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Influence of handling stress and fasting on estimates of ammonium excretion by tadpoles and fish: recommendations for designing excretion experiments

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Abstract

Excretion rate estimates are important for linking consumers to biogeochemical processes. Short-term incubations in chambers are a common approach for studies. This, however, may result in inaccuracies due to a well-documented decline in excretion with time, which is often attributed to fasting. An alternative explanation, however, is that excretion slows during recovery from handling stress. Whereas shorter incubations may reduce fasting effects, longer incubations can allow for recovery from handling. Although both views have merit and are not mutually exclusive, attempts to identify optimal incubation times are lacking. We examined effects of handling stress and fasting on NH_4^+ excretion by omnivorous tadpoles and predatory fish. Fasting did not influence tadpole excretion for at least 6 h. Rather, handling stress appeared to cause initially elevated excretion, and thus longer incubations were appropriate. Fasting and handling stress influenced fish excretion, and intermediate incubations (~30 min) appeared most appropriate. We also found that ammonium measurements were confounded by increasing concentrations of excretory products in longer incubations. We recommend that studies include incubation time trials and correct for potential matrix effects. Effects of handling and fasting likely vary with organism and experimental procedures, and both can be accounted for using the procedures we describe.

Introduction

Consumer communities in freshwater systems influence nutrient cycling in many ways (Wallace and Webster 1996; Vanni 2002). Excretion by consumers, in particular, is an important source of dissolved nutrients for microbes and primary producers (Vanni 2002; Cross et al. 2005). A recent surge of interest in the link between consumers and cycling of limiting nutrients in freshwater systems has fueled a demand for accurate estimates of excretion rates of a variety of aquatic organisms.

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While excretion rates can be estimated using bioenergetic or mass-balance models (e.g., Randall and Wright 1987), many investigators have opted to measure excretion directly by incubating organisms in containers and measuring changes in nutrient concentrations in the surrounding water (e.g., Vanni et al. 2002; Hall et al. 2003). While there is evidence that both procedures yield similar results, there are only a handful of studies that allow for meaningful comparison (Vanni 2002). More than three decades ago, Ikeda (1977), a zooplankton ecologist, noted "in most of the studies on respiration and excretion of zooplankton so far reported, the experimental procedures appear to be determined by each worker's convenience." This situation seems to have changed little since this observation. In our experience, as well as that of Ikeda (1977), one outstanding weakness in the choice of method for estimating excretion rates is the lack of understanding of the effects of incubation duration.

Our recent investigations of excretion rates of neotropical tadpoles and fish showed that their excretion represented a significant source of NH_4^+ in neotropical streams (Whiles et al. 2006; McIntyre et al. in press). However, during experiments

where > 100 different tadpoles, representing a range of body sizes, were incubated for different lengths of time (e.g., 15 min versus 2 h), we found that excretion rates varied considerably and systematically as a function of incubation time (e.g., 3-6 × higher rates in shorter incubations; Fig. 1; Whiles et al. unpubl. data). In his review of the topic, Vanni (2002) noted this same issue, hypothesized that this was due to incipient fasting, and considered values derived from long incubation times inaccurate. Vanni's (2002) hypothesis is well supported by numerous observations of declines in excretion rate with time since feeding in many aquatic organisms [e.g., Mayzaud 1976 (marine copepods and a chaetognath), Regnault 1981 (a crangonid shrimp), Quetin et al. 1980 (a mysid shrimp), Pickering at al. 1982 (salmonid fish), Boucher-Rodoni and Mangold 1985 (cephalopod), Torres et al. 1996 (larval sciaenid fish), Devine and Vanni 2002 (benthic insects and oligochaetes), Glaholt and Vanni 2005 (centrarchid fish); a quick perusal of the literature will provide numerous additional examples]. The effect of incipient fasting on ammonium excretion can be particularly evident for taxa with high protein diets.

In the case of omnivorous or herbivorous fishes and tadpoles, with low protein diets, relatively long guts, and slow gut-passage times, a rapid decline in ammonium excretion once feeding stops may not be observed. For example, Chakraborty and Chakraborty (1998) studied the effect of different levels of protein content of food rations on ammonium excretion by Indian major carp (*Labeo rohita*) fingerlings and showed that excretion rates of ammonium continued to increase for at least 8 h following cessation of feeding. Similarly, Nutall and Richardson (1991) showed that ammonium excretion by European carp (*Cyprinus carpio*) continued to rise 10 d following cessation of feeding. In such cases, following the logic of Vanni (2002), a short incubation time would result in an underestimate of actual ammonium excretion. We expect a scenario similar to omnivorous fishes, such as carp, for macrophagous tadpoles feeding on periphyton and associated detrital particles (S.M. Secor, Univ. of Alabama, pers. comm.). Given this expectation, why, in our excretion experiments with tadpoles, did we observe a consistent, rapid decline as a function of incubation time (Fig. 1)?

In this study, we test an alternative to the hypothesis that the decline in ammonium excretion that we observed was due only to incipient fasting. Our alternative hypothesis is that higher excretion rates that are often observed during the initial stages of incubations are due to an initial elevation in ammonium release associated with capture and containment, followed by a gradual return to resting levels. There is ample reason to suspect that high, initial excretion rates by some aquatic vertebrates may be due to stress associated with capture, handling, and the initial introduction into incubation containers. For tadpoles, in particular, there are a number of studies suggesting elevated rates of NH_4^+ excretion as a response to handling or when attacked by predators (Kiesecker et al. 1999; Manteifel et al. 2005; Wilding and Kerschbaum 2007). For freshwater fishes, ammonium excretion increases in response to exercise have been documented, apparently due to an increase in the gill-diffusing capacity for ammonium (Walsh 1998). For salmonids, in particular, a 4-fold increase in ammonium excretion for several h following exhaustive activity has been shown (Randall and Wright 1987). Given that aquatic vertebrates are subject to physical stress during capture, resulting in heightened activity, a stress-related spike in rates of ammonium excretion immediately following handling and during initial confinement should be anticipated. For some taxa, particularly predators with rapid gut-passage times, this hypothesized spike could be confounded with a reduction in excretion rates due to fasting. For others, this hypothesized stress-induced spike may be uncoupled from changes in excretion rates due to cessation of feeding.

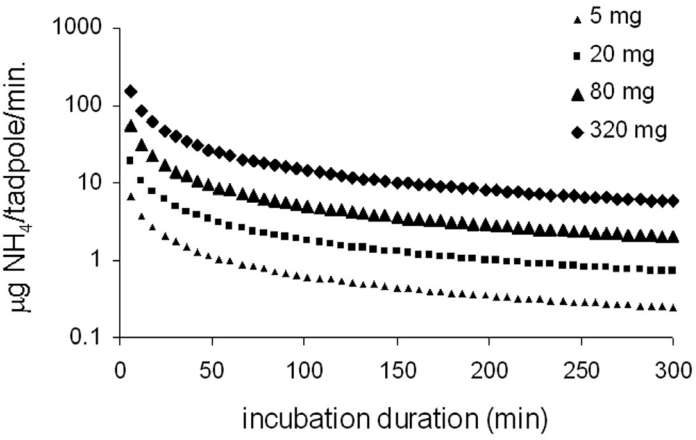

Fig. 1. Modeled excretion rates for different sized tadpoles in Panamanian streams. Lines are predicted excretion rates over time based on a multiple regression model performed on values obtained from 110 individual tadpoles incubated in chambers for time periods ranging from 18 min. to 5 h. Multiple regression model was highly significant ($R^2 = 0.69$, $P < .0001$).

We tested our hypothesis via experiments that specifically assessed the effects of stress and hunger on the time-course of excretion rates of tadpoles and fish held in incubation containers. Based on our past observations that tadpole excretion rates obtained from short incubation times (e.g., 20 min) were much higher than those obtained from longer incubations (e.g., 1-3 h), we specifically predicted that a stress response associated with capture and placement in the incubation chambers would cause high initial rates of NH_4^+ excretion. These initial high rates would then gradually slow as excretion rates returned to resting levels. We also predicted that the trajectory of change of excretion rates with time would be independent of feeding regime (i.e., fasting would not be a factor during the 6-h duration of our experiments). While assessing the influences of stress and handling, we also examined the potential for increasing concentrations of salts and dissolved organics in longer incubations to confound ammonium

estimates (e.g., potential matrix effects). Although we focus on omnivorous tadpoles and a predatory fish, our goal was to outline an approach for assessing the effect of incubation duration on organisms used in experiments designed to measure excretion rates for ecological studies.

Materials and procedures

We collected spring peeper (*Pseudacris crucifer*) and western chorus frog (*P. triseriata*) tadpoles from a fishless pond in Jackson County, IL on 19 May 2006. Tadpoles ranged from 20-40 mm in total length and had not developed limbs. We electroshocked brook trout (*Salvelinus fontinalis*) from beaver ponds adjacent to the East River, Gunnison County, CO on 8 July 2007 and 18 June 2008. For each experiment, tadpoles and fish were immediately placed in groups of ~25 individuals in holding tanks containing only filtered pond or stream water. No food was placed in holding containers. Holding tanks consisted of ~19 L plastic buckets for tadpoles and 30 L plastic coolers placed in the stream for brook trout, with galvanized hardware cloth (6.4 mm mesh) suspended ~10-15 cm above the bottom. The hardware cloth functioned as a screen that separated animals from their feces to ensure that coprophagy did not occur. Holding containers were randomly assigned fasting periods of 0, 1, 2, and 6 h for tadpoles and 0, 1, 2, and 4 h for fish. A longer maximum period of fasting was used for tadpoles (6 h versus 4 h for trout) because of their longer gut tracts. Fasting periods represented elapsed time before measurements of NH_4^+ excretion rates were initiated.

At the termination of each fasting period, groups of 16 tadpoles or fish were removed from their holding chambers, randomly placed in clear, plastic 60 mL centrifuge vials (tadpoles) or 2 L plastic bags (fish) for ~10, 30, 60, and 120 min (one individual per vial or bag). Tadpole total body length measurements (nearest mm) were made by holding tubes against a ruler when tadpoles were not moving, such that no additional handling occurred. Tadpole length was converted to ash-free dry mass (AFDM) using length-mass relationships developed by us following procedures of Benke et al. (1999). Fish standard length (nearest mm) and wet body mass (WM; nearest 0.1 g) were measured in the field after incubations were finished. Individual mass was used to calculate mass-specific excretion rates.

A subset of 3-4 individuals from each fasting treatment were placed in vials or bags and immediately removed to correct for NH_4^+ that might be introduced with animals. Values for these vials were averaged and subtracted from the NH_4^+ concentrations measured in the 10-120 min incubations.

Following removal of tadpoles and fish, NH_4^+ concentrations in the water remaining in the centrifuge vials were immediately measured following method of Holmes et al. (1999), as modified by Taylor et al. (2007). Modifications in Taylor et al. (2007), correct errors in the methods used to adjust for matrix effects and background fluorescence. All fluorometric measurements were made within 5 h of the termination of each incubation trial using a Turner Designs 10-AU or Trilogy fluorometer (Turner Designs, Inc.).

During the trout experiment, we also examined the degree to which compounds released by fish (e.g., dissolved organic matter and salts) affected measurements of NH_4^+ excretion rates (matrix effects) by estimating NH_4^+concentrations using a deionized water standard curve and the standard additions method described by Taylor et al. (2007). The standard additions method involves adding serial spikes of NH_4^+ to sample water to create a standard curve that adjusts for matrix effects. Matrix effects are caused by substances other than the analyte (e.g., dissolved organic matter and salts) that suppress or intensify the response signal (Taylor 1989). Preparing a standard curve by adding different amounts of NH_4^+ to deionized water does not adjust for matrix effects as accurately or precisely.

Excretion rates across time (= slopes) for fasting treatments were compared with analysis of covariance (ANCOVA, α = 0.05) performed on log-transformed data.

Assessment

Average tadpole NH_4^+ excretion rates (± 1 SE) for fasting treatments ranged from a low of 0.28 ± 0.029 μg/mgAFDM/min for the 2 h treatment to a high of 0.33 ± 0.03 for the 1 hour treatment. Average fish NH_4^+ excretion rates (± 1 standard error) ranged from a low of 0.198 ± 0.023 μg/gWM/min for the 4 h fasting treatment to a high of 0.342 ± 0.040 for the treatment with no fasting. For both fish and tadpoles across all treatments, there was a significant, exponential decline in excretion rates with incubation time; the slopes of these relationships were similar (ANCOVA interaction term, tadpoles: $P = 0.96$; fish: $P = 0.33$; Figs. 2,3). For tadpoles, elevations of slopes did not differ among treatments (ANCOVA, $P = 0.18$), indicating there was no effect from fasting on tadpole excretion rates during the 6 h duration of the experiment (Fig. 2). Rather, initially high rates appeared linked to a short-term increase in NH_4^+ excretion during the initial period of incubation. For fish, elevations of slopes declined significantly as fasting time increased (ANCOVA treatment term, $P = 0.002$), indicating there was a fasting effect on fish excretion rates (Fig. 3). However, the exponential decline with increasing incubation was due only partly to fasting, as similar patterns were evident in the no fasting and fasting treatments.

Average fish excretion rates (± 1 SE) estimated with deionized water standard curves (0.347 ± 0.059 μg/g/min), and hence, not corrected for matrix interference, were 1.7× higher than rates corrected for matrix interference with the standard additions method (0.199 ± 0.024 μg/g/min). All fish excretion rates estimated with the standard additions method declined with incubation time; whereas, rates estimated with a deionized water standard curve did not decline with time (ANCOVA interaction term, $P = 0.003$) (Fig. 4), indicating that correcting for matrix effects is more important with longer incubations.

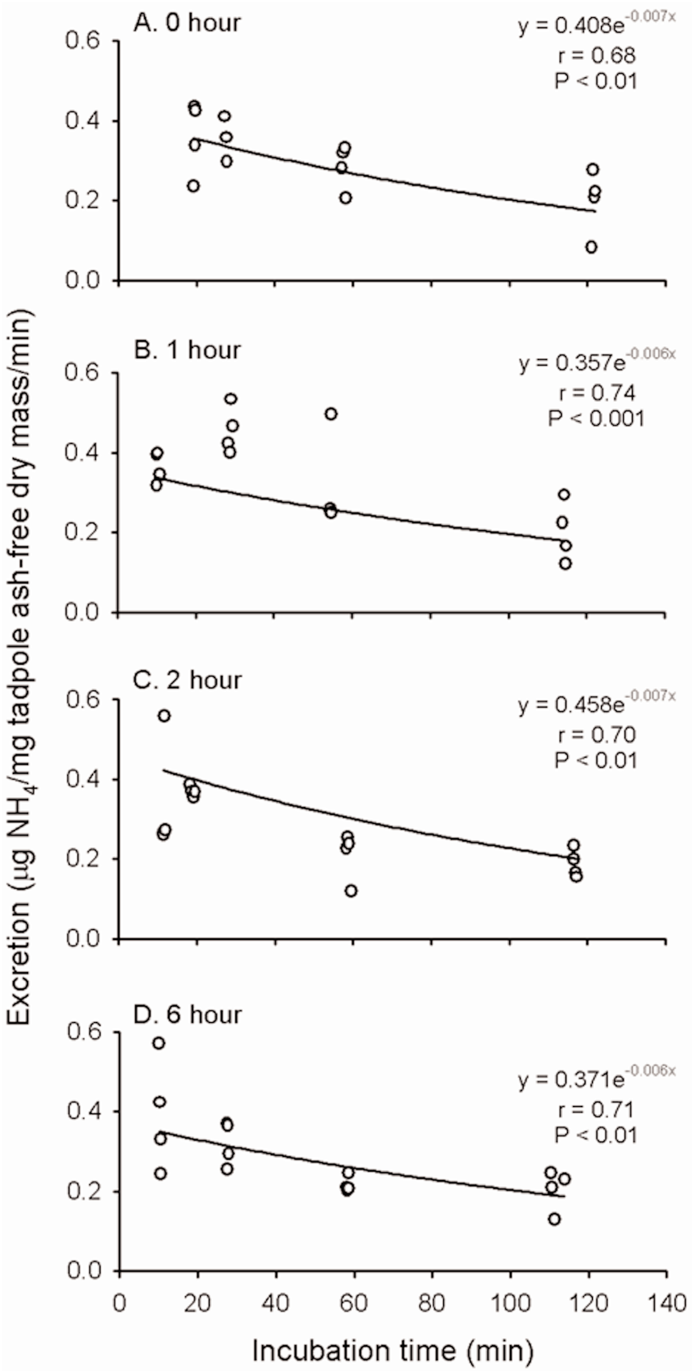

Fig. 2. Excretion versus time for tadpoles fasting for 0 (A), 1 (B), 2 (C), or 6 (D) h. For each fasting treatment, replicates of 4 individuals were removed and ammonium was measured in chambers at ~10, 20, 60, and 120 min. Slopes for all treatments are similar (ANCOVA, $P = .96$), and there was no effect of fasting (slope elevations do not differ [ANCOVA, $P = .18$]).

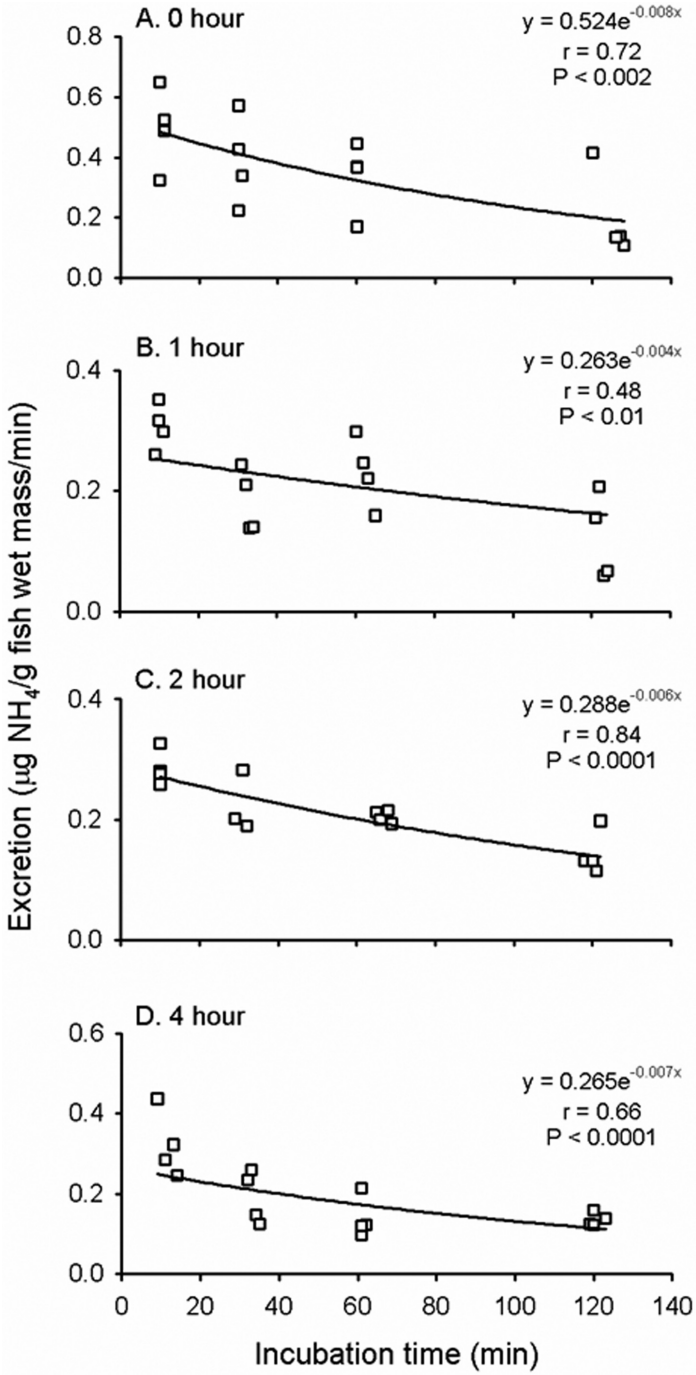

Fig. 3. Excretion versus time for fish fasting for 0 (A), 1 (B), 2 (C), or 4 (D) h. For each fasting treatment, replicates of 4 individuals were removed and ammonium was measured in plastic bags placed in a cooler at ~10, 20, 60, and 120 min. Slopes for all treatments are similar (ANCOVA interaction term, $P = .33$), and there was a significant effect of fasting on excretion (ANCOVA treatment term, slope elevations differ [ANCOVA, $P = .002$]). Note different y-axis scales for some treatments.

Discussion

A number of studies have shown elevated rates of NH_4^+ excretion by tadpoles related to handling stress or when attacked by predators (Kiesecker et al. 1999; Manteifel et al. 2005; Wilding and Kerschbaum 2007). Other studies have

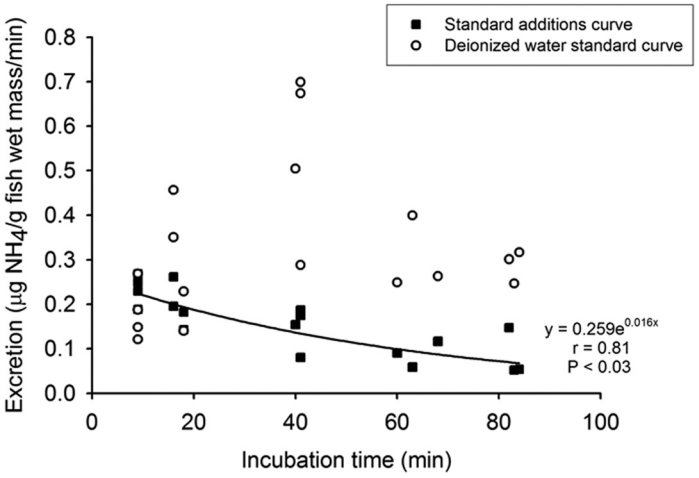

Fig. 4. Excretion rates for fish measured using deionized water standard curves and the method of standard additions that corrects for matrix effects caused by excreted dissolved organic matter and salts that can intensify the fluorometric response signal. The slopes are significantly different (ANCOVA interaction term, $P = .003$).

shown an increase in ammonium excretion by fish following intensive exercise (Randall and Wright 1987). Thus, handling stress is a logical explanation for initially high excretion rates when tadpoles, fish, and presumably other aquatic organisms, are placed into incubation chambers. Accordingly, an initial elevation in metabolic rates and excretion should be anticipated, followed by a decline as the study animal acclimates. The degree of handling stress, and thus the magnitude of the initial spike in excretion, will undoubtedly vary with type of organism (e.g., invertebrate versus fish), size of organism relative to the chamber, method of capture, and other, related specifics of a given study. As such, a study design that accounts for changes in excretion rates over time, as presented here, should be used for any new investigations of excretion rates, as it will allow the investigator to assess and account for the effects of incubation length on results, which can be substantial. For example, given a brook trout biomass of 6.9 g WM/m^2, the excretion rate for brook trout based on short incubations (10 min) would be 2.6 µg NH_4-N/m^2/min versus 0.97 µg NH_4-N /m^2/min for longer incubations (120 min), or a 2.7-fold difference.

When fasting effects are not evident, as was the case with tadpoles in our study, investigators can use the time series relationships derived from this experimental design to estimate baseline excretion rates using a non-linear regression model (Seber and Wild 1989) of the form:

$$Y = \alpha + \beta e^{-\gamma t} + \varepsilon$$

where Y is the excretion rate at time t, α is the baseline excretion rate, β is the increase caused by systematic factors (e.g., handling stress) contributing to initially high excretion rates, γ is the rate at which the effect of handling stress decays over time, and ε is a normally distributed error term. Note that average excretion rate for this model would be $\alpha + \beta$ at $t = 0$, while it approaches α as $t \to \infty$. Using pooled data from all treatments in our tadpole experiment (because fasting treatments did not influence slopes in this case) in this model with PROC NLIN in SAS 9.1 (SAS Institute Inc. 2005), we obtained the following estimates and 95% confidence intervals for the three parameters: $\hat{\alpha}$ = 0.0833 (–0.3012, 0.4679), $\hat{\beta}$ = 0.3340 (–0.0030, 0.6710) and $\hat{\gamma}$ = 0.0096 (–0.0105, 0.0297). The overall model was highly significant ($F_{2,60} = 24.53$, $P < 0.0001$) and the mean value of $\hat{\beta}$ suggests that handling stress produced a substantial increase in the excretion rate over the baseline rate $\hat{\alpha}$. We also found that the confidence intervals of the model parameters for the tadpole incubations were broad. This argues for even longer incubations than two h in cases when fasting is not an issue, which would extend the observation times until the excretion rate was closer to its asymptotic baseline value of α; excretion rates in our experiment were greater than $\hat{\alpha}$ = 0.0833 even after 2 h had elapsed. Longer incubations would help determine if decay in excretion rates is truly exponential or of some other form. However, one potential problem with longer incubations in closed chambers (i.e., not flow-through) is that nitrogenous waste can build up and decrease the diffusion gradient for excretion, thereby slowing the animal's excretion rate. Variability in model parameters can also be reduced by taking more frequent measurements; in the case of our tadpole study, an additional measurement at 90 min may have provided a greater level of precision.

A different model is needed when fasting reduces excretion, as in our brook trout example. In this case, suppose that well-fed and unstressed fish have an excretion rate of α. Fasting might produce an exponential decline in the excretion rate to some level below α, say $\alpha - \delta$, where δ is the maximum reduction in the excretion rate. Handling stress could also increase initial excretion rates, similar to the previous model for tadpoles. Considering both factors, the excretion rate could then be described as

$$Y = \alpha - \delta(1 - e^{-\theta(t+t_s)}) + \beta e^{-\gamma t} + \varepsilon$$

where θ is rate at which the fasting effect increases over time and t_s is the fasting time, with the other parameters defined as before. The objective here is to estimate α, the excretion rate free of the fasting and handling effects. This could prove difficult using a single experiment, because excretion rates would be influenced by both effects throughout the measurement period. However, the experimental design we present includes different fasting periods, providing information on the time course of handling and fasting effects, as well as α and δ. We fitted the above model to the trout data (combined across all fasting treatments) and obtained the following estimates and 95% confidence intervals: α = 0.4464 (0.3180, 0.5748), δ = 0.3033 (0.1934, 0.4131), θ = 0.0174 (0.0058, 0.0291), β = 0.1526 (0.0621, 0.2431) and γ = 0.0309 (–0.0144, 0.0763). The overall model was highly significant ($F_{4,59} = 21.77$, $P < 0.0001$). These results indicate a significant effect of fasting on excretion

(the interval for δ does not overlap zero). The half-life of the the fasting and handling stress effects were 39.8 and 22.4 min, respectively. The excretion values in the experiment that appear closest to our estimated α occurred for incubation times ~30 min, but this, of course, was unknown until the model was fitted.

In cases where fasting reduces excretion rates but handling stress is not a factor, short incubations (e.g., durations less than when rates begin to decline from fasting) could be used. However, we suspect that few animals are indifferent to handling, and an experimental design like we present here is still needed to initially assess potential handling stress effects and optimal incubation periods for a given animal.

Fish and tadpoles can also excrete compounds, such as dissolved organic matter and salts, which can interfere with nutrient analyses by either suppressing or intensifying the response signal of the samples compared with standards prepared in deionized water (e.g., matrix effects). We found that this effect increased with incubation time and was not corrected for using standard curves made with deionized water, but was corrected for using the method of standard additions. We attribute the higher rates obtained from the deionized water standard curve to excretion and accumulation of salts that are known to intensify the response signal in fluorometric analyses (Holmes et al. 1999; Taylor et al. 2007). Given the results of our experiments, we cannot recommend shorter incubation times to avoid matrix effects. Rather, longer incubations that account for handling stress, corrected for matrix effects using the standard additions method, are likely most accurate.

Comments and recommendations

The development of simple, field-based protocols for measuring excretion rates by aquatic consumers have greatly increased our understanding of the roles of consumers in nutrient cycling in freshwater ecosystems. Our results, using omnivorous tadpoles and predatory brook trout, suggest that estimates based on short incubation periods, particularly in cases where fasting for a few hours does not change rates, may result in serious overestimates of excretion rates, and may thus inflate the roles of these consumers in N mineralization. On the other hand, results of other studies that have focused on omnivorous fishes (Nutall and Richardson 1991; Chakraborty and Chakraborty 1998) indicate that incubation times as long as 8 h or more may underestimate excretion rates. Clearly, no single incubation period is optimal for all study organisms. Assuming that changes in ammonium excretion rates are linked to general metabolic rates, this is also likely the case for assessments of phosphorus excretion, which are often measured in similar fashion.

More than three decades ago, Ikeda (1977, pg. 251) provided a cautionary statement regarding the effect of incubation time on the accuracy of excretion experiments: "there exists a good chance of obtaining anomalous ... rates by means of short incubation periods. A longer incubation time might solve this problem, but would also introduce another factor: a starvation effect..." We similarly caution investigators involved in studies of excretion rates to carefully assess the effects of incubation time and technique on the results of their excretion experiments. It is our experience that investigators involved in such studies often use short incubation times (e.g., ~10-15 min in some cases; e.g., McIntyre et al. in press) to reduce the effect of confinement and fasting on excretion rates. We recommend that investigators use an experimental design like the one we present here and measure excretion rates at multiple incubation times to assess handling stress and fasting effects for the specific organisms of interest. These data, in turn, can then be analyzed with nonlinear regression techniques to produce more realistic estimates of excretion rates and identify optimal incubation periods. Additionally, potential matrix effects should be accounted for when longer incubations are used.

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