

## THE BIOLOGICAL BASIS OF SEXUAL BEHAVIOUR IN AMPHIBIA

### I. THE EXPERIMENTAL INDUCTION OF THE MATING REFLEX (COUPLING) IN *XENOPUS LAEVIS* (THE SOUTH AFRICAN CLAWED TOAD) BY MEANS OF PREGNANCY URINE AND OF ANTERIOR PITUITARY EXTRACTS, WITH THE PRODUCTION OF FERTILISED OVA.

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(With One Plate.)

#### I. INTRODUCTION.

THE close relationship of the gonads to the mating reflex in Amphibia has long been recognised (Nussbaum, 1907; Steinach, 1910; Brossard and Gley, 1929), but as no work on the role of the pituitary-like substances found in the urine of pregnant women or on the effect of anterior pituitary extracts seems to have been undertaken in this connection, it was decided to investigate this problem.

Recently Ruch (1934) drew attention to the fact that injection of pituitary suspensions (whether prepared from male or female frogs) will induce amplexus if both the male and the female frogs are injected. He also claimed that there was no sex specificity of the pituitary hormone. Quantitatively appropriate injections of the pituitary suspension of either sex will produce amplexus.

*Xenopus laevis* (the Platanna or the South African clawed toad) is a very suitable amphibian in which to investigate the mechanism of the mating reflex, on account of the ease with which the animal can be maintained in the laboratory. Moreover, all sexual activity disappears completely as soon as the animals are removed from the pond even at the height of the breeding season. Coupling pairs can, however, be recovered from the pond during the rainy months of the breeding season (July to September in South Africa). Such pairs are by no means common, and their sexual activity ceases a few hours after they have been brought into the laboratory. In such couples as the present author has recovered from the pond, the male was seen embracing the lumbar region of the female with his forelimbs.

II. MATERIALS AND METHODS.

In order to investigate the reflex, male and female toads fresh from the pond were brought into the laboratory at regular intervals from August 1934. The animals were fed with minced meat twice a week, and the water of their containers was changed daily.

(a) *Preparation of pituitary-like substance from pregnancy urine.*

The technique of the extraction of the pregnancy urine has been described by Katzman and Doisy (1932). The urine was collected in a large flask in which 0.5–1.0 ml. of chloroform had previously been placed.

Two to four litres were collected at a time, and after glacial acetic acid had been added until the urine was acid to methyl red, the acidified urine was filtered by means of a Buchner funnel to facilitate filtration. To the filtered urine was added a saturated solution of benzoic acid in acetone (50 ml. per litre of urine). For this purpose the urine was placed in a tall measuring cylinder and stirred vigorously with a stout glass rod to ensure adequate mixing and precipitation while the benzoic acid-acetone mixture was being added. The urine thus treated was placed in a refrigerator at about 4° C. overnight. The precipitate was filtered off the next morning, thoroughly washed with acetone, and then centrifuged to remove and discard the supernatant fluid. The acetone insoluble residue was dried by means of an air current from a foot-bellows, or else left for a few hours to dry by itself when the residue was too powdery to permit the use of the bellows. The dried residue, after having been weighed, was dissolved in distilled water (25 ml. per litre of urine). The water insoluble residue was dried with alcohol and ether and weighed again. In this way the amount of acetone insoluble residue which went into solution in water could be determined. The aqueous solution has a white, opaque appearance, and can be used immediately for injection. Alternatively, the acetone insoluble residue can be stored as a dry powder in the refrigerator until required for use.

Control extracts of male urine were collected and prepared in the same way.

(b) *Preparation of anterior pituitary extract.*

The method of preparation of acid extracts of anterior pituitary has been described by Bellerby (1933). The pituitaries in all cases were removed within 1–2 hours after the animals had been killed at the Maitland abattoirs, situated half an hour by car from the laboratory. Immediately on their removal the glands were placed on ice. The anterior lobes were carefully dissected out, weighed, cut up into fine pieces and then ground with coarse sand. The whole mixture was then extracted, for 24 hours, with 1 per cent. acetic acid one and a half times the volume of original tissue. The next day the mixture was centrifuged, the supernatant fluid decanted and neutralised with 40 per cent. NaOH. A flocculent precipitate comes down at the neutral point. This precipitate is removed by centrifuging, and the supernatant fluid is ready for injection.

Sheep and goat pituitaries were used, two to three dozen pituitaries being prepared at a time.

Control extracts of sheep and goat brains were prepared in the same way.

### III. EXPERIMENTAL.

Pairs of toads (a male and a female in each pair) were used in all these experiments. The injections were given intraperitoneally.

After injection the toads were placed in flat-bottomed circular or rectangular glass vessels. Water sufficient to cover the toads was added. The rectangular vessels measured 11 in.  $\times$  5 in.  $\times$  5 in. and could hold two pairs of toads comfortably. The larger circular vessels were 12 in. in diameter and could accommodate three to four pairs of toads. The smaller ones were 9 in. in diameter and held two pairs each. The glass lids were arranged so as to admit fresh air to the containers, without allowing the toads to escape. During the day the toads were examined at 1- or 2-hourly intervals. After 6.0 p.m. a reading was taken at midnight, and again the following morning at 8.0 a.m.

Coupling pairs were gently removed and placed in separate containers for further observation.

A preliminary series of experiments was carried out to determine the amount of extract necessary to provoke the coupling response.

Coupling pairs were pithed and examined.

### IV. RESULTS.

#### (a) *The effect of injection of pregnancy urine extracts.*

These experiments were carried out during August, September, October and December, 1934; and January and February, 1935.

Urine was collected from women whose recent impregnation (1-2 months) was diagnosed by the *Xenopus* test for pregnancy as described by Shapiro and Zwarenstein (1933, 1934, 1935). Such urine was found to yield more potent extracts than that from pregnant women near term.

The amount of acetone insoluble residue which is water soluble varies considerably in different women, and in the same woman at different times.

A preliminary experiment was carried out to determine the provocative dose. This was found to be independent of the amount of solids in solution. A dose of 0.5 ml. extract gave a response of the order of 70 per cent. when urine from early pregnancy was used.

Three groups of animals were injected. In the first group both the male and the female toads were injected (120 pairs).

In the second group, females only and not males were injected (37 pairs).

In the third group, males only and not females were injected (24 pairs).

Coupling began 10-12 hours after injection, and lasted 10-48 hours. Ninety pairs in the first group exhibited the mating reflex, *i.e.* a 75 per cent. response.

Eleven pairs reacted in the second group, *i.e.* a 30 per cent. response. No response at all was observed in the third group. Fifteen of the animals in the first group which had failed to couple were pithed and examined post-mortem. In thirteen of these the ovaries were severely atrophied. A few showed ova in the oviducts.

In all cases of successful coupling, fertilised ova were recovered and tadpoles reared from them.

Repeated injections with male urine extract gave no response (coupling or ovulation).

In all, the mating reflex was induced in 101 different pairs of toads in the laboratory at the time of the pond breeding season as well as midway between breeding seasons.

*(b) The effect of injection of anterior pituitary extracts.*

The data of an experiment performed to determine the amount of anterior pituitary extract which would provoke the coupling reflex are recorded in Table I below.

Table I. *To determine the dose of anterior pituitary extract which will provoke the mating (coupling) reflex in Xenopus laevis. September 1934.*

No. of pairs injected	Dose in mg. fresh tissue	Mating response	
		Positive	Negative
15	32	0	15
14	64	2	12
14	128	8	6
23	192	17	6

Both the male and the female partners were injected with the same amount of extract. A dose of 192 mg. (as can be seen from the table above) provoked a 74 per cent. response. Coupling was observed 10-12 hours after the injection was given, but in rare cases this was seen after 2 hours.

In all cases coupling was eventually accompanied by oviposition, and eggs oviposited during coupling were fertilised. Tadpoles were reared from them.

The experiments were repeated during January and February of 1935. These months are in point of time roughly midway between two breeding seasons, and the weight of the ovaries relative to the body weight of the animals is lowest at this time of the year (Shapiro and Shapiro, 1934). Much larger doses (*e.g.* 500 mg. fresh tissue) were necessary to obtain a response of the order of 74 per cent. previously recorded with doses of 192 mg. fresh tissue. Whether this represents a seasonal variation in the sensitivity of the animal to the extract, and one which can be correlated with ovarian changes in the sexual cycle of *Xenopus* will be discussed in the sequel. Fertilised eggs were again obtained and tadpoles reared from them.

In all, the mating reflex was induced in thirty-nine different pairs of toads by means of anterior pituitary extracts. The reflex was thus evoked in the laboratory

at the height of the pond breeding season as well as at a point in time midway between two breeding seasons.

Control experiments with brain extracts gave negative results in all cases. These toads were pithed and examined post-mortem. The ovaries in all cases were in good condition.

(c) *The mating reflex.*

Toads brought in fresh from the pond swim around actively and make repeated attempts to escape from their containers. Even when they are temporarily quiescent, the slightest sound acts as a stimulus to renewed activity in the form of very vigorous swimming and kicking with the hindlimbs. Within a few hours after injection with pregnancy urine or anterior pituitary extract, however, a marked change is visible in the behaviour of the toads. They become much more quiescent and are not so easily disturbed. It is even possible to walk by them quite noisily, and remove the glass lids of their containers without disturbing them. This phase of depression is especially marked in the female toad.

The passive behaviour of the female is an important event in the successful establishment of the coupling reflex. The male is the active partner but he would be unable to effect his lumbar embrace of the female unless she exhibited a marked degree of passivity. When he is about to embrace the female, the male can be heard croaking loudly as he swims about. Croaking is, however, occasionally heard in animals which do not couple. The male swims towards the hind end of the female, his forelimbs resting gently upon the posterior half of her dorsal surface. If he is disturbed at this stage it may take several hours for the reflex to reappear. This reveals the importance of the passive role of the female which has been seen to maintain an immobile attitude for over half an hour. The forelimbs of the male rest lightly alongside the flanks of the female. Soon, however, the embrace is tightened (see Pl. I, fig. 1 a) and the male's finger tips meet or almost meet on the ventral surface of the female (see Pl. I, fig. 1 b). The coupling is now fairly firmly established and force must be used to separate the pair.

The first stage in the mating reflex is completed without oviposition, 1-2 hours or more elapsing before oviposition begins.

When the female is thus embraced by the male the pair does not swim about much. The male, however, can be seen executing forward movements of his body without changing the position of his forelimbs. This is generally accompanied by bodily tremors. His ventral aspect stimulates the cloaca of the female and also a portion of her back. He performs this movement seven or eight times at intervals of a few seconds. When sufficiently stimulated the female begins to swim forwards. As soon as this happens the male arches his back and forces his pubis against the female cloaca, and with this manœuvre an ovum can be seen escaping from the female cloaca and rolling along the belly of the male in a groove formed by two folds of skin on his ventral surface. The ovum rolls on past the cloaca of the male and settles on the floor of the vessel.

Such ova were recovered and were found after a few hours to be fertilised.

Several hundred ova are deposited during the period of coupling, which lasts from 10-48 hours.

The embrace of the male weakens after a variable time, so that when the female swims forward the male is detached. Such pairs may, however, begin to couple again later.

(d) *The fertilised ova.*

Ova in the containers of coupling pairs of toads were sucked up in a pipette and transferred to small glass crucibles for examination under a low-power dissecting microscope. The earliest stage seen was the fertilisation membrane, and at all stages development can easily be followed on account of the large size of the ovum. Frequently the first segmentation can be seen by the naked eye. The yolk-plug stage is easily recognisable macroscopically. The development of the ovum and the larva into the tadpole stage corresponds to the description given by Bles (1905). The fertilised eggs are kept in glass crucibles in ordinary tap water which is changed daily. When the tadpoles develop from the larvae they are transferred to a larger dish in which some pond material, including green plants, has been placed. In this way tadpoles have been reared with a minimum amount of trouble for 4-5 months. Metamorphosis has not yet been observed.

It is important to observe that the tadpoles can be reared in the laboratory by this technique not only at a time corresponding to the breeding season in the pond, but also midway between breeding seasons at the height of summer.

V. DISCUSSION.

(a) The lumbar embrace of *Xenopus laevis* appears to be similar to that of the tailless Batrachians, *Pelobates*, *Discoglossus*, *Alytes*, *Pelodytes*, *Bombinator* (Rostand, 1934). Important differences exist in the details of the reflex. It is quite unlike the embrace of the toad *Bufo* which, like that of the common frog or of the tree frog, is axillary.

Thomas has suggested that, at least in France, there is a connection between the pupil and the mode of embrace. The horizontal pupil corresponds to the axillary embrace and the vertical or triangular pupil to the lumbar embrace. In the South African clawed toad, however, the lumbar embrace is associated with a circular pupil. There does not seem therefore to be any relation between the pupil and the mode of embrace.

The manner in which the male embraced the female after they had both been injected with appropriate doses of extract did not differ in any way from the embrace induced by pregnancy urine extracts.

(b) It is clear that a maximum percentage of coupling took place when both the male and the female were injected with pregnancy urine extract. This was also the case with anterior pituitary extracts. The response was of the order of 75 per cent. When females alone were injected the coupling response was of the order of 30 per cent. When the males only were injected no coupling was observed. This may have been due to the fact that the uninjected females in the third group were not suf-

ficiently quiescent to permit of the male embrace. The 30 per cent. response in the second group suggests, on the contrary, that some stimulus from the female activates the uninjected male. The nature of this activation and the details of the mechanism are being investigated.

(c) The physiological properties of the gonadokinetic substances extracted from pregnancy urine are known to be similar to those of anterior pituitary extracts in many respects. If the kidneys are regarded as a renal route of excretion of excess pituitary hormone during pregnancy, the experiments described above lend support to the view that the anterior lobe of the pituitary is involved in the mating reflex.

(d) Coupling pairs of toads which had been embracing for some time were pithed and their abdominal organs examined. A marked hyperaemia of the testes in the male, and of the ovaries and the oviducts in the female, was seen. The anal labia, which distinguish the female, were also markedly congested. These effects were produced both by pregnancy urine and by anterior pituitary extracts.

These facts suggest that the extracts activate the gonads of the injected animals. The extracts, however, are known to have gonad-stimulating properties apart from the mating reflex, *e.g.* ovulation can be induced in females by injecting either extract. The possibility therefore arises that the hyperaemia of the gonads may be an effect concomitant with, but independent of the principle responsible for the mating reflex. Alternatively the same substance may be acting in different ways to produce in the one case ovulation and oviposition, and in the other case, a lumbar embrace. Evidence for the view that the same chemical substance which stimulates ovulation activates a second ovarian mechanism concerned with the mating reflex will be presented in another paper (this *Journal*).

It should be noted that coupling begins before oviposition.

(e) The elaborate pattern of motor behaviour initiated by injection of the extracts described above suggests that the nervous system is intimately involved in the response.

Goltz (quoted by Schrader, 1887), Tarchanoff (1887), Steinach (1910) and Lindeboom (1928) have indicated the importance of the central nervous system in this connection. Kahn (1921), Wacholder (1923) and Lullies (1923) have drawn attention to changes in the electrical excitability of the frog's tissues during clasping, while Lindeboom (1928) has shown marked differences in muscular excitability in the amphibian forelimbs (flexor carpi radialis muscle) before and after rut.

Steinach (1910) considers that the clasping reflex in the male is normally inhibited by an inhibitory centre which he located between the medulla oblongata and the midbrain. Langhans, working in Steinach's laboratory, localised this inhibitory centre more accurately in the distal portions of the corpora bigemina and the hind brain. During the breeding season the inhibitory centre is itself inhibited, and the clasping reflex thus appears in the male frog. This hypothesis also explains the appearance of the reflex in the animal which has had its medulla divided experimentally.

In view of the experiments described above it can be suggested that the anterior pituitary-like substance extracted from the urine of pregnancy or the anterior

pituitary extract acts by inhibiting the inhibitory centre in the corpora bigemina and the hind brain, thus releasing the clasping reflex in the male toad. In the female the mode of action of the extract appears to be quite different. She is physiologically depressed, whereas the male is stimulated.

It is possible, however, that the extract does not act directly on the clasping reflex inhibitory centre, but does so only through the intermediation of the gonads. This problem is being investigated experimentally.

(f) Hogben and Slome (1931) have demonstrated the close endocrine relationship which exists between colour change (expansion or contraction of the melanophores) in *Xenopus* and the different parts of the pituitary.

In the experiments of the present author, dark as well as pale toads were used. Coupling appeared to be quite independent of the chromatic responses of the animals.

(g) It was observed that larger doses of anterior pituitary extract were required midway between breeding seasons to produce responses of the same magnitude as those obtained at the time of the breeding season. The experiments of Honikman, Shapiro and Zwarenstein (1934), however, show that great differences in potency (with ovulation and oviposition as the index) exist in different batches of pituitaries extracted at the same time. It would therefore be premature to attribute the larger doses required to provoke the mating reflex midway between breeding seasons to the less sensitive gonads of the toads at such times.

(h) Control extracts with the urine of adult males, and with sheep and goat brain extracts, gave negative results.

## VI. SUMMARY.

1. The complete disappearance of all sexual activity in *Xenopus laevis*, male or female, is observed under laboratory conditions.

2. A technique is described whereby the mating reflex (coupling) can be established in the laboratory during the pond breeding season as well as midway between breeding seasons, by means of injections of human pregnancy urine extracts, or by acid extracts of the anterior lobe of the pituitary gland of sheep and goats.

3. The mating reflex has been induced in 101 different pairs of toads with pregnancy urine extracts and in 39 different pairs of toads with anterior pituitary extracts.

4. The reflex induced by anterior pituitary extracts in no way differs from that produced by pregnancy urine extracts.

5. The possibility that the extract acts through the intermediation of the gonads is considered.

6. The mating reflex is described, and attention is drawn to the passive role of the female, while the male is stimulated to activity.

7. It is suggested that a stimulus from the female reflexly activates the male to clasping activity.

8. Fertilised ova from coupling pairs have been reared successfully to an advanced tadpole stage (4-5 months). Tadpoles can be reared also midway between breeding seasons.

9. The injected extract, acting possibly through the intermediation of the gonads, may be suggested to inhibit the clasping reflex inhibitory centre in the brain described by Steinach and others.

10. The mating reflex appears to be independent of the pituitary mechanism associated with chromatic response in *Xenopus laevis*.

11. The larger doses (500 mg. fresh tissue) of anterior pituitary extract required to produce in summer a response of the same magnitude as that produced by smaller doses (192 mg. fresh tissue) in winter cannot be attributed to a diminished sensitivity of the ovaries.

12. Control extracts of male urine and of sheep and goat brains repeatedly gave negative results.

#### VII. ACKNOWLEDGMENTS.

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#### EXPLANATION OF PLATE I.

Fig. 1 a. Dorsal view of the lumbar embrace during coupling in *Xenopus laevis*. Fertilised ova can be seen.

Fig. 1 b. Ventral view of the lumbar embrace. Note the fertilised ova.

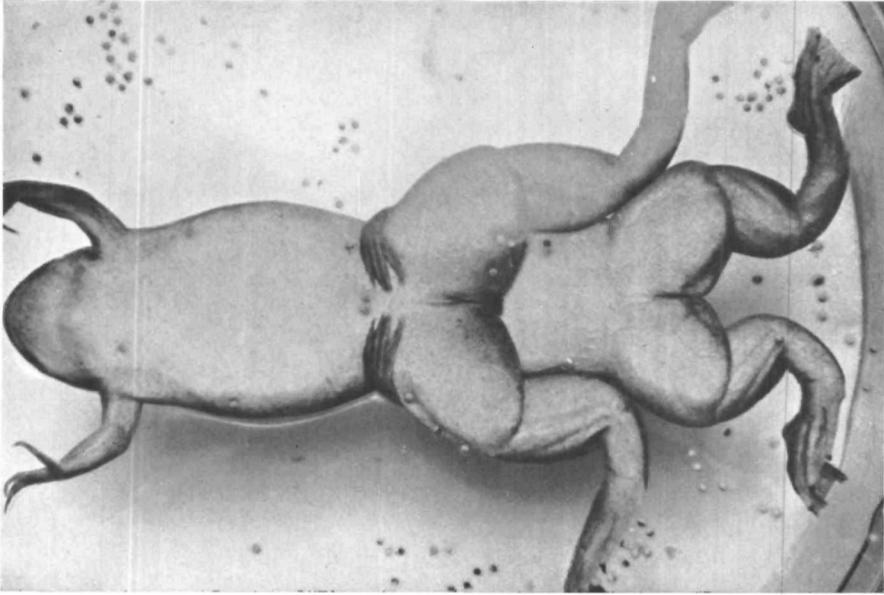


Fig. 1b.

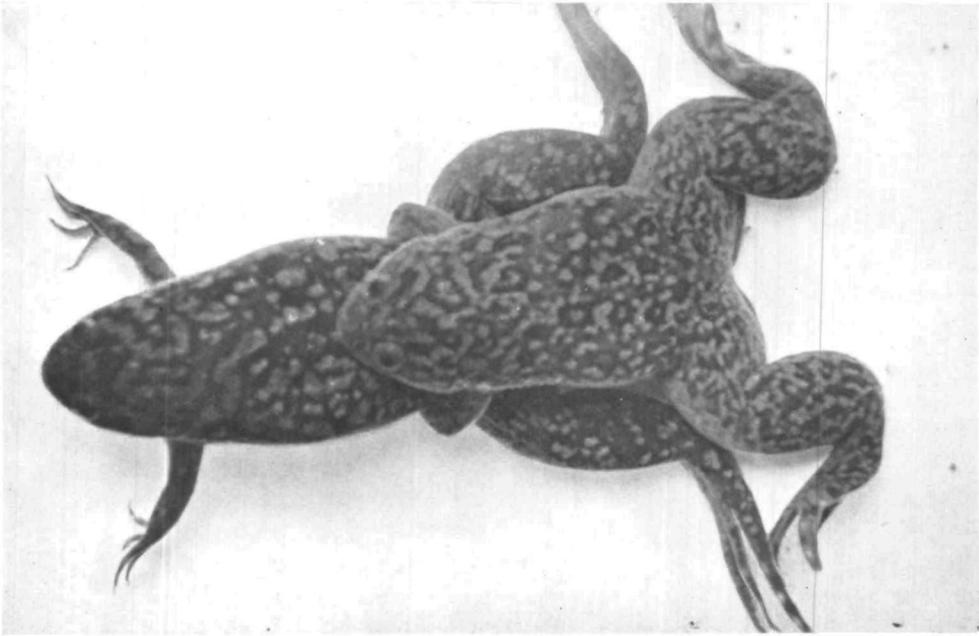


Fig. 1a

