Adaptations to Hypoxia in Freshwater Triclads, with particular reference to *Dendrocoelum lacteum* (Müller)

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Thesis Summary

Preliminary observations on triclads in rivers, suggested that individuals in some populations were more tolerant to low PO_2 than others. In particular, those from organically enriched sites were best at tolerating these conditions. This study attempted to describe these environmental differences in more detail and tried to relate them to any possible physiological, biochemical or genetic differences between different populations.

This work has concentrated mainly on *Dendrocoelum lacteum* (Müller) and to a lesser extent *Polycelis tenuis* Ijima. All populations in this study were from the River Kelvin System situated on the west coast of Scotland, near Glasgow. Two sites were chosen with apparently large differences in organic input, and hence PO_2; the Allander Water, (N.G.R. NS 539769) situated downstream from a sewage effluent input and the River Kelvin (NS 568664), downstream from a weir. Diurnal and seasonal measurements of PO_2 were estimated and this showed that there were considerable differences in PO_2 between sites i.e. the Allander Water site was hypoxic more frequently and for longer periods of time than the River Kelvin site.

The tolerance of triclads to low PO_2 was measured in open and closed systems. These results indicated that *Polycelis tenuis* and *Dendrocoelum lacteum* were capable of surviving prolonged exposure to hypoxia and anoxia and that LT_{50} values were affected by temperature, time of year, number of animals present, and nutritional state. Intraspecific differences were only considered for *D. lacteum*, and here individuals from the Allander Water had significantly higher LT_{50} values than those from the River Kelvin ($P<0.05$). In general, animals lived longer at a low PO_2 after acclimation to hypoxia, but the

intraspecific differences that persisted between populations before acclimation were also observed after acclimation. It should also be noted that these intraspecific differences also persisted in the F1 generations. Hence, there may be both environmental and genetic components in these differences.

Oxygen consumption was measured in closed and open respirometers for *D. lacteum* at various temperatures and PO_2. Intraspecific differences in oxygen consumption were observed at two experimental temperatures (10 and 20°C) by using Analysis of Covariance on regression equations, relating oxygen consumption to body mass. Triclads were also found to have higher respiration rates in closed respirometers and during the hours of darkness. There were differences between populations in ability to "regulate" the Pc (as defined Herreid, 1980). Animals from the Allander Water had a Pc which was considerably lower than for Kelvin animals at 10°C, but as triclads were not able to maintain oxygen independence at higher temperatures, Pc points at these temperatures could not be established. An "oxygen debt" was generated in animals from both sites, with animals from the Allander Water exhibiting a larger debt and payment taking a shorter time than Kelvin worms.

A red pigment, which appears to be haemoglobin, has been found in *D. lacteum*. This pigment may act as an oxygen store and aid in facilitated diffusion of oxygen across the body wall. The generation of a pigment was only observed during the summer months, possibly due to hypoxic stress, with the appearance of reddish-purple worms. It has been found in triclads from both sites but a significantly higher proportion of triclads possessed it at the hypoxic site (Allander 60-80%, Kelvin < 10% population). Early investigations have shown that the

pigment is a water insoluble compound, can donate oxygen, contains haematin and may be similar in structure to tissue or myo-haemoglobins

During anoxia, stores of glycogen are utilised and triclads shrink visibly. There was no intraspecific variation in glycogen utilisation, although there was more glycogen in animals from the River Kelvin. Triclads in this study have been shown to use anaerobic pathways distinct from, or in addition to, glycolysis. The end products from anaerobic metabolism were investigated using H.P.L.C. (High Precision Liquid Chromatography) on triclads kept under anoxic conditions for at least 24 hours. This showed that the main end products accumulated under anoxia were succinate, propionate, and acetate. There was no difference in the end products triclads from either site generated. These end products would be compatible with the kinds of anaerobic pathways that have been described for parasitic helminths (Cestoda and Trematoda) and it would therefore seem possible that triclads are facultative anaerobes utilising these or similar metabolic processes.

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And many races of living beings must have died out and been unable to reproduce themselves.
For whatever you see absorbing oxygen, it is cunning, valour, or finally nimbleness, which has protected and preserved that race from its origin.

Lucretius 99-55 B.C. (De rerum natura, v, 888)

CHAPTER 1

CHAPTER 1

Introduction

"Life is an active equilibrium between the living organism and its surroundings, an equilibrium which can be maintained only if the environment suits the particular animal or plant, which is then said to be 'adapted' to that environment. If an animal is placed in an environment which differs too greatly from that to which it is adapted, the equilibrium breaks down; a fish out of water will die."

John Maynard Smith (1977)

1.1 The Role of Adaptation

Individuals are mortal; therefore life must continue by the replacement of one generation of organisms with another. Every individual, however, will not succeed in leaving progeny i.e. the ability to reproduce and leave surviving offspring is not shared equally by all the members of a population. Members of a population vary in their ability to survive and reproduce. Genetic variation in the ability to reproduce and survive, and changing selection pressures can lead to changes in the characteristics of the population from one generation to another. These changes are, for a large part adaptive. This is because differing abilities to leave offspring frequently reflect differing abilities of individuals to cope with their surroundings.

Selection pressures will constantly operate to make all organisms maximally suited to occupy their particular niche. These selection pressures may involve predation, and physico-chemical factors. As there is no stability in any of the environmental constraints which constitute an ecological niche, all being subject to random and regular changes, natural selection will act on chance mutations (i.e. a variation in form, and or, function) to afford an organism a marginally improved chance of surviving to maturity to pass on these heritable qualities. Selection will only promote actual change in the morphology, physiology, or biochemistry of the organism, if environmental conditions (biotic or abiotic) are altered in some way.

Within any ecological system the effects of such pressures may be observed in terms of adaptation. The three types of response to changes in the environment; evolutionary, acclimatory, and reflex, relate to the three principle time scales of environmental change and hence adaptation. Evolutionary or genotypic adaptations have been described by many authors and many well known examples exist (Bradshaw *et al.*, 1965; Antonovics and Bradshaw, 1970; Kettlewell, 1973). Cain and Sheppard (1950) studied the common snail *Cepaea nemoralis* and noted the number of bands, shell colour, and the background colour of its habitat. They were then able to relate selective predation by the Song Thrush, *Turdus philomelos*, on those individuals that were conspicuous on differing backgrounds. They found selective predation with the morphs varying in frequency in their various habitats until equilibrium was reached.

Phenotypic adaptations, or acclimations, are changes within the lifetime of an individual organism in response to particular environmental circumstances, and they disappear when these

circumstances no longer exist. It is important to distinguish here between two terms which often appear in the literature, i.e acclimation and acclimatisation. In this instance, acclimatisation will be defined as a physiological response to natural changes in the environment. Acclimation, on the other hand, is used only for physiological changes; often this comprises of one factor, caused by experimentally induced stress.

Phenotypic adaptations usually involve changes in enzyme systems and may be completed within a relatively short time (less than 24 hours for many small animals). Examples of acclimatory adaptations are widespread (reviews in Alderice, 1972; Newell, 1970; Prosser, 1972). Spellberg (1972) and Graham (1972) examined temperature acclimations within species while Billings *et al.* (1971) also showed a differential capacity for such acclimation between species. Fry, Brett, and Clawson (1942), worked on thermal tolerance in goldfish acclimated to different temperatures. They demonstrated that by acclimating two populations of goldfish to 24 and 37.5^{o}C the thermal range of the fish could be extended.

More generally it may be necessary for an organism to respond to a change in the environment in an immediate (reactive) way. The immediate response of higher animals to environmental events involve reflexes of various degrees of complexity. Behaviour can be regarded as rapid response to environmental change. Behaviour may be instinctive (genotypic) or learned (phenotypic) and has often been regarded as distinct from physiology. However, as it often relates to an environmental change and involves coordinated actions by the animal, it must have some physiological basis. Examples of rapid changes to environmental stress are again widespread, Holmes (1940) described

rapid colour changes in the cuttlefish *Sepia officianalis* which correspond to the surrounding environment. Anti-predator responses are also excellent examples of reflex adaptations; young sticklebacks follow erratic escape movements (Humphries and Driver, 1971) while moths have been demonstrated to fall passively downward in response to the sonar clicks of bats (Roeder, 1965).

There is a considerable amount of information in the literature describing the changes in physiology correlated with changes in ecological pressures; this indeed has been the main subject matter of Physiological Ecology. The assumption is that the physiology of the organism is adapted to the environment. However, in order to substantiate these claims based on correlational techniques, it is necessary to demonstrate that physiological differences are correlated with differences in fitness and to show that they have a genetic basis. Physiological Ecology should properly extend from observations on physiology and its implications for survivorship to the biochemical and genetic basis of these differences.

The task of fulfilling all the requirements listed above are immense and this thesis will concentrate on providing a synthesis of observations at several levels; from ecological to physiological to biochemical, and hopefully to genetic. There is very little precise information available on any of these levels for freshwater triclads, but their suitability as an experimental animal (abundance, restricted movement, and benthic nature) makes them an excellent choice. The aims of this project were to identify any adaptations to hypoxia in two species of triclads, *Dendrocoelum lacteum* (Müller) and *Polycelis tenuis* Ijima. A comparative study of triclads living in different oxygen regimes was carried out by examining their mortality, respiration, biochemistry and morphology. An attempt was made to distinguish between

proximate (an immediate physiological response) and ultimate (involving a genetic component) responses and the physiological and biochemical basis of these differences was also considered. In conclusion, it is hoped that this thesis will, by integrating ecology, physiology, and genetics make a contribution to a better understanding of Evolutionary Physiological Ecology.

CHAPTER 2

Chapter 2

Literature Review

2.1 Introduction

This chapter reviews the information available from previous studies on the taxonomy, ecology, behaviour and zoogeography of the two experimental animals, *Dendrocoelum lacteum* and *Polycelis tenuis*. Information on other species will, however, also be included where relevant. Also included in this chapter is a review of general adaptations to hypoxia, (behavioural, physiological, and biochemical) with particular reference to the invertebrates.

2.2 Triclad Taxonomy

Dana (1766) made the first valid description of a freshwater triclad. It is true, however, that prior to this Linnaeus (1746) had described a worm, *Hirudo alba*, which was probably *Dendrocoelum lacteum*. Müller (1774) first described *Planaria torva*, *Dendrocoelum lacteum* and *Polycelis nigra* and included them with their parasitic relatives in the genus *Fasciola*, but this he later changed (Müller 1776) to the generic name *Planaria*. Kirby (1794) recognised two species within the *Planaria*, *Hirudo alba* and *Hirudo nigra*, which are now commonly referred to as *Dendrocoelum lacteum* and *Polycelis nigra*. Oersted (1844) instigated the new genus *Dendrocoelum* in which he placed the previously named *Fasciola lacteum*, where it has remained unchanged to the present day as *Dendrocoelum lacteum* (Table 2.1).

Table 2.1

Classification of British Freshwater Flatworms

Phylum	Platyhelminthes
Class	Turbellaria
Order	Seriata
Suborder	Tricladida

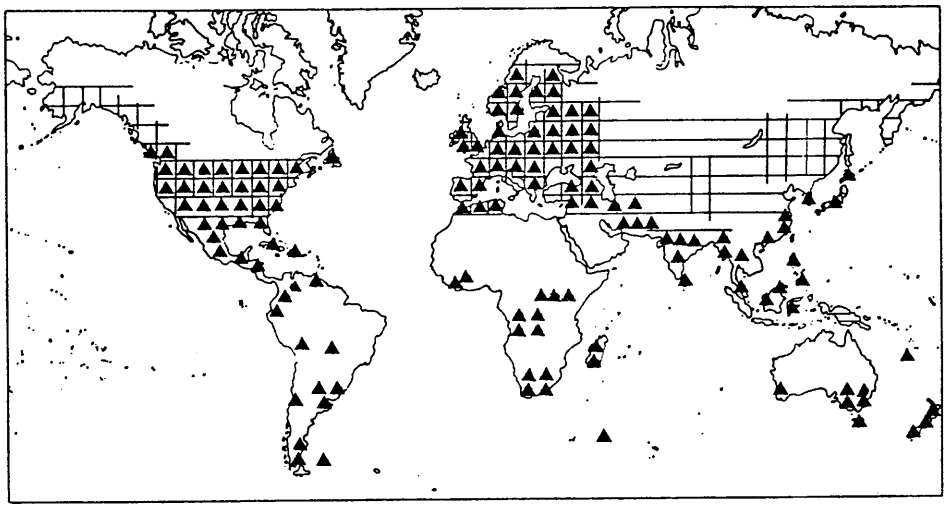
Infraorder	Paludicola
Family	Dugesidae
Genus	*Dugesia*
	D. tigrina
	D. polychroa
	D. lugubris
Family	Planariidae
Genus	*Planaria*
	Pl. torva
Genus	*Polycelis*
	P. nigra
	P. tenuis
	P. felina
Genus	*Phagocata*
	Ph. vitta
	Ph. woodworthi
Genus	*Crenobia*
	C. alpina
Family	Dendrocoelidae
Genus	*Dendrocoelum*
	D. lacteum
Genus	*Bdellocephala*
	B. punctata

Ehrenberg (1831) formed a new genus (*Polycelis*) for the species *Hirudo nigra* and subsequent studies by Ijima (1884) distinguished two separate species, *Polycelis nigra* and *Polycelis tenuis*, on the basis of colour and penial structure. Reynoldson (1948) strengthened these observations, as he not only looked at triclad morphology but also included ecological data. Dahm (1958) believed that cytological studies could supplement both morphological and ecological differences found in triclads and lead to a better understanding of triclad taxonomy. Le Moigne (1962) and Benazzi (1963), confirmed a distinct difference based on karyology, as well as those based on colour, penial structure and ecology. A third species, *Polycelis hepta*, was suggested by Hansen-Melander *et al.* (1954), but this was later disputed by Lender and Le Moigne (1960) and Benazzi (1963). Dalyell (1814) first named the third member of this genus, *Polycelis felina*, which, after various name changes through the years, was returned by Kenk (1974) to the original. At present, therefore, there are three distinct British *Polycelis* species Reynoldson (1978).

2.3 Distribution

Freshwater triclads can be found distributed throughout all the major continents of the world, although they are not found in the Arctic or Antarctic. The *Dugesiidae* demonstrate this broad distributional range (Fig 2.1), while the *Planariidae* and *Dendrocoelidae* are generally restricted to North America, Europe and the northern tip of Africa. A single *Polycelis* species has, however, been reported in New Guinea (Fig 2.1). The distribution of triclads has been recorded for the Americas (Ball, 1969; 1971), Europe (Dahm, 1967), and for Japan (Kawakatsu, 1965). Kenk (1974) has also provided a very

Fig 2.1 The World distribution of freshwater planarians - Dugesiidae, triangles; Planariidae, cross-hatching; and Dendrocoelidae, vertical hatching. (After Ball and Reynoldson 1981).



comprehensive world list of all known triclad species. There have also been many studies dealing with the distribution of triclad species in Britain (Carpenter, 1928; Reynoldson, 1958a; Reynoldson and Bellamy, 1970; and Wright, 1972).

The distribution of *Polycelis tenuis* has been difficult to assess due to the difficulties early researchers had in differentiating between *Polycelis tenuis* and *Polycelis nigra*. The distribution of the latter being somewhat uncertain. Kenk (1974) has identified those publications in the available literature that erroneously described *Polycelis nigra* as *Polycelis tenuis*. From this work we can establish that *Polycelis tenuis* is an indigenous European species found in many Western European countries including, France, Germany, Holland, (Lascomb, 1974; Ijima, 1884; Hartog, 1962) and other Eastern European countries and Western Siberia (Kenk, 1974). In Britain it is a very common species, although as with all other lentic species it is not found north of the Great Glen Fault.

The *Dendrocoelidae* is primarily a Eurasian group, with many diverse and bizarre forms being found in the region of lake Baikal, Siberia (Porfirjeva, 1973, 1977). In Britain, *Dendrocoelum lacteum* has often been found with *Polycelis tenuis* and its distribution is very similar, although the range of *Dendrocoelum lacteum* does extend further north. The range of *Dendrocoelum lacteum* in Britain has been carefully studied (Reynoldson and Young, 1966) and has been found to correlate closely with that of the crustacean *Asellus aquaticus*. Both species are generally found in productive, often organically enriched, lakes or slow-flowing rivers with high levels of calcium.

The distribution of triclads can be affected by a number of environmental variables. Reynoldson (1958b, 1958c) concluded that there

was a significant correlation between the amount of dissolved solids and hardness of a lake with the total abundance of triclads. Other factors that have been shown to influence triclad distribution, include competition, temperature and water velocity (Reynoldson, 1966a; Pattee *et al.*, 1973; Wright, 1974). Many recent studies of the biogeography of the group have tried to explain distributional patterns in terms of continental drift and plate tectonics (Ball, 1974; 1975). This is because triclads have no resistant or dispersal phases in their life cycle and are therefore often useful as indicators of palaeogeographical relationships.

The dispersal of individuals from one population to another will be vey important when considering the genetic composition of each population. Passage of individuals downstream would seem a common occurrence under conditions of spate, but populations from rivers a few miles apart may be genetically isolated. It was thought unlikely, that any aided form of dispersal could occur (Reynoldson, 1966b). However, there are isolated cases of passive dispersal of freshwater triclads by birds (Dahm, 1958; Reynoldson, 1966a), and egg capsules in floodwaters (Leloup 1944). *Polycelis tenuis* may also have been dispersed by fish restocking and by students engaged in freshwater field courses (Reynoldson and Jaques, 1976).

2.4 Behaviour and Ecology

Polycelis tenuis is a lake-dwelling (lentic) species, which can be commonly found in the quieter stretches of rivers. It is a perennial, iteroparous species that begins to produce oval shaped cocoons in February when temperatures rise above 5^{o}C (Calow and Woollhead 1977). The size of the population is regulated primarily by

competition for food, both intraspecifically and interspecifically (Reynoldson, 1964; Reynoldson and Bellamy 1971). This can occur even when food, comprising largely of oligochaetes, is abundant. Interspecific competition can also occur, primarily with the closely related species *Polycelis nigra*. This may cause competitive exclusion, which can force one or other species to vacate its niche or reduce the level of competition (Reynoldson and Sefton, 1972). This reduction can be achieved by one species altering its diet to depend heavily on a particular prey species, referred to as a "food refuge" (Ball and Reynoldson, 1981). Competition may not only instigate the use of "food refuges" but may also lead to changes in behaviour. *Dendrocoelum lacteum* and *Bdellocephala punctata* have been shown to be "sit and wait" predators feeding on active prey. *Polycelis tenuis* tends to be a "search out" predator that seeks out damaged or dying prey (Bellamy and Reynoldson 1974, Calow and Woollhead 1977). The competition for food is most intense during the reproductive season and it is at this time that numbers are most severely reduced.

Many early studies noted that predation on triclads was common (Moquin-Tandon, 1826; Harding, 1910) and proposed that this may be an important factor in regulating numbers of triclads. Young and Reynoldson (1965) disputed this, as in the laboratory very few animals would attempt to predate *Polycelis tenuis*. Only some dragonfly nymphs (Taylor, 1960), sticklebacks (Whitehead, 1922) and the water beetle *Dytiscus marginalis* (Young, 1963) ate any worms. This may be due to protection from rod-like structures called rhabdoids which can be extruded from the epidermis (Jennings, 1957). It was also suggested by Hyman (1951) that cannibalism is commonplace, a view supported by Root (1960) and Armstrong (1964). Reynoldson and Young (1963), however, found that in most species there was little evidence for cannibalism,

although *Polycelis tenuis* and *Dugesia lugubris* did show some cannibalistic behaviour in the laboratory. Predation is therefore less important than interspecific competition for food as a density regulating agent (Davies and Reynoldson, 1971). However, Davies (1969) demonstrated that predation can take a heavy toll on triclad populations, but only in small weedy ponds. Under these circumstances predation from dragonfly nymphs and newt larvae may be intense enough to cause a severe depletion in numbers, which may even lead to extinction.

A variety of parasites are found on triclads including Protozoa, larvae of Trematoda, Cestoda and Nematoda (Reynoldson, 1978). Various protozoans have been reported by Reynoldson (1950) as being parasites of many species of lake-dwelling triclads. Two species, *Urceolaria mitra* and *Trichodina steinii* were found living as epizoites on *Polycelis* species, particularly on *Polycelis tenuis* (Whitehead, 1915). Spontaneous tumours have also been found in other triclads particularly on *Dugesia* species (Pickavance, 1968), but it is unlikely that it would play any part in regulating numbers within a population.

Dendrocoelum lacteum is a sexually-reproducing, semelparous species commonly found in slow-running rivers as well as in its more common lentic habitat. Cocoon production begins in early January when temperatures rise above 1.5^{o}C. Dendrocoelid cocoons are large, 1-3 mm in diameter, and contain an average of eight young, whereas *Polycelis tenuis* cocoons contain only two or three individuals (Ball and Reynoldson 1981). The reasons for these differences are discussed from an energetics viewpoint in Calow and Woollhead (1979).

The *Dendrocoelidae*, in contrast to the scavenging *Planariidae* and *Dugesiidae*, are active hunters using an anterior "sucker-like"

organ to grip and subdue prey. Chemoreception, important in the *Planariidae*, therefore plays a much lesser role in detection of prey. The role of mucous secretion in the capture of prey has been investigated by De Silva (1976) and Pickavance (1971) who found that mucus acted as a trap for prey in the species *Dugesia tigrina* and *Dendrocoelum lacteum*. Once a prey item has been caught many other animals, including other species, come to feed (Ball and Reynoldson, 1981). The feeding process is lengthy, often taking over thirty minutes, and consists of the penetration of prey by the pharynx, aided by enzymatic secretions, and the removal of fluid and tissues into the gut. Digestion has been studied by Jennings (1959, 1962), who discovered that particles were phagocytosed for intracellular digestion, and there was no evidence of intraluminar digestion. Storage products were not thought to involve glycogen (Hyman, 1951), but Gelei (1936) and Jennings (1957) showed that this was not true. Glycogen was found in gut and mesenchyme cells, together with fat whereas protein reserves were located in the sphere cells of the gut (Jennings, 1957). Jennings (1957) also reported that it was the stored glycogen that was first utilised under starvation. Brand (1936) agreed with this and also showed a seasonal cycle in the polysaccharide content of *Planaria torva*.

2.5.1 Behavioural Adaptations to Hypoxia

Behavioural adaptations to hypoxia, are perhaps the simplest form of adaptation; yet, in many ways, the simplest solution is often the most effective. The ability to utilise anaerobic pathways, synthesise respiratory pigments or regulate respiration rates are made redundant, if behavioural responses can either prevent or restrict the extent of hypoxic conditions experienced by the triclads.

Behavioural adaptations can take many forms; many normally aerobic animals are able to survive long periods of oxygen depletion as resistant stages (cysts or eggs) whose metabolism is much reduced. When animals such as nematodes, rotifers, or tardigrades encounter extreme conditions detrimental to life i.e extreme dryness, cold, or anoxia, they enter a state called cryptobiosis (Keilin, 1959; Crowe and Cooper, 1971; Hinton, 1971). In this "death-like" state they shut down all metabolic processes. Only when more favourable conditions arise, with the return of oxygen and warmth, do they return to their normal activities.

A number of invertebrates will, in response to hypoxia, increase their surface area for gas exchange (Dimmock, 1977). There are also many well known adaptations for air-breathing in aquatic animals, *Planorbis corneus* and *Lymnaea stagnalis* being good examples of animals that live in water, yet surface periodically in stagnant water to replenish their lungs with fresh air. Many other authors have shown similar traits in other animals (review Carter, 1931; Jones, 1961; Taylor *et al.*, 1973, 1977), which can enable them to escape adverse conditions by evading their hostile environment.

Mobile organisms can respond to hypoxic conditions by

moving throughout their environment to a more suitable location. Studies of this response in fish are well documented. Shephard (1955) described the escape responses of young speckled trout *Salvelinus fontinalis* in oxygen-deficient water. There was an immediate increase in activity with exhaustive attempts to surface. As the amount of oxygen in solution fell, the fish remained quiescent at the bottom of the flask, activity being restricted to laboured respiratory movements. With the onset of acute hypoxia, the fish finally went into violent and uncoordinated leaps, before cessation of respiration. Jones (1952) described similar responses in sticklebacks, minnows and brown trout, while Gamble (1971) showed that some species of amphipod, given the choice of oxygenated or de-oxygenated water, move into the former.

The plecopteran nymph *Acroneuria facifica* also has elaborate behaviour patterns, that coincide with the onset of hypoxia (Knight & Gaufin, 1963). The general behaviour pattern under reduced oxygen conditions begins with hyperactivity leading to a posture, referred to, as a "stilted" stance. In this position, the legs are rigid and the body is held high above the normal level. With further decreases in oxygen, rapid undulatory body movements or pumping motions occur. These movements reached a maximum at about 60% air saturation (Benedetto, 1970). After this peak, there was a decrease in the number of pulsations corresponding with decreasing oxygen concentrations. This phase was then followed by a period of frenzied activity before the stonefly lapsed into unconsciousness and death.

The larvae of *Cloen dipterum* (Ephemeroptera) also have elaborate behavioural responses to combat hypoxia. Nagell (1977a) states that in aerated water larvae moved towards warmth and darkness, but under anoxia the opposite is true. During the winter ice covers

many ponds resulting in anoxic conditions for many months. The reversal in behaviour during anoxia greatly increases the survival of the larvae, attracting them to the underside of the ice. Here, oxygen levels are at their highest and during periods of thawing, oxygenated melt water can percolate through the ice which is then probably utilised by the larvae (Nagell, 1977b).

Similar movements towards the surface under oxygen stress are common amongst other groups of animals. These movements are often associated with the change from a negative to a positive phototactic response. Fry (1971) suggested that the surfacing of fish towards the light was triggered by the stress of low oxygen concentration. Other animals that have also shown similar types of response include dragonfly larvae (Wallengren, 1914), the zygopteran larvae of *Calopteryx* species (Zahner, 1959), and some species of mayfly larvae (Elliot, 1968).

2.5.2 Physiological Adaptations to Hypoxia

The ability to regulate oxygen consumption rates over a wide range of oxygen tensions has often been used to diagnose hypoxic tolerance. While metabolic "regulators" are clearly more suited to low oxygen environments than an organism whose oxygen consumption varies directly with environmental partial pressure, (i.e. a "conformer") these states are merely two ends of a spectrum with many intermediate forms (Mangum and Van Winkle, 1973).

Many authors have tried to quantify the degree of regulation of a species, and hence hypoxic tolerance; Mangum and Van Winkle (1973) and Bayne (1973) proposing alternative mathematical answers to this problem. Most authors, however, still make use of the critical oxygen

tension (Pc) shown by the animal, i.e. that point at which the animal changes from a regulator to a conformer. Clearly a low Pc point would be indicative of hypoxic tolerance, but such a staightforward interpretation is often dangerous as abrupt transition points often do not exist (Taylor *et al.*, 1977), and because metabolic regulation varies not only interspecifically but also within species as well (Bayne, 1971a).

Pc points are, however, useful as long as their shortcomings are borne in mind and the experimental conditions under which animals are examined are precisely defined. Many authors have listed factors that affect an organism's Pc point (review Herreid, 1980) and a brief summary will only be given here. Newell *et al.* (1978) demonstrated that the Pc point of an organism will increase with a rise in temperature and can change a regulating organism into a conformer (Herreid, 1980). There are both internal (activity, stress, amount of respiratory pigment) and external factors (salinity, time of day) that can also affect the Pc point (Bayne, 1971b, 1973; Sander, 1973; Spoek, 1974; Taylor, 1976).

If an animal has a low Pc, it does necessarily mean that it actively compensates for hypoxic conditions. Small animals with a large surface area do not have any problems with oxygen conductance (see below) until a very low external PO_2 is reached. Rogers (1962) demonstrated that this is indeed the case for certain nematode species. Regulation therefore may be "passive" as in the case of nematodes and small animals with a large surface area, or "active" where the conductance changes to offset the fall in PO_2.

Conductance will be different for every species and can be viewed as a number of additive resistances. For example, in a

freshwater triclad there would be only resistance offered by cell membranes and cytoplasm, whereas a crab would have resistance offered by circulation, ventilation, cardiac output, respiratory pigment affinities as well as its cell membranes and cytoplasm. (Herreid, 1980). A high conductance would therefore mean a low P_c point and *vice versa*. Active regulators must therefore increase their O_2 conductance when the external PO_2 is falling. This can be achieved in a number of ways; respiration can be maintained by increased ventilatory movements (Herreid *et al.*, 1979) or the affinity of respiratory pigments can increase (Vos *et al.*, 1979). Herreid (1980) stated that invertebrate blood generally has a low oxygen carrying capacity and a high oxygen affinity. Although this view is not universally shared (Mangum, 1980) many species do not utilise their respiratory pigments at rest, and it is only during exercise or hypoxia that their contributions significantly increase. The concentration of pigments may also alter. Fox (1954) stated that in *Daphnia* the haemoglobin concentration of the blood increases or decreases according to whether the water in which the animal lives contains a high or low concentration of dissolved oxygen. This view was supported by Weider and Lampart (1985) who isolated clones of *Daphnia pulex* and established relationships between low PO_2 and the amount of haemoglobin synthesised. Bayne (1971) found another alternative, the mussel *Mytillus edulis* when regulating in response to anoxia, increased its cardiac output and this also increased its oxygen conductance.

2.5.3 Biochemical Adaptations to Hypoxia

The most commonly adopted strategy for dealing with hypoxia is to supplement or replace aerobic respiration by an alternative

metabolism for which molecular oxygen is not essential. This alternative metabolism is termed fermentation or anaerobiosis. In this section, I will briefly review the major biochemical adaptations associated with hypoxia. The strategies associated with anaerobiosis are, however, discussed in detail by Hochachka and Somero (1973, 1984), de Zwaan and Wijsman (1976), and by Hochachka (1980).

It was thought until recently that the response of invertebrates to declining oxygen tensions necessitated a "switchover" from aerobic to anaerobic metabolism. There is, however, no low critical PO_2 at which "switching" occurs and in low oxygen conditions both aerobic and anaerobic metabolism may proceed simultaneously (Wells, 1980). The pH of invertebrate fluids falls sharply at very low PO_2 conditions and this has a marked effect on the affinity and the rate constants of various key enzymes, tending to favour those which participate in anaerobiosis.

Both aerobic and anaerobic metabolism depend on glucose or glycogen to generate energy. These energy producing processes generate their energy primarily from the high energy bonds of adenosine triphosphate (ATP). This involves the splitting of ATP into adenosine diphosphate (ADP) and inorganic phosphate (P_i) which releases the chemical energy that meets the energy requirements of the cell. The conversion of glucose into acceptable energy forms (ATP) is carried out in all animals by the process of glycolysis which involves the breaking down of six-carbon sugars into three-carbon sugars and phosphoenolpyruvate. It is only after this intermediary compound that the end products vary according to the circumstances.

In the presence of oxygen, carbohydrates are catabolised along two basic pathways: the glycolytic pathway and the Krebs' cycle.

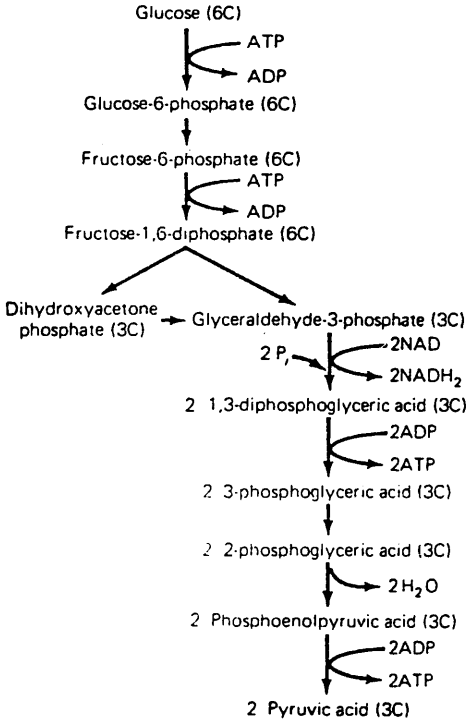
These pathways are described in a simple form in Fig 2.2. It is important to note that in glycolysis one molecule of glucose is converted into two molecules of pyruvic acid with the following results: two molecules of ATP have been used and four have been formed, resulting in a net increase of two molecules of ATP per molecule of glucose. The pyruvic acid which was formed in glycolysis is then oxidised by the Krebs' cycle. The reactions are well known and need not be reviewed here. It is sufficient to point out that two more molecules of ATP are generated per molecule of glucose and CO_2 is generated.

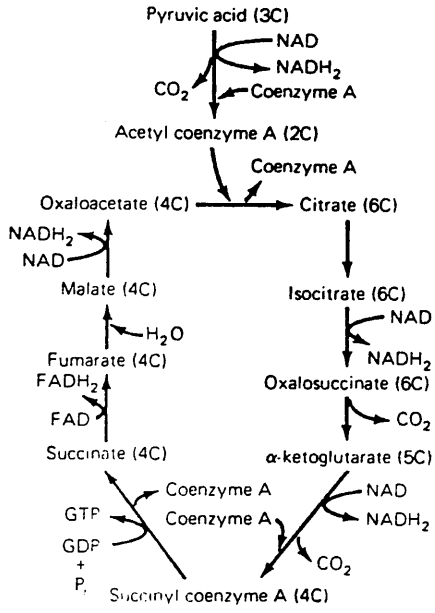
In the discussion so far, we have not mentioned molecular oxygen. This is because molecular oxygen is not needed at any stage in glycolysis or in Krebs' cycle. It is only in the electron transport chain (Fig 2.2) that oxygen acts as a terminal electron acceptor, with the final end product being water. If oxygen is denied, the electron transport chain ceases to operate and all members of the chain remain in their reduced state. The number of ATP molecules formed from the respiratory-chain phosphorylations per molecule of glucose is 32, which added to the substrate-level phosphorylations makes a total of 36 molecules of ATP per molecule of glucose.

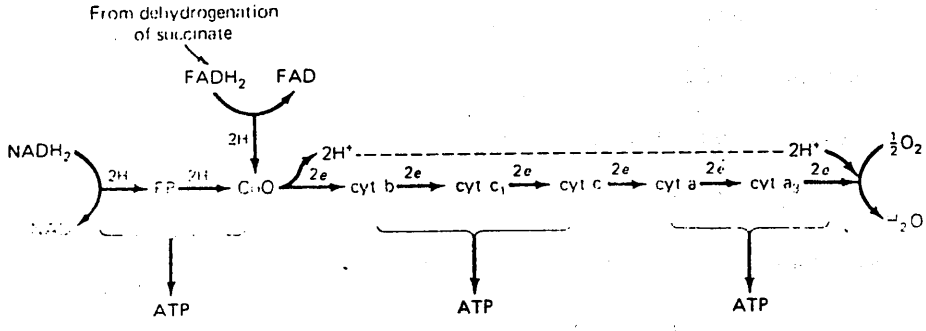
The simplest solution to short-term hypoxia, after exhausting all available oxygen stores may be to utilise fermentative energy yielding reactions. This is a strategy adopted by many animals including crustaceans, gastropods and some bivalves (Gäde, (1983). Many crustaceans accumulate lactic acid during hypoxia, indicating that anaerobic metabolism is supplementing or replacing aerobic metabolism (Teal and Carey, 1967; Bridges and Brand, 1980; Wheatly and Taylor, 1981). A comparable muscle metabolism is found in the mantle of the octopus. Here, octopine dehydrogenase takes over the role of lactate dehydrogenase with the subsequent production of octopine (Calow, 1981;

Fig 2.2 Top - The Glycolytic Pathway and Krebs' Cycle

Bottom - The Electron Transport Chain


Glucose (6C)
ATP
ADP
Glucose-6-phosphate (6C)
Fructose-6-phosphate (6C)
ATP
ADP
Fructose-1,6-diphosphate (6C)
Dihydroxyacetone phosphate (3C)
Glyceraldehyde-3-phosphate (3C)
2 P_i
2NAD
$2NADH_2$
2 1,3-diphosphoglyceric acid (3C)
2ADP
2ATP
2 3-phosphoglyceric acid (3C)
2 2-phosphoglyceric acid (3C)
$2H_2O$
2 Phosphoenolpyruvic acid (3C)
2ADP
2ATP
2 Pyruvic acid (3C)


Pyruvic acid (3C)
NAD
$NADH_2$
CO_2
Coenzyme A
Acetyl coenzyme A (2C)
Coenzyme A
Oxaloacetate (4C)
Citrate (6C)
$NADH_2$
NAD
Malate (4C)
Isocitrate (6C)
NAD
$NADH_2$
H_2O
Fumarate (4C)
Oxalosuccinate (6C)
$FADH_2$
FAD
CO_2
Succinate (4C)
α-ketoglutarate (5C)
Coenzyme A
NAD
GTP
Coenzyme A
$NADH_2$
GDP
+
P_i
CO_2
Succinyl coenzyme A (4C)


From dehydrogenation of succinate
$FADH_2$
FAD
2H
$NADH_2$
2H
2H
2H+
2H+
$\frac{1}{2}O_2$
2e
2e
2e
2e
2e
2e
cyt b
cyt c_1
cyt c
cyt a
cyt a_3
H_2O
ATP
ATP
ATP

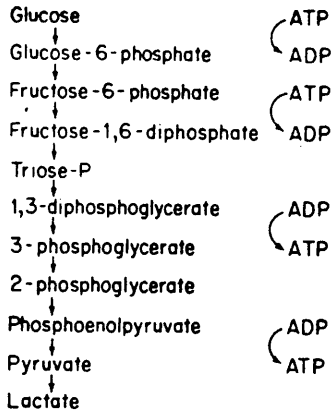
Fields, 1983).

In utilising lactate fermentation (Fig 2.3), oxygen deprivation will exclude the production of ATP by the respiratory-chain phosphorylations; i.e. 32 of the 36 molecules of ATP produced by aerobic catabolism will be lost. The yield of ATP can however be slightly increased by using glycogen as the main fuel. Glycogen and glucose are interchangeable fuels, but whereas glucose uses one molecule of ATP to enter glycolysis, glycogen does not. This therefore results in a 50% increase in ATP yield. Also in many anaerobic tissues, the breakdown of glycogen proceeds at a faster rate than the aerobic rate. This "Pasteur effect" will help to compensate for the lower energy yield under anaerobiosis. In lactate fermentation energy production can be maintained as long as the concentration of lactate does not become too high, since lactic acid is potentially toxic. To alleviate this problem, lactate can either be excreted, as in some parasites, or removed to another place in the body that has enough oxygen to permit aerobic oxidation. This strategy, however, depends on the eventual return to oxygenated conditions and the subsequent conversion of lactic acid back to pyruvic acid and the replenishment of glycogen stores. This is the "oxygen debt" generated during hypoxia which must now be paid back. This is not the only reason for the build up of an oxygen debt, replenishing oxygen stores or respiratory pigments, and replacing high energy phosphate bond reservoirs (e.g. creatine or arginine phosphate) used in anaerobiosis must also be accounted for (Ellington, 1983).

The pattern of oxygen debt varies between and within species (Von Brand & Mehlman, 1953; Bayne and Livingston, 1977; McMahon & Russell-Hunter, 1978), and falls into five categories (Fig 2.4). On

Fig 2.3 Top - Lactate fermentation

Bottom - Anaerobic pathway in bivalve molluscs (succinate pathway)


Glucose
ATP
Glucose-6-phosphate
ADP
Fructose-6-phosphate
ATP
Fructose-1,6-diphosphate
ADP
Triose-P
1,3-diphosphoglycerate
ADP
3-phosphoglycerate
ATP
2-phosphoglycerate
Phosphoenolpyruvate
ADP
Pyruvate
ATP
Lactate

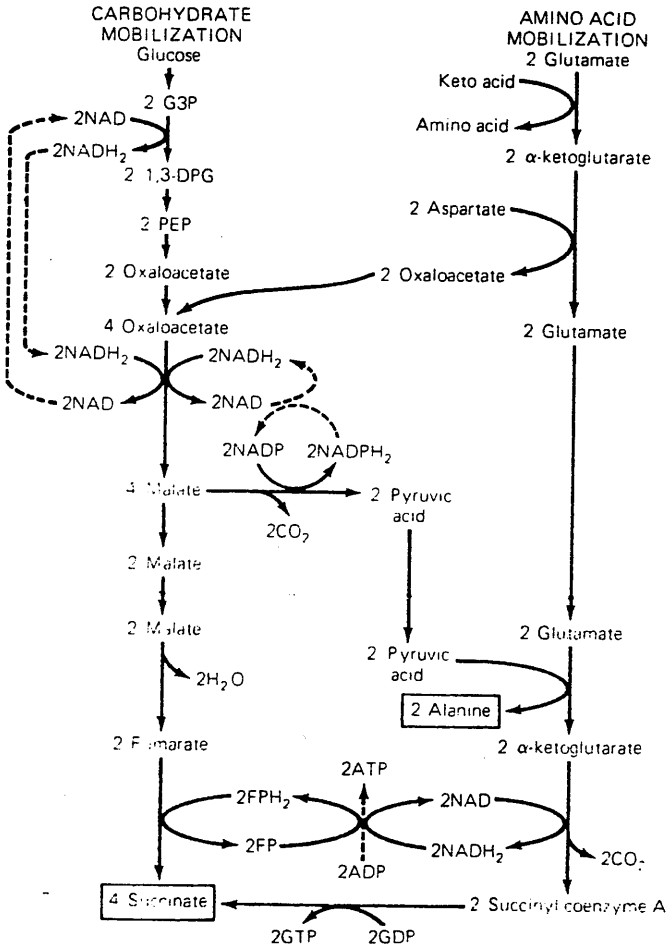
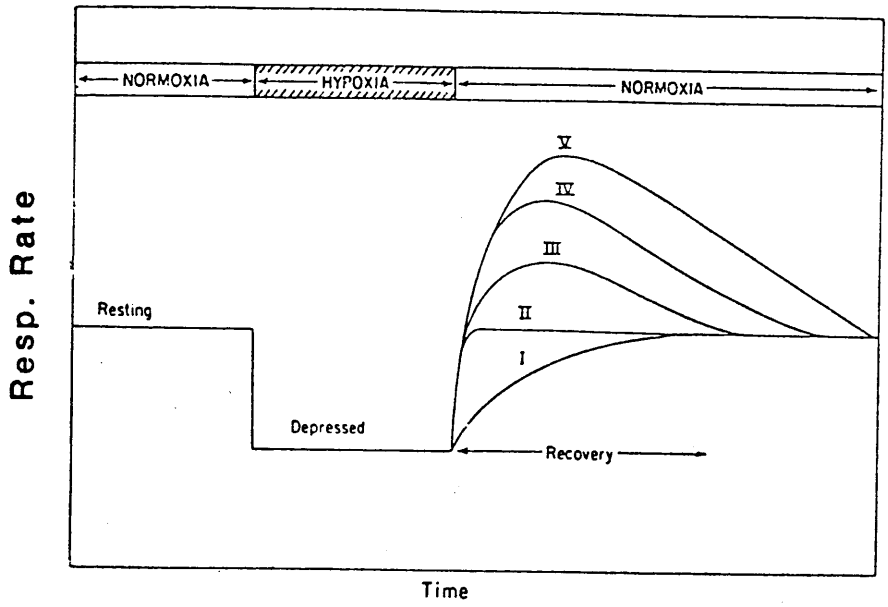

CARBOHYDRATE MOBILIZATION
Glucose
2 G3P
2NAD
2NADH2
2 1,3-DPG
2 PEP
2 Oxaloacetate
4 Oxaloacetate
2NADH2
2NADH2
2NAD
2NAD
2NADP
2NADPH2
4 Malate
2 Pyruvic acid
2CO2
2 Malate
2 Malate
2H2O
2 Fumarate
AMINO ACID MOBILIZATION
2 Glutamate
Keto acid
Amino acid
2 α-ketoglutarate
2 Aspartate
2 Oxaloacetate
2 Glutamate
2 Pyruvic acid
2 Glutamate
2 Alanine
2 α-ketoglutarate
2ATP
2FPH2
2FP
2NAD
2NADH2
2CO2
2ADP
4 Succinate
2 Succinyl coenzyme A
2GTP
2GDP

Fig 2.4 Five types of recovery after a period of hypoxia :

Curve I - Negative oxygen debt
Curve II - No oxygen debt
Curve III - Subnormal oxygen debt
Curve IV - Normal oxygen debt
Curve V - Supernormal oxygen debt

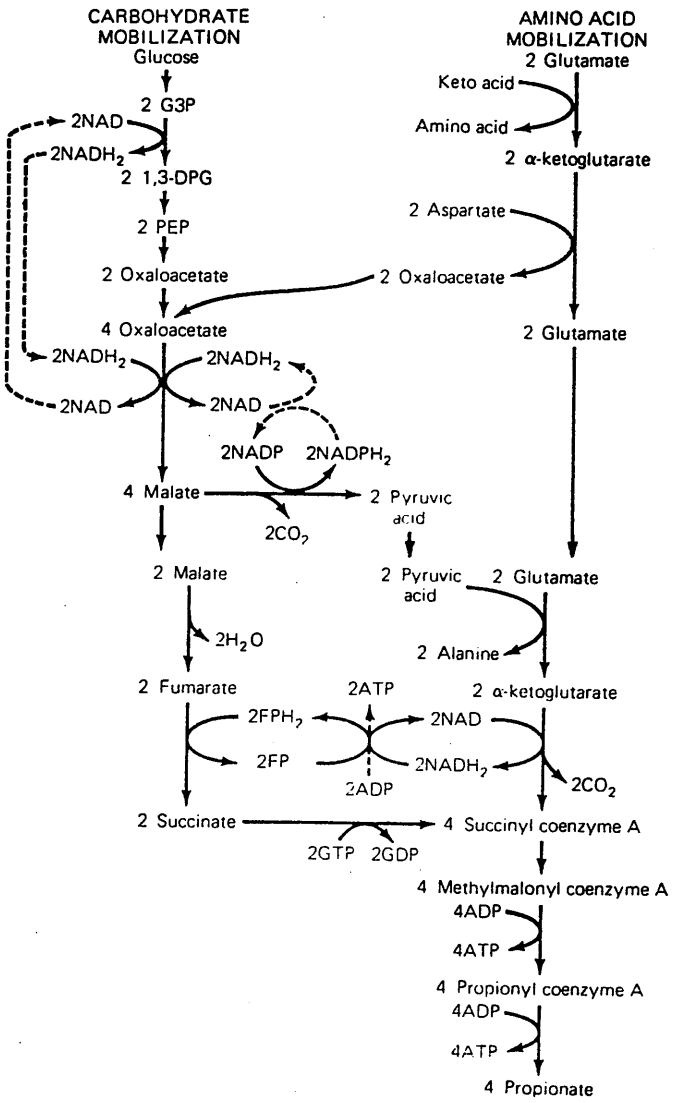
The scales on the graph are in relative units (After Herreid, 1980)


NORMOXIA
HYPOXIA
NORMOXIA
V
IV
III
II
I
Resting
Depressed
Recovery
Resp. Rate
Time

return to normoxia the first response (type I) shows a negative oxygen debt. This condition is observed in many invertebrates (Von Brand, 1946) and in some diving mammals (Dejours, 1975). This is because alterations made in the aerobic enzymes during anoxia take time to revert back to their original form. Type II shows no oxygen debt and this is characteristic of animals that either do not accumulate anaerobic end products or excrete them (Spoek, 1974; Badman & Chin, 1973). The third response to hypoxia (type III) shows a partial repayment of the oxygen debt which correspond to animals that excrete or oxidise part of their anaerobic wastes (McMahon & Russell-Hunter, 1978). Total repayment is seen in type IV with animals converting their anaerobic end products back into storage molecules during recovery (Taylor *et al.*, 1977) while an excess repayment (Type V) is typical of an animal stressed or active during hypoxia (Butler *et al.*, 1978).

There are many alternatives to lactic acid fermentation with accumulations of some the following end products: alanine, strombine, acetate, propionate, and succinate (Reviews by Fields, 1983; De Zwaan & Wijsman, 1976). The most common alternative end products are, however, succinate and propionate and these accumulate in many animals during hypoxia. The succinate pathway (Fig 2.3) is common in mussels (Ahmad & Chaplin, 1979; Hochachka *et al.*, 1973) and insect larvae (English *et al.*, 1981; Whilps and Zebe, 1976) and has distinct energetic advantages over anaerobic glycolysis. If, for each molecule of glucose metabolised two molecules of aspartate and alpha-ketoglutarate are also mobilised, then six molecules of ATP will be produced. This is a considerable improvement over the two molecules produced in anaerobic glycolysis but is less than the possible ten molecules of ATP produced in the propionate pathway (Fig 2.5) This pathway is utilised by many animals that demonstrate a marked ability to live anaerobically for indefinite

Fig 2.5 The Propionate pathway common in helminth parasites.


CARBOHYDRATE MOBILIZATION
Glucose
2 G3P
2NAD
2NADH2
2 1,3-DPG
2 PEP
2 Oxaloacetate
4 Oxaloacetate
2NADH2
2NADH2
2NAD
2NAD
2NADP
2NADPH2
4 Malate
2 Pyruvic acid
2CO2
2 Malate
2H2O
2 Fumarate
2FPH2
2FP
2ATP
2ADP
2NAD
2NADH2
2CO2
2 Succinate
2GTP
2GDP
4 Succinyl coenzyme A
4 Methylmalonyl coenzyme A
4ADP
4ATP
4 Propionyl coenzyme A
4ADP
4ATP
4 Propionate
AMINO ACID MOBILIZATION
2 Glutamate
Keto acid
Amino acid
2 α-ketoglutarate
2 Aspartate
2 Oxaloacetate
2 Glutamate
2 Pyruvic acid
2 Glutamate
2 Alanine
2 α-ketoglutarate

periods. These include mostly helminth parasites which produce mainly acetate and propionate (De zoeten *et al.*, 1969; Lahoud *et al.*, 1971; Van Vugt *et al.*, 1979).

Since the alternative succinate/propionate pathways are so much more efficient at producing energy for use within cells than the lactate-producing one, a question arises as to why the former has not replaced the latter. This question is discussed by Gnaiger (1983) who stated that during short-term hypoxia, thermodynamically inefficient pathways (lactate pathway) are used to sustain high metabolic rates for brief periods. Alternatively when long-term hypoxia occurs low sustained energy production processes (succinate/propionate pathways) are more efficient (Calow, 1981).

CHAPTER 3

CHAPTER 3

Spatial, Temporal, and Microhabitat Variations in the PO_2 Content of a River

3.1 Introduction

Most organisms that inhabit fresh waters require a good supply of oxygen. Under normal circumstances, the partial pressure of oxygen in water (PO_2 - measured in Torr) is maintained close to saturation at about 155 Torr (Laurie, 1942; Höll, 1955). It is therefore rare in natural habitats for oxygen availability to be limiting except in the hypolimnion of lakes. In many cases, especially at the lower end of the concentration scale, oxygen levels do determine the distribution of many animals in various freshwater habitats (Hubault, 1927; Hawkes and Davies, 1970; Davis, 1975; Davies and Hawks 1981). Oxygen is a very important chemical parameter, as it acts not only as a regulator of metabolic processes, it also gives a good indication of lake or river conditions. Animals present in freshwater ecosystems, particularly in organically enriched areas with low oxygen tensions, may therefore be expected to show pronounced adaptation for this mode of life.

The concentration of oxygen in water at any given time is dependent upon many factors, eg. the temperature of the water (due to oxygen being more soluble at lower temperatures) the partial pressure of the gas (PO_2) in the atmosphere in contact with the water, the concentration of dissolved salts in the water and biological activity (especially during the summer). According to Reid and Wood (1976) there

are three main sources of input for dissolved oxygen in rivers. These are ground water and surface run-off, photosynthesis, and physical aeration. The contributions from each source are far from equal and may vary greatly with time of day, season, current velocity, stream morphology, temperature and biological activity. Ground water and surface run-off provide little oxygen to the river since water issuing from subterranean channels or springs is typically low in dissolved oxygen, often to the point of being anaerobic. Photosynthesis can, however, produce large increases in PO_2 where rich growths of algae and higher plants are present. Oxygen can also be gained by direct diffusion at the surface. This is usually slow, but can be enhanced by surface water agitations such as riffles, waterfalls and turbulences due to obstructions.

Oxygen depletion is a fairly common event in most organically enriched systems. Downing (1967) has suggested four main reasons for this. Firstly, the biochemical oxygen demand (B.O.D.) which depends on the carbon content of the stream and on the number of micro-organisms present varies widely with pollutional load. Large increases in waste products will vastly increase the number of micro-organisms present and hence reduce the amount of dissolved oxygen. Also nitrogenous material present in the effluent is oxidised as it passes downstream taking oxygen out of the water. The respiration of muds and slimes also contributes, but the most significant loss arises from macrophytic respiration (Dawson *et al.*, 1982).

The vast majority of data on oxygen levels come from extensive investigations carried out in lakes. (Delorme, 1982; reviews Hutchinson, 1957; Welch, 1952; Macan and Worthington 1974). In contrast, relatively few studies have been carried out in lotic

habitats (Hawks, 1962; Hynes, 1970). Dissolved oxygen levels in river systems have been studied with regard to distribution and organic loading (Macan, 1961; Schwoerbel, 1972; Decamps and Pujol, 1977). A typical river maintains oxygen saturation with aeration roughly balancing depletion (Höll, 1955). The effects of organic enrichment can cause a severe reduction in oxygen levels for some distance downstream of its source (Downing, 1967; Gessner, 1961; Dratnal and Kasprzak, 1980; Mason 1981) and during the summer months, the decomposition of organic matter, coupled with the high water temperatures, causes increased bacterial, algal and macrophytic growths. This may then result in large diel fluctuations in oxygen content (Butcher *et al.*, 1927; Whitney, 1942; Lavandier and Capblanco, 1975; Grant and Hawkes, 1982;). Garey and Rahn (1970) pointed out that although photosynthesis and aquatic respiration are the main causes of such diurnal variations, they are dependent on many other factors such as solar radiation, cloud cover, and water temperature. Hubault (1927) and Neel (1951) demonstrated that many rivers also show seasonal fluctuations in oxygen content, an oxygen "sag" in autumn increasing throughout spring to hyperoxic conditions followed by a decline in summer. It should be noted, however, that these variations are not large in clean rivers and it is only in organically enriched streams that severe deficits can occur.

A comparative study of two sites was therefore carried out to assess the effects of organic loading on the dissolved oxygen content of one river system. The sites were chosen to reflect differing oxygen regimes and hence differing physiological stresses. The first site, on the Allander Water, was situated downstream of a large sewage outlet, producing a fall in oxygen content. The second, a well-oxygenated site on the River Kelvin, was located at the base of a relatively large

weir. Measurements of oxygen content were made seasonally and diurnally to estimate the extent and duration of hypoxia (low oxygen levels) at each site.

3.2 Description of the Sites

Dendrocoelum lacteum and *Polycelis tenuis* have a widespread distribution throughout central Scotland. The selection of sites was therefore concerned mostly with suitability of habitat. After careful consideration it was decided to carry out the study at two sites in the Glasgow area; the River Kelvin and one of its tributaries, the Allander Water, situated near the Firth of Clyde (Fig 3.1). The two sites chosen would have to reflect markedly differing dissolved oxygen levels; a well-oxygenated site situated at the base of a weir, and an oxygen deficient habitat.

According to criteria set up by the Department of the Environment (1972), and based on a biological survey on both sites (Table 3.1), both rivers would be categorised as Class C. These are generally organically enriched rivers in which the variety of macroscopic invertebrate fauna is restricted and the community is dominated by the isopod *Asellus aquaticus*. Although some Amphipoda may be present, Trichoptera and Ephemeroptera are relatively rare.

The geology of the catchment area will exert a strong influence on the physical and chemical characteristics of any running-water system. The geology of the Kelvin catchment area for both the River Kelvin and Allander Water is similar in nature. The rivers rise on igneous porphryte rocks, and then flow over limestone and intrusive basalt rocks. Water from igneous rocks is usually low in dissolved salts, containing only small amounts of calcium, magnesium, and potassium as bicarbonates, sulphates, and chlorides. Silica is usually the commonest constituent of such rivers. Sedementary rocks, on the other hand usually contain much higher amounts of dissolved solids with notably large amounts of calcium bicarbonate.

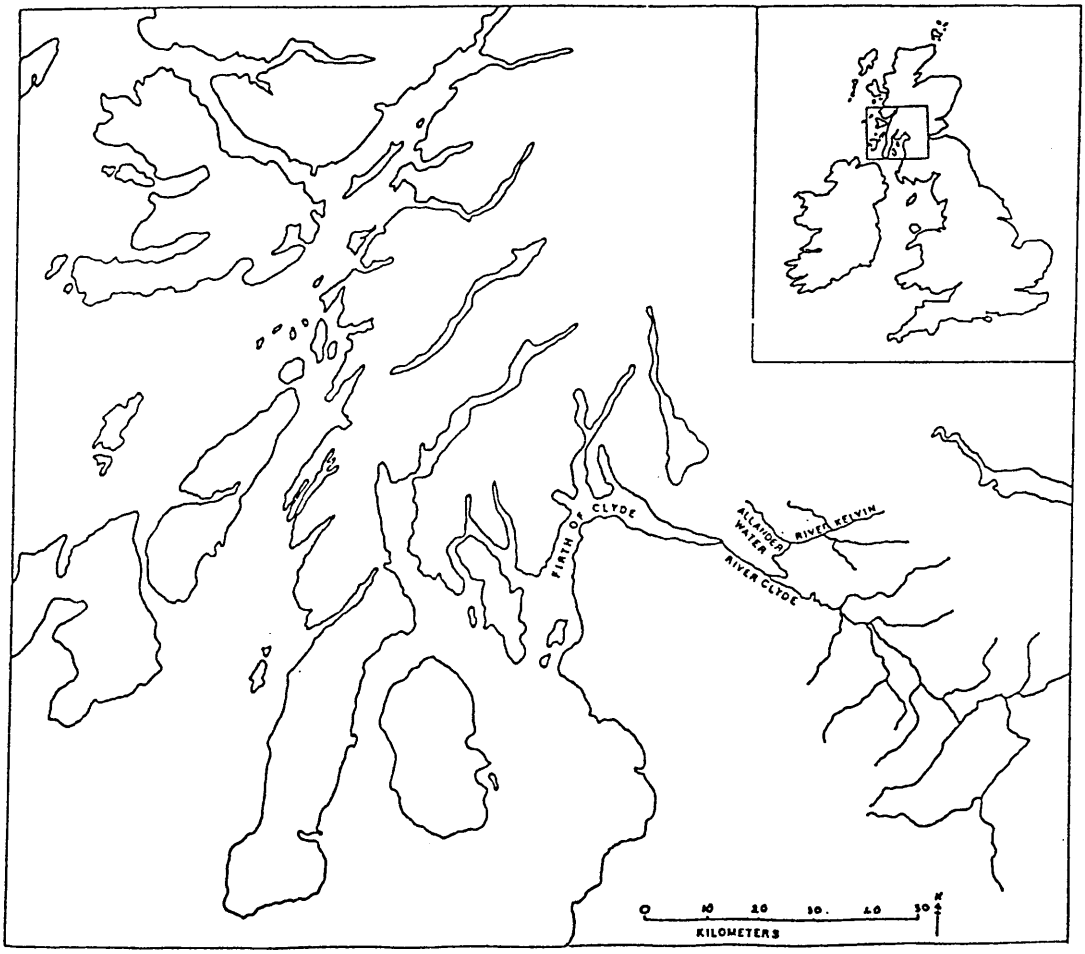

FIRTH OF CLYDE
ALLANDER WATER
RIVER KELVIN
RIVER CLYDE
0 10 20 30 40 50
KILOMETERS
N

Table 3.1

Species lists for the Allander Water and River Kelvin

For July 1983

Flora

Allander Water	River Kelvin
Potamogetan natans	*Potamogetan pectinatus*
Potamogetan crispus	*Cladophora glomerata*
Sparganium emersum	
Callitriche stagnalis	
Eurhynchium riparioides	
Cladophora sp.	

Fauna

Allander Water	River Kelvin
Dendrocoelum lacteum	*Dendrocoelum lacteum*
Polycelis tenuis	*Polycelis tenuis*
Dugesia lugubris	*Dugesia lugubris*
Dugesia polychroa	*Dugesia polychroa*
Erpobdella octoculata	*Erbobdella octoculata*
Glossiphonia complanata	*Glossiphonia complanata*
Glossiphonia heteroclita	*Glossiphonia heteroclita*
Baetis rhodani	*Theromyzon tessulatum*
Ecdyonurus dispar	*Trocheta bykowski*
Ephemerella ignita	*Baetis rhodani*
Chironominae	*Chironominae*
Orthocladiinae	*Tandypodinae*
Tandypodinae	*Naididae*
Tubificidae	*Lymnaea peregra*
Lumbricidae	*Asellus aquaticus*
Naididae	
Lumbriculidae	
Asellus aquaticus	
Gammarus pulex	
Copepoda	
Limnius volkmari	
Oulimnius tuberculatus	

The River Kelvin rises in marshy ground near the village of Kelvinhead (Scotland) and flows in a south-westerly direction for approximately 30km towards the Clyde Estuary. The Allander Water, a tributary of the River Kelvin, is formed by the confluence of the Lecher and Auldmurroch burns at Carbeth and flows for about 12 km towards the outskirts of Glasgow where the rivers join. Although the catchment area of the Kelvin to the north is hilly, the main river and many of its tributaries flow through fairly flat land resulting in a rather sluggish flow rate. This poor flow is further reduced during the summer when vast macrophytic growths appear along virtually the whole length of the River Kelvin and in many of its tributaries. This does not, however, affect the site on the River Kelvin, due to the sparging nature of the weir (Fig 3.2). The most predominant weed is *Sparganium emersum* which appears around May and by late July chokes most of the river.

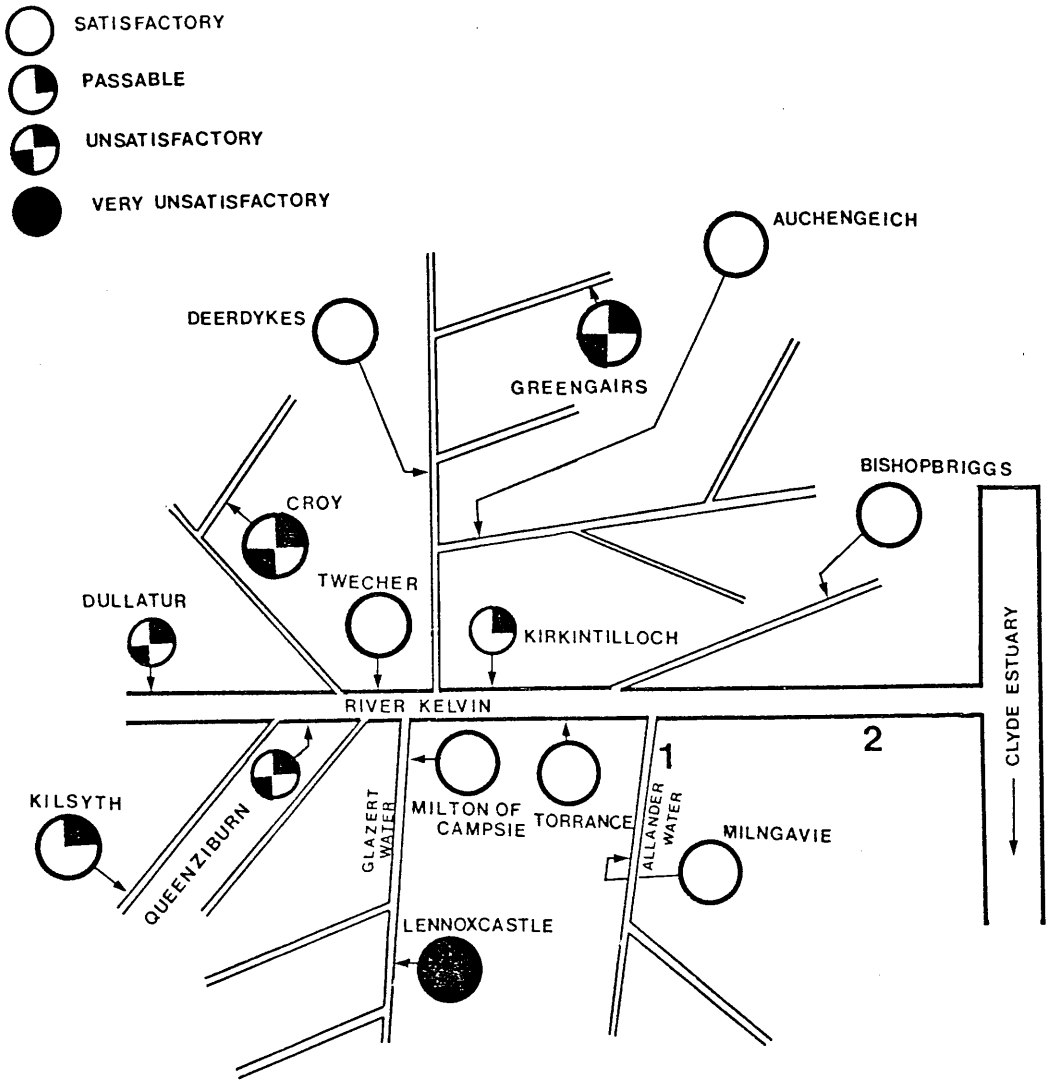
The main source of pollution in the River Kelvin and its tributaries are the local authority sewage works (Fig 3.3). Although most of these tend to produce "good quality" effluent, characterised by low biochemical oxygen demands and suspended solids, there is very little dilution available in the receiving streams and in some extreme cases a large proportion of flow in the stream consists of sewage effluent (Clyde River Purification Board, 1975). It is only as the river enters Glasgow that the gradient increases sufficiently to allow some replenishment of oxygen.

The two sites selected were chosen to reflect different environmental states and hence differing oxygen regimes. A full description of the sites is given below:-

Fig 3.2 Photographs of the Allander Water and River Kelvin sites during summer demonstrating the mass macrophytic growths present in the Allander.

Fig 3.3 Diagramatic map of the River Kelvin Catchment Area showing all the Local Authority Sewage Works and the quality of effluent they produce. (After C.R.P.B., 1986 in prep.)

SEWAGE WORKS



Site 1 : The Allander Water - (British Ordinance Survey National Grid Reference NS 539769)

This site, situated on the Allander Water, is between Milngavie and the junction with the River Kelvin and is approximately 1 km downstream of an effluent outlet for the Milngavie Sewage Works. These works, although producing effluent of a satisfactory quality, are becoming volumetrically overloaded due to a major expansion in local housing. The River is about 10m wide and has a depth that varies between approximately 0.5 and 1.0 metres deep. There is a stony substrate which becomes covered with *Cladophora* and sewage fungus in the summer and the river has some tree cover along its banks.

Site 2 : The River Kelvin - (NS 568664)

The site on the River Kelvin is in the centre of Glasgow, near Glasgow University. It is both wider (20m) and deeper (1 - 3m) in the centre and has a considerable flow rate. The geology of the bed is similar to that of the Allander Water with a stony substrate and a good deal of tree cover. This site was downstream from a large number of sewage discharges. However, between the site and the last sewage input were a number of large weirs. These re-oxygenate the river, reduce the amount of suspended solids and, as a result, lower the B.O.D. of the river. However, during the spring, as the level of the water fell, pools formed and isolated communities of flora and fauna. Mass algal blooms of *Cladophora glomerata* were prevalent which caused large fluctuations in PO_2.

3.3 Materials and Methods

3.3.1 Recording Techniques

A water quality survey was undertaken to assess the levels of certain pollutants and the extent of fluctuations throughout one year. The chemical parameters measured were :-

1) PO_2
2) Temperature
3) Biochemical Oxygen Demand
4) pH
5) Suspended Solids
6) Nitrate
7) Ammonia

The chemical properties of the two rivers were monitored to establish any differences in organic loading between the two rivers. These differences could then be considered in terms of the amount of oxygen present at each site throughout the season. Many of the parameters mentioned above can be directly related to organic enrichment and hence low levels of PO_2. High levels of nitrate, ammonia, and a high B.O.D. and temperature would be reflected in low oxygen levels. The B.O.D. of a river was calculated by a standard technique which measured the amount of oxygen consumed (over 5 days) by bacteria present in the water. The chemical analysis of water used standard water analysis techniques (Golterman *et al.*, 1978; Mackereth, Heron, & Talling, 1978), to measure the parameters described above over one season. Samples were collected at approximately monthly intervals and analysed immediately on returning to the laboratory to observe any seasonal variations present.

PO_2 was recorded using a Radiometer E5046 oxygen probe (Radiometer, Copenhagen) in its thermostated cell, coupled to a

Strathkelvin 781B portable oxygen meter (Strathkelvin Instruments, Glasgow). The oxygen electrode was calibrated in air at the ambient temperature and in a zero PO_2 solution. To set zero, an anoxic solution was prepared from a sodium tetra-borate solution (0.01 molar) to which a few crystals of sodium sulphite had been added. Fully saturated conditions were achieved by allowing the electrode to stabilise in air, then setting the meter to read 160 Torr. The values recorded were subsequently corrected to compensate for variations in barometric pressure and water temperature. PO_2 (mm Hg or Torr) was then obtained from the following standard formula:-

$$PO_2 = (b-vp) \times 20.946/100$$

where b is the barometric pressure in Torr and vp is the water vapour pressure at a given temperature obtained from tables (Dejours, 1981) and 20.946 is the percentage of oxygen in the atmosphere. Water samples were collected using 10ml syringes which were placed under the water, cleared of air bubbles and then slowly filled.

Once samples had been collected, and prior to removal from the water, a cap was placed over the syringe nozzle to prevent any subsequent entry of oxygen. The sample was immediately injected into the electrode cell at air temperature and left until a stable reading was observed (45 secs). Corrections for barometric pressure and for water temperature, recorded on a Digitron Digital Thermometer (model 4706), were made as follows :-

3.3.2 Field Corrections

Example 1 : Corrections for barometric pressure.

$$PO_2 = (b-vp) \times 0.2093$$

Assumed PO_2 = 160 torr, assumed air temp. is 10°C then vp = 9.209

$$160 = (b - 9.209) \times 0.2093$$

$$b = 773.67 \text{ mm Hg}$$

Therefore assumed barometric pressure is 773.67 mm Hg. Let actual barometric pressure be 762 mm Hg, then the assumed barometric pressure was 101.9% greater than this. The oxygen electrode was therefore calibrated to 157 torr.

Example 2 : Corrections for water temperature. Let the actual water temperature = 4°C

then PO_2 = (Actual b - vp at water temp) x 0.2093

= (773.67 - 6.101) x 0.2093

= 160.65

This is then the true value at which the meter was calibrated for field barometric pressure and temperature. The results would therefore have to be multiplied by a factor of 160.65/157.

As the terms % air saturation and Torr are used throughout this thesis, an explanation of their inter-relationship is necessary. The term 100% air saturation means that at a constant experimental temperature, all the oxygen that can diffuse from the atmosphere into the water has done so, and a state of equilibrium now exists. Hyperoxia or supersaturation only arises with an increase in oxygen accompanied by a sudden rise in temperature which prevents the oxygen from escaping back to the atmosphere. To find out the comparative value in Torr, the simple equation in Example 1 can be used. A close approximation of 100% air saturated water at about 20°C would be about 155 Torr, and 78 Torr would be approximately 50% air saturation.

3.3.3 Sampling Programme

Oxygen levels were monitored in various habitats throughout both rivers. Samples were collected from :-

i. Water Column - just under the surface of the water at both riffle (faster flowing waters about midstream) and edge sites (slower running water about 1m. from the bank).

ii. Bed - taken as close to the bed as possible without introducing any sediment (about 2-5cm away). Samples were taken only at edge sites (about 1m out).

iii. Understone - taken with syringes fitted with fine capillary tubing. Both syringe and tubing were placed underwater, inserted under a marked stone and a sample extracted. Again only edge sites were examined.

Seasonal fluctuations in PO_2 were examined at both sites over a twelve month period. Oxygen and temperature measurements were made in the water column of the two rivers approximately 1 m from the bank (edge) between mid-day and 14.00 each day. Samples were collected at two week intervals throughout the spring and summer and approximately monthly thereafter. Diurnal measurements were made over a similar period in the various microhabitats listed in Table 3.2, with the exception of the "understone" habitat which was only examined on a few occasions throughout the season.

Table 3.2

Summary of the Sampling Programme Undertaken

Sites	Allander Water	River Kelvin
Habitat		
Column Riffle	Diurnal (+)	Diurnal (+)
Column Edge	Seasonal (+)	Seasonal (*)
Bed Edge	Seasonal/diurnal (+)	Seasonal/diurnal (*)
Under Stone Edge	Seasonal/diurnal (+)	Seasonal/diurnal (*)

+ - Always Open Water * - During Summer In Pools

3.4 Results

3.4.1 Water Analysis

The differences observed in water quality (Fig 3.4) are summarised in Appendix 1. Fluctuations in PO_2 were observed at both sites over the twelve months sample period. The Allander Water demonstrated the typical picture of an organically enriched stream, with the water being fully saturated with oxygen during the winter months and tending to become hypoxic over the summer months. Normoxic water conditions were present from late September until April when hypoxia arose and these conditions persisted until the following October. It is important to note that for four months the oxygen content of the river was below 50% air saturation.

The River Kelvin was expected to show a completely different oxygen regime, remaining saturated throughout the year due to the turbulent effects of the weir. However, this was not found to be the case (Fig 3.4). Fully saturated, normoxic conditions, were present in the Allander Water from September until the end of April. However, during May until the end of August a mirror image of the oxygen conditions exhibited in the Allander Water was observed. The River Kelvin remained supersaturated for many months with values of 150% air saturation measured during July.

Water temperatures (Fig 3.4) in the Allander Water over the twelve month period showed the normal cyclical pattern associated with many streams and rivers. The water temperature rose from a low of about $4^{o}C$ in midwinter (Jan/Feb) to a peak of $19^{o}C$ during the summer. The temperature readings of the River Kelvin followed an almost identical pattern to that of the Allander Water with similar maximum and minimum

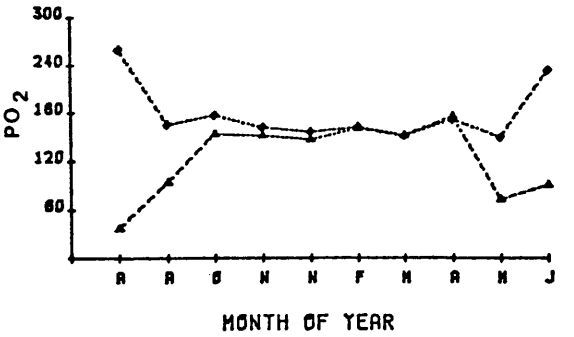
Fig 3.4 Graphs of the physical and chemical parameters measured over one season in 1983. These included :-

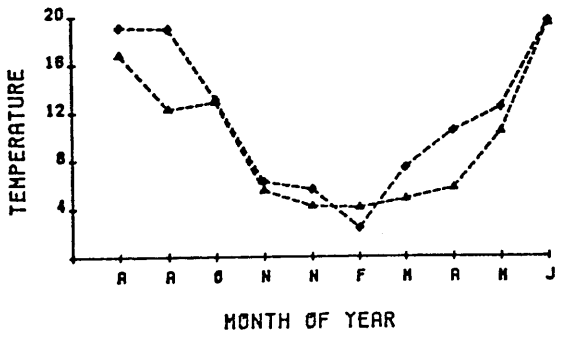
1) PO_2 —
2) Temperature
3) B.O.D.
4) pH
5) Suspended Solids
6) Nitrate
7) Ammonia

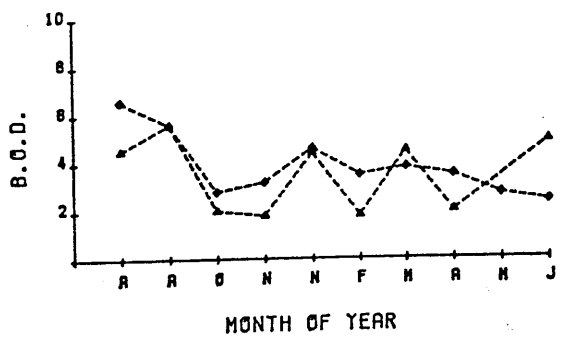
Diamonds = The River Kelvin

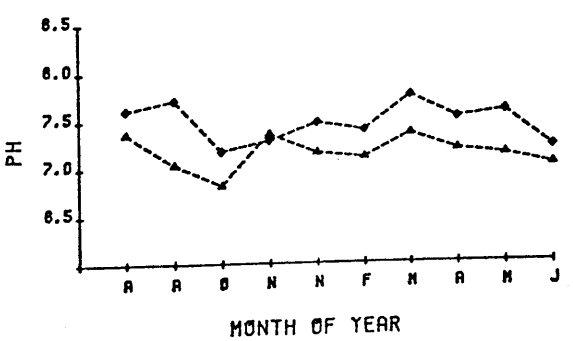
Triangles = The Allander Water

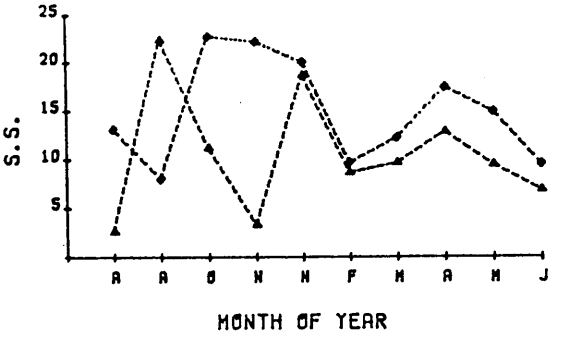
Results for B.O.D., S.S., Ammonia, and Nitrate are given in mgl^{-1}

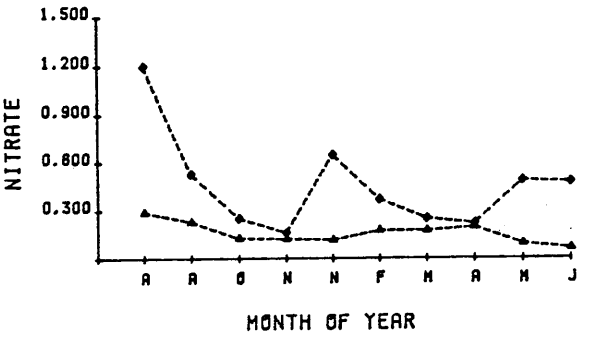

300
240
180
120
60
PO_2
A A S O N D J F M A M J
MONTH OF YEAR

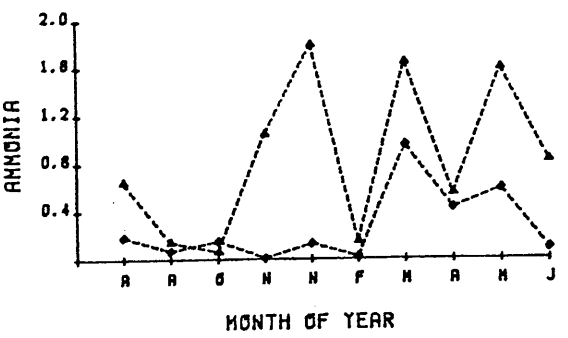

20
16
12
8
4
TEMPERATURE
A A S O N D J F M A M J
MONTH OF YEAR


10
8
6
4
2
B.O.D.
A A S O N D J F M A M J
MONTH OF YEAR


8.5
8.0
7.5
7.0
6.5
PH
A A S O N D J F M A M J
MONTH OF YEAR


S.S.
25
20
15
10
5
A A S O N D J F M A M J
MONTH OF YEAR


NITRATE
1.500
1.200
0.900
0.600
0.300
A A S O N D J F M A M J
MONTH OF YEAR


AMMONIA
2.0
1.8
1.2
0.8
0.4
A A S O N D J F M A M J
MONTH OF YEAR

readings. Fluctuations in B.O.D., however, showed greater differences, with the Allander Water remaining lower throughout most of the year. The only exception to this was during the summer when values at the Allander water rose to well above that of the River Kelvin. The Allander Water was also markedly more acidic than the River Kelvin with little fluctuation throughout the year.

Large fluctuations were observed in the quantity of suspended solids (i.e. the weight of dried particulate matter filtered from 1 litre of stream water) at both sites over the season; the River Kelvin showing large peaks in October and April whereas the Allander Water peaked only once, in April. Nitrate levels were very stable throughout the year in the Allander Water site, but large fluctuations were observed in the River Kelvin. The levels of ammonia in the River Kelvin remained low throughout the winter months but increased during the spring and summer. In contrast, the Allander Water demonstrated large fluctuations in the levels of ammonia throughout the year. The concentrations of ammonia were also observed to be far higher than those in the Kelvin.

3.4.2 Diurnal Fluctuations in PO_2

Diurnal variations in PO_2 were examined at both sites and the results have been plotted for typical days during the summer and winter (Fig 3.5). The River Kelvin unexpectedly followed the pattern of an organically enriched stream, with extensive diurnal variations. During the day until late afternoon the PO_2 increased until the water became supersaturated. As the evening approached, the PO_2 fell until normoxic conditions prevailed. It was only as the night progressed that the water, with the continual fall in PO_2, became hypoxic. As dawn

Fig 3.5 PO_2 values for a typical summer and winter day for the River Kelvin and Allander Water (Note differences in scale).

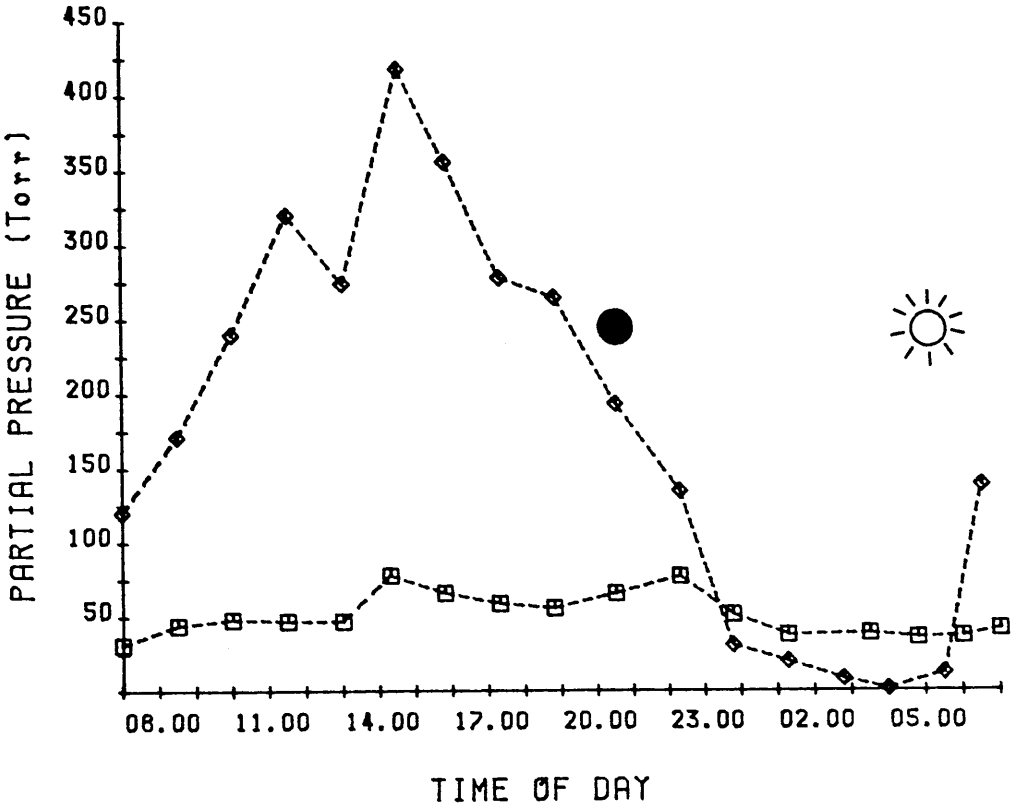
Sun = Sunrise

Moon = Sunset

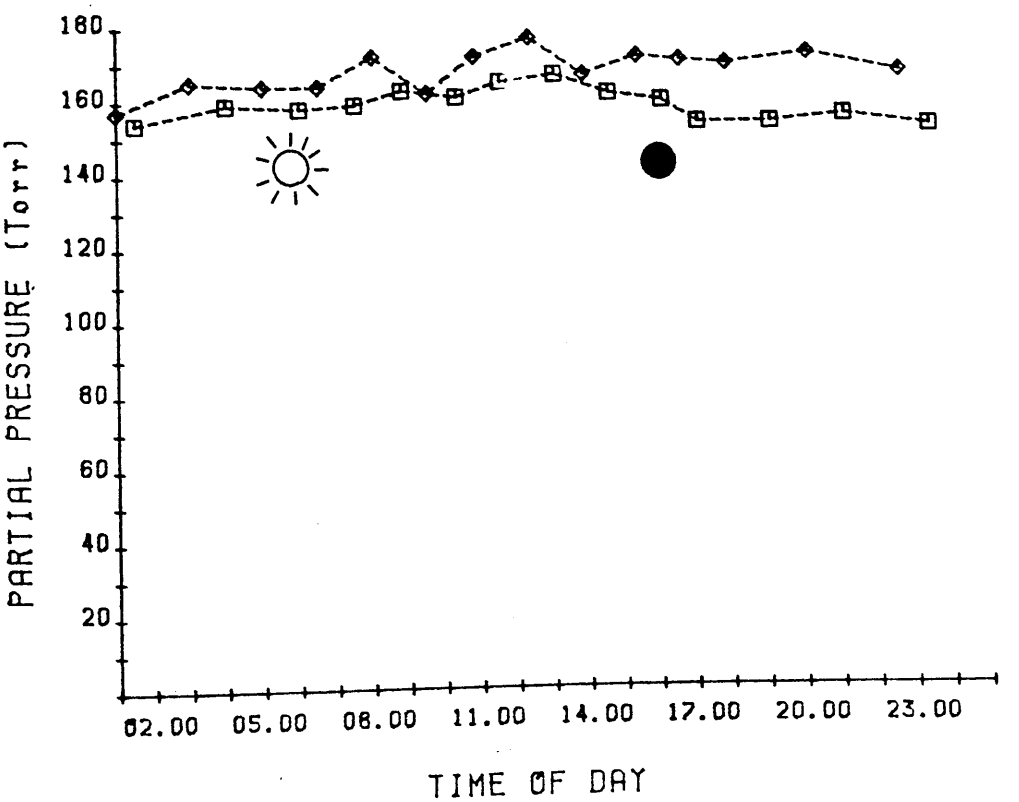
Diamonds = River Kelvin

Squares = Allander Water

Summer



Winter



approached, the levels of oxygen continued to decline until conditions became almost anoxic. After dawn the PO_2 quickly returned to normal levels.

The Allander Water, did not follow this cyclic pattern, but maintained hypoxic conditions throughout the hours of light and darkness. There were, however, slight variations in PO_2 that corresponded to those observed in the River Kelvin. The results for a typical day during the winter (Fig 3.5) show no such variations in PO_2 with saturated conditions being maintained at both sites throughout the day. These stable, diurnal PO_2 values were also found to occur during any spates in summer.

Figure 3.6 shows diurnal variations present in the Allander Water and River Kelvin during one season. The plot for each site was split into two sections corresponding to the average PO_2 values recorded during the hours of light and darkness. There was a distinct separation between the two PO_2 readings (light and dark hours) during the spring and summer months in the River Kelvin, but there was no significant difference during the winter months. Hyperoxic conditions ... River Kelvin were present during the spring and summer but only during the daylight hours. Hypoxia also occurred over the same period but only during the hours of darkness.

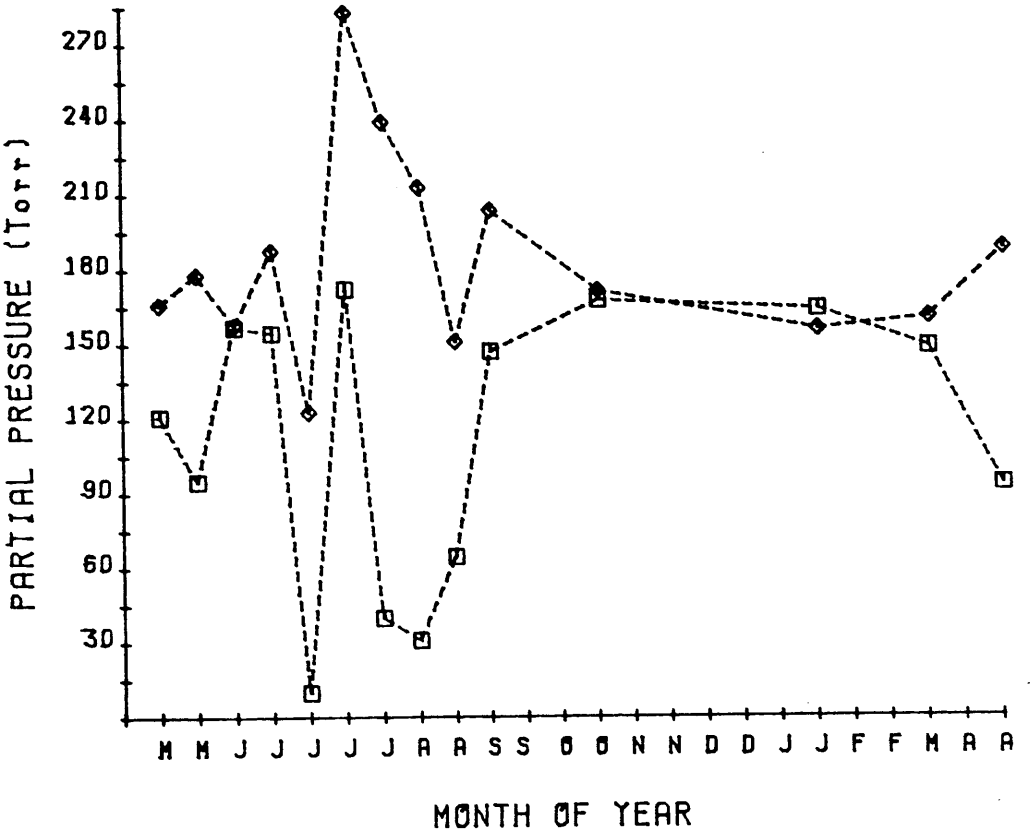
There was very little difference between the plots of PO_2 variations between the hours of light and darkness in the Allander Water throughout the year. Spring was the only time of year when significant differences ($P<0.05$) in the levels of PO_2, between the hours of light and dark, appeared. Unlike the River Kelvin, however, supersaturation did not occur. Acute hypoxia was present at the Allander Water site during the hours of light and darkness and as

Fig 3.6 Diurnal variations in PO_2 over one season - split into average PO_2 values measured over light and dark periods.

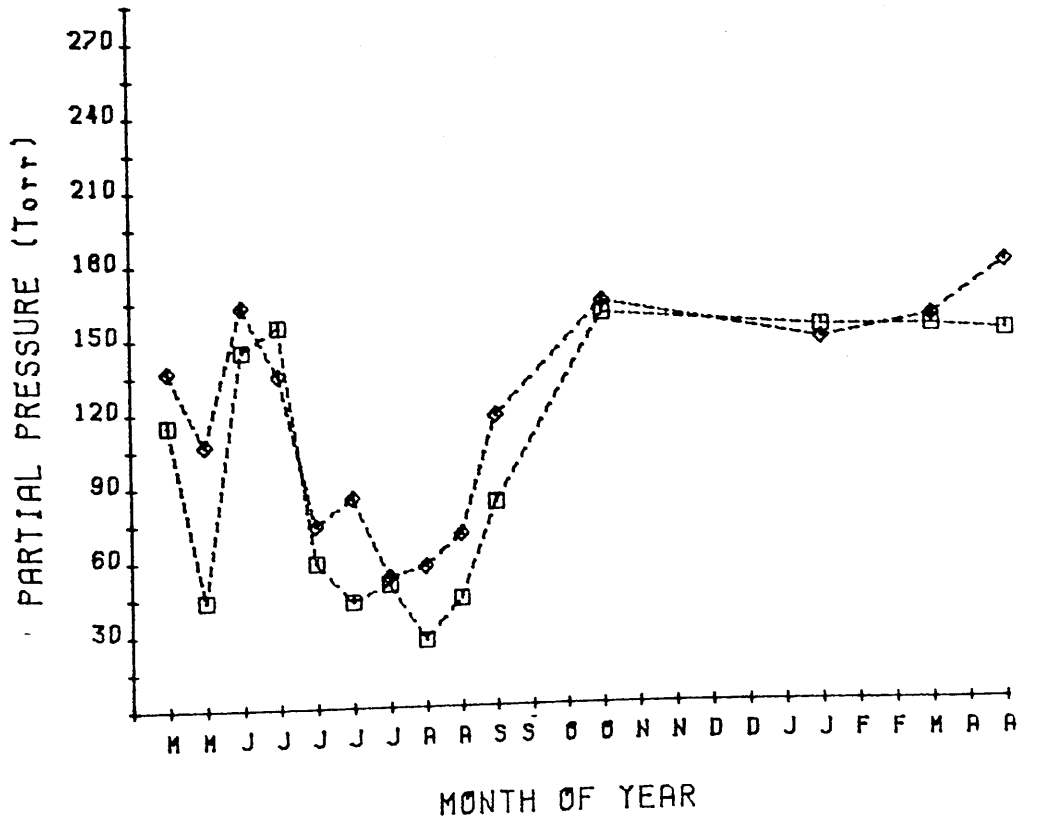
Diamonds = Light Hours

Squares = Dark Hours

River Kelvin



Allander Water



conditions deteriorated towards the end of summer, values approaching 20 Torr were recorded. The results obtained over the winter months were again very similar to those for the River Kelvin with saturated oxygen levels being recorded throughout winter and no differences being observed in PO_2 between the hours of light and darkness.

3.4.3 PO_2 Variations Within Stream Microhabitats

Variations in PO_2 within various microhabitats were measured over 24 hour periods during spring and winter. The results for the River Kelvin (Fig 3.7) shows that diurnal variation occurred during spring in two of these habitats; "understone" and "bed". The PO_2 readings for both these habitats followed a similar pattern except that the understone values had a tendency to lag a few hours behind bed values. This was especially prominent just after dawn, when PO_2 levels taken from the bed did not reach equilibrium until much later in the day. Readings taken directly under the weir in the main body of the river ("midstream") exhibited only slight variations and maintained values close to saturation throughout the day. During a similar period in the winter there was virtually no difference in the PO_2 content of the River Kelvin within the various habitats mentioned above.

The results from the Allander Water (Fig 3.8) demonstrated limited diurnal variations in all habitats. Midstream readings were generally above those recorded at bed and understone habitats throughout the hours of darkness. During the daylight hours, PO_2 values recorded at bed level were greater than midstream readings and this situation was maintained until dusk. PO_2 values recorded in the understone habitat were consistently lower than the other two habitats. Hypoxic conditions were present through the night in all habitats until

Fig 3.7 Microhabitat variation in the River Kelvin during May (Top) and January (Bottom).

Squares - Midstream

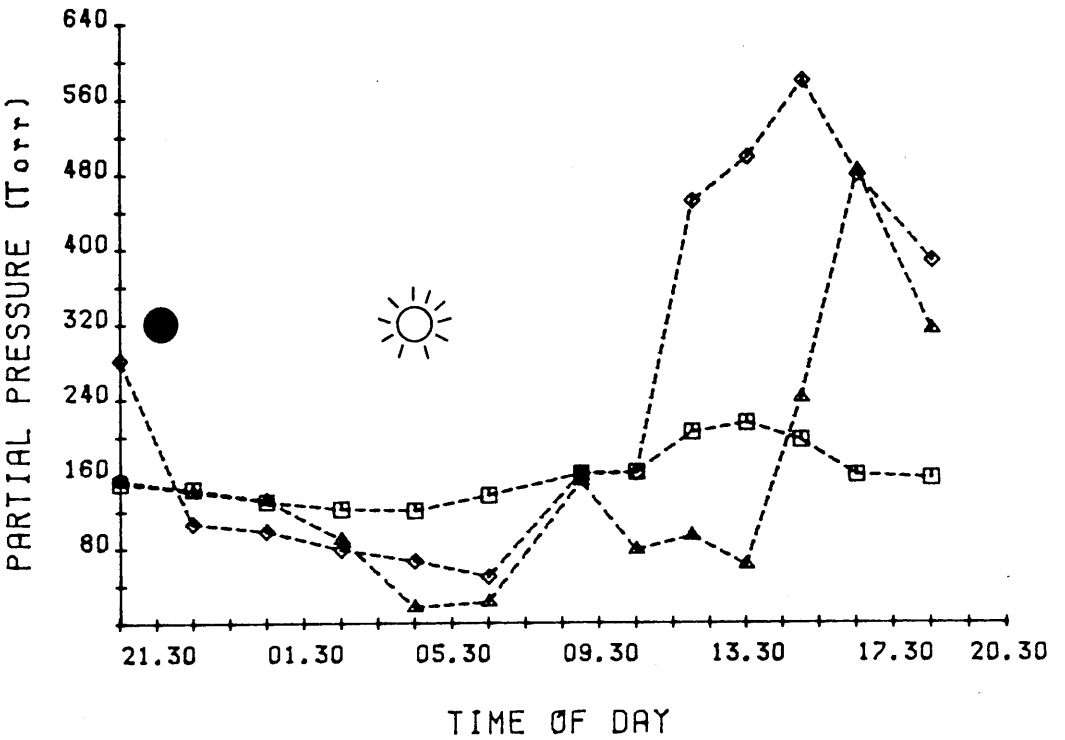
Diamonds - Bed

Triangles - Understone

Sun - Sunrise

Moon - Sunset

River Kelvin



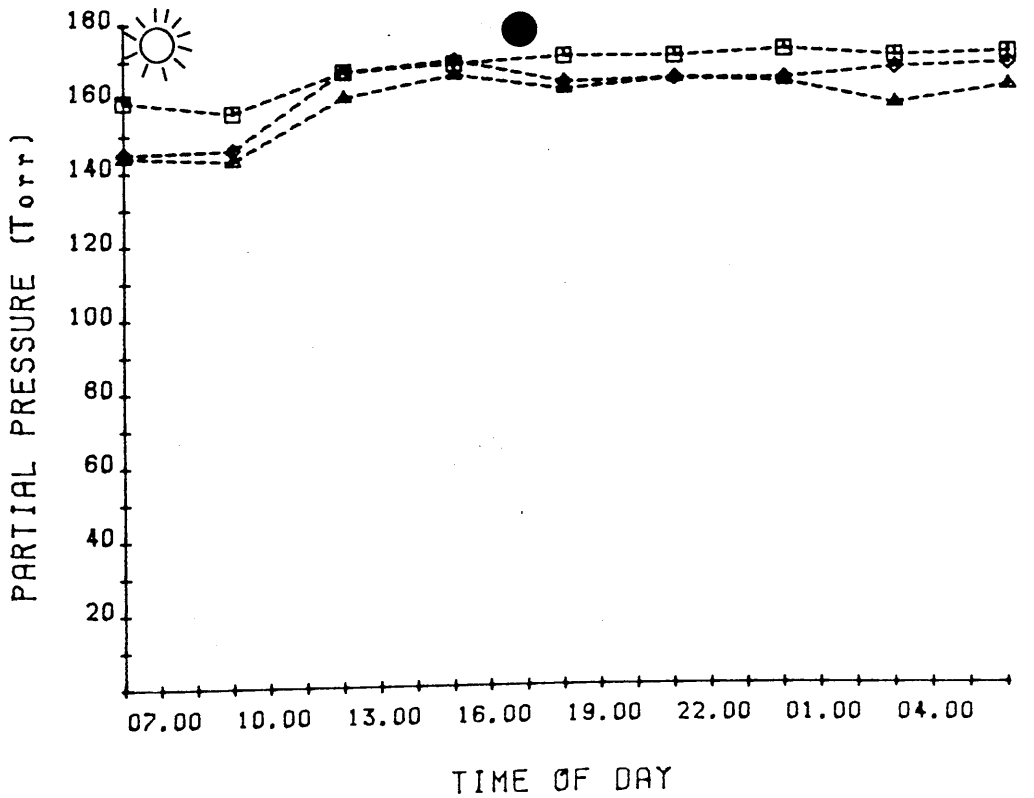


Fig 3.8 Microhabitat variation in the Allander Water during May (Top) and January (Bottom).

Squares - Midstream

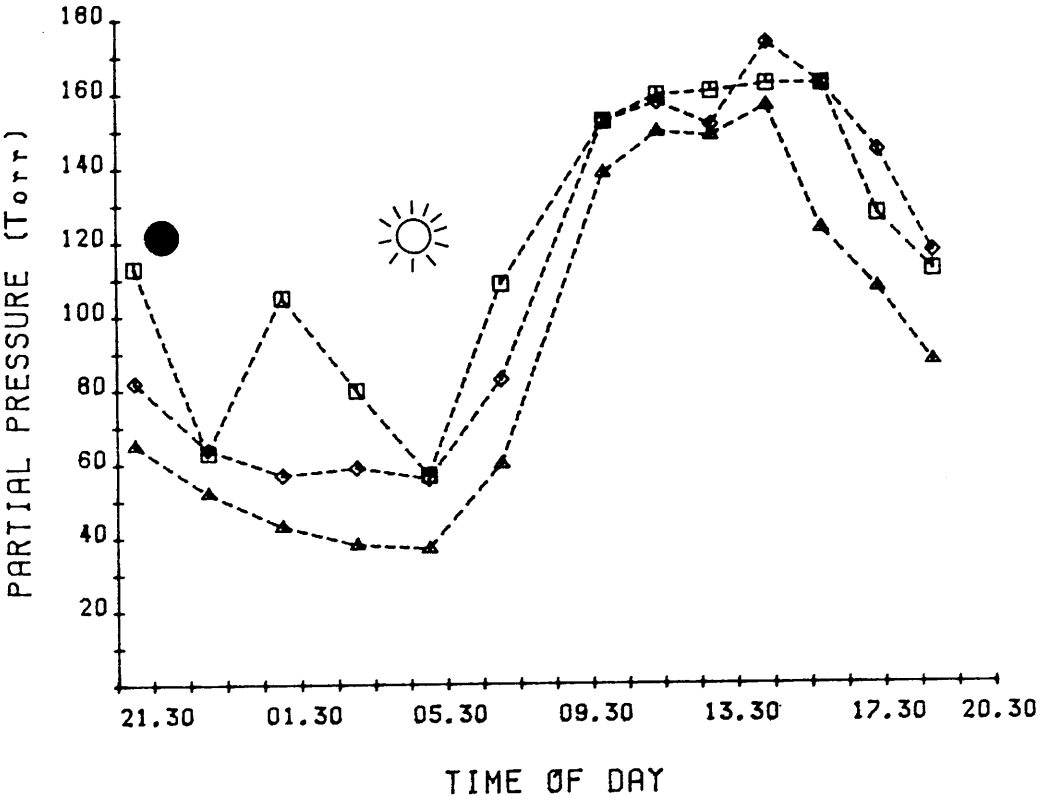
Diamonds - Bed

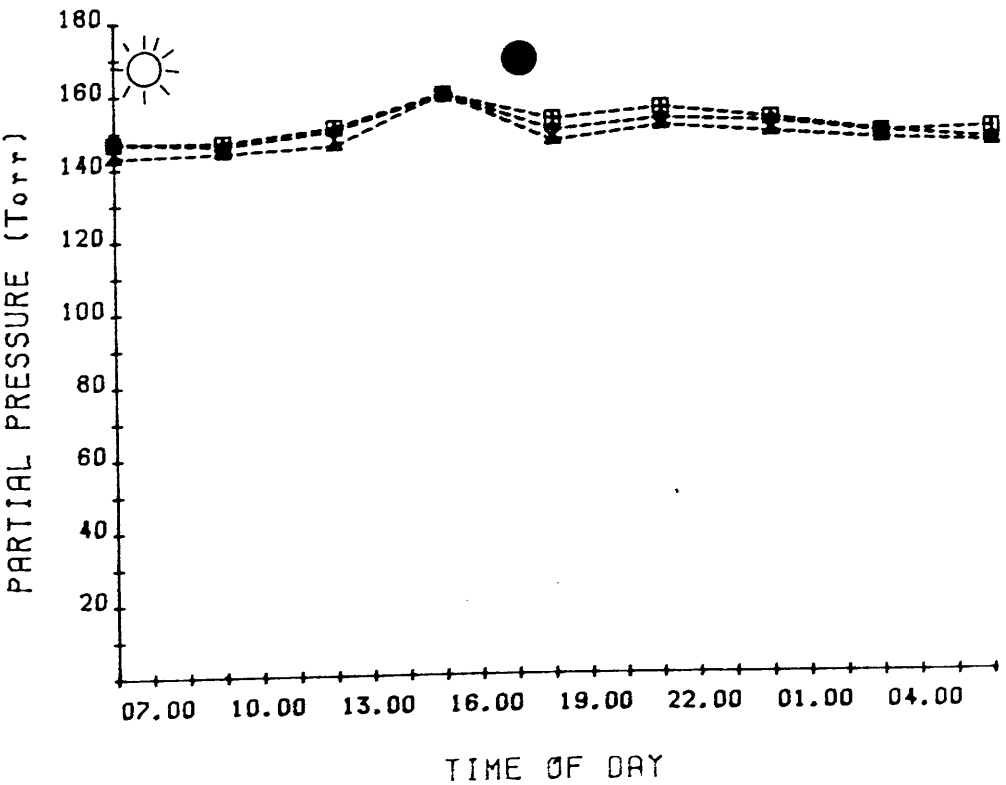
Triangles - Understone

Sun - Sunrise

Moon - Sunset

Allander Water





photosynthetic replenishment of oxygen occurred the following day. PO_2 values during winter remained almost constant throughout the 24 hour period at saturation. Again, as with the River Kelvin during this period of time, there were no differences in PO_2 between the various microhabitats.

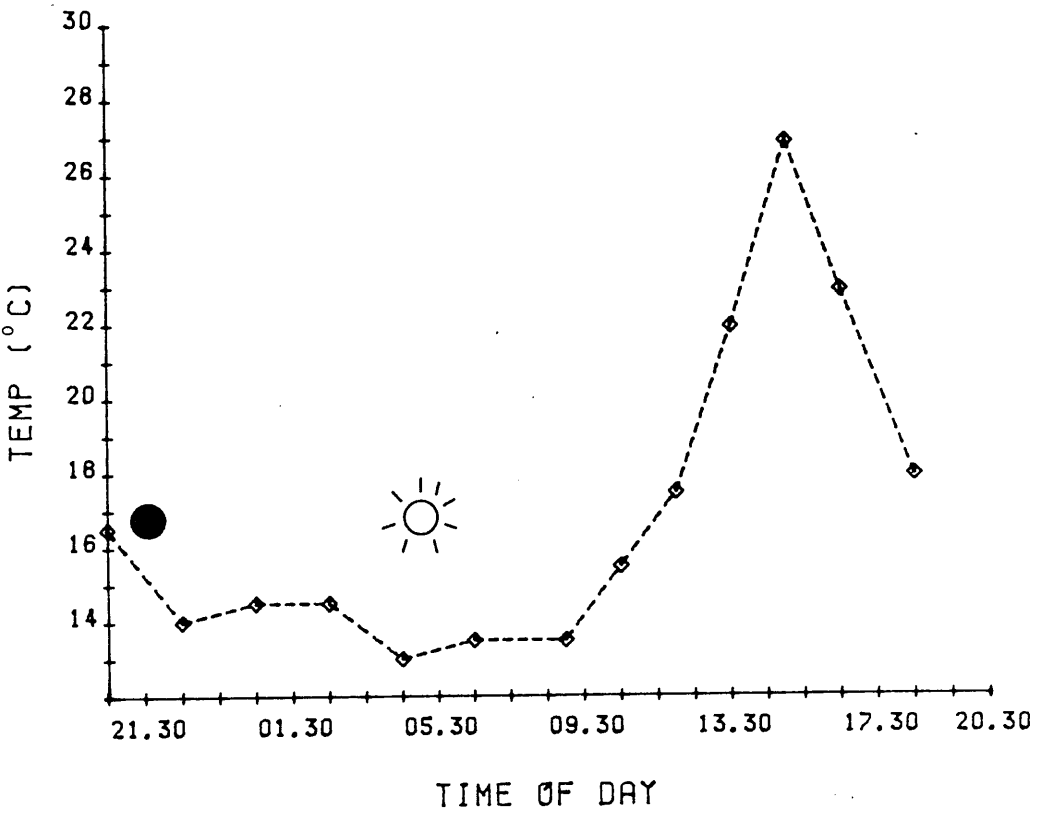
Diurnal variations in temperature were also noted in the River Kelvin (Fig 3.9) during the spring months, while measurements taken during the winter showed no such variations. Recorded values were also below zero for a considerable part of the day. Temperature readings from the Allander Water (Fig 3.10) were very similar to those from the River Kelvin. The only differences observed were in the extent of diurnal variation during the spring, with the River Kelvin demonstrating far wider fluctuations. As observed in the River Kelvin, the temperature of the water during the winter in the Allander Water remained low, but above zero, and showed little diurnal variation.

Fig 3.9 Temperature readings for a day in May (Top) and January (bottom) from the River Kelvin.

Sun - Sunrise

Moon - Sunset

River Kelvin



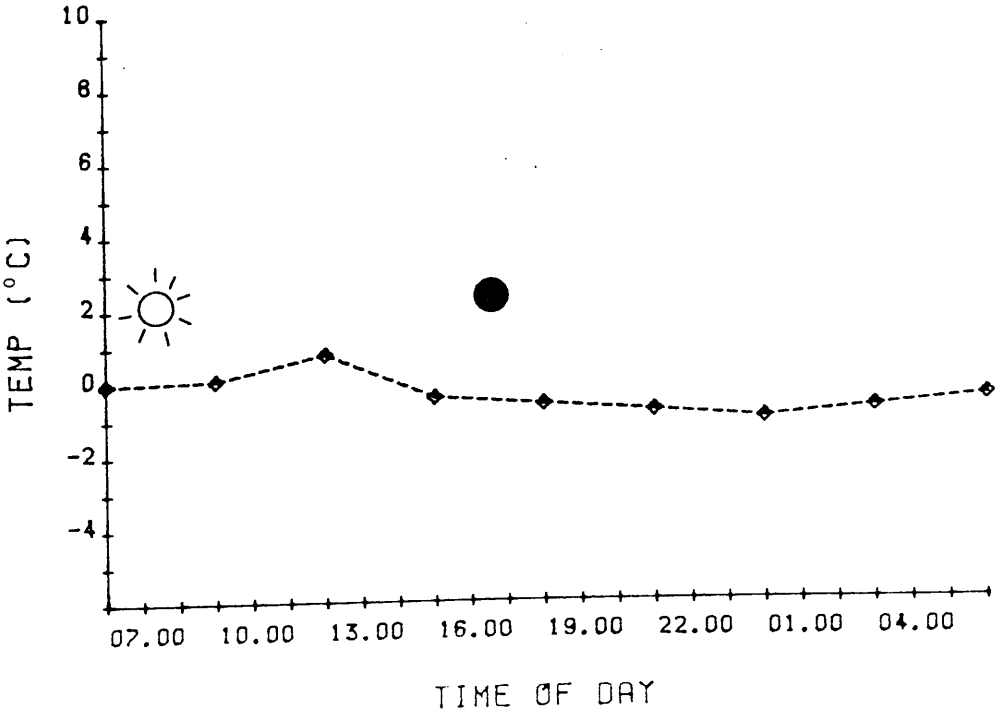
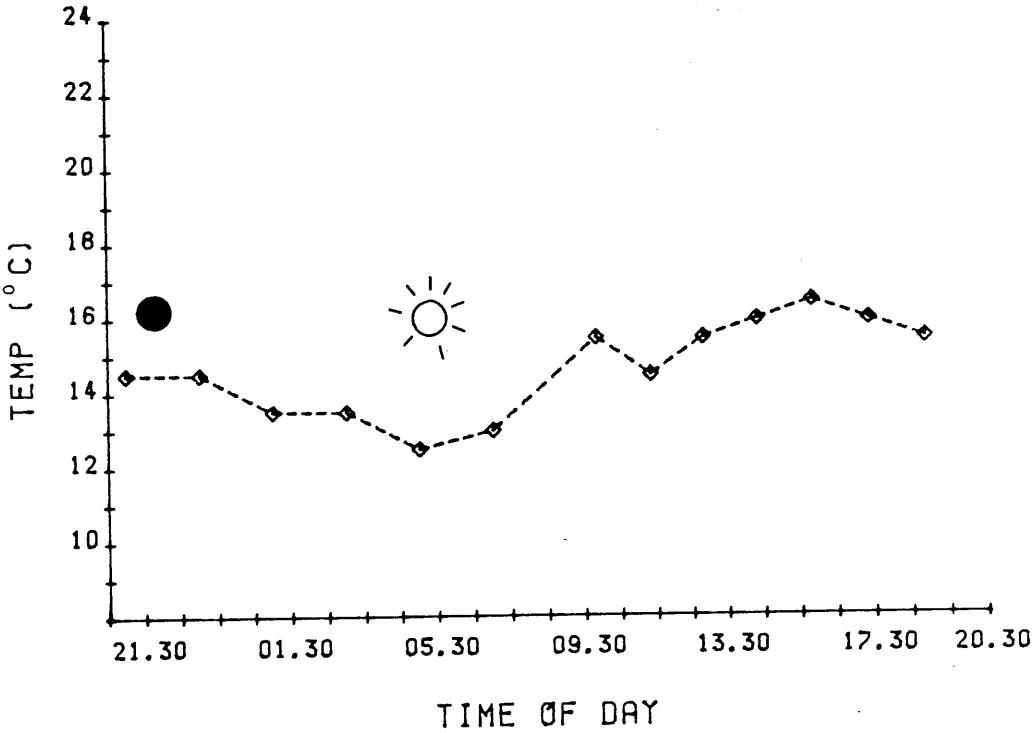


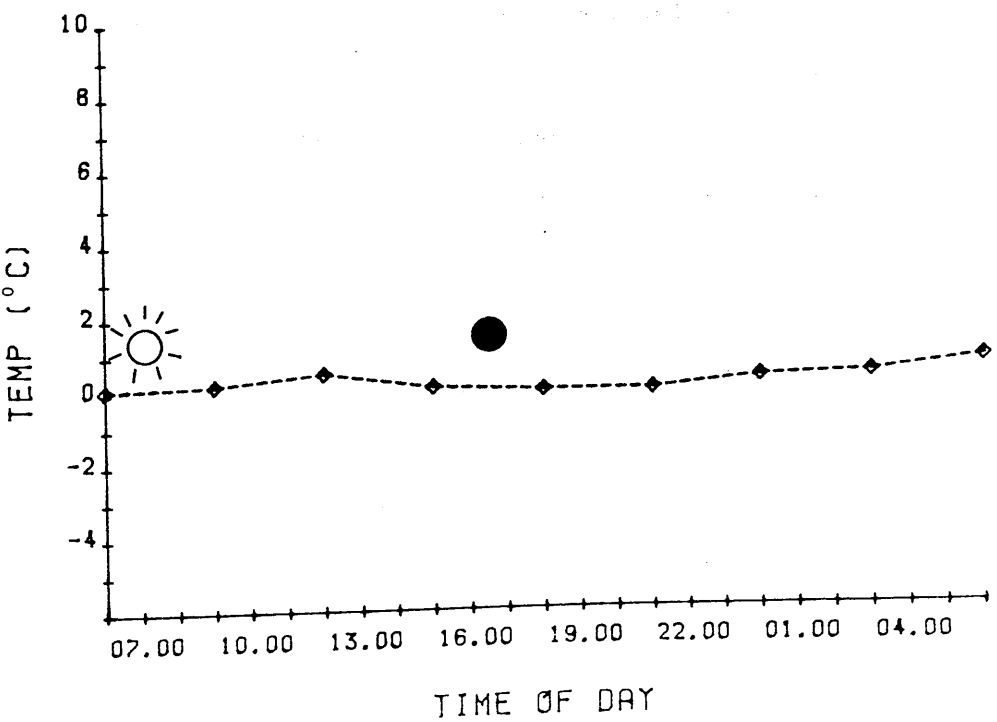
Fig 3.10 Temperature readings for a day in May (Top) and January (bottom) from the Allander Water.

Sun - Sunrise

Moon - Sunset

Allander Water





3.4 Discussion

Seasonal variations in PO_2 are characteristic of most streams (Hubault, 1927; Neel, 1951), but this situation may be amplified by a heavy organic load. The results obtained showed that both rivers were organically enriched. The biochemical oxygen demand (B.O.D.) of the rivers were relatively stable throughout the year, only increasing as might be expected during the summer. This was due to higher temperatures and the increase in the numbers of micro-organisms at this time. Average B.O.D. measurements were lower in the Allander Water than in the River Kelvin throughout most of the year but the levels rose sharply during the summer due to the heavy organic loading from the local sewage works.

The Allander Water was shown to have a lower pH, which remained moderately stable throughout the year, but fell noticeably during late summer. This occurred as a result of the local authority sewage works increased organic load arising from lower water levels and hence less dilution in the receiving streams. The release of humic acids leaching out of the soil, also predominantly occurring at this time of year, may also have contributed. The levels of suspended solids found in the rivers may simply reflect differing flow rates. The River Kelvin had numerous storm overflows which react quickly to rises in the water level washing large amounts of organic and other materials into the river. The River Kelvin would therefore show a marked increase in suspended solids at these times. Differences between the sites were particularly pronounced during the winter when spates were frequent. During the spring as rainfall returned to normal, the B.O.D. for both rivers became similar with the River Kelvin remaining slightly higher.

Nitrogen can exist in solution in three forms: ammonia,

nitrite and nitrate. Ammonia levels were noticeably greater in the Allander Water than the River Kelvin reflecting the high level of organic input from the local Sewage Works and agricultural land surrounding this site. In running waters nitrate is the form in which most nitrogen is held, except in conditions of organic enrichment. The nitrate form is the one most available for plant growth. The main sources of nitrate entering into streams are from rainfall and from land surface drainage. It has been shown that drainage from agricultural land produces large amounts of nitrate (Sawyer, 1947). Normally the concentration of nitrate in streams is low because the ions are rapidly taken up by plants (Minckley, 1963). There are seasonal differences in uptake as reported by Shadin (1956) who reported values of over 1mg per litre in rivers during the winter when plants are not actively growing and values that approach zero in the summer.

The results obtained for the River Kelvin and Allander Water would seem to fit the explanations given above by Sawyer (1947), Minckley (1963) and Shadin (1956). Both sites are linked to agricultural land, with the River Kelvin having the largest drainage area. Lower nitrate values in the Allander water were observed throughout the year, with only a slight increase in winter. These results might have been expected considering the large biomass of aquatic vegetation found at this site. The River Kelvin follows a similar trend but is richer in nitrate and shows a distinct peak in November. This rise may, however, be related to surface run-off and winter spates.

The seasonal fluctuations in temperature followed the ambient air temperatures. Diurnal variations in temperature during the summer reflect the amount and intensity of sunshine. These conditions were not

observed, however, during the winter months with the River Kelvin exhibiting subzero values. The subzero temperatures measured were very unusual and may be explained by the water cooling while being constantly agitated by the weir. Small ice crystals form on any particulate matter in suspension giving the water a very viscous appearance. Ice is therefore prevented from forming, until temperatures of two or three degrees below zero are reached.

The stable PO_2 values recorded during the winter months were due to lower temperatures; directly by increasing the amount of oxygen present and indirectly due to the reduced metabolism of flora and fauna. The increased frequency of spates which increased mixing within the water column would also have increased the amount of oxygen in solution. Hyperoxic conditions during the spring at the River Kelvin were probably due to a combination of the intense photosynthetic activity of high densities of algae, associated with the effects of higher temperatures and light intensities (Dawson *et al.*, 1982).

The effects of organic enrichment were demonstrated at the Allander Water with very low oxygen levels maintained for many months throughout the summer. The Allander Water site is fairly shallow and has a very large biomass of benthic organisms. Owens and Edwards (1963) calculated that in rivers less than 1 metre deep, 50% of the total oxygen consumption may be attributed to mud respiration, particularly if there were large number of animals present. According to Dawson *et al.* (1982), plants can also affect the oxygen content of a stream by photosynthesis and respiration during spring and summer, but also more importantly when they become moribund in late summer and start to decompose. This was the situation which occurred at the Allander Water with its massive covering of macrophytic growths. Edwards (1962) also

pointed out that in many shallow streams some of the oxygen generated by macrophyte photosynthesis does not dissolve into the water and was lost to the atmosphere in the form of bubbles. This problem can be further exacerbated, as in the case of the Allander Water, by many emergent macrophyte forms losing much more of their photosynthetic oxygen to the atmosphere. Such aerial growth also reduces the the amount of light available for aquatic photosynthesis. These factors, together with a reduced flow rate and higher summer temperatures, increased the rate of oxidation of surplus organic matter and microbial activity which resulted in a more intense depletion of oxygen.

Macrophytic photosynthesis and respiration will also have an important role in diurnal variation of PO_2. On most occasions when large variations occur, dense growths of aquatic plants have been observed. It is therefore surprising that the Allander Water showed little if any of this variation. During the summer months the water levels and flow rates were low. Dense growths of macrophytes were present, many of which were partly out of the water, thus reducing photosynthetic re-aeration. This dense covering may have also prevented sunlight penetrating the macrophytic growths to allow algal photosynthesis to occur.

The Kelvin site did, however, show diurnal periodicity in its oxygen levels. Such variations were first noticed in rivers by Butcher *et al.* (1927) and were described more fully, relating the effects of organic enrichment, by Grant and Hawks (1982). Schmassman (1957) also stated that large diurnal variations are often associated with organic enrichment which encourages algal growth in the presence of a fairly high oxygen demand. At the Kelvin site, large algal mats are formed during spring and summer and these tend to be pushed towards the bank by the current. As summer progresses, the water level drops and pools

are frequently formed in which many animals are trapped. These pools may be large in size and are separated from the main river by the mats of algae. These pools show very large diurnal variations in PO_2 (8-580 Torr) during spring and summer, related to the photosynthetic activity of the pool flora and to the respiration of both flora and fauna. Photosynthesis during the day produced more oxygen than the respiratory demands of the pool inhabitants required, producing hyperoxic conditions. At night, however, photosynthesis ceased and the respiration of the flora and fauna in the pools gradually depleted the available oxygen.

Temperature variations within the pool had a significant effect on this oxygen cycle. During the day, as the water temperature increased, gas solubility decreased and the metabolic rates of the flora and fauna present rose. Since the Q_{10} (Temperature Coefficient) for photosynthesis is lower than that for respiration (1.5 as compared with 2-3) (Hoar, 1966), the effects on respiration will be somewhat greater than on photosynthesis. Truchot and Duhamel-Jouve (1980) suggest that diffusion may limit the PO_2 increase during the day and decrease during the night with photosynthesis also inhibiting the increase in PO_2 by high oxygen levels (Warburg effect).

An interesting comparison may be made between the temporary pools formed in the River Kelvin and the normal situation found within marine intertidal rock pools. Both kinds of pools are separated from large water bodies for various periods of time and are therefore more susceptible to environmental change. Intertidal rock pools, immersed by the sea twice daily, exhibit large diurnal variability in temperature, salinity, pH, and oxygen tensions. (Naylor and Slinn, 1958; Daniel and Boyden, 1975; Morris and Taylor, 1983). The PO_2 of the water is

normally the most variable factor, as Truchot and Duhamel-Jouve (1980) demonstrated in their study of rock pools on the north-west coast of France, with large diurnal variations in PO_2 occurring and values near zero being recorded.

Intertidal rock pools also demonstrate seasonal variations in PO_2, related to the onset of rapid algal growth. Since the physico-chemical conditions within the pools are closely linked with the biomass of the flora and fauna, this may account for some of the observed differences in the time at which seasonal maximum and minimum values were observed (Morris and Taylor 1983). The existence of oxygen gradients within river pools demonstrated in the microhabitat results for the Allander Water, would seem to have similar causes as those found in intertidal rock pools. Both show that the PO_2 of water at the sides of the pools and at the bottom was higher than those near the surface. This would appear to be the result of algae which cover most of the sides and bottom of the pool.

Seasonal and diurnal variations must have a marked effect on the organisms present in freshwater pools, particularly benthic ones. By examining local variations in PO_2 within the pools during the periods of greatest stress we can obtain an accurate picture of the tolerance of hypoxia of many animals. The microhabitat results from the Allander Water do show limited diurnal fluctuations, samples being taken during spring before mass macrophyte growth has occurred. All three areas of sampling bed, understone and midstream followed the same pattern, with microhabitat levels showing the lowest values, due to community respiration beneath the stone.

A different situation was observed in the River Kelvin with the midstream sample showing no diurnal variation, the weir keeping

oxygen levels close to saturation. The microhabitat values showed the same general trends as the substrate readings. PO_2 levels were, however, lower during darkness with values approaching anoxia, and were maintained at this level for far longer. This can be explained by diffusion gradients, oxygen taking considerably longer to diffuse under a stone than the substrate with community respiration explaining the anoxic conditions during the night.

CHAPTER 4

Nature, if it meet with unfavourable conditions,
like any other seed out of its region,
always fails to thrive.

Dante (Paradiso, VIII, 139)

CHAPTER 4

Hypoxia Tolerance in Triclads

4.1 Introduction

Depletion of oxygen in a river, as demonstrated in the previous chapter, is a very common occurrence as a result of organic enrichment. The community of such habitats will therefore be restricted to animals tolerant of low PO_2. Organically enriched habitats will tend to have characteristic communities associated with the degree of organic loading; indeed this is the basis of many pollution indices which relate the known tolerance of different benthic invertebrate taxa to the pollutional state of the river by using simple scores or indices (Trent Biotic Index, Woodiwiss, 1964; The Graham Index, Graham, 1965; and The Chandler Score, Chandler, 1970).

In heavily polluted areas only very resistant animals will survive under anoxic stress. This may only be short-term, minutes or hours for some animals, while others can extend this to days or weeks and in some cases survive indefinitely. This diversity of response to anoxia has been generally categorised into three broad groups (Hill, 1976). Some animals have an essential need for a constant supply of oxygen. These animals on encountering anoxia will die within minutes, or at the very most, a few hours, and are termed obligate aerobes. Facultative anaerobes can survive anoxia for substantial periods, ranging from days to weeks, yet do not survive indefinitely. The third group, obligate anaerobes, survive indefinitely under anoxia and are killed or debilitated in the presence of oxygen. These groupings are not strict; as some animals, mostly Protozoa, are equally

capable of living with or without oxygen but are also termed facultative anaerobes.

Many invertebrates, from a wide range of phlya including cephalopod molluscs, adult insects, and decapod crustaceans, are obligate aerobes and die quickly without oxygen. At the other end of the spectrum are animals capable of surviving anoxia indefinitely, the obligate anaerobes. These are animals in which anaerobic pathways meet all the energetic needs of the animal indefinitely. There are very few animals that are true obligate anaerobes but animals that live in the bottom sediments, the sulfide system (prokaryotes, ciliates, Platyhelminthes, and Aschelminthes) and anaerobic bacteria are classed as such. Internal parasites have often been termed obligate anaerobes but this classification is now unclear. Internal parasites, such as *Ascaris lumbricoides* and *Hymenolepis diminuta* have often been the subject of controversy regarding their oxygen requirements. Many authors suggest that these animals should be considered obligate anaerobes as the adult lives under virtual anoxia and they are adversely affected by culture under aerobic conditions (Laser, 1944; Krotov, 1968). Crompton *et al.*, (1965), however, showed that in the small intestine of the domestic duck there was an oxygen partial pressure of 25mm Hg close to the villi declining to zero in the centre of the lumen. This would give nematode parasites living close to the villi access to plenty of oxygen. This view was supported by Smith (1969) who stated that all internal parasites are facultative anaerobes equiped to live in regions of low and possibly variable oxygen tensions.

By far the commonest group are the facultative anaerobes; species that can survive at least a day without oxygen uptake and

many examples are found in most phyla. Many invertebrate species are capable of relatively long anoxic survival, although some animals may not be able to support all their vital functions anaerobically. These animals utilise internal oxygen stores, in the same way as diving mammals support heart and central nervous functions during dives (Hill, 1976). The effect of anoxia on marine intertidal animals has rarely been studied outside the soft shore environment and has mostly been concerned with burrowing animals (Teal and Carey, 1967; Hill, 1981; Shumway and Scott, 1983). Recently, however, rock pool environments have been studied in relation to hypoxic stress, and the tolerance of some species established (Ritz, 1980; Theede, 1973; Agnew and Taylor, 1986; Morris and Taylor, 1983, 1985).

Fox (1954) studied the mud fauna at the bottom of North American lakes and found that for many months during the long summers, the water was totally devoid of oxygen. Yet, the mud harbours a fauna including, *Tubifex*, *Chironomus* larvae, ostracods, and the bivalve *Pisidium*. Cole (1921) has kept *Chironomus* in sealed jars, with no oxygen, and found them to remain alive for up to 50 days and active *Cyclops* have been taken from water containing no oxygen (Huss, 1913). Aquatic insect larvae have also been extensively studied, with some groups appearing relatively intolerant e.g. mayflies, stoneflies, caddis-flies, (Nagell and Fagerström, 1978; Benedetto, 1970; Nebeker, 1972) while others, dragonflies and alder flies, are much more tolerant (Cairns and Dixon, 1971; Mackenthun, 1966). Other insect orders, such as Hemiptera (bugs), Coleoptera (beetles) and Diptera (true-flies) do not show any characteristic degree of tolerance within their groups, each having species with widely differing capabilities.

In general, the "worms" have been found to have the highest degree of tolerance with many forms from different phyla being

remarkably tolerant. Mann (1956, 1961) has demonstrated that many species of leech are capable of prolonged survival under acute hypoxia and anoxia. The oligochaetes, *Tubifex tubifex* and *Branchiura sowerbyi*, have also been shown to demonstrate extensive anaerobic capacities surviving anoxia for over four weeks (Aston, 1973). Por and Masry (1968) investigated the anoxic survival of large numbers of the nematode, *Eudorylaimus andrassyi*, and the tubificid worm, *Eurilyodrilus heuscheri*. These worms survived for eight months in nature and over six months in the laboratory in a medium totally devoid of oxygen.

Previous studies on freshwater triclads were equivocal, some authors claiming triclads were intolerant of hypoxia others claiming to have provided evidence suggesting a limited capability for hypoxic and anoxic survival. Bunge (1888, 1889) carried out many experiments and found that *Dendrocoelum lacteum* and *Planaria torva* survived for two days under strictly anoxic conditions. Rode (1925) reported much longer survival times but there is some doubt whether the techniques employed yielded a totally oxygen free environment. Hyman (1919a) showed that free living turbellarians could survive in environments containing high concentrations of cyanide. In the absence of any formal knowledge of cytochrome systems at that time, regarding the specific effects of cyanide on aerobic metabolism, incorrect conclusions seem to have been drawn. There seems no doubt, however, that the survival of these organisms was made possible by a well developed anaerobic metabolism (Bryant, 1982). Other studies by Hammen and Lum (1962) showed that planarians could take up radioactively labelled carbon from bicarbonate and incorporate it into organic acids. Russier-Delholm (1974) and Abbott (1960) determined the lethal level of oxygen in three species of asexually reproducing lotic triclads. The results showed

that most worms died when oxygen levels fell to between 3-6% of air saturation. More recent work, however, has shown that *Cura pinguis*, an Australian freshwater triclad, possesses high concentrations of cytochrome b, which is almost always found in organisms capable of reducing fumarate to succinate and that these triclads excrete reduced respiratory end products into their environment (C. Bryant, Pers. Comm).

As freshwater triclads have been shown by many authors to exist under conditions of organic pollution and hence low PO_2 (Macan, 1962; Holsinger, 1966; Gaufin, 1958), it is to be expected that they would be tolerant of hypoxic stress. The degree of tolerance established may be related to environmental factors such as the oxygen stress of the habitats the animals came from. This study aimed to compare the hypoxic tolerance between and within various species of triclads from completely different oxygen backgrounds. It was also hoped to consider whether any subsequent differences observed were physiologically or genetically based, by examining these differences through F1 and F2 generations.

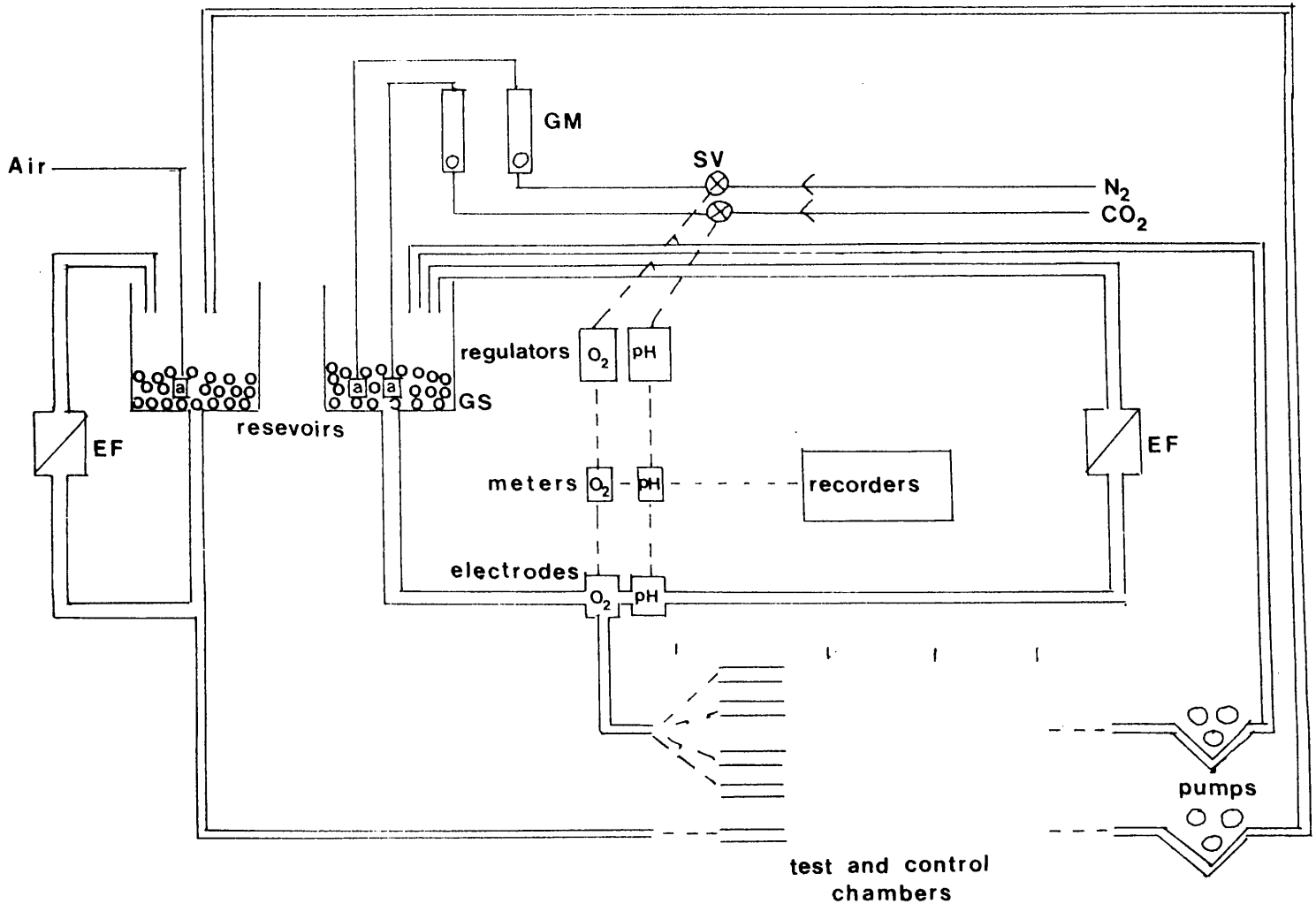
4.2 Materials and Methods

4.2.1 General

Triclads were collected from the underside of stones and vegetation from the two sampling sites (see Chap 3). All of the triclads were collected by hand using a fine paintbrush, those damaged being discarded. After collection, they were transferred to stock tanks (60x40x30 cm^3) with a running supply of copper-free tap water. The triclads were kept in a constant temperature room (10^oC), under a 12 hour light/dark cycle and maintained at approximately 100% air saturation. All of the triclads were fed until five days before the start of experimentation. Triclads were found to grow and reproduce normally on a diet of *Asellus aquaticus* and *Tubifex* sp. (Calow and Woollhead, 1977; Reynoldson, 1978) and so this was used throughout experimentation. When the animals were required for an experiment, they were removed from the stock tanks and placed into groups of ten individuals in plastic sandwich boxes (28x16x8 cm^3). The animals were kept in these boxes, without food, for one week at the required experimental temperature. Sixty triclads were usually tested per group during each experiment unless otherwise stated. During experimentation mortality values were recorded twice daily. Death, in this study, was defined as having occurred when the animal ceased to respond to mechanical stimulation and no longer adhered to the glass sides of the chambers or bottles.

4.2.2 Open System

Preliminary experiments on tolerance were based on a flow-through system. In this open system (Fig 4.1) ten animals were placed into each experimental and control chamber and the PO_2 level selected.


Air
GM
SV
N_2
CO_2
regulators
O_2
pH
a
a
a
GS
resevoirs
EF
EF
meters
O_2
pH
recorders
electrodes
O_2
pH
pumps
test and control
chambers

Water was then circulated in two complete circuits; a fast flowing inner loop that filtered the water and regulated oxygen and pH levels, and a slower outer loop spliced off the inner that incorporated the test chambers (glass syringes). Various oxygen concentrations were achieved by using a regulator which monitored changes in voltage output from an oxygen meter. The regulator opened a solenoid valve to allow nitrogen to enter the inner loop reducing the PO_2, and closed it when the preset PO_2 was achieved. Oxygen could then diffuse back into the system, as the reservoirs were open to the atmosphere, and the PO_2 increased until it passed the preset value, when the cycle recommensed. Fine control was brought about by gap meters to control gas flow. The regulation of pH would have been brought about in a similar method but this was not found to be necessary due to the natural buffering capabilities of the water used. The control chambers were fed from a reservoir which was constantly aerated, and flow rates through the experimental chambers were adjusted using peristaltic pumps. Tests determining the levels of accuracy established fluctuations of only 1-2% oxygen saturation at 50 Torr which was easy to maintain for long periods.

The results (See Sect. 4.3) from this particular method, however, showed it to be quite unsuitable for the experimental animals involved, as their tolerance to hypoxia was much greater than had initially been expected. Initial trials were set at PO_2 values of about 20-40% air saturation. This, however, proved to be too high, since the triclads survived indefinitely (> 30 days). Triclads were also found to exhibit prolonged survival under conditions of acute hypoxia (15 Torr) and even this experiment took approximately one month to complete. Acute hypoxia (10-20 Torr) was both difficult to maintain and highly expensive in nitrogen using the existing apparatus. Anoxic

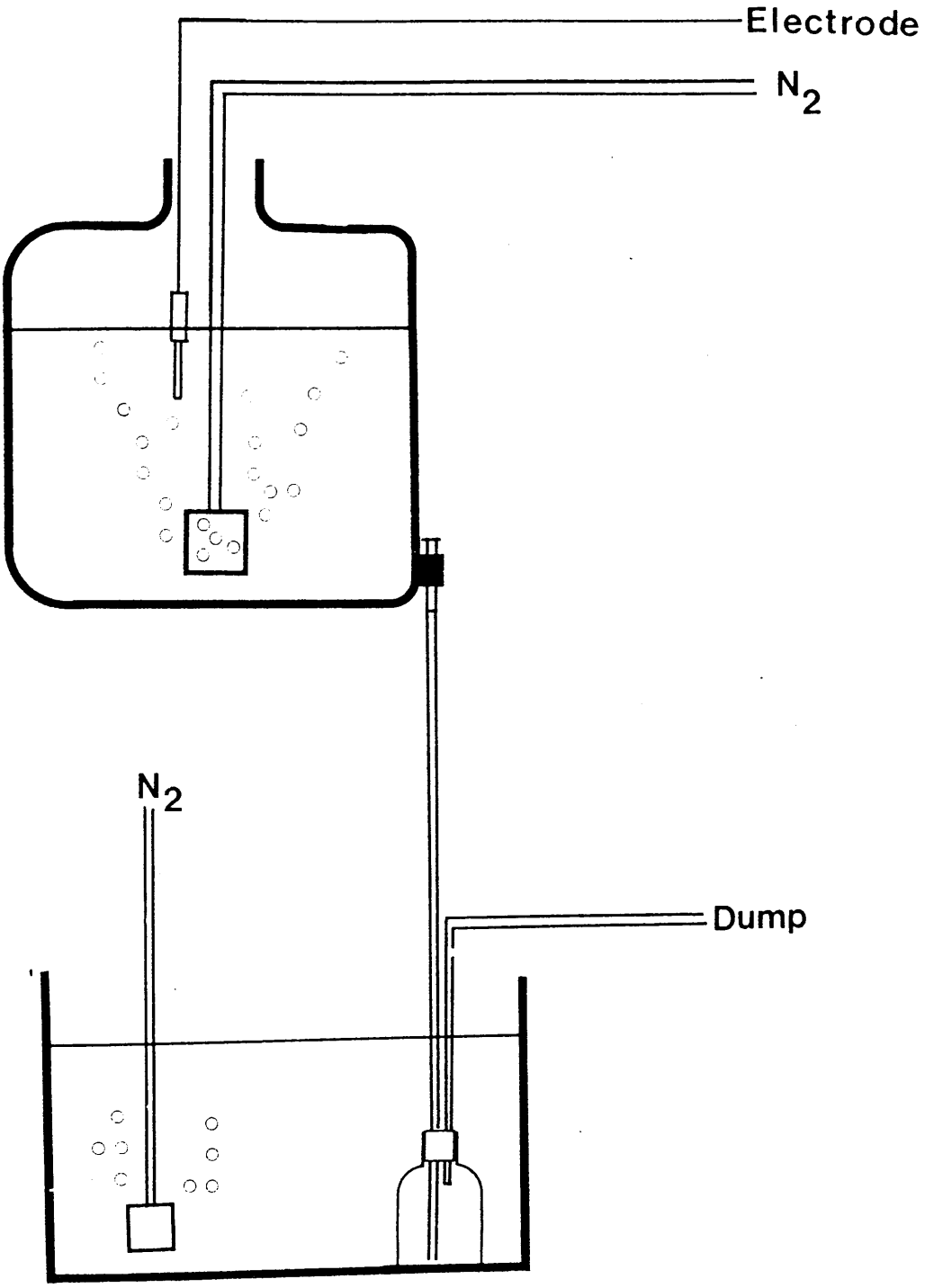
conditions, were therefore never examined and an alternative (closed system) and simpler method was adopted utilising the previous data for comparative purposes.

4.2.3 Closed System

The closed system involved the use of 500ml reagent bottles with ground glass seals and 28 ml universal containers with rubber seals. Vaseline was applied around the seals and the containers were completely immersed in hypoxic water. Experiments assessing the quality of the seals showed no significant increase in PO_2 over a one week period; an oxygen electrode (Radiometer E5046) coupled to a Strathkelvin 781 Oxygen Meter was used to determine the level of dissolved oxygen in solution. Hypoxic copper-free water was changed every two days to prevent the accumulation of toxic end products and to prevent respiration lowering the PO_2 significantly in hypoxic tests.

Initial trials were carried out at 10^{o}C in reagent bottles to assess the survival of three species of triclads from the River Kelvin (*Dendrocoelum lacteum*, *Dugesia lugubris*, and *Polycelis tenuis*). Intraspecific differences in populations from the two sample sites were also examined in *Dendrocoelum lacteum* and *Polycelis tenuis* with the former being more extensively studied. Water of various % air saturation values (0%,10%,20%,30%,40%, and 100%) was introduced into the reagent bottles, using the apparatus shown in Fig 4.2. The reason for using % air saturation in this case was that it made the comparisons of mortality at different PO_2's easier to interpret (see Chap 3). Water in a 10 litre container was set to the required PO_2 and the water was then passed into bottles containing the worms under water of approximately the same PO_2. Complete deoxygenation (< 1 Torr) was achieved by bubbling nitrogen through the water in the 10 litre

Fig. 4.2 Apparatus for introducing water of various oxygen tensions into the experimental chambers.


Electrode
N_2
N_2
Dump

reservoir until a stable zero reading was recorded. Other factors that were considered to be important in survivorship were also monitored. These were, size of animal, nutritional state, the effect of acclimation and the resistance to anoxia of the F1 generation. Experiments on animals kept individually were also carried out and these included the effect of temperature (5,10,15, and 20°C) and seasonal effects. These experiments all had the same general experimental protocol but with slight modifications, as summarised below.

Estimations of the length of triclads were made by watching triclads as they travelled freely across 1cm graph paper. They were then put into a size category small, medium, or large.

Dendrocoelum lacteum	0-5mm	small
	6-11mm	medium
	12-19mm	large

Populations of *Dendrocoelum lacteum*, from both sites, were also kept in separate tanks and acclimated to 40% and 100% air saturation for 50 days under a normal feeding regime. During this time mortality occurred (approximately 10-20%) in the low PO_2 tank. In assessing the effect of nutrition on survival, large numbers of *Dendrocoelum lacteum* were isolated, starved for two months and kept at 100% saturation. *Dendrocoelum lacteum* cocoons from the two stock populations (Allander Water and River Kelvin) were placed in separate tanks and the resulting young reared normally. This produced an F1 generation and it was hoped to examine the tolerance of an F2 generation, but due to contamination within stock tanks, this was not possible.

4.2.4 Analysis of Results

Many different methods have been used in an attempt to analyse data generated from tolerance experiments; time to first death,

time to last death and various other estimates of activity (Wieser and Kanwisher, 1959; Hutchinson , 1961; Preece, 1970). These methods, in the past, have not always been satisfactory and in an attempt to standardise data, measurements of tolerance are now generally expressed in graphical terms on the basis of LT_{50} values. The LT_{50} is an estimate of the time taken for 50% of the sample to die, measured by plotting cumulative mortality against time which generally produces a sigmoidal curve. An LT_{50} value can then be interpolated from the 50% mortality level. In an attempt to express confidence limits around this value by subjecting the data to regression analysis, various transformations can be carried out on the data including logarithmic, probit and probability analysis which convert the tolerance curve into a straight line (Bliss, 1938; Finney, 1947; Litchfield and Wilcoxan, 1949). This therefore enables comparisons between different groups with some degree of confidence.

The methods chosen to present the data in this thesis are approximate LT_{50} values estimated graphically and analysis of variance. In estimating LT_{50} values, no transformations were carried out and the data (expressed as % accumulated mortality) were plotted against time. This produced an S-shaped curve, from which you could read off the approximate time at which 50% of the sample died (i.e. the LT_{50}). To examine differences within groups or species the data were sorted into the number of animals dying between each time category i.e. if two animals were dead at 40 hours and one at 56 hours, the three data points would be 40,40,56. When the tolerance data were plotted in this way, the shape of the distribution was observed. Transformations were carried out where necessary, although a simple logarithmic transformation was all that was normally required. Differences between groups were then established by using a 1 or 2-way analysis of variance

test on the data. If this difference was found to be significant, the differences within the groups could then be determined by Scheffé's Test (Steel and Torrie, 1980). In Scheffé's Test, the variance of one sample is compared with another using a pooled estimate of the standard deviation. The data generated from this analysis will be presented by underscoring as follows:-

Population means used in the following calculations are drawn in line and underscored as follows. It should be noted that on many occasions the population mean and LT_{50} vary considerably from one another.

Groups	1	2	3	4	5	6
	13.3	14.6	18.8	19.9	24.0	28.8

This would mean that there was no significant difference (P>0.05) between the groups joined by a line i.e. there was no, significant difference between groups 1, 2, 3, and 4. There was also no significant difference between groups 3, 4, and 5, and groups 5 and 6. Significant differences, however, existed between the groups not joined by a line i.e. between groups 1,2 and 5 or 6, or between groups 4 and 6.

4.3 Results

4.3.1 Open system

The results from an initial trial on the tolerance of *Dendrocoelum lacteum* at 10°C from the Allander Water at 10% air saturation were compared with animals (groups of ten) kept in reagent bottles in Fig 4.3. Throughout experimentation both sets of controls (triclads maintained in open and closed systems at 100% air saturation) had minimal mortality (<2%). LT_{50} values were estimated graphically from the mortality curves and found to be very similar, 310 and 319 hours for the open and closed systems respectively. To investigate whether this difference was significant, a 1-way analysis of variance was carried out. This showed that there was no significant difference between the two groups ($P>0.05$) and hence there was no difference in measuring tolerance in either an open or closed system.

4.3.2 Closed System

The closed system incorporated two types of container, one for group tests, the other for individuals. In order to establish any possible difference between these two methods, a comparison was made examining groups of five triclads with individuals of *D. lacteum* from the Allander Water under anoxic conditions at 10°C. The results of this test are shown in Fig 4.4. LT_{50} values were calculated at 201 and 282 hours for the groups and individuals respectively. A 1-way analysis of variance showed that individual triclads survived significantly longer ($P<0.05$) than triclads tested in groups.

4.3.3 Interspecific differences in Tolerance

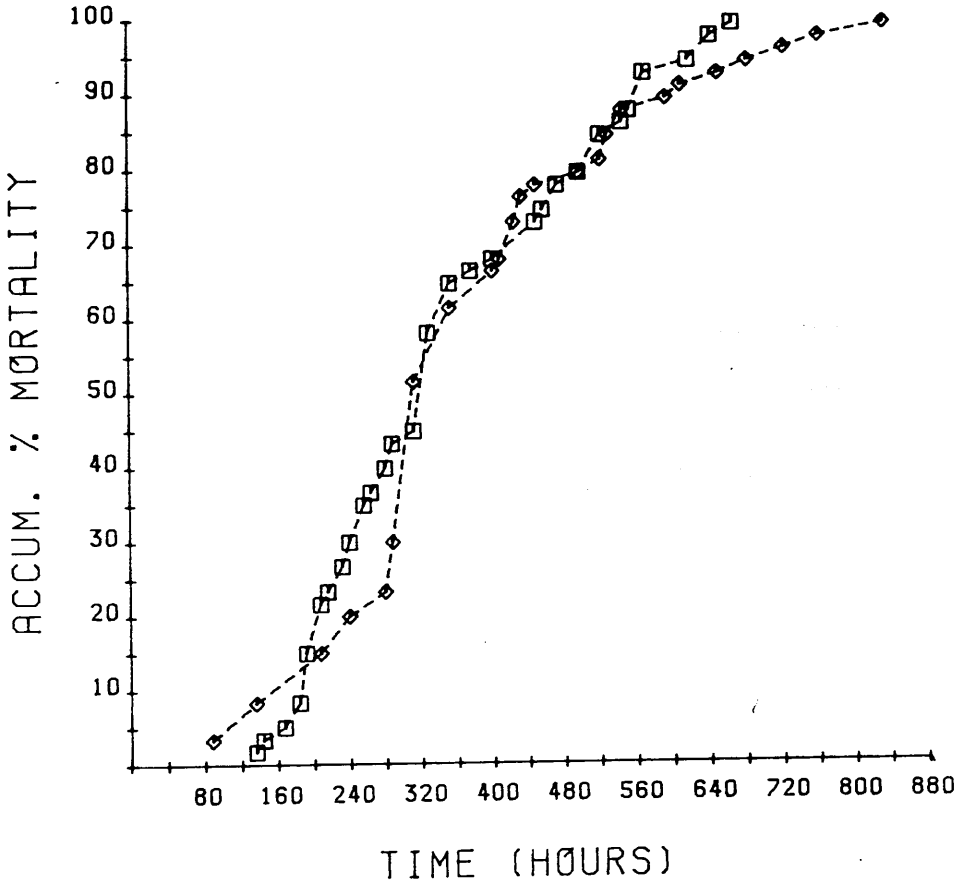
Interspecific differences were examined under anoxia at 10°C

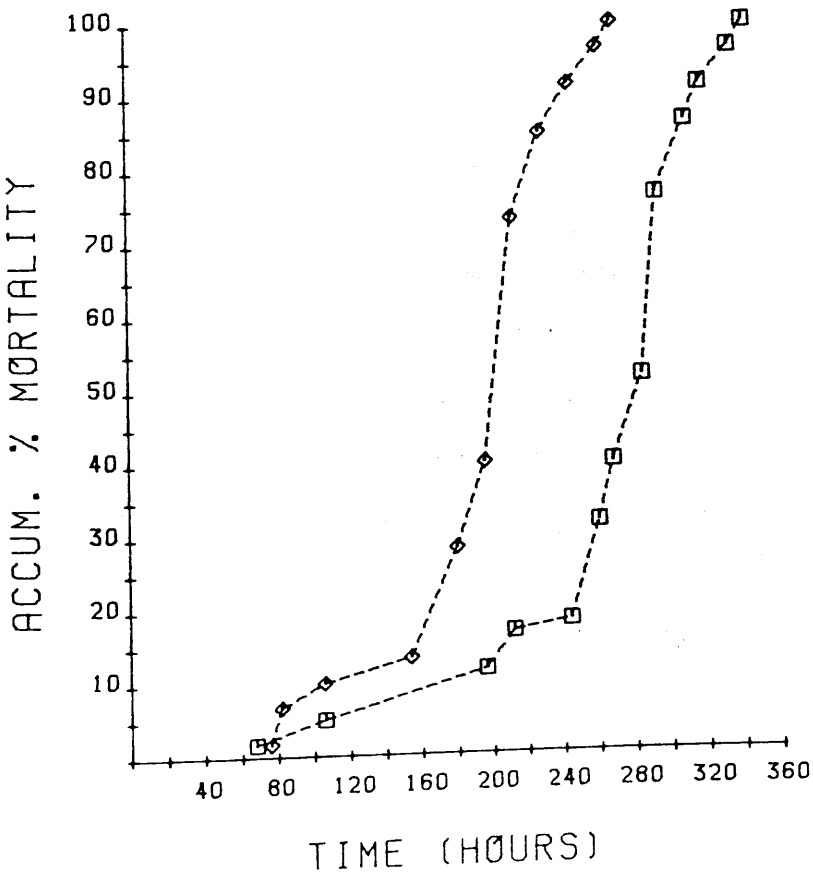
Fig 4.3 Mortality curves for *D. lacteum* from the Allander water at 10°C and at 10% saturation in an open and closed system.

Diamonds - represent the open system.

Squares - represents the closed system.

Fig 4.4 Mortality curves comparing the tolerance of individual *D. lacteum* from the Allander Water (Squares) with those kept in groups (Diamonds) at 10°C under anoxia.


ACCUM. % MORTALITY
100
90
80
70
60
50
40
30
20
10
80
160
240
320
400
480
560
640
720
800
880
TIME (HOURS)


ACCUM. % MORTALITY
100
90
80
70
60
50
40
30
20
10
40
80
120
160
200
240
280
320
360
TIME (HOURS)

between three species of triclads *D. lacteum*, *D. lugubris*, and *P. tenuis*) from the River Kelvin. Control groups for each species were maintained under similar conditions at 100% air saturation. The results presented in Fig 4.5 show that *Dugesia lugubris* survived the longest with an LT_{50} of 342 hours while *Dendrocoelum lacteum* and *Polycelis tenuis* survived 250 and 109 hours respectively. No mortality was observed in the controls and an analysis of variance showed that there was a significant difference ($P<0.05$) between the experimental groups. Scheffé's test was therefore carried out with the following results.

	Mean Survival Time (Hours)
Dugesia lugubris	337
Dendrocoelum lacteum	209
Polycelis tenuis	121

121 209 337

Significant differences were therefore found to exist between all of the groups.

4.3.4 Intraspecific difference in Tolerance

Unfortunately, only two of the above-mentioned species (*D. lacteum* and *P. tenuis*) could be screened for any intraspecific differences in tolerance of hypoxia as *D. lugubris* did not occur in sufficient numbers at the Allander Water site. The results of screening both species at 5°C under anoxia from the experimental populations are shown in Fig 4.6. The LT_{50} values for *P. tenuis* were 109 and 116 hours for the Kelvin and Allander populations respectively with *D.lacteum* surviving slightly longer with LT_{50} values of 250 hours for triclads from the River Kelvin and 278 hours for the Allander Water. A 1-way analysis of variance showed a significant difference ($P<0.05$) between

Fig 4.5 Mortality curves for three species of triclads from the River Kelvin, measured under anoxic conditions at 10°C.

D. lacteum - Diamonds

D. lugubris - Triangles

P. tenuis - Squares

Fig 4.6 Mortality curves measured at 5°C under anoxia demonstrating intraspecific differences in tolerance to hypoxia in two species of triclad.

River Kelvin

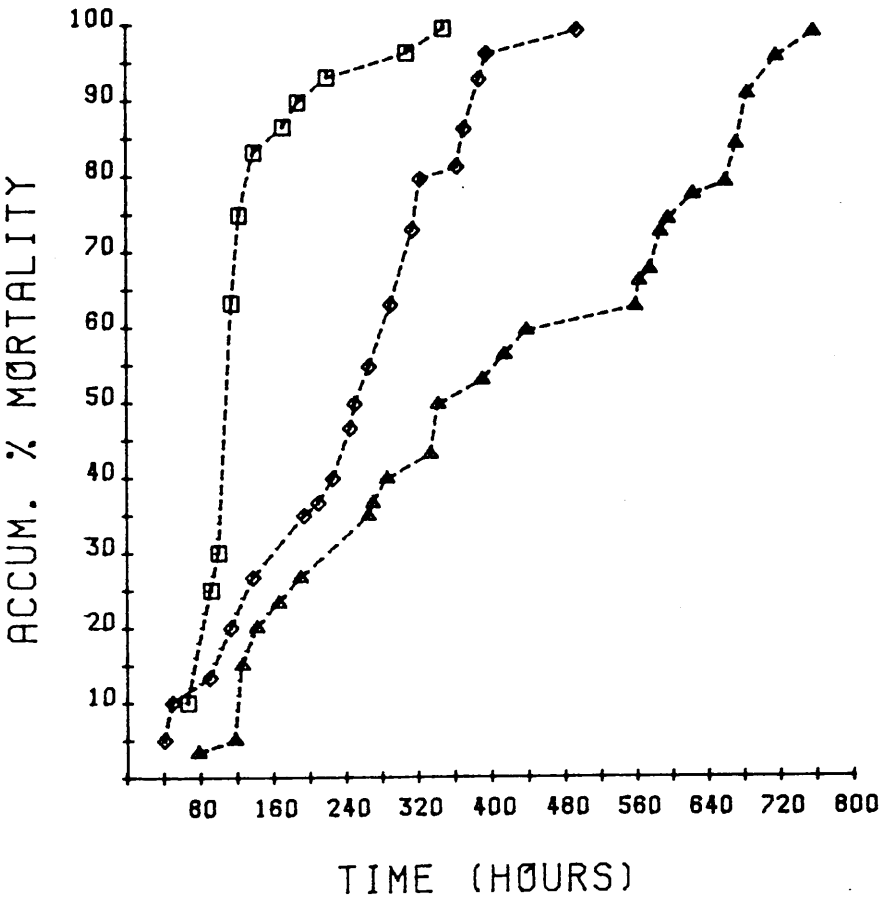
D. lacteum - Diamonds with a broken line.

P. tenuis - Diamonds with a solid line.

Allander Water

D. lacteum - Squares with a broken line.

P. tenuis - Squares with a solid line.


ACCUM. % MORTALITY
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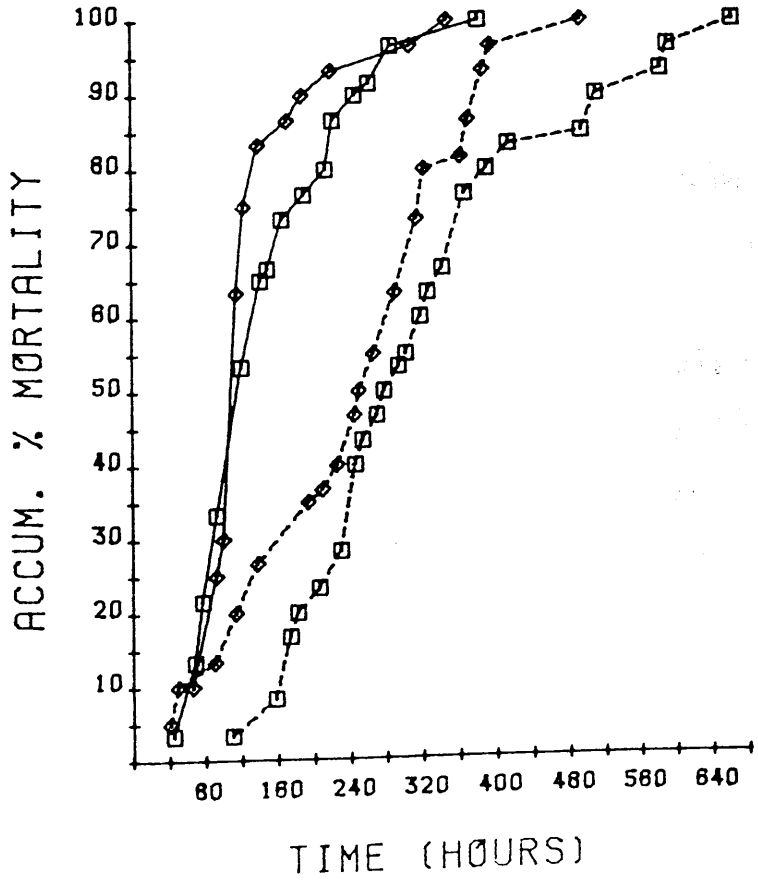
640
720
800
TIME (HOURS)


ACCUM. % MORTALITY
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90
80
70
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50
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20
10
80
160
240
320
400
480
560
640
TIME (HOURS)

the groups and the results of a Scheffé's test are therefore shown below.

	Mean Survival Time (Hours)		
D. lacteum - Kelvin	209	*P. tenuis* - Kelvin	121
D. lacteum - Allander	290	*P. tenuis* - Allander	130

121 130 209 290

Because a significant difference ($P<0.05$) was found only in *D. lacteum*, it was decided to concentrate on this species in all subsequent experiments.

In order to assess the tolerance of the two populations of *D. lacteum* over a range of oxygen tensions, an experiment was set up to examine the tolerance of groups at 10°C under various PO_2 tensions that might be encountered in the field. The results for the River Kelvin and Allander Water are shown in Fig 4.7. These results showed that *D. lacteum* from the River Kelvin survived much better at a higher PO_2, with LT_{50} values of 56, 117, and 267 hours, at 0, 10, and 20% air saturation. Similar results were obtained for triclads from the Allander Water, except that their survival times were extended (LT_{50} values of 129 and 319 hours at 0, and 10% air saturation). There was no significant mortality when these animals were maintained at over 20% air saturation. All the control animals, which were kept under similar condition at 100% saturation, survived the experimental period (30 days). The differences observed at both sites were examined by Anovar and found to be significant ($P<0.05$).

The results of Scheffé's test are shown below for the river Kelvin.

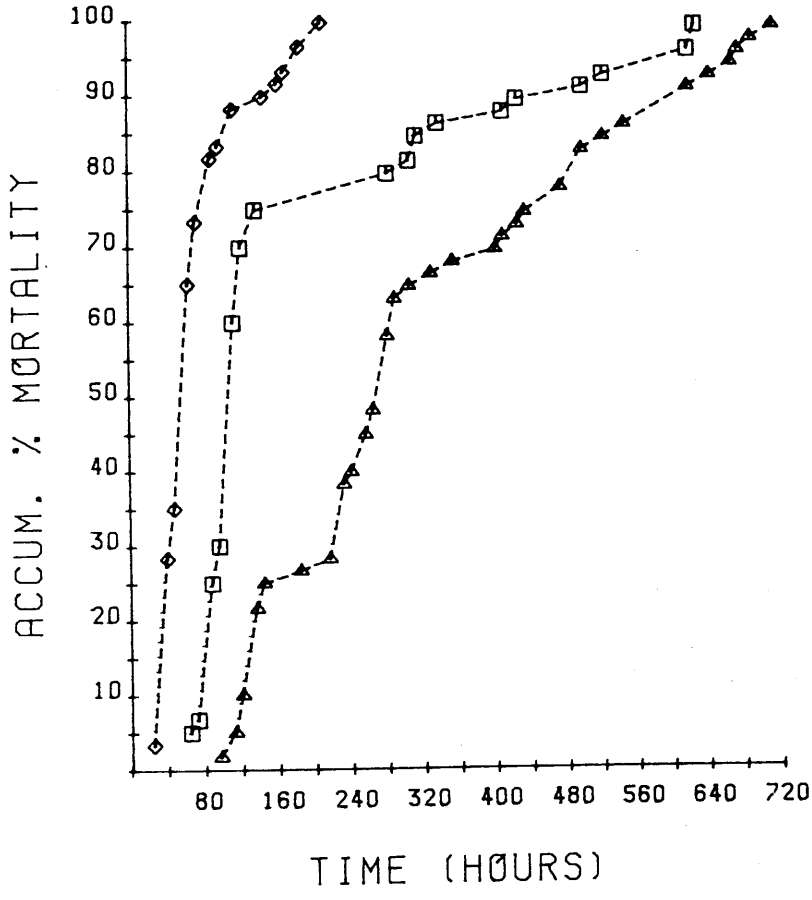
	Mean Survival Time (Hours)
D. lacteum - 0%	65

Fig 4.7 Mortality curves at 10°C for the River Kelvin at 0,10 and 20% saturation (Top) and Allander Water (Bottom) at 0 and 10% saturation.

0% saturation - Diamonds with a broken line.

10% saturation - Squares with a broken line.

20% saturation - Triangles with a broken line.


ACCUM. % MORTALITY
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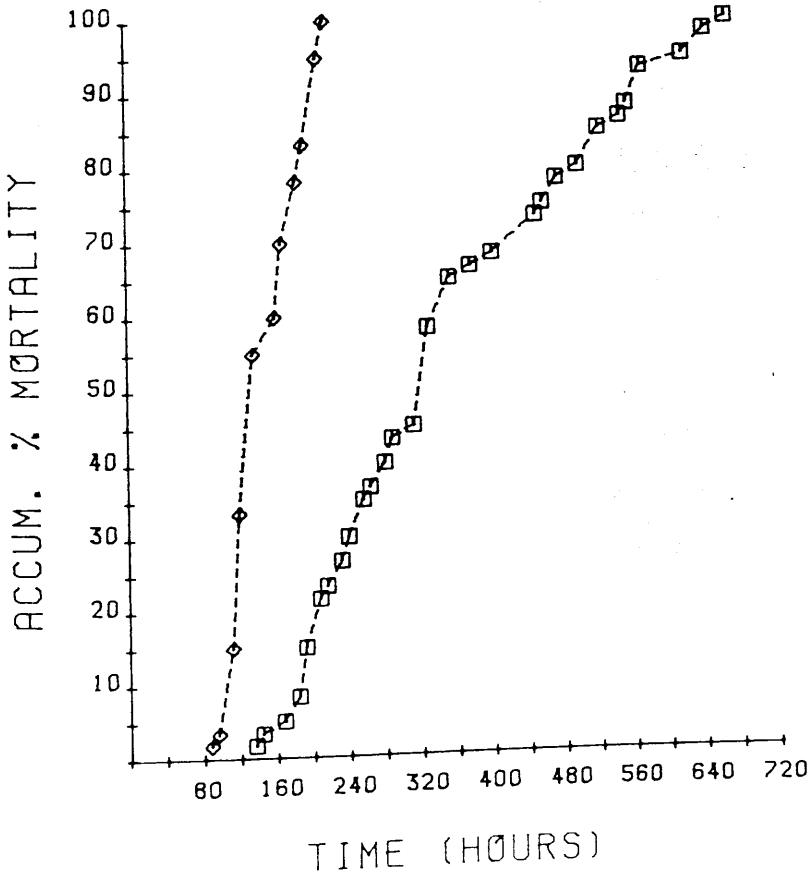
TIME (HOURS)


ACCUM. % MORTALITY
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TIME (HOURS)

D. lacteum - 10%	144
D. lacteum - 20%	272

65 144 272

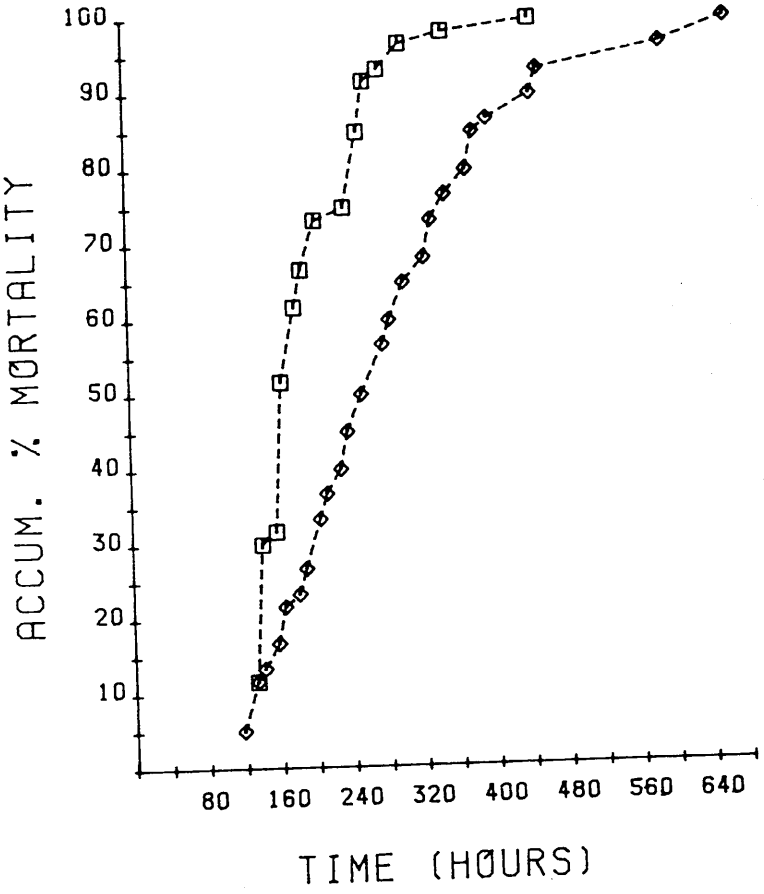
A similar experiment to the one described above was carried out under hyperoxic conditions. *D. lacteum* from both sites were screened at 500 Torr and their behaviour monitored. There was no apparent difference in behaviour and no mortality occurred over a 24 hour period.

4.3.5 Environmental factors affecting Tolerance

It was also considered that the effects of starvation on triclads might have a pronounced effect on their survival rate. Triclads from the River Kelvin were therefore starved for eight weeks and then assessed for tolerance under anoxic conditions at 5°C with suitable controls. The results obtained for this experiment are shown in Fig 4.8 and demonstrate that prolonged starvation does affect the LT_{50} of a species with values of 253 and 164 hours being estimated for normal and starved animals. An analysis of variance showed that starvation significantly reduced the tolerance of triclads to anoxia ($P<0.05$).

The physiological state of the triclads may also exert an influence on survivorship. In an attempt to assess this aspect of survivorship, triclads were collected in summer after cocoon production (June) and in winter (November) and screened under anoxic conditions at 5°C. The results are shown in Fig 4.9 and show LT_{50} values of 250 and 278 hours for Kelvin and Allander triclads in summer whereas in winter these values were reduced to 197 and 246 hours. An analysis of variance on the data was significant ($P<0.05$) and so Scheffé's Test was carried

Fig 4.8 Mortality curves for *D.lacteum* under anoxia at 5°C, from the River Kelvin. The triclads were starved for eight weeks (Squares) and fed normally (Diamonds).


ACCUM. % MORTALITY
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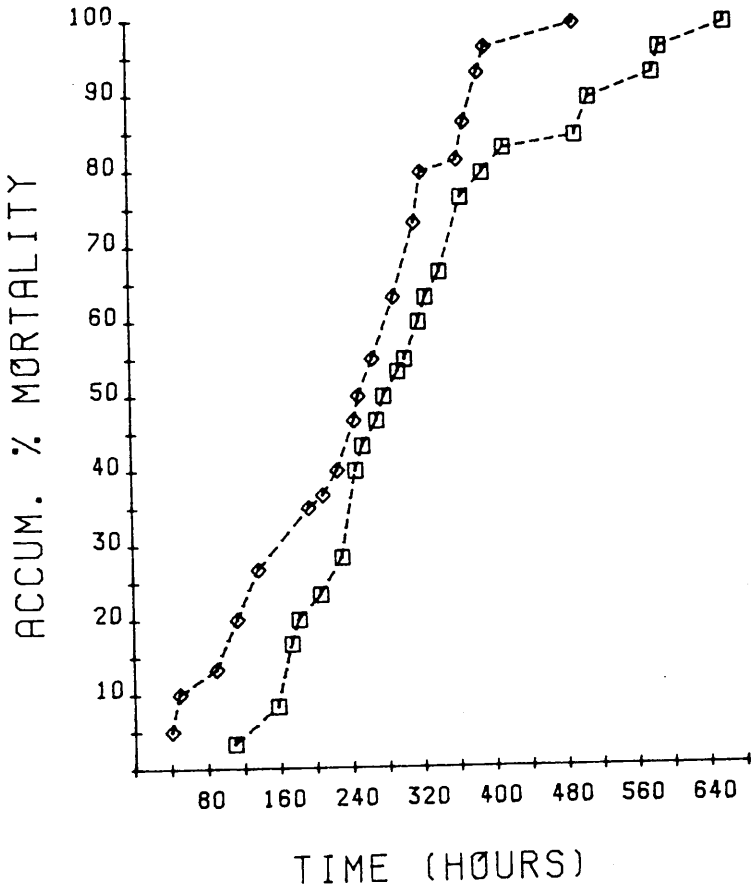
TIME (HOURS)

Fig 4.9 Mortality curves for *D. lacteum* at 5°C under anoxia during summer (Top) and winter (Bottom).

Triclads from the River Kelvin - Diamonds.

Triclads from the Allander Water - Squares.


ACCUM. % MORTALITY
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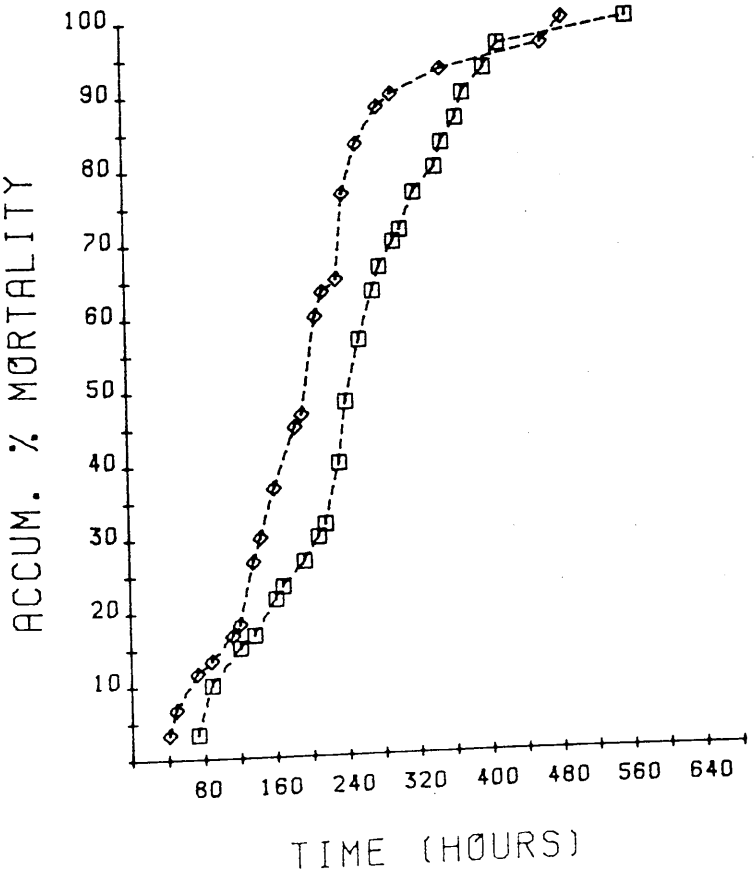
TIME (HOURS)


ACCUM. % MORTALITY
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TIME (HOURS)

out with the following results.

Mean Survival Time (Hours)

Summer		Winter	
D. lacteum - Kelvin	209	*D. lacteum* - Kelvin	180
D. lacteum - Allander	290	*D. lacteum* - Allander	233

180 209 233 290

As can be seen above there was no significant difference in tolerance to anoxia between Kelvin triclads and Allander triclads during the summer or winter, but the significant intraspecific difference noted in the summer does not persist during the winter.

The effects of temperature on the survival of *D. lacteum* under anoxia were also examined, covering the approximate range of temperatures found in the field (5, 10, 15, and 20°C). The results are presented in Fig 4.10 and show that the tolerance of triclads from both sites decreased as temperature increased. A list of LT_{50} values is given below.

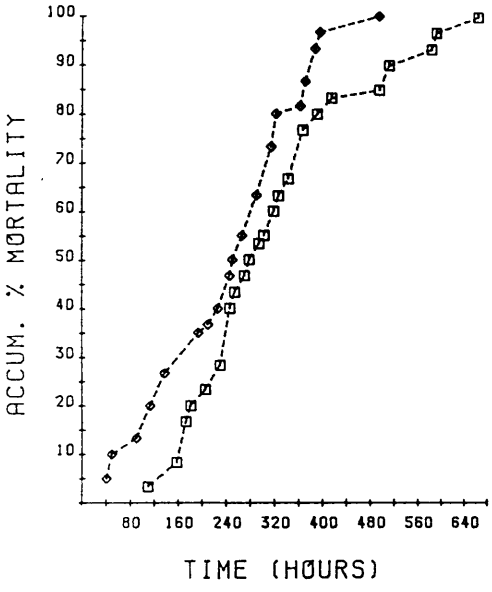
	LT_{50} Values			
	5°C	10°C	15°C	20°C
D.lacteum - Kelvin	250	227	107	50
D.lacteum - Allander	278	261	142	64

A Two-way analysis of variance was carried and showed that a significant difference ($P<0.05$) in mortality existed, both between sites and across the range of temperatures examined. There was, however, a significant interaction which showed that the main effects of one of the variables examined was not the same at different levels

Fig 4.10 Mortality curves for *D. lacteum* from the River Kelvin (Diamonds) and Allander Water (Squares) under anoxia and at various Temperatures.

Top left - 5°C Top right - 10°C

Bottom left - 15°C Bottom right - 20°C


ACCUM. % MØRTALITY
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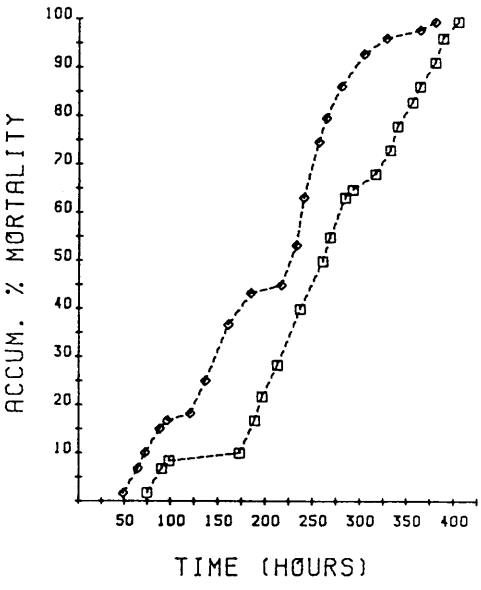
TIME (HØURS)


ACCUM. % MØRTALITY
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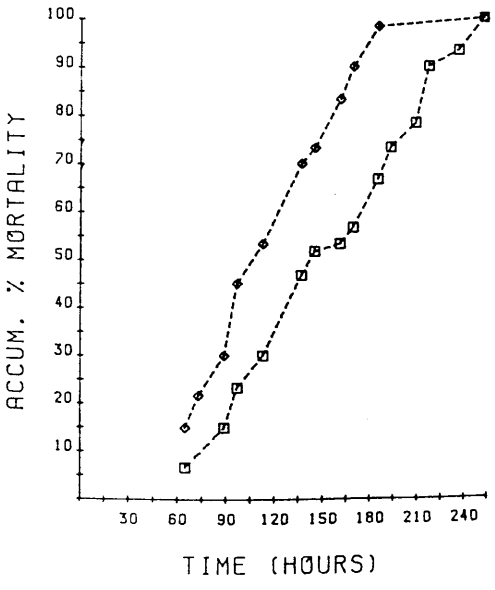
TIME (HØURS)


ACCUM. % MØRTALITY
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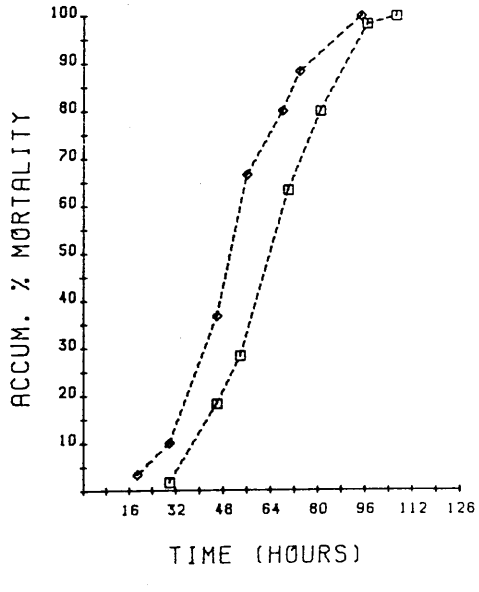
TIME (HØURS)


ACCUM. % MØRTALITY
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TIME (HØURS)

of the second variable. i.e. the survivorship of *D. lacteum* from both sites did not follow the same pattern of mortality at different temperatures. In order to examine these differences a 1-way analysis of variance was also carried out, followed by Scheffé's Test with the following results.

	Mean Survival Time (Hours)			
	5°C	10°C	15°C	20°C
D.lacteum - Kelvin	209	187	111	55
D.lacteum - Allander	290	252	147	69

55 69 111 147 187 209 252 290

This shows that there was a marked decrease in tolerance with an increase in temperature and that there was a significant difference between Allander and Kelvin triclads at 5 and 10°C but not at 15 or 20°C. There was also no significant difference within Allander or Kelvin triclads at 5 and 10°C.

4.3.6 Effect of Acclimation and Size on Tolerance

The effect of any size differences between animals i.e. age, may also have some affect on the survivorship of triclads. An experiment was therefore set up with groups of 30 animals at 10°C under anoxia to monitor size differences as well as the effect of acclimation on the mortality of triclads. Unfortunately, no small animals could be examined since the mortality rates of triclads at this stage in their life cycle were very high and this would mask any possible constructive interpretation of the results. The results (Fig 4.11) show that medium-

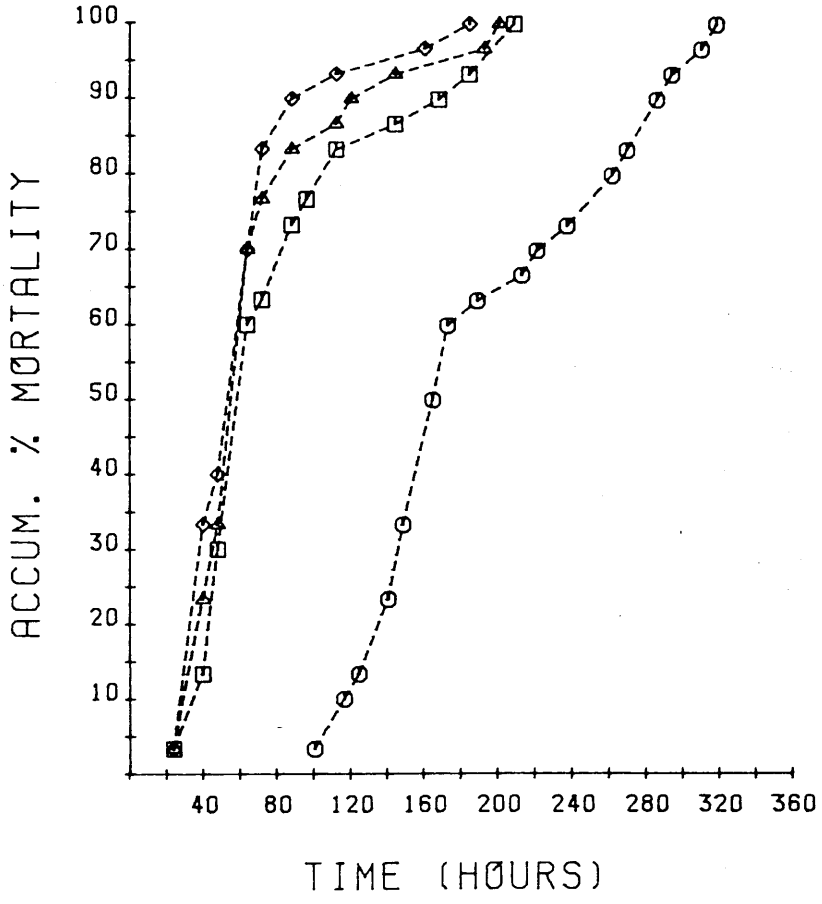
Fig 4.11 Mortality curves at 10°C under anoxia for acclimated and various size classes of *D. lacteum* from the River Kelvin (Top) and the Allander Water (Bottom) and after Acclimation.

Medium sized Triclads - Diamonds

Large Triclads - Squares

Triclads Acclimated to 100% air saturation - Triangles

Triclads Acclimated to 40% air saturation - Octagons


ACCUM. % MORTALITY
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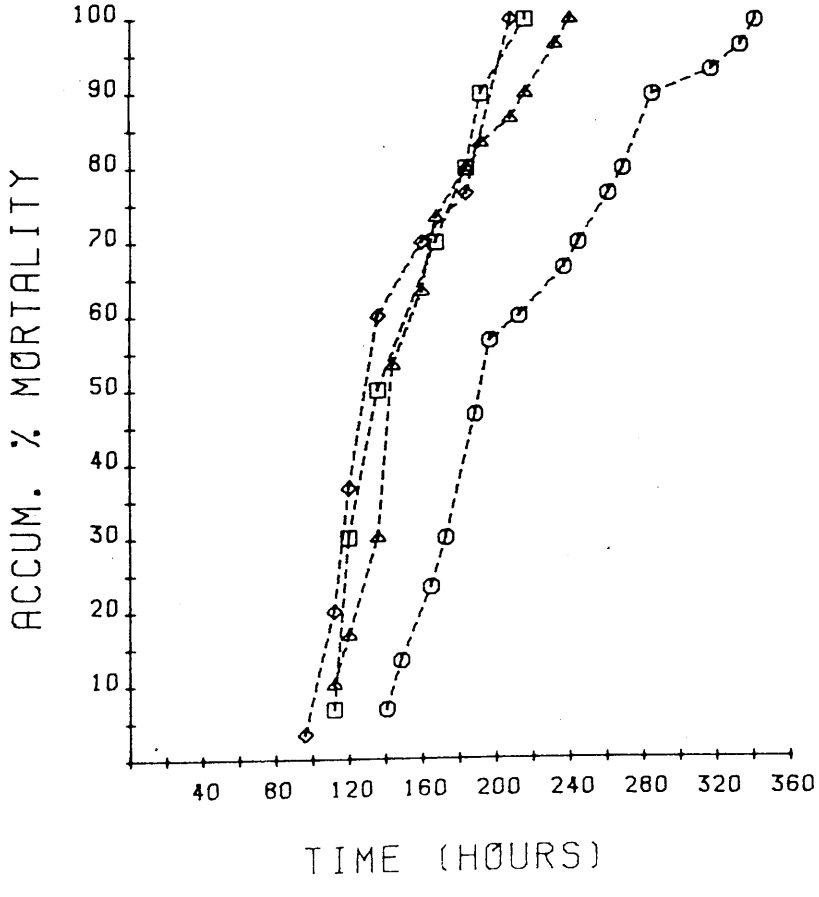
TIME (HOURS)


ACCUM. % MORTALITY
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TIME (HOURS)

sized, large, and acclimated animals (100%) from each site have similar LT_{50} values. The LT_{50} values from the River Kelvin were 54, 59, and 55 hours for medium, large, and triclads acclimated to 100%. Triclads acclimated to 40% air saturation survived much longer with a LT_{50} of 165 hours. Triclads from the Allander water had similar results with 129, 136, 143, and 191 hours for the same groups. The triclads acclimated to 40% air saturation showed an increase in tolerance especially those triclads from the River Kelvin. These results were shown to be significant ($P<0.05$) by analysis of variance and within group comparisons were therefore carried out by Scheffé's Test (See below).

	Mean Survival Time (Hours)		
Kelvin		Allander	
D. lacteum - Medium	60	*D. lacteum* - Medium	146
D. lacteum - Large	72	*D. lacteum* - Large	150
D. lacteum - Acc. 100%	65	*D. lacteum* - Acc. 100%	156
D. lacteum - Acc. 40%	184	*D. lacteum* - Acc. 40%	210
60 65 72	184	146 150 156	210

There was no significant difference in mortality in animals from both sites with respect to size or triclads acclimated to 100% air saturation. The only difference occurred in animals acclimated to 40% air saturation which survived significantly longer at both sites.

4.3.7 Tolerance of the F1 generation

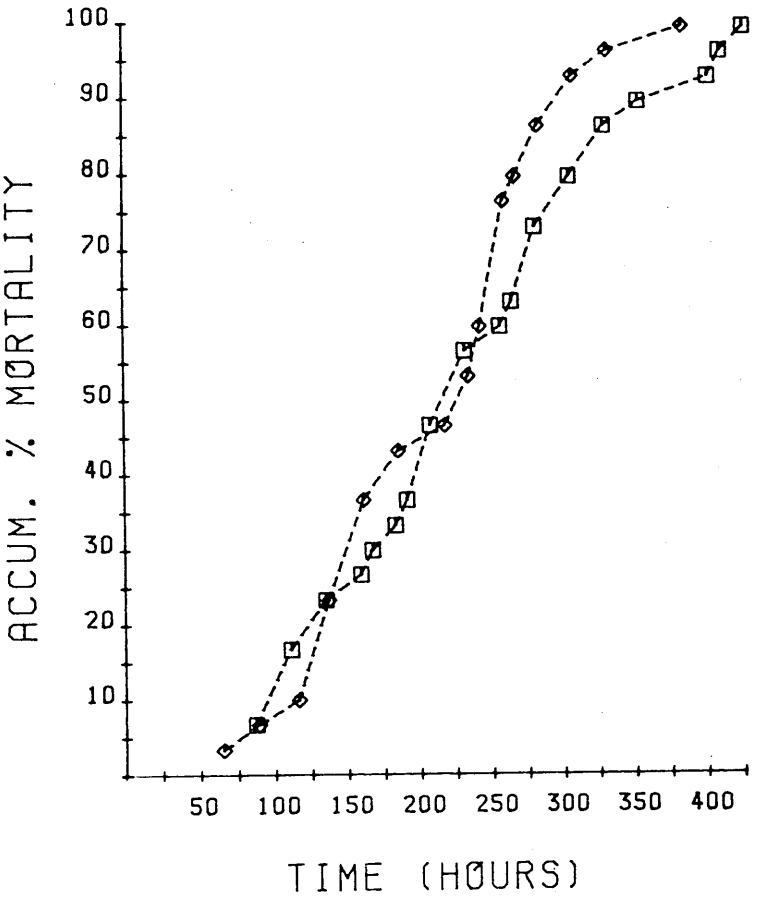
The results mentioned above indicate that triclads can adapt quickly to hostile environments, but are the population differences observed genetically or physiologically based? In order to investigate this problem, triclads were bred in the laboratory under a constant

feeding and oxygen regime (100%). The resulting worms were then screened under anoxic conditions at 10°C with recently collected material. The results for both sites are shown in Fig 4.12. LT_{50} values of 225 and 215 hours were estimated for the control and F1 generations from the River Kelvin with Allander Water triclads surviving 245 and 264 hours respectively. An analysis of variance was carried out on the data from both sites and there was found to be no significant differences between control and F1 generations ($P>0.05$). A significant difference was, however, shown to exist between the two sites with the control and F1 from the River Kelvin being significantly different from the control and F1 generation from the Allander Water ($P<0.05$).

Fig 4.12 Mortality curves measured at 10°C under anoxia for *D. lacteum* from the River Kelvin (Top) and Allander Water (Bottom) for control and F1 generations.

Control - Diamonds

F1 generation - Squares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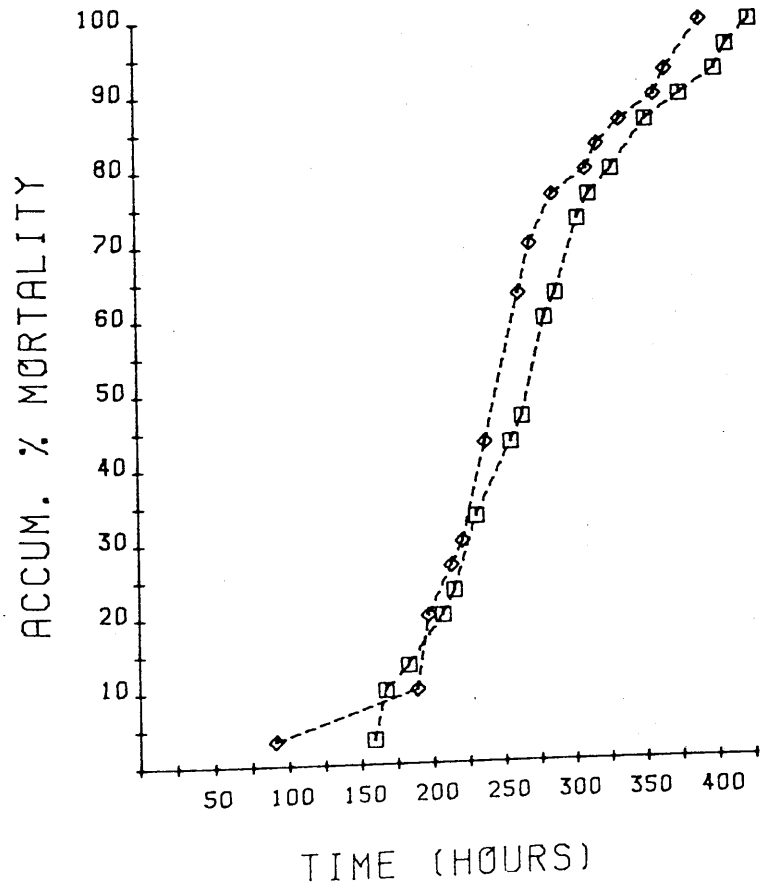
TIME (HØURS)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TIME (HØURS)

4.4 Discussion

There was no apparent difference in the results obtained using either open or closed systems to determine the tolerance of triclads. Differences were noted, however, in the screening of individual or group samples. The experimental protocol was designed to minimise any release of toxic end products on the death of the animals by constantly replacing the water supply. These toxins might, however, have adversely affected triclads in the same apparatus, since triclads kept individually survived significantly longer than those kept in groups. Another possible explanation for these results may be that the small residual amount of oxygen, left under anoxic conditions (<1 Torr), could be utilised to extend an individual's survival far longer than the survival of a large group of animals. This, does not mean, however, that the data collected for groups are invalid provided the comparisons were made intraspecifically, as intraspecific differences were found to persist in both group and individual cases, the only difference being that triclads tested individually survived significantly longer.

Many different species of "worms" exhibit a very great resistance to anoxia e.g. *Tubifex*, while other groups, notably many polychaetes succumb to asphyxiation in containers with varying amounts of oxygen still present (von Brand, 1946). It has been shown in the present study that triclads are capable of enduring prolonged exposure to anoxia. Interspecific differences in tolerance to anoxia are related to physiological and biochemical differences between species reflecting the types of habitat in which the animals had been located. *D. lugubris* is often found in small heavily polluted ponds or ditches (Mettrick *et al.*, 1970) and these habitats will undergo wide diurnal fluctuations in

PO_2 and animals inhabiting them may suffer acute hypoxia. This may help to explain the higher degree of tolerance to hypoxia found in *D. lugubris*. *D. lacteum*, and to a lesser extent *P. tenuis*, are often found in the quieter stretches of organically enriched rivers, with the distribution of the former being closely linked to the pollution tolerant *Asellus aquaticus* (Reynoldson and Young, 1966). These two species, therefore, may not have been expected to show the same degree of tolerance as *D. lugubris* as their habitats have a much more stable oxygen regime.

Interpopulation, or intraspecific variation has rarely been recorded and most previous work on tolerance has simply been to monitor interspecific differences. In the present study two populations of *D. lacteum*, from the River Kelvin and Allander Water, have been shown to exhibit differences in hypoxic and anoxic tolerance. The reason for this divergence was possibly due to the hypoxic stress imposed by the Milngavie Sewage Works which produces a high level of organic input to the Allander Water and this reduces the PO_2 levels significantly (See Chap 3). This selection pressure may have been enough to separate both populations into two distinct groupings, "tolerators" and "non-tolerators"; the difference in LT_{50} values reflecting different proportions of resistant individuals. Angus (1981) demonstrated a similar situation in populations of the mosquitofish (*Gambusia affinis*). He compared three populations of mosquitofish for phenol tolerance from various phenol polluted and non polluted sources. Differing LT_{50} values were found to reflect differing proportions of resistant individuals with phenol tolerant populations containing 67-80% resistant fish and non-tolerant populations containing only between 23-27%. This phenol tolerance was found to depend on the possession of a genetically determined, rapidly acting detoxification mechanism.

Are the intraspecific differences mentioned above genetically or physiologically based or even a combination of both? Experiments constructed to assess the effects of acclimation on triclads showed very significant increases in tolerance in triclads from both sites which were acclimated to a low PO_2. There was, however, no significant difference between worms acclimated to 100% air saturation or within various different size classes. Differences in tolerance might have been expected in animals of different sizes since smaller animals would have higher metabolic rates (on a per gram basis) and smaller energy stores. Perramon *et al.* (1983) selectively bred two populations of Japanese quail (*Coturnix coturnix japonica*), one resistant and one susceptible to acute hypoxic nitrogen challenge. He concluded that within these populations body surface was an important parameter in hypoxic survival, smaller animals surviving longer than the larger ones and that genetic differences with respect to hypoxia could be one of the most important causes of variation in the results.

The intraspecific differences in tolerance mentioned above were found to persist to an F1 generation which would suggest a genetic component. By estimating the length of time that the environmental stress may have acted on the triclads, it may then be possible to establish whether enough generations have passed to initiate a genetic basis for these differences. A similar study on populations of the Amargosa pupfish (*Cyprinodon nevadensis*) separated into rivers and desert springs about 4000-5000 years ago by falling water levels have shown genetic differences with respect to temperature and oxygen tolerances (Hirshfield *et al.*, 1980). McMahon (1975) demonstrated a much shorter time scale of 54 generations for the probable genetic differences in heat tolerance of the freshwater pulmonate pond snail

Physa virgata. In a similar situation, Holland *et al.* (1974) reported the formation of genetically different, thermally induced, race of the the bluegill sunfish *Lepomis macrocherus* in an area receiving the heated effluent of a power plant.

The sewage works, located on the Allander water, has been in existence for approximately 60 years (Sewage Dept., Strathclyde regional Council; Pers. Comm.) hence inducing a strong selection pressure for "tolerators", for only about 60 generations. This therefore, may have been sufficient time for a genetic adaptation, but to be sure an F2 generation of tolerant triclads would also have to be screened. This was unfortunately not possible, due to the contamination of holding tanks, and so the present results suggest a physiological component in the tolerance of triclads to anoxia, but a genetic basis also seems likely and cannot be ruled out.

It is clear that the resistance to lack of oxygen depends in many cases on the physiological state of the triclads. Any condition which lowers the energy requirements of these animals will tend to increase the anaerobic resistance. The less active the animal, the lower the anaerobic metabolism required to sustain life and the fewer toxic end products that can accumulate in the tissues or surrounding environment. This accumulation of toxic end products is what usually limits the anaerobic resistance of an animal. Strategies for tolerance may include a very much reduced metabolism, including cessation of movements (Knight & Gaufin, 1963) and in an extreme case cryptobiosis (Hinton, 1971). Triclads, exposed to anoxia, were carefully observed and a consistent pattern of behaviour was noted, regardless of species. On introduction to an acutely hypoxic environment, triclads became very active but this stage lasted only a matter of 5-10 minutes. The animals then stopped moving and remained quiescent for the rest of the

experiment, or until death. It was also observed on some occasions that at very low PO_2, *D. lacteum* attached to the sides of the container by its anterior "sucker" and released its lower body and tail outwards into the body of the water. This may have had the effect of doubling the surface area available for gaseous exchange, as well as creating currents in the water to replenish the exhausted oxygen supply. The only noticeable effect of the experiments on the animals was a gradual shrinking in size during the course of the experiment. This loss of tissue, usually from the tail end first, was most marked under anoxic conditions and during this time the animals were seen to shrink visibly. This was probably related to the catabolism of high energy glycogen reserves stored predominantly at the rear of the animal. A similar situation was noted by Crompton and Smith (1963) when examining the flatworm *Phaenocora unipunctata* under anoxia. They stated that there was no obvious change in colour in worms after one week but worms became very much smaller, shrunken at the posterior end, having apparently metabolised a considerable portion of their reserve substances.

Nutrition, would also be a very important factor in determining the energy requirements of the animals. Many animals demonstrate an increase in oxygen consumption immediately after a meal. This has often been referred to as Specific Dynamic Action (S.D.A.) and different foods have different magnitudes of effect. Nelson *et al.* (1977) noted a 40% increase in metabolic rate in tubificids after eating. This effect is, however, only short term and subsides shortly after the meal. Pütter (1908) studied more long term effects of starvation and showed that well fed leeches (*Hirudo medicinalis*) died more rapidly under anoxia than leeches starved for many months. Hyman (1919b) showed this situation to be reversed in planarians; with an

increased metabolic rate observed immediately after feeding, reaching a minimum about two weeks later, after which a progressive and marked increase was observed. This was substantiated by the results of the present study with normally fed *D. lacteum* surviving significantly longer than its starved counterpart.

The temperature at which an experiment was carried out would also be important, as the metabolic rate of the animal would rise with any appreciable increase in temperature. It is generally accepted that for every increase in temperature of 10°C there is a doubling of metabolic rate (Wells, 1980). This would mean that at higher temperatures energy reserves were being utilised at a faster rate than would otherwise have been necessary. The results for *D. lacteum* subjected to anoxia at various different temperatures confirmed this with a marked decrease in tolerance associated with any increase in temperature. It was interesting to note that there was no significant difference in tolerance of triclads between 5 and 10°C, this would suggest that there was only a minimal increase in metabolic rate over this temperature range. This may be due to acclimation in the field, river temperatures at this time of the year were fluctuating around 10°C. Intraspecific differences demonstrated at 5 and 10°C do not persist at 15 and 20°C, this may have been due to the sampling time and hence too few data points i.e. the time between measurements was too long and did not allow enough data points to be obtained for comparative purposes.

Seasonal variations in tolerance were examined to investigate whether the physiological condition of the triclads was important. Triclads were tested in summer, after breeding, which was considered to lower their tolerance to hypoxic stress. This was not found to be the

case since there was no significant difference between triclads from the River Kelvin and Allander Water during the summer or winter. Intraspecific differences noted in the summer did not persist during the winter. This may have been due to acclimatory effects, the PO_2 of the Allander Water remained much lower during the summer months and triclads therefore became acclimated to these conditions at this site. Another possible explanation could be that, during the winter, the population at both sites, was composed of different percentages of "tolerators" and "non-tolerators". During the summer the PO_2 at the Allander water site drops and the "non-tolerators" are killed off. This would then explain the greater differences between the sites during the summer months.

CHAPTER 5

CHAPTER 5

Oxygen Consumption in *Dendrocoelum lacteum*

5.1 Introduction

The respiratory physiology of triclads has been extensively studied, over the past 70 years, by many workers using a variety of different techniques. Allan (1919) and Calow and Woollhead (1977) used variations on the Winkler method, Fraps (1930) used the manometric method of Warburg and Lovtrop (1953) and Pederson (1956) made use of the Cartesian Diver method. During this time it had been generally assumed that anaerobic metabolism was of little or no importance (von Brand, 1945) and so the methods used were largely concerned with the measurement of aerobic respiratory exchange i.e. oxygen consumption. Only Child (1920) and Allen (1920) used a different method, which involved assessing the production of carbon dioxide as an estimate of metabolic rate; all the others measured oxygen consumption.

Many factors can affect the respiratory rate of an animal in a variety of different ways (reviews in Prosser and Brown, 1961; Newell, 1970). These factors can be conveniently classed into two groups; endogenous and environmental. The effect of endogenous factors such as size (Kleiber, 1932; Hemmingsen, 1950, 1960; Newell, 1970), activity (Fry and Hart, 1948; Newell and Roy, 1973) and nutritional state (Marsden, 1973; Calow, 1974) have been studied in great detail in many different animals. Triclads, like most other animals, have demonstrated an increase oxygen consumption which increases with weight (Allen, 1918; Whitney, 1942) but respiratory rate per unit weight was

higher for smaller triclads than for larger ones (Hyman, 1919b). A linear relationship between oxygen consumption and size measured in terms of "plan area" was also noted by Calow (1977).

Many studies have shown a reduction in respiratory rate during starvation (Child, 1919; Hyman, 1919b; Calow and Woollhead, 1977). Bolen (1937) also found similar reductions which he attributed to a reduction in Specific Dynamic Action. Calow and Woollhead (1977) agreed with this, but added that differences in locomotory strategies adopted during food shortages exerted a modifying effect.

The effects of reproduction on the respiratory rates of triclads has also been studied. Woollhead (1979) examined the respiration rates of five species of sexually reproducing triclads and concluded that there was no significant difference in respiration rates between reproducing and non-reproducing worms. A similar situation was observed in asexually reproducing triclads with no significant difference in respiration rates regardless of reproductive condition (Pederson, 1956; Calow, Beveridge, and Sibly, 1979).

Triclads have no respiratory structures and rely exclusively upon diffusion through the body surface for the exchange of respiratory gases and this holds true for terrestrial as well as aquatic species (Hyman, 1951). There have, however, been significant differences in theoretical and experimental results calculated for the rate of oxygen diffusion through a triclad's integument (Chapman, 1980). It would appear, from the experimental results from this study, that triclad integument is less permeable than naked muscle (Coward, 1968) or that the mucus secreted by triclads onto the under-side of their bodies may act as a diffusion barrier by increasing the diffusion distance and decreasing the oxygen conductance much in the same way as Ultsch and

Gros (1979) described in the gills of carp.

The influence of environmental factors on the respiration rates of freshwater triclads has, in general, not been as well documented as for the endogenous factors. The effect of oxygen availability, however, has been reasonably well established. Lund (1921) found no change in the rate of oxygen consumption in *Dugesia dorotocephala* down to 1/3rd air saturation. Hyman (1929), working on the same species, later added further support to Lund's work. Abbott (1960) working on *Phagocata gracilis* and *Dugesia tigrina* observed no difference in respiration rates down to oxygen tensions of about 1/15th air saturation, which is approximately 10 Torr. At this oxygen tension Russier-Delholm (1974) showed that the oxygen consumption of *Dugesia tigrina* and *Polycelis tenuis* was reduced to about 1/4 of its value at 100% air saturation.

Temperature was considered to be the most important controlling factor which operates on the respiration rate of poikilotherm animals (Newell, 1970). Little work has, however, been carried out on the respiratory physiology of freshwater triclads at different temperatures. Behre, (1918) found an increase in oxygen consumption in *D. dorotocephala* after transferring it from a low to a higher temperature, although this effect was later modified by acclimation.

Since there is an extensive range of literature available on the respiration of triclads, this chapter will not concentrate on the respiratory physiology of triclads *per se*, but rather on any intraspecific differences that may occur between the two experimental populations of *D. lacteum*. These differences might possibly explain the differential mortality demonstrated in the previous chapter.

5.2 Materials and Methods.

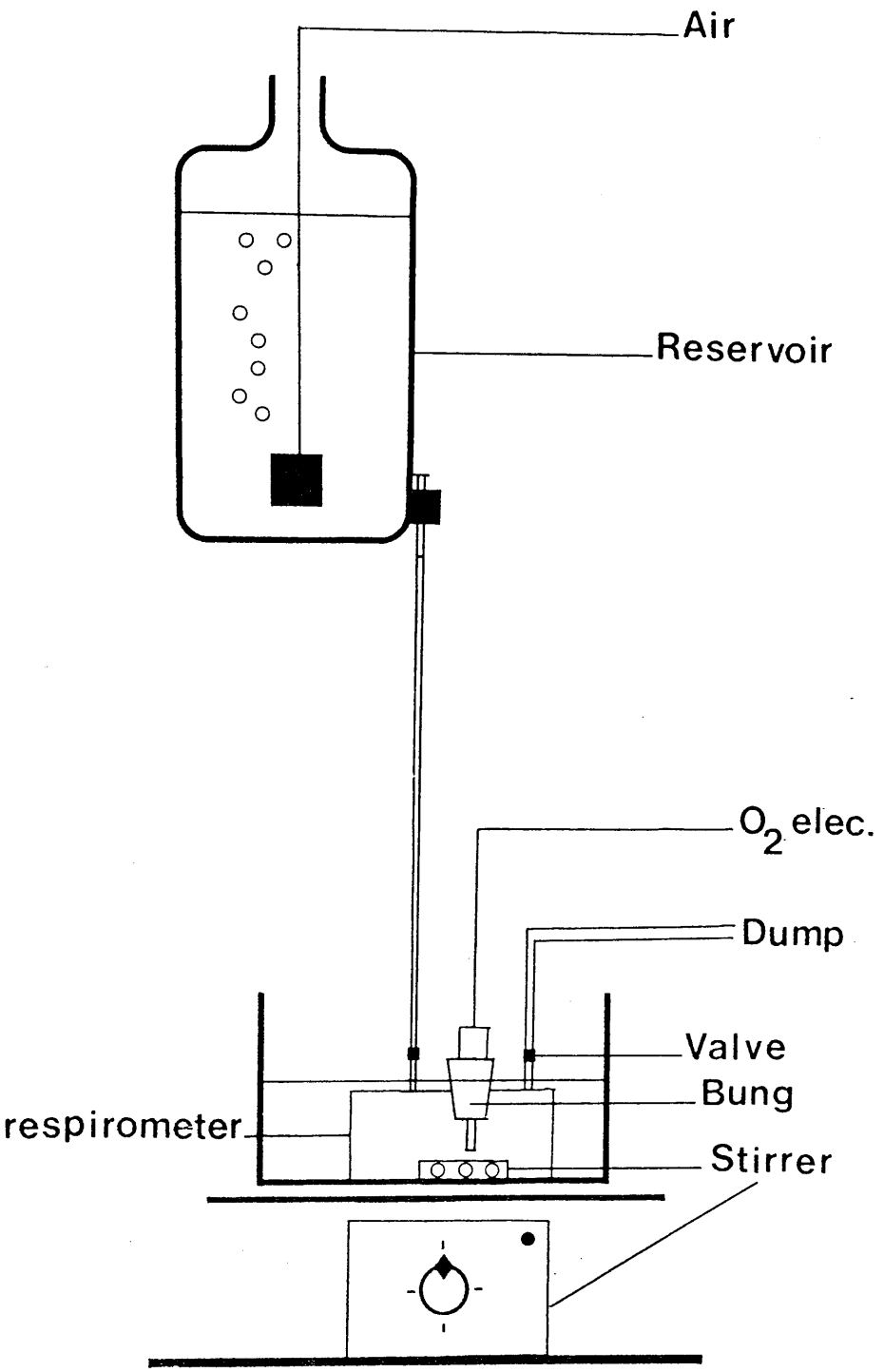
5.2.1 General

Specimens of *Dendrocoelum lacteum* were collected as required from the two study sites and maintained as described in Chapter 4. The triclads were fed normally until one week prior to experimentation. At this time the animals were starved and kept at their experimental temperature. This, it was hoped, would avoid any effect of Specific Dynamic Action or acclimation which might affect the measured rates of oxygen consumption. All experiments were carried out in constant temperature rooms with the apparatus left to acclimate for three days before the experiment commenced. At the end of all experiments the dry weight was recorded after drying the animals in an oven at 55^{o}C for 48 hours. Oxygen consumption rates were then calculated and plotted against dry body weight on double logarithmic paper and regression analysis and analysis of covariance carried out for intraspecific comparisons of the data.

5.2.2 Closed Respirometer

Two methods of measuring oxygen consumption were employed, a closed respirometer constructed from clear perspex and a flow through respirometer. The closed respirometer (Fig 5.1) had a volume of 56ml and was fitted with mushroom valves at the inlet and outlet ports which closed automatically on the cessation of irrigation. Copper-free water, at the required temperature, was passed by a gravity feed from a 10 litre reservoir to the respirometer. The oxygen tension of the reservoir was maintained at 100% air saturation by continuous aeration of the water and the respirometer contained a small magnetic stirrer to prevent any stratification or local depletion of oxygen during the

Fig 5.1 Closed respirometer used for measuring oxygen debts and Pc points.


Air
Reservoir
O_2 elec.
Dump
Valve
Bung
respirometer
Stirrer

experiment. The stirrer was enclosed in a small circular perspex box to protect the animal from injury and was kept at a constant speed during experimentation. There was some heat production from the stirrer mechanism which might have affected the PO_2 but this was prevented by placing the respirometer under water at the experimental temperature and insulating the respirometer with polystyrene. A check on the pH of the water was made at the end of experimentation under anoxic conditions and was found to show little variation from the control maintained at 100% air saturation.

During experimentation the apparatus was blacked out to prevent any disturbance to the animals during the experiment. The animals were left in the apparatus with a constantly flowing supply of normoxic water for 24 hours before the experiment commenced. At the end of this time, the valves at the reservoir were closed and the mushroom valves in the ports closed. During the next few days (48-72 hours), the animal was allowed to deplete the available oxygen, with the rate of depletion being monitored by an oxygen electrode (Radiometer E5046; Radiometer, Copenhagen) coupled to an oxygen meter (Strathkelvin 781B; Strathkelvin Instruments, Glasgow) and a chart recorder (Tekman TE200/1; Tekman Ltd., England). In a further set of experiments standard oxygen consumption rates were measured and animals were then placed into chambers and maintained under anoxic conditions for twelve hours. On returning the animal to normoxia the oxygen consumption rate was again measured and by subtracting the second reading from the first an estimate of the accumulated oxygen debt was assessed. A distinct disadvantage of this system was that recordings could only be made on single animals and hence data aquisition was slow. A second method was therefore used in which groups of animals were placed in individual flow-through respirometers.

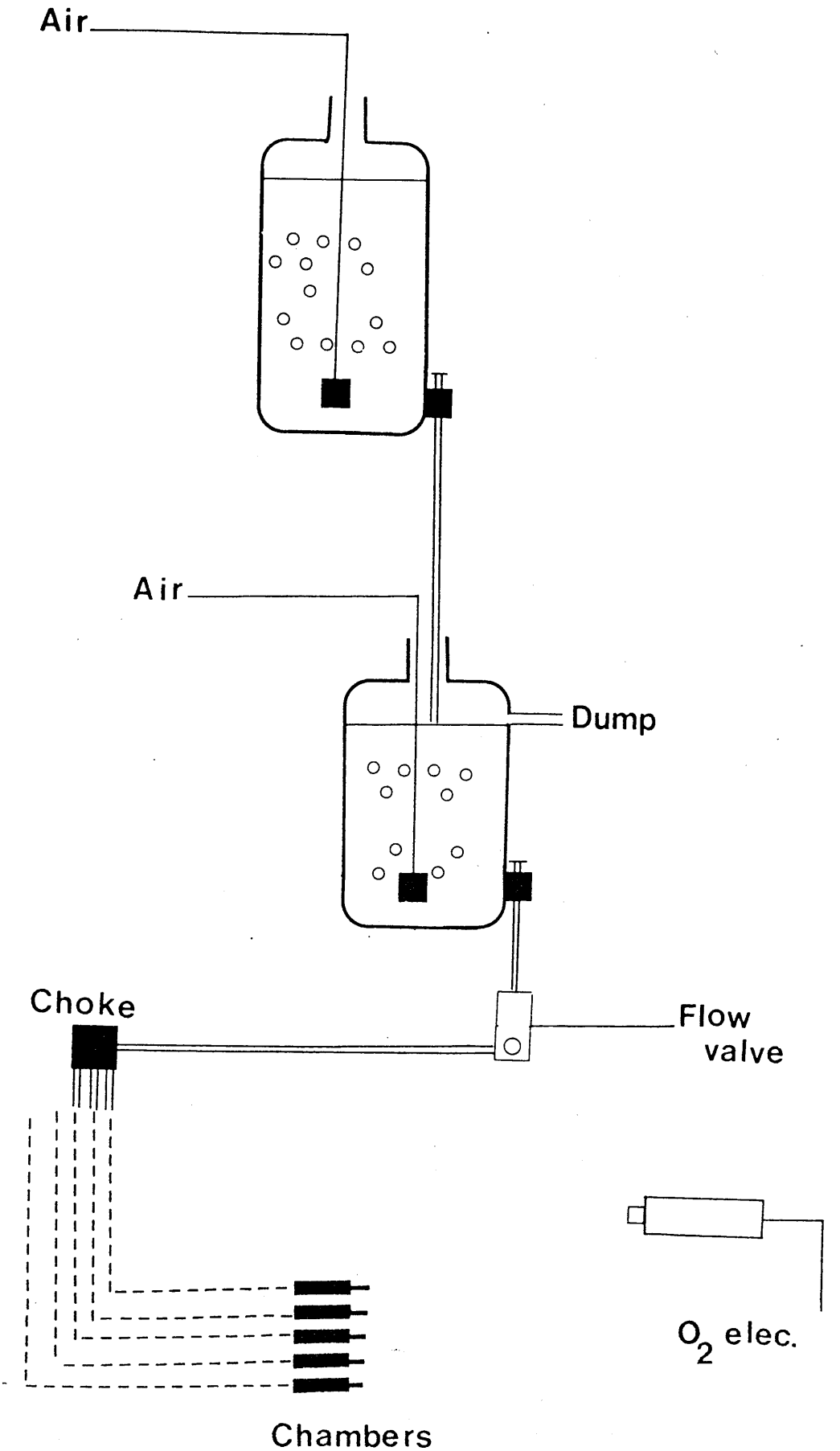
5.2.3 Open Respirometer

This respirometer was, in the first instance, similar in construction to the apparatus used for screening triclads (Chapter 4). After initial trials, however, large discrepancies were established between replicates. This was traced to a temperature effect, the water being heated as it passed through the peristaltic pump. Modifications were therefore carried out which enabled the apparatus to operate by a gravity feed and this was found to give far more stable and repeatable PO_2 readings.

The apparatus (Fig 5.2) consisted of two 10 litre reservoirs, the first supplying the second to maintain a constant head of water. The flow-rate from the first reservoir was regulated until it was supplying slightly more water than the second reservoir was losing. In the side of the second reservoir was a dump which allowed any excess water to drain away. Both reservoirs were constantly aerated and maintained at 100% air saturation at the required temperature. The water was then passed from the second reservoir through tygon tubing to a flow valve which then led to the experimental chambers.

Triclads which had no visible signs of injury were selected and placed into the chambers under water to prevent any air bubbles forming. The flow valve was then opened and water passed down the tygon tubing and out via a rubber bung (size 7) with half a hypodermic needle (19 guage) inserted into it. The bungs were then inserted into the backs of the syringes and placed on a platform which inclined slightly upwards. Syringe needles, cut off at about halfway with pieces of tygon tubing attached, were then placed onto the front of the syringes. Water was seen to drip from the ends of the tygon tubing attached to the needles and the flow rate was then adjusted to between 3 and 6ml per

Fig 5.2 An open respirometer used for monitoring population differences in oxygen consumption at various temperatures.


Air
Air
Dump
Choke
Flow
valve
O_2 elec.
Chambers

hour. The triclads were then left in the respirometer to acclimatise to the experimental conditions for 12 hours.

The flow rate through the respirometers had to be slow enough to set up an oxygen gradient in the test chambers but not too slow to result in hypoxic or anoxic conditions. Differences in PO_2 between experimental and control chambers of about 20 Torr were normal. The flow rate was measured by placing 2 ml pipettes sealed at the tips under each experimental chamber. The water dripping from each chamber was collected in the pipettes and the amount of water collected in one hour established. The chambers consisted of ten glass syringes (2ml), nine experimental (each containing one animal) and one control chamber (without an animal) and were set side by side and inclined slightly upwards. Samples were extracted from an experimental chamber using a 50ul microsyringe and these were then introduced into a thermostated cell (at the required temperature) containing a Radiometer electrode. This was then repeated for the control chamber. After each experiment the chambers were thoroughly cleaned with 100% alcohol and rinsed with distilled water.

The sampling protocol was :-

$$E_1,\ C,\ E_1,\ C,\ E_1,\ C,\ E_2,\ C,\ \text{etc.}$$

where E_1 is experimental chamber 1, E_2 experimental chamber 2 and C is the control. Oxygen consumption rates were then calculated from the following formula:-

$$\dot{V}o_2 = \frac{(PIo_2 - PEo_2) \times Bo_2 \times F}{Wt} \times 22.414$$

$\dot{V}O_2$ - Oxygen consumption rate - μl O_2/h/mg dry or fresh wt.

BO_2 - Solubility coefficient - μmol/l/Torr

PIO_2 - Input (Torr) F - Flow rate - l/h

PEO_2 - Exit (Torr) Wt - Fresh or Dry weight - mg

Experiments were carried out, in the flow-through system, to assess what effect differences in animal size might have on the oxygen consumption of *D. lacteum*. These experiments were carried out at two experimental temperatures (10 and 20°C) to examine the effects of increased metabolic rate on the oxygen consumption of triclads. Measurements of oxygen consumption were also made on triclads with a standard metabolic rate i.e. maintained at 100% air saturation at 10 and 20°C, in total darkness. This would allow an accurate examination of any intraspecific differences that might exist between populations of *D. lacteum* from the River Kelvin and Allander Water. Pc points were estimated from data collected using the closed system and the existence and extent of an oxygen debt established. It is well known that in many species of animals the rate of oxygen consumption may show considerable variation during a 24 hour period (Shirley and Finney, 1977, McCorkle *et al.*, 1979). An experiment was therefore included that investigated oxygen consumption during periods of light and darkness.

5.3 Results

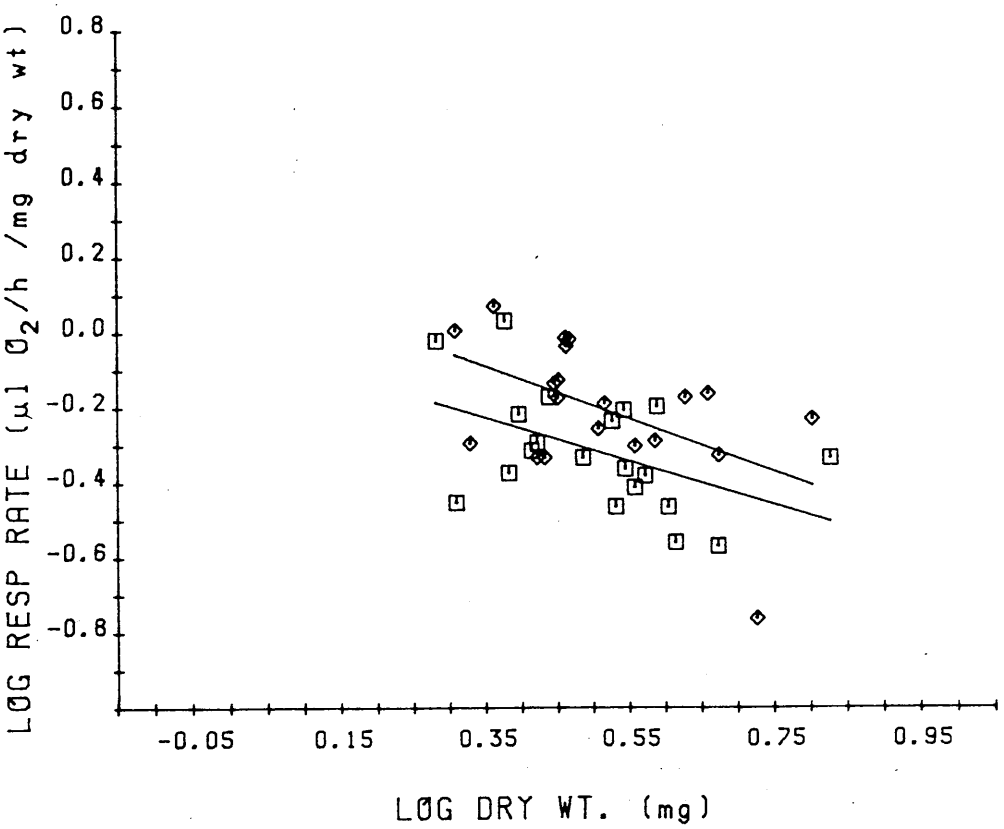
5.3.1 Influence of Size and Temperature

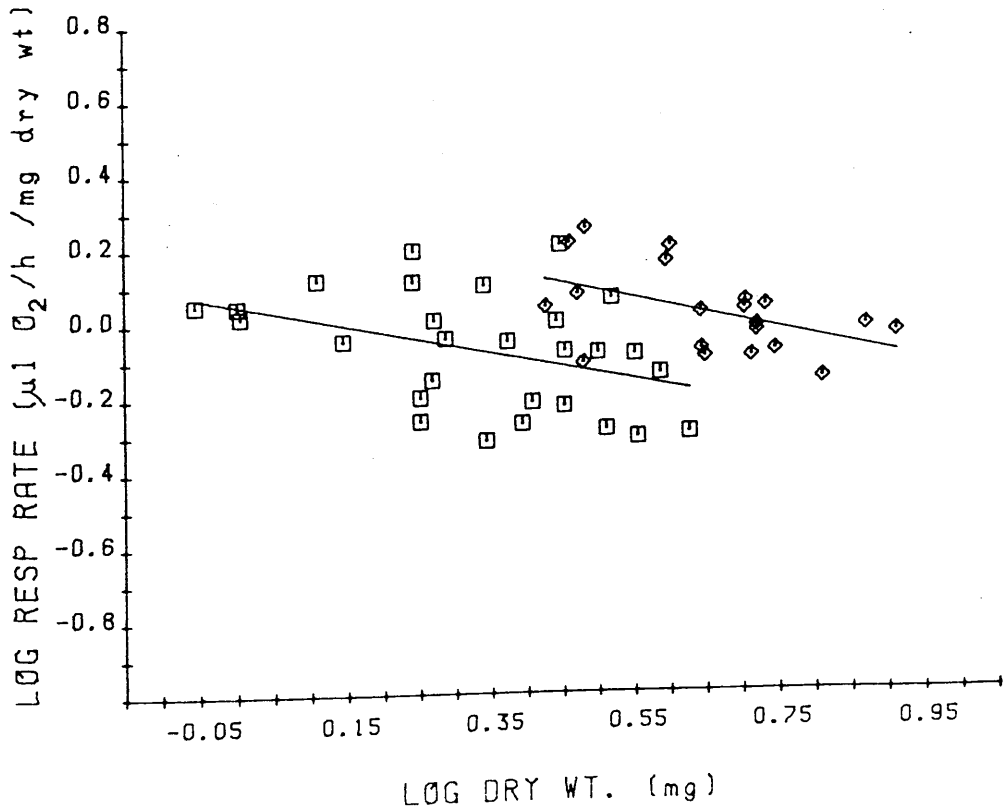
Oxygen consumption rates for *D. lacteum* were measured using the open system at two different temperatures (10 and 20°C) and the results were plotted on double-log graph paper against dry weight. The results shown in Fig 5.3 demonstrate a decrease in oxygen consumption per unit weight with an increase in size. Straight lines were plotted through the data, fitted by regression analysis (least squares) and these data were then examined using covariance analysis to investigate any intraspecific differences in oxygen consumption that may have been present. There was considerable scatter in the oxygen consumption of *D. lacteum* from both sites at 10°C, but there were significant correlations (r-values ($P<0.05$)) in both populations at 10 and 20°C and the resulting regression equations were calculated and are expressed below:-

	Allander Water	River Kelvin
10°C	$\log Y = -0.579 \times \log X - 0.019$	$\log Y = -0.699 \times \log X + 0.162$
20°C	$\log Y = -0.369 \times \log X + 0.054$	$\log Y = -0.418 \times \log X + 0.301$

An analysis of covariance was carried out on the regression equations described above to determine if any significant differences existed between any of the groups. There was no significant difference in the slope of both populations ($P>0.05$) at the two experimental temperatures. Despite the scatter, there was a significant difference ($P<0.05$) in the elevation of the regression equations between triclads from the River Kelvin and Allander Water at both 10 and 20°C. A significant difference was also noted between triclads from the Allander Water at 10 and 20°C and between triclads from the River

Fig 5.3 Oxygen consumption rates for *D. lacteum* from the River Kelvin (Diamonds) and the Allander Water measured at 10°C (Top) and 20°C (Bottom).


LOG RESP RATE (µl O_2/h /mg dry wt)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-0.05
0.15
0.35
0.55
0.75
0.95
LOG DRY WT. (mg)


LOG RESP RATE (µl O_2/h /mg dry wt)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-0.05
0.15
0.35
0.55
0.75
0.95
LOG DRY WT. (mg)

Kelvin at 10 and 20°C. This difference was again restricted to the elevation of the regression equations with no significant difference being observed in the slope ($P>0.05$).

The rate of any biochemical reaction is dependent upon the ambient temperature and a method commonly used to quantify the degree of dependence is the estimation of Q_{10} where,

$$Q_{10} = \left(\frac{R1}{R2}\right)^{10/(T1-T2)}$$

The approximate Q_{10} values were calculated from the regression equations for the respiration rates of triclads at 10 and 20°C from both the Allander Water and River Kelvin and were found to be 1.54 and 1.97 respectively.

5.3.2 Influence of Activity

It is generally accepted that triclads are negatively phototaxic and are far more active during the night than during the day (Kenk, 1974). An experiment was therefore constructed to examine the influence of activity on respiration by comparing oxygen consumption rates of triclads (n=10) under periods (12 hours) of light and dark conditions. The results of this experiment are shown in Fig 5.4 and demonstrate that there was a significant increase ($P<0.05$) in the rates of oxygen consumption during the hours of darkness which was almost double the values obtained during daylight hours.

5.3.3 Influence of declining PO_2

The oxygen consumption rates of five individuals of *D. lacteum* from both the Allander Water and River Kelvin were measured over a wide range of oxygen tensions at 10°C. The results of a typical trace, for triclads from each site, is shown in Fig 5.5. These results

Fig 5.4 Oxygen consumption rates of *D. lacteum* during periods of light and darkness.

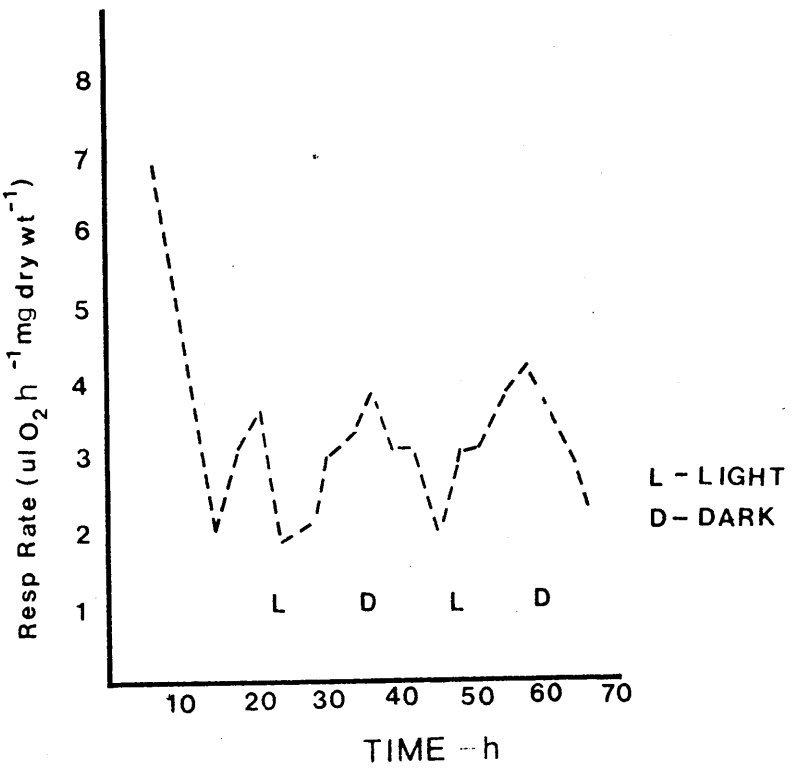
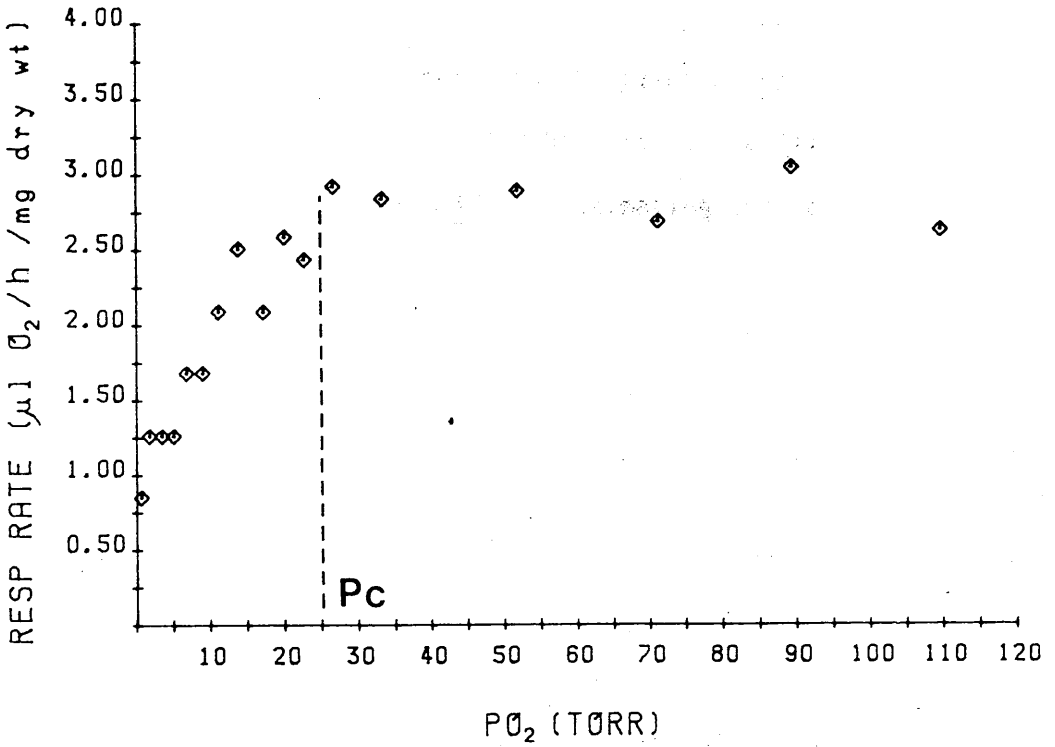
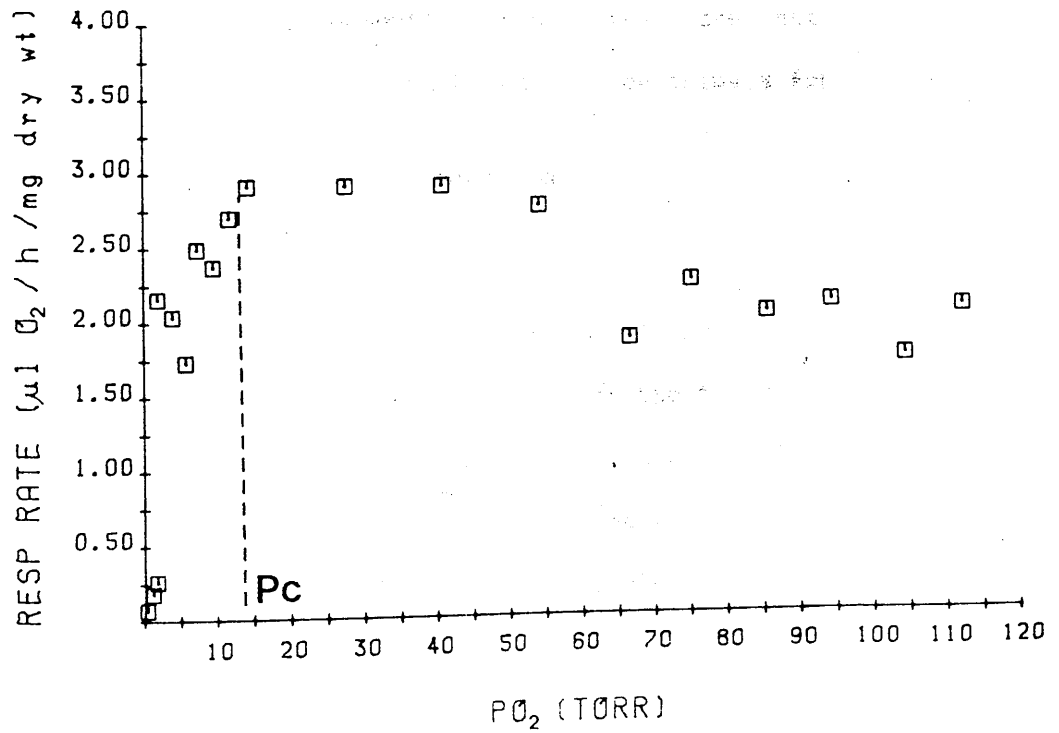

Resp Rate (ul O_2 h^{-1} $mg\ dry\ wt^{-1}$)
8
7
6
5
4
3
2
1
L
D
L
D
10
20
30
40
50
60
70
TIME – h
L – LIGHT
D – DARK

Fig 5.5 The effect of hypoxia on the oxygen consumption rates of *D. lacteum* from the River Kelvin (Top) and Allander Water (Bottom) at 10°C.


RESP RATE (µl O_2/h /mg dry wt)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
Pc
10
20
30
40
50
60
70
80
90
100
110
120
PO_2 (TORR)


RESP RATE (µl O_2 / h /mg dry wt)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
Pc
10
20
30
40
50
60
70
80
90
100
110
120
PO_2 (TORR)

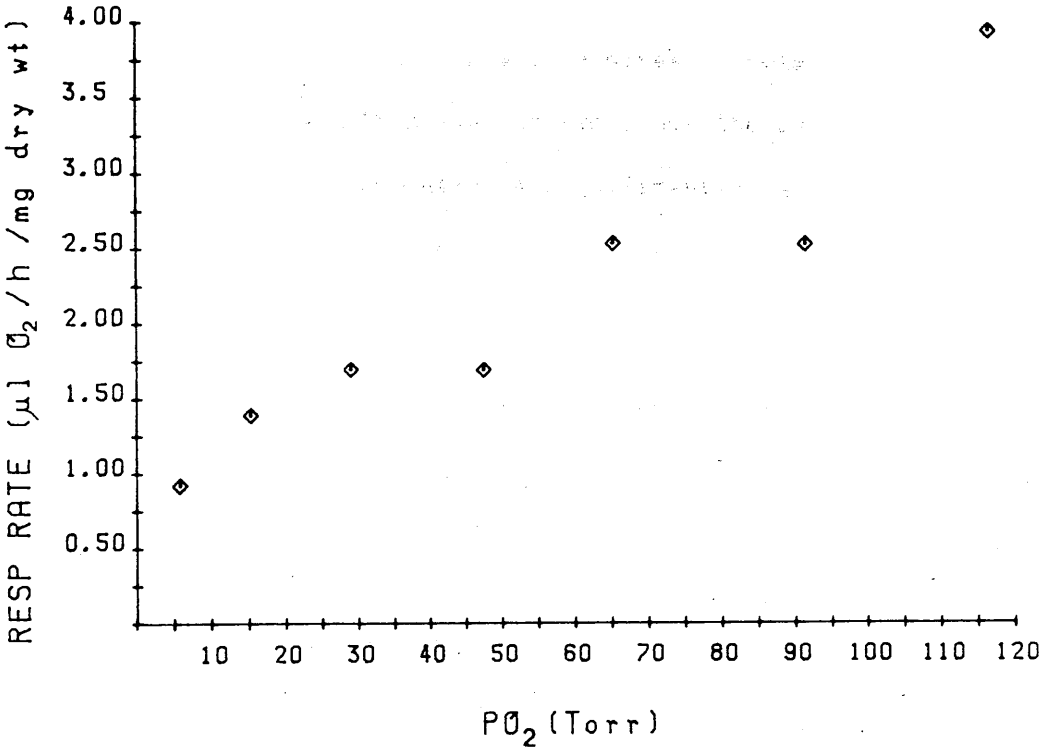
clearly demonstrate that *D. lacteum* from the Allander water and River Kelvin were exhibiting "respiratory independence" i.e. triclads were able to maintain their rate of oxygen consumption independent of the ambient oxygen tension over a wide range of PO_2. Various authors have used different techniques to assess the Pc point of an animal. Mangum and Van Winkle (1973), Tang (1933) and Bayne (1973) proposed alternative mathematical methods of estimating the critical oxygen tension (Pc) but neither of these methods have proved to be very successful. Pc points were therefore simply estimated by eye and were assessed to be between 10-20 Torr for the Allander Water and between 20-30 Torr for the River Kelvin. Average Pc values were estimated for both sites and a T-test carried out. The Pc points established for the Allander Water were significantly lower than those obtained for the River Kelvin ($P<0.05$).

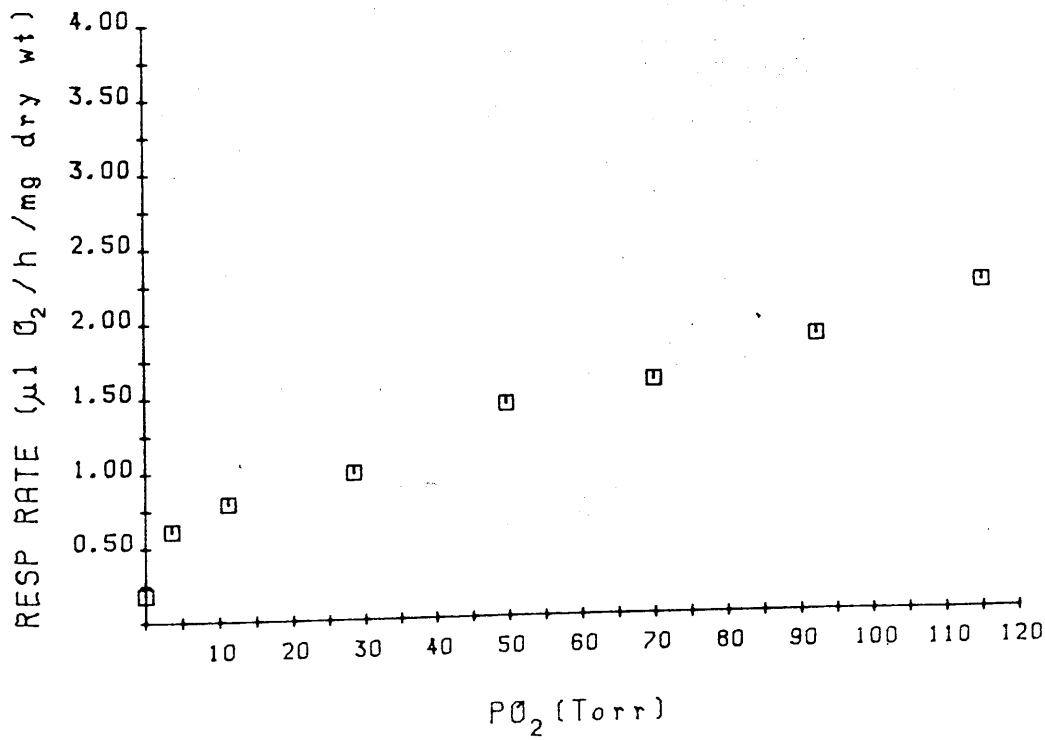
A similar experiment was also carried out at $20^{o}C$ on *D. lacteum* and the results of the oxygen consumption of a typical animals are shown in Fig 5.6. These results demonstrate that the rate of oxygen consumption in *D.lacteum* was now dependent on the PO_2 of the water (i.e it was acting as a "conformer"). It was, therefore, not possible under these circumstances to estimate Pc points for animals from either site.

5.3.4 Influence of Anoxia

The effect of exposure to hypoxia and anoxia with any subsequent accumulation of an "oxygen debt" would perhaps indicate differences in metabolic activity between the two populations of *D. lacteum*. Animals were therefore placed in closed respirometers, left to settle for twelve hours, and then an estimate of their rates of oxygen consumption established. These measurements were later used as a standard to be compared with future oxygen consumption rates measured

Fig 5.6 Effects of hypoxia on the oxygen consumption rates of *D. lacteum* from the River Kelvin (Top) and Allander Water (Bottom) at 20°C.


RESP RATE (µl O_2 /h /mg dry wt)
4.00
3.5
3.00
2.50
2.00
1.50
1.00
0.50
10 20 30 40 50 60 70 80 90 100 110 120
PO_2 (Torr)


RESP RATE (µl O_2 /h /mg dry wt)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
10 20 30 40 50 60 70 80 90 100 110 120
PO_2 (Torr)

after 12 hours exposure to anoxia. The results of this experiment are shown in Fig 5.7 and demonstrate that at 10 and 20°C an oxygen debt was accumulated in both populations. At 10°C there was no significant difference in the size of the debt accumulated in animals from either of the two populations; animals from both sites increased their oxygen consumption by about 75%. This was, however, not the case at 20°C with the Allander triclads accumulating a significantly greater ($P<0.05$) oxygen debt (175% increase) than triclads from the River Kelvin (140% increase).

The duration of the period of recovery from exposure to anoxia was monitored in a separate experiment (Fig 5.8) and it was demonstrated that in both populations repayment was virtually complete within four hours of the animals returning to normoxic conditions.

Fig 5.7 The % increase in the rate of oxygen consumption in *D. lacteum* exposed to anoxia for 12 hours at 10 and 20°C. Resp. rates are expressed as a % increase above the normal resting rate of oxygen consumption in this species. Values are means ± standard deviations.

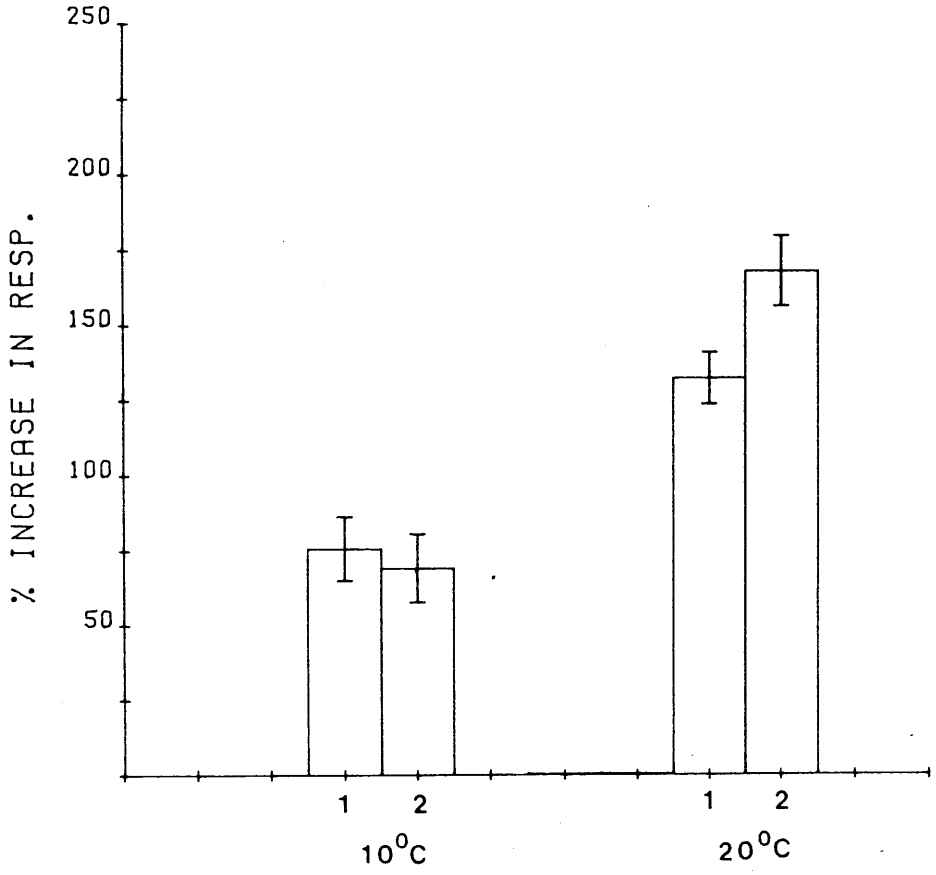
Site 1 - River Kelvin

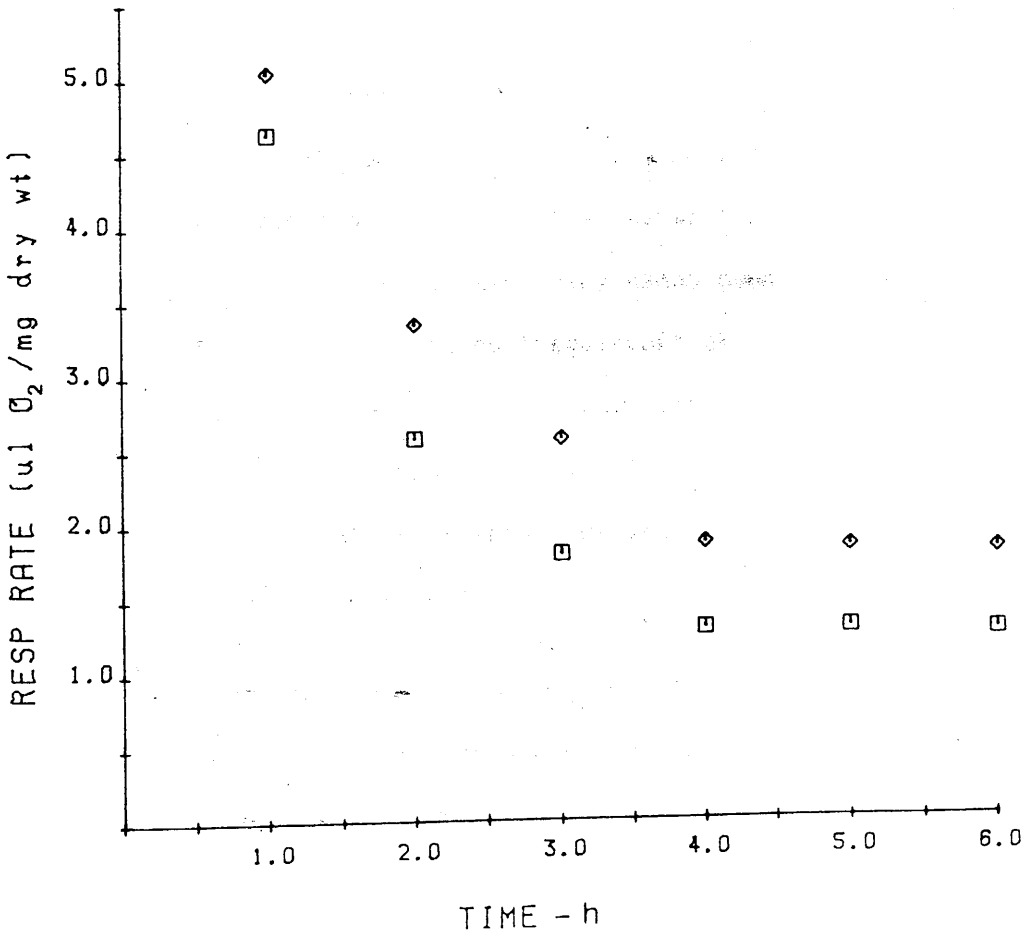
Site 2 - Allander Water

Fig 5.8 The rate of oxygen consumption in *D. lacteum* at 20°C during the period of recovery from exposure to anoxia for a period of twelve hours.

River Kelvin - Diamonds

Allander Water - Squares


% INCREASE IN RESP.
250
200
150
100
50
1
2
1
2
10°C
20°C


RESP RATE (ul O2/mg dry wt)
5.0
4.0
3.0
2.0
1.0
1.0
2.0
3.0
4.0
5.0
6.0
TIME - h

5.4 Discussion

Many authors have established correlations between the rate of oxygen consumption of aquatic animals and the their ecology (Whitney, 1942; Prosser, 1973). Two main conclusions have emerged from much of this work: that many inhabitants of fast-flowing streams have higher rates of oxygen consumption than their more lentic counterparts and that inhabitants of oxygen deficient water are often able to maintain a steady rate of oxygen consumption in the face of falling oxygen tensions until a critical oxygen tension (Pc point) is reached, below which oxygen consumption falls rapidly (Mann, 1956).

It has been demonstrated that some animals e.g. nematodes (Rogers, 1962), polychaetes (Dales *et al.*, 1970), echinoderms (Johansen and Peterson, 1971), crustaceans (McMahon *et al.*, 1978; Bradford and Taylor, 1981) and some bivalves (Taylor and Brand, 1975),) exhibit a high degree of respiratory independence. Other species e.g. the crayfish *Pacifastacus leniusculus* and shore crab *Carcinus maenas* are dependent on the ambient PO_2 and are classed as "conformers" (Herreid, 1980). These definitions are not strict as metabolic regulation not only varies among species, but can also vary among members of the same species (Bayne, 1971a). Thus the terms "regulator" or "conformer" which can be affected by many other factors e.g. temperature, activity, salinity, size, and circulation (Herreid, 1980) must not be used to label different species, rather it should be used on individuals with the experimental conditions precisely defined.

The terms "regulator" or "conformer" as described above and used freely in present-day literature are misleading in that they place animals firmly into one or other of these categories with no intermediate forms. These terms are merely two ends of a spectrum of

possible responses for a particular species with most species lying somewhere between (Mangum and Van Winkle, 1973). Also the term "regulators" suggest that regulation is an active process brought about by a change in oxygen conductance i.e. changes in ventilation and or changes in circulation. This is not always the case, some animals are passive "regulators". These animals are usually small in size with a large surface area and can operate by diffusion allowing the animal to show respiratory independence down to very low PO_2 values (Rogers, 1962; Moss, 1970). This may be the situation which was occurring with *D. lacteum*, being flat and having a very large surface area, the rate of oxygen diffusion into the tissues would be ample to meet normal aerobic demands. Triclads could also, like some of their parasitic relatives, utilise anaerobic metabolic pathways when oxygen tensions fell to supplement some of their energy requirements. This may even occur under relatively high PO_2 conditions giving the appearance of being good "regulators" even at very low PO_2 values (Moss, 1970).

Active "regulators" do demonstrate a change in their oxygen conductance to offset a falling PO_2. An increase in ventilation rate is a common respiratory response to hypoxia in many aquatic animals e.g. in crustaceans (Wolvekamp and Waterman, 1960) and in many fishes (Hughes and Shelton, 1962). The level of oxygen utilisation will also exert a major influence as demonstrated in some bivalves. It will depend on the oxygen carrying capacity of the blood and water and the relation between their volume flow rates. This means that if the blood flow rate is inadequate to remove all the oxygen made available by the respiratory current, oxygen utilisation will be low. To maintain oxygen independence, therefore, a slight increase in ventilation can occur with a much greater increase in cardiac output (Taylor and Brand, 1975). For these reasons, in this thesis, the terms oxygen dependent or

independent will be used as these terms give a clearer indication of the respiratory capabilities of the animals involved.

Triclads from the Allander water were shown to have a lower Pc point (10-20 Torr) than triclads from the River Kelvin (20-30 Torr) and therefore demonstrated a greater degree of respiratory independence than triclads from the River Kelvin. The fact that triclads from the Allander Water were able to maintain respiratory independence over a wide range of PO_2 may reflect stresses present in their natural environment. The Pc point was seen to disappear or be displaced at higher temperatures; i.e. triclads were no longer regulating their oxygen consumption and had lost their respiratory independence. This situation is not unusual and many different invertebrate groups follow similar trends e.g. crustaceans (Wiens and Armitage, 1961), annelids (Cosgrove and Schwartz, 1965) and starfish, (Johansen and Peterson, 1971).

Differences in the oxygen consumption rates between animals from the River Kelvin and Allander Water were observed at both experimental temperatures (10 and 20^{o}C). The intraspecific differences observed in oxygen consumption, as described above, may be caused by the flow characteristics of the habitats each population occupies. Studies by Mann (1956) and Ambühl (1961) showed that the consumption of oxygen by some animals increased in relation to the flow characteristics of the rivers they inhabited. The River Kelvin, at the base of a large weir was much more turbulent in comparison to the Allander Water and animals living there would possibly have a higher oxygen consumption rate. This difference may disappear if the animals were acclimated to laboratory conditions for a few months prior to experimentation.

In this study it was also shown that smaller animals had a greater oxygen consumption rate per unit area than larger triclads. These results agreed with the work previously carried out by Allen (1918) and Hyman (1919b) whereas Whitney (1942) showed that some species e.g. *Polycelis nigra* did not conform to this relationship and oxygen consumption was actually lower per unit weight in smaller worms than in larger ones. The results of Whitney (1942) for *P. nigra* were very unusual, deviating from the normal response found in most other animals. It can only be assumed that experimental error was involved and that any subsequent study would resolve this problem.

The differences in the rates of oxygen consumption at 10 and 20°C were caused by an increase in metabolic rate associated with the rise in temperature. The calculated values of Q_{10} for both species show that the metabolic rate of Allander triclads was slightly more temperature independent than triclads from the River Kelvin. The rate of activity and consequently the rate of oxygen consumption nearly always increases logarithmically with temperature with a Q_{10} of approximately 2.0 (Prosser and Brown, 1961; Newell, 1970). A low Q_{10} value (1.2-1.4) was often found in environments showing considerable temperature fluctuations (Newell, 1973). This could not explain the results found in this study, since triclads from the River Kelvin were located in habitats that experienced considerable temperature variations had a higher Q_{10} than triclads from the more thermally stable Allander Water. As only two experimental temperatures were examined it is hard to attach any ecological significance to these limited results.

The influence of activity has been extensively studied by many authors and only a very brief review will be presented here. Many

animals demomstrate a marked increase in oxygen consumption at higher activity levels (Fry, 1957; Newell, 1973; Brown, 1979). This has therefore allowed many authors to separate an active from a standard or quiescent respiration rate (Newell and Northcroft, 1965; Newell and Pye, 1970; reviews Newell, 1969; 1970). Various activities can therefore be monitored e.g swimming in the amphipod *Gammarus oceanicus* and these activities can then be directly related to any increase in oxygen consumption. A lot of work has also been carried out in relation to circadian rhythms in oxygen consumption. Triclads have been shown to be more active at night (Kenk, 1974). This is not surprising as triclads are nocturnal predators and animals in this study have been shown to exhibit cyclical variations in the rate of oxygen consumption that corresponded to the 12 hour light and dark cycles imposed. McCorkle *et al.* (1979) and Shirley and Finney (1977) have demonstrated similar trends in clams and periwinkles which they also related to changes in levels of activity.

Theoretical calculations can be made to determine the amount of oxygen available to triclads solely by diffusion under saturated or hypoxic conditions (Chapman, 1980). These values can then be compared with actual oxygen consumption data at various oxygen tensions and temperatures and the comparison of the two would indicate whether any active "regulation" of the animal's oxygen consumption was occurring or whether the Pc point was simply set by the constraints resulting from limitations of operating by diffusion.

According to Chapman (1980), the amount of oxygen which can diffuse through two surfaces under normoxic conditions to a distance of half the thickness of the animal was 1.1×10^{-4} ml per min^{-1}. He assumed an oxygen consumption rate of 8×10^{-6} ml min^{-1} for *D. lacteum*, whereas in this study oxygen consumption rates were found to be 5.18 x

10^{-5} and 4.11×10^{-5} ml min^{-1} for average sized Kelvin and Allander triclads respectively. At these values, and using the theoretical values of Chapman (1980) triclads would have no problems at maintaining normal oxygen consumption rates down to about 38 Torr at $10^{o}C$. If, however, only 1 side of the animal was available for the uptake of oxygen as would perhaps seem likely with the bottom surface of the animal covered in mucus, then the Pc would be about 80 Torr. The value calculated for diffusion into the animal for two sides is fairly consistent with the Pc values found in triclads from the River Kelvin but is about three times greater than the Pc of Allander triclads. It should also be noted that observations were made in Chapter 3 of triclads attaching themselves to the wall of a container by an anterior "sucker" and detaching the rest of their body from the wall which then wafted into the body of the hypoxic water. This could be interpretated as triclads actively trying to increase their surface area for oxygen uptake by revealing both surfaces of their body.

In Chapman's (1980) calculations, muscle was used as a "standard" tissue in assessing diffusion rates through a triclad's integument and some of the observed differences shown in this study may be attributable to this fact. The intraspecific differences observed could possibly be linked to either an increase in conductance i.e. the Allander triclads were able to actively regulate to compensate for the falling PO_2 or to the utilisation of an anaerobic metabolism to supplement aerobic energy production. The evaluation of these two alternatives will be discussed in subsequent chapters and will not be discussed further here.

Triclads have been shown to demonstrate considerable tolerance to hypoxic and anoxic conditions. The ability to tolerate

hypoxia would involve the generation of anaerobic end products. These products can be excreted, oxidised or converted back into storage compounds such as glycogen at the cost of increased energy utilisation (i.e. an "oxygen debt") on return to normoxia. Triclads were shown to generate a large oxygen debt under anoxia which was paid back quickly on returning to normoxic conditions. There were intraspecific differences in the size of the debt generated with triclads from the Allander Water accumulating a larger debt but only at 20°C. This could have been caused by many factors e.g. Allander triclads being less active or by having a more efficient anaerobic metabolism. It has also been shown that the replenishment of oxygen stores within body fluids and respiratory pigments can cause increased oxygen debts (Herreid, 1980). The two sets of data i.e. 10 and 20°C samples were obtained at different times of year, early spring and summer for animals at 10 and 20°C respectively. During the summer animals in the Allander Water were seen to develop a purple/red colouration which could possibly represent a respiratory pigment and this could have caused an increase in the oxygen debt accumulated. This will be discussed in more detail in Chapter 6.

CHAPTER 6

Chapter 6

A Respiratory Pigment Present in *Dendrocoelum lacteum*

6.1 Introduction

At present there are four known respiratory pigments which can be found in the Invertebrata. These are, in order of the frequency in which they occur, haemoglobin, haemocyanin, haemerythrin, and chlorocruorin. When combined with oxygen each of these pigments appears respectively, a red, blue, pink, or green colour. These pigments can carry oxygen in the blood or coelomic fluid or can be stored in the tissues. The pigments resemble each other, in that they consist of a conjugated protein, linked to a prosthetic group containing a metal ion but this is where the general similarities end. Haemoglobin and chlorocruorin are the only pigments to have a similar prosthetic group called haem. This is a porphyrin group linked to an atom of ferrous iron. In haemocyanin, the prosthetic group is not a porphyrin and is attached to copper. A similar condition can also be found in the prosthetic group in haemerythrin which is again not a porphyrin but in this case does contain iron. These pigments are found in blood, haemolymph, or coelomic fluid. They can also be located in erythrocytes, muscles, or nerve ganglia. Another oxygen-combining pigment is found in the tissues, typically in the muscle cells of mammals and birds and is termed myoglobin. Myoglobin has a monomeric structure, unlike haemoglobin which is tetrameric and therefore has a much greater molecular weight.

Haemerythrin, is a straight-chain compound which contains two

atoms of iron. These are attached directly to the protein, rather than the situation found in haem. It is found inside cells called corpuscles, which generally circulate in the coelomic fluid. Haemerythrin is only found in the representatives of four different phyla, the Sipuncula, some brachiopods, Annelida, and in the Priapulida. The apparent scarcity of the pigment haemerythrin, and the phylogenetic links of triclads with haemoglobin found in other Turbellaria would suggest that the pigment occurring in *D. lacteum* was of a haemoglobin type.

Haemoglobin consists of two parts, a haem group coupled to a protein moiety called 'globin'. The haem is composed of a ring of carbon, hydrogen, and nitrogen atoms, called "porphyrin", and has an iron atom at its centre. Haem on its own when oxidised, can bind oxygen so tightly to it that it can no longer act as a reversible oxygen carrier. To prevent this from occurring the iron molecule is protected in a protein cavity. This prevents the oxygen from forming a tight bond with the iron molecule and the reaction can then be reversible. The haem part of haemoglobin is a constant entity in all haemoglobins from Protozoa to man, but the variation in the amino acid sequencing of the globin results in many functional differences being observed.

Haemoglobin is not restricted to blood and other body fluids. It is also commonly found in solution in some species and in erythrocytes in others. The location of haemoglobin in tissues has been extensively reviewed by Fox (1955). In certain nematodes e.g. *Ascaris* there is haemoglobin both within the body wall and in the pseudocoelom. In the haemoglobin-containing flatworms, however, the pigment is located primarily in the parenchymal tissue. (Manwell, 1960). There are therefore, two main types of haemoglobin molecule; intracellular (generally low molecular weight) i.e within erythrocytes or tissues

(myoglobin) or extracellular (high molecular weight), where the haemoglobin molecule is dissolved within the body fluids.

The occurrence of haemoglobin has been observed in several groups of invertebrates (Walshe, 1950; Fox, 1955; Prosser and Brown, 1961; and Wigglesworth, 1965). Haemoglobin has, however, only been documented in a relatively few number of genera of the Turbellaria (Mosely, 1874; Francotte, 1883; Fox, 1955; Crompton and Smith, 1963), and has never been observed in freshwater triclads. Mosely (1874) and Reynoldson (T.B. Reynoldson, pers. comm.) have observed, however, that in some situations *Dendrocoelum lacteum* exhibits a red colouration which could suggest the presence of haemoglobin-like compound.

In the present study a red colouration was occasionally observed in certain specimens of the triclad *Dendrocoelum lacteum*. This could possibly indicate the presence of a red coloured pigment, a haemoglobin or a haemerythrin-like compound. The presence of this pigment was only observed during the warmer summer months when oxygen levels were very low (Chap 3).

The presence of a respiratory pigment under such conditions is not uncommon. Scholander (1960) demonstrated that mud-dwelling worms, some insect larvae (chironomids), certain crustaceans that are commonly found in stagnant pools (*Daphnia*), intestinal nematodes and maggots (*Ascaris* and *Gastrophilus*) and also many mammals exposed to high altitudes can develop higher concentrations of oxygen carrying pigments. It has also been well established by a number of authors that under such conditions of oxygen stress, the synthesis of haemoglobin can occur (Fox, 1945, 1954; Weber, 1980; Weider and Lampart, 1985). This apparent synthesis of a "respiratory pigment" as described above may occur in *D. lacteum* as an aid in oxygen deficient environments.

An examination of the "pigment" was therefore undertaken to investigate whether the pigment had any potential respiratory properties. Experiments were therefore set up to establish the presence and location of iron and haematin within the bodies of the triclads. If this presence was established, any pigment present would be extracted for analysis. The optical properties could then be established which would then positively identify the pigment and electron micrographs coupled to X-ray analysis would be used to assess more precisely the distribution of the pigment.

6.2 Materials and Methods

6.2.1 Light Microscopy

To examine the distribution of the pigment, starved (7 days) specimens of *D. lacteum* were collected which exhibited a strong red colouration (Fig 6.1). These animals were immediately fixed in a standard Bouin's solution for twenty four hours and were then transferred through several changes of 70% alcohol to a 90% alcohol solution for a further twenty four hours. This was followed by two further changes in 90% alcohol, each for two hours. The triclads were then placed in a solution of 100% alcohol for two hours; this process was repeated and followed by two changes in Histoclear for 2 and 1 hour respectively. The animals were then placed into paraffin wax in an oven at 56^{o}C for two hours. The wax was changed, and after a further three hours the animal was set into a block for cutting. Sections were cut at 7μm transversely through the posterior end of the triclad just behind the pharynx.

Three techniques were used to establish the presence of iron, Heidenhains Iron Haematoxylin (Drury and Wallington, 1967), Mayer's Haemalum and Eosin (Drury and Wallington, 1967), both of which establishes the presence of ferric iron by staining iron a blue/black colour and a sodium nitroprusside-benzidine technique (Pickford, 1934) which reacts with haematin compounds staining them black.

6.2.2 Electron Microscopy

All the solutions and triclads used in this procedure were first cooled to 4^{o}C before fixation. The triclads were placed in 3% gluteraldehyde in 0.2m cacodylate buffer at 4^{o}C for three hours. The organisms were then washed in a fresh 0.2M cacodylate rinse overnight.

Fig 6.1. Specimens of *D. lacteum* taken from the Allander Water during the summer (Top) and during the winter (Bottom).

This was followed by two further rinses of fresh 0.2M cacodylate rinse solution. The animals were then placed in a 50:50 rinse solution of 4% osmium tetroxide and distilled water, i.e. a 2% OsO_4 solution, for three hours. This solution was topped up with distilled water and the animals placed into two changes of fresh distilled water (10 mins each). The triclads were then stained with 0.5% aqueous uranyl acetate. Dehydration was achieved through an alcohol series i.e. 30%, 50%, 70%, 90%, absolute, with 10 minute changes and three changes in absolute alcohol. The triclads were then routinely embedded in Araldite. Sections were cut with a L. K. B. Ultratombe (L.K.B. Ultratombe Products, Sweden) at 500 Angstroms and these sections were mounted on a Formvar coated copper grid. The grids were double stained with a 2% methanolic uranyl acetate and lead citrate solution. They were then examined using a A. E. I. 801 Transmission Electron Microscope operating at 60Kv.

A similar procedure was followed for specimens being used in the X-ray analytical experiments, except that no heavy metal staining was employed. These sections were mounted in copper coated slot grids and examined in a JEOL 100C transmission electron microscope with a Link System 290 X-ray analyser fitted.

6.2.3 Spectrophotometric Analysis

Specimens of *D. lacteum* were collected which exhibited a strong red colouration. To examine if this pigment would dissolve into solution an individual worm was placed in a 1ml Eppendorf tube containing 500µl of distilled water and was ground up using the blunted end of a glass rod. The resulting solution was then centrifuged in a Damar/I.E.C. Centra-4X centrifuge (Damar/I.E.C. U.K. Ltd, England) at 10,000 r.p.m. This resulted in a clear coloured supernatant with a red

coloured pellet sitting at the bottom of the Eppendorf. The supernatant showed no spectral peaks on a Pye-Unicam SP800 spectrophotometer (Pye-Unicam Ltd., England) and was it therefore assumed that the pigment present in the pellet was either water insoluble or that mucus produced within the triclad was preventing the pigment going into solution.

Various extraction procedures were therefore examined i.e. using various detergents Digitonin, Sodium cholate, Sodium dodecyl sulphate (SDS), and SDS in sodium hydroxide. Other methods were also considered e.g using an enzyme (hyaluronidase) to release the pigment into solution from any mucus present. Alcohols and chloroform were also tried but only two extraction procedures resulted in any of the pigment going into solution. These involved a similar process as mentioned above, but instead of using 500 µl of distilled water as a medium, 500µl of 2% SDS was used. The triclad was then ground up and centrifuged as described previously and the spectral characteristics of the pigment examined. This method did not extract all the pigment into solution and so a mixture of 2% SDS in 0.1M NaOH was tried. This was very successful and immediately brought all the pigment into solution for spectral analysis.

6.2.4 Presence of a pigment

The colour change observed in *D. lacteum*, i.e. the generation of a red colouration in contrast to its normal white colouration was most evident in those individuals from the oxygen deficient Allander Water site. In order to quantify this difference, two locations on each river were selected and 100 worms from each were examined for the obvious presence of a red colouration. This experiment was conducted during summer (July) when triclads would be expected to have most pigment.

6.3 Results

6.3.1 Light Microscopy

Ferric iron was found to be distributed throughout the triclad, with the greatest concentrations appearing around the gut and pharynx (Fig 6.2). Haematin was, however, not as prevalent as ferric iron, but again the greatest accumulations were found around the pharynx and gut. This would suggest that some ferric iron occurs in compounds other than haematin.

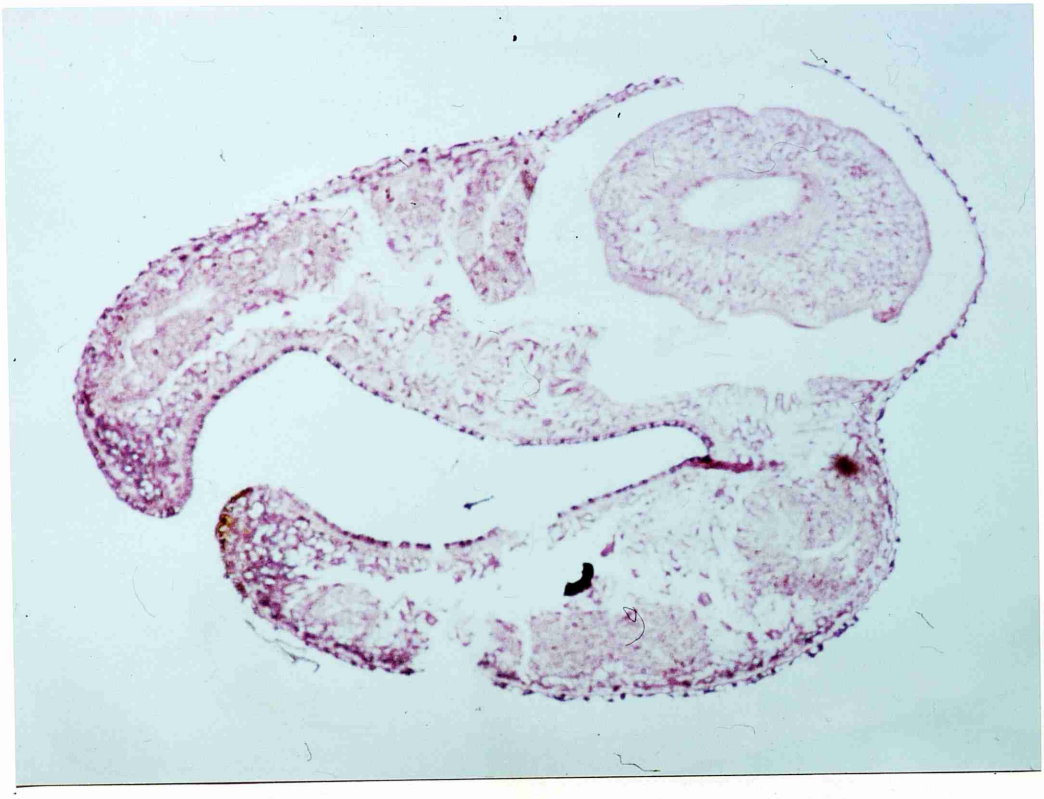
6.3.2 Electron Microscopy

Dark granular structures were observed in areas surrounding the gut (Fig 6.3) which may be associated with the haemoglobin molecule. It was worth noting that on some occasions large numbers of mitochondria were observed in the same areas as these granules. To further investigate the composition of these granules, an X-ray probe from a JEOL 100C transmission electron microscope with a Link System 290 X-Ray microanalyser fitted, was positioned onto one of these granules and an elemental analysis carried out. The results showed that a considerable amount of iron (double peaks E_1 and E_2) was present together with some phosphorus-containing compounds. (Fig 6.4). The copper and titanium peaks were artefacts coming from the instrument itself and the grid on which the animal was mounted.

6.3.3 Spectrophotometric analysis

A spectrophotometric analysis was carried out on the pigment extracted from *D. lacteum*. The absorption spectrum (Fig 6.5) showed three distinct peaks at 500, 375, and 322nm. This absorption spectrum differed markedly from that of haemoglobin which shows a typical double

Fig 6.2. Sections of *D. lacteum* showing the pharynx and gut (Top right) at low x16 magnification (Top) and at a higher magnification (x160) seen below. The top plate shows the general distribution of the pigment arround the gut and the bottom plate demonstrating the exact location and approximate density of the concentration of ferric iron. The stain used was Haemalum and Eosin and stains iron a blue/black colour.



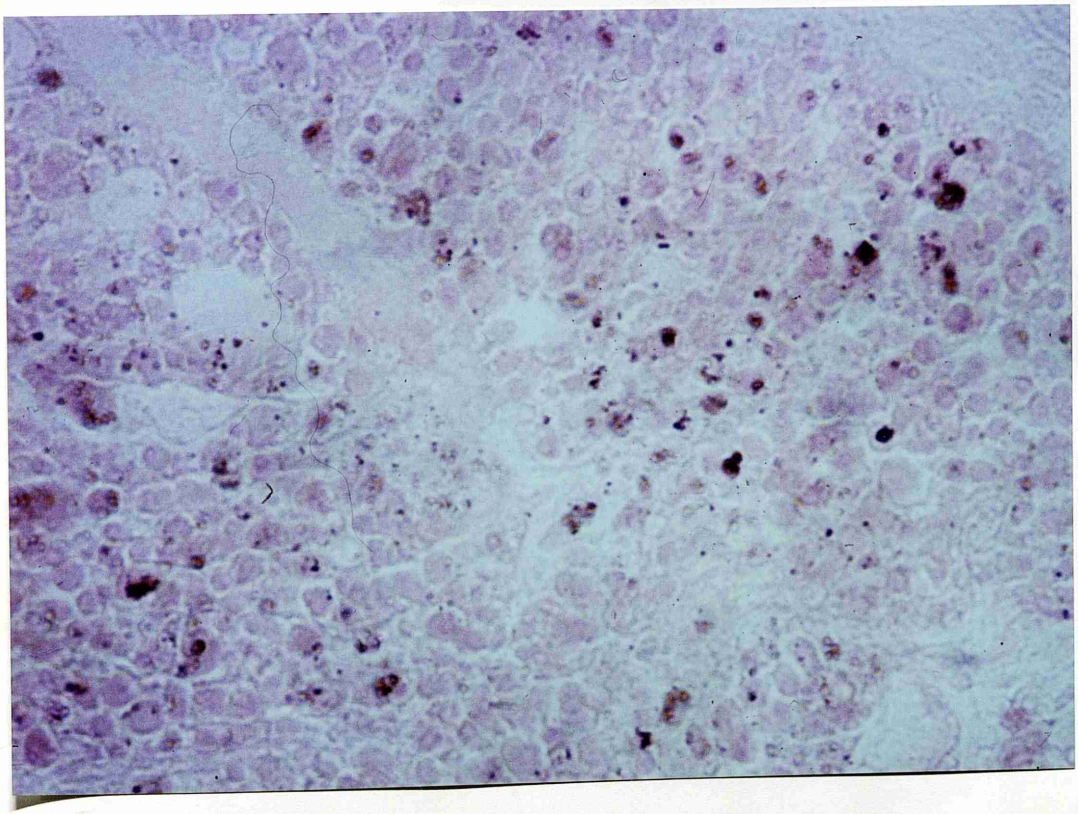
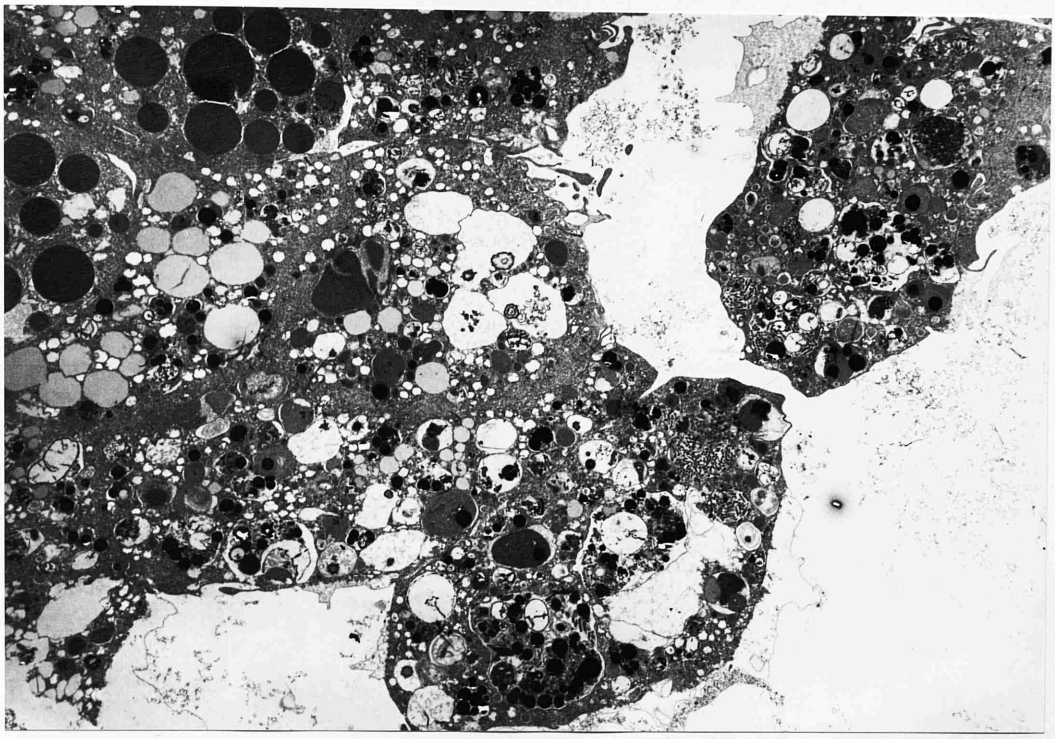


Fig 6.3 This figure shows dense granular material at low (x 1700) and high (x 40000) magnifications. They show a section from the edge of the gut inwards with the small dense granular structures present in the top photograph magnified in the bottom one.



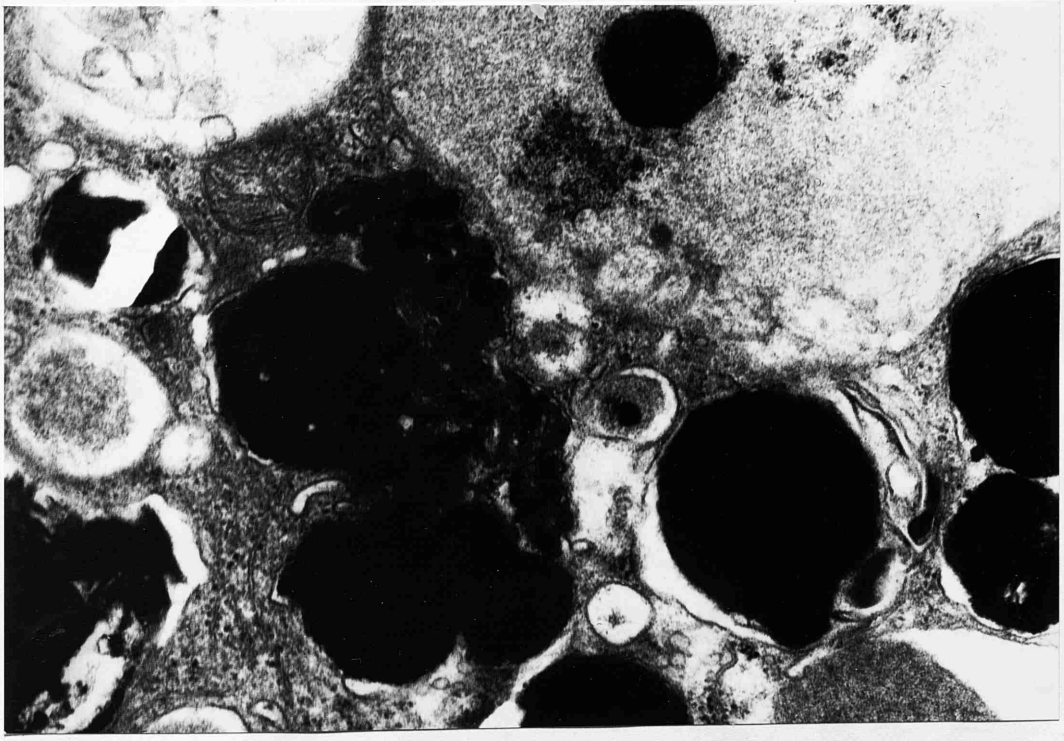


Fig 6.4 The results of an X-ray analysis of a section of gut from a specimen of *D. lacteum* from the Allander Water. The peaks correspond to,

A - Aluminium B - Silica C - Phosphorus D_1, D_2 - Titanium E_1, E_2 - Iron F_1, F_2 - Copper

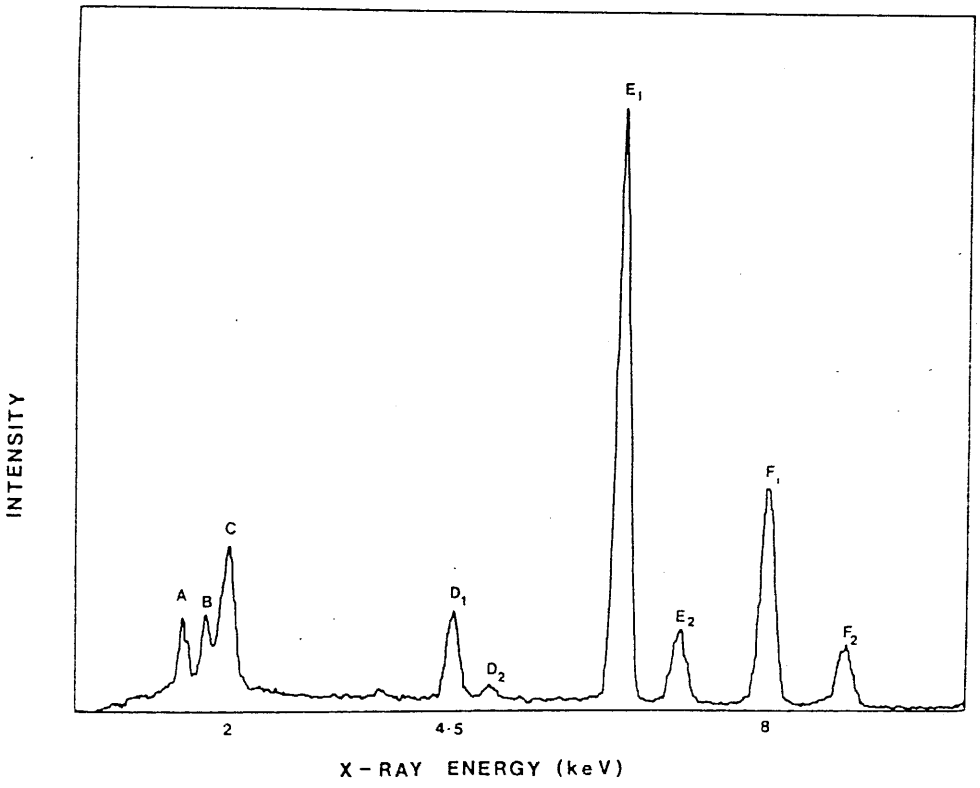
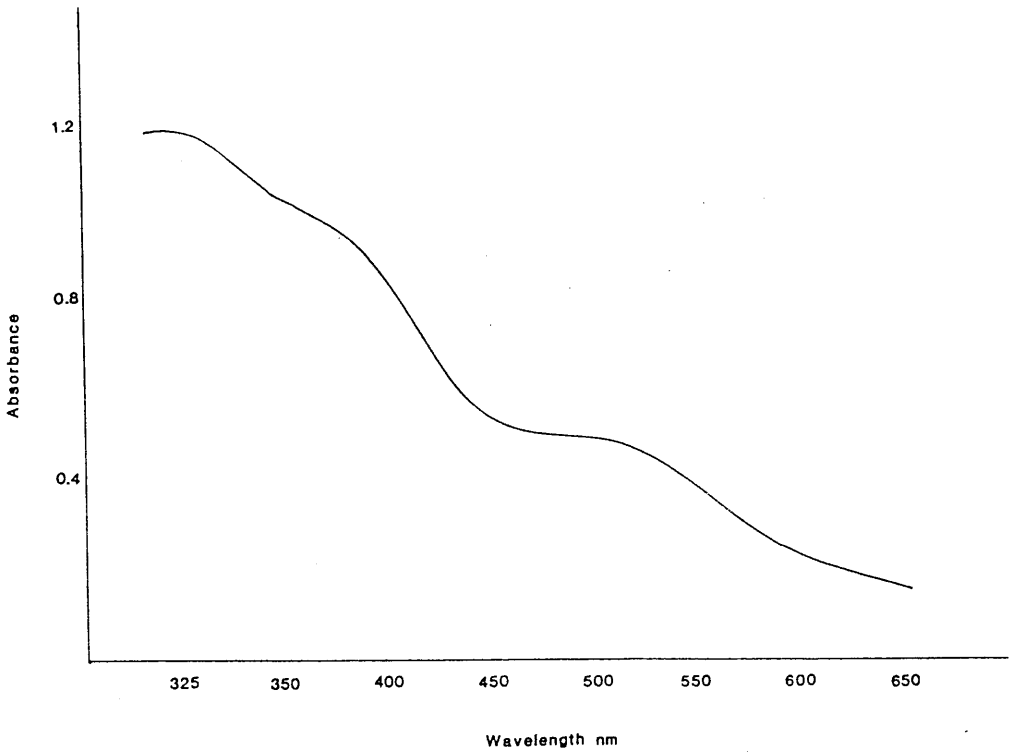
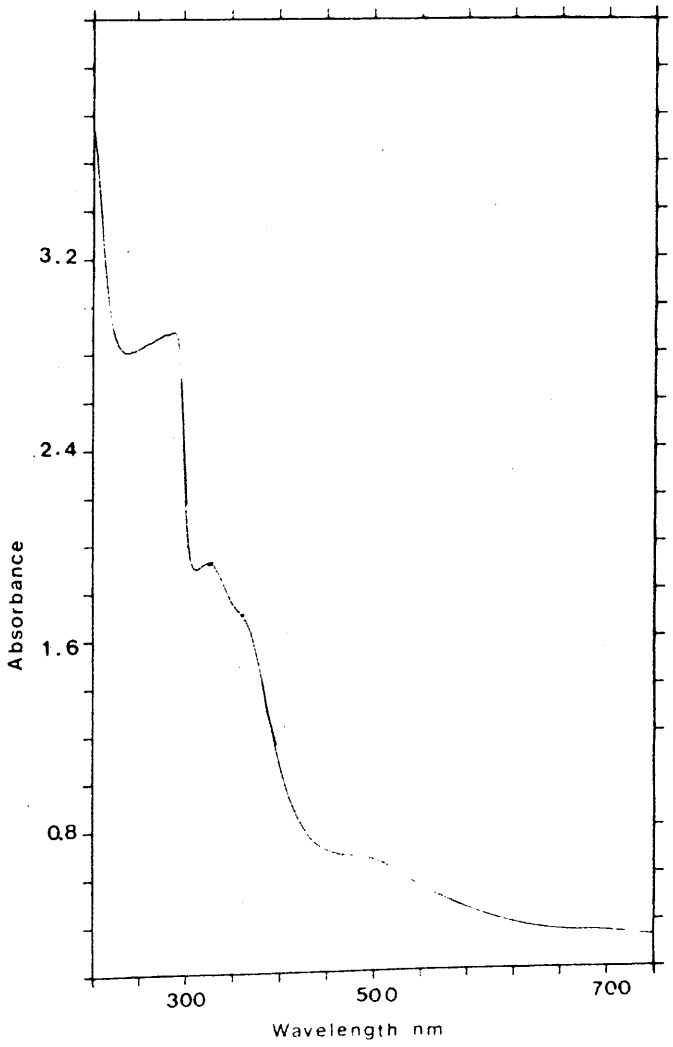

INTENSITY
A
B
C
D1
D2
E1
E2
F1
F2
2
4·5
8
X-RAY ENERGY (keV)

Fig 6.5 The spectral properties of the pigment extracted from a specimen of *D. lacteum* from the Allander water.

Fig 6.6 The spectral properties of a pigment (Haemerythrin) extracted from a priapulid.


1.2
0.8
0.4
Absorbance
325
350
400
450
500
550
600
650
Wavelength nm


3.2
2.4
1.6
0.8
Absorbance
300
500
700
Wavelength nm

peak at about 570 and 545nm. (Prosser and Brown, 1961) It was noticed, however, that the absorption spectrum of haemerythrin was quite similar to that obtained from the *D. lacteum* extract. To compare the two spectra directly, a sample of haemerythrin from a priapulid was extracted in distilled water, centrifuged and the absorption bands analysed (Fig 6.6). Surprisingly, the absorption spectrum was very similar to the spectrum from the pigment extracted from *D. lacteum*. The peaks of the spectrum from the pigment extracted from *D. lacteum* almost exactly coincided with those obtained for priapulid haemerythrin (Fig 6.6). These results could, however, have been an artefact due to the method of extraction i.e. the use of SDS is known to cause the dissociation of large molecules into subunits. To investigate this possibility, samples of human haemoglobin were examined after extraction with 0.2% SDS in 0.1m Na OH. The results were almost identical to those obtained for the pigment from *D. lacteum* i.e peaks at 500, 380 and 320nm.

Experiments were also carried out to determine the absorption spectrum of oxygenated and deoxygenated versions of the pigment found in *D. lacteum*. Sodium diothionite was added to the pigment in solution and the resulting spectrum observed. The only difference observed, which was noticed on a few occasions, was that the peak at 500nm disappeared. If nitrogen or argon was introduced into the solution the same effect was observed. This method had one distinct advantage over the sodium diothionite in that oxygen could be reintroduced back into the solution by shaking the mixture vigorously and the new spectrum examined. De-oxygenation would on occasion remove the peak at 500nm but on the introduction of oxygen the peak located at 500nm was never found to return.

6.3.4 Presence of pigment

The results of a random examination of triclads (*D. lacteum*) from the Allander water and River Kelvin showed that there was a significant difference between the sites in the quantity of worms with a strong red pigmentation. In the Allander Water, 68% of *D. lacteum* collected had a red colouration. This contrasted with the results from the River Kelvin with only 10% of individuals of *D. lacteum*, exhibiting a red colour during the summer.

6.4 Discussion

The results from examining triclad tissues with light and electron microscopes showed that the location of iron and haematin was very similar to that observed in the flatworm genus *Phaenocora* (Crompton and Smith, 1965; Young and Harris, 1973). In this genus, the distribution of the pigment was observed to be mostly around the gut and pharynx of the animal. This was very similar to the situation in *D. lacteum* and may provide useful circumstantial evidence for the presence of a haemoglobin-like compound.

Unfortunately, it was not possible to establish the true spectral properties of this pigment due to the problems of extraction. These problems were thought to involve the denaturing of the haemoglobin molecule by the alkaline SDS solution rendering it useless as an oxygen carrier. There are haemoglobin molecules, however, which are insoluble in water. It was interesting to note the presence of a non water-soluble haemoglobin molecule which has been found in the nuclei of rat and rabbit liver (Manwell, 1960).

The spectral properties of haemoglobin obtained after using the same extraction process as for the triclad pigment were found to be almost identical. This might suggest that the pigment present in *D. lacteum* was a haemoglobin-like molecule which had been denatured into smaller subunits; the absorption spectrum of which resembled priapulid haemerythrin. The pigment in *D. lacteum* did give up its oxygen on some occasions at low oxygen levels but was unable to operate reversibly due to being denatured by the strong alkaline solution.

Until the pigment found in *D. lacteum* can be effectively extracted and solubilised without affecting its respiratory function i.e. without denaturing it, then, unfortunately it can not be

positively identified as a tissue haemoglobin. The circumstantial evidence, colour, phylogenetic links, production under low PO_2, distribution, as described above would, however, leave little doubt that this pigment is probably similar in structure to haemoglobin.

It has been established that many animals can synthesize haemoglobin (see review of Fox, 1955). A considerable amount of work has been carried out on the cladoceran *Daphnia pulex* which has been shown to synthesize haemoglobin in water of a low oxygen content (Fox, 1948, 1955). Prosser and Brown (1961) demonstrated that *Daphnia* can synthesize the haemoglobin it requires within 10 days of encountering any hypoxic conditions. Weider and Lampart (1985) have shown intraspecific differences in the production of haemoglobin. They also stated that physiological differences existed between two clones of *Daphnia*, one clone produced the least amount of haemoglobin and was least tolerant of low oxygen levels. *D. lacteum*, like *Daphnia*, may be synthesizing haemoglobin in response to the hypoxic conditions associated with Allander Water site. Triclads present in the River Kelvin have no need of a respiratory pigment as they live in a well-oxygenated environment at the base of a weir and do not encounter sustained hypoxia. Triclads at this site, therefore, would not be expected to have any pigment. The results obtained in respect of tolerance to hypoxic conditions (Chap 4) i.e. Allander Water triclads being more tolerant of hypoxia than Kelvin triclads, would also add weight to this supposition that certain triclads under hypoxic stress were synthesizing a respiratory pigment much in the same way as *Daphnia pulex*.

If haemoglobin is present in *D. lacteum*, how would it function? Many animals can use haemoglobin as an oxygen store.

Arenicola marina, *Tubifex tubifex* and the aquatic larvae of some midges use their haemoglobin as an oxygen store to be utilised when external PO_2 declines and hypoxic or anoxic conditions arise (Chapman, 1980). Dales and Warren (1980) have shown that the polychaete *Cirriformia tentaculata*, which lives in marine burrows, use haemoglobin as an oxygen store to be utilised under hypoxic stress. Triclads can be found in environments which lack oxygen (Chap 3), living under rocks in groups of fifty or more animals. Community respiration under these circumstances can make PO_2 values almost anoxic for 6-8 hours per day during a hot summer (see Chap 3) and an oxygen store present in triclads under these circumstances would be very advantageous.

Another possible role for the presence of haemoglobin in *D. lacteum* would be for facilitated diffusion. In this process the rate of oxygen transport across a membrane can be enhanced by the presence of haemoglobin and myoglobin (Scholander, 1960; Kreuzer, 1970). This process is brought about by an oxygen gradient being established in a haemoglobin solution. When this occurs, oxygen molecules are passed down from one haemoglobin molecule to the next in a chain or "bucket brigade" fashion (Scholander, 1960). Provided the oxygen is used up at one end and is available at the other, a steady state system is set up which results in the facilitation of oxygen transport through the chain. In Chapter 5, a theoretical calculation for the diffusion of oxygen through a triclads integument was compared with actual oxygen consumption data. In animals from the Allander water, the rate of oxygen consumption was maintained until much lower PO_2 values were recorded i.e. these animals had a low Pc (10-20 Torr). Animals from the River Kelvin, however, were not able to maintain their oxygen consumption rates down to these low levels (Pc 20-30 Torr). This higher Pc in Kelvin triclads was the approximate level calculated by Chapman

(1980) that diffusion would no longer meet the animals respiratory requirements. Triclads from the Allander water were, however, extracting more oxygen at these low PO_2 values and this may involve the presence of haemoglobin which can then allow facilitated diffusion to occur.

CHAPTER 7

CHAPTER 7

Anaerobic metabolism in *D. lacteum*

7.1 Introduction

Various invertebrate groups have been shown to live in oxygen deficient environments and have highly developed anaerobic capabilities. These groups include, nematodes, trematodes, cestodes, annelids, molluscs, and some arthropod species (Hammen, 1969; Coles, 1970; Mangum, 1970; Mangum and Van Winkle, 1973; Saz, 1971). The three most studied groups are the helminths (particularly the parasitic helminths), intertidal bivalve molluscs, and more generally, benthic invertebrates which burrow into the bottom sediment. Within these groups a spectrum of possible anaerobic strategies are encountered ranging from obligate anaerobes to facultative anaerobes depending on the oxygen stress of their habitats and the resulting biochemical pathways utilised.

For animals to survive under anoxic conditions they must have a biochemistry suitably adapted to provide sources of fermentable storage energy and be able to maintain oxidation-reduction (Redox) potentials capable of generating high-energy phosphate compounds (ATP). The biochemical adaptations which are often found in facultative anaerobes may include; the deletion of certain key enzymes e.g. lactate dehydrogenase to avoid metabolic dead ends, the modification of the kinetic properties of various key branchpoint enzymes which allows an efficient transfer from aerobic to anaerobic metabolism and the coupling of other substrate-level phosphorylations to the glycolytic reactions which increases the yield of energy-producing compounds (Hochachka and Somero, 1973).

The phylum Platyhelminthes contains a heterogeneous collection of forms that occupy many diverse habitats; some are parasitic, while others are free-living. The free-living Platyhelminthes are found in marine, freshwater and terrestrial habitats. It is hardly surprising therefore that with all the diversity of habitats encountered, almost all helminths exhibit a marked capacity for anaerobic respiration. Indeed, the parasitic helminths, have been called "the best of animal anaerobes" (Hochachka, 1980). Parasitic forms have evolved elaborate metabolic adaptations which have allowed them to survive in hostile environments such as the gastrointestinal tract of mammals which have often been shown to be totally devoid of oxygen. These parasitic helminths can produce a multiplicity of anaerobic end products, the commonest of which are succinate, propionate, carbon dioxide and acetate with lactic acid also being produced on some occasions.

As mentioned above, a storage form of energy is essential for survival under sustained anoxia. In the standard anaerobic pathway, (anaerobic glycolysis) energy is stored in in the form of glycogen, and indeed glycogen is utilised in many helminths (Fairbairn, 1970; Saz, 1971). In addition, it has become clear that certain amino acids (leucine, valine, and iso-leucine) are an important potential source of energy in some helminths and these animals, under hypoxic stress, can produce end products such as isovalerate, isobutyrate and methylbutyrate (Bueding and Yale, 1951; Coles, 1970; Fairbairn, 1970).

In parasitic helminths CO_2 plays a critical role. This is because CO_2 is both fixed as a potential substrate source and is released as an anaerobic end product during fermentation. Carbon dioxide fixation is now known to be an integral part of anaerobic

metabolism in trematodes, nematodes, cestodes and many other invertebrate groups (Hochachka, 1980). CO_2 fixation has been linked to the activity of the two key enzymes, PEPCK (phosphoenolpyruvate carboxykinase) and PK (pyruvate kinase) with PEPCK activity being highest in species showing the most effective CO_2 fixation. When the concentration of CO_2 is high, PK is inhibited and PEPCK catalyses the carboxylation of PEP (phosphoenolpyruvate) to oxaloacetate (OXA) which can then be converted to malate, fumarate, succinate and ultimately propionate. A reduction in CO_2 raises PK activity while simultaneously reducing PEPCK activity and this results in the formation of pyruvate which is then ultimately converted to lactate or acetate. Thus under different conditions the proportions and concentrations of various end products may vary, as well as the actual end products themselves.

Freshwater triclads have been shown in the present study to withstand prolonged periods of exposure to anoxia (Chap 4) and the survival of these animals could only have been achieved through an efficient anaerobic metabolism. The animals were observed in experiments to assess hypoxic and anoxic tolerance (Chap 4) to shrink to about half their previous size in a matter of days. This would suggest a massive utilisation of storage products. The identification of any reduced metabolic end products, if present in *D. lacteum*, and the monitoring of potential energy stores (glycogen) during anoxia would give a good indication of the anaerobic pathways utilised. Tests were therefore carried out on animals from both sites kept under anoxic conditions for at least 24 hours to investigate the composition and location of any end products generated.

7.2 Materials and Methods

7.2.1. Anaerobic End Products

In order to investigate the accumulation of anaerobic end products, triclads were placed in sealed glass chambers and kept under anoxic conditions at 20°C for various lengths of time (usually 24 h). At the end of this time, the animals were removed from their chambers and placed into a chilled 1ml Eppendorf tube. Samples of the water in which the worms were kept were also collected and both the triclads and water samples were then immediately frozen in liquid nitrogen. These animals were weighed and stored in a freezer for subsequent analysis. The tissues of the triclads may have required deproteinisation to extract any end products and so two methods of extraction were initially employed.

a) Five previously weighed triclads were placed into a chilled 1ml Eppendorf tube containing 525µl of chilled distilled water. The animal was ground up with the blunt end of a glass rod and the resulting suspension centrifuged in a Damar/IEC Centra-4x centrifuge (Damar/IEC U.K. Ltd., England) at 10,000 rpm for 5min. The supernatant was extracted, frozen in liquid nitrogen and stored in a freezer for subsequent analysis by an H.P.L.C. (High Precision Liquid Chromatography) System (Gilson, France) coupled to a chart recorder (Tekman TE200/1; Tekman Ltd., England) with an organic acid column (Bio-Rad ammex ion exclusion column HPX - 87H). At all times during the experiment the animals and apparatus were kept in a chilled environment (<4°C). Individual triclads were also tested with a similar procedure except that the quantity of reagents was divided by five.

b) A similar procedure was carried out to the one described above except that instead of adding 525µl of distilled water, only 250µl was added together with 250µl 0.3M PCA (Perchloric acid). This was later neutralised with 25µl of 2.5M $KHCO_3$. This solution would be more effective at breaking down the animal tissues to release any metabolic end products into solution. After extraction the same procedures were followed as described above.

After trials of both methods, both were found to give identical results and so procedure a) was used throughout all subsequent experiments. Samples of water, in which the animals were kept, required no treatment and were simply frozen for analysis.

The analysis of samples in an H.P.L.C. System involved injecting 25µl of sample into the apparatus by the use of a microsyringe. To identify the sample, the time taken for the sample to travel along the column, reach the optical sensor set at 215nm, and produce peaks on the chart recorder was compared to the retention times of standard organic acid solutions. During these experiments the column was continuously flushed (1ml/min) with 0.07N H_2SO_4. Unfortunately the common anaerobic end products succinate and lactate could not be separated by the method described above as their retention times were almost identical and so lactate determinations were made by the standard enzymatic method described by Gutmann and Wahlfeld (1974) using a spectrophotometer to measure the absorption produced at 340nm.

The experiments on lactate accumulation involved triclads being kept for 24 hours under anoxic conditions at 20°C. The animals were then ground up as described above, centrifuged and the supernatant extracted. A control group and an experimental group were examined. The reagents used for each group are listed below:-

	Control	Samples
Hydrazine-glycine buffer	2.5ml	2.5ml
NAD solution	0.2ml	0.2ml
Extract	-	0.2ml
Perchloric acid	0.2ml	-

These solutions were left in a water bath at 37^{o}C for 1 hour, and the change in optical density measured in a spectrophotometer at 340nm. The amount of lactate accumulated could be read from a calibration curve which had been previously prepared.

7.2.2 Glycogen Estimation

Triclads from the River Kelvin and Allander Water were subjected to anoxic conditions at 20^{o}C for various lengths of time. At the end of specified lengths of time, three triclads were taken out of their chambers, frozen in liquid nitrogen and stored in a deep freeze. Ultimately, all the animals were analysed for changes in the concentration of glycogen during anoxia. Weighed, deep frozen triclads were placed into small test tubes with one volume (500µl) of 60% (W/V) K OH. The test tubes were then placed into a boiling water bath and the tissues dispersed with a glass rod. Heat was applied until all the tissue was well dispersed. Two volumes of 90% ethanol were added and the resulting solution thoroughly mixed. Glycogen was then allowed to precipitate out over several hours and this mixture was centrifuged at 10,000g for 10min. The supernatant was removed and the pellet washed with 60% ethanol and centrifuged repeatedly until the washings had a pH of about 7. The pellet was then washed with chloroform/methanol 1/4 (V/V) and finally dried. This pellet was redissolved in water, heated if necessary, and centrifuged again. Finally estimations were made of the amount of glycogen dissolved in the water by the Anthrone method

after Seifter *et al.* (1950).

7.2.3 Ammonia Estimations

Samples from the water in which ten triclads (*D. lacteum*) were kept during exposure to 24 hours of anoxia were tested for the presence of nitrogenous waste products. The concentration of ammonia was investigated using standard methods as described in Chaney and Marback, (1966).

7.3 Results

7.3.1 Anaerobic End products

Triclads from both sites (River Kelvin and Allander Water) were tested for the accumulation of many commonly produced anaerobic end products. As described previously the H.P.L.C. System could not distinguish between the presence of succinate or lactate and therefore an enzymatic method of estimating lactate accumulation was employed. The results, shown in Table 7.1 clearly demonstrate that lactic acid, although present in the tissues of *D. lacteum* (approximately 1mM), was neither excreted into the surrounding water nor accumulated in the tissues during a 24 hours period of anoxia.

A second experiment, similar to the first, was employed to determine the presence of lactate dehydrogenase. This was carried out by adding a known concentration of lactic acid to the extract with and without the presence of any external LDH. These results showed that an increase in absorption was found only when an external source of LDH was added. The amount of lactate measured was estimated on a calibration curve, and found to be about 82% of the amount added. LDH could therefore only have been present in very small amounts in *D. lacteum* suggesting that this metabolic pathway was not of major importance. Any subsequent differences in the succinate/lactate peak in the H.P.L.C. System between control and experimental animals (anoxia 24 hours) can probably be attributed mainly to changes in succinate concentration.

There was no intraspecific variation in the anaerobic end products generated, with animals from both sites producing the same end products. The accumulation of anaerobic end products was restricted to the tissues with no detectable excretion of any end products into the

Table 7.1

Lactate Determinations

a) Excreted Products

	ABSORBANCE	
Time (Hrs)	Blank (Av. Values)	Sample (Av. Values)
1	0.292	0.292
2	0.296	0.298
5	0.298	0.298
48	0.290	0.291

b) Tissue extracts

	ABSORBANCE	
	Control (Av. Values)	Experimental (Av. Values)
	0.519	0.494
	0.569	0.558
	0.507	0.554
	0.541	0.569
Anoxia	0.562	0.558
48 hrs.	0.629	0.494
	0.573	0.590
	0.552	0.523
	0.610	0.620

There was no significant difference between groups at $P>0.05$.

water surrounding the animals. The results of a typical run on an H.P.L.C. System with two similarly sized triclads, one kept under normoxia the other under anoxia for 24 hours, are shown in Fig 7.1. The first peak that appears on both traces was the protein front, which was observed in all samples. The main differences between the two traces was the appearance of propionate and acetate in the extract from an animal kept under anoxia. There were, however, subtle differences in the concentrations of the end products before and after 24 hours anoxia. Concentrations were calculated by comparing peak heights with standards of known concentration (1mM). After 24 hours under anoxia the concentration of of oxaloacetate and pyruvate increased slightly while the concentration of butyrate, fumarate and succinate fell with malate concentrations remaining about constant.

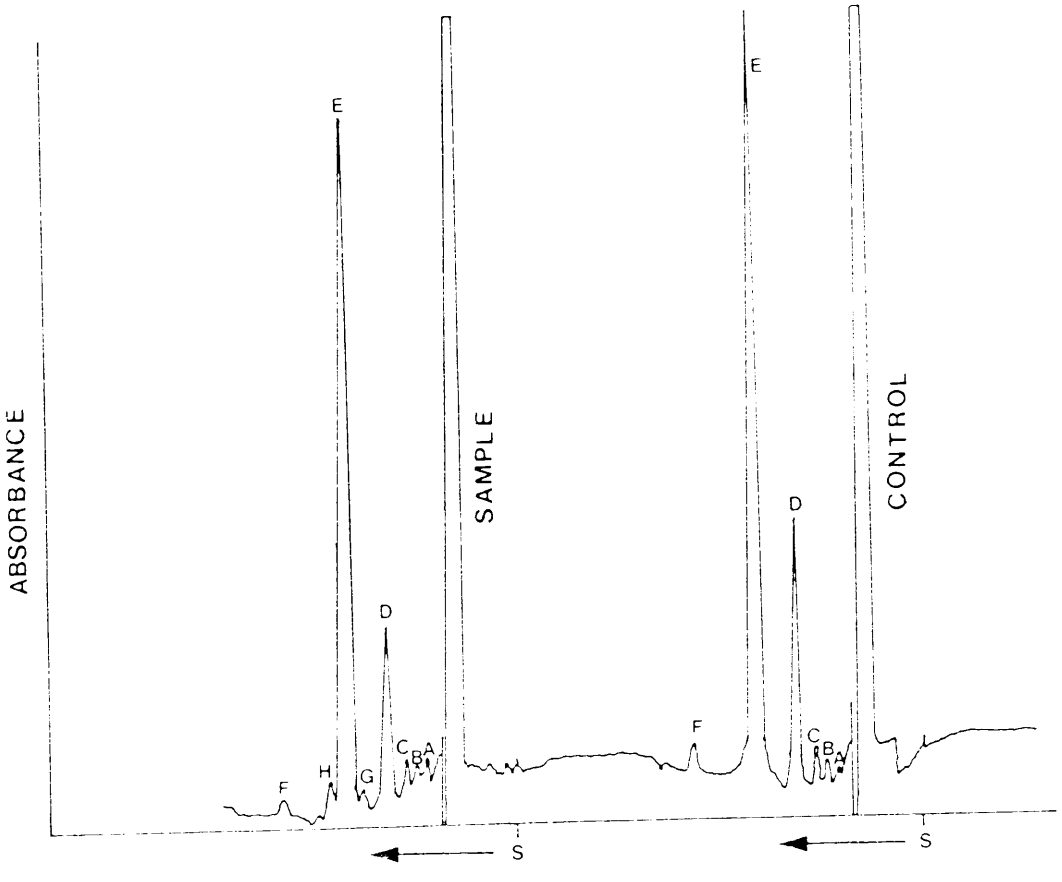
7.3.2 Glycogen Estimation

The results of experiments examining glycogen utilisation during periods of exposure to anoxia are shown in Fig 7.2. These results showed that triclads from the River Kelvin had significantly greater stores of glycogen, ($P<0.05$) and that the energy stores present in animals from both sites, were rapidly depleted under anoxic conditions. There was, however, no significant difference ($P>0.05$) in glycogen content between triclads from the River Kelvin at the start and after 6 hours anoxia. Triclads from the Allander Water, however, showed no significant ($P>0.05$) utilisation of glycogen until after 12 hours of anoxic conditions. The control animals which were kept in air saturated water did not have glycogen levels significantly different ($P>0.05$) from animals sampled at the start of the experiment. The amount of glycogen utilised in animals during a 24 hours period under anoxia was estimated to be about 75% of the total reserves in Kelvin

Fig 7.1 The absorption peaks of anaerobic end products from triclads kept under normoxic (Control) and 24 hours anoxic (Sample) conditions. The peaks represent:-

A - Oxaloacetate B - Pyruvate C - Malate D - Succinate

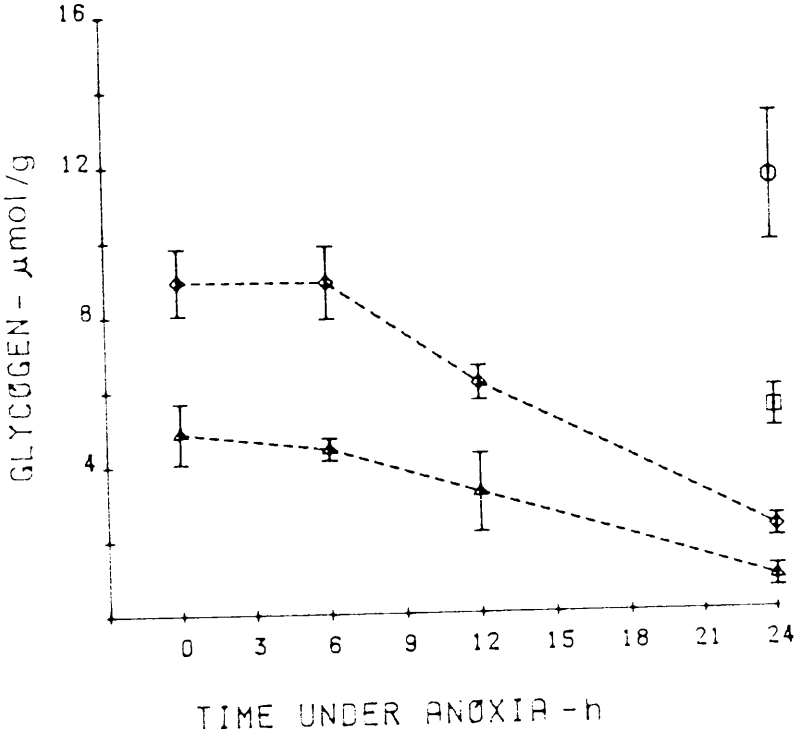
E - Fumerate F - Butyrate G - Acetate H - Propionate



TIME AFTER INPUT OF SAMPLE – S

MINS.

Fig 7.2 Graph showing the decline in the glycogen content (Fresh wt.) of *D. lacteum* from the River Kelvin (Diamonds) and Allander Water (Triangles) after exposure to anoxia for varying duration. Controls for the River Kelvin (Octagon) and Allander Water (Squares) were kept in air saturated water for 24 hours. Values are means ∓ S.D.


16
12
8
4
GLYCOGEN - µmol/g
0
3
6
9
12
15
18
21
24
TIME UNDER ANOXIA - h

triclads and 82% of the available energy resources in Allander triclads.

7.3.3 Ammonia Estimation

Ten triclads were left under normoxic conditions for 24 hours and the amount of ammonia excreted into the water established. Over this 24 hour period the amount of ammonia excreted into solution was estimated to be 0.94µg NH_3-Nl^{-1}. mg dry wt^{-1}. day^{-1}.

7.4 Discussion

The ability of freshwater triclads to respire anaerobically has been recognised for many years. Hyman (1919a) reported that free-living Turbellaria could survive in environments containing high concentrations of cyanide. Carbon dioxide fixation, a phenomenon associated with a marked capacity for anaerobic survival has also been observed in freshwater triclads e.g *Dugesia tigrina* (Hammen and Lum, 1962; Bryant and Smith, 1963) and cytochromes of the b-group, which are almost always found in organisms capable of reducing fumarate to succinate, are present in *Cura pinguis*, an Australian freshwater planarian (C. Bryant, Pers. Comm.).

The nature of metabolic pathways utilised during anaerobiosis in freshwater triclads is, however, largely unknown but recent studies on the anaerobic metabolism of facultative invertebrate anaerobes have shown two distinct types of anaerobic metabolism. These are, ecologically induced hypoxia brought about by a gradual depletion in oxygen within the environment and a depletion of oxygen caused by high activity levels. The former leads to a need for a lower rate of ATP production over long periods and the development of fumarate-succinate pathway with succinate and propionate as major end products. Bursts of activity require a higher rate of energy production and generally have lactate and or octopine as end products (review Zebe *et al.*, 1980). The present study would suggest that *D. lacteum* experiences considerable periods of environmental hypoxia and the metabolic pathways utilised may therefore be very similar to the pathways observed in its parasitic relatives the Cestoda and Trematoda.

The results from the present study would indicates that *D. lacteum* produces significant quantities of propionate and acetate under

anoxic conditions. Ammonia is the major excretory product in triclads (Hyman, 1951) and indeed ammonia was found to be excreted in this study. No lactic acid was accumulated in *D. lacteum* suggesting that at the PEP branchpoint PEPCK was converting phosphoenolpyruvate (PEP) to oxaloacetate by CO_2 fixation rather than being converted by PK to pyruvate which could then lead to the formation of lactate, acetate or ethanol. This is not unusual in helminths, lactic acid may or may not accumulate depending on the species studied which suggests that LDH (lactate dehydrogenase) also may or may not be present (Hochachka and Somero, 1973). Lactate dehydrogenase has been reported in *D. lacteum* (Livingston *et al.*, 1983). A small amount of lactic acid may accumulate in the first few hours associated with the initial increase in activity caused by placing the the animal into an anoxia environment. Similar results have been demonstrated for *Tubifex tubifex* (Seub *et al.*, 1983).

In *Ascaris* and *Fasciola*, PEP is converted by PEPCK to oxaloacetate by CO_2 fixation. Once this occurs, the oxaloacetate is converted into malate, which then inhibits PK activity. Malate then undergoes simultaneous oxidation and reduction (Bryant, 1982). The oxidation of malate occurs *via* the malic enzyme and the pyruvate dehydrogenase system. The resulting product is acetate, and reducing equivalents which drive the reduction sequence. In this reduction sequence fumarate derived from malate is converted to succinate by the activity of the fumarate reductase system which may then be converted into propionate. The end products of the malic enzyme branch are acetate and at the fumarate reductase branch succinate and propionate. These were the anaerobic end products found to accumulate in *D. lacteum* and it would seem probable that the metabolic pathway described above for *Fasciola* is very similar to the pathway investigated in *D. lacteum*.

Several annelids and various bivalves also survive during periods of hypoxia by employing a similar mode of anaerobic energy production. Under these conditions, some species exhibit an increased consumption of carbohydrates (Pasteur effect) accompanied by the accumulation or excretion of organic acids e.g. succinate, propionate and acetate (Crenshaw and Neff, 1969; De Zwann, 1977). Badman and Chin (1973) observed this phenomenon in the bivalve *Pleurobema coccineum* with a rapid decrease in glycogen and glucose from 24-48 hours, but this levelled off after 48 hours indicating only a partial Pasteur effect. Gäde *et al.* (1975) failed to demonstrate a Pasteur effect in the bivalve *Anodonta cygnea* while Zs.-Nagy (1973) observed only normal (aerobic) glycogen consumption in the same species over an eight day period of time. The Pasteur effect therefore only seems to give a significant increase in glycogen consumption within the first 24 hours of anoxia (De Zwann and Wijsman, 1976).

Glycogen is a very common storage product in helminths (Fairbairn, 1970; Saz, 1971) as well as many other types of invertebrate (Von Brand, 1946). Does the anaerobic survival of invertebrate animals depend on, the amount of carbohydrate supplies accumulated and a means of preventing toxic end products accumulating? Von Brand (1927) demonstrated a correlation between the amount of stored glycogen and resistance to anoxia. In the present study, *D. lacteum* was not observed to utilise carbohydrates for at least the first six hours; 12 hours in animals from the Allander Water. This may have been caused by a small residual amount of oxygen being left in the apparatus, but this does not explain the intraspecific differences in utilisation. These experiments were carried out in July when most individuals from the Allander Water possessed a respiratory pigment (Chap 6). Intraspecific differences in the presence of pigment and

hence of an oxygen store may have enabled triclads from the Allander Water to maintain an aerobic metabolism for longer periods before turning to anaerobiosis and the breakdown of their energy stores. Approximately 80% of the stored glycogen was utilised in 24 hours whereas Seub *et al.* (1983) found that *Tubifex tubifex* only utilsed 40% of its glycogen reserves in 18 hours. It has been established, however, that the glycogen content of triclads is lowest at this time of year (Boddington and Mettrick, 1971; Mettrick and Boddington, 1972). This reduction in glycogen can be attributed mainly to the energetic costs of reproduction. In triclads from the Allander water site the utilization of glycogen under hypoxic stress may have depleted further this store. This would account for the lower glycogen levels observed in *D. lacteum* from this site, even though the food availability at this site was considerably better than at the River Kelvin (Chap 3). The Pasteur effect may also have been occurring in *D. lacteum* which would accelerate the rate of depletion of the stored glycogen. Future experiments could extend the period of anoxia to investigate if the rate of utilization glycogen would fall.

If triclads do have similar anaerobic metabolic pathways to their parasitic relatives, the Trematoda and Cestoda, what are their purpose? Under normal circumstances, triclads frequently encounter hypoxic conditions in their environment (Chap 3), but only on very rare occasions are they likely to encounter complete anoxia. It is possible that the capacity for sustained anaerobic metabolism is a fundamental property of the turbellarian group derived from distant ancestors inhabiting an ancient anoxic environment, "the sulphide system" (Bryant 1982). In this totally anoxic environment Platyhelminthes and Aschelminthes are found in abundance with the primitive turbellarian orders Acoela and Rhabdocoela most prominent. Triclads may therefore

have inherited this facility for anaerobic survival from their more primitive ancestors. This facility might be used in the freshwater triclads to aid aerobic metabolism under hypoxia, but it is only in their parasitic relatives that we can see the complete utilization of this process. This therefore raises interesting evolutionary possibilities that parasitic forms were pre-adapted for life at low PO_2 before they encountered their hosts.

CHAPTER 8

8.1 General Discussion

The many factors which can affect the hypoxic tolerance of triclads have been discussed in the previous chapters. The main conclusions from these chapters have provoked a variety of interesting questions which will now be examined in more detail e.g. "Is environmental hypoxia the stimulus for intraspecific variation in anoxic tolerance ?", "Can adaptations to a hostile environment occur over relatively short time scales ?", "What is the mechanism by which tolerance is achieved ?" and "Is the observed intraspecific variation in tolerance physiologically or genetically based ?"

Triclads are often found in waters which suffer from organic enrichment (Gaufin, 1958; Holsinger, 1960; Macan, 1962) and these habitats have been shown to undergo considerable reductions in PO_2 (Gessner, 1961; Downing, 1967). The hypoxic and anoxic tolerance of triclads from an organically enriched site (Allander Water) situated downstream from a major sewage works were compared with triclads from a well oxygenated site located at the base of a large weir (River Kelvin).

The PO_2 content of both sites were examined and the results showed that large differences were present. These differences were observed predominantly during the spring and summer months. The River Kelvin was shown to be hyperoxic during spring and summer while the Allander Water was hypoxic during the same period. These differences in PO_2 may have been the stimulus for producing hypoxia tolerant triclads at the Allander Water site. However, in order to accurately assess the stresses imposed on triclads, PO_2 measurements were also recorded diurnally within various microhabitats. The results from this study

showed even greater differences in the PO_2 content of the rivers; the River Kelvin had large diurnal variations in PO_2 during the spring and summer while the Allander Water remained relatively stable. The Allander Water did, however, have consistently lower PO_2 readings, often <50 Torr, which were maintained for many months throughout the summer.

The animals present at both sites would therefore be subjected to vastly different oxygen regimes and the biochemistry and physiology of triclads located at each site would reflect this bias. This indeed has been the basis of many pollution indices which score the tolerance of various benthic invertebrates to the pollutional state of a river (Graham, 1965; Chandler, 1970). The animals taken from the River Kelvin were subjected for short periods to a variety of oxygen tensions, ranging from anoxia to 500 Torr. To survive under such conditions, they must have had an efficient short-term anaerobic metabolism capable of sustaining the energy requirements of the animal until normoxic conditions or an oxygen store, or perhaps both. Triclads from the Allander Water needed to endure prolonged exposure to hypoxia and this may have necessitated a different solution to the one described above.

The results from previous studies on the tolerance of triclads were equivocal with some authors claiming triclads are intolerant of hypoxia (Abbott, 1960; Russier-Delholm, 1974) while others have claimed a limited capability for anaerobiosis (Hyman, 1919a; Rode, 1925). In the present study, triclads from both sites were shown to survive considerable periods of hypoxia and anoxia and therefore must have been utilising some form of anaerobic metabolism. Mortality was, however, affected by a number of different factors with

LT_{50} values being influenced by temperature, time of year, number of animals present and nutritional state.

Intraspecific variations in tolerance to hypoxic and anoxic stress were established for *D. lacteum* with triclads from the Allander Water surviving significantly longer than their counterparts from the River Kelvin. How did these differences arise and were they physiologically or genetically based or both? It now seems likely that within each of these populations there are two separate sub-populations, composed of hypoxia tolerant and non-tolerant triclads. The percentage of "tolerators"' and "non-tolerators" present at each site would determine the mean tolerance of triclads for that particular site. Angus (1981) has carried out a study on various populations of the mosquitofish (*Gambusia affinis*) to establish phenol tolerance. He discovered that in populations tolerant of high concentrations of phenol the population consisted of between 60-80% of resistant fish. In non-tolerating populations only 23-27% of the population was tolerant. A very similar situation may exist in *D. lacteum* with a far higher proportion of hypoxia tolerant triclads being present in the Allander Water. It is interesting to note at this point that there was a significant difference, between the sites, in the number of triclads which contained a respiratory pigment. During the spring and summer <10% of animals present in the River Kelvin had a respiratory pigment whereas between 60-80% of the triclads present in the Allander Water possessed a pigment.

This raises a very interesting question, *viz* could the intraspecific variation in tolerance to hypoxia be linked to the presence or absence of a respiratory pigment? This pigment was found predominantly in triclads from the Allander Water and was only present during the summer months when hypoxic stress is greatest. The

intraspecific differences in mortality found in this study adds some support to this theory. These results showed that *D. lacteum* from the Allander Water differed only from its counterparts from the River Kelvin during the summer. During the winter there was no significant difference in mortality rates. This could be correlated with the presence of a respiratory pigment.

Tolerance of hypoxia has many contributing factors, the presence of a respiratory pigment may help to explain some of the intraspecific differences observed in *D. lacteum*. A respiratory pigment may have been synthesized in the triclads present in the Allander Water during the spring and summer. It is common for certain animals i.e. the cladoceran *Daphnia pulex* to synthesize haemoglobin in water of a low oxygen content (Fox, 1948; 1955).

Nutrition also affects mortality with better fed triclads surviving longer under anoxic conditions than starved triclads. Von Brand (1927) demonstrated that the amount of stored glycogen correlated with survival i.e. animals with a larger carbohydrate store survived longer. Triclads from the Allander Water have an abundant food supply and may develop larger stores of glycogen than their Kelvin counterparts. This was shown not to be the case as Kelvin triclads had a larger glycogen store. During the winter, however, triclads from the Allander Water may accumulate larger glycogen stores due to larger food supplies. These supplies would then be depleted during the sustained hypoxia of the summer months. This would help to explain any intraspecific differences which may persist throughout the year.

Do the intraspecific adaptations to hypoxia (respiratory pigment) present in triclads from the Allander Water have a physiological or genetic basis? The hypoxic stress that occurs in the

Allander water due to the effects of a local sewage works, has only been present for about 60 years. McMahon (1975) demonstrated that a genetic difference in the heat tolerance of a freshwater snail was possible after only 54 generations. In the present study experiments acclimating triclads to low PO_2 values demonstrated a significant increase in resistance, compared with normoxic controls. This effect was observed in triclads from both sites. The acclimation process may have been a stimulus for haemoglobin synthesis which could allow prolonged survival by aiding oxygen uptake under hypoxic conditions through facilitated diffusion. The observation that the tolerance of an F1 generation of triclads from both sites was similar to the tolerance of their parental stock demonstrated that a genetic component could not be excluded.

Intraspecific differences in tolerance to low PO_2 and anoxia have now been established and a possible solution postulated; but what is the mechanism for anaerobic survival? Triclads at both sites were capable of prolonged exposure to anoxic conditions and produced similar metabolic end products (succinate, acetate and propionate). These end products are very similar to those observed in other helminth groups and are generally regarded as being required for prolonged anoxic survival (Fairbairn, 1971; Saz, 1971). There were no obvious intraspecific differences in the end products generated and a detailed study would be required to determine any differences in the proportions or concentrations of any end product produced.

If the anaerobic metabolism of animals present at both sites was very similar, then any intraspecific differences in tolerance would most likely to be linked with the presence of a respiratory pigment. The purpose of the respiratory pigment seems to have two main uses.

Firstly to act as a limited oxygen store, which would enable a far higher proportion of Allander triclads to operate aerobically at very low PO_2 tensions for far longer than Kelvin triclads and to aid in the facilitated diffusion of oxygen across the body wall. The use of a respiratory pigment as an oxygen store, even for a relatively short period, would help to explain intraspecific differences in tolerance to anoxia. Most of the tolerance experiments were carried out under anoxia and the presence of a respiratory pigment would be of more use under hypoxic conditions. In the field, acute hypoxia occurs regularly at the Allander Water site and anoxic conditions are rare. Under these conditions the synthesis of a respiratory pigment would be of great benefit. As triclads proved to be very tolerant to hypoxia and the intraspecific differences to hypoxia were maintained under anoxia, most experiments for speed and convenience were carried out at this level. An experiment measuring the utilisation of glycogen in triclads from both sites subjected to anoxia for 24 hours supports this theory. It was found that glycogen utilisation does not effectively start until 12 hours after the onset of anoxia in triclads from the Allander water. Triclads from the River Kelvin, however, started to utilise their stores after only six hours. This difference could be explained by the presence of a respiratory pigment in Allander triclads. This suggested that oxygen was being utilised from an oxygen store (respiratory pigment) and that either there was none present in Kelvin triclads or that the size of the store was much smaller.

Intraspecific differences in the Pc point established for animals from both populations can now be explained by the diffusion of oxygen aided by the presence of a respiratory pigment (Scholander, 1960). As the PO_2 declined, triclads maintained their oxygen consumption rate i.e. were able to 'passively regulate', due to their

small size and low metabolic requirements. Below 30 Torr the oxygen consumption rate of triclads from the River Kelvin started to decline. Triclads from the Allander Water, however, were able due to fascilitated diffusion to maintain their oxygen consumption over a greater PO_2 range which resulted in a much lower Pc in animals from this site during the summer.

The triclads in this study seem to have adapted very quickly to a new environment i.e. acute hypoxia. It is interesting to consider whether triclads have evolved specific physiological/biochemical adaptations to this new environment or, could a more likely explanation be that they were they already pre-adapted to anoxia and merely exploiting a suitable habitat. Bryant (1982) has suggested that helminths, particularly the parasitic forms, were pre-adapted to their hypoxic host environment. This would suggest that triclads may have inherited alternative anaerobic pathways from their more primitive ancestors which enabled them to survive limited periods of anoxic stress. The evolution of a respiratory pigment predominantly in triclads from the Allander Water may have occurred over a much shorter time scale. This ability to synthesize a respiratory pigment seems to be induced by low PO_2 levels and the proportions of triclads with this pigment indicate the tolerance of the animal at that particular site.

This study has attempted to answer many of the questions mentioned above, but in doing so, many more questions have been raised which subsequent studies may attempt to answer. The study, although concentrating on certain aspects of biology, attempts to answer one of the fundamental questions in the study of biology itself, "How do animals adapt and evolve to a changing environment." It is hoped that this thesis has demonstrated that it can only be by the integration of the many different facets of biology (behaviour, ecology, physiology,

biochemisty etc.) that biologists can begin to understand the mechanism and relationships by which adaptation occurs.

BIBLIOGRAPHY

Abbott, B,J. (1960). A note on the oxygen tolerance and temperature tolerances of the triclads *Phagocata gracilis* and *Dugesia tigrina*. *Va. J. Sci. New Ser.*, 11, 1-8.

Agnew, D.J. and Taylor, A.C. (1986). Seasonal and diurnal fluctuations of some physico-chemical parameters of a boulder shore. *Ophelia*, in Press.

Ahmad, T.A., and Chaplin, A.E. (1979). Seasonal variation in the anaerobic metabolism of the mussel *Mytilus edulis* (L.). *Comp. Biochem. Physiol.*, 64B, 351-356.

Alderice, D.F. (1972). Factor combinations. In: *Marine Ecology*, Vol. 1 (3). Ed, Kinne, O., Wiley Interscience; London.

Allen, G.D. (1918). Quantitative studies on the rate of respiratory metabolism in Planaria. I. The effect of potassium cyanide on the rate of oxygen consumption. *Am. J. Physiol.*, 48, 93-120.

Allen, G.D. (1919). Quantitative studies on the rate of respiratory metabolism in Planaria. I. The rate of oxygen consumption during starvation, feeding, growth and regeneration in relation to the method of susceptability to potassium cyanide as a measure of the rate of metabolism. *Am. J. Physiol.*, 49, 420-473.

Allen, G.D. (1920). The rate of carbon dioxide production by pieces of planaria in relation to the theory of metabolic gradients. *Anat. Rec.*, 17, 347-348.

Ambühl, H. (1961). Die strömung als physiologischer und ökologischer faktor. *Verh. int. Verein theor. angew. Limnol.*, 14, 390-395.

Angus, R.A. (1983). Phenol tolerance in populations of mosquitofish from polluted and nonpolluted waters. *Trans. Am. Fish. Soc.*, 112, 794-799.

Antonovics, J. and Bradshaw, A.D. (1970). Clinal patterns at a mine boundry, *Heredity*, (Edinburgh) 23, 219-38.

Armstrong, J.T. (1964). The population dynamics of the planarian, *Dugesia tigrina*. *Ecol.*, 45, 361-365.

Aston, R.J. (1973). Tubificids and water quality: a review. *Environ. Pollut.*, 5, 1-10.

Badman, D. and Chin, S. (1973). Metabolic responses of the fresh-water bivalve, *Pleurobema coccineum* (Contrad.) to anaerobic conditions. *Comp. Biochem. Physiol.*, 44B, 27-32.

Ball, I.R. (1969). An annotated checklist of the freshwater Tricladida of the Nearctic and Neotropical regions. *Can. J. Zool.*, 47, 59-64.

Ball, I.R. (1971). Systematic and biogeographical relationships of some *Dugesia* species (Tricladida, Paludicola) from Central and South America. *Am. Mus. Novit.*, 2472, 1-25.

Ball, I.R. (1974). A contribution to the phylogeny and biogeography of the freshwater triclads (Platyhelminthes Turbellaria). In: *Biology*

of the Turbellaria, Ed. Riser, N.W. and Morse, M.P. pp. 339-401, McGraw-Hill, New York

Ball, I. R. (1975). Nature and formulation of biogeographical hypothesis. *Syst. Zool.*, 24, 407-430.

Ball, I.R. and Reynoldson, T.B. (1981). *British Planarians*. Cambridge University Press. 141pp.

Bayne, B. (1971a). Ventilation, the heart beat and oxygen uptake by *Mytilus edulis* L. in declining oxygen tension. *Comp. Biochem. Physiol.*, 40A, 1065-1085.

Bayne, B. (1971b). Oxygen consumption by three species of lamellibranch mollusc in declining ambient oxygen tension. *Comp. Biochem. Physiol.*, 40A, 955-970.

Bayne, B. (1973). The responses of three species of bivalve mollusc to declining oxygen tensions at reduced salinity. *Comp. Biochem. Physiol.*, 45A, 793-806.

Bayne, B. and Livingston, D. (1977). Responses of *Mytilus edulis* L. to low oxygen tension: acclimation of the rate of oxygen consumption. *J. Comp. Physiol.*, 114, 129-142.

Behre, E.H. (1918). An experimental study of acclimation to temperatures in *Planaria drotocephala*. *Biol. Bull. mar. biol. Lab., Woods Hole*, 35, 277-317.

Bellamy, L.S. and Reynoldson, T.B. (1974). Behaviour in competition for food amongst lake-dwelling triclads. *Oikos*, 25, 356-364.

Benedetto, L. (1970). Observations on the oxygen needs of some species of European Plecoptera. *Int. Rev. Gesamten Hydrobiol.*, 55, 505-510.

Benazzi, M. (1963). Il problema sistemàtico della *Polycelis* del gruppo *nigra-tenuis* alla luce di ricerche citologiche è genetiche. *Monit. Zool. Ital.*, 70, 288-300.

Billings, W.D., Godfrey, P.J., Chabot, B.F., & Bourque, D.P. (1971). Metabolic acclimation to temperature in arctic and alpine ecotypes of *Oxyria digyna*. *Arct. Alp. Res.*, 3, 277-89.

Bliss, C.I. (1938). The determination of the dosage-mortality curve from small numbers. *Q. Jl. Pharm. Pharmac.*, 11, 192-216.

Boddington, M.J. and Mettrick, D.F. (1971). Seasonal changes in the chemical composition and food reserves of the freshwater triclad *Dugesia tigrina* (Platyhelminthes: Turbellaria). *J. Fish. Res. Board Can.*, 28, 7-14.

Bolen, H. (1937). Specific dynamic action in planaria. *J. Exp. Zool.*, 75, 389-412.

Bradford, S.M. and Taylor, A. (1982). The respiration of *Cancer pagurus* under normoxic and hypoxic conditions. *J. Exp. Biol.*, 97, 273-288.

Bradshaw, A.D., McNeilly, T.S., & Gregory,, R.P. (1965).

Industrialisation, evolution and development of heavy metal tolerance in plants. *5th Symp. Brit. Ecol. Soc.*, 327-43.

Brand, T. von, (1927). Stoffbestand und Ernährung einiger Polychaeten und anderer mariner. *Z. vgl. Physiol.*, **5**, 643.

Brand, T. von, (1936). Studies on the carbohydrate metabolism in Planarians. *Physiol. Zool.*, 9, 530-541.

Brand, T. von, (1945). The anaerobic metabolism of invertebrates. *Biodynamica*, 5, 165-292.

Brand, T. von (1946). Anaerobiosis in inve ebrates. *Biodynamica*, Monographs No. , Normandy, Missouri.

Brand, T. von and Mehlmann, B. (1953). Relations between pre- and post-anaerobic oxygen consumption and oxygen tension in some fresh water snails. *Biol. Bull. mar. biol. Lab., Woods Hole*, **104**, 301-312.

Bridges, C.R. and Brand, A.R. (1980). The effect of hypoxia on oxygen consumption and blood lactate levels of some marine Crustacea. *Comp. Biochem. Physiol.*, 65A, 399-409.

Brown, A.C. (1979). Oxygen consumption of the sandy-beech whelk *Bullia digitalis* Meuschen at different levels of activity. *Comp. Biochem. Physiol.*, 62A, 673-675.

Bueding, E. and Yale, H.W. (1951). Production of methylbutyric acid by bacteria-free *Ascaris lumbricoides*. *J. Biol. Chem.*, 193, 411-423.

Bunge, G. (1888). Ueber das Saeurstoffbedürfniss der Schammbewahner. Hoppe-Seyler's *Z. Physiol. Chem.*, 12, 565.

Bunge, G. (1889). Weitere Untersuchungen uber die Athmung der Wurmer. Hoppe-Seyler's *Z. Physiol. Chem.*, 14, 318.

Bryant, C. (1982). The biochemical origins of helminth parasitism. In *Biology and control of endoparasites*. Academic Press.

Bryant, C. and Smith. M.J.H. (1963). Some aspects of the intermediary metabolism in *Fasciola hepatica* and *Polycelis nigra*. *Comp. Biochem. Physiol.*, 9, 189-194.

Butcher, R.W., Pentelow, F.T.K., & Woodley, J.W.A. (1927). The diurnal variations of gaseous constituents of river waters. *Biochem. J.* 21, 945-57.

Butler, P., Taylor, E. and McMahon, B. (1978). Respiratory and circulatory changes in the lobster (*Homarus vulgaris*) during long term exposure to moderate hypoxia. *J. Exp. Biol.*, 73, 131-146.

Cain, A.J., & Sheppard, P.M. (1950). Selection in the polymorphic land snail *Cepaea nemoralis*. *Heredity* (Edinburgh), 4, 275-94.

Cairns, J. and Dickson, K. (1971). A simple method for the biological assessment of the effects of waste discharges on aquatic bottom-dwelling organisms. *J. Water Pollut. Control Fed.*, **43** (5), 755-772.

Calow, P. (1974). Some observations on locomotory strategies and their metabolic effects in two species of freshwater gastropods *Ancylus fluviatilis* Müll. and *Planorbis contortus* Linn. *Oecologia*, 16, 149-161.

Calow, P. (1977). The joint effect of temperature and starvation on the metabolism of triclads. *Oikos*, 29, 87-92.

Calow, P. (1981). *Invertebrate biology*. John Willey & Sons, New York.

Calow, P. and Woollhead, A.S. (1977). Locomotory strategies in freshwater triclads and their effects on the energetics of degrowth. *Oecologia*, 27, 353-362.

Calow, P. and Woollhead, A.S. (1979). Energy partitioning strategies during egg production in semelparous and iteroparous triclads. *J. Anim. Ecol.*, 48, 491-499.

Calow, P., Beveridge, M. and Sibly, R. (1979). Heads and tails: Adaptational aspects of asexual reproduction in freshwater triclads. *Am. Zool.*, 19, 715-727.

Carpenter, K.E. (1928). On the distribution of freshwater Turbellaria in the Aberystwyth district, with especial reference to two ice-age relics. *J. Ecol.*, 16, 105-122.

Carter, G.S. (1931). Aquatic and aerial respiration in animals. *Biol. Rev.*, 5, 1-35.

Chandler, J.R. (1970). A biological approach to water quality management. *Wat. Pollut. Control*, 69, 415-421.

Chaney, A.L. and Marback. E.P. (1962). Modified reagents for the determination of urea and ammonia. *Clin. Chem.*, 8, 130-132.

Chapman, G. (1980). *The body fluids and their functions*. 2nd. Edit. Edward Arnold, London

Child, C.M. (1919). A comparative study of carbon dioxide production during starvation in planaria. *Am. J. Physiol.*, 48, 231-257.

Child, C.M. (1920). Some considerations concerning the nature and origin of physiological gradients. *Biol. Bull. mar. biol. Lab., Woods Hole*, 39, 102-142.

Clyde River Purification Board (1975). *Water Quality: A baseline report.*

Cole, A.E. (1921). Oxygen supply of certain animals living in water containing no dissolved oxygen. *J. Exp. Zool.*, 33, 293.

Coles, G.C. (1970). Some biochemical adaptations of the swamp worm *Alma emini* to low oxygen levels in tropical swamps. *Comp. Biochem. Physiol.*, 34, 481-489.

Coward, S.J. (1968). The relation of surface and volume to so-called physiological gradients in planaria. *Dev. Biol.*, 18, 590-601.

Cosgrove, W. and Schwartz, J. (1965). The properties and function of

the blood pigment of the earthworm, *Lumbricus terrestris*. *Physiol. Zool.*, 38, 206-212.

Crenshaw, M.A. and Neff, J.M. (1969). Decalcification at the mantle of the mantle-shell interface in molluscs. *Am. Zool.*, 9, 881-889.

Crompton, D.W., Shrimpton, D.H. and Silver, I.A. (1965). Measurements of the oxygen tension in the lumen of the small intestine of the domestic duck. *J. Exp. Biol.*, 43, 473-478.

Crompton, D.W. and Smith, M.H. (1963). Haemoglobin in a flatworm. *Nature, Lond.*, 197, 1118-1119.

Crowe, J.H. and Cooper, A.F. (1971). Cryptobiosis. *Sci. Am.*, 225, (6), 30-36.

Dales, R.P. and Warren, L.M. (1980) Survival of hypoxic conditions by the polychaete *Cirriformia tentaculata*. *J. mar. biol. Ass., U.K.*, 60, 509-516.

Dalyell, J.G. (1814). *Observations on some interesting phenomena on animal physiology exhibited by several species of planaria.* Edinburgh. 146 pp.

Dana, J.P.M. (1766). De hirudinis nova specie, noxa, remediisque adhibendis. *Mélang. phil. math. Soc. r. Turin*, 3, 199-205.

Dahm, A.G. (1958). *Taxonomy and Ecology of Five Species groups in the Family Planariidae (Turbellaria, Tricladida, Paludicola)*. Malmo.

Dahm, A.G. (1967). Tricladida et Temnocephalida. In: *Limnofauna Europaea*. Ed. J. Illies. G. Fischer, Stuttgart. pp 14-17.

Dales, R., Mangum, C. & Tichy, J. (1970). Effects of changes in oxygen and carbon dioxide concentrations on ventilation rhythms in onuphid polychaetes. *J. mar. biol. Ass. U.K.*, 50, 365-380.

Daniel, M.J. & Boyden, C.R. (1975). Diurnal variations in physico-chemical conditions within intertidal rock pools. *Field Stud.*, 4, 161-176.

Davies, L.J. & Hawks, H.A. (1981). Some effects of organic pollution on the distribution and seasonal incidence of Chironomidae in riffles in the River Cole. *Freshw. Biol.*, 11, 549-555.

Davies, R.W. (1969). Predation as a factor in the ecology of triclads in a small weedy pond. *J. Anim. Ecol.*, 38, 577-584.

Davies, R.W. and Reynoldson, T.B. (1971). The incidence and intensity of predation on lake-dwelling triclads in the field. *J. Anim. Ecol.*, 40, 191-214.

Davis, J.C. (1975). Minimal dissolved oxygen requirements of aquatic life with emphasis on Canadian species. *J. Fish. Res. Board. Can.*, 32, 2295-2332.

Dawson, F.H., Kern-Hanson, U., Westlake, D.F. (1982). Water plants and the oxygen and temperature regimes of lowland streams. In: *Studies on aquatic vascular plants*. eds. J.J. Symoens, S.S. Hooper, & P.

Compere, 214-221, Royal Botanical Society of Belgium, Brussels.

Decamps, H. & Pujol, J.Y. (1977). Influences humains sur les benthos d'un ruisseau de montagne dans les Pyrénées. *Bull. Ecol.*, 8 (3), 349-358

Dejours, P. (1975). *Principles of Respiratory Physiology*. North-holland, Amsterdam.

Dejours, P. (1981). *Principles of Comparative Respiratory Physiology*. 2nd revised edition , North holland, Amsterdam. 253pp.

Delorme, L.D. (1982). Lake Erie oxygen; the prehistoric record. *Can. J. Aquat. Sci.*, 39, 1021-1029.

Department of the Environment (1972). *Report of a river pollution Survey of England and Wales*. London H.M.S.O.

Dimnock, R. (1977). Effects of eviscertion on oxygen consumption by *Stichopus parvimensis* Clark (Echinodermata, Holutheroidea). *J. Exp. Mar. Biol. Ecol.*, 28, 125-132.

Downing, A.L. (1967). The effect of pollution on the oxygen balance of rivers. *Wat. Waste Treat.*, 11, 360-364.

Dratnal, E. & Kasprzak, K. (1980). The response of the invertebrate fauna to organic pollution in a well oxygenated Karst stream exemplified by the Pradnika Stream (South Poland). *Acta Hydrobiol.*, 22, 263-278.

Drury, R.A. and Wallington, E.A. (1967). *Carleton's histological technique*. 4th Ed., Oxford University Press.

Edwards, R.W. (1962). Some effects of plants and animals on the conditions in fresh-water streams with particular reference to their oxygen balance. *Int. J. Air Wat. Pollut.*, 6, 505-520.

Ehrenberg, C.G. (1831). Animalia evertebrata exclusis insectis. In: *Symbolae Physicae*. Ed. Hemrich, F.G. and Ehrenberg, C.G., 4, series prima cum tabularumdecade prima.

Ellington, W.R. (1983). The recovery from anaerobic metabolism in invertebrates. *J. Exp. Zool.*, 228, 431-444.

Elliot, J.M. (1968). The daily activity pattern of mayfly nymphs (Ephemeroptera). *J. Zool.*, 155, 201-221.

Englisch, H., Opalka, B. and Zebe, E. (1982). The anaerobic metabolism of the midge *Chaoborus crystallinus*. *Insect biochem.*, 12, (2), 149-155.

Fairbairn, D. (1970). Adaptations and loss of genetic capacity in helminth parasites. *Biol. Rev.*, 45, 29-64.

Fields, J.H.A. (1983). Alternatives to lactic acid: possible advantages. *J. Exp. Zool.*, 228, 445-457.

Finney, J. (1947). *Probit analysis*. Cambridge University Pess. 256pp.

Fox, H.M. (1945). The oxygen affinities of certain invertebrate haemoglobins. *J. Exp. Biol.*, 21, 161-165.

Fox, H.M. (1948). The haemoglobin of *Daphnia*. *Proc. Roy. Soc. B.*, 135, 195.

Fox, H.M. (1954). Oxygen and haem in invertebrates. *Nature, Lond.*, 174, 355.

Fox, H.M. (1955). The effect of oxygen on the concentration of haem in invertebrates. *Proc. Roy. Soc. Lond.*, 143B, 203-214.

Francott, P. (1883). Note sur l'anatomie et l'histologie d'un Turbellarié rhabdocoèle. *Bull. Acad. r. Belg., Cl. Sci.*, 6, 723-735.

Fraps, M. (1930). Studies on respiration and glycolysis in *Planaria*. I. Methods and certain basic factors in respiration. *Physiol Zool.*, 3, 242-270.

Fry, F.E.J. (1957). The aquatic respiration of fish. In: *The physiology of fishes*. Ed. Brown, M.E., Vol 1, Academic Press, New York.

Fry, F.E.J. (1971). The effect of environmental factors on the physiology of fish. - In: *Fish physiology*. Ed. Hoar, W.S. and Randall, D.J., Vol 6, Academic Press, New York.

Fry, F.E.J., Brett, J.R., & Clawson, G.H. (1942). Lethal limits for temperature for young goldfish. *Rev. Can. Biol.*, 1, 50-6.

Fry, F.E.J. and Hart, J.S. (1948). The aquatic respiration of fish. In: *The physiology of fish. Vol I, - Metabolism*. Ed. Brown, M. E. Academic Press, New York. 1-63.

Gäde, G. (1983). Energy metabolism of arthropods and molluscs during environmental and functional anaerobiosis. *J. Exp. Zool.*, 228, 415-429.

Gäde, G., Wilps, H., Kluytmans, J.H. and Zwann, A. de. (1975). Glycogen degredation and end products of anaerobic metabolism in the freshwater bivalve *Anodonta cygnea*. *J. Comp. Physiol.*, 104, 79-85.

Gamble, J. (1971). The responses of marine amphipods *Corophium arenarium* and *C. volutator* to gradients and to choices of different oxygen concentrations. *J. Exp. Biol.*, 54, 275-290.

Garey, W.f. & Rahn, H. (1970). Gas tensions in tissues of trout and carp exposed to diurnal changes in oxygen tension of the water. *J. Exp. Biol.*, 52, 575-582.

Gaufin, A.R. (1958). The effects of pollution on a midwestern stream. *Ohio J. Sci.*, 58, (4) 197-208.

Gelei, J. von (1936). Tanulmanyok a *Dendrocoelum lacteum* Oerstead. szovettanarol. A Magyar Tudomanyos Akademia Kiadvanya Budapest. cited in: Brand, T. von. Studies on the carbohydrate metabolism in Planaria. *Physiol. Zool.*, 9, 530-541.

Gessner, F. (1961). Der Sauerstoffhaushalt des Amazonas. Int. Rev.

Gesamten Hydrobiol., 46, 542-561

Gnaiger, E. (1983). Heat dissipation and energetic efficiency in animal Anoxibiosis: economy contra power. *J. Exp. Zool.*, 228, 471-490.

Golterman, H.L., Clymo, R.S., & Ohnstad, M.A.M. (1978). *Methods for phyical and chemical analysis of freshwaters.* I.P.B. Handbook No. 8. 2nd. Ed.

Graham, J.B. (1972). Low temperature acclimation and the seasonal temperature sensitivity of some marine fishes. *Physiol. Zool.*, 45, 1-13.

Graham, T.R. (1965). *Annual report for the lothians Purification Board for 1965.*

Grant, I.F. & Hawks, H.A. (1981). The effects of diel oxygen fluctuations on the survival of the freshwater shrimp *Gammarus pulex. Environ. Pollut.*, 28, 53-66.

Gutmann, I. and Wahlfeld, A.W. (1974). *Methods of enzymatic analysis*, 2nd Ed., Academic Press, New York.

Hammen, C.S. (1969). Metabolism of the oyster *Crassotrea virginica. Am. Zool.*, 9, 309-318.

Hammen, C.S. and Lum, S.C. (1962). Carbon dioxide fixation in marine invertebrates. *J. Biol. Chem.*, 237, 2419-2422.

Hansen-Melander, E., Melander, Y. and Reynoldson, T.B. (1954). A new species of freshwater triclad belonging to the genus *Polycelis*, *Nature, Lond.*, 173, 354-355.

Harding, W.A. (1910). A revision of the British leeches. *Parisitol.*, 3, 130-201.

Hartog, C. den, (1962). De Nederlandse platwormen (Tricladida). *Wet. Meded. k. ned. natuurh. Veren.*, 42, 1-40.

Hawks, H.A. (1962). Biological aspects of river pollution, In: *River pollution. Causes and effects*, (Ed by L. Klein), pp. 311-437. Butterworths, London

Hawks, H.A., & Davies, L.J. (1970). Some effects on the organic enrichment on benthic invertebrate communities in stream riffles. In: *The scientific management of animal and plant communities for conservation*, eds. E. Duffy, and A.S. Watt, 271-293, Blackwell, Oxford.

Hemmingsen, A.M. (1950). The relationship of standard energy metabolism to total fresh weight of living organisms. *Rep. Steno. Meml. Hosp. Copenhag.*, 4, 7-58.

Hemmingsen, A.M. (1960). Energy metabolism as related to body size and respiratory surfaces, and its evolution. *Resp. Steno. Meml. Hosp. Nord. Insulin Lab.*, 9, 1-10.

Herreid, C.F. (1980). Review: Hypoxia in Invertebrates. *Comp. Biochem. Physiol.*, 67A, 311-320.

Herreid, C., O'Mahoney, P. and Shag, G. (1979). Cardiac and respiratory response to hypoxia in the land crab *Cardisoma guanhumi* (Latreille). *Comp. Biochem. Physiol.*, 63A, 145-151.

Hill, B. (1981). Respiratory adaptations of three species of *Upogebia* (Thalassinidea, Crustacea) with special reference to low tide periods. *Biol. Bull. mar. biol. Lab., Woods Hole*, 60, 272-279.

Hill, R.W. (1976). *Comparative physiology of animals*. Harper and Row, New York.

Hinton, H.E. (1971). Reversible suspension of metabolism. In: *From Theoretical Physics to Biology*, ed. by M Marois, Paris, Editions du Centre National de la Recherche Scientifique, 446pp.

Hirshfield, M., Feldmeth, C. and Soltz D. (1980). Genetic differences in Physiological tolerance of Armagosa Pupfish (*Cyprinodon nevadensis*) populations. *Science, N.Y.*, 207, 999-1001.

Hoar, W.S. (1966). *General and comparative physiology*, first edition. Prentice Hall.

Hochachka, P.W. (1980). *Living without Oxygen*. Harvard University Press, London, England.

Hochachka, P.W., Fields, J. and Mustafa, T. (1973). Animal life without oxygen: basic biochemical mechanisms. *Am. Zool.*, 13, 543-555.

Hochachka, P.W. and Somero, G.N. (1973). *Strategies of Biochemical Adaptations*. W.B. Saunders Co. Torronto.

Hochachka, P.W. and Somero, G.N. (1984). *Biochemical Adaptations*. Princeton University Press, Princeton, New Jersey.

Höll, K. (1955). Chemische Untersuchungen an kleinen Fliessgewässern. *Verh. int. Verein. theor. angew. Limnol.* 2 360-372.

Holland, W.E., Smith. M.H., Gibbons, J.W. and Brown, D.H. (1974). Thermal tolerances of fish from a reservoir receiving heated effluent from a nuclear reactor. *Physiol. Zool.*, 47, 110-118.

Holmes, W. (1940). The colour changes and patterns of *Sepia officinalis* L. *Proc. Zool. Soc. Lond.*, 110, 17-35.

Holsinger, J.R. (1960). A preliminary study of the effects of organic pollution of Bankers Corner Cave. *Va. Int. J. Speleol*, 123, 53-57.

Hubault, E. (1927). Contribution à l'étude des les invértébres torrenticoles. *Bull. Biol. suppl.*, 9, 1-38

Hughes, G.M. and Shelton, G. (1962). Respiratory mechanisms and their nervous control in fish. *Adv. comp. Physiol. Biochem.*, 1, 275-364.

Humphries, D.A., & Driver, P.M. (1971). Protean defence by prey animals. Oecologia, 5, 285-302.

Huss, H. (1913). Können die Cyclopiden intramolekular atmen ? *Int Rev.*

Gesamten Hydrobiol., 6, 38.

Hutchinson, E.G. (1957). A treatise on limnology. Vol. 1., ed., J.Wiley & Sons Inc., New York.

Hutchinson, V.H. (1961). Critical thermal maxima in salamanders. *Physiol. Zool.*, 34, 92-125.

Hyman, L.H. (1919a). On the action of certain substances on oxygen consumption (2) : Action of potassium cyanide on planarians. *Am. J. Physiol.*, 48, 340-371.

Hyman, L.H. (1919b). Physiological studies on Planaria. I. Oxygen consumption in relation to feeding and starvation. *Amer. J. Physiol.*, 49, 377-419.

Hyman, L.H. (1929). The effect of oxygen tension on the oxygen consumption in planaria and some echinoderms. *Physiol. Zool.*, 2, 505-534.

Hyman, L.H. (1951). *The Invertebrates.* II. Platyhelminthes and Rhyncocoela, the acoelomate bilateria. Mcgraw-Hill, New York. pp. 572.

Hynes, H.B.N. (1970). *The Ecology of running waters.* Liverpool University Press, Liverpool.

Ijima, I. (1884). Untersuchungen über den Bau und die Entwicklungsgeschichte der Süsswasser-Dendrocoelen (Tricladen). *Z. Wiss. Zool.*, 40, 359-464.

Jennings, J.B. (1957). Studies on feeding, digestion and food storage in free-living flatworms. (Platyhelminthes, Turbellaria). *Biol. Bull. mar. biol. Lab., Woods Hole*, 112, 63-80.

Jennings, J.B. (1959). Observations on the nutrition of the land planarian *Orthodemus terrestris* (O.F. Müller). *Biol. Bull. mar. biol. Lab., Woods hole*, 117, 119-124.

Jennings, J.B. (1962). Further studies on feeding and digestion in triclad Turbellaria. *Biol. Bull. mar. biol. Lab., Woods hole*, 123, 571-581.

Johansen, K. and Peterson, J. (1971). Gas exchange and active ventilation in a starfish, *Pteraster tesselatus. Z. vergl. Physiol.*, 71, 365-381.

Jones, J. (1961). Aspects of respiration in *Planorbis corneus* L. and *Lymnaea stagnalis* L. (Gastropoda = Pulmonata). *Comp. Biochem. Physiol.*, 4, 1-29.

Jones, J.R.E. (1952). The reactions of fish to water of low oxygen concentrations. *J. Exp. Biol.*, 29, 403-415.

Kawakatsu, M. (1965). On the ecology and distribution of freshwater planarians in the Japanese islands, with special reference to their vertical distribution. *Hydrobiol.*, 26, 349-408.

Keilin, D. (1959). The problem of anabiosis or latent life: history and

current concept. *Proc. Roy. Soc. Lond.*, 150B, 149-191.

Kenk, R. (1974). Index to the genera and species of the freshwater triclads (Turbellaria) of the world. *Smithson. Contrib. Zool.*, 183, 1-90.

Kettlewell, H.B.D. (1973). *The evolution of melanism*. Claredon press, Oxford.

Kirby, W. (1794). Descriptions of three new species of *Hirudo*; with an additional note by G. Shaw. *Trans. Linn. Soc. Lond.*, 2, 316-320.

Kleiber, M. (1932). Body size and metabolism. *Hilgardia*, 6, 315-353.

Knight, A.W. and Gaufin, A.R. (1963). The effect on water flow, temperature, and oxygen concentration on the plecopteran nymph, *Acroneuria facifica* Banks. *Utah academy Proc.*, 40, 2, 175-184.

Kreuzer, F. (1970). Facilitated diffusion of oxygen and its possible significance: a review. *Respir. Physiol.*, 9, 1-30.

Krotov, A.I. (1968). Effect of physiological conditions of Ascarids on their survival time in an atmosphere of oxygen. *Bull. Exp. Biol. Med.*, U.S.S.R. 49, 996-998.

Lahoud, H., Prickard, R.K., McManus, W.R. and Schofield, P.J. (1971). Volatile fatty acid production by the adult liver fluke *Fasciola hepatica*. *Comp. Biochem. Physiol.*, 38B, 379-391.

Lascombe, C. (1974). Recherches ecologiques et biogéographiques sur deux espèces jumelles de planaires d'eau douce dans la région lyonnaise. *Bull. mens. Soc. linn. lyon*, 43, 168-176.

Laurie, E.M.O. (1942). The dissolved oxygen of an upland pond and its inflowing stream, at Ystumtuen, North Cardiganshire, Wales. *J. Ecol.*, 30, 357-382.

Lavandier. P. & Capblanco, J. (1975). Influence des variations d'oxygène dissous sur les invertébrés benthiques d'un ruisseu des Pyrénées Centrales. *Annls. Limnol.*, 11, 101-106.

Laser, H. (1944). The oxidative metabolism of *Ascaris suis*. *Biochem. J.* 38, 333-338.

Leloup, E. (1944). Recherches sur les triclades dulcicoles épigés de la forêt de soignes. *Mém. Mus r. Hist. nat. Belg.*, 102, 3-112

Le Moigne, A. (1962). Etudes de formules chromosomique de quelques *Polycelis* (Turbellariés, Triclades) de la région Parisienne. *Bull. Soc. Zool. Fr.*, 87, 259-270.

Lender, T. and Le Moigne, A. (1960). Les organes musculoglandulaires de *Polycelis tenuis* Ijima (Turbellarie, Triclade). *Bull. Soc. Zool. Fr.*, 85, 90-99.

Linnaeus, C. (1746). *Systema suecica*. Stockholm 411pp.

Litchfield, J.T. and Wilcoxon F.W. (1949). A simplified method of evaluating dose-effect experiments. *J Pharm. exper. Ther.*, 96, 99-

113.

Livingston, D., Zwann, A. de., Leopold, M. & Marteijn, E. (1983). Studies on the phylogenetic distribution of pyruvate oxidoreductases. *Biochemical Syst. Ecol.*, **11**, 415-425.

Lovtrop, E. (1953). Studies on Planarian respiration. *J. Exp. Zool.*, 124, 427-434.

Lund, E.J. (1921). Oxygen concentration as a limiting factor in the respiratory metabolism of *Planaria agilis*. *Biol. Bull. mar. biol. Lab., Woods Hole*, **41**, 203-220.

McCorkle, S., Shirley, T.C. & Dietz, T.H. (1979). Rhythms of activity and oxygen consumption in the common pond clam, *Ligumia subrostrata* (Say). *Can. J. Zool.*, 57, 1960-1964.

McMahon, R. (1976). Effluent-induced interpopulation variation in the thermal tolerance of *Physa virgata* Gould. *Comp. Biochem. Physiol.*, 55A, 23-28.

McMahon, R. and Russel-Hunter, W.D. (1978). Respiratory responses to low oxygen stress in marine littoral and sublittoral snails. *Physiol. Zool.*, 51, 408-424.

McMahon, R., Butler, P. & Taylor, E. (1978). Acid base changes during recovery from disturbance and during long term hypoxic exposure in the lobster *Homarus vulgaris*. *J. Exp. Zool.*, **205**, 361-370.

Macan, T.T. (1961). Factors that limit the range of freshwater animals. *Biol. Rev.*, 36, 151-198.

Macan, T.T. (1962). Biotic factors in running water. *Schweiz. Z. Hydrol.*, 24, 386-407.

Macan, T.T. & Worthington. E.B. (1974). *Life in lakes and rivers*. Collins, London.

Mackereth, F.J.H., Heron, J., & Talling, J.F. (1978). *Water analysis: some revised methods for limnologists*. F.B.A., Scientific Publication No. 36.

Macenthun, K.M. (1966). Biological evaluation of polluted streams. *J. Wat. Poll. Cont. Fed. J.*, 38, 241.

Mangum, C. (1970). Respiratory physiology in annelids. *Am. Sci.*, 58, 641-647.

Mangum, C. (1980). Respiratory functions of haemocyanins. *Am Zool.*, 20, 19-38.

Mangum, C. and Van Winkle, W. (1973). Responses of aquatic invertebrates to declining oxygen conditions. *Am. Zool.*, 13, 529-541.

Mann, K.H. (1956). A study of the oxygen consumption of five species of leech. *J. Exp. Biol.*, 33, 615-626.

Mann, K.H. (1961). The life history of the leech *Erpobdella testacea*

(Sav) and its adaptive significance. *Oikos*, 12, 164-169.

Manwell, C. (1960). Comparative physiology: blood pigments. *Ann. Rev. Phys.*, 22, 191-244.

Marsden, I.D. (1973). Influence of starvation on temperature relationships in *Carcinus maenas*. In: *Effects of temperature on heterothermic organisms*. Ed. Weiser, W. Springer-Verlag, Berlin.

Mason, C.F. (1981). *Biology of freshwater pollution*. ed, Longman.

Maynard Smith, J. (1977). *The theory of evolution*. reprint 3rd. edition, Penguin books, England.

Mettrick, D.F. Boddington, M.J. and Gelder, S.R. (1970) Distribution of freshwater triclads (Platyhelminthes : Turbellaria) in central southern Ontario. *Proc. Conf. Great Lakes Res.*, 13, 71-81.

Mettrick, D.F. Boddington, M.J. (1972). The chemical composition of some freshwater Turbellarians. *Carib. J. Sci.*, 12, 1-7.

Minkley, W.L. (1963). The ecology of a spring stream Doe Run, Meade County, Kentucky. *Wildl. Monogr. Chestertown*, 11, 124pp.

Moquin-Tandon, A. (1826). *Monographie de la famille des* Hirudineés. Montpellier, Paris.

Morris, S. & Taylor, A.C. (1983). Diurnal and seasonal variation in physico-chemical conditions within intertidal rock pools. *Estuarine, Coastal Shelf Sci.*, 17, 339-355.

Morris, S. and Taylor, A.C. (1985). The respiratory response of the intertidal prawn *Palaemon elegans* (Rathke) to hypoxia and hyperoxia. *Comp. Biochem. Physiol.*, 81A, 633-639.

Mosely, H.N. (1874). On the anatomy and history of the land planarians of Ceylon, with some accounts of their habitats, and description of two new species and with notes on the anatomy of some European aquatic species. *Phil. Trans. R. Soc.*, 164, 105-172.

Moss, G. (1970). The excretory metabolism of the endoparasitic diagenean *Fasciola hepatica* and its relationship to its respiratory metabolism. *Parasit.*, 60, 1-19.

Müller, O.F. (1774). *Vermium Terrestrium et Fluviatilium, seu Animalium Infusoriorum. Helminthicorum et Testaceorum, non Marinorum, Succincta Historia*. I (2). Havinae et Lipsiae.

Müller, O.F. (1776). *Zoologicae Danicae Prodromus seu Animalium Daniae et Noregiae Indigenorum Characteres, Nomina et Synonyma Imprimis Popularium*. Havinae.

Nagell, B. (1977a). Phototactic and thermotactic responses facilitating survival of *Cloen dipterum* (Ephemeroptera) larvae under winter anoxia. *Oikos*, 29, 342-347.

Nagell, B. (1977b). Survival of *Cloen dipterum* (Ephemeroptera) larvae under anoxic conditions in winter. *Oikos*, 29, 161-165.

Nagell, B. and Fagerström, T. (1978). Adaptations and resistance to anoxia in *Cloen dipterum* (Ephemeroptera) and *Nemoura cinerea* (Plecoptera). *Oikos*, 30, 95-99.

Naylor, E. & Slinn, D.J. (1958). Observations on the ecology of some brachish water organisms in pools at Scarlett Point, Isle of Man. *J. Anim. Ecol.*, 27, 15-25.

Nebeker, A.V. (1972). Effect of low oxygen concentrations on survival and emergence of aquatic insects. *Trans. Am. Fish Soc.*, 4, 675-679.

Neel, J.K. (1951). Interrelations of certain physical and chemical features in a head-water limestone stream. *Ecol.*, 32, 368-91.

Nelson, S.G., Knight, A.W. and Li, H.W. (1977). The metabolic cost of food utilisation and ammonia production by juvinile *Macrobranchium rosenbergii* (Crustacea: Palaemonidae). *Comp. Biochem. Physiol.*, 57A, 67-72.

Newell, R.C. (1969). Effect of fluctuations in temperature on the metabolism of intertidal invertebrates. *Am. Zool.*, 9, 293-307.

Newell, R.C. (1970). *Biology of intertidal animals*. Logos Press, London.

Newell, R.C. (1973). Factors affecting the respiration of intertidal invertebrates. *Am. Zool.*, 13, 513-528.

Newell, R. and Northcroft, H.R. (1965). The relationship between cirral activity and oxygen uptake in *Balanus balanoides*. *J. Mar. Biol. Ass. U.K.*, 45, 387-403.

Newell, R. and Pye, V.I. (1970). Seasonal changes in the effect of temperature on the oxygen consumption of the winkle *Littorina littorea* (L.) and the mussel *Mytilus edulis* L. *Comp. Biochem. Physiol.*, 34, 367-383.

Newell, R., Johnson, L. and Kofoed, L. (1978). Effects of environmental temperature and hypoxia on the oxygen consumption of the suspension-feeding gastropod *Crepidula fornicata* L. *Comp. Biochem. Physiol.*, 59A, 175-182.

Newell, R.C. and Roy, A. (1973). A statistical model relating the oxygen consumption of a mollusc to activity, body size, and environmental conditions. *Physiol. Zool.*, 46, 253-275.

Oersted, A.S. (1844). *Entwurf einer systematischen Eintheilung und speciellen Beschreibung de Plattwürmer, auf microscopische Untersuchungen gregründet*. Copenhagen. pp. 96.

Owens, M., & Edwards, R.W. (1964). A chemical survey of some English rivers. *Proc. Soc. Wat. Treat. Exam.*, 13, 134-144.

Pattee, E., Lascombe, C. and Delholm, R. (1973). Effects of temperature on the distribution of turbellarian triclads. In: *Effects of temperature on ectothermic organisms*. Ed. Wieser, W., Springer-verlag, Berlin pp. 201-207.

Pederson, K.J. (1956). On the oxygen consumption of *Planaria vitta* during starvation, the early phase of regeneration and asexual reproduction. *J. Exp. Zool.*, 131, 123-135.

Perramon, A., Stupfel, M.,Mérat, P., Demaria Pesce, V., Gourlet, V. and Thierry, H. (1983). Evidence of genetic differences in acute hypoxia survival. *Aviat. Space. Environ. Med.*, 54, 127-131.

Pickavance, J.R. (1968). *The ecology of Dugesia tigrina; an American immigrant planarian.* Unpublished Ph.D. Thesis, University of Liverpool.

Pickavance, J.R. (1971). The diet of the immigrant planarian *Dugesia tigrina* (Girard) I. Feeding in the laboratory. *J. Anim. Ecol.*, 40, 623-635.

Pickworth, F.A. (1934). A new method of study of the brain capillaries and its application to the regional localisation of mental disorder. *J. Anat., Lond.* 69, 62-72.

Por, F. and Masry, D. (1968). Survival of a nematode and an oligochaete species in the anaerobic benthal of Lake Tiberias. *Oikos*, 19, 2.

Porfirjeva, N.A. (1973). *The planarian fauna of Lake Baikal.* Kazanski University, Kazan. 192pp.

Porfirjeva, N.A. (1977). *Planarians of Lake Baikal.* Akademia Nauk SSSR Novosibirsk. 206pp.

Preece, G.S. (1970). Salinity and survival in *Bathyporeia pilosa* Lindstrom and *B. pelagica* (Bate) *J. Exp. Mar. Biol. Ecol.*, 5, 234-245.

Prosser, C.L. (1973). *Comparative animal physiology.* 3rd edn. W.B. Saunders; Philadelphia.

Prosser, C.L. and Brown, F.A. (1961). *Comparative animal physiology.* Ed. Saunders Co., Philadelphia. 688pp

Pütter, A. (1908). Der staffwechsel des blutegels (*Hirudo medicinalis* L.) *Z. allg. Physiol.*, 7, 16.

Reid, G.K. & Wood, R.D. (1976) *Ecology of inland waters and estuaries.* D. Van Nostrand Company, New york.

Reynoldson, T.B. (1948). British species of *Polycelis* (Platyhelminthes). *Nature, Lond.*, 162, 620-621.

Reynoldson, T.B. (1950). Natural population fluctuations of *Urceolaria mitra* (Protzoa, Peritricha) epizoic on flatworms. *J. Anim. Ecol.*, 19, 106-118.

Reynoldson, T.B. (1958a). The quantitative ecology of lake-dwelling triclads in northern Britain. *Oikos*, 11, 125-141.

Reynoldson, T.B. (1958b). Observations on the comparative ecology of lake-dwelling triclads in Southern Sweden, Finland, and Northern Britain. *Hydrobiol.*, 12, 129-141.

Reynoldson, T.B. (1958c). Triclads and lake typology in Northern Britain - Qualitative aspects. *Verh. int. Verein theor. angew. Limnol.*, 13, 320-330.

Reynoldson, T.B. (1964). Evidence for intra-specific competition in field populations of triclads. *J. Anim. Ecol.*, Jubilee Symposium, 33 (suppl.), 187-201.

Reynoldson, T.B. (1966a). The distribution and abundance of lake-dwelling triclads - towards a hypothesis. In: *Advances in ecological research*. Ed. Cragg, J.B., 3, pp. 1-71.

Reynoldson, T.B. (1966b). Preliminary laboratory experiments on recruitment and mortality in triclad populations. *Verh. int. Verein theor. angew. Limnol.*, 16, 1621-1631.

Reynoldson, T.B. (1978). *A key to British species of freshwater triclads*. F.B.A. Key, No. 23 (2nd Ed).

Reynoldson, T.B. and Bellamy, L.S. (1970). The status of *Dugesia lugubris* and *D. polychroa* in Britain. *J. Zool.*,, 162, 157-177.

Reynoldson, T.B. and Bellamy, L.S. (1971). Intraspecific competition in lake-dwelling triclads. A labratory study. *Oikos*, 22, 315-328.

Reynoldson, T.B. and Jaques, R.M. (1976). Changes in the triclad fauna of some North Wales lakes during the 20 years up to 1973. *Nature in Wales*, 15, 73-80.

Reynoldson, T.B. and Young, J.O. (1963). The food of four species of lake-dwellind triclads. *J Anim. Ecol.*, 32, 175-191.

Reynoldson, T.B. and Young, J.O. (1966). The relationship between the distribution of *Dendrocoelum lacteum* and *Asellus aquaticus* in Britain and Fennoscandia. *Verh. int. Verein theor. angew. Limnol.*, 16, 1633-1639.

Reynoldson, T.B. and Sefton, A.D. (1976). The food of *Planaria torva* (Müller) (Turbellaria, Tricladida), a laboratory field study. *Freshw. Biol.*, 6, 383-393.

Ritz, D.A. (1980). Tolerance of intertidal amphipods to fluctuating conditions of salinity, oxygen, and copper. *J. mar. biol. Ass. U.K.*, 60, 489-498.

Rode, P. (1925) Résistance des planaires a la privation d'oxygène en fonction de l'éthologie. *C.R. Soc. Biol. Paris*, 92, 1269.

Roeder, K.D. (1965). Moths and ultrasound. *Sci. Am.*, 212 (4), 94-102.

Rogers, W. (1962). *The Nature of Parasitism*. Academic Press, New York.

Root, R.B. (1960). An estimate of the intrinsic rate of natural increase in the planarian *Dugesia tigrina*. *Ecol.*, 41, 369-372.

Russier-Delholm, R. (1974) Etude comparative de la tolérance aux baises tensions d oxygène chez deux planaires d'eux douce *Dugesia tigrina* et *Polycelis felina*. *Annls. Limnol.*, 10, 311-322.

Sander, F. (1973). A comparative study of respiration in two tropical marine polychaetes. *Comp. Biochem. Physiol.*, **46A**, 311-323.

Sawyer, C.N. (1947). Fertilisation of lakes by agricultural and urban drainage. *J. New Engl. Wat. Wks. Ass.*, **61**, 109-27.

Saz, H.J. (1971). Facultative anaerobiosis in the invertebrates: pathways and control systems. *Am. Zool.*, **11**, 125-135.

Schmassmann, H.J. (1951). Untersuchungen über Stoffhaushalt fliessender Gewässer. *Schweiz. Z. Hydrol.*, **13**, 300-335.

Scholander, P.F. (1960). Oxygen transpo through haemoglobin solutions. *S ɔce*, **131**, 585-590.

Schwoerbel, J. (1972). Produktionsbiologische aspekte in Fliessgewässern. *Verh. Dtche. Zool. Ges.*, **65**, 57-64.

Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). The estimation of glycogen with the Anthrone method. *Arch. Biochem. Biophys.*, **25**, 191-200.

Seub, J., Hipp, E. & Hoffmann, K.H. (1983). Oxygen consumption, glycogen content and the accumulation of metabolites in *Tubifex* during aerobic-anaerobic shift and under progressing anoxia. *Comp. Biochem. Physiol.*, **75A**, 557-562.

Shadin, V.I. (1956). Life in rivers. (Russian) *Jizni presnih vod S.S.S.R.*, **3**, 113-256.

Shepard, M.P. (1955). Resistance and tolerance of young speckled trout (*Salvelinus fontinalis*) to oxygen lack, with special reference to low oxygen acclimation. *J. Fish. Res. Board. Can.*, **12**, (3) 387-446.

Shirley, T.C. and Finney, A.M. (1977). Circadian rhythm of oxygen consumption in the marsh periwinkle, *Littorina irrotata* (Say.). *Comp. Biochem. Physiol.*, **59A**, 339-342.

Shumway, S.E. and Scott, T.M. (1983). The effects of anoxia and hydrogen sulphide on survival, activity and metabolic rate in the coot clam *Mulina lateralis* (Say). *J. Exp. Mar. Biol. Ecol.*, **71**, 135-146.

Silva, P.K. de (1976).The importance of mucus of *Dendrocoelum lacteum* (Müller) (Turbellaria, Tricladida) in community existence. *Arch. Hydrobiol.*, **78**, 363-374.

Spellberg, I.F. (1972). Temperature tolerances of Southeast Australian reptiles examined in relation to reptile thermoregulatory behaviour and distribution. *Oecologia*, **9**, 23-46.

Spoek, G. (1974). The relationship between blood haemocyanin level, oxygen uptake, and the heart-beat and scaphognathite-beat frequencies in the lobster *Homarus gammarus*. *Neth. J. Sea. Res.*, **8**, 1-26.

Steel, R.G. and Torrie, J.H. (1980). *Principles and proceedures of*

statistics. McGraw-Hill, New York.

Tang, P.S. (1933). Oxygen consumption as a function of oxygen pressure. *Q. Rev. Biol.*, 8, 260-274.

Taylor, A. C. (1976). The respiratory responses of *Carcinas maenas* to declining oxygen tension. *J. Exp. Biol.*, 65, 309-322.

Taylor, A.C. and Brand, A.R. (1975). A comparative study of the respiratory responses of the bivalves *Arctica islandica* (L.) and *Mytilus edulis* L. to declining oxygen tension. *Proc. R. Soc. Lond.*, 190B, 443-456.

Taylor, E., Butler, P. and Sherlock, C. (1973). The respiratory and cardiovascular changes associated with the emmersion response of *Carcinus maenas* (L.) during environmental hypoxia, at three different temperatures. *J. Comp. Physiol.*, 86, 95-115.

Taylor, E., Buttlet, P. and Al-Wassia, A. (1977). Some responses of the shore crab *Carcinas maenas* (L.) to progressive hypoxia at different acclimation temperatures and salinities. *J Comp. Physiol.*, 122, 391-402.

Taylor, M.C. (1960). *An ecological study of the triclad population of an Anglesey lake*. Diss. Ph.D., University of Wales.

Teal, J.M. amd Carey, F.G. (1967). The metabolism of marsh crabs under conditions of reduced oxygen pressure. *Physiol. Zool.*, 40, 83-91.

Theede, H. (1973). Comparative studies on the influence of oxygen deficiency and hydrogen sulphide on marine bottom invertebrates. *Neth. J. Sea Res.*, 7, 244-252.

Truchot, J.P. & Duhamel-Jouve, A. (1980). Oxygen and carbon dioxide in the marine environment: Diurnal and tidal changes in rock pools. *Respir. Physiol.*, 39, 241-254.

Ultsch, G.R. and Gros, G. (1978). Mucus as a diffusion barrier to oxygen: Possible role in O_2 uptake at low pH in carp (*Cyprinus carpio*) gills. *Comp. Biochem. Physiol.*, 62A, 685-689.

Vos, J., Bernaerts, F., and Declier, W. (1979). Aerobic and anaerobic respiration of adult *Artemia salina* L. acclimated to different oxygen concentrations. *Comp. Biochem. Physiol.*, 62A, 545-548.

Vugt, van, F. Mere, van der, P. and Bergh, van den, S.G. (1979). The formation of propionate and acetate as terminal processes in the energy metabolism of the adult liver fluke *Fasciola hepatica*. *Int. J. Biochem.*, 10, 11-18.

Wallengren, H. (1914). Physiologisch-biologische Studien über die Atmung bei den Arthropoden III. - *Lund Univ. Arsskr.*, 10: nr. 8.

Walshe, B.M. (1950). The function of haemoglobin in *Chironomus plumosus* under natural conditions. *J. Exp. Biol.*, 27, 73-95.

Weber, R.E. (1980). Functions of invertebrate haemoglobins with special reference to adaptations to environmental hypoxia. *Am. Zool.*, 20, 79-101.

Weider, L.J. and Lampert, W. (1985). Differential response of *Daphnia* genotypes to oxygen stress: respiration rates, haemoglobin content and low oxygen tolerance. *Oecologia*, 65, 487-491.

Welch, P.S. (1952). *Limnology*, 2nd ed, Mcgraw Hill Book Co., New York, N.Y., 538p.

Wells, R.M.G. (1980). *Invertebrate Respiration.* Studies in Biology, No. 127, Arnold, 72pp.

Wheatly, M.G. and Taylor, E.W. (1981). The effect of progressive hypoxia on heart-rate, ventilation, respiratory exchange, and acid-base status in the crayfish *Austropotamobius pallipes*. *J. Exp. Biol.*, 92, 125-141.

Whitehead, H. (1915). On a epizoic infusorian, *Trichodina steinii* Cl. et L. found on Turbellaria. *J. Quekett Microsc. Cl. Ser.*, 2, 12, 545.

Whitehead, H. (1922). The British freshwater planarians. *Essex. Nat.*, 21, 1-20.

Whitney, R.J. (1942). Diurnal fluctuations of oxygen and pH in two small ponds and a stream. *J. Exp. Biol.*, 19, 92-99.

Wiens, A.W. and Armitage, K. (1961). The oxygen consumption of the crayfish *Orconectes immunis* and *Orconectes nais* in response to temperature and to oxygen saturation. *Physiol. Zool.*, 34, 39-54.

Wieser, W. and Kanwisher, J. (1959). Respiration and anaerobic survival in some sea weed-inhabiting invertebrates. *Biol. Bull. mar. biol. lab., Woods Hole*, 128, 594-600.

Wigglesworth, V.B. (1965). *The principles of insect physiology.* Methuen, London.

Wilps,H. and Zebe, E. (1976). The end-products of anaerobic carbohydrate metabolism in the larvae of *Chironomus thummi thummi*. *J. Comp. Physiol.*, 113, 263-272.

Wolvekamp, H.P. and Waterman, T.H. (1960). Respiration. In: *The physiology of Crustacea*. Ed. Waterman, T.H., Vol 1, New york, Academic Press, pp. 35-100.

Woodiwiss, F.S. (1964). The biological system of stream identification used by the Trent Water Board. *Chemistry Industry*, 14, 443-447.

Woollhead, A.S. (1979). *Energy-partitioning strategies in triclad flatworms with different life cycles.* Unpublished Ph.D. Thesis, Universty of Glasgow.

Wright, J.F. (1972). The distribution of stream-dwelling triclads (Platyhelminthes) in North Wales. *Freshw. Biol.*, 2, 57-64.

Wright, J.F. (1974) Some factors affecting the distribution of *Crenobia alpina* (Dana), *Polycelis felina* (Dalyell) and *Phagocata vitta* (Duges) (Platyhelminthes) in Caernarvonshire, North Wales. *Freshw. Biol.*, 4, 31-59.

Young, J.O. (1963). *A quantitative ecological study of Dendrocoelum lacteum (Müller) (Turbellaria, Tricladida)*. Ph.D thesis, University of Wales.

Young J.O. and Harris, J.H. (1973). Another instance of haemoglobin in a flatworm (*Phaenocora typhlops* Vejdovsky 1880). *Freshw. Biol.*, 3, 85-88.

Young, J.O. and Reynoldson, T.B. (1965). A laboratory study of predation in lake-dwelling triclads. *Hydrobiol.*, 26, 307-313.

Zahner, R. (1959). Über die Bindung der mitteleuropäischen *Calopteryx*-arten (Odonata, Zygoptera) an den Lebensraum des strömende Wassers. *Int Ges. Hydrobiol.*, **44**, 51-130.

Zebe, E., Grieshaber, M. & Shöttler, U. (1980). Biotopbedingte und funktionsbedingte anaerobiose. Der energiestoffwechsel wirbelloser tiere bei sauerstoffmangel. *Biol. Z.*, 10, 175-182.

Zoeten, de, L.W., Posthuma, D. and Tipker, J. (1969). Intermediary metabolism of the liver fluke *Fasciola hepatica*. I Biosynthesis of propanoic acid. Hoppe Selyers. *Z. Physiol. Chem.*, 350, 683-690.

Zs.-Nagy, I. (1973). Adenosine phosphate concentrations and carbohydrate consumption in the tissues of *Anodonta cygnea* L. (Mollusca, Pelecypoda) under normal and anoxic conditions. *Acta. Biochim. biophys. Acad. Sci. Hung.*, 8, 143-151.

Zwaan, A. de. (1977). Anaerobic energy metabolism in bivalve molluscs. *Oceanogr. Mar. Biol. Ann. Rev.*, 15, 103-187.

Zwaan, A. de. and Wijsman, T.C.M. (1976). Anaerobic metabolism in Bivalvia. *Comp. Biochem. Physiol.*, 54B, 313-324.

APPENDICES

Appendix 1

Chemical Analysis of Water Samples from the River Kelvin And Allander Water.

Chemical Analysis of the River Kelvin

Date	18/8/83	30/8/83	4/10/83	14/11/83	24/11/83	14/02/84
Water Temp (^{o}C)	19.1	19.0	13.1	6.2	5.6	2.40
PO_2 (Torr)	260	165	177	162	157	163
B.O.D.	6.58	5.60	2.80	3.2	4.60	3.50
Suspended solids	13.1	8.0	22.8	22.3	20.1	9.7
pH	7.61	7.71	7.17	7.28	7.47	7.39
Ammonia	0.185	0.07	0.15	0.01	0.13	0.02
Nitrate	1.20	0.53	0.25	0.16	0.65	0.37

	15/3/84	12/4/84	15/5/84	07/6/84
Water temp	7.4	10.5	12.5	19.3
PO_2	152	172	150	234
B.O.D.	3.8	3.5	2.7	2.4
S.S.	12.3	17.5	15.0	9.6
pH	7.75	7.52	7.59	7.21
Ammonia	0.957	0.43	0.59	0.09
Nitrate	0.25	0.22	0.49	0.48

Chemical Analysis of the Allander Water

Date	18/8/83	30/8/83	4/10/83	14/11/83	24/11/83	14/02/84
Water Temp (^{o}C)	16.8	12.3	12.9	5.5	4.2	4.1
PO_2 (Torr)	37	94	154	152	147	161
B.O.D.	4.5	5.6	2.0	1.8	4.4	1.8
Suspended solids	2.6	22.4	11.2	3.3	18.7	8.7
pH	7.36	7.03	6.81	7.35	7.15	7.1
Ammonia	0.65	0.14	0.06	1.05	1.80	0.15
Nitrate	0.29	0.23	0.125	0.12	0.115	0.175

	15/3/84	12/4/84	15/5/84	07/6/84
Water temp	4.8	5.7	10.4	19.5
PO_2	152	176	73	91
B.O.D.	4.5	2.0	-	4.9
S.S.	9.7	12.9	9.5	6.9
pH	7.35	7.18	7.13	7.02
Ammonia	1.65	0.55	1.60	0.83
Nitrate	0.175	0.195	0.09	0.06

Results for B.O.D., S.S., Ammonia, and Nitrate are given in mgl^{-1}