Transgenerational Variation in Metabolism and Life-History Traits Induced by Maternal Hypoxia in *Daphnia magna*[*]

Sarah J. Andrewartha
Warren W. Burggren[†]
Developmental and Integrative Biology, Department of Biological Sciences, University of North Texas, 1155 Union Circle #305220, Denton, Texas 76203

Accepted 5/10/2012; Electronically Published 8/2/2012

ABSTRACT

Hypoxic stress can alter conspecific phenotype and additionally alter phenotypes of the filial generation, for example, via maternal or epigenetic processes. Lasting effects can also be seen across development and generations even after stressors have been removed. This study utilized the model of rapidly developing, parthenogenetic *Daphnia* to examine the intraspecific variability of response of exposure of a parental generation to hypoxia (4 kPa) within a single clone line across development, across broods, and across generations. Body mass across development and reproductive output were monitored in the parental generation and the first three broods of the first filial generation (which were not directly exposed to hypoxia). O_2 consumption across a wide Po_2 range (normoxia to anoxia) was assessed to determine whether exposure of the parental generation to hypoxia conferred hypoxia tolerance on the offspring and whether this transgenerational, epigenetic phenomenon varied intraspecifically. Differences in mass occurred in both the parental generation (hypoxia-exposed smaller during brood 1 and brood 2 neonate production) and the filial generation (e.g., brood 1 and 2 neonates from hypoxic mothers were initially smaller than control neonates). However, differences in mass were not accompanied by changes in reproductive output (assessed by brood number and neonate size). At day 0, first filial generation brood 1 neonates from hypoxia-exposed mothers had a higher metabolic rate than control neonates. However, this effect, like that of body mass, dissipated with development within a brood but also with subsequent broods. An isometric scaling exponent for $\dot{V}O_2$ was repeatedly observed across a wide Po_2 range (21–2 kPa) throughout neonatal development.

[*] This paper was submitted in response to a call for papers for a Focused Issue on "Intraspecific Variation in Physiology and Behavior."

† Corresponding author; e-mail: burggren@unt.edu.

Physiological and Biochemical Zoology 85(6):625–634. 2012. 1522-2152/2012/8506-1134$15.00. DOI: 10.1086/666657

Introduction

Although not the first to recognize the "tyranny of the golden mean," the seminal paper by Bennett (1987) encouraged ecologists and physiologists to view intraspecific (i.e., interindividual) variability as a tool to be exploited rather than as confounding background noise. Subsequently, many studies have focused on a snapshot view of variability between individuals (e.g., Sinervo et al. 1991; Chappell et al. 2007; Bozinovic et al. 2009). While useful in across-population analysis and determining correlations between individual physiology and relevant variables (e.g., morphology, habitat, diet, etc.), the concept of intraspecific variation can be applied in an even broader sense, encompassing variation within a species across developmental time (within individuals or cohorts). For example, temporal changes in heart rate variation across embryonic development are indicative of maturation of nervous control and increasing endothermic capabilities (see Andrewartha et al. 2011 for review), and the flight capabilities of honeybees are affected by individual age and behavioral development (whether nurse or foraging caste; Vance et al. 2009). Indeed, intraspecific physiological variation can even be viewed in an epigenetic context—that is, in the form of nongenetic, transgenerational transfer of phenotype (see Ho and Burggren 2010 for review of physiological epigenetic phenomena).

Intraspecific variation in morphological, physiological, and behavioral phenotype arising from genetic or other origins undeniably influences many parameters of interest to physiologists, such as rate of oxygen consumption ($\dot{V}O_2$), reproductive output, growth, and development (e.g., Boratynski and Koteja 2009; Millidine et al. 2009; Biro and Stamps 2010; McKechnie and Swanson 2010). Even parthenogenetic individuals from the same clone line exhibit variability during controlled laboratory experiments. For example, egg mass of parthenogenetically reproducing Bynoe's gecko (*Heteronotia binoei*) varied by nearly threefold within some clones. While females from some clone lines selected laying temperatures within a relatively narrow range (~2°–3°C), the selected laying temperature within other clones ranged by as much as 22°C (Andrewartha et al. 2010). These and other studies highlight that interindividual variation persists even within a single clone line, although whether this variation manifests through alterations in epigenome, maternal effects, or subtle environmental differences is not yet understood.

Stressors such as hypoxia have the potential to alter the conspecific phenotype (e.g., Wiggins and Frappell 2000; Seidl et al. 2005), and, through maternal or epigenetic processes, they

can alter filial generation phenotypes (Agrawal et al. 1999; LaMontagne and McCauley 2001; Ho and Burggren 2010; Mikulski and Pijanowska 2010; Tong et al. 2011). The water flea *Daphnia* is a fast-reproducing, parthenogenetic model that provides an opportunity to remove genetic variability and focus on intraspecific variability within a single clone line in response to a stressor (in this case hypoxia) not only across development but also between broods and between generations. The current study, then, moves beyond the typical interindividual variation snapshot to examine the effect of an acute (neonatal day 0–6) hypoxic stress on *Daphnia magna* mass simultaneously across development and multiple reproductive events. Further, this study aims to examine mass and rate of oxygen consumption ($\dot{V}O_2$) across development and reproductive output of the first filial generation (not itself directly exposed to the stressor). Exposure of a parental generation (P) of *Daphnia* to chronic hypoxia decreases adult body size but does not alter the number of neonates produced per brood (Seidl et al. 2005). We therefore hypothesize that neonate size will be reduced when the P generation is exposed to hypoxia because neonate size, determined by maternal carapace size (e.g., Lampert 1993; Seidl et al. 2005), is certainly smaller following hypoxic exposure. This effect may potentially continue in the F_2 generation (where there is no direct hypoxia exposure) if the F_1 individuals are still smaller when reproducing. Exposing adult *D. magna* to hypoxia (~4 kPa) reduces overall $\dot{V}O_2$ and critical Po_2 (P_{crit}; Wiggins and Frappell 2000; Seidl et al. 2005). Therefore, we additionally hypothesize that the first brood (B1) of the first filial generation (F_1) may also demonstrate a reduction in overall $\dot{V}O_2$ compared with control animals.

Material and Methods

Animals and Husbandry

Stock cultures of *Daphnia magna* were maintained in 1-L aquaria at an ambient temperature (T_a) of 21.5°C and a photoperiod of 14L : 10D in ADaM medium (Klüttgen et al. 1994) with a pH of ~7. The medium was slowly bubbled with atmospheric air (~14 mL min^{-1}) to ensure normoxic conditions. Dead animals and carapaces were removed, and three-fourths of the medium was replaced weekly. All animals (stock and experimental) were fed every second day at 10:00 a.m. with algae (*Scenedesmus* sp.). The bottom of the medium was gently agitated every other day to redisperse any settled algae.

Parental (P) Generation Experimental Protocol

Synchronized clonal animals were obtained by a process similar to that described by Seidl et al. (2005). A single female was kept under normoxic conditions, and her first two broods were discarded. The nine parthenogenetic offspring from her third brood were isolated and allowed to develop in normoxia. The resulting 150 parthenogenetic offspring from the third broods of these original offspring represent the parental (P) generation for this study. The P generation was divided into two acclimation conditions: control and hypoxia (4 kPa).

P neonates were exposed to an acclimation condition from days 0 to 6 to allow Hb production to plateau (reaches steady state at ~3–6 d in adult females; Zeis et al. 2003; Paul et al. 2004; Seidl et al. 2005).

Approximately 25 individual day 0 P neonates were selected at random and transferred into fresh medium (with algae) in 250-mL Tupperware containers equipped with a gas outlet (hole for gas release) and inlet (aquarium tubing reaching the bottom). Three containers (i.e., 3 × 25 individuals) were then placed within one of two sealed 20-L glass chambers (used as gas reservoirs) with a small opening at one upper corner to allow pumped-in gas to escape. Gas within each reservoir was maintained at either normoxic (21 kPa) or hypoxic (4 kPa) levels. A small gas pump inside each reservoir delivered either normoxic or hypoxic (4 kPa) gas to the bottom of each chamber (via the inlet) at a rate of ~24 mL min^{-1}. Identical equipment and culture conditions were ensured for normoxic and hypoxic exposure conditions. The chambers remained undisturbed in their gas reservoirs for the 6-d exposure period, apart from feeding with algae on day 2 and day 4 of exposure. The oxygen content of the external reservoir was periodically monitored during the experimental period, and preliminary testing indicated that the desired gaseous steady state (either 4 or 21 kPa) of the chambers returned within 1 h after feeding.

On day 6 of development and hypoxic exposure, all animals were transferred into 20 mL of fresh normoxic medium in individual 30-mL plastic medicine cups. Thus, the exposure period occurred before the animals were reproductively active (first eggs were present in the brood chamber on day 8). Animals were checked daily at 10:00 a.m., the release dates and numbers of neonates in the first three broods of the F_1 generation were recorded, and the medium was replaced after each brood. Animals were fed every second day at 10:00 a.m. with algae, and the bottom of the medium was gently agitated every other day to redisperse any settled algae. Twelve to fifteen randomly chosen individuals were photographed every second day, and carapace length (L_c in mm) was determined using ImageJ 1.44p software (National Institutes of Health, Bethesda, MD). Dry mass in micrograms was then calculated according to the regression $\ln(\text{mass}) = 3.05 + 2.16 \ln L_c$, which is specific for *D. magna* (Kawabata and Urabe 1998).

F_1 Generation Experimental Protocol

The release date and number of neonates was recorded for the first three broods (B1–B3) of each individual P female. On a given day, day 0 neonates were randomized within P exposure (and brood) groups and transferred into 50 mL of fresh medium in 150-mL plastic drinking cups at a density of 1 neonate mL^{-1}. Animals were checked daily at 10:00 a.m., and the release dates and numbers of neonates in the first broods of the F_2 generation were recorded.

Length and Mass Determination. Following respirometry (see below) day 0–6 neonates were photographed under a stereomicroscope and their length and mass determined as above.

From day 8 onward, 15 randomly selected individuals from each brood were photographed every second day, and carapace length (L_c in mm) was determined using ImageJ 1.44p software (National Institutes of Health). Dry mass was then calculated as described above.

Respirometry. Rate of oxygen consumption ($\dot{V}O_2$ in μg mL^{-1} h^{-1}) was determined on groups of day 0, 2, 4, and 6 neonates in ~0.2-mL closed respirometers at 22°C. The respirometers were purpose built from a borosilicate glass vial. A 23-gauge needle sealed with silicone into the polyethylene lid allowed pressure release. The entire lid was further covered with Loctite Fun-Tak mounting putty (Henkel, Johannesburg, South Africa) to ensure oxygen impermeability. A planar O_2-sensitive spot sensor mounted inside the respirometer allowed Po_2 to be determined using an OXY-4 four-channel O_2 meter (Loligo Systems ApS, Hobro, Denmark). The respirometers and fiber optic cables attached to the OXY-4 were mounted within a water bath, and ambient temperature (T_a) was controlled by a TMP-REG temperature analyzer and regulator (Loligo Systems ApS) and an external water bath (Isotemp 3016D, Fisher Scientific, Pittsburgh, PA). Data were collected and temperature regulation integrated with AutorResp (ver. 1.0.0) software (Loligo Systems ApS) and later analyzed with Chart (ver. 5) software (AD Instrument, Colorado Springs, CO).

To eliminate microbial respiration, respirometers were cleaned with 100% ethanol and allowed to air-dry before each measurement. The system was then calibrated with air-equilibrated medium and a zero solution (saturated sodium sulfite solution) at the beginning and end of each day. One chamber, devoid of neonates, was designated as the blank to control for any remaining microbial respiration. The blank was filled with freshly autoclaved, air-equilibrated media and sealed. The remaining three respirometers were filled with freshly autoclaved, air-equilibrated medium. Given the small size of the neonates, 6–28 animals, depending on age, were placed in each respirometer. Respirometer Po_2 was then continuously monitored from normoxia at the start of measurement down to near anoxia (≤1 kPa) caused by the consumption of O_2 by the *Daphnia* within the respirometers. $\dot{V}O_2$ was then calculated using the following equation:

$$\dot{V}O_2 = \frac{\text{neonate}\Delta Po_2}{\Delta t}(P_B - P_S) \times \beta O_2 \times \text{vol} \times 0.2093 \times 22.39, \quad (1)$$

where P_B is the barometric pressure (kPa), P_S is the saturation vapor pressure of water (3.63 kPa at 22°C), βo_2 is the capacitance of water for oxygen (0.07 μmol L^{-1} kPa^{-1} at 22°C), vol is the chamber volume minus the neonate volume (in liters, assuming 1 g wet mass = 1 mL), 0.2093 is the fractional concentration of oxygen in well-aerated water, and 22.39 is the conversion factor for micromoles to microliters. $\dot{V}O_2$ was determined after correcting for any measurable microbial respiration in the blank respirometer. Mass-specific $\dot{V}O_2$ was determined by dividing the product of equation (1) by body mass as determined above.

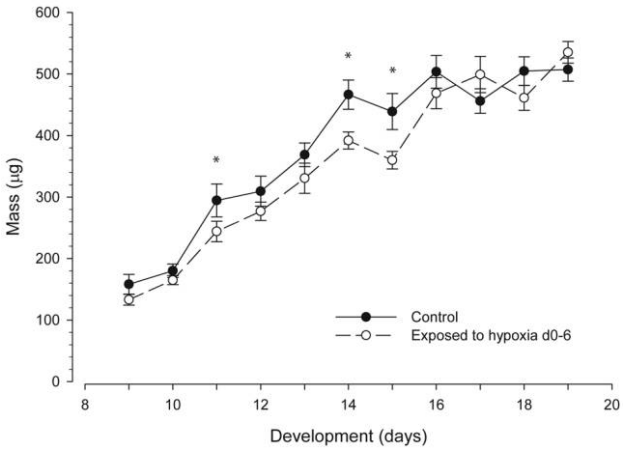

Figure 1. Body mass (μg) changes in the parental generation (P) of *Daphnia magna* following exposure to hypoxia (~4 kPa) or normoxia (control) from day 0 to day 6. Mean values ± 1 SEM are presented. *N* values are in parentheses. An asterisk indicates a significant pairwise difference in animals exposed to hypoxia or normoxia within the parental (P) generation on a given day of development.

Statistical Analysis

The effect of hypoxic exposure and development on P generation mass was analyzed by two-way ANOVA with Holm-Sidak method post hoc comparisons using SigmaStat (ver. 3.5) software. The effects of hypoxic exposure and brood on neonate release date within the 3-d brood window and on mass were tested in separate mixed models (PROC MIXED) with animal as a random factor and Bonferroni post hoc comparisons using SAS (ver. 9.1) software (Littell et al. 1998). An "unstructured" variance structure, which makes no assumption regarding equal variances or correlations, was used following the recommendations for repeated-measures analyses by Littell et al. (1998). Similarly, the effects of maternal gas exposure (normoxia or hypoxia), brood, and development (day) on F_1 neonate mass were tested for using a mixed model approach (PROC MIXED) with unstructured variance structure and Bonferroni post hoc comparison separating the animals by brood. The effects of maternal gas exposure (normoxia or hypoxia), brood, development (day), and Po_2 on F_1 neonate $\dot{V}O_2$ were tested for using a mixed model approach with unstructured variance structure and Bonferroni post hoc comparison separating the data by day using SAS (ver. 9.1) software. Initially, all models were run with all interaction terms included, and nonsignificant interactions were removed from the final models. Statistical significance was assumed at $P < 0.05$.

Results

Parental Generation

Exposure to chronic hypoxia significantly reduced body mass in the parental generation ($P < 0.03$, ANOVA), but the magnitude of the effects depended on age. On days 9 and 10,

Table 1: Development of carapace length of parental (P) and first filial (F_1) generation *Daphnia magna* following exposure of the P generation to 4-kPa hypoxia during neonatal days 0–6

	Parental generation (P)		First filial generation (F_1)					
			Brood 1 (B1)		Brood 2 (B2)		Brood 3 (B3)	
Day	Control carapace length (mm)	Carapace length of *Daphnia* exposed to hypoxia (mm)	Control carapace length (mm)	Carapace length of *Daphnia*, P exposed to hypoxia (mm)	Control carapace length (mm)	Carapace length of *Daphnia*, P exposed to hypoxia (mm)	Control carapace length (mm)	Carapace length of *Daphnia*, P exposed to hypoxia (mm)
0	...	...	1.03 ± .02 (66)	.98 ± .02 (85)[a]	.92 ± .01 (78)	.87 ± .01 (94)[a]	.93 ± .01 (117)	.9 ± .01 (97)
2	...	...	1.29 ± .01 (67)	1.12 ± .01 (52)[a]	1.17 ± .02 (91)	1.11 ± .01 (82)[a]	1.12 ± .01 (99)	1.07 ± .01 (90)
4	...	...	1.55 ± .02 (42)	1.36 ± .01 (54)[a]	1.59 ± .02 (51)	1.41 ± .02 (59)[a]	1.56 ± .02 (62)	1.62 ± .02 (77)
6	...	...	1.77 ± .01 (87)	1.72 ± .02 (100)	1.72 ± .02 (84)	1.51 ± .01 (109)[a]	1.75 ± .01 (126)	1.78 ± .01 (108)
8	...	...	1.98 ± .05 (15)	2.15 ± .06 (13)	2.26 ± .05 (15)	2.41 ± .04 (15)	1.78 ± .03 (15)	1.87 ± .03 (15)
9	2.51 ± .10 (12)	2.33 ± .07 (12)	...	...	...	...	...	...
10	2.69 ± .07 (12)	2.58 ± .05 (12)	2.67 ± .10 (15)	2.61 ± .08 (15)	2.71 ± .11 (15)	2.45 ± .09 (15)	2.58 ± .08 (15)	2.53 ± .06 (15)
11	3.35 ± .14 (12)	3.08 ± .10 (12)[a]	...	...	...	...	...	...
12	3.44 ± .12 (12)	3.28 ± .09 (12)	3.42 ± .11 (15)	3.50 ± .09 (15)	3.14 ± .06 (15)	3.00 ± .05 (15)	3.09 ± .08 (15)	3.13 ± .10 (15)
13	3.75 ± .09 (12)	3.55 ± .12 (12)[a]	...	...	...	...	...	...
14	4.17 ± .10 (15)	3.86 ± .06 (15)[a]	3.85 ± .13 (15)	3.89 ± .05 (15)	3.69 ± .08 (15)	3.56 ± .09 (15)	...	...
15	4.05 ± .12 (15)	3.71 ± .07 (15)	...	...	...	...	...	...
16	4.32 ± .10 (15)	4.18 ± .10 (15)	4.16 ± .11 (15)	4.38 ± .10 (15)	4.09 ± .07 (15)	3.98 ± .09 (15)	...	...
17	4.13 ± .08 (15)	4.30 ± .12 (15)	...	...	...	...	...	...
18	4.33 ± .09 (15)	4.15 ± .09 (15)	4.50 ± .08 (15)	4.42 ± .08 (15)	...	...	...	...
19	4.35 ± .08 (15)	4.46 ± .07 (15)	...	...	...	...	...	...

Note. Data are mean ± SEM (*N*). Ellipses indicate that length was not measured for a cohort on a specific day.

[a]Indicates a significant difference from control within a cohort on a specific day. Note that variation in sample size occurs because animals from day 0 to day 6 originate from $\dot{V}O_2$ experiments, whereas from day 8 onward, animals were individually photographed.

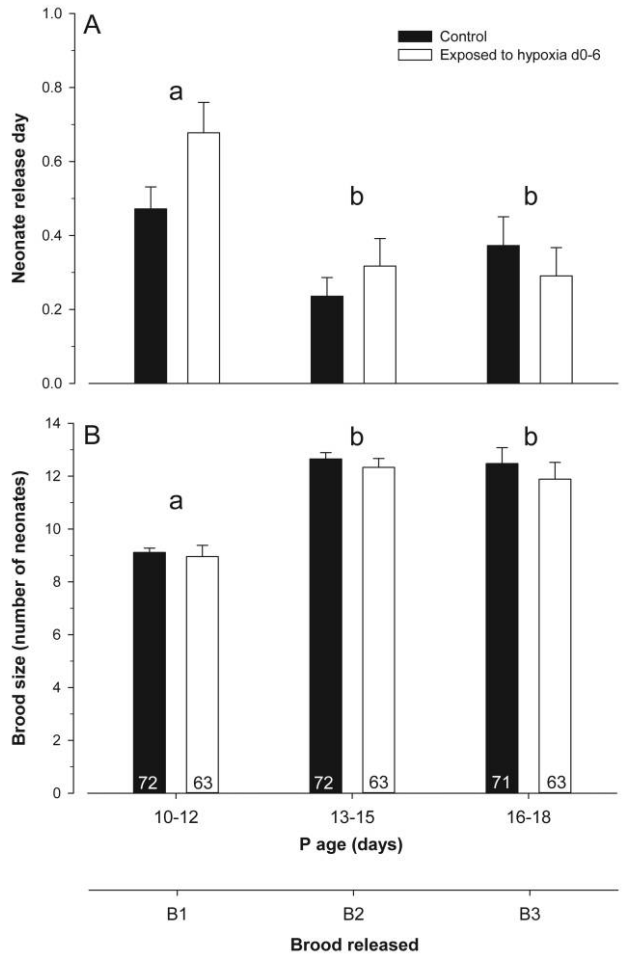

Figure 2. Neonate release date within the 3-d brood window (*A*) and number of neonates (*B*) in the first three broods from parental generation (P) of *Daphnia magna* following exposure to hypoxia (~4 kPa) or normoxia (control) from day 0 to day 6. Mean values ± 1 SEM are presented. *N* values are indicated at the bottom of the bars. Different lowercase letters indicate a difference in neonate number among broods. No effect of hypoxia on neonate number was observed.

immediately after hypoxia (4 kPa) exposure (which occurred from day 0 to day 6), there was no difference in body mass between hypoxia-exposed and control *Daphnia*. By day 11, *Daphnia* that were exposed to hypoxia from day 0 to day 6 were ~17% smaller at day 11 and days 14–16 than those reared exclusively in normoxia (i.e., control; $P < 0.001$; fig. 1; table 1). All P animals released each of their broods of neonates within a 3-d window for each brood; B1 during days 10–12, B2 during days 13–15, and B3 during days 16–18 (fig. 2). Release date for each brood within the window was not affected by hypoxic exposure. However, both normoxic and hypoxic B2 and B3 neonates were released earlier than B1 neonates within their respective windows (fig. 2). Hypoxic and normoxic mass for P animals increased (~62% and 58%, respectively) between B1 (days 10–12) and B2 (days 13–15) production and plateaued between B2 and B3 (days 16–18). Concurrently, neonate production followed a similar pattern, increasing between B1 and B2 (by ~ 42% ± 3% and 62% ± 7%, respectively) and then plateauing (figs. 1, 2). Hypoxia exposure did not result in a reduction in the number of neonates produced for the first three broods. However, there were ~50% more neonates in B2 and B3 from both normoxic and hypoxia-exposed females compared with B1 (fig. 2). Hypoxia-exposed females produced B1 and B2 neonates that were ~7% and 11% smaller, respectively, than control neonates at day 0. There was, however, no difference in the size of B3 neonates from hypoxia-exposed compared to control mothers at day 0 (fig. 3). Thus, hypoxia exposure from day 0 to day 6 decreased mass at day 11 and days 14–16, coinciding with the production of B1 and B2 neonates. The resulting B1 and B2 neonates (F_1 generation) were smaller from hypoxia-exposed mothers. However, by days 16–18, parental generation mass was statistically identical, and there

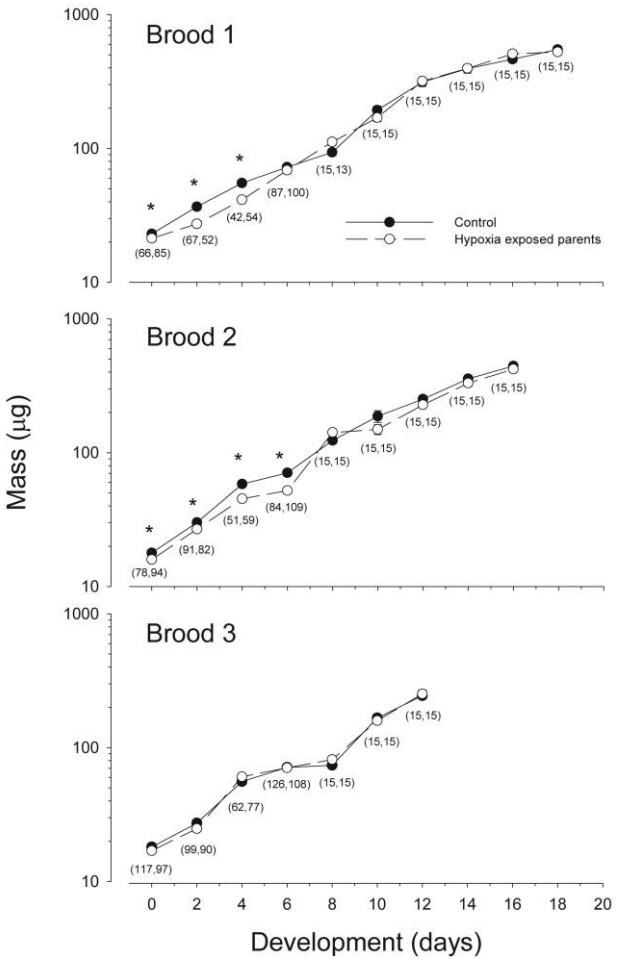

Figure 3. Log mass of the first three broods (B1–B3) of the F_1 generation of *Daphnia magna* whose mothers were exposed to either hypoxia (~4 kPa) or normoxia (control) as neonates (days 0–6). Mean values ± 1 SEM are presented. *N* values are in parentheses (control, P hypoxia-exposed). An asterisk indicates a significant pairwise difference between F_1 animals from mothers (P) exposed to normoxia (control) or hypoxia on a given day.

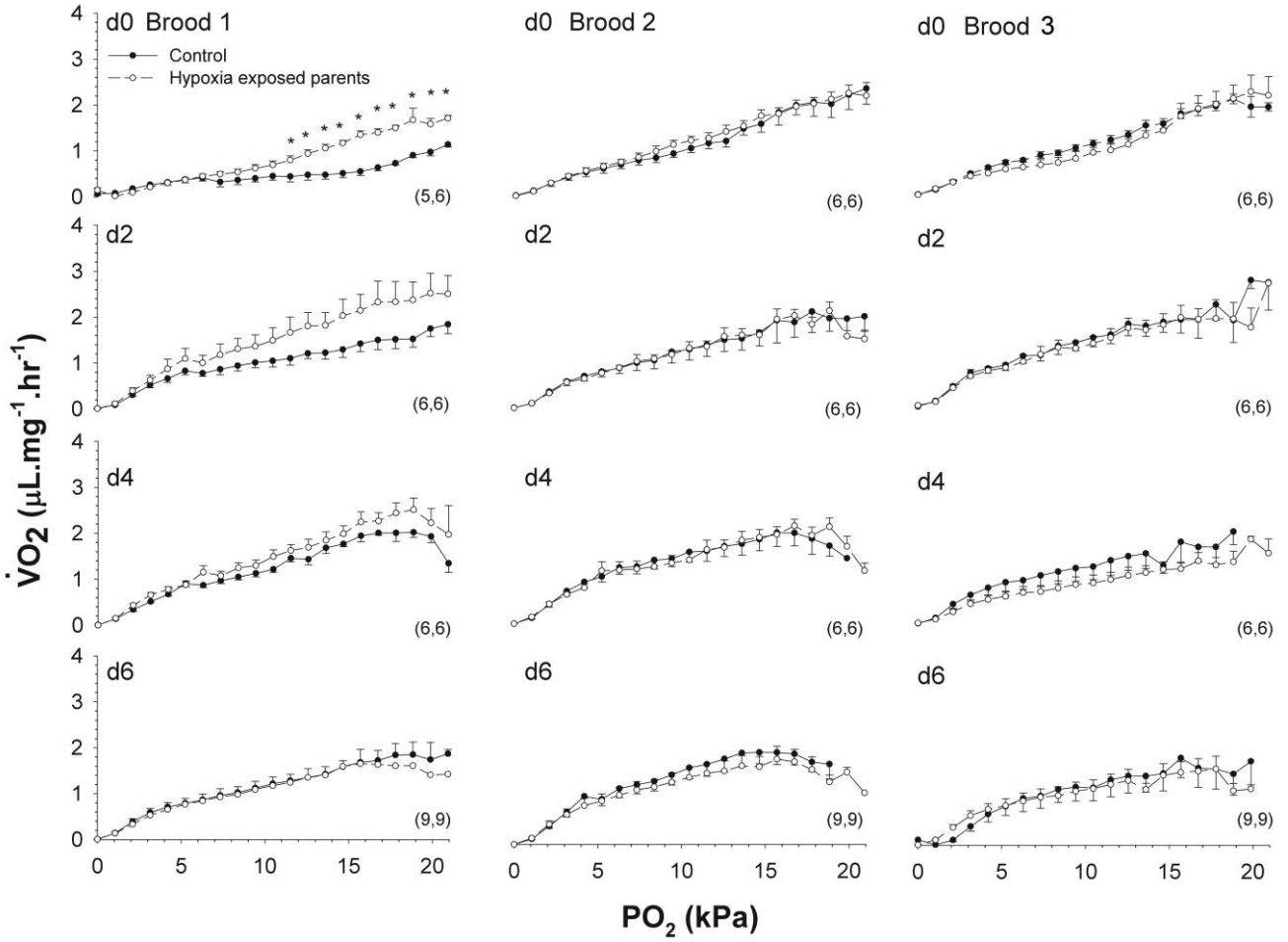

Figure 4. $\dot{V}O_2$ as a function of Po_2 for the first three broods (B1–B3) of neonatal *Daphnia magna* whose mothers were exposed to either hypoxia (~4 kPa) or normoxia (control) as neonates (days 0–6). Mean values ± 1 SEM are presented. *N* values are in parentheses (control, P hypoxia-exposed). An asterisk indicates a significant pairwise difference between F_1 animals from mothers (P) exposed to normoxia (control, filled symbols) or hypoxia (open symbols) on a given day of development at a given Po_2.

was correspondingly no effect of maternal hypoxia exposure on the neonate mass of the brood (B3) produced at this time (figs. 1, 3).

F_1 Generation

The mean growth rate for the three broods was similar (0.23 ± 0.02, 0.24 ± 0.05, and 0.25 ± 0.03 μg d^{-1}, respectively, for B1, B2, and B3) across the first 12 d of development (fig. 3; table 1). However, overall F_1 mass differed among the broods ($P < 0.001$), with post hoc testing revealing that B1 (from both normoxic and hypoxic mothers) were larger than B2 and B3 individuals across the first 8 d of development. In B1, the difference in mass between neonates from mothers exposed to hypoxia or normoxia ($P = 0.015$) was due to differences at days 0–4 of development. In B2, mass differences persisted to day 6 ($P < 0.001$), and in B3, no mass differences due to P exposure was observed ($P = 0.975$; fig. 3), indicating that the transgenerational effect "washed out" with increasing numbers of broods.

$\dot{V}O_2$ of neonates was influenced by maternal exposure conditions ($P < 0.001$), Po_2 ($P < 0.001$), brood number ($P < 0.001$), and day of development ($P < 0.001$). Further, significant interactions between day of development and Po_2 ($P < 0.001$) and brood and day of development ($P < 0.001$) also influenced $\dot{V}O_2$ (fig. 4). Bonferroni post hoc comparisons revealed that B1 day 0 neonates whose mothers had been exposed to hypoxia had higher $\dot{V}O_2$'s at Po_2's from 11.5 up to 21 kPa than control neonates. Further closer examination of $\dot{V}O_2$ across development in one brood (B1) from one clone line reveals the constancy of the scaling exponent across a wide Po_2 range from normoxia to severe hypoxia: that is, 1.085 at $Po_2 = 21$ kPa; 1.094 at $Po_2 = 10$ kPa, and 1.105 at $Po_2 = 2$ kPa (fig. 5).

Release date for the first brood for the animals from B1 to B3 of the F_1 generation was not affected by maternal hypoxic exposure. However, B1 animals released their first brood ~1 d

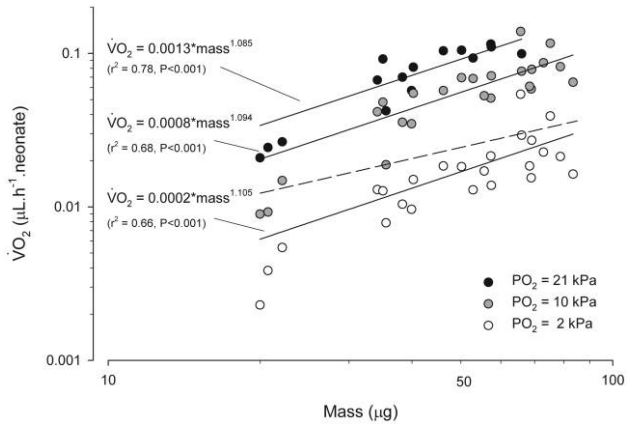

Figure 5. Scaling of log $\dot{V}O_2$ as a function of log mass in the brood 1, control population of neonatal *Daphnia magna*. Each circle represents an individual data point. Solid lines represent significant regressions for each Po_2 level with a scaling exponent that is not different from 1, and the dashed line indicates the predicted relationship with a scaling exponent of 0.75.

earlier than B2 and B3 animals (fig. 6). Maternal hypoxia exposure did not affect the number of neonates produced, but B1 animals (which came from broods with smaller numbers; see fig. 2) produced smaller broods than B2 and B3 animals (fig. 6).

Discussion

Neonatal Hypoxic Exposure Has Lasting Costs during High Energy Demand

Exposure to severe hypoxia (4 kPa) during the first 6 d of development significantly reduced mass at day 11 and days 14–15 (fig. 1). Although hemoglobin concentration [Hb] was not measured in this study, the animals exposed to hypoxia were visibly red, unlike the pale controls, following exposure (at day 7). This very likely indicates an increase in Hb production. Exposure to similar hypoxic levels in adult *Daphnia* reveals that Hb production plateaus at ~3–6 d in adult females (Zeis et al. 2003; Paul et al. 2004; Seidl et al. 2005). The small but significant reduction in body mass resulting from hypoxic exposure (fig. 1) may have been due to the diversion of energy away from growth and into Hb production. Further, the significant pairwise differences between control and hypoxia-exposed animals occurred during the periods of increased energy demand when most females were producing their first (day 11) and second (days 14–15) broods (figs. 1, 2). Reproduction (as measured by brood size and timing of release) may thus be prioritized ahead of growth even though overall reproductive output (offspring size dependent on maternal carapace length; e.g., Seidl et al. 2005) may be reduced.

There was manifest intraspecific variation in phenotype across development in response to stressor exposure. Whereas the mass of hypoxia-exposed individuals initially follows the developmental trajectory of control animals, during production of the first two broods, they are unable to sustain growth at control levels because reproduction is prioritized over growth, and they follow an altered trajectory (see Burggren and Warburton 2005 for review on developmental trajectories), resulting in altered phenotype (smaller mass). A further temporal variation occurs when the hypoxia-exposed animals were able to sustain growth (i.e., attain mass equal to that of control animals) during B3 neonate production. The reduction in body mass of hypoxia-exposed P animals (day 11 and days 14–15) does not appear to represent slower development because the average release date of B1 and B2 neonates was not altered by hypoxic exposure (fig. 2). Release date was affected by parental age, however, because B2 and B3 neonates released earlier than B1 animals within their respective 3-d brood windows (fig. 2). Thus, although all broods were released within a similar 3-d period (i.e., absolute range remained unchanged), a temporal variation occurred, with more females releasing their B2 and

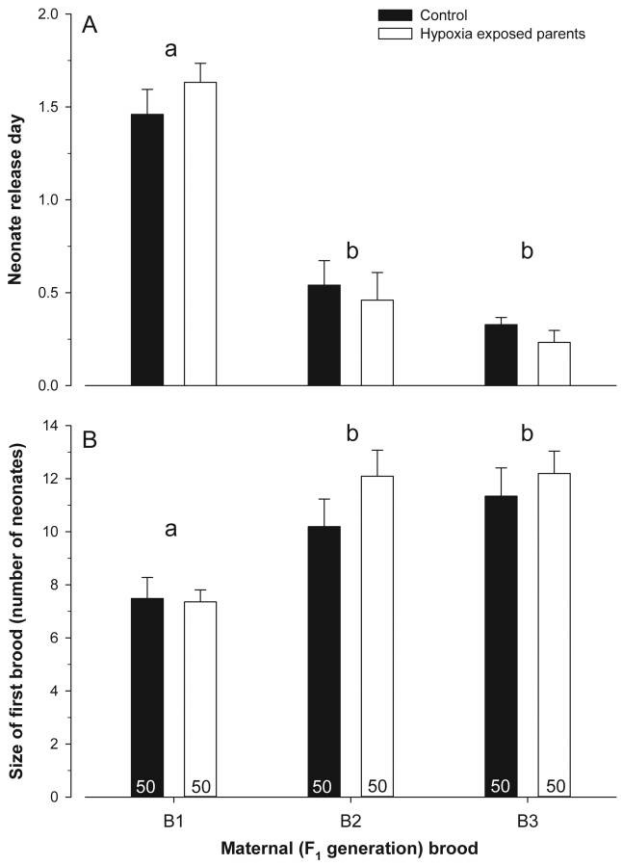

Figure 6. Neonate release date within the 3-d brood window (*A*) and number of neonates (*B*) from the first broods (B1 of the F_2 generation) of B1–B3 of the F_1 generation of *Daphnia magna* following exposure to hypoxia (~4 kPa) or normoxia (control) from day 0 to day 6. Mean values ± 1 SEM are presented. *N* values are indicated at the bottom of the bars. Different lowercase letters indicate a difference in neonate number among broods. No effect of hypoxia on neonate number was observed.

B3 young earlier within the 3-d window and more B1 neonates being released later within the window (fig. 2).

The overall pattern of concurrent increase in P mass (both hypoxic and normoxic) and brood size (number of neonates) between B1 and B2 and plateau between B2 and B3 suggests that brood size is dependent on adult mass (or carapace length; figs. 1, 2). Indeed, previous studies have demonstrated that maternal carapace length (which may be influenced by hypoxia) rather than severe chronic hypoxia (~4 kPa) itself has the larger influence on brood size (e.g., Seidl et al. 2005). In our study, hypoxia exposure decreased carapace length by ~17% (cf. control) at day 11 and days 14–15, precisely when B1 and B2 neonates were being produced (table 1). Surprisingly, however, a decrease in brood size due to hypoxia exposure (or due to the indirect effect of maternal carapace length decrease) in B1 and B2 was not observed (figs. 1, 2).

The mass of B1 and B2 neonates produced by mothers exposed to hypoxia was significantly reduced compared to that of controls. By days 16–18, when the third brood was released, there was no difference in P generation mass and similarly no difference in B3 neonate mass at day 0 (figs. 2, 3). Thus, similar to previous studies, neonate mass (or length) depends on P mass (or length; e.g., Lampert 1993; Seidl et al. 2005), which in turn was altered by hypoxia exposure in this study. The P generation responded to the hypoxic stress through altered phenotype (decrease in mass) during times of increased energy demand (production of B1 and B2), and consequently those animals were unable to allocate as much energy to reproduction, with a reduction of ~7% and 11% in total reproductive output during their first two broods compared with control animals. Interestingly, by days 17–19, when B3 was being released, the hypoxia-exposed P animals had returned to the developmental trajectory of the control P animals. Potentially, lower reproductive output during B1 and B2 was the inherent cost to these animals to maintain growth at a level similar to that of control animals.

Parental Hypoxia Exposure Effects "Wash Out" across F_1 Broods and Development

The dissipation ("washout") of an epigenetically induced phenotype, when even studied has typically been examined across generations (e.g., Drake et al. 2005; Johannes et al. 2009; Leon and Moser 2010; see, e.g., Curley et al. 2011 for a recent review). Far less frequently examined is the washout of the phenotype across multiple reproductive events in the corresponding multiple F_1 generations. One of the most interesting (and surprising) findings in this study was that there is a washout of the effect of maternal hypoxia exposure not only across development (within a brood) but also across subsequent broods (fig. 3), disappearing by brood 3. Thus, intraspecific variation occurs across a complex matrix of population, development, brood, and generation.

The washout is exemplified in the first filial generation, where B1 and B2 neonate mass is smaller (at day 0), but by the time B3 is produced the effect of the stressor has been mitigated and no mass differences are observed (fig. 3). Washout also occurs across development. Although B1 and B2 neonates from hypoxia-exposed mothers were initially smaller, by 6 and 8 days of development, respectively, they had attained masses similar to those of their control counterparts (fig. 3). A return to the developmental trajectory of control populations (i.e., catch-up growth) often comes at a price that may last well into adulthood (e.g., Ozanne and Hales 2004; Criscuolo et al. 2008; Sloboda et al. 2011). This cost does not appear to be reproductive, however, with B1 and B2 individuals from hypoxia-exposed mothers (who experienced the catch-up growth) producing the same number of neonates as control animals in their own first broods (fig. 6). Potentially, a trade-off could still be apparent, with a reduction in neonate size (not measured in this study) reducing overall reproductive output in the first broods of the F_2 generation or with effects becoming more marked further downstream with later broods.

Intraspecific variation across development, brood, and generation also occurred with O_2 consumption (fig. 4). The higher $\dot{V}O_2$ in day 0 B1 neonates whose mothers were exposed to hypoxia (fig. 4) is contrary to expectation, given that hypoxia-acclimated adults have a lower $\dot{V}O_2$ compared with control animals (Seidl et al. 2005). There was no indication that neonates from a hypoxia-exposed mother are any better prepared for hypoxia than their normoxic counterparts. The relatively higher $\dot{V}O_2$ of day 0 B1 neonates could potentially be linked to catch-up growth of the hypoxic animals. However, if this were the case, we would expect it to persist until day 4 in B1 neonates and also during the first 6 d in B2 neonates, where no elevation of $\dot{V}O_2$ compared with control animals occurred.

Isometric Scaling in Normoxic to Near Anoxic Environments

Contrary to expectations based on general findings for metabolic scaling, mass-specific $\dot{V}O_2$ in neonatal *Daphnia* did not decrease across development and the attendant increase in body mass. This pattern of isometric or near isometric scaling (as opposed to allometric scaling) has been shown across five phyla of pelagic animals, including *Daphnia magna* (see Glazier 2006 for review). The constant swimming behavior of pelagic animals and high predation risks resulting in life histories that favor rapid growth and reproduction have been suggested as likely explanations for the high scaling exponent of $\dot{V}O_2$, which has been proposed to be evolutionarily malleable in pelagic animals (see Glazier 2006 and references within). In recent years many studies have drawn attention to the inability of an interspecific allometric scaling exponent to explain mass relationships in a multitude of circumstances (e.g., Riisgard 1998; Bokma 2004; Glazier 2006). This evidence for the repeatability of the intraspecific scaling exponent highlights the potential for intraspecific scaling exponents to explain mass relationships even under widely varying environmental conditions.

Conclusions

This study on clonal populations of *D. magna* has revealed that intraspecific variation spurred by stressors like hypoxia occurs across development, across multiple broods, and across generations in genetically identical populations (clones). Moreover, the effects can dissipate over developmental time or with successive productions of broods. Developmental physiological studies have tended to pool data across cohorts (e.g., broods) and successive generations. Doing so does not yield "inaccurate" data and will continue to power experimental design in years to come. However, as physiological studies delve further and further into epigenetic, transgenerational phenomena (Ho and Burggren 2010), progressively sophisticated experimental designs that take into account these additional sources of variation will become increasingly important.

Acknowledgments

Support for this study was provided by National Science Foundation operating grant IOS-1025823 to W.W.B. We thank Mallory Burdick for administrative assistance and Aradhana Sahoo for assistance with data collection.

Literature Cited

Agrawal A.A., C. Laforsch, and R. Tollrian. 1999. Transgenerational induction of defences in animals and plants. Nature 401:60–63.

Andrewartha S.J., N.J. Mitchell, and P.B. Frappell. 2010. Does incubation temperature fluctuation influence hatchling phenotypes in reptiles? a test using parthenogenetic geckos. Physiol Biochem Zool 83:597–607.

Andrewartha S.J., H. Tazawa, and W.W. Burggren. 2011. Embryonic control of heart rate: examining developmental patterns and temperature and oxygenation influences using embryonic avian models. Respir Physiol Neurobiol 178:84–96.

Bennett A.F. 1987. Interindividual variability: an underutilized resource. Pp. 147–169 in M.E. Feder, A.F. Bennett, W.W. Burggren, and R.B. Huey, eds. New directions in ecological physiology. Cambridge University Press, Cambridge.

Biro P.A. and J.A. Stamps. 2010. Do consistent individual differences in metabolic rate promote consistent individual differences in behavior? Trends Ecol Evol 25:653–659.

Bokma F. 2004. Evidence against universal metabolic allometry. Funct Ecol 18:184–187.

Boratynski Z. and P. Koteja. 2009. The association between body mass, metabolic rates and survival of bank voles. Funct Ecol 23:330–339.

Bozinovic F., J.M. Rojas, B.R. Broitman, and R.A. Vásquez. 2009. Basal metabolism is correlated with habitat productivity among populations of degus (*Octodon degus*). Comp Biochem Physiol A 152:560–564.

Burggren W.W. and S.J. Warburton. 2005. Comparative developmental physiology: an interdisciplinary convergence. Annu Rev Physiol 67:203–223.

Chappell M.A., T. Garland, J.F. Robertson, and W. Salzman. 2007. Relationships among running performance, aerobic physiology and organ mass in male Mongolian gerbils. J Exp Biol 210:4179–4197.

Criscuolo F., P. Monaghan, L. Nasir, and N.B. Metcalfe. 2008. Early nutrition and phenotypic development: "catch-up" growth leads to elevated metabolic rate in adulthood. Proc R Soc B 275:1565–1570.

Curley J.P., R. Mashoodh, and F.A. Champagne. 2011. Epigenetics and the origins of paternal effects. Horm Behav 59: 306–314.

Drake A.J., B.R. Walker, and J.R. Seckl. 2005. Intergenerational consequences of fetal programming by in utero exposure to glucocorticoids in rats. Am J Physiol 288:R34–R38.

Glazier D.S. 2006. The 3/4-power law is not universal: evolution of isometric, ontogenetic metabolic scaling in pelagic animals. BioScience 56:325–332.

Ho D.H. and W.W. Burggren. 2010. Epigenetics and transgeneration transfer: a physiological perspective. J Exp Biol 213:3–16.

Johannes F., E. Porcher, F. Teixeira, V. Saliba-Colombani, M. Simon, N. Agier, A. Bulski, et al. 2009. Assessing the impact of transgenerational epigenetic variation on complex traits. PLoS Genetics 5:e1000530, doi:10.1371/journal.pgen.1000530.

Kawabata K. and J. Urabe. 1998. Length-weight relationships of eight freshwater planktonic crustacean species in Japan. Freshwater Biol 39:199–205.

Klüttgen B., U. Dülmer, M. Engels, and H.T. Ratte. 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Res 28:743–746.

LaMontagne J.M. and E. McCauley. 2001. Maternal effects in *Daphnia*: what mothers are telling their offspring and do they listen? Ecol Lett 4:64–71.

Lampert W. 1993. Phenotypic plasticity of the size at first reproduction in *Daphnia*: the importance of maternal size. Ecology 74:1455–1466.

Leon D.A. and K.A. Moser. 2010. Low birth weight persists in South Asian babies born in England and Wales regardless of maternal country of birth: slow pace of acculturation, physiological constraint or both? analysis of routine data. J Epidemiol Community Health, doi:10.1136/jech.2010.112516.

Littell R.C., P.R. Henry, and C.B. Ammerman. 1998. Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 76:1216–1231.

McKechnie A.E. and D.L. Swanson. 2010. Sources and significance of variation in basal, summit and maximal metabolic rates in birds. Curr Zool 56:740–756.

Mikulski A. and J. Pijanowska. 2010. When and how can *Daphnia* prepare their offspring for the threat of predation? Hydrobiologia 643:21–26.

Millidine K.J., J.D. Armstrong, and N.B. Metcalfe. 2009. Juvenile salmon with high standard metabolic rates have higher energy costs but can process meals faster. Proc R Soc B 276: 2103–2108.

Ozanne S.E. and C.N. Hales. 2004. Lifespan: catch-up growth and obesity in male mice. Nature 427:411–412.

Paul R.J., B. Zeis, T. Lamkemeyer, M. Seidl, and R. Pirow. 2004. Control of oxygen transport in the microcrustacean *Daphnia*: regulation of haemoglobin expression as central mechanism of adaptation to different oxygen and temperature conditions. Acta Physiol Scand 182:259–275.

Riisgard H.U. 1998. No foundation of a "3/4 power scaling law" for respiration in biology. Ecol Lett 1:71–73.

Seidl M.D., R.J. Paul, and R. Pirow. 2005. Effects of hypoxia acclimation on morpho-physiological traits over three generations of *Daphnia magna*. J Exp Biol 208:2165–2175.

Sinervo B., R. Hedges, and S.C. Adolph. 1991. Decreased sprint speed as a cost of reproduction in the lizard *Sceloporus occidentalis*: variation among populations. J Exp Biol 155:323–336.

Sloboda D.M., M. Hickey, and R. Hart. 2011. Reproduction in females: the role of the early life environment. Hum Reprod Update 17:210–227.

Tong W., Q. Xue, Y. Li, and L. Zhang. 2011. Maternal hypoxia alters matrix metalloproteinase expression patters and causes cardiac remodeling in fetal and neonatal rats. Am J Physiol 301:H2113–H2121, doi:10.1152/ajpheart.00356.2011

Vance J.T., J.B. Williams, M.M. Elekonich, and S.P. Roberts. 2009. The effects of age and behavioral development on honey bee (*Apis mellifera*) flight performance. J Exp Biol 212:2604–2611.

Wiggins P.R. and P.B. Frappell. 2000. The influence of haemoglobin on behavioural thermoregulation and oxygen consumption in *Daphnia carinata*. Physiol and Biochem Zool 73:153–160.

Zeis B., B. Becher, T. Lamkemeyer, S. Rolf, R. Pirow, and R.J. Paul. 2003. The process of hypoxic induction of *Daphnia magna* hemoglobin: subunit composition and functional properties. Comp Biochem Physiol B 134:243–252.