Michael Hubbard

Best regards,
Margaret Friesen

EFFECTS OF TEMPERATURE AND COLD STORAGE ON DEVELOPMENT TIME AND VIABILITY OF EGGS OF THE BURROWING MAYFLY *HEXAGENIA RIGIDA* (EPHEMEROPTERA: EPHEMERIDAE)

M.K. FRIESEN, J.F. FLANNAGAN, AND S.G. LAWRENCE
Freshwater Institute, Department of Fisheries and Oceans, Winnipeg, Manitoba R3T 2N6

Abstract

Can. Ent. 111: 665–673 (1979)

Eggs of *Hexagenia rigida* McDunnough transferred directly from 20° to 36°, 32°, 28°, 24°, 20°, 16°, 12°, and 8°C were monitored for start of hatch, hatching rate, success of hatch, and hatching period. Eggs did not hatch at 36°C or 8°C. There was a direct relationship between temperature and start of hatch (from day 7 at 32°C to day 77 at 12°C). Over 90% hatch occurred at all temperatures except 12°C. Hatching periods ranged from about 6 days at the higher temperatures to over 80 days at 16°C and 12°C. Hatching occurred at 36°C when eggs were transferred in increments of 4°C every 2 days.

Storage capabilities of eggs in "early", "middle", and "late" stages of embryonic development were tested at 12°C and 8°C using a direct or stepwise transfer method. Hatching parameters monitored were hatch rate, success of hatch, and hatching period up to 90% hatch of total number of eggs. Middle stage eggs transferred in a stepwise manner could be held at 8°C for up to 52 weeks with least effect on hatching parameters. Middle and late stage eggs could be stored for up to 16 weeks at 8°C using the direct transfer method, and early stage eggs, which were affected most by cold storage, could be held for 2 days at 12°C with minimal effect on hatching parameters. The storage of eggs, shown here to be possible, is potentially useful for providing such material for bioassays throughout the year.

Burrowing mayflies of the genus *Hexagenia* are an important food item for many fish species (Neave 1932; Hoopes 1960). The nymph has been used in bioassays (Carlson 1966; Fremling 1970, 1975; Oseid and Smith 1974; Surber and Bessey 1974), but little work has been done on the egg stage. Eggs of *Hexagenia rigida* McDunnough were found to be convenient to work with as bioassay material in the toxicity testing of a whole effluent where lethal and sublethal effects on the embryonic development and hatching characteristics were monitored (Friesen in prep.). Although viable eggs can be obtained parthenogenetically (Friesen and Flannagan 1976) or using artificial insemination (Friesen in prep.), these sources are unreliable and a method of storing eggs, which are available in large numbers during the brief emergence period, would be useful in providing material for bioassays throughout the year.

Laboratory studies on the eggs of other *Hexagenia* species (Neave 1932; Spieth 1938; Hunt 1951; Flattum 1963; Fremling 1967) and in other mayfly genera (Bohle 1969, 1972; Benech 1972; Elliott 1972, 1978) showed that duration of embryological development is temperature dependent. Hunt (1951) and Flattum (1963) found that the eggs of *H. limbata* could be held at low temperatures for a prolonged period of time with hatch occurring when returned to room temperatures. Flannagan (in press) in a study on the life histories of *H. limbata* and *H. rigida* in Lake Winnipeg, suggests that overwintering may occur in the egg stage when eggs are laid late in the summer.

This paper describes two studies on the effects of temperature on the hatching parameters of eggs of *H. rigida*: the effects of a range of temperatures, and the storage capabilities of eggs at different stages of embryonic development at temperatures near the lower temperature limit for hatching.

Materials and Methods

Eggs were obtained from female imagines collected at the Red River, Winnipeg, Manitoba on 4 and 6 July 1976. Twenty-five females were used for each of the studies. Eggs were dissected into thiosulphate dechlorinated water, mixed and aliquots

put into approximately 50 ml of dechlorinated water in glass Petri dishes. Care was taken to avoid clumping of eggs. Eggs were then exposed to the various temperature treatments (see respective studies below), observations were made at scheduled intervals and numbers of hatched eggs (i.e. egg cases from which nymphs had completely freed themselves) were recorded. Counts were made using a dissecting microscope at ×25 magnification. Observations were stopped when no further hatch was expected, i.e. eggs were either brown or deteriorating internally indicating death. Hatching parameters monitored were: start of hatch, hatching rate (i.e. number of nymphs hatched per day), percentage hatch, and hatching period.

In study I there were three dishes per treatment and in study II there were two dishes per treatment. Number of eggs per dish ranged from 275 to 2082 but was usually between 400 and 1000 eggs per dish. Percentage hatch of total number of eggs per dish was calculated and all other analyses on hatching percentages were performed on arcsin transformed data. Probits were estimated from log-probit graph paper.

Study I. Petri dishes of eggs were transferred to each of the following temperatures (°C)[1]: 36±1°, 32±1°, 28±1°, 24±1°, 20±1°, 16±1.5°, 12±1°, and 8±1.5°. Checks for hatched eggs were made as follows: 20° to 36°—daily for start of hatch, daily for 7 days after first hatch, and then periodically until no further hatch was expected; 16°—at least every 2 days for first hatch and at least every 2 days thereafter until no further hatch was expected; 12°—every 2 days for first hatch, at least every 2 days for 6 weeks thereafter, and then weekly for 5 months; 8°—weekly for 10 months.

In addition, some eggs were gradually transferred from 20° to 28°, 32° and 36° at 4° increments every 2 days. Hatching was monitored as described above.

Study II. Eggs were allowed to develop at 20° to various stages of embryonic development and were then transferred to 12° or 8° directly, or to 8° in steps of 4°. They were returned to 20° (directly or in steps) after various time periods, and hatch was monitored every 2 days till 90% of total number of eggs had hatched. Hatch of eggs incubated only at 20° was used as the control. Embryonic stages tested were: "early" where only yolk was visible, "middle" where the embryo was discernible, and "late" where the appendages and eyespots were distinguishable.

Eggs transferred directly to 12° and 8° were 1/2, 8 1/2, and 12 1/2 days old and are referred to as early, middle, and late stage eggs, respectively. Eggs were held at 12° for 2 days and 8 weeks before being returned directly to 20°. Eggs were held at 8° for 2 days, 16 and 41 weeks before being returned directly to 20°.

Eggs transferred in steps to 8° (4° every 2 days) were 1/2, 6 1/2, and 12 1/2 days old, and are also referred to as early, middle, and late stage eggs, respectively. These eggs were returned to 20° after 8, 16, 41, and 52 weeks in 4° increments every 7 days (eggs stored for 52 weeks were held at 12° for 5 days instead of 7).

Results and Discussion

Study I. Eggs hatched at all temperatures except 36° and 8°, indicating that the upper threshold for hatching lies between 32° and 36° and the lower threshold between 8° and 12°. All hatching parameters monitored, namely, start, hatching rate, percentage hatch, and hatching period were affected by temperature (Fig. 1). Start of hatch was related directly to temperature ranging from first hatch by day 7 at 32° to first hatch by day 77 at 12°. A similar relationship was found for *H. limbata* where first hatch ranged from day 10 at 32° to day 127 at 12°, with no development at 7° (Flattum 1963). Hatching rates at 32° to 20° were similar with hatch nearly

[1] and similarly throughout the study.

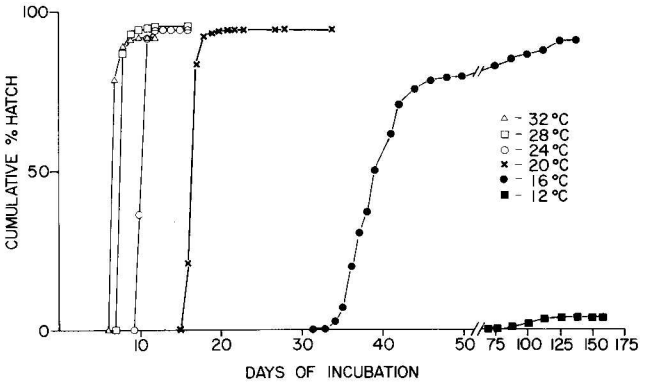


FIG. 1. Cumulative percentage hatch of *H. rigida* eggs versus days of incubation at constant temperatures.

complete within 4 days after first hatch, and were much slower at 16° and 12°. Total hatching periods were shortest at the higher temperatures (6 days at 32°, 28°, and 24°, 23 days at 20°, and over 80 days at 16° and 12°). Longer hatching periods at lower temperatures has also been recorded in *Baëtis rhodani* (Benech 1972; Elliott 1972). The mean total percentage hatch and standard deviation at 32° was 90.5±0.01%, at 28° was 94.8±0.00%, at 24° was 94.1±0.11%, at 20° was 94.6±0.37%, at 16° was 90.5±0.05%, and at 12° was 4.5±0.12%. The reduced percentage hatch at 32°, 16°, and 12° was probably due to the proximity to the respective temperature limits for hatching. Days to first hatch and percentage development per day were plotted against temperature and a hyperbola and sigmoid curve, respectively, were obtained (Fig. 2). Formulae were calculated as in Davidson (1944). These curves are typical of many insect development–temperature relationships and have been discussed by Andrewartha and Birch (1954), Wigglesworth (1965), and Howe (1967). The favourable temperature range for hatching, estimated from the straight portion of the percentage development curve, occurs between 16° and 32°. The temperature of the Red River during peak emergence and egg-laying of *H. rigida* falls near the middle of this range (M.K. Friesen unpub. data) and eggs laid at this time would be expected to hatch in the same season provided temperature is the only determining factor.

In eggs transferred stepwise to 36°, 32°, and 28°, start and hatching rates were similar to those recorded at constant temperatures above 20° (Fig. 3). Final percentage hatch at 36° was 73.3±0.54%, at 32° was 93.6±0.01%, and at 28° was 93.9±0.03%. This apparent elevation in the upper temperature limit in stepwise transferred eggs and increase in success of hatch at 32° may have occurred because eggs had undergone considerable development before reaching 36° and the exposure period to the unfavourable temperature was therefore relatively brief. Healthy development for short intervals at temperatures which are harmful under continuous exposure has been recorded in other insects (Andrewartha and Birch 1954). The increase in success hatch may also have been due to acclimation (Prosser and Brown 1965) during the stepwise transfer and/or transfer of eggs when they were in a less sensitive point of embryonic development.

Study II. In eggs incubated only at 20°, 90% of total number of eggs hatched within 4 days after first hatch (final hatch was 93.8%). The hatching pattern at 20° was a steep sigmoid curve when cumulative percentage hatch of total number of eggs was graphed against time (Fig. 4), and was a straight line when cumulative percentage of "total" eggs hatched (see below) converted to probits was plotted

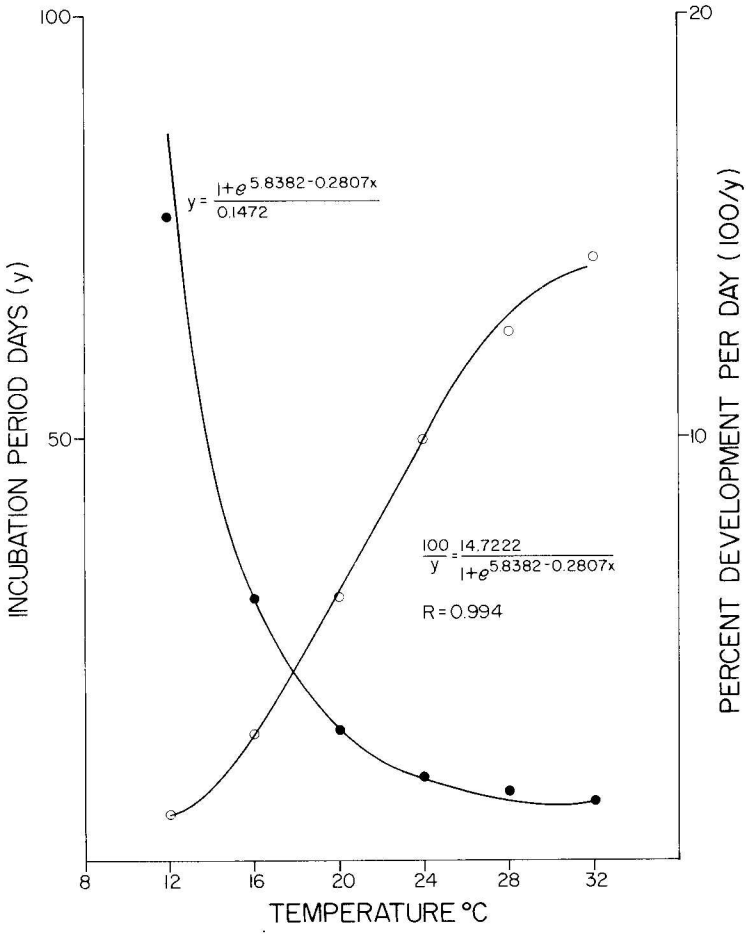
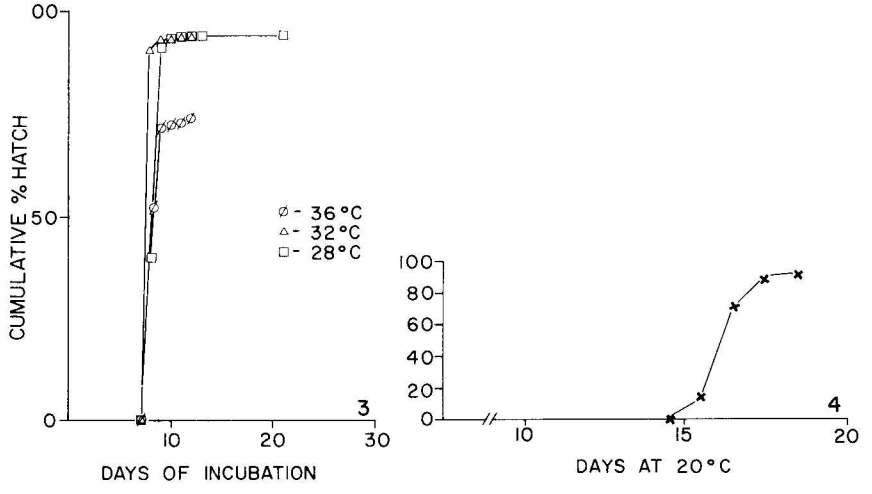


FIG. 2. Relationship between temperature and development of eggs of *H. rigida* calculated using day to first hatch.



FIGS. 3-4. 3, cumulative percentage hatch versus days of incubation of *H. rigida* eggs transferred stepwise to 28°, 32°, and 36°C. 4, cumulative percentage hatch of *H. rigida* eggs versus days of incubation at 20°C.

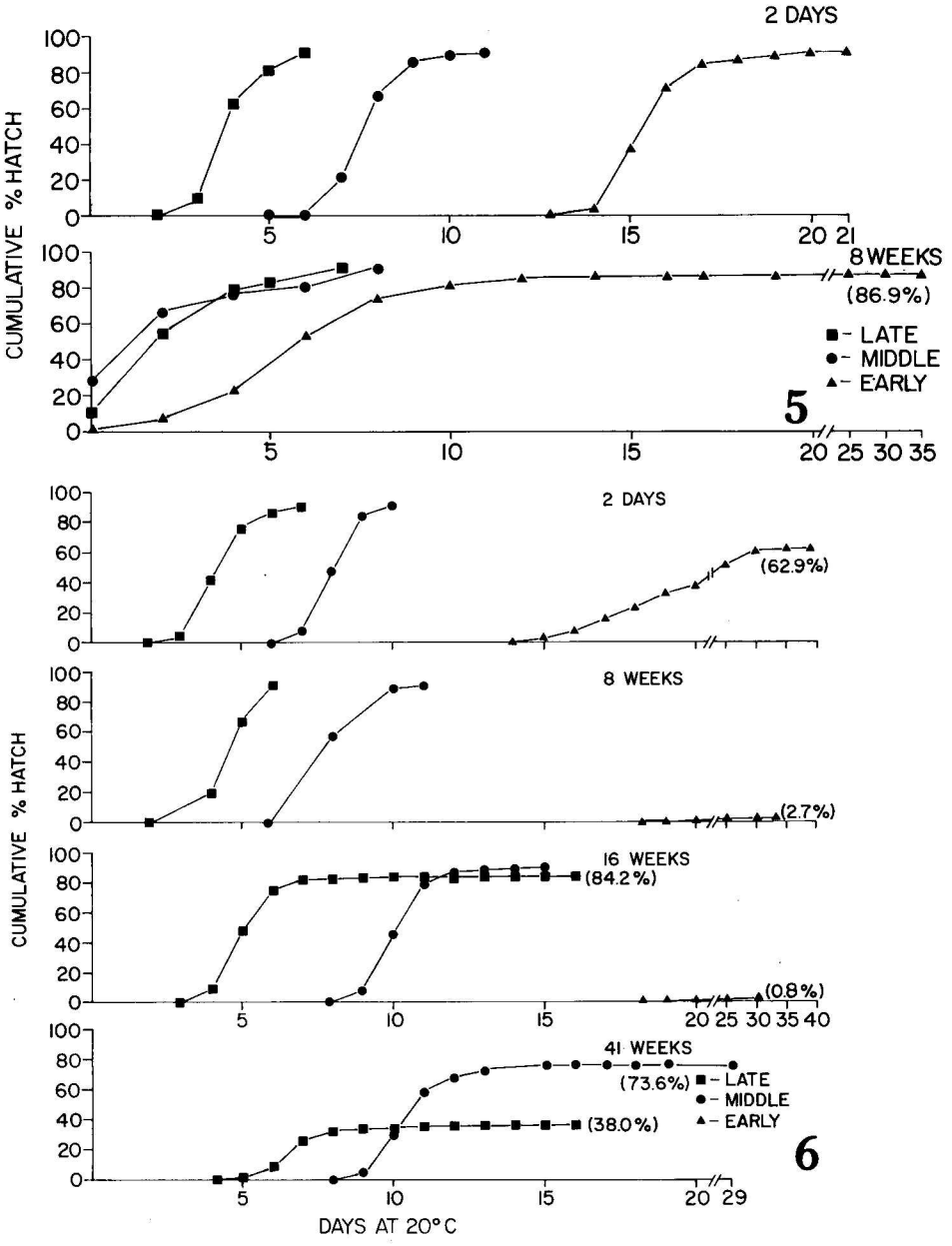
Table I. Goodness of fit values and F test values comparing slopes of regression lines (determined by plotting percentage hatch of "total" hatch transformed to probits versus log days) describing hatching patterns of eggs of *H. rigida* in different embryonic stages of development at 20°C after storage at 12°C and 8°C for various periods

	R^2						F					
	0.99 (1 degree freedom)											
20°C	Early	D.F.	Mid	D.F.	Late	D.F.	Early	D.F.	Mid	D.F.	Late	D.F.
							12°C direct					
2 days	0.99	4	1.00	1	0.99	1	5.56*	1,5	4.14*	1,2	0.44*	1,2
8 weeks	0.87	5	0.99	2	0.99	2	1.53*	1,6	82.9	1,3	29.9	1,3
							8°C direct					
2 days	0.96	12	0.98	1	1.00	2	11.53	1,13	0.01*	1,2	0.02*	1,3
8 weeks	0.88	4	1.00	0	1.00	0	24.12	1,5	7.72*	1,1	16.96*	1,1
16 weeks	0.96	2	0.99	2	1.00	2	9.01*,†	1,3	0.60*	1,3	1.74*	1,3
41 weeks	No hatch	—	0.98	7	0.97	7	No hatch	—	15.16	1,8	5.72	1,8
							8°C stepwise					
8 weeks	0.99	1	1.00	1	1.00	0	15.32*	1,2	1.73*	1,2	18.04*	1,1
16 weeks	0.98	5	1.00	2	0.97	5	0.22*	1,6	3.27*	1,3	7.43	1,6
41 weeks	1.00	0	0.96	3	0.96	1	14.44*,†	1,1	5.02*	1,4	0.08*	1,2
52 weeks	0.96	1	0.99	4	0.99	4	5.11*,†	1,2	2.05*	1,5	75.22	1,5

* Indicates slope is not significantly different from slope of the control at 5% level.

† Indicates samples with low total percentage hatch.

against the logarithm (base 10) of days. For the probit conversions, the values of 90%, hatch of total number of eggs or, of total hatch if hatch was less than 90%, were considered at 100% hatch, i.e. "total" hatch, and all other values in the sample were adjusted accordingly. This creates a bias against samples with less than 90% hatch since the tail (present due to the extended hatching time of the last few eggs) is included in calculations and may cause the regression line to slant upwards.



FIGS. 5-6. 5, cumulative percentage hatch of *H. rigida* eggs versus days at 20°C after being stored at 12°C for 2 days and 8 weeks. 6, cumulative percentage hatch of directly transferred eggs of *H. rigida* versus days at 20°C after being stored at 8°C for 2 days, 8, 16, and 41 weeks.

Goodness of fit and comparisons of slopes of the regression lines are given in Table I.

Hatching parameters of middle and late stage eggs held at 12° were not affected after 2 days storage (Fig. 5). The hatching period in early stage eggs was slightly extended but this was due to a small number of eggs and the hatching rate was not significantly different from the control (Table I). After 8 weeks at 12°, hatching had already begun in late and middle stage eggs and hatching rates were altered significantly upon return to 20° (Fig. 5). This disruption in synchrony of development may be due to the ability of some eggs to develop better at low temperatures than others (Richards 1957).

In eggs stored at 8° hatching rates were affected by storage time, egg stage, and method of transfer. Time to first hatch tended to increase the longer eggs were held in storage, indicating that embryonic development was not occurring at 8° and that there may be an adjustment period when eggs are returned to 20°. Success of hatch was reduced the longer the storage, and differed with egg stage and method of transfer. Stepwise transferred eggs had consistently higher hatch than directly transferred eggs in the respective egg stages, with middle stage eggs having the highest hatches. Overall, hatching periods were extended the longer the storage period (except when total hatch was low) but there was no obvious quantitative relationship.

Hatching parameters of middle stage eggs transferred stepwise to and from 8° were least affected of the egg stages studied (Fig. 7). Hatching rates were not significantly different from the control at any storage times studied (Table I) and these samples always had the highest percentage hatch for each storage period. Success was reduced at 41 and 52 weeks to 88.9% and 71.8%, respectively.

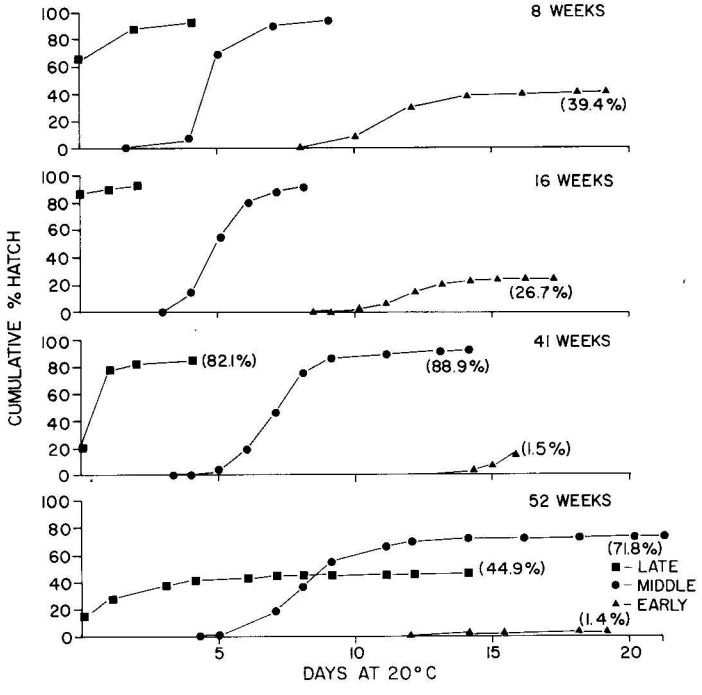


FIG. 7. Cumulative percentage hatch of stepwise transferred eggs of *H. rigida* versus days at 20°C after being stored at 8°C for 8, 16, 41, and 52 weeks.

Middle and late stage eggs transferred directly could be stored for up to 16 weeks without affecting hatching rates (Table I) although success of hatch was reduced somewhat in late stage eggs (84.2%) (Fig. 6). Late stage eggs transferred in steps could be stored for up to 41 weeks without affecting hatching rates, but, it must be noted that these rates were determined using the latter part of the hatching period, since hatch had already started by the time samples were returned to 20° (Fig. 7). Early stage eggs were affected most by cold storage. Percentage hatch in stepwise transferred eggs ranged from 39.4% after 8 weeks to 1.4% after 52 weeks, and in direct transferred eggs from 62.9% after 2 days to no hatch after 41 weeks (Figs. 6, 7). Although hatching rates of some of these samples were not found to be significantly different from the control (Table I), storage of this egg stage would not be useful due to the low percentage hatch.

To determine the usefulness of this cold storage method as a means of providing eggs for toxicity testing on a year round basis, studies comparing the sensitivity of eggs to toxicants after various storage times are needed.

Conclusions

Start, hatching rate, per cent, and hatching period of eggs of *H. rigida* are affected by temperature. For eggs transferred directly to a constant temperature and allowed to develop there, the upper temperature limit for hatching lies between 32° and 36° and the lower limit between 8° and 12°.

Eggs can be stored at temperatures near the lower temperature limit for hatching for prolonged periods and still have hatch occur at 20°. Success of storage varies with temperature, length of storage period, stage of embryonic development of the eggs at time of transfer, and transfer method.

The combination found to be best for long-term storage (up to 52 weeks) was use of middle stage eggs stored at 8° and transferred in a stepwise manner to (4° every 2 days) and from (4° every 7 days) 8°. Eggs in middle and late stages of development could be stored for up to 16 weeks at 8° using the direct transfer method. Short-term storage (2 days to less than 8 weeks) is also possible at 12° using the direct transfer method.

Acknowledgments

We thank Mr. D.A. Duncan and Ms. S.L. Leonhard for technical assistance, Ms. L. Davies and Mr. L. Taite for drafting of figures, and Dr. T.G. Galloway and Dr. J. Mathias for useful criticism of the manuscript.

References

Andrewartha, H.G. and L.C. Birch. 1954. The Distribution and Abundance of Animals. University of Chicago Press, Chicago, Ill. 782 pp.

Benech, V. 1972. Etude expérimentale de l'incubation des oeufs de *Baëtis rhodani* Pictet. *Freshwat. Biol.* **2:** 243–52.

Bohle, H.W. 1969. Untersuchungen über die Embryonalentwicklung und die embryonale Diapause bei *Baëtis vernus* Curtis und *Baëtis rhodani* (Pictet) (Baëtidae, Ephemeroptera). *Zool. Jb. Abt. Anat. Ontog.* **86:** 493–575.

——— 1972. Die Temperaturabhängigkeit der Embryogenese und der embryonalen Diapause von *Ephemerella ignita* (Poda) (Insecta, Ephemeroptera). *Oecologia (Berl.)* **10:** 253–268.

Carlson, C.A. 1966. Effects of three organophosphorus insecticides on immature *Hexagenia* and *Hydropsyche* of the Upper Mississippi River. *Trans. Am. Fish. Soc.* **95:** 1–5.

Davidson, J. 1944. On the relationship between temperature and rate of development of insects at constant temperatures. *J. Animal Ecol.* **13:** 26–38.

Elliott, J.M. 1972. Effect of temperature on the time of hatching in *Baëtis rhodani* (Ephemeroptera: Baëtidae). *Oecologia (Berl.)* **9:** 47–51.

——— 1978. Effect of temperature on the hatching time of eggs of *Ephemerella ignita* (Poda) (Ephemeroptera: Ephemerellidae). *Freshwat. Biol.* **8:** 51–58.

Flannagan, J.F. The burrowing mayflies of Lake Winnipeg, Manitoba, Canada. *Proc. Second Int. Conf. Eph.* In press.

Flattum, R. 1963. Apparent effects of refrigeration on the rate of embryonic development of mayfly (*Hexagenia limbata*) eggs. *Proc. Minn. Acad. Sci.* **31:** 82–83.

Fremling, C.R. 1967. Methods of mass-rearing *Hexagenia* (Ephemeroptera: Ephemeridae). *Trans. Am. Fish. Soc.* **96:** 407–410.

——— 1970. Mayfly distribution as a water quality index. U.S. Environ. Prot. Agency. *Water poll. Contr. Res. Ser.* 16030 DQH 11/70. 39 pp.

——— 1975. Acute toxicity of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to nymphs of mayflies (*Hexagenia* sp.). *U.S. Fish. Wildl. Serv., Invest. Fish Control* 58. 8 pp.

Friesen, M. K. and J. F. Flannagan. 1976. Parthenogenesis in the burrowing mayfly *Hexagenia rigida* (Ephemeroptera). *Can. Ent.* **108:** 1295.

Hoopes, D.T. 1960. Utilization of mayflies and caddisflies by some Mississippi River fishes. *Trans. Am. Fish. Soc.* **89:** 32–34.

Howe, R.W. 1967. Temperature effects on embryonic development in insects. *A. Rev. Ent.* **12:** 15–42.

Hunt, B.P. 1951. Reproduction of the burrowing mayfly *Hexagenia limbata* (Serville), in Michigan. *Fla Ent.* **34:** 59–70.

Neave, F. 1932. A study of mayflies (*Hexagenia*) of Lake Winnipeg. *Contr. Can. Biol. Fish.* **7**: 177–201.

Oseid, D.M. and L.L. Smith, Jr. 1974. Factors influencing acute toxicity estimates of hydrogen sulfide to freshwater invertebrates. *Water Res.* **8:** 739–746.

Prosser, C.L. and F.A. Brown Jr. 1965. Comparative Animal Physiology. 2nd ed. Saunders, Philadelphia, Pennsylvania. 688 pp.

Richards, A.G. 1957. The generality of temperature effects on developmental rate and on oxygen consumption in insect eggs. *Physiol. Zool.* **37:** 199–211.

Spieth, H.T. 1938. A method of rearing *Hexagenia* nymphs (Ephemerida). *Ent. News* **49:** 29–32.

Surber, E.W. and W.E. Bessey. 1974. Minimum oxygen levels survived by stream invertebrates. *Bull. Va polytech. Water Resour. Res. Cent.* 81. 52 pp.

Wigglesworth, V.B. 1965. The Principles of Insect Physiology. 6th ed. Methuen, London. 741 pp.

(Received 10 September 1978)