SEX RATIO AGENT IN *Cadra cautella* (WALKER) -- ITS EXPRESSION BY TEMPERATURE, GENETICS, AND MASS PRODUCTION OF THE VIRGIN FEMALE MOTHS FOR SEX PHEROMONE STUDY

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PROLOG: Mass production of virgin female moths of *Cadra cautella* (Walker) for sex pheromone study: Females of the almond moth, *Cadra cautella* (Walker), and the flour moth, *Anagasta kühniella* Hübner, emit a sex pheromone to which the males of both species respond. Our collaborators in Kyoto University started the sex pheromone studies in these species, and finally they isolated and identified the chemical structure of their sex pheromone as cis-9, trans-12 tetradecadienyl acetate (1 and 2). This was the first identification of insect pheromone in Japan.

At the start of the project they requested me to design the mass production of female moths of *C. cautella*, and I presented two kinds of design. One was the system to produce only female moths using a special strain of *C. cautella* named FT-strain which carried the sex ratio agent. In this strain, only female moths emerged when they were reared at 20°C, while both sexes emerged in equal numbers when the rearing temperature was 30°C (3). This character is very useful skipping the steps to separate females from males. However, their speed of development became slow and it took more than 60 days at 30°C from oviposition to adult emergence, and we had to wait for more than two months to obtain the first sample of female moths. Furthermore, for the mass production we needed wide space in temperature controlled room which was not easily accomodated in our laboratories.

Another system was to produce both sexes and separate females from males by hand. When they were reared at 30°C and 70% R.H., the duration from oviposition to adult emergence was about 28 days and we needed relatively shallow space for mass production. Our collaborators hurried to obtain samples for their chemical study and we started the project with this system. The system is diagrammatized in Figure 1.

Each step of life cycle of the moth was already analysed and summarised by Takahashi (4) especially with respect to the effect of population density upon the speed of development, mortality, and reproduction. From the results of this study the most productive system was designed as follows: Eggs were easily obtained by keeping adults in a glass vial without any food.

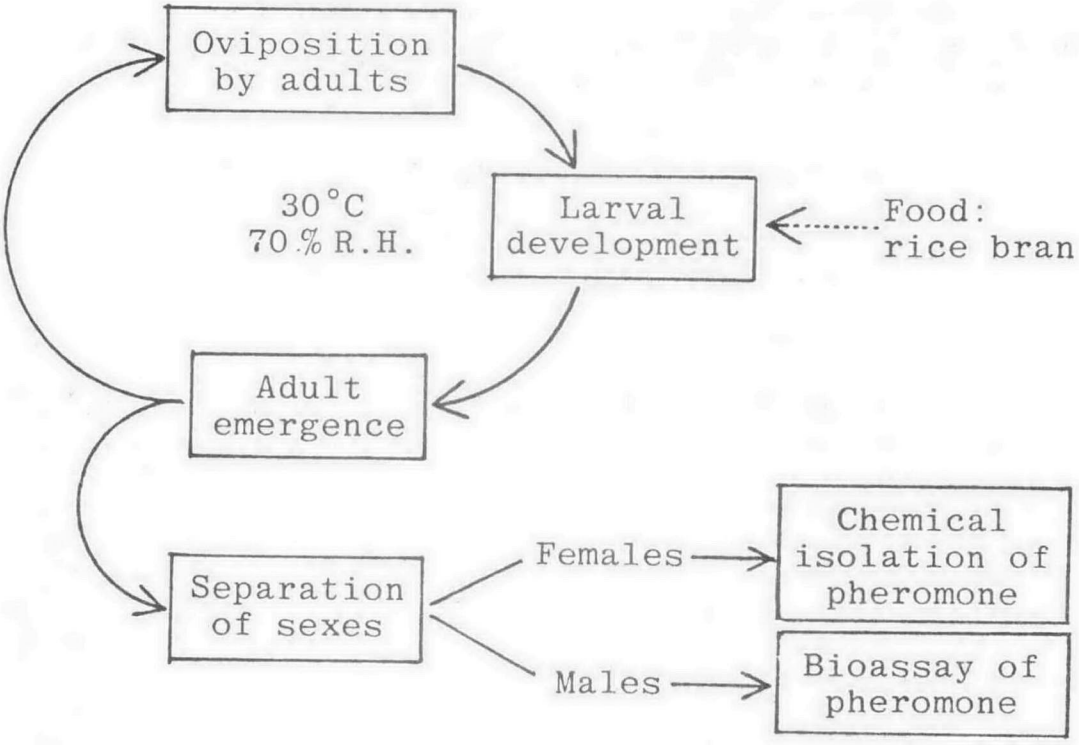

Figure 1. The rearing system of the moths at 30°C

Eggs deposited in a vial were cleaned up from debris, scales, and dead bodies by a soft blow. The rearing with the initial density of 30 eggs per 1 gr of rice bran at a food depth of less than 2 cm was recommended to produce maximum number of moths. But, the wide variation was observed in developmental period at such high density and it wasted labor for collecting moths frequently from vials. Therefore, the density of 20 eggs per 1 gr of rice bran was employed. Practically 8,000 eggs were reared in 400 gr of bran in a vial (27 x 35 x 6 cm).

About 4 weeks later many moths emerged every day. After anesthetization by ether gas they were sexed by hand. Females were stored in a solvent, hexane. However, Dr. Kuwahara, one of our collaborators, found that the solvent did not exhibit any sex pheromone activity to male moths in bioassay. The behavioral study of moths demonstrated the reason why the solvent did not contain the sex pheromone (5).

The sex pheromone was already detectable in female pupae of grayish color which had begun their eclosion. The pheromone content in a female increased up to a maximum after 3 hours from emergence and maintained this level until death if the moths did not mate. The sensitivity of the male moths to the sex

pheromone increased gradually with their age up to the 7th day from their emergence, while their mortality increased greatly after about the 5th day. Most of the moths emerged during a short period of a few hours around the sunset. They copulated within 2 hours after their emergence though their pheromone content in a female and the sensitivity of males to pheromone were not fully developed. When female moths copulated their pheromone activity disappeared rapidly in 4 or 5 hours after the beginning of copulation.

Therefore, the pheromone activity could not be expected in female moths collected in the morning unless their copulation was inhibited. From this fact, some modification was proposed in the mass production system, such as changes of circadian rhythm of moths by changing light and dark period in a day. But, their behavior of emergence was not so strict in responding to light stimuli. The program was, then, changed to the system in which the special character of sex ratio agent was fully utilized. The system is diagrammed in Figure 2.

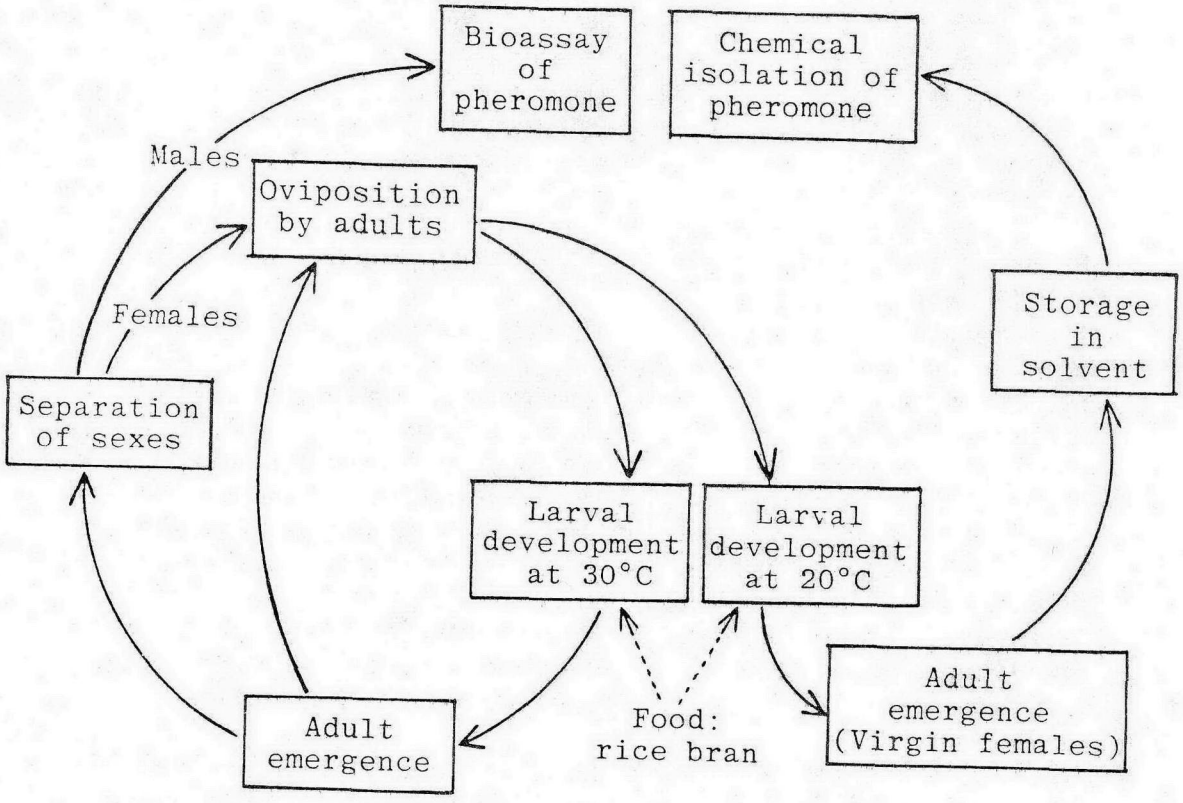

FIGURE 2. The rearing system of the virgin female moths employing their sex ratio agent

An economical rearing method of virgin female moths using FT-strain was established by Takahashi et al. (6). Fundamental methods were similar to those obtained at 30°C and the rearing density did not modify the degree of sex ratio manifestation. About 7,000 eggs were obtained from the moths reared from 200 eggs on 20 gr of bran at 30°C. Using these eggs female moths were raised at about 20°C, 70% R.H. The maximum number of their emergence within a week was obtained at the density of 20-30 eggs per 1 gr of bran at a food depth of less than 2 cm. Practically the rearing was conducted by 10,000 eggs in 400 gr of bran within a vial (27 x 35 x 6 cm) at 20±<5°C. During this project we produced about 1,200,000 unmated female moths and isolated 6 mg of sex pheromone (2).

EXPRESSION OF SEX RATIO AGENT IN *C. cautella* BY TEMPERATURE: The FT-strain of *C. cautella* had been maintained under a constant temperature of 30°C for about 8 years in the laboratory. The sex ratio of adults of this strain was greatly apart from the normal ratio of 1:1 when they were reared at lower temperature. When 150 eggs were reared in 30 gr of rice bran at several combinations of temperature and atmospheric moisture, the number of female and male moths emerged were shown in Table 1. The sex ratio was 1:1 at 30°C even though the percentage of emergence changed with the change of moisture. At 20°C only a few males emerged at every moisture condition while females emerged at almost the same number to those at 30°C. At 25°C, however, some males emerged and Dr. Mutuura found that their copulatory organs were malformed in variable degree. Several abnormal forms of the male copulatory organs were shown in Figure 3. They were abnormal formation of vinculum and of the valva of one side; degeneration of valva and of aedoeagus; pseuohermaphroditic male with a bursa copulatrix; and a super male having a supernumerary valva. The occurrence of these abnormal forms was shown in Table 2 when the insects were reared at 22.5°C and 25°C. Even the apparently normal males had some malformations in their copulatory organs and emerged in retarded days than females. Thus, the effect of low temperature was different on males and females. Male gonad development and the formation and growth of male copulatory organs were especially influenced. The characteristics of the abnormal sex ratio in FT-strain was a consequence of high mortality of males in their larval and pupal stages. The abnormal male adults died faster than normal ones and they could not copulate with females.

SENSITIVE STAGE IN THE DEVELOPMENTAL PERIOD TO LOW TEMPERATURE STIMULATION PROMOTING THE SEX RATIO AGENT: The insects reared at 30°C were exposed to 20°C in various developmental stages for 5 days, but the sensitive stage to produce abnormal males was not detected due to the weak and short duration of stimulation of low

Table 1. Number of female and male moths of FT-strain reared at several combinations of temperature and atmospheric moisture (Takahashi and Mutuura, 1964)

	Atmospheric moisture (% R. H.)											
	29～33			49～57			71～77			82～87		
Sex	♀	(♂)	♂	♀	(♂)	♂	♀	(♂)	♂	♀	(♂)	♂
Temperature												
35 °C	0	0	0	0	0	0	11	0	7	1	0	0
30 °C	0	0	0	38	0	51	57	0	41	6	0	12
25 °C	0	0	0	30	7	19	41	13	31	22	3	18
20 °C	0	0	0	49	0	0	40	2	1	14	0	0
15 °C	0	0	0	0	0	0	0	0	0	0	0	0

♀, ♂ : Apparently normal females and males.

(♂) : Apparently abnormal males.

Atmospheric moisture was controlled by $CaCl_2$ (29～33%), $Ca(NO_3)_2$ (49～57%), NaCl (71～77%), and KCl (82～87%), respectively, and 150 eggs were reared on 30 gr rice bran.

Table 2. Occurrence of abnormal forms of male copulatory organs when they were reared at low temperature. (Takahashi & Mutuura, 1964)

Temperature	Days after oviposition	Number of females	Number of males						
			Total	Near normal *	Abnormal valva of one side	Malformed vinculum	Degeneration of valva and aedoeagus	Super male	Pseudo-hermaphroditic male
22.5°C	49～52	110	1	1					
	53～56	124	29	11	2		15	1	
	57～60	36	24	8			14		2
	61～64	16	14	6	1		7		
	65～73	4	7	2			5		
	Total	290	75	28	3	0	41	1	2
25°C	38～40	75	13	6	1	2	4		
	41～44	119	78	34	13	1	29		1
	45～48	14	39	17	2		19		1
	49～52		16	2	3		10		1
	57～58	1							
	Total	209	146	59	19	3	62	0	3

* no males were found with the complete copulatory organs.

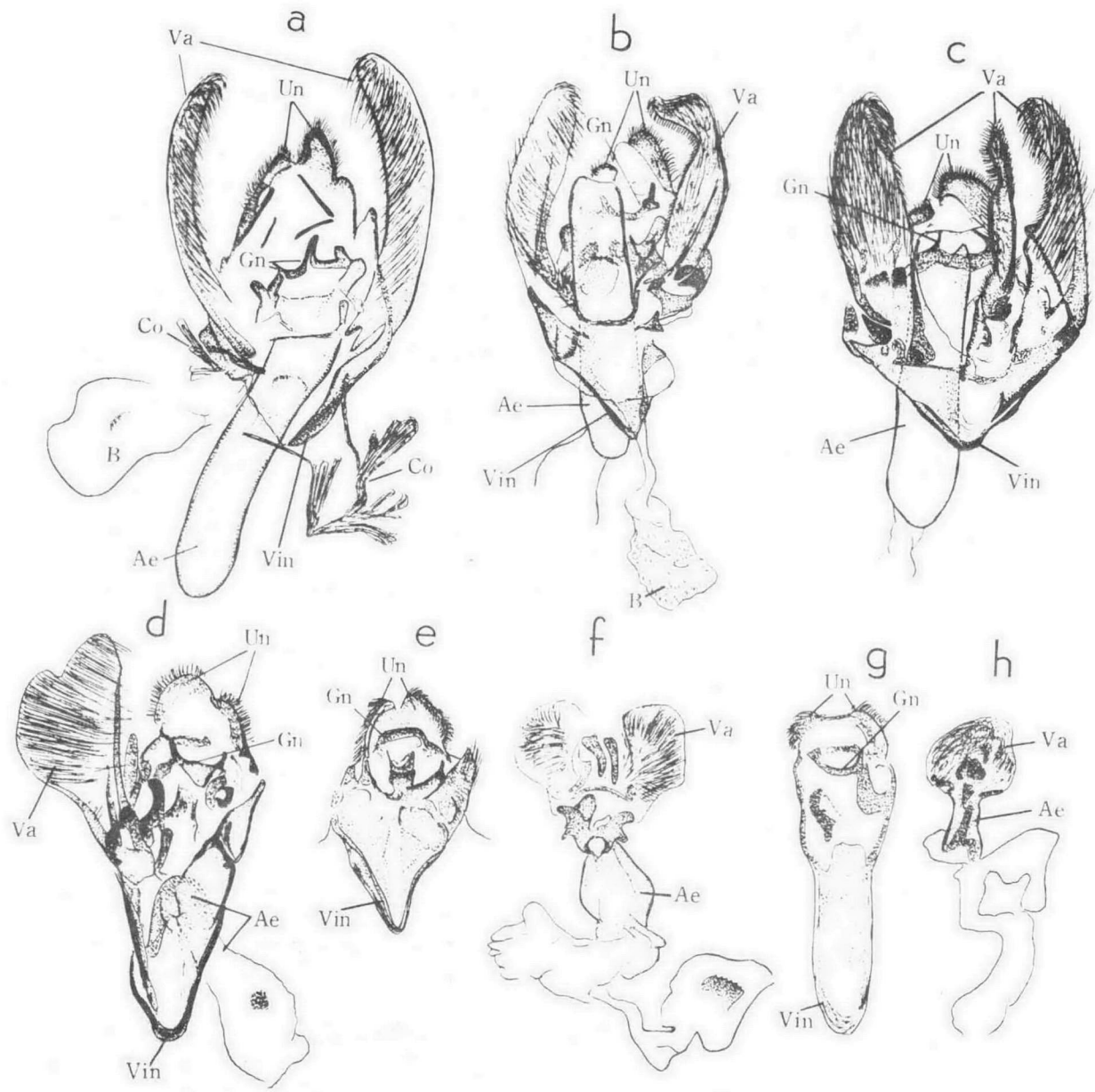

FIGURE 3. Abnormal forms of male copulatory organs in *Cadra cautella* reared at 25°C. Drawn by A. Mutuura (Takahashi and Mutuura, 1964)

a, b: Pseudohermaphroditic males.
c: Degeneration of the valva of one side.
e-h: Degeneration of male copulatory organs. They apparently resemble female, but they have no female characters.
Ae, Aedoeagus; B, Bursa copulatrix; Co, Crest; Gn, Gnathos; Un, Uncus; Va, Valva; Vin, Vinculum.

temperature (3). Takahashi et al. (6) studied again with a little strong stimulation of low temperature (15°C for 10 days). The occurrence of abnormal male copulatory organs was observed in every larval and pupal stage especially from 4th instar to prepupae as shown in Table 3, and the critical stage was not detected. For the practical production of virgin female moths we had to keep insects at low temperature in their whole developmental stages to promote effectively their sex ratio agent.

GENETICS OF THE SEX RATIO AGENT: Takahashi and Kuwahara (7) collected some strains of *C. cautella* and reared at 30°C and 20°C. They were S-strain supplied from Hatano Tobacco Experiment Station in Kanagawa Pref., N-strain collected at a granary in Kyoto City, M-strain collected at a bakery in Osaka City, and W-strain collected at a granary in Kyoto University from where FT-strain was collected 14 years ago. Amongst 5 strains FT- and M-strains express strong abnormality in sex ratio at 20°C. In W-strain males were fewer than females and some abnormal males were found. However, S- and N-strains were normal in their sex ratio. Their sex ratios were rather stable in every season and in every strain. Therefore, it was concluded that the character of sex ratio agent can be frequently observed in some strains and is inherited from generation to generation.

CROSSING EXPERIMENT OF FT-STRAIN WITH N-STRAIN: To examine the mode of inheritance of the sex ratio agent Takahashi and Kuwahara (7) conducted the crossing experiments between FT-strain and strains of normal sex ratio. There were no big differences in the percentages of egg hatching and adult emergence in the crossings between FT- and N-strains (Tables 4 and 5). On the other hand, some abnormal sex ratio and abnormal male copulatory organs were found in some crossings at 20°C (Table 6). However, they could not find clear genetic relation in this crossing experiment.

CROSSING EXPERIMENT OF FT-STRAIN WITH S-STRAIN: The crossing experiment was again conducted between FT- and S-strains. In this case the fecundity was normal in every combination of crossing among strains and hybrids though it was clearly governed by the size of female body following the relation found in FT-strain (Fig. 4). However, a unidirectional incompatibility was found in the rate of egg hatching in the crossing between the two strains (Table 7). The crossing of FT-female x S-male showed very high mortality before egg hatching while normal rate of egg hatching was found in the crossing of S-female x FT-male. The high egg mortality was also found in some hybrid crossings and back crossings. The mortality rate was at the same degree at 30°C and 20°C and this phenomenon was not related to that of sex ratio agent. This will be discussed elsewhere.

Table 3. Effects of low temperature on the sex ratio of moths treated at various developmental stages (Takahashi et al. 1968)

Starting date of treatment in days after oviposition	Approximate stage of development when treated		Replicates	Total number of emerged moths ♀	(♂)	♂
0	Egg		3	15	0	2
2	Larval instar	1st	3	103	28	26
5		2nd	3	81	9	49
8		3rd	3	115	76	28
11		4th	3	119	91	4
14		5th	3	108	74	6
17	Prepupa*		3	98	92	1
20	Pupa		3	119	69	31
Control (no treatment)			4	176	2	146

One hundred eggs were reared on 40 gr rice bran at 30°C. Vials were kept in 15°C for 10 days. *: treated for 11 days.

♀ and ♂ : apparently normal females and males.

(♂) : apparently abnormal males.

Table 4. Percentage of egg hatching in the crossing between FT-strain (F) and N-strain (N) at 20°C and 30°C. (Takahashi & Kuwahara, 1970)

♀ \ ♂	F			N			F_1(F♀ × N♂)			F_1(N♀ × F♂)		
	Number of eggs	Number hatched	%	Number of eggs	Number hatched	%	Number of eggs	Number hatched	%	Number of eggs	Number hatched	%
F												
20°C	649	484	74.6	412	269	65.3	748	545	72.9	600	336	56.0
30°C	778	618	79.4	1013	708	69.9	723	551	76.2	600	276	46.0
N												
20°C	656	516	78.7	489	346	70.8	800	552	69.0	400	274	68.5
30°C	1200	1004	83.7	945	430	45.5	550	394	71.6	432	286	66.2
F_1(F♀ × N♂)												
20°C	400	306	76.5	600	374	62.3	600	480	80.0	558	394	70.6
30°C	400	239	59.8	600	418	69.7	600	483	80.5	604	354	58.6
F_1(N♀ × F♂)												
20°C	819	490	59.8	600	349	58.2	600	370	61.7	800	419	52.4
30°C	600	471	78.5	600	356	59.3	754	477	63.3	800	403	50.4

Table 5. Percentage of adult emergence from eggs hatched in the crossing between FT-strain (F) and N-strain (N) at 20°C and 30°C. (Takahashi & Kuwahara, 1970)

♀ \ ♂	F			N			F_1(F♀ x N♂)			F_1(N♀ x F♂)		
	Number of larvae	Adults emerged	%	Number of larvae	Adults emerged	%	Number of larvae	Adults emerged	%	Number of larvae	Adults emerged	%
F												
20°C	484	262	54.1	269	215	79.9	545	380	69.7	336	209	62.2
30°C	618	559	90.5	708	632	89.3	551	476	86.4	276	242	87.7
N												
20°C	516	396	76.7	346	221	63.9	552	486	88.0	274	226	82.5
30°C	1004	882	87.8	430	384	89.3	394	349	88.6	286	269	94.1
F_1(F♀ x N♂)												
20°C	306	299	74.8	374	276	73.8	480	369	76.9	394	346	87.8
30°C	239	208	87.0	418	357	85.4	483	405	83.9	354	338	95.5
F_1(N♀ x F♂)												
20°C	490	352	71.8	349	278	79.7	370	277	74.9	419	321	76.6
30°C	471	438	93.0	356	328	92.1	477	442	92.7	403	366	90.8

Table 6. Number of emerged moths in the crossing between FT-strain (F) and N-strain (N) at 20°C and 30°C. (Takahashi & Kuwahara, 1970)

♀ \ ♂		F			N			F_1(F♀ × N♂)			F_1(N♀ × F♂)		
		♀	(♂)	♂	♀	(♂)	♂	♀	(♂)	♂	♀	(♂)	♂
F	20°C	220	33	9	117	11	87	263	41	76	143	37	29
	30°C	270		289	310		322	227		249	123		119
		χ^2=94.32*			χ^2=1.85			χ^2=39.99*			χ^2=14.35*		
N	20°C	229	41	126	107	1	113	247	2	237	128	27	71
	30°C	458		424	203		181	181		168	145		124
		χ^2=3.83			χ^2=1.11			χ^2=0.09			χ^2=0.37		
F_1(F♀ × N♂)													
	20°C	147	16	66	157	17	102	176	17	176	178	13	155
	30°C	98		110	172		185	202		203	169	1	168
		χ^2=12.90*			χ^2=4.73*			χ^2=0.37			χ^2=0.14		
F_1(N♀ × F♂)													
	20°C	237	28	87	171	4	103	161	34	82	191	29	101
	30°C	230		208	174		154	229		213	190		176
		χ^2=17.73*			χ^2=4.39*			χ^2=2.73			χ^2=3.99*		

The sex ratio at 20°C was compared with that at 30°C by χ^2-test, in which the males having abnormal copulatory organs(♂) were included in the number of males.

* : significant at 95 % level.

Table 7. Percentage of egg hatching in the crossing between FT-strain (F) and S-strain (S) at 20°C and 30°C. (Takahashi & Kuwahara, 1970)

♀ \ ♂	F			S			F_1(F♀ x S♂)			F_1(S♀ x F♂)		
	Number of eggs	Number hatched	%	Number of eggs	Number hatched	%	Number of eggs	Number hatched	%	Number of eggs	Number hatched	%
F												
20°C	524	413	78.8	532	34	6.4	862	605	70.2	863	2	0.2
30°C	500	409	81.8	1488	85	5.7	483	313	64.8	546	3	0.5
S												
20°C	276	198	71.7	418	376	90.0	840	653	77.7	608	428	70.4
30°C	678	523	77.1	416	390	93.8	768	615	80.1	519	364	70.1
F_1(F♀ x S♂)												
20°C	568	227	40.0	710	171	24.1	798	462	57.9	647	10	1.5
30°C	869	498	57.3	692	151	21.8	549	347	63.2	519	19	3.7
F_1(S♀ x F♂)												
20°C	525	138	26.3	404	186	46.0	440	293	66.6	484	163	33.7
30°C	503	144	28.6	950	672	70.7	429	301	70.2	458	171	37.3

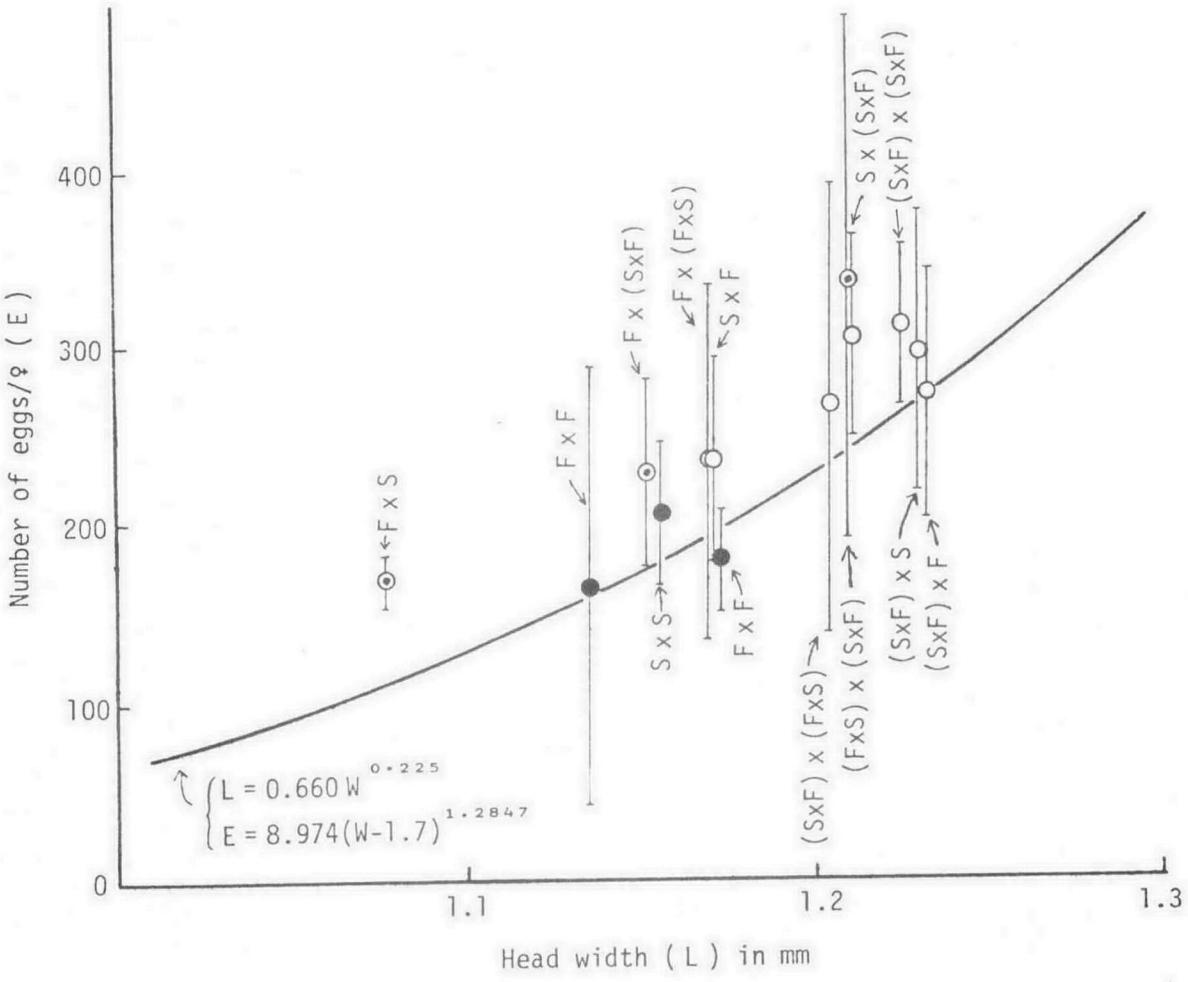

FIGURE 4. Number of eggs deposited by a female moth in relation to her head width in the crossings between FT-strain (F) and S-strain (S). (Takahashi and Kuwahara, 1970)

The larvae of each crossing were reared at 20°C and 30°C and adults emerged at a similar survival rate in every crossing (Table 8). At 30°C the sex ratio was normal but the abnormal sex ratios were found in some crossings at 20°C (Table 9). The results showed much clear relation of maternal inheritance than in the case of crossing between FT- and N-strains.

INJECTION OF THE HAEMOLYMPH OF FT-STRAIN TO THE LARVAE OF NORMAL STRAIN: There are some records that the sex ratio agent in *Drosophila* is spirochete which can be transmitted by injection of haemolymph (8). Trials to transfer the abnormal sex ratio agent were made by injecting larval haemolymph of the FT-strain into larval body of a normal strain (Table 10). However, these attempts were unsuccessful as shown in Table 11.

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Table 8. Percentage of adult emergence from eggs hatched in the crossing between FT-strain (F) and S-strain (S) at 20°C and 30°C. (Takahashi & Kuwahara, 1970)

♀ \ ♂	F			S			F_1(F♀ x N♂)			F_1(N♀ x F♂)		
	Number of larvae	Adults emerged	%	Number of larvae	Adults emerged	%	Number of larvae	Adults emerged	%	Number of larvae	Adults emerged	%
F												
20°C	413	266	64.4	34	16	47.1	605	383	63.3	2	1	50.0
30°C	409	388	94.9	85	64	75.3	313	264	84.3	3	2	66.7
S												
20°C	198	187	94.4	376	328	87.2	653	544	83.3	428	348	81.3
30°C	523	386	73.8	390	357	91.5	615	518	84.2	364	314	86.3
F_1(F♀ x S♂)												
20°C	227	136	59.9	171	119	69.6	462	287	62.1	10	2	20.0
30°C	498	419	84.1	151	124	82.1	347	248	71.5	19	3	15.8
F_1(S♀ x F♂)												
20°C	138	118	85.5	186	174	93.5	293	244	83.3	163	154	94.5
30°C	144	138	95.8	188	182	96.8	301	261	86.7	171	151	88.3

Table 9. Number of emerged moths in the crossing between FT-strain (F) and S-strain (S) at 20°C and 30°C. (Takahashi & Kuwahara, 1970)

♀ \ ♂		F			S			F_1(F♀ × S♂)			F_1(S♀ × F♂)		
		♀	(♂)	♂	♀	(♂)	♂	♀	(♂)	♂	♀	(♂)	♂
F	20°C	290	109	5	52	19	12	357	149	43	16	3	4
	30°C	272		272	73	1	108	191		212	2		4
		χ^2=45.57*			χ^2=11.62*			χ^2=29.58*			χ^2=1.34		
S	20°C	359		395	259	1	258	275		269	348	1	361
	30°C	628		654	285		279	273		245	341		315
		χ^2=0.36			χ^2=0.03			χ^2=0.49			χ^2=1.20		
F_1(F♀ × S♂)													
	20°C	85	48	3	67	20	32	387	67	125	2	1	0
	30°C	208		211	62		62	151		109	3		5
		χ^2=6.81*			χ^2=0.97			χ^2=5.99*			χ^2=0.03		
F_1(S♀ × F♂)													
	20°C	123		163	174		163	121		144	103		124
	30°C	139		131	171		182	135		157	107		138
		χ^2=4.00*			χ^2=0.70			χ^2=0.02			χ^2=0.14		

The sex ratio at 20°C was compared with that at 30°C by χ^2-test, in which the males having abnormal copulatory organs(♂) were included in the number of males.
* : significant at 95 % level.

Table 10. Emergence from the larvae of N-strain which were injected the haemolymph of FT-strain larvae.

(Takahashi & Kuwahara, 1970)

Haemolymph was obtained from	injected into	Number of larvae injected	Number of adults emerged	Mark of moths
FT ♀	N ♀	61	26	(A)
FT ♀	N ♂	44	12	(B)
FT ♂	N ♂	39	10	(C)

Table 11. Number of adults emerged from eggs which were obtained by the crossing of moths emerged in the experiment shown in Table 10 and by their crossing. (Takahashi & Kuwahara, 1970)

Mark of parents in Table 10 and in this table ♀ x ♂	Reared at 20°C			Reared at 30°C			
	Number of eggs	Number of moths emerged ♀	♂	Number of eggs	Number of moths emerged ♀	♂	Mark of moths
A x B	509	99	100	467	68	61	(D)
A x C	430	79	83	287	65	68	(E)
D x D	700	128	122				
E x E	650	89	65				