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On a green pigment in the blood-serum of subadult lacustrine *Galaxias* (Pisces; Galaxiidae)

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Abstract. A green pigment was discovered in the serum of the almost colourless blood of subadult specimens of *Galaxias maculatus*. It is no longer present in adults which have red blood with haemoglobin-filled erythrocytes. The pigment probably is a large molecule with a strong negative charge functioning in transport of respiratory gases. Similarities with and differences from other fish species lacking haemoglobin or with haemoglobin in addition to non-red blood pigments are discussed.

Key words. Green blood, green plasma pigment, juvenile blood, green-blooded fishes, haemoglobinless fishes, Galaxiidae, *Galaxias maculatus*.

Introduction

During electrophoretic studies of the blood of galaxiids in southern Chile, a striking phenomen was noticed: the blood of subadult stages of lacustrine *Galaxias maculatus* (the name "*maculatus*" is used with reservation; see Busse & Campos 1987) is nearly colourless, while the separated blood serum is light green (Busse 1991).

Colourless blood has been known for more than 60 years from the so-called "ice fishes" or "bloodless fishes" in Antarctic waters. However, it failed to attract the attention of biologists until it was reported by Ruud (1954) in the notothenioid fish family Channichthyidae endemic to Antarctic waters (see also Andriashev 1965: 527). Apart from channichthyids with no haemoglobin, other Antarctic fishes with a moderate to extreme reduction in haemoglobin were studied by some authors with respect to the blood cells, haemoglobin contents and physiological as well as systematic implications (Ruud 1958, Martsinkevitch 1958, Tyler 1960, Kooyman 1963, Andriashev 1963, 1965). Recently the blood of several Antarctic fish species has been examined extensively by different authors in its varying degrees of reduction of the amount of haemoglobin, oxygen affinities (i. e. Bohr and Root effects) and other blood parameters (di Prisco 1988, di Prisco et al. 1988, 1990, 1991, Kunzmann 1991 a, b). They also show correlations of the blood properties with the specific physiological demands of ecological conditions. If there is haemoglobin, most species have more than one kind of it with different properties but some have only one (Kunzmann 1991, Kunzmann et al. 1992, D'Avino et al. 1992). No comment is made whether or not some non-red plasma pigment is present. In ice fishes there is at least no green plasma pigment (Kunzmann, pers. comm.).

Green blood serum is also known from fishes like *Clinocottus analis* (Fang & Bada 1988, 1990). Also the eel *Anguilla anguilla* has green blood-serum but in combination with normal haemoglobin bearing erythrocytes, so that the blood as a whole is red coloured at least in the post-larvae and adults.

Blood pigments other than red ones were described for some vertebrates like frogs (Barrio 1965) and reptiles (Greer & Raizes 1969, Mertens 1975).

The presence of a green plasma pigment representing the main protein fraction of the blood and virtually no haemoglobin seems to be new for vertebrates and is similar to the situation in some invertebrates.

Materials and Methods

Galaxiids were collected at Lago Riñihue (Prov. Valdivia, Chile) in March 1990 initially for serological comparison of adult specimens of different populations. During these studies it was noticed that the translucent larvae, which may attain a relatively large size compared with adults (Fig. 1), have virtually colourless blood. The blood of the larvae was sampled by heart puncture (Busse & Campos 1987), centrifuged and run electrophoretically in two different ways. The first one was performed with an agarose gel (Beckman): high resolution electrophoresis (HRE) of the Paragon system for clinical purposes, but slightly modified by using a small, self designed electrophoretic chamber for use in the field. Here individual samples were taken with a capillary tube, then sealed and centrifuged. After breaking the tube just at the interphase the supernatant could be directly applied to the gel. Since such small samples of one to few microliters are difficult to store, the rest had to be pooled for transport after freezing and keeping on dry ice. In the laboratory the samples were treated by the second method, an electrophoresis in a discontinuous gel of polyacrylamide (disc. electrophoresis or PAGE) and stained by Coomasie blue; see Busse & Campos (1987). Additionally samples of the green blood serum of the eel *Anguilla anguilla* were included in the study.

Results

After centrifugation the blood separates into two fractions. The bottom fraction, containing the cellular part, is smaller in subadult *Galaxias maculatus* than in adults and of a very faint yellow or orange colour, while the supernatant fraction is greater

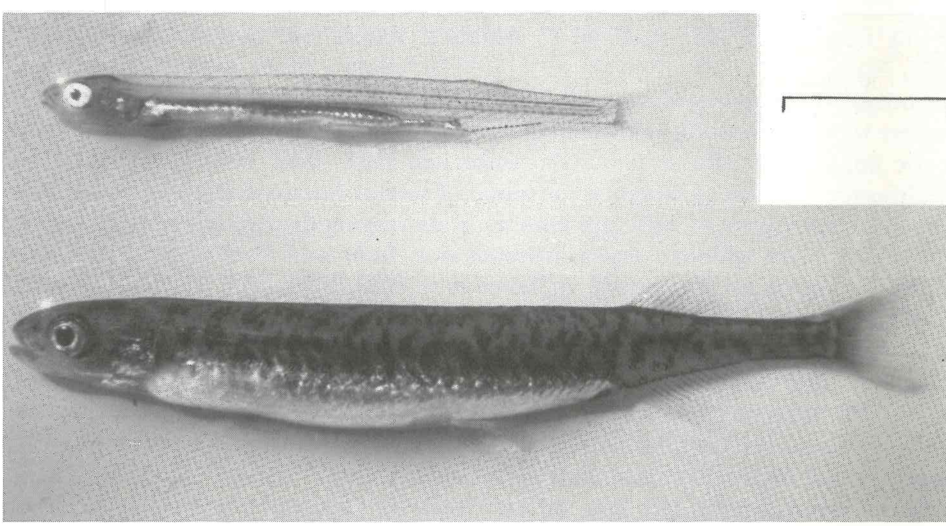

Fig. 1: Juvenile and adult specimen of lacustrine *Galaxias maculatus*. The adult is one of the few individuals surviving the first spawning season. Normally there is less size difference between adults and juveniles. Scale is 1 cm.

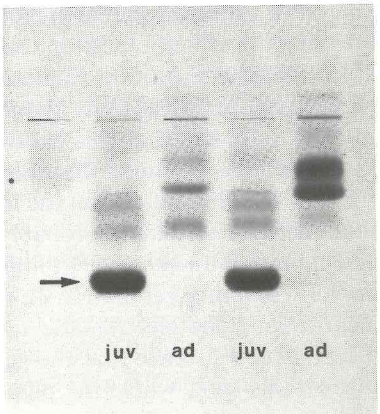

Fig. 2: Electropherograms (HRE, Paragon system) of the blood of juvenile lacustrine *Galaxias maculatus* alternating with adult ones (the last lane from a haemolytic sample). Only in juveniles the green pigment is detectable and represents the main protein fraction (arrow). As it runs far anodically it must be a protein with a strong negative charge. In some individuals it may form a double band.

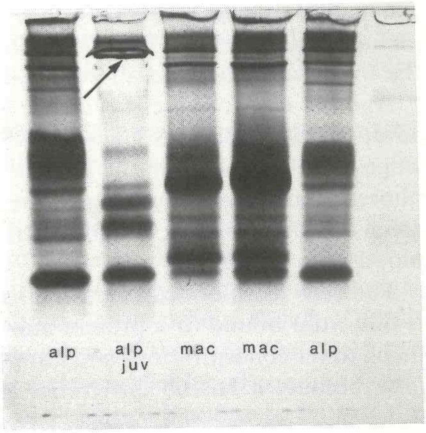

Fig. 3: Electropherogram (PAGE, disc. electrophoresis) of the blood serum of lacustrine and diadromous *Galaxias*. alp: adult specimens of lacustrine population; alp juv: juvenile specimens of lacustrine population; mac: adult specimens of diadromous population. The arrow indicates the position of the green chromoprotein. The band appears "empty", a common staining defect of too high concentrations. In the middle of this light area a narrow green band was visible before staining. This corresponds to the interface between the stacking gel and separating gel. Probably the protein is too large to enter the separating gel.

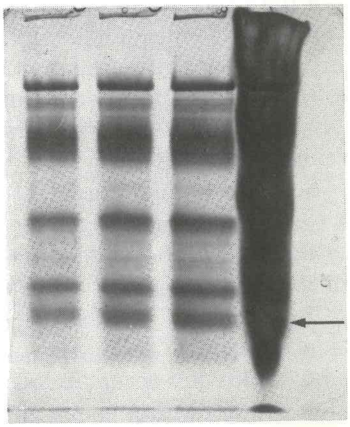

Fig. 4: Electropherogram as in fig. 3 but with blood serum of the eel *Anguilla anguilla* at different concentrations. The last lane (right) was overloaded by a factor of 10 to 20 times the amount of the first three. Only in the last lane the green pigment was visible before staining (arrow). Therefore in the former lanes the first heavier band (same level of the arrow) corresponds to the biliverdin bearing protein. According to its high mobility it must be a smaller molecule than the green pigment of *Galaxias*. According to its density relative to other bands it represents a smaller fraction than the galaxiids' chromoprotein.

and has a light transparent green colour. As these colours are to some extent complementary the whole blood appears colourless.

In the high resolution electrophoresis method (HRE) the green pigment formed the heaviest band due to its abundance and also was the fastest running fraction (Fig. 2). This indicates that this substance is a molecule with a high negative netcharge. In the second method, the polyacrylamide gel electrophoresis (PAGE), the green fraction failed to pass from the stacking part of the gel to its separating part. Therefore this green substance probably is a very large protein molecule (or a smaller molecule bound to a large protein molecule). It is too large to enter the pores of the mesh of the separating gel matrix. In the interface there is probably such a great antagonism among the electrostatic forces of the molecule in the electric field and the mechanical resistance that the gel is altered at this point and looks as if it was torn just on this spot, where the pigment accumulates as a very narrow green line. This narrowness makes the band clearly visible even before staining, because it is compressed into a small volume with resulting high concentration. A typical result of exceedingly high concentrations is that the band stains only on its borders, so that the middle appears empty (the concentration ratio versus stain density is far beyond its linearity range). This is evident in the electropherogram shown in fig. 3. In fig. 2 the lower concentration allowed full staining.

Another electrophoretic evidence is that some of the bands of the blood-serum of adult *Galaxias* (Busse & Campos 1987) are already present in the subadult or larval stage (Fig. 3). Therefore the juveniles may be recognized by means of their electrophoretic pattern as belonging to the lacustrine population. On the other hand, the green component of larval blood-serum disappears during metamorphosis, when red blood cells appear.

The eel's red-blooded post larval stage also bears a green serum pigment, which is biliverdin bound to a protein molecule. Its electrophoretic study (Fig. 4) showed much lower concentrations and a smaller size of the molecule than in *Galaxias maculatus* because it has no difficulties to enter the separating gel.

Discussion

In the translucent juvenile *Galaxias maculatus* neither vessels filled with red blood nor red gills are visible. However, nobody, including myself, concluded from this fact that they lack haemoglobin. Only when I extracted the blood it occurred to me that no haemoglobin was present.

Many questions arise about the nature and function of the green chromoprotein of juvenile *Galaxias maculatus*. Its similarities and differences to other blood pigments are discussed.

Some other fish species, like *Clinocottus analis* or the eel *Anguilla* (Fig. 4), also have a green plasma pigment: biliverdin bound to an albumin-like protein, as a stage in the catabolism for binding and transporting the tetrapyrrole moiety of degraded haemoglobin (Fang et al. 1988). The differences from *Galaxias* are the following: 1. In subadult *Galaxias* there is no haemoglobin as a probable source for haem catabolism. 2. Their green pigment is a much bigger molecule than bile-pigment compounds and occurs in much higher concentrations. 3. It disappears when haemoglobin is appearing at the adult stage.

These facts suggest a respiratory function of the larval green pigment, especially as it is replaced by erythrocytal haemoglobin in adults (Figs. 2 and 3). There is also a striking resemblance to chlorocruorin or even haemocyanin, but these respiratory pigments are only known from invertebrates, being also big molecules of the plasma (or haemolymph).

It has been argued that the loss of haemoglobin in the evolution of channichthyid fishes may be due to: 1. the low temperature of Antarctic waters, with the consequent high solubility of respiratory gases, and 2. the increased viscosity due to low temperature, which can be compensated by reducing the cellular part of the blood (see Kunzmann 1991 a, b, and references therein). This may to some extent also apply to galaxiids which live in cool and well oxygenated waters. The gas dissolving capacity of their environment (including the water of their own body fluids) must be lower, however, because it is not as cold as the Antarctic Seas. This difficulty may be compensated by a plasmatic gas transport pigment (like the green one), unless the galaxiids' small size by itself is not enough to make a gas transport pigment unnecessary.

The problem of gas transport is less critical the smaller an organism is and in fish in general it is less critical than in terrestrial vertebrates. Some fishes are highly capable of anaerobic metabolism. Under pathologic circumstances even a carp, *Cyprinus carpio*, can still be alive after having lost all its haemoglobin (Schlicher 1927, see also Demoll et al. 1936: 200).

Independently from the role the green pigment might have, it is also evident that larvae have less respiratory efficiency than adults. When oxygen content of water drops, larvae are the first to die.

Although the ontogeny of the blood of other galaxiids (e. g. *Brachygalaxias*) was not studied in detail, it is evident that, contrary to *Galaxias*, they have red blood even in the early stages of development. They live in little ponds and streams among vegetation, roots etc. This is completely different from the more pelagic shoals of *Galaxias maculatus*. In the lacustrine (or estuarine) environment there must be a selective pressure to remain as long as possible in an almost transparent larval condition, perhaps less visible for predators, so that the lack of haemoglobin may be part of the adaptive response. Moreover, it is interpreted as a compromise between the poorer gas carrying physiology and the chance of being as invisible as possible for potential predators. Indeed this larvae condition covers an important part of their life span. Sexual maturity, spawning and death commonly follow soon after metamorphosis in *G. maculatus*.

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Zusammenfassung

Es wurde ein grünes Pigment im Blutplasma der Jungfische von *Galaxias maculatus* entdeckt. Es handelt sich dabei um ein großes Proteinmolekül mit einer hohen negativen Nettoladung. Es wird die Hypothese aufgestellt, daß es sich um ein Atemgas-Transportpigment handeln könnte, welches nach der Metamorphose verschwindet und durch das Hämoglobin der Erythrocyten ersetzt wird. Es werden Ähnlichkeiten und Unterschiede zu anderen Fischen diskutiert, welche ebenfalls kein Hämoglobin haben, oder, sofern sie welches besitzen, zusätzlich andere Pigmente im Serum aufweisen.

Resumen

Un pigmento verde fué descubierto en la sangre casi incolora de peces juveniles de *Galaxias maculatus*. Debe ser una molécula grande de proteina con una carga altamente negativa. Puede tratarse de un medio de transporte de gases respiratorios, ya que después de la metamorfosis desaparece mientras aparecen los eritrocitos cargados de hemoglobina, que le dan el color rojo a la sangre del adulto. Se discuten semejamzas y diferencias con respecto a otros peces carentes de hemoglobina o, poseedores de pigmentos plasmáticos adidionalmente a la hemoglobina.

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