FACTORS AFFECTING REPRODUCTION AND THE IMPORTANCE OF ADULT SIZE ON REPRODUCTIVE OUTPUT OF THE MIDGE CHIRONOMUS TENTANS

PAUL K. SIBLEY,*† GERALD T. ANKLEY,‡ and DUA N E A. BEN OIT†
†Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada
‡Mid-Continent Ecology Division, U.S. Environmental Protection Agency, 6201 Congdon Boulevard, Duluth, Minnesota 55804

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Abstract—We conducted two separate tests to evaluate the influence of several factors that could affect estimation and interpretation of effects on reproductive output of Chironomus tentans in sediment toxicity tests. Specifically, the influence of adult size, mating frequency in males (♂), and age of both males and females (♀) at first mating success (number of successful matings), fecundity (number of eggs/female), percentage hatch, and number of offspring (number of hatched eggs) was assessed. In the first experiment, the influence of adult size on reproductive output was determined by growing midges fed a low (0.29 mg/individual/d) and a high (0.42 mg/individual/d) amount of food to produce small (S) and large (L) adults, respectively. The adults were then mated in one of four scenarios: S♂ × S♀, S♂ × L♀, L♂ × S♀, and L♂ × L♀. An increase in male weight at a constant female weight had no significant effect on fecundity, whereas an increase in female weight yielded 49.5 and 60.7% increases in fecundity when mated with low- and high-fed males, respectively. Similarly, mean number of offspring (fecundity × percent hatch) increased by 19.8 and 48.3% when male weight was increased and female weight was held constant and by 141.9 and 180.4% when female weight was increased and male weight was held constant. In the second experiment, conducted at a single feeding rate, fecundity increased significantly with an increase in female weight but not male weight. The number of offspring was not significantly influenced by adult weight. The age of males or females at first mating, the frequency with which males were mated (up to seven consecutive days), and the proportion of males successfully mating had no effect on fecundity or number of offspring; however, large increases in variability about mean estimates were observed in some endpoints for older males. These results suggest that adult female size is the most important factor affecting reproductive output in C. tentans, and that enhanced performance of the life-cycle test with respect to all reproductive endpoints might be achieved if males are not mated for more than five consecutive days.

Keywords—Chironomus tentans Life-cycle test Adult size Mating frequency Fecundity

INTRODUCTION

Toxicity in contaminated sediments often occurs at sublethal levels, which may become apparent only after extended (chronic) periods of exposure. However, many tests used to assess sediment toxicity emphasize lethality associated with short-term exposures, a situation that may appreciably reduce the effectiveness of sediment risk assessments. To provide a stronger basis upon which to assess sublethal toxicity in contaminated sediments and, thereby, enhance the risk assessment process, test methods that incorporate sensitive and ecologically relevant endpoints are needed [1]. This philosophy was one of the primary motives underlying the recent development of two life-cycle tests using the amphipod Hyalella azteca [2] and the midge Chironomus tentans [3]. These tests incorporate a suite of measurement endpoints that facilitates comprehensive, weight-of-evidence evaluations of sediment toxicity [2–5], and each has recently been incorporated into standard methods by the U.S. Environmental Protection Agency [6] and the American Society for Testing and Materials [7].

An important feature of these tests is the capacity to measure endpoints related to the incorporation of reproductive endpoints in sediment toxicity test protocols offer several advantages [3], but perhaps none more important than the enhanced ecological relevance rendered by the ability to interpret toxicity in terms of population-level effects [5]. The potential interpretive power gained by incorporating reproductive endpoints in sediment (and other) toxicity tests is perhaps best illustrated in relation to emerging concerns regarding the impact of endocrine-disrupting substances on reproduction in wildlife [8,9]. In this context, the C. tentans life-cycle test has been used to evaluate the effects of nonylphenol, a putative endocrine-disrupting compound, on emergence and reproduction [10].

Proper interpretation of toxicological information derived from reproductive endpoints requires a thorough understanding of the reproductive biology and life history of the organism in question and of factors that could affect expression of related endpoints. Considerable information is available on many aspects of the ecology, life history, and general biology of C. tentans [11–16]. In the context of sediment toxicity testing, this information has been used to augment efforts aimed at assessing factors that could affect the survival and growth responses of C. tentans in sediment toxicity tests [17–20]. However, these studies have predominantly focused on the 10-d test, and to our knowledge, considerably less effort has been made to assess factors that could affect the expression of endpoints in the life-cycle test, particularly those associated with reproductive output [3,5]. In addition, although assessment of reproduction in the life-cycle test requires several manipulations of adult midges (e.g., frequent mating of males, delayed mating), the effect of these manipulations on reproductive output has not been determined. The objective of the current study, therefore, was to evaluate the relationships between selected biological and procedural aspects of the C. tentans life-cycle
test (e.g., adult weight, mating frequency, age at first mating) and reproductive output.

MATERIALS AND METHODS

Study 1: Influence of adult size on reproductive output

This study was conducted to test the null hypothesis that adult size, as influenced by food supply, does not affect reproductive output (fecundity or number of offspring) of *C. tentans*. To test this hypothesis, we mated adults derived from larvae grown at two feeding levels, which were established to achieve as large a difference in the size and weight of adults as possible. The high feeding level was 0.46 mg/individual/d (5.5 mg/beaker/d) of TetraMin® fish food (TetraWerke, Melle, Germany), which corresponds approximately to the recommended feeding rate in the 10-d and life-cycle tests with this midge [6]. The low feeding rate was 0.29 mg/individual/d (3.5 mg/beaker/d). This feeding rate corresponds to the minimum amount required by larvae to achieve a weight (0.5 mg ash-free dry wt [AFDW]) that consistently yields high emergence, but that produces small adults relative to those produced under the recommended feeding rate [5,20].

The study was conducted using the method described by Benoit et al. [3] in the exposure system also described by Benoit et al. [21]. The test was initiated with 12 first-instar larvae (<24 h old), added to each 300-ml, tall-form beaker containing 100 ml of clean reference sediment from West Bearskin Lake (MN, USA) [16]. The temperature of the exposure system was maintained at 23 ± 0.5°C, with a photoperiod of 16:8 h (light:dark).

Based on the two food treatments, a total of four mating scenarios were evaluated: (1) low-fed males mated with low-fed females, (2) low-fed males mated with high-fed females, (3) high-fed males mated with low-fed females, and (4) high-fed males mated with high-fed females. To assess these scenarios, two batches of 24 beakers were initiated 10 d apart in the same exposure chamber and under identical exposure conditions [3]. Within each batch of 24, two groups of 12 received one of the two food treatments. Within each group of 12, three sets of four beakers were pooled to serve as a source of males/females for each of the four mating scenarios. Thus, between the two batches, a total of eight beakers (four sets per batch) at each food level were combined to supply adults to assess reproductive endpoints, and these are considered as the replicates (*n* = 3) for statistical purposes. This design, in which the two batches were off-set temporally and the beakers combined for collecting adults, was necessitated by the fact that peak emergence of males typically occurs several days in advance of females. Had all the beakers been initiated simultaneously, insufficient overlap in emergence of males and females would have occurred, producing too few pairs to adequately assess the four mating scenarios. Male weight was determined primarily from the first set of 24 beakers during early stages of emergence, whereas adult female weight was determined primarily from the second set during later stages of emergence. The weight of adult females was determined after the egg mass had been oviposited to maximize the number of egg masses available for assessment of reproductive endpoints. All weights are expressed as AFDW, as determined by drying individual adults at 90°C for 24 h, then burning at 500°C for 2 h [18].

Study 2: Influence of mating frequency and age on reproductive output

This study was conducted to assess the potential effect of mating frequency (males) and age at first mating (males and females) on reproductive output in *C. tentans*, and to further evaluate the relationship between adult weight and reproductive output as assessed in the first study using a different experimental design (single feeding level). The experimental conditions and procedures used in this study were identical to those described above, except that a single feeding rate of 0.5 mg/individual/d of TetraMin fish food was used for all beakers and that two sets of 48 beakers containing clean sediment were initiated 10 d apart. The two sets of 48 beakers were divided into six groups of eight (16 in total), with each group being used as a pooled source of adults for one of the six treatments used to assess the influence of mating frequency and age at first mating on reproductive output (see below).

To assess male mating frequency (e.g., number of successive mating events a male can undertake that yields a viable egg mass), individual adult males, obtained from each group of 16 beakers, were mated with virgin females (<1 d old), obtained from the same group of beakers, at a frequency of once per day. Each male was monitored for the duration of its life span [3]. To assess the relationship between age at first mating and reproduction, individual males and females, obtained from each group of 16 beakers, were held separately for 1 to 6 d (males) or 1 to 5 d (females) before mating. After the specified holding interval, each male or female was mated with a virgin female or male (<1 d old), obtained from the same group of beakers from which the delayed male or female was taken. In both assessments, oviposited egg masses were removed to a separate holding system, and the eggs were counted using the estimation method described by Benoit et al. [3]. For both endpoints, each group of 16 beakers served as a separate pool for obtaining emerged males and females, and each group was considered to be a replicate (*n* = 6) for statistical purposes.

Additional endpoints monitored in both studies included larval survival (determined at 20 d), percentage emergence, and percentage hatch. The latter was determined by incubating the eggs at 23°C for 6 d, after which unhatched eggs were counted and expressed as a percentage of the original number counted in the egg rope.

Statistical analysis

In both studies, differences between treatments for each endpoint were evaluated using a one-way analysis of variance. Percentage mortality of larva (first study) was arcsine transformed to ensure compliance with the assumption of normal distribution and equal variance. In the second study, relationships between individual variables (e.g., adult weight vs duration of emergence period, adult weight vs fecundity) were analyzed using bivariate linear regression. In all analyses, results were considered significant at *p* ≤ 0.05.

RESULTS

Influence of adult size on reproductive output

In the first study, mean survival of larvae at 20 d was significantly lower (*p* = 0.001) in the low-food treatment (63.3%) than in the high-food treatment (85%) (Table 1). Conversely, AFDW of larvae at 20 d was significantly greater in the high-food treatment than in the low-food treatment when
expressed as mean total AFDW per replicate \((p < 0.001)\), but not when expressed as mean individual dry weight \((p = 0.133)\) (Table 1). Mean emergence was 71\% in the high-food treatment and 64.7\% in the low-food treatment. The mean AFDW of both males and females was significantly greater \((p < 0.001)\) in the high-food treatment than in the low-food treatment (Table 1).

Mean fecundity (number of eggs/female) and total number of offspring (number of eggs \( \times \) percentage hatch) increased with an increase in total AFDW of adults in the four mating scenarios (Table 2); however, because of high variability associated with mean estimates of these two endpoints in the lowest mating scenario, these trends were not statistically significant. In the low-weight mating scenario, mean fecundity and total offspring were 519 and 265, respectively, whereas in the high-weight mating scenario, these trends were not statistically significant. In the low-weight mating scenario, mean fecundity and total offspring were 903 and 743, respectively. The difference between the low- and high-weight mating scenarios represented increases of 74.0 and 180.4\% in fecundity and total offspring, respectively. In cross-treatment pairings, mean fecundity and total offspring were 562 and 513, respectively, when low-weight females were mated with high-weight males \((0.46 \times 0.29)\); this represented increases of 8.3 and 48.3\%, respectively, over the low-food treatment. Similarly, mean fecundity and total offspring were 776 and 641, respectively, when high-weight females were mated with low-weight males \((0.29 \times 0.46)\), corresponding to increases of 49.5 and 141.9\%, respectively, over the low-food treatment and of 27.7 and 19.8\%, respectively, over the 0.46 \( \times \) 0.29 treatment. No significant difference was found between matings scenarios with respect to hatch success, despite much lower hatch (51.1\%) in the lowest-weight mating scenario.

In the second study, no significant difference was found between the six groups of beakers with respect to survival (range, 72.4–81.3\%), male or female AFDW (range, 0.54–0.67 mg/individual for males, 0.65–0.79 mg/individual for females), or emergence (range, 54.2–68.2\%). Thus, for regression analysis, the data for each of these endpoints were combined, resulting in an experiment-wide mean of 77\% for survival, a mean AFDW of 0.59 and 0.72 mg/individual for males and females, respectively, and a mean emergence of 60.9\%.

Based on the combined data, the dry weight of newly emerged males \((r^2 = 0.04, p = 0.023)\) and females \((r^2 = 0.19, p < 0.001)\), as well as fecundity \((r^2 = 0.10, p < 0.001)\), exhibited small but significant increases with an increase in time from the first recorded emergence (Fig. 1). Similarly, a significant increase \((r^2 = 0.32, p < 0.001)\) was found in fecundity with increasing female dry weight (Fig. 2). In contrast, no apparent relationship was found between male weight and either fecundity \((r^2 < 0.001, p = 0.63)\) or total number of offspring sired \((r^2 < 0.001, p = 0.86, \text{data not shown})\).

### Table 1. Mean (± standard deviation) survival and weight (ash-free dry wt) of larvae and adults and percentage emergence of *Chironomus tentans* at a low (0.29 mg/individual/d) and high (0.46 mg/individual/d) feeding regime

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival</th>
<th>Larval weight (mg)</th>
<th>Adult individual weight (mg)</th>
<th>Emergence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Individual</td>
<td>Biomass</td>
<td>Male</td>
</tr>
<tr>
<td>Low food</td>
<td>63 (4.1)</td>
<td>1.3 (0.5)</td>
<td>7.4 (1.1)</td>
<td>0.40 (0.03)</td>
</tr>
<tr>
<td>High food</td>
<td>85 (0)</td>
<td>1.7 (0.5)</td>
<td>14.5 (3.7)</td>
<td>0.6 (0.04)</td>
</tr>
</tbody>
</table>

* Difference between treatments is significant \((p = 0.05)\).

### Table 2. Mean (± standard deviation) fecundity, percent hatch, and total number of offspring in *Chironomus tentans* in relation to four mating scenarios derived from a low (0.29 mg/individual/d) and high (0.46 mg/individual/d) feeding regime

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. egg masses</th>
<th>Mean no. of eggs</th>
<th>Mean % hatch</th>
<th>Mean total offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.29 ( \times ) 0.29</td>
<td>5</td>
<td>519 (320)</td>
<td>51.1 (44)</td>
<td>265 (311)</td>
</tr>
<tr>
<td>0.46 ( \times ) 0.29</td>
<td>11</td>
<td>562 (96)</td>
<td>91.2 (7.7)</td>
<td>513 (108)</td>
</tr>
<tr>
<td>0.29 ( \times ) 0.46</td>
<td>14</td>
<td>776 (65)</td>
<td>82.6 (5.9)</td>
<td>641 (18)</td>
</tr>
<tr>
<td>0.46 ( \times ) 0.46</td>
<td>19</td>
<td>903 (206)</td>
<td>82.2 (5.0)</td>
<td>743 (181)</td>
</tr>
</tbody>
</table>

* \( \hat{\sigma} = \text{male}, \hat{\varphi} = \text{female} \).
Factors affecting reproduction in *Chironomus tentans*  

**Fig. 2.** Fecundity (number of eggs/female) in relation to ash-free dry weight (AFDW) of adult female *C. tentans* at a single feeding rate (0.46 mg/individual/d). Data points represent individual females. $R^2 = 0.32, p < 0.001$.

**Fig. 3.** Change in (A) fecundity (number of eggs/female), (B) percentage hatch, and (C) number of offspring in *C. tentans* as a function of the lifetime number of matings for males ($n = 20$ for one to three mating events, $n = 18$ for four mating events; $n = 10$ for five mating events; $n = 5$ for six mating events; and $n = 2$ for seven mating events). Error bars represent standard error of the mean.

**Table 3.** Effect of age at first mating in adult *Chironomus tentans* (no. of days a male or female is delayed from mating) on mean ($\pm$ standard deviation) number of males/females successfully mating, fecundity (number of eggs/female), percentage hatch, and total number of offspring

<table>
<thead>
<tr>
<th>Delay time</th>
<th>$n^a$</th>
<th>% Mating$^b$</th>
<th>No. of eggs</th>
<th>% Hatch</th>
<th>Total offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>13</td>
<td>100</td>
<td>1106 (161)</td>
<td>88.7 (24.9)</td>
<td>1004 (349)</td>
</tr>
<tr>
<td>2 d</td>
<td>15</td>
<td>100</td>
<td>1030 (127)</td>
<td>88.3 (18.8)</td>
<td>919 (256)</td>
</tr>
<tr>
<td>3 d</td>
<td>14</td>
<td>100</td>
<td>900 (161)</td>
<td>95.6 (2.9)</td>
<td>860 (156)</td>
</tr>
<tr>
<td>4 d</td>
<td>14</td>
<td>91.7 (20.4)</td>
<td>875 (171)</td>
<td>96.9 (3.0)</td>
<td>846 (158)</td>
</tr>
<tr>
<td>5 d</td>
<td>14</td>
<td>89.0 (17.0)</td>
<td>975 (154)</td>
<td>92.9 (6.2)</td>
<td>909 (163)</td>
</tr>
<tr>
<td>6 d</td>
<td>11</td>
<td>75 (42)</td>
<td>1128 (260)</td>
<td>95.3 (5.5)</td>
<td>1080 (289)</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>7</td>
<td>91.7 (16.7)</td>
<td>1103 (292)</td>
<td>79.0 (22.0)</td>
<td>910 (446)</td>
</tr>
<tr>
<td>2 d</td>
<td>7</td>
<td>100</td>
<td>1614 (595)</td>
<td>98.6 (1.8)</td>
<td>1595 (600)</td>
</tr>
<tr>
<td>3 d</td>
<td>17</td>
<td>88.8 (17.2)</td>
<td>1109 (165)</td>
<td>60.17 (48.7)</td>
<td>722 (599)</td>
</tr>
<tr>
<td>4 d</td>
<td>11</td>
<td>47.2 (52.1)</td>
<td>1113 (424)</td>
<td>73.4 (26.5)</td>
<td>896 (622)</td>
</tr>
<tr>
<td>5 d</td>
<td>5</td>
<td>100</td>
<td>1182 (306)</td>
<td>70 (28.2)</td>
<td>768 (484)</td>
</tr>
</tbody>
</table>

$^a$ Total number of male or female flies across all replicates used in each assessment.  

$^b$ Indicates that female produced an egg mass.
successfully mating (yielding an egg mass). The proportion of males that successfully mated was not significantly related to delay time, although both a decline (25%) and increased variability associated with this endpoint were observed after 5 d.

**DISCUSSION**

**Influence of adult size on reproductive output**

In this study, we assessed the relationship between the size of adults at emergence and reproductive output in *C. tentans* in two separate studies. In the first study, this was achieved by mating adults derived from two distinct size classes established by manipulating larval food supply (low- and high-food levels). In the second approach, we measured reproductive output as a function of variation in the size of adults grown with a single food supply. With the exception of survival in the first study, survival, dry weight of larvae, and emergence met or exceeded the respective performance criteria for a control sediment [6]. The lower survival in the low-food treatment of the first study was not unexpected, because this food level (0.29 mg/individual/d) is only marginally greater than that reported for *C. tentans* in contaminated sediments from the Great Lakes [5,20]. Although the importance of male size and nutrient donations have been well documented, the precise contribution of these nutrients to reproductive output is not clear. In male fruit flies (*Drosophila* sp.), it has been hypothesized that amino acids transferred with the ejaculate are utilized by females to synthesize proteins in the somatic tissue outside the ovary to provide additional yolk protein and, thus, to increase egg production [35].

An interesting trend observed during the second study was a small, but significant, increase in the size of females and fecundity with an increase in time from first emergence. As discussed above in relation to larval dry weight estimation, this trend most likely reflects a temporal increase in the per-capita amount of food and space available to larvae that have not emerged because of reduced competition for these resources. The resulting increase in the size of larvae would be expected to yield an increase in the size of adult females and, ultimately, an increase in fecundity [5,20]. The size of males also increased significantly as the time from first emergence increased, but this relationship was weaker than that for females. More importantly, no apparent relationship was found between percentage hatch or total number of offspring and time from first emergence, suggesting that the temporal increase in male size had no effect on reproduction. The reduced influence of time on male weight can be explained by the fact that males emerge before females, such that the larvae corresponding to males would not experience the same degree of competitive release as those corresponding to females.

From a practical standpoint, increased fecundity at later stages of the life-cycle test could affect interpretation of toxicity using this endpoint, because this would inflate estimates of mean fecundity and, thus, reduce the likelihood of detecting a statistical difference from control sediments. This phenomenon has also been observed in relation to dry-weight estimates, which become inflated at intermediate levels of survival [19,22]. This problem could be addressed using two approaches. First, the period over which emergence is monitored could be restricted to a specific period of time (e.g., two weeks), thereby minimizing the occurrence of this phenomenon. Second, rather than expressing fecundity as the mean number of
eggs per female, it could be expressed as the mean total number of eggs per replicate, either by taking the average of the total number of eggs produced in each replicate or by dividing the total number of eggs by the original number of organisms placed in the test chambers [6].

**Influence of mating frequency and age on reproductive output**

In the *C. tentans* life-cycle test, it is often necessary to hold both males and females during periods when a mate is not available. This situation most often arises during early stages of emergence, when males predominate, or during later stages of emergence, when females predominate. In the current study, reproductive output (fecundity, percent hatch, and number of offspring) was not affected by the length of time that a male or female was held before mating. However, the decline in the proportion of males that successfully mated, as judged by the failure of the paired female to produce an egg mass the following day, and the increased variability after 5 d may be important in terms of test performance. That is, the reduction in mating success among males, albeit small, may be symptomatic of a general decline in overall health because of aging, an effect that might be expected to correlate with male size, as discussed above. In midges, larger males have been shown to live longer than smaller males [36,37], although this may not necessarily translate into increased lifetime reproductive success [36]. In our study, the average, nonmated male lived 7.3 (±1.24 SD) d. Although we did not quantify the relationship between male dry weight and age, it is plausible that those males failing to copulate after 6 d fell into the lower distribution of the size range for males. Downe [38] also suggested that older males of the midge *C. riparius* experienced lower mating success, although in that study, as in the current one, the relationship between the age and size of adults was not determined. A similar trend was also reported by Taylor et al. [28], who found that copulation success in males of the stonefly *Megarchys* sp. declined with increased age.

In female *C. tentans*, no decline was found in either the proportion of females successfully mating or fecundity as a function of age. The low proportion of females mating on the fourth day resulted from a single replicate in which three females failed to produce an egg mass; in all other replicates for this treatment, mating success was greater than 85%, which was comparable to the other values. The average life span of mated females was 5.9 (±1.48 SD) d, which suggests that females of *C. tentans* are equally receptive and fecund over the duration of their life span, at least in the laboratory. Downe [38] found that female *C. riparius* were also equally receptive to mating throughout adult life. From a practical standpoint, therefore, holding females for an extended period of time should not affect assessment of fecundity in the *C. tentans* life-cycle test.

In the assessment of mating frequency, the number of successive times that a male was mated had no significant effect on fecundity. This finding provides additional evidence supporting the principal role of females in determining fecundity of *C. tentans*. Similarly, no significant effect of mating frequency on the proportion of males successfully mating, hatch success, and number of offspring sired was observed. From a practical standpoint, however, the decline and increased variability associated with the seventh mating event for both endpoints may be worth noting. A similar decline was indicated in older males in the delay study. Collectively, these data may indicate that male *C. tentans* could experience reduced reproductive performance at later stages of the adult male life span.

An important consideration in the current study is that males were mated at a frequency of once per day, a stipulation dictated primarily by our objective of following the *C. tentans* life-cycle test protocol. As such, we did not evaluate the potential effect of shorter periods between mating events, as might be expected to occur in nature. An important question, therefore, is whether males could be mated more frequently than once per day, and, if so, what effect would this have on reproduction. Few studies have been conducted with midges, but numerous studies on Lepidoptera have demonstrated that the interval between mating events can have a profound effect on the amount and quality of ejaculate produced by a male [33–34]. For example, Rutkowski et al. [39] showed that the size of spermatophore and the volume of associated nutrients in male alfalfa butterflies (*Colias eurytheme*) that remated within 1 h of a previous mating were approximately half the normal values. In the present study, we observed two instances in which a single male was accidentally placed in the presence of two females. In both cases, two egg masses were oviposited the following day, but in each case, one egg mass contained 40 to 50% fewer eggs compared to the other. Clearly, the single male successfully inseminated both females; however, the smaller size of the second egg mass suggests that the copulations occurred relatively close together in time. If so, this trend is consistent with the studies cited above, and it suggests that a refractory period, characterized by diminished reproductive output, likely occurs in male *C. tentans*. Interestingly, the percentage of eggs that hatched in each case was greater than 95%. This suggests that sperm quantity was probably not a limiting factor and may provide evidence that the female regulated reproductive output in these instances. Similar trends have been observed in some species of Lepidoptera and may occur in response to a limited supply of nutrients associated with the ejaculate [39].

In the *C. tentans* life-cycle test, adults are not supplied with a source of food during the emergence period, so the increase in variation associated with some endpoints in older males may have reflected a decline in their nutritional state. Feeding is not conducted during emergence in the life-cycle test, because it is assumed that adult midges do not feed and that the energy and nutrients required to complete reproduction are obtained solely during the larval stage. However, adults of many Chironomidae, including *C. tentans*, feed on various natural substrates, most notably nectar and honeydew [42]. In nature, it hypothesized that males use this food as a source of energy for swarming and/or increasing investment in female reproductive effort by increasing the quantity and quality of nutrients transferred with the spermatophore during mating. A similar rationale was offered by Goff [43] to explain feeding in adults of *C. riparius*. Thus, in the absence of food, the successive transfer of nutrients by frequently mated males, without replenishment, could deplete somatic and gametic tissue reserves gained during larval growth, thereby reducing the probability of reproductive success during later stages of the adult life span. In this sense, it may be practical to consider feeding adults during a life-cycle test to enhance reproductive performance, particularly during later stages of adult life. Although the benefit of feeding adults remains to be determined in *C. tentans*, precedent for its application exists in studies with mosquitoes [44].
CONCLUSIONS

Based on evidence from two separate experiments, fecun-
dity in *C. tentans* was predominantly determined by the fe-
male, as egg production was strongly correlated with the size of females and not males. In addition, both the size of newly 
emerged females and fecundity increased as the time from first 
emergence increased. From a toxicological standpoint, this 
latter finding may be important with respect to the interpre-
tation of reproductive data, because it could reduce the like-
lihood of detecting a true difference between treated and con-

trol sediments. The influence of female size on fecundity as a 
function of emergence duration could be minimized either by 
restricting the length of the monitoring period during emer-
gence or by expressing fecundity as total number of eggs per 
replicate rather than per individual. In both the delay and mat-
ing frequency assessments, the age of males and females had 
no significant influence on fecundity. Similarly, hatch success 
and total number of offspring sired were not affected by the 
age and mating frequency of males. However, increased var-
iability associated with estimates of percentage hatch and num-
erical of reproductive endpoints in the life-cycle test might 
best be achieved if males are held for no more than 5 d. Doing 
so, could decrease bias and variability associated with mean 
estimates of reproductive endpoints (fecundity, percent hatch, 
and total offspring), thereby increasing the statistical power 
with which to discriminate between treatments.

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