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Part 1: Laboratory culture of *Centroptilum triangulifer* (Ephemeroptera: Baetidae) using a defined diet of three diatoms

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HIGHLIGHTS

- A reproducible culture method for a parthenogenetic mayfly species for use in toxicity tests.
- The culture method has been used for over 9 generations.
- Young have been successfully tested in toxicity tests with high control survival and growth.
- Methods for culturing diatoms that can be used for culture and testing of *Centriptilum triangulifer*.

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ABSTRACT

Development of methods for assessing exposure and effects of waterborne toxicants on stream invertebrate species is important to elucidate environmentally relevant information. Current protocols for freshwater invertebrate toxicity testing almost exclusively utilize cladocerans, amphipods or chironomids rather than the more typical aquatic insect taxa found in lotic systems. *Centroptilum triangulifer* is a parthenogenetic mayfly occurring in depositional habitats of streams and rivers of the Eastern U.S. and Canada. *C. triangulifer* is an ideal stream insect for toxicity testing under field and laboratory conditions because of its short life cycle, parthenogenetic mode of reproduction, and it represents a group considered sensitive to environmental stressors. In this study, a colony of *C. triangulifer* was reared using a defined diet of three diatoms, *Mayamaea atomus var. permitis*, *Nitzschia cf. pusilla*, and *Achnanthidium minutissimum*. Percent survival ($\geqslant$80%), fecundity measurements ($\geqslant$1000 eggs) and pre-egg laying weights were used as indicators of overall colony health and fitness in our laboratory water (Lab-line) and in Moderately Hard Reconstituted Water (MHRW). Lab-line reared *C. triangulifer* had average survival rate of 92.69% for eleven generations and 82.99% over thirteen generations. MHRW reared *C. triangulifer* had an average survival rate of 80.65% for four generations and three generations of fecundities greater than 1000 eggs per individual. Pre-egg laying weight and fecundity were highly correlated and a best-fit model equation was derived to estimate egg counts for future generations. Establishment of this culturing protocol provides a more ecologically relevant species for toxicity testing and aids in further stressor identification for stream bioassessments.

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1. Introduction

Interest exists in the development of standardized methods for assessing traditional aquatic stream invertebrates with a greater range of sensitivities in order to make a more representative toxicity dataset. Mayflies (Ephemeroptera), stoneflies (Plecoptera), and caddisflies (Trichoptera), or "EPTs" have been recognized as sensitive biological indicators of overall stream health and are important taxa in many stream bioassessment protocols (Barbour et al., 1999; Maxted et al., 2000; Klemm et al., 2003). Many field studies have shown that mayflies are particularly sensitive to anthropogenic disturbance (Pond, 2010) and have been negatively affected or extirpated entirely in stream habitats affected by coal mining activities, where total dissolved solids (TDS) are high (>700 $\mu S\ cm^{-1}$) as a result of land-use disturbances (Kennedy et al., 2003; Pond et al., 2008; Fritz et al., 2010; Pond, 2010).

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For example, the mayfly *Isonychia bicolor* was shown to be more sensitive to coal processing effluent than the cladoceran *Ceriodaphnia dubia* (Echols et al., 2010). In addition to proving to be a valuable indicator in field studies, mayflies have also been used worldwide in many controlled toxicological experiments for various anthropogenic stressors (Tabak and Gibbs, 1991; Diamond et al., 1992; Lowell et al., 1995; Goetsch and Palmer, 1996; Chadwick, 2002; Irving et al., 2003; Buchwalter and Luoma, 2005; Brinkman and Johnston, 2008; Mebane et al., 2008; Conley et al., 2009). Extensive studies showed that EPT taxa were some of the most sensitive macroinvertebrates to high salinity (TDS) in Australia (Kefford et al., 2003; Hickey et al., 2008). Studies have also shown that EPT taxa were the most sensitive macroinvertebrate to salinity regardless to global location (Dunlop et al., 2008; Kefford et al., 2012). Chronic mortality in these mayflies was measured as a 21-d LC50, and ranged from 0.9 to 2.7 mS cm^{-1} (Hassell et al., 2006). Growth and survival of two mayfly species from Australia, *Cloeon* sp and *Centroptilum* sp., showed a complete mortality at 10 mS cm^{-1} electrical conductivity. *C. triangulifer* has the potential to be a model organism for these types of studies due to its sensitivity to particular salts like sodium chloride (Hassell et al., 2006; Struewing et al., 2013).

The goal of this study was to develop a defined culture method for *C. triangulifer* so that this stream insect could be used in toxicity tests. *C. triangulifer* was chosen as our experimental organism because of preliminary culture success at the Stroud Water Research Center (Sweeney and Vannote, 1984). *C. triangulifer* is an obligate parthenogenetic mayfly that inhabits slow flow or depositional areas in streams throughout the northeastern United States and eastern Canada (Gibbs, 1973; Sweeney and Vannote, 1984; Funk et al., 2006). Their life cycle is about 30 d at 25 °C from newly hatched larvae to emergent adult. They lay about 1000–2200 eggs per female and larvae normally feed on periphytic algae and diatoms (Sweeney and Vannote, 1984). These characteristics make C. *triangulifer* an ideal insect for laboratory rearing for ecotoxicological testing and biomonitoring.. Current culturing protocols of *C. triangulifer* at Stroud utilize acrylic plates (6.5 × 23 × 0.15 cm) roughened with sand paper colonized by periphyton using local streams as a periphyton and water source (Sweeney and Vannote, 1984; Sweeney et al., 1992; Standley et al., 1994; Hassell et al., 2006; Conley et al., 2009; Xie et al., 2010; Xie and Buchwalter, 2011; Conley et al., 2011; Kim et al., 2012). However, the use of a localized sources is difficult to standardize and is not readily transferrable to other laboratories. Therefore the need to develop a defined culture method so acute and chronic sensitivity can be reliably measured has become paramount to future studies.

This paper describes the methods used to maintain a colony of *C. triangulifer* on a defined diet of cultured diatoms that can be reproduced in a laboratory, Two primary endpoint criteria were considered for developing a successful culture method for *C. triangulifer*. These endpoints were used to establish a culture with an average of ⩾80% survival and to maintain an average fecundity measured as 1000–2200 eggs per individual. The ⩾80% survival endpoint was base on standard culture survival for EPA test organisms used in acute and chronic testing (States Environmental Protection Agency, 2002a; States Environmental Protection Agency, 2002b) and 1000–2200 fecundity is based on fecundities from nymphs feeding on field collected diatoms (Sweeney and Vannote, 1984).

2. Materials and methods

2.1. Culturing diatoms as a standard food source

The following diatoms were cultured for a standard *C. triangulifer* food source: *Mayamaea atomus var. permitis* (Hustedt) Lange-Bertalot (Source: Carolina Biological Supply Co., Burlington, NC), *Nitzschia* cf. *pusilla* (Kützing) Grunow emend. Lange-Bertalot (Source: Carolina Biological Supply Co., Burlington, NC), and *Achnanthidium minutissimum* (Kützing) Czarnecki (Source: University of Texas Cultured Collection of Algae, Austin, TX). All three diatoms were taxonomically confirmed using conventional light microscopy by an EPA certified diatom taxonomist and an independent diatom taxonomist. These three diatoms were chosen based on relatively small size and abundance from previous studies using native stream diatoms (White Clay Creek, Avondale, PA, USA) (Sweeney and Vannote, 1984) and personal communication from David Funk at Stroud Water Research Center.

2.2. Water used in culturing diatoms and C. triangulifer

Two types of water were used to culture the diatom stocks:

(1) Lab-line: Dechlorinated tap water was supplemented with calcium chloride to obtain a hardness of 160–180 mg L^{-1} as $CaCO_3$ (electrical conductivity of 440–510 μS cm^{-1}).
(2) Moderately Hard Reconstituted Water: Water produced using MILLIPORE Super-Q® System water and reagent grade chemicals to reach a hardness of 80–100 mg L^{-1} and alkalinity of 60–70 mg L^{-1} as $CaCO_3$ (States Environmental Protection Agency, 2002a; States Environmental Protection Agency, 2002b) and aged for at least 3 d before being used (electrical conductivity of 290–350 μS cm^{-1}).

All waters produced diatom stock cultures but Lab-line was used as the main water in rearing the diatom stocks for this experiment. All glassware were cleaned with soap and acid washed with hydrochloric acid.

2.3. Culture of diatom stock solutions

For each diatom species stock prepared, a 2 L flask containing 1 L MHRW or Lab-line was autoclaved with a Teflon® coated stir bar. Any water lost during the autoclaving process was replaced with sterile autoclaved water of the same type. Water was allowed to equilibrate for at least 24 h or hours hrs before adding nutrient solutions and diatom cultures. An amount of 0.4 mL each of Kent® – Proculture Professional F/2 Algal Culture Formula A and Kent® – Proculture Professional F/2 Algal Culture Formula B were added along with 30 mg of sodium metasilicate to each flask. Twenty milliliters (12 mg ± 5% dry weight) of settled diatoms from each of the three diatom stocks were added to individual flasks and placed on a stir-plate. They were stirred moderately and diatoms were allowed to grow for 10 d at 25 °C with 16 h light/8 h dark schedule, under a fluorescent light source of 4306 Lux (128.18 mol m^{-2} s^{-1}) total light – T8 Standard 32 watt fluorescent bulbs. At the end of 10 d of growth, diatom stocks were checked microscopically for contamination and viability (intact chloroplasts) then stored at 4 °C until needed for colonization of diatom slides. Refrigerated stocks were held at 4 °C for no more than two months before being replaced with fresh cultures.

2.4. Mixed diatom food preparation for early nymph stage

A 10 mL calibrated pipette was used to transfer equal volumes of each settled diatom species into a sterile 50 mL polypropylene centrifuge tube (Corning® 430921). Autoclaved Lab-line water was added until the total volume was 50 mL. The centrifuge tube was then capped and shaken vigorously to obtain a homogenous mixture and a dry weight of 73 mg (±5%) diatoms/50 mL Lab-line was determined. Several tubes were prepared in advance and stored in 4 °C until needed. The tubes were shaken to homogenize the diatoms before pipetting into mayfly containers.

2.5. Colonization of mixed freshwater diatom slides for late nymph stage

Seventy-five glass microscope slides either frosted or pre-etched with coarse grade sandpaper were placed on the bottom of a 15 L autoclavable (polysulfone) container containing 3 L of Lab-line water or MHRW and covered with a semi-transparent plastic lid. The container with glass slides is autoclaved (15 min at 121 °C and 18 psi, liquid cycle) and allowed to equilibrate for at least 24 h. Next, 0.5 mL each of nutrient Kent® stocks A and B were added along with 90 mg of sodium metasilicate. Then 15 mL of settled diatoms from each diatom stock culture) were added to each 15 L container and lids were then covered with plastic wrap in order to prevent contamination and evaporation. Diatom slides were maintained at 22 °C ± 2 °C under a total light intensity of 4306 Lux (128.18 mol m^{-2} s^{-1}) of continuous "cool-white" fluorescent lighting using standard T8 32 watt fluorescent bulbs and a light cycle of 16 h light and 8 h dark. Diatoms were allowed to grow on the slides for 7 d then the slides were checked for diatom colonization. Diatom growth on the slides continued for approximately 2 weeks prior to being used as a food source for the mayfly culture. Nutrients for the diatom slide cultures were renewed weekly (0.5 mL of Stocks A and B and 90 mg of sodium metasilicate) until a density of multiple cell layers of diatoms were observed microscopically on the slides using a compound microscope. In order to reduce contamination, forceps were decontaminated either with 10% bleach or heat sterilized before removing slides.

2.6. Centroptilum triangulifer nymphal rearing method

In 2009 the U.S. EPA Aquatic Research Facility in Cincinnati, OH received *C. triangulifer* eggs from Stroud Water Research Center for initial culture development. The genetic composition of these eggs was from one clone (WCC-2) isolated from White Clay Creek in Chester County, PA (39°51′N. 75°47′W) with conductivities between 130 and 400 μS cm^{-1} by Stroud Water Research Center (Funk et al., 2006). The current method for rearing *C. triangulifer* has been split into two phases based on growth. The first phase is from hatching to visibility of about 2 mm. The second phase is from early visibility at about 2 mm to reproductive adulthood. The early nymphs must feed within 12 h in order to ensure survivability since preliminary results showed some high mortality in early instars. This may be due to the higher culture temperature than other investigators use (Sweeney and Vannote, 1984; Sweeney et al., 1992; Standley et al., 1994; Buchwalter and Luoma, 2005; Conley et al., 2009; Xie et al., 2010; Xie and Buchwalter, 2011). As a result, we modified our method after generation 12 to improve the survivability of the newly hatched nymphs.

2.7. Egg collection and storage

One or more vials of freshly collected eggs of *C. triangulifer* were placed into a 25 °C incubator until hatching (all cultures should be started with fresh eggs if possible). Eggs can be stored at 4 °C and transferred into a 10 °C incubator for 3 d then moved into a 25 °C incubator until hatching, if freshly collected eggs are not available. Add 200 μL of mixed diatoms to the vial to provide food to newly hatched nymphs.

2.8. Early nymphal rearing method phase

Upon hatching, the nymphs were split into a circular (Pyrex® 150 × 75 No. 3140) 1 L container. The container was then filled with 500 mL of the water used for the culture (500 mL of MHRW, or 500 mL of Lab-line) and a water line marked at 500 mL was made and checked every other day for evaporation. Deionized water was used to replace the water lost from evaporation by filling to the 500 mL watermark line. The nymphs in this container were fed 8 mL of mixed diatoms (73 mg (±5%) diatoms/50 mL Lab-line determined by dry weight measurements) the day of set up. Forty nymphs were collected on the 5th day from this dish using a disposable transfer pipet and placed into a new circular (Pyrex® 150 × 75 No. 3140) 1 L container with 500 mL of chosen water type (Lab-line or MHRW). Containers were aerated, marked for water level and covered with a plastic bag or acrylic cover. Water changes were performed twice weekly using an aspirator under a dissecting scope to remove 80% of the water then replaced with appropriate water type. These containers were fed 8 mL of mixed diatoms after each water change. When the nymphs become visible to the naked eye at about 1–2 mm, they were transferred to the late nymphal rearing method (phase 2).

2.9. Late nymphal rearing method phase

Eight colonized diatom slides were placed into a 15 L mayfly culture container with 4 L of chosen water type (Lab-line or MHRW) with aeration via an air stone (low aeration) prior to the addition of animals to avoid injury. If MHRW is used, it becomes necessary to include nutrients for diatom growth (0.4 mL each of Kent® – Proculture Professional F/2 Algal Culture Formula A and Kent® – Proculture Professional F/2 Algal Culture Formula B plus 30 mg of sodium metasilicate). Forty 1–2 mm long nymphs of *C. triangulifer* were transferred to the container from the early nymphal containers. Slides were replaced once a week, or as needed due to mayfly grazing, and water changes were made twice a week. Prior to adult emergence the container lids were covered in nylon netting to prevent escape. Culture containers were maintained in environmental chambers (Percival Model 166LLVL) for rearing under the following conditions: 16 h photoperiod, 25 °C, and relative humidity at 50%).

2.10. Adult collection

Adult *C. triangulifer* emerged in 25–30 d at 25 °C after hatching and were collected in the morning after the molt from subimago to adult. Adults were weighed on a Cahn® microbalance to the nearest 0.00001 g. After weights were recorded, *C. triangulifer* were held by the wings with forceps with their abdomen touching autoclaved Lab-line water in a 25 mL vial in order to release the eggs. Oviposition typically occurred at the moment the abdomen touched the water. Once ovipositing was complete, eggs were either held in 4 °C which slows embryonic development until fecundity measurements were recorded or were placed in 25 °C environmental chamber for initiating the next generation. Eggs from two to three females were used to start the next generation of cultures. The eggs from twenty percent of vials collected from each generation were randomized and either counted manually using a dissecting microscope at 10× magnification or by using ImagePro® software to determine fecundity.

2.11. Statistics

Pre-egg laying weight and fecundity data were analyzed using a regression analysis and a Bonferroni adjusted *t*-tests (compared endpoints among generations) following ANOVA using SAS/STAT® software.

3. Results

3.1. Nymphal survival on a mixture of three diatoms

3.1.1. Lab-line water

C. triangulifer were reared under the current protocol using the three diatoms as a food source for 13 generations in Lab-line water. Two generations (F1 and F2) were not included in Table 1 because they were reared using much smaller scale containers and lower numbers of individuals and F11 was reared in only MHRW. Ten of the 13 generations were above the set criteria of over 80% survival from the 1st instar (hatchling) to adult emergence (Table 1). The 13 generations had an average survival rate of 92.69% which exceeded the 80% survival criteria. The overall average survival rate for the 13 generations reared on Lab-line was 82.99%. There seemed to be two different periods of mortality taking place in the study during the first two weeks after hatching or right before molting to the subimago. In two generations (F4 and F7) where mortality was high in Lab-line water; early instars were affected most with an average mortality of 69.65%. To address this mortality issue, the early nymphal rearing phase was developed after generation F12. This modification has resulted in minimal early nymphal mortality. In all generations tested in Lab-line, the average mortality of the nymphs molting into the subimago or subimago failing to molt to the adult was 4.69%. The critical period of the two weeks after hatching from the egg determined rearing success. The early nymphs must feed within 12 h in order to ensure survivability. Providing a solution of diatoms to the culture container perhaps enhances the availability of diatoms versus having to scrape the food source from a slide. As a result, we modified our method after generation 12 to improve the survivability of these early nymphs.

3.1.2. MHRW

C. triangulifer was also reared with this method using the three diatoms as a food source for 4 generations in MHRW water. Two of the four generations were above the set survival criteria at 96.7% survival from the 1st instar (hatchling) to adult emergence (Table 2). The overall average survival rate for all four generations reared on MHRW was 80.65%. In two generations (F10 and F12) where mortality was high in MHRW water, the early instars were affected the most with an average mortality of 29.64%. It should be noted that all MHRW data was prior to the institution of the early nymphal rearing phase method and more research is needed.

3.2. Relationship between pre-egg laying weight and fecundity

There were significant differences among generations reared in Lab-line water in both pre-egg laying weight and egg count ($P < 0.05$, Bonferroni adjusted *t*-tests following ANOVA) (Fig. 1). Generation F1 was significantly different from all other generations with the lowest weight and fecundity ($P < 0.0001$). Generation F2, F7, and F10 were not significantly different from each other but were significantly different from all other generations with the largest weights and fecundities. Both the mean and median egg count were between 1000–2200 for each generation except F1. Generation F4 results were not included in this analysis because the weights were not obtained prior to egg laying despite the fact that the corresponding fecundity was measured.

Pre egg laying weight and fecundity were highly correlated ($p < 0.0001$, *R* squared (R^2) = 0.7175) and a best fit model to predict egg counts from the weight of pre-egg laying females was determined (Fig. 2). The best fit model equation was egg count = 495 684 * w + 20 (MS error = 195) where w = weight in grams. With this equation the fecundity of *C. triangulifer* can be predicted with 95% confidence. This is equivalent to solving the regression equation for pre-egg laying weight when the egg count is 1390 (100 + 2 *MSE): pre-egg laying weight = (1390 − 20)/495 684 = 0.002764 g. Therefore, a pre-egg laying *C. triangulifer* with a weight $\geqslant$0.0028 g has a 95% confidence of having greater than 1000 eggs and therefore exceeding the set fecundity criteria. There were not significant differences among generations F12–F15 reared in Lab-line water in both pre-egg laying weight and egg count ($p < 0.05$, Bonferroni adjusted *t*-tests following ANOVA) (Fig. 3). All fecundities for these generations were derived from using the regression equation (Fig. 2). All generations had median and mean egg counts greater than 1000.

There were significant differences among generations reared in MHRW water in both pre-egg laying weight and egg count ($p < 0.05$, Bonferroni adjusted *t*-tests following ANOVA) (Fig. 4). All fecundities for MHRW were derived using the regression equation (Fig. 2). Generation F13 was significantly different from all other generations with the lowest weight and fecundity ($P < 0.0001$). All generations except F13 had median and mean egg counts >1000.

4. Discussion

A great deal of interest and resources are being utilized to evaluate the extent to which humans and wildlife are being exposed to various chemical stressors in the aquatic environment. The goal of our study was to establish a defined protocol and endpoint criteria for successfully rearing *C. triangulifer* in order to standardize conditions for toxicological testing.

Our results for *C. triangulifer* showed that it can be reared for continuous generations using a standardized diet of three diatoms, *Mayamaea atomus var. permitis*, *Nitzschia cf. pusilla*, and *A. minutissimum*. It appears that both Lab-line water and Moderately Hard

Table 1
Survival of *C. triangulifer* in culture over 13 generations in Lab-line water using *Mayamaea atomus var. permitis*, *Nitzschia* cf. *pusilla*, and *Achnanthidium minutissimum*.

Generation	Initial	Early nymph stage		Emerging subimago stage		Total survival from 1st instar nymph to reproductive adult	
	# nymphs added	# dead	% mortality	# dead	% mortality	# adult survival	% total survival
F3	157	3	1.91	7	4.46	147	93.63
F4	150	101	67.30	0	0.00	49	32.66
F5	153	2	1.31	0	0.00	151	98.69
F6	159	6	3.77	0	0.00	153	96.23
F7	150	108	72.00	2	1.33	40	26.67
F8	103	0	0.00	1	0.97	102	99.03
F9	111	0	0.00	16	14.41	95	85.59
F10	321	23	7.17	34	10.59	264	82.24
F12	60	4	6.67	7	11.67	49	81.67
F13	40	0	0.00	5	12.50	35	87.50
F14	40	0	0.00	2	5.00	38	95.00
F15	80	0	0.00	0	0.00	80	100.00
F16	80	0	0.00	0	0.00	80	100.00

Table 2
Survival of *C. triangulifer* in culture over 4 generations in Moderately Hard Reconstituted Water (MHRW) using *Mayamaea atomus var. permitis*, *Nitzschia* cf. *pusilla*, and *Achnanthidium minutissimum*.

Generation	Initial	Early nymph stage		Emerging nymph stage		Total survival from 1st instar nymph to reproductive adult	
	# nymphs added	# dead	% mortality	# dead	% mortality	# adult survival	% total survival
F10	50	16	32.00	3	6.00	31	62.00
F11	120	7	5.83	1	0.83	112	93.33
F12	55	15	27.27	3	5.45	37	67.27
F13	108	0	0.00	0	0.00	108	100.00

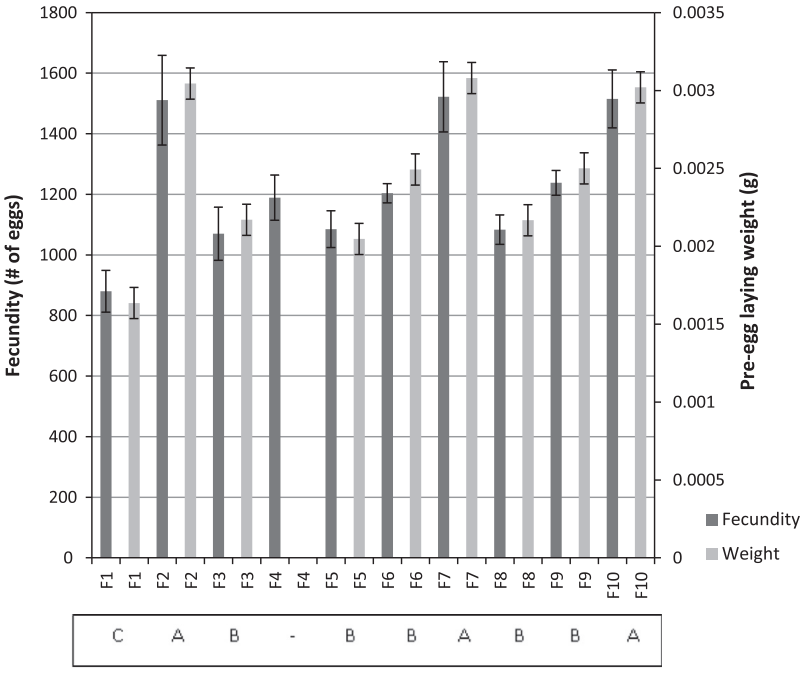

Fig. 1. The relationship between pre-egg laying live mass and fecundity for 10 generations in Lab-line water. Means with the different letters are significantly different ($p < 0.05$; Bonferroni adjusted *t*-tests following ANOVA).

Reconstituted Water (MHRW) can produce similar results in culture success using these diatoms. However, additional research is necessary in using MHRW as a culture media in order to show a similar consistency in meeting the 80% survival criteria.

Our criteria before starting the culture was to achieve a survival rate ⩾80% for multiple generations and in Lab-line water it was achieved for 11 out of the 13 generations and in MHRW it was achieved in 2 out of 4 generations. In both types of water where the generations did not meet the >80% survival rate, mortality happened in the very early instars, which may indicate an issue of food quality, food availability, organism fitness, or water chemistry. In order to try and eliminate the early instar mortality, smaller containers were used with food being provided as free settling diatoms versus diatoms adhered to a slide. The advantage of the smaller containers is that the newly hatched nymphs can be viewed under a dissecting microscope and thus monitored during this critical life stage. It was found that nymphs must feed within 12 h in order to ensure survivability. Moreover, providing diatoms loosely in the culture container enhanced the availability of diatoms versus having to scrape the food source from a slide. The results of this method advancement improved the survivability of these early nymphs to 100% survival since Generation F12 in Lab-line water (Table 1).

The results show that an 1000 egg count fecundity can be expected in both Lab-line water and MHRW using the standardized diet of the three diatoms. Fecundity for twelve generations of Lab-line reared *C. triangulifer* showed that both mean and median egg count were >1000 for nine generations. Fecundity for three generations reared in MHRW had both mean and median egg counts >1000. This indicates that fecundity of ⩾1000 eggs in *C. triangulifer* can be successfully maintained using the mixed diatom food source under continuous culture in both types of water. This study used live weights of pre-egg laying *C. triangulifer* to compare exact weight with number of eggs o. Previous studies have used postmortem adults to collect egg counts for weight and fecundity studies (Sweeney and Vannote, 1984). Using the live *C. triangulifer* gives the advantage of more accuracy in number of eggs that are actually deposited by the corresponding individual. Fecundity and weight were highly correlated statistically ($p < 0.0001$, R squared = 0.7175) as expected. Previous studies have shown that weight and fecundity are highly correlated using stream cultured plates (Sweeney and Vannote, 1984; Sweeney et al., 1992; Standley et al., 1994; Hassell et al., 2006; Conley et al., 2009; Xie et al., 2010).

Regression analysis showed that the fecundity of *C. triangulifer* can be predicted from their pre-egg laying weight for future generations. A pre-egg laying weight of 0.002764 grams must be achieved in order to give at least 1000 eggs with 95% confidence. As a result, adult pre-egg laying weight can be used as an indicator or endpoint of culture health.

A successful in-house culture method for *C. triangulifer* allows continued development toward a new standard test organism with ecological relevance. Using the culture method and the young from several of the generations described in this paper, comparative

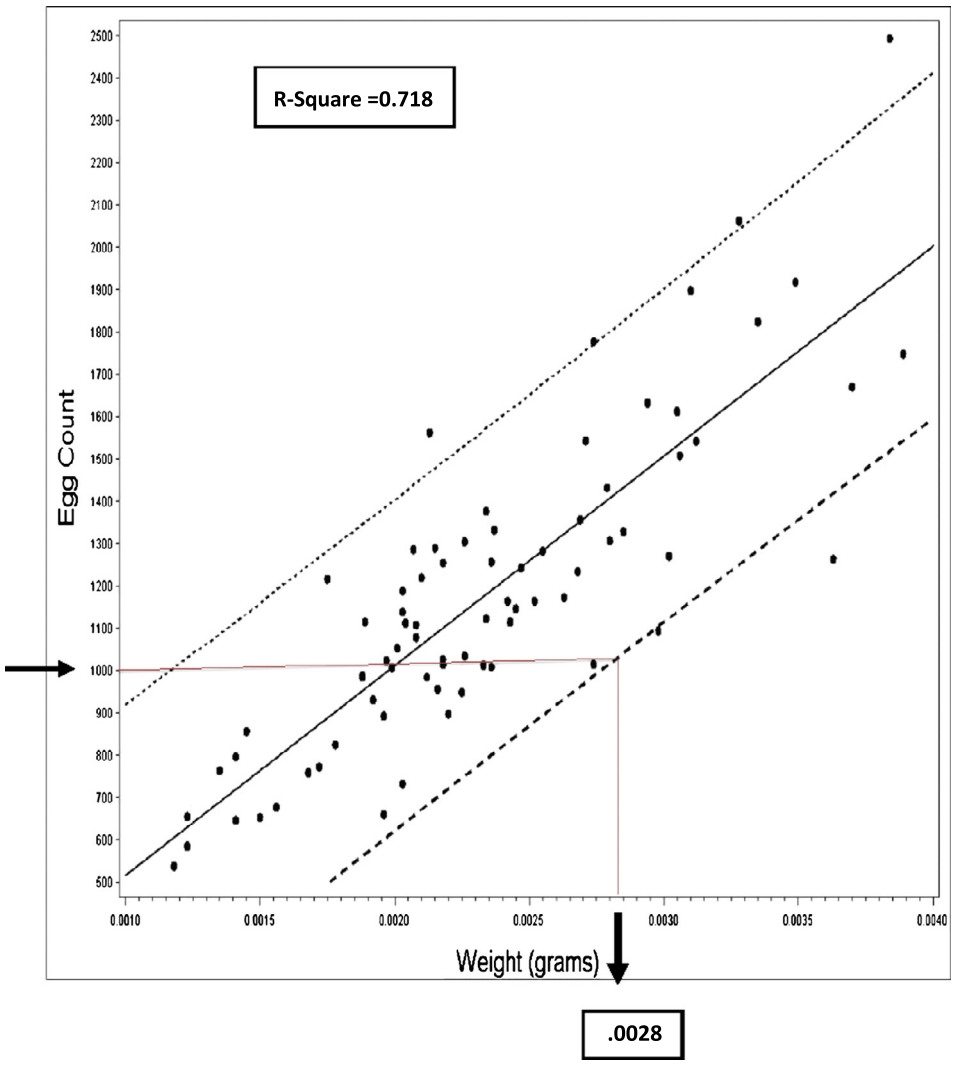

Fig. 2. Regression relationship between pre-egg laying live mass and fecundity. Line and arrows indicate weight of adults at which 1000 eggs or greater will be produced.

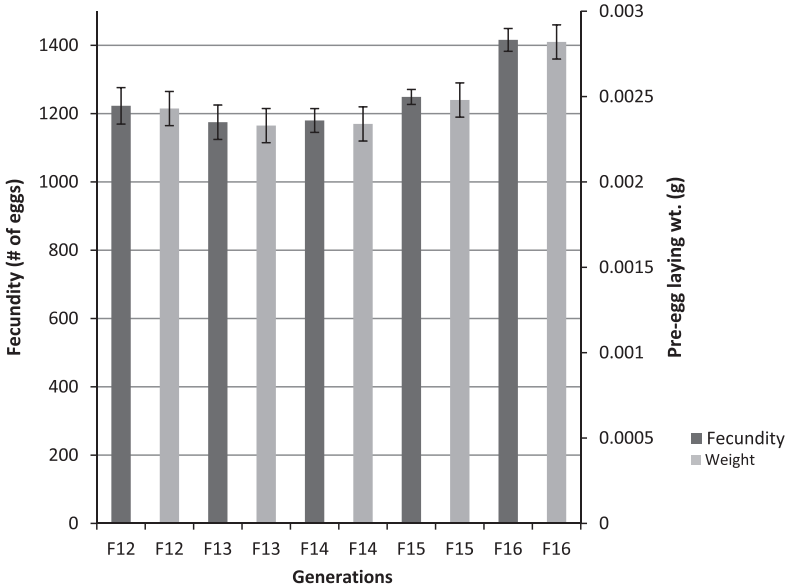

Fig. 3. The relationship between pre-egg laying live mass and fecundity using regression equation for F12–F16 generations of *C. triangulifer* in Lab-line water. Means with the different letters are significantly different ($p < 0.05$; Bonferroni adjusted *t*-tests following ANOVA).

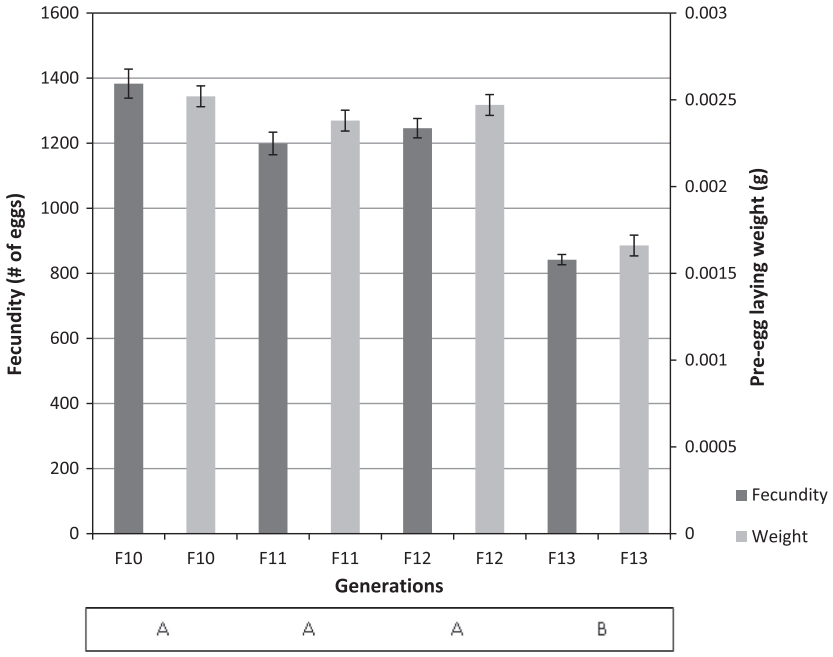

Fig. 4. The relationship between pre-egg laying live mass and fecundity using regression equation for 4 generations of *C. triangulifer* in Moderately Hard Reconstituted Water (MHRW). Means with the different letters are significantly different ($p < 0.05$; Bonferroni adjusted *t*-tests following ANOVA).

testing was conducted to assess exposure sensitivity to several reference toxicants Standard toxicity test organisms, including *C. dubia* and *Daphnia magna,* were tested alongside *C. triangulifer* using three reference toxicants to access sensitivity differences in both acute and chronic tests (Struewing et al., 2013). The culture information provided in this paper and the test methods developed in Struewing et al. (2013) will allow the ecotoxicology community to use *C. triangulifer* as a new test organism for testing the toxicity of a variety of chemicals and environmental samples.

One species of mayfly cannot possible represent all of the possible toxicant sensitives found in all the EPT. Future studies may include laboratory testing of field collected EPTs along with using cultured *C. triangulifer* to explore a greater range of diversity in toxicant sensitivities. The work of collecting steam insects and testing them in a laboratory has yielded excellent results for determining toxicant sensitivities especially with salinity (Kefford et al., 2003; Hassell et al., 2006; Hickey et al., 2008; Dunlop et al., 2008; Echols et al., 2010; Kefford et al., 2012). One disadvantage of using field collected EPTs is making sure the test conditions replicate their environmental conditions so other variables are not contributing to mortality or growth effects. The information gained from both field collected EPTs and from cultured *C. triangulifer* will help elucidate many toxicological and water quality problems and help us better understand the critical role EPTs play in stream ecology. Future plans are to develop culture protocols for other EPT species to represent certain geographic locations. Many attributes need to be considered when choosing a new cultured EPT: Short life cycle, mating habits, water parameters, functional feeding, food supply and quality, and diapausing conditions. Not all EPTs are ideal for laboratory culturing due to not meeting one of these attributes. However, there are many EPTs that can be cultured given the proper conditions and this will give researches an advantage in determining toxicant sensitives that are more representative of natural populations.

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