THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

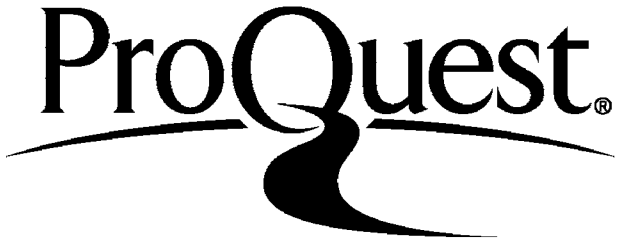
OCTOBER 1958

THE METABOLISM OF *ARTEMIA SALINA* (L.)

BY

BARBARA M. GILCHRIST

Bedford College, University of London



ABSTRACT

Some aspects of the metabolism of Artemia have been investigated. Experiments have been made with animals of different stocks, obtained from different localities.

The growth of Artemia is influenced not only by the sex of the animal, but also by the stock from which it is derived. Likewise, the effect of the salinity of the medium on growth varies with sex and stock. The growth of Artemia is retarded in brine with a low dissolved oxygen content.

Changes in body proportions occur with increase in size of Artemia; in particular, the abdomen becomes relatively longer. Body form is also influenced by the salinity of the external medium. The extent to which this occurs varies with the sex of the animal and the stock from which it is derived.

The oxygen consumption of Artemia has been measured in relation to the size and sex of the animal and also to the salinity of the medium and its dissolved oxygen content. Females have the same rate of oxygen uptake in sea water as in more concentrated brine. Males in sea water have a higher rate of oxygen uptake than in concentrated brine. This difference has been related to the greater area of the second antennae of males in sea water.

The colours of Artemia are due mainly to haem compounds and carotenoids. Haemoglobin occurs in solution in the blood; the role of this pigment in the life of Artemia has been investigated. The

colours of the eggs are due to differences in total haem content of the egg shell. The red colour of the nauplius and of certain adults is due to carotenoids; some of these have been identified.

CONTENTS

THE METABOLISM OF *ARTEMIA SALINA* (L.)

INTRODUCTION

The brine shrimp, *Artemia salina* (L.), was first described just over two hundred years ago. In 1756, while visiting salt works at Lymington on the south coast of England, Dr. Schlosser was amazed to see in a 'pan' containing very concentrated brine, "des millions d'insectes les plus agiles du monde."

These brine shrimps, classified today as Crustacea : Anostraca, live under extreme environmental conditions; they are found only in highly saline lakes and pools in many parts of the world. In the laboratory, *Artemia* will flourish in sea water, and yet it is never found in the sea itself nor even in moderately saline waters. The reasons for its absence from such habitats would appear to be ecological rather than physiological; *Artemia* cannot survive in the presence of predators. It only begins to flourish when concentrations of brine are reached which few other animals can tolerate.

The saline waters in which brine shrimps are found differ widely both in composition and concentration. Such an environment must greatly influence the mode of life of *Artemia*. In this thesis, I have attempted to find to what extent the metabolism of *Artemia* is influenced by factors in the external environment. These extrinsic factors may be both physico-chemical and biotic; in particular, the influence of the salinity of the medium and its dissolved oxygen

content on the metabolism of Artemia has been investigated. Before drawing conclusions from such investigations, it is essential to know to what extent the metabolism is influenced by intrinsic factors. These include not only the size and sex of the animal, but its genetical constitution. This latter factor is particularly important in studies on Artemia. Although it is generally agreed that there is only one species in the genus Artemia, individuals from populations of brine shrimps from different localities have widely different chromosome numbers ranging from diploid to octoploid, polyploid and polysomic individuals.

I use the term metabolism in a wide sense, as being a measure of the active processes in operation within an organism. One of the most obvious results of such active processes is the phenomenon of growth. Thus, the influence of both intrinsic and extrinsic factors on the growth of Artemia has been investigated. Since form is a function of growth, this aspect of the metabolism of Artemia has also been studied.

In its narrowest sense, metabolic rate means only the rate of oxygen consumption; this gives a measure of the energy cost of the active processes going on within the animal. The oxygen consumption of Artemia has been measured in relation to the size and sex of the animal, and also to certain environmental factors such as the salinity of the medium and its dissolved oxygen content.

Not all aspects of the metabolism of Artemia are discussed.

Information on some topics has been acquired solely from the literature and not as the result of personal observation. To avoid confusion as to information which has been derived from the literature and that derived from personal observation and experiment, a list of the main advances resulting from my own work on the metabolism of Artemia is included in this thesis.

ACKNOWLEDGEMENTS

It is a pleasure to record my appreciation of the encouragement and advice I have received from Professor H. Munro Fox, F.R.S. His interest, enthusiasm and helpful criticism have been the main factors promoting my own interest in the field of experimental ecology.

To my colleague, Dr. J. Green, I am grateful for much helpful discussion and advice generously given on many occasions.

In connection with experiments on oxygen consumption, I have benefited from the advice of Professor Kaj Berg, Copenhagen, and on the statistical treatment of the results I have been advised by Miss M.A. Creasy. It is a pleasure to thank them both for their assistance.

I should also like to thank Professor G. Petit, Directeur du Laboratoire Arago, Banyuls-sur-mer, and Professor P. Mathias, Directeur de la Station Zoologique de Sète, for hospitality and assistance.

Finally, I wish to record my appreciation of grants received from the Central Research Fund of the University of London which made possible the field work in the south of France.

MATERIAL AND METHODS

Eggs of Artemia salina were obtained from salt works in Europe, North Africa and America. From these, cultures were maintained of animals from the following five sources:-

San Diego, California

Great Salt Lake, Utah

La Palme, south-west France

Arzeu, Algeria

Cagliari, Sardinia

In agreement with Linder (1941) and in the absence of conclusive evidence to the contrary, only a single species of Artemia, namely A. salina (L.), is recognised in this thesis. The animals from the localities listed above will, therefore, be referred to in the text as follows:-

Californian stock (diploid, bisexual)

Utah stock (diploid, bisexual)

La Palme stock (diploid, parthenogenetic)

Algerian stock (diploid, bisexual)

Cagliari stock (diploid, bisexual)

Details of experimental methods will be described in the relevant part of the text, and only general methods are described here.

CULTURE METHODS

Stock cultures of Artemia were maintained in various concentrations of brine in a constant-temperature room at 25°C. The diatom Phaeodactylum tricornutum Bohlin, formerly Nitzschia closterium f. minutissima Allen & Nelson (Hendey, 1954), and a marine species of Chlamydomonas were given as food. These were grown in 'Miquel-Allen solution', which is a medium of sea water further enriched with inorganic salts (Allen & Nelson, 1910). The cultures were kept in a north window, except for a few months in winter when they were kept in front of a mercury vapour strip lamp. When required, the food was separated from the culture medium by centrifugation, and re-suspended in brine of the same concentration as that in which Artemia was reared.

In experiments involving controlled feeding, the density of a suspension of Chlamydomonas in the experimental brine was measured with a Medical Research Council grey-wedge photometer (King et al. 1948), using a red filter. At the start of the experiments the Chlamydomonas suspension was adjusted to an optical density* of about 0.15. More Chlamydomonas was added daily to restore the optical density to 0.15. This was done by centrifuging the algal culture, thus separating the Chlamydomonas from the medium, and adding the required amount of algae to the experimental dishes with a paint brush.

* The optical density is the logarithm of the ratio of the intensity of incident light to that of light transmitted through 1 cm. of a solution or suspension.

In all controlled feeding experiments the concentration of Artemia was adjusted to the size of the individuals. Three concentrations were employed in relation to three approximate size groups as shown below:-

Artemia per ml. brine	Length, mm.
1 in 10	hatching to 3.0
1 in 50	3.0 to 6.0
1 in 100	over 6.0

Various concentrations of brine were made by dissolving commercial sea salt in sea water and filtering the solution. The salinity of the media was determined by titration with a standard solution of silver nitrate (Harvey, 1955). Throughout this thesis the symbol S‰ is used to denote the salinity of a medium in grams of salt per litre of water, and the word 'brine' is used in its widest sense to mean a 'salt water'.

In all experiments on growth and form, standard culture methods were used starting with newly liberated nauplii. Under the culture conditions already described for stock animals, females of Artemia are viviparous, and actively swimming nauplii are liberated from the brood pouch of the parent. These nauplii were collected from females reared in the experimental brines; they were then transferred to dishes containing a volume of experimental brine appropriate to the number of nauplii available, to give a concentration of one nauplius

per 10 ml. of brine. With increasing size of the animals, the concentration of brine shrimps was adjusted and controlled feeding was given throughout the experiments as described above.

A number of animals was withdrawn from the experimental dishes at frequent intervals during the experiments. The individuals were lightly narcotised, measured and, on complete recovery from narcosis, were returned to their respective experimental dishes. Numbers of such experiments were made, all under standard conditions, until sufficient data were obtained.

LINEAR MEASUREMENTS

All measurements were made on animals lightly narcotised with chloroform. It was found that one or two drops of chloroform in a watch glass containing brine was the most satisfactory narcotic. In this, animals remain motionless for a few minutes, but the heart continues to beat, the blood circulates and recovery is complete within ten minutes.

Narcotised animals were placed ventral side down on a microscope slide and supported in a drop of the experimental medium. In males, the large second antennae were bent forward to extend in front of the head and in females with a well developed brood pouch, the measurements were made with the animal lying in a cavity slide. These precautions insured that the animals lay horizontally on the slide. Total length, from the anterior margin of the head in front of the ocellus to the base of the caudal furcae, was measured with

a Vernier scale on the mechanical stage of the microscope. Similarly, the length of the abdomen was measured from, and including, the first genital segment to the base of the caudal furcae. With a calibrated eye-piece micrometer, the abdomen width was measured at the level of the posterior termination of the heart. The eye-piece micrometer was also used to measure the length of the caudal furcae from base to tip.

GROWTH AND FORM

"The form of an object is defined when we know its magnitude, actual or relative, in various directions; and Growth involves the same concepts of magnitude and direction, related to the further concept of Time." (Thompson 1942).

The phenomena of growth and form are intimately related to one another; the form of an organism is largely determined by its rate of growth in various directions. Thus, form is a function of growth and the study of the rate of growth of an organism is a necessary preliminary to the study of form.

The young of Artemia hatch from eggs as nauplius larvae, and gradually grow by the addition of somites and the development of the characteristic features of the adult. There is no metamorphosis in the life history of Artemia. Increase in size takes place after moulting and so the process of growth occurs in a series of instars. The general features of growth and adult form are described first, while details of hatching and pre-adult instars will be discussed in a subsequent section of this thesis.

Since Artemia is found in nature in saline waters of widely different salt concentrations, dissolved oxygen content and temperature, reference to a 'normal' environment has no meaning in studies on Artemia. It is not possible, therefore, to investigate the factors which influence the 'normal' growth and form of the animal, but the ways in which growth and form vary under different conditions can be investigated.

GROWTH

Previous work

Few studies have been made on the rate of development of *Artemia*. It is stated by a number of authors (Martin & Wilbur, 1921; Heath, 1924; Bond, 1933; Warren, 1938) that the adult size of *Artemia* varies inversely with the salinity of the medium, and, in laboratory bred animals, that sexual maturity is attained in three to four weeks. According to Jensen (1918), *Artemia* from the Great Salt Lake, reared in the laboratory at room temperature in brine of unspecified salinity, is sexually mature within 18 to 21 days after hatching. Barigozzi (1939) measured the average time of development, from hatching to sexual maturity, in three populations of *Artemia* cultured in the laboratory at 18 to 20°C in an artificial sea water. No difference in time of development in the different populations was observed; the animals of the bisexual race from America and the parthenogenetic females from the south of France and north Italy all reaching sexual maturity in 22 to 28 days.

In these experiments no actual measurements were made and neither temperature nor the amount of food given was controlled. Barigozzi (1939) himself suggests that the variations in time taken to reach maturity may be due to variations in feeding, although it was supposed that excess food was available all the time.

More precise experiments on growth rate have been made by Weisz (1946), who was primarily interested in the problem of

segment formation in relation to size and shape of Artemia. Nauplii were obtained from "commercial air-dried cysts" and the rate of development, from the time of hatching to sexual maturity, was measured in brines of different salt concentration. The experiments were made at room temperature, corresponding to an average water temperature of 21 to 22°C. According to Weisz, a sigmoid curve of growth is obtained, irrespective of the salinity of the medium (figure 1). It should be noted, however, that the units of length are on the abscissa and those of time on the ordinate. This does not result in a sigmoid curve of growth as generally accepted. If figure 1 is redrawn in the form of a normal growth curve with units of time on the abscissa and length on the ordinate, the curves are no longer sigmoid (figure 2).

Weisz (1946) distinguishes three phases of growth in Artemia. A thoracic phase, from the time of hatching to the appearance of the eleventh thoracic segment, and an abdominal phase from the appearance of the twelfth to nineteenth segments. After this stage, a variable number of non-segmental stages follows before sexual maturity is reached. It can be seen from figure 1, that the time taken to reach sexual maturity is inversely related to the salinity of the medium, but the total length of individuals of Artemia at equivalent stages of development is identical. Thus, the thoracic phase of growth is complete when Artemia has a total length of 1.75 mm., irrespective of the salinity of the medium. According to Weisz (1946),

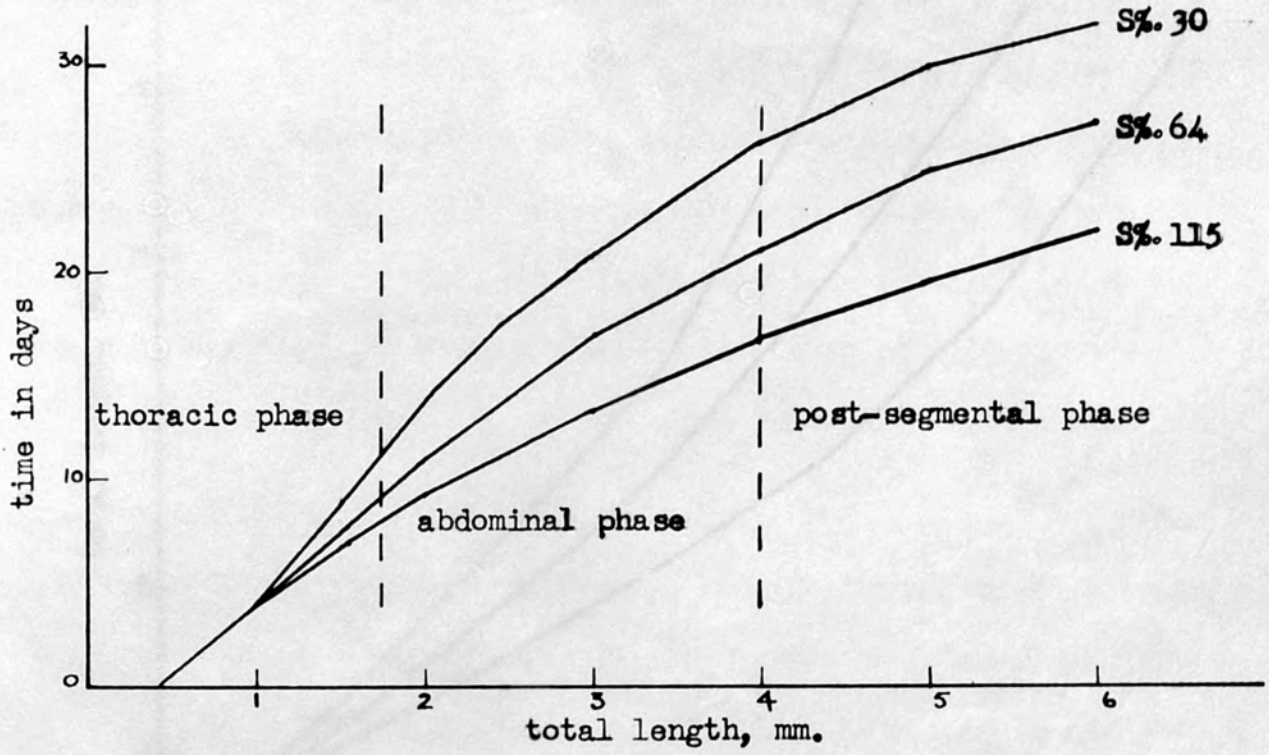


Figure 1. Rate of growth of Artemia, from hatching to sexual maturity, in different concentrations of brine at 21-22°C. (after Weisz, 1946)

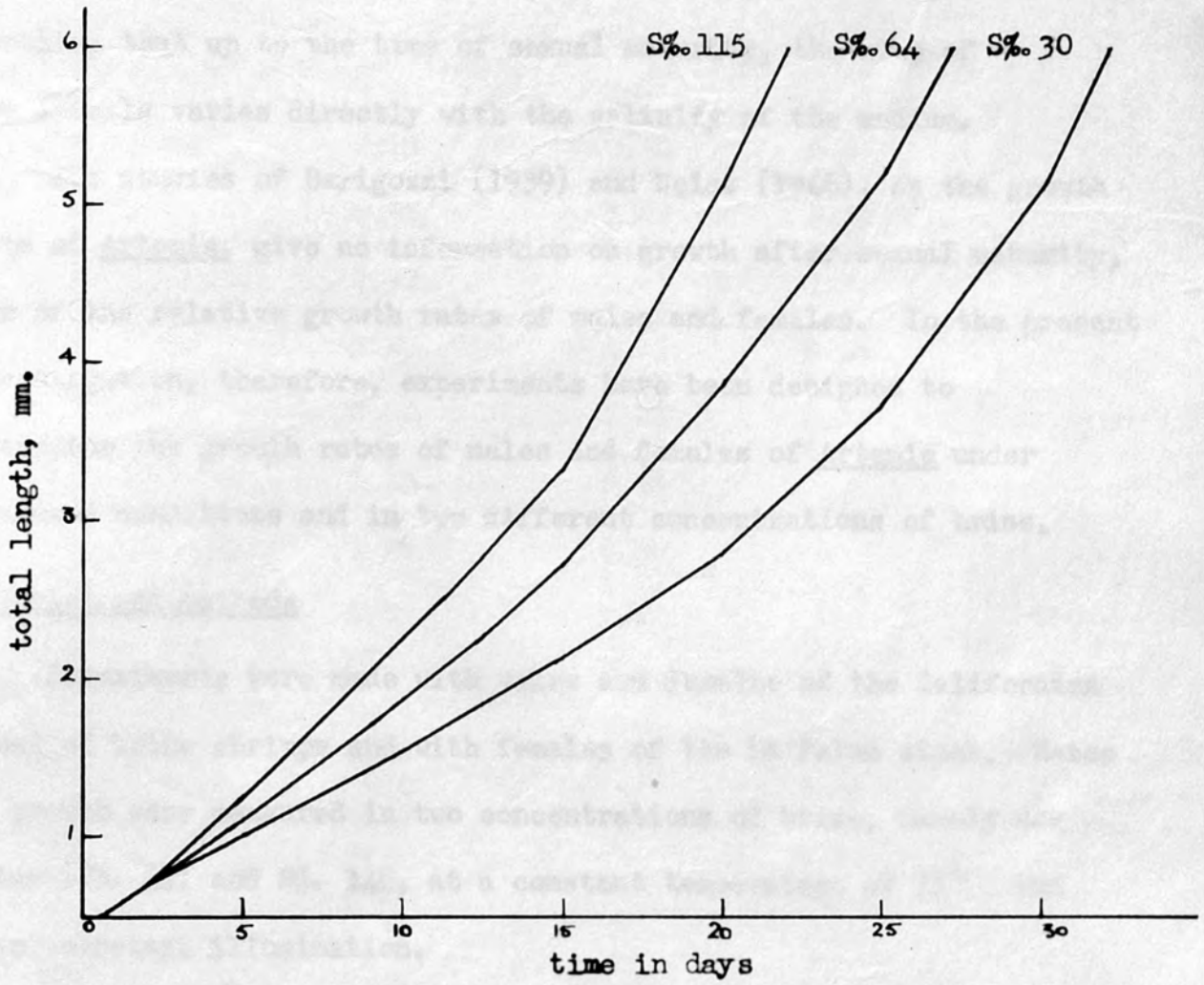


Figure 2. Growth of *Artemia*, from hatching to sexual maturity, in different concentrations of brine, at 21 to 22°C. (redrawn from Weisz, 1946)

therefore, the rate of development of Artemia increases with increasing concentration of brine, sexual maturity being reached in about 32 days in dilute brine (S‰ 30) and in about 22 days in concentrated brine (S‰ 115). It follows then, from Weisz's results, that up to the time of sexual maturity, the size of the animals varies directly with the salinity of the medium.

The studies of Barigozzi (1939) and Weisz (1946), on the growth rate of Artemia, give no information on growth after sexual maturity, nor of the relative growth rates of males and females. In the present investigation, therefore, experiments have been designed to determine the growth rates of males and females of Artemia under standard conditions and in two different concentrations of brine.

Material and methods

Experiments were made with males and females of the Californian stock of brine shrimps and with females of the La Palme stock. Rates of growth were measured in two concentrations of brine, namely sea water (S‰ 35) and S‰ 140, at a constant temperature of 25°C. and under constant illumination.

The animals were grown under standard culture conditions already described (pages 6 - 8), and measurements were made at frequent intervals. The average length of Artemia at any one time was based on measurements made on thirty individuals.

Results

The results of these experiments are shown in tables 1 and 2,

TABLE 1

Growth of females of Artemia salina in two concentrations of brine at 25°C. (Californian stock)
Each value represents the mean length of 30 individuals; standard errors have been inserted for the comparison of certain means.

Time in days	Total length in millimetres			
	Californian stock		La Palme stock	
	S‰ 35	S‰ 140	S‰ 35	S‰ 140
1	0.65	0.53	0.53	0.58
2	0.86	-	0.77	0.84
3	-	0.89	1.06	1.08
4	-	-	1.47	1.22
5	1.68	1.42	1.61	1.73
6	2.06	-	2.08	1.96
7	-	2.87	2.32	2.41
8	2.10	-	3.43	2.95
9	-	3.12	3.51	3.89
10	-	-	4.98	4.49
11	3.06	4.18	5.23	-
12	4.36	-	-	5.45
13	-	4.96	5.95	-
14	-	-	6.09	6.40
15	-	5.50	7.25	-
16	6.50	-	-	7.11
17	-	-	7.35	-
18	-	-	-	7.98
19	-	6.50	9.00	-
20	7.24	-	-	8.70
21	-	7.06	9.40	-
22	-	-	-	8.96
23	-	7.25	9.80	-
25	-	7.51	-	-
26	8.50	-	-	-
27	-	7.55	10.50	-
28	-	-	-	10.50
29	-	7.82	10.60	-
30	-	-	-	10.90
33	9.26±0.09	8.23±0.11		
37	-	8.69		
40	9.82	-		
44	-	8.76		
47	10.50	-		
51	-	8.99		
55	10.70	-		
61	11.20	-		

These results are represented graphically in figures 3 to 9.

TABLE 2

Growth of males of Artemia salina in two concentrations of brine at 25°C. (Californian stock)
Each value represents the mean length of 30 individuals; standard errors have been inserted for the comparison of certain means.

Time in days	Total length, mm. S‰ 35	S‰ 140
1	0.65	0.53
2	0.86	-
3	-	0.89
5	1.68	1.42
6	2.06	-
7	-	2.87
8	2.10	-
9	-	3.12
11	3.06	4.18
12	4.10	-
13	-	4.96
15	-	5.01
16	5.83	-
18	5.89	-
19	-	6.06
20	6.35	-
21	-	6.50
25	-	6.86
26	7.35	-
27	-	6.99
29	-	7.02
33	7.80 ± 0.12	7.41 ± 0.11
37	-	7.61
40	8.26	-
44	-	7.94
47	8.42	
55	8.84	
61	9.00	

These results are represented graphically in figures 3 to 9.

and in figures 3 to 9. It can be seen that the extent to which the growth rate of Artemia is influenced by the salinity of the medium varies not only with the stock of animals but also the sex. One feature is constant however, irrespective of the concentration of brine or race of animals: eggs first appear in the median brood pouch of the females when the latter are 18 to 21 days old, sexual maturity having been attained 14 to 17 days after hatching.

In figures 3 and 4, the growth of females and males of the Californian stock is compared in two concentrations of brine. In both concentrations the growth rate of sexually mature females is greater than that of males, the difference being more pronounced in sea water. This confirms an observation made by Artom (1907 a), that in equal concentrations of brine, males have a shorter total body length than females. When the growth of females of this stock is compared in the two media (figure 5), it is found that the rate of growth is greater in the more dilute medium. In males, the difference in growth rate in the two media is less obvious (figure 6), but if the mean lengths of animals 33 days old are compared statistically, it is found that males in sea water are significantly longer than males in the more concentrated brine. With 58 degrees of freedom and 't' = 2.36, the probability that the difference is due to chance is less than 5%. Thus, in both males and females of the Californian stock, the size of sexually mature individuals is inversely related to the salinity of the medium.

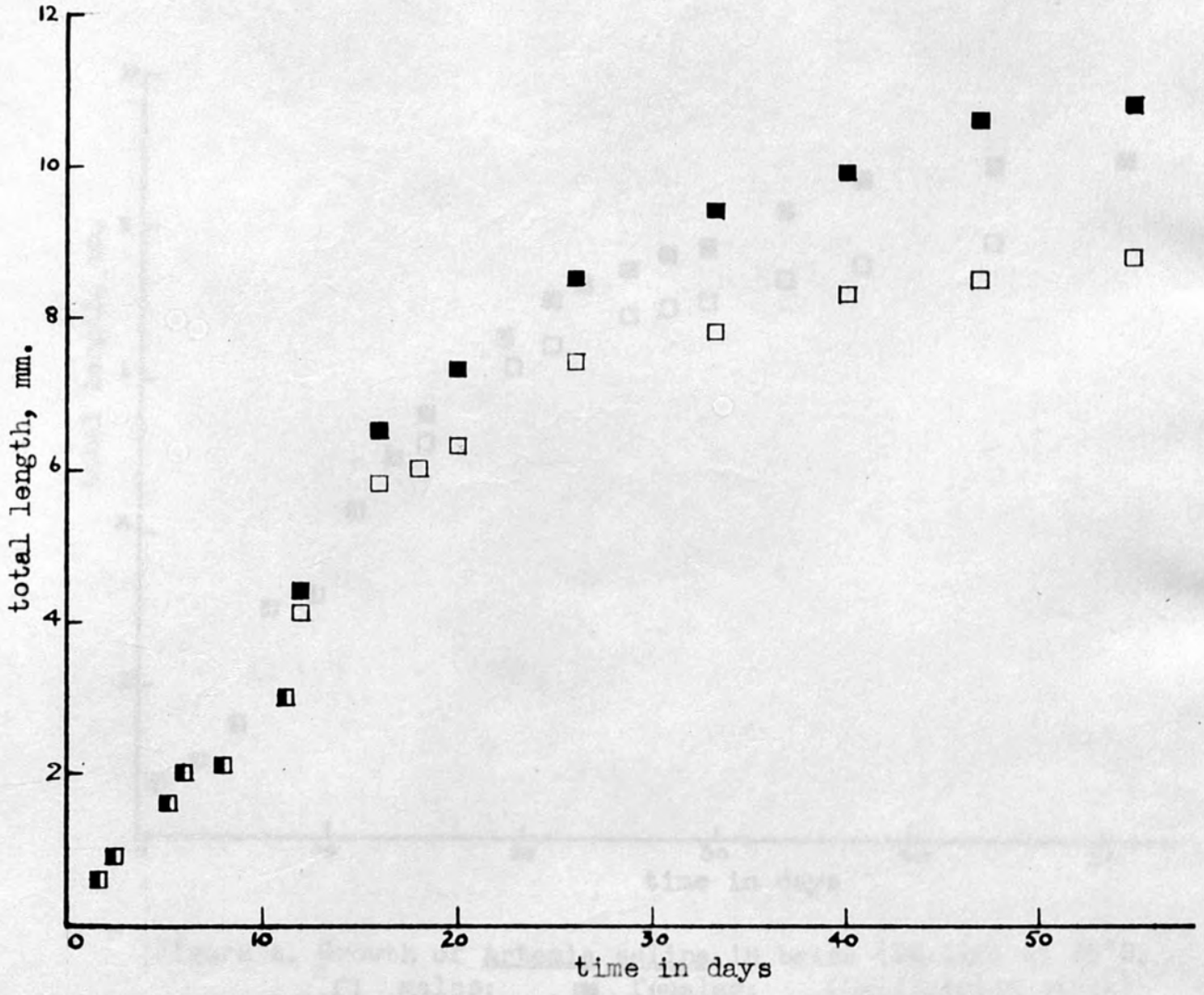


Figure 3. Growth of Artemia salina in sea water (S‰ 35) at 25°C.
□ males; ■ females; (Californian stock)

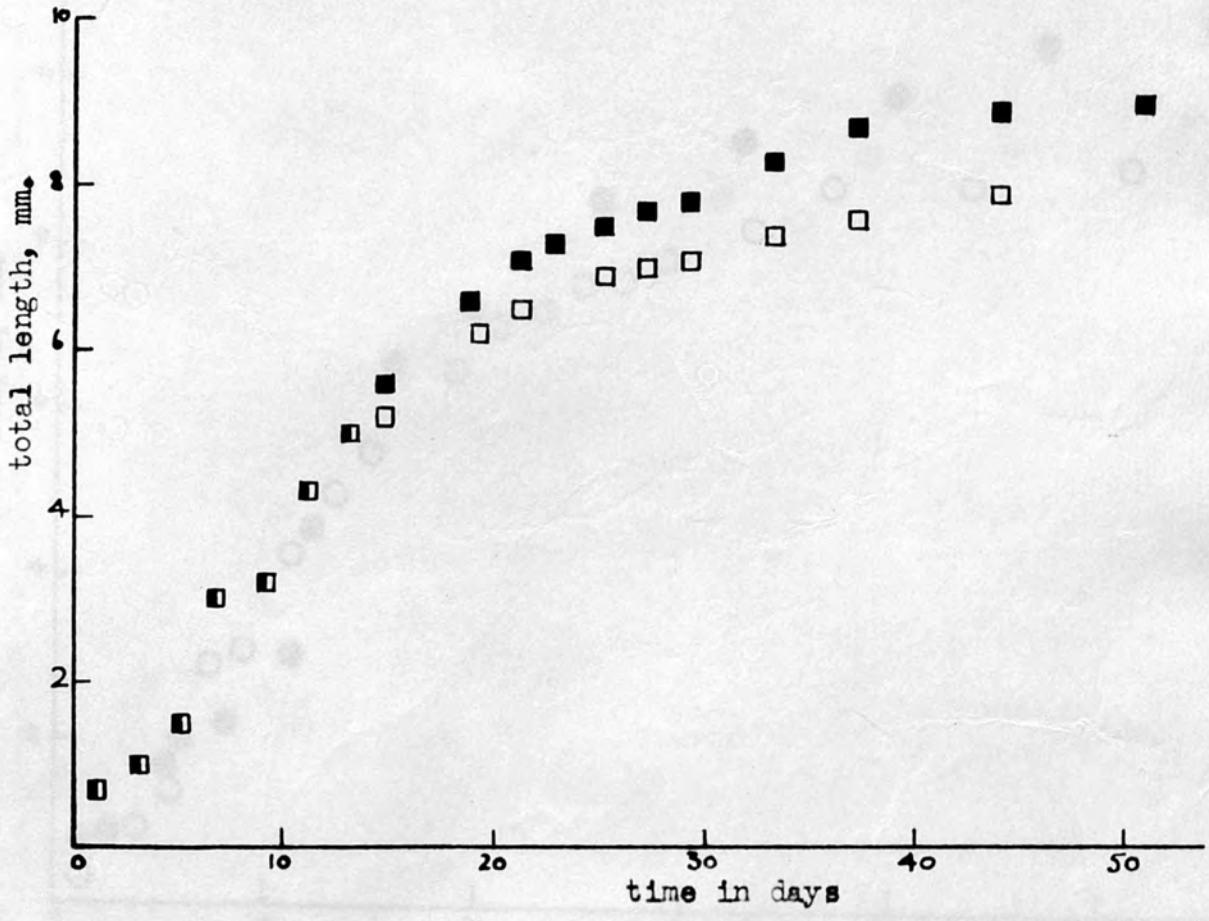


Figure 4. Growth of Artemia salina in brine (S‰.140) at 25°C.
□ males; ■ females; (Californian stock)

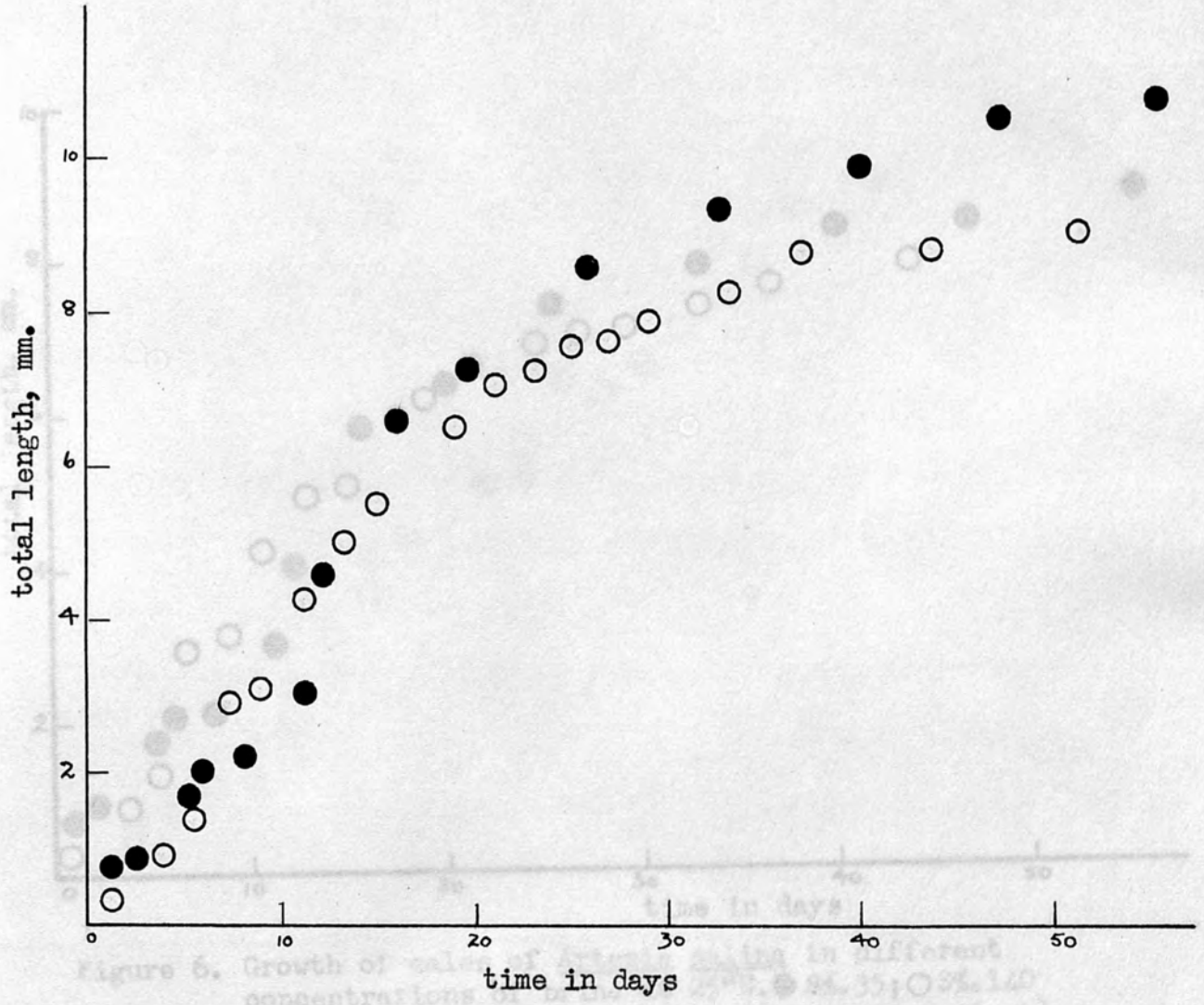


Figure 5. Growth of females of Artemia salina in different concentrations of brine at 25°C. ● S‰ 35; ○ S‰ 140. (Californian stock)

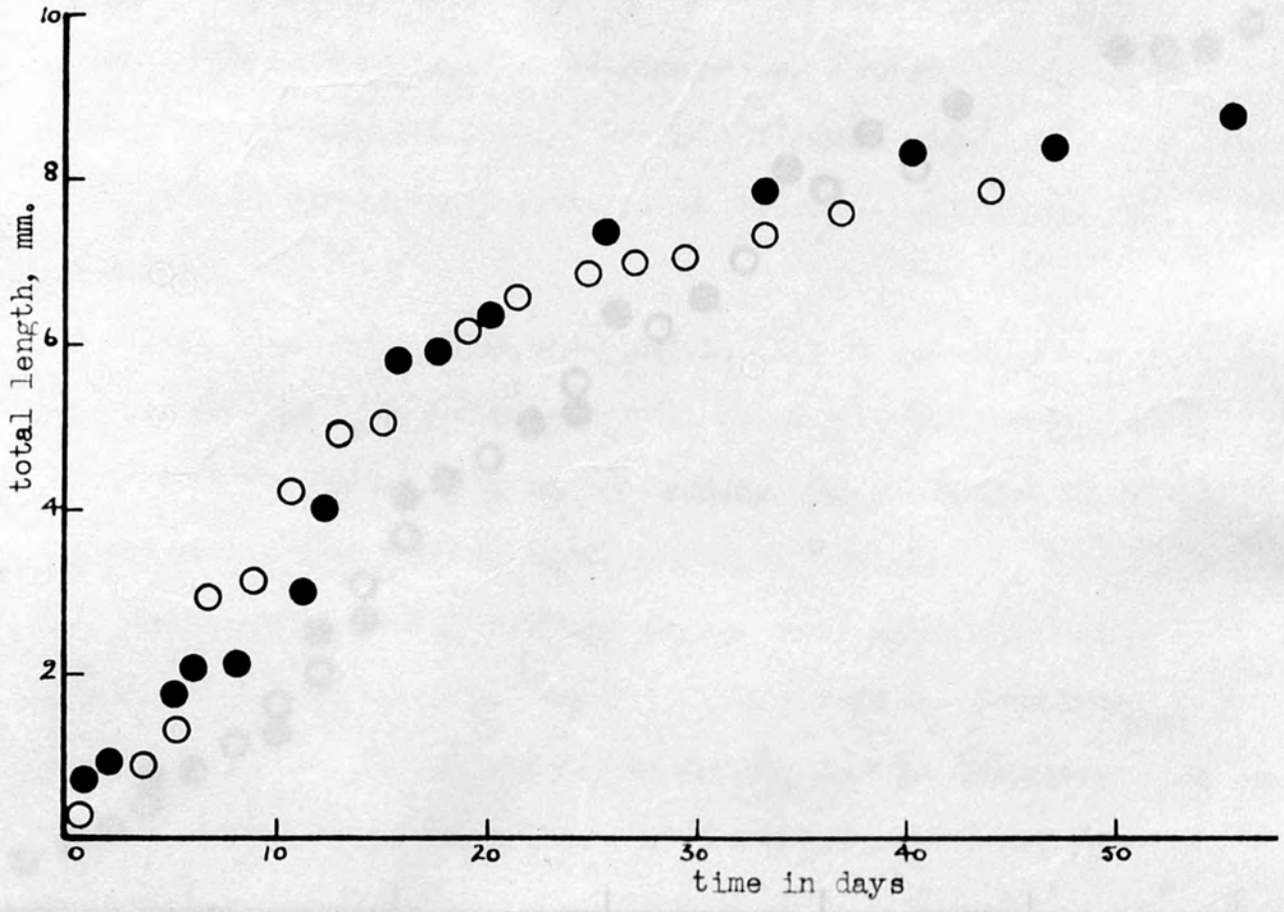


Figure 6. Growth of males of Artemia salina in different concentrations of brine at 25°C. ● S‰ 35; ○ S‰ 140 (Californian stock)

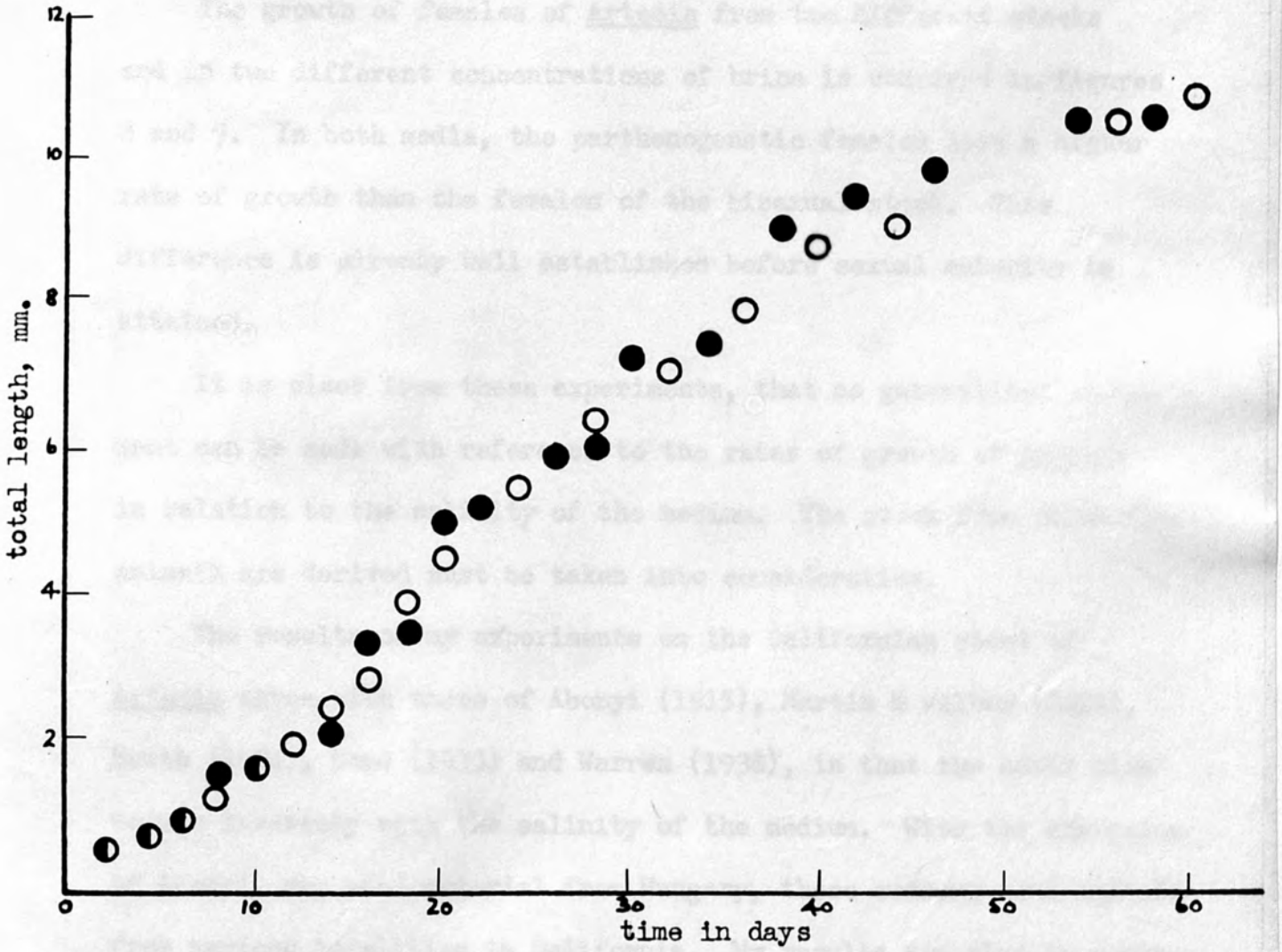


Figure 7. Growth of parthenogenetic females of Artemia salina in different concentrations of brine at 25°C. ● S‰ 35; ○ S‰ 140;
(La Palme stock)

In figure 7, the growth of parthenogenetic females of Artemia from La Palme is compared in two different concentrations of brine. No difference in rate of growth in the two media was found.

The growth of females of Artemia from two different stocks and in two different concentrations of brine is compared in figures 8 and 9. In both media, the parthenogenetic females have a higher rate of growth than the females of the bisexual stock. This difference is already well established before sexual maturity is attained.

It is clear from these experiments, that no generalised statement can be made with reference to the rates of growth of Artemia in relation to the salinity of the medium. The stock from which the animals are derived must be taken into consideration.

The results of my experiments on the Californian stock of Artemia agree with those of Abonyi (1915), Martin & Wilbur (1921), Heath (1924), Bond (1933) and Warren (1938), in that the adult size varies inversely with the salinity of the medium. With the exception of Abonyi, who used material from Hungary, these authors used animals from various localities in California. My results are also in agreement with those of Barigozzi (1939), in that there is no difference in the time taken to reach sexual maturity between females of the bisexual Californian stock and parthenogenetic females from southern France. On the other hand, the parthenogenetic females have a more rapid rate of growth and are thus larger in size when sexual maturity is attained.

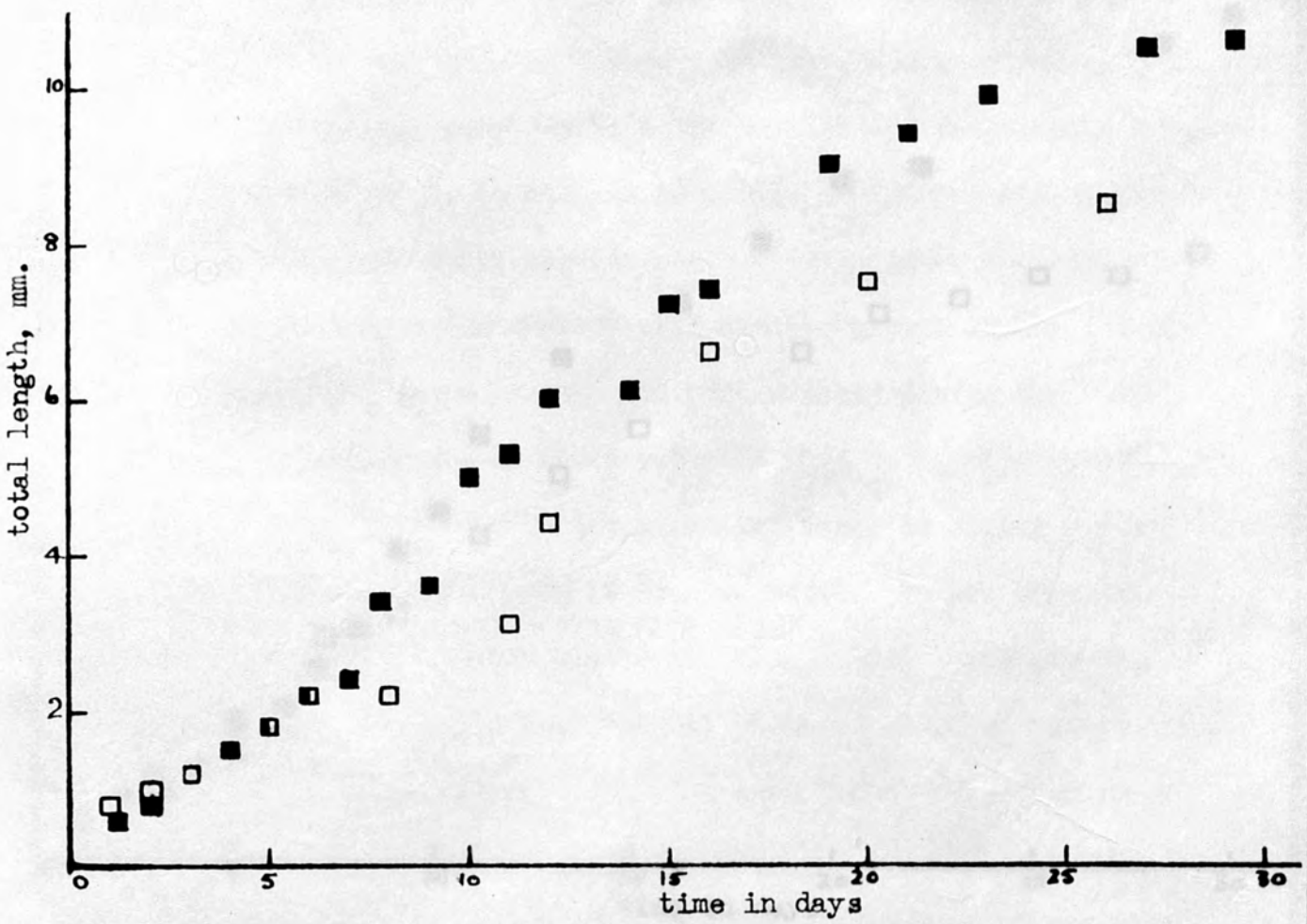


Figure 8. Growth of females of Artemia salina, from different localities, in sea water (S‰.35) at 25°C. □ California; ■ La Palme.

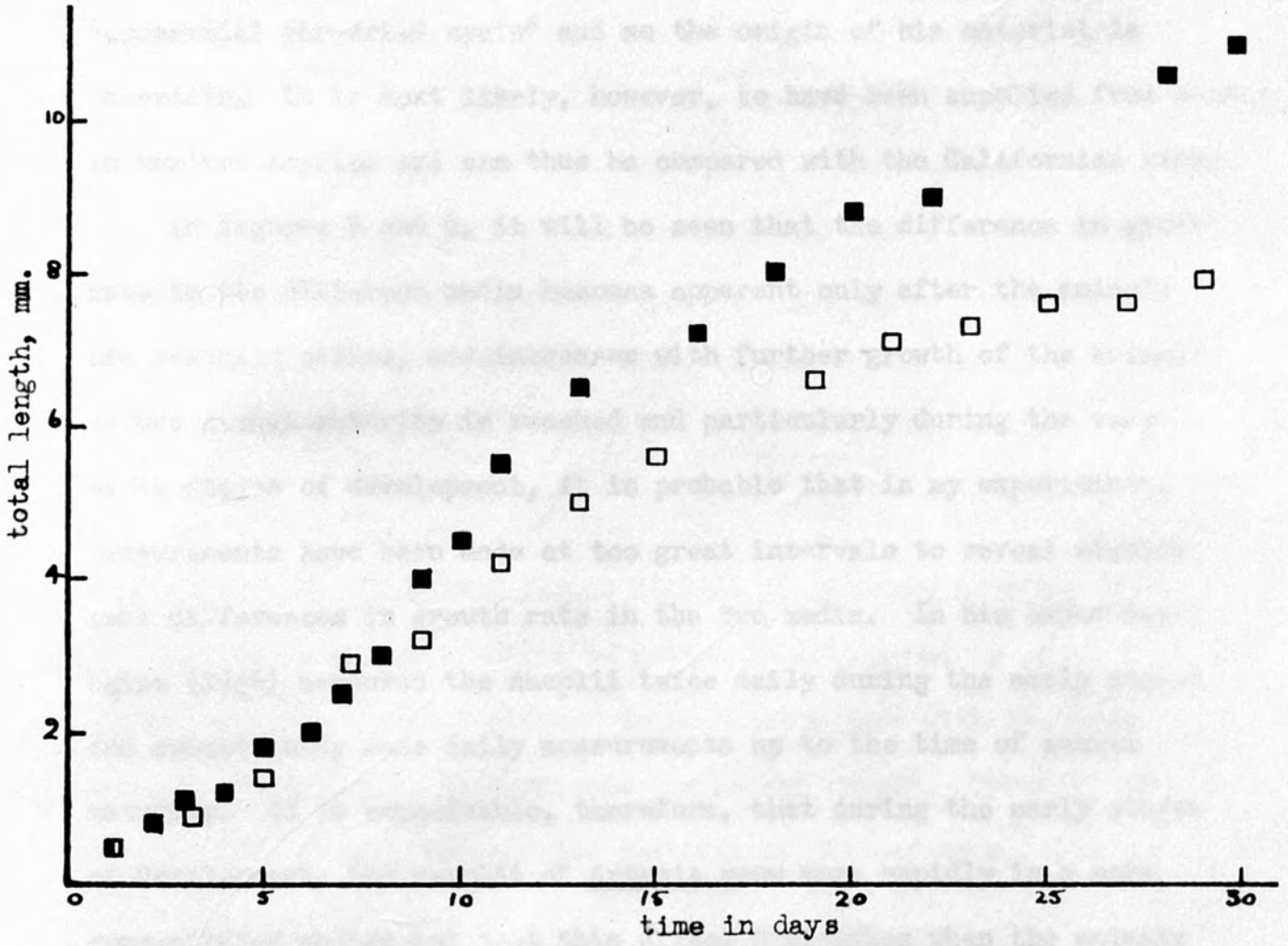


Figure 9. Growth of females of Artemia salina, from different localities, in brine (S‰.140) at 25°C. □ California; ■ La Palme.

The conclusions of Weisz (1946), that up to the time of sexual maturity the growth rate of the animals varies directly with the salinity of the medium, seem to contradict the findings of other workers. His experiments were made with nauplii obtained from "commercial air-dried cysts" and so the origin of his material is uncertain. It is most likely, however, to have been supplied from sources in western America and can thus be compared with the Californian stock.

In figures 5 and 6, it will be seen that the difference in growth rate in the different media becomes apparent only after the animals are sexually mature, and increases with further growth of the animals. Before sexual maturity is reached and particularly during the very early stages of development, it is probable that in my experiments, measurements have been made at too great intervals to reveal significant differences in growth rate in the two media. In his experiments, Weisz (1946) measured the nauplii twice daily during the early stages and subsequently made daily measurements up to the time of sexual maturity. It is conceivable, therefore, that during the early stages of development, the nauplii of Artemia grow more rapidly in a more concentrated medium but that this effect diminishes when the animals become sexually mature and with further growth an inverse relationship between size and salinity is established. It should also be noted in the experiments of Weisz, that the time taken by Artemia to reach sexual maturity was about 32 days in the least concentrated medium (S‰ 30) at a temperature of 21 to 22°C. In my own experiments, females

become sexually mature in sea water (S‰ 35) at 14 to 17 days after hatching when reared at a temperature of 25°C. This suggests that the sea-water-yeast suspension, on which Weisz fed his experimental animals, is not as satisfactory food for Artemia as the green alga Chlamydomonas. Further, the results of Weisz's experiments are difficult to reconcile with those of Eliassen (1952), who found that the oxygen consumption of nauplii and immature stages of Artemia was higher in less concentrated media. Thus, according to Eliassen, the metabolism of the nauplii of Artemia is inversely related to the salinity of the medium, but according to Weisz (1946), the growth rate of nauplii is directly related to the salinity of the medium.

Influence of the dissolved oxygen content of brine on growth.

The brine shrimp is found in nature in saline waters which differ widely in concentration. Other factors vary with the salt content of the water, in particular dissolved oxygen. There is an inverse relationship between the salinity of the medium and its dissolved oxygen content.

Experiments were made to determine the effect of dissolved oxygen on the growth of Artemia. Animals of the Californian stock were reared in sea water at 25°C. under standard conditions and, when 16 days old, equal numbers of males and females were put into each experimental vessel. These were 450 ml. conical flasks, containing 440 ml. of sea water and forty-four individuals of Artemia. In order

to maintain a low dissolved oxygen content of the sea water, the initial oxygen pressure was reduced by bubbling nitrogen through the water in the experimental flasks. Control experiments were set up with shallow open dishes, covered with glass plates to prevent evaporation, containing aerated sea water and animals in the same proportion as in the conical flasks. The experiments were made in the dark at 25°C. and lasted for 3 weeks. During this period large numbers of nauplii were produced. These were removed every other day and the adults put back into fresh sea water of the same dissolved oxygen content as before. The dissolved oxygen content of the sea water was measured every other day by the syringe-pipette modification of the Winkler method (Fox & Wingfield, 1938). During the experiments, Artemia was fed on the diatom Phaeodactylum tricornutum. An equal volume of a thick suspension was added daily to each vessel.

The results of these experiments are summarised in table 3. The mean length is based on measurements made on ten individuals. It is clear that the growth rate of females is retarded by the low oxygen content of the sea water; the growth rate of males, however, is not significantly slower in oxygen-deficient water. These results also confirm the conclusions from earlier experiments, that females in air-saturated sea water have a more rapid rate of growth than males in the same medium.

Among other phyllopod Crustacea, the effect of oxygen on growth has only been investigated in Daphnia magna Straus; lack of oxygen retards the growth of parthenogenetic females (Green, 1956 a).

Table 3. Influence of dissolved oxygen on the growth of Artemia

	% air saturation of sea water					
	87 - 92			15 - 20		
	mean total length (mm. with S.E.)					
	initial	final	% increase	initial	final	% increase
males	5.7	8.0±0.17	40.4	5.7	7.7±0.21	35.1
females	6.3	9.3±0.13	47.6	6.3	8.1±0.14	28.6
males	5.7	8.1±0.10	42.1	5.7	7.9±0.17	38.6
females	6.3	9.3±0.15	47.6	6.3	8.3±0.15	31.8

It has been shown in figures 5 and 6 that the adult size of Artemia, from the Californian stock, varies inversely with the salinity of the medium. The dissolved oxygen content of brine also varies inversely with salt concentration. It may be, therefore, that the the smaller size of adults in the more concentrated brine is in part, if not entirely, an effect of a low dissolved oxygen content of the medium. Whether due directly to the salt concentration or the dissolved oxygen content, the effects are more marked in females than in males.

ADULT FORM IN RELATION TO SIZE AND THE SALINITY OF THE MEDIUM

In the majority of Crustacea, growth only occurs after moulting. At this time the integument is soft and, as the result of the osmotic intake of water, the body swells and increases in size. Among branchiopod crustaceans, the increase in size is rapid. In Daphnia magna the process is completed in less than a minute (Edlén, 1937), and a female of Daphnia obtusa Kurz can increase in length from 1.3 to 1.6 millimetres in about ten seconds (Green, 1956a).

If growth in the Crustacea is dependent on the uptake of water, the osmotic gradient between the blood and the external medium is likely to be an important factor in this process and thus, growth may be regarded as a function of the external medium. Further, if one accepts the thesis of D'Arcy Thompson (1942) that form is a function of growth, the relationship between adult form and the external medium forms a logical sequence to studies of growth.

It is suggested by Höber (1899) and by Loeb (1905) that the modifications of form undergone by some phyllopod Crustacea, subsequent upon changes in the salinity of the medium in which they live, can be referred to the dependence of growth on the uptake of water. Among these phyllopods, Artemia is most famous. The variations in form of brine shrimps, in relation to the salinity of the environment, have been the subject of many investigations and constitute an aspect of the biology of Artemia most extensively

studied. In spite of this, the picture is confused and in the literature many contradictory results are found. The main contributions to the subject are now reviewed.

Previous work

About the middle of the nineteenth century, several new species of Artemia were described from different parts of the world. An important morphological character used in the determination of species was the size of the caudal furcae and the number of setae on them. With the publication of the results of his observations on the variability of Artemia, Schmankewitsch (1875) excited much interest and threw an entirely new light upon the systematics of brine shrimps. His conclusions are widely reported in the literature, but they are sometimes exaggerated and misinterpreted. In view of this, they will be described here in some detail.

Schmankewitsch (1875) published an account of observations he made on brine shrimps occurring in a salt lagoon on the edge of the Black Sea near Odessa. The lagoon was divided by a dam into upper and lower parts. The salt water in the upper part was less concentrated than in the lower part, in which the water was saturated with salt. In 1871, during a spring flood, the wall separating the two parts of the lagoon broke down and water from the upper part overflowed into the lower, thereby reducing the density of the water in this region. Thus, the water in the lower part of the lagoon now

had a density of 8°Baume (about S‰ 75) and at this time large numbers of individuals of Artemia salina appeared. Later in this year, 1871, the wall separating the upper and lower parts of the lagoon was repaired, and gradually, as the result of evaporation over a period of three years, the concentration of salt in the water of the lower lagoon increased until finally, the salt crystallised out. The rate of increase in concentration of the brine in the lower part of the lagoon is indicated below:-

Season		Density °B.	Approx. S‰
spring	1871	8.0	75
summer	1872	14.0	140
	1873	18.0	190
August	1874	23.5	240
September	1874	25.0	270

Concurrent with these changes in the salinity of the water, Schmankewitsch (1875) observed certain progressive changes in the form of the brine shrimps. At the beginning of his period of observation, in 1871, Schmankewitsch noted that the caudal furcae were well developed, each bearing from eight to twelve setae. With the increase in the salinity of the medium, however, and in the course of several generations of brine shrimps, the caudal furcae became reduced and bore fewer setae, until finally, all trace of these furcae was lost. These changes are illustrated in figure 10. Thus,

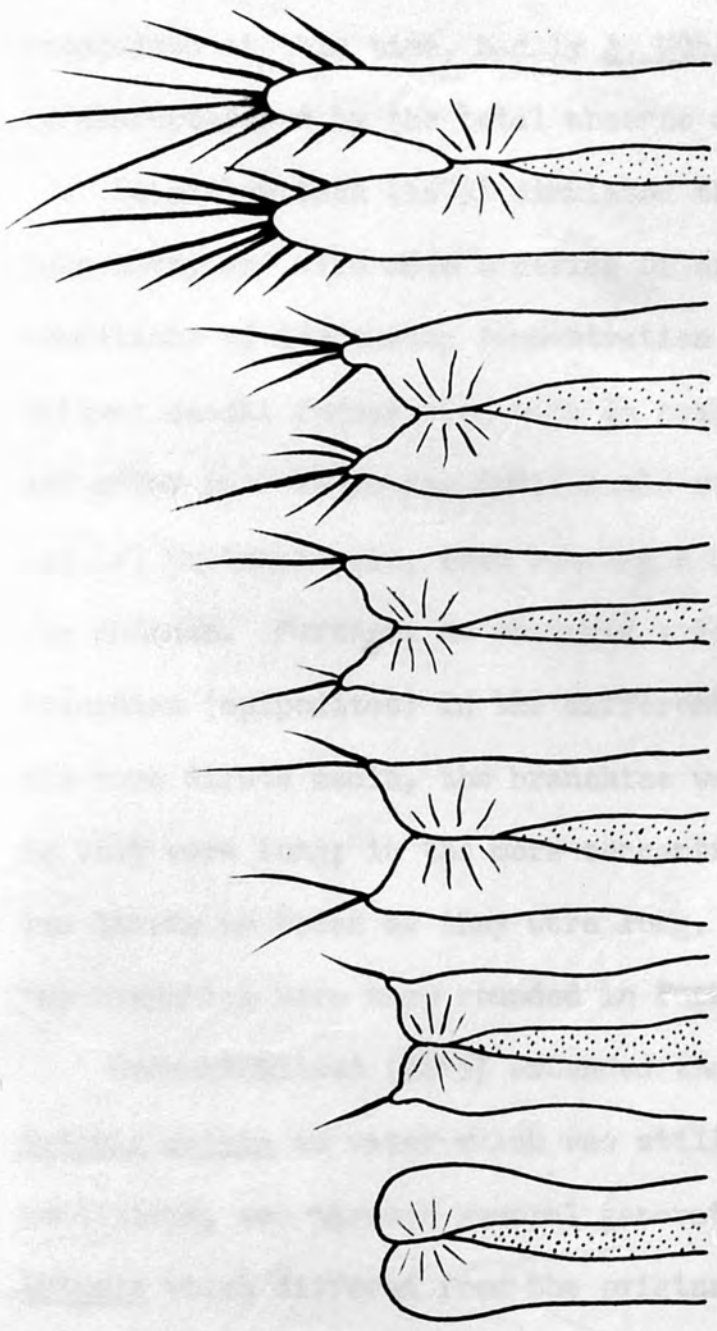


Figure 10. Post-abdomen of Artemia in relation to different concentrations of brine. (After Schmankewitsch, 1875)

Schmankewitsch concluded that under certain environmental conditions, Artemia salina comes to resemble another species of Artemia recognised at this time, namely A. Mühlhausenii Miln. Edw.*, which is characterised by the total absence of caudal furcae.

Schmankewitsch (1875) simulated these conditions in the laboratory and also made a series of experiments under reverse conditions of decreasing concentration of the medium. Animals without caudal furcae were kept in brine which was gradually diluted, and after several weeks, individuals were found in which a pair of conical protuberances, each bearing a seta, appeared at the end of the abdomen. Further, he observed a change in the shape of the branchiae (epipodites) in the different media. In Artemia salina in the more dilute media, the branchiae were about one half as broad as they were long; in the more concentrated media, they were about two thirds as broad as they were long. Thus, in concentrated brine the branchiae were more rounded in form.

Schmankewitsch (1875) extended these experiments by keeping Artemia salina in water which was still further diluted. Under these conditions, and through several generations, he obtained a form of Artemia which differed from the original in the segmentation of the

*According to Daday de Deés (1910), the brine shrimp found by Schmankewitsch was Artemia Mühlhausenii S. Fischer, and is regarded by Daday de Deés as synonymous with A. salina (L.) var. Köppeniana (S. Fisch.)

abdomen. The abdomen of Artemia salina consists of two genital segments followed by six post-genital segments and the caudal furcae. In the third generation of animals reared in progressively diluted brine, Schmankewitsch obtained forms in which the last abdominal segment appeared to be divided into two segments, so that there were nine abdominal segments. He points out that this is the abdominal segmentation characteristic of the fresh water genus Branchipus (Schaeff), and he goes so far as to suggest that Artemia from very dilute brine gradually comes to resemble a new species of Branchipus, the only character separating the two genera being the division of the last abdominal segment in Branchipus. In this respect, however, Schmankewitsch overlooked a very important point; the sexual characters of the males of Branchipus and Artemia are distinctly different, in particular in the form of the second antennae, and he produces no evidence that these characters are modified by the salinity of the medium.

In a subsequent publication (1877), Schmankewitsch further elaborates his ideas and suggests that, in relation to his own experiments, there are two species of Artemia; a number of 'varieties' and 'forms' of these also occur, which, under the influence of the environment, come to resemble one another very closely. Schmankewitsch summarises the characteristic features of these as follows:-

Artemia salina Milne-Edwards*

- occurs in brine of density 5 to 12°Baume (about S‰ 45 to 125)
- caudal furcae variable in length, each bearing 4 to 12 setae
- ratio of length of thorax to abdomen 5:6 or 5:7

Artemia salina Milne-Edwards variety 'a'

- occurs in brine of density 12 to 20°Baume (about S‰ 125 to 210)
- caudal furcae present, each bearing 8 to 12 setae
- ratio of length of thorax to abdomen 5:8

Artemia salina Milne-Edwards variety 'b'

- occurs in brine of density 4°Baume (about S‰ 35)
- caudal furcae large, each bearing 12 to 22 setae
- abdomen shorter and stouter than in the other forms
- last abdominal segment apparently divided by a transverse ring

Artemia Mühlhausenii S. Fischer, first 'form'

- occurs in brine of density 20 to 23°Baume (about S‰ 210 to 250)
- caudal furcae absent or weakly developed, with 0 to 3 setae
- ratio of length of thorax to abdomen 5:8

Artemia Mühlhausenii S. Fischer, second 'form'

- occurs in brine of density 23 to 24°Baume (about S‰ 250 to 260)
- ratio of length of thorax to abdomen 5:9 or 5:10

Schmankewitsch (1877) stresses the fact that Artemia salina variety 'a', in the upper range of concentration of brine in which it occurs, approaches the form of Artemia Mühlhausenii, and that variety 'b' closely approximates to the genus Branchipus. He did not claim, as is sometimes suggested in the literature, to have created new genera, or to have created one species from another.

* Synonymous with Artemia salina (L.) var. arietina (S. Fisch.) of Daday de Deés (1910)

The validity of Schmankewitsch's observations cannot be doubted and, as will be shown in the following pages, they are confirmed by many later investigators. It is his interpretation of these observations which cannot be accepted. He assumes that different species of Artemia exist, although the evidence for this is most inconclusive. His observations make it clear that nearly all the characters used in the determination of species of Artemia are variable under the influence of the external medium and, therefore, are of no taxonomic value.

In 1894, Bateson criticised the work of Schmankewitsch and contributed some further observations on the subject of variability. He collected material from west and central Asia, from localities differing in chemical composition and salt concentration. Observations were made only on well preserved females of Artemia bearing brood pouches: poorly preserved material was discarded. His main conclusions are:-

1) that specimens from the same locality show great variability in development of caudal furcae and setae,

2) that forms bearing few setae on the caudal furcae are, on the whole, found in waters of a high specific gravity, and

3) that large caudal furcae, on the whole, bear many setae and small caudal furcae bear few setae.

Thus, in general, his conclusions support those of Schmankewitsch (1875), but Bateson points out that the relationship between body

form and the salinity of the medium is not a close one. He concludes that each locality seems to have a particular form of Artemia, differing from other forms in colour, size and the average number of setae on the caudal furcae; he does not consider these differences to be especially related to the salinity of the medium.

The observations of Schmankewitsch (1875, 1877) are further confirmed by Anikin (1898) and Samter & Heymons (1902) on material from near the Black Sea and Caspian Sea respectively. These authors, however, do not agree with Schmankewitsch's interpretation of his observations. It is stressed by Anikin, that the differences in body form, obtained under the influence of the physico-chemical composition of the medium, do not result in new species, but should be regarded as 'ontogenetic' differences which are only maintained while the operative factor in the environment persists. He also points out that certain specific characters of males of Artemia do not change under the influence of the environment. The main contribution to the subject made by Samter & Heymons (1902), is their insistence that in any particular concentration of brine, all variations in form of Artemia may occur, although the majority of individuals are affected in a particular way. They do not doubt, however, that the concentration of the medium influences the size and shape of Artemia.

Much of this early work on the influence of the salinity of the medium on body form of Artemia is incomplete in that numerical

data are lacking. In some cases this is because no measurements of body proportions were made, and in others, it must be assumed that measurements were made but not published. Likewise, many of the observations were made on natural populations of brine shrimps, in media subject to considerable fluctuations in concentration. Such is the case with regard to the observations of Kellogg (1906) on a new species of Artemia, A. franciscana*, which he described from California. He says that differences in body form "are apparent in this new Artemia and evidently bear a definite relation to the different densities of the pools in which the Artemias are living". No measurements appear to have been made either on body proportions or on the fluctuations in concentration of the medium.

The observations of Artom (1907a, 1907b) cannot, however, be criticised on this basis. He made extensive measurements on brine shrimps in a natural population near Cagliari, Sardinia, over a period of several years, during which changes occurred in the salinity of the medium. The results of his experiments agree with those of Schmankewitsch (1875) and Samter & Heymons (1902) in demonstrating that the salinity of the environment influences the growth of various parts of the body of Artemia, both in males and females. The numerical data published do not, in fact, show

*Synonymous with Artemia salina var. arietina (S. Fisch.) of Daday de Deés (1910)

this very clearly, as Artom has not taken into account the possibility that variation in body form might occur in relation to the size of Artemia. In any one concentration of brine, the body proportions are given of a number of animals differing considerably in size, and Artom attempts to allow for this by correcting all measurements to a standard length of thorax of 3.6 mm. This is valid only if it is shown that there is no variation in body proportions with size; Artom gives no evidence that body proportions remain constant over the size range of animals investigated.

Artom (1907a) disagrees with Samter & Heymons (1902), who state that the external salinity does not influence body form in a constant and characteristic way. He says that particular concentrations effect variations which are constant and characteristic, particularly in females of Artemia, and to a lesser extent in males.

In order to make this review of the literature complete, reference must be made to the observations of Evans (1913). These were made on material of unspecified origin, and were mainly concerned with the hatching and survival of Artemia in different concentrations of brine. Evans states that no variation in form, as described by Schmankewitsch (1875), was found. In particular, the caudal lobes were uniform in size and bore the same number of setae in all concentrations of the medium. No measurements

appear to have been made and no information is given as to how long the animals were kept in a particular concentration of brine while observations were being made.

One of the most interesting contributions to this subject is that made by Abonyi (1915), who made experiments on brine shrimps in Hungary. He found that changes in form which resulted from increased concentration of the medium, could occur during the life of an animal, but took place mainly in the course of three or four generations. He confirmed experimentally, that as the salinity of the medium increases, the following changes occur in Artemia:-

1) the caudal furcae become shorter and bear fewer setae and,

2) the abdomen becomes relatively longer in relation to the rest of the body.

Thus, not only is the rate of growth of a whole animal retarded in very saline water, but the specific rates of growth of particular parts of the body are influenced by changes in the concentration of the medium. Abonyi (1915) has attempted to express this conclusion numerically. By plotting graphically the length of the abdomen as a percentage of the length of the rest of the body, against the density of the brine, he obtained a regular curve along which the varieties and species of Artemia recognised at this time, are found to occupy fairly well defined regions (figure 11). From this, Abonyi concludes that the density of the brine is 'specific'; a

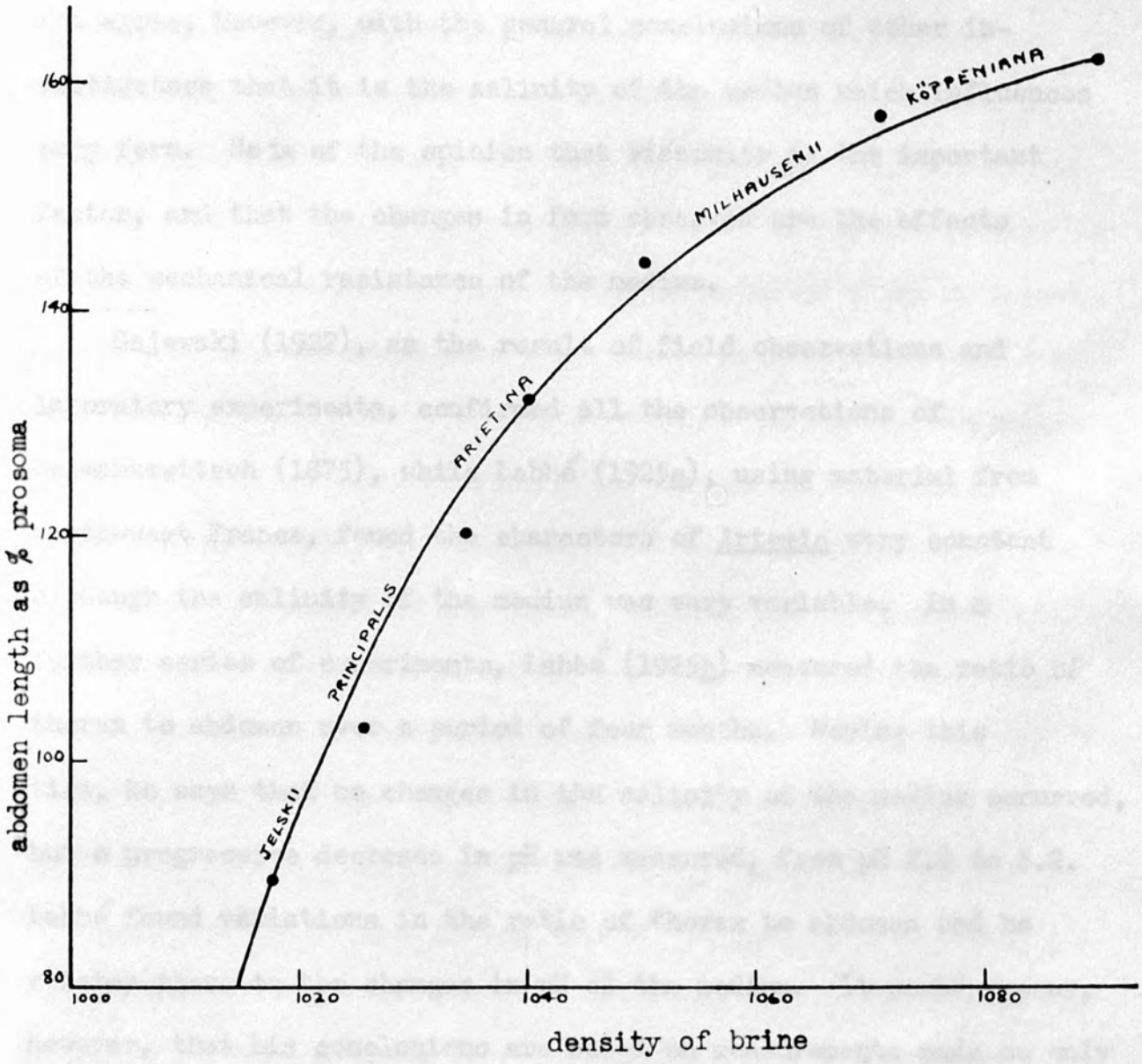


Figure 11. Length of abdomen, expressed as a percentage length of prosoma*, of Artemia from brines of different densities. (after Abonyi, 1915)

*prosoma = head + thorax

particular variety of Artemia is more or less identifiable by the density of the brine in which it is found. Abonyi (1915) does not agree, however, with the general conclusions of other investigators that it is the salinity of the medium which influences body form. He is of the opinion that viscosity is the important factor, and that the changes in form observed are the effects of the mechanical resistance of the medium.

Gajewski (1922), as the result of field observations and laboratory experiments, confirmed all the observations of Schmankewitsch (1875), while Labbé (1925a), using material from north-west France, found the characters of Artemia very constant although the salinity of the medium was very variable. In a further series of experiments, Labbé (1925b) measured the ratio of thorax to abdomen over a period of four months. During this time, he says that no changes in the salinity of the medium occurred, but a progressive decrease in pH was measured, from pH 8.6 to 8.2. Labbé found variations in the ratio of thorax to abdomen and he relates these to the changes in pH of the medium. It would appear, however, that his conclusions are based on measurements made on only four individuals of Artemia.

Finally in this review, the conclusions of Bond (1933), Kuenen (1939) and Weisz (1946) on the variation of Artemia from California must be mentioned. Bond (1933) points out that most of the measurements on body proportions have been made on fixed material,

and he suggests that many of the changes of form described are the result of shrinkage during the process of fixation. From measurements made on animals from natural populations and on laboratory bred animals, Bond concludes that "no variable Artemia exists in the western United States", and that this Artemia remains constant in form whatever the salinity of the medium. In the laboratory experiments, brine shrimps were reared in media of three different concentrations, and measurements were made on a total of seventeen individuals, the number of specimens measured in each concentration being nine, two and six. Further, the animals in the most dilute medium averaged 9.60 mm. in length while those in the concentrated medium averaged only 6.85 mm. Thus, not only are the numbers of animals measured inadequate, but body proportions are compared in animals of very different total lengths.

With regard to the experiments of Kuenen (1939) on the variation of form of Artemia, little can be said. No reference to these experiments is made in the text of his publication, but the results are expressed graphically in the form of two points joined by a straight line, representing the ratio of the thorax to abdomen as a function of the concentration of the brine. This line has a positive slope only slightly at an angle to the horizontal, indicating that the abdomen becomes relatively slightly longer in the more concentrated media, but without the numerical data from which this line has been compiled, no conclusions should be drawn.

In his experiments on the growth and segmentation of Artemia in different concentrations of brine, Weisz (1946) did not observe any effect of the salinity of the medium on the relative body proportions. These experiments were limited to measurements of the growth of animals from hatching to sexual maturity. What changes in form, if any, might have occurred had the experiments been continued, cannot be predicted.

From this review of previous work, it is clear that no attempts have yet been made to investigate changes in body form during the growth of Artemia under constant conditions. It seems that this should constitute a preliminary study to the investigation of changes in body form in relation to the salinity of the medium. Experiments have been designed, therefore, to investigate this problem of body form in relation to size and sex of Artemia, as well as in relation to the salinity of the medium.

Materials and methods.

Cultures of Artemia from five different stocks were maintained under standard conditions of population density, temperature and feeding as already described (pages 6 - 8). The animals were reared in two different concentrations of brine, S‰ 35 (sea water) and S‰ 140; only animals derived from parents reared in these brines for at least two generations were used in studies on variation of form. Linear measurements were made on narcotised animals as described on page 8 .

Results

For convenience, the results of the experiments on body form will be described in two parts; firstly, the influence of size and sex of Artemia on body form, and secondly, the influence of the salinity of the medium on body form. The numerical data relating to these experiments will be found in Appendix I, tables A to G; the data are represented graphically in the text in figures 12 to 35.

The influence of size and sex of Artemia on body form.

In figures 12 to 15 it is shown that in females of Artemia, the abdomen becomes relatively longer as the total length of the animals increases; this is so in females of the five stocks investigated, both in sea water and in concentrated brine. For any particular length of female, however, the percentage ratio of abdomen to prosoma is not the same in females from different stocks. In both concentrations of brine, females of the Californian and Utah stocks have a shorter abdomen than those of the same total length from La Palme; these, in turn, have a shorter abdomen than females of the same total length from Cagliari and Algeria. Thus, the difference in relative length of abdomen, in females from California and Algeria reared under the same standard conditions, is very marked.

In males of Artemia, from the American stocks, the abdomen

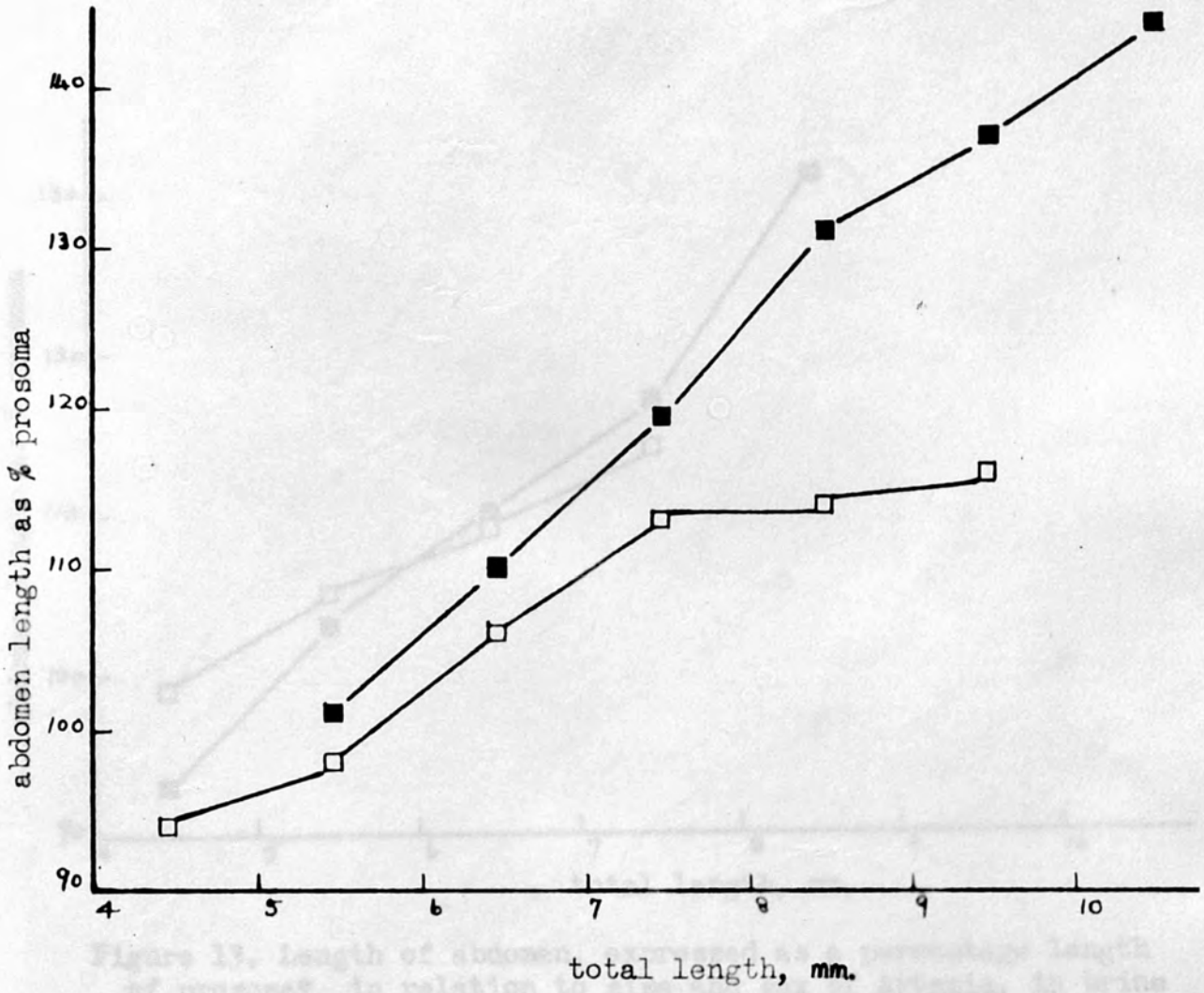


Figure 12. Length of abdomen, expressed as a percentage length of prosoma*, in relation to size and sex of Artemia, in sea water (S‰.35) at 25°C. ■ females; □ males; Algerian stock.

* prosoma = head + thorax

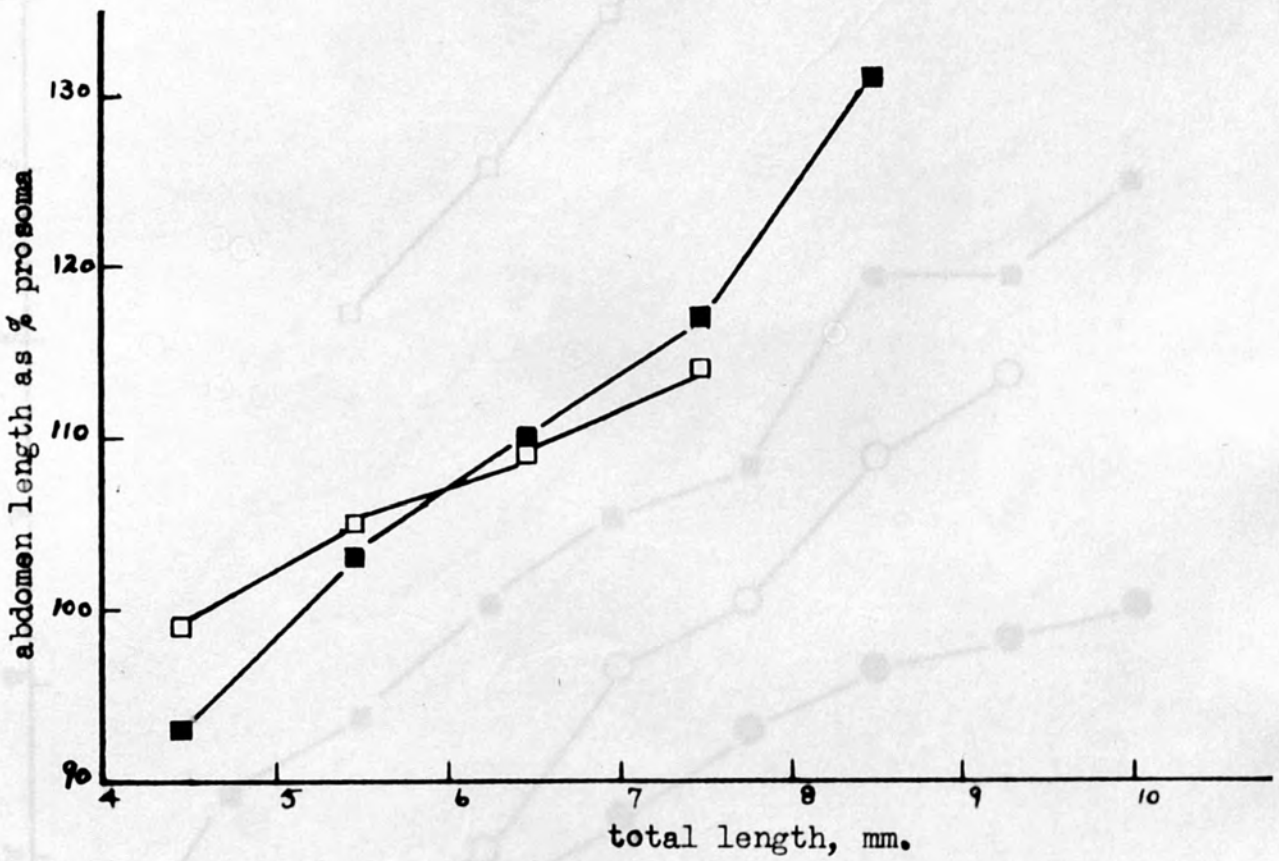


Figure 13. Length of abdomen, expressed as a percentage length of prosoma*, in relation to size and sex of Artemia, in brine of salinity S‰140 at 25°C. ■ females; □ males; Cagliari stock

* prosoma = head + thorax

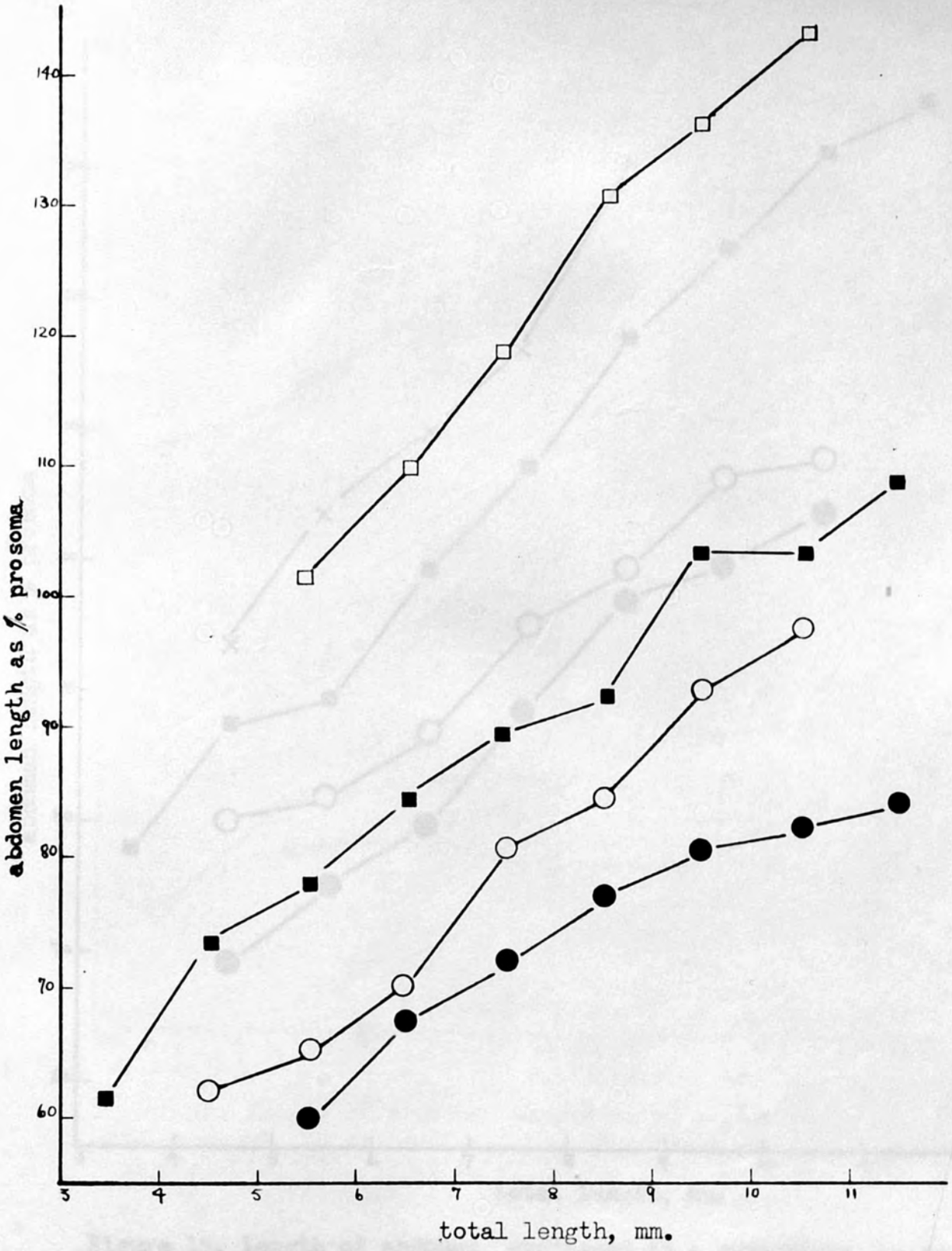


Figure 14. Length of abdomen, expressed as a percentage length of prosoma*, in females of Artemia from four different stocks, in sea water (S‰ 35) at 25°C.
● California; ○ Utah; ■ La Palme; □ Algeria.

*prosoma = head + thorax

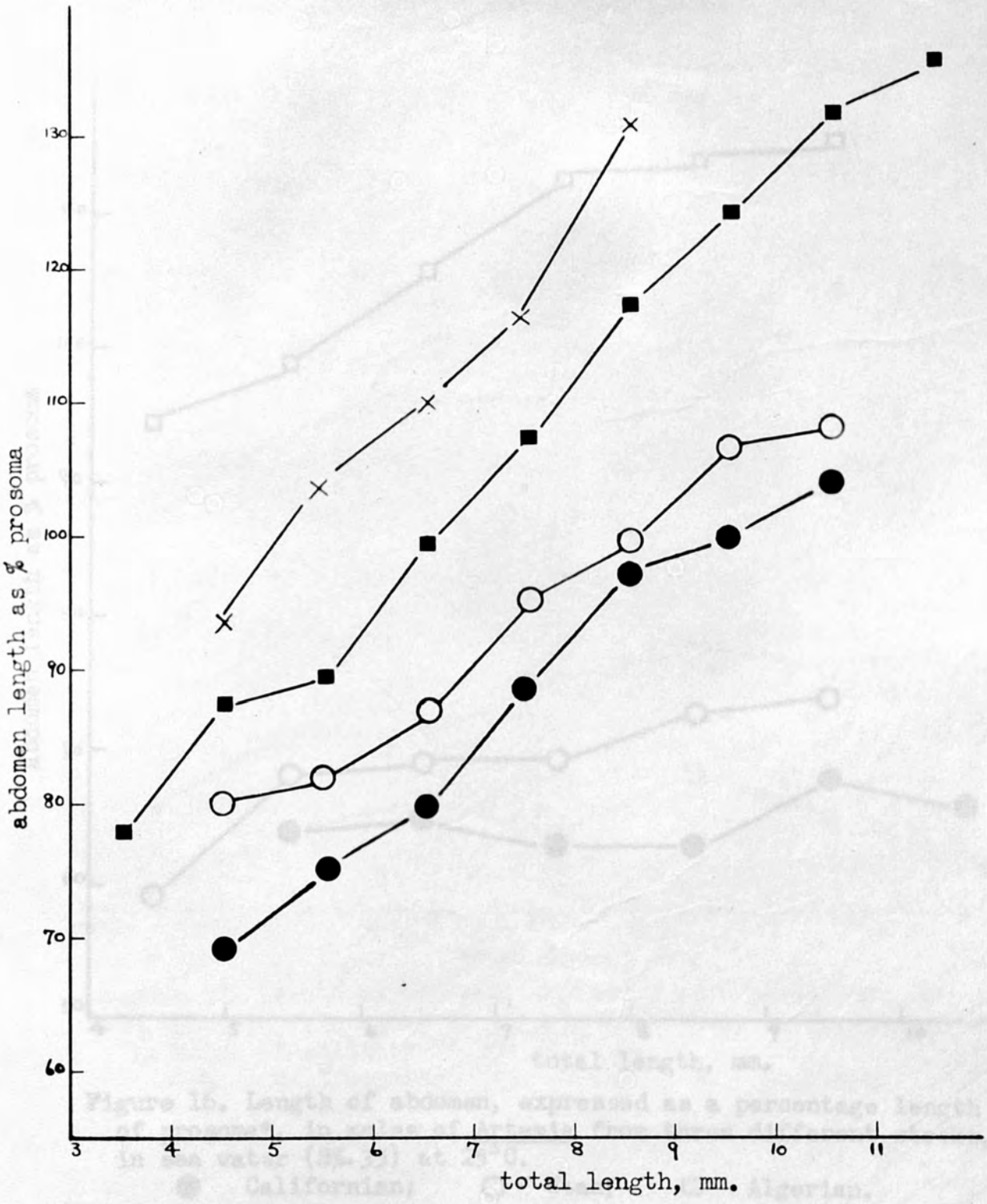


Figure 15. Length of abdomen, expressed as a percentage length of prosoma*, in females of Artemia from four different stocks, in brine (S‰ 140) at 25°C.
● California; ○ Utah; ■ La Palme; × Cagliari.

*prosoma = head + thorax

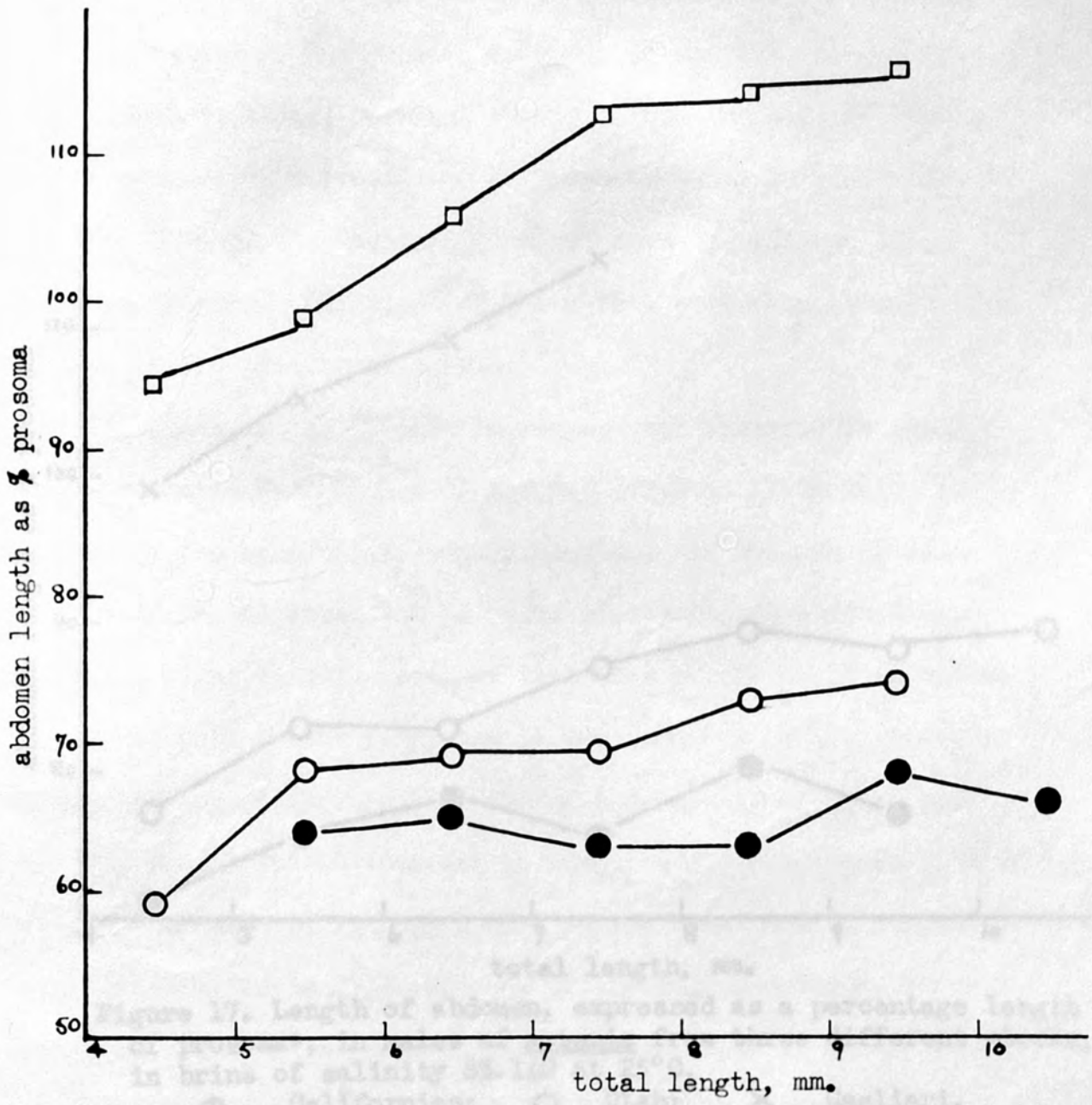


Figure 16. Length of abdomen, expressed as a percentage length of prosoma*, in males of Artemia from three different stocks, in sea water (S‰ 35) at 25°C.

● Californian; ○ Utah; □ Algerian.

* prosoma = head + thorax

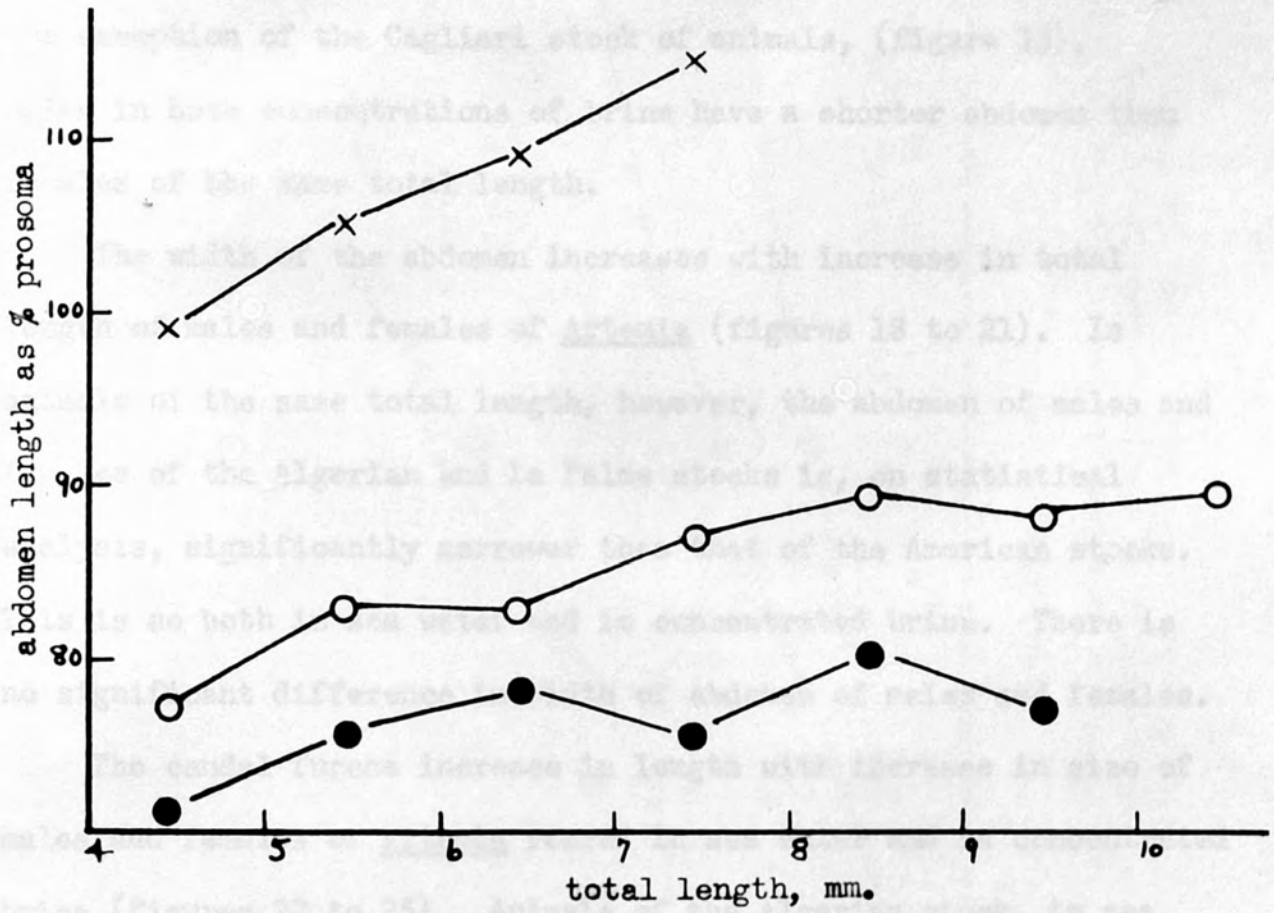


Figure 17. Length of abdomen, expressed as a percentage length of prosoma*, in males of Artemia from three different stocks, in brine of salinity S‰ 140 at 25°C.

● Californian; ○ Utah; × Cagliari.

* prosoma = head + thorax

increases in length only very slightly relative to the increase in total length of the animals (figures 16 and 17). Those from Algeria and Cagliari, however, show a marked increase in length of the abdomen relative to the increase in total length. With the exception of the Cagliari stock of animals, (figure 13), males in both concentrations of brine have a shorter abdomen than females of the same total length.

The width of the abdomen increases with increase in total length of males and females of Artemia (figures 18 to 21). In animals of the same total length, however, the abdomen of males and females of the Algerian and La Palme stocks is, on statistical analysis, significantly narrower than that of the American stocks. This is so both in sea water and in concentrated brine. There is no significant difference in width of abdomen of males and females.

The caudal furcae increase in length with increase in size of males and females of Artemia reared in sea water and in concentrated brine (figures 22 to 25). Animals of the Algerian stock, in sea water, have much shorter caudal furcae than those of other brine shrimps investigated (figures 22 and 24). With regard to the number of setae on the caudal furcae, this is significantly higher in animals of the American stocks than of the European stocks in both media (figures 26 to 29).

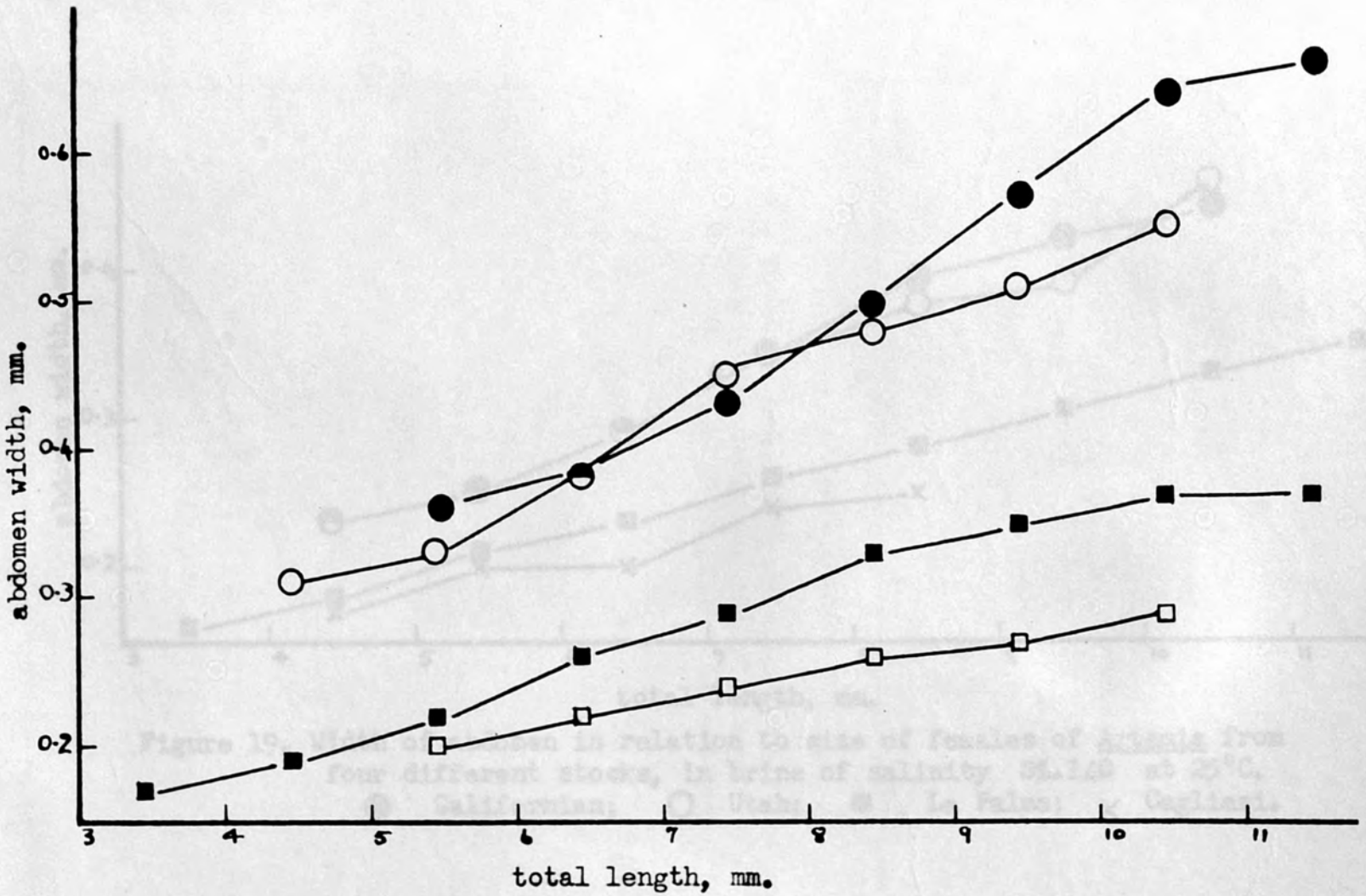


Figure 18. Width of abdomen in relation to size of females of Artemia from four different stocks, in sea water (S‰35) at 25°C.
● Californian; ○ Utah; ■ La Palme; □ Algerian.

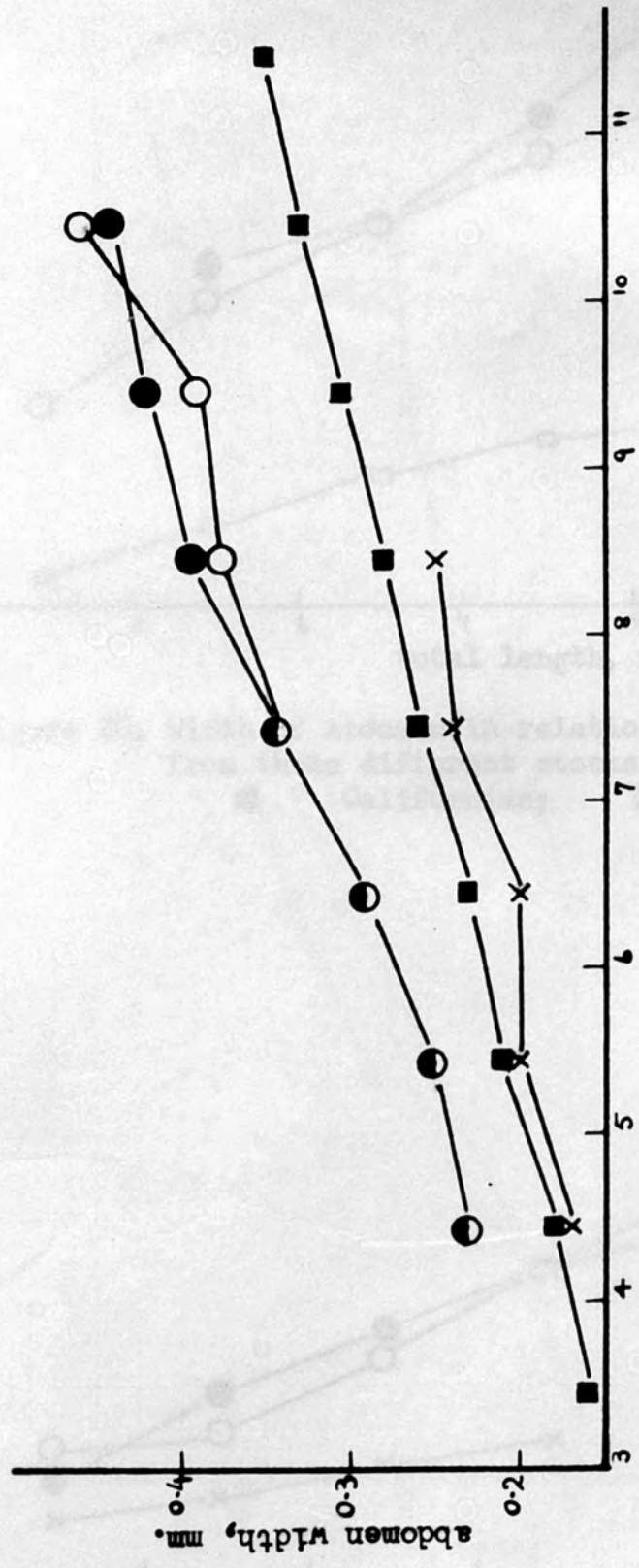


Figure 19. Width of abdomen in relation to size of females of Artemia from four different stocks, in brine of salinity S‰ 140 at 25°C.
● Californian; ○ Utah; ■ La Palme; × Cagliari.

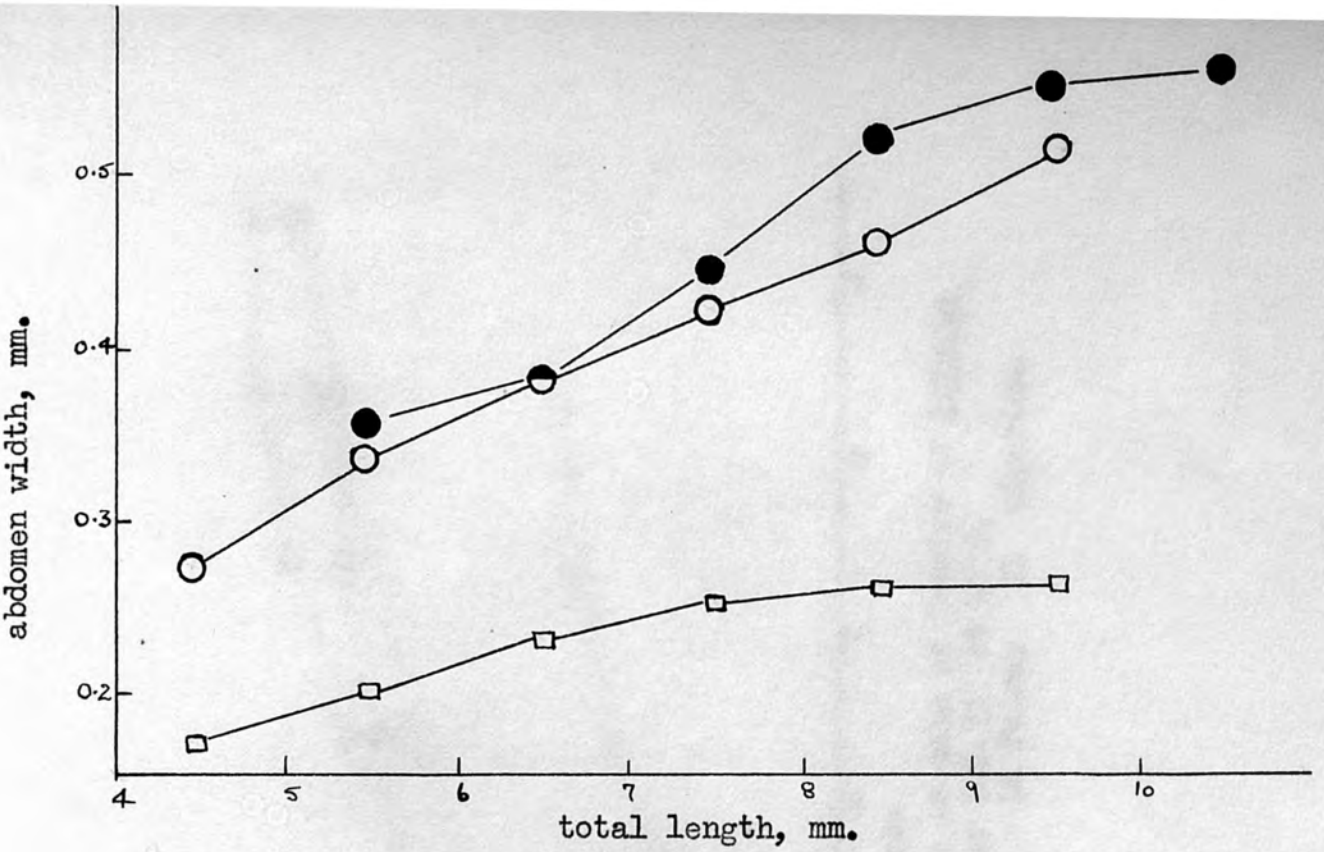


Figure 20. Width of abdomen in relation to size of males of Artemia from three different stocks, in sea water (S‰ 35) at 25°C.
● Californian; ○ Utah; □ Algerian.

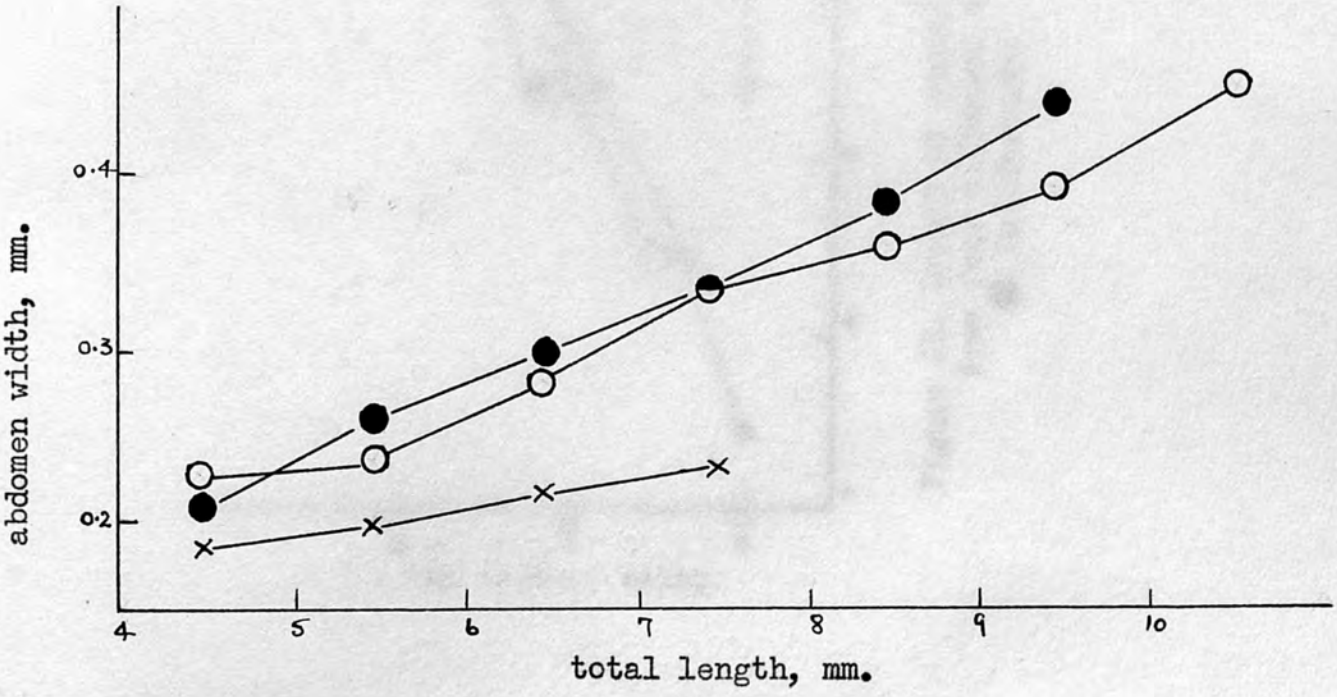


Figure 21. Width of abdomen in relation to size of males of Artemia from three different stocks, in brine (S‰ 140) at 25°C.
● Californian; ○ Utah; × Cagliari.

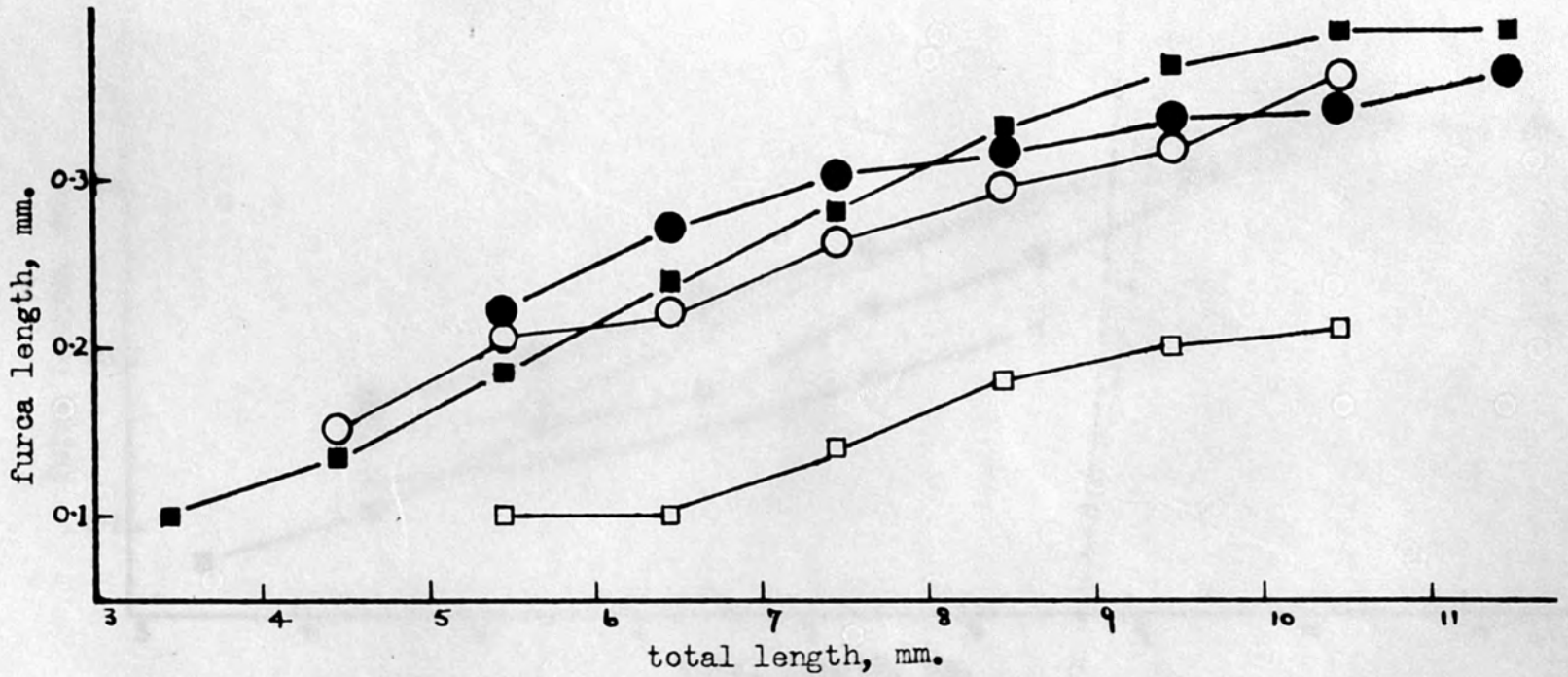


Figure 22. Length of caudal furca in relation to size of females of Artemia from four different stocks, in sea water (S‰.35) at 25°C.
● Californian; ○ Utah; ■ La Palme; □ Algerian.

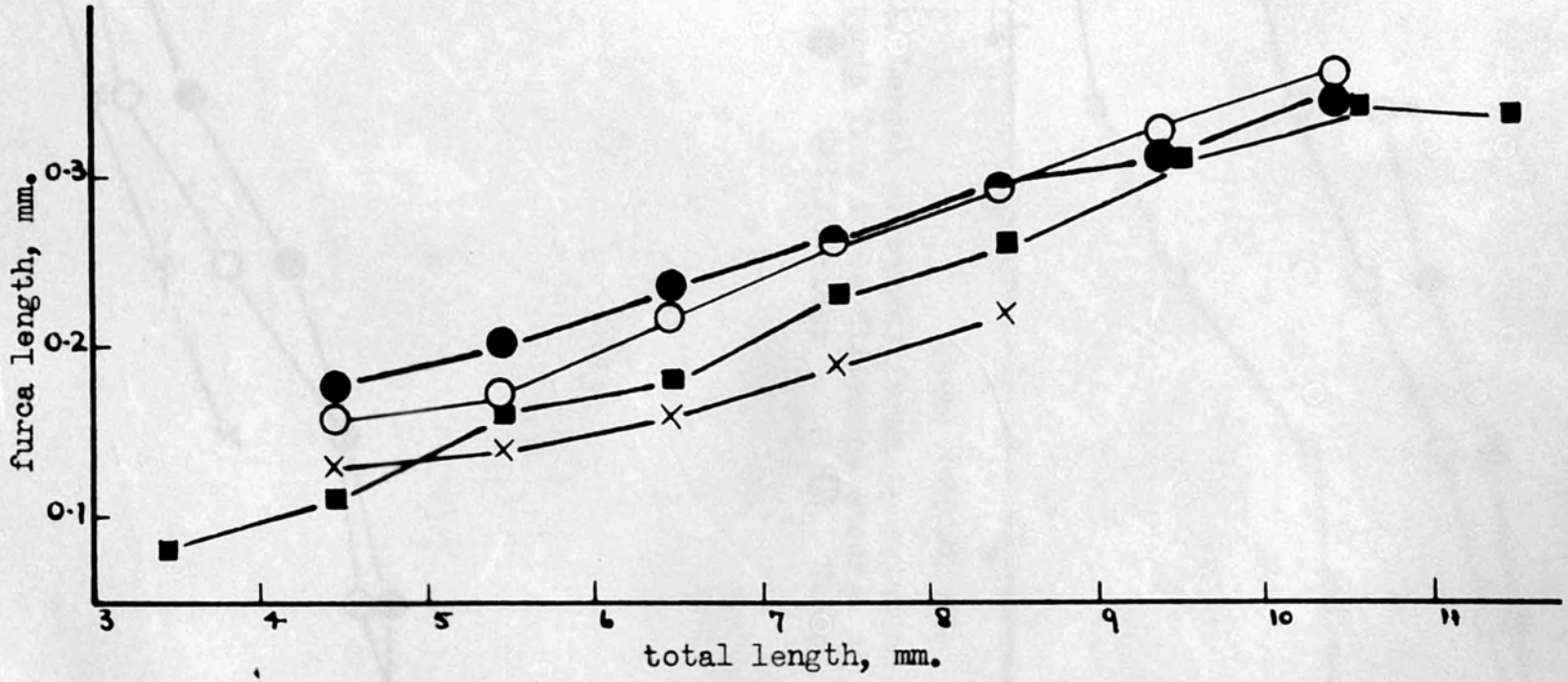


Figure 23. Length of caudal furca in relation to size of females of Artemia from four different stocks, in brine of salinity S‰.140 at 25°C.
● Californian; ○ Utah; ■ La Palme; × Cagliari.

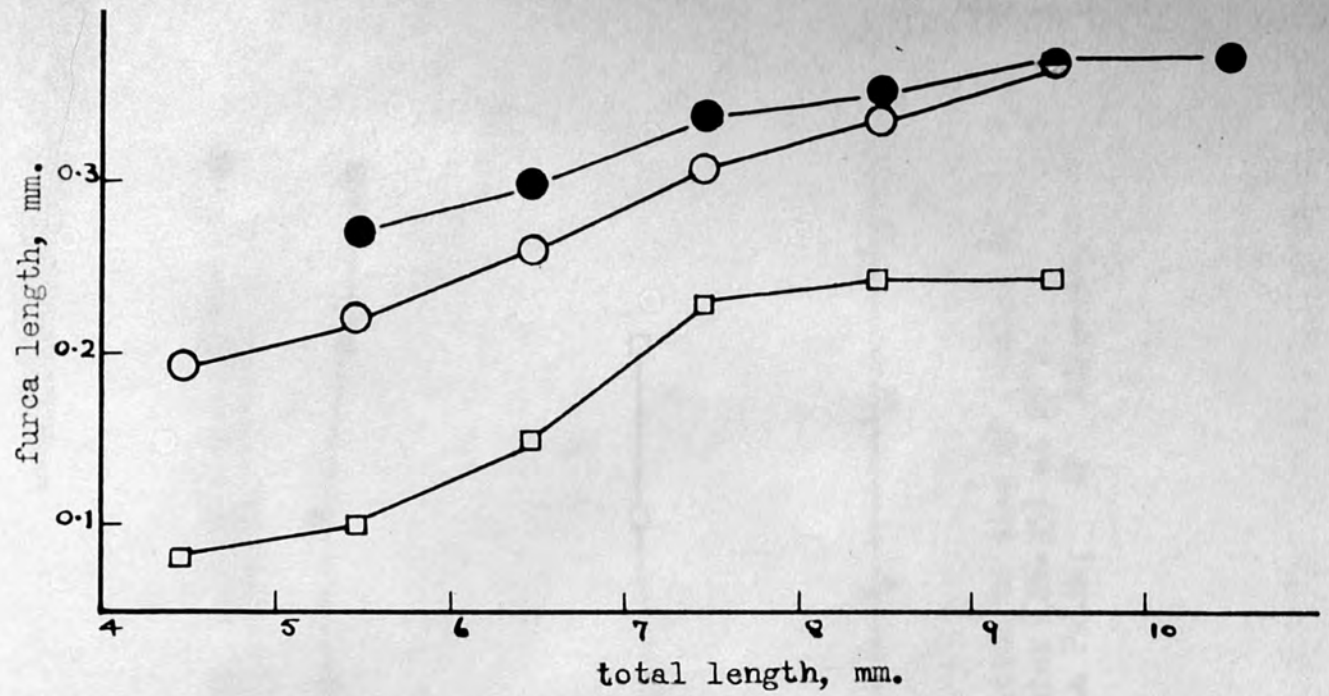


Figure 24. Length of caudal furca in relation to size of males of Artemia from three different stocks, in sea water (S‰.35) at 25°C.
● Californian; ○ Utah; □ Algerian.

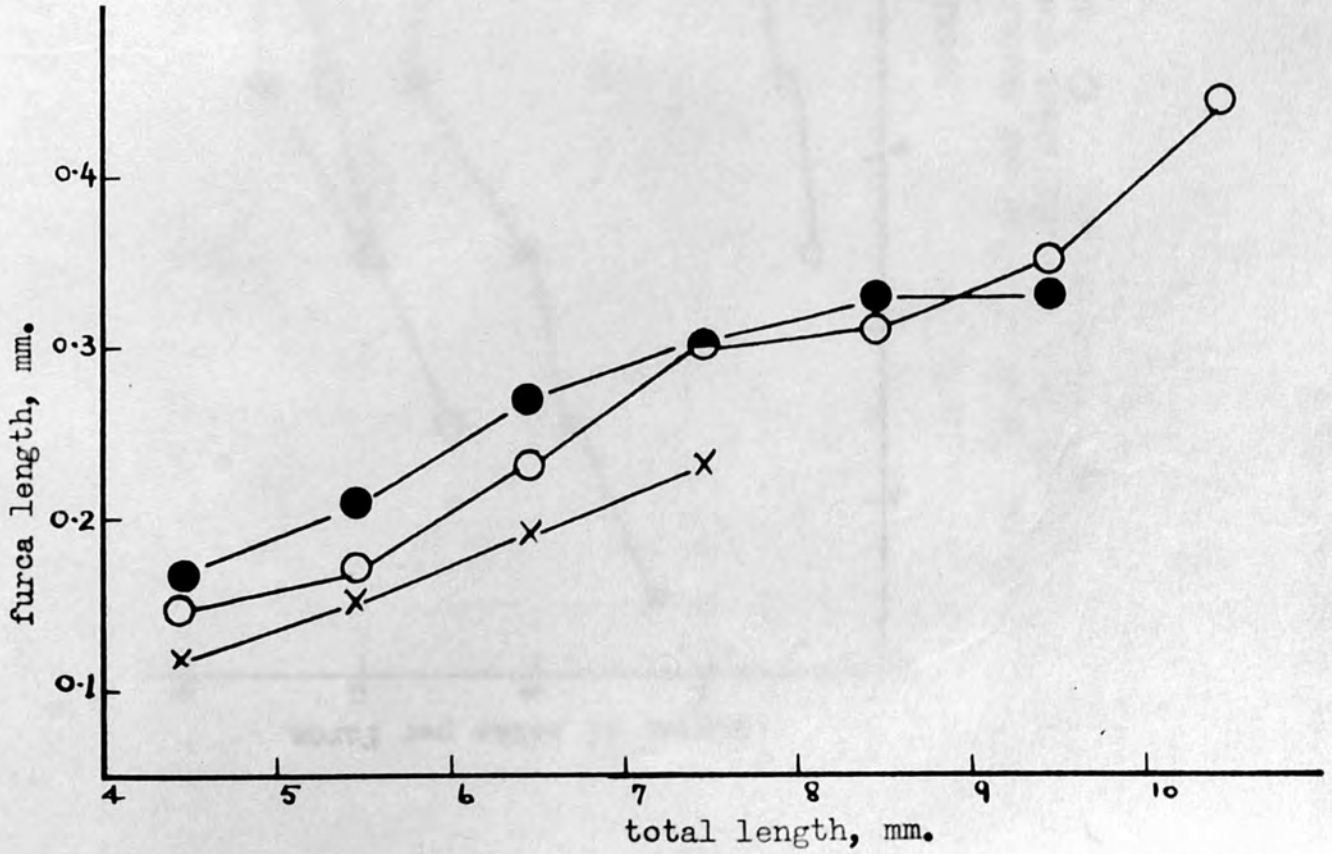


Figure 25. Length of caudal furca in relation to size of males of **Artemia** from three different stocks, in brine (S‰.140) at 25°C.
● Californian; ○ Utah; × Cagliari.

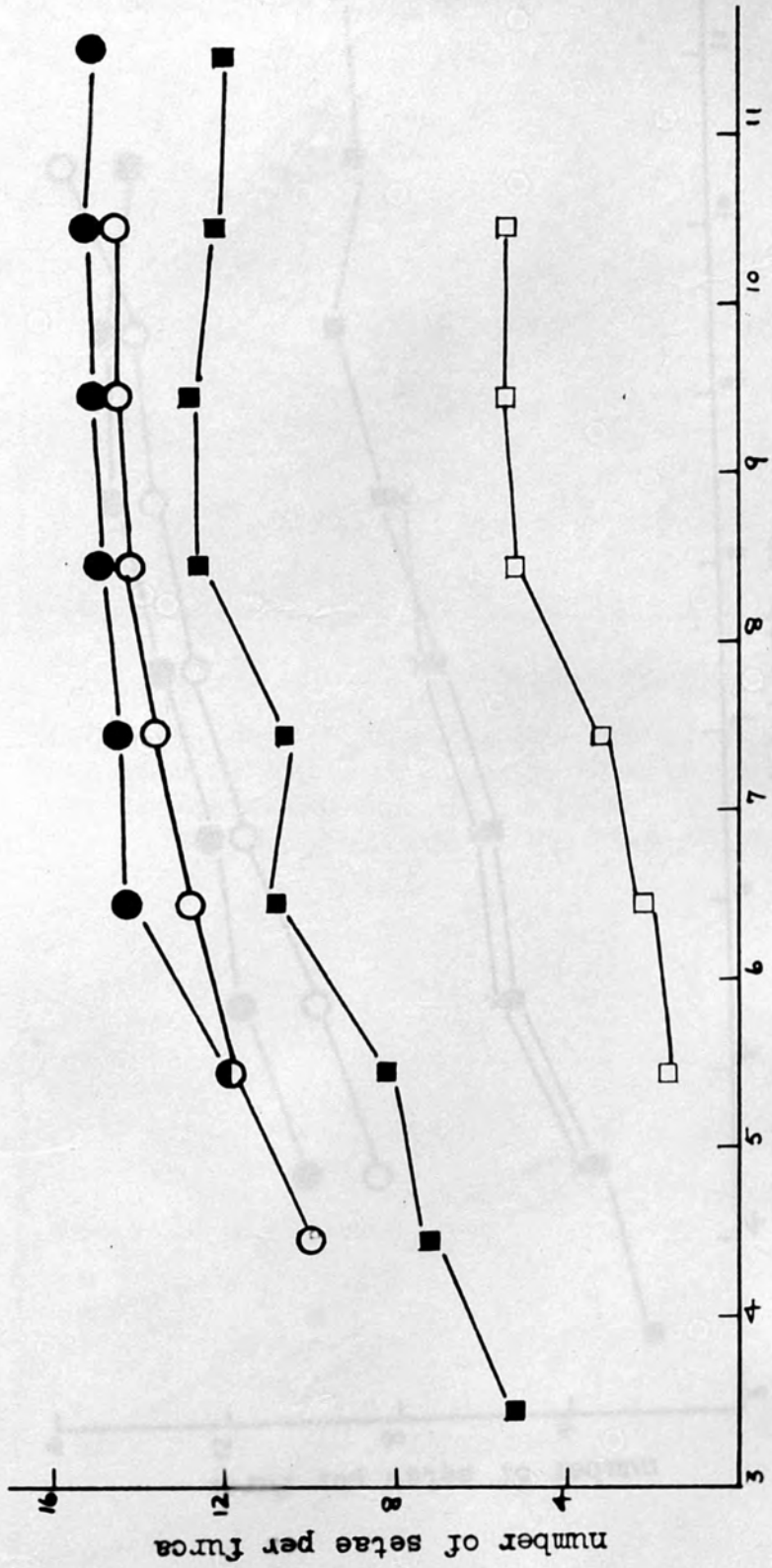


Figure 26. Number of setae per caudal furca in relation to size of females of Artemia from four different stocks, in sea water (S‰ 35) at 25°C.
● Californian; ○ Utah; ■ La Palme; □ Algerian.

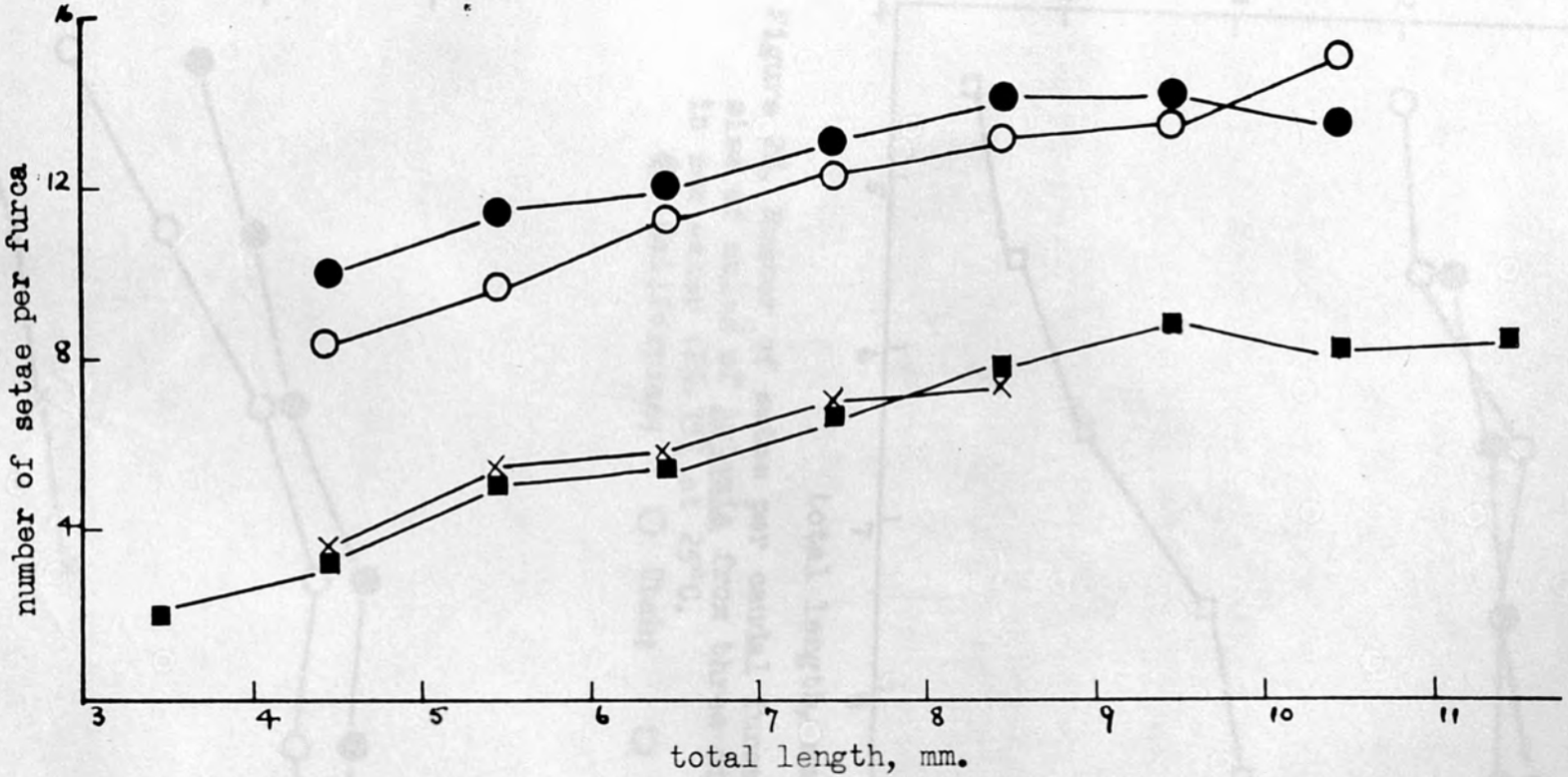


Figure 27. Number of setae per caudal furca in relation to size of females of Artemia from four different stocks, in brine of salinity S‰.140 at 25°C.
● Californian; ○ Utah; ■ La Palme; × Cagliari.

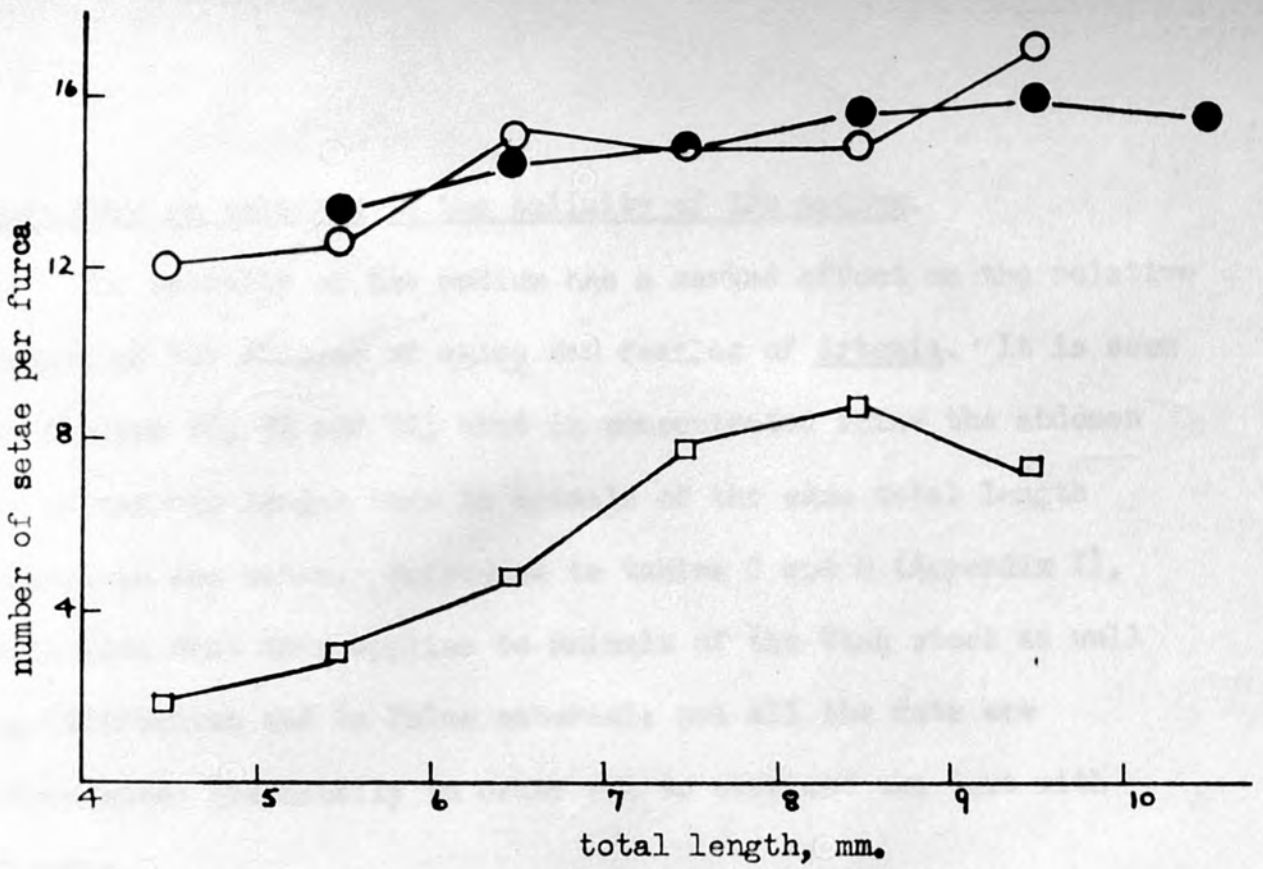


Figure 28. Number of setae per caudal furca in relation to size of males of Artemia from three different stocks, in sea water (S‰.35) at 25°C.
● Californian; ○ Utah; □ Algerian.

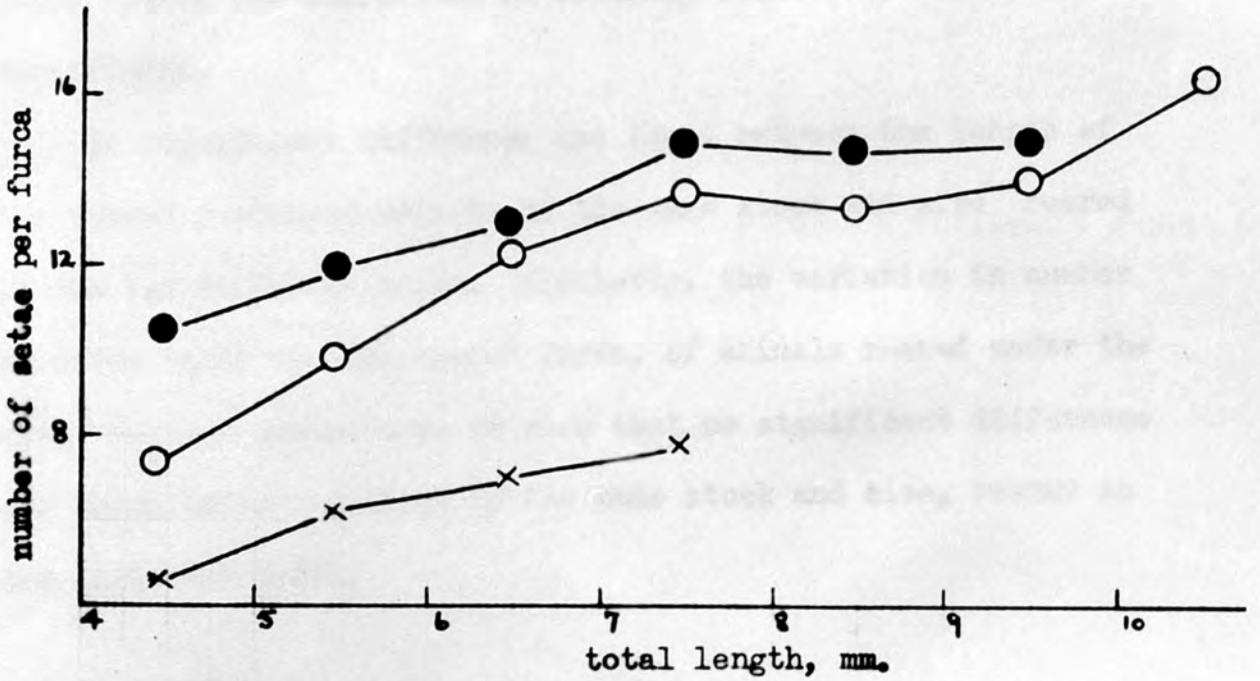


Figure 29. Number of setae per caudal furca in relation to size of males of Artemia from three different stocks, in brine of salinity S‰.140 at 25°C.
● Californian; ○ Utah; × Cagliari.

Body form in relation to the salinity of the medium.

The salinity of the medium has a marked effect on the relative length of the abdomen of males and females of Artemia. It is seen in figures 30, 31 and 32, that in concentrated brine the abdomen is relatively longer than in animals of the same total length reared in sea water. Reference to tables C and D (Appendix I), will show that this applies to animals of the Utah stock as well as Californian and La Palme material; not all the data are represented graphically in order not to overload the text with figures.

The abdomen of brine shrimps reared in sea water is wider than that of animals reared in a more concentrated medium (figures 33, 34 and 35; also 18 to 21). In the case of the La Palme stock, the difference in width is doubtfully statistically significant.

No significant difference was found between the length of the caudal furcae of animals of the same stock and size reared in the two different media. Similarly, the variation in number of setae borne by each caudal furca, of animals reared under the same standard conditions, is such that no significant difference was shown between animals of the same stock and size, reared in two different media.

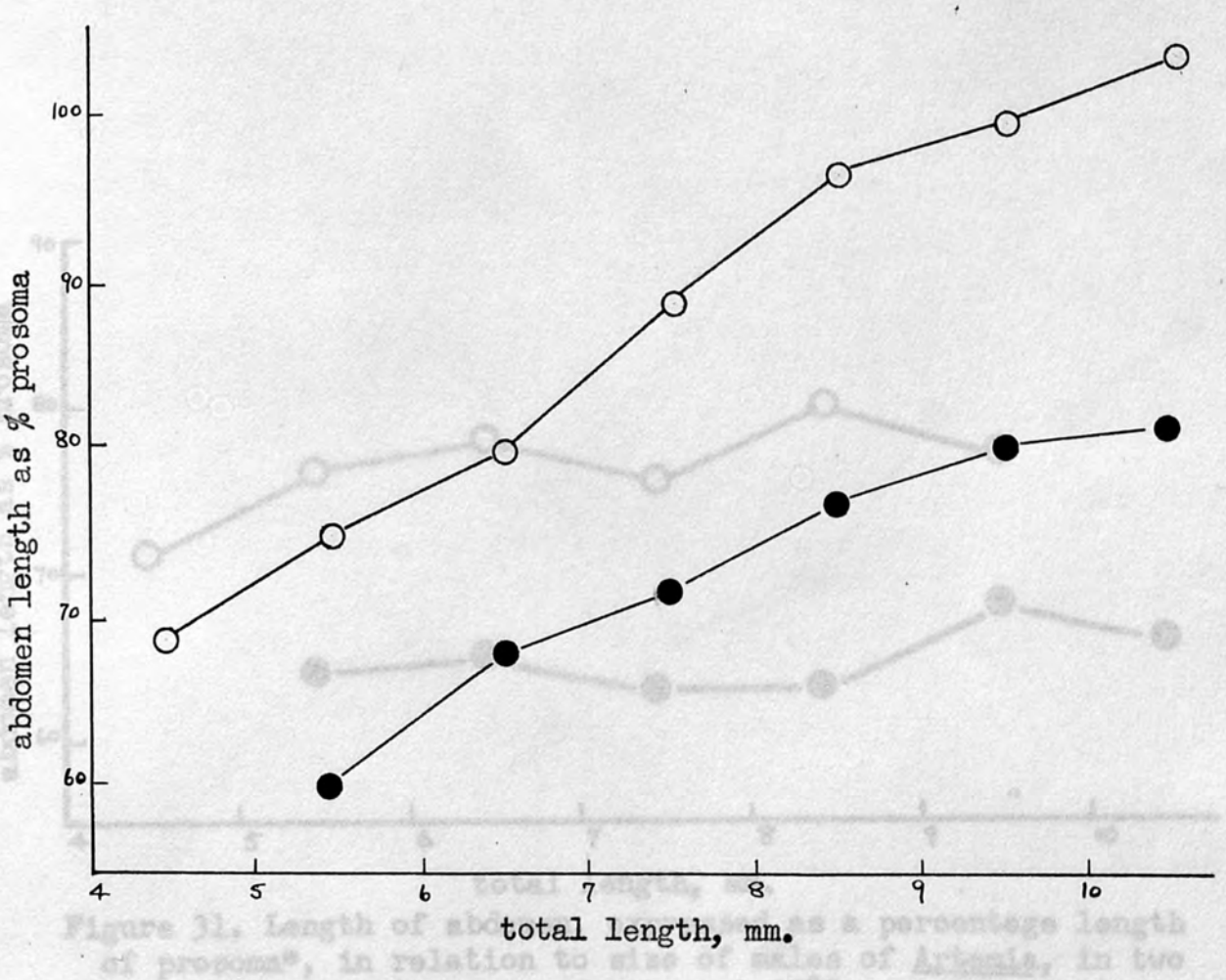


Figure 30. Length of abdomen, expressed as a percentage length of prosoma*, in relation to size of females of Artemia, in two different concentrations of brine at 25°C. Californian stock.
● S‰ 35; ○ S‰ 140.

*prosoma = head + thorax

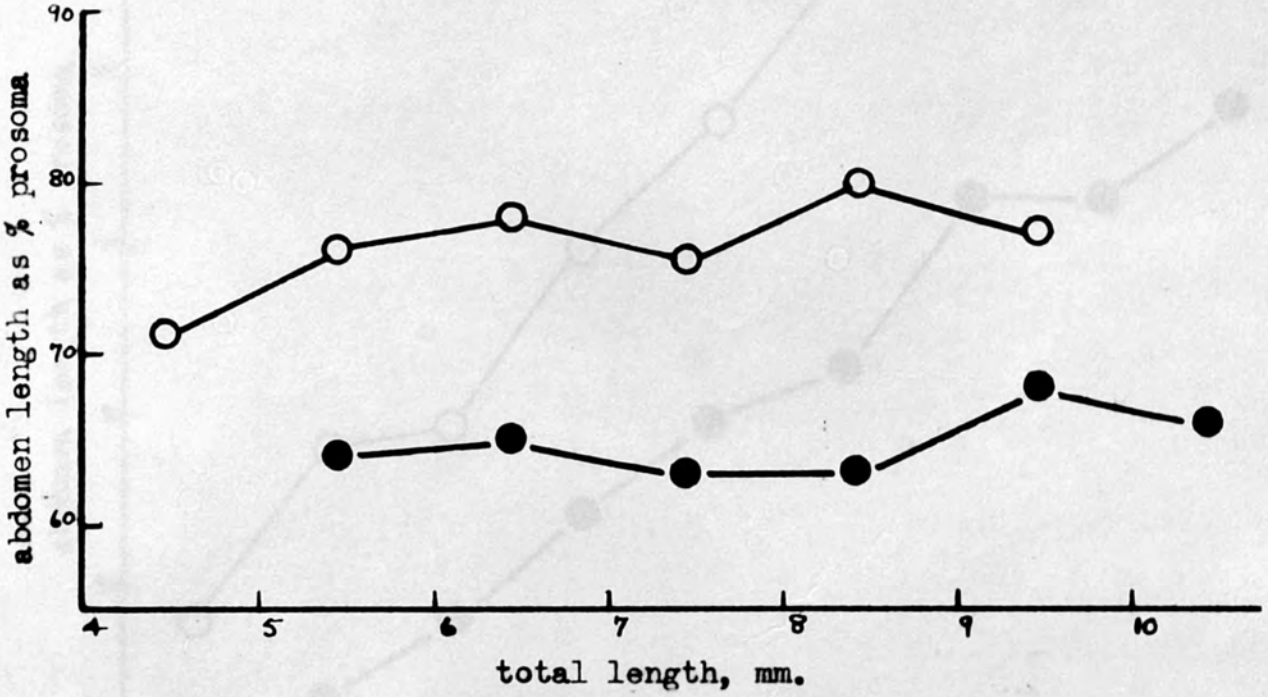


Figure 31. Length of abdomen, expressed as a percentage length of prosoma*, in relation to size of males of Artemia, in two different concentrations of brine at 25°C.
● S‰ 35; ○ S‰ 140; Californian stock.

* prosoma = head + thorax

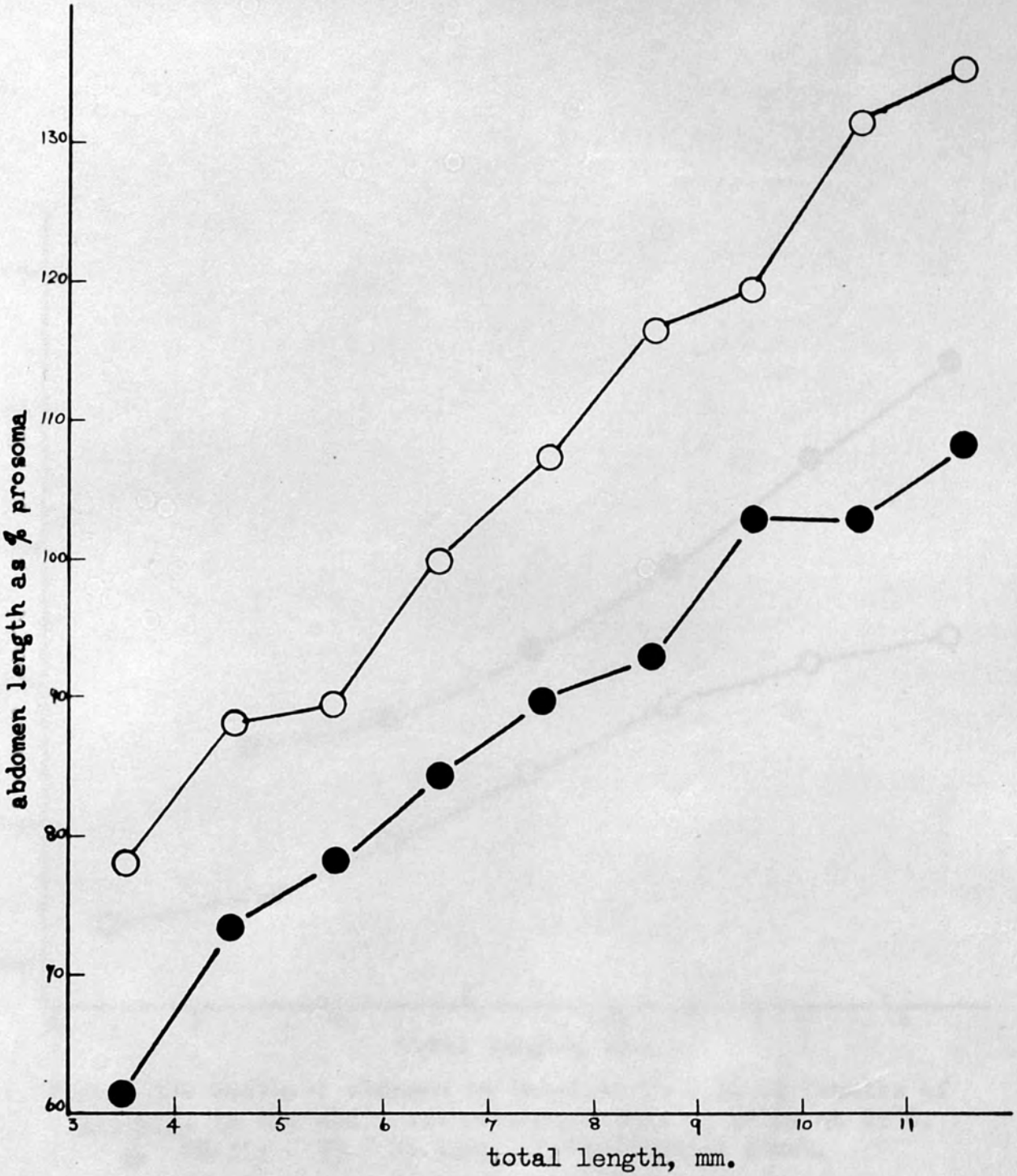


Figure 32. Length of abdomen, expressed as a percentage length of prosoma*, in relation to size of parthenogenetic females of Artemia, in two different concentrations of brine at 25°C.
● S‰.35; ○ S‰.140; (La Palme stock)

*prosoma = head + thorax

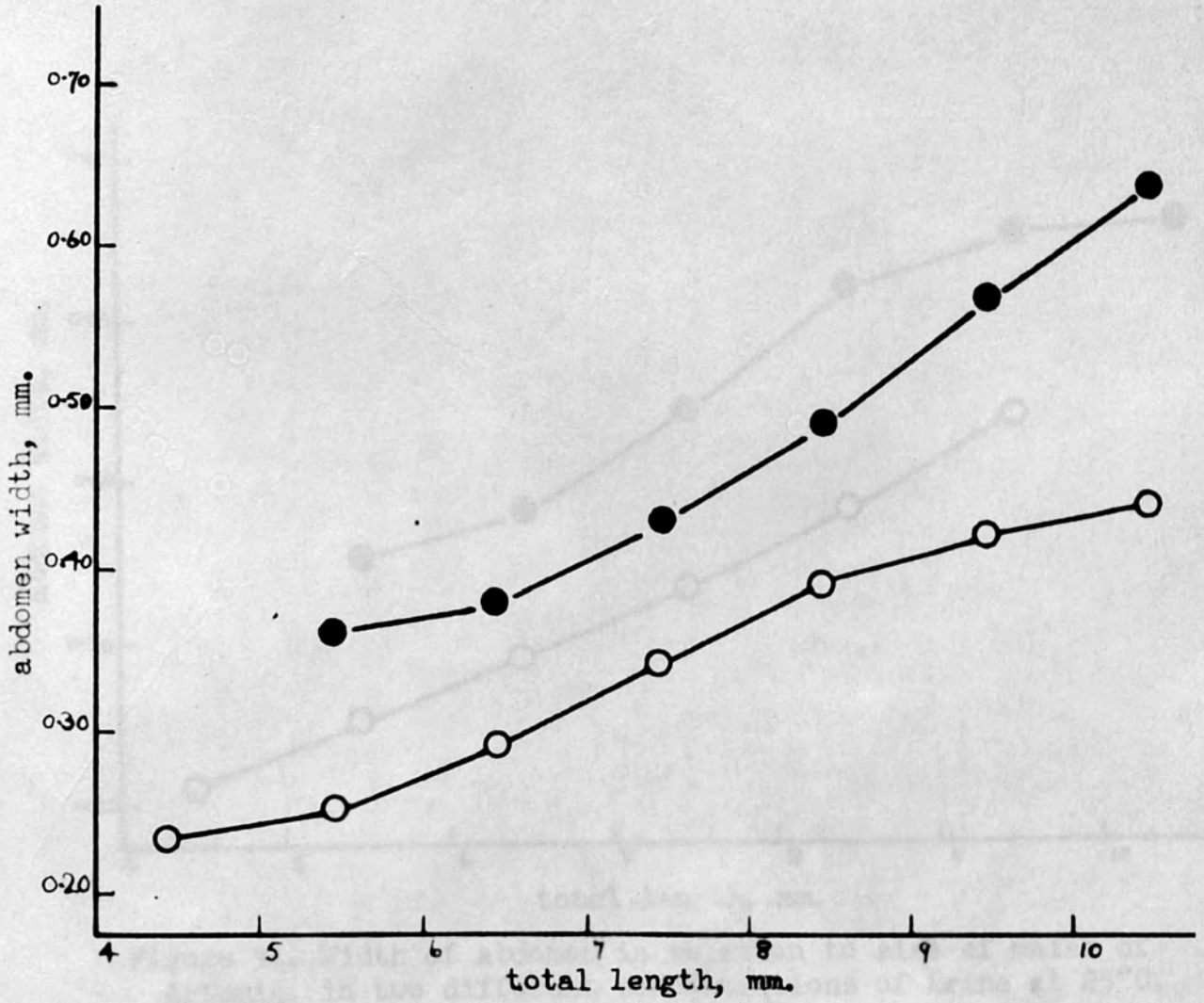


Figure 33. Width of abdomen in relation to size of females of Artemia, in two different concentrations of brine at 25°C.
● S‰ 35; ○ S‰ 140; Californian stock.

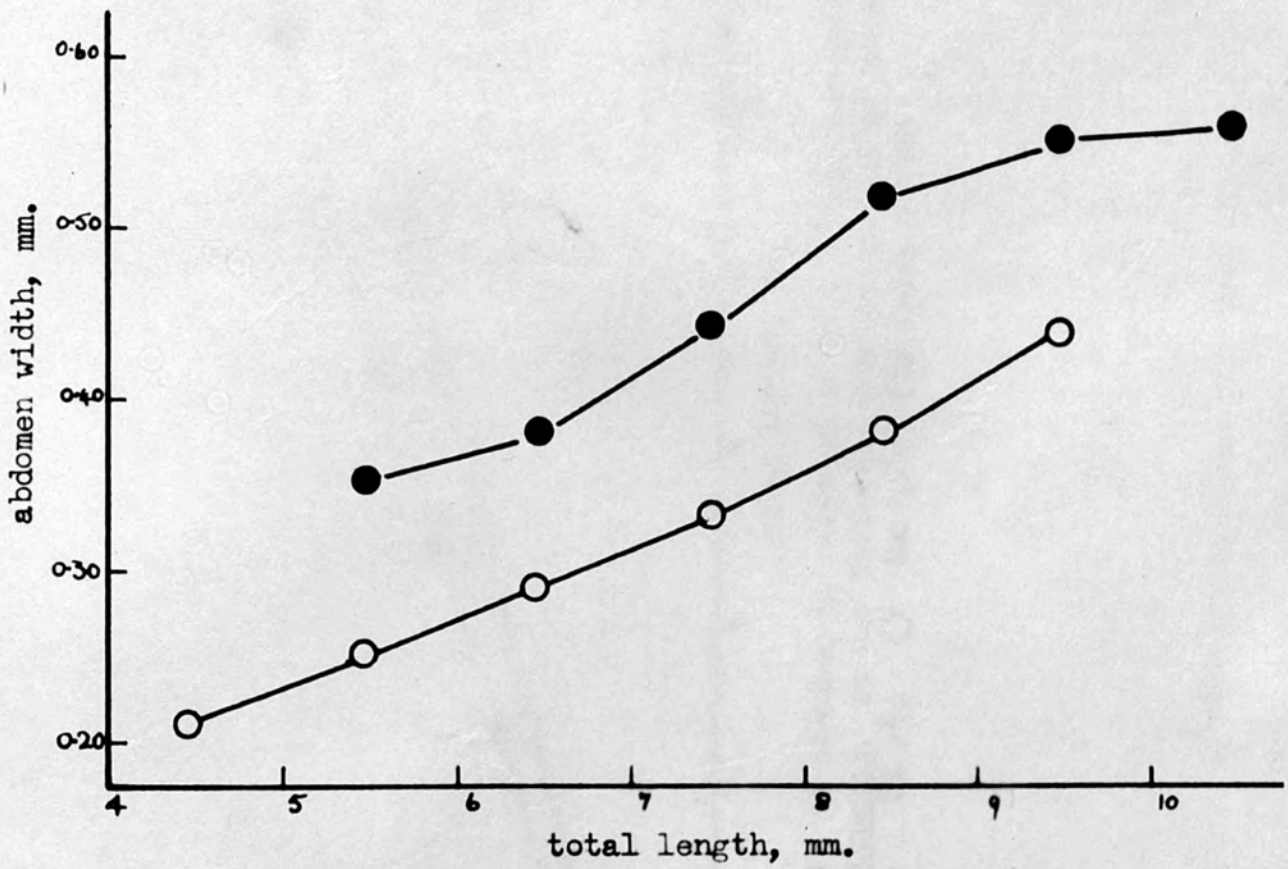


Figure 34. Width of abdomen in relation to size of males of Artemia, in two different concentrations of brine at 25°C.
● S‰.35; ○ S‰.140; Californian stock.

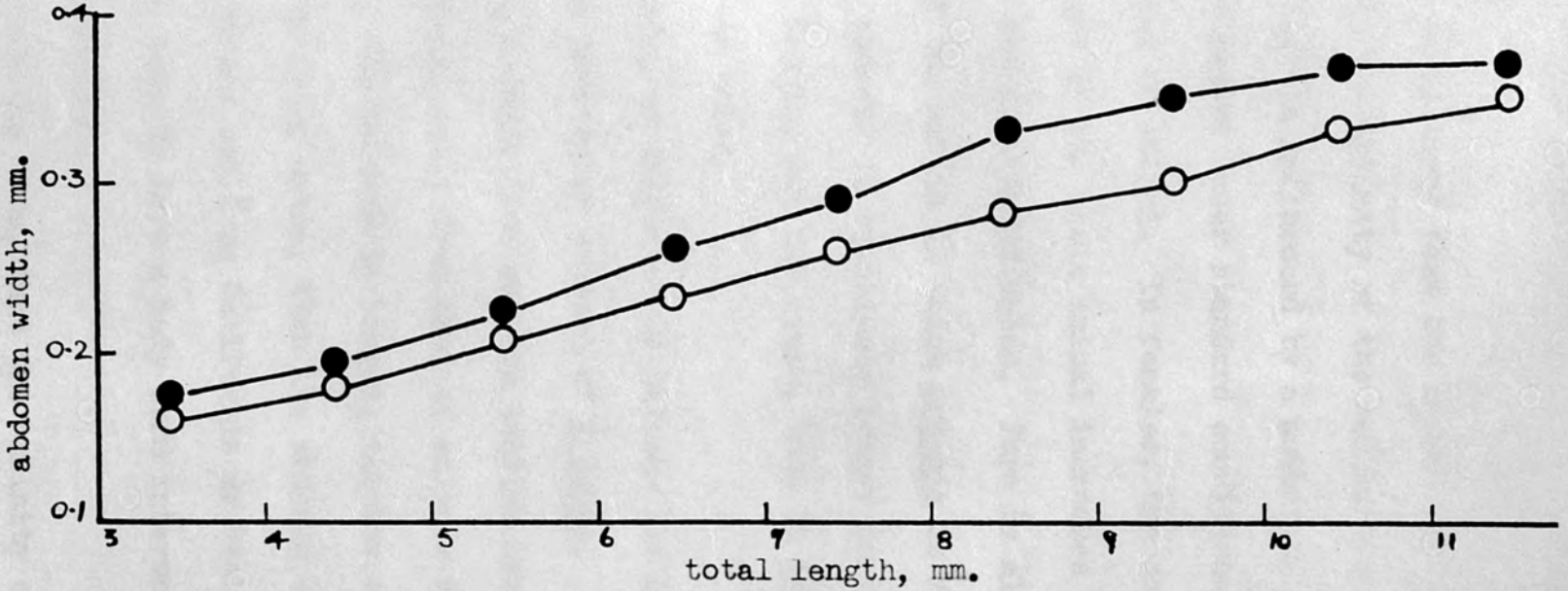


Figure 35. Width of abdomen in relation to size of parthenogenetic females of Artemia, in two different concentrations of brine at 25°C.
● S‰ 35; ○ S‰ 140; (La Palme stock)

Discussion

Thus, it is clear that one cannot generalise about the influence of the salinity of the medium upon the body form of Artemia. Form is influenced by a number of intrinsic factors when animals are reared under standard conditions: it varies with size, sex and stock of animal. In females, the abdomen becomes relatively longer as the whole animal increases in length, and is wider than the abdomen of males. Form is also influenced by the salinity of the medium in which Artemia is reared; in concentrated brine, the abdomen is relatively longer in individuals of the same total length, sex and stock, than it is in individuals reared in sea water.

It seems, as suggested by Bateson (1894), that each locality has its own particular pattern of Artemia. Under standard culture conditions, animals from western America have a body form which differs significantly from that of animals from north Africa. In the latter, the abdomen is longer, thinner and bears shorter caudal furcae, with fewer setae, than the abdomen of animals of the same total length and sex from California or Utah. Animals from south-west France seem to have a body form intermediate between the two extremes described.

It is obvious then, that the salinity of the medium is not the only factor which brings about changes in body form of Artemia. It is suggested by Thompson (1942), that the density of the medium in which Artemia lives is 'specific' to a particular body form; the

stronger the brine, the longer and thinner the abdomen. This suggestion is not supported by the results of my experiments. It has been shown that both males and females of the Algerian stock reared in sea water (S‰ 35) have a relatively longer and thinner abdomen than that of animals of the Californian stock reared in concentrated brine (S‰ 140).

It is frequently stated in the literature that no 'variable' Artemia exists in the western United States, and that the form which does occur keeps its characters constant irrespective of the salinity of the medium. This conclusion has been drawn from the results of the field observations and laboratory experiments made by Bond (1933). Since much has been inferred from these results, they are given in full below (table 4) so that they may be critically analysed.

Table 4. Body proportions of Artemia from natural and experimental brines. (Bond, 1933)

Natural brines				
Specific gravity	Total length, mm.	thorax / abdomen	No. caudal setae	No. specimens averaged
1.088	10.35	1.15	33.4	12
1.110	10.15	1.15	30.5	53
1.175	7.11	1.08	29.2	11
1.240	6.75	1.10	31.0	1
Specimens reared in the laboratory				
1.025	9.60	1.04	31.2	9
1.125	7.12	0.99	29.5	2
1.240	6.85	1.01	30.3	6

It can be seen that in both series of measurements, in natural brines and in laboratory reared animals, the average length of the animals covers a wide range. Thus the ratio of the length of the thorax to the length of the abdomen is compared in animals of different sizes. It has already been shown from the results of my own experiments, that this ratio itself varies with size. By reference to tables A and B, (Appendix I), it can be seen that a female in the 9.0 to 9.9 mm. length group, reared in sea water, has an abdomen which is 81% the length of the rest of the body; a female in the 6.0 to 6.9 mm. length group, reared in concentrated brine, has an abdomen which is 80% the length of the rest of the body. Thus, there might appear to be no difference in the relative length of abdomen of females reared in the two media. It is only when the factor of size is taken into account and animals in the same size groups are compared, that the real influence of the salinity of the medium on body form of Artemia becomes apparent. From the data published by Bond (1933), it is not possible to compare animals of the same size in different media. His conclusion, that the abdomen remains constant in length relative to the thorax, is not valid; the brine shrimps of the western United States, like those of Europe and North Africa, are 'variable' under the influence of the salinity of the medium.

EGGS AND PRE-ADULT INSTARS

It is well known that both bisexual and parthenogenetic populations of brine shrimps are established in many parts of the world. There seems to be no correlation between geographical distribution and the occurrence of males.

In America, all the brine shrimp populations of California and Utah are bisexual; males are abundant all the year round. It is stated by Siebold (1877), Samter & Heymons (1902), Jensen (1918) and Relyea (1937) that isolated females of Artemia from the Great Salt Lake are capable of reproducing parthenogenetically. I have been unable to confirm this; fifty females of the Utah stock, isolated when ten days old and reared in isolation for three months, produced neither viable eggs nor nauplii during this period. When they were put with males, all these females produced nauplii viviparously within a week. A similar result was obtained by Artom (1906a) with isolated females from the bisexual population from Cagliari, Sardinia; during five months of isolation, not a single embryo was produced by these females.

Bisexual populations, in which males are as abundant as females, are recorded from south Spain, North Africa, Egypt, Palestine and Odessa. Almost exclusively parthenogenetic populations are found in south France, North Africa, the shores of the Adriatic and in Transcaspia. A single male individual of Artemia from Molla-Kary, Trans-

caspia, is described by Samter & Heymons (1902), and Mathias (1934a) found two males in a parthenogenetic population near Sète in southern France. I have examined many hundreds of brine shrimps of this population over a period of years, both in the field and in the laboratory: during this time I have found only one male.

It must not be thought that there are only two main types of brine shrimps, bisexual and parthenogenetic. The individuals of one population may differ from those of another in respect of their chromosome numbers. Thus, among both bisexual and parthenogenetic types, there may be diploid, tetraploid, octoploid and polyploid strains (Goldschmidt, 1952).

Although such variety is to be found both in chromosome number and presence or absence of males, the mode of reproduction of brine shrimps seems to be fairly uniform. Reproduction may be either ovo-viviparous, in which actively swimming nauplii are liberated from the brood pouch of the female, or it may be oviparous, eggs being liberated from the brood pouch. In natural populations of brine shrimps, the pattern of reproduction appears to be oviparity during the spring, autumn and winter, and viviparity during the period July to September. This seasonal variation in mode of reproduction is described for populations from Capodistria, Trieste (Siebold, 1873), Cagliari (Artom, 1906a; 1906b), south France (Joly, 1840; Artom, 1931; Mathias, 1932) and California (Lochhead, 1941). In the 'salines' near Sète, Artemia is only

viviparous when the temperature of the water is above 25°C. (Mathias, 1937).

I have found that in laboratory bred populations of Artemia, kept at 25°C. and adequately fed, viviparous reproduction predominates all the year round. There is evidence, however, that temperatures above 28°C. and below 15°C. cause the animals to reproduce oviparously (Miss E. Jordan, personal communication).

Very little precise information is available on the reproductive cycle of Artemia. From the paired ovaries, eggs pass into a pair of lateral pouches and thence to a median brood pouch or ovisac. Here fertilisation occurs, embryonic membranes envelop the egg and some development takes place. Finally, either eggs or nauplii are liberated from the maternal brood pouch. After the liberation of each batch of young, the female moults. I have found that well fed females of Artemia, reared in sea water at 25°C. produce up to 200 nauplii every four days.

It is frequently stated that two types of egg are produced by females of Artemia; hard-shelled, resistant eggs, which will only hatch after a period of desiccation, and thin-shelled eggs which hatch without previous drying (Mathias, 1932; Whitaker, 1940; Jennings & Whitaker, 1941). It has been found by Lochhead (1941), however, that the hard shelled eggs may lie dormant in brine for many months and then hatch. Further, I have found that the same type of egg is capable of hatching whether previously dried or not. A batch of eggs laid by females in sea water was divided into two

groups. One group was left in the medium and the other was removed and allowed to dry slowly in air as the sea water evaporated. After three weeks desiccation, the eggs were put into sea water and 36 hours later actively swimming nauplii hatched from the eggs. At this time, the eggs which had been left in the medium had not hatched, but they did so about three weeks later. It would be unwise to assume from this single observation that the period of desiccation had hastened the development of the eggs which were removed from the medium. It does show quite clearly, however, that the same type of egg will hatch whether it has been subjected to a period of desiccation or not; the statement that two types of egg are produced should, in the meantime, be accepted with hesitation.

The eggs of Artemia, when liberated from the maternal brood pouch, consist of an outer shell enclosing egg membranes within which is the embryo. According to Dawydoff (1928), the embryo is in the form of a blastula, and in this state it may survive for long periods during which the eggs may be desiccated. When the eggs are returned to brine, development proceeds to a nauplius state before hatching occurs.

The enclosing egg membranes of Artemia were first described by Claus (1886), who thought they were secreted by the shell glands in the maternal brood pouch. This suggestion had already been made by Buchholz (1864) and Spangenberg (1875) as to the egg membranes of the freshwater anostracan Branchipus. The origin and nature

of the egg membranes of another species of fresh water anostracan, Chirocephalus diaphanus Prévost, has been investigated by Mawson & Yonge (1938). They found that two membranes are present; an inner chitinous one secreted by the epithelium of the oviduct, and an outer, wrinkled, non-chitinous membrane, the 'shell', secreted by the uterine or shell glands in the maternal brood pouch. According to Hall (1953), a third membrane is present in the eggs of Chirocephalus, lying between the two described by Mawson & Yonge (1938).

Two membranes within the outer 'shell' of the egg of Artemia are described by a number of authors (Boone & Baas Becking, 1931; Jennings & Whitaker, 1941; Weisz, 1947; Myint, 1956). According to Lochhead (1941), the nature of the membranes depends upon whether reproduction is oviparous or viviparous. In the former, two membranes are present, a thick, non-chitinous pigmented outer layer secreted by the maternal shell glands, and an inner chitinous layer secreted by the embryo. Thus, the egg has an outer 'shell' and a single inner membrane. Lochhead's observations are not in accord with those of the authors mentioned above, who describe an outer 'shell' and two inner membranes. When viviparous young are produced, Lochhead (1941) found that at least two separate membranes are formed, both thin and transparent, of chitin and 'cuticulin' mixed. He suggests that the inner membrane is secreted by the embryo and the outer one by the maternal shell glands.

The outer shell of the eggs of Artemia contains both calcium and haematin (Needham & Needham, 1930), and may vary in colour from pale cream to very dark brown (Joly, 1840; Boone & Baas Becking, 1931; Mathias, 1932). In collaboration with Dr. J. Green, I have recently found that the pale eggs of Artemia contain less haem than dark eggs. It may be, therefore, that variations in colour of the eggs are due to variations in haem content. Further, I have observed in the field that eggs laid in concentrated brine are darker than eggs laid in more dilute media. In sea water cultures in the laboratory, I have never seen dark brown eggs of Artemia: those eggs that are occasionally laid are always pale cream in colour. This subject will be discussed in detail in a later section of this thesis dealing with the pigments found in Artemia (page 158).

The ability of phyllopod eggs to withstand extreme environmental conditions is well known (Mathias, 1929). Many phyllopods inhabit small, temporary pools which are liable to dry up periodically, and thus, the eggs must be able to withstand not only desiccation but extremes of temperature.

After fourteen years drying in a desiccator, phyllopod eggs have been successfully hatched (Wolf, 1903), while Needham (1931) reports that Artemia eggs exposed to phosphorous pentoxide, a desiccating agent, at less than a half millimetre of pressure for several days, developed normally when put into brine. Mathias (1932) considers that the percentage of eggs which hatch is a function of

the length of the period of desiccation. After 3 hours desiccation at 28°C. he found that only a few eggs of Artemia hatched, but after 5 days drying at 28°C. about 60% of the eggs hatched twenty-four hours after they were put into sea water. Mathias (1932) considers three hours desiccation to be the minimum period required to promote the hatching of the eggs of Artemia; he does not mention the optimum period of desiccation. It is well known, however, that a high percentage hatch can be obtained with eggs that have been dried for many years. According to Dempster & Hanna (1956), dried eggs of Artemia sealed in ampoules under high vacuum, retained full viability for four years, whereas nearly all control eggs, stored in screw top jars for a similar period, failed to hatch. In my own experience, at least 60% of eggs of Artemia kept dry for ten years will hatch when put into sea water. The percentage of eggs that hatch declines after this length of time and I have been unable to promote hatching of eggs that have been dry for twenty years.

The dry eggs of Artemia can survive extremes of temperature. Hatching is not impaired in dry eggs kept at 83°C. for four hours (Mathias, 1934b) and 26% of eggs hatch after one hour at 103.5°C. (Hinton, 1954). The lowest recorded temperature to which the eggs of Artemia have been subjected is -190°C; after twenty-four hours at this temperature neither the percentage nor the rate of hatching is impaired (Whitaker, 1940).

The hatching of nauplii from the eggs of Artemia takes place in two phases (Jennings & Whitaker, 1941; Weisz, 1947). The embryo emerges from the egg head first, enclosed in a transparent membrane; it appears to remain attached to the shell by its posterior end. As pointed out by Weisz (1947), however, the embryo is attached not to the shell, but to a second membrane which is usually left behind in the shell after the embryo has emerged. If the embryo is shaken vigorously, the shell drops free and a crumpled outer membrane remains hanging from the tail of the embryo. This phase, the emergence of the nauplius from the egg, is complete in eighteen to twenty hours after the dry eggs have been put in sea water. During the second phase, the transparent membrane enclosing the embryo gradually softens and appears to rupture as the result of the movements of the larval appendages. This second phase, the hatching of the nauplius from the membrane, is complete in twenty-four to thirty hours after the eggs are put in sea water.

Both temperature and the salinity of the medium influence the hatching of the eggs of Artemia. The optimum temperature for hatching is 30°C. (Boone & Baas Becking, 1931); temperatures above and below this delay hatching. At 30°C. the embryos begin to emerge about ten hours after dry eggs are placed in sea water.

More information is available on the influence of the salinity of the medium on hatching. The experiments of Jensen (1918) and Barigozzi (1939), show that the time taken for the emergence of the

nauplius from the egg is directly related to the salinity of the medium. At 18 to 20°C. emergence from the egg in dilute brine (S‰ 30) is complete in forty-eight hours; in more concentrated brine (S‰ 75) emergence is complete in five days. No nauplii emerge after twenty days in brine of salinity S‰ 160. Jennings & Whitaker (1941), on the other hand, found that the percentage of nauplii which emerge from the eggs is the same in all concentrations of brine, but the numbers which subsequently hatch vary with the salinity of the medium. It is seen in figure 36, that about 95% of the embryos which emerge also hatch in brines of 50% to 225% sea water. In lower concentrations the percentage decreases until finally, in distilled water, no nauplii hatch.

Jennings & Whitaker (1941) also found that the rates of emergence and hatching are influenced by the salinity of the medium. Over a wide range of concentrations of brine (from 25% to 125% sea water) the rates of emergence and hatching are practically constant (figure 37). In the range 150% to 225% sea water, the rate of hatching decreases with increasing salinity of the medium; in dilute media emergence is accelerated and hatching retarded.

The emergence and hatching of nauplii of Artemia are influenced not only by the concentration of the medium, but also by its composition. It has been shown by a number of authors that the nauplii of Artemia are only able to hatch and survive in solutions containing particular salt combinations (Boone & Baas Becking, 1931; Jacobi &

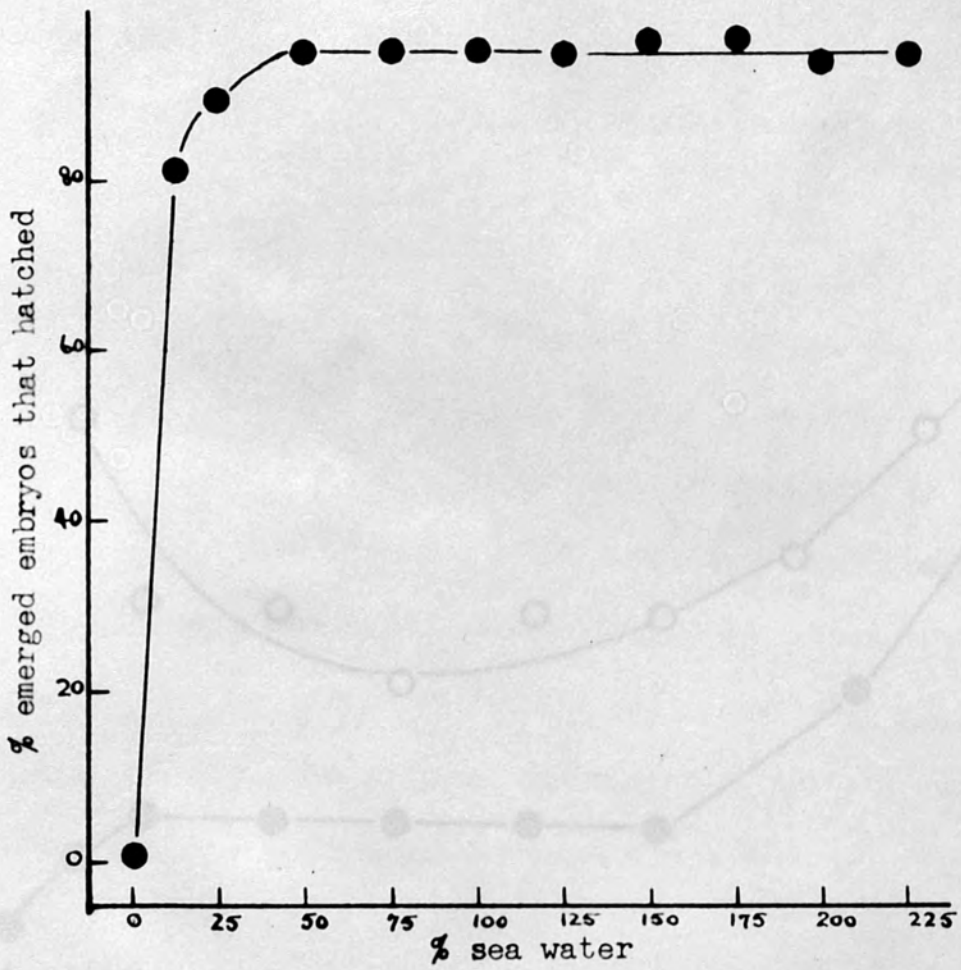


Figure 36. Percentage of emerged embryos of Artemia hatched in various concentrations of brine at 25°C. (after Jennings & Whitaker, 1941)

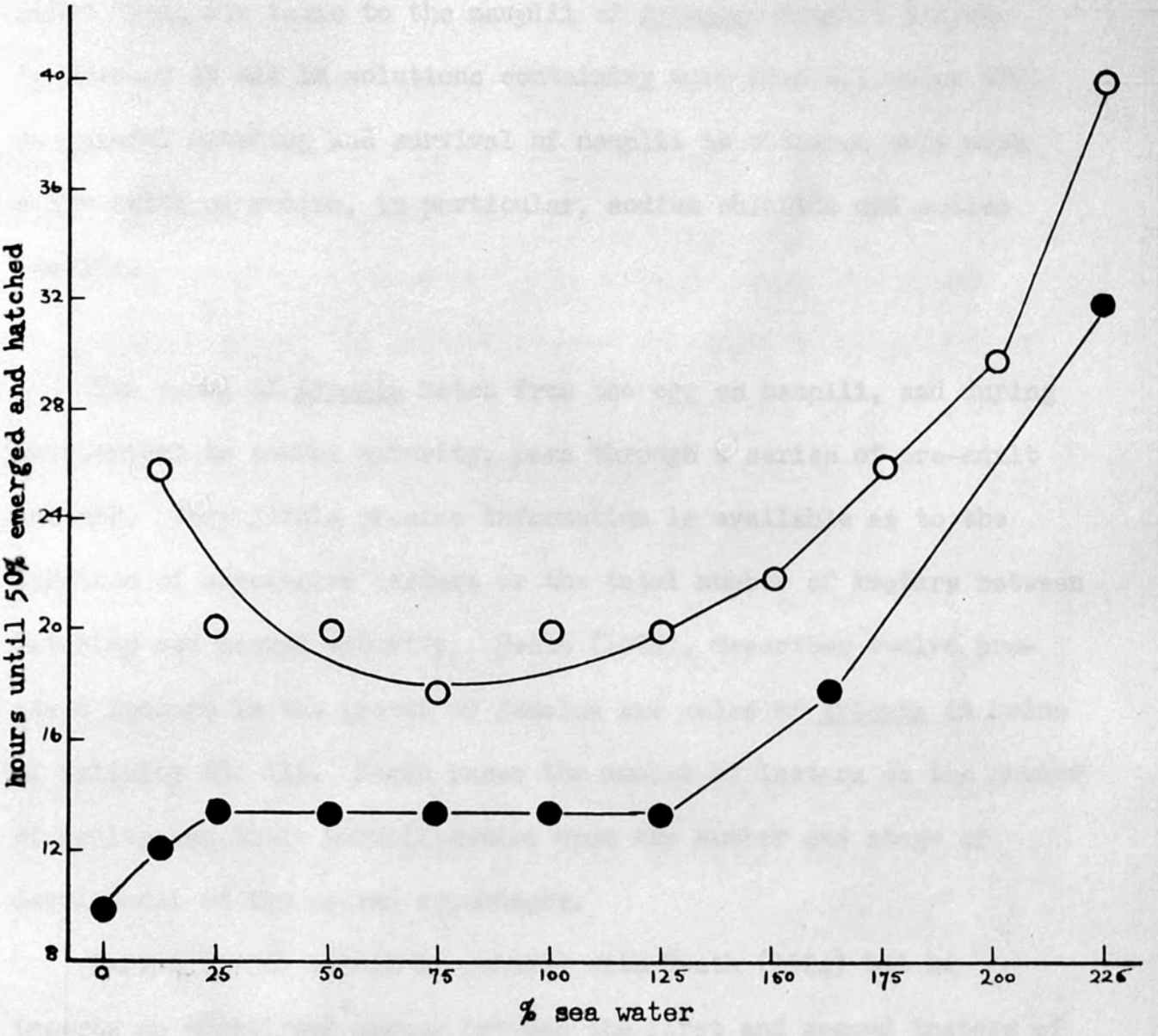


Figure 37. Rates of emerging and hatching of embryos of Artemia in various concentrations of brine at 25°C.
● emerged; O hatched. (after Jennings & Whitaker, 1941)

Baas Becking, 1933; Baas Becking, Karstens & Manner, 1936). The chlorides of calcium and magnesium, while permitting life for a short time, are toxic to the nauplii of Artemia; nauplii failed to develop at all in solutions containing more than 0.1 molar KCl. Successful hatching and survival of nauplii is obtained only with a few salts of sodium, in particular, sodium chloride and sodium bromide.

The young of Artemia hatch from the egg as nauplii, and during development to sexual maturity, pass through a series of pre-adult instars. Very little precise information is available as to the duration of successive instars or the total number of instars between hatching and sexual maturity. Heath (1924), describes twelve pre-adult instars in the growth of females and males of Artemia in brine of salinity S‰ 115. Heath bases the number of instars on the number of moults and their identification upon the number and stage of development of the paired appendages.

Warren (1938) agrees in general with Heath (1924) but he inserts an additional instar between the first and second instars of Heath. It is not clear from his work whether Warren observed a moult to have occurred between the first and second instars described by Heath. I have observed that the nauplius of Artemia, on hatching, shows no sign of segmentation of the abdomen; soon after hatching, however, the abdomen elongates and external signs of segmentation become visible although no moult appears to have taken place.

According to Weisz (1946) a range of from twelve to sixteen moults occurs between hatching and sexual maturity of Artemia in brine of salinity S‰ 115, while in a more dilute medium (S‰ 30), from twenty-five to twenty-nine moults occur. Thus a staging of larval development according to moults can only apply for a precise concentration of the external medium.

It is clear that there is little information on the stages of growth of Artemia up to sexual maturity. Data on the number of pre-adult instars is inadequate, and no information is available as to the duration of successive instars under controlled conditions. I am not aware of any work that has been done on other Anostraca with respect to stages of growth. This is in marked contrast to other branchiopods, particularly the Cladocera. As a result of the investigations of many workers, much detailed information is available on the growth of Daphnia and the factors which influence the number and duration of the pre-adult and adult instars (for references, see Green, 1956a).

OSMOTIC AND IONIC REGULATION

Brine shrimps are found in saline waters which differ greatly in concentration and composition. The successful colonisation of such environments would seem to involve almost complete independence of the external medium or the ability to adapt to wide variations in its concentration. This could be achieved either by maintaining a high degree of impermeability, or by having some regulatory mechanism which would adjust the composition of the body fluid in relation to changes in the concentration of the external medium.

The work of several authors, on the survival of Artemia in various media, suggests that brine shrimps are not entirely impermeable to the surrounding medium (Martin & Wilbur, 1921; Boone & Baas Becking, 1931; Jacobi & Baas Becking, 1933; Baas Becking, Karstens & Manner, 1936). More recently, Croghan (1958a) has confirmed and extended these earlier observations. Successful survival of Artemia is only obtained in media in which sodium chloride or sodium bromide predominate; certain substances, particularly potassium, are highly toxic although the toxicity of potassium ions can be antagonised by sodium ions.

Clearly, Artemia is not insensitive to the chemical composition of the external medium; it is not impermeable. This is confirmed by Ussing (in Krogh, 1939) who studied the exchange of heavy water from the external medium into the animal. A slow rate of exchange

was measured, showing that the permeability of Artemia to water is low. Nevertheless, Artemia is permeable, and thus its survival in highly saline waters must be due to the presence of a regulatory mechanism which maintains the concentration of the haemolymph at a relatively constant level.

There is much evidence indicating that the haemolymph of Artemia is hypotonic to the external medium. Martin & Wilbur (1921), as the result of determinations of the ash weight in Artemia, conclude that the very low weight obtained could only be accounted for had the concentration of body fluids been much less than that of the surrounding medium. Measurements of the osmotic pressure of the blood of Artemia have been made by Medwedewa (1927), Warren, Kuenen & Baas Becking (1938), Kuenen (1939) and Plattner (1955). These authors all agree that the blood is maintained hypotonic to the external environment, even in very concentrated media (S‰ 285), and that most of the osmotic pressure is due to sodium and chloride ions. Recently, Croghan (1958b) has extended these investigations to cover a wider range of concentrations of brine and to determine in greater detail the chemical composition and osmotic pressure of the haemolymph in relation to that of the external medium. The results of these experiments by Croghan are shown in figures 38 and 39. They confirm the findings of previous workers that the blood of Artemia is hypotonic to the external medium. It is at once seen that the

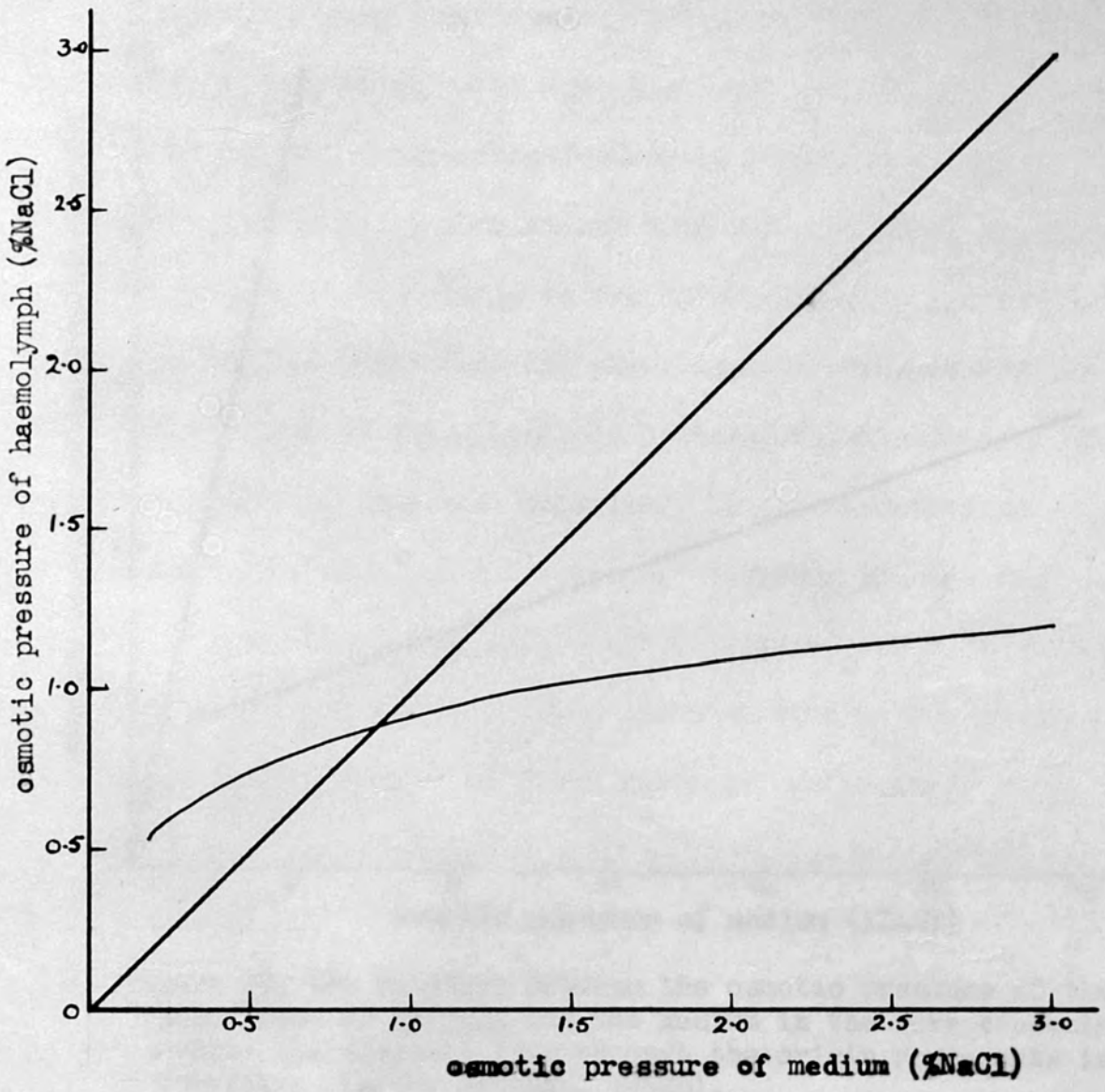


Figure 38. The relation between the osmotic pressure of the haemolymph of *Artemia* and the medium in the more dilute media. The diagonal line through the origin represents isotonicity. (after Croghan, 1958b)

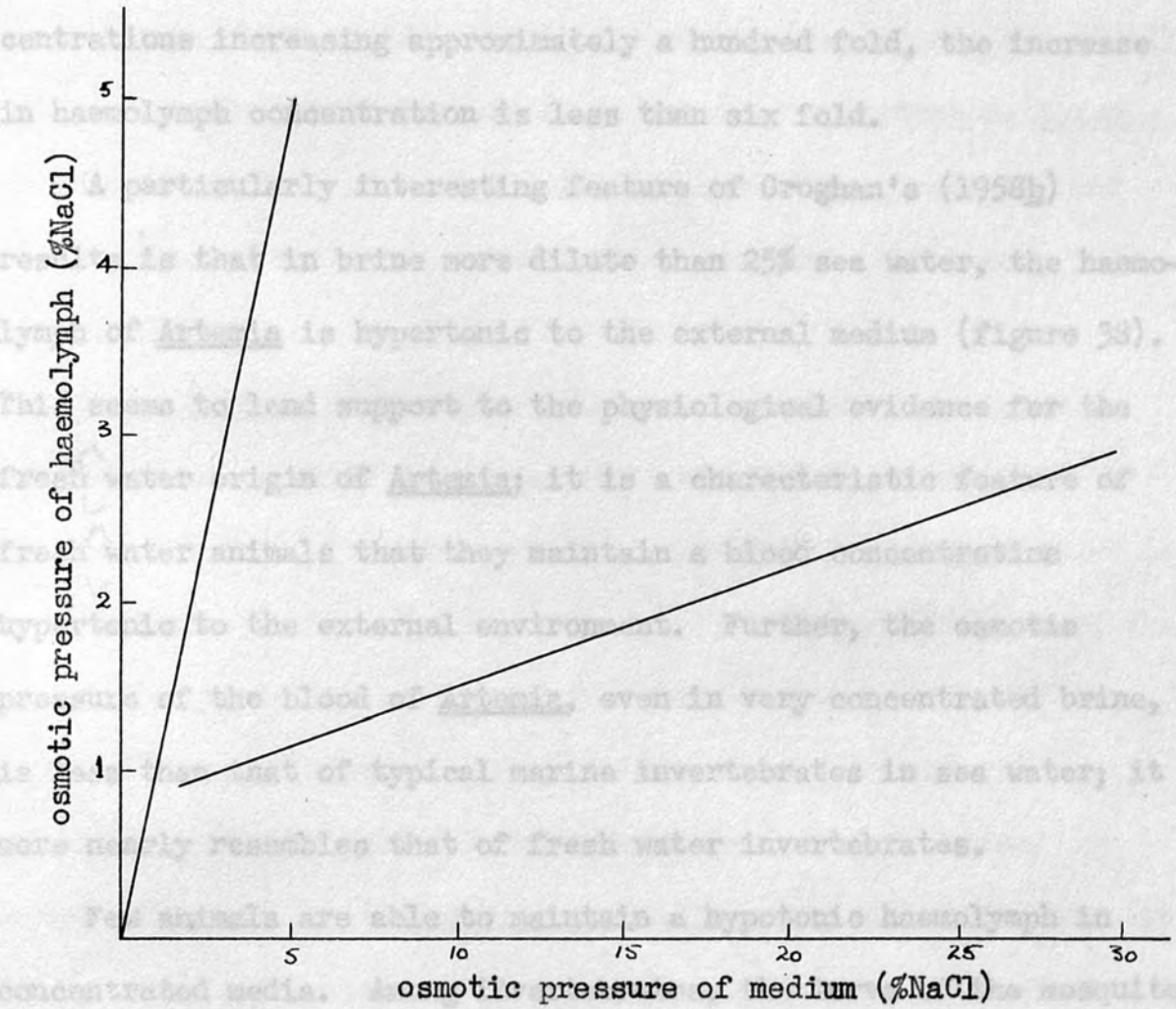


Figure 39. The relation between the osmotic pressure of the haemolymph of Artemia and the medium in the more concentrated media. The diagonal line through the origin represents isotonicity. (after Croghan, 1958b).

haemolymph concentration remains relatively constant and independent of the surrounding medium. Over a range of external concentrations increasing approximately a hundred fold, the increase in haemolymph concentration is less than six fold.

A particularly interesting feature of Croghan's (1958b) results is that in brine more dilute than 25% sea water, the haemolymph of Artemia is hypertonic to the external medium (figure 38). This seems to lend support to the physiological evidence for the fresh water origin of Artemia; it is a characteristic feature of fresh water animals that they maintain a blood concentration hypertonic to the external environment. Further, the osmotic pressure of the blood of Artemia, even in very concentrated brine, is less than that of typical marine invertebrates in sea water; it more nearly resembles that of fresh water invertebrates.

Few animals are able to maintain a hypotonic haemolymph in concentrated media. Among invertebrates, the larva of the mosquito Aedes detritus Edw. can maintain its blood hypotonic to the external medium up to an external concentration of about 10% NaCl (Beadle, 1939). Some brackish water prawns, in particular Palaemonetes varians (Leach), when in sea water, have a blood concentration hypotonic to the external medium (Panikkar, 1941). Many grapsoid crabs, which can live successfully either in sea water or fresh water, maintain the blood hypotonic to a concentrated medium and hypertonic to dilute media (Schlieper, 1935; Conklin & Krogh, 1938;

Krogh, 1939; Jones, 1941). The ability of marine teleosts to maintain a blood concentration hypotonic to sea water is well known (Smith, 1930; 1932). Until the recent work of Croghan (1958), it was assumed that the mechanism of osmotic regulation in Artemia is similar to that of marine teleosts, water being retained and salt eliminated against a concentration gradient (Schlieper, 1930; Pantin, 1931).

Since Artemia maintains a relatively constant haemolymph osmotic pressure while in a hypertonic medium, a mechanism for the active uptake of water against a concentration gradient must be in operation. It has been demonstrated by Croghan (1958b) that the gut of Artemia is the most permeable part of the animal, and in a subsequent paper (Croghan, 1958c) he has shown that the gut epithelium actively takes up water from the external medium. In common with many Crustacea, Artemia swallows the medium in which it lives. This swallowing, both oral and anal, has been described and discussed by Fox (1952), who considers that the anal intake of water acts as an enema, stretching the gut wall muscles and facilitating defaecation. The oral swallowing of the medium by Artemia is clearly important to the animal as a source of water. In concentrated media, there might be a danger of the animal becoming dehydrated as the result of the osmotic gradient between the gut fluid and the haemolymph; the prevention of this demands exceptional powers of water transport of the part of the gut epithelium. It is

shown by Croghan (1958c), however, that although Artemia may swallow a medium very hypertonic to the haemolymph, the osmotic pressure of the gut fluid is always markedly lower than that of the external medium. Thus, not only water but also salts must be actively taken up by the gut epithelium. This mechanism of salt uptake will clearly help to solve the problem of water uptake from the gut against a steep gradient. The removal of salts from the gut fluid, with a resultant lowering of osmotic pressure, will reduce the gradient between gut fluid and haemolymph to one which can be overcome by the gut epithelium. Croghan (1958c) suggests that the net gain of water to Artemia is considerable, as he has demonstrated that dyes suspended in the medium and taken into the gut lumen are rapidly concentrated. Any excess water passing into the haemolymph is presumably excreted by the maxillary glands, although there is no experimental evidence in support of this.

Thus a mechanism is present in the gut epithelium of Artemia for the active uptake of sodium chloride and water from the gut fluid. To maintain the concentration of the haemolymph hypotonic to that of the external medium, salt taken up by the gut epithelium and passed into the haemolymph must be eliminated. Using silver staining techniques, Dejdar (1930) and Croghan (1958d) have shown that the branchiae (epipodites) of the first ten pairs of thoracic limbs of Artemia become white in silver nitrate solution, and subsequently turn black when put in developer solution. Such staining

of localised parts of crustacean cuticle has been interpreted as indicating the site of active chloride uptake (Koch, 1934; Krogh, 1939). In Artemia, however, this is unlikely to apply, but it does indicate that the branchiae of the first ten pairs of limbs are permeable. Since silver stain is limited to the branchiae, it follows that the permeability of the external cuticle is limited to that of the branchiae. It is likely, therefore, that these structures are responsible for the elimination of salt from Artemia. It is interesting to note that the first ten pairs of branchiae of the fresh water anostracan Chirocephalus also stain with silver, which suggests that this may be the site of salt uptake (Dejdar, 1930; Panikkar, 1941b; Croghan, 1958d).

Further evidence of the function of the branchiae of Artemia in salt elimination is provided by experiments in which the branchiae are destroyed by treatment with potassium permanganate (Croghan, 1958d), the animals subsequently being placed in dilute media of different salt concentration. Measurements of the haemolymph osmotic pressure of these animals show that they have entirely lost the ability to osmoregulate. The haemolymph is isotonic with the external medium up to an external concentration of about 75% sea water. This appears to be the maximum concentration of salt that the tissues of Artemia can tolerate.

There is, therefore, a striking similarity in the mechanisms of osmotic and ionic regulation between Artemia and marine teleosts. It has been demonstrated by Keys (1931) and by Keys & Willmer (1932),

that chloride is secreted by the gills of many marine teleosts, and Smith (1930; 1932) has shown that the gut of marine teleosts is the site of water uptake. Thus, in both Artemia and marine teleosts salt is secreted by the gills and water regulation takes place in the gut.

It has been shown by Croghan (1958b) that the haemolymph of nauplii of Artemia is hypotonic to sea water; a mechanism for osmoregulation must be present. In the nauplii, however, the limb buds are just beginning to develop and there are no branchiae. A conspicuous structure present in the nauplius of Artemia and absent in the adult, is the neck or dorsal organ. As the result of his early work on vital staining and staining with silver nitrate, Dejdar (1930) considers that the dorsal organ of the nauplius and the branchiae of the adult are part of the same physiological system. He concludes that they are respiratory organs, the dorsal organ of the nauplius being replaced functionally by the branchiae as the adult limbs develop. Croghan (1958b) confirms these observations on the staining properties of the dorsal organ of the nauplius, but he considers that this structure is concerned with the excretion of sodium chloride. With the development of the adult limbs, the dorsal organ is functionally replaced by the branchiae and so it gradually degenerates. It is interesting to note, as pointed out by Panikkar (1941b), that in those phyllopods which

have no branchiae on the adult limbs, as in Leptodora, the dorsal organ is retained.

THE OXYGEN CONSUMPTION OF *ARTEMIA*

The ability of *Artemia* to regulate the osmotic and ionic concentration of its blood has been discussed (page 87). Brine shrimps maintain a relatively constant and markedly hypotonic blood osmotic pressure over a wide range of concentrations of the external medium. In order to maintain this water and salt balance energy must be expended, and it seems reasonable to suggest that the steeper the osmotic gradient between internal and external environments, the more the energy that will be expended. It has been stressed by Peters (1935), however, that the total mass of tissue responsible for the water and salt regulation of an animal is generally small relative to the total bulk of the animal's tissues; changes in metabolism of the osmo-regulatory tissue are largely masked by the metabolic activity of the whole animal. More recently, Potts (1954) has estimated theoretically the total osmotic work performed by some fresh and brackish water animals. In the case of the grapsoid crab, *Eriocheir sinensis* Milne-Edw. when in fresh water, only about 0.5% of the total metabolic energy is concerned with osmotic work. Thus, variations in the osmotic work performed by an animal may not be apparent from measurements of total metabolic work.

Brine shrimps are found in media of widely differing concentrations and it has already been suggested that the animals

must perform more osmotic work in concentrated than in dilute media. It is of interest, therefore, to know whether such differences in energy expenditure influence the respiratory demands of Artemia.

The oxygen consumption of Artemia in brines of different concentrations has been measured by Kuenen (1939) and by Eliassen (1952); their results are not in agreement. Kuenen (1939) found that the rate of respiration in concentrated brine (S‰ 116) was one and a half times as rapid as in a more dilute medium (S‰ 29). On the other hand, Eliassen (1952) found a decrease in oxygen consumption as the salinity of the external medium increased. This effect was most marked in the nauplii and became less apparent with increasing size of the animals.

In view of these conflicting results it seemed advisable to attempt to discover whether there are any measurable differences in demand for oxygen by brine shrimps living in different concentrations of brine.

Two important factors must be taken into account in determining the rate of oxygen consumption of an animal. Firstly, the effect of the oxygen concentration of the external medium on the oxygen consumption of the animal must be determined. If oxygen consumption is dependent upon oxygen concentration, experiments to compare the oxygen uptake of an animal in two different media must be made at the same level of oxygen concentration. Secondly,

the relationship between oxygen consumption and the size of the animal must be considered. The importance of the 'size factor' in comparative studies on oxygen consumption has been extensively investigated (Weymouth, Crismon, Hall, Belding & Field, 1944; Kleiber, 1947; Ellenby, 1951; Berg, 1952 & 1953; Zeuthen, 1953 & 1955; Bertalanffy, 1957). It has been shown by Bertalanffy & Krywienczyk (1953), studying the oxygen consumption of Artemia in one concentration of brine, that oxygen uptake is proportional to the square of the body length. Presupposing that body proportions in Artemia do not change with increasing size (an assumption which they do not substantiate), they conclude that oxygen uptake in Artemia is proportional to the surface area.

When comparing the oxygen consumption of brine shrimps reared in different salinities, surface area, expressed as the square of a linear dimension, can only be used as a standard for comparison if it has been established that the shape of the animals remains constant over the size range and in the different media. Further, the use of the two-thirds power of the body weight as an estimate of surface area (Rubner, 1883) is only justified if both specific gravity and body shape are constant over the size range and in the different media. These factors, therefore, are considered in conjunction with experiments on the oxygen consumption of Artemia.

OXYGEN CONSUMPTION IN RELATION TO OXYGEN CONCENTRATION

Materials and methods.

Experiments to determine the influence of the concentration of dissolved oxygen in the medium upon the oxygen consumption of Artemia were made with laboratory bred females of the Californian and La Palme stocks. The animals were reared in brines of the same salinity as those in which the oxygen consumption was to be determined. Only animals between two and three weeks old were used, and thus the experiments were not complicated by the presence of eggs or embryos in the brood pouch of the females.

The oxygen uptake of Artemia was measured in a 20 ml. all-glass hypodermic syringe (figure 40), using a method similar to that described by Ewer (1942). The exact volume of the respiratory chamber was determined as follows. The respiratory chamber was filled up to a fixed mark with M/240 potassium iodate solution and the nozzle of the syringe was stoppered with a piece of glass rod. The outside of the syringe and glass rod were washed with distilled water to remove any traces of the iodate solution. The contents of the respiratory chamber were then expelled into a conical flask and the syringe was twice rinsed with distilled water. To the solution in the flask were added 2.5 ml. ortho-phosphoric acid and 2.5 ml. 5% potassium iodide solution. The solution of potassium iodate was then titrated against N/40 sodium thiosulphate solution, using starch as indicator. Thus, from the mean of three determinations,

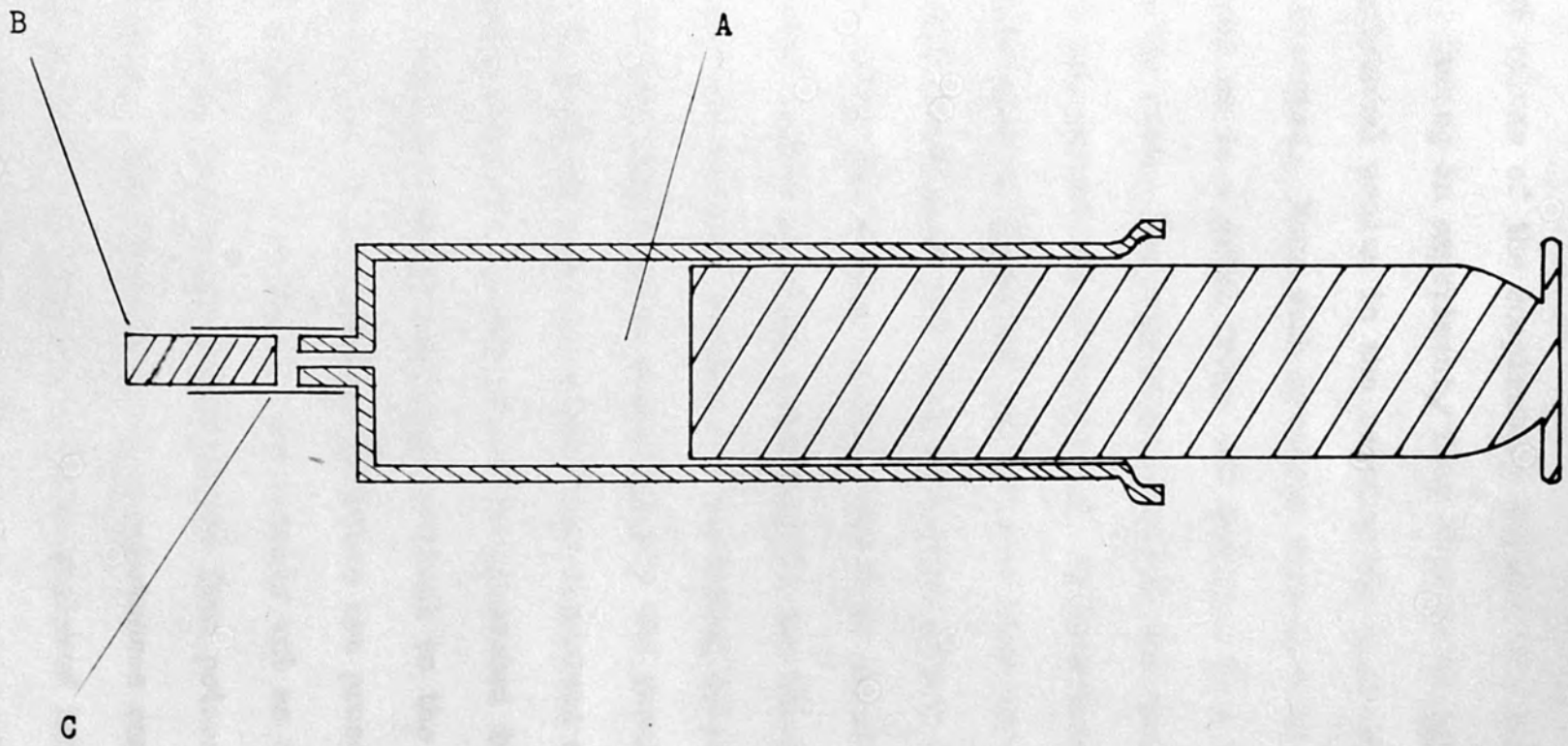


Figure 40. Respiratory vessel. A, respiratory chamber. B, glass nozzle. C, rubber tubing.

the exact volume of the respiratory chamber was measured for each syringe. During an experiment, four females of Artemia were put in the experimental medium in the respiratory chamber, and the glass rod was inserted. Four such syringes were used at a time and each was clipped on to a metal frame and immersed in a water bath at 25°C. in the dark. The oxygen consumption was measured in two different concentrations of brine, S‰ 35 (sea water) and S‰ 210. All measurements of dissolved oxygen were made by the syringe-pipette modification of the Winkler method (Fox & Wingfield, 1938).

When using the Winkler method, which is based on the oxidation of potassium iodide solution resulting in the liberation of iodine, it is important to know whether any oxidising substances are present in the sample which might falsify the results of the method. If such substances, other than dissolved oxygen itself, are present, then the amount of iodine liberated during a Winkler analysis would not be entirely proportional to the amount of dissolved oxygen in the sample. Nitrites are present in sea water in small amounts and although they usually act as reducing agents, they will cause the liberation of iodine from potassium iodide in acid solution. The presence of such substances can be demonstrated by taking a sample of the medium to be analysed into the syringe pipette without the first Winkler reagent, manganous chloride, and then continuing as for a normal analysis by adding the other Winkler reagents. If a blue colour is obtained on the addition of starch

to the final solution, then iodine has been liberated from iodide. In this 'blank' analysis, the liberation of iodine could only have been brought about by oxidising substances, such as nitrites, in the sample. In the experiments on the oxygen consumption of Artemia, therefore, when a sample of the medium was withdrawn for dissolved oxygen analysis, a second sample was withdrawn, the 'blank'. This 'blank' was treated as described above, omitting the addition of manganous chloride from the procedure, and any blue colour obtained on the addition of starch was titrated against sodium thiosulphate solution. The amount of sodium thiosulphate used in the 'blank' analysis was then subtracted from the amount used in the titration of the sample. In this way, the amount of iodine liberated by nitrites or similar substances in the medium, was taken into account in the calculation of the dissolved oxygen content of the sample.

At intervals of approximately one hour throughout the experiment, two samples of brine were withdrawn from the respiratory chamber using the syringe-pipette described by Fox & Wingfield (1938). The glass rod is removed from the nozzle of the syringe and the nozzle of the syringe-pipette is inserted in its place. Since the respiratory chamber is in the form of a syringe, the plunger of the syringe slides in to compensate for the volume of brine withdrawn. In this way a series of measurements of oxygen uptake can be made without bringing air into contact with the brine

in the respiratory chamber. The exact volume of sample withdrawn in the syringe-pipette is known and thus the decrease in volume of brine in the respiratory chamber can be calculated.

Each experiment lasted about four hours. The animals were then removed from the respiratory chamber and rinsed rapidly in distilled water. They were put into crucibles of known weight, dried for fifteen hours at 100°C. and weighed. Certain precautions had to be taken in determining the dry weight of the animals. The weight of the crucibles was determined as follows. They were kept overnight in an oven at 100°C. and then transferred to a desiccator to cool. After exactly 30 minutes, the crucibles were removed from the desiccator and immediately weighed. Precisely the same procedure was followed in determining the weight of the crucibles plus the animals. In this way accurate measurements were obtained.

The oxygen consumption during each respiratory period was calculated in millilitres of oxygen per gram dry weight per hour. The average oxygen concentration of the brine during a respiratory period was calculated from the initial and final concentrations.

Results

The results of experiments on the influence of the oxygen concentration of the medium upon the oxygen uptake of Artemia are shown in figures 41 and 42. The numerical data from which these figures are compiled will be found in Appendix II (tables A to D).

The oxygen consumption of females of the Californian stock reared in sea water, remains relatively constant until the concentration of dissolved oxygen in the medium has fallen to 2.75 ml./l. (figure 41). At oxygen concentrations below this, the oxygen consumption falls steadily. For females reared in the concentrated medium, there is no significant difference in oxygen uptake until the external concentration has fallen to 1.25 ml./l. At concentrations below this, oxygen consumption decreases with decreasing external concentration.

Parthenogenetic females of the La Palme stock, reared in sea water, have a constant oxygen consumption, independent of the oxygen concentration of the external medium, until the external concentration has fallen to 1.25 ml./l. (figure 42). At concentrations below this, oxygen consumption falls steadily. A similar result was obtained with parthenogenetic females reared in the more concentrated medium. At oxygen concentrations below 1.25 ml./l., the oxygen consumption decreases with decreasing external oxygen concentration. Thus, the parthenogenetic females in both media, have an 'independent' metabolism until the external oxygen content falls to 1.25 ml./l.; below this level, metabolism is dependent upon the external concentration of oxygen.

There appears to be a physiological difference between the females of the two stocks. The metabolism of the Californian females, reared in sea water, becomes 'dependent' at a higher external

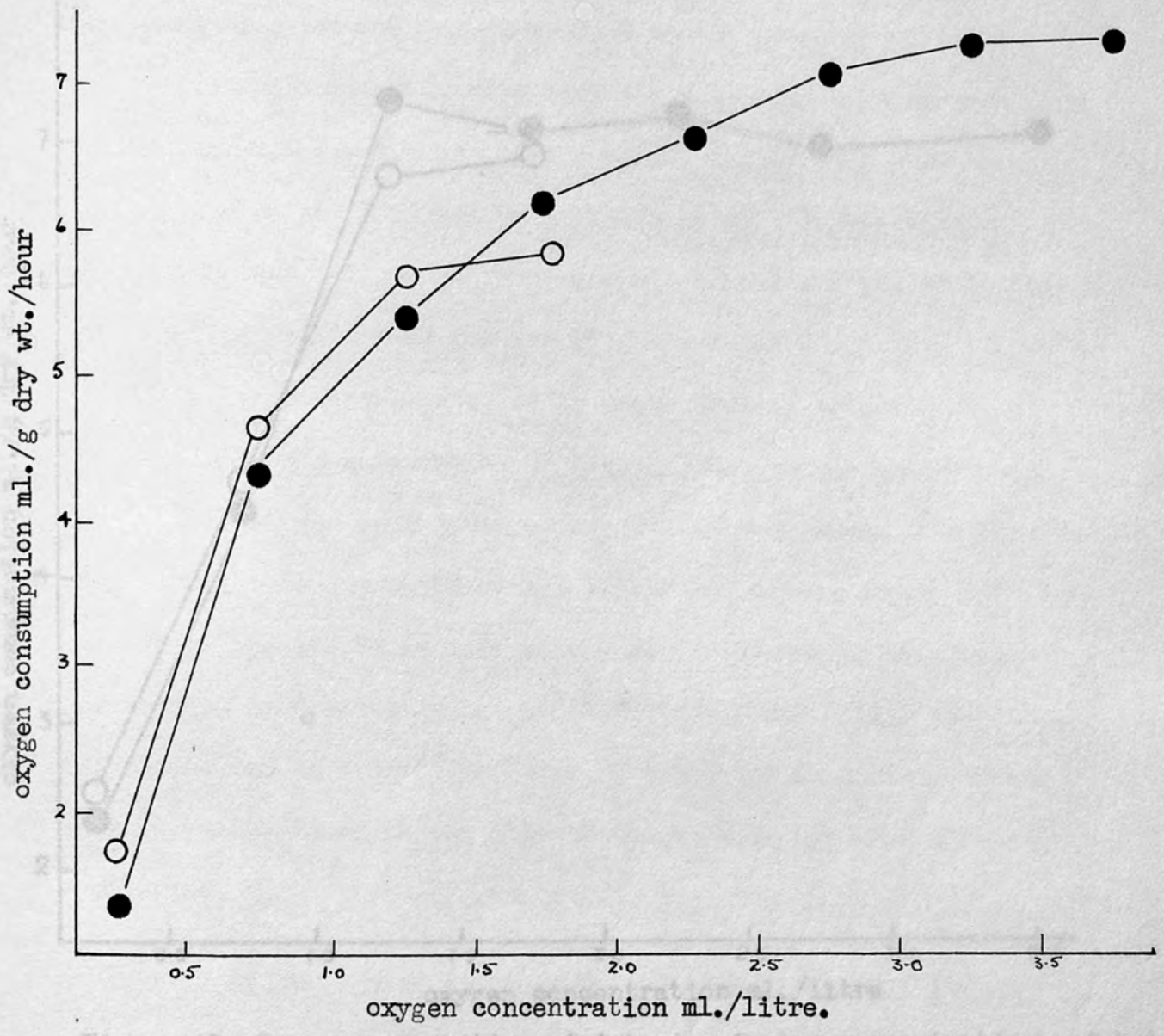


Figure 41. Oxygen consumption of Artemia, in two concentrations of brine, in relation to the oxygen content of the medium. ● S‰ 35; ○ S‰ 210. (Californian stock)

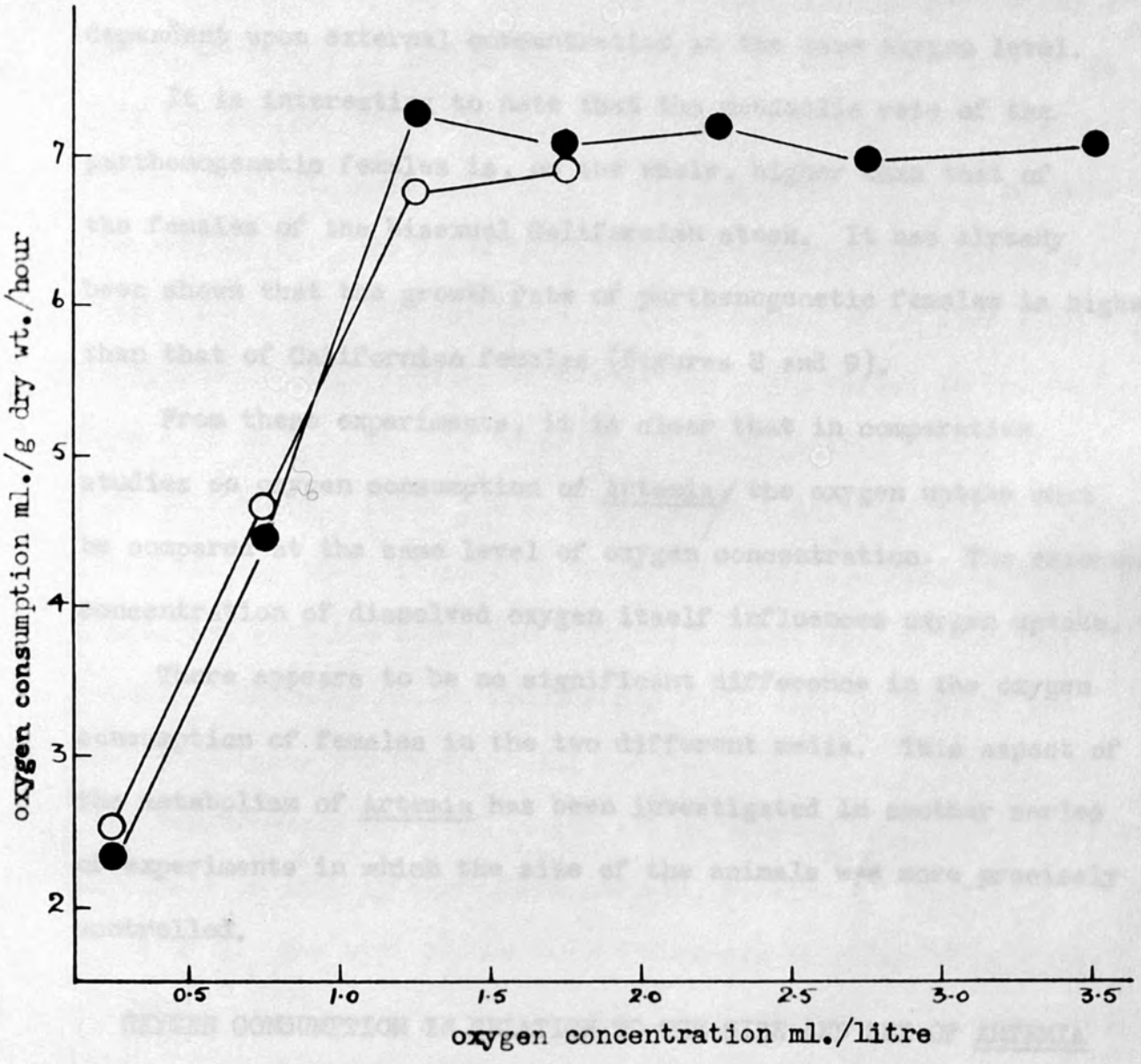


Figure 42. Oxygen consumption of Artemia, in two concentrations of brine, in relation to the oxygen content of the medium. ● S‰. 35; ○ S‰. 210. (La Palme stock)

concentration of dissolved oxygen than does the metabolism of the parthenogenetic females in the same medium. In the more concentrated medium, the metabolism of females of both stocks becomes dependent upon external concentration at the same oxygen level.

It is interesting to note that the metabolic rate of the parthenogenetic females is, on the whole, higher than that of the females of the bisexual Californian stock. It has already been shown that the growth rate of parthenogenetic females is higher than that of Californian females (figures 8 and 9).

From these experiments, it is clear that in comparative studies on oxygen consumption of Artemia, the oxygen uptake must be compared at the same level of oxygen concentration. The external concentration of dissolved oxygen itself influences oxygen uptake.

There appears to be no significant difference in the oxygen consumption of females in the two different media. This aspect of the metabolism of Artemia has been investigated in another series of experiments in which the size of the animals was more precisely controlled.

OXYGEN CONSUMPTION IN RELATION TO THE SIZE AND SEX OF ARTEMIA AND TO THE SALINITY OF THE EXTERNAL MEDIUM

Material and methods

Experiments were made with males and females of the Californian stock reared at 25°C. in brines of the same salinity as those in

which the oxygen consumption was to be determined, namely S‰ 35 and S‰ 140. Experiments were made on two size groups, small and large animals as defined below. Small males and females reared in sea water varied in length from 3.82 to 4.80 mm. and from 3.95 to 4.82 mm. respectively; those reared in concentrated brine (S‰ 140) varied in length from 3.27 to 3.63 mm. and from 3.40 to 3.67 mm. respectively. Large males reared in sea water varied in length from 6.27 to 7.25 mm. and those in the stronger brine from 5.51 to 6.80 mm. Large females reared in sea water varied in length from 6.75 to 7.60 mm. and those in the concentrated medium from 5.42 to 6.25 mm. These two size groups were selected because they represent the smallest size at which the sexes are readily distinguished with the naked eye, and the largest size of immature animals. Sexually mature females may carry eggs or embryos and thus complicate the experimental results.

Measurements of total length and of abdomen length and breadth were made on lightly narcotised animals, as already described (page 8). The area of the second antennae of males of Artemia, in arbitrary units, was measured with a planimeter. The narcotised animals were laid ventral side down on a microscope slide and supported in a drop of the experimental medium. The large second antennae were bent forward to extend in front of the head as shown in figure 43. Using a prism and mirror attached to a microscope,

the image of the head was projected on to white paper and the outline of the antennae traced. It was found more convenient to trace the outline on paper and then determine the area of the antennae with a planimeter, rather than to determine the area directly from the projected image. Commencing at A in figure 43 and proceeding in an anti-clockwise direction to B, the outline of each antenna was traced with the point of the planimeter. At B, a straight line was drawn back to A. In this way the approximate area of each antenna was determined in arbitrary units; the average of the measurements for each antenna was taken as representing the 'antennal area' of the individual. Only the area of the apical 'joint' distal to the line AB was measured, because the region just proximal to this line is frequently telescoped into the basal 'joint' and its area cannot easily be measured.

The dissolved oxygen content of brine of salinity S‰ 140 at 25°C. is approximately 2.8 ml./l. and in order to measure oxygen consumption at the same level of oxygen concentration in the two media, the oxygen content of sea water had to be reduced from about 4.9 ml./l. (100% air saturation). This was done by bubbling nitrogen through the sea water until the dissolved oxygen content was approximately 2.8 ml./l. All measurements of dissolved oxygen were made by the syringe-pipette modification of the Winkler method (Fox & Wingfield, 1938).

Experiments on oxygen consumption were made with animals

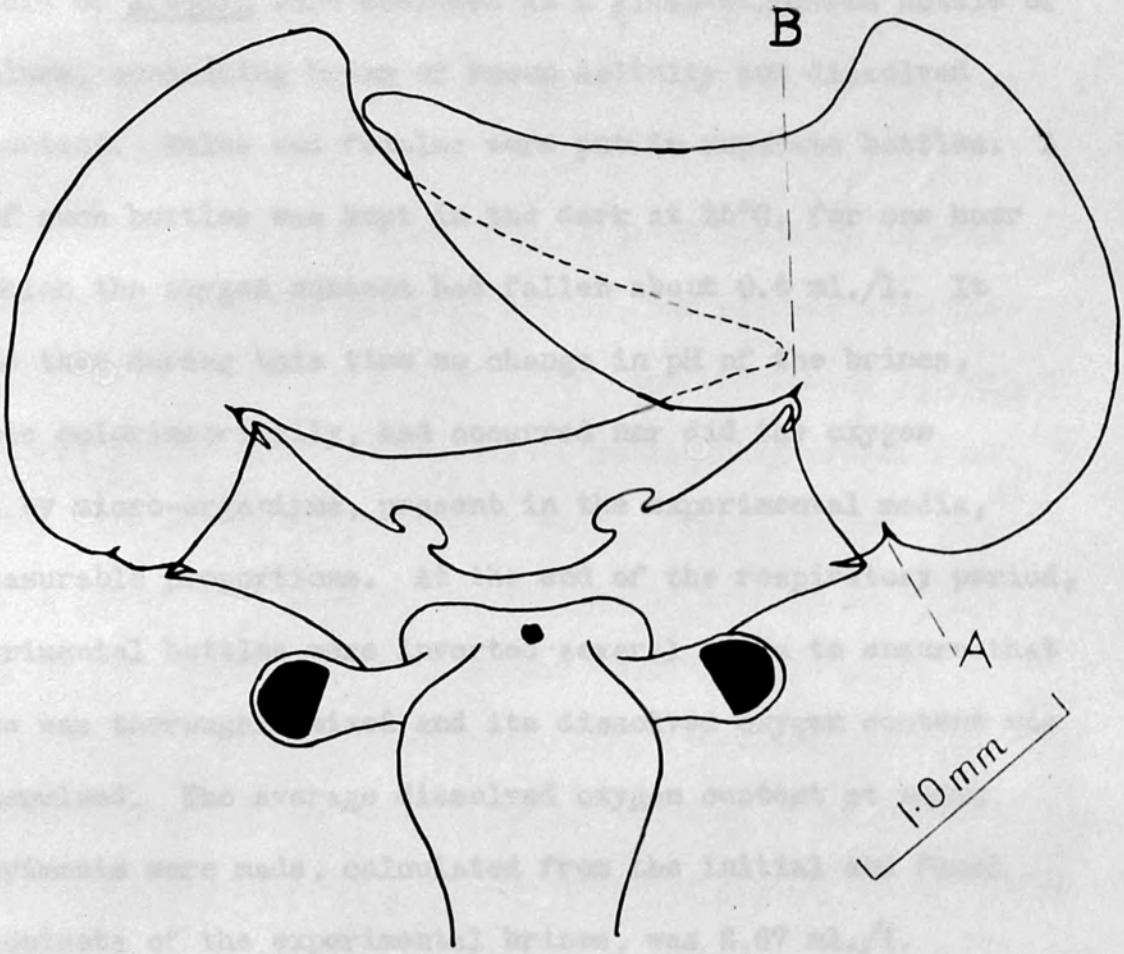


Figure 43. Head of male Artemia, dorsal view. The **second** antennae have been bent forward in front of the **head.**

which had been starved overnight in a medium of the same salinity, dissolved oxygen concentration and temperature as that to be used in the experiments. In each experiment, twenty small or ten large individuals of Artemia were enclosed in a glass-stoppered bottle of known volume, containing brine of known salinity and dissolved oxygen content. Males and females were put in separate bottles. A number of such bottles was kept in the dark at 25°C. for one hour during which the oxygen content had fallen about 0.4 ml./l. It was found that during this time no change in pH of the brines, measurable colorimetrically, had occurred nor did the oxygen consumed by micro-organisms, present in the experimental media, reach measurable proportions. At the end of the respiratory period, the experimental bottles were inverted several times to ensure that the brine was thoroughly mixed and its dissolved oxygen content was then determined. The average dissolved oxygen content at which all experiments were made, calculated from the initial and final oxygen contents of the experimental brines, was 2.67 ml./l.

The animals from each bottle were lightly narcotised and their linear dimensions measured, also the area of the second antennae of the males. On recovery from narcotisation, the animals were rinsed rapidly in distilled water, to remove salt water from between the limbs, carefully dried on filter paper and weighed. They were then dried for 15 hours at 100°C. and again weighed. The same precautions in determining the dry weights were taken as already described on page 104. Oxygen consumption was expressed as cubic millimetres of

oxygen per animal per hour, in relation to dry weight. Ten experiments were made in each salinity for each size group of males and of females, amounting to eighty experiments in all.

Results

The results of these experiments are expressed in figures 44 to 51.

The variability in body form of Artemia in relation to size and to the salinity of the external medium, has already been discussed (page 31). In concentrated media, the abdomen of Artemia is relatively longer than in more dilute media; body proportions are not the same in the two different media. Thus, to use the square of a linear dimension as an estimate of surface area, according to the 'surface law' of Rubner (1883), would not give reliable results. Similarly, the other long established concept that the two-thirds power of the wet weight can be used as an estimate of surface area, can only apply if the specific gravity of an animal remains constant with size and in different media.

Any change in the specific gravity of Artemia will be reflected in the ratio of dry weight to wet weight for a constant size of animal. This ratio will also show if there are differences in the water content of animals in relation to differences in size of animal and to the media. According to Schlieper (1936), the amount of oxygen consumed by tissues is directly related to their water content.

The ratio of dry weight to wet weight, in relation to total length of females of Artemia reared in different concentrations of brine, is shown in figure 44. The ratio does not differ significantly in animals reared in the two media, and although there is an indication that small females have a higher ratio of dry weight to wet weight, thus implying a higher specific gravity, this difference is not statistically significant over the size range investigated. The regression coefficients and standard errors, for the straight lines fitting the data, are -0.0030 ± 0.0016 for females reared in sea water and -0.0038 ± 0.0022 for those reared in the concentrated brine.

In figure 45, the ratio of the dry weight to wet weight of males of Artemia, in relation to total length, is expressed graphically. The regression coefficients and standard errors for the straight lines fitting the data are -0.0037 ± 0.001 and -0.0059 ± 0.003 for males in sea water and in brine of salinity S‰ 140 respectively. If the intercepts of the regression lines on the ordinate are compared, it is found that males reared in sea water have a significantly greater water content than males reared in more concentrated brine. On statistical analysis, 't' = 2.70 and with 36 degrees of freedom, a value for P, the probability that the difference is due to chance, of 0.01 is obtained. It is also seen that large males contain relatively more water than small males in both media.

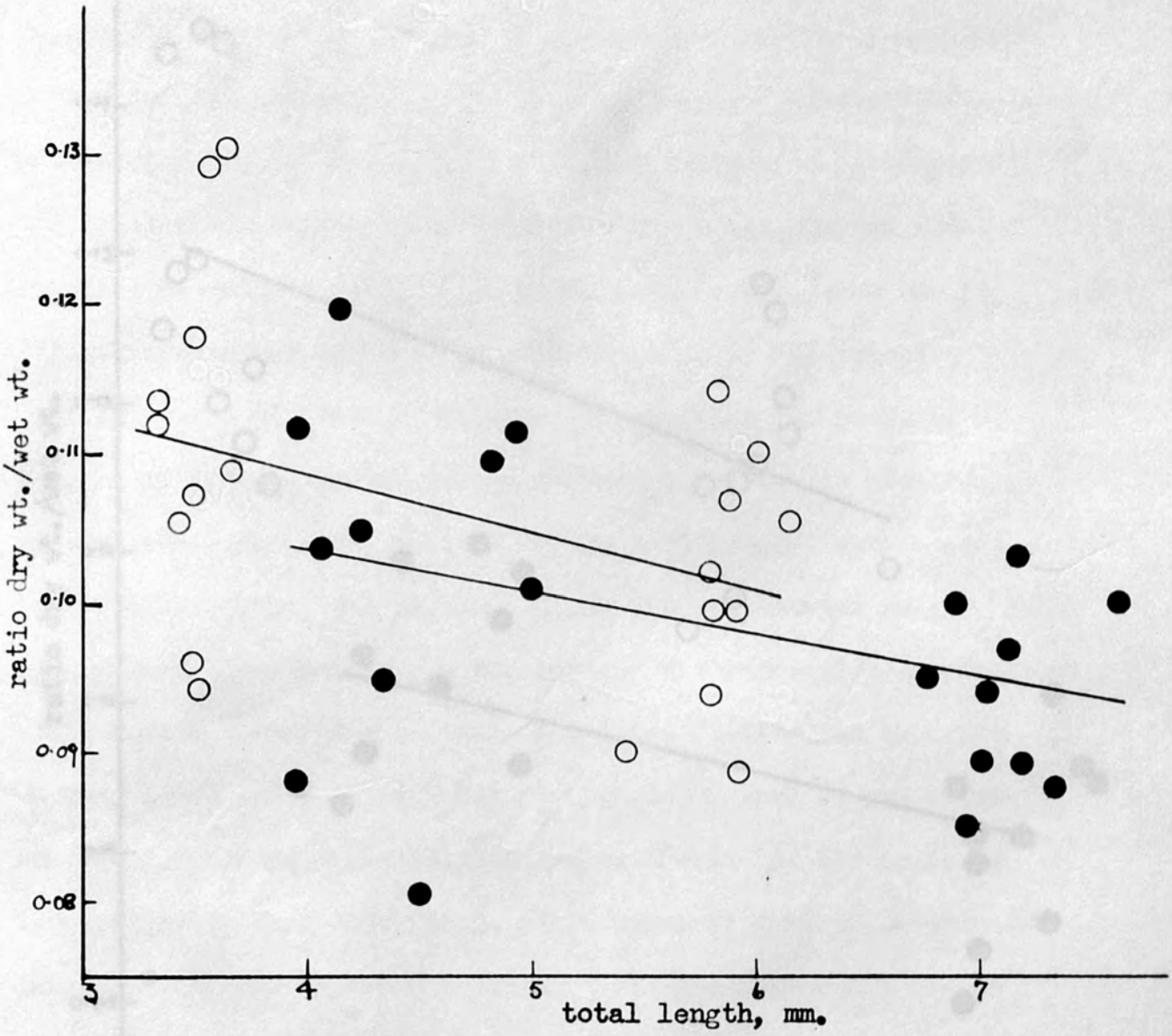


Figure 44. Ratio of dry weight to wet weight in relation to total length of females of Artemia reared in different salinities.
● S‰. 35; ○ S‰. 140.

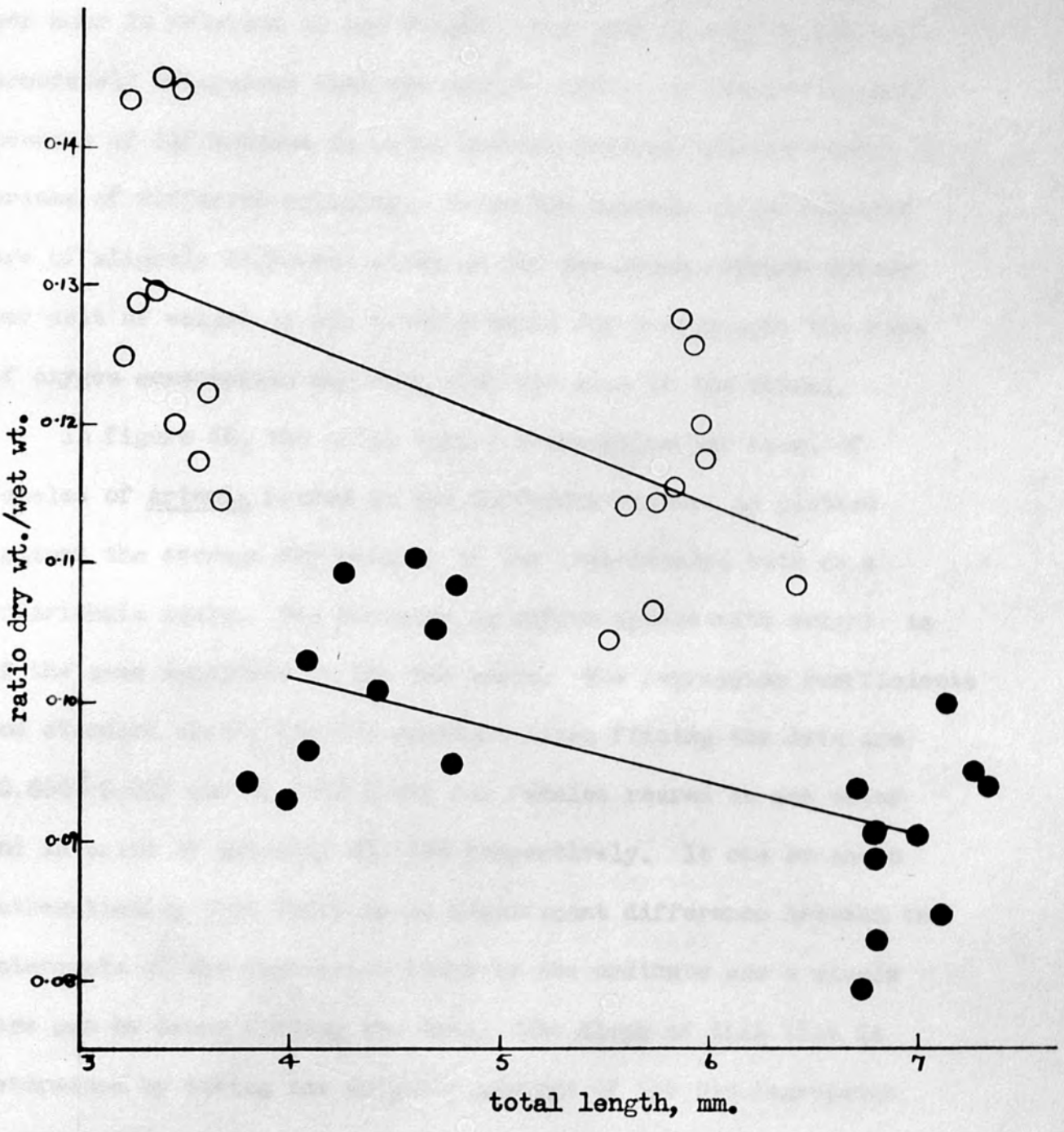


Figure 45. Ratio of dry weight to wet weight in relation to total length of males of *Artemia* reared in different salinites. ● S‰ 35; ○ S‰ 140.

In view of the above results, therefore, oxygen consumption is expressed in terms of the total oxygen uptake per individual per hour in relation to dry weight. Not only is dry weight more accurately determined than wet weight, but it is relatively independent of differences in water content between animals reared in brines of different salinity. Since the animals to be compared are of slightly different sizes in the two media, oxygen uptake per unit of weight is not a valid basis for comparison; the rate of oxygen consumption may vary with the size of the animal.

In figure 46, the total oxygen consumption per hour, of females of Artemia reared in two different brines, is plotted against the average dry weights of the individuals, both on a logarithmic scale. The increase in oxygen uptake with weight is of the same magnitude in the two media. The regression coefficients and standard errors for the straight lines fitting the data are $+0.604 \pm 0.083$ and $+0.721 \pm 0.041$ for females reared in sea water and in brine of salinity S‰ 140 respectively. It can be shown mathematically that there is no significant difference between the intercepts of the regression lines on the ordinate and a single line can be drawn fitting the data. The slope of this line is determined by taking the weighted average of the two regression coefficients. This average line fitting the data has a regression coefficient of +0.662 and has been drawn in figure 46. Thus, total oxygen consumption of females of Artemia, over the size range

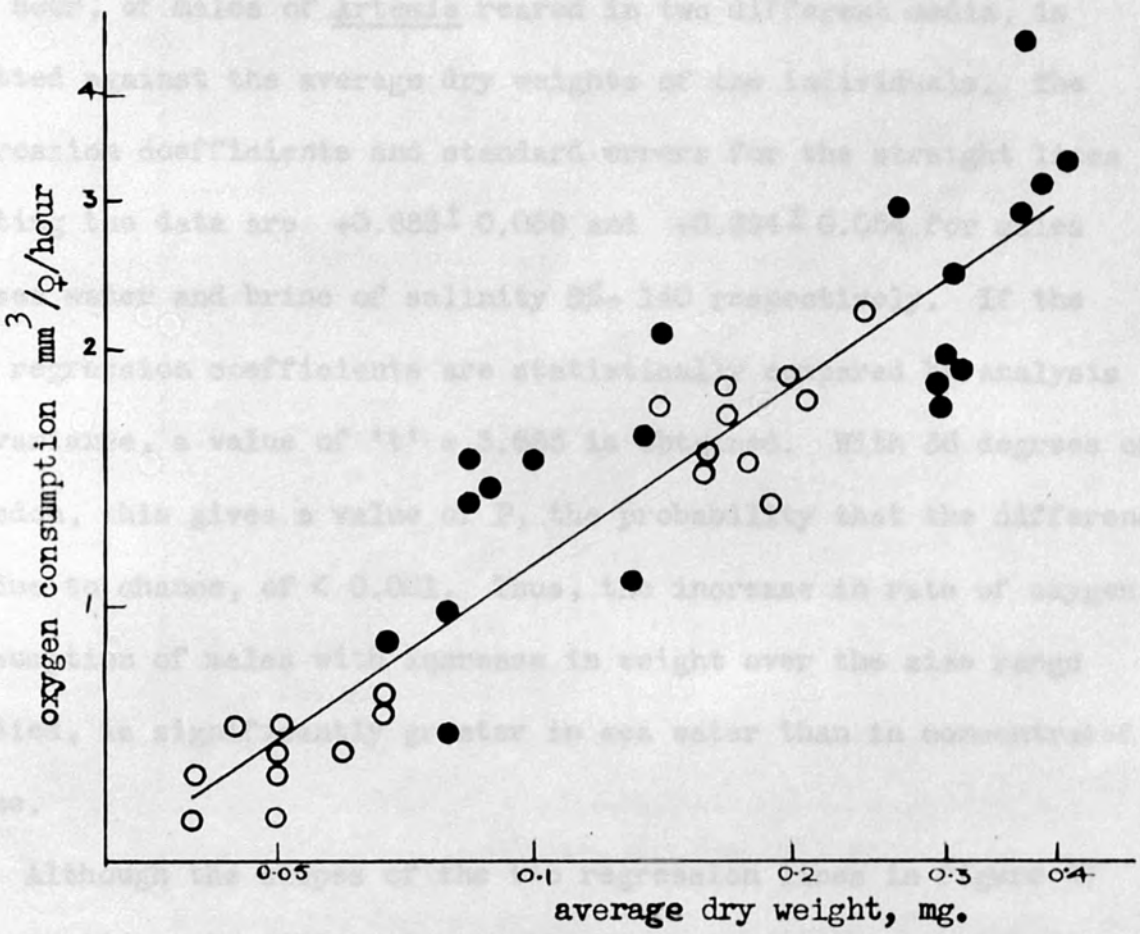


Figure 46. Oxygen consumption of females of Artemia reared in different salinities in relation to dry weight, both on a logarithmic scale. ● S‰ 35; ○ S‰ 140.

investigated, is proportional to the two-thirds power of the dry weight. The rate of oxygen consumption is the same for females reared in sea water and in concentrated brine (S‰ 140).

In figure 47 the total oxygen consumption, per individual per hour, of males of Artemia reared in two different media, is plotted against the average dry weights of the individuals. The regression coefficients and standard errors for the straight lines fitting the data are $+0.883 \pm 0.039$ and $+0.624 \pm 0.054$ for males in sea water and brine of salinity S‰ 140 respectively. If the two regression coefficients are statistically compared by analysis of variance, a value of 't' = 3.885 is obtained. With 36 degrees of freedom, this gives a value of P, the probability that the difference is due to chance, of < 0.001. Thus, the increase in rate of oxygen consumption of males with increase in weight over the size range studied, is significantly greater in sea water than in concentrated brine.

Although the slopes of the two regression lines in figure 47 are significantly different, and the increase in oxygen uptake is occurring at different rates in the two media, it is of interest to know whether the magnitude of oxygen uptake of males is greater in sea water than in the more concentrated brine. By means of a 't' test, the values of x (dry weight) have been calculated, above and below which the two regression lines are significantly different at the 5% level of significance. It is found that males of 0.16 mg. dry weight and over have a higher rate of oxygen consumption in

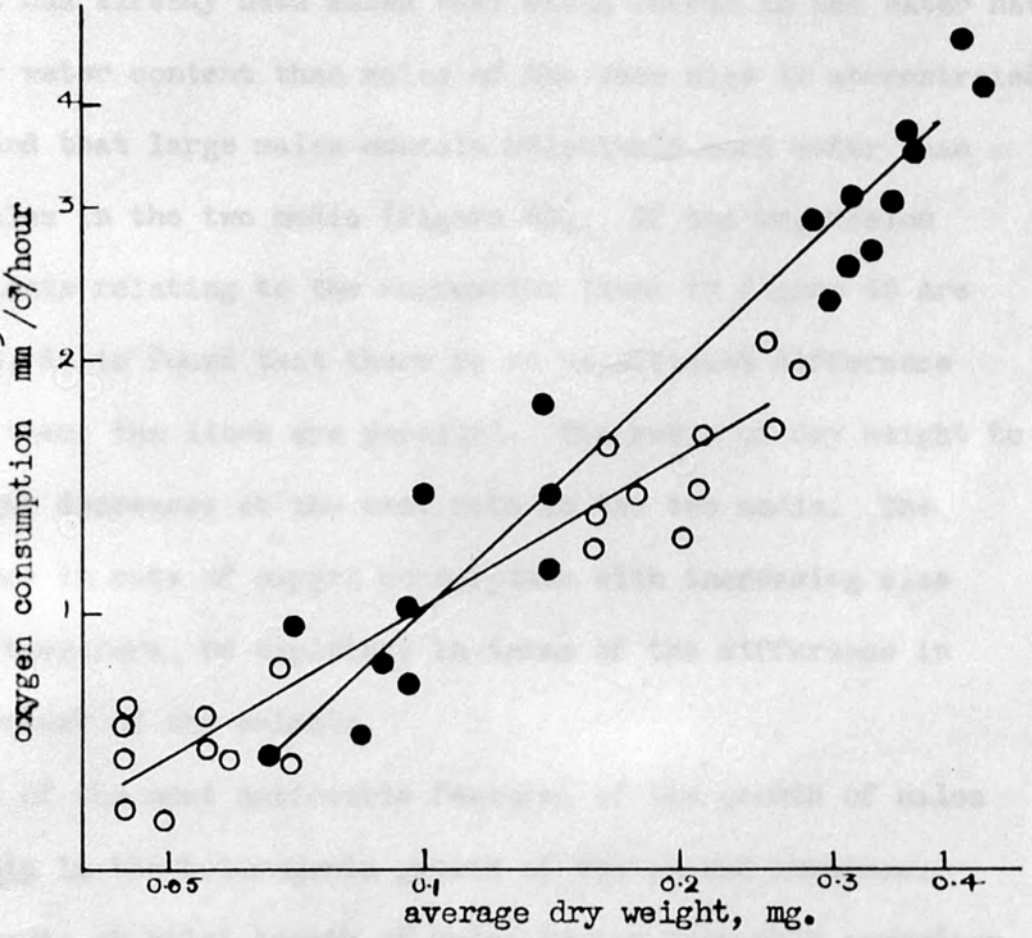


Figure 47. Oxygen consumption of males of Artemia reared in different salinities in relation to dry weight, both on a logarithmic scale. ● S‰ 35; ○ S‰ 140.

sea water than those of the same dry weight in the concentrated brine. In the case of smaller individuals, there is no significant difference in magnitude of oxygen uptake in the two media.

It has already been shown that males reared in sea water have a higher water content than males of the same size in concentrated brine, and that large males contain relatively more water than small males in the two media (figure 45). If the regression coefficients relating to the regression lines in figure 45 are compared, it is found that there is no significant difference between them: the lines are parallel. The ratio of dry weight to wet weight decreases at the same rate in the two media. The difference in rate of oxygen consumption with increasing size cannot, therefore, be explained in terms of the difference in water content of the animals.

One of the most noticeable features of the growth of males of Artemia is the heterogonic growth of the second antennae. Measurements of total length of males do not take this appendage into account. As will be seen in figure 43, the second antennae have a large surface area relative to the size of the animal. Any difference in the relative growth of the antennae in the two experimental brines might account for the difference in rate of oxygen uptake. In figure 48 the antennal area, expressed in arbitrary units, of males reared in sea water, is compared with that of males reared in brine of salinity S‰ 140. These measurements were made

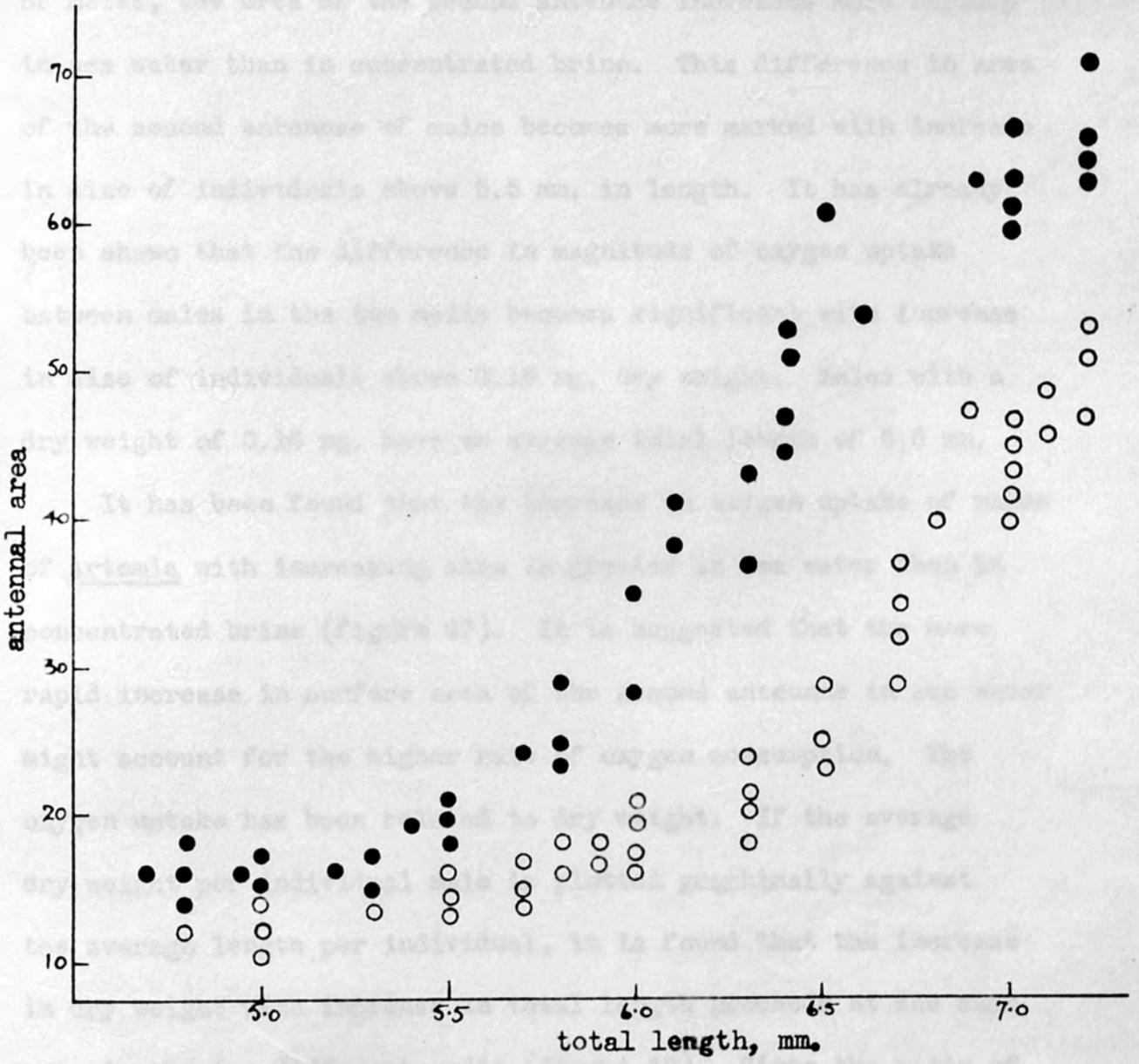


Figure 48. Area of second antennae of males of Artemia, expressed in arbitrary units, in relation to total length of animals reared in two different salinities. ● S‰ 35; ○ S‰ 140.

over the same size range of animals as selected for experiments on oxygen consumption. It can be seen that with increasing size of males, the area of the second antennae increases more rapidly in sea water than in concentrated brine. This difference in area of the second antennae of males becomes more marked with increase in size of individuals above 5.5 mm. in length. It has already been shown that the difference in magnitude of oxygen uptake between males in the two media becomes significant with increase in size of individuals above 0.16 mg. dry weight. Males with a dry weight of 0.16 mg. have an average total length of 5.5 mm.

It has been found that the increase in oxygen uptake of males of Artemia with increasing size is greater in sea water than in concentrated brine (figure 47). It is suggested that the more rapid increase in surface area of the second antennae in sea water might account for the higher rate of oxygen consumption. The oxygen uptake has been related to dry weight. If the average dry weight per individual male is plotted graphically against the average length per individual, it is found that the increase in dry weight with increase in total length proceeds at the same rate in the two different media (figure 49). Since the ratio of dry weight to wet weight changes with size at the same rate in the two media (page 116), the change in wet weight must also occur at the same rate. Thus, although the surface area of the second antennae of males reared in sea water is increasing more rapidly

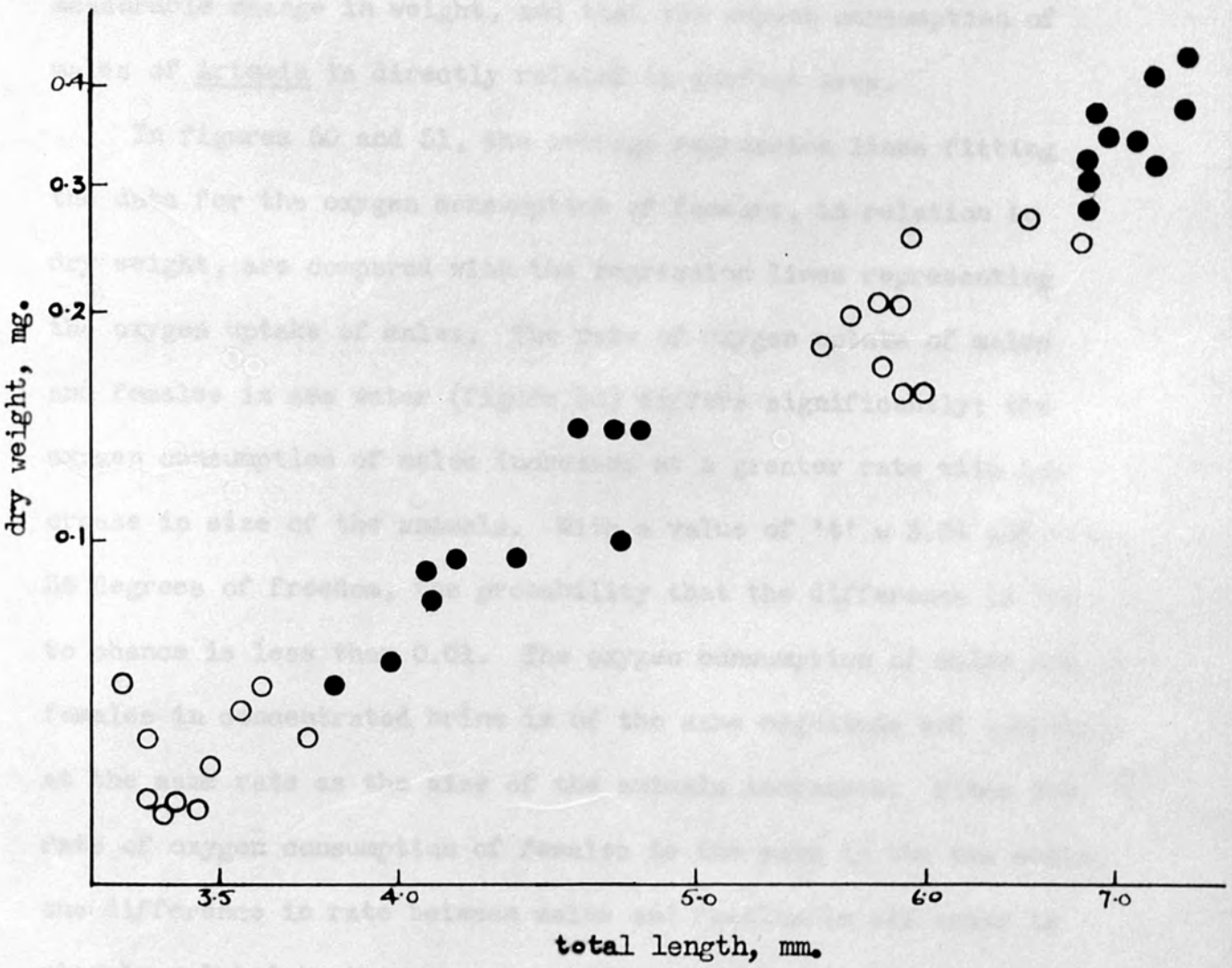


Figure 49. Relationship of **total** length to dry weight of males of **Artemia** reared in two different salinities, both on a logarithmic scale. ● S‰ 35; ○ S‰ 140.

than in males reared in concentrated brine, there is no reflection of this in either wet or dry weights. It seems, therefore, that a change in shape is involved which is not accompanied by a measurable change in weight, and that the oxygen consumption of males of Artemia is directly related to surface area.

In figures 50 and 51, the average regression lines fitting the data for the oxygen consumption of females, in relation to dry weight, are compared with the regression lines representing the oxygen uptake of males. The rate of oxygen uptake of males and females in sea water (figure 50) differs significantly; the oxygen consumption of males increases at a greater rate with increase in size of the animals. With a value of 't' = 3.04 and with 36 degrees of freedom, the probability that the difference is due to chance is less than 0.01. The oxygen consumption of males and females in concentrated brine is of the same magnitude and increases at the same rate as the size of the animals increases. Since the rate of oxygen consumption of females is the same in the two media, the difference in rate between males and females in sea water is clearly related to the greater surface area of the second antennae of males in this medium.

Discussion

The experiments of Bertalanffy & Krywienczyk (1953) have shown that the oxygen consumption of Artemia is proportional to the

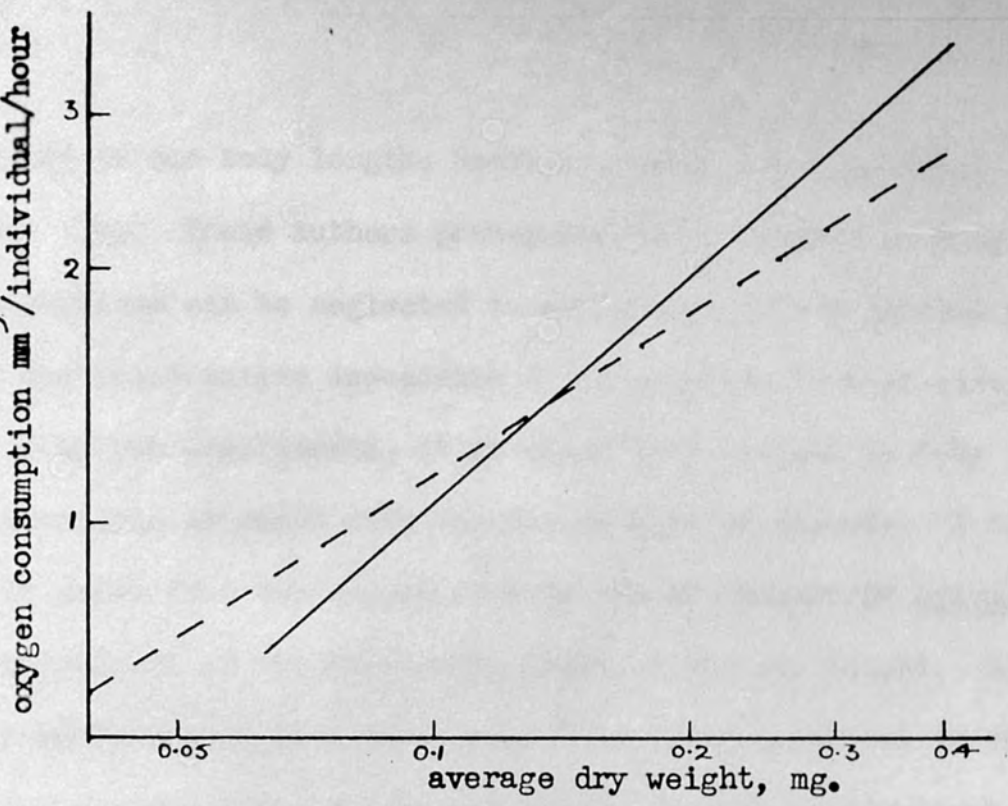


Figure 50. Regression lines relating oxygen consumption to dry weight of males and females of Artemia reared in sea water (S‰ 35). ——— males; — — females.

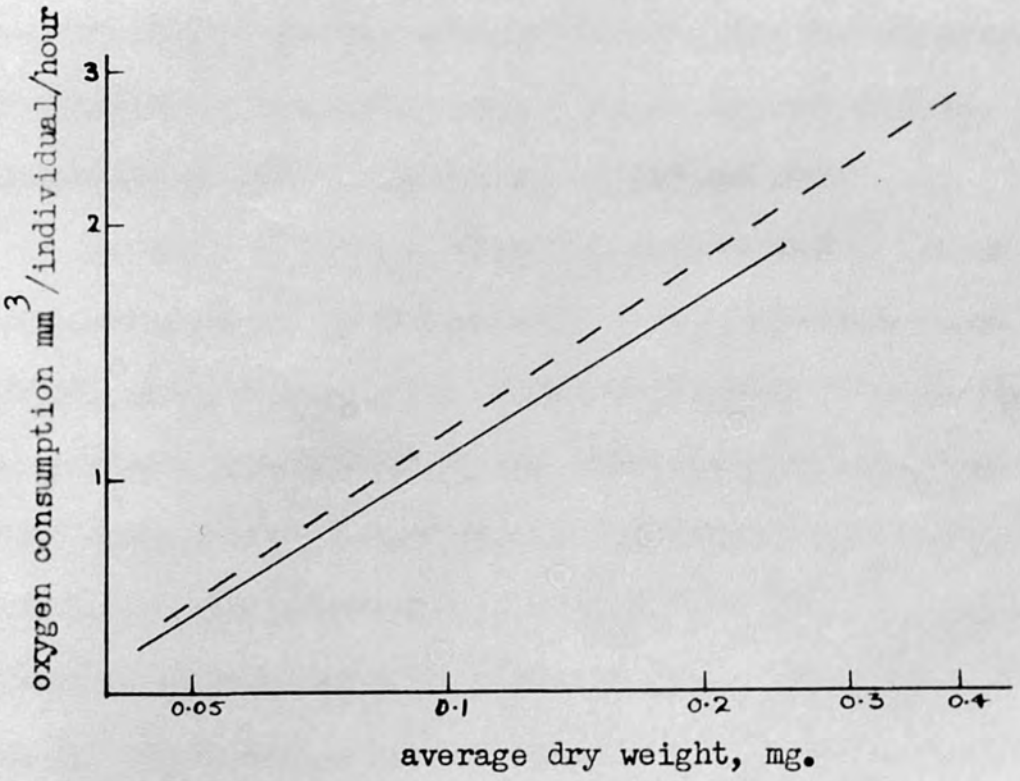


Figure 51. Regression lines relating oxygen consumption to dry weight of males and females of Artemia reared in brine of salinity S‰ 140. ——— males; — — females.

square of the body length; metabolic rate is proportional to surface area. These authors presuppose that "changes in body proportions can be neglected in comparison to the general trend of the quantitative dependence of respiration on body size." From my own experiments, it is clear that changes in body proportions do occur with increasing size of animals. I have also shown that the oxygen consumption of females of Artemia is proportional to the two-thirds power of the dry weight. Since the surface area of a particular body is proportional to the two-thirds power of its volume and since, for any particular body, volume will be proportional to weight, the surface area will be proportional to the two-thirds power of body weight. It seems, therefore, that in females of Artemia, the oxygen consumption is proportional to surface area and that changes in body proportions in relation to size and salinity, do not detract from the general dependence of oxygen consumption on surface area.

In males of Artemia reared in concentrated brine (S‰ 140), oxygen consumption is proportional to the two-thirds power of dry weight, as in females. The oxygen consumption of males reared in sea water is proportional to the 0.883 power of dry weight, a value which differs significantly from two-thirds. Both the specific gravity and body shape of males change, not only with differences in size but also in different media. This might justify the rejection of the surface rule. However, the surface

area of the second antennae of males has been measured and it has been shown that the difference in oxygen uptake of males in the two media is related to the difference in surface area. Thus, both in males and females of Artemia, oxygen consumption is proportional to surface area.

Kuenen (1939) found that the oxygen consumption of Artemia gracilis Verrill is higher in concentrated brine than in more dilute media. He regards this Californian brine shrimp as a distinct species, characterised by the absence of two lateral hairs on each abdominal segment; this conclusion is rejected by Linder (1944). Further, on examining animals from my cultures of Californian material, I found two lateral hairs present on each abdominal segment. The difference in our results on oxygen consumption cannot, therefore, be explained on the grounds that the material was different. It can perhaps be explained by considering certain differences in experimental methods. In his experiments, Kuenen (1939) compared the time taken by 200 mg. of animals to consume 0.1 cubic centimetre of oxygen at the "level where the oxygen pressure in the water was 90% of saturation" in each of the three experimental brines. The three concentrations of brine at which oxygen consumption was measured were S‰ 29, S‰ 58 and S‰ 116 approximately. The dissolved oxygen content of brines of salinity S‰ 29 and S‰ 116 when 90% saturated with air at 25°C. will be approximately 4.0 and 2.7 ml./l. respectively.

Thus, oxygen uptake is being compared at different oxygen pressures. Secondly, very little information is given by Kuenen (1939) about the size and sex of the animals used. He says that males and females were used separately to avoid "riding positions" and copulations taking place during the experiments. This suggests that the males and females were sexually mature and reproducing. It would be of interest to know how much of the oxygen consumed by the females and how much of the weight of the animals was due to eggs or embryos in the maternal brood pouch. Finally, it is stated by Kuenen (1939) that no difference in rate of oxygen consumption was found between males and females and so it must be assumed that the data for males and females have been combined in the graphic presentation of the results. In view of the scattering of the points on the graph, and of the results of my own experiments, it would be interesting to know which points referred to males and which to females. Kuenen (1939) concludes from his experiments, that the oxygen consumption of Artemia is greater in the higher concentrations of brine. No statistical data are given, but on analysis, his results show no statistically significant difference in the rate of oxygen consumption of animals in brine of salinity S‰ 29 and S‰ 58, or between animals in brine of salinity S‰ 58 and S‰ 116. There is, however, a significant difference between the rate of oxygen consumption in the two extreme concentrations of brine.

My results probably agree with those of Eliassen (1952), although it is difficult to compare them as he does not distinguish between males and females in his experiments relating to adult animals. Measuring the oxygen uptake per gamma of nitrogen of the tissues, over a wide size range of Artemia from nauplius to adult, he obtained a three-phasic curve. There is first of all a relatively large fall in oxygen consumption with increasing size of the nauplii, followed by a smaller decrease in the animals of middle size and finally, a more marked fall in oxygen consumption as the animals become mature. He finds this three-phasic curve for animals reared in three different concentrations of brine S‰ 10, S‰ 35 and S‰ 50. Oxygen consumption decreases with increasing salinity of the medium, but this effect is most marked in the younger nauplii and fades as the size of the animals increases. My results can only be compared with the third phase of Eliassen's curve, in which he shows graphically the curve for oxygen consumption in brine of salinity S‰ 50 lying just below that for oxygen consumption in sea water. As no statistical analysis of the results is given it is not possible to say whether the difference is real or not.

Experiments have been made by many authors to measure the rate of oxygen consumption of certain invertebrates in media of different salt concentration. Schlieper (1929), found that the oxygen uptake of the shore crab, Carcinus maenas, increases as the

salt concentration of the external medium decreases. A similar relationship between salinity and oxygen uptake is described by Beadle (1931) for the planarian Gunda ulvae. Studying the rate of oxygen uptake of the brackish water amphipod Gammarus chevreuxi in water of different salinities, Lowenstein (1935) found that the respiratory rate was about 20% lower in sea water than in 25% sea water.

In these experiments described above, the animals concerned were transferred to media of different salt concentration and the rate of oxygen uptake was compared in the different media. These experiments are not comparable to those made on Artemia. In the latter, the animals were not subjected to a change in the salt concentration of the medium. Oxygen consumption was measured in a medium of the same salinity as that in which the animals had been reared through several generations.

My experiments on the oxygen consumption of Artemia in different concentrations of brine may be compared to those of Schwabe (1933) on the grapsoid crab Eriocheir sinensis. This crab can live both in sea water and fresh water. Schwabe (1933) found no difference in oxygen consumption between animals in fresh water and in brines of salinity S‰ 15 and S‰ 32. Recently, Lumbye (1958) has measured the oxygen consumption of a gastropod mollusc, Theodoxus fluviatilis, in brackish and fresh waters. He found that the respiratory rate of individuals from the fresh water population

was at the same level as individuals from the brackish water population.

These experiments on Eriocheir, Theodoxus and Artemia are comparable in that for each, the oxygen consumption of individuals inhabiting waters of a particular salt concentration is compared to that of individuals inhabiting waters of a different salt concentration. It is interesting that in all three cases, there is no difference in rate of oxygen consumption in the different media.

The mechanism of osmoregulation in Artemia has already been discussed (pages 87-96). Brine shrimps maintain a blood concentration which is hypotonic to the external medium over a wide range; the principle involved is similar to that of marine teleosts. It has been shown by Conklin & Krogh (1938) that the blood of Eriocheir sinensis is hypotonic to the external medium when the crab is in concentrated sea water. The mechanism of osmotic regulation in Eriocheir has not been investigated, but it may be similar to that of Artemia, involving the active excretion of salt and the uptake of water against a concentration gradient. Such active processes must surely involve the expenditure of energy. Yet both in Eriocheir and Artemia no difference in respiration is measured in animals in media of different salt concentration. It is suggested by Conklin & Krogh (1938) that this indicates that the active transport of ions is kept up all the time.

Ion transport can be brought about by processes other than active transport; both free and exchange diffusion systems may be involved. Ussing (1949) uses the term 'exchange diffusion' to describe a system in which there is an exchange of specific ions across a membrane separating two compartments in which the ion concentration is not in electro-chemical equilibrium. The specific ion crosses the membrane by combining with a carrier molecule, the exchange particle, which is part of the membrane. Ions attached to the exchange particle could exchange with ions present in the solutions on either side of the membrane. Croghan (1958e) has shown that remarkably rapid ionic fluxes occur between the medium and the haemolymph of Artemia. He shows that this high rate of exchange could be the result of the active transport of ions, involving an expenditure of energy which he regards as well within the metabolic capabilities of Artemia. On the other hand, his results indicate that the influx and efflux of sodium are not independent processes; sodium ions must be present in the external medium before sodium efflux will occur. Croghan (1958e) suggests that there is a 1 : 1 exchange of sodium ions and also of chloride ions between the haemolymph of Artemia and the external medium. Such an exchange is characteristic of exchange diffusion systems.

Thus, exchange diffusion simulates active transport, but it is a passive process: no energy is required. Another important

difference in the two processes is that exchange diffusion cannot bring about a net transport of ions; the ionic flux is about the same in both directions. Exchange diffusion cannot, therefore, be the process whereby ion transport occurs in the osmoregulatory mechanism of Artemia. Here a net transport of ions must take place in order to maintain the concentration of the haemolymph hypotonic to the external medium.

It seems, therefore, that in Eriocheir, Theodoxus and Artemia the metabolic activity associated with osmotic work is small in relation to total metabolic activity and is not measurable by the methods employed to determine the rate of oxygen consumption. It may be, on the other hand, that although the total oxygen uptake is the same in animals in different media, the distribution of the available energy may differ according to the needs of the animals. It has already been shown that individuals of Artemia reared in concentrated brine have a slower rate of growth and are smaller than individuals reared in more dilute brine (pages 15-28). Further, I have made observations on natural populations of brine shrimps, in media of different salinity, with regard to the reproductive activity of the animals. Large numbers of females were collected from brine reservoirs of salt works at La Palme, in south-west France, and the percentage of females carrying eggs or embryos estimated. It can be seen from table 5 that with increasing salinity of the medium, the parthenogenetic females are smaller in

size and the number carrying eggs or embryos is reduced. Although no counts of eggs were made, it was obvious that those females which were reproducing in the concentrated brine carried fewer eggs than those in more dilute brine. These observations suggest that increased activity in connection with osmoregulation may be compensated for by curtailing other metabolic processes such as growth and reproduction.

Table 5. The influence of the salinity of the medium on size and reproduction in a natural population of parthenogenetic females of Artemia salina.

S‰	% females with eggs or embryos	mean total length mm.	number measured
115	72	8.42	229
140	49	7.85	225
160	15	6.88	140

THE PIGMENTS OF *ARTEMIA*

Many references to the colours of *Artemia* are to be found in the literature; most frequent reference is made to the red colour of the adults. In his original description of the brine shrimp, Schlosser (1756) says that the red colour of the animals tinted the water in the tanks in which they were found; both Rackett (1812) and Baird (1850) also refer to the red colour of British brine shrimps. Likewise, in certain Siberian lakes (Pallas, 1777), the Great Salt Lake, Utah (Packard, 1883) and in brine pools in Algeria (Blanchard, 1891) *Artemia* is described as being intensely red.

The colour of the nauplius of *Artemia* has also been referred to in the literature. According to Thompson (1829), on hatching from the egg the embryo "appears of an orange colour"; both Packard (1883) and Jensen (1918) describe the nauplius of *Artemia* as "blood red" in colour.

Of particular interest are the references to variations in the red colour of *Artemia* in relation to the salt concentration of the medium. The redness of adults becomes more intense as the salinity of the external medium increases (Payen, 1836; Joly, 1840; Schmankewitsch, 1877; Kellogg, 1906). Artom (1905) found the adults of *Artemia* red at high concentrations of brine, and the nauplii red both at high and low concentrations of the medium.

A few authors describe a variety of colours of Artemia. Salmon pink females with greenish head and limbs are described by Ermakow (1928), who says that males have a green body with reddish legs. Reference to green brine shrimps is also made by Bond (1933), who thought the colour of Artemia was dependent upon its food, and by Lochhead & Lochhead (1941).

Although so many references have been made to the colours of Artemia, little information is available as to the nature of the pigments concerned. Haem compounds are known to occur in Artemia. Haematin is found in the shells of the eggs (Needham & Needham, 1931); haemoglobin may occur in solution in the blood (Lochhead & Lochhead, 1941) and a haemochromogen has been identified from the gut (Phear, 1955). A second group of pigments in Artemia has been referred to in the literature, the carotenoids. Needham & Needham (1930) obtained an alcohol-ether extract of an orange pigment from embryos of Artemia; Needham (1931) calls this pigment a "lipochrome or crustaceorubin". With reference to the orange pigment sometimes found in certain cells of the limbs of Artemia, Lochhead & Lochhead (1941) say the pigment "is presumably a carotenoid, possible astacin or one of its derivatives". No identification of carotenoids in Artemia has been made by these authors.

It seems, therefore, that the red and green colours of Artemia may be due either to haem compounds or to carotenoids, or

to both. The work now to be described was undertaken in order to find to what extent these pigments are responsible for the colours of Artemia, both adults and nauplii, and to obtain information on the causes of colour variation in Artemia. The work will be described in two parts, the first part will be concerned with the haem compounds and the second with carotenoid pigments.

HAEM COMPOUNDS

1. HAEMOGLOBIN IN SOLUTION IN THE BLOOD.

Introduction

The first published record of the occurrence of haemoglobin in the Crustacea is that of Lankester (1869) who identified it in the blood of a fresh water anostracan, Chirocephalus diaphanus Prévost. Lankester observed the absorption bands of oxyhaemoglobin with a microspectroscope. Subsequent to Lankester's report, Claus (1886) states that haemoglobin is abundant in Artemia. He gives no evidence of having identified the pigment, and it is probable that he assumed the red colour was due to haemoglobin as the result of Lankester's work on Chirocephalus. The determination of haemoglobin in solution in the blood of Artemia was first made by Lochhead & Lochhead (1941) who identified it spectroscopically.

It is known that haemoglobin is widespread among entomostracan Crustacea. Until recently, the pigment was recorded from all the major groups of the Entomostraca except free-living

copepods and the Branchiura (for review of literature see Fox, Gilchrist & Phear, 1951). Recently, haemoglobin has been found in solution in the blood of free-living harpacticoid copepods (Fox, 1957a) and also in Dolops ranarum (Stuhlmann), a branchiuran found in Lake Victoria, East Africa (Fox, 1957b). Thus haemoglobin, in solution in the blood, is recorded from all the major divisions of the Entomostraca.

Among Entomostraca, much is known about the haemoglobin of the cladoceran Daphnia. The factors which influence synthesis and breakdown, the variations in concentration of the pigment and its functions have been extensively studied by Fox and his colleagues (Fox, 1948; Fox, Hardcastle & Dresel, 1949; Fox et al, 1951; Fox & Phear, 1953; Green, 1956b). The work to be described was done in the laboratory and in the field, to find what role haemoglobin plays in the life of the brine shrimp and to what extent the pigment is responsible for the red colour of the animals.

Oxygen and haemoglobin concentration

The ability to gain and lose haemoglobin in response to the oxygen content of the water in which they live is a property of phyllopod Crustacea (Fox, 1954). When the present work was begun, quantitative measurements of the haemoglobin concentration in the blood had been published only for Daphnia (Fox, 1948; Fox et al, 1951), and it was of interest, therefore, to obtain quantitative

data for another phyllopod.

The experiments were made with males and females of the Californian stock cultured in sea water at 25°C. and fed with the diatom Phaeodactylum tricornutum and a marine Chlamydomonas.

The haemoglobin content of the blood of Artemia was measured by a modification of the index method (Fox, 1948; Fox et al, 1949). The colour of the blood of the animal is matched with the colour of a known dilution of human blood, the standard, contained in a glass wedge-shaped trough. This trough is placed between the microscope lamp and the mirror in such a way that the colour of the standard is projected onto the microscope field to cover about a third of the field. This colour is matched with that of the blood of the animal seen in the remaining part of the field. The trough stands on a paper scale divided into 160 arbitrary units, and is so arranged that its narrow end, when seen through the microscope, corresponds to 0 on the scale and its wide end to 160. The standard haemoglobin solution is 0.1 ml. blood in 37.5 ml. distilled water, with the addition of a trace of saponin to ensure that haemolysis is complete and a drop of sodium bicarbonate solution to prevent the breakdown of haemoglobin to haematin. When this standard solution has been put in the wedge-shaped trough, a drop of octyl alcohol is added; this reduces the 'creep' up the narrow end of the trough.

When measuring the haemoglobin content of the blood of

Artemia, the standard haemoglobin solution was diluted by half; the solution was made by adding 0.1 ml. blood to 75 ml. distilled water, and the paper scale on which the trough stands read from 0 to 80 units. *Artemia* was narcotised with chloroform and each of ten individuals was placed ventral side down in a little sea water on a glass slide. The colour of the oxyhaemoglobin in solution in the blood at the side of the gut, on a level with the posterior termination of the heart, was matched with the standard solution. The width of the abdomen at this point was measured, also the total length of the animal from the anterior margin of the head in front of the ocellus to the base of the caudal furcae.

The haemoglobin *index* of a population is the mean of the haemoglobin *value* measured for each of ten individuals. As has been pointed out by Green (1956*b*), the haemoglobin value of an individual is the combination of the haemoglobin concentration and the depth through which it has been measured. Thus the haemoglobin value of each animal varies as the depth through which it is measured. The depth through which the value is measured will depend upon the width of the abdomen of *Artemia*. In all the experiments on *Artemia* the haemoglobin index has been corrected to a standard width of abdomen of 0.45 mm., and thus the corrected indices represent haemoglobin concentration.

In laboratory experiments the dissolved oxygen was measured by the syringe-pipette modification of the Winkler method (Fox &

Wingfield, 1938). In the field, dissolved oxygen was determined by Whitney's (1938) syringe-pipette modification of the Winkler method. No adjustment was made in the field for the presence of oxidising or reducing substances in brine which may falsify the results of the Winkler method; the values obtained, therefore, represent approximate dissolved oxygen content.

a) Synthesis of haemoglobin in response to a low dissolved oxygen content of the water.

Preliminary experiments (Fox, 1949) have shown that Artemia cultured for two to three weeks in sea water only 10 to 20% saturated with air synthesizes more haemoglobin than in well aerated water. The experiments described below were made to demonstrate quantitatively this increase in response to a paucity of oxygen.

In order to maintain a low oxygen content of sea water, the initial oxygen pressure was reduced by bubbling nitrogen through the water in the experimental vessels. These were 450 ml. conical flasks containing 440 ml. of sea water and forty-four individuals of Artemia. Control experiments were set up with shallow open dishes containing sea water and animals in the same proportion as in the conical flasks. Animals 16 days old were used, equal numbers of males and females being put into each experimental vessel. Experiments were made in the dark at 25°C. and lasted for 3 weeks. During this period large numbers of nauplii were produced. These were removed every other day and the adults put

back into fresh sea water at the same temperature and of the same dissolved oxygen content as before. During the experiments Artemia was fed daily with Phaeodactylum; an equal volume of a thick culture was added to each experimental vessel.

The results of these experiments are summarised in table 6. Initially, no haemoglobin was detectable in any animals when examined with a microspectroscope. The results show that Artemia, like other phyllopods, synthesizes more haemoglobin when cultured in water with a low dissolved oxygen content than with a high one, and that females synthesize more of the pigment than males in a given period. The influence of the low oxygen content of the water on the growth of males and females of Artemia has already been commented upon (page 28).

b) Loss of haemoglobin in well-aerated water

Preliminary experiments had shown that when individuals from a red population of Artemia, with much haemoglobin in the blood, were kept for 2 to 3 weeks in well aerated water they became paler in colour. Experiments were made, therefore, to measure quantitatively the loss of haemoglobin from the blood.

Male and female individuals from a red population of Artemia were put into vessels containing sea water through which air was bubbled continuously for twenty days, at room temperature (19 to 22°C.). In this way the oxygen content of the water was maintained at approximately 90% air saturation. The animals were fed on

Table 6. Synthesis of haemoglobin by *Artemia salina* in oxygen-deficient sea water at 25°C.

% air saturation of sea water		87 - 92		15 - 20	
sex		male	female	male	female
	experiment 1.				
mean total length (mm. with S.E.)	initial	5.7	6.3	5.7	6.3
	final	8.0±0.17	9.3±0.13	7.7±0.21	8.1±0.14
mean abdomen width (mm.)	initial	0.34	0.38	0.34	0.38
	final	0.48	0.58	0.41	0.41
haemoglobin index (with S.E.)	final	<15	< 15	39±1.6	47±3.4
	experiment 2.				
mean total length (mm. with S.E.)	initial	5.7	6.3	5.7	6.3
	final	8.1±0.10	9.3±0.15	7.9±0.17	8.3±0.15
mean abdomen width (mm.)	initial	0.34	0.38	0.34	0.38
	final	0.47	0.58	0.48	0.49
haemoglobin index (with S.E.)	final	<15	<15	39±0.6	49±1.6

Phaeodactylum; the diatom culture was added to each of the experimental dishes in equal proportions.

The results of one experiment are summarised in table 7. The data are the means of measurements on ten animals, and, as before, haemoglobin indices have been corrected to a standard width of abdomen of 0.45 mm. It can be seen that in well-aerated water Artemia loses haemoglobin, and that females lose more than males in a given time.

Table 7. Loss of haemoglobin by Artemia salina in well aerated sea water.

		males	females
mean total length (mm.)	initial	7.7	8.1
	final	8.3	9.7
mean abdomen width (mm.)	initial	0.41	0.41
	final	0.44	0.52
haemoglobin index	initial	38	47
	final	18	<15

Thus the brine shrimp gains or loses haemoglobin in solution in its blood in response to a low or high dissolved oxygen content of the medium in which it lives. Females gain and lose the pigment more rapidly than males.

In Daphnia magna, the only other entomostracan in which haemoglobin synthesis and breakdown have been measured quantitatively, males gain and lose the pigment more rapidly than females (Green,

1955; 1956b). As pointed out by Green (1956b) it is perhaps not surprising that males of Daphnia magna gain haemoglobin more rapidly than females; males have a higher metabolism (MacArthur & Baillie, 1929) and females lose haemoglobin from solution in the blood as they pass the pigment into the eggs (Dresel, 1948). In females of Artemia, the passage of haemoglobin from the blood into eggs has not been observed; in sea water, adult males have a higher rate of oxygen consumption than females (page 126), and so it is surprising that females gain and lose haemoglobin more rapidly than males.

Salinity of the medium and haemoglobin concentration

During my first visit to salt works in the south of France, I noticed that in concentrated brines Artemia was redder than in more dilute brines. This has already been commented upon by a number of previous workers (Payen, 1836; Joly, 1840; Schmankewitsch, 1877; Artom, 1905; Kellogg, 1906), but no suggestion is made as to the cause of the difference in colour.

On examining samples of brine shrimps, from the different concentrations of brine in the salt works, with a microspectroscope the absorption bands of oxyhaemoglobin were clearly visible. Experiments were made, therefore, to determine to what extent haemoglobin was responsible for the differences in colour of the animals. Observations were made on the haemoglobin content of

Artemia in brines of different salinity and on the dissolved oxygen content of these brines.

Artemia was collected from brine reservoirs at La Palme (Aude) in southern France. Approximate determinations of salinity were made in the field with a hydrometer, and samples of brine were subsequently titrated with a standard solution of silver nitrate. Dissolved oxygen in the brine was determined in the field using Whitney's (1938) syringe-pipette modification of the Winkler method. The haemoglobin index, mean length and mean abdomen width of thirty animals from each of four different salinities was determined. The indices were corrected to a standard width of abdomen of 0.3 mm.

The results are shown in table 8. It is clear that as the salinity of the brine increases, and its dissolved oxygen content decreases, the haemoglobin concentration in the blood of Artemia increases. Thus, the higher the salinity of the medium, the redder are the animals.

Table 8. Salinity of brine and haemoglobin concentration of blood for natural populations of Artemia salina at 24 to 25°C.

salinity (S‰)	oxygen (ml./l.)	haemoglobin index	total length (mm.)	abdomen width (mm.)
245	1.64	46	7.2	0.26
185	2.29	33	7.7	0.26
160	2.82	21	8.3	0.30
115	3.30	<15	8.5	0.30

These results were confirmed by observations at three other

salt works in the south of France. In all cases it was found that the increased red colour of Artemia in the higher salinities was due to the increased haemoglobin content of the blood.

In view of my results, the observations of Joly (1840) are of particular interest. He found that not only was Artemia red in concentrated brine, but when these red animals were transferred to a more dilute medium the red colour gradually disappeared. The more dilute medium would have a higher dissolved oxygen content than the concentrated brine, and thus the loss of haemoglobin, associated with the increase in oxygen, would account for the loss of red colour.

Function of haemoglobin in Artemia

It is known that the additional haemoglobin synthesized by the cladoceran Daphnia in oxygen deficient water has several functions (Fox et al, 1951). It aids survival, enables more food to be gathered, causes more energetic swimming and an increased egg production. Experiments were made, therefore, to see whether the additional haemoglobin in the blood of Artemia is of functional significance.

a) Viability in water deficient in oxygen

The time of survival of red and pale animals in sea water containing little dissolved oxygen was compared. The experiments were made with animals from the Californian stock, reared at 25°C.

and fed on Phaeodactylum. From this stock, red and pale populations were obtained by keeping animals for approximately 3 (Three) weeks in sea water containing little or much dissolved air. The presence of haemoglobin in the blood was detected with a microspectroscope.

Experiments were made by the method already described for Daphnia magna (Fox et al, 1951). In each experiment three vessels were used. Two of them, (a) and (b), were 3 l. stoppered bottles completely filled by sea water with a low dissolved oxygen content, and the third vessel (c) was an open dish of sea water with a much higher oxygen content. In each experimental bottle the initial oxygen content of the sea water was lowered, by bubbling nitrogen through it, to a level just above that at which the haemoglobin of Artemia becomes deoxygenated, since it is important to ensure that the haemoglobin is in the oxygenated state during the experiments.

The oxygen content of the sea water at which the blood of Artemia becomes deoxygenated was determined as follows. The method used was a modification of that described by Fox (1945). About twenty animals with much haemoglobin in the blood were enclosed in a stoppered glass tube completely filled with sea water at 25°C. The two absorption bands of oxyhaemoglobin were observed through the glass tube with a hand spectroscope; the moment that the two bands were no longer visible the oxygen

content of the water in the tube was determined. It was found, as the result of ten determinations, that the average dissolved oxygen content at which the blood of Artemia becomes deoxygenated is 0.6 ml./l. at 25°C.

Into each of the three experimental vessels were put five male and female individuals of Artemia, red animals in one bottle (a), pale animals in another (b), and an equal number of red and pale in the open dish (c) containing well-aerated sea water. Experiments were made in the dark, at room temperature (21 to 22°C.), and no food was given. When 50% of the animals were dead in one of the bottles the experiment was terminated. The dissolved oxygen content of the water in the vessels was determined at the beginning and end of the experiment by the syringe-pipette micro-Winkler method.

The results of three experiments are given in table 9, from which it is seen that red animals survive longer than pale animals. Thus, Artemia with haemoglobin in the blood is more viable in poorly aerated sea water than animals with no detectable amount of the respiratory pigment. No difference in survival time was observed between males and females. In control experiments, (c), the fact that only one animal out of thirty died, indicates that starvation was not the cause of death in the experimental bottle (b).

b) Influence of haemoglobin on the oxygen uptake of Artemia.

It is known that the haemoglobins of certain invertebrates

Table 9. Viability of *Artemia salina*, with much and with no detectable haemoglobin, in sea water with a low dissolved oxygen content, at 21 to 22°C.

		oxygen concentration (ml./l.)			animals alive at stated hours from the beginning				
expt.	vessel	initial	final	haemoglobin	0 h.	29 h.	35 h.	53 h.	
1	a	1.21	0.88	present	10	10	10	10	
	b	1.19	0.95	undetectable	10	8	7	4	
	c	4.65	3.80	both	10	10	10	10	
					0 h.	29 h.	35 h.	53 h.	
2	a	0.92	0.85	present	10	10	10	10	
	b	0.97	0.92	undetectable	10	9	8	5	
	c	4.65	3.80	both	10	10	10	10	
					0 h.	28 h.	31 h.	36 h.	52 h.
3	a	1.07	0.73	present	10	10	10	10	10
	b	1.02	0.91	undetectable	10	9	8	6	4
	c	4.30	4.16	both	10	10	10	9	9

function in oxygen transport by the blood when the animals are in water saturated with air, as well as at lower oxygen concentrations. This is so in Tubifex (Dausend, 1931) and in Lumbricus (Krüger, 1938; Johnson, 1942). In others, the haemoglobin only functions in oxygen transport when the animals are in water not saturated with air, as in the larvae of Chironomus (Ewer, 1942) and Tanytarsus (Walshe, 1947).

Experiments were made to find whether the haemoglobin of Artemia functions in oxygen transport by the blood, and if so, to determine the range of dissolved oxygen concentrations over which the pigment functions. This work was done at the Laboratoire Arago, Banyuls-sur-mer, from where it was possible to collect brine shrimps with much haemoglobin in the blood. These animals are parthenogenetic, and so all experiments were made with females. The oxygen consumption of normal red animals was compared with that of others whose haemoglobin had been rendered functionless by conversion to carboxyhaemoglobin. The experiments were made in brine of the same salinity as that from which the animals were collected in the field, S‰ 195. It was found, however, that if natural brine was used, the oxygen uptake by micro-organisms in the brine falsified the results. To avoid this, artificial brine was made by dissolving commercial sea salt in sea water. The animals were kept in this medium for 48 hours after collection in the field before they were used in experiments.

To determine the oxygen consumption, approximately thirty animals were enclosed in a glass-stoppered bottle of known volume (about 70 ml.), completely filled with brine of known dissolved oxygen content. A number of such bottles were placed in a water-bath at 23.5°C. in the dark for one hour, during which time each bottle was inverted every ten minutes to ensure that the animals were well distributed in the brine. After one hour, during which the oxygen content of the brine was lowered by about 0.5 ml./l., the oxygen concentration in each bottle was determined by the syringe-pipette micro-Winkler method. The animals were then removed from the bottles, rinsed twice in distilled water, carefully dried on filter-paper and weighed. By varying the initial oxygen content of the brine, a range of oxygen concentrations was obtained. The average oxygen concentration of the brine at which the respiratory rate was measured was calculated from the initial and final concentrations.

The treatment with carbon monoxide was as follows. About 200 animals were enclosed in a stoppered bottle in well-aerated brine to which sufficient carbon-monoxide-saturated brine had been added to make the partial pressure of carbon monoxide one-sixth that of the dissolved oxygen.* This was obtained by adding 1 ml. of carbon-monoxide-saturated brine to 22 ml. of air-saturated

* It was assumed that the relative solubilities of oxygen and carbon monoxide are the same in brine as in fresh water.

brine, and proportionately less in partially aerated brine. In this way the amount of carbon monoxide is sufficient to render the haemoglobin functionless but is unlikely to inhibit cellular respiration (Ewer & Fox, 1940). Preliminary experiments showed that after treatment for one hour, in the dark, with carbon monoxide the haemoglobin of Artemia is converted to carboxy-haemoglobin and remains in this state for the duration of the experiment. Animals so treated, with functionless haemoglobin, were then transferred to the experimental bottles as described for normal animals. At the end of each experiment the presence of carboxyhaemoglobin in the blood was checked as follows. Animals were placed on a glass slide in a few drops of a solution of sodium dithionite, and as this solution contains no dissolved oxygen, any oxyhaemoglobin present in the blood would be quickly deoxygenated. Carboxyhaemoglobin, however, is not affected and since two absorption bands were still visible with the micro-spectroscope the haemoglobin was still in the carboxy state.

The rate of oxygen uptake by Artemia, with and without functional haemoglobin, at various concentrations of dissolved oxygen, is given in figure 52 and in Appendix III, tables A and B. Since air-saturated brine of salinity S‰ 195 has an oxygen content of approximately 2 ml./l. at 25°C., it will be seen that the haemo-globin of Artemia functions in oxygen transport at air saturation

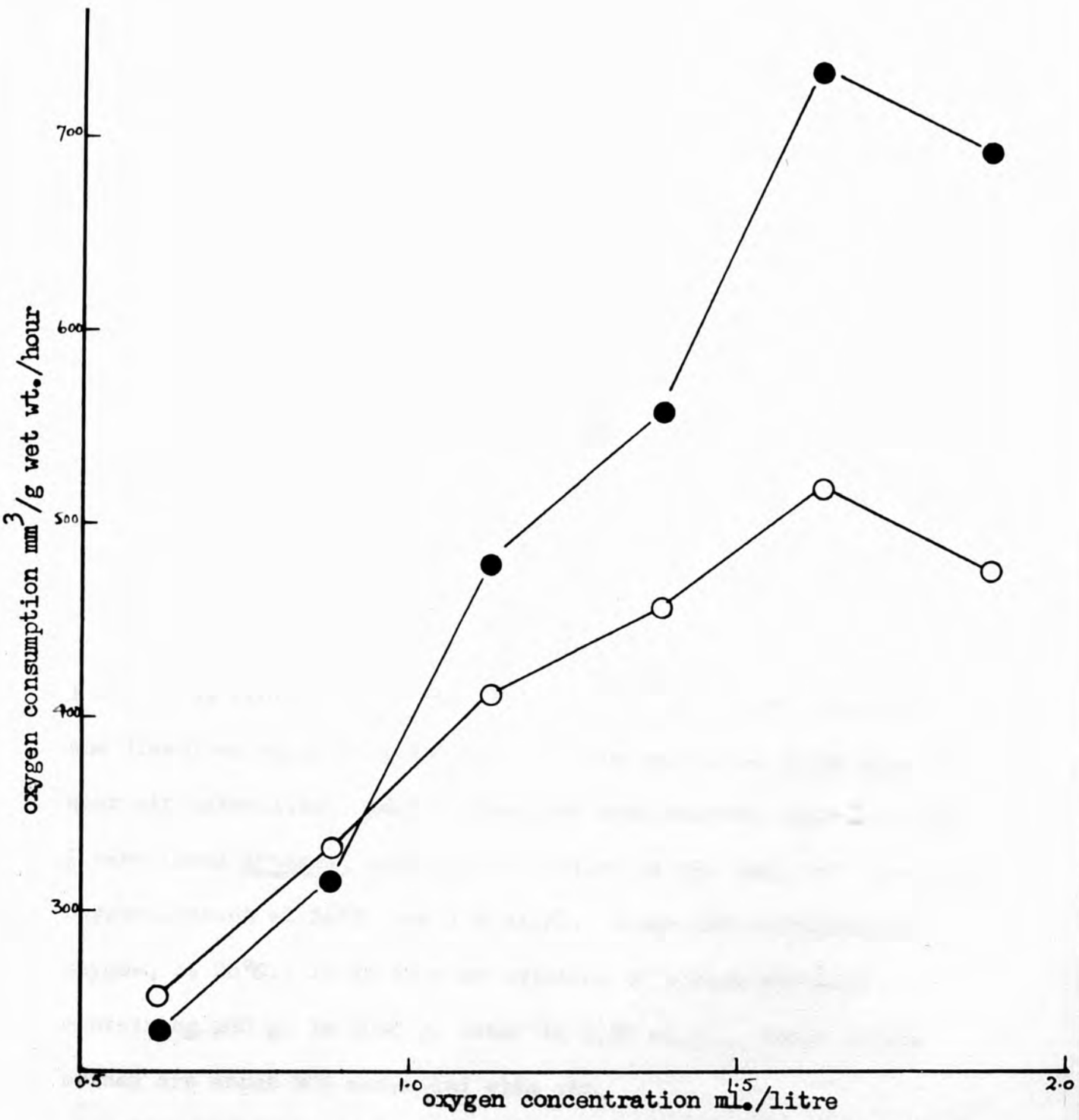


Figure 52. Oxygen consumption of *Artemia*, with and without functional haemoglobin, in brine (S‰ 195) at 23.5°C.
● normal animals; ○ those with carboxyhaemoglobin.

of the brine. It must be remembered, however, that although the brine is air saturated its dissolved oxygen content is low; it is not surprising, therefore, to find that the haemoglobin functions at this oxygen level. If animals are collected from less concentrated brines, the amount of haemoglobin in the blood is very small, and in those found in more dilute brines (less than S‰ 125) the pigment is generally not detectable. It would seem, therefore, that in such brines, with a dissolved oxygen content greater than 3 ml./l., haemoglobin is not necessary to the animals.

Below a dissolved oxygen content of about 1.05 ml./l. there is no significant difference in the oxygen uptake of animals with and without functional haemoglobin. Whether in nature Artemia is ever subjected to an oxygen pressure below that equivalent to 1 ml./l. is doubtful. In the salt works which I have investigated, the dissolved oxygen content of the brine was found to be always near air saturation. Even in the most concentrated brine in which I have found Artemia, with a salt content of S‰ 280, the dissolved oxygen content at 25°C. was 1.0 ml./l. Since the solubility of oxygen, at 25°C., in an aqueous solution of sodium chloride containing 280 g. in 1000 g. water is 1.25 ml./l., these strong brines are about 80% saturated with air.

The work described above has shown that Artemia is able to vary the amount of haemoglobin in solution in its blood according

to the dissolved oxygen content of the medium in which it lives. Since brine shrimps are found in nature in waters of widely different salt content, they must also be subjected to different amounts of dissolved oxygen. Animals in more concentrated brines will have less available oxygen than those in more dilute brines; they will, therefore, synthesize more haemoglobin and so become redder in colour. It seems, then, that the differences in red colour of Artemia in different concentrations of brine, as observed by many investigators, can be interpreted in terms of the gain and loss of haemoglobin.

It has also been shown that the additional haemoglobin enables Artemia to obtain more oxygen from the external medium than animals with no detectable blood pigment. This would seem to be essential to their survival on concentrated brines, for experiments described above have shown that in sea water containing little dissolved oxygen animals with no measurable amount of haemoglobin do not survive as long as those with much haemoglobin. So, in concentrated brines where the oxygen content is always low, even when the brine is fully saturated with air, the haemoglobin of Artemia functions in oxygen transport by the blood, thus enabling the animals to obtain enough oxygen for survival.

The range of concentrations of brine within which I have found Artemia in nature is S‰ 112 to S‰ 280, but until concentrations

above S‰ 125 are reached there is generally no detectable haemoglobin in the blood, although some individuals may be reddish in colour. This colour is due to carotenoid pigments. In brines above S‰ 125, Artemia synthesizes more and more haemoglobin as the dissolved oxygen in the medium decreases. It would seem, therefore, that the ability to synthesize haemoglobin is a property of Artemia essential to its existence in very saline water.

2. Haematin in the eggs of Artemia

Haematin in the eggs of Artemia has been identified by Needham & Needham (1930). These authors soaked eggs overnight in dilute alkali and so obtained a dark green solution; the fragments of egg shell remaining were colourless. This solution had the absorption spectrum characteristic of alkali haematin.

Reference has already been made to the variability in colour of the eggs of Artemia (page 79). Experiments were made, therefore, to see to what extent haematin in the egg shells is responsible for the differences in colour of the eggs. These experiments were made in collaboration with Dr. J. Green.

The absorption spectrum of haematin is rather diffuse, but it becomes very distinct when haematin is reduced and combined with pyridine, thus forming a pyridine haemochromogen. It was decided, therefore, to prepare a pyridine haemochromogen from the eggs of Artemia. This would give an estimate of the total haem content of

the eggs. We have never detected haemoglobin in the eggs or the nauplii or Artemia, and so the total haem will include haem compounds in the shell of the egg and cellular haem enzymes, cytochromes, in the tissues of the nauplii.

The method used to measure total haem was the same as that described by Green (1956b). By this method, the intensity of the α-band of a standard solution of pyridine haemochromogen is compared with that of a solution of pyridine haemochromogen prepared from the eggs of Artemia. The intensity of the α-bands of the two solutions is matched using the comparator microspectroscope method of Elliot & Keilin (1934). The standard solution of pyridine haemochromogen is placed in a wedge-shaped trough and put in front of the side aperture of the microspectroscope. The trough stands on a platform attached to the microscope tube and its position is read on a scale fixed to the platform. This scale measures 100 mm. and by moving the trough on the scale, absorption bands of different intensities are obtained.

The standard solution of pyridine haemochromogen was prepared as follows. A haemoglobin solution was made containing 0.1 ml. blood in 75 ml. distilled water. A pinch of the reducing substance sodium dithionite was added to 40 ml. of this haemoglobin solution, together with 10 ml. pyridine. The mixture was gently agitated to ensure proper mixing. If less pyridine is used the solution is cloudy, and there is also a danger that all the haem is not converted to the haemochromogen.

The pyridine haemochromogen solution was prepared from the eggs of Artemia as follows. One thousand eggs were counted and homogenized with a pinch of sodium dithionite and 2 ml. of pyridine. This homogenate was put into a small glass tube and the volume of the homogenate was made up to a fixed mark on the tube with pyridine. The tube was then left undisturbed for exactly three minutes to allow the fragments of shell and the excess sodium dithionite to settle. In this way a clear supernatant solution of pyridine haemochromogen was obtained; 1 ml. of this solution was transferred to a $1\frac{1}{2}$ x $\frac{3}{8}$ inch flat-bottomed tube and the tube was placed on the surface of the condenser of the microscope. The same flat bottomed tube was used in all the estimations of total haem; it was held in place on the condenser by racking down a 33 mm. objective until the surface of the lens of the objective was just in contact with the rim of the tube containing the haemochromogen solution.

The trough containing the standard haemochromogen solution and the tube containing the sample to be matched were each illuminated by a lamp, the intensity of whose light could be regulated. One lamp was placed opposite the side aperture of the microspectroscope so that the beam of light passed through the standard solution in the trough and so to the microspectroscope. The sample solution in the tube on the condenser was illuminated by a microscope lamp whose light beam was reflected from the

microscope mirror up through the condenser and so through the solution in the tube. The illumination of the two spectra was equalised by adjusting the intensities of the two light beams. The trough was then moved along the scale until the absorption bands of pyridine haemochromogen from the two solutions were of equal intensity. Three readings were made for each sample and the mean of these readings represents the haem content of the sample.

The results obtained by this method were verified using a Unicam S.P. 500 spectrophotometer in the following way. It was previously determined that the absorption maximum of the pyridine haemochromogen solution was at 557 mμ. A measure of the strength of a solution of pyridine haemochromogen was obtained by determining the intensity of its absorption at a wave length of 557 mμ. During preliminary experiments it was found that the intensity of absorption of the haemochromogen solutions decreased markedly the longer they were kept. As a precaution, therefore, in all the determinations of total haem, measurements both on the comparator microspectroscope and on the spectrophotometer were made after exactly the same interval of time since the preparation of the solutions.

Estimations of total haem content were made on two different samples of eggs of Artemia. One sample was obtained from a sea water culture of parthenogenetic females of the La Palme stock.

It was observed one day that a large number of eggs had been laid; these were pale cream in colour and were lying on the bottom of the culture vessel. The total haem content of these eggs was compared with that of eggs collected from salt works at La Palme. These eggs were collected from a part of the salt works where the brine is concentrated (about S‰ 180 to S‰ 200); they were darker in colour than the eggs laid in the sea water cultures.

The results of these experiments are shown in table 10. The comparator microspectroscope method is referred to as method 1, and the spectrophotometer determinations as method 2. The actual measurements obtained by these two methods are not comparable since different scales of measurement are used, but the ratio of the total haem content in pale and dark eggs is comparable in the two methods.

Table 10. Comparison of the total haem content of pale and dark coloured eggs of Artemia salina.

	total haem content of eggs		
	mean scale readings		ratio
	pale eggs	dark eggs	pale : dark
experiment 1			
method 1	7.3	28.7	1 : 3.9
method 2	56	200	1 : 3.6
experiment 2			
method 1	7.5	29.7	1 : 4.0
method 2	50	176	1 : 3.5

From these results it is clear that dark eggs contain from three and a half to four times as much haem as pale eggs; it is probable,

therefore, that the variations in colour of the eggs of Artemia are due to different concentrations of haem in the egg shell.

It is interesting to speculate upon the source of this haem which is found in the eggs. As has already been mentioned (page 79), I have never seen dark coloured eggs in a sea water culture of Artemia. Those eggs which are occasionally laid in this medium are pale in colour. On the other hand, the majority of eggs collected in the field from the edges of salt 'pans' containing concentrated brine are dark brown in colour. This suggests that females in concentrated brine have more haem available to put into their eggs than females in sea water. It has already been shown that females in concentrated brine have more haemoglobin in solution in the blood than those in more dilute media. It may be, therefore, that in Artemia the haem found in the egg shell is derived from the haemoglobin in the blood. In concentrated brine more haemoglobin is synthesized and so more is available to be passed into the egg shell; in aerated sea water little or no haemoglobin is synthesized and so there is little available to be passed to the eggs.

In the cladoceran Daphnia, it is known that haemoglobin passes from the blood into the parthenogenetic eggs (Dresel, 1948). This occurs when females are losing haemoglobin in response to an increase in the dissolved oxygen content of the water. It is not known whether there is a continuous 'turn-over' of haemoglobin in Daphnia

kept in poorly aerated water. No haemoglobin has ever been detected in the eggs or nauplii of Artemia, but it may be that haem is passed from the blood to the shell glands in the maternal brood pouch, and so to the egg shell. It is interesting to note, as the result of my own observations in the laboratory and in the field, that Artemia tends to be viviparous when kept in dilute media and oviparous in concentrated media. Thus the conditions which promote the synthesis of haemoglobin seem also to promote the production of eggs; haem can then be passed to the eggs. In sea water cultures eggs are rarely produced and haemoglobin rarely synthesized; those eggs which are occasionally produced contain very little haem. It may be then that there is a parallel between the passage of haemoglobin from the blood into the parthenogenetic eggs of Daphnia, and the passage of haem from the blood of Artemia into the egg shells.

CAROTENOIDS IN ARTEMIA

It has been shown that the red colour of Artemia in concentrated brine is due mainly to haemoglobin in solution in the blood. This pigment is synthesised in response to the low dissolved oxygen content of the brine.

Brine shrimps are frequently reddish-orange in colour when cultured in well aerated sea water and when there is no detectable haemoglobin in the blood. Likewise, the nauplii of Artemia are bright red or orange in colour and yet I have never detected haemoglobin in nauplii. These colours are due to carotenoid pigments. In well fed cultures of brine shrimps in sea water, I have regularly observed that females are orange in colour, while males are greenish in colour. There is apparently a sexual difference in the extent to which carotenoids are stored, or in their chemical constitution.

Carotenoid pigments are most abundant in the phagocytic storage cells (Lochhead & Lochhead, 1941) or 'fat' cells of Artemia. These occur mainly in the limbs, labrum and trunk. Occasionally, certain cells of the exopodites of the limbs are reddish in colour, particularly in males; Lochhead & Lochhead (1941) suggest that this pigment is "possibly astacin or one of its derivatives". The only other reference to carotenoids in

Artemia is that of Needham & Needham, (1930). They extracted an orange pigment from nauplii and refer to it as 'crustaceorubin'.

Little information is available as to the identity of the carotenoids found in other branchiopods. Those occurring in Daphnia magna Straus, have been identified and some of the causes of the variation in carotenoid content of different populations have been determined (Green, 1957).

In the following pages an account is given of preliminary experiments made to identify the carotenoid pigments present in Artemia. This work has been done in collaboration with Dr. J. Green.

Material and methods.

Carotenoid pigments were extracted from eggs, newly hatched nauplii and adults of Artemia of the bisexual Californian stock. The animals were reared in sea water; some cultures were fed on the green alga Chlamydomonas and others on a suspension of yeast. Carotenoids were also extracted from Chlamydomonas.

The reagents used in the extraction of the pigments were all of B.D.H. Analar quality.

The following procedure was adopted in the extraction and identification of the carotenoid pigments. The material, whether eggs, nauplii or adults, was concentrated in a mortar and any excess sea water drained off. The material was then ground up

thoroughly with acid-washed sand, and the pigments extracted with acetone. In the case of the eggs of Artemia, the material was reground many times and the pigments extracted with acetone until finally, the extract was colourless.

The acetone extract was filtered and the filtrate put into a separating funnel; an equal volume of petrol-ether was added and the pigments were transferred to the petrol-ether phase by the addition of water.

The pigments in the petrol-ether phase were then separated into hypophasic and epiphasic carotenoids by partition between petrol-ether and 90% methanol. An equal volume of 90% methanol was added to the petrol-ether extract in a separating funnel; those carotenoid pigments with epiphasic properties remain in the petrol-ether phase and those with hypophasic properties go into the 90% methanol. The two groups of pigments can thus be separated.

The hypophasic fraction was evaporated to dryness in a water bath, under a stream of nitrogen to prevent oxidation of the pigment, and the residue was dissolved in a suitable solvent, usually petrol-ether but occasionally benzene or ether, and subjected to chromatography. The epiphasic fraction was dried, when necessary, over anhydrous sodium sulphate and then chromatographed.

Both hypophasic and epiphasic fractions were chromatographed on alumina (aluminium oxide for chromatographic adsorption analysis)

columns in petrol-ether. The alumina was previously activated by drying in an oven overnight at 80°C. Some carotenoid pigments became adsorbed onto the column and some gradually washed through in the solvent. Any fractions which washed through were collected in small conical flasks. Those pigments which adsorbed onto the column were slowly separated from each other and finally eluted by gradually 'developing' the column with increasing amounts of ether or acetone in petrol-ether. In this way, a number of fractions was separated.

Some of these fractions were rechromatographed on the same or on different adsorbents, such as calcium hydroxide or calcium carbonate, in order further to purify the fractions.

Finally, the various fractions were evaporated to dryness in a water bath, under a stream of nitrogen, and then re-dissolved, generally in carbon disulphide or hexane, more rarely in ethanol or methanol. The absorption maxima of the various fractions were then determined with a Unicam S.P. 500 spectrophotometer.

Occasionally, the extracted pigments had to be kept for several hours before their absorption maxima could be determined. Such extracts were always kept under nitrogen in a corked tube or flask and put in a light-proof cupboard.

Results.

Carotenoid pigments in the eggs of Artemia

The carotenoid pigments extracted from the eggs of Artemia

showed entirely epiphasic properties when partitioned between petrol-ether and 90% methanol. The petrol-ether extract was chromatographed on alumina, and a single broad pink band of pigment appeared at the top of the column. This band moved slowly down the column and finally washed through with petrol-ether. The absorption spectrum of this extract in petrol-ether showed a single broad maximum from 466 to 468 mμ.

Some of the original acetone extract from the eggs of Artemia was divided into three parts, evaporated to dryness and the residues dissolved in carbon disulphide, hexane and pyridine respectively. The absorption maxima of these solutions were as follows:-

solvent	absorption maxima mμ.
carbon disulphide	500 to 504
hexane	462
pyridine	488 to 489

These figures agree well with those given by Goodwin & Srisukh (1949) and by Vevers (1952) for esterified astaxanthin.

Carotenoid pigments from the nauplii of Artemia

The acetone extract from newly hatched nauplii was put into a separating funnel, an equal volume of petrol-ether was added and by the addition of distilled water, the pigments were taken into the petrol-ether phase. On partition between petrol-ether

and 90% methanol, the pigments showed entirely epiphasic properties. The petrol-ether extract was then chromatographed on alumina.

On the several occasions upon which this experiment was made, two bands of pigment always appeared on the column. The lower of the two bands washed straight through the column with petrol-ether and the upper band slowly moved down the column with 80 to 90% ether in petrol-ether and was finally eluted with 100% ether. When the absorption spectra of these two fractions were determined it was found that the maxima occurred at the same wave length, indicating that only one carotenoid was present. The absorption maxima are given below.

solvent	absorption maxima mμ.
carbon disulphide	500
hexane	466
petrol-ether	468

Thus, the only carotenoid pigment identified in the newly hatched nauplii of Artemia is esterified astaxanthin.

It is reasonable to assume that the esterified astaxanthin identified in the eggs of Artemia resides in the nauplius within the egg and is not in the egg shell. It has already been shown that the colour of the egg shell is due to haematin (page 162).

Carotenoid pigments in the adults of Artemia

Acetone extracts of algal-fed mixed cultures of males and

females of Artemia were taken into petrol-ether and the pigments partitioned between petrol-ether and 90% methanol. Both hypophasic and epiphasic pigments were present.

1. Hypophasic pigments.

The hypophasic extract in 90% methanol was diluted with distilled water and the pigments taken into petrol-ether. The petrol-ether extract was chromatographed on activated alumina. Three bands of pigment were adsorbed onto the column, a lower yellowish-orange band (a), a middle yellowish-green band (b) and an upper pale orange band (c).

The lower band (a) moved slowly down the column and was eluted with 10% acetone in petrol-ether. The absorption spectrum of this pigment in carbon disulphide showed a maximum at 501 mμ, and in hexane the maximum was at 465 mμ. The position of these maxima correspond to those of esterified astaxanthin as identified from the eggs and nauplii of Artemia. Astaxanthin esters are characteristically epiphasic on partition between petrol-ether and 90% methanol; this was so in the case of extracts from the eggs and nauplii. In the adults of Artemia they appear to be hypophasic. We have repeated these experiments many times, always with the same result, and we have recently heard that hypophasic esters, although uncommon, are occasionally identified (personal communication, Dr. Cheesman).

It is of interest to record that in one series of experiments,

carotenoid pigments were extracted only from males. No hypophasic astaxanthin esters were identified from this extract although the pigment was identified in the epiphase.

The middle band of pigment (b) was eluted with 80% acetone in petrol-ether. The absorption spectra of this fraction, in various solvents, showed maxima at the wave lengths given below:-

solvent	absorption maxima mμ.		
carbon disulphide		473	(501-2)
ethanol		444	
methanol	(420)	442	(471)

These figures agree well with those given by Karrer & Jucker (1950) for xanthophyll epoxide.

The absorption spectrum of xanthophyll epoxide is very similar to that of violaxanthin, which has the following absorption maxima:-

solvent	absorption maxima mμ.		
carbon disulphide	440	470	501
ethanol	417	442	471
methanol	415	440	469

According to Karrer & Jucker (1950), the two pigments can be distinguished by dissolving them each in chloroform, to which a few drops of concentrated hydrochloric acid have been added. Xanthophyll epoxide is converted to flavoxanthin, with absorption maxima in carbon disulphide at 478 and 449 mμ; violaxanthin is converted

into auroxanthin, with absorption maxima in carbon disulphide at 454 and 423 mμ. This test was made on fraction (b) and the resulting pigment, in carbon disulphide, had an absorption spectrum with maxima at 449 and 478 mμ. This confirms the identity of xanthophyll epoxide.

The upper pale orange band (c) gradually faded on the column and we were not able to elute it.

2. Epiphasic pigments.

The epiphasic extract was chromatographed on activated alumina in petrol-ether. Three bands of pigment appeared on the column, a lower orange band (a), a middle red-orange band (b) and an upper orange band (c).

The lower band of pigment (a) was eluted with 2% acetone in petrol-ether and had the following absorption maxima:-

solvent	absorption maxima mμ.	
carbon disulphide	485	
hexane	449	(476)

The chromatographic behaviour of this pigment and the position of its absorption maxima indicate that it is β-carotene.

The middle band of pigment (b) was eluted from the column with carbon disulphide at 503 mμ. This pigment appears to be esterified astaxanthin.

The upper band of pigment (c) was eluted with 10% acetone in petrol-ether, and in carbon disulphide the absorption spectrum had

an ill-defined maximum between 494 and 504 mμ. Clearly, this fraction is a mixture of carotenoids, possibly of γ-carotene (absorption maximum in carbon disulphide at 496 mμ) and of esterified astaxanthin. One of the chromatographic properties of γ-carotene is that it is more strongly adsorbed onto the column than is β-carotene, and so it appears nearer the top of the column.

A second epiphasic extract of the carotenoid pigments from adults of Artemia was chromatographed in petrol-ether on a mixture of activated calcium hydroxide and an inert material ('ballotini' grade 4; tiny glass balls of various grades which are manufactured for spraying onto cinema screens). Two bands of pigment appeared on the column, a lower orange band and an upper pinkish band.

The lower band was eluted from the column with 2% acetone in petrol-ether and the first and last fractions of the eluted pigment were kept separate. The absorption maxima of the first fraction were as follows:-

solvent	absorption maxima mμ.
carbon disulphide	483 to 485
hexane	449 (476)

From its position on the column and its absorption spectra, the pigment appears to be β-carotene. The last fraction of the lower band had an absorption maximum in hexane at 453 mμ. This figure agrees fairly well with the absorption maximum of echinenone,

represented graphically by Goodwin (1952). Although at first thought to be a pigment characteristic of echinoids, echinenone now appears to be widely distributed in algae and many marine invertebrates. It may be obtained by the oxidation of β-carotene and this fact may account for the appearance of a pigment similar to echinenone, together with β-carotene on the chromatogram. The identity of this fraction must be regarded as uncertain; it requires further investigation.

The upper band of pigment from the second epiphasic extract was eluted with 5% acetone in petrol-ether and its absorption spectrum in carbon disulphide had a main peak at 502 mμ. This pigment appears to be an astaxanthin ester.

Thus, the following carotenoid pigments have been identified from the adults of Artemia, β-carotene, esterified astaxanthin and xanthophyll epoxide. The identity of γ-carotene and echinenone is uncertain.

Carotenoid pigments in Chlamydomonas

The brine shrimps used in the above experiments were fed on a marine species of Chlamydomonas, and so it was of importance to know which carotenoids were available to the animals in their food. When these experiments were made, no information was available in the literature as to the carotenoid pigments in Chlamydomonas.

Using methods as already described for the separation and identity of carotenoids from Artemia, we have identified a number of these pigments from Chlamydomonas. These carotenoids are listed

in table 11 and are arranged in the order in which they were adsorbed onto the column. The identity of the pigments is based upon their chromatographic behaviour and the position of their absorption maxima, using the figures in Karrer & Jucker (1950) as a standard for comparison.

Since this work was done, Sager & Zalokar (1958) have published a list of carotenoid pigments which they have extracted from Chlamydomonas reinhardi. They separate fifteen different fractions, but the only carotenoids identified are xanthophyll and α - and β -carotene. The wave lengths of the absorption maxima given for many of the unidentified fractions are very similar and it seems most unlikely that they represent separate pigments.

Thus, it seems that all the carotenoid pigments identified from the adults of Artemia, with the exception of esterified astaxanthin, are available in the food of the animals. Some of these pigments would, in fact, be present in algal cells in the guts of the animals when the latter were ground up in acetone.

Esterified astaxanthin has not been identified from Chlamydomonas, but it is the only carotenoid identified from the eggs and nauplii and it is abundant in the adults. It seems, therefore, that Artemia can alter the carotenoids which it takes in. Another point of interest, suggesting that Artemia can selectively concentrate certain carotenoids, is the fact that although α - and β -carotene have been identified from Chlamydomonas, of these two pigments only β -carotene has been found in the adults of Artemia.

Table 11. Carotenoid pigments identified from Chlamydomonas.

fraction	solvent	absorption maxima mμ.			identity
epiphase 1.	carbon disulphide	(469)	495	(527)	γ -carotene
	hexane		460		
epiphase 2.	carbon disulphide		477/8		α -carotene
epiphase 3.	hexane		450		β -carotene
hypophase 1.	carbon disulphide		472	504	xanthophyll epoxide
hypophase 2.	carbon disulphide		474	505	xanthophyll epoxide + xanthophyll ?
hypophase 3.	carbon disulphide		470	501	violaxanthin
	ethanol		442	(469)	
hypophase 4.	carbon disulphide		467/8	499	?

The distribution of the carotenoid pigments identified from Artemia and from Chlamydomonas is summarised in table 12. These results must be regarded as a preliminary study of the carotenoids of Artemia. Nothing is known as to how esterified astaxanthin gets into the eggs. The ovary of Artemia is never orange or red or blue-green in colour as is found in Daphnia. Carotenoids may, however, be present in the ovary linked to a protein and possibly colourless or only faintly blue-green in colour. On the other hand, the pigment may enter the egg while the latter is in the brood pouch of the female; the shell glands in the maternal pouch are sometimes orange-brown in colour. Further discussion is clearly fruitless until more information on the subject is available.

Table 12. Distribution of carotenoid pigments identified from Artemia and from Chlamydomonas.

Carotenoid pigment	Chlamydomonas	Artemia		
		adults	nauplii	eggs
α -carotene	+	-	-	-
β -carotene	+	+	-	-
γ -carotene	+	+?	-	-
xanthophyll	+	-	-	-
xanthophyll epoxide	+	+	-	-
violaxanthin	+	-	-	-
esterified astaxanthin	-	+	+	+

Water-soluble green pigment in the blood of Artemia

It has frequently been noticed that males of Artemia, and more rarely females, are green in colour. This is particularly noticeable in yeast-fed animals. It is not known whether the yeast itself plays some part in the production of the green colour, or whether the paucity of carotenoid pigments in yeast-fed animals renders the green colour more conspicuous.

The green colour resides in the blood of Artemia. Green blood has occasionally been observed in a number of branchiopod Crustacea (Lochhead & Lochhead, 1941; Fox, 1955). This green pigment in the blood of Artemia has been identified as a carotenoid-protein (Fox, 1955), and variations in concentration of a carotenoid-protein in the blood of Daphnia magna have been studied by Green (1957). He has shown that the amount of the pigment in the blood increases rapidly after the eggs have been laid, then it maintains a steady level and finally falls when the next brood of eggs is laid. It is suggested that the loss of green colour in the blood is due in part, at least, to the passage of the pigment into the ovary.

We have attempted on several occasions to confirm that the green colour of the blood of Artemia is due to a carotenoid-protein; the results have not been conclusive. When several animals, with bright green blood, are treated with protein denaturing agents such as acetone or concentrated acid, the green colour of the blood

disappears, but the blood does not become orange in colour as would be expected were a carotenoid present.

A small quantity of green blood was obtained by gently squeezing a large number of yeast-fed males of Artemia through fine bolting silk. This green blood, in water, had an absorption maximum at 413 mμ, and lesser peaks at 540 and 674 mμ.

An equal volume of acetone was added to some of the green blood collected as described above, and the mixture was well shaken. A small amount of protein was precipitated, but there was no change in colour of the blood. Petrol-ether was added, together with some distilled water and the mixture was again thoroughly agitated. The blood remained green and the petrol-ether phase was colourless. A few drops of concentrated hydrochloric acid were added; the petrol-ether phase became yellow-orange in colour. Seemingly, the link between the protein and the prosthetic group had been broken.

The absorption spectrum of the petrol-ether phase showed a maximum at 376 mμ. Clearly, the pigment separated from the protein does not have the properties characteristic of a carotenoid. The pigment is more firmly attached to a protein than are the majority of carotenoids; the link was not broken by acetone, but only by concentrated acid. Further, the absorption maximum in petrol-ether does not correspond to that of any known carotenoid pigment.

On the other hand, it is known that certain bile pigment

chromoproteins are very firmly bound together, the prosthetic group being set free only after treatment with concentrated acid (Lemberg & Legge, 1949). Further, the absorption spectrum of the green blood of Artemia, in water, is not unlike that of some bile pigments (Gray, 1953). It seems, therefore, that the green colour of the blood occasionally found in Artemia, may not be due to a carotenoid-protein such as is found in the blood of Daphnia and several other branchiopods. The possibility that the colour is due to a bile pigment linked with a protein must be further investigated.

General discussion on pigments of Artemia

It has been shown that the red colour of Artemia is due to two types of pigment, haemoglobin and carotenoids.

Haemoglobin is present in solution in the blood of adult brine shrimps; the amount of the pigment present is inversely related to the dissolved oxygen content of the external medium. Thus, in very concentrated brine, containing little dissolved oxygen even when fully saturated with air, Artemia is redder in colour than in more dilute media. Haemoglobin has not been identified in the nauplii; these are bright red in colour due to a carotenoid, esterified astaxanthin.

It is of interest to relate these facts to references in the literature to the red colour of Artemia. As already mentioned (page 146), it is stated by Payen (1836), Schmankewitsch (1877) and

Kellogg (1906) that Artemia becomes redder as the salinity of the external medium increases. This colour is clearly the result of the synthesis of haemoglobin. Likewise, the red animals described by Joly (1840), when transferred to a more dilute medium, became less red as the result of the loss of haemoglobin from the blood. This loss is associated with the increase in amount of dissolved oxygen in the more dilute medium.

When Packard (1883) and Jensen (1918) describe the nauplii of Artemia as "blood red", the colour is not, in fact, due to haemoglobin but to a carotenoid pigment. It is interesting to note that Artom (1905) found the nauplii of Artemia red in colour both in high and in low concentrations of brine. This observation is again in agreement with the facts described above; the colour is due to a carotenoid pigment and not to haemoglobin.

Reference should be made to the statement of Bond (1933) that the colour of Artemia is dependent upon its food. This is in part true, since brine shrimps fed on green algae store more carotenoids and are, therefore, redder in colour than those animals which are fed on yeast. Yeast-fed animals, particularly males, are green in colour. On the other hand, much of the red colour of Artemia is due to haemoglobin and variations in the amount of haemoglobin are not related primarily to the food of Artemia but to the dissolved oxygen content of the external medium.

GENERAL DISCUSSION

Discussion relating to specific aspects of the metabolism of *Artemia* has been inserted in the relevant parts of the text. It remains, therefore, to comment upon the implications and inter-relationships of the work described in this thesis.

Not all aspects of the metabolism of *Artemia* have been discussed; feeding and nutrition have been omitted. Food, however, must be regarded as the primary source of energy, the 'fuel', for all metabolic activities. From food energy is derived for growth, reproduction and the many chemical and physiological processes in operation within an animal. In my own experiments, I have attempted to ensure that excess food was always available to *Artemia*; the amount given was controlled and standardised so that the initial 'fuel' supply available was the same in all comparative studies on metabolism.

The metabolism of *Artemia* is influenced by the inherent properties of the animal. Important among these intrinsic factors are age, sex and genetic constitution. Likewise, metabolism is influenced by extrinsic factors, properties of the external environment. In the life of *Artemia*, the influence of the salinity of the medium and its dissolved oxygen content is significant.

The phenomenon of growth is perhaps the most obvious result of the metabolic activity of an animal. If the length of *Artemia* is expressed graphically in relation to time, a curve of growth

results. By comparing curves of growth, information is obtained on the growth rate of an animal under different conditions. The salinity of the external medium and its dissolved oxygen content influence the rate of growth of Artemia; a high salt content and a paucity of oxygen retard growth. This statement, however, must be modified in relation to certain intrinsic factors. The growth rate of females is retarded to a greater extent than that of males, while the growth rate of parthenogenetic females from southern France is not retarded in concentrated brine. Clearly, size is dependent upon many variables other than age.

The form of an animal is the direct result and consequence of growth; the two phenomena are intimately related. It is not surprising, therefore, to find that factors which influence the growth of Artemia are also responsible for variations in body form. The genetic constitution of Artemia seems to be the most important intrinsic factor modifying the effects of the external medium on body form. Brine shrimps from different localities, cultured under standard conditions in the laboratory, do not look alike. Body proportions vary markedly, and yet only a single species in the genus Artemia is recognised. In the absence of precise morphological characters, on which to base the separation of species, this seems advisable. Not only do body proportions vary between animals from different localities, but the extent to

which body form is influenced by the salinity of the external medium, is related to the genetic constitution of the population.

Growth and form are not the only metabolic processes in Artemia influenced by the genetic make-up of the animal. Physiological differences occur between animals from different localities. The oxygen consumption of parthenogenetic females from southern France, when measured under standard conditions, is higher than that of females from a bisexual population derived from California. The higher metabolic activity of the parthenogenetic females is also reflected in their curve of growth; they have a more rapid rate of growth than females of the bisexual stock when reared under standard culture conditions. It seems, therefore, that local geographical races of brine shrimps should be recognised; the morphological and physiological characteristics of these races must be considered in comparative studies of the metabolism of Artemia.

Changes in metabolic activity are generally reflected in the oxygen consumption of an animal. In Artemia, oxygen consumption is proportional to body surface; it is influenced not only by the external concentration of dissolved oxygen but also by the age and sex of the animal. The oxygen uptake of brine shrimps reared in concentrated media is of the same magnitude as that of animals reared in more dilute media. The energy requirements of

Artemia are likely, however, to be greater in strong brines since osmotic work is being done against a steeper gradient. The distribution of available energy is perhaps modified according to the metabolic needs of the animal. In strong brines, more energy is needed for osmotic work and so other metabolic processes are curtailed; egg production is reduced and growth is retarded. It is of interest to note that in strong brine, the growth of males is retarded less than that of females; this may be because they do not require to expend such a large proportion of energy on reproduction.

The paucity of dissolved oxygen in concentrated brines stimulates the synthesis of haemoglobin in Artemia. The respiratory pigment enables brine shrimps to obtain more oxygen from the external medium; without haemoglobin, Artemia is unable to survive in very saline waters. We know little about the haem metabolism of brine shrimps, but the conditions which stimulate haemoglobin synthesis appear, indirectly, to stimulate oviparous reproduction. Haem is abundant in the eggs of Artemia, and it may be that the haemoglobin in solution in the blood is the source of the haem in the eggs.

It is clear that the metabolic activities of Artemia are closely inter-related; they cannot be considered as separate functions. It has been said by the eighteenth century anatomist,

Xavier Bichat, that "la vie est l'ensemble des fonctions qui résistent à la mort." Evidence in support of this aphorism may be found in the metabolic activities of Artemia. Those few activities which have so far been investigated seem to be directed towards the survival of Artemia in its own particular habitat.

SUMMARY OF THE MAIN CONTRIBUTIONS MADE IN THIS THESIS TO THE STUDY OF THE METABOLISM OF *ARTEMIA SALINA* (L.)

1. The literature relevant to the present work on the metabolism of *Artemia* has been critically reviewed. The results of previous authors have, in part, been re-interpreted in the light of recent work.

2. Factors influencing the growth of *Artemia* have been investigated in animals derived from five different stocks of brine shrimps, reared in two different concentrations of brine.

3. The growth of *Artemia* is retarded in very concentrated brines and also by a low dissolved oxygen content of the medium. The effect is more marked on females than on males.

4. Growth is influenced by certain intrinsic factors, in particular, the sex of the animal and the stock from which it is derived.

5. The factors influencing body form in *Artemia* have been investigated.

6. With increase in total length of *Artemia*, the abdomen becomes relatively longer; the extent to which the ratio of the length of abdomen to prosoma increases, varies with the sex

and the genetic constitution of the animal.

7. The width of the abdomen, length of caudal furcae and the number of setae on the furcae, in relation to the size of Artemia, vary according to the stock from which the animal is derived.

8. Brine shrimps reared in a concentrated medium have a relatively longer and narrower abdomen than animals of the same total length reared in dilute media. The influence of the external medium is modified by the sex of the animal and its genetic constitution.

9. The oxygen consumption of parthenogenetic females from southern France is higher than that of females from the bisexual Californian stock.

10. The oxygen consumption of Artemia is influenced by the oxygen concentration of the external medium.

11. The relation between oxygen consumption and size of Artemia has been investigated. The ratio of dry weight to wet weight has been measured and its importance in relation to oxygen consumption discussed.

12. The oxygen consumption of Artemia has been compared in animals reared in different concentrations of brine.

13. Females of *Artemia* reared in sea water have the same rate of oxygen consumption as those reared in concentrated brine.

14. The increase in oxygen uptake with increase in size of males of *Artemia* is greater in sea water than in concentrated brine.

15. The area of the second antennae of males increases more rapidly with increase in size of animals reared in sea water than in concentrated brine. The higher rate of oxygen consumption of males reared in sea water is related to the larger area of the second antennae in this medium.

16. The colours of *Artemia* are due mainly to two types of pigment, haem compounds and carotenoids.

17. The gain and loss of haemoglobin in solution in the blood of *Artemia*, in response to a low or high dissolved oxygen content of the medium, has been measured quantitatively.

18. Females of *Artemia* gain and lose the pigment more rapidly than males.

19. In natural populations, *Artemia* is redder in colour in proportion to the increasing salinity of the medium. This is the result of the synthesis of haemoglobin in response to the paucity of oxygen in the concentrated media.

20. Brine shrimps with haemoglobin in solution in the blood are more viable in poorly aerated sea water than animals with no detectable amount of the respiratory pigment.

21. The haemoglobin of Artemia functions in oxygen transport by the blood when animals are in concentrated brines, either fully or partially saturated with air.

22. The colour of the eggs of Artemia is due to haematin. Eggs laid in sea water are pale cream in colour and those laid in concentrated brine are dark brown in colour.

23. There is approximately four times as much haem in the eggs laid by females in concentrated brine as in those laid in sea water. The source of the haematin in the eggs is discussed.

24. The nauplii of Artemia are red in colour due to a carotenoid pigment, esterified astaxanthin.

25. Carotenoid pigments have been separated and identified from the adults of Artemia. The carotenoids available in the food of the brine shrimps have also been identified.

26. A water soluble green pigment occurs in the blood of Artemia; the nature of this pigment is discussed.

REFERENCES

Abonyi, A. 1915 Experimentelle Daten zum Erkennen der Artemia Gattung. Z. wiss. Zool. 114, 95-168.

Allen, E.J. & Nelson, E.W. 1910 On the artificial culture of marine plankton organisms. J. Mar. biol. Ass. U.K. 8, 421-474.

Anikin, W.P. 1898 Einige biologische Beobachtungen über die Crustaceen der Gattung Artemia. Zool. Zbl. 6, 757-760.

Artom, C. 1905 Osservazioni generali sull' Artemia salina Leach delle Saline di Cagliari. Zool. Anz. 29, 284-291.

Artom, C. 1906a Ricerche sperimentali sul modo di riprodursi dell' Artemia salina Lin. di Cagliari. Biol. Zbl. 26, 26-32.

Artom, C. 1906b Osservazioni e raffronto tra le Artemie sessuate et le Artemie partenogenetiche. Biologica, Torino 1, 1-4.

Artom, C. 1907a La variazione dell' Artemia salina (Linn.) di Cagliari sotto l'influsso della salsedina. Mem. R. Accad. Torino 57, 221-254.

Artom, C. 1907b Ricerche sperimentali sulla variazione dell' Artemia salina Lin. di Cagliari. Biologica, Torino 1, 247-264.

Artom, C. 1931 L'origine e l'evoluzione della partenogenesi attraverso i differenti biotipi di una specie collettiva (Artemia salina L.) con speciale riferimento al biotipo diploide partenogenetico di Sète. Mem. R. Accad. Ital. 2, 1-57.

Baas Becking, L.G.M., Karstens, W.K.H. & Manner, M. 1936 Salzeffekte und Milieu bei Artemia salina L. nebst Bemerkungen über ionenantagonismus. Protoplasma 25, 32-40.

Baird, W. 1850 The Natural History of the British Entomostraca. London : Ray Society.

Barigozzi, C. 1939 La biologia di Artemia salina Leach studiata un aquario. Atti Soc. ital. Sci. nat. 78, 137-160.

Bateson, W. 1894 Materials for the study of variation. London : Macmillan.

Beadle, L.C. 1931 The effect of salinity changes on the water content and respiration of marine invertebrates. J. exp. Biol. 8, 211-227.

Beadle, L.C. 1939 Regulation of the haemolymph in the saline water mosquito larva Aedes detritus Edw. J. exp. Biol. 16, 346-362.

Berg, K. 1952 On the oxygen consumption of Ancylidae (Gastropoda) from an ecological point of view. Hydrobiologia 4, 225-267.

Berg, K. 1953 The problem of respiratory acclimatization. Hydrobiologia 5, 331-350.

Bertalanffy, L. von 1957 Quantitative laws in metabolism and growth. Quart. Rev. Biol. 32, 217-231.

Bertalanffy, L. von & Krywienczyk, J. 1953 The surface rule in Crustaceans. Amer. Nat. 87, 107-110.

Blanchard, R. 1891 Résultats d'une excursion zoologique en Algérie. Mém. Soc. zool. Fr. 4, 208-245.

Bond, R.M. 1933 Observations on Artemia 'franciscana' Kellogg, especially on the relation of environment to morphology. Int. Rev. Hydrobiol. 28, 117-125.

Boone, E. & Baas Becking, L.G.M. 1931 Salt effects on eggs and nauplii of Artemia salina L. J. gen. Physiol. 14, 753-763.

Buchholz, R. 1864 Branchipus Grubii v. Dybowski. Schr. phys.-ökon. Ges. Königsb. 5, 93-108.

Claus, C. 1886 Untersuchungen über die Organisation und Entwickelung von Branchipus und Artemia nebst vergleichenden Bemerkungen über andere Phyllopoden. Arb. zool. Inst. Univ. Wien 6, 267-370.

Conklin, R.E. & Krogh, A. 1938 A note on the osmotic behaviour of Eriocheir in concentrated and Mytilus in dilute sea-water. Z. vergl. Physiol. 26, 239-241.

Croghan, P.C. 1958a The survival of Artemia salina (L.) in various media. J. exp. Biol. 35, 213-218.

Croghan, P.C. 1958b The osmotic and ionic regulation of Artemia salina (L.). J. exp. Biol. 35, 219-233.

Croghan, P.C. 1958c The mechanism of osmotic regulation in Artemia salina (L.) : the physiology of the gut. J. exp. Biol. 35, 243-249.

Croghan, P.C. 1958d The mechanism of osmotic regulation in Artemia salina (L.) : the physiology of the branchiae. J. exp. Biol. 35, 234-242.

Croghan, P.C. 1958e Ionic fluxes in Artemia salina (L.). J. exp. Biol. 35, 425-436.

Daday de Deés, E. 1910 Monographie systématique des Phyllopodes Anostracés. Ann. Sci. nat. Zool. (9), 11, 91-489.

Dausend, K. 1931 Über die Atmung der Tubificiden. Z. vergl. Physiol. 14, 557-608.

Dawydoff, C. 1928 Traité d'Embryologie comparée des Invertébrés. Paris : Masson.

Dejdar, E. 1930 Die Korrelationen zwischen Kiemensäckchen und Nackenschild bei Phyllopoden. Z. wiss. Zool. 136, 422.

Dempster, R.P. & Hanna, G.D. 1956 Preserving Artemia eggs in high vacuum. Aquarium J. 27, 112-113.

Dresel, E.I.B. 1948 Passage of haemoglobin from blood into eggs of Daphnia. Nature, Lond., 162, 736.

Edlén, Å. 1937 Experimentelle Wachstumsstudien an Daphnia magna. K. fysiogr. Sällsk. Lund Förh. 49, 1-29.

Eliassen, E. 1952 The energy metabolism of Artemia salina in relation to body size, seasonal rhythms and different salinities. Univ. Bergen Årb. naturv. R. 11, 1-18.

Ellenby, C. 1951 Body size in relation to oxygen consumption and pleopod beat in Ligia oceanica L. J. exp. Biol. 28, 492-507.

Elliot, K.A.C. & Keilin, D. 1934 The haematin content of horseradish peroxidase. Proc. Roy. Soc. B, 114, 210-222.

Ermakow, H.B. 1928 cited in Brehm, V. 1938 Die Rotfärbung von Hochgebirgsseeorganismen. Biol. Rev. 13, 307-318.

Evans, T.J. 1913 Observations on Artemia salina. Nature, Lond., 92, 385-386.

Ewer, R.F. 1942 On the function of haemoglobin in Chironomus. J. exp. Biol. 18, 197-205.

Ewer, R.F. & Fox, H.M. 1940 On the function of chlorocruorin. Proc. Roy. Soc. B, 129, 137-153.

Fox, H.M. 1945 The oxygen affinities of certain invertebrate haemoglobins. J. exp. Biol. 21, 161-165.

Fox, H.M. 1948 The haemoglobin of Daphnia. Proc. Roy. Soc. B, 135, 195-212.

Fox, H.M. 1949 Haemoglobin in Crustacea. Nature, Lond. 164, 59.

Fox, H.M. 1952 Anal and oral intake of water by Crustacea. J. exp. Biol. 29, 583-599.

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

Fox, H.M. 1957a Haemoglobin in Crustacea. Nature, Lond., 179, 148.

Fox, H.M. 1957b Haemoglobin in Branchiura. Nature, Lond., 179, 873.

Fox, H.M., Gilchrist, B.M. & Phear, E.A. 1951 Functions of haemoglobin in Daphnia. Proc. Roy. Soc. B, 138, 514-528.

Fox, H.M., Hardcastle, S.M. & Dresel, E.I.B. 1949 Fluctuations in the haemoglobin content of Daphnia. Proc. Roy. Soc. B, 136, 388-399.

Fox, H.M. & Phear, E.A. 1953 Factors influencing haemoglobin synthesis by Daphnia. Proc. Roy. Soc. B, 141, 179-189.

Fox, H.M. & Wingfield, C.A. 1938 A portable apparatus for the determination of oxygen dissolved in a small volume of water. J. exp. Biol. 15, 437-445.

Gajewski, N. 1922 Uber die Variabilität bei Artemia salina. Int. Rev. Hydrobiol. 10, 139-159 and 299-308.

Goldschmidt, E. 1952 Fluctuation in chromosome number in Artemia salina. J. Morph. 91, 111-131.

Goodwin, T.W. 1952 The comparative biochemistry of the carotenoids. London : Chapman & Hall.

Goodwin, T.W. & Srisukh, S. 1949 Some observations on astaxanthin distribution in marine Crustacea. Biochem. J. 45, 268-270.

Gray, C.H. 1953 The bile pigments. London : Methuen.

Green, J. 1955 Studies on a population of Daphnia magna. J. Anim. Ecol. 24, 84-97.

Green, J. 1956a Growth, size and reproduction in Daphnia (Crustacea : Cladocera). Proc. zool. Soc. Lond., 126, 173-204.

Green, J. 1956b Variation in the haemoglobin content of Daphnia. Proc. Roy. Soc. B, 145, 214-232.

Green, J. 1957 Carotenoids in Daphnia. Proc. Roy. Soc. B, 147, 392-401.

Hall, R.E. 1953 Observations on the hatching of eggs of Chirocephalus diaphanus. Proc. zool. Soc. Lond., 123, 95-109.

Harvey, H.W. 1955 The chemistry and fertility of sea waters. Cambridge : University Press.

Heath, H. 1924 The external development of certain phyllopods. J. Morph. 38, 453-483.

Hendey, N.I. 1954 Note on the Plymouth 'Nitzschia' culture. J. Mar. biol. Ass. U.K. 33, 335-339.

Hinton, H.E. 1954 Resistance of the dry eggs of Artemia salina L. to high temperatures. Ann. Mag. nat. Hist. 7, 158-160.

Höber, R. 1899 Bedeutung der Theorie der Lösungen für Physiologie und Medizin. Biol. Zbl. 19, 272.

Jacobi, E.F. & Baas Becking, L.G.M. 1933 Salt antagonism and effect of concentration in nauplii of Artemia salina L. Tijdschr. ned. dierk. Ver. 3, 145-153.

Jennings, R.H. & Whitaker, D.M. 1941 The effect of salinity upon the rate of excystment of Artemia. Biol. Bull., Wood's Hole 80, 194-201.

Jensen, A.C. 1918 Some observations on Artemia gracilis, the brine shrimp of Great Salt Lake. Biol. Bull., Wood's Hole 34, 18-25.

Johnson, M.L. 1942 The respiratory function of the haemoglobin of the earthworm. J. exp. Biol. 18, 266-277.

Joly, N. 1840 Histoire d'un petit crustacé (Artemia salina Leach) auquel on a faussement attribué la coloration en rouge des marais salans méditerranéens, suivi de recherches sur la cause réelle de cette coloration. Ann. Sci. nat. (2), 13, 225-290.

Jones, L.L. 1941 Osmotic regulation in crabs. J. cell. comp. Physiol. 18, 79-92.

Karrer, P. & Jucker, E. 1950 Carotenoids. London : Elsevier.

Kellogg, V.L. 1906 A new Artemia and its life conditions. Science 24, 594-596.

Keys, A.B. 1931 Chloride and water secretion and absorption by the gills of the eel. Z. vergl. Physiol. 15, 364-388.

Keys, A. & Willmer, E.N. 1932 "Chloride secreting cells" in the gills of fishes, with special reference to the common eel. J. Physiol. 76, 368-378.

King, E.J., Gilchrist, M., Wootton, I.D.P., Donaldson, R., Sisson, R.B., Macfarlane, R.G., Jope, H.M., O'Brien, J.R.P., Peterson, J.M. & Strangeways, D.H. 1948 Determination of haemoglobin. V. Precision of colorimetric methods. Lancet 2, 563.

Kleiber, M. 1947 Body size and metabolic rate. Physiol. Rev. 27, 511-541.

Koch, H.J. 1934 Essai d'interprétation de la soi-disant 'réduction vitale' de sels d'argent par certains organes d'arthropodes. Ann. Soc. Sci. méd. nat. Brux. B, 54, 346-361.

Krogh, A. 1939 Osmotic regulation in aquatic animals. Cambridge: University Press.

Krüger, F. 1938 Neue Versuche über die Bedeutung des Haemoglobins im Blute des Regenwurmes. Zool. Anz. 11 Supplementband, 141-146.

Kuenen, D.J. 1939 Systematical and physiological notes on the brine shrimp, Artemia. Arch. néerl. Zool. 3, 365-449.

Labbé, A. 1925a Quatre générations d'Artemia arietina S. Fisch. C. R. Acad. Sci., Paris 180, 1697-1699.

Labbé, A. 1925b Les courbes de croissance d'Artemia arietina S. Fisch. C. R. Acad. Sci., Paris 180, 1872-1874.

Lankester, E.R. 1869 Abstract of a report on the spectroscopic examination of certain animal substances. J. Anat., Lond., 4, 119-129.

Lemberg, R. & Legge, J.W. 1949 Hematin compounds and bile pigments. London : Interscience.

Linder, F. 1941 Contributions to the morphology and the taxonomy of the Branchiopoda Anostraca. Zool. Bidr. Uppsala, 20, 103-302.

Lochhead, J.H. 1941 Artemia, the 'brine shrimp'. Turtox News, 19, 41-45.

Lochhead, J.H. & Lochhead, M.S. 1940 The egg shells of the brine shrimp, Artemia. Anat. Rec. 78, Suppl. 1, 75-76.

Lochhead, J.H. & Lochhead, M.S. 1941 Studies on the blood and related tissues in Artemia (Crustacea Anostraca). J. Morph. 68, 593-632.

Loeb, J. 1905 Studies in general physiology. Chicago.

Löwenstein, O. 1935 The respiratory rate of Gammarus chevreuxi in relation to differences in salinity. J. exp. Biol. 12, 217-221.

Lumbye, J. 1958 The oxygen consumption of Theodoxus fluviatilis (L.) and Potamopyrgus jenkinsi (Smith) in brackish and fresh water. Hydrobiologia, 10, 245-262.

MacArthur, J.W. & Baillie, W.H.T. 1929 Metabolic activity and duration of life. J. exp. Zool. 53, 221-268.

Martin, E.G. & Wilbur, B.C. 1921 Salt antagonism in Artemia. Amer. J. Physiol. 55, 290-291.

Mathias, P. 1929 Résistance à la chaleur de l'oeuf des Crustacés phyllopodes. Bull. Soc. zool. Fr., 54, 460-463.

Mathias, P. 1932 Sur le développement de l'oeuf d'un Crustacé phyllopode (Artemia salina L.). C. R. Acad. Sci., Paris 194, 1195-1197.

Mathias, P. 1934a Sur la biologie de l'Artemia salina L. (Crustacé phyllopode). Ann. Sci. nat. Zool. 17, 433-439.

Mathias, P. 1934b Résistance au froid et à la chaleur de l'oeuf d'Artemia salina L. (Crustacé phyllopode). Congrès de Soc. Savantes, Paris 67, 157-161.

Mathias, P. 1937 Biologie des Crustacés phyllopodes. Actualités sci. industr. No. 447, 1-106.

Mawson, M.L. & Yonge, C.M. 1938 The origin and nature of the egg membranes in Chirocephalus diaphanus. Quart. J. micr. Sci. 80, 553-565.

Medwedewa, N.B. 1927 Über den osmotischen Druck der Hämolymphe von Artemia salina. Z. vergl. Physiol. 5, 547-554.

Myint, T. 1956 New details of excystment of Artemia salina Leach. Proc. La Acad. Sci. 19, 24-28.

Needham, J. 1931 Chemical embryology. 3 vols. Cambridge : University Press.

Needham, J. & Needham, D.M. 1930 On phosphorus metabolism in embryonic life. J. exp. Biol. 7, 317-348.

Packard, A.S. 1883 A Monograph of the phyllopod Crustacea of North America, with remarks on the order Phyllocarida. U.S. Geol. Geogr. Surv. : Wyoming & Idaho, 12th. Ann. Rep. Pt. 1, 295-592.

Pallas, P.S. 1777 cited in Kuenen, D.J. & Baas Becking, L.G.M. 1938 Historical notes on Artemia salina (L.). Zoöl. Meded. 20, 222-230.

Panikkar, N.K. 1941a Osmoregulation in some palaemonid prawns. J. Mar. biol. Ass. U.K. 25, 317-359.

Panikkar, N.K. 1941b Osmotic behaviour of the fairy shrimp Chirocephalus diaphanus Prévost. J. exp. Biol. 18, 110-114.

Pantin, C.F.A. 1931 The origin of the composition of the body fluids in animals. Biol. Rev. 6, 459-482.

Payen, 1836 Note sur des animaux qui colorent en rouge les marais salans. Ann. Sci. nat. Zool. 6, 219-226.

Peters, H. 1935 Über den Einfluss des Salzgehaltes im Aussenmedium auf den Bau und die Funktion der Exkretions organe dekapoder Crustacean (nach Untersuchungen an Potamobius fluviatilis und Homarus vulgaris). Z. Morph. Ökol. Tiere 30, 355-381.

Phear, E.A. 1955 Gut haems in the invertebrates. Proc. zool. Soc. Lond., 125, 383-406.

Plattner, F. 1955 Der osmotische Druck von Artemia salina. Pflüg. Arch. ges. Physiol. 261, 172.

Potts, W.T. 1954 The energetics of osmotic regulation in brackish and fresh-water animals. J. exp. Biol. 31, 618-630.

Rackett, T.H. 1812 Observations on Cancer salinus. Trans. Linn. Soc. Lond. (Zool.) 11, 205-206.

Relyea, G.M. 1937 The brine shrimp of Great Salt Lake. Amer. Nat. 71, 612-616.

Rubner, M. 1883 Ueber den Einfluss der Körpergrösse auf Stoff- und Kraftwechsel. Z. Biol. 19, 536-562.

Sager, R. & Zalokar, M. 1958 Pigments and photosynthesis in a carotenoid-deficient mutant of Chlamydomonas. Nature, Lond., 182, 98-100.

Samter, M. & Heymons, R. 1902 Die Variationen bei Artemia salina Leach, und ihre Abhängigkeit von äusseren Einflüssen. Abh. preuss. Akad. Wiss. 2, 1-62.

Schlieper, C. 1929 Über die Einwirkung niederer Salzkonzentrationen auf marine Organismen. Z. vergl. Physiol. 9, 478-514.

Schlieper, C. 1930 Die Osmoregulation wasserlebender Tiere. Biol. Rev. 5, 309-356.

Schlieper, C. 1935 Neuere Ergebnisse und Probleme aus dem Gebiet der Osmoregulation wasserlebender Tiere. Biol. Rev. 10, 334-360.

Schlieper, C. 1936 Die Abhängigkeit der Atmungsintensität der Organismen vom Wassergehalt und dem kolloidalen Zustand des Protoplasmas. Biol. Zbl. 56, 87-94.

Schlosser, 1756 Extrait d'une lettre de M. le Docteur Schlosser concernant un petit Insecte peu connu. Observations périodiques sur la physique, l'histoire naturelle et les beaux arts, de Gautier, p. 58. Paris.

Schmankewitsch, W.J. 1875 Ueber das Verhältniss der Artemia salina Miln. Edw. zur Artemia Mühlhausenii Miln. Edw. und dem Genus Branchipus Schaeff. Z. wiss. Zool. Suppl. 25, 103-116.

Schmankewitsch, W.J. 1877 Zur Kenntniss des Einflusses der äusseren Lebensbedingungen auf die Organisation der Thiere. Z. wiss. Zool. 29, 429-494.

Schwabe, E. 1933 Über die Osmoregulation verschiedener Krebse (Malacostracen). Z. vergl. Physiol. 19, 183-236.

Siebold, C. von 1873 Ueber Parthenogenesis der Artemia salina. S. B. bayer. Akad. Wiss. 3, 168-196.

Siebold, C. von 1877 Ueber die München gezüchtete Artemia fertilis aus dem grossen Salzee von Utah. Verh. schweiz. naturf. Ges. 59, 267-280.

Smith, H.W. 1930 The absorption and excretion of water and salts by marine teleosts. Amer. J. Physiol. 93, 480.

Smith, H.W. 1932 Water regulation and its evolution in the fishes. Quart. Rev. Biol. 7, 1-26.

Spangenberg, F. 1875 Zur Kenntniss von Branchipus stagnalis. Z. wiss. Zool. 25, Suppl. 1, 1-64.

Thompson, d'A.W. 1942 On growth and form. Cambridge : University Press.

Thompson, J.V. 1829 Development of Artemia Salinus, or brine shrimp. Zoological Researches, and illustrations; Cork 1, Memoir 6, 103-110.

Ussing, H.H. 1949 Transport of ions across cellular membranes. Physiol. Rev. 29, 127-155.

Vevers, H.G. 1952 The biology of Asterias rubens L. III. Carotenoid pigments in the integument. J. Mar. biol. Ass. U.K. 30, 569-574.

Walshe, B.M. 1947 The function of haemoglobin in Tanytarsus (Chironomidae). J. exp. Biol. 24, 345-351.

Warren, H.S. 1938 The segmental excretory glands of Artemia salina Linn., var. principalis Simon (the brine shrimp). J. Morph. 62, 263-289.

Warren, H., Kuenen, D.J. & Baas Becking, L.G.M. 1938 On the relation between internal and external medium in Artemia salina (L.) var. principalis Simon. Proc. K. Akad. Wet. Amst. 41, 873-878.

Weisz, P.B. 1946 The space-time pattern of segment formation in Artemia salina. Biol. Bull., Wood's Hole 91, 119-140.

Weisz, P.B. 1947 The histological pattern of metameric development in Artemia salina. J. Morph. 81, 45-95.

Weymouth, F.W., Crismon, J.M., Hall, V.E., Belding, H.S. & Field, J.F. 1944 Total tissue respiration in relation to body weight. A comparison of the kelp crab with other crustaceans and with mammals. Physiol. Zoöl. 17, 50-71.

Whitaker, D.M. 1940 The tolerance of Artemia cysts for cold and high vacuum. J. exp. Biol. 83, 391-399.

Whitney, R.J. 1938 A syringe pipette method for the determination of oxygen in the field. J. exp. Biol. 15, 564-570.

Wolf, E. 1903 Dauereier und Ruhezustände bei Kopepoden. Zool. Anz. 98, 98-108.

Zeuthen, E. 1953 Oxygen uptake as related to body size in organisms. Quart. Rev. Biol. 28, 1-12.

Zeuthen, E. 1955 Comparative physiology (respiration). Annu. Rev. Physiol. 17, 459-482.

APPENDIX I

Table A. Body proportions in relation to size of *Artemia salina* in sea water (S‰ 35) at 25°C. (Californian stock).

	length group mm.	length, mm. abdomen	length, mm. prosoma*	abdomen/prosoma x 100	abdomen width, mm.	furca length, mm.	setae per furca	number measured
females	5.0 - 5.9	2.14	3.54	60	0.36	0.22	11.9	5
	6.0 - 6.9	2.60	3.80	68	0.38	0.27	14.2	25
	7.0 - 7.9	3.05	4.23	72	0.43	0.30	14.3	36
	8.0 - 8.9	3.64	4.75	77	0.49	0.31	14.7	43
	9.0 - 9.9	4.24	5.21	81	0.57	0.33	14.8	54
	10.0 - 10.9	4.71	5.74	82	0.64	0.34	15.0	64
	11.0 - 11.9	5.13	6.09	84	0.66	0.36	15.0	30
	12.0 - 12.9	5.61	6.48	86	0.71	0.41	15.5	8
males	5.0 - 5.9	2.19	3.43	64	0.35	0.27	13.3	18
	6.0 - 6.9	2.53	3.87	65	0.38	0.30	14.3	48
	7.0 - 7.9	2.83	4.50	63	0.44	0.34	14.9	62
	8.0 - 8.9	3.23	5.12	63	0.52	0.35	15.6	85
	9.0 - 9.9	3.72	5.50	68	0.55	0.37	16.0	38
	10.0 - 10.9	4.05	6.10	66	0.56	0.37	15.6	8

* prosoma = head + thorax

APPENDIX I

Table B. Body proportions in relation to size of *Artemia salina* in brine (S‰ 140) at 25°C. (Californian stock).

	length group mm.	length, mm. abdomen	length, mm. prosoma*	abdomen/prosoma x 100	abdomen width, mm.	furca length, mm.	setae per furca	number measured
females	4.0 - 4.9	1.93	2.79	69	0.23	0.17	10.0	9
	5.0 - 5.9	2.36	3.16	75	0.25	0.20	11.5	27
	6.0 - 6.9	2.83	3.54	80	0.29	0.23	12.0	35
	7.0 - 7.9	3.49	3.90	89	0.34	0.26	13.0	48
	8.0 - 8.9	4.13	4.24	97	0.39	0.29	14.0	55
	9.0 - 9.9	4.65	4.62	100	0.42	0.31	14.0	20
	10.0 - 10.9	5.15	4.95	104	0.44	0.34	13.5	6
males	4.0 - 4.9	1.89	2.67	71	0.21	0.16	10.5	13
	5.0 - 5.9	2.32	3.06	76	0.25	0.21	12.0	38
	6.0 - 6.9	2.76	3.52	78	0.29	0.27	13.2	54
	7.0 - 7.9	3.14	4.14	76	0.33	0.30	14.8	66
	8.0 - 8.9	3.68	4.62	80	0.38	0.33	14.4	26
	9.0 - 9.9	3.99	5.19	77	0.44	0.33	14.7	6

* prosoma = head + thorax

APPENDIX I

Table C. Body proportions in relation to size of *Artemia salina* in sea water (S‰ 35) at 25°C. (Utah stock)

	length group mm.	length, mm. abdomen	length, mm. prosoma*	abdomen/prosoma x 100	abdomen width, mm.	furca length, mm.	setae per furca	number measured
females	4.0 - 4.9	1.82	2.88	63	0.31	0.15	9.8	5
	5.0 - 5.9	2.15	3.24	66	0.33	0.20	11.8	15
	6.0 - 6.9	2.60	3.82	68	0.38	0.22	12.9	26
	7.0 - 7.9	3.31	4.08	81	0.45	0.26	13.6	24
	8.0 - 8.9	3.83	4.54	84	0.48	0.30	14.2	23
	9.0 - 9.9	4.49	4.82	93	0.51	0.32	14.5	18
	10.0 - 10.9	5.12	5.21	98	0.55	0.36	14.5	9
males	4.0 - 4.9	1.75	2.95	59	0.27	0.19	12.0	2
	5.0 - 5.9	2.21	3.27	68	0.33	0.22	12.6	16
	6.0 - 6.9	2.63	3.81	69	0.38	0.26	15.0	35
	7.0 - 7.9	2.99	4.35	69	0.43	0.31	14.9	32
	8.0 - 8.9	3.48	4.78	73	0.46	0.34	15.0	19
	9.0 - 9.9	3.92	5.30	74	0.51	0.37	17.2	5

*prosoma = head + thorax

APPENDIX I

Table D. Body proportions in relation to size of *Artemia salina* in brine (S‰ 140) at 25°C. (Utah stock)

	length group mm.	length, mm. abdomen	length, mm. prosoma*	abdomen/prosoma x 100	abdomen width, mm.	furca length, mm.	setae per furca	number measured
females	4.0 - 4.9	2.03	2.54	80	0.23	0.16	8.4	7
	5.0 - 5.9	2.44	2.97	82	0.25	0.17	9.7	27
	6.0 - 6.9	2.96	3.42	87	0.29	0.22	11.4	16
	7.0 - 7.9	3.59	3.79	95	0.34	0.26	12.3	28
	8.0 - 8.9	4.14	4.18	99	0.37	0.29	13.1	46
	9.0 - 9.9	4.83	4.48	107	0.39	0.32	13.6	38
	10.0 - 10.9	5.38	5.00	108	0.46	0.35	15.0	11
males	4.0 - 4.9	2.09	2.70	77	0.22	0.15	7.4	7
	5.0 - 5.9	2.41	2.91	83	0.24	0.17	9.9	26
	6.0 - 6.9	2.87	3.45	83	0.28	0.23	12.1	24
	7.0 - 7.9	3.46	3.96	87	0.33	0.30	13.7	45
	8.0 - 8.9	3.92	4.41	89	0.36	0.31	13.4	34
	9.0 - 9.9	4.32	4.93	88	0.39	0.35	14.2	18
	10.0 - 10.9	4.85	5.46	89	0.45	0.44	16.4	5

*prosoma = head + thorax

APPENDIX I

Table E. Body proportions of parthenogenetic females of *Artemia salina* in relation to size and to the salinity of the medium at 25°C. (La Palme stock)

	length group mm.	length, mm. abdomen	length, mm. prosoma*	abdomen/prosoma x 100	abdomen width, mm.	furca length, mm.	setae per furca	number measured
	3.0 - 3.9	1.29	2.11	61	0.17	0.10	5.2	21
	4.0 - 4.9	1.84	2.52	73	0.19	0.14	7.2	38
females	5.0 - 5.9	2.32	2.98	78	0.22	0.19	8.3	56
	6.0 - 6.9	2.90	3.46	84	0.26	0.24	10.9	72
S‰ 35	7.0 - 7.9	3.46	3.85	90	0.29	0.27	10.6	50
	8.0 - 8.9	4.30	4.31	93	0.33	0.31	12.5	21
	9.0 - 9.9	4.78	4.66	103	0.35	0.37	12.7	31
	10.0 - 10.9	5.22	5.06	103	0.37	0.39	12.0	26
	11.0 - 11.9	5.83	5.34	109	0.37	0.39	12.0	11
	3.0 - 3.9	1.57	2.02	78	0.16	0.08	2.0	13
	4.0 - 4.9	2.06	2.33	88	0.18	0.11	3.2	81
males	5.0 - 5.9	2.56	2.89	89	0.21	0.16	5.0	69
	6.0 - 6.9	3.17	3.17	100	0.23	0.18	5.5	76
S‰ 140	7.0 - 7.9	3.80	3.53	108	0.26	0.23	6.6	45
	8.0 - 8.9	4.46	3.80	117	0.28	0.26	7.7	28
	9.0 - 9.9	5.23	4.23	124	0.30	0.31	8.6	24
	10.0 - 10.9	5.87	4.45	132	0.33	0.34	8.1	7
	11.0 - 11.9	6.48	4.77	136	0.35	0.33	8.1	6

*prosoma = head + thorax

APPENDIX I

Table F. Body proportions in relation to size of Artemia salina in sea water (S‰ 35) at 25°C. (Algerian stock)

	length group mm.	length, mm. abdomen	length, mm. prosoma*	abdomen/prosoma x 100	abdomen width, mm.	furca length, mm.	setae per furca	number measured
females	5.0 - 5.9	2.83	2.46	101	0.20	0.10	1.8	11
	6.0 - 6.9	3.27	2.98	110	0.22	0.10	2.2	29
	7.0 - 7.9	3.98	3.34	119	0.24	0.14	3.3	24
	8.0 - 8.9	4.80	3.67	131	0.26	0.18	5.1	18
	9.0 - 9.9	5.44	3.97	137	0.27	0.20	5.4	40
	10.0 - 10.9	6.00	4.16	144	0.29	0.21	5.3	15
males	4.0 - 4.9	2.20	2.35	94	0.17	0.08	1.8	6
	5.0 - 5.9	2.66	2.72	98	0.20	0.10	3.0	12
	6.0 - 6.9	3.27	3.09	106	0.23	0.15	4.8	32
	7.0 - 7.9	3.92	3.47	113	0.25	0.23	7.9	28
	8.0 - 8.9	4.51	3.96	114	0.26	0.24	8.9	39
	9.0 - 9.9	4.87	4.21	116	0.26	0.24	7.3	7

*prosoma = head + thorax

APPENDIX I

Table G. Body proportions in relation to size of Artemia salina in brine (S‰ 140) at 25°C. (Cagliari stock)

	length group mm.	length, mm. abdomen	length, mm. prosoma*	abdomen/prosoma x 100	abdomen width, mm.	furca length, mm.	setae per furca	number measured
females	4.0 - 4.9	2.27	2.45	93	0.18	0.13	3.7	6
	5.0 - 5.9	2.76	2.67	103	0.20	0.14	5.4	34
	6.0 - 6.9	3.31	3.00	110	0.20	0.16	5.8	50
	7.0 - 7.9	3.94	3.37	117	0.24	0.19	7.0	37
	8.0 - 8.9	4.59	3.50	131	0.25	0.22	7.4	10
males	4.0 - 4.9	2.28	2.31	99	0.19	0.12	4.7	12
	5.0 - 5.9	2.78	2.64	105	0.20	0.15	6.1	62
	6.0 - 6.9	3.31	3.04	109	0.21	0.19	7.0	48
	7.0 - 7.9	3.81	3.34	114	0.23	0.23	7.6	17

*prosoma = head + thorax

APPENDIX II

Table A. Oxygen consumption of *Artemia* (Californian stock) in sea water at 25°C. at various concentrations of dissolved oxygen.

oxygen concentration ml./l.	oxygen consumption (ml./g dry wt. / hour) separate values						mean and S.E.
4.00 - 3.51	8.40	6.06	5.93	8.62	8.02	7.48	7.31 ± 0.31
	6.81	7.02	8.31	6.45			
3.50 - 3.01	8.15	7.06	7.43	6.91	7.41	7.85	7.30 ± 0.17
	7.02	6.58					
3.00 - 2.51	6.76	7.43	7.16	6.10	6.77	7.21	7.07 ± 0.17
	8.10	6.58	7.20	7.39			
2.50 - 2.01	5.86	6.83	6.06	5.95	8.08	8.10	6.62 ± 0.40
	5.29	5.26	8.14				
2.00 - 1.51	5.45	7.32	4.82	5.84	6.09	8.06	6.19 ± 0.35
	7.78	5.45	6.08	5.91	3.95	7.00	
	6.73						
1.50 - 1.01	7.30	6.32	6.67	4.88	4.24	3.55	5.41 ± 0.34
	6.96	5.85	6.03	4.41	5.40	4.16	
	3.62	6.28					
1.00 - 0.51	2.64	5.24	5.73	5.00	3.78	4.08	4.30 ± 0.31
	6.48	5.79	4.10	2.85	4.10	4.76	
	3.88	1.98					
0.50 - 0.01	1.78	1.50	1.13	1.70	0.60	1.69	1.42 ± 0.15
	1.28						

These results are represented graphically in figure 42.

APPENDIX II

Table B. Oxygen consumption of Artemia (Californian stock) in brine (S‰ 210) at 25°C. at various concentrations of dissolved oxygen.

oxygen concentration ml./l.	oxygen consumption (ml./g dry wt. / hour) separate values						mean and S.E.
2.00 - 1.51	6.18	6.46	4.80	4.78	5.98	6.12	5.81 ± 0.27
	6.37						
1.50 - 1.01	5.14	5.24	9.46	6.00	6.96	3.68	5.72 ± 0.65
	5.18	4.11					
1.00 - 0.51	4.20	4.38	5.05	3.68	6.05	6.00	4.88 ± 0.37
	5.68	3.20	5.42	3.18	6.85		
0.50 - 0.01	2.12	1.41	1.51	2.20	1.43	1.94	1.81 ± 0.13
	2.04						

These results are represented graphically in figure 42.

APPENDIX II

Table C. Oxygen consumption of Artemia (La Palme stock) in sea water at 25°C. at various concentrations of dissolved oxygen.

oxygen concentration ml./l.	oxygen consumption (ml./g dry wt. / hour) separate values						mean and S.E.
4.00 - 3.01	6.84	6.73	7.44	7.78	6.46	7.01	7.21 ± 0.13
	7.97	7.59	6.97	7.29	7.34	7.09	
3.00 - 2.51	7.70	6.38	7.42	7.25	6.70	7.55	7.08 ± 0.14
	7.20	6.40	6.30	7.52	7.86	6.94	
	7.00						
2.50 - 2.01	7.42	7.33	6.49	6.74	8.74	8.45	7.22 ± 0.38
	6.72	5.94	5.45	8.12	5.48	9.80	
2.00 - 1.51	5.00	9.34	7.65	8.80	6.20	8.15	7.14 ± 0.53
	6.65	5.33					
1.50 - 1.01	6.55	8.10	5.73	8.57	7.40	7.80	7.28 ± 0.28
	6.92	7.01	7.46				
1.00 - 0.51	5.14	5.42	5.40	4.87	5.15	4.11	4.53 ± 0.36
	6.06	4.83	3.38	2.25	5.46	2.32	
0.50 - 0.01	3.90	1.43	2.41	1.41	3.70	3.27	2.35 ± 0.38
	1.56	1.24					

These results are represented graphically in figure 43.

APPENDIX II

Table D. Oxygen consumption of *Artemia* (La Palme stock) in brine (S‰ 210) at 25°C. at various concentrations of dissolved oxygen.

oxygen concentration ml./l.	oxygen consumption (ml./g dry wt. / hour) separate values					mean and S.E.
2.00 - 1.51	6.23	6.50	7.50	8.20	6.71	6.98 ± 0.21
	7.10	6.45	6.64	7.48		
1.50 - 1.01	7.54	6.13	7.50	6.24	6.36	6.74 ± 0.23
	7.13	6.30				
1.00 - 0.51	6.00	3.65	5.90	6.30	3.82	4.57 ± 0.40
	3.80	3.32	4.55	4.62	2.30	
	6.00					
0.50 - 0.01	1.64	2.80	3.08	1.43	4.10	2.50 ± 0.41
	1.98					

These results are represented graphically in figure 43.

APPENDIX III

Table A. Oxygen consumption of *Artemia salina* in brine (S‰ 195), at 23.5°C., at various concentrations of dissolved oxygen. Normal animals.

oxygen concentration ml./l.	oxygen consumption (mm^3/ g. wet wt./hour) separate values	mean and S.E.
2.00 - 1.76	594, 705, 704, 702, 756, 632, 765, 616, 724, 748	693 ± 18.5
1.75 - 1.51	772, 743, 795, 802, 782, 628, 613, 695, 755, 784, 757	739 ± 19.7
1.50 - 1.26	482, 646, 781, 455, 439, 533, 500, 439, 636, 640	555 ± 36.3
1.25 - 1.01	529, 425, 345, 494, 523, 553, 606, 417, 474, 565, 446, 368	479 ± 23.3
1.00 - 0.76	342, 334, 296, 288, 300, 320, 284, 328, 342, 330	316 ± 7.1
0.75 - 0.51	197, 305, 224, 180, 196, 273 297, 262, 183, 240	236 ± 14.8

APPENDIX III

Table B. Oxygen consumption of *Artemia salina* in brine (S‰ 195), at 23.5°C., at various concentrations of dissolved oxygen. Carbon-monoxide-treated animals.

oxygen concentration ml./l.	oxygen consumption (mm^3/ g. wet wt./hour) separate values	mean and S.E.
2.00 - 1.76	596, 594, 514, 340, 419, 307, 503, 486, 548, 366	467 ± 32.9
1.75 - 1.51	403, 563, 491, 519, 597, 595, 570, 550, 518, 536	534 ± 18.1
1.50 - 1.26	469, 506, 467, 500, 403, 582, 408, 557, 414, 403, 378	462 ± 20.5
1.25 - 1.01	273, 413, 388, 405, 373, 409, 421, 436, 475, 456, 475, 413, 435, 485, 413, 407, 446	419 ± 11.6
1.00 - 0.76	368, 407, 487, 425, 364, 356, 315, 355, 340, 348, 223, 294, 290, 324	342 ± 18.0
0.75 - 0.51	241, 319, 196, 276, 234, 276, 296, 260, 242, 280	262 ± 11.1

Subsidiary matter submitted in support of candidature

Bishop, A. & Gilchrist, B.M. 1944 A method for collecting the sporozoites of Plasmodium gallinaceum by feeding infected Aedes aegypti through animal membranes. Nature, Lond. 153, 713

Bishop, A. & Gilchrist, B.M. 1946 Experiments upon the feeding of Aedes aegypti through animal membranes with a view to applying this method to the chemotherapy of malaria. Parasitology, 37, 85-100

Bishop, A., Birkett, B. & Gilchrist, B.M. 1947 The response of blood inoculated and sporozoite induced infections of Plasmodium gallinaceum to drugs. Parasitology, 38, 163-174

Gilchrist, B.M. & Haldane, J.B.S. 1947 Sex linkage and sex determination in a mosquito, Culex molestus. Hereditas, 33, 175-190

Fox, H.M., Gilchrist, B.M. & Phear, A.E. 1951 Functions of haemoglobin in Daphnia. Proc. Roy. Soc. B., 138, 514-528

Fox, H.M., Phear, A.E. & Gilchrist, B.M. 1953 Influence of temperature and iron on haemoglobin synthesis by Daphnia. Nature, Lond. 171, 347

(*Reprinted from Nature, Vol.* 171, *p.* 347, *February* 21, 1953)

Influence of Temperature and Iron on Hæmoglobin Synthesis by *Daphnia*

Daphnia, in common with other branchiopod Crustacea, synthesizes hæmoglobin in response to a deficiency of dissolved oxygen in the surrounding water[1]. The hæmoglobin is in solution in the blood. In poikilothermal animals, chemical processes in the body normally proceed more rapidly at a high external temperature, and we have found that in *Daphnia* hæmoglobin synthesis is quicker in warm water. We wished, in addition, to know whether, at the lower of two temperatures, *Daphnia* would eventually acquire as much hæmoglobin as at the higher temperature (the low oxygen contents of the waters being maintained at the same level) or whether at the higher temperature a greater amount of hæmoglobin would be synthesized. The second alternative proved to be correct. In one experiment with *D. obtusa*, in water containing approximately 1 ml. dissolved oxygen per litre, the hæmoglobin content of the blood increased, in 28 days, five-fold at 17° C. but seven-fold at 28° C. After that there was no further increase. One cause of the greater synthesis at the higher temperature is probably the low oxygen content of the tissues, due to a high metabolic rate.

There are great differences in the iron content of natural fresh waters in which *Daphnia* lives. It has been found to vary between 50 mgm./l. and no iron detectable by dipyridyl (before or after reducing). The question thus arises as to whether the addition of iron salts to water containing little, or no, detectable iron would increase the quantity of hæmoglobin synthesized by *Daphnia* in response to oxygen deficiency. Our work has shown this to be the case. In one experiment, at 20° C., in water with no detectable iron and a low dissolved oxygen content of 0·9 ml./l., the blood hæmoglobin increased seven-fold in sixteen days (deriving its iron no doubt from algal food) and then remained steady for a further twelve days; whereas in similar water, to which 4 mgm./l. iron was added every other day, the increase in sixteen days was between nine-fold and ten-fold, with subsequent maintenance of this level for twelve days.

It was found that ferrous salts are more effective than ferric salts in augmenting hæmoglobin synthesis. This is curious for the following reason. A hæmochromogen is present in the gut lumen of *Daphnia*[2].

At the low oxygen content of the outside water necessary for hæmoglobin synthesis by *Daphnia*, this hæmochromogen can be seen with a spectroscope to be in the reduced state, the α-absorption band showing clearly. Now, the redox potential for the reduction of various hæmochromogens is known to be well below that at which the ferric ion is reduced to the ferrous state[3]. It is thus probable that in the gut lumen of *Daphnia* iron is in the ferrous state, even if swallowed as a ferric salt. The explanation of the greater effect of ferrous salts in increasing hæmoglobin synthesis seems to be as follows. The ferric salt added to the experimental water is gradually converted to ferric hydroxide and precipitated. The ferrous salt is also converted to ferric hydroxide, but in a more finely divided state which remains longer in suspension, and is thus available to *Daphnia* for a longer time.

A full account of these results will appear in the *Proceedings of the Royal Society*.

H. Munro Fox
Elizabeth A. Phear
Barbara M. Gilchrist

Bedford College,
University of London.
Dec. 17.

[1] Fox, H. Munro, Gilchrist, B. M., and Phear, E. A., *Proc. Roy. Soc.*, B, **138**, 514 (1951).
[2] Fox, H. Munro, *Proc. Roy. Soc.*, B, **135**, 195 (1948).
[3] Dixon, M., "Multi-enzyme Systems", 73 (Cambridge, 1951).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

A METHOD FOR COLLECTING SPOROZOITES OF *PLASMODIUM GALLINACEUM* BY FEEDING INFECTED *AEDES ÆGYPTI* THROUGH ANIMAL MEMBRANES

By Dr. ANN BISHOP and BARBARA M. GILCHRIST

(*Reprinted from* NATURE, *Vol.* 153, *page* 713, *June* 10, 1944)

A Method for Collecting Sporozoites of *Plasmodium gallinaceum* by Feeding Infected *Aedes aegypti* through Animal Membranes

THE well-known difference between the resistance of blood-inoculated and mosquito-induced malaria indicates that sporozoites, or the stages arising immediately from them, are very resistant to the action of the known anti-malarial drugs. Since a true prophylactic drug, that is, one which will prevent sporozoite-induced infections, is the most urgent requirement in the chemotherapy of malaria, a method for testing drugs directly on sporozoites is of great importance. Hitherto the only method of doing this was to obtain sporozoites by the dissection of salivary glands from infected mosquitoes, which is both laborious and unsatisfactory, as the sporozoites are always mixed with and may be protected by fragments of the salivary gland cells. Thus it is difficult to obtain them in uniform suspension and to be sure that they are all exposed equally to the action of the admixed drugs being tested.

In order to overcome these difficulties, we began to work upon the possibility of obtaining sporozoites free from gland tissue by feeding infected mosquitoes (*Aedes ægypti*) through animal membranes. Gordon[1] had shown that mosquitoes would feed through a fresh animal membrane, and Yoeli[2] had infected *Anopheles elutus* with *Plasmodium falciparum* by inducing the mosquitoes to gorge upon infected blood through a prepared rabbit-skin membrane.

The type of membrane which we have found to be the most successful is one made of chicken skin. We prepared it by soaking the skin of a 1–3 week old chick in absolute alcohol for at least 30 minutes, washing it in running tap water and stretching it over a piece of glass tubing 2·5 cm. × 6 cm., and keeping it in place, until dry, by a thin rubber band. On drying, the skin is thin and parchment-like and adheres firmly round the glass tubing, making a water-tight seal.

The highest rates of gorging are obtained when the blood in the membrane (1·5–3 c.c. of heparinized chick blood) is warmed to 41–42° C. and kept warm by a surrounding water-jacket filled with water at that temperature. Moistening the outer side of the membrane, that is, that presented to the mosquitoes,

with saliva also increases the rate of gorging as compared with a dry surface. The apparatus is placed upon the mosquito-netted top of a jar containing *Aedes ægypti* so that the surface of the membrane

INFECTIVE CYCLE OF *Plasmodium gallinaceum*, BY *Aedes ægypti*, THROUGH ANIMAL MEMBRANES.

rests upon the netting. Gorging is effected in the dark in an incubator at 28° C., in a moist atmosphere.

We have infected *Aedes ægypti* with *Plasmodium gallinaceum* by feeding mosquitoes through a membrane upon heparinized chick blood heavily infected with gametocytes. The rate and intensity of infection, as assessed by oocyst counts on the fourth day, were equal to those obtained from mosquitoes of the same age-group gorged on the same chicken immediately prior to drawing the blood for the membrane experiment.

What is, however, more important for our work, is that we have been able to demonstrate that infected mosquitoes, when they gorge through a membrane, extrude sporozoites into the blood, and that these sporozoites are viable, since the blood containing them when injected into clean young chicks produces infections typical of mosquito transmission.

Mosquitoes, which became infected as a result of gorging through a membrane upon blood containing gametocytes of *P. gallinaceum*, have ejected sporozoites through a membrane into uninfected chick blood, and this blood has proved infective when injected into a clean chick. The cycle of development as shown in the accompanying chart has, therefore, been completed.

In obtaining sporozoites from infected *Aedes ægypti* only batches of mosquitoes which have been proved, by oocyst count on the fourth–fifth day, to be heavily infected are used. The rate of gorging through a membrane of infected mosquitoes (that is, mosquitoes which had already had one, the infective, blood-meal) is generally not so high as when mosquitoes gorge for the first time, but usually at least 50 per cent gorge, and in certain batches the rate has been as high as 90 per cent.

Our practice is to allow the mosquitoes thirty minutes in which to gorge upon 1·5–3 c.c. of heparinized blood from clean chicks, and then to pipette out the blood from the membrane and shake it well to ensure that the sporozoites are evenly distributed. The blood (0·4–0·5 c.c. per bird) is injected intravenously into 5–10 day old chicks. Groups of three or four chicks injected with blood from membranes through which batches of 35–53 mosquitoes had gorged showed parasites in their peripheral blood on the sixth–seventh day. This incubation period is, in our experience, similar to that in chicks bitten by two to four heavily infected mosquitoes. In one experiment, blood from which forty-five mosquitoes had gorged produced infections of such intensity in six chicks that five died on the seventh day, post-mortem examination revealing enormous numbers of

exoerythrocytic schizonts in the capillaries of the brain.

Infected Aedes remain infective after gorging through a membrane and can be induced to eject their sporozoites by this method on more than one occasion. Thus a batch of thirty-nine infected mosquitoes ejected sporozoites through a membrane into clean blood on the ninth day after infection. From this blood five chicks were infected. Six days later, the twenty-one Aedes surviving gorged again through a membrane upon clean blood, and the blood in the membrane proved infective to the two chicks inoculated with it, parasites being found in the peripheral blood of each chick on the sixth day.

Experiments are now being made upon the applicability of this method for testing the action of antimalarial drugs directly upon sporozoites and the developmental stages arising from them, and the results will be reported later.

ANN BISHOP*.
BARBARA M. GILCHRIST.

Molteno Institute of Biology and Parasitology,
University of Cambridge.

* Member of the Scientific Staff of the Medical Research Council.

[1] Gordon, R. M., *Ann. Trop. Med. Parasitol.*, **16**, 424 (1922).
[2] Yoeli, M., *Riv. Malariolog.*, **17**, 62 (1938).

PRINTED IN GREAT BRITAIN BY FISHER, KNIGHT AND CO., LTD., ST. ALBANS

[FROM PARASITOLOGY, VOL. 37, NOS. 1, 2, JANUARY 1946.]

PRINTED IN GREAT BRITAIN

EXPERIMENTS UPON THE FEEDING OF *AËDES AEGYPTI* THROUGH ANIMAL MEMBRANES WITH A VIEW TO APPLYING THIS METHOD TO THE CHEMOTHERAPY OF MALARIA

BY ANN BISHOP* AND BARBARA M. GILCHRIST

From the Molteno Institute, University of Cambridge

(With 2 Figures in the Text)

CONTENTS

I. INTRODUCTION

The primary object of our experiments upon the feeding of *Aëdes aegypti* through membranes was, as stated in a previous communication (Bishop & Gilchrist, 1944), to obtain sporozoites free from all trace of glandular tissue in order to test the action of drugs directly upon them. Hitherto the only method of obtaining sporozoites was by dissection of the salivary glands, and by this method it is difficult to be certain that all the sporozoites are freed from cellular tissue and equally exposed to the admixed drugs.

Gordon (1922) had succeeded in making *Stegomyia* (=*Aëdes*) *calopus* feed through the skin of a bat, though they never fed to repletion even on whole blood. Woke (1937) had induced *Aëdes aegypti* to feed through a rat-skin membrane upon whole blood or certain fractions of it, and Yoeli (1938) had caused *Anopheles elutus* to gorge through prepared rabbit-skin membranes upon blood containing gametocytes of *Plasmodium falciparum*, and obtained infection of the mosquitoes.

We decided to find out if *Aëdes aegypti* infected with *Plasmodium gallinaceum* would, when fed through a membrane, eject viable sporozoites, and if so, if they would be ejected in numbers sufficiently numerous for experimental purposes.

II. STRAINS OF *AËDES AEGYPTI* AND *PLASMODIUM GALLINACEUM* USED

Three strains of *Aëdes aegypti* have been used in the experiments described herein. One strain, referred to as the B strain, was that used by James & Tate (1938) as a vector for *Plasmodium gallinaceum* and was given to them by Prof. E. Brumpt, Faculté de Médecine, Paris: the second strain, referred to as the G strain, was the gift of Prof. R. M. Gordon of the Liverpool School of Tropical Medicine and was used as a vector for *P. gallinaceum* by Lumsden & Bertram (1940); and the third, the C strain, was the gift of Dr Coatney of the National Institute of Health, U.S.A.

The strain of *P. gallinaceum* was obtained from Prof. E. Brumpt in 1937.

* Member of the Scientific Staff of the Medical Research Council.

III. THE METHOD OF FEEDING *AËDES AEGYPTI* THROUGH A MEMBRANE

(1) *Type of membrane*

The first problem in setting out to obtain viable sporozoites by feeding infected mosquitoes through a membrane was to discover the most satisfactory type of membrane, and the optimum conditions for inducing *Aëdes aegypti* to feed through it. In his experiments Woke (1937) used the skins of week-old rats. Yoeli (1938) took the skin from a rabbit's ear and, after soaking it in 96 % alcohol, washed it and dried it stretched over a glass cylinder. In our experience the skin of young chickens not more than 2–3 weeks old has produced the best membranes. Whereas the skin of young rats, whether fresh or fixed in alcohol, tears easily and does not adhere to the glass cylinder over which it is stretched, chicken skin is extremely strong and adherent when prepared by soaking in absolute alcohol for 30 min. or longer, followed by prolonged washing in water. It is then dried, stretched over one end of the glass cylinder (6 × 2·5 cm.) to be used in the feeding experiments, and held in place by a rubber band. The skin dries to form a thin, parchment-like membrane which adheres so firmly to the outer surface of the glass cylinder that it does not come loose even if soaked in water, and is absolutely watertight. Such membranes can be stored on their cylinders for long periods, and can be used several times if they are washed after use and resoaked in absolute alcohol before being dried.

Cellophane membranes were tried, but found to be unsuitable.

(2) *Method of feeding*

The mosquitoes to be fed are placed in glass cylinders approximately 12 × 9 cm. Both ends of the cylinders are covered with mosquito-netting held in place with strong rubber bands. In the net covering the top of the cylinder a small hole is made which can be closed with a cotton-wool plug. Through this hole the mosquitoes are introduced. To provide the damp atmosphere necessary to successful gorging the mosquito-cylinders are stood over Petri dishes containing damp filter paper.

Experiments have proved (p. 87) that the highest proportions of *A. aegypti* feed through a membrane when the temperature of the blood at the commencement of the feeding experiment is approximately 40–42°C. We have devised, therefore, a water jacket surrounding the membrane cylinder, to prevent the temperature of the blood from falling rapidly. The water jacket is made partly of rubber and partly of glass (Fig. 1), the rubber base being held in place round the membrane cylinder and the outer cylinder of the water jacket by strong rubber bands. The initial temperature of the water jacket should be approximately 48°C., as heat is lost in warming up the apparatus. The blood, at 40–42°C., is poured into the membrane cylinder when the temperature of the water jacket has reached that temperature. The apparatus is then stood on the top of the mosquito cylinder so that the surface of the membrane rests upon the net.

Feeding is performed in the dark at 28°C. and, unless otherwise stated, the mosquitoes are allowed 30 min. in which to feed.

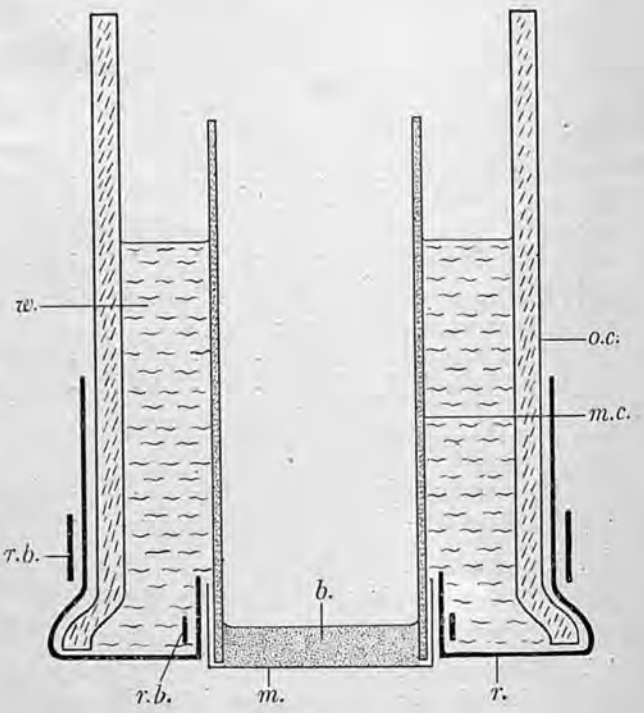


Fig. 1. *o.c.* outer cylinder of water jacket; *m.c.* membrane cylinder; *w.* water; *r.b.* rubber band; *r.* rubber base of water jacket; *b.* blood, or test fluid; *m.* chicken skin membrane.

IV. THE EFFECT OF AGE UPON FEEDING IN *AËDES AEGYPTI*

(1) *Feeding upon chickens*

In experimental work with sporozoites it is essential that the mosquitoes should be infected at the earliest age at which they will feed readily, so that the greatest number possible should survive until the salivary glands become infected. In order to discover the earliest age for successful feeding under the conditions available to us, we made a series of experiments with female mosquitoes of different age groups increasing in intervals of 24 hr. from emergence, with a minimum of 0–24 hr. and a maximum of 72–96 hr. In each series of experiments all the mosquitoes used were derived from the same batch of eggs and were bred under the same conditions. The mosquitoes in all the groups of the same series, therefore, were similar except in age. From the time of emergence until the first blood meal

they were given no food, but were allowed access to water.

In feeding mosquitoes upon chickens the mosquitoes were applied to the breast of the chicken in a biting box. The experiments were performed in the dark at 28° C., in a moist atmosphere, and the mosquitoes were allowed 30 min. in which to feed. In assessing the numbers which had fed, only those which had gorged fully were counted.

The results (Table 1) show that in both the B and C strains there is a gradual increase in the proportion of mosquitoes gorging up to a maximum at 72–96 hr.

Table 1. Aëdes aegypti. *The effect of age upon the proportions gorging upon a chicken*

	B strain			C strain		
Age of *Aëdes* in hours	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
0–24	3	8/126	6	1	0/51	0
24–48	3	56/135	41	1	26/73	36
48–72	3	104/137	76	1	89/94	95
72–96	1	23/29	79	1	74/79	94

Table 2. Aëdes aegypti. *The effect of age upon the proportions gorging through membranes*

	B strain			C strain			G strain		
Age of *Aëdes* in hours	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
0– 24	4	13/258	5	3	11/209	5	1	0/47	0
24– 48	4	89/302	29	2	22/152	14	1	3/46	7
48– 72	4	151/301	50	2	59/159	37	1	2/42	5
72– 96	2	105/193	54	2	124/170	73	1	10/33	30
96–120	—	—	—	3	190/284	67	—	—	—
120–144	—	—	—	2	132/186	71	—	—	—

The 48–72 hr. group was the youngest in which a high proportion of the mosquitoes gorged. Above the 72–96 hr. group little consistent increase in the proportions gorging has been found.

Seaton & Lumsden (1941), working under carefully controlled conditions of temperature and humidity, found that when virgin female *A. aegypti* were offered blood meals from the human forearm the proportions gorging increased through the age groups from 0–24 hr. to 72–96 and 96–120 hr.

When infecting *A. aegypti* with *Plasmodium gallinaceum* by feeding on infected chickens we therefore, whenever possible, use mosquitoes 48–96 hr. old.

(2) *Feeding through membranes*

The experiments were similar to those with chickens, with the exception that the mosquitoes were offered blood at 40–42° C. through a membrane according to the method described above. Much wider variation was observed in the same age groups in the different series of experiments with mosquitoes fed through membranes than on chickens, but when the percentages of all the age groups are compared (Table 2), the proportions gorging are found to increase with increase in age as in the experiments with chickens. This increase in proportions gorging with increase in age was found in all three strains of *A. aegypti*.

Whereas in feeding *A. aegypti* directly on a chicken the earliest age groups in which a high proportion of mosquitoes gorged was 48–72 hr., when fed through a membrane 72–96 hr. from emergence was the earliest age at which gorging was consistently high.

V. THE EFFECT OF A HEAT GRADIENT UPON THE PROPORTION OF *AËDES AEGYPTI* FEEDING THROUGH A MEMBRANE

Preliminary experiments showed that when *A. aegypti* were offered blood through a membrane the proportions of mosquitoes which gorged was much greater if the blood was warmed to 40–42° C. than if its temperature was that of the environment. The question therefore arose whether the mosquitoes were attracted to warmed blood by the presence of a heat gradient, or whether the heat given off from the blood provided a microclimate of optimum temperature for gorging.

In experiments designed to elucidate this question mosquitoes of the same age derived from the same batch of eggs and bred under similar conditions were collected into five groups (with the exception of one experiment in which only four groups were used). Although all the groups of mosquitoes in each particular experiment were of the same age, the age of the mosquitoes in different experiments

varied from 72–96 to 120–144 hr. No food was given to the mosquitoes between the time of their emergence and of the experimental feeding. The experiments were performed in the dark, at 28°C., in a saturated atmosphere.

Fresh heparinized chick blood was used for feeding the mosquitoes.

Using water-jacketed membrane cylinders containing fresh heparinized chick blood on top of cylinders of female *A. aegypti* in the manner previously described, a series of experiments was set up as follows:

A. Temperature of environment 24°C. Temperature of blood 24°C. Temperature gradient 0.

B. Temperature of environment 28°C. Temperature of blood 28°C. Temperature gradient 0.

28°C. is a more favourable temperature to gorging than 24°C. and thus more than compensated for the slightly smaller gradient.

That in those experiments where the blood was heated to 42°C. it was the heat gradient which accounted for the high proportions of mosquitoes which gorged, and not the presence of a warm microclimate immediately below the membrane, is proved by the fact that when the temperature of the blood and the environment were both high (37°C.) the rate of gorging was much lower than when a heat gradient was present.

Since all differences other than that of temperature were eliminated in these experiments it can be concluded that a heat gradient is an important factor in attracting *A. aegypti* to its food.

Table 3. Aëdes aegypti. *The effect of a heat gradient upon the proportions gorging*

Temperature of blood ...	24°C.			28°C.			37°C.		
Temperature of environment ...	24°C.			28°C.			37°C.		
	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
	6	26/430	6·0	6	35/439	8·0	5	74/393	19

Temperature of blood ...	42°C.					
Temperature of environment ...	24°C.			28°C.		
	No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
	6	224/436	51	6	285/403	71

C. Temperature of environment 37°C. Temperature of blood 37°C. Temperature gradient 0.

D. Temperature of environment 24°C. Temperature of blood 42°C. Temperature gradient 24–42°C.

E. Temperature of environment 28°C. Temperature of blood 42°C. Temperature gradient 28–42°C.

It will be seen from Table 3 that in those cylinders where the temperature of the blood was the same as that of the environment the proportion of mosquitoes which gorged was low, though it increased with the rise in temperature of the environment. But in those cylinders in which there was a gradient between the temperature of the blood and that of the environment the proportion which gorged was high. In the cylinders where this heat gradient was greatest (24–42°C.) the proportion of mosquitoes which gorged was not so high as in those where the gradient was 28–42°C. The explanation may be that

VI. *AËDES AEGYPTI*. THE EFFECT OF THE APPLICATION OF SALIVA TO THE OUTER SURFACE OF THE MEMBRANE ON THE PROPORTION GORGING ON BLOOD

In feeding *Aëdes aegypti* upon fowls, Lumsden & Bertram (1940) found that if mosquitoes showed a reluctance to feed they could often be induced to do so if saliva were applied to the skin of the fowl. In the light of these observations it seemed possible that an application of saliva to the outer side of the membrane might increase the proportion of *A. aegypti* feeding through it. Preliminary experiments (Bishop & Gilchrist, 1944) seemed to indicate that an increase in the proportion of mosquitoes feeding did occur when saliva was applied to the membrane. Further experiments, however, in which females of the same age group and strain were offered fresh heparinized chick blood from the same

source under identical conditions, with the exception that in one case the outer surface of the membrane was dry whereas in the other it was moistened with saliva (Table 4), proved that saliva had little effect upon the proportion of mosquitoes feeding through the membranes.

Table 4. Aëdes aegypti. *The effect of moistened (saliva) membrane upon proportions gorging*

Membrane moistened with saliva			Dry membrane		
No. of experiments	No. gorged/ no. of mosquitoes	% gorged	No. of experiments	No. gorged/ no. of mosquitoes	% gorged
9	270/474	57	9	239/469	51

VII. THE USE OF THE MEMBRANE TECHNIQUE IN A STUDY OF THE FEEDING REACTIONS OF *AËDES AEGYPTI*

In studying the feeding mechanism of mosquitoes the technique used by earlier workers (Fülleborn, 1908; Kadletz & Kusmina, 1929; MacGregor, 1930, 1931; Roy & Gosh, 1940) was that of the capillary tube. By this method the fluid on which the mosquito is to be fed is put into the tube and this is slipped over the mouthparts of the mosquito. MacGregor (1931) maintained that when this technique was used on mosquitoes with the proboscis unsheathed the results obtained were comparable with those from true biting; but Roy & Gosh (1940) considered that such results were irregular and not comparable with natural feeding. It seemed to us possible that a technique such as is offered by the use of prepared membranes, in which the act of feeding involves piercing and is similar to natural biting, and in which the factors involved can be carefully controlled, might yield useful information on the feeding reactions of mosquitoes.

That the destination of different kinds of foods in the internal organs of the mosquito was not the same had been noted by many workers. Nuttall & Shipley (1903) were the first to show that when *Anopheles maculipennis* were fed on food containing sugar, and killed immediately after the feed the three diverticula were filled with the fluid. Wright (1924) confirmed this, and found that after a blood meal little of this fluid was found in the diverticula, the greater part being in the stomach. According to Kadletz & Kusmina (1929), who used the capillary tube technique, blood was directed to the crop, stomach and diverticula whereas sugary fluids were directed to the crop and diverticula.

MacGregor (1930, 1931) described two methods of feeding in mosquitoes; true biting and 'discontinuous suction'. According to him, when mosquitoes feed by biting through the skin of the host the destination of the blood imbibed is the stomach, whereas that of water, fruit saps or other sugar-containing fluids ingested by discontinuous suction is the diverticula. He believed that mosquitoes exercise a 'selective and voluntary control' in regard both to the aspiration and destination of the fluids ingested. Using the capillary tube technique with unsheathed proboscis, he found that mosquitoes sometimes would imbibe blood when they had refused honey, and sometimes the reverse reaction would occur. When blood containing a trace of honey was offered to *Aëdes aegypti* by the capillary technique it was despatched to the diverticula, instead of passing directly to the stomach as would be the case with pure blood.

Roy & Gosh (1940) compared the results of feeding mosquitoes upon honey, glucose or citrated blood imbibed from wet cotton-wool and by forcible feeding by the capillary tube technique. In *A. aegypti* the sugary fluids, when given by the former method, were directed mainly to the diverticula which were fully distended though a trace might be found in the stomach, whereas citrated blood was directed primarily to the stomach, though a trace was found in the diverticula. When these foods were fed forcibly to *A. aegypti* the results were inconsistent. Blood ingested naturally by biting was directed to the stomach and diverticula, all of which were fully distended.

Yoeli & Mer (1938) fed *Anopheles elutus* upon solutions of haemoglobin, suspensions of blood cells, and serum through animal membranes 'in order to prevent their penetration into the diverticulum of the mosquitoes, which occurs regularly when the anophelines do not feed on an animal'. They give no data to support this assertion.

We decided to use the technique of membrane feeding in studying the reactions of *A. aegypti* to different types of food and, by comparing the results of feeding similar fluids through a membrane and in open drops, to obtain more light upon the problem of whether the method of feeding or the nature of the food determines its destination in the mosquito.

In each of the feeding experiments the mosquitoes used were females of the same strain and age group. Approximately equal numbers were put into each of two biting cylinders. On top of one cylinder a membrane apparatus was stood containing the test fluid at 40–42°C., and on the other, as a control, a similar apparatus, also at 40–42°C., containing heparinized whole blood freshly drawn from a chick. Feeding was effected in the dark, at 28°C., in a moist atmosphere, and the time allowed was 30 min.

Since some of the fluids tested were colourless, and could not therefore be detected easily in the alimentary canal of the mosquitoes, it was first

ascertained whether the addition of cochineal to whole blood had any effect upon the proportion of *A. aegypti* which gorged. Five drops of an aqueous solution of cochineal were added to 3–5 c.c. of the test fluids. A series of experiments was performed, according to the method described above, in which the test fluid was heparinized whole chick blood coloured with cochineal. The addition of cochineal had no inhibitive effect upon feeding, in fact in the small series (Table 5) of experiments which was performed the mean percentage of mosquitoes feeding on blood and cochineal was higher than that of those feeding on blood alone.

All the mosquitoes which ingested blood, or blood and cochineal, were fully gorged, with the exception of two (Table 5) in which the stomach was only partly filled. No trace of blood was found in the diverticula of any of the mosquitoes.

Table 5. Aëdes aegypti. *Comparison of feeding reactions to whole blood and whole blood + cochineal*

	Whole blood		Whole blood + cochineal		% fully gorged	
No. of experiments	Proportion containing blood	Distribution of blood and amount ingested	Proportion containing fluid	Distribution of fluid and amount ingested	Whole blood	Whole blood + cochineal
9	41/87 = 47 %	39 + S 2 (+) S	51/91 = 56 %	51 + S	45	56

+ S = stomach fully gorged. (+) S = trace or small quantity of fluid in the stomach.

Table 6. Aëdes aegypti. *Comparison of feeding reactions to whole blood and plasma*

	Whole blood		Plasma		% fully gorged	
No. of experiments	Proportion containing fluid	Distribution of fluid and amount ingested	Proportion containing fluid	Distribution of fluid and amount ingested	Whole blood	Plasma
4	84/126 = 67 %	84 + S	13/124 = 10 %	13 (+) S	67	0

+ S = stomach fully gorged. (+) S = trace or small quantity of fluid in the stomach.

(1) *Chick plasma*

A comparison was next made of the feeding reactions of *A. aegypti* to the two main components of blood, in order to discover whether plasma and corpuscles were equally attractive to the mosquitoes.

Plasma was prepared by centrifuging fresh, heparinized chick blood and removing the clear, supernatant fluid. A few drops of cochineal solution were added to the plasma and to the whole blood to be used as a control. The temperature of the fluids offered was 40–42° C. Although a normal proportion of the control mosquitoes gorged fully upon the whole blood, it appeared by direct examination that none of those offered plasma had ingested any, but upon dissection a trace was found in the stomachs of 13 out of 42 mosquitoes in one experiment (Table 6). In none of the mosquitoes was either blood or plasma found in the diverticula. All the mosquitoes which ingested whole blood were fully gorged.

These results suggest that some factor is missing from plasma which induces or enables *A. aegypti* females to gorge upon whole blood. The feeding reactions of *A. aegypti* to washed corpuscles therefore were studied.

(2) *Washed corpuscles*

Corpuscles were prepared by centrifuging fresh, heparinized chick blood and removing the plasma. The corpuscles were washed twice in citrate saline solution and suspended in this fluid, the proportion of saline to corpuscles being equal to that of the plasma removed. Heparinized whole chick blood was used as a control. In all four experiments (Table 7) some of the mosquitoes which were offered washed corpuscles ingested them, though the mean percentage was lower than of those which ingested whole blood. Whereas those mosquitoes which imbibed plasma ingested only a trace, all those which ingested washed corpuscles gorged fully. The ingested food, irrespective of whether it was corpuscles alone or whole blood, was present in the stomach and never in the diverticula.

Since mosquitoes gorged fully upon washed corpuscles whereas they either failed to feed upon plasma or ingested only a trace of it, it seemed probable that the corpuscles contained some substance attractive to the mosquitoes. If this were so the rupturing of the corpuscles by haemolysis might liberate more of the attractive substance and so induce a greater proportion of mosquitoes to gorge.

(3) *Haemolysed blood*

Haemolysed blood was prepared by the rapid freezing and thawing of heparinized chick blood.

The viscid solution of haemoglobin in plasma was freed from the stroma by centrifuging. The proportion of mosquitoes which ingested haemolysed blood was smaller than of the controls which ingested whole blood (Table 8). Whereas, however, almost all the mosquitoes which ingested normal blood gorged fully, of those which ingested haemolysed blood the number which gorged fully was smaller than of those which contained a mere trace of fluid visible only on dissection. Since the viscosity of blood haemolysed by this method is very great it seemed possible that this physical property might account for the small proportion of mosquitoes which gorged diluted none of the mosquitoes ingested blood (Table 8).

It seemed possible that the greater facility of ingestion by *A. aegypti* of whole as compared with haemolysed blood might be explained by the mechanical factor of the presence in whole blood of particulate matter in the form of corpuscles. In order to study the mechanical effect upon gorging of the addition of small particles to haemolysed blood, a suspension of rice starch granules in a solution of haemoglobin in plasma was offered in a membrane to *A. aegypti*, but none of the mosquitoes ingested it.

Table 7. Aëdes aegypti. *Comparison of feeding reactions to washed corpuscles and whole blood*

	Whole blood		Washed corpuscles suspended in citrate saline		% fully gorged	
No. of experiments	Proportion containing blood	Distribution of blood and amount ingested	Proportion containing corpuscles	Distribution of corpuscles and amount ingested	Whole blood	Washed corpuscles
4	88/136 = 65%	86 + S 2 (+) S	50/130 = 38%	50 + S	63	38

+ S = stomach fully gorged. (+) S = trace or small quantity of fluid in the stomach.

Table 8. Aëdes aegypti. *Comparison of feeding reactions to haemolysed and whole blood*

	Whole blood		Haemolysed blood				% fully gorged	
No. of experiments	Proportion containing blood	Distribution of blood and amount ingested	Fluids	No. of experiments	Proportion containing fluid	Distribution of fluid and amount ingested	Whole blood	Haemolysed blood
5	157/227 = 69%	156 + S 1 (+) S	Haemoglobin in plasma	6	178/341 = 52%	52 + S 126 (+) S	69	15
			Diluted 1 : 2	2	7/72 = 10%	7 (+) S		
			Diluted 1 : 4	1	23/73 = 32%	11 + S 12 (+) S		
4	59/121 = 49%	57 + S 2 (+) S	Haemoglobin in water	4	47/144 = 33%	38 + S 9 (+) S	47	26
			Haemoglobin in water diluted 1 : 2	1	0/24 = 0%	0	—	0

+ S = stomach fully gorged. (+) S = trace or small quantity of fluid in the stomach.

to repletion. Dilution of the fluid with water, however (Table 8), caused a decrease, not an increase, in the proportion of mosquitoes which imbibed the fluid, and of these few gorged to repletion.

As a further test of the influence of viscosity upon feeding, a solution of haemoglobin in water was prepared by haemolysing washed corpuscles in water and centrifuging off the stroma. The proportion of water to corpuscles was the same as that of the plasma removed. The proportion of mosquitoes which fed on this fluid was smaller than in the controls (Table 8), but of those which ingested the fluid a greater proportion gorged fully than upon haemoglobin in plasma. When the solution was

In none of the mosquitoes, whether fed upon heparinized whole blood, haemolysed blood, washed corpuscles or plasma, was any trace of the fluid found in the diverticula; the stomach alone contained it.

(4) *A comparison of the results of feeding* A. aegypti *through a membrane and from open drops*

In order to discover whether the method of feeding or the nature of the food determines its destination to stomach or diverticula, the results were compared when female *A. aegypti* of the same age group were offered similar fluids (*a*) through a membrane and (*b*) from a drop on a glass slide. The

initial temperature of the fluid in the membrane was 40–42°C., and since a water jacket at the same temperature was used the fall in temperature was slow; whereas although the temperature of the fluid in the drops in the first place was 42°C., it fell rapidly to that of the environment (28°C.).

When *heparinized blood* was offered in drops (Table 9) a much smaller proportion of *A. aegypti* fed upon it than when it was offered through a membrane. The only difference in conditions was that of the temperature of the fluids and the method by which the food was offered. All the mosquitoes found in the diverticula of mosquitoes which imbibed it through a membrane.

Of the small proportion (3/66) of *A. aegypti* which ingested haemoglobin in water from drops, 2 had a trace in the stomach and the third a trace in both stomach and diverticula. None of the mosquitoes ingested it through a membrane.

The results with *plasma* were similar whether it was offered in drops or through a membrane (Table 9). Only a small proportion of mosquitoes ingested a trace of it and this was found in the stomach.

Table 9. Aëdes aegypti. *Comparison of feeding reactions to fluids in drops or through membranes*

		Drops			Membrane		
			Distribution of fluid in mosquitoes			Distribution of fluid in mosquitoes	
No. of experiments	Test fluid	Proportion containing fluid	S	D	Proportion containing fluid	S	D
3	Blood	18/108	16 +	0	74/147	74 +	0
			2 +	2 (+)			
1	Haemoglobin in plasma	0/46	0	0	26/53	8 +	8 +
						18 (+)	0
1	Haemoglobin in water	3/66	1 (+)	1 (+)	0/61	0	0
			2 (+)	0			
1	Plasma	7/19	7 (+)	0	7/22	7 (+)	0
			(Total number of mosquitoes dissected 73)				
4	Glucose	139/207	56 (+)	56 +	20/202	12 (+)	0
			0	17 +		0	2 (+)
						3 (+)	3 +
						3 (+)	3 (+)
			(Total number of mosquitoes dissected 42)				
2	Honey	80/93	5 (+)	5 +	0/91	0	0
			0	37 +			
1	Washed corpuscles in 8% glucose	8/26	0	2 +	6/17	6 +	0
			3 (+)	3 +			
			3 +	3 (+)			
1	Blood + honey	37/41	37 (+)	37 +	5/37	5 (+)	0

Unless otherwise stated all mosquitoes in each experiment were dissected.

\+ = fully gorged.
(+) = small quantity or trace of fluid ingested.
S = stomach.
D = diverticulum.

which imbibed blood from the drops gorged fully and, with the exception of 2 which had a trace of blood in the ventral diverticulum, blood was found only in the stomach. The stomachs of the mosquitoes which fed through the membrane were distended with blood and the diverticula empty.

When *haemoglobin in plasma* was offered in drops (Table 9) none of the mosquitoes ingested it, whereas 26 imbibed it through a membrane, 18 containing a trace of fluid in the stomach and the remaining 8 having both stomach and diverticula fully distended. This was the only occasion on which blood or any component part of blood was

With few exceptions, therefore, the destination of blood, whether whole or haemolysed, and of plasma is the stomach, and is the same whether the method of intake involves true biting or discontinuous suction.

Since fruit juices and other sugar-containing fluids normally are imbibed by discontinuous suction and not by 'biting', and since their destination when thus ingested is the diverticula, the results of feeding female *A. aegypti* of the same age groups upon honey or a saturated solution of glucose by discontinuous suction (i.e. from open drops) and by biting (i.e. through a membrane) were compared.

Both honey and glucose were coloured with cochineal and warmed to 40–42°C.

When saturated glucose solution was offered in drops a high proportion of mosquitoes gorged fully upon it, with the exception of one experiment when gorging was poor (Table 9). The diverticula of these mosquitoes were fully distended with the fluid and the stomach of most individuals contained a trace. In marked contrast to these results few or none of the mosquitoes offered glucose through a membrane imbibed it, and then almost without exception in small quantities only. The glucose was found in either stomach or diverticula or in both.

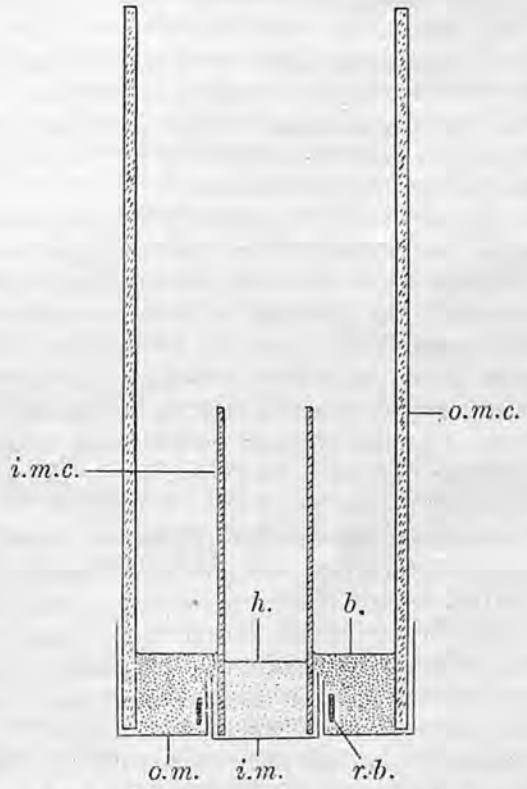


Fig. 2. *o.m.c.* outer membrane cylinder; *i.m.c.* inner membrane cylinder; *b.* blood; *r.b.* rubber band; *h.* honey; *o.m.* outer membrane; *i.m.* inner membrane.

None of the mosquitoes imbibed honey through a membrane though the proportion which gorged on it from drops was high (Table 9). In these the diverticula were fully distended with honey and, in a few mosquitoes on dissection, a trace was found in the stomach also.

These experiments demonstrate that whereas *A. aegypti* will gorge freely on honey or glucose solution from drops, either they are unaware of these fluids when presented in a membrane, or find difficulty in aspirating them. It is difficult to believe that these solutions are less palatable when offered by this method.

In order to compare the reactions of the same mosquitoes under identical conditions to carbohydrates and blood we prepared two concentric membrane tubes (Fig. 2) with honey and cochineal in the inner tube and blood in the outer tube. The outer tube was surrounded by a water jacket at 40–42°C. and the apparatus stood on a cylinder of female *A. aegypti* of the same age group at 28°C. Twenty-three of the mosquitoes gorged fully on the blood which was directed to the stomach only. No trace of honey was found in the stomach or diverticula of these mosquitoes, or in those which did not imbibe blood. This experiment was repeated using a saturated solution of glucose coloured with cochineal in the outer membrane and blood in the inner membrane. Twenty-four out of 52 mosquitoes gorged fully in 30 min. and these contained blood in the stomach only. No trace of glucose was found either in the stomach or diverticula of these mosquitoes or of those which failed to ingest blood. When this experiment was repeated with glucose in the inner membrane and blood in the outer, 23 out of 54 mosquitoes gorged fully. One of these mosquitoes contained only glucose. The ventral diverticulum was fully distended with the fluid, but no trace of it was present in the stomach. Twenty-two mosquitoes contained blood in the stomach only, no trace of glucose being found in stomach or diverticulum, and the remaining 31 contained neither blood nor glucose.

As it seemed possible that an olfactory factor might be responsible for this selection of foods the following experiment was performed. Two membrane tubes were arranged one inside the other, the outer one containing saturated glucose solution coloured with cochineal and the inner one whole blood. The outer surface of the membrane containing the blood came into close juxtaposition to the glucose solution which was only 1 mm. or so in depth. It was hoped that if mosquitoes were attracted to blood by some odour which was given out through the membrane this might impregnate the glucose solution and render it attractive to the mosquitoes. But of the 39 mosquitoes in the biting cylinder none gorged on the glucose.

The effect of mixing blood or its components with glucose solution or honey was next studied. A small proportion of *A. aegypti* (Table 9) imbibed a suspension of corpuscles in 8% glucose from drops. In 5 out of 8 the ventral diverticulum was fully distended, 3 of these having traces of the suspension in the stomach also, whereas in the remaining 3 the stomach was fully distended and a trace was found in the ventral diverticulum. In all 6 mosquitoes which imbibed the suspension through a membrane the stomachs were fully distended, no trace being found in the diverticulum. A high proportion of mosquitoes imbibed a mixture of blood and honey from drops, and in these the diverticula were fully

distended with the fluid and the stomach contained a trace; but when a similar mixture was offered through a membrane the proportion which imbibed it was small and only a trace was ingested which was located in the stomach.

A comparison of the feeding reactions of *A. aegypti* of the same age group to plasma, honey and a mixture of these substances when offered through a membrane gave some anomalous results (Table 10).

Table 10. Aëdes aegypti. *Comparison of feeding reactions to honey, plasma, and a honey-plasma mixture fed through membranes*

No. of experiments	Test fluid	Proportion containing fluid	Distribution of fluid in mosquitoes dissected	
			S	D
4	Honey	0/174	0	0
2	Plasma	11/83	11 (+)	0
1	Honey + water 1 : 1	1/48	0	1 (+)
			(Total number of mosquitoes dissected 62)	
3	Honey + plasma 1 : 1	57/157	27 (+)	27 +
1	Honey + plasma 5 : 1	6/32	0	6 +

Unless otherwise stated all mosquitoes in each experiment were dissected.

\+ = fully gorged.
(+) = small quantity or trace of fluid ingested.
S = stomach.
D = diverticulum.

Whereas none of the mosquitoes ingested honey and few ingested plasma and then only in small quantities, in two of the experiments the proportion of mosquitoes which ingested a mixture of these fluids was high and in each case the mosquitoes were fully gorged. In one experiment all the mosquitoes were dissected and in 21 out of 56 the diverticula were fully distended with the fluid, and the stomachs contained a trace of it. In each of the other experiments only 3 of the mosquitoes which had gorged fully were dissected, but the distribution of the fluid in the mosquitoes' organs was similar to that in the previous experiment. This increase in the proportion of mosquitoes which gorged on the mixture of honey and plasma as compared with either of the component parts was not due merely to the dilution of the honey, for when the results of experiments with similar dilutions of honey and water, and honey and plasma, were compared it was found that whereas only 1 out of 48 mosquitoes gorged on the honey and water, 21 out of 56 gorged on the plasma and honey.

(5) *Discussion of the results from feeding experiments*

From an analysis of these experiments upon the feeding reactions of *A. aegypti* the following deductions appear to be warrantable. Whole blood, when offered through a membrane, is ingested more consistently than either plasma, haemolysed blood, or washed corpuscles suspended in saline, and the mosquitoes almost invariably gorge fully upon it. *A. aegypti* gorge fully on washed corpuscles also, but the probability of gorging is less than with whole blood. A much smaller and more variable proportion of mosquitoes ingest haemolysed blood and of these many imbibe only a small quantity of the fluid. Plasma is seldom ingested, and then only in small quantities. The destination of all these fluids when ingested through a membrane is the stomach, with the exception that in a few mosquitoes fed upon haemolysed blood the ventral diverticulum also was fully distended.

The proportion of *A. aegypti* which ingest whole blood or haemolysed blood from drops is much smaller than the proportion ingesting them through membranes. The proportion ingesting plasma is equally poor from drops or membranes. When ingested from drops the destination of all these fluids is primarily the stomach, as is the case when they are ingested through membranes, though a trace of the fluid may be found in the diverticula also.

In comparing the results of other workers using a membrane technique we find that Gordon (1922) induced *A. aegypti* to feed upon serum, washed red cells, and whole blood, either from open watch glasses or through the skin of a bat, but by neither method did the mosquitoes feed to repletion on any of these fluids. Woke (1937) fed *A. aegypti* through membranes upon red cells suspended in Ringer's solution, plasma, serum, and rabbit haemoglobin in water, but he gives no data of the proportion of mosquitoes which fed nor of the amount of fluid imbibed. As he was studying the effect of food upon egg production it is to be concluded that the mosquitoes ingested sufficient of the fluid for it to be visible without dissection.

An analysis of the results of feeding *A. aegypti* upon glucose or honey shows that their reactions to these fluids are the reverse of those with blood. The proportion of mosquitoes imbibing glucose or honey from drops is high whereas few imbibe glucose and none honey through a membrane. When imbibed from drops the primary destination of these fluids is the ventral diverticulum which is usually fully distended, but traces of the fluids may be found in the stomach also. Glucose imbibed through a membrane may be present in the diverticula or stomach or both; but of the few mosquitoes which

gorged fully it was the diverticula, not the stomach, which were fully distended.

From these results, therefore, it can be concluded that it is the nature of the food, not the method of feeding, which determines its destination whether into the stomach or diverticula of the mosquito.

The results from feeding experiments with mixtures of blood or plasma and honey or glucose are variable, but as a whole they agree with those which MacGregor (1931) obtained with the capillary tube technique, i.e. in the majority of mosquitoes fed upon such mixtures, whether from drops or through membranes, the ventral diverticulum is fully distended, and the stomach contains a small quantity of the fluid.

Two problems have arisen in the course of these feeding experiments for which there are as yet no explanations: first, why *A. aegypti* should gorge freely on glucose or honey from drops and yet seldom imbibe the former and never the latter fluid through a membrane; and secondly, why they should gorge through a membrane upon a mixture of plasma and honey whereas they do not imbibe honey and seldom plasma, and then only in small quantities, when offered to them separately under identical conditions. The absence of a sustained heat gradient may account for the smaller proportion of *A. aegypti* which gorges from drops of blood as compared with blood through a membrane; but this explanation will not hold for the glucose or honey experiments since the mosquitoes fail to gorge on these fluids through a membrane in the presence of a well-sustained heat gradient, whereas they gorge freely upon them from drops in which the heat gradient is not sustained. Also, when the same mosquitoes are offered a choice of blood or honey, or blood and glucose, under identical conditions (i.e. through concentric membranes, p. 93) the blood is ingested and the honey or glucose neglected. It has been observed in a few instances that *A. aegypti* is able to ingest glucose through a membrane, whilst the ingestion of a mixture of plasma and honey has been observed in many mosquitoes. Both these fluids were directed into the diverticula as in discontinuous feeding. There appears therefore to be no mechanical difficulty in imbibing through a membrane fluids which are destined to the diverticula. The results of the feeding experiments with these fluids suggest that the factor or factors which attract *A. aegypti* to feed upon glucose or honey from drops do not operate through a membrane.

Fülleborn's (1908, 1932) contention based upon experiments with the capillary tube technique, that in *Anopheles maculipennis* aspiration of fluids once begun is automatic and continues until the stomach is fully distended, is not confirmed by our observations upon feeding *Aëdes aegypti* through membranes. These mosquitoes never gorged fully upon plasma and infrequently upon haemolysed blood. They were able to stop aspirating a fluid when only a trace had been ingested.

VIII. THE INFECTION OF *AËDES AEGYPTI* WITH *PLASMODIUM GALLINACEUM* THROUGH ANIMAL MEMBRANES

Yoeli (1938) first proved that mosquitoes (*Anopheles elutus*) could be infected with the malaria parasite by feeding them through a membrane upon defibrinated blood containing crescents of *Plasmodium falciparum*. He found that the blood was not only infective when freshly drawn, but remained so when kept for 5 hr. at 23–27° C., or for 72 hr. in an ice box.*

In the following infection experiments with *P. gallinaceum* the Brumpt strain of *Aëdes aegypti* was used almost exclusively. The rate and intensity of infection in this strain proved very variable even when mosquitoes of the same age group were fed directly upon infected chickens whose blood contained a high proportion of gametocytes of both sexes. A high rate of exflagellation was not a guarantee that mosquitoes gorged upon the chick from which the blood was drawn would become well infected. The proportion of mosquitoes infected and the intensity of their infection may both be low when allowed to gorge on such birds, or the attempt to infect may fail completely.

In the experimental infection of *Aëdes aegypti* with *Plasmodium gallinaceum* by feeding through a membrane, heparinized blood containing a high proportion of gametocytes was used immediately after it had been drawn from the chick. The blood in the membrane apparatus was warmed to 40–42° C. and the mosquitoes were fed in the dark at 28° C., 30 min. being allowed for feeding. A small sample of the blood was kept under observation in order to see whether exflagellation occurred and, if so, after what period of time it began. The mosquitoes which gorged were kept 4–5 days at 28° C. after which their stomachs were examined for oocysts. *Aëdes aegypti* which had gorged in this manner upon blood containing gametocytes of *Plasmodium gallinaceum* became infected (Table 11), and the infections developed normally. Sporozoites were found in the salivary glands and proved to be infective when injected into a chick.

The rate and intensity of infection in mosquitoes

* After our preliminary communication in *Nature*, Prof. R. M. Gordon informed me, in a personal communication, that he and Dr S. Bertram had been working on somewhat similar lines in 1939, and had infected mosquitoes, reared under sterile conditions, with *Plasmodium gallinaceum* by allowing them to gorge on sterile blood through sterile membranes made of sausage skin. A. B.

fed through a membrane on infected blood was compared with that of mosquitoes of the same age group fed directly upon the infected chick from which the blood was drawn. In these experiments one batch of mosquitoes was fed directly upon the chick at 28°C. in the dark, and the second batch was fed through a membrane, under similar conditions of temperature and humidity, upon blood drawn immediately after the first batch had gorged. The gametocytes, therefore, were at a similar stage of development in both experiments.

Whilst in some experiments (Table 11, Exps. 3–5) the rate of infection obtained by the two methods was approximately the same, in others the number of mosquitoes infected when fed through a membrane was lower than when fed directly on a chicken. In many of the mosquitoes which had gorged through a membrane, the number of cysts on the stomach was as great as on those which gorged directly on a chicken.

Table 11. Aëdes aegypti. *Rate and intensity of infection with oocysts of* Plasmodium gallinaceum *when fed* (*a*) *through a membrane*, (*b*) *on a chicken*

	(*a*) Through membranes		(*b*) On chickens	
	Proportion of *A. aegypti* infected	No. of cysts per stomach	Proportion of *A. aegypti* infected	No. of cysts per stomach
1	3/9	>50, 3, >50	—	—
2	4/7	16, 3, 5, 1	—	—
3	10/20	13, 4, 30, 7, >50, 2, 9, 11, 7, 5	6/13	3, 6, 18, 20, 7, 3
4	3/5	3, 1, 6	5/7	1, 13, 1, 11, 10
5	4/8	1, 1, 2, 3	3/7	1, 1, >50
6	15/20	1, 4, 13, 8, 2, 12, 2, 38, 4, 3, 7, 12, 22, 5, >50	8/8	10, 13, 10, 12, 14, 17, >50, >50
7	1/16	1	4/7	1, 3, 11, 1
8	1/16	2	4/8	3, 4, 2, 19
9	5/13	3, 2, >50, >50, >50	8/8	28, 2, 5, 2, 50, >50, >50, >50
10	1/9	9	4/8	15, 7, 9, 1
11	11/12	1, 1, 1, 2, 30, 4, 2, 17, 5, 47, 35	—	—
12	9/12	1, 3, 8, 16, 1, 4, 2, 1, 10	—	—
13	8/12	2, 3, 1, 1, 1, 5, 1, 5	—	—

In all the experiments recorded in Table 11 in which *Aëdes aegypti* were fed through a membrane, exflagellation was observed in the blood. The length of time before exflagellation began, and the number of male gametocytes exflagellating per field varied widely in different samples of blood. In some experiments exflagellation began before the mosquitoes had access to the blood in the membrane, in others it did not begin until about halfway through the feeding experiment, and in others was not seen until after the membrane apparatus had been removed from the mosquito cylinder. Observations were made in order to discover whether the occurrence of exflagellation in the blood during or before the feeding of the mosquitoes had an inhibitory effect upon the rate of infection; or whether blood in which exflagellation had ceased before it was ingested remained infective. In Exp. 4, Table 11, exflagellation had started before the mosquitoes had access to the blood, and had ceased before the membrane apparatus was removed from them, but infection occurred. In Exp. 6 though no exflagellation was seen until 10 min. after the blood was offered to the mosquitoes, many exflagellating parasites were seen between this period and the end of the feeding experiment; yet the rate and intensity of infection in the mosquitoes which had ingested the blood was relatively high. It was possible, however, that the majority of the mosquitoes infected had gorged before exflagellation became frequent.

The rate and intensity of infection in mosquitoes which had gorged (*a*) before exflagellation could be seen in the blood, (*b*) whilst it was plentiful, and (*c*) after it could no longer be found, were compared. Freshly drawn, heparinized blood containing a high proportion of gametocytes, but in which no exflagellating forms could be seen, was placed in a membrane apparatus upon a cylinder of *A. aegypti* for 10 min. At the time of its removal a few exflagellating forms were found in the blood. Eleven out of 12 of the mosquitoes gorged during the 10 min. became infected (Table 11, Exp. 11) and the intensity of infection was relatively high. The same sample of blood was placed upon a second cylinder of mosquitoes of the same age group. The number of exflagellating forms present was very great during the whole period of 25 min. that the mosquitoes had access to the blood. Nine out of 12 mosquitoes became infected during this period

(Table 11, Exp. 12) and the intensity of infection was moderate. The same sample of blood was kept at +4°C. for 20 hr. after which it was warmed to 40°C. and examined for exflagellation, but none was found either then or at subsequent examinations. Eight out of 12 (Table 11, Exp. 13) of the mosquitoes of the same age group as in the two previous experiments became infected when gorged through a membrane upon the blood, though the intensity of infection was low.

From these observations it can be concluded that though exflagellation may be very frequent at the time of the ingestion of blood by mosquitoes it does not prevent their infection, and blood in which exflagellation has been frequent but has ceased remains infective. It is possible that fertilization of the female gametes had taken place before the blood was ingested by the mosquito, and that the zygotes remained viable. No attempt has been made in the experiments described herein to discover the proportion of male gametocytes undergoing exflagellation. It might therefore be suggested that infection takes place by means of male gametocytes which do not exflagellate until ingested by the mosquitoes; but in Exps. 6 and 12 the numbers of exflagellating parasites was so great that it seemed improbable that there were many mature male gametocytes which were not exflagellating.

IX. A METHOD FOR OBTAINING VIABLE SPOROZOITES OF *PLASMODIUM GALLINACEUM* BY FEEDING INFECTED *AËDES AEGYPTI* THROUGH ANIMAL MEMBRANES

Little is known as yet of the sensitivity of sporozoites *in vitro* to antimalarial drugs, though much evidence has been amassed of the greater resistance to the drugs of sporozoite induced malaria infections of man than of blood-inoculated malaria. It was observed by Yorke & MacFie (1924), in the treatment of general paralysis of the insane, that blood-inoculated infections of malaria and sporozoite-induced infections differed in their response to quinine. The inefficacy of the drug upon sporozoite-induced infections, unless administered for 10–14 days after infection, was attributed to its lack of action upon the sporozoites themselves. A similar lack of response in sporozoite-induced infections to prophylactic treatment with antimalarial drugs in doses highly effective upon blood-inoculated infections was first observed in avian malaria by Russell & Nono (1932) and Tate & Vincent (1933) with plasmoquine, and Kikuth & Giovannola (1933) and Tate & Vincent (1934) with atebrin. These observations have been confirmed by many other workers.

The problem of the nature of this resistance of sporozoite-induced infections of malaria to antimalarial drugs has been complicated by the more recent discovery, in avian malaria, of an exo-erythrocytic cycle of development. Evidence now indicates that in avian malaria, and by analogy probably in human malaria, the sporozoites on entry into the body of the host undergo a phase of development in the tissues (cryptozoites of Huff, Coulston & Cantrell (1943), primary tissue phase of Davey (1944)) before entering erythrocytes. A true causal prophylactic might, therefore, act on the sporozoites, or the primary tissue phase, or on both. A complete knowledge of the mode of action of any antimalarial drug which had been proved to eradicate sporozoite-induced infections in experimental animals would involve a study of its action upon sporozoites *in vitro* and upon the primary tissue phase in culture. By such *in vitro* methods it would be possible to compare the action of a drug upon the sporozoites and primary tissue phase of the species of malaria used in the experimental animal (e.g. *Plasmodium gallinaceum* in chicks) with its action upon the sporozoites and primary tissue phase of any of the species of *Plasmodium* of man. In order to approach such a study it is essential that a method should be devised whereby sporozoites can be obtained free from gland tissue and bacterial contaminants. The method in general use whereby sporozoites are obtained for injection is by dissecting out the infected glands from the mosquito and teasing them apart or grinding them up. Such a method is laborious, and though suitable for infection experiments is unsuitable for the study either of the direct action of drugs upon sporozoites or for tissue culture, for it is impossible to ensure that all the sporozoites are free from traces of tissue, and bacterial contaminants may be present.

Having devised a method whereby *Aëdes aegypti* would gorge readily upon heparinized chick blood, we decided to see whether this technique might be applied successfully to the collection of sporozoites. It appeared to us that success or failure would depend upon the stage in the process of biting at which infected mosquitoes ejected sporozoites. MacGregor (1931) saw no definite sign of salivation when mosquitoes were fed upon freshly drawn blood in capillary tubes, and he considered it probable that saliva was ejected only in the process of incision and not during the act of drawing up the blood. He considered that its function was probably that of a lubricant during actual incision. Gordon & Lumsden (1939) state that 'fluid, presumably salivary secretion, is injected into the tissues at various stages of penetration', but they also noted in one case that it was ejected directly into a capillary.

If, as MacGregor believed, the saliva is merely a lubricant and ejected only during penetration then it seemed unlikely that sporozoites would be ejected

into the blood in the membrane tube when infected mosquitoes gorged; but if saliva is ejected into the capillaries during aspiration then sporozoites should be ejected into the blood when infected mosquitoes gorge through a membrane.

The blood used in feeding infected mosquitoes through a membrane was taken from uninfected chicks preferably not more than 4–5 weeks old. Whereas young chicks infected with *Plasmodium gallinaceum* frequently die a few days after the infection becomes patent, either from the intensity of the infection in the red blood corpuscles, or from the occlusion of the capillaries of the brain by exo-erythrocytic parasites, older birds may survive the acute stage of the infection and lapse into a chronic state, though they may die later from exo-erythrocytic parasites in the brain. Since older birds thus show some degree of immunity to infection with *P. gallinaceum* their blood was not used for the collection of sporozoites.

0·5 c.c. of the blood to be used in the membrane was injected intravenously into a clean chick, in order to prove that no accidental infection of the blood had occurred. These control chicks have invariably remained uninfected.

The heparinized chick blood was put into the membrane apparatus at 40–42° C. according to the usual method, and stood upon the cylinder of infected mosquitoes. In each experiment each chicken was inoculated with 0·5 c.c. blood, and the amount used in the membrane cylinder varied from 1·5 to 4 c.c. according to the number of mosquitoes available and the number of chicks to be inoculated.

It is essential that the *Aëdes aegypti* used for the collection of sporozoites should be well infected. In order to ensure this a few mosquitoes from each batch were examined for oocysts on the 4th or 5th day after being fed on an infected chicken, and only batches in which 50% or more of the sample examined was infected were retained for use. Mosquitoes which had been infected 8–12 days were most frequently used.

The mosquitoes were allowed 30 min. at 28° C. in which to feed upon the blood through the membrane. The blood was then pipetted out of the membrane cylinder, the inner surface of the membrane being washed with a few drops of saline and the washings added to the blood. The blood was shaken well in order that the sporozoites should be evenly distributed, and injected intravenously into chicks, each chick receiving 0·5 c.c. The birds were examined for the appearance of parasites in the peripheral blood from the 5th day onwards.

In each infection experiment chicks of the same age group were used, the ages ranging from 3 to 18 days. Owing to wartime difficulties it has not been possible to keep to one breed, but no difference in infectivity was found amongst the breeds used.

The proportion of *A. aegypti* which had fed through the membrane was counted. In assessing these numbers it did not appear to be necessary to dissect the mosquitoes which had not gorged, as experience in feeding experiments (pp. 89–93) had shown that, almost invariably, when offered whole blood either they gorged fully or not at all. It was improbable, therefore, that mosquitoes which did not appear to the naked eye to contain blood had ingested any. As in the early stages of the work it was of interest to compare the approximate number of infected mosquitoes used in each experiment with the number of chicks infected from them, the period of incubation, and the intensity of the resulting infections, the salivary glands of some, or all, of the mosquitoes which had fed were examined (Table 12).

Table 12. Aëdes aegypti *infected with* Plasmodium gallinaceum. *Incubation period in chicks inoculated intravenously with sporozoites ejected through a membrane*

	Mosquitoes			Chicks	
	Proportion gorged	No. examined	No. infected	Proportion infected	Day of appearance of parasites in peripheral blood
1	49/84 = 58%	9	8	3/3	7, 7, 7
2	35/57 = 61%	10	6	3/3	6, 6, 6
3	45/72 = 63%	5	5	6/6	7, 7, 7, 7, 7, 7
4	15/32 = 47%	15	5	2/2	6 Died on 5th day. Exo-erythrocytic forms found in liver and spleen
5	21/41 = 51%	7	7	3/3	6, 6, 6
6	18/110 = 16%	18	11	2/5	7, 10
7	21/52 = 40%	8	4	7/7	6, 7, 6, 7, 7, 6, 7

The incubation period in chicks injected intravenously with 0·5 c.c. of blood containing sporozoites was usually 6–7 days (Table 12) which was similar to that in chicks of the same age group bitten by 1–4 infected mosquitoes (Table 13). In only one experiment (Table 12, Exp. 6) did some of the chicks

Table 13. Aëdes aegypti *infected with* Plasmodium gallinaceum. *Incubation period in chicks infected directly by mosquito bite*

No. of infected *Aëdes* gorged on chicken	Intensity of infection of salivary glands	Day of appearance of parasites in peripheral blood of chicken
1	++++	7th
1	++++	Negative
1	++	7th
1	++++	7th
2	++++ ++	7th
2	++++ +++	8th
2	++++ ++++	6th
2	+++ ++	6th
3	++++ ++++ +++	7th
3	+++ ++ +	6th
3	++++ +++ +	6th
4	++++ ++++ +++ +++	7th

\+ glands lightly infected with sporozoites.
++ glands moderately infected with sporozoites.
+++ glands heavily infected with sporozoites.
++++ glands very heavily infected with sporozoites.

Table 14. Aëdes aegypti *infected with* Plasmodium gallinaceum. *Infection experiments in chicks to prove that sporozoites are ejected through a membrane on more than one occasion*

1st gorge			2nd gorge			3rd gorge		
Proportion of *Aëdes* gorged	Proportion of chicks infected	Day of appearance of parasites in peripheral blood	Proportion of *Aëdes* gorged	Proportion of chicks infected	Day of appearance of parasites in peripheral blood	Proportion of *Aëdes* gorged	Proportion of chicks infected	Day of appearance of parasites in peripheral blood
39/53 = 74%	4/4	6, 6, 6, 7	21/43 = 49%	2/2	6, 6	—	—	—
114/168 = 68%	2/2	6, 6.	59/88 = 67%	4/4	6, 6, 6, 6	29/53 = 55%	3/4	7, 7, 9

inoculated fail to become infected; but failure to infect sometimes occurs when chicks are bitten by heavily infected mosquitoes.

In many chicks the first sign of infection in the peripheral blood was the appearance of minute uninucleate parasites in the erythrocytes, in others schizonts were the first forms to appear, and occasionally mature gametocytes were found before asexual parasites. Adler & Tchernomoretz (1943) have described the appearance of gametocytes before mature schizonts in mosquito-induced infections of *Plasmodium gallinaceum* in fowls after suppressive treatment with quinine. They found that gametocytes which had attained, or almost attained, their full dimensions, were clearly recognizable before the first cycle of erythrocytic schizogony was completed, and conclude that their only source was the exo-erythrocytic parasites.

The course of the infection following intravenous injection of sporozoites collected by the membrane technique was similar in intensity and duration to that resulting from the bite of infected mosquitoes. In young chickens death frequently occurred on the 2nd–5th day after the infection became patent, and exo-erythrocytic parasites were found at autopsy in the brain and internal organs. In Table 12, Exp. 3 five out of six of the chicks died a few hours after uninucleate parasites had first appeared in the peripheral blood, and the capillaries of the brain were found to be occluded by enormous exo-erythrocytic parasites. Presumably these birds received a very heavy dose of sporozoites. In one bird (Table 12, Expt. 4) which died accidentally on the 5th day, before parasites had appeared in the peripheral blood, exo-erythrocytic forms were found in the liver and spleen.

Attempts to demonstrate sporozoites in smears made from the blood in the membrane failed, but the bulk of the blood was relatively very great. An attempt therefore was made to see if mosquitoes would be attracted to and gorge from a much smaller quantity of blood contained in a smaller membrane. A membrane approximately 1 cm. in diameter was made, by the usual method, over a piece of glass tubing. It was surrounded by a small water jacket at 40–42° C., and 0·2 c.c. of heparinized chick blood was placed in the membrane tube. Nineteen out of 40 *Aëdes aegypti* gorged through the membrane on the blood, and sporozoites were found in films made from the blood. A little of the blood was diluted in citrate saline solution and injected intravenously into a 9-day-old chick. Parasites were found in the peripheral blood on the 7th day, and death occurred on the 9th day, the infection being very heavy.

Sporozoites were obtained from the same infected

mosquitoes on more than one occasion by membrane feeding (Table 14). The mosquitoes were used for the second or third feed only after the blood from the previous meal was digested, and the proportion which gorged was not significantly less than in previous experiments in which mosquitoes had gorged through a membrane for the first time.

X. SUMMARY

1. Membranes prepared from chicken skin provide a suitable medium through which *Aëdes aegypti* females may be induced to gorge.

2. Under suitable conditions the proportion of female *A. aegypti* which will gorge through membranes, though more variable than when a living chick is offered, is great enough for experimental purposes.

3. It is shown that the gorging reaction in *A. aegypti* is provoked by a heat gradient between the environment and the food-limiting membrane.

4. The feeding reactions of *A. aegypti* towards whole blood, fractions of blood, and other substances have been studied. It was found that (*a*) whole blood, and red corpuscles in saline *when ingested through membranes* go directly into the stomach which becomes fully distended; (*b*) haemoglobin in plasma or distilled water is ingested to a lesser degree than whole blood or red corpuscles in saline, and plasma alone is rarely ingested, but all these pass to the stomach; (*c*) sweet solutions containing glucose or honey are seldom imbibed through membranes and pass to the stomach or diverticula, but only the diverticula are fully distended.

When offered as *open drops* (*a*) blood is seldom ingested, but if ingested passes to the stomach; (*b*) haemoglobin in plasma or water, or plasma alone, are very rarely ingested, but pass mainly to the stomach; (*c*) sweet solutions containing honey or glucose, or mixtures of blood and honey are readily ingested and pass mainly to the diverticula which become fully distended, though traces may be found in the stomach.

5. *Aëdes aegypti* may be infected with *Plasmodium gallinaceum* by allowing them to gorge on drawn infected chicken blood through a membrane. Infection rates comparable to those obtained when the mosquitoes are fed directly on living chickens may be obtained by this method.

6. If infected mosquitoes are allowed to gorge upon uninfected blood through membranes they eject viable sporozoites into the blood. When young chicks are injected intravenously with blood so infected, infections are produced which in period of incubation and intensity are comparable with those resulting from the bites of infected mosquitoes.

7. The ejection of sporozoites through membranes in this manner provides a ready means of obtaining sporozoites free from glandular tissue.

8. Sporozoites collected by this method will be suitable for *in vitro* experiments upon the action of drugs on sporozoites, and also as a source of material for studying in tissue cultures the developmental stages of the malaria parasite arising directly from the sporozoite.

We wish to thank Prof. D. Keilin, F.R.S., and Dr P. Tate for helpful criticism and advice during the course of the work.

REFERENCES

Adler, S. & Tchernomoretz, I. (1943). *Ann. Trop. Med. Parasit.* **37**, 148.

Bishop, A. & Gilchrist, B. M. (1944). *Nature, Lond.*, **153**, 713.

Davey, D. G. (1944). *Nature, Lond.*, **153**, 110.

Fülleborn, F. (1908). *Arch. Schiffs- u. Tropenhyg.* **12**, Beih. 8.

Fülleborn, F. (1932). *Arch. Schiffs- u. Tropenhyg.* **36**, 169.

Gordon, R. M. (1922). *Ann. Trop. Med. Parasit.* **16**, 425.

Gordon, R. M. & Lumsden, W. H. R. (1939). *Ann. Trop. Med. Parasit.* **33**, 259.

Huff, C. G., Coulston, F. & Cantrell, W. (1943). *Science*, **97**, 286.

James, S. P. & Tate, P. (1938). *Parasitology*, **30**, 128.

Kadletz, N. A. & Kusmina, L. A. (1929). *Arch. Schiffs- u. Tropenhyg.* **33**, 335.

Kikuth, W. & Giovannola, A. (1933). *Riv. Malariol.* **12**, 657.

Lumsden, W. H. R. & Bertram, D. S. (1940). *Ann. Trop. Med. Parasit.* **34**, 135.

MacGregor, M. E. (1930). *Trans. R. Soc. Trop. Med. Hyg.* **23**, 329.

MacGregor, M. E. (1931). *Trans. R. Soc. Trop. Med. Hyg.* **24**, 465.

Nuttall, G. H. F. & Shipley, A. E. (1903). *J. Hyg., Camb.*, **3**, 166.

Roy, D. N. & Gosh, S. M. (1940). On *J. Malaria Inst. India*, **3**, 253.

Russell, P. F. & Nono, A. M. (1932). *Philipp. J. Sci.* **49**, 595.

Seaton, D. R. & Lumsden, W. H. R. (1941). *Ann. Trop. Med. Parasit.* **35**, 23.

Tate, P. & Vincent, M. (1933. *Parasitology*, **25**, 96.

Tate, P. & Vincent, M. (1934). *Parasitology*, **26**, 523.

Woke, P. A. (1937). *Amer. J. Hyg.* **25**, 372.

Wright, W. R. (1924). *Ann. Trop. Med. Parasit.* **18**, 77.

Yoeli, M. (1938). *Riv. Malariol.* **17**, 62.

Yoeli, M. & Mer, G. G. (1938). *Trans. R. Soc. Trop. Med. Hyg.* **31**, 437.

Yorke, W. & MacFie, J. W. S. (1924). *Trans. R. Soc. Trop. Med. Hyg.* **18**, 13.

(*MS. received for publication* 1. iv. 1945.—Ed.)

[FROM PARASITOLOGY, VOL. 38, No. 3, JULY 1947.]
 PRINTED IN GREAT BRITAIN

THE RESPONSE OF BLOOD-INOCULATED AND SPOROZOITE-INDUCED INFECTIONS OF *PLASMODIUM RELICTUM* TO DRUGS*

BY ANN BISHOP†, BETTY BIRKETT AND BARBARA M. GILCHRIST,
Molteno Institute, University of Cambridge

CONTENTS

I. INTRODUCTION

The difference in response to antimalarial drugs of infections of avian malaria induced by sporozoites and by blood-inoculation is well known. Whereas Roehl (1926) found that plasmoquine in dilutions of 1/1500 to 1/50,000 prolonged the incubation period significantly in blood-inoculated infections of *Plasmodium praecox* (=*P. relictum*), Russell & Nono (1932) found that it had no action, even when given in the maximum tolerated dose, upon infections induced by sporozoites. Tate & Vincent (1933*a*) confirmed this observation, and also found (Tate & Vincent, 1934) that atebrin, though highly effective upon blood-inoculated infections, as shown by Kikuth (1932, 1933), was without action upon sporozoite-induced infections. Not only did this drug, when given in the maximum tolerated dose both before and after infection, fail to prolong the incubation period significantly, but in one bird in which drug treatment was continued until the 10th day after infection, parasites appeared in the erythrocytes during treatment. This observation was confirmed in a later, unpublished experiment, in which they observed a few parasites, from the 8th–14th day after infection, in a canary which had received one 5 mg. dose of atebrin‡ prior to infection with sporozoites of *P. relictum*, and sixteen consecutive daily doses subsequently. The parasites which appeared during atebrin treatment, or immediately after it had ended, were devoid of pigment granules and stained very lightly with Leishman's stain. Such abnormal parasites, when injected into clean birds gave rise to normal infections of pigmented parasites.

Kikuth & Mudrow (1939) observed that in birds infected with liver suspensions containing exo-erythrocytic stages of *P. cathemerium*, parasites appeared in the peripheral blood during treatment with quinine or atebrin. Observations of a similar nature in infections of *P. gallinaceum* in fowls were made by Adler & Tchernomoretz (1941), who found that merozoites arising from exo-erythrocytic schizonts could penetrate into the erythrocytes although the host was receiving daily doses of 150 mg./kg. of quinine. These small trophozoites did not, however, develop further unless the quinine treatment was interrupted, or the dose was reduced to 50 mg./kg.

In infections of *P. relictum* induced by blood-inoculation Tate & Vincent (1934) found that the effective range of doses of atebrin, when given on six consecutive days including the day of inoculation, was 5–0·33 mg. As a similar regimen of 5 mg. doses failed to inhibit the appearance of parasites in the red blood corpuscles of birds infected with sporozoites, it was apparent that in such infections invasion of the red blood corpuscles could take place in the presence of fifteen times the dose suppressive

* This work has been financed by the Medical Research Council.

† Member of the Scientific Staff of the Medical Research Council.

‡ In Tate & Vincent's (1934) experiments, and in those described in the following account, the doses of drug were calculated in mg./20 g. of body weight of bird.

to blood-inoculated infections. A possible explanation was that in sporozoite-induced infections erythrocytic parasites resistant to atebrin were produced during treatment with the drug. When, however, in further unpublished experiments, Tate & Vincent inoculated such parasites intramuscularly into clean canaries and treated them from the day of inoculation, in one case with ten consecutive daily doses each of 5 mg. of atebrin, and in the second case with six similar daily doses, the birds were sterilized of their infections, whereas a normal, though slight, attack developed in an untreated control bird. The appearance of parasites in the erythrocytes during treatment with atebrin could not therefore be ascribed to the development of true drug-resistance since they showed a normal sensitivity to the drug on being transferred to a new host.

The discovery made since Tate & Vincent's experiments, in several species of avian malaria, that sporozoites undergo a cycle of development in the cells of the reticulo-endothelial system prior to the invasion of the erythrocytes, offers an explanation of the difference in reaction to antimalarial drugs of infections induced by sporozoites and by blood-inoculation. The parasites observed by Tate & Vincent during treatment with atebrin, and by Kikuth & Mudrow during treatment with atebrin or quinine, may be presumed to have arisen from exo-erythrocytic parasites unaffected by the drug, as suggested by the latter workers, but their ability to invade erythrocytes in birds receiving fifteen times the dose of atebrin suppressive to blood-inoculated infections seemed to warrant further investigation.

II. METHODS

The following experiments were performed upon the two strains (A and G) of *P. relictum* used by Tate & Vincent (1934). No difference has been observed in the reaction of these strains to atebrin (mepacrine), plasmoquine or quinine. Strain A has the advantage of a high rate of gametocyte production throughout the year, whereas in strain G it falls off during the winter months (Bishop, 1943). The two strains, therefore, have been used indiscriminately, the choice depending upon which produced the greater number of gametocytes at the time required.

The canaries were inoculated with sporozoites either by the bite of infected mosquitoes, or by the intramuscular or intravenous injection of a suspension of sporozoites in saline, prepared from freshly dissected salivary glands. No difference in resulting infection was observed from the use of these three methods, though the incubation period following intravenous injection frequently was at least one day less than when the other methods were used.

The drugs were given to the canaries orally, by catheter tube, in aqueous solution, the dose being calculated in mg./20 g. of body weight of bird. Unless otherwise stated, the doses were given once daily, the first being given immediately after inoculation.

In the early experiments a water-soluble preparation of atebrin (Bayer) was used, but later mepacrine hydrochloride, also water-soluble, was used. The bases of these compounds are identical, but the German product is called atebrin, and the British mepacrine. The mepacrine powder was kindly supplied to us by Imperial Chemical (Pharmaceuticals) Ltd.

The maximum dose of atebrin (or mepacrine) which canaries will tolerate when given orally on six or more successive days is 5 mg. The maximum tolerated dose of quinine hydrochloride, when given under similar conditions is 5 mg. in the majority of birds, but this dose may produce severe though not fatal symptoms of toxicity in some individuals. In our experience 2·5 mg. is the highest dose tolerated by all birds.

With regard to the toxicity of plasmoquine simplex (Bayer), our experience was similar to that of Tate & Vincent (1933*a*, 1933*b*). The highest dose which could be given with safety to all canaries was 0·32 mg.

The drugs were made up freshly daily or on alternate days.

III. THE EFFECT OF PROLONGED TREATMENT WITH ATEBRIN (MEPACRINE) UPON SPOROZOITE-INDUCED INFECTIONS OF *PLASMODIUM RELICTUM*

When daily doses of 5 mg. of atebrin or mepacrine were given to canaries inoculated with sporozoites of *P. relictum*, parasites appeared in the erythrocytes of the peripheral blood during treatment, and after an incubation period not significantly longer than in the untreated control birds (Table 1). The cytoplasm of the parasites, as described by Tate & Vincent (1934), stained very lightly with Giemsa's stain and frequently was vacuolated. The nuclei stained with normal intensity with Giemsa's stain. The parasites were, as noted by Tate & Vincent, devoid of pigment granules. Whereas the majority of the parasites were small and uninucleate, individuals with two, four, six or eight nuclei were not infrequent. Many of these multinucleate parasites were so large that they filled the greater part of the red blood corpuscle, though they never attained to the size of the normal fully grown schizont, and they were less robust in appearance. Parasites actually undergoing schizogony were also found. Besides these parasites some were also present which were shrunken and stained very darkly and were obviously severely affected by the drug.

Infections in birds undergoing prolonged treatment with atebrin or mepacrine varied in intensity, some being so slight that it was necessary to examine several or even many microscopic fields ($\frac{1}{12}$ in. objective × 10 ocular) before a parasite could be found, whereas in others parasites were present in each, or each alternate field, and at the peak of one infection there was an average of seven parasites per field.

Table 1. *The effect of atebrin or mepacrine upon sporozoite-induced infections of* Plasmodium relictum *in canaries*

No. of bird	Dosage*	Period (in days) of infection in red blood corpuscles	Day when killed	Post-mortem examination of internal organs for exo-erythrocytic parasites
		Atebrin		
4238	15 × 5	10–15	—	—
4354	14 × 5	8–12	14	0
4453	17 × (2 × 2·5)	12–17	—	—
5108	9 × 5	8–14	—	—
5109	9 × 5	7–9	9	Liver ++, spleen +, marrow +
		Mepacrine		
5351	9 × 5	7–9	9	Spleen +, brain (+)
5352	14 × 5	8–14	—	—
5362	8 × 5	6–8	8	Liver +++, spleen +++, kidney ++, brain +, lung ++
5420	9 × 5	7–10	—	—
5680	11 × (2 × 2·5)	7–11	—	—
5392	21 × 0·32	7–11	—	—
5436	8 × 0·32	6–7	7	Liver +++, spleen ++, kidney +++, lung +, brain (+)
5451	8 × 0·32	6–8	8	Liver +, spleen ++
		Controls		
4237		8–14	—	—
4353		6–13	—	—
5107		8–14	—	—
5354		8	9	0
5419		5–12	—	—
5452		6–8	8	Liver +, spleen +

* Dosage is calculated in mg./20 g. of body weight of bird. The first dose was given on the day of inoculation. 15 × 5 means that doses of 5 mg. were given on fifteen consecutive days. 17 × (2 × 2·5) means that doses of 2·5 mg. were given twice daily on seventeen consecutive days.

(+) = very slight infection.
\+ = slight infection.
++ = moderate infection.
+++ = heavy infection.

Several of the birds under treatment with atebrin or mepacrine were killed whilst the infections were patent. Exo-erythrocytic parasites in all stages of schizogony were found in impression smears of the internal organs in five out of six of the birds examined (Table 1). They were most prevalent in the liver and spleen, though they were also found in some birds in the kidney, lung, marrow and brain.

It seemed possible that, since the drug was administered once daily, its concentration in the blood might fall to a low level during the 24 hr. between doses, and that merozoites arising from exo-erythrocytic schizonts might enter erythrocytes and undergo some development there before the blood-concentration rose again after further dosing. Little variation, however, in size of parasite, or in the proportion of multinucleate to uninucleate forms was found in films made 6 hr. before dosing, immediately before the drug was given and 2–3 hr. later. When the 5 mg. dose of atebrin or mepacrine was divided into two doses each of 2·5 mg., one being given between 9.30 and 10 a.m. and the other between 5 and 6 p.m., uninucleate and multinucleate forms were found in one of the two birds treated. In the remaining bird only uninucleate and binucleate forms were found.

If the administration of the drug was stopped whilst parasites were present in the peripheral blood

(Table 1, bird 5108) there was no increase in the intensity or duration of the infection as compared with birds treated for longer periods nor of the proportion of multinucleate to uninucleate parasites. The parasites remained devoid of pigment granules during the period corresponding to that of normal, acute infection, but those found later at infrequent intervals, when the birds had passed into a chronic state of infection and no drug had been given for some weeks, were normal in appearance and possessed pigment granules.

Experiments (Table 2) confirmed the efficacy of atebrin and mepacrine upon infections of *P. relictum*

Table 2. *The effect of atebrin or mepacrine upon blood-inoculated infections of* Plasmodium relictum *in canaries*

No. of bird	Dosage		Day of appearance of parasites in peripheral blood
3887	6 × 5	A	Sterilized
3888	6 × 5	A	33
3889	6 × 0·32	A	19
3890	6 × 0·32	A	33
5325	6 × 0·32	M	14
5520	6 × 0·32	M	16
5541	6 × 0·32	M	13
5655	6 × 0·32	M	25
5656	6 × 0·32	M	21
4281	28 × 0·32	A	40
4352	35 × 0·32	A	Died on 56th day, still negative
5359	23 × 0·32	M	35
3891	6 × 0·16	A	5
3892	6 × 0·16	A	5

A = atebrin. M = mepacrine.

induced in canaries by inoculation with infected blood. As Tate & Vincent (1934) report, not only the maximum tolerated dose (5 mg.), when given on six consecutive days beginning with the day of inoculation, prolonged the period of incubation significantly or sterilized the bird of its infection, but doses as small as 0·32 mg. were definitely effective. Doses below 0·32 mg. were without effect. When daily treatment with 0·32 mg. of atebrin or mepacrine (Table 2, birds 4281, 4352 and 5359) was prolonged beyond the normal period of incubation in blood-inoculated birds no parasites appeared in the erythrocytes during treatment, but in two cases a very light infection of normal parasites developed 12 days after it had ended.

When sporozoite-induced infections were treated daily, for a period exceeding that of normal incubation, with the minimum dose effective upon blood-inoculated infections (0·32 mg.), parasites appeared in the erythrocytes during treatment (Table 1, birds 5392, 5436 and 5451). The infections were heavier than the majority of those occurring during treatment with the maximum tolerated dose (5 mg.), the number of parasites per field averaging 3–10, but they were devoid of pigment granules and similar in appearance to those occurring in birds receiving 5 mg. doses. Though the majority were uninucleate, multinucleate parasites and schizonts breaking up into eight merozoites were present. In birds treated with the smaller doses, as in those which received the larger doses, parasites were found which were severely affected by the drug, their cytoplasm being shrunken and deeply stained.

Tate & Vincent's observation that the appearance of parasites in the peripheral blood of canaries infected with sporozoites, during treatment with atebrin, was not due to drug resistance was confirmed. Two normal birds were inoculated intramuscularly with parasites which had appeared in a sporozoite-infected bird during treatment with 5 mg. doses of atebrin. One bird received six daily doses of 0·64 mg. of atebrin and the other 0·32 mg. The former bird was sterilized of its infection and in the latter the infection was retarded until the 18th day after inoculation. Untreated control birds inoculated from the same source produced normal though light infections. Parasites arising from sporozoite-induced infections during treatment with atebrin are therefore as sensitive to the drug on inoculation into a new host as those occurring in normal blood-inoculated infections. Any drug-resistance there may be is of a purely transitory nature which is lost on inoculation into a new host.

It is impossible, after intramuscular inoculation, to study the immediate effect upon the parasites of introduction into a new host, as even in untreated birds they do not appear in the blood in any appreciable number before the 5th or 6th day. After intravenous injection with heavily infected blood parasites can be found in the peripheral blood of the new host immediately. By this method, therefore, it is possible to compare the immediate effect upon parasites of inoculation into a normal untreated bird, with inoculation into a bird, previously uninfected, but which has received the same number of doses of drug as the original host, and in which therefore the blood-concentration of drug is presumably roughly the same.

Blood was taken on the 8th day after infection with sporozoites, from a canary which had received eight daily doses of 0·32 mg. of mepacrine. In smears made at the time when the blood was withdrawn ten or more parasites, devoid of pigment granules and for the most part uninucleate, were present per field. The blood (0·15 c.c.) was diluted with an equal amount of citrate saline solution and injected slowly into the toe vein of a canary which

had previously received eight daily doses each of 0·32 mg. of mepacrine. The drug-treatment of the recipient was continued for a further 10 days. A few vacuolated parasites devoid of pigment granules were found 24 hr. after inoculation, and single similar parasites on the 3rd and 7th days. Thereafter the blood remained free from parasites until the bird's death 10 days after drug treatment had ended.

Table 3. *The effect of quinine* 2HCl *upon sporozoite-induced infections of* Plasmodium relictum *in canaries*

No. of bird	Dosage	Period (in days) of infection in red blood corpuscles	Day when killed	Post-mortem examination of internal organs for exo-erythrocytic parasites
5402	15 × 5	7–14	15	0
5417	8 × 5	7–8	8	Liver +++, spleen, kidney (+), marrow (+)
5489	7 × 5	5–7	7	Liver ++, spleen (+), kidney +
5679	10 × (2 × 2·5) + 2 × 5	7–12	—	—
5394	10 × 2·5	7–10	10	0
5408	8 × 2·5	6–8	8	Liver +(+), spleen +++, kidney +
		Controls		
5401		7–9	9	—
5419		5–12	—	—
5488		6–7	7	Spleen +

In a further experiment equal volumes (0·05 c.c.) of heparinized blood from a canary which had been inoculated intravenously with sporozoites 8 days previously, and which had received daily doses of 0·32 mg. of mepacrine in the intervening period, were injected intravenously into two canaries. The blood contained an average of eight parasites per field, some of which were multinucleate and all devoid of pigment granules. One of the recipients had previously received eight daily doses of 0·32 mg. of mepacrine, so that the blood-concentration of drug was presumably roughly the same as that of the donor bird, whereas the second bird was untreated. The former recipient received fourteen further doses of the drug and during this period single multinucleate parasites, vacuolated and devoid of pigment granules, were found in the peripheral blood on four occasions. After a period of 6 days following the termination of treatment, during which no parasites were found, a slight infection of normal parasites developed. In the untreated recipient bird parasites, normal in appearance and containing pigment granules, were found within 24 hr. of inoculation, and a heavy infection developed. The fully-grown schizonts were normal in size and in number of merozoites.

These experiments therefore show that the parasites arising from sporozoite infections during treatment with mepacrine are capable of normal development immediately after being transferred into a normal host, and that the absence of pigment granules and the small size of the schizonts are due to the presence of the drug. Though parasites may be numerous in the erythrocytes of the peripheral blood of a bird inoculated with sporozoites and undergoing treatment with mepacrine, they are incapable of producing an attack in a fresh host if this is undergoing treatment with the drug. They may, however, give rise to an infection of normal parasites when drug treatment has ceased.

IV. THE EFFECT OF PROLONGED TREATMENT WITH QUININE HYDROCHLORIDE UPON SPOROZOITE-INDUCED INFECTIONS OF *PLASMODIUM RELICTUM*

The effect of quinine hydrochloride upon sporozoite-induced infections of *P. relictum* when dosage is prolonged beyond the normal period of incubation was studied for comparison with atebrin.

In birds receiving daily doses each of 5 mg. (Table 3) parasites were found in the erythrocytes of the peripheral blood during treatment. They appeared on the 5th–7th day and were present for a period approximately equal to that of acute infection in untreated birds. The number of parasites per field varied from two to six, and, though the greater proportion were uninucleate, schizonts and mature gametocytes of both sexes were found. The schizonts were smaller than normal, the average number of merozoites being eight. In birds receiving daily doses of 2·5 mg. comparatively heavy infections occurred in the erythrocytes during treatment, up to thirty parasites being found per field. In some birds receiving this dose the proportion of schizonts and mature gametocytes was high, and the former frequently contained 12–16 merozoites.

The parasites, unlike those occurring during treatment with atebrin, contained pigment granules, and though some were vacuolated and stained less intensely than normal, many, including gametocytes, were normal in appearance.

In order to discover whether the growth of the parasites was due to a fall in the blood-concentration of the drug during the 24 hr. period between doses, a bird (Table 3, bird 5679) was given quinine twice daily beginning with the day of inoculation with sporozoites. It was not possible to give two doses each of 5 mg. daily for several days, as severe toxic symptoms developed, but doses of 2·5 mg. twice daily were well tolerated. Neither mature schizonts nor gametocytes were found during this regimen, uninucleate and young multinucleate forms alone being seen, but when, on the 4th and 5th day of patent infection, the dosage was changed to one single dose of 5 mg. daily, mature schizonts and gametocytes appeared.

In birds infected with sporozoites and undergoing treatment with quinine, exo-erythrocytic schizonts were found in three out of four killed whilst the infections were patent.

In infections of *P. relictum* induced by the inoculation of infected blood six consecutive daily doses of 2·5 mg. of quinine will retard the appearance of parasites in the peripheral blood for a significant period. In twenty-five birds thus treated the average prepatent period was 13 days as compared with 5–7 days in the untreated controls. When 2·5 mg. of quinine was given daily for 20 days, beginning with the day of inoculation, to a bird inoculated intramuscularly with infected blood, no parasites were found during treatment.

It is obvious from these experiments that parasites can appear in the erythrocytes of the peripheral blood of birds infected with sporozoites, during treatment with quinine in doses sufficient to suppress infections induced by blood-inoculation, but that full development only occurs when the drug is given once daily—a regimen which presumably allows a decrease in blood concentration of drug sufficient to enable many parasites to reach maturity during the 24 hr. periods between doses.

Blood (0·2 ml.) containing thirty parasites per field in all stages of development was taken from a sporozoite-infected canary which had received eight daily doses of 2·5 mg. of quinine, and was injected intravenously into a bird which had received similar quinine treatment and which was subjected to eight further daily doses. A few vacuolated parasites were found in the peripheral blood during the first 4 days following inoculation, but thereafter the blood remained free from parasites until 4 days after drug treatment was discontinued. A very low-grade infection then developed which lasted for 6 days, after which no parasites were found. A similar volume of blood from the same source produced a heavy infection within 48 hr., when injected intravenously into an untreated bird. This infection persisted for 17 days.

It can be concluded, therefore, that, although parasites which appear during treatment with quinine in the peripheral blood of birds infected with sporozoites may develop into fully grown schizonts, they are incapable of multiplying when introduced into the blood of a fresh host whilst this also is receiving quinine treatment. In this respect they resemble the parasites arising during treatment with atebrin.

V. THE EFFECT OF PROLONGED TREATMENT WITH PLASMOQUINE UPON SPOROZOITE-INDUCED INFECTIONS OF *PLASMODIUM RELICTUM*

Although Tate & Vincent (1933*a*) found that plasmoquine, even when given in the maximum tolerated dose, had little or no preventive action upon sporozoite-induced infections of *P. relictum*, parasites were not found in the peripheral blood during treatment. They appeared, however, within 3 or 4 days after this had ceased. Kikuth & Mudrow (1939) observed in infections of *P. cathemerium* induced by sporozoites, or by suspensions of organs heavily infected with exo-erythrocytic parasites, that plasmoquine given during the period of normal incubation not only prevented the appearance of parasites in the peripheral blood, but appeared to inhibit to some extent, though never entirely, the development of exo-erythrocytic parasites.

In Tate & Vincent's experiments drug-treatment in no case exceeded 11 days. The effect of prolonged treatment with plasmoquine (Table 4) of canaries

Table 4. *The effect of plasmoquine upon sporozoite-induced infections of* Plasmodium relictum *in canaries*

No. of bird	Dosage	Period (in days) of infection in red blood corpuscles	Day when killed	Post-mortem examination of internal organs for exo-erythrocytic parasites
5355	25 × 0·32	32–41	—	—
5363	21 × 0·32	31–35	—	—
5415	8 × 0·32	—	8	Liver (+)
5491	7 × 0·32	—	7	0
5412	18 × 0·02	7–11	—	—
		Controls		
5354	—	8–9	9	0
5364	—	7–12	—	—
5411	—	7–14	—	—
5414	—	6–8	8	Spleen (+), marrow (+)
5488	—	6–7	7	Spleen +, liver +

heavily infected with sporozoites, was studied in order to discover whether parasites would ultimately appear in the peripheral blood during treatment. When doses of 0·32 mg. (M.T.D.) were given daily for periods of 21 or 25 days to birds infected with sporozoites, no parasites appeared in the peripheral blood during treatment, but a very low grade infection of normal parasites developed a week or 10 days after it had ended. With doses of 0·02 mg. parasites appeared in the peripheral blood from the 7th–11th day. The majority of the parasites were small and uninucleate, but some schizonts also were seen, though these never contained more than eight merozoites. Gametocytes, smaller than normal and with diffuse nuclei, also were found. The parasites contained pigment granules.

Doses of 0·02 mg. of plasmoquine, when given on six successive days beginning with the day of inoculation, were, in our experience, as in that of Roehl (1926) and Tate & Vincent (1933*a*, 1933*b*), effective upon infections induced by blood-inoculation. Even doses of 0·01 mg. delayed the appearance of parasites until the 14th day or longer in four out of five birds. When daily doses of 0·02 mg. of plasmoquine were given to a bird inoculated with infected blood no parasites appeared in the blood during the period of drug treatment (22 days following inoculation) but a few were seen 12 days after treatment had ended.

In sporozoite-induced infections plasmoquine, therefore, when given in the maximum tolerated dose though it does not prevent infection, is able to prevent merozoites arising from exo-erythrocytic schizonts from penetrating into the erythrocytes at least in numbers sufficient to be demonstrated by direct observation, but in doses of 0·02 mg. it is incapable of preventing this, though such doses will inhibit the multiplication of parasites of erythrocytic origin.

Two canaries heavily infected with sporozoites and undergoing treatment with 0·32 mg. of plasmoquine (Table 4, birds 5415 and 5491) were killed at a time when exo-erythrocytic parasites were present in the majority of sporozoite-infected birds treated with atebrin or quinine, and in the controls, but prolonged search revealed only a few exo-erythrocytic forms in the liver of one of the birds. The other organs were all negative on examination. The numbers of exo-erythrocytic schizonts present in sporozoite-infected control birds, or in such birds treated with atebrin or quinine are, however, so variable that observations based on two birds only are not significant.*

* In a personal communication Dr D. G. Davey told A.B. that he has evidence that pamaquin (plasmoquine) has an inhibitive effect upon the development of exo-erythrocytic forms of *Plasmodium relictum* and *P. cathemerium* in canaries, though the drug does not eradicate them completely.

VI. DISCUSSION

Our experiments with atebrin, quinine and plasmoquine upon sporozoite-induced infections of *P. relictum* confirm the observations of Kikuth & Mudrow (1939) upon *P. cathemerium* that plasmoquine is more effective against infections induced by sporozoites than either of the other drugs. No parasites were found in the erythrocytes during treatment with the maximum tolerated dose when this was continued beyond the normal period of incubation. Whether this is due to an inhibitive effect of the drug upon the development and multiplication of exo-erythrocytic parasites arising from the sporozoites, so that the number of merozoites entering red blood corpuscles is so few that they escape detection in the blood films, or whether the concentration of drug in the plasma is lethal to the merozoites, causing them to die before they can effect an entry into the erythrocytes, has not been proved.

Atebrin and quinine in the maximum tolerated dose not only fail to prevent merozoites arising from exo-erythrocytic schizonts from penetrating into the erythrocytes, but allow a small proportion to develop there. That the parasites in the erythrocytes differ greatly in the degree to which they are affected by the drug may be due to a difference in the rate of its absorption by individual corpuscles, with the result that intracorpuscular parasites are exposed to different drug-concentrations, or there may be a difference in drug-susceptibility amongst the parasites themselves.

The growth and rate of division of parasites surviving in the erythrocytes appear to be affected by the drugs, as few develop into schizonts, and those that do contain a smaller number of merozoites than normal. This observation is in agreement with that of Lourie (1934*b*) who found that the growth of *P. cathemerium* was retarded by therapeutic doses of quinine and that the number of merozoites formed in the erythrocytes was 3–6 as compared with 15–16 in untreated infections. The mode of action of atebrin upon *P. relictum* must, however, differ from that of the other two drugs as it prevents the formation of pigment even in mature parasites, whereas they do not. The effect of atebrin upon pigment granules had been noted previously by other workers. Thus James (1934) observed a disappearance of pigment granules in the parasites of patients undergoing treatment with atebrin; Tate & Vincent (1934) noted its absence in *P. relictum*, and Hewitt & Richardson (1943) described a similar effect in *P. lophurae*. According to Hewitt & Richardson, the pigment granules already formed within the parasites are extruded or dissolved on treatment of the host with the drug. But our experiments show that atebrin also definitely prevents pigment

formation in parasites which are growing inside the erythrocytes. The metabolic processes of the parasite must therefore be fundamentally affected by the drug.

Doses of atebrin exert an effect upon *P. relictum* for a longer period than quinine. Thus in sporozoite-induced infections, if atebrin treatment is terminated during the period corresponding to that of the acute attack the appearance of the parasites does not revert to normal nor does the intensity of infection increase. This, presumably, is due to the slow rate of excretion of the drug. The tissues of birds which have received a course of atebrin are stained yellow for some days, or even longer, after treatment has ceased. In the fowl, according to Kelsey and co-workers (1943), quinine is rapidly excreted, practically all the drug disappearing from the tissues within 24 hr. Rapidity of excretion of the drug would explain the difference in results when sporozoite-induced infections are treated with 2·5 mg. of quinine once and twice daily. In the former cases mature schizonts with 12–16 merozoites and fully grown gametocytes are found, whereas in the latter only immature parasites are present. In the former case the blood-concentration of the drug presumably falls sufficiently low between doses to allow the development of the parasites to proceed whereas in the latter case it inhibits it almost completely. A single daily dose of 5 mg. of quinine produces a concentration over the 24 hr. period sufficient to inhibit growth and development to some extent, but not so completely as a 2·5 mg. dose twice daily.

In sporozoite-induced infections merozoites are continually cast into the plasma by the breaking-up of mature exo-erythrocytic schizonts. They enter the erythrocytes where they grow and develop. In birds under treatment with atebrin or quinine, or small doses of plasmoquine, the number of parasites which are able to develop is reduced, and the process is retarded to varying degrees by the action of the drugs. It has been observed by Hewitt & Richardson (1943), and confirmed by our observations, that in the case of all three drugs the young ring stage is the one most rapidly attacked. But though the mortality rate of the young merozoites may be high, their numbers are continually reinforced throughout the period corresponding to that of the acute attack by fresh merozoites arising directly from the exo-erythrocytic schizonts. When the surviving parasites are inoculated into a new host which is also receiving drug the delaying effect upon growth and multiplication, and the lethal action of the drug upon young stages, continue, but the parasite numbers are no longer reinforced by fresh merozoites from exo-erythrocytic schizonts. The infection therefore fails to become patent during treatment, though it may after this has ceased.

When infections induced by the inoculation of infected blood from untreated canaries are treated with therapeutic doses of antimalarial drugs the same mechanism will operate. The number of birds sterilized of such infections when treated with mepacrine or plasmoquine, even in the maximum tolerated dose, is variable; whilst with quinine we have never succeeded in eradicating an infection. The inability of quinine, when given for prolonged periods, to sterilize blood-inoculated infections of *P. cathemerium* had been noted by Lourie (1934*a*). After treatment with therapeutic doses of an effective drug for six consecutive days a delay in the appearance of parasites occurs which is variable even amongst birds treated with the same amount of drug. It may be as great as forty or more days and is followed by a light infection of short duration as first noted by Brünn (1926). In some birds only a few parasites are found on one or two days, but this slight infection renders the bird immune to further infection on reinoculation with parasites of the same strain.

There are three possible explanations of the method by which the infection survives the action of the drug. First the inoculum may, as observed in infections of *P. gallinaceum* in the chick (James, 1939), contain exo-erythrocytic parasites circulating in the leucocytes of the peripheral blood, which would enter the endothelial cells and so resist the action of the drugs. Against this hypothesis it may be stated that we have never seen exo-erythrocytic parasites of *P. relictum* in films made from canaries inoculated with infected blood, though the experience of one of us (A.B.) embraces several thousand of these infections. Exo-erythrocytic forms have been found free in the peripheral blood of canaries infected with sporozoites, but they are extremely rare.

Secondly, erythrocytic parasites may enter the reticulo-endothelial cells soon after they are introduced into the host and before they have been damaged by the drug, and thus constitute a source of fresh erythrocytic parasites when the blood concentration of the drug has fallen sufficiently low to allow of their survival. Sterilization of an infection would occur if the drug eradicated all the erythrocytic parasites before they had time to enter the reticulo-endothelial cells. Coulston & Manwell (1941) have shown that in *P. circumflexum* exo-erythrocytic parasites may be derived from erythrocytic forms, and Corradetti (1938) states that he found exo-erythrocytic forms of *P. relictum* in the internal organs of canaries infected by blood-inoculation and killed as soon as parasites appeared in the peripheral blood. Porter (1942), however, failed to find them in four strains of *P. relictum* maintained in canaries by blood-inoculation. Our own experience shows that in blood-inoculated infections of *P. relictum* exo-erythrocytic parasites are extremely rare. In

spite of prolonged search we have never found them in the internal organs of birds undergoing treatment with drugs, nor in birds with chronic infections. As, however, even in sporozoite-infected birds the period during which exo-erythrocytic parasites can be found is a very limited one, we inoculated a series of birds intravenously with heparinized blood heavily infected with parasites and killed them at times ranging between the 1st day, when the infection was already well established in the blood, and the 11th day when it was passing into the chronic phase. In spite of prolonged examination of many impression smears of the internal organs no large exo-erythrocytic forms similar to those occurring in sporozoite-induced infections were found. A few small uninucleate, non-pigmented parasites were found in the macrophages of the spleen of a bird killed on the 3rd day, also a four-nucleate non-pigmented form, but this was extracellular in position. In a bird killed on the 5th day an unpigmented, uninucleate parasite was found in a macrophage of the spleen. From these experiments it is apparent that exo-erythrocytic parasites are extremely rare in the G strain of *P. relictum* when it is transmitted by blood-inoculation. That exo-erythrocytic parasites if present must be very few is also proved by the fact that erythrocytic parasites are not seen in the peripheral blood if drug-treatment is prolonged beyond the period of normal incubation as in the case of sporozoite-induced infections, and the time required to build up a patent infection after drugging has ceased may be long. This period, however, seems to depend to some extent upon the nature of the drug, as the delay in appearance of parasites caused by therapeutic doses of quinine is usually much smaller than that caused by plasmoquine or mepacrine. Lourie (1934*a*) occasionally observed a single parasite in canaries infected with *P. cathemerium* and undergoing daily intraperitoneal injections of 1 mg. of quinine for periods exceeding 10 weeks. It is possible that the numbers of exo-erythrocytic parasites produced may vary from strain to strain, which would account for the greater facility with which Corradetti appears to have found them as compared with our experience.

The third possible explanation of the persistence of blood-inoculated infections after suppressive treatment with antimalarial drugs is that some of the erythrocytic parasites may be able to withstand much greater concentrations of drugs. If this be so, the resistant parasites must be very few as they are not apparent during drug treatment or for a considerable period after it is ended. Moreover, the maximum tolerated dose of plasmoquine or atebrin will not invariably eradicate an infection which can be effectively suppressed with a 1/32 and 1/16 dilution of the drugs respectively. The variability in parasite resistance would therefore need to be very wide for individuals to survive so much greater a concentration of drug than is lethal to the majority. Such a resistance to the action of the drug, if it exists, is not passed on to future generations of parasites arising directly from the survivors of drug treatment, as infections derived from them are as sensitive to the action of the drug as those produced from an untreated strain.

SUMMARY

1. When sporozoite-induced infections of *Plasmodium relictum* are treated with atebrin (mepacrine) in doses suppressive to blood-inoculated infections, for periods exceeding that of normal incubation, parasites devoid of pigment appear in the erythrocytes of the peripheral blood. A small proportion of these may develop into schizonts, but the number of merozoites produced is fewer than normal.

2. A similar phenomenon occurs when quinine is substituted for atebrin, but the drug appears to be excreted more rapidly so that the inhibitive effect upon growth is less marked.

3. When plasmoquine is given in the maximum tolerated dose to birds infected with sporozoites, parasites are not seen in the peripheral blood during treatment, though the infection is not sterilized. With smaller doses parasites, including a few small schizonts, are found in the erythrocytes during treatment.

4. Merozoites produced in the erythrocytes during drug treatment are not capable of further multiplication when inoculated into uninfected birds having a similar blood-concentration of drug, so long as treatment continues, though they develop normally when transferred to untreated birds.

5. No indication of drug resistance has been observed.

6. The possible mechanisms by which blood-inoculated infections are eradicated, or resist eradication, are discussed.

We wish to thank Miss Vincent (Mrs Thorpe) and Dr Parr Tate for access to their preliminary unpublished experiments, and the latter for helpful criticism.

REFERENCES

ADLER, S. & TCHERNOMORETZ, I. (1941). *Ann. Trop. Med. Parasit.* **35**, 241.

BISHOP, A. (1943). *Parasitology*, **35**, 82.

BRÜNN, W. (1926). *Zbl. Bakt.* **98**, 507.

CORRADETTI, A. (1938). *Riv. Parassit.* **2**, 23.

COULSTON, F. & MANWELL, R. D. (1941). *Amer. J. Hyg.* **34**, 2*c*, 119.

HEWITT, R. I. & RICHARDSON, A. P. (1943). *J. Infect. Dis.* **73**, 1.

JAMES, S. P. (1934). *Trans. R. Soc. Trop. Med. Hyg.* **28**, 3.

JAMES, S. P. (1939). *Trans. R. Soc. Trop. Med. Hyg.* **32**, 763.

KELSEY, F. E., OLDHAM, F. K. & GEILING, E. M. K. (1943). *J. Pharmacol.* **78**, 314.

KIKUTH, W. (1932). *Dtsch. med. Wschr.* **58**, 50.

KIKUTH, W. (1933). *Zbl. Bakt.* **127**, 172.

KIKUTH, W. & MUDROW, L. (1939). *Z. ImmunForsch.* **95**, 285.

LOURIE, E. M. (1934*a*). *Ann. Trop. Med. Parasit.* **28**, 151.

LOURIE, E. M. (1934*b*). *Ann. Trop. Med. Parasit.* **28**, 255.

PORTER, R. J. (1942). *J. Infect. Dis.* **71**, 1.

ROEHL, W. (1926). *Arch. Schiffs- u. Tropenhyg.* Beiheft 3, **30**, 11.

RUSSELL, P. F. & NONO, A. M. (1932). *Phillip. J. Sci.* **49**, 595.

TATE, P. & VINCENT, M. (1933*a*). *Parasitology*, **25**, 96.

TATE, P. & VINCENT, M. (1933*b*). *Parasitology*, **25**, 411.

TATE, P. & VINCENT, M. (1934). *Parasitology*, **26**, 523.

(*MS. received for publication* 18. VII. 1946.—Ed.)

B. M. GILCHRIST AND J. B. S. HALDANE

SEX LINKAGE AND SEX DETERMINATION IN A MOSQUITO, CULEX MOLESTUS

Separat ur Hereditas XXXIII (1947)

Printed in Sweden

SEX LINKAGE AND SEX DETERMINATION IN A MOSQUITO, CULEX MOLESTUS

BY *B. M. GILCHRIST* AND *J. B. S. HALDANE*
LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE, AND UNIVERSITY COLLEGE, LONDON

(*Read to the Mendelian Society on March 26, 1946*)

IN most animals so far studied in which sex is genetically determined there is a chromosomal difference between the sexes. One sex possesses two homologous sets of chromosomes. The other sex has one such complete set, and another set in which one or more members are absent or replaced by chromosomes of a different type. In some groups the male has only a haploid chromosome set. There are however a number of groups in which sex is wholly or mainly determined genetically, but in which no chromosomal differences between the sexes have been seen. These include all vertebrates except mammals and birds. In the *Nematocera*, the primitive suborder of the *Diptera* to which *Culex* belongs, some families have sex chromosomes, while others have not. According to the review of WOLF (1941), sex chromosomes are found in the *Phryneidae*, *Thaumaleidae*, *Scatopsidae*, and *Fungivoridae*, while no difference between the sexes has been found in *Bibionidae*, *Itoninidae*, *Culicidae*, *Melusinidae*, *Tendipedidae*, *Tipulidae*, *Limoniidae*, and *Cylindrotomidae*. In addition sex chromosomes are unknown in the *Chironomidae*. In the *Sciaridae* and *Cecidomyidae* the somatic nuclei of the sexes differ, but their germ lines do not, so that the chromosomal difference is concerned with sex development rather than determination (v. WHITE, 1945).

It has often been thought that where no sexual difference has yet been found, a further study would reveal it. However no structural differences have been discovered in the large chromosomes of the *Urodela*, or in the giant polytene chromosomes of the salivary and other glands of the *Culicidae* and *Chironomidae* (BAUER, 1935, 1936; PHILIP, 1942; SUTTON, 1942). The last author examined the giant chromosomes of the Malpighian tubules of *Culex pipiens*.

Most genetical work has been done on animals with two *X* chromosomes in one sex, and an *X* and a *Y* in the other; and there has been a tendency to regard the results obtained in them, and particularly in

Drosophila species, as of universal validity. In this communication we present facts which are best explained on the hypothesis that in *Culex molestus* sex is determined by a single pair of genes, maleness being dominant over femaleness, and that another gene pair located in the same chromosome shows linkage of the usual type, that is to say partial coupling or repulsion, with them.

Systematic position and origin of stock. — White-eyed individuals appeared in a stock of *Culex* (*Culex*) *molestus* FORSSKÅL, which is very closely allied to the commoner *Culex* (*Culex*) *pipiens* L. It was originally regarded as a biological race of *C. pipiens* in which the adults do not hibernate, the females lay fertile eggs without a blood meal, and pairing can take place in a small space. However MARSHALL and STALEY (1937) give reasons for regarding this autogenous race as a separate species, a nomenclature now generally adopted. Among other differences, *C. molestus* bites human beings far more readily than *C. pipiens*. The subject has been fully reviewed by MARSHALL (1938). It must be remembered that *C. molestus* and *C. pipiens* give fertile hybrids (TATE and VINCENT, 1936).

Our stock of *C. molestus* originated from larvae collected by Mr. P. G. SHUTE, malaria officer, Ministry of Health, from a platform sump at Old Street Underground Station, London. The stock has been maintained under laboratory conditions since March 1944 by Dr. A. BISHOP, Molteno Institute, Cambridge, whom we have to thank for supplying us with egg rafts from this stock.

In a cage containing several hundred mosquitoes 4 white-eyed females and 10 white-eyed males were found, and all other white-eyed individuals were descended from them. The eye pigment is completely lacking, and no differences in other organs or in the shape or size of the eye have been noticed. The white eye can be seen in the first larval instar and in all later stages, however the numbers in Table 1 are counts of imagines.

Methods. — The experimental work was entirely carried out by GILCHRIST in the Department of Entomology of the London School of Hygiene and Tropical Medicine, HALDANE being responsible only for planning and interpretation. The mosquitoes were bred in a constant temperature room at 24° C—25° C, at a relative humidity of 70 % —80 %. In these conditions the life cycle was completed in 18 to 21 days.

Larvae were reared in enamel bowls containing tap water into which food, consisting of powdered dog biscuits and stabilized wheat

embryo (»Bemax») was sprinkled. The water was changed daily and fresh food added. The first pupae appeared 10 to 12 days after the eggs hatched, and all larvae pupated within the next 3 days. Pupae were removed daily, and put into a 3×1 inch specimen tube. At this stage the normal and white-eyed individuals were separated, and the sexes were separated to a large extent. This was possible since males pupated about 24 hours earlier than females, so virgins could be obtained without isolating each individual. Up to 30 pupae could be placed in one tube without interfering with emergence. The tubes were plugged with cotton wool, and a strip of paper inserted into each which was held in place by the plug, and on which the newly hatched imagines could settle.

Paired matings were made in similar specimen tubes not more than 3 days after females had emerged. On the day after mating 1—2 cm. of water was run into the tube, and eggs were generally laid in the next 4 days. The number of eggs in a raft was counted, and it was transferred to a bowl, where the eggs hatched in 36 to 48 hours. The pre-imaginal mortality varied from about 5 % to 15 %, except in two series where it rose over 20 % owing, we have reason to believe, to accidental contamination with »D. D. T.» in dust.

Sex ratio and single factor ratios. — Counts of the flies derived from three rafts laid by the original white-eyed females with unknown mates suggested the form of sex linkage which was afterwards found, and systematic paired matings, with some mass matings, were undertaken. The results of all matings in which both parents were known are given in Table 1. Expectations are given in italics where segregation occurred. *w* symbolizes the recessive gene for white eye, and + its normal allelomorph; *M* symbolizes the dominant gene for maleness, and *m* its allelomorph. 36 of the 132 rafts were derived from matings of several females with one or more males. The remainder were from paired matings. The mean number of eggs per raft was 86,3. The means for normal and white-eyed mothers are 86,38 and 86,27 respectively, a very close equality.

The first two lines refer to stocks in which brother—sister mating was carried on for three generations in each case. The next two lines show the results of reciprocal crosses between them. It will be noted that the pre-imaginal mortality does not differ significantly in the three genotypes $\frac{+}{+}$, $\frac{+}{w}$, and $\frac{w}{w}$. The remainder of the flies recorded in this

TABLE 1.

Mother	Father	Rafts	Eggs	Imagines	Normal ♀	White ♀	Normal ♂	White ♂	Remarks
$\frac{m+}{m+}$	$\frac{m+}{M+}$	9	702	636	310	0	326	0	
$\frac{mw}{mw}$	$\frac{mw}{Mw}$	9	728	646	0	308	0	338	
$\frac{m+}{m+}$	$\frac{mw}{Mw}$	2	137	124	61	0	63	0	Normal parent from inbred line.
$\frac{mw}{mw}$	$\frac{m+}{M+}$	2	138	118	53	0	65	0	
$\frac{mw}{mw}$	$\frac{m+}{M+}$	1	73	64	36	0	28	0	Parents from F_2 with white grandmother.
$\frac{mw}{m+}$	$\frac{mw}{Mw}$	4	297	262	69 (72)	75 (72)	64 (59)	54 (59)	Mother's mother white.
$\frac{mw}{m+}$	$\frac{mw}{Mw}$	2	147	145	46 (40)	34 (40)	33 (32,5)	32 (32,5)	Parents from F_2 with white grandmother.
$\frac{m+}{mw}$	$\frac{mw}{Mw}$	13	1222	901[1]	233 (226)	219 (226)	227 (223,5)	220 (223,5)	Mother's father withe.
$\frac{mw}{mw}$	$\frac{mw}{M+}$	24	2104	1623	41 (47,1)	711 (704,9)	824 (816,5)	47 (54,5)	Father's mother white.
$\frac{mw}{mw}$	$\frac{mw}{M+}$	7	548	491	14 (17,0)	257 (254,0)	209 (206,2)	11 (13,8)	Parents from F_2 with white grandmother.
$\frac{mw}{mw}$	$\frac{m+}{Mw}$	10	981	854	409 (411,5)	30 (27,5)	39 (26,0)	376 (389,0)	Father's father white.
?$\frac{m+}{mw}$	$\frac{m+}{Mw}$	1	86	79	36 (36,6)	3 (2,4)	0 (2,5)	40 (37,5)	Parents both said to be normal with white father.
$\frac{mw}{m+}$	$\frac{mw}{M+}$	39	3511	3331	851 (902,1)	847 (795,9)	1584 (1581,9)	49 (51,1)	Parents' mother white.
$\frac{m+}{mw}$	$\frac{m+}{Mw}$	9	722	631	267 (272,8)	24 (18,2)	176 (180,6)	164 (159,4)	Parents' father white.
		132	11396	9905	4934		4969		

table were derived from earlier matings in which the normal parent was often heterozygous.

The sex ratio is very close to unity. The grand total of Table 1 is $50{,}18 \pm 0{,}50$ % of males. For the first 50 families on the record $\chi^2 = 51{,}02$, expectations being calculated on a basis of equality. This is very close to the value of 50 expected if deviations from equality

[1] Including two gynandromorphs.

were wholly due to sampling. Where the ratio is aberrant, a sex-linked lethal might at first sight be postulated. Thus one family consisted of 41 ♀, 22 ♂, and it might be thought that a lethal gene had killed off about 19 potential males. But as 63 out of 67 eggs gave imagines, this is impossible.

Before discussing the segregation of white eye we must refer to the family whose maternal origin is prefixed by a query. This family segregated as if from $\frac{mw}{mw}$♀ × $\frac{mw}{M+}$♂. It is of course possible that a mistake was made as to the mother's eye colour. It is also possible that she was a mosaic, her eyes being dark, but her ovaries homozygous for *w*. All the other 109 segregating families segregated in approximately the expected ratios. Here the absence of normal males and the large number of white-eyed males are equally unexpected. This family will be referred to as the exceptional family.

The gene *w* for white eye is fully penetrant, and fully recessive on crossing. Since the sex ratio is unity we can legitimately add the data for the two sexes even when there was sex-linkage. The grand total for all back-crosses of known or presumed heterozygotes, including the exceptional family, is 2244 +, 2109 *w*, or $48{,}45 \pm 0{,}76$ % white-eyed. The ratio is much the same when the total is subdivided into groups such as the progeny of white-eyed females and normal sons of white mothers.

The offspring from all crosses of known heterozygotes *inter se* is 2878 +, 1084 *w*, or $27{,}36 \pm 0{,}71$ % white-eyed, a significant excess above 25 %. If the exceptional family is included, this becomes 27,91 %.

The single factor ratios are very steady from one family to another. Reckoning expectations of white-eyed mosquitoes as $^1/_2$ and $^1/_4$, the 19 single raft cultures from heterozygous mothers gave $\chi^2 = 11{,}23$, the 26 from heterozygous fathers gave $\chi^2 = 20{,}91$; and the 27 with both parents heterozygous gave $\chi^2 = 19{,}63$. This would be increased to 56,12 with $n = 28$, were the exceptional family included. With this exception, the variation between families is below that expected on a basis of sampling alone, though not significantly so.

Linkage. — Table 1 shows clearly that there is only one kind of heterozygous female, but two kinds of heterozygous male. The data agree with the hypothesis that females are *mm*, males *Mm*, and that *M* and *w* are closely linked, with a small recombination frequency *x*. Thus the two types of heterozygous male may be symbolized as $\frac{mw}{M+}$

TABLE 2.

Mother	Father	Expected types of segregation			
		$+\,♀$	$w\,♀$	$+\,♂$	$w\,♂$
$\frac{m+}{mw}$	$\frac{mw}{Mw}$	1	1	1	1
$\frac{mw}{mw}$	$\frac{mw}{M+}$	x	$1-x$	$1-x$	x
$\frac{mw}{mw}$	$\frac{m+}{Mw}$	$1-x$	x	x	$1-x$
$\frac{m+}{mw}$	$\frac{mw}{M+}$	$1+x$	$1-x$	$2-x$	x
$\frac{m+}{mw}$	$\frac{m+}{Mw}$	$2-x$	x	$1+x$	$1-x$

and $\frac{m+}{Mw}$, according as they derived the gene w from the mother or father. The expectations from the different types of segregating mating are given in Table 2.

The recombination frequency x is most simply calculated from the number of cross-overs among the progeny of heterozygous males of known parentage, mated to white-eyed females. These number 113 out of 2001, giving $x = 0{,}056$. However a somewhat better estimate can be obtained from all the families showing linkage. If we assume that the mother of the exceptional family bred as $\frac{mw}{mw}$, we have $1-x : x :: 2862 : 185$; and $1-x : 1+x : 2-x : x :: 1027 : 1011 : 1851 : 73$. Applying the method of maximum likelihood, the logarithm of the likelihood is: —

$$L = 258 \log x + 1027 \log (1+x) + 3873 \log (1-x) + \\ + 1851 \log (2-x) + C.$$

Differentiating and putting $\frac{dL}{dx} = 0$, we have $7009\,x^3 - 7470\,x^2 - \\ - 7801\,x + 516 = 0$. So $x = 0{,}0626$. If the exceptional family is assigned to its putative mother we have $7009\,x^3 - 7434\,x^2 - 7760\,x + \\ + 516 = 0$, whence $x = 0{,}0629$. The expectations of Table 1 are calculated from the former value.

Were the uncertainty in the value of x wholly due to sampling we should have for its standard error,

$$\sigma^{-2} = \frac{-d^2L}{dx^2} = \frac{258}{x^2} + \frac{1027}{(1+x)^2} + \frac{3873}{(1-x)^2} + \frac{1851}{(2-x)^2} = 71648$$

whence $\sigma = 0{,}0037$. However most of the uncertainty is not due to sampling, as appears when we calculate χ^2. Taking $x = 0{,}0626$, we have $\chi^2 = 66{,}70$ for the 33 cultures in which a ratio of $1-x : x$ is expected, and $\chi^2 = 152{,}11$ for the 33 half-cultures of one sex in which a ratio of $2-x : x$ is expected. The sampling distribution of χ^2 deviates from its classical form when expectations are small, but the probability that the above values should be due to sampling is extremely small. Most of the information about x is derived from back-cross families, and as χ^2 has twice its expected value, the amount of information is halved. The remaining information is even more reduced, so the standard error is about $0{,}006$, or the recombination is $6{,}26 \pm 0{,}6$ %.

The cause of the divergences between different cultures is unknown. There were a few cultures with very high recombination. Thus two heterozygous brothers with white-eyed mates gave 16 cross-overs out of 99, and 12 out of 103. However brothers can differ greatly. Thus two brothers gave 11 out of 107 and 0 out of 96. The probability of obtaining so great a divergence by chance is $\frac{192!\,107!}{203!\,96!} = 6{,}94 \times 10^{-}$ Other pairs of brothers show almost as large differences. The most obvious hypothesis is that there are inversions in the neighbourhood of the genes concerned which are occasionally lost by crossing over. This is however hard to reconcile with the data, and the question can only be decided by further genetical and cytological work.

Gynandromorphs. — Three gynandromorphs were found, *A* in a mass culture not included in Table 1, and *B* and *C* in separate cultures from $\frac{mw}{m+}\,♀ \times \frac{mw}{Mw}\,♂$. A search of the literature revealed two other records (MARSHALL, 1938; WEYER, 1938) of gynandromorphism in this species, and 12 in all other mosquito species both in Britain and abroad. Of these, 7 were in the genus *Aëdes* (EDWARDS, 1917; BRELJE, 1923; SHUTE, 1926; MARSHALL, 1938; SMYLY, 1942), 4 in the genus *Culex* (BEDFORD, 1914; MARSHALL, 1938; MIDDLEKAUFF, 1945), and 1 in *Theobaldia* (CLASSEY, 1942).

Our three gynandromorphs are described in Table 3. The mouth parts have not been included, as it has been found impossible to identify the stylets or their relative positions from the mounted heads. Fig. 1 shows the head of specimen *B*. Usually one appendage of a pair is

TABLE 3.

Organ	Left side	Right side
Gynandromorph A.		
Antenna	♂ normal	Torus as in normal ♀, flagellum hairier than normal ♀, less so than normal ♂.
Palp	♂ normal	Length intermediate between normal ♂ and ♀, 3rd, 4th, and 5th joints distorted, thickened, hairy.
Eye	Normal	Normal, slightly larger than left.
Fore leg, midleg	♂ normal	♀ normal
External genitalia	♂ normal	♀ normal
Gonads	Normal ovary	Normal ovary
Spermathecae	Two (normal ♀ has three)	
Gynandromorph B.		
Antenna	♀, hairier than normal.	♂ normal
Palp	♀ normal	♂ normal
Eye	Pigmented save for 15 white facets on median posterior margin.	White, no pigment.
Fore leg	♂ normal	♂ normal
Midleg	4th tarsal segment longer than 5th, claws toothed (as in ♂).	4th tarsal segment as long as 5th, claws toothed.
External genitalia	♀ normal	♂ normal
Gonads	Normal testis	Normal testis.
Spermathecae	Two	
Gynandromorph C.		
Antenna	♂ normal	♀ normal
Palp	Length between ♂ and ♀, distorted, thick, hairy.	♂ normal
Eye	Pigmented	Mainly pigmented, but groups of white facets give mottled appearence.
Wing	Smaller	Larger
Fore leg, midleg	♂ normal	♀ normal
External genitalia	♂ normal	♀ normal
Gonads	One ovary, one testis, but not certain on which side situated.	
Spermathecae	Two	

female, the other male, but it is not uncommon for one to be male and the other intermediate, as if a hormone diffused from the male regions. In *C* it will be noted that the right palp was male, the male appendage being on the left in other segments. The sex of the gonads has little relation to that of external organs.

Gynandromorphs *B* and *C* were members of otherwise normal cultures from $\frac{m+}{mw}\text{♀} \times \frac{mw}{Mw}\text{♂}$. Their interpretation is difficult because we do not know the sex of the eyes. Gynandromorphs in *Drosophila* generally arise by the loss of one *X* chromosome from some cells of

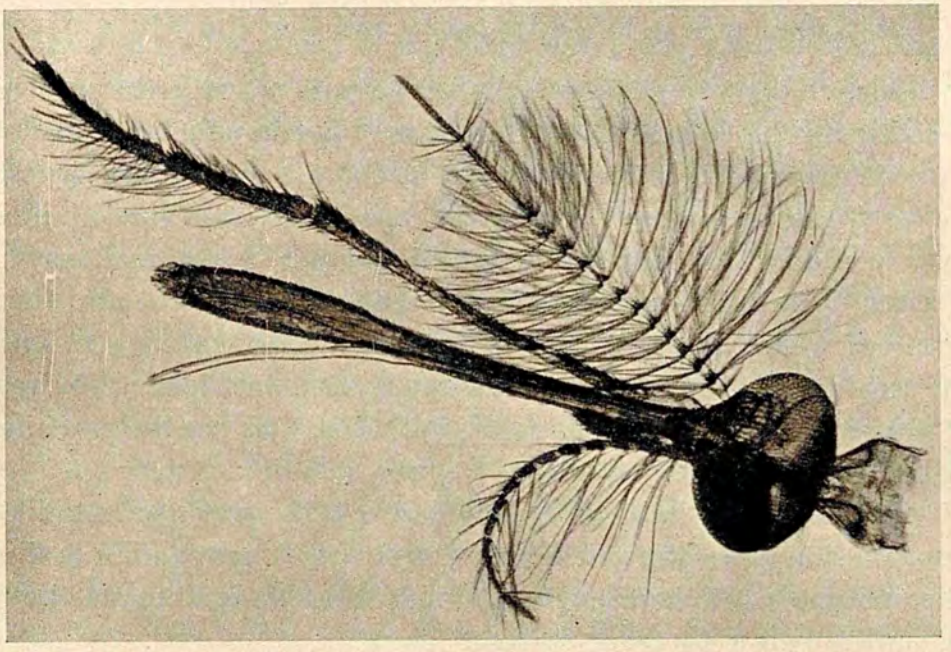


Fig. 1. Head of gynandromorph *B*. Explanation in Table 3.

what would otherwise be a female (*XX*). In a mosquito it seems quite likely that the loss of one of the six chromosomes would be fatal, and most unlikely that it would give normal female or male organs. We can at once rule out the possibility of dispermy alone. This would give a mosaic for sex but not for eye colour. On the other hand if a polar body were fertilized by a spermatozoön, and the female pronucleus by another, each providing nuclei for about half the body, the facts are explicable if one part is $\frac{m+}{mw}$ and the other $\frac{mw}{Mw}$, or if one part is $\frac{mw}{mw}$ and the other $\frac{mw}{M+}$. Such double fertilization seems to occur in Lepidoptera (GOLDSCHMIDT and KATSUKI, 1931).

If the gynandromorphs arose from a single diploid nucleus this

must have been $\frac{m+}{Mw}$. Elimination of a chromosome will not account for them unless we make the unlikely assumption that tissues with the gene *M* alone are female. But they can be explained on either of two hypotheses. Somatic crossing over may have occurred, as it does in *Drosophila*, giving rise to $\frac{m+}{m+}$ and $\frac{Mw}{Mw}$ regions. Or the two daughters of one chromosome may have gone to one pole at an early mitosis, those of its homologue going to the other. It is clear that such an event would be facilitated by somatic pairing. Either hypothesis implies that $\frac{M}{M}$ is viable, as it is in *Ambystoma*, and that the white eye regions were male. This would have been in agreement with the appendages in *B* and with the palp in *C*. When genes appear which affect sexually dimorphic organs it will be possible to decide between these hypotheses.

The influence of eye colour on behaviour. — Normal imagines show a well-marked reaction when an opaque object such as a hand or a piece of paper is passed between the source of light and the cage. Mosquitoes which have settled on the netting of the cage at once become very active, making a loud buzzing when in flight. The reaction is of short duration but striking in intensity. In contrast, white-eyed imagines show no such activity.

When a bright light was switched off leaving a dimly lit room the normal imagines showed no such activity. The reaction is thus one to the moving contour between a bright and dark area of the visual field, and not to a decrease in light intensity.

However preliminary experiments by Mr. T. R. RAO in the Department of Entomology of the London School of Hygiene and Tropical Medicine have shown a response of both normal and white-eyed *C. molestus* to light intensity. Two mosquito cages, one covered with black paper, were placed about 12 inches apart in a room in daylight, and connected by a cardboard tunnel. About 100 mosquitoes, male and female, were put into the darkened cage, and the number of mosquitoes which had entered the lighted cage was counted at intervals. During the daytime both types showed little activity, and remained in the darkened cage. At dusk most of them came out into the uncovered cage. The difference in behaviour between the two types is exactly similar to that found by KALMUS (1943) between normal and white-eyed members of *Drosophila* species. Both types show phototaxis, but the white-eyed forms do not respond to moving visual contours, as their

ommatidia are not isolated from one another by pigment, and visual acuity is thus reduced to nil. However the light receptors are quite functional when it is irrelevant whether or not they are isolated.

In the larval and pupal stages eye pigment is wholly absent in $\frac{w}{w}$ individuals; nevertheless no difference was found between their behaviour and that of normal individuals. Both showed a well marked shadow response when an opaque object was passed over the water; individuals suspended from the surface film immediately swam downwards. This reaction was also observed when, in a dimly lit room, a bright light placed near a bowl of larvae or pupae was switched off. It was therefore a response to light intensity, not to a moving contour.

Discussion. — The main result obtained is that in this species a pair of genes for which both sexes are diploid yet show a partial or incomplete linkage with sex. This phenomenon is not very rare. It is found in *Drosophila* species for the gene *bobbed*, which however at most crosses over very rarely with the sex genes (PHILIP, 1935). It occurs in the beetle *Phytodecta variabilis* (DE ZULUETA, 1925). Here the same sets of genes for natural polymorphism are found both in the *X* and *Y* chromosomes, and crossing over is very rare, if it occurs at all. In this species GALAN (1931) found an unequal pair of chromosomes in the male. So here, as in *Drosophila*, sex is determined by a chromosomal section, but the section of the *X* and *Y* chromosomes which is homologous contains homologous genes.

AIDA (1921) discovered partial sex-linkage in the Cyprinodont teleost *Aplocheilus latipes*, and it has since been found in other Cyprinodonts, notably *Lebistes reticulatus* where WINGE has studied it extensively. He found no unequal chromosomes in the male, nor did any of the numerous sex-linked genes which he found behave like those of *Drosophila* or mammals, where the *Y* chromosome does not carry them. On the contrary WINGE and DITLEVSEN (1938) were able to obtain viable »*YY*», or *MM* individuals provided that the *Y* chromosomes came from different lines. This suggests that the gene for maleness is often coupled with a recessive lethal, but not always with the same one. The fact that either sex may be heterogametic within a group of crossable species, or even within one species (WINGE, 1934), suggests that sex is here determined by genes, and not by chromosome segments.

The only species in which a number of genes showing both types of sex-linkage have been described is man (HALDANE, 1936, 1941).

Here the genes in that part of the *X* which has no homologue in the *Y* show the classical type of sex-linkage, while those which can cross over between the *X* and the homologous segment of the *Y* show partial sex-linkage, detectable in the progeny of heterozygous males as in *Culex*.

The strongest evidence for the determination of sex by a single gene is provided by the work of HUMPHREYS (1945) on the axolotl *Ambystoma mexicanum*. Here the male is normally *ff*, the female *Ff*. By grafting testes which were later removed, HUMPHREYS transformed *Ff* individuals into males. The mating *Ff* ♀ × *Ff* ♂ gave 3 ♀ : 1 ♂, and some of the females gave all female progeny with normal males, and were therefore *FF*. In this species femaleness therefore seems to be due to a single completely dominant gene. It is suggested that where sex is genetically determined in *Pisces*, *Amphibia*, many *Nematocera*, and probably *Reptilia*, it is usually determined by a single gene, even if we accept SVÄRDSON's (1945) presumptive evidence for a chromosome fragment determining sex in *Coregonus lavaretus*, which is not conclusive.

The fact that a worker so experienced as SUTTON in detecting small differences between homologous polytene chromosomes failed to find them in *Culex* suggests that if they exist they are at most of the slight nature associated with some single gene differences in *Drosophila*. An inversion which would allow several genes to act as a unit, or a deficiency or duplication of more than perhaps a single band would probably have been detected. The same applies to *Chironomus* and other Nematoceran species which have been extensively studied. A definite proof of the existence and viability of *MM* cells would strongly support the hypothesis of a single gene. In any case the sex-determining mechanism has as much right to be described as a gene as have such genes as *Bar*, *Delta*, or *Moiré* in *Drosophila melanogaster*.

On the assumption that sex is determined by a gene, or a small chromosomal abnormality which is not lethal when homozygous, and on the further hypothesis that somatic crossing over or abnormal mitosis occurs, we can perhaps explain the occurrence of spanogyny, or shortage of females, which ROUBAUD (1933) and TATE and VINCENT (1936) have observed in *Culex pipiens*. As a result of somatic crossing over mosquitoes or testes of genotype *MM* may arise, and their progeny will be entirely male. Spanandry could also be explained if other genes could determine sex, as they do in *Lebistes*. Lethal genes closely coupled with *m* could produce a shortage of females, but probably not families of 100 ♂ and 1 ♀ as reported by TATE and VINCENT.

Crossing over has so far only been detected in the spermatogenesis of *Culex*. It does not occur, except very rarely at mitosis, in *Drosophila* males. A comparison of its frequency in the sexes of *Culex* must await the discovery of two linked genes.

Although sex determination by a single gene is simpler than by a chromosome, and appears to be primitive in vertebrates, it cannot be assumed to be a primitive character in the *Nematocera*, if only because chromosomal sex determination is found in insect orders more primitive than the *Diptera*. Although the *Nematocera* are more primitive than the *Brachycera*, it is entirely possible that their ancestors had a sex determining mechanism like that of *Drosophila*. For STURTEVANT (1945) describes an autosomal recessive gene, *transformer*, in *Drosophila melanogaster* which has no effect on males, but transforms *XX* or even *XXY* zygotes which would otherwise be females, into males of normal morphology, apart from small size of the testes, and normal behaviour, but sterile. If a similar gene, or the same one with suitable modifiers, produced fertile males, it would be possible to secure a race in which the chromosomes of the sexes would be alike, and femaleness would be due to a single dominant gene.

The gene for white eyes in *Culex* is probably homologous with that in *Drosophila*, where there is only one locus giving this effect. If other sex-linked genes in *Drosophila* have sex-linked homologues in *Culex* it may be possible to homologize the whole or parts of the sex-determining chromosome of *Culex* and the *X* of *Drosophila*. If so this will be an argument for the primitive character of the condition in *Culex*, which could obviously evolve into the *Drosophila* mechanism by a simple process. So long as crossing over occurs between the chromosomes carrying *M* and *m*, or so long as *MM* testes occur with appreciable frequency, the chromosomal regions round the *M* locus are not shielded from natural selection. If, as the result of inversions, suppression of crossing over in the male, and suppression of somatic crossing over, these regions are shielded, recessive lethals, and ultimately deficiencies, will accumulate in them, and the *M* chromosome will degenerate into a nearly functionless *Y*. It is a striking fact that this has not happened in many large and successful groups.

The high variability of crossing over is the more striking since DE WINTON and HALDANE (1935) in *Primula sinensis* and SPURWAY (1945) in *Drosophila subobscura* found that crossing over was hardly more variable than was to be expected as a result of sampling, and decidedly less so than single factor segregation.

Our results emphasize the need for a widespread comparative study of sex-linkage. It is most unlikely that *Homo sapiens* is the only species in which both types of sex-linkage are found for a number of genes; and the partial type found in *Culex* may be far commoner than has been supposed.

We have to thank Professor P. A. BUXTON for allowing this work to proceed in his department, and for his interest in it; Dr. M. J. D. WHITE for photographing the head of a gynandromorph; and Dr. H. SPURWAY for suggesting the hypothesis here put forward as to the origin of the gynandromorphs.

Summary. — White eye in *Culex molestus* is a recessive character giving normal Mendelian ratios. It is partially linked with sex, showing $6{,}3 \pm 0{,}6$ % recombination. There are no sex chromosomes, but maleness appears to be due to a single dominant gene in the same chromosome as that for white eye. Crossing over is unusually variable. Three gynandromorphs, two being mosaic for eye colour, are described. These latter cannot be due to chromosomal elimination, and may be due to somatic crossing over.

LITERATURE CITED.

1. AIDA, T. 1921. On the inheritance of color in a fresh-water fish *Aplocheilus latipes*, TEMMICK and SCHLEGEL, with special reference to sex-linked inheritance. — Genetics 6, 554.
2. BAUER, H. 1935. Der Aufbau der Chromosomen aus den Speicheldrüsen von *Chironomus thummi* KIEFER. — Z. Zellforsch. 23, 280.
3. — 1936. Beiträge zur vergleichenden Morphologie der Speicheldrüsenchromosomen. — Zool. Jb. (Phys.) 56, 239.
4. BEDFORD, G. A. H. 1914. A curious mosquito. — Trans. Roy. Soc. S. Afr. 4, 143.
5. BRELJE, R. v. D. 1923. Ein Fall von Zwitterbildung bei *Aëdes mergenanus*. (*Diptera*: *Culicidae*.) — Arch. mikr. Anat. 100, 317.
6. CLASSEY, E. W. 1942. Gynandromorphism in *Theobaldia annulata* SCHRANK. (*Diptera*: *Culicidae*.) — Entomologist 75, 181.
7. EDWARDS, F. W. 1917. Notes on *Culicidae* with descriptions of new species. — Bull. ent. Res. 7, 201.
8. GALAN, F. 1931. Estudios sobre la espermatogénesis del coleóptero, *Phytodecta variabilis*. — Eos. 7, 461.
9. GOLDSCHMIDT, R. and KATSUKI, K. 1931. Vierte Mitteilung über erblichen Gynandromorphismus und somatische Mosaikbildung bei *Bombyx mori* L. — Biol. Zentbl. 51, 58.
10. HALDANE, J. B. S. 1936. A search for incomplete sex-linkage in man. — Annals of Eugenics 7. I.

11. HALDANE, J. B. S. 1941. The partial sex-linkage of recessive spastic paraplegia. — Journ. Genet. XLI, 2 and 3.
12. HUMPHREYS, R. R. 1945. Sex determination in Ambystomid salamanders. A study of the progeny of females experimentally converted into males. — Am. Journ. Anat. 76, 33.
13. KALMUS, H. 1943. The optomotor responses of some eye mutants of *Drosophila*. — Journ. Genet. 45, 206.
14. MARSHALL, J. F. 1938. The British mosquitoes. — London.
15. MARSHALL, J. F. and STALEY, J. 1937. Some notes regarding the morphological and biological differentiation of *Culex pipiens* LINNAEUS and *Culex molestus* FORSSKÅL. (*Diptera*: *Culicidae*.) — Proc. Roy. Ent. Soc. Lond. (A). 12, 17.
16. MIDDLEKAUFF, W. W. 1945. Gynandromorphism in recently collected mosquitoes. — J. Econ. Ent. 37, 297.
17. PHILIP, U. 1935. Crossing over between the *X* and *Y* chromosomes in *Drosophila melanogaster*. — Journ. Genet. 31, 341.
18. — 1942. An analysis of chromosomal polymorphism in two species of *Chironomus*. — Journ. Genet. 44, 129.
19. ROUBAUD, E. 1933. Assai synthétique sur la vie du moustique commun (*Culex pipiens*). L'évolution humaine et les adaptations biologiques du moustique. — Ann. Sci. nat. Zool. 16, 5.
20. SHUTE, P. G. 1926. Intersexual form of *Ochlerotatus punctor* KIRBY var. *mergenanus*. — Entomologist 59, 12.
21. SMYLY, W. J. P. 1942. A gynandromorph of *Aëdes aegypti* L. (*Stegomyia fasciata*). — Proc. R. Ent. Soc. Lond. (A). 17, 111.
22. SPURWAY, H. 1945. The genetics and cytology of *Drosophila subobscura*. 1. Element A. Sex-linked mutants and their standard order. — Journ. Genet. 46, 268.
23. STURTEVANT, A. H. 1945. A gene in *Drosophila melanogaster* that transforms females into males. — Genetics 30, 297.
24. SUTTON, E. 1942. Salivary gland type chromosomes in mosquitos. — Proc. Nat. Acad. Sci. 28, 268.
25. SVÄRDSON, G. 1945. Chromosome studies in *Salmonidae*. — Reports from the Swedish State Institute of Fresh-Water Fishery Research, 23. Stockholm.
26. TATE, P. and VINCENT, M. 1936. The biology of autogenous and anautogenous races of *Culex pipiens* L. (*Diptera*: *Culicidae*.) — Parasitology 28, 115.
27. WEYER, F. 1938. Ein Zwitter von *Culex pipiens*. — Zool. Anz. 123, 184.
28. WHITE, M. J. D. 1945. Animal cytology and evolution. — Cambridge.
29. WINGE, Ö. 1934. The experimental alteration of sex chromosomes into autosomes and vice versa, as illustrated by *Lebistes*. — C. R. Lab. Carlsberg 21, 1.
30. WINGE, Ö. and DITLEVSEN, E. 1938. A lethal gene in the *Y* chromosome of *Lebistes*. — C. R. Lab. Carlsberg 22, 203.
31. DE WINTON, D. and HALDANE, J. B. S. 1935. The genetics of *Primula sinensis*. III. Linkage in the diploid. — Journ. Genet. 31, 67.
32. WOLF, E. 1941. Die Chromosomen in der Spermatogenese einiger Nematoceren. — Chromosoma 2, 192.
33. DE ZULUETA, A. 1925. La herencia ligada al sexo en el coleoptero *Phytodecta variabilis*. — Eos. 1.

CONTENTS.

Hereditas

☐ Hereditas,

a periodical devoted to the publication of original research in heredity, is published by the MENDELIAN SOCIETY *at Lund, Sweden. All contributions are written in English, German or French, illustrated, when necessary, with adequate text figures and plates. Each volume contains about 500 pages, issued in four numbers.*

☐ Subscriptions

should be sent to the Editor. Price ***Twenty-five (25) Swedish Kronor per volume.*** *Post free. (The price of Vols. I—X is 10 Swedish Kronor per volume, of Vols. XI—XXIII, 15 Sw. Kr., and of Vols. XXIV—XXV, 20 Sw. Kr.)*

Robert Larsson, Editor

3, Adelgatan Lund, Sweden

LUND 1946. BERLINGSKA BOKTR.

Functions of haemoglobin in *Daphnia*

By H. Munro Fox, F.R.S., Barbara M. Gilchrist and Elizabeth A. Phear

Reprinted without change of pagination from the
Proceedings of the Royal Society B, *volume* 138, 1951

Reprinted without change of pagination from the
Proceedings of the Royal Society, B, *volume* 138, 1951

Functions of haemoglobin in *Daphnia*

By H. Munro Fox, F.R.S., Barbara M. Gilchrist and Elizabeth A. Phear
Zoology Department, Bedford College, University of London

(*Received* 7 *April* 1951)

In *Daphnia* a manifold increase in the haemoglobin content of the blood is known to result from oxygen deficiency in the water; pale animals become red. The functions of this additional haemoglobin have now been studied experimentally. In poorly aerated water *Daphnia* benefits from it in the following ways:

(1) The additional haemoglobin increases length of life; red individuals survive longer than pale ones.

(2) The additional haemoglobin enables more food to be gathered. Red *Daphnia* clears a suspension of graphite or of *Chlorella* more quickly than pale *Daphnia*, and normal red animals clear suspensions quicker than others whose haemoglobin has been inactivated as an oxygen carrier by carbon monoxide. The rate of clearance is proportional to the concentration of blood pigment; even a small increase in haemoglobin has a beneficial effect. The more rapid gathering of food is accomplished by a quicker movement of the thoracic limbs.

(3) The additional haemoglobin causes *Daphnia* to swim more energetically.

(4) The additional haemoglobin results in an increased egg production; parthenogenetic females lay fewer eggs in the brood pouch if treated with carbon monoxide.

(5) Previous work had shown that the extra haemoglobin passing from blood into eggs assists reproduction in another way; it accelerates embryonic development.

Introduction

Among the Crustacea haemoglobin is widespread in the Entomostraca but unknown in the Malacostraca. It is found dissolved in the blood plasma of Notostraca (Regnard & Blanchard 1883), Conchostraca (Fox 1949*a*), Anostraca and Cladocera (Lankester 1871), parasitic Copepoda (van Beneden 1880) and Rhizocephala (Pérez & Bloch-Raphaël 1946). It occurs also in the parthenogenetic eggs of *Daphnia* (Teissier 1932). The only entomostracan groups in which haemoglobin has not yet been found are the Branchiura, and the non-parasitic Cirrepedia and Copepoda.*

In the parasitic Copepoda haemoglobin is known in *Lernaeocera*, which feeds on fishes, having, of course, haemoglobin in their blood. Although it has been shown that the parasite's haemoglobin is distinct from that of the host (Fox 1945*a*), the host's blood might provide the parasite with protohaem for haemoglobin synthesis. We were therefore interested to find haemoglobin in the blood of a parasitic copepod, *Mytilicola intestinalis* Steuer, whose host, the mussel, *Mytilus edulis* L., does not possess the pigment.

The quantity of haemoglobin in the blood of *Daphnia* increases greatly when the animal finds itself in water with a low content of dissolved oxygen (Fox 1948). The same is true of the parthenogenetic eggs of *Daphnia*, which receive their haemoglobin from the blood stream while they are still forming in the ovaries (Dresel 1949). Haemoglobin increases likewise in the blood of *Triops* (Fox 1949*b*), *Leptestheria*

* Even such a brilliantly red copepod as *Arctodiaptomus bacillifer* Koelb, of Alpine lakes, when examined with the microspectroscope shows no oxyhaemoglobin bands, but contains only a carotenoid pigment soluble in acetone.

and *Artemia* (Fox 1949*a*) when the animals are in water deficient in oxygen, and to the list can now be added *Branchipus stagnalis* L. and *Tanymastix perrieri* Daday.

One of the reddest of the Cladocera is *Ilyocryptus sordidus* (Lièven). The colour is due to haemoglobin in the blood, and it is significant that this animal lives buried in mud—an unusual habitat for a cladoceran—where the oxygen content of the water must be very low. If the young are raised in the laboratory on unicellular algae in well-aerated water without mud, they grow into pallid adults. When these are given mud they immediately burrow into it, and within a week become bright red.

In human beings the low pressure of atmospheric oxygen on mountains results in an increase in blood haemoglobin. This is of functional importance, and one would expect that the increase in quantity of haemoglobin in the blood of Crustacea under comparable circumstances of deficient oxygen would likewise be of functional significance. We have now made extensive experiments with *Daphnia* to test this supposition.*

Methods

The species of *Daphnia* used were *D. magna* Straus, *D. pulex* (De Geer), *D. obtusa* Kurz and *D. curvirostris* Eylmann,† according as they were available.

Red animals with much haemoglobin, pink ones with less, and pale ones with hardly any of the respiratory pigment were produced at will by keeping *Daphnia*, of whatever initial colour, for a week or ten days in waters of different dissolved oxygen contents. For cultures in well-aerated water, open dishes were used. For cultures deficient in oxygen, conical flasks containing different quantities of water, thus having different surface areas, were used, nitrogen first being bubbled through the water to reduce the dissolved oxygen content to near the desired value. After *Daphnia* and algal food had been added, an equilibrium became established between oxygen consumed and oxygen coming in from the atmosphere. The cultures were kept in the dark to avoid photosynthesis. The concentration of animals was one *D. magna*, or two of the other species, per 4 ml. of water. The unicellular alga *Chlorella* was given as food, at a standard concentration as described below. The oxygen content of the culture water was determined every second day by the method of Fox & Wingfield (1938).‡

Chlorella vulgaris Beij was used as food for *Daphnia*. In spite of the disadvantage of this alga being non-motile and therefore falling gradually to the bottom of the vessel, it is an excellent food and it has the advantage of being easily cultured.

* A preliminary report of the results has been published (Fox, Gilchrist & Phear 1950).

† The occurrence of *Daphnia curvirostris* in this country was first pointed out to us by the late Mr D. J. Scourfield shortly before his death, since when we have found it in various localities in and around London. The species was named by Eylmann (1887). In Britain it has previously been confused with *D. pulex* and *D. obtusa*. An account of the characters and distribution in this country of *D. curvirostris* by Mr D. S. Johnson, of this Department, will shortly be published in a paper on the British species of *Daphnia*.

‡ In the description of the method it is stated on p. 439 that the diameter of the nozzle-bore in the syringe-pipette is 1 mm. This should be 0·4 mm.; with a wider nozzle too much dissolved oxygen is admitted with the reagents. We now find it advantageous to use 75% instead of pure phosphoric acid, owing to the high viscosity of the latter.

Chlorella was grown on agar slopes in front of a mercury strip lamp; the nutrient medium was that of Pearsall & Loose (1936). The alga was added to *Daphnia* cultures or experimental flasks with a paint brush direct from the agar slope, and was then stirred up daily with the brush. The density of *Chlorella* in suspension in the water was determined with an M.R.C. grey-wedge photometer (King *et al.* 1948), using a red filter. Cultures and experiments were started with the suspension of *Chlorella* at an optical density* of about 0·25. This gives a *Chlorella* suspension of approximately the same concentration as that previously found (Fox, Hardcastle & Dresel 1949, p. 392) to be optimal for *Daphnia* at the particular degree of crowding which we used. More *Chlorella* was added daily to restore the optical density to 0·25.

The haemoglobin content of *Daphnia* was estimated by the index method (Fox 1948; Fox *et al.* 1949). It was found more convenient, however, to take 0·1 ml. instead of 0·2 ml. of the worker's blood and to add it to 37·5 ml. water; this gives a sufficient volume of the standard haemoglobin solution. When low haemoglobin values are measured, the solution is diluted with an equal quantity of water. Haemoglobin values less than 15 cannot be estimated; when such values had to be included with others above 15 to get an index, they were taken as equal to 10.

In order to study the rate of feeding in *Daphnia*, experiments were made on the rate of clearance of graphite suspensions by the animals. This method has been used by Jørgersen (1949) with mussels; on his advice we used Aquadag, grade S.† The graphite concentration was measured at the beginning and end of experiments with the M.R.C. photometer, using the no. 2 green filter.

When carbon monoxide was used to inactivate the haemoglobin of *Daphnia* as an oxygen carrier, its concentration in the water was such that the ratio of the partial pressure of carbon monoxide to oxygen was 1:6. This is obtained by adding 1 ml. of water saturated with carbon monoxide to 22 ml. of aerated water, and proportionally less carbon monoxide for partially aerated waters. This precaution avoids a possible inhibition of cytochrome oxidase‡ by too much carbon monoxide. During or at the end of experiments the presence of carboxyhaemoglobin in the blood was checked by examining with a microspectroscope individual *Daphnia* in a solution of sodium dithionite ($Na_2S_2O_4$). This solution contains no dissolved oxygen; the consequence is that the oxyhaemoglobin in the blood quickly becomes deoxygenated as some of its oxygen diffuses out into the solution and the rest is used by the animal. Carboxyhaemoglobin is not affected, and if the two adsorption bands do not fade, the haemoglobin is all in the carboxy state. This treatment is apparently innocuous to the animal, which can subsequently be returned to the experimental flask.

The experiments were all made at room temperature. This is, of course, variable, but the variations affected experimental flasks and controls alike.

* The optical density is the logarithm of the ratio of the intensity of incident light to that of light transmitted through 1 cm. of a solution or suspension.

† Kindly given by Acheson Colloids Ltd., London.

‡ Cytochrome itself was seen with a microspectroscope in the mandibular muscles of *Leptestheria mayeti* (Simon) from Algeria. This seems to be the first recorded instance of cytochrome in the Entomostraca.

Increase in haemoglobin

In water deficient in dissolved oxygen the haemoglobin content of the blood of *Daphnia* increases. It is of interest to know whether the haemoglobin concentration is proportional to oxygen deficit at all percentage air saturations of the water at which *Daphnia* can live. Figure 1 shows the haemoglobin indices of two series of cultures of *D. pulex* in waters of various oxygen contents, all receiving the same

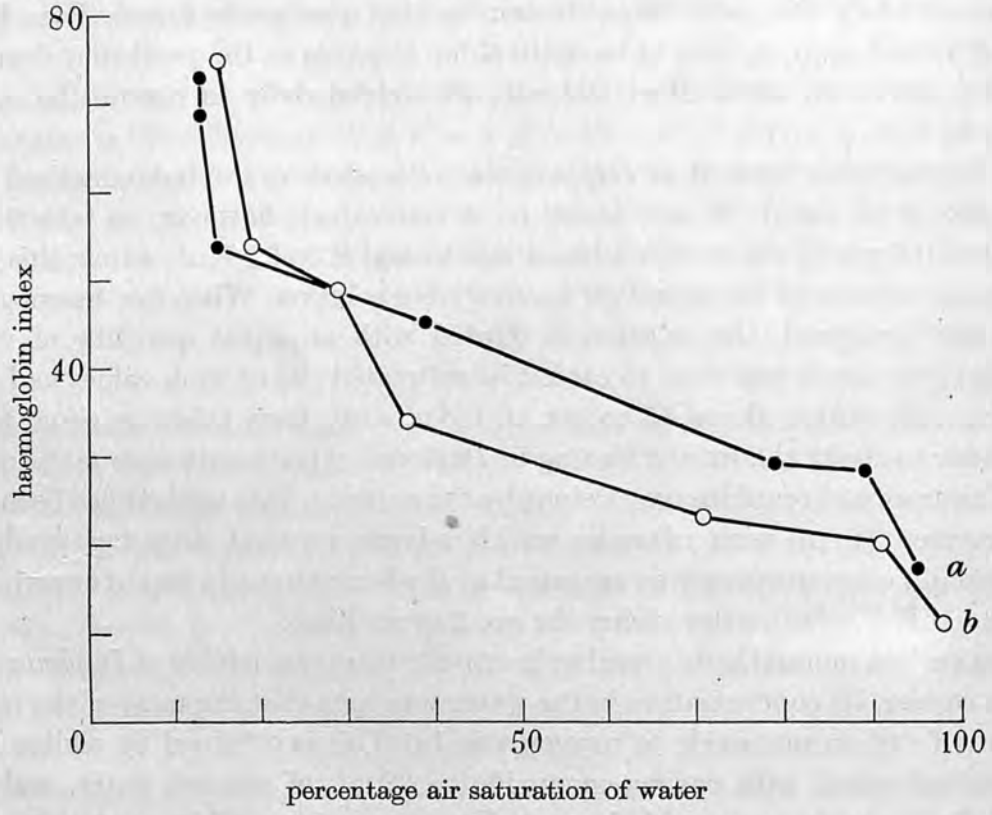


Figure 1. Relation of haemoglobin concentration in blood of *Daphnia pulex* to oxygen content of water (expressed as percentage of the air-saturation value) in two series of seven cultures each, which lasted: *a*, 7 days, and *b*, 9 days, at 22 to 23° C. Each point gives the haemoglobin index for one culture at the end of the period. Initial haemoglobin index: 36. Some cultures lost, others gained haemoglobin.

standard *Chlorella* feeding. The original haemoglobin index in all the cultures was 36 and they had been kept, for 7 and 9 days respectively, in the dark. Oxygen was determined every other day in each culture and the mean values obtained. It is seen that the quantity of haemoglobin in the blood of animals is inversely proportional to the oxygen content of the water; and that even a small deficit of oxygen below air saturation results in some increase in haemoglobin over the value in air-saturated water. In series *a* of figure 1 the culture with water of the lowest oxygen content gave a haemoglobin index which was nearly 4 times that of the most aerated culture. It is probable that had the culturing been continued longer than a week, the multiple would have been greater, for whereas the highest index in the culture series was 73, in nature the same species, *Daphnia pulex*, has been found with an index of 128. The index of this species in nature has been found to vary twelvefold, but this was among different populations (Fox 1948, pp. 198, 199).

Survival in water deficient in oxygen

The results of the experiments made to test the value of haemoglobin to *Daphnia* in aiding the animals to avoid death in semi-anaerobic conditions are summarized in table 1. Three experiments were made; they were carried out as follows. Pale and red *D. magna* were first prepared from the same stock by keeping animals for 10 days in water containing much or little dissolved air. At the end of this time the haemoglobin index of red populations was over 70, and of pale ones less than 15 (that is, too low to be measured). For each experiment three vessels were used. Two of them (*a*, *b*) were 3 l. stoppered bottles completely filled by water with the same low content of dissolved oxygen, and the third vessel (*c*) was an open shallow

Table 1. Survival of *Daphnia magna* with much and with little haemoglobin in water deficient in dissolved oxygen

exp.	temp. (° C)	vessel	oxygen conc. (ml./l.) initial	oxygen conc. (ml./l.) final	colour	haemoglobin index	animals alive at stated hours from beginning				
							0 h.	21 h.	24 h.	26 h.	
1	19·5	*a*	1·6	0·8	red	72	20	20	20	20	
		b	1·5	0·9	pale	<15	20	15	12	9	
		c	5·4	5·2	both	both	20	19	19	19	
							0 h.	45 h.	78 h.	93 h.	
2	22·5	*a*	1·5	0·5	red	75	20	20	20	20	
		b	1·5	0·7	pale	<15	20	19	16	10	
		c	5·1	4·8	both	both	20	20	20	20	
							0 h.	18 h.	21 h.	42 h.	45 h.
3	23·5	*a*	1·4	0·5	red	75	20	20	20	20	20
		b	1·5	0·5	pale	<15	20	17	15	12	10
		c	5·0	4·7	both	both	20	20	20	20	20

dish of water in which the oxygen content was much higher. Before the experiments the oxygen in the water of the bottles was reduced, by bubbling nitrogen through the water, to a value a little above that at which the oxyhaemoglobin in the blood of *D. magna* can be seen to become deoxygenated. At 17° C. this occurs at an oxygen partial pressure of 28 mm. of mercury (Fox 1945*b*), which corresponds to 1·2 ml. dissolved oxygen per litre of water. Into each of the three vessels were put twenty individuals of *D. magna*, red animals into one bottle (*a*), pale ones into the other bottle (*b*), and ten red with ten pale into the open control dish (*c*). It was estimated that the volume of water in the bottles was sufficiently large for the animals not to reduce the oxygen content of the water rapidly during the course of the experiment; clearly, if the dissolved oxygen were quickly reduced to a value at which the haemoglobin in the animals' blood was completely deoxygenated, the respiratory pigment could not function. The amount of decrease in dissolved oxygen in the course of each experiment is entered in table 1. No food was given to the animals. The three vessels in each experiment were subject to the same changes of room temperature; the extreme temperatures, in different experiments, were 19 and 25° C.

Each experiment was continued until 50 % of the animals were dead in one of the vessels. It is seen from table 1 that, in each of the three experiments, when 50 % of the pale animals were dead in bottle *b*,* no red ones had died in *a*. This proves that the extra haemoglobin which *Daphnia* synthesizes in conditions of oxygen paucity is of value to the animals in resisting death from lack of oxygen.

The almost complete absence of deaths in the open control dishes (*c*), with water of moderately high oxygen content, shows that in the bottles (*a*, *b*) animals did not die of starvation. It also shows that pale animals are not intrinsically less viable.

Rate of feeding

Clearance of suspensions

Daphnia feeds on unicellular organisms—algae, flagellates and bacteria—suspended in the water. Vortex currents produced by the thoracic limbs bring the food to the animal. The organisms are collected by the setae on the limbs, and moved forward ventrally to the jaws. It is well known to aquarium keepers that *Daphnia* will clear turbid water; suspensions of both nutritive and non-nutritive matter are collected and swallowed. We have made use of this faculty of *Daphnia* in a quantitative study of the rate of feeding of the animals, by measuring the progressive clearance of suspensions. First we set out to find whether—as would be expected—the rate of feeding diminishes progressively as the dissolved oxygen content of the water falls to low values. This was found to be so. We then went on to investigate whether extra haemoglobin in the blood aids the feeding process in water deficient in oxygen.

The first method used was to measure the rate of clearance by *Daphnia* of suspensions of colloidal graphite. In each experiment a series of 100 ml. conical flasks was used. Each flask was completely filled with graphite suspension having an optical density of about 0·25. The water in the separate flasks was adjusted to various low-oxygen contents by bubbling nitrogen through it. The precise oxygen content, and the precise optical density of the graphite suspension, in each flask was then measured in turn, *Daphnia* was added (two individuals of *D. obtusa* and *D. curvirostris*, or one of *D. magna* per 2 ml. water), and the flask was corked and put in the dark for 3 hr. At the end of this time the oxygen and graphite were measured again.

Preliminary experiments had shown that it was advisable to put cotton-wool in the bottom of the flasks in order to catch falling particles. In each experiment a blank flask was kept without *Daphnia*, to measure the spontaneous clearance of the suspension by deposition. (It was found that if the mother suspension is prepared on the previous day, less graphite is deposited during an experiment.) The graphite diminution in the blank was deducted from those found for the flasks with *Daphnia*.

Figure 2 gives the results of two experiments which show that the rate of graphite clearance by *Daphnia* diminishes progressively with declining oxygen content of

* It is not known why such different lengths of time were required for a 50 % mortality of pale animals in the different experiments.

the water. The next step was to see whether different concentrations of haemoglobin in the blood affect the rate of clearance. Preliminary experiments were made with red and pale animals, obtained by culturing *Daphnia* from the same stock with little or much dissolved air. These experiments indicated that the haemoglobin does intervene; at low oxygen contents of the water animals with more haemoglobin

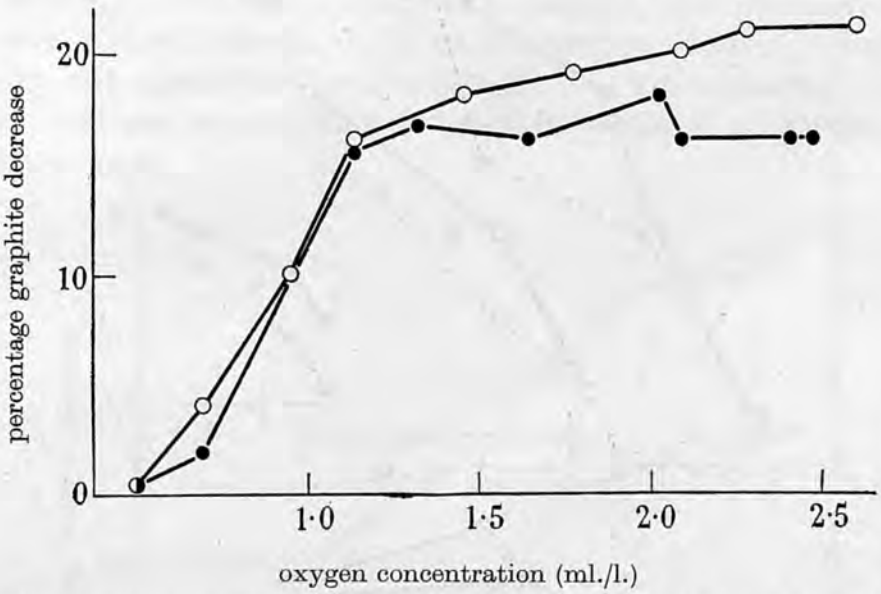


FIGURE 2. Rate of clearance of a graphite suspension by *Daphnia obtusa* in waters of various oxygen contents. Two series of experiments are shown, lasting 3 hr. at 20° C. Each point represents a flask.

removed more graphite. Confirmation was sought by making a series of experiments with red *Daphnia*, in which graphite clearance by normal animals was compared with that by others treated with carbon monoxide to inactivate haemoglobin. This was considered to be a better method, because the animals which were compared differed only by the presence or absence of carboxyhaemoglobin, whereas animals cultured in two different ways may differ otherwise than by the possession of much or little haemoglobin. The results of the six experiments made with *Daphnia* with and without carboxyhaemoglobin, in water of low oxygen content, are given in figure 3. It is seen that at low values of dissolved oxygen the curves for normal animals are above those for animals with carboxyhaemoglobin; the normal animals removed more graphite.

It would seem that haemoglobin enables *Daphnia* to remove suspended particles more quickly, and thus presumably helps them to feed more efficiently, in water deficient in oxygen. Additional experiments with red *Daphnia* in waters with various higher oxygen contents showed that here carbon monoxide does not measurably diminish the rate of graphite clearance.

Experiments were next made to compare the effect, on the rate of collection of suspended particles by *Daphnia* at low oxygen concentrations, of different amounts of haemoglobin in the blood. *D. pulex* was first cultured in waters of various oxygen contents to obtain populations of different degrees of redness, having a range of haemoglobin indices. The rate of graphite clearance by samples of the different

populations was then measured. Three experiments were made and the results are shown in figure 4. It is seen that the rate of graphite clearance in water containing little oxygen is proportional to the amount of haemoglobin in the animal's blood,

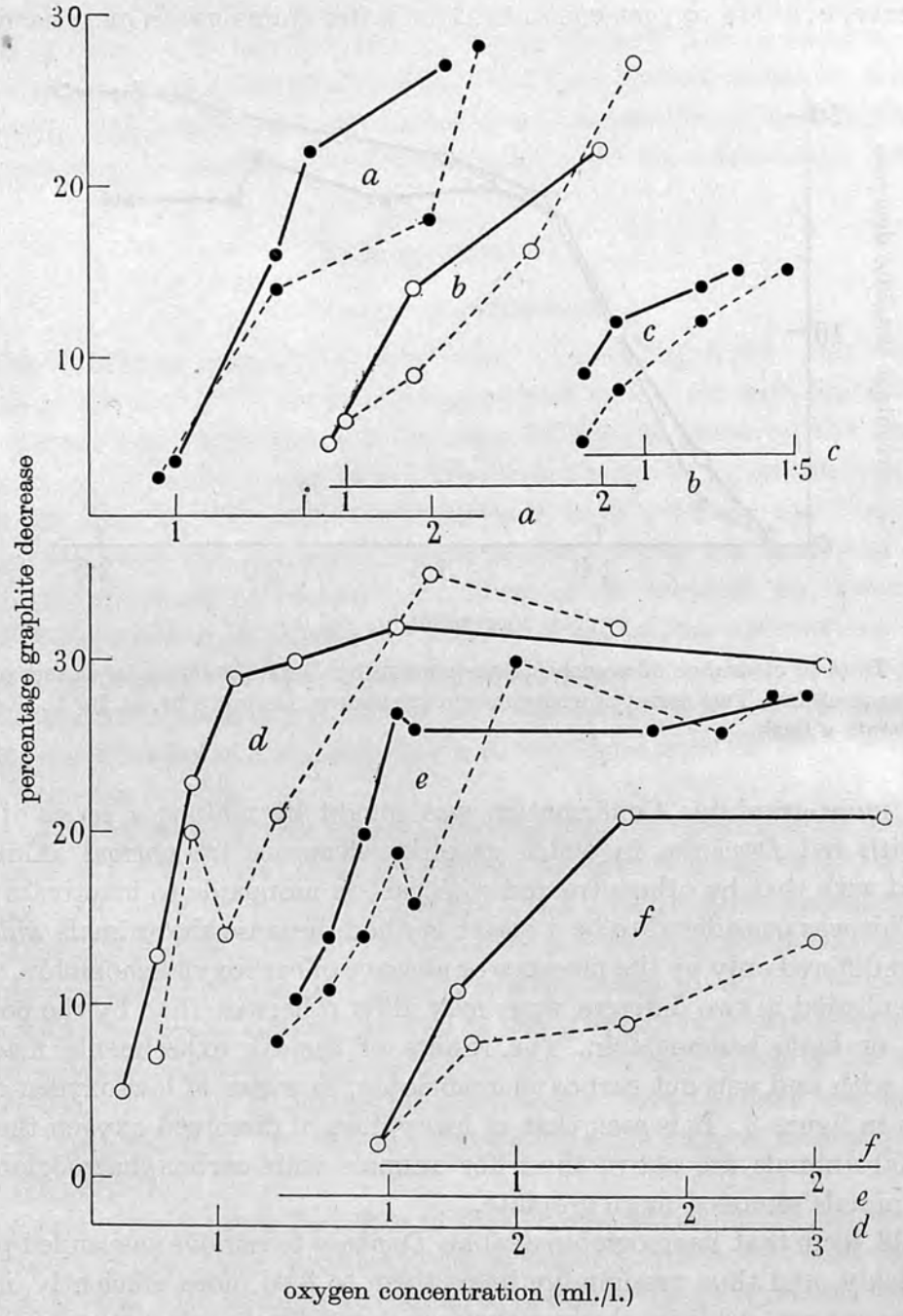


FIGURE 3. Rate of clearance of a graphite suspension by red *Daphnia* with and without functional haemoglobin, in waters of various oxygen contents. Exps. *a*, *b*, *D. curvirostris*; exps. *c*, *d*, *e*, *f*, *D. magna*. —— normal animals; ------ those with carboxyhaemoglobin. Experiments lasted 3 hr. Exps. *a*, *b*, 18° C.; *c*, *d*, *e*, *f*, 25° C. Each point represents a flask.

and that even a small quantity of the respiratory pigment assists feeding under these circumstances. It will be noted that, as before, more graphite is removed at the higher oxygen content of the water than at the lower.

Graphite is, of course, not a food for *Daphnia*, and it was thought worth while to find out the effect of haemoglobin on the rate at which a real food, namely *Chlorella*, is removed from water. The duration of the experiments was much longer than with graphite, for it takes more time for *Daphnia* to clear a suspension of *Chlorella* than of graphite. There are several reasons for this. In the first place, *Chlorella* cells are bigger; their length is 5 to 7μ, whereas the graphite particles measured 1 to 2μ. In the second place, *Chlorella* settles on the bottom, although it was stirred up periodically with a paint brush and so brought back into suspension, together with the faeces consisting to a considerable extent of uninjured or only partly digested cells (Lefèvre 1942).

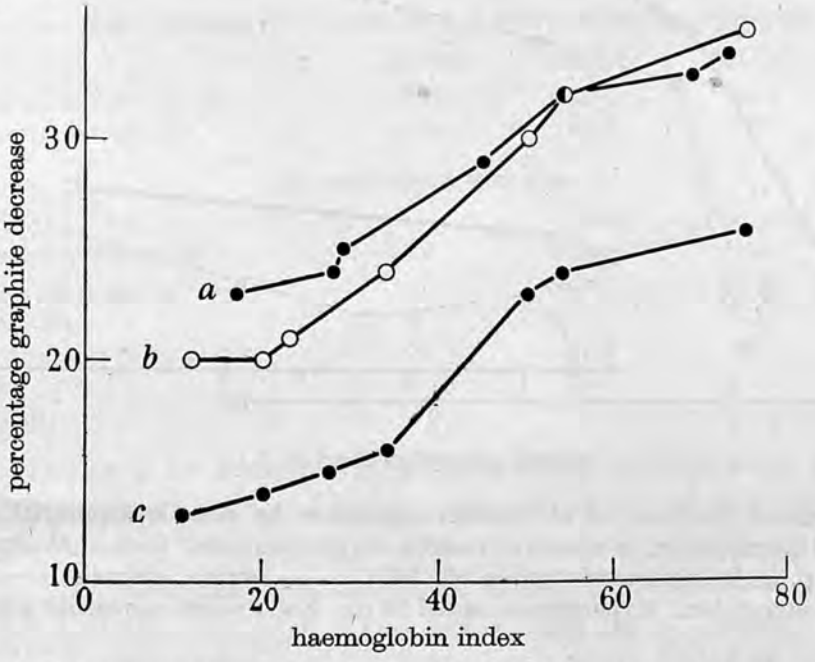


FIGURE 4. Rate of clearance of a graphite suspension, in water deficient in dissolved oxygen, by *Daphnia pulex* with various concentrations of haemoglobin in the blood. Three experiments are shown, *a* and *b* with 1·5 ml./l. and *c* with 1·2 ml./l. dissolved oxygen. Experiments lasted 3 hr. at 21° C. Each point represents a flask.

As in the case of graphite clearance, experiments were first made to compare *Chlorella* clearance by red and pale *Daphnia*. These preliminary experiments indicated that at low oxygen contents of the water *Daphnia* with much haemoglobin eats *Chlorella* more quickly than *Daphnia* with little haemoglobin. After that, the rate of *Chlorella* clearance by normal red *Daphnia* was compared with that by animals from the same population treated with carbon monoxide. These experiments were carried out as follows. A number of 100 ml. conical flasks was prepared containing water of different low oxygen contents. *Daphnia* was added in the usual concentration of one animal to 2 ml. water and *Chlorella* to an optical density of about 0·25. Then the uncorked flasks were left in the dark for 24 hr. to attain a steady oxygen content. After this, *Chlorella* was added again to reattain the optical density of 0·25; then the oxygen was measured and carbon monoxide was added in the usual amount to half the number of flasks, thus starting the experiment. The *Chlorella* was stirred every few hours. At the end of a further 24 hr. *Chlorella* and

oxygen were measured again, and the mean oxygen content of each flask over the 24 hr. calculated. An imperfection of this type of experiment is that the carbon monoxide gradually diffuses into the air from the water surface, so that the haemoglobin does not remain in the carboxy state for the whole 24 hr. This was revealed by a dithionite test made at intervals on single animals as described above. A greater concentration of carbon monoxide cannot be used, and the flasks cannot be stoppered as in the short graphite experiments because in 24 hr. the *Daphnia* and *Chlorella* (in the dark) would reduce the oxygen content of the water.

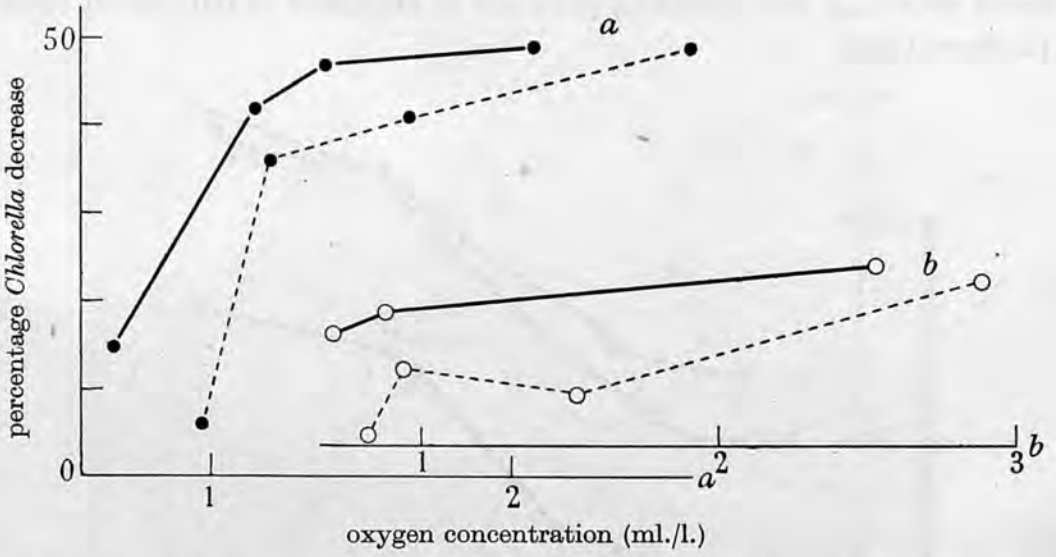


FIGURE 5. Rate of clearance of a *Chlorella* suspension by red *Daphnia* with and without functional haemoglobin, in waters of various oxygen contents. Exp. *a*, *D. obtusa*, index 66, 25° C.; exp. *b*, *D. curvirostris*, index 65, 24° C. —— normal animals; ----- those with carboxyhaemoglobin. Experiments lasted 24 hr. Each point represents a flask.

The results of two experiments are shown in figure 5. It is seen from the curves that, in spite of the carboxyhaemoglobin not having persisted for 24 hr., normal *Daphnia* clears more *Chlorella* at low oxygen values than *Daphnia* with inactivated haemoglobin. The haemoglobin favours uptake of *Chlorella* in poorly aerated water as it favours graphite clearance.

Limb movements

Daphnia collects its food by movements of the thoracic limbs. Since we had found that in poorly aerated water *Daphnia* clears a suspension quicker when there is more haemoglobin in its blood, it appeared likely that a more rapid movement of the limbs was responsible. This was tested as follows. By modifying a technique of Scourfield (1900), a loop at the end of a wire was fixed by agar to the back of individual *Daphnia*. The rate of movement of the thoracic limbs could then easily be measured with a stop-watch. This was done with normal red animals and with others treated by carbon monoxide to inactivate the haemoglobin. The observations were made at 21° C. in water containing 1·27 ml./l. oxygen, i.e. 20 % saturated with air. On each of 4 days, five animals with carboxyhaemoglobin were compared with five normal animals. Different animals were used on the different days. With each

animal the number of seconds for 100 movements was measured 10 times. The means for each day with and without carboxyhaemoglobin are entered in table 2, from which it is seen that the movement was faster with normal haemoglobin. The analysis of variance shows that the time for 100 movements was significantly greater after carbon monoxide treatment. Thus in poorly aerated water the food-catching limb movement is faster when there is more haemoglobin in the blood.

TABLE 2. INFLUENCE OF FUNCTIONAL HAEMOGLOBIN ON THE RATE OF FOOD-CATCHING LIMB MOVEMENTS OF RED *DAPHNIA MAGNA* IN WATER CONTAINING LITTLE DISSOLVED OXYGEN

(*a*) mean number of seconds for 100 thoracic limb movements

	1st day	2nd day	3rd day	4th day
treatment 1: without CO	36·1	23·4	31·7	32·2
treatment 2: with CO	47·1	34·5	39·6	40·0

(*b*) analysis of variance

source of variance	D.F.	mean square	variance ratio	*P*
between treatments	1	889·3	23·55	<0·001
between days	3	270·1	7·15	
interaction day/treatment	3	8·3		
residual	32	37·8		

TABLE 3. INFLUENCE OF FUNCTIONAL HAEMOGLOBIN ON THE RATE OF SWIMMING MOVEMENTS OF RED *DAPHNIA MAGNA* IN WATER CONTAINING LITTLE DISSOLVED OXYGEN

(*a*) mean number of seconds for 20 antennal movements

	1st day	2nd day	3rd day	4th day
treatment 1: without CO	5·35	7·58	6·49	5·75
treatment 2: with CO	5·99	8·27	7·05	6·09

(*b*) analysis of variance

source of variance	D.F.	mean square	variance ratio	*P*
between treatments	1	6·11	8·27	<0·001
between days	3	20·76	28·11	
interaction day/treatment	3	0·12		
residual	72	0·74		

A further study was made of the influence of haemoglobin on the swimming movements, which are made by the antennae. In these experiments the animals swam freely. On each of 4 days ten normal red animals and ten other red animals treated with carbon monoxide were studied. The water, at 20° C., contained 1·27 ml./l. oxygen. For each animal ten measurements were made of the time in seconds for twenty antennal movements. The mean times for the ten animals of each day and treatment are given in table 3. In spite of considerable absolute differences in times from day to day, the analysis of variance shows a highly significant difference between the treatments. Thus haemoglobin enables *Daphnia* to swim faster in conditions of oxygen scarcity.

Egg production

If well fed, the parthenogenetic female of *Daphnia* lays eggs in her brood pouch after each moult, which occurs, in *D. obtusa*, at intervals of between 2 and 3 days at a temperature of 22° C. We noticed that in poorly aerated water *Daphnia* lays fewer eggs than in well-aerated water. This is proved by experiments summarized in figure 6. It is seen that progressively fewer eggs are produced as the oxygen content of the water decreases. In experiments *a* to *d* *Daphnia* was kept for 4 days in flasks with various oxygen contents, the animals being fed on *Chlorella* at the standard concentration, maintained by daily additions. At the end, the average number of eggs per female was estimated.

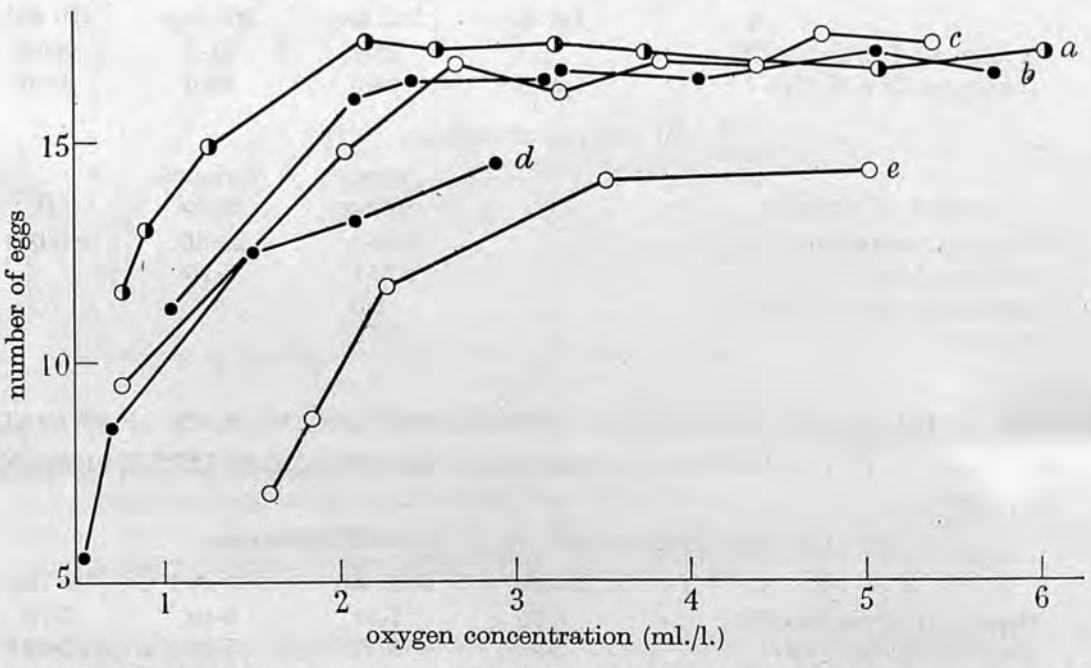


Figure 6. Number of eggs produced by *Daphnia obtusa* at various oxygen contents of the water. Curves *a* to *e* are separate experiments: *a*, *b*, *c*, 20° C.; *d*, 17° C.; *e*, 16° C. Each point represents a flask or cylinder.

In water deficient in oxygen *Daphnia* tends to congregate at the air surface. It is probable that animals near the surface are not always exposed to such a low dissolved oxygen concentration as that given by an analysis of the water. An experiment was therefore made in which cylinders were used instead of flasks, and *Daphnia* was kept from the surface by bolting silk on a wire ring a little beneath the surface. The water under the silk was moved hourly in the daytime by a glass stirrer in order to keep the *Chlorella* in suspension. This is experiment *e* in figure 6; its curve is to the right of the others, indicating that the decline in egg production really occurs at a rather higher oxygen content of the water than *a* to *d* apparently show.

We next studied the question of whether haemoglobin intervenes in egg production under conditions of oxygen deficiency. Does the additional haemoglobin which is synthesized in response to oxygen deficit result in a lesser diminution of egg production than would otherwise be the case? To answer this question red *Daphnia* was kept for several days, with and without carbon monoxide, in waters of different

dissolved oxygen contents, and fed with *Chlorella*. After one or two preliminary days to allow the oxygen values to become steady in the flasks, the experiments proper, with and without carbon monoxide, were begun and lasted 2 days. In determining the final egg numbers, females carrying embryos with eyes were not counted because the eggs from which these late embryos developed would have been formed in the ovaries, or may have been laid in the brood pouch, before the carbon monoxide was added. The experiments are open to the same criticism as those on *Chlorella* clearance, namely, that carboxyhaemoglobin was only present for part of

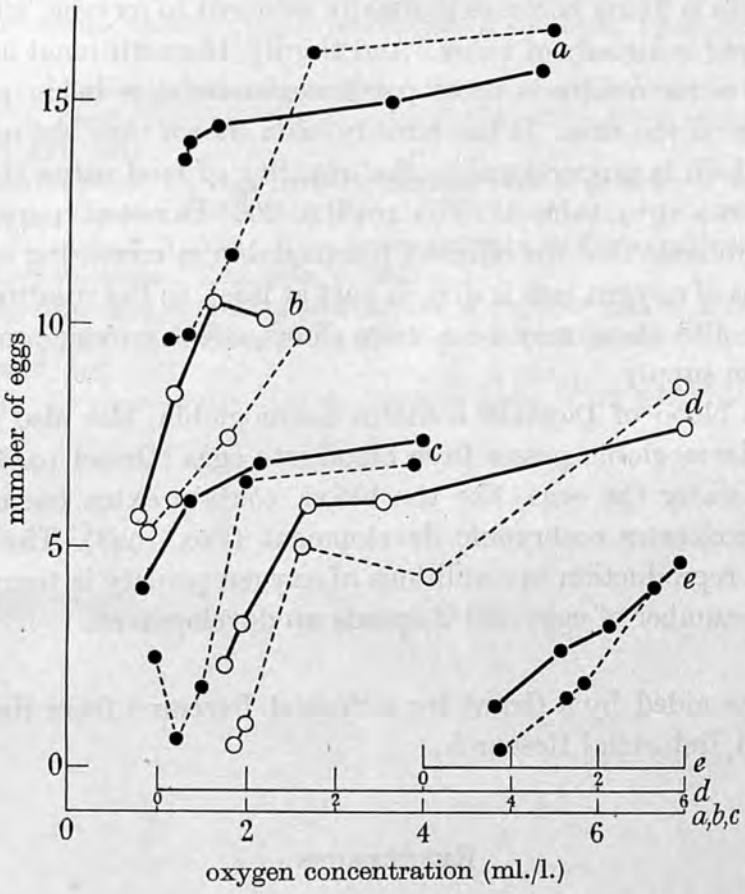


FIGURE 7. Number of eggs produced by red *Daphnia* with and without functional haemoglobin, in waters of various oxygen contents. Exps. *a*, *c*, *d*, *e*, *D. curvirostris*; exp. *b*, *D. obtusa*. —— normal animals; ----- those with carboxyhaemoglobin. *a*, 20° C.; *b*, 24° C.; *c*, 25° C.; *d*, *e* 21° C. Each point represents a flask.

the time, and indeed dithionite tests showed this to be so. Nevertheless, the results of the five experiments that were made, which are given in figure 7, indicate that *Daphnia* with haemoglobin inactivated, if only for part of the time, produces even fewer eggs in oxygen-deficient water than *Daphnia* with functional haemoglobin.

Thus, the extra haemoglobin synthesized in water deficient in oxygen assists egg production.

CONCLUSION

We have now shown that the extra haemoglobin which appears in the blood of *Daphnia* as a result of oxygen deficiency in the water has several functions. First, it increases the time of survival of the animals in semi-anaerobic conditions; it

enables them to respire adequately. Failure to demonstrate this in earlier experiments (Fox 1948) may have been due to the experiments not being continued long enough. Secondly, the extra haemoglobin enables *Daphnia* to gather more food in water deficient in oxygen; the muscular work of moving the thoracic food-catching limbs adequately requires sufficient oxygen, which under these conditions can only be obtained through the haemoglobin. The rate of feeding when oxygen is scarce was found to be proportional to the haemoglobin content of the blood, even a small amount of haemoglobin assisting food gathering; this means that when the water in which *Daphnia* is living becomes gradually deficient in oxygen, the first haemoglobin synthesized is already of value. And thirdly, the additional haemoglobin in poorly aerated water results in more parthenogenetic eggs being produced than would otherwise be the case. It has already been shown that the number of eggs formed by *Daphnia* is proportional to the quantity of food eaten (Ingle, Wood & Banta 1937; Banta 1939, table 31; Fox 1948, p. 202; Fox *et al.* 1949, table 1), and it is therefore probable that the effect of haemoglobin in increasing egg production under conditions of oxygen lack is due, in part at least, to the resulting more abundant nutrition. But there may be a more direct effect on egg production of an adequate oxygen supply.

Not only the blood of *Daphnia* contains haemoglobin, but also the parthenogenetic eggs. Haemoglobin passes from blood into eggs (Dresel 1948), and thus in poorly aerated water the eggs, like the blood, contain extra haemoglobin. This haemoglobin accelerates embryonic development (Fox 1948). The assistance of haemoglobin to reproduction in conditions of oxygen paucity is therefore twofold: it increases the number of eggs and it speeds up development.

This work was aided by a Grant for a Special Research from the Department of Scientific and Industrial Research.

References

Banta, A. M. 1939 Studies on the physiology, genetics, and evolution of some Cladocera. *Publ. Carneg. Instn*, no. 513.

van Beneden, E. 1880 De l'existence d'un appareil vasculaire à sang rouge dans quelques Crustacés. *Zool. Anz.* **3**, 35, 55.

Dresel, E. I. B. 1948 Passage of haemoglobin from blood into eggs of *Daphnia*. *Nature*, **162**, 736.

Eylmann, E. 1887 Beitrag zur Systematik der europäischen Daphniden. *Ber. naturf. Ges. Freiburg i. B.* **2**, 61.

Fox, H. M. 1945*a* Haemoglobin in blood-sucking parasites. *Nature*, **156**, 475.

Fox, H. M. 1945*b* The oxygen affinities of certain invertebrate haemoglobins. *J. Exp. Biol.* **21**, 161.

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

Fox, H. M. 1949*a* Haemoglobin in Crustacea. *Nature*, **164**, 59.

Fox, H. M. 1949*b* On Apus: its rediscovery in Britain, nomenclature and habits. *Proc. Zool. Soc. Lond.* **119**, 693.

Fox, H. M., Gilchrist, B. M. & Phear, E. A. 1950 Functions of haemoglobin in *Daphnia*. *Nature*, **166**, 609.

Fox, H. M., Hardcastle, S. M. & Dresel, E. I. B. 1949 Fluctuations in the haemoglobin content of *Daphnia*. *Proc. Roy. Soc.* B, **136**, 388.

Fox, H. M. & Wingfield, C. A. 1938 A portable apparatus for the determination of oxygen dissolved in a small volume of water. *J. Exp. Biol.* **15**, 437.

Ingle, L., Wood, T. R. & Banta, A. M. 1937 A study of longevity, growth, reproduction and heart rate in *Daphnia longispina* as influenced by limitations in quantity of food. *J. Exp. Zool.* **76**, 325.

Jørgersen, C. B. 1949 The rate of feeding by *Mytilus* in different kinds of suspensions. *J. Mar. Biol. Ass. U.K.* **28**, 333.

King, E. J., Gilchrist, M., Wootton, I. D. P., Donaldson, R., Sisson, R. B., Macfarlane, R. G., Jope, H. M., O'Brien, J. R. P., Peterson, J. M. & Strangeways, D. H. 1948 Determination of haemoglobin. V. Precision of colorimetric methods. *Lancet*, **2**, 563.

Lankester, E. R. 1871 Ueber das Vorkommen von Haemoglobin in den Muskeln der Mollusken und die Verbreitung desselben in den lebendigen Organismen. *Pflüg. Arch. ges. Physiol.* **4**, 315.

Lefèvre, M. 1942 L'utilisation des algues d'eau douce par les Cladocères. *Bull. biol.* **76**, 250.

Pearsall, W. H. & Loose, L. 1936 The growth of *Chlorella vulgaris* in pure culture. *Proc. Roy. Soc.* B, **121**, 451.

Pérez, C. & Bloch-Raphaël, C. 1946 Note préliminaire sur la présence d'un pigment respiratoire chez le *Septosaccus Cuénoti* (Duboscq.) *C.R. Acad. Sci., Paris*, **223**, 840.

Regnard, P. & Blanchard, B. 1883 Note sur la présence de l'hémoglobine dans le sang des Crustacés branchiopodes. *Zool. Anz.* **6**, 253.

Scourfield, D. J. 1900 The swimming peculiarities of *Daphnia* and its allies with an account of a new method of examining living Entomostraca and similar organisms. *J. Quekett Micr. Cl.* (2) **7**, 395.

Teissier. G. 1932 Le pigment des œufs de *Daphnia pulex*. *C.R. Soc. Biol., Paris*, **109**, 813.

PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, CAMBRIDGE
(BROOKE CRUTCHLEY, UNIVERSITY PRINTER)