CAUSES FOR VARIATION IN THE HAEMOGLOBIN CONTENT OF *DAPHNIA* IN NATURAL WATERS

by

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HISTORICAL INTRODUCTION

The red colour shown sometimes by populations of Daphnia in ponds was described as early as 1758 by Swammerdam and also by Linnaeus who had seen red Daphnia in such abundance that it created the impression of blood. This was no exaggeration; I have seen the same myself on one occasion.

It was not until over a hundred years later that Lankester (1871) discovered the cause of this red colouring to be haemoglobin dissolved in the blood.

Later (Teissier 1932) it was found that not only the blood but also the parthenogenetic eggs within the brood pouch of Daphnia contain haemoglobin.

Since Lankester's discovery, several workers have investigated the factors responsible for this redness in Daphnia. Fritzsche (1917) considered that it was caused by good nutrition. Schultz (1928) in his experimental work, found that the pigment was developed only in those cultures which were kept in the light.

Both of these workers were under the impression that carotene was responsible for the colour. Banta (1939) believed that intra-vitam staining from the water was the cause of it.

Experiments were carried out by Verne (1923), who realised that the pigment was haemoglobin. He set up three sets of Daphnia cultures. To one set he added fragments of leaves or algae rich in chlorophyll together with a trace of an organic iron salt. To the second he added cultured zoogloea devoid of chlorophyll, while the third was similar to the second except for the addition of iron. Only the first of the series produced haemoglobin. Verne therefore concluded that chlorophyll was necessary for the production of haemoglobin in Daphnia.

In 1947, Fox (1948) showed that the haemoglobin of Daphnia is synthesized more readily in poorly than in highly aerated water. Since 1947, Fox has carried out and sponsored much experimental work on the problem and the following account and discussion is mainly concerned with this.

The light experiments of Schultz were repeated by Fox. His results did not confirm those of Schultz but contradicted them. In darkness the increase of haemoglobin was greater and its loss slower than in the light.

"The effect of light and darkness on the haemoglobin content of Daphnia blood might be (a) a direct one on the animal, or (b) the result of more green algal food in the light, or (c) a higher oxygen content of the lighted cultures caused by algal photosynthesis and an oxygen deficit in the dark brought about by bacteria and by the Daphnia themselves."

Experiments showed that there was no direct effect of light on the animals. They showed also that whether the animals were fed on green or non-green food made no difference. However a difference in oxygen concentration was found to have a large effect and to account for the cultures in darkness producing the more haemoglobin.

A low oxygen content of the water was found to increase haemoglobin production in D. magna Straus, D. pulex (De Geer), D. obtusa Kurz and even in the

normally colourless lake species, D. hyalina Leydig. It was also found that a number of other crustacean genera are affected in a similar way (Fox, Gilchrist & Phear 1951). By means of cultures maintained at various oxygen levels it was shown (Fox et al. 1951) that there is an inverse relationship between haemoglobin content and oxygen concentration at all percentage air saturations of the water at which Daphnia can live.

As already mentioned, no effect of different kinds of food on haemoglobin production was revealed. Animals fed on yeast or Gonium produced similar amounts of haemoglobin. The possibility, in the light and dark experiments, of bacterial food in the dark providing a better diet than algal in the light, was considered. However, egg production was higher in the lighted cultures and a high egg number is evidence of good nutrition (Ingle, Wood & Banta 1937; Banta 1939; Fox 1948; Fox, Hardcastle & Dresel 1949).

The quantity of available food was, however,

found to have an effect and, up to a certain level, to increase haemoglobin production (Fox et al. 1949). Relevant factors other than food and oxygen were sought; those which might be present in natural waters being first investigated. Fox (1948) cultured pale Daphnia in water taken from a pond in which the natural population was unusually red. The result of this experiment indicated the presence in the pond water of some factor, other than oxygen, which could stimulate the synthesis of haemoglobin.

In 1949 a similar experiment was conducted. This time standard conditions of feeding and oxygen concentration were maintained. Pale Daphnia in this case did not become redder. It was therefore supposed that the factor which, in the earlier experiment, stimulated haemoglobin production was solely good nutrition.

However, it is not necessary to conclude this, I feel. The pond from which water was taken in the earlier experiment may have contained a stimulating substance. That utilised in the later experiment may, on the other hand, have possessed no stimulating factor other than a low oxygen concentration. Actually, in the

later experiment, the animals were not the same colour in both waters but were actually redder in their own. This surely implies that there was a stimulant in their own water, and that the population inhabiting it was pale on account of a high oxygen content in this pond.

It was suspected that heavy rainfall caused Daphnia in ponds to become paler. This was tested (1948) by adding tap water to a water butt containing D. pulex. Eight days after the water had been added, the haemoglobin content of the population had fallen measurably. It seemed that a stimulating substance had been diluted, unless the oxygen content had merely been raised by disturbance of the water in filling the butt.

The species of Daphnia which tend to become red are often found in waters which are to a greater or lesser extent polluted with organic matter. As ducks are one source of pollution, an experiment was designed to test the effect of duck faeces (1948). One set of pale D. magna was cultured in a suspension of duck faeces and another set in water whose oxygen content

was reduced by hydrogenation and to which algae were added as food. The animals supplied with faeces became the redder. This result implied that faeces were a stimulating factor.

As in the case of the pond-water experiments, it was thought that better nutrition (possibly bacterial) might be causing the result. However, more recent culture experiments (unpublished work), in which feeding as well as oxygen was standardised, gave similar results. These showed that the faeces of ducks (and hamsters) were in themselves effective in stimulating haemoglobin production at low oxygen concentrations. At a high oxygen content of the water, faeces were found ineffective. Since the egg production of the animals with or without faeces was similar, the result could not have been due to a better diet being produced by faeces with Chlorella than by Chlorella alone (Ingle, Wood & Banta 1937; Banta 1939; Fox 1948; Fox et al. 1949).

Similar experiments were designed to test the effect of iron salts and also of vitamin B_{12}, both of which intervene in the haemoglobin synthesis of Man. At

low oxygen concentrations both of these acted as stimulants. Iron was effective in the ferrous or ferric state. Ferrous iron did not remain in the reduced state for long. It was, however, slightly more effective than ferric iron. The reason for this was that the former on hydrolysis forms a suspension of finer particles that the latter. It therefore remained longer in suspension.

Another factor which has recently been shown in this laboratory to be effective (unpublished work) is temperature. A higher temperature produces redder Daphnia. The probable cause for this is that metabolism is raised at the higher temperature. The internal oxygen content of the animal is therefore lowered and haemoglobin production augmented.

AIMS OF THE WORK

It is known, therefore, that, under experimental conditions, oxygen, temperature, iron, faeces, vitamin B_{12} and quantity of food can all affect haemoglobin production in *Daphnia*. The aim of this work was to investigate whether these factors operate also in natural populations and to look for any hitherto unsuspected factors.

No simple correlations were expected to emerge from the field results since there must be an interaction in ponds between many factors which in laboratory work are controlled.

FACTORS CHOSEN FOR INVESTIGATION IN PONDS

In selected ponds, measurements were made of the oxygen content, temperature and dissolved iron concentration of the water. These were factors considered likely to show some correlation with the haemoglobin content of *Daphnia* populations.

Measurements were also made of temporary hardness (alkalinity), total calcium and magnesium ion content (permanent hardness) and, in a few ponds, of oxidation-reduction potential.

General observations were also made on these ponds. These included the nature of the pond bed, the approximate depth and exposure to sunlight, the colour and turbidity of the water, the quantity and kind of vegetation (when present) and of macrofauna when abundant. The presence of domestic or wild animals which might defecate into the water was also noted.

There are other factors, apart from those already discussed, which intervene in the haemoglobin synthesis of Man and which might act in a similar fashion in Daphnia. Among these are the trace elements cobalt and copper. Apart from measurements made by Fox of the copper content in a few ponds (personal communication) this has not been studied. One would expect. however, that sufficient of these substances would be available from the mixed diet available in a pond and that varying amounts dissolved in the water would be of no significance.

SAMPLING THE DAPHNIA POPULATION

On a sample of ten large parthenogenetic female Daphnia, quantitative measurements of the haemoglobin content, the body length and the number of young within the brood pouch were made. The species was identified and notes were made of abundance, the colour of the gut contents, the presence of epibionts or parasites and, in D. obtusa, of the length of the shell-spine.

METHODS

1. ANALYSES MADE ON POND WATER

(a) OXYGEN

Methods employed

The sample was collected by means of a cradle, which contained two connected bottles and was lowered by means of a string handle to approximately the mid-depth of the water in the region sampled. The capacity of the first bottle was 40 ml. The second bottle was larger, having a capacity of 75 ml., so that the smaller bottle was flushed through with twice its volume of water before the sample was collected in it. The sample bottle possessed a ground-glass conical stopper which, when put on the bottle, displaced the surface water without entrapping air bubbles.

For determining the oxygen content of the water the Winkler method was used. By means of pipettes, 0.4 ml. of Winkler's Reagent was added, followed by 0.4 ml. of manganous chloride solution. At one

time the syringe pipette designed by Mortimer was used. This instrument, though convenient, was heavy to carry.

The strength of the reagents used was as follows:-

(a) Manganous chloride solution: 100 g. pure crystalline $MnCl_2.4H_2O$ in 200 ml. distilled water.

(b) Winkler's Reagent: 100 g. KOH in 200 ml. distilled water + 60 g. KI.

The stoppered sample bottle, containing a precipitate of higher oxides of manganese, was carried back to the laboratory. After the addition of 0.8 ml. 50% sulphuric acid, a sample of the resulting iodine solution was removed and titrated against an $\frac{N}{40}$ solution of sodium thiosulphate. The latter was standardised every few weeks against a solution of potassium iodate.

I found it convenient to use the micrometer syringe burette (Fox and Wingfield 1938) for the titrations, since I was already using this instrument

for some laboratory work. I used the syringe pipette as an ordinary pipette to withdraw portions of iodine solution from the sample bottle. The volume of my portions titrated was thus equal to that of the barrel plus the dead space of the pipette.

In July, 1951, I started to use the colorimetric modification devised by Johnson and Whitney (1939). With this the whole analysis could be made in the field. The method saved my carrying numerous sample bottles back and forth to the laboratory for titration and was particularly useful for the work in Denmark.

The solution of iodine formed on acidification was shaken with chloroform in a 10 ml. stoppered glass cylinder. The resulting violet colour of the chloroform extract was then compared with glass comparator disks made by the British Drug Houses Ltd.

As the partition coefficient of iodine in chloroform and water varies with the amount of potassium iodide present, I re-calibrated the glass disks, using the amount and strength of reagents to

which I was accustomed. For this, titrations were made by the normal Winkler technique at the same time as the colorimetric method was used on each iodine solution. Appendix 1 shows the calibration data. The provisional line was used to correct all my results. The regression line was afterwards found statistically and found to be almost the same.

This method gave the oxygen content to within 0.2 ml./l. which was less accurate than the titration method but sufficiently so for my purpose.

For any pond whose water was suspected of containing reducing or oxidising substances in amounts liable to invalidate the results produced by the normal Winkler method, a modification of the technique was used. This was the sample-blank modification (Ellis, Westfall & Ellis 1946; Adams, Barnett & Keller 1943).

Oxidising substances, such as nitrites and ferric iron, liberate iodine from the potassium iodide in the Winkler's Reagent and thus give a falsely high

result. Reducing substances such as ferrous iron, sulfites and various organic compounds by chemical action or adsorption remove some of the liberated iodine. Too low a reading is therefore produced.

Two samples in place of one are taken. To the Winkler's Reagent, before use, sufficient of a stock iodine solution is added to give the reagent a faint yellow tinge. This reagent is used for both samples. To one only, however, is the usual addition of manganous chloride made afterwards. The other - or "blank" - therefore differs from the true "sample" in that no precipitate is formed by the dissolved oxygen in the water. In both samples, however, reducing or oxidising substances present have the same effect. The difference in the results obtained is therefore equal to the oxygen content of the water.

Estimation of Minimum Oxygen Content Considered

At one time I considered making an estimate of the minimum daily oxygen content reached in a pond. My intention was to measure the oxygen directly at the time of its expected maximum and then to measure the oxygen consumption of the water. The latter would have been done by air-saturating a sample and leaving it in a pond in a blackened stoppered bottle. After a standard length of time the oxygen content of the sample would be again measured. Thus the oxygen consumption of the water per hour would be found. From a knowledge of the number of dark hours during the night, the minimum oxygen content reached could then be estimated.

This project was abandoned, however, for two reasons. Firstly: unless the blackened bottle was collected the next morning, oxygen would be consumed more rapidly within it during the day, on account of the higher temperature, than at night. The result obtained for the rate of consumption of oxygen would not, therefore, apply strictly to the night alone but would give too high a value.

Secondly: the sample bottle provides an unnatural environment for the microflora of the water contained within it. This might lead to an increased and altered bacterial activity (ZoBell 1940) and again a false impression of the oxygen consumption of the water would be obtained.

Vertical Gradient of Oxygen Concentration and Depth of Sampling

It is possible for there to be a vertical gradient of the oxygen concentration inaa pond. One might be expected in still weather, in summer or winter, in a pond which has no agents such as ducks to stir the water. On account of this possibility, I have, since March, taken samples for oxygen analysis from approximately mid-depth, assuming that this would generally give a mean value for the pond. The earlier samples were taken at a standard depth from the surface.

I have, in a few cases, tested for the presence of an oxygen gradient. I first did this in the very

shallow water of North Pond, Great Missenden, for I was surprised that water with so large a surface-to-volume ratio should have such a low percentage air saturation with oxygen.

For this I used a syringe pipette (Fox & Wingfield 1938) and carried back to the laboratory tubes containing the iodine solution formed on the addition of phosphoric acid. These samples were titrated against sodium thiosulphate some hours later in the laboratory. It was not a highly accurate method but was sufficiently so to indicate the absence of a steep oxygen gradient. It showed, also, that even 0.25 cm. below the surface film the water was only about 25% saturated with oxygen. The results are shown in table I.

TABLE I. North Pond, Great Missenden, a pond situated in a sheltered dingle. May 29, 1951, two hours after sunrise. Morning fine with no sun; rather windy.

DISTANCE BELOW SURFACE (cm.)	OXYGEN CONTENT (ml./l.)
0 . 25	1. 98
1 . 5	1. 67
4 . 0	1. 22
5 . 0 to 5 . 5	1. 43

Measurements made in Denmark on Praestevang II, on the other hand, indicated a considerable gradient (see table 2).

TABLE 2. Praestevang II, Hillerød, a large forest pond surfaced with Lemna. August 31, 1951, three hours before sunset. Day still and warm, cloudy with some rain.

DISTANCE BELOW SURFACE (cm.)	OXYGEN CONTENT (ml./l.)
8	3.9
23	3.0
30	1.3

In Praestevang II a comparison was made also between different regions at a constant depth (45 cm.) and constant distance from the edge (1.3 m.). The whole pond was at this time covered with Lemna. Region III differed from the other regions in lying under the shade of a hanging beech branch. Table 3 expresses these results.

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TABLE 3.

REGION	OXYGEN CONTENT (ml./l.)
1	3.0
2	2.2
3	1.4

These measurements show that, in a large pond, the oxygen content may vary at different levels and also in different regions. It would be of interest to know whether the individual Daphnia is restricted to regions of a certain oxygen concentration or swims throughout the pond. We know that in a culture flask of low oxygen content Daphnia rises to the surface (Fox et al. 1951). In Praestevang II it was necessary to collect Daphnia by throwing far into the pond a large net which was drawn back through the water by means of a rope. I could not tell, therefore, whether or not animals were present at all depths.

Diurnal Variation of Oxygen Concentration and Time of Sampling

Unlike the population in a culture flask, *Daphnia* in a pond is subjected to a diurnal variation of both light intensity and temperature. During the day, photosynthesis of the phytoplankton causes oxygen to be liberated into the water. The respiration of both plant and animal life within the pond has the reverse effect of using up the dissolved oxygen and this process continues throughout the twenty-four hours.

Generally, the effect of photosynthesis in the daytime masks that of respiration. The oxygen content of the water therefore rises during the day and a maximum is expected at about dusk or soon afterwards (Whitney 1942). During the night the oxygen concentration falls and a minimum is expected at about dawn.

It might be the mean daily oxygen content or the length of time during which oxygen concentration falls below a certain level which would affect haemoglobin production. To find the actual diurnal changes

within a pond the ideal method would be to use a continuously recording apparatus. As this was not feasible, the best plan seemed to be to make measurements at the times of expected maximum and expected minimum.

With this end in view my earlier samples were taken at sunrise and sunset. This was soon altered to a standard time (two hours) after sunrise and a standard time (two hours) before sunset. This seemed to be a legitimate compromise between what was most convenient and what, under ideal weather conditions, would give the truest results. It would be impossible to predict the exact time of maximum and minimum in the English or Danish climate.

Temperature was recorded whenever oxygen samples were taken. The other factors which were measured: iron content, temporary hardness and calcium and magnesium content were not expected to have any significant diurnal variation (Whitney 1942). Water samples for these analyses were, however, taken at the time of oxygen sampling for the sake of convenience.

(b) TEMPERATURE

In my earlier work I took the temperature of the surface water. In the case of temperature, however, as with oxygen content, it is possible for a vertical gradient to occur. I therefore changed to mid-depth recording. This was done by leaving the thermometer for one minute in the second bottle of the sampling cradle.

(c) IRON

For measuring the iron content of water α-α'-dipyridyl was used. This method was chosen since, by means of it, both ferric and ferrous iron can be simply measured. However, the proportion of ferric to ferrous ions is liable to change during the time between collecting the sample and its analysis. Measurements of total dissolved iron only were therefore made in the laboratory. A rough qualitative test was made in the field for the presence of ferrous iron by noting whether a pink colour appeared on the addition of excess α-α'-dipyridyl to a sample of water taken from

mid-depth.

A-a'-dipyridyl gives a red colouration with ferrous ions at a pH of 3.5 to 8.5. A solution of sodium sulphite is used to reduce ferric ions to the ferrous state. This reduction, in the case of a pure ferric iron solution, is completed within 15 minutes. However, certain forms of insoluble iron are gradually dissolved in the process. If the solution is left after the addition of the reagents, therefore, for more than 15 minutes the colour may continue to deepen and increasingly higher readings be obtained. It is necessary, therefore, to measure the samples either when the increase has ceased or after a standard time. My measurements were all made exactly 15 minutes after the addition of the last reagent.

Pond water for iron analysis was collected in bottles with glass or bakelite lids. A bottle was washed three times in the water before the sample was taken. Surface water was taken but any scum was avoided.

The sample was not filtered but was allowed to stand for at least several hours, during which time there was often a slight deposition of suspended matter on the bottom of the vessel. Care was taken not to include any of this in the portions withdrawn by pipette for analysis, since so doing was found to give a higher reading on account (presumably) of iron adsorbed on the surfaces of the particles.

To a 10 ml. portion of water the following reagents were added by pipette:-

1.0 ml. α-α'-dipyridyl solution (0.5% solution in absolute alcohol).

0.1 ml. 5N hydrochloric acid.

1.0 ml. sodium sulphite solution (10% solution in distilled water, prepared on the day of analysis).

The contents of the test-tube was well mixed with a glass rod and the time was noted.

Periodically, a check was made to ensure that the reagents were iron-free by carrying out the procedure on 10 ml. of distilled water.

A Duboscq colorimeter was used to match the colour of the sample against that of a prepared solution of known iron content. Two standard solutions of ferric ammonium sulphate were made up by the method described in the B.D.H. Book of Organic Reagents for Analytical Use (1949). These contained, respectively, 8.0 and 0.8 mg./l. of iron and were found to have remained stable during the whole period of the work.

A range of temporary iron standard solutions with the reagents added was made up for use in analysis. I found, however, that these standards, kept in glass-stoppered bottles, gradually increased in iron concentration. A provisional measurement on the pond sample, therefore, was made, using the appropriate provisional standard. Then a fresh standard was prepared in order to correct the result.

(d) TEMPORARY HARDNESS

Samples were collected in the same way as for iron analysis. Measurement of temporary hardness was carried out as soon as possible on returning to the

laboratory. The water was filtered through No.1 Whatman filter paper and 10 ml. portions were titrated against $\frac{N}{100}$ hydrochloric acid, using "4.5" indicator (prepared by the British Drug Houses Ltd.)

(e) CALCIUM + MAGNESIUM ION CONTENT

This was measured by the "Versene" method of Biedermann and Schwarzenbach, revised by Betz and Noll (1950). Samples were collected and filtered as for temporary hardness. To a 10 ml. portion of filtered sample, 0.1 ml. buffer solution was added. The titrating flask was shaken and 0.2 ml. indicator was added. The sample was then titrated against a solution of ethylene-diamine-tetra-acetic acid. The reagents were supplied ready for use by the British Drug Houses Ltd.

The method is a sensitive one but great care is needed with the end-point as the reaction takes place slowly.

(f) OXIDATION-REDUCTION POTENTIAL

Approximate measurements of "redox" potential were made by means of the dye, m-carboxy-phenol-indo-2:6-dibromophend which is reduced at a potential of $E_0' = 0.250$ Volts at pH 7. At first I carried samples back for testing in the laboratory. The samples were first saturated with nitrogen in order to remove any free dissolved oxygen and make the samples more comparable.

During nitrogenation, however, gases other than oxygen might also be removed. I therefore used the dyes in the field in water samples collected with the precautions used in sampling for oxygen analysis.

II. MEASUREMENTS MADE ON DAPHNIA

(a) HAEMOGLOBIN

Haemoglobin content was measured by the technique devised by Fox. As the method has been fully described (Fox 1948) I shall give only the essence of it in this thesis.

The ten specimens selected for examination are laid in a row on a glass slide placed on the stage of the microscope. The tint of oxyhaemoglobin near the base of the second antenna of each animal in turn is then compared with a diluted standard made from the worker's blood. The standard is contained in a wedge-shaped trough placed before the microscope mirror. A daylight lamp is placed beyond the trough and the image of the haemoglobin solution is made to fill the upper third of the field of view. The trough is moved over a scale in arbritary units until the colour of its image matches that of the animal under observation. The mean of the readings for ten animals is known as the haemoglobin index.

(b) BODY LENGTH

The length of each animal was measured from the front of the head to the base of the shell-spine. A micrometer grid was placed in a X6 eyepiece of the microscope and used with a 50 mm. objective. Calibration showed that each square was 1.00 X 1.00 mm. Measurements were approximated to the nearest 0.1 mm.

(c) NUMBER OF OFFSPRING

When the other measurements and observations had been made on them, the specimens were placed under a binocular microscope. A pair of fine needles was used to release the offspring from each brood pouch so that they could be counted.

SELECTION OF PONDS

I decided to study a selection of ponds throughout a year and, besides this, to visit as many other ponds as possible, even if once only. Ponds in different districts were visited. In England, these were in Surrey, London and Bedfordshire. In Denmark ponds near Hillerød, Zealand, were studied.

The ponds visited throughout the seasons were in two sets. In each set ponds fairly close to one another were chosen. This was in order that analyses could be made on them all within one hour. By this means a comparison could be made between their respective oxygen concentrations under similar weather conditions and at almost the same time of day.

One such group of four ponds was selected at Newdigate, Surrey, in April, 1951. One of these (Blanks Farm Pond) was chosen because it was a duck pond and because, in February, the haemoglobin index had been high. The three other ponds (Round Pond, Long Pond and

BLANKS FARM POND, NEWDIGATE,
September, 1952. (See p.32 + fig. 12, p.69)

Wilderness Pond) were chosen on account of their proximity to one another and because the species was the same as that in Blanks Farm Pond, namely D. obtusa.

This choice of ponds was rather unfortunate since, during spring and summer, the population of each pond was pale, having a haemoglobin index of less than 40 and mostly under 20, at which level accurate measurements of index cannot be made. Also, by September, the population in Round Pond and Long Pond had died out, though in November D. pulex appeared in the latter.

These Newdigate ponds were visited at weekly intervals whenever possible.

The other group was visited every two months from June, 1951, to July, 1952. These ponds were in Whipsnade Zoological Park, Bedfordshire. Of these, three, (Holly Frindle I, II and III) were in the enclosures of various breeds of duck and swan, one (Hippo Pond) of a pair of Hippopotamus and another (1X) of White-tailed Gnu. In these the species was D. obtusa with sometimes D. magna occuring with it.

HIPPO POND, WHIPSNADE,
July, 1952. (See this page+p.54)
In the foreground, Hippopotamus faeces are seen afloat.

Another set of ponds which I studied in a similar way though during one season only (August 1951) was in Zealand, Denmark. One of these ponds (Praestevang II) was a large pond in a beech forest. The others (Lysthus II, III and IV) formed part of a series of cement ponds formerly built by Professor Wesenberg-Lund. Praestevang II, Lysthus II and Lysthus IV were covered with a carpet of Lemna.

The species in these Danish ponds was D. pulex and the haemoglobin indices were surprisingly high (81, 60, 72 and 111 respectively). I shall now mention some of the other ponds on which similar but less frequent and in some case but a single analysis was carried out.

In January and February, 1951, six ponds in London and Surrey were investigated. An analysis was made also at the Wellcome Research Institution in several tanks which were maintained at a temperature of 25°C. and contained D. magna. In all these the index exceeded 50.

During April, 1951, I visited, without making

analyses, eighteen ponds in Surrey. In all these the populations ranged from colourless to pink. The species was mostly D. obtusa but occasionally D. curvirostris or the two species together.

The iron content of two ponds at Great Missenden, Middlesex, had been previously measured (personal communication from H. Munro Fox) and found to be exceedingly high. These ponds (North Pond and South Pond) were therefore analysed on several occasions in May and June. The ponds contained very shallow water which was opaque and reddish with colloidal iron. The populations of D. obtusa were moderately red.

In June, 1951, I paid the first of six visits to Whipsnade. Apart from those ponds used for regular analysis there were 16 others containing Daphnia. Measurements were made on samples from these populations but no analyses were made. This was repeated on most of these ponds on later visits to the Park.

In two of these ponds (XXl and XXll) there was a vigorous population of D. atkinsoni from December to

May. This is of interest since there are few other British records for this species (unpublished work: D.S. Johnson).

In Denmark, apart from four ponds on which analyses were carried out, other ponds were found containing D. pulex ranging from very red to pale pink in colour. Pale D. magna populations were found in two ponds. D. longispina was found in 10 ponds, including Praestevang II, where it was co-existent with D. pulex. In these populations there was no obvious haemoglobin in the blood, though in two ponds (Tern Pond and Fredensborg Pond) the animals had a slight pink appearance which might have been due to haemoglobin. It might, however, have been caused by some other haem compound in the blood (Fox 1948). An attempt was made to reveal haemoglobin in dried specimens brought back by conversion to a haemochromagen and with the use of a microspectroscope failed to show it. In October, 1951, a Newdigate pond (Copse Pond) was found containing D. pulex which was pinker than D. obtusa in nearby and similar ponds. Analyses were therefore made on this. D. magna in a cattle pond at Landbeach, Cambridge, in

June, 1951, were an amazing sight on account of the density of the population, and the index was 63 but many individuals appeared white on account of the parasitic protozoan, Thelohania. More spectular was D.magna at Hampton, May 1952 (see p.59).

PRAESTEVANG II, DENMARK,

August, 1951. (See pp.20+35)

Lemna covers most of the water surface.

RESULTS OF THE WORK

I OF ANALYSES MADE ON POND WATER

(a) OXYGEN

On figure 1 haemoglobin index corrected for size is plotted against mean daily oxygen concentration in ponds. Each point represents either a different pond or the same pond at a different season. The values for mean daily oxygen content were obtained from successive morning and evening recordings. A negative relationship between the two variables is indicated and is better seen on figure 2 where the mean index for each 1 ml./l. range of oxygen is shown. With this is a histogram which shows the number of results from which each mean was calculated. It can be seen from this that at oxygen concentrations below 3 ml./l. indices lie above 40 while at concentrations higher than this they lie below 20.

Figure 3, plotted in a similar way, shows the variation of haemoglobin content with minimum oxygen concentration. In figure 4, plotted in the same way as figure 2, a relationship similar to that shown by the latter graph is seen, and this seems to be similar to that shown in the laboratory with cultures of Daphnia

FIGURE 1.
VARIATION OF HAEMOGLOBIN CONTENT WITH MEAN DAILY OXYGEN CONTENT IN PONDS

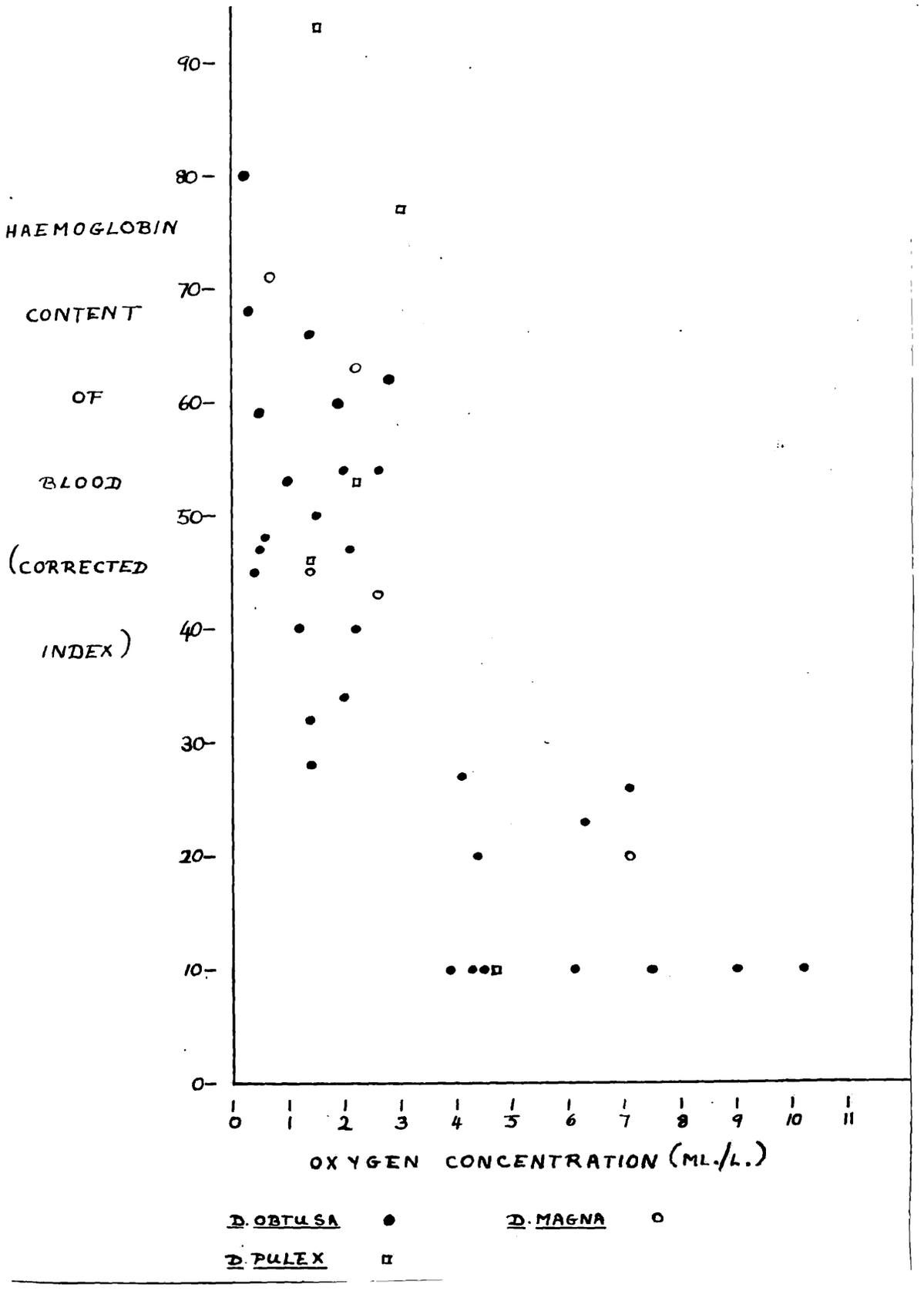


FIGURE 2.
VARIATION OF HAEMOGLOBIN CONTENT WITH MEAN DAILY OXYGEN CONTENT IN PONDS.

EACH POINT REPRESENTS THE MEAN HAEMOGLOBIN CONTENT FOR A CERTAIN RANGE OF OXYGEN CONCENTRATION. THE HISTOGRAM INDICATES THE NUMBER OF RESULTS FROM WHICH EACH MEAN IS DERIVED.

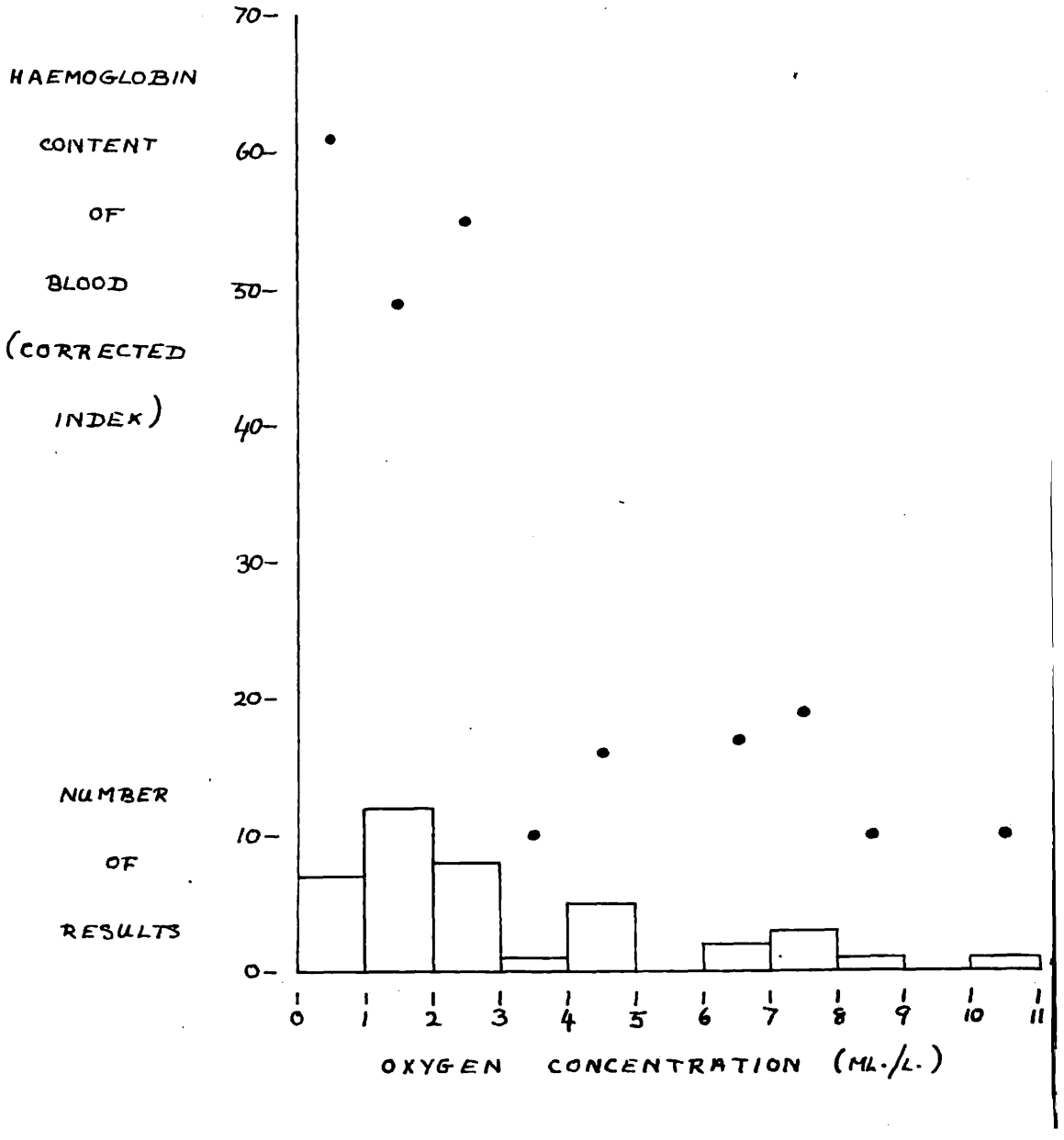


FIGURE 3.
VARIATION OF HAEMOGLOBIN CONTENT WITH MINIMUM OXYGEN CONTENT IN PONDS

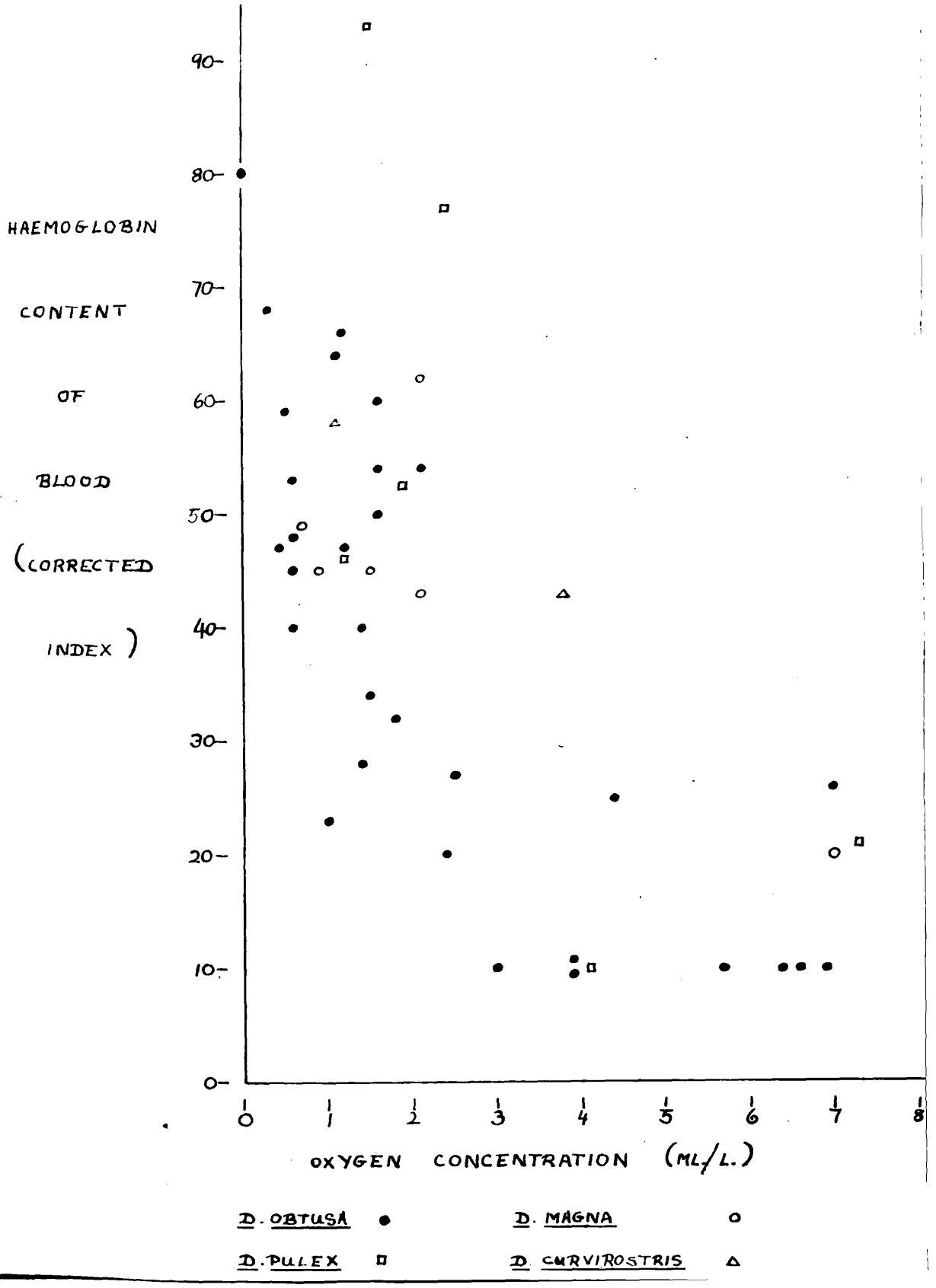
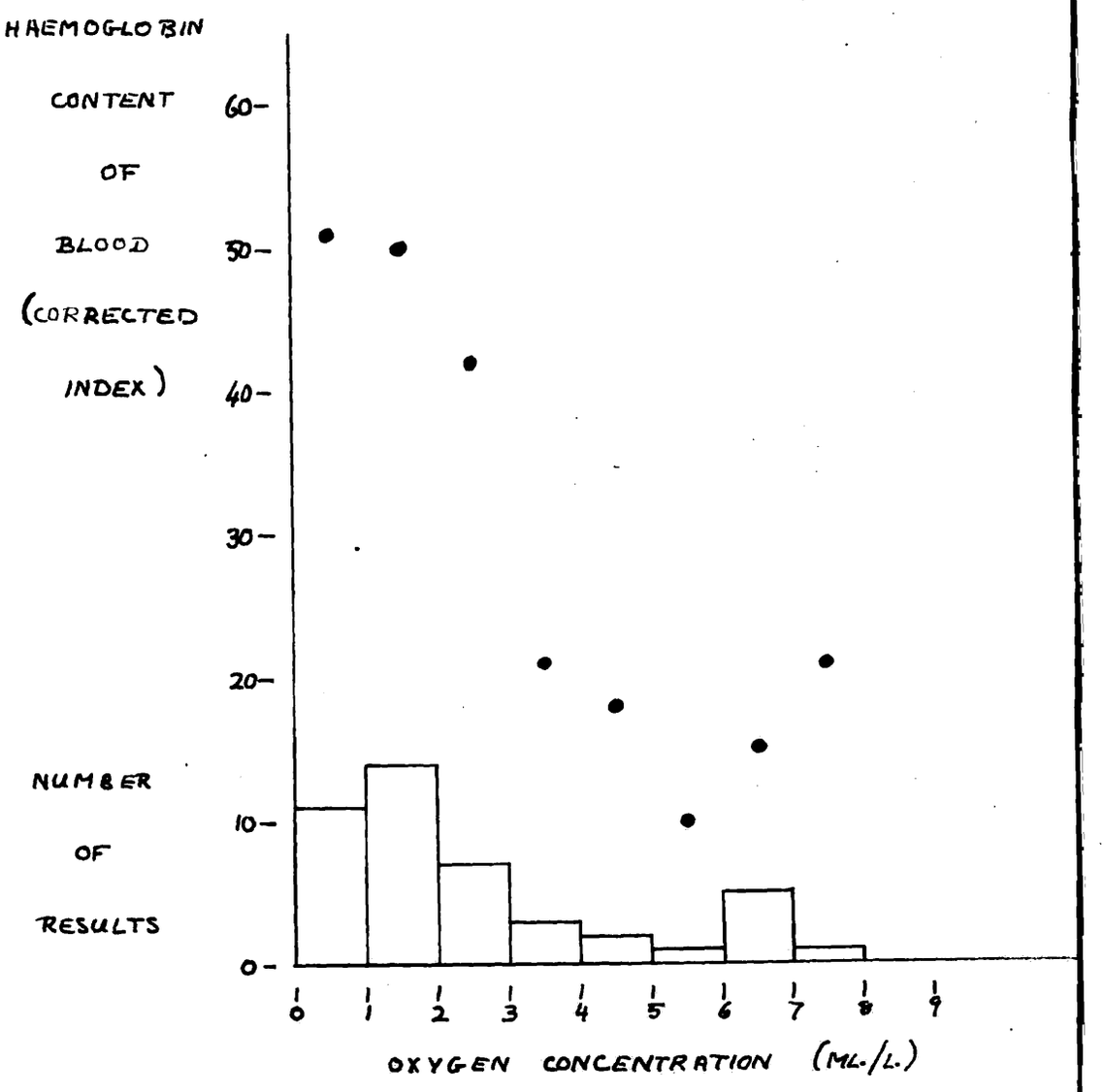


FIGURE 4.
VARIATION OF HAEMOGLOBIN CONTENT WITH MINIMUM OXYGEN CONTENT IN PONDS

EACH POINT REPRESENTS THE MEAN HAEMOGLOBIN CONTENT FOR A CERTAIN RANGE OF OXYGEN CONCENTRATION. THE HISTOGRAM INDICATES THE NUMBER OF RESULTS FROM WHICH EACH MEAN IS DERIVED.



maintained at different oxygen concentrations (Fox et al. 1951). Many of the points on figures 1 and 3 are averages of two or more successive measurements. The period of the year during which these were taken for each pond are given in appendix II from which the graphs were plotted. Results which have been omitted are those made during very exceptional circumstances, for instance those for Blanks Farm Pond in February when there was a gale and the water of the pond was flooded across the nearby road. The oxygen content, normally low, was on this occasion as high as 6 ml./l. Also omitted are the results from the first visit to Whipsnade (June, 1951) since on this occasion the length of the animals was not measured and the corrected index could not, therefore, be calculated.

There are two things which probably lessen the correlation shown by these graphs. One is that results are included for different species, though D. obtusa predominates. I have reason to suppose (see p.82) that the indices for D. pulex populations are considerably higher than they would be in the case of D. obtusa.

The other is that indices below 20 have been given the arbitary value of 10 as it was considered inaccurate to measure below 20. I think now that this was a mistake.

Since the haemoglobin index of a population in a single pond varies throughout the year (see p.72), one could seek a correlation between haemoglobin and oxygen concentration for a single pond. This would appear to have the advantage that less variables would interfere with the relationship. This is probably not the case, however. Consider figure 12. I have found similar and often greater fluctuations of the various factors in other ponds.

However there does seem to be a definite correlation with mean oxygen content shown in Blanks Farm Pond and in the four ponds most studied at Whipsnade. The graphs on figure 13 illustrate this.

FIGURE 5.
VARIATION OF HAEMOGLOBIN CONTENT WITH IRON CONTENT IN PONDS

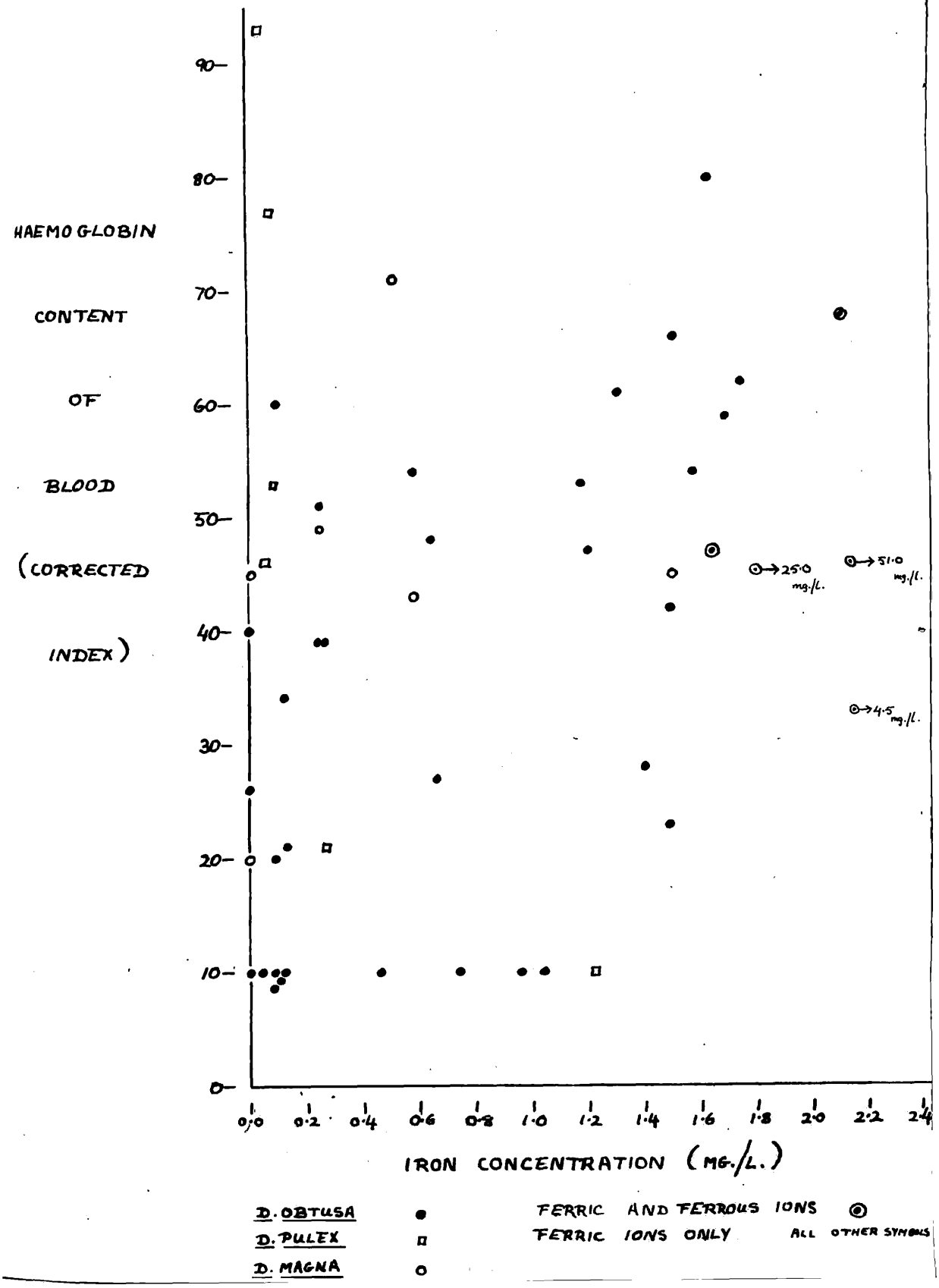
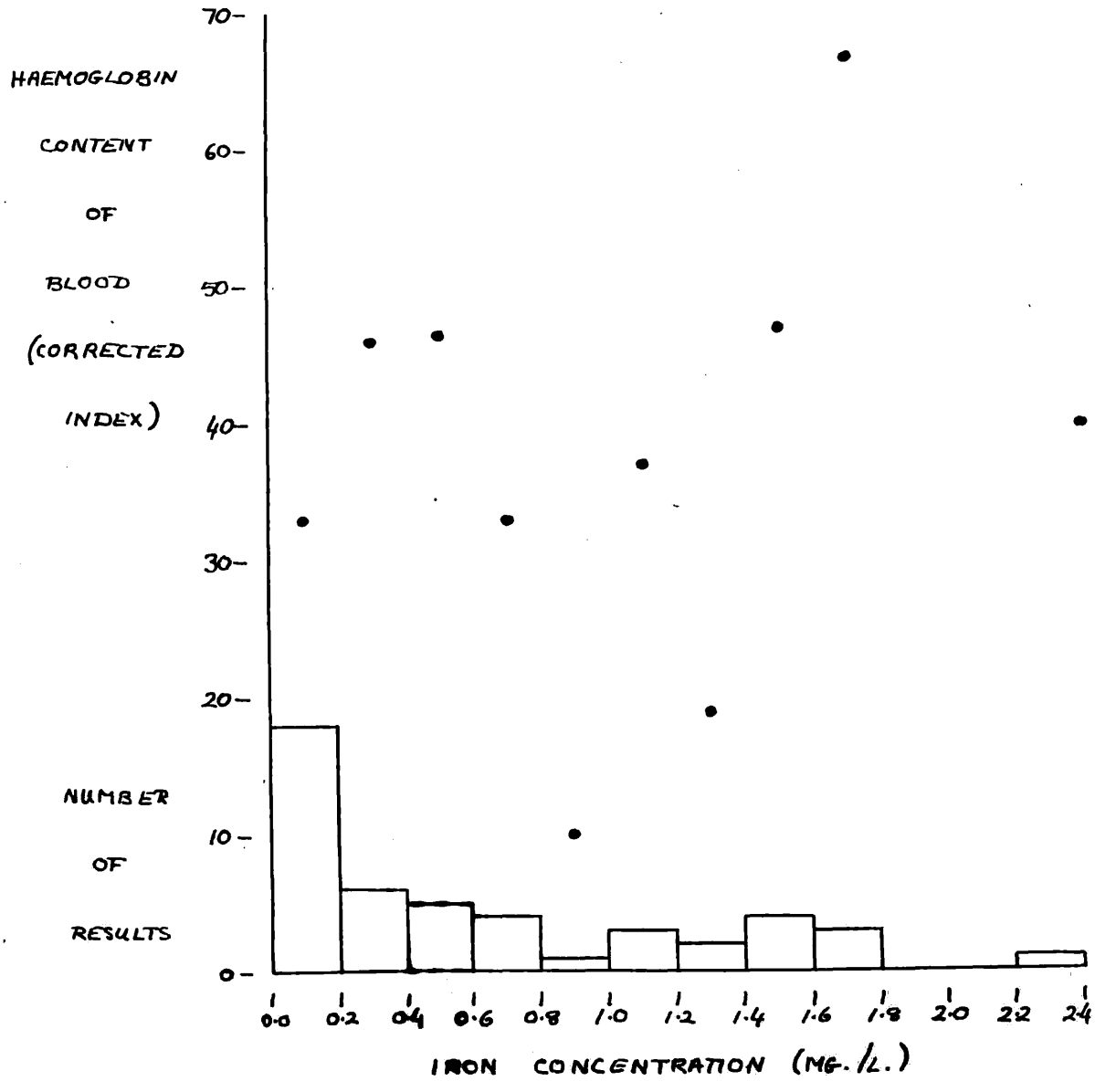


FIGURE 6.
VARIATION OF HAEMOGLOBIN CONTENT WITH IRON CONCENTRATION IN PONDS

EACH POINT REPRESENTS THE MEAN HAEMOGLOBIN CONTENT FOR A CERTAIN RANGE OF OXYGEN CONCENTRATION. THE HISTOGRAM INDICATES THE NUMBER OF RESULTS FROM WHICH EACH MEAN IS DERIVED.



(b) IRON

The total concentration of ferric and ferrous ions in pond waters is plotted, on figure 5, against haemoglobin content. In most ponds, ferric iron only was detected. A different symbol indicates those where ferrous iron was also present.

The concentration in most ponds lay between 0.00 and 2.00 mg./l. Also marked on this graph (in red ink) are some values of exceptionally high iron content, much of which was in the ferrous state.

The results have been grouped for 0.2 mg./l. ranges of iron concentration on figure 6. Little or no correlation is evident from this. For instance, D. obtusa with an index as high as 43 was found in June in a pond with no detectable iron in the water, while among the lowest iron contents were the Danish ponds containing very red D. pulex.

I found ferrous iron in only about four ponds. In one of these, Lily Pond, the water was highly polluted with organic matter and the total content of dissolved iron was 2.1 mg./l. In the other ponds the

FIGURE 7.

VARIATION OF HAEMOGLOBIN CONTENT WITH IRON CONCENTRATION IN PONDS OF SIMILAR MEAN DAILY OXYGEN CONTENT

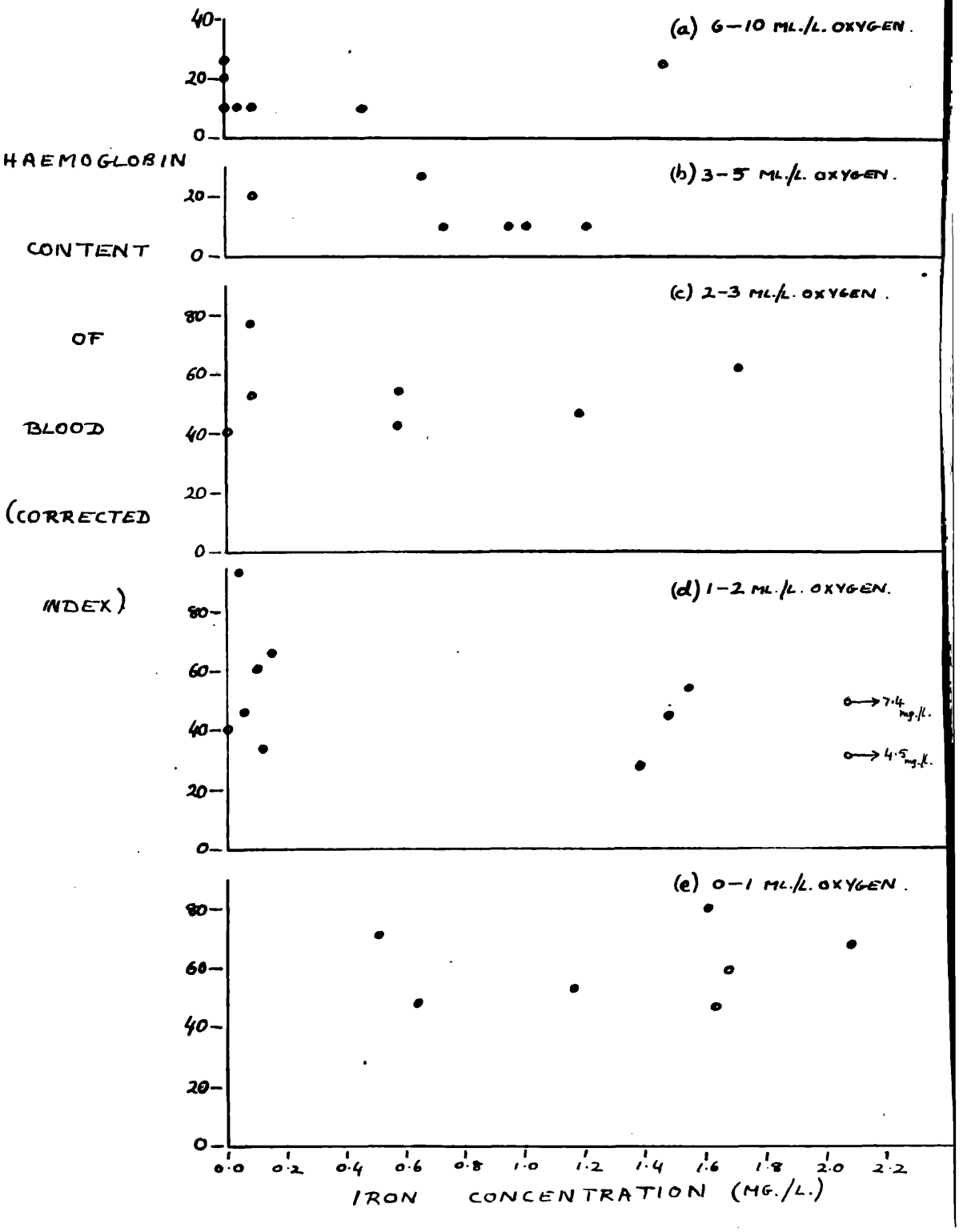


FIGURE 8.

WEATHER CONDITIONS FROM MARCH 18TH TO APRIL 4TH, 1952.

DATA FOR KEW, FROM AIR MINISTRY'S "DAILY WEATHER REPORT"

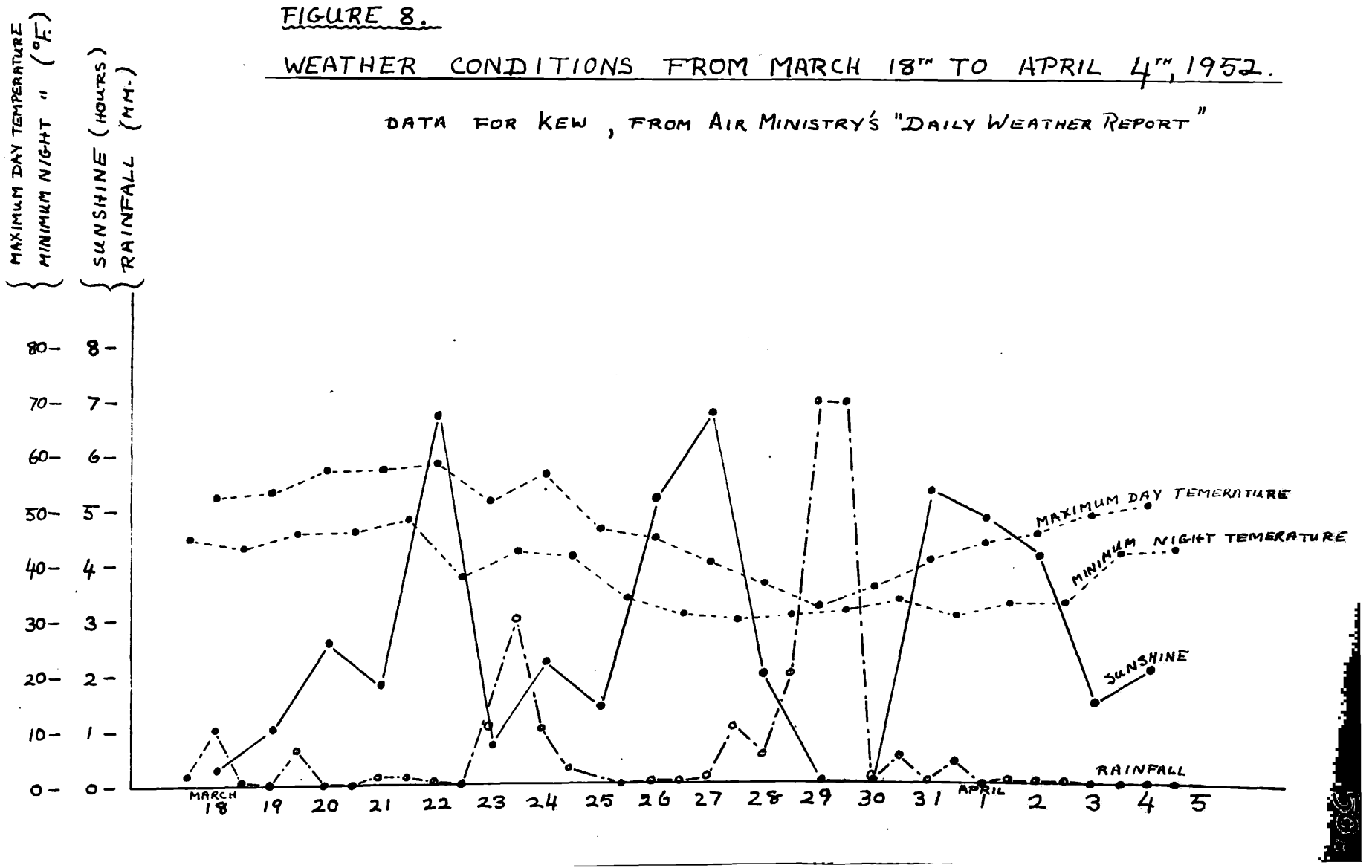
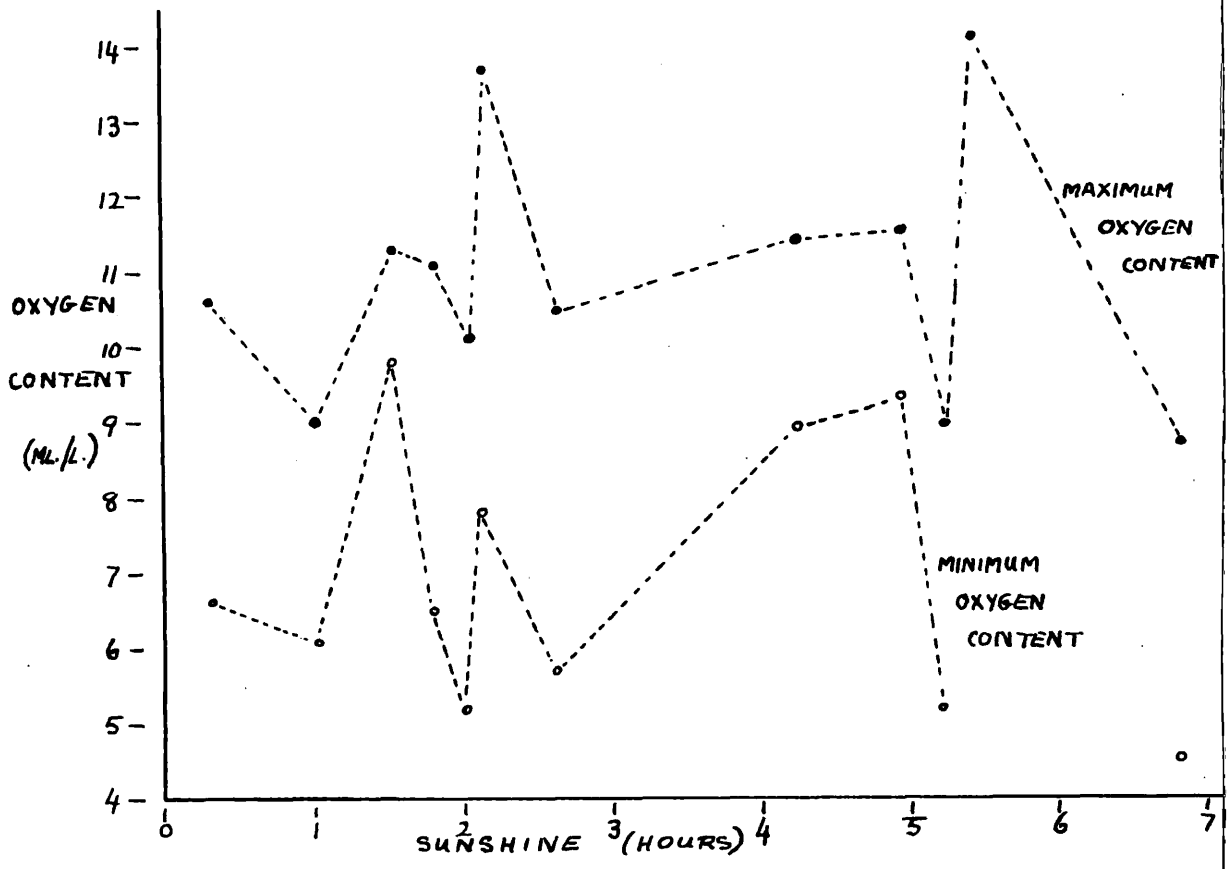


FIGURE 9.

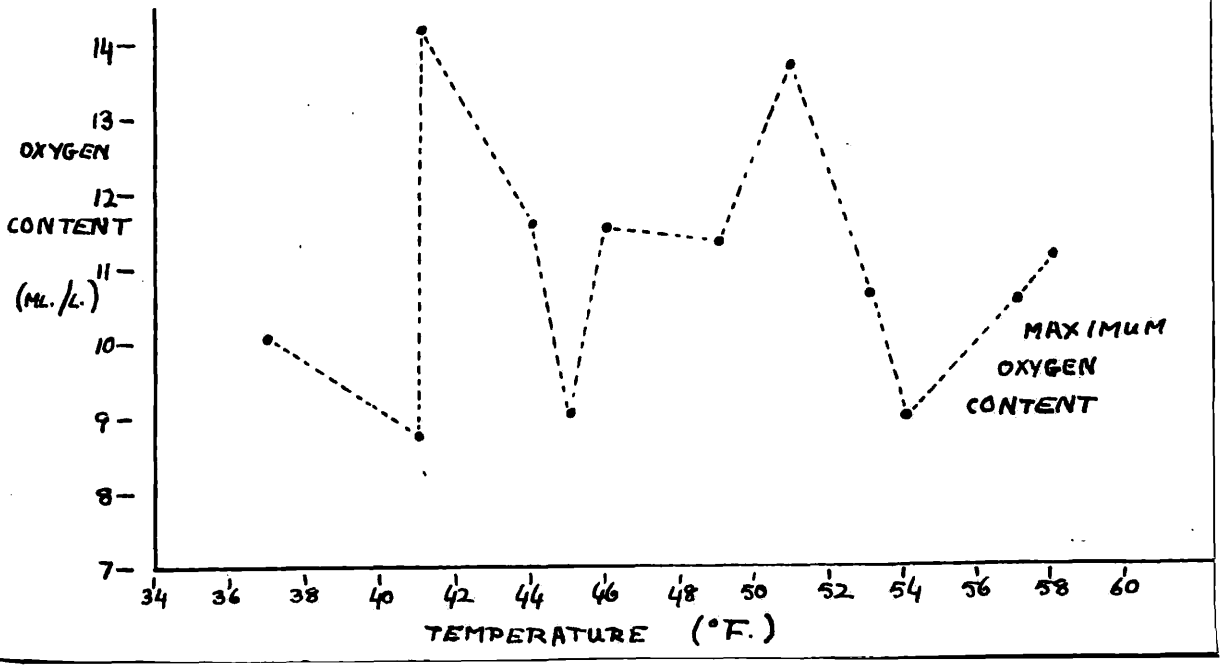
(a) VARIATION OF OXYGEN CONCENTRATION IN A POND (POOL VI)

WITH NUMBER OF HOURS SUNSHINE.



(b) VARIATION OF OXYGEN CONCENTRATION IN POOL VI

WITH MAXIMUM DAILY AIR TEMPERATURE.



water was very shallow and was reddish brown and opaque with colloidal ferric hydroxide. It was in these ponds that the iron content was very high (up to 51 mg./l.). Despite a low oxygen content, however, the haemoglobin indices were not above 65 (D. obtusa). Perhaps colloidal iron cannot be readily utilised by Daphnia.

Since oxygen is an important factor, its effect may mask that of iron in ponds. On figure 7, therefore, results covering ponds of similar mean daily oxygen content have been plotted. In none of the five oxygen ranges does any correlation become evident. There seems however some indication that at the highest oxygen concentrations iron content is low, and at the lowest, the iron content is higher.

A further means of examining iron results is by considering the seasonal changes which occur in a single pond, but the large seasonal changes in oxygen content render this form of comparison of little value. It is interesting to note, however, that considerable fluctuations in iron concentration do occur within ponds (see, for example, figure 12).

(c) TEMPORARY HARDNESS

A number of analyses of temporary hardness were made during the early part of the work. Values found in ponds, expressed as calcium carbonate equivalent, varied from 63 to 380 mg./l. Considerable changes occurred even within a single pond. No correlation with haemoglobin content became evident and the analyses were discontinued.

(d) CALCIUM & MAGNESIUM ION CONTENT

Here again there appeared to be no correlation with haemoglobin content. The range found in ponds was from 71 to 516 mg./l. as calcium carbonate equivalent.

There seemed to be no correlation, either, between the sum of the two forms of hardness in a pond and haemoglobin index.

These results do not give sufficient evidence to prove that temporary and permanent hardness are not relevant factors. They have no obvious effects, however, and it was not expected that they should have. A list of the results for "permanent" and temporary hardness are given in appendix 1V.

(e) FAECES

Where analyses were made on ponds obviously polluted by visiting animals, the oxygen content was low and reducing substances were present, necessitating the use of the modified Winkler method for oxygen determination. In the Hippopotamus pond at Whipsnade, for example (see photograph p.34) there was at times no detectable oxygen in the early morning.

However a low oxygen concentration was found also in ponds devoid of visiting animals. This was probably produced by much bacterial activity at the mud surface or by the lessening of photosynthesis on account of shading.

(f) OXIDATION-REDUCTION POTENTIAL

A low "redox" potential is caused by decaying organic matter, ferrous iron, hydrogen sulphide, ammonia, some nitrogen compounds and a low oxygen content of the water (Allgeier, Hafford & Juday 1941). A few measurements were made in ponds since it was hoped that the presence of some hitherto unsuspected

factor might be indicated.

A low potential (below E_0' = +0.250 Volts at pH7) was found, by the field method (though not detected by the nitrogenation method), to exist in North Pond and South Pond, Great Missenden, and in Hippo Pond, Whipsnade. The potential was too high to reduce the same dye in Danish ponds where the oxygen content was low and the Daphnia population red. No value was derived from these results.

II OF MEASUREMENTS MADE ON *DAPHNIA*

(a) BODY LENGTH

Assuming that the diameter of the blood sinuses varies directly with length in *Daphnia*, the larger the individual, the higher its haemoglobin index for a given concentration of haemoglobin in the blood. The index, therefore, is a measure of the total amount of haemoglobin in the animal but not of its concentration in the blood.

On the assumption mentioned, one can compare the haemoglobin concentration in the blood of different populations by correcting the index to that for a standard body length. I have used this method and chosen 2.0 mm. as the standard length.

It has been shown (personal communication from J. Green) that there is a direct relationship in *D. magna* between body length and breadth (in the region where the index is measured). How far it is permissable to compare one species with another on this basis is discussed under the section on species (p.82).

In some cases difference in size between the individuals of different populations is due to a difference in age. This no doubt causes errors in the results. In a pond in Denmark, containing Daphnia pulex, I noticed a striking difference in redness between the immature females and the larger mature females. The latter were very much paler. In only one other pond (at Newdigate; again containing D. pulex) have I seen this obvious difference.

(b) NUMBER OF OFFSPRING

It has been shown (Ingle, Wood & Banta 1937; Banta 1939; Fox 1948; Fox 1949) that the number of offspring produced in the brood pouch by parthenogenesis is indicative of the nutrition of a Daphnia population. In a population of low egg number, therefore, one might suppose that the diet was poor enough to retard the synthesis of haemoglobin.

Yet poor nutrition is not the only factor which produces small broods in Daphnia. Low temperature (Johnson 1951), darkness (Schultz 1928; Johnson 1951) and crowding (Pratt 1943) are all factors which reduce it. One need not necessarily expect, therefore, a correlation between low egg number and low haemoglobin index.

Moreover a low oxygen content of the water is another factor which reduces egg production (Fox et al. 1951) and also increases haemoglobin production. From this one would expect a correlation between low egg number and high index.

Contradicting this, however, is the fact that the presence of functional haemoglobin in Daphnia increases egg production (Fox et al. 1951).

We know (Dresel 1948) that in a mature parthenogenetic female the index varies with the stage of instar. This is on account of haemoglobin being passed into the eggs from the blood. The index,when the eggs are newly laid,falls to two-thirds of that at the embryo stage. One might suppose that the index would fluctuate more widely in an animal with many than in one with few eggs. Perhaps, though, each receives less haemoglobin in a large than in a small brood. Whichever may be the case, however, will have little bearing on my results for index since,in a sample of ten animals,there will be animals at all stages of instar.

The index method was modified by Fox, Hardcastle & Dresel (1949) to increase its accuracy. Parthogenetic

females all at the same instar stage were selected for measurement. This refinement was not considered necessary for my work.

The range of egg number which I have encountered has been from 0 to 50 (D. atkinsoni in December. That of one individual was 76) and my results seem to confirm the conclusion drawn by Fox (1948) that "neither in nature nor in the laboratory is there any direct relation between number of young and haemoglobin content of the mother's blood".

(c) OTHER OBSERVATIONS

There appears to be no correlation between abundance and haemoglobin. Animals were red and scarce in Long Pond, Lysthus 1X, Praestevang 11, and Hippo Pond, while they were red and very abundant in Landbeach Pond, Lysthus 11, 111 and 1V. I have never seen such abundance as at Hampton in May, 1952, when D. magna was present in a storage tank for waste water. Many animals were caught above the surface film and drifts of them moved by the wind gave the appearance of streaks of blood.

There seems to be no correlation evident from the results of gut colour, length of shell-spine or the presence of epibionts. Red animals may be clean or smothered with the rotifer, Brachionus. Their guts may be of various colours (i.e. their diet various) and the shell-spine of various lengths.

In the case of animals parasitized with the sporozoan, Thelohania, however, I suspected a correlation. Diseased individuals tended to have a higher haemoglobin index. They also possessed fewer eggs than healthy animals.

THE INFLUENCE OF WEATHER ON CONDITIONS IN PONDS

The extent of photosynthesis taking place during the day is dependent partly on the nature of the algal flora and partly on weather conditions. With higher temperatures and longer periods of sunshine, the rate of photosynthesis and consequent liberation of oxygen would be expected to be greater.

Weather may also have a direct effect on the oxygen content of a pond. At lower temperatures oxygen becomes more soluble in water and more tends to diffuse in at the surface. Air-saturated water at 20, 15, 10, 5 and 0°C. contains respectively 6.4, 7.0, 7.9, 8.9 and 10.2 ml./l. oxygen. Again, at lower temperatures one would expect the rate of oxygen consumption by the plants, animals and bacteria of the pond to be less.
Wind and heavy rain may causing mixing of the water in a pond and thus aerate it.

These are some of the reasons for which weather has a bearing on haemoglobin synthesis. Because of weather changes, analyses of oxygen content were made

as often as possible on those ponds which were studied most, and a note was made of the weather on each occasion.

INVESTIGATION MADE IN A SINGLE POND

During March and April, 1952, I made recordings of the oxygen content within a single pond (Pool V1, Bedford College Botany Garden) over a period of three weeks. The aim of this work was to obtain an indication of how much correlation one might expect between oxygen concentration and meteorological changes, and of the value of weather notes made when my samples were taken.

On twelve occasions during this period, I made measurements at two hours after sunrise and two hours before sunset. The results may be seen on table 4.

TABLE 4. Oxygen concentration in Pool V1 on different days during a three-week period.

DATE	MORNING		EVENING		DIURNAL RISE IN OXYGEN CONTENT (ml./l.)
	OXYGEN CONTENT (ml./l.)	TEMPERATURE C°	OXYGEN CONTENT (m./l.)	TEMPERATURE C°	
March 18	6.6	7.8	10.6		4.0
19	6.1	7.5	9.0	9.3	2.9
20	5.7	8.4	10.5	10.5	4.8
21	6.5	8.9	11.1	11.0	4.6
26	5.2	5.2	9.0	8.0	3.8
27	4.6	3.9	8.8	7.3	4.2
28	5.2	3.3	10.1		4.9
29	6.5	2.3			
31			14.2	5.5	
April 1	9.4	3.5	11.6	7.3	2.2
2	9.0	4.2	11.5	5.9	2.5
3	9.8	3.8	11.3	7.3	1.5
4	7.8	6.2	13.7	9.4	5.9

During this period there was a considerable variety of weather conditions. These are illustrated by figure 8. This shows that day-to-day changes in maximum and minimum temperature were gradual but that there were sudden changes in the amount of both sunshine and rainfall, while on two days there was snow.

On figure 9 (a) I have plotted maximum oxygen content against number of hours sunshine. The minimum oxygen value of the same day is plotted on the same graph. This shows no obvious correlation. A correlation can be seen from a comparison of the two graphs, however. These follow one another, thus showing that the evening oxygen content is dependent to a considerable degree on that of the previous morning. It can also be seen from this graph that diurnal rise in oxygen content is not proportional to the amount of sunshine.

The possible effect of changes in air temperature were also considered. On figure 9 (b) diurnal rise in oxygen content is plotted against maximum air temperature. Again there appears to be no correlation.

FIGURE 10.

WEATHER CONDITIONS FROM FEBRUARY, 1951, TO MARCH, 1952.

DATA FOR WISLEY, SURREY, FROM AIR MINISTRY'S MONTHLY WEATHER REPORT.

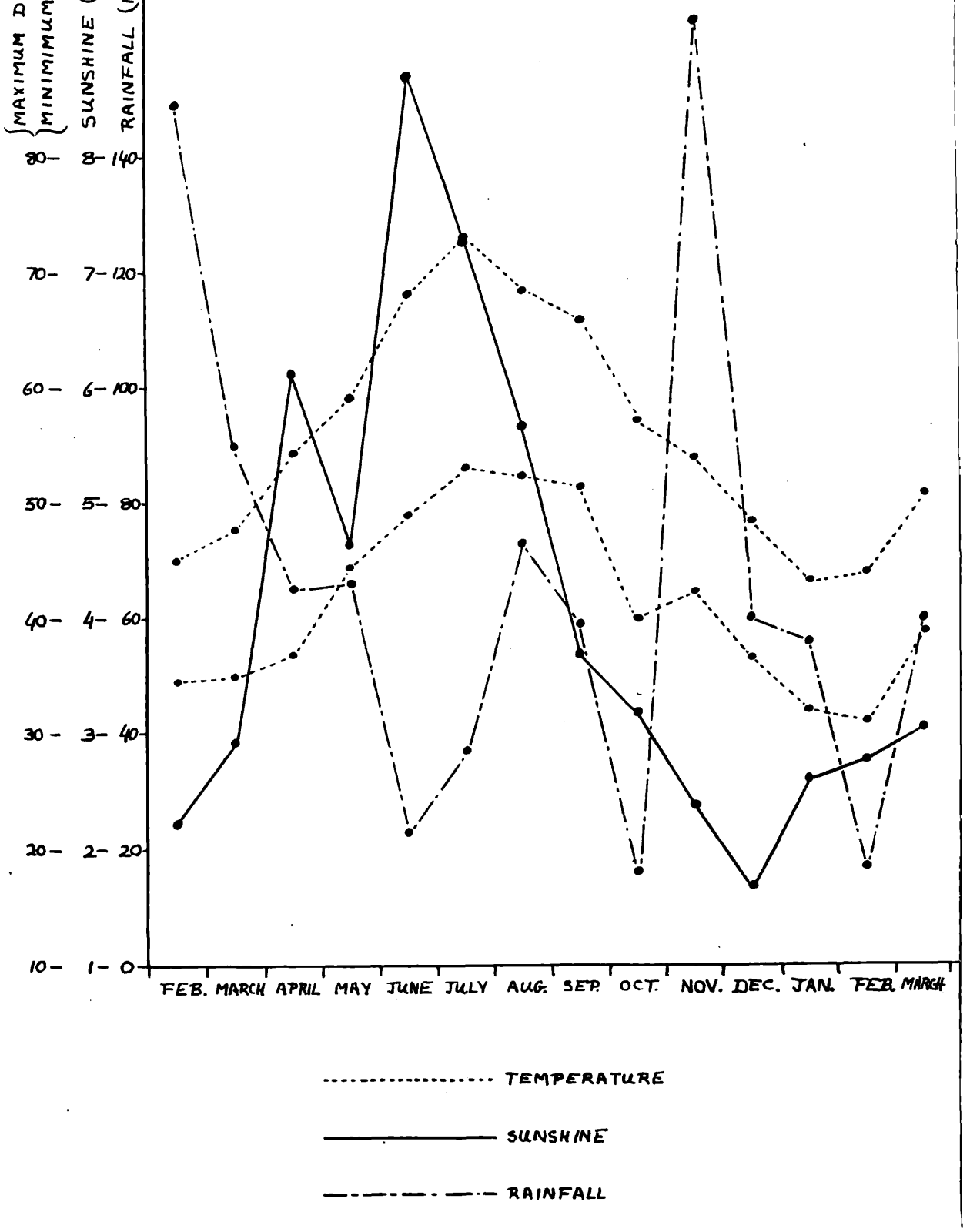
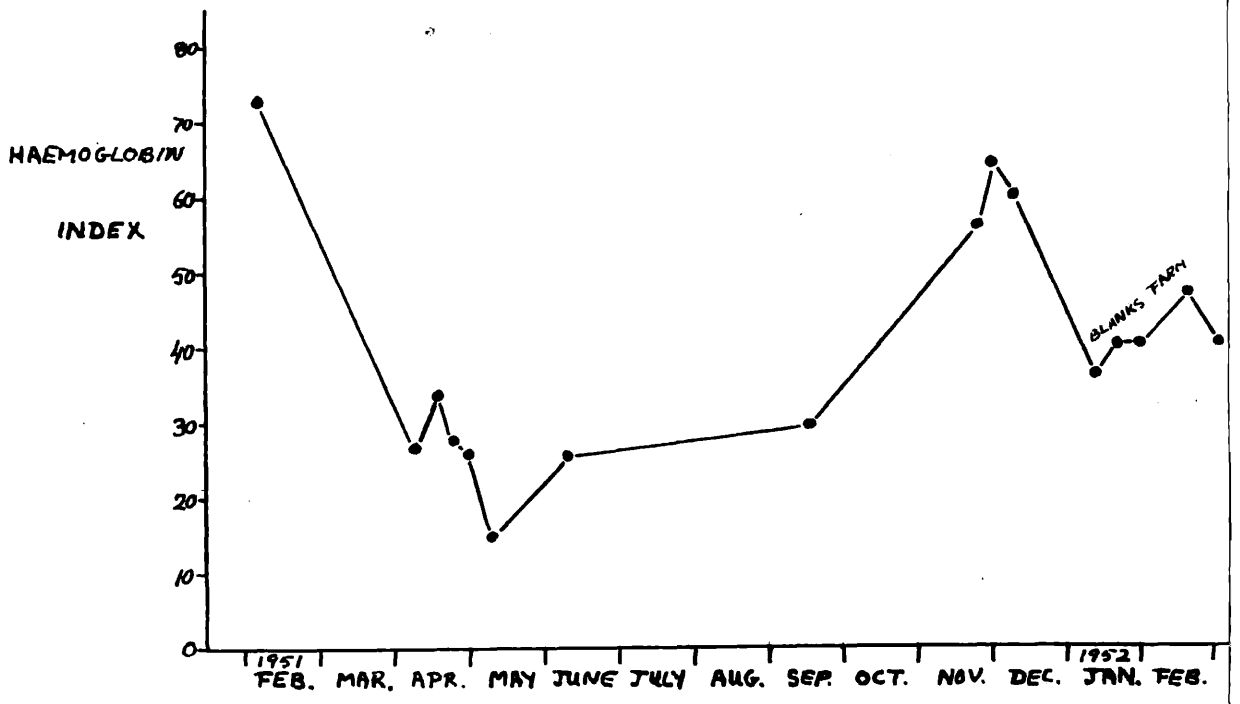
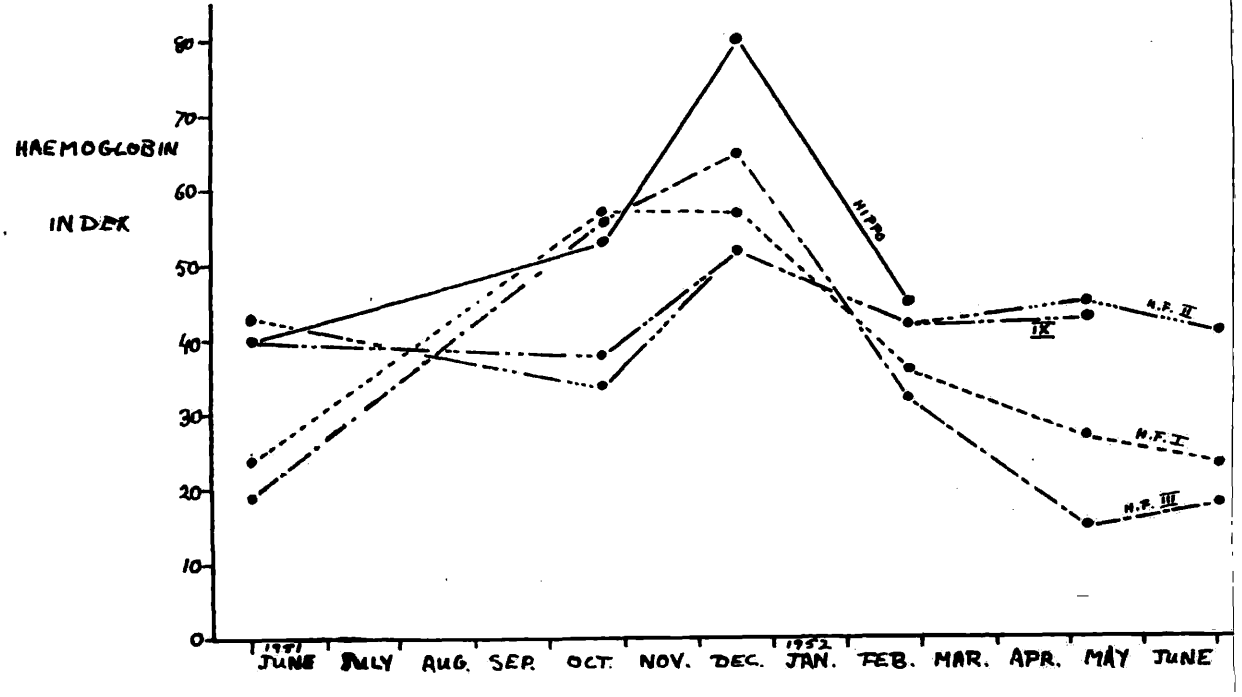


FIGURE 11

SEASONAL VARIATION IN HAEMOGLOBIN INDEX OF D. OBTUSA IN SIX PONDS.





The work shows that considerable changes in oxygen content on different days are possible. These, however, are not in any simple way dependent on the weather conditions - sun, temperature and rainfall. The concentration reached by the end of a day depends considerably on that at the beginning of the day. Likewise, that in the morning depends on that of the evening before. Day to day variations must to some extent depend on the interaction of climatic factors and also on biological fluctuations within the pond. These latter are probably the more important.

The results of this work indicate that it is valuable to have oxygen recordings for a pond on as many different days as possible. They suggest, too, that little importance should be attached to notes which I made on the weather at the times of sampling.

Another point of interest is that on each day there was a significant diurnal rise in oxygen. This emphasizes the need for making two recordings per day or at least of noting the time of day when but a single analysis can be made.

SEASONAL CHANGES IN HAEMOGLOBIN PRODUCTION

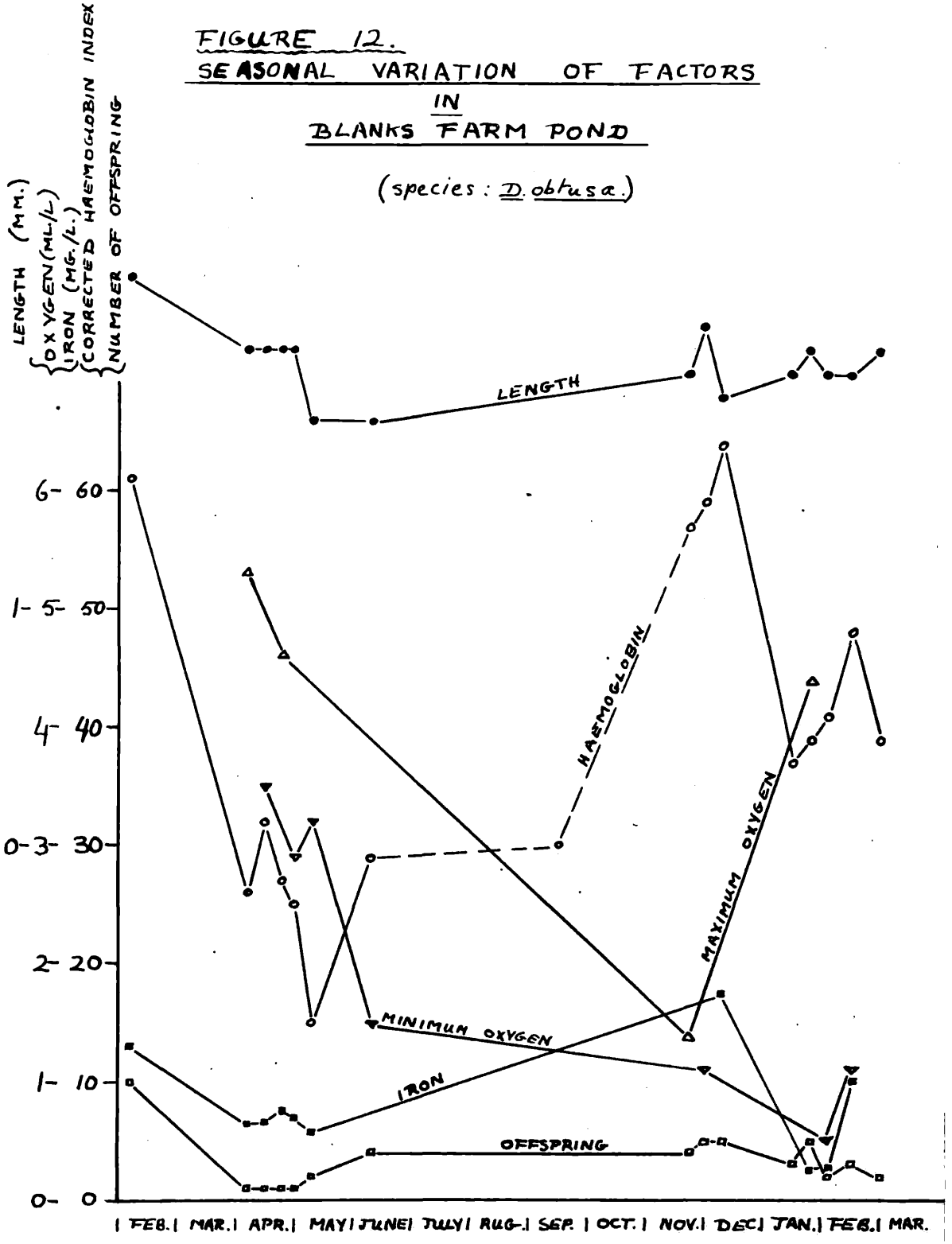
The haemoglobin index of a Daphnia population probably depends mainly on the average oxygen content within the pond during the previous week or ten days.

It is of interest to consider whether this average oxygen content and in fact whether any other relevant factor varies with the season of the year. Figure 10 shows the seasonal change in sunshine, temperature and rainfall for Wisley, Surrey, from February, 1951, to March, 1952. Data for Whipsnade follow similar curves but the rainfall was slightly higher and the temperature slightly lower. The amount of sunshine was about the same.

These curves show that in winter there is far less sunshine than in summer. Not only is the length of day much shorter but the intensity of the light and heat reaching the earth during the periods of sunshine is less. Temperatures are therefore distinctly lower in winter. Rainfall tends to be much higher in winter

FIGURE 12.
SEASONAL VARIATION OF FACTORS
IN
BLANKS FARM POND

(species : D. obtusa.)



but this is a more variable factor. Though no correlation between weather factors and oxygen content emerged from the work carried out over a period of three weeks, it seems likely that there would be seasonal changes of the kind discussed under the section on weather (p.61). Apart from oxygen, other dissolved substances in the water may be affected by prolonged weather conditions. The possibility that rain dilutes salts (such as iron) has already been mentioned (p. 6). In ponds, however, rain may add salts to the water by washing them in from the surrounding soil. (Nicol 1935; Atkins & Harris 1925; Savage 1935).

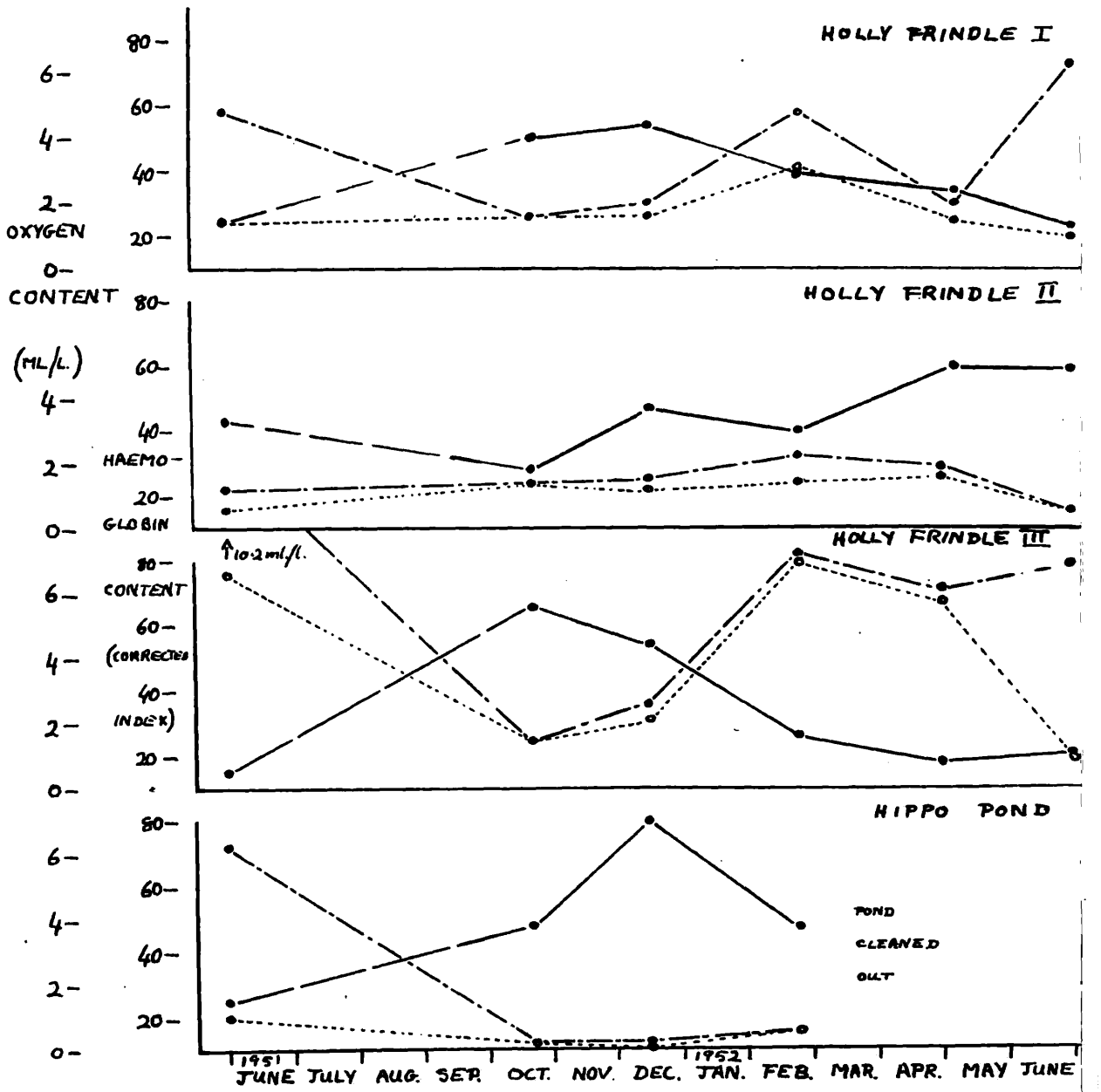
Affects of the climate on the algal flora of a pond may affect Daphnia not only by alterations in photosynthetic activity but also regarding food supplies. One might expect, for instance, that in winter there would be insufficient food supplies to maintain a high level of haemoglobin production. Again, we would expect low temperatures to reduce haemoglobin production by a direct effect on the animals.

A number of factors are therefore interacting. Which of these will override others will depend on the

FIGURE 13.

SEASONAL VARIATION OF HAEMOGLOBIN CONTENT AND OXYGEN CONCENTRATION IN FOUR PONDS AT WHIPSNADE.

(SPECIES: D. OBTUSA)



nature of the pond and on the climatic nature of the year.

It seems that there is a seasonal change in haemoglobin content common to populations in many ponds. This change takes the form of a winter rise and a summer fall in redness. Fox (1948) measured the haemoglobin at regular intervals in two ponds during part of the year. His results were the first to show that fluctuations in a single population are possible. They also suggest that the amount rises to a maximum in winter.

Figure 11 shows the variation in haemoglobin content in six ponds which I have visited at intervals over a period of a year. The lower graph shows the Whipsnade group of ponds, studied from June, 1951, to July, 1952. The upper graph shows one of the Newdigate group. In each of these ponds a significant rise in colour in the winter months is shown.

HAEMOGLOBIN PRODUCTION IN DIFFERENT SPECIES OF DAPHNIA

Fox (1948) discovered that the environment can act differently on two species of Daphnia present together in a pond. He found that in one pond D. obtusa was redder than D. pulex while in other ponds D. obtusa was the paler of the two species. He noticed the same phenomenon with the species D. magna and D. obtusa, for in one pond the former and in another the latter possessed the greater concentration of haemoglobin in the blood.

It would be interesting, yet not surprising, to find that different species of Daphnia vary in the amount of haemoglobin synthesized under similar conditions. It seems strange, however, that it should be possible for either of a pair of species to be the redder. "Whatever it is in water or food that influences the quantity of haemoglobin, the factor acts differentially on two species in one pond and its relative effect on two species differs in different localities" (Fox 1948).

A possible explanation for this would be that the populations in these different localities belong to physiological races differing with respect to their haemoglobin-forming mechanisms. A point in favour of this is that pale D. obtusa from different localities took very different lengths of time to become red when cultured in poorly aerated water (personal communication from B.M. Gilchrist).

Another explanation could be that more than one factor is operating in one of a pair of ponds. Perhaps one species is more susceptible than the other to this second factor. Although I have been unable to find any correlation in the field between haemoglobin content and any factor other than oxygen, I cannot deny the likelihood that other factors, such as those effective in laboratory experiments, do interact in the field.

It is not necessary to assume the existence of races or of a second factor, however. Supposing oxygen alone were effective, curves relating haemoglobin index to oxygen content for the two species might cross at some point. At an oxygen concentration above this

point one species would be the redder. At an oxygen content below the point it would be the paler.

Other possible explanations are concerned with the state of the populations. The size of the animals was measured by Fox. That of D. pulex and D. obtusa was the same. D. magna was considerably larger than D. obtusa and when the size discrepancy was allowed for the relative concentrations of haemoglobin in the blood of these two species were still quite different in the two ponds. It is possible, however, that one population might have been in a different state of maturity, nutrition or health which might influence haemoglobin production. It has been shown, for instance, that the haemoglobin content of the blood varies with the age of the individual in D. magna (personal communication from J. Green).

Being interested in this comparison between species, I made measurements, whenever possible, on a sample of each of two species occurring together. I made six such measurements in the case of D. magna and D. obtusa and one for D. obtusa with D. curvirostris.

It can be seen from table 5 that the haemoglobin index of D. obtusa is in every case the lower. However, if size is taken into account it becomes evident that the concentration of haemoglobin in the blood of this species is the same as or higher than that of D. magna. The single measurement for D. obtusa and D. curvirostris shows the haemoglobin concentration in the former species to be again the greater.

TABLE 5. Comparison between the haemoglobin content of the blood of two species inhabiting the same pond.

POND	DATE	SPECIES	HAEMO-GLOBIN INDEX	NUMBER OF OFF-SPRING	LENGTH IN mm.	HAEMOGLOBIN INDEX FOR LENGTH OF 2.0 mm.
Holly Frindle III	Oct.20,1951	(D.obtusa	56	1	1.7	66
		(D.magna	61	0	2.7	45
Holly Frindle III	Dec.17,1951	(D.obtusa	65	13	2.4	54
		(D.magna	79	8	3.7	43
Hippo	Oct.20,1951	(D.obtusa	53	12	2.2	48
		(D.magna	69	0	3.0	46
	"	(D.obtusa	26	4	1.9	27
		(D.magna	48	19	3.7	26
Holly Frindle III	Feb.25,1952	(D.obtusa	32	14	2.5	26
		(D.magna	45	29	4.4	20
	Oct.20,1951	(D.obtusa	17	8	1.8	19
		(D.magna	33	35	3.3	20
Pool VII	Dec.3, 1951	(D.obtusa	64	11	2.0	64
		(D.curvirostris	70	12	2.4	58

On other occasions when I have found D. obtusa and D. magna mixed, the latter species has been too scarce for measurements to be made.

I have found no such case as Fox found of D. magna exceeding D. obtusa in index. One of the suggestions I put forward to explain his results was that the curves relating haemoglobin to oxygen for the two species might cross at some point (see p.74). My results cover a considerable range of haemoglobin index, a range of 17 to 65 for D. obtusa and 33 to 79 for D. magna but relative differences in haemoglobin content over this range give no evidence in favour of this suggestion.

It seems that D. obtusa possesses a slightly redder blood than D. magna under the same conditions. This is with two assumptions, however. Firstly that it is justifiable to correct for length between different species: justifiable to assume that the ratio of length to the depth of the blood sinus at the base of the second antenna is the same for both species.

The other assumption is that both species occupy the same ecological niche within the pond throughout night and day. The oxygen content within a pond can vary greatly with depth and in different regions (see p.19). Perhaps D. obtusa inhabits regions of lower oxygen than D. magna. Both are known to grub on the bottom for food and to swim nearer the surface when the water becomes poorly aerated. To prove a real difference between the species, therefore, controlled experiments would be required.

D. obtusa and D. pulex I have never found together except in the Leg of Mutton Pond, Hampstead, where each inhabited a different region. I became interested in comparing these species, however, partly because of the results of Fox (1948) and partly in an attempt to explain my own results. By looking at figure 1 one can see that almost all values for populations with a high index are for D. pulex. In these cases the index (uncorrected) ranges from 60 to 111 for a mean daily oxygen range of 1 to 3 ml./l. In ponds of this oxygen content containing D. obtusa, on the other hand, indices range from 30 to 60 only.

These results for D. pulex, however, are all

from Denmark and it is possible that the ponds contained some factor which I did not discover. In these ponds there was a considerable oxygen gradient (see table 2). It is possible that D. pulex spent much time near the pool bed where there was less oxygen than at mid-depth where I measured it. In Praestevang II I was unable to tell which regions Daphnia occupied. In the other ponds (Lysthus II, III and IV), however, D. pulex was abundant at all depths. Moreover it seems unlikely that this species should have a preference for the bottom regions since it is associated with open ponds with an oxygen content probably higher than that of the smaller more turbid pools associated with D. obtusa.

In a pond in England (Copse Pond) I found D. pulex and it was pinker than D. obtusa in neighbouring and similar ponds, although the oxygen content was high.

Another explanation could be that D. pulex synthesizes more haemoglobin than D. obtusa under the same conditions just as D. obtusa appears to have redder blood than D. magna. Moreover Fox (1948) mentions

only one case where D. obtusa is redder than D. pulex in the same pond and more than one of the opposite case.

I considered therefore that it would be worth making a laboratory comparison between these two species such as I mentioned would be necessary in a true comparison between D. magna and D. obtusa.

LABORATORY COMPARISON BETWEEN D. PULEX AND D. OBTUSA

The method of culturing followed was that which has been developed in this department (Fox et al. 1951). The chief modification made was that each experimental flask, instead of containing a single species, contained an equal number of the two species D. pulex and D. obtusa. This ensured that the conditions for both were identical.

Method

Twenty parthenogenetic females of each species were cultured, in each of several 100 ml. conical flasks, in filtered water from Regent's Park Lake. Chlorella vulgaris Beij was used as food and was added each day to restore the optical density of the suspension to 0.25 after that which had settled on the bottom had been stirred into suspension with a pipette. A grey-wedge photometer was used to measure the optical density, a reading of about 50 being used.

Chlorella was cultured on agar slopes containing glucose and salts in proportions recommended by

Pearsall and Loose (1937). Slope making and subculturing were performed by standard techniques.

By filling the flasks to an appropriate level in the neck, a low oxygen content was soon reached and maintained within the cultures. The oxygen content was measured one or more times during each experiment by the method of Fox and Wingfield (1938) (see appendix V). The flasks were kept in a dark cupboard in order to prevent oxygenation by the photosynthesis of Chlorella.

On the second day of each experiment 0.5 ml. of an approximately 1% solution of ferrous ammonium sulphate was added to each flask in order to speed the rise in haemoglobin production.

Throughout the experiments animals from the same sources were used. D. pulex was obtained from Copse Pond, Newdigate and D. obtusa from Pool V1, Bedford College Botany Garden.

Four separate experiments were set up and were continued for respectively 10, 8, 18 and 9 days.

Approximate measurements were made on the animals of index, body length and number of offspring before the experiments were set up. Large individuals, almost colourless and with apparent freedom from parasites were chosen.

The methods already described (p.30) were used for measuring the haemoglobin content and length of the animals. The number of offspring within the brood pouch was estimated without dissection, since when an experiment was to be continued, it was important not to damage the specimens.

Measurements of breadth were taken just behind the fornix on specimens propped dorsal side upwards on a slide. For this a micrometer grid was placed in a X6 eyepiece. Calibration of the grid showed each square to be 0.500 X 0.500 mm. when a 33 mm. objective was used. For the measurement of length when it was to be compared with breadth the objective was changed for a 16 mm. Each square was then found to be 0.157 X 0.157 mm.

The previous experience of the animals varied in the different experiments. In the preliminary experiment (1) specimens were straight from the field. Those used in experiments 2 and 3 had been cultured for three and five weeks, respectively, in filtered lake water with Chlorella as food and a change of water each week. During these experiments, Daphnia reproduced, and some were killed by protozoan or fungal parasites. The animals measured at the end, therefore, were not all necessarily the originals. I do not think that losses through disease should vitiate the results since only those measurements made on specimens free from parasites, and in all other respects normal, were used. It did not appear that one or other species was the more susceptible to parasitism. The size of the sample of each species from any one flask was that of the species in least abundance.

In experiment 3, animals measured after 8 days were set up again as experiment 3A for a further 5 days and then, after another measurement, for a final 5 days.

In the case of experiment 4, animals at the same age for both species were required. Large egg-bearing females were selected and after two days the neonatae were pipetted off and cultured for seven days.

TABLE 6.

EXP.	VESSEL	D. pulex HAEMOGLOBIN INDEX	D. pulex LENGTH (mm.)	D. pulex HB.INDEX FOR LENGTH OF 2.0mm.	D. pulex NO.OF OFF-SPRING	D. obtusa HAEMOGLOBIN INDEX	D. obtusa LENGTH (mm.)	D. obtusa HB.INDEX FOR LENGTH OF 2.0mm.	D. obtusa NO.OF OFF-SPRING	NO.OF EACH SP.IN SAMPLE
1	a	33	2.4	28	5	20	2.2	18	8	7
	b	29	2.4	24	0	18	2.2	16	3	7
	c	29	2.3	25	3	14	2.1	13	8	5
Mean:		30	2.4	26	3	17	2.2	16	6	Total=19
2	a	72	2.3	63	7	33	2.1	32	9	4
	b	60	2.6	41	16	43	2.5	34	19	6
Mean:		66	2.5	52	12	38	2.3	33	14	Total=10
3	a	81	2.1	77	10	58	1.8	65	9	30
	b	73	2.1	70	8	53	1.8	59	7	17
	c	63	2.5	50	8	48	2.0	48	11	10
	d	78	2.1	74	6	44	2.0	44	6	8
	e	68	2.2	62	5	45	2.0	45	7	6
Mean:		73	2.2	67	7	50	1.9	52	8	Total=71
3A	a	86	2.3	75	8	68	2.2	62	13	17
	b	70	2.5	56	11	48	2.1	46	17	15
	c	68	2.5	54	12	60	2.0	60	12	16
Mean:		74	2.4	62	10	59	2.1	54	14	Total=48

EXP.	VES-SEL	D. pulex HAEMO-GLOBIN INDEX	D. pulex LENGTH (mm.)	D. pulex HB.INDEX FOR LENGTH OF 2.0mm.	D. pulex NO.OF OFF-SPRING	D. obtusa HAEMO-GLOBIN INDEX	D. obtusa LENGTH (mm.)	D. obtusa HB.INDEX FOR LENGTH OF 2.0mm.	D. obtusa NO.OF OFF-SPRING	NO.OF EACH SP.IN SAMPLE
3B	a	67	2.3	54	10	48	2.4	40	11	11
	b	64	2.6	49	12	47	2.3	41	16	10
	c	60	2.5	48	5	48	2.2	44	12	8
	Mean:	64	2.5	50	9	48	2.3	42	13	Total=29
4	a	35	2.5	28	17	23	2.2	21	10	11
	b	38	2.5	30	13	23	2.1	22	9	10
	c	37	2.5	30	14	30	2.2	27	13	11
	Mean:	37	2.5	29	15	25	2.2	23	11	Total=32
4A	a	49	2.6	38	18	30	2.2	27	12	8
	b	51	2.6	39	12	28	2.2	25	8	9
	c	51	2.6	42	12	25	2.3	22	12	9
	Mean:	50	2.6	40	14	28	2.2	25	11	Total=26

The preliminary experiment (1) gave a result indicating that D. pulex synthesized more haemoglobin than D. obtusa within each flask. The average for the three flasks of index for the two species was 30 and 17 respectively and of corrected index 26:16 on 19 animals. Feeding had been spasmodic during this experiment and that is why the rise in haemoglobin was small.

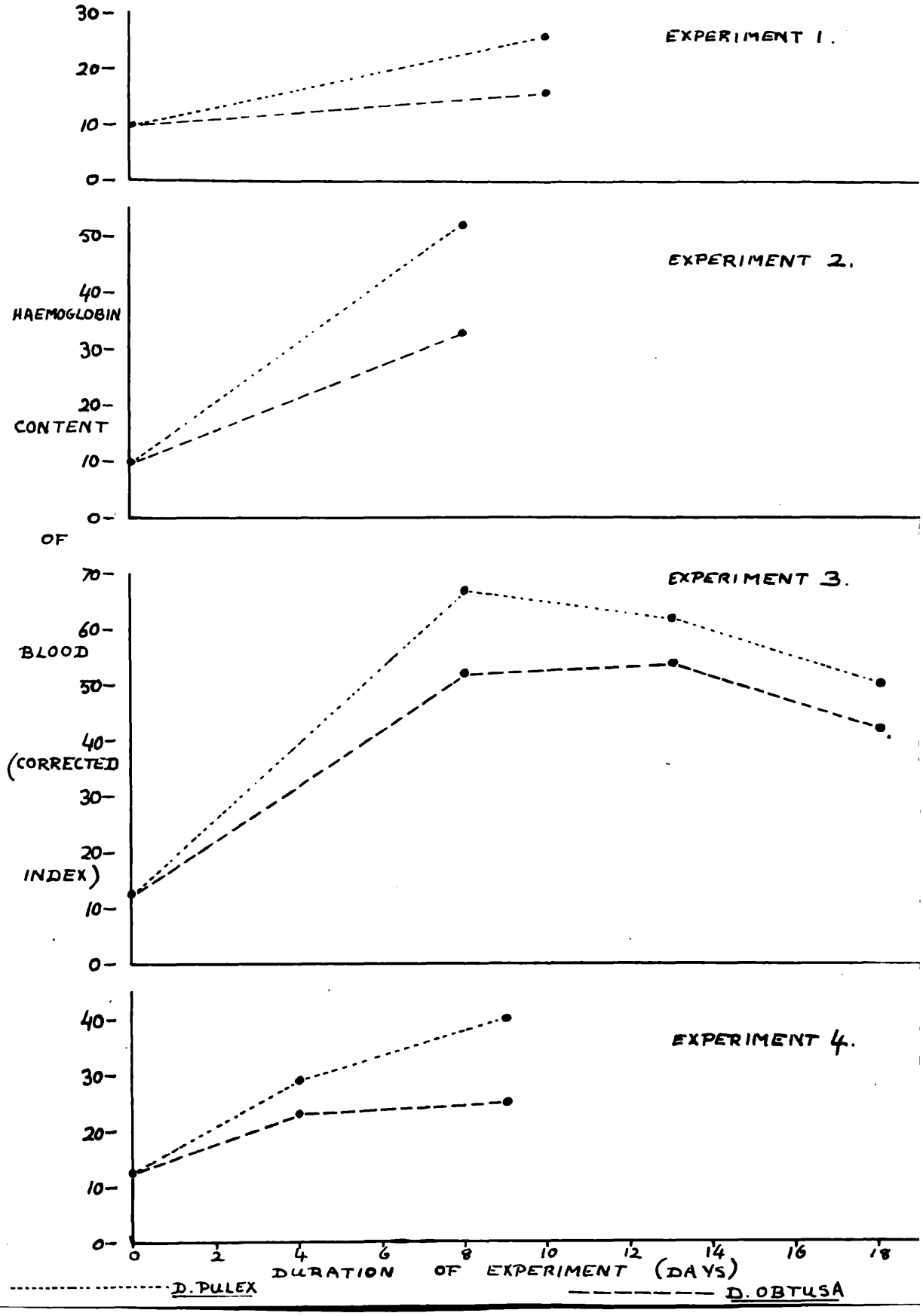
In experiment 2 feeding was standardised and the indices rose considerably. The mean ratio for index was 66:38 and for corrected index 52:33.

These results indicated that, after eight or ten days under similar conditions, D. pulex synthesized a great deal more haemoglobin than D. obtusa.

I then wanted to know whether this was due solely to a difference in rate of gain or was indicative of a final state of affairs. Experiment 3 was therefore continued for 18 days during which time measurements were made on the 8th, 13th and 18th days. On each occasion D. pulex contained the greater concentration of blood haemoglobin, though the difference between the species lessened slightly after 8 days.

FIGURE 14

HAEMOGLOBIN CONTENT OF D. PULEX AND D. OBTUSA UNDER SIMILAR CONDITIONS



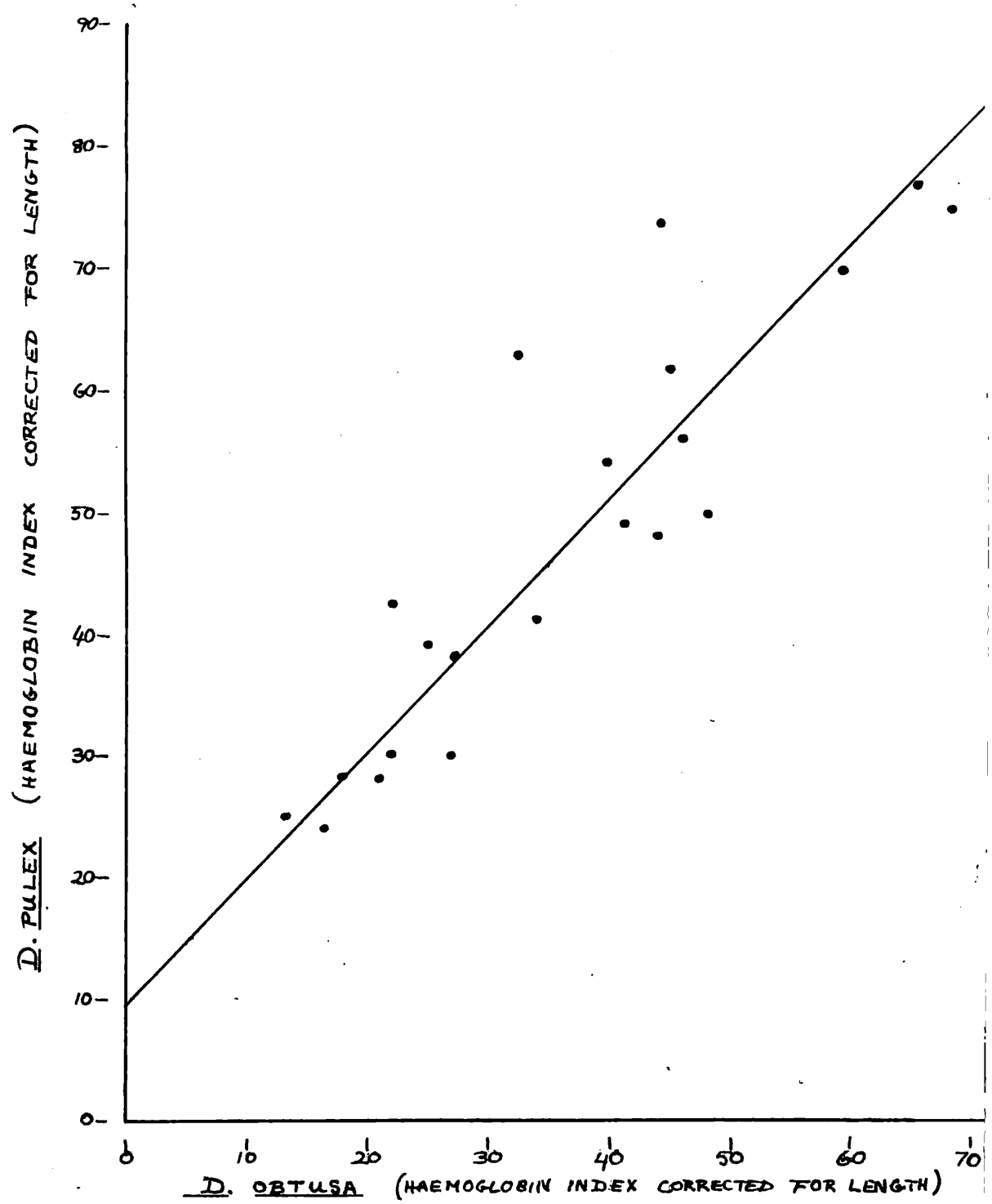
In these experiments the individuals of the two species were not necessarily of the same age. It has been shown (personal communication from J. Green) that haemoglobin concentration varies with age in D. magna and I have noticed the same in the case of D. pulex in the field.

In experiment 4, neonatae of the same age for both species were employed. This experiment lasted 9 days and measurements were made on the 4th and 9th days. The results obtained confirmed those of the previous experiments.

Figure 14 illustrates the mean results of each of the four experiments after the indices have been corrected to a size of 2.0 mm.

The corrected indices for each flask are represented on figure 15. Those of the two species within a flask are plotted one against the other. The points appear to represent a straight line. There again there is no evidence that at one end of the index range the redder species is D. pulex and at the other D. obtusa.

FIGURE 15.
COMPARISON OF HAEMOGLOBIN CONTENT OF D. PULEX WITH THAT OF D. OBTUSA UNDER THE SAME CONDITIONS



EACH POINT REPRESENTS A DIFFERENT VESSEL.

I shall now mention the results of the other measurements made at the end of each experiment.

The egg number in most experiments was slightly greater in D. obtusa than in D. pulex though in experiment 4 the reverse was the case. Better nutrition cannot therefore account for the higher haemoglobin production of D. pulex.

The length was in every case the greater in D. pulex. A higher haemoglobin index would therefore be expected in this species if the concentration of blood haemoglobin were the same in both species. Correction of index to that for a size of 2.0 mm. lessend slightly but not considerably the species difference.

It is important to consider whether this correction to a standard size gives an adequate comparison between the blood haemoglobin concentration of the two species. It was shown by Green (personal communication) that, in D. magna, the width of the body at the point where the index is measured is proportional to the length (from front of head to base of spine). One might

expect this to be the case also in other species. Even making this assumption, however, it would not be legitimate to assume that the proportion of length to breadth is in the same ratio for different species. For a given length one might be fatter than the other.

In order to test this, 22 specimens from each species, from experiment 4A, were measured for length and breadth. These measurements are listed in the appendix(VI). The averages for each flask are given here.

FLASK NO.	D. pulex			D.obtusa			NUMBER OF EACH SPECIES
	LENGTH in mm.	BREADTH in mm.	LENGTH ÷ BREADTH	LENGTH in mm.	BREADTH in mm.	LENGTH ÷ BREADTH	
1	2.61	0.683	3.82	2.24	0.612	3.66	6
2	2.61	0.686	3.80	2.15	0.613	3.51	8
3	2.55	0.679	3.76	2.26	0.650	3.48	8
		Mean:	3.79		Mean:	3.55	Total=22

In every case the ratio of length to breadth is greater in D. pulex. For a given length this species would be thinner than D. obtusa.

This difference reinforces the hypothesis that D. pulex has the redder blood. The indices in the case of D. pulex have been corrected to slightly too low a value by assuming that the proportions of the two species were similar.

Figures 1 and 3 of the field results for haemoglobin and oxygen content should now be reconsidered in the light of these results. If the graphs were replotted with each value for D. pulex "corrected" to that which D. obtusa would have in the same pond, a better correlation would be obtained.

Figure 15 may be used for this. For example the highest index is 111 for D. pulex in Lysthus IV. Corrected to a size of 2.0 mm. this becomes 93. Corresponding on figure 15 to 93 for D. pulex is 78 for D. obtusa. If a further correction is made for the different size proportions of the species we have $78 \times \frac{3.55}{3.79}$ giving a value

of 73. In a similar way the next highest index, 81 for D. pulex in Praestevang II, would be reduced to 61.

A further point of interest about this difference in haemoglobin production is that another distinction is found between these two recently separated species (Scourfield 1942).

DISCUSSION

I shall not repeat here the discussion given in the historical introduction to this thesis, but will modify it where possible in relation to my own results.

The field results for oxygen concentration confirm those of Fox for Daphnia under laboratory conditions; the lower the oxygen content of the water, the higher the haemoglobin index. There is considerable scatter in the results, however, as might be expected in a natural environment. This could be accounted for by day-to-day fluctuations in oxygen concentration, by the existence of different physiological races of Daphnia or by the presence of some stimulating agent in ponds other than a low oxygen content.

Day-to-day fluctuations of oxygen content were shown by the work on Pool V1. The attempt made to correlate these with weather factors was not successful. This implies that recordings of weather at times of sampling are of little or no value except, perhaps, when the weather is exceptional.

In spite of these fluctuations, different ponds compared at the same time tended to keep the same order of oxygen content. In the Newdigate group of ponds, for example, Round Pond had always the highest and Blanks Farm Pond the lowest concentration. Long Pond and Wilderness Pond had similar oxygen contents, sometimes one and sometimes the other being the higher.

No confirmation for the effect of dissolved iron was found. It is likely that, in a natural environment, iron is seldom the limiting factor in haemoglobin synthesis, the mixed diet probably providing an adequate supply.

No correlation with calcium + magnesium, temporary hardness or oxidation-reduction potential was apparent. I have not sufficient measurements to prove the complete absence of any effect but it seems unlikely that there should be any.

No correlation was found of haemoglobin content with number of offspring (which confirms the conclusion of Fox) or with body length.

I suspected that Daphnia parasitised by Thelohania possessed redder blood than healthy individuals.

Species of Daphnia were found to differ in the amount of haemoglobin synthesized under similar conditions. In six ponds containing both D. magna and D. obtusa the haemoglobin concentration of the blood was either the same in both species or higher in D. obtusa. A laboratory comparison between D. pulex and D. obtusa showed D. obtusa to be always the paler species. Considering the existence of these specific differences, it would be of interest to compare the capacities of haemoglobin production in all pond species of Daphnia. This distinction between D. pulex and D. obtusa provides a likely cause for the unexpectedly high haemoglobin content found in natural populations of D. pulex in both Denmark and England.

I should now expect to find the reddest populations of Daphnia most commonly in winter; in ponds polluted by animals or by decaying organic matter or in shaded situations; and where the species is Daphnia pulex.

SUMMARY

1. Previous work on the haemoglobin of *Daphnia* is briefly reviewed and discussed.
2. *Daphnia* ponds in London, Surrey, Bedfordshire and in Denmark were investigated. The species was mostly *D. obtusa* but, in some cases, *D. pulex*, *D. magna*, *D. curvirostris* or *D. atkinsoni*.
3. Methods used in the analysis of oxygen, iron, calcium + magnesium and temporary hardness are described.
4. Samples for oxygen determination were taken at a standard depth (mid-depth) and at standard times (two hours after sunrise and two hours before sunset).
5. A vertical gradient of oxygen concentration was found to occur in some ponds. Regional variation in a large pond was also found.
6. A negative relationship was found between the haemoglobin content of *Daphnia* and both mean and minimum daily oxygen concentration in ponds.
7. No correlation of haemoglobin production with other factors was discovered.

8. Ponds polluted by animal faeces generally have a low oxygen concentration and a red Daphnia population. A low oxygen content may be found also in ponds not visited by animals.

9. There is a seasonal change in haemoglobin production common to many natural populations of Daphnia, the highest production being in winter. This is associated with changes in oxygen concentration.

10. Estimations of oxygen content were made twice daily in a single pond during a three-week period. Attempts made to correlate the results with weather conditions proved unsuccessful.

11. In cases where D. magna occurred together with D. obtusa in a pond, the latter species possessed either the same or a greater concentration of haemoglobin in the blood.

12. D. pulex was not found together with D. obtusa. It was noticed, however, that for a given oxygen concentration D. pulex was the redder species.

13. Experiments were designed to compare haemoglobin production in D. pulex and D. obtusa under identical conditions. Cultures containing both species were used. D. pulex developed and maintained a higher concentration of haemoglobin in the blood

than D. obtusa under the same conditions.

14. For a given body length, the breadth of D. pulex is less than that of D. obtusa in the region where measurements of haemoglobin content are made.

15. The occurrence of D. atkinsoni in two ponds at Whipsnade is recorded.

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APPENDIX I.

Calibration of colorimetric disks used in the estimation of oxygen content.

DISC READING	OXYGEN CONCENTRATION (ml./l.)
1.9	3.25
3.8	5.85
3.0	4.50
1.7	3.05
0.3	0.82
3.8	6.00
0.2	0.42
3.8	5.00
4.3	5.70
4.5	5.85
2.6	3.68
4.6	6.15
1.4	2.40
4.4	5.73
5.2	6.76
8.6	12.2
3.4	4.18
4.6	6.01

DISC READING	OXYGEN CONCENTRATION (ml./l.)
1.9	2.76
0.9	0.99
1.1	1.46
0.9	1.09
3.4	4.23
4.6	5.88
8.6	10.79
6.4	7.63
6.8	8.52
6.0	6.91
5.8	6.65

APPENDIX II.

Minimum and mean daily oxygen concentration in ponds, listed in order of decreasing haemoglobin content of *Daphnia* population.

POND	DATE	SPECIES	CORRECTED HAEMOGLOBIN INDEX	MINIMUM OXYGEN CONTENT (ml./l.)	MEAN OXYGEN CONTENT (ml./l.)
L.1V	Aug.	p	93	1.5	1.5
Hippo	Dec.	0	80	0.0	0.2
Pr.11	Aug.	p	77	2.4	3.0
Hampton	May	m	71	-	0.7
Lily	Jan.	0	68	0.3	0.3
H.F.111	Oct.	0	66	1.5	1.4
Pool V11	Dec.	0	64	1.1	
Land	June	m	63*	-	2.2
B.F.	Dec.	0	62	2.1	2.8
H.F.11	May	0	60	1.6	1.9
H.F.11	July	0	59	0.5	0.5
Pool V11	Dec.	c	58	1.1	-
H.F.1	Dec.	0	54	1.6	2.0
H.F.111	Dec.	0	54	2.1	2.6
B.O.	Jan.	0	53	0.6	1.0
L. 111	Aug.	p	53	1.9	2.2
H.F.1	Oct.	0	50	1.6	1.5
W. 11	Jan.	m	49	0.7	-

POND	DATE	SPECIES	CORRECTED HAEMOGLOBIN INDEX	MINIMUM OXYGEN CONTENT (ml./l.)	MEAN OXYGEN CONTENT (ml./l.)
Hippo	Oct.	O	48	0.0 ?	0.0 ?
Gnu	May	O	48	0.6	0.6
H.F.11	Dec.	O	47	1.2	2.1
Hippo	Feb.	O	47	0.5	0.5
B.111	June	O	46*	0.6	1.0
Hippo	Oct.	m	46	0.0 ?	0.0 ?
L.11	Aug.	p	46	1.2	1.4
N.P.	May	O	45	0.6	0.4
W.111	Jan.	m	45	0.9	-
H.F.111	Oct.	m	45	1.5	1.4
Pool 1V	Dec.	c	43	3.8	-
H.F.111	Dec.	m	43	2.1	2.6
H.F.11	June	O	43*	0.6	1.2
Gnu	Oct.	O	40	0.6	1.2
H.F.11	Feb.	O	40	1.4	2.2
H.F.1	May	O	34	1.5	2.0
S.P.	May	O	32	1.8	1.4
H.F.11	Oct.	O	28	1.4	1.4
B.F.	April-June	O	27	2.5	4.1
H.F.111	Feb.	O	26	7.0	7.1
Lily	April	O	25	4.4	-
Hippo	June	O	25*	1.0	6.2
H.F.1	June	O	24*	1.4	4.8

POND	DATE	SPECIES	CORRECTED HAEMOGLOBIN INDEX	MINIMUM OXYGEN CONTENT (ml./l.)	MEAN OXYGEN CONTENT (ml./l.)
H.F.1	July	O	23	1.0	6.3
Copse	Dec.	p	21	7.4	-
H.F.111	Feb.	m	20	7.0	7.1
H.F.111	July	O	20	2.4	4.4
H.F.111	June	O	under 20*	6.6	10.2
Long	April-June	O	under 20	3.9	4.3
Round	April-June	O	under 20	6.9	9.0
Wild	April-June	O	under 20	3.9	4.5
Pool V1	April-May	O	under 20	6.4	7.5
H.F.111	May	O	under 20	5.7	6.1
Long	Dec.	p	under 20	4.1	4.7
Wild	Dec.	O	under 20	3.0	3.9

* Index not corrected for size. These values are not plotted in figures 1 - 4.

APPENDIX III.

Concentration of iron (ferric and ferrous ions) in ponds, listed in order of decreasing haemoglobin content.

POND	MONTH	SPECIES	CORRECTED HAEMOGLOBIN INDEX	IRON CONTENT (mg./l.)
L. 1V	Aug.	p	93	0.04 ap.
Hippo	Dec.	0	80	1.62
Pr.11	Aug.	p	77	0.08 ap.
Hampton	May	m	71	0.51
Lily	Jan.	0	68	2.10**
H.F.111	Oct.	0	66	1.5 ap.
Land.	June	m	63*	0.09
B.F.	Dec.	0	62	1.74
B.F.	Feb.	0	61	1.30
H.F.11	May	0	60	0.10
H.F.11	July	0	59	1.69
H.F.1	Dec.	0	54	1.57
H.F.111	Dec.	0	54	0.58
B.O.	Jan.	0	53	1.17
L.111	Aug.	p	53	0.09 ap.
S.P.	June	0	53*	46.**
B.O.	Jan.	0	51	0.25
H.F.1	Oct.	0	50	7.4** ap.

POND	MONTH	SPECIES	CORRECTED HAEMOGLOBIN INDEX	IRON CONTENT (mg./l.)
W.11	Jan.	m	49	0.25
Gnu	May	0	48	0.64
H.F.11	Dec.	0	47	1.20
Hippo	Feb.	0	47	1.64**
B.111	June	0	46*	20.**
L.11	Aug.	p	46	0.06 ap.
N.P.	May	0	45	25.**
N.P.	June	0	45	51.**
W.111	Jan.	m	45	0.01
H.F.111	Oct.	m	45	1.5 ap.
H.F.111	Dec.	m	43	0.58
H.F.11	June	0	43*	0.00
H.Tr.	May	0	42	1.49
Gnu	Oct.	0	40	2.4 ap.
H.F.11	Feb.	0	40	0.00 Tr.
B.F.	Jan.	0	39	0.26
B.F.	March	0	39	0.25
H.F.1	May	0	34	0.12
S.P.	May	0	32	4.49
H.F.11	Oct.	0	28	1.4 ap.
B.F.	April-June	0	27	0.66
H.F.111	Feb.	0	26	0.00 Tr.

POND	MONTH	SPECIES	CORRECTED HAEMOGLOBIN INDEX	IRON CONTENT (mg./l.)
Hippo	June	O	25*	0.00
H.F.1	June	O	24*	0.00
H.F.1	July	O	23	1.49
Copse	Dec.	p	21	0.27
B.O.	April	O	21	0.13
H.F.111	Feb.	m	20	0.00 Tr.
H.F.111	July	O	20	0.09
H.F.111	June	O	20*	0.00
Long	Apl.-June	O	under 20	0.74
Round	Apl.-June	O	"	0.04
Wild	Apl.-June	O	"	1.02
Pool V1	Apl.-May	O	"	0.09
H.F.111	May	O	"	0.46
Wild	Dec.	O	"	0.96
Long	Dec.	p	"	1.22
Wayside	April	O	"	0.12
Red Ho.	April	O	"	0.11
G.R.	May	O	"	0.08

* Index not corrected for size. These values are not plotted in figures 5 - 7.

Tr. = Trace.

ap.= approximately.

**Iron partly ferrous.

APPENDIX IV.

Temporary hardness and total magnesium + calcium ion content in ponds, listed in order of decreasing haemoglobin content of *Daphnia* population.

POND	DATE	CORRECTED HAEMOGLOBIN INDEX	TEMPORARY HARDNESS (mg./l.)	CALCIUM + MAGNESIUM CONTENT (mg./l.)
L.B.	June 16	63*	380	516
W.1	Jan. 4	61	147	140
B.F.	Feb. 5	61	185	182
S.P.	June 27	53*	167	-
B.O.	Jan. 29	53	150	110
B.O.	Feb. 12	53	73	101
W.P.	May 29	49*	106	126
W.11	Jan. 23	49	143	146
W.111	Jan. 23	45	318	174
W.P.	June 27	45*	197	200 approx.
C.M.	Feb. 12	44	203	154
A.P.	Jan. 25	41	92	480
O.P.	Jan. 25	39	113	150
S.P.	Jan. 29	30*	72	107
H.T.	May 7	29	-	277
B.F.	April 18	22	-	303
N.P.	May 14	22	155	104
L.P.	May 2	21	188	200

POND.	DATE	CORRECTED HAEMOGLOBIN INDEX	TEMPORARY HARDNESS (mg./l.)	CALCIUM + MAGNESIUM CONTENT (mg./l.)
S.P.	May 14	21	82	89
W.P.	April 18	under 20	100	71
R.P.	April 18	"	181	160
L.P.	April 18	"	129	87
W.P.	April 22	"	-	89
L.P.	April 22	"	-	84
R.P.	April 22	"	-	109
B.F.	April 22	"	-	207
W.P.	April 30	"	63	133
R.P.	April 30	"	114	137
B.F.	April 30	"	80	117
P.V1	May 3	"	219	199

* Index not corrected for size.

APPENDIX V.

Oxygen content of vessels containing cultures of D. pulex and D. obtusa mixed.

EXP.	DURATION OF EXP. IN DAYS	DAY OF ANALYSIS	OXYGEN CONTENT AS % AIR SATURATION OF EACH VESSEL				
			1	2	3	4	5
1	10	2nd	37	51	36		
		9th	8	16	8		
2	8	2nd	24	22			
		8th	11	13			
3	8	3rd	22	16	18	16	15
		7th	13	9	14	10	10
3A	5	3rd	19	29	25		
3B	5	5th	-	16	16		
4	4	-	all probably about 20				
4A	5	4th	18	19	15		

APPENDIX VI.

Measurements of length and breadth of D. pulex and D. obtusa.

VESSEL	D. PULEX		D. OBTUSA	
	LENGTH (mm.)	BREADTH BEHIND FORNIX (mm.)	LENGTH (mm.)	BREADTH BEHIND FORNIX (mm.)
1	2.65	0.676	2.10	0.597
	2.65	0.723	2.30	0.659
	2.70	0.676	2.25	0.627
	2.75	0.723	2.40	0.627
	2.50	0.692	2.00	0.550
	2.40	0.597	2.15	0.613
2	2.65	0.676	2.30	0.644
	2.65	0.692	2.15	0.628
	2.60	0.706	2.10	0.613
	2.70	0.692	2.20	0.676
	2.50	0.676	2.00	0.597
	2.60	0.676	2.10	0.582
	2.65	0.723	2.15	0.582
	2.40	0.628	2.10	0.582

VESSEL	D. PULEX		D. OBTUSA	
	LENGTH (mm.)	BREADTH BEHIND FORNIX (mm.)	LENGTH (mm.)	BREADTH BEHIND FORNIX (mm.)
3	2.70	0.738	2.25	0.659
	2.65	0.676	2.35	0.676
	2.75	0.738	2.40	0.691
	2.65	0.706	2.25	0.644
	2.30	0.596	2.20	0.582
	2.60	0.676	2.30	0.676
	2.40	0.676	2.30	0.691
	2.20	0.596	2.00	0.582