Survival of Cloeon dipterum (Ephemeroptera) larvae under anoxic conditions in winter

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Larvae of *Cloeon dipterum* L. are commonly found in ponds. In Sweden, ponds, especially small ones, are anoxic for 3–4 months in winter. In laboratory experiments, about 50% of larvae survived 130 d of anoxia at 0°C; the most resistant surviving 155 d. Undissociated H_2S (1.4 mg l^{-1}) in the anoxic water did not reduce survival. Severely starved larvae survived anoxia for only 16 d (LT_{50}). Probably larvae survive anoxia by using an anaerobic metabolism. The larvae are facultative anaerobes and will use oxygen if it is available in winter. Resistance to anoxia is a necessary prerequisite for the occurrence of the species in small ponds in regions with long and cold winters.

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Личинки *Cloeon dipterum* L. обычно встречаются в прудах. В Швеции, особенно в маленьких прудах, устанавливается бескислородный режим в течение 3–4 зимних месяцев. В лабораторных условиях около 50% личинок переносили бескислородный режим при 0° до 130 дней, а большинство выживало до 155 дней. Недиссоциированный H_2S (1,4 мг $л^{-1}$) в бескислородной воде не снижал устойчивости личинок. Сильно голодавшие личинки выдерживали бескислородный режим лишь 16 дней (LT_{50}). Очевидно личинки выживают в бескислородных условиях с помощью анаэробного дыхания. Личинки являются факультативно анаэробными и могут использовать кислород зимой, если он доступен. Устойчивость к бескислородным условиям – необходимая предпосылка для возможности расселения этого вида в маленьких прудах в районах с длинной и холодной зимой.

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1. Introduction

Small ponds frequently become anoxic and contain H_2S during winter when they are covered by ice (Lindeman 1942, Elgmork 1959, Kühlmann 1960, Sikorowa 1968). Only specially adapted animals can survive long periods under such conditions. But not a great deal is known about survival mechanisms of overwintering fauna in small ice-covered ponds (Elgmork 1959, Danks 1971, Sawchyn and Gillott 1975).

Larvae of the mayfly *Cloeon dipterum* L. commonly develop in small ponds. In Sweden there is only one generation per year. Adults emerge in June and the larvae live in the ponds from August to June.

In association with oxygen consumption measurements on *Cloeon* larvae (Nagell 1973, 1974, 1975), a laboratory investigation of the resistance of larvae to anoxia was initiated. Very high resistance to anoxia at 0°C was observed in preliminary experiments for winter larvae but not for summer larvae. On the basis of this observation the following experiments were performed. A study was also carried out on the environmental conditions and behaviour of larvae in small ponds in winter (Nagell 1977).

The following four aspects were investigated: (1) Resistance to anoxia of larvae collected in summer; (2) Resistance to anoxia of larvae which, during the autumn, had been prepared under natural conditions for overwintering; (3) Resistance of larvae, prepared to overwinter like those above, to anoxia combined with low concentrations of H_2S; (4) Resistance to anoxia of larvae prepared to overwinter which had been severely starved before the experiment.

2. Material and methods

2.1. Experiments performed

The aspects outlined above were studied in a series of experiments as shown in the following scheme. The numbers in the scheme refer to the curves of Fig. 2.

	Autumn larvae		Summer larvae
	Not starved before experiment	Starved before experiment	Not starved before experiment
Anoxia	2,3	8	no curve
Anoxia and H_2S . . .	4,5	–	–
Aerated	6,7	9	no curve

There were 170 larvae in the experiments of curve 2, 150 for curve 4, and 100 in the remaining experiments. Information of the chemical conditions of the experiments is given in Tab. 2.

2.2. Experimental larvae

Larvae were collected from the same pond near Uppsala in central Sweden. The pond was situated in a mixed forest mainly of conifers and was 5 × 7 m and 1.5 m deep. Summer larvae were collected at the beginning

of September in water at 14°C and acclimated to 1°C for one week. They were about 4 mm long and most were in stage L III as defined by Cianciara (1977). Autumn larvae ready to overwinter were collected in November in water at about 2°C just before the pond was frozen over. They were about 6 mm long and most were in stage L VI. They had begun their period of "no growth" (Bretschko 1965) and had ceased eating. Autumn larvae were acclimated to 1°C over one week. The severely starved larvae used in some experiments were kept without food at 1°C in aerated water for 20 wk before use.

2.3. Experimental method

Larvae were kept in 500 ml Erlenmeyer flasks fitted with glass stoppers. The underside of the stopper was spherical and penetrated by a 1 mm diameter capillary. This allowed a rapid and bubble-free closing of the flask. After temperature equilibration the capillaries were sealed by slipping rubber caps over their free outer ends. Four stoppers were constructed to permit in situ measurements of redox potential and were provided with a thin agar salt bridge and a 20 × 1 mm platinum electrode. Larvae could cling to pieces of cotton netting placed at the bottom of the flasks.

Only 10 larvae were kept in each flask to reduce the risk of poisoning by dead decomposing larvae. No food was provided as it was observed that larvae did not eat while overwintering in nature. Larvae were examined about once a week in their flasks under strong illumination. Flasks were opened only when all 10 larvae were dead. The criterion of death was that larvae had come to rest on their sides or backs and showed no movement of their legs or antennae. About 7 d after death the larvae showed signs of decomposition.

Anoxia in the flasks was obtained as follows: Flasks were filled with lake water (conductivity = 390 μs, 20°C), bubbled with nitrogen for 5 min and stoppered. A solution of 20 mg glucose in 0.5 ml water was injected with a hypodermic needle through the capillary and the flasks were immersed in water at 0.0°C for one week. Glucose was added to stimulate respiration of bacteria in the water and thereby eliminate the last traces of oxygen. The establishment of anoxic conditions was controlled in a number of test flasks by injection of Winkler reagents. When no oxygen was detected, the larvae, in a minimum volume of anoxic water (0.5 ml), were put into the flasks under nitrogen. Flasks were stoppered and placed in the water bath. Fe^{2+} ions added in two anoxia experiments (11.2 mg l^{-1}, see Tab. i) bound all H_2S that was formed. At this concentration Fe^{2+} ions quantitatively precipitate sulfide ions to solid FeS.

In two experiments (curves 3 and 5) anoxic water was obtained directly through a tube inserted below the ice of an anoxic pond. The water (conductivity = 340 μs, 20°C) was pumped out and transferred, without contact with air, into flasks filled with nitrogen.

Tab. 1. Concentrations at the end of the experiments of total Fe, total sulfide ($n = 6$), undissociated H_2S ($n = 6$) and pH (median value, $n = 6$). The total sulfide values also include the portion of S bound as FeS.

Experiment (Curve Fig. 2)	Fe(mg l^{-1})	Total sulfide (mg l^{-1})	H₂S (mg l^{-1})	pH start	end
2	0.1	none	none	7.2	6.8
3	1.6 + 11.2	3.3 ± 1.0	trace	7.1	6.8
4	0.1	3.1 ± 0.5	1.4 ± 0.2	7.3	6.8
5	1.6	3.3 ± 0.4	1.1 ± 0.2	7.1	6.8
8	0.1 + 11.2	2.6 ± 0.8	trace	7.2	7.0

Flasks were maintained at 0.0°C by placing them in water and crushed ice in an insulated, darkened container. A stream of air bubbles was used to slowly circulate the water. The container was placed in a constant temperature room at 2°C.

2.4. Statistical treatment

A nonparametric statistical test was used to determine whether the results of experiments with non-starved autumn larvae were significantly different, i.e. experiments in anoxia, in anoxia combined with H₂S, and in aerated water. Statistically the experiments were designed to include several flasks, usually ten, so as to obtain a dispersion of values. Fig. 2 shows the results condensed as mean distributions. When determining whether these were significantly different, however, all individual distributions of the flasks were used.

Significance was tested by a multiple comparison procedure based on Kruskal-Wallis rank sums (Hollander and Wolfe 1973). The lethal time value used for ranking was a trimmed mean value; calculated from the estimated times (t_i) of death of the larvae that died as number 3 to number 7 in each flask. Thus extreme values are excluded.

$$m_i = \frac{t_{i_3} + t_{i_4} + t_{i_5} + t_{i_6} + t_{i_7}}{5}$$

Times of death (t_i, days) were estimated by interpolation, since the flasks were only observed once a week. When less than 7 larvae died during an experiment it was not possible to calculate the m_i value. Instead, the t_{i_5} value was used as a rank criterion. In a few cases in experiments 6 and 7, where only 2 or 3 larvae died, t_{i_3} or even t_{i_2} values were used for ranking.

3. Results

3.1. Conditions in the experimental flasks and in natural ponds

Tab. 1 shows the concentrations of Fe, total sulfide, H₂S and pH at the end of the experiments. Concentrations of total sulfide were similar in different experiments. Addition of Fe²⁺ did not seem to inhibit formation of sulfide. The most toxic of the sulfur compounds

was probably undissociated H₂S. Its molecule is small and uncharged and can penetrate the cuticle more easily than the corresponding ions.

Sulfide was not formed in two of the anoxia experiments (curves 2 and 8). Possibly H₂S-producing bacteria were not present and other anaerobic bacteria predominated.

The redox potential first declined and then gradually stabilized at about –240 mV (Fig. 1). In the flasks with added Fe²⁺ ions there was an initial delay but the potential finally stabilized at about –240 mV.

Measurements were also made in some *Cloeon*-inhabiting ponds near Uppsala. For comparison between the conditions in the experiments and in nature, a few values are given in Tab. 2. Values for the laboratory experiments are in close agreement with those for the ponds.

3.2. The survival of the larvae

Larvae were mostly motionless in anoxia, but were not torpid. When irritated by tapping on the flasks the larvae crawled or swam away. Practically no gill movements were observed during the many hours of observation.

All summer larvae were dead within 40 h of anoxia at 0°C. Thus summer larvae were not very resistant to anoxia. All 100 summer larvae survived in the aerated control experiment.

Fig. 2 shows the results of the other survival experiments. Curves 2, 4, 3 and 5 show a very high resistance
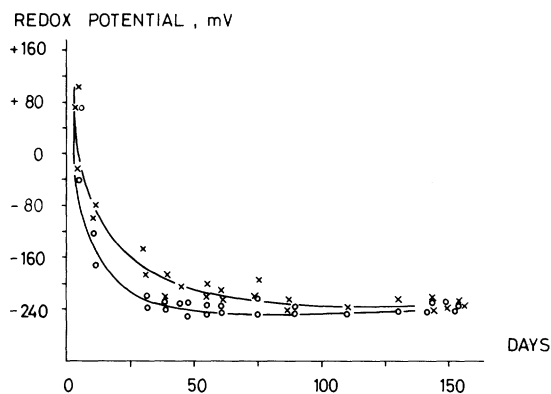

Fig. 1. Redox potentials measured in two flasks with Fe²⁺ added (crosses) and in two flasks without Fe²⁺ (circles).

Tab. 2. Concentrations of total Fe, total sulfide and undissociated H_2S at the end of winter, and of pH both at the end and the beginning of winter in four *Cloeon* inhabiting ponds near Uppsala. At the end of the winter the redox potential was -150 to -200 mV.

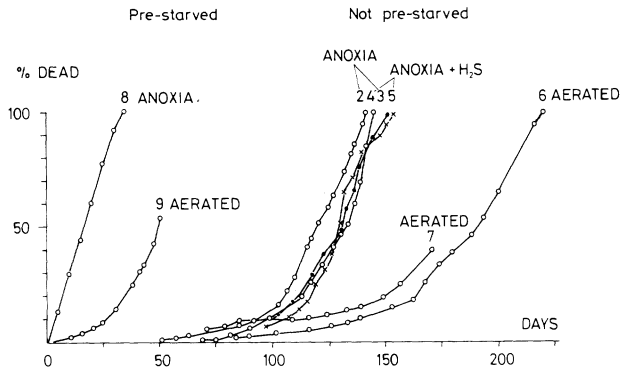| Pond	Fe (mg l^{-1})	Total sulfide (mg l^{-1})	H_2S (mg l^{-1})	pH start	pH end
A	7.5	2.2	1.3	6.5	5.7
B	4.3	0.5	0.1	6.5	6.2
C	4.2	0.8	0.05	6.8	6.3
D	6.1	1.0	0.2	7.4	6.9

Fig. 2. Survival vurves of experiments, with year of measurements in brackets. Curve 2 (1972): No H_2S present. 3 (1974): All H_2S bound as FeS. 4 (1973) and 5 (1974): Undissociated H_2S present. 6 (1972) and 7 (1974): Control experiments in aerated water. 8 (1972): Larvae starved 5 months before the experiment, all H_2S bound as FeS. 9 (1972): Control experiment to 8 in aerated water.

to anoxia in those larvae about to overwinter. The four curves combined have a median lethal time (LT_{50}) of about 130 d. LT_{50} values for these curves are remarkably similar for the three years 1972–74.

Fig. 3 shows the results of statistical comparisons between curves 2 to 7. There is no significant difference between curves 2 to 5, which shows that H_2S at these concentrations did not influence survival. But strictly, comparison should be restriced to larvae from the same autumn population, i.e. between curves 3 and 5.

Curves 2 to 5 (anoxia) differ significantly from curves 6 and 7 (aerated water), except for the comparison between curves 7 and 4. But this may be due to the fact that experiment 7 was ended too soon: curve 7 in Fig. 2 is too short for a reliable ranking.

Curves 6 and 7 (aerated water) do not differ significantly. The LT_{50} of them is about 185 d. The difference between the LT_{50} of larvae in anoxia and in aerated water is thus about 55 d.

The correlation between severe pre-starvation and lowered resistance to anoxia is shown in the difference between curves 8 and 2–5. The LT_{50} for pre-starved larvae is only 16 d.

Parts of the larvae taken from the ponds during winter were violet in colour, especially the gills and between the abdominal segments. This was not due to H_2S because it also appeared in the H_2S-free experiment.

4. Discussion

In this study it was essential that anoxia was maintained in the flasks. All measurements and observations, including redox measurements (Fig. 1), show that this was attained.

The chemical and physical parameters during the experiments were similar to those in natural ponds (Tabs 1, 2). Thus resistance is comparable to that of larvae in the natural environment. This was confirmed in a survival experiment carried out in a pond where larvae had spent 138 d of anoxia at 0°C. During this time 82% of the larvae died (Nagell 1977), a value which is within the limits of curves 2–5.

The results demonstrate clearly that summer larvae are only slightly resistant to anoxia, but larvae prepared to overwinter are very resistant.

Resistance is developed during autumn. Factors which are known to induce adaptations in insects for the winter are low temperature and short daylength (Danilevskii 1965). In the laboratory *Cloeon* larvae become resistant after acclimation for six weeks at 1°C and a daylength of 12 h. Acclimation for only one week does not induce resistance to anoxia, nor does six weeks acclimation at 10°C under similar conditions (Nagell and Fagerström, unpubl).

According to the definition given by Mansingh (1971) the overwintering larvae are in a state of diapause: they

Curve	2a	2b	3	4	5	6	7
2a							
2b	2						
3	14	16					
4	21	23	7				
5	15	17	05	7			
6	(50)	(52)	(36)	(29)	(35)		
7	(45)	(47)	(30)	23	(30)	5	

A

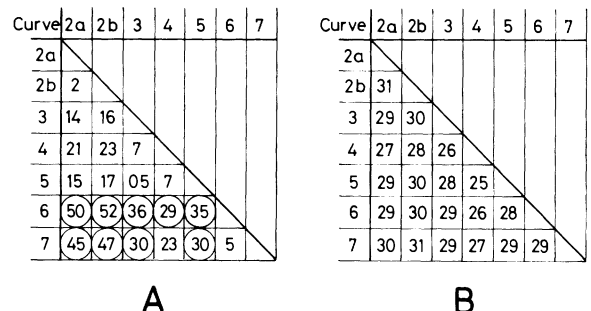| Curve	2a	2b	3	4	5	6	7
2a							
2b	31						
3	29	30					
4	27	28	26				
5	29	30	28	25			
6	29	30	29	26	28		
7	30	31	29	27	29	29	

B

Fig. 3. Results of pair-wise comparisons between curves based on Kruskal-Wallis rank sums. The difference between two curves is significant if certain critical values are exceeded. A: Absolute differences in mean ranks. Circled values are significant. B: Critical values of absolute differences in mean ranks at 5% significance level. Curve 2 of Fig. 2. is divided into two parts in the test to correspond to experiments with 100 and 70 larvae.

do not eat and show enhanced resistance against an adverse environmental condition.

Resistance to anoxia is probably associated with an anaerobic metabolism. During overwintering the larvae are attached to the underside of the ice where oxygen-rich melt water is sometimes available. Experiments have shown that larvae will use this oxygen (Nagell 1977) and so while the larvae are overwintering they are facultative anaerobes. An anaerobic metabolism vital for the survival of an overwintering beetle has been reported by Conradi-Larsen and Sømme (1973).

Anaerobic glycolysis is the best understood energy-yielding anaerobic pathway. It is less efficient in yielding ATP than aerobic processes. Probably animals that sustain anoxia for long periods do not utilize this pathway in its simplest form. More efficient pathways for such animals have been discussed by Hochachka et al. (1973); they suggest the simultaneous catabolism of carbohydrate and amino acids as a means by which the ATP yield can be increased without seriously reducing the cell. However, the coupling between these two processes has not yet been explored in detail.

The LT_{50} of larvae with access to oxygen is about 50% greater than for larvae under anoxic concitions. This is not surprising considering the higher efficiency of aerobic metabolism and which also includes catabolism of compounds that cannot be used under anaerobic conditions, e.g. fat.

Sixteen weeks exposure to H_2S did not affect survival, even though it is very toxic and has an effect similar to cyanide. Few studies have been made on the toxicity of H_2S to macroscopic aquatic animals. Adelman and Smith (1970) have reviewed the literature for fish. Most studies on aquatic invertebrates have been reviewed by Oseid and Smith (1974). In the experimental part of their paper they show that larvae of *Hexagenia limbata* (Serville) were more resistant to H_2S during winter. However the experiments are not directly comparable with mine, because they were performed at 10° and 15°C. The crucian carp *Carassius carassius* L. is very resistant to H_2S in winter (Blăzka 1958).

Resistance to H_2S and anoxia are probably not unusual among animals living in environments where anoxia is of regular occurrence e.g. in the profundal zone of highly productive lakes, in very deep lakes or in small rich ponds. Interesting is how these animals maintain their internal redox balance for long periods under the influence of such a strong reducing agent as H_2S. Severe starvation considerably reduces resistance to anoxia, because the stored energy reserves are reduced and are therefore not available for anaerobic metabolism.

In summary, the *Cloeon* larvae survive anoxia during winter by anaerobic metabolism of substances which are probably stored during the autumn. The potential for anaerobic metabolism is aquired with the falling water temperatures and shortening days of the autumn. Larvae are facultative anaerobes while overwintering because they will use oxygen when it becomes available

(e.g. from melt water). This anaerobic metabolism is most likely a necessary prerequisite for the species to inhabit small ponds in northern temperate regions.

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