Low Temperature and Polyploidy Result in Larger Cell and Body Size in an Ectothermic Vertebrate

Adam Hermaniuk[1,*]
Mariusz Rybacki[2]
Jan R. E. Taylor[1]
[1]Institue of Biology, University of Białystok, Białystok, Poland; [2]Department of Evolutionary Biology, Kazimierz Wielki University, Bydgoszcz, Poland

Accepted 11/25/2015; Electronically Published 1/13/2016

ABSTRACT

Previous studies reported that low temperatures result in increases in both cell size and body size in ectotherms that may explain patterns of geographic variation of their body size across latitudinal ranges. Also, polyploidy showed the same effect on body size in invertebrates. In vertebrates, despite their having larger cells, no clear effect of polyploidy on body size has been found. This article presents the relationship between temperature, cell size, growth rate, and body size in diploid and polyploid hybridogenetic frog *Pelophylax esculentus* reared as tadpoles at 19° and 24°C. The size of cells was larger in both diploid and triploid tadpoles at 19°C, and triploids had larger cells at both temperatures. In diploid and triploid froglets, the temperature in which they developed as tadpoles did not affect the size of their cells, but triploids still had larger cells. Triploid tadpoles grew faster than diploids at 19°C and had larger body mass; there was no clear difference between ploidies in growth rate at 24°C. This indicates better adaptation of triploid tadpoles to cold environment. This is the first report on the increase of body mass of a polyploid vertebrate caused by low temperature, and we showed relationship between increase in cell size and increased body mass. The large body mass of triploids may provide a selective advantage, especially in colder environments, and this may explain the prevalence of triploids in the northern parts of the geographic range of *P. esculentus*.

Keywords: polyploidy, triploids, cell size, body size, temperature-size rule, *Pelophylax esculentus*.

*Corresponding author; e-mail: adamher@uwb.edu.pl.

Physiological and Biochemical Zoology 89(2):118–129. 2016. 1522-2152/2016/8902-5090$15.00. DOI: 10.1086/684974

Introduction

Body size is one of the most important traits of organisms and significantly affects both physiological processes and individual fitness (Peters 1983). Body growth may occur either by increasing cell size or cell number or by a combination of both (Kozłowski et al. 2003; Hessen et al. 2013). In organisms with a fixed cell number, body growth can happen only through changes in cell size (van Voorhies 1996), which in turn may also influence body size in organisms with variable cell numbers (Blanckenhorn and Llaurens 2005; Hessen et al. 2013). It has been proved that cell size strongly correlates with genome size, and this pattern seems to be universal for both plants and animals (Bennett 1987; Gregory 2001*a*, 2001*b*). The clearest demonstration of the positive correlation between genome size and cell size comes from polyploids that arise as the result of duplication of entire chromosome sets (Gregory and Mable 2005; Mable et al. 2011). On the contrary, the effect of polyploidy on body size is much more complex. Polyploid invertebrates may reach maturity at larger sizes compared to diploids, but examples of this are not numerous (Weider 1987; Walsh and Zhang 1992). However, in vertebrates, with few exceptions, no apparent effect of polyploidy on body size has been found, indicating that polyploids composed of larger cells regulate their size through changes in cell number (Fankhauser 1945; Swarup 1959; Mahony and Robinson 1980).

For decades it has been recognized that polyploids (both plants and animals) are more abundant in severe environments, especially at higher latitudes and altitudes, such as arctic and alpine habitats (Löve 1953; Levin 1983; Otto et al. 2007). These observations suggest that polyploids possess some physiological traits that allow them to better adapt to colder environments compared to their diploid counterparts. Laboratory studies that have compared life-history traits in diploid and polyploid individuals of various animal species have confirmed the greater tolerance of the polyploids to low temperature, but the reasons for this phenomenon are not entirely clear (Schultz 1982; Dufresne and Hebert 1998). The most common explanation is the idea that the increased heterozygosity provided by an "extra" genome provides metabolic flexibility to cope with a wide range of environmental conditions (Otto and Whitton 2000). It is also possible that possession of a higher quantity of enzymes per cell improves metabolic efficiency in polyploids at low temperatures (Dufresne and Hebert 1998).

The link between low temperature, cell size, and body size may be the key to understanding another biological phenomenon, that is, that the majority of ectotherms (from protists to amphibians) grow more slowly at low temperatures but finally become larger than their conspecifics growing at higher temperatures (the pattern called the temperature-size rule [TSR];

Atkinson 1994; Angilletta et al. 2004; Hessen et al. 2013). A possible consequence of the TSR is the considerable variation of body size in ectothermic animals across latitudinal ranges—larger organisms inhabit areas in higher latitudes (Bergmann's rule; originally referred exclusively to endotherms but also applies to ectotherms), as has been observed even in intraspecific comparisons (Chown and Gaston 2010). Possible mechanisms of the TSR and Bergmann's size clines in ectotherms are still debated, and both ultimate and proximate explanations have been offered (van Voorhies 1996; Partridge and Coyne 1997; Angilletta et al. 2004). Considering the ultimate explanations, optimization models can predict conditions in which a smaller body size at higher temperatures maximizes fitness (Atkinson and Sibly 1997; Kozłowski et al. 2004). Common-garden experiments have revealed that individuals from colder environments still exhibit larger sizes at maturity than those from warmer environments, and this provides genetic arguments that a larger body size may be adaptive in colder areas (Partridge and French 1996). Also, laboratory experiments on *Drosophila melanogaster* mimicking the action of natural selection for cold survival resulted in increased cell size and body size (Partridge et al. 1994). According to the proximate explanations, some general physical constraints, operating at cellular and molecular levels, play a causal role in TSR and Bergmann size clines in ectotherms. Van der Have and de Jong (1996) argued that cell growth is more sensitive to thermal constraints than cell division. This means that cells will be smaller after dividing at higher temperatures, resulting in a smaller overall organism. Other arguments point to the relationship between cell size and the availability of oxygen. Woods's (1999) biophysical model assumes that the maximal size of a cell is limited by the oxygen concentration at its center, which decreases with increasing temperature, especially in water habitats. At the organismal level, this implies that ectotherms raised at higher temperatures attain a smaller final size because of biophysical constraints at the cellular level.

Given that there is a general trend of body size increase with latitude (in temperature gradient) in ectotherms and that there is increasing incidence of polyploidy along the same clines, polyploids seem to be perfect models to test whether large cell size may have morphological and physiological consequences at low temperatures. Most of the studies that have investigated whether the temperature-related effects on body size are the consequences of changes occurring at the cellular level have concerned diploid invertebrates (van Voorhies 1996; see Arendt 2007 for review) and, to a lesser extent, diploid vertebrates (Arendt and Hoang 2005; Arendt 2006, 2007; Goodman and Heah 2010). Only a few authors have investigated the relationship between cell size and body size in polyploid vertebrates (Fankhauser 1945; Swarup 1959; Mahony and Robinson 1980). We are aware of only one study that has examined the combined effect of polyploidy and temperature on growth rate and body size in vertebrates. That study (Licht and Bogart 1989) was devoted to the embryonic growth and body size of the hatchlings of *Ambystoma laterale-texanum* complex (mole salamanders).

This article examines the effect of temperature on the cell size of different tissues, the growth pattern, and the body size of diploid and triploid edible frogs *Pelophylax esculentus* (Linnaeus 1758) reared at low (19°C) and high (24°C) water temperatures. We measured the size of erythrocytes in tadpoles and frogs, epidermal cells in tadpoles, and hepatocytes in frogs. The size of erythrocytes is the most often measured cell size in vertebrates and serves to discriminate between diploid and triploid frogs (Günther 1977; Polls Pelaz and Graf 1988), but the data on the size of cells from other tissues in amphibians are very scarce. We tested following hypotheses: (i) triploids comprise larger cells than diploids, (ii) a low temperature will increase the size of cells in triploids and diploids, and (iii) a larger cell size will result in a larger body mass of triploid and diploid tadpoles at low temperature. Since low temperature and polyploidy may affect cell size in the same direction, we expected that triploid tadpoles reared at lower temperature would reach the largest body size among other relatives.

The edible frog (*P. esculentus*) is a natural bisexual hybrid between the pool frog *Pelophylax lessonae* (genotype LL) and the marsh frog *Pelophylax ridibundus* (RR). In most of its European range, *P. esculentus* occurs as a diploid form (LR) and lives in sympatry with one of the parental species. It reproduces by hybridognesis, a mechanism in which the parental genome of the species present in the population is eliminated before meiosis and gametes produced by a hybrid are clonal (L or R); when fertilized by gametes of a related species, the progeny is hemiclonal (for review, see Graf and Polls Pelaz 1989). In northern parts of the geographical range of the *Pelophylax* species complex (in Sweden, Denmark, northern Germany, northern Poland), diploid hybrids coexist with allotriploid hybrids (LLR and/or LRR) and often form all-hybrid populations (see Plötner 2005 for a review). Although the manner of reproduction that allows for the existence of all-hybrid populations is relatively well understood (Christiansen 2009), it is not clear why these populations with triploid individuals inhabit mainly the northern part of Europe. According to Pruvost et al. (2013), hybrids, especially the two triploid types, have higher fitness under cold conditions than do both parental species. Similarly as in triploid and diploid *P. esculentus*, the occurrence of polyploid amphibian species in areas of greater climate severity, in contrast with their close diploid relatives inhabiting areas where climatic conditions are milder, was found in green toads (*Bufo viridis* complex) and in the gray tree frog complex (*Hyla versicolor* and *Hyla chrysoscelis*; Stöck et al. 2001; Otto et al. 2007).

Material and Methods

Population Sources and Crossing Procedure

During the two breeding seasons of 2009 and 2010, we crossed 19 pairs of *Pelophylax lessonae* (LL) males and *Pelophylax esculentus* females (LR or LRR). The males were collected from three ponds, from mixed *lessonae-esculentus* populations (Poznań district, Poland), and the females were derived from an all-hybrid *esculentus-esculentus* population from six ponds (Szczecin district, Poland). Ploidy and genome compositions of the parents were preliminarily determined on the basis of morphological

indexes (DP/CI = length of the *digitus primus*/length of the *callus internus*, T/CI = length of tibia/length of the *callus internus*, and the shape of the *callus internus*) and erythrocyte size, which is in most cases a reliable method (Günther 1977; Polls Pelaz and Graf 1988). The final confirmation of the parental genome composition was derived from offspring genotypes determined by chromosome inspection (as described below). We used artificial fertilization to obtain offspring, stimulating the female with the luteinizing salmon hormone (LHRH; Bachem Bioscience) as described by Berger et al. (1994). Sperm suspension was obtained directly from testes, dissected from the male once killed by decapitation.

Pelophylax esculentus females produce gametes of various sizes (small, medium, and large), related to different ploidy levels (Czarniewska et al. 2011). To obtain the highest percentage of diploid offspring, triploid females (LRR) were crossed with diploid males (LL), and only eggs classified as small (haploid R gametes in most cases) were taken after fertilization for further rearing. A similar procedure was applied to reach the highest percentage of triploid offspring, with the exception that diploid females (LR) were used instead of triploid ones and only large eggs (diploid LR gametes in the most cases) were taken for further rearing. In this way, we obtained 9 crosses of diploid (LR) and 10 crosses of triploid (LLR) progeny that originated from different parents. In one cross, in which we expected on the basis of egg size only LLR specimens, the mixed progeny, including LR, appeared. Another interesting result was the pentaploid specimen that was found among all-triploid siblings obtained from a diploid female *P. esculentus* (LR) crossed with a diploid male *P. lessonae* (LL). This was the first observation of pentaploidy within a *P. esculentus* hybrid complex. The detailed description of this unique individual, including erythrocyte size, DNA content, and microsatellites analysis, was published elsewhere (Hermaniuk et al. 2013). The pentaploid specimen and the cross with mixed genotype of offspring were excluded from further procedures. In this study we used diploid (LR) and triploid (LLR) individuals that belonged to 18 (9 + 9) different crosses, 8 in 2009 and 10 in 2010.

Rearing of Tadpoles and Froglets

Tadpoles. Eggs and hatchlings were raised in plastic litter trays filled with aerated tap water at room temperature until tadpoles reached developmental stage 25 according to Gosner (1960; the stage of free swimming and independent feeding). At this stage, on the median date of May 20, a group of 140 healthy-looking individuals from each cross, diploid or triploid, was randomly selected. Half of these were raised at 19°C in one tank and the second half at 24°C in another tank, and two tanks from each cross were placed next to each other. These two temperatures approximated the range of mean temperatures measured in ponds (microhabitats) inhabited by the tadpoles of green frogs (*P. esculentus* and *P. lessonae*). The grand mean water temperature, measured every 15 min in three ponds in northeast Poland (54°15′N), was 16.6°C at the beginning of tadpoles' development and was 23.0°C in the second half of development (A. Hermaniuk, unpublished data). In southern Sweden (60°30′N), the tadpoles experienced the average water temperature of 19.4°C (Orizaola and Laurila 2009*b*; see also Negovetic et al. 2001).

Until metamorphosis, the development of all animals was conducted in exactly the same tanks (50 cm × 35 cm × 30 cm) placed in a climate chamber set to 16°C (±1°C). Temperature controllers (Thermostab TS 500, Aquael) equipped with heaters were responsible for maintaining an appropriate water temperature (accuracy ±0.25°C) in the tanks. Lamps with 70-W bulbs were installed above each tank. Water was aerated permanently by using air stones and filters (PZC 300, Aquael) that additionally purified the water from food remains and feces. A natural photoperiod was maintained throughout the period of larval development; the day length was increasing from the beginning of the experiment and then decreasing, as in outdoor conditions. Tadpoles were fed ad lib. with a powder mix consisting of one part dried nettle and one part commercial fish food (Supervit, Tropical). A volume of 0.5 cm^3 of food was supplied to each tank twice a day. Half of the water from each tank was renewed three times a week with aged tap water stored for at least 24 h at room temperature. The tanks were inspected daily, and dead larvae and food remains were removed.

To determine the increase in the body mass of diploid and triploid tadpoles at two temperatures soon after hatching, when the growth rate is high, we weighed them in 2009 in stages 26, 31, and 34 of development (Gosner 1960) to within 0.1 mg (with the WPA 71 scale, Radwag). Before weighing, all animals were put on blotting paper to remove water that covered the body. Overall, we weighed 356 individuals from all 8 crosses. At each stage, 10 randomly caught larvae were weighed. The following stages were treated as achieved when at least 5 out of 10 tadpoles in the tank reached the particular stage.

To assess the rate of development, we also analyzed the time to metamorphosis. The moment of the appearance of at least one forelimb was defined as the beginning of metamorphosis and corresponded to Gosner's stage 42. The number of days from the beginning of the experiment (Gosner's stage 25) to this stage was used as a measure for time to metamorphosis. All 373 tadpoles from eight crosses were weighed at Gosner's stage 42.

The size of erythrocytes and epidermal cells was measured in tadpoles at similar stages of development as described below. The ploidy of all tadpoles used for determination of growth rate and cell size was confirmed by chromosome analysis (see below).

Froglets. At the beginning of the metamorphosis (Gosner's stage 42), all tadpoles were transferred to terrariums placed in the climate chamber. Individuals from each cross were kept until the metamorphosis at two temperatures, and after reaching this stage they were still reared in separate containers but all at the same air temperature of 23°C. Terrariums (84 cm × 50 cm × 40 cm) were covered with lids made of netting. They were equipped with a litter tray filled with tap water and houseplants, which increased humidity; the rest of the bottom was covered with gravel. A basking spot lamp (SUN GLO 75 W, Exo Terra) was installed above each terrarium. Froglets were fed ad lib. with

earthworms and crickets. Water was changed in each terrarium two times a week. A natural photoperiod was maintained until mid-October and remained unchanged afterward. About 3 mo after metamorphosis, the froglets were used for the measurements of metabolic rates (to be described elsewhere) and thereafter killed for determination of the size of hepatocytes and erythrocytes. The ploidy of all froglets was confirmed as described below.

Karyotyping

The genome composition of tadpoles and froglets was determined using the AMD/DAPI method (fluorescence double-staining technique), which enables discrimination between R and L chromosomes due to the fluorescence of AT-rich pericentromeric heterochromatin regions in R chromosomes (Ogielska et al. 2004). To obtain metaphase plates, inner intestine epithelial tissue fragments were used. Tadpoles were immersed in 15 mL of 0.05% colchicine (Sigma-Aldrich) 1 d before tissue preparation. Froglets were peritoneally injected with 0.5 mL of 0.3% colchicine 24 h before dissection. Chromosome counting was done on three to five complete metaphase plates from each individual (see Hermaniuk et al. 2013 for further details).

Measurements of Cell Sizes

Erythrocytes. We used blood smears from a cut tail (tadpoles) and fingertip (froglets) to measure the erythrocyte area as described in Hermaniuk et al. (2013). The measurements of erythrocytes were performed in 2009 and 2010, in 158 tadpoles from 18 crosses at similar stages of development (Gosner stages 33–35) and in 115 froglets from 15 crosses.

Epidermal Cells and Hepatocytes. The tips of tadpole tails were used to measure the size of epidermal cells. Livers dissected from froglets were used to analyze the size of hepatocytes. Microscopic slides were prepared in accordance with the following procedure: one pad of the tissue (tail and liver) from each animal was fixed in 10% phosphate-buffered formalin, dehydrated in ethanol and xylene, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin for microscopic examination. Cross-sectional areas of epidermal cells and hepatocytes (μm^2) with a clearly visible nucleus, 30 cells per individual, were measured using MultiScanBase, version 14.02. The areas of epidermal cells were analyzed in the material collected in 2010, which included 70 tadpoles from nine crosses. All individuals were at a similar stage of development (Gosner stages 33–35). Hepatocytes were analyzed on the material derived from 2009 and 2010, which included 57 froglets from 12 crosses.

Statistical Analyses

Cell size, time to metamorphosis, and mortality were analyzed by means of ANOVA model that included ploidy (diploid, triploid) and water temperature (19°, 24°C) as fixed factors and the cross nested in ploidy as the random factor. The model also composed the interaction between ploidy and temperature, as well as between temperature and cross nested in ploidy. As for tadpoles, the tests performed for froglets also included the temperature of the water in which they were developing as larvae. The area of epidermal cells, area of erythrocytes, and time to metamorphosis were log transformed, and mortality was arcsine transformed before tests to fulfill the assumptions of ANOVA. Body mass was tested for each stage of development separately with the same ANOVA model. The course of the effect of ploidy × temperature interaction with advancing stage was used as a means of analyzing differences in growth rates between diploid and triploid tadpoles. We did not apply a single model for all the data, with the age or stage as a covariate. The variance of body mass increased greatly with the stage and age, which would require log transformation of the data. However, log transformation distorted the comparison of the growth rates between the two ploidies.

All tests were performed using SAS, version 9.3 (SAS Institute, Cary, NC). ANOVAs were performed using the MIXED procedure based on the restricted maximum likelihood method. Mean values are reported with standard errors (SE) throughout the study.

Results

Cell Size

Tadpole mean cell areas, both erythrocytes and epidermal cells, differed greatly between ploidies (ANOVA, $F_{1,16} = 251.61$, $P < 0.001$ and $F_{1,7} = 20.29$, $P = 0.003$, respectively). We also found a significant effect of temperature on the area of erythrocytes and epidermal cells ($F_{1,16} = 22.51$, $P < 0.001$ and $F_{1,7} = 8.60$, $P = 0.022$, respectively), and we did not find any effect of ploidy × temperature interaction ($F_{1,16} = 0.12$, $P = 0.732$ and $F_{1,7} = 2.79$, $P = 0.139$, respectively). Triploids had a larger area of erythrocytes and epidermal cells at both temperatures, and both diploids and triploids had larger cells at 19°C (fig. 1).

In froglets, ANOVA revealed a highly significant interploidy difference of the cell areas, in both erythrocytes and hepatocytes ($F_{1,13} = 146.62$, $P < 0.001$ and $F_{1,10} = 57.89$, $P < 0.001$, respectively), a nonsignificant effect of the temperature in which froglets developed as larvae ($F_{1,13} = 1.57$, $P = 0.232$ and $F_{1,10} = 0.87$, $P = 0.372$, respectively), and a nonsignificant effect of ploidy × temperature interaction ($F_{1,13} = 0.10$, $P = 0.755$ and $F_{1,10} = 1.33$, $P = 0.276$, respectively). Triploids had larger areas of erythrocytes and hepatocytes than their diploid relatives (fig. 2). No cases of endopolyploidy were recorded in the intestine epithelial tissue of inspected diploid and triploid tadpoles or froglets.

Tadpole Growth Rates

The development time from fertilization until stage 25 was similar in all 18 crosses (13.0 ± 0.3 and 12.6 ± 0.3 d in diploid and triploid crosses, respectively; *t*-test, $t_{16} = 1.08$, $P = 0.297$). After stage 25, when the experiment at two temperatures commenced, growth patterns for diploid and triploid tadpoles were

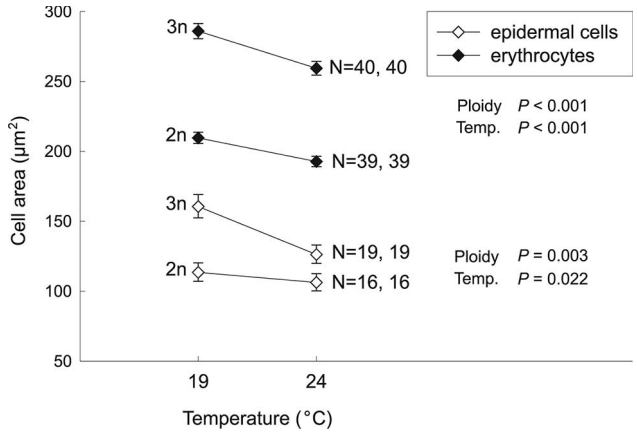

Figure 1. Area of erythrocytes and cross-sectional area of epidermal cells in diploid (2n) and triploid (3n) tadpoles of *Pelophylax esculentus* in relation to water temperature. The points are adjusted means (±SE) from two ANOVAs with ploidy and temperature as the main factors (see "Statistical Analyses"); the interaction terms were nonsignificant in these ANOVAs. N = sample size.

different. Comparisons of body masses at three developmental stages (fig. 3) by means of ANOVA (for each stage separately) revealed an increasing effect of the interaction between ploidy and temperature with advancing stage ($F_{1,6} = 0.21$, $P = 0.662$; $F_{1,6} = 3.11$, $P = 0.129$; $F_{1,5} = 11.46$, $P = 0.019$, for stages 26, 31, and 34, respectively). The interaction was significant in stage 34 but not in previous stages. Body masses of diploid and triploid tadpoles in stage 34 did not differ at 24°C ($F_{1,6} = 4.98$, $P = 0.067$), in contrast with 19°C, where triploids had significantly higher masses both in the same stage 34 ($F_{1,5} = 30.28$, $P = 0.002$) and at a similar age (in stage 31; $F_{1,6} = 13.74$, $P = 0.010$; table 1). We concluded, therefore, that triploids enjoyed faster growth than diploids at 19°C and the difference between two ploidies was less pronounced at 24°C. This is supported by the comparison of the slopes of regression lines of body mass versus age in figure 3 (each fitted to three averages for each temperature/ploidy). Triploid tadpoles grew 31.5% and 13.0% faster than diploids at 19° and 24°C, respectively, and growth rates of triploid tadpoles were similar at the two temperatures.

Body masses of triploid tadpoles at a given temperature, compared at the same developmental stage (26, 31, and 34), were always higher than in diploid individuals, except for the mentioned stage 34 at 24°C (table 1). The mortality of tadpoles between hatching and stage 34 was very low, 1.4% ± 0.5% on average ($n = 16$; eight crosses at each of the two temperatures) and was not affected by ploidy or temperature ($F_{1,6} = 0.53$, $P = 0.495$ and $F_{1,6} = 0.01$, $P = 0.941$, respectively).

Metamorphosis

ANOVA revealed that ploidy and temperature significantly affected the body mass attained at metamorphosis ($F_{1,6} = 12.51$, $P = 0.012$ and $F_{1,6} = 30.55$, $P = 0.001$, respectively), whereas there was no significant effect of the interaction between ploidy and the temperature ($F_{1,6} = 1.74$, $P = 0.236$). Triploids were bigger at both temperatures, and both diploids and triploids were bigger at 19° than at 24°C (fig. 4). Comparison of body masses at two temperatures separately revealed a significant difference between the two ploidies at 19° but not at 24°C (table 1).

The age at metamorphosis was significantly dependent on the temperature ($F_{1,6} = 361.30$, $P < 0.001$), but ploidy did not affect the larval development time ($F_{1,6} = 0.49$, $P = 0.511$). Both diploid and triploid tadpoles developed much faster at a higher temperature (fig. 4). The interaction between ploidy and temperature was not significant ($F_{1,6} = 4.01$, $P = 0.092$).

The mortality of tadpoles between stage 34 and metamorphosis differed between the two temperatures but not between ploidies ($F_{1,6} = 27.75$, $P = 0.002$ and $F_{1,6} = 0.17$, $P = 0.692$, respectively, with a nonsignificant interaction between the two factors, $F_{1,6} = 0.53$, $P = 0.495$). Mortality was higher at 24°C (32.8% ± 3.8%) than at 19°C (6.3% ± 2.3%).

Discussion

Cell Size

The results of our study showed that both ploidy and temperature significantly affected cell size. Triploid individuals, both tadpoles and froglets, had larger cells of the tested tissues compared to diploids (figs. 1, 2), which was, at least in part, a consequence of the larger genome of triploid cells. LLR triploids have 43% more DNA in erythrocyte nuclei than LR diploids (Ogielska et al. 2004). The large size of erythrocytes in polyploid animals is a well-documented fact, and measurement of erythrocyte size is the easiest method for distinguishing diploids from polyploids in fish and amphibians (Austin and Bogart 1982; Polls Pelaz and Graf 1988; Matson 1990; Ballarin et al. 2004). However, only a few studies have reported that cells

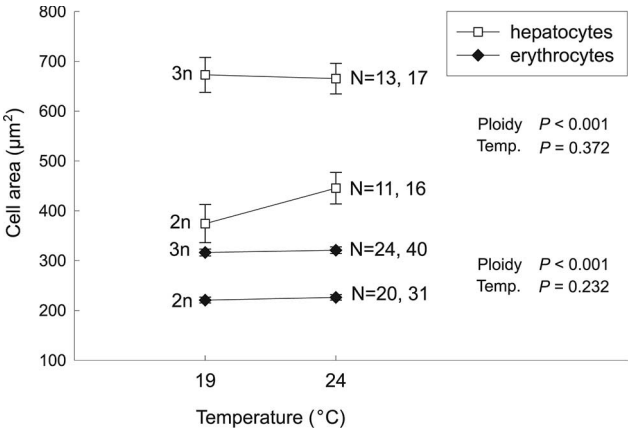

Figure 2. Area of erythrocytes and cross-sectional area of hepatocytes in diploid and triploid froglets *Pelophylax esculentus* in relation to the temperature of the water in which they developed as larvae. The points are adjusted means (±SE) from two ANOVAs with ploidy and temperature as the main factors (see "Statistical Analyses"); the interaction terms were nonsignificant in these ANOVAs. N = sample size.

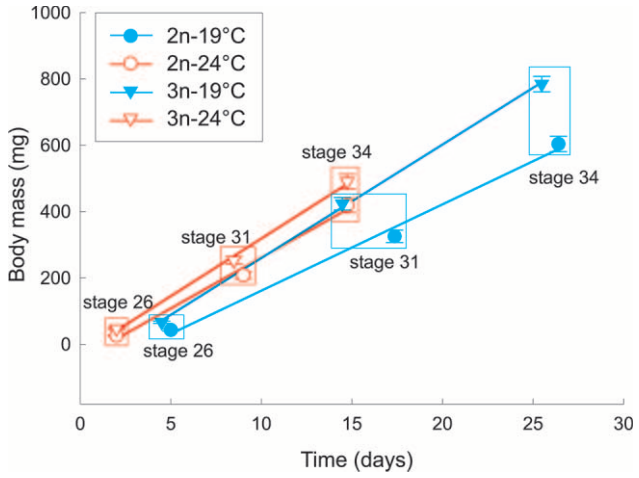

Figure 3. Growth rate of diploid (2n) and triploid (3n) *Pelophylax esculentus* tadpoles in early phase of development at two water temperatures. Points are mean body masses of tadpoles at Gosner's stages 26, 31, and 34 (adjusted means ± SE from ANOVA; see table 1); body masses are shown against time elapsed from stage 25, when tadpoles begin free swimming and independent feeding. The slopes of the lines at 24°C are 30.7 and 34.7 mg d^{-1} in diploid and triploid tadpoles, respectively, and at 19°C are 26.0 and 34.2 mg d^{-1}, respectively.

of other tissues are also larger in polyploid vertebrates than in their diploid counterparts (Fankhauser 1945; Swarup 1959; Suresh and Sheehan 1998). The larger hepatocytes and epidermal cells (unexplored tissues in *Pelophylax esculentus* until now) in triploids described in this study suggest that the whole body of polyploid *P. esculentus* is composed of larger cells, which is supported by the fact that cell size in amphibians is positively correlated between different tissues (Kozłowski et al. 2010).

Our results showed that water temperature strongly affected the size of erythrocytes and epidermal cells in tadpoles. Both diploids and triploids had larger cells at lower temperature (fig. 1). This observation is consistent with research conducted on a wide range of diploid ectotherms that possessed larger cells at lower temperatures than their conspecifics reared at higher temperatures (van Voorhies 1996; Blanckenhorn and Llaurens 2005; Arendt 2007). It is not clear how temperature may induce changes in cell sizes. A recent study on *Daphnia* has shown that both nucleus and genome size increased in individuals raised at 10°C compared with those reared at 20°C (Jalal et al. 2013). The authors of this study suggested that larger cell size at low temperature could be partly attributed to the enlarged nucleus and that DNA condensation was the most likely cause of the low-temperature response. It has been revealed that large-scale chromatin condensation occurs in ontogenesis for the control of the nucleocytoplasmic ratio at cell enlargement (Vinogradov 2005). More recently, Jalal et al. (2015) documented that both nucleus size and DNA condensation varied with temperature in *Drosophila melanogaster*, while DNA content appeared to be constant.

Tadpoles in our study were developing at two constant water temperatures, while in natural ponds tadpoles experience diurnal and seasonal variation of temperature. The average daily temperature range in ponds inhabited by *P. esculentus* in NE Poland was 5.1°C in the beginning of tadpoles' development and was 7.7°C in the second half of development (A. Hermaniuk, unpublished data). An interesting question is whether variable temperatures may have different impact on cell size of tadpoles than constant temperatures, although this subject requires further study. In a study on *D. melanogaster*, either a higher mean temperature or daily variation of temperature (±4°C) caused flies to develop smaller cells relative to their body sizes, but the effect of thermal fluctuations was much weaker in magnitude than the effect of mean temperature (Czarnoleski et al. 2013).

Interestingly, we did not observe any temperature effect on cell size in froglets a few months after metamorphosis when

Table 1: Body masses (mg) of diploid and triploid *Pelophylax esculentus* tadpoles in selected stages of development, reared at two water temperatures

Temperature (°C) and ploidy	Stage			
	26	31	34	42
19:				
3n	66.6 ± 3.2 (20)	423.5 ± 18.8 (34)	784.3 ± 23.3 (21)	1385.9 ± 59.0 (112)
2n	43.3 ± 2.9 (38)	325.3 ± 18.7 (34)	604.0 ± 23.1 (23)	1124.3 ± 59.3 (105)
F	29.0	13.7	30.3	9.78
df	1, 6	1, 6	1, 5	1, 6
P	.002	.010	.003	.020
24:				
3n	47.4 ± 1.9 (39)	252.8 ± 10.6 (33)	490.1 ± 22.5 (23)	1059.6 ± 41.3 (78)
2n	26.4 ± 2.0 (38)	208.5 ± 10.8 (30)	419.1 ± 22.5 (23)	923.3 ± 41.4 (78)
F	57.8	8.52	4.98	5.43
df	1, 6	1, 6	1, 6	1, 6
P	<.001	.027	**.067**	**.059**

Note. Stage 42 = metamorphosis. Values are adjusted means and standard errors from ANOVA, with ploidy and cross nested in ploidy as factors. Numbers of individuals are given in parentheses. Nonsignificant differences ($P > 0.05$) between two ploidies at a given temperature are in bold.

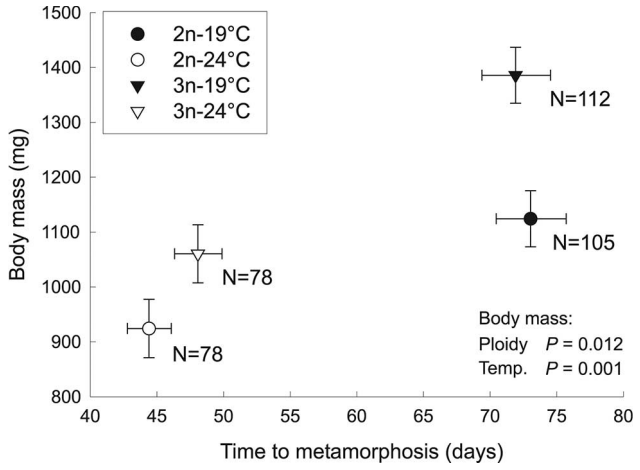

Figure 4. Body mass at metamorphosis (Gosner stage 42) versus time to metamorphosis in diploid and triploid tadpoles of *Pelophylax esculentus*. Adjusted means (±SE) from ANOVA. N = sample size.

animals shifted their environment from aquatic to terrestrial and were placed in common temperature (fig. 2). Cell size constraints (also TSR) seem to be most noticeable in aquatic habitats where the solubility of oxygen decreases with temperature. This may imply selection for smaller species at high water temperatures and also explain the gigantism of many polar taxa (Chapelle and Peck 1999). In marine invertebrates (e.g., in copepods and other major groups of crustaceans), the general pattern of enlarged adult body size at low temperatures reflects enlarged cell and genome size, whereas among terrestrial invertebrates, various responses are common, including both larger and smaller body size in colder areas (Hessen et al. 2013).

Body Mass versus Cell Size in Tadpoles

Our measurements of cell size in tadpoles revealed a larger size in triploid than in diploid tadpoles and a larger cell size at lower than at higher temperature. Comparison of these measurements and the course of body masses in tadpoles indicates that cell size directly affects their body size. First, the body mass of triploid tadpoles was significantly larger than in diploids at all developmental stages at 19°C and at the first stages at 24°C (table 1). Second, both diploid and triploid tadpoles reared at lower temperature, besides having larger cells, reached a larger body mass at metamorphosis (fig. 4). The largest body size at metamorphosis found in triploid tadpoles reared at 19°C was in agreement with the largest cell size of triploids developing at that temperature. It should be noted that the large size of our *P. esculentus* triploid (LLR) tadpoles was not caused by the gene dosage effect, that is, by an excess of L genomes over R genomes, because the parental *Pelophylax lessonae* (LL) is smaller than *P. esculentus* (Berger 2008).

Earlier studies on *P. esculentus* have revealed that triploids (LLR) have a larger body length than diploids (LR) at the end of metamorphosis (Gosner's stage 46; table A1). Different results from ours and those of the above authors have been reported in a recent article by Pruvost et al. (2013), who reared tadpoles of *P. esculentus* complex at two temperatures, 18° and 24°C. They did not find differences in body mass between diploid (LR) and triploid (LLR) *P. esculentus* at metamorphosis. Only LRR metamorphs tended to be heavier than diploids (LL and LR) at 24°C, which was interpreted as a weight effect of the *Pelophylax ridibundus* genome (R), the largest species in the water frog complex. The difference between ours and Pruvost et al.'s (2013) study is that we weighed tadpoles at the beginning of metamorphosis (stage 42) while Pruvost et al. did so at the end of metamorphosis (stage 46; after weight loss due to tail resorption). Nevertheless, body masses at stages 42 and 46 are strongly correlated irrespective of rearing temperature, and the loss of body mass at that time is an invariable percentage of the maximum premetamorphic mass, at least in diploid frogs, including *P. esculentus* (Negovetic et al. 2001; Álvarez and Nicieza 2002; Orizaola and Laurila 2009*a*). The most plausible explanation for the discrepancy might be the very restrictive feeding of tadpoles in Pruvost et al.'s (2013) study. This led to a very long time to metamorphosis in diploid and triploid *P. esculentus*, for example, 35% longer in diploids reared at 24°C as compared with diploids in our study at 24°C. The prolonged development of tadpoles was associated with a very low body mass of metamorphs, about half of the mass recorded in our study at 24°C (when corrected for body mass loss between stages 42 and 46 after Orizaola and Laurila 2009*a*).

Although there are examples of increased body size of polyploids versus diploids in invertebrates (Weider 1987; Walsh and Zhang 1992), a larger size for triploid *P. esculentus* is exceptional among polyploid vertebrate ectotherms, as follows from the scarce studies on that group. Tetraploid frogs *Neobatrachus* and triploid *Gasterosteus aculeatus* (three-spine sticklebacks), despite their larger cells, are of approximately the same size as their diploid counterparts (Swarup 1959; Mahony and Robinson 1980). Likewise, tri-, tetra-, and pentaploid eastern newts *Notophthalmus viridescens* are no larger at hatching and metamorphosis than diploid forms at the same developmental stages (Fankhauser 1945). It has also been found in the two latter studies that the increase in a size of cells is met by a decrease in cell number. The only example similar to our results of a positive correlation between cell size (only erythrocytes were analyzed) and body size in polyploid vertebrates has been presented in tetraploid *Cobitis biwae* (spinous loach; Schultz 1980).

Low temperature had a dramatic impact on tadpoles by increasing the age when tadpoles reached metamorphosis and their body mass at that time. The larger body mass of diploid and triploid tadpoles at 19°C at the end of their linear segment of growth (on stage 34; fig. 3) is at least partly explained by their larger cells (fig. 1). Similarly, an increased body mass of diploid metamorphs in the cold has been reported in numerous studies on larval development in amphibians, including *P. esculentus* (Licht and Bogart 1989; Negovetic et al. 2001; Watkins and Vraspir 2006; Pruvost et al. 2013). Studies of the contribution of cell size to body size at different temperatures in diploid vertebrates have reported very mixed results. Fish

are the only group that have been studied extensively in this respect, but the majority of studies have reported embryonic growth only, comparing fish at hatching (for review see Arendt 2007). Hatchlings incubated in colder water have usually been larger and have consisted of larger cells (Arendt 2007). In *Dicentrarchus labrax* (sea bass), low temperature increases the age but not body mass of metamorphs, and they are built of larger cells (Ayala et al. 2001). In the lizard *Anolis carolinensis*, larger cells have been produced in hatchlings from cooler treatments, but hatchling body size is unaffected by temperature (Goodman and Heah 2010).

To our knowledge, our study is the first to report an increase in the body mass of a polyploid vertebrate caused by low temperature and that the increase in cell size contributes to increased body mass. This effect was shown in tadpoles from the stage of independent feeding to metamorphosis. The only comparable example from vertebrates that we are aware of comes from *Ambystoma*. Large (triploid) eggs have given rise to hatchlings larger than from small (mainly diploid) eggs only at low temperature, and the body size of hatchlings from large and small eggs is larger at cold temperatures than at warm temperatures (significant interaction between egg size and temperature; Licht and Bogart 1989). Nothing has been reported, however, about the subsequent course of body size in these *Ambystoma* larvae.

Growth Rate and Body Mass–Ecological Background

Characteristically, at the initial linear segment of growth, triploid tadpoles grew at the same rate at 19° and 24°C (fig. 3). In contrast, diploid tadpoles grew at a lower rate that was most pronounced at the lower temperature. This suggests that decreasing temperature has a lower impact on mechanisms controlling development in our LLR triploids than in diploids. Consequently, LLR triploids of *P. esculentus* are better adapted to cold environments, which may elucidate their high proportion in populations at high latitudes (Plötner 2005). The most plausible explanation for the wide temperature tolerance in triploid tadpoles is their increased heterozygosity resulting from the additional genome; this heterozygosity may manifest, for example, in more different forms of enzymes available for polyploids (Otto and Whitton 2000). The other possibility is that a higher quantity of enzymes per cell improves the metabolic processes of the polyploids at low temperatures (Dufresne and Hebert 1998).

Also, the larger size of LLR triploids in comparison to diploids revealed in our study (table 1; figs. 3, 4) may have significant ecological consequences. Larger body mass at earlier stages of development reduces predation risk and enhances competitive ability in many species of tadpoles (Travis et al. 1985; Semlitsch 1990). Numerous studies have confirmed that size at metamorphosis is crucially important for amphibians and enhances survival and fecundity in later life stages (Morey and Reznick 2001; Altwegg and Reyer 2003). Therefore, larger triploid *P. esculentus* froglets may survive better than diploid froglets, assuming that differences in body mass between two ploidies at the beginning of metamorphosis (our study) and at the end of metamorphosis (table A1) continue for longer in terrestrial life. This assumption is supported by the fact that diploid *P. esculentus* individuals metamorphosing at a large size are larger at maturity (Altwegg and Reyer 2003). Unfortunately, we could not test this assumption in our froglets, because too many uncontrolled factors determined their growth rate.

Benefits arising from larger body size in amphibians become particularly important in the northern part of species ranges. Northern populations, in cold environments, undergo prolonged hibernation periods, and *P. esculentus* hibernates mainly on land (Holenweg and Reyer 2000). Metabolic rate in ectotherms is a function of ambient temperature; thus, hibernating froglets seem not to be at risk of depleting endogenous energy stores, as long as snow provides insulative cover and air temperature is stable. Variable winter temperatures with periodic spells of increased temperatures result in enhanced mortality of *P. esculentus* (Anholt et al. 2003). Increased metabolic rate at warmer temperatures may risk exhaustion of energy reserves before frogs can begin feeding again in spring. Although diploid and triploid *P. esculentus* froglets do not differ in metabolic rates when corrected for body mass (A. Hermaniuk and J. R. E. Taylor, unpublished data), larger individuals have lower mass-specific metabolic rates. Larger on average triploid froglets, with lower mass-specific rates, may survive longer on body reserves. Consistent with this reasoning, the larger *P. esculentus* has a higher overwinter survival rate than smaller *P. lessonae* in the same habitat (Anholt et al. 2003).

Freeze tolerance and freeze avoidance via supercooling are important mechanisms for dealing with subzero temperatures in a diverse array of ectothermic animals that are terrestrial hibernators (Ramlov 2000). *Pelophylax esculentus* exhibits modest supercooling with a crystallization temperature ranging from −0.8° to −1.4°C and survives moderate freezing (Voituron et al. 2005). Freezing tolerance in *P. esculentus* is significantly correlated with body mass, and larger individuals exhibit lower ice accumulation rates (expressed as percent of body water frozen vs. time; Voituron et al. 2005). This may pose a selective advantage for the larger triploid *P. esculentus* compared to diploid individuals, enhancing winter survival in cold climates. The observation that survival of *P. esculentus* from metamorphosis until the following spring is positively related to size at metamorphosis (Altwegg and Reyer 2003) supports this reasoning.

The larger body size of triploid tadpoles may also have other selective advantages. It has been proved that *P. esculentus* frogs do not compensate their small size at metamorphosis by enhancing their postmetamorphic growth (Altwegg and Reyer 2003). This might be especially true in northern habitats, where the season suitable for growth after metamorphosis is very short and body mass increase during terrestrial life might not be sufficient to ensure survival through the winter. This is presumably connected with decreasing temperatures at the end of the growing season resulting in a lower activity of froglets and their arthropod prey. Under such conditions, attaining a high body mass at the end of the aquatic phase of life is of crucial importance.

We conclude that the higher body mass of polyploid tadpoles may provide selective advantages under both aquatic and terrestrial conditions, especially in colder environments. This may explain the observation that triploid individuals of *P. esculentus*, LLR form in particular, prevail in the north. However, it has to be stressed that triploid individuals cannot replace diploids in any population because their presence depends on diploid gametes produced by diploid females (Christiansen 2009).

Acknowledgments

We would like to thank Anna Koszewnik, Marta Strzałka, Małgorzata Lewoc, Emilia Murawska, and Natalia Gawrysiuk for their assistance with laboratory work and maintenance of tadpoles and frogs. We would also like to thank Maria Ogielska and Piotr Kierzkowski for their support and advice during initial karyotyping of animals. We are grateful to Jan Kozłowski and two anonymous reviewers for valuable comments on an earlier draft of the manuscript. This study was supported by a grant from the Polish Ministry of Science and Higher Education (grant N N304 048734) awarded to J.R.E.T. The procedures concerning animals adhered to principles of animal care and to specific national laws (approval DOP-4200/II-8/238/09 from the Ministry of Environment and permit 29/2009 from the Local Ethical Committee in Białystok). Adam Hermaniuk is a beneficiary of the project "Scholarships for PhD students from Podlaskie Voivodeship." The project is cofinanced by the European Social Fund, Polish Government, and Podlaskie Voivodeship. We declare no conflicts of interest.

APPENDIX

Table A1: Comparison of body length (mm) of diploid (LR) and triploid (LLR) *Pelophylax esculentus* at the end of metamorphosis (Gosner's stage 46)

		LR		LLR		
No.	Female no.	Length	*N*	Length	*N*	Source
1	4/90	20.5	7	21.7	4	Berger and Roguski 2002, table 3
2	8/99b	23.6	1	27.2	1	Berger and Roguski 2002, table 3
3	12/78	17.1	10	20.0	4	Berger and Roguski 2002, table 3
4	20/85a	15.2	4	16.2	4	Berger and Roguski 2002, table 3
5	12/98	20.8	4	23.1	7	Berger and Roguski 2002, table 3
6	15/77	22.7	36	26.0	5	Berger and Truszkowski 1980, table 5
7	12/78	16.6	8	20.0	4	Berger and Truszkowski 1980, table 5
8	...	24.1	28	27.4	8	Uzzell et al. 1975, table 3
9	RL-S	16.3	5	17.9	5	Berger and Günther 1988, table 7

Note. All metamorphs were the offspring of *P. esculentus* (LR) females and *Pelophylax lessonae* (LL) males. All nine females listed produced both LR and LLR progeny in the same clutches of eggs. Mean body lengths of LR and LLR individuals were 19.7 and 22.2 mm, respectively (paired-sample *t*-test, $t_8 = 7.42$, $P < 0.001$). N = sample size.

Literature Cited

Altwegg R. and H.-U. Reyer. 2003. Patterns of natural selection on size at metamorphosis in water frogs. Evolution 57: 872–882.

Álvarez D. and A.G. Nicieza. 2002. Effects of temperature and food quality on anuran larval growth and metamorphosis. Funct Ecol 16:640–648.

Angilletta M.J., T.D. Steury, and M.W. Sears. 2004. Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. Integr Comp Biol 44:498–509.

Anholt B.R., H. Hotz, G.D. Guex, and R.D. Semlitsch. 2003. Overwinter survival of *Rana lessonae* and its hemiclonal associate *Rana esculenta*. Ecology 84:391–397.

Arendt J. 2006. The cellular basis for phenotypic plasticity of body size in western spadefoot toad (*Spea hammondi*) tadpoles: patterns of cell growth and recruitment in response to food and temperature manipulations. Biol J Linn Soc 88: 499–510.

———. 2007. Ecological correlates of body size in relation to cell size and cell number: patterns in flies, fish, fruits and foliage. Biol Rev 82:241–256.

Arendt J. and L. Hoang. 2005. Effect of food level and rearing temperature on burst speed and muscle composition of western spadefoot toad (*Spea hammondii*). Funct Ecol 19:982–987.

Atkinson D. 1994. Temperature and organism size: a biological law for ectotherms? Adv Ecol Res 25:1–58.

Atkinson D. and R.M. Sibly. 1997. Why are organisms usually bigger in colder environments? making sense of a life history puzzle. Trends Ecol Evol 12:235–239.

Austin N.E. and J.P. Bogart. 1982. Erythrocyte area and ploidy determination in the salamanders of the *Ambystoma jeffersonianum* complex. Copeia 2:485–488.

Ayala M.D., O. López-Albors, F. Gil, A. García-Alcázar, E. Abellán, J.A. Alarcón, M.C. Álvarez, et al. 2001. Temperature effects on muscle growth in two populations (Atlantic and Mediterranean) of sea bass, *Dicentrarchus labrax* L. Aquaculture 202:359–370.

Ballarin L., M. Dall'Oro, D. Bertotto, A. Libertini, A. Francescon, and A. Barbaro. 2004. Hematological parameters in *Umbrina cirrosa* (Teleostei, Sciaenidae): a comparison between diploid and triploid specimens. Comp Biochem Physiol A 138:45–51.

Bennett M.D. 1987. Variation in genome form in plants and its ecological implications. New Phytol 196:177–200.

Berger L. 2008. European green frogs and their protection. Ecological Library Foundation, Prodruk, Poznań.

Berger L. and R. Günther. 1988. Genetic composition and reproduction of water frog populations (*Rana* kl. *esculenta* Synklepton) near nature reserve Serrahn, GDR. Arch Nat Lands 28:265–280.

Berger L. and H. Roguski. 2002. Characteristics of diploid and triploid kin-tadpoles of Polish water frogs (*Rana esculenta* complex). Biol Lett 39:29–42.

Berger L., M. Rybacki, and H. Hotz. 1994. Artificial fertilization of water frogs. Amphibia-Reptilia 15:408–413.

Berger L. and J. Truszkowski. 1980. Viability and inheritance of characters in water frogs (*Rana esculenta* complex) in agrocenoses. Genet Pol 21:309–323.

Blanckenhorn W.U. and V. Llaurens. 2005. Effects of temperature on cell size and number in the yellow dung fly *Scathophaga stercoraria*. J Therm Biol 30:213–219.

Chapelle G. and L.S. Peck. 1999. Polar gigantism dictated by oxygen availability. Nature 399:114–115.

Chown S.J. and K.J. Gaston. 2010. Body size variation in insects: a macroecological perspective. Biol Rev 85:139–169.

Christiansen D.G. 2009. Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). BMC Evol Biol 9:1–16.

Czarniewska E., M. Rybacki, M. Pabijan, and L. Berger. 2011. Large eggs and ploidy of green frog populations in Central Europe. Amphibia-Reptilia 32:149–158.

Czarnoleski M., B.S. Cooper, J. Kierat, and M.J. Angilletta. 2013. Flies developed small bodies and small cells in warm and thermally fluctuating environments. J Exp Biol 216: 2896–2901.

Dufresne F. and P.D.N. Hebert. 1998. Temperature-related differences in life-history characteristics between diploid and polyploid clones of the *Daphnia pulex* complex. Ecoscience 5:433–437.

Fankhauser G. 1945. The effects of changes in chromosome number on amphibian development. Q Rev Biol 20:20–78.

Goodman R. and T.P. Heah. 2010. Temperature-indiced plasticity at cellular and organismal levels in the lizard *Anolis carolinensis*. Integr Zool 5:208–217.

Gosner K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Graf J.-D. and M. Polls Pelaz. 1989. Evolutionary genetics of the *Rana esculenta* complex. Pp. 289–301 in R.M. Dawley and J.P. Bogart, eds. Evolution and ecology of unisexual vertebrates. New York State Museum, Albany.

Gregory T.R. 2001*a*. The bigger the C-value, the larger the cell: genome size and red blood cell size in vertebrates. Blood Cell Mol Dis 27:830–843.

———. 2001*b*. Coincidence, coevolution, or causation? DNA content, cell size, and the C-value enigma. Biol Rev 76:65–101.

Gregory T.R. and B.K. Mable. 2005. Polyploidy in animals. Pp. 427–517 in T.R. Gregory, ed. The evolution of the genome. Elsevier, San Diego, CA.

Günther R. 1977. Die Erythrocytengröße als Kriterium zur Unterscheidung diploider und triploider Teichfrösche, *Rana "esculenta"* L. (Anura). Biol Zbl 96:457–466.

Hermaniuk A., N.B.M. Pruvost, P. Kierzkowski, and M. Ogielska. 2013. Genetic and cytogenetic characteristics of pentaploidy in water frogs. Herpetologica 69:36–45.

Hessen D.O., M. Daufresne, and H. Leinaas. 2013. Temperature-size relations from the cellular-genomic perspective. Biol Rev 88:476–489.

Holenweg A.-K. and H.-U. Reyer. 2000. Hibernation behavior of *Rana lessonae* and *R. esculenta* in their natural habitat. Oecologia 123:41–47.

Jalal M., T. Andersen, and D.O. Hessen. 2015. Temperature and developmental responses of body and cell size in *Drosophila*: effects of polyploidy and genome configuration. J Therm Biol 51:1–14.

Jalal M., M.W. Wojewodzic, C.M.M. Laane, and D.O. Hessen. 2013. Larger *Daphnia* at lower temperature: a role for cell size and genome configuration? Genome 56:511–519.

Kozłowski J., M. Czarnoleski, and M. Dańko. 2004. Can optimal resource allocation models explain why ectotherms grow larger in the cold? Integr Comp Biol 44:480–493.

Kozłowski J., M. Czarnoleski, A. Francois-Krassowska, S. Maciak, and T. Pis. 2010. Cell size is positively correlated between different tissues in passerine birds and amphibians, but not necessarily in mammals. Biol Lett 6:792–796.

Kozłowski J., M. Konarzewski, and A.T. Gawelczyk. 2003. Cell size as a link between noncoding DNA and metabolic rate scaling. Proc Natl Acad Sci USA 100:14080–14085.

Levin D.A. 1983. Polyploidy and novelty in flowering plants. Am Nat 122:1–25.

Licht L.E. and J.P. Bogart. 1989. Embryonic development and temperature tolerance in diploid and polyploid salamanders (genus *Ambystoma*). Am Midl Nat 122:401–407.

Löve A. 1953. Subarctic polyploidy. Hereditas 39:113–124.

Mable B.K., M.A. Alexandrou, and M.I. Taylor. 2011. Genome duplication in amphibians and fish: an extended synthesis. J Zool 284:151–182.

Mahony M.J. and E.S. Robinson. 1980. Polyploidy in the Australian leptodactylid frog genus *Neobatrachus*. Chromosoma 81:199–212.

Matson T.O. 1990. Erythrocyte size as a taxonomic character in the identification of Ohio *Hyla chrysoscelis* and *H. versicolor*. Herpetologica 46:457–462.

Morey S. and D. Reznick. 2001. Effects of larval density on postmetamorphic spadefoot toads (*Spea hammondii*). Ecology 82:510–522.

Negovetic S., B.R. Anholt, R.D. Semlitsch, and H.-U. Reyer. 2001. Specific responses of sexual and hybridogenetic European waterfrog tadpoles to temperature. Ecology 82:766–774.

Ogielska M., P. Kierzkowski, and M. Rybacki. 2004. DNA content and genome composition of diploid and triploid water frogs belonging to the *Rana esculenta* complex (Amphibia, Anura). Can J Zool 82:1894–1901.

Orizaola G. and A. Laurila. 2009*a*. Intraspecific variation of the temperature-induced effects on metamorphosis in the pool frog (*Rana lessonae*). Can J Zool 87:581–588.

———. 2009*b*. Microgeographic variation in temperature-induced plasticity in an isolated amphibian metapopulation. Evol Ecol 23:979–991.

Otto C.R.V., J.W. Snodgrass, D.C. Forester, J.C. Mitchell, and R.W. Miller. 2007. Climatic variation and the distribution of an amphibian polyploid complex. J Anim Ecol 76:1053–1061.

Otto S.P. and J. Whitton. 2000. Polyploid incidence and evolution. Annu Rev Genet 34:401–437.

Partridge L., B. Barrie, K. Fowler, and V. French. 1994. Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. Evolution 48:1269–1276.

Partridge L. and J.A. Coyne. 1997. Bergmann's rule in ectotherms: is it adaptive? Evolution 51:632–635.

Partridge L. and V. French. 1996. Thermal evolution of ectotherm body size: why get big in the cold? Pp. 265–292 in I.A. Johnston and A.F. Bennett, eds. Animals and temperature: phenotypic and evolutionary adaptation. Cambridge University Press, Cambridge.

Peters R.H. 1983. The ecological implications of body size. Cambridge University Press, New York.

Plötner J. 2005. Die westpaläarktischen Wasserfrösche. Laurenti, Bielefeld.

Polls Pelaz M. and J.-D. Graf. 1988. Erythrocyte size as an indicator of ploidy level in *Rana* kl. *esculenta* before and after metamorphosis. Alytes 7:53–61.

Pruvost N.B.M., D. Hollinger, and H.-U. Reyer. 2013. Genotype-temperature interactions on larval performance shape population structure in hybridogenetic water frogs (*Pelophylax esculentus* complex). Funct Ecol 27:459–471.

Ramlov H. 2000. Aspects of natural cold tolerance in ectothermic animals. Hum Reprod 15:26–46.

Schultz R.J. 1980. Role of polyploidy in the evolution of fishes. Pp. 313–340 in W.H. Lewis, ed. Polyploidy: biological relevance. Plenum, New York.

———. 1982. Competition and adaptation among diploid and polyploid clones of unisexual fishes. Pp. 103–119 in H. Dingle and J.P. Hergmann, eds. Evolution and genetics of life histories. Springer, New York.

Semlitsch D.R. 1990. Effects of body size, sibship, and tail injury on the susceptibility of tadpoles to dragonfly predation. Can J Zool 68:1027–1030.

Stöck M., D. Frynta, W.-R. Grosse, C. Steinlein, and M. Schmid. 2001. A review of the distribution of diploid, triploid and tetraploid green toads (*Bufo viridis* complex) in Asia including new data from Iran and Pakistan. Asiat Herpetol Res 9: 77–100.

Suresh A.V. and R.J. Sheehan. 1998. Muscle fibre growth dynamics in diploid and triploid rainbow trout. J Fish Biol 52:570–587.

Swarup H. 1959. Effect of triploidy on the body size, general organization and cellular structure in *Gasterosteus aculeatus* (L.). J Genet 56:143–155.

Travis J., W.H. Keen, and J. Julianna. 1985. The role of relative body size in a predator-prey relationship between dragonfly naiads and larval anurans. Oikos 45:59–65.

Uzzell T., L. Berger, and R. Günther. 1975. Diploid and triploid progeny from diploid female of *Rana esculenta* (Amphibia Salientia). Proc Acad Natl Sci Phila 127:81–91.

van der Have T.M. and G. de Jong. 1996. Adult size in ectotherms: temperature effects on growth and differentiation. J Theor Biol 183:329–340.

van Voorhies W.A. 1996. Bergmann size clines: a simple explanation for their occurrence in ectotherms. Evolution 50: 1259–1264.

Vinogradov A.E. 2005. Genome size and chromatin condensation in vertebrates. Chromosoma 113:362–369.

Voituron Y., P. Joly, M. Eugéne, and H. Barré. 2005. Freezing tolerance of the European water frogs: the good, the bad, and the ugly. Am J Physiol Reg 288:1563–1570.

Walsh E.J. and L. Zhang. 1992. Polyploidy and body size variation in a natural population of the rotifer *Euchlanis dilatata*. J Evol Biol 5:345–353.

Watkins T.B. and J. Vraspir. 2006. Both incubation temperature and posthatching temperature affect swimming performance and morphology of wood frog tadpoles (*Rana sylvatica*). Physiol Biochem Zool 79:140–149.

Weider L.J. 1987. Life history variation among low-arctic clones of obligately parthenogenetic *Daphnia pulex*: a diploid-polyploid complex. Oecologia 73:251–256.

Woods H.A. 1999. Egg-mass size and cell size: effects of temperature on oxygen distribution. Am Zool 39:244–252.