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Development of an easily made artificial sediment that reduces experimental variability

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Chironomus riparius toxicity tests are commonly performed on natural, thus variable, sediments. The introduction of transcriptomics in ecotoxicology increased the necessity for reduced experimental variability. To this purpose we developed an easily made artificial sediment and monitored larval development. We showed that larval development was synchronized and that the days when specific larval stages are reached can be identified. We conclude that the newly developed artificial sediment will facilitate the use of transcriptomics in ecotoxicology.

Keywords: Chironomus riparius, artificial sediment, larval development

The first microarrays produced to study gene expression were constructed about 15 years ago (Schena *et al.* 1995, Lockhart *et al.* 1996). Ever since, the field of transcriptomics has been evolving rapidly. Microarrays are nowadays affordable and can be obtained commercially for a growing number of model organisms. This is clearly visible in the field of toxicology, where microarrays have become a standard tool to elucidate the mode of action of toxicants at the molecular level (Andrew *et al.* 2003), as well as to identify toxicant specific gene expression profiles (Hamadeh *et al.* 2002). With some delay, transcriptomics also found its way into ecotoxicology.

Although the first ecotoxicogenomical studies focussed on stress responses in model organisms (Momose & Iwahashi 2001, Seki *et al.* 2002), recent gene expression studies are performed on ecologically relevant non-model organisms, such as *Folsomia candida* (Timmermans *et al.* 2007) and *Daphnia magna* (Poynton *et al.* 2007). It is expected that with the current technological breakthroughs in the next generation sequencing technologies, the number of gene expression studies performed on non-model organisms will increase rapidly (Vera *et al.* 2008, Bellin *et al.* 2009). One of the organisms for which currently hardly any sequence data is available, but for which a microarray is currently being developed is the non-biting midge *Chironomus riparius*. This chironomid species is ecologically relevant

due to its widespread distribution, numerical abundance and importance as prey (Armitage *et al.* 1995). In addition, *C. riparius* is also widely used in ecotoxicology for acute, chronic and life cycle testing of chemicals according to standardized test protocols (Ristola *et al.* 1999, Béchard *et al.* 2008, Leon Paumen *et al.* 2008).

One of these protocols is OECD guideline 218, which describes how to perform a sediment-water chironomid toxicity test using spiked sediment (OECD 2004). To reduce variability within and between experiments, the guideline recommends the use of artificial sediment consisting of 75% quartz sand, 20% kaolin clay and 5% peat. This artificial sediment is a great improvement compared to natural sediments, however, as it contains peat, it still varies inevitably between batches. When studying changes in gene expression upon exposure to toxicants, it is necessary to standardize experimental conditions to such an extent that the observed changes are as little as possible influenced by other variables than the toxicant. Therefore we aimed to develop an improved artificial sediment that would be consistent over time and that could easily be produced with well-defined commercially available components. To test whether larval development would be better synchronized on the newly developed artificial sediment, larval growth and development were monitored. Synchronizing larval development will allow to identify the days when specific larval stages are reached.

MATERIALS AND METHODS

Test organism and culturing conditions

Chironomus riparius larvae used in the experiments originate from the University of Amsterdam's in-house laboratory culture. This culture has been maintained at 20 ± 1 °C, 65% relative humidity and L16:D8 photoperiod. The culture was fed a mixture of Trouvit® (Trouw, Fontaine-les-Vervins, France) and Tetraphyll® (Tetrawerke, Melle, Germany) in a weight ratio of 20:1. This mixture was also used as food for all subsequent experiments.

Experimental design

Experiment 1: Growth test with various artificial sediments

A 14-day growth experiment with *C. riparius* larvae was preformed to test the suitability of various artificial sediment compositions. The artificial sediments were made according to the OECD 218 guideline (OECD, 2004), with slight modifications. All sediments consisted of 75% quartz sand (Sibelo NV, Mol, Belgium), 20% Kaolin clay (Keramikos, Haarlem, The Netherlands) and 5% organic matter. As organic matter, grounded peat (Tuincentrum Het Oosten, Aalsmeer, The Netherlands), cellulose fibers (homogenized unbleached paper) and α-cellulose powder (Sigma-Aldrich, St. Louis, MO, USA) were used. $CaCo_3$ was used to adjust pH of the artificial sediments to 7.0 ± 0.5. Deionised water was added to obtain a moisture content of the artificial sediment of 50%.

Three feeding rates were tested per artificial sediment: 0.25, 0.5 and 1.0 mg food/larva/day. To ensure a homogenous distribution of the food in the sediment, the total amount of food for the entire duration of the test (35, 70 and 140 mg) was added to a glass bottle together with the artificial sediment and placed overnight on a roller bank (20 rpm). Aliquots of 60 g of this homogenized food-sediment mixture were added to each of three replicate 400-ml glass beakers. For the peat containing artificial sediment an additional treatment was included with twice the amount of sediment per beaker (120 g). These beakers were then carefully topped up with 250 ml of Dutch Standard Water (DSW; deionised water with 200 mg/l $CaCl_2*2H_2O$, 180 mg/l $MgSO_4*H_2O$, 100 mg/l $NaHCO_3$ and 20 mg/l $KHCO_3$; pH 8.2) and allowed to settle for 4 h before aeration was turned on. The test beakers were conditioned for 1 week under the same conditions which prevailed in the subsequent test, after which 10 first-instar larvae, <24 h old, were added per beaker. The larvae were allowed to settle on the sediment for 4 h before aeration was restarted. After 14 days of incubation with constant aeration, the sediment was sieved through a 425 micron sieve. Larvae were collected and photographed. Using analySIS® software (Soft Image System, GmbH) the body length (BL) of the larvae was determined.

Experiment 2: Larval development in the new artificial sediment

A 14-day growth experiment with artificial sediment containing α-cellulose powder as organic matter and a feeding rate of 0.5 mg food/larva/day was conducted as previously described. The experiment was started with 36 replicates. Three days after introduction of the first-instar larvae to the test beakers, monitoring of larval development was initiated. Each day three replicates were sieved, the larvae photographed and their body length, head capsule width and head capsule length determined using analySIS® software.

Statistical analysis

Results from the 14-day growth experiment with different artificial sediments were checked for normality using Shapiro-Wilk's W test and tested for homogeneity of variance using Leven's test, after which they were compared at a 5% significance level using an one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test. The statistical analyses were performed using SPSS® 17 for windows.

RESULTS & DISCUSSION

Growth test with various artificial sediments

Three artificial sediment compositions were tested for larval development, containing either cellulose fibers, α-cellulose powder or grounded peat as organic matter. For the peat-containing sediment an additional treatment was included that had an increased sediment thickness commonly used in our laboratory for natural sediments. Feeding level had a much stronger impact on lar-

val development than the composition of the artificial sediment (Fig. 1). For the four sediments a significant increase in larval growth was observed with increasing food levels; cellulose fiber ($F_{2,54}$ = 132.406, P<0.001), α-cellulose powder ($F_{2,55}$ = 52.029, P<0.001), grounded peat ($F_{2,51}$ = 74.211, P<0.001) and thicker layer of grounded peat ($F_{2,50}$ = 122.466, P<0.001). This finding is in agreement with Ristola *et al.* (1999) who tested four feeding levels on four natural sediments differing in organic content and particle size distribution. They observed that larval growth was greatly enhanced with increasing food levels in all natural sediments.

No significant differences in larval growth were observed between the three artificial sediment compositions tested. The only sediment where the larvae grew significantly less was the thicker peat containing sediment. This was the case for all three feeding levels; 0.25 mg/larva/day ($F_{3,68}$ = 6.464, P = 0.001), 0.5 mg/larva/day ($F_{3,71}$ = 20.842, P<0.001) and 1.0 mg/larva/day ($F_{3,71}$ = 12.696, P<0.001). A possible explanation could be that due to the thickness of the sediment, approximately 1 cm as to the 4 mm of the other treatments, part of the food was not available to *C. riparius* larvae, that mostly forage on the sediment surface and the layer just below it.

Since larval growth did not differ between the three artificial sediment compositions, it was decided to continue with the most standardized of the three, the α-cellulose containing sediment. This artificial sediment has the benefit that α-

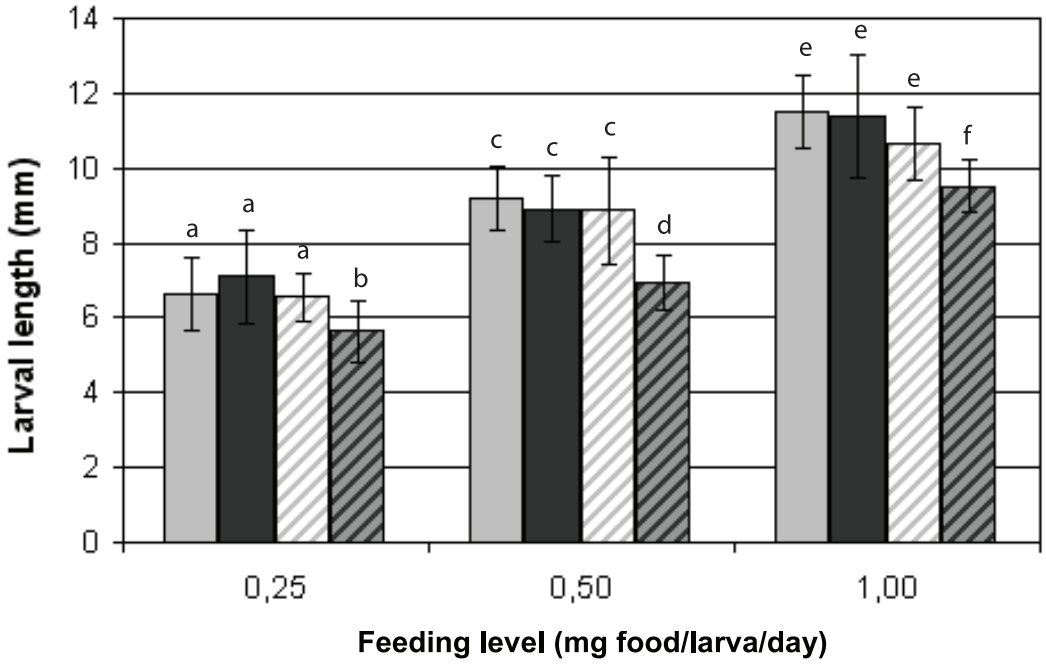

Figure 1. Mean (± SD) larval length after 14 days at three feeding regimes, *i.e.*, 0.25, 0.5 and 1.0 mg food/larva/day, in artificial sediment containing 5% organic matter composed of cellulose fibers (solid light grey; 60 g ww), α-cellulose powder (solid dark grey; 60 g ww) or peat (hatched light grey; 60 g ww, and hatched dark grey; 120 g ww).

cellulose can be obtained commercially, thus allowing an easy and quick production of artificial sediment, while ensuring reproducibility in time. The suitability of α-cellulose had already been advocated by Ribeiro *et al.* (1999) and Fleming *et al.* (1998), using other proportions of sand, clay and α-cellulose.

Larval development in the new artificial sediment

To test whether larval development would be better synchronized on the newly developed artificial sediment, a 14-day growth experiment (0.5 mg food/larva/day) was conducted where larval development was monitored on a daily basis. Three parameters were noted for each larva: body length, head capsule length and head capsule width. Previously, Watts & Pascoe (2000) compared *C. riparius* and *Chironomus tentans* development and plotted body length vs. head capsule width. These graphs did not yield distinct groups, so clustering was necessary. In this study we decided to plot head capsule length vs. width (Fig. 2). In this way all larvae fell into one of four distinct groups corresponding to the four larval instars of *C. riparius* (Fig. 2) making clustering unnecessary. In future experiments, Figure 2 will allow the determination of the instar stage of larvae that due to toxicant exposure have a delayed development.

With the clear classification of larvae into one of the four instars, we were able to identify the days when certain stages were reached under control conditions (Fig. 3). This knowledge will be useful in future ecotoxicogenomics stud-

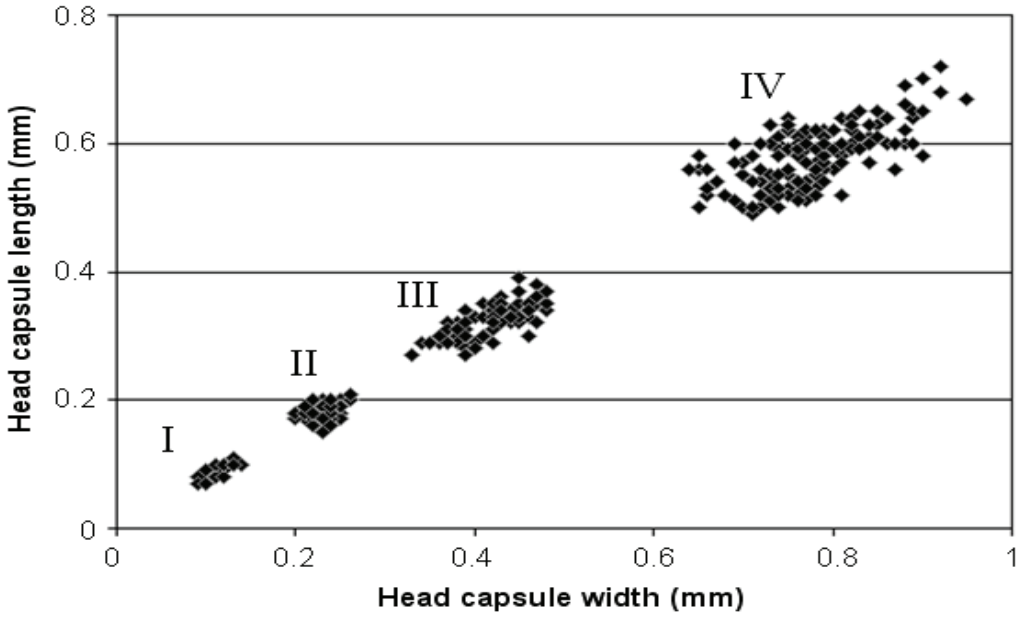

Figure 2. Head capsule development. Larvae are distinctly grouped according to the four larval instars when head capsule width is plotted against length. Experiment was performed in artificial sediment with 5% α-cellulose at 0.5 mg food/larva/day.

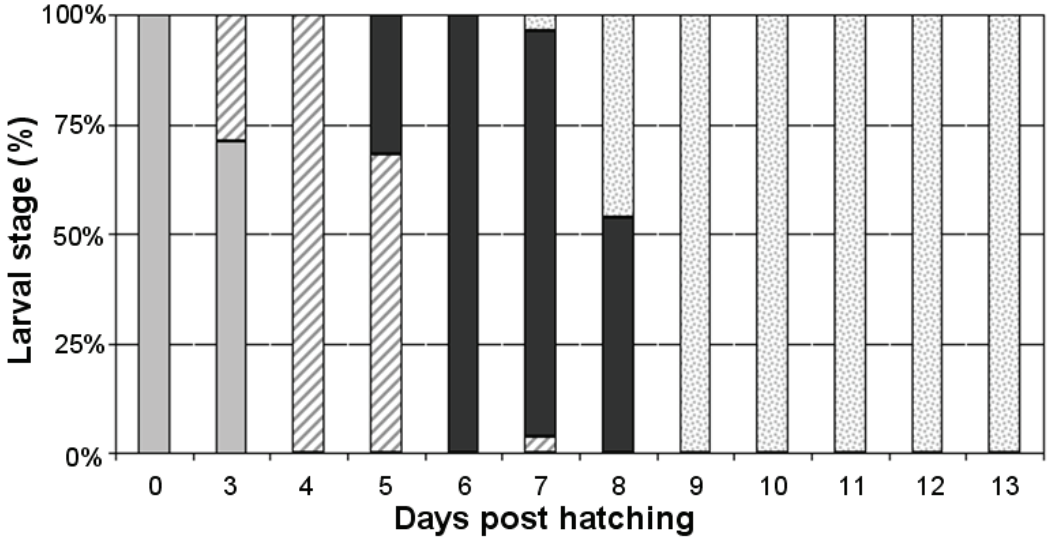

Figure 3. Larval development over time indicated by percentage of larvae being instar I (grey), II (hatched), III (black) or IV (whitish). Experiment was performed in artificial sediment with 5% α-cellulose at 0.5 mg food/larva/day.

ies that will require larvae of specific stages. We therefore conclude that both the availability of the newly developed standardized sediment as well as the synchronization of larval development growing on it, will be of great assistance in the performance of future ecotoxicogenomics work with *C. riparius*.

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