STUDIES ON ANIMAL CAROTENOIDS

II. CAROTENOIDS IN THE REPRODUCTIVE CYCLE OF THE BROWN TROUT

By D. M. STEVEN, *Department of Zoology, University of Edinburgh*

(*Received* 14 *March* 1949)

(With Three Text-figures)

In the first paper of this series (Steven, 1948) it was shown that the tissues of the trout (*Salmo trutta* Linn.) normally contain β-carotene, lutein and astacene. Lutein and astacene occur as esters in the red and yellow chromatophores of the skin, and may also be present in the muscles as free hydroxy-carotenoids. Carotene is present in the liver, which also contains xanthophylls but no astacene. The ripening oocytes, however, contain all three types of carotenoid. Red and yellow chromatophores first appear during the larval period of development before the young fish has commenced to feed independently, and appear to obtain their pigment from the carotenoids laid down in the yolk. This paper records the chemical changes and distribution of these substances from the maturation of the oocytes to the stage of metamorphosis of the larva.

Adult trout were obtained alive from a reservoir near Edinburgh. Some ova were obtained from ripe fish in the laboratory, and fertilized with sperm from males of the same batch. Larger numbers were supplied by the Howietoun and Northern Fisheries, Stirling, as 'eyed' ova, and were used for most of the experiments on the larval period of development. Ova and larvae were reared in batches of about 100 each in glass aquarium jars, which were immersed nearly to the rim in a larger tank through which passed a rapid flow of water taken direct from the mains supply. This arrangement gave satisfactory temperature stabilization for the period of the experiments, from January to the end of March. Temperature variation was less than 1° C. for periods of more than a week, and remained within the range 4–6° C. for the whole period.

Carotenoids were estimated with a photoelectric colorimeter by the method described in detail by Steven (1948).

THE MOBILIZATION OF CAROTENOIDS IN SPAWNING FEMALES

The ovaries of mature female brown trout vary greatly in size at different seasons of the year. In Britain the species spawns in late autumn, and during the winter months following the ovaries remain minute. In April and May they contribute about 1 % to the total body weight, and the oocytes which will ripen in the following autumn are each about 1 mm. in diameter and bright orange in colour. By October the combined ovaries may constitute up to 10 % of the body weight; the individual ova at the time of spawning are 5–6 mm. in diameter and weigh about 0·1 g. The changes found in their carotenoid content during the ripening period are summarized

in Table 1. The concentration of ovarian pigments, expressed as μg./g. of fresh tissue, was found to remain more or less constant during the period of maturation. The total amount, however, increases greatly as the oocytes enlarge, and can be regarded as an increase in the carotenoid content of each oocyte in proportion to its increase in size. The ratios of β-carotene, lutein and astacene also remained approximately constant during the maturation period.

Table 1

A. *The distribution of carotenoids in unripe and spawning female trout*

	Fish no.	Wt. of fish (g.)	Wt. of ovaries (g.)	Ovaries wt./Body wt. %	Total carotenoids (μg./g.), estimated as lutein		
					Ova	Muscles	Skin and fins
Unripe females (May)	1	204	1·56	0·76	152	32	135
	2	253	1·90	0·68	114	17	166
	3	188	1·31	0·70	125	30	140
Spawning females	4	227	44	17·6	105	Trace?	172
	5	316	76	24	120	0	129
	6	208	48	23	102	0	155

B. *The detailed composition of ovary carotenoids*

		Carotenoids (μg./g. fresh tissue)			
		β-Carotene	Lutein	Astacene	Total carotenoids (estimated as lutein)
Small oocytes (May)	Fish no. 1	4	22	140	152
	Pooled sample from 8 fish	2·5	20	117	108
Near-ripe oocytes (Sept.)	Pooled sample from 3 fish	2·5	13	160	112
Ripe ova (Nov.)	Fish no. 5	3·5	15	126	129
	Fish no. 6	2·0	24	137	155
	Pooled sample of 5 fish	3·5	18	124	102

It was shown previously (Steven, 1948) that the muscles of trout may contain up to about 30 μg./g. of astacene and 3·5 μg./g. of lutein. Those of ripe female fish examined shortly before or after spawning, however, yielded no carotenoids whatever. It seems clear that the free astacene and lutein of the muscles are transferred to the oocytes during the last few weeks before spawning, at the time when the latter are growing most rapidly. The astacene and lutein esters in the chromatophores of the skin, on the other hand, are not depleted, since the skins of ripe female fish yielded the same amounts of these pigments as did the skins of males and non-spawning females. The carotenoid content of the skin appears to be maintained at a constant level throughout the year in both sexes. If it is assumed that the muscles constitute about 60 % of the total body weight, those of a well-pigmented fish may contribute rather more than half the total lutein and astacene found in the ripe ova. Since the chromatophores are unaffected, and the fish does not possess any other considerable reserve of these pigments, the rest is presumably obtained direct from the food eaten during the months when the oocytes are increasing in size.

THE DISTRIBUTION OF CAROTENOIDS DURING THE LARVAL PERIOD

The distribution of carotenoids as between the yolk and the body of the embryo was measured at various stages from the time of hatching until metamorphosis. The procedure adopted with slight modifications for separating the yolks from the embryos was that described by Gray (1926). Batches of about fifty larvae were narcotized with 2 % urethane, their lengths measured individually to the nearest 0·5 mm., adherent water removed by drying lightly between sheets of filter-paper, and the group weighed as a whole. The yolk sacs were removed from the embryos, which were then washed with 0·7 % NaCl, again dried lightly with filter-paper and reweighed. The washing detached any droplets of pigmented yolk fat adhering to the embryos. These droplets were collected from the surface of the saline solution and returned to the yolk fraction. The embryos and yolks were then ground separately with anhydrous sodium sulphate, extracted exhaustively with petroleum ether containing about 2 % of methyl alcohol, and the carotenoids estimated by the usual method. In most experiments only the total carotenoid content of each fraction was measured, the result being expressed in terms of a standard solution of lutein, which was prepared from a crystalline sample supplied by Prof. L. Zechmeister. In some cases, however, the astacene and lutein were separated and estimated individually.

In order to detect any possible loss of pigment during the separation of embryos from their yolk sacs, and to obtain information on the carotenoid content of the whole larva at all stages of development, a second batch of about fifty larvae was taken from the same tank at the time of each experiment, weighed, dried and the total pigments extracted and estimated.

The carotene content of the yolk is much less than the lutein and astacene, and larger batches of up to 200 larvae were required to obtain reasonably accurate estimates of it.

The information obtained from this series of experiments is expressed in Table 2 and Fig. 1, which illustrate the following points:

(1) Lutein and astacene are transferred progressively from the yolk to the embryo during the larval period without apparent loss.

(2) The transfer of carotenoids is relatively delayed compared with the general development of the embryo or the rate of utilization of the yolk. At the time of hatching the embryo constitutes about 19 % of the total weight of the larva, but contains only about 7 % of the carotenoids. During the larval period, however, the rate of transfer of pigment increases steadily relative to the weight increase of the embryo, and at metamorphosis all the pigment is in the body of the embryo.

(3) Lutein and astacene are apparently transferred in constant ratio throughout the larval period.

(4) Although carotene constitutes about 2 % of the initial yolk carotenoids, none was detected in the embryo at any stage.

The rate of transfer of lutein and astacene corresponds closely with the visible development of xanthophores and erythrophores in the skin of the embryo. These types of chromatophore are not usually apparent at the time of hatching, although lipoid containing cells of typical embryonic chromatophore pattern can be demonstrated in the fins by staining with Sudan IV or Sudan Black by the method described by Baker (1945). The first pale yellow xanthophores can usually be seen a few days after hatching on the tail, dorsal and future adipose fins. They increase rapidly in

Table 2

A. *The transference of carotenoids from yolk to embryo during development at* 4–6° C.

Stage	% carotenoids in embryo	$\frac{\text{Embryo wt.}}{\text{Larva wt.}}$ %	Total larval carotenoids (μg./larva)	Length of posterior end of embryo (mm.) (dorsal fin to tail)	Total length of embryo (mm.)
1. Freshly fertilized ova	0	0	9·8	0	0
2. Hatching	6·9	19	10·2	7·5	15·0
3.	9	33	8·0	8·5	17·5
4.	25	44	10·4	9·0	19·5
5.	48	68	7·3	11·5	23·0
6.	64	81	10·8	13·0	24·0
7.	80	89	12·0	14·0	24·5
8. Metamorphosis	100	100	10·0	15·0	25·0

B. *The detailed composition of embryo and yolk carotenoids* (μg./*larva*)

Stage	Yolk			Embryo		
	β-Carotene	Lutein	Astacene	β-Carotene	Lutein	Astacene
2	0·2	1·5	8·1	0	0·2	1·0
5	0·05?	1·0	4·6	0	1·3	4·4
8	0	0	0	0	1·9	11·2

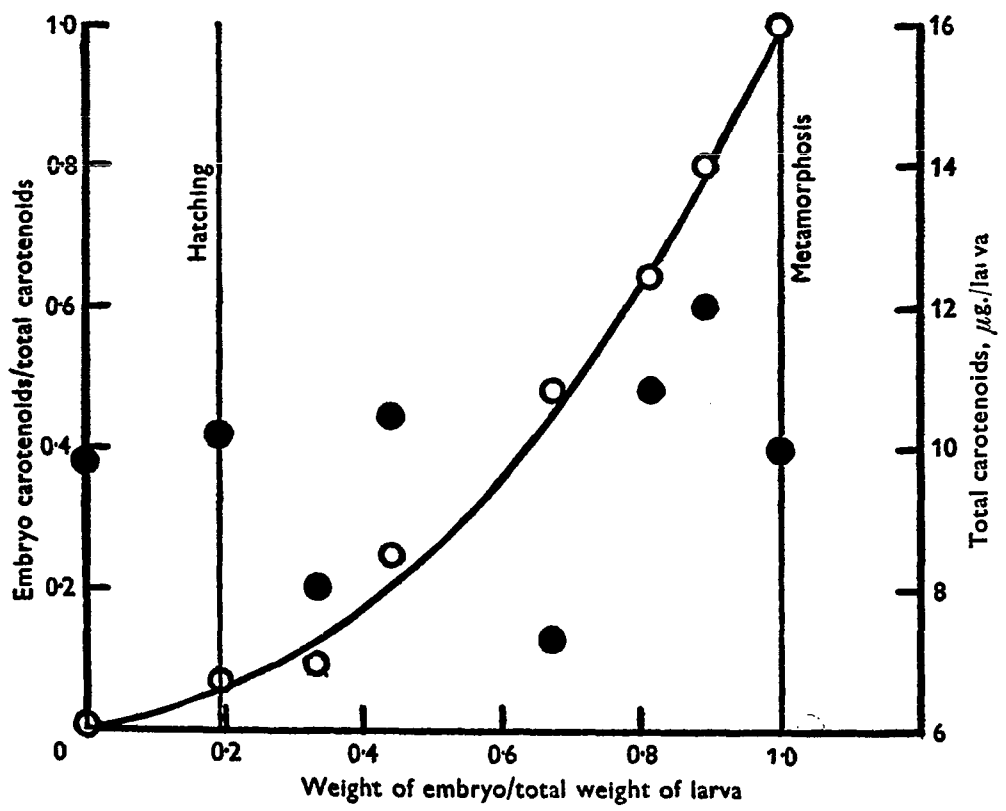

Fig. 1. To show the relation between the rate of transference of carotenoids from the yolk to the embryo and the general growth rate of the embryo (open circles). (The filled circles represent the values obtained for total larval carotenoids (yolks + embryos) at different stages from hatching to metamorphosis.)

number and intensity of pigmentation; and during the second week of larval life it is possible to distinguish the erythrophores, which appear first orange and then red. By metamorphosis both types are numerous, and distributed in accordance with the characteristic colour pattern of adult trout, erythrophores being confined principally to the red areas of the adipose and tail fins, while xanthophores are generally distributed over the surface of the body. Red spots along the lateral line are not, however, apparent during larval life.

The lutein and astacene of the yolk are in the form of free hydroxy-carotenoids. In the embryo, however, they were found to be esterified. In order to determine the distribution of these esters in the embryonic body, a number of larvae close to

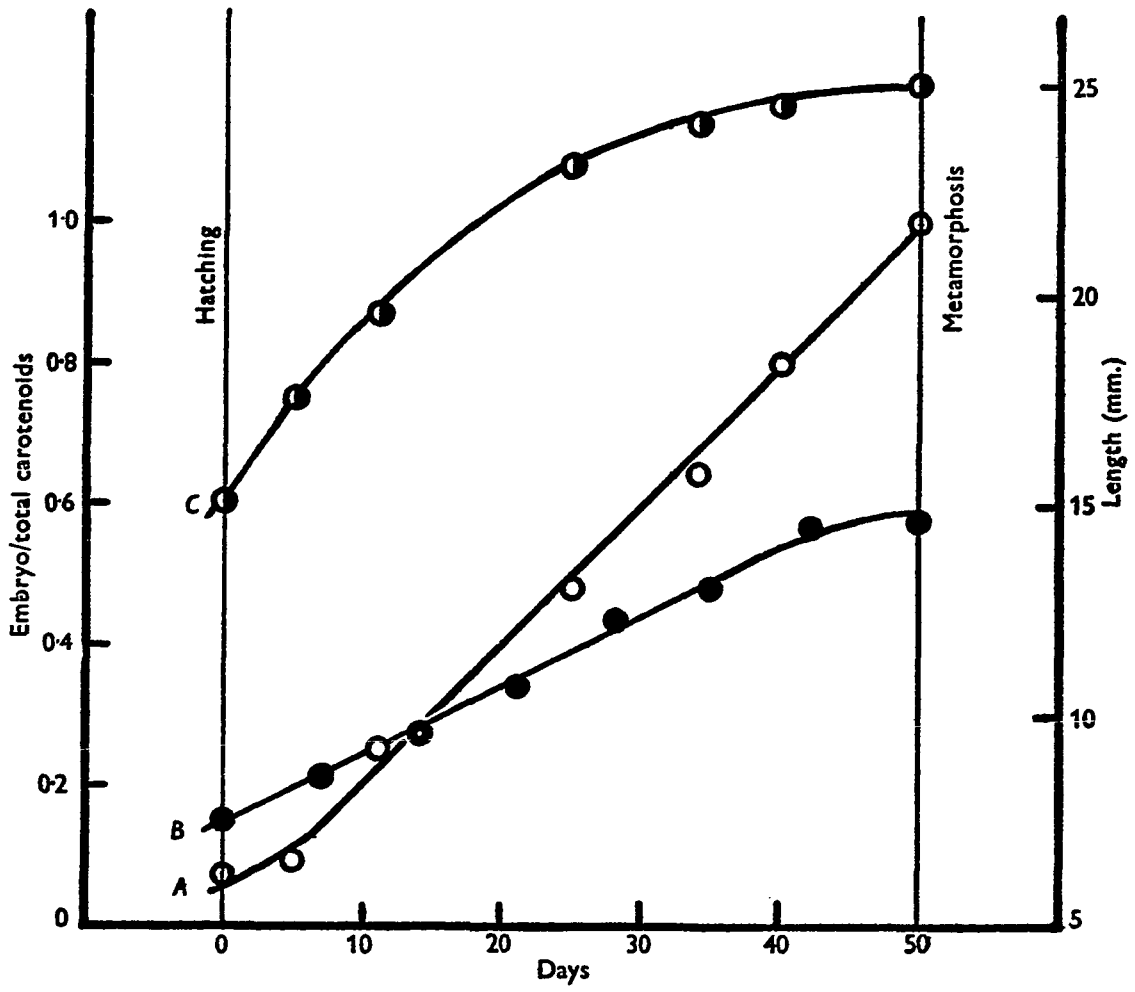

Fig. 2. To compare the rate of transference of carotenoids from yolk to embryo (A) with the growth in length of the posterior end of the embryo (B) and the growth in length of the whole embryo (C).

metamorphosis were skinned and the carotenoids of the whole skin, together with the fins, estimated separately from those of the rest of the carcass. The skin and fins were found to contain 64 % of the total carotenoids of the embryo. This represents the fraction of pigment laid down in the xanthophores and erythrophores by the end of the larval period. It was not possible to analyse further the distribution of the remaining fraction of pigment among the tissues of the rest of the carcass, but this, too, was in the esterified form. Further experiments will be required to establish the actual site where esterification takes place, which may prove to be in the yolk before the pigments are transferred, or possibly in the liver or in a variety of tissues of the embryo.

Although the transfer of lutein and astacene from the yolk is at first delayed relative to the general development of the embryo as measured by increase in weight, the process bears a general resemblance to the growth in length of the posterior end of the body, measured from the leading edge of the dorsal fin to the tip of the tail. Both are nearly linear with respect to time during the larval period, whereas the overall growth rate of the embryo, measured either as growth in length or weight, shows progressive deceleration (Fig. 2). As is well known, development of the head region is relatively precocious in Vertebrates. Most of the carotenoid containing chromatophores, however, develop in the skin of the posterior end of the body, particularly on the tail and adipose fins, and the similarity between the rate of transfer of pigment from the yolk and the growth of the posterior end may simply express the fact that the speed of transfer of pigments is related to the development of the cells which are to receive them.

THE EFFECT OF REMOVAL OF CAROTENOIDS FROM THE YOLK

Newly hatched larvae were sucked into lengths of glass tubing wide enough to hold them comfortably but prevent them from turning, and kept in water with their head ends directed downward. Being more buoyant than the aqueous part of the yolk, the oil globules containing the carotenoid rise in the course of a few minutes to the posterior end of the yolk sac, which projects from the body of the embryo. In many larvae almost

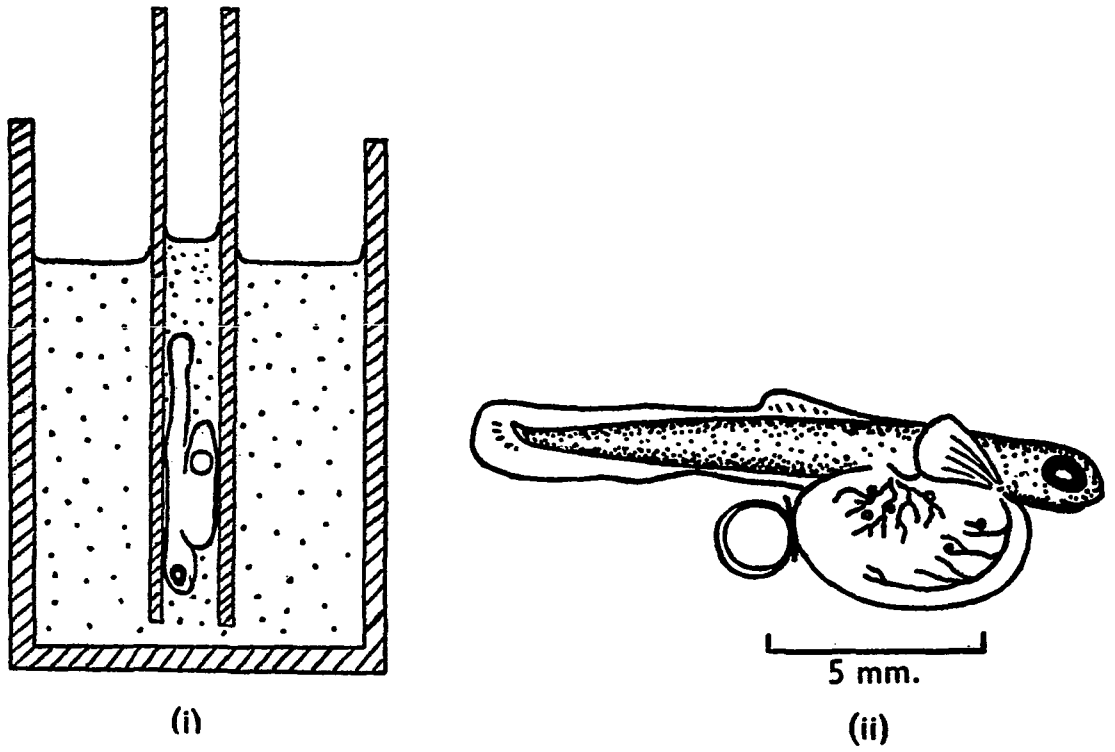

Fig. 3. Operation for removal of fat droplets containing carotenoids from the yolk sacs of trout larvae. (i) Alevin held vertically in tube. Fat globule rising to posterior end of yolk sac. (ii) Alevin with main fat globule of yolk ligatured. Small droplets of fat adhering to vitelline vessels.

all the pigment is contained in a single large globule. The larvae were narcotized with 2 % urethane while still in the head-down position. They were then removed from the tubes, and the oil globule ligatured with fine silk thread and cut away from the rest of the yolk (Fig. 3). The ligature was removed after a few days. By this operation almost the whole of the lipoid fraction and carotenoids were removed with a minimum amount of other yolk substances. In a second series of larvae a similar fraction of the yolk was ligatured

and removed in the same way, omitting the preliminary period in the vertical position, so that the pigment and oil globules were undisturbed and remained within the reduced yolk sac.

It proved possible by this operation to remove about 90 % of the lipoid and carotenoid of the yolk. Small fat droplets, some of them containing pigment, did not rise to the posterior end of the yolk sac, but remained attached to the walls of the vitelline blood vessels, from which they could not be detached by more severe procedures, such as centrifuging at low speeds, without killing the larvae. The amount of lipoid and carotenoid removed from the second series of larvae was less than 5 % of the total yolk content.

Post-operative mortality of both experimental and control series of larvae was about the same, and due mainly to rupture of the yolk sac or invasion by water at the site of the wound. The survivors of both series developed at the same apparent rate, though as noticed by Gray (1928) they were slightly smaller than unoperated larvae kept at the same temperature. Xanthophores and erythrophores developed in the control series as in normal larvae. Those from which the lipoid and carotenoid had been removed, however, developed very few chromatophores, and those which did appear contained little pigment. Pale yellow chromatophores were generally distributed over the fins and body surface before metamorphosis, but could not be differentiated as xanthophores and erythrophores. The experiment was not continued beyond this stage. The type of diets on which alevins are usually reared, such as chopped liver, yolk of egg or live Entomostraca, all contain considerable amounts of carotenoids, and an attempt to wean them on a low carotenoid diet of egg albumen, cod muscle and oatmeal proved unsuccessful.*

DISCUSSION

Hartmann, Medem, Kuhn & Bielig (1947) have recently analysed the chemical constituents of the ova of the rainbow trout, *S. irideus* Gibb. They found them to contain the same three carotenoids as the brown trout used in my investigations, but in considerably smaller amounts and in different relative proportions. 13,500 ova weighing 600 g. yielded only 350 μg. of pigment, estimated as lutein; and after separation the amounts of lutein, astacene and β-carotene were estimated as 43·4, 7·2 and 12·6 μg./100 g. of fresh ova respectively. Although the method of separation used by them involved several stages of purification by chromatographic adsorption and subsequent elution of the pigment fractions, a process which usually involves considerable loss of carotenoids by oxidation, the discrepancy between their values and those found by myself (Steven, 1948) for the brown trout appear to be too great to be accounted for solely by differences of procedure. MacWalter & Drummond (1933) found that the ova of rainbow trout contain less carotenoid and more vitamin A than those of brown trout, and it seems reasonable to conclude that the two species do in fact differ in the amounts and proportions of carotenoids laid

* Since this paper was sent to press a number of larvae, from which the carotenoids had been removed, have been reared successfully on live Enchytraeus. At the time of writing (June 1949) they completely lack xanthophores and erythrophores and are exceptionally pale in colour. In all other respects (growth rate, behaviour, etc.) they appear to be normal.

down in the ova, though not in their nature. The difference is greatest in the case of astacene, which is the most abundant carotenoid in the ova of *S. trutta*, but the least in *S. irideus*.

In this connexion it is important not to overlook the possibility that qualitative and quantitative differences found in different investigations may be due to differences in the diet of spawning female fish. Ova from different females vary considerably in carotenoid content, and it might be possible, as in the case of the hen, to produce ova with little or no pigment by supplying the fish a carotenoid-free diet for several months before spawning. There appears to be no experimental evidence on this point in the case of fish, although other experiments of mine (Steven, 1948) indicate that trout cannot synthesize any carotenoid and must therefore depend upon their food for their whole supply. It is worth noting, however, that the conditions under which trout are commonly reared in hatcheries, where they often receive a diet of a single foodstuff, such as horseflesh, may provide just the type of conditions required for producing ova with abnormally low carotenoid contents.

The most important feature of the investigations of Hartmann and his colleagues, however, is their discovery that in the rainbow trout astacene acts as a fertilization hormone (*Befruchtungsstoffe*) in a manner similiar to the action of echinochrome A in the sea urchin, *Arbacia pustulosa*. Concentrations of colloidal solutions of $1:10^5$ were found to be sufficient to activate spermatozoa and to sustain positive chemotaxis. Lutein and β-carotene had no such effect. No experiments have been described to test whether any of the carotenoids has a similar role in *Salmo trutta*. My findings emphasize the importance of the pigments in another function during the reproductive cycle. The amounts of lutein and astacene in the ova of brown trout are much greater than one would expect to be necessary if they acted solely as fertilization hormones. Moreover, all the available reserves of the parent female, principally the free hydroxy-carotenoids of the muscles, are mobilized and transferred to the ripening oocytes. The lutein and astacene esters of the chromatophores in the skin, however, are not depleted, and the external colour pattern of the fish is therefore unaffected. The pigments of the ova are not destroyed during development, but are transferred without loss into the body of the embryo, where a large proportion can be recovered as esters from the skin and fins towards the end of the larval period. The most striking characteristic of the whole process is the emphasis on maintaining the colour pattern of the skin, and the importance of the carotenoids in this respect both to the spawning female and the developing larva.

The experiments in which up to 90 % of the yolk carotenoids were removed shortly after hatching support the view that these pigments are not essential for any vital physiological process during the larval period, or at least that the amount provided greatly exceeds any such requirement. The large amounts laid down in the ova provide the embryo with sufficient lutein and astacene to develop the colour pattern characteristic of the species during the larval period, before the young fish has commenced to seek its own food, a provision which may confer an important selective advantage. Removal of the carotenoids merely inhibits development of the colour pattern, but does not otherwise appear to affect the larva.

The distribution of β-carotene during the reproductive cycle differs from that of lutein and astacene. As shown previously (Steven, 1948), it is found in considerable amounts only in the livers of adult trout, and is not one of the pigments of the chromatophores. It is present in ripening oocytes and in freshly laid ova in smaller concentrations than lutein and astacene, but was not found in the bodies of embryos at any stage of development. It seems likely that it functions solely as a precursor of vitamin A, and is converted and utilized in the latter form by the embryo. This was the view of MacWalter & Drummond (1933), who claimed that the carotenoid concentration falls by about half during larval development, while the vitamin A increases. They were unaware at that time, however, that the ova of trout contain more than one type of carotenoid, which they considered to be similar to but not identical with β-carotene. Their estimates of the carotenoid content of ova were based upon measurements of the absorption of petroleum extracts at 480 mμ. Since, however, the absorption maxima of all three carotenoid fractions lie close to this wave-length, one would not expect a decrease of the β-carotene content to be easily detectable in the presence of much larger amounts of lutein and astacene, neither of which have ever been shown to act as precursor substances of vitamin A. This point clearly requires reinvestigation with the more sensitive methods now available.

SUMMARY

1. The free lutein and astacene in the muscles may contribute about half the carotenoid found in the ova of spawning female trout. The esterified forms of these pigments in the chromatophores of the skin are not, however, depleted.

2. Lutein and astacene are transferred without apparent loss from the yolk of the egg to the body of the embryo, principally during the later part of the larval period. The rate of pigment transfer appears to be related to the growth rate of the posterior end of the embryo, and corresponds closely with the visible development of xanthophores and erythrophores.

3. Removal by operation of about 90 % of the yolk carotenoids results in larvae which are slightly smaller than normal and with very few pale chromatophores, but with no other apparent defect.

4. Lutein and astacene in the yolk are the free hydroxy-carotenoids, but are esterified in the embryo. About two-thirds of the pigment of the embryo was found to be in the skin and fins at metamorphosis.

5. β-Carotene, which is present in freshly spawned ova, was not detected in the embryo at any stage of development.

I am grateful to Prof. James Ritchie for his care in reading this manuscript and for many helpful suggestions.

REFERENCES

BAKER, J. R. (1945). *Quart. J. Micr. Sci.* **85**, 1.
GRAY, J. (1926). *J. Exp. Biol.* **4**, 215.
GRAY, J. (1928). *J. Exp. Biol.* **6**, 110.
HARTMANN, M., MEDEM, F. G., KUHN, R. & BIELIG, H.-J. (1947). *Z. Naturforsch.* **2**, 330.
MACWALTER, R. J. & DRUMMOND, J. C. (1933). *Biochem. J.* **27**, 1415.
STEVEN, D. M. (1948). *J. Exp. Biol.* **25**, 369.