Phosphorus Release Patterns and the Effects of Reproductive Stage and Ecdysis in *Daphnia magna*[1]

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Rates of continuous phosphorus release by individual *Daphnia magna* (0.3–0.9 mg dry weight) were measured using high performance liquid chromatography components in conjunction with a specially designed incubation flow cell. Within the temporal resolution of the technique (3 min), phosphorus release often appeared pulsed rather than only steady. We found significant variation in release rates among the different stages of an individual's parthenogenetic reproductive cycle. Rates observed for animals carrying more mature embryos were lower than those earlier in the reproductive cycle. Rates of phosphorus release observed at and after ecdysis averaged 6.7 times higher than rates observed at other times.

Key words: phosphorus, excretion, reproductive stage, ecdysis, *Daphnia magna*

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Les taux de mise en liberté continue de phosphore par des *Daphnia magna* individuelles (0,3–0,9 mg de poids sec) ont été mesurés à l'aide de composantes de chromatographie en phase liquide à haute performance, de concert avec une cellule d'incubation avec débit spécialement conçue. Dans les limites de résolution temporelle de la technique (3 min), la mise en liberté de phosphore semble souvent pulsée plutôt que régulière. Parmi les différents stades du cycle reproducteur parthénogène d'un individu, nous observons une variation significative des taux de mise en liberté. Les taux observés chez des animaux portant des embryons plus développés sont inférieurs à ceux dont le cycle reproducteur n'est pas aussi avancé. Les taux de mise en liberté du phosphore observés pendant et après la mue sont en moyenne de 6,7 fois plus élevés que ceux observés à d'autres périodes.

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THE importance of herbivorous zooplankton in controlling available phosphorus concentration in the epilimnion of stratified lakes has been recognized for some time (e.g. Ketchum 1967; Ganf and Blazka 1974; Richey 1979; Scavia 1979; Lehman 1980a, b). Although forms of phosphorus released by zooplankton have been partially characterized (e.g. Pomeroy

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et al. 1963; Peters and Lean 1973) and rates of this release have been approximately determined (e.g. Barlow and Bishop 1965; Hargrave and Green 1968; Peters and Rigler 1973; Lehman 1980a, b; Scavia and Gardner 1982), mechanisms controlling phosphorus release have not been elucidated.

We here explore two aspects of phosphorus release by *Daphnia magna*. First, we demonstrate that release of phosphorus is sometimes pulsed. Second, we demonstrate dependence of phosphorus release on the parthenogenetic reproductive cycle of *Daphnia magna*. Animals carrying embryos in late stages of development have relatively depressed rates, whereas animals at and after ecdysis have dramatically increased rates. These results have implications for both the design of experiments and nutrient regeneration in the natural environment.

Materials and methods — Continuous soluble reactive phosphorus (SRP) release rates by individual *Daphnia magna* were measured using a flow-through incubation cell (Gardner and Scavia 1981) in conjunction with high performance liquid chromatographic plumbing. Tygon tubing was substituted for Teflon whenever possible, to minimize phosphorus sorption.

Low-nutrient water (LNW), containing 20 mg $CaCl_2 \cdot 2H_2O$, 10 mg $MgSO_4$, and 20 mg $NaHCO_3$ per litre of distilled, deionized water (Lehman 1980a), and all reagents were pumped through the system with a Technicon proportioning pump. Reagents (Murphy and Riley 1962, as adapted for Technicon AutoAnalyzer) were mixed with water coming from the outlet end of the incubation flow cell in a Technicon AutoAnalyzer manifold. Before reaching the manifold, however, LNW passed through an Altex sample injection valve allowing flow to be diverted from the incubation cell to a bypass loop, to obtain a LNW base line or to change animals. This system is similar to that described by Gardner and Scavia (1981) for measurement of continuous release of ammonia plus primary amines, except nitrogen analyzer and pump are replaced by autoanalyzer components.

Absorbance was recorded with time to provide a continuous record of SRP concentration in the outflowing water. To quantify rates, we replaced the LNW input reservoir by a vial containing standard PO_4 solution prepared with LNW. After a fixed period of time (usually 10 min) the input tube was removed from the standard PO_4 solution, rinsed with distilled, deionized water, and placed back in the original reservoir. An absorbance plateau was observed for the standard solution, followed by a blank base line response after the standard solution had passed through the system.

A stock culture of *Daphnia magna* was maintained in the laboratory under constant fluorescent lighting at 20°C in an aerated tank (ca. 4 L) of filtered Huron River (near Ann Arbor, MI) water. Food supply was maintained through addition of algal cells (*Chlamydomonas reinhardti*) obtained by centrifuging portions of a semicontinuous culture grown on full-strength unbuffered WC medium (Guillard and Lorenzen 1972) without silicate at 20°C under continuous light (~700 lx).

Two experiments were performed. The first examined the kinetic mode (i.e. steady or pulsed) of SRP release and compared results for animals fed algal cells prepared two different ways with respect to phosphorus. The second experiment compared release rates for individuals at different stages in their reproductive cycles.

In the first experiment, animals were maintained initially for ca. 24 h at a high ($>2 \times 10^5$ cell $\cdot$ mL^{-1}) concentration of *Chlamydomonas reinhardti* (~35 $\mu m^3 \cdot cell^{-1}$). Then animals, for one treatment, were transferred to a culture containing 2×10^5 to 4×10^5 cell $\cdot$ mL^{-1} for 3 h prior to release rate determinations. A second set of animals was transferred to a culture containing 2×10^5 to 4×10^5 cell $\cdot$ mL^{-1} of P-starved algae and also held for 3 h prior to use. P-starved algae were prepared by diluting 400 mL of the original stock algal culture with 1200 mL of WC media lacking P; this was repeated every other day for 6 d prior to their use. Lack of growth following the final dilution indicates that cells were starved for P. A total of 14 animals fed normally cultured cells and 17 fed P-starved cells were analyzed at room temperature (25–27°C).

In the second experiment, three groups of four to eight animals (2.5–2.9 mm long; 0.2–0.7 mg dry weight) were removed at different times from the stock culture. SRP release rates were measured at 20°C during different stages of their reproductive cycles beginning at least 24 h after removal from the stock culture. Individuals were maintained in separate beakers (~100 mL) in which LNW and algal food (*C. reinhardti*) were replaced daily. The concentration of cells in each beaker was maintained in excess of 5×10^5 cells $\cdot$ mL^{-1} (~18.5×10^6 $\mu m^3 \cdot mL^{-1}$) to ensure maximal feeding rates. The incipient limiting food level for *Daphnia magna* is in the range 2.8×10^6 to 6×10^6 $\mu m^3 \cdot mL^{-1}$ (Kersting and van der Leeuw 1976).

Before release rate measurements, each animal was examined in a depression slide under a dissecting microscope to determine length, measured with an ocular micrometer from tip of the helmet to base of the posterior spine, and reproductive state, including stage and number of embryos present in brood chamber. Embryos were staged as follows: stage I represents eggs prior to any visible differentiation aside from the formation of fat cells; stage II is from differentiation until eyespots become visible; and stage III is from this point until the embryos are released. This classification is similar to that used by Stross et al. (1961) and Edmondson (1955). The short stage between release of young and ecdysis was classified as premolt (P). After this examination (30–90 s) the animal was rinsed in LNW, placed in the incubation flow cell, and the valve switched to direct water past the animal.

SRP release in both experiments was measured for ~30 min to observe release patterns. Recorder response was integrated for the period 5–10 min after beginning in the first experiment and for the period 4–14 min in the second experiment to quantify release rates immediately after the animal was removed from the food. Recall that animals were fed continuously until removed for measuring release rates.

SRP release rates, which in the first experiment were related to wet weight, were converted to dry weight basis by a constant factor (dry weight:wet weight $\pm$ SE = 0.100 ± 0.005; $n = 47$). Wet weights were determined by gently drying each animal with tissue (ca. 3 min) and weighing on a Sartorius six-place balance (precision = ca. 1 μg). Dry weights were determined on animals dried for 48 h at 60°C in preweighed Al planchetts.

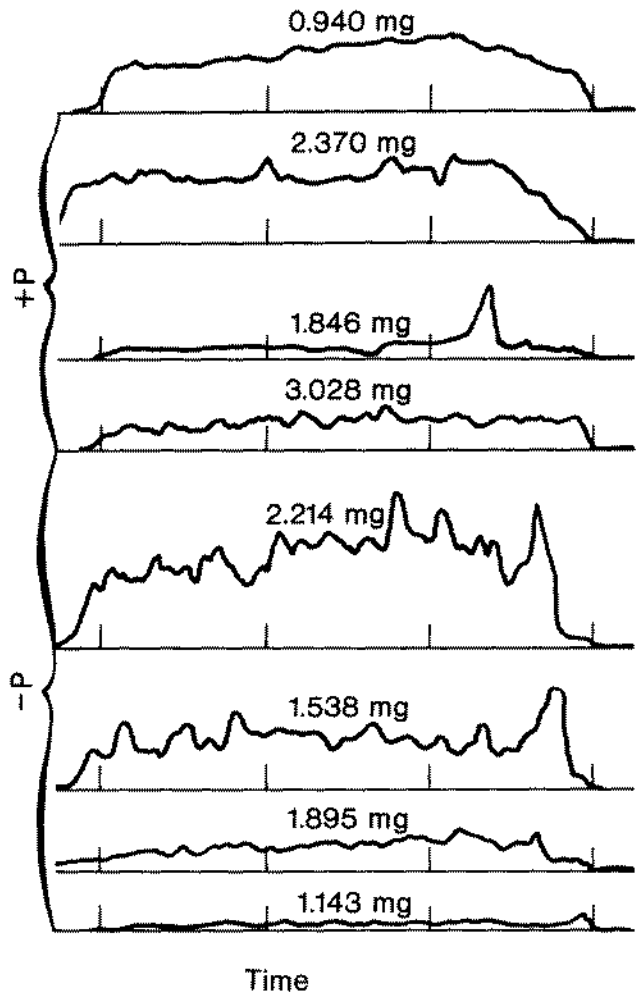

FIG. 1. Representative continuous traces of soluble reactive phosphorus release for 30 min after animals were removed from their food. +P represents animals fed for 3 h on algae grown on full-strength medium. −P represents animals fed for 3 h on P-starved algae. Animal wet weight is indicated on each trace. Height of trace is proportional to SRP release per individual.

Weight-specific release rates in the second experiment were based on a length–weight relationship determined from 30 animals. Each experimental animal's length and dry weight were determined to ensure that it was consistent with the previously determined relationship. Then, the animal's dry weight for each stage of the reproductive cycle was estimated from its length measured at that stage.

Results and discussion — Phosphorus release, in both experiments, was often pulsed. Representative continuous traces of SRP release from the first experiment are shown in Fig. 1. Taylor and Lean (1981) found similar sporadic releases of ^{32}P by the ciliated protozoan, *Strombidium viride*, and the rotifer, *Keratella chochlearis*, although our observed pulsing appears to be at high frequency. These results contrast with the nonpulsed NH_4 release rates observed previously for *D. magna* fed a soyflour/bacteria mixture (Gardner and Scavia 1981). In most cases, pulsing here appeared superimposed upon a base rate; hence it is reasonable to assume

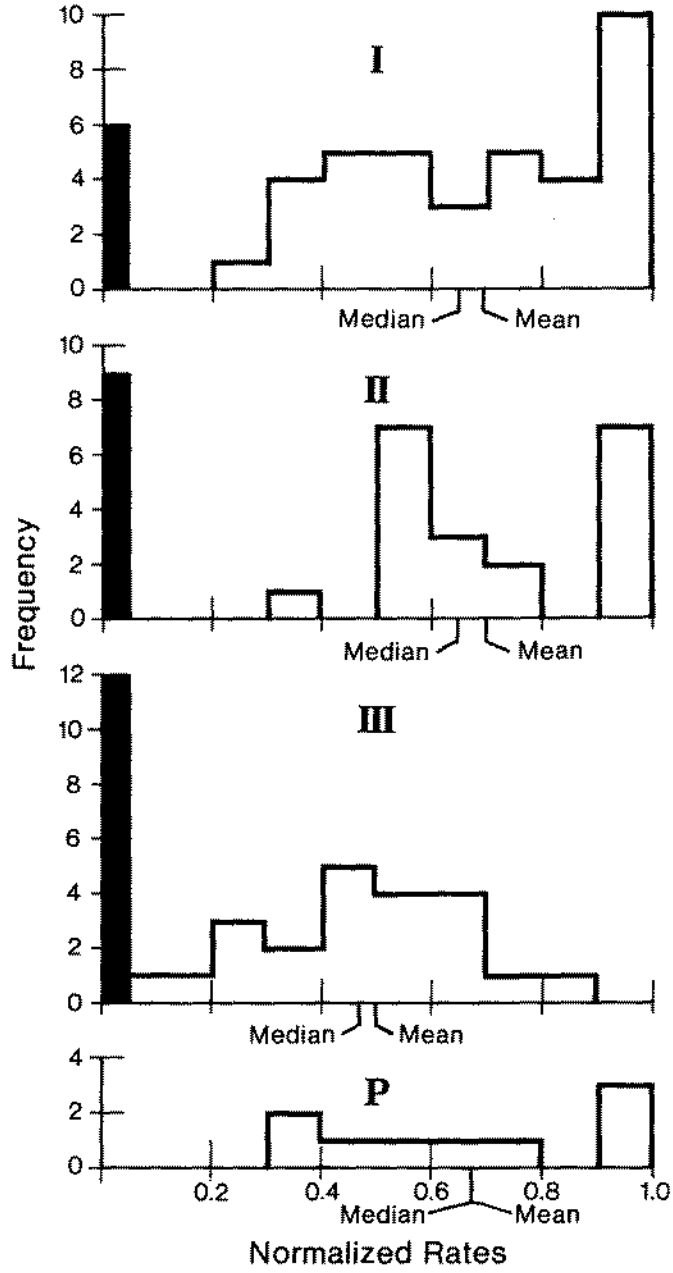

FIG. 2. Histograms of normalized release rates for each reproductive stage. Shaded areas are for measurements below detection. Rates were normalized by dividing each rate by maximum rate obtained for that animal. See text for description of stages I, II, III, and P.

phosphorus excretion is continuous when calculating concentration gradients near zooplankton (e.g. Jackson 1980; Lehman and Scavia 1982a, b). Pulses, possibly from P associated with fecal material as Taylor and Lean (1981) suggest, would provide additional short-term increases in release rates and thus discrete patches of higher concentration. This may be particularly important in cladocerans which produce diffuse feces that break up immediately upon release into the water. The fecal pulses may be most important during periods when animals' feeding rates are high because of increased egestion, and possibly less efficient assimilation, associated

with increased ingestion.

Differences were not discernible betwen 5–10-min rates for animals fed on algae conditioned in two different ways with regard to phosphorus (t-test, two-tailed, 29 df, $P > 0.1$), which suggests that P release kinetics in our experiments were not affected greatly by the way the algae were prepared. Animals fed for 3 h should have replaced the contents of their guts; however, they probably would not have adjusted metabolically to differences in P content of the food.

Mean release rates (± SE), averaged over all animals and reproductive states, for the three groups of animals in the second experiment (1.72 ± 0.222 nmol $P \cdot mg^{-1} \cdot h^{-1}$, $n = 19$; 0.43 ± 0.026, $n = 41$; and 0.29 ± 0.017, $n = 26$) were significantly different ($P < 0.01$, t-test for means with unequal variance estimates). The only known difference among the three groups is their time of removal from mass culture for preconditioning (13 d between first two groups and 19 d between second two groups). Fungal masses were observed in the appendages on some animals from the mass culture near the end of the experiment. Although these were not observed for experimental animals, changing conditions in the mass culture may have been reflected in the decreasing mean rates of these groups. The rates for animals in the first group are similar to those suggested for similar size daphnids by Peters and Rigler (1974) and within the range of those measured for *Daphnia magna* under a different feeding regime (Scavia and Gardner 1982). Because we are concerned here only with differences within the reproductive cycle and to facilitate statistical comparisons, rates for each animal were normalized to the maximum rate measured over all reproductive stages of that animal. Normalized rates (Fig. 2) for animals carrying stage III embryos were statistically lower than those carrying either stage I or stage II embryos (Mann–Whitney U-test, one-tailed, $P < 0.01$) or those in the premolt condition ($P < 0.025$). Differences in normalized rates for premolt, stage I, and stage II animals were not statistically discernible. The ratios of mean normalized rates from stages I, II, and premolt to stage III are, respectively, 1.50, 1.57, and 1.48.

Other physiological and behavioral processes vary with reproductive state. Rates of assimilation (Schindler 1968) and filtration (Nauwerk 1959) are higher for ovigerous than for nonovigerous Cladocera. Filtration rates (Christensen 1973) and carotenoid and hemoglobin content (Green 1956a, 1957) vary with stage of embryonic development in *Daphnia*. Although alteration in filtering appendage movements prior to release of young (Burns 1968) and passage of hemoglobin from blood to eggs prior to molting and extruding eggs into the brood chamber (Green 1956a) suggest mechanisms affecting these processes, mechanisms leading to reduction in phosphorus release for animals carrying more mature embryos are, at this time, unknown.

On four occasions during our experiments and twice during experiments described by Scavia and Gardner (1982), animals in the premolt condition molted in the incubation flow cell during measurement of SRP release. In all cases, SRP release increased dramatically over the rates determined prior to molting. Release rates increased by an average factor of 6.7 (SE = 2.10, $n = 6$, range = 2.7–16.5) over premolt rates. Because, from the above analysis, rates for animals in the premolt condition are not statistically different from the highest nonmolting rates expected during any reproductive cycle, release rates at ecdysis are likely the highest rates the animals attain. Peters and Rigler (1973) estimated, for *Daphnia rosea*, that loss of P at ecdysis due to the carapace alone could account for only a few percent of overall loss; however, they were not able to estimate loss with molting or other fluids. Lee and Buikema (1979) observed molting *Daphnia pulex* to be more sensitive to the effects of chromate during toxicity studies. They suggest that the increased sensitivity may be related to increased chromate uptake during the rapid size increase after molting or to calcium deficiency in the new exoskeleton. Our elevated release rates may be related to permeability of the exoskeleton immediately after molting. The new exoskeleton may be permeable to interior body fluids allowing P to be lost more readily with fluid exchange associated with the dramatic size increase.

To our knowledge, the dramatic increase in phosphorus release at and after ecdysis has not been observed previously, but may have experimental and environmental implications. The higher release rates do not represent discrete injections of P into the surrounding environment, but rather sustained elevated release rates for periods ranging from 15 min to greater than 2 h in our experiments.

A simple, but instructive calculation demonstrates the potential bias introduced if elevated release rates during molting are not considered. Stages I, II, and III each require approximately equal portions of total embryonic development time (Green 1956b). Christensen (1973) observed that the period between release of young and ovulation in the next clutch was approximately equal to that of embryonic development of one stage in *Daphnia magna*. If we assume that P release rates are elevated for only one-half of the time between release of young and ovulation and that the elevated rate is, as we observed, 6.7 times the average rate (R) during stages I, II, and III, then the average rate over the whole reproductive cycle would be

$$\frac{1}{4}\left(3.5R + 0.5 \times 6.7R\right) = 1.7R$$

This rough calculation, showing a 70% difference, demonstrates the impact on inferences drawn from experiments where some of the animals may have molted. If no animals have molted, extrapolation of laboratory estimates to the field may underestimate true flux of P to the water. If an unusually high proportion of the animals molted, true flux may be overestimated.

More important perhaps is the influence these intermittent elevated release rates would have on nutrient heterogeneity. If microenvironments in fact have bearing on algal production and species competition (Turpin et al. 1981) and if zooplankton are important mediators of such heterogeneity (e.g. Goldman et al. 1979; Lehman and Scavia 1982a, b), then focusing elevated release rates into discrete periods of time (i.e. during molt) may also have important implications for algal community production and structure.

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