

## Overwintering at low oxygen concentrations in the mayfly *Leptophlebia vespertina*

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Brittain, J. E. and Nagell, B. 1981. Overwintering at low oxygen concentrations in the mayfly *Leptophlebia vespertina*. – Oikos 36: 45–50.

The survival of *L. vespertina* (L.) nymphs in three humic forest ponds in southern Norway was investigated. The main water bodies of the ponds were apparently anoxic for part of the winter due to ice cover and organic decomposition. However, laboratory experiments showed that the nymphs are unable to survive long-term anoxia even at low temperatures. Their oxygen level for 95% sustained survival at 0.3°C is 0.4–0.5 mg O<sub>2</sub> l<sup>-1</sup>. Although most of the water body is anoxic for several months, oxygen is present in the shore areas and also, during thaws, in the water layer immediately under ice. Laboratory experiments demonstrated that the nymphs are both positively phototactic and negatively thermotactic in low oxygen concentrations. Coupled with an ability to withstand short-term anoxia, this results in a movement of nymphs into the shore areas, where temperatures approach 0°C, percolation of meltwater containing oxygen occurs and food is available. Under such conditions the nymphs can survive and even grow slowly.

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Исследовали выживаемость нимф *L. vespertina* (L.) в трех лесных, богатых органикой озерцах в южной Норвегии. Основная часть воды в озерцах несомненно анаэробна в течение части зимнего периода вследствие наличия ледового покрова и развития процессов разложения. Однако, лабораторные опыты показали, что нимфы неспособны переносить длительные анаэробные условия, даже при низких температурах. Содержание кислорода при 95% выживаемости и 0,3°C составляло 0,4–0,5 мг O<sub>2</sub>/л. Хотя основная масса воды анаэробна в течение нескольких месяцев, кислород имеется в прибрежных участках и также во время оттепели в слое воды непосредственно под льдом. Лабораторные опыты показали, что нимфы имеют положительный фототаксис и отрицательный термотаксис при низкой концентрации кислорода. Обладая способностью к перенесению кратковременных анаэробных условий, нимфы в результате этого мигрируют в прибрежные участки, где температура приближается к 0°C и имеет место перколяция талой воды, содержащей кислород, а также имеется доступная пища. При этих условиях нимфы могут жить и даже медленно расти.

Accepted 27 March 1980

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## 1. Introduction

The presence of the mayfly, *Leptophlebia vespertina* (L.), in three humic ponds with winter oxygen depletion was first observed by Dr K. Elgmork during the latter part of the 1970–71 winter when nymphs occurred in plankton samples. Further investigations in the ponds confirmed that *L. vespertina* was a constant member of the aquatic fauna (Brittain 1974) and that the main water masses were anoxic in at least one pond during winter ice cover. Mayflies are generally considered to be unable to survive anoxic conditions, although recently Nagell (1977a) has demonstrated survival of the mayfly *Cloëon dipterum* under anoxia, both in the field and in the laboratory. Earlier studies of respiration in *L. vespertina* by Fox et al. (1937) and Kjellberg (1972) were not concerned with tolerance to anoxia. Thus the aim of the present investigation was to ascertain whether *L. vespertina* can survive anoxia at temperatures approaching 0°C and if not how it manages to maintain populations in habitats apparently anoxic for several months.

## 2. Habitat descriptions

The three water bodies, cabin ponds A and B and Lille Damtjern (subsequently referred to as CA, CB and CC) are situated in the Vassfaret area about 100 km NW of Oslo, Norway (UTM NN 252083). Ponds CA and CB are adjacent while CC is 150 m distant. They are all surrounded by peat bog and mixed forest, mainly of Norway spruce. Hydrographical data are given in Tab. 1. The largest pond CC has a small outflow; otherwise water exchange is mainly by seepage to and from the surrounding bogs. CA and CB are the same as ponds 1 and 2 in Nagell and Brittain (1977). The ponds are unpolluted except through precipitation (Elgmork et al. 1973). They are usually ice covered from October until May. No fish are present.

## 3. Materials and methods

### 3.1. Experiments

Four experiments were carried out to investigate if the nymphs were able to develop resistance to anoxia under

certain conditions. In experiment 1 the effect of long acclimation to 0.1°C and a daily reduction in the photoperiod was studied. Experiment 2 was based on nymphs acclimated to low oxygen concentrations for a long period. In experiment 3 the effect of starvation was studied in nymphs otherwise treated as in experiment 1. Experiment 4 was carried out with nymphs collected during the winter from under the ice in pond CC, to see if the nymphs had developed any resistance under natural conditions.

It was of interest to determine the oxygen level for 95% sustained survival of the nymphs, i.e. the lowest oxygen concentration at which 95% of the nymphs would survive continuously. This was carried out with nymphs acclimated as in experiment 1.

To obtain an idea of the nymphs' ability to move into areas favourable for survival during overwintering, a series of tests were made of their phototactic and thermotactic responses under high and low oxygen concentrations.

### 3.2. Nymphs

All nymphs were collected from pond CC during October, except those used in experiment 4 which were collected during February. Nymphs used in experiments on resistance to anoxia were acclimated to 0.1°C for 11 wk with a daily photoperiod decreasing from 9h to 5h during that time. The nymphs were fed prior to experiments 1 and 2. In experiment 2 the nymphs were subjected to oxygen concentrations which varied daily between 1.5 and 0.4 mg l<sup>-1</sup> during the last 4 wk of acclimation. In experiment 3 the nymphs were starved during the last 8 wk. In experiment 4 they were not acclimated. In the thermotactic and phototactic experiments the nymphs were acclimated at 0.1°C for 8 wk and thereafter at 2.4°C for 3 wk. The body lengths, excluding cerci, of the nymphs were 5–7 mm in experiment 4 and 6–8 mm in the remainder.

### 3.3. Methods

Oxygen sampling and analyses were carried out mainly as described in Nagell and Brittain (1977). Samples were taken through a 6 mm diameter brass tube 2 m long and with a stopcock at the top. The upper 1.5 m were insulated with polystyrene. The bottom 5 cm was

Tab. 1. Hydrographical data from three Vassfaret localities. Chemical data are taken from Brittain (1974) and are averages of values recorded on five occasions throughout the ice-free period.

	Altitude (m a.s.l.)	Area (m <sup>2</sup> )	Max. depth (m)	Conductivity (μS cm <sup>-1</sup> )	pH	KMnO <sub>4</sub> (mg l <sup>-1</sup> )	Ca <sup>2+</sup> (mg l <sup>-1</sup> )
Cabin Pond A (CA) .....	795	1800	3.0	10.0	5.1	42.9	0.9
Cabin Pond B (CB) .....	795	6100	2.1	11.8	4.8	38.3	0.7
Lille Damtjern (CC) .....	792	20000	1.5	11.5	5.3	23.9	0.9

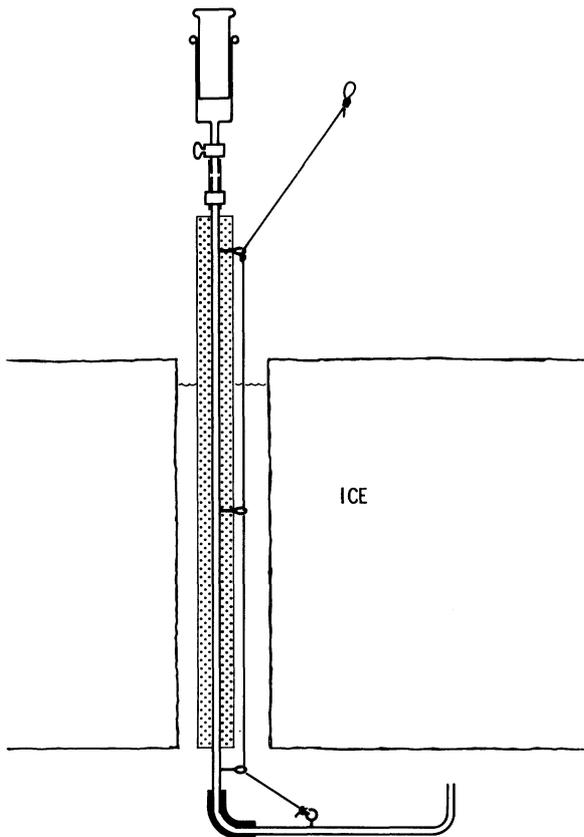


Fig. 1. The field water sampler in the sampling position. The insulation around the brass tube is indicated by the dotted shading.

bent at right angles permitting sampling from the underside of the ice. The sampling apparatus and method were modified from that figured in Nagell and Brittain (1977) as the sampling tube was not fixed in the ice. The tube could be bent at a joint of PVC tubing by a wire alongside the tube (Fig. 1). At first the whole tube was held straight and inserted through a 10 cm hole bored in the ice. Then the lower part (50 cm long) was bent upwards at the joint into a horizontal position. In that way samples could be taken in undisturbed water just below the ice some 40 cm from the actual hole. Samples were drawn up into a 100 ml all-glass syringe. To clean the system 100 ml was first withdrawn prior to taking a bubble-free 75 ml sample. Winkler reagents were immediately added into the syringe. Thus the sample was preserved without any contact with air. Samples for sulphide analyses (Methylene blue method – Anon. 1971) were also preserved directly in the syringe.

Resistance to anoxia was studied in 500 ml flasks with nine nymphs in each flask and seven flasks per experiment. The flasks were fitted with ground-glass stoppers which were closed bubble-free. The nymphs could cling to pieces of cotton netting. Anoxic water was obtained by bubbling nitrogen into a container closed to the air

for 30 min. Bacterial removal of any trace of oxygen was induced by adding 20 mg glucose  $l^{-1}$ . The water was then forced by nitrogen into previously nitrogen filled flasks. The flasks were stoppered and immersed in water for one week at 0.1°C. By experience the flasks were now known to be anoxic, although the absence of oxygen was controlled in three flasks. The nymphs were then added in 0.5 ml of nitrogen bubbled water and the flasks were closed bubble free. The nymphs were subjected to anoxia for 24 h at 0.1°C and then allowed to recover for 72 h in oxygen rich water at the same temperature. Further details are given in Nagell (1977a).

The investigation of the oxygen level for sustained survival was carried out in an apparatus described by Nagell and Larshammar (1981). Briefly, the apparatus consists of two containers each with 80 l dechlorinated Stockholm tap water. The oxygen concentration of the water in them was kept constant, one at very low level and the other at a low level, by slowly bubbling through mixtures of nitrogen and air. Constant gas mixing was achieved by a sensitive manometric device connected to each container. Without bubbling, the oxygen concentration in the water diminished due to bacterial respiration. Each 8 h a 10 s pulse of carbon dioxide was given to keep the pH of the water at a suitable level. The oxygen concentration was kept constant to within  $\pm 0.01$  mg  $l^{-1}$  and the pH between 7.25 and 7.10 during the 180 h and 140 h the experiments lasted. The water in the containers was forced out by a slight constantly maintained pressure into a system of precision capillaries fused together to mix the water from the containers in constant proportions. The system gave five different oxygen concentrations: The low level, the very low level and in between mixtures 1:3, 1:1, 3:1. The water flow through each outlet was 100 ml  $h^{-1}$ . A small chamber (vol. 10 ml) in which 10 nymphs were exposed to the water was attached to each outlet. They were given old apple leaves as food and substrate. Survival was measured at intervals by careful examination of the larvae without opening the chambers. For each exposure time the oxygen concentration necessary for 95% survival was estimated by linear log-probit regression (Bliss 1935) in which the oxygen concentration in each

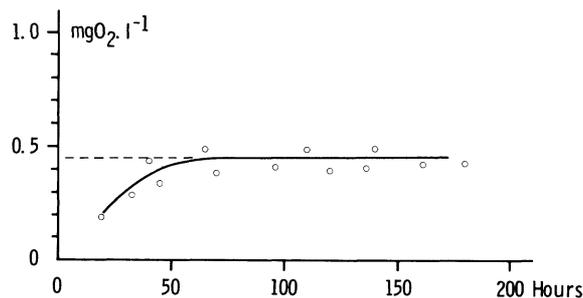


Fig. 2. Oxygen level for 95% sustained survival of *L. vespertina* nymphs at 0.3°C. The mean  $\pm$  S.D. of the values beyond 90 h was  $0.44 \pm 0.04$  ( $n = 7$ ).

of the five chambers was converted to logarithms and percent survival to probits. The values obtained were then plotted against exposure time (Fig. 2).

The apparatus and methods used in the thermotactic and phototactic experiments are described in Nagell (1977b). The experiments were carried out in a temperature and light gradient at both high and low oxygen concentrations. At low oxygen concentration they were carried out in a perspex tube of length 100 cm and diameter 5 cm. The oxygen concentration at the start of the experiment was  $0.4 \text{ mg l}^{-1}$  and at the finish  $0.3 \text{ mg l}^{-1}$ . The experiment in high oxygen concentration ( $13.5 \text{ mg l}^{-1}$ ) was carried out in an open trough. The trough and the tube were divided into five equal zones, with a temperature gradient as follows:  $4.2^\circ\text{C}$  in zone 1,  $2.6^\circ\text{C}$  in zone 3 and  $1.8^\circ\text{C}$  in zone 5. In the light gradient experiment the temperature was constant at  $2.4^\circ\text{C}$ . The light intensity was 25 lux in zone 1, 4 lux in zone 3 and  $<1 \text{ lux}$  in zone 5. In one of the light gradient experiments an air bubble was introduced into zone 3 after 5 h. The oxygen content of the bubble was sufficient to give an oxygen concentration of  $4.2 \text{ mg l}^{-1}$  in the water volume of zone 3. In each run 50 nymphs were used. They were introduced into the middle of zone 3, kept there for 30 min and then very carefully released.

#### 4. Results

In connection with zooplankton studies, Elgmork, Eie and Halvorsen (pers. comm.) recorded oxygen and temperature at 0.5 m intervals over the deepest parts of the three ponds from October 1973 to March 1975 using a 0.5 l Ruttner sampler. Anoxic conditions occurred in all ponds during winter ice cover. In CA the whole water column was anoxic during February and March. In CB and CC only the water immediately under the ice contained oxygen and on 23 March 1974 oxygen concentrations at 5–10 cm below the ice of these two ponds were  $0.8 \text{ mg l}^{-1}$  (6.2% saturation) and  $1.7 \text{ mg l}^{-1}$  (12.6), respectively. Our own measurements from CB are given in Tab. 2. The data show that on that occasion, when

Tab. 2.  $\text{O}_2$  and  $\text{H}_2\text{S}$  (as total sulphide) concentrations in pond CB on 1 April 1978 immediately under the lake ice at the centre of the pond and at two locations about 1 m from the edge of the pond. Duplicate samples were taken at each location.

	$\text{O}_2$ ( $\text{mg l}^{-1}$ )	$\text{O}_2$ (% saturation)	$\text{H}_2\text{S}$ ( $\text{mg l}^{-1}$ )
Centre	0.0	0.0	1.0
	0.0	0.0	0.6
Edge 1	2.6	19.8	0.0
	2.6	19.8	
Edge 2	1.8	13.4	0.0
	1.6	11.9	

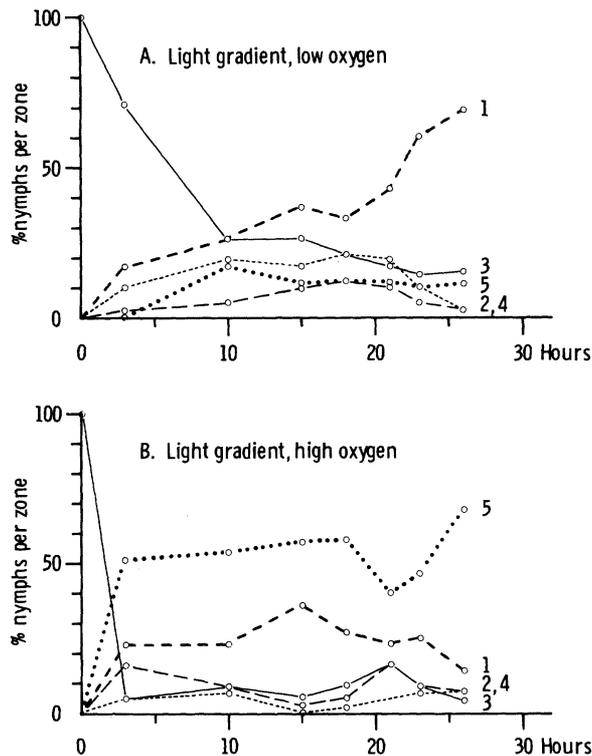


Fig. 3A, B. Movement of *L. vespertina* nymphs in a light gradient. Zone 1 is the brightest at 25 lux and zone 5 the darkest at  $<1 \text{ lux}$ .

there was no thaw, anoxic conditions prevailed at the centre of the pond even just under the ice, but at two sites ca. 1 m from the shore oxygen was present.

No nymphs survived in any of the anoxia experiments. Thus the nymphs are unable to survive 24 h in anoxia, even nymphs taken directly from the pond in February. Therefore, a change to anaerobic metabolism, such as occurs in *Cloëon dipterum* (Nagell 1977a, 1980), does not appear to take place. Nevertheless there is some resistance to shorter periods of anoxia. In fact 21 out of 45 nymphs exposed to anoxia for 8 h at  $0.1^\circ\text{C}$  survived. From Fig. 2 it follows that the oxygen level for 95% sustained survival at  $0.3^\circ\text{C}$  lies between  $0.4$  and  $0.5 \text{ mg O}_2 \text{ l}^{-1}$ . This should be the minimum oxygen concentration at which overwintering is possible.

The results from the phototactic experiment (Fig. 3A, B) showed clearly that the nymphs are positively and negatively phototactic in low and high oxygen concentrations, respectively. The thermotactic experiments (Fig. 4A, B) suggested that they are negatively thermotactic in low oxygen concentrations. The movements of the nymphs in the different experiments indicate that light has a more overriding influence than temperature. From Fig. 5 it can be seen that the air bubble introduced into zone 3 strongly attracted the nymphs.

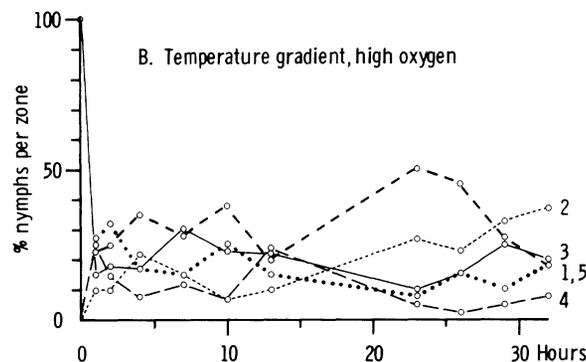
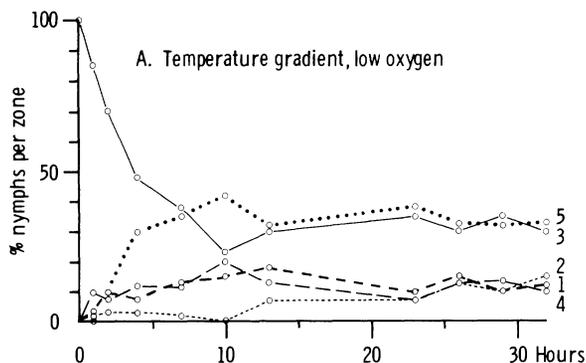


Fig. 4A, B. Movement of *L. vespertina* nymphs in a temperature gradient. Zone 1 is the warmest at 4.2°C and zone 5 the coldest at 1.8°C. Low oxygen = 0.3–0.4 mg l<sup>-1</sup>; high oxygen = 13.5 mg l<sup>-1</sup>.

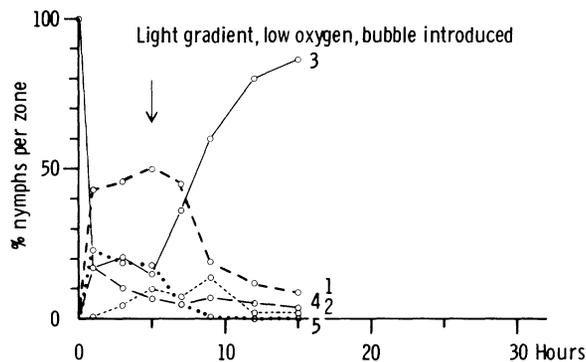


Fig. 5. Movement of *L. vespertina* nymphs in a light gradient. The arrow indicates the introduction of an air bubble in zone 3. Zone 1 is the brightest at 25 lux and zone 5 the darkest at <1 lux. Oxygen concentration, except in zone 3, was 0.3–0.4 mg l<sup>-1</sup>.

## 5. Discussion

The laboratory results clearly showed that *L. vespertina* nymphs are unable to withstand prolonged anoxia even at low temperatures. Therefore there must be areas within all three ponds where there is sufficient oxygen to permit survival, i.e. concentrations greater than

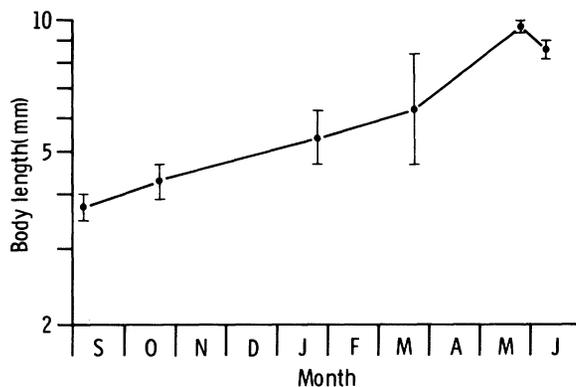


Fig. 6. Growth of *L. vespertina* nymphs in Damtjern (CC). Mean body lengths are given  $\pm 95\%$  confidence limits. Emergence took place during June.

0.4–0.5 mg l<sup>-1</sup> (ca. 4% saturation). In addition, it is reasonable to assume that there are adaptive mechanisms, probably behavioural, which enable the nymphs to find such areas. In the laboratory experiments the nymphs were observed to be surprisingly active at low temperatures even swimming and crawling at 0.1°C. This can also be seen by the rapid increase in numbers in zone 3 after the introduction of an air bubble. Such locomotory ability at low temperatures and their apparently effective gills which function both as respiratory organs and as ventilators (Eastham 1936), together with their ability to withstand short-term anoxia, should give the nymphs a good chance to find oxygen rich areas in the ponds. According to the experiment this takes place primarily according to light intensity, although also with low temperature, as the directing stimulus. Active movement of nymphs towards the shallower, lighter and colder water of the shore zone at the end of the winter has been observed by Kjellberg (1972). Nagell and Brittain (1977) demonstrated percolation of melt water containing oxygen down through minute cracks in the pond ice when temperatures rose above zero, thus producing a thin water layer containing oxygen immediately under the ice. The measurements in CB indicate that there is also a greater chance of there being oxygen near the edge of the ponds, where additional seepage from surrounding terrain probably occurs. Nymphs in CC have the extra possibility of moving out of the pond and into the outflow stream where oxygen concentrations will be higher. In fact the highest densities of *L. vespertina* occur near the outflow of CC. The importance of low temperatures for survival of the nymphs should be stressed. The movement of the nymphs to the region immediately under the ice and the areas near the shore has two advantages. Firstly, the presence of oxygen is more likely in these areas and secondly temperatures are near 0°C instead of 3–4°C in the deeper waters of the ponds, thus reducing oxygen demand. However a position of the nymphs on the underside of the ice is unfavourable due

to the absence of food. The field nymphal growth curve (Fig. 6) shows that the nymphs grow, albeit very slowly, during the winter. This agrees well with observations in the laboratory when the nymphs were being acclimated, as they both consumed food and grew even at 0.1°C. Furthermore the nymphs collected in February contained food in their guts. In the ponds actively swimming nymphs have been taken in holes bored in the ice. This means therefore that no diapause, as defined by Mansingh (1971), is involved in the survival strategy.

Several representatives of other freshwater insect groups, such as Odonata, Coleoptera and Trichoptera, overwinter in these ponds. These species must also have evolved physiological and/or behavioural mechanisms to survive the low oxygen concentrations during the period of winter ice cover.

*Acknowledgements* – We are grateful to Dr K. Elgmork for making us aware of this phenomenon and for the loan of his cabin during the fieldwork. L. M. Sættem gave valuable assistance in the field.

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