

The production of haemoglobin by small pond *Daphnia pulex*: intraspecific variation and its relation to habitat

DIANA L. ENGLE Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan, U.S.A.

SUMMARY. 1. Haemoglobin production was measured for two populations of *Daphnia pulex*, one from a temporary pond (GR Pond) and one from a permanent pond (Rash Pond). Surface water in Rash Pond remains high in oxygen, while by late summer very little oxygen remains in GR Pond. Haemoglobin synthesis was induced in the laboratory by artificial oxygen deficiency.

2. Reproductive state influenced the level of haemoglobin in *Daphnia*. Females with parthenogenetic eggs had higher total body haemoglobin than ephippial females. Clutches laid by pale *Daphnia* immediately before exposure to low oxygen conditions were aborted. Offspring produced by haemoglobin-rich females later in the treatment survived.

3. Haemoglobin production was higher for Rash Pond *Daphnia*. Visual predators exist in GR Pond, but are absent in Rash Pond. Also, *Daphnia* are smaller in GR Pond. Visual predation may have selected against high levels of haemoglobin in the temporary pond. Thus oxygen availability in nature is not necessarily a good predictor of haemoglobin production by *Daphnia*.

4. Feeding behaviour was altered in low oxygen conditions. Browsing was dominant in low oxygen treatments while *Daphnia* in control treatments filtered algae normally. Browsing may increase access to sedimented iron necessary for haemoglobin synthesis.

Introduction

Daphnia increase the synthesis of haemoglobin when low oxygen conditions are encountered. Elevated levels of haemoglobin prolong survival, increase swimming and feeding activity, speed development of eggs, and allow increases in clutch size (Fox, 1948; Fox, Gilchrist

& Phear, 1951; Kring & O'Brien, 1976; Heisey & Porter, 1977). Species of *Daphnia* may vary considerably in the ability to synthesize haemoglobin (Chandler, 1954; Fox, 1948; Green, 1956). These differences are not necessarily associated with different levels of oxygen stress in nature. Often, two species from the same habitat display variation in haemoglobin content. Green (1956) proposed three explanations for this phenomenon. First, behavioural differences may separate the two species spatially such that they encounter different oxygen

Correspondence: Diana L. Engle, Division of Environmental Research, Academy of Natural Sciences of Philadelphia, 19th and the Parkway, Philadelphia, Pennsylvania 19103, U.S.A.

conditions. Second, a physiological difference, such as in metabolic rate, might stimulate one species to produce more or less haemoglobin than another. Third, although two species may occupy the same microhabitat and have similar physiologies, there may exist inherent, perhaps genetic, differences in ability to respond to low oxygen.

Intraspecific variation in haemoglobin content has also been noted in some habitats. Prepas & Rigler (1978) studied the vertical distribution of haemoglobin-rich and pale *D. pulex* over time in Lake Crawford, Ontario, and concluded that they comprised two spatially separate populations, the red *Daphnia* occupying a deeper stratum near an anaerobic chemocline where dissolved oxygen was 1 mg l^{-1} or less. The vertical segregation of pale and red *D. pulex* was also observed in two thermally stratified lakes in southern Michigan (Third Sister Lake, R. M. Dorazio, pers. comm.; Hamilton Lake, C. E. Goulden, pers. comm.). It is not clear whether this type of stratification stems initially from behavioural differences or from genetic variation in abilities to produce haemoglobin.

The complexity of factors effecting haemoglobin synthesis by *Daphnia* makes it difficult to assess the extent to which patterns of oxygen availability determine this biosynthetic capability. In order to define the connection between habitat type and patterns of oxygen consumption, typical lake invertebrates have been contrasted with typical pond invertebrates (Berg, Jonasson & Ockelman, 1962; Calow, 1975; Heisey & Porter, 1977). This distinction may not always be valid. Small pond *Daphnia* may be exposed to very different oxygen regimes depending on whether they inhabit permanent or temporary bodies of water. Temporary pond *Daphnia* are often exposed to extremely low and unvarying oxygen levels during drying due to minimal water mixing, highly organic substrata and large bacteria populations (Moore & Burn, 1968). *Daphnia* in permanent pond, however, are usually capable of occupying surface strata where dissolved oxygen is high. In this investigation, I compared haemoglobin production by two populations of *Daphnia pulex*, one from a temporary pond and one from a permanent pond. In this way I was able to test for intraspecific variation in haemoglobin production by one pond species

of *Daphnia* and to see if such differences were correlated to oxygen availability in the two habitats.

Materials and Methods

Daphnia pulex Leydig were collected from two ponds in southeastern Michigan in mid-August 1982. The first pond, Rash Pond, is a permanent shallow body of water in an abandoned gravel pit located in the University of Michigan Mathaei Botanical Gardens, Ann Arbor, Michigan. It has a maximum depth of 2 m and a maximum area of 1000 m^2 (Brambilla, 1980). At the time of sampling, dissolved oxygen in Rash Pond decreased from slightly below saturation (7.5 mg l^{-1}) at the surface to as low as 1 mg l^{-1} near the bottom. The second pond, GR Pond, is a heavily shaded temporary pond approximately 300 m^2 in area located in the E. S. George Reserve, Pinckney, Michigan. It forms from run-off and precipitation during spring thaw and dries completely by late summer. Its substratum is thickly covered with leaf litter and decaying material. Its depth in mid-August 1982 was less than 60 cm. Dissolved oxygen in GR Pond was less than 1 mg l^{-1} throughout the water column.

Laboratory clones of *D. pulex* were started by isolating ovigerous females into beakers of filtered pond water (Whatman glass fibre, GF-F). *Chlamydomonas reinhardtii* Dang from a unialgal batch culture (grown at 20°C under 16:8 LD cool fluorescent light) was provided as food. Over several days the clones were acclimated to an artificial medium at pH 7.6 (MWC medium, see Lehman, 1976). Animals were also kept at 20°C on a 16:8 LD light cycle. Later, an experimental stock for each population was begun by raising twenty-four neonates (each from a different clone) in individual shell vials with 15 ml of MWC medium and *C. reinhardtii* at a concentration of 1×10^5 cells ml^{-1} . The neonates were transferred each day to fresh algal suspension (hereafter called culture medium).

Haemoglobin induction experiments

Three consecutive experimental trials were carried out on each population. Each trial consisted of a pre-treatment period of 10 days

in which neonates were raised to maturity in near-saturated oxygen conditions followed by a treatment period of 10 days in which the adult *Daphnia* were subjected to low oxygen. Total body haemoglobin was assayed on day 21 of each experimental trial.

The first offspring produced by the experimental stocks served as the source of neonates for Trial 1. The neonates were pooled and randomly sorted into 600 ml beakers of culture medium (forty neonates per beaker). Every 24 h an aliquot from each pretreatment container was measured for absorbance at 500 nm with a Hitachi Model 100-80 spectrophotometer to determine algal density. *C. reinhardtii* was then added to readjust food concentration to 1×10^5 cells ml^{-1} . Offspring produced by *Daphnia* in the pretreatment beakers (typically on day 9 or 10) in trial 1 and trial 2 were pooled and served as the source of neonates to begin trials 2 and 3, respectively.

On day 10, treatment bottles were prepared. Glass reagent bottles (500 ml) were filled with culture medium. For low oxygen treatments, dissolved oxygen was reduced to 3 mg l^{-1} by bubbling the culture medium with compressed N_2 . An oxygen electrode (Orion Research Model 97-08) inserted into the neck of the bottles measured dissolved oxygen during bubbling. Low oxygen bottles were kept firmly stoppered. Control bottles were not bubbled and were left unstoppered to allow exchange with room air. Adult *Daphnia* from each set of pretreatment beakers were pooled and randomly transferred into treatment bottles (two low oxygen bottles, one control bottle) until each contained thirty-five animals. All bottles were kept at 20°C and in the dark to prevent photosynthesis.

Daphnia were kept in treatment bottles for 10 days. Every 24 h, algal concentration in each treatment bottle was measured and adjusted (1×10^5 cells ml^{-1}) as described for pretreatment beakers. Any carcasses, moults, and shed ephippia were removed. When necessary, I readjusted dissolved oxygen in the low oxygen bottles with a special bubbling device (see Fox, 1948) that allowed circulation of culture medium past a stream of nitrogen gas, but protected animals from contact with bubbles. In the same way, air was gently bubbled into control bottles daily for a few minutes to

maintain oxygen at near saturation. After 5 days, dissolved oxygen was reduced to 2 mg l^{-1} in low oxygen bottles for the rest of the treatment period.

On day 21 of each trial, all adult *Daphnia* were removed from treatment bottles, measured with an ocular micrometer (head shield to base of tail spine) and sorted into vials with respect to four reproductive states:

Eggs: presence of eggs or early embryos (head bulge, but no eye spots) in the brood chamber.

Mid/Late: presence of mid embryos (double red eye spots) or late embryos in the brood chamber.

Barren: not reproducing, ovaries unenlarged and inactive.

Ephippial: stages of formation, or presence, of ephippium.

Females with enlarged ovaries that had moulted, but not yet laid eggs, were included in the with-eggs category. Total body haemoglobin was measured using a modification of the carboxyhaemoglobin method of Crider, Wilhm & Harmon (1982). Samples consisted of three to fifteen *Daphnia* of the same reproductive state.

Length-dry weight relationships were established for each population for three categories of adult animals from the laboratory clones: fecund (eggs or embryos); barren; and ephippial (see Bottrell *et al.*, 1976; Richman, 1958). All *Daphnia* used in the analysis had full guts and a high lipid content (visual index; Tessier & Goulden, 1982). Dry weights of single animals were found to the nearest 0.01 mg on a Cahn Model 29 Electrobalance. The resulting regression formulae allowed me to predict individual dry weight from length with an average standard error of 18%. Each haemoglobin value was divided by the total dry weight of *Daphnia* included in the same and converted to nmol HbCO per mg dry weight.

Results

Haemoglobin content of Daphnia

Levels and patterns of haemoglobin production within each population were similar in trials 1-3. Therefore, for each population the results of the three trials were pooled. Kruskal

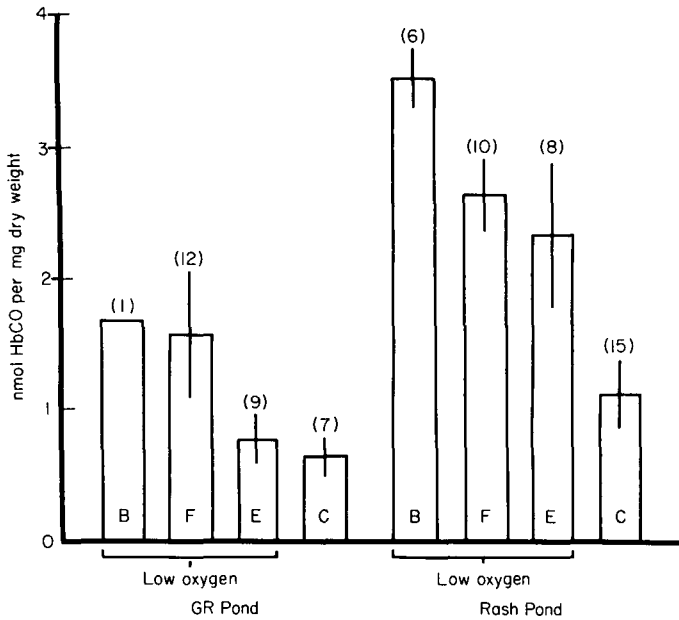


FIG. 1. Mean haemoglobin content (nmol carboxyhaemoglobin mg^{-1} dry weight) for barren (B), fecund (F), ephippial (E) and control (C) *Daphnia pulex*. Results shown are those pooled from all three experimental trials. The number of samples assayed for each category is given in parentheses. Error bars represent 95% confidence intervals around the means.

Wallis tests (Sokal & Rohlf, 1969) indicated that for both ponds there was no significant difference in haemoglobin content between females with eggs and females with mid or late embryos (for GR Pond, $P=0.570$; for Rash Pond, $P=0.088$). These two categories were therefore pooled and treated as one category, 'Fecund,' in all subsequent analyses. Similar tests showed that reproductive state (fecund, barren, ephippial) had no significant effect on the haemoglobin content of *Daphnia* in control

treatments (for GR Pond, $P=0.448$; for Rash Pond, $P=0.860$). These categories were also pooled for each population and treated as one, 'Control' in further analysis. Overall trends in haemoglobin content with respect to reproductive state were the same for Rash Pond and GR Pond (Fig. 1). Rash Pond *Daphnia* produced significantly more haemoglobin than those from GR Pond in every category (fecund, $P=0.006$; ephippial, $P=0.0005$; control, $P=0.001$).

TABLE 1. Length of adult (assayed) *Daphnia pulex*. Trials 1–3 pooled.

Population	Category	No. of samples	Mean length (mm)	Standard error
GR Pond	Fecund	12	2.121	0.026
	Barren	1	(2.030)	—
	Ephippial	9	1.997	0.024
	Control	7	2.027	0.035
Rash Pond	Fecund*	10	2.344	0.040
	Barren	6	2.216	0.026
	Ephippial†	8	2.118	0.033
	Control‡	14	2.237	0.061

*Significantly larger than GR Fecund: Kruskal Wallis, $\chi^2=10.44$, $P=0.0012$.

†Significantly larger than GR Ephippial: Kruskal Wallis, $\chi^2=5.33$, $P=0.0209$.

‡Significantly larger than GR Control: Kruskal Wallis, $\chi^2=4.06$, $P=0.0439$.

GR Pond

Fecund females had a significantly higher haemoglobin content than either ephippial ($P=0.001$) or control females (148% increase, $P=0.0004$). The haemoglobin content of ephippial females was not significantly different from that of the control females (24% increase, $P=0.153$). The single sample of barren females falls within one SE of the mean haemoglobin content for fecund females.

Rash Pond

Barren females had the highest mean haemoglobin content and were significantly different from fecund ($P=0.009$), ephippial ($P=0.002$), and control *Daphnia* ($P=0.001$). Fecund females had the next-highest levels of haemoglobin. Although they contained significantly more haemoglobin than control animals (134% increase, $P=0.0001$), they were not significantly different from ephippial females ($P=0.155$). In contrast to the results from GR Pond, ephippial females from Rash Pond had a significantly higher haemoglobin content than control females (108% increase, $P=0.001$).

Length of *Daphnia*

Low oxygen treatments. In both populations fecund females formed the largest size class and ephippial females were the smallest (see Table 1). Kruskal Wallis tests (Sokal & Rohlf, 1969) showed that fecund females from GR Pond were significantly larger than ephippial females ($P=0.004$) from the same pond. Rash Pond fecund females were significantly greater in length than both barren ($P=0.017$) and ephippial females ($P=0.004$). *Daphnia* from Rash Pond were significantly larger than GR Pond *Daphnia* in every category considered.

Control treatments. *Daphnia* assayed from the control treatments for both populations were comprised of similar percentages of fecund, ephippial and barren females. Within both groups of control animals, the same trends for body size were observed. Fecund females were largest, barren females were next-largest and ephippial females were smallest. Statistical tests on length were not performed between reproductive categories within control groups. However, when analysed as

two groups, Rash Pond control females were significantly larger than GR Pond control females (see Table 1).

Weight of *Daphnia*

Dry weight followed the same trends as body length with one exception: GR Pond ephippial *Daphnia* weighed more on average (165 μg) than Rash Pond ephippial *Daphnia* (76 μg). In every other category, however, Rash Pond *Daphnia* exceeded GR Pond *Daphnia* in weight.

Discussion

Maternal haemoglobin and egg development

The level of haemoglobin in the blood of parthenogenetically reproducing *Daphnia* fluctuates over the course of each instar. Up to one-third of an ovigerous female's haemoglobin passes into ovaries and eggs just before moulting takes place and eggs are laid into the brood chamber (Fox, Hardcastle & Dresel, 1949; Green, 1956; Dresel, 1948). As the eggs develop, more haemoglobin is produced by the female. Haemoglobin is thus lowest in a female early in an instar and greatest late in an instar just before mature embryos are released. In this study I detected less haemoglobin in females carrying eggs (or about to lay eggs) than in females carrying mid or late embryos. This was true in both low oxygen and control bottles, but the differences were not statistically significant. The average haemoglobin content of ephippial females was lower than that of fecund females. Absence of haemoglobin in ephippial eggs (Fox, 1948) may have contributed to this difference.

Within the first 3 days of oxygen deprivation, I observed that almost all moulting was accompanied by abortion of undeveloped eggs or embryos. This suggests that maternal haemoglobin is necessary for development of parthenogenetic eggs under low oxygen conditions. The aborted clutches were laid before the females had elevated levels of blood haemoglobin and did not receive the same reserve of maternal haemoglobin as later clutches did. Development of these first eggs may have been hindered by inadequate respiration once the parents were placed in low

oxygen conditions (Fox, 1948). Offspring produced by haemoglobin-rich females later in the treatment period were vigorous and usually highly pigmented.

Haemoglobin content and metabolic rate

Variation in haemoglobin production among *Daphnia* in uniform oxygen conditions may sometimes be explained by differences in metabolic rate, related to the age, size and sex of individuals (Green, 1956). The inverse relationship between respiration rate and body size among *Daphnia* (Hoshi & Yajima, 1970) suggests that haemoglobin production will also be inversely related to body size. Green (1956) used this relationship to explain his observation that when pairs of pond-dwelling species of *Daphnia* were compared in low oxygen tests, the smaller species in each pair produced a higher concentration of haemoglobin. Others have studied the relationship between metabolic rate and haemoglobin content by varying temperature (Kobayashi, 1974, 1981; Hoshi *et al.*, 1978). Fox & Phear (1953) discovered that when oxygen concentrations were held constant, *Daphnia* synthesized more haemoglobin at a higher temperature (presumably due to a higher metabolic rate).

In my experiment, the relationship between body size and haemoglobin synthesis (Green, 1956) did not hold true. Rash Pond *Daphnia* were significantly larger (Table 1) in every category than GR Pond *Daphnia*—yet they produced more, not less, haemoglobin in low oxygen conditions. (All assayed animals were of the same age and sex.) Body sizes cannot account for differences between reproductive categories. Overall, ephippial females were lowest in body length and weight, yet they displayed the lowest haemoglobin levels. Also, barren *Daphnia*, which produced the highest haemoglobin levels, were intermediate in length and dry weight.

It is not clear why low oxygen conditions induced a greater increase in haemoglobin content for ephippial females from Rash Pond (108% increase relative to control or 'basal' level) than for GR Pond ephippial females (24% increase). Although ephippial females from Rash Pond exceeded those from GR Pond in length, they weighed less. Their greater response to low oxygen conditions may

have been due, in part, to a higher metabolic rate.

Feeding behaviour

A striking difference in feeding behaviour was observed between *Daphnia* in low oxygen conditions and those in control conditions. *Daphnia* in the control bottles were usually evenly distributed in the water column and appeared to filter the culture medium in a normal fashion. In contrast, the *Daphnia* in low oxygen bottles foraged on the bottoms and sides of their treatment bottles. At the end of any 24 h period nearly 100% of the animals in low oxygen bottles would be clinging firmly to or combing the walls of their container. Vigorous beating of filtering appendages dislodged particles of detritus, which were usually collected and ingested. If disturbed by bubbling activity, the *Daphnia* only temporarily invaded the water column, quickly adhering to glass surfaces again. The appearance of the test bottles supported these observations. The bottoms of control bottles were consistently covered with an undisturbed layer of sedimented algae and the sides grew cloudy with algal wall growth. In contrast, wall growth was removed in low oxygen bottles, a thin deposit of faecal pellets lined the bottom, and undigested algal material was absent.

The term 'browsing' has been adopted by MacMahon & Rigler (1963) to contrast this foraging behaviour with normal suspension feeding. Data on the conditions under which *Daphnia* become bottom feeders suggests that browsing is an adaptation to bodies of water in which pelagial food sources vary in time but where the benthos provides a highly caloric supply of bacteria-rich sedimented or detrital food (Papinska, 1981; Rodina, 1963). Horton *et al.* (1979) studied the relationship between food supply and browsing in *D. pulex* and found that when the concentration of suspended algae dropped below 1×10^4 cells ml^{-1} , up to 70% of the animals sunk out of the water column to forage in bottom sediments. The high haemoglobin content of *Daphnia* in low oxygen conditions may have enabled them to remove algae at rates 2–2.5 times greater than the pale *Daphnia* in control conditions (Kring & O'Brien, 1976). Food concentration in low oxygen bottles may have decreased during each 24 h period to some critical threshold

value more rapidly than in control bottles and may have stimulated the change in feeding behaviour.

A shift from filter feeding to browsing may actually promote haemoglobin synthesis by cladoceran zooplankton. *Daphnia* are clearly able to ingest colloidal precipitates of ferric hydroxide and addition of iron compounds has been shown to increase haemoglobin production in low oxygen conditions (Gellis & Clark, 1935; Fox & Phear, 1953; Hoshi & Kobayashi, 1972). In laboratory cultures and in nature, iron in the water column typically sinks as fine precipitates of ferrous and ferric hydroxide (Fox & Phear, 1953; Wetzel, 1975). Browsing by *Daphnia* on the bottoms of ponds or lakes may increase their access to a source of inorganic iron less available through feeding activity higher in the water column.

Haemoglobin content and habitat differences

Based on patterns of oxygen availability in the ponds, the results of my experiment are surprising. In late summer, *Daphnia* in GR Pond have no oxygenated refuge and must survive nearly anoxic conditions in order to complete the production of resting eggs. *Daphnia* in Rash Pond, however, are capable of visiting surface waters where oxygen content is high. It is reasonable to expect that haemoglobin production would be greater in GR Pond *Daphnia*. However, Rash Pond *Daphnia* surpassed GR Pond *Daphnia* in haemoglobin synthesis, in both low and high oxygen (control) conditions. This suggests that a difference, perhaps genetically based, may exist in the basal level of haemoglobin in *D. pulex* from the two localities (Fox, 1948; Green, 1956; Heisey & Porter, 1977). What difference, other than oxygen regime, might underlie the differences in haemoglobin production by *Daphnia* from GR Pond and Rash Pond?

Visual predation may have selected against high basal levels of haemoglobin in GR Pond. In summer, GR Pond supports a population of salamander larvae (*Ambystoma*) which prey visually on *Daphnia* (Brambilla, 1980). Rash Pond, however, contains no visual predators. In many cases, haemoglobin content in *Daphnia* may be directly related to visual predation. Vertebrate predators detect red *Daphnia* more quickly and at greater distances than pale

Daphnia (Vinyard & O'Brien, 1975). Landon & Stasiak (1983) suggested that the absence of visual predators in Arco Lake allowed *D. pulex* to maintain a relatively high basal haemoglobin level above the thermocline.

In general, visual predation tends to exclude large zooplankton from lakes and ponds (Brooks, 1968; Brooks & Dodson, 1965; Dodson, 1970; Kerfoot, 1974; Wells, 1970). Predation experiments by Brambilla (1980) indicate that salamander larvae prefer the largest *D. pulex* available. The fact that GR Pond *Daphnia* were significantly smaller than Rash Pond *Daphnia* supports the suggestion that visual predation is an important selective factor in GR Pond.

The results of this experiment indicate that when races of the same species of *Daphnia* differ in the ability to produce haemoglobin in low oxygen conditions, the differences will not necessarily correspond to patterns of oxygen availability in nature. Habitat variables (such as oxygen supply, temperature, and visual predation) may interact in complex ways with physiological variables (such as metabolic rate) in determining the extent to which *Daphnia* will synthesize haemoglobin. Additionally, it is not clear what role genetic differences, perhaps involving the mechanism for haemoglobin induction, may play in regulating this biosynthetic trait.

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