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HEMOGLOBIN POLYMORPHISM AND ITS ONTOGENY IN SEA-RUNNING AND LANDLOCKED ATLANTIC SALMON (SALMO SALAR L.)

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1. Introduction

The use of biochemical characteristics in taxonomic problems has gained ground rapidly during the last few years. It is well known that proteins can provide indirect information of the degree of genetic homology between animals because the amino acid sequence of a protein is a direct translation of the genetic information coded into DNA molecules. A single protein can provide data only about a small fraction of the genetic material but Wilson & Kaplan (1963) have postulated that to estimate the overall degree of genetic homology, studies of five to ten protein will be sufficient (cf. Zuckerkandl, 1965; Dayhoff, 1969).

Because amino acid sequence determinations are with the present techniques very laborious a number of other methods such as immunological, chromatographical and electrophoretical have been applied in the study of proteins. Especially enzymes, plasma, muscle and egg proteins and hemoglobins have proved to be of value in studies of animal classification, relationship and evolution. The usefulness and limitations of biochemical characteristics in taxonomic studies have been discussed e.g. by Engle & Woods (1960), Sibley (1960). Alston & Turner (1963), Dessauer & Fox (1963), Manwell (1963b), Wilson & Kaplan (1963), Cushing (1964), Leone (1964), Tsuyuki, Roberts & Vanstone (1965a), Manwell & Kerst (1966), Fujino (1969), Koehn (1969), Love (1970).

Some of the first applications in which hemoglobin electrophoresis has been applied in systematics were identification of cod (*Gadus morhua*) and whiting (*G. merlangus*) populations (e.g. Sick, 1961, 1965a, b; Frydenberg, Møller, Naevdal & Sick, 1965), distinguishing sibling species of frogs (Bertini & Rathe, 1962), detection of a new sibling species of sea cucumber (Manwell & Baker, 1963), and detection of hybrid centrarchid fishes (Manwell, 1963b).

Hemoglobins have been found to be polymorphic in a great number of vertebrates and invertebrates (reviewed by Gratzer & Allison, 1960) and this phenomenon has been observed in most fish species so far examined. There are many reasons for the existence of multiple hemoglobins (e.g. Manwell & Schlesinger, 1966), but most important for taxonomic investigations is hemoglobin polymorphism, which results from intraspecific variation during development and from genetic polymorphism.

The hemoglobin of some fish species has been found to undergo an ontogenetic development, which is restricted to a retively short larval or fetal phase (Manwell, 1957, 1958a, b, 1963a; Adinolfi & Chieffi, 1958; Wilkins, 1963; Golovanenko, 1964; Vanstone, Roberts & Tsuyuki, 1964; Callegarini, 1966), or has proceeded during a great part of the life cycle (Hashimoto & Matsuura, 1960b; Vanstone *et al.* 1964; Koch, Bergström & Evans, 1964, 1966; Wilkins & Iles, 1966; Yamanaka, Yamaguchi, Hashimoto & Matsuura, 1967).

Intraspecific variations in hemoglobin electrophoretic pattern have been found in some fish species (Sick, 1961; Tsuyuki, Uthe, Roberts & Clarke, 1966; Westrheim & Tsuyuki, 1967; Naevdal, 1968; Schlotfeldt, 1968; Tsuyuki, Roberts, Lowes, Hadaway & Westrheim, 1968) and the study of gene frequencies for polymorphic hemoglobins has proved to be useful until now at least in taxonomic investigations of cod and whiting populations.

Schumann (1959) obtained with agar gel electrophoresis two components for the Atlantic salmon (*Salmo salar*). Koch *et al.* (1964) observed by starch gel that the hemoglobins of Atlantic salmon of Scandinavian origin split into two groups of multiple components and a size dependent developmental pattern in the appearance and disappearance of the fractions was manifest. Later a shift correlated with size in the relative quantity of the two groups of the multiple components was observed (Koch *et al.* 1966). Wilkins (1968) has studied the hemoglobins of salmon originating from Scotland, Greenland and Canada with a different electrophoretic technique. He confirmes the existence of multiple components and the ontogenetic variations.

The aim of the present study was to examine further and in detail the electrophoretic pattern and its ontogeny of the hemoglobin of the Atlantic salmon and especially of fish originating from Bay of Bothnia river systems and landlocked salmon from Lake

Saimaa (Eastern Finland) by scanning and integrating in detail the hemoglobin patterns. This seemed to be especially important because the multiple components obtained by starch gel electrophoresis have been suggested by Koch, Bergström, Bodarwé-Schmitz & Evans (1968), Wilkins (1968) and Koch (1969) to be structurally different hemoglobins. Moreover intraspecific ontogenetic variations as well as genetic polymorphism must be known if hemoglobin patterns are used in taxonomic studies. It has also been examined whether the hemoglobin patterns of sea-running salmon, landlocked salmon and brown trout (*Salmo trutta*) confirm the suggestion made by Seppovaara (1962) on the basis of meristic and morphometric characters, that the landlocked salmon in Lake Saimaa is a salmon and not a brown trout population (in Finnish »siikalohi») as earlier suggested.

2. Material and methods

2.1. Test animals

A total of 195 individual blood samples were collected in different seasons of the years 1966—1969 from sea-running Atlantic salmon and landlocked salmon from Lake Saimaa.

Of the 142 sea-running salmon examined, seven wild specimens varying in length from 50.0 to 79.0 cm, were obtained from the mouth of the River Oulujoki (Bay of Bothnia), eight specimens between 48.0—95.0 cm were captured outside the River Iijoki (Bay of Bothnia) prior to their entry in fresh water, and one 78.0 cm long individual was caught from the Sea of Ahvenanmaa. The fish originating from the River Oulujoki were transported to the Montta fish hatchery of Oulujoki Osakeyhtiö at Muhos where the blood samples were taken. These salmon specimens were probably fish, which had been reared at Montta fish hatchery from spawn either of River Luleälv origin or River Skellefteå origin (Bay of Bothnia, Sweden), and then released as smolts in the River Oulujoki. The original River Oulujoki salmon population has become extinct as a consequence of the power plants. On the other hand, salmon from the River Iijoki were most likely of the original strain from this river. Four of these specimens were transported to the Hollola fish hatchery of Kalataloussäätiö at Hollola where they were reared in ponds. Blood samples from these fish were also taken at the Hollola fish hatchery.

Blood samples from 104 hatchery reared sea-running salmon specimens between 3.5—17.3 cm (age 2—29 months), and seven specimens between 24.5—48.0 cm (age 3—5 years) were taken at Montta fish hatchery. The latter were not released in the sea as smolts, but were left in the hatchery and reared in freshwater ponds. All the fish from Montta were of River Luleälv or River Skellefteå stocks.

15 salmon specimens between 27.0—34.0 cm (age 3—4 years), originating from Montta, were reared for two years in a net-container in a bay at Koukkusaari at Kotka (Gulf of Finland).

All 53 landlocked salmon specimens examined were hatchery reared but all the spawn were taken from wild parent fish, which were captured outside the River Pielisjoki, Lake Saimaa. 44 fish of the size 5.0—51.0 cm (age $^1/_2$—5 years) originated from Kontiolahti fish hatchery of Pohjois-Karjalan Maanviljelysseura at Kontiolahti, and blood from nine specimens between 37.0—47.0 cm were taken at Hollola fish hatchery.

A total of twenty specimens, reared at Montta, Hollola and Kontiolahti hatcheries, were marked with Carlin tag so that it was possible to take several blood samples from the same individual.

The length of the fish is given as total length to the nearest millimetre.

2.2. Sampling of the blood

The blood was taken from fish anesthetized with M.S. 222 (tricaine methanesulfonate, Sandoz, Basle, Switzerland). From small individuals (less than 10 cm) the samples were usually taken by cutting off the caudal peduncle and sampling the emerging blood

directly in heparinized micro-hematocrit capillaries (Clay-Adams, no. A-2930, 75 mm in length, 1.1—1.2 mm inside diameter, N.Y., U.S.A.). When taking blood from very small specimens (less than 6 cm) very thin pre-heparinized hematocrit capillaries were used (Clay-Adams, no. A-2937, 0.5—0.6 mm inside diameter).

From larger specimens, which were to be kept alive, the blood was taken at the beginning of the study by heart puncture with a 1-ml hypodermic syringe and 16 or 18 gauge needle. Blood was drawn from the heart or associated vessels by inserting the needle into the center of the isthmus on a line drawn between the origins of the pectoral fins. The syringe and the needle were rinsed with a five per cent solution of heparine.

Because this method is relatively slow and requires the preparation of syringes and needles it was changed over to a faster and more convenient method, slightly modified from that described by Larsen & Snieszko (1961). The tip of a heparinized microhematocrit capillary tube was drawn out to a sharp constricted end in a pinpoint flame. Only the very tip of the capillary is allowed to be heaten to prevent the destruction of the anticoagulant. The operculum of an anesthetized fish was lifted and the pointed tube was then used to puncture the branchial membrane and penetrate the heart or associated structures. The area of puncture was kept free from water to avoid dilution of the blood sample. As the puncture was made the blood surged into the capillary tube. When necessary, this was facilitated by mouth suction with a polyethylene tube. If any body fluid emerged into the tube, the sample was discarded and a new puncture was made. To expose the entire sample to anticoagulant the sample was forced to the opposite end by tapping the tube or by mouth suction with the polyethylene tube. After that the tube was sealed. When the fish returned to water the bleeding stopped quickly.

The blood taken was never pooled but handled individually even with the smallest fish.

Nearly all sampling was done in the field and the blood samples were kept all the time packed in thermos flasks filled with ice-water mixture.

2.3. Preparation of the hemoglobin solution

At the beginning of this study the method described by Koch *et al.* (1964) was used. The collected blood was centrifuged for 1—2 min at 20 000 r.p.m. in the Beckman Spinco Microfuge (Model 152, Beckman Instruments). This was done at + 2 °C to prevent overheating of the sample, which could damage the hemoglobin. Plasma was removed and blood corpuscles were admixed with a paste of Sephadex G.25 (Pharmacia, Uppsala, Sweden) in distilled water. After centrifuging this mixture for 1—2 min in the Microfuge a clear concentrated supernatant hemoglobin solution directly suitable for electrophoresis could be removed on top of the Sephadex paste.

As the above mentioned method was quite complex and slow a very simple and rapid micro method for the preparation of blood was developed. This suits very well for small specimens, which contain only 1—2 drops of blood.

The micro-hematocrit tubes, in which blood were collected were sealed with clay (Seal-Ease, Clay-Adams, N.Y., U.S.A.), and centrifuged at + 2 °C with an International hematocrit centrifuge for 5 min at 11 000 r.p.m. The hematocrit was determined and the plasma and the buffy layer was carefully removed with a very thin glass capillary. The erythrocytes were washed directly in the same hematocrit tube three times with three volumes of Alsever's solution (Alsever & Ainslie, 1941). The solution was blown with a thin capillary under the corpuscule mass and recentrifuged.

When washed with Alsever's solution the corpuscules do not form a »jelly», which is often the case when salmon erythrocytes are washed with a saline solution. If this happens, it is impossible to obtain hemolysed blood in a short time (cf. Wolf, 1959; Koch *et al.* 1964). It was found that the washing step can be omitted before hemolysis of the erythrocytes because this did not result in any observable changes in electrophoretic patterns, probably because the remaining amount of plasma proteins after centrifugation is very small (cf. Hashimoto & Matsuura, 1960 a; Westrheim & Tsuyuki, 1967).

The corpuscules were hemolysed in the same hematocrit capillary with one or two

volumes of distilled water. With this method nothing was lost of the blood sample as all the steps were done in the same hematocrit capillary.

All the samples were centrifuged as soon as possible, usually within one day after bleeding, as it was found that already a storage of some days even in a refrigerator resulted in changes in the hemoglobin electrophoresis pattern. For the same reason the blood samples or already separated hemoglobin solution were never frozen.

2.4. Starch gel electrophoresis

A horizontal micro starch gel electrophoresis method slightly modified from that described by Koch *et al.* (1964) was used. Tris-HCL buffer of pH 8.2 and 0.005 M was used as bridge buffer. The gel buffer was a ten-fold dilution of the same. The starch gel used was of reagent grade and supplied by Connaught, Toronto, Canada (Starch-Hydrolysed for gel electrophoresis, Lot 216-1). The amount of starch needed for 100 ml buffer solution was 10.2 g as indicated by the manufacturer. Starchbuffer solution was heated over a naked flame and stirred continuously until the viscosity had fallen well below the initial maximum (Smithies, 1959). The degassed gel was poured evenly over four standard microscope slides in a tray of the size 11 × 14 × 2 cm. The uncovered gels were allowed to set and cool at room temperature for four hours.

The adherence of the starch gel layer to the microscope slides is of great importance. The slides were washed thoroughly, held 12 hours in ether-alcohol solution, rinsed with distilled water and, as suggested by Koch *et al.* (1964), just before use, de-greased by a wash in reagent grade iso-propanol. Slides and the tray were placed in an oven at 100 °C for a while as the gel will adhere better to warm slides.

The slides were cut out individually from the starch gel mass with a point of a fine scalpel. Each gel was sliced separately to a standard 1.2 mm thickness by peeling the unwanted upper gel layer off with an apparatus and technique described by Koch *et al.* (1964) and Poulik (1966).

A transverse slit was cut in the gel 28 mm from the cathodic end (cf. Koch *et al.* 1964). Slits were made by pressing a microscope cover slip into the gel with a special device (Koch *et al.* 1964) and by pushing the slide forwards with the same apparatus so that the cover slip opens a slit in the gel. With this technique a very thin, straight and well defined starting line with uniform depth (0.8 mm) and width (15 mm or if two hemoglobin samples were run simultaneously on the same gel slide 8 mm wide) could be easily made. The slit did not reach to the edge of the gel or the microscope slide under the gel.

The hemoglobin solution was run from a micro-pipette into the slit along the cover slip. The cover slip was removed and the slit was allowed to close so that petroleum jelly was not needed to seal the slit. Only about 0.3—0.5 μl hemoglobin solution was needed to a slit of 15 mm breadth and a half of this volume to a 8 mm slit, respectively. The hemoglobin sample was always so small that it did not spread from the closed slit on the gel. The gels and also the slit were coated with a strip of very thin polyethylene in order to prevent evaporation from the surface of the gel. Gel ends which served as contacts were left free. Air bubbles between the gel and the polyethylene strip were avoided.

The perspex glass electrophoretic tank used was a modification (Koch *et al.* 1964) of that described by Wieme (1961). In this tank model the gel and the bridge buffer are connected by two capillary plates so that all intermediary agents are done away with. Platinum plates (20 × 5 × 0.3 mm) were used as electrodes.

The power supply was LKB (Model 3276, Stockholm, Sweden), which assures constant voltage. Three slides, each containing mostly two hemoglobin samples, were run at the same time. The running time was 60 min. The power supply showed at the start a current of 7—8 mA at 270 V (36 V/cm), but this changed during the run and soon a current of 14—16 mA was reached. Preliminary experiments had established that maximum separation occurs under those conditions.

In order to prevent overheating of the gel the electrophoresis was performed in a cold room at + 2 °C. The buffer solution was also precooled and the gel in the tank was surrounded with cooling basins filled with crushed ice and water.

The gels were stained immediately after the electrophoresis in a solution of Amido Black

10B (250 mg of dye in 1 000 ml of 10 % acetic acid) for one hour. Excess stain was removed in several washings of 10 % acetic acid. Destained gels were transparent with only a trace of bluish background.

Comparisons among gels were made on the basis of protein distribution, i.e. the number of bands, the relative distance migrated and the relative concentration. To make any sense of the comparisons densitometer tracing was made for each gel at 580—650 mμ wavelength by means of an integrating densitometer using transmitted light (densitometer model DD2, micrograph model BD2, integrator model BC1, Kipp & Zonen, Delft, Holland). The height of the densitometer curve depicting a fraction was assumed to correspond to the amount of dye bound by the fraction. The distance of migration for each component was measured from the origin to the maximum of optical density.

The area of each integrated fraction was calculated as a percentage of the whole anodal or cathodal area. The total anodal area was also computed as a percentage of the both areas. Calculations were made at the Computing Centre, University of Helsinki.

Identification of the hemoglobin bands were eventually ascertained by means of o-dianisidine reagent (Owen, Silberman & Got, 1958) and benzidine-method of Smithies (1959). After the electrophoresis run the gels were sliced lengthwise in two identical halves. One half was stained with dye and the other with o-dianisidine or benzidine reagent.

The gels were preserved by immersing them in a solution of 5 % glycerine in 2 % acetic acid and water for about one day. Then the gels were dried on filter paper strip as described by Koch *et al.* (1964). These gel strips can be scanned by reflectance and stored in plastic bags under normal humidity conditions.

3. Results

3.1. Hemoglobin pattern and its ontogeny

The hemoglobins of 142 wild- and hatchery reared sea-running Atlantic salmon and 53 landlocked salmon from Lake Saimaa were analysed with starch gel electrophoresis. As a whole more than 300 electrophoretic runs were made as a number of samples were run in duplicate. All gels were densitometred and scannings from 99 sea-running and 51 landlocked salmon hemoglobins were also integrated. All of the gels were not integrated because this was not possible at the beginning of this study.

The hemoglobins of all investigated salmon showed two groups of components, one migrating to the anode (called the A group) and the other to the cathode (called the C group). The number of components in the A group varied from one to five and in the C group from four to eight, respectively.

An ontogenetic change was found in the hemoglobin pattern and both the number and relative intensity of the observed fractions varied in a definite, regular manner during growth. This developmental change, which was first observed in the Atlantic salmon by Koch *et al.* (1964), was beautifully visualized in electrophoretic runs made with two or three hemoglobin samples taken from the tagged fish at intervals from one to three years.

As it was found difficult to interpret visually the patterns visible in gels, the evaluations were made from integrated densitometric curves. As independent hemoglobin components were considered those, the relative concentration of which exceeded 1 % counted from the total anodic or cathodic areas of components.

Fig. 1 gives a diagrammatic representation of the main hemoglobin patterns according to the number of components. The anodic bands are tentatively numbered from A1 to A5 and the cathodic bands from C1 to C8, respectively, according to their order of appearance during the ontogenetic development of the hemoglobin. It should be stressed that even if the patterns in Fig. 1 represent certain stages in the ontogenetic development of the hemoglobin, the illustrated patterns are not definitive but represent only arbitrary stages of a continuous process. In some individuals intermediate patterns were observed. This concerns especially the pat-

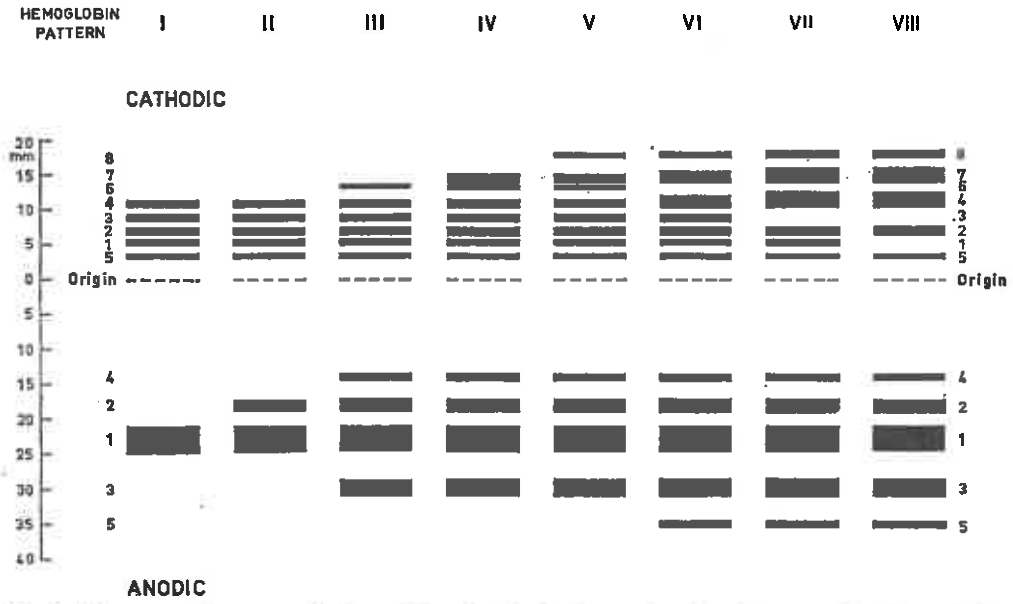

Fig. 1. Diagrammatic representation of the starch gel electrophoresis patterns of the hemoglobins of the Atlantic salmon.

terns III, IV and V of group A in which the component A3 or A4 or in some specimens both of them were missing. These rare patterns have however been omitted from the discussion. Similarly the missing of component C5 in one fish of a length of 3.7 cm was not taken as an independent hemoglobin type.

The hemoglobin patterns in salmon originating from Bay of Bothnia river systems and landblocked salmon from Lake Saimaa were identical and the patterns were the same in both sexes.

Table 1 shows the correlation between the hemoglobin patterns and mean lengths and length ranges of salmon. Because it was not possible to obtain continuous series of lengths the mean lengths and length ranges representing the hemoglobin types can be presented only in outline.

A continuous development of the hemoglo-

Table 1. Mean lengths and length ranges of sea-running and landlocked Atlantic salmon illustrating the different hemoglobin patterns.

Hemoglobin pattern	Sea-running salmon			Landlocked salmon		
	No. of observations	Mean length (cm)	Length range (cm)	No. of observations	Mean length (cm)	Length range (cm)
I	13	5.6	3.5—12.4	4	5.6	4.8— 6.6
II	13	8.1	3.7—16.0	7	5.6	4.4— 7.5
III	11	12.8	8.0—16.0	4	17.6	16.5—19.0
IV	12	14.9	9.6—29.6	3	16.8	16.0—17.5
V	30	19.3	8.8—37.5	9	23.2	17.5—28.0
VI	7	36.9	31.3—52.0	8	26.4	25.0—28.0
VII	9	69.0	48.0—95.0	11	43.0	37.0—51.0
VIII	4	77.5	74.0—82.0	5	44.7	39.0—50.0

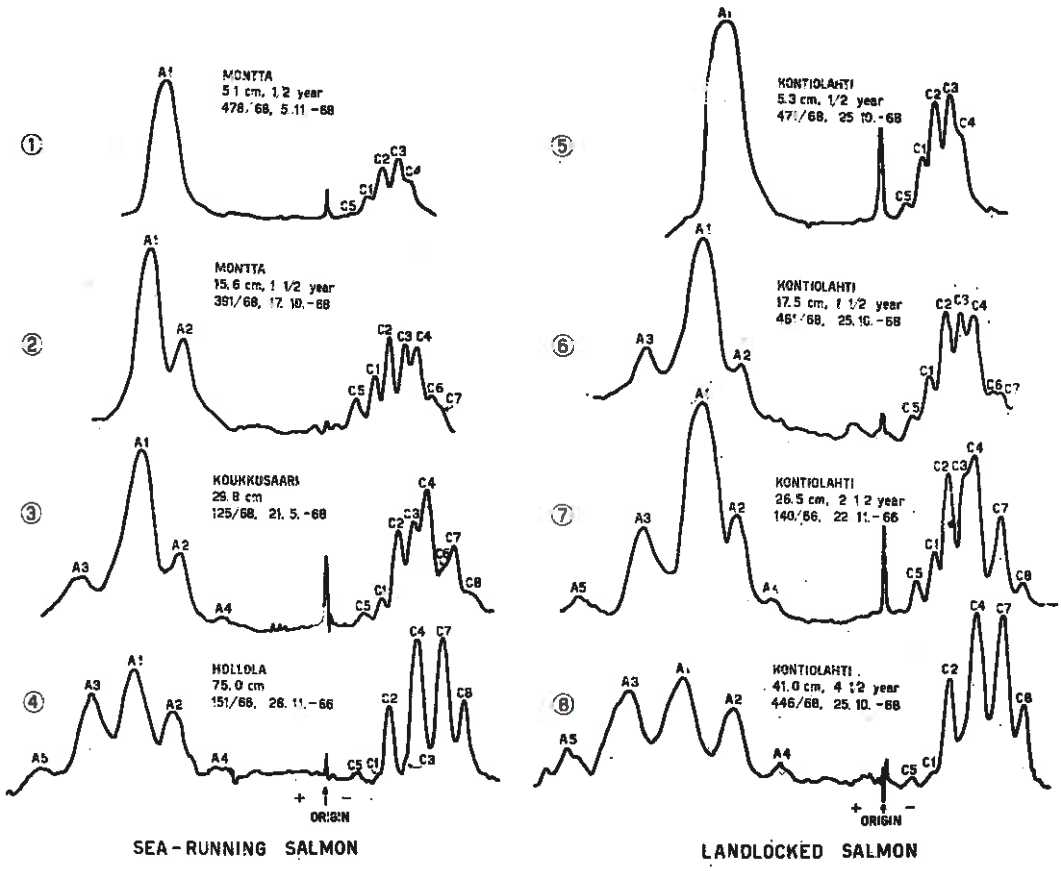

Fig. 2. Original densitometric curves of sea-running and landlocked Atlantic salmon specimens of different lengths.

bin pattern along with increasing length was observed (Table 1). It seems as if in the mean lengths representing the hemoglobin types I to V there were not any major differences between sea-running and landlocked salmon. On the other hand the mean lengths of especially the hemoglobin patterns VII and VIII in landlocked salmon were considerably smaller than in sea-running salmon, i.e. for the same size the hemoglobin pattern was more advanced in large landlocked salmon when compared with sea-running salmon. This suggests that the ontogenetic development of hemoglobin had proceeded with increasing size faster in landlocked than in sea-running salmon.

The hemoglobin pattern I, which can be called the primary or larval pattern, was the simplest of the observed patterns and its duration in terms of length of the fish was also the shortest. The hemoglobin type VI represented a culmination stage in the development of the hemoglobin complex, because the first component (C6) disappeared. The hemoglobin pattern VIII, which is illustrated with five anodic and five cathodic components, can be called the final or adult pattern.

Fig. 2 shows the original densitometric curves of four sea-running and four landlocked salmon specimens of different length. It is interesting that the smaller the fish the shorter is the distance between the components C1—C4. This suggests that there might have been a preceding hemoglobin pattern of the type I with only two or perhaps only one component in the C group.

Table 2. Mean concentrations (± SEs) and frequencies of the anodic components in different length groups of sea-running and landlocked Atlantic salmon.

n	Length (cm) Mean	Range	Fraction A5 Mean ± SE	Freq.	Freq. %	Fraction A3 Mean ± SE	Freq.	Freq. %	Fraction A1 Mean ± SE	Freq.	Freq. %	Fraction A2 Mean ± SE	Freq.	Freq. %	Fraction A4 Mean ± SE	Freq.	Freq. %
Sea-running salmon																	
17	4.7	3.5 — 5.7	—	—	—	—	—	—	94.5 ± 1.8	17	100.0	4.9 ± 1.6	6	35.3	—	—	—
9	9.1	8.0 — 9.6	—	—	—	2.0 ± 1.9	1	11.1	88.7 ± 2.9	9	100.0	8.3 ± 1.4	8	88.9	—	—	—
10	11.0	10.0 — 11.8	—	—	—	—	—	—	78.8 ± 3.6	10	100.0	17.4 ± 2.6	10	100.0	1.9 ± 0.7	5	50.0
23	13.4	12.0 — 14.8	—	—	—	0.9 ± 0.6	2	8.7	83.9 ± 2.0	23	100.0	12.4 ± 1.7	21	91.3	0.8 ± 0.4	3	13.0
8	15.7	15.0 — 17.2	—	—	—	3.0 ± 1.9	2	25.0	76.6 ± 4.5	8	100.0	17.5 ± 3.8	7	87.5	0.8 ± 0.6	2	25.0
9	28.7	27.0 — 30.5	—	—	—	12.4 ± 1.7	9	100.0	65.1 ± 2.2	9	100.0	16.8 ± 1.5	9	100.0	3.6 ± 1.0	7	77.8
8	33.5	31.3 — 37.5	1.0 ± 0.6	2	25.0	19.8 ± 3.2	7	87.5	55.0 ± 4.2	8	100.0	19.1 ± 1.1	8	100.0	3.0 ± 0.5	8	100.0
5	50.2	48.0 — 53.0	3.4 ± 1.2	5	100.0	27.6 ± 2.5	5	100.0	40.0 ± 1.6	5	100.0	22.6 ± 2.5	5	100.0	4.2 ± 0.8	5	100.0
4	73.0	70.0 — 75.0	3.5 ± 0.8	4	100.0	26.8 ± 1.9	4	100.0	41.8 ± 1.4	4	100.0	22.8 ± 1.4	4	100.0	3.3 ± 0.5	4	100.0
6	81.7	77.0 — 95.0	7.3 ± 1.6	6	100.0	29.7 ± 3.0	6	100.0	38.5 ± 3.0	6	100.0	19.0 ± 2.3	6	100.0	3.0 ± 0.5	6	100.0
Landlocked salmon																	
11	5.7	4.4 — 7.5	—	—	—	—	—	—	95.3 ± 1.8	11	100.0	4.1 ± 1.2	7	63.2	—	—	—
10	17.4	16.0 — 19.0	—	—	—	14.1 ± 1.2	10	100.0	70.6 ± 2.8	10	100.0	12.1 ± 1.9	10	100.0	1.5 ± 0.5	6	60.0
14	26.2	24.5 — 28.0	1.6 ± 0.6	6	42.9	22.7 ± 1.4	14	100.0	53.4 ± 1.9	14	100.0	17.1 ± 1.0	14	100.0	2.7 ± 0.6	13	92.9
8	40.4	37.0 — 42.5	9.0 ± 1.2	8	100.0	35.8 ± 1.5	8	100.0	34.8 ± 0.6	8	100.0	15.4 ± 0.8	8	100.0	2.6 ± 0.2	8	100.0
8	46.7	44.0 — 51.0	9,9 ± 1.2	8	100.0	37.3 ± 0.9	8	100.0	34.5 ± 1.7	8	100.0	13.8 ± 0.7	8	100.0	2.3 ± 0.3	8	100.0

Table 3. Mean concentrations (± SEs) and frequencies of the cathodic hemoglobin components in different length groups of sea-running and landlocked Atlantic salmon.

Sea-running salmon

n	Length (cm) Mean	Range	Fraction C5 Mean ± SE	Freq.	Freq. %	Fraction C1 Mean ± SE	Freq.	Freq. %	Fraction C2 Mean ± SE	Freq.	Freq. %	Fraction C3 Mean ± SE	Freq.	Freq. %
17	4.7	3.5 — 5.7	2.6 ± 0.4	16	94.1	10.7 ± 0.9	17	100.0	30.7 ± 0.7	17	100.0	34.1 ± 0.9	17	100.0
9	9.1	8.0 — 9.6	3.7 ± 0.4	9	100.0	10.6 ± 0.9	9	100.0	24.8 ± 1.6	9	100.0	27.1 ± 1.6	9	100.0
10	11.0	10.0 — 11.8	2.9 ± 0.5	10	100.0	9.3 ± 1.3	10	100.0	20.1 ± 1.9	10	100.0	25.8 ± 1.6	10	100.0
23	13.4	12.0 — 14.8	4.7 ± 0.5	23	100.0	12.3 ± 0.5	23	100.0	23.5 ± 0.6	23	100.0	23.5 ± 0.7	23	100.0
8	15.7	15.0 — 17.2	5.6 ± 0.9	8	100.0	14.5 ± 1.5	8	100.0	25.9 ± 1.3	8	100.0	23.6 ± 1.5	8	100.0
9	28.7	27.0 — 30.5	2.6 ± 1.5	9	100.0	6.8 ± 1.0	9	100.0	17.4 ± 1.6	9	100.0	15.3 ± 2.2	8	88.9
8	33.5	31.3 — 37.5	3.4 ± 0.1	8	100.0	6.3 ± 0.9	8	100.0	17.1 ± 1.7	8	100.0	12.8 ± 1.4	8	100.0
5	50.2	48.0 — 53.0	1.6 ± 0.4	5	100.0	2.2 ± 0.6	4	80.0	14.4 ± 1.0	5	100.0	2.4 ± 1.3	2	40.0
4	73.0	70.0 — 75.0	1.5 ± 0.4	4	100.0	0.8 ± 0.4	2	50.0	10.3 ± 0.2	4	100.0	—	—	—
6	81.7	77.0 — 95.0	1.5 ± 0.2	6	100.0	0.8 ± 0.6	4	66.7	12.8 ± 1.4	6	100.0	—	—	—

Landlocked salmon

11	5.7	4.4 — 7.5	3.4 ± 0.4	11	100.0	13.3 ± 0.5	11	100.0	31.4 ± 1.0	11	100.0	33.4 ± 0.7	11	100.0
10	17.4	16.0 — 19.0	4.9 ± 0.5	10	100.0	11.8 ± 0.7	10	100.0	24.8 ± 0.8	10	100.0	19.3 ± 1.1	10	100.0
14	26.2	24.5 — 28.0	3.2 ± 0.3	14	100.0	6.4 ± 0.8	14	100.0	22.0 ± 0.6	14	100.0	14.7 ± 0.8	14	100.0
8	40.4	37.0 — 42.5	1.9 ± 0.3	8	100.0	1.5 ± 0.4	6	75.0	17.8 ± 1.2	8	100.0	—	—	—
8	46.7	44.0 — 51.0	1.9 ± 0.1	8	100.0	1.8 ± 0.5	4	50.0	15.8 ± 0.6	8	100.0	0.7 ± 0.7	1	12.5

Sea-running salmon

n	Length (cm) Mean	Range	Fraction C4			Fraction C6			Fraction C7			Fraction C8		
17	4.7	3.5 — 5.7	16.9 ± 0.7	17	100.0	—	—	—	—	—	—	—	—	—
9	9.1	8.0 — 9.6	20.3 ± 1.2	9	100.0	4.8 ± 2.0	4	44.4	2.6 ± 1.4	3	33.3	0.9 ± 0.6	2	22.2
10	11.0	10.0 — 11.8	19.2 ± 1.1	10	100.0	10.5 ± 1.5	9	90.0	5.1 ± 1.5	6	60.0	3.1 ± 1.2	5	50.0
23	13.4	12.0 — 14.8	19.9 ± 0.5	23	100.0	7.3 ± 0.9	21	91.3	3.2 ± 0.5	17	73.9	1.4 ± 0.4	11	47.8
8	15.7	15.0 — 17.2	19.0 ± 2.1	8	100.0	5.0 ± 1.3	7	87.5	1.3 ± 0.5	4	50.0	0.1 ± 0.1	1	12.5
9	28.7	27.0 — 30.5	24.9 ± 1.4	9	100.0	7.4 ± 1.2	8	88.9	12.9 ± 1.7	9	100.0	7.2 ± 1.9	8	88.9
8	33.5	31.3 — 37.5	25.9 ± 1.5	8	100.0	3.1 ± 1.2	4	50.0	19.3 ± 2.2	8	100.0	8.0 ± 2.2	8	100.0
5	50.2	48.0 — 53.0	33.6 ± 3.0	5	100.0	—	—	—	32.6 ± 1.8	5	100.0	9.8 ± 1.4	5	100.0
4	73.0	70.0 — 75.0	33.5 ± 1.3	4	100.0	—	—	—	34.5 ± 1.7	4	100.0	16.3 ± 0.4	4	100.0
6	81.7	77.0 — 95.0	34.3 ± 2.0	6	100.0	—	—	—	31.2 ± 2.2	6	100.0	15.2 ± 1.3	6	100.0

Landlocked salmon

11	5.7	4.4 — 7.5	14.9 ± 1.3	11	100.0	—	—	—	—	—	—	—	—	—
10	17.4	16.0 — 19.0	24.5 ± 1.0	10	100.0	7.3 ± 1.5	10	100.0	3.1 ± 1.1	6	30.0	0.4 ± 0.2	3	30.0
14	26.2	24.5 — 28.0	29.3 ± 0.6	14	100.0	1.8 ± 0.7	6	42.9	15.9 ± 1.3	14	100.0	2.6 ± 0.3	14	100.0
8	40.4	37.0 — 42.5	35.9 ± 0.8	8	100.0	—	—	—	30.4 ± 0.7	8	100.0	10.0 ± 0.6	8	100.0
8	46.7	44.0 — 51.0	34.1 ± 0.9	8	100.0	—	—	—	31.6 ± 1.2	8	100.0	11.1 ± 0.6	8	100.0

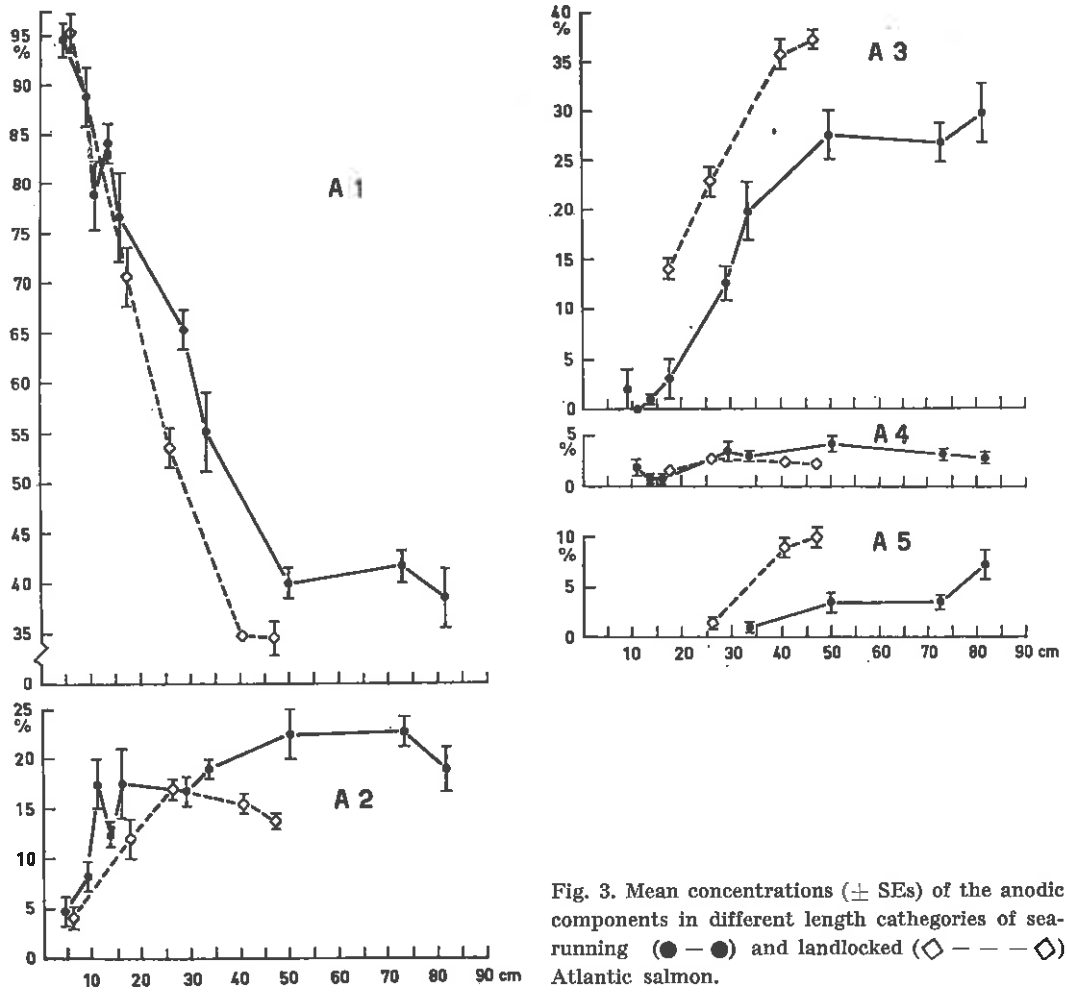

Fig. 3. Mean concentrations (± SEs) of the anodic components in different length cathegories of sea-running (● — ●) and landlocked (◇ — — — ◇) Atlantic salmon.

3.2. The ontogenetic development of the components

The ontogenetic development of the hemoglobin pattern described only in outline above cannot, however, give any real idea of the ontogeny of the different components. To elucidate this the sea-running salmon has been divided into ten and landlocked salmon into five length cathegories and the mean concentration of each component has been calculated of these (Tables 2 and 3). The values appear also in Figs 3 and 4.

Fish in the first sea-running salmon group were 3—5 months, in the second group 12 and 17 months, in the third, fourth and fifth groups 17 months old. Fish in the first landlocked salmon group were 5 months, in the second group 17 months, in the third group 30 months, and in the fourth and fifth groups 53 months old. This means that the fish were of the same age in the third, fourth and fifth group of sea-running and the second group of landlocked salmon and even the sampling time was about the same, but the growth rate has been faster in landlocked salmon.

Individual differences in the relative intensity of different hemoglobin components were quite small in fish of the same length, especially in the cathodic group, in which the separation was sharper than in the anodic group. This suggests that the ontogenetic development of the hemoglobin proceeds in a regular manner in Atlantic salmon. Onto-

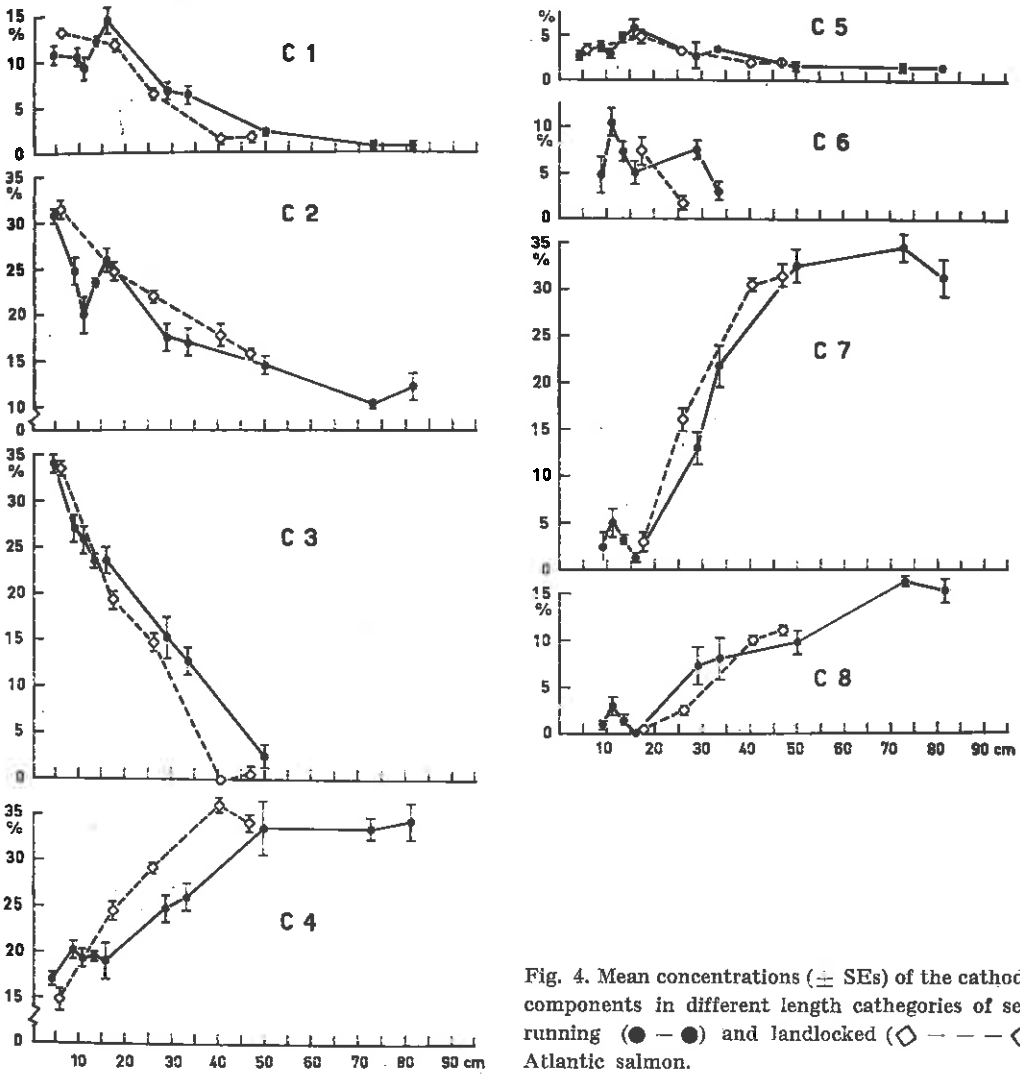

Fig. 4. Mean concentrations (± SEs) of the cathodic components in different length cathegories of sea-running (● — ●) and landlocked (◇ — — — ◇) Atlantic salmon.

genetic development takes places during the whole life cycle of the salmon even if most changes, when compared with the increase of the length of the fish, occur in juvenile fish, of a length below 20 cm.

In the relative concentration of some anodic and cathodic components of sea-running salmon of about 12—16 cm an abrupt change was observed, which clearly deviated from the overall trend of concentration of these components (Figs 3 and 4). In the anodic components the greatest changes took place in the fourth length cathegory (mean length 13.4 cm), and in the cathodic components in the fifth length cathegory (mean length 15.7 cm), respectively. Those components, which are conspicuous in juvenile fish and which exhibit a trend to smaller concentration with increasing length, increased in the mean concentration. This was especially clear in components A1, C1 and C2, in which the increase was about 5—6 %. The change was on opposite direction in those components, which increase in concentration with increasing length, and was greatest in components A2, C7 and C8.

The concentration of those components, which are conspicuous in juvenile fish, in-

creased particularly clearly. So the hemoglobin pattern of fish 14—16 cm in length (in these the increase was the greatest) was at the same developmental stage as in fish of the length of 8—9 cm, i.e. they exhibited a less developed pattern than expected for their length. This suggests that the hemoglobin pattern had rejuvenated for a short duration. A particular interest lies in the fact that this change occurs approximately in the length group, in which the smolting already is taking place.

The above mentioned changes were observed in fish of the third, fourth and fifth length cathegories. The 41 specimens comprising these groups were (save for two specimens) of the same origin, reared at Montta hatchery in the same circumstances and were also of the same age (17 months). The sampling took also place at the same time in the autumn 1968. The only thing, which differed in these particular length groups was that eight of the total of ten fish in the third group had been fed with Clark's New Age Fish Feed dry pellets (Dansk Ørredfoder AS, Brande, Denmark), when 27 of a total of 31 fish in the fourth and fifth length groups had been fed with Forelli (Vaasan Höyrymylly Oy, Helsinki, Finland) and Ewos (Ewos Ab, Södertälje, Sweden) dry pellets. Salmon fed with Clark were besides of being small also highly anemic (mean hematocrit 21.9 %; mean hemoglobin concentration 2.2 g/100 ml). These values differed significantly ($P < 0.001$) from those obtained with fish fed Forelli (mean hematocrit 53.7 %; mean hemoglobin 8.0 g/100 ml) and Ewos (mean hematocrit 55.9 %; mean hemoglobin 8.5 g/100 ml) (Westman & Sumari, in preparation). The hematocrit and hemoglobin concentration were, however, normal in fish in the fourth and fifth length groups, in which the observed change in the concentration of components was greatest.

No change in the overall hemoglobin pattern could, however, be noticed in fish afflicted with severe nutritional anemia.

Special attention was paid to the fact that the described changes occured about at the same strength and in the same length cathegories in both anodic and cathodic hemoglobin components. As already mentioned in »Methods» the relative concentration of each component has been calculated separately from the total anodic or cathodic concentration, so that the values obtained for the anodic components are independent of the values of the cathodic components.

Whether this phenomenon described above also occurs in landlocked salmon remains uncertain as the material was incomplete.

The proportion of those components, which are in high concentration in juvenile fish decreased with increasing length of fish faster in landlocked salmon and those components which had a trend to greater concentration with increasing length increased faster in landlocked salmon, respectively (Figs 3, 4). This is also reflected in the faster ontogenetic development rate of the hemoglobin pattern of the landlocked salmon when compared with the sea-running salmon as already mentioned (Table 1, Fig. 1).

Save for the differences in the rate of the ontogenetic development mentioned above it was not possible to find any component or pattern characteristic to either sea-running or landlocked salmon. No individual differences in the pattern could be neither observed, i.e. there were no intraspecific hemoglobin variants in the material examined.

The ontogenetic development of each component is presented in outline to clarify the observed variations.

Component A1 was found in all examined specimens. In the smallest individuals it was the only anodic component. This suggests that A1 is ontogenetically the most primitive hemoglobin component. Its concentration decreases rapidly with increasing length. This is more rapid in landlocked salmon (Fig. 3). In all examined sea-running specimens A1 was, however, the most intense fraction during the whole life cycle and its relative concentration did not fall below 30 % in any examined fish. But in many landlocked salmon above 37 cm and in all of above 44 cm length the component A3 exhibited a higher concentration than A1.

Component A2 appears soon after component A1 and was lacking only in the smallest individuals. A2 exhibited in landlocked salmon the highest concentration (about 25 %) in fish between 25—30 cm but in searunning salmon (about 30 %) not until in fish between 50—75 cm in length.

Component A3 was not present in juveniles. It exhibited an increase in concentration with increasing length and in sea-running salmon

it attained the greatest concentration (37—38 %) in fish above 50 cm and in landlocked salmon (41—44 %) already in fish above 40 cm, respectively.

Component A4 was not present in juveniles and appeared about at the same time as component A3. Its concentration did not exceed 10 % in sea-running salmon and 6—8 % in landlocked salmon.

Component A5 appeared in sea-running salmon about at the length of 34 cm but in landlocked salmon it was already observed at the length of 25 cm. It increaded in intensity during growth and this was much faster in landlocked than in sea-running salmon. Consequently the greatest observed concentration of component A5 (15 %) was observed in sea-running salmon above 70 cm but in landlocked salmon already at the length between 40—50 cm.

Component C1 decreased in concentration with increasing length save for an abrupt increase in sea-running salmon of the length between 15—17 cm. C1 was only faintly present or totally absent in landlocked salmon over 40 cm and sea-running salmon over 50 cm.

Component C2 was present in all fish examined. It was at highest concentration (35 %) in the smallest individuals and decreased in concentration with increasing length to about 15 % in the largest landlocked salmon. C2 represents with C3 the major cathodic juvenile hemoglobin components.

Component C3 exhibited maximum intensity in the smallest individuals and decreased with increasing length. It was totally absent already in all landlocked salmon over 37 cm but in sea-running salmon not until the fish were above 50 cm.

Component C4 was with the components A1, A2 and C5 (excluding one individual) the only component present in all examined fish. C4 was present at a concentration of 15 % in the smallest fish and developed in landlocked salmon during the growth from 20 to 50 cm and in searunning salmon from 25 to 50 cm to be the major adult hemoglobin component of the C group. In sea-running salmon above 50 cm the concentration of C4 and C7 was about the same but in individuals over 75 cm component C4 exhibited a small increase in concentration.

As can be seen from the densitometric curves (Fig. 2) components C3 and C4 are in a way »twin» components. The latter is in small fish only a faint swelling in the side of component C3 but the concentration of C3 decreases and the concentration of C4 increases with increasing length and in fish over 25 cm component C3 can be seen in turn as a small swelling at the side of component C4 until it is finally covered by C4.

Component C5 was present excluding one small specimen in all examined fish. This component showed as a whole only small fluctuations in the relative concentration. Because of the constancy it does not considerably influence the overall development of the hemoglobin pattern.

Component C6 represents a kind of an intermediate component of small duration in terms of length of the fish. It was not present in the smallest specimens and decreased very soon so that it was already absent in landlocked salmon of the length 25—30 cm and in sea-running salmon at 35—40 cm, respectively.

Component C7 was not found in juveniles but was present in strong concentration in all adults. It developed during the growth to 40 cm in landlocked salmon and to 50 cm in sea-running salmon to as high concentration as component C4, i.e. to 30—35 %. Components C6 and C7 represent also »twin» components as did components C3 and C4.

Component C8 is the fastest cathodic component. It was not present in juveniles. C8 was not regularly observed in landlocked salmon until at the length of 20—25 cm and little larger sea-running salmon. A trend to increase in concentration with length was observed but this was much more slower than in component C7. Component C8 developed to the highest concentration in landlocked salmon of 48—51 cm (11—13 %) and in sea-running salmon of 75 cm (18—20 %).

3.3. The proportion of anodic and cathodic hemoglobins

Along with the ontogenetic development in the hemoglobin pattern, i.e. in the number and relative concentration of the different components, the proportion of total anodic hemoglobin (called Hb A) was found to decrease and total cathodic hemoglobin (called Hb C) to increase with increasing lengt. This trend, which was first observed in Atlantic salmon by Koch *et al.* (1966), was

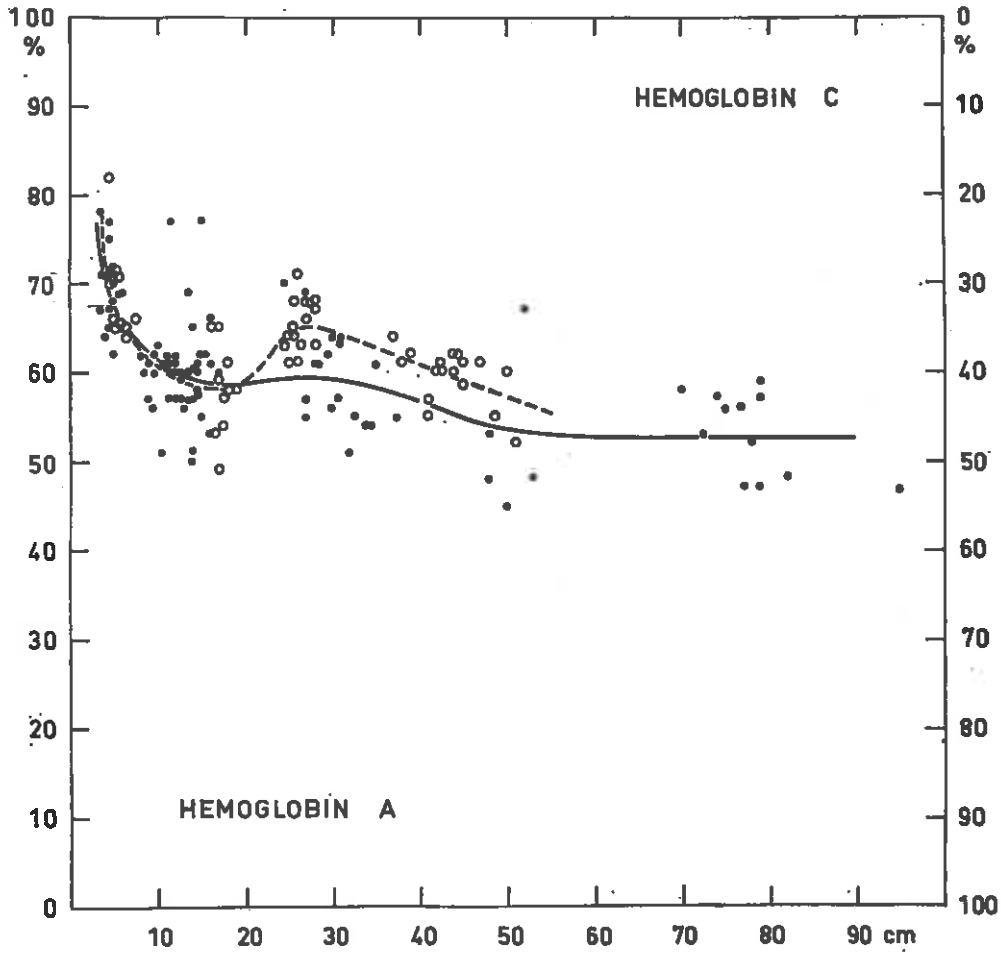

Fig. 5. Proportion of anodal (Hb A) and cathodal (Hb C) migrating hemoglobins of sea-running (● — ●) and landlocked (○ — — — ○) Atlantic salmon of different length.

found to be mainly a result of the very fast decrease of the concentration of the main juvenile component A1. The other appearing anodic components did not increase in concentration as much as A1 decreased. Of the cathodic components particularly C4 and C7 increased in concentration. Fig. 5 gives an outline of the proportion of anodal and cathodal hemoglobins as a function of the length of the fish. During the period of growth from 3.5 to 10.0 cm, the proportion of Hb A decreased from about 71 % to about 60 %. The trend of the curve suggests that in very small individuals the proportion of the Hb A will reach 100 % asymptotically and Hb C will be virtually lacking. Concomitant with this the distance between the cathodic components C1—C4 becomes shorter as already mentioned.

Of particular interest was the fact that a significant trend to higher concentration of Hb A was observed in landlocked salmon during the growth from about 20 to about 28 cm. The mean value of Hb A increased from about 58 % to 65 % ($P < 0.001$). During subsequent growth the proportion of Hb A decreased again and in the length group of 40—45 cm the mean value of Hb A was about 60 % ($P < 0.001$). This change seems to result to a great extent from the fast increase in the concentration of the components A2, A3 and A5 and of the decrease of the components C1 and C2 (Figs 3, 4).

A small increase in the proportion of Hb A was also found in sea-running salmon during the growth from about 17 to 31 cm.

4. Discussion

4.1. Discussion of the methods

Blood sampling is most frequently done by excising the caudal fin. This method cannot, however, be used if repeated samplings are desired, if valuable fish or a large number of fish are to be examined. Other methods such as heart puncture, puncture of the caudal artery or vein (Itazawa, 1957; Snieszko, 1960; Steucke & Schoettger, 1967), drawing of blood from the Cuverian ducts (Sano, 1960), repeated puncture or cannulation of the dorsal aorta (Schiffman, 1959; Conte, Wagner & Harris, 1963; Smith & Bell, 1964; Thurston, 1967), have been succesfully used but these methods are relatively slow and require the preparation of syringes and needles. As often in taxonomic work sampling of large numbers of individuals are to be done, the sampling and also the preparation of blood must be rapid and simple. The sampling method described by Perkins (1957), Suyehiro (1958) and Larsen & Snieszko (1961), in which pointed capillary tubes were used for cardiac punctures, was found to be very fast and practical, especially when taking blood samples from small valuable fish, which should be kept alive. There is no loss of the sample as it is taken directly into the hematocrit capillary, where it can also be washed and hemolysed for electrophoresis after the hematocrit determination as described in the present paper.

Steucke & Schoettger (1967) have lately described a very practical modification of the above mentioned method, in which an incision is first made into the sinus venosus and the blood sample is then taken with a capillary. By this method the possibility that body fluids may dilute the blood sample is very small and no pointed capillary tubes are needed.

When hemoglobin separation was made in microfuge tubes dry EDTA (ethylene diaminetetraacetate) powder was also tried as anticoagulant as suggested by Smith & Bell (1964, 1967). As this was found to cause irregularities in the hemoglobin electrophoretic pattern it was omitted.

The micro starch gel electrophoresis method described by Koch *et al.* (1964), which was used with slight modifications in the present study, provides an exceptionally sharp and reproducible resolution of salmon hemoglobin in a short time. It is superior especially when great number of specimens are to be analysed within a short time as is usually the case in taxonomic studies. The high quality of the electropherograms is largerly dependent on the very thin starch gel layer (1.2 mm), on the thin and straight starting line, the insertion of the sample directly into the gel without any supporting medium, the direct contact between the bridge buffer and the gel and the relatively high voltage gradient, which shorten the time of electrophoresis run.

As Smithies (1959) has pointed out the sample should be inserted into the gel without any supporting material, so that adsorption effects are completely abolished. However, severe electrodecantation effects have resulted in horizontal gels and if the gel is to be sliced before staining there is no plane, along which a true distribution of the protein zones can be observed (Smithies, 1959). This difficulty is overcome in the method used in this study were thin gels need not to be sliced after the electrophoresis. They are stained as a whole and the components taking the dye are observed through the gels by tansmitted light instead of being observed by reflected light only at the cut surfaces. The advantage of this technique has been discussed by Smithies (1959).

A great advantage of the method used is that the sample can be very small, only about 0.2—0.5 µl. This micro starch gel electrophoresis method combined with the described micro method of blood sampling and preparation make possible the individual examination of even the smallest fish specimens.

4.2. Multiple hemoglobins of Atlantic salmon

As pointed out by Manwell & Schlesinger (1966) multiple hemoglobins may be the result of developmental sequences (larval, embryonic or fetal hemoglobins), of tissue specificity (muscle and blood hemoglobins), of genetically based individual variation (»abnormal» hemoglobins), or of an intrinsic property of the hemoglobin of all the individuals of a given species (e.g. human Hb A and Hb A_2) (cf. Schroeder, 1963).

With techniques as diverse as e.g. electrophoresis, chromatography, fingerprinting, alkali denaturation and oxygen affinity tests multiple hemoglobins have been demonstrated in many vertebrate species. Gratzer & Allison (1960) and Muller (1961) have reviewed the whole field.

Hemoglobins of several species of salmonids have been shown to separate by electrophoresis into two, three or four components or to one or two groups of multiple components depending on the method and especially on the stabilizing media used. The hemoglobins of Pacific salmon, genus *Oncorhynchus*, split by means of electrophoresis on paper into two components (Buhler & Shanks, 1959) and by free boundary electrophoresis into two to four components depending on species (Hashimoto & Matsuura, 1959a, b, 1960a). But by electrophoresis on starch or polyacrylamide gel it has been possible to demonstrate the existence of as many as 22 hemoglobin components (e.g. Tsuyuki & Gadd, 1963; Vanstone *et al.* 1964; Tsuyuki *et al.* 1965a, b). Similarly two or three hemoglobin components have been observed by means of paper, agar gel and free moving boundary electrophoresis with brown trout (*Salmo trutta*) rainbow trout (*S. gairdneri*), brook trout and arctic char (*Salvelinus fontinalis* and *S. alpinus*), grayling (*Thymallus thymallus*) and some *Coregonine* fishes (Schumann, 1959; Buhler & Shanks, 1959; Hashimoto & Matsuura, 1959a, 1960a; Buhler, 1963). But in these species as well as in lake trout (*Salvelinus namaycush*) and in Dolly Varden (*S. malma*) a great number of components can be resolved by starch gel electrophoresis (Tsuyuki & Gadd, 1963; Tsuyuki *et al.* 1965a, 1966; Yamanaka *et al.* 1967; Westman, unpublished data). Using cellulose acetate membranes Burke (1965) reported six hemoglobin bands for rainhow trout, four bands for brown trout and three bands for brook trout.

The situation is quite similar in Atlantic salmon. Schumann (1959) found that the hemoglobins of Atlantic salmon separate into two components on agar but lately Haen & O'Rourke (1968) obtained on agar as much as seven different hemoglobin components. The difference in these studies may result from different stages in the ontogenetic development of the hemoglobin of the examined salmon or perhaps the hemoglobin in the latter study had dissociated into subunits. Two components of different electrophoretic mobility are resolved on paper (Koch *et al.* 1964). By means of starch gel electrophoresis the hemoglobins of Atlantic salmon split, however, into two groups of multiple components. As many as 13 hemoglobin fractions, five of which were anodal and eight cathodal, were obtained by Koch *et al.* (1964) and also in the present study. Wilkins (1968) obtained with a different technique in salmon nine anodic and seven cathodic components giving a total of 16 components. The number of bands appears to depend very much on the buffer type and its ionic strength (e.g. Koch, Wilkins, Bergström & Evans, 1967).

It should be pointed out that the existence of two groups of multiple components in Atlantic salmon depends, however, on the stage of the ontogenetic development of the hemoglobin. In specimens of the length 3—4 cm the anodic hemoglobin is still represented by only one component (A1).

When Koch *et al.* (1964) separated by centrifugation the two hemoglobin components obtained with paper electrophoresis and electrophoresed these again, now on starch gel, the two fractions on paper corresponded to the anodal and cathodal groups of multiple components observed by direct electrophoresis on starch gel. Each group gave the expected number of fractions.

The reproducibility of the results obtained with starch gel and the occurrence of repeatable variations in identically treated samples seem to exclude the occurrence of *in vitro* artefacts as an explanation of the observed phenomena (cf. Koch *et al.* 1964; Wilkins, 1968). The components were identified as hemoglobins by means of o-dianiside- and benzidine reagents.

As the molecule sieve effect of the paper and agar gel electrophoresis is negligible, the proteins are separated mainly according to their charge. In starch gel electrophoresis the protein molecules are separated according to their electric charge, molecular size and possibly shape (e.g. Smithies, 1959, 1962; McDougall & Synge, 1966; Ogston, 1966; Porter, 1966).

This would mean that the anodic and cathodic hemoglobin components of Atlantic salmon have identical electrophoretic charges but different molecular sizes, which may be

taken as an evidence of that salmon has only two structurally different hemoglobins. Thus the great number of components obtained on starch gel would not represent hemoglobins, which are synthesized under different growth and physiological conditions but might be polymers or fractions of bigger molecules.

The hemoglobins of a number of animal species have been found to have a tendency to polymerize (discussed e.g. by Riggs, 1965). Polymerization seems not, however, to be correct explanation for the multiple components in Atlantic salmon. Wilkins (1968) has observed with preliminary elution experiments that human and adult salmon hemoglobins have about the same molecular weight (ca 70 000) and in the hemolysates of the salmon seem not to be any larger hemoglobin molecules. Further Wilkins has observed that at least those fractions analysed seemed to have two major globin components and different fractions had different pairs of components. According to Wilkins it seems likely that fractions analysed differ in one or more of their constituent polypeptide chains and not in varying states of oxidation or of molecular aggregation.

It is, however, difficult to explain why these structurally different hemoglobins of Atlantic salmon are not revealed on paper or agar gel electrophoresis.

Koch *et al.* (1968) and Koch (1969) have also made an attempt to clarify the nature of fractions obtained with starch gel by means of isoelectric focusing in a natural pH gradient with the method of Svensson (1961, 1962) and Vesterberg & Svensson (1966). With this method it was possible to separate a large number of bands (about 20) from the hemolysate of the Atlantic salmon on the basis of their respective isoelectric points. Koch *et al.* (1968) supposed that these bands were molecular species of hemoglobins and not either polymers or fractions of bigger molecules.

There seem, however, to be still some discrepancies in the results or rather in the interpretation of the results obtained with paper electrophoresis and electrofocusing, i.e. with techniques, in which the stabilizing media are inert. According to my opinion Koch *et al.* (1968) and Koch (1969) showed by electrofocusing that the different hemoglobin bands have different isoelectric points and on this evidence they seem not to be polymers. More evidence should, however, be obtained before all these components can be taken to be different hemoglobins.

As such it is not impossible that Atlantic salmon could possess as many as 20 structurally different hemoglobins, but such a great number of hemoglobins would be, according to the present knowledge, unique in the animal kingdom. The greatest number of hemoglobins have been until now found, as far as I know, in white-tailed deer (*Odocoileus virginianus*), which had nine distinguishable hemoglobins (Kitchen, Putnam & Taylor, 1967). Especially interesting was the observation that two different α-chains, six different β-chains and one γ-chain was identified. This means that the heterogeneity of the white-tailed deer hemoglobins is based upon a variety of combinations of these numerous polypeptide chains. Further Baglioni & Sparks (1963) found that the tadpole of the bullfrog, *Rana catesbeiana*, possesses four hemoglobins and the adult frog four other hemoglobins. Rumen & Love (1963) reported six hemoglobins of the sea lamprey (*Petromyzon marinus*).

In this connection it is interesting to note that chum salmon (*Oncorhynchus keta*), whose hemoglobins split on paper electrophoresis into two components but on starch gel into two groups of multiple components, possesses only two hemoglobins. These differ in such properties as solubility, absorption spectrum, heat coagulability, amino acid composition and oxygen dissociation curve (Hashimoto & Matsuura, 1959a, b, c, d; Eguchi, Hashimoto & Matsuura, 1960; Hashimoto, Yamaguchi & Matsuura, 1960). Similarly, despite of the multiple nature of the mouse hemoglobin obtained with starch gel electrophoresis there appears to be only two hemoglobins with different amino acid sequences in their β-chains (Schwartz & Gerald, 1967).

A possible explanation to the great number of hemoglobin components might be the fractionation of bigger molecules. The hemoglobin molecule of higher vertebrates, from teleost fish to mammals, is a tetramer (e.g. Ingram, 1963), and the molecule has a tendency to dissociate already under mild conditions into halves or even into quarter molecules, i.e. individual polypeptide chains (discussed e.g. by Goldberg, 1961; Schroeder, 1963; Antonini, 1965; Riggs, 1965; Gilbert, 1966). The ontogenetic development of the

hemoglobin of Atlantic salmon would perhaps result from the different tendency of the molecule to dissociate and reassociate to form molecular hybrids during the growth of the fish. This could in turn result from a number of factors, e.g. changes in enzyme activities during the growth and in different physiological stages (cf. Riggs, 1965).

If Atlantic salmon possesses two hemoglobins and the tetramer hemoglobin molecule is composed of two different kind of polypeptide chains then three polypeptide chains are necessary to explain these and their primary structure would be controlled by three different structural genes. If, however, salmon possesses 20 hemoglobins, then a minimum of seven polypeptide chains is needed and if they would structurally resemble the human hemoglobins, in which α-chain is common for Hb A, Hb A_2 and fetal hemoglobin (e.g. Ingram, 1963), then a far greater number of different polypeptide chains would be needed. At this point the number of polypeptide chains and hemoglobins of the Atlantic salmon remains an open question.

4.3. Ontogeny of the hemoglobin

In all sufficiently studied mammals and a number of other vertebrates the fetus or larva has had a hemoglobin that is biochemically distinct from that of the adult (reviewed by Gratzer & Allison, 1960; Muller, 1961). Besides the fetal hemoglobin the occurrence of a distinct embryonic hemoglobin type has been described in a number of vertebrates (e.g. Halbrecht & Klibanski, 1956; Manwell, Baker, Roslansky & Foght, 1963; Huehns *et al.* 1964; Kleihauer, Brauchle & Brandt, 1966; Schmid & Thein, 1967). This embryonic, fetal or larval hemoglobin normally disappears completely shortly after the birth or metamorphosis as in frogs (Riggs, 1951; Baglioni & Sparks, 1963) and is replaced by adult hemoglobin.

Ontogenetic studies have revealed that also some fish species possess distinct larval, fetal or juvenile and adult hemoglobins. This phenomenon has until now been found at least in *Scorpaenichthys marmoratus* (Manwell, 1957), *Raja binoculata* (Manwell, 1958a), *Squalus suckleyi* (Manwell, 1958b, 1963a), *Petromyzon planeri* (Adinolfi & Chieffi, 1958), *Embiotica lateralis* (Manwell, 1960), *Clupea harengus* (Wilkins, 1963), *Acipenser güldenstadti* (Golovanenko, 1964), *Oncorhynchus kisutch* (Vanstone *et al.* 1964) and *Lepomis gibbosus* (Callegarini, 1966). On the other hand in some fish species the hemoglobin has been found to undergo a gradual ontogenetic change during a great part of the life cycle. This has been observed in some species of the genus *Oncorhynchus* (Hashimoto & Matsuura, 1960b; Vanstone *et al.* 1964), in *Salmo trutta* (Koch *et al.* 1966; Westman, unpublished), in *Salvelinus fontinalis* and *Salmo gairdneri* (Yamanaka *et al.* 1967) and in above mentioned *Acipenser güldenstadti* (Golovanenko, 1964) and *Clupea harengus* (Wilkins & Iles, 1966).

The hemoglobin of the Atlantic salmon undergoes a dual ontogenetic development. First the hemoglobin pattern undergoes ontogenetic development comprising changes both in the number and in the relative intensities of the anodic and cathodic components. Concomitant with this the amount of the total anodal hemoglobin decreases with increasing size of the fish and the amount of cathodal hemoglobin increases, respectively. Even if the greatest ontogenetic changes in both the hemoglobin pattern and the Hb A/Hb C ratio occur in juvenile salmon it is of particular interest that the development proceeds throughout the whole life cycle.

In the present study the mean lengths of salmon corresponding to the eight observed hemoglobin pattern have been determined. Instead of length, different stages in the life history of the Atlantic salmon can be compared with the hemoglobin patterns, of course only in broad outline. Patterns I and II are associated with the juvenile salmon living in its natal river before smolting, patterns III and IV are associated with the parr-smolt transformation and the migration from the river to sea or lake, patterns VI—VIII correspond with the life phase in sea or lake including the migration of sexually mature salmon back to their native rivers. However, it should be pointed out that the changes in habitat cannot be considered as a direct reason to the change in the hemoglobin pattern. The ontogenetic development of the hemoglobin of landlocked salmon kept for their whole life in hatchery and of sea-running salmon artificially retained in fresh water (e.g. fish No. 3 in Fig. 2) was also found to be normal (cf. Koch *et al.* 1964).

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ıdl, 1960). As an explanation
:ygen affinity of the Hb F in
ınstone *et al.* (1964) suggested
›lting young Pacific salmon
ıtic salmon) leave their well
/ers and move to the sea where
ıay lower the oxygen tensions
that they need a hemoglobin
oxygen affinity. According
Pacific- and Atlantic salmon
y need a larval hemoglobin
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nding oxygen because the
he egg as well as the hatched
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wning gravels, depending of
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ally injured or infested with
oportion of juvenile Hb A is
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4.5. The application of hemoglobin electrophoresis to taxonomic studies of the salmon

It has been postulated that the genes for hemoglobin of vertebrates evolved from a common ancestral gene (discussed by Ingram, 1961, 1963). The number of differences in the amino acid sequence of a given polypeptide chain between two animal species is an expression of mutations reflecting the time, at which the two species branched off from a common ancestor (e.g. Ingram, 1961, 1963; Zuckerkandl & Pauling, 1962; Zuckerkandl, 1965). The primary structure of hemoglobin has only a low capacity of environmental modification (Zuckerkandl, 1965), which makes this protein useful for classifications at the species, generic and higher taxonomic levels. On the other hand this also restricts the use of hemoglobin e.g. in population studies.

In most taxonomic studies hemoglobins are analysed by electrophoresis and the obtained patterns are compared. If these are identical the compared specimens are considered identical in this respect. If valid interspecific comparisons are made using the electrophoretic patterns, intraspecific ontogenetic variations and intraspecific genetic polymorphism must be known (cf. Baker, Manwell, Labisky & Harper, 1966) as well as the restrictions of electrophoresis.

Intraspecific variations in hemoglobin electrophoretic pattern have been found in some fish species (Sick, 1961; Tsuyuki *et al.* 1966, 1968; Westrheim & Tsuyuki, 1967; Naevdal, 1968; Schlotfeldt, 1968). The best known case is the intraspecific variation in the electrophoretic mobility of cod (*Gadus morhua*) hemoglobin between samples from different localities, first described by Sick (1961). This could be explained with a co-dominant allelic gene hypothesis providing a valuable basis in taxonomic studies of this species at the subspecific level (e.g. Moller & Sick, 1963; Frydenberg *et al.* 1965; Sick, 1965a, b; Møller, 1966). Rattazzi & Pik (1965) found that the polymorphic hemoglobin bands of cod differed by a single peptide in the chymotryptic digest of the globin.

When electropherograms are compared it must be kept in mind that such mutations in hemoglobin molecules, which do not involve a change in the charge, e.g. a mutation from one neutral amino acid residue to another, are not

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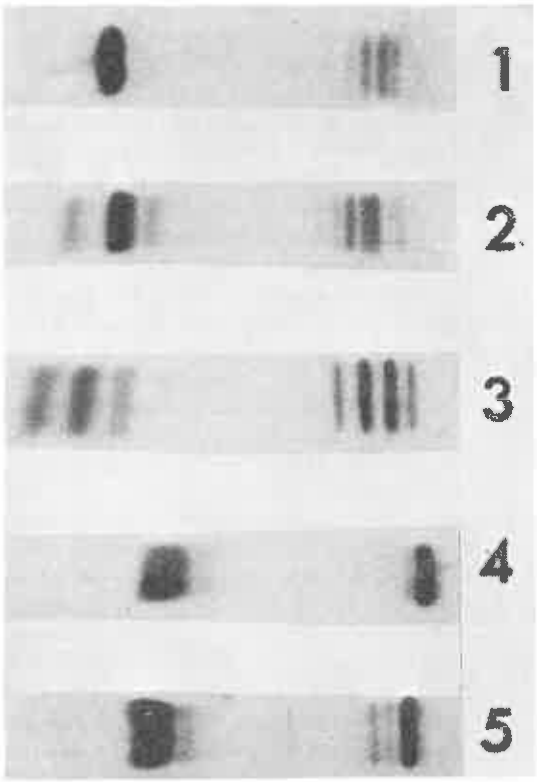

Fig. 6. Starch gel hemoglobin electrophoresis patterns of three Atlantic salmon (1—3) and two brown trout (4 and 5). Sample 1 is a 8 cm long sea-running salmon, sample 2 a 25 cm long landlocked salmon and sample 3 a 75 cm long sea-running salmon. Brown trouts are 33 cm (5) and 70 cm (4) in length. Anode to the left.

recognised with electrophoresis. There are some examples of hemoglobins, which have identical electrophoretic mobility but which are biochemically different. Manwell (1963c) found that adult hemoglobin in *Ichthyomyzon unicuspis* and *Petromyzon marinus* have identical electrophoretic behaviour but show significant differences in oxygen equilibrium and Bohr effect and small differences in the primary structure. Similarly one *Rana catesbeiana* tadpole hemoglobin has the same electrophoretic mobility as a frog hemoglobin but these hemoglobins have a completely different pattern of peptides as revealed by fingerprinting (Baglioni & Sparks, 1963).

One of the aims of the present study was to examine by means of the hemoglobin patterns and their ontogenetic development if the landlocked salmon of Lake Saimaa is really a salmon as suggested by Seppovaara (1962) on the basis of meristic and morphometric characters or a brown trout population as earlier thought. As can be seen from the Fig. 6 the hemoglobin pattern of landlocked salmon was markedly different from that of the brown trout originating from Lake Saimaa but exactly the same as in sea-running salmon. The hemoglobin of brown trout separated on starch gel usually into a single anodal componend migrating on the mean identically with component A1 in Atlantic salmon and to a group of cathodal components. The pattern of this cathodal hemoglobin differed also from the cathodal hemoglobin pattern in landlocked salmon so that the smallest salmon possessing only a single anodal component could be separated from brown trout. The electrophoretic pattern of the cathodal hemoglobin of brown trout seems to be irregular.

These observations support the suggestion by Seppovaara (1962) that in the Lake Saimaa there is a landlocked salmon population. It seems not, however, justified to give to this landlocked salmon a scientific name *Salmo salar saimensis* as proposed by Seppovaara (1962). This would imply that landlocked salmon is a subspecies of sea-running salmon. Koli (1969) has pointed out that no such characteristics, which would be typical for this landlocked salmon population, have been described. So it seems more justified, at least at this moment, to consider this landlocked salmon as well as other landlocked salmon populations as ecological races and use only the scientific name *Salmo salar* (cf. Koli, 1969).

Hemoglobin electrophoresis can be used to identify landlocked salmon as well as sea-running salmon from brown trout. A much smaller material was needed when starch gel electrophoresis of hemoglobins was applied than when meristic and morphometric characters were used (Seppovaara, 1962) to identify the landlocked salmon population. The starch gel technique, with its better resolution, is superior to agar of paper electrophoresis in this respect (cf. Yamanaka *et al.* 1967).

Because no differences in the hemoglobin patterns of sea-running and landlocked salmon were observed it seems likely that in the hemoglobin genotype structural genes are homo-

geneous. Because hemoglobin is a very old protein, successful amino acid substitutions occur very seldom, approximately one every seven million years (Zuckerkandl, 1965).Landlocked salmon population in Lake Saimaa seems to be phylogenetically so young, i.e. it has been landlocked only after the last Ice Age, that mutations in the genes coding for the primary structure of the hemoglobin have not yet occurred or at least not such mutations, which can be revealed by means of starch gel electrophoresis. The different sea-running salmon populations seem also to be homogeneous as to the hemoglobin genotype. There is a good deal of evidence that different rivers have local salmon populations and they are maintained by the return of the fish to their natal stream so that there is not any significant exchange of genetic material between populations (e.g. Harden Jones, 1968). No differences in the hemoglobin patterns in Atlantic salmon originating from different rivers were observed neither in the present study or by Koch *et al.* (1964, 1966) and Wilkins (1968). Scandinavian salmon seems to be homogeneous with Scottish salmon (Koch *et al.* 1967) and the latter in turn with salmon originating from Canada and Greenland (Wilkins, 1968). The results obtained by electrophoresis suggest that the hemoglobin genotype of Atlantic salmon is homogeneous over its range.

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SUOMEN KALATALOUS 25—43 FINLANDS FISKERIER

25 SJÖBLOM, VEIKKO: Rannikkomme syyskutuinen silakka vuonna 1964 ja sen runsauteen vaikuttavista tekijöistä
Sammandrag: Den höstlekande strömmingen vid våra kuster år 1964 och faktorer som inverkar på nämnda fiskbestånds riklighet.
Summary: Autumn-spawning Baltic herring (Clupea harengus L.) on the Finnish coast in 1964 and factors affecting fluctuations in its abundance. 23 s. (1966).

26 SJÖBLOM, VEIKKO: Rekisteröityjen kalastusalusten toiminta-alue ja kalastuspaikkojen valinnan ekologiset perusteet
Sammandrag: De registrerade fiskebåtarnas verksamhetsområde och de ekologiska grunderna för val av fiskeplatser
Summary: The operating area of registered fishing vessels and ecological background of the choice of fishing places. 26 s. (1966).

27 SJÖBLOM, VEIKKO: Meriveden kumpuaminen ja Porkkalan niemi
Sammandrag: Uppvälvningen av havsvattnet och Porkala udd
Summary: Upwelling of the sea water and the cape of Porkkala. 12 s. (1967).
Suomen kalatalous 1—24 Finlands fiskerier, Sisältö — Innehåll — Contents. 4 s. (1967).

28 HINTIKKA, NIILO: Kalansaalis, kalastajat ja kalastuksessa käytetyt veneet sekä pyynti rekisteröidyillä aluksilla Suomessa vuosina 1964 ja 1965
Sammandrag: Fiskfångsten, fiskarna och de vid fisket använda båtarna samt fångsten ombord på registrerade fiskefartyg i Finland åren 1964 och 1965
Summary: Catch of fish, fishermen, boats used in fishing and fishing with registered vessels in Finland years 1964 and 1965. 28 s. (1967).

29 HURME, SEPPO: Lounais-Suomen lohi- ja taimenjoet
Sammandrag: Lax- och laxöringsåar i sydvästra Finland
Summary: Salmon and trout rivers in southwestern Finland. 17 s. (1967).

30 AIRAKSINEN, KARI: Varmavirran muikku
Sammandrag: Siklöjan i Varmavirta-strömmen (Norra Saimen)
Summary: The vendace (Coregonus albula L.) of Varmavirta in the northern part of Lake Saimaa (SE-Finland) 32 s. (1967).

31 AHLGRÉN, SIMO: Kalastus Näsijärvellä
Sammandrag: Fisket vid Näsijärvi-sjö
Summary: Fisheries at the lake Näsijärvi. 54 s. (1967).

32 SILVO, OLAVI E. J.: Alustavia tutkimuksia eräiden herbisidien myrkyllisyydestä nuorille karpin poikasille (Cyprinus carpio L.)
Sammandrag: Preliminära undersökningar angående några herbisiders toxicitet för karpyngel (Cyprinus carpio L.)
Summary: Preliminary investigations on the toxicity of some herbicides to young carp (Cyprinus carpio L.). 28 s. (1967).

33 SJÖBLOM, VEIKKO: Helsingin edustan vedet ennen jätevesien johtamista saaristoon (1962—63) ja ensimmäisenä keväänä (1964) Finnånlahden jätevesijohdon käyttöönoton jälkeen
Summary: On the sea water outside Helsinki before and after the discharge of sewage into the island area. 46 s. (1968).

34 SILVO, OLAVI E. J.: Vesikasvien torjuntaan käytettävän parakvatin vaikutus kaloihin, kasviplanktoniin ja veden happipitoisuuteen sekä sen häviäminen vedestä
Sammandrag: Effekten på fisk, växtplankton och syrehalt i vattnet av för vattenvegetationbekämpning använt paraquat samt dettas försvinnande ur vattnet
Summary: The influence of paraquat, a herbicide used in aquatic weed control, upon fish, phytoplankton and the oxygen content of water and its disappearance from the water. 20 s. (1968).

35 SILVO, OLAVI E. J.: Tutkimus parakvatin akuutista myrkyllisyydestä kirjolohen (Salmo gairdneri Richardson) poikasille
Sammandrag: En undersökning angående akut toxicitet av paraquat för unga regnbågsforeller (Salmo gairdneri Richardson)
Summary: A research on the acute toxicity of paraquant to young rainbow trout (Salmo gairdneri Richardson). 8 s. (1968).

36 HÄSÄNEN, ERKKI & VEIKKO SJÖBLOM: Kalojen elohopeapitoisuus Suomessa vuonna 1967
Sammandrag: Kvicksilverhalt i fisk i Finland år 1967
Summary: Mercury content of fish in Finland in 1967. 24 s. (1968).

37 SEPPOVAARA, OSSI: Nieriä (Salvelinus alpinus L.) ja sen kalataloudellinen merkitys Suomessa
Sammandrag: Rödingen (Salvelinus alpinus L.) och dess fiskeriekonomiska betydelse i Finland
Summary: Char (Salvelinus alpinus L.) and its fishing industrial importance in Finland. 75 s. (1969).

38 SEPPOVAARA, OSSI: Ison-Saimaan kalat ja kalastus
Sammandrag: Fiskar och fiske i Stora-Saimen
Summary: Fish and fishing in the lake Iso-Saimaa. 84 s. (1969).

39 HURME, SEPPO: Oulujärvi lohivetenä
Sammandrag: Ule träsk som laxförande vattendrag
Summary: Oulujärvi as a salmonoid lake. 26 s. (1969).

40 WESTMAN, KAI, OLLI SUMARI & JORMA J. LAINE: Comparative dry diet feeding experiment on rainbow trout (Salmo gairdneri Richardson) in floating net-containers
Yhteenveto: Kuivarehuilla verkkoaltaissa suoritettu kirjolohen (Salmo gairdneri Richardson) vertaileva kasvatuskokeilu
Sammandrag: Jämförande torrfoderförsök med regnbågslax (Salmo gairdneri Richardson) i flyttande nät-bassänger. 46 s. (1969).

41 HINTIKKA, NIILO: Kalansaalis, kalastajat, kalastuksessa käytetyt veneet, pyynti rekisteröidyillä aluksilla sekä kotimaisen kalan jalostus ja kauppa Suomessa vuonna 1966
Sammandrag: Fiskfångsten, fiskarna, de vid fisket använda båtarna, fångsten ombord på registrerade fartyg samt förädlingen av och handeln med inhemsk fisk i Finland år 1966.
Summary: Catch of fish, fishermen, boats used in fishing, fishing with the registered vessels and processing and commerce of domestic fish in Finland 1966. 45 s. (1969)

42 SILVO, OLAVI E. J. & VEIKKO SJÖBLOM: Jatkotutkimuksia parakvatin vaikutuksista ja kulkeutumisesta vedessä
Sammandrag: Förtsatta undersökningar över effekterna av paraquat och dess vandringsvägar i vattnet
Summary: Further studies on the effects and movements of paraquat in the water. 30 s. (1969).

43 SUMARI, OLLI & KAI WESTMAN: Haukikantojen hoito
Sammandrag: Vården av gäddbestånden
Summary: The management of northern pike (Esox lucius L.) populations. 24 s. (1969).

44 TUUNAINEN, PEKKA: Relations between the benthic fauna and two species of trout in some small Finnish lakes treated with rotenone. 53 s. (1970).

45 WESTMAN, KAI: Hemoglobin polymorphism and its ontogeny in sea-running and landlocked Atlantic salmon (Salmo salar L.) . 28 s. (1970).