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Polyploid unisexual salamanders have higher tissue regeneration rates than diploid sexual relatives

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Abstract

Differences in genome composition are known to influence cell division and tissue growth, yet few studies have compared tissue growth between closely related taxa that vary in ploidy and genome composition. Whether cellular mechanisms scale to a functional trait such as tissue regeneration is important for understanding the ecological interactions between polyploids and closely related diploid taxa. We studied regeneration in unisexual *Ambystoma* salamanders, an ancient unisexual (all-female) lineage in which most individuals are triploids consisting of combinations of two or more distinct genomes from their sexual relatives. We discuss the aspects of haploid genome size and polyploidy that may contribute to variation in tissue regeneration and hypothesize that higher ploidy or variations in genome composition in unisexual *Ambystoma* would result in increased tissue regeneration compared to diploid sexual relatives, as polyploidy is generally associated with faster limb regeneration. We tested this hypothesis by comparing tail regeneration rates over 4 months between polyploid unisexual salamanders and sympatric diploid sexual salamanders under standardized laboratory conditions. Consistent with our prediction, unisexual *Ambystoma* regenerated tail tissue at approximately twice the rate of the sexual species. This result provides a physiological difference between unisexual and sexual salamanders that could influence their coexistence.

Introduction

Differences in genome size or structure can produce drastic changes in phenotype, potentially increasing cell size (Comai, 2005), increasing cell cycle time (Davies & Rees, 1975) or decreasing growth rate (Hessen, Daufresne & Leinaas, 2013). These changes in size and growth rate may translate to phenotypic differences between competing taxa that vary in ploidy or haploid genome size. This ecological scenario is common in taxa with both sexual and asexual reproductive modes, where asexuals often show patterns of niche differentiation from parental sexual species (Kokko, Heubel & Rankin, 2008). In these systems, the relationship between genome size, polyploidy, and phenotype is especially important for understanding possible mechanisms that determine coexistence between these competing forms.

One phenotype of interest in natural systems is tissue regeneration, a trait of adaptive value in a variety of taxa (Bely & Nyberg, 2010). Here, we define tissue regeneration as the replacement of lost body parts, which can vary extensively across vertebrates groups (Alvarado, 2000). There are various hypotheses for differences between species in rates of regeneration, including the effects of phylogenetic inertia or adaptive benefits (reviewed in Bely & Nyberg, 2010). Additionally, factors such as age, body size, and reproductive mode have been shown to influence regeneration rates at an individual level (Seifert *et al.*, 2012; Krois *et al.*, 2013).

Caudates (salamanders and newts) are model organisms for studying tissue regeneration, as they are able to regrow a variety of complex structures (Voss, Epperlein & Tanaka, 2009; reviewed in Brockes, 2015). Importantly, appendage injuries are common in natural salamander populations (Semlitsch & Reichling, 1989), and these injuries can have quantitative negative effects on an animal's ability to reproduce and defend itself from predators (Bernardo & Agosta, 2005).

Interspecies variation in appendage regeneration rate does occur between species within the salamander genus *Ambystoma*, in which four diploid species display limb regeneration rates that are tightly correlated with body size (Young, Bailey & Dalley, 1983). Additionally, the genus *Ambystoma* offers a sympatric polyploid example with which to compare diploid regeneration rates. Unisexual *Ambystoma* is a polyploid, all-female lineage that reproduces through kleptogenesis, a reproductive mode in which unisexual females generally reproduce clonally while occasionally producing offspring with increased ploidy or substituted genomes that come directly from males of sympatric, sexual *Ambystoma* species (Bogart *et al.*, 2007).

An increased number of genomes may provide a regenerative advantage by resulting in higher amounts of RNA and protein production (de Godoy *et al.*, 2008; Neiman *et al.*, 2009). This combined with the known positive correlation between asexuality and higher tissue regeneration rates supports unisexual *Ambystoma* as having a higher capacity for

tissue regeneration compared to sexual, diploid species (Hypothesis 1; Krois *et al.*, 2013). However, three lines of evidence could support lower regeneration rate in unisexuals (Hypothesis 2): a negative relationship between regeneration rate and haploid genome size (Sessions & Larson, 1987), decreased growth rate among cells with larger cell volume (Hessen, Daufresne & Leinaas, 2013), and that larger genomes are correlated with a longer cell cycle time (Davies & Rees, 1975). However, the relationship between regeneration and genome composition (the combinations of genomes from different parental species) is generally unknown. Here, we compare the regeneration rates of diploid and polyploid salamanders to investigate a potential link between increased ploidy, genome composition, and regenerative ability in these animals. Under Hypothesis 1, we predict that unisexual *Ambystoma* will have greater tissue regeneration rate compared to sympatric sexual species. For Hypothesis 2, we predict that sexual individuals will display an equal or greater regeneration rate compared to unisexuals.

Materials and methods

We collected six egg masses for both small-mouthed salamanders, *A. texanum* (Matthes, 1855; henceforth 'smallmouths'), and unisexual *Ambystoma* salamanders (henceforth 'unisexuals'), from the same two wetlands in Crawford County, Ohio. We compared sexual and unisexual individuals from the same site instead of comparing individuals with different levels of ploidy within only unisexuals to make a direct comparison between groups predicted to be in local competition, as unisexuals are the dominant and most abundant *Ambystoma* in these locations. Variation in ploidy among unisexuals exists, but a single ploidy (either triploid or tetraploid) is most common at individual sites in Crawford County (Denton, unpublished). We collected eggs masses that were at least 5 m apart to avoid collecting egg masses from the same female, and the number of embryos per egg mass for both groups is similar (Petranka, 1998). Hatched larvae were housed individually in plastic containers (450 mL) with dechlorinated, treated water (Tetra Blackwater extract and Tetra Fungus Guard, Blacksburg, VA, USA). We conducted 50:50 water changes every other day, and all salamanders were kept in a closed room at the same temperature 72–75°F with a 12-h light/dark cycle. Individuals were fed equal amounts of size-appropriate invertebrates throughout development (brine shrimp, daphnia and crickets).

Sixty days after salamanders metamorphosed from the water (~ 10–12 months old), we conducted tissue removal procedures (Ohio State IACUC protocol #2012A00000039). We first recorded the snout-vent (posterior) length to the nearest 0.1 mm (SVL), weight to the nearest 0.01 g and tail length to the nearest 0.1 mm (from posterior vent to tail tip). We then removed 40% of the original tail length from each animal to control for differences in individual size. We chose to remove tail tissue as an alternative to limb amputation due to the simplicity of viewing the plane of amputation at the tail tip (Voss *et al.*, 2013). Before tissue removal, the unisexual salamanders had longer tails proportional to their larger size (mean tail length = 23.4 mm for smallmouths and 29.5 mm for unisexuals). We extracted DNA from the unisexual tail tissues using a Qiagen DNeasy Kit (Qiagen, Valencia, CA, USA) and assayed a set of single nucleotide polymorphisms that identified the genomic composition of each unisexual (Greenwald & Gibbs, 2012). All unisexual individuals used in trials were identified as triploids with one genome derived from the blue-spotted salamander, *A. laterale*, and two genomes derived from the Jefferson salamander, *A. jeffersonianum*.

We measured regrowth from the middle of the plane of amputation to the distal tip of the newly formed tail tissue (Fig. 1). We first recorded tail regrowth 2 weeks after the tail clipping, and measurements were taken every 7 days thereafter until the individual grew back at least 100% of the original tail length that had been removed. Two investigators recorded two measurements weekly using a digital caliper (± 0.02 mm accuracy), and the mean of these two numerical values was used for analyses. Overall, 30 smallmouths and 10 unisexuals were successfully reared and measured. We calculated the mean number of weeks until full tail regrowth for all salamanders from each egg mass, and used a two sample *t* test to determine if smallmouths and unisexuals differed in time to full regrowth ($N = 6$ egg masses for both, assuming unequal variances).

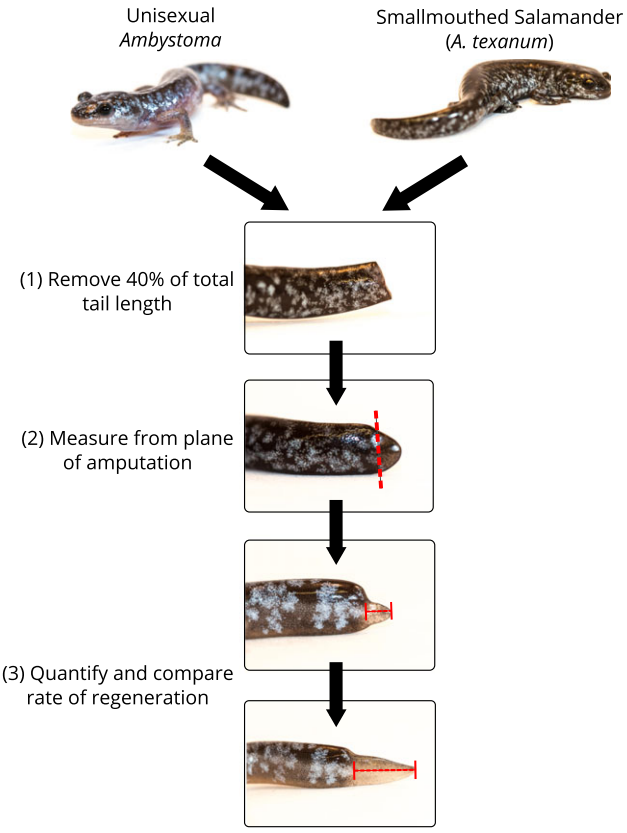

Figure 1 General methodology for tissue regeneration comparison and representative individuals of both study groups: unisexual *Ambystoma* (left) and a diploid sexual species, *A. texanum* (right). The vertical dashed line represents the original plane of amputation and the horizontal dashed line represents the length measurement taken to measure regeneration.

We assessed differences in regeneration rates (mm of growth/week) using two separate analyses. To account for the variation in SVL/weight and the potential effects of relatedness between individuals from a single egg mass, we used nested analyses of variance (ANOVA) with SVL or weight as covariates. In each model, an individual's egg mass identity was nested (one of five different egg masses) within type (smallmouth or unisexual). The regeneration rates were log-transformed to fit the assumptions of normally distributed residuals and homoscedasticity of variance. We confirmed a linear relationship between our covariate and response using Pearson correlations (all $P < 0.01$) and visual inspections of the data. Additionally, we compared the log-transformed regeneration rates between groups (smallmouth or unisexual) using egg mass as the unit of measurement with a *t* test. This approach is a more conservative solution to potential pseudo-replication among relatives, but comes at the sacrifice of not including covariation of SVL/weight.

Results

In support of Hypothesis 1, unisexuals reached complete regrowth 36% faster than smallmouths (mean number of weeks ± SE = 7.6 ± 1.1 for unisexuals, 11.9 ± 0.5 for smallmouths; $P < 0.001$, $t = -6.33$, d.f. = 10; Fig. 2). The raw means for regeneration rate were 0.85 ± 0.03 and 1.76 ± 0.27 (mm/week ± SE) for smallmouths and unisexuals respectively. Unisexuals had both longer SVL (Mean ± SE: 34.6 ± 0.6 mm for unisexuals, 28.7 ± 0.5 mm for smallmouths) and greater weight (Mean ± SE: 1.80 ± 0.1 g for unisexuals, 0.93 ± 0.1 g for smallmouths). Because animal size is a potential explanatory factor for regrowth rate in *Ambystoma* (Young, Bailey & Dalley, 1983), we analyzed the regrowth rates using both SVL and weight. Models with either SVL or mass as covariates and egg mass identity nested within type (unisexual or smallmouth) explained a similar amount of variation (both R^2 values > 0.80) and showed that unisexuals had significantly higher regeneration rates once snout-vent length differences between the unisexuals and sexuals were taken into account ($P < 0.001$, $F_{1,27} = 38.54$). There was no significant effect of egg mass in the model. The mean estimates scaled to a standard SVL of 30.18 mm were 0.73 ± 1.10 (mm/week ± SE) and 3.81 ± 1.22 for smallmouths and unisexuals respectively. Accounting for the larger size of unisexual juveniles resulted in regeneration rates that were 1.4–5 times faster among unisexuals compared to the sexual species. This same result was repeated when using the mean regeneration rate of all individuals from a single egg mass ($N = 6$ for smallmouths, $N = 6$ for unisexuals) in a *t* test ($P < 0.001$, $T = -7.72$, d.f. = 10).

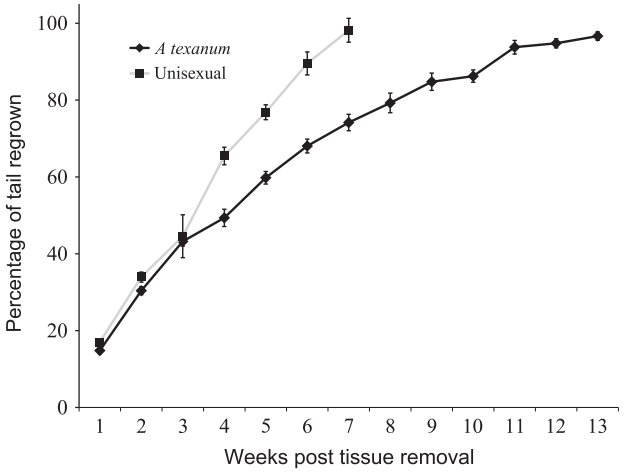

Figure 2 Percentage of tail regrown by sexual, diploid small-mouthed salamanders *Ambystoma texanum* and unisexual, polyploid *Ambystoma* over time. Data are displayed until point where means were within one standard deviation of 100%, and each measurement point is displayed with bars representing ± 2 SE.

Discussion

Despite the potential for polyploid salamanders to regenerate more slowly due to larger genomes and cell sizes, the polyploid unisexuals regenerated at almost double the rate observed in their diploid, sexual counterparts. Both groups show similar regeneration rates up until 3 weeks post-removal, which was also the point at which the majority of animals were switched from a brine shrimp diet to larger *Daphnia.* There is little known concerning the effect of nutrient composition on cell growth in polyploids, but there is empirical evidence for phosphorus limitation in polyploid asexuals (Neiman *et al.*, 2009; Neiman, Kay & Krist, 2013a; Hessen *et al.*, 2010). For this study, the composition of phosphorus and other nutrients was not compared between the brine shrimp and *Daphnia* diets.

At present, it is unclear whether faster regeneration in polyploid unisexuals is due to differences in ploidy alone, the increased diversity in unisexual genomes due to the presence of alleles from two species leading to a 'hybrid vigor' effect as observed in plant allopolyploids (Chen, 2007), or a combined effect of both factors. The suggestion that genome diversity *per se* may be important stems from this result being the opposite of that of Sessions & Larson (1987), who showed that diploid plethodontid salamanders with larger genomes had lower rates of limb regeneration. In contrast to the gradient of genome sizes among plethodontids, the unisexuals here were allopolyploids with multiple haploid nuclear genomes from two species that have recorded haploid genome sizes similar to the mean values from Sessions & Larson 1987 (see also Licht & Lowcock, 1991). The haploid genome sizes of both the smallmouths (*C* value: 27.1) and the two species that are present in the unisexuals (*C* value for *A. laterale* = 29.2, *C* value for *A. jeffersonianum* = 28.8) are similar (Licht & Lowcock, 1991). However, the unisexuals in this study did not include genomes from smallmouths, which would allow for a direct comparison of the contributions from different nuclear genomes. For example, a comparison could be made between diploid sexual species such as *A. laterale* (LL) or *A. jeffersonianum* (JJ) and unisexuals with corresponding nuclear genomes at varying ploidy (i.e. LJJ, LJJJ, LLJ, LLLJ). Unisexual polyploids that include smallmouth genomes have similar developmental rates to diploid, sexual smallmouths (Licht & Bogart, 1987), but the link between developmental rate and regeneration ability is unclear. Tissue

regeneration could be strongly influenced by a single gene or pathway within the *A. jeffersonianum* and *A. laterale* genomes of the unisexuals, but the only other intra-specific comparison of tissue regeneration within *Ambystoma* shows little variation between four species, including smallmouths, when SVL is considered as a covariate (i.e. larger species displayed faster limb regrowth; Young, *et al.*, 1983). Therefore, the most likely explanation for the difference in regeneration rate seems to be the influence of ploidy or interactions between the hybrid genomes rather than the influence of the particular nuclear genomes incorporated in the unisexual.

Tissue regeneration mechanisms may benefit from the dosage effects of multiple gene copies across haploid genomes or from 'hybrid vigor' (Osborn *et al.*, 2003). Unisexuals with combinations of varying levels of ploidy and genome composition (i.e. one *A. laterale* genome and two *A. jeffersonianum* genomes vs. one *A. laterale* genome and three *A. jeffersonianum* genomes) represent a tractable system for teasing apart these alternative hypotheses. The higher regeneration rates in polyploid salamanders may also provide further evidence for the importance of RNA in tissue regeneration. For example, transcript availability was cited as a possible explanation for higher regeneration rates by polyploid New Zealand mud snails *Potamopyrgus antipodarum* when compared to diploid counterparts (Krois *et al.*, 2013). Because protein production is a necessary component of tissue growth, it is plausible that a higher quantity of RNA could result in a faster regeneration rate, and there is generally a positive association between ploidy and bodily RNA content (Neiman *et al.*, 2009; Neiman, Kay & Krist, 2013b). A predicted greater amount of RNA in polyploid salamanders offers a possible explanation as to why the diploid sexual salamanders were unable to regenerate at the higher rate of polyploid salamanders.

Our study provides the first comparative analysis of closely related vertebrate taxa which vary in genome composition and ploidy. Due to the direct, negative fitness costs of an injury, such as tail amputation (reviewed in Bernardo & Agosta, 2005), differences in tissue regeneration rate could influence the competition between co-occurring species such as those in this study. This consequence is especially relevant among unisexual taxa, which act as sexual parasites on sympatric species and where coexistence between groups is often difficult to explain (Schley, Doncaster & Sluckin, 2004). The interspecies differences in regeneration ability described here and within the genus *Plethodon* (Sessions & Larson, 1987) justify an expanded survey of regeneration ability among salamander species, especially considering the support for taxa-specific protein relationships with regeneration ability (Garza-Garcia, Driscoll & Brockes, 2010). Species-specific differences associated with genome size, ploidy, or genome diversity that may contribute to competitive dynamics between species occupying similar niches could ultimately reveal the molecular mechanisms underlying salamanders' superior limb regeneration compared to other vertebrates.

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