Phosphorus use and excretion varies with ploidy level in *Daphnia*

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Ploidy affects the amount of DNA per cell. Because phosphorus (P) is required for building DNA, polyploids should have higher P content and be more sensitive to dietary P limitation. Such differences could be due to some combination of differential incorporation and excretion of P. We tested these predictions using diploids and polyploids of the zooplankter *Daphnia pulex*. After manipulating the P content of natural seston, we fed it to diploids and polyploids and quantified growth and %P. Although neither growth nor %P differed significantly with ploidy, polyploids tended to grow faster on P-spiked seston. Laboratory experiments on four genotypes using *Scenedesmus* algae cultured under low (LoP) and high (HiP) phosphorus supplies revealed significant P treatment by ploidy interactions in both growth and %P, with polyploids growing faster in HiP and containing more P in LoP. Radiotracer assays revealed that polyploids incorporated significantly more $^{33}$P and excreted significantly less $^{33}$P compared with diploids. Ploidy-specific P use could interact with environmental P supply to affect the fitness of genotypes differing in ploidy level. Further, these results indicate unique ecological functions (i.e. P recycling) of lineages differing in ploidy level that should be generally applicable to taxa that have undergone whole-genome duplications.

KEYWORDS: ecological stoichiometry; genome size; nutrient limitation; polyploidy; radiotracers; sub-Arctic

INTRODUCTION

Polyploids are eukaryotic organisms that possess two or more complete sets of chromosomes (Stebbins, 1947). Mayr (1963) proposed polyploidization as a rapid driver of evolutionary change. Much work has since focused on the evolutionary mechanisms underlying the origin and maintenance of polyploidy (Solís and Solís, 2000; Wendel, 2000; Comai, 2005; Otto, 2007; Leitch and Leitch, 2008), identifying whole-genome duplication
to be a potent evolutionary driver (Selmecki et al., 2015). Yet, it is clear that, despite significant progress in genetics and genomics, we are far from a complete understanding of the role of polyploidy in determining the evolutionary success of taxa, notably in animals. Identifying and understanding differences in the ecology and physiology of taxa differing in ploidy level could provide answers (Solitis et al., 2010; Madlung, 2013; Ramsey and Ramsey, 2014).

Lewis (Lewis, 1985) was among the first to hypothesize a relationship between nutrient stress and genome size, observing that haploid algae dominated over diploids under nutrient scarcity. Using this logic, Hessen et al. (Hessen et al., 2010) predicted that lineages with large genomes should have higher demand for elements that constitute DNA. Further, they suggested that a higher phosphorus (P) demand for DNA in polyploids could tradeoff with an allocation of P to RNA and the protein synthesis machinery (i.e. growth). Although scarce in the biosphere (micromolar concentration; Macia, 2005), P is ubiquitous and performs critical functions within living systems (often in millimolar concentrations). Hence, P limitation is quite prevalent in herbivorous invertebrates including Daphnia (Sterner and Elser, 2002). The physicochemical properties of P predispose it to be a critical component of many key compounds (Westheimer, 1987) that carry out basic biochemical processes, including nucleic acids that transmit genetic information, nucleotides that are precursors in DNA and RNA synthesis and ribosome biogenesis. These central roles of P in the structure and functioning of the genome suggests that P demand can vary with genome size and that larger genomes may be more susceptible to P limitation. More generally, P use should vary with ploidy level.

Hessen et al. (Hessen et al., 2008) compared the genome sizes of copepods and cladocerans that have similar niches and body sizes, yet differ markedly in somatic P content and growth rate and found that fast growing (P-rich) cladocerans have significantly smaller genomes. Because P is required for both DNA and RNA, they suggested that this trend could be driven by selection for smaller genomes in fast growing taxa. In a recent study, Neiman et al. (Neiman et al., 2013) studied triploid and tetraploid lineages of the freshwater snail Potamogeton frigidus and found strong interactions between P and ploidy. Tetraploids experienced a more than 2-fold reduction in growth under P limitation. Moreover, P content in tetraploid individuals was more sensitive to changes in dietary P compared with triploid counterparts. These observations indicate that environmental supply of P can be an important selective factor determining the abundance and distribution of cytotypes differing in ploidy level. Similarly, Jalal et al. (Jalal et al., 2014) found significant reductions in the haploid genome size of Daphnia magna growing under P limitation for 35 generations compared with a lineage grown under P-replete conditions. These studies, thus, strongly indicate differential P demand, but how P use, and excretion varies with ploidy level have yet to be tested.

Here, we first test whether polyploids of Daphnia have higher specific P content and whether growth of polyploids is more sensitive to changes in environmental P supply in two different experiments. More importantly, we directly tested whether polyploids ingest, and/or retain more P compared with diploids using radiotracer (32P) assays. One experiment was conducted on multiple genotypes of field-caught individuals cultured in semi-natural conditions. Another set of experiments was conducted on a smaller subset of genotypes in controlled laboratory conditions.

**METHOD**

**Study system**

We studied diploid and (allo)polyploid lineages of obligately parthenogenetic Daphnia pulex sensu lato in a sub-Arctic rock pool system in Churchill, Manitoba, Canada (described in Weider and Hebert, 1987), that has been monitored for the last ~30 years (Weider et al., 2010). Prior work (Weider, 1987; Weider and Hebert, 1987; Wilson and Hebert, 1992; Dufresne and Hebert, 1995) has established that this complex contains distinct, obligately parthenogenetic diploid and polyploid lineages. While most polyploids are melanic (Weider, 1987), non-melanic polyploids are also present. As there may be metabolic costs related to melanin synthesis (Hessen, 1996), we focused on comparing non-melanic polyploid lineages with non-melanic diploid lineages to understand the role of ploidy in affecting phosphorus use.

**Daphnia growth and phosphorus content on natural seston**

Water (~20 L, 80 µm filtered to remove grazers) from 12 rock pools was brought to the laboratory, half of it spiked with phosphate (50 µg of P L⁻¹) and incubated overnight. A sample (~500 mL) was filtered (GF/F, Whatman, Inc., Maidstone, UK) to determine particulate carbon content (an indicator of food quantity) in both spiked and unspiked water from each rock pool. These filters were dried and brought back to Oklahoma State University for quantification of total carbon using an elemental analyzer (Elementar, Inc., Mannheim, Germany). Total particulate carbon in the rock pools varied considerably (range = 118.2–424.5 µg L⁻¹), as
did the molar carbon:phosphorus (C:P) ratio of seston (range = 332–585). Spiking resulted in a marked decrease in average seston C:P (range = 99–128).

Animals were collected from the rock pools using an 80-μm dip net and brought to the laboratory at the Churchill Northern Studies Centre. Approximately 10 adult individuals were placed in a drying oven (60°C) for determination of phosphorus content. We determined %P of wild-caught daphniids in triplicate, in batches of two to three individuals (APHA, 1992). Ten gravid females were placed individually in 50-mL centrifuge tubes with filtered (80 μm) water from their respective rock pools. Water was replaced every day until neonates were released (within a maximum of 3 days). Upon birth, three neonates were genotyped using cellulose acetate electrophoresis as described in Weider and Hebert (Weider and Hebert, 1987). These analyses, based on the presence of either unbalanced heterozygous genotypes at specific polymorphic allozyme loci (i.e. indicating more than two alleles) or the distinct presence of three or more alleles at monomeric loci, revealed that six pools contained polyploids, while five contained diploids. Further, we also used the extensive prior allozyme datasets (Wilson, 1989; Wilson and Hebert, 1992) on these clones to help verify clonal identities and ploidy levels. While we recognize that further ploidy-level analyses are needed to ascertain whether diploid allozyme genotypes were indeed diploid (Vergilino et al., 2009), allozyme characterization of ploidy level in this system has been quite reliable. Only 3 of 36 genotypes initially characterized as diploids were ascertained to be polyploids (Wilson, 1989; Wilson and Hebert, 1992) using microdensitometry (Beaton and Hebert, 1989).

Per clone, eight less than 12-h-old neonates from the same mothers were photographed at 4-fold magnification (Veho Discovery Camera VMS-001, UK). Three neonates were individually transferred to 50-mL centrifuge tubes with P-spiked water from respective rock pools, while another three were transferred to tubes with control (non-spiked) water. Water was replaced daily for 3 days. After 72 h, individuals were photographed again. Length of daphniids was measured from the top of the head to the base of the tail spine using Imagej software (Schneider et al., 2012). Forty randomly chosen images were used to quantify body depth and total area, to test for potential differences in growth response between diploids and polyploids that are not captured by total length measurements. We found no significant effect of ploidy on body depth (F1,39 = 0.35; P = 0.55), or area (F1,39 = 0.09; P = 0.75), and thus used total length as a reliable indicator of growth (McCauley, 1984).

Daphnia growth and phosphorus content on *Scenedesmus*

We intensively studied four genotypes (two diploid, two polyploid) in the laboratory. The ploidy level of these genotypes was confirmed using microdensitometry (Beaton and Hebert, 1989). All genotypes were maintained at ~20°C, and 16:8 h light:dark cycle in artificial (COMBO) medium (Kilham et al., 1998) and fed *Scenedesmus acutus* algae cultured under high phosphorus supply (C:P~100) at a concentration ~1 mg C L\(^{-1}\) day\(^{-1}\). *Scenedesmus acutus* was cultured in semi-continuous chemostats under P-sufficient (50 μM) and P-deficient (5 μM) conditions to generate the experimental diet differing in P content. These conditions produced algae with C:P ratio ~150 (hereafter termed 'HiP') and ~750 (hereafter termed 'LoP'), respectively. After ~7 days, when chemostats reached stable state, algal outflow was collected, its C content determined spectrophotometrically (Beckman Coulter DU700, Fullerton, CA, USA) and used to feed experimental animals. Growth assays were performed as described above on HiP or LoP algae supplied at 1 mg C L\(^{-1}\) day\(^{-1}\). To determine the amount of phosphorus contained in diploid and polyploid genotypes in HiP and LoP conditions, 10 <12-h-old neonates were placed in triplicate 1-L glass jars and fed either HiP or LoP algae at 1 mg C L\(^{-1}\) day\(^{-1}\) for 3 days. At the end of 3 days, daphniids were dried at 60°C for 48 h, weighed to the nearest mg, acid digested and %P was determined spectrophotometrically (APHA, 1992).

Dual radiotracer assays

To test whether ploidy level affects material use physiology, we performed dual radiotracer (\(^{14}\)C/\(^{33}\)P; DeMott et al., 1998) assays on two diploid and two polyploid genotypes (i.e. clones). Animals were maintained in standard laboratory conditions (20°C, 18:6 h light:dark cycle) and fed 1 mg C L\(^{-1}\) day\(^{-1}\) of high P *S. acutus* algae in COMBO medium. Experimental individuals were third clutch granddaughters of a single parthenogenetically reproducing individual.

HiP and LoP algae from ~7-day-old chemostats were collected and centrifuged (at 3500 rpm for 30 min) and resuspended in 200 mL of COMBO (no N and P) at 1 mg C L\(^{-1}\). Radioisotopes \(^{14}\)C (as bicarbonate) and \(^{33}\)P (as orthophosphate) were spiked simultaneously in the amount of 3.7 and 5.55 MBq L\(^{-1}\), respectively, and incubated for 72 h (following DeMott et al., 1998; He and Wang, 2007). Algal suspensions were incubated in a shaker at 20°C, 16:8 h light:dark cycle for 72 h. Two milliliters of algal culture were filtered through a GF/F filter and radioactivity quantified.
After radiolabelling algae, younger than 12-h-old granddaughters of each genotype were isolated individually in 50-mL jars. Individuals were divided into two groups and fed algae of different qualities (HiP or LoP) at the same concentration (1 mg C L\(^{-1}\) day\(^{-1}\)) for 5 days. After 5 days, 10 individuals were starved for 2 h and placed together in 200-mL jars containing radiolabeled algae (HiP or LoP). This setup was replicated three times per C:P treatment for each genotype. After 40 min, three animals were pipetted out, rinsed and transferred to scintillation vials for estimation of radioactivity; while another three individuals were rinsed and immediately transferred to jars with corresponding (i.e. HiP or LoP) unlabeled algae. After 4 h, these three animals were pipetted out for radioactivity counting as above. The medium was changed hourly to minimize the recycling of \(^{33}\text{P}\) and \(^{14}\text{C}\). Thus, we corrected growth on a per-carbon basis for subsequent analyses. Both diploids and polyploids grew faster when feeding on natural seston spiked with \(^{33}\text{P}\)-spiked seston (Fig 1; \(F_{1,64} = 74.24; P < 0.001\)). However, we found no differences between ploidy levels (\(P = 0.2\)), or ploidy-specific responses to altered diets, although polyploids appeared to grow marginally faster than diploids on P-spiked seston (\(F_{1,64} = 3.26; P = 0.07\)). P content did not differ significantly between diploids and polyploids (\(F_{1,32} = 0.53; P = 0.47\)).

In contrast, growth of the four laboratory genotypes on \(Scenedesmus\) was significantly affected by a P treatment by ploidy interaction (\(F_{1,20} = 5.98; P = 0.02\); Fig. 2a). Post

**RESULTS**

We measured the growth of 68 individuals from 12 separate rock pools in both natural (\(n = 34\)) and P-spiked (\(n = 34\)) treatments. Body size was not significantly different between diploids and polyploids at the start of the growth experiment (\(F_{1,64} = 0.01; P = 0.72\)). Growth rate was strongly correlated with total particulate carbon in both natural (\(y = 0.0017x + 0.1; r^2 = 0.67; P < 0.001\)) and spiked seston (\(y = 0.0014x + 0.63; r^2 = 0.48; P < 0.001\)). Thus, we corrected growth on a per-carbon basis for subsequent analyses. Both diploids and polyploids grew faster when feeding on natural seston spiked with P (Fig. 1; \(F_{1,64} = 74.24; P < 0.001\)). However, we found no differences between ploidy levels (\(P = 0.2\)), or ploidy-specific responses to altered diets, although polyploids appeared to grow marginally faster than diploids on P-spiked seston (\(F_{1,64} = 3.26; P = 0.07\)). P content did not differ significantly between diploids and polyploids (\(F_{1,32} = 0.53; P = 0.47\)).

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**Phosphorus recycling**

These experiments were designed to quantify the rate of P excretion by diploids and polyploids by measuring the uptake of recycled P by co-existing algae. Following the experimental design of Sterner (Sterner, 1986), we created two chambers separated from each other by a 0.2-μm nitrocellulose filter (Millipore, MA, USA) that only allowed the transfer of dissolved P. The upper chamber was a 70-mL white plastic cup that fitted the mouth of a 150-mL Borosil® beaker that served as our lower chamber. The bottom of the cups (upper chamber) were removed and fitted with the filters. The upper and lower chambers were filled with 25 and 70 mL of modified COMBO (Kilham et al., 1998; without P), respectively. The upper chamber was inoculated separately with 20 similarly sized individuals of one of the four genotypes. All individuals were starved for 2 h, and fed \(^{33}\text{P}\)-labelled HiP or LoP algae for 4 h at a concentration of 3 mg C L\(^{-1}\) prior to experiments. The upper chambers received either HiP or LoP unlabeled algae at a concentration of 3 mg C L\(^{-1}\). The lower chambers for all jars were inoculated with 200 μL of unlabeled LoP algae. Each treatment was replicated three times. After 24 h, the lower chamber was homogenized by stirring and 2 mL of algae were pipetted out and filtered through a GF/F filter for radioactivity counting. Two control jars for each treatment were set up without any \(Daphnia\) in the upper chamber. Mass-specific log-transformed DPM data were used for analyses.

![Fig. 1. Change in length (mm) of individual diploid (open circles) and polyploid (filled squares) \(Daphnia\) fed natural seston from respective rock pools spiked with 50 μg of phosphorus (P) L\(^{-1}\) HiP or control (LoP). Because particulate carbon (C) was significantly different among rock pools, and length gain was strongly correlated to particulate C, length gained per μg of seston C is represented. Symbols indicate the mean and error bars represent ± 1 SD.](image-url)
Analyses revealed that polyploids grew faster than diploids only in HiP conditions, whereas no differences were found in LoP conditions. Similarly, phosphorus content of daphniids was significantly affected by a P treatment by ploidy interaction ($F_{1,20} = 5.15; P = 0.03$; Fig. 2b). Post hoc tests revealed that polyploids contained more P than diploids only in LoP conditions, whereas no differences were observed in HiP, where both polyploids and diploids contained equally high P compared with LoP conditions.

Analyses of radiotracer data clearly revealed that polyploids incorporated significantly more C ($F_{1,20} = 5.96; P = 0.02$; Fig. 3a) and P ($F_{1,20} = 22.46; P < 0.0001$; Fig. 3b) after 40 min of feeding on radiolabeled algae. After 4 h, both diploids and polyploids retained more C ($F_{1,20} = 31.12; P < 0.0001$; Fig. 3c) and P ($F_{1,20} = 11.81; P = 0.003$; Fig. 3d) under low P conditions. Furthermore, polyploids contained more C ($F_{1,20} = 6.83; P = 0.01$) and P ($F_{1,20} = 14.58; P = 0.001$).

We found a significant P treatment by ploidy interaction in the amount of $^{33}$P recycled by daphniids back to algae ($F_{1,20} = 26.67; P < 0.0001$; Fig. 4). While both diploids and polyploids recycled more P to algae in HiP conditions, more $^{33}$P was found in algae co-existing with diploids in HiP conditions, meaning that polyploids retained P more efficiently.

**DISCUSSION**

Together, these results indicate that diploid and polyploid daphniids respond to changes in phosphorus supply quite differently in growth, P content, P intake and P excretion. The directions of these differences appear to be consistent with the hypothesis that polyploids have higher P demand compared with diploids. It is worth noting that, regardless of ploidy level, striking effects of P supply on *Daphnia* growth were observed, indicating that P-supply treatments employed in this study compare P-replete and P-limited *Daphnia*.

While both growth and %P did not differ statistically between diploids and polyploids cultured in natural seston, trends were consistent with expectations. Polyploids (1.12%) contained more P than diploids (1.09%) although there was considerable variation. Such variation is perhaps not surprising because several other ecological parameters most likely vary among ponds. Despite large variation, food quantity in the rock pools (total particulate carbon) was not related to %P of daphniids ($P = 0.78$) although food quantity was strongly correlated with growth rate. The range of particulate carbon available to daphniids in these rock pools is in the linear phase of the growth curve (the curve plateaus at $\sim 700 \mu g \ C \ L^{-1}$; Lampert, 1978). Per unit carbon available, polyploids grew somewhat faster than diploids in natural seston experimentally supplemented with phosphate, but again, this effect was not statistically significant (Fig. 1). It is important to note that 3 of the 12 rock pools had C concentrations near the incipient limiting concentration for *Daphnia* (i.e. 100 $\mu g \ C \ L^{-1}$; Lampert, 1987). Thus, we acknowledge that these observations on field-caught individuals raised on natural seston are not suitable for making robust inferences about differences in material demand between diploid and polyploid daphniids.

Results from controlled laboratory conditions, albeit on only four unique genotypes (two diploid, two polyploid), lend support to the hypothesis that ploidy level alters material demand. After experimentally controlling food quantity, interactive effects of dietary P and ploidy level were perceptible on the growth and physiology of daphniids. Polyploids grew significantly faster than
diploids in *Scenedesmus* containing higher P, while both cytotypes grew much slower in low P conditions (Fig. 2a). Moreover, polyploids contained significantly more P than diploids, particularly in P-limited conditions (Fig. 2b). These results are consistent with previous work by Elser et al. (Elser et al., 2000) on multiple populations of Arctic and temperate *D. pulex* in N. America. Although they did not explicitly test for ploidy-level variation, the authors acknowledge it as an underlying factor based on ecological conditions selecting for rapid growth during the shorter Arctic growing season, and the latitudinal distribution of polyploid daphniids (Beaton and Hebert, 1988; Adamowicz et al., 2002). Indeed, Van Geest et al. (Van Geest et al., 2010) studied Arctic and temperate populations of *D. pulex* in Europe and found that Arctic (polyploid) daphniids grew faster than temperate (diploid) counterparts and that such rapid growth is supported by increased allocation of P to RNA.

The previous portion of this study and prior work represent growing evidence on the differential phenotypic consequences to P limitation among lineages differing in ploidy level. However, comparatively little is known about the physiological mechanisms that underlie such variation. Results from dual radiotracer (\(^{14}\text{C}\) and \(^{32}\text{P}\)) experiments, for the first time, illuminate the strikingly different physiological responses of diploids and polyploids in the use of material resources under both P-replete and P-limited conditions.

After 40 min of feeding on dual-labeled algae, polyploids had incorporated considerably more \(^{14}\text{C}\) and \(^{32}\text{P}\) regardless of algal stoichiometry (Fig. 3a and b). A number of phenotypic differences could underlie these observations.
First, polyploids may have a higher consumption rate. In Daphnia, consumption rate is affected by the beat rate of filtering appendages, area of the filter and mesh size (Lampert and Brendelberger, 1996). Thus, higher consumption of algal cells by polyploids may be a result of faster appendage beat rate, larger filters and smaller mesh size. Another possibility is that polyploids may have a larger gut volume compared with diploids. Given the striking phenotypic differences between diploid and polyploid daphniids (Weider, 1987; Beaton and Hebert, 1997), differences in the morphology of feeding structures may well also be different and warrants further study.

Strong effects of dietary stoichiometry and ploidy level were observed in $^{14}$C and $^{33}$P activities after 4 h. Under P-limited (high C:P) conditions, both cytotypes contained more $^{14}$C and $^{33}$P. Stoichiometric theory predicts that under such imbalances, daphniids should retain less C and more P (Sterner and Elser, 2002). Experimental individuals retaining more C in high C:P conditions indicate that 4 h may not have been enough to invoke physiological accommodations, which are often apparent after 12 h (see Roy Chowdhury et al., 2014). Regardless, we found major differences in the amount of $^{14}$C and $^{33}$P between diploid and polyploid genotypes after 4 h (Fig. 3c and d). Higher activities of $^{14}$C and $^{33}$P in polyploids are likely a function of differential ingestion because the proportion of ingested $^{14}$C and $^{33}$P retained did not differ with ploidy level ($^{14}$C: $F_{1,20} = 0.35; P = 0.55$; $^{33}$P: $F_{1,20} = 0.03; P = 0.95$).

While strong differences in ingestion of $^{14}$C and $^{33}$P between cytotypes may indicate an important mechanism that differs in material processing, the question of whether the cytotypes use P differently, remains an open question. The results from the $^{33}$P recycling experiment reveal important patterns. Despite diploids containing significantly less $^{33}$P after 40 min and 4 h with algae co-existing with diploids contained more recycled $^{33}$P after 24 h (Fig. 4). This result strongly indicates that diploids are inefficient in P use compared with polyploids, likely reflecting lower somatic demands for P. Again, these results are similar to those found by Elser et al. (Elser et al., 2000) where temperate D. pulic excreted significantly more P than Arctic lineages. Increased demands for P in polyploids could partly reflect the material costs of increased DNA, but could also reflect increased demands for RNA to maximize growth. In any case, there will obviously be tradeoffs between diploid and polyploids related to the availability of P.

These observations on a few genotypes, from one local (yet, spatially structured) population (Weider et al., 2010) have obvious limitations. Whether the costs of P use are realized in the wild remains an open question, although evidence is growing. For example, a long-term field study on plants (Smarada et al., 2013) found that polyploids were more frequent in P-fertilized plots. Interactions between ploidy level and environmental P supply may be important in the world experiencing unprecedented changes to the global P cycle, and thereby provide important insights into understanding longstanding questions about the costs and benefits of polyploidy, and the role of polyploidy in the evolutionary fate of taxa.

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