Studies on temperature acclimation in the freshwater pulmonate mollusc Lymnaea stagnalis (L.)

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STUDIES ON TEMPERATURE ACCLIMATION IN THE FRESHWATER PULMONATE MOLLUSC *LYMNAEA STAGNALIS* (L.)

by

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P.T.C. Harrison

ABSTRACT

This work is concerned with processes of thermal acclimation in the freshwater pulmonate, Lymnaea stagnalis. Three physiological functions were studied: heart rate, rate of oxygen consumption and assimilation efficiency. Seasonal changes in rate-temperature curves of the first two processes were investigated and compared with alterations induced by exposure to constant temperatures in the laboratory. Simple comparisons were made to determine whether season affected assimilation efficiency. The aims of the investigation were to show whether the measured physiological functions exhibited acclimatory responses, to determine the precise nature and inter-relationship of any such adaptations, and to suggest possible mechanisms responsible for the changes.

It was found that changes in the heart rate-temperature relation were induced both by season and by laboratory acclimation. Results of the seasonal study showed apparent capacity adaptations, so that winter animals had a higher heart rate than summer animals at temperatures between 15 and 25°C, and also resistance adaptations, which gave summer animals increased resistance to heat and winter animals greater tolerance of cold. Laboratory acclimation induced resistance adaptations at both temperature extremes but capacity adaptation was absent. Observed bimodality in heart rate-temperature curves of both studies indicated that control of heart rate is complex. These results are discussed further with reference to changes in physiological mechanisms.

Seasonal changes occurred in the size-rate regression for oxygen consumption and in the general shape of the rate-temperature curves. There was evidence for a 'reverse acclimation' in response to seasonal changes in temperature. These seasonal responses were not produced, however, by exposure to constant temperature in the laboratory. It is proposed that the observed changes resulted essentially from reproductive activity and seasonal changes in dietary conditions. Hormonal influences are thought to be most important in mediating these changes.

No significant differences were found in the assimilation efficiencies of winter and summer snails. Results of this and other studies suggest that the assimilation function does not show acclimatory changes in response to either temperature or season.

The results are discussed in relation to the known biology of Lymnaea stagnalis and with reference to fundamental aspects of temperature acclimation.

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I was in receipt of an S.R.C. studentship throughout the course of this research.

CONTENTS

Additional Material (back cover pocket): Reprints of two papers containing results derived from the present study:-

Harrison, P.T.C. (1977) Seasonal changes in the heart rate of the freshwater pulmonate Lymnaea stagnalis (L.). Comp. Biochem. Physiol. 58A, 37-41.

Harrison, P.T.C. (1977) Laboratory induced changes in the heart rate of Lymnaea stagnalis (L.). Comp. Biochem. Physiol. 58A, 43-46.

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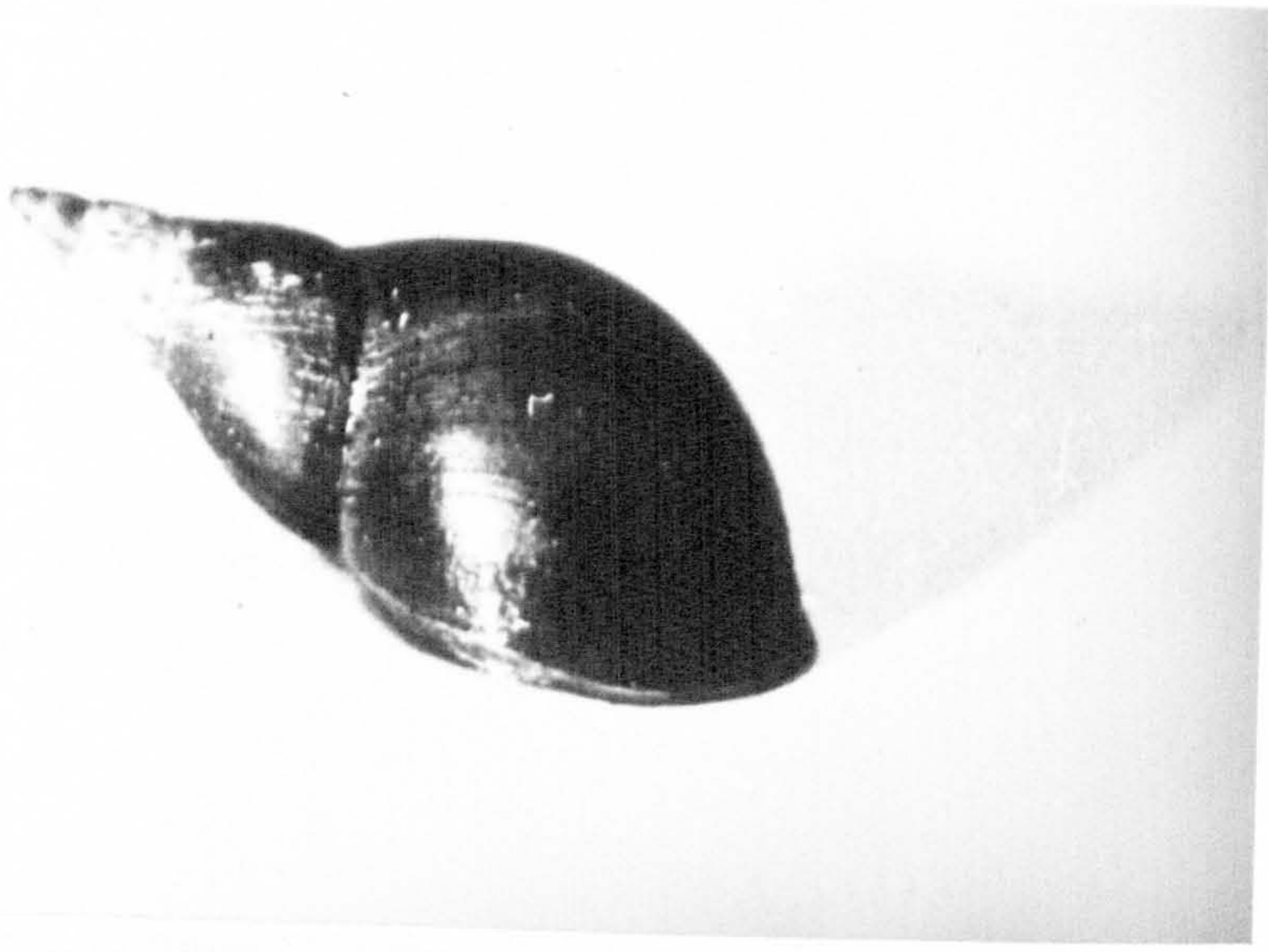
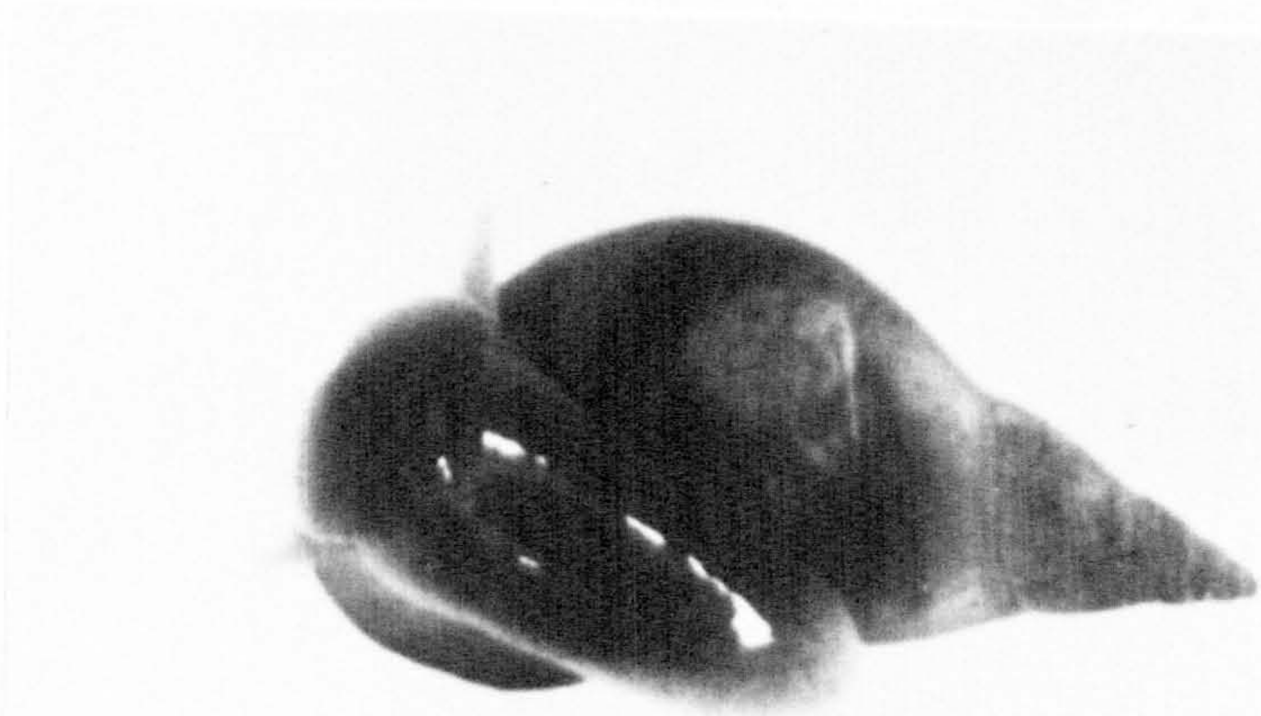




CHAPTER I

INTRODUCTION

Brief aims of the study

It is now well established that poikilotherms frequently exhibit compensatory changes in various metabolic rate functions in response to temperatures encountered in various spatial and temporal habitats. Such adaptations may also be induced by exposure to constant conditions in the laboratory, and are frequently described by the term 'thermal acclimation'.

This study aims to determine whether seasonal changes occur in three basic physiological processes of the great pond snail Lymnaea stagnalis (L.) (heart rate, oxygen consumption and assimilation) and to discover if any of these changes may be induced by exposure to constant temperature in the laboratory. It is also intended to identify some physiological mechanisms responsible for the observed changes and to relate the findings to the known ecology of the animal.

Thermal acclimation

It is accepted that, in general terms, temperature affects the rate of physiological processes in a similar manner to the way in which it influences chemical reaction velocity. Snyder (1906) found that the heart rate of a nudibranch mollusc exhibited changes with temperature according to the formula of Arrhenius: $\log K = -\frac{A}{T} + B$, where A and B are constants and K is the measured rate at T^{o} Absolute. Krogh (1914), in reviewing the influence of temperature on respiratory exchange, distinguished between the influence upon the central nervous system and that upon the metabolic processes in the tissues themselves. When

studied under standard conditions with nervous influences abolished, the effect of temperature on the metabolism of an animal was indeed shown to be regular. Crozier (1924) attempted to standardize respiration measurements using 'critical thermal increments', being defined as log CO_2 production/$\frac{1}{T^o}$ Abs. Later studies (Crozier and Stier, 1926) showed various modifications of this rate-temperature characteristic according to seasonal or experimental conditions. Belehradek (1930) reviewed the application of mathematical formulae (including Berthelot's exponential rule, the Q_{10} relation and the Van't Hoff-Arrhenius Law) and deduced that attempts to apply such chemical temperature-velocity formulae to biological data were invalid. Nevertheless, Crozier and Stier's data had suggested that real changes in metabolic rate-temperature relations could be induced by changes in external conditions.

Despite Belehradek's and, later, Mellanby's (1940) criticisms of the use of Q_{10} in analysis of rate-temperature relations, this remained popular. Rao and Bullock (1954), holding rigidly to the use of Q_{10}, considered in more detail the effects of size and adaptation temperature on metabolism and quoted several cases where Q_{10} was found to increase with increased temperature of adaptation. Later studies stressed the importance of considering the whole metabolic rate-temperature curve in such comparisons (for example, Precht, 1958). With regard to effects of body size on respiration rate, various theories were proposed, stating that metabolic rate was proportional to surface area (the 'Surface Law'), body weight or volume, or an intermediate function. Expressed in the form $R = aW^b$, where R = rate, W = size (e.g. weight) and a and b are constants, the value of the exponent 'b' was generally

found to lie between 0.67 and 0.75 (see Kleiber's (1961) review on relations between body size and metabolic rate, also Zeuthen, 1947, 1953; Hemmingsen, 1950, 1960; Bertalanffy, 1951 and Rao and Bullock, 1954).

Acceptance of true and constant relationships between metabolism and size allowed more meaningful comparisons to be undertaken, and acclimation studies became popular in the analysis of metabolic rate-temperature relations and the search for causal factors in temperature adaptation.

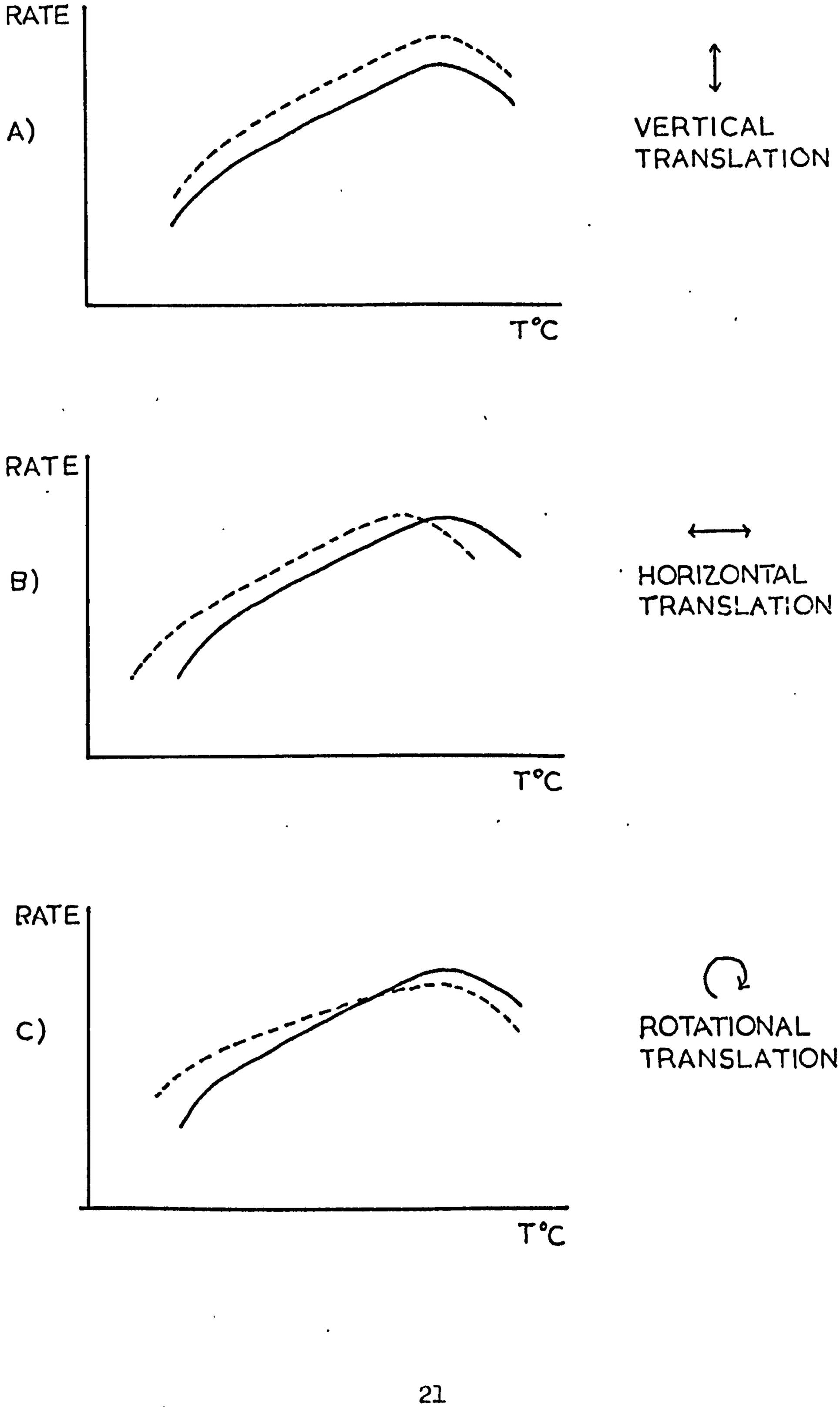
Studies on acclimation effects in poikilotherms have been well reviewed by various authors. Initial interest lay in the differences found between arctic and tropical species. Although exposed to widely different environmental temperatures, cold-blooded animals can maintain comparable levels of activity at different latitudes. This was first fully investigated by Fox and Wingfield (Fox, 1936, 1938, 1939; Wingfield, 1939; Fox and Wingfield, 1937).

Respiratory movements and heart-beat of various crustaceans were observed, also oxygen consumption rates of polychaete worms and echinoderms, and gill movements of scallops. Various adaptations were observed in these animals according to their latitude of origin. Similar studies on arctic and tropical species were performed by Scholander et. al. (1953), who investigated comparative rates of oxygen consumption in various animals, including fish, crustaceans, molluscs and insects. These authors in most cases found very considerable, though not complete, metabolic adaptation in the arctic forms relative to tropical forms. More recently, Wohlschag (1957), Vernberg and Vernberg (1964) and Lee and Fenchel (1972) have investigated latitude effects in further detail.

Seasonal and laboratory-induced changes in metabolic rate functions have been broadly investigated in many different poikilotherms and these studies have been extensively reviewed, for example, by Bullock (1955), Precht et. al. (1955, 1973); Precht (1958); Prosser (1958); Fry (1958, 1964), Kinne (1964); Newell (1966); McWhinnie (1967); Weiser (1973) and Vernberg and Vernberg (1975). Segal's (1961) review pays specific attention to acclimation in the Mollusca. Most authors attempt physiological explanations for their findings and Newell in particular (1969, 1973, 1975) has proposed some possible mechanisms for respiratory acclimation, whilst biochemical changes at the subcellular level have been detailed, for example, by Hochachka and Somero (Hochachka, 1965, 1967, 1973; Somero, 1969; Hochachka and Somero, 1968, 1973).

The earliest and most useful reviews on non-genetic compensation in poikilotherms were written by Bullock (1955), Prosser (1955) and Precht et. al. (1955). These formed a sound basis for later work and were followed by similar reviews written by Fry (1958), Precht (1958), Prosser (1958) and Kinne (1964). These articles are particularly useful for explanations and definitions of terms. Initially there was much confusion over the use of terms describing metabolic temperature compensation, and 'adaptation', 'acclimation' and 'acclimatization' all had common usage in the literature. Various descriptions were also given to different types of metabolic compensation. In general the terms adopted by Precht (1958) will be used in this study, except that 'acclimation' is to be considered in all senses as particular subjection or adjustment to temperature and use of the term 'acclimatization', which is normally applied to seasonal changes, will be avoided.

FIG. 1 THEORETICAL CHANGES IN R-T CURVES CAUSED BY THERMAL ACCLIMATION



Acclimation responses are considered to comprise of two basic and separate features, namely 'capacity adaptation' and 'resistance adaptation'. Changes in the shape of rate-temperature curves caused by acclimation may consist of vertical or horizontal translation, a rotation of the whole curve, or a combination of such movements (see Fig. 1). As a result of these changes there may be an adaptation within the normal temperature range, manifesting itself as an apparent vertical shift in the curve, called a 'capacity adaptation', or there may be adaptations to extreme temperatures at either end of the rate-temperature curve, called 'resistance adaptations', which result in increased or reduced sensitivity to high or low temperatures. Resistance adaptations may be coupled with capacity adaptations, but the former are more common than the latter. Few papers exist in which both adaptation types are examined together in the same species. Precht (1958) and Prosser (1958) both categorised capacity adaptations according to the comparative shapes of resultant rate-temperature curves, including 'ideal', 'missing', 'inverse'/'reverse' and other types. Here, capacity adaptations, if present, will be referred to simply as 'normal' or 'reverse'.

This study will show whether apparent thermal acclimation of any type occurs in response to season and, if it occurs, whether it can be reproduced by subjecting the experimental animals to constant temperatures in the laboratory. It will be seen that several factors other than temperature are important in observed seasonal changes.

The biology of Lymnaea stagnalis (L.)

1) Occurrence and general habitats:

The habitat of Lymnaea stagnalis in Britain is described by

Boycott and Oldham (1936) and briefly by McDonald (1969). This freshwater pulmonate snail, commonly called the great pond snail (Class: Gastropoda; Sub-class: Pulmonata; Order: Basommatophora; Family: Lymnaeidae), has a preference for large, open habitats, though it is not uncommon in cattle ponds and occurs sometimes in fairly swift rivers, especially in the south-east of England. It occurs throughout England except in the West, over most of Ireland and a few places in the South of Scotland. The chief considerations for aquatic Mollusca are calcium, for shell growth, and water which is not badly polluted by decaying organic matter. Foul media and food shortage are the most common growth-inhibiting factors in L. stagnalis. Effects of over-crowding have been studied by Turner (1927) and Thomas et. al. (1975). Generally speaking northern temperate freshwater molluscs are active, grow and breed only in late spring and summer. Often the most important factor to the snails is the temperature in the shallow weedy water at the edge of the pond or river. Lymnaea stagnalis mostly lives in this zone, except in the colder months. The water here readily warms up in the sunshine until, particularly in summer, the mean temperature may exceed that of the air. Some temperature characteristics of the pond where the animals for the present study were collected are presented in a later section of this chapter. In winter L. stagnalis can endure low temperatures, and although the snails normally hibernate in the mud or deeper water during the colder months, they are sometimes seen crawling about under ice and may be frozen into ice for a long time without being killed.

With regard to reproductive activity, the animals can breed long before attainment of full size. The breeding season ~~begins~~ begins in spring

and is continuous throughout summer, during which time the gonads increase greatly in size. The somatic growth of L. stagnalis begins at a moderate rate, after which follows a period of quickened growth until the older the animal the slower the growth. This results in a sigmoidal growth curve which is very much related to season. Young snails appear able to assimilate food and grow only when the water attains a temperature of about 11°C, (Taylor, 1894; Ellis, 1926; Crabb, 1929; Wilbur and Owen, 1964; Berrie, 1965). Maximum shell length normally attained by L. stagnalis is approximately 5 cm.

Lymnaea stagnalis obtains its food from three main sources: feeding on rooted submerged vegetation, raking small pieces of food from the surface film, and scraping material from rocks, etc. In addition, these animals are known to feed on dead animal remains and in this sense may be considered to be omnivorous. Certainly the diet is very varied.

Being a pulmonate, L. stagnalis is able to respire atmospheric oxygen and does so even when dissolved pO_2 is high. When the oxygen tension of the pulmonary cavity falls to a low level a negative geotropic response obtains (mediated by paired statocysts) and the snail ascends to the surface. When the cavity is filled the geotropic reaction becomes positive and the snail descends. When oxygen tension is low, in summer for example, the snail must visit the surface very frequently. There may, therefore, be a conflict in behaviour between feeding and refilling the pulmonary cavity (Jones, 1961; McDonald, 1969). Strategic aspects of time allocation in the ecology of freshwater pulmonate snails have been studied in some detail by Townsend (1975). Bovbjerg (1975) studied the behaviour of Lymnaea stagnalis in relation to three experimental environmental factors, namely presence or absence

of vegetation, of animal carrion and a thermal gradient. In the presence of single factors the snails responded with a kinesis to an aggregation on vegetation, a negative thermotaxis from high temperature and a strong chemotaxis towards carrion. In combination, vegetation tended to slightly mask the aggregations on carrion in cooler water.

The life-span of L. stagnalis in the natural situation is normally one or two years, although some suggestions of three to four years have been made (Barrie, 1965; McDonald, 1969). Normally the adults lay eggs in the latter part of the summer, the eggs hatch in the autumn and the newly hatched snails survive the winter, resume growth and reach sexual maturity by the end of summer (McDonald, 1969). The animals never pass the winter as eggs (Boycott and Oldham, 1936). Certainly in the present investigation it was noted that over-wintering populations consist essentially of very small snails, entering their first year of growth, and larger animals passing into their second year. Natural death appears to be the result of expansion of the gonad and degenerative changes in those internal organs not directly connected with reproduction (McDonald, 1969).

2) Lymnaea stagnalis as an experimental animal:

The great pond snail is common in South-East England and is easy to collect, store and feed. Moreover, it has been extensively studied and there are many reports on its use and its requirements for storage in laboratory conditions. Perhaps the most useful of these is that published by Noland and Carriker (1946) who observed the New World sub-species Lymnaea stagnalis appressa during twenty generations in laboratory culture. These authors described simple storage

conditions, consisting essentially of large tanks of aerated 'hard' water, regularly changed and furnished with some fine sand which is used in trituration of food in the gizzard. The snails were fed with lettuce leaves and, weekly, wheat cereal cooked in milk, supplemented with a balanced mixture of salts. Subsequent authors have suggested that lettuce alone is sufficient food. The present study adopts Noland and Carriker's basic method but the lettuce diet is supplemented with small amounts of TetraMin fish food (Tetra Werke, W. Germany), which consists of various natural dried substances and contains in all 46% protein, 5% fat and 8% fibre. Water used is London tap-water, which has a high salt content. This is chlorinated, but Noland and Carriker showed chlorination to be unimportant. More refined storage systems and associated influences of various artificial conditions, including food, light and temperature are discussed by Van der Steen (1967, 1969).

In agreement with Noland and Carriker's findings, L. stagnalis is shown to be an excellent experimental animal which, moreover, can be easily marked using alcohol-based fibre-tipped pens.

3) Processes under investigation:

(a) Heart rate

The anatomy of molluse hearts and the nature of blood circulation is described by Darwin (1876), Hill and Welsh (1966) and, specifically for Lymnaea stagnalis, by Bekius (1972).

All pulmonate molluscs have an open circulatory system in which movement of blood is at least partially dependent on a simple two chambered heart which receives blood from various sinuses and pumps

it out through arteries to circulate and again make its way back to the sinuses. The heart of L. stagnalis lies in a pericardium which is located on the left side of the body. It consists of a thin-walled auricle, which lies dorso-caudally and receives blood from the veno-pulmonalis, and a thick-walled ventricle which lies medio-ventrally and pumps blood into the aorta. The circulation is regulated by a pair of valves in the constriction between the two chambers and a crescent-shaped aortic valve which lies in the beginning of the aorta. It has been suggested (Krijgsman and Davaris, 1955) that the total volume of the pericardial fluid plus auricular contents must remain constant. When the auricle contracts the contents are transferred to the ventricle, a change which involves no alteration in the volume of the system; when the ventricle contracts its contents are transferred to the aorta and to preserve the total volume of the heart an equal volume of blood is sucked into the auricle from the veins, the pericardial fluid acting as intermediary.

The heart is the major, but not the only, propulsive mechanism for the circulation of blood. The buccal mass, for example, contains numerous venous spaces located within the muscles, and eating results in blood transport in this organ. The same holds for the gizzard, and movement of the entire animal may have an even greater impact on circulation.

The general physiology of the molluscan heart, including innervation mechanisms, has been discussed by various authors, most notably Carlson (1905a, b, c) and, more recently, Hill and Welsh (1966), Kerkut (1967) and Cardot (1971). It is accepted that heart beat is of myogenic origin, and there are localized pacemaker

regions. Existence of direct neuronal stimulation is debated, but certainly cardioregulation by neurohumoral agents is proved, these substances including acetylcholine, 5-hydroxytryptamine and catechol amines.

(b) Respiration

Basic aspects of respiration in pulmonates are adequately described by Fusser and Krüger (1951) and Chiretti (1966). Although oxygen uptake usually takes place through the lung, cutaneous respiration, by general diffusion through the moist skin, is always important to a greater or lesser extent depending on the animals' habitat and level of activity. The lung has evolved as a highly vascularized part of the mantle cavity in which relatively large quantities of blood may be brought into close proximity with air which fills the cavity. Pulmonates possess various modifications which minimize evaporation from the respiratory surface and also have a pumping mechanism for the renewal of air. The opening of the respiratory (mantle) cavity is reduced to a narrow hole, the pneumostome, and the muscular floor contracts rhythmically to aid gaseous exchange. Species such as Lymnaea visit the surface frequently to take in air, but are able to live for long periods of time (indeed for generations: see Noland and Reichel, 1943) completely submerged. In this case the mantle cavity functions as a gill, being filled with water rhythmically drawn into and expelled from the body. The blood of Lymnaea stagnalis does not contain a respiratory pigment.

The general relationship between respiration rate and body size has already been described as $R = aW^b$, or $\log R = b \log W + \log a$. b is the slope of the log size-rate regression and may vary from about

0.45 to 1.00. It is known that season can influence this parameter. Seasonal variations also occur in actual rates of oxygen consumption. Such changes will be discussed further in Chapter 3. Direct effects of temperature on metabolism have been previously discussed, but in Lymnaea stagnalis increased temperature, leading to increased rates of respiration, also causes more regular visits to the surface to replenish the air supply. Although, for example, a rise in temperature may cause a two-fold increase in oxygen consumption, the animal must surface more than three times as often at the higher temperature (McDonald, 1969).

Other factors affecting respiration in pulmonates include starvation and oxygen tension. It is known that in many poikilotherms starvation results in reduction in the rate of oxygen consumption. Changes in oxygen tension cause various metabolic responses. Some species of freshwater snail maintain their consumption relatively unchanged with decreasing oxygen content of the water until a critical point is reached, whilst in other species the respiratory rate decreases gradually in response to oxygen supply. Lymnaea stagnalis is thought to fall into the former category, with increased pulmonary uptake compensating for reduced cutaneous respiration (Jones, 1961).

Newell and Roy (1973) and Newell et. al. (1976) attempted to relate many variable factors affecting oxygen consumption in multiple regression equations. This was done for Ligia oceanica and the intertidal gastropod Littorina littorea.

(c) Digestion and assimilation

The morphology of the alimentary system of L. stagnalis has been described in detail by Carriker (1946, 1947) and briefly by

McDonald (1969). Carbohydrate metabolism in molluscs has been discussed by Goddard and Martin (1966) and Goudsmit (1972), and assimilation efficiency in various gastropods has been studied by Grahame (1973), Richardson (1975), Hargrave (1970, 1971), Kofoed (1975a, b) and Calow (1970). The diet and feeding habits of L. stagnalis have been previously discussed.

The digestive system is described briefly as follows: The mouth, bounded by oral lappets, opens into the buccal cavity housing the tongue-like odontophore and chitinous radula. The radula, lying over the heavily muscularized odontophore serves as a powerful abrasive tool to rasp and scrape food. Pieces of food are then passed into the buccal cavity where they are mixed with mucus which contains the digestive enzyme amylase which is produced by the granular cells in the secretory ducts of the salivary glands. Food particles pass from the buccal cavity through the oesophagus and the crop to the strongly muscularized gizzard, where sand acts as an effective tool for grinding food. The food particles are filtered out and passed through ciliated ducts into the bilobed hepatopancreas (midgut gland or disgestive gland) for intracellular digestion. Extracellular digestion occurs in the lumen of the disgestive tract. Indigestible material and large food particles are gathered into mucus strings which are carried out into the ciliated intestine where they are compressed into pellets and passed through the anus to the exterior. The two main mechanisms responsible for the passage of food through the digestive tract are ciliary action and muscular contraction. With the exception of the anterior portion of the buccal cavity and the gizzard the entire digestive tract is ciliated.

The occurrence of digestive enzymes in gastropods varies considerably (see Owen, 1966). In some species the only extracellular enzymes appear to be amylase and possibly a cellulase, whilst others' gastric juices contain proteases, lipases and carbohydrases. Which enzymes other than amylase are present in Lymnaea stagnalis is not known, but certainly the terrestrial pulmonate Helix pomatia contains a wide range of digestive enzymes (Myers and Northcote, 1958).

The collection site

The main collection site for animals used in these studies was a small shallow pond in open parkland at Chase Cross, near Romford, Essex (lat. 51° 36' N, long: 0° 12' E). This water body is approximately 80 yards long and 40 yards wide and is roughly rectangular with shallow grassy or muddy banks. It is bordered on one edge by scrubland but is not overhung by trees. Most of the aquatic vegetation is found near the extremities of the pond, being particularly dense at the Eastern and Northern edges. It is stocked with coarse fish, mostly rudd, roach, carp and perch.

General meteorological data for the area were collected about ¾ mile due South of the pond. Also, various temperature recordings of the pond and environs were made at different times of the year to determine relative temperature conditions of the pond. The meteorological data is shown in graphical form in Fig. 2a. The mean monthly temperature, mean maximum and minimum temperatures and extreme maximum and minimum temperatures are shown here for the period April 1975 to May 1976. Figure 2b is constructed from data derived from recordings made on seven days throughout the period August 1975 to July 1976 of maximum and minimum temperatures in the area of collection in the pond, and also

Fig. 2 (a) Local air temperature data for the period April 1975 to May 1976

Legend:

-.-.-.- Mean monthly temperature

——— Mean maximum and minimum temperatures

..... Extreme maximum and minimum temperatures

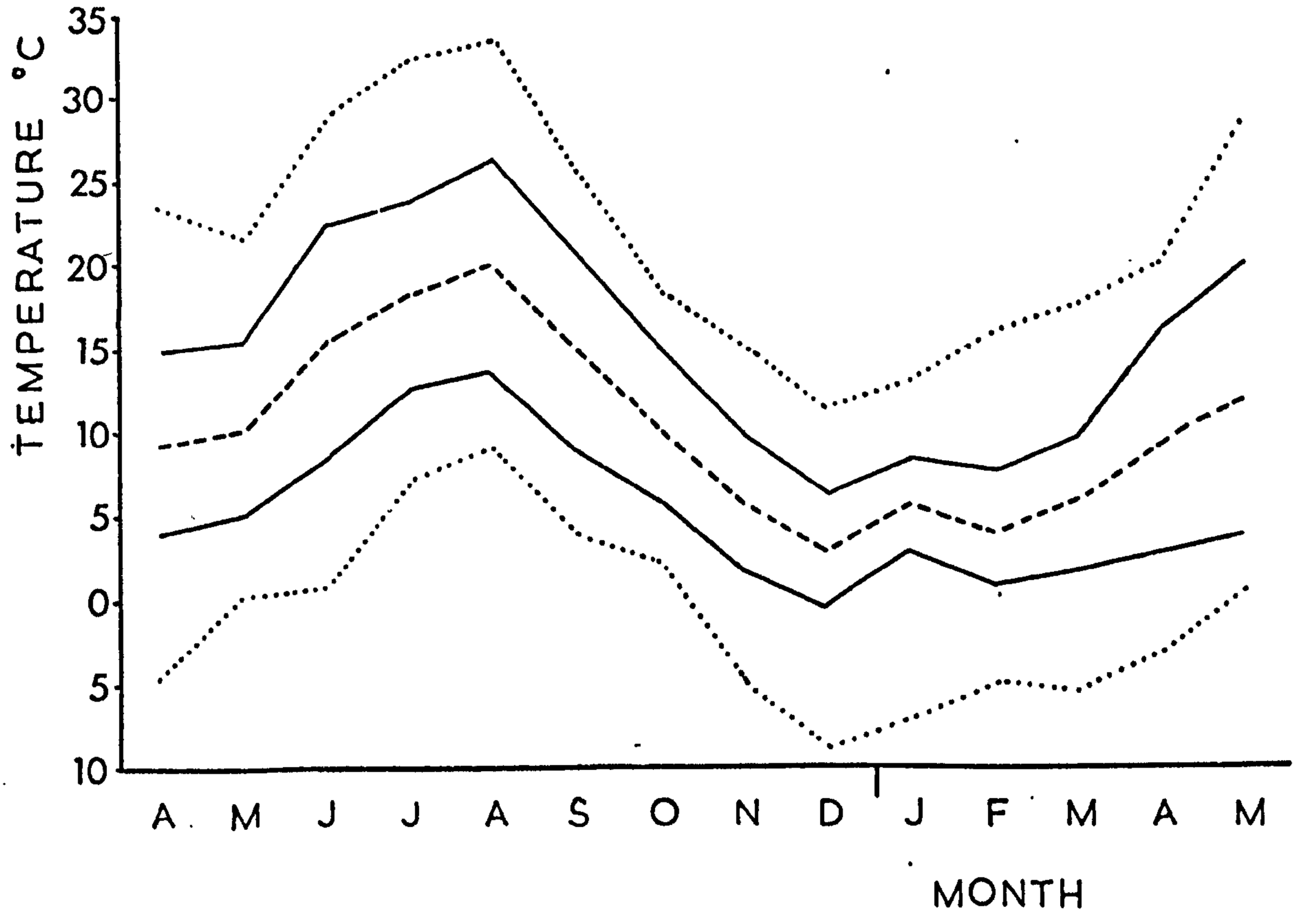
Fig. 2 (b) Maximum and minimum air and pond temperatures on seven occasions during the period August 1975 to July 1976

Legend:

——— } Maximum and minimum air temperatures

——— }

 Maximum and minimum water temperatures


TEMPERATURE °C
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30
25
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15
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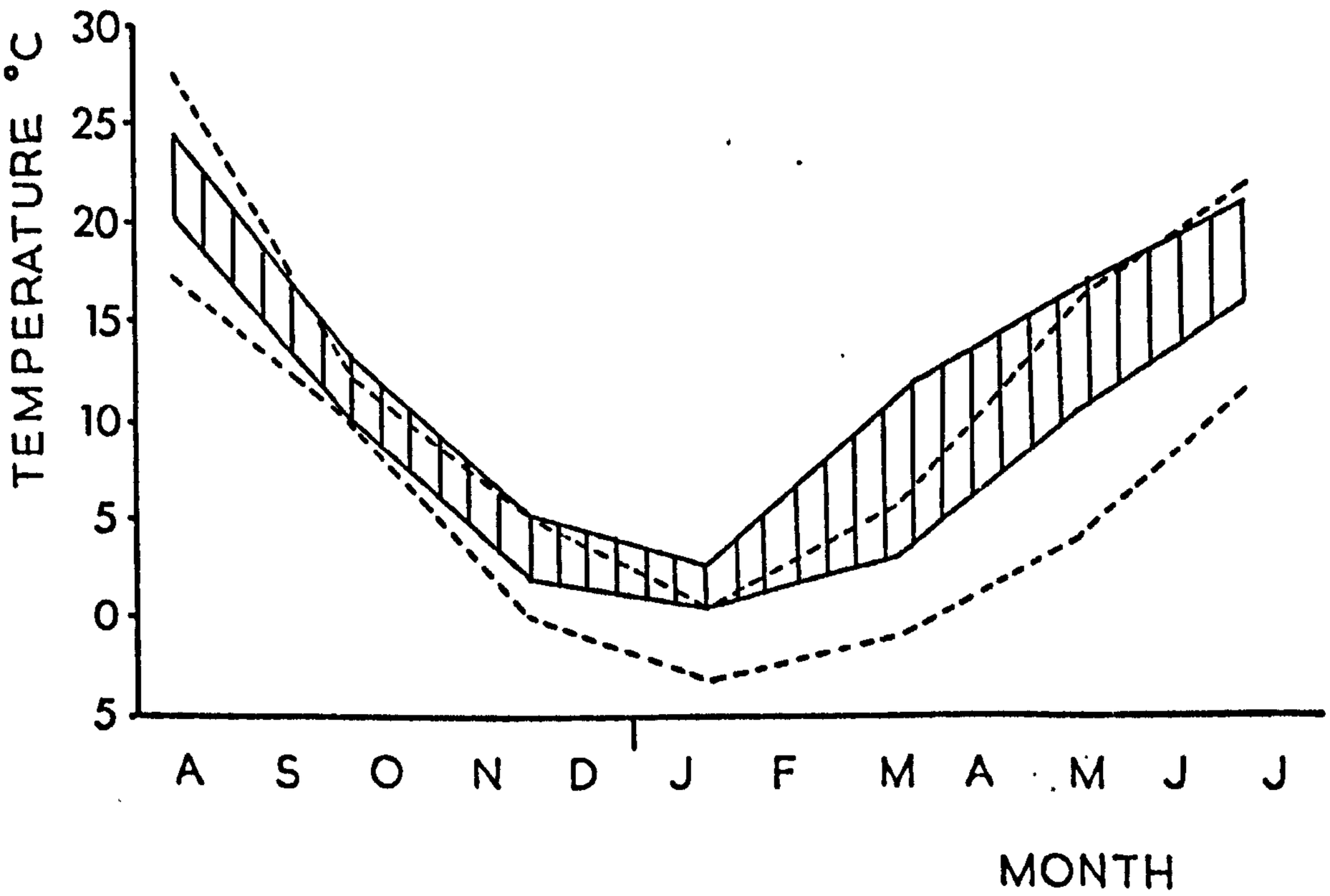
5
0
5
10
A M J J A S O N D J F M A M
MONTH


TEMPERATURE °C
30
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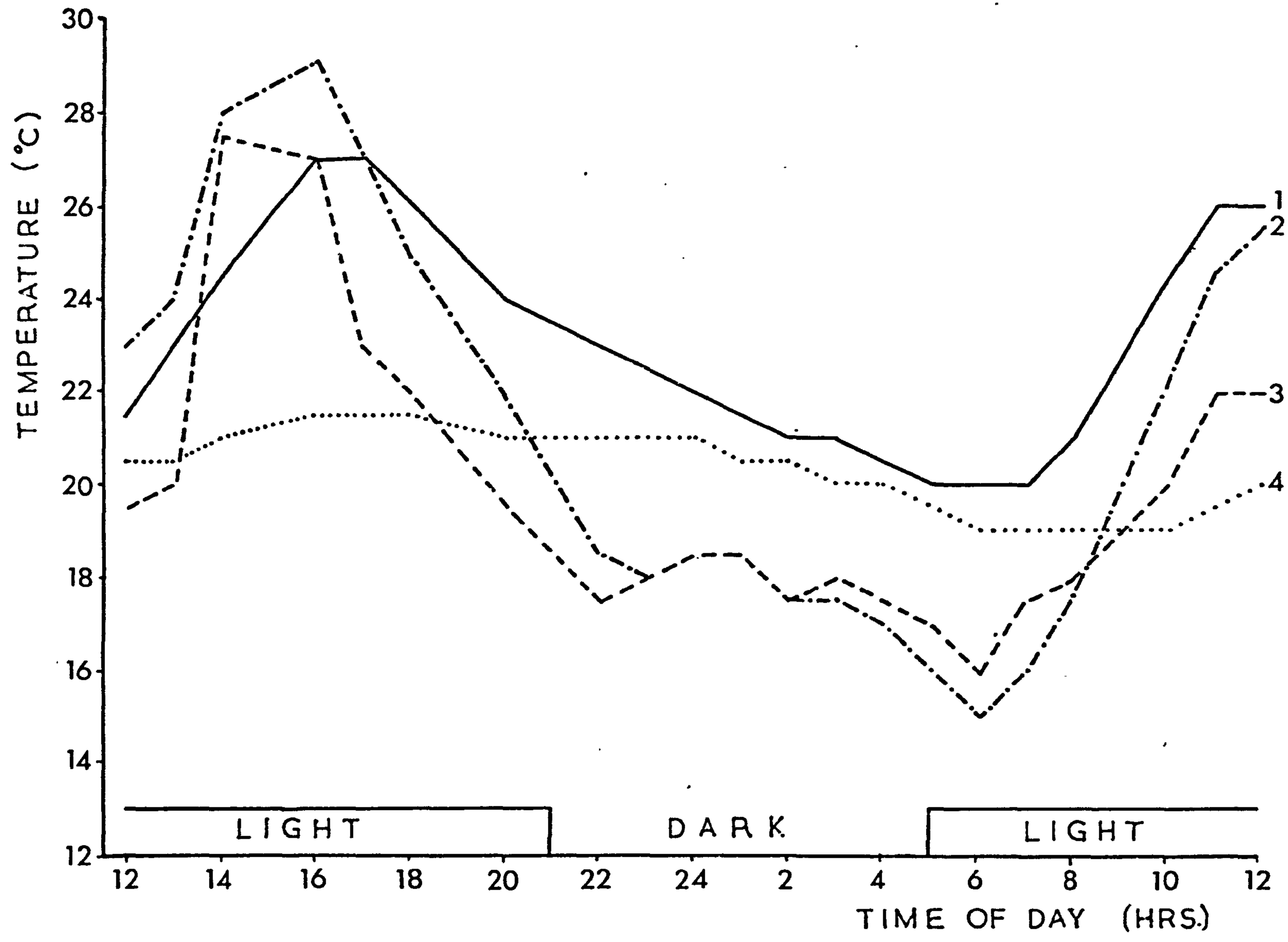
MONTH

Fig. 3 Various pond temperature characteristics recorded over a period of 24 hours (11th August 1975)

Legend:

1. _____ Area of collection
2. -.-.-. Extreme edge of pond
3. ------ Air temperature
4. ..... Mud temperature (10 cm depth)


TEMPERATURE (°C)
30
28
26
24
22
20
18
16
14
12
1
2
3
4
LIGHT
DARK
LIGHT
12
14
16
18
20
22
24
2
4
6
8
10
12
TIME OF DAY (HRS.)

maximum and minimum air temperatures. This was done to determine the approximate relationship between air temperature and water temperature so that the data shown in Fig. 2a could be better related to the pond situation. It is seen that the range of temperatures in the pond is generally slightly less than the range of air temperatures and that in the warmer months the former range lies within the latter. Also the pond temperatures tended to be higher than measured air temperatures. This is to be expected since water has a high thermal capacity and so retains heat during the night whilst absorbing heat from sunlight during the day. The meteorological data, then, is a good guide to pond temperature except that the range of mean maximum and minimum temperatures shown here (Fig. 2a) is likely to be slightly greater than that actually encountered in the pond. Extreme maximum and minimum air temperatures would likewise be somewhat moderated in the pond situation.

To follow in more detail the diurnal relationship between air temperature and pond temperature, recordings were made over 24 hours on a warm sunny summer day when maximum temperature fluctuations were likely to occur. Recordings were made of air temperature, mud temperature (at 10 cm depth at the edge of the pond), temperature of water in the main area of collection of the snails (about 6 ft. from the edge, well vegetated) and temperature of water at the extreme edge of the pond (shallow, no vegetation), using standard thermistor probes connected to a portable chart recorder. These data are shown in Fig. 3. It is seen that maximum diurnal temperature change occurs in water at the extreme edge of the pond. Clearly this area suffers most rapid heating and cooling through the day. Snails were generally not found here. Mud temperature, however, shows little temperature change and is therefore a moderating influence. The temperature of water in the area

of collection follows quite closely the diurnal changes in air temperature but is nearly always higher than air temperature, by a margin of about 3 or 4 degrees. Minimum temperature occurs shortly after dawn and maximum temperature in mid-afternoon. It is also seen, as before, that the range of air temperatures (16-28°C) is considerably greater than the range of temperatures in the area of collection (20-27°C).

In conclusion it may be stated that the meteorological (air temperature) data give a good indication of general seasonal changes in pond temperature, but the mean pond temperature (at area of collection of snails) is always slightly higher than mean air temperature, especially in winter, and the range of temperature is less particularly with regard to minimum temperatures.

Snails were collected using a broad 'shrimping' net. They were placed immediately in a Dewar vacuum flask containing a quantity of pond water and transported as soon as possible to the laboratory.

Animals used in the simple seasonal study of assimilation efficiency were collected from the Grand Union Canal at Mile End, London (lat. 51° 31' N, long. 0° 2' W). No temperature data are available.

Data Analysis

Statistical analysis of experimental data was performed with reference to three basic texts (Moroney, 1956; Simpson et. al., 1960; Snedecor and Cochran, 1967). Means and standard deviations were calculated according to standard formulae incorporating correction, where necessary, for small sample size. Comparison of means was

performed using the 'students' t-test'. 95% confidence intervals ($\bar{x} \pm \frac{t.\sigma}{\sqrt{n}}$) were plotted as error bars on graphs etc. to aid comparison of data points. The standard level of significance used throughout was p = 0.05 (95% level).

Further data processing, which is described in more detail in the relevant chapters, included correlation and regression analysis. Computer programs (Fortran language) were written as necessary. Other facilities used included a Hewlett-Packard programmable calculator with graph-plotter.

CHAPTER II

ACCLIMATION OF HEART RATE

INTRODUCTION

This section is concerned with seasonal and laboratory-induced changes in the relationship between heart rate and temperature in Lymnaea stagnalis. Like most physiological processes in poikilotherms, mollusc heart rate is increased by heating and slowed by cooling, within physiological ranges. Early investigations were undertaken by Snyder (1906), who attempted to quantify the relationship between heart rate and temperature in the nudibranch Phyllirhoe sp. using Arrhenius' formula, and by Crozier and Arey (1919) and Skramlik (1929). Many studies have since been made to investigate heart rate-temperature relations in various invertebrate animals. However, comparatively little information is available regarding thermal acclimation of this function either seasonally or as produced in the laboratory. The present study investigates the relationships between heart rate and temperature in L. stagnalis and determines whether these relationships change with season or are affected by thermal acclimation in the laboratory. Possible physiological mechanisms involved in control of heart rate are discussed.

BASIC METHOD AND MATERIALS

In the main seasonal and laboratory studies, heart rate was measured over full ranges of temperature, from 5°C to at least 30°C at intervals of 5°C. Peaks in the heart rate-temperature curves were more accurately defined, where necessary, by further recordings at 2.5°C intervals.

Snails used in these investigations were collected as required from the Essex pond described in Chapter I. They were removed to the laboratory and, where necessary, stored until use in an outside tank which was supplied with a slow flow-through of tap water to prevent stagnation. The animals were fed ad libitum with lettuce leaves and Tetramin fish food and were supplied with small quantities of fine sand, as previously described. Measurement of heart rates was normally completed within ten days for any particular group of animals. Random samples of ten or twenty snails were used for each experimental temperature. The shell length of each animal was measured with calipers.

The snails were mounted for observation by pushing the spire of the shell into a piece of plasticene held in a small glass stand, and measurements were carried out in aerated water at constant temperature. Temperature control was achieved by passing water from a Grant pumping water bath through coils of plastic tubing attached to the inside walls of the glass observation vessel. The mercury-contact thermostat thermometer was mounted inside this tank. Heart rate was measured by direct observation through the shell, the animals being illuminated from beneath by means of a 15 watt microscope lamp. The apparatus is shown in Fig. 4 and Plate 2 (b). The described method of mounting and observing the snails is an improvement on that used by Tsukuda and Ohsawa (1959) in their studies on heart rate-temperature relations in a red snail Physa sp.

Preliminary observations revealed that an initial increase in heart rate caused by the sudden increase in light intensity following switching on of the illuminating lamp was reduced to negligible

Fig.4 Heart-rate observation tank

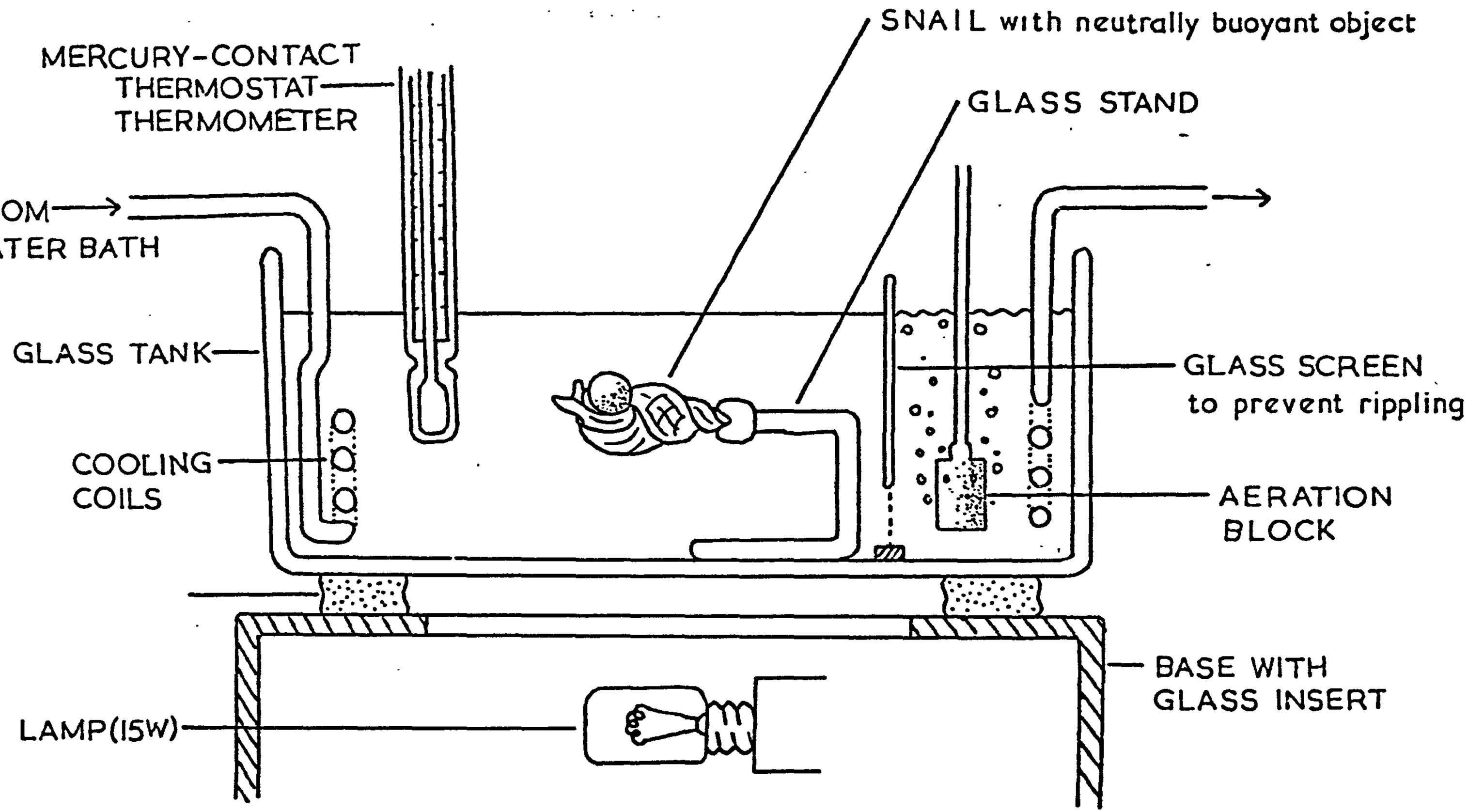
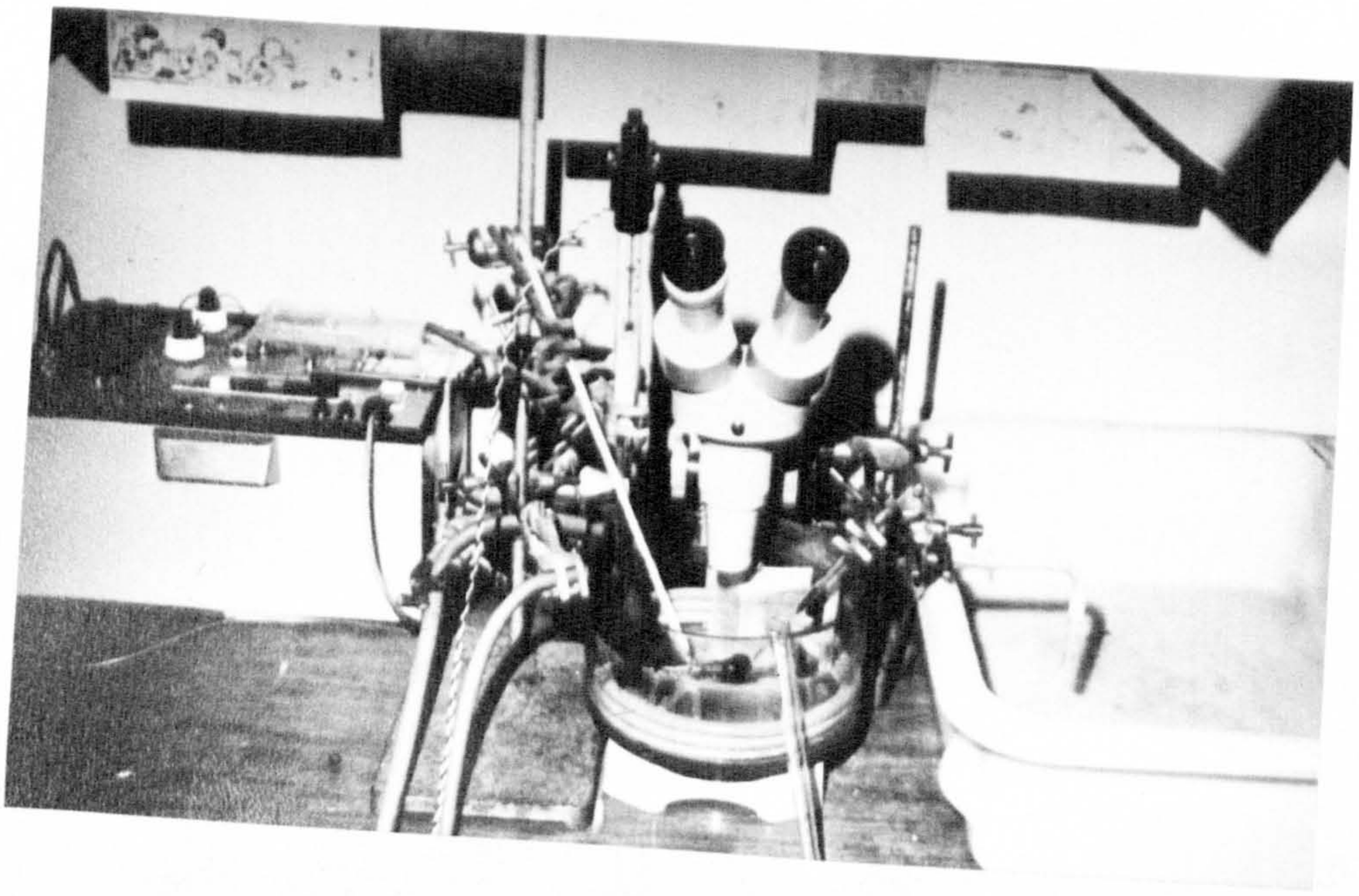


Plate 2: Heart rate observation apparatus

a) General view showing water change siphons, reservoir and chart recorder.

b) Close-up view showing cooling coils, thermometer, thermostat and thermistor, with snail in position. The air stone is hidden behind the ripple screen. N.B. Water was removed from the apparatus for the purpose of the photograph.



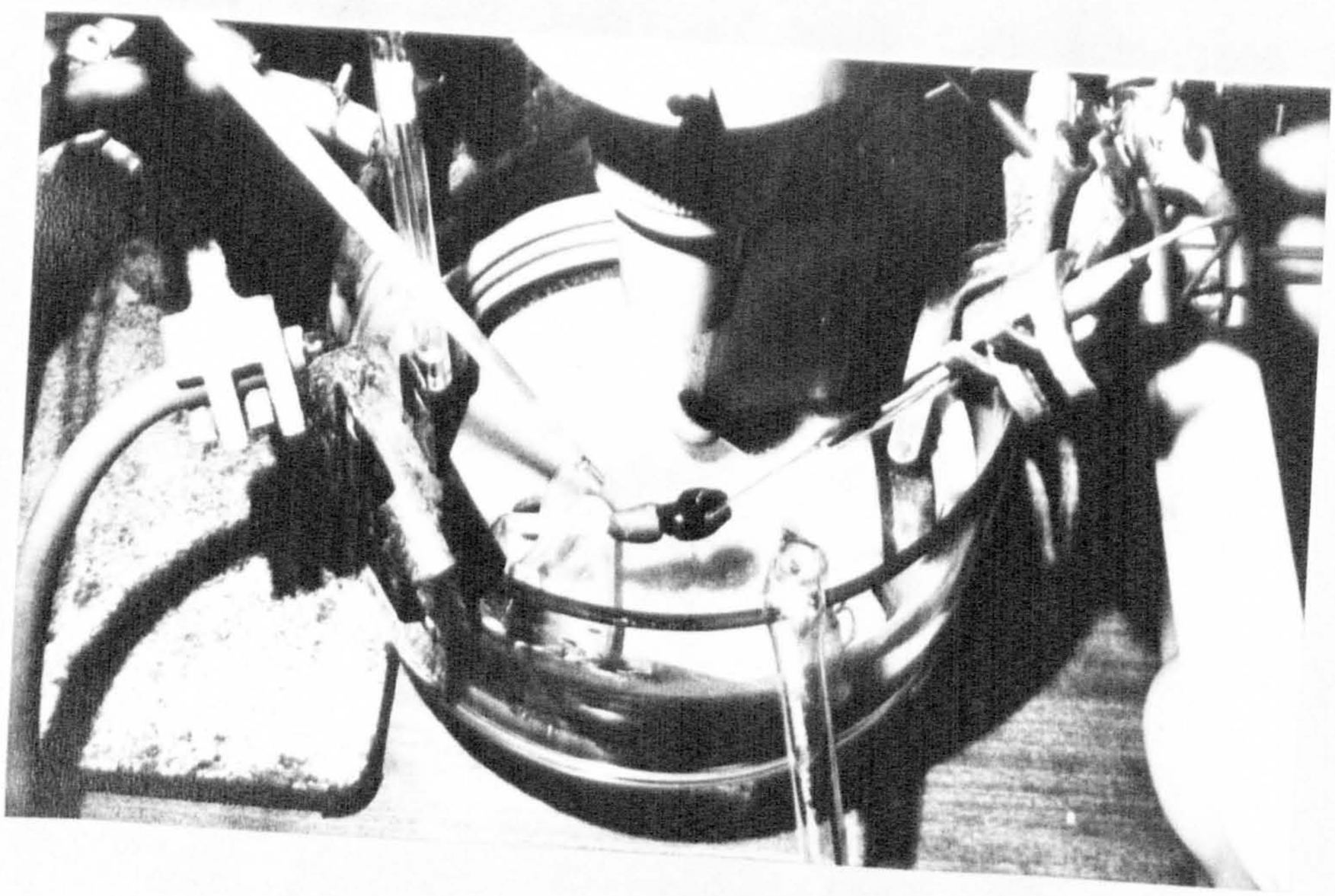
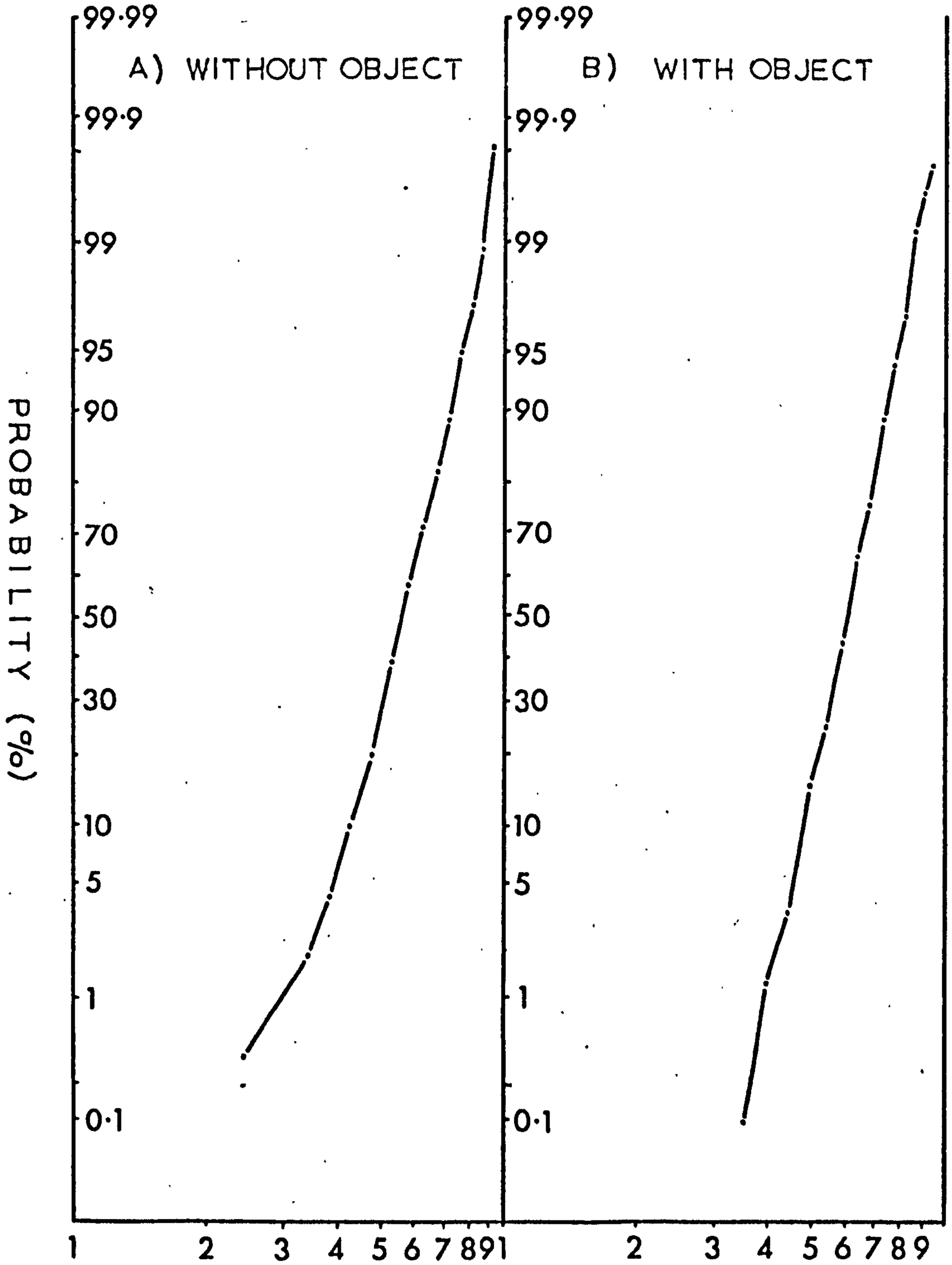


Table 1: Change in heart rate following switching-on of lamp

	½-minute interval number									
	1	2	3	4	5	6	7	8	9	10
Mean (no. of beats in 30 secs)	6.32	6.24	6.27	6.19	6.12	6.18	6.06	5.97	6.05	6.00
S.D.	1.38	1.32	1.27	1.29	1.21	1.16	1.22	1.19	1.07	1.11

Mean number of beats in 30 seconds with Standard Deviation.

FIG. 5 HEART BEAT FREQUENCY PROBABILITY PLOT



proportions within five minutes. These results, shown in Table 1, were derived from 30-second beat counts measured over five minutes at 10°C using 106 animals. To prevent the snails from extruding the foot and obscuring the heart from view, a small, neutrally buoyant, object was introduced to each animal under study. This was constructed from compressed Parafilm rolled into a ball, weighted with plasticene and covered with aluminium foil. Such practice resulted in a previously obtained normal distribution of heart rate measurements becoming skewed. A logarithmic transformation, however, returned the distribution to the normal form, as indicated in Fig. 5. The results for these graphs were derived from half-minute heart beat counts over a five-minute period for a random sample of 106 animals, measured at 10°C. The straight line in this log-probability plot implies normality (see Lewis and Taylor, 1967). All heart rate measurements and related statistics will therefore be expressed in logarithms (base 10), except in the preliminary experiments.

PRELIMINARY EXPERIMENTS

The first of the experiments described here was undertaken to investigate the direct dependence of heart rate on temperature. This was done by subjecting snails to gradual changes in experimental temperature. The second preliminary investigation seeks to determine the nature of changes in heart rate induced by rapid increases in exposure temperature. These initial studies were performed in order to verify that the described basic experimental method was practicable and free from erroneous assumptions; also to determine the required time lapse before measuring heart rates following immersion of the snail.

I). Effects of Slow Temperature Changes on Heart Rate

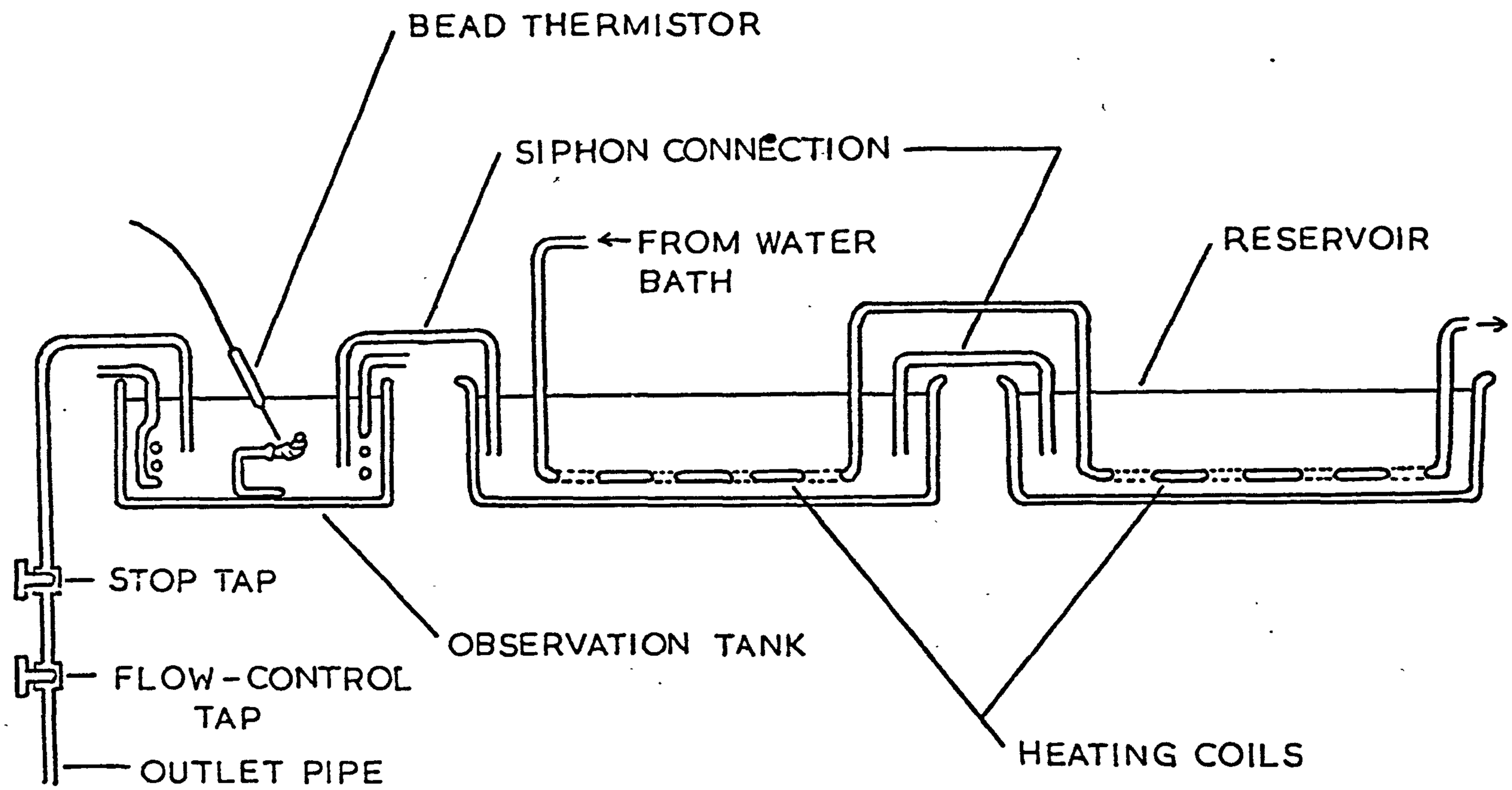
Introduction

When performing experiments concerning adaptation of metabolic functions it is necessary to complete recordings in as short a time as possible, before re-adaptation can occur. Measurement of heart rate can be performed very quickly. It is important, however, to ensure that any response to a temperature change is fully developed before measurements are begun, to allow for initial hystereses (see Spaargaren et. al., 1977). This experiment shows how heart rate of L. stagnalis changes as ambient temperature is slowly and steadily increased through a large range of temperature.

Method and Materials

The method of observation of heart rate is as described in the main methods section. The apparatus was adapted, however, to enable the temperature of water contained in the observation tank to be suitably raised. A microthermistor was incorporated for fast and accurate recording of temperature close to the snail. The calibration curve for this bead thermistor is shown in Appendix 1. The apparatus used in this investigation is shown in Fig. 6 and Plate 2 (a). The essential features of the apparatus are the siphons, which enabled water in the observation tank to be removed and replaced without physically disturbing the snail under study. The animals used in this study were collected in March 1975 and stored at 10°C until required. Experimental procedure was as follows: The temperature of water in the observation tank was controlled initially to 10°C, and that in the reservoir tanks controlled to 40°C. Heart rate recordings were then begun. After two

Fig.6 Slow temperature change apparatus



minutes the water bath controlling the observation tank temperature was switched off and the stop tap on the outlet siphon opened, with the flow-rate tap already adjusted to give the required rate of change. This caused a drop in water level in the observation tank, resulting in water, heated to 40°C, flowing from the reservoir tanks into the observation tank and raising the experimental temperature. Mixing was achieved by aeration. Heart rate recordings were continued until the water in the observation tank reached a temperature of about 35°C, this operation taking approximately eight minutes. Water temperature was monitored during this time on a Washington multi-channel chart recorder. An 'event marker' was used to record heart beats on the same chart. This procedure was repeated for five animals of different size.

Results

Information recorded on the Washington recorder was redrawn in simple graphical form, making use of the appropriate thermistor calibration curve (Appendix 1). The original traces were split into sub-units of 0.7 mins, for which mean temperature and heart rate were calculated. These graphs, represented in Fig. 7, show how temperature and heart rate changed with time for the five snails investigated. Shell length, initial heart rate at 10°C and temperature for peak heart rate are shown in Table 2.

Discussion

It has been shown in this study that heart rate in *L. stagnalis* is extremely temperature-dependent and that an upper thermal limit in the physiological range is marked by a decline in heart rate as

FIGURE 7 HEART RATE CHANGES INDUCED BY SLOW INCREASE OF EXPOSURE TEMPERATURE

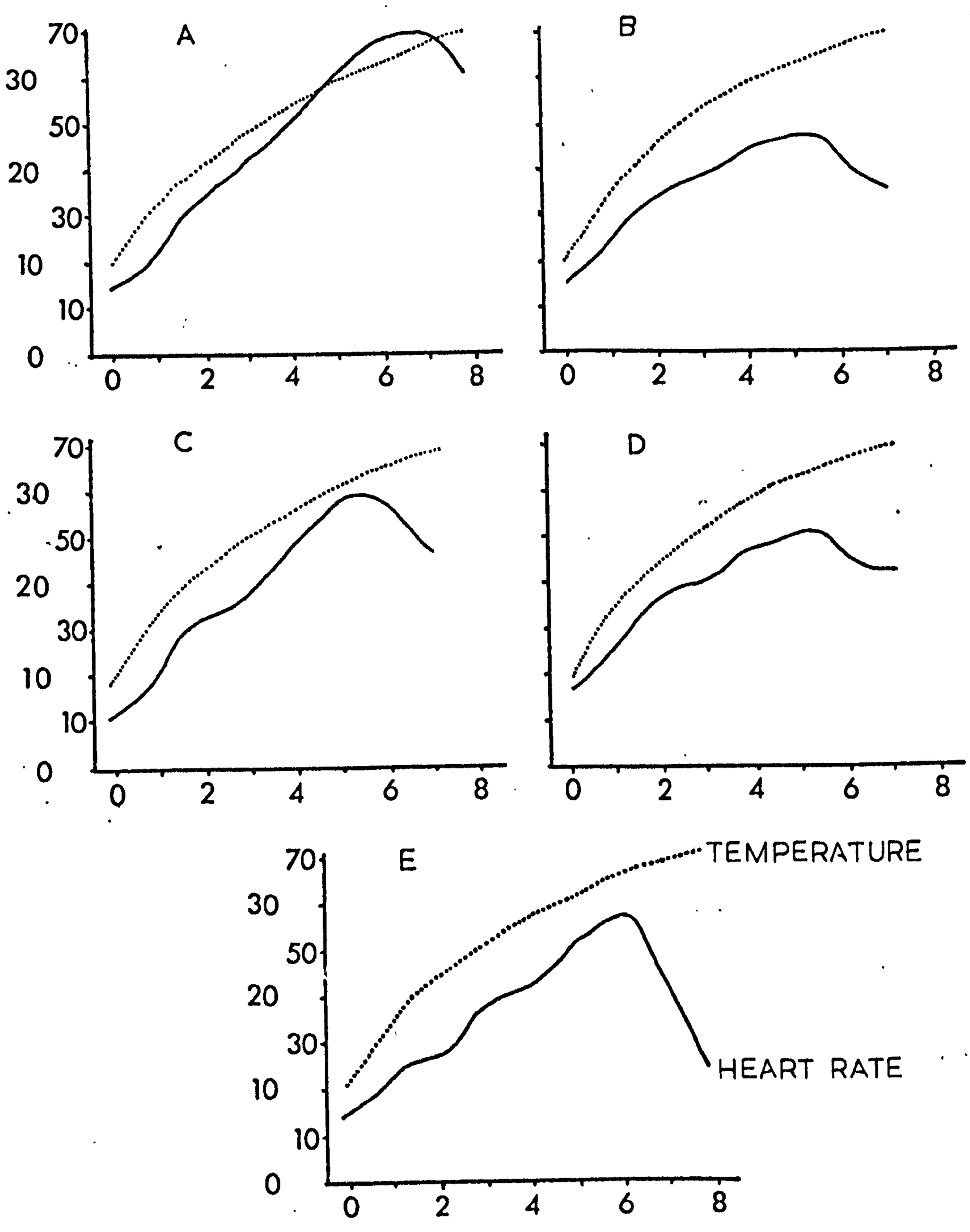


Table 2: Shell length and initial heart rate of animals used in Preliminary Experiment I

Animal No.	Shell length (cm)	Initial heart rate at 10°C (beats/min)
A	1.54	15.0
B	2.37	11.5
C	2.09	15.5
D	1.88	14.5
E	2.13	15.5

temperature increases beyond this point (Fig. 7). Orr (1955) describes in some detail the physiological effects of high temperature on heart beat in the frog Rana pipiens. The slow temperature increases in the present investigation were closely followed by changes in heart rate. All five graphs shown are similar in shape, with differences in the relative gradients of the temperature and heart rate curves, and in actual peak heart rate values. The temperature for peak heart rate, to be referred to as the upper thermal limit (U.T.L.) was similar for all snails studied here, ranging between 30.8 and 33.5°C. No obvious association is evident between observed differences and size of animal used.

II. Effects of Rapid Temperature Changes on Heart Rate

Introduction

The previous study showed that slow increases in temperature, within a physiological range, were quickly and smoothly followed by increases in rate of heart beat. The chosen method for the main investigation, however, necessarily involved sudden temperature changes, resulting from rapid immersion of animals into the temperature-controlled observation tank. The experiments described here investigate heart rate responses to abrupt increases in exposure temperature. Segal (1962) and Spaargaren et. al. (1977) have described some such changes in invertebrate heart rates, and it is known that a degree of 'hunting' or oscillation of rate frequently occurs following sudden temperature changes. It was necessary to determine if such oscillations occur in L. stagnalis and if so, to discover their duration so that sufficient time could be allowed, in the experimental method proper, for stabilization of heart rate before commencement of recordings.

Method

The method for observation of heart rate was as previously described. Stepwise increases in experimental temperature were achieved by adding to the observation tank 500 ml aliquots of water heated to 50°C. Mixing was achieved by aeration. Five animals, which were collected in March 1975 and stored at 10°C, were used in this investigation. Water in the observation tank was controlled to 10°C, the pumping water bath being switched off immediately before addition of the hot water. Two consecutive temperature increases were induced, from 10°C to approximately 18°C and thence to about 25°C. Heart rate was recorded for two minutes before addition of the first aliquot of heated water. The second aliquot was added 4½ minutes later and heart rate followed for a further 4½ minutes. As before, temperature was measured using a microthermistor and recorded simultaneously with heart beats on a Washington multi-channel recorder. The experiments were repeated on animals acclimated to 5 and 20°C; these results are not shown here but will be briefly discussed below.

Results

Results are shown in graphical form in Fig. 8. Heart rates were calculated for each succeeding 30 second period during the experiment. Temperature was continually recorded. The original trace was transcribed using the thermistor calibration curve previously obtained (see Appendix 1). The shell length of animals used here and their initial heart rates at 10°C are shown in Table 3. The results show considerable individual variation but do indicate certain regular tendencies with no obvious size effects.

FIGURE 8 HEART RATE CHANGES INDUCED BY RAPID INCREASES IN EXPOSURE TEMPERATURE

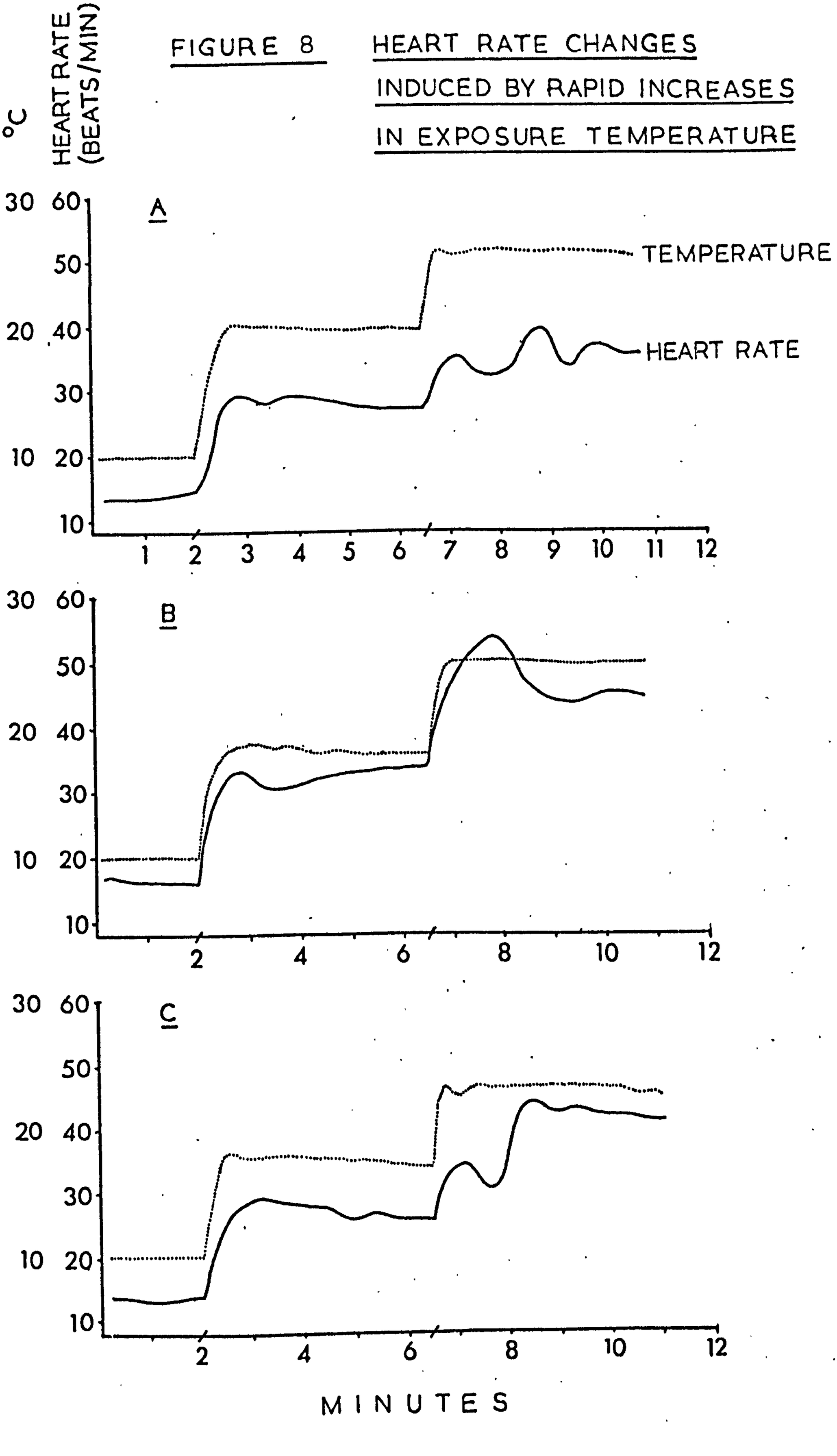


FIGURE 8 (CONTINUED)

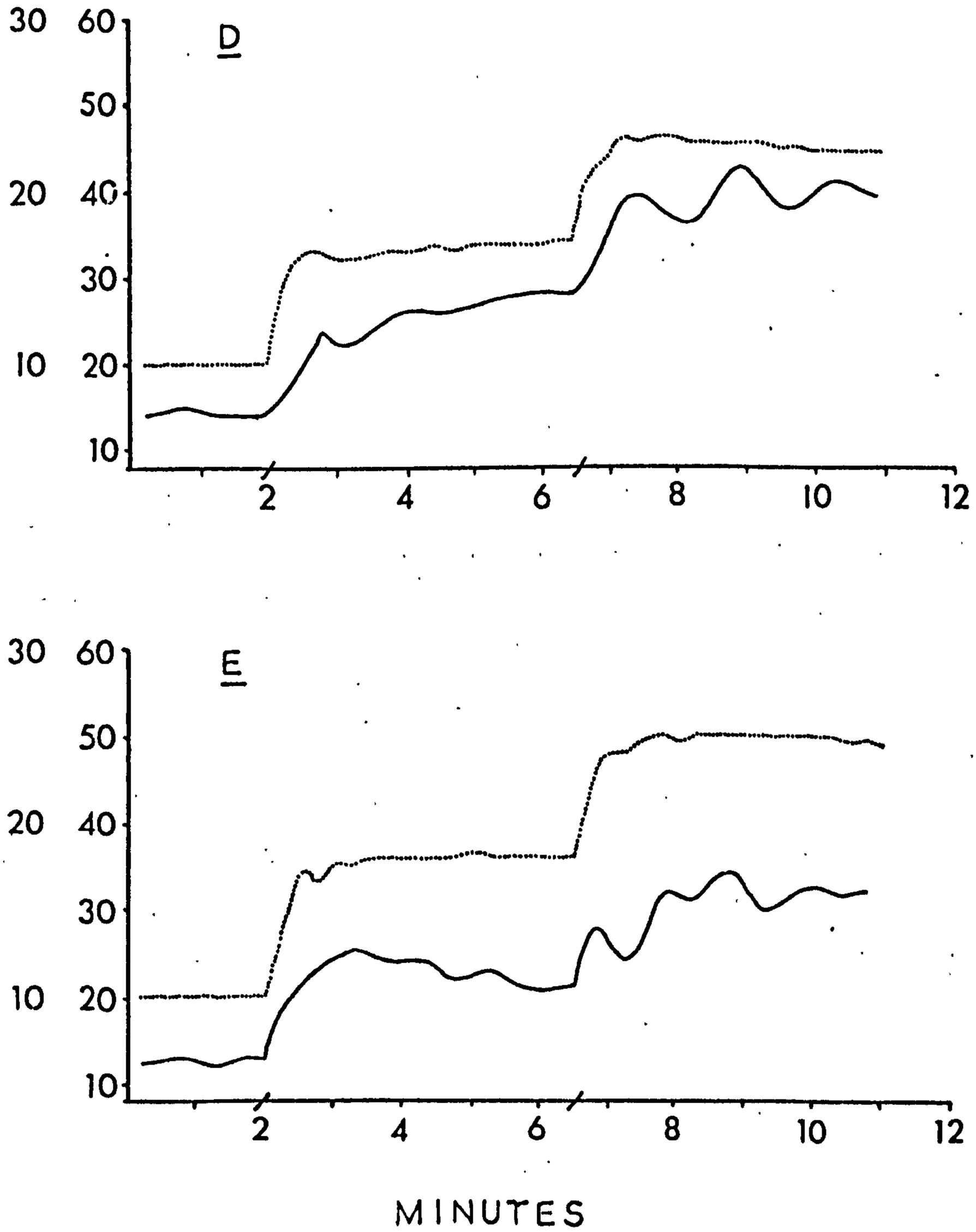


Table 3: Shell length and initial heart rate of animals used in Preliminary Experiment II

Animal No.	Shell length (cm)	Initial heart rate at 10°C (beats/min)
A	2.29	13.5
B	1.84	16.0
C	1.74	13.5
D	1.62	14.5
E	2.04	12.5

Discussion

Results of the present investigation show that rapid temperature changes are not followed smoothly by changes in heart rate, but instead induce fluctuation of heart rate frequently preceded by an overshoot reaction, especially at higher temperatures. These oscillations, however, appear to be short-lived and become damped and extinguished within about four minutes of the causal rise in temperature. An overshoot response is especially marked in Fig. 8 (b), following the second temperature increase. 'Hunting' is well illustrated in Fig. 8 (a). Similar results were also obtained from experiments performed on animals acclimated to 5 and 20°C. Overshoot and hunting reactions were more apparent in the latter and were invariably greater following the second temperature rise.

Various activity rates of poikilotherms, following a rapid change in temperature, frequently show an overshoot or undershoot before stabilizing at a level characteristic of the new temperature (Grainger, 1956, 1958). This initial response to temperature change is much neglected and little understood. Overshoot reactions and subsequent effects in the heart rate of molluscs have been investigated by Segal (1962), and Spaargaren et. al. (1977) have studied similar reactions in marine and brackish-water crustaceans. It is difficult to interpret rate oscillations in terms of functional significance; it is more probable that an abrupt temperature change causes a temporary imbalance in certain physiological systems (nervous and perhaps hormonal), and these fluctuations are merely incidental to the adjustments that are forced upon the animal.

Segal (1962) found that the initial response of the gastropod Acmaea limatula to abrupt changes of 4°, 9° and 14°C (cf. 8°C temperature increases in the present study) was an overshoot after an increase and an undershoot after a decrease in temperature. He found also that the peak frequency usually was attained within a minute and rarely later than two minutes after the temperature change. Such findings are in agreement with those of the present study. In addition he found that the greater the temperature increment the longer the heart-rate takes to stabilize at the new level, and, as described here, the same temperature increment causes a greater overshoot at higher temperatures.

Thus, although this study has shown that fluctuations in heart rate normally die out within four minutes of an abrupt temperature change in stated conditions, this period could be longer at higher temperatures. It was therefore decided to allow a ten-minute lapse before measurement of heart rate in the following experiments.

Conclusions

It has already been established that 10 minutes is ample time for a snail to recover from the sudden changes in light intensity encountered during the experiments. The preliminary observations described here show, firstly, that within a physiological range heart rate smoothly and rapidly follows a gradual rise in experimental temperature and, secondly, that ten minutes should be sufficient time for the extinction of fluctuations in heart rate following sudden temperature changes.

It is concluded that the described basic method of heart rate measurement is valid, but that a ten minute lapse should be allowed after immersion of each animal into the observation tank before commencement of heart beat recordings. This finding was incorporated into the meth od used in the main investigations.

SEASONAL CHANGES IN HEART RATE

Introduction

Little information is available regarding seasonal adaptation of the heart rate function in poikilotherms. Barcroft and Izquierdo (1931) and Stier and Taylor (1937) have shown distinct seasonal changes in the heart rate responses of frogs, and Segal (1956) found that the heart rate of the limpet Acmaea limatula varied seasonally over a range of temperatures between 9 and 29°C, having a peak in winter and a minimum in summer. Further, Crozier and Stier (1924) found clear seasonal changes in Q_{10} for heart beat frequency in the slug Limax maximus. Conversely, Rüth (1974) stated that for several species of anuran the temperature-dependence of heart beat was independent of season of the year, and Pickens (1965), following experiments with the mussels Mytilus edulis and M. californiansis, could reach no positive conclusions with regard to seasonal adaptation of the heart-rate function.

This study aims to establish the precise relationship between heart rate and temperature in Lymnaea stagnalis, and to determine whether this relationship changes with season.

Method

Snails were collected approximately every eight weeks, from April 1975 to May 1976. They were stored and fed until use as previously described. Each animal was allowed ten minutes to adjust to a new experimental temperature before heart beats were recorded for two minutes using a hand-counter and stop-watch. Shell lengths were carefully measured. Recordings were begun as soon as possible after collection and normally were completed within ten days.

Results

Heart beat frequencies were recorded and transformed to logarithms. The mean, standard deviation and 95% confidence limits of the data were then calculated. In addition, correlation coefficients for $\log_{10}$ shell length against $\log_{10}$ heart rate were calculated for each seasonal sample of animals at each experimental temperature. Size was found to change considerably with season (see Fig. 10) and it was necessary to determine the relationship, if any, between size and heart rate. The values of these correlation coefficients are shown in Table 4. It is seen that only thirteen of the seventy computations proved to be significant at the 95% level of significance. Therefore it is concluded that size-effects were minimal in these experiments.

The heart rate data are shown in Table 5 and derived rate-temperature curves, with 95% confidence intervals, are illustrated in Fig. 9. It was found that heart rate increased with temperature up to a peak, the upper thermal limit, beyond which it declined (as seen in Preliminary Experiment I) and the beats became erratic, resulting in a

Table 4: Values of the correlation coefficient (r) for log heart rate against log shell length

	Month							
Te °C	April	June	August	October	Nov-Dec	February	March	May
5	-0.32	0.14	0.28	-0.13	-0.09	-0.58	-0.14	-0.33
10	-0.33	0.22	-0.51	0.02	-0.17	-0.29	<u>-0.71</u>	-0.51
15	-0.46	-0.16	<u>-0.85</u>	-0.13	0.08	<u>-0.72</u>	-0.61	<u>-0.65</u>
20	-0.10	-0.20	-0.39	-0.48	0.14	<u>-0.68</u>	<u>-0.87</u>	-0.49
25	0.26	-0.44	-0.05	-0.54	-0.07	<u>-0.89</u>	<u>-0.75</u>	-0.47
27.5	-	-	-	-	-0.22	-0.34	0.05	-
30	-0.46	<u>-0.74</u>	-0.41	-0.50	-0.55	<u>-0.94</u>	-0.52	-0.55
32.5	0.20	0.00	-0.48	-0.16	-0.29	<u>-0.89</u>	-0.35	<u>-0.75</u>
35	-0.14	<u>0.70</u>	-0.44	-0.19	-	-	0.16	0.13
37.5	-	0.35	0.19	0.08	-	-	-	0.34
40	-	-	-0.08	-	-	-	-	
N	10	10	10	10	8	10	10	10

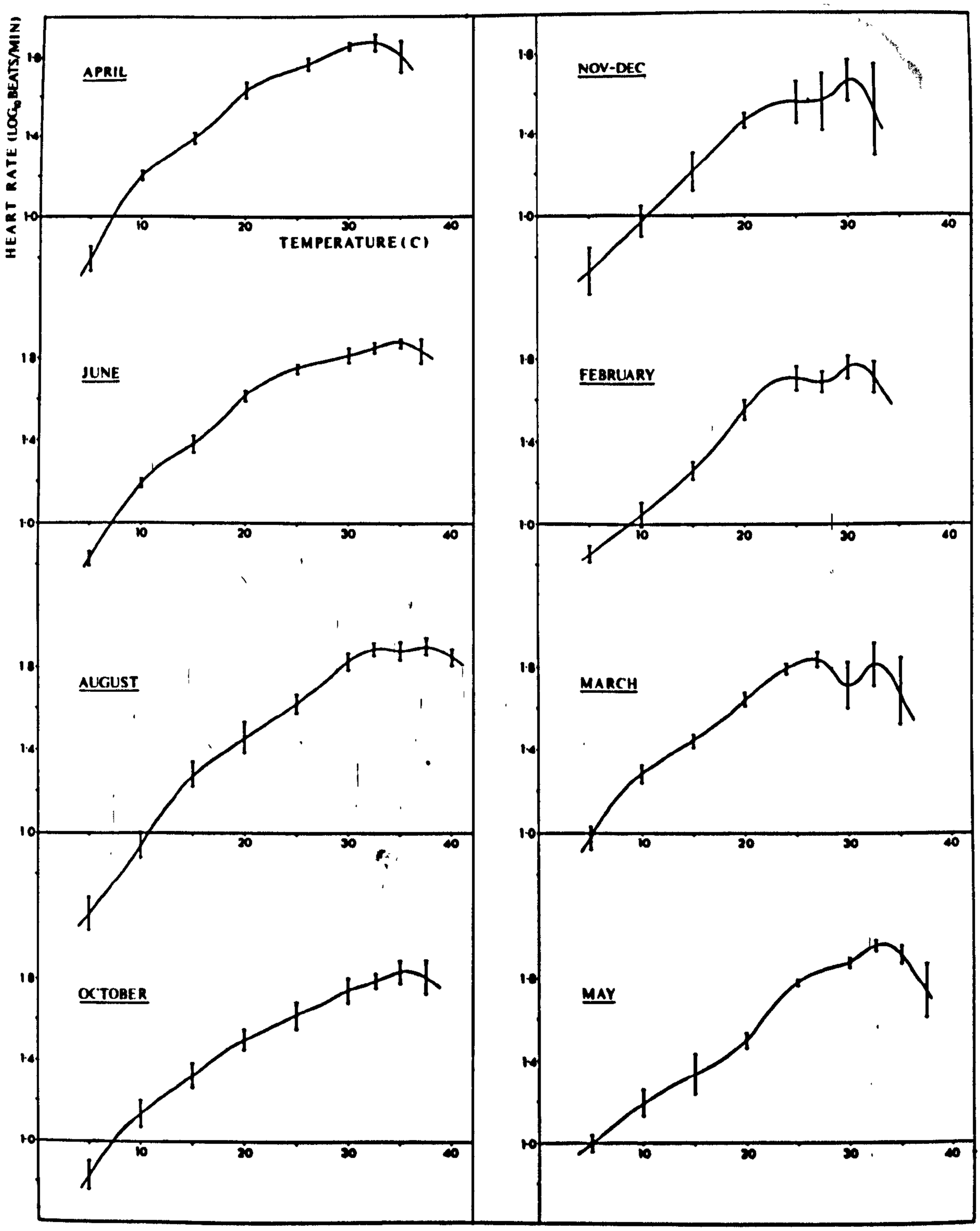
Underlined values are significant at the P = 0.05 level

TABLE 5: Seasonal heart rate data

Month	Experimental temperature (°C) 5	10	15	20	25	27.5	30	32.5	35	37.5	40	N	Mean shell length (log_{10} cm)
APRIL	0.79 ±0.06	1.20 ±0.03	1.39 ±0.02	1.63 ±0.04	1.76 ±0.03	-	1.85 ±0.02	1.87 ±0.04	1.79 ±0.08		-	10	0.296 ±0.043
JUNE	0.83 ±0.04	1.20 ±0.02	1.39 ±0.04	1.62 ±0.02	1.76 ±0.02	-	1.82 ±0.03	1.86 ±0.02	1.88 ±0.02	1.86 ±0.02	-	10	0.450 ±0.046
AUGUST	0.61 ±0.02	0.94 ±0.06	1.28 ±0.06	1.46 ±0.07	1.62 ±0.05	-	1.83 ±0.04	1.89 ±0.02	1.88 ±0.04	1.90 ±0.04	1.85 ±0.04	10	0.448 ±0.015
OCTOBER	0.81 ±0.07	1.14 ±0.06	1.33 ±0.06	1.50 ±0.05	1.61 ±0.06	-	1.74 ±0.06	1.78 ±0.03	1.82 ±0.05	1.80 ±0.08	-	10	0.492 ±0.022
NOV-DEC	0.72 ±0.11	0.97 ±0.07	1.21 ±0.11	1.45 ±0.03	1.55 ±0.12	1.55 ±0.14	1.66 ±0.10	1.51 ±0.23	-	-	-	8	0.169 ±0.040
FEBRUARY	0.85 ±0.04	1.05 ±0.06	1.21 ±0.04	1.55 ±0.05	1.71 ±0.06	1.69 ±0.05	1.76 ±0.05	1.71 ±0.07	-	-	-	10	0.109 ±0.050
MARCH	0.98 ±0.06	1.29 ±0.04	1.45 ±0.03	1.65 ±0.03	1.79 ±0.02	1.84 ±0.04	1.72 ±0.11	1.82 ±0.11	1.68 ±0.16	-	-	10	0.142 ±0.062
MAY	1.02 ±0.04	1.20 ±0.06	1.34 ±0.06	1.50 ±0.04	1.77 ±0.01	-	1.87 ±0.02	1.95 ±0.03	1.91 ±0.04	1.73 ±0.13	-	10	0.330 ±0.056

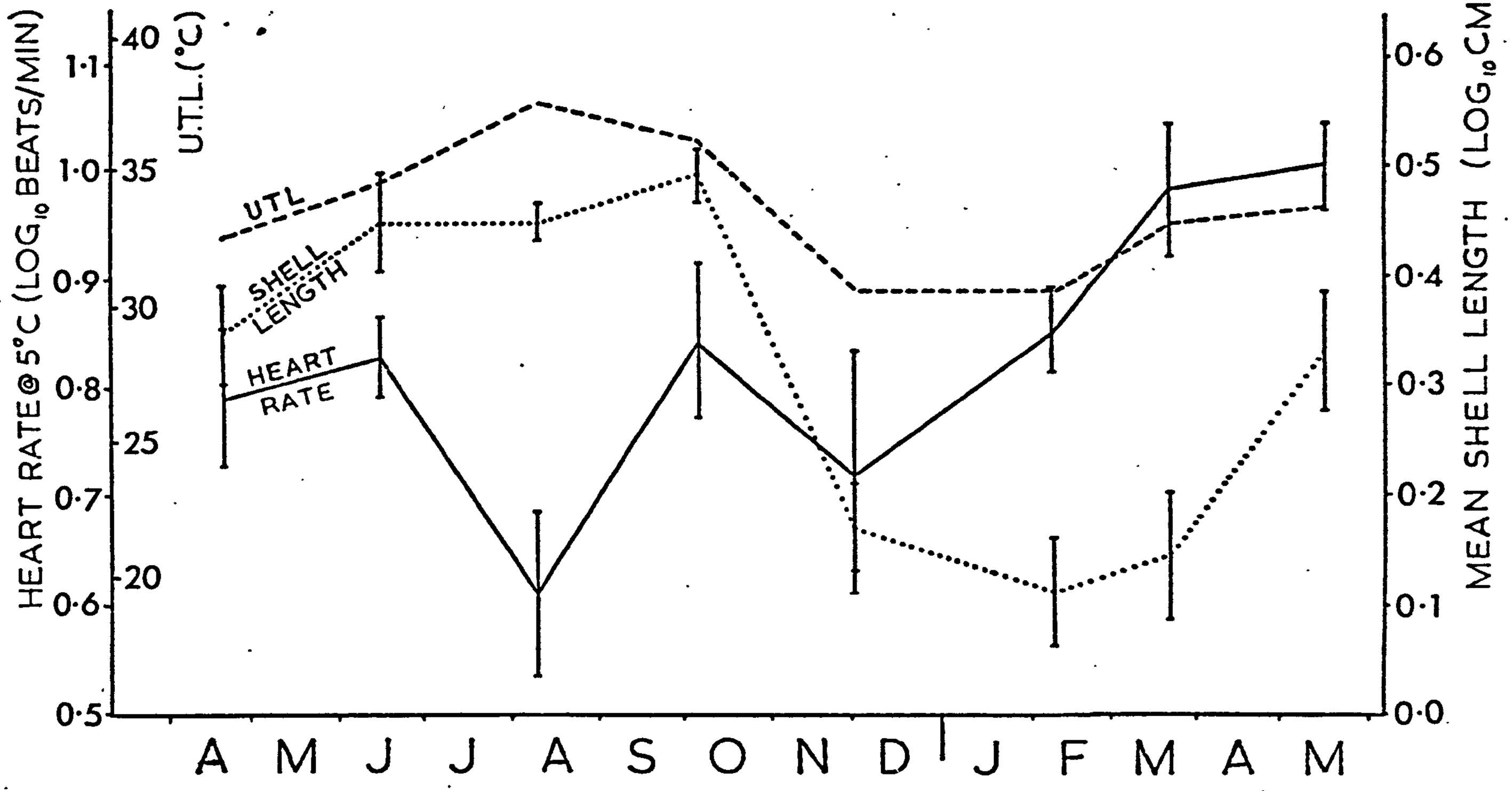
Mean log_{10} heart rates for each season at each experimental temperature with sample sizes and mean log_{10} shell length (±S.D.). 95% confidence limits of the means are given.

FIG. 9 SEASONAL HEART RATE-TEMPERATURE CURVES



Mean values with 95% confidence limits

FIGURE 10 SUMMARY OF SEASONAL CHANGES



general increase in variability of the data. Fig. 10 is a summary of the year's results in graphical form. It shows how upper thermal limit (UTL), heart rate at 5°C (an indicator of low-temperature sensitivity) and mean $\log_{10}$ shell length varied with season (April 1975 to May 1976).

Discussion

Fig. 9 shows changes in the relationship between heart rate and temperature through the course of the year, the most distinct differences occurring at either end of the curves. There is a clear shift in the low temperature portion of the curve and a change in the position of the peak. The shape of the peak also changes. In addition there is evidence for translation of the curves between 15 and 25°C.

Comparison of the March and August curves provides an example of translation. The $\log_{10}$ heart-rate in March exceeds that in August by 0.17 at 15°C, 0.19 at 20°C and by 0.17 at 25°C (see Table 5). All these differences are significant at the 95% level of confidence. The summer heart rate-temperature curve therefore shows a depression compared to that recorded in winter. (Seasonal temperature data are shown in Fig. 2). This vertical translation of the curve represents a capacity adaptation and is equivalent to Prosser's (1958) acclimation type II. There is not, however, a clear and straightforward seasonal progression in the shift of the rate temperature curves, and only when temporally well-spaced curves are compared do significant changes become apparent. Miller and Mizell (1972) likewise found distinct winter-summer differences in the heart rate of Rana pipiens but stated

that during intervening months the response was intermediate with no predictable pattern.

The changes occurring at the temperature extremes provide evidence for seasonal resistance adaptation. The temperature for peak heart rate (UTL) varies considerably with season. For example, comparing August with February, the UTL in summer is approximately 37.5°C, whereas in winter the peak occurs at about 30.5°C. Warm adapted animals are therefore more tolerant of high exposure temperatures. The determination of a lower thermal limit is more problematic because this is indicated not by a peak but by a disproportionate drop in heart rate at low temperature. However, if resistance adaptation to low temperature is present, then winter animals will exhibit a considerably higher heart rate at 5°C than summer animals. A comparison shows that the mean $\log_{10}$ heart rate of August animals at 5°C is 0.37 lower than that of March animals, a significant difference ($P < 0.01$). This change in rate at low temperature is not due solely to the general vertical translation of the curve discussed earlier, since comparison of the same months' results shows a displacement of only 0.17 $\log_{10}$ beats/min in the 15 - 25°C temperature range. It is concluded that the mechanisms of capacity and resistance adaptation act together to produce significant seasonal differences in heart rate at extreme low temperature.

Figure 10 shows how upper thermal limit increases through spring and summer to reach a maximum in August, then falls to a minimum in December and February. Heart rate at 5°C shows a more erratic variation with season, exhibiting a minimum in August and increasing through autumn and winter to show a maximum in spring. These two sets of

results therefore do not exactly concur. For example, a minimum UTL does not coincide with a maximum rate at 5°C. This finding appears to contradict Precht's (1958) suggestion that adaptation to low temperature necessarily incurs changes in heat tolerance, and vice versa. It infers, instead, that changes in heat and cold tolerance are not inseparably linked but may each be controlled by separate mechanisms.

Further evidence that no single factor is responsible for the observed seasonal changes in heart rate is provided by the occurrence of a bimodality in the rate-temperature curve at certain seasons. Figure 9 clearly shows a progression in the development of a secondary peak at lower temperature. This first becomes apparent in November-December, reaches a maximum in March and declines by June. The possibility of this and other effects being caused by changes in the size distribution of animals through the year is ruled out because there is no strong correlation between heart rate and size (see Table 4). Figure 10 shows further evidence that size is not a major influence on heart rate. Comparing the June and August results, the change in mean log shell length is insignificant but the UTL increases by 3°C and log heart rate at 5°C decreases by 0.22, a significant change. Moreover, a large and significant increase in size from March to May has no significant effect on the heart rate parameters illustrated.

Discussion of the results has so far stressed the importance of seasonal temperature changes, but other factors may well be involved. Breeding condition may be considered an important factor affecting heart rate, although Segal (1956) found in Acmaea limatula that size of gonad did not contribute to the variation in heart rate between samples. Food availability may affect the heart rate-temperature relation also.

Various anomalous results were obtained by Pickens (1965) from field and laboratory-acclimated populations of the mussels Mytilus californianus and M. edulis. He suggested that although temperature compensation in heart rate could not be ruled out, a more likely explanation for the rate changes was that they reflected adjustments to different food conditions.

The main findings of the present study are that Lymnaea stagnalis exhibits seasonal changes in the heart-rate temperature relation resulting in capacity and resistance adaptations which are due to physiological adjustments controlled by more than one independent mechanism. Temperature may or may not be a direct causal factor.

Similar results have been quoted by other workers. Segal (1956), studying the intertidal mollusc Acmaea linatula, found that both high and low level animals had faster rates in winter than in summer at any temperature between 9 and 29°C and therefore exhibited clear seasonal capacity adaptations. Similarly Pickens (1965) found that in the summer, heart rates of Mytilus edulis and M. californianus acclimatised to colder conditions were always higher than those in warm water populations measured at the same temperatures. Distinct seasonal changes have been observed in the shape of the heart rate-temperature curves of excised and intact frogs' hearts, with changes also in the position of the peak (Barcroft and Izquierdo, 1931; Smith, 1951; Stier and Bock, 1966; Miller and Mizell, 1972 and Harri and Talo, 1975a, b). A study by Stier and Taylor (1937) on the behaviour of the heart of Rana pipiens at high temperature showed that the upper limit of temperature at which normal contraction was maintained varied with season and thus revealed a resistance adaptation. Lang (1910, cited by Crozier and Stier, 1924), found different heart rate Q_{10}'s according

to season in the terrestrial gastropod Helix.

Conclusions

With regard to possible errors incurred in this investigation, it is assumed that no changes in heart rate-temperature relations occurred during the short storage period before measurement, induced either by changes in nutritional status or by a different temperature regime. Size effects, which appeared to be negligible, were ignored.

To summarize the results, it has been found that the seasonal rate-temperature curves showed translation between 15 and 25°C. This capacity adaptation was most distinct when comparing winter and summer animals. During intervening months the response was intermediate with no clear seasonal progression. Upper thermal limit and heart rate at 5°C (an indicator of cold-sensitivity) both changed considerably with season, illustrating resistance adaptations at both temperature extremes. Changes in heat and cold tolerance appear to be controlled by separate mechanisms. The occurrence of a secondary peak in winter and spring curves was considered to be further evidence for the complexity of heart rate control. This investigation has shown, therefore, that time of year considerably influences the shape of the heart rate-temperature curve, but the precise seasonal influences are not known. Although the results have been exclusively related to temperature effects, other factors such as daylength, reproductive status and food availability may also be important. It is likely that an inter-relationship of environmental factors is responsible for the complex changes observed.

LABORATORY-INDUCED CHANGES IN HEART RATE

Introduction

It has been shown that Lymnaea stagnalis exhibits seasonal adaptations of the heart rate-temperature relation, although the precise factors responsible for these changes could not be specified. Use of terms such as 'capacity adaptation' and 'resistance adaptation' implies adjustment to temperature alone. This investigation seeks to determine whether changes in heart rate function can be induced by thermal acclimation in the laboratory.

Studies on acclimation of heart rate are relatively few, and from the work published it appears that some poikilotherms do exhibit acclimatory responses in this rate-temperature relation (Mellanby, 1940; Segal, 1956; Tsukuda and Ohsawa, 1959), whereas others do not (Ahsanullah and Newell, 1970; Widdows, 1973; Weathers, 1975). Rarely have laboratory studies been directly compared with seasonal observations however. Temperature may or may not be the most important factor affecting seasonal adaptations, but certainly it is known (Fry, 1958) that direct effects of temperature may be opposed or reinforced by the effects of other environmental conditions. It is of special interest to isolate which of the observed seasonal changes, if any, may be induced by acclimation to temperature alone.

Materials and Methods

Animals were collected in March 1975 and groups of twenty or more animals stored in small tanks of aerated water in Fisons environmental cabinets at 5, 10, 15, 20 and 25°C (all c. ± 1°C). Lighting regimes were maintained at 12 hours light:12 hours dark and the snails

fed ad libitum with lettuce and TetraMin fish food, as before. The animals were allowed to acclimate to these conditions for 3 - 6 weeks. Random samples of 10 or 20 animals were then taken and heart rates measured according to the methods previously described. Shell size was recorded. With regard to the required time for complete acclimation, a period of 3 to 4 weeks is normally considered sufficient, although shorter times have been reported for various metabolic rate functions.

Results

Heart beat frequencies were recorded, transformed to logarithms and the mean, standard deviation and 95% confidence intervals calculated. Correlation coefficients for $\log_{10}$ shell length against $\log_{10}$ heart rate were also calculated for each acclimation group at each experimental temperature. These values are shown in Table 6. It is seen that only 7 of 43 size-rate correlations proved to be significant at the 95% level. It was therefore concluded that size-effects were minimal in these experiments. Such findings are in agreement with those of the seasonal study. The basic heart rate data are shown in Table 7 and the five rate-temperature curves derived from these are represented, with 95% confidence intervals, in Fig. 11. It is seen that these are of generally similar shape except for a bimodality observed in the Ta: 20°C curve. (Ta = temperature of acclimation). In order to show more clearly the differences between these curves, the graphs for the Ta: 5, 15 and 25°C animals are superimposed in Fig. 14. Figure 13, which is essentially a summary of results in graphical form, shows $\log_{10}$ heart rate at 5°C (an indicator of cold sensitivity), upper thermal limit

Table 6: Values of the correlation coefficient (r) for log heart rate against log shell length

	Acclimation temperature (Ta °C)				
Te °C	5	10	15	20	25
5	<u>-0.56</u>	0.00	-0.10	0.20	0.35
10	-0.42	0.00	-0.33	0.24	0.00
15	<u>-0.61</u>	0.14	-0.11	0.14	0.00
20	<u>-0.59</u>	-0.37	<u>-0.59</u>	-0.33	0.00
25	-0.33	<u>-0.57</u>	-0.39	0.24	0.34
27.5	-0.18	-	-	-	-
30	0.10	<u>-0.56</u>	-0.26	0.22	-0.10
32.5	-0.11	-0.26	-0.30	0.32	-0.13
35	-	-0.32	0.45	0.00	0.03
37.5	-	-	-	-0.10	<u>0.55</u>
40	-	-	-	-	0.04
N	20	20	20	10	10

Underlined values are significant at the P = 0.05 level

Table 7: Laboratory acclimation heart rate data.

T_a °C	Experimental temperature (T_e °C) 5	10	15	20	25	27.5	30	32.5	35	37.5	40	N	Mean shell length (cm)
5	0.94 ± 0.02	1.14 ± 0.02	1.33 ± 0.02	1.52 ± 0.02	1.64 ± 0.02	1.62 ± 0.05	1.57 ± 0.04	1.51 ± 0.06	-	-	-	20	1.96 ± 0.26
10	0.83 ± 0.04	1.08 ± 0.03	1.26 ± 0.03	1.52 ± 0.03	1.68 ± 0.02	-	1.79 ± 0.02	1.81 ± 0.02	1.74 ± 0.04	-	-	20	2.06 ± 0.29
15	0.83 ± 0.05	1.09 ± 0.03	1.36 ± 0.03	1.57 ± 0.03	1.72 ± 0.02	-	1.81 ± 0.02	1.77 ± 0.03	1.76 ± 0.04	-	-	20	1.91 ± 0.32
20	0.78 ± 0.03	1.07 ± 0.04	1.28 ± 0.04	1.48 ± 0.05	1.65 ± 0.05	-	1.77 ± 0.05	1.74 ± 0.07	1.83 ± 0.04	1.68 ± 0.11	-	10	2.32 ± 0.12
25	0.58 ± 0.06	0.97 ± 0.06	1.32 ± 0.06	1.48 ± 0.03	1.65 ± 0.03	-	1.75 ± 0.03	1.80 ± 0.03	1.82 ± 0.02	1.78 ± 0.04	1.75 ± 0.06	10	2.87 ± 0.14

Mean $\log_{10}$ heart rate for each acclimation temperature (Ta) at each experimental temperature (Te) with sample size (N) and mean shell length. Variability of data represented by 95% confidence intervals for heart rate means and by standard deviation for shell length.

Figure 11: Heart rate-temperature curves for each acclimation temperature (Ta)

Mean values with 95% confidence intervals

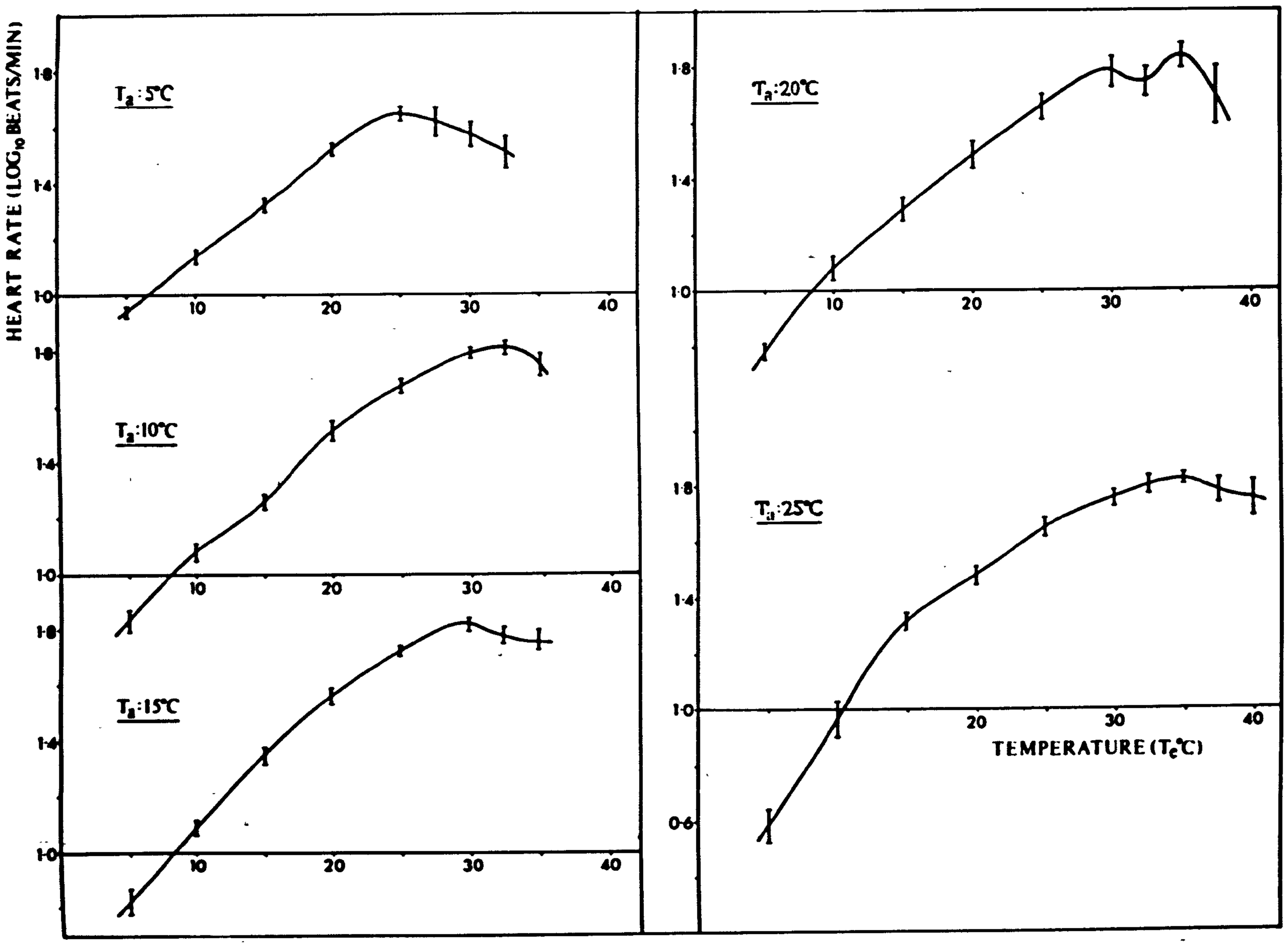

HEART RATE (LOG10 BEATS/MIN)
Ta:5°C
Ta:10°C
Ta:15°C
Ta:20°C
Ta:25°C
1·8
1·4
1·0
0·6
10
20
30
40
TEMPERATURE (Tc°C)

FIGURE 12 COMPARISON OF T_a: 5, 15 & 25 °C CURVES

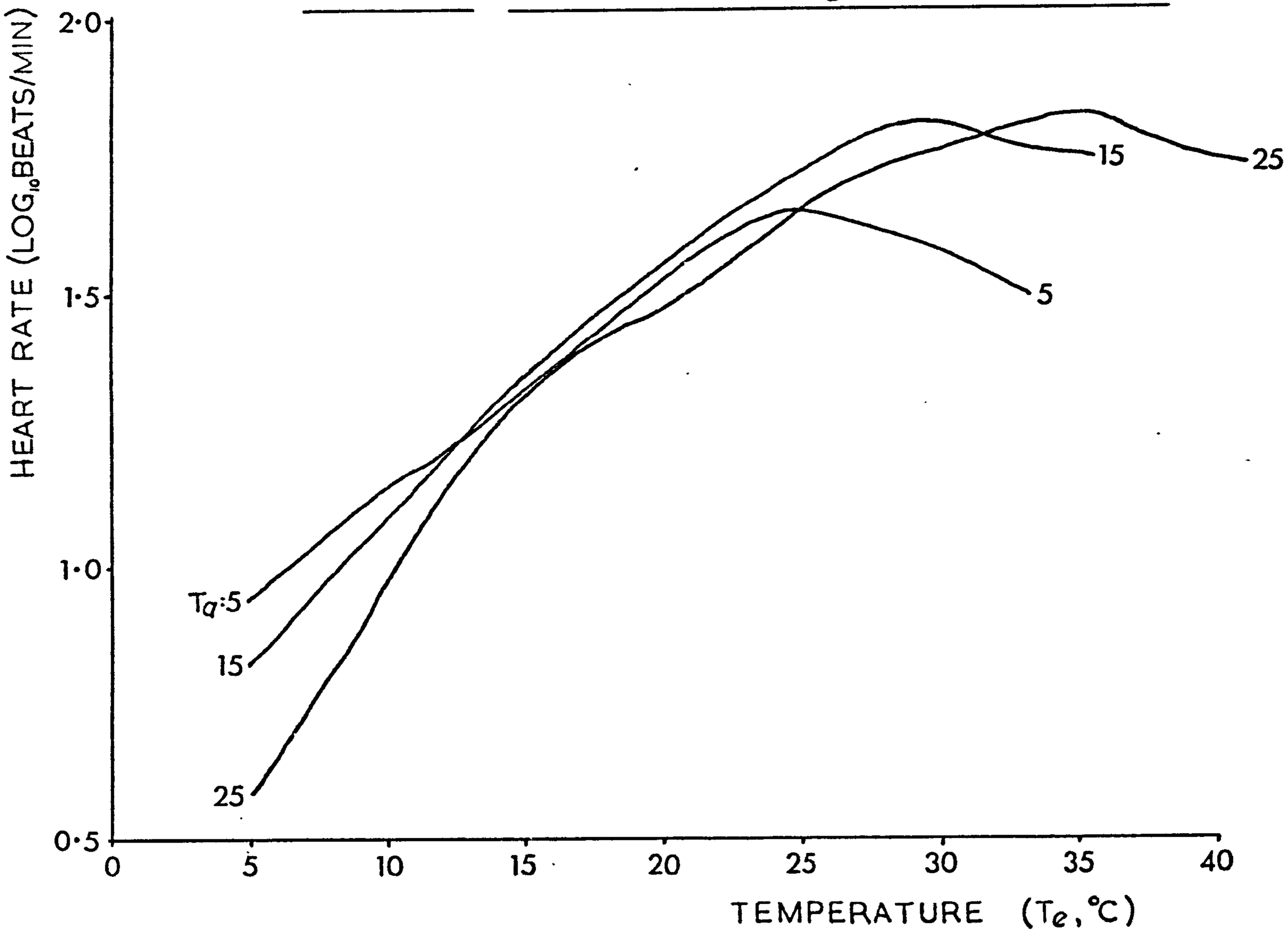


FIGURE 13 SUMMARY OF ACCLIMATION RESULTS

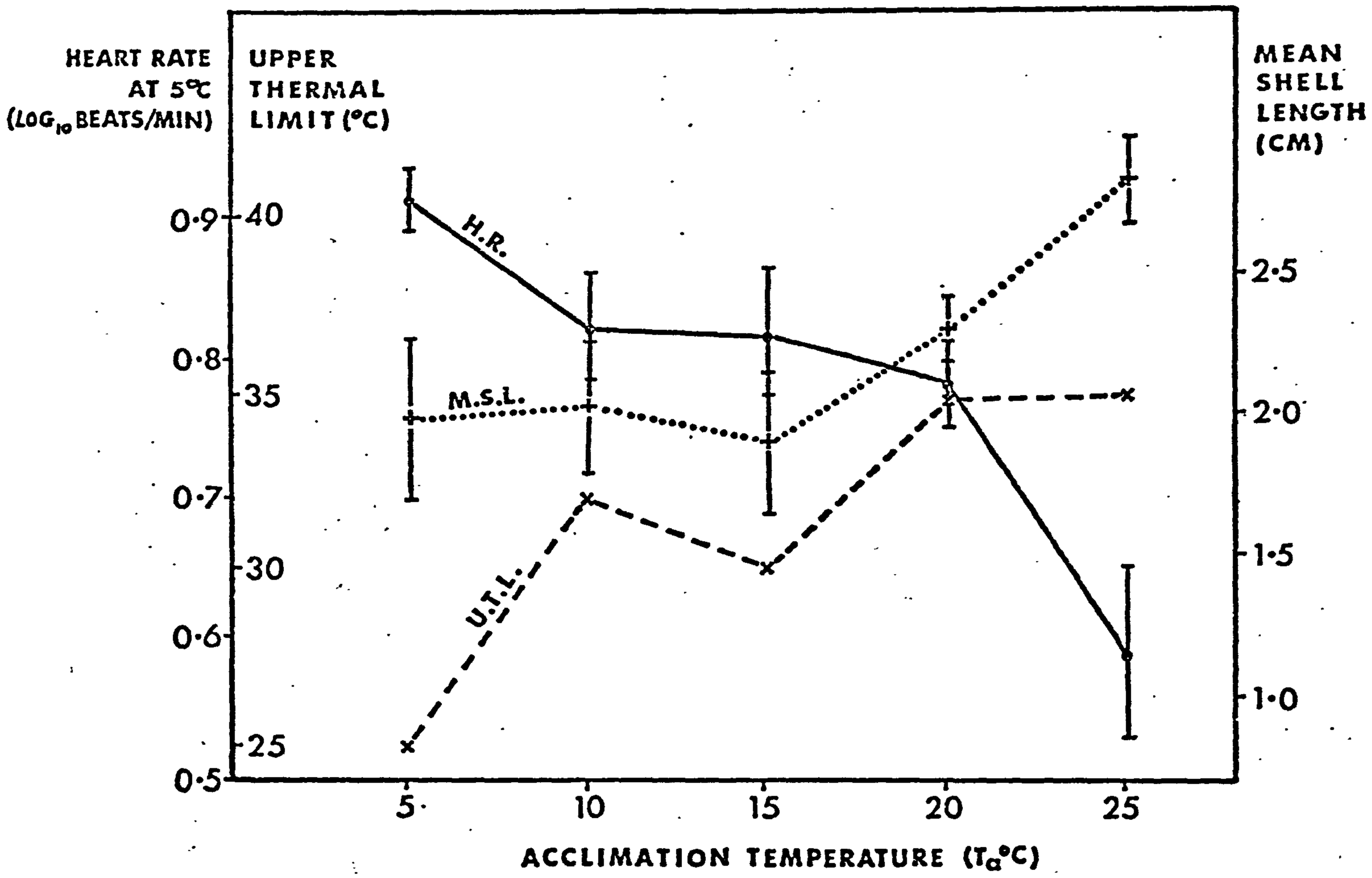
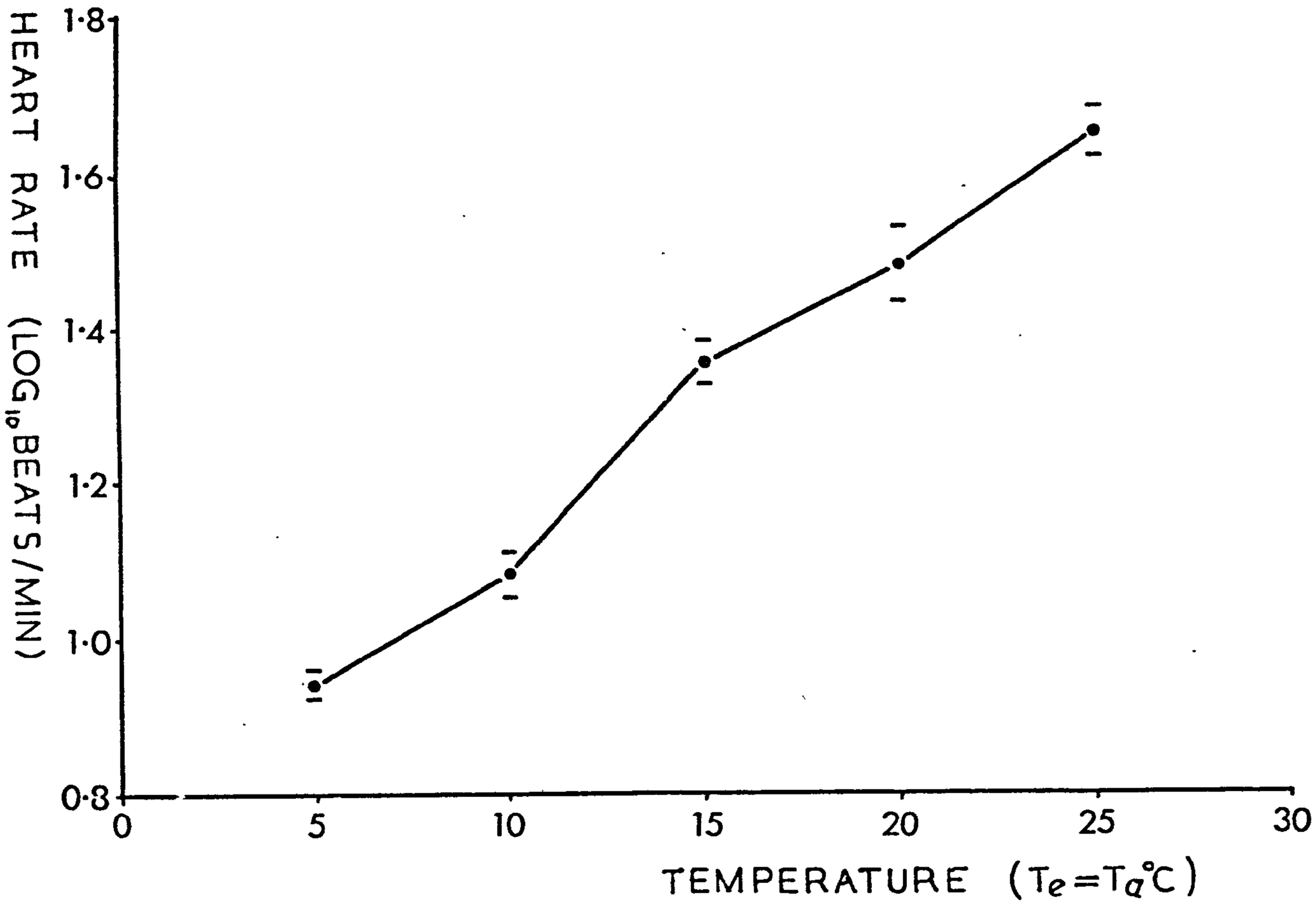


FIGURE 14 ACCLIMATED R-T CURVE



(UTL) and mean shell length for each acclimation group. Figure 14 is an acclimated rate-temperature (R-T) curve and shows heart rate for each acclimation group measured at the respective acclimation temperatures (Te = Ta).

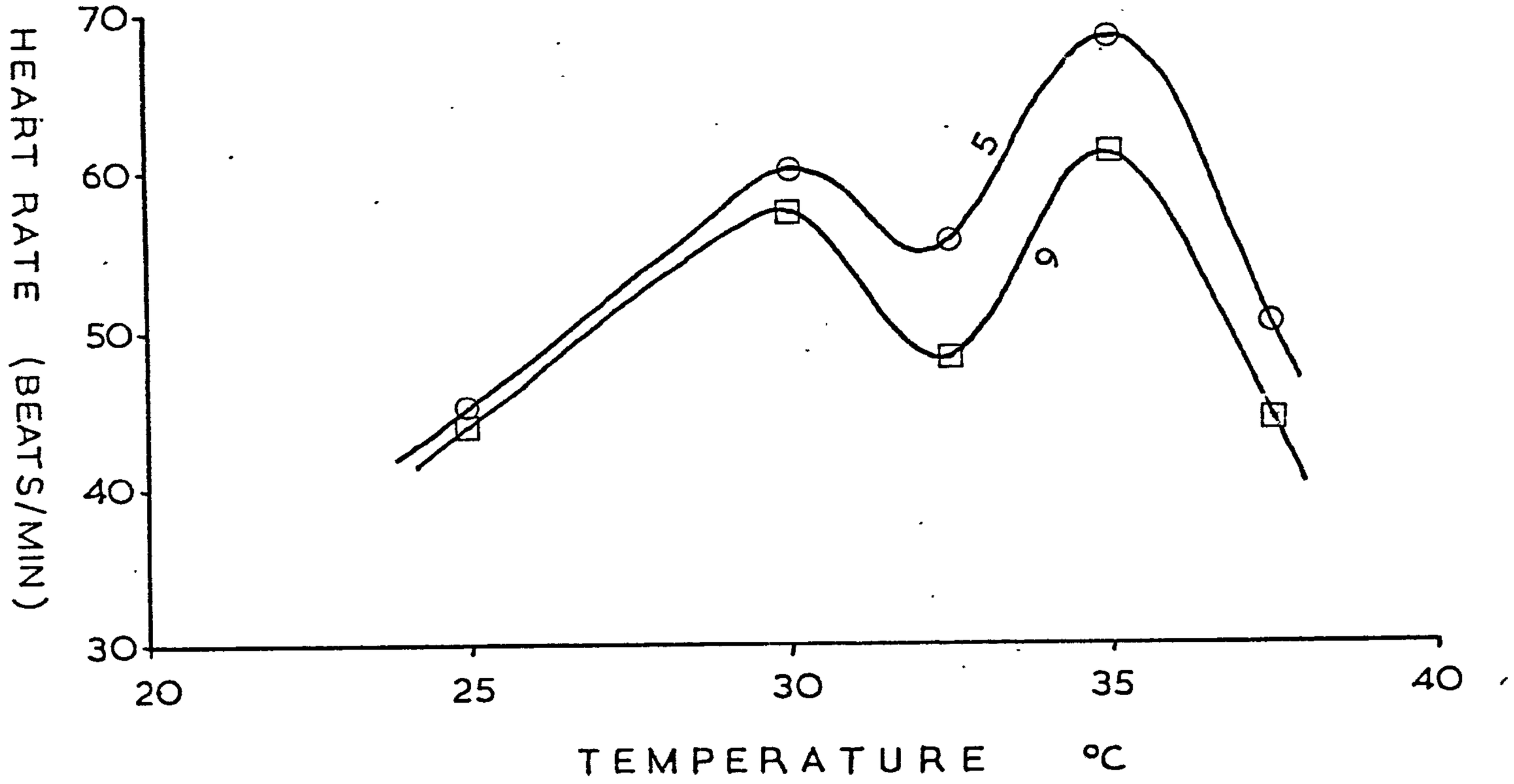
Discussion

The simple heart rate-temperature curves illustrated in Fig. 11 show how the relationship between heart rate and experimental temperature (Te) varies according to the temperature of acclimation (Ta). The most obvious changes occur at each end of the curves. Direct comparison of the Ta: 5, 15 and 25°C curves is facilitated by the superimposition shown in Fig. 12. Between Te: 15 and 25°C there is little difference between the curves, but beyond these limits clear resistance adaptations are evident. Comparison of the data points between 15 and 25°C for all five curves (Table7) shows that confidence intervals overlap the means in most cases, indicating an absence of clear capacity adaptation. The temperature for peak heart rate (the upper thermal limit, UTL) and heart rate at 5°C, however, change considerably with temperature of acclimation. An increase in Ta generally results in a rise in UTL. The upper thermal limit for Ta: 5°C is 25°C and for Ta: 15°C it is 30°C, rising to 35°C for the Ta: 25°C group. Acclimation therefore changes tolerance of high temperatures. Heart rate at 5°C, which gives an indication of sensitivity to low temperature, increases as Ta decreases and this suggests that acclimation to low temperatures results in a resistance adaptation to low temperature. Thermal acclimation therefore affects both upper and lower limits of the rate-temperature curve and effectively extends the viable temperature

range. These results are summarised in Fig. 13. Mean shell length is also indicated here. It is seen that somewhat larger animals were used in the Ta 20 and 25°C groups. However, it has already been shown that size has little effect on heart rate and Fig. 13 gives further evidence for this assertion. Comparing the Ta: 5 and 15°C results, for example, animal size is not significantly different yet both UTL and heart rate at 5°C differ considerably. A significant difference in size between Ta: 15 and 20°C animals results in no real change in heart rate at 5°C. The acclimated rate-temperature curve shown in Fig. 14 is similar in shape to the normal curves of Fig. 11 and further illustrates that capacity adaptation of rate is absent. For perfect or total acclimation the slope of this line would be zero; heart rate would be equal for each acclimation temperature. According to Bullock (1955) the acclimated rate-temperature curve shows a slope indicating the degree of sensitivity to temperature, a length indicating temperature range and a shape at the ends indicating the sharpness of ecologic limits.

It is apparent from the results of this investigation that acclimation to temperature alone results in resistance adaptation at each temperature extreme, but there is no evidence for capacity adaptation. Previous studies have produced conflicting results. Capacity adaptation of heart rate resulting from thermal acclimation has been shown to occur in the crested newt Triturus cristatus (McLanby, 1940), in the limpet Acmaea limatula (Segal, 1956), and in a freshwater snail, Physa sp. (Tsukuda and Ohsawa, 1959). Conversely, however, no acclimatory changes have been found in the heart rate responses of crabs Carcinus maenas (Ahsanullah and Newell, 1970), various anurans (Rüth, 1974; Weathers, 1975) or mussels Mytilus edulis (Widdows, 1973).

FIG. 15 UPPER REGIONS OF T_a: 20°C CURVES (5 & 9 WEEKS)



It appears that factors controlling compensatory responses in the heart rate-temperature relation differ between species so that no generalisation can be made regarding the induction of capacity adaptation of this function by temperature acclimation.

A further result of interest is the occurrence of a double peak in the rate-temperature curve for Ta: 20°C (Fig. 11), similar to that sometimes observed in the seasonal study. Measurements were repeated on the same group of animals four weeks later (total acclimation time then 9 weeks) and a similarly shaped curve was obtained (see Fig. 15), indicating that it was not an artefact of measurement. Further, the lower peak, at 30°C, corresponds to that of the Ta 15°C curve and the upper peak, at 35°C, corresponds to that of the Ta: 25°C curve. It appears that separate factors are responsible for the position of the maximum. These factors are temperature-induced and at intermediate temperatures of acclimation may occur together to affect the upper region of the heart rate-temperature curve.

Further discussion of the results in comparison with those of the seasonal study will be considered in the final section of the chapter.

Conclusions

All conditions, except temperature, were held constant for all groups of animals in the environmental cabinets. Lighting regimes were maintained at 12 hours light:12 hours dark in all cases. The assumption that photoperiod effects were equal in all cases ignores any possible inter-relationships between photoperiod and temperature, and further does not account for possible physiological changes in all animals induced by the sudden change from spring lighting conditions

(as encountered by the animals before collection) to the artificial 12:12 lighting conditions. The nutritional aspect is also important here. Animals were fed ad libitum and snails maintained at high acclimation temperature were seen to consume food at a faster rate than cold acclimated ones. Any restriction in food rationing, however, may have resulted in starvation in certain groups and would have been practically more difficult. Relationships between temperature and feeding rates and possible effects on metabolic functions will be discussed in the last chapter. As in the seasonal study, size effects, which appeared negligible, were ignored. The assumptions listed here may have resulted in some misinterpretation of the derived results. Further, if the experiments were repeated using animals collected at a different time of year, identical results may not have been obtained.

To summarise the findings presented in this section it may be stated that acclimation to a wide range of temperatures resulted in resistance adaptations at both ends of the heart rate-temperature curves, but there was no evidence for capacity adaptation. Resistance adaptations were indicated by general increases in upper thermal limit and reductions in heart rate at 5°C (indicator of cold sensitivity) for increased temperature of acclimation. The Ta: 20°C curve showed a bimodality with maxima occurring at 30 and 35°C, these peaks corresponding to those of the Ta: 15 and 25°C rate-temperature curves respectively. This result suggests that more than one factor is responsible for the position of the UTL and that these are not equally affected by acclimation to constant temperature.

GENERAL DISCUSSION

Experimental Findings

It was seen in the seasonal investigation that animals exhibited both resistance and capacity adaptations of the heart rate function. The seasonal curves showed distinct translation between 15 and 25°C and also clear changes in UTL and heart rate at 5°C. The adaptations were most obvious when comparing winter (February) and summer (August) animals. Experimental acclimation to temperature in the laboratory, however, produced only resistance adaptations; there was no evidence for capacity adaptation between 15 and 25°C. The general shapes of the R-T curves were similar in both studies. It appears that thermal acclimation produces the changes in thermal limits of the heart rate function also found at different times of year, but that capacity adaptation occurs only in seasonally adapted animals. This suggests either that a longer time course is required for the response or that other factors, such as fluctuation of temperature and photoperiod or reproductive status of the animal, are important. Indeed, it is proper not to over-estimate constant temperature effects in seasonally acclimated animals. Reproductive activity substantially affects an animal's activity, behaviour and physiology. In many species the metabolic rate becomes very high during the breeding season, which often coincides with warmer environmental temperatures, and some species undergo a winter rest phase.

The occurrence of a bimodality in the rate-temperature curves of both studies is a most interesting feature and further suggests that control of heart rate is complex and dependent on more than one mechanism.

Possible physiological processes involved in heart-rate control are discussed below.

Possible Heart-Rate Control Mechanisms

The relationship between heart rate and temperature has been shown to be very flexible, canging with season in the natural situation, and with acclimation temperature in laboratory studies. It is pertinent to briefly discuss some general physiological mechanisms involved in the control of the heart rate function.

It is generally accepted that molluscan hearts lack direct neural control but have instead a diffuse generalised myogenic pacemaker mechanism. However, electrical stimulation of visceral nerves or ganglia (Silvey, 1968) has revealed cardioregulatory effects in most cases, with both excitatory and inhibitory nerve fibres controlling frequency and amplitude of beat (Hill and Welsh, 1966). These effects imply the involvement of neurohormones. Known neurohormonal transmitters include acetylcholine, an inhibitor, and 5-hydroxy-tryptamine, an excitatory transmitter (Hill and Welsh, 1966). Kale and Rao (1973) showed that neurosecretions of the nervous system of earth-worms have an important role in the control of metabolic activities during cold acclimation, and Lagerspetz and Tirri (1968) have indicated that high heat resistance of heart beat of the freshwater mussel Anodonta, induced by seasonal acclimation, is associated with elevated 5-hydroxytryptamine concentrations in the heart. An annual cycle of activity has indeed been observed in the neurosecretory cells of the mollusc Viviparus (work by Gorff, quoted by Gersh, 1958), where granules were found in some ganglion cells. They were numerous in

summer but few in winter.

Carter (1933) has investigated effects of endocrine substances on the form of the heart rate-temperature curve in the frog. He concluded that observed seasonal changes in this relationship were indeed of endocrine origin and that the effective organ was the thyroid, by increase of its activity in the summer. Further, according to Smith (1951), the seasonal changes in the rate-temperature curves closely parallel the cycles of thyroid activity described by Sklower (1925) and Meisenheimer (1936).

It is known that pulmonate molluscs possess both nervous and non-nervous endocrine organs (Boer and Joosse, 1975) which similarly may play important roles in the control of heart rate in Lymnaea stagnalis.

Other factors which may influence heart rate are changes in the speed of conduction in the myogenic pacemaker mechanism and change in the structure and/or function of the heart muscle. The gross effects of temperature on various aspects of nerve activity have been studied by Hodgkin and Katz (1913), Carter (1931), Gasser (1931), Kerkut and Ridge (1962) and Vislobokov (1975). The latter worker, studying L. stagnalis, found interneuronal differences but stated that the most suddenly changeable parameters were velocity and duration of the action potential. Carten and Sulze (1913) showed that the behaviour of isolated nerves of frogs at low temperature depends upon the previous thermal history of the animals. A review by Lagerspetz (1974) records that the conduction velocity of the compound action potential of peripheral nerves has shown compensatory acclimation to temperature in a fish, a snail and a crab. Single septate giant fibres of earthworms show compensatory temperature acclimation of conduction properties,

the form of the action potential and of the axonal cable properties. The synthesis of acetylcholine receptor molecules may also be affected by temperature acclimation. Neuromuscular transmission in the frog, following acclimation to the cold, shows increased resistance to low temperature and some indications of temperature compensation. Lagerspetz also verifies that changes in neurosecretion appear to be involved in temperature acclimation.

Evidence for gross changes in muscular responses at different temperatures has been provided by Rao and Singh (1907), who found in a frog and in Mytilus that muscle tone increases with temperature up to 20°C, then declines between 35 and 40°C and increases again up to 45 - 46°C. This bimodal property of the muscle tone-temperature curve is of particular interest when considering the double-peaked heart rate-temperature curves obtained in the present study. Decrease of temperature was found to decrease the rate of rise in tension and to increase the latent period of the response to acetylcholine. Hadju (1951) found apparent changes in the temperature dependence of frog musculus sartorius according to season. Muscles of summer frogs developed maximal tension at a higher temperature than winter frogs. The shapes of the temperature dependence curves were similar, but that of the summer animals was shifted to the right by 5°C on the temperature axis. Similarly Precht (1960) has shown that muscle from a warm adapted frog still contracts, when stimulated directly, at a temperature at which muscle from a cold adapted frog will no longer respond. Benthe (1954) found that the excitability of the isolated foot of Lymnaea stagnalis changed according to acclimation temperature and, further, that the temperature at which muscle membrane becomes polarised is higher in the case of warm adapted animals. Further information regarding adaptation of poikilotherm muscle structure and function has come to light through the work of

Johnston et. al. (1975). At 1°C the myofibrillar ATPase activity of cold acclimated fish was 2.8 times higher than that of warm acclimated fish. The log plots of activity versus temperature were found to be significantly different. In addition to differences in enzyme activity, myofibrils from cold and warm acclimated fish had different thermo-stabilities, implying resultant resistance adaptations. The result also provided strong evidence for a change in either structure or accessibility of the active site in response to temperature acclimation.

This brief survey of the literature has indicated that the complex changes in heart rate-temperature curves observed in the present investigation, including development of secondary peaks, may result not from any single mechanism but from several working together. Hormones or neurohormones produced in different amounts according to time of year or acclimation temperature, changes in conduction properties of muscles and nerves and changes in muscle performance and structure may all affect heart rate and lead to considerable capability for seasonal and temperature-induced adaptation of this rate function.

Conclusions

Seasonal acclimation induced both capacity and resistance adaptations in the heart rate-temperature curves. Acclimation to constant temperatures in the laboratory resulted in resistance adaptations at each temperature extreme, but there was no evidence for capacity adaptation in the mid-temperature range. It is proposed that factors other than temperature are responsible for seasonal capacity adaptations.

A bimodality in the rate-temperature curves of both studies suggests that control of heart rate is complex and dependent upon more than one mechanism. Possible control factors include neuro-hormonal changes and alterations in heart muscle structure and function.

CHAPTER III

ACCLIMATION OF RATE OF OXYGEN CONSUMPTION

INTRODUCTION

Rate of oxygen consumption is the simplest and most frequently used indicator of total metabolism. Much information is available regarding adaptation of this function to temperature and season. Studies on seasonal variations of oxygen consumption in relation to exposure temperature have been undertaken on a wide range of poikilotherms, including a large number of gastropod molluscs. It is found that seasonal changes do occur in most cases. This investigation aims to establish for Lymnaea stagnalis the precise relationship between oxygen consumption, temperature and body size at different times of the year and to determine how and why seasonal changes occur. Further it is intended to show which of these changes, if any, may be produced by acclimation to constant temperature in the laboratory. In view of the large amount of work done on acclimation of oxygen consumption it is perhaps surprising that only rarely have both seasonal and laboratory studies been performed on the same organism.

BASIC METHODS AND MATERIALS

Oxygen consumption was measured using a Gilson respirometer (Model GR 20). This apparatus was originally designed for measuring small changes in gas volume resulting from chemical reactions, but has many applications and has been used to measure respiration rates of both isolated tissues and whole animals.

FIGURE 16 ILLUSTRATION OF THE PRINCIPLE OF THE GILSON DIFFERENTIAL RESPIROMETER

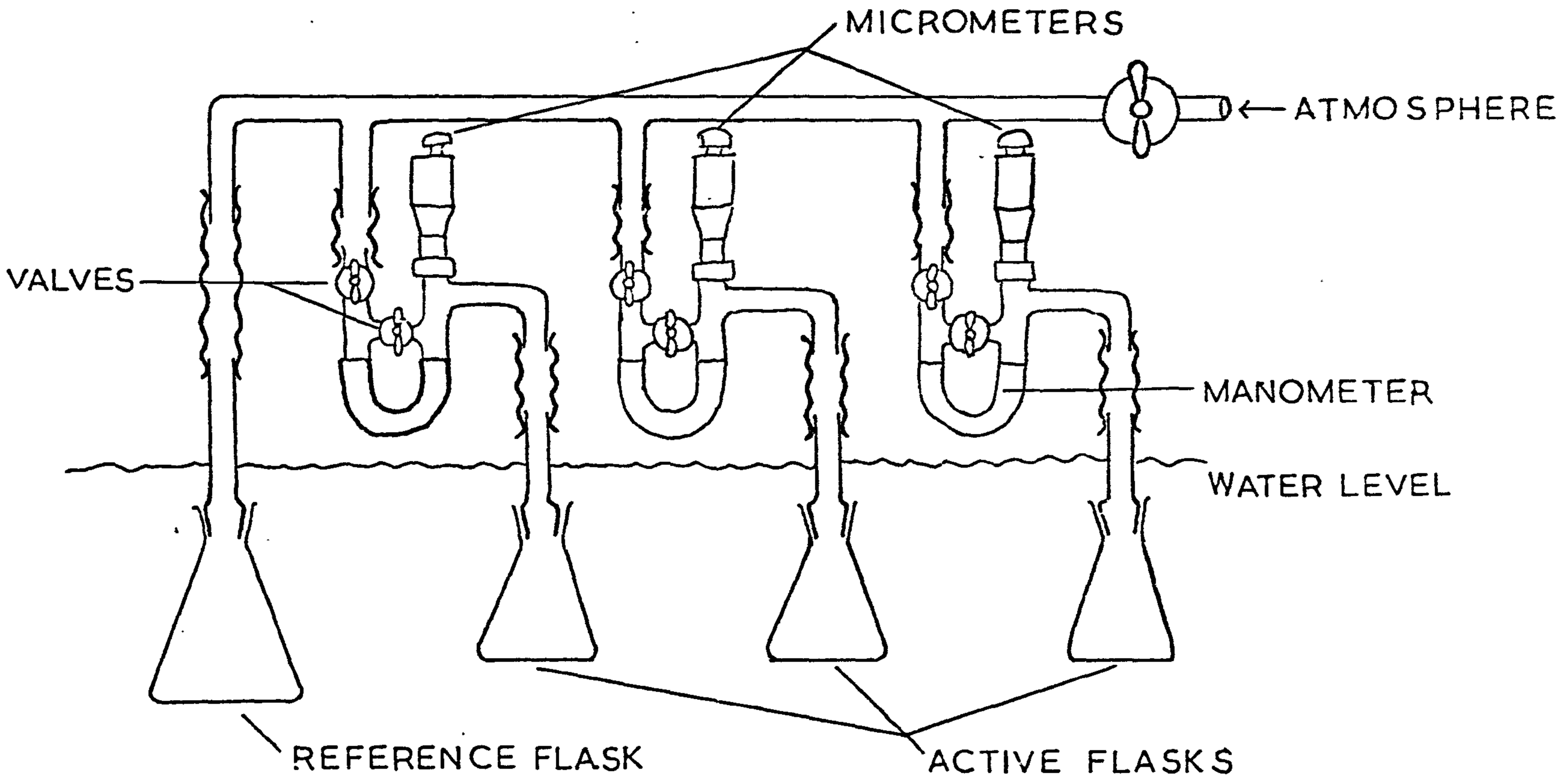
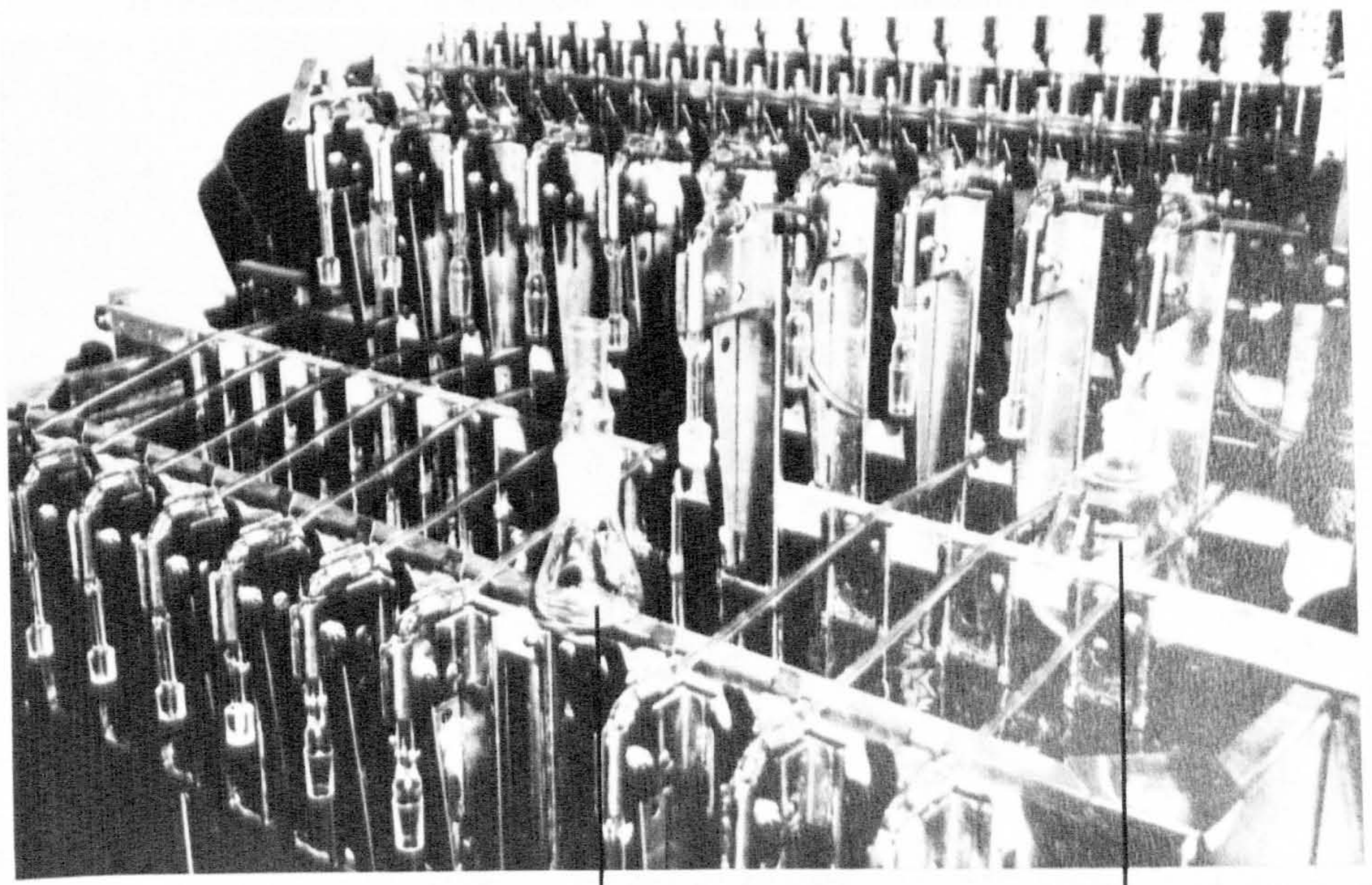


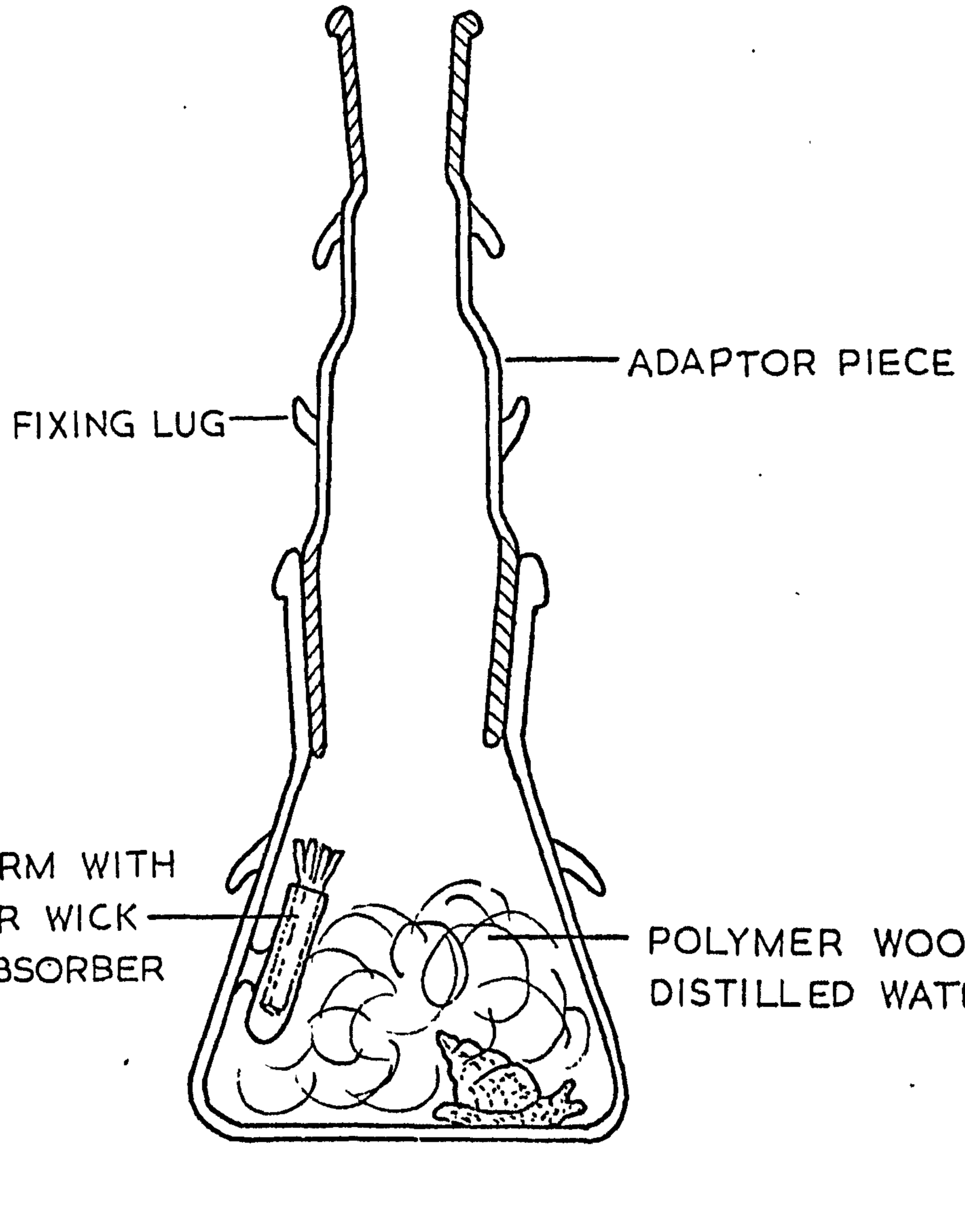
PLATE 3 THE GILSON DIFFERENTIAL RESPIROMETER



The machine consists essentially of twenty simple Warburg-type manometers mounted in racks. Temperature in the flasks is controlled by immersion in a large water bath which has heating and cooling facilities. The respiration vessels contain a side-arm into which is placed a carbon dioxide absorber. An animal placed in the respiration vessel consumes oxygen and the carbon dioxide evolved is collected in the absorber. A reduction in volume of the gas in the system results, causing a change in the level of the manometer fluid. This change is compensated for and measured by use of a micrometer screw attached to a membrane in the manometer. This is calibrated in microlitres. The volume change recorded over a chosen period of time is corrected for temperature and pressure using a basic expression derived from Charles' and Boyles' Laws. An illustration of the principle of the Gilson apparatus is shown in Fig. 16.(from Umbrecht et. al., 1964). Plate 3 is a photograph of the actual machine used.

It is important to note that when very small volume changes are being investigated the apparatus is particularly sensitive to temperature change. Many of the complications in the method result from necessary compensatory procedures and adjustments. A small quantity of distilled water must be added to each respiration vessel and to the reference flask (see Fig. 16 and Plate 3) which is connected to the distal end of the manometers. This ensures that a 'wet' gas volume is compared to another 'wet' gas reference volume. Marked instability results when attempting to run dry. Fortunately the snails used in this investigation do require a water-saturated atmosphere to remain damp and maintain respiration. The volume of the reference flask is approximately the same as the total volume of all the respiration vessels in use. This

FIGURE 17 MODIFIED RESPIRATION VESSEL



is necessary because not all of the enclosed gas volume is submerged in the controlled water bath and changes in ambient temperature therefore affect the respirometer. It has been empirically determined that when the active flasks are all of the same size the effect of ambient temperature changes are minimized by making the gas volume of the reference flasks equal to the sum of the gas volumes of the active flasks. Accurate volume measurements are not necessary; approximations using nominal values are adequate. Original respiration vessels supplied with the equipment were not used because the necks were too narrow to permit the introduction of some of the larger snails used in the study. Slightly larger vessels with wider necks, and an adaptor piece, were specially manufactured for use in these investigations (see Fig. 17 and Plate 3). These had a volume of about 50 ml. A reference flask of suitable volume was used according to the number of flasks in use, normally ten. At least one control vessel was included in the manometer racks to monitor volume changes caused by temperature fluctuation. If the controls registered large deviations then the results for that experiment were discarded. Normally, however, careful control of laboratory heating and ventilation resulted in a thermally stable environment with only small volume changes being detected in the control flasks.

When water bath temperature is changed, as when new experimental temperatures are selected, it is very important to allow sufficient time for the system to attain thermal and vapour pressure equalisation. According to the manufacturers instructions this period is at least fifteen minutes. Equilibration periods of one hour were allowed in the following investigations.

The respiration vessels were prepared for use in the following manner. First the flasks were carefully cleaned and autoclaved to destroy any microorganisms present. A small piece of polymer wool was then introduced into each vessel and moistened with 1.4 ml of pasteurized distilled water. It has been stated that a small volume of water is required in the respiration vessels to improve stability of the recordings. For simplification of technique it was decided to measure the rate of aerial oxygen consumption of the snails, and the volume of water added was sufficient to provide moisture and a humid atmosphere for the animals. It is known that Lymnaea stagnalis has the inherent ability to respire atmospheric oxygen and apparently does so even in the natural situation when dissolved pO_2 is high (Jones, 1961; McDonald, 1969). To the side-arm of each flask was then added 0.8 ml of 10% KOH solution - a carbon dioxide absorber. A small wick of rolled and serrated filter paper was added to each side arm to increase the surface area of the absorber and improve its efficiency. The snails could then be introduced into the flasks (except the controls), taking care not to allow contact with the KOH. The adaptor pieces were added and the labelled flasks attached to the appropriate numbered manometers of the Gilson apparatus. Seals were made airtight using lanolin, and springs or bands fixed where necessary. Figure 17 shows a respiration vessel prepared in the described manner.

To begin the experiment the racks of respiration vessels were lowered into the water bath and after the one-hour equilibration period the appropriate valves were turned and measurement of oxygen consumption begun. Micrometer readings for each flask, including the controls, were taken every five minutes for thirty five minutes except when respiration

rate was very low (e.g. small animals at low temperature), when recordings were made every ten minutes for seventy minutes. Laboratory temperature recordings were also made at these intervals and atmospheric pressure was measured on a laboratory mercury barometer at the end of the experiment. A graph was drawn for each flask's results of recorded volume against time. A straight line was then fitted by eye to these points and the gradient measured. This value represents the rate of oxygen consumption in microlitres per hour and was corrected for temperature and pressure using the following multiplying factor:-

$$\frac{273 \times Pb}{(t + 273) \times 760}$$

where t = temperature in ^{o}C
and Pb = barometric pressure in mm Hg.

At the end of the experimental period the flasks were raised from the water bath and the next experimental temperature selected by adjustment of the mercury-contact thermostat thermometer. Oxygen consumption was measured over a full range of experimental temperatures, from 5 to 30°C at intervals of 5°C, and from 30 to 37.5°C or 40°C at intervals of 2.5°C. Normally three runs were performed each day, the temperature increment between succeeding experiments usually being 5°C. It was necessary to choose a constant procedure and the order of exposure temperature used was as follows: 20, 25, 30; 15, 10, 5; 32.5, 35, 37.5 (40). A random sample of nine animals was normally used for measurement although there were occasional mortalities during the course of the experiments. The same animals were of necessity used throughout the nine or ten experimental runs of each condition. At the end of the series each animal was taken from the apparatus, the surplus moisture removed using paper tissue and the shell length and

fresh weight recorded. The snails were then killed by heating in water, the soft tissues extracted with forceps and dried on filter paper and the 'wet' tissue weight determined. The tissues were then placed on glass slides and dried for 24 hours at 65°C, then weighed again to obtain tissue 'dry' weight.

A computer program was written to calculate correlation and regression coefficients, using the 'least squares' method, for log oxygen consumption against log shell length, log fresh weight, log tissue weight and log tissue dry weight for all conditions. This was done in an attempt to standardise the results for animal size and to show how the size-rate relation changed with exposure temperature and season or temperature of acclimation. It is known (see Chapter I) that the size-rate relation is normally of the following form: $m = a\ w^b$, where m = metabolic rate, w = weight or other measure of size, and a and b are constants. Expressed in logarithms this equation has the form: $\log m = \log a + b \log w$, where b, the exponent, is the gradient of the log size-rate regression. It is normal to express body size in terms of tissue dry weight, but it was found that fresh weight yielded the greatest number of significant correlations with oxygen consumption. Although measurement of fresh weight incurred inevitable errors resulting from inconsistencies in the removal of excess moisture from the snails, it was clearly more accurate than the determination of dry weight, where considerable difficulty was encountered in removing all body tissues from frequently thin and brittle shells. A small error in the estimate of dry weight (which is smaller than fresh weight by a factor of about 20) would result in a large percentage error, whereas measurement of fresh weight is relatively insensitive to methodological

inconsistencies. Furthermore, tissue water content may in itself be an important factor in metabolic rate. Fresh weight was therefore chosen as the best representative of size and henceforth all discussion of size effects will use fresh weight as the basic measure.

SEASONAL CHANGES IN THE RATE OF OXYGEN CONSUMPTION

Introduction

Studies on seasonal variations of oxygen consumption in relation to temperature have been performed on various gastropod molluscs, including intertidal limpets (Davies, 1966 , 1967), marine winkles (Newell and Pye, 1970), freshwater limpets (Berg, 1951; Berg *et. al.*, 1958; Burky, 1971; McMahon, 1973; Calow, 1975), freshwater snails (Duškova, 1934; Berg, 1961; Calow, 1975) and terrestrial snails (Blazka, 1955). It is found that seasonal changes do occur in most cases. The selective value of seasonal metabolic adaptation in freshwater gastropods has been discussed, for example, by Burky (1971), Calow (1975) and Russell-Hunter (1977), although actual mechanisms concerned were not considered.

The aim of this investigation is to determine the precise effects of temperature on oxygen consumption in *L. stagnalis* at different seasons of the year, to show whether significant changes do occur, and also to assess the influence of body size on this relationship. Various influences on the control of growth and metabolism will be briefly discussed.

Method

Animals used in this study were collected from the field approximately every eight weeks. They were removed to the laboratory and, as in the heart rate study, stored until use in an outside tank supplied with a slow flow-through of tap water. The animals were fed as previously described. Respiration was measured as soon as possible after collection, the recordings usually being completed within five days. Oxygen consumption was determined over a full range of experimental temperatures from 5°C to 37.5 or 40°C. A random sample of nine animals was normally used. After the experiments the animals were killed and their size determined as described.

Results

1) The effects of body size on oxygen consumption:

The equations of the regression lines relating $\log_{10}$ oxygen consumption and $\log_{10}$ fresh weight were determined at each experimental temperature (Te) for each seasonal group of animals. Associated values of the correlation coefficient were also recorded. These data are shown in Table 8. Further analyses were performed only on data derived from significant size-rate correlations.

It can be seen from Table 8 that only October, February and May results yielded significant correlations ($p \leqslant 0.05$) at all, or nearly all, experimental temperatures. However, all seasons yielded significant correlations at Te 10 and 20°C. The gradient (b) of these regressions are shown with calculated standard errors in Table 9. These values are seen to vary according to season, although there is no clear pattern to the results. Values at Te 10°C ($0.37 < b < 1.12$) are generally lower

Table 8: Regression equations for seasonal oxygen consumption data.

Month	Te °C	Regression equation	r	P	N
April	5	log y = 1.66 + 0.52 log x	0.89	**	9
(1975)	10	log y = 1.56 + 0.37 log x	0.70	*	9
	15	log y = 1.81 + 0.31 log x	0.87	**	9
	20	log y = 1.90 + 0.72 log x	0.80	**	9
	25	log y = 2.07 + 0.58 log x	0.58	N.S.	9
	30	log y = 1.98 + 0.59 log x	0.77	*	9
	32.5	log y = 2.13 + 0.75 log x	0.84	**	9
	35	log y = 2.11 + 0.29 log x	0.39	N.S.	9
	37.5	log y = 1.84 + 0.01 log x	0.01	N.S.	9
June	10	log y = 1.74 + 1.12 log x	0.72	*	9
	15	log y = 1.97 + 0.79 log x	0.57	N.S.	9
	20	log y = 2.15 + 0.87 log x	0.81	**	9
	25	log y = 2.38 + 0.86 log x	0.75	*	9
	30	log y = 2.32 + 0.25 log x	0.16	N.S.	9
	32.5	log y = 2.30 + 0.02 log x	0.02	N.S.	9
	35	log y = 2.17 - 0.03 log x	-0.03	N.S.	9
	37.5	log y = 2.12 - 0.01 log x	-0.01	N.S.	9
	40	log y = 0.85 -10.80 log x	-0.42	N.S.	9
August	5	log y = 0.81 - 1.98 log x	-0.13	N.S.	8
	10	log y = 1.82 + 0.87 log x	0.92	**	8
	15	log y = 1.94 + 0.80 log x	0.60	N.S.	8
	20	log y = 1.94 + 1.04 log x	0.96	***	8
	25	log y = 2.26 + 0.40 log x	0.45	N.S.	8
	30	log y = 2.21 - 0.21 log x	-0.15	N.S.	8
	32.5	log y = 2.25 + 0.38 log x	0.45	N.S.	8
	35	log y = 2.32 + 0.12 log x	0.10	N.S.	8
	37.5	log y = 2.15 - 0.15 log x	-0.16	N.S.	8
	40	log y = 2.13 + 0.36 log x	0.28	N.S.	8

Table 8 (continued)

October	5	log y = 1.37 + 1.06 log x	0.86	**	8
	10	log y = 1.80 + 0.66 log x	0.85	**	8
	15	log y = 2.03 + 0.67 log x	0.85	**	8
	20	log y = 1.81 + 2.02 log x	0.96	***	8
	25	log y = 2.17 + 1.23 log x	0.81	**	8
	30	log y = 2.21 + 0.90 log x	0.80	**	8
	32.5	log y = 2.13 + 1.34 log x	0.92	**	8
	35	log y = 2.12 + 1.11 log x	0.85	**	8
	37.5	log y = 2.00 + 1.07 log x	0.93	***	8
	40	log y = 1.78 + 0.74 log x	0.66	N.S.	8
Nov - Dec	5	log y = 1.22 + 0.43 log x	0.47	N.S.	7
	10	log y = 1.62 + 0.56 log x	0.90	**	7
	15	log y = 1.96 + 1.00 log x	0.96	***	7
	20	log y = 1.43 + 0.46 log x	0.64	N.S.	7
	25	log y = 1.76 + 0.47 log x	0.68	N.S.	7
	30	log y = 1.46 + 0.12 log x	0.16	N.S.	7
	32.5	log y = 2.28 + 0.97 log x	0.82	*	7
	35	log y = 1.31 + 0.03 log x	0.03	N.S.	6
	37.5	log y = 1.47 + 0.24 log x	0.30	N.S.	6
February (1976)	5	log y = 1.17 + 0.29 log x	0.73	*	9
	10	log y = 1.52 + 0.63 log x	0.83	**	10
	15	log y = 1.85 + 0.90 log x	0.90	***	9
	20	log y = 1.97 + 0.80 log x	0.75	*	10
	25	log y = 2.03 + 0.72 log x	0.93	***	10
	30	log y = 2.20 + 0.87 log x	0.96	***	10
	32.5	log y = 2.01 + 0.71 log x	0.82	**	9
	35	log y = 1.49 + 0.55 log x	0.91	**	8
	37.5	log y = 1.34 + 0.36 log x	0.75	*	8

Table 8 (continued)

March	5	log y = 1.85 + 1.07 log x	0.90	***	9
	10	log y = 1.68 + 0.97 log x	0.97	***	9
	15	log y = 1.93 + 0.83 log x	0.83	**	9
	20	log y = 2.06 + 0.68 log x	0.67	*	9
	25	log y = 2.24 + 0.82 log x	0.77	*	9
	30	log y = 2.20 + 0.68 log x	0.84	**	9
	32.5	log y = 2.16 + 0.72 log x	0.68	*	9
	35	log y = 2.20 + 0.97 log x	0.60	N.S.	9
	37.5	log y = 1.69 + 0.63 log x	0.44	N.S.	9
May	5	log y = 1.58 + 0.94 log x	0.84	**	9
	10	log y = 1.81 + 0.55 log x	0.79	*	9
	15	log y = 1.94 + 0.44 log x	0.73	*	9
	20	log y = 2.28 + 1.16 log x	0.69	*	9
	25	log y = 2.35 + 0.62 log x	0.90	***	9
	27.5	log y = 2.42 + 0.60 log x	0.85	**	9
	30	log y = 2.42 + 0.58 log x	0.86	**	9
	32.5	log y = 2.35 + 0.39 log x	0.70	*	9
	35	log y = 2.33 + 0.44 log x	0.69	*	9
	37.5	log y = 2.31 + 0.88 log x	0.75	*	9

y = Rate of oxygen consumption (ul/hr)

x = Fresh weight (gm)

N = Number of animals used

r = Correlation coefficient

P = Degree of significance of r

*** : Significant at the P = 0.001 level

** : Significant at the P = 0.01 level

* : Significant at the P = 0.05 level

N.S.: Not Significant

Table 9: Slopes of the seasonal size-rate regressions at Te 10 and 20°C.

Month	b ± standard error Te 10°C	Te 20°C	N
April	0.37 ± 0.06	0.72 ± 0.09	9
June	1.12 ± 0.41	0.87 ± 0.23	9
August	0.87 ± 0.14	1.04 ± 0.11	8
October	0.66 ± 0.15	2.02 ± 0.21	8
Nov-Dec	0.56 ± 0.03	-	7
February	0.63 ± 0.04	0.80 ± 0.07	10
March	0.97 ± 0.02	0.68 ± 0.05	9
May	0.55 ± 0.09	1.16 ± 0.27	9

N = No. of animals

Table 10: Sizes of animals used in the seasonal oxygen consumption study.

Month	Mean fresh weight ($\log_{10}$ gm)	
April	-0.33 ± 0.17	(0.47 gm)
June	-0.01 ± 0.07	(0.98 gm)
August	0.05 ± 0.11	(1.12 gm)
October	0.13 ± 0.25	(1.34 gm)
Nov-Dec	-0.97 ± 0.26	(0.11 gm)
February	-1.18 ± 0.36	(0.07 gm)
March	-0.02 ± 0.19	(0.10 gm)
May	-0.26 ± 0.20	(0.55 gm)

Mean size of animals used, with standard deviation.

Antilogs of the means are shown in parentheses.

than those at To 20°C ($0.68 < b < 2.02$). It is evident therefore that both exposure temperature and seasonal factors affect the slope of the regressions relating log metabolism and log body weight. In this respect the data resemble those described for Lymnaea palustris and L. pereger (Berg and Ockelmann, 1959), Littorina littorea (Newell and Pye, 1970; Newell and Roy, 1973) and several other invertebrates (see Rao and Bullock, 1954). The results certainly suggest that it is not possible to assume a common value of b for general application to the data.

2) The effects of season on the relation between temperature and rate of oxygen consumption:

It has been shown that oxygen consumption is dependent upon animal size. For a true comparison of levels of oxygen consumption at different seasons it is necessary, therefore, to correct the results for size, especially since it is found that mean size of animals varies considerably throughout the year (see Table 10). The raw data, however, do give an estimation of the actual metabolism-temperature relationships for the snail populations at each season of the year. The mean rates of oxygen consumption of seasonally collected animals, with 95% confidence intervals, are shown in Table 11, and in graphical form in Fig. 18. The large scatter of data, indicated by the broad confidence intervals, largely reflects the mixed sizes of animals used in the experiments. There is some indication that seasonal changes in the form of the curves result in regions of reduced temperature sensitivity.

Direct comparison of the results can best be made following correction of the data to the calculated rate of oxygen consumption for

Table 11: Seasonal oxygen consumption rate data

	Experimental temperature (Te °C)										
	5	10	15	20	25	30	32.5	35	37.5	40	N
April	1.49 ±0.07	1.43 ±0.07	1.71 ±0.05	1.66 ±0.11	1.88 ±0.13	1.79 ±0.09	1.89 ±0.11	2.03 ±0.09	1.84 ±0.21	-	9
June	-	1.73 ±0.08	1.96 ±0.07	2.13 ±0.06	2.37 ±0.06	2.32 ±0.08	2.30 ±0.06	2.17 ±0.13	2.12 ±0.09	1.83 ±0.22	9
August	1.63 ±0.11	1.86 ±0.09	1.97 ±0.12	1.99 ±0.10	2.28 ±0.08	2.20 ±0.13	2.27 ±0.08	2.33 ±0.11	2.14 ±0.08	2.14 ±0.12	8
October	1.50 ±0.26	1.89 ±0.16	2.11 ±0.16	2.07 ±0.44	2.33 ±0.32	2.33 ±0.24	2.30 ±0.31	2.26 ±0.27	2.14 ±0.24	1.87 ±0.24	8
Nov-Dec	0.81 ±0.23	1.08 ±0.16	0.99 ±0.27	0.99 ±0.18	1.31 ±0.18	1.34 ±0.19	1.34 ±0.30	1.30 ±0.25	1.26 ±0.19	-	7
February	0.83 ±0.12	0.77 ±0.20	0.83 ±0.26	1.02 ±0.28	1.17 ±0.20	1.17 ±0.24	1.17 ±0.26	0.86 ±0.20	0.93 ±0.16	-	9

Table 11 (continued)

March	0.76 ±0.18	0.69 ±0.15	1.14 ±0.14	1.37 ±0.15	1.40 ±0.18	1.51 ±0.12	1.44 ±0.16	1.22 ±0.24	1.05 ±0.21	-	9
May	1.33 ±0.18	1.67 ±0.11	1.82 ±0.09	1.97 ±0.26	2.19 ±0.11	2.27 ±0.11	2.25 ±0.09	2.21 ±0.10	2.08 ±0.18	-	9

Mean rates of oxygen consumption ($\log_{10}$ ul/hr) with 95% confidence limits and sample size (N).

Figure 18: Seasonal oxygen consumption rate-temperature curves.

Mean values with 95% confidence intervals.

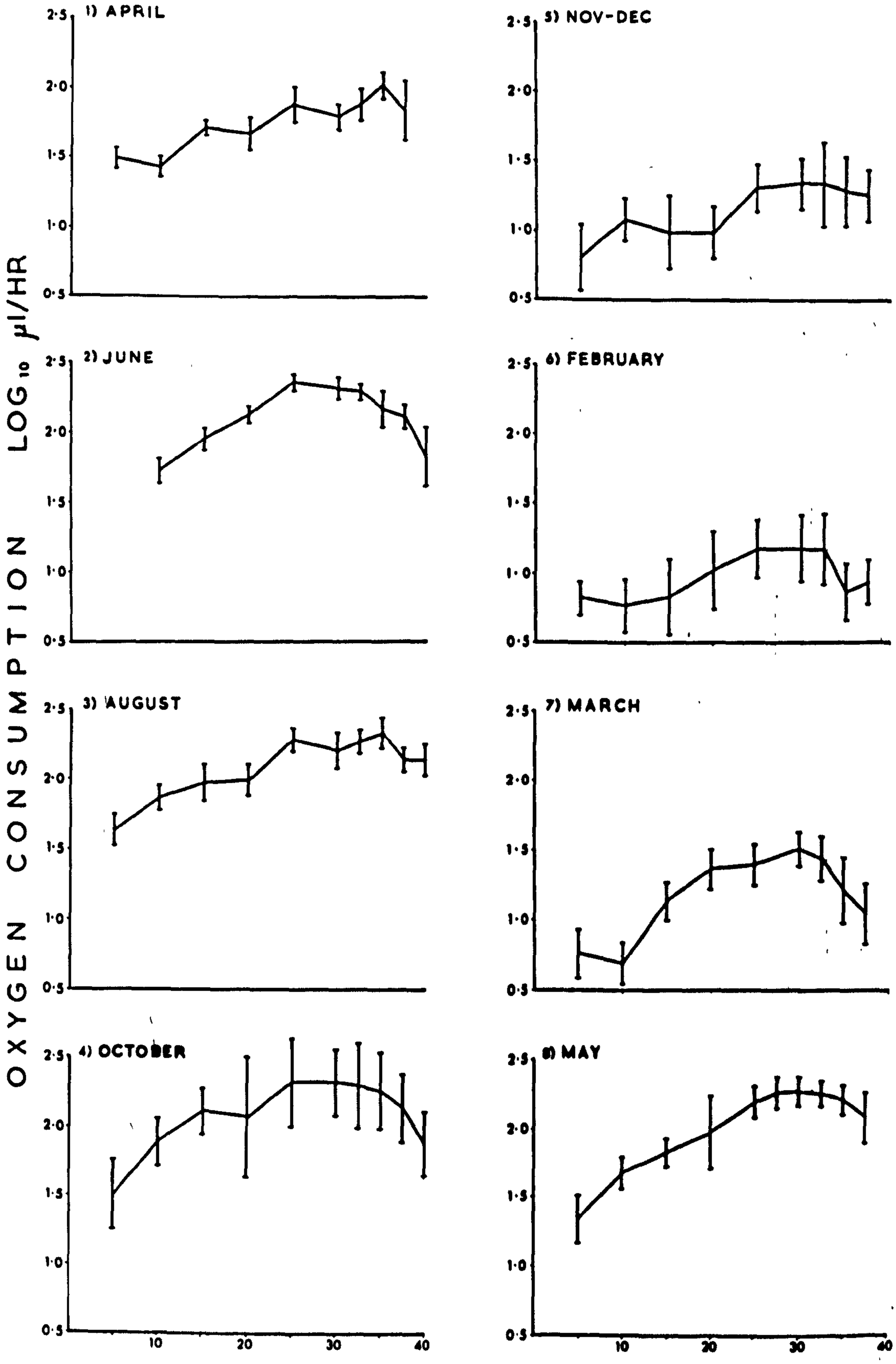

1) APRIL
2) JUNE
3) AUGUST
4) OCTOBER
5) NOV-DEC
6) FEBRUARY
7) MARCH
8) MAY
2·5
2·0
1·5
1·0
0·5
OXYGEN CONSUMPTION LOG10 µl/HR
10
20
30
40
TEMPERATURE °C

Figure 19: Comparison of October, February and May size-corrected oxygen consumption rate-temperature curves.

Legend: △-·-·-·-△ October

▲——————▲ February

●——————● May

Standard errors (derived from computed regression analyses) are shown on the February and May curves only.

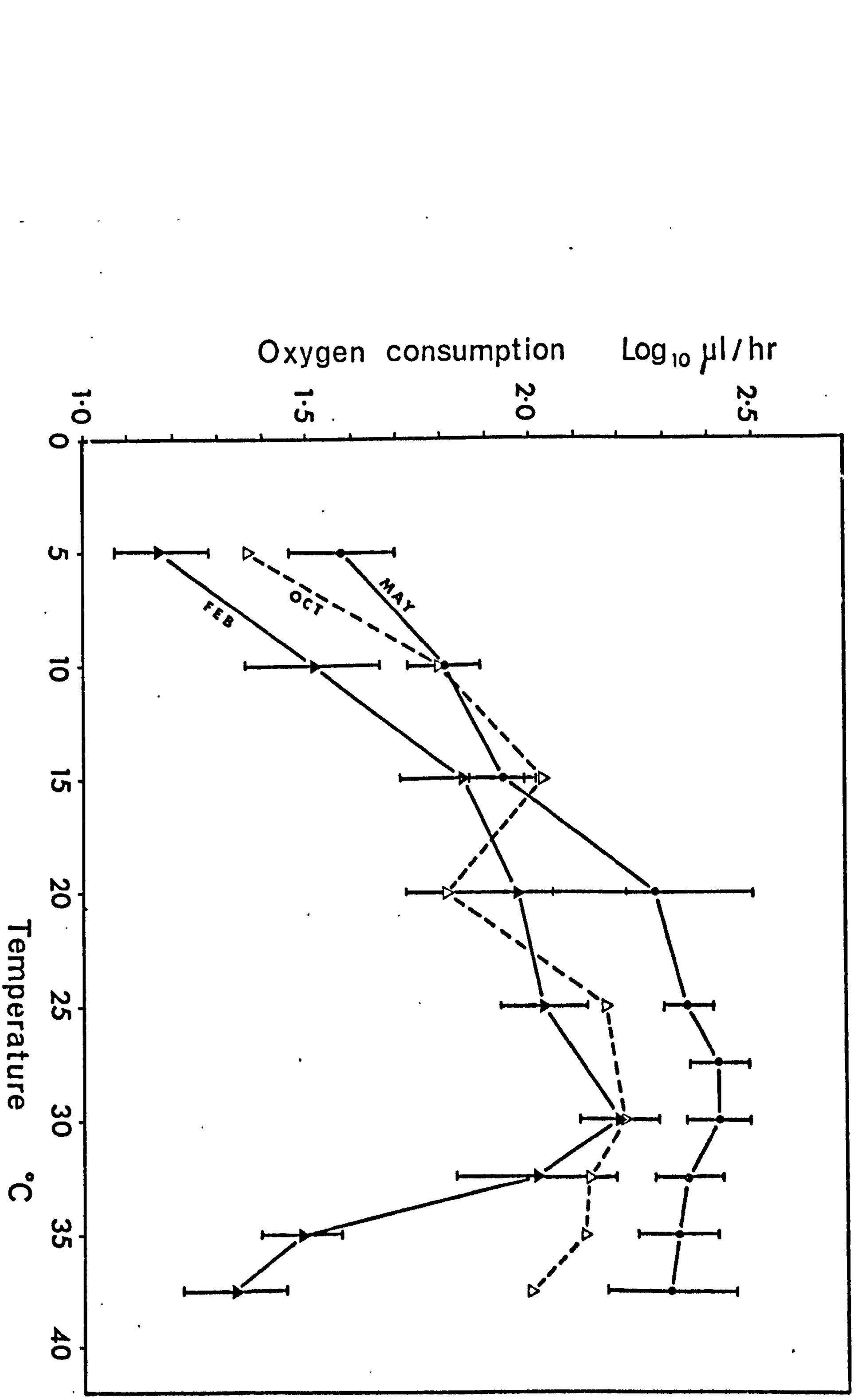
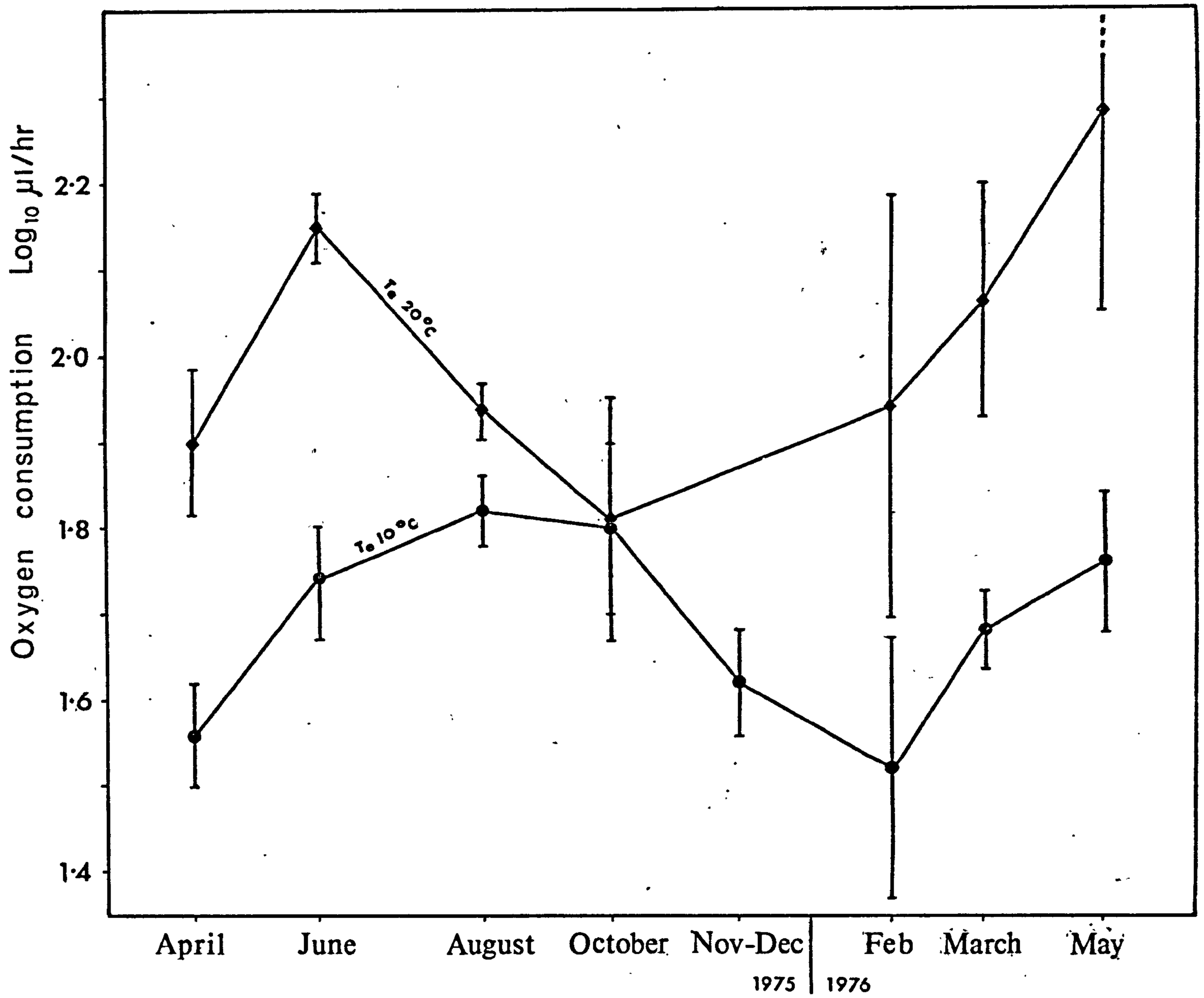

Oxygen consumption Log10 µl/hr
1.0
1.5
2.0
2.5
0
5
10
15
20
25
30
35
40
Temperature °C
FEB
OCT
MAY

Figure 20: Seasonal changes in weight-specific rates of oxygen consumption at Te 10 and 20°C.

Legend: ●——● Te: 10°C

♦——♦ Te: 20°C

Oxygen consumption rates for 1 gm fresh weight animals with standard errors derived from the regression analyses.


Oxygen consumption Log10 µl/hr
2·2
2·0
1·8
1·6
1·4
Te 20°C
Te 10°C
April
June
August
October
Nov-Dec
Feb
March
May
1975
1976

an arbitrary-sized animal of 1 gm fresh weight. Such comparisons were only possible where significant regressions were obtained. Figure 19 shows size-corrected rate-temperature curves derived from the results obtained from snails collected in October, February and May. It is seen that the May curve lies well above the February curve, with the October results intermediate. The February curve also appears most sensitive to high temperatures. Seasonal changes in weight-specific rates of oxygen consumption at two exposure temperatures, Te 10 and 20°C, are shown in Fig. 20. Oxygen consumption at 10°C shows a general increase from February to August with minimal values occurring between November and February and maximal values between August and October. At Te 20°C this general trend is maintained, but the maximum and minimum values for oxygen consumption occur somewhat earlier in the year, in June and October respectively.

Neither the size-corrected curves nor the uncorrected rate-temperature curves give any clear indication of resistance adaptations at either extreme of the temperature range.

Discussion

It is known from the studies of Zeuthen (1947, 1953), Hemmingsen (1950, 1960) and many other workers, that the slope 'b' of the common regression line relating log metabolism to log body weight in poikilotherms frequently approximates to 0.75. There are, however, many instances where significant deviations from this mean value occur in response to different conditions (see Chapter I). Berg and Ockelmann (1959), for example, found in various freshwater gastropods that 'b' varied between about 0.45 and 1.00 according to season, and

showed that these slopes of the size-rate regression lines for Lymnaea palustris and L. peregor were greater in June than in August. The results of the present study indicate that both time of year and exposure temperature influence the slope of this regression in Lymnaea stagnalis. Values are generally higher at Te 20°C than at Te 10°C and they vary considerably with season (Table 9). Similar results were obtained by Newell and Pye (1970), who showed for the intertidal snail Littorina littorea that a reduction in slope of the weight-specific oxygen consumption (b-1) occurred at all experimental temperatures with the onset of warmer conditions. Newell and Roy (1973) produced a statistical model for the metabolism of this animal which predicted an increase in the slope of the size-rate regression with increasing exposure temperature. Bayne (1973) found seasonal changes in b in the mussel Mytilus edulis. Such changes in the size-rate regression may well reflect different seasonal patterns of somatic growth, or size-dependent reproductive growth and activity. It is known that the rate of growth in L. stagnalis is not uniform but sigmoidal (Taylor, 1894) and, moreover, that at the approach of the breeding season, which commences in spring and is continuous through the summer, the sexual organs increase greatly in size, sometimes occupying the greater part of the body cavity (Ellis, 1926; Berrie, 1965). Berg and Ockelmann (1959) suggested that seasonal variation of 'b' in freshwater snails may be caused by a comparatively greater increase, during the breeding season in the oxygen consumption of the larger reproductive individuals than of the smaller ones. In autumn, when reproduction declines, 'b' then decreases.

Figure 18 shows no distinct pattern in the shapes of the uncorrected rate-temperature curves, but there is some indication that seasonal changes in the form of the curves result in regions of reduced temperature sensitivity over the ranges of temperature prevailing in the environment. The occurrence of such areas of temperature-independence in invertebrates has been described by Newell and Northcroft (1967; see also Davies and Tribe, 1969), by Riddle (1977) in work on two terrestrial snails, and by Percy and Aldrich (1971), who studied changes in respiration rates of various mollusc tissues.

The vertical translation of seasonal size-corrected rate-temperature curves, shown in Fig. 19, may be interpreted as a form of capacity adaptation. It appears to represent a reverse acclimation (see Precht, 1958; Prosser, 1958) since oxygen consumption during the cool months is generally less than during the warmer months when measured over the same range of temperature (see Fig. 2 for seasonal meteorological data). The results illustrated in Fig. 20 show that oxygen consumption at Te 10°C and 20°C was generally higher in the warm months than in the cold; which is further evidence for an apparent reverse adaptation type in response to seasonal changes in environmental temperature. The occurrence of such apparently anomalous adaptational responses is frequently encountered in freshwater molluscs and has been discussed, for example, by Berg et. al. (1958), Burky (1971), Calow (1975), McMahon (1973) and Russell-Hunter (1977). It has also been found in the terrestrial snail Helix pomatia (Blazka, 1955). Berg et. al. (1958) found that oxygen consumption of the freshwater limpet Ancylus fluviatilis was higher in spring and early summer than at other seasons of the year and proposed that this increased oxygen consumption, accompanied by a fall in growth rate, was most probably caused by

reproductive activity. Indeed, Calow (1975) found that respiratory metabolism in A. fluviatilis rose approximately 1.5 times during months when oviposition occurs. Burky (1971), finding reverse acclimation in the stream limpet Ferrissia rivularis, concluded that higher summer respiration rates reflected increased assimilation, due to growth and/or reproduction. McMahon (1973) obtained similar results for the freshwater limpet Laevapex fuscus, but showed that at low oxygen tensions winter animals have a higher respiration rate than summer animals. He concluded that apparent reverse acclimation in this limpet may be a side-effect of a more general physiological change in winter-conditioned animals which involves the survival of the over-wintering limpet in contact with reducing mud. It is not known whether Lymnaea stagnalis shows similar changes, but certainly in winter it is to be found in similar conditions. Barnes and his co-workers (1963, 1965) have shown that seasonal changes in oxygen uptake of barnacles are related to changes in composition and activity of the body tissues, the most noticeable changes taking place during spring as food reserves are laid down. A study by Hunter and Popovich (1977) on Cepaea nemoralis showed seasonal changes in organic carbon and the C:N ratio in the major food storage organs. Burky's (1971) data on energy balance emphasised the adaptive importance of reverse acclimation at times of low energy input during winter food storage. Certainly it is known that starvation can suppress metabolism (see Marsden, 1973). Russell-Hunter (1977) further discusses respiratory acclimation of pulmonate snails in the context of energy budgets of natural populations and their physical environments. Changes in oxygen consumption apparently resulting from reproductive activity may simply be caused by gross changes in weight of the sexual

organs, as previously discussed. Other aspects of growth may also be important. It can be seen (Table 10) that the mean size of animals used in the present study increased rapidly through spring, the start of the period of reproductive activity. The sharp decline in mean body size from October to November and December indicates the change from one generation of snails to the next. Mortality of the large mature animals begins in autumn and the over-wintering population consists mostly of much smaller animals. These sudden changes in mean size may also affect observed metabolic responses. Weiser et. al. (1970), looking at seasonal controls of respiration in the terrestrial snail Arianta arbustorum found that the Q_{10} of oxygen consumption varied with season and state of activity of the animal, but that temperature relationships of the respiration of foot and hepatopancreas tissues did not vary with season or other ecological factors. It was concluded that changes in temperature relationships are centrally regulated. It was also found that a metachronous course of temperature dependency of animals activity was correlated with seasonal changes of temperature. This evidence seems to suggest hormonal influences.

With regard to possible errors, the snails may have been affected by their short period in storage, during which temperatures were undoubtedly different from those of the pond from which they were taken, and during which time also the animals were fed ad libitum. Further, it was found that numbers of animals in the pond varied considerably with season, and whereas in summer a good assortment of snails could be collected, during winter the population was very much depleted and few in excess of requirements could be taken. A more detailed account of possible errors is given in the 'General Discussion' section of this chapter.

Conclusions

Results indicate that time of year and exposure temperature both influence the slope of the size-rate regression, this value being generally higher at higher exposure temperatures and varying considerably with season. The seasonal changes may well reflect different patterns of somatic growth, or size-dependent reproductive growth and activity. The shape of the simple oxygen consumption rate-temperature curves changed with season and there was evidence for regions of reduced temperature sensitivity within normal environmental temperature ranges. Weight-specific oxygen consumption was generally higher in the warm months than in the cold, and this was considered to be evidence for an apparent reverse acclimation response to seasonal changes in environmental temperature, although other environmental and physiological factors were thought to be of considerable importance here.

LABORATORY-INDUCED CHANGES IN THE RATE OF OXYGEN CONSUMPTION

Introduction

It has been shown that seasonal changes in the oxygen consumption of *L. stagnalis* involve changes in the size-rate regression and apparent reverse capacity adaptation of the oxygen consumption-temperature relation. It appeared likely, however, that seasonal temperature effects were not the most important factors affecting these functions. The purpose of this investigation is to show whether snails subjected to constant temperatures in the laboratory reveal adaptations in the oxygen consumption rate-temperature relation similar to those seen in

seasonally acclimated snails. It is of interest to isolate those changes, if any, which may be induced by acclimation to temperature alone.

Much information is available concerning laboratory-induced changes in oxygen consumption in a wide range of poikilothermic organisms, and this has been well reviewed (e.g. Bullock, 1955; Precht, 1958; McWhinnie, 1967; Weiser, 1973). With regard to gastropods, laboratory acclimation studies have been performed, for example, by Calow (1975), Kirbirger (1953), Newell and Pye (1970), Roy (1963, 1969) and Weiser et. al. (1970). See also Segal's (1961) review on acclimation in molluscs.

Method

Animals were collected from the field in spring 1975 and groups of twenty or more were stored in aerated water in environmental cabinets controlled to 5, 10 and 20°C (all c. ± 1°C). Lighting regimes were maintained at 12 hours light:12 hours dark. The animals were acclimated to each condition for at least six weeks before measurements were begun. Random samples of nine animals were used, recordings usually being completed within five days. The snails were returned to the cabinets when not in use. Oxygen consumption was measured, using the Gilson respirometer, over a full physiological range of temperatures according to the method previously described. The animals were killed immediately after experimentation and their size determined. Regression analyses were performed, as before.

Results

1) The effects of body size on oxygen consumption:

As in the seasonal study, the equations of the regression lines relating $\log_{10}$ oxygen consumption to $\log_{10}$ fresh weight were determined at each experimental (exposure) temperature (Te) for each acclimation temperature (Ta). The associated values of the correlation coefficients and their significance were also recorded. These data are shown in Table 12. Further analyses were performed only on data derived from significant size-rate correlations.

Only at Te 10°C were significant correlations obtained for all three acclimation conditions ($p \leqslant 0.05$). Table 13 shows values of the slope 'b' of these regressions, with standard errors. It appears that the slope of the regressions relating log metabolism to size is not significantly affected by temperature of acclimation and that values obtained are all close to the commonly derived figure of 0.75 (see Zeuthen, 1953; Hemmingsen, 1960).

2) The effects of temperature acclimation on the rate of oxygen consumption:

The mean rates of oxygen consumption of laboratory acclimated animals at a variety of exposure temperatures between 5 and 37.5°C are shown, with 95% confidence intervals, in Table 14, and in graphical form in Fig. 21. These values are uncorrected for size. Increased temperature of acclimation affects the form of the rate-temperature curves, and regions of reduced temperature sensitivity are evident.

Table 12: Regression equations for laboratory acclimation oxygen consumption data.

T_a °C	T_e °C	Regression equation	r	P	N
5	5	$\log y = 1.46 + 1.12 \log x$	0.80	**	9
	10	$\log y = 1.73 + 0.80 \log x$	0.78	*	9
	15	$\log y = 1.97 + 0.60 \log x$	0.47	N.S.	9
	20	$\log y = 1.94 + 0.53 \log x$	0.34	N.S.	9
	25	$\log y = 1.85 + 0.38 \log x$	0.12	N.S.	9
	30	$\log y = 2.31 + 0.75 \log x$	0.84	**	9
	32.5	$\log y = 1.78 - 0.08 \log x$	-0.02	N.S.	9
	35	$\log y = 1.83 + 0.04 \log x$	0.02	N.S.	9
10	5	$\log y = 1.63 + 0.24 \log x$	0.47	N.S.	9
	10	$\log y = 1.73 + 0.77 \log x$	0.70	*	9
	15	$\log y = 1.93 + 1.25 \log x$	0.78	*	9
	20	$\log y = 1.95 + 0.58 \log x$	0.49	N.S.	9
	25	$\log y = 2.21 + 0.74 \log x$	0.46	N.S.	9
	30	$\log y = 2.10 + 0.68 \log x$	0.59	N.S.	9
	32.5	$\log y = 2.26 + 0.81 \log x$	0.87	**	9
	35	$\log y = 1.72 + 0.29 \log x$	0.18	N.S.	9
	37.5	$\log y = 2.54 + 2.03 \log x$	0.92	***	9
20	5	$\log y = 1.19 - 0.40 \log x$	-0.17	N.S.	9
	10	$\log y = 1.59 + 0.67 \log x$	0.67	*	9
	15	$\log y = 1.90 + 1.11 \log x$	0.52	N.S.	9
	20	$\log y = 1.92 + 0.67 \log x$	0.49	N.S.	9
	25	$\log y = 2.03 + 0.18 \log x$	0.18	N.S.	9
	30	$\log y = 1.53 - 2.46 \log x$	-0.44	N.S.	9
	32.5	$\log y = 1.78 - 1.15 \log x$	-0.35	N.S.	9
	35	$\log y = 2.11 + 0.04 \log x$	0.02	N.S.	9
	37.5	$\log y = 2.13 + 0.32 \log x$	0.13	N.S.	9

*** $p \leq 0.001$

** $0.001 < p < 0.01$

* $0.01 < p < 0.05$

N.S. Not Significant ($p > 0.05$)

Table 13: Slopes of the laboratory acclimation size-rate regressions at Te 10°C.

Ta (°C)	b ± standard error	N
5	0.80 ± 0.15	9
10	0.77 ± 0.08	9
20	0.83 ± 0.07	9

N = number of animals used

b = slope of the regression

Table 14: Laboratory acclimation oxygen consumption rate data

	Experimental temperature (Te °C)									
Ta °C	5	10	15	20	25	30	32.5	35	37.5	N
5	1.08 ±0.11	1.47 ±0.08	1.77 ±0.10	1.74 ±0.12	1.78 ±0.22	2.07 ±0.07	1.80 ±0.22	1.82 ±0.14	1.47 ±0.31	9
10	1.54 ±0.05	1.43 ±0.10	1.45 ±0.15	1.72 ±0.11	1.92 ±0.14	1.84 ±0.10	1.95 ±0.04	1.61 ±0.15	1.77 ±0.20	9
20	1.28 ±0.08	1.41 ±0.05	1.66 ±0.08	1.77 ±0.05	1.99 ±0.04	1.95 ±0.27	2.03 ±0.12	2.10 ±0.06	2.17 ±0.19	9

Mean rates of oxygen consumption ($\log_{10}$ µl/hr) with 95% confidence intervals. N = sample size.

Fig. 21: Oxygen consumption rate-temperature curves for each acclimation temperature.

Mean values with 95% confidence intervals

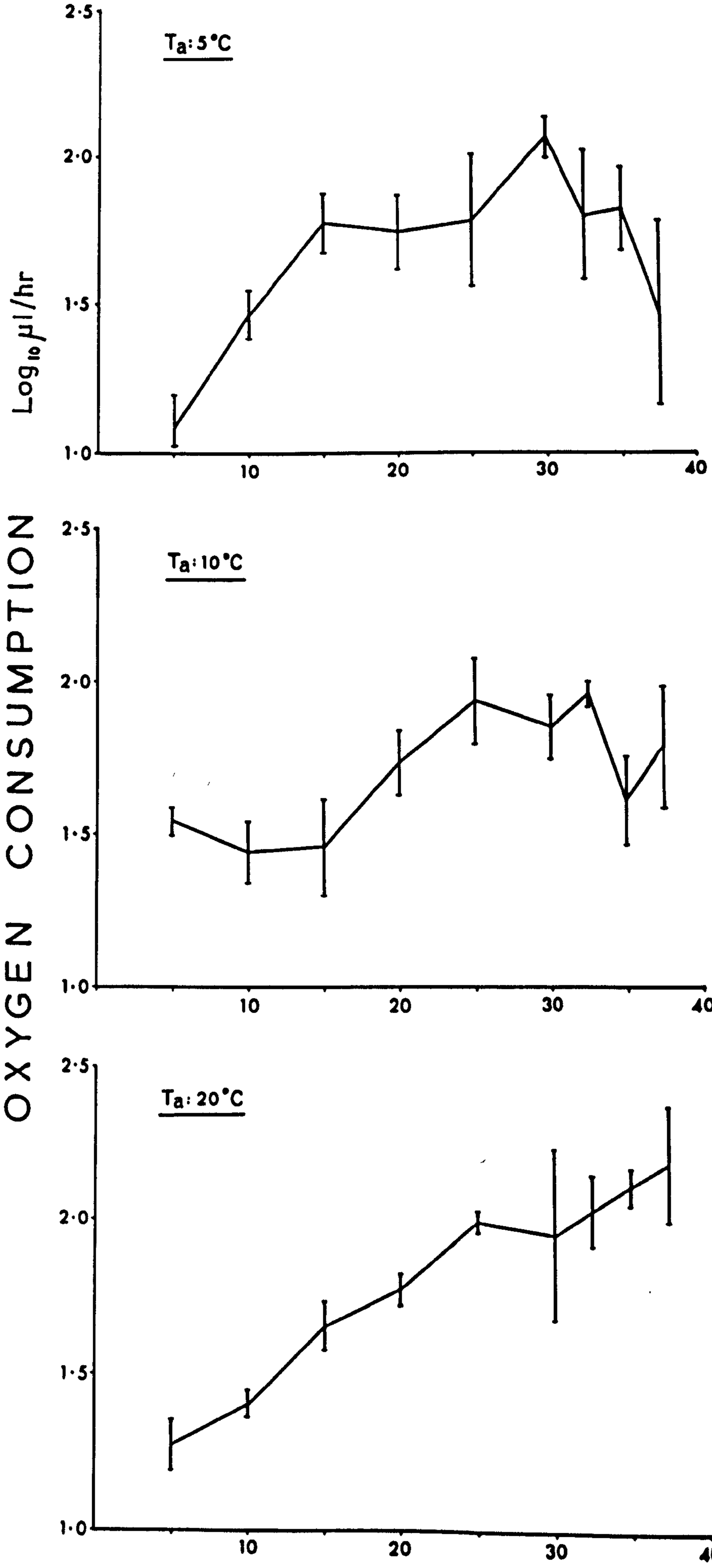

Ta: 5°C
Ta: 10°C
Ta: 20°C
2·5
2·0
1·5
1·0
10
20
30
40
Log10 µl/hr
OXYGEN CONSUMPTION
TEMPERATURE °C

Table 15: Sizes of animals used in the laboratory acclimation oxygen consumption study.

$Ta^{o}C$	Fresh weight (log_{10} gm)	
5	-0.33 ± 0.17	(0.47 gm)
10	-0.38 ± 0.07	(0.42 gm)
20	-0.21 ± 0.11	(0.62 gm)

Mean size of animals used, with standard deviations.

Antilog values of means in parentheses.

Table 16: Weight specific rates of oxygen consumption of laboratory-acclimated animalsat Te $10^{o}C$

Ta (^{o}C)	log_{10} µl/hr ($\pm$ standard error)
5	1.73 ± 0.06
10	1.73 ± 0.09
20	1.59 ± 0.04

High temperature of acclimation also appears to increase resistance to high experimental temperatures. These changes are complex with no clear progression or pattern.

Table 15 shows that the mean sizes of animals used in the acclimation groups differed considerably, and in order to make a valid comparison between results it was necessary to correct for body size. This was only possible using the Te 10°C results. Oxygen consumption was calculated for an arbitrary sized animal and 1 gm fresh weight, as before. Acclimation-induced changes in weight-specific rates of oxygen consumption at Te 10°C are shown in Table 16. The rates for Ta 5 and 10°C are identical. The oxygen consumption of the Ta 20°C animals is lower, but not significantly so ($p > 0.05$).

Discussion

It was shown in the seasonal study of oxygen consumption that both exposure temperature and time of year influenced the slope of the size-rate regression. The present brief investigation shows no significant difference between values of 'b' ($p > 0.05$) for each acclimation group measured at Te 10°C. The mean value is 0.80, which is close to the commonly quoted figure of 0.75 for the slope of the regressions relating poikilotherm metabolic rate to temperature.

Figure 21 shows no distinct pattern in the shapes of the rate-temperature curves for each Ta (uncorrected for size), but the Ta 5 and 10°C curves do exhibit ranges of temperature over which the rates of oxygen consumption remain virtually unchanged, and in this respect the results concur, to some degree, with those of the seasonal study. There is evidence also for a resistance adaptation at the upper

temperature extreme: the peak rate of the Ta 10°C curve occurs at a slightly higher temperature (32.5°C) than that of the Ta 5°C curve, and the peak of the Ta 20°C occurs at 37.5°C or above. Although these findings are not conclusive it is clear that increased temperature of acclimation does affect the shape of the oxygen consumption rate-temperature curves. Similar findings were reported by Newell and Pye (1970a, b) following seasonal and laboratory studies on the marine winkle Littorina littorea.

With regard to the influence of thermal acclimation on oxygen consumption for size-corrected data, there is no evidence for any adaptatory responses in the present investigation. At Te 10°C there ~~is~~ are no significant differences between mean oxygen consumption of the three acclimation groups. Studies by Calow (1975), have shown that the freshwater limpet Ancylus fluviatilis exhibits 'reverse' acclimation when adapted to 4° and 18°C, whereas the snail Planorbis contortus exhibits 'normal' temperature acclimation responses under these conditions. It appears that different species of gastropods exhibit different responses.

It should be noted that the amount of information which could be derived from the results was severely restricted by the lack of good correlation between oxygen consumption and size. The reasons for this are not known, but it is suggested that unnatural conditions of constant temperatures and daylength, in addition to different activity states and stresses of experimentation may have disrupted the physiological response of animals under investigation.

In consideration of further possible errors it is important to realise that during storage at constant temperature the animals in all cases were given food in excess of their requirements. In the natural

situation snails may respond to seasonal changes in food availability by showing changes in growth and metabolic rate, and these changes would be reflected in the results of the seasonal study. The laboratory investigation presumes that feeding ad libitum, rather than by fixed ration, is the most logical regime to follow when seeking a 'constant' procedure although, clearly, animals at higher acclimation temperatures are likely to consume considerably more food than those at low temperatures. It would be interesting to investigate the effects of different feeding regimes on the measured metabolism of the animals. Certainly it is known that starvation often affects ectotherm metabolism.

Further complicating factors in the laboratory studies, where temperature is theoretically the only valuable, are changing reproductive state and activity. It was observed that animals stored at high acclimation temperatures had a faster growth rate than those stored at low temperature. This may be considered an acceptable side-effect of temperature conditions, but associated with somatic growth is an increase in size of gonads and attainment of sexual maturity. This is an aspect of the animals physiology which cannot easily be analysed and corrected for. Effects of reproductive development during storage on the measured metabolism of the animal is not known. Lastly, it is possible that the original date of collection of the acclimated animals is important and that different results may have been achieved if, for example, summer animals were used. Certainly, transfer of animals from, in this case, cool and thermally fluctuating conditions, to constant temperatures, with equinoctial daylengths, may have had important physiological effects which cannot here be account for. Further errors incurred in this study, including acute effects of procedure, will be considered in the errors section of the 'General Discussion' of this chapter.

Conclusions

Acclimation to constant temperature results in changes in the shapes of the oxygen consumption rate-temperature curves, with regions of apparent reduced temperature sensitivity and changes in the position of the upper thermal limit. There is no evidence for capacity adaptations of the oxygen consumption function, nor for changes in the value of the slope of the size-rate regression.

GENERAL DISCUSSION

Experimental Findings

It was shown in the seasonal study of oxygen consumption that time of year affects the slope of the calculated size-rate regression and also the level of weight-specific oxygen consumption rates. It was found that summer animals generally exhibit higher rates of oxygen consumption than winter animals. Rate-temperature curves uncorrected for size showed regions of reduced temperature sensitivity within the normal ambient range. The seasonal change in the oxygen consumption-temperature relation was described as a 'reverse acclimation' since increasing seasonal temperature apparently induced higher rates of respiration when measured at the same experimental temperature. It was suggested, however, that this response was probably not a direct adaptation to temperature but rather a result of summer reproductive activities, growth and changes in tissue composition, adaptations to different oxygen tensions, winter food storage, or any combination of these factors. The laboratory study was undertaken in order to show which responses in the oxygen consumption-temperature relation, if any,

could be induced by acclimation to constant temperature alone. It was found that laboratory acclimation did induce changes in the shapes of the rate-temperature curves, with regions of reduced temperature sensitivity again evident, but there was no evidence for capacity adaptation of any sort, nor was the slope of the size-rate regression apparently affected by different temperatures of acclimation. It is therefore assumed that temperature alone is not important in the seasonal induction of 'reverse acclimation', although diurnal temperature fluctuations and the range of these oscillations may be relevant factors (see Newell, 1969; Widdows, 1976). Of the other possible influences listed above it is likely that reproductive changes and growth, involving also alterations in tissue composition, are most important, especially since the size-rate regression was affected by season but not by laboratory acclimation. These responses may be triggered by changes in daylength. Seasonal changes in dietary conditions are probably important also.

Temperature effects may have direct influence on the shapes of the rate-temperature curves and on the appearance of temperature-independent regions in courses of both studies, but no positive conclusions may be drawn concerning these results.

Possible Factors Involved in Oxygen Consumption Rate Changes

It is appropriate here to consider in more detail some factors which may be directly or indirectly responsible for observed seasonal changes in oxygen consumption. Particular attention is to be paid to growth and sexual development and the associated role of hormones.

Seasonal changes in food storage organs have been described by Barnes and associates (1963, 1975) in work on barnacles, and by Hunter and Popovich (1977) in studies on the terrestrial gastropod Cepaea nemoralis (see also Krüger, 1960; Goddard and Martin, 1966; Goudsmit, 1972). Barnes et. al. found that seasonal changes in oxygen consumption were directly related to changes in composition and activity of the body tissues with major changes taking place during spring as food reserves are laid down. Clearly, if growth is allometric and results in different ratios of tissues with different activities, then seasonal alterations in size-rate regressions and weight-specific oxygen consumption rates will result. Such is the situation observed in the present study, although similar results were not reproduced by acclimation to constant temperature in the laboratory, suggesting that such growth is dependent upon seasonal triggering factors such as daylength changes. This perhaps implies the involvement of hormonal mechanisms. It is thought that seasonal changes in tissue composition result essentially from changes in individuals' energy partitioning in response to over-wintering and periods of peak reproductive activity. These changes in tissue composition, often with alterations in the carbon and nitrogen contents, are frequently of such magnitude that the calorific value of the whole organisms may increase or decrease (see Hunter and Popovich, 1977). Changes in whole animal calorific value have been associated with a variety of environmental and physiological changes, two of which are particularly relevant here. Prus (1970) observed that high calorific values are usually associated with periods preceeding prolonged diapause or stages preceeding the output of gametes, and Russell-Hunter (1970), has observed that the C:N ratio of

snail tissue increases prior to over-wintering as energy-rich substrates are stored, and that this ratio decreases prior to reproduction. Hunter and Popovich (1977) showed that the digestive gland and mature albumen gland have higher than normal carbon content and far lower than normal nitrogen content. These authors considered seasonal changes in the C and N component of these glands as compared with other tissues to be consistent with their role in energy storage. Carbon stored in the digestive gland of Cepaea nemoralis was found to be utilised by the hibernating snail as an energy reserve, whilst that of the albumen gland passed into the eggs. Veldhuijzen and Van Beek (1976) have shown for Lymnaea stagnalis that the mantle, digestive gland/ovotestis and muscle fraction are very important in the storage and mobilisation of polysaccharides. Polysaccharide synthesis was seen to be stimulated by high haemolymph-glucose levels, being induced directly or via hormone producing organs. Further investigations (Veldhuijzen and Cuperus, 1976) showed that hormones from the Dorsal Bodies of the cerebral ganglia of Lymnaea stagnalis stimulate synthesis of galactogen in the albumen gland but that synthesis of glycogen in the mantle is independent of Dorsal Body Hormone and is influenced directly by the glucose levels of the haemolymph. Seasonal build-up of food storage tissues, then, is dependent upon hormonal influences associated with egg production, and also upon dietary conditions.

Other aspects of growth in pulmonates have been recently studied, for example, by Widjenes and Runham (1977), Geraerts (1976) and Geraerts and Joosse (1975). The specific control of somatic growth in invertebrates as a whole has been poorly investigated (see Highnam and Hill, 1969). Recently, however, much work has been done on molluscs,

and especially on Lymnaea stagnalis. Preliminary studies by Geraerts and Joosse (1975) showed that the dorsal bodies (DB) of the cerebral ganglia produce an endocrine substance, the dorsal body hormone (DBH) that stimulates vitellogenesis and growth of the female accessory sex organs. Geraerts (1976) then investigated roles of other endocrine centres, namely the LGC*, the caudodorsal cells (CDC), bright green cells (BGC) and the lateral lobes (LL). Cauterisation of the neurosecretory LGC of rapidly growing juvenile snails resulted in a markedly retarded body growth, which could be restored by implantation of cerebral ganglia containing LGC. Cauterisation of the LL resulted in giant growth, suggesting that the LL have a growth retarding effect. It was found that the effect of the growth hormone of the LGC on the female organs requires the presence of the gonadotropins produced by the DB and CDC. The LGC growth hormone was also seen to have an indirect effect, via growth and metabolism, on feeding activity. Widjenes and Nunham (1976, 1977) found similar effects for the neurosecretory medial cells of the slug Agriolimax reticulatus. It is seen, therefore, that general control of somatic growth in Lymnaea stagnalis is mediated by hormonal influences. It is not known precisely what triggers activity of the neurosecretory organs, but certainly seasonal environmental conditions are likely to be important. Neither is it known what metabolic side-effects this activity has on oxygen consumption. Probably more important than somatic growth in the observed rapid seasonal changes in body weight and changes in oxygen consumption is the disproportionate growth of the reproductive organs during spring and summer. The seasonal trends in the growth of these organs in L. stagnalis has been recorded by Berrie (1965). Development of the gonad shows rapid

* LGC = light green cells

changes, leading to full maturation, which appear to be related to season. The timing of these rapid changes in the gonads of the first year snails corresponds well with expansion of the albumen gland (see earlier discussion), the start of secretion of the spermatotheca and with the field observation that egg-laying starts during spring. Growth of parts of the reproductive system not directly involved in these events is more continuous. The rapid development of the gonads has been shown to be a result of hormonal influences, and in L. stagnalis the neurosecretory bodies responsible have been identified (Geraerts, 1976; Geraerts and Algera, 1975; Geraerts and Bohlken, 1976; Geraerts and Joosse, 1975). It was shown that the dorsal body hormone (DBH) directly or indirectly controls vitellogenesis and the growth of the female accessory sex organs. The neurosecretory CDC produce a hormone which controls ovulation and the LL are thought to produce one or more hormones which accelerate male and female development and stimulate the rate of ovipository activity. Observed seasonal reduction in growth appears to be related to the stimulation of female reproductive activity. Both the frequent production of egg masses and body growth demand large quantities of metabolites and energy, and this probably makes the two processes antagonistic.

Thus it is seen that increase in size of food storage organs, general somatic growth and growth of the reproductive organs all exhibit seasonal changes mediated by hormonal actions and interactions. Such changes must directly or indirectly induce alterations in other physiological processes including respiration rates. The complex seasonal changes observed in the oxygen consumption rate-temperature relation in L. stagnalis may therefore be partly explained by various

growth factors. In addition, seasonal changes in food intake may have a direct effect on oxygen consumption. Certainly it is known that starvation can suppress metabolic rate in poikilotherms (see Marsden, 1973) and it is agreed that starvation is an important ecological factor which may be implicated in seasonal variations in metabolism. Nopp (1965) showed in the pulmonate Arianta arbustorum that starved animals had a lower oxygen consumption than well fed ones, and if starvation was continued for four days then the rate fell to aestivation level. This effect also was observed in isolated hepatopancreas and gonad tissue following several weeks starvation (Nopp and Farahat, 1967). In studies on the effects of starvation on the haemolymph-glucose levels of Lymnaea stagnalis, Veldhuijzen (1975) showed that during starvation (up to 15 days) haemolymph-glucose remained at the same constant level, but reproduction and growth stopped, resulting in decreased metabolic rates. At the restart of feeding there was a temporary rise in the haemolymph-glucose level, which appeared to be the stimulus for the start of reproduction and growth. Seasonal changes in the quality and ingestibility of available food may also be of importance in observed respiration changes. Changes in respiratory quotient (RQ) according to the substrate utilised would affect rates of oxygen consumption.

This discussion has been concerned with possible explanations for the seasonal 'reverse acclimation' observed in this study. It has been shown that hormonal and dietary influences are probably most important. However, the masked existence of other capacity adaptations of the oxygen consumption-temperature relation, with changes at the sub-cellular level, cannot be discounted. This will be discussed further in the final chapter.

Errors and Assumptions of the Method

Various errors are incurred in the described method of use of the Gilson differential respirometer. Actual physical conditions in the respiration vessels are important in this respect. Movement of the snail inside the vessel was somewhat restricted and this might have affected the activity of the animal. The amount of handling of the animals immediately before the experiment is also important in this respect. Certainly activity is a very important consideration when measuring total metabolic rates of animals, but is very difficult to control in the experimental situation. No attempt was made here to account for or control activity, and this probably resulted in greater variability of the oxygen consumption data. Effects of different states of activity have been considered by various authors from an early date when differences between 'standard' or 'basal' and 'active' rates were described (see Fry, 1947). Newell (1973) briefly reviews such effects in ectotherms and also describes the influence of non-activity on acclimatory responses. Ideally, if an animal shows continuous activity of some description, the oxygen consumption rate and activity level should be simultaneously monitored. Oxygen consumption then can be plotted as a function of locomotory or other activity and by extrapolation the respiration rate of the organism at zero or maximum activity be recorded. It has been shown (Newell, 1973) that experimental temperature often affects 'standard' and 'active' rates differently. Metabolism of quiescent Littorina littorea, for example, is at a low level and markedly independent of experimental temperature. Such effects could account for some of the unexpected and anomalous features of the rate-temperature curves shown in this study. For example, regions of

reduced temperature sensitivity may result from general inactivity at those temperatures. Newell, however, states that most organisms are to a greater or lesser extent active in a respirometer and therefore 'active' temperature-dependent rates of oxygen consumption are mostly obtained.

A further important aspect of physical conditions inside the respiration vessels concerns the limited amount of water present. It is true that in the natural situation Lymnaea stagnalis does breath atmospheric oxygen even when the pO_2 in solution is high and that the lung accounts for up to 40% of total uptake even in the most favourable conditions for cutaneous respiration (Jones, 1961). It would nevertheless be interesting to know whether different results would have been obtained if the animals' respiration had been measured under water. Simultaneous measurement of aquatic and aerial rates of oxygen consumption for each individual specimen would have been a difficult procedure and the Gilson apparatus in its simple form could not have been used.

The length of time that the animals were subjected to stressful experimental conditions was unavoidably long owing to the necessary time for thermal equilibration of the apparatus and the length of the experiment proper. In all investigations concerning acclimation to temperature it is important to reduce effects of re-adaptation by minimising times of exposure to experimental temperatures. The long exposure times incurred using the Gilson apparatus may be considered a major error although it is true that the normal time course of temperature acclimation is measured in days or even weeks. Also it was necessary to measure oxygen consumption of the same group of animals

successively at (usually) three different experimental temperatures in order that the complete range of experimental temperatures could be studied in a suitably short time. It was therefore necessary to leave animals in the respiration vessels between experiments. The snails were thus subjected to many stressful temperature changes and 'rest' periods of various lengths which may have affected the animals' metabolism. Moreover, they were unable to feed during this time. Although a constant order of Te's was adopted (see methods section) it is likely that animals' reaction to changed temperature was important and may have varied according to their previous thermal history, possibly resulting in unknown changes in the measured rate-temperature curves.

The actual construction and conformation of the apparatus may have led to further minor errors. For example, the volume of the respiration vessels was quite large compared to the size of the animals. This possibly resulted in diffusion effects delaying absorption and reducing the efficiency of the CO_2 absorber. The Gilson is supplied with a shaking mechanism which is intended to aid mixing. Needless to say this could not be used in the present study. There are some connective parts of the manometers which are not temperature controlled but are exposed to ambient conditions, resulting in the machine recordings being sensitive to external temperature fluctuations. The precautions taken to record and minimise these effects are described in the Methods section. As a result of these efforts it is unlikely that external temperature influences had any measurable effect on the respiration rates recorded.

A small error in the values of mean oxygen consumption over each experimental period may have resulted from the practice of fitting straight lines by eye to the graph plots for each individual snail.

In the results section it was described how regression equations were calculated for the size-rate data using various parameters of size. The most frequently significant regression was found to be that between log oxygen consumption and log fresh weight, and this was chosen for use throughout the study. It should be noted, however, that errors are incurred in fresh weight measurement, resulting from difficulties in removing all excess moisture from the snails. A standard procedure was adopted and constant errors assumed. A further drawback in the use of fresh weight as the correlate is the influence of shell weight. It is known that the tissue weight - shell weight ratio changes slightly with size and according to environmental conditions (see Taylor, 1894; Nolan and Von Brand, 1954; Eckblad, 1971) and this may affect to a small degree relations between the regression equations obtained.

Effects of storage and feeding regimes have been discussed previously in the relevant sections.

Conclusions

It has been shown that there are seasonal changes in the size-rate regression and in the general shape of the seasonal oxygen consumption rate-temperature curves, and there is evidence for 'reverse acclimation' in response to seasonal changes in temperature, with

weight-specific rates being generally higher in the warmer months than in the cold. Acclimation in the laboratory, however, resulted only in obvious changes in the shape and range of the oxygen consumption rate-temperature curves. There was no evidence for capacity adaptations of any type. It is proposed that the seasonal changes resulted essentially from growth and changes in tissue composition, reproductive growth and activity, and seasonal changes in dietary conditions. Hormonal influences, forming the link between physiology and environment, are thought to be most important, with adaptation to temperature alone being relatively unimportant.

CHAPTER IV

SEASONAL STUDY OF ASSIMILATION EFFICIENCIES

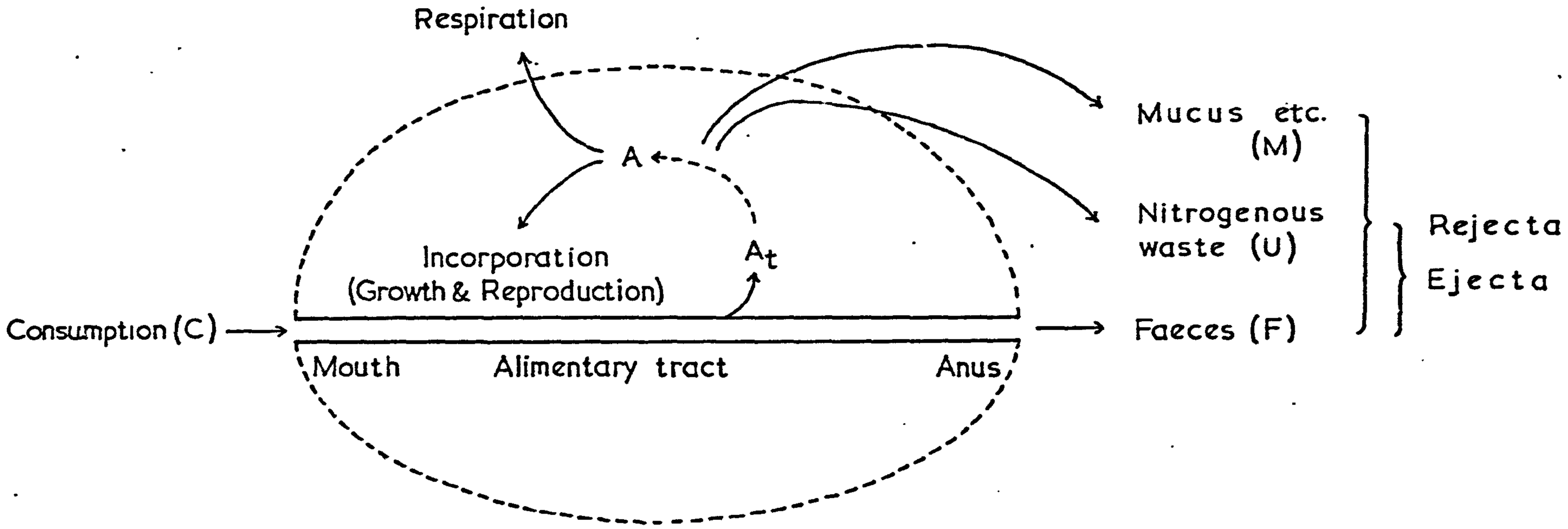
INTRODUCTION

Seasonal and laboratory-induced changes have been observed in the heart beat and oxygen consumption rate functions of L. stagnalis. Acclimation of heart rate is considered to be important in the maintenance of energy supply to the tissues, and changes in oxygen consumption are related to seasonal differences in amounts of energy consumed by the animal. Assimilation efficiency determines the degree of utilisation of food and therefore is also important in the overall energy budget. It is of interest to determine whether seasonal changes occur in this function, especially since any such changes are likely to be caused by alterations in the activity of digestive enzymes. Certainly it is known that these enzymes can reveal acclimatory changes (for example, Mews, 1957).

Studies on the efficiency of assimilation of food have been undertaken on numerous organisms. The total calorific contents of food ingested and material egested are measured and assimilation efficiency calculated using simple equations such as those described by Petrusewicz and Macfadyen (1970). These authors defined assimilation efficiency as the ratio of consumption less rejection to total consumption, in energy terms. The present study adopts this basic definition, which may be written:

$A = (C - (F + U + M))/C$, where A = assimilation efficiency, C = total consumption, F = true faeces, U = urine (nitrogenous excretion) and M = other metabolised products (notably mucus in this instance).

FIG. 22 SCHEMATIC REPRESENTATION OF THE USE OF ASSIMILATION TERMS



$$A_t = (C - F)/C$$

$$A = (C - (F+U+M))/C$$

$$C = \text{Rejecta} + \text{Respiration} + \text{Incorporation}$$

The fraction F + U will be referred to as 'ejecta', and F + U + M described by the term 'rejecta'. Reference to 'true assimilation' will imply the strict definition of this term; that is the total quantity of energy passed through the gut wall which becomes available to the animal, irrespective of its eventual fate. A true assimilation efficiency would be simply defined as $A_t = (C - F)/C$. This ratio is sometimes called the 'digestive efficiency' (see Petrusewicz and Macfadyan, 1970) but is seldom used because of practical difficulties encountered in separating from the true faeces the components of nitrogenous excretion and other metabolised products such as mucus, digestive enzymes and gut lining. Figure 22 summarises the use of terms in this study.

Calorific values of food and faeces are commonly assumed to be equal and gravimetric methods are used to determine assimilation efficiencies in terms of dry weight. Assimilation efficiencies may also be determined by chemical analysis of organic carbon content (Kofoed, 1975a; Hunter, 1975). It is often very difficult, however, to collect all the faeces produced by an animal under study and so indigestible tracer substances, such as chromic oxide, are sometimes used to label food. If the concentration of marker substance and amount of food consumed is known, then measurements of the strength of marker in the faeces permits calculation of the total quantity produced daily (Edin, 1926, in Milner, 1967; McGinnis and Kasting, 1964). Conover (1966) has developed a fast and simple method analogous to this which assumes that only the organic fraction of food ingested is affected by the digestive process, and requires only measurement of the ash-free dry weight:dry weight ratios of food and faeces. Ash content here corresponds to the

indigestible marker substance. The critical assumption regarding the digestive process, however, does not always hold true (see Richardson, 1975). Techniques involving radioactive tracers have recently been developed. These use substances which may be partly incorporated into the tissues, such as ^{85}Sr (Hubbell et. al., 1965), ^{32}P (Paris and Sikora, 1967), ^{65}Zn (Williamson, 1975) and ^{131}I (Marshall and Orr, 1955), or elements which are totally assimilatable, such as ^{3}H or ^{14}C (Arnold, 1971; Brenner et. al., 1976; Kofoed, 1975a, b; Tsikhon-Lukania and Sorokin, 1965). Calow and Fletcher (1972) have devised a radiotracer method using ^{14}C and ^{51}Cr which is analogous to indicator methods and is less tedious and time-consuming than normal ^{14}C techniques. This, unlike normal gravimetric and radiotracer methods, does not require the quantitative collection of faeces.

Such variety of techniques has enabled assimilation to be measured in numerous species of invertebrates, both in the laboratory and in the field, from zooplankton (Conover, 1966) to grasshoppers (Wiegert, 1965) and including various gastropod molluscs (Calow and Fletcher, 1972; Kofoed, 1975a, b; Richardson, 1975; Tsikhon-Lukania and Sorokin, 1965).

Although the acute effects of temperature on assimilation efficiency are frequently described, long term effects of temperature acclimation have been investigated relatively seldom (Precht et. al., 1973). Neither have seasonal changes in this function been adequately described, yet it is clear that assimilation, like any other metabolic process, is liable to be influenced not only by acute temperature changes but also by long-term seasonal/acclimation conditions. The aim of this investigation is to determine whether Lymnaea stagnalis exhibits any form of seasonal compensation (capacity adaptation) in efficiency of

assimilation. Winter and summer-adapted animals were compared at an experimental temperature of 15°C, using a ^{14}C tracer method involving the quantitative collection of all metabolic products. The use of a radioactive marker, although time-consuming, does allow more precise analysis of food utilisation over a short period of time and the method chosen, based on the work of Kofoed (1975a, b), enabled ingested food to be subsequently partitioned into respired, incorporated and rejected components. ^{14}C was considered the best isotope to use because carbon is one of the most abundant elements in organic material and is incorporated into most metabolic substances. It is assumed that the ^{14}C label, once incorporated into the food, behaves as ^{12}C and when ingested is liable to follow the same pathways as any other carbon atoms.

METHODS AND MATERIALS

The method used to determine assimilation efficiency in Lymnaea stagnalis was based, in its essential details, on the work of Kofoed (1975a, b and personal communication). It differs by having the capacity of monitoring food utilisation by individual animals throughout any required experimental period. Food used was the green alga Chlorella ellipsoidea, labelled using NaH $^{14}CO_3$. Liquid scintillation techniques were used for the detection of radioactivity in respired, rejected and incorporated components. Nitric acid digestion methods were used as necessary to break down organic material.

Culture and Labelling of Food Material

During the course of the studies, lettuce has been used as the main food material for L. stagnalis in storage. Initially, therefore, the

feasibility of labelling lettuce leaves with ^{14}C was investigated (using one of the methods described by Sudia and Linok, 1961). It was considered necessary here, however, to obtain a uniform labelling of material: this is difficult to achieve with vascular plants. Consequently a common freshwater unicellular alga, Chlorella ellipsoidea was chosen as the food material. This may be easily labelled by introducing the radioactive tracer, in suitable form, into the culture medium. Planktonic algae are not usual food items for L. stagnalis, but this animal usually obtains food by scraping material from rocks and submerged vegetation and it was found that C. ellipoidea, presented on a surface, was readily fed upon.

Chlorella ellipsoidea (obtained from the Culture Centre of Algae and Protozoa, Cambridge) was cultured in 1 L conical flasks containing approximately 800 ml of culture medium under constant aeration. The medium used was that first described by Beijerinck which, according to Nichols (1973), is suitable for the culture of all Chlorophyceae. It was prepared from three basic stock solutions and a micronutrient solution, containing the following salts in the specified quantities:

Stock I (100 ml/L medium)

$NH_4 NO_3$	1.5 g/L
$K_2 HPO_4$	0.2 g/L
$MgSO_4 7H_2O$	0.2 g/L
$Ca Cl_2 2H_2O$	0.1 g/L

Stock II (40 ml/L)

$KH_2 PO_4$	9.07 g/L

Stock III (60 ml/L)

$K_2 HPO_4$	11.61 g/L

Micronutrients (1 ml/L)	
$H_3 BO_3$	1.0 g/100 ml
$CuSO_4 \ 5H_2O$	0.15 g
EDTA*	5.0 g
$ZnSO_4 \ 7H_2O$	2.2 g
$MnCl_2 \ 4H_2O$	0.5 g
$Fe SO_4 \ 7H_2O$	0.5 g
$Co Cl_2 \ 7H_2O$	0.15 g
$(NH_4)_6 Mo_7O_{24} \ 4H_2O$	0.10 g

The micronutrients were dissolved one at a time in 100 ml warm deionised water. Following the addition of each, the pH was adjusted to 5 with KOH pellets. The final culture solution had a pH ca. 6.5.

Deionised water, used to make up the stock solutions and the final culture medium, was pasteurised at 70°C before use to prevent inclusion of viable microorganisms in the algal cultures. The culture flasks were sealed with polymer wool, and sterile conditions maintained at all times. Flasks were sterilised by autoclaving for several hours before use. Cultures were initiated by the innoculation of 1 ml of strong culture. Maximum strength developed in two to three weeks, and samples of these cultures were labelled as required. The cultures were constantly aerated with air pre-saturated with water vapour to minimise evaporative losses. They were maintained at temperatures between 15 and 22°C and illuminated by both natural and artificial (fluorescent) lighting.

The method of labelling the algae with ^{14}C was essentially identical to that described by Arnold (1971), Brenner et. al. (1976) and Kofoed (1975a) (see also Rigler, 1971) involving the introduction of small aliquots of $NaH \ ^{14}CO_3$ solution into the culture medium. Kofoed used 26.7 µCi per

* EDTA = Ethylene diamine tetracetic acid

litre of sea water, in which he cultured various species of green algae. A larger amount of label was found to be necessary for these investigations (see sub-section 5) and the equivalent of 80 µCi/L was used.

The isotope, obtained from the Radiochemical Centre, Amersham, had the following specifications: Volume: 0.5 ml. Activity: 1 mCi in 1/60 mmole NaH $^{14}CO_3$ (≡ 898 µCi/mg). 1 µl therefore contained 2 µCi. 50 ml samples of algal suspension were removed from the culture as required and introduced into a 100 ml glass stoppered bottle to which was added 4 µCi (2 µl) NaH $^{14}CO_3$ solution, using a Hamilton 10 µl syringe. The bottle was then immediately re-stoppered and placed under artificial lighting.

Preliminary experiments were conducted to determine the time course of uptake of ^{14}C by the algae. A 50 ml culture sample was labelled with 4 µCi of sodium bicarbonate and 10 ml subsamples removed after 16, 24 and 40 hours. These were centrifuged for 15 minutes at moderate speed. The supernatant was then removed and the algal cells resuspended in deionised water. The samples were re-centrifuged and the liquid again poured off before digesting the algae in nitric acid and analysing in a scintillation counter according to the method described in the 'Procedure' section of this chapter. The net count rate (uncorrected for quenching) for 10 µl aliquots of algal digest lay between 7,000 and 8,000 counts per minute (c.p.m.) for all three samples (see Appendix 2). It was concluded that the label was taken up within the first 16 hours and that culture samples could be adequately labelled on the evening before their proposed use. Indeed, Arnold (1971) considered 1 hour to be sufficient time for ^{14}C in sodium bicarbonate to be taken up by algae.

Apparatus for Investigating Food Utilisation

The essential features of the apparatus are shown in diagrammatic form in Fig. 23. Plate 4 is a photograph of the apparatus in use. It consists of three closed chambers constructed from truncated dropping funnels, in a vertical arrangement. The top chamber (a) is the water reservoir and contains a thermometer for monitoring the temperature of the system. This chamber is connected, via a glass stopcock, to the respiration chamber (b) which contains the snail under study and approximately 40 ml of water. Chambers (a) and (b) are temperature-controlled. To achieve this, polyethylene tubing ($\frac{1}{8}$" diameter) was wrapped tightly around the outside of the thin glass chambers, fixed with Araldite and connected to a Grant pumping water bath. Water from the bath was also passed through a series of wide cooling coils inside a small trough of deionised water, used to refill the reservoir. The mercury-contact thermostat/thermometer of the water bath was adjusted to give the required temperature of 15°C in the reservoir chamber. The setting of this thermometer was somewhat dependent upon the ambient room temperature and was periodically re-adjusted as necessary. The respiration chamber is connected, via a glass stopcock, to the acidification chamber (c) which itself leads into a replaceable collection tube (d). The respiration and acidification chambers each have an inlet aeration tube and an outlet tube. The reservoir also has an inlet tube for equalisation of pressures. All three inlet tubes are connected via flow rate control valves to a water vapour-saturated, CO_2-free filtered air supply provided by a continuous rating electric pump. Rate of flow through each aeration tube was approximately 0.8 L/hr (measured using a soap-bubble flow meter).

Fig. 23 Diagram of assimilation apparatus

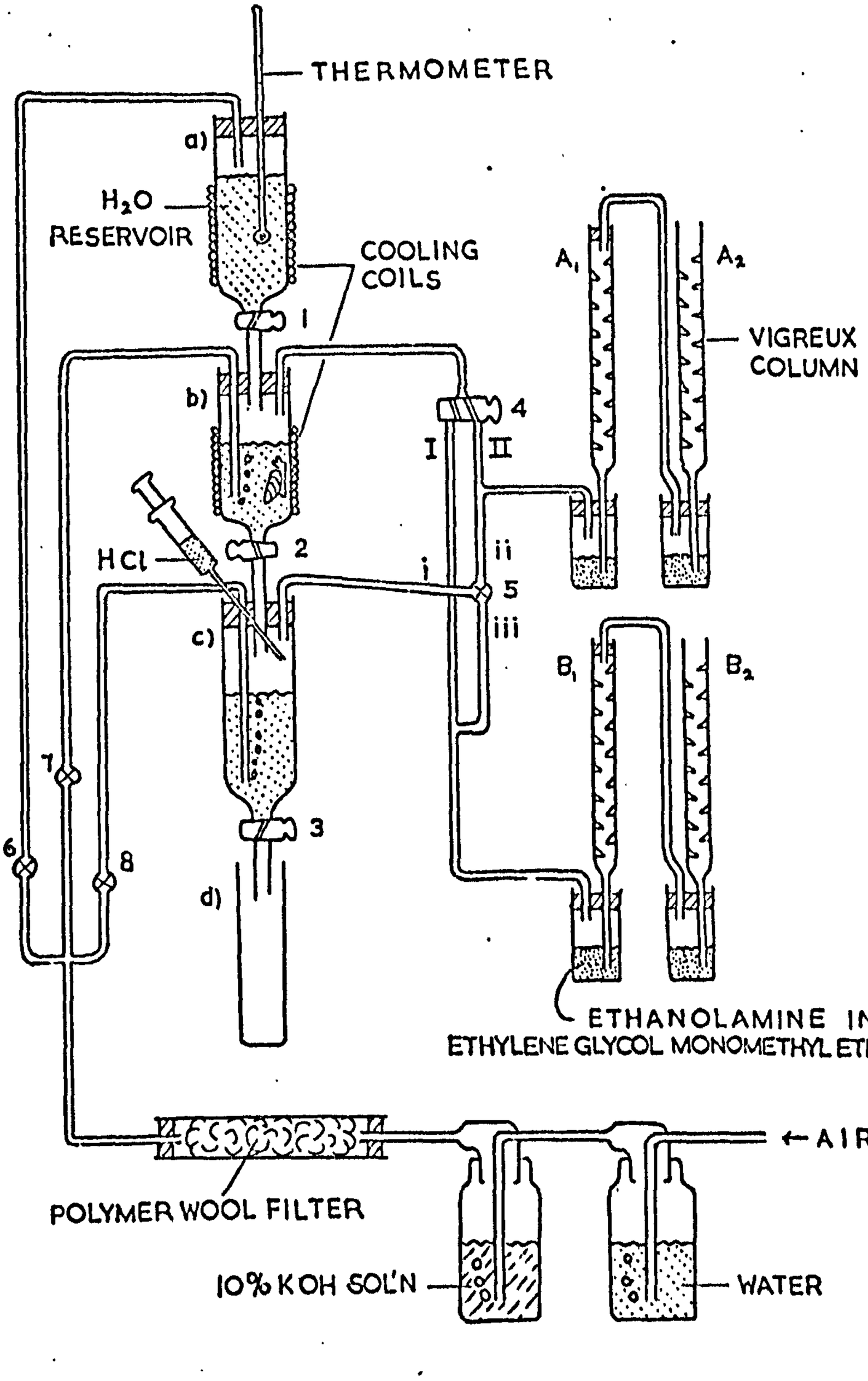
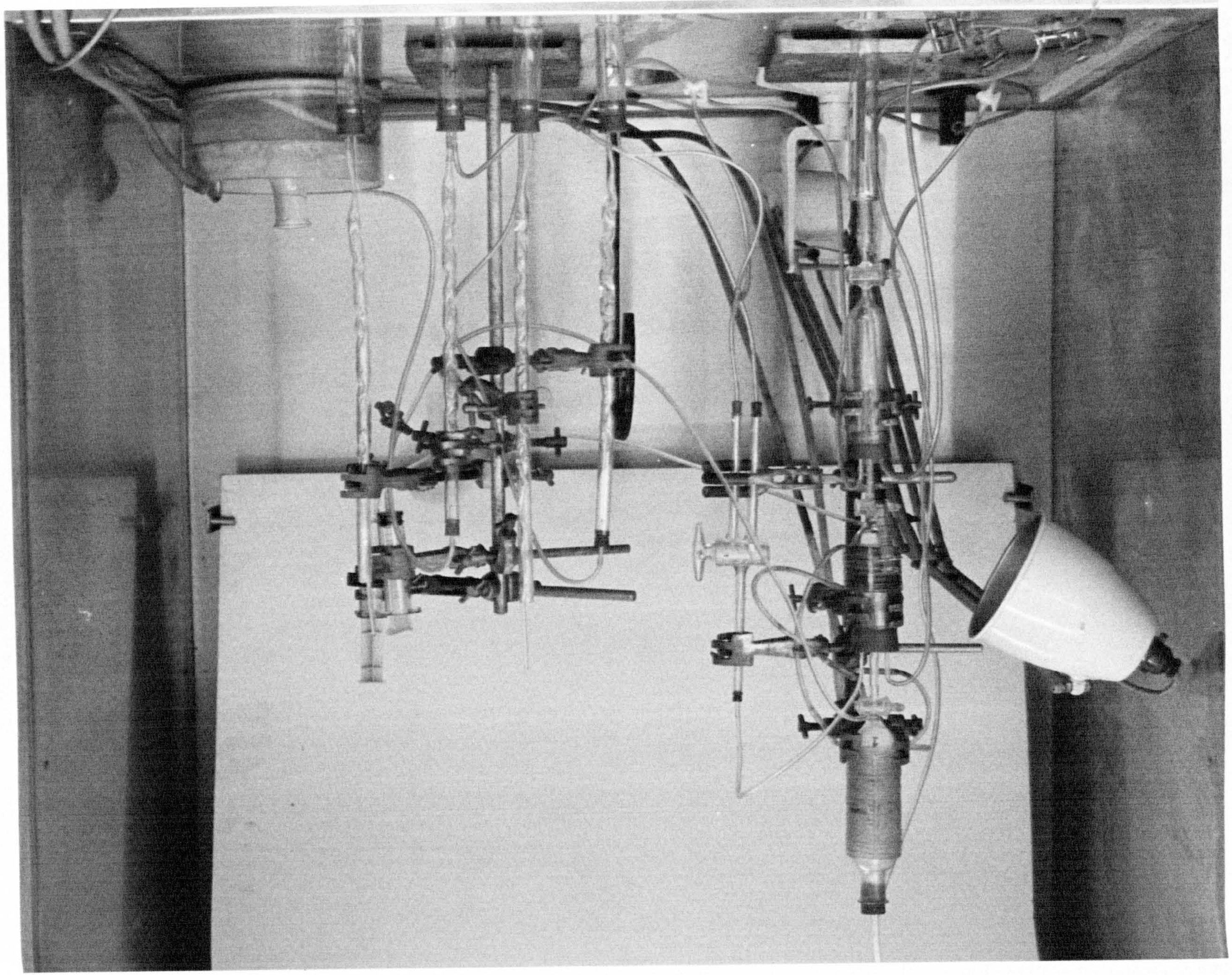


Plate 4: Assimilation apparatus in use.



The rubber stopper of the acidification chamber is pierced by a syringe needle to which may be attached a 2 ml disposable syringe, for injection of acid. The outlet tubes from the respiration and acidification chambers are connected via two two-way valves to two pairs of vigreux columns (constructed from ¼" glass tubing) approximately 30 cm tall. The bottom reservoirs, containing a CO_2 absorber (ethanolamine in ethylene glycol monomethyl ether, 1:2 v/v), consist of stoppered 3" x 1" glass specimen tubes. Connective piping for the apparatus is made from ¼" glass and polyethylene tubing.

The respiration and acidification chambers both have adjustable rinsing tubes (not shown in diagram) with angled constricted nozzles through which deionised water can be forced from large (20 ml) plastic syringes. These are used to remove any material (faeces, mucus) which becomes attached to the inside of the chamber walls.

Seals and joints were made air-tight, where necessary, by the application of Dow-Corning high-vacuum silicone grease.

Procedure

1) Brief outline of the method:

Snails were studied singly. Each was fed with labelled algae filtered onto a Millipore filter, then the snail was removed to the respiration chamber of the apparatus described above. Air was slowly and continuously bubbled through the water contained there and collected in a pair of vigreux columns containing CO_2 absorber. At predetermined intervals material, including dissolved and particulate matter, was drained from the respiration chamber (which was subsequently refilled from the reservoir) into the acidification chamber where a quantity of HCl was

added, thereby liberating any CO_2 dissolved in the water. This was collected in the same aliquots of absorber which were then removed and analysed by scintillation counting according to the method of Jeffay and Alvarez (1961), described below. Rejected materials (faeces, urine, mucus) were collected and centrifuged. The solid material was digested and analysed according to the rapid nitric acid procedure of Pfeffer et. al. (1971), described below. The liquid 'filtrate' was also analysed for radioactive content using an adapted method. Quenching was estimated and corrected for in all cases.

At the end of the experiment the snail was digested using the nitric acid technique. CO_2 released by oxidisation of the carbonate shell material was also collected and analysed. By summing the respired, rejected and incorporated (+ gut content) components the total quantity of material ingested could be calculated in terms of radioactive carbon (in decompositions per minute, d.p.m.). Using these results assimilation efficiencies could be calculated and these and other features of the digestion, metabolic and assimilation processes followed with time.

2) Notes on scintillation techniques:

^{14}C is a beta-emitter, these emanations being physically the same as electrons. The range of penetration of beta particles is small compared to that of gamma-rays, for example, and this necessitates the use of scintillators which convert the energies of ionising rays into photons which may be easily detected by photo-sensitive devices and converted into countable electronic pulses. Scintillation techniques were used throughout this study in conjunction with a Tracerlab scintillation counter (type: spectromatic/corumatic 100a). This machine,

which had automatic cycling and printout facilities, was set to the following conditions for optimum counting efficiency of a low activity ^{14}C standard:

Coarse gain:	4	} Total gain 7.2
Fine gain:	1.8	
Window:	5000 mV	
Threshold:	0250 mV	
Voltage:	3875 V	

Quenching, the tendency of sample or scintillant material to absorb photons before detection, was measured in all cases using internal standardisation with standard ^{14}C toluene. This contained 2 µCi in 5 ml, equivalent to 4×10^{-4} µCi/ml. 1 µCi = 3.7×10^{4} d.p.s.; therefore 1 µl of standard toluene = 888 d.p.m. A 1:51 dilution was also prepared which had a specific activity of 174 d.p.m./µl. Amount of 'spike' used was dependent upon the activity of the sample under analysis.

The following formula was used as a guide to indicate necessary length of time for counting particular sample activities:

$t = (Ed + 2B)/e^2 E^2 d^2$, where E = absolute efficiency,

d = sample disintegration rate, B = background rate and

e = standard erro required for observation (for example 5%)

All chemicals used, unless otherwise stated, were of scintillation grade obtained from Koch-Light Laboratories Ltd., Colnbrook.

3) Analysis of absorbed $^{14}CO_2$, including preparation and use of a PPO-toluene based scintillator:

This method of absorbing and analysing $^{14}CO_2$ was first described by Jeffay and Alvarez (1961).

A solution of the absorber ethanolamine in ethylene glycol monomethyl ether (1:2 v/v) was prepared. A scintillation medium was also made up;

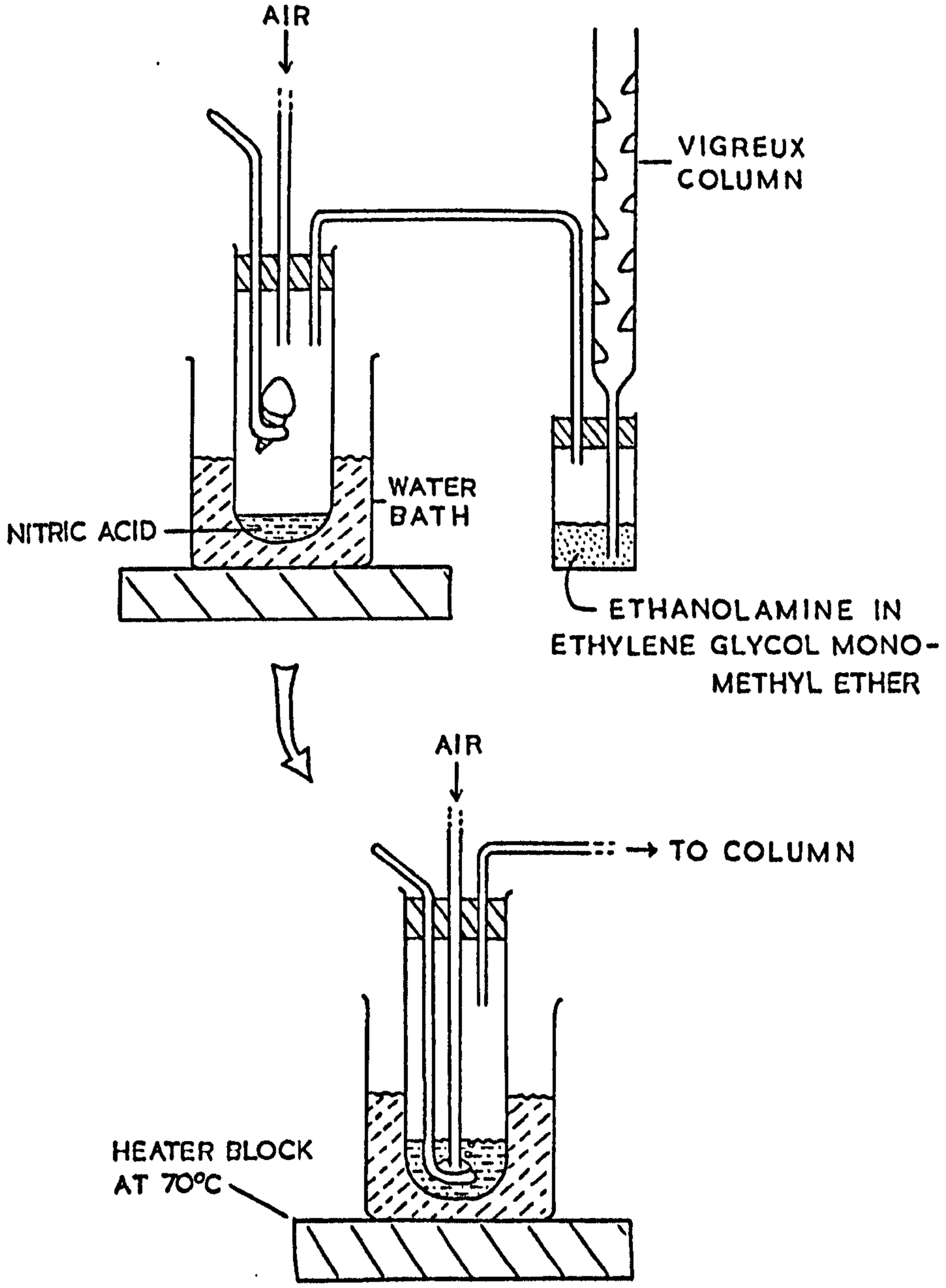
this consisted of a toluene:ethylene glycol monomethyl ether solution (2:1 v/v) containing 5.5 grams per litre of P.P.O. (2,5-diphenyl-oxazole). It was stored in the dark until use in an amber bottle (a Jensoms rapid dispenser). 3 ml of the ethanolamine solution to be analysed was transferred as required into a glass counting vial containing 15 ml of the scintillator solution, giving a final solution of ethanolamine, ethylene glycol monomethyl ether and toluene in the ratio 1:7:10 (v/v).

The background count of each vial with blank scintillator was determined by counting for 100 minutes immediately before use. The 3 ml sample of ethanolamine solution was then added and the vial re-counted until 20,000 counts were recorded. The net count rate was calculated. Quenching was estimated by adding an aliquot of standard ^{14}C toluene, using a Hamilton 10 µl or 100 µl syringe, and re-counting. The previously determined net count rate was divided by the fraction of known activity added which was actually detected. A simple proportional factor was applied to give total activity, in d.p.m., for the whole absorbar sample.

4) The rapid nitric acid digestion technique, including preparation and use of a Butyl-PBD-dioxan based scintillator:

A 'rapid procedure for scintillation counting of animal tissues using a nitric acid digestion procedure and a dioxan-based scintillator' is described by Pfeffer et. al. (1971). This method was adapted for use in the present study for analysis of the radioactive content of algae, faeces (plus other rejecta) and whole snails. The present study thus differs from that of Kofoed (1975a, b), who combusted samples in a crucible and trapped the resultant $^{14}CO_2$ in ethanolamine for subsequent analysis using the method described above.

Fig. 24 Snail digestion apparatus



The scintillator mixture was made up by dissolving 10 gms of Butyl-PBD (2 (4'-t-Butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole) and 60 gms of naphthalene in a litre of 1,4-dioxan. It was stored in an amber bottle until use.

To digest algal samples (10 ml) and faeces etc., solid material (not exceeding 1 gm in weight) was isolated by centrifugation and 1 ml of conc. nitric acid (analytical grade) added. The mixture was heated for about 5 minutes at 70°C in a Dri-block heater and then made up to 10 ml with 0.75 M Tris-buffer (2-amino-2-(hydroxymethyl)-1,3-propanediol). 1 ml of this preparation was added to 10 ml of scintillator in a glass counting vial whose background count had previously been established. The net count rate was calculated and the degree of quenching determined by adding an aliquot of standard toluene and recounting. The sample count was then corrected for volume and quenching.

Digestion of whole snails was performed using the apparatus shown in Fig. 24. The snail to be digested was lowered into 1 ml (1.5 ml if snail weight > 1 gm) of conc. nitric acid (Analytical grade). Air was bubbled through the acid and thence through 8 ml of CO_2 absorber contained in a Vigreux column. This solution was then analysed for ^{14}C content according to the method previously described. The digest was neutralised by making up to 10 ml (or 15 ml) with 0.75 M Tris. 1 ml of this preparation was added to 10 ml of dioxan-based scintillator, and counting and quench correction performed as before.

Efficiency of recovery of radioactive material from a standard digestion process:

Pfeffer et. al. (1971) investigated the efficiency of recovery of added standard counts from tissue digests using glycine-1-^{14}C and glycine

2-^{3}H. Spiked tissues were digested and counted according to the methods described above. The overall mean recovery of activity was quoted as 99.3 ± 2.9% for ^{14}C (and 97.8 ± 3.7% for ^{3}H). A similar investigation was undertaken for the present study, again using glycine-1-^{14}C (obtained from the Radiochemical Centre). A dilution was prepared such that 20 µl of glycine solution contained on average approximately 8,470 c.p.m. (see Appendix 3). 20 µl aliquots of this solution were injected just beneath the foot of each of five snails which had been recently killed by rapid freezing. The snails were then digested in acid in the manner described above, and the digests analysed in the normal way. Calculated efficiency of recovery was 87.2% (see Appendix 4) which is slightly lower than that given by Pfeffer et. al. (1971). It is not correct to assume that ^{14}C incorporated into tissues and in faeces would be recovered with exactly equivalent efficiency, but one may broadly conclude that the digestion technique employed does yield a high percentage recovery of labelled material and is suitable for use in these investigations. Since it is not possible to quote an exact figure for all digestions, the efficiency of recovery of ^{14}C using this technique will be assumed to be 100% in all cases (see Discussion section).

5) Preliminary Work:

A preliminary experiment was performed to check the practicality of the technique, to locate any design faults in the apparatus and to determine the correct amount of tracer to be used. A complete run was performed according to the methods and procedures previously described except that only 2 µCi (1 µl) of NaH $^{14}CO_3$ were used to label the 50 ml aliquot of algal culture. Samples of rejecta and expired CO_2 were removed

after 1, 3, 5, 7, 9, 11, 23, 26 and 29 hours.

Analysis of the results showed that i) the amount of tracer used should be increased ii) the experiment should be continued for as long a period as practicable, although it was not necessary to sample at such frequent intervals iii) 8 ml of absorber is sufficient to trap the CO_2 produced through any period investigated. Several minor modifications to apparatus and methodology were necessary. The final full experimental procedure is described below.

6) Full Procedure:

Snails were taken from the Grand Union Canal, Mile End, in August and November 1976. The November snails were stored in a small tank on the roof of Queen Mary College Biological Sciences building until their use in February (beginning February 16th). The August animals were studied soon after collection (beginning August 4th). In all cases the snails were fed ad libitum with lettuce leaves and Tetramin fish food as previously described, until individually required.

On the day preceding experimentation a snail was taken from its tank and put in a separate container, without food, to give the animal a 16 hour 'starvation period'. Also at this time a 50 ml sample of algal culture was labelled with ^{14}C in the manner previously described.

The experimental procedure proper was begun by filtering a 20 ml sample of labelled algae through a membrane filter (Millipore: 0.45 μ, 47 mm diameter) using a Stefi filter and water vacuum pump. The algae were rinsed by passing a further 20ml of deionised water through the filter which was then removed and placed in a plastic petri dish. The snail under investigation was measured (shell length) and weighed (fresh weight, excess

moisture removed) and then placed on the moist filter where it was allowed to feed at room temperature, for 20 minutes. Meanwhile a further 10 ml of labelled algal culture were centrifuged, then re-suspended in deionised water and centrifuged again. The liquid was removed and the algal cells digested in nitric acid and analysed for ^{14}C content as described in a previous sub-section. This was done to check that sufficient amounts of label had indeed been taken up by the algae. At the end of the feeding period the snail was rinsed in clean deionised water and wiped with tissue paper. Twenty minutes was considered adequate time for the snail to ingest enough food without being sufficiently long for digestive processes to have taken significant effect (see Kofoed, 1975b).

The snail was then placed in the respiration chamber (b) of the apparatus (see Fig. 23). 8 ml of absorber (ethanolamine in ethylene glycol monomethyl ether, 1:2 v/v) were pipetted into vigreux column A_1, and 4 ml into column A_2. Tap 4 was set to position II (II open), tap 5 turned to position ii (ii closed) and the air pump switched on. Tap 1 was opened fully and valve 6 gently opened until sufficient water (about 40 ml) was forced from the reservoir (a) into the respiration chamber (b). Tap 1 and valve 6 were then shut off. Valve 7 was slowly opened until air began bubbling through the water contained in b. This continuously swept respired air into the first pair of vigreux columns (A) where expired CO_2 was trapped in the ethanolamine solution. At the end of the first prescribed period (1 hour), tap 2 was opened, allowing water and rejects from the respiration chamber to fall into the acidification chamber (c). The former chamber was then rinsed by syringing (see 'Apparatus' section). Tap 4 was closed briefly to force any remaining

water from b. Tap 2 was then shut and valve 7 closed. Tap 4 was switched to position I (I open) and tap 5 to position iii (iii closed). The respiration chamber was refilled with water by opening tap 1 and valve 6, as before. Valve 7 was opened and regulated to again give the required slow air flow through the respiration chamber, now sweeping air into the second pair of vigreux columns (B_1 and B_2), filled with 8 ml and 4 ml of ethanolamine solution respectively, as before. The syringe was removed from the hypodermic needle embedded in the stopper of the acidification chamber and filled with 2 ml of 1N HCl. It was then replaced and the acid injected. This lowered the pH of the water contained here, resulting in the liberation of dissolved CO_2. Valve 8 was opened to give a slow flow of air sweeping this gas into the first pair of vigreux columns (A), where it was absorbed. After 20 minutes tap 3 was opened, allowing the liquid and rejects to be ejected into the collection tube (d). The respiration chamber was rinsed by syringing as necessary. The collection tube was then sealed and stored at $4^{\circ}C$ until analysis. Valve 8 and tap 3 were then closed. The vigreux columns (A) were rinsed by applying 2 ml of ethanolamine solution slowly to the open ends of the columns. After allowing a few minutes for draining, the liquid was removed from the bottom containers and transferred to a single tube (total volume of absorber: 16 ml) for subsequent analysis according to the method described in a previous sub-section. New bottom containers were fitted and the columns refilled with absorber as required. At the end of the second (3 hours) and subsequent (9, 24 and 33 hours) prescribed periods similar procedures were followed (see below). At the end of the final experimental period (total, 48 hours) tap 5 was switched to position iii (iii closed), tap 4 turned to the closed position and tap 2 opened so that the final

quantity of liquid and rejecta was ejected into the acidification chamber. Valve 7 and tap 2 were then closed. Acid was added and valve 8 opened as before. Finally tap 3 was opened and the faecal material and liquid collected. The snail was then removed from the apparatus, placed in a sealed specimen tube and stored, until analysis, at -12°C. The entire apparatus was thoroughly rinsed with deionised water before introducing the next snail. The full list of operations is given in brief below:-

BEGIN	Set 4 to position II
	Set 5 to position ii
	Open 1
	Open 6
	Close 6
	Close 1
(1)	Open 7 - regulate
At end of timed period:	Open 2 - syringe (b)
	Close 4
	Open 4 (II)
	Close 2
	Close 7 ..... end of 1
	Switch 4 to position I
	Switch 5 to position iii
	Open 1
	Open 6
	Close 6
	Close 1
(2)	Open 7 - regulate
	Add acid
	Open 8 - regulate
	Open 3 - syringe (c)
	Close 3
	Close 8
	Rinse columns and change absorber.
	Fill syringes.

At end of timed period: Switch 5 to position ii

Open 2 - syringe (b)

Close 2

Close 7 ..... end of 2

Switch 4 to position I

Open 1

Open 6

Close 6

Close 1

(3) ..... Open 7 - regulate

Add acid

Open 8 - regulate

Open 3 - syringe (c)

Close 3

Close 8

Rinse columns and change absorber.

Fill syringes.

At end of timed period: Switch 5 to position iii

Open 2 - syringe (b)

Close 2

Close 7 ..... end of 3

Switch 4 to position I

Open 1

Open 6

Close 6

Close 1

(4) ..... GO TO (2) ..... continue

TO END (48 hours) Switch 5 to position iii

Turn off 4

Open 2

Close 7

Close 2

Add acid

Open 8 - regulate

Open 3

Close 3

Close 8

Rinse columns, collect absorber.

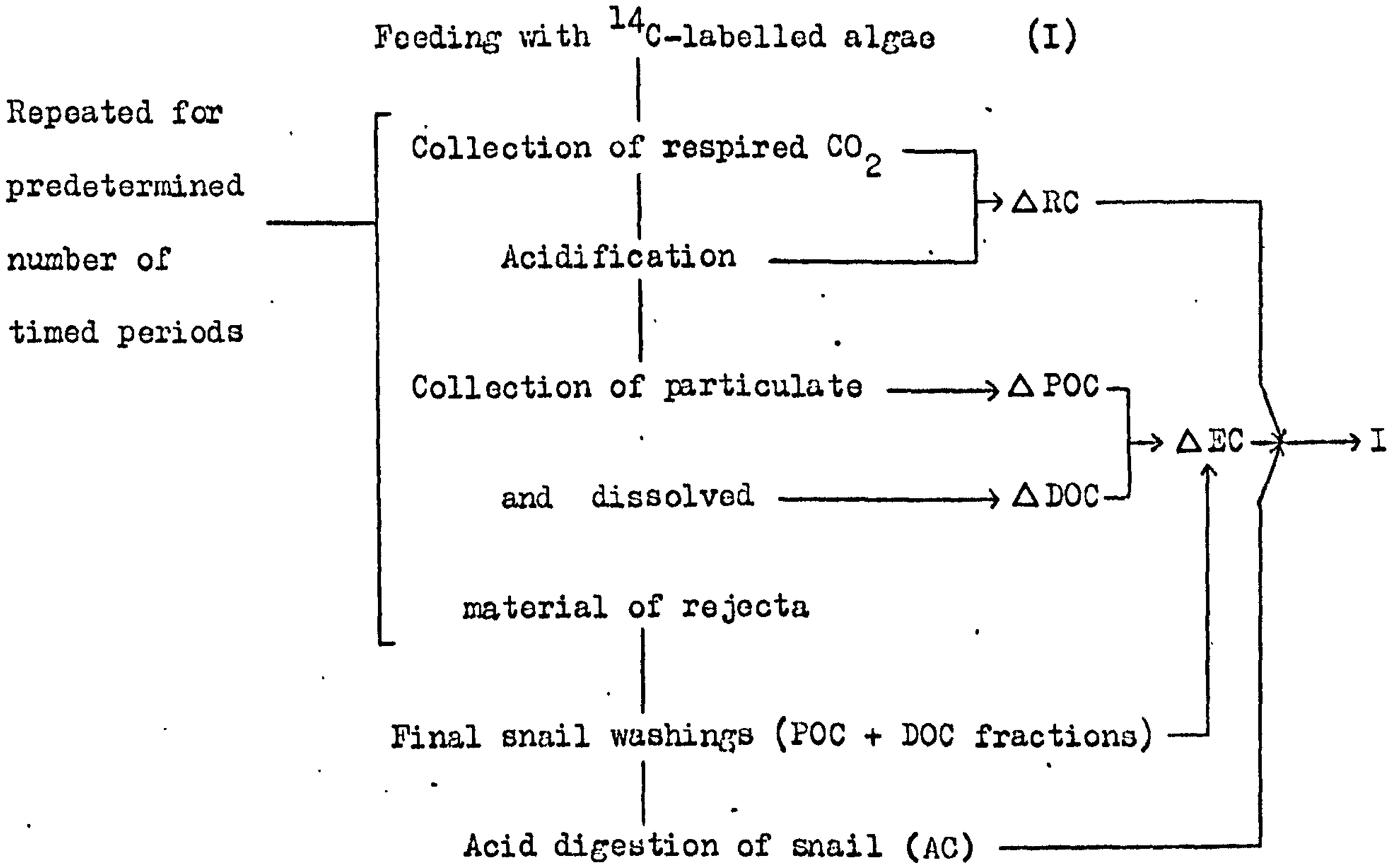
FINISH.

For analysis, the samples of faeces and other rejecta (including nitrogenous excretion and mucus) were removed from cold storage and the solid material separated from the liquid by slow centrifugation. The solid material was then digested in nitric acid according to the method previously described. Pfeffer et. al. (1971) stated that the dioxan-based scintillator incorporated in the digestion technique is able to hold up to 4.5 ml of H_2O per 10 ml of solvent. This afforded a method whereby the liquid 'filtrate' could be simply analysed by adding samples directly to the scintillant mixture. 3 ml aliquots were used and the vials counted as before.

It was found that rejecta samples for the first 1 hour period sometimes contained proportionately large quantities of ^{14}C compared with subsequent periods, yet it had been shown that following a meal, faeces are usually not produced for several hours (see Appendix 5). It was concluded that any radioactivity detected in the first sample must have been due to the presence of labelled algae which had not properly been removed from the snail prior to its being placed in the apparatus (see also Malone and Nelson, 1969). Consequently all first samples were discounted and the amount of ^{14}C excreted in the first hour periods assumed to be zero.

The frozen snails were thawed, washed, and then digested in nitric acid, as described. The washings were also analysed. The total amount of labelled material remaining in the gut or incorporated into the snail tissues (including shell) was determined for each animal.

Figure 25: Schematic diagram showing full experimental procedure of assimilation study.



$$I = \sum (\Delta RC + \Delta EC) + AC$$

Legend:

I = Total ^{14}C ingested

ΔRC = Respired ^{14}C fraction

ΔPOC = Particulate organic carbon fraction

ΔDOC = Dissolved organic carbon fraction

ΔEC = Total ^{14}C fraction of rejecta (ΔPOC + ΔDOC)

AC = Incorporated ^{14}C (+ remaining gut contents)

A schematic diagram of the entire procedure is shown in Fig. 25.

All measured scintillation count rates were corrected for quenching and dilution and the results analysed to give a description of the relationships between total ingested carbon and the amounts contained in the rejecta and respired components during consecutive periods of time through 48 hours. As previously described, total ingested material was not measured initially but was calculated as the sum of total amounts respired, rejected and incorporated in the snail (see Fig. 22), plus final gut contents. Actual amounts of carbon digested and metabolised are assumed to be proportional to the quantities of ^{14}C detected, presented in terms of decompositions per minute (N.B. 1 μCi = 2.22×10^6 d.p.m.).

Food utilisation characteristics, including assimilation efficiency, were compared for each group of eight winter and summer-adapted snails. Variability of the data was more accurately assessed as necessary using the arcsin transformation for proportions (Bliss, cited by Snedecor and Cochran, 1967). Multiple regression analysis was used to isolate the effects on assimilation efficiency of body size and amount of food ingested and to estimate values of this function corresponding to standard (mean) values of the other two variables.

RESULTS

Individual and cumulative totals of ^{14}C contained in the rejecta and respired components were calculated (d.p.m.) and likewise the proportions of amounts collected as percentages of the total rejecta and respired fractions after 48 hours. These data are shown in Tables 17-20 (summer animals) and 21-24 (winter animals). Table 25 shows total amounts

Table 17: Individual and cumulative totals (d.p.m.) of ^{14}C respired (summer animals).

Snail No.	Period 1 (1 hr.)	2 (3 hrs.)	3 (9 hrs.)	4 (24 hrs.)	5 (33 hrs.)	6 (48 hrs.)
1	50	58 (108)	107 (215)	116 (331)	30 (361)	36 (397)
2	89	225 (314)	647 (961)	475 (1436)	261 (1697)	174 (1871)
3	106	128 (234)	378 (612)	523 (1135)	183 (1318)	142 (1460)
4	144	258 (402)	902 (1304)	773 (2077)	286 (2362)	199 (2562)
5	730	2133 (2863)	3219 (6082)	7216 (13298)	9221 (15220)	459 (16679)
6	225	496 (721)	1050 (1171)	4152 (5923)	769 (6682)	650 (7342)
7	1079	2565 (3644)	8220 (11864)	11298 (23162)	3819 (26981)	3385 (30566)
8	310	759 (1069)	1409 (2478)	3052 (5530)	1516 (7046)	1215 (8261)

Cumulative totals are shown in parentheses

Table 18: Cumulative proportions of ^{14}C respired as percentages of total amount respired in 48 hours (summer animals).

Snail No.	Period 1	2	3	4	5	6
1	12.6	27.2	54.2	83.4	90.9	100
2	4.8	16.8	51.4	76.8	90.7	100
3	7.3	16.0	41.9	77.7	90.3	100
4	5.6	15.7	50.9	81.1	92.2	100
5	4.4	17.2	36.5	79.7	91.3	100
6	3.1	9.8	24.1	80.7	91.1	100
7	3.5	11.9	38.8	75.8	88.3	100
8	3.8	12.9	30.0	66.9	85.3	100
Mean	5.6%	15.9%	41.0%	77.8%	90.0%	100%
S.D.	2.9	4.9	10.1	4.7	2.1	-
C.I.	2.4	4.1	8.4	3.9	1.8	-

Table 19: Individual and cumulative totals (d.p.m.) of ^{14}C in rejecta (summer animals).

Snail No.	Period 1 (hr.)*	2 (3 hrs.)	3 (9 hrs.)	4 (24 hrs.)	5 (33 hrs.)	6 (48 hrs.)	Final washings
1	(939)	401	768 (1169)	424 (1593)	245 (1838)	266 (2104)	454 (2558)
2	(1239)	403	968 (1371)	695 (2066)	15962 (17758)	287 (18045)	569 (18614)
3	(1847)	365	470 (835)	599 (1434)	474 (1908)	401 (2309)	302 (2611)
4	(12988)	22888	12563 (35451)	16611 (52062)	1262 (53324)	1077 (54401)	260 (54661)
5	(4667)	1924	146535 (148459)	13286 (161745)	6616 (186351)	909 (169270)	761 (170031)
6	(7071)	3693	620 (5313)	58175 (63488)	9389 (72877)	872 (73749)	586 (74335)
7	(23288)	19043	284757 (303800)	35061 (338861)	33323 (372184)	18830 (391014)	1016 (392030)
8	(4839)	3931	2467 (6398)	5931 (12329)	61820 (74149)	10550 (84698)	5236 (89934)

Cumulative totals in round parenthises

* 1ST HOUR REJECTA IGNORED (see Methods, sub-section 6)

Table 20: Cumulative proportions of ^{14}C of rejecta as percentages of total amounts detected after 48 hours, including final washings (summer animals).

	Period				
Snail No. 1 (1 hr.)*	2 (3 hrs.)	3 (9 hrs.)	4 (24 hrs.)	5 (33 hrs.)	6 (48 hrs.)
1	-	15.7	45.7	60.2	71.9	82.2
2	-	2.2	7.4	11.1	95.4	96.9
3	-	14.0	32.0	54.9	73.1	88.4
4	-	41.8	64.8	95.2	97.6	99.1
5	-	1.1	87.3	95.1	99.0	99.6
6	-	5.0	7.1	85.4	98.0	99.2
7	-	4.9	77.5	86.4	94.9	99.7
8	-	4.4	7.1	13.7	82.4	94.2
Mean	-	11.1%	41.1%	62.8%	89.0%	94.9%
S.D.	-	12.1	30.8	32.2	10.7	6.0
C.I.	-	10.1	25.8	26.9	8.9	5.0

Table 21: Individual and cumulative totals (d.p.m.) of ^{14}C respired (winter animals).

Snail No.	Period 1 (1 hr.)	2 (3 hrs.)	3 (9 hrs.)	4 (24 hrs.)	5 (33 hrs.)	6 (48 hrs.)
A	37	67 (104)	94 (198)	97 (295)	31 (326)	30 (356)
B	253	1656 (1909)	4155 (6064)	7138 (13202)	2386 (15588)	2479 (18067)
C	227	723 (950)	3041 (3991)	5065 (9047)	1389 (10436)	1244 (11680)
D	213	760 (973)	5721 (6694)	8006 (14700)	1961 (16661)	1500 (18161)
E	174	148 (322)	75 (397)	115 (512)	46 (558)	57 (615)
F	94	116 (210)	218 (428)	445 (873)	143 (1016)	86 (1102)
G	342	646 (988)	4213 (5201)	4847 (10048)	1418 (11466)	1649 (13115)
H	135	556 (691)	4474 (5165)	11404 (16569)	4010)(20579)	2323 (22902)

Cumulative totals in parentheses

Table 22: Cumulative proportions of ^{14}C respired as percentages of total amount respired in 48 hours (winter animals)

Snail No.	Period 1	2	3	4	5	6
A	10.4	29.2	55.6	82.8	91.6	100
B	1.4	10.6	33.6	73.1	86.3	100
C	1.9	8.1	34.2	77.5	89.3	100
D	1.2	5.4	36.9	90.9	91.7	100
E	28.3	52.4	64.6	83.3	90.7	100
F	8.5	19.0	38.8	79.2	92.2	100
G	2.6	7.5	39.7	76.6	87.3	100
H	0.6	3.0	22.6	72.3	89.9	100
Mean	6.9%	16.9%	40.8%	78.2%	89.8%	100%
S.D.	8.8	15.6	12.4	3.9	2.0	-
C.I.	7.4	13.0	10.4	3.3	1.7	-

Table 23: Individual and cumulative totals (d.p.m.) of ^{14}C in rejecta (winter animals)

Snail No.	Period 1 (1 hr.)*	2 (3 hrs.)	3 (9 hrs.)	4 (24 hrs.)	5 (33 hrs.)	6 (48 hrs.)	Final Washings
A	(669)	272	2772 (3044)	375 (3419)	387 (3806)	374 (4180)	36 (4216)
B	(1124)	449	43970 (444419)	22706 (67125)	1044 (68169)	24817 (92986)	341 (93327)
C	(931)	1644	180645 (182289)	63722 (246011)	36663 (282674)	19240 (301914)	147 (302061)
D	(1012)	650	67926 (68576)	58366 (126942)	24009 (150951)	1983 (152934)	504 (153438)
E	(2398)	269	911 (1180)	737 (1917)	408 (2325)	642 (2967)	42 (3009)
F	(2529)	254	287 (541)	5766 (6307)	2591 (8898)	603 (9501)	50 (9551)
G	(374)	455	35028 (35483)	17775 (53258)	25112 (78370)	17521 (95891)	188 (96079)
H	(4320)	2007	261140 (263147)	59128 (322275)	48126 (370401)	3186 (373587)	473 (374060)

Cumulative totals in round parentheses

* 1st hour excretions discounted (see Methods, sub-section 6)

Table 24: Cumulative proportions of ^{14}C of rejecta as percentages of total amounts detented after 48 hours, including final washings (winter animals).

	Period					
Snail No.	1	2	3	4	5	6
A	-	6.4	72.2	81.1	90.3	99.1
B	-	0.5	47.6	71.9	73.0	99.6
C	-	0.5	60.3	81.4	93.6	100.0
D	-	0.4	44.7	82.7	98.4	100.0
E	-	8.9	39.2	63.7	77.3	98.6
F	-	2.7	5.7	66.0	93.2	99.5
G	-	0.5	36.9	55.4	81.6	99.8
H	-	0.5	70.3	86.2	99.0	99.9
Mean	-	2.6%	47.1%	73.6%	88.3%	99.6%
S.D.	-	3.1	20.1	10.3	9.2	0.5
C.I.	-	2.6	16.8	8.6	7.7	0.4

Table 25: Total amounts of material respired, contained in rejecta, remaining in the snail and, by summation, ingested (estimated after 48 hours) for all animals, in d.p.m.

Snail No.	Total respired	Total rejecta	Total incorporated (+ gut contents)	.°. Total ingested (sum of components)
Summer				
1	397	2558	729	3684
2	1871	18614	1785	22270
3	1460	2611	1155	5226
4	2562	54661	1540	58763
5	16679	170031	14173	200883
6	7342	74335	3370	85047
7	30566	392030	19882	442478
8	8261	89934	5164	103360
			Mean $\log_{10}$:	4.662
Winter				
A	356	4216	143	4715
B	18067	93327	37284	148678
C	11680	302061	10074	323815
D	18161	153438	22902	194501
E	615	3009	226	3850
F	1102	9551	582	11235
G	13115	96079	10854	120048
H	22902	374060	11674	408636
			Mean $\log_{10}$:	4.746
			Overall mean:	4.704

Fig. 26: Cumulative proportions of ^{14}C respired and contained in rejecta as percentages of respective total amounts at 48 hours (summer animals).

Legend: ——— Respired CO_2

——·——·—— Rejecta

FIGURE 26

SUMMER

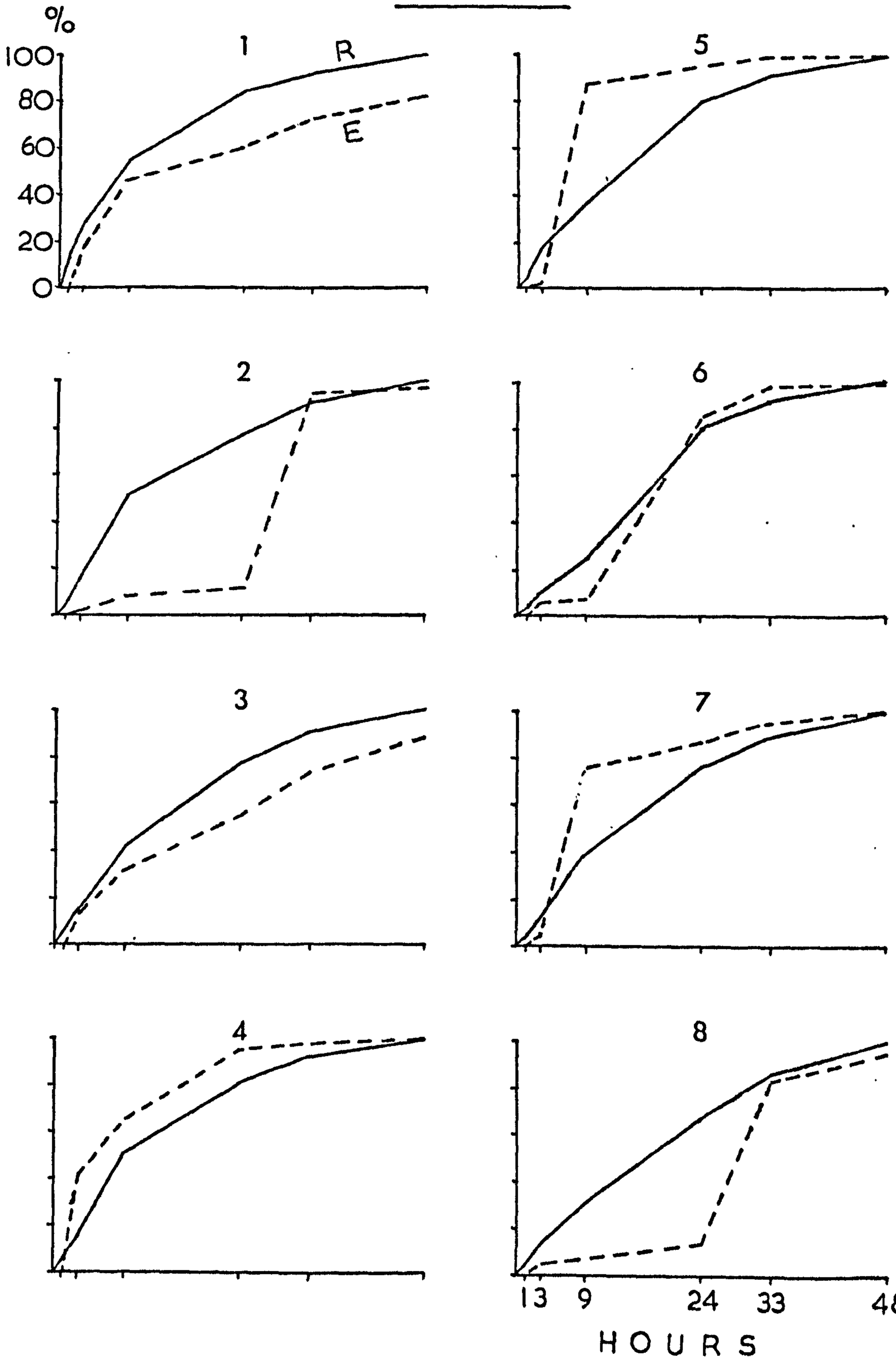


Fig. 27: Cumulative proportions of ^{14}C respired and contained in rejecta as percentages of respective total amounts at 48 hours (Winter animals).

Legend: ——— Respired CO_2

- - - - - - - Rejecta

FIGURE 27

WINTER

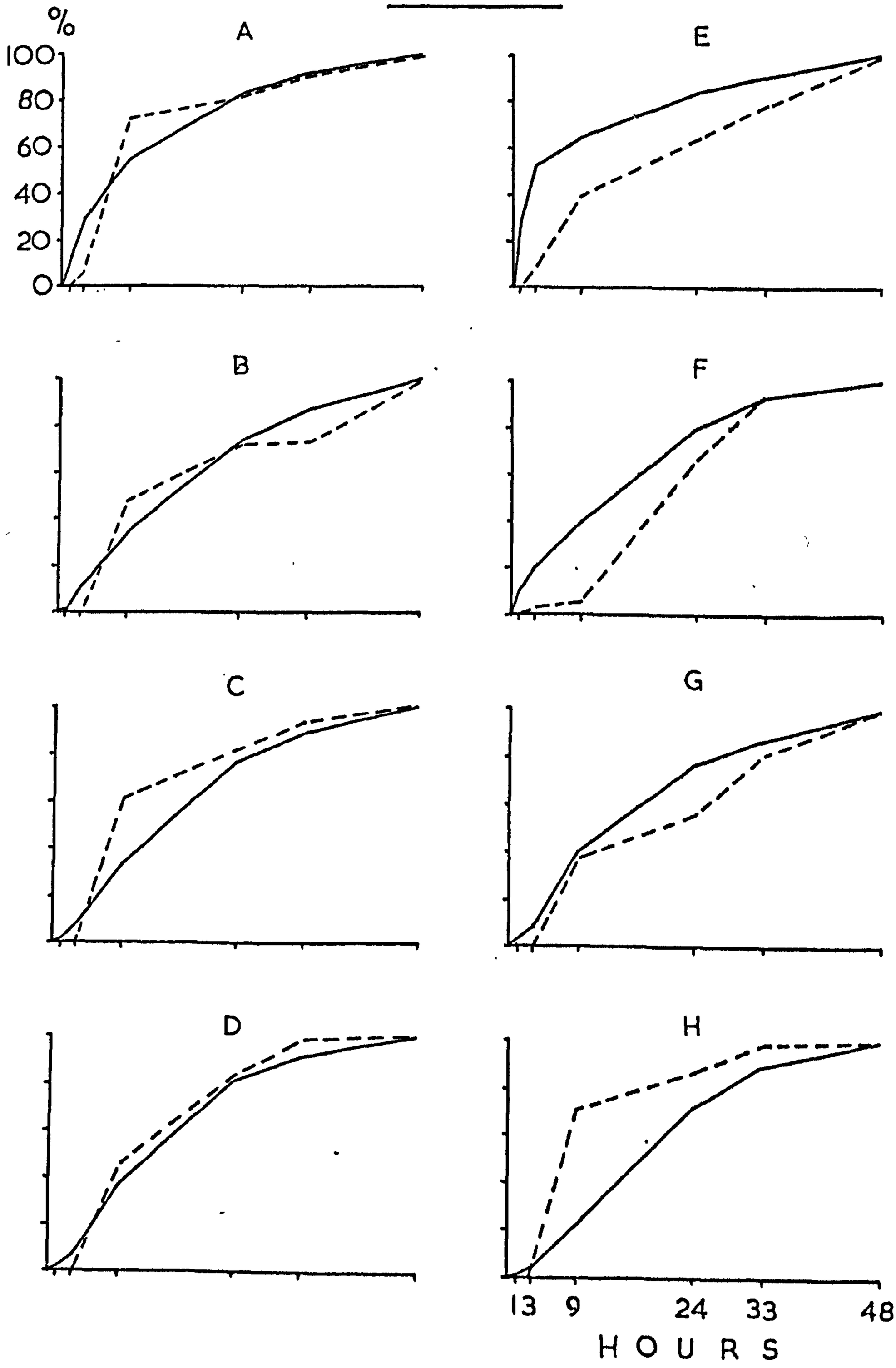


Fig. 28: Mean percentages of ^{14}C respired and contained in rejecta against time for both summer and winter groups.

Legend: R = Respired CO_2 curve

E = Rejecta curve

Dotted lines show 50% values of R and E for both groups.

FIGURE 28

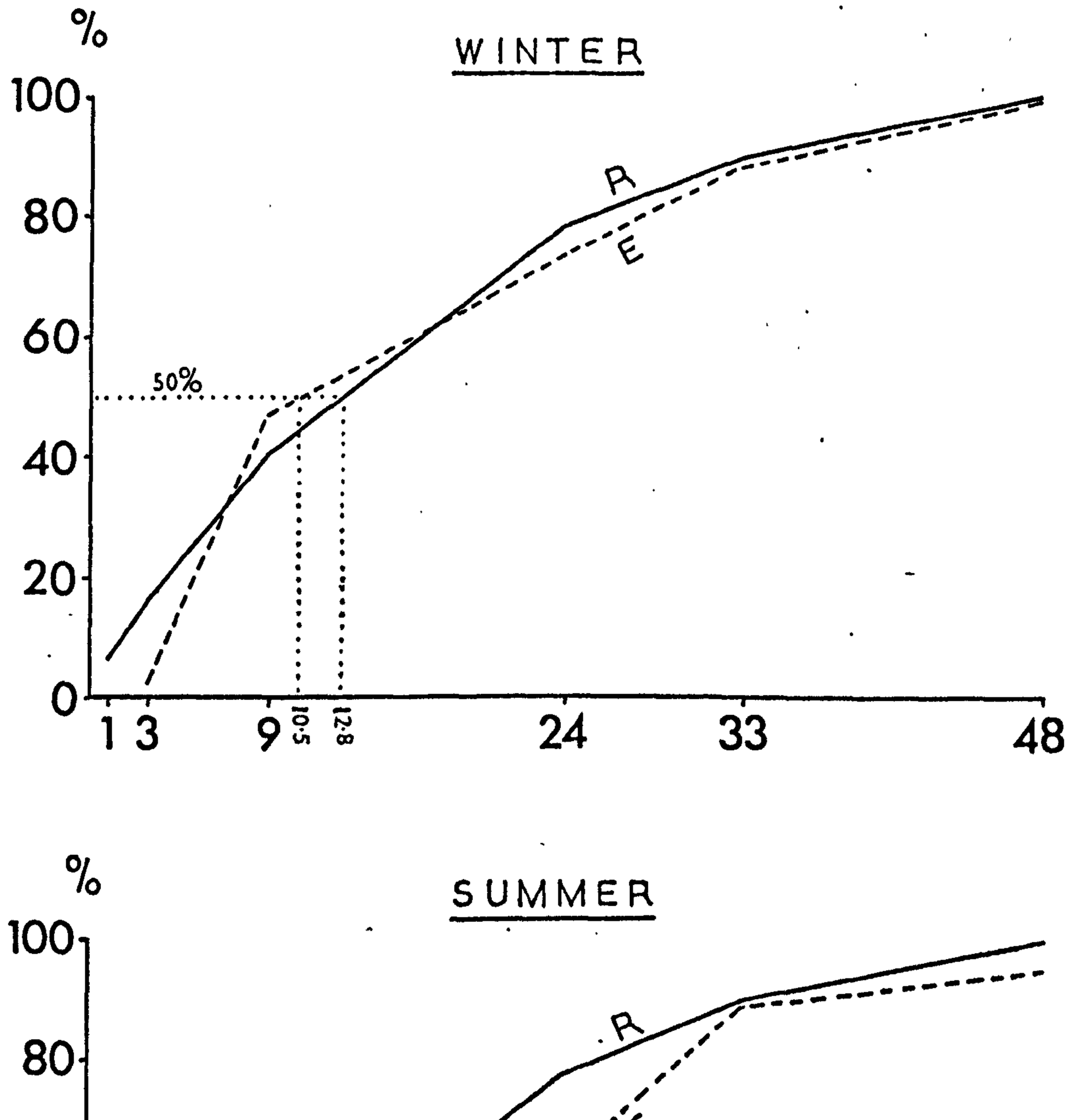

WINTER
%
100
80
60
40
20
0
R
E
50%
1 3 9 10·5 12·8 24 33 48
SUMMER
%
100
80
60
40
20
0
R
E
50%
1 3 9 12·8 15·2 24 33 48
HOURS

Table 26: Further analysis of selected percentage results using the arcsin transformation for propostions.

Percentage of total rejecta produced, after 24 hours.

Summer (August)			Winter (Feb - March)		
Snail No.	%	Transformed value	Snail No.	%	Transformed value
1	60.2	50.9	A	81.1	64.2
2	11.1	19.5	B	71.9	58.0
3	54.9	47.8	C	81.4	64.5
4	95.2	77.3	D	82.7	65.4
5	95.1	77.2	E	63.7	53.0
6	85.4	67.5	F	66.0	54.3
7	86.4	68.4	G	55.4	48.1
8	13.7	21.7	H	86.2	68.2
Mean:	53.8	= 65.1%	Mean:	59.5	= 74.2%
S.D.	21.7	(U.C.L: 90.5%)	S.D.	6.7	(U.C.L.: 82.3%)
C.I.	18.2	(L.C.L: 33.9%)	C.I.	5.6	(L.C.L: 65.3%)

U.C.L. = Upper Confidence Limit

L.C.L. = Lower Confidence Limit

Table 27: Values of the ratio $\frac{I - E}{I} \times 100\%$ (assimilation efficiency) for summer animals.

	Period					
Snail No.	1	2	3	4	5	6	Including final washings
1	-	89.6	69.7	58.8	52.4	45.5	30.6
2	-	98.2	93.9	90.7	20.3	19.0	16.4
3	-	93.0	84.0	72.6	63.5	55.8	50.0
4	-	61.0	39.7	11.4	9.3	7.4	7.0
5	-	99.0	26.1	19.5	16.2	15.7	15.4
6	-	95.7	93.8	25.3	14.3	13.3	12.6
7	-	95.7	31.3	23.4	15.9	11.6	11.4
8	-	96.2	93.8	88.1	28.3	18.1	13.0
Mean	-	91.1%	66.5%	48.7%	27.5%	23.3%	19.6%
S.D.	-	11.7	27.7	30.5	18.5	16.4	13.2
C.I.	-	9.8	23.2	25.5	15.5	13.7	11.0

Table 28: Values of the ratio $\frac{R}{I} \times 100\%$ for summer animals.

Snail No.	Period 1	2	3	4	5	6
1	1.4	2.9	5.8	9.0	9.8	10.8
2	0.4	1.4	4.3	6.5	7.6	8.4
3	2.0	4.5	11.7	21.7	25.2	27.9
4	0.2	0.7	2.2	3.5	4.0	4.4
5	0.4	1.1	1.6	3.6	7.6	8.3
6	0.3	0.8	2.1	7.0	7.9	8.6
7	0.2	0.8	2.7	5.2	6.1	6.9
8	0.3	1.0	2.4	5.4	6.8	8.0
Mean	0.7%	1.7%	4.1%	7.7%	9.4%	10.4%
S.D.	0.6	1.3	3.2	5.5	6.2	6.8
C.I.	0.5	1.1	2.7	4.6	5.2	5.7

Table 29: Values of the ratio $\frac{R}{(I - E)} \times 100\%$ for summer animals.

Snail No.	Period 1	2	3	4	5	6	Including final washings
1	-	3.2	8.3	15.3	18.7	23.7	35.3
2	-	1.4	4.6	7.2	37.4	44.2	51.2
3	-	4.8	13.9	29.9	39.7	50.0	55.8
4	-	1.1	5.5	30.7	43.0	59.5	62.9
5	-	1.1	6.1	18.5	46.9	52.9	53.9
6	-	0.8	2.2	27.7	55.2	64.7	68.3
7	-	0.8	8.6	22.2	38.4	59.5	60.5
8	-	1.0	2.6	6.1	24.0	44.2	61.5
Mean	-	1.8%	6.5%	19.7%	37.9%	49.8%	56.2%
S.D.	-	1.4	3.6	9.1	11.0	12.1	9.4
C.I.	-	1.0	3.0	7.6	9.2	10.1	7.9

Table 30: Values of the ratio $\frac{I - E}{I} \times 100\%$ (Assimilation efficiency) for winter animals.

	Period					
Snail No.	1	2	3	4	5	6	Including final washings
A	-	94.2	35.4	27.5	19.3	11.3	10.6
B	-	99.7	70.1	54.9	54.1	37.5	37.2
C	-	99.5	43.7	24.0	12.7	6.8	6.7
D	-	99.7	64.7	34.7	22.4	21.4	21.1
E	-	93.0	69.4	50.2	39.6	22.9	21.8
F	-	97.7	95.2	43.9	20.8	15.4	15.0
G	-	99.6	70.4	55.6	34.7	20.1	20.0
H	-	99.5	35.6	21.1	9.4	8.6	8.5
Mean	-	97.9%	60.6%	39.0%	26.6%	18.0%	17.6%
S.D.	-	2.6	19.4	13.1	14.1	9.3	9.2
C.I.	-	2.2	16.2	11.0	11.8	7.8	7.7

Table 31: Values of the ratio $\frac{R}{I}$ x 100% for winter animals.

Snail No.	Period 1	2	3	4	5	6
A	0.8	2.2	4.2	6.3	6.9	7.6
B	0.2	1.3	4.1	8.9	10.5	12.2
C	0.1	0.3	1.2	2.8	3.2	3.6
D	0.1	0.5	3.4	7.6	8.6	9.3
E	4.5	8.4	10.3	13.3	14.5	16.0
F	0.8	1.9	3.8	7.8	9.0	9.8
G	0.3	0.8	4.3	8.4	9.6	10.9
H	0.0	0.2	1.3	4.0	5.0	5.6
Mean	0.9%	2.0%	4.1%	7.4%	8.4%	9.4%
S.D.	1.4	2.5	2.6	3.0	3.2	3.6
C.I.	1.2	2.1	2.2	2.5	2.7	3.0

Table 32: Values of the ratio $\frac{R}{I - E}$ x 100% for winter animals.

Snail No.	Period 1	2	3	4	5	6	Including final washings
A	0.8	2.3	11.9	17.7	35.9	66.5	71.3
B	0.2	1.3	5.8	16.2	19.4	32.5	32.6
C	0.1	0.3	2.7	11.7	25.2	52.9	53.7
D	0.1	0.5	5.3	21.9	38.4	43.5	44.2
E	4.5	9.0	14.8	26.5	36.6	69.9	73.1
F	0.8	1.9	4.0	17.8	43.3	63.7	65.4
G	0.3	0.8	6.1	15.1	27.7	54.2	54.7
H	0.0	0.2	3.7	19.0	53.2	65.1	66.2
Mean	0.9%	2.0%	6.8%	18.2%	35.0%	56.0%	57.7%
S.D.	1.4	2.7	4.0	4.2	10.0	12.0	13.2
C.I.	1.2	2.3	3.3	3.5	8.4	10.0	11.0

Fig. 29: Graphs of the ratios $\frac{I - E}{I} \times 100\%$, $\frac{R}{I} \times 100\%$ and $\frac{R}{I - E} \times 100\%$ against time for summer animals.

Legend:

——— $\frac{I - E}{I} \times 100\%$ (assimilation efficiency) (100% scale)

- - - - - - - $\frac{R}{I - E} \times 100\%$ (50% scale)

. . . . . $\frac{R}{I} \times 100\%$ (50% scale)

FIGURE 29

SUMMER

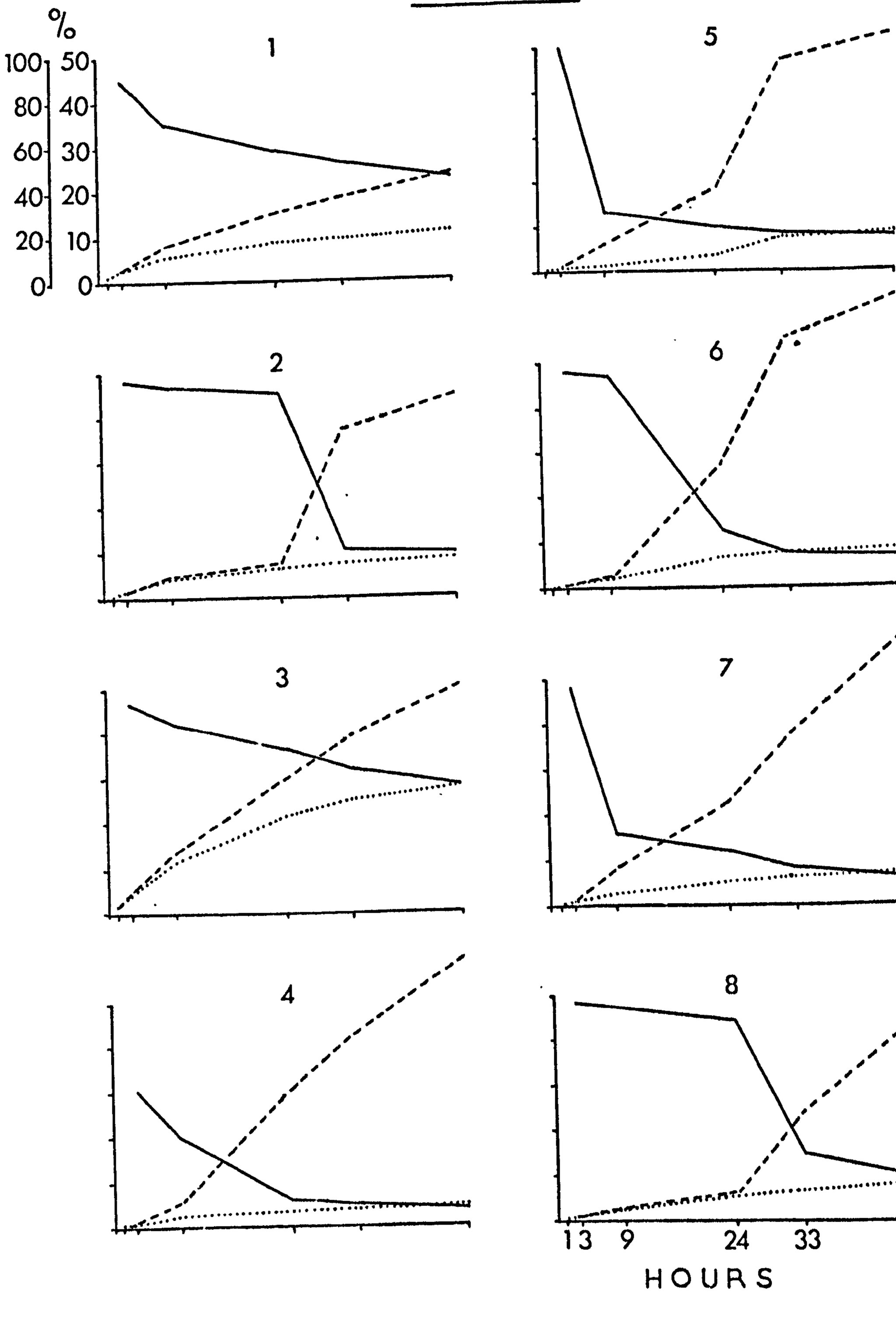


Fig. 30: Graphs of the ratios $\frac{I - E}{I} \times 100\%$, $\frac{R}{I} \times 100\%$ and $\frac{R}{I - E} \times 100\%$ against time for winter animals.

Legend:

——— $\frac{I - E}{I} \times 100\%$ (assimilation efficiency) (100% scale)

..... $\frac{E}{I - E} \times 100\%$ (50% scale)

..... $\frac{R}{I} \times 100\%$ (50% scale)

FIGURE 30

WINTER

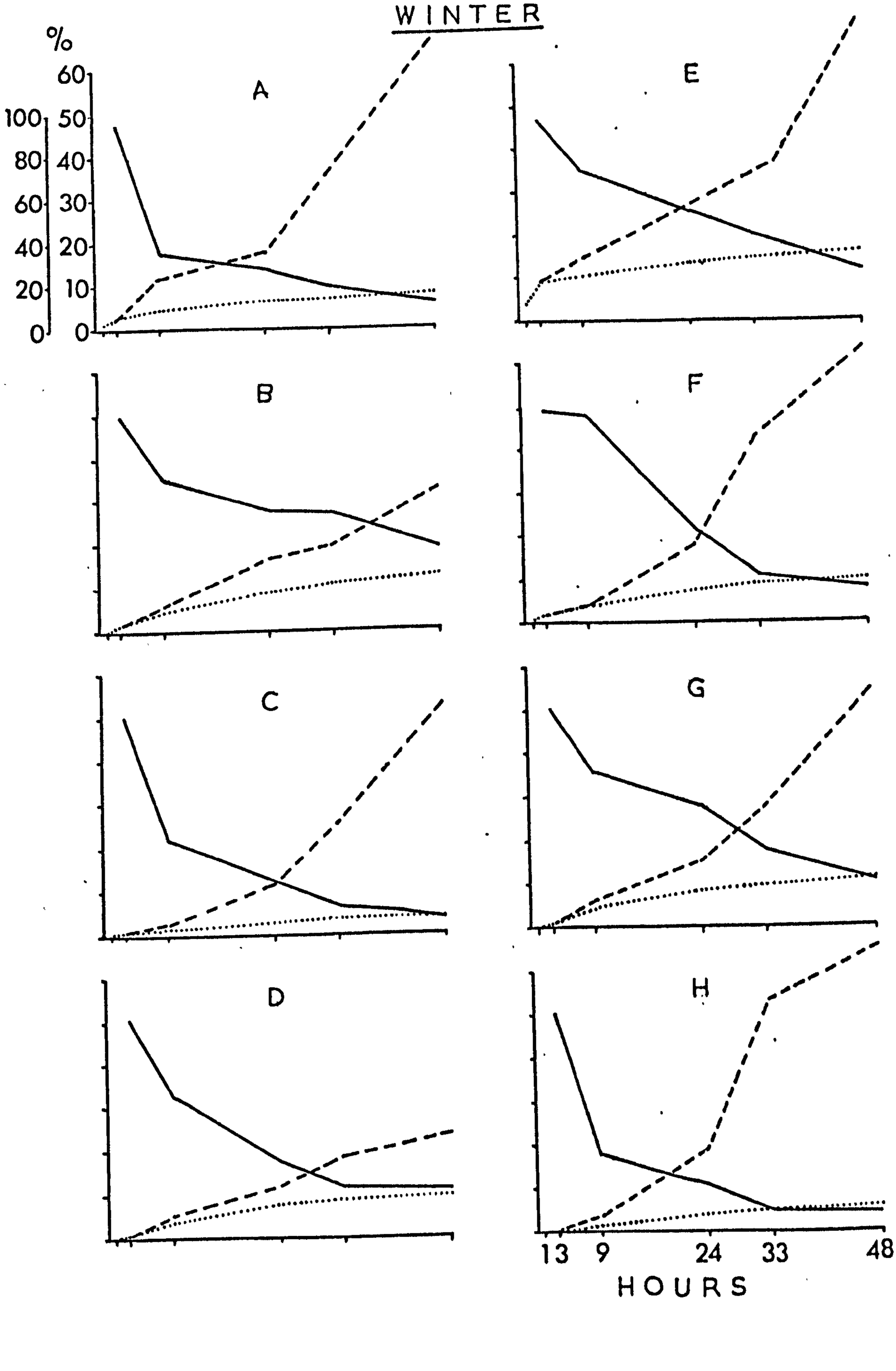


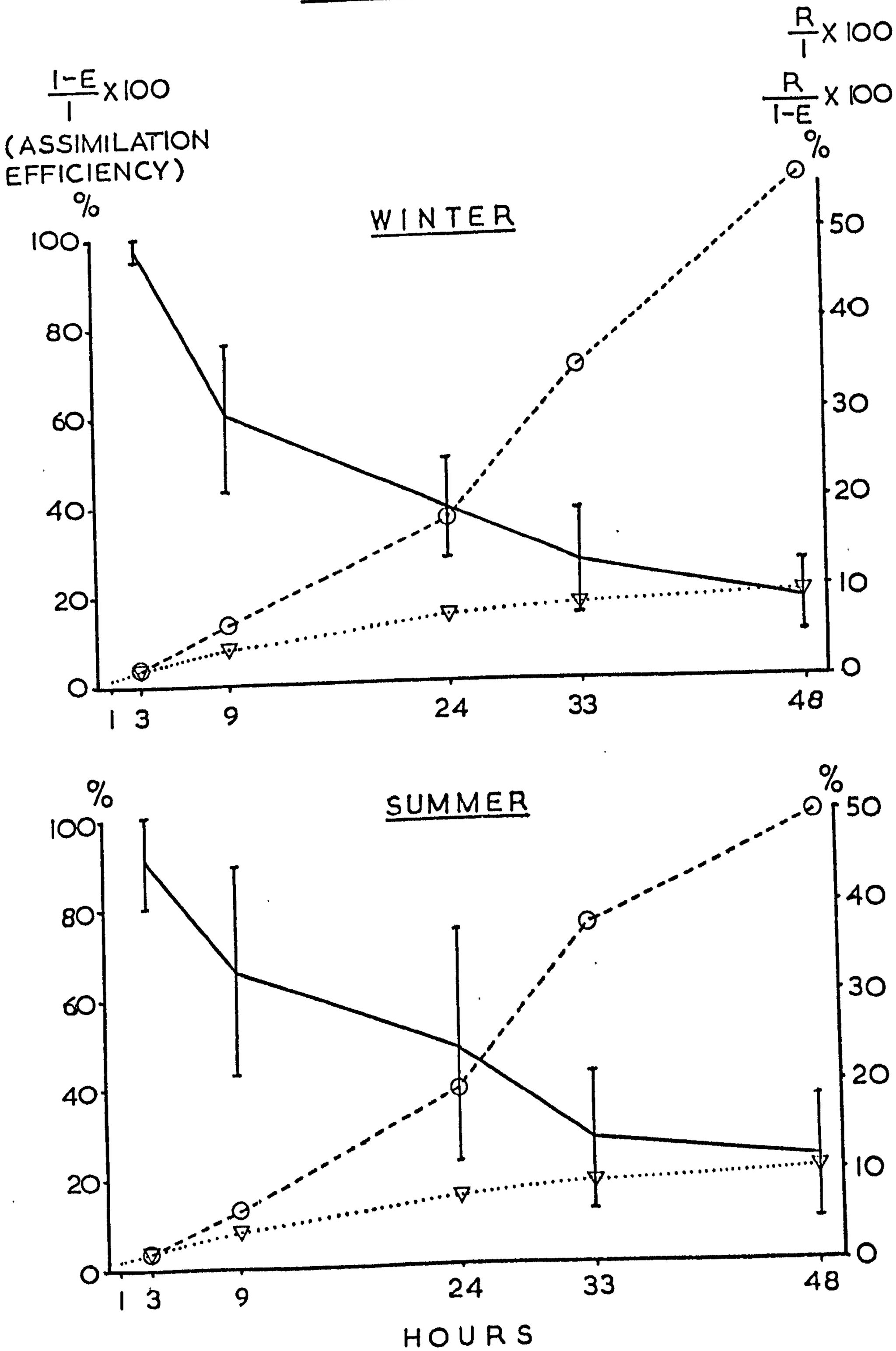
Fig. 31: Mean values of $\frac{I - E}{I} \times 100\%$, $\frac{R}{I} \times 100\%$ and $\frac{R}{I - E} \times 100\%$ against time for winter and summer animals.

Legend: ——— $\frac{I - E}{I} \times 100\%$ (assimilation efficiency) (100% scale)

⟳ - - - - ⟲ $\frac{R}{I - E} \times 100\%$ (50% scale)

▽ . . . . . ▽ $\frac{R}{I} \times 100\%$ (50% scale)

FIGURE 31


$\frac{I-E}{I}$ X 100
(ASSIMILATION EFFICIENCY)
%
$\frac{R}{I}$ X 100
$\frac{R}{I-E}$ X 100
%
WINTER
100
80
60
40
20
0
50
40
30
20
10
0
1 3
9
24
33
48
SUMMER
%
%
HOURS

respired, rejected and remaining in each snail after 48 hours and, by summation, amounts ingested. Figures 26 and 27 show the percentage results, in graphical form, for each group of eight animals. In all cases it is seen that $^{14}CO_2$ production is rapid initially but soon begins to level off as time progresses. These respiration curves are of generally smooth shape and there are no obvious differences between the summer and winter results. Cumulative percentages of ^{14}C of the rejecta show a more erratic variation with time, especially in the summer animals where in two cases (Nos. 2 and 8) the majority of material is not egested until 24-33 hours after the start of the experiment. Figure 28 shows mean percentages of amounts respired and rejected against time for both summer and winter animals. The respiration curves are virtually identical and are very smooth, having levelled off by 33 hours such that between 33 and 48 hours the increases in respired ^{14}C amount only to about 10% of the total. 50% of total measured respired ^{14}C is in both cases collected within the first 12.8 hours of the experiment (see dotted lines). The two curves representing percentages totally rejected are also similar but show particular differences in the first 24 hours. 50% of total rejecta production occurs within 15.2 hours in the summer animals, but only 10.5 hours are necessary for production of the same proportion in winter animals. Variability of the data for proportions of rejecta is seen to be very large (Tables 20 and 24) so that even at 24 hours (period 4), where an apparently large difference exists between means of untransformed data, the confidence intervals do overlap the means. Even when an arcsin transformation for proportions is applied to the results (Table 26), there is no significant difference between these data points.

Using the data shown in Tables 17, 19, 21, 23 and 25 it was possible to calculate the following ratios for each snail at successive time intervals:-

$\frac{I-E}{I} \times 100\%$, $\frac{R}{I} \times 100\%$ and $\frac{R}{I-E} \times 100\%$, where I = total ingested, E = rejecta and R = amount respired. $\frac{I-E}{I} \times 100\%$ is assimilation efficiency, the main function being studied. $\frac{R}{I} \times 100\%$ is the percentage of ^{14}C totally ingested which has been respired and $\frac{R}{I-E} \times 100\%$ is the ratio of amount respired to amount apparently available for metabolism in any particular period of time. These data, with means, standard deviations and 95% confidence intervals, are shown in Tables 27-32, and in graphical form in Figs. 29-31. Figures 29 and 30 show considerable variability in the shapes of the curves in both groups of snails. Assimilation efficiency always has an apparent high value initially, but falls steeply as material is egested. A steady value, approximating to the actual assimilation efficiency for each animal is normally not achieved before 33 hours. Time clearly has a very significant effect on estimation of this function. Values of the function $\frac{R}{I} \times 100\%$ form smoother curves, but no generalications can be made at this stage regarding summer-winter differences. The lines showing $\frac{R}{I-E} \times 100\%$ against time are generally linear or slightly sigmoid in shape in both groups of animals. Again, no obvious differences are apparent. Figure 31 shows the mean values of these functions against time and allows easier comparison between the winter and summer groups. 95% confidence limits are plotted on the assimilation efficiency curves. All three lines are similar for each group. The lines representing $\frac{R}{I} \times 100\%$ against time are virtually identical for the winter and summer animals. Both $\frac{R}{I-E}$ curves are sigmoid in shape, but the summer results show a more accentuated curve from 24 to 48 hours, perhaps suggesting that this function is attaining an equilibrium value sooner in the summer animals than the winter snails.

The assimilation efficiency results in both cases, but notably in the winter group, show an initial steep decline which evens out by 33 hours. The confidence limits indicate that there are no winter-summer differences between data points on these curves. These statistics (shown here and in the appropriate tables) are based on untransformed data. As previously stated, however, it is more correct to apply an arcsin transformation to percentage data when calculating means, standard deviations, etc. Such transformations and subsequent data analysis are shown in Table 33 for the three calculated ratios at 24 and 48 hours. Mean assimilation efficiencies at 48 hours, for example, are transformed from 19.6% to 18.3% (summer animals) and from 17.6% to 16.7% (winter animals). Calculated confidence intervals, however, are again seen to overlap the means. Similarly, the values of the ratios $\frac{R}{I}$ and $\frac{R}{I - E}$ of 48 hours for the winter and summer snails are not significantly different. At 24 hours, where the greatest difference between assimilation efficiencies exists, confidence intervals overlap the mean in both the transformed and the untransformed data.

Table 34 shows the dimensions of the snails studied in terms of $\log_{10}$ shell length (cm) and $\log_{10}$ fresh weight (gm), with means and standard deviations. It is evident that the mean size of the summer animals was considerably larger than that of the winter animals ($P < 0.01$ using 'students' t' test, $\log_{10}$ shell length and $\log_{10}$ fresh weight). In summer animals assimilation efficiency (at 48 hours) was not found to be significantly correlated with body size, but was correlated with total material ingested ($r = -0.769$, $P < 0.05$, $\log_{10}$ d.p.m. ingested vs. assimilation efficiency). Amount of food ingested is expected to show a correlation with body size, and this is indeed shown. In the summer

Table 33: Further analysis of selected efficiency results using the arcsin transformation for proportions.

Assimilation efficiency (24 hours):

Summer (August)			Winter (Feb - March)		
Snail No.	%	Transformed value	Snail No.	%	Transformed value
1	58.8	50.1	A	27.5	31.6
2	90.7	72.2	B	54.9	47.8
3	72.6	58.4	C	24.0	29.3
4	11.4	19.7	D	34.7	36.1
5	19.5	26.2	E	50.2	45.1
6	25.3	30.2	F	43.9	41.5
7	23.4	28.9	G	55.6	48.2
8	88.1	69.8	H	21.1	27.4
Mean:	44.4	= 49.0%	Mean:	38.4	= 38.6%
S.D.:	19.5	(U.C.L. 76.0%)	S.D.:	7.9	(U.C.L. 50.0%)
C.I.:	16.3	(L.C.L. 22.2%)	C.I.:	6.6	(L.C.L. 27.8%)

Assimilation efficiency (48 Hours):

Summer (August)			Winter (Feb - March)		
Snail No.	%	Transformed value	Snail No.	%	Transformed value
1	30.6	33.6	A	10.6	19.0
2	16.4	23.9	B	37.2	37.6
3	50.0	45.0	C	6.7	15.0
4	7.0	15.3	D	21.1	27.4
5	15.4	23.1	E	21.8	27.8
6	12.6	20.8	F	15.0	22.8
7	11.4	19.7	G	20.0	26.6
8	13.0	21.1	H	8.5	17.0
Mean:	25.3	= 18.3%	Mean:	24.2	= 16.7%
S.D.:	8.9	(U.C.L. 29.4%)	S.D.:	6.8	(U.C.L. 24.8%)
C.I.:	7.5	(L.C.L. 9.3%)	C.I.:	5.7	(L.C.L. 10.1%)

Table 33 (continued)

(R/I) x 100% (24 hours):

Summer (August)			Winter (Feb - March)		
Snail No.	%	Transformed value	Snail No.	%	Transformed value
1	9.0	17.5	A	6.3	14.5
2	6.5	14.8	B	8.9	17.4
3	21.7	27.8	C	2.8	9.6
4	3.5	10.8	D	7.6	16.0
5	3.6	10.9	E	13.3	21.4
6	7.0	15.3	F	7.8	16.2
7	5.2	13.2	G	8.4	16.9
8	5.4	13.4	H	4.0	11.5
Mean:	15.5 =	7.1%	Mean:	15.4 =	7.1%
S.D.:	5.1	(U.C.L. 11.5%)	S.D.:	3.4	(U.C.L. 9.9%)
C.I.:	4.3	(L.C.L. 3.8%)	C.I.:	2.9	(L.C.L. 4.7%)

(R/I) x 100% (48 hours):

Summer (August)			Winter (Feb - March)		
Snail No.	%	Transformed value	Snail No.	%	Transformed value
1	10.8	19.2	A	7.6	16.0
2	8.4	16.9	B	12.2	20.4
3	27.9	31.8	C	3.6	0.9
4	4.4	12.1	D	9.3	17.8
5	8.3	16.7	E	16.0	23.6
6	8.6	17.1	F	9.8	18.2
7	6.9	15.2	G	10.9	19.3
8	8.0	16.4	H	5.6	13.7
Mean:	18.2 =	9.8%	Mean:	17.5 =	9.0%
S.D.:	5.5	(U.C.L. 15.0%)	S.D.:	3.7	(U.C.L. 12.4%)
C.I.:	4.6	(L.C.L. 5.5%)	C.I.:	3.1	(L.C.L. 3.2%)

Table 33 (continued)

R/(I - E) x 100% (24 hours):

Summer (August)			Winter (Feb - March)		
Snail No.	%	Transformed value	Snail No.	%	Transformed value
1	15.3	23.0	A	17.7	24.9
2	7.2	15.6	B	16.2	23.7
3	29.9	33.2	C	11.7	20.0
4	30.7	33.7	D	21.9	27.9
5	18.5	25.5	E	26.5	31.0
6	27.7	31.8	F	17.8	25.0
7	22.2	28.1	G	15.1	22.9
8	6.1	14.3	H	19.0	25.8
Mean:	25.7 = 18.8%		Mean:	25.2 = 18.1%	
S.D.:	7.1	(U.C.L. 27.6%)	S.D.:	3.1	(U.C L. 21.8%)
C.I.:	6.0	(L.C.L. 11.4%)	C.I.:	2.6	(L.C.L. 14.8%)

R/(I - E) x 100% (48 hours):

Summer (August)			Winter (Feb - March)		
Snail No.	%	Transformed value	Snail No.	%	Transformed calue
1	35.3	36.5	A	71.3	57.6
2	51.2	45.7	B	32.6	34.8
3	55.8	48.3	C	53.7	47.1
4	62.9	52.5	D	44.2	41.7
5	53.9	47.2	E	73.1	58.8
6	60.3	55.7	F	65.4	54.0
7	60.5	51.1	G	54.7	47.7
8	61.5	51.7	H	66.1	54.4
Mean:	48.6 = 56.3%		Mean:	49.5 = 57.8%	
S.D.:	5.5	(U.C.L. 64.1%)	S.D.	7.8	(U.C.L. 68.9%)
C.I.:	4.6	(L.C.L. 48.2%)	C.I.	6.6	(L.C.L. 46.3%)

Table 34: Dimensions of snails studied.

Snail No.	Shell length (cm)	$\log_{10}$ shell length	Fresh weight (gm)	$\log_{10}$ fresh weight
		Summer (August)		
1	3.09	0.490	1.312	0.118
2	2.78	0.444	1.220	0.086
3	3.75	0.574	2.110	0.324
4	3.15	0.498	1.301	0.114
5	3.73	0.572	2.508	0.400
6	3.83	0.583	2.663	0.425
7	3.00	0.477	1.658	0.220
8	3.29	0.517	2.055	0.313
	Mean ($\log_{10}$)	0.519		0.250
	S.D.	0.048		0.126
		Winter (Feb - March)		
A	2.53	0.403	0.975	-0.011
B	2.32	0.365	0.635	-0.197
C	2.10	0.322	0.460	-0.337
D	2.30	0.362	0.643	-0.192
E	2.38	0.377	0.613	-0.213
F	2.13	0.328	0.438	-0.359
G	1.95	0.290	0.327	-0.485
H	3.79	0.579	3.383	0.529
	Mean ($\log_{10}$)	0.378		-0.158
	S.D.	0.083		0.291
	Overall mean:	0.449		0.046

Table 35: Multiple regression analysis relating assimilation efficiency, body size and food ingested.

Summer: $\hat{Y}_1 = 43.50 - 15.07\ x_1 + 89.23\ x_2$

Winter: $\hat{Y}_2 = 27.99 + 0.53\ x_1 - 34.12\ x_2$

where $\hat{Y}$ = assimilation efficiency (%)

x_1 = material ingested ($\log_{10}$ d.p.m.)

x_2 = shell length ($\log_{10}$ cm.)

Overall mean amounts ingested: 4.70 ($\log_{10}$ d.p.m.)

Overall mean shell length: 0.45 ($\log_{10}$ cm.)

When $x_1 = 4.70$ and $x_2 = 0.45$:

Summer	$\hat{Y}_1 = 12.8\%$	}	$\hat{Y}_1$ and $\hat{Y}_2$ not significantly different ($p > 0.05$)
Winter	$\hat{Y}_2 = 15.1\%$	}	

animals the value of the correlation coefficient for $\log_{10}$ shell length vs. $\log_{10}$ d.p.m. ingested, although not significant at the 5% level, was high, at 0.510. Multiple regression analysis was therefore performed on the data, relating assimilation efficiency, body size and material ingested for both the winter and the summer animals, according to the method described by Snedecor and Cochran (1964). The resulting regression equations are shown in Table 35. Substituting the actual overall mean values of $\log_{10}$ shell length and $\log_{10}$ d.p.m. ingested (see Tables 25 and 34) into these equations, the values 12.8% and 15.1% are obtained for assimilation efficiencies of summer and winter animals respectively. All other variables being equal it appears that winter animals have a higher efficiency of assimilation than summer animals. A χ^2-test, however, reveals that these figures are not significantly different at the $P = 0.05$ level.

DISCUSSION

Limitations of the Method

1) Assumptions:

An initial assumption is made whenever ^{14}C is used in the determination of assimilation efficiencies. This carbon isotope is presumed to behave in an identical fashion to its more common counterpart, ^{12}C. Moreover, the ratios of labelled carbon found in metabolised components are presumed to be proportional to the calorific contents of these materials.

With regard to experimental procedure, the assumption that labelled material is recovered from the acid digests with 100% efficiency will

cause some error in the magnitude of the final results. Pfeffer et. al. (1971), using the same digestion technique with different material, quoted a figure of 99.3 ± 2.9% for efficiency of recovery of ^{14}C from the digest, but preliminary experiments showed here that this figure is probably lower. Actual values undoubtedly vary according to amount and type of material being digested. In order to establish a constant and straightforward procedure in this study the 100% value was used throughout. The net result of this assumption is effectively to reduce estimates of amounts rejected and incorporated into tissues (or remaining in the gut), and thereby increase by a small proportion the estimated values of assimilation efficiency. These errors will also be reflected in the variability of the data.

Further errors are incurred, in the regression analysis, by the assumption that the alga labelling procedure is equally efficient in all cases and that the figure for material ingested, in d.p.m., is actually proportional to the weight of alga ingested. Analysis of labelled algae sub-samples showed that activity did vary between cultures, and it is likely that cultures varied in cell density. Ideally this latter feature would have been measured, enabling the actual number of cells ingested by the snails to have been calculated. Unfortunately, when the experiment was begun the possible importance of actual weight of food ingested had not been realised.

The effects of algal respiration and bacterial action through the course of the experiments are assumed to be negligible.

2) Other errors:

Construction of the apparatus used in this series of experiments necessarily involved the use of much glass and polythene tubing, resulting in fairly large volumes of potential 'dead space'. The main vessels were also of relatively large volume. This, combined with diffusion effects, could delay the collection of all ^{14}C O_2 respired in any particular period. The magnitude of error caused by these effects, however, is considered to be very small. The effects would be similar in every case and undoubtedly have reduced to negligible proportions by the end of the experimental periods.

A major requirement in the experimental technique was that all labelled material should be removed from the fed snails immediately prior to their being placed in the assimilation apparatus. Analysis of the first hour's results showed that this was not always successfully achieved. Labelled algae tended to become lodged beneath the retracted foot of the snail and this was only released when the snail began to move about inside the respiration chamber. Consequently the first hour's rejecta results were discarded and the actual amount rejected in this period assumed to be zero, since it had been shown that faeces, the major component, are normally not produced for several hours after a meal (see Results section). It is possible, however, that labelled algae remained attached to some snails even after the first period. This might have been released through succeeding periods or could have remained right to the end of the experiment. Retention of labelled material is thus a possible source of major error.

In the calculation of assimilation efficiency and of the ratio $R/(I - E) \times 100\%$ it is necessary to know the value of I, the total amount ingested. As described in the methods section, this figure is

calculated indirectly by summing amounts rejected (faeces, urine, mucus, etc.), respired and remaining as food in the gut or incorporated in each snail, assuming no other losses. This is perhaps an unusual procedure: total ingesta is normally measured directly at the beginning of the experiment. However, the latter method is very time consuming and is liable to considerable errors. Kofoed (1975) measured total ingested material using both approaches and thereby achieved independent estimates of I. He found fairly good aggrement between figures, but showed that the sum of respired, rejected and incorporated components was generally slightly less, by about 5%, than the initial estimate of consumption. Calculation of total material ingested is therefore liable to some error, which will be reflected to a small degree in the assimilation efficiency results.

In long-term feeding experiments re-ingestion of mucus and defaecated material can have significant effects on final results. In the present investigation the design of the apparatus made it difficult, but not impossible, for a snail to ingest its own faeces (which normally fell into a small well at the base of the respiration chamber). There was no restriction whatever on possible re-ingestion of mucus which occurs in the natural situation when a snail is feeding at the water surface or on a solid surface over which it has already moved. Re-ingestion of defaecated material is likely to slightly increase apparent efficiency of assimilation. If mucus containing assimilated ^{14}C is ingested a similar effect will result. The time scale of faeces and metabolite prodiction will also be affected by re-ingestion of these rejected materials (see following sub-sections).

Further errors may have been induced by inevitable small changes in experimental temperature throughout the course of the experiments,

especially during the summer when diurnal temperature fluctuations in the laboratory were large. Also it would have been advisable to repeat the experiments with more than eight animals, but this was not possible in the time and with the equipment available. Increased duplication would also have improved the statistical significance of the results quoted in Appendices 2 to 5.

3) The 'assimilation efficiency' formula:

It will be remembered (see Introduction) that assimilation was defined as consumption less rejecta (A = C - (F + U + M)), where the component termed rejecta includes faeces, nitrogenous excretion and other metabolised products such as digestive enzymes, mucus and gut lining. It is clear, therefore, that assimilation efficiency in this sense does not take proper account of material incorporated into urine, mucus, etc. Theoretically it would be more satisfactory to calculate a 'true' assimilation efficiency, the so-called 'digestive efficiency' which is defined as the ratio of consumption less faeces alone to consumption: D.E. = (C - F)/C. In practice, however, it is only 'assimilation efficiency' which can be easily determined. These ratios normally differ by only a few percent (Petrusewicz and Macfadyen, 1970) since nitrogenous excretion contains very little organic carbon, and amounts contained in mucus, enzymes, etc., are usually small compared to the amounts defaecated. However, freshwater snails do secrete large amounts of mucus in association with feeding and locomotory movements and it is possible over 48 hours that significant amounts of assimilated ^{14}C could be incorporated into this mucus, resulting in an underestimate of the true 'assimilated' fraction. Indeed, Hargrave (1970, 1971) has shown for

Hydrobia sp. that the loss of carbon material as mucus can account for as much as 9% of total assimilated carbon after 24 hours. Neglect of these losses must result in error by underestimate of true assimilation. Kofoed (1975b), again working with Hydrobia, was able to calculate mucus secretion by extrapolation of results, presuming certain conditions to be true. Such methods, however, were not possible in the present investigation.

4) Time factors:

When this series of experiments was first planned it was realised that the investigations needed to be completed within a fairly short period, before acclimation processes could begin to affect the animals' responses. Gravimetric methods were considered to be necessarily of too long duration and so tracer methods were adopted whereby estimates of assimilation efficiencies could be accurately achieved in a short time. It was initially believed that experiments could be run for as short a period as six hours for aquequate information to be obtained, especially since Noland and Carriker (1946) stated the time for passage of food from mouth to anus to be about two hours. Analysis of results of preliminary experiments, however (see Materials and Methods section), showed that a much longer time was required. Consequently animals were each studied for 48 hours after feeding. Analysis of ^{14}C production in respired CO_2 and total rejecta showed that probable minimum period for study was 33 hours. Significant amounts of particulate organic carbon (POC) were still being produced up to this time and small amounts even beyond this period. This seems a long period, especially considering the previously stated gut passage times for Lymnaea stagnalis, but Kofoed (1975a), working with the

small marine snail Hydrobia ventrosa, found similar results and Calow and Fletcher (1972) allowed 96 hours for complete retrieval. Kofoed suggested that this prolonged release of labelled material was not solely due to faecal production, since mean passage time in his actively feeding snails was 30 - 40 minutes (Fenchel, 1972) and complete emptying time ranged from 90 minutes to 3 hours (Kofoed, 1975a). He proposed that excretion of POC probably took place mainly in the form of mucus. In the present investigation, however, faecal material was evident in rejecta collected up to, and sometimes beyond, 33 hours. There are several possible explanations for this observation; these concern experimental technique and the digestive physiology of Lymnaea stagnalis. Firstly, although gut passage times in snails are normally much shorter than those implicated by the results of the present experiment, it must be stressed that these times are normally measured for continuously fed animals. This is certainly true for Kofoed's (1975a, b) studies, in which labelled meals were followed by ingestion of unlabelled food. In the present study, however, it was not possible to feed animals whilst contained within the experimental apparatus. Consequently the snails remained unfed for the duration of the 48 hours experiments and it is likely that passage of the labelled material was retarded by the absence of newly ingested food. The nature of digestive processes in Lymnaea stagnalis has been outlined by McDonald (1969) (see Chapter I). Food particles pass from the buccal cavity through the oesophagus and the crop to the strongly muscular gizzard, where sand acts as an effective tool in grinding the food into minute particles. These particles are filtered out and passed through ciliated ducts into the bilobed hepatopancreas, otherwise known as the liver, midgut gland, or digestive gland, for intracellular digestion.

Extracellular digestion occurs in the lumen of the digestive tract. Indigestible material and large food particles are gathered into mucus strings which are carried into the ciliated intestine where they are compressed into pellets and passed through the anus to the exterior. According to Calow (1970), who studied digestion in Lymnaea peregra (see also Carriker, 1946, on L. stagnalis), the faecal string produced can be clearly divided into the gizzard string (containing indigestible material derived directly from the gizzard), the caecal string (a mucoid cementing string derived from the caecum), and the liver string (containing material derived directly from the liver). Rates of passage of these different components of the faeces may vary greatly, especially in the absence of newly digested material. Further, the two main mechanisms responsible for the passage of food through the digestive tract are ciliary action and muscular contraction. This latter process may be inhibited by a lack of recently ingested food. Re-ingestion of faeces produced in an earlier period may also be responsible for apparent delays in defaecation. As described in a previous sub-section, design of the apparatus made it difficult but not impossible for a snail to re-ingest its own faeces. Such re-ingestion would result in prolonged faecal production. Temperature effects are also important in this respect. An experimental temperature of 15°C was chosen for the present study because it was felt that this is a temperature likely to be experienced, in the natural situation, by both summer and winter animals. It should be remembered, however, that unless otherwise stated most authors quote results for studies at room temperature (18 - 20°C). This difference in experimental temperature may partly explain observed slowing of defaecation rates in the present study. Also it must be stated that unnatural conditions in the experimental apparatus may have affected the time course of digestive processes in the animals under investigation.

It has been seen that time factors are very important in consideration of POC production and these, in turn, grossly affect estimated values of assimilation efficiency. Ideally this function should be measured when all food material is egested, considering also the effects of other excretions and secretions. It appears in the literature, however, that time effects are seriously underestimated, and assimilation efficiences are frequently measured after very short periods when faecal production may be far from complete. For example, Kofoed (1975a) 'standardised' some of his assimilation efficiency results by measuring assimilation after only 1.5 hours, despite his findings that particulate organic carbon was still being detected, albeit in small amounts, up to 24 hours and beyond. Measurement of assimilation efficiency before all faeces are liberated leads to considerable overestimate of this function. In the present investigation, for example, mean values for assimilation efficiency (summer) ranged from 90% at 3 hours to 50% at 24 hours and 11% at 48 hours. These results will be further discussed in the Conclusions section. Clearly, as time progresses, the apparent value of assimilation efficiency will fall sharply initially as faecal material is egested and then continue to fall more slowly as assimilated carbon is lost in secreted mucus and excreted urine. A period of measurement must therefore be chosen in which faecal production is the most important consideration but which takes into account the time course of other losses. It was also important in these experiments that animals should not have sufficient time to re-adapt to the experimental temperature. As already stated, a 48 hour period was eventually chosen here, although a 33 hour period was considered. It should be noted in this respect that a shorter period is often considered sufficient for acclimation of certain functions, so that

the present investigation cannot be taken as providing conclusive evidence regarding seasonal adaptation of assimilation efficiency. It was observed, however, that most changes in digestive processes occurred in the first 24 hours, so possible errors due to re-adaptation are perhaps less than suspected.

5) Suggestions for improvement of the method:

The design and construction of the apparatus described here necessarily depended to a certain extent on the amount of time and material available. These conditions also imposed various restrictions on the method, and the experiments were essentially of a preliminary nature. Both apparatus and technique are open to improvement. Previous parts of the discussion have indicated possible practical improvements, and these are briefly reviewed below.

With regard to apparatus design it would be preferable to have less 'dead space'. This could be achieved by having vessels better suited to the size of the animals contained in them, and also to reduce the length pf tubing used. This would also help to achieve better temperature control. A major flaw in these experiments was the occasional tendency of animals to re-ingest their own faeces. It was tried, without success, to introduce a fine grid into the respiration chamber to entirely prevent this. The problem is difficult to completely overcome because it was found that the snails also produce large quantities of mucus, some of which becomes attached to the chamber walls and occasionally causes faecal material also to adhere here, within reach of the snail.

In considering experimental technique several improvements are possible. Firstly, it would be preferable to obtain an independent

estimate of actual radioactivity ingested and also the total number of algal cells eaten by the snails. This process would be very time consuming but would supply invaluable additional data. Also it is essential to ensure that all labelled material is removed from the animals prior to their placement in the apparatus since this is a potential source of major error. Difficulties involved in the estimation of efficiencies of chemical digestion of snails, faeces, etc, have already been discussed. In this study a 100% efficiency of recovery was assumed although this was actually thought to be an over-estimate. Ideally, further experiments would be performed to give more accurate estimates for all digestions undertaken. With regard to the previous discussion on the effects of mucus production on the calculation of assimilation efficiency, it would be advisable to attempt separation of mucus and ejecta, either physically or theoretically (see Kofoed, 1975b). Also, if possible, it would be preferable to have unlabelled food available to the snails whilst contained in the apparatus. Problems would result from photosynthetic and/or bacterial action (although it may be possible to use a bacteriocide) and clogging of the apparatus. In this study snails were subjected to a pre-study 16 hour starvation period, which may have influenced the results to some degree. This could be investigated. Lastly, because of the large variability found in the data, it would be preferable to look at a larger number of animals. This would, however, necessitate building further apparatus and running the experiments in duplicate or triplicate.

Theoretically the method here described has wide applications and is suitable for all small aquatic animals. Animals can be taken individually from any situation and assimilation efficiencies measured at

controlled temperature for a given short period. It would have been of interest in this study, for example, also to have compared animals from different constant temperature acclimation regimes and at several different experimental temperatures. Additional studies could be performed beyond the quoted period of study by following elimination rates of labelled material from the animals and thereby also derive an estimate of metabolic rate (see Petruszewicz and Macfadyen, 1970). Also, this method could be used in conjunction with Calow and Fletcher's (1972) radiotracer method involving ^{14}C and ^{51}Cr, which enables a distinction to be made between faecal material derived from food, and that derived from metabolic secretions.

CONCLUSIONS

Interpretation of the Results

Results of this study have shown that at Te 15°C quantitative measurements of various digestive processes, including assimilation efficiency, reveal no significant differences between winter and summer animals. The ratios of ^{14}C respired to amounts ingested are virtually identical in the two groups (see Figs. 29, 30, 31). There is, perhaps, an indication that initial rates of defaecation are higher in the first 24 hours in winter animals than in summer animals, resulting in small differences here in the assimilation efficiency and $\frac{R}{I-E} \times 100\%$ curves, but these differences are not statistically significant. Following a steep decline, the assimilation efficiency curves show only small changes between the 33 and 48 hour markers and, as previously discussed, it is

assumed that measurement of assimilation after 48 hours yields the best estimate. There is no significant difference ($p > 0.05$) between these estimates of assimilation efficiency. The function $\frac{R}{I - E} \times 100\%$ yields S-shaped curves with time, and in both seasons these remain fairly steep throughout the 48 hour periods, suggesting that assimilated material is not stored but is rapidly utilised and respired. From the graphs (see Figs. 29 - 31) it appears that this function would attain the 100% value much sooner in winter animals than in summer animals, but no real conclusions can be drawn here. It would have been interesting to follow this function for a longer period and to have performed size regressions to standardise the results, since this function seems a good estimate of metabolic rate for known quantities of 'assimilated' food. As such it could have been used to complement the results of the previous chapter.

Although it is not possible to state that wide variability in the data, owing to small sample size, is not hiding real differences, it must be concluded here that no differences exist between winter and summer animals in the efficiencies of digestion and assimilation measured at 15°C. This does not rule out the possibility that resistance adaptations occur whereby, for example, summer animals may maintain efficient assimilation of food at higher temperatures than winter snails. Testing of this hypothesis would need further investigations. The conclusion that no capacity adaptation of assimilation efficiencies occurs is perhaps not surprising since rarely is there wastage of energy in natural systems and there is no reason to suspect that digestive efficiencies should ever be less than the maximum obtainable for any given temperature within the physiological range. With regard to the biology of L. stagnalis it is known that in summer, when food is

plentiful, reproductive activity is also at a peak and much energy is used in gonadal development, food storage and egg production. In winter, although activity rates are low, so also are potential food supplies. In both seasons, therefore, the snail must use its food reserves to the full and at all times maintain optimum digestive and assimilation efficiencies.

Further Discussion

In most poikilotherms there are temperatures below and above which no feeding occurs. Within that range, temperature changes may have profound effects on feeding rates, assimilation levels and other digestive functions. However, these acute temperature effects are by no means simple. Hubendick (1957) has shown that raised temperature increases the rapidity of eating movements in Lymnaea stagnalis (although McDonald (1973) suggests that this is countered by a decrease in the time available for feeding, owing to respiratory requirements and the necessary division of available time into feeding and visiting the water surface for air). According to Hubendick a fall in ingestion rate, as may occur through drop in temperature, allows a compensatory increase in assimilation efficiency. Assimilation efficiency, therefore, may appear to be related to temperature because of changed food input (see later paragraphs). Richardson (1975) working on a land snail Cepaea nemoralis, showed that consumption rates were temperature-dependent over a measured range of 5 - 20°C but found that assimilation efficiency in fact did not vary with temperature. A study on herbivorous lizards (Harlow et al., 1976) showed that raised temperature increased digestive efficiency, but this was apparently due to thermoregulation responses by the lizards. Hassanali

(personal communication), using gravimetric methods to study the effects of temperature on growth, food intake and assimilation efficiency in Lymnaea stagnalis, has shown that food intake is directly related to temperature by a highly significant regression, and also that a correlation does exist between assimilation efficiency and temperature, being highest at 21.0°C and lowest at 4.5°C.

According to Precht et. al. (1973), general rules about the degree of utilisation of food can not be formulated because the temperature requirements of different species vary so greatly. However, these authors do reach various conclusions regarding food consumption. These are: that the same quantity of food is used to reach a given stage at all temperatures which permit growth and development, that more food is used to reach a given stage at high temperatures than at low ones and that a dependence with an optimum temperature exists.

Feeding rates and assimilation efficiencies also depend to a large extent on the quality of food and the age and physiological condition of the animals (Precht et. al., 1973). Richardson (1975), working on Copaea nemoralis showed that in spite of the independence of assimilation efficiency from temperature, both this function and feeding rates were dependent on the nature of the food substrate. This is indeed a common finding in invertebrates (see Welch, 1968; Kofoed, 1975a; Tsikhon-Lukanin et. al., 1965; Arnold, 1971). Also, amount of food consumed may affect the efficiency of assimilation of that food. It is possible that a small meal is more efficiently assimilated than a large meal. If this is true, then changing seasonal conditions of food availability would be important, in addition to previously discussed effects of temperature on feeding rates. Effects of food ration size on growth and metabolism in

fish have been broadly studied (for example, De Silva and Baltontin, 1974; Elliott, 1975a, b). Thompson and Bayne (1974) investigated relationships between growth, metabolism and food in the mussel Mytilus edulis and showed that growth efficiency, obtained by an integration of ingested ration, assimilated ration and metabolic rate, increased hyperbolically with ingested food ration to reach a maximum, after which efficiency decreased as ration was further increased. These results do suggest that there may be an optimum ration size for highest efficiency of assimilation in L. stagnalis.

The effect of thermal acclimation on food utilisation has been investigated relatively seldom. As previously discussed, such investigations encounter the basic difficulty that the time needed to ascertain results is fairly long, and also that animals often refuse to feed after a temperature change. Weiser (1965) performed food ingestion experiments on the woodlouse Porcellio scaber. Animals acclimated to 8.6°C were placed in experimental chambers at 20°C and their food uptake compared with that of 20°C animals. The cold adapted isopods did not equal the higher food consumption of the 20°C animals until between the third and fourth day. Increased synthesis of digestive enzymes could not account for this. With regard to seasonal changes, Richardson (1975) showed that, as might be expected, ingestion rate of the land snail Cepaea nemoralis was maximal in the summer, with a peak in late May. Widdows and Bayne (1971) investigated acclimation of assimilation efficiency, in the edible mussel Mytilus edulis. Although complete acclimation of oxygen consumption and filtration rate occurred within 14 days, there was no change in assimilation efficiency within the 28-day experimental period. The present findings appear to be in agreement with

this, although seasonal changes and not experimental ones were investigated. It should be noted that work has also been done on temperature adaptations in the digestive enzymes (Hews, 1957), but it is clear that any effects observed here are not necessarily reflected in actual recorded values of assimilation efficiency.

It is perhaps of value to review some results for actual values of assimilation efficiency, bearing in mind the discussed influences of temperature and, especially, food types. It should be remembered that the food used in the present study, Chlorella ellipsoidea, is probably not a usual food item for L. stagnalis and may therefore have yielded lower values for assimilation efficiency than would have been achieved using a different material. This study has shown that assimilation efficiency of Lymnaea stagnalis approximates to between 17.6 and 19.6% (untransformed data), or 16.7 to 18.3% (arc-sin transformation) or 12.8 to 15.1% (multiple regression analysis with substitution of mean body size and mean number of counts ingested). Most authors quote the simple mean of the percentage values, which in this investigation therefore is c. 18%. Hassanali (personal communication), using gravimetric methods, found mean assimilation efficiency at 17°C to be considerably higher than this, quoting a figure of 52%. Food used in Hassanali's study, however, was lettuce, which might well be more digestible for L. stagnalis than is Chlorella. Other findings for gastropod molluscs give various results. Calow and Fletcher (1972) quoted values between 85 and 97% for assimilation of various foodstuffs by Ancylus fluviatilis, a freshwater limpet, and Planorbis contortus, a snail. Gravimetric determination of assimilation efficiency in Littorina littorea, fed with Ulva lactuca, gave a mean value of 57% (Grahame, 1973). Kofoed's (1975a, b) work with Hydrobia ventrosa showed that efficiency of assimilation in this animal is normally

high (60.7% for diatom food) but that a Chlorococcus sp. was assimilated with an efficiency of only 8%. This is an important observation with regard to the findings of the present study. Tsikhon-Lukanin et. al. (1965) found assimilation efficiency in the operculate freshwater snail Valvata piscinalis, for ingestion of Chlorella pyrenoides, to vary between 18% and 63%, with a mean of 37%. Richardson (1975) and Williamson (1975) showed that assimilation efficiency in Cepaea nemoralis fed with natural foods was also about 37%. Mason (1970) studied assimilation of beech litter by woodland snails and quoted a mean value of 49.1% for assimilation efficiency. In a general study of assimilation efficiencies in aquatic consumers, Welch (1968) quotes a range of values between 13.7% and 88%. It is seen, therefore, that although this study yielded an apparently low value for assimilation efficiency in Lymnaea stagnalis compared with most other quoted results, the mean value of 18% is an acceptable figure, especially when taking into account different methods, circumstances and degree of digestibility of food.

To conclude, the evidence here presented suggests that ingestion rates are extremely dependent on temperature, but that assimilation efficiencies are less affected by this function within physiological ranges. The nature of food ingested has a large effect on both ingestion rates and on assimilation efficiencies. Bearing in mind the limitations in the described method, including storage and feeding of animals before use, notably in the case of the 'winter' animals, one may state that the results of this and other studies suggest that assimilation efficiency in L. stagnalis does not show acclimation responses to either temperature or season. Seasonal changes may occur 'in the field', however, because of changes in food type and availability.

CHAPTER V

DISCUSSION

EXPERIMENTAL FINDINGS

Seasonal changes in the heart rate-temperature relation of Lymnaea stagnalis included both capacity and resistance adaptations. Exposure to constant temperatures in the laboratory resulted only in resistance adaptations. In the oxygen consumption study seasonally collected animals showed apparent 'reverse' acclimation, with summer animals having a higher rate of respiration than winter animals. There was no clear evidence for resistance adaptation but significant changes occurred in the size-rate regression and temperature-insensitive regions of the curves were apparent. The laboratory acclimated animals did not exhibit capacity adaptations of any kind, nor were there measured changes in the size rate regressions. However, temperature-insensitive regions again occurred in the rate-temperature curves and there was some evidence for resistance adaptations at the upper temperature extreme. A seasonal study only was made of assimilation efficiency, measured at a single experimental temperature ($15^{\circ}C$). Despite methodological problems it appeared that seasonal changes in assimilation efficiency and other features of food utilisation did not occur. Similar studies by other authors agree with this finding.

Observed rate-changes have been discussed in relation to some general physiological processes. It is of interest now to consider possible inter-relationships between the measured functions and to review the fundamental processes involved in metabolic adaptation.

FUNCTIONAL INTER-RELATIONSHIPS

Thermal acclimation may be considered an evolved physiological capability related to an animal's ecology. If a poikilotherm is subjected

in its natural environment to great extremes of temperature or to very wide seasonal or diurnal ranges of temperature it would appear advantageous for the animal to compensate for these in some way so that the overall balance between energy income and expenditure is unaffected. When individual processes such as heart rate and respiration are studied, one should consider the roles and inter-relationship of observed adaptations in the overall energy budget, and also relate the findings to the known ecology of the animal.

Heart rate is a function of fundamental importance which may be directly measured. Maintenance of controlled blood flow is essential in the transport of food and oxygen to the tissues and therefore the heart rate function might be expected to show good acclimatory responses. It has been shown that extremes of temperature cause irregularities in heart beat and a fall in rate. This would result in an inability to pump sufficient blood to the tissues and death would eventually ensue. It is apparent, then, that resistance adaptations in heart rate help the survival of the animal in a seasonal environment. These changes in resistance to upper and lower temperature extremes appeared to be only indirectly linked. Snails adapted to low winter temperatures had a lowered upper thermal limit whilst those adapted to high summer temperatures showed reduced resistance to cold. However, a maximum U.T.L. did not necessarily correspond to a minimum in cold adaptation, nor did a minimum U.T.L. coincide with a maximum rate at low temperature. This suggested that separate mechanisms are responsible for the control of rate-temperature relations at extreme temperatures. One particular mechanism, or several in association, may be 'switched in' to the exclusion of others. Certainly in the present studies the appearance of discrete peaks in the heart rate-temperature curves, and the development

of double peaks in some, suggested that there may be a switching system in operation which is dependent upon acclimation (see Chapter II). Similar results have been described for various other functions, and a favourite explanation for discrete switching in rate functions concerns isozymes which become 'active' according to temperature of acclimation. This aspect of temperature adaptation will be further discussed later.

Seasonally induced capacity adaptation in the heart rate function is perhaps more difficult to explain, especially considering the observed 'reverse' acclimation of oxygen consumption. If increased total oxygen consumption occurs in summer with respect to winter rates measured at the same temperature, heart rate too might be expected to show a comparative increase in summer to convey oxygen at a faster rate to the tissues, but the opposite of this was found. It appears that oxygen consumption and heart rate are not directly or obviously linked. The relationship between these functions has been considered by many authors. Experiments were performed, for example, by Von Brand and Mehlman (1953) to investigate pre- and post-anaerobic consumption in Lymnaea stagnalis. Oxygen transport by circulation was thought to be a possible limiting factor on the rate of oxygen consumption, but no positive conclusions were possible concerning this relationship. Baskin and Allen (1963) studied regulation of respiration in the heart of the clam mussel Tivella stultorum and showed that changed oxygen tensions affected the oxygen consumption of the tissues but not the rate of beating of the ventricle. Oxygen tension thus appeared to be unimportant in the control of heart rate, within normal limits. Widdows (1973), working on Mytilus edulis, similarly found no direct correlation between heart rate and oxygen consumption, although Bayne (1971) did show that a reduction in pO_2 can cause increased amplitude of beat. Dejours et al. (1970) compared blood convection requirements with the oxygen

concentration of the inspired medium and the post-pulmonary or post-branchial blood/haemolymph in various warm- and cold-blooded animals. It was found that convection of oxygen molecules between the ambient medium and the tissues takes place in two stages: convection of the O_2-containing ambient medium and convection of the O_2-containing internal medium. Rates of convection vary according to 1) factors which determine the oxygen concentration and pressure of the oxygen carrying medium, the nature of the medium and variables which determine the exact amount of oxygen in the medium (for example, barometric pressure and partial pressure of O_2, air temperature, salinity, blood temperature) and 2) factors which determine the oxygen demand (for example, size of animal, activity, ambient temperature and acclimatory condition. In addition, nutritional status may be important. Changes in haemolymph glucose levels (Veldhuijzen, 1975) may have direct or indirect effects on both oxygen consumption and rate of heart beat.

It is concluded that although heart rate does not necessarily provide complete information on the apportioning of energy input and expenditure (Coleman, 1974), it is a function of fundamental physiological importance which must be maintained at appropriate levels in spite of seasonal changes in the ambient temperature range. Seasonal compensations in heart rate result in both capacity and resistance adaptations so that effects of consistant high or low temperatures are reduced, and there is effective resistance to extrems temperatures. Total oxygen consumption, on the other hand, appears more to reflect seasonal changes in growth and activity and therefore shows an increase during the breeding season and in the season of maximum growth (spring and summer), with reductions during the inactive winter months. These changes result in the observed apparent 'reverse' acclimation of the oxygen consumption rate function. There

appears to be little direct dependence between heart rate and oxygen consumption. It should be remembered, however, that measurement of the oxygen consumption of a live animal is not a very sensitive technique since total respiration is dependent upon a multiplicity of factors. Adaptations shown by particular tissues may be masked by other effects.

As mentioned previously, no seasonal differences were found in the efficiency of assimilation of food by L. stagnalis, measured at the same temperature. Since no parallel alterations in assimilation efficiency occur, it is assumed that long term increases in oxygen consumption, in summer for example, must be accompanied by increased food intake. This seems reasonable because the period of maximum oxygen consumption corresponds with natural food abundance. In winter, although food is scarce, activity levels are also low. There appears to be no necessary functional relationship between oxygen consumption and assimilation efficiency. Possible effects of acute changes in the level of food input should not be overlooked, however. It is generally accepted that small meals are assimilated with greater efficiency than larger ones. This is due simply to the mechanics of digestion and assimilation. Complex changes in enzyme function, for example, are not implicated. Welch (1968) described for a variety of aquatic animals how a decrease in ingestion allows a compensatory increase in assimilation efficiency, and vice versa. In L. stagnalis there is a behavioural conflict between feeding and surfacing for respiratory purposes. Increased temperature or activity increases respiration rate and thereby causes a reduction in the amount of time available for feeding. This may result in a reduction in food input and lead to increased assimilation efficiency apparently associated with increased respiratory rates. However, higher temperatures also lead to

faster ingestion rates which would tend to counteract the incurred reduction in feeding time. Seasonal changes in food type and availability, temperature and oxygen requirements are likely to have complex effects on these relationships, but net changes are probably small.

Growth and energy balance are often considered to be good indicators of overall metabolic compensation in an animal. It would be possible using the results of the present study to calculate an approximate energy budget for Lymnaea stagnalis, making various assumptions regarding digestibility, availability and calorific content of food, using mean feeding rates and oxygen consumption values derived from the seasonal measurements, and taking R.Q. and calorific equivalent values from the literature (for example, Colley and Gentry, 1964. See also Slobodkin, 1962; Phillipson, 1966; Mason, 1970; Elliott and Davison, 1975). Growth (see Bertalanffy, 1964) is effectively the summation over a given period of time of total energy input and output, although care must be taken to ensure that account is made of gamete and egg (yolk) production, otherwise energy output may be underestimated. Measurement of growth rate is normally only possible over long periods of time (days or weeks) although the degree of incorporation of labelled ^{14}C measured in the present assimilation study could be used as an indicator of actual growth if gut content effects could be eliminated and full account be made of losses due to secretion, and respiration of storage carbohydrates. Dehnel (1955) studied rates of growth of gastropods as a function of latitude to determine whether this function, like oxygen consumption, showed compensation according to geographic distribution. Rates of growth of embryos and larvae from northern populations were found to be from two to nine times greater than for southern populations of the same species at a given comparable temperature. Several derived growth curves showed apparent

temperature-indie pendent rates with very low Q_{10} values. The results showed that growth was apparently linked with oxygen consumption, but other factors must have been responsible for some of the observed changes. Welch (1968) made a comparative study of growth efficiencies and assimilation efficiencies for aquatic consumers, using energy budgets derived from the literature. He found that gross growth efficiency (growth/ ingestion) demonstrated a non-linear correlation with assimilation efficiency and considered the latter a function of the former. Since in the present study assimilation efficiency showed no acclimatory changes with season it must be concluded that overall growth efficiencies are also independent of time of year, although they are likely to be affected by acute changes in exposure temperature. Changes in food input and respiration rate will also affect growth functions. Certainly growth efficiency is known to be affected by ration size. Thompson and Bayne (1974), working with Mytilus edulis, found growth efficiency to increase hyperbolically with increasing ingested ration to reach a maximum, after which it decreases as ration is further increased. Paloheimo and Dickie (1966) found similar results. Fish fed ad libitum had a somewhat greater metabolic rate than fish fed a maintenance ration, although the relationship between metabolism and body size remained unchanged. As previously discussed, the nature of food ingested may also affect assimilation and growth efficiencies. Seasonal studies have been made on growth rate in L. stagnalis (Taylor, 1894; Berrie , 1965a; see also Calow, 1973). These show that growth rate is always fastest in spring (corresponding to the middle portion of the size-age graph) resulting in a sigmoid growth curve. Low temperatures and food shortage may inhibit growth in winter whilst increased food supplies and an absence of metabolically costly activities may result in increased growth in spring.

Summer growth rate is low because metabolic rate is increased (reverse acclimation of oxygen consumption) owing essentially to reproductive activities.

Newell, Roy and their co-workers have considerably advanced our knowledge of the inter-relationship of factors affecting metabolic adaptation in poikilotherms by formulating mathematical models. Multiple regression equations were calculated for various seasonally changing functions such as temperature, activity, oxygen consumption, body size and food ration. It was then possible to 'predict' adaptational responses for given conditions (see Roy, 1969; Newell and Roy, 1973; Newell, 1975; Newell et al., 1976). Such models are likely to become more complex as further variables are incorporated into the equations.

MECHANISMS

Temperature effects may, in some cases, act directly on the physiological processes of poikilotherms, but hormonal influences, triggered by various environmental factors, are also very important.

Early texts on thermal acclimation (see Chapter I) tended to be largely descriptive, but later reviews often dealt in more detail with physiological mechanisms involved in acclimatory processes, with special emphasis on changes at the biochemical level. Much work was done too on adaptive changes in the nervous system (for example, Lagerspetz, 1973, 1974) and neuroendocrine control of temperature adaptation through the action of transmitters (for example, Harri, 1973; Kale and Rao, 1973). The use of isolated tissues was a popular approach in investigating thermal acclimation since whole body reactions, including control systems, are thereby excluded. Later, tissue homogenates were used in studying the effects of temperature on sub-cellular mechanisms. It was found that some enzymes from cold

acclimated organisms show higher levels of activity than those extracted from warm acclimated ones (see Prosser's (1962) review and Precht et al., 1973). Much work has subsequently concentrated on effects of temperature acclimation on enzyme systems (for example, Hochachka, 1967 and 1973; Hochachka and Somero, 1968; Rao, 1967; Somero, 1969). Newell (1967) meanwhile had investigated temperature effects in suspensions of mitochondria, the sites of oxidative reactions, extracted from poikilotherm tissues. Evidence was obtained supporting earlier findings which indicated a degree of temperature-independence in poikilotherm metabolism (Newell and Northcroft, 1967) and suggesting that acclimation to constant temperatures affects the range over which such metabolic homeostasis occurs (see also Johnson and Newell, 1973; Pye, 1973; Weiser, 1973).

Sizer (1943) was one of the first experimentalists to attempt to apply the available information on enzymes to the interpretation of the effects of temperature on biological systems. He suggested that knowledge of each enzymic component of the respiratory chain and its activation energy would greatly facilitate interpretation of respiratory and other physiological processes. Temperature effects on metabolism could then be more easily understood. Umbarger (1961) proposed an environment-dependent control of enzyme activity. He showed how competition of various enzyme systems for a limiting substrate or co-factor could produce a change in metabolic pattern with a change in environmental conditions. Hochachka's (1973) review outlines some currently accepted strategies and mechanisms of enzyme adaptation to temperature. Basically, compensatory strategies of enzymes take into account the fact that temperature-dependence of enzyme-catalysed reactions is determined strictly by thermodynamic parameters, and lead to the elaboration of enzymes with altered turnover rates and/or Q_{10}.

Under subsaturating substrate conditions enzymic reaction rates are determined by the kinetic properties of the catalyst, in particular the enzyme-substrate (E–S) affinity. Generally speaking, as temperature rises, E–S affinity drops, allowing apparent temperature-independent rates. Starvation will also affect this relationship by reducing substrate levels. This explains the importance of nutritional status in metabolic adaptation (Newell and Bayne, 1973; Marsden, 1973). Thus the Q_{10} for any given enzyme reaction can be regulated by changing the E–S affinity, the substrate concentration, or both. Most previous attempts to explain the role of enzymes in long-term adaptation to temperature considered activation energies, thermal optima for maximum velocities and heat denaturation (see Hochachka and Somero, 1968). It appears now that acclimation involves three main control processes; 1) control of enzyme activity, 2) control of the level of enzyme types already present and 3) control of the level of enzyme variants (known as isozymes) uniquely suited to function at particular environmental temperatures. Such enzyme variants induced during short-term acclimation appear to be selected during evolutionary adaptation to temperature. Hochachka (1965), for example, identified five lactate dehydrogenase (LDH) isozymes in the liver of cold-adapted fish. Newell's (1973) review deals further with the isozyme principle in thermal acclimation. It appears that synthesis of isozymes may be induced in response to ionic or pH changes in the cellular environment, by hormonal or nervous stimuli induced by seasonal changes in photoperiod or temperature (see following paragraphs). Behrisch (1973) and Shaklee et al. (1977) have more fully investigated molecular mechanisms of enzyme adaptations. Berisch suggested that there are two main sites at which selective pressure is greatest during evolution to cold, these being 1) enhanced enzyme-ligand affinity

at low temperature, and 2) reduced sub-unit binding energy, which permits allosteric behaviour of control enzymes at low temperatures. Shaklee *et al.*, observing temperature acclimation in fish, found few changes in isozyme pattern in this instance but showed that enzymes in different pathways frequently exhibited changes in opposite directions. This indicated that major metabolic re-organigations were occurring. Low and Somero (1976) and Hoffmann (1976) investigated pyruvate kinases and found that structural rigidity was positively correlated with acclimation temperature. These enzymes can exist in two or more temperature-dependent confrontational states, and the enthalpy of activation was found to be correlated with species' acclimation temperature. Precht *et al.* (1973) state that in addition to the activity, concentration and conformation of enzymes contained within them, the actual number and size of mitochondria may also depend on acclimation temperature. Newell and Bayne's (1973) review describes the possible influences of variations in mitochondrial membrane permeability influencing substrate levels and thereby affecting enzyme reaction rates. Hazel (1973) also investigated the regulation of cellular function by temperature-induced alterations in membrane composition, in particular the lipid composition of these systems. His review summarises the evidence for temperature-induced alterations in membrane lipid composition, discusses the implications of such alterations for the regulation of membrane function, and deals with the regulatory aspects of the lipid biosynthetic pathway which may account for the temperature sensitivity of lipid and membrane composition.

Newell (1975) attempted to relate measured cellular processes with acclimation responses of whole animals (*Littorina littorea*). He concluded that only by detailed quantitative comparisons between the metabolism of intact animals and those of subcellular components can biochemical events

be related to those occurring in the whole organisms.

In previous discussions of the results (see individual chapters) much emphasis was placed on the possible importance of daylength changes (and the associated role of hormones) in metabolic adaptation, It would be interesting to monitor which of the observed seasonal changes in heart rate and respiration could be produced by controlled changes in daylength conditions; also to investigate possible interactions between temperature and daylength. Roberts (1961) for example, investigated the influence of photoperiod upon thermal acclimation in fish. He found evidence that alteration in daylength induced extensive physiological responses and that peculiar seasonal differences in oxygen consumption were obtained in response to different adaptation photoperiods. Various general works are available on the physiology of diurnal rhythms and biological 'clocks' (for example, Sweeney and Hastings, 1960; Harker, 1964; Wilkins, 1965; Bünning, 1967). It is known that in very many cases, observed daily and seasonal physiological, morphological, and behavioural changes are related directly or indirectly to daylength changes. Further, there may in some cases be subtle inter-relationships between light and temperature fluctuation in the triggering of seasonal physiological changes. Even in short-term experiments the importance of circadian rhythms should not be underestimated. Pollard and Larimer (1977), for example, found a degree of rhythmicity in the measured heart rate of a crayfish. Heart rate, and, indeed, oxygen consumption measurements of the present study may well have been affected by similar indiginous factors. Furthermore, in the present study, laboratory-acclimated animals were subjected to constant temperature conditions, yet it is unusual in the natural situation to encounter long-term constant temperatures. In recent years more emphasis has been placed

on investigating acclimation to fluctuating temperatures, especially when studying animals which are known to experience such conditions (see Newell, 1969; Widdows, 1976).

The described effects of fluctuating environmental conditions such as daylength and temperature are undoubtedly produced by endocrine mechanisms. Hormones are known to form an important link between environment and physiology and appear to be very important in seasonal adaptations. They may also be involved in direct temperature effects. Endocrine substances may influence synthesis of proteins by genes and therefore affect enzyme concentrations or they may have direct effects by stimulating or inhibiting enzyme activity or altering membrane permeability (Precht et al., 1973). A variety of hormonal effects have been described for various animals (see Janowsky's review, 1964). Some effects of neurohormones, specifically in relation to heart rate control were discussed in Chapter II. Kale and Rao's (1973) studies showed that the respiration rate of worms (Lampito mauritii) injected with extracts of nervous tissue from cold-acclimated animals was higher than that of control individuals. It was suggested therefore that secretions of the nervous system play an important role in controlling metabolic activity during cold acclimation. Clarke (1964) had postulated the importance of neuroendocrine systems in the regulation of metabolism following his studies on locusts. He postulates that a change in environmental temperature is sensed by receptors, information is fed to the CNS and, through the release of hormones, that level of metabolism is reached which is the most economical under the environmental conditions imposed. Harri's (1973) detailed investigation on neural control of temperature adaptation in Rana temporaria showed how levels of transmitter 5-hydroxytryptamine and adrenaline changed with season and, to a lesser extent, acclimation temperature. Levels of 5HT were

highest during summer and lowest during winter, the opposite being the case for adrenaline, but these seasonal changes were shown not to be simple effects of temperature. It was concluded that increased utilisation of these amines in the cold may initiate and control the peripheral, adaptive changes associated with cold acclimation. With regard to gross neuronal or neuromuscular responses in live animals, Ohsawa and Tsukuda (1956) found seasonal changes, and Ohsawa (1956) found experimental acclimation in the temperature-foot extrusion response of Nodilittorina sp. Cook (1971) described experiments to measure habituation of reflex actions in Lymnaea stagnalis. It would be interesting to investigate effects of temperature acclimation on such neuronal responses.

The known endocrine substances of Lymnaea stagnalis and their effects on growth and metabolism were described in Chapter III. It is thought that many of the observed seasonal changes in heart rate and respiration are caused by the action of hormones, which, as discussed, may act directly or indirectly on net enzyme activity, or induce the synthesis of isozymes.

The gross physiological effects observed in the present experiments must essentially be dependent on biochemical changes, although these changes may be very complex and dependent upon a variety of factors. Enzymes may show alterations directly induced by temperature and these may be enforced or opposed by other influences on cell chemistry, notably hormonal changes.

CONCLUSIONS

The results indicate that if acclimation responses are observed in one gross metabolic function, they are not necessarily to be found in another. Certain adaptations may even appear to be antagonistic. Further, seasonal adaptations may include direct temperature effects but are usually

not entirely dependent upon these. Apparently, metabolic adaptations are often mediated by hormonal and neurohormonal agents whose production may be stimulated by temperature, daylength or other environmental variables. These agents act at the subcellular level through enzyme reactions to produce the observed physiological responses. Temperature effects alone may directly influence enzyme structure and function, notably through the induction of enzyme affinity.

Some physiological responses (for example, acclimation of heart rate) are important for the survival of the animal and may have repercussions on metabolic efficiencies, whilst others(for example, reverse acclimation of oxygen consumption) are apparently important in the maintenance of metabolic rates appropriate to the animals' behaviour and activity at different developmental stages. The life history itself is adapted to seasonal changes in resource availability.

Weiser's (1973) review is particularly interesting with respect to the inter-relationship of components which may be important in the control of ectotherm metabolism by temperature, including fine-control and feedback mechanisms. He states that different reactions are found in different organisms and that too little is known of the detailed ecology of specific ectotherms to permit the construction of an adequate model of the subtle inter-relationship existing between temperature on the one hand and metabolism, development and behaviour on the other. The present study was an attempt to relate seasonal and temperature-induced reactions in specific metabolic functions to the animals known ecology and physiology. No obvious inter-relationships were seen to exist between the acclimation responses of heart rate, oxygen consumption and assimilation efficiency, and it is clear that further studies, particularly on biochemical aspects, would have been necessary to provide a more complete picture of overall

metabolic acclimation. Also, to enable a fuller understanding of the gross adaptations shown by Lymnaea stagnalis, it would be necessary to obtain more detailed information on the animals' ecology. For example, it would be interesting to investigate changes in energy input during the life cycle associated with seasonal changes in food availability and ingestion rates. Information on the amount of energy spent searching for food and visiting the surface for air, and the precise effects of temperature and food availability on these conflicting behaviour patterns, would also prove useful. Further, it would be interesting to determine how and when somatic and reproductive growth are partitioned, and relate this information to the measured seasonal changes in oxygen consumption.

It was suggested earlier in the Discussion that the acclimation of metabolic functions to seasonal temperature changes might stabilise the energy budget and energy partitioning. Certainly this is likely to be of adaptive significance to an animal. However, such adaptations were not seen to occur in Lymnaea stagnalis, primarily because of 1) changing environmental conditions such as food availability and oxygen supply, and 2) changes in activity levels at different stages in the life cycle, notably as a result of reproductive changes. These factors appeared to largely over-ride direct temperature compensation effects. In summary, oxygen consumption of L. stagnalis showed an apparent seasonal 'reverse' acclimation which was related to changes in activity, but no seasonal changes were observed in the assimilation efficiency function. Heart rate showed ~~that~~ the best 'classical' features of thermal acclimation, including very clear resistance adaptations which appeared to be wholly temperature-dependent. A most important finding of this study was that acclimation to temperature alone produced few of the complex changes which were observed

through the course of a year. This suggests that hormonal influences, probably triggered by external factors such as daylength and associated with the animal's stage of development, are most important.

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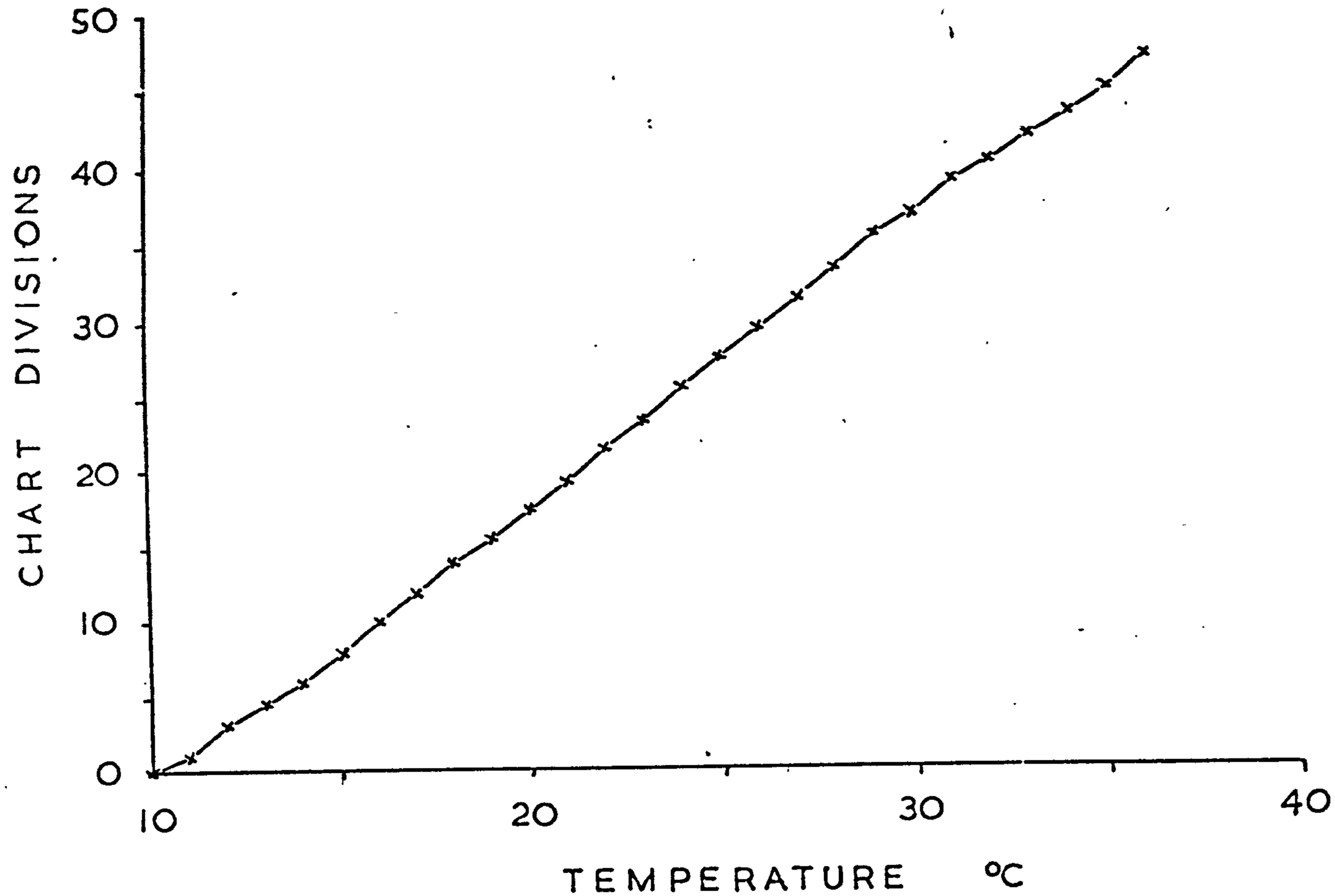
APPENDIX I THERMISTOR CALIBRATION



APPENDIX 2

Uptake of ^{14}C by Chlorella ellipsoides

Time after introduction of label	Background count (c.p.m.)	Activity in 10 ul of algal digest (c.p.m.)	Net count rate (c.p.m.)
16 hours	28	8,000	7,972
24 hours	27	7,986	7,959
40 hours	28	7,414	7,386

χ^2 -test shows no significant difference between net counts at the 95% level.

APPENDIX 3

Absolute efficiency of counting system using P.B.D. dioxan-based scintillant with addition of 10 µl (8880 d.p.m.) standard ^{14}C toluene.

Vial No.	Background count (c.p.m.)	Count rate (c.p.m.)	Net count (c.p.m.)
1	27.4	8134.2	8106.8
2	25.5	8163.6	8138.1
3	22.8	8300.3	8277.5
4	26.7	8010.6	7983.9
5	22.6	8121.2	8098.6

Mean: 8120 c.p.m.

$$\text{Counting efficiency} = \frac{8120}{8880} \times 100\% = \underline{\underline{91.5\%}}$$

APPENDIX 4

Efficiency of recovery of active material from standard digest.

Snails used:	No.	Length (cm)	Fresh weight (gm)
	1	3.01	1.323
	2	2.97	1.642
	3	2.57	0.893
	4	2.71	1.118
	5	2.79	1.306
Control	6	2.58	0.907

Estimate of activity to be injected into snails

Addition into each vial of 20 µl diluted glycine-1-^{14}C solution (maximum estimated activity 5×10^{-3} µCi = 11,100 d.p.m.)

Tube	Background (c.p.m.)	Count (c.p.m.)	Net (c.p.m.)
1	26.7	7949.2	7922.5
2	27.7	7929.0	7901.3
3	25.5	7508.8	7483.3
4	23.4	7793.4	7770.0
5	23.4	7682.5	7659.1

Mean: 7747 c.p.m.

Estimatedefficiency of counting system = 91.5% (see Appendix 3)

$\therefore$ Mean count contained in 20 µl glycine solution = $\frac{7747}{0.915}$ = 8467 d.p.m.

Appendix 4 (continued)

Analysis of 'spiked' - snail digests

Snail No.	Background	Count (c.p.m.)	Net count (c.p.m.)	+ 888 d.p.m. standard toluene (1 ul)	Increase (c.p.m.)	% Recovery	Digest volume (ml)	Net count dilution and quench corrected (d.p.m.)
1	25.7	279.4	253.7	553.0	299.3	33.7	9.5	7876
2	26.0	317.8	291.8	600.8	309.0	34.8	8.6	7211
3	26.3	285.5	259.2	535.4	276.2	31.1	10.1	8418
4	23.9	281.4	257.5	581.6	324.1	36.5	9.7	6838
5	23.6	242.1	218.5	486.7	268.2	30.2	9.1	6584
(Control)								
6	22.6	23.0	-	-	-	-	-	-

Mean: 7385 d.p.m.

Mean activity added = 8467 d.p.m.

∴ Efficiency of recovery = (7385/8467) x 100 = 87.2%

APPENDIX 5

Rates of faeces production

Time after meal (mins.)		Number of snails having produced faeces
20		0
40		0
60	(1 hour)	0
80		0
100		0
120	(2 hours)	0
140		2
160		2
180	(3 hours)	3
200		4
220		4
240	(4 hours)	5
260		6
280		6
300	(5 hours)	6

Results for eight snails fed on lettuce for twenty minutes at room temperature (c. 20°C).

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SEASONAL CHANGES IN THE HEART RATE OF THE FRESHWATER PULMONATE *LYMNAEA STAGNALIS* (L.)

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Abstract—1. *Lymnaea stagnalis* (L.) exhibits seasonal changes in the heart rate–temperature relation.

2. Seasonal acclimatization induces capacity adaptations, whereby winter animals show a higher heart rate than summer animals at any temperature between 15 and 25°C, and also resistance adaptations, which give summer animals increased tolerance to heat and winter animals greater resistance to cold.

3. The appearance of a bimodality in the rate–temperature curves at certain seasons of the year illustrates that control of heart rate is complex.

4. Some possible modes of action of heart rate control mechanisms are discussed.

INTRODUCTION

It is now well established that poikilotherms frequently exhibit compensatory changes in various metabolic rate functions in response to the temperatures encountered in different latitudes, seasons and micrographic areas. Such adaptations may also be induced by laboratory acclimation. Literature concerning these phenomena has been well reviewed, for example by Bullock (1955), Fry (1958), Kinne (1964), McWhinnie (1967), Newell (1966), Precht (1958) and Segal (1961).

It is known that the heart rate of poikilotherms increases with temperature, within physiological ranges, but little information is available regarding seasonal adaptation of this function. Evidence that such compensation does occur has been provided by Barcroft & Izquierdo (1931) and Stier & Taylor (1937), who found distinct seasonal changes in the heart rate responses of frogs, by Segal (1956), who showed that the heart rate of the limpet *Acmaea limatula* has a peak in winter and a minimum in summer, and by Crozier & Stier (1924), who found evidence for seasonal changes in the Q_{10} for heart beat frequency in the slug *Limax maximus*.

This paper aims to establish for the pond snail *Lymnaea stagnalis* (L.) the precise relationship between heart rate and temperature and to determine whether this relationship changes with season.

MATERIALS AND METHODS

Heart rate was measured over a full range of temperatures from 5 to 30°C at intervals of 5°C. Peaks in the rate–temperature curves were more accurately defined where necessary by further recordings at 2.5°C intervals.

Snails were collected approximately every 8 weeks from a large shallow pond in Essex. They were removed to the laboratory and stored until use in an outside tank supplied with a slow flow-through of tap water. The animals were fed *ad libitum* with lettuce leaves and Tetramin fish food. Heart rates were measured as soon as possible after collection, the recordings normally being completed within 10 days. Random samples of 10 animals were used for each experimental temperature. The shell length of each animal was measured.

The snails were mounted by pushing the spire into a piece of plasticene held in a small glass stand, and measurements were carried out in aerated water at constant temperature. Heart rate was measured by direct observation through the shell (Tsukuda & Ohsawa, 1959), the animals being illuminated from beneath using a 15 W lamp. Preliminary experiments revealed that, following immersion, any fluctuations in heart rate caused by the sudden temperature change (Segal, 1962), or by the change in light intensity, ceased within 10 min. A 10 min lapse was

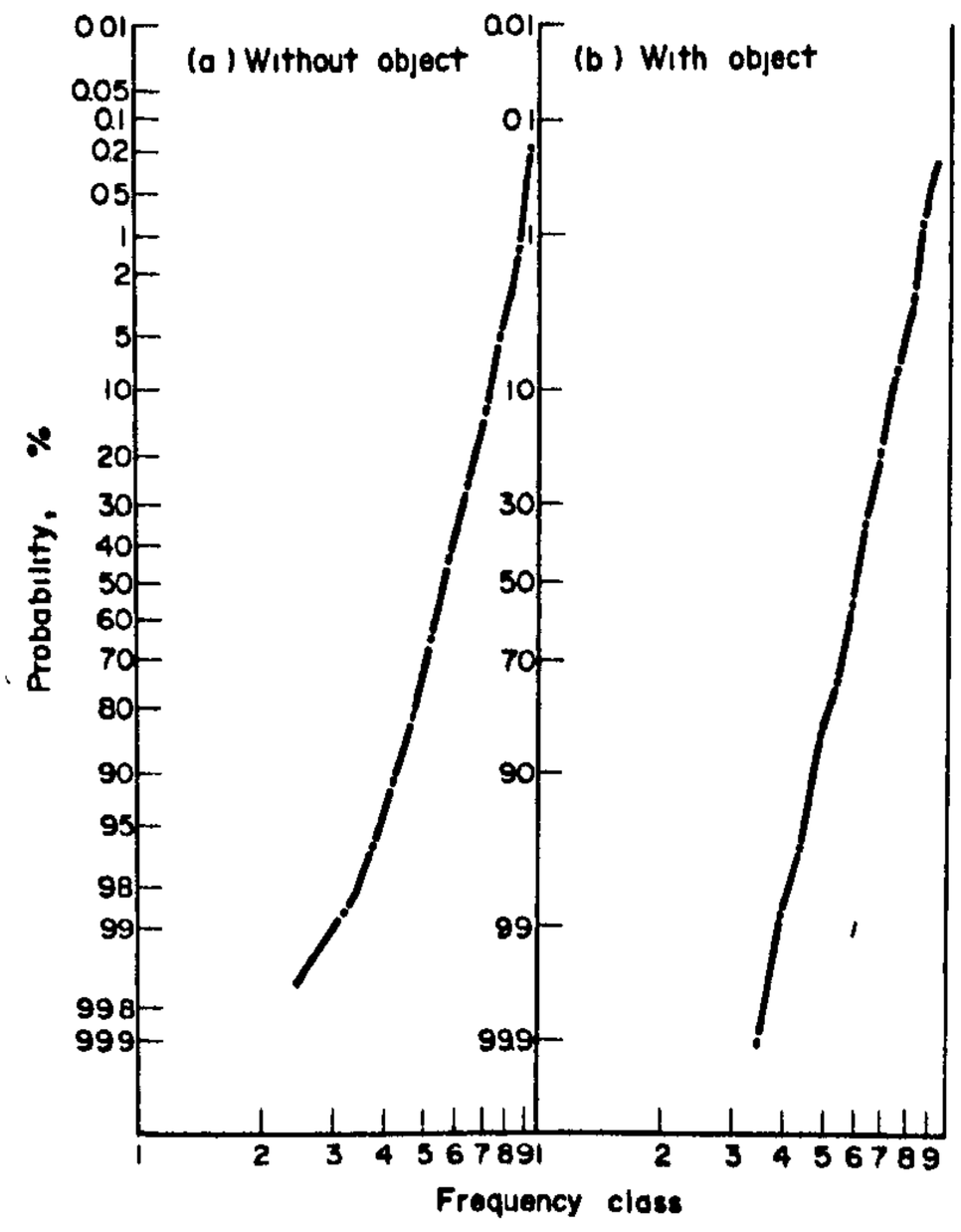

Fig. 1. Log-probability plot of the distribution of 10 half-minute heart rate values for samples of 106 animals with and without an object on which to cling, measured at 10°C.

Table 1. Heart rate results

Month	Experimental temperature (°C) 5	10	15	20	25	27.5	30	32.5	35	37.5	40	N	Mean shell length (log_{10}cm)
April	0.79 ±0.06	1.20 ±0.03	1.39 ±0.02	1.63 ±0.04	1.76 ±0.03	—	1.85 ±0.02	1.87 ±0.04	1.79 ±0.08	—	—	10	0.296 ±0.043
June	0.83 ±0.04	1.20 ±0.02	1.39 ±0.04	1.62 ±0.02	1.76 ±0.02	—	1.82 ±0.03	1.86 ±0.02	1.88 ±0.02	1.86 ±0.02	—	10	0.450 ±0.046
August	0.61 ±0.08	0.94 ±0.06	1.28 ±0.06	1.46 ±0.07	1.62 ±0.05	—	1.83 ±0.04	1.89 ±0.03	1.88 ±0.04	1.90 ±0.04	1.85 ±0.04	10	0.448 ±0.015
October	0.84 ±0.07	1.14 ±0.06	1.33 ±0.06	1.50 ±0.05	1.61 ±0.06	—	1.74 ±0.06	1.78 ±0.03	1.82 ±0.05	1.80 ±0.08	—	10	0.492 ±0.022
Nov–Dec	0.72 ±0.11	0,97 ±0.07	1.21 ±0.11	1.45 ±0.03	1.55 ±0.12	1.55 ±0.14	1.66 ±0.10	1.51 ±0.23	—	—	—	8	0.169 ±0.040
February	0.85 ±0.04	1.05 ±0.06	1.26 ±0.04	1.55 ±0.05	1.71 ±0.06	1.69 ±0.05	1.76 ±0.05	1.71 ±0.07	—	—	—	10	0.109 ±0.050
March	0.98 ±0.06	1.29 ±0.04	1.45 ±0.03	1.65 ±0.03	1.79 ±0.02	1.84 ±0.04	1.72 ±0.11	1.82 ±0.11	1.68 ±0.16	—	—	10	0.142 ±0.062
May	1.02 ±0.04	1.20 ±0.06	1.34 ±0.06	1.50 ±0.04	1.77 ±0.01	—	1.87 ±0.02	1.95 ±0.03	1.91 ±0.04	1.73 ±0.13	—	10	0.330 ±0.056

Mean log_{10} heart rates with 95% confidence limits, sample size (N) and mean shell lengths.

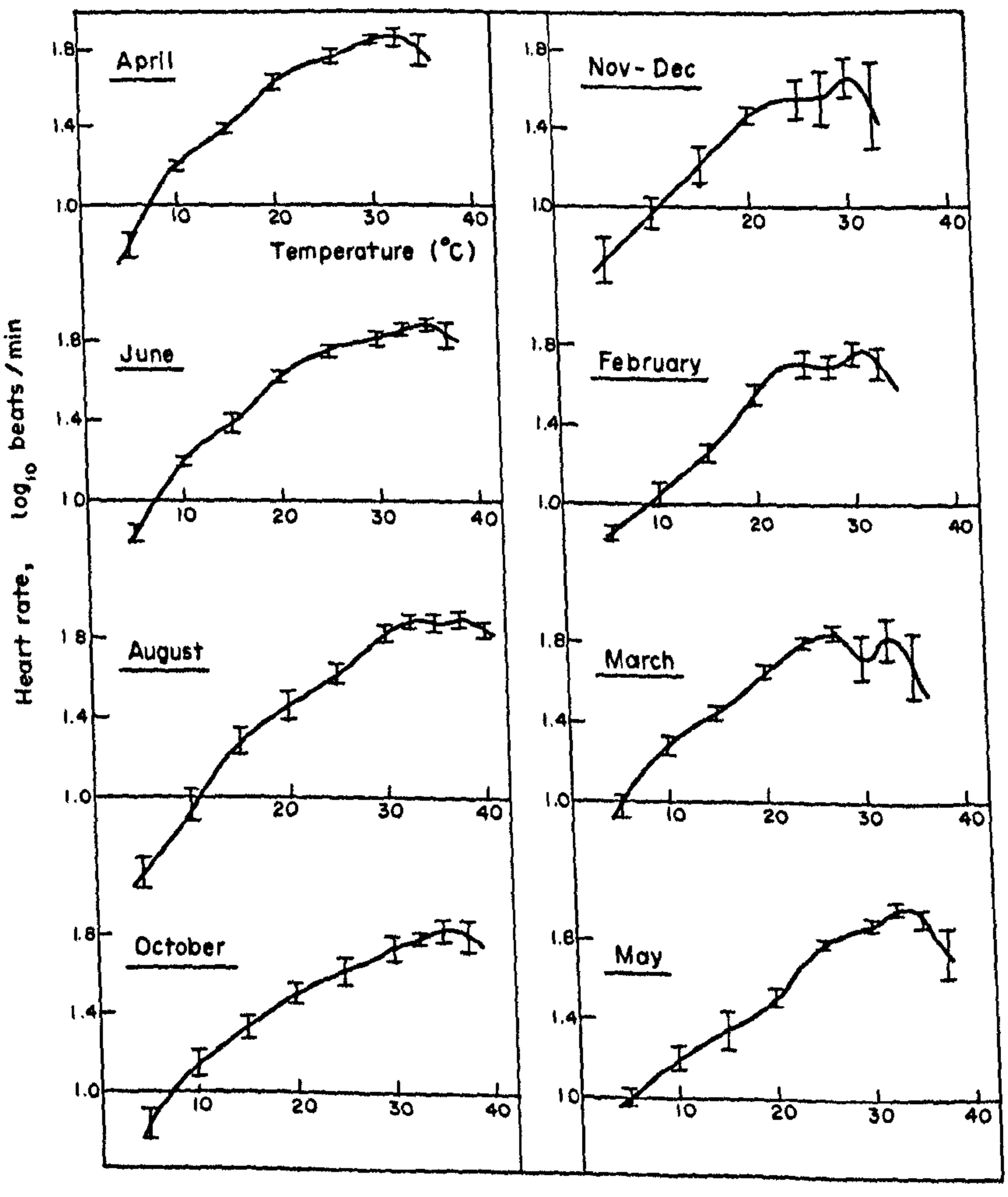

Fig. 2. Seasonal heart rate–temperature curves with 95% confidence limits.

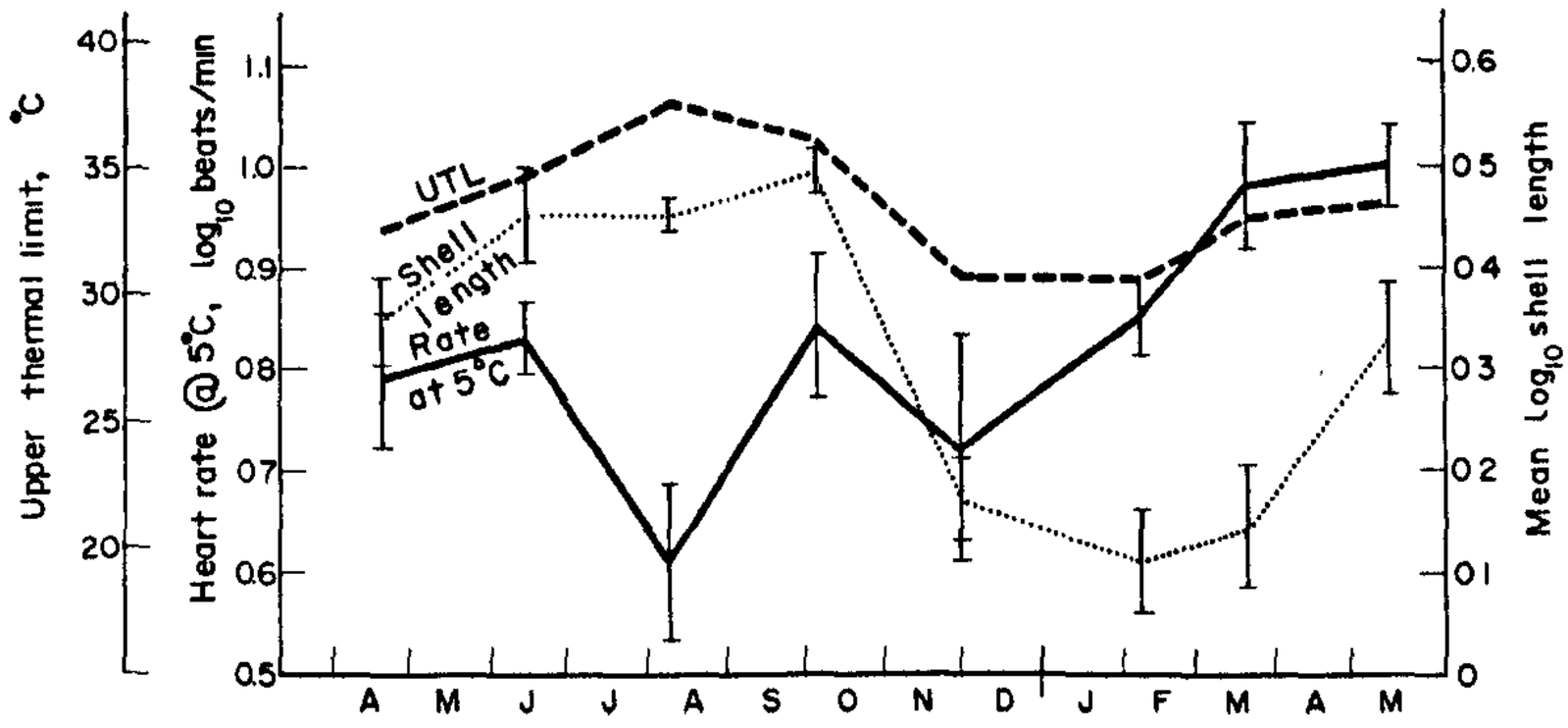

Fig. 3. Summary of seasonal changes in upper thermal limit (U.T.L.), heart rate at 5°C and mean $\log_{10}$ shell length.

therefore allowed for stabilization of heart rate before recordings were begun on each animal. Also, to prevent the animals from extruding the foot and thereby obscuring the heart from view, a small neutrally buoyant object was introduced to the snails. This resulted in the previously obtained normal distribution of heart rate measurements becoming skewed, but a logarithmic transformation returned the distribution to the normal form. Figure 1 shows that only those animals provided with an object exhibited this normality, as indicated by the straight line "b" (Southwood, 1966). All heart rate measurements and related statistics are therefore expressed in terms of the logarithms of heart beat frequency.

RESULTS

Heart beat frequencies were recorded, transformed to logarithms and the mean, standard deviation and 95% confidence intervals calculated. An analysis of the data, which were gathered at 2 monthly intervals from April 1975 to May 1976, is shown in Table 1 and the 8 seasonal rate–temperature curves obtained are represented in Fig. 2. It was found that heart rate increased with temperature up to a peak beyond which the rate lessened and the beats became erratic, causing a general increase in variability of data above this upper thermal limit. Figure 3 is a summary of the year's results in graphical form. It indicates how the upper thermal limit (U.L.T.), heart rate at 5°C and mean $\log_{10}$ shell length varied with season.

DISCUSSION

A comparison of the rate–temperature curves in Fig. 2 shows changes in the relationship between heart rate and temperature through the course of the year, the most distinct differences occurring at either end of the curves. There is a clear shift in the low temperature portion of the curve and a change in position of the peak. The precise nature of the peak also changes. In addition there is evidence of translation of the curves between 15 and 25°C.

Comparison of the March and August curves provides an example of translation. The $\log_{10}$ heart rate in March exceeds that in August by 0.17 at 15°C, by 0.19 at 20°C and by 0.17 at 25°C (Table 1). All these differences are significant at the 95% level of confidence. The summer heart rate–temperature curve thus shows a depression compared to that recorded in winter. This vertical translation of the curve represents a "capacity adaptation" (Precht, 1958) and is equivalent to Prosser's (1958) acclimation type II. There is not, however, a clear and straightforward seasonal progression in the shift of the rate–temperature curve, and only when temporally well-spaced curves are compared do significant changes become apparent. Miller & Mizell (1972) likewise found distinct winter–summer differences in the heart rate of *Rana pipiens* but stated that during intervening months the response was intermediate with no predictable pattern.

The changes occurring at the temperature extremes provide evidence for seasonal "resistance adaptations" (Precht, 1958). The temperature for peak heart rate (U.T.L.) varies considerably with season. For example, comparing August with February, the U.T.L. in high summer is approx 37.5°C, whereas in winter the peak occurs at about 30.5°C. Warm adapted animals are therefore more tolerant of high temperatures. The determination of a lower thermal limit is more problematic because this is implicated not by a peak but by a disproportionate drop in heart rate at low temperature. However, if resistance adaptation to low temperature is present, then winter animals will exhibit a considerably higher heart rate at 5°C than summer animals. A comparison shows that the mean $\log_{10}$ heart rate of August animals at 5°C is 0.37 lower than that of March animals, a significant difference ($P < 0.01$). This change in rate at low temperature is not due solely to the general vertical translation of the curve discussed earlier, since comparison of the same months' results shows a displacement of only 0.17 $\log_{10}$ beats/min in the 15–25°C temperature range. It is concluded that the mechanisms of capacity and resistance adaptation act together to produce significant seasonal differences in heart rate at extreme low temperature.

Figure 3 summarizes the seasonal changes in U.T.L. and heart rate at 5°C. The upper thermal limit increases through spring and summer to reach a maximum in August, then falls to a minimum in December and February. Heart rate at 5°C shows a more erratic variation with season, exhibiting a minimum in August and increasing through autumn and winter to show a maximum in spring. These two sets of results therefore do not exactly concur. For example, a minimum U.T.L. does not coincide with

a maximum rate at 5°C. This appears to contradict Precht's (1958) suggestion that adaptation to low temperature necessarily incurs changes in heat tolerance, and vice versa. It infers, instead, that changes in heat and cold tolerance are not inseparably linked but could each be controlled by separate mechanisms.

Further evidence that no single factor is responsible for the observed seasonal changes in the heart rate of *Lymnaea stagnalis* is provided by the occurrence of a bimodality in the rate–temperature curve at certain seasons. Figure 2 clearly shows a progression in the development of a secondary peak at a lower temperature. This first becomes apparent in November–December, reaches a maximum in March and declines by June. The possibility of this and other effects being caused by changes in the size distribution of animals through the year is ruled out because there is evidence that no strong correlation exists between heart rate and size. Shell size was found to change considerably with season, but only 13 of 71 size–rate correlations proved to be significant at the 95% level of significance. Figure 3 reveals further evidence that size is not a major influence on heart rate. Comparing the June and August results, the change in mean log shell length is insignificant but the U.T.L. increases by 3°C and $\log_{10}$ heart rate at 5°C decreases by 0.22, a significant change. Moreover, a large and significant increase in size from March to May has no significant effect on the heart rate parameters illustrated. Breeding state may also be considered an important factor affecting heart rate, but Segal (1956) found for the limpet *Acmaea limatula* that size of gonad did not contribute to the variation in heart rate between samples. It is therefore proposed that the observed seasonal changes in heart rate are predominantly due to physiological adjustments, which are controlled by more than one mechanism. They result in significant seasonal capacity and resistance adaptations.

Similar results have been quoted by other workers. Segal (1956), working with the intertidal mollusc *Acmaea limatula*, found that both high and low level animals had faster rates in winter than in summer at any temperature between 9 and 29°C, thereby exhibiting a capacity adaptation. Distinct seasonal changes have been observed in the shape of the heart rate–temperature curve of excised and intact frogs' hearts, with changes also in the position of the peak (Barcroft & Izquierdo, 1931; Carter, 1933; Harri & Talo, 1975*a*, *b*; Miller & Mizell, 1972; Smith, 1951 and Stier & Bock, 1966). A study by Stier & Taylor (1937) on the behaviour of the heart of *Rana pipiens* at high temperature showed that the upper limit of temperature at which normal contraction was maintained varied with season and thus revealed a resistance adaptation. Evidence of seasonal changes in the heart rate–temperature relationship of the gastropod *Helix* sp. was presented by Lang (1910, quoted by Crozier & Stier, 1924), who found different Q_{10}'s according to time of year.

With regard to the possible mechanisms concerned in the control of heart rate, it has been shown in frogs that seasonal changes in the heart rate–temperature relation are of endocrine origin and caused by increased activity of the thyroid in the summer (Carter, 1933; Smith, 1951). It is known that pulmonate molluscs possess both nervous and non-nervous endocrine organs (Boer & Joose, 1975) which similarly may be important in the control of heart rate. Kale & Rao (1973) showed that neurosecretions of the nervous system of earthworms have an important role in the control of metabolic activities during cold acclimation, and Lagerspetz & Tirri (1968) have indicated that high heat resistance of heart beat of the freshwater mussel *Anodonta*, induced by seasonal acclimatization, is associated with elevated 5-hydroxytryptamine concentrations in the heart. An annual cycle of activity has indeed been observed in the neurosecretory cells of the mollusc *Viviparus* (work by Gorff quoted by Gersh, 1959). Other important factors involved may be temperature-induced changes in the speed of conduction in the myogenic and pacemaker mechanisms, and changes in the structure and function of the heart muscle. Evidence for such possibilities is provided by Garten & Sulze (1913), Kerkut & Ridge (1962), Lagerspetz (1974) and Vislobokov (1975), who studied effects of temperature on nervous mechanisms, and Benthe (1954), Hadju (1951), Johnston *et al.* (1975) and Rao & Singh (1907) who have investigated changes in muscle performance and structure. Such variety of control mechanisms could result in the observed complexity of seasonal adaptation of the heart rate function.

SUMMARY

Heart rates of *Lymnaea stagnalis* (L.), collected seasonally, were measured over full physiological ranges of temperature. Size was shown not to be a major influence of heart rate.

The seasonal heart rate–temperature curves showed translation between 15 and 25°C. This capacity adaptation was most distinct when comparing winter and summer animals. During intervening months the response was intermediate with no clear seasonal progression.

The upper thermal limit and heart rate at 5°C, an indicator of cold sensitivity, both changed considerably with season, illustrating resistance adaptation at both temperature extremes.

The occurrence of a secondary peak in winter and spring rate–temperature curves was considered to be evidence for the complexity of seasonal heart rate control. Several possible mechanisms of control were discussed.

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LABORATORY INDUCED CHANGES IN THE HEART RATE OF *LYMNAEA STAGNALIS* (L.)

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Abstract—1. Thermal acclimation induces significant changes in the heart rate–temperature relation of *Lymnaea stagnalis* (L.).

2. Resistance adaptations occur at both temperature extremes but there is no evidence for capacity adaptation.

3. An acclimation temperature of 20°C induces a bimodality in the rate–temperature curve similar to that sometimes found in seasonally adapted snails.

4. Various similarities exist in the changes in heart rate function induced by seasonal and experimental acclimations. Capacity adaptation, however, is present only in seasonally adapted snails, suggesting that acclimation to temperature alone is not sufficient to produce this response.

INTRODUCTION

A previous paper (Harrison, 1977) showed that *Lymnaea stagnalis* (L.) exhibits seasonal adaptation of the heart rate temperature relation. The present investigation seeks to determine the nature of changes in the heart rate function induced by thermal acclimation in the laboratory.

Studies on the acclimation of heart rate are relatively few, and from the work published it appears that some poikilotherms exhibit acclimatory responses in this rate–temperature relation (Mellanby, 1940; Segal, 1956; Tsukuda & Ohsawa, 1959) whereas others do not (Ahsanullah & Newell, 1970; Weathers, 1975; Widdows, 1973). Rarely have seasonal and experimental changes been directly compared, however. Fry (1958) stated that the direct effects of temperature may be opposed or reinforced by the effects of other environmental identities. It has been shown that seasonal changes in the heart rate–temperature relationship of *L. stagnalis* are complex and involve both capacity and resistance adaptations. It is of interest to isolate which, if any, of these seasonal changes may be induced by temperature alone.

MATERIALS AND METHODS

Animals were collected in Spring 1975 and groups of 20 or more animals were stored in aerated water in environmental cabinets at 5, 10, 15, 20 and 25°C (all ±1°C). Lighting regimes were maintained at 12 hr light: 12 hr dark and the snails were fed *ad libitum* with lettuce and Tetra Min fish food. The animals were allowed to acclimate to these temperatures for 3–6 weeks. Random samples of 10 or 20 animals were then taken and heart rates measured over a full physiological range of temperature according to the method described in the previous paper (Harrison, 1977).

RESULTS

Heart beat frequencies were recorded, transformed to logarithms and the mean, standard deviation and 95% confidence intervals calculated. An analysis of the data is shown in Table 1 and the 5 heart rate–temperature curves obtained are represented, with 95% confidence intervals, in Fig. 1. Figure 2 is a superimposition of three of the five rate–temperature curves, without error bars, to aid comparison. Figure 3 shows $\log_{10}$ heart rate at 5°C, upper thermal limit and mean

Table 1. Heart rate results

	Experimental temperature (T_e°C)												
T_a°C	5	10	15	20	25	27.5	30	32.5	35	37.5	40	N	Mean shell length (cm)
5	0.94 ±0.02	1.14 ±0.02	1.33 ±0.02	1.52 ±0.02	1.64 ±0.02	1.62 ±0.05	1.57 ±0.04	1.51 ±0.06	—	—	—	20	1.96 ±0.26
10	0.83 ±0.04	1.08 ±0.03	1.26 ±0.03	1.52 ±0.03	1.68 ±0.02	—	1.79 ±0.02	1.81 ±0.02	1.74 ±0.04	—	—	20	2.06 ±0.29
15	0.83 ±0.05	1.09 ±0.03	1.36 ±0.03	1.57 ±0.03	1.72 ±0.02	—	1.81 ±0.02	1.77 ±0.03	1.76 ±0.04	—	—	20	1.91 ±0.32
20	0.78 ±0.03	1.07 ±0.04	1.28 ±0.04	1.48 ±0.05	1.65 ±0.05	—	1.77 ±0.05	1.74 ±0.07	1.83 ±0.04	1.68 ±0.11	—	10	2.32 ±0.12
25	0.58 ±0.06	0.97 ±0.06	1.32 ±0.06	1.48 ±0.03	1.65 ±0.03	—	1.75 ±0.03	1.80 ±0.03	1.82 ±0.02	1.78 ±0.04	1.75 ±0.06	10	2.87 ±0.14

Mean $\log_{10}$ heart rates with 95% confidence limits, sample size (N) and mean shell lengths.

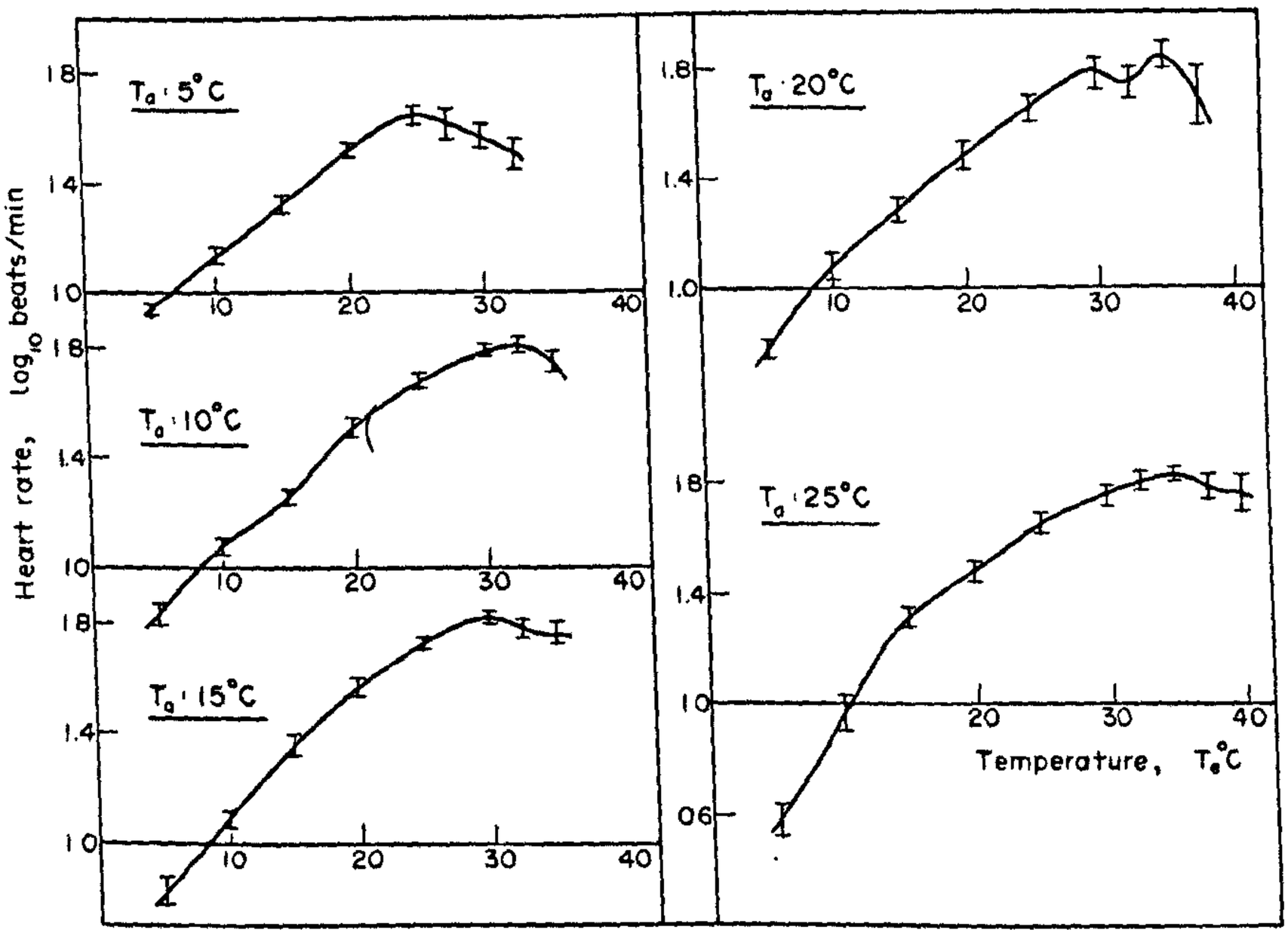

Fig. 1. Heart rate–temperature curves for each T_a with 95% confidence limits.

shell length for each acclimation temperature. Figure 4 is an acclimated rate–temperature curve (Bullock, 1955) which illustrates the degree of compensation of rate.

DISCUSSION

A comparison of the rate–temperature curves in Fig. 1 shows clear changes in the relationship between heart rate and experimental temperature (T_e) according to the temperature of acclimation (T_a). These changes occur predominantly at either end of the curves; there is little evidence of change in the mid-regions. The superimposition of the T_a: 5, 15 and 25°C curves in Fig. 2 shows this resistance adaptation occurring at each temperature extreme. The temperature for peak heart rate, the upper thermal limit (U.T.L.), changes considerably with acclimation temperature. An increase in T_a results in a rise in U.T.L. The U.T.L. for T_a: 5°C is 25°C, 30°C for T_a: 15°C and 35°C for Ta: 25°C animals. Acclimation to heat therefore increases tolerance to high temperature. Heart rate at 5°C, which gives an indication of sensitivity to low temperature, increases as T_a decreases and indicates that acclimation to cold results in a resistance adaptation to low temperature. Thermal acclimation thus affects both upper and lower limits of the rate–temperature curve and effectively extends the viable temperature range. Figure 3 summarises these changes and show that in all cases sensitivity to low temperature is increased as temperature of acclimation increases. Also, with the exception of the T_a: 15°C results, the U.T.L. is raised by acclimation to higher temperatures.

Figure 3 also shows that size is not a major influence on heart rate. For example, comparing the T_a: 5 and 15°C results animal size is not significantly different, yet both U.T.L. and heart rate at 5°C differ considerably. Further, of 43 size–rate correlations, performed for each T_e at all T_a's, only 7 were significant at the 95% level. These findings are in agreement with those of a previous paper (Harrison, 1977).

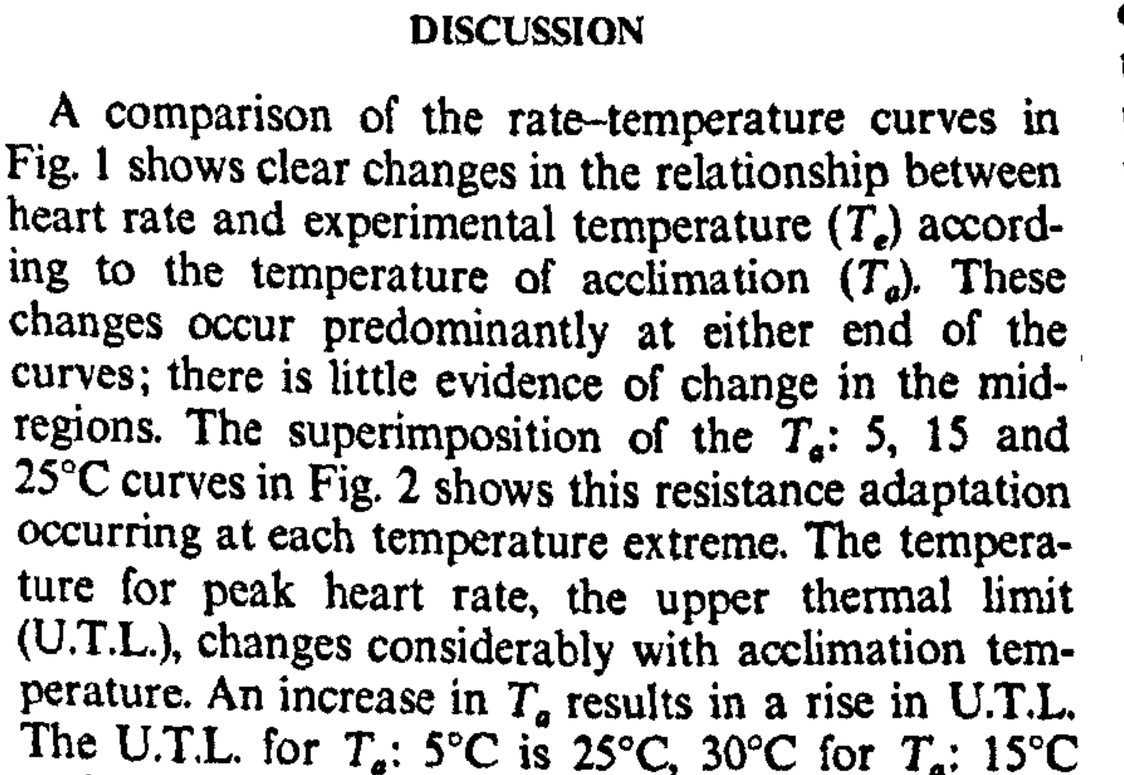

Fig. 2. Superimposition of T_a: 5, 15 and 25°C curves.

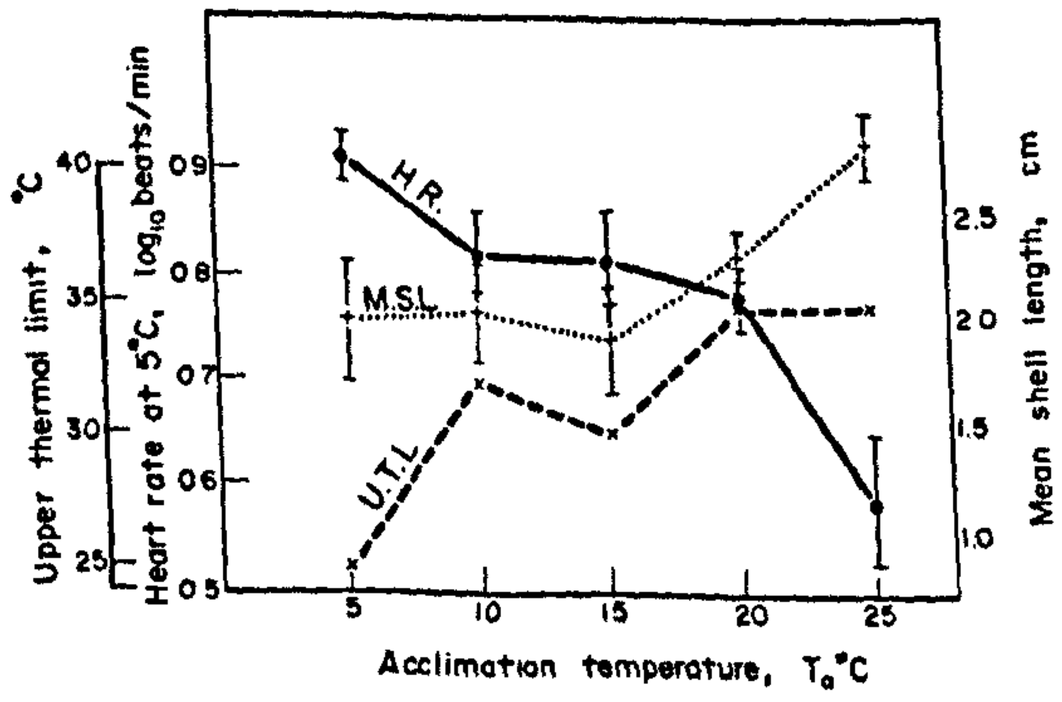

Fig. 3. Summary of changes in upper thermal limit (U.T.L.), heart rate at 5°C and mean shell length according to temperature of acclimation.

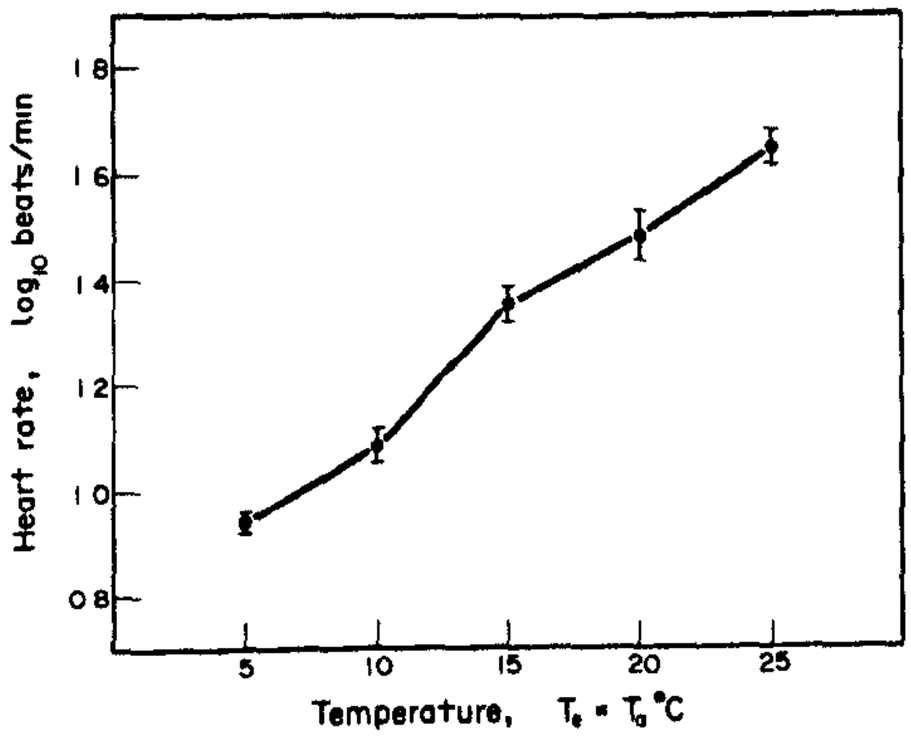

Fig. 4. Acclimated rate–temperature curve showing heart rate where $T_a = T_e$.

The acclimated rate–temperature curve shown in Fig. 4 is similar in shape and gradient to the normal curves of Fig. 1 and further illustrates that capacity adaptation of rate is absent. For perfect or total acclimation the slope of this line would be zero; heart rate would be equal for each acclimated temperature (Bullock, 1955).

It is concluded that acclimation to temperature alone results in resistance adaptations at each temperature extreme, but there is no evidence for capacity adaptation. The failure of laboratory acclimation to induce capacity adaptation of heart rate has likewise been found in the crab *Carcinus maenas* (Ahsanullah & Newell, 1970) in various anurans (Rüth, 1974; Weathers, 1975) and in the mussel *Mytilus edulis* (Widdows, 1973). Conversely, however, such adaptation has been shown to occur in several poikilotherms, for example, the crested newt (Mellanby, 1940), the limpet *Acmaea limatula* (Segal, 1956) and in a freshwater snail, *Physa* sp. (Tsukuda & Ohsawa, 1959). It appears that factors controlling compensatory responses of heart rate differ between individual species so that no generalization can be made regarding the induction of capacity adaptation of the function by temperature acclimation.

Comparing the findings of this investigation with those of the previous study of seasonal changes in heart rate in *Lymnaea stagnalis* (Harrison, 1977) it is apparent that thermal acclimation to a large extent mimics the changes in lethal limits found at different seasons, but that capacity adaptation occurs only in seasonally adapted animals, suggesting either that a longer time course is required for this response, or that other factors such as fluctuation of temperature or photoperiod are important. This perhaps implies that the capacity adaptation response is dependent upon hormonal changes, as discussed in the previous paper. The occurrence of bimodality in rate–temperature curves in both studies reinforces the assertion that control of heart rate is complex. The rate–temperature curve for T_a: 20°C animals (Fig. 1) shows a double peak similar to that encountered through winter and spring in the seasonally adapted snails. Measurements were repeated four weeks later (total acclimation time 9 weeks) and an identically shaped curve obtained, indicating that this is a real feature of the curve and not an artefact of measurement. Further, the lower peak, at 30°C, corresponds to that of the T_a: 15°C curve and the upper peak, at 35°C, corresponds to that of the T_a: 25°C curve. It appears that separate factors are responsible for the position of the maximum, that these mechanisms are temperature-induced and at intermediate temperatures may occur together, certainly in the same population and probably in the same individual. The nature of these mechanisms, however, can not here be further elucidated.

SUMMARY

Heart rates of laboratory acclimated snails (*Lymnaea stagnalis*) were measured over full physiological ranges of temperature. Five acclimation temperatures were used; 5, 10, 15, 20 and 25°C, and heart rates were measured according to the method described in a previous paper (Harrison, 1977). Size was shown not to be a factor of importance.

The heart rate–temperature curves obtained showed resistance adaptations at both temperature extremes. Increase in temperature of acclimation in most cases resulted in an increase in upper thermal limit and a reduction in the heart rate at 5°C, an indicator of cold sensitivity. There was no evidence for capacity adaptation.

The T_a: 20°C curve showed a bimodality with maxima occurring at 30 and 35°C, these peaks coinciding with those of the T_a: 15 and 25°C rate–temperature curves respectively. The T_a: 20°C curve was similar in shape to the rate–temperature curves obtained during winter and spring in a seasonal study of changes in the heart rate–temperature relation (Harrison, 1977).

Further comparisons between the seasonal and laboratory acclimation studies showed that resistance adaptations were present in both cases but capacity adaptation was found only in the former, suggesting that thermal acclimation alone was not sufficient to produce a capacity adaptation response in heart rate.

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