of sap by continuous feeding.

The testes of oleander-injected males demonstrated delayed differentiation, as demonstrated by the persistence of some germaria in 5-day-old pupal testes; germaria were completely absent by this time from the saline-injected control group. Defects observed in spermatogenesis caused by oleander treatment included degeneration of the follicle envelopes, which was virtually complete as early as the 5-day pupal stage and which contributed to the very disorganized arrangements of spermatozoan cysts in the later developmental stages. In addition, the cysts of the oleander-treated group appeared diffuse at the 9-day pupal stage and had indistinctly stained spermatozoa by the adult stage.

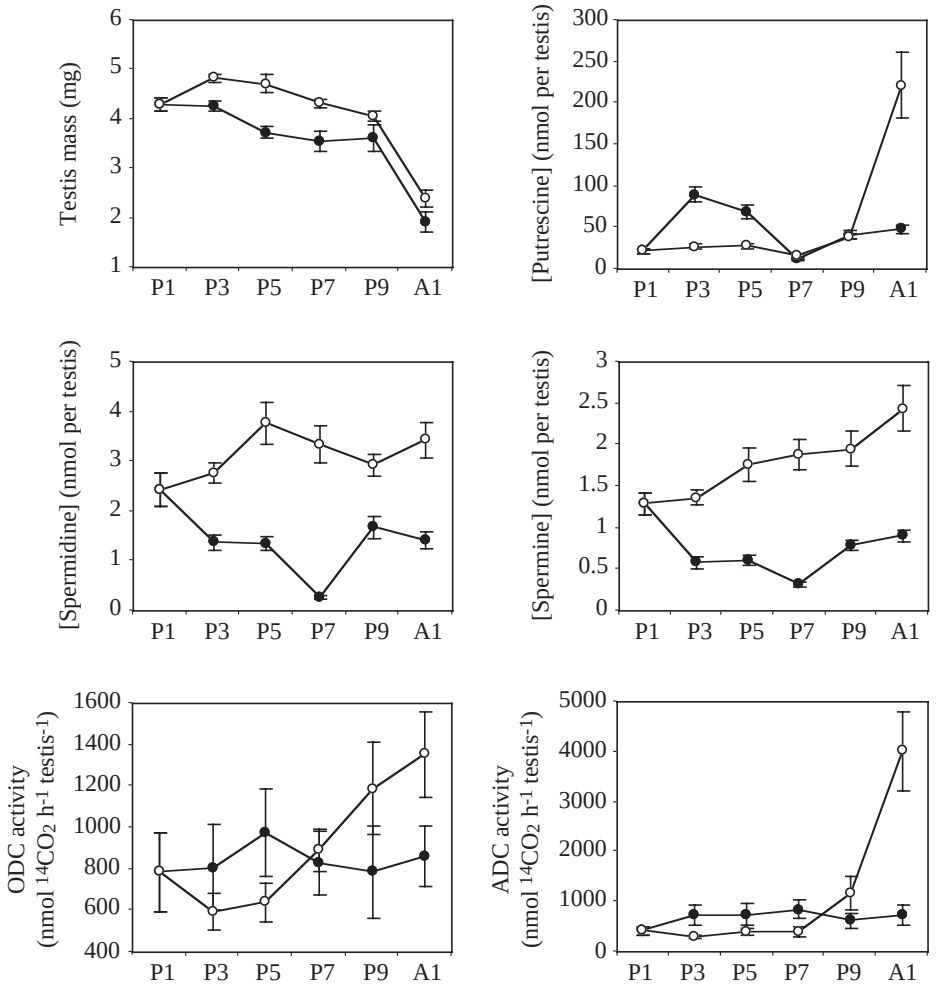

Fig. 2. Changes in testis wet mass, putrescine, spermidine and spermine levels and ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) activities during pupal–adult development of *Helicoverpa assulta* following injection with saline or oleander sap. ○, oleander-sap-injected; ●, saline-injected. P1, 1-day-old pupa; P3, 3-day-old pupa; P5, 5-day-old pupa; P7, 7-day-old pupa; P9, 9-day-old pupa; A1, 1-day-old adult. The injections were carried out at the 2-day-old pupal stage. Enzyme activity is expressed in units of nmoles of $^{14}CO_2$ released per hour. The data points represent means ± S.D. of three samples, each made up of the combined testes from seven animals.

Since developing lepidopteran testes undergo dramatic changes in volume and mass associated with water influx/efflux during physical elongation and maturation of the spermatozoa (Bodnaryk, 1989), we examined the effect of oleander treatment on the wet mass of the testes. The gross developmental abnormalities described above were found to be associated with decreased testis wet mass throughout pupal–adult development relative to controls (Fig. 2). Although we are not aware of any other published studies demonstrating sterility in lepidopteran males caused by administration of sap from oleander or other plants in the Apocyanaceae family, the flower extract of *T. nerifolia* has been shown to induce sterility in the male red cotton bug *Dysdercus similis* (Pyrrhocoridae; Heteroptera), accompanied by anatomical defects, including incomplete testis follicles, thinner vasa deferentia and smaller accessory glands (Raju et al., 1990). These effects on male sexual development, and particularly on spermatogenesis, are similar to those induced by injecting α-difluoromethylornithine (DFMO) and α-difluoromethylargine (DFMA) into *H. assulta* pupae (Jeong and Kown, 1996).

The present study shows that the effects of injection of oleander sap on fertility and spermatogenesis in *H. assulta* males are accompanied by changes in levels of the major polyamines as well as in the activities of ornithine decarboxylase and arginine decarboxylase. It is well known that putrescine levels increase under various physical and chemical stresses, together with somewhat enhanced ornithine decarboxylase activity in plants and animals (Ekker and Sourkes, 1985; Watts et al., 1991; Loevkvist-Wallstroem et al., 1995; Torrigiani et al., 1997; Mautes et al., 1999). The initial increase in testis putrescine levels in the oleander-

injected group, together with increases in the activities of ornithine decarboxylase and arginine decarboxylase (Fig. 2) compared with saline-injected controls are consistent with a stress response induced by compounds in the oleander sap. However, during mid-to-late pupal development in the oleander-injected group, ornithine decarboxylase and arginine decarboxylase activities and putrescine levels remain unchanged or decrease, whereas the activities of these enzymes in saline-injected controls undergo significant inductions, and putrescine increases to a level that is four- to fivefold greater than that of the oleander-treated group. In contrast to the effect of oleander injection on putrescine levels, spermidine and spermine levels decrease significantly following injection and remain at levels substantially below those of the saline-injected control group throughout pupal and larval development (Fig. 2).

In conclusion, ingestion of the milky sap of the common oleander *Nerium indicum* affects fertility in males but not in females of the oriental tobacco budworm *Helicoverpa assulta*. These male-specific fertility effects are associated with profound abnormalities in spermatogenesis and concomitant departures from normal polyamine metabolism, the most notable aspect of which is substantially reduced levels of spermidine and spermine. With regard to the latter issue, since putrescine levels are initially elevated following oleander injection and putrescine is present in substantial molar excess compared with spermidine and spermine in the testes of all developmental stages and all treatment groups, we conclude that the primary effect of oleander injection on decreased spermidine and spermine levels is not at the level of putrescine or the enzymes involved in its biosynthesis. Our findings do not distinguish between two possible explanations for the effects of oleander treatment on spermidine and spermine levels: (i) that a component of oleander sap could be directly inhibiting one of the enzymes used in common during the conversion of putrescene to spermidine and of spermidine to spermine and (ii) that all the observed effects on polyamine metabolism are derivative and that the primary effect of oleander treatment in *H. assulta* males is a disruption of an early fundamental process in the normal program that guides testicular development and spermatogenesis. Further studies are necessary to elucidate the mechanistic basis of the effects of oleander sap on spermatogenesis in *H. assulta* males and to identify the active component or components causing these effects, which are of potential practical significance.

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