SHEILA M. HARDCASTLE.
BEDFORD COLLEGE FOR WOMEN:

THE INFLUENCE OF NUTRITION ON THE HAEMOGLOBIN CONTENT OF DAPHNIA

ABSTRACT OF THESIS

The blood of pond-living species of Daphnia contains haemoglobin, which often gives the animals a pink or red colour. The quantity of haemoglobin is very variable; a Daphnia species may be colourless, pink or red in different ponds, or in the same pond at different times. Daphnia may gain or lose considerable quantities of haemoglobin in a few days, as a result of decrease or increase in the amount of dissolved oxygen in the water.

In the work reported in this thesis, I have found that the haemoglobin content of Daphnia blood is also influenced by the quantity of available food. The number of parthenogenetic young produced was used as an indication of the state of nutrition. Haemoglobin production did not take place in starved animals even with very little oxygen. At low oxygen concentrations an increase in the amount of food added caused an increase in haemoglobin synthesis to a certain maximum value, above which greater quantities of food had no effect.

It had been thought that pale Daphnia placed in a water from a pond which contained red Daphnia produce more haemoglobin than in their own water, at the same oxygen concentration. I have found that this is really due to differences in nutrition.

It was also thought that the presence of chlorophyll in food caused greater haemoglobin formation. I have shown that pale Daphnia fed on colourless and on green organisms, such as Gonium

cont'd...

and yeast, Chlorella and Prototheca, Chlamydomonas and Polytoma do not produce significantly different amounts of haemoglobin. Equal quantities by weight of Chlamydomonas and Polytoma were used.

I have investigated a number of ponds containing red and colourless Daphnia at different seasons. An estimate of the maximum and minimum oxygen concentration in various ponds was obtained indirectly by finding the oxygen consumption of pond waters in stoppered bottles in light and in darkness. An estimate of the amount of phytoplankton present was also obtained by this means. Possible correlations between the haemoglobin content of the Daphnia and the oxygen content and alkalinity of the pond waters, and the seasons, were also studied.

THE INFLUENCE OF NUTRITION ON THE HAEMOGLOBIN CONTENT OF *DAPHNIA*.

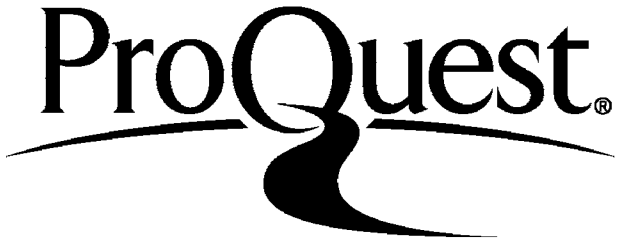
by

Sheila M. Hardcastle. B.Sc.

A thesis submitted to the Faculty of Science of the University of London, for the degree of Master of Science.

Bedford College for Women,

May, 1948.



CONTENTS

1. INTRODUCTION

Haemoglobin is found in several Entomostraca, but its presence is especially interesting in Daphnia as this animal shows large fluctuations in the quantity of haemoglobin, apparently without any correlated change in vitality or survival power in adverse surroundings (Fox 1947, 1948).

The red colour of Daphnia in ponds or ditches had been described by Swammerdam (1758) and Baird (1849) but it was not identified as haemoglobin until 1871 by Ray Lankester. It is present in the species of Daphnia inhabiting ponds and ditches. There are three common species of Daphnia found in these localities, namely D.magna Straus, D.pulex (De Geer) and D.obtusa Kurz. (Scourfield and Harding 1941, Scourfield 1942). Although there is great variation in the amount of haemoglobin in the blood of Daphnia, there are comparatively few references to the colour of Daphnia in the literature.

Haemoglobin is found in solution in the blood plasma, and it was also discovered in the eggs of D.pulex in the brood pouch (Tessier 1932). Smith (1915) discusses the bright reddish orange colour of Daphnia kept in crowded conditions at low temperature, and the blood red colour of ponds, but he did not attribute the colour to haemoglobin. Ramner (1933) similarly describes the massing of D.pulex to form red swarms. Banta (1939) considered the red colour of Daphnia to be caused by an intra-vitam staining obtained from the water.

The red colour of Daphnia has often been associated with good nutrition, although the colour has not always been attributed to haemoglobin. Fritzsche (1917) thought that the red colour of Daphnia is due to carotene and he ascribed its formation to good nutrition. Schulz (1928) was also of this opinion. Verne (1923) studied the synthesis of haemoglobin in Daphnia and concluded that the appearance of haemoglobin depends on the presence of chlorophyll (or products of its disintegration).

It is important to discover the factors responsible for the formation of haemoglobin in such variable quantities, and the reasons for its loss in the laboratory and in nature.

More than one factor is involved in the synthesis of haemoglobin in Daphnia. Fox (1948) found that Daphnia in the laboratory will lose haemoglobin in well aerated water, whereas the quantity of haemoglobin in the same Daphnia in water deficient in dissolved oxygen remained steady or rose. In other experiments with pale Daphnia, oxygen lack was found to be a factor not only in preventing haemoglobin loss but also in its synthesis. Pale Daphnia in conditions of low oxygen gained haemoglobin, while in aerated water the quantity of haemoglobin was not found to change significantly.

Although oxygen deficiency is thus a factor influencing the haemoglobin content of Daphnia, haemoglobin synthesis was also observed in certain pond waters even when aerated. In addition to this the faeces of ducks were found to promote haemoglobin formation more than oxygen deficiency alone. In

one experiment Fox (1948) found that the amount of haemoglobin had increased to over three times its original value in the presence of faeces, but it had only doubled in hydrogenated water of a similar low oxygen content. Since certain pond waters in which Daphnia is red are able to induce haemoglobin production even when the oxygen concentration is not low, this suggests that there may be some other factor in pond water responsible for haemoglobin production. This factor may be capable of acting independently, or it may come into operation only in conditions of oxygen lack.

The object of this present investigation is to find the factor, which, in combination with oxygen lack, induces haemoglobin synthesis in Daphnia. My work has been concerned mainly with the influence of nutrition on the haemoglobin production of Daphnia. The influence of different quantities of algal food on the haemoglobin index has been investigated. The effect of the presence of chlorophyll in food on the haemoglobin content of Daphnia has also been considered.

The problem has also been approached by making field observations in different localities over a period of several months, of the oxygen concentration or oxygen consumption of the pond water, the alkalinity of the water and the haemoglobin index of the Daphnia present.

2. METHODS

The quantity of haemoglobin in Daphnia or the

"haemoglobin index" has been estimated by the method devised by Fox (1948) using ten of the biggest Daphnia taken from a population. The colour of oxyhaemoglobin close to the base of the second antenna of each animal in turn is matched against a standard solution of diluted blood from the worker's finger. The diluted blood solution is placed in a trough before the mirror of a microscope, and the image of the haemoglobin solution is made to fill the upper third of an evenly illuminated field of vision in the microscope. The colours are matched by moving the trough to right or left. The trough stands on a scale, so that the narrow end seen through the microscope corresponds to 0 and the wide end to 160 arbitrary units. For pale populations the standard haemoglobin solution is diluted to one half strength, the scale reading from 0 to 80 units. The values for each of the 10 individuals are averaged and thus the haemoglobin index for the population is obtained.

Inaccuracies in the method are caused by individual variations in the Daphnia of a population, and by the personal error in estimation.

Daphnia of above a certain size were taken at random from a population. The inaccuracy caused by the individual variation in Daphnia taken in this way was tested by taking two lots of 10 Daphnia from each of several different populations, and finding the haemoglobin index of each. The difference between the two values was found to vary in different localities. In seven populations the results were

104 and 98, 93 and 86, 72 and 61, 60 and 56, 48 and 47, 40 and 39, 29 and 27. It follows that the error of the method is less than 10%.

It has been shown (Dresel, unpublished) that the haemoglobin content of Daphnia in which the parthenogenetic young are at the egg stage is less than the haemoglobin content of Daphnia carrying well developed embryos. The haemoglobin content gradually increases as the eggs develop in the brood pouch. It has been shown that the accuracy of the method is greater when Daphnia are taken in which the parthenogenetic young are at the same stage of development.

In later experiments, therefore, Daphnia have been used in which the parthenogenetic young were at the same stage of development. In order to obtain comparable results two stages have been considered, the egg stage and the eyed-embryo stage, in which the embryos have one or two eyes. The first ten animals of the correct size and with young at the required stage were taken, in order to avoid selecting particular animals.

The personal error in estimation was tested by determining the haemoglobin index of the same 10 Daphnia three times. Although the individual values obtained were not indentical, the mean values were approximately the same, values of 44, 46 and 46 being obtained. In addition the average index obtained from 10 D. obtusa was estimated using strong and dilute haemoglobin solutions successively. The same average value of 41 was obtained.

When the haemoglobin index has been estimated, the length of each Daphnia is measured from the forehead to the base of the posterior shell spine, using a micrometer eyepiece and a 1 inch or 2 inch objective. Details are noted of the colour of the liquid in the space between gut wall and peritrophic membrane (Chattom 1920), the gut content, the quantity of fat, the size of the shell spine and any other interesting feature. The number of eggs or embryos in the brood pouch of each Daphnia is then counted, after dissecting them out in most cases, and the colour of the young is noted.

Dissolved oxygen measurements are made in all cases by the syringe-pipette micro-Winkler method of Fox and Wingfield (1938). The method is modified by the preliminary use of sodium azide, as in some pond waters the presence of nitrite causes an inaccuracy in the result. The presence of nitrites can be shown by making an oxygen determination without the addition of manganous chloride solution, a blue colour appearing if nitrites are present. This error is avoided by filling the dead space of the pipette with 1% solution of sodium azide which reacts with nitrites with the production of nitrogen.

In all experiments Daphnia is kept in containers which allow a small surface area in comparison with the volume of the water, so that diffusion of oxygen into the medium is reduced. Conical and round-bottomed flasks of 250 cc. capacity were found to be suitable for this purpose.

By varying the volume of water used, and hence the surface area, different oxygen concentrations can be obtained. For oxygen concentrations of more than 50% air saturation, it was found satisfactory to use conical flasks, and to begin the experiment in aerated water, the oxygen concentration being reduced rapidly by the Daphnia and the added food organisms. For low oxygen concentrations Daphnia is enclosed in round-bottomed flasks filled to various levels in the neck with water of low oxygen content, so that the surface area is extremely small in proportion to the volume of water used. The respiration of Daphnia is sufficient to counteract the diffusion over the surface, and daily oxygen determinations during an experiment show that the oxygen content does not vary much. In all experiments a series of flasks is used with different surface area/volume ratios. The number of Daphnia and the amount of food added is proportional to the volume of pond water used. A series of oxygen concentrations is thus obtained, which can be compared with a similar series in a different pond water. This enables the haemoglobin production of Daphnia in pond waters of varying oxygen consumption to be compared, at the same percentage air saturation.

Flasks containing Daphnia are kept im the dark, to avoid oxygen production by algal photo-synthesis.

3. THE FEEDING OF DAPHNIA:

(a) Previous Work

Smith (1915) used Protococcus successfully as food for

Daphnia, but it was thought that Cladocera are unable to digest cellulose (Naumann 1921) and so to feed on unicellular algae other than flagellates. Detritus of an organic origin was considered to be more important than algal food. The results of Pacaud (1939) indicate that flagellates of the nannoplankton are better nourishment than detritus of various kinds. Von Dehn (1930) showed that cells of Stichococcus had to pass several times through the gut, before the contents were leeched out of the only partly digested cellulose cell wall.

Various workers have since kept Daphnia in a healthy condition on various strains of algae. Chlorella has been used (Woltereck 1928, Pratt 1943), Scenedesmus was found to be a successful food (Schulz 1928), and Gonium has also been used (Mortimer 1936, Pacaud 1939). Pacaud (1939) after trying various algae concluded that Daphnia are better nourished on a mixed diet, Chlamydomonas agloeformis and Gonium pectorale being used.

The suitability of many species of algae as food for Daphnia has been experimentally investigated and discussed by Lefèvre (1942), many species being found to be of nutritional value. The most favourable species, namely Phaecus pyrum, Chlorella vulgaris, Gonium pectorale and Synura uvella, differ morphologically and chemically, but have in common a small size, fragile membrane, and a tendency to remain easily suspended in a culture.

Foods not containing chlorophyll have also been used

for rearing Daphnia. Verne (1923) used "zooglées" without chlorophyll. Bacteria from a manure extract were found to be suitable food by Hutchinson (1933). Banta (1939) considered pure strains of bacteria difficult to manipulate, although various bacterial cultures have been used successfully (Salimovskaya-Rodina 1940). Yeasts have also been used by the same author, who found that Daphnia can feed exclusively on these micro-organisms and can be cultivated to a mature state, and produce normal offspring. All species of micro-organisms were not found to meet the nutritional requirements of Daphnia, and moreover some proved to be quite unsuitable, for example Bacterium prodigiosum and B.violaceum. On emulsions of Azotobacter and Torula, Daphnia attained maturity, produced offspring, showed "an excellent reddish - pink colour" and accumulated reserves. Saccharomyces cerevisiae and S.ellipsoideus were claimed to be successful food materials. Yeasts, however, were not considered by Anderson and Jenkins (1942) to meet the nutritional requirement of Daphnia.

The concentration of food material used is important. High concentrations of bacteria are unsuitable, probably as these accelerate poisoning with the waste of bacterial growth (Salimovskaya - Rodina 1940). Digestion of algal cells is more complete if a dilute suspension is used, so that passage through the alimentary canal is slow (Lefèvre 1942).

In nature the food of Daphnia usually consists of detritus with its contained micro-organisms. The food is separated from the gut wall by a peritrophic membrane (Chatton 1920). The space between the peritrophic membrane and the gut wall in the anterior part of the mid-gut is coloured green with chlorophyll or a product of its disintegration in Daphnia which have been feeding on green food. I have used the colour of this space as an indication of the state of nutrition of Daphnia fed on organisms containing chlorophyll.

I have used three green organisms as food for Daphnia, Gonium pectorale Müll., Chlamydomonas incisa Pringsh., both members of the Order Volvocales, and Chlorella vulgaris Beij., a member of the order Chlorococcales.

As foods without chlorophyll I have used baker's yeast, Polytomella caeca Pringsh., and Polytoma uvella Ehrbg., two members of the order Volvocales, and Prototheca Zopfii Krüg. a colourless member of the order Chlorococcales. These organisms, with the exception of yeast and Polytomella, were cultured.

(b) Culture Methods:

In my experiments large quantities of food organisms were required, but the above species vary greatly in the amount of growth which is obtained in an artificial medium. Pure strains of all the organisms used were kept on agar slopes, the strains being maintained by subculturing every two or three weeks. Some species, such as Chlorella

vulgaris and Prototheca Zopfii, were grown entirely on agar slopes, as very thick cultures can be obtained in this way. Liquid media or soil water media were used when these conditions favoured growth.

Culture vessels of Pyrex glass were used whenever possible. All glassware was cleaned in a mixture of potassium dichromate and sulphuric acid and then washed carefully to remove all traces of the cleaning solution. Glass distilled water was used in the making up of all media.

Agar slopes were prepared in large test tubes or in flat bottles in order to obtain a large surface, these containers being found more suitable than petri-dishes. The required constituents in solution, together with sufficient agar to make a 1.5% solution were heated in a steam chamber until the agar dissolved. The liquid was then transferred into the tubes without moistening the inner parts of the rims, and the tubes were plugged with cotton wool. These were then autoclaved, cooled and finally allowed to solidify in an oblique position.

The quality of the slopes was improved by autoclaving the nutrient materials and agar separately (Pringsheim 1946a). A concentrated solution of nutrient substances, and a mixture of agar and water were autoclaved, together with the test tubes required. The agar was soaked overnight before heating. After sterilization the agar and nutrient solutions were mixed and transferred rapidly

to the auto-claved tubes. The tubes were then steamed for 15-20 minutes before sloping to ensure complete sterilization. Agar slopes prepared in this way are more elastic. They do not break when shaken, and are not easily penetrated by a wire loop such as is used for innoculation (Pringsheim 1946a).

There is some discussion as to the length of time required for complete sterilization. Many authors state that a pressure of 15 - 20 lbs. for 20 - 30 minutes is necessary in order to sterilise media. This intensive heating is not considered necessary by Pringsheim. Autoclaving is carried out at a pressure of two atmospheres, the pressure is allowed to remain at two atmospheres for two minutes when the source of heat is turned off (Pringsheim 1946a). I have used both methods of sterilization. Autoclaving at 15 lbs. pressure for 20 minutes was not found to cause the quality of the medium to deteriorate noticeably. Heating in a steam chamber for half an hour on three consecutive days was used as an alternative method of obtaining pure cultures.

A soil-water medium was not used extensively as a culture medium. It was considered preferable to use a medium of known composition which could be reproduced exactly. Soil-water media are however valuable, for as Pringsheim (1946a) has shown, many species show prolonged development in a medium containing soil. Subcultures of many Volvocales, such as Gonium and Chlamydomonas, often

fail to multiply in liquid media, but motility can be restored by transference to a soil-water medium (Pringsheim 1946a).

Soil-water cultures were prepared in jam jars as described by Pringsheim (1946b). For chlorophyll containing organisms which prefer an alkaline medium, such as Gonium and Chlamydomonas, calcium carbonate was placed in the bottom of the jar followed by an inch of ordinary garden soil, which had been previously dried and sifted. Glass distilled water was then added, to within an inch of the top of the jar, and the jars covered with grease proof paper. For organisms such as Polytoma which do not contain chlorophyll, 4 or 5 wheat grains were placed in the bottom of the jar with calcium carbonate, before the addition of the soil. The jars are heated in a steam chamber for one hour, on three consecutive days.

Each food organism may be considered separately. (a) Gonium pectorale. Different media were tried for culturing Gonium pectorale, but it was found to grow well in Chu's medium No.10 (Chu 1942) with the addition of a small quantity of beef extract. A 0.03 - 0.04% concentration of beef extract was found to be adequate. The pure strain was kept on agar in a similar medium. Gonium was found to grow better in liquid culture at a pH of 6.8 - 7.2. The pH of the medium varied with the quantity of beef extract added, but was adjusted by the addition of small quantities of sodium carbonate solution.

Although Gonium multiplies well, it is short lived in pure liquid culture, and it sometimes loses its colour rapidly and disappears (Pringsheim 1946a). Gonium grows well in soil - water cultures in which the strain can be maintained. I made subcultures of Gonium from soil - water cultures into pure liquid media, and in this way thick cultures of Gonium were obtained. Cultures of Gonium were kept in a healthy state by subculturing at intervals from a fresh strain of Gonium obtained from a soil - water medium.

b) Chlamydomonas incisa was grown on agar in a medium of the following composition:-

Sodium acetate Beef extract	0.02%
Bacto - Tryptone Yeast extract	0.04%
Agar	1.5%

The pH of the medium was adjusted to 7.5 by the addition of sodium carbonate solution. Chlamydomonas was also grown in liquid medium in soil-water culture.

c) Chlorella vulgaris was cultured in bacteria-free condition on agar slopes, using the medium described by Pearsall and Loose (1937). This medium, which contains a high percentage of glucose, supports rich growths of Chlorella. It is necessary not to autoclave above 11 lbs. pressure, in order to prevent charring of the glucose.

d) Polytoma uvella was grown on agar in the following medium:-

Sodium acetato Bacto-Tryptone	0.1%
Yeast extract	0.2%
Agar	1.5%

The pH of the medium was adjusted to 7.5, as previously. Polytoma was also grown in soil-water cultures.

e) Prototheca Zopfii was grown successfully on agar slopes, of the following composition:-

Malt extract	2%
Glucose	5%
Agar	1.5%

The media used for Chlamydomonas, Polytoma and Prototheca were suggested by Pringsheim.

Cultures of algae containing chlorophyll were not exposed to direct sunlight. They were given continuous artificial illumination from a 100 watt. bulb at a distance of 20-30 cms. When the cultures had almost attained maximum growth they were transferred to a north window, where they obtained illumination over a shorter period of time. To avoid overheating of the cultures, and the drying out of the media, a water screen was placed between the lamp and the culture flasks or tubes. Two cylindrical vessels were placed one inside the other, the space between being filled with water, and the bulb being suspended inside the inner one. The water screen

was cooled by means of a coil of copper tubing, through which a constant stream of tap water was allowed to run (Pringsheim 1946a). This was found to be more satisfactory than a direct system of cooling in which the bulb was immersed im running water, as algae tended to accumulate on the glass.

Cultures of colourless organisms were grown in the dark.

In controlled feeding experiments, it is essential to be able to give exactly the same amount of food to comparable experimental flasks. It is also an advantage to know the exact amount of food that has been added. Algae which are grown in liquid culture can be used directly. To avoid the addition of culture medium to the experimental flasks a known volume of algae was centrifuged, and the culture medium was then removed. The concentrated algal cells were washed into the flasks with a little of the pond water. Oxygen tests made before and after the addition of algae showed that this method of addition made no significant difference to the oxygen content of the flasks.

Algae were not washed directly from the agar slopes into the flasks containing Daphnia, as in this way the exact amount of food added is not known. I have washed algae from agar with filtered pond water, or filtered Regent's Park lake water. Filtered pond water was used if this was practicable, the water being taken from the

pond which was being used in the experiment. In this way a concentration of algal cells was obtained in a relatively small amount of water. A known volume of this algal suspension was then centrifuged, and the concentrated algal cells were washed into the flasks as before.

The algal suspensions were made up each day. When liquid cultures are used, many cultures are needed for each experiment. Although it is possible to feed flasks equally, or in a known ratio, when using different algal suspensions from agar, or different culture flasks of algae each day, it is not possible to have quantitatively the same feeding in different experiments, unless some method of comparing different algal cultures or algal suspensions is used.

An artificial standard was used consisting of solutions of potassium chromate and nickel sulphate with the addition of kieselguhr. In this way a green suspension was obtained which closely resembled an algal suspension. The algal suspension and standard were compared using a colorimeter. This standard was used for Chlorella grown on agar slopes, and also for Gonium in liquid culture. In the case of Chlorella, the algal suspension in lake water was adjusted until it matched the standard, the same volume of algal suspension was therefore added each day. Algal cultures of Gonium were compared with a similar standard, but differences in the algal cultures were

compensated by the addition of different volumes of algae to the experimental flasks.

A more exact method of feeding involves the counting of the number of algal cells in a certain volume of the algal suspension, using a Fuchs Rosenthal haemacytometer. This method was used when comparing the relative food values of Chlamydomonas and Polytoma. Suspension of Chlamydomonas and Polytoma were obtained by shaking the cells from agar into lake water. The number of algal cells was then counted in a certain volume of a diluted sample of each of these suspensions, the algae being immobilised by dilution in a weak solution of formalin. When the number of cells per cu.mm. in each algal suspension was known, the volumes of each algal suspension to be added to the experimental flasks could be calculated. This will be described more fully later.

Several preliminary experiments were made to determine the optimum concentration of algae to be added to the Daphnia cultures, and the frequency of feeding required for good nutrition. For this purpose Daphnia was required with an empty gut. Daphnia have been placed in a kieselguhr suspension to remove the gut contents (Von Dehn 1930) but the emptying of the gut by inert powders was found unsuitable by Lefèvre (1942). Rapid evacuation of the gut by placing Daphnia in a warmer medium and continual fasting to obtain completely empty guts were found by Lefèvre (1942) to weaken Daphnia so that they were

not suitable for experiments.

The Daphnia used in my tests had been placed in successive large volumes of tap water and it was found that in a short time their guts were completely or mainly evacuated. Such Daphnia was then placed in flasks to which different concentrations of Gonium pectorale were added. The condition of the animals was judged by examination of the gut contents and the colour of the liquid in the space between the peritrophic membrane and the gut wall. It was found that daily additions of algae maintained Daphnia in a better fed condition than feeding on larger quantities of algae at the beginning of an experiment, or every second or third day. The exact quantity added varied with the volume of the liquid in the flask, and the culture used.

4. THE INFLUENCE OF POND WATERS CONTAINING RED DAPHNIA IN NATURE ON HAEMOGLOBIN SYNTHESIS:

When pale Daphnia was placed in water from a pond in which the Daphnia was very red, the haemoglobin index of the pale Daphnia increased, although the amount of dissolved oxygen was not at all low (Fox 1948). The faeces of ducks were found to promote haemoglobin formation more than oxygen deficiency alone (Fox 1948). These facts suggested that there may be some other factor in pond water in which Daphnia is red, and in duck faeces, responsible for haemoglobin production. I have compared the haemoglobin production in pond waters from different localities.

Fifty pale D. obtusa from Chingford Common were placed in 200 cc. of pond water from different localities, Parrot House pond water, Gull pond water, and Brent park pond water being used. The water before use was filtered through very fine bolting silk, in order to remove any Daphnia present, but to leave the water as unchanged as possible. The Daphnia from Chingford Common had been kept in the laboratory for five days in their own water, during which time the haemoglobin index was reduced. The experimental flasks were kept in duplicate in the dark at 15°C and 5°C. The low temperature was chosen as field work had shown that with the onset of cold weather two populations of Daphnia had changed from practically colourless to pale pink.

Gonium pectorale was added daily. Parrot House pond water was pale green because of the presence of algae in the water, but it was considered that the quantity of Gonium added would be more than sufficient for the needs of the Daphnia. The water in each flask was changed twice during ten days, fresh pond water being fetched from each locality. Daphnia was examined after seven days, and detailed observations were made on Daphnia taken at random from the experimental flasks at 15°C and 5°C on the 11th and 12th days respectively (Table 1).

During the experiment recordings were made of the Daphnia in the localities from which the pond waters were taken (Table 11).

TABLE 1

The effect on the haemoglobin index of keeping pale *D.obtusa* for 11-12 days in various pond waters which in nature contain red *Daphnia*. *Gonium pectorale* was used as food. *Daphnia* was taken at random from the flasks.

Experiment No.	1	2	3	4	5	6	7	8	9	10	11	12
Temperature °C.	15						5					
Pond	Brent		Parrot		Gull		Brent		Parrot		Gull	
Oxygen, % air sat. Last day	74	86	56	61	83	90	71	80	36	47	83	73
Haemoglobin index.												
1st day	28	28	28	28	28	28	28	28	28	28	28	28
Last "	39	41	60	67	33	34	27	25	24	25	28	24
Number of Young												
1st day	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Last "	9.7	7.1	15.3	14.4	3.4	3.1	0.9	0.3	0.1	0.9	0.6	0.3
Size, mm. Last day	2.1	2.1	2.3	2.2	2.0	2.0	2.0	1.8	1.9	2.0	1.8	2.0

TABLE 11

Data concerning Daphnia inhabiting the pond waters used in the experiments of Table 1. Obtained at the same time, on Daphnia taken at random from the populations.

Pond	Brent	Parrot / Gull		
Species	D. magna	D. magna	D. pulex	D. obtusa
Haemoglobin index	98	90	49	63
Number of young	0.2	3.5	4.2	4.8
Size, mm.,	4.1	4.9	1.9	2.0

At 15°C the haemoglobin index rose in experimental Daphnia in the laboratory in all waters, but especially in Parrot House pond water, although in these flasks the oxygen concentration was somewhat lower. At 5°C there was no effect on the haemoglobin index, although the oxygen content of the flasks was approximately the same as at the higher temperature. In the Parrot House pond water flasks, the oxygen concentration was lower at 5°C than at the higher temperature. Haemoglobin production does not occur at 5°C, a higher temperature favours its formation.

There was also a difference in the average size attained by Daphnia in the different flasks, and in the number of eggs and embryos produced. At 5°C there was a small increase in average size of not more than 0.2 mm. and the number of parthenogenetic young was the same or less than the average number found in nature. At 15°C the average size had increased by 0.2 to 0.5 mm. The Daphnia kept in Parrot House pond water had attained the greatest size. The number of parthenogenetic young produced in all Daphnia at 15°C was greater than the average number found in nature. The production of young in Parrot House pond water was much greater than in other pond waters.

It can be seen from these results that although more haemoglobin was produced by Daphnia in Parrot House pond water at 15°C than in Brent park or Gull pond water, the experimental Daphnia attained a greater size and produced more young. Similarly the haemoglobin index of Daphnia in

Brent park pond water at 15°C was slightly greater than in Gull pond water but again the average number of parthenogenetic young and the size were greater than the values obtained for Daphnia in Gull pond water.

Fat globules were formed in all Daphnia, their occurrence being more common in Daphnia kept at low temperature.

The duration of the above experiment was 11-12 days, although even after 7 days some haemoglobin production had been observed. The water in the flasks had also been changed twice during this period. The above experiment in Parrot House pond water was repeated for a shorter period of time, and without a change of water.

Pale D. obtusa from Chingford Common were again used in Parrot House pond water, and as a control pale Daphnia were kept in their own water under similar conditions. The flasks were set up in the following way, giving a range of oxygen concentrations.

110	D. obtusa	in	220	cc.	water
100	"	"	200	"	"
90	"	"	180	"	"
80	"	"	160	"	"
70	"	"	140	"	"
60	"	"	120	"	"

in Chingford and Parrot House pond water. The pond waters were filtered through fine bolting silk in the same way, and were air saturated before use. Gonium pectorale was

added daily. The temperature varied from 17 - 18.5°C. On the 6th or 7th day the haemoglobin index of Daphnia taken at random from each flask was found and other observations were also made.

A range of oxygen concentrations was obtained in each pond water, so that Daphnia in both pond waters could be compared at the same oxygen concentration. Although the oxygen concentration was not very low, varying from 50 - 100% air saturation, haemoglobin production was found to have occurred in Parrot House pond water, but not in Chingford water at the same oxygen concentration (Table 111). A change of water during the experiment was found to be unnecessary, 6-7 days being sufficient for measurable haemoglobin formation.

The average number of parthenogenetic young in all flasks was greater than the average found in nature, but as in the previous experiment, the Daphnia in Parrot House pond water produced a considerably greater number of eggs or embryos than the Daphnia in their own water. The average size of Daphnia in Parrot House pond water was also slightly greater.

Before exact comparisons can be made, the experimental Daphnia must be in the same condition. A higher haemoglobin index was obtained for Daphnia in Parrot House pond water in both experiments, but these Daphnia were producing more young and were slightly larger than Daphnia in their own water or in another pond water in the

TABLE 111

The difference in haemoglobin production in pale *D. obtusa* kept for 6-7 days in its own water and in water of another pond which in nature contained red *Daphnia*. *Gonium pectorale* was used as food. *Daphnia* was taken at random from the flasks.

Experiment No.	1	2	3	4	5	6	7	8	9	10	11	12
Water	Own pond						Another pond					
Oxygen, % air sat.												
Last day	86	91	95	101	102	103	53	73	89	89	93	101
Haemoglobin index												
1st day	26	26	26	26	26	26	26	26	26	26	26	26
Last "	23	24	24	26	22	21	42	37	34	35	30	26
Number of Young												
1st day	0	0	0	0	0	0	0	0	0	0	0	0
Last "	2.9	1.7	2.0	3.2	2.8	1.5	11.6	11.4	10.1	9.7	9.6	10.4
Size, mm. Last day	2.1	2.0	2.0	2.1	2.0	2.0	2.1	2.1	2.1	2.1	2.1	2.1

same experiment. These animals must therefore have been receiving better nutrition than those with which they were being compared, as an increase in the amount of food available causes a corresponding increase in the egg production of Daphnia (Ingle, Wood and Banta 1937). The Daphnia present in Parrot House pond when the water samples were taken had a high average number of parthenogenetic young of 35 (Table 11), so that in nature the red Daphnia present in the pond were obtaining good nutrition.

The pond water used in these experiments was filtered through fine bolting silk as explained previously, and algae already present in the water were not removed. Parrot House pond water contained several different species of algae, but Gonium pectorale was added daily to each flask, and comprised the bulk of the food material. The difference in nutrition in Parrot House pond water flasks was not only a quantitative difference, but also a qualitative one, as it has been shown that Daphnia are better nourished when fed on two strains of algae, than when reared on a single pure strain (Pacaud 1939). A single species of algae probably does not supply all the elements needed by the animal.

These results are similar to those obtained previously (Fox 1948), as a greater increase in haemoglobin production occurred when pale Daphnia were placed in water which in nature contained red Daphnia, than when they were

placed in their own water. But the conditions of nutrition in the two cases, as indicated by the number of young and size, were not the same.

The haemoglobin production of Daphnia in its own water, and in water from a pond containing red Daphnia in nature, was compared under the same feeding conditions, in water of low oxygen content. Pale D. pulex from Golder's Hill park was placed in its own water, and in Miflin pond water previously deoxygenated by nitrogen to the same percentage air saturation. Miflin pond water was used, as in nature the pond contained D. pulex with a haemoglobin index of 110. The flasks were arranged as follows:-

155	D. pulex	in 310 cc. own water	) With Gonium
160	"	320 cc. "	) pectorale
163	"	325 cc. "	) proportional to
165	"	330 cc. "	) the volume of
			) water.

Similar flasks were assembled in Miflin pond water. Determinations of the oxygen content of the water were made during the experiment. Gonium pectorale was added daily. After 6 or 7 days observations were made on Daphnia taken at random from the experimental flasks (Table IV).

Haemoglobin production took place in Daphnia in both waters, and the average number of parthenogenetic young formed was greater than the average in nature. Examination of the gut space showed that a gut space

TABLE IV

The difference in haemoglobin production in pale D. pulex kept for 6-7 days in its own water and in another pond water, which contained red Daphnia in nature. Gonium pectorale was used as food. Daphnia was taken at random from the flasks.

Experiment No.	1	2	3	4	5	6	7	8
Water	Own pond				Another pond			
Oxygen, % air sat.								
2nd day	12	10	9	13	12	13	17	25
4th "	7	8	6	13	10	12	10	48
6th "	7	11	10	29	12	12	11	62
7th "		7	34			10		55
Average Oxygen, % air sat.	9	9	15	18	12	12	13	45
Haemoglobin index								
1st day	25	25	25	25	25	25	25	25
Last "	56	46	42	43	38	32	33	26
Number of Young								
1st day	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Last "	1.9	3.1	1.1	2.0	2.5	1.7	3.5	0.8
Size, mm. last day	1.9	2.1	2.2	2.2	2.0	2.1	2.1	2.1

originally pale yellowish-green became bright yellowish-green. This feeding was therefore considered to be adequate.

It is important to note that the haemoglobin content of Daphnia in Miflin pond water is not greater than in their own water at the same oxygen concentration. The average number of parthenogenetic young produced, and the average size measurements are approximately the same for the animals in both waters. Thus Daphnia placed in a pond water which in nature contains red Daphnia does not produce more haemoglobin than in its own water at the same oxygen concentration, when under equal feeding conditions.

In this experiment less haemoglobin was produced by Daphnia in Miflin pond water than in their own pond water at the same oxygen concentration. This may be the result of a change of medium. It has been shown that a sudden transference from one water to another causes a weakening of the race, if the difference between the two waters is great (Warren 1900).

The results obtained previously, which suggested the existance of some factor in pond waters which contain red Daphnia in nature, capable of inducing haemoglobin formation were therefore probably the result of differences in nutrition. Subsequent experiments have been carried out on the same plan (Dresel unpublished) which support this conclusion.

It was therefore considered necessary to investigate the influence of nutrition, quantitatively and qualitatively on haemoglobin synthesis.

5. THE INFLUENCE OF NUTRITION ON HAEMOGLOBIN SYNTHESIS

a) Previous work:

The red colour of Daphnia has been associated with good nutrition, although the colour has not always been attributed to haemoglobin. The blood of pale, starved Daphnia was found to become a reddish colour, when the animals were fed on algae (Fritzsche 1917), but the oxygen content of the medium was not discussed. Fritzsche concluded that the red colour of Daphnia is due to carotene, and is only found in well nourished animals with a high blood osmotic pressure. Schulz (1928) accepted the observations of Fritzsche, and considered that the red colour of Daphnia is due to carotene. He states that the red colour is never produced in darkness, even under good feeding conditions.

Verne (1923) studied the synthesis of haemoglobin in Daphnia. Pale D.pulex which had lost their colour in the laboratory were used. His experiment was divided into three series:-

(1) With the addition of leaf debris, or algae rich in chlorophyll macerated in the water. A trace of iron was added.

(2) With cultures of "zooglœes" without chlorophyll, and without detectable iron.

(3) the previous medium, with the addition of iron.

After three weeks, haemoglobin was present in the Daphnia of series (1), but was not present in the Daphnia of series (2) or (3). Verne states that "zooerythrine"

(with reactions similar to the carotinoids of Decapod Crustacea) caused a reddish colour in the Daphnia of all series. He concluded from this that haemoglobin formation depends on the presence of chlorophyll or of its breakdown products in the medium. Verne does not give details of his experiments.

As a result of his Daphnia experiments Verne (1924) suggests that animals in general, which are able to break down chlorophyll as far as porphyrin, use this in haemoglobin formation, although he does not show that Daphnia breaks down chlorophyll to a porphyrin.

The influence of different quantities of algal food, and the effect of the presence of chlorophyll in food have been considered.

b) The Influence of Minimal Nutrition

The haemoglobin production was compared in fed and not fed D.pulex from Golder's Hill Park pond in their own water. Gonium pectorale was used as food. The culture flasks were kept in the dark, and oxygen determinations were made at intervals during the experiment. The gut contents and gut liquids were periodically examined to ensure that feeding was adequate.

Preliminary experiments were made in which Daphnia was kept in conical flasks in previously aerated water. For example, in one case 60 Daphnia in 240 cc. 55 in 220 cc. and 50 in 200 cc. in duplicate flasks, without food, and again with Gonium pectorale as food. This provided Daphnia in

flasks at a range of oxygen concentrations, varying from 78 - 94% air saturation. The original haemoglobin index was 25. Oxygen determinations were made during the experiment, and on the sixth day, the Daphnia of each flask were examined. Two flasks were selected at an oxygen concentration of 82% air saturation, the haemoglobin indices were found not to have changed significantly being 27 and 24 for fed and not fed Daphnia respectively. The oxygen concentration was therefore too high for haemoglobin synthesis. The Daphnia were fed at the beginning of the experiment only.

Ingle, Wood and Banta (1937) have shown that well fed Daphnia grow more rapidly and reproduce in greater numbers than animals on limited food. The number of young produced has been used as an index of the general metabolic condition of Daphnia (Anderson and Jenkins 1942). The eggs formed in one instar are deposited in the brood pouch after ecdysis, so that the growth in, for instance, instar 5 must be correlated with the number of young produced in instar 6. The duration of each instar is known to increase with the age of Daphnia: at room temperature the first three instars of D.pulex last one day, the fourth instar 1½ days, the 5th instar two days and subsequent instars last longer (Anderson, Lumer and Zupancic 1937).

In a feeding experiment of 12-13 days, the influence of different concentrations of food is shown clearly in the average number of parthenogenetic young produced. In the same experiment, after 6-7 days the

number of parthenogenetic young formed is less, but the values obtained are in the same ratio, and show the influence of different concentrations of food (Table V11). The duration of some experiments of 6-7 days was therefore sufficient for the influence of feeding to be shown by the average number of parthenogenetic young, although for quantitative feeding experiments a period of 12-13 days was allowed.

The pale Daphnia used in experimental work have generally had a small average number of young. It has been shown that when well-fed Daphnia are transferred to a medium with less food, they promptly approach a level of reproductive activity about equal to that of animals kept at the lower feeding value (Dunham 1938). It is therefore also satisfactory to use pale Daphnia from nature which have a high average number of young.

In the experiment described above the average egg numbers were 1.6 and 0.2, and the average sizes of the animals 2.0 mm. and 1.9 mm. for algal-fed and not-fed Daphnia respectively. Although the number of young and average size are thus slightly greater for Daphnia fed on Gonium, the addition of a large quantity of algae at the beginning of an experiment does not maintain Daphnia in a well fed condition, as shown by the colour of the gut space. A heavy mortality occurred when Daphnia was not fed.

The above experiment was repeated in conditions of oxygen lack. Pale D.pulex was placed in its own water

previously deoxygenated by nitrogen to an oxygen concentration of approximately 10% air saturation. Larger numbers of animals were used than in the previous experiment, for example 130 Daphnia were placed in 260 cc. own water. The experiment was divided into two series, half of the animals were not fed, while additions of Gonium pectorale were made to the remaining flasks, twice during the experiment. After six days observations were made on Daphnia taken at random from the flasks (Table V).

The haemoglobin index of Gonium-fed animals rose from 18 to 46 in amounts inversely proportional to the oxygen content of the medium. A small amount of haemoglobin was produced in not-fed animals. A few parthenogenetic young were formed in algal-fed Daphnia, the average size was slightly greater than that of starved individuals, and fat globules were produced. No fat globules and no parthenogenetic young were produced in not-fed Daphnia.

The gut liquid of algal-fed Daphnia remained a yellowish-green as in nature, and did not become a bright yellowish green, characteristic of Daphnia which have been supplied with abundant algal food. Although feeding in this experiment was very low, as indicated by the small number of young produced, it was sufficient to show that more haemoglobin is produced in Daphnia which are fed, than in starved animals, at the same oxygen concentration.

Flasks containing Daphnia were kept in the dark to avoid oxygen production by algal photosynthesis. The

TABLE V

The effect of feeding with *Gonium pectorale* on the haemoglobin index of *D.pulex* kept in its own water. *Daphnia* was taken at random from the flasks.

Experiment No.	1	2	3	4	5	6
Food	None			Gonium		
Oxygen, % air sat.						
1st day	14	22	13	12	19	13
2nd "	14	12	30	17	13	23
4th "	23	23	53	9	14	34
6th "	30	44	69	13	20	49
Average Oxygen, % air sat.	20	25	41	13	17	30
Haemoglobin index						
1st day	18	18	18	18	18	18
6th "	30	31	23	44	46	30
Number of Young						
1st day	3.1	3.1	3.1	3.1	3.1	3.1
6th "	0	0	0	0.5	0.9	0.2
Size, mm. 6th day	1.9	2.0	1.7	2.2	2.1	2.5

haemoglobin production obtained under these circumstances directly contradicts the observation of Schulz (1928), that a red colour never appears in darkness even with good nutrition.

The experiment was again repeated in conditions of oxygen lack, D.pulex being placed in its own water and in another pond water, previously deoxygenated by nitrogen to the same percentage air saturation. The flasks were arranged as follows:-

155	D.pulex	in 310 cc. own water	With Gonium pectorale proportional to the volume of water.
160	"	320 cc. "	
163	"	325 cc. "	
165	"	330 cc. "	
160	"	320 cc. "	Not fed.
165	"	330 cc. "	

Similar flasks were assembled in another pond water, namely Miflin pond water. Some of the results of this experiment have been considered earlier (Table IV). Determinations of the oxygen content of the water were made during the experiment. Gonium pectorale was added daily. After 6 or 7 days observations were made on Daphnia taken at random from the experimental flasks. The complete results (including the results expressed in Table IV) are summarised in Table VI.

No haemoglobin production was observed in starved Daphnia, and no parthenogenetic young were formed.

TABLE VI (including results given in Table IV.)

The effect of feeding with Gonium pectorale on the haemoglobin index of D.pulex kept for 6-7 days in its own water and in another pond water. Daphnia was taken at random.

Experiment No.	1	2	3	4	5	6	7	8	9	10	11	12
Water	Own pond						Another pond					
Food	None		Gonium				None		Gonium			
Oxygen, % air sat.												
2nd day	9	9	12	10	9	13	14	27	13	13	17	25
4th "	15	13	7	8	6	13	16	14	10	12	10	48
6th "	6	12	7	11	10	29	12	15	12	12	11	62
7th "				7	34					10		55
Average Oxygen, % air sat.	10	11	9	9	15	18	14	19	12	12	13	45
Haemoglobin index												
1st day	25	25	25	25	25	25	25	25	25	25	25	25
Last "	27	30	56	46	42	43	24	22	38	32	33	26
Number of Young												
1st day	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Last "	0	0	1.9	3.1	1.1	2.0	0	0	2.5	1.7	3.5	0.8
Size, mm. last day	1.9	1.9	1.9	2.1	2.2	2.2	1.8	1.8	2.0	2.1	2.1	2.1

Haemoglobin production took place in Daphnia which had been fed on algae, and the average number of parthenogenetic young formed was larger than the average in nature. Examination of the gut space showed that a gut space originally pale yellowish-green, became colourless or very pale in starved Daphnia, but in Gonium-fed animals became a bright yellowish-green. This feeding was therefore considered to be adequate.

It was also noticed that feeding on Gonium resulted in the formation of abundant or very abundant fat globules, the Daphnia at the beginning of the experiment having no fat or very little fat, and the starved individuals having no visible fat globules. This may not be a direct effect of feeding, but may be a result of overcrowding in the fed cultures (Smith 1915) as the mortality in these flasks was negligible.

Feeding is essential over a period of 6-7 days as judged by the rate of survival of fed and not fed Daphnia. In flasks to which Gonium had been added, very few or no deaths occurred. The number of young produced indicated that the animals were well fed. Nutrition is therefore essential to maintain Daphnia in a fit metabolic condition for haemoglobin synthesis.

Whether minimal nutrition alone is necessary, or whether an increase in food would cause a further increase in haemoglobin production requires investigation. It is also necessary to consider nutrition from a qualitative

aspect, to find out whether the haemoglobin synthesis is being influenced by chlorophyll or its derivatives in the food.

c) The Influence of Increasing Concentrations of Algal Food on the Haemoglobin Index

Pale D. obtusa was placed in its own water previously filtered through No.1 Whatman filter paper to remove algae and other small organisms. The water was deoxygenated by nitrogen to an oxygen concentration of approximately 20% air saturation before use. The experiment was divided into four series, so that four different concentrations of food could be used, each series consisting of three flasks. The flasks contained different volumes of pond water, and had different surface areas, so that a range of oxygen concentration was obtained in each series.

The number of Daphnia in each flask was proportional to the volume of pond water used, so that all the experimental animals were under exactly the same conditions. The ratio of 1 Daphnia: 2 cc. pond water was maintained in all flasks, for example, 165 Daphnia were placed in 330 cc. pond water, 160 Daphnia were placed in 320 cc. pond water. The oxygen concentration of the water in each flask was determined every second day.

The duration of the experiment was 12-13 days, during which time two algae were used as food. Gonium pectorale was used for the first eight days, and Chlorella vulgaris for the remaining four or five days. Chlorella was washed

from agar in filtered lake water, which, like the culture liquid of Gonium was removed by centrifuging. At the end of eight days the water in each flask was changed, so that the two algae were not present in the flasks at the same time.

The volume of algal food added to the flasks in the four series increased in the ratio of 1: 2: 4: 8. A flask containing 330 cc. of pond water and 165 Daphnia received 4, 8, 16 or 32 cc. of centrifuged algal solution daily, according to the series in which it was placed. In each series the three flasks contained different volumes of water. If the flask did not contain 330 cc. of pond water, the volume of algal solution added was adjusted proportionally, so that the Daphnia in the three flasks of each series received exactly the same amount of food.

On the 6th or 7th day, observations were made on Daphnia taken at random from each flask. The water in the flasks was then replaced by fresh pond water, filtered as before, and deoxygenated by nitrogen to the original oxygen concentration. A replacement of water was considered necessary to remove the excretory products of Daphnia, and to prevent too large an accumulation of algae in the water, as this would cause the oxygen concentration to fall to very low limits. When the water change was made the number of survivors was noted. Any loss of Daphnia was compensated where possible by a decrease in the volume of the water, so that the relationship between the number of

Daphnia and the volume of the water remained approximately constant.

On the 12th and 13th day, observations were made on Daphnia taken at random from each flask. Daphnia in which the brood pouch contained embryos possessing eyes were examined at the same time. The results are given in Tables V11 (a) and (b).

The influence of quantitative algal feeding can be seen in the results obtained from Daphnia taken at random from the different flasks (Table V11a). After 6-7 days the average number of parthenogenetic young present was greater than at the beginning of the experiment. The number of young increased in proportion to the amount of food added, as far as the third feeding value, after which doubling of this concentration of food did not cause a greater production of parthenogenetic young. The increase in the haemoglobin content of the Daphnia at the same feeding value was proportional to the oxygen concentration of the water. When results at the same average oxygen concentration are considered, it is seen that the haemoglobin index increased with increasing concentration of food to the third feeding value, after which a further increase in nutrition did not cause more haemoglobin production.

After 12-13 days the same result was obtained, increasing concentrations of food caused the haemoglobin index and number of parthenogenetic young to increase to

TABLE VII

The influence of different concentrations of algal food on the haemoglobin production of D.obtusa kept in its own water for 12-13 days. Gonium pectorale and Chlorella vulgaris were used as food. (a) Daphnia was taken at random from the experimental flasks.

Experiment No.	1	2	3	4	5	6	7	8	9	10	11
Food Ratio		1			2		4			8	
Oxygen % air sat.											
2nd day	14	14	23	12	16	14	15	20	10	8	11
4th "	9	15	22	14	16	9	11	18	5	5	11
6-7th "	6	5	33	6	9	3	5	13	0.9	1	3
9th "	4	9	34	3	13	2	3	10	4	2	2
10th "	5	3	12	5	13	9	5	8	7	12	17
12-13th	7	6	12	2	7	3	3	5	6	7	7
Average Oxygen % air sat.	8	9	23	7	12	7	7	12	6	6	9
Haemoglobin index											
1st day	21	21	21	21	21	21	21	21	21	21	21
6-7th "	40	40	31	55	48	65	62	70	65	72	59
Last "	59	56	52	65	58	74	78	83	84	82	81
Number of Young											
1st day	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
6-7th "	0.9	0.8	0.7	1.6	1.6	4.1	4.2	4.6	3.8	1.5	5.7
Last "	2.3	2.0	2.3	3.8	5.4	8.7	8.7	13.5	7.2	7.1	8.9
Size, mm.											
6-7th day	1.9	2.0	1.9	2.0	2.1	2.0	2.0	2.1	1.9	2.0	2.1
Last "	2.0	2.0	2.0	2.3	2.2	2.2	2.1	2.4	2.1	2.1	2.1

TABLE VII (continued)

b) Daphnia with eyed-embryos were taken from the experimental flasks.

Experiment No.	1	2	3	4	5	6	7	8	9	10	11
Food Ratio	1			2		4			8		
Average O_2. % air sat: as in Table VII(a).	8	9	23	7	12	7	7	12	6	6	9
Haemoglobin index											
1st day	21	21	21	21	21	21	21	21	21	21	21
Last "	60	58	53	69	51	37	87	87	94	89	87
Number of embryos											
1st day	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Last "	2.3	2.1	1.9	7.4	5.9	8.2	7.2	7.2	6.6	5.7	7.9
Size, mm.											
Last day	2.0	1.9	1.9	2.2	2.2	2.1	2.0	2.3	2.0	2.0	2.1

the third feeding value. The difference between the average number of young produced in Daphnia at the second and third feeding values was slight, and not as great as previously, but there was no increase in either the number of young produced or in the haemoglobin index at the fourth feeding series.

Daphnia in which the parthenogenetic young were at the eyed-embryo stage showed the same result (Table VIIb). An increase in the amount of food added caused a corresponding increase in the number of embryos and in the haemoglobin index, to a maximum at the third feeding value. Doubling of this concentration of food resulted in no further change. The haemoglobin production of Daphnia at different feeding values must be compared at the same oxygen concentration.

The gut space of the experimental Daphnia which was originally pale yellow or pale green became yellowish green as a result of algal feeding.

The fat content of the Daphnia was increased during the experiment. At the beginning of the experiment, Daphnia had no fat globules, but after 12-13 days the fat globules were frequent or very common in all animals. The fat content was approximately the same at all feeding values. This suggests that fat production is not a direct result of feeding.

The above experiment was repeated, again using D.obtusa in its own water previously filtered through

No.1 Whatman filter paper, and deoxygenated by nitrogen to an oxygen concentration of approximately 20% air saturation. The experiment was divided into five series, five different concentrations of food were used, and each series consisted of three flasks. As in the previous experiment, in order to obtain a series of oxygen concentrations the volumes of pond water used were varied from 200-210 cc. so that different surface areas were obtained in the different flasks. Slightly larger surface areas were used at higher feeding values. The number of animals in each flask was, as in the previous experiment, half the volume in cc. of the pond water present. Oxygen analyses of the water were made every day, as far as possible.

Chlorella vulgaris was used as food during the whole experiment. Chlorella was washed from agar in filtered lake water, and centrifuged before adding to the experimental flasks. The Chlorella suspensions in lake water were made freshly each day. Approximately equal suspensions were obtained by comparing the algae in lake water with a standard green suspension of the following composition:-

4 cc. saturated nickel sulphate solution

0.52 cc. " Potassium chromate solution

0·2 gm. kieselguhr

Distilled water to increase the volume to 20 cc.

The algal suspension and the above standard were compared with the aid of a colorimeter. Readings were taken rapidly before the algal cells or kieselguhr began to settle. In this way Daphnia was fed approximately

equally every day.

The volume of algal suspension added to the flasks in the five series increased in the ratio of 1: 2: 4: 8: 16. A flask containing 200 cc. of pond water and 100 Daphnia was supplied with 2, 4, 8, 16 or 32 cc. of centrifuged algal suspension daily, depending on the feeding value. In each series the exact volume of algal solution that was added varied according to the amount of water and the number of Daphnia present. For example:-

105 D. obtusa in 210 cc. own water plus 2.1 cc. algae
110 " 220 cc. " plus 2.2 cc. "

The concentrations of algal food used in this experiment cannot be compared with those of the previous experiment, as the volumes of water used in the experimental flasks was not the same. In this experiment also, one organism, Chlorella vulgaris was used as food, whereas in the previous experiment Genium pectorale and Chlorella vulgaris were used. The concentrations of the algal suspensions also varied in the two experiments.

After six days the water in each flask was replaced by fresh pond water, filtered and deoxygenated as previously, to the original oxygen concentration.

After 12-13 days, observations were made on the Daphnia in all flasks. Flasks in which the average oxygen concentration of the water was approximately the same were examined on the same day. At the lower feeding values, Daphnia with parthenogenetic young at the eyed-embryo

stage were examined (Table VIIIa). At the higher feeding values dufficient animals were not available with parthenogenetic young at the eyed-embryo stage, so that observations were made on Daphnia carrying parthenogenetic eggs (Table VIIIb).

It can be seen from Table VIIIa, that the haemoglobin index rose from 58 to 77 with an increase in the feeding ratio of 1 to 4, at an average oxygen concentration of 19 - 20% air saturation. An increase of food therefore causes a corresponding increase in haemoglobin production, at the same average oxygen concentration. The average number of embryos produced showed a slight increase at the third feeding value.

The results of haemoglobin production at higher concentrations of food in the same experiment are expressed in Table VIIIb. It will be seen from the experiment numbers, that for flasks 6, 8, and 9 observations were made on Daphnia carrying eyed-embryos and also on Daphnia carrying eggs. It will be seen from these results that the haemoglobin content of Daphnia carrying eggs was less than that of Daphnia carrying eyed-embryos. The values for the haemoglobin indices obtained at the higher concentrations of food, using Daphnia carrying eggs, were therefore less than those obtained at the lower feeding values, in which observations were made on Daphnia with eyed-embryos in the brood pouch.

Although the haemoglobin indices were less, the same

TABLE V111

The influence of different concentrations of Chlorella vulgaris on the haemoglobin production of D.obtusa kept in its own water for 12-13 days. (a) The parthenogenetic young of Daphnia were at the eyed-embryo stage.

Experiment No.	1	2	3	4	5	6	7	8	9
Food Ratio		1			2			4	
Oxygen, % air sat:									
2nd day	10	10	8	8	8	22	30	18	21
3rd "	8	12	14	10	12	25	18	20	24
4th "	9	14	23	12	15	34	22	26	27
6th "	15	8	29	16	13	40	44	31	40
7th "	10	15	9	11	14	18	7	22	22
8th "	12	15	10	8	11	17	7	19	20
10th "	13	21	23	8	9	31	2	25	38
11th "	18	24	30	11	10	34	19	25	44
12-13th"	19	26	33	8	11	31	26	22	29
Average Oxygen % air sat.	13	16	20	10	11	28	19	23	29
Haemoglobin index									
1st day	21	21	21	21	21	21	21	21	21
Last "	62	62	58	65	64	49	77	54	62
Number of embryos									
1st day	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3
Last "	9.6	9.4	9.7	9.0	9.6	13	8.8	10.3	13.3
Size, mm. last day	2.2	2.3	2.3	2.2	2.3	2.4	2.3	2.3	2.4

TABLE VIII (continued)

(b) The parthenogenetic young of *Daphnia* were at the egg stage.

Experiment No.	6	8	9	10	11	12	13
Food Ratio	2	4		8		16	
Oxygen % air sat.							
2nd day	22	18	21	22	28	16	11
3rd "	25	20	24	19	27	22	17
4th "	34	26	27	29	42	32	35
6th "	40	31	40	39	52	43	34
7th "	18	22	22	17	16	16	32
8th "	17	19	20	13	14	20	33
10th "	31	25	38	11	11	16	16
11th "	34	25	44	22	11	20	22
12-13th	31	22	29	28	14	22	21
Average Oxygen. % air sat.	28	23	29	22	24	23	25
Haemoglobin index							
1st day *	20	20	20	20	20	20	20
Last "	41	45	44	60	51	51	51
Number of eggs.							
1st day ⁂	5.1	5.1	5.1	5.1	5.1	5.1	5.1
Last "	10.2	14.7	15.3	13.3	13.8	19.2	17.2
Size, mm. last day	2.3	2.3	2.4	2.4	2.4	2.5	2.4

⁂ Observations on *Daphnia* taken at random from the flasks.

result was obtained. At an oxygen concentration of 23 - 24% air saturation, the haemoglobin indices for Daphnia at feeding values of 4, 8 and 16 were 45, 51, or 60, and 61 respectively. At the same oxygen concentration therefore, an increase in the amount of food added caused an increase in haemoglobin formation to the fourth feeding value, above which a greater concentration of food caused no further haemoglobin synthesis.

The increase of food also caused a corresponding increase in the number of parthenogenetic eggs produced. At the higher feeding values the animals were also of a slightly larger average size.

The influence of Chlorella feeding was also shown by the colour of the gut space. In all animals the gut space which was originally pale yellowish-green became a much brighter yellowish-green colour, as a result of feeding on algal cells containing chlorophyll. There was also a change in the fat content. At the beginning of the experiment Daphnia had no or very occasional fat globules, but at the end of the experiment the amount of fat had increased slightly, the animals containing frequent or few fat globules. As in the previous experiment, the increase in fat was approximately the same at each feeding value.

Nutrition is essential for haemoglobin production. It can be concluded from these experiments, that increasing concentrations of food above this necessary minimal value cause increasing amounts of haemoglobin synthesis, to a

certain maximum of food, above which further increase has no effect. The number of parthenogenetic young produced, which is used as an indication of the state of nutrition of the animal, increased similarly with increasing concentrations of food to this maximum value. This maximum value of food varies for the species of algae used, the maximum concentration being less for Conium and Chlorella than for Chlorella alone. This is probably caused by differences in the stored products of the two algae. It has been shown that Daphnia is better nourished on a mixture of two organisms than on a single species (Pacaud 1939).

d) The Influence of Chlorophyll or Products of its Disintegration on the Haemoglobin Content

It has been claimed that the formation of haemoglobin in Daphnia depends on the presence of chlorophyll in the medium (Verne 1923). Daphnia progressively losing haemoglobin in the laboratory, however, may have its gut lumen green with chlorophyll (Fox 1948). Pale Daphnia in nature often has a bright green gut space. It may be suggested that these facts indicate that chlorophyll is not important in haemoglobin formation, but the oxygen concentration of the water is not considered. As oxygen lack is one factor in haemoglobin formation, it is possible that oxygen lack is essential for haemoglobin synthesis.

To test the influence of chlorophyll on haemoglobin

production, Daphnia have been fed on organisms which possess chlorophyll, and also on organisms which do not contain chlorophyll, and the amount of haemoglobin produced in these animals has been compared at the same oxygen concentration. Colourless organisms have been used by previous workers for feeding Daphnia (see above).

In a preliminary experiment, I used Gonium pectorale as food containing chlorophyll, and as food lacking chlorophyll I used Polytomella caeca, a colourless flagellate of the order Volvocales, and baker's yeast. Pale D.obtusa was placed in its own water, filtered through No.1 Whatman filter paper, to remove the few algae present. After deoxygenation by nitrogen to an oxygen concentration of approximately 15% air saturation, the following flasks were assembled:-

160 D.obtusa in 320 cc. own water)
163 " 325 ") in 4 series
165 " 330 ")

Series 1) No food added
2) With the addition of Polytomella caeca
3) " " Gonium pectorale
4) " " yeast.

The initial and final oxygen concentrations were determined. After 6 days observations were made on Daphnia in the flasks of series 1) and 2). After 7 days the Daphnia of series 3) and 4) were examined. The results are summarised in Table 1X.

TABLE IX

The effect of chlorophyll on the haemoglobin index of *D. obtusa* kept in its own water for 6-7 days. *Gonium pectorale* was used as food with chlorophyll, *Polytomella caeca* and baker's yeast as colourless foods. *Daphnia* was taken at random.

Experiment No.	1	2	3	4	5	6	7	8	9	10	11	12
Food	None			Polytomella			Gonium			Yeast		
Oxygen, % air sat.												
1st day	10	10	11	12	10	13	12	12	15	12	15	14
Last "	11	11	20	8	12	16	6	8	12	6	7	9
Average oxygen, % air sat.	11	11	16	10	11	15	9	9	14	9	11	12
Haemoglobin index												
1st day	27	27	27	27	27	27	27	27	27	27	27	27
Last "	25	26	26	26	27	25	35	41	38	38	34	38
Number of Young												
1st day	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Last "	0.2	0	0.1	0.1	0	0.1	3.6	5.0	4.7	0.4	0.1	0.5
Size, mm. Last day	1.6	1.7	1.6	1.7	1.8	1.7	2.1	2.1	2.0	1.7	1.8	1.7

No haemoglobin was produced in starved or in Polytomella-fed Daphnia. Nutrition is essential for haemoglobin formation, as has been shown previously. Polytomella is either not a suitable food, or insufficient quantities were used, as indicated by the failure of the Daphnia to produce young, and their small average size when compared with Daphnia fed on Gonium.

Haemoglobin was produced in animals fed on Gonium and on yeast. At the same oxygen concentration the amount of haemoglobin produced in Daphnia fed on Gonium and on yeast was the same.

Very slight mortality was observed among the Daphnia in Gonium and in yeast-fed flasks. With daily additions of Gonium, the average number of parthenogenetic young was maintained at the value found in nature. The average size of Daphnia was bigger than in the other flasks, and the bright green gut space indicated good feeding. The Daphnia which had been fed on yeast were of small average size, and very few young were produced, so that feeding must have been at a minimal value.

The parthenogenetic young produced in Gonium-fed Daphnia were bright pink or pale pink, whereas in other flasks the young produced were green. Fat production was observed in Gonium-fed and in yeast fed Daphnia. At the beginning of the experiment the Daphnia had no or very few fat globules, but after 6-7 days frequent or common fat globules were present in Gonium-fed and yeast-fed animals.

Although the amount of haemoglobin produced in Daphnia fed on Gonium and on yeast was the same, the animals were not receiving equal nutrition, as indicated by the difference in the average size and in the number of parthenogenetic young produced.

The storage product of yeast is not the same as of the other food organisms. Polytomella contains abundant starch grains (Doflein 1916, Kater 1925). Gonium also stores starch, the storage of starch and volutin being characteristic of the Volvocales (Pascher 1927). Yeast stores mainly glycogen (Gwynne-Vaughan and Barnes 1927).

To continue the study of the influence of chlorophyll on haemoglobin production, two nearly related organisms were chosen of approximately the same size, and with an similar stored products as possible. These were two members of the order Chlorococcales and family Chlorellaceae, the green Chlorella vulgaris and Prototheca Zopfii, a colourless member of the same family. The average diameters of ten individuals of Chlorella and Prototheca were 7 and 8 micra respectively.

In Chlorella vulgaris the percentage of the dry weight of the different substances present is known; 1-2% is fat, 15-20% is starch, while 50-65% of reserve carbohydrate is referred to under the group hemi-cellulose (Pearsall and Loose 1937). Prototheca also contains starch and fat, and is a close parallel to Chlorella (Chodat 1913, Fritsch 1935). Both organisms were grown on agar, from which the cells were

washed in filtered lake water, before adding to the experimental flasks.

Pale D.obtusa was placed in Regent's Park lake water, previously filtered through No.1 Whatman filter paper. The water was deoxygenated by nitrogen to an oxygen concentration of approximately 15% air saturation before use. The experiment was divided into four series.

160 D.obtusa in 320 cc. lake water)
163 " " 325 cc. " ") in 4 series
165 " " 330 cc. " ")

Series 1) No food added
2) With the addition of Gonium pectorale
3) " " " Chlorella vulgaris
4) " " " Prototheca Zopfii.

Gonium pectorale was included in the series to compare the relative food value of Gonium and Chlorella.

The initial and final oxygen concentrations were determined. After 6-7 days the Daphnia in all flasks were examined, the Chlorella-fed and Prototheca-fed Daphnia were examined on the same day. The results are summarised in Table X.

After 6 days, no haemoglobin was produced in starved animals, very few or no parthenogenetic young were formed and there was no increase in the average size of the animals. Haemoglobin was produced in all fed animals, although different numbers of parthenogenetic young were formed in Daphnia fed on green and on colourless food.

TABLE X

The effect of chlorophyll on the haemoglobin index of D. obtusa kept in Regent's Park lake water for 6-7 days. Gonium pectorale and Chlorella vulgaris were used as foods with chlorophyll, Prototheca Zopfii as food without chlorophyll. Daphnia was taken at random.

Experiment No.	1	2	3	4	5	6	7	8	9	10	11	12
Food	None			Gonium			Chlorella			Prototheca		
Oxygen, % air sat.												
1st day	18	19	20	8	10	11	11	11	14	9	9	7
Last "	48	51	53	5	7	11	6	7	7	4	4	6
Average oxygen, % air sat.	33	35	37	7	9	11	9	9	11	7	7	7
Haemoglobin index												
1st day	24	24	24	24	24	24	24	24	24	24	24	24
Last "	25	25	24	48	51	55	52	48	44	42	42	39
Number of young												
1st day	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Last "	0	0	0.1	3.3	3.1	2.8	3.8	4.0	5.0	0.1	0.1	0.1
Size, mm. Last day	1.9	1.7	1.7	2.0	2.0	2.0	2.1	2.0	2.1	1.8	1.8	1.9

At the same oxygen concentration there is no difference in the amount of haemoglobin produced by Daphnia fed on Gonium and on Chlorella. The difference in the number of parthenogenetic young produced is also slight, the value being higher than the average value of the experimental Daphnia in nature. The increase in the average size of Daphnia fed on Gonium and on Chlorella also indicates good nutrition. Gonium and Chlorella are therefore equally good as food organisms for Daphnia.

Slightly more haemoglobin is produced by the animals fed on Chlorella than is formed by those fed on the colourless Prototheca. There is also a big difference in the number of parthenogenetic young possessed by the Daphnia, those fed on Chlorella contain more young than in nature, while the Daphnia fed on Prototheca contain very few parthenogenetic young. This difference of nutrition is also emphasised by a consideration of the average size. The average size of Daphnia fed on Chlorella is greater than the average size of Daphnia fed on Prototheca.

It is interesting to compare the results obtained on starved Daphnia and those fed on Prototheca, when it will be seen that feeding in the Prototheca flasks must have been minimal.

The change in the colour of the gut space is also interesting. In starved Daphnia, and Daphnia fed on Prototheca the gut space lost its colour becoming uniformly pale, while in Daphnia fed on Gonium or Chlorella the gut

space became a bright yellowish-green. The change in fat content was also noted. At the beginning of the experiment the Daphnia had no fat globules. After 6-7 days the starved animals were still without visible fat globules, but the Daphnia fed on Gonium, Chlorella and Prototheca had common or very common fat globules. The reason for the production of fat requires investigation.

Although Chlorella and Prototheca are nearly related, and are said to contain similar storage products, Daphnia fed on Chlorella receive better nutrition as shown by the difference in the production of young. An increase in nutrition causes an increase in haemoglobin synthesis (see previous), so it is possible that the slight difference in haemoglobin content between Chlorella-fed and Prototheca-fed Daphnia may be a result of the better nutrition of the animals fed on Chlorella.

A pair of nearly related organisms is therefore needed, one of which contains chlorophyll, which when fed to Daphnia will cause the production of equal numbers of parthenogenetic young.

Another pair of organisms was chosen, Chlamydomonas incisa and Polytoma uvella both members of the order Volvocales and family Chlamydomonadaceae. Chlamydomonas contains chlorophyll. Polytoma does not contain chlorophyll, but is a yellowish colour due to the presence of carotene and xanthophyll (Pringsheim and Mainx 1926).

The storage products of the two flagellates are the

same. As members of the order Volvocales they contain starch and volutin (Pascher 1927). Although Polytoma has no chloroplast, it still produces starch as well as volutin (Jirovec 1926, Pringsheim 1927).

In order to compare the haemoglobin production of Daphnia fed on green and on non-green organisms, equal quantities of these two organisms must be used. Measurements of the cells of Chlamydomonas incisa and Polytoma uvella show that the Chlamydomonas species is of a larger average size. The average size of ten individuals of Chlamydomonas incisa and Polytoma uvella was 18 x 11 micra and 13 x 7 micra respectively. In order to overcome this difference of size, equal quantities of food by weight were added to the experimental flasks. The dry weight of a certain known number of cells of each of the two organisms was found. From these results it is possible to calculate the number of cells to add to Daphnia fed on Chlamydomonas and on Polytoma, so that the Daphnia are receiving equal weights of the two organisms. This method, which was used for both organisms, will be described more fully.

A suspension of algal cells grown on agar was made in filtered Lake Windermere water. The suspension was strained through fine bolting silk, to ensure that no small particles of agar had been washed from the slopes, and was then shaken well to distribute the cells equally. To 1cc. of this suspension was added 1cc. of 10% formalin to immobilise the algae, and 4 cc. of lake water to dilute the

sample. The number of cells in this diluted sample was then found using a haemacytometer. Stages in the life history of the organisms were present in the suspensions, but in haemacytometer counts, divided cells were considered as one individual, as they were surrounded by a single membrane. From these results the number of cells in a certain volume of the original suspension can be calculated.

The algae were weighed in centrifuge tubes. These were dried at 102° C. and weighed to a constant weight. Into two or three tubes was placed 5cc. of the original suspension of algae. The tubes were then centrifuged to remove the lake water, and the tubes containing algal cells were dried at 102° C until repeated weighings were identical. The average dry weight of a known number of cells of the two organisms can be found from these results. The results are summarised in Table XI.

It was found that :-

Weight of Chlamydomonas : weight of Polytoma = 5:1

In order to obtain equal feeding by weight

Volume of Chlamydomonas : volume of Polytoma = 5:1

for suspensions of the two organisms containing the same number of cells.

Suspension of the organisms were made each day, and the number of cells in each suspension was found using a haemacytometer. The volume of each solution to add to the experimental flasks, so that Daphnia was fed equally by weight, was then calculated.

TABLE XI

Results of the day weights of Chlamydomonas incisa and Polytoma uvella from 5cc. of suspensions of the 2 organisms. Results of haemacytometer counts give the number of cells in the suspensions.

Organism	Chlamydomonas		Polytoma		
Sample No.	1	2	1	2	3
Weight of centrifuge tube, gm.	18.5047	17.3559	17.7922	17.6572	17.6147
" " " plus centrifuged cells, gm.	18.5159	17.3669	17.7952	17.6599	17.6176
∴ " centrifuged cells, gm.	0.0112	0.0110	0.0030	0.0027	0.0029
∴ Average weight of centrifuged cells in 5 cc. suspension, mg.	11.1		2.9		
Number of cells in 1 cmm. suspension (haemacytometer count)	3552		4875		
∴ Number of cells in 5 cc. suspension	17.76×10^6		24.38×10^6		
Weight of 10^8 cells, mg.,	62.8		11.9		

∴ Weight of Chlamydomonas: Weight of Polytoma = 628 : 119

= 5.3 : 1

Pale D.obtusa was placed in its own water, previously filtered through No.1 Whatman filter paper, and deoxygenated by nitrogen to an oxygen concentration of approximately 20% air saturation. The flasks were assembled in the following way,

3 flasks of Series 1) 105 D.obtusa in 210 cc.own water) in 2 series
" 2) 110 " " 220 cc. " ")
With the addition of Chlamydomonas incisa
" " " Polytoma uvella.

Daphnia was fed daily as described above. Oxygen analyses were made every second day. After 6-7 days, observations were made on Daphnia in all flasks. Daphnia in which the parthenogenetic young were at the egg stage and at the eyed- embryo stage were considered i.e. two results were obtained for the Daphnia of each flask where possible. The flasks in which the average oxygen concentrations of the water were comparable, were examined on the same day. The results are expressed in Table XII(a) and (b).

It can be seen from the results for Daphnia carrying parthenogenetic eggs, that there is no difference in the haemoglobin content of Daphnia fed on Chlamydomonas and on Polytoma at the same oxygen concentration. The average egg numbers show that the animals were receiving comparable nutrition, Daphnia fed on Chlamydomonas do not have more eggs than those fed on Polytoma. Average size results for Daphnia fed on Chlamydomonas and on Polytoma were the same.

TABLE XII

The effect of chlorophyll on the haemoglobin index of D.obtusa kept in its own water for 6-7 days. Chlamydomonas incisa was used as food with chlorophyll, and Polytoma uvella as food without chlorophyll.

(a) The parthenogenetic young of Daphnia were at the egg stage.

Experiment No.	1	2	3	4	5	6	7	8
Food	Chlamydomonas				Polytoma			
Oxygen, % air sat.								
2nd day	13	16	18	25	12	20	21	36
4th day	5	5	8	17	8	8	23	48
6th "	3	2	9	39	7	8	39	60
7th "	4	2			7			75
Average Oxygen, % air sat.	6	6	12	27	9	12	28	54
Haemoglobin index								
1st day	27	27	27	27	27	27	27	27
Last "	51	49	42	35	54	44	37	28
Number of eggs								
1st day	20.9	20.9	20.9	20.9	20.9	20.9	20.9	20.9
Last "	5.5	4.7	4.9	4.8	6.2	4.3	2.5	3.2
Size, mm. last day	2.0	2.1	2.1	2.1	2.2	2.2	2.1	2.2

TABLE XII (continued)

b) Daphnia were examined in which the parthenogenetic young were at the eyed-embryo stage, or in which the embryos had just been released.

Experiment No.	2	3	4	5	6	7	8
Food	Chlamydomonas			Polytoma			
Oxygen, % air sat. average see Table XIIa)	6	12	27	9	12	28	54
Haemoglobin index							
1st day	29	29	29	29	29	29	29
Last "	59	48	37	57	62	38	29
Number of embryos							
1st day	19.8	19.8	19.8	19.8	19.8	19.8	19.8
Last "	0	2.1	1.7	0	3.5	2.1*	1.7
Size, mm. Last day	2.0	2.0	2.1	2.0	2.1	2.0	2.0

* A few of the embryos in the animals considered did not possess eyes.

This also indicates that the animals were receiving approximately equal nutrition.

A similar result was obtained for Daphnia in which the parthenogenetic young were at the eyed-embryo stage. The amount of haemoglobin produced in Daphnia fed on Chlamydomonas was not greater than that produced in Daphnia fed on Polytoma. It can therefore be concluded that when Daphnia is receiving equal nutrition, the presence of chlorophyll in the food does not cause greater haemoglobin formation.

The influence of feeding also caused a change in the colour of the gut space. The gut space of Daphnia at the beginning of the experiment was pale yellowish-green or pale green. The gut space became yellowish-green or bright yellowish-green in Daphnia fed on Chlamydomonas, but it became pale yellow or pale in Daphnia fed on Polytoma.

A change also took place in the fat content of the animals. At the beginning of the experiment Daphnia with parthenogenetic eggs in the brood pouch had very occasional or few fat globules, but after 6-7 days at low oxygen concentrations, Daphnia fed on Chlamydomonas had frequent fat globules, whereas Daphnia fed on Polytoma had no or very occasional fat globules. A similar result was obtained for Daphnia in which the parthenogenetic young were at the eyed-embryo stage. The number of fat globules increased from very few to frequent or common in Chlamydomonas-fed

animals, while in Polytoma-fed animals there were no or very occasional fat globules. The number of survivors was high, and approximately the same in the flasks of the two series.

It can be concluded from the above feeding experiments that:-

a) Nutrition is essential for haemoglobin synthesis.

b) At low oxygen concentrations, an increase in the amount of algal food added, causes an increase in haemoglobin synthesis to a certain maximum value, above which a further increase of food has no effect.

c) When Daphnia is receiving equal nutrition, a food containing chlorophyll does not cause more haemoglobin production than a food not containing chlorophyll, at the same oxygen concentration.

6. THE CORRELATION BETWEEN THE HAEMOGLOBIN CONTENT OF DAPHNIA FROM VARIOUS LOCALITIES AND CERTAIN ENVIRONMENTAL FACTORS.

The haemoglobin index of a certain population of Daphnia may vary greatly during the year. Continued field observations were made of several localities over a period of time, and it was hoped to correlate certain environmental factors with the haemoglobin index of the population.

The oxygen concentration of the pond water is an important factor in haemoglobin synthesis, but there is no method by which continuous oxygen recordings can be obtained in the field. The oxygen concentration of pond water was measured directly using the field apparatus designed by

Whitney (1938). Recordings were made twice during the day, in the early morning and in the evening, when the minimum and maximum oxygen concentrations were obtained.

An estimate of the maximum and minimum oxygen concentrations which are possible can be obtained indirectly, by enclosing the pond water in stoppered bottles at a fixed temperature for a constant period of time. The oxygen content of the water falls as a result of the respiration of the contained organisms, such as bacteria, algae and Protozoa. The difference in oxygen concentration between the initial oxygen content and the oxygen content after the water has been enclosed for a certain length of time is known as the "oxygen consumption" of the water. When the bottles are kept in the dark, this difference in oxygen concentration gives a value for the maximum amount of oxygen consumed by the respiration of the organisms present. If, however, the bottles are illuminated, so that photosynthesis can take place, the results obtained give a value for the minimum amount of oxygen consumed, the balance between the amount of oxygen produced during photosynthesis, and that consumed during respiration. The difference between the values of oxygen consumption obtained in the light and in the dark is the amount of oxygen produced as a result of photosynthesis. Under constant conditions of temperature and light the amount of oxygen produced in this way can be used as an estimate of the quantity of phytoplankton present in the water. Both of these methods have been used to determine

the range of oxygen concentration possible in pond water.

The alkalinity of the water was chosen as another factor for investigation.

a) THE HAEMOGLOBIN CONTENT OF DAPHNIA AND THE OXYGEN CONCENTRATION OF POND WATER

1. Direct Oxygen Recordings:

The syringe-pipette micro-Winkler method of Fox and Wingfield (1938) which has been used for all estimations of dissolved oxygen, is not suitable for use in the field. A syringe pipette method for the determination of oxygen in the field was devised by Whitney (1938). The apparatus consists of a syringe pipette with a maximum capacity of 20 ccs. and a burette of 1 cc. capacity with a U-shaped delivery end, so that it can be attached to the pipette.

The method is modified by the preliminary use of sodium azide, as in some pond waters the presence of nitrites causes an inaccuracy in the result, as explained previously. A known water sample of approximately 10cc. was taken directly from the pond by means of the syringe. The water sample was always taken in the same place, the region of the pond being chosen where Daphnia was plentiful. The sample was taken at a fixed distance below the surface. A gauze was used over the end of the cannula to prevent Daphnia from being sucked into the pipette with the pond water. The usual oxygen reagents were then added. The iodine solution in the pipette was titrated directly against a standard sodium thiosulphate solution in the burette, which was coupled to the end of the syringe

pipette (Whitney 1938).

As low oxygen concentration induces haemoglobin formation (Fox 1948) it is possible that a correlation may exist between the oxygen limits of the pond water and the haemoglobin index of the population. It was considered necessary to find the amount of variation in oxygen content which could be obtained between ponds containing Daphnia with the same haemoglobin index, before extending the observations to ponds in which the haemoglobin indices of Daphnia varied greatly.

Three localities were therefore selected containing Daphnia with approximately the same haemoglobin index, and observations were made on the Daphnia from each source. Daphnia was collected from the region of the pond from which oxygen samples were to be taken. Recordings of oxygen concentration were made twice during the day, in the early morning when the minimum oxygen concentration was obtained, and in the late afternoon when a maximum oxygen concentration had been reached. Results were obtained from the three localities on two days during which the weather conditions were almost identical, the weather being bright and sunny during the day, very warm in the afternoon and with slight morning mist on the second day. Observations were then repeated on Daphnia from the three localities, to ensure that the haemoglobin index had not altered significantly during these few days. The results are expressed in Table XIII.

The three habitats chosen varied in several respects,

TABLE XIII

Morning and evening determinations of the oxygen concentration of three pond waters. Observations were made on Daphnia taken at random from the populations, before and after the recordings of the oxygen concentration were made.

Locality		Big Butler	Long Butler	Tank
Species		D.pulex	D.pulex	D.obtusa
Haemoglobin index				
1st day		52	59	57
7th "		44	49	57
Number of Young				
1st day		2.8	18.9	0.2
7th "		3.8	0	0.6
Size, mm.				
1st day		2.1	2.4	1.7
7th "		2.0	1.8	1.6
Oxygen Conc, % air sat.				
4th day	a.m.	15	0.05	1.9
	p.m.	39	35	20
6th "	a.m.	23	0.9	2.0
	p.m.	64	27	36
Time G.M.T. of recording.				
4th day	a.m.	8.30	9.0	10.0
	p.m.	4.10	4.40	5.25
6th "	a.m.	8.0	8.40	9.27
	p.m.	3.33	4.9	5.35

such as in the amount of surface area, in depth and in vegetation. Big Butler is a large pond with no overhanging trees and very clear water. Long Butler has many overhanging trees which almost completely shade the water and the pond has a rich growth of phytoplankton. The third locality that was chosen was a large concrete trough, which had been untouched for a long period of time, and contained a flourishing population of Daphnia.

Although the haemoglobin index of Daphnia in the three localities did not vary greatly, the results obtained on the same day show quite a variation in the maximum and minimum oxygen limits for the ponds (Table XIII). The results obtained for each locality on two days with similar weather conditions are also variable. The afternoon maximum of oxygen concentration in ponds must be reached at different times, depending on the amount of shade cast on the water by overhanging trees, and the quantity of phytoplankton in the water. The morning minimum value will also change less in a pond which does not receive morning sun. Before exact comparisons can be made, it is therefore necessary to have more frequent recordings of the oxygen concentration of the pond water, particularly around the maximum and minimum values. A method is needed by means of which continuous oxygen recordings can be made.

2. ESTIMATION OF THE OXYGEN CONSUMPTION OF POND WATER

(1) Previous work

The method of enclosing a sample of water in blackened and ordinary glass bottles, has been used by investigators

of marine plankton, to obtain an estimate of the plankton productivity in regions where currents produced variations from day to day greater than the growth and decrease of the plankton algae (Gaarder and Gran 1927, Gran 1927). The production of phytoplankton is estimated quantitatively by enclosing natural sea water in light and dark bottles. This method has also been employed in tropical shallow water areas as a gauge of the productivity (Riley 1938, 1939, 1941). Similar experiments have been made to find the compensation point using cultures of a single diatom. Marshall and Orr (1928) experimented with cultures of Coscinosira polychorda in Loch Striven, while Jenkin (1937) used cultures of Coscinodiscus excentricus in the English Channel. A similar method was used to determine the carbon assimilation of Chlorella in Lake Windermere. (Loose, Pearsall and Willis 1934).

The enclosure of natural water in light and dark bottles has also been used in culture experiments, in which the productivity has been estimated after the addition of phosphates and nitrates (Gaarder and Gran 1927, Gran 1927).

(ii) Method:

The oxygen consumption of pond water is found by enclosing the water in glass bottles of approximately 40 cc. capacity with solid ground glass stoppers. Some of the bottles were blackened on the outside to exclude light. The bottles were maintained in a thermostat at 20°C for a period of 20 hours. The temperature of the thermostat was kept constant by a mercury-toluene control.

A balance of temperature was maintained between an electrical heating system, and a cooling coil of lead piping through which a stream of cold water was allowed to flow. During the summer months the water in the cooling coil was too warm to reduce the temperature of the thermostat to 20°C. The water was therefore first cooled by passing through a coil of lead tubing which was submerged in water surrounded by solid carbon dioxide.

The pond water to be examined was collected and immediately filtered through bolting silk to remove organisms above a certain size, such as large members of the zooplankton. Collections of Daphnia were made from the region of the pond from which the water sample was obtained. The water was always taken from the same region of the pond, if Daphnia was plentiful in that region. The water was then allowed to stand overnight in a cool place, in a glass dish with a large surface area in proportion to volume. This was necessary as sufficient time to determine the oxygen consumption of the water was not available on the day of collection.

Pond waters vary greatly in their capacity for removing dissolved oxygen, and most pond waters require dilution before the oxygen consumption can be found. There is a difference of opinion as to the best dilution water to employ. In estimations of the quantity of organic matter in sewage effluents many dilution waters have been used (Standard Methods of Water Analysis 1946).

The necessary characteristics of a dilution water are that it should itself have a very low oxygen consumption, and that it should contain no caustic or harmful materials. Surface lake water from Lake Windermere was found to be a satisfactory dilution water. It was used after filtration through No.1 Whatman filter paper, and it was found to have a very low oxygen consumption. Some pond waters require considerable dilution, and the best dilution to employ so that the oxygen concentration does not fall below 30-40% air saturation in the bottles during 20 hours was found by experience. It is advisable to set up two or more dilutions of the pond water, so that the most satisfactory dilution can be achieved. Some pond waters require no dilution.

The pond water, after dilution if necessary, was aerated for half an hour in a water bath at 20 - 21°C. This was to ensure that aeration was not carried out at a temperature below 20°C, as the temperature change to 20°C on placing in the thermostat would cause the excess oxygen to be given off in the form of small bubbles, thus causing an error in the result. The initial oxygen concentration was determined, and the water was poured gently into four bottles, two of which were blackened to exclude the light. After making sure that no bubbles had been trapped around the rim of the bottles the stoppers were fixed firmly, and the bottles were placed in the thermostat for 20 hours. At the end of this period the oxygen concentration of the water in each bottle was determined. The bottles were shaken before being opened. From these results the average oxygen concentration

of pond water enclosed in light and dark bottles was obtained. The loss or gain of oxygen in light and in darkness was then calculated in cc. of oxygen per litre.

When estimations of oxygen consumption were carried out, the haemoglobin index of the Daphnia of the pond was also determined. Other observations of the number of young, size, fat content, gut content and the colour of the gut liquid were made at the same time. The percentage of different species present was noted, and also other features such as the percentage of males, ephippial females and parasitised individuals.

(iii) Discussion of Results

The results of the oxygen consumption of pond waters were obtained in the following way:-

1) For Undiluted Pond Water or Dilution Water

e.g. Windermere dilution water.

Initial oxygen concentration	= 6.49	cc/L
Final average oxygen concentration in light	= 6.25	"
" " " " in darkness	= 6.20	"
Oxygen consumption in light = (6.49 - 6.25)	= 0.24	"
" " " darkness = (6.49 - 6.20)	= 0.29	"
Oxygen produced by algae in water = (0.29 - 0.24)	= 0.05	"

2) For Diluted Pond Water.

e.g. Rat Hill pond water diluted 1 in 6 with the above Windermere water.

Initial oxygen concentration of diluted sample = 6.29 cc/L

Final average oxygen concentration of diluted sample in light = 5.89 cc/L

" " " " darkness = 5.45 "

Oxygen consumption of diluted sample in light = (6.29 - 5.89) = 0.40 "

" " " " " darkness = (6.29 - 5.45) = 0.84 "

Since Rat Hill Pond water was diluted 1 in 6,

1 litre undiluted pond water plus 5 litres dilution water = 6 litres diluted pond water

∴ Oxygen consumption of undiluted pond water = (oxygen consumption of diluted pond water x 6) - (oxygen consumption of dilution water x 5)

i.e. Oxygen consumption of undiluted pond water in light

= (0.40 x 6) - (0.24 x 5) = 1.2 cc/L

Oxygen consumption of undiluted pond water in darkness = (0.84 x 6) - (0.29 x 5) = 3.6 cc/L

Oxygen produced by algae in water = (3.6 - 1.2) = 2.4 cc/L

The final average oxygen concentration of the water obtained is the mean of two values. The difference between the values of the oxygen concentration obtained in the two bottles is caused by the unavoidable inclusion in the bottles of slightly different numbers of organisms. The difference between the two values is not great. In estimations of the oxygen consumption in the dark of pond waters from ten different localities, the final oxygen concentrations in cc. of oxygen per litre in each of the two bottles were, 6.6 and 6.6, 3.3 and 3.2, 5.5 and 5.5, 5.2 and 5.3, 5.8 and 5.9, 6.4 and 6.2, 6.1 and 6.3,

3.1 and 5.2, 5.3 and 5.3, 3.5 and 3.1. The error of the method is therefore generally less than 5%. In occasional results the difference may be as great as 10%.

Occasional results of oxygen consumption were taken from several ponds and are summarised in Table XlV. The Daphnia of these ponds varied considerably in haemoglobin index. Comparisons can be made between the results obtained for the same species of Daphnia, but the results do not show any correlation between the haemoglobin index of the population and the oxygen consumption of the water. This is not surprising as the results of the oxygen consumption of the water indicate the maximum and minimum oxygen concentrations which are possible in the pond owing to the respiration and photosynthesis of the small plankton organisms present in pond water. The results do not take into account the nature of the pond, for example, the surface area, and hence the amount of oxygen uptake over the surface, the depth, the amount of shade and the respiration of other aquatic organisms. Ponds 2 and 3 given in Table XlV contain D.pulex with indices of 36 and 110 respectively. The results of oxygen consumption are approximately the same 0.9 and 0.9, 0.86 and 1.08 cc. of oxygen per litre respectively. Pond 3 is however a much deeper pond, and it has a smaller surface area than pond 2. It is also shaded by overhanging bushes and trees. Pond 4 referred to in Table XlV has water with a high oxygen consumption. It is a very shallow duck pond, and has a large surface area, so that the oxygen uptake

TABLE XIV

Recordings of the haemoglobin index of *D.pulex* and *D.obtusa* from several different localities and the oxygen consumption of the pond waters. *Daphnia* was taken at random.

Locality	1	2	3	4	5	6
Species present	*D.pulex*			*D.obtusa*		
Haemoglobin index	25	36	110	19	53	58
Number of Young	0.3	3.5	2.7	1.0	1.4	4.0
Size, mm.,	3.2	2.2	2.2	1.4	1.5	1.8
Oxygen consumption cc./L						
In light	5.1	0.9	0.9	1.0	1.3	13.8
" darkness	5.8	0.9	1.1	1.1	1.4	18.7
O_2 produced by algae						
cc O_2/L	0.7	0	0.2	0.1	0.1	4.9

over the surface must be very large, and the actual oxygen concentration of the water would probably never fall to these limits.

It is impossible therefore to compare results of the oxygen consumption of pond water taken in many localities, as different factors are influencing the actual oxygen values obtained in the pond, and thus the amount of haemoglobin present in Daphnia. I have therefore found the oxygen consumption of the water of certain ponds at regular intervals over a period of time. The same, or very similar additional environmental factors should theoretically influence each result, making a comparison of the change in haemoglobin index and oxygen consumption possible.

Five ponds were chosen, and monthly observations were made on the Daphnia population, the oxygen consumption of the water being found at the same time. Unfortunately, owing to the very hot summer of 1947 four of the populations died out, so that a second pond was chosen at a later date. Monthly results were obtained for Rat Hill pond containing D.magna and occasionally D.obtusa, and for Big Butler pond containing D.pulex. The results are summarised in Tables XV and XVI respectively.

When the results for Rat Hill pond are considered (Table XV Frig.1a) it will be seen that there is a correlation between the haemoglobin index of Daphnia and the oxygen consumption of the water in darkness over the period June to October. Increase in oxygen consumption is followed by an

TABLE XV

Monthly recordings of the change in the haemoglobin index of *D.magna* and *D.obtusa*, and the oxygen consumption and alkalinity of the water of Rat Hill pond, Chippenham. *Daphnia* was taken at random from the population.

Date	1947							1948	
	26/6	27/7	24/8	16/9	6/10	9/11	10/12	5/1	2/2
D.magna									
Haemoglobin index	55	55	95	94	77	48	45	45	38 *
Number of young	0.9	26.9	3.2	5.5	0	11.5	29.3	39.0	0
Size, mm.,	3.9	5.4	3.8	4.2	3.5	4.4	5.4	5.3	3.5
D.obtusa									
Haemoglobin index	19	29	55						40
Number of young	1.0	8.6	1.8						21.2
Size, mm.,	1.4	1.9	1.4						2.3
%age population	6.5	17.5	0.01						18.3
Oxygen consumption cc.O_2/L.									
In light	1.0	-3.9	1.2	2.9	2.2	2.6	3.9	4.8	3.6
" darkness	1.1	3.5	3.8	5.0	2.5	2.5	4.6	4.9	3.8
O_2 produced by algae cc.O_2/L.	0.1	7.4	2.6	2.1	0.3	-0.1	0.7	0.1	0.2
Alkalinity N x 10^4	54	30	60	72	73	51	33	32	31

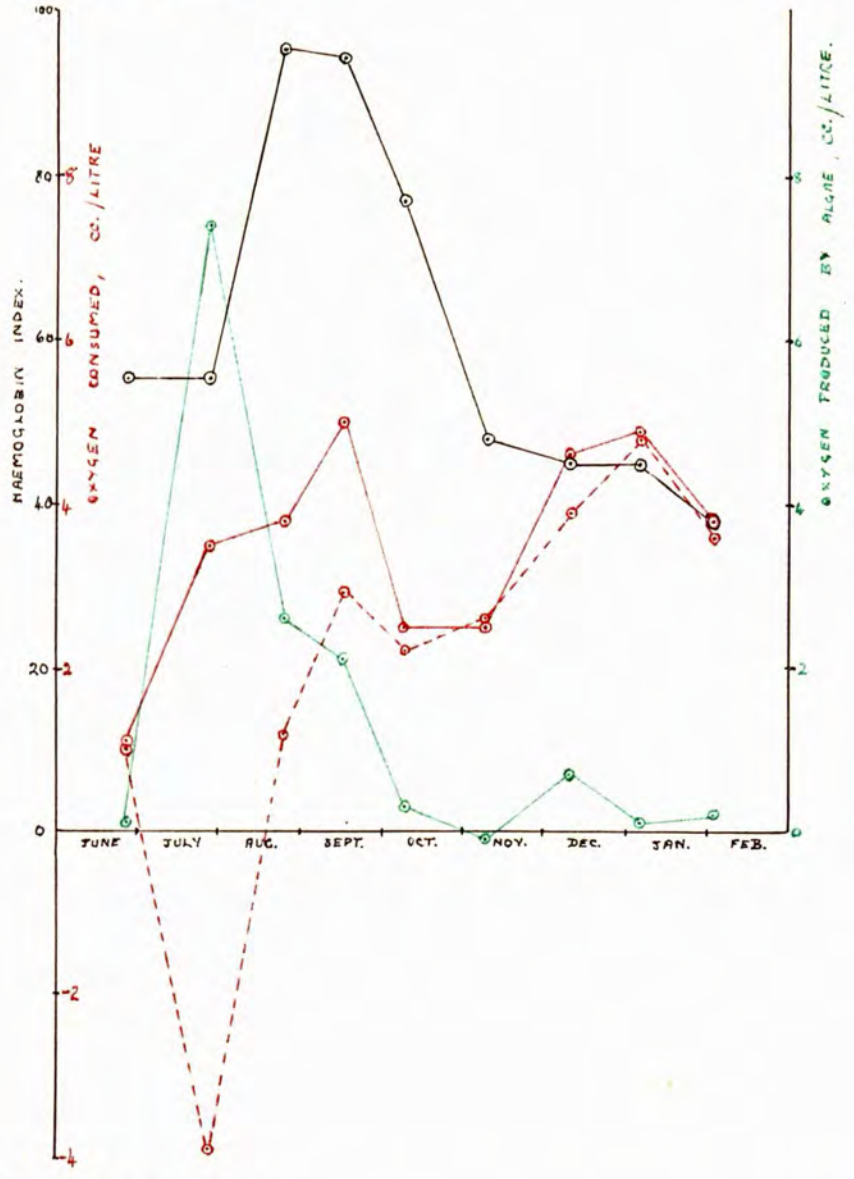
* *Daphnia* used were immature

TABLE XVI

Monthly recordings of the change in the haemoglobin index of D.pulex and the oxygen consumption and alkalinity of the water of Big Butler pond, Chippenham. Daphnia was taken at random from the population.

Date	1947			1948	
	6/10	9/11	10/12	5/1	2/2
D.pulex					
Haemoglobin index	44	36	36	38	29
Number of Young	3.8	3.5	4.0	16.5	4.9
Size, mm.,	2.0	2.2	2.0	3.5	2.3
Oxygen Consumption cc. O_2/L.					
In light		0.9	3.8	1.9	3.7
" darkness		0.9	3.5	2.3	3.7
O_2 produced by algae cc O_2/L.		0	-0.3	0.4	0
Alkalinity N x 10^4		43	31	48	35

(a) RAT HILL POND



(b) BIG BUTLER POND

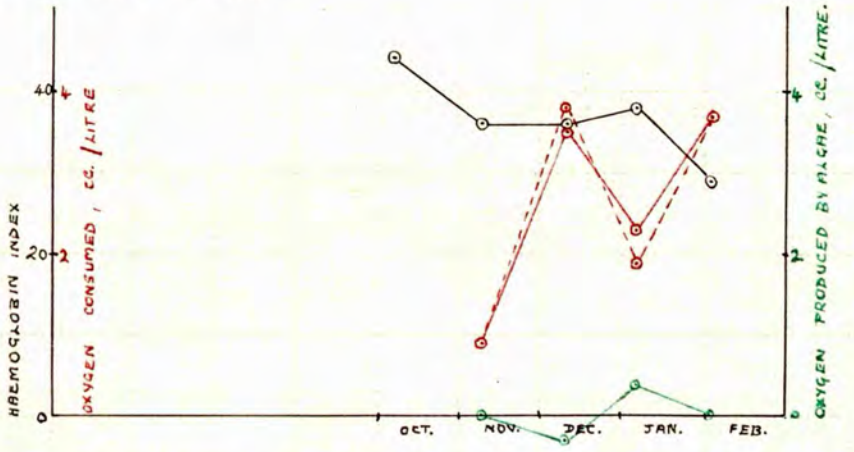


FIG. 1. MONTHLY RECORDINGS OF THE HAEMOGLOBIN INDEX OF DAPHNIA AND THE OXYGEN CONSUMPTION OF THE POND WATER IN LIGHT AND IN DARKNESS OF (a) RAT HILL POND CONTAINING D. MAGNA, AND (b) BIG BUTLER POND CONTAINING D. PULEX. DATA FROM TABLES XV AND XVI.

——— HAEMOGLOBIN INDEX.
——— OXYGEN PRODUCED BY ALGAE.
- - - - - OXYGEN CONSUMED IN LIGHT.
——— " " " DARKNESS.

increase in haemoglobin index, and conversely decrease in oxygen consumption is followed by a decrease in the amount of haemoglobin present. But increased oxygen consumption of the water in darkness in July is not correlated immediately with an increased haemoglobin index. There are two possible explanations. It may be that there is a certain time lag in the effect of the lower oxygen concentration in the pond, but as the increase in algae, bacteria and other organisms in the water must be a gradual increase, the lowering of the oxygen concentration in the pond during hours of darkness must also be gradual. It would therefore influence the haemoglobin content of Daphnia at the same time, as experimental results do not support the idea of a prolonged time lag in the effect of low oxygen. The lack of correlation in July is more probably due to the high oxygen concentration possible during the day, as shown by the results of the oxygen consumption of the water in light. This is supported by the fact that in August, September and October, the difference between the values of oxygen consumption in light and in darkness is not as great, and a correlation exists between the oxygen consumption in darkness and the haemoglobin index. The range of oxygen concentration, and not only the minimum value of oxygen reached appears to be important in determining the amount of haemoglobin present.

When the results of November to February are considered for Rat Hill pond this correlation is not found. Although the oxygen consumption increased, there was no corresponding rise in haemoglobin index. The results from the second pond

show that for the months October to February, although the oxygen consumption of the water fluctuated the haemoglobin content did not change greatly (Table XVI Fig.1b).

The correlation between the haemoglobin index of Daphnia and the oxygen consumption of the water in Rat Hill pond existed over a period of time when the weather conditions were remarkably uniform, and the temperature fairly high. They were obtained during the warm summer months when there was practically no rainfall, the only important change taking place in the ponds was a decrease in the volume of the water as a result of evaporation. The weather conditions from November to February were very different, there was heavy rainfall and many stormy periods, the temperature was lower, and during this time there was no correlation between the haemoglobin index of Daphnia and the oxygen consumption of the water.

There are two possible explanations. The oxygen content of the pond water was influenced directly by these climatic factors, by the addition of large amounts of air-saturated rain water and by wave action on the surface of the pond. The oxygen concentration was also influenced indirectly by the effect of temperature changes on the metabolic rate of the organisms present in the water. The respiratory and photosynthetic activity of these organisms was estimated at the same temperature of 20°C. In winter when the temperature of the pond is 5°C or less, the

metabolic rates of the organisms present will be correspondingly lower, so that the change in oxygen concentration that they cause, and which influences the haemoglobin content of Daphnia will be less than that indicated by results of the oxygen consumption of the water.

The low temperature may also be acting directly on the metabolic processes of Daphnia. At low temperature such as 5°C haemoglobin formation does not take place in Daphnia even at low oxygen concentrations (Table 1). It is probable therefore that conditions of low oxygen in the pond would cause a smaller increase, or no increase in haemoglobin concentration, if the temperature of the pond water was too low.

The increase in the oxygen consumption of the water of Rat Hill pond in December did not cause any increase in the haemoglobin index of Daphnia. Similarly the changes in the oxygen consumption of the water of Big Butler pond are not correlated with alterations in the haemoglobin content of the animals present. It is probable that this lack of correlation may be the result of the greater influence of the weather on the oxygen concentration of the pond and Daphnia present, than in the preceding months. The oxygen concentration was probably higher than the results suggest.

A method is needed by which continuous oxygen results can be obtained in the field.

The amount of oxygen produced by the algae as a result of photosynthesis may be used as an indication of the

productivity of the pond water. In Rat Hill pond the increase in phytoplankton associations present in the water in July is connected with a big increase in the number of young in D.magna, and a corresponding increase in the number of young in D.obtusa. (Table XV Fig.2a). The number of young in D.pulex from Big Butler pond, over a period of five months is correlated directly with the quantity of algae in the water (Table XVI Fig.2b).

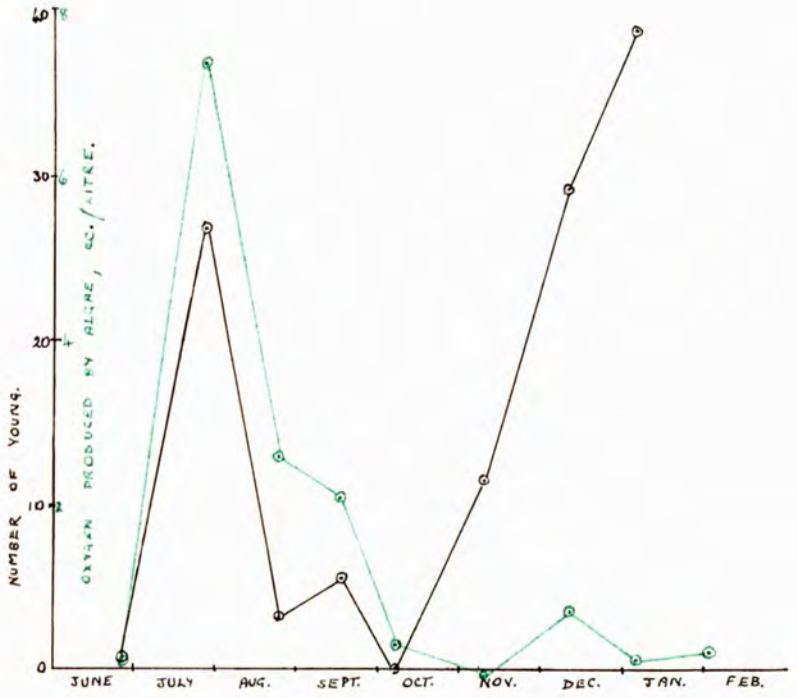
The correlation is not however constant, as an increase in the number of young in Daphnia from Rat Hill pond in December and January, is not correlated with an abundance of algae in the water. This is not surprising as the food of Daphnia is obtained from many sources. These results do show however that phytoplankton organisms are important constituents of the food of Daphnia, as was found by Pacaud (1939).

In order to study the correlation between the haemoglobin index of Daphnia and the oxygen concentration of the pond water, a method is needed by means of which continuous results of oxygen content can be obtained in the field.

Direct recordings were made of the morning and evening oxygen concentrations in three localities in which the haemoglobin content of Daphnia did not differ greatly. The results were found to be variable. More frequent recordings of the oxygen content of the water are therefore needed, to determine more exactly the maximum and minimum values of oxygen concentration in the pond.

Results of the oxygen consumption of pond waters must

(a) RAT HILL POND.



(b) BIG BUTLER POND

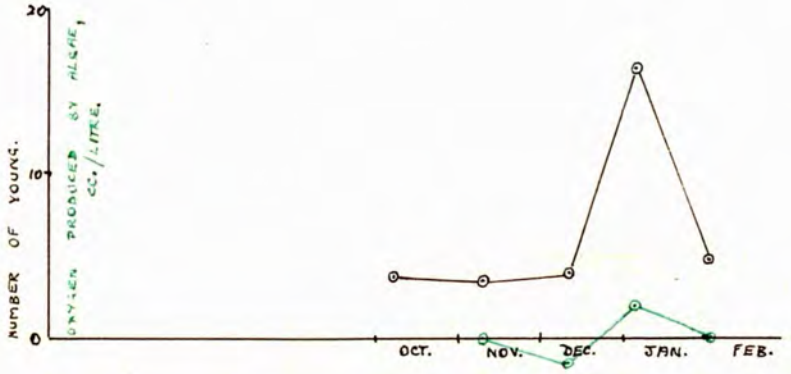


FIG. 2. MONTHLY RECORDINGS OF THE NUMBER OF PARTHENOGENETIC YOUNG OF DAPHNIA AND THE QUANTITY OF PHYTOPLANKTON IN THE WATER OF (a) RAT HILL POND CONTAINING D. MAGNA, AND (b). BIG BUTLER POND CONTAINING D. PULEX. DATA FROM TABLES XV AND XVI.

——— NUMBER OF YOUNG.
——— OXYGEN PRODUCED BY ALGAE.

be obtained in the same localities over a period of time. The values obtained for the oxygen consumption of a pond water over a period of time when weather conditions were fairly uniform suggests that a direct correlation exists between the haemoglobin content of Daphnia and the oxygen consumption of the pond water. The influence of climatic conditions probably accounts for the lack of correlation in the later part of the year.

b) THE HAEMOGLOBIN CONTENT OF DAPHNIA AND THE ALKALINITY OF POND WATER:

The alkalinity of pond water is estimated by titrating a known volume of water against dilute sulphuric acid, using methyl orange as an indicator. The end point is determined by comparison with a standard end point of methyl orange in glass distilled water, saturated with carbon dioxide.

The haemoglobin index of Daphnia and the alkalinity of the pond water were found in many localities (Table XVIIa). These results show that when the haemoglobin index and alkalinity are compared in different ponds, there is no correlation between the two values.

The haemoglobin index of Daphnia and the alkalinity of the pond water were therefore studied every month over a period of time, in three localities containing D. obtusa (Table XVIIb). These results show that a decrease in the haemoglobin index is accompanied by a decrease in alkalinity, although in pond 3 the correlation is not as close as in ponds 1 and 2.

Recordings of alkalinity were made for Rat Hill pond

TABLE XVII

The haemoglobin index of *Daphnia* from different localities, and the alkalinity of the pond water.

a) Occasional observations in several localities.

Locality	1	2	3	4	5	6	7
Species	*D. pulex*	*D. obtusa*				*D. magna*	
Haemoglobin index	49	40	53	55	68	93	98
Alkalinity $Nx10^4$	35	45	63	60	9	46	29

b) Monthly observations in three localities containing *D. obtusa*.

Locality	1			2			3			
Observation	1	2	3	1	2	3	1	2	3	4
Haemoglobin index	55	41	41	40	33	31	86	47	58	77
Alkalinity $Nx10^4$	6.0	5.9	5.5	4.5	3.2	2.8	69	43	95	63

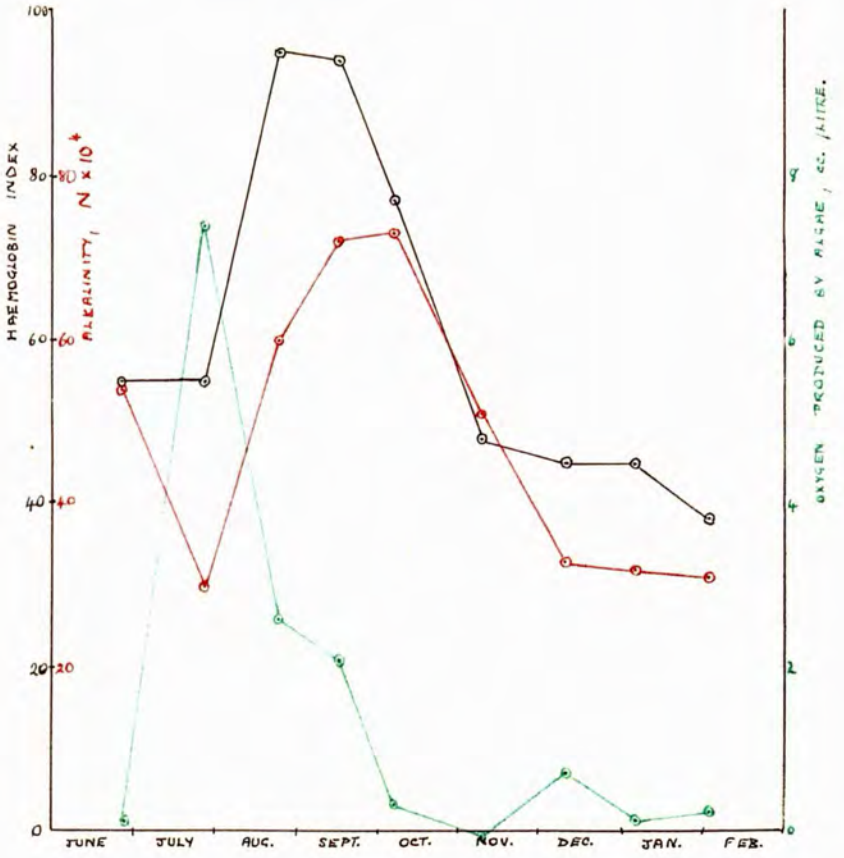
and Big Butler pond every month, at the same time as determinations of the oxygen consumption of the pond water (see previous Tables XV and XVI).

In Rat Hill pond (Table XV, fig 3a) parallel changes occur in the haemoglobin index and alkalinity, over a period of eight months. In Big Butler pond (Table XVI fig.3b) changes in the haemoglobin index and alkalinity are slight, so that no correlation can be recognised.

When the alkalinity and the quantity of phytoplankton in the water are considered for Rat Hill pond (Table XV, fig 3a), an indirect correlation is seen to exist between them. An increase in the quantity of algae present, causes an increase in the amount of carbon dioxide removed from the water, and a corresponding decrease in alkalinity therefore takes place. This indirect correlation does not exist over the winter months, in either Rat Hill pond on Big Butler pond (fig.3a and b). It is probable that the results are being influenced by climatic conditions. For example, heavy rainfall over the period October-December would account for the decrease in alkalinity.

The above results are difficult to interprete. It is possible that under constant environmental conditions, an increase in the oxygen consumption of the water in darkness would indicate a lowering of the minimum oxygen concentration in the pond, and hence a higher haemoglobin content of the Daphnia present. An increase in the oxygen consumption of the water in darkness may be caused by an increase in the quantity of phytoplankton present. An increase in the

(a) RAT HILL POND.



(b) BIG BUTLER POND.

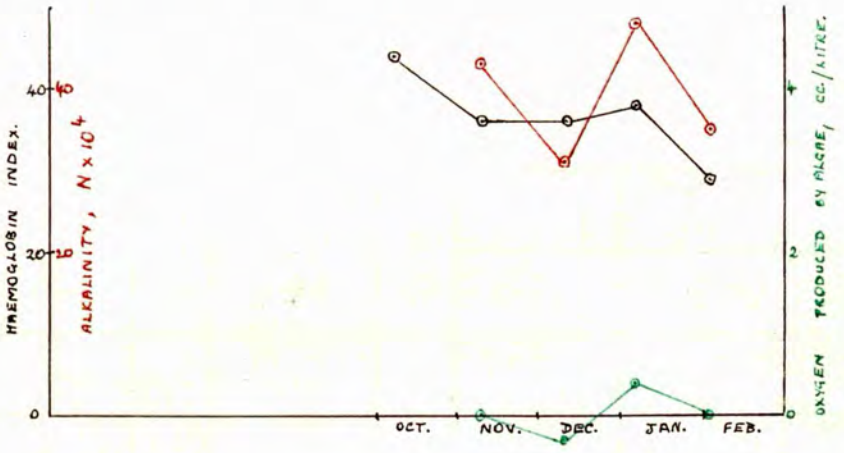


FIG. 3. MONTHLY RECORDINGS OF THE HAEMOGLOBIN INDEX OF DAPHNIA, THE ALKALINITY OF THE WATER AND THE QUANTITY OF PHYTOPLANKTON IN THE WATER OF (a). RAT HILL POND CONTAINING D. MAGNA, AND (b). BIG BUTLER POND CONTAINING D. PULEX. DATA FROM TABLES XV AND XVI.

HAEMOGLOBIN INDEX.
ALKALINITY OF WATER.
OXYGEN PRODUCED BY ALGAE.

amount of phytoplankton in the water would cause a decrease in the alkalinity, by the removal of carbon dioxide used in photosynthesis. In this way the alkalinity of the water and the haemoglobin content of Daphnia would appear to be directly correlated, and indirectly correlated with the amount of phytoplankton in the water.

The above values are influenced by climatic conditions, so that definite conclusions cannot be made.

It has been thought that there is a seasonal change in the haemoglobin content of Daphnia. It is more correct to say that the haemoglobin content is influenced by changing climatic and environmental conditions, which may or may not coincide with different seasons.

7. SUMMARY

1. Pale *Daphnia* placed in a pond water which contains red *Daphnia* in nature do not produce more haemoglobin than in their own water, at the same oxygen concentration, when under equal feeding conditions.

2. Nutrition is essential for haemoglobin production. Starved animals do not produce haemoglobin even under conditions of oxygen lack.

3. At low oxygen concentrations, an increase in the amount of algal food added, causes an increase in haemoglobin synthesis to a certain maximum value, above which greater quantities of food have no effect.

4. The presence of chlorophyll in the food does not cause an increase in haemoglobin synthesis. Pale *Daphnia* fed on green organisms and on organisms without chlorophyll such as *Gonium* and yeast, *Chlorella* and *Prototheca*, *Chlamydomonas* and *Polytoma* do not produce significantly different amounts of haemoglobin. Equal quantities by weight of *Chlamydomonas* and *Polytoma* were used.

5. Direct recordings of the oxygen concentration of pond waters were made in the morning and in the evening, in three localities in which the haemoglobin content of *Daphnia* did not vary greatly. Before exact comparisons can be made it is necessary to have more frequent recordings of the oxygen concentration of the pond water, particularly around the maximum and minimum values.

6. Results of the oxygen consumption of pond waters must

be obtained in the same localities over a period of time. A direct correlation was obtained between the haemoglobin index of Daphnia and the oxygen consumption of the pond water over a period of time when weather conditions were remarkably uniform. The influence of climatic conditions probably accounts for the lack of correlation in the later part of the year.

7. Possible correlations between the haemoglobin index of Daphnia, the alkalinity of the water and the amount of phytoplankton present in the water are discussed.

REFERENCES.

Anderson, B.G. and Jenkins, J.C.(1942). Events in the life span of Daphnia magna. Biol. Bull. 83, 260.

Anderson, B.G.Lumer, H.and Zupancic, L.J. Jr. (1937). Growth and variability in Daphnia pulex. Biol.Bull 73, 444.

Baird, W.(1849). The Natural History of the British Entomostraca Ray. Soc., London.

Banta, A.M. (1939). Studies on the physiology, genetics, and evolution of some Cladocera. Paper No.39, Dept. of Genetics, Carnegie, Inst. Wash.

Chatton, E.(1920). Membranes peritrophiques des Drosophiles et des Daphnies. Bull. Soc. zool. Fr. 45, 265.

Chodat, R. (1913). Monographie d'Algues en culture pure. Matér. Flore Cryptog. Suisse, 4, Fasc.2.

Chu, S.P.(1942). The influence of the mineral composition of the medium on the growth of planktonic algae.I. Methods and cultural media. J.Ecol. 30, 284.

von Dehn, M.(1930). Untersuchungen über die Verdanung bei Daphniden. Z.vergl. Physiol. 13, 334.

Doflein, F.(1916). Polytomella agilis. Zool. Anz.47, 273.

Dunham, H.H. (1938). Abundant feeding followed by restricted feeding and longevity in Daphnia. Physiol. Zool. 11,399.

Fox, H.M.(1947). Daphnia haemoglobin. Nature, 160, 431.

Fox, H.M.(1948). The Haemoglobin of Daphnia. Proc. Roy. Soc. B. 135 in the press.

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

Fritsch,F.E.(1935). The Structure and Reproduction of the Algae. I. Cambridge.

Fritzsche,H.(1917). Studien über Schwankungen des osmotischen Druckes der Körperflüssigkeit bei Daphnia magna. Int.Rev. Hydrobiol. 8, 22, 125.

Gaarder,T.and Gran, H.H.(1927). Investigations of the Production of Plankton in the Oslo Fiord. Rapp. Cons. Explor. Mer. Copenh. 43, 3.

Gran, H.H.(1927). The production of plankton in coastal waters off Bergen. Rep. Norweg. Fish Invest. 3,(8).

Gwynne- Vaughan, C.H.I and Barnes, B.(1927). The Structure and Development of the Fungi. Cambridge.

Hutchinson, G.E.(1933). The Magnesium Tolerance of Daphnidae and its ecological significance. Int. Rev. Hydrobiol 25, 90.

Ingle, L. Wood, T.R.and 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.

Jenkin, P.M.(1937). Oxygen Production by the diatom Coscinodiscus excentricus Ehr. in relation to submarine illumination in the English Channel.

J.Mar.biol.Ass.U.K. 22, 301.

Jirovec O.(1926). Protozoenstudien I. Arch.Protistenk. 56, 280

Kater, J. McA. (1925). Morphology and life history of Polytomella citri sp.n. Biol.Bull. Wood's Hole, 49, 213.

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.Fr.Belg.76,250

Loose, L. L. Pearsall, W.H.and Willis, F.M.(1934). Carbon assimilation by Chlorella in Windermere. Proc. Leeds. phil. lit. Soc. 2, 11, 519.

Marshall S.M. and Orr. A.P. (1928). The photosynthesis of diatom cultures in the sea. J.Mar.biol. Ass. U.K. 15, 321.

Mortimer C.H.(1936). Experimentelle und cytologische Untersuchungen über den Generationswechsel der Cladoceran. Zool. Jahrb. Abt. Allg. Zool. 56, 324.

Naumann, E. (1921). Spezielle Untersuchungen über die Ernährungsbiologie des tierischen Limnoplanktons. Acta. Univ. Lund. 17, No.4

Pacaud, A. (1939). Contribution à l'écologie des Cladocères. Bull. biol. Suppl. 25.

Pascher, A.(1927). Volvocales - Phytomonadinae. Süsswasserfl. Deutschlands, Oesterreichs u.d. Schweiz, 4.

Pearsall, W.H. and Loose, L.(1937). The growth of Chlorella vulgaris in Pure Culture. Proc. Roy. Soc.B., 121, 451.

Pratt, D.M. (1943). Analysis of population development in Daphnia at different temperatures. Biol. Bull. 85, 116.

Pringsheim, E.G. (1927). Enthält Polytoma Stärke ? Arch. Protistenk. 58, 281.

Pringsheim E.G.(1946a) Pure Cultures of Algae. Their Preparation and Maintenance. Cambridge.

Pringsheim E.G.(1946b). The Biphasic or Soil- Water Culture method for growing Algae and Flagellata. J.Ecol. 33, 193.

Pringsheim, E.G. and Mainx, F.(1926). Untersuchungen an Polytoma uvella Ehrbg., insbesondere über Beziehungen zwischen chemotactischer Reizwirkung und chemischer Konstitution. Planta 1, 583.

Ramner, W. (1932). Einwirkung der Uebervölkerung auf Daphnia pulex. Zool. Anz. 101, 41.

Riley, G.A.(1938). Plankton studies I. J.Mar.Res.1, 335.

Riley, G.A.(1939). Plankton studies 11. J.Mar.Res.2. 145.

Riley, G.A.(1941). Plankton studies 111. Bull. Bingham oceanogr. Coll. 7, Art 3.

Riley, G.A.(1941) Plankton studies lV. Bull. Bingham oceanogr. Coll.7, Art.4

Salimovskaya-Rodina, A.G.(1940). Bacteria and Yeasts as food for Cladocera (Daphnia magna). C.R.Acad. Sci. U.R.S.S. 29, No.3.

Schulz, H. (1928). Ueber die Bedeutung des Lichtes im Leben niederer Krebse. Z.vergl. Physiol. 7, 488.

Scourfield,D.J.(1942). The "Pulex" forms of Daphnia and their separation into two distinct series, represented by D.pulex (De Geer) and D.obtusa Kurz. Ann. Mag. nat Hist. 9, 202.

Scourfield,D.J. and Harding J.P.(1941). A key to the British species of Cladocera with notes on their ecology. Freshwater Biol. Assn. of the Brit. Emp. Sc., Pub. No.5.

Smith, G. (1915). The life cycle of Cladocera, with remarks on the physiology of growth and reproduction in the Crustacea. Proc. Roy. Soc.B. 88, 418.

Swammerdam. J.(1758). The Book of Nature, London.

Tessier, G. (1932). Le pigment des oeufs de Daphnia pulex. C.R.Soc. Biol. Paris, 109, 813.

Verne, J. (1923). Les pigments rouges et la formation de l'hémoglobine chez les Daphnies. Bull. Soc. Zool. Fr. 48, 140.

Verne, J. (1924). Hémoglobine et chlorophylle. Bull. Soc. Zool. Fr. 49, 526.

Warren, E. (1900). On the Reaction of Daphnia magna Straus to certain Changes in the environment. Quart J. micr. Sci. 43, 199.

Whitney, R.J.(1938). A Syringe Pipette Method for the Determination of Oxygen in the Field J.exp. Biol. 15, 564.

Wolterecк R.(1928). Lebensraum, Nahrung und Körperform bei pelagischen Cladoceren. Biol. Zbl. 48, 522.