NERVOUS CONTROL OF CHROMATOPHORES IN TELEOST FISHES

III. LOCAL TEMPERATURE RESPONSES IN THE MINNOW (*PHOXINUS PHOXINUS* (L.))

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INTRODUCTION

Changes ot skin colour with temperature are remarkably consistent in reptiles; high temperatures cause paling of the skin while low temperatures induce darkening. The same rule generally applies to the Amphibia (Parker, 1948), although exceptions have frequently been noted. But in teleost fishes the responses are often ill-defined, unpredictable and even contradictory. There is a large literature of observations on these and other groups of animals, (listed in Pye, 1961).

Von Frisch (1911 *b*) suggested that complex factors such as respiratory stress are involved when a fish is immersed in water of different temperatures. He therefore removed minnows (*Phoxinus*) from the water, giving them an oral supply of cool aquarium water to breathe, and restricted temperature changes to a separate jet of water playing on to the flank. Under these conditions a remarkably powerful and consistent reaction was elicited. An area of skin warmed to 35° C. showed maximum melanophore dispersion, appearing black, while cooling to 2° C. produced maximum aggregation to give a very pale skin colour. Both effects were extremely local in extent and could be produced simultaneously by playing jets on to opposite flanks or on to adjacent areas of the same flank. Both were completely reversible at the inter-mediate temperature of 15° C.

Following surgical section of the chromatic motor tracts in the sympathetic chain anterior to vertebra 15 (Fig. 1) these responses disappeared in the anterior, chromati-cally paralysed region of the body and were occasionally replaced by weaker responses in the opposite direction, that is by paling at high temperatures and darkening at low temperatures. The posterior, chromatically intact, region was unaffected by the nerve section and continued to give the strong normal response.

Smith (1928) repeated these experiments on the killifish (*Fundulus heteroclitus*) and was able to confirm them except for one point. Following section of the chromatic motor tract, the 'reversed' responses of *Fundulus* melanophores were always distinct and regular. Smith further showed that in the normal fish high temperatures below 28° C. produced no response, while temperatures above 29° C. produced a maximum response regardless of the application time. On the other hand reversed responses

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following nerve-section were graded with temperature and occurred more rapidly at more extreme temperatures.

Spaeth (1913 a, b) found that the melanophores borne on scales, which had been removed from *Fundulus* to a physiological solution, showed a graded response to temperature in the 'reversed' direction. This was confirmed by Smith who emphasized the analogies between this response and that of surgically paralysed melanophores in the living fish.

Both Smith and von Frisch concluded that the response following section of the chromatic motor nerves is a direct reaction of the melanophores as independent effectors, which is overriden in the intact fish by an opposing influence of the nervous system. Smith inclined to the view that this latter influence involved a neuron reflex or one mediated through the sympathetic nervous system, although von Frisch had earlier rejected this in favour of an unspecified change in melanophore physiology following denervation. Neither worker could demonstrate the existence of a reflex pathway. Further investigations of these effects by the present author have not led to conclusive settlement of this point but have revealed several new and interesting features of the response.

METHODS

In the present experiments water from a constant-pressure head was divided into two tubes, one of which led through either a heating (40° C.) or a cooling (0° C.) bath. The two flows were recombined, in proportions determined by two reciprocally coupled taps, in a single glass nozzle where the temperature of the mixture was measured with a thermocouple. This arrangement produced a jet whose temperature could be raised or lowered (depending which bath was used) from room temperature with no change in pressure. Thermal inertia was very low and the temperature could be adjusted rapidly and accurately to within 0·1° C.

With this apparatus the experiments of von Frisch were repeated and confirmed. Operative procedures followed those described by Healey (1948), which represent an improvement on the simpler techniques of von Frisch by permitting section of the spinal cord at any level and also section of the sympathetic chain within the body cavity without damaging the aorta. By these means the experimental animals could be kept alive and apparently healthy for chronic observation.

At first minnows were obtained from Lake Blaenmelindwr, Cardiganshire, and later from the London area.

Nerve section

Complete section of the spinal cord was performed at a variety of levels both anterior and posterior to the level of the 15th vertebra (Fig. 1). In the former case melanophore responses disappeared, or were slight and reversed, over the whole surface of the fish. This appears to be a condition not investigated by either von Frisch or Smith, probably because survival times were short and the immediate post-operative condition is one of extreme darkening (Healey, 1948). Animals used in this experiment were maintained in an upright position over a white background for several days before thermal testing, in order to allow recovery from initial trauma and to achieve some humoral paling (Healey, 1948). The extent of this paling did not appear to affect the responses or the lack of them.

Fish treated by spinal-section posterior to vertebra 15 continued to show strong responses over the whole body surface, as described by von Frisch. The extent and level of section were determined in both experiments by post-mortem dissection, but no further differences of response could be detected following sections at widely differing levels either anterior or posterior to the 15th vertebra.
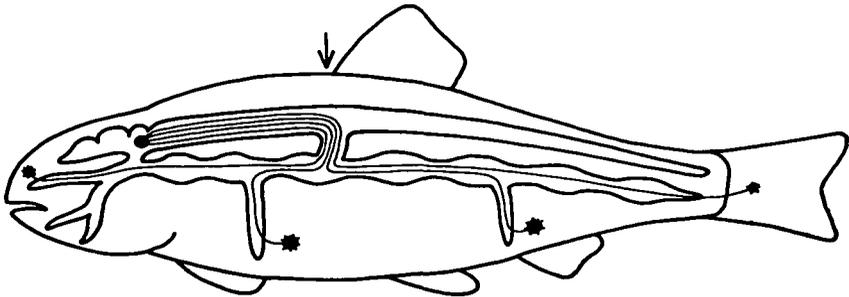

Fig. 1. The pathways of melanophore-aggregating nerve fibres in *Phoxinus* after von Frisch (1911*a*). The arrow indicates the level of the 15th vertebra.

Bilateral section of the sympathetic chain within the body cavity (Fig. 1) was performed on several further fish. Since vertebra 15 lies at the posterior level of the body cavity this resulted in post-operative darkening, and subsequent chromatic paralysis, of the whole body anterior to the point of section. Thermal responses remained normal in the posterior region but disappeared or were slightly reversed in the anterior region. After 7 days on a white background these fish assumed a pale tint over the whole body surface and the sharp boundary between normal and chromatically paralysed regions was difficult to distinguish by eye. It could then be made clearly visible by warming (or rather less clearly by cooling) the skin to provoke normal strong chromatic responses posteriorly and slight reversed ones anteriorly.

Occasionally incomplete section of the sympathetic chain produced a fish which was chromatically paralysed over one anterolateral quadrant, and in one case, by performing section just at the point where the chain entered the haemal canal (just posterior to vertebra 15), only the posterior half of the body was chromatically paralysed. In these cases normal temperature responses remained in all parts of the skin where nervous control of the melanophores was intact, but disappeared or were reversed in paralysed regions.

The lateralis X and the much larger lateral cutaneous X nerves were also cut at an anterior level, just behind the point where they join and submerge to enter the medulla. These two nerves were cut singly and together, both unilaterally and bilaterally, without affecting normal chromatic responses to temperature in any part of the body surface.

Responses of isolated skin

The thermal responses of paralysed melanophores in *Phoxinus* were described by von Frisch (1911*b*) as absent or weak and reversed. Smith (1928) found stronger reversed responses in *Fundulus*, but Smith & Smith (1935) reported a lack of response in *Phoxinus*. All these authors agreed that the effect represents an independent activity of the melanophore cells which is overriden in the intact fish by a strong

opposing influence of the nervous system. If this is so, the weak response in *Phoxinus* may represent either a truly weaker reaction or restraint by the humoral control system which is absent from *Fundulus*.

In the present experiments some variation was found. Minnows from Wales showed distinct but weak responses following nerve-sections while those from London showed no reaction at all. Tests were made between 0° C. and 35° C. at widely varying intervals after nerve-section. An attempt was then made to repeat on *Phoxinus* the isolated scale experiments which Spaeth and Smith had performed on *Fundulus*. As stated previously (Pye, 1964a), single scales removed from *Phoxinus* do not bear undamaged melanophores but small pieces of skin can be isolated in Ringer solution and remain chromatically responsive to a variety of stimuli for some time. Several such pieces from each of 12 London fish were observed during both slow and quick changes between 2 and 35° C. in a variable-temperature microscope stage. The majority showed clear 'reversed' responses (pigment aggregation at high temperatures; dispersion at low temperatures) for up to an hour. The reactions gradually became sluggish and finally ceased although the melanophores still reacted immediately to the addition of adrenalin. No sharp threshold temperatures were observed; the response appeared to occur gradually over the whole temperature range, as described by Smith.

Thus melanophores which showed no response following nerve-section often showed clear responses when isolated from the rest of the body. These observations confirm the accounts of earlier workers and lend some support to the suggestion that the difference between *Fundulus* and *Phoxinus* lies in humoral restraint in the latter.

Responses under anaesthetic

No changes could be distinguished in any of the thermal responses when minnows were maintained under total anaesthesia. This was induced either by urethane or by Sandoz MS222 (Tricaine methansulphonate). Following initial anaesthesia in a dish (0·5 % solution of urethane or 1/12,500 of MS222), further anaesthetic was administered orally (0·25 % of urethane or 1/12,500–1/20,000 of MS222) and further diluted by the addition of water if necessary. By this means complete anaesthesia could be maintained and controlled for several hours with steady respiratory movement and quick recovery when returned to an aquarium. All the foregoing nerve-section experiments were repeated in this way and confirmed. The complete control of the animal allowed more refined experiments to be attempted, including microscopical observation of effector response. All subsequent experiments were performed by this technique.

Threshold temperatures

The continuously variable jet temperature and anaesthetized fish permitted slow and precise measurement of threshold temperatures to be made while the melanophores were being observed microscopically. Although the macroscopic appearance of colour change appeared to be fairly gradual and spread over several degrees, different groups of melanophores were found to respond at different temperatures and to produce their complete response over remarkably small temperature ranges. Maximum dispersion and recovery commonly occurred in small groups of cells for a rise and fall of less than 1° C. As the temperature was gradually raised different types of melanophores always responded in the same order. This was best seen in the tail

where at least four groups could be distinguished, closely related to the groupings of Healey (1951) (Fig. 2). This is illustrated by the following observations from a fairly typical experiment:

(i) Large melanophores of the lateral stripe and a patch at the base of the caudal fin (Healey's *A* and *L.S.* areas), 26–28° C.

(ii) Large melanophores forming the dorsal pattern, 29–31° C.

(iii) Small melanophores of the dorsal 'ground colour' (Healey's *D.S.* area), 32–35° C.

(iv) Small melanophores of the region just dorsal to (i) (Healey's *B* area). No response at 35° C.

Recovery occurred in the reverse order and at the same temperatures during gradual re-cooling. Temperatures above 35° C. were never used as they were liable to produce irreversible effects.
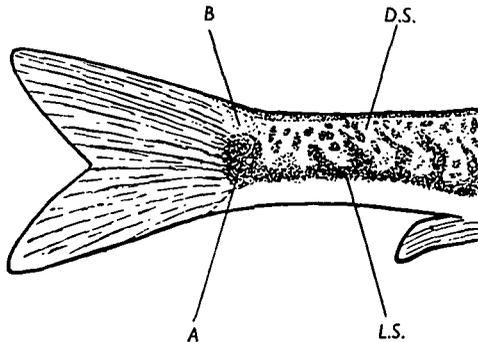

Fig. 2. Diagram of the tail region of *Phoxinus* after Healey, 1951. Region *A*, enlarged tail region of lateral stripe; *B*, region of small melanophores; *D.S.*, small melanophores on the dorsal surface. *L.S.*, large melanophores on the lateral stripe.

The high-temperature threshold of individual melanophores showed no tendency to change during experiments of up to an hour, but the thresholds did change from day to day. Determinations on the same region of the same fish on consecutive days varied inexplicably by as much as 6° C. That these changes were not due to varying levels of pituitary melanophore-aggregating hormone in the blood was established by intraperitoneal injections of pituitary extracts during a series of prolonged threshold estimations. A whole minnow pituitary ground up in Ringer is sufficient to bias all the melanophores of the recipient to complete aggregation for about 1 hr., but this affected neither the responses to high temperatures nor their thresholds. An enormous dose, consisting of one plaice (*Pleuronectes*) pituitary extracted in acid, produced complete aggregation for several hours and for a while abolished responses to temperature. But some time before recovery from the injection could be detected visually in the resting condition of the melanophores at room temperature, the thermal dispersion responses returned at the same temperature thresholds as before. Both these doses are abnormally large, for they produce paling in a normal fish swimming on a black background, whereas in an untreated fish, even after prolonged exposure to a white background, nervous adaptation to a black background is rapid (Healey, 1948).

Low-temperature responses in *Phoxinus* appeared to occur just above 0° C. where control of the jet temperature, using ice for cooling, was not practicable. It is not

possible at present to state whether similar threshold phenomena apply to this response. They were later found to be present in other species of teleosts (Pye, 1964b) and there is no reason to doubt their existence in the minnow.

Correlation with respiratory movements

During a series of acute surgical experiments it was necessary to make a longitudinal incision of 1 cm. into the body cavity of an anaesthetized fish. It was found that if the incision were made at a lateral level instead of through the soft belly, excessive trauma caused violent shaking of the fins for some minutes, followed by death. At the instant when respiratory movement ceased the temperature responses also stopped abruptly. This remarkable coincidence was observed several times and the correlation seemed too close to be fortuitous.

In later experiments, where it was necessary to remove all humoral influences, the ventral aorta was opened or the heart was extirpated from several anaesthetized fish. Again normal responses were obtained, as the temperature was varied rapidly up and down, until the moment of cessation of opercular movements. Then the melanophores 'froze' in whatever condition they happened to have reached.

In order to investigate these effects by a repeatable method, the concentration of the oral supply of anaesthetic to intact fish was increased so that coma deepened to the point where breathing stopped. The fish could then be resuscitated by changing to an oral supply of water alone, or in obstinate cases by administering a little carbon dioxide in a few drops of 'flat' soda water. The same correlation with temperature responses was observed. If the temperature of the jet had been changed in either direction just before the opercular movements stopped, melanophore responses were halted halfway. When movements began again on resuscitation, the melanophore response immediately proceeded to completion, and could again be elicited repeatedly. This non-terminal experiment was repeated many times on many fish and the effect was consistently observed. The same effect was also seen in minnows treated with Rogetine or with atropine (Pye, 1964a) and also in several other species of teleost (Pye, 1964b). The speed of cessation and resumption of response cannot be attributed to anoxia, for this shows as a melanophore aggregating response about 30–60 min. after death—described by von Frisch (1911b) as Anämieaufhellung.

Conclusions

There is no reason to doubt that the thermal responses of paralysed or 'isolated' melanophores are direct reactions of the cells as independent effectors. But the powerfull overriding influence in the intact fish is difficult to explain. A thermal reflex as proposed by Smith would imply that the response could be abolished by cutting the sensory pathway alone. Nerve-section experiments show that the response disappears only when the chromatic motor tracts are interrupted; this occurs for all the melanophores paralysed by a section at any level of the tracts and for no others. No sensory pathway exists within the cranial nerves, at least for the trunk and tail. Neuron reflexes are also ruled out by the results of sections within the central nervous system. Thus the only possible pathway for afferent sensory tracts would be the exact reverse of the motor tracts so that the two would always be cut together. The extremely

localized nature of the response would then demand a system of one-to-one connexions somewhere in the brain. There is little justification for proposing such a complex arrangement to explain a response which has no adaptive significance, since it cannot be induced in a submerged fish or (for the high-temperature response) at normal sublethal temperatures. Indeed it does not seem permissible to postulate *any* new anatomical units whose only function could be to mediate this response.

An alternative hypothesis, consistent with these arguments, is to suppose that extreme temperatures interfere with the normal motor excitation of the melanophores in the skin. This proposal is only tenable if the melanophores, like most and possibly all other autonomic effectors, are controlled by a double, opposed, tonic motor system. Thus extreme high temperatures might preferentially block the melanophore-aggregating fibres to give complete pigment dispersion, while very low temperatures could similarly block the dispersing fibres to give complete aggregation. The threshold temperatures at any time might then be expected to depend on the balance of tonus currently existing within the two systems. The precise correlation between melano-phore responsiveness and respiratory rhythm may find a possible explanation if the chromatic motor tonus and respiratory rhythm share a common pace-maker. Unfortu-nately these suggestions cannot be tested directly at present, since no one has yet succeeded in recording chromatic motor action potentials.

Two other observations are of interest in this connexion, although no great reliance can be placed on either of them at this stage. First, it is possible to obtain normal high-temperature responses over the whole body surface of *Phoxinus* by heating the sympathetic chain within the body cavity. This was exposed as for the sympathectomy operation and a fine jet of warm Ringer was directed on to it. The responses were immediate and occurred at normal threshold levels. But despite the application of cooling jets to the observed area of skin and monitoring of the peripheral temperature by subcutaneous thermocouples, the possibility of thermal conduction through the tissues or by the blood-stream (heated in the aorta) cannot be entirely discounted.

Secondly, Smith (1928) showed that bilateral section of the optic nerves abolished normal temperature responses in *Fundulus*. If motor tonus were to subside in the absence of sensory input, the melanophores would be free to act independently, although their motor connexions would be anatomically intact. An attempt was made here to repeat this experiment on London minnows. The responses were clearly disrupted in several fish but no clear-cut reversal could be demonstrated. This may have been partly because these fish did not show a clear reversal after section of the motor tracts (see above) and in any case it was not possible to make observations more than 30 hr. after blinding operation, when recovery may not have been complete. Also, as von Frisch (1911 c) showed the roof of the diencephalon in *Phoxinus* acts as an accessory photoreceptor in mediating chromatic responses to illumination (this has not been shown for *Fundulus*), and sensory input may not therefore have been eliminated. A more revealing experiment might be to test for temperature responses in complete darkness in a stock of minnows which showed clear reversal after section of the motor nerves.

The only claim for the hypothesis of tonic imbalance at extreme temperatures is that, together with several unsubstantiated corollaries, it is the only one which can explain all features of the observed responses. Drug effects must be excepted from this, but as

shown elsewhere (Pye, 1964a) they are self-contradictory and cannot be interpreted by analogy with their effects in mammals. The hypothesis depends entirely on the presence of a double opposed innervation whose existence has not been proven although it is indicated by a great deal of circumstantial evidence. Direct tests of the theory are difficult to envisage unless chromatic action potentials can be detected and their influence on the melanophores indisputably demonstrated. However, the suggestion that chromatic motor tonus is related both to sensory input and to respiratory rhythm would bear further examination. Temperature thresholds may bear some relation to background colour in the active fish but no way of testing this could be devised. Further information of this kind would be valuable in attempting to understand the powerful temperature response and the control of teleost chromatophores in general.

SUMMARY

1. Observations of earlier workers on the chromatic responses to local heating or cooling of the skin of *Phoxinus* have been confirmed. They have also been found to hold true for anaesthetized fish, in which finer control and observation are possible.

2. The results of a series of nerve-section experiments are held to exclude any possibility that the responses of the intact fish are mediated by a nervous reflex from thermoreceptors in the skin.

3. The responses of melanophores following section of the chromatic motor tracts, or when isolated from the body, are considered to be independent cellular responses.

4. Normal responses in the intact fish show clear temperature thresholds and are completely dependent upon continuation of the respiratory rhythm.

5. Possible physiological mechanisms for mediating these non-adaptive responses are discussed and a new hypothesis is put forward.

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