CHAPTER IV

RESULTS

1. Biological study

1.1 Observations on life duration

The median embryonation periods of both species C and species E were 2 days (2-3) which are not significantly different from each other (Table 2). The median larval period of species C was 9 days (8-10) which is significantly shorter than that of species E (median = 11, range 9-12) (Table 3). The median pupation period of both species C and species E were 2 days (1-2) in males and females (Table 4). The median longevity of the adult females of species C was 18 days which was significantly longer than species E (17 days) (Table 5). However, the median longevity of the adult males of species C was 10 days which was significantly shorter than species E (20 days) (Table 6).

1.2 Ability of free mating in a 30-cm cage

Study of mating behavior showed that the adult males of *An. minimus* species C failed to mate (0% insemination rate) with the females of neither species C nor species E. The adult males of species E could mate with the females of species C, with the insemination rates of 18.71%, but this was significantly lower than the species E control cross (Table 7).

Table 2. Duration of 100 eggs of *An. minimus* species C and species E developing to hatching first instar larvae in laboratory.

Duration (days)	Number of larvae hatched	
	Species C	Species E
2	69	75
3	24	16
Median	2	2
(range)	(2-3)	(2-3)

Mann-whitney U Test = 1.349, $P > 0.05$

Table 3. Duration of 100 larvae of *An. minimus* species C and species E developing to pupae in laboratory.

Duration (days)	Number of larvae pupated	
	Species C	Species E
8	2	0
9	72	3
10	20	30
11	0	46
12	0	9
Median	9	11
(range)	(8-10)	(9-12)

Mann-whitney U Test = 10.928, $P < 0.05$

Table 4. Duration of 100 pupae of *An. minimus* species C and species E developing to emerging adults in laboratory.

Duration (days)	Number of pupae emerged			
	Species C		Species E	
	Female	Male	Female	Male
1	5	15	3	10
2	32	43	27	56
Median (range)	2[a] (1-2)	2[b] (1-2)	2[a] (1-2)	2[b] (1-2)

[a] Mann-whitney U Test = 0.438, $P > 0.05$

[b] Mann-whitney U Test = 1.477, $P > 0.05$

Table 5. Longevity of 100 adult females of *An. minimus* species C and species E in laboratory rearing with 5% sugar solution.

Age (days)	Number of dead mosquitoes			
	Species C	Cumulative	Species E	Cumulative
5	1	1	2	2
6	1	2	4	6
7	1	3	3	9
8	3	6	7	16
9	1	7	2	18
10	1	8	6	24
11	3	11	2	26
12	3	14	3	29
13	0	14	5	34
14	7	21	3	37
15	5	26	4	41
16	5	31	4	45
17	5	36	14	59
18	24	60	8	67
19	7	67	5	72
20	4	71	4	76
21	2	73	3	79
22	2	75	2	81
23	1	76	2	83
24	3	79	3	86
25	0	79	2	88
26	3	82	3	91
27	0	82	2	93
28	2	84	2	95
29	1	85	1	96
30	1	86	1	97
31	1	87	1	98
32	0	87	1	99
33	0	87	1	100
34	4	91	0	
35	0	91	0	
36	1	92	0	
37	4	96	0	
38	4	100	0	
Median	18		17	
(range)	(5-38)		(5-33)	

Mann-whitney U Test = 2.742, $P < 0.05$

Table 6. Longevity of 100 adult males of *An. minimus* species C and species E in laboratory rearing with 5% sugar solution.

Age (days)	Number of dead mosquitoes			
	Species C	Cumulative	Species E	Cumulative
4	2	2	2	2
5	4	6	2	4
6	10	16	3	7
7	9	25	3	10
8	16	41	2	12
9	2	43	1	13
10	13	56	1	14
11	3	59	4	18
12	11	70	2	20
13	1	71	3	23
14	2	73	2	25
15	0	73	4	29
16	4	77	1	30
17	1	78	7	37
18	2	80	5	42
19	2	82	4	46
20	5	87	5	51
21	0	87	6	57
22	1	88	5	62
23	1	89	7	69
24	1	90	2	71
25	0	90	1	72
26	1	91	6	78
27	1	92	4	82
28	2	94	2	84
29	1	95	2	86
30	0	95	2	88
31	1	96	3	91
32	1	97	1	92
33	1	98	1	93
34	1	99	2	95
35	1	100	2	97
36	0		3	100
Median (range)	10 (4-35)		20 (4-36)	

Mann-whitney U Test = 6.029, $P < 0.05$

Table 7. Insemination rates of *An. minimus* species C and species E after free mating in a 30-cm cage.

Type of mosquitoes	% Insemination rate (No. positive for sperms in spermatheca / No. dissected)
species C (control)	0 (0/134)
species E (control)	53.97 (68/126)*
species E female x species C male	0 (0/124)
species C female x species E male	18.17 (26/139)*

* Chi-Square test = 35.9, $P < 0.05$

2. Hybridization study

Crossing experiments between the progeny *An. minimus* species C (KAN strain) and the laboratory ISG strain (species E) revealed that hybrid progeny were obtained from both directions of crosses. The egg hatching rates among the six crosses of C female x E male varied significantly from 80.9% to 99.0% ($P < 0.05$) (overall 91.1%); among the four crosses of E female x C male also varied significantly from 80.4% to 95.0% ($P < 0.05$) (overall 87.8%) (Table 8); among the five crosses of species E control ranged from 89.9% to 95.9% ($P > 0.05$) (overall 92.6%), and of the five crosses of species C control ranged from 92.1% to 97.9% ($P > 0.05$) (overall 95.5%). The pupation rates calculated from the first instar among the six crosses of C female x E male ranged from 89.8% to 97.8% ($P > 0.05$) (overall 94.2%); among the four crosses of E female x C male ranged from 95.1% to 100% ($P < 0.05$) (overall 98.4%); among the five crosses of species E control ranged from 66.9% to 90.9% ($P < 0.05$) (overall 81.9%); among the five crosses of species C control ranged from 71.0% to 94.4% ($P < 0.05$) (overall 84.8%). The adult emergence rates calculated from the pupal stage among the six crosses of C female x E male ranged from 94.3% to 100% ($P > 0.05$) (overall 97.6%); among the four crosses of E female x C male ranged from 98.8% to 100% ($P > 0.05$) (overall 99.4%); among the five crosses of species E control ranged from 93.1% to 100% ($P < 0.05$) (overall 97.8%); the five crosses of species C control ranged from 91.9% to 100% ($P < 0.05$) (overall 98.0%). The sex ratios of the emerging adult of all crosses and controls were not significantly different ($P > 0.05$).

In C female x E male crosses, the hybrid males (5-7 days old) showed the completely sterile testes (51/51) and atrophied accessory glands (Fig. 4), whereas the

ovaries of the hybrid females looked normal. In addition, the external terminalia of these males was never completely rotated and they failed to copulate with female by the artificial mating.

In E female x C male crosses, the hybrid males (5-7 days old) showed partially sterile testes (50/50) in which most spermatozoa were abnormal (enlarged head) and inactive (Fig. 5), whereas the ovaries of the hybrid females looked normal. The hybrid males failed to inseminate with KAN and ISG females, but had a little success with the hybrid females and there females produce F_2 hybrids. However, the egg hatching rate, the pupation rate and the adult emergence rate were significantly low (Table 9). The testes of the F_2 hybrid males were partially sterile with abnormal spermatozoa (Fig. 6). In addition, the external terminalia of these males was never completely rotated and they failed to copulate with females by the artificial mating. The ovaries of F_2 hybrid females looked normal.

When the F_1 hybrid females from both directions of crosses were backcrossed with either species C or species E males, they produced viable eggs. The egg hatching rates among the four crosses of (E x C)F_1 x C ranged from 83.7% to 92.9% ($P > 0.05$) (overall 89.2%); among the three crosses of (E x C)F_1 x E ranged from 88.6% to 96.7% ($P > 0.05$) (overall 92.3%); among the four crosses of (C x E)F_1 x E ranged from 91.8% to 99.1% ($P > 0.05$) (overall 94.6%); among the three crosses of (C x E)F_1 x C ranged from 91.2% to 98.4% ($P > 0.05$) (overall 95.8%). The pupation rates calculated from the first instar among the four crosses of (E x C)F_1 x C ranged from 60.9% to 84.8% ($P < 0.05$) (overall 70.7%); among the three crosses of (E x C) F_1 x E ranged from 83.9% to 93.5% ($P > 0.05$) (overall 88.9%); among the four crosses of (C x E)F_1 x E ranged from 66.2% to 98.4% ($P < 0.05$) (overall 86.4%);

among the three crosses of (C x E)F_1 x C ranged from 77.1% to 84.6% ($P < 0.05$) (overall 81.1%). The adult emergence rates calculated from the pupal stage among the four crosses of (E x C)F_1 x C ranged from 89.4% to 97.0% ($P > 0.05$) (overall 94.8%); among the three crosses of (E x C)F_1 x E ranged from 94.5% to 100% ($P > 0.05$) (overall 97.6%); among the four crosses of (C x E)F_1 x E ranged from 82.0% to 96.8% ($P < 0.05$) (overall 88.7%); among the three crosses of (C x E)F_1 x C ranged from 92.3% to 95.1% ($P > 0.05$) (overall 93.4%). In addition, the male progeny obtained from backcrosses showed various degrees of abnormality of testes and accessory glands (Table 10).

Table 8. Results from interstrain cross between *An. minimus* species E and species C.

Crosses[1]	No. of broods	Eggs		Hatching rate[2]	Pupation rate[2]			Emergence rate[2]		
		Total	Average/brood		Female	Male	Total	Female	Male	Total
Interstrain crosses										
C x E	6	462	77.0	91.1[ab]	42.2	43.7	85.9	41.8	42.0	83.8
E x C	4	402	100.5	87.8[a]	41.0	45.3	86.3	41.0	44.8	85.8
Control crosses										
E x E	5	610	122.0	92.6[b]	37.5	37.4	74.9	37.0	36.4	73.4
C x C	5	639	127.8	95.5[c]	37.9	43.2	81.1	37.6	42.3	79.9

[1] All crosses are female x male.

[2] Each rate was calculated by (total number of individuals that reached each developmental stage) / (total number of eggs) x 100.

The same letter in each column indicated no significantly difference (all $P > 0.05$).

Table 9. Results from fertility tests of backcrosses between the hybrids and the parental strains and of the hybrids.

Crosses[2]	No. of broods	Eggs		Embryo -nation rate	Hatching rate[3]	Pupation rate[3]			Emergence rate[3]		
		Total	Average/ brood			Female	Male	Total	Female	Male	Total
E x (E x C)F_1	3[4]	366	122.0	0	0						
C x (E x C)F_1	1[4]	132	132.0	0	0						
(E x C)F_1 x (E x C)F_1	8[5]	767	95.9	n.d.	15.9[c]	4.0	4.0	8.0	3.9	3.8	7.7
(C x E)F_1 x (C x E)F_1	8[4]	0	0								
(E x C)F_1 x E	4	425	106.3	n.d.	89.2[a]	33.9	28.7	62.6	32.5	26.6	59.1
(E x C)F_1 x C	3	273	91.0	n.d.	92.3[ab]	35.9	45.8	81.7	34.8	45.1	79.9
(C x E)F_1 x E	4	522	130.5	n.d.	94.6[b]	35.2	41.2	76.4	33.1	35.2	68.3
(C x E)F_1 x C	3	356	118.7	n.d.	95.8[b]	42.1	34.3	76.4	39.0	31.5	70.5

[1] n.d., not determined.

[2] All crosses are female x male.

[3] Each rate was calculated by (total number of individuals that reached each developmental stage) / (total number of eggs) x 100.

[4] No sperm were observed in the spermathecae of any of these females.

[5] Small numbers of spermatozoa were found in the spermathecae of 3 females.

The same letter in each column indicated no significantly difference (all $P > 0.05$).

Table 10. Abnormality of male progeny from backcrosses.

Abnormity	Male progeny from			
	(E x C) x E (n = 33)	(C x E) x E (n = 39)	(E x C) x C (n=29)	(C x E) x C (n =34)
1. partially sterile with abnormal and inactive spermatozoa (Fig. 7, 10)	63.6 %	51.3%	62.1%	55.9%
2. small accessory glands and sterile testes (Fig. 8, 11)	24.2%	25.6%	37.9%	44.1%
3. atrophied testes and accessory glands and the external terminalia never completely rotated (Fig. 9)	12.2%	23.1%	-	-

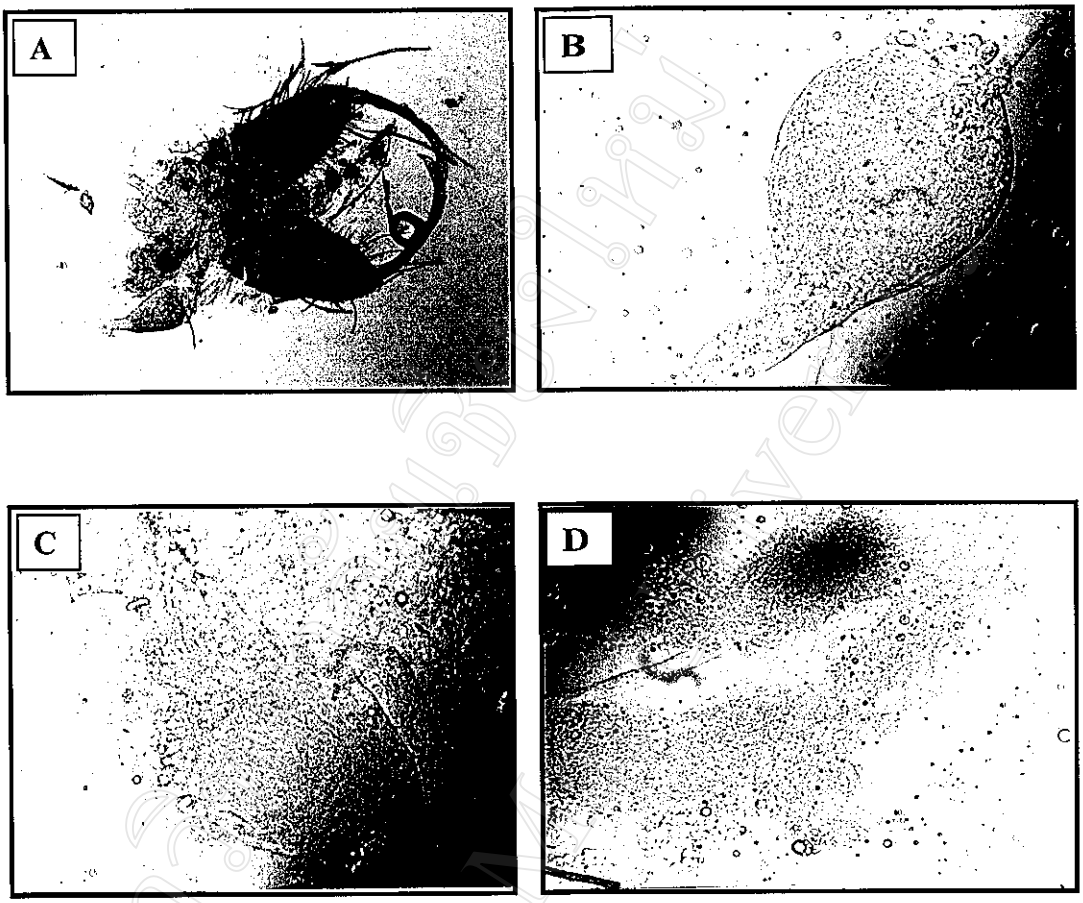

Fig. 4. Internal sex organs of F_1 hybrid males from species C female x species E male, showing the atrophied accessory glands (A) and sterile testes (B-D).

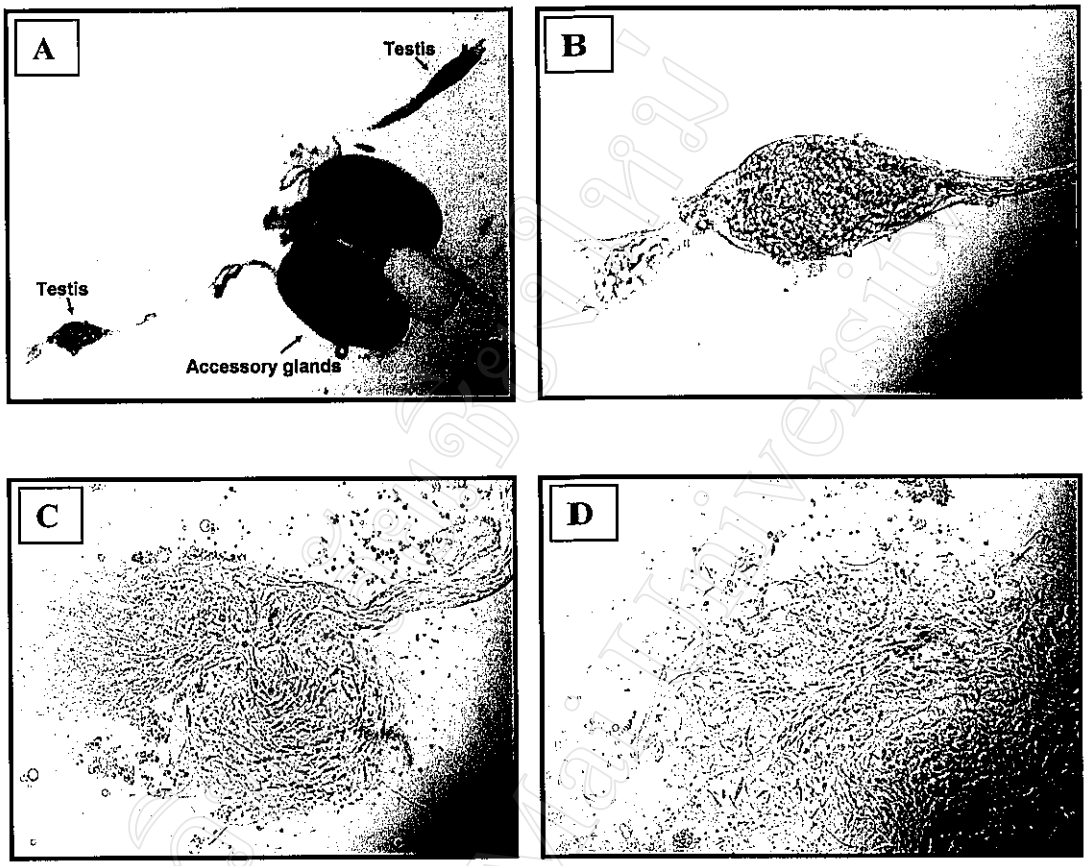

Fig. 5. Internal sex organs of F_1 hybrid males from species E female x species C male, showing the normal-sized accessory glands (A), and the testes with enlarged-head spermatozoa (B-D).

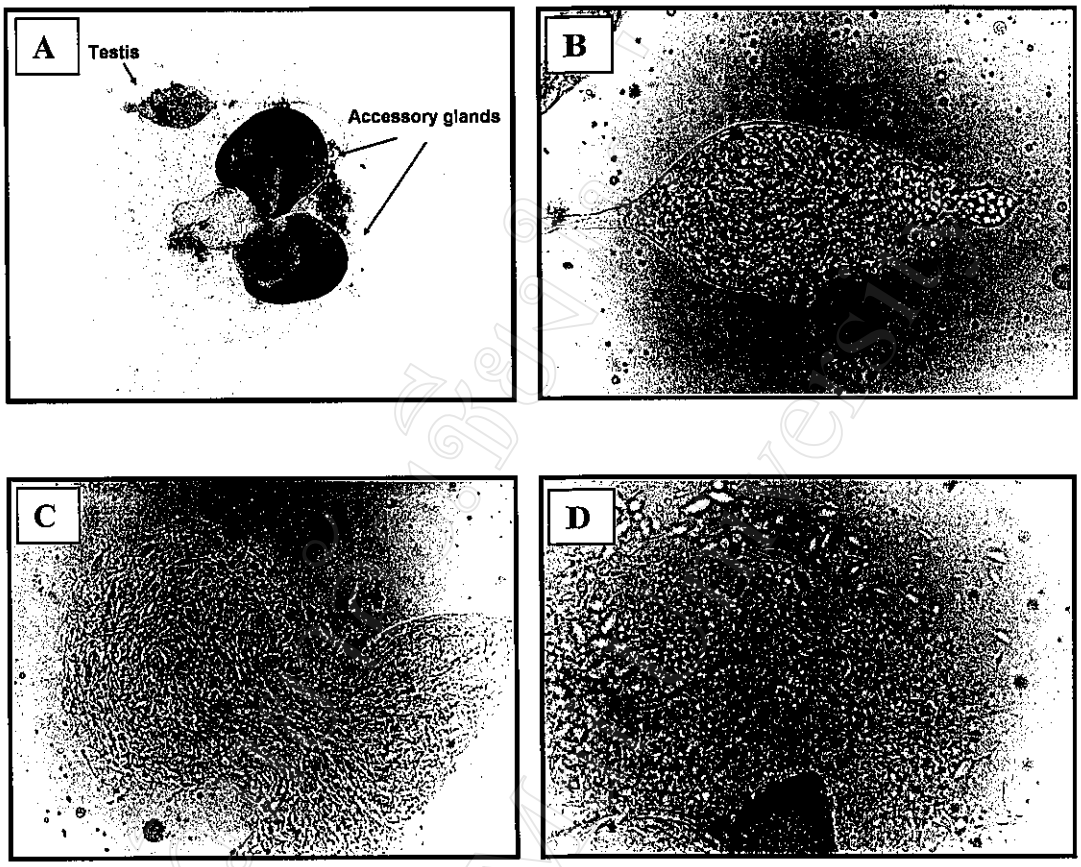

Fig. 6. Internal sex organs of F_2 hybrid males from (E female x C male) F_1 hybrid, showing the normal-sized accessory glands (A), and testes with enlarged-head spermatozoa (B-D).

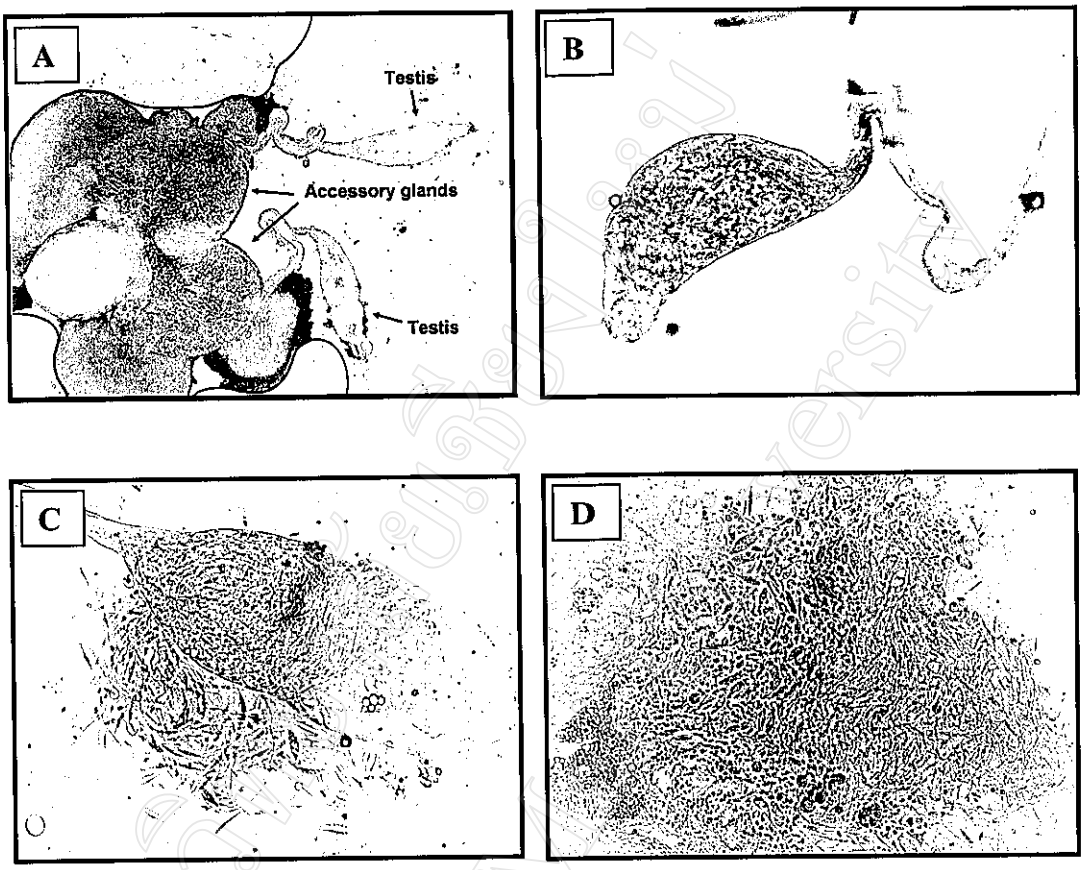

Fig. 7. Internal sex organs of male progeny obtained from backcrosses of both (C x E) x E and (E x C) x E, showing the normal-sized accessory glands (A), and the testes with enlarged-head spermatozoa (B-D).

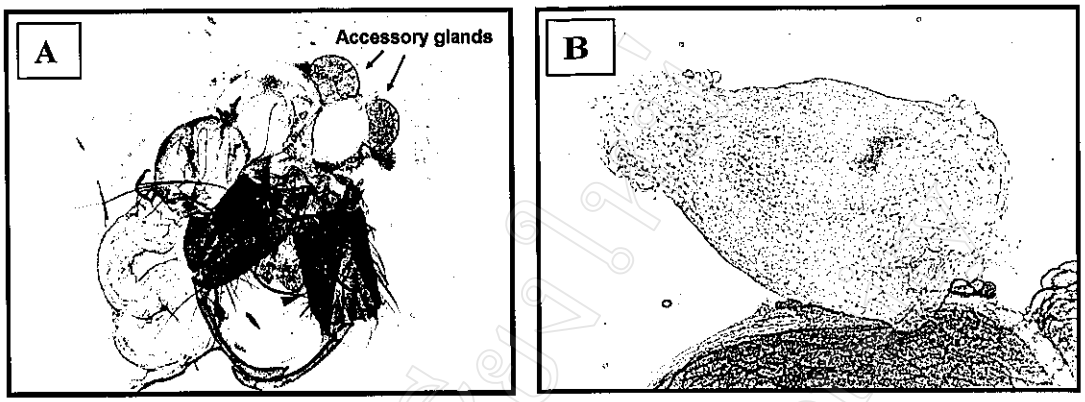

Fig. 8. Internal sex organs of male progeny obtained from backcrosses of both (C x E) x E and (E x C) x E, showing small accessory glands (A) and sterile testes (B).

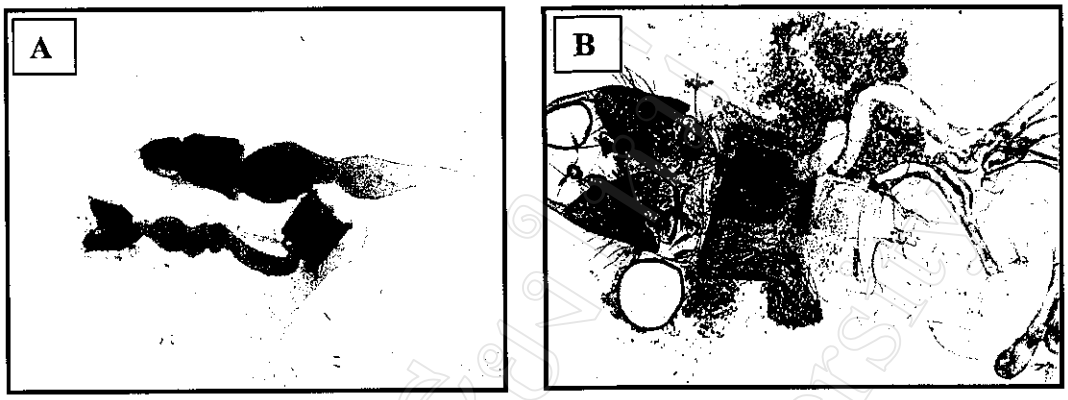

Fig. 9. Internal sex organs of male progeny obtained from backcrosses of both (C x E) x E and (E x C) x E, showing the atrophied testes and accessory glands.

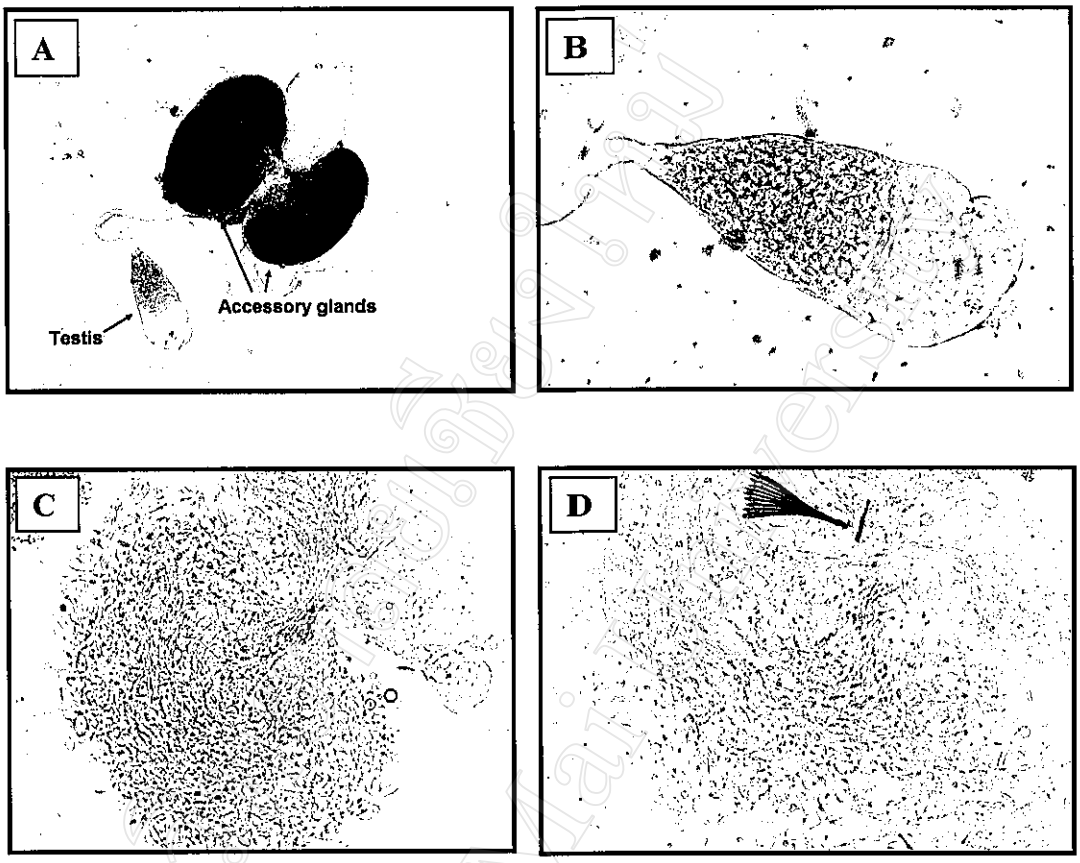

Fig. 10. Internal sex organs of male progeny obtained from backcrosses of both (C x E) x C and (E x C) x C, showing the normal-sized accessory glands (A), and the testes with enlarged-head spermatozoa (B-D).

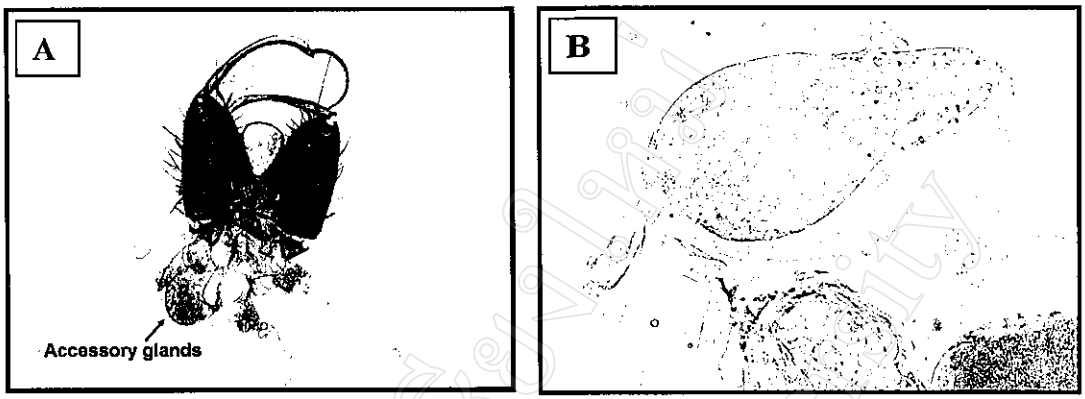

Fig. 11. Internal sex organs of male progeny obtained from backcrosses of both (C x E) x C and (E x C) x C, showing small accessory glands (A) and sterile testes (B).

3. Study of polytene chromosomes

The salivary gland polytene chromosomes of F_1 hybrid larvae from species E female x species C male showed partial asynapsis on identified arms (2R, 3R and 3L) and fixed heterozygous inversion on 3R arm (Fig. 12) whereas those of F_1 hybrids from species C female x species E male showed fixed heterozygous inversion on 3L arm (Fig. 13).

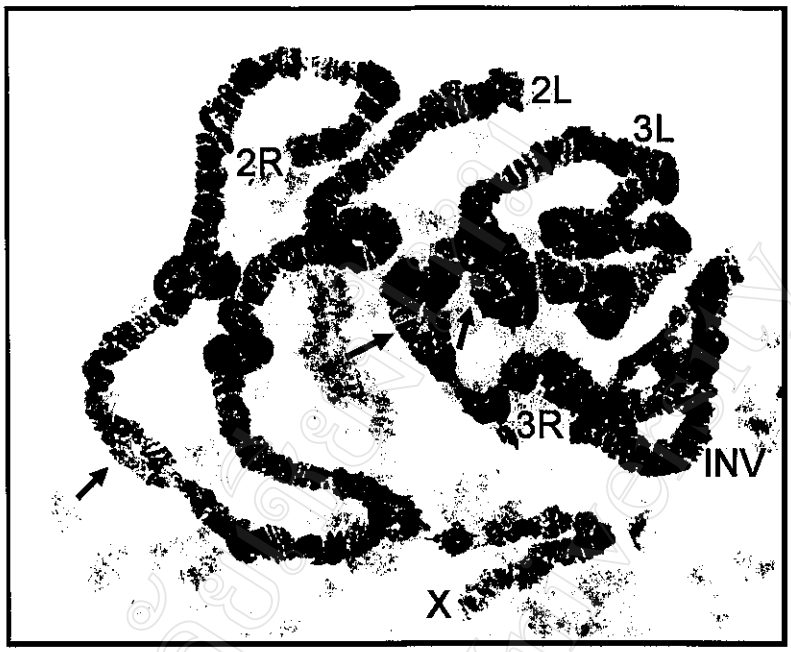

Fig. 12. The salivary gland polytene chromosome of F_1 hybrid larvae from species E female x species C male. The positions of asynapsis are indicated by arrows. Fixed heterozygous inversion (INV) was observed on arm 3R.

Fig. 13. The salivary gland polytene chromosome of F_1 hybrid larvae from species C female x species E male. The position of fixed heterozygous inversion is indicated by arrow. (C = centromere)