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COLD STORAGE EFFECTS ON EGG HATCH IN LABORATORY-REARED *CULICOIDES VARIIPENNIS SONORENSIS* (DIPTERA: CERATOPOGONIDAE)

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ABSTRACT. The effects of cold storage (5°C) on the hatching rates of laboratory-reared *Culicoides variipennis sonorensis* eggs were examined. Mortality increased with storage time. Average maximum embryo survivorship for 4 trials was 55.0 ± 4.2 (±SEM) days. Alternating daily cycles of high and then low mean hatching rates occurred and possibly were due to location differences in temperature within the temperature-controlled rearing system. During cold storage at 5°C, *C. v. sonorensis* eggs may be kept for *ca.* 28 days with an anticipated hatching rate of about 50%.

INTRODUCTION

A biting midge, *Culicoides variipennis sonorensis* Wirth and Jones, has been reared continuously at the Arthropod-borne Animal Diseases Research Laboratory since 1957 (Jones 1960, 1964; Jones et al. 1969; Akey et al. 1978; Hunt 1994). The demand for large numbers of *C. v. sonorensis* for vector competence studies with bluetongue viruses and other infectious animal agents (Jones and Foster 1974, 1978; Tabachnick 1991) requires efficient and economical production methods (Hunt 1994).

Egg storage, sometimes for extended periods, is advantageous for maintaining laboratory colonies of *C. v. sonorensis* or other arthropod vectors. One way of delaying embryonic development is to maintain the eggs at low temperatures (Jones 1964, Tubergen et al. 1978, Koch 1981, Goll et al. 1989). Cryogenic storage of *C. v. sonorensis* eggs has not been successful to date (R. A. Nunamaker, personal communication). The current study was designed to examine the effects of cold storage on the hatching rates of *C. v. sonorensis* eggs in the laboratory.

MATERIALS AND METHODS

Test insects: Eggs were obtained from a long-established colony of *C. v. sonorensis* (Jones 1960). Insect-rearing procedures and equipment designed for large-scale production were used (Hunt 1994).

Experimental design: Female adults, 24 to 48 h old, were allowed to feed on defibrinated sheep blood, without additives, through a reinforced silicone membrane attached to an artificial blood-feeding apparatus (Hunt and McKinnon 1990). Freshly deposited eggs, 0 to 8 h old, were collected during scotophase on a moist substrate consisting of absorbent cotton, filter paper, and deionized water. Four groups (trials) of *ca.* 70,000 eggs each were stored in plastic Petri dishes and maintained in darkness at *ca.* 5°C.

For trials 1 and 2, 3 batches of *ca.* 50 eggs each were randomly removed daily from the original group by flushing the eggs from the oviposition paper using a small pipet and deionized water, with the aid of a dissecting microscope. The eggs were placed on 7.0-cm-diam filter papers, and a paper was placed in each of 3 newly prepared rearing pans. The remainder of the eggs was returned to the refrigerator for continual daily sampling.

Trial 3 tested the possible influence of both light and changes in temperature. Seventy samples of several hundred eggs each were randomly obtained by cutting small sections of filter paper from the original group of eggs. Each egg sample was placed in a single plastic Petri dish, wrapped with aluminum foil, and stored at 5°C. Daily, 3 batches of *ca.* 50 eggs each were randomly removed from one sample and set up as described previously for trials 1 and 2. The remaining unused eggs were discarded daily.

During trials 1–3, 3 white enamel pans (25.4 cm wide, 41.9 cm long, 6.4 cm high) (replicates) were established each day. Because the developmental time for *C. v. sonorensis* eggs (oviposition to hatch) is 45.2 ± 0.8 (mean ± SEM) h at 27°C (G. J. Hunt, unpublished data), the rearing pans were maintained for 48 h at a constant temperature of 26 ± 1.5°C and a photoperiod of 13:11 (D:L) hours. An insect-rearing rack accommodated up to 7 rearing pans on each of 5 aluminum shelves (198.1 cm wide, 61.0 cm long, 5.1 cm high). Pan temperature was maintained by 2 650-W rubber heating strips (2.54 cm wide) connected in parallel and bonded to the underside of each aluminum shelf. The first group of 3 rearing pans for each trial was started on the left side of a shelf, whereas the 2nd con-

secutive group was set up on the right side. Thereafter, each 48-h-long group of pans was set up alternating between the left and right sides of the shelf. After 48 h, 3 filter papers (replicates) containing eggs were removed from their respective rearing pans and the proportions of hatched eggs were determined. Each trial was continued daily until no eggs hatched.

For trial 4, eggs and rearing pans were processed and maintained as were those for trials 1 and 2, except the 3 groups of eggs were established in one newly prepared rearing pan each day because of space limitations. Due to the possibility that minor variations in the oviposition time could have affected egg hatching rates substantially during trials 1–3, the time period for ensuring complete egg hatching was increased from 48 to 60 h.

Data analysis: The proportion of eggs hatching each day was transformed to arcsines and analyzed by a 2-way analysis of variance (ANOVA) (SuperANOVA 1989). A runs test for analyzing trend data (runs up and down for randomness) (Sokal and Rohlf 1981) was used to detect significant alternations in the daily mean hatching rates.

RESULTS AND DISCUSSION

The daily mean egg hatching rates (±SEM) of *C. v. sonorensis* for trials 1–4 are presented in Fig. 1. Progressively increasing mortality rates were observed as the age of the eggs increased. The longest survivorship of the embryos during trials 1, 2, 3, and 4 was 47, 50, 57, and 66 days, respectively. There were significant differences in the mean hatching rates among the 4 trials ($F = 154.45$; df = 3,438; $P = 0.0001$). There was a significant difference in the mean hatching rates from day to day ($F = 108.58$; df = 64,438; $P = 0.0001$). During all trials, the mean hatching rate was high and then low, alternating from day to day; in particular, between days 1 and 28 for trial 1, between days 1 and 36 for trial 2, between days 1 and 21 for trial 3, and between days 1 and 44 for trial 4.

We postulated 2 possible causes for this alternating cycle of high and low daily hatching rates during trials 1 and 2. The original groups of refrigerated eggs were repeatedly exposed to white illumination and to a room temperature of *ca.* 21°C when random samples of eggs were being obtained for the 3 replicates daily. Both of these variables associated with the handling of the eggs occurred for no more than a few minutes each day. Although trial 3 was designed to minimize these factors, the up and down cycle of daily hatching rates was observed again. The lengthening of the time period for complete egg hatching during trial 4 produced a similar hatching pattern as during trials 1–3. The runs up and down test showed that the mean hatching rates during the entire lengths of trials 1 and 4 were significantly different from random expectations ($t_s = 2.03$, $P < 0.05$ and $t_s = 3.28$, $P < 0.05$; respectively). The daily mean hatching rates during the entire lengths of trial 2 ($t_s = -0.11$, $P > 0.05$) and trial 3 ($t_s = 0.32$, $P > 0.05$) conformed to random expectations. Nevertheless, tests of selected periods during trial 2 and trial 3 (between days 1 and 36 and between days 1 and 21, respectively) showed significant departures from randomness ($t_s = 2.06$, $P < 0.05$ and $t_s = 2.22$, $P < 0.05$, respectively).

Our experimental design for trial 3 did not support our hypothesis of the causes of the alternating cycle because controlling light and temperature during the handling of the eggs had no effect. It is unlikely that *C. v. sonorensis* exhibited a circadian rhythm in egg hatching controlled by photoperiodism, even though circadian egg hatching has been reported for a few species of hematophagous insects, including *Mansonia uniformis* (Theobald) (Laurence 1960), *Mansonia titillans* Walker (Nayar et al. 1973), and *Triatoma infestans* (Klug) (Lazzari 1991).

We therefore evaluated temperature differences between groups of pans as a possible cause of the up and down hatching cycle. Temperatures were recorded daily for the pans in trial 3. Figure 2 shows the differences in the daily mean hatching rates between pairs of consecutive days vs. the differences in the daily pan temperatures for those days. A temperature increase of 1 ± 0.5°C between 2 days resulted in a mean hatching rate increase of up to 25%. Likewise, when the temperature of the pans decreased between days, there was a decrease in the mean hatching rate in the respective pans. Thus, the hatching of eggs originating from cold storage appears to be remarkably sensitive to subtle changes in temperature. We believe that these changes were the most likely cause of the high and low hatching pattern that was observed during trials 1–4.

We recorded a temperature of 26.3 ± 0.4°C

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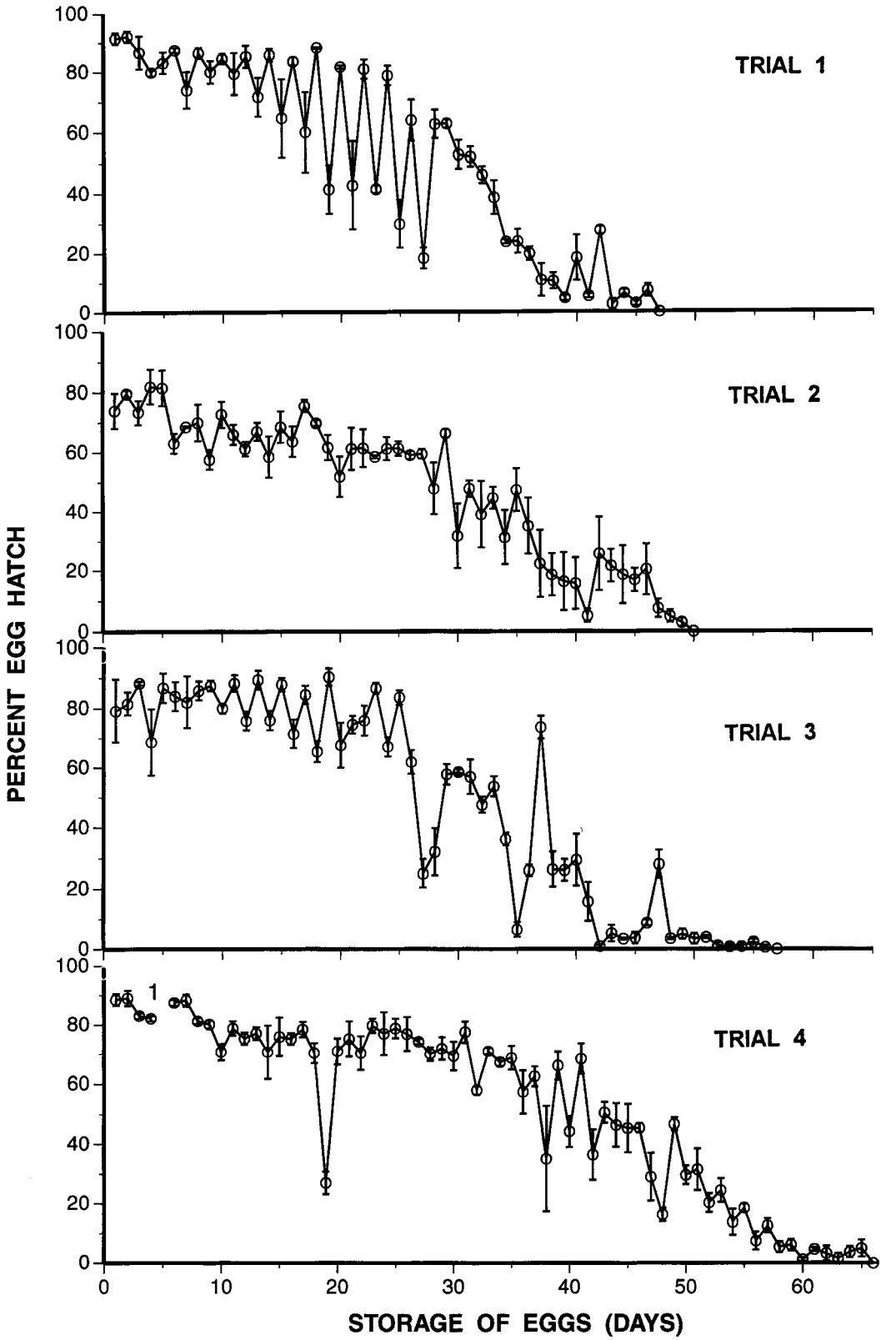
Fig. 1. Effects of cold storage (5°C) on the viability of laboratory-reared *Culicoides variipennis sonorensis* eggs. Points represent the average percent ($n = 3$) of the daily egg hatch. Vertical bars represent standard errors of the mean. 1 = missing data.


TRIAL 1
TRIAL 2
TRIAL 3
TRIAL 4
PERCENT EGG HATCH
STORAGE OF EGGS (DAYS)
0
20
40
60
80
100
0
10
20
30
40
50
60

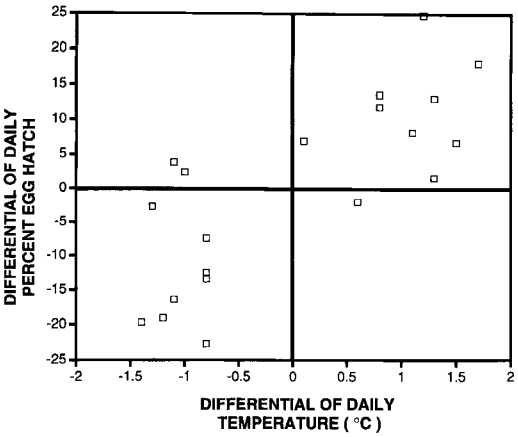


Fig. 2. Relationship between differences of the daily egg hatching rates and differences of the daily temperatures of *Culicoides variipennis sonorensis* during trial 3.

on the left side of the shelf during the 57 days of trial 3, which was significantly higher than the right side mean temperature of 25.8 ± 0.3°C (t = 5.73; df = 1,55; $P < 0.01$). Although the significantly higher temperatures of pans on the left side were recorded throughout the experiment, we observed the alternating hatching cycle predominantly during the first 21 days. The high egg mortalities observed during the latter portions of the study presumably reduced our ability to detect alternating hatching rates because fewer available live embryos were present to allow temperature to stimulate hatching.

In summary, we determined that cold storage of laboratory-reared *C. v. sonorensis* eggs at 5°C provided a hatching rate of at least 50% for eggs up to 28 days old. Also, significant responses of daily egg hatching rates to slight temperature differences in the insect-rearing rack were observed. The storage of eggs at low temperature may have conditioned *C. v. sonorensis* eggs to be very sensitive to small differences in temperatures that stimulate hatching. A location effect within the insectary equipment appears to have produced the cyclic high and low hatching rates.

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