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CONTROL OF SEX IN CLADOCERA. III. LOCALIZATION OF THE CRITICAL PERIOD FOR CONTROL OF SEX[1]

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Every young female of *Moina macrocopa* has the possibility of producing in the first adult instar either of two types of eggs. She may produce (1) sexual eggs, or (2) parthenogenetic eggs. The former are haploid and do not develop unless fertilized. The latter are diploid and develop at once into females or males. The normal or usual course is the production of parthenogenetic females. Sexual eggs and males are called forth by very special and quite diverse environmental conditions.[2,3,4] The experimenter can, by appropriate treatment during the period of development, cause an animal, which would normally produce female progeny, to produce males. It is obvious, then, that the sex of the forthcoming parthenogenetic eggs is undetermined during the early growth stages of the mother, but definitely fixed at some period before the eggs are laid.

Before crucial work on the control of sex in this species could be done, it was necessary to determine with some exactness the position, in terms of the developmental stages of the mother, of this critical period in the development of the eggs during which they are amenable to control of sex. The present paper presents some of these data (for first broods only) for one species of Cladocera, *Moina macrocopa*.

Experimental.—Since the length of the developmental period of *M. macrocopa* varies greatly with temperature, having a higher temperature coefficient ($Q_{10} = 2.39$, for the temperature interval 20° to 30°C.) than most common Cladocera,[5] it was necessary to have definite morphological units as an index of the age, rather than time units. Since the number of preadult instars of a daphnid is constant, regardless of the temperature, and since the different instars present marked differences in length of the animals, even though lacking strictly structural dissimilarity, the instar was chosen as the index of the age of the animals. The young females of *M. macrocopa* when released from their mother's brood chamber have an

average length of 0.591 mm. Following the first moult, the length of the female increases to 0.717 mm. The third instar animal (following the second ecdysis) has an average length of 0.892 mm. During the fourth instar, at the beginning of which the first clutch of eggs passes from the ovaries to the brood chamber, the female has an average length of 1.090 mm. There are, then, two juvenile instars, and one adolescent instar before the first adult instar; and these can be distinguished readily by size. The average lengths for the first, second, third and fourth (first adult) instars were based on 333, 256, 387 and 182 measurements, respectively. The durations of the instars are not equal. At 20°C. the length of life to maturity (first three instars) is about 65 hours; 38 hours at 25°; and 30 hours at 30°. The fourth instar, at the beginning of which the parthenogenetic eggs pass into the brood chamber and at the end of which the young are released, is equal to approximately 70 per cent of the combined length of the three earlier instars. Hence a young female at 20°C. produces her first young when about 114 hours old; at 30°C. this time is in the neighborhood of 50 hours.

While the length of the animals does not change appreciably during an instar, certain differences in the appearance of the ovary are apparent. These changes in the ovary are slight during the first and second instars, and consist mainly in the relative, though slight, increase in size of the ovary and the increasing prominence of the egg nuclei. But during the third instar the changes are more conspicuous. The nuclei are plainly visible during the first two-thirds of this instar but during the middle third they are becoming obscured by the deposition of yolk granules within the eggs. During the latter part of this instar the nuclei are completely hidden by the large amount of yolk present. These changes in the amount of yolk in the eggs are, of course, accompanied by changes in the size and shape of the entire ovary, which becomes distended and lobed due to the increased mass of the contained eggs. Unfortunately in this species the yolk is not highly colored as in some other species, and so does not give well-defined landmarks within the instar.

It was known from some preliminary experiments,[2] using elapsed time as an index of the age of the animals, that the sex of the developing eggs was not definitely fixed until quite late in the development of the mother. These experiments were conducted as follows: One or more individuals of a large brood (20 or more) of newly released parthenogenetic young were isolated in bottles containing 75 cc. of culture water. The remaining young of the same brood were placed in a single bottle containing the same amount of culture water. It was known from earlier work[2,3] that females crowded together would produce a high percentage of males. In the present experiments animals were removed at various times from the crowded bottle and isolated in the usual amount of culture medium.

Mothers not crowded at all, and mothers crowded for any length of time up to 31 to 46 hours (17° to 19°C.) and then isolated, failed to produce males. Mothers crowded 48 to 76 hours gave 45.0 per cent males.

TABLE 1

Experiments to localize the critical period previous to which control measures are ineffective.

Mothers crowded and later isolated at definite times to determine at how late a period the crowding must exist in order to be effective. The numbers of mothers isolated at each hour period are in parentheses. The "numbers of hours" were determined by frequent examination of the mothers used in these experiments to determine just when the eggs were laid. Production of males by mothers (in each case by a single mother) isolated 10, 8 and 6 hours, respectively, before the eggs were laid is exceptional.[7] Since these experiments were conducted at 25–26°C. and since an adult instar at this temperature occupies about one half the time required at 20°C. the times in hours given in this table have been multiplied by two so as to make the times directly comparable with those obtained at 20° as given in Table 2.

NUMBER OF HOURS BEFORE EGG LAYING WHEN MOTHERS WERE ISOLATED, I.E., WHEN CROWDING CEASED	EXPERIMENT NO. 2826 SEX OF YG. ♀	♂	2827 SEX OF YG. ♀	♂	2828 SEX OF YG. ♀	♂	2832 SEX OF YG. ♀	♂	TOTAL YOUNG SEXED ♀	♂	AVE. % ♂
	(5)				(2)		(2)		(9)		
12	11	0			5	0	17	0	33	0	0
			(1)		(1)		(4)		(6)		
11			5	0	3	0	39	0	47	0	0
					(4)		(2)		(6)		
10					14	5	14	0	28	5	15
					(2)		(2)		(4)		
9					12	0	13	0	25	0	0
											5
	(1)		(1)		(3)		(2)		(7)		
8	4	0	7	0	12	5	14	0	37	5	12
			(2)		(6)		(1)		(9)		
7			13	0	26	0	6	0	45	0	0
					(4)		(3)		(7)		
6					19	0	12	6	31	6	16
	(1)		(2)		(4)		(2)		(9)		
5	3	0	16	0	19	0	10	0	48	0	0
	(3)		(2)		(6)				(11)		
4	1	10	4	11	20	4			25	25	50
	(4)		(5)		(2)		(2)		(13)		
3	7	6	18	12	0	7	8	0	33	25	43
		69 % ♂'s		35 % ♂'s		66 % ♂'s		20 % ♂'s			47.5
			(4)		(6)		(9)		(19)		
2			20	8	12	17	31	11	63	36	36
	(4)		(2)		(11)		(3)		(20)		
1	3	9	16	0	8	51	17	3	44	63	59
0 (not isolated)	29	78	191	69	177	259	102	51	499	457	47.8
		73 % ♂'s		27 % ♂'s		59 % ♂'s		33 % ♂'s			
									958	622	
									1580		

Mothers not isolated but left in the crowded bottles until after the eggs of the first clutch were laid (72 to 96 hours) gave 34.1 per cent males among the 1066 young sexed. These experiments[6] while not significant as to time because of the lack of temperature control, indicated that the sex of the eggs is not determined until late in their ovarian development.

A second series of experiments, involving 5995 sexed young, was conducted in a similar manner, but the age of the animals was determined by attained instars, which made the record independent of the temperature. Females from the same brood, having been originally heavily crowded and hence if undisturbed destined to produce a large percentage of males, were isolated, several at each of the following stages—first instar, second instar, early third instar, late third instar (a short time before the eggs were laid), and early fourth instar (a short time after the eggs were laid). A number remained as controls, i.e., were not isolated at all. These age classes gave, respectively, the following percentages of males: 0, 0, 0, 9.0, 57.6 and 41.2. These data localized the critical period for the control of sex near the end of the adolescent instar. The 9.0 per cent males produced by those isolated in the latter part of the third instar were probably produced by mothers which were actually near the end of the third instar.

Attention was now directed toward a more critical analysis of the time relations near the end of the third instar. While, as indicated above, there are some morphological changes in the developing eggs due to the deposition of yolk, these are not marked enough to serve as landmarks in critically determining the stage of development of the animals within this instar so that time intervals had to be used again. But as the time periods were short, the temperature variations were slight. These later series of experiments were conducted in like manner to those just discussed, but the isolations were made at definite time periods during only the latter half of the third instar; and the actual time of egg-laying was determined by occasional microscopic examination of some of the mothers. A summary of the results of one of these series is shown in Table 1.

Except for three exceptional occurrences of males,[7] no males were produced by the 57 mothers isolated more than four hours before egg-laying. Those isolated 4 hours before egg-laying produced 50 per cent males; those isolated 3, 2 and 1 hours before egg-laying produced 43, 36 and 59 per cent males, respectively. The average male production for all those mothers isolated 4 or less than 4 hours before egg-laying was 47.5 per cent; while those mothers not isolated at all produced substantially the same male percentage, 47.8 per cent. Other experiments showed similar results. It is clear that isolation of mothers more than 4 hours before egg-laying (at 20° to 22°C.) ordinarily prevents male production by previously crowded mothers; but that after this time isolation is without effect upon the sex of the forthcoming young.

Other experiments were planned in such a way that the animals which had previously been under conditions in which only female production ordinarily occurs could be subjected almost immediately to an environment which influences male production, i.e., crowding, instead of relieving them of this condition, as in the series of experiments just reported. This was done in the following manner. A double row of bottles was prepared in advance. Into one row of bottles was placed a large brood of animals, three (occasionally only two) animals per bottle; each of the bottles in the second row contained a large population of animals of the same species. At each successive hour during the last nine to twelve hours of the third

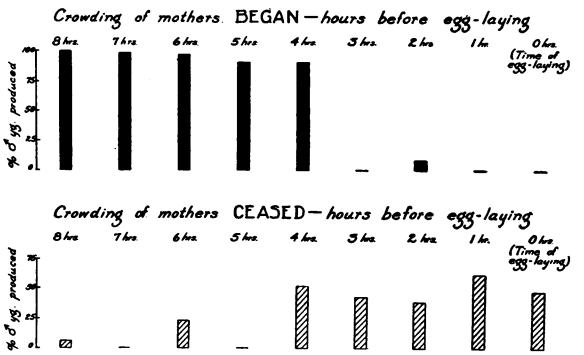

FIGURE 1

A diagrammatic presentation of the results of the series of experiments tabulated in Tables I and II. The shaded series of polygons show the results of a series of experiments in which the crowding was less effective than in the series represented by the black polygons, hence the shaded polygons are less tall than the black polygons. The one small black polygon and the two small shaded polygons indicate the appearance of small numbers of males where they ordinarily do not occur.

instar the three animals previously isolated in one of the first row of bottles were placed in the corresponding crowded bottles. These test mothers were confined within a section of glass tubing, the lower end of which was covered by a piece of silk bolting cloth to keep the animals in and to allow free interchange of the excretory products between the animals outside and those inside the tube. After all of the animals had reached the fourth instar and the eggs were laid, they were returned to their original bottles and kept until the first brood of young was released. These young were then sexed. Table 2 gives the detailed data for three such experiments. The average male production of the 68 mothers crowded until after egg-laying, from times beginning from 12 to 4 hours before egg-laying, is 77.9

per cent. The percentages of males for the different time classes for all three experiments taken together show a very striking change between four and three hours previous to egg laying. The eight animals subjected to crowding for four hours gave 82 per cent males, while the six animals crowded for only three hours before egg laying failed to produce any males

TABLE 2

Experiments to localize the critical period beyond which sex is no longer subject to control measures.

Mothers reared isolated and then subjected to crowded conditions, beginning at definite times, to determine how early the crowding must exist in order to be effective. The "numbers of hours" were determined by examination of control females (from the brood of mothers used in each experiment) to learn just when their eggs were laid. Some variation in actual time of egg-laying occurs. Presumably the female in Experiment 543 which was crowded for only two hours before the eggs were supposed to have been laid, and yet produced some males, was actually four hours or more from her actual time of laying when first subjected to the crowded conditions.

NUMBER OF HOURS BEFORE EGG LAYING WHEN CROWDING BEGAN	EXPERIMENT NO. 541 SEX OF YG. ♀ ♂		542 SEX OF YG. ♀ ♂		543 SEX OF YG. ♀ ♂		TOTAL YOUNG SEXED ♀ ♂	AVE. % ♂'s	
12	(3) 0 19				(2) 17 0		(5) 17 19	53	
11	(3) 19 16				(2) 2 14		(5) 21 30	59	
10	(3) 0 23		(3) 22 0		(3) 2 21		(9) 24 44	65	
9	(3) 0 18		(3) 23 9		(3) 25 1		(9) 48 28	35	
8	(3) 0 19	92 % ♂'s	(3) 0 33		(3) 0 27	73 % ♂'s	(9) 0 79	100	77.9
7	(3) 0 29			69 % ♂'s	(3) 1 25		(6) 1 54	98	
6	(3) 0 32		(3) 5 20		(3) 0 58		(9) 5 110	96	
5	(2) 0 22		(3) 0 27		(3) 17 30		(8) 17 79	82	
4	(2) 0 31		(3) 10 42		(3) 13 33		(8) 23 106	82	
3	(2) 29 0	0 % ♂'s	(2) 35 0	0 % ♂'s	(2) 32 0	18 % ♂'s	(6) 96 0	0	4.8
2	(2) 29 0		(6) 93 0		(2) 20 12		(10) 142 12	8	
							394 561		
							855		

at all.[8] This result, together with the results of experiments discussed in earlier paragraphs of this paper and of other series of experiments not here recorded, clearly indicates that the sex of the developing egg is subject to control only during a period localized at about four hours (at 20°C.) before the time it leaves the ovary and passes to the brood chamber. Figure 1 shows diagrammatically the results of the series of experiments tabulated in tables 2 and 3.

Discussion.—The parthenogenetic egg of cladocerans is diploid whether the egg produces a female individual[9,10] or a male.[10,11,12] In *Moina* the first and only maturation division in the parthenogenetic egg occurs just after the egg is laid and there is no reduction in the number of chromosomes.[11,13] Allen's findings indicate that the spindle and the small chromosomes appear shortly (probably an hour or less) before the eggs are laid. We have a situation here, then, in which the sex of the parthenogenetic egg is fixed by external environmental conditions effective for a period ending (at 20°C.) about three and a half hours before the chromosomes appear or the spindle of the single maturation division is formed.

It has so far proved impossible to determine as definitely as we should like the time of fixation (for sectioning and cytological study) of a given mother with reference to the time at which she would have laid her eggs. The actual time of egg-laying of sisters reared in the same culture bottle was learned and used as a means of calculating the stage at which fixations were presumably made. But these sisters do not all lay their eggs at once. In fact, their times of egg-laying may vary over a range of as much as four hours. In preparing material upon which Dr. Allen's[10] cytological observations were made the average time of egg-laying of the control sisters was assumed to be the time at which those fixed would have laid their eggs. But obviously such staging is only approximate. Further, this is very difficult cytological material. Few of the egg-bearing mothers which are fixed yield cytologically useful material. These few were of the calculated stage of 1 hour or less before egg-laying. The fact that spindle formation was found only in cases in which the time of fixation was presumably about $^1/_2$ hour before egg-laying, and not in the presumably earlier stages which Dr. Allen studied, inclines the authors to believe that the beginning of spindle formation is probably actually near the calculated time—about $^1/_2$ hour before egg-laying. It is still possible, however, that this event begins to occur earlier, i.e., nearer the experimentally determined critical period for sex-control (approximately 4 hours before the eggs are laid) than the present observations indicate. To the authors it seems improbable that the localization of the critical period can be narrowed down much further or that, on the other hand, the stage at which animals are killed for cytological examination can be much more precisely determined.

Another consideration is of interest with reference to the time relation between the critical period for sex-control and the beginning of the maturation division. The experimentally determined critical period (about 4 hours before the eggs are to be laid) is, we think, approximately correct for the *external* environment. But we have no information nor do we know any feasible means of obtaining precise information concerning the the location of the critical period for the *internal* environment of the ovarian eggs. With the apparent time of spindle formation located at about $^1/_2$ hour before the eggs are to be laid and about $3^1/_2$ hours after the critical period for the external environment, it is still possible that the critical period for the internal environment coincides with or immediately precedes the beginning of spindle formation. For it is possible that the internal environment retains the effects of crowding for $3^1/_2$ hours after the actual crowding has ceased; and conversely that only in animals which have been crowded as much as (or more than) $3^1/_2$ hours does the internal environment reach sufficient saturation of the effects of crowding to affect the maturation division. If this supposition is correct, with reference to the *internal* environment, the critical period may readily be about $^1/_2$ hour before the eggs are to be laid and thus coincide with the time of initiation of the maturation division.

The fact that external environmental influences operative *previous* to the formation of the maturation spindle control the sex of *Moina* eggs is perhaps consistent with any of the following interpretations: (1) A direct action of the environment on the egg and not through a chromosome mechanism. (2) An effect through the directing of a sex chromosome complex. (3) An effect through a more deeply seated and fundamental mechanism to which the sex chromosome mechanism is subordinate.

1. A direct action of the environment on the egg, and not on the behavior of sex chromosomes during the maturation division, might seem to apply. But it is difficult to understand how this action can be accomplished without the more frequent appearance of sex intergrades (only one having been recognized in our *Moina* material). The extent of the crowding, i.e., the concentration of the influential excretory products, acts to give varying numbers of males, but the males so produced are normal; and the sex intergrades that have appeared in cladocerans, so far as critically studied, behave as mendelian dominants[14,15] in sexual reproduction and so are clearly genic in inheritance.

A further difficulty with this type of explanation is the wide, almost universally demonstrated occurrence of the sex-chromosome mechanism in the different groups of animals. It seems improbable that Cladocera, at least eight species of which are subject to sex control by environmental means,[2] lack such a mechanism; and it seems improbable that such a mechanism, if present, would be generally over-ridden by environmental

influences in even so specialized a group as the Cladocera, unless indeed there is a mechanism more fundamental than the sex chromosome mechanism.

2. Inasmuch as the cytology of this group has not been exhaustively studied, one is left free to suggest that the manner of sex-determination in *Moina* may not be greatly different from that found by Morgan[16] in phylloxerans and aphids in which the parthenogenetic males are diploid, their chromosome complement being the result of the elimination during maturation in oögenesis of half the X-chromosomes. The male so produced gives only female-determining sperm inasmuch as the sperm containing no X-chromosomes degenerate. Certainly Cladocera, like the phylloxerans and aphids, have diploid parthenogenesis and the males so produced are diploid. The fertilized egg produces only female young. There is a suggestion in the paper of Chambers[17] on *Simocephalus* that only a portion of the sperm (presumably the female determiners) survive, but of this Chambers was not convinced. But so far no studies are at hand to indicate that the male has a chromosome complement different from that of the female. The only present indication from the cytological side is Allen's[10] report of 22 as the chromosome count for eggs produced by *Moina macrocopa* mothers under conditions which ordinarily cause them to produce a high proportion of males. If this chromosome number applies to the male, as well as the female, then the interpretation suggested above will not apply unless the sex-determining mechanism is of the WZ type. But this latter suggestion involves such additional assumptions (without known cytological precedent), in order to harmonize with the facts of the life cycle, that it fails to retain plausibility.[18]

Schrader[19] has recently reviewed the cytological information concerning the sex chromosomes in crustaceans. He cites evidence that in two Malacostraca, *Cambarus* and *Gammarus*, there are sex chromosomes, with indications that the male is the heterozygous sex. However, with the copepods, constituting an order of animals more nearly related to Cladocera, the somewhat fragmentary cytological evidence suggests that in some species the female is the heterozygous sex and has the XO-condition. Should the female Cladoceran prove to be heterozygous for sex, with the XO-condition, the cytological implications of our results might be somewhat as follows. The usual parthenogenetic Cladoceran egg would then presumably have, as its one maturation division, an equational division, the egg retaining the full autosomal set of chromosomes and the one X-chromosome and would produce a female. The parthenogenetic egg destined to become a male would, on the other hand, have to achieve a duplication of the X-chromosome. In this case the sperm should be of one sort and, in order to harmonize with the fact that the fertilized sexual egg always produces a female, one must assume that sexual eggs of

only one sort, without an X-chromosome, are produced (or are hatchable).

If a sex chromosome complex is present in Cladocera, either of the above theoretical explanations would appear to fit the observed facts but, except for the first mentioned hypothesis, they involve somewhat heterodox assumptions and, so far as Cladocera cytology is concerned, none of them have any supporting evidence. Any type of explanation which assumes a chromosome mechanism as a basis for sex-determination in Cladocera must take into account the fact that something in the mother's environment influences the distribution of the sex-chromosomes in the one (non-reductional) maturation division of the parthenogenetic egg.

3. Numerous workers have suggested or implied an inadequacy of the sex chromosome hypothesis. It is theoretically possible that there may be, in our material and elsewhere, a mechanism more fundamental than the sex chromosome complex to which the latter (very wide and possibly universal in its occurrence) is subservient and of which it is merely the visible index.

Whatever the theoretical explanation, the parthenogenetic egg of *Moina macrocopa* may develop into either a female or a male, depending upon environmental conditions and the environmental factors which control the determination of sex in *Moina macrocopa* are effective during a period which antedates the formation of the maturation spindle and the appearance of definitive chromosomes apparently by more than three hours (20° to 23°C.).

The knowledge of the location of the critical period was of extreme value in later experiments in which agencies other than crowding were used in controlling sex.

Summary.—1. *Moina macrocopa* has two juvenile instars, and one adolescent instar, before it becomes adult. The first clutch of eggs passes from the ovary to the brood chamber within the first few minutes after the beginning of the first adult instar.

2. The critical period for the control of sex is about four hours (20°C.) before the eggs are laid.

3. Possible mechanisms of sex-determination in this species are discussed.

[1] From the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, N. Y.

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[4] Banta, A. M., *Zeit. ind. Abs. Vererb.*, **40**, 28 (1925).

[5] Brown, L. A., *Proc. Soc. Exp. Biol. Med.*, **25**, 164 (1927).

[6] Tabulated in Banta and Brown, 1923, Table 3.

[7] Three unexpected cases of production of males (in each case by a single mother) occurred in Experiments 2828 and 2832 (Table 1). In one of these experiments the mother was isolated 10, in the other, 8 and 6 hours before egg laying. All the mothers isolated in these experiments were placed in vials to permit ready observation of the time of release of the young. In view of the very great rarity of male production by mothers isolated at such times it is believed that the mothers in these experiments were isolated in too small a quantity of culture medium and that consequently they "crowded" themselves, i.e., were influenced by their own excretory products sufficiently to induce male production in these three cases. Earlier experiments (Banta and Brown, 1929) had shown that individual mothers reared in 7.5 cc. of culture medium gave normal male production for crowded mothers. Other experiments conducted precisely like those tabulated in Table 1 confirmed our opinion that the unexpected male production by these three mothers was not due to the effects of the earlier crowding.

If one prefers not to accept the male production by three of the 57 mothers isolated more than four hours before egg-laying as exceptional, it is still clear that there is a vast difference in male production between mothers isolated more than four hours before egg-laying (produced 5 per cent males) and those isolated four or less than four hours before egg-laying (produced 47.5 per cent males). The location and significance of the critical period remains unaltered.

[8] The one mother, which produced some males (12) after supposedly having been crowded for only two hours before egg-laying, presumably was an irregular one which was actually four or more hours from egg-laying when first subjected to the crowding effect.

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[10] Allen, E., *Science, N. S.*, **67**, 18 (1928).

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[17] Chambers, E., *Biol. Bull.*, **25**, 134 (1913).

[18] If the WZ type is assumed as the condition, the female is WZ and the male ZZ. Parthenogenetically produced females are WZ. But to get the parthenogenetically produced males a differential maturation involving the passing of the W to the polar body and a reduplication of the Z, or a transformation of the W to the Z type, would be necessary in order to arrive at the ZZ condition. Further, since all sexual eggs hatch as females, the mature sexual eggs should contain only W to account for which another differentiatial maturation must be assumed.

[19] Schrader, F., *The Sex Chromosomes*, Berlin, Borntraeger (1928).