SOME PHYSIOLOGICAL ASPECTS OF REPRODUCTION IN XIPHOPHORUS MACULATUS

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Live-bearing fishes are being used extensively in biological research and instruction in genetics, embryology, endocrinology, pigmentation studies, and other fields to which these fishes can readily be adapted. Turner (1937) has carefully worked out the reproductive cycle of *Xiphophorus* from the viewpoint of ova development, period of fertilization, and superfetation. The present study is concerned with the reproductive cycle in this fish from the viewpoint of spermatozoa viability, selection and competition and the duration of ova production. Experiments have been conducted to show the number of young that can be born during the reproductive activity of the female and to show the duration of life of spermatozoa within the female genital tract and the competition and selection involved.

Animals and Their Treatment

The fishes used in this experiment were kept in aquaria 20 × 25 × 20 cm., which had a layer of sand on the bottom about two inches deep. Each aquarium contained ample amounts of *Vallisneria* or *Sagitarria* planted along the back wall and two sides, allowing full observation from the front. Young fishes were kept in larger aquaria measuring 30 × 26 × 26 cm. until almost sexually mature; then they were segregated according to sex in the smaller type of aquarium. Prior to giving birth to young, gravid females were placed in aquaria of the smaller type heavily planted with hornwort (*Ceratophyllum*). After the young were born, the females were removed to other aquaria of the same size. The young were counted in the late afternoon of the day they were born and then returned to the same aquaria. No more than three sexually mature fish were kept in one aquarium.

The diet for mature fish throughout the experiment consisted of white worms, mosquito larvae, Daphnia, cyclops, prepared dry food and a dried and ground mixture of strained liver, spinach and bran flakes. Young fish were fed "micro worms" (Nematoda), prepared dry food, Daphnia and white worms. Mature fish were fed once a day. Young fish were fed three or four times daily.

Two kinds of water were used. More commonly the water was taken directly from the tap and allowed to stand in earthenware crocks or porcelain-lined tubs for about two weeks before being used. At other times water was used that had been filtered through charcoal. This was allowed to remain about a week in the tubs before being used. No adverse effect of the water used was noted during the

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course of the experiments. A routine schedule was maintained for cleaning the aquaria whereby each aquarium was thoroughly cleaned once each month.

The fish were kept in a greenhouse which has crude temperature control. In the winter months the temperature averaged about 75° F. during the day. During the last week of February, however, the steam supply was drastically reduced, and the temperatures were somewhat lower. During the summer months the control was much less perfect.

During the eight-months period that the fishes were kept under these conditions only three fishes died. Two of these, males of unknown age, slowly wasted away; the other, a female, died suddenly a few days before she should have given birth to her first brood of young. The reasons for these deaths could not be determined.

The species used in these experiments were *Xiphophorus maculatus, Xiphophorus variatus* and *Xiphophorus helleri—Xiphophorus maculatus* hybrids. Various color forms of *X. maculatus* were used. These included forms without any peduncular or caudal pattern, the one-spot pattern, the crescent pattern, the wag pattern, and the stippled, spotted, red, blue and red-bellied color forms. Matings were usually made between color forms and identity of young based upon color patterns.

Experiments and Results

The experiments were of two types. In one experiment (Group I) female fish were isolated from contact with any male fish and records kept of the number of broods of young produced by them during the period of isolation. In some cases females were used which had been kept in aquaria for some time with males of their own species (Group I-A). Whether these females had given birth prior to being isolated cannot accurately be determined; therefore, the number of broods produced by these females is not considered as maximum. In another group (Group I-B) virgin females were used. After fertilization was accomplished these fish were isolated as in the previous experiment.

Another group of fish was kept in contact with males of their own species but of different color form and males of different species (Group II). In some cases the contact consisted only of the brief period of courtship and insemination (Group II-A). In other cases, (Group II-B), the contact with the male was constant except for the time that the female was removed for a few days prior to giving birth to a brood of young. (The term brood is used as defined by Turner (1937) to indicate a group of growing and differentiating ovocytes of approximately equal development up to the time of fertilization and also the embryos produced by the fertilization of these ova up to the time of birth.)

In some cases copulation was observed, but no young were produced by these females. These cases will be considered as Group III.

Group I-A

A group of five females was kept isolated in small aquaria throughout the experiment. Records were kept of the number of broods, the number of young produced by these females and the interval of time between broods. These females had already been inseminated when the experiments began, and it is not known

whether broods were produced by the females prior to this time. Therefore, the number of broods produced by these females cannot be considered as maximum. The results of this experiment are recorded in Table I. These results show a fairly consistent trend; that is, the number of young in the last brood is smaller than the usual number of young in the broods of each female. Fishes 0–1, 0–2, 0–4 and 0–5 were kept in contact with males after it was believed that they had produced their last broods.

At autopsy these fish showed the following results:

0–1. Fifty-three days after delivery of the fifth brood of one embryo and after 18 days of contact with male B-20, autopsy showed 16 large, deep amber ova; two large, light-colored ova; 16 embryos well developed; one embryo only slightly developed; and many small, white ova in the ovary.

0–2. Fifty-three days after delivery of the fifth brood of one embryo and after 18 days of contact with male M-10, autopsy showed 23 large, deep amber ova; two embryos about one-half developed; and many small, white ova in the ovary.

TABLE I

Reproduction records of females of Group I-A

Females	Number in Brood A	Interval	Number in Brood B	Interval	Number in Brood C	Interval	Number in Brood D	Interval	Number in Brood E
0–1	8	37	30	35	30	31	33	37	1
0–2	8	36	24	33	26	36	8	30	1
0–3*	19	30	9	35	8	30	9		
0–4	16	36	7	31	1				
0–5	8	35	6						
0–6	7	32	26	32	23	33	4		

* Female 0–3 was kept in constant contact with male 0–20.

0–4. Ninety-eight days after delivery of the fourth brood and after 20 days of contact with male M-11, autopsy showed about 40 large, deep amber ova; several small, white ova; but no embryos.

0–5. One hundred and thirty-one days after delivery of the second brood and after 20 days of contact with male M-12, autopsy showed 34 embryos about three-quarters developed; several small, white ova; but no large, deep amber ova.

The mean interval between broods for these fish was 33½ days.

It seems justified to assume that these females ceased to produce young because of lack of viable spermatozoa within the ovary. With the possible exception of 0–4, none had reached the end of the reproductive period. Whether successful insemination was accomplished with female 0–4 is, of course, open to question. Copulation was observed, but it does not necessarily follow that the female was inseminated.

Group I-B

In seven matings the females gave birth to a limited number of broods. They were kept isolated in small, heavily planted aquaria and examined every day for

TABLE II

Reproduction records of females of Group I-B

Mating		Number of Broods	Total number of young	Time in days*	Days from last brood to autopsy
Female	Male				
C-1	× C-20	4	56	134	34
C-5	× R-10	3	58	103	29
C-7	× K-20	3	56	101	33
K-2	× K-20	3	48	97	73
K-3	× B-20	3	60	104	66
K-6	× C-15	4	79	132	40
N-3	× P-20	3	71	102	66

* From insemination to birth of last brood.

young. At the end of the experiment these females were autopsied and the condition of the ovary noted. These data are recorded in Table II. Evidently, the viable spermatozoa that were introduced at the insemination were depleted. Although the females were capable of producing more broods, none were produced because the lack of viable spermatozoa in the ovary or genital tract made fertilization impossible.. Popular literature about aquarium fishes sets the upper limit for the number of broods produced in isolation at eight broods. This limit was not reached in these experiments. In all instances autopsy revealed that the ovary contained large, deep amber ova; small, white ova; but no embryos.

TABLE III

Reproduction records of females of Group II-A

Female	Insemination date	Interval to Brood A	Interval to Brood B	Interval to Brood C	Interval to Brood D	Interval to Brood E
C-1	II-6	38	32	32	33	
C-2	VI-6	48				
C-3	VI-7	45				
C-4	VI-12	42				
C-5	III-15	41	31	31		
C-6	V-15	42	29			
C-7	III-13	40	31	30		
C-8	IV-26	50	31			
Ca-2	II-11	40	32	30	32	28
Ck-1	VI-6	37				
K-1	II-6	57	31	31	27	
K-2	II-4	39	26	32		
K-3	II-4	42	32	30		
K-5	V-31	44				
K-6	II-4	40	32	28	32	
N-2	IV-26	42	30			
N-3	II-6	39	31	33		
P-3	VI-5	31				
P-5	V-18	35				

Group II-A

Soon after the experiments were begun it was noted that the virgin females usually did not give birth to their first brood until about 40 days after fertilization. Since copulation had been observed in each case and the female isolated from all contacts immediately after insemination, accurate dating of the period between insemination and birth of the brood was possible. Table III shows the periods in days between insemination and birth of first brood.

This table shows that for the cases listed the average period between insemination and birth of first brood was 41.7 days. When this period is compared with the periods between subsequent broods for these females, it is evident that some factor is involved which delays the birth of the first brood. What this factor is was not ascertained in this study.

That it is due to some delayed action on the part of spermatozoa is not plausible in the light of one experiment in which a female was inseminated by two males at different times. Forty-two days after insemination by the first male the female gave birth to her first brood of young. On that day the female was fertilized by the second male. Thirty days after this second insemination the second brood was born. Of the 41 young, 28 were of the same phenotype as the second male. In this case the spermatozoa fertilized ova that developed in the normal thirty-day period.

Group II-B

Several attempts were made to establish the degree of competition between the spermatozoa of different males. In some cases females were used which had been inseminated by males of their own species or hybrid type. After giving birth to broods of young, these females were placed in constant contact with males of different species or males of the same species but of a phenotype different from the original male. The results of these experiments are as follows:

1. A *X. maculatus* female, K-1, was inseminated by a male *X. variatus,* V-20. Four days after the birth of the second brood from this mating (a period of 92 days from insemination) the female was placed in constant contact with a male *X. maculatus*. During the contact with this male, two broods of young were born. Although the males differed phenotypically and the young produced from ova fertilized by spermatozoa of the *X. maculatus* male could have readily been distinguished from young produced from ova fertilized by the *X. variatus* male, no young could be assigned to the *X. maculatus* male. Copulation was observed after the second male was introduced. Whether insemination took place cannot be ascertained.

2. A *X. maculatus* female, N-2, was fertilized by a *X. maculatus* male, B-20. On the day the first brood was born contact was made with a *X maculatus,* male, 0–21. In the second brood 28 of the 41 young were of the same phenotype as male 0–21 and distinct from the phenotype of male B-20.

3. A hybrid *X. helleri–X. maculatus* female was kept in constant contact with a male *X. maculatus,* K-20. No other fish were kept with this pair. During 136 days of contact, the female gave birth to four broods of 56 young. Two of the fourth brood of fifteen showed the phenotype of male K-20. A fifth brood of nine contained no young of the same phenotype as male K-20.

4. A similar experiment was set up with a brood sister of the female described in (3) above. This female had previously given girth to two broods of young before being placed with male 0–22. During 107 days of constant contact with male 0–22 the female hybrid gave birth to four broods of 52 young. Three young of the fourth brood showed the phenotype of male 0–22.

5. A third hybrid female was placed in contact with a male *X. maculatus,* W-1, after having been inseminated by a male hybrid from the same brood. During 116 days of contact, the female had given birth to four broods. None showed the phenotype of male W-1. The male died before the fifth brood of seven young was born. None of this brood had the phenotype of male W-1.

These experiments, although incomplete and lacking reciprocal crosses, show that once a female has been fertilized by a male of either the same species or a different species, the spermatozoa of the second male can fertilize ova while the spermatozoa of the first male are still viable and fertilizing ova which are in the same broods. These experiments also give an indication of the degree of competition that exists among viable spermatozoa within the ovaries of female viviparous fishes.

A pair of *X. maculatus* was kept in a small aquarium isolated from other fishes to serve as controls and to establish what degree of relationship, if any, existed between constant insemination and the number of young and interval between broods. These results are listed in Table I for female 0–3. At autopsy 42 days after the birth of the last brood this female contained 33 large ova of a very pale, almost transparent color. Usually ova at autopsy are deep amber in color. After development begins the deep amber ova remain the same color and do not change until the embryos are evacuated from the female's body. If any of the yolk material remains at birth, it soon changes to an opaque yellow. No autopsy revealed embryos developing from ova of the pale, transparent type.

Group III

The normal procedure for mating fish in all these experiments was to place the female in a small aquarium which had been occupied for at least a day by an isolated male. In most cases the courtship began almost immediately or as soon as the male was aware of the female in the aquarium. Copulation usually took place after twenty minutes or less of courtship. One lasting contact was considered as sufficient, and an effort was made to limit the mating to only one contact. In some cases females never produced young, although copulation with a lasting contact was observed. Females C-8, N-1, N-4, P-2 and P-4 had contact with only one male but never produced young from these matings. Female C-8 was later mated with another male, C-23, and produced broods from this mating. Females Ca-1, Ck-1, K-4 and P-1 had contact with two males in rapid succession. This was an attempt to have spermatozoa from two different males introduced into the genital tract of the female at as nearly the same time as possible. In two cases, females Ck-1 and P-1, copulation with the second male was accomplished within two minutes after copulation with the first male. Female Ck-1 was later mated with another male, L-20, and produced broods of young.

Whether this failure to produce broods of young can be attributed to lack of insemination and subsequent fertilization is open to question because other factors such

as immaturity may have been responsible. It is noteworthy to mention here that all four attempts to inseminate females with the spermatozoa of two males met with failure. In the total of nine cases of failure in insemination seven males were used (males R-10, B-20, O-21, V-10, C-23, S-10 and X-15). In other matings males R-10, B-20, O-21, V-10, C-23 and X-15 were shown to have produced viable spermatozoa. Male S-10 died before being mated again. Autopsy of female Ca-1 showed that the ovary contained 41 large, deep amber ova. The remainder of this group of females is being used in other experiments and cannot be autopsied at this time. Attempts at hybrid matings of female *X. maculatus* and male *X. helleri* met with failure; no copulation was observed.

Discussion

An attempt has been made, by means of controlled mating, to determine what some of the factors are that affect reproduction in *X. maculatus*. It has been shown by Turner (1937, 1940), Hopper (1943), Wolf (1931) and others that a definite ovarian cycle exists in the female of this species. The cycle may be briefly outlined as follows:

1. Upon completion of development within the follicles of the ovary, embryos are evacuated to the ovarian cavity from which they descend the short genital tract (oviduct) to the exterior of the female's body.
2. As this brood is developing within the ovary, a group of ova which is approximately equal in number to the embryos is becoming larger, accumulating yolk material and approaching a state in which fertilization is possible.
3. A few days after the brood is born the ova are fertilized in the ovarian follicles by the spermatozoa within the ovary. Winge (1922) shows a photomicrograph of spermatozoa lying ready in the ovary of a *Lebistes* female. The heads of the spermatozoa are as near the immature egg as possible.
4. A third group of ova begins to enlarge and the cycle is continued. Bellamy (1924) states that female *X. maculatus* are capable of producing as many as ten broods.

To my knowledge no female *X. maculatus* that has not been inseminated by a male of the same or closely allied species has given birth to young. Hubbs and Hubbs (1946) report an interesting case of *Mollienisia formosa* in which no males of the species are known except from experiments of masculinizing females with gonadotrophic hormones. The female of the species has never been known to reproduce parthenogenically but must first mate with a male of another species before giving birth to young. The young show no signs of paternal inheritance. Hubbs and Hubbs regard this as a species permanently fixed diploid requiring only the stimulus of spermatozoa to initiate development.

That a competition and selection exist among the spermatozoa within the ovary and genital tract of the female is shown in the experimental data for Group II-B. In these experiments various hybrid matings were attempted. These are as follows: (1) a female *X. maculatus* with a male *X. variatus* and male *X. maculatus*, (2) a female *X. maculatus* with two male *X. maculatus* of different phenotypes, (3) three female *X. helleri–X. maculatus* hybrids with three *X. maculatus* males of different phenotypes.

In the first mating the *X. maculatus* male never successfully fertilized any ova of two broods totaling 59 in number, while the male *X. variatus* fertilized ova producing 117 young. The *X. variatus* male was in contact with the female only long enough to effect one insemination; the *X. maculatus* male was placed with the female 92 days after insemination by the *X. variatus* male and four days after the birth of the second brood. This male remained in constant contact with the female for 55 days until the birth of the fourth brood. If the *X. maculatus* spermatozoa had fertilized any of the 59 ova of the third and fourth broods, the young would have shown the crescent tail pattern which was carried by the male. Gordon (1947) has shown that a series of seven dominant autosomal allelic genes controls the pattern of peduncular and caudal pigmentation in *X. maculatus*. Throughout these experiments these data have been used to establish identity and paternal relationship.

All the surviving fish of the first brood produced from this *X. maculatus–X. variatus* cross have differentiated as males. There are differences in the sex-determining mechanisms of the two species. In *X. variatus* the female is the homogametic sex; in domestic *X. maculatus* the male is the homogametic sex. Gordon (1944) confirms the findings of Bellamy (1936) and Kosswig (1935) that all the hybrids from matings of domestic male *X. maculatus* and female *X. variatus* are males. The hybrid mating described in these experiments uses the two heterogametic sexes. The fact that only ten of the twenty young have survived to sexual maturity suggests that other factors may be involved. Perhaps chance has produced such a sex ratio. None of the young from the subsequent broods have reached sexual maturity, and the small number of young in the first brood does not justify any conclusion.

In the second mating described the first male was in contact with the female for a period of 26 days. Forty-two days after the female was introduced into the aquarium containing the male the first brood was born. Beginning on this day the female was placed in contact with the second male for a period of 29 days. On the following day the second brood of 41 was born. Twenty-eight of this brood had the peduncular pattern of the second male; thirteen resembled the first male. The spermatozoa of the second male succeeded in fertilizing the majority of the ova of the second brood. Reference to the other matings in which only one male was used leads to the thought that without intervention of the spermatozoa of the second male, the spermatozoa of the first male would have fertilized the majority of all the ova of the second brood. This indicates that a competition exists among spermatozoa within the ovary or genital tract of the female. The subsequent selection, which is a product of this competition, might have its foundations in the different ages or different quantities of spermatozoa, differential placement within the genital tract, or more subtle differences in viability, size or other factors.

In those experiments in which hybrid females were used, an attempt was made to ascertain the degree of competition between spermatozoa of different species. These females were of a strain that has been bred in commercial hatcheries to include the *X. maculatus* gene for red body color. Gordon (1946) has traced the development of a similar strain which has been developed to include the *X. maculatus* gene for the comet tail pattern in the cytoplasm of the swordtail, *X. helleri*. The red swordtail-platyfish hybrids have been bred by back crosses to the wild-type swordtail to produce a fish that is identical in body form and size, behavior and taxonomic

characters of dorsal fin ray count and lateral line scale count with the wild-type swordtail. In these experiments the females used had previously been inseminated by a male of their own type (in reality a brood brother). They were then placed with *X. maculatus* males. In two of these matings hybrid young carrying the color patterns of the *X. maculatus* males were produced. The third mating was unsuccessful in producing any hybrid young. In one case two hybrids were produced after 136 days of contact; in the other three hybrids were produced after 137 days of constant contact. These periods of contact before hybrid young were produced compared favorably with the periods of 97 to 145 days for the production of young by females in isolation (Groups I-A and I-B). In light of these data there is an indication that the spermatozoa of the *X. maculatus* male are selected against, and that they can fertilize ova only when the spermatozoa of the *X. helleri* male are partially depleted or reduced in number below a critical point.

Another factor which should be considered here is the difference between the courtship and mating behavior of these two species. Clark *et al.* (1948) after a study of their behavior concluded that differences between these fishes do exist and that such psychological barriers can effectively prevent their hybridization in natural situations.

These experiments on the effect of spermatozoa viability, competition and selection have certain general implications as they stand, regardless of the fact that the analyses of the underlying mechanisms are incomplete or entirely wanting. These may be summarized briefly as: (1) offering suggestions concerning the regulation of the reproduction of *X. maculatus* in natural habitats; (2) pointing out a barrier that exists in nature between two sympatric species of livebearing Cyprinodontes; (3) supporting the concept that there are simple social factors such as competition and selection, in operation below the level usually regarded as social, and that these factors can operate in small and subtle ways.

Summary

1. Experiments have been conducted which show that *Xiphophorus maculatus* females which have been successfully inseminated can continue to give birth to young after isolation from contact with male fishes. Although no maximum limit has been established, three to five broods of young has been shown to be the general trend.

2. Females ceased to produce young because of the lack of viable spermatozoa within the ovary. After the spermatozoa within the ovary cease to fertilize ova, the female can be inseminated again and can again produce broods of embryos.

3. By hybrid matings support has been given to the concept of isolating factors between *X. helleri* and *X. maculatus* in nature. Where attempts have been made to inseminate female *X. helleri* with *X. maculatus* spermatozoa, the results were very poor or failed completely.

4. The period of time between insemination and birth of the first brood is longer than the periods of time between subsequent broods produced from the same insemination.

5. Copulation by a female with two male fish in rapid succession failed to produce broods of embryos in these experiments.

6. The general implications of these experiments are suggested.

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