THE CHROMATIC FUNCTION IN XENOPUS LAEVIS

A THESIS PRESENTED FOR
THE DOCTORATE IN PHILOSOPHY IN THE
UNIVERSITY OF CAPE TOWN

- - - - - - - -

BY

DAVID SLOME M.A.

- - - - -

-CONTENTS.-

- - -0- - -

PART I.

THE NORMAL RESPONSES OF XENOPUS LAEVIS.

- - -0- - -

PART 1.

THE NORMAL RESPONSES OF XENOPUS LAEVIS.

In few Amphibia is the extent of visible pigmentary effector activity as striking as in the case of the South African clawed toad. Nevertheless, there is no account on record of the phenomena of colour response in this species. The effector organs are, as in most other Anura, of three categories: (a) epidermal melanophores, (b) dermal melanophores, (c) xantholeucophores. Of these the second, as is general among those vertebrates that display the property of colour response, are the principal agents in the visible, that is, macroscopic effect. In an investigation intended to extend previous researches into the mechanism of co-ordination which underlies the pigmentary responses of Amphibia, it became necessary to make a thorough study of the normal responses of Xenopus. Since the dermal melanophores can be examined without difficulty in the living animal by microscopic inspection of the web between the toes, special attention was paid to them. In the course of the investigation results have emerged that open up a new field of problems in connection with the co-ordinating mechanism of Amphibian colour response. These results are the subject of the present communication.

At an early stage it became evident that, whereas light reflected from the background is a comparatively unimportant factor, as compared with other external conditions such as humidity, temperature, etc., in the case of the common frog of Great Britain, for Xenopus the light reflected from the background is the all important factor. The word background here, as elsewhere in this paper, is not used in a strictly literal sense, but - to avoid periphrasis - corresponds more precisely to the immediate field of vision of the animal. Two categories of animals were, therefore, employed in working out the normal reactions of the dermal melanophores, namely normal and eyeless. In the case of the latter the complete eyeball was removed. A careful study of the dermal melanophores in the living condition, as indicated above, early made it evident that eyeless animals are macroscopically speaking dark, but not so dark as the normal dark animal that has been kept on a black background, failing, moreover, to display pallor when kept on a white background; further, that the dermal melanophores of the eyeless animal were never as completely expanded as those of the seeing animal kept on a dark background. This necessitated a more definite classification of the condition of the dermal

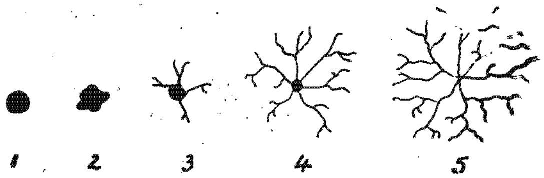


Fig. 1. Key to the Melanophore Index of *Xenopus laevis*

melanophores than that employed previously, and arbitrary numerical symbols were applied to five stages of contraction and expansion, which suggested themselves for convenient identification. These are indicated in the text figure.

The containers used for the experiments were square tin receptacles, 32 cm. broad and 7 cm. deep either (a) varnished so as to present a bright metallic surface hereunder referred to as "neutral background", or (b) and (c) painted with a hard black and white enamel respectively. These are referred to as "black" and "white background", though experiment showed that the field of vision of the animal was almost uniquely defined by the sides of the vessel, only slightly by the floor, and not at all by the cover, which was a piece of thin flat glass 35 cm. square, either unpainted or painted white or black as indicated in the text. The three types of covers were used to dim the incident light to varying extent. In no case were they light-proof. Experiments were undertaken with glass vessels painted on the outside to test any possible effect of the enamel.

In the tables recording the experimental findings set forth below the margin of error cited is the standard deviation of the mean value for each series of observations, i.e. $\sigma / \sqrt{n}$ where σ is the mean square root deviation of the series and n the number of individuals in each series. If x_1 and x_2 are the standard deviations of the mean values M_1 and M_2 of two series of observations, then $\sqrt{x_1^2 + x_2^2}$ is the standard deviation of the mean difference. If the mean difference is three times the standard deviation of the mean difference, the probability integral tables gives 270 : 1 as the odds in favour of significance.

It has long been known that, if the eyes are removed, the characteristic white background response is not given by Amphibia or Fishes. The initial experiment brings out a new fact, namely, that the eyeless animal does not give the black background response. The statistical data set forth bring out in sharp relief the impression gained from the macroscopic appearance of the animals, namely, that the eyeless Platanna on a black background is visibly paler than the normal animal on a black background, although the eyeless animal on a white background is much darker than the normal animal on a white background. It will be shown later that, in complete darkness, the normal animal is not dark but assumes the intermediate condition of the eyeless animal. The next point to call for attention was a more searching analysis of what was, at first, a somewhat indefinite impression gained from the macroscopic appearance of the animals, namely, that the most extreme pallor was found in animals placed on a white background in dim light, whereas the most extreme darkening of the skin was manifest in animals placed on a black background in bright light. This seeming paradox appeared to be consonant with observations on Urodele larvae, which have been placed on record by Laurens (1915-16), who found that eyeless individuals in bright light become darker, and that seeing individuals at first become darker and then paler. Laurens distinguishes between a primary or non-visual response to light by expansion of the melanophores, and a secondary response superimposed upon the former, dependent upon the eyes and acting in the opposite direction. The data set forth in Tables in connection with Experiments II and III show that, in Xenopus, there is a slight degree of primary reactivity of the type seen in Urodele larvae, that is, analogous to the Reptilian type of photic reaction, but the possible range of this primary reactivity is very small compared with the secondary or co-ordinated activity, which is dependent upon the eye. Moreover, the threshold of illumination requisite for the manifestation of the primary response is much higher than for the secondary, so that, in dim light, the secondary response on white background is not diminished by the primary reactivity, whereas in bright light the black background response is reinforced by the primary reactivity.

Experiment 1.

Exposed for 24-48 hours in diffuse light, 10 days after operation in case of eyeless toads. Temperature 19-20°C.

	White background with glass tops.	Black background with glass tops.
Eyed.	1.90 ± 0.04	4.54 ± 0.07
No. of individuals.	48	48
Eyeless	3.13 ± 0.13	3.13 ± 0.15
No. of individuals.	48	48

Experiment II.

Exposed for 24 hours to diffuse light at 19-20°C., fourteen days after removal of eyes in case of eyeless animals. Eight individuals in each batch.

	White covers		Black covers	
	White Background	Black Background	White Background	Black Background.
Eyed.	2.0 ± 0.00	4.6 ± 0.13	1.65 ± 0.16	4.25 ± 0.15
Eyeless.	3.13±0.30	3.0 ± 0.31	2.75 ± 0.18	2.75 ± 0.23

The consistent shift seen with the dimmer illumination (black covers) towards greater pallor is seen in another experiment in which the background was a varnished metallic surface which reflected light to a considerable extent. This is set forth in the table in connection with Experiment III.

Experiment III.

Exposed 24-48 hours diffuse light at 19-21°C., eyeless fourteen days after operation. Eight toads in each batch. Neutral, that is metallic background.

	Glass cover	White cover	Black cover.
Eyed.	2.6 ± 0.24	1.5 ± 0.25	2.4 ± 0.35
Eyeless.	3.4 ± 0.17	2.4 ± 0.17	2.4 ± 0.17

Before proceeding to give direct critical proof of this restricted degree of primary reactivity, which consistently emerged in our earlier experiments, we may pause to comment upon one issue. The intermediate condition of the eyeless animals in the early stages after operation might be attributed to stimulation resulting from necrotic changes in the degenerating fibres of the optic nerves. The eyeless animals have, therefore, been repeatedly tested, and an experiment carried out on an extensive scale after the lapse of sufficient time to ensure complete degeneration of the optic fibres, is here included to demonstrate the fact that conclusions drawn from experiments on animals fourteen days after operation was not invalidated by the possibility stated.

Experiment IV.

Exposed 24 hours in diffuse light at 16°C., six weeks after removal of eyes of eyeless toads.

	Black background Black tops.	White background White tops.
Eyed.	4.82 ±0.07	1.8 ±0.08
No. of individuals.	28	27
Eyeless.	2.8 ± 0.13	3.5 ±0.13
No. of individuals.	28	27

Experiment V.

At 17° C., six weeks after operation in case of eyeless toads.

	Complete darkness 60 hours.	Bright illumination 10 hours.
Eyed, on white background 12 toads.	2.7 ± 0.24	2.1 ± 0.08
Eyed, on black background, 12 toads	3.0 ± 0.18	5.0 ± 0.18
Eyeless, 48 toads.	3.0 ± 0.11	3.8 ± 0.09

The facts tabulated under Experiment V provide conclusive evidence first, for the fact that the neutral condition of the melanophores is intermediate, and that, superimposed on the major visual photic response, there is in Xenopus a slight degree of primary reactivity of the Chameleon type. We see that the normal toad on a white background becomes darker, and on a black background paler in complete darkness than in light, further, that the eyeless animal is significantly darker in bright light. Using the same standard of arbitrary numerical symbols to denote different degrees of melanophore expansion, we may say that the range of the primary reactivity is at a maximum only ten per cent of the range of secondary reactivity.

Humidity is not a significant factor in colour change in Xenopus, as shown in the following experiment. The point is hardly worthy of further investigation, as the animal will not live very long out of water, and if placed in a dry container the animal is kept moist by its own skin secretion for many hours.

Experiment VI.

Eyeless frogs for twenty-four hours on neutral background in diffuse light at 17°C.

	Mean Value	No. of Toads
Wet	3.4 ±0.18	16.
Dry	3.1 ± 0.15	16

Of the effect of temperature it is sufficient to say that in Xenopus laevis that between 15°C. and the lethal upper limit (about 35° C.) temperature has no significant effect. Below 10°C. there is a definite tendency to darkening of the ski

Thus the general type of effect is similar to that seen in most vertebrates which display chromatic response. But the noteworthy feature of Xenopus is the wide range of temperature over which the chromatic function remains unaffected as regards the final response.

From the data presented above it appears that (1) The dermal melanophores of normal individuals of Xenopus laevis are contracted when the animals have been kept for twenty-four hours on a white background between 15°C. and 30°C. They are expanded after exposure to a black background for the same period, and in complete darkness they assume an intermediate degree of expansion, preserved independently of the background in diffuse light by the eyeless animal. (2) This visual response is superimposed upon a very restricted degree of primary reactivity of the Reptilian type, not dependent on the eye.

The conclusions which have been set forth above were confirmed by experiments carried out after a lapse of twelve months. The only new point which emerges is that the equilibrium value for eyeless animals a year after operation is rather lower, i.e. they tend to approximate more to the condition of the pale animal as far as the microscopic appearance of the dermal melanophores is concerned. Macroscopically they shew no perceptible difference. The following experiment was undertaken after an analysis of the time factor in colour change had emphasised the necessity of prolonged exposure in establishing the true condition of equilibrium. Illumination was continuous and the record was taken after three weeks exposure. The mean temperature was 16° C.

	White Background.		Black background.	
	Bright	Dim	Bright	Dim
Normal.	1.25 ± 0.09	1.13 ± 0.07	4.54 ± 0.09	4.58 ± 0.12
No. of Toads	24	23	24	24
Eyeless	2.8 ± 0.15	2.15 ± 0.17	2.9 ± 0.09	1.9 ± 0.16
No. of Toads	20	20	20	20

For the sake of clarity it may be as well to restate the conclusions of the foregoing observations as follows.

SUMMARY OF PART 1.

(i) That, within a wide range of temperature (5°- 30°C) normal individuals of <u>Xenopus laevis</u> become dark if placed in light in a situation such that the field of vision is occupied by a surface which absorbs light ("black background" and become pale if placed in light in a situation such that the field of vision is occupied by a surface which scatters and reflects light ("white background").

(ii) That eyeless individuals do not display this type of reactivity, but, like normal animals subjected to prolonged exposure to total darkness, remain in an intermediate condition due to the partial expansion of the dermal melanophore

(iii) That in eyeless animals the melanophores are slightly more expanded in light than in darkness, so that there is a "primary" reactivity of the pigmentary effector system independent of the eyes and tending in the opposite direction of the "secondary" co-ordinated response for which the eye is the receptor organ and much less considerable in extent than the visual response.

(iv) Since the threshold of illumination for the "primary" reaction to light is higher than that for the secondary, the optimum condition for pallor in normal animals is dim light reflected from a light-scattering surface occupyin the field of vision, while the optimum condition for darkening is a light-absorbing visual field with bright illumination from above.

PART II.

THE TIME FACTOR IN THE CHROMATIC RESPONSES OF XENOPUS LAEVIS.

- - -0- - -

PART II.

THE TIME FACTOR IN THE CHROMATIC RESPONSES OF XENOPUS LAEVIS

The time taken for any response in an organism to occur must be referred to three separate entities: the latent period of the receptor, the time occupied by the propagation of the disturbance set up in the receptor through the co-ordinating mechanism, and the time which elapses in bringing the effector organ itself into action. Since we know the order of magnitude of the second, if the co-ordinating mechanism happens to be nervous an hypothesis of nervous co-ordination must involve certain limitations to the time factor in any type of response. The reaction time of the melanophore itself can be determined from perfusion experiment Krogh gives data with reference to the frog. Allowing a full hour for the reaction time of the melanophore the maximum requisite for a response controlled entirely by the nervous system will be of the same order of magnitude.

Disregarding for the present the primary reactivit which may depend (a) on direct response of the pigmentary effector organ to stimulus, or (b) a co-ordinated response for which the receptor is some photo-sensitive end-organ in the skin. The "secondary" reactivity on the other hand must involve a co-ordinating system which may be endocrine, nervous, or both, and the fact that eyeless animals are neither completely pale nor completely dark implies that the <u>white-background</u> and <u>black-background</u> response involve separate entities in the co-ordination of stimulus and response. Now this two-fold character of the co-ordination may be explained by either of two hypotheses (a) and (b) or a combination of both. The two hypotheses are:

(a) That there are two separate photo-receptive elements in the eye with different thresholds of excitation tending to produce in the one case inhibition, in the other case excitation, of the same mechanism of discharge (endocrine or nervous).

(b) That there are two separate mechanisms of discharge acting in opposite senses on the pigmentary effector, and that which predominates over the other is determined by the number of photo-receptors stimulated.

For the purpose of clearer exposition it is proposed to call the mechanism of discharge underlying, on the second hypothesis, the white background response W, and the mechanism of discharge underlying the black-background response B, and it may here be said that experimental evidence to be submitted at a later stage justifies the conclusion that B is identifiable with the secretion of the melanophore stimulant of the pituitary gland. A question which at once presents itself on the basis of the facts hitherto elicited is whether W is nervous or endocrine on the assumption that hypothesis (b) is correct, and a study of the time factor in the process was undertaken as an initial step.

The further analysis of the visual response from this angle opens up the possibility of discriminating between hypotheses (a) and (b), as well as placing on record for the first time a quantitative treatment of this aspect of pigmentary effector activity. A study has therefore been made of the time relation involved in:

(i) Passing from the black -and white-background response to the intermediate phase in total darkness.

(ii) Transition to the white-and black-background response from the intermediate condition consequent on prolonged exposure to darkness.

(iii) Complete reversal of the white-and black-background response in both directions.

The results of the investigation will be considered in this order. But before proceeding it may be advisable to sound a note of caution. In interpreting these results which are presented in graphical form, it must be borne in mind that the numerical symbols applied to different configurations of the dermal melanophores are quite arbitrary, and therefore, though some insight may be obtained from a consideration of the intervals which elapse between equilibrium conditions and the intercalation of subnormal or supernormal phases, no significance can legitimately be attached to the gradients of the curves.

A. Transition to the Intermediate Condition in Darkness.

The first problem to be investigated was the time taken by dark and pale animals to reach the intermediate condition in complete darkness. Previous experience had shown that this was considerable, and that pale toads required a more protracted exposure to darkness than dark ones before equilibrium was established, i.e. the eyeless condition was attained. In the first experiment (fig.2) eleven pale toads in white pots and eleven dark toads in black pots were placed in a large light-proof and well-ventilated box in the dark-room. The use of the box disposed of the necessity of exposing any individual to light for more than a few seconds when observations were taken. In this experiment there are three significant features which at once emerge from the graphical record. First, that the dark animals attain the eyeless

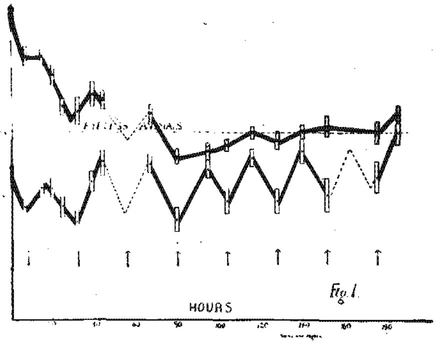
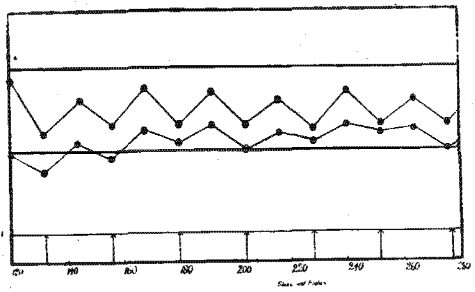


Fig. 2. Dark room experiment at 22 C. Upper series toads in black containers (originally dark).Lower series originally pale toads in white containers. Margin of error, standard deviation of mean of each series. The arrows represent twenty-four hour intervals (midnight to midnight).

equilibrium much more readily than the pale ones. Second, that there is a decided tendency for the melanophores to be more contracted at midnight, suggesting a diurnal variation analogous to that recorded for the primary photic reaction of Crustacea by Keeble and Gamble ( ). Third, that dark animals, after reaching the intermediate condition within 40 to 50 hours, become considerably paler than the eyeless animals, and remain so until about 120 hours after beginning of experiment, that is to say, they do not settle down to the eyeless equilibrium condition till about the same time as the toads which were initially pale. To obtain further confirmation on the last two points, i.e. the diurnal variation and the subnormal phase of the initially dark toads, the experiment was repeated at a different temperature. In the second experiment twelve toads in each series were employed, initially pale animals being placed in black pots and initially dark animals in white pots immediately before placing in the dark-room. In this second experiment the time required to attain equilibrium on the part of the initially pale toads was even more protracted than in the first. A part only of the experiment is reproduced, but the diurnal variation was consistent throughout the entire series of observations. Now, while the variation is not significantly outside the range of probable error for two successive points on the curve, the complete consistency of the two curves, and the perfect regularity of the result, leaves no doubt as to the



Part of an experiment showing transition from pale (lower) and dark (upper) condition to the intermediate state in darkness. It is inserted to show the regular diurnal variation. Arrows twenty-four hour intervals (midnight to midnight).

Fig. 3. Part of an experiment showing transition from pale (lower) and dark (upper) condition to the intermediate state in darkness. It is inserted to show the regular diurnal variation. Arrows twenty-four hour intervals (midnight to midnight).

authenticity of this diurnal change, which it is not without interest to note is in the same sense as the normal diurnal rhythm resulting from the primary response, and thus suggests that the mechanism involved is a "conditioned" one in Pavlov's terminology. In the second experiment the subnormal phase of the initially dark toads was quite pronounced, and complete equilibrium of the initially pale animals took about a fortnigh

B. Transition from Darkness to White and Black Background.

Two experiments were carried out on the same two series of animals as were employed in those represented in Figs 2 and 3. Fig. 4 is a continuation of Fig.2, the origin being taken as the point of illumination to continuous diffuse artificial light. On a black background the animals attain their final equilibrium in about an hour, surpass it seemingly, and then settle down to their final equilibrium about the time when the series placed on white background attained maximum pallor asymptotically - 25-30 hours after the beginning of the experiment. In a repetition of this experiment on the second series of individuals to ascertain whether the apparent existence of a supernormal phase on the series placed on black backgrounds was the fortuitous result of reinforcement by the diurnal variation, the existence of the supernormal phase was very decisively confirmed as the following protocol makes evident.

Time	White background	Black background.
hr. min.		
0 00	2.7 ± 0.22	2.5 ±0.19
1 50	2.75±0.18	3.5 ±0.15
3 00	2.5 ±0.19	4.1 ±0.15
4 00	2.4 ±0.14	4.2 ±0.16
5 30	2.3 ±0.13	4.4 ±0.15
6 55	2.18±0.16	4.4 ±0.15
7 55	2.18±0.10	4.6 ±0.14
21 25	2.0 ±0.00	4.5 ±0.15
24 55	2.0 ±0.00	4.4 ±0.19
Equilibrium value		
150 hours	1.9 ± 0.08	4.25±0.12

It is worth while pausing at this point to compare the time relations of the transition from darkness to white and black background respectively, and the reverse operation as recorded in the preceding section. From these experiments it is seen that the time taken to reach equilibrium in passing from the black-background equilibrium to the intermediate state is significantly greater than in the reverse process, viz., in passing from the intermediate condition characteristic of equilibrium in darkness to the black-background response in light.

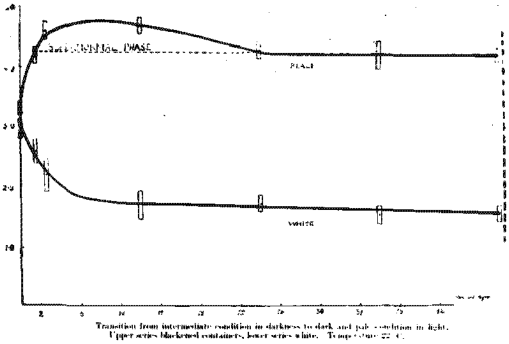


Fig. 4. Transition from intermediate condition in darkness to dark and pale condition in light. Upper series blackened containers, lower series white. Temperature 22°C.

It is further seen that the time taken to pass from the white-background equilibrium to the intermediate condition in darkness is significantly greater than the time taken to pass from the intermediate condition in darkness to white-background equilibrium in light. Finally, it is seen that the time required to pass from black-background equilibrium to the intermediate condition is much less than the time required to pass from the white-background equilibrium to the intermediate condition in darkness. Also that the time required to pass from intermediate condition in darkness to black-background equilibrium in light is less than the time required to pass from intermediate condition in darkness to white-background equilibrium in light. These conclusions may be epitomised by the aid of the following symbolism where b, w and i represent respectively the equilibrium conditions for black background, white background, and darkness, the arrows the direction of the process, and the usual conventions for inequalities being employed.

$$b \longrightarrow i > i \longrightarrow b$$

$$w \longrightarrow i > i \longrightarrow w$$

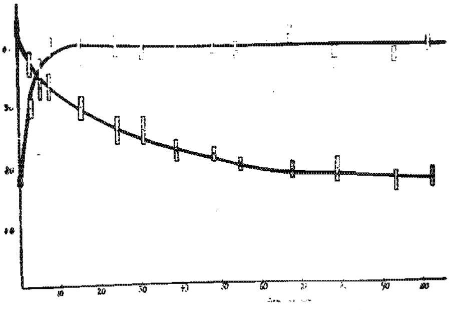
$$b \longrightarrow i < w \longrightarrow i$$

$$i \longrightarrow b < i \longrightarrow w$$

These results are totally inexplicable in terms of hypothesis (a) set forth in the introduction, and the existence of two separate mechanisms of discharge is reinforced by the existence of the subnormal and supernormal phases. For, if mechanisms B and W are both brought into play when the eye is illuminated, the final result depending on the number of photo-receptors stimulated, the slower rate at which W subsides would necessitate a subnormal phase in passing from black background i light to darkness, while the slower rate of development of W would necessitate a supernormal phase in passing from the intermediate condition in darkness to the black-background equilibrium.

C. Reversal of Background Response.

There remains to consider what happens when animals which have attained the white-background equilibrium are transferred to a black background and vice versa. Here, as before (see Fig. 4), mechanism W comes into play much more slowly than mechanism B; in other words, equilibrium is reached more rapidly in passing from white to black than from black to white. But, as would be expected, it takes much longer to attain equilibrium in passing from these extremes than in passing from the intermediate state to either condition. Not only is this the case, but the time taken to pass from the intermediate level to the equilibrium state when the backgrounds are reversed in light is significantly greater than the time taken to pass from intermediate condition of animals that have



Reversal from black to white and white to black background. Temperature 17 C.

Fig. 5. Reversal from black to white and white to black background. Temperature 17°C.

been kept in darkness to either type of background equilibrium. So that, if i_1 and i_2 respectively denote the intermediate condition of the melanophores of an animal kept in darkness and an animal which is in process of passing from black to white background in light or vice versa, then

$$i_2 \longrightarrow w > i_1 \longrightarrow w,$$

and $$i_2 \longrightarrow b > i_1 \longrightarrow b.$$

On the other hand, it takes less time to pass from white-background equilibrium to the black-background equilibrium in light than it does to pass from the white background to the intermediate equilibrium in darkness or $w \longrightarrow b < w \longrightarrow i_1$. And on the hypothesis of two separate efferent mechanisms B and W, this result is again explicable, since the first reaction involves the subsidence of W only, whereas the second result involves the subsidence of W and the subsidence of B.

Two experiments were performed and the results were perfectly consonant. Fig. 5 is based on the same series of animals as those used in the experiment on which Fig. 3 is based. It will be noticed that there is no supernormal phase in passing from white-background equilibrium to black-background equilibrium and this would be expected in terms of hypothesis b that may now formulated in more explicit terms.

The entire series of data here presented are, it is submitted, incompatible with the hypothesis that a single efferent mechanism, viz., the fluctuating secretion of the melanophore stimulant of the pituitary gland, underlies the background response of Xenopus and, it may be added, of other Amphibia. There may or may not be two sets of receptors whose activation tend to produce opposing results. For the present it is an economy to assume that the number rather than quality of receptors brought into play is the significant factor. But, in any case, there must be separate efferent mechanisms - using the term efferent without restriction as to endocrine or nervous character of the process - underlying the white- and black-background responses. It would then be possible to outline a working hypothesis in some such terms as these. When an animal kept in total darkness for some time is placed on a black background the small amount of light reflected stimulates some photo-receptors, bringing into play mechanism B, i.e. increased output of pituitary secretion, and mechanism W at present unknown. At first the effect of B overrides W, but as more photo-receptors are brought into play, i.e. when the field of vision is occupied by a light-scattering surface, the output of B reaches a limit before W so that W overrides B.

Alternatively the small amount of light reflected from a light-absorbing surface may inhibit W more than B, but as more receptors are brought into play the inhibition of B becomes more complete than the inhibition of W. In any case the salient points which emerge from the study of the time relations of pigmentary effector activity of <u>Xenopus</u> point to the following conclusions. First, that a different efferent mechanism, endocrine or nervous, underlies the black- and white-background responses of the intact animal. Second, that the protracted development of both types of response is consistent with an endocrine rather than a nervous mechanism. Thirdly, the existence of a diurnal variation in darkness, comparable in extent and direction to the normal diurnal rhythm of primary reactivity, is suggestive of the intervention of nervous agencie in the primary reaction. Evidence submitted at a later stage will shew that this suggestion is misleading.

SUMMARY OF PART II.

An analysis of the time relations of the secondary response involved in the transition (i) from the black to white background response and vice versa, (ii) from the intermediate condition in darkness to the white or black background response and vice versa provides evidence that the dermal melanophores of Xenopus laevis are subject to a double endocrine control.

PART III.

THE RELATION OF THE PITUITARY GLAND TO PIGMENTARY EFFECTOR ACTIVITY IN XENOPUS LAEVIS.

- - -0- - -

PART III.

THE RELATION OF THE PITUITARY GLAND TO PIGMENTARY EFFECTOR ACTIVITY IN XENOPUS LAEVIS.

Xenopus laevis possesses a great advantage over the European Common Frog in experiments involving operative procedure. It is much more viable in the laboratory. It recovers well from operative disturbance. It is easily fed on raw meat. It lives within a wider range of temperature. In seeking to elucidate two separate endocrine factors in the secondary photic response the first issue which presented itsel for enquiry was the identification of the agency controlling th black background response. The secretion of the posterior lobe of the Pituitary Gland has already been proved to be significant to darkening of skin in Amphibia generally. Accordingly at an early stage a large stock of hypophysectomise animals was prepared for future use. A few preliminary remarks must therefore be made with reference to operative procedure. At the same time a brief reference may be made to the technique employed for estimating the potency of extracts of the posterio lobe of the pituitary gland.

(a) <u>Operative removal of the Pituitary Gland</u>. The method

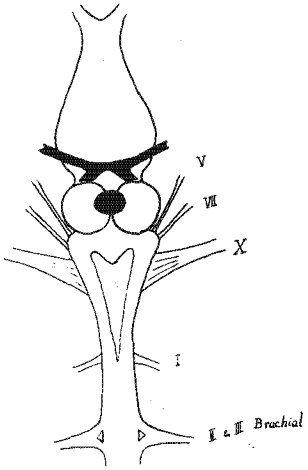


Fig. 6. Diagram of dorsal surface of brain of Xenopus showing (in black) the relative positions occupied by optic chiasma and Pituitary gland on ventral surface.

employed was in principle essentially similar to that described by Hogben for the removal of the pituitary gland in other Anura and in Urodeles. There are however certain morphological details which render the operation of hypophysectomy more difficult in Xenopus laevis. These are preeminently the thickness of the bones of the skull and the adherence of the mucous membrane of the mouth to the floor of the skull. In Xenopus it is not possible to see the outline of the pituitary gland through the thin translucent base of the skull as in Anura and Urodeles. To locate its position without extensive exposure it is necessary to apply the dental drill to a spot in the middle of the anterior margin of the common Eustachian orifice. Before doing so a median incision is made in the mucou membrane which is scraped away from base of skull. This procedure usually leads to loss of a good deal of blood if other precautions are not taken. Bleeding can be prevented by cauterising two large superficial vessels with a small electric cautery. Owing to the adherence of the mucous membrane it is easy to tear away large masses of tissue in applying the dental drill. It was found advisable to keep the mucous membrane stretched by covering the ventral surface of the mouth with a pad of rubber. No mortality occurs when these precaution are taken if the operation is performed in the cooler months, but a high mortality occurred in the ensuing weeks during November to March. In either case animals surviving the first three weeks after operations live indefinitely in the laboratory and have been kept under observation for more than two years. After three weeks they were fed regularly once a week. The water was changed after feeding. Most of the operated animals employed in this investigation had been kept in the laboratory after operation for a much longer period of time than those which have been tested in previous investigations of the same kind.

In preparing stocks of operated animals it was decided at the outset to prepare equal numbers of individuals in which the whole gland or the anterior lobe only had been removed. The latter were originally intended to serve as controls. It will be seen later that they provided ulterior evidence bearing on the role of endocrine agencies in controlling pigmentary changes in Xenopus laevis.

(b) Injection of Pituitary Extracts. In experiments involving injection of pituitary extracts a new departure has been made. Hogben and Winton have recorded the effects of injecting posterior lobe extract into normal and hypophysectomised frogs. Their comparison of the reaction of the two groups was based on the minimal dose requisite to produce a macroscopic effect. In experiments recorded in this section a comparison of the effects on injecting pituitary extract into normal and hypophysectomised Platanas was based on a more refined procedure developed in the course of this investigation and already described in detail by Hogben and Gordon (1930). This method involves plotting a curve for the variation of the melanophore index with time after injection. It is a more delicate instrument for two reasons. First, as has been shown in the preceding sections the microscopic appearance of the melanophores can be described with greater precision than the macroscopic effect: Secondly, both the intensity and persistence of the effect are determined by the amount of active substance injected. The first of these perhaps merits further comment. Although it is true to say that in equilibrium conditions the background response of the dermal and epidermal melanophores is the same in Xenopus laevis, the rate of change is not the same for both. Furthermore though both react to pituitary extracts in the same sense their respective thresholds are not identical. For this reason it is possible to encounter in experiments involving injection of pituitary extracts considerable discrepancies between the macroscopic appearance and the microscopic state of the dermal melanophores. There is no doubt that these discrepancies can be explained in the main by two circumstances. One is that the epidermal melanophores do contribute significantly to the macroscopic effect. The other is that epidermal melanophores are relatively much more numerous in some individuals than others. In some animals they are so few in number that they are difficult to find. In others they are more numerous than the dermal melanophores. It is for this reason that attention has been directed almost exclusively to the dermal melanophores. To what extent the Xantholeucophores play a significant part in the macroscopic effect it is difficult to say. No such consistency in their behaviour as described in Rana could be elucidated successful in Xenopus laevis. In very pale animals that have been kept for a long time on a white background and generally speaking in hypophysectomised animals they are seen to be completely expanded. They are sometimes seen to be very completely expanded in animals whose macroscopic appearance is as dark as possible. These discrepancies may be due to the fact that the rate of reaction is different from that of the melanophores or to the influence of agencies which do not exercise a noticeable effect upon the latter. It has not been possible to arrive at any satisfactory conclusions concerning the behaviour of the Xantholeucophores. In many animals they are so inconspicuous that it is not convenient to observe how they behave.

(c) Effects of operative removal of the gland. After complete removal of the pituitary gland Xenopus laevis remains pale on a black background indefinitely. Hypophysectomised animals and control series have been kept under observation for a period of more than two years. In the course of parallel investigations

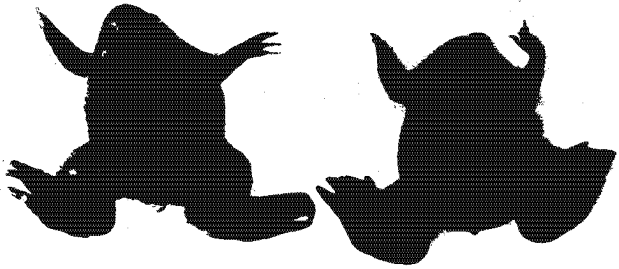


Fig. 6. Two toads.

Right - Normal animal kept on a black background for three weeks.

Left - Hypophysectomised animal kept on black background since operation (twenty-five months carried out in this laboratory in connection with metabolic changes accompanying pituitary removal and pigmentary effector activity stocks of from two to three hundred animals have been kept under observation. Though the macroscopic appearance of hypophysectomised toads when kept in a black background indicates maximal pallor, a study of the microscopic appearance indicates that normal animals kept on a white background are paler. That is to say there is a slight but not necessarily significant difference in the melanophore index of the two groups. The melanophore index of normal toads after prolonged exposure to white background is significantly lower than the melanophore index of the completely hypophysectomised animals also placed on a white background. This difference is shown in the accompanying table which also reveals an additional circumstance bearing on the role of the pituitary gland in relation to the primary response. The primary response is completely independent of the pituitary gland.

Reactions of normal and hypophysectomised toads.

16°C. Continuous illumination ten days.

	White background	Black background	Darkness.
Normal Toads	1.25 ± 0.09	4.54 ± 0.09	
Number	24	24	
Hypophysectomised (both lobes) (x)	1.64 ± 0.13	1.40 ± 0.13	1.30 ± 0.13
Number	14	15	15
Hypophysectomised (y) (Anterior lobe only)	4.4 ± 0.13		
Number	13		

(x) Nine months after operation. (y) Four months after operation.

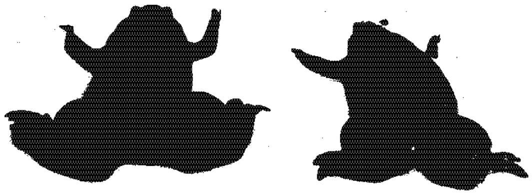


Fig. 7. Two toads.

Right - Normal animal kept on a white background for three weeks.

Left. - Toad with the anterior lobe of the pituitary removed twenty months previously kept on white background.

This slight difference in the behaviour of normal animals exposed to a white background and hypophysectomised animals exposed to the same conditions would be inexplicable on the assumption that the co-ordination of colour response in Xenopus is solely determined by the activity of the posterior lobe of the pituitary gland. On the other hand the experiments already recorded have conclusively shown that there are two endocrine agencies involved in the pigmentary responses of Xenopus. It is evident that what has been called the B mechanism corresponds to the activity of the posterior lobe of the Pituitary gland whose importance in other amphibia is well established. This conclusion is confirmed by subsequent experiments on the effect of injection. Two hypotheses would account for the new fact which is brought to light in the foregoing table. If the removal of the pituitary gland as a whole involved the elimination of the W mechanism it would be expected that the normal animal would in favourable circumstance be capable of more extreme pallor than the completely hypophysectomised animal.

The simplest hypothesis would then be that mechanism W is identifiable with the anterior lobe of the gland. Alternatively it may well be that the activity of some other endocrine organ is determined by the presence of the pituitary gland. In this connection it may be mentioned that removal of the pituitary gland in tadpoles is accompanied by involution of the suprarenal cortex (Smith).

That one or other of these hypotheses is correct is strongly confirmed by the behaviour of animals in which the anterior lobe alone was removed. This will be seen from the last series of the foregoing table. Removal of the anterior lobe in Xenopus is not so easy to perform as in Rana. It adheres by its anterior margin. In consequence it is easy to leave a small portion behind. In animals in which it was seen to have been completely removed remained dark on a white background for five or six months after operation. Partial recovery occurred in some individuals. It could then be generally established by postmortem examinations that regeneration had occurred. On the other hand two animals kept alive for over two years after removal remained with their melanophores fully expanded in optimal conditions for pallor. (Fig. 7). A few experiments on Rana Fuscigula did not yield certain confirmatory results. They were carried out in hot months when the animal does not survive the operation for long though in all cases they remained dark in optimal conditions for pallor. Some of them assumed an intermediate condition for a day or two preceding death. Were it not for the fact that it has been possible to study the reactions of animals surviving for a long period after operation it might be possible to attribute this result to operative disturbance of the posterior lobe. The long continued survival of the operated animals makes it highly unlikely that such an explanation is valid. It would thus appear that in Xenopus laevis the white background response depends upon the presence of the anterior lobe of the pituitary gland and that the black background response depends upon the presence of the posterior lobe.

It seemed desirable to extend the scope of the investigation to include the effect of removing one or both lobes of the pituitary in eyeless individuals. From the accompanying protocol the results of such experiments are seen to be entirely consonant with the conclusions stated above:

Diffuse light 17° C.

	No. of animals.	Melanophore Index.	
		White background	Black backgrou
Eyeless: Pituitary intact	21	3.7	3.5
Eyeless: Anterior lobe removed (x)	21	4.9	4.7
Eyeless: completely hypophysectomised (x)	12	1.4	1.4

(x) Two months after operation

(d) <u>Injection of Posterior lobe Extracts.</u> Injection of an extract of the posterior lobe of the pituitary gland in Xenopus

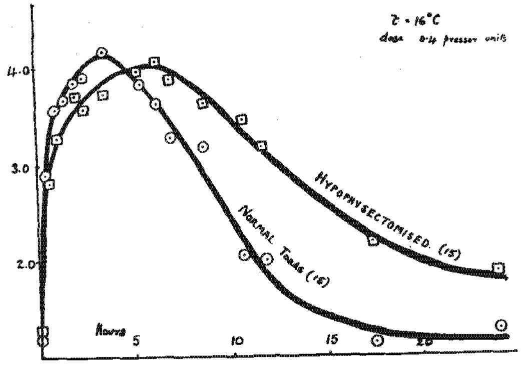


Fig. 8. Effect of injection of Pitressin into fifteen hypophysectomised and fifteen normal toads. Dose .4 Pressor Units. Temperature 16°C.

as in Rana evokes expansion of the melanophores. Experiments on the effect of injecting pituitary extracts into normal and hypophysectomised toads revealed a peculiar circumstance. The hypophysectomised animal has a lower threshold and the duration of the darkening produced is much greater than is the case with the normal. This conclusion was so unexpected that a long series of experiments were carried out. In some of these commercial extracts were employed, in others extracts prepared from fresh bovine glands prepared in the laboratory were used. The intraperitoneal method was adopted. All animals used in these experiments had been previously kept for a week on a white background. The commercial extract chiefly employed was that known as Pitressin (Parke Davis). This is a highly concentrated extract of the pressor substance. The separated oxytocic principle Pitocin has no action on the melanophores. Three representative protocols will be cited.

(1) Fifteen normal and fifteen hypophysectomised toads each received a dose equivalent to .4 pressor units of Pitressin. For the normal group a maximal effect was reached at about four hours with a melanophore index of 4.1. The maximal effect for the hypophysectomised toads was not reached until six hours from injection had elapsed, the melanophore index being 4.0. Twelve hours after injection the melanophore index of the hypophysectomised group was 3.1 and of the normal 2.0 (See Fig 8)

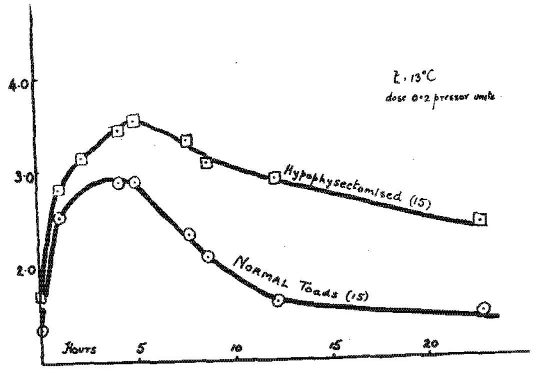
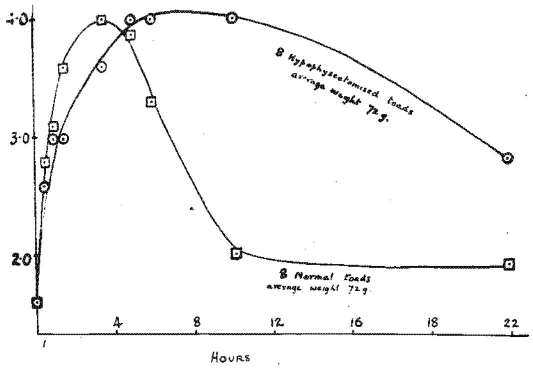


Fig. 9. Illustrating effect of injection of Pitressin into fifteen hypophysectomised and fifteen normal toads. Dose .2 Pressor Units. Temperature 13°C.

(2) To emphasise the difference in threshold a lower dosage was administered in a precisely parallel experiment (Fig 9). In this experiment .2 pressor units were injected into each individual. The maximal value of the melanophore index of hypophysectomised group was 3.6. That of the control group was 2.9. Thirteen hours after injection the hypophysectomised group had reached 3.0 the normal group had come down to 1.7.

There is an obvious objection to any conclusive deduction from the foregoing experiments. The posterior lobe of the pituitary gland has been shown by Krogh and his collaborators to control the state of tone of the capillaries. In this investigation as in Krogh's experiments it was frequently observed that the capillaries of hypophysectomised animals were dilated. It would therefore be expected that injection of pituitary extracts containing the pressor principle would constrict the peripheral vessels of the normal animal to a greater extent. Constriction of the vessels would naturally tend to mask the effect of the melanophore stimulant B. This ambiguity in the preceding experiments can be eliminated. Hogben and Gordon (1930) have conclusively shown that the pressor and melanophore activities of posterior lobe extracts depend on different substances. The pressor activity can be completely destroyed leaving the melanophore activity intact. Indeed the melanophore activity of extracts



Melanophore only: pressor destroyed. Dose $\frac{1}{50}$ cc. Parke Davis pitressin.

Fig. 10. Effect of injection of Pitressin treated for destruction of pressor principle into eight hypophysectomised and eight normal toads. (average weight 72 grams.) Dose equivalent of one-fiftieth cc. Pitressin. Temp. 15°C.

is increased when the pressor substance is destroyed. This would be expected from the considerations which have been advanced in the foregoing remarks. To place the issue on a more secure foundation experiments were conducted to test the action of extracts in which the pressor substance had been destroyed.

(3) To an aqueous solution of Pitressin an equal quantity of 2.5 Molar Sodium Hydroxide was added. This was allowed to stand for three hours in the cold, neutralised with Hydrochloric acid, and diluted to the appropriate strength for injection. The dose injected into eight normal and eight hypophysectomised toads (females average weight 72 grams) was equivalent to one-fiftieth cc. Pitressin. As will be seen from Fig. 10, the melanophore index of the hypophysectomised group ten hours after injection was 4.0, that of the normal group had come down to 2.0. Twenty two hours after injection the melanophore index of the hypophysectomised was 2.8.

The results of the experiments recorded above might seem to be in conflict with earlier observations on the injection of posterior lobe extracts into normal and hypophysectomised frogs. Hogben and Winton (1923) found that the minimal dose for hypophysectomised frogs was higher than for normal ones. Several explanations of this discrepancy might be advanced. One which is specially relevant to what follows is that in Hogben and Winton's experiments animals were injected immediately after operation. All the animals employed in the foregoing experiments had been living in the laboratory for nine months. There seems to be only one apparent explanation of the greater sensitivity of Xenopus to pituitary extracts after its pituitary gland has been removed. If the removal of the anterior lobe of the Pituitary gland eliminates the W mechanism which has been postulated in Part II of this communication , it is easy to see why this result should occur. In the hypophysectomised animal the B substance injected into the circulation is not antagonised by the presence of W substance in excess. On the other hand it is not reinforced by the B substance already present in the normal animal. Thus the hypotheses that two endocrine agencies are involved in the colour responses of Xenopus laevis is fully compatible both with the observations on the effects of injection of Pituitary extract on normal and hypophysectomised frogs as recorded by Hogben and Winton, and with the contrary result obtained in experiments on Xenopus laevis as set forth in this communication. In any case the peculiar fact that the hypophysectomised clawed toad has a greater rather than a lower sensitivity to posterior lobe extracts as compared with the normal animal reinforces from an unexpected source the conclusion that the W mechanism is closely associated in some way with a part of the pituitary gland and almost certainly not the same as that which is identifiable with the B mechanism.

(e) Injection of Anterior Lobe Extracts. Conclusive evidence identifying the secretory activity of the anterior lobe with the W mechanism whose existence was established in Section 2 could be obtained by evoking pallor in the dark animal by injection of anterior lobe extracts. It has not been possible hitherto to establish this reaction in an entirely satisfactory manner. It will therefore suffice to state briefly what measure of success has been achieved. On various occasions extracts of the anterior lobe of Xenopus and of the Ox have evoked a complete macroscopic pallor. Hitherto such results have only been obtained with the use of extracts prepared in 1% Acetic-Ringer. On the other hand samples prepared in an identical manner have not produced wholly uniform results. It is therefore necessary to speak of them with caution.

There are three features of a long series of experiments which merit comment. First it may be stated with confidence that the reaction when it was obtained was tissue specific. Secondly in most experiments where no macroscopic changes were recorded extracts of anterior lobe evoked a very definite fall in the melanophore index, after a latent period of 24 to 56 hours. Parallel injections of pale animals showed that this latent period is attributable to the presence of the B. substance from which no anterior lobe preparations , however carefully prepared, are ever completely free. Finally it may be stated with some assurance that the failures encountered were not due to a high concentration of the B substance in the extracts prepared. Ox glands dissected out within half-an-hour of killing were in all cases employed and additional precautions to prevent infiltration with posterior lobe secretions did not increase the intensity of the result. Three explanations of the incomplete success which attended this part of the investigation may be proferred. One is that an autocoid is invariably present but that it is a highly unstable substance. This is fully consonant with the very variable quality of extracts prepared from the anterior lobe to evoke the Ovarian reaction of Zondek and Ascheim. Again, it is possible that a very small amount of the active substance postulated is ever present in the gland. This suggestion is in agreement with the very slow rate of development of the white background reaction. It was not confirmed however by a parallel series of tests of extracts from the anterior lobes of dark animals and pale animals in which on the present hypothesis the anterior lobe should have been active. One other possibility emerges. In the frog the pars tuberalis is represented by two plaques lying quite separately from the pituitary as a whole on the tuber cinereum. Strictly speaking the operation of total hypophysectomy cannot therefore be performed in the frog. In Urodeles there is no separate pars tuberalis. It is represented by two anterior horns of basophil cells continuous with the pars anterior and firmly attached to the tuber cinereum. Sections of the pituitary gland of Xenopus revealed no trace of the separate paired tuberalis elements of the frog. On the other hand they elucidated a feature of the gross morphology of the gland already referred to in the section on operative procedure. I am indebted to Mrs. Rimer for the preparation of a series of sections which she permitted me to examine. They show that the pars anterior of the pituitary of Xenopus is firmly attached to the tuber cinereum by a forward prolongation of basophil cells. It is difficult to escape the conclusion that the pituitary of Xenopus is of the Urodele type. In that case what has been called the anterior lobe of the Clawed Toad represents the pars anterior together with the pars tuberalis of the Frog. An extract of the pars tuberalis of the Ox alone injected into a dark animal evoked complete macroscopic pallor. The difficulties of obtaining sufficiently large quantities of Ox pituitaries have prevented an extensive confirmation of this experiment. It is legitimate tentatively to state the conclusion that the W substance is located mainly in the Pars Tuberalis, and this conclusion would harmonise the slight discrepancy between the effects of pituitary removal in Xenopus and Rana recorded in a preceding section.

SUMMARY OF PART III.

1. Xenopus laevis can be kept alive indefinitely after removal of the whole pituitary (complete hypophysectomy) or of the anterior lobe alone. Such animals have been kept under observation in the laboratory for a period of a little more than two years.

2. Removal of the whole gland abolishes the black background response.

3. Removal of anterior lobe alone abolishes the white background response.

4. Normal animals after prolonged exposure to a white background are slightly paler than completely hypophysectomised animals in the same situation. This is explicable on the assumption that the W mechanism is directly or indirectly dependent on the activity of the anterior lobe as suggested by 3

5. Injection of posterior lobe extracts into completely hypophysectomised clawed toads produces darkening as in frogs. The removal of the whole gland renders the animal more sensitive and not less sensitive to the injection of pituitary extracts. This could be explained on the assumption that B substance introduced into circulation is not antagonised by the presence of W substance already there. Such an explanation implies that the W. mechanism is dependent in some way on the

anterior lobe of the pituitary gland as indicated in 3 and 4.

6. The primary response is independent of the pituitary gland.

PART IV.

THE RELATION OF THE NERVOUS SYSTEM TO COLOUR RESPONSE IN XENOPUS LAEVIS.

- - -0- - -

PART IV.

THE RELATION OF THE NERVOUS SYSTEM TO COLOUR RESPONSE IN XENOPUS LAEVIS.

Lister and the earlier workers on Amphibian colour change concluded that the disturbances propagated from the receptors to the C.N.S. travelled thence by nerves which supply the melanophores themselves.

The work of Pouchet (1876), Parker (1906), Wyman (1924), Redfield (1918) and Hogben and Mirvish (1927) provides very definite experimental evidence for the view that the melanophores of fishes and reptiles are innervated. There is however no direct histological support for this conclusion as applied to Amphibia, and, as pointed out by Hogben, the experimental evidence is both equivocal and conflicting. On the other hand there is at the same time conclusive evidence that the pituitary gland plays an essential role in the co-ordinatio of colour change in Amphibia and strong presumptive evidence derived from analysis of the time factor to shew that the predominating agency or agencies are of an endocrine nature. The evidence presented in parts 2 and 3 of this communication makes it well nigh impossible to entertain the view that the pituitary secretion is a static condition essential to the proper exercise of a dynamic nervous control, a hypothesis, which must not be overlooked. It is now proposed to bring forward direct evidence (a) to shew that the secondary or eye-co-ordinated response in Xenopus is independent or more strictly, can be exercised independently, of the peripheral nervous system (the optic nerves being, naturally excluded), (b) whether the peripheral nervous system, in the same sense, plays any significant role in the control of the <u>primary</u> response.

Before however proceeding to describe experiments undertaken with these objectives in view, it is perhaps advisable to reiterate an essential condition that must be borne in mind in the interpretation of all such procedures as involve section or stimulation of nerves. The entire nervous system of a vertebrate may be looked upon as the seat of impulses which control the tone of blood vessels. It is impossible to stimulate or transect the Central nervous system at any level, or a peripheral spinal nerve without affecting in some way the blood supply of the area innervated by the nerve stimulated or cut off from control by the section of its efferent fibres. It is therefore evident, as insisted by Langley and by Hogben, that (1) the results of stimulation are only unequivocal if it is possible after cutting off the circulation to produce an effect which would not occur in response to cutting off the circulation of itself; and (2) the results of section may inform us that a given response can occur independently of the peripheral nervous system, but cannot possibly provide conclusive evidence that a response is not due to the stimulus of a circulating autacoid, since nerve section will affect the amount of blood circulating through the area cut off from the C.N.S.

(a) Relation of the Nervous System to the Primary Response.

In striking contrast with the slow development and subsidence of the secondary or co-ordinated photic response for which the eye is the receptor organ, the primary response which is best studied in the eyeless animals occupies between thirty minutes and three quarters of an hour. From the time relations of the primary response we have nothing to guide us in deciding whether it is (a) a simple reflex, (b) partly reflex and partly endocrine, or (c) a direct reaction of the melanophores themselves to light. At first sight the last hypothesis seems the most likely; but it has been shewn by Parker that there are photoreceptors in the skin of Amphibia, since eyeless frogs shew phototropic behaviour. Unpublished experiments by Zoond in this laboratory prove that this conclusion applies to Xenopus laevis. The second hypothesis may be excluded by the data presented in the preceding section in which it has been shewn that the primary response is manifest in the completely hypophysectomised animal. It is now necessary to decide between the first and the last hypotheses.

If there is a peripheral nerve supply to the melanophores of Xenopus a simple reflex depending upon photoreceptors in the skin might be the explanation of the primary response. To test this the spinal cord was completely destroyed in twenty four animals. The temperature of the room was 19°C. The webs were spread out and twelve animals were placed in darkness, the others being placed in white containers in bright light. At the end of one hour the conditions of the webs of the two series was recorded, the figures being:

Darkness	2.1	0.14
Light	2.4	0.14

The two series were then reversed and a second record taken after two hours. This resulted in the following data:

Darkness	1.9	0.14
Light	2.25	0.19

The consistency of this result seems to yield fairly conclusive evidence in favour of the view that the primary response is completely independent of the C.N.S. as well as of the pituitary. This amplifies a previous statement that the primary response of Xenopus is essentially like the Reptilian response to light. The photic response of Reptiles is due to direct action of the light on the melanophores without the intervention of any system of co-ordination.

(b) Effects of Nerve Section on the Secondary Response.

The precautions necessary in the interpretation of experiments on nerve section have been mentioned. An experimental precaution too often overlooked by previous workers is the desirability of working with animals that have completely recovered from operative disturbances. In selecting Xenopus for a more extensive investigation of the control of Amphibian colour change, benefit has been derived from the experience of Professor Jolly whose work has shewn that Xenopus is particularly well suited for survival experiments involving section of nerve trunks. Various types of experiments were carried out. Three only will be described in detail.

(1) Section of the sciatic plexus.

The entire sciatic plexus of one side (left) was destroyed in six toads. After three weeks the incision had healed, and the circulation of the web was not noticeably deranged on the operated side. The following readings were taken, the initial time being three weeks after operation. Where readings were taken at midnight the fact has been noted, as demonstrating the "diurnal variation" elsewhere described.

Time	Left Leg (Paralysed)	Right Leg (normal)
.00	2.16	2.16
Transferred to Black background (0.00)		
2.30	3.82	3.82
5.00	4.33	4.33
13.00 (midnight)	4.0	4.0
24.30	4.5	4.5
Transferred to White background (24.30)		
32.00	2.0	2.0
37.00 (midnight)	1.6	1.6
50.00	2.0	2.0
61.30 (midnight)	1.5	1.5
76.30	1.6	1.6
Transferred to Black background (76.30)		
81.30	3.7	3.7
120.00	4.8	4.8
Transferred to White background (120.00)		
216.00	1.3	1.3
Transferred to Black background (216.00)		
276.00	4.0	4.0
288.00	4.16	4.16

From these data it is seen that there is complete agreement between the behaviour of the two limbs even as regards the diurnal variation, which at first sight suggests a conditioned reflex. To make certain that the limbs react at the same rate further records were taken at shorter intervals six weeks after operation.

On white background

Time	Right Leg	Left (operated) leg
0.00	1.5	1.5
Transferred to Black background		
1.00	3.0	3.0
3.00	3.8	3.8
4.30	4.0	4.0
9.30 (midnight)	3.8	3.8
19.30	4.3	4.5
22.30	4.6	4.6
31.30 (nearly midnight)	4.0	4.0
Six days later on Black background		
0.00	4.0	4.0
Transferred to White background		
6.00	2.2	2.2

Again the diurnal variation is well shewn in both legs, and there is no discrepancy as regards the rate of reaction. This is not surprising, since the circulation in the operated limb seemed to be restored to its normal condition macroscopically.

(ii) Section of Spinal Cord and Sympathetic chain

Three animals survived the operation of transection of the cord at the level of the 7th, 8th and 9th vertebra together with section of the sympathetic chain . Their behaviour was uniform, and it is therefore permissible to give the figures in averages.

Time (reckoned from three weeks after operation)	
0.00	4.3
Transferred to White background.	
2.30	2.0
5.0	1.7
13.00 (midnight)	1.7
24.30	1.7
32.00	1.3
37.00 (midnight)	1.3
50.00	1.7
61.30 (midnight)	1.3
76.30	1.6
Transferred to Black background.	
81.30	3.0
120.00	3.7
216.00	4.0
Transferred to White background	
276.00	3.3
288.00	1.3

Again we see the diurnal variation to be independent of the peripheral nervous system. In this experiment which was carried on with repeated transference from white to black background, there are two features which call for comment. The first is that the black background response is abnormally sluggish, the second is that though macroscopically the operated animals were indistinguishable from normal ones, the black background response never proceeds to the limit of completion reached in normal toads. It is obvious that the sluggishness can only be interpreted in terms of circulatory disturbance which was evident from the dilation of the blood vessels.

In observing the responses of animals in which the C.N.S. was cut at the level of the 2nd and 3rd vertebrae, it was noted that a complete macroscopic reaction to white background by pallor did not occur. The melanophore index varied between 5 - 3.0, when the animals were transferred from black to white background and vice versa. The operation was accompanied by a considerable general disturbance of the circulatory system as indicated by the state of the capillaries in the web. Where a double endocrine mechanism exists, it is impossible to predict whether a generalised condition of capillary dilation would reinforce the black background or the white background response. On the other hand another possibility must be taken into consideration. If the W. mechanism depends on the suprarenal cortex section of the spinal cord above the level, where the preganglionic nerve supply of the adrenals emerges, would interfere with its action. Removal of the pituitary gland in tadpoles has been shewn by various workers to be accompanied by involution of the suprarenal cortex. The effects of removing the anterior lobe might conceivably be attributed to the activity of the suprarenal cortex. Injection of adrenaline was found to have little effect on colour response in Xenopus when physiological doses were employed. Alkaline, neutral, acid and alcoholic extracts of fresh bovine suprarenal cortex produced no pallor in dark animals.

Whatever is the true explanation of this discrepancy, the incomplete responses of operated animals after spinal transection certainly cannot be used to support the view that the melanophores of Xenopus are directly innervated like those of Amphibia and Fishes. Nerve section and spinal transection in particular in Fishes and Reptiles is accompanied by purely segmental disturbances. The segmental phenomena have been recorded photographically by Hogben and Mirvish (1927).

The generalised disturbance which was observed in these experiments might therefore be used with more cogency as an argument against rather than in favour of the hypothesis that the melanophores of Amphibia are directly innervated.

(iii) Section of the Optic Nerves.

In view of the recent work of Perkins (1928) on Crustacean chromatophores it seemed worth while to compare the effects of section of the optic nerve with the effects of total removal of the eyes. The results of experiments undertaken with this end in view indicate that the eye exercises its influence on colour response in Xenopus laevis through impulses initiated in the retina and transmitted by the optic nerves to the brain.

	No. of animals.	White background	Black background.
Normal Toads.	12	1.5	4.6
Eyeless Toads x	12	3.3	3.4
Toads with optic nerves severed at Chiasma x	12	3.7	3.7

x 3 Weeks after operation.

The above record was taken at 15°C. in diffuse light. Aqueous extracts of the eye did not yield results analogous to those recorded by Perkins.

(c) Discussion:

In a recent communication Kropp (1927) has reopened the question of nervous control of the pigmentary effector system of Amphibia. He describes experiments which he regards as incompatible with a conclusion stated elsewhere by Hogben (1924) to the effect that "the regulation of colour response by fluctuating pituitary secretion is adequate to interpret all the accredited phenomena in Amphibia". He appears also to imply that Hogben denies the existence of nervous control of the pigmentary effector system. As Dr. Kropp's communication contains no new data bearing on this problem with one exception to be mentioned later, it seems desirable to reaffirm somewhat more explicitly the position taken up by Hogben and confirmed by the results of this enquiry.

The independent work of P.E. Smith, Hogben, Hogben and Winton, Krogh and his pupils, Houssay and others has now established beyond doubt the fact that the pituitary gland is essential to the rhythm of colour change in adult and larval Amphibia. The fact that the eyes are the receptors for the light reaction (which in the European frog is apparently less important than the temperature and humidity responses) is also established. A large literature dealing with data bearing on the supposed direct innervation of amphibian melanophores reveals complete disagreement between the testimony of different workers with regard to the most vital points. Given the fact that pituitary secretion is necessary to the expansion of Amphibian melanophores, and the direct evidence of Krogh and Houssay, who have shewn that the quantity of circulating hormone is rarely if ever in normal circumstances much below the necessary threshold for expansion, it follows that reducing the blood supply of a region of the body, when the melanophores are expanded, may evoke contraction, while increasing it in the pale animal may call forth expansion of the melanophores of the part affected. Since the stimulation of any nerve in the vertebrate body generally evokes vasoconstriction, while section of nerves is usually followed by dilatation, no experiments can conclusively prove the direct innervation of the melanophores unless the effects of stimulating or of excluding the influence of vasoconstrictor fibres is eliminated.

Kropp's paper like the earlier work of Hooker gives ample illustration of the importance vasomotor effects entering into the interpretation of his results. Thus he finds after transection of the sciatic nerve "for two days after the operation the operated leg reacts normally <u>except for a slight lag in time"</u> (p. 293 italics inserted). On p. 297 he notes that after section of the sciatic the operated leg did not become as pale as its fellow after injection of adrenaline into the circulation. On p. 304 he states that "no change was observed following simple transection of the sciatic plexus.... synchronous colour changes occurred with only a slight lag comparable in every way to that which followed cutting the sciatic nerve". On p. 306 after describing the effect of spinal transection in the tail of the tadpole, he notes that the expansion of the melanophores "is not permanent as in Fundulus" which has an undoubted innervation of the pigmentary effector system".

All the foregoing experiments provide suggestive indications that the phenomena with which Kropp was dealing are capable of quite a different interpretation from that which he advocates. The line of evidence upon which he chiefly relies is derived from stimulation of the sciatic plexus and its associated sympathetic components. It is difficult to follow the reasoning on p. 306, where Kropp states that "these cases shew conclusively that coordination of the melanophores occurs through sympathetic fibres." In these experiments the sciatic nerve alone, the sympathetic roots associated therewith alone, an the entire plexus were stimulated. In all cases some degree of pallor was obtained. When the entire plexus was stimulated the maximal effect occurred. The pigment cells shewed "a very disorganised appearance" and "they appeared to be broken". There is nothing to indicate that this effect was a reversible one. On what grounds does Kropp assume that these are not vasomotor effects? He tells us, "when circulatory disturbance was unavoidable extreme care was exercised with the control leg. Barring the actual stimulation or cutting the control leg was subjected to the same operations and conditions as the experimental leg". From this it would appear that Kropp has entirely failed to appreciate the fundamental issue. It is impossible to cut or stimulate any peripheral trunk without affecting its vasomotor components. The procedure which Kropp adopted could not possibly avoid the intervention of circulatory disturbances. These were uncontrolled however great the care adopted to subject the "control" leg to similar treatment. Curiously Kropp gives no indication of having made personal observations on the dilatation of the capillaries of the web such as have been recorded by Krogh and by Hogben (Pigmentary Effector System IV p. 264 and 265). Had he done so he would have observed, one presumes, direct evidence of the ubiquitous presence of vasomotor components in all peripheral nerves.

There are two minor points in Kropp's thesis that demand brief comment. Referring to observations on the retinal pigment cells he states "it is remarkable that the melanophores of the operated eye do not expand with those of the control eye, if the adaptation to darkness is co-ordinated through the presence of pituitrin in the blood". As a matter of fact, Hogben and Winton have recorded the observation that the retinal pigment cells do not react to pituitary extract in the same way as the melanophores of the skin. This criticism has therefore no evident bearing on the question. The second point arises in connection with the following quotation which suffices to shew that Kropp has not read with great care the series of papers which he cites in his literature list more especially one in whic Hogben deals specifically with every issue raised in his paper (Pigmentary Effector System II). "In the Frog" he writes on p. 312 "stimulation with a weak faradic current of the cloaca, lips, surface of the mouth and nictitating membrane of the eye causes blanching over the entire animal within five minutes. These results are in entire accord with the conclusions of

Hogben ('24) regarding melanophore contraction under similar conditions, and, like Hogben, I conclude that this type of co-ordinated melanophore reaction is definitely brought about by means of adrenalin secreted into the blood stream." There are three comments which it seems appropriate to make in this connection. First that Hogben has never recorded melanophore contraction under similar conditions. Secondly, it certainly does not occur in Rana fuscigula or in Xenopus laevis the only two species of Amphibia that have been accessible for the present investigation, nor, it would seem does it occur in Rana temporaria the species on which the work of Hogben and Winton and of Hogben were based. Third, in none of his publications has Hogben expressed or apparently entertained the belief that adrenal secretion plays any significant part in the regulation of the chromatic function in Amphibia. On the other hand, if Dr. Kropp's observations were correct, it should provide him with a critical experiment which would be adequate to settle the question. In the chameleon stimulation of the cloaca does *reversibly* produce generalised pallor within a few minutes both in the intact animal and in the posterior severed portion of the trunk after evisceration, so that all circulatory effects are excluded. Hogben and Mirvish (1928) have recorded this phenomenon in the chameleon with photographic reproduction of a typical experiment. Photographs of similar phenomena in the frog would go far to disposing of any remaining doubt as to the direct innervation of Amphibian melanophores. Till such are produced it suffices to reaffirm the conclusion stated by Hogben (p. 265 pt. cit): "it is not easy to prove a negative, and to deny that amphibian melanophores are subject to direct innervation would not be justified, but in the light of the new evidence one can approach the question without a strong pre-disposition in favour of the positive attitude."

It is a pity that Dr. Kropp has recorded no single experiment in which the attempt has been made to demonstrate nervous intervention in the absence of the influence of the pituitary, more especially in view of the fact that the technique of hypophysectomy in Amphibia is a comparatively simple procedure.

SUMMARY OF PART IV.

1. The section of the entire nerve supply of the hind limb in Xenopus does not interfere with normal colour response.

2. At no level is Spinal transection accompanied by segmental chromatic changes.

3. Section of the optic nerves at the chiasma evokes the same result as removal of the eyes.

4. The primary response is not dependent on the C.N.S. it is a direct reaction of the melanophores to incident illumination.

5. The diurnal rhythm is not dependent on the peripheral nerve supply of the skin.

6. There is no need to assume that the nervous system plays any part in Amphibian colour change other than in transmitting the stimulus from the eye to the pituitary gland.

PART V.

GENERAL CONCLUSIONS.

- - -0- - -

PART V.

GENERAL CONCLUSIONS.

The results of this investigation may now be summarised as follows. Colour change in Xenopus is mainly determined by photic stimuli. The present investigation has been restricted to an analysis of the behaviour of the dermal melanophores. By the use of a quantitative method it has been shown that the dermal melanophores of Xenopus laevis have a slight "primary" reactivity to incident light. This is independent of the Central Nervous System and of the organs of internal secretion. It is a direct response of the pigmentary effector organ, and is exactly analogous to the reptilian response. The "primary" reaction is of small magnitude compared with the "secondary" reaction for which the retina is the receptor organ. This is determined by the light scattering or light absorbing property of surfaces which occupy the field of vision. The secondary reaction is of the same type as the "background" response of fishes. An analysis of the time factor on the other hand shows that it is not primarily determined by peripheral nervous control. Impulses transmitted by the optic nerve to the brain bring into action two endocrine systems at different levels of intensity of photic stimulation. One of these is the secretion of the posterior lobe of the pituitary gland whose role in amphibian colour response is already firmly established both for Urodeles and Anura. The other has not been identified or recognised in any previous investigation. Evidence has been brought forward from various sources to show that it can be identified with the "anterior lobe" of the pituitary gland. Some uncertainty arises on account of inadequate existing information concerning the homologies of the anterior lobe in Xenopus laevis. It appears that in Xenopus laevis the anterior lobe includes in addition to the pars anterior the pars tuberalis whose separate identity has only been recently recognised by the work of Tilney and Atwell and there is some reason for believing that the pars tuberalis is the essential constituent which determines the "white background" response.

BIBLIOGRAPHY.

Atwell. (1919) "The Development of the Hypophysis in the Anura", Anat. Rec., vol. xv.

Atwell. (1921) "The Morphogenesis of the Hypophysis in the Tailed Amphibia," Anat. Rec., vol xxii.

Hogben. (1923) "A Method for the Removal of the Pituitary in Frogs and Toads," Quart. Journ. Exp. Physiol., vol.xiii.

Hogben. (1924) "The Pigmentary Effector System IV.," Brit.Journn. Exp. Biol. Vol i., No.2

Hogben. (1924) "The Pigmentary Effector System" Edinburgh .

Hogben and Crew. (1923) "Studies on Internal Secretion II.," Brit. Journ. Exp. Biol., vol i., No. I.

Hogben and Gordon. (1930) "Studies on the Pituitary VI" ibid (in the press)

Hogben and Mirvish. (1928) "The pigmentary effector system .V. The nervous control of excitement pallor in reptiles." British Journ. Exp. Biol 5,

Hogben and Winton. (1922) "Studies on the Pituitary. I. The Melanophore Stimulant in Posterior Lobe Extracts," Biochem. Journ. vo. xvi;

Hogben and Winton. (1922) "The Pigmentary Effector System I.," Proc. Roy. Soc. B., vol. xciii.

Hogben and Winton. (1922) "The Pigmentary Effector System II.," Proc. Roy. Soc. B., vol xciv.

Hogben and Winton. (1923) "The Pigmentary Effector System III.," Proc. Roy. Soc. B., vol xcv.

Keeble and Gamble. (1904-6) "The Colour Physiology of the Higher Crustacea," Phil Trans. Roy. Soc. B., vol cscvi-cscviii

Krogh. (1922) The Anatomy and Physiology of Capillaries, Yale University Press.

Kropp (1927) "The control of the melanophores in the Frog." Jour Exp. Zool. 49.

Langley (1921) "The Autonomic Nervous System, Part I.," Cambridge.

Laurens. (1914) "The reactions of normal and eyeless amphibian larvae to light". Science, 39.

Laurens (1914) "The reactions of the melanophores of Amblystoma larvae". Proc. Soc. Exp. Biol. Med. 12.

Laurens.(1914) "The reactions of normal and eyeless amphibian larvae to light". Journal Exp. Zool.16.

Laurens (1915) "The reactions of the melanophores of Amblystoma larvae". Jour. Exp. Zool 18.

Laurens (1916) "The reactions of the melanophores of Amblystoma larvae. The supposed influence of the pineal organ". Jour. Exp Zool 20.

Laurens (1917) "The reactions of the melanophores of Amblystoma tigrinum larvae to light and darkness." Journ. Exp. Zool 23.

Parker (1903) "The Skin of the Eyes as Receptive Organs to the Reactions of Frogs to Light," Amer. Journ. Physiol x.

Parker (1906) "The Influence of Light and Heat on the Movement of the Melanophore Pigment especially in Lizards", Journ. Exp. Zool. Vol,, iii.

Parker and Lanchner. (1922) "The responses of Fundulus to white, black and darkness." Amer. Journ. Physiol 61.

Perkins. (1928) "Colour changes in Crustaceans, especially in Palaemonetes." Journ. Exp. Zool 50.

Pouchet (1872) "Sur les rapides changements de coloration provoques experimentalement chez les crustaces et sur les colorations bleues des poissons." Journ. Anat. Phys. 8.

Pouchet (1876) "Des changements de coloration sous l'influence des nerfs." Journ. Anat. Physiol 12.

Redfield (1916) "The coordination of chromatophores by harmones" Science, 43.

Redfield (1918) "The physiology of the melanophores of the horne toad Phrynosoma." Journ. Exp. Zool. 26.

Slome and Hogben (1928) "The chromatic function in Xenopus laevi South African Journ. Sci. 25.

Slome and Hogben (1928) "The Time Factor in the Chromatic Responses of Xenopus Laevis. Trans. Roy. Soc. South Africa. Vol. XVII. Part 2.

Smith (1920) "The Pigmentary Growth and endocrine disturbances induced in the anuran tadpole by the early ablation of the Pars. buccalis of the hypophysis." Amer. Anat. Mem. No. II.

Swingle. (1921) "The Relation of the pars intermedia of the Hypophysis of Pigmentation Changes in Anuran Larvae". Journ. Exp. Zool Vol. xxxiv.

Wyman. (1924) "Blood and Nerve as Controlling Agents in the Movements of the Melanophores." Journ. Exp. Zool. xxxix.

- - -0- - -