STUDIES ON THE HAEMATOLOGY, PHYSIOLOGY
AND BIOCHEMISTRY OF THE BLOOD OF THE
LAMPREY GEOTRIA AUSTRALIS GRAY

This thesis is presented for the degree of
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Submitted by

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Scanning electron micrograph of red blood cells from larvae of Geotria australis Gray. x 3500.
I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any University.

David J. Macey
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PUBLICATIONS

Some of the work reported in this thesis has been published or accepted for publication.


ABSTRACT

Various cytological, physiological and biochemical properties of the blood have been examined in different life cycle stages of the lamprey *Geotria australis*.

Initial experiments to determine physiologically realistic upper temperatures yielded an ultimate incipient lethal temperature for larvae of 28.3°C. Since this value is comparatively low, it helps explain the restriction of *G. australis* to more southern rivers in Australia.

The haematocrit (46.2%), red blood cell number (1.231 x 10^6 cells mm^-3) and haemoglobin concentration (11.8 g 100 ml^-1) of adult *G. australis* are more typical of comparable stages in holarctic lampreys than are those of their ammocoetes (41.5%, 1.809 x 10^6 cells mm^-3, 11.1 g 100 ml^-1). During metamorphosis, the pattern of change in haemopoietic sites, haemoglobin electropherograms and the proportion of mature erythrocytes, indicate that erythrocytes containing larval and adult haemoglobins always originate in different structures. The molecular weights (c. 17000) and pI values (5.1-6.4) of *G. australis* haemoglobins are similar to those of other lampreys. The P_{50} of larval blood is very low, while that of adult blood is more comparable to that of other lampreys (cf. 0.92 mm Hg for ammocoete and 10.3 mm Hg for adult at pH 7.75 and 15°C). Increases in temperature do not affect the Bohr shift (range -0.16 to -0.27) but are accompanied by a shift of the oxygen dissociation curve to the right.

The major plasma iron binding proteins have molecular weights of 354,000 in the ammocoete and 296,000 in the adult and contain 20 and 4 subunits respectively. The larval IBP is thus ferritin-like while that of the adult is transferrin-like, features consistent with their respective pI values, Fe/protein ratios and ultrastructure. Total
plasma iron was 19,760 µg 100 ml$^{-1}$ in larvae and 34 µg 100 ml$^{-1}$ in adults. Iron granules were present in the columnar cells of the posterior intestine in small or negligible amounts in the Petromyzonidae and in very large amounts in the Mordaciidae. While some iron was found in the same location in the Geotriidae, it was also present in very large concentrations elsewhere in the body.

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1. **INTRODUCTION**

1.1 **Life cycle and taxonomy**

The lampreys (Petromyzoniformes), together with the hagfishes (Myxiniformes), are the only extant representatives of the Agnatha (Heintz, 1963; Bardack and Zangerl, 1971; Hardisty, 1979; Jarvik, 1980). While the two groups share some common features, they are both highly specialised in a number of different respects (Hubbs and Potter, 1971; Hardisty, 1979). Moreover, the life cycles of the Myxiniformes and Petromyzoniformes are vastly different.

The hagfishes are an entirely marine group which feed on dead or dying marine invertebrates and fish (Strahan, 1963) and do not have a distinct larval stage (Adam and Strahan, 1963; Hardisty, 1979). Since the osmotic pressure of their blood that is attributable to the presence of electrolytes is approximately 1,000 mOsmoles, and therefore similar to that of full strength sea water (35%), a situation unique amongst lower vertebrates, it has been suggested that this group originated and has subsequently remained in a marine environment (Lutz, 1975). In contrast to the hagfishes, the lampreys possess a microphagous larval stage which develops and lives in fresh water (Hardisty and Potter, 1971a; Potter, 1980a). Lampreys also undergo a radical metamorphosis which, in the case of anadromous species, is followed by a marine parasitic phase in which the adults feed predominantly on teleost fishes (Hardisty and Potter, 1971b). Moreover, the osmotic pressure of the blood of the post-metamorphic stage of anadromous parasitic species is approximately only one third of full strength sea water, a feature which was considered by Lutz (1975) to reflect a fresh water origin of the Petromyzoniformes. Some anadromous parasitic species have given rise to landlocked parasitic forms which also utilize the tissue and
body fluids of teleost fishes as a source of food (Applegate, 1950; Hardisty and Potter, 1971b; Smith, 1971; Smith and Tibbles, 1980). There are, however, some fresh water parasitic species which have no living anadromous counterpart. Moreover, the most numerous of lamprey species are the non-parasitic or brook lampreys which never leave fresh water and do not feed after metamorphosis (Hardisty and Potter, 1971c; Potter, 1980b). Each of the species with this type of life cycle are believed to have been derived from a particular anadromous or fresh water parasitic species. For this reason, Zanandrea (1959) coined the term paired species for these closely related species which possess divergent life cycles.

There are currently 39 described species of lampreys (Potter, 1980a). The 35 Northern Hemisphere species were all placed in the family Petromyzontidae by Hubbs and Potter (1971) and Potter (1980b), and in the subfamily Petromyzontinae by Bailey (1980). Seven of these species have an anadromous parasitic life cycle, with three having given rise to landlocked forms, and a further seven are parasitic in fresh water (Potter, 1980b). The remaining twenty one species are nonparasitic. The four Southern Hemisphere lampreys were placed in two families (Mordaciidae or Geotriidae) by Hubbs and Potter (1971) and Potter (1980b), and in two subfamilies (Mordaciinae or Geotriinae) by Bailey (1980). The former of these two taxonomic schemes is adopted in this thesis. The single genus in the Mordaciidae contains three species. *Mordacia mordax* (Richardson, 1846) is found only in south-eastern Australia, including Tasmania, and *Mordacia lapicida* (Gray, 1851) is confined to Chile. Both species have an anadromous parasitic life cycle (Potter and Strahan, 1968). The third mordaciid species, *Mordacia praecox* Potter, 1968, is nonparasitic and is believed to have arisen from *M. mordax* with
which it is sometimes found in the same river systems (Potter, 1980b). The Geotriidae is represented by the single species, *Geotria australis* Gray, 1851, which is present in the southern rivers of both east and west coasts of South America and throughout New Zealand. In Australia, it has been found in rivers from the south-west eastwards to Lake King in Victoria and it is also abundant in Tasmania (Potter and Strahan, 1968).

Lampeys have an antitropical distribution, being found only to the north and south of approximately the 20°C annual isotherm in the Northern and Southern Hemispheres respectively (Hubbs, 1952; Lanzing, 1957).

It is evident from the fossil lampeys, *Mayomyzon piekoensis*, from the upper Pennsylvanian, which was described by Bardack and Zangerl in 1968, that the morphology of lampeys has not altered markedly during the last 280 million years.

1.2 Larval phase

Larval lampeys (ammocoetes) are relatively sedentary animals which live in burrows in the soft substrates of streams and rivers (Hardisty, 1944; Potter, 1970, 1980a; Malmqvist, 1980). The average duration of larval life varies amongst species, a feature which is reflected by differences in the total body length at the onset of metamorphosis. Thus, the duration of the larval phase in *Lampetra fluviatilis* has been estimated as 4½ years with the onset of metamorphosis occurring at a mean length of approximately 100 mm (Hardisty and Huggins, 1970), whereas comparable values for its non-parasitic derivative are 6½ years and 110-160mm (Hardisty, 1961; Hardisty and Potter, 1971a; Bird and Potter, 1979a). Within any one species, the length of the larval period is dependent not only on age but also on growth rate and therefore presumably the conditions in the river system (Potter, 1980a).
The food of ammocoetes consists predominantly of algae, such as diatoms, and other microorganisms and detritus which are drawn into the pharynx in a combined respiratory and nutritive water current from water surrounding the burrow (Moore and Potter, 1976a,b; Moore and Mallatt, 1980; Rodgers, Glenn and Potter, 1980). The microscopic food particles are trapped in intricate mucous strands produced in the pharynx and then passed backwards to the intestine (Sterba, 1953; Mallatt, 1979, 1981; Moore and Mallatt, 1980).

While ammocoetes are photophobic and usually remain burrowed during the day (Young, 1935; Harden Jones, 1955; Sterba, 1962; Reynolds and Casterlin, 1978), there is now good evidence that they sometimes emerge at night (Gritsenko, 1968; Long, 1968; Manion and Smith, 1978). Movement by these relatively sedentary animals enables them to select more favourable conditions, a feature that would be of considerable importance after disturbance by heavy flooding (Potter, 1970; Malmqvist, 1980).

1.3 Metamorphosis

Metamorphosis is usually initiated during the summer months in both the Northern and Southern Hemispheres and is remarkably synchronous in any given population (Hardisty and Potter, 1971b; Bird and Potter, 1979a,b; Potter, 1980a). While the main morphological and physiological changes associated with the development of adult characteristics are usually completed within 3-4 months (Youson and Connelly, 1978; Potter, Wright and Youson, 1978; Bird and Potter, 1979a), some animals do not start feeding for a further three to six months (Moore and Potter, 1976b; Potter and Beamish, 1977). The delay is associated with the onset of "winter" conditions, with feeding only being initiated when temperatures start rising. Energy is provided during the non-trophic period by the mobilisation
of reserves, particularly lipid, accumulated over the final few years prior to the onset of metamorphosis (Lowe, Beamish and Potter, 1973; Moore and Potter, 1976b; Youson, Lee and Potter, 1979; Bird and Potter, 1981). The profound anatomical and physiological alterations that take place at this time can generally be related to the change from a relatively sedentary filter feeding habit to a more active predatory existence (Potter, Wright and Youson, 1978; Bird and Potter, 1979a,b; Youson and Potter, 1979; Potter, Hilliard and Bird, 1980).

1.4 Adult phase

The fully-metamorphosed young adult lampreys migrate downstream to either a lacustrine or a marine environment where feeding commences (Hardisty and Potter, 1971b). Attachment to the hosts, which are predominantly teleost fishes, is accomplished by the use of a suctorial disc (Dawson, 1905; Hardisty and Potter, 1971b). Destruction and cytolysis of the host tissue is achieved through the action of a tongue-like piston and enzymatic secretions (lamphredin) from the buccal glands (Dawson, 1905; Lennon, 1954). The composition of the food varies among species. Thus, while Petromyzon marinus is essentially a sanguiniphore (Farmer, Beamish and Robinson, 1975), several Lampestra species feed more on muscle tissue (Bahr, 1933; R.J. Beamish, 1980; Youson, 1981a). The adult trophic period, which varies among species, lasts for between approximately four and thirty months (Hardisty and Potter, 1971b; F.W.H. Beamish, 1980). At the commencement of the final phase of the life cycle, feeding ceases and the animal starts migrating upstream during which time sexual maturation takes place (Larsen, 1980). Spawning occurs during the spring or summer, the precise timing being related to water temperatures (Hardisty and Potter, 1971b).
1.5 Life cycle of Geotria australis

Despite the very wide geographical distribution of Geotria australis, there have only been a few papers providing details of the life cycle of this species (Potter et al., 1980). Much of the data on this species comes from studies undertaken in New Zealand by Maskell (1929, 1930, 1931, 1932). In many respects the life cycle of the Southern Hemisphere G. australis parallels that of anadromous parasitic species of lampreys in the Northern Hemisphere. However, while the general pattern exhibited by the life cycle is similar, there are some important differences in terms of morphological changes, particularly during metamorphosis and the adult fresh water phase (Potter and Strahan, 1968; Potter et al., 1980).

As in holarctic lampreys, larval G. australis burrow in the sand and debris of streams and rivers (Maskell, 1929) feeding on the algae, detritus and microorganisms in the water and on the substrate surrounding their burrow (Rogers et al., 1980). A feature unique to larval G. australis is the presence of two diverticula at the junction of the oesophagus and the anterior intestine, a situation that can be compared with that in the Mordaciidae in which the ammocoetes possess a single diverticulum and with the Petromyzonidae in which no well-defined diverticulum is found (Maskell, 1929, 1932; Potter and Strahan, 1968; Strahan and Maclean, 1969; Potter, 1980b; Youson, 1981a).

The duration of larval life is between three and five years with many animals probably entering metamorphosis at 3½ years of age (Potter, 1980a). This short larval life can be related to the shorter total length of this species at the onset of metamorphosis (Potter, 1980a). Thus, the mean length of 90 mm found in ammocoetes of G. australis at the start of metamorphosis (Potter et al., 1980) can
be compared with means of 100 mm in *L. fluviatilis* (Hardisty and Huggins, 1970), 120-130 mm in *Mordacia mordax* (Potter, 1970), and 120-150 mm in *Petromyzon marinus* (Potter et al., 1978; Potter, 1980a). The earliest signs of metamorphosis in *G. australis* in south-western Australia can be seen in late January (Potter et al., 1980), which corresponds to the situation in the Northern Hemisphere where metamorphosis is initiated during June and July (Potter, 1980a). However, the five to six months duration of this non-feeding period in *G. australis* is comparatively long compared with the period of metamorphosis in holarctic species which can be as short as four months (Applegate, 1950; Lowe et al., 1973; Potter et al., 1978; Bird and Potter, 1979a; Potter et al., 1980). While all three genera of lampreys follow a similar pattern of change during this transition from larva to adult, certain common structures develop at different rates (Potter et al., 1980).

During metamorphosis, the second dorsal fin becomes separated from the caudal fin, a feature which has often been used as a diagnostic character for the Geotriidae (Potter and Strahan, 1968; Potter et al., 1980). In addition, besides developing the silvery sheen characteristic of all anadromous species of lampreys towards the end of metamorphosis, *G. australis* also develops two dorso-lateral longitudinal blue/green bands (Potter et al., 1980).

As in other anadromous parasitic species, the downstream migration, which takes place in late July and early August, is remarkably synchronous in any particular river system, a feature which can be correlated with marked increases in fresh water discharge (Potter et al., 1980). Information on the parasitic phase of all species of anadromous lamprey is very limited and in *G. australis* is basically restricted to two papers by Ivanova-Berg (1968) and Potter, Prince...
and Croxall (1979) which deal with lampreys taken from the waters of South Georgia. These papers show that *G. australis* is found considerable distances from their natal streams and suggest that they are present at night in large groups in the surface waters where they become a major food source of the grey headed albatross (Potter *et al.*, 1979). Adult *G. australis* return to fresh water between late June and early August, the precise time depending on the prevailing fresh water discharge rates (Potter *et al.*, unpublished data).

*G. australis* increases greatly in length from approximately 90 mm in the downstream migrant to just over 600 mm in the early upstream migrant, which corresponds to an increase in weight from 0.9 to 230 g respectively. This suggests that adult lampreys remain at sea for periods longer than a single year (Potter *et al.*, 1979). As sexual maturity approaches during the upstream migration, *G. australis* undergoes a second radical change in its appearance (Maskell, 1929; Potter and Strahan, 1968), a change that is so marked that it has been termed a second metamorphosis (Dendy and Oliver, 1901). The pronounced morphological changes in the life cycle have produced considerable taxonomic confusion and have lead to a great proliferation of genera and species based on individuals at different stages of development (Potter and Strahan, 1968). This confusion was finally resolved by Strahan (1959) who found that the four alleged species referred to by Holly (1933) were, in fact, all stages in the life cycle of *G. australis*.

Upon entering fresh water the bright blue/green coloration displayed by adults of the downstream migrant and marine phase stages is soon replaced by a uniform grey/black dorsal surface and a slightly lighter ventral surface (Maskell, 1929). Towards the end of the migration, by which time the adults have sometimes travelled several hundred miles upstream (Potter and Strahan, 1968), a pouch or gular
sac has developed on the ventral surface of the males between the disc and the second gill pore. Concomitant with this change is an enormous enlargement of the oral disc of the males (Maskell, 1929; Potter and Strahan, 1968; Potter, 1980b). This form of sexual dimorphism, paralleled to a lesser extent by species of the Mordaciidae, is far more extreme than in holarctic genera (Potter, 1980b). During this period, considerable shortening of the body takes place in both sexes, presumably due in part to catabolism of body tissues. In common with other lampreys, *G. australis* does not feed after the onset of the upstream migration (Maskell, 1929; Hardisty and Potter, 1971b). The adults presumably die after spawning as no evidence has been found of any adults returning to the sea. Such a journey would be virtually impossible in view of the marked degeneration of all tissues except those concerned with reproduction (Maskell, 1929).

The preceding account gives a brief outline of the life cycle of lampreys, and in particular, *G. australis*, that is relevant to the work carried out on various aspects of the physiology of the blood of *G. australis*. 
2. **COLLECTION AND MAINTENANCE OF ANIMALS**

*Geotria australis* were obtained from tributaries of the Warren and Donnelly rivers in south-western Australia, which are located at Lat. 34°S, Long. 116°E (Fig. 1). The ammocoetes and metamorphosing individuals (for description of stages 1-7 see Potter, Hilliard and Bird, 1980) used in the current study were collected with an electric fish shocker between April 1978 and December 1980 (Fig. 2a). In view of the fact that marked changes occurred in the blood during stage 7, this stage in metamorphosis was separated into early and late categories in Section 4 which deals with changes in the blood during metamorphosis. The downstream migrants were collected from the trays that filter the river water to a trout hatchery. Some of these latter animals were held in the laboratory in full strength sea water (35%) for a month after prior acclimation for four days to firstly 12% and then 24% sea water. A dip net was used in September, October and November of 1977, 1978, 1979 and 1980 to obtain adults from in front of a weir which was impeding their migration towards their spawning grounds (Fig. 2b). Since it has proved impossible to catch later migratory stages in the field, a limited number of adults were also kept in the laboratory for 8-10 months under light/dark and temperature regimes paralleling those found in the field.

The haematological study (Section 4) also utilised 29 larval *G. australis* and 18 larval *Mordacia mordax* collected in January 1978 from the Ringarooma River in Tasmania (Lat. 41°S, Long. 148°E).
Fig. 1a  A map showing the general area in Australia (arrow) from which the majority of animals of the various life cycle stages of *G. australis* were collected.

b  A more detailed map of the area in Fig. 1a (c) showing the location of the Warren and Donnelly rivers and their tributaries. 'P' refers to the place where both the Tranbikie hatchery and weir used in the collection of downstream migrant and adult stages respectively, are situated.
Fig. 2a  Carey Brook near Pemberton showing a typical area from which larval and early metamorphosing stages of Geotria australis were collected.

b  Adults of Geotria australis scaling one of the many obstructions encountered on their upstream migration.
3. LETHAL TEMPERATURES OF AMMOCOETES

3.1 Introduction

Lampreys have an antitropical distribution (Hubbs, 1952), the southern-most limit in the Northern Hemisphere approximating to the 20°C isotherm line (Lanzing, 1957). Furthermore, recent work has shown that this pattern of distribution can be related to the temperature tolerance of the larval stages. For example, the incipient lethal temperatures for ammocoetes of four species, representing three genera acclimated at 15°C, were found to range from 28.0 to 30.5°C (Potter and Beamish, 1975). Contrasting with the reasonably comprehensive data on lethal temperatures in the ammocoetes of holarctic species (Petromyzonidae) is the absence of any such information for the exclusively Southern Hemisphere family, the Geotriidae. This family, which is believed to be monotypic (Potter and Strahan, 1968), is widely distributed, occurring in New Zealand and the southern regions of South America and Australia (Hubbs and Potter, 1971; Potter, 1980b). Together with the members of the Mordaciidae, the only other extant lamprey family, Geotria australis, has been regarded as exhibiting such marked morphological differences from the Petromyzonidae that the two groups are believed to have been isolated for a long period of time (Hubbs, 1952).

This study was initiated to compare the lethal temperatures of the larva of Geotria australis with those of holarctic species using identical experimental regimes. Such data are of interest in view of both the apparent antiquity of the Geotriidae and the unusual conditions which pertain in the rivers of south western Australia from which these animals were obtained (Lake, 1971). Values for the upper limits of temperature tolerance were also essential for the construction of physiological experiments based on a range of different
temperature regimes.

3.2 Materials and methods

Ammocoetes of Geotria australis were placed in tanks containing well-aerated water and a natural substrate into which they readily burrowed. The temperature was then raised or lowered at a rate of 1°C per day to either 5, 15 or 25°C. These acclimation temperatures were held at ±0.1°C for three to four weeks before ten animals were transferred to each of a series of experimental tanks maintained at a given temperature ±0.1°C.

The times of both emergence and death were plotted on three cycle log probability paper to establish in each experiment the median time of emergence and of death. Calculations were then made for each acclimation temperature of the temperature at which 50% of the animals would die within 96 hours (Lₜ 50). A calculated regression line was then drawn through the three Lₜ 50's, or incipient lethal temperatures, until it met a line drawn upwards at 45°C from the origin of the lethal and acclimation temperature axes (Fry, 1947). The point of intercept provides a value known as the ultimate incipient lethal temperature.

3.3 Results and discussion

After the direct transfer to experimental tanks from acclimation temperatures of 15 and 25°C, the majority of animals entered the substrate within a few minutes. Although animals acclimated at 5°C generally burrowed within ten minutes, the period immediately following their transfer was characterised by convulsions interposed with bouts of rapid swimming. In contrast to the relatively rapid burrowing that took place in most experiments, exceptions were observed at high experimental temperatures. For example, animals acclimated at
5 and 25°C did not enter the substrate at 28 and 32°C respectively.

In cases where death occurred in animals that had burrowed, the ammocoetes almost invariably first came out of the substrate, swam briefly and then lay on their sides. At any given experimental temperature a clear correlation could be seen between the time of emergence and the resistance time (Fig. 3). For example, all animals acclimated at 25°C had emerged and died within 1,380 minutes at 30°C, whereas at 28°C the first animal did not emerge until the experiment had been under way for 2,925 minutes and the death of the last animal did not occur until 13,899 minutes (≈10 days) had elapsed.

No significant difference (P > 0.05) was found between the time at which the smallest (50 to 60 mm) and largest (80 to 90 mm) animals died at any of the three experimental temperatures, a finding similar to that reported by Potter and Beamish (1975) for holarctic species.

The plot for the median time to death against experimental temperatures yielded similar curves for each experimental series representing the results for the three acclimation temperatures (Fig. 4). The $L_{50}$ for acclimation temperatures of 5, 15 and 25°C were calculated as 27.3, 27.5 and 28.2°C respectively. A regression line, relating the three incipient lethal temperatures for each acclimation value was calculated and extended to meet the line drawn upwards at 45°C from the origin (Fig. 5). The ultimate incipient lethal, represented by the point of intersection, was 28.3°C, a value which falls just below that of the species of lamprey studied by Potter and Beamish (1975). For example, the ultimate incipient lethals for _Lampetra planeri_ and _Petromyzon marinus_ examined in the summer were 29.4 and 31.4°C. That _Geotria australis_ has a similar but slightly lower ultimate incipient lethal temperature than holarctic species is further borne out by the fact that the single incipient lethal
Fig. 3  Median and range for the emergence and resistance times of ammocoetes of *Geotria australis* at different experimental temperatures after acclimation at 25°C. Prior to death, all animals first burrowed and then emerged apart from at 31°C where two animals did not enter the substrate at any stage.
Fig. 4  Median resistance time for ammocoetes of *Geotria australis* in relation to acclimation and experimental temperature.
Fig. 5 Comparisons between incipient lethal and acclimation temperature for ammocoetes of *Geotria australis* with those of four holarctic species taken from the data of Potter and Beamish (1975). The ultimate incipient lethal temperature is represented by the point at which the regression line relating incipient lethals met a line drawn upwards from the origin at 45° (Fry, 1947).
values for *Lampetra lamottenii* and *Icthyomyzon fossor*, based on animals acclimated at 15°C, were 29.5 and 30.5°C compared with the 27.5°C obtained for *G. australis* in this study.

The similarity in lethal temperatures of the Petromyzonidae and Geotriidae, together with strong indications that this homogeneity is extended to the other Southern Hemisphere family, the Mordaciidae (Potter and Beamish, 1975), indicate that a similar factor or factors limiting temperature tolerance have been retained in all lamprey groups during the long periods they have apparently been isolated. The inability of the larvae of any species of lamprey to survive at temperatures much above 30°C may be due, amongst other factors, to the presence of weak bonds in thermo-labile enzymes which tend to dissociate at high temperatures (Hochachka and Somera, 1973) or to the "melting" of cellular and subcellular membrane lipids so that they become freely permeable (Prosser, 1973).

A point of marked contrast between larval lampreys and teleosts is found in the relationship between incipient lethals and experimental temperatures. For example, Fry (1971) has stated that in teleosts a rise of 3°C in acclimation temperature generally results in a 1°C rise in incipient lethal. In larval lampreys, however, no such marked difference was found. For example, the rise in incipient lethal in *G. australis* acclimated at 5 and 25°C was only from 27.3 to 28.2°C. This small rise over 20°C is typical of other larval lampreys (Potter and Beamish, 1975) and may be related to the low metabolic rate of ammocoetes (Hill and Potter, 1970; Potter and Rogers, 1972).

In conclusion, it is worth noting that the ultimate incipient lethal of 28.3°C in larval *G. australis* almost certainly accounts for the current restricted distribution of this species to the rivers in the very southernmost region of Western Australia. This view is
based on the fact that even in the Swan River, where no lampreys have been recorded for several years, water temperatures approaching 30°C (Latitude 32°S) have been frequently recorded (Spencer, 1956; Chubb, personal communication).
4. ERYTHROCYTE AND HAEMOGLOBIN MEASUREMENTS

4.1 Introduction

Work on several species of lampreys has shown that both their ammocoetes and adults possess at least two different haemoglobins and that the change from larval to adult haemoglobins occurs during metamorphosis (Adinolfi, Chieffi and Siniscalco, 1959; Marwell, 1963; Uthe and Tsyuki, 1967; Potter and Nicol, 1968; Beamish and Potter, 1972; Potter and Brown, 1975). The differences between the haemoglobins of the two life cycle stages are paralleled by differences in the oxygen dissociation curves of the haemoglobins, erythrocyte suspensions and whole blood (Marwell, 1963; Bird, Lutz and Potter, 1976). Since the affinity for oxygen is greater in the ammocoete than the adult, Marwell (1963) has suggested that this "reflects the mud dwelling habits" of this stage in the life cycle. The alterations that occur during metamorphosis are paralleled by changes in the sites where blood cell formation takes place (Percy and Potter, 1976, 1977). The latter workers also proposed a scheme for the pattern of development of the erythrocytes and other blood cells, which they subsequently developed in greater detail (Percy and Potter, 1981). Although differences have been found between the nuclear/cytoplasmic ratio of the mature erythrocytes of the larval and upstream migrant stages (Potter, Robinson and Brown, 1974), it is not known whether this reflects a change that takes place during metamorphosis or later in adult life. It is evident, however, from other studies that various blood parameters, such as haematocrit and haemoglobin concentration, change during both metamorphosis and the upstream spawning migration (Ivanova Berg and Sokolova, 1959; Korzhuev and Glazova, 1967; Beamish and Potter, 1972; Potter and Beamish, 1978).

Apart from such studies as the investigation of the electro-
phoretic properties of the haemoglobins of *Mordacia* spp. and *Geotria australis* (Potter and Nicol, 1968), most of the published work relating to lamprey blood cells has been performed on Northern Hemisphere species. Moreover, the data are the cumulative product of different studies on restricted aspects of the blood, and on different species and life cycle stages, a feature which, particularly in the context of metamorphosis, makes it difficult to determine precisely how the various changes that take place at this time are interrelated.

The work described in this section was undertaken to measure changes in the blood of larval, metamorphosing and adult representatives of the Southern Hemisphere lamprey *Geotria australis* collected from the same geographical area. The parameters recorded were haematocrit, the concentration and electrophoretic characteristics of the haemoglobins, the size, number and haemoglobin content of erythrocytes, and the relative abundance of immature members of the erythrocyte series. Supplementary measurements have also been given for larval *G. australis* and *Mordacia mordax* caught in Tasmania. Several of the measurements represent the first data on some of the blood parameters in one or more of the major different phases in the life cycle of lampreys. Emphasis has been placed on ascertaining whether there are seasonal trends and on the relationship between the various parameters. Wherever possible, comparisons have also been made between the measurements for *G. australis* from Western Australia and those obtained for comparable stages in the life cycle of other Southern Hemisphere populations and more particularly with those recorded for the lampreys of the Northern Hemisphere.

4.2 Materials and methods

After anaesthesia in benzocaine, large ammocoetes (> 60 mm)
metamorphosing stages and downstream migrants of *Geotria australis* were clamped immediately in a vertical position and their tail cut off just behind the cloaca. Blood was then collected in capillary tubes from the main vessel in the immediate post-cloacal region. It is worth noting that no significant differences were found between the haematocrits, red blood cell numbers and haemoglobin concentrations in animals anaesthetized with benzocaine and those treated with MS 222 (Calbiochem) and carbon dioxide. Moreover, there was also no significant difference in the three blood parameters (P > 0.05) between samples in which the blood was collected from the caudal region as described above or by cardiac puncture. The former technique was preferred since greater problems were encountered obtaining blood by the second method, a feature related to the small size of even the largest *G. australis* ammocoetes. In adult *G. australis* which are very much larger, it was more convenient to extract blood by cardiac puncture. Although it was difficult to ascertain macroscopically the sex of the smaller ammocoetes used in this study, a record was kept of the sex of the largest ammocoetes, metamorphosing stages, downstream migrants and adults in which the type of gonad was more clearly distinguishable.

The haematocrits (Ht) are based on blood samples collected in heparinised microhaematocrit tubes which were spun at 16,000 G for 5 min. The number of erythrocytes in the blood of each animal (RBC) is based on the mean of four separate counts made in a christalite cell (Hawksley). A cyanmethaemoglobin method (Sigma Chemical Co., 1975) was used to determine haemoglobin concentration (Hb).

In the monthly samples of ammocoetes, measurements of haematocrit and haemoglobin concentration began in April 1978 while counts of the red blood cell number were not initiated until September 1978 (Fig. 6).
Insufficient blood could be obtained from ammocoetes to permit measurements of red blood cell number and haemoglobin concentration to be made on samples from the same animal. There was on some occasions enough blood in larvae, however, to enable both haematocrit and either red blood cell number or haemoglobin concentration to be determined on blood taken from individual animals. In adult stages, the volume of blood obtained was sufficient to permit all the above three measurements to be recorded for 36 individual animals. Blood extracted from some other animals that were being used for other purposes enabled a few additional measurements to be recorded for one or two of the parameters.

Data on the above measurements have been used to calculate the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) using the following equations, in which Ht = % of erythrocytes 100 ml$^{-1}$ of blood; Hb = haemoglobin concentration in g 100 ml$^{-1}$ of blood and RBC = millions of erythrocytes mm$^{-3}$.

\[
\text{MCV} = \frac{\text{Ht} \times 10}{\text{RBC}} \quad \text{MCH} = \frac{\text{Hb} \times 10}{\text{RBC}} \quad \text{MCHC} = \frac{\text{Hb} \times 10^2}{\text{Ht}}
\]

It should be noted that because of the problem of obtaining enough blood for making a series of different measurements on each ammocoete, the MCH for larvae has been calculated using the following equation \[\text{MCHC} \times \text{MCV} = 100 \ \text{MCH} \ (\text{Bishop, 1978}).\]

The diameter and thickness of erythrocytes were measured in suspensions of whole blood prepared in the manner described by Potter et al. (1974). The relative numbers of the different cell types in the red blood cell lineage were ascertained in smears treated by Wright's stain using an Ames Hema-Tech Slide Stainer and a modification of the identification procedures for the erythrocyte series.
outlined by Percy and Potter (1976). The area of the erythrocyte and its nucleus and cytoplasm were measured from photographs of known magnification using a Kent planimeter. For convenience, the nuclear/cytoplasmic ratio is given as the value x10^2.

Comparisons of the various blood parameters in larval and adult lampreys are based mainly on ammocoetes which had been feeding in rivers and on early upstream migrants that had only recently completed their marine trophic phase (Table 1). In the case of adults, more restricted comparisons were made with the downstream migrants that had not fed for the approximately 5-6 month period of metamorphosis and with the non-trophic upstream migrants held in the laboratory for several months.

Sections of larval, metamorphosing and upstream migrant *G. australis* were prepared in the manner described by Percy and Potter (1976) to examine the degree of haemopoiesis occurring in the larval (nephric fold and typhlosole) and adult haemopoietic sites (fat column).

Haemoglobin solutions for electrophoretic studies were prepared by washing the blood four times in 0.6% NaCl and centrifuging at 800 G. The blood was lysed in distilled water, frozen overnight and then concentrated using an ultrafiltration membrane (Amicon). The haemoglobin was run on two 7.5% acrylamide gels, one of which was stained with Coomassie Brilliant Blue G250 and the other with 0-dianisidine. The concentration of the acrylamide was lowered on some additional gels in an attempt to increase the resolution of the bands in the electrophorograms of the haemoglobins of metamorphosing animals.

The sample sizes for the measurements of the various parameters in larvae and early upstream migrant adults are given in Table 1. The
total number of metamorphosing animals used in the current study, which contained an approximately equal number for each stage, was 147. Blood was taken from 26 downstream migrants and measurements on adults held in the laboratory for 8-10 months are based on blood collected from six individuals.

4.3 Results

In none of the blood parameters was there a significant difference (P > 0.05) between the males and females in either samples of large larvae, or in the downstream or upstream migrants, or in any of the stages in metamorphosis. For this reason, the data for the two sexes have been pooled in the case of each of the monthly samples of ammocoetes and each of the stages in metamorphosis, and in both the early and laboratory-held upstream migrant adults.

Values for ammocoetes and adults

The red blood cell number, haematocrit and haemoglobin concentration of ammocoetes did not show a consistent pattern of seasonal change (Fig. 6). Moreover, the mean values obtained for a particular month in one year occasionally varied significantly from those recorded for the same month in another year. For example, the mean red blood cell number of 1.646 x 10^6 cells mm^-3 in December 1979 was significantly lower (P < 0.001) than the mean of 2.056 x 10^6 cells mm^-3 recorded in the December of the previous year. There were also a number of cases when the mean in one month varied greatly from those in the preceding and following months. Such a situation was found, for example, with the value for the haematocrit in April 1979 and with the haemoglobin concentration in October 1979.

In view of the absence of clearly defined seasonal trends in the above parameters in larvae, the following comparisons between ammocoetes and early upstream migrants are based in the case of the
former life cycle stage on pooled values for all months (Table 1, Fig. 7).

The number of red blood cells in a given volume of blood was greater in ammocoetes than the early upstream migrant adults. Thus, the mean of $1.809 \times 10^6$ cells mm$^{-3}$ for the number of erythrocytes in larvae was significantly greater ($P < 0.001$) than the $1.231 \times 10^6$ cells mm$^{-3}$ in early upstream migrants (Table 1, Fig. 7). However, the converse situation pertained with haematocrit and haemoglobin concentration in which the respective larval values were 41.5% and 11.1 g 100 ml$^{-1}$, compared with 46.2% and 11.8 g 100 ml$^{-1}$ in the adult (Table 1, Fig. 7). Both these sets of measurements showed a significant difference ($P < 0.001$ and $P < 0.02$ respectively) between the two life cycle stages. In the case of all three blood measurements, however, the ranges for the values for the ammocoetes and adults overlapped.

The values for the number of red blood cells and haematocrit in ammocoetes caught in Western Australia did not differ significantly ($P > 0.05$) from the $1.862 \times 10^6$ cells mm$^{-3}$ and 38.6% obtained for larvae of the same species caught in Tasmania.

In the measurements made on blood smears (Table 1, Fig. 9), the mean area of the mature erythrocytes in ammocoetes (105.2 $\mu m^2$) was significantly smaller ($P < 0.01$) than that found in early upstream migrant adults (117.3 $\mu m^2$). A highly significant difference was also found between their respective nuclear areas (18.5 and 15.4 $\mu m^2$), cytoplasmic areas (87.2 and 102.0 $\mu m^2$) and nuclear/cytoplasmic ratios (21.2 and 15.1). The above values for total cell area, nuclear area and nuclear/cytoplasmic ratio of erythrocytes from pooled samples of ammocoetes caught in Western Australia are similar to the respective values of 100.8 $\mu m^2$, 17.8 $\mu m^2$ and 21.4 obtained for red blood cells
Table 1. The mean (+95% confidence limits) and sample size (n) for the various measurements made on ammocoetes and early upstream migrant adults of *Geotria australis*.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Ammocoete</th>
<th></th>
<th>Adult</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>±95%</td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>Body length (mm)</td>
<td>79.1</td>
<td>0.672</td>
<td>517</td>
<td>660.0</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>0.65</td>
<td>0.016</td>
<td>517</td>
<td>245.0</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>41.5</td>
<td>0.437</td>
<td>418</td>
<td>46.2</td>
</tr>
<tr>
<td>Red blood cell number (10⁶ cells mm⁻³)</td>
<td>1.809</td>
<td>0.034</td>
<td>169</td>
<td>1.231</td>
</tr>
<tr>
<td>Haemoglobin conc. (g 100 ml⁻¹)</td>
<td>11.1</td>
<td>0.174</td>
<td>187</td>
<td>11.8</td>
</tr>
<tr>
<td>Erythrocyte area (µm²)</td>
<td>105.2</td>
<td>1.517</td>
<td>223</td>
<td>117.3</td>
</tr>
<tr>
<td>Erythrocyte nuclear area (µm²)</td>
<td>18.5</td>
<td>0.312</td>
<td>223</td>
<td>15.4</td>
</tr>
<tr>
<td>Erythrocyte cytoplasmic area (µm²)</td>
<td>87.2</td>
<td>1.237</td>
<td>223</td>
<td>102.0</td>
</tr>
<tr>
<td>Erythrocyte nuclear/cytoplasmic ratio x 10²</td>
<td>21.3</td>
<td>0.443</td>
<td>223</td>
<td>15.1</td>
</tr>
<tr>
<td>Mean corpuscular volume (µm³)</td>
<td>228.4</td>
<td>5.012</td>
<td>153</td>
<td>369.5</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin (pg)</td>
<td>63.7</td>
<td>-</td>
<td>-</td>
<td>94.1</td>
</tr>
<tr>
<td>Mean corpuscular haemoglobin conc. (%)</td>
<td>27.9</td>
<td>0.666</td>
<td>106</td>
<td>25.5</td>
</tr>
</tbody>
</table>
Fig. 6 The mean value (±95% confidence limits) for red blood cell number (RBC), the haematocrit (Ht), and haemoglobin concentration (Hb) of large larval Geotria australis (60-102 mm) caught in sequential monthly samples.
Fig. 7 The mean value (±95% confidence limits) for the haematocrit (Ht) and red blood cell number (RBC) of large larvae (L), metamorphosing stages 1-7, downstream migrants (DM) and early upstream migrant adults (A). Stage 7 is separated into early (7E) and late (7L) categories.
Fig. 8  The mean value (±95% confidence limits) for the percentage of those immature members of the erythrocyte series (middle and late erythroblasts and very early erythrocytes) which can be distinguished amongst the cells in Romanowsky-type stained blood smears of *Geotria australis*. Life cycle stages represented are large larvae (L), metamorphosing stages 1-7, downstream migrants (DM) and early upstream migrant adults (A). Stage 7 is separated into early (7E) and late (7L) categories.
Fig. 9  The mean value (±95% confidence limits) for the total area and the nuclear/cytoplasmic (n/c) ratio of mature erythrocytes in large larvae (L), metamorphosing stages 1-7, downstream migrants (DM) and early upstream migrant adults (A) of Geotria australis. Metamorphosing stage 7 is separated into early (7E) and late (7L) categories.
in a single sample of larval *G. australis* collected in Tasmania.

Paralleling the situation with erythrocyte area in Western Australian populations, the diameter of the erythrocyte in blood suspensions was significantly greater (P < 0.01) in the adult (12.8 μm) than in the larvae (12.4 μm). In the case of thickness, the mean values for the adult (4.1 μm) were greater than those of the larvae (3.3 μm) at a higher level of significance (P < 0.001).

The mean corpuscular volume of 228.4 μm$^3$ calculated for larvae was significantly lower (P < 0.001) than the 369.5 μm$^3$ calculated for adults. Moreover, there was no overlap between the individual values for the two life cycle stages. Consistent with this finding is the fact that the mean corpuscular haemoglobin of 63.7 pg in ammocoetes was much lower than the 94.1 pg obtained for adults (Table 1). However, the mean corpuscular haemoglobin concentration of 27.9% in the ammocoete was significantly greater (P < 0.001) than the 25.5% found in the adult (Table 1).

**Relationship between blood parameters in ammocoetes and adults**

In ammocoetes, a significant correlation (P < 0.001) was found between red blood cell number (RBC) and haematocrit (Ht) and between haemoglobin concentration (Hb) and haematocrit (Figs 10, 11). The relationships can be expressed by the following equations.

\[ \text{RBC} = (0.673 + 0.0276 \times \text{Ht}) \times 10^6 \quad (r = 0.531; \ n = 102) \]
\[ \text{Hb} = 7.08 + 0.097 \times \text{Ht} \quad (r = 0.382; \ n = 91) \]

The larger volume of blood that could be obtained from adults also enabled the red blood cell number and the haemoglobin concentration to be measured on samples taken from the same animal. In the early upstream migrants, the red blood cell number was significantly correlated (P < 0.001) both with haematocrit and haemoglobin concen-
tration. The data on these relationships and on that between haemoglobin concentration and haematocrit can be expressed by the following equations.

\[
\text{RBC} = (0.075 + 0.0260 \times \text{Ht}) \times 10^6 \quad (r = 0.881; \quad n = 39)
\]

\[
\text{Hb} = -0.21 + 0.25 \times \text{Ht} \quad (r = 0.930; \quad n = 37)
\]

\[
\text{RBC} = (0.205 + 0.0944 \times \text{Hb}) \times 10^6 \quad (r = 0.857; \quad n = 33)
\]

When the results from animals held in the laboratory for 8-10 months were added, the correlation coefficient increased greatly (see equations below) (Figs 12, 13, 14). The main effect of adding these data is to increase the range in the values for each parameter due to the fact that they undergo changes during the period corresponding to the spawning run (see later).

\[
\text{RBC} = (0.0210 + 0.0280 \times \text{Ht}) \times 10^6 \quad (r = 0.941; \quad n = 44)
\]

\[
\text{Hb} = -0.95 + 0.26 \times \text{Ht} \quad (r = 0.962; \quad n = 41)
\]

\[
\text{RBC} = (0.138 + 0.1031 \times \text{Hb}) \times 10^6 \quad (r = 0.925; \quad n = 36)
\]

**Changes in metamorphosis**

During metamorphosis, both the haematocrit and the number of red blood cells declined initially and then rose to levels approaching those found in ammocoetes (Fig. 7). The lowest mean haematocrit of 23.5% recorded at stage 4 may be compared with the overall mean of 41.5% obtained for ammocoetes and the 39.3% for the late representatives of stage 7. The mean haematocrit for animals caught on their downstream migration (33.9%) was significantly lower \((P < 0.02)\) than that found in the latter part of stage 7, and the values fell precipitously to 4.9% in those animals acclimated and held in 100% sea water for a further month. Although the minimum mean value for the number of erythrocytes \((1.187 \times 10^6 \text{ cells} \ mm^{-3})\) was reached at stage 5, rather than at stage 4 as with the haematocrit, the subsequent peak was also reached in late stage 7 when the mean number was \(1.674 \times 10^6\).
Fig. 10  The relationship between red blood cell number (RBC) and haematocrit (Ht) in large larval Geotria australis (60-102 mm).

The equation for the relationship is
\[ \text{RBC} = (0.673 + 0.0276 \text{Ht}) \times 10^5 \ (r = 0.531; \ n = 102). \]
Fig. 11 The relationship between haemoglobin concentration (Hb) and haematocrit (Ht) in large larval Geotria australis (60-102 mm).

The equation for the relationship is

\[ Hb = 7.08 + 0.097 \ (r = 0.382; \ n = 91). \]
Fig. 12 The relationship between red blood cell number (RBC) and haematocrit (Ht) in upstream migrant adult Geotria australis using animals caught in the field and held in the laboratory.

The equation for the relationship is

\[ RBC = (0.0210 + 0.0280 \times Ht) \times 10^6 \quad (r = 0.941; \quad n = 44). \]
Fig. 13 The relationship between haemoglobin concentration (Hb) and haematocrit (Ht) in upstream migrant adult Geotria australis using animals caught in the field and held in the laboratory.

The equation for the relationship is

$$\text{Hb} = -0.95 + 0.26 \text{Ht} \ (r = 0.962; \ n = 41).$$
Fig. 14 The relationship between red blood cell number (RBC) and haemoglobin concentration (Hb) in upstream migrant adult *Geotria australis* using animals caught in the field and held in the laboratory.

The equation for the relationship is

\[
RBC = (0.138 + 0.1031 \text{ Hb}) \times 10^6 \quad (r = 0.925; \quad n = 36).
\]
cells mm$^{-3}$.

Although the early erythroblast of lampreys cannot be clearly distinguished in Romanowsky-type stained material (Percy and Potter, 1976, 1981), the medium and larger-sized erythroblasts are clearly recognizable. These cells have a basophilic cytoplasm and a relatively large leptochromatic nucleus. In late erythroblasts or early erythrocytes, the cytoplasm starts to become slightly eosinophilic and the chromatin is more condensed and uniformly basophilic within the nucleus which has undergone a reduction in size. In contrast to these two groups of "immature" red blood cells are the more "mature" erythrocytes in which the cytoplasm is much more markedly eosinophilic and the chromatin is compacted within a nucleus which has a basophilic consistency and is much reduced in size (pachychromatic). In the oldest erythrocytes, the nuclear material becomes even more compacted. The work of Percy and Potter (1976, 1981) has shown that during erythrocyte development there is a progressive increase in erythrocyte size, a feature which aided the categorisation of stages in red blood cell maturation in the current study.

The proportion of immature members of the erythrocyte series in the total blood cell population fell from a mean of 6.5% at the beginning of metamorphosis to 0.9% at stage 2 (Fig. 8). Values rose to 4.6% at stage 3 and 9.6% at stage 4, after which they increased rapidly to reach 38.2% in early stage 7. The proportion of immature cells dropped abruptly to 8.5% in late stage 7 animals and to 2.0% in the downstream migrants. The fact that no immature red blood cells were found in early upstream migrants indicates that erythropoiesis has ceased at this stage. Since blood cell formation was still taking place in the fat column, it seems probable that many of
the new cells being formed at this time were granulocytes. Such a view would be consistent with the decrease in the relative number of erythrocytes and the rise in the relative number of particularly the neutrophils during the spawning run of *L. fluviatilis* (Percy and Potter, 1976).

In mature erythrocytes, the total area in blood smears declined progressively from values of 101.6 μm² at stage 1 to 81.8 μm² in early stage 7, before rising precipitously to 105.7 μm² in late stage 7 animals and 116.1 μm² in downstream migrants (Fig. 9). The nuclear/cytoplasmic ratio in mature erythrocytes increased from mean values of between 21.7 and 23.4 in stages 1-3 to 25.7 in early stage 7, before declining markedly to 19.0 at late stage 7 and 18.3 in the downstream migrants.

Since one of the two major haemoglobins in the adult migrated in gels to a position intermediate between those of the two major ammocoete haemoglobins, and the difference between the electrophoretic mobility of the latter two was very small, it was not possible to ascertain precisely when the haemoglobins changed from predominantly the larval to adult type. It was possible, however, to obtain a reasonably accurate assessment as to when this occurred from the time of appearance and degree of the intensity of staining of the second adult component. The blood clearly contained predominantly larval haemoglobins until at least stage 6 and does not possess mainly adult haemoglobins until late stage 7. The downstream migrant still contained traces of larval haemoglobins (Fig. 15).

**Changes in blood parameters in adults held in the laboratory**

An examination of the haematocrit, haemoglobin concentration, and red blood cell number for early upstream migrants and for the limited number of animals held for 8-10 months in the laboratory,
Fig. 15 Polyacrylamide gel electropherograms of haemoglobins of metamorphosing stages (1-7), downstream migrant (DM) and adult (A) Geotria australis.
indicate very strongly that these blood parameters decline during the spawning run. For example, the respective haematocrits and haemoglobin concentrations of about 15% and 4 g 100 ml\(^{-1}\) in the latter group of animals were approximately one third of typical values found at the commencement of the upstream migration (Table 1). While these values may approximate closely those found under field conditions, especially since in other species the haematocrit and haemoglobin concentration are known to decline during the spawning migration (Ivanova Berg and Sokolova, 1959; Potter and Beamish, 1978), the inability to obtain from the field adults that are approaching sexual maturity has made it impossible to verify this opinion.

4.4 Discussion

The absence of any clearly defined seasonal trends in haematocrit, red blood cell number and haemoglobin concentration in larval *Geotria australis* is perhaps surprising in view of the results of haemopoietic and haematological studies carried out on other species. For example, haemopoietic activity declines during the winter in *Lampetra* spp. in English rivers (Percy and Potter, 1977) and haematocrits increase during the spring and early summer in *Petromyzon marinus* in Canadian rivers (Potter and Beamish, 1978). The apparent anomaly provided by *G. australis* can probably be attributed to one or a combination of factors. Firstly, the temperatures of the water in the winter in south-western Australia rarely fall below 10\(^\circ\)C, whereas lower temperatures are maintained for long periods in the areas of England, and more particularly Canada from which the animals in the above studies on *Lampetra* and *Petromyzon* were collected. Thus, since summer temperatures are comparable in all three regions, the reduced temperature range found in the streams in south-western Australia may
well be reflected by a less pronounced seasonal change in haemopoietic activity. Secondly, the effects on blood cell number due to any pattern of change in haemopoietic sites would tend to become slightly obscured if the lamprey erythrocyte tends to have a long life, and there is now evidence to support the view that this is the case (Percy and Potter, 1977). Thirdly, the movements of ammocoetes, allied with the effects of large discharges of fresh water on sampling sites, has meant that it was not always possible to sample precisely the same population of G. australis every month. Indeed the latter feature could well be the reason for the fact that the blood parameters on a few occasions differed significantly from those recorded in the preceding and following months. Despite these reservations, however, it still seems reasonable to assume from the data that there can at most be only a small amount of seasonal change in the blood within a given population of ammocoetes of G. australis in south-western Australia.

The mean haematocrits of 41.5 and 38.6% given in this paper for larval G. australis from Western Australia and Tasmania respectively, are much greater than those recorded for the ammocoetes of other species. For example, values of between 22 and 29% have been obtained for the larvae of Ichthyomyzon hubbsi and the landlocked and anadromous forms of P. marinus (Potter, Hill and Gentleman, 1970; Beamish and Potter, 1972; Potter and Beamish, 1978). The situation is paralleled by differences in the haemoglobin concentration. Thus, values of 11.1 g 100 ml\(^{-1}\) in G. australis may be compared with 5.6 g 100 ml\(^{-1}\) in anadromous P. marinus (Potter and Beamish, 1978) and 7.4 g 100 ml\(^{-1}\) in I. hubbsi (Potter et al., 1970). Since we obtained similar but more variable results using heart puncture, the high values are not apparently related to differences in technique. This
view is supported by the fact that much more typical haematocrits of 26.6% were recorded using the same method on ammocoetes of *M. mordax* caught in Tasmania. The relatively very large number of erythrocytes, and therefore the amount of haemoglobin present in larval *G. australis*, will increase the oxygen capacity of its blood. Such a characteristic may well be of value to ammocoetes living in many areas in south-western Australia where the very sluggish flow and the tendency for pools to form in many of the rivers during the summer months almost certainly leads to reduced oxygen tensions in and immediately above the animal's burrow. Since similar environmental situations are not generally found in Tasmania, and the larvae of *M. mordax* have a much lower haematocrit than those of *G. australis* in the rivers of this island, it is postulated that the unusually high oxygen capacity of larval *G. australis* was evolved in environments containing reduced oxygen tensions.

Paralleling the presence of a higher haematocrit in the larvae of *G. australis* than in those of other species, the number of erythrocytes per unit volume given in this section for the ammocoete of *G. australis* (1.809 x 10^6 cells mm^{-3}) is greater than the 1.137 x 10^6 cells mm^{-3} found in *L. planeri*, which represents the only data on this blood parameter in any other species of lamprey (Likovsky, 1972).

In contrast to the situation in ammocoetes, the haematocrit and haemoglobin concentration in the early upstream migrant adults are much closer to those of comparable stages in at least two other species. Thus, the mean haematocrit of 46.2% in *G. australis* is similar to the 36.5 and 39.4% recorded respectively for *L. fluviatilis* by Ivanova Berg and Sokolova (1959) and Abou-Seedo (1977) and the 44.7% given for the anadromous form of *P. marinus* by Potter and Beamish (1978). These values are, however, rather lower than the
53.0% recorded for *Caspioomyzon wagneri* (Korzhuev and Glazova, 1967). The mean haemoglobin concentration of 11.8 g 100 ml\(^{-1}\) found in adult *G. australis* lies between the 10.9 g 100 ml\(^{-1}\) recorded for *P. marinus* (Potter and Beamish, 1978) and the 13.0 and 13.4 g 100 ml\(^{-1}\) found in *L. fluviatilis* (Ivanova Berg and Sokolova, 1959; Abou-Seedo, 1977) and the 13.0 g 100 ml\(^{-1}\) recorded for *C. wagneri* (Korzhuev and Glazova, 1967). The haematocrit for upstream migrating adults (46.2%) was significantly greater than the 33.9% of recently metamorphosed animals caught during their downstream migration (P < 0.001) and the 39.3% in animals collected towards the end of the last stage (7) in metamorphosis (P < 0.05). Since the haematocrits declined rapidly in downstream migrants held in the laboratory, it would appear important that the animal commences parasitic feeding as soon as possible after the completion of the non-trophic period that constitutes metamorphosis in order that energy sources are maintained at a sufficiently high level to support haemopoiesis. In this context, it is noteworthy that in *P. marinus*, which had not fed for approximately ten months since the commencement of metamorphosis, the haematocrit had declined to 10.1%, but after feeding in fresh water for a period before migrating to sea, it rose rapidly to 14.6% and later to 21.4% (Potter and Beamish, 1978).

The number of red blood cells in early upstream migrant adults of *G. australis* (1.231 \times 10^6 cells mm\(^{-3}\)) is lower than the 1.657 \times 10^6 cells mm\(^{-3}\) recorded for comparable stages of *L. fluviatilis* (Ivanova Berg and Sokolova, 1959). Much lower red blood cell numbers of 0.33 \times 10^6 cells mm\(^{-3}\) and approximately 0.36 \times 10^6 cells mm\(^{-3}\) have been recorded by Kisch (1951) and Gage (1888) for the blood of adult *P. marinus*. By contrast, the mean cell volume given by Kisch (1951) for the single adult *P. marinus* he examined (710 \mu m\(^3\)) is very much greater than the 189 \mu m\(^3\) recorded for the early upstream migrant of
*L. fluviatilis* by Ivanova Berg and Sokolova (1959) and the 370 $\mu m^3$ given in the present study for a comparable stage in *G. australis*. The latter estimate for the volume of the erythrocyte of adult *G. australis* is much higher than the 228 $\mu m^3$ recorded for the larva of this Southern Hemisphere species.

It is evident from blood cell suspension data that the diameter of living erythrocytes in the adults of different species of lampreys are similar. Thus, the value of 12.8 $\mu m$ for *G. australis* can be compared with 11.6 $\mu m$ in *L. fluviatilis* (Potter et al., 1974) and means of 13-14 $\mu m$ and 14.2 $\mu m$ in *P. marinus* (Thompson, 1887; Gage, 1888). Likewise, in the larvae, diameters of 12.4 $\mu m$ for *G. australis* may be compared with 11.2 $\mu m$ in *L. fluviatilis* (Potter et al., 1974) and a value of 13.4 $\mu m$ for an unidentified larvae of a North American species (Gage, 1888). In comparison with the relatively small differences in the diameter of living erythrocytes in larval and adult lampreys, their thickness differs to a much larger degree. Thus, in *G. australis* for example, the maximum thickness was approximately one third greater in the adult than the ammocoete and a similar situation has been recorded by Gage (1888). Thus, at least in *G. australis*, the larger erythrocyte volume found in the adult than the ammocoete can be attributed mainly to a greater thickness of the cell.

The mean cell haemoglobin in adult *G. australis* (94.1 pg) is approximately half the amount recorded for a single adult of *P. marinus* (Kisch, 1951; Riggs, 1972).

The mean area of the erythrocytes of larval *G. australis* (105.2 $\mu m^2$) was very similar to that recorded by Potter et al. (1974) for larval *L. fluviatilis* (109.8 $\mu m^2$). However, unlike the situation in the latter species where the area of the erythrocytes of ammocoetes
and adults were virtually identical, the area of the erythrocytes in the early upstream migrants of *G. australis* (117.3 \( \text{m}^2 \)) was significantly greater (P < 0.01) than that of larvae. Since the area of the mature erythrocyte in fully metamorphosed downstream migrants (116.1 \( \text{m}^2 \)) did not differ significantly from those of the upstream migrants (P > 0.05), but was highly significantly different from that of larvae (P < 0.001), the area of the mature erythrocyte of post-metamorphic stages in *G. australis* are apparently consistently greater than those of their ammocoetes. The presence of significantly greater (P < 0.001) nuclear areas (17.6 \( \mu\text{m}^2 \)) and nuclear/cytoplasmic ratios (18.3) in the erythrocytes of downstream migrants than in adults (15.4 \( \mu\text{m}^2 \) and 15.1), probably reflects their recent maturation during the terminal part of metamorphosis. However, it is almost certainly relevant that the values for the nuclear area and the nuclear/cytoplasmic ratio of both the young mature erythrocytes of downstream migrants, as well as those of the upstream migrants, were significantly smaller than those of the larvae. Indications that the blood data recorded for *G. australis* in Western Australia may be generally representative of this species in other geographical regions is provided by the similar values obtained for the various erythrocyte measurements and the red blood cell number and haematocrit in larvae taken from this area and from a rather different environment in Tasmania.

The decline in haematocrit and the number of red blood cells during the early stages of metamorphosis in *G. australis* suggests either that erythropoiesis is declining or that erythrocytes are being destroyed. However, it seems possible that both factors are operating since observations have shown that the activity of the main larval haemopoietic sites, i.e. the typhlosole and nephric fold of the kidney, declines during metamorphosis in *Lampetra* spp., (Percy and Potter, 1976, 1977), and that erythrophagocytosis
by the Kupffer cells of the liver is very extensive at this time (Percy and Potter, 1981). The pronounced rise in haematocrit and red blood cell number towards the end of metamorphosis implies that at this stage the rate of development of those red blood cells that will function in the young adult have increased. Such a view is consistent with studies on *Lampetra* spp. which have demonstrated that the main site of haemopoiesis in the adult, namely the fat column lying above the nerve cord, becomes increasingly active during metamorphosis (Percy and Potter, 1977). In this context, it is relevant that the less detailed study of haemopoietic sites in metamorphosing *G. australis* has shown that the pattern of change over is similar in this species to that described for *Lampetra* spp.

The tendency in *G. australis* for the area of the erythrocytes with an eosinophilic cytoplasm to decline through the early part of stage 7 in metamorphosis and for their nuclear/cytoplasmic ratio to increase during the same period, indicate that the erythrocyte population contains a decreasing proportion of fully mature cells. This is consistent with the fact that the proportion of immature red blood cells, i.e. cells corresponding to the erythroblast and very early erythrocyte of Percy and Potter (1976), rose during metamorphosis from less than 5% at stages 2 and 3 to approximately 38% in the early part of stage 7. The subsequent marked fall in the proportion of immature erythrocytes in late stage 7 and the downstream migrants, allied with a concomitant pronounced rise in erythrocyte area and a decline in erythrocyte nuclear/cytoplasmic ratio, indicate that the mature erythrocytes found in the early part of the marine phase are developed in the terminal part of metamorphosis. It is thus highly relevant that mature erythrocytes are characterised by possessing an eosinophilic cytoplasm, which indicates the presence
of haemoglobin, and that it is in the latter part of stage 7 and in the downstream migrants that the haemoglobin electropherograms first show that adult haemoglobin clearly predominates in the blood. On the basis of the above observations on the interrelationship of various blood parameters in *G. australis*, and the parallels between changes in haemopoietic sites in *Geotria* and those in *Lampetra* spp. (Percy and Potter, 1976, 1977, 1981), it can now reasonably be postulated that larval haemoglobins are normally present only in erythrocytes originating from the typhlosole and nephric fold, and that adult haemoglobins are found only in erythrocytes derived from the fat column.
5. EFFECT OF TEMPERATURE ON THE OXYGEN DISSOCIATION CURVES OF THE BLOOD OF LARVAL AND ADULT GEOTRIA AUSTRALIS

5.1 Introduction

Both lampreys and hagfishes contain many highly specialised features, such as those associated with their feeding mechanisms (Hubbs and Potter, 1971). Yet, there is also little doubt that the lampreys and hagfishes have retained some primitive features. In this context, it is almost certainly of significance that their haemoglobins differ from those of all other vertebrates. Thus, while the haemoglobins of all gnathostomes are tetrameric (Perutz, 1969; Lehninger, 1975), those of the hagfishes are predominantly monomeric in the oxygenated state (Bannai, Sugita and Yoneyama, 1972; Bauer, Engels and Paleus, 1975), as they also are to a large degree in dilute solutions and at neutral pH in the case of lampreys (Riggs, 1972). Under various conditions, the haemoglobins of lampreys aggregate in solutions to form dimers and tetramers (Riggs, 1972). Although Riggs postulated in 1972 that "it is very doubtful that lamprey haemoglobin ever dissociates to any significant extent at the high protein concentrations which must exist in the red cell", he now believes that this view may be incorrect (Riggs, pers. comm.).

Most of the studies of oxygen dissociation curves of lampreys have been performed on haemoglobin solutions (e.g. Wald and Riggs, 1951; Gibson, 1955; Briehl, 1963; Love and Rumen, 1963; Antonini et al., 1964; Behlke and Scheler, 1970a,b,c; Dohi, Sugita and Yoneyama, 1973; Johansen, Lenfant and Hanson, 1973). While Manwell (1963) and Potter, Hill and Gentleman (1970) provided data on the oxygen dissociation curves of erythrocyte suspensions, the only comparable study on the whole blood of lampreys was the work of Bird, Lutz and Potter (1976) on Lampetra fluviatilis. Although the latter
study provided data on blood from both the larvae and adults, the experiments were restricted to a single temperature.

The study described in this section was undertaken to acclimate animals at three very different physiologically realistic temperatures and then record at the same respective temperatures the oxygen dissociation curves and the Bohr effect produced by the unique lamprey haemoglobins in whole blood. The investigation also concentrated on evaluating the differences between the curves of both the larval and adult stages of *Geotria australis*. The data were compared with those recorded at a single temperature for ammocoetes and adults of the Northern Hemisphere lamprey *L. fluviatilis* (Bird *et al.*, 1976). Such comparisons were considered particularly relevant in view of the unusually high haematocrits found in larval *G. australis*, a feature that may have evolved as an adaptation for life in an oxygen-depleted environment.

5.2 Materials and Methods

After capture the animals were taken to the laboratory where they were held for at least two days under temperature and light conditions paralleling those existing in the field. Separate groups of animals were acclimated to each of the temperatures at which the subsequent blood experiments were to be performed by either raising or lowering the temperature by 1°C every two days. They were held for two to three weeks at each of the three experimental temperatures (5, 15 and 25°C) before they were used to provide blood. The larvae were always maintained in laboratory aquaria supplied with food and a natural silt substrate into which they would readily burrow. The adults, which do not feed during their upstream migration, were kept in large tanks containing large stones and other structures to provide cover and a water supply that was well aerated and filtered.
All animals were anaesthetised in MS222 (Calbiochem). Blood was extracted from the caudal vessels of larvae (Bird et al., 1976) and by cardiac puncture from adults as described previously (Section 4.2). In order to obtain sufficient blood for each oxygen dissociation curve in the ammocoete, it was necessary to pool the blood from approximately 15-20 animals to obtain the 0.25 ml required.

Oxygen dissociation curves were determined using the "electrolytic" method of Longmuir and Chow (1970) in the manner described in the study of the blood of *Lampetra fluviatilis* (Bird et al., 1976). The blood was maintained within the apparatus at the temperature to which the animals had been acclimated, i.e. 5, 15 or 25°C. The relationship between the P_{50} and pH at each of the experimental temperatures was expressed in the form of regression equations for the blood of both larval and adult lampreys. The slope in these equations corresponds to the Bohr effect (Table 2, Fig. 18). Regression equations were also calculated in the same manner as those given in Table 2 at successive 10% intervals in saturation. This enabled the P_{10}, P_{20}, P_{30}, etc., at each experimental temperature to be calculated and thus provide composite oxygen dissociation curves for larval (Fig. 16) and adult blood (Fig. 17) at a pH of 7.75. This latter pH was chosen to enable interspecific comparisons to be made between the composite curves of *G. australis* and the single curves of larval and adult blood of *L. fluviatilis* recorded at this pH by Bird et al. (1976). The total number of curves plotted for larval and adult *G. australis* at the three temperatures and within the pH range 6.8-8.2 are given in Table 2.

The Hill plots given by Log (Y/100-Y) vs Log P_{02}, where Y = percentage saturation of the blood and P_{02} = partial pressure of oxygen, yielded curved lines (Fig. 19). The lines were found to be
best represented by a cubic equation, from which the slope (n) at 35, 50 and 80% levels of oxygen saturation were then calculated (Table 3).

5.3 Results

The data for the blood of *G. australis* show that the oxygen dissociation curves of this lamprey are influenced by temperature and that there are marked differences between the two major life cycle stages (Figs 16, 17). Thus, values for the $P_{50}$ at a pH of 7.75 and respective temperatures of 5, 15 and 25°C were 0.57, 0.92 and 1.19 mm Hg in the case of ammocoetes and 6.86, 10.33 and 19.02 mm Hg with adults.

The Bohr effect, calculated over the pH range 6.8-8.2, did not differ significantly ($P > 0.01$) between the blood of ammocoetes at each of the three temperatures (Fig. 18, Table 2). Thus, respective values for ammocoetes at 5, 15 and 25°C were -0.24, -0.27 and -0.24. While the Bohr effect in adults changed from -0.23 and -0.25 at 5 and 15°C respectively to -0.16 at 25°C (Fig. 18, Table 2), these values also did not differ significantly ($P > 0.05$). The Bohr effect in larval and adult blood of *L. fluviatilis* at 10°C did not differ significantly ($P > 0.05$) from that of the comparable life cycle stage of *G. australis* measured at 5 and 15°C.

The curvilinear nature of the Hill plots obtained for both the major life cycle stages of *G. australis* is illustrated by the data shown for one set of determinations on larval and adult blood at a pH of 7.75 and a temperature of 15°C (Fig. 19). Determination of the slope (n) in the Hill plots made at 35, 50 and 80% oxygen saturation from oxygen dissociation curves representing a range of pH values showed that at these three saturations the n values were independent
Fig. 16  Oxygen dissociation curves for the blood of larval
Geotria australis at a pH of 7.75 and temperature of
5°C, 15°C and 25°C.

Inset: A comparison of larval (*) and adult blood (●)
at a pH of 7.75 and 15°C.
Fig. 17 Oxygen dissociation curves for the blood of adult

*Geotria australis* at a pH of 7.75 and temperatures

of 5°C, 15°C and 25°C.
Fig. 18  The Bohr effect in larval and adult blood of *Geotria australis* at 5°C, 15°C and 25°C. The Bohr effect for larval and adult blood of *Lampetra fluviatilis* is represented by dashed lines.
Table 2. The Bohr effect equations for the blood of larval and adult *Geotria australis* at temperatures of 5, 15 and 25°C and over the pH range of 6.8 to 8.2.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>°C</th>
<th>BOHR EFFECT EQUATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>Log $P_{50}$ = 1.6117 - 0.2396 pH (n = 18 r = 0.6377)</td>
</tr>
<tr>
<td>LARVA</td>
<td>15</td>
<td>Log $P_{50}$ = 2.0407 - 0.2681 pH (n = 31 r = 0.7089)</td>
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<td></td>
<td>25</td>
<td>Log $P_{50}$ = 1.9090 - 0.2364 pH (n = 16 r = 0.6004)</td>
</tr>
<tr>
<td>ADULT</td>
<td>5</td>
<td>Log $P_{50}$ = 2.6338 - 0.2319 pH (n = 17 r = 0.7861)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>Log $P_{50}$ = 2.9532 - 0.2502 pH (n = 29 r = 0.6542)</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>Log $P_{50}$ = 2.5571 - 0.1649 pH (n = 13 r = 0.7600)</td>
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</table>
Fig. 19 Hill plots calculated from single oxygen dissociation curves for larval (●) and adult (■) blood of *Geotria australis* at a pH of 7.75 and 15°C.
Table 3. The mean values ± 1 standard error for the slopes (n) in Hill plots for the blood of larval and adult *Gaotria australis* at three oxygen saturations (35, 50 and 80%) and three temperatures (5, 15 and 25°C).

<table>
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<th>±1 S.E.</th>
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<tr>
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<td>35</td>
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<td>13</td>
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</tbody>
</table>
of pH in both larval and adult blood at 5, 15 and 25°C. In both blood from ammocoetes and early upstream migrants, n increased with the degree of oxygen saturation (Table 3). For example, at 15°C, the respective values for 35, 50 and 80% saturation were 0.65, 0.86 and 1.37 in larval blood and 0.90, 1.22 and 1.97 in adult blood. Furthermore, n increased with temperature. Thus, the respective values at 50% saturation and temperatures of 5, 15 and 25°C were 0.63, 0.86 and 0.99 in larval blood and 1.08, 1.22 and 1.36 in adult blood. The values at a given saturation and temperature were always lower in larvae than upstream migrants. For example, at 80% and 25°C, the n value was 1.58 in ammocoetes and 2.46 in adults.

5.4 Discussion

The data presented in this paper show that with an increase in temperature from 5 to 15 to 25°C, the oxygen dissociation curve of both the larval and adult blood of Geotria australis shifts to the right, paralleling the situation found in many other vertebrates, including fishes (see e.g. Grigg, 1969). This shift represents a weakening of the bond between haemoglobin and oxygen with the result that oxygen is given up more readily. Such a feature would be of physiological significance to lampreys since, as with poikilotherms in general (Schmidt-Nielsen, 1979), the metabolic rate increases with a rise in temperature (Hill and Potter, 1970; Johansen et al., 1973; Claridge and Potter, 1975). In other words, it is of advantage to lampreys to have a more rapid delivery of oxygen to the tissues at higher temperatures.

In G. australis, the oxygen dissociation curve of the blood of the larvae at a given pH lay well to the left of that of the adult at each of the three experimental temperatures (cf. Figs 16 and 17). While oxygen dissociation curves can be shifted to the left by
exposure of the animal to experimentally-induced hypoxia (see Wood, 1980), it should be emphasised that our larvae were maintained in burrowed conditions approximating those normally found in the field. Moreover, the difference in the oxygen affinity, which reflects a change from larval to adult haemoglobins (Potter and Nicol, 1968), is almost certainly related to differences in the environment and the modes of life of the two life cycle stages. Thus, the ammocoete is a relatively sedentary burrowing microphagous animal living in the soft deposits of rivers (Potter, 1980a), whereas the adult is more active attacking marine teleosts and undertaking long migrations (Hardisty and Potter, 1971b). The high oxygen affinity of larval blood can be thus considered as an adaptation to enable larval lampreys to obtain oxygen from a surrounding environment which may be rather depleted in oxygen. By contrast, the much lower oxygen affinity of adult blood may be an adaptation to the higher environmental oxygen tensions that are found in the open waters of the sea and the faster-flowing regions of river systems frequented by the adults of anadromous parasitic species. It also has the effect of producing a greater oxygen delivery pressure which would be of particular advantage during the upstream migration when the animal has to overcome the effects of downstream flow and any natural or artificial barriers.

In the context of the change in position of the oxygen dissociation curve with life cycle stage and temperature, it is relevant to calculate the heat of combination of oxygen with haemoglobin (ΔH) between 5 and 15°C and 15 and 25°C. The respective values were -34.41 and -18.37 kJ mole⁻¹ in the larvae and -27.26 and -42.32 kJ mole⁻¹ in the adult. Thus, in contrast to the situation in adult blood, ΔH in ammocoetes is therefore lower between 15 and 25°C than
between 5 and 15°C. This implies that, while in general a shift to the right with increasing temperature is usually of value to poikilo-therms, it may be advantageous at higher temperatures to restrict the degree to which this shift occurs in burrowed ammocoetes which may be extracting oxygen from an environment depleted in oxygen.

It is evident from our superimposition of the data for *L. fluviatilis* on the graph showing the Bohr effect in *G. australis* (Fig. 18) that the oxygen affinity of blood in larvae of the Southern Hemisphere species differs markedly from that of ammocoetes of the holarctic lamprey. Thus, at a pH of 7.75, the *P*<sub>50</sub> for *L. fluviatilis* (1.95 mm Hg) is much greater than that found in *G. australis* either at 5°C (0.57 mm Hg) or at 15°C (0.92 mm Hg). It is in fact even greater than the *P*<sub>50</sub> found in *G. australis* at 25°C (1.19 mm Hg). The above data thus provide strong indications that at 10°C the *P*<sub>50</sub> of blood from larval *L. fluviatilis* is approximately 2½ times greater than that of larval *G. australis*. It is also evident from the Bohr effect graph that this statement is true over a range of pH values (Fig. 18). Moreover, a comparison of the larval oxygen dissociation curves for *G. australis* at 5 and 15°C with that given by Bird *et al.* (1976) for *L. fluviatilis* at 10°C provide every indication that the oxygen affinity is much greater in the Southern Hemisphere lamprey over a wide range of *P*<sub>O<sub>2</sub></sub> values.

The exceptionally high affinity of blood from larval *G. australis* parallels the situation found with the haematocrit and haemoglobin concentration. For example, a haematocrit of 41.5% and a haemoglobin concentration of 11.1 g 100 ml<sup>-1</sup> in ammocoetes of *G. australis* (Section 4) may be compared with haematocrits of 22-29% and haemoglobin concentrations of 5.6 - 7.4 g 100 ml<sup>-1</sup> in various other species of lampreys (Potter *et al*., 1970; Potter and Beamish, 1978; Section
4). A high affinity of the blood for oxygen, allied with a very high haemoglobin concentration, which increases the oxygen capacity, are features that would be of advantage to an animal living in oxygen-depleted environments. Such a situation may well exist in the ammocoete habitats found in the rivers of south-western Australia where flow virtually ceases during the summer to produce on occasions isolated pools in which ammocoetes are sometimes found.

Although there is also some evidence that the oxygen affinity of the blood of adult *G. australis* is also greater than that of adult *L. fluviatilis*, the differences are much less pronounced. This inference is based on the fact that at a pH of 7.75, the *P*<sub>50</sub> for *L. fluviatilis* (10.67 mm Hg) at 10°C is not intermediate between those of *G. australis* at temperatures of 5°C (6.86 mm Hg) and 15°C (10.33 mm Hg). These comparisons emphasise that, as shown in Fig. 18, the oxygen affinity of blood from adult river lampreys at 10°C is very similar to that of adults of the Southern Hemisphere lamprey at 15°C over a pH range encompassing physiologically realistic values.

The data on the Bohr shift in *G. australis* showed that, particularly in the larval stage of this species, the effect was independent of temperature. Moreover, the range in Bohr effects of -0.24 to -0.27 over the temperature range of 5-25°C encompasses the value of -0.25 recorded for the blood of larval *L. fluviatilis* examined at 10°C (Bird *et al.*, 1976). The above values are similar to the -0.23 and -0.25 recorded for adult blood of *G. australis* at 5 and 15°C respectively, and the -0.22 found with adult blood of *L. fluviatilis* at 10°C. Although these values were greater than the -0.16 found in adult *G. australis* at 25°C, the reduced magnitude of the slope was not significantly different.
A comparison by Schmidt-Nielsen (1979) of the Bohr shift in a wide range of mammals showed that this effect exhibited a pronounced tendency to decrease in magnitude with increasing body weight. However, despite the fact that the adults of *L. fluviatilis* and *G. australis* attain weights which are respectively about 30 and 250 times greater than those of their larvae, the Bohr effect in the blood of the two life stages is very similar. The similarity of the Bohr effect in ammocoetes and adults, particularly at 15°C and below, is also striking in view of differences in the degree of activity exhibited during the two stages and in the oxygenation properties of their respective haemoglobins.

The data now collected for lamprey blood, which indicate that at least in the larvae and adults of *G. australis* and *L. fluviatilis*, the Bohr effect is less than -0.30. The values are therefore markedly different from those found in haemoglobin solutions. For example, haemoglobin solutions have yielded Bohr effects of -0.70 in larval and adult *Petromyzon marinus* (Wald and Riggs, 1951; Manwell, 1963), -0.45 for adult *Ichthyomyzon unicuspis* (Manwell, 1963) and -0.41 for adult *Lampetra tridentata* (Johansen et al., 1973).

The increase in the n values obtained from Hill plots with an increase in oxygen saturation, which occurred in both larvae and adults and at all three temperatures, parallels the situation found in the blood of *L. fluviatilis* (Bird et al., 1976). The n values at a given saturation also increase with a rise in temperature. The data for both *G. australis* and *L. fluviatilis* show that n values are less in larval than adult blood at any given oxygen saturation level and temperature. It is also evident that values for n <1.0 are typically found at all temperatures in larval blood at oxygen satura-
tions below 50% and the same generalisation applies to adult blood below 35%. While n values <1.0 could indicate negative or hindering haem-haem interactions (Manwell, 1963), Riggs (pers. comm.) has suggested that it could be the result of functional heterogeneity amongst the haemoglobins.
6. **IRON LEVELS AND MAJOR IRON BINDING PROTEINS IN THE PLASMA OF AMMOCOETES AND ADULTS**

6.1 **Introduction**

Work on several species of lampreys has shown that the profound external changes that take place during metamorphosis are paralleled by equally extensive physiological and biochemical modifications. For example, the erythrocytes of the young adult exhibit different morphological characteristics from those of ammocoetes and are also produced in different sites within the body (Potter, Robinson and Brown, 1974; Percy and Potter, 1976, 1977; Section 4). These changes, which take place over several weeks, are paralleled by alterations in the haemoglobins and a marked shift in the oxygen dissociation curve of the blood (Adinolfi, Chieffi and Siniscalco, 1959; Manwell, 1963; Uthe and Tsyuki, 1967; Potter and Nicol, 1968; Beamish and Potter, 1972; Potter and Brown, 1975; Bird, Lutz and Potter, 1976; Sections 4 and 5).

The iron ultimately found in haemoglobin is transported in the blood of gnathostomatous vertebrates bound to the specific iron-transport protein transferrin (Morgan, 1980). In contrast to the detailed information on the blood related to oxygen transport in the ammocoete and adult, no studies have compared the properties of the transferrins present in adult lampreys (Boffa et al., 1967a,b; Webster and Pollara, 1969) with the iron-transport molecules found in ammocoetes. The study described in this section was therefore undertaken to isolate and compare for the first time some of the chemical characteristics of the main iron-binding proteins of larval and adult lampreys, using as a source of material the Southern Hemisphere lamprey *Geotria australis*. 
6.2 Materials and Methods

After anaesthesia in benzocaine, large ammocoetes (total length >60 mm) were clamped in a vertical position and their tail cut off just behind the cloaca. Blood was then collected in heparinised capillary tubes from the main vessel in the immediate post-cloacal region. Plasma was obtained by spinning the tubes in a Clements micro-haematocrit centrifuge at 16000 G for 5 minutes. Due to the small amount of blood obtained from each individual, which weighed ≤0.9 g, the results for larvae are based on samples of blood pooled from 20-30 animals, except in the case of total plasma iron for which the determination required only a very small volume of blood. In adults, which are very much larger than ammocoetes (total length >500 mm), the blood was extracted by cardiac puncture and spun in a M.S.E. bench centrifuge at 500 G for 10 minutes. The plasma was stored at -20°C.

Although it was difficult to ascertain macroscopically the sex of the smaller ammocoetes used in this study, a record was kept of the sex of large ammocoetes and adults in which the type of gonad was more clearly distinguishable.

Total plasma iron was determined by the method of Olson and Hamlin (1969) using an atomic absorption spectrophotometer (Perkin-Elmer 503).

Plasma iron binding proteins were labelled by the addition of 40 μl of $^{59}$Fe Cl$_3$ in 0.1 M HCl (2 μCi, i.e. 74 kBq) to 300 μl of plasma (Webb and Chrystal, 1981). After incubation for two hours at 4°C, an aliquot of 0.1 M NaHCO$_3$ was added to neutralize the acid. Any samples in which precipitation occurred were immediately discarded. Partial purification of both the ammocoete and adult plasma iron-
binding proteins, which are subsequently referred to as IBP, was achieved by molecular weight exclusion chromatography followed by ion-exchange chromatography.

Labelled plasma was applied to a Sepharose 6B column (1.8 x 25.0 cm), equilibrated with 0.054 M triethanolamine/HCl buffer (containing 0.125 M NaCl) at pH 7.1 (Huebers et al., 1976). Ultraviolet adsorption at 280 nm was followed continuously using a flow-through cell in a modified Hitachi spectrophotometer. Fractions of 2 ml were collected and monitored for $^{59}$Fe content using a Searle $\gamma$-counter. The $^{59}$Fe-containing fractions were collected and dialysed overnight against 0.05 M Tris/HCl buffer pH 8.2 before being applied to a column (2.0 x 20 cm) of DEAE Sepharose CL-6B equilibrated with the dialysis buffer. Elution by the starting buffer (2 bed volumes) was followed by the application of a gradient of 0.05 M Tris/HCl pH 8.2 to 0.5 M Tris/HCl pH 8.2. The eluted $^{59}$Fe fractions were concentrated over a membrane filter (Amicon UM10). All separations were carried out at 4°C.

Molecular weight determinations of purified labelled IBP's from both ammonocytes and adults were carried out using molecular weight exclusion chromatography on a Sepharose 6B column (2.5 x 90.0 cm), equilibrated with 0.05 M Tris/HCl pH 7.5 and calibrated using the following proteins: thyroglobulin (molecular weight 669,000), ferritin (443,000), catalase (232,000) and alkaline phosphatase (140,000) as markers. The regression equation relating log molecular weight and $K_{av}$ was calculated using the method of least squares.

The molecular weight of the subunits was determined by electrophoresis using 7.5% acrylamide gels in the presence of sodium dodecyl sulphate (SDS) (Weber and Osborn, 1969). A short incubation period of five minutes at room temperature was sufficient time to dissociate the proteins for subunit analysis. The gels were calibrated with a
Dalton Mark VI marker kit (Sigma) containing bovine albumin (molecular weight 66,000), egg albumin (45,000), pepsin (34,700), trypsinogen (24,000), β-lactoglobulin (18,400) and lysozyme (14,300). Gels were stained by soaking overnight in 3.5% aqueous perchloric acid solution containing 0.05% Coomassie Brilliant Blue G-250 (Eastman) and destained in 7% aqueous acetic acid. The regression equation relating log molecular weight and the relative mobility of the standards was calculated using the method of least squares. Since trypsinogen lies slightly off a line drawn through the other standards (Sigma, 1977; Fig. 21b), it was omitted from the calculation.

Isoelectric focussing was carried out in 10% acrylamide gels using Ampholine pH range 3-10 (LKB). The anode buffer was 5% H₃PO₄ while the cathode buffer was 5% ethylenediamine. Focussing was achieved using 0.5 x 14.0 cm tubes at 100 V overnight. The gels were then cut into 3 mm slices and eluted overnight in sealed tubes with 3 ml H₂O, after which the pH of the H₂O was determined. The $^{59}$Fe content was determined for each slice to identify the IBP.

The protein content of the IBP was determined using the Coomassie Brilliant Blue G-250 (Eastman) assay of Bearden (1978) which employed bovine serum albumin as a protein standard. The iron content of the IBP was measured using atomic absorption spectrophotometry.

Preparations of larval and adult IBP and also horse spleen ferritin were negatively stained with 1% aqueous phosphotungstic acid (Richter, 1959) and then placed on Formvar coated grids and examined with a Phillips 301 electron microscope.

6.3 Results

No significant difference (P > 0.05) was found between the
molecular weights and isoelectric points of the iron binding proteins (IBP) of the two sexes in the upstream migrant stage. Similarly, no significant difference ($P > 0.05$) was present between the concentration of iron in the plasma of males and females in either ammocoetes or the early upstream migrants. For these reasons, the data for both sexes in both life cycle stages have been pooled in this study.

Three distinct fractions were obtained on molecular weight exclusion chromatography of plasma from both ammocoetes and adults of *Geotria australis* (Fig. 20a,c). While the values for the optical density of Fractions II and III were relatively consistent, the height of Fraction I varied greatly, particularly in adults (Fig. 20a,c).

Fraction II always contained at least 75% of the $^{59}$Fe label in both larvae and adults (Fig. 20). Since the $^{59}$Fe label peak occurred at a slightly greater elution volume in the adult than in the ammocoete (Fig. 20a,c), the major plasma IBP of the adult had a smaller molecular size than that of the ammocoete. The remainder of the $^{59}$Fe label occurred in a different position in larval and adult profiles. Thus, in the ammocoete, the peak occurred between Fractions I and II, whereas in the adult it was found towards the latter part of Fraction III (Fig. 20a,c). Since the proportion of the $^{59}$Fe label found under the curve corresponding to Fraction I was very small in ammocoetes and not present in adults, the variation in Fraction I mentioned earlier does not influence the interpretation of the results in terms of IBP. When detected, free $^{59}$Fe was found to elute at or slightly beyond the bed volume.

In both life cycle stages, application of the pooled $^{59}$Fe portion of Fraction II to the ion exchange column produced only the one peak containing $^{59}$Fe which eluted with the starting buffer (Fig. 20b,d).
Although this always took place after the void volume, which shows that some adsorption had occurred, the greatest optical density was found later in the ammocoete than in the adult. No further peaks were obtained on eluting with the Tris/HCl gradient system.

The molecular weight of the purified non-dissociated plasma IBP determined by exclusion chromatography on a calibrated 6B column was greater for larvae than adults (Fig. 21a). Thus, a molecular weight of approximately 354,000 for ammocoete IBP can be compared with approximately 296,000 for adult IBP. A single subunit was obtained for both ammocoete and adult IBP on electrophoresis in the presence of the denaturing agent SDS. However, while the subunit of the ammocoete IBP had a molecular weight of approximately 18,000, that of the adult subunit was approximately 78,000 (Fig. 21b).

In isoelectric focussing of ammocoete IBP labelled with $^{59}$Fe in the usual manner, two distinct bands were observed whose isoelectric points (pI) corresponded to 5.1 and 5.7 (Fig. 22). This and subsequent determinations confirmed that the label was not equally distributed between the two bands, but occurred in a ratio of 60:40. However, when the plasma was saturated with excess FeCl$_2$ before labelling and subsequent purification, only one band corresponding to an isoelectric point of 5.1 was obtained. Thus, while the ammocoete contains only one major IBP, it can exist in at least two forms which may represent different degrees of iron saturation. The adult IBP displayed only one band which corresponded to an isoelectric point of 8.6 (Fig. 23).

In view of the difference between the pI of the ammocoete IBP (5.1, 5.7) and the pH of the ion exchange column (8.2), it is surprising that larval IBP eluted in the starting buffer. However, it is worth reiterating that the elution peak was delayed in comparison
with that of the adult, which had a higher pI (8.6). It is also known from $^{59}$Fe counts that much iron was lost from the protein at this stage, presumably by binding to the column resin.

Assay by Coomassie Brilliant blue G-250 of the IBP showed that only small amounts were recovered from whole plasma taken from both life cycle stages. The mean concentration of IBP recovered in ammocoetes was 230 $\mu$g ml$^{-1}$ of plasma, while that of adults was 299 $\mu$g ml$^{-1}$ of plasma. However, while the total amount of IBP recovered was similar for both life cycle stages, the amount of iron bound to the IBP differed greatly. For example, samples which gave an IBP concentration of 387 $\mu$g ml$^{-1}$ for an ammocoete and 348 $\mu$g ml$^{-1}$ for an adult yielded 7.5 and 0.5 mg Fe g$^{-1}$ IBP respectively. From these data and those given for molecular weights, it can be calculated that in the ammocoete IBP, the molecular ratio of iron to protein is 48:1, while in the adult the ratio is much lower, i.e. 2.7:1. The above values can be compared with those obtained for total iron in whole plasma (Table 4). In ammocoetes, the calculated value of 150 $\mu$g iron 100 ml$^{-1}$ of plasma obtained from protein and iron assays of purified IBP is very much lower than the mean value of 19,760 $\mu$g iron 100 ml$^{-1}$ of plasma determined in whole plasma (Table 4). This considerable difference confirms that much iron has been lost during the preparative procedures. While this feature may be due in part to a weak affinity for iron by larval IBP, it could also be due to the presence of non-specific iron which binds to the ion exchange column as has been found to occur in studies of plasma from patients with pathological cases of iron overload, such as for example occurs in thalassemia (Hershko et al., 1978). In the adult, the similarly calculated iron level of 15 $\mu$g 100 ml$^{-1}$ of plasma can be compared with a mean value of 34.6 $\mu$g 100 ml$^{-1}$ obtained for total iron in
Fig. 20  Elution profiles of the plasma of ammocoetes (a, b) and adults (c, d) of Geotria australis on Sepharose 6B (a, c) and DEAE Sepharose CL-6B (b, d).

Optical density profile ———, $^{59}$Fe profile -----.

The major fractions eluting on molecular weight exclusion chromatography are indicated by Roman numerals.
Fig. 21 Molecular weight determination of (a) the major plasma iron binding protein of ammocoetes and adults of *Geotria australis* using molecular weight exclusion chromatography; and (b) the respective sub-units using polyacrylamide gel electrophoresis in the presence of S.D.S. $K_{av} = \frac{V_e - V_o}{V_t - V_o}$. $R_f = $ relative mobility.
Fig. 22 Result of analysis of fractions from isoelectric focussing of purified iron binding protein from larval *Geotria australis*. Both pH (△) and $^{59}$Fe (○) of each fraction are shown.
Fig. 23  Result of analysis of fractions from isoelectric focusing of purified iron binding protein from adult *Geotria australis*. Both pH (▲) and $^{59}$Fe content (●) of each fraction are shown.
Table 4. The mean, 1 standard error, range and sample size (n), for total iron in the plasma of ammocoetes and early upstream migrant adults of *Gorria australis*.

<table>
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<th>STAGE</th>
<th>MEAN μg/100 ml</th>
<th>1 STANDARD ERROR</th>
<th>RANGE</th>
<th>n</th>
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<td>AMMOCOETE</td>
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<td>2,977</td>
<td>5,320 - 52,750</td>
<td>16</td>
</tr>
<tr>
<td>ADULT</td>
<td>34.6</td>
<td>1.55</td>
<td>17.0 - 59.2</td>
<td>35</td>
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</table>
whole plasma. Since a small amount of protein is known to be lost during ion exchange procedures, the difference between these later two values is not as marked as was the case with ammocoetes. From these data, it would thus appear that the adult IBP binds iron strongly.

Under the transmission electron microscope, the purified IBP of ammocoetes showed both the light halo characteristics of ferritin and the clear circular shape of apoferritin (Richter, 1959; Bessis, 1973). Since structures giving the latter appearance were by far the more abundant, it would appear that much of the ferritin had lost its iron during the preparative procedures. No ferritin or apoferritin-like structures were observed in adult IBP.

6.4 Discussion

The data presented in this section demonstrate that different proteins are used to bind iron in the plasma of the larval and upstream migrant adult stages of the Southern Hemisphere lamprey Geotria australis.

The major IBP in the adult bears some similarities with the transferrin of gnathostomes, even though it has a molecular weight of 296,000. The crucial point is, however, that the molecule is a tetramer composed of subunits of 78,000 and this monomeric molecular weight is the same as that of a typical transferrin (Aisen and Litowsky, 1980). Moreover, the molecule can be easily dissociated using SDS.

The presence of a tetrameric molecule in adult lampreys helps to explain why the Fe/protein ratio (2.7:1) is greater than is theoretically possible (2:1) in other vertebrates. That the ratio is not 8:1 can be attributed to incomplete saturation of the molecule, a
situation found with gnathostome transferrins in which saturation levels range from 20-60% (Morgan, 1980).

The pI of 8.6 obtained with isoelectric focussing for the IBP of adult _G. australis_ is very much higher than the pI found for example in human transferrin which ranges from 5.0 to 5.6 depending on the iron content (Aisen, 1980). Moreover, the value for the tetrameric IBP in adult _G. australis_ is similar to the 9.1 to 9.2 obtained for the monomeric transferrin-like molecule of adults of the Northern Hemisphere lamprey _Petromyzon marinus_ (Boffa et al., 1967a,b; Webster and Pollara, 1969). The high pI recorded for _P. marinus_ has been attributed to the absence of neuraminic acid on the protein carbohydrate chains (Boffa et al., 1967a).

The presence of a transferrin or transferrin-like molecule with a molecular weight of 78,000 in gnathostomes, as well as in _P. marinus_ and also _Eptatretus stoutii_, a representative of the other and highly divergent extant agnathan group, the hagfishes (Aisen, Liebman and Sia, 1972), suggests that such a situation was present in the very early vertebrates. However, since the gnathostome molecule is composed of two very similar amino acid sequences, other workers have also suggested that the current form of transferrin in gnathostomes represents the product of gene duplication early in its evolution (MacGillivray, Mendey and Brew, 1977; Aisen, 1980).

The data demonstrate that the major IBP in larval _G. australis_ is an oligomeric protein similar to gnathostome ferritin (Harrison, Clegg and May, 1980). The ammocoetes of this Southern Hemisphere lamprey thus differ from adult lampreys, hagfishes (Aasa, 1973) and gnathostomes (Morgan, 1980) in which transferrin is the major IBP. However, it should be noted that in the chromatographic data (Fig. 20) the area under the peak corresponding to ferritin is asymmetric
and encompasses the region where the transferrin peak is found in adults. Thus, the possibility cannot be excluded that the larvae contains a subsidiary iron binding protein whose characteristics are similar to those of the adult. Since the very high concentrations of iron found in larval plasma would lead to complete iron saturation of transferrin and hinder labelling, determination of whether or not transferrin is present in larval plasma would be difficult. While ferritin is found in the blood of gnathostomes, the levels are normally very low and the importance of this molecule in this group of vertebrates resides much more in its role as a means of storing iron in tissues (Munro and Linder, 1978). However, elevated levels of ferritin have been found in the blood of humans suffering from idiopathic haemochromatosis (Powell and Halliday, 1980).

The ferritin found in the plasma of larval G. australis has a molecular weight of approximately 354,000 and is composed of 20 subunits, each of which has a molecular weight of 18,100. Although this molecule resembles that found in gnathostomes, it must be recognised that in this latter group of vertebrates, tissue ferritin has a slightly greater molecular weight (c. 443,000) and is composed of a larger number (24) of subunits (Harrison et al., 1980). Moreover, although there is some evidence for the presence of two types of subunits of slightly different size in the ferritin produced in different tissues of the body in mammals (Munro and Linder, 1978), the electrophoretic data indicate that all subunits of ammocoete IBP have an identical size. The pI values of the two Fe labelled bands on isoelectric focussing were 5.1 and 5.7, which fall within the range found for tissue ferritin in gnathostomes (Bomford and Munro, 1980). Further indirect support for the view that iron is carried in a ferritin-like
molecule comes from the extraordinarily high levels of Fe in the blood (19,760 µg 100 ml⁻¹). These levels are consistent with such a Fe-binding molecule having a very large number of binding sites. The small number of binding sites on transferrin thus preclude the candidature of this molecule as the main IBP. Furthermore, no evidence was found with molecular weight exclusion chromatography for ⁵⁹Fe being bound to low molecular weight fractions. On the basis of the Fe/protein ratios, which were as high as 48:1, even after purification during which appreciable amounts of labelled iron were lost, it is evident that the iron binding protein in larval _G. australis_ blood had a large number of iron binding sites. In mammals, ferritin has been shown to have the capacity to bind up to 4,500 atoms of iron per molecule of protein (Harrison _et al._, 1980). Finally, the ultrastructural characteristics revealed by the electron microscope provided yet additional proof that a ferritin-like molecule is present in the plasma of larval _G. australis_.

The difference in the type of IBP molecule found in the blood of larval and adult _G. australis_ is paralleled by vast differences in the total amount of iron carried in the plasma of the two stages in the life cycle. Although no data are available for total plasma iron in any other species of lamprey or even hagfish, the values for each of the two life cycle stages in _G. australis_ can be compared with those reported for different gnathostomatosus groups. In general, the levels in adult _G. australis_ (35 µg 100 ml⁻¹) tend to lie slightly below the lower values found in other vertebrates (Morgan, 1980). For example, the total plasma iron levels in two other lower vertebrates, namely the teleosts _Tinca vulgaris_ and _Tinca tinca_, are 61 and 91 µg 100 ml⁻¹ respectively (Hevesy, Lockner and Sletten, 1964; van Dijk _et al._, 1975). By contrast, the plasma iron levels in larval
*G. australis* (19,760 μg 100 ml⁻¹) are far greater than those reported for any other vertebrate (Morgan, 1980).

Ammocoetes of *G. australis* possess a very much higher haemoglobin concentration (11.1 g 100 ml⁻¹) than those reported for larvae of other lamprey species, a feature that may reflect an adaptation to reduced environmental oxygen tensions (Section 4). It can be postulated that the very high levels of plasma iron in larvae may therefore be required to maintain the large amount of haemoglobin found in the blood. If this is the case, larval *G. australis* would be an atypical vertebrate in that it relies on iron transported as ferritin for the iron that is synthesised into haemoglobin. Since in *G. australis* the blood of adults has a similar haemoglobin concentration (11.8 g 100 ml⁻¹) to that of their ammocoetes (Section 4), but contains little or no ferritin, the interrelated physiological mechanisms involved in iron transport and haemoglobin synthesis are different in larval and upstream migrant stages.
7. DISTRIBUTION OF IRON-CONTAINING GRANULES IN LAMPERYS

7.1 Introduction

During the last twenty years, evidence has accumulated which shows that the chemical characteristics of the haemoglobins and the position of the oxygen dissociation curves of the blood of the larvae and adults differ markedly in several species of lampreys (see e.g. Manwell, 1963; Potter and Nicol, 1968; Uthe and Tsyuki, 1967; Bird, Lutz and Potter, 1976; Sections 4 and 5). Recent haematological studies have also revealed very striking differences between both the levels of iron and the major iron binding protein in the blood of the two life cycle stages of Geotria australis (Section 6). Thus, the mean total plasma iron concentration was 19,760 \( \mu g \) 100 ml\(^{-1}\) in ammocoetes compared with only 34 \( \mu g \) 100 ml\(^{-1}\) in adults, and the main iron binding plasma protein is ferritin-like in the ammocoete and transferrin-like in the adult (Section 6).

The current histological study was undertaken to examine the precise locations where iron deposits are found in larval, metamorphosing and adult \textit{G. australis}. Comparisons are also made between representatives of the three petromyzoniform families to help determine whether differences in such features as blood iron level, haemoglobin concentration, and the time when erythrocyte destruction takes place, are related to differences between life cycle stages and taxa.

7.2 Materials and Methods

Representatives of stages in the life cycle of several species of lampreys were collected from various Northern and Southern Hemisphere rivers (Table 5). The results reported in this study are based on a minimum of five representatives of each life cycle stage for each species. In the case of \textit{G. australis}, which was studied in most
Table 5. Data on the locality from which the various life cycle stages of each of the representatives of the three lamprey families were collected for the study on iron-containing granules.

<table>
<thead>
<tr>
<th>FAMILY</th>
<th>SPECIES</th>
<th>LIFE CYCLE STAGE</th>
<th>RIVER AND COUNTRY</th>
<th>LATITUDE</th>
<th>LONGITUDE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Ichthyomyzon greeleyi</em></td>
<td>Ammocoete</td>
<td>Tributary of French Broad, North Carolina, U.S.</td>
<td>36°N</td>
<td>83°W</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metamorphosing)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Petromyzonidae</td>
<td></td>
<td>Ammocoete</td>
<td>Ocqueoc, U.S.</td>
<td>45°30'N</td>
<td>84°W</td>
</tr>
<tr>
<td></td>
<td><em>Petromyzon marinus</em></td>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lampetridae</td>
<td><em>Lampetra lamottenii</em></td>
<td>Ammocoete</td>
<td>Tributary of Lake Erie</td>
<td>42°N</td>
<td>83°W</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metamorphosing)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lampetridae</td>
<td><em>Lampetra zanadrea</em></td>
<td>Ammocoete</td>
<td>Po, Italy</td>
<td>45°N</td>
<td>10°E</td>
</tr>
<tr>
<td>Lampetridae</td>
<td><em>Lampetra fluviatilis</em></td>
<td>Ammocoete</td>
<td>Teme, U.K.</td>
<td>51°N</td>
<td>4°W</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metamorphosing)</td>
<td>Vistula, Poland</td>
<td>52°N</td>
<td>20°E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lampetridae</td>
<td><em>Lampetra planeri</em></td>
<td>Ammocoete</td>
<td>Honduu, U.K.</td>
<td>52°N</td>
<td>3°30'W</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metamorphosing)</td>
<td>Highland Water, U.K.</td>
<td>51°N</td>
<td>1°30'W</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geotriidae</td>
<td><em>Geotria australis</em></td>
<td>Ammocoete</td>
<td>Tributary of Wanganui, New Zealand</td>
<td>43°S</td>
<td>172°E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ammocoete</td>
<td>Ringerooma, Tasmania, Australia</td>
<td>41°S</td>
<td>148°E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metamorphosing)</td>
<td>Warren and Donelly, Western Australia</td>
<td>34°S</td>
<td>116°E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ammocoete</td>
<td>Limay, Argentina</td>
<td>39°S</td>
<td>68°W</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metamorphosing)</td>
<td>Donguil, Chile</td>
<td>39°S</td>
<td>73°W</td>
</tr>
<tr>
<td>Mordaciidae</td>
<td><em>Mordacia mordax</em></td>
<td>Ammocoete</td>
<td>Ringerooma, Tasmania, Australia</td>
<td>41°S</td>
<td>148°E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metamorphosing)</td>
<td>Donguil, Chile</td>
<td>39°S</td>
<td>73°W</td>
</tr>
<tr>
<td></td>
<td><em>Mordacia lacca</em></td>
<td>Ammocoete</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Mordacia lapicida</em></td>
<td>Ammocoete</td>
<td></td>
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</tr>
</tbody>
</table>
detail, a total of 150 animals were used.

For light microscopy the animals were placed in 10% formalin or Bouin's fixative. Segments of the body were removed from the region of the liver and the anterior, middle and posterior parts of the trunk. Transverse sections of paraffin-embedded material were cut at 3 and 6 μm, treated with Perl's Prussian blue and counterstained with safranin (Culling, 1974). Bathophenanthroline (see Hukill and Putt, 1962) were also used to substantiate that structures which stained blue with Perl's Prussian blue contained iron.

For electron microscopy, segments of the body were fixed overnight in 5% gluteraldehyde in 0.5M phosphate buffer and then postfixed in 2% aqueous OsO₄ for one hour before dehydration and embedding in Epon 812. Thin sections were stained with uranyl acetate and lead citrate before examination on a Phillips 301 electron microscope.

7.3 Results

Transverse sections treated with Perl's Prussian blue revealed marked differences between the amount of iron deposited in the body of amphicoetes representing the three lamprey families.

Amongst the larvae of Northern Hemisphere lampreys, no iron was detected in either Lampetra zanandrei or Petromyzon marinus. Very small amounts were present in some but not all of the amphicoetes of Ichthyomyzon greeleyi, Lampetra lamottenii, Lampetra fluviatilis and Lampetra planeri. In these latter four species, the iron was always restricted to the columnar cells lining the apex of the typhlosole and the opposing wall of the intestine in the most posterior region of the trunk. The iron appeared as dark blue granules near the luminal border of the cells where they could often be seen to be located in groups within vacuoles.
Iron was also restricted to the intestine in the larvae of the two parasitic species of the Southern Hemisphere family Mordaciidae. However, in the two *Mordacia* species, iron was present in vastly greater quantities than in the Petromyzonidae and it also occurred, but in slightly less quantity, in the columnar cells of the intestine in the middle as well as the posterior regions of the trunk (Fig. 24a). Although the iron was most concentrated in the epithelial lining of the apex of the typhlosole and the opposite inner wall of the intestine, it was found in decreasing quantities in the epithelial cells away from this centre of focus (Fig. 24a). While the iron sometimes appeared as small dark and rounded blue granules in thinner sections (Fig. 24b), the concentration of these was generally so great that in 6 μm sections they often produced much larger and irregularly-shaped blue masses. The iron deposits were most pronounced in the upper middle region of the cell (Fig. 24b).

In *G. australis*, iron was found in slightly greater quantities but in a similar position in the intestinal cells of the posterior intestine (Fig. 24c) to that described for the Petromyzonidae. Clearly defined discrete granules predominated in the upper middle part of the intestinal cells whereas less numerous but much larger accumulations of iron granules were found in various regions of the cell. However, iron was also deposited in large but variable amounts in other areas of the body of larvae of the Geotriidae (Table 6), particularly in the fat column (Fig. 24d) and nephric fold (Fig. 24e). The amount of iron in the nephric fold ranged from moderate deposits in Argentinian ammocoetes to very concentrated deposits in larvae from Chile and south-western Australia (Table 6). In the fat column, which lies above the nerve cord and is a structure unique to lampreys (Potter, Percy and Youson, 1978), the amount ranged from moderate deposits in Argentinian
animals to large deposits in individuals from Chile and south-western Australia (Fig. 24d, Table 6). Although some iron was found in adipose tissue in a few other areas of the body, such as around the notochord and dorsal aorta, virtually none was present in association with the fat cells in the musculature or beneath the skin. As was the case with the iron in the intestine of *Mordacia* spp. (Figs 24a,b), the iron granules were either discrete or formed dark and much larger irregular blue patches (Fig. 24e). Traces of iron were detected in the cells of the larval opisthonephros and in the hepatocytes of the liver of some larval *G. australis* (Table 6).

During the metamorphosis of lampreys, the adipose tissue which extends throughout the body cavity becomes mobilised as an energy source (Youson, Lee and Potter, 1979). At the same time, the larval opisthonephros, which is located in the more anterior part of the body cavity and lies under part of this adipose tissue, undergoes involution and is replaced posteriorly by an adult opisthonephros (Youson, 1981b). As the fatty tissue becomes depleted in the nephric fold of *G. australis*, much iron remains in the same region. In the region above the recently-formed adult opisthonephros of downstream migrants, this produces a small but very concentrated region of iron in the dorsal part of the body cavity (Fig. 25a). In the fat column of *G. australis*, in which the fat is not as extensively mobilised during metamorphosis as in the nephric fold, the concentration of iron remains at a similar level throughout the transition from larva to young adult. However, the amount of iron in the liver increased during metamorphosis from a trace in the ammocoete (Fig. 25b) to a greater amount by stage 5 (Fig. 25c), and finally to very large concentrations in the downstream migrant (Fig. 25d). Under high magnification, the iron in the hepatocytes of larvae and the first few stages in metamorphosis can often be
Fig. 24a A transverse section through the posterior region of the intestine of larval _Nordasia mordax_ showing the presence of abundant iron in the intestinal columnar cells and in the intestinal contents. t, typhlosole; a, adipose tissue of nephric fold. x 100.

In this and subsequent figures the iron has a blue coloration as a result of its reaction with Perl's Prussian blue.

b Iron granules in the columnar cells of the posterior region of the intestine of larval _Nordasia mordax_. x 1770.

c Iron granules in the columnar cells at the apex of the typhlosole of the posterior intestine of larva of _Geotria australis_ collected in south-western Australia. x 500.

d Iron granules in the fat column of larval _Geotria australis_. n, notochord. x 150.

e Extensive deposits of iron granules in the adipose tissue of the nephric fold of the posterior region of the trunk of larval _Geotria australis_. Note the almost complete absence of iron in the columnar epithelial cells of the intestine compared with the situation in _Nordasia mordax_ (see Fig. 24a). i, intestinal lumen. x 150.

f A high power photograph of part of the adipose tissue shown in Fig. 1e, illustrating the discrete nature of the iron granules and the lipid deposits of the fat cells. x 2900.
Fig. 25a A dense area of iron deposition above the recently-formed adult opisthonephric kidney (k) of a downstream migrant of Geotria australis. Note that the intestine has developed the folds characteristic of the adult condition. x 190.

b, c & d Transverse sections through the liver of an ammocoete (b), metamorphosing stage 5 (c) and downstream migrant (d) of Geotria australis showing the marked and progressive darkening of the liver which occurs during metamorphosis as a result of the accumulation of iron. x 150.
Table 6. The relative density of iron deposits in various structures in larval *Geotria australis*, ranging from traces (+) to very large accumulations (++++)

<table>
<thead>
<tr>
<th>RIVER AND COUNTRY</th>
<th>ADIPOSE TISSUE</th>
<th>ADIPOSE TISSUE</th>
<th>ADIPOSE TISSUE</th>
<th>ADIPOSE TISSUE</th>
<th>ADIPOSE TISSUE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FAT COLUMN</td>
<td>NEPHRIC FOLD</td>
<td>LARVAL OPISTHONEPHROS</td>
<td>LIVER</td>
<td>INTESTINE</td>
</tr>
<tr>
<td>Warren Donnelly, Western Australia</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Ringarooma, Tasmania</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Wanganui, New Zealand</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>Limay, Argentina</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>Donguil, Chile</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>++</td>
</tr>
</tbody>
</table>
seen as discrete granules, but by the conclusion of metamorphosis they have become so concentrated that in thick sections they form large blue masses. Despite the presence of extremely large amounts of iron in the liver in the downstream migrant, much smaller quantities were detectable in upstream migrants, and these were principally located in the cells around the outer edge of the liver. Moreover, iron was not detected in spawning-run animals in any of the other sites in which it was found in downstream migrants.

During the metamorphosis of Lampetra spp., the amount of iron in the hepatocytes and kupffer cells of the liver also increased but never approached the concentration found at the end of metamorphosis in G. australis. Iron was also found in the liver of downstream migrants of P. marinus and in upstream migrants of L. fluviatilis.

Examination with the transmission electron microscope of the various regions of larval G. australis where iron-containing bodies are seen with the light microscope showed that the precise location of the iron deposits varied. For example, the iron deposits in the liver were present as irregular black masses inside the cell, while in the nephric fold, they occurred both as irregular deposits in the cytoplasm of lipid cells (Fig. 26a) and as round or oval deposits in the intercellular spaces (Fig. 26b). There was also considerable variation in the structure of the granules. In some cases, they were relatively electron-light and appeared to be composed of numerous dense cores (Fig. 27a), while in others the deposits showed variable electron density with little or no discernable internal structure (Fig. 27b). Following the detailed descriptions and interpretations by Jacobs (1980) based on mammalian material, it is assumed that the relatively light structures represent aggregations of ferritin while the denser deposits represent haemosiderin.
Fig. 26a  Transmission electron micrograph of iron granules (dark areas) in the cytoplasm of a lipid cell in the nephric fold of larval \textit{Geotria australis}.  x 19,380.

b  Transmission electron micrograph of a different area in the nephric fold to that shown in Fig. 26a, showing an iron granule present in the intercellular space.  x 15,300.
Fig. 27a High magnification transmission electron micrograph of a relatively electron-light iron granule in larval Geotria australis showing the presence of numerous dense cores. x 309,400.

b Electron micrograph of a leucocyte which has ingested iron granules of two different densities. x 8,500.
7.4 Discussion

This study has shown that the sites where iron-containing granules are prevalent varies amongst the larval stages of different species of lampreys and in general the differences correspond to the three families. This view is based on the contrast between the similar pattern found within six species representing three genera of Petromyzonidae and that present in Mordacia spp., and the very different situation existing in the monotypic Geotriidae collected from five widely separated geographical localities.

Before discussing the above differences in patterns of iron deposition, it is important to consider first the function of iron in vertebrates. The major proportion of iron taken in through the gut wall is utilised for the synthesis of haemoglobin (Jacobs, 1980). A small amount of iron also forms an important component of myoglobin, the cytochromes and other iron-containing enzymes, and a certain amount of iron can be stored as either ferritin or haemosiderin in various parts of the body (May and Williams, 1980; Wixom, Prutkin and Munro, 1980).

The larvae of species of the Petromyzonidae and Mordaciidae are characterised by haematocrits of between 22 and 29% (Potter, Hill and Gentleman, 1970; Potter and Beamish, 1978; Section 4). Although data on haemoglobin concentrations are less comprehensive, the relationship between this blood parameter and haematocrit in P. marinus and I. greeleyi suggests that the haemoglobin concentration of larval petromyzonid and mordaciid species generally lies between 5 and 8 g 100 ml\(^{-1}\) (Potter et al., 1970; Potter and Beamish, 1978). The absence or iron in structures other than the intestine in the larvae of the Petromyzonidae and Mordaciidae indicates that iron uptake in the ammocoetes of these families is regulated within the intestinal
epithelium, and that this metal is assimilated into the circulation in concentrations that can maintain these haemoglobin levels without apparently producing iron overload in other tissues. Indeed, it is almost certainly significant that in both these families the major concentration of iron occurs posteriorly in the trunk in absorptive cells which line the apex of the typhlosole and the opposing wall of the intestine as these cells are known to be constantly extruded (Hansen and Youson, 1978a,b).

The accumulation of iron in the form of granules within the vacuoles of intestinal epithelial cells, which subsequently become extruded into the faeces, provides a similar means of removing iron from the body to that described by Morton and Wimsatt (1980) for the vampire bat, Desmodus rotundus. This latter species, which is an obligate sanguiniphore, has the problem of eliminating iron from a diet in which the intake of this metal has been estimated as 800 times that of man.

The much greater amount of iron in the intestinal epithelium of the larvae of the Mordaciidae than in those of the Petromyzonidae, when considered in conjunction with the absence of deposits of iron in other regions of the body of both groups, suggests that the two Mordacia species representing the Southern Hemisphere family had ingested a larger amount of iron. However, although the intestinal epithelium of those larval G. australis which were collected from the same larval habitats as larval M. mordax possessed a far smaller amount of iron, exceptionally large amounts of iron were found in other sites in the body of the former species. A mechanism has thus apparently been evolved in G. australis for transferring large amounts of iron from the intestinal lumen into the blood. In this context, it may be relevant that the haematocrit and haemoglobin concentration of
larval *G. australis* (41.5% and 11.1 g 100 ml⁻¹) are much greater than the comparable values suggested earlier as typical of the Petromyzoni-dae and Mordaciidae (see Section 4). Recent studies have also shown that the plasma of *G. australis* carries a much greater concentration of iron than any other vertebrate that has so far been investigated (Section 6). There is therefore, apparently a relationship in larval *G. australis* between a relatively very high haemoglobin concentration and plasma iron level and the presence of exceptionally large deposits of iron.

In essence, the above comparisons between the Geotriidae and Mordaciidae argue that the mechanisms for eliminating assimilated iron through cellular extrusion from the intestinal lining have been greatly reduced in *G. australis*. However, the data on the amount of iron stored in the body suggest that, while an increased uptake of iron into the blood may have adaptive significance in terms of haemoglobin synthesis, there has been a concomitant reduction in the ability to regulate the actual amount of iron assimilated. The storage of iron in excess of immediate needs in adipose tissue has the advantage of taking iron away from areas where it might be toxic. Although there are no data on iron levels in the habitats where the samples of *G. australis* used in this study had been collected, it can be proposed that the larvae with the smallest iron deposits, i.e. those from Argentina, came from rivers in which the amount of iron was less than in those where it was greatest, i.e. south-western Australia.

It is now relevant to consider the possible significance of the changes that occur in the liver during metamorphosis. In *Lampetra* spp., the Kupffer cells lining the sinusoids in the liver are capable of extensive erythrophagocytosis and these ultimately contain dark particles (Percy and Potter, 1981). The results of the current study
with Perl's Prussian blue and bathophenanthrol ine have shown that these particles, and also those in the hepatocytes, almost certainly represent haemosiderin produced from the breakdown of haemoglobin. The above views are consistent with the observation that during the metamorphosis of *Lampetra* spp. changes occur in the sites of haemopoiesis and in the type of haemoglobins (Potter and Brown, 1975; Percy and Potter, 1977), and that in *G. australis* these are paralleled by a change in the morphological characteristics of the erythrocytes (Section 4). In other words, the data strongly suggest that metamorphosis is a period during which there is a very extensive breakdown of larval erythrocytes and haemoglobins and that this is reflected by the accumulation of mainly haemosiderin in the Kupffer cells and hepatocytes within the liver.

The much greater accumulation of iron in the liver of *G. australis* during metamorphosis than occurs in the liver of *Lampetra* spp. at a comparable stage may be related to the higher haematocrit in the larva as this would imply that a greater number of erythrocytes would be broken down at this time. The difference could also reflect differences between the vast amount of iron stored in larval *G. australis* and the absence of conspicuous iron deposits outside the intestine in ammocoetes of *Lampetra* spp., as is typically the case in the larvae of all other lampreys. Although during metamorphosis in *G. australis* iron is retained in the body cavity, the amount of adipose tissue lying above the developing adult opisth nephros regresses markedly at this time. It thus seems possible that, when these changes are occurring, iron is almost certainly discharged into the blood system and that the liver becomes the organ for the subsequent filtration of this metal. The progressive marked accumulation of iron in liver cells during metamorphosis, irrespective of its origin, may be related to the loss
of the bile duct that occurs at this time (Bertolini, 1965; Sidon in Youson, 1981c).

It is evident from the current study that larval *G. australis* could provide a useful model for investigating iron overload in vertebrates. Certainly, the large and varied form of the iron deposits provides an excellent opportunity for examining the relationship between the different types of iron bodies which bear a close resemblance to those seen in iron overload situations in humans, such as occurs in idiopathic haematochromatosis and transfusional siderosis (Jacobs, 1980). It would also be of value to determine the mechanisms involved in regulating the amount of iron assimilated through the intestine into the blood stream of larval lampreys. In view of the difference that apparently exists between the ammocoetes of *G. australis* and *M. mordax*, it would thus be pertinent to examine these two species collected from the same environment.
8. RELATIVE PROPORTIONS OF BLOOD CELLS AND SOME PROPERTIES OF LARVAL AND ADULT HAEMOGLOBINS

8.1 Introduction

Data on the relative number of the different blood cell types of lampreys are relatively scant. The only person to attempt to provide actual counts of cells other than erythrocytes is Likovský (1972), but the recent work by Percy and Potter (1976, 1981) suggest that the designation of some of his cell types is dubious. While a number of workers have drawn attention to differences between the relative number of some cell types in blood smears from larval and adult lampreys (e.g. Raunich, 1947), the data are not comprehensive.

Previous work on the chemical characterisation of lamprey haemoglobin has apparently been carried out only on adults. Thus, molecular weights of between 16,272 and 18,400 have been recorded for the adults of Petromyzon marinus, Lampetra fluviatilis and Lampetra japonica (Allison et al., 1960; Rumen and Love, 1963; Li and Riggs, 1970; Dohi, Sugita and Yoneyama, 1973; Zelenick, Rudloff and Braunitzer, 1979). In the first two of these species the pI values for the haemoglobins were given as 4.5 to 6.6.

During the study of the numbers of erythrocytes as a percentage of the total blood cell number in G. australis (Section 4), an investigation was made of other types of blood cells. Likewise, as a complement to the study of electrophoretic patterns of haemoglobins and oxygen dissociation curves of whole blood, which showed that the properties of the haemoglobins of larva and adult differed greatly, an investigation was made of their molecular weights and pI values. The results of the preliminary studies on blood cell types and the chemical characteristics of haemoglobins are reported in this Section.
8.2 Materials and Methods

Blood was obtained from large ammocoetes, the seven stages in metamorphosis and adults using the method described in Section 4.

For identification and enumeration of cell types, the blood was allowed to drip directly onto a microscope slide, after which it was immediately smeared and air dried. Data on differential cell counts were obtained by counting the cells in every fifth field from one edge of the slide until a minimum of either 500 cells or ten fields had been covered. Haemoglobin solutions for molecular weight and pI determinations were prepared using the procedures described in Section 4. Isoelectric focussing was carried out in a similar way to that described in Section 5, except that the material was not labelled. Moreover, after focussing, the position of the haemoglobin bands in the gel were recorded photographically from which their respective pI values were calculated.

For the molecular weight studies, the haemoglobins were purified, cleaned and washed as described above and then purified further by molecular weight exclusion chromatography on a Sephadex G-75 column (3.5 x 40 cms) in 0.05 M Tris/HCl pH 7.5. The two major ammocoete haemoglobins were separated from each other by ion exchange chromatography on a Whatman DE-52 column (2.0 x 20 cms), equilibrated with 0.005 M Tris/HCl pH 8.6 and eluted with 0.03 M Tris/HCl pH 8.6. Molecular weight determinations were carried out using both SDS polyacrylamide gel electrophoresis as described in Section 6, and exclusion chromatography on a Sephadex G-100 column (2.5 x 90.0 cms) equilibrated with 0.05 M Tris/HCl pH 8.6 and calibrated with alkaline phosphatase (molecular weight 140,000), bovine albumin (66,000), ovalbumin (45,000), myoglobin (16,890), and cytochrome c (12,380). Regression equations for both systems were calculated as described in Section 6.
8.3 Results

*Geotria australis* possessed all the cell types described for *Lampetra* spp. by Percy and Potter (1976, 1977, 1981). As in *Lampetra* spp., it was not possible to differentiate between small lymphocytes, thrombocytes and stem cells. Following the above authors (1981), these cell types have been placed together as the small lymphocyte/thrombocyte/stem cell (SLTS) group. Likewise, the slightly larger cells that lead to the erythrocyte and granulocyte series are considered to represent an intermediate (I) group of cells in which the lineages cannot be differentiated.

The total number of cells comprising the SLTS group, I group, monocytes, neutrophils and eosinophils expressed as a percentage of the total cell count differed significantly (P<0.01) amongst larval, metamorphosing, downstream migrant and early upstream migrant stages of *G. australis*. The percentage of these cells rose from 0.42% in the ammocoete to 0.73% in metamorphosing individuals to 1.53% in downstream migrants and finally to 3.87% in early upstream migrants (Table 7).

Although the proportion of the above five groups of cells in the total blood cell complement approximately doubled at each stage of the life cycle, the percentage of each of the individual cell types showed much more variability (Table 7). Thus, while the relative proportion of SLTS group, I group and monocytes each rose between successive stages in the life cycle, the most pronounced rise in the SLTS group was during the transition to the young adult, whereas both I group cells and monocytes increased dramatically in the early upstream migrants. The proportion of neutrophils remained relatively constant at 0.33 to 0.37% of the total cell population until the early upstream migrant phase when they rose to 1.59%. By contrast,
Table 7. The mean values for all SLTS group, I group, monocytes and granulocytes, and each individual cell group expressed as a percentage of the total cell count.

<table>
<thead>
<tr>
<th></th>
<th>ALL SLTS GROUP, I GROUP, MONOCYTES AND GRANULOCYTES AS A PERCENTAGE OF THE TOTAL CELL COUNT</th>
<th>SLTS GROUP</th>
<th>I GROUP</th>
<th>MONOCYTES</th>
<th>NEUTROPHILS</th>
<th>EOSINOPHILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMMOCOETES</td>
<td>0.42</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
<td>0.35</td>
<td>0.03</td>
</tr>
<tr>
<td>METAMORPHOSING STAGES 1-7</td>
<td>0.73</td>
<td>0.22</td>
<td>0.02</td>
<td>0.12</td>
<td>0.33</td>
<td>0.04</td>
</tr>
<tr>
<td>DOWNSTREAM MIGRANTS</td>
<td>1.53</td>
<td>0.93</td>
<td>0.05</td>
<td>0.15</td>
<td>0.37</td>
<td>0.03</td>
</tr>
<tr>
<td>ADULTS</td>
<td>3.87</td>
<td>1.03</td>
<td>0.33</td>
<td>0.93</td>
<td>1.59</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Fig. 28 Molecular weight determination of the major haemoglobins from ammocoete and adult *Geotria australis* using molecular weight exclusion chromatography.
the percentage of eosinophils fell from between 0.03 and 0.04% in the larval, metamorphosing and downstream migrant stages to 0.01% in the early upstream migrant (Table 7).

Isoelectric focussing of solutions of ammocoete haemoglobins yielded two major bands whose mean isoelectric points based on several determinations were 5.25 and 6.4. By contrast a single major band was found in adult haemoglobin solutions, corresponding to an isoelectric point of 5.1.

The molecular weight of each of the two larval and one adult haemoglobins, as determined by molecular weight exclusion chromatography, was 22,000 (Fig. 28). After electrophoresis in the presence of the denaturing agent SDS of both the two ammocoete and one adult haemoglobin produced two bands in virtually identical positions. The first band, which stained the more heavily, corresponded to a mean molecular weight (1 standard error) of 16,700 (±300) while the second corresponded to a mean molecular weight of 32,000 (±400).

8.4 Discussion

Any comparison of differential blood cell counts among lower vertebrates suffers from the problem that in many cases the morphology and the function of various cells have received different nomenclature and interpretations (Ellis, 1977). However, the comprehensive descriptions by Percy and Potter (1976; 1977; 1981) for blood smears of Lampetra fluviatilis and Lampetra planeri prepared using Romanowsky-type stains enable some comparisons to be made. Thus, in Geotria australis, the blood cell types found in smears are essentially the same as those described for the above two species. In addition, the data in the current study help confirm the observation by the above authors (1976) that basophilic granulocytes are not found at any
stage of the life cycle in the Petromyzoniformes.

The different proportions of individual cell types found in each stage probably reflects to some extent the different conditions that *Geotria australis* experiences during its life cycle. Thus, the lowest contribution of cells other than erythrocytes to the total blood cell complement (0.42%) occurs in the larval stage during which the animal remains in the same environment (Potter, 1980). The highest value (3.87%) occurs during the upstream migration. By contrast, when the adults have to overcome both the physical barriers imposed by dams and natural obstructions which can inflict physical damage (Hilliard, Pass and Potter, 1979), and the problems imposed by both re-entry into fresh water (Morris, 1971). Moreover, in the case of *G. australis*, this species has also to withstand the effects of gram negative bacteria to which they are particularly susceptible (Hilliard et al., 1979).

Prior to the adult stage, the most pronounced rise in individual cell proportions occurs in the SLTS group and monocytes which may be related to the extensive catabolism which occurs during metamorphosis (Percy and Potter, 1976, 1981).

While a detailed discussion of the individual blood cell properties is not possible, some of the trends seen in *G. australis* are paralleled in other lamprey species. Thus, in this study and those of Raunich (1947) and Likovský (1972), neutrophils are the most common granulocyte in both larval and adult stages. Moreover, eosinophils are more common in the blood of ammocoetes than they are in the blood of adults in which they are comparatively rare (Raunich, 1947; Likovský, 1972).

Data for all the molecular weight and pI values for lamprey haemoglobins have been restricted to the adult stage in the life cycle. The molecular weight of 16,700 recorded for each of the major
haemoglobins in the larvae and adult stages of *G. australis* on S.D.S. electrophoresis falls within the range of 16,272 to 18,400 given by a number of authors using a variety of techniques on the haemoglobins of other lamprey species (Allison et al., 1960; Rumen and Love, 1963; Li and Riggs, 1970; Dohi, Sugita and Yoneyama, 1973; Zelenik, Rudloff and Braunitzer, 1979). The second minor band corresponding to a molecular weight 32,000 using SDS almost certainly represents a dimerisation of the molecule which is known to occur in lamprey haemoglobins (Riggs, 1972; Dohi et al., 1973). The tendency for the molecule to self associate to form dimers and tetramers almost certainly also accounts for the high molecular weight (22,000) recorded on molecular weight exclusion chromatography. Using the technique, the haemoglobin is in a dynamic, rather than a fixed state as in S.D.S. electrophoresis, and thus a limited association between molecules would account for an elevated value that is not a direct multiple of 16,700.

The acidic pI values recorded for the two major ammocoete and the single adult haemoglobin parallel the values of 4.5 to 6.6 recorded for the multiple haemoglobins of adult *Petromyzon marinus* (Rumen and Love, 1963), and the 5.6-5.7 recorded for a single haemoglobin from adult *L. fluviatilis* (Allison et al., 1960). While the values for lampreys are lower than the 6.8 to 7.0 generally recorded for vertebrates (Prosser, 1973), they are very similar to those recorded for *Myxine glutinosa*, a member of the other extant agnathan group (Quast and Vesterberg, 1968).
9. **CONCLUDING REMARKS**

The data presented in this thesis on the haematology, physiology and biochemistry of the blood of *Geotria australis* show that, in many respects, their properties differ markedly from those of the ammocoetes of Northern Hemisphere species. It would also appear from the limited information for *Mordacia mordax* that the other Southern Hemisphere family (Mordaciidae) is in these respects much more similar to holarctic species. By contrast, the characteristics of the blood of adult *G. australis* are similar to those of Northern Hemisphere species.

The values for the red blood cell number, haematocrit and haemoglobin concentration in larval *G. australis* (see Section 4) are far higher than those recorded for comparable stages of the Petromyzonidae and the Mordaciidae, whereas those for adults of the Geotriidae are similar to those found in the two other lamprey families. Similarly, the $P_{50}$ of whole blood of larval *G. australis*, at a comparable temperature and pH, is very much lower than that found in larval *Lampetra fluviatilis*. By contrast, the $P_{50}$ of the adult stage is only slightly lower than that of adult *L. fluviatilis* (Section 5). The studies on iron metabolism (Section 6) also show that the ammocoetes of *G. australis* have a very high plasma iron level compared with their adults and, in this case, also in other vertebrates. Moreover, amongst the Petromyzoniformes, they are unique in containing large deposits of iron in the adipose tissue of the nephric fold and fat column (Section 7).

The very high haematocrit and haemoglobin concentration in larval *G. australis* indicate that there have been strong selection pressures favouring the evolution of a high oxygen affinity and oxygen capacity of the blood. This suggests that the oxygen tensions in the environment in which the ammocoetes of *G. australis* live are lower than those
in which the larvae of other species are found. It is thus probably relevant that water flow ceases in many parts of the rivers in south-western Australia from which the majority of the animals used in this study were collected. This feature would have the effect of reducing the influx of oxygenated water into the immediate regions surrounding and within the ammocoete burrow where the oxygen has been depleted by the animal's respiratory activities. The high levels of iron in the plasma are seen as reflecting an adaptation to maintain high haemoglobin concentrations, rather than as having a more specific role to play in the physiology of the animal.

The atypical aspects of the physiology of the blood of larval *G. australis* merit further attention, particularly in relation to the ability of the ammocoetes of this species to tolerate low environmental oxygen tensions. It would also be of interest to see whether the presence of a much lower *P*<sub>50</sub> than in other species is reflected by a much lower metabolic rate and if so whether this influences growth rates. In addition, the possession of high levels of a ferritin-like protein in the plasma and relatively large quantities of iron granules in the adipose tissue, could provide a useful model for studies of iron overload in vertebrates.

As is the case in other anadromous parasitic species, the adults of *G. australis* live in the relatively well oxygenated water of the sea during their trophic phase or in the main body of the water column in river systems on their upstream migration. These similarities among environments in which adult lampreys are found is paralleled by similarities in the respiratory properties of their blood. However, it would be of value to ascertain the way in which the blood of *G. australis* is adapted to the problems of a spawning run that may last for as long as a year. For example, since erythropoiesis
apparently ceases early in the upstream migration, the question of
the duration of life of erythrocytes is thus raised and how the
animal compensates for the red blood cell destruction that occurs on
the upstream migration.

The results reported in this thesis have extended the limited
comparisons that have been made between the haemoglobins of larval
and adult lampreys to a contrast between their molecular weight and
pI values. However, it is still evident that most of the work on
lamprey haemoglobins have been carried out on adults. Thus, further
studies on the larval haemoglobins would be of value in enabling the
functional significance of their biochemical and physiological
properties to be ascertained in terms of the mode of life of ammo-
coetes. The data collected during metamorphosis on changes in
erythrocytes, haemoglobins and haemopoietic sites illustrate the
value of lampreys as a model for changes similar to those found at
birth in mammals. In this context, it is also relevant to note that
further work is required to elucidate the functional significance of
life cycle changes in blood cells other than the erythrocytes. For
example, no obvious reason can be given at present for the difference
in the incidence of eosinophils in larvae and adults.

One final point is worth emphasising. The data in this thesis
show that, although the living lampreys are regarded as a relatively
homogeneous group (Potter, 1980b; Bailey, 1980), the different
families exhibit quite marked differences in some aspects of their
biology. In the case of the blood of larval G. australis, an attempt
has been made in this thesis to relate these to the environment in
which the animal lives. If future studies confirms that this is a
valid relationship, it could illustrate one of the reasons for the
success of the Petromyzoniformes. For example, while fossil evidence
suggests that the evolution of lampreys has been relatively conservative for the last 280 million years (Bardack and Zangerl, 1971), this group has still retained a pronounced ability to adapt to different environmental conditions.
REFERENCES


