LXXXIII. THE METABOLISM OF DEVELOPING SALMON EGGS.

II. CHEMICAL CHANGES DURING DEVELOPMENT.

BY FREDERICK RONALD HAYES.

*From the Department of Oceanography,
The University of Liverpool.*

(Received April 8th, 1930.)

INTRODUCTION.

THERE is much evidence in the literature of embryonic metabolism which goes to show that during the course of its development the embryo makes use of foodstuffs as energy sources in the order carbohydrates, protein, fat. With this idea in mind Needham and Murray [see Needham, 1927] have recently ascertained the wet and dry weights, and the constitution, concentration, combustion, and absorption of yolk materials, of the developing embryo. The peak of carbohydrate combustion and absorption was found to be on or before the 4th day. With protein there was no correlation whatever between absorption and combustion; the peak of combustion at the 9th day came just at the trough of absorption, and by the time the peak of absorption was reached on the 16th day combustion had ceased to be a factor. A similar statement can be made for fat, the absorption of which rose to a peak by the 9th day, dropped to a trough by the 15th day and then rose until the end of development. Thus fat and protein complement each other during the later stages as sources of material for the formation of living tissue; their curves are reciprocals. It is probable that the cells of the blastodermal blood vessels which absorb the yolk and white into the embryo, have periods of varying permeability to water-soluble and fat-soluble substances. Of the end-products of protein combustion ammonia reaches a peak first, then urea, and finally uric acid. At the time of transference from protein to fat as a chief source of energy the cost of constructing 100 mg. of living tissue is greatest. The results of these workers are mentioned because they suggest the explanation to so many of the observations here recorded. It cannot be stated at present exactly to what extent the fish resembles the bird in the chemistry of its development. The scarcity of carbohydrate in fish eggs accounts for one difference.

Chemical analyses of Atlantic salmon (*Salmo salar*) eggs are not available but Greene [1921, 1, 2] removed mature ova from the body cavity of the king salmon (*Oncorhynchus tshawytscha*) and determined the following percentage composition: water 57.93, protein (alcohol precipitate) 26.79, neutral fat 10.42, phospholipins 2.0, organic extractives 1.42, glucose 0.09, inorganic ash

0.65. For trout eggs (*Trutta fario*) Fauré-Fremiet and Garrault [1922, 1, 2] have found the following percentage composition: water 58.5, protein (total nitrogen $\times 6.25$) 29.81, neutral fats 4.20, phospholipins 3.46, cholesterol 0.58, carbohydrates 0.34, ash 1.25. They were unable to find glycogen in the egg, but determined that the reducing sugar isolated was glucose.

Some recent work, which will be further discussed, has led to the conception that an aquatic egg is essentially different from a terrestrial one in two ways. First the products of its protein combustion are excreted through the shell, and second the supply of water for the developing embryo is unlimited. The latter idea has been discussed at length in the foregoing paper [Hayes, 1930]. Protein metabolism is held to be limited in terrestrial eggs by some factor connected with the increasing concentration of nitrogenous end-products. Thus protein is a minor source of energy in terrestrial embryonic development, and a major source in aquatic embryos. Another explanation is possible, using the ever attractive theory of recapitulation. Up to about the 7th day the chick embryo develops like a fish. It combusts and absorbs chiefly protein. Then the vitelline membrane (corresponding to the fish egg shell) finally disappears and its place is taken by the serosa. The embryo gradually loses its dependence on protein, ceases to be a fish, changes into a bird. The fish at this stage hatches and continues to rely on protein as the main source of food supply during larval development.

MATERIAL AND METHODS.

Eggs of the Atlantic salmon (*Salmo salar*) were procured in the eyed stage, reared, weighed, and dried as described in the first paper of this series. The dried material was then analysed for protein and fat.

Fat was extracted in a Soxhlet apparatus, the eggs having been previously pulverised; this latter procedure was unnecessary with the larvae. Extraction was allowed to proceed in the usual way for 3 hours with the siphon operating every 5 minutes, re-extraction of some of the samples having shown that this period was of sufficient duration to remove all the fatty constituents. After extraction the solvent was distilled off and the residue and extract dried to a constant weight. The extract was always slightly heavier than the residue loss. The former has provided the data recorded, except in one or two cases of obvious experimental error in which the residue loss, appropriately corrected, has been used.

The fat values given are the result of carbon tetrachloride extraction. The relative merits of ether and carbon tetrachloride as fat extractives have been discussed by several workers. Johnstone [1918] found 1.0 to 1.62 % of nitrogenous substances in carbon tetrachloride extracts of herring tissue. Bull [1928] preferred to use ether for similar work. It is always desirable, and particularly so in studies of developmental mechanics, to differentiate between lower nitrogenous substances such as protein building stones, aminolipins, etc., and the true proteins. Greene [1921, 1] calls the former substances "organic

extractives" to distinguish them from the neutral fats and phospholipins. In more general analyses they should be placed with the fats rather than with the proteins. Carbon tetrachloride is to be preferred as an extractive if it will remove these lower substances and thus permit a more accurate estimate of the proteins in the residue. If its extract contains slightly more nitrogen than ether extract of similar tissue it is fair to assume that this excess represents the simplest organic nitrogenous substances present. Differences in extractive power may be entirely due to temperature. In ordinary procedure ether extracts at slightly below its boiling point of 35°, while carbon tetrachloride extracts at slightly below 77°. Greene considered it necessary to modify his apparatus in order to ensure the highest possible temperature for extraction.

The first point was to determine whether there was a quantitative difference between the two types of extracts, and this was done using eggs of the common lump sucker (*Cyclopterus lumpus*). Three samples were extracted with ether, then dried, weighed and extracted with carbon tetrachloride. The averaged results showed that 98.5 % of the total extract was removed by the ether and the remaining 1.5 % by the carbon tetrachloride. Estimations showed that the percentage dry weight of nitrogen in ether extracts is 0.287, and in carbon tetrachloride extracts it is 0.354. Combining these results we find that 19 % of the total nitrogen is contained in the carbon tetrachloride fraction. In other words the 1.5 % extra fat which the carbon tetrachloride removes contains 6.73 % nitrogen, a figure which is not far from the proportion in the egg as a whole. This extra nitrogen, however, is not capriciously taken from the main store of protein. Longer or repeated extraction will never remove any more, so that it must represent a specific type of compound in the egg—some product of protein breakdown. In a developing system such rearrangements are of the greatest interest, so that carbon tetrachloride, which differentiates to a greater extent than ether, is to be preferred as an extractive. It must be further noted that the nitrogen removed is in any case a relatively small fraction, amounting with carbon tetrachloride to 0.924 and with ether to 0.707 % of the total in the egg. Thus in about 25 % of the dried tissue extracted as fat considerably less than 1 % of the nitrogen is included. All these figures have been checked by another method, namely the analysis of eggs for nitrogen before and after extraction. The two sets of results showed close agreement.

Nitrogen was estimated by the Kjeldahl method, using 0.5 g. of dry fat-free substance. For percentage values it was necessary to apply a correction for the extracted fat. After some consideration it was decided to follow the classic practice of stating that the protein factor 6.25 is incorrect for fish tissues, pointing out why it is incorrect, and then using it. The question of non-protein nitrogen is discussed elsewhere.

The ash was determined by gently heating 0.5 g. of dry fat-free substance in a lightly covered silica crucible for 1 hour, then applying intense heat until the ash became colourless. Bruce [1924] has pointed out that some of the

volatile chlorides are driven off during the heating and some phosphorus in organic combination may be extracted with the fat, but these small quantities are within the limits of experimental error.

The Winkler method was used to determine the oxygen requirements of the developing eggs and larvae. A large receptacle was filled with tap water and allowed to stand until equilibrium with the atmosphere had been reached. Water was then siphoned from the receptacle into a 6-litre jar of accurately known capacity, from which samples were taken for oxygen estimation. One group of 1000 eggs was used for all the tests, these being placed in the jar and the lid sealed on, avoiding included air. Sometimes a bubble appeared in the jar during the course of the experiment, probably released from the slightly supersaturated water, but the error so introduced was not great enough seriously to affect the results. The duration of each test with eggs was 2 hours, a period in which less than 10 % of the available oxygen was used. The suddenly increased oxygen requirements after hatching necessitated a change in the routine, and 200 larvae were allowed to breathe for one hour in the jar. Burfield [1928] showed that the oxygen consumption rate of plaice eggs decreases in a closed vessel owing to the small quantities of carbon dioxide produced. Respiration was normal with a greatly diminished oxygen supply. The analyses here given indicate that the respiratory quotient is almost constant throughout development, so that by the standard methods adopted any decrease in oxygen consumption is the same in all cases and can be disregarded. During experiments the large jar was kept immersed in a tank to ensure standard temperature conditions. The eggs or larvae were kept frequently in motion by turning over the jar every 15 minutes. At the conclusion the lid was removed and water samples taken. A siphon reached to the bottom of the sampling bottle and water was allowed to flow over the top until three times its volume had passed through. Then the siphon was removed and the bottle stoppered without included air, so that the sample taken had not progressed toward equilibrium with the atmosphere. All determinations were made in duplicate, and care was taken frequently to standardise the sodium thio-sulphate solution against potassium permanganate solution, which was itself frequently standardised against potassium tetra-oxalate. Infrequently one of the readings was wide of the mark and was discarded; in other cases the figures quoted are averaged from all four determinations.

PROTEIN VARIATIONS DURING DEVELOPMENT.

The results of the protein determinations are shown in col. 3, Table I, and in Fig. 1. The first important point is that the larva has a larger proportion of protein than the egg. The sudden rise at hatching can only mean that the protein is relatively more concentrated in the larva than it is in the shell and perivitelline fluid. But an analysis of egg capsules three weeks before hatching shows that the protein and water content are higher than in the egg as a whole. Some of the protein in the egg capsule has been destroyed before hatching by

the secreted enzyme. The rise in the total protein of the egg just before hatching is due to the method of calculation, which assumes that all the nitrogen is protein-nitrogen, which is never quite true and sometimes not as approximately true as at other times. Nitrogen is present in forms which are extracted with the fat (see next section). The measured protein content of the larva rises because yolk-protein of low nitrogen content is transformed into living tissue-protein of high nitrogen content. The sudden percentage decrease before hatching is merely a reflection of the greatly increased water content.

Fig. 1.

Table I. *Variations in the chemical composition and oxygen consumption during development.*

1 Days after fertilisation	2 Material	3 Protein %	4 Fat %	5 Remainder %	6 Smoothed remainder %	7 Oxygen consumption
36	e	21.98	3.98	5.49	5.45	0.060
40	e	21.42	4.94	5.19	5.31	0.075
44	e	21.17	4.81	5.17	5.17	0.068
46	s	22.30	—	—	—	—
50	e	20.15	4.93	5.87	4.97	0.066
55	e	21.20	5.01	3.04	4.80	0.055
60	e	21.15	5.00	4.70	4.63	0.052
65	e	21.43	4.94	4.48	4.46	0.049
65	l	24.54	3.00	6.71	5.49	—
70	e	20.35	4.47	4.63	4.29	—
70	l	22.60	4.20	5.15	5.16	0.433
76	l	22.90	4.06	4.79	4.77	—
79	l	22.50	4.06	4.89	4.58	0.471
86	l	21.05	5.83	3.17	4.12	—
90	l	22.10	5.63	1.62	3.86	0.729
94	l	20.45	4.87	3.73	3.59	0.629
100	l	19.96	5.16	2.53	3.20	0.956
106	l	17.80	5.41	2.94	2.82	—
111	l	17.36	5.06	3.13	2.49	—
116	l	16.91	5.18	2.54	2.18	—

Each oxygen figure is the requirement of 1000 individuals in 2 hours. Absolute figures represent the weights for 100 individuals in grams. For "remainder" calculations before hatching the averaged ash value of 0.16 g. or 1.25 % is used; after hatching 0.134 g. or 1.25 %. In col. 2 e means eggs; s, shells; l, larvae.

The activity of the free-swimming larva is shown by the rapid rate at which the protein is burned after hatching. The suddenly increased rate of combustion at the 100th day followed by a diminution 10 days later, marks this as a critical period in development. Morphological considerations offer no solution. Now Needham [1927] found that in the chick the point of maximum protein combustion is also the point at which the greatest quantity of substance is burned per unit of new tissue formed (this is not necessarily true on theoretical grounds), and it is just at this period that the transfer from fat to protein is going on. He cites Terroine, Trautman and Bonnet's results showing that such a transference results in an extra energy loss of 23 %. In the salmon fat begins to disappear at this point, and it also corresponds to the second growth rate maximum in the trout. A morphological examination shows that there is still an abundance of protein in the yolk sac. It is the physico-chemical properties of the organism and not a lack of food which determine why it should suddenly start to dilute its protein diet with fat.

The "remainder" values to be found in Table I and in Fig. 2, have been found by subtracting the total of the other values from the total weight of the egg or larva or from 100 (col. 5), and then smoothing these values into two straight lines corresponding to the periods before and after hatching (col. 6). (The values for total weight and water content are given by Hayes [1930], Table I.) The smoothed values were calculated using the simplest case of the method of moments where

$$y = a + bx$$

$$a = \frac{1}{2l} m_0$$

$$b = \frac{3}{l} \cdot \frac{1}{2l} \cdot \frac{m_1}{l}$$

y = vertical ordinate; x = distance from centre of range; $2l$ = range; m_0 = total area (sum of the frequencies); and m_1 = first moments about the centre of the range. The method is described by Elderton [1906]. The curves in Fig. 2 have been plotted along the smoothed values, although the points shown represent only the unsmoothed figures. (The ash content showed no significant variations during development. Its average for 100 eggs was 0.160 g.; for 100 larvae 0.134 g. The percentage throughout development was 1.25. These constants have been used to calculate the "remainder.")

Since the "remainder" values involve so many sources of error a high degree of conformity between the smoothed and unsmoothed values cannot be expected, but the points show clearly that at the time of hatching the slope of the curve suddenly steepens. The possible carbohydrate content of the eggs is small and cannot explain the "remainder." Water, fat and ash contents have been determined directly and any possible error could not vary with the developmental stage. With the protein, however, the procedure of multiplying the total nitrogen by 6.25 (or any other factor) involves the assumption that the

relation of nitrogen to protein is the same in different stages of development and in different egg constituents. From the entrance of the spermatozoon until the larva begins to take outside food there is nothing added to the egg except water, so that all the nitrogen must have been there from the beginning although more of it was missed in the earlier stages than later. Levene [1901] has shown that the nucleoproteins of the cod egg, which are especially rich in nitrogen, and moreover are required for the formation of cell nuclei, increase during development. The period of salmon development considered is one of rapid growth, and differences in constitution between embryo and yolk show with increasing clearness in successive analyses. Probably increasing nucleoprotein explains the form of the "remainder" curves. Stated otherwise the curves show that in the system as a whole the amount of protein corresponding to one unit of nitrogen is greater in the earlier stages than later, while the method of analysis assumes that it does not vary.

Fig. 2.

Since this difference between yolk and embryonic protein is a true one it is interesting to gain some idea of its magnitude. By what figure should the total nitrogen (after fat extraction) be multiplied in order to find the total embryonic or yolk protein? It is necessary to assume that substances other than water, protein, fat and ash are present in such small quantities that they may be disregarded. In col. 5, Table I, there is a "remainder" of 5.49 for the 36-day eggs. This means that on the above assumptions the correct protein value should be 27.47 instead of the 21.98 shown in col. 3. To obtain this value a factor of 7.81 must be used. From cols. 2 and 3, Table II [Hayes, 1930], the weight of the embryo and yolk at this age may be obtained and expressed as percentages; they are 1.76 and 98.24 respectively. If the same data are sought for the 116-day larva it is found that the larval protein factor necessary is 7.19, the embryonic weight 41.8, and the yolk weight 58.2. Let x = the embryonic

protein factor, and let y = the yolk factor, and the following equations are true:

$$\begin{aligned} 1.76x + 98.24y &= 100 \times 7.81 \\ 41.8x + 58.2y &= 100 \times 7.19. \end{aligned}$$

The solution gives x a value of 6.36 and y a value of 7.82, which means that the embryonic protein contains 15.7 % nitrogen and the yolk-protein 12.8 %. Embryonic and yolk weights within the egg and other values could be obtained by the use of these factors, but such calculations are not justified without confirmatory evidence. Research in the choice of a factor in such investigations as this is obviously most desirable.

Fig. 3.

FAT VARIATIONS DURING DEVELOPMENT.

The fat extraction results are shown in col. 4, Table I, and in Fig. 3. The absolute and percentage values rise steadily until a few days before hatching, then decline suddenly. Hatching produces a further drop due to the loss of the shell and perivitelline fluid. During larval development the fat maximum is reached at the 106th day, following which both percentage and absolute values decrease. There is every indication that fat is being formed from protein during the early stages and again during the greater part of larval development. Since many fish eggs have very small quantities of fat their ability to synthesise it is a matter of very great importance in larval nutrition. A fat increase during development has been found in trout eggs by Tangl and Farkas [1904] and Gortner [1913], and in plaice eggs by Dakin and Dakin [1925]. With the plaice the amount of fat involved is exceedingly small, amounting to only 0.085 % in the beginning and 0.307 % at the end. Fig. 3 shows that there is a large decrease in late hatching eggs, owing to the necessity for the embryo to burn fat under the special conditions of hatching. Taken in relation to the protein the form of the fat curve suggests that there is one succession of energy sources in the egg and another in the larva.

OXYGEN CONSUMPTION DURING DEVELOPMENT.

The oxygen consumption records are given in col. 7, Table I, and the decrease in hatching is illustrated in Fig. 2. The figures serve plainly to emphasize what appears from all the other experiments; first that hatching produces a complete change in the conditions of development; and second that life processes drop to a low ebb under the unfavourable conditions previous to hatching. The great activity of the free-swimming larva causes its oxygen requirements to be about 10 times as great as in the egg stage.

Fig. 4.

Kawajiri [1925] used the Winkler method to investigate the oxygen requirements of the Japanese land-locked salmon (*Oncorhynchus masou*). The variations in the egg stage were negligible, the curve showing a slight trough between the 5th and 10th days. A newly hatched larva consumed 5 times as much oxygen as an egg and the rate of absorption rose rapidly until the end of development. *Fundulus* eggs have also been studied by Scott and Kellicott [1916] and by Hyman [1921]. Their results agree except on one point, the decrease before hatching. Scott and Kellicott failed to find a decrease, but Hyman contradicts this result. Both found that at the time of gastrulation there is a marked rise while during the formation of the embryo there is no further increase. Another sudden rise when the heart begins to beat is followed, according to Hyman, by a gradual decrease until hatching. In the larval stages the oxygen requirements are greatly increased. Both workers followed the same method, namely the estimation of carbon dioxide by the p_H changes of the water in which the eggs were breathing.

THE NATURE OF GROWTH.

In the later stages of egg development there is a gradual increase in water. It is important to know to what extent salmonid eggs are completely closed systems with respect to water, but there is some conflict in the data. In the salmon according to Rnnstrom [1920] and in the trout according to Svetlov [1929] there is a decrease in osmotic pressure during development, showing

that water is taken up. Gray [1920] found that in the trout the osmotic pressure of the yolk remains constant during development. Kronfeld and Scheminzky [1926] follow the conceptions of Tangl in regarding the egg as a completely closed system. Since the results given here agree with those of some other workers and have been obtained by direct measurement they may be accepted provisionally.

It had been believed that different osmotic pressures existed in the egg contents, but Svetlov [1929] found (on adequate theoretical grounds) that the embryonic osmotic pressure is the same as that of the yolk. Assuming that substances necessary for growth do not enter the egg from the environment, facts are now at hand for a revised story of the process of embryo building.

As the embryo grows the proportion of salt remains equal to that of the yolk, but that of the organic material is much lower. For building operations there must be a preferential selection of salt. For combustion on the other hand there is an exclusive selection of organic material. Within the egg little movement is possible and combustion amounts approximately to the minimum requirements for basal metabolism. It follows that if the water content of the yolk remained constant its proportion of salt would necessarily decrease. By some property, at the nature of which we can only guess, it maintains a constant salt content by expelling water into the perivitelline fluid which (as Svetlov showed) steadily increases during development. The decline after the first growth rate maximum shows that the increased concentration of colloidal material has changed the properties of the yolk. It cannot become indefinitely more concentrated—there is a plateau in the curve showing yolk water losses (Kronfeld and Scheminzky's fig. 2). In subsequent development the embryo becomes drier and takes more nearly equal proportions of salt and organic material. It also begins to burn fat. Speaking of the chick Needham [1927] states, "Out of 100 g. of protein in its diet the embryo can store away 98; out of 100 g. of carbohydrate 82; but out of 100 g. of fat only 43." If this relationship were true in the fish as well it would offer an explanation of the results. The embryo must maintain its level of water as constant as possible, and the mechanism adopted would be to use an energy source of which a large proportion was burned rather than built into new tissue. At the same time the enzyme which dissolves the shell is secreted and its unfavourable effects appear. At hatching the embryo is suffering from malnutrition, both embryo and yolk are lacking in water, but both have maintained their salt content. In the larval condition the embryo immediately begins to recover. It takes up water rapidly, and salt in proportion, so that the yolk loses water for a short time. The ultimate balance is restored by the greatly increased activity in the free-swimming state until finally the yolk has returned to its early water content level. This point has been shown to correspond to the second growth rate maximum in the trout (Kronfeld and Scheminzky). In the salmon the stabilisation of the water content comes just before the second change to fat as a source of energy. The embryo has grown so rapidly that the conditions of

the later egg stages have repeated themselves and the reaction is the same—the selection of fat as building material. There is a decrease in the growth rate and the larva first begins at this time to seek outside food.

These considerations bring the question of the succession of energy sources one step nearer to its solution. Attention is focussed on the salt content, and a fundamental question is to discover why it must remain constant and what happens when it is experimentally changed.

SUMMARY.

1. In the developing salmon the protein decreases in the earlier egg stages and increases before hatching; in the larval stage it is burned rapidly. It has been calculated that embryonic protein contains 15.7 and yolk protein 12.8 % of nitrogen.

2. The fat content rises to two peaks, the first 10 days before hatching, and the second 6 weeks after. The decreases after the maxima are believed to show a change from protein to fat as a source of energy.

3. Variations in oxygen consumption indicate that metabolic processes drop to a low ebb before hatching and greatly increase in the free-swimming larva.

I am very grateful for the inestimable help of Prof. James Johnstone and Dr R. J. Daniel under whose direction this research has been carried out. Prof. J. M'Lean Thompson kindly permitted me to improvise hatching apparatus under especially favourable conditions in the Department of Botany, University of Liverpool. Much of the material used was provided through the courtesy of Mr J. G. D. Watts, Superintendent for the Dee Fishery Board. I also wish to thank the Nova Scotia I.O.D.E. and the International Education Board for financial assistance.

REFERENCES.

- Bruce (1924). *Biochem. J.* **18**, 469.
 Bull (1928). *J. Mar. Biol. Assoc.* **15**, 207.
 Burfield (1928). *Brit. J. Exp. Biol.* **5**, 177.
 Dakin and Dakin (1925). *Brit. J. Exp. Biol.* **2**, 293.
 Elderton (1906). Frequency curves and correlation. (C. and E. Layton, London.)
 Fauré-Fremiet and Garrault (1922, 1). *Compt. Rend. Acad. Sci.* **174**, 1375.
 ——— (1922, 2). *Bull. Soc. Chim. Biol.* **4**, 379.
 Gortner (1913). *J. Amer. Chem. Soc.* **35**, 632.
 Gray (1920). *J. Physiol.* **54**, 68.
 Greene (1921, 1). *J. Biol. Chem.* **48**, 59.
 ——— (1921, 2). *J. Biol. Chem.* **48**, 429.
 Hayes (1930). *Biochem. J.* **24**, 723.
 Hyman (1921). *Biol. Bull.* **40**, 32.
 Johnstone (1918). *Ann. Rep. Lanc. Sea-Fish. Lab.* **27**, 36.
 Kawajiri (1925). *J. Imp. Fish. Inst. Tokyo*, **21**, 18.
 Kronfeld and Scheminzky (1926). *Roux's Arch. Entwkgmechan.* **107**, 129.
 Levene (1901). *Bull. U.S. Fish. Comm.* **19** (1899), 153.
 Needham (1927). *Brit. J. Exp. Biol.* **5**, 43.
 Rünstrom (1920). *Acta Zool. (Stockh.)*, **1**.
 Scott and Kellicott (1916). *Anat. Rec.* **2**, 531.
 Svetlov (1929). *Roux's Arch. Entwkgmechan.* **114**, 771.
 Tangl and Farkas (1904). *Pflüger's Arch.* **104**, 624.