J. N. Am. Benthol. Soc., 2000, 19(1):169–175

Comparison of gut fluorescence and gut dry mass techniques for determining feeding periodicity in lotic mayflies

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Abstract. Gut tube dissection and gut fluorometry are methods that allow assessment of mayfly gut contents for the determination of diel feeding periodicity. We conducted laboratory experiments that quantitatively compared the field applicability of the 2 methods. Our study demonstrated that larval mayflies could be frozen for up to 6 mo without affecting the levels of chlorophyll *a* extracted from their guts. The ability to freeze mayflies and the possibility of working with reasonably small numbers of larvae, negligible background chlorophyll *a* levels, and flexible extraction times gives gut fluorescence comparable flexibility to preserving mayflies for the dry mass technique. Direct comparisons of the 2 gut content techniques revealed similar gut evacuation rates of *Baetis* larvae fed an ad libitum supply of *Navicula*. The maximum time between samples for either technique should be ≤4–8 h to estimate diel feeding periodicity accurately. Fluorometry and dry mass techniques were equally suitable to field studies that quantified algal feeding by mayfly grazers, but the quantification of gut mass using fluorometry was not useful for a scraper-collector that consumed large amounts of fine particulate organic matter along with algae. Thus, the gut fluorometry technique is a simple alternative to the detailed microscopic work required for the dry mass technique when algae are the primary food, but the dry mass technique is the more reliable measure of gut fullness when the primary food source is unknown or changes through time.

Key words: gut mass, lotic mayfly, dry mass, fluorometry, chlorophyll *a*, diel periodicity.

There are currently 2 methods that provide a rapid assessment of material in the guts of lotic macroinvertebrates. One technique involves weighing the gut tube to obtain the dry mass of its contents (Scrimgeour et al. 1991, Culp and Scrimgeour 1993, Scrimgeour et al. 1994). This technique requires training for the microscopic dissections, but the direct measurement of gut contents is quick. The 2nd technique uses fluorometry (Cowan and Peckarsky 1990, 1994), and was originally developed to evaluate zooplankton gut contents (Mackas and Bohrer 1976). Gut fluorometry estimates gut fullness based upon the amount of total pigments (chlorophyll *a* and phaeopigments) in whole or partially dissected individuals. Although gut tube dissection and gut fluorometry have been used in field studies to examine mayfly feeding periodicity (Culp and Scrimgeour 1993, Cowan and Peckarsky 1994), a quantitative comparison of the 2 techniques is needed.

We examined the applicability of the 2 techniques to study diel feeding periodicity for larvae of *Baetis tricaudatus* and *Heptagenia* sp., 2 common mayflies that are scraper-collectors (Merritt and Cummins 1984). Our objectives were 1) to improve the field applicability of these gut analysis techniques, and 2) to determine whether the detection of patterns in feeding periodicity depended on the technique used to collect the data or the taxa involved. We hypothesized that the 2 methods would indicate similar feeding patterns for larvae that fed primarily on algae, but different patterns for larvae that fed on a periphytic

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matrix of algae and fine particulate organic matter (FPOM).

Methods

General laboratory procedures

Baetis larvae (3–6 mm body length) were collected from Big Hill Springs, Alberta, in April to May 1993 for all preliminary laboratory experiments. Prior to experiments, larvae were maintained for up to 2 wk under a constant temperature (10°C) and light regime (16:8 h light: dark cycle) in vigorously aerated chambers (250 mL). A maximum of 10 larvae were held in each container where they were fed an ad libitum supply of *Navicula* diatoms cultured on Plexiglas blocks (Scrimgeour et al. 1994). Each larva was measured to the nearest 0.1 mm under 25× magnification prior to determining the mass of chlorophyll *a* (chl *a*) in gut contents. Gut content mass was determined using the fluorometry technique of Cowan and Peckarsky (1990) and the dry mass technique of Scrimgeour et al. (1994). Both techniques measure feeding activity in terms of changes in the food mass in the gut. The fluorometry technique required larvae to be macerated and transferred to a plastic centrifuge tube with 10 mL of 90% ethanol (Nusch 1980). Pigments from whole, macerated larvae were extracted for 5 min at 80°C and cooled in the dark for ≥2 h. After pigment extraction, fluorescence was measured with a Turner Designs model 10–005 fluorometer, and chl *a* content was expressed as ng of chl *a* per individual mayfly.

Dry gut mass (DM) of *Baetis* larvae was estimated by removing the gut tube by dissection. Larvae were placed in a wax dissecting dish, the heads were removed, and the gut walls were pulled from the other internal tissues (Scrimgeour et al. 1994). Gut contents were then separated from the gut wall, placed on preweighed filters, and dried at 40°C for 48 h. Samples were reweighed to the nearest 0.001 mg with a Cahn C-30 microbalance. Correction for the weight of the gut tube was not required because gut contents were separated from the gut wall (Scrimgeour et al. 1994).

Laboratory experiments

Laboratory experiments to evaluate the 2 gut mass techniques included: 1) determination of background levels of chl *a* in *Baetis* guts; 2) examination of the effect of extraction and sample storage times on chl *a* yields; and 3) a comparison of gut clearance rates of *Baetis* larvae measured by the fluorometry and DM techniques. Preliminary gut fluorometry experiments were conducted prior to these experiments to determine the appropriate sample size, n ($n = s^2/D^2\bar{X}^2$ where s^2 = the sample variance, $\bar{X}$ = the sample mean, and D = the index of precision, Elliott 1977) for estimating *Baetis* gut fullness. These preliminary experiments indicated that, to achieve a 20% level of precision, the sample size should be 11 for fed and 13 for starved larvae. Thus, a minimum sample size of 15 larvae was set for all subsequent experiments.

The attachment of diatom cells to the larval exoskeleton may result in background levels of chl *a* in *Baetis* with guts cleared as a result of starvation. To examine if epizoic chl *a* increased with larval size, *Baetis* larvae ($n = 77$) that had been starved for 36 h in chambers free of algae were examined for background chl *a*, and the relationship between chl *a* and body size was determined by linear regression (Sokal and Rohlf 1995).

To determine if chl *a* extraction time affected the amount of pigment extracted, we examined the fluorescence values of mayflies for extraction times of 2 h and 19 h. The short extraction period allowed the completion of analyses within 1 d. The long extraction time represented overnight extraction. Larvae for these experiments were allowed to feed ad libitum on *Navicula* diatoms for 2 d, after which they were randomly assigned to 2-h ($n = 22$) or 19-h ($n = 21$) extraction periods. A *t*-test was used to determine if chl *a* yields differed significantly between the 2 extraction times.

The ability to freeze and store mayfly larvae would greatly increase the utility of the gut fluorometry technique, so the effect of long-term sample storage on chl *a* levels in mayfly guts was examined over 6 mo. Baetis larvae ($n = 103$) were fed as described above and each individual was frozen immediately at −40°C. Larvae were subsequently selected at random after 0 (i.e., no freezing) 1, 7, 17, 60, 130, and 193 d of freezing. The selected larvae were thawed quickly (<1 min) and immediately analysed for chl *a*. The effects of storage time were tested with a Model I, 1 way ANOVA with 7 time periods (Sokal and Rohlf 1995).

Gut clearance rates of *Baetis* larvae as determined by both gut mass techniques were examined so that suitable field sampling intervals could be estimated quantitatively. *Baetis* larvae were maintained in the feeding chambers for a 36-h period with an unlimited supply of *Navicula*. All algal patches were then removed, and individuals were randomly selected at 1, 2, 4, 8, and 24 h after the end of feeding. *Baetis* larvae were either immediately frozen for later chl *a* analysis or preserved in 10% formalin for DM determination. To determine if these gut fullness techniques produced similar gut clearance rates, the proportion of material remaining in *Baetis* guts was regressed against time since feeding. Slopes of these curves were compared with an ANCOVA (Sokal and Rohlf 1995) after linearizing with a $\log_{10}(x) - \log_{10}(y)$ transformation.

Diel feeding periodicity of Baetis and Heptagenia in the field

Three field studies were undertaken to examine whether patterns in diel foraging periodicity were similar regardless of the type of gut mass analysis used and the taxa involved. For all field sampling, mayflies were collected from replicate riffles over a 24-h period at 3- to 6-h intervals using a U-net sampler (mesh size = 250 μm) (Scrimgeour et al. 1993). *Baetis* (4.0–6.5 mm total length) were collected from Jumpingpound Creek (17–18 July 1993), a 4th-order stream (mean annual discharge = 0.51 m^3/s) located in the foothills of the Rocky Mountains (lat 51°9'N, long 114°31'W). Mayflies were also collected twice from the larger South Saskatchewan River (mean annual discharge = 259 m^3/s) near Saskatoon, Saskatchewan (lat 52°15'N, long 106°37'W) on 6–7 July 1993 (*Baetis* and *Heptagenia*) and 21–22 April 1994 (*Baetis* only).

Individuals were randomly assigned for gut content quantification using either the DM or fluorometry technique. Individuals selected for the DM technique were preserved in 10% formalin, whereas those for fluorometry were frozen immediately and stored in the dark prior to analysis. One-way ANOVAs (i.e., 4–10 time periods) assessed whether gut fullness differed over the 24-h sampling periods. Data were log(x) transformed to equalize variances.

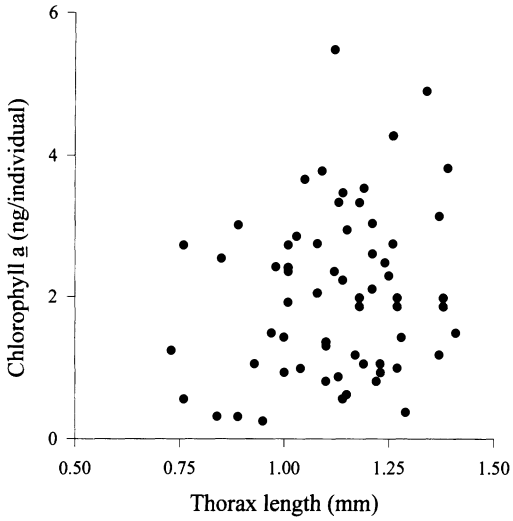

FIG. 1. Relationship between the amount of chlorophyll *a* extracted from *Baetis* larvae starved for 36 h and larval size (thorax length).

Results

Laboratory experiments

The amount of chl *a* in *Baetis* guts did not differ with length of extraction time, duration of sample storage or with larval length. Chlorophyll *a* concentrations extracted from *Baetis* starved for 36 h ranged from 0.25 to 5.5 ng/individual, but no relationship was observed between mayfly size and chl *a* concentration (Fig. 1, $r^2 = 0.03$, $F_{1,75} = 3.22$, $p = 0.08$). Furthermore, although the mean (±1 SE) background chl *a* for starved *Baetis* (2.12 ± 0.14 ng/individual) was higher than the detection limit of the fluorometer (0.8 ± 0.4 ng), *Baetis* mayflies fed on *Navicula* contained at least 10× more chl *a* (25.8 ± 4.9 ng/individual) than starved larvae. Thus, a correction factor for chl *a* background levels was not applied in further experiments.

Ethanol extraction time did not significantly affect the amount of chl *a* recovered from fed *Baetis* larvae. Chlorophyll *a* yields did not differ significantly whether extraction continued for 2 or 19 h (2 h = 21.1 ± 2.7 ng/individual, 19 h = 17.8 ± 2.5 ng/individual; $t_{20} = 1.21$, $p = 0.24$). Similarly, chl *a* levels did not differ between fresh larvae (day 0) and larvae that had been frozen at −40°C for up to 6 mo (Fig. 2, $F_{6,96} = 0.18$, $p = 0.98$).

Gut fullness measured as chl *a* and DM de-

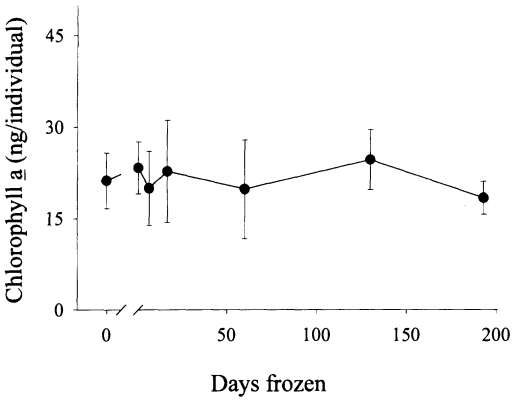

FIG. 2. Chlorophyll *a* biomass (mean ±1 SE) extracted from the guts of fed *Baetis* mayflies after storage for up to 6 mo at −40°C (day 0 larvae were not frozen).

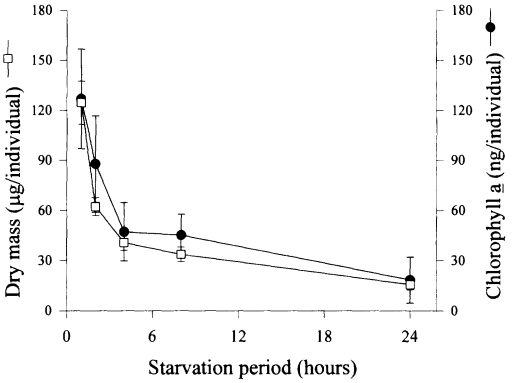

FIG. 3. Gut clearance (mean ±1 SE) of *Baetis* larvae at 10°C over 24 h as measured by either the fluorometry method or dry mass method.

clined rapidly over a 24-h period (Fig. 3). These relationships were non-linear, negative exponential functions, which indicated that most (>65%) gut mass was lost within the first 4 h. There was no significant difference in relative rates of gut clearance (i.e., log proportion of gut mass remaining vs log starvation time) between the 2 techniques (ANCOVA, homogeneity of slopes: $F_{1,6} = 0.005$, $p = 0.95$). In addition, the proportion of material in the gut was not significantly affected by whether gut mass was measured by DM or gut fluorescence ($F_{1,7} = 1.12$, $p = 0.33$). After 24 h, both techniques indicated similar levels of gut fullness relative to initial gut contents (i.e., ~13–14% of initial amounts).

Diel feeding periodicity of Baetis and Heptagenia in the field

Observed patterns of gut fullness for *Baetis* larvae were similar for both gut content techniques (Fig. 4). In Jumpingpound Creek, DM and chl *a* content of gut contents were significantly affected by time of day in July 1993 (single factor ANOVA: chl *a* − $F_{9,154} = 6.99$, $p < 0.001$; DM − $F_{9,138} = 52.7$, $p < 0.001$), indicating a nocturnal feeding periodicity (Fig. 4A). *Baetis* larvae were also nocturnal in the South Saskatchewan River during July (Fig. 4B) when gut masses were higher at night (Tukey's HSD, $p < 0.05$). Both techniques showed that gut fullness was significantly affected by time (single factor ANOVA: chl *a* − $F_{3,62} = 10.04$, $p < 0.001$; DM − $F_{9,62} = 44.31$, $p < 0.001$), with maximum gut fullness occurring at night. Although both techniques showed concordance in peak foraging period, chl *a* levels declined more slowly than levels of DM in the gut with the onset of daylight.

In contrast to the nocturnal foraging patterns observed in July, *Baetis* foraging was distinctly diurnal (Fig. 4C) in the South Saskatchewan River in April 1994. Chlorophyll *a* and DM were significantly affected by time (single factor ANOVA: chl *a* − $F_{6,91} = 3.59$, $p = 0.003$; DM − $F_{6,101} = 9.72$, $p < 0.001$) and comparisons of treatment means showed that gut masses were significantly higher during the day than at night (Tukey's HSD, $p < 0.05$). As noted for *Baetis*, there was a strong concordance in feeding periodicity between the techniques.

Diel periodicity of *Heptagenia* sp. differed between the 2 gut mass techniques (Fig. 5). The DM technique showed that gut fullness was significantly affected by time of day (single factor ANOVA: $F_{3,54} = 18.03$, $p < 0.001$) with peak feeding occurring at night, whereas the fluorometry technique showed no difference through time (single factor ANOVA: $F_{3,27} = 0.10$, $p = 0.96$).

Discussion

Quantification of diel activity patterns and rates of food intake are important components of many ecological studies that aim to identify the spatial and temporal domain within which predators and their prey interact (Kohler and

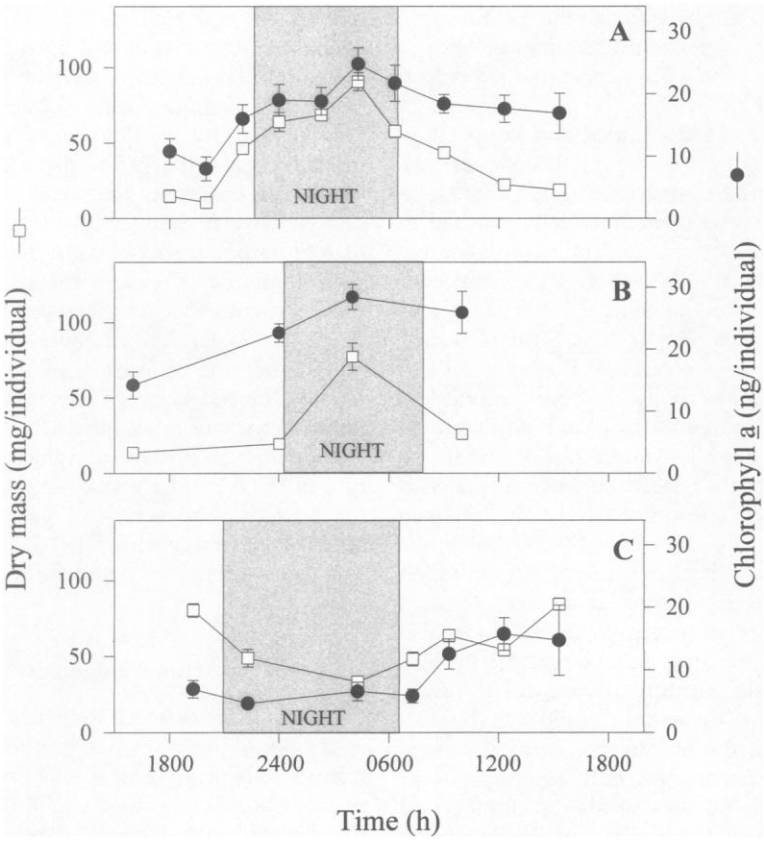

FIG. 4. Gut fullness (mean ±1 SE) of *Baetis* larvae over a 24-h period as measured by either the fluorometry method or dry mass method in (A) Jumpingpound Creek, July 1993, (B) the South Saskatchewan River, July 1993, and (C) the South Saskatchewan River, April 1994.

McPeek 1989, Allan et al. 1991, Culp and Scrimgeour 1993, Cowan and Peckarsky 1994). These data are often the basis for subsequent work requiring a considerable investment of resources. Thus, researchers must understand the limitations of alternative assessment tools (i.e., gut fluorometry and DM techniques) for quantifying food intake by macroinvertebrates in lotic ecosystems.

A primary objective of this study was to im-

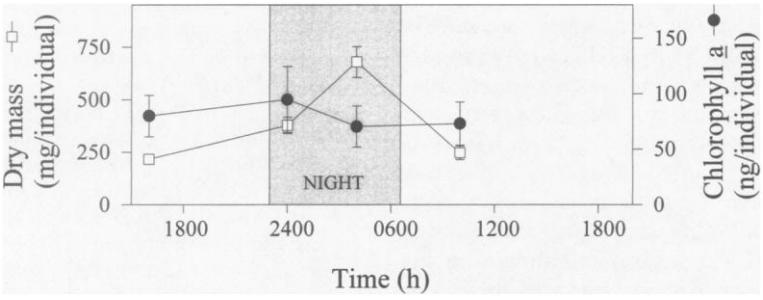

FIG. 5. Gut fullness (mean ±1 SE) of *Heptagenia* larvae over a 24-h period as measured by either the fluorometry or dry mass method in the South Saskatchewan River, July 1993.

prove the field applicability of the gut fluorometry method by determining sources of error in the technique, including concerns related to background chl *a* levels in the gut, variations in extraction time of gut samples, and sample degradation during freezer storage (Nicolajsen et al. 1983). Our study first demonstrated that background chl *a* levels from algal cells attached to the exoskeleton, or retained in the gut for extended periods after cessation of feeding, contributed <10% to the total chl *a* content and were unrelated to larval size. Similar results were obtained by Cowan and Peckarsky (1990) for *Baetis bicaudatus* (body only background levels = 3–6 ng/individual). Second, although extraction times for algal samples reported in the literature typically ranged between 6 and 24 h (e.g., Nusch 1980, Jespersen and Christoffersen 1987, Cowan and Peckarsky 1990, 1994), we found shorter extractions (i.e., 2 h) provided good results for macerated larvae when the first 5 min of extraction occurred at 80°C. We also observed that freezing *Baetis* larvae for up to 6 mo did not significantly reduce chl *a* yields, thereby allowing increased flexibility in the storage and processing of samples. Third, the number of larvae needed for both techniques was low (n = 15), and was similar to numbers of *Baetis bicaudatus* used by Cowan and Peckarsky (1990). Although our results provided useful guidelines, we caution that researchers should confirm the appropriate sample sizes for their study animals, and check background chl a levels for species populations with a large size range.

Diel feeding periodicity of lotic macroinvertebrates in the field is usually determined by collecting individuals at intervals of up to 6 h (Winterbourn 1974, Allan et al. 1991, Culp and Scrimgeour 1993, Cowan and Peckarsky 1994). Our laboratory feeding trials of *Baetis* larvae fed an ad libitum supply of *Navicula* showed that most of the meal (65%) was digested within the first 4 h. Similar clearance rates for *Baetis* have been measured using gut fluorescence (Cowan and Peckarsky 1994) or by visual estimates (Williams and Levens 1988). Thus, the appropriate sampling interval for these larvae should be 4–8 h for diel foraging studies at temperatures around 10°C. Clearly, sampling should be sufficiently frequent to detect changes in feeding rate such that the intervals between collections is less than the period required for gut evacuation (Baars and Helling 1985, Wang and Conover 1986, Ramcharan and Sprules 1988).

Our study indicated that both fluorometry and DM techniques were suitable to quantify feeding activity of *Baetis* and perhaps other mayfly grazers that primarily ingest algal material. For example, there was strong concordance between changes in gut mass measured using fluorometry and DM in *Baetis* larvae from Jumpingpound Creek. Concordance between the techniques was also observed for *Baetis* from the South Saskatchewan River. In contrast, observed patterns of feeding periodicity for *Heptagenia* differed between the techniques. Thus, fluorometry will be of little use for scraper-collectors such as *Heptagenia*, which ingest a mixture of FPOM and algae, or in situations where the primary food source of the invertebrate is unknown. In these situations, the DM technique is a more robust and reliable measure of gut fullness.

Acknowledgements

We are grateful to G. Richards for assistance in the field. The work was supported by funds from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Environment Canada (National Water Research Institute) to JMC. GJS was supported by postdoctoral fellowships from NSERC, the Izaak Walton Killiam Memorial Fund, and funds from the Sustainable Forest Management Network.

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Received: 1 December 1998
Accepted: 17 January 2000