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The relationship between nucleolar organizer size and growth in *Chironomus riparius* larvae (Diptera:Chironomidae)

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Abstract. Chironomid larvae possess giant polytene chromosomes. When genes on these chromosomes undergo transcription, they are visible as puffs. The nucleolar organizer region (NOR), visible as an especially large puff, shrinks when a larva is at particular developmental stages or is subjected to chemical stress. However, whether reduced NOR size is indicative of reduced growth is unknown. Therefore, we conducted 2 experiments to examine the relationship between NOR size and chironomid growth under controlled laboratory conditions. In the 1st experiment, we quantified the effect of ration quality on larval growth and NOR size. In the 2nd, we determined whether NOR size varied as a function of recent growth, independently of larval size. The combined results demonstrated that NOR size varied as a function of prepupal development and was positively correlated with a chironomid's most recent growth rate, independently of its biomass. The finding that NOR size is related to growth validates its use as a biomarker of sublethal stress. NOR size also has potential value as a measure of instantaneous growth state of field-collected larvae and, thus, may provide a surrogate measure useful for estimating secondary production in natural populations.

Key words: nucleolar organizer region, Chironomidae, growth, polytene, chromosome, puff, biomarker.

Midge larvae are typical of Diptera in possessing giant polytene chromosomes that form when chromatid strands replicate but fail to divide. When genes on these chromosomes undergo transcription, the normally condensed chromatid strands uncoil and characteristic puffs appear. Puffs are sites of ribonucleic acid (RNA) synthesis (reviewed by Daneholt 1975), and the rate of transcription is proportional to the size of the puff (Pelling 1964 cited by Grossbach 1977, Daneholt et al. 1969).

The nucleolar organizer region (NOR), visible as an especially large puff, is an area of preribosomal RNA synthesis (reviewed by Case and Daneholt 1977). Its relative size has been used as an indicator of sublethal stress (Bentivegna and Cooper 1993, Hudson and Ciborowski 1996) because it shows marked reductions (Michailova et al. 1998, 2001a, b, 2003, 2006, 2012) and transcriptional activity (Planello et al. 2007) when

larvae are exposed to contaminated sediments or to elevated levels of heavy metals.

Pelling and Beermann (1966, p. 395) reported that "condensed [nucleolar] organizers appear almost exclusively under conditions when metabolic activity is at a minimum" and that "in rapidly growing *Chironomus* larvae, the nucleolar organizer regions are most frequently found in an extremely puffed condition." These observations suggest that NOR size is intimately linked to growth, but to our knowledge, this relationship has not been established experimentally.

We report the results of 2 growth experiments done to examine how NOR size changes as a function of *Chironomus riparius* growth under controlled laboratory conditions. We expected to find a strong positive correlation between NOR size and growth. Specifically, because of the short latency of the NOR (Planello et al. 2007), we expected NOR size to reflect an individual's most recent growth rate. In both experiments, we used ration quality as a limiting factor to growth so that any observed changes in NOR size could not be interpreted as responses to toxins.

Methods

Organism collection and maintenance

We obtained *C. riparius* egg masses from a laboratory culture maintained by Environment Canada (Burlington, Ontario). To maintain genetic diversity, the culture population has been supplemented with individuals hatched from egg masses obtained from other North American testing laboratories annually or biennially since its inception. The incidence of larval mouthpart deformities, which becomes elevated in inbred laboratory populations, was no higher than reference levels for *Chironomus* species in the Laurentian Great Lakes (D. Milani, Environment Canada, personal communication). We incubated egg masses upon arrival at room temperature until they hatched. We used only 1st-instar larvae ($\leq$24 h old) in experiments.

Experimental design

We conducted 2 independent experiments simultaneously and ran 2 sequential 14-d trials (each with 4 replicates/treatment) in each experiment.

Uniform-ration experiment.—In this experiment, we used a uniform feeding regime to quantify the effect of ration quality on larval growth and NOR size. We provided larvae with an optimum daily food ration of 1.0 mg/larva as determined by Naylor and Rodrigues (1995). Food consisted of a mixture of ground Nutrifin® fish food flakes (the food typically used in *Chironomus* cultures) and methylcellulose, which served as a source of biomass that is consumed by larvae but has no nutritive value (Burt 1998). We regulated ration quality by varying the proportions of each constituent. Treatments followed a geometric series of nutritional quality (0, 12.5, 25, 50, and 100% Nutrifin) and ranged from having no nutritional value (0% Nutrifin) to greatest nutritional value (100% Nutrifin). At the conclusion of the experiment, we measured survival, biomass, and NOR size of each surviving larva. We expected growth (measured as individual final biomass) to increase as ration quality increased. We also expected NOR size to vary as a function of ration quality. Better-fed larvae would be growing faster at the end of the experiment and would exhibit larger NOR than poorly fed larvae.

Variable ration experiment.—In this experiment, we used a variable feeding regime to determine if NOR size varied as a function of recent growth independently of larval size. The experiment followed a 2 × 2 factorial design. The 2 factors were experimental period and ration quality. The time course of this experiment was divided into early (days 0–7) and late

(days 8–14) periods. Ration quality was either low (12.5% Nutrifin) or high (100% Nutrifin). Thus, larvae in a treatment received a ration of either low or high quality for the early part of the trial, after which ration quality was either kept the same or changed to the complementary treatment. At the conclusion of the experiment, we measured survival, biomass, and NOR size. We set up additional jars ($n = 4$) of low and high ration quality and harvested them at the midpoint of the experiment (day 7) to provide a 2nd measure of biomass to estimate the growth rate of chironomids during the late period.

The potential for new tissue elaboration is proportional to the amount of food consumed and assimilated, so we expected the final biomass of individual larvae to reflect the quality of the ration with which they were provided averaged over the early and late periods. Therefore, we expected larvae given a uniformly low or high quality ration to be small or large, respectively, and larvae whose ration was changed at the midpoint of the trial to be intermediate in size.

Unlike larval final biomass, which we expected to depend on an individual's entire feeding history, we expected larval growth rates at the conclusion of the experiment to reflect the quality of the ration that they had been given during the late period only. Therefore, we expected larvae switched from a high- to low-quality ration to be growing slowly at the end of the late period, and we expected the converse for larvae switched from a low- to high-quality ration. We expected NOR size to reflect recent growth rates and, thus, the quality of the ration provided during the late period only.

Experimental procedures

Treatment jars were 12 × 12 × 15 cm, 2-L glass containers prewashed with 10% HNO_3 and triple rinsed with distilled water. Jars contained 500 g of washed, fine silica sand and 1 L of water aerated for 48 h prior to the addition of larvae. Ionic concentration of the water was standardized by adding salts to deionized water ($CaCl_2$: 79.2 mg/L, $MgSO_4$: 44.5 mg/L, $NaHCO_3$: 85.6 mg/L, KCl: 2.8 mg/L; Bedard et al. 1992). Each jar was covered, continuously aerated using a branching capillary tube system (Corkum and Hanes 1989), and kept at 21 ± 2°C on a 16:8 h light:dark photoperiod in a walk-in environmental chamber. Mean (± SE) temperature, dissolved O_2 concentration, and pH were 21.8 ± 0.1°C, 5.9 ± 0.4 mg/L, and 8.0 ± 0.1, respectively.

We randomly selected groups of 50 newly hatched larvae from egg masses and placed 1 group in each treatment jar. We added food daily to each treatment jar.

At the conclusion of the trial, we emptied the jar contents and recovered larvae on a 250-µm-mesh sieve. We hand-picked the larvae from the retained sand with fine forceps, individually blotted them on paper toweling to remove excess water, counted them, and immediately preserved them in chilled (4°C) Carnoy's solution (3:1 volume/volume absolute ethanol/glacial acetic acid). We poured off the Carnoy's solution and replaced it with fresh preservative after 10 min, 1 h, and 24 h. Total handling time for each jar (time elapsed between first and last individual preserved) was constant among trials and treatments (uniform ration experiment: 3.5 ± 0.3 min, $n = 40$; variable ration experiment: 5.0 ± 1.3 min, $n = 32$).

Biomass and growth rate measurements

We placed preserved larvae on their sides in a Petri plate and digitally photographed them with a color video camera (Hitachi, Chiyoda, Tokyo, Japan) equipped with a macro lens and a Pixelsmart® video card (Pixelsmart®, Toronto, Canada). We measured total body length, from the anterior part of the head to the posterior edge of the last abdominal segment, to the nearest 0.01 mm using Mocha® imaging analysis software (version 1.2; Jandel Scientific, Corte Madera, California). We converted body length to biomass with the length–dry mass conversion formula $M = aL^b$ (Burgherr and Meyer 1997), where M is individual biomass (mg), L is body length (mm) and, a and b are constants equal to 0.00068 and 2.620, respectively, derived from a length–mass regression equation for *Chironomus* spp. (Benke et al. 1999). We calculated growth (mg/d), where g represents the mean growth rate of larvae in a single jar, with the formula $g = \ln(M_F/M_I)/t$ where M_F is mean final biomass, M_I is mean initial biomass, and t is the number of days between biomass measurements (Hauer and Benke 1991).

We noticed considerable variation in development among individuals of the same age while photographing the larvae. Because the pattern and timing of puff activation may be associated with development (e.g., Ashburner 1967, Santa-Cruz et al. 1978) and NOR expression depends on larval developmental stage (Kiknadze et al. 1989), we scored each individual according to its developmental stage before extracting the salivary glands. We assigned each larva to 1 of 3 classes (early = stage 0–3, intermediate = stage 4–6, late = stage 7–9) based on the 9 developmental phases of the 4th instar illustrated by Wülker and Götz (1968).

Polytene chromosome extraction and staining

We extracted polytene chromosomes from salivary glands and stained them with a modification of the aceto-orcein technique (M. G. Butler, North Dakota State University, personal communication). To extract the salivary glands from a preserved chironomid, we placed a specimen in a depression slide filled with 50% acetic acid and viewed it under the low power of a dissection microscope. We removed the head and severed the body just below the 2nd thoracic segment. We extruded the salivary glands anteriorly from the thorax using gentle pressure, carefully isolated and transferred them to a microscope slide, and stained them with aceto-orcein (1 mg orcein:50 mL of 60% acetic acid). After 6 min, we triple rinsed the glands with 50% acetic acid (2 drops placed over each gland and immediately drawn off with bibulous paper) to remove any excess or unbound stain. Afterward, we rinsed salivary glands with a drop of 15% lactic acid, followed immediately with a drop of 25% lactic acid. We transferred the glands to a clean slide and covered them with a drop of 45% lactic acid. We placed a coverslip on top, and squashed the glands with a press designed and constructed to deliver an even and consistent amount of pressure. We allowed the preparation to sit for 10 min at room temperature to dry before rimming the edges of the coverslip with nail polish. We stored the slide in a freezer until it could be photographed digitally.

Measurements of NOR size

Previous investigators have characterized puff size by calculating the relative nucleolar diameter (Bentivegna and Cooper 1993, Hudson and Ciborowski 1996). Puff size is proportional to chromosome size, and chromosome size corresponds to the size of an individual (Bentivegna and Cooper 1993). Therefore, measurements taken from different-sized larvae can be standardized by measuring both NOR and chromosome size.

We also used diameter as a measure of NOR size. The NOR of *C. riparius* is found on chromosome G (Michailova et al. 2006; Fig. 1A–C; color version available online from: http://dx.doi.org/10.1899/12-168.s1). We measured NOR diameter at the center of the Dd region. We used the diameter of the A1b band as a measure of chromosome size. This band is furthest away from the NOR and Balbiani rings (BRs), making it least likely to be structurally affected by changes in their size and activity (Zhimulev et al. 1981).

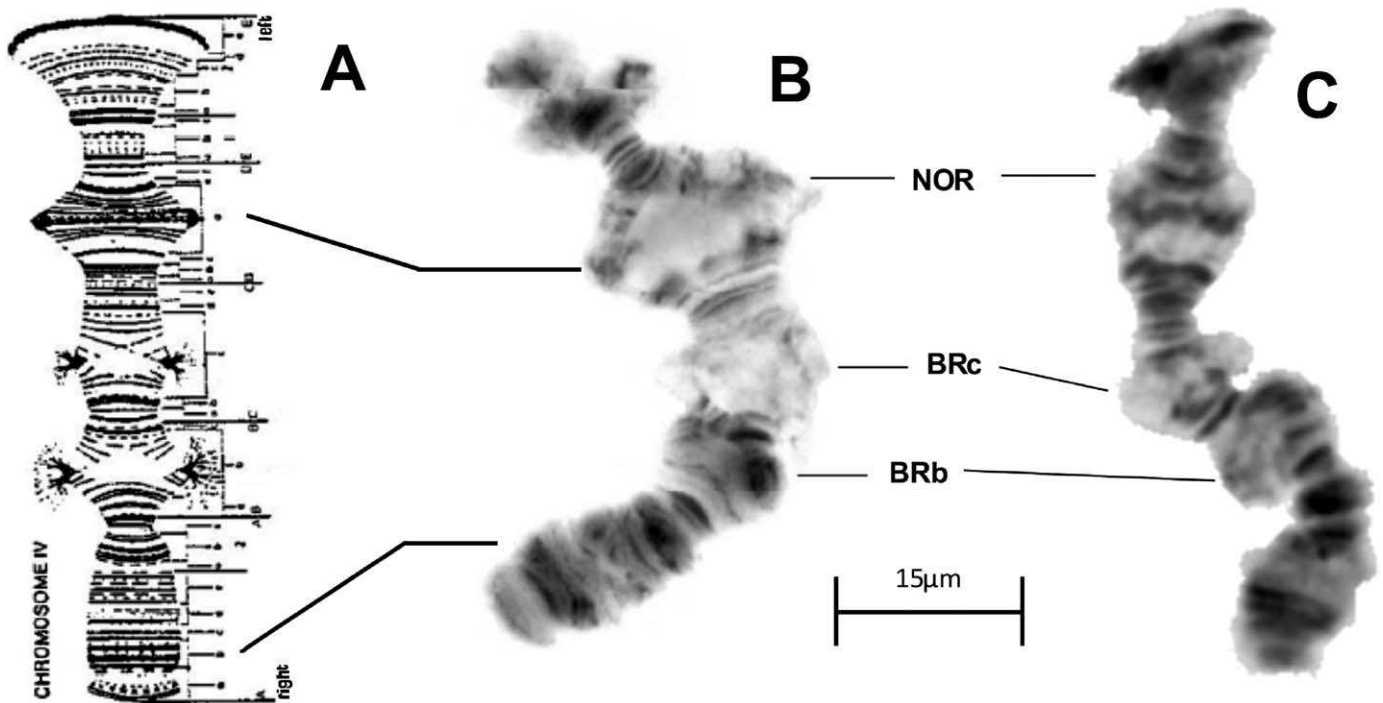We viewed polytene chromosomes at 400× magnification with a Meiji compound microscope (Meiji

FIG. 1. Chromosomal map (Keyl and Strenzke 1956) (A) and digital images taken at 400× magnification (B, C) of the IVth chromosome (G arm) of *Chironomus riparius*. Lines indicate the location of the Dd and A1b bands used as measures of nucleolar organizer region (NOR) and chromosome size, respectively. Also indicated are the Balbiani rings (BRb and BRc). NOR size was correlated with larval growth rate. *Chironomus riparius* larvae growing rapidly (B) had larger NOR than larvae growing slowly (C) during the late period of the variable-ration experiment.

Techno America, San Jose, California) attached to a SPOT Insight[TM] Firewire 2 Megapixel Color Mosaic digital video camera (SPOT Imaging Solutions, Sterling Heights, Michigan). We photographed 3 to 6 chromosomes/larva and measured NOR diameter from the digital images to the nearest 0.1 μm using UTHSCSA ImageTool (version 3.0; UTHSCSA, San Antonio, Texas). We calculated the mean NOR diameter for a single chironomid. We excluded individuals from which <3 NORs were measured from further analyses. For each replicate jar, we calculated mean NOR diameter from measures taken from 2 to 6 individuals. To account for variability in size among individuals and because good-quality stains were difficult to obtain from individuals <9 mm (JJHC, personal observation), we systematically chose the 3 largest and median-sized larvae in each jar for analysis.

Statistical analyses

We ran statistical analyses in STATISTICA (version 6.1; StatSoft, Inc., Tulsa, Oklahoma). We tested statistical significance of differences among treatment groups and trials for chironomid survival, biomass, and growth using planned-comparison analysis of variance (ANOVA). We tested the statistical significance of differences among treatment groups and trials for NOR size (controlling for chromosome size) using planned-comparison analysis of covariance (ANCOVA). We adjusted all reported NOR values to a mean chromosome size of 9.2 μm, the greatest area of overlap among treatments. Statistical significance of differences in the proportion of larvae in each developmental stage was tested using a χ^2 contingency test. We report values as mean $\pm$ SE, and α was set at 0.05 for all tests.

Results

Uniform-ration experiment

Survival and biomass.—As expected, survival was lowest in the starvation (0% Nutrifin) treatment (3.5 $\pm$ 2.5%, $n = 8$; planned comparison, $F_{1,30} = 52.45$, $p < 0.001$). Because only a few very small larvae survived in the 0% Nutrifin treatment they were not included in further analyses. Survival was homogeneous among all other treatments (45.0 $\pm$ 3.2%, $n = 32$; planned comparisons, $p > 0.05$). Larval survival was significantly lower in trial 1 (34.3 $\pm$ 3.4%, $n = 16$) than in trial 2 (55.8 $\pm$ 4.1%, $n = 16$) ($F_{1,30} = 11.88$, $p <$
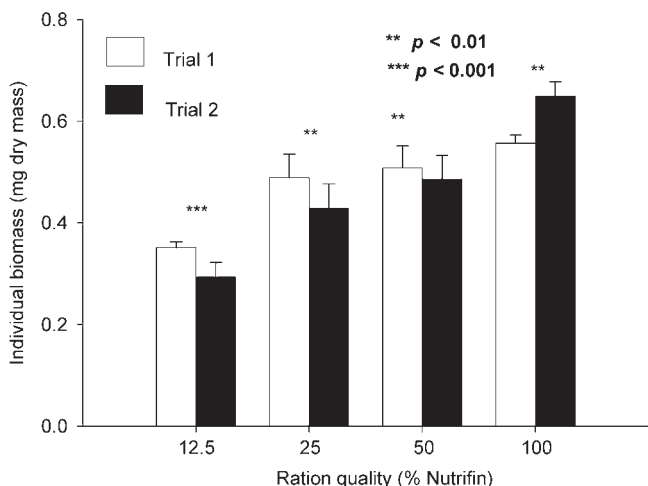
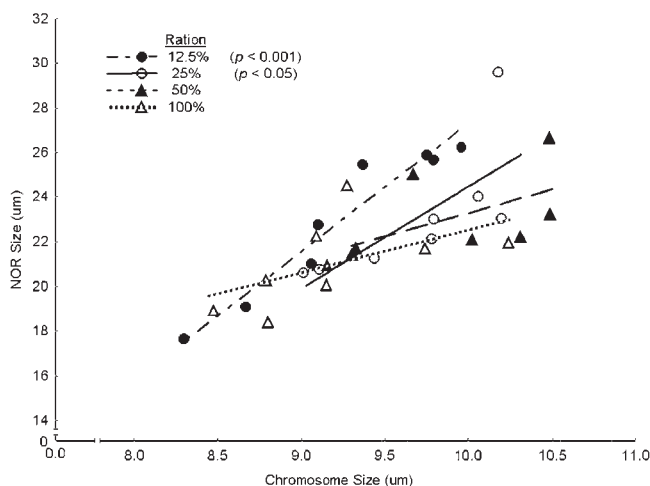FIG. 2. Mean (+1 SE) biomass of midge larvae (mg dry mass) at the end of the uniform-ration experiment.

FIG. 3. Nucleolar organizer region (NOR) size at the end of the uniform-ration experiment as a function of chromosome size at each of 4 different ration qualities. Each point represents the mean for all larvae from a single replicate jar.

0.001). Individual biomass was significantly different among treatments (planned comparisons, $p < 0.01$) and increased as ration quality increased (Fig. 2). Individuals provided with a low-quality ration (12.5% Nutrifin) were smallest, weighing 0.32 ± 0.02 mg ($n = 8$) on average, whereas those kept on the highest quality ration (100% Nutrifin) were largest, weighing 0.60 ± 0.02 mg ($n = 8$) on average. Biomass did not differ between trials ($F_{1,24} = 0.22$, $p > 0.1$).

Ration quality also influenced development. Two lines of evidence support this conclusion. First, by the conclusion of the experiment, 7 individuals had pupated, and 6 of these had been fed 1 of the 2 highest quality rations (50 and 100% Nutrifin). Second, based on the assignment of individuals to 1 of 3 prepupal developmental classes (done prior to extracting the salivary glands), the proportion of individuals intermediate or late in development increased as diet quality increased (Table 1; χ^2 contingency test, $\chi^2 = 30.56$, df = 6, $p < 0.001$).

TABLE 1. Percentage of *Chironomus riparius* larvae (from which nucleolar organizer region (NOR) size measurements were obtained) at the end of the uniform-ration experiment assigned to each of 3 developmental classes based on the development of their imaginal discs.

Diet quality (% Nutrifin)	Developmental class		
	Early	Intermediate	Late
12.5% ($n = 41$)	100	0	0
25% ($n = 41$)	92.7	4.9	2.4
50% ($n = 45$)	77.8	20.0	2.2
100% ($n = 46$)	58.7	30.4	10.9

NOR size.—A total of 906 chromosomes was photographed and measured from 173 individuals. NOR size was significantly correlated with chromosome size (regression analysis, $R^2 = 0.49$, $F_{1,30} = 28.71$, $p < 0.001$). Larger chromosomes had larger NOR size (Fig. 3). A test for parallelism revealed that this relationship was homogeneous across larvae fed different quality rations ($F_{3,16} = 0.81$, $p > 0.1$). NOR size was significantly larger in trial-1 larvae (22.54 ± 0.49 µm, $n = 16$) than in trial-2 larvae (20.64 ± 0.42 µm, $n = 16$) (ANCOVA, $F_{1,23} = 10.70$, $p < 0.01$). However, contrary to our expectations, NOR size did not differ significantly among larvae fed different quality rations (ANCOVA, $F_{3,23} = 2.34$, $p > 0.1$). Both NOR and chromosome size were independent of larval biomass (linear regression: $R^2 = 0.01$, $F_{1,30} = 0.3$, $p > 0.1$; $R^2 = 0.09$, $F_{1,30} = 3.04$, $p > 0.05$; respectively).

We contrasted the NOR of the various developmental stages, independent of treatment (Fig. 4). Larvae that were early in their development had a significantly larger NOR than those larvae who were in the intermediate or late stages of prepupal development (planned comparison, $F_{1,169} = 14.89$, $p < 0.001$).

Variable-ration experiment

Survival and biomass.—Survival did not differ among treatments ($49.8 \pm 3.4\%$, $n = 32$; ANOVA, $F_{3,24} = 0.42$, $p > 0.1$). Survival was significantly lower in trial 1 ($37.6 \pm 4.1\%$, $n = 16$) than in trial 2 ($62.0 \pm 3.4\%$, $n = 16$) (ANOVA, $F_{1,24} = 19.75$, $p < 0.001$).

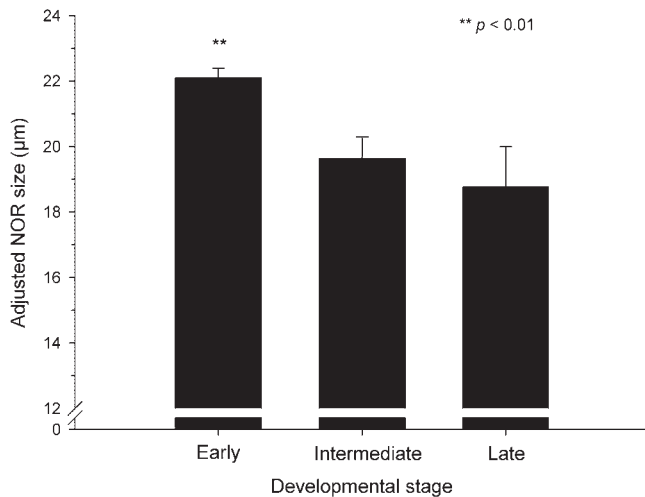Both early and late ration quality significantly affected biomass (Fig. 5). Midway through the

FIG. 4. Mean (+1 SE) nucleolar organizer region (NOR) size for larvae at different stages of prepupal development (early, $n = 141$; intermediate, $n = 25$; late, $n = 7$). Values are adjusted for a chromosome size of 9.2 μm.

experiment, larvae provided with a low-quality ration were ½ the size of larvae that were provided with a high-quality ration (0.027 ± 0.003 mg, $n = 4$ vs 0.054 ± 0.007 mg, $n = 4$) (1-tailed t-test, $t = -3.39$, df = 6, $p < 0.05$). Larvae fed a low-quality ration during the early period also were significantly smaller at the conclusion of the experiment than larvae fed a high-quality ration during the early period (ANOVA, $F_{1,24} = 61.54$, $p < 0.001$). Regardless of the quality of the ration they received during the early period, larvae provided with a low-quality ration during the late period were significantly smaller at the conclusion of the experiment than larvae fed a high-quality ration
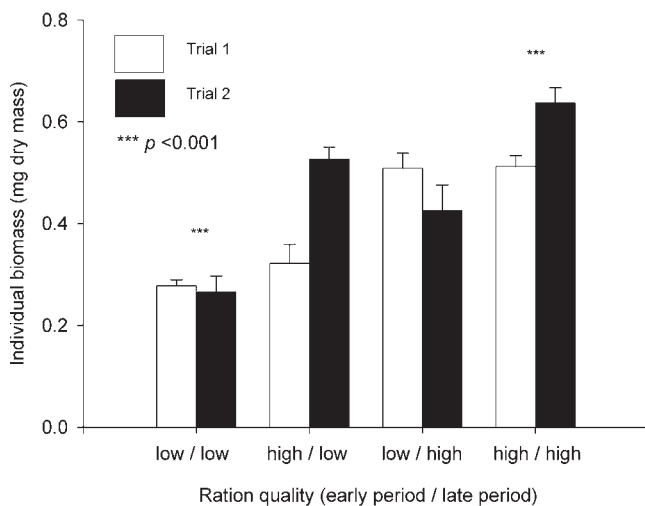

FIG. 5. Mean (+1 SE) biomass of midge larvae at the end of the variable-ration experiment.
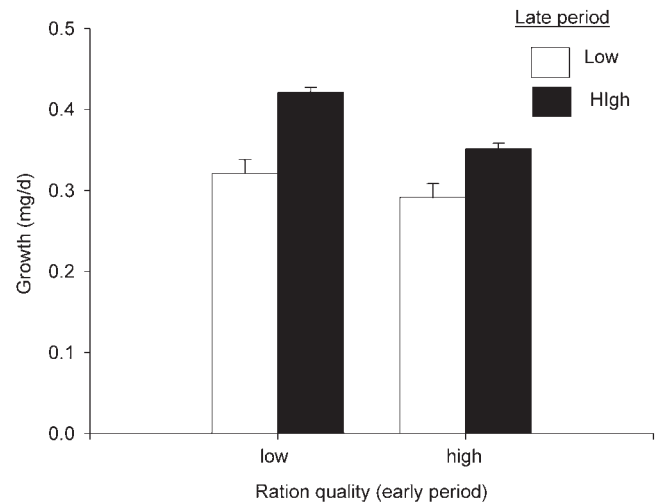

FIG. 6. Mean (+1 SE) growth rate of midge larvae during the late period of the variable-ration experiment ($n = 4$).

during the late period (ANOVA, $F_{1,24} = 34.79$, $p < 0.001$). Individual biomass was significantly lower during trial 1 (0.41 ± 0.12 mg, $n = 16$) than during trial 2 (0.46 ± 0.15 mg, $n = 16$) (ANOVA, $F_{1,24} = 7.05$, $p < 0.05$).

In accordance with our expectations, planned comparisons showed that larval size at the end of the study reflected the nutritional quality of the ration provided averaged over both the early and late periods. Larvae given a low-quality ration throughout the study were small, larvae given a high-quality ration were large, and larvae whose ration was changed at the midpoint of the study were intermediate in size. Larval development proceeded at a similar rate across treatments (χ^2 contingency test, $\chi^2 = 8.17$, df = 6, $p > 0.1$).

Recent growth rate (day 8–14).—Growth rates during the late period were calculated only for trial 2 ($n = 4$) because no initial biomass measurements (day 7) were obtained for larvae from trial 1. As expected, larval growth during the late period reflected the nutritional quality of the ration provided during that period. Larvae given a high-quality ration were growing 25% faster than larvae given a low-quality ration (ANOVA, $F_{1,12} = 38.16$, $p < 0.001$; Fig. 6). However, the quality of the ration provided during the early period also significantly affected growth during the late period. Larvae given the low-quality ration early were growing 15% faster than larvae provided with a high-quality ration early (ANOVA, $F_{1,12} = 14.59$, $p < 0.01$). Also in line with our expectations, although larvae from the reciprocal treatments were similar in size at the end of the experiment (planned comparison, $F_{1,24} = 1.89$, $p > 0.1$; see Fig. 5), larvae switched from a
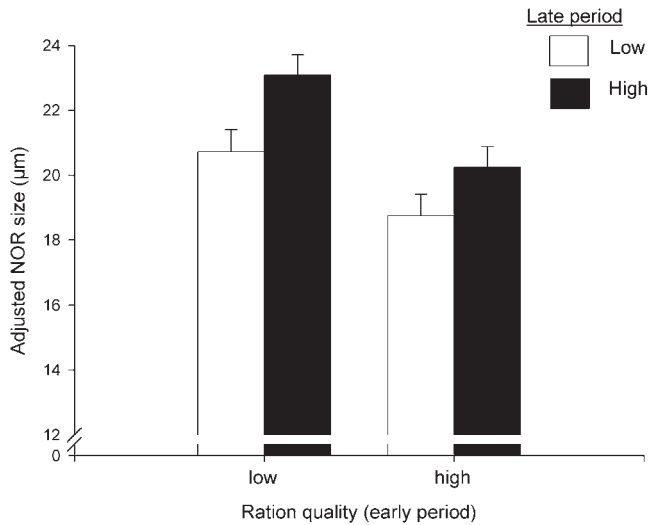
FIG. 7. Mean (+1 SE) nucleolar organizer region (NOR) size of larvae at the conclusion of the variable-ration experiment ($n = 4$). Values are adjusted for a chromosome size of 9.2 μm.
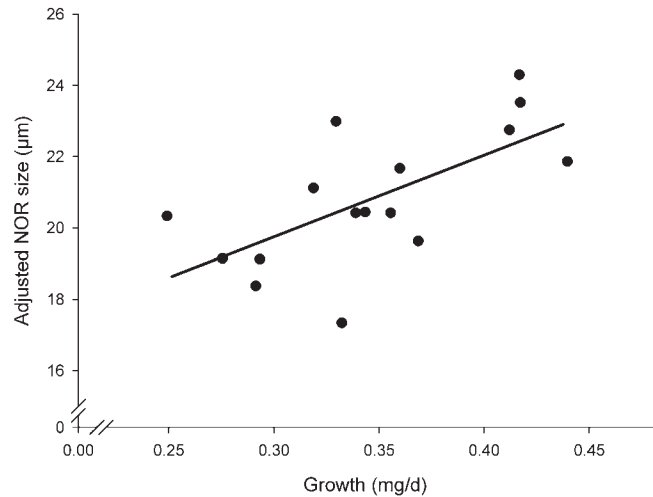

FIG. 8. Scatterplot showing the relationship between nucleolar organizer region (NOR) size and growth rate during the late period of the variable-ration experiment ($n = 16$). NOR values are adjusted for a chromosome size of 9.2 μm. Each point represents the mean for a single replicate jar.

low- to high-quality ration had a significantly higher growth rate during the late period than larvae switched from a high- to low-quality ration (planned comparisons, $F_{1,12} = 49.97$, $p < 0.001$).

NOR size.—A total of 495 chromosomes was photographed and measured from 93 individuals. NOR size was significantly correlated with chromosome size (regression analysis, $R^2 = 0.29$, $F_{1,14} = 5.68$, $p < 0.05$). Larger chromosomes had larger NOR. A test for parallelism revealed this relationship was homogeneous across all 4 treatments ($F_{1,8} = 0.008$, $p > 0.1$). NOR size was significantly affected by the quality of both early and late rations (Fig. 7). When the quality of the ration provided during the early period was low, larvae had a significantly larger NOR at the conclusion of the experiment (ANCOVA, $F_{1,11} = 14.72$, $p < 0.01$). However, if larvae were given low-quality food during the late period, they had a significantly smaller NOR at the conclusion of the experiment than larvae fed a high-quality ration over the same interval (ANCOVA, $F_{1,11} = 8.74$, $p < 0.05$).

NOR size and recent growth rate.—We found a significant positive relationship between NOR size and recent growth rate (regression analysis, $R^2 = 0.43$, $F_{1,14} = 10.53$, $p < 0.01$; Fig. 8). Larvae growing rapidly during the late period had larger NOR than larvae growing slowly during the late period (Fig. 1B, C).

Discussion

We conducted 2 experiments to examine the relationship between NOR size and chironomid

growth under controlled laboratory conditions. The uniform-ration experiment revealed that ration quality influenced chironomid growth and development. It also demonstrated that NOR size was independent of larval biomass, but was developmentally dependent. In the variable-ration experiment, both early and late ration quality affected chironomid biomass and growth, and NOR size reflected an individual's recent growth rate. The combined results demonstrated that NOR size varied as a function of prepupal development and was positively correlated with a chironomid's most recent growth rate, independently of its biomass.

Uniform-ration experiment

Initial comparison of the expected and observed relationships gave results inconsistent with expectations. NOR size did not vary as a function of ration quality. However, further inspection suggests that chironomid growth rates at the end of the experiment also did not vary as a function of ration quality. Typical larval chironomid growth is nonlinear. The rate of mass accrual decreases as biomass increases (Stites and Benke 1989). In the very late stages of prepupal development, 4th-instar larvae may even lose biomass (Vos et al. 2002). Thus, as larvae approach maximum size, they allocate increasing amounts of energy to pupation rather than to growth. Eventually, growth ceases altogether (growth is restricted to the larval instars; Butler 1984) and metamorphosis takes place.

Laboratory-reared *C. riparius* larvae attain a length of 12.5 to 12.6 mm (Perry et al. 2002). At the conclusion of the uniform-ration experiment, only 9% of larvae fed a low-quality ration of 12.5% Nutrifin were >12.6 mm. In contrast, 57% of the larvae fed an optimal ration of 100% Nutrifin were >12.6 mm. Presumably, these large individuals were approaching, or had already reached, their maximum size. Furthermore, many of these larvae were late in development and approaching metamorphosis. Therefore, both larval size and the proportion of larvae whose growth had begun to slow increased as a function of ration quality.

We suggest this created a situation in which recent growth rates were similar, on average, among treatments, but for different reasons. Most individuals fed a low-quality ration were growing slowly because of food limitation, whereas most individuals fed a high-quality ration were growing slowly because they had attained maximum size and development. Thus, NOR size was invariable because recent growth rates were invariable. Unfortunately, the uniform-ration experimental design precluded obtaining direct measures of recent growth during the late period. However, the finding that NOR size varied as a function of prepupal stage in 4th-instar larvae is consistent with this scenario and findings that NOR expression is reduced during larval molts (Kiknadze et al. 1989).

Variable-ration experiment

The relationship between NOR size and growth rate was most clearly demonstrated by the results from the variable-ration experiment. Chironomids in the reciprocal treatments displayed very different NOR size despite being similar in size and development. The differences in NOR size between the 2 treatments reflected differences in recent growth rate, and as expected, larvae recently fed a high-quality ration had larger NOR. The finding that early ration quality had an effect on growth rates during the late period was not surprising. Food deprivation early in development can influence several life-history traits including survival, growth, and reproduction (Metcalfe and Monaghan 2001). With respect to growth, many organisms exhibit accelerated growth rates following unfavorable conditions or a period of resource limitation in an attempt to compensate or catch-up (Metcalfe and Monaghan 2001). This response is especially pronounced when individuals are nutritionally deprived early in life (e.g., Mangel and Munch 2005, Dmitriew et al. 2007). Thus, compensatory growth probably explains why, despite being starved early, larvae fed a high-quality ration only

during the late period had higher growth rates and higher NOR size than larvae fed a high-quality ration throughout. As in the uniform-ration experiment, larvae fed a high-quality ration throughout had similar NOR size to larvae fed a low-quality ration throughout. Direct measures of growth confirmed that these larvae also were growing at comparable rates.

Regression analysis showed a significant linear relationship between NOR size and growth rate. The regression explained 43% of the observed variation, but we suggest the relationship is actually much stronger. First, although NOR size was measured on an individual basis and then averaged, we measured growth (change in total biomass) at the level of the population (replicate jar). As a result, we were unable to account for among-individual variation in growth. This limitation may have increased the variability of our measures, particularly in the low-quality ration and reciprocal treatments because individual differences in growth are magnified under more stressful conditions (Koehn and Bayne 1989). Second, our measure of growth reflected the average growth rate of larvae over a 7-d period. However, a significant difference in NOR activity and size is observable in as little as 12 h after exposure to Cd (Planello et al. 2007). Much explanatory power probably was lost by our decision to relate a short-term putative growth indicator (NOR size) to a measure of growth estimated over a 7-d interval. Both of these concerns could be addressed in future studies by rearing and feeding larvae individually and taking measurements of growth and NOR size more frequently.

NOR size as an indicator of stress

The NOR is a specialized puff that displays reduced size, which is indicative of reduced transcriptional activity, in chironomid larvae exposed to toxic stress. Hudson and Ciborowski (1996) found that the proportion of *Chironomus salinarius* group larvae exhibiting NOR regression increased with exposure to greater degrees of sediment contamination. Field-collected and laboratory-reared *C. riparius* larvae exposed to elevated levels of Cr, Cu, Zn, Pb, and Al exhibit regressed NOR (Michailova et al. 1998, 2001a, b, 2003, 2006, 2012). In addition to decreasing in size, the NOR of *C. riparius* also showed lower levels of preribosomal RNA synthesis following exposure to Cd (Planello et al. 2007). Based on our results, the NOR of *C. riparius* also responds to generalized environmental stress and shows decreased size in response to lower ration qualities. However, even in the presence of abundant food and favorable

environmental conditions, NOR expression may be inhibited by the presence of contaminants, as described above. Our results also agreed with previous findings that NOR size varies as a function of prepupal development (Kiknadze et al. 1989). Balbiani rings—another specialized class of puffs—also change in size in response to toxic chemicals (Aziz et al. 1991, Bentivegna and Cooper 1993, Michailova et al. 1998, 2001a, b, 2003, 2006, 2012) and modify their activity throughout development (Santa-Cruz et al. 1978).

Most importantly, NOR size reflects a chironomid's most recent growth rate, independently of its biomass. This finding is consistent with the observation by Pelling and Beermann (1966) that metabolically active chironomid larvae have extremely puffed NOR, whereas inactive larvae have condensed NOR. Our results also build on the findings of Kiknadze (1978) that NOR size depends strongly on 4th-instar developmental stage (cited in Kiknadze et al. 1989). To our knowledge, our study is the first experimental demonstration of the correlation between NOR size and growth rate. Because puff size is related to transcriptional activity (Pelling 1964 cited by Grossbach 1977, Daneholt et al. 1969) and the NOR is responsible for the synthesis of preribosomal RNA (reviewed by Case and Daneholt 1977), the relationship between NOR size and growth rate is consistent with other studies linking RNA synthesis to growth. In bacteria, total RNA and ribosomal RNA (rRNA) concentrations increase as growth rates increase (Sutcliffe 1970, Binder and Liu 1998). The RNA:deoxyribonucleic acid (DNA) ratio is positively related to growth in bacteria (Kemp et al. 1993) and invertebrates (McKee and Knowles 1989, Dahlhoff and Menge 1996, Vrede et al. 2002, Schlechtriem et al. 2008) and is an increasingly commonly used indicator of growth and nutritional condition in fish (Buckley et al. 1999).

Our results validate the continued development and use of the NOR as a biomarker of sublethal stress by showing a direct relationship of this suborganismal response to unfavorable changes (slowed/reduced growth) at the individual level. Our results have important potential implications for assessing chironomid growth and chironomid secondary production in natural populations. Midge larvae are often easy to collect in the field, but multiple generations/y, nonrecognizable cohorts, and rapid growth rates (e.g., Benke 1998) necessitate the use of time-consuming and labor-intensive methods to estimate secondary production (Waters 1977). In some instances, samples must be taken no less than every 3 d (Benke 1984). Given that NOR size reflects a chironomid's recent—

and probably instantaneous—growth rate, it could potentially provide a surrogate measure of growth for larvae collected in the field that, combined with a measure of population biomass, could be used to estimate secondary production from a single sample. If multiple estimates of NOR size can indeed be extrapolated to estimates of chironomid secondary production, the NOR may ultimately serve as a sublethal biomarker that permits one to correlate changes in response to stress at the suborganismal level (NOR size) with changes at the individual (growth rate) and population (secondary production) level.
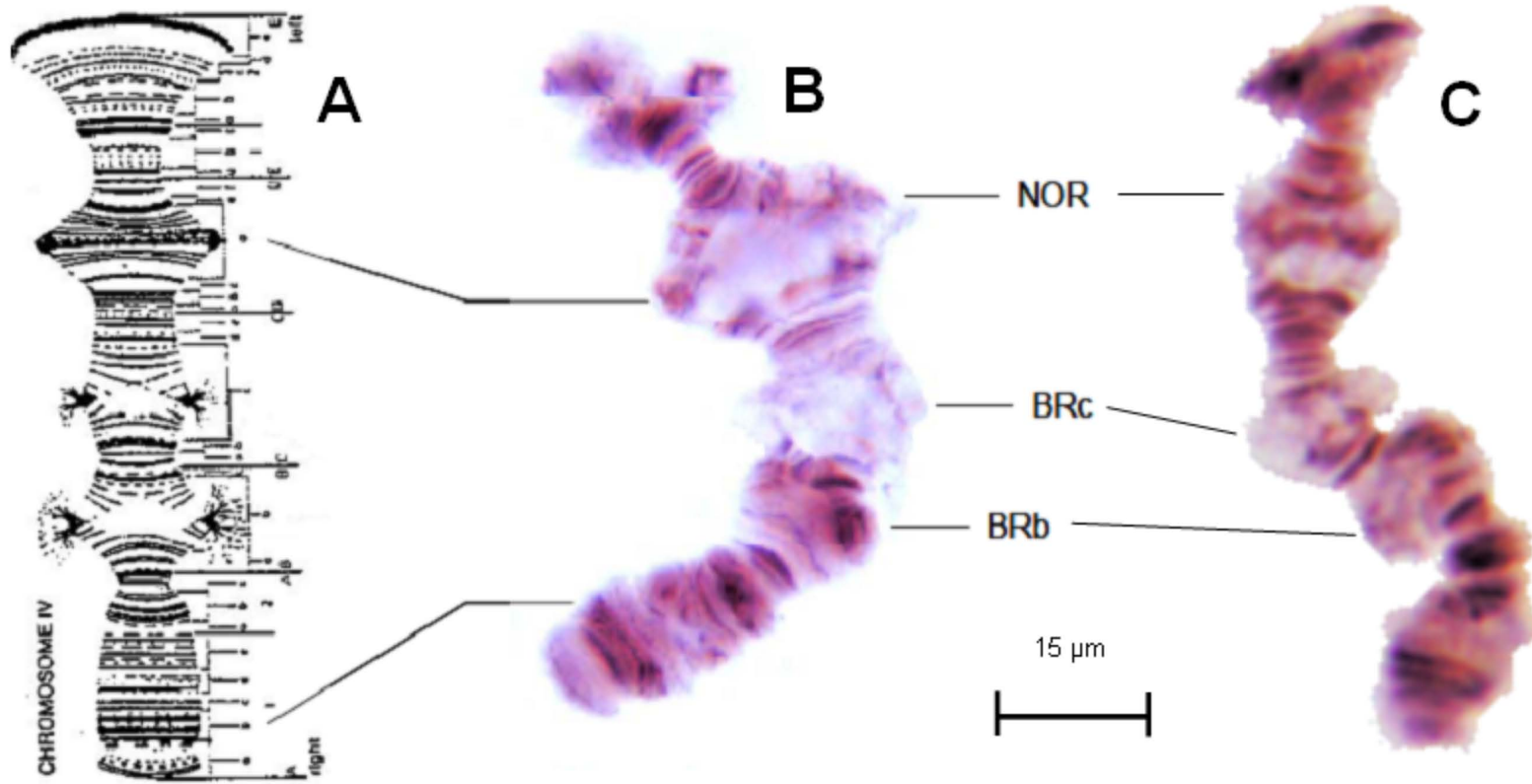
Acknowledgements

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A   **B**   **C**

CHROMOSOME IV

NOR

BRc

BRb

15 µm