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The haemoglobin of *Daphnia*

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Daphnia is often coloured pink or red by haemoglobin in solution in the blood. This applies to pond-living but not to lacustrine species. A *Daphnia* species may vary from red to colourless in different ponds or in the same pond at different times. In the laboratory individual *Daphnia* can be seen to lose or gain haemoglobin in the course of a few days.

Daphnia loses haemoglobin in well-aerated water and gains haemoglobin in water containing little dissolved oxygen.

Abundance of parthenogenetic young may be taken as a criterion of good nutrition; judged by this standard, good nutrition alone does not result in abundance of haemoglobin. Nor is chlorophyll in food a cause of haemoglobin production.

There is a haemochromogen in solution in the intestinal fluid of *Daphnia*. Like helicorubin in the snail it shows reversible oxidation. In quantity it is proportional to the haemoglobin of the blood, which suggests that it may be an excretory product of haemoglobin. Yet it occurs in a lake plankton species lacking the blood pigment.

Occasionally one of the two excretory shell glands of *Daphnia* contains concentrated haemoglobin. This pathological condition may be an indication that haemoglobin is normally excreted as such by the shell glands.

The presence of haemoglobin in the blood of *Daphnia* suggests a respiratory function. The increase in quantity of the haemoglobin in response to oxygen deficiency, just as in man, supports this thesis. Nevertheless, there appears to be no such function. Animals whose haemoglobin is functionally inactivated with carbon monoxide are as vigorous and survive as well as untreated animals, at all concentrations of air dissolved in the water.

Haemoglobin is present in the parthenogenetic eggs of *Daphnia* as well as in the blood. Respiratory conditions in the brood pouch of parthenogenetic females are not good. This suggests an importance of haemoglobin in parthenogenetic development. Experiments in which the haemoglobin was functionally inactivated by carbon monoxide showed that the respiratory pigment of the egg does have a favourable influence on late stages of the parthenogenetic embryo.

Fertilized eggs, in ephippia, contain no haemoglobin. Nevertheless, they develop as well in water deficient in oxygen as in aerated water.

1. Introduction

Those species of *Daphnia* which live in ponds or ditches are often pink or even red. Swammerdam (1758) described the colour of the 'Arborescent Water Flea' as being 'like that of beef, which has been some time steeped in water'. He relates how he

saw so many in one pool 'that the water appeared as if changed into blood; which, indeed, terrified me at first'. The aspect of such ponds is well described by Baird (1850). The colour is due to haemoglobin in solution in the blood of the animals. Lake-dwelling species of the genus have no pink tint.

In the Crustacea haemoglobin is widespread among Entomostraca, but it is unknown in Malacostraca. Ray Lankester (1871) discovered the haemoglobin of *Daphnia* and *Chirocephalus* with the spectroscope. The blood of Apus (*Triops*) is red with haemoglobin (Regnard & Blanchard 1883). Lochhead & Lochhead (1941) found the pigment in *Artemia* and I have found it in the ostracod *Cypria ophthalmica* (Jurine). The blood of the conchostracan *Limnadia* is described as bright red (Klunzinger 1864) and haemoglobin may well be responsible. Haemoglobin occurs in the blood of some parasitic copepods (van Beneden 1880; Fox 1945*b*) but has not been found in free-living members of this order. It is found also in a parasitic cirripede (Pérez & Bloch-Raphäel 1946).

The fact is familiar to aquarium keepers who use *Daphnia* as fish food that water fleas are sometimes red, sometimes pink, and at other times have hardly a trace of reddish tint, or none at all. But it is not known to most zoologists that any one of the several pond species may be either red or colourless, and still less is it known that one and the same individual can gain or lose haemoglobin to a surprising extent in the course of a few days. There is, indeed, no other animal known which shows these great changes. Nor does *Daphnia* appear to suffer in vigour from being anaemic. Clearly it is of importance to discover the factors responsible for this rapid and considerable synthesis and loss of haemoglobin. It is equally interesting to know the function of a haemoglobin so variable in quantity, and to find out where it is formed and how it is got rid of when it diminishes.

2. Situation, properties and fluctuation of haemoglobin

(1) *Haemoglobin in blood and eggs*

In Britain there are three common species of *Daphnia*, namely *D. magna* Straus, *D. pulex* (De Geer) and *D. obtusa* Kurz, which are found in ponds or ditches (Scourfield & Harding 1941; Scourfield 1942). The haemoglobin content of these species varies widely. There are other species, inhabiting open waters or lakes, which are not visibly coloured by haemoglobin. In the species named above, the haemoglobin is localized in two tissues: it is found in solution in the blood plasma and it is present in the eggs. There is no haemoglobin in the muscles. A pink *Daphnia* examined under the microscope with a spectroscopic ocular shows the α- and β-bands of oxyhaemoglobin. The animal can be bled white by teasing it with fine needles; if then all blood is washed from the interstices of the muscles, no trace of the absorption bands remains. When now pyridine and sodium hydrosulphite are added, no haemochromogen band appears, proving that not only is there no haemoglobin in the muscles but the latter contain no other haem compounds detectable in this way. This is not unexpected since the limb muscles of other

crustaceans, such as crabs, contain so little haem that it can only be shown clearly with pyridine in considerably greater thickness of muscle than is available from one bled *Daphnia*.

Teissier (1932) discovered that the eggs of *D. pulex* in the brood pouch contain haemoglobin. As he points out, this is the only known occurrence of haemoglobin in an ovum. Since then an analogous case has been reported: the eggs of the marine echiuroid worm *Urechis caupo* contain a pigment, urechrome, which a vacuum reversibly changes from yellow to pink (Horowitz 1940). In *Daphnia pulex*, *D. obtusa* and *D. magna* the parthenogenetic eggs are more often than not coloured green by a carotenoid-protein pigment. The tint varies very considerably, no doubt with the particular algal food of the mother, and Teissier has shown that feeding *Daphnia* artificially with carotene-free 'farine lactée' produces eggs without the pigment. In these eggs the pink colour of oxyhaemoglobin is visible, and *Daphnia* in certain ponds has such pink eggs. But in all *Daphnia* of the three species named, no matter how green are the eggs, the bands of oxyhaemoglobin can be seen in a single egg with the microspectroscope. This is so even when the blood is so pale that no bands are visible in it when a single animal is examined.

(2) *Properties of the blood haemoglobin*

The following physico-chemical data are available on the haemoglobin of *Daphnia* blood.

(i) Its molecules appear to be of two sizes, with weights respectively one-half and six times that of mammalian haemoglobin (Svedberg & Eriksson-Quensel 1934).

(ii) The uptake of oxygen by *Daphnia* haemoglobin occurs at oxygen pressures which are not quite so low as in the case of other invertebrates. Yet, at the temperatures at which these animals live, the requisite pressures are much lower than for human haemoglobin at blood heat. For example, the oxygen pressures (in mm. of mercury) for 50% oxyhaemoglobin at 17° C are 3·1 for *D. magna* and 0·6 for *Chironomus riparius* in the absence of carbon dioxide; in the presence of 1% carbon dioxide the value for *Daphnia magna* is 4·9. For man at 37° C and pH 7·4 the value is 27 mm. But the oxygen pressure in the water outside *Daphnia* need fall no lower than 28 mm. (1·18 ml. oxygen per litre at 17° C) to cause deoxygenation of the haemoglobin in the animal's blood, since there is a steep oxygen gradient across the body wall (Fox 1945*a*).

(iii) Species of the genus *Daphnia* have different haemoglobins, the wave-length of the α-band axis for the oxyhaemoglobins of *D. magna*, *D. pulex* and *D. obtusa* being respectively 5766, 5764 and 5761 A (Fox 1945*b*, 1946).

(3) *Measurement of haemoglobin in the blood*

In order to estimate changes in the haemoglobin content of *Daphnia* blood, in nature and under laboratory conditions, a quantitative method was devised. This enables the haemoglobin concentration to be measured in single individuals. The

comparison standard consists of 0·2 ml. of blood from the worker's finger diluted in 75 ml. distilled water, with the addition of a trace of saponin for complete haemolysis and a drop of sodium bicarbonate solution to avoid breakdown to haematin. Of this solution, 50 ml. are put into a wedge-shaped optical glass trough, 10 cm. long, 4 cm. across at the wide end and 4 cm. high. A trace of octyl alcohol is added to the diluted blood to lessen the creep up the narrow end of the trough. The trough is placed before the mirror of a microscope. The optical disposition is as follows: daylight lamp with water-bath and ground-glass screen in front of it. trough, concave mirror, condenser screwed up, 16 mm. objective and × 6 eyepiece. The image of the haemoglobin solution is made to fill the upper third of the evenly illuminated field of vision in the microscope. Ten large *Daphnia*, taken at random from a population, are laid in a row, out of water but damp, on a microscope slide without a cover-slip. The tint of oxyhaemoglobin close to the base of the second antenna* of each animal in turn is matched with the standard diluted blood by sliding the trough to right or left. The trough stands on a paper scale so that its narrow end when seen through the microscope corresponds to 0, its wide end to 160 arbitrary units. (For pale populations the standard haemoglobin solution is diluted to one-half strength; the scale then reads from 0 to 80 units.) The values for each of the ten individuals are averaged and thus the *haemoglobin index* for the population is obtained.

Tests of the accuracy of the method were made by finding the haemoglobin indices of nine different populations, each in duplicate; that is, two lots of ten individuals were measured from each population. The results were 104 and 97, 93 and 86, 73 and 61, 60 and 56, 48 and 47, 46 and 44, 41 and 41, 40 and 39, 29 and 27. It follows that the error of the method is less than 10%. The method depends upon the constancy of the haemoglobin content of the observer's blood, but the small daily or longer-period fluctuations in haemoglobin content are not likely to be great compared with other unavoidable experimental errors. Obviously a mixed solution of dyes imitating the colour of haemoglobin would be preferable if such could be found. When the haemoglobin index had been determined the average individual size was estimated by measuring the length of each of the ten animals, from forehead to base of posterior shell spine, using an eyepiece micrometer with a 50 mm. objective. The number of eggs or young in each brood pouch was then counted, and various details of egg colour, food, fat, etc., were noted.

(4) *Variation of blood haemoglobin in nature*

The highest haemoglobin indices I have found in nature are 128 for *D. pulex*, 103 for *D. obtusa* and 118 for *D. magna*. These are, of course, averages of ten individuals; the highest single individual value was 150 for *D. pulex*. The size of

* This position was chosen because a considerable thickness of blood space can here be seen by transparency without interference of the alimentary canal. This blood space is the front end of the 'loge intestinale' of Hérouard (1905), who by mistake gives it the name 'ventrale' on p. 221, line 11 from below.

D. pulex and *D. obtusa* is usually about the same but *D. magna* is generally bigger; the average length of individuals in the three populations whose haemoglobin indices are given above were 2·0, 2·2 and 3·3 mm., but *D. magna* may have an average length up to 4·5 mm. This means that while the indices of *D. pulex* and *D. obtusa* are comparable, in *D. magna* the depth of blood measured is greater, so that this species has relatively less haemoglobin than its index would suggest. The lowest indices found in nature for all three species were less than 10, below which figure there is no measurable pink colour, so that no precise value can be given. If we suppose the index of such a population of *D. pulex* to be 10, it follows that the haemoglobin content of the blood in this species can vary 12-fold.

The haemoglobin content of a population in a pond changes as time passes. In one pond the indices for *D. magna*, at intervals from September of one year to April of the next, were 45, 57, 81, 56 and 42. In another pond *D. pulex* between August and December had successive indices of 36, 50, 62 and 72. In both cases the maximum change in half a year was twofold. When there are two species in a pond their indices at one and the same time may differ considerably, although the sizes of individual are the same. In one case the index for *D. pulex* was 49, for *D. obtusa* 63. In other ponds, on the contrary, the former species had more haemoglobin than the latter. In yet another pond *D. magna* had 81, *D. obtusa* 103; the latter species is much the smaller, so obviously its blood had a considerably greater haemoglobin content. But this is not a specific character, for in a different pond *D. obtusa* had much less haemoglobin than *D. magna*, even after allowing for their size discrepancy. 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. Yet in a given pond two species can show parallel changes; for instance, in one case at bimonthly intervals the indices for *D. magna* and *D. obtusa* were 81 and 103, 56 and 97, 42 and 66.

3. Causes of gain and loss of haemoglobin

(1) *Previous work*

Fritzsche (1917) was under the impression that the red colour of *Daphnia* is due to carotene and that the redness is to be ascribed to good nutrition. Schultz (1928) accepted the opinion of Fritzsche and showed experimentally to his satisfaction that the red colour appears only in light and is never formed in cultures kept in darkness. Banta (1939) believed the red colour 'to be of the nature of intra-vitam staining obtained from the water'.

Verne (1923), recognizing that the red colour is due to haemoglobin, studied the cause of its synthesis. Many workers who have kept *Daphnia* alive in the laboratory have noticed that, in the course of a week or so, red populations get noticeably paler. Eventually the same individuals and their offspring become quite colourless. Verne started with *D. pulex* which had become 'incolores' in 8 days; I myself have never seen such a quick complete decoloration. He made three series of

cultures: (1) with 'débris de feuilles, ou des algues riches en chlorophylle, en macération dans l'eau. J'ajoutais des traces d'un sel organique de fer'; (2) 'Cultures de zooglées sans chlorophylle, ne montrant pas de fer décélable'; and (3) the last-mentioned medium with the addition of iron. At the end of 3 weeks (1) had haemoglobin, (2) and (3) had none. From this he concluded that the appearance of haemoglobin depends on the presence of chlorophyll or of its breakdown products.

Verne gives no experimental details or quantitative data. I have quoted in his own words his statements of how the experiments were made because his conclusion is far-reaching. This is so both from the standpoint of biochemistry, implying that chlorophyll with its pyrrol groups supplies the building stones of protoporphyrin, and from that of medicine, since there have been proprietary preparations on the market claiming to benefit anaemia through chlorophyll derivatives. Indeed Verne, in a further publication (1924), elaborates a thesis derived from his *Daphnia* experiments that animals in general which are able to break down chlorophyll in their intestine as far as porphyrin use the latter for building up haemoglobin. He had not, incidentally, shown that *Daphnia* breaks down chlorophyll to a porphyrin.

(2) *Haemoglobin synthesis due to oxygen lack*

The first experiments I made were designed to test the effects of light and darkness on the haemoglobin content of *Daphnia* blood. In a typical experiment *D. pulex* with an initial haemoglobin index of 61 was used. As usually happens in the laboratory, this value fell in the course of days, but it fell much more quickly in light than in darkness. After 21, 29, 34 and 45 days in light and darkness respectively the indices were 23 and 52, 16 and 53, 25 and 44, 20 and 36. But darkness or dim light did not always slow down the loss of haemoglobin; it sometimes resulted in a gain. In one experiment *D. pulex* started at 67; after 4 days in bright light and in shade the indices were respectively 66 and 87. In another case, starting with an index of 33, 4 days in bright light caused no change, whereas 4 days in shade raised the value to 59. This contradicts Schultz (1928) referred to above.

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 the dissolved oxygen content of the water does indeed affect the haemoglobin content of *Daphnia*. In one case the initial index of *D. pulex* was 62. The animals were divided into two lots, through one of which air was bubbled. The air was bubbled into a wide glass tube closed below with gauze and suspended in the vessel containing the *Daphnia*. The object of this procedure was to prevent the bubbles from hitting the animals. Next day the air saturation* of

* Dissolved oxygen was measured by the syringe-pipette micro-Winkler method of Fox & Wingfield (1938).

the water in this lot was 91 % and the haemoglobin index had fallen to 48. The other moiety was in a conical flask with little surface. Respiration had reduced its air saturation next day to 9 %, while the index remained virtually steady at 65. The fall in haemoglobin due to aeration was in this case unusually fast.

Other experiments showed that oxygen lack not only may prevent the loss of haemoglobin but it can be a factor in its synthesis. In one experiment *D. pulex* had an initial haemoglobin index of 21. The population was divided into two halves, through one of which air was bubbled. After 4 days this had an air saturation of 82 % while the other half had a value of 21 %. The haemoglobin indices had become respectively 19 and 56. The amount of haemoglobin was thus more than doubled in 4 days by decreasing the available oxygen. Further experiments (e.g. (*b*) on p. 203) confirmed this result.

Since dissolved oxygen influences the haemoglobin content of *Daphnia* it seems probable that the oxygen content of the water is the explanation of the effects of light and darkness; the cultures in the light contained more oxygen, thanks to algal photosynthesis. I have been unable to find a direct effect of light on the amount of haemoglobin in *Daphnia* blood. For instance, the loss of haemoglobin by red *Daphnia* kept in pond water lacking algae is the same in light and darkness. Nor does green food affect the blood pigment; pale *D. obtusa*, some of which were fed on yeast and others on the green alga *Gonium pectorale*, both lots being kept in darkness for 5 days in water of a low oxygen content, gained haemoglobin to the same extent.

(3) *Influence of nutrition*

My results contradict the conclusion of Verne, for the animals which lost haemoglobin in the window were feeding on algae, while those which gained it in the dark had fewer or eventually no algae in their food. It was once doubted whether *Daphnia* can digest cellulose and thus feed on unicellular algae other than flagellates (Naumann 1921), but various workers have shown that *Daphnia* can be cultured on pure strains of algae (cf. Mortimer 1936; Lefèvre 1942). Algal cells can become colourless by digestion in the gut of *Daphnia* while the cell walls still retain their shape and their cellulose reaction (von Dehn 1930); the cell contents are leached out. The food of *Daphnia*, which in nature often consists of much dark detritus and a few algal cells, is seen under the microscope to be confined in the gut within a peritrophic membrane (Chatton 1920). The wide space in the front half of the midgut between this membrane and the gut wall is often coloured green with chlorophyll, or a breakdown product of chlorophyll, showing with a microspectroscope the characteristic intense absorption band at the red end of the spectrum. This pigment may be of a brilliant green and there is so much of it that it must accumulate gradually from the sparse algal cells scattered among the detritus in the gut. The green pigment is perhaps adsorbed on a colloid, which may be the swallowed secretion of the big labral glands, which Cannon (1922) showed not to be mucus. Be this as it may, *Daphnia* progressively losing haemoglobin in the laboratory very often has its gut lumen green with chlorophyll. In nature the pond

water may be a green soup of algae while the *Daphnia* in it has colourless blood. Yet this is by no means always the case; *Daphnia* in another green pond may be pink or red. And *Daphnia* in clear water containing few algae may have pale blood with little haemoglobin but bright green intestinal fluid. All this indicates that chlorophyll as food is not responsible for haemoglobin.

It has often been thought that red blood in *Daphnia* implies good nutrition. Fritzsche (1917) was of this opinion. It cannot, however, really be the case that red blood is just due to good food. In the light and dark experiments there was at least as much algal food in the light as bacterial in the dark. Yet it can be objected that the bacterial food may have been more nutritious, especially as the digestion of algal cell walls is apparently not easy. But there is an indication to the contrary; the lighted cultures were better fed, since they produced more eggs. In the first experiment quoted above (p. 200), the mean number of parthenogenetic eggs or young per mother after 21, 29, 34 and 45 days in light and dark respectively were 6·2 and 0·8, 4·6 and 0·7, 5·2 and 0·9, 3·4 and 0·6. There were more eggs in the light, and this was typical of other cases. It is well known that good feeding gives large broods of Cladocera. To take one instance, *D. pulex* with a mean egg count of 4·1 was kept in a laboratory window with and without abundance of an alga, *Chromulina Rosanoffii*, as food. After 8 days both lots were swimming actively but the mean egg numbers were 12·6 and 0. Thus, as judged by egg production, the lighted cultures in my first experiments were better nourished, yet it was those in the dark which retained or formed more haemoglobin.

Yet, much haemoglobin is not necessarily correlated with an impoverished state, for in nature red *Daphnia* may or may not carry numerous eggs. To take an example, a population of red *D. magna* in nature with a haemoglobin index of 90 had a mean egg number of 35. In another pond *D. magna* of index 86, which is sensibly the same, carried only an average of three eggs. Both observations were made in autumn. I have known individual *D. magna* with very pale blood bearing a record number of over 90 eggs in the brood pouch. Thus neither in nature nor in the laboratory is there any direct relation between number of young and haemoglobin content of the mother's blood.

Feeding does intervene in haemoglobin synthesis, however, to this extent that minimal nutrition is necessary; starved *Daphnia* does not produce the blood pigment under circumstances when well-fed animals do so. This was found in experiments in which pale *D. obtusa* was kept for 5 days (in the dark) in water of low oxygen content (*a*) with *Gonium pectorale* given daily as food, and (*b*) without this food. With the food haemoglobin was synthesized; without food there was a heavy mortality without any haemoglobin synthesis in the survivors.

(4) *Pond water and haemoglobin synthesis*

Pink or red *Daphnia* kept in the laboratory in water nearly saturated with air usually lose their haemoglobin, becoming gradually paler. In water deficient in dissolved air similar *Daphnia* lose haemoglobin much less rapidly, or they may

retain it. In other experiments, however, animals in aerated water did not lose haemoglobin, and those kept in partially anaerobic conditions increased their blood pigment, becoming redder. These experiments, resulting in the retention of haemoglobin in aerated water and its increase with slight oxygen deficiency, were made in water from a pond in which *D. pulex* was unusually red; the haemoglobin index was 128. This suggested that some other factor in the water in addition to oxygen deficiency may intervene in haemoglobin synthesis. The idea was supported by experiments made with this same pond water, in the course of which the haemoglobin increased even when the amount of dissolved oxygen was not very low. For example, when the initial haemoglobin index of *D. pulex* was 37, after 5 days in water from this pond, as much as 84% saturated with air, the index rose to 47, while 5 days in the same water at 48% air saturation, which is not a very low value, raised the index to 66. There is apparently a factor in this water other than oxygen deficiency which stimulates haemoglobin synthesis.

Abundant rain-water falling into a pond with red *Daphnia* has on more than one occasion given the impression of causing the animals to become paler. In one case I measured such an effect. A water butt in a garden had a population of red *D. pulex* with an index of 91. The butt was half empty and I added enough tap water to increase the water volume by one-third. Eight days later the haemoglobin index had dropped to 77. Apparently a haemoglobin-stimulating substance in the water had been diluted but perhaps the oxygen content of the water had merely been raised.

(5) *Haemoglobin synthesis promoted by duck faeces*

The species of *Daphnia* which can become red with haemoglobin are found in waters that are to a greater or less extent polluted with organic matter. Ducks are one of the sources of pollution. Accordingly experiments were made to discover whether duck faeces contain a substance that is able to stimulate haemoglobin synthesis.

Very pale *D. magna* with a haemoglobin index of 11, which had been feeding on algae for months in the laboratory window, were put into water with a suspension of duck faeces. Six days later their haemoglobin index was 54, while that of the stock culture in the window was unchanged. The faeces suspension, owing no doubt to bacterial action, was only 39% saturated with air at the end of the 6 days, while the stock *Daphnia* culture was 140% supersaturated as a result of algal photosynthesis. Was it the oxygen deficiency or the duck faeces that had stimulated haemoglobin synthesis?

This question was studied as follows. Pale *D. magna* with an index of 17 were put into (*a*) a suspension of duck faeces, (*b*) water through which hydrogen was slowly and continuously bubbled (with the precaution mentioned above to avoid disturbing the animals), and to which algae were added for food. After 5 days the haemoglobin indices had risen to (*a*) 56, (*b*) 36, while the average air saturations, from six estimations made during the 5-day period, were (*a*) 26%, (*b*) 18%. The

haemoglobin had increased over threefold in presence of the faeces, but it had only doubled in the hydrogenated water, although the oxygen content of the latter was lower than that of the former. In another experiment the initial index was 36; after 10 days the indices became (*a*) 69, (*b*) 54 and the average air saturations, from two estimations daily, were (*a*) 22 %, (*b*) 16 %.

It is clear that duck faeces contain something which promotes haemoglobin formation over and above the effect of oxygen deficiency. Is this just better nutrition, or is it a specific substance?

4. Breakdown and excretion of haemoglobin

(1) *Synthesis and breakdown*

In vertebrate animals the sites of haemotopoiesis are known, but not as yet in *Daphnia*. The precursor of haemoglobin might be a porphyrin, but I have been unable to detect porphyrin either in individual *Daphnia* or in mass extracts. In man, as in *Daphnia*, oxygen deficiency in the environment stimulates haemoglobin formation (Campbell 1926), and the same is true of fishes (Schlicher 1926; Ozolius 1936), but the causal factor acting in the bone marrow of man or the spleen of fishes is unknown; in *Daphnia* we could obviously not yet expect to know the train of causes between oxygen lack in the water and haemoglobin production in the animal.

In the vertebrates the haem of haemoglobin is continuously broken down to bile pigment and iron, and the bile pigment is excreted. Does *Daphnia* also get rid of its haemoglobin thus? I have not been able to detect any bile pigment in *Daphnia*. There are, however, two indications of other possible modes of exit of haemoglobin from the cladoceran body, namely, an occasional pathological condition of haemoglobinuria and the normal presence of a haemochromogen in the lumen of the gut.

(2) *Haemoglobinuria*

The excretory organs of *Daphnia* are a pair of relatively large, clearly visible maxillary glands, often called shell glands. As in all Crustacea these excretory organs are closed internally and they are bathed in blood. Klotzsche (1913) noticed that occasionally one of the two shell glands of *Daphnia* is bright red. A microspectroscope shows that the red substance is oxyhaemoglobin. This is very much more concentrated than the pigment in the blood. In a colourless population, too, a few individuals may have a pink shell gland; the imperceptible haemoglobin of the blood has accumulated there. The haemoglobin fills a part, or the whole, of the convoluted tubule. A microscope shows that the red substance is in the kidney's lumen; the cells of the wall appear free of it. A red shell gland is only occasionally found. Most populations are devoid of it and when it occurs it is usually found in less than 0·1 % of individuals. Exceptionally I have found it in nature in 2 % of *D. magna*.

Isolating individuals with a red gland shows that they are usually less viable than normal ones. It also shows that quite often the red colour disappears in the course of a day or two; the animals recover. I have never seen an individual with both shell glands affected; doubtless, as in man, at least one functional kidney is essential.

The impression made by the haemoglobinuria is that haemoglobin has accumulated in the shell gland owing to a blockage of the outlet. It suggests that perhaps haemoglobin as such is normally excreted in small quantities by the maxillary glands. However, affected animals in a red population are anaemic compared with normal ones, which seems to argue against this hypothesis.

(3) *A haemochromogen in the alimentary canal*

If a normal pink or red *D. magna* is examined under the microscope with a spectroscopic ocular, in a drop of water beneath the cover-slip of a compressorium, it shows two strong bands of oxyhaemoglobin in the blood. In a short time these bands fade and vanish, as the animal's respiration deoxygenates the haemoglobin. The process can be much hastened by introducing a little sodium hydrosulphite solution beneath the cover-slip with a fine pipette. If the specimen is so arranged on the microscope stage that the field of vision under the high power is mostly filled by part of the anterior end of the midgut, then as the two oxyhaemoglobin bands fade a narrower band appears between them. This is first seen when the oxyhaemoglobin bands are half gone, and it becomes progressively stronger. It is a narrow band like that of cytochrome *b*. The wave-length of its axis, measured with the Zeiss spectroscopic ocular, calibrated with a neon lamp, is at 563 mμ. A much weaker band can be seen at 533 mμ. The pattern of absorption bands shows that the substance is a haemochromogen. If now (in the absence of hydrosulphite) the cover-slip is raised for a moment to admit air, these two bands vanish as the oxyhaemoglobin reappears. The haemochromogen thus reacts reversibly with dissolved oxygen: in the oxidized form the strong bands go, when reduced again they reappear. For laboratory convenience I refer to this pigment as *daphniarubin* on the analogy of helicorubin, a haemochromogen in the crop liquid of snails which is reversibly oxidizable and reducible.

Helicorubin, in the crop liquid of the snail *Helix pomatia*, has a double α-band with axes at 563 and 558 mμ; its β-band is at 532 mμ. Clearly the two bands of daphniarubin correspond to the longer wave α-band of helicorubin and to its β-band. Exploration with the microspectroscope shows that daphniarubin is confined to the midgut, and to that part of it where there is a space between peritrophic membrane and wall, namely, in the front part of the midgut. It is also in the anterior paired gut diverticula. In most individuals the pigment cannot be traced further back than opposite the heart; but occasionally it can be followed to the posterior bend of the intestine. In such cases the peritrophic membrane encloses little food and the gap between it and the gut wall can be seen to extend further back than usual. The bands of daphniarubin can be seen with the microspectroscope in the intestine

of *Daphnia* after it has been dissected out of the animal in a solution of sodium hydrosulphite. But as soon as the isolated intestine is torn and teased with needles the bands vanish. Clearly the pigment has leaked away. This shows that it is in solution in the liquid contents of the intestine; it is not situated in the gut wall. There is no daphniarubin in the eggs.

In helicorubin the reversible oxidation occurs only in an acid medium (Dhéré & Vegezzi 1917). When *Daphnia* is put for some hours into a dilute solution of bromothymol blue this indicator accumulates in the gut lumen, where it is much more concentrated than in the water outside. The mode of accumulation may be similar to that of chlorophyll discussed above. The indicator shows (without allowing for protein and other errors) that the anterior three-quarters of the midgut has a pH varying with individuals from 6·0 to 6·8, while the posterior quarter has 6·6 to 7·2. These figures agree sensibly with those found previously by von Dehn (1930) and Hasler (1935); they show that the front part of the gut of *Daphnia* has a suitable pH for the reversible oxidation of a pigment like helicorubin.

The amount of daphniarubin present in the intestine is proportional to the haemoglobin content of the animal's blood. Red *Daphnia* have most, pink ones have less. In pale or colourless individuals no daphniarubin can be detected in a single individual, though piling one on another may reveal it. The proportionality between daphniarubin and the blood haemoglobin suggests that the former may perhaps be the excretory product of the latter. Moreover, daphniarubin, with an α-band again at 563 mμ, is present also in the fairy shrimp *Chirocephalus diaphanus* Prévost, all along the gut; anaerobic conditions beneath a cover-slip reveal it through the microspectroscope. There must be less of the pigment here than in red *Daphnia* since the bands are no stronger although the animal is much bigger. *Chirocephalus* has haemoglobin in its blood (Lankester 1871), though the quantity is generally small.

There are, however, arguments against the suggestion that daphniarubin is the excretory product of haemoglobin in *Daphnia* and *Chirocephalus*. *Daphnia hyalina* Leydig is found in lake plankton; its blood is colourless. Heaping eggless individuals of this species on top of one another and examining them with the microspectroscope by transmitted light fails to show oxyhaemoglobin, but daphniarubin reveals itself after a few minutes of autoreduction. Here is daphniarubin without haemoglobin. Another objection is the fact that helicorubin is found in the snail's crop although the snail has no haemoglobin. But the snail's heart and buccal mass have cytochrome and there are haem compounds in liver, foot and elsewhere. The crayfish *Astacus pallipes* Lereboullet has a haemochromogen like daphniarubin in its hind gut liquid, with the α-band at 561 mμ. Here again there is no haemoglobin in the animal, but it has cytochrome, especially in the heart, and its liver contains haem. In the snail and crayfish the haemochromogen may represent the excretion of cytochrome and other haem compounds, in some water fleas and in the fairy shrimp perhaps that of haemoglobin.

(4) *Haematin in blood*

Under the heading of haemoglobin excretion one other point must be mentioned. Populations of *Daphnia magna* are sometimes found which are brown. This colour may be due to a greyish green tint of the eggs combined with an orange gut liquid and pink blood. But the brown aspect cannot always be accounted for thus. When *Daphnia* is squashed *en masse* the liquid, clarified by filtering through kieselguhr, is usually pink owing to haemoglobin, but occasionally it is brown. The intensity of the α-band of oxyhaemoglobin in such a filtrate from brown *D. magna* was found to match the intensity of the α-band in a much paler, and of course pinker, dilution of my own blood. But the haemochromogen formed in the *Daphnia* filtrate by the addition of pyridine and sodium hydrosulphite was considerably more concentrated than that derived from my diluted blood. Thus the brown colour is due, in part at least, to a haem compound which supplied the excess of pyridine haemochromogen.

This haem compound in the blood of brown *Daphnia* might be methaemoglobin or haematin. To test the first possibility, the filtered *Daphnia* extract was divided into two lots, to one of which sodium hydrosulphite was added. If methaemoglobin were present it would thus be reduced to haemoglobin. Carbon monoxide was then passed through both lots. There was no increase either in pink colour or in the intensity of the absorption bands in the lot which had been treated with hydrosulphite, which showed that there had been no methaemoglobin there. It is therefore probable that the brown blood was coloured by haematin.

This haematin is not necessarily an excretory product of the haemoglobin, but it is noteworthy as an alternative haem compound in the blood. Four months previously a particular brown population of *D. magna* had been light pink, with a haemoglobin index of 42. So far as could be judged, the index of the brown populations was 25, but obviously this could not be accurately determined in view of the disturbing brown colour.

5. Has the haemoglobin a function?

(1) *The blood pigment is apparently functionless*

A low concentration of dissolved oxygen causes *Daphnia* to increase the haemoglobin content of its blood. The haemoglobin of *Ceriodaphnia laticaudata* has a higher oxygen affinity than that of *D. magna* (Fox 1945*a*), and the former lives in fouler water which is likely to contain less oxygen. These facts suggest that the blood pigment of the Cladocera is of use, and perhaps of vital importance.

When *Daphnia* is enclosed in a corked tube full of water the respiration of the animals gradually removes the dissolved oxygen from the water and after a time the haemoglobin in the blood becomes deoxygenated. The disappearance of the two absorption bands in the more or less crowded *Daphnia* population can be observed with a hand spectroscope. In one such experiment with *D. obtusa* the haemoglobin lost its oxygen in 20 min. The animals continued swimming, however,

for a long time after this. An hour later many were actively swimming, and some were still swimming at the end of 5 hr. Such preliminary experiments showed that deoxygenation of the haemoglobin in the blood has no immediately fatal effect.

A series of experiments was next made to compare the activity and survival, in water containing little dissolved oxygen, of normal animals and of animals whose haemoglobin had been rendered functionless for oxygen transport by carbon monoxide. Ten experiments were made, in each of which twenty animals with carboxyhaemoglobin were enclosed in one stoppered bottle completely full of water, twenty untreated animals in another. The bottles had a capacity of 3 l., large enough for the respiration of the animals to have little effect on the concentration of dissolved oxygen. The initial oxygen content of the water was the same in each bottle; by previous bubbling with nitrogen it had been reduced to a value little above that at which the blood of *Daphnia* loses its oxygen (1·18 ml./l. at 17° C, see p. 197). The average oxygen concentration at the beginning of the experiments was 1·40 and at the end 1·13 ml./l. The experiments lasted 30 to 45 hr. Preliminary tests showed that the oxyhaemoglobin of *Daphnia* is rapidly converted into carboxyhaemoglobin by immersing the animals for less than 1 min. in aerated water 5 % saturated with carbon monoxide. This low proportion of carbon monoxide to oxygen should not affect cytochrome oxidase if present. The animals were left for 5 min. The water in the 3 l. bottle into which the treated animals were then put for the experiment was 1 % saturated with carbon monoxide to avoid dissociation of the carboxyhaemoglobin, and both this bottle and the control were kept in the dark. At the end of the experiment animals with carboxyhaemoglobin were examined with the microspectroscope in sodium hydrosulphite solution to see that there was no fading of the absorption bands in the blood. In these ten experiments there were 184 survivors of the 200 animals with carboxyhaemoglobin and 189 survivors of the 200 with oxyhaemoglobin. The difference is negligible and there was no visible difference in the activity of the two lots of survivors. The blood pigment appears thus to be unimportant in respiration. It may, of course, have some other function.

Artemia salina usually contains quite small amounts of haemoglobin. It is improbable that such low concentrations of blood pigment can be functional in respiration when the much more concentrated haemoglobin often present in *Daphnia magna* is apparently not so used. In *Artemia* the quantity of haemoglobin varies greatly in different individuals of a population. In some cases the oxyhaemoglobin bands can easily be seen in a single individual, in others the bands are faint or invisible. This variability argues against functional importance.

(2) *Function of haemoglobin in parthenogenetic eggs*

If the blood pigment is functionless in respiration, is the haemoglobin in the eggs of use? The microspectroscope shows that the pond species *Daphnia magna*, *D. pulex* and *D. obtusa* have haemoglobin in the eggs within the brood pouch, even when the blood is more or less colourless. The lake plankton species *D. hyalina*, with

quite colourless blood, also has a trace of haemoglobin in the eggs. In this species the oxyhaemoglobin bands can be discerned with the spectroscopic ocular in a pile of parthenogenetic egg-bearing females heaped up moist on a microscopic slide, if examined quickly before the pigment is deoxygenated. A similar pile of eggless females shows no oxyhaemoglobin bands.

Respiratory conditions are not ideal in the brood pouch. This is clear from the following observations. If an individual of *D. magna*, *D. pulex* or *D. obtusa*, lightly held in a compressorium, is allowed to deoxygenate its haemoglobin by its own respiration, then the oxyhaemoglobin absorption bands fade first in the eggs and then in the blood. This might mean that the blood pigment has a higher affinity for oxygen than the haemoglobin of the eggs. But this is not so, for if the eggs are brought outside the brood pouch before the experiment begins by gently pressing on the brood pouch with a needle, then the oxyhaemoglobin bands in the eggs fade at the same time as those of the blood, or slightly later. It may then be that, enclosed as they are in the brood pouch, the eggs need their haemoglobin for respiration during development.

The experiments with carboxyhaemoglobin described on p. 208 had a dual purpose. They were designed also to test the importance of the egg haemoglobin in development. The *D. magna* used were parthenogenetic females with eggs, but not embryos, in the brood pouch. At the end of each experiment most individuals carried embryos, all of which were at the same stage of development in a given mother, but the stage reached in the different mothers varied, doubtless owing to initial differences in the degree to which cleavage had progressed. Moreover, the experiments were purposely varied in duration from 30 to 45 hr. so that different embryonic stages should be attained. The stage reached was recorded for each mother at the end of an experiment. The following easily recognizable arbitrary stages were used: (1) uncleaved eggs, (2) cleaved eggs, (3) headless embryos, (4) embryos with head but no eyes, (5) two red eyes, antennae not free, (6) two red eyes, antennae free, (7) double black eye, (8) single black eye. Table 1 groups together the number of mothers whose young reached the various developmental stages at the end of the ten experiments with and without carboxyhaemoglobin. It is clear that up to stage 5 haemoglobin makes no difference in rate of development, but in the last three stages embryos without the functional respiratory pigment lag behind. χ^2 for the eight pairs of values is 60·25, giving a probability of less than 0·01 that the difference between the two series is due to chance.

Haemoglobin seems thus to have a function in embryonic development. Nevertheless carbon monoxide treated young show themselves to be just as active and

Table 1. Numbers of *Daphnia magna*, the embryos of which reached various stages of development

stage	1	2	3	4	5	6	7	8
with CO	2	10	15	76	33	38	6	4
without CO	1	14	18	71	38	14	19	13

viable as normal ones when kept for several days after hatching. Moreover, although the egg haemoglobin seems only to intervene in late embryonic stages, yet the quantity of haemoglobin in the egg diminishes as development proceeds. This is best seen in *D. magna* and *D. pulex* with colourless blood and with so little haemoglobin in the eggs that the oxy-bands can only just be seen. As the embryos develop these bands disappear.

(3) *Absence of haemoglobin from ephippial eggs*

Considering the relatively large amount of haemoglobin in the parthenogenetic eggs, it is surprising to find no trace of the pigment in fertilized eggs taken out of the ephippium. This is so even with the most red blooded *D. magna*, *D. pulex* and *D. obtusa*. These fertilized eggs, however, although lacking haemoglobin, contain abundant haem. This is shown by treating the eggs with pyridine and hydrosulphite, upon which a strong haemochromogen α-band appears.

The absence of haemoglobin in the fertilized eggs of *D. magna*, *D. pulex* and *D. obtusa* and its presence in the parthenogenetic eggs of these species would seem to accord with the relatively better respiratory conditions in which the fertilized eggs can develop, since they are not enclosed in the brood pouch. Yet this suggestion is not valid, for the fertilized eggs develop just as well at low as at high concentrations of dissolved oxygen. Experiments were made in which ephippia of *D. obtusa*, just freed from the mother, were put into three stoppered bottles completely filled with a relatively large volume of water having a dissolved oxygen content of 1·3 ml./l. at 18° C, i.e. only 18% saturated with air. After 11 days the oxygen content of the water was then 1·2 ml./l. The young hatched at the same time as those out of other ephippia from the same population which had been kept in well-aerated water, namely on the 10th and 11th days.

It might be suggested that the apparent lack of utility of haemoglobin in the blood of *Daphnia*, and its function, even if a minor one, in the development of parthenogenetic embryos, implies that the pigment in the blood is an overflow from a supply to the eggs. If this were so one might expect to find a different amount of blood pigment in parthenogenesis than in bisexual reproduction, since ephippial eggs lack the pigment. Yet in point of fact parthenogenetic females have no more blood haemoglobin than ephippial females in the same population.

At one period in the early history of animals haemoglobin must have appeared for the first time, a mere by-product of some metabolic chemical reaction. In mammals, in fishes and in *Daphina* the production of haemoglobin is increased by oxygen deficit; perhaps this was a condition of its first appearance. Initially the haemoglobin would be useless. Then its potentially useful capacity for reversible oxygenation would in certain animals have been utilized. May it not be that in the blood of *Daphnia* haemoglobin still appears to-day as a mere by-product of semi-anaerobic metabolism?

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Mitotic activity in the adult male mouse, *Mus musculus* L. The diurnal cycles and their relation to waking and sleeping

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The diurnal cycles of mitotic activity in the ear epidermis of the adult male mouse have been determined by the removal of earclips at 2 hr. intervals throughout the 24 hr. The mice used were between 3 and 4 months old, and were of the Kreyberg white label and Strong's *CBA* strains. A considerable degree of individual variation was found, but on the average the maximum mitotic activity was at 06.00 and 14.00 hr. and the minimum mitotic activity at 10.00 and 20.00 hr.

This observation was confirmed by killing groups of mice, each group consisting of five males, at the same 2 hr. intervals throughout the 24 hr. Similar variations in the mitotic activity of the ear epidermis were observed, and, in addition, similar cycles were evident in the mid-dorsal epidermis of the back, the stratified epithelium of the oesophagus, the lining epithelium of the epididymis, and the proliferating zone of the duodenal mucosa. In this last tissue the rate of cell division never fell to a very low figure, and in the proliferating centres of the intestinal lymph nodules and in the seminiferous tubules of the testis there was no trace of a cycle since the rate of cell division remained constantly high.

A study was also made of the spontaneous bodily activity of the mice throughout the 24 hr., and by comparing the average figures so obtained with the average figures for epidermal mitosis, it proved possible to make the significant correlation that when the animals are at rest mitotic activity is at a maximum and that when they are awake and active it is at a minimum.

This correlation permits an explanation of the individual variation in mitotic activity, since there is also a high degree of individual variation in spontaneous bodily activity. It also permits an explanation of the contradictory results which have been reported in the past regarding diurnal mitosis rhythms in mice, since it is evident that the rhythms of bodily activity must be strongly affected by differences in the age, sex and condition of the animals used, in the season of the year, and in the routine of the laboratory.