**UNIWERSYTET W BIAŁYMSTOKU**

Wydział Biologiczno-Chemiczny

Adam Hermaniuk

# The impact of cell size on metabolic rate, growth rate and development in amphibians: A case study on the diploid and triploid edible frogs (*Pelophylax esculentus*)

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*The selection forces working for the diminution of genome and cell size may be very powerful and may change these cell characters in a comparatively short time...*

Henryk Szarski. 1983. Journal of Theoretical Biology

***I dedicate this dissertation to my mom, my wife, Katarzyna; and my son, Tymon. Without your love, patience and support, this thesis would not have been possible.***

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# Contents

# Wielkość komórek a tempo metabolizmu i rozwoju diploidalnych i triploidalnych żab wodnych (*Pelophylax esculentus*)

## Streszczenie

Wśród organizmów wielokomórkowych obserwuje się znaczne zróżnicowanie wielkości komórek, co w dużej mierze związane jest z wielkością genomu. Wiadomo też, że mniejsze komórki mają wyższe tempo metabolizmu w przeliczeniu na jednostkę masy niż komórki większe. Według jednej z hipotez, tempo metabolizmu całego organizmu powinno być odzwierciedleniem tempa metabolizmu pojedynczych komórek, które składają się na organizm. Poprzez związek z tempem metabolizmu, rozmiar komórek powinien również wpływać na tempo podziałów komórkowych, a co za tym idzie – na tempo wzrostu i rozwoju. Porównania międzygatunkowe nie dają jednak jednoznacznej odpowiedzi co do związku pomiędzy wielkością komórek, tempem metabolizmu na poziomie całego organizmu i tempem wzrostu. U zwierząt zmiennocieplnych, najsilniejszy wpływ na rozmiary komórek mają temperatura i wielkość genomu (zwłaszcza poliploidalność). Powiększenie rozmiarów komórek spowodowane przez niską temperaturę może być przyczyną geograficznej zmienności wielkości ciała; w chłodniejszych środowiskach większość zwierząt zmiennocieplnych rośnie wolniej, ale osiąga większe rozmiary ciała (reguła zależności wielkości ciała od temperatury otoczenia; TSR). U organizmów poliploidalnych nieliczne przykłady wskazują, że większe genomy i komórki mogą powodować wzrost wielkości ciała, jak również mogą obniżać tempo metabolizmu przeliczone na jednostkę masy. Natomiast wpływ temperatury na wielkość komórek somatycznych u zwierząt poliploidalnych prawdopodobnie nigdy wcześniej nie był badany.

Celem mojej pracy doktorskiej było zbadanie związku pomiędzy wielkością komórek, wielkością ciała, tempem wzrostu i tempem metabolizmu u płazów. Obiektem moich badań były diploidalne i triploidalne żaby wodne, *Pelophylax esculentus*, które różniły się wielkością genomu i wielkością erytrocytów, co wykazano w poprzednich pracach. Badanie konsekwencji zróżnicowania wielkości komórek w obrębie jednego gatunku omija problem różnej historii ewolucyjnej gatunków porównywanych w analizach międzygatunkowych. W pierwszej części mojej pracy skupiłem się na wpływie poliploidii i temperatury na wielkość komórek i wielkość ciała kijanek hodowanych w 19 °C i 24 °C. Wykazałem, że ploidia i temperatura istotnie wpływa na wielkość komórek kijanek (erytrocytów i komórek naskórka). Kijanki di- i triploidalne

miały większe komórki w 19 °C, a komórki triploidów były większe niż u diploidów w obu temperaturach. Średnia wielkość erytrocytów pentaploidalnej żabki, która nieoczekiwanie pojawiła się w jednej z krzyżówek, nie była jednak proporcjonalnie większa od tej u triploidów, czego można było oczekiwać na podstawie wielkości genomu. U diploidalnych i triploidalnych żabek temperatura wody, w której żabki rozwijały się jako kijanki, nie wpływała na wielkość ich komórek. Triploidy nadal jednak posiadały większe komórki (hepatocyty i erytrocyty). W dalszej części mojej pracy testowałem hipotezę, że osobniki zbudowane z mniejszych komórek mają wyższe tempo metabolizmu niż osobniki o porównywalnej masie ciała, ale zbudowane z większych komórek. Zgodnie z oczekiwaniami, diploidalne kijanki miały wyższe standardowe tempo metabolizmu (SMR) niż triploidy. Co ciekawe, ploidia nie wpływała jednak na SMR u żabek. Na podstawie uzyskanych wyników, jak również w oparciu o obszerny przegląd literatury, wnioskuję, że wielkość komórek może mieć większe znaczenie dla SMR w wodzie niż na lądzie, ponieważ ilość tlenu zawartego w wodzie jest mniejsza, a jego dostępność w stosunku do zapotrzebowania maleje wraz ze wzrostem temperatury. W wodzie poliploidy zbudowane z większych komórek (o mniejszej powierzchni w stosunku do objętości) mogą być niewystarczająco zaopatrywane w tlen, wobec czego wykazują niższe tempo metabolizmu. Wykazałem również, że temperatura wody, w której hodowane były kijanki, nie miała wpływu na SMR żabek po metamorfozie, co było spójne z brakiem wpływu temperatury wody na wielkość komórek u żabek.

Kijanki obu ploidii były większe i rozwijały się dłużej w niskiej temperaturze, co miało związek z ich większymi komórkami. Nie było to dotychczas obserwowane u płazów i wskazuje, że wielkość komórek może mieć istotne znaczenie w wyjaśnieniu TSR. Wykazałem też, że poliploidia/wielkość komórek wpływa na rozmiar ciała kijanek, ale ich tempo wzrostu zależy w dużej mierze od temperatury. Triploidy rosły szybciej niż diploidy w 19 °C i miały większą masę ciała, ale w 24 °C nie było wyraźnej różnicy w tempie wzrostu pomiędzy ploidiami. Odrzuciłem więc hipotezę, że triploidalne kijanki, zbudowane z większych komórek, rosną wolniej i rozwijają się dłużej w danej temperaturze. Wydaje się, że większe komórki triploidów nie są czynnikiem ograniczającym ich wzrost w niskiej temperaturze.

Wyniki mojej pracy doktorskiej wyraźnie wskazują, że zróżnicowanie wielkości komórek, wywołane przez czynniki środowiskowe i cytogenetyczne, może odgrywać

istotną rolę w fizjologii zmiennocieplnych kręgowców. Po raz pierwszy wykazałem, że poliploidia/wielkość komórek może różny sposób wpływać na tempo metabolizmu całego organizmu w obrębie jednego gatunku w zależności od środowiska rozwoju – wodnego lub lądowego. Wykazałem, że niska temperatura w połączeniu z poliploidią skutkuje większymi rozmiarami komórek, jak również większymi rozmiarami ciała, co było pierwszą taką obserwacją u kręgowców. Największą masę ciała osiągnęły kijanki triploidalne w niskiej temperaturze, co może być potencjalnie korzystne na wyższych szerokościach geograficznych i może tłumaczyć częstsze występowanie osobników triploidalnych w północnych częściach geograficznego zasięgu *P. esculentus*.

# Abstract

Among multicellular organisms, substantial variation in cell size has been demonstrated, largely related to the size of the genome. It has been shown that small cells have higher mass-specific metabolic rates (MRs) than do larger cells. One hypothesis predicts that the metabolic rate of a whole organism should reflect the metabolic rates of the individual cells that ultimately constitute that organism. Via the link with metabolic rate, cell size should also affect the rate of mitotic divisions and therefore the organismal growth rate and development. However, the interspecific comparisons do not always reveal the links between cell size, whole-body MR and growth rate. Of all tested factors, temperature and genome size (especially polyploidy) produce the strongest effects on cell size in ectotherms. The increase in cell size induced by low temperature may explain the pattern of geographic variation of body size; in colder environments, the majority of ectotherms grow more slowly but mature at larger body sizes (the temperature-size rule, TSR). Regarding polyploids, there is some, albeit scarce, evidence that their larger genomes and cells may increase the whole body size and decrease mass-specific MR. The impact of temperature on the size of somatic cells in polyploid animals has presumably never been studied.

The objective of my thesis was to investigate the links between cell size, body size, growth rate and whole-body metabolic rate in amphibians. My research objects were diploid and triploid edible frogs, *Pelophylax esculentus*, which had previously been shown to differ in genome size and erythrocyte size. Studying the consequences of cell size variation within one species overcomes the problem of inter-specific comparisons associated with the different evolutionary histories of the compared species. In the first part of the thesis, I focused on the effect of polyploidy and temperature on the cell size and body size in tadpoles that were reared at 19 °C and 24 °C. I found that ploidy and temperature significantly affected cell size in tadpoles (erythrocytes and epidermal cells). The cells were larger in both diploid and triploid tadpoles at 19 °C than at 24 °C, and triploids had larger cells than diploids at both temperatures. The mean erythrocyte size in a pentaploid froglet, which was unexpectedly found in one of the crosses, was not proportionally larger than that in triploids, as might be expected on the basis of genome size. 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 (hepatocytes and erythrocytes). In the

second part of the thesis, I tested the hypothesis that individuals composed of smaller cells have a higher MR than individuals of a comparable body size but composed of larger cells. As I expected, diploid tadpoles had a higher standard metabolic rate (SMR) than triploids. Interestingly, in froglets, ploidy did not affect the SMR. Based on this result and an extensive review of the literature, I suggest that cell size may have more consequences for whole-body metabolic rates in aquatic than in terrestrial habitats because oxygen is less available in water, and its availability in relation to oxygen demand decreases with increasing temperatures. In water, polyploids composed of larger cells (with a less favorable surface-to-volume ratio) may be more vulnerable to insufficient oxygen supply and display lower MRs. I also found that water temperatures in which tadpoles were reared had no effect on the SMR of froglets after metamorphosis, consistent with no effect of these temperatures on cell size.

Tadpoles of both ploidies were larger and developed over a longer time period at the lower temperature, which was associated with larger cells. This result has not been observed in amphibians before and indicates that variation in cell size may be important in explaining the TSR. I was able to demonstrate that polyploidy/cell size affects body size in tadpoles but that their growth rate largely depends on temperature. At 19 °C, triploids grew faster than diploids and had larger body mass, whereas at 24 °C there was no clear difference between ploidies in growth rate. Thus, I rejected the hypothesis that triploid tadpoles, composed of larger cells, grow more slowly and develop over a longer time period at a given temperature. It seems that the larger cells of triploids are not a limiting factor for their growth at lower water temperatures.

My thesis clearly demonstrates that the variation in cell size induced by internal and external factors may play a significant role in the physiology of ectothermic vertebrates. I present the first report of two distinct effects of polyploidy/cell size on the whole-body metabolic rate within a single species developing in two different habitats, aquatic and terrestrial. I was also able to demonstrate, for the first time in vertebrates, that the combination of low temperature and polyploidy results in larger cells and body size. Considering the potential advantages of large body size at higher latitudes, the highest body mass of triploid tadpoles at lower temperature found in my study may explain the observation that triploid individuals of *P. esculentus* prevail in the in the northern parts of its geographic range.

# Introduction

The observed substantial variation in cell size among animal species, determined to a large degree by genome size, may have crucial consequences for physiological functions affecting various parameters of the organism, such as body size or the whole-animal metabolic rate (MR) (Gregory 2001; Dufresne and Jeffery 2011; Kozłowski et al. 2003). On the cellular level, cell size is important because small cells have higher mass-specific metabolic rates than do larger cells (Goniakowska 1970; Monnickendam and Balls 1973). One of the explanations for this phenomenon is that the relatively higher ratio of membrane surface to cell volume in smaller cells requires more energy for phospholipids turnover and to maintain the ionic gradient between the cytoplasm and the cell's surroundings (Rolfe and Brown 1997; Konarzewski and Książek 2013). The complementary approach points to the importance of cell membranes in exchanging important components between the cytoplasm and its surroundings, especially to the oxygen supply required to maintain the metabolism of a cell. Hence, the relatively larger area of exchange in smaller cells coupled with the shorter distance of diffusion could enhance their metabolic rate (Szarski 1983; Czarnoleski et al. 2013, 2015).

According to the cell metabolism hypothesis, the whole-body metabolic rate can be considered as the sum of the metabolic rates of the constituent cells. Thus, at the organismal level, individuals built of smaller cells should have a higher MR than individuals of a comparable body size but composed of larger cells (Davison 1955; Kozłowski et al. 2003). This prediction, however, was not always upheld when interspecific comparisons were performed within different vertebrate classes. Significant negative correlations between mass-specific MR and cell size were found in mammals, birds and one group of reptiles (Vinogradov 1995; Gregory, 2002; Starostová et al. 2009). In amphibians, only among salamanders was there a significant correlation between cell size and mass-specific MR (Gregory 2003). Via the link with MR, cell size is inversely correlated with the rate of mitotic division, which should affect the organismal growth rate. This effect has been found in amphibians, insects and crustaceans but not in mammals or birds (Angilletta et al. 2004; Gregory 2005).

Large variations in cell size are also observed within species and complexes of closely related species that include both diploid and polyploid forms. The latter are originated as a consequence of duplication of entire chromosome sets. It has been shown that the significant increase in the genome size in polyploids increases their cell

size (Gregory 2001; Choleva and Janko 2013). However, contrary to expectations, in the majority of studies, the differences in cell size between diploids and polyploids do not reveal a link between cell size and mass-corrected MR (Mable et al. 2011; Choleva and Janko 2013).

Of all the physical parameters that have been tested, temperature is the most dramatic in its effect on cell size in ectothermic animals. It is documented that for ectotherms (fish and invertebrates were mainly tested), low temperatures result in larger cells, which may increase their body size (van Voorhies 1996; Blanckenhorn and Llaurens 2005; Arendt 2007; Hessen et al. 2013). Larger cells, through their potential effect on the rate of mitotic division and therefore on growth rate, may explain the phenomenon that individuals raised at low temperatures generally grow more slowly but eventually become larger than their conspecifics raised at higher temperatures (the temperature-size rule, TSR) (Atkinson, 1994; Angilletta et al. 2004). TSR may in turn be associated with the observed pattern of ectotherm body size along climate gradients (larger individuals inhabit colder environments), even at the intra-specific level (Chown and Gaston 2010). Although possible mechanisms of the TSR in ectotherms are still under debate, physical constraints operating at the cellular and molecular levels are identified as proximate explanations (Van der Have and de Jong 1996; Woods 1999; Angilletta et al. 2004). Alternatively, ultimate hypotheses can predict conditions in which delay maturation and larger body size at lower temperatures increases fitness (Atkinson and Sibly, 1997, Kozłowski et al., 2004; Angilletta et al. 2004). It is also intriguing that polyploid organisms (which have larger cells than their diploid relatives) are often more abundant in harsh or extreme environments, especially at higher latitudes and altitudes, such as arctic and alpine habitats (Löve 1953; Stöck et al. 2001; Otto et al. 2007). However, larger cells in polyploids rarely produce larger body sizes. No apparent effect of polyploidy on body size has been found in vertebrates (Benfey 1999; Mable et al. 2011; Choleva and Janko 2013), but studies on the effect in this group (excluding fish) are very scarce. Moreover, to my knowledge, the effect of temperature on the size of somatic cells in polyploid animals has not yet been studied (only two papers reported the impact of temperature on egg size in polyploid females; Dufresne and Hebert 1998; Licht and Bogart 1989).

In summary, the studies presented above indicate that variation in cell size (induced by both internal and external drivers) may play an important role in an

evolutionary context, as a potential determinant of MR, growth rate and body size, although the various papers provide a mixed picture. Additionally, the consequences of cell size variation in polyploids have received very little attention (Choleva and Janko 2013). For this reason, **I studied the relationships between cell size, growth rate, body mass and MR in diploid and triploid individuals of a hybridogenetic species**, the edible frog, *Pelophylax esculentus*. Studying the consequences of variation in cell size within one species overcomes the problems of interspecific comparisons, which involve unpredictable variables that may arise from unknown evolutionary history of the different species that are analyzed.

*P. esculentus* is a natural bisexual hybrid between the pool frog, *P. lessonae* (genotype LL), and the marsh frog, *P. ridibundus* (RR). In most of its European range, it occurs in a diploid form (LR) and reproduces by hybridogenesis with one of the parental species (Graf and Polls Pelaz 1989). In some populations, diploid hybrids coexist with allotriploid hybrids (LLR and/or LRR), especially in central and northern parts of the species complex range (Plötner, 2005). As objects of my research, I chose diploid (LR) and triploid (LLR) individuals that differed from each other in genome size and erythrocyte size (Ogielska et al. 2004; Kierzkowski et al. 2011).

In **Chapter I** of my dissertation, I focused on the effect of polyploidy/genome size on cell size. Previous studies revealed that triploid edible frogs have larger erythrocytes than diploids, serving as a useful preliminary method for ploidy discrimination (Günther, 1977; Polls Pelaz and Graf, 1988). It was not known, however, whether cells of other tissues conform to this pattern. To answer this question, I also measured hepatocytes and epidermal cells. I hypothesized that triploids, with their larger genomes, would have larger cells than diploids. A single case of pentaploidy that is described in **Chapter II** enabled me to examine the effect of an additional increase in the genome size on cell size and metabolic rate. In **Chapter I**, I also examined the effect of temperature on cell size and growth rate in diploid and triploid tadpoles that were reared at two different temperatures. I tested the hypothesis that low temperature would increase the size of cells and the whole body size in diploid and triploid individuals and that both ploidies would develop more slowly at the lower temperature (see the TSR above). Due to the lower MR, larger cells should have a lower rate of mitotic divisions, which in turn should result in a slower growth rate in triploids at a given temperature. Since low temperature and polyploidy may affect cell size in the

same direction, I expected that triploid tadpoles reared at a lower temperature would reach the largest body size among the animals studied. This should be associated with their prolonged development. In **Chapter I**, I showed that erythrocytes were not the only cells larger in triploids; therefore, in **Chapter III**, I tested the hypothesis that triploid tadpoles and froglets composed of larger cells will have lower standard metabolic rates (SMRs) than diploid relatives composed of smaller cells. Tadpoles in my study were kept at two water temperatures until their metamorphosis, but after reaching this stage, all individuals were transferred to terraria at same air temperature. This allowed me to determine whether thermal differences during larval development affect the froglets' cell size and SMR after metamorphosis (**Chapter I and III**). It must be noted that I compared hybrids with different genome affiliation (LR and LLR) in my analysis. Therefore, the effect of cell size may potentially be affected by the genome dosage in LLR individuals (the relative excess of the L genotype). To test whether the genome dosage affects the morphology, I compared linear measurements of hind legs in LLR froglets with those in LL and RR froglets (**Chapter III**).

# Chapter I

## Low temperature and polyploidy result in larger cell and body size in an ectothermic vertebrate

Illustration by J.J. Grandville, from Jean de La Fontaine *Bajki*, PIW, 1986

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**My contribution:** contribution to the concept of the study and to developing the experimental design, collecting water frogs in the field, participation in crossing frogs, maintenance of amphibians, morphometric measurements, karyotyping, measurements of cell sizes, data analysis, manuscript preparation.


The pool frog, *Pelophylax lessonae*, on the cover was photographed by Adam Hermaniuk in Biebrza National Park.

# 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

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## 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*.

## 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];

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

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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 ($\mu m^2$) 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

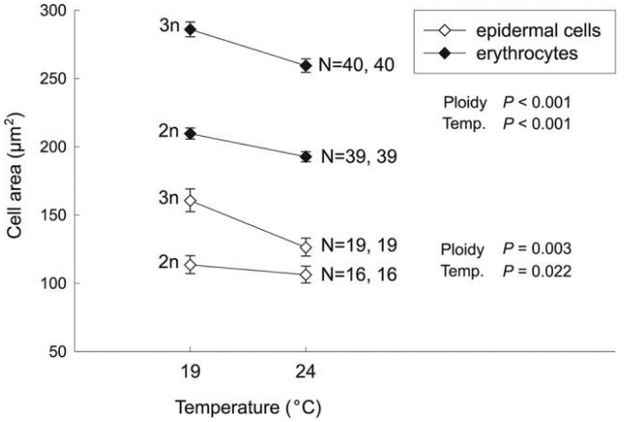


Figure 1. Area of erythrocytes and cross-sectional area of epidermal cells in diploid (2n) and triploid (3n) tadpoles of *Pelophylax esculentus* in relation to water temperature. The points are adjusted means (±SE) from two ANOVAs with ploidy and temperature as the main factors (see "Statistical Analyses");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

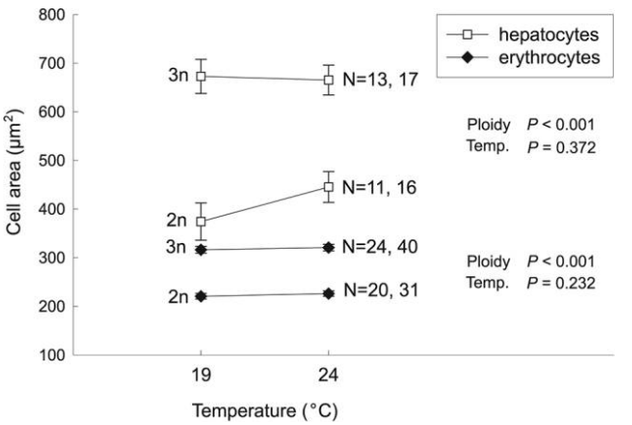


Figure 2. Area of erythrocytes and cross-sectional area of hepatocytes in diploid and triploid froglets *Pelophylax esculentus* in relation to the temperature of the water in which they developed as larvae. The points are adjusted means (±SE) from two ANOVAs with ploidy and temperature as the main factors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

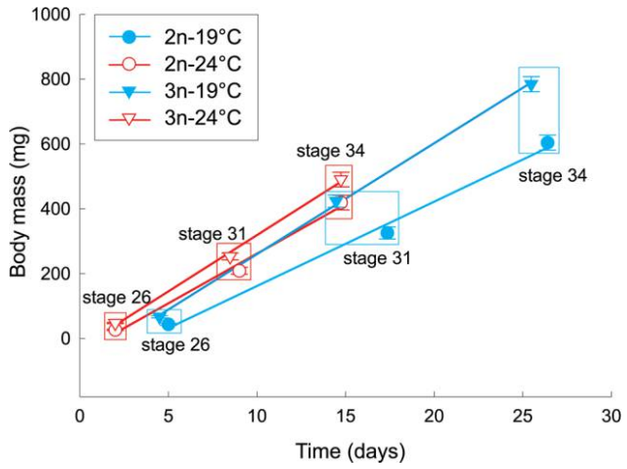


Figure 3. Growth rate of diploid (2n) and triploid (3n) *Pelophylax esculentus* tadpoles in early phase of development at two water temperatures. Points are mean body masses of tadpoles at Gosner's stages 26, 31, and 34 (adjusted means ± SE from ANOVA; see table 1); body masses are shown against time 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.

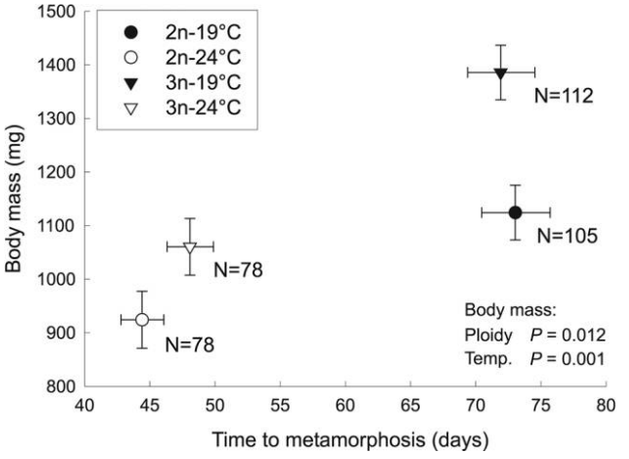


Figure 4. Body mass at metamorphosis (Gosner stage 42) versus time to metamorphosis in diploid and triploid tadpoles of *Pelophylax esculentus*. Adjusted means (±SE) from ANOVA. $N$ = sample size.

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).

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## 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.

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# Chapter II

## Genetic and cytogenetic characteristics of pentaploidy in water frogs

Illustration by J.J. Grandville, from Jean de La Fontaine *Bajki*, PIW, 1986

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# GENETIC AND CYTOGENETIC CHARACTERISTICS OF PENTAPLOIDY IN WATER FROGS

ADAM HERMANIUK[1,4], NICOLAS B. M. PRUVOST[2], PIOTR KIERZKOWSKI[3], AND MARIA OGIELSKA[3]

[1]*Institute of Biology, University of Białystok, ul. Świerkowa 20B, 15–950 Białystok, Poland*
[2]*Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*
[3]*Laboratory of Evolutionary and Developmental Biology of Vertebrates, Wrocław University, Zoological Institute, ul. Sienkiewicza 21, 50-335 Wrocław, Poland*

ABSTRACT: We describe a pentaploid froglet (LLLRR; three *Pelophylax lessonae* and two *Pelophylax ridibundus* genomes) that has never been reported before within the Water Frog (*Pelophylax esculentus*) hybrid complexes. The pentaploid specimen was found among almost all triploid siblings obtained from a diploid female *P. esculentus* (LR) crossed with a diploid male *P. lessonae* (LL). We confirmed ploidy levels of the parents and the offspring by karyotyping, microsatellite analysis (18 loci), and measurements of DNA content and erythrocyte size. Microsatellite analysis indicated that the pentaploid originated from a tetraploid ovum (LLRR) fertilized by a haploid sperm (L). Surprisingly, the erythrocytes of the pentaploid were not proportionally larger than in triploids, despite a higher DNA content. Only 6.7% of the erythrocytes were distinctly large, whereas the others varied strongly in shape and size; besides typical ovoid mature erythrocytes there were small, tear-shaped, or enucleated ones. We discuss the possibility of loss of some cytoplasm by large erythrocytes as a result of mechanical damages during circulation through the narrow vessels; when the erythrocytes achieve a relatively higher surface-to-volume ratio, they may function more effectively in a proper gas exchange.

*Key words*: Cell size; Enucleated erythrocytes; Genome size; Hybridogenesis; Microsatellites; *Pelophylax esculentus*; Polyploidy

THE WATER FROG, *Pelophylax esculentus* (genus *Rana* until Frost et al., 2006), is a natural bisexual hybridogenetic hybrid between the Pool Frog, *P. lessonae* (genotype LL), and the Marsh Frog, *P. ridibundus* (RR; for review, see Graf and Polls Pelaz, 1989). Both species and the hybrid can be freely crossed artificially, giving progeny of various viability (Berger, 1988; Berger et al., 1994). In most of its European range, *P. esculentus* appears as a diploid form (LR) and lives in sympatry with one of the parental species. In some populations, diploid hybrids coexist with allotriploid hybrids (LLR and/or LRR), especially in central and northern parts of the species complex range (Mikulíček and Kotlík, 2001; Rybacki and Berger, 2001; Borkin et al., 2004; Plötner, 2005). Moreover, in the northern part of the range (Sweden, Denmark, northern Germany, northern Poland), diploids and triploids often form all-hybrid populations (Christiansen and Reyer, 2009; Arioli et al., 2010). Spontaneous cases of tetraploidy were reported in some populations, but it is not known if they are of any importance for the genetic dynamic of these populations (Borkin et al., 2004; Christiansen, 2009). This study is the first report describing a single case of pentaploidy in hybridogenetic Water Frogs that arose among almost all-triploid offspring obtained from a *P. esculentus* female crossed with a *P. lessonae* male.

It is well documented that DNA content is strongly and positively correlated with erythrocyte size in different groups of vertebrates (Gregory, 2001; Sessions, 2008). The clearest demonstration of positive correlation between genome size and cell size are polyploids that result from the duplication of entire chromosome sets (Gregory, 2003; Mable et al., 2011). Cell size, however, is not always directly proportional to genome size, as is observed in animals with higher ploidy levels (Mable et al., 2011). Erythrocytes in polyploid amphibians are proportionally bigger than in diploids but are often less than a theoretical factor (for example, less than two in tetraploids), as might be expected with the duplication of entire chromosome sets (Deparis et al., 1975; Matson, 1990; Mable et al., 2011). Further-

[4] CORRESPONDENCE: e-mail, adamher@uwb.edu.pl

more, in some research authors have reported that the proportion of abnormal erythrocyte shapes (dumbbell shaped, U shaped, tear shaped, and round) increased with increasing ploidy levels (Liu et al., 2003; Lu et al., 2009). An unusually high percentage of atypical enucleated erythrocytes (> 80%)—probably caused by an extremely large genome size—were described in plethodontid salamanders of the genus *Batrachoseps* (Villolobos et al., 1988; Mueller et al., 2008). The examples mentioned above may demonstrate a relationship between large genomes and the occurrence of abnormal erythrocytes that have originated independently in two different groups of animals (in polyploids and in plethodontid salamanders). Abnormal erythrocytes may result from a random breakage of large cells during circulation, causing the loss of some cytoplasm or even nuclei (Villolobos et al., 1988). Moreover, smaller, more variable in shape, or enucleated erythrocytes, besides having reduced problems with circulation, have a more favorable surface-to-volume ratio that could be advantageous in a proper gas exchange.

In this study, we compared erythrocyte sizes of the pentaploid and its triploid siblings raised under the same laboratory conditions. Such an approach reduced the possible influence of environmental factors, which can mask a potential ploidy effect. Measurement of erythrocyte size is also the easiest and reliable method for distinguishing diploids from triploids in *P. esculentus* complex (Günther, 1977; Berger et al., 1978; Polls Pelaz and Graf, 1988). The results obtained by Kierzkowski (2004) in a large sample of Water Frogs with known genotypes (110 diploids and 64 triploids) demonstrated that discrimination between diploid and triploids was possible for 99.4% individuals. Furthermore, the pentaploid froglet (as revealed by karyotyping) provided an opportunity to test the efficiency of microsatellite analysis in identifying higher ploidy levels of Water Frogs. Molecular analysis also allows us to determine unambiguously the participation of each of the parents in terms of ploidy and genomic composition of their gametes.

The objective of our research was to characterize the pentaploid froglet in comparison to its triploid siblings, which enabled us (1) to examine the effect of a major increase in DNA content on erythrocyte size, and (2) to examine the efficiency of microsatellite analysis and measurements of erythrocyte size in correct identification of pentaploidy in Water Frogs.

## Materials and Methods

### *Animals and Crossing Experiments*

In a larger project, aimed at comparing growth and metabolic rates in diploid (2N) and triploid (3N) frogs, we crossed 16 pairs of male *P. lessonae* and diploid female *P. esculentus* to obtain a high frequency of 3N offspring (Czarniewska et al., 2011). In one of the crosses one pentaploid (5N) froglet occurred. The father derived from a mixed *lessonae–esculentus* population (Rogaczewo Wielkie, Poznań district, Poland, 52°02′23″N, 16°50′21″E; datum = WGS84) and the diploid mother derived from an all-hybrid *esculentus–esculentus* population (Wysoka Kamieńska, Szczecin district, Poland, 53°49′18″N, 14°50′37″E). We determined ploidy and genome compositions of the parents on the basis of morphological indices and erythrocyte size, and confirmed them later by microsatellite analysis. To obtain offspring, we used artificial fertilization, stimulating the female with the luteinizing salmon hormone (LHRH, Bachem Bioscience Inc.; Berger et al., 1994). We obtained sperm suspension directly from testes, dissected from the anesthetized male (MS 222).

To obtain the highest percentage of triploid offspring, we took only eggs classified as large (98.7% of the whole clutch) for further rearing. When tadpoles began to feed (Stage 25; Gosner, 1960) we selected 40 healthy-looking individuals for further rearing. Among the 21 individuals that completed metamorphosis, only 7 (lab numbers 13/09, 15/09, 34/09, 35/09, 45/09, 51/09, 52/09) survived the next 3 mo, when we sacrificed them for analyses. We kept tadpoles and juvenile frogs under laboratory conditions with controlled temperatures (24 and 23°C, respectively) and natural photoperiods. All animals were fed ad libitum.

### *Karyotyping*

We carried out karyotyping for all seven survivors. A day before tissue preparation we injected each animal peritoneally with 0.5 mL of 0.3% colchicine (Sigma). To obtain metaphase plates, we squashed inner-intestine epithelial tissue fragments under a cover slip in a drop of 70% acetic acid. We stained the chromosomes with the use of the AMD/DAPI method (fluorescence double-staining technique), which enables discrimination between R and L chromosomes because of the fluorescence of AT-rich pericentromeric heterochromatin regions in R chromosomes (Heppich et al., 1982). We used chromosomes of the 10th pair, which were easily distinguishable by their secondary constriction (NOR region), as a genome composition marker (Ogielska et al., 2004). Chromosome counting was done on 3–5 complete metaphase plates from each individual. We examined the slides with a Nikon (Eclipse E600) microscope equipped with a fluorescence lamp with appropriate filters. The microscope was connected to a digital camera.

### *Measurements of Erythrocyte Size and DNA Content*

We made blood smears from a cut fingertip on microscopic slides, air dried them for 1 h, and stored them in darkness at room temperature. We acquired images of the erythrocytes with the use of a microscope (Zeiss Jena) with a camera connected to a computer equipped with the Multiscan CSS computer program. Long and short axes of 30 randomly chosen undamaged ellipsoid erythrocytes were measured for each individual. We calculated the erythrocytes' area from the $ab\pi/4$ formula, where $a$ and $b$ denote long and short axes, respectively.

We measured DNA content in erythrocyte nuclei of the pentaploid, in one triploid sibling, and three adult *P. lessonae* (diploid genome) as a reference. We stained blood smears of all specimens in the same series according to Feulgen's method with Schiff reagent (Ogielska et al., 2004). Images of the manually selected erythrocyte nuclei were acquired with a standard video camera, optically coupled with an upright Zeiss Axioskop 20 microscope and processed by the computer image analysis system KS400 (Carl Zeiss Vision). We calculated the integrated optical density (IOD) for each analyzed nucleus according to the formula:

$$\mathrm{IOD} = S \times D,$$

where $S$ is the area of the nucleus and $D$ is optical density. Optical density is expressed as

$$D = \log I_0/I_1,$$

where $I_0$ represents mean background brightness, and $I_1$ is mean brightness of the nuclei.

For each smear, we measured 60 undamaged and properly stained nuclei and calculated mean IOD values.

### *Analysis of the Size of Erythrocytes*

We tested normality of distributions of erythrocyte area in each specimen with the Shapiro–Wilk test. Because some measurements showed nonnormal distributions (in the pentaploid and one triploid), we did not compare centers of distribution and tested the differences in dispersion of erythrocyte sizes between pentaploid and every single triploid with the use of Aly's permutation test (for details see Good, 2005). To combine significance levels ($P$ values) obtained from six pairs of comparisons, we used Jost's formula (Jost, 2008). It gave us the true probability that a set of $P$ values was produced by chance. We calculated these statistics using Rundom Pro 3.14 software (Jadwiszczak, 2009).

### *Microsatellites*

We analyzed nine individuals altogether (two parents and seven of their offspring). The starting material consisted of toes fixed in ethanol 80° (parents) and dry muscle with no fixation (offspring). We extracted DNA with the use of the Qiagen Biosprint™ 96 DNA Blood Kit following the supplier's protocol.

We used a set of 18 microsatellite primer pairs run in four primer mixes: Primer Mix 1A (PM1A): CA1b6, Ga1a19 redesigned (Arioli et al., 2010), RlCA1b5, RlCA5 (Garner et al., 2000), Rrid064A (Christiansen and Reyer, 2009); Primer Mix 1B (PM1B): Re2caga3 (Arioli et al., 2010), Res16, Res20 (Zeisset et al., 2000), RlCA2a34 (Christiansen and Reyer, 2009); Primer Mix 2A (PM2A): ReGA1a23, Rrid169A, Rrid059A redesigned (Christiansen

and Reyer, 2009), Res22 (Zeisset et al., 2000), Rrid013A (Hotz et al., 2001); and Primer Mix 2B (PM2B): Re1Caga10 (Arioli et al., 2010), RlCA18 (Garner et al., 2000), RlCA1a27, Rrid135A (Christiansen and Reyer, 2009). We ran polymerase chain reaction (PCR) products for fragment-length analysis on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ) and scored the alleles with the Genemapper software v3.7 (Applied Biosystems).

Loci Res20, RlCA1a27, and RlCA18 were species specific for *P. lessonae*, and Re2caga3, Res22, and Rrid169A were species specific for *P. ridibundus*. The other 12 loci amplified in both *lessonae* and *ridibundus* genomes (Christiansen, 2005, 2009; Arioli et al., 2010).

Seven of the primers showed a genome dosage effect that enables the detection of the presence of multiple copies of an allele in the genome by comparing the ratio of the heights of the peaks (allele 1:allele 2; Christiansen, 2005). We then corrected the ratio by the slope between the top of each peak obtained for a diploid specimen. In order to assess the ploidy level of the specimens and to determine their genomic composition, we plotted the values against expected values of allele ratios of 1:1 (expected ratio = 1 for diploids), 1:2 and 2:1 (0.5 and 2 for triploids), or 3:2 (1.5 for pentaploids). When the number of values allowed an estimate of the variance we ran a one-tailed one-sample *t*-test, with the use of the program R (version 2.15.1; R Development Core Team, 2012), to test if there was a significant difference between the value of the corrected ratio of the putative pentaploid and the mean of the diploid or triploid values. Following the microsatellite alleles from the parents to the offspring allowed us to determine the contribution of each parents unequivocally in term of genome composition and of ploidy of the gametes they produced.

## Results

### *Karyotyping*

Among seven analyzed offspring, one was pentaploid with 65 chromosomes, other froglets were triploids with 39 chromosomes. We determined the genome composition on the basis of fluorescent pericentromeric regions of the 10th pair of chromosomes. We classified all triploids as LLR and the pentaploid as LLLRR (Fig. 1).

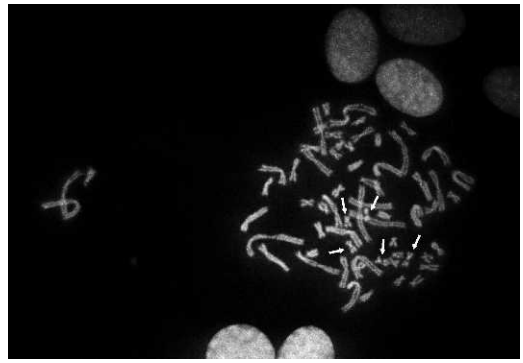

Fig. 1.—Metaphase plate of the pentaploid (5N = 65 chromosomes, genome LLLRR) *Pelophylax esculentus* specimen stained with the AMD/DAPI method. Diagnostic chromosomes No. 10, with characteristic secondary constriction, are indicated by arrows.

### *Erythrocyte Size and DNA Content*

Contrary to expectations, erythrocytes of the pentaploid individual were not proportionally larger than those in triploids; median values of area in the 5N individual were actually lower (Fig. 2). The distributions of erythrocyte area in the pentaploid deviated significantly from normality ($W = 0.92$, $P = 0.031$); distribution was positively skewed with long right "tails" representing the biggest cells.

We found a significantly higher dispersion of erythrocyte area in the 5N animal in comparison with every single triploid (at significance level $\alpha = 0.05$; Fig. 2). The probability that such a set of $P$ values was produced by chance was lower than 0.0001. Erythrocyte area in the pentaploid ranged between 115 and 558 $\mu m^2$, and between 252 and 392 $\mu m^2$ in triploids. The size of 30% of erythrocytes in the pentaploid were not uniform and formed two extremes: large erythrocytes (area $> 400$ $\mu m^2$), and small erythrocytes (area range: 115–165 $\mu m^2$) that represented 6.7% and 23.3% of all erythrocytes, respectively. The area of the remaining 70% of ellipsoid erythrocytes ranged between 188 and 392 $\mu m^2$.

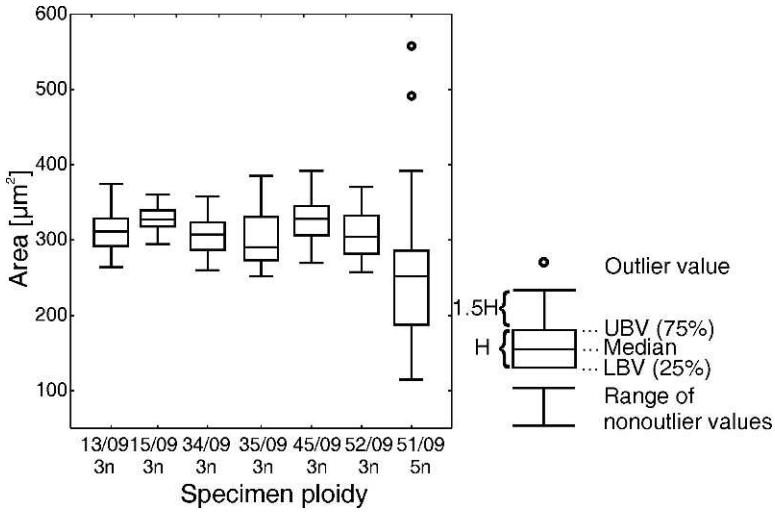


FIG. 2.—Statistics of erythrocyte size for triploid and pentaploid *Pelophylax esculentus* froglets. UBV = upper box value, 75th percentile; LBV = lower box value, 25th percentile; H = height of the box; nonoutlier values are those located between UBV + 1.5H and LBV + 1.5H; outlier values are greater than 1.5H.

The most striking feature of the pentaploid blood was the variety of unusual shapes of erythrocytes (Fig. 3). We observed tear-shaped, dumbbell-shaped, and U-shaped cells and enucleated cells.

Despite the variation of shapes, DNA content in all blood cells was at the pentaploid level. The IOD value for the diploid genome (2C), measured for three *P. lessonae*, averaged 4.40. Therefore a theoretical IOD value for the haploid genome (1C) would be 2.20. As expected, the measured IOD value for the triploid specimen (No. 52/09) was 6.86 (3C), and for the pentaploid specimen 10.94 (5C).

*Microsatellites*

After successful amplification, the 18 microsatellite loci gave complete multilocus genotypes for the two parents and seven offspring (Table 1).

Microsatellite analysis was consistent with the fact that among the seven offspring analyzed, six were triploid (LLR), and one specimen was a LLLRR pentaploid. We showed that the *P. lessonae* father was a typical haploid gamete donor, whereas the *P. esculentus* mother passed, in the case of the specimen 51/09, two copies of each of her L and R genomes (LLRR). We detected triploi-

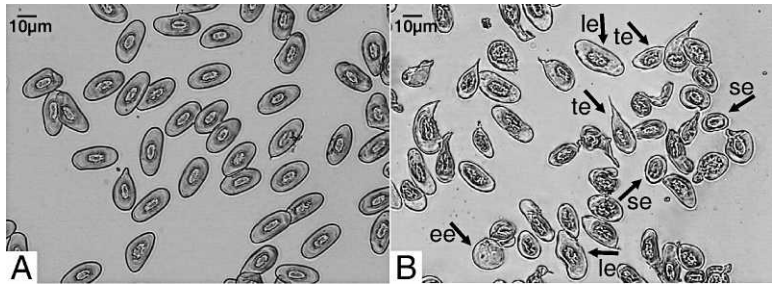


FIG. 3.—Erythrocytes of the triploid (A) and pentaploid (B) *Pelophylax esculentus* froglets. Note the different types of erythrocytes in the pentaploid animal: small (se), tear-shaped (te), enucleated (ee), and large (le).

TABLE 1.—Microsatellite multilocus genotypes of the two parents and of the seven offspring; Pl. indicates the ploidy; Gen. indicates the specific genotype composition; A1, A2, and A3 indicate first, second, and third alleles, respectively. *Pelophylax lessonae*–specific alleles are in italics and *P. ridibundus*–specific alleles are nonitalicized.

| | | | CA1b6 | | | Ga1a19 | | RlCA1b5 | | | RlCA5 | | | Rrid064A | | | RlCA2a34 | | | Res16 | | | Res20 | | | ReGA1a23 | | | Rrid059A | | | Rrid013A | | | Re1Caga10 | | | RlCA18 | | | Rrid135A | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | Pl. | Gen. | A1 | A2 | A3 | A1 | A2 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 |
| Male | 2N | LL | *78* | *82* | | *195* | | *118* | | | *256* | *260* | | *242* | | | *140* | *156* | | *121* | | | *120* | *126* | *123* | *133* | | | *278* | | | *296* | | | *97* | | | *179* | *186* | | | | |
| Female | 2N | LR | *78* | *92* | | *195* | *201* | *118* | *132* | | *232* | *256* | | *225* | *242* | | *106* | *145* | | *121* | *127* | | *124* | | | *98* | *125* | | | *280* | *315* | | *281* | *296* | | *97* | *135* | | *191* | | | *203* | *236* |
| 13/09 | 3N | LLR | *78* | *92* | | *195* | *201* | *118* | *132* | | *232* | *256* | | *225* | *242* | | *106* | *145* | *156* | *121* | *127* | | *124* | *126* | *98* | *123* | *125* | | *278* | *280* | *315* | *281* | *296* | | *97* | *135* | | *179* | *191* | | *203* | *236* |
| 15/09 | 3N | LLR | *78* | *82* | *92* | *195* | *201* | *118* | *132* | | *232* | *256* | *260* | *225* | *242* | | *106* | *140* | *145* | *121* | *127* | | *120* | *124* | *98* | *125* | *133* | | *278* | *280* | *315* | *281* | *296* | | *97* | *135* | | *186* | *191* | | *203* | *236* |
| 34/09 | 3N | LLR | *78* | *82* | *92* | *195* | *201* | *118* | *132* | | *232* | *256* | *260* | *225* | *242* | | *106* | *145* | *156* | *121* | *127* | | *120* | *124* | *98* | *125* | *133* | | *278* | *280* | *315* | *281* | *296* | | *97* | *135* | | *179* | *191* | | *203* | *236* |
| 35/09 | 3N | LLR | *78* | *92* | | *195* | *201* | *118* | *132* | | *232* | *256* | | *225* | *242* | | *106* | *145* | *156* | *121* | *127* | | *124* | *126* | *98* | *125* | *133* | | *278* | *280* | *315* | *281* | *296* | | *97* | *135* | | *186* | *191* | | *203* | *236* |
| 45/09 | 3N | LLR | *78* | *82* | *92* | *195* | *201* | *118* | *132* | | *232* | *256* | *260* | *225* | *242* | | *106* | *140* | *145* | *121* | *127* | | *124* | *126* | *98* | *123* | *125* | | *278* | *280* | *315* | *281* | *296* | | *97* | *135* | | *179* | *191* | | *203* | *236* |
| 52/09 | 3N | LLR | *78* | *82* | *92* | *195* | *201* | *118* | *132* | | *232* | *256* | *260* | *225* | *242* | | *106* | *140* | *145* | *121* | *127* | | *124* | *126* | *98* | *123* | *125* | | *278* | *280* | *315* | *281* | *296* | | *97* | *135* | | *179* | *191* | | *203* | *236* |
| 51/09 | 5N | LLLRR | *78* | *92* | | *195* | *201* | *118* | *132* | | *232* | *256* | | *225* | *242* | | *106* | *140* | *145* | *121* | *127* | | *120* | *124* | *98* | *125* | *133* | | *278* | *280* | *315* | *281* | *296* | | *97* | *135* | | *186* | *191* | | *203* | *236* |

Loci Re2Caga3, Rrid169A, and Res22 are monomorphic for the R alleles 212, 189, and 116, respectively, in all specimens except for the father. Locus RlCA1a27 is monomorphic for the L allele 111 in all specimens.

dy in the offspring directly, by the number of peaks for at least three loci displaying three peaks in the PCR product analysis. Pentaploidy could not be directly detected by the number of allele peaks because both parents were diploid and the alleles transmitted in more than one copy would give only one and the same peak in the fragment-length analysis (Fig. 4). Hence, the microsatellite peaks only showed that the *P. lessonae* father donated a haploid L sperm, and the *P. esculentus* mother produced some eggs carrying both the L and R genomes, but with an unknown number of copies. Only the ratio of the heights of peaks for the alleles with dosage effect can provide information on the number of copies. Seven of the analyzed loci (CA1b6, Ga1a19, Res16, Res20, RlCA2a34, ReGA1a23, and Rrid059) displayed the genome dosage effect. In each locus, such comparison of the ratio can only be made between the same pair of alleles (e.g., in Table 1, for alleles 120 and 124 in loci Res20, the comparison could only be made between specimen 51/09 and two other siblings).

We were able to compare the mother and its seven offspring in the case of loci Res16, Ga1a19, and CA1b6, using the peak height ratios of the alleles 121 (L specific) and 127 (R specific), 195 (L) and 201 (R), and 78 (L) and 92 (R), respectively. Here we only present details for the one locus, but the results of the other loci are fully congruent with the following conclusion. The corrected height ratio for alleles 121 and 127 in locus Res16, for six offspring specimens (13/09, 15/09, 34/09, 35/09, 45/09, 52/09), gave values clustering around 2, with a 99% confidence interval of 1.901 ± 0.086, whereas the offspring specimen 51/09 showed a significantly different value of 1.503 (one-sample *t*-test $t_5 = 18.541$, $P < 0.001$; Fig. 5). This result allows us to conclude that six individuals carried two copies of allele 121 for one copy of allele 127, whereas specimen 51/09 had three copies of allele 121 for two of alleles 127. This example, together with the congruent results of other loci, allowed us to determine unequivocally that the male always provided haploid L sperm that combined with diploid LR ova results in

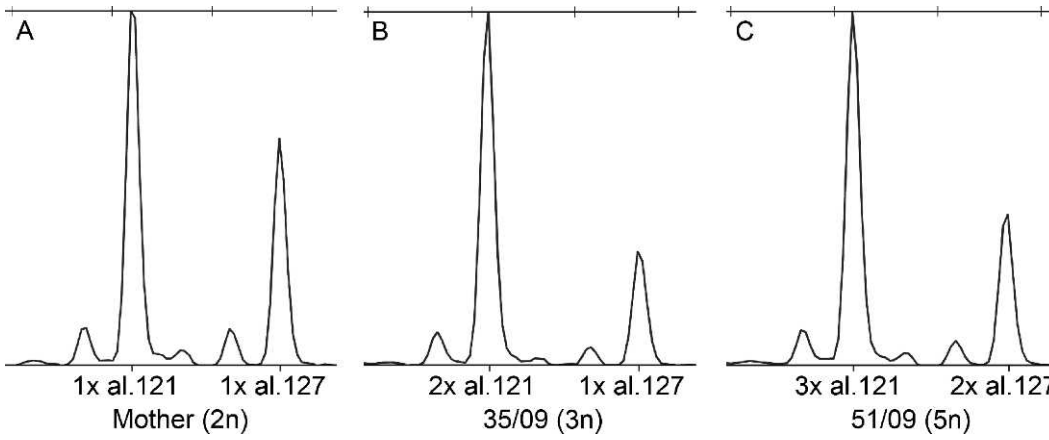


FIG. 4.—Amplification patterns of the alleles 121 (L-genome specific) and 127 (R-genome specific) of the microsatellite locus Res16 for (A) a diploid, (B) a triploid, and (C) a pentaploid specimen.

the triploid offspring, and combined with a tetraploid LLRR ovum results in the pentaploid specimen.

## DISCUSSION

*Pelophylax esculentus* is a bisexual hybrid that reproduces by hybridogenesis; in this process one of the parental genomes 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 semiclonal (Graf and Polls Pelaz, 1989). One of the well-documented phenomena observed in *P. esculentus* is the produc-

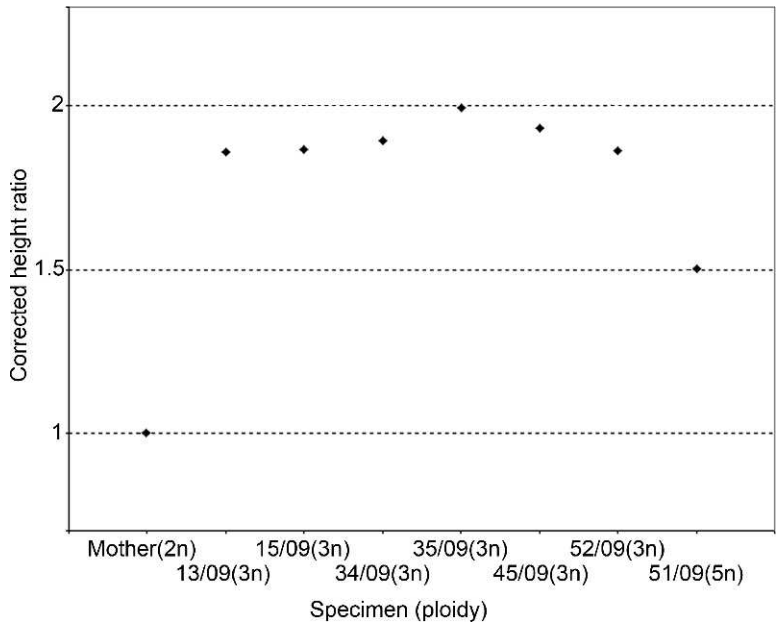


FIG. 5.—Peak height ratios of a female and the seven polyploid offspring for the microsatellite locus Res16. Plotted are ratios of the height of peak 121 on the height of peak 127, relative to the same ratio in the diploid female which was set to 1. Diamonds are the calculated values, dotted lines represent the expected values for 2N (= 1), 3N (= 2), and 5N (= 1.5).

tion of eggs of various sizes (small, medium, and large), ploidy, and genome composition by hybrid females (Berger, 1988; Czarniewska et al., 2011). As a rule, large eggs are diploid and give rise to triploid progeny, as was also confirmed in our study. Until now it was thought that diploid *P. esculentus* females from all-hybrid populations produce mostly R and LR eggs (Christiansen, 2009). We showed that one female also produced a tetraploid LLRR egg that gave rise to the pentaploid offspring. Such genome composition of a gamete may originate from suppression of both meiotic divisions and retention of all tetrads in a single nucleus, or suppression of the second division in an already tetraploid oocyte I, which might be a consequence of chromosomal reduplication (Fankhauser, 1945; Sessions, 1982; Otto and Whitton, 2000). The pentaploid individual developed properly as a tadpole and then as a froglet and displayed no abnormalities characteristic of Water Frog progeny (Berger, 1967; Ogielska, 2009). Unfortunately, we detected the unusual ploidy level only after the death of the animal and for this reason we did not raise the pentaploid further to see how long it could survive and which type of gamete it could produce.

Triploid Water Frogs have significantly larger (30–50%) erythrocytes than diploids, a characteristic that is often used for preliminary assessment of ploidy (Günther, 1977; Polls Pelaz and Graf, 1988). We expected that the pentaploid specimens would have significantly larger erythrocytes than their triploid siblings, but instead we found a high variability of their shapes and sizes and thereby, the mean value of area of the cells was lower than in triploids. Despite their different sizes and shapes, DNA content measurements indicated 5C values in all analyzed erythrocytes in the pentaploid frog, and we thereby excluded tissue mosaicism, as was the case of 1N/3N erythrocytes described in *P. esculentus* by Berger and Ogielska (1994). We also excluded the possibility that abnormal erythrocyte shapes observed in the pentaploid froglet were artifacts formed during the blood smear because within hundreds of smears of diploids and triploids only the pentaploid specimen had such abnormalities. More variable or generally smaller erythrocyte sizes than expected from theoretical calculations were also observed in pentaploid newts of the genus *Pleurodeles*, tetraploid *Hyla versicolor*, and tetraploid *Bufo viridis* (Deparis et al., 1975; Matson, 1990; Stöck and Grosse, 1997). Nevertheless, the mean size of pentaploid *Pleurodeles* newt cells were significantly bigger than triploid ones, and slightly bigger than tetraploid ones. Another observation was that 5N newts do not regulate hematocrit and hemoglobin values and are slightly anemic (Deparis et al., 1975). We conclude that the mean size of erythrocytes is not a good marker for determination of pentaploidy in Water Frogs, but high variability of the cell sizes and shapes could be a strong clue for the presence of ploidy level higher than 3N.

Enucleated erythrocytes, extremely unusual among nonmammalian vertebrates, have been reported in plethodontid salamanders of the genus *Batrachoseps*. All species of this genus have miniaturized or attenuated body form and large genome size in comparison with other vertebrates (Villolobos et al., 1988). Mueller et al. (2008) suggested that such cells may have evolved in response to a physical constraint against the circulation of large, fully nucleated cells through the circulatory system of a miniaturized/attenuated animal. The presence of similar unusual tear-shaped, very small, and enucleated erythrocytes in the peripheral blood of the pentaploid *P. esculentus* described herein suggests that mechanism of their formation is not necessarily adaptive and might be purely mechanical, as was proposed by Villolobos et al. (1988). We believe that during circulation through narrow vessels, large erythrocytes may become deformed, and after that may lose some cytoplasm in the process of amitotic division, as was proposed by Emmel (1924) and supported by Cohen (1982). Gibiński's (1947) observations of the origin of enucleated and tear-shaped cells from mature erythrocytes in Water Frogs under anoxemia conditions might confirm the above assumption.

Concerning the suitability of the different loci to detect pentaploidy, we cannot recommend any specific loci. Their ability to detect higher ploidy levels directly depends on the genotype of the parents, when analyzing some

offspring, and/or on the heterozygosity of the specimens, when analyzing population genetics data (Ramsden et al., 2006). Nevertheless we advise researchers who use microsatellites to define the genome composition of Water Frogs to check their data carefully from a genome dosage effect point of view. By calculating and plotting the different peak height ratios for all combinations of alleles (if possible normalized by dividing them by the same ratio for a diploid individual, and keeping in mind that those comparisons can only be made for the same pair of alleles), one could find clustering values corresponding to different genomic compositions. The large spread of such ratios with discrete groups is a very strong clue for the presence of different ploidy types. Authors of previous studies detected and separated LR diploids (with an L:R ratio of 1) from two types of triploids (LLR and LRR) easily, by showing that diploids cluster in a discrete group in between the two other clusters formed by LLR and LRR frogs (with ratios of 2 and 0.5, respectively; Christiansen 2005, 2009; Christiansen and Reyer 2009, 2010; Arioli et al., 2010). Here we show that pentaploid individuals can be detected in the same way, but that that their ratio of 1.5 (respectively 0.67) places them in between diploid and triploid categories, with the risk of blurring the clusters when looking at a large number of frogs.

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# Chapter III

## Metabolic rate of diploid and triploid edible frog, *Pelophylax esculentus,* correlates inversely with cell size in tadpoles but not in frogs

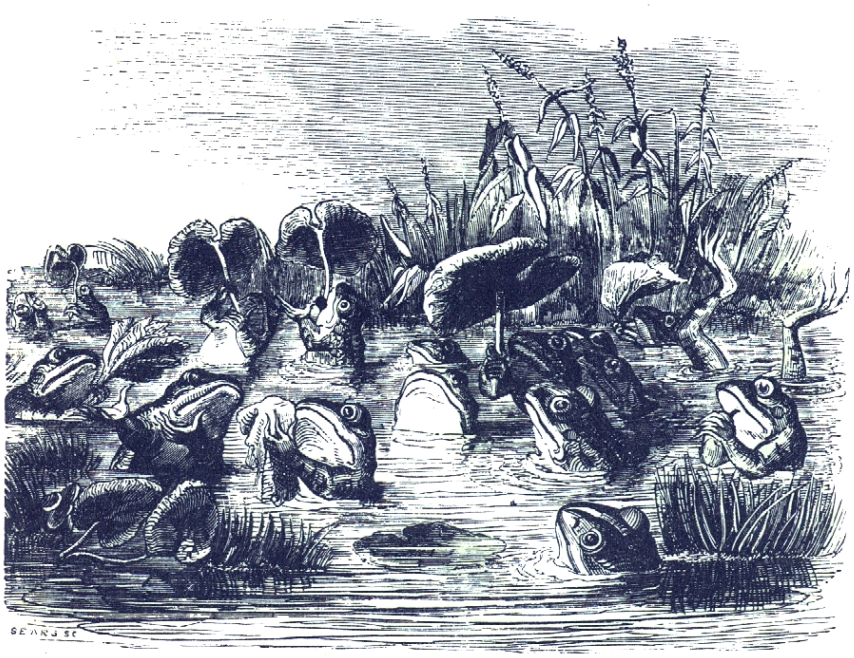

Illustration by J.J. Grandville, from Jean de La Fontaine *Bajki*, PIW, 1986

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**My contribution:** contribution to the concept of the study and to developing the experimental design, collecting water frogs in the field, participation in crossing frogs, maintenance of amphibians, morphometric measurements, karyotyping, metabolic measurements, data analysis, manuscript preparation.

# Metabolic Rate of Diploid and Triploid Edible Frog *Pelophylax esculentus* Correlates Inversely with Cell Size in Tadpoles but Not in Frogs

**Adam Hermaniuk**[1,*]
**Mariusz Rybacki**[2]
**Jan R. E. Taylor**[1]
[1]Institute of Biology, University of Białystok, ul. Ciołkowskiego 1J, 15-245 Białystok, Poland; [2]Department of Evolutionary Biology, Kazimierz Wielki University, Al. Ossolińskich 12, 85-067 Bydgoszcz, Poland

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## ABSTRACT

In multicellular organisms, cell size may have crucial consequences for basic parameters, such as body size and whole-body metabolic rate (MR). The hypothesis predicts that animals composed of smaller cells (a higher membrane surface–to–cell volume ratio) should have a higher mass-specific MR because a large part of their energy is used to maintain cell membranes and ionic gradients. In this article, we investigated the link between cell size and MR in diploid and triploid tadpoles and froglets of the hybridogenetic frog *Pelophylax esculentus*. In our previous study, we showed that triploids had significantly larger cells (erythrocytes, hepatocytes, and epidermal cells were measured). Therefore, we hypothesized that triploid tadpoles and froglets would have a lower standard metabolic rate (SMR). Our study demonstrated for the first time two distinct effects of polyploidy/cell size on MR within a single species developing in both aquatic and terrestrial habitats. As we hypothesized, diploid tadpoles had a higher SMR than triploids, whereas in froglets, ploidy did not affect the SMR. We also found that the water temperatures in which tadpoles were reared had no effect on the SMR of froglets after metamorphosis. Based on our results and other reports, we suggest that cell size may have more consequences for whole-body MR in aquatic habitats than in terrestrial habitats because oxygen is less available in water and its availability in relation to oxygen demand decreases with temperature.

*Keywords:* polyploidy, triploids, metabolic rates, oxygen limitation.

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

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## Introduction

Among animal species, there is large variation in the genome size that determines an equally large variation in cell size (Gregory 2001; Dufresne and Jeffery 2011). Cell size may have crucial consequences for physiological functions affecting various parameters of the organism, such as body size or the whole-animal metabolic rate (MR; Kozłowski et al. 2003). Consequently, the variation in cell size may also affect scaling exponents of interspecific allometries of MR because the increase in body size may occur by increasing either the cell size or the cell number or a combination of both (Davison 1955; Kozłowski et al. 2003; Starostová et al. 2009). The importance of cell size arises from the observation that small cells have higher mass-specific MR than do larger cells (Goniakowska 1970; Monnickendam and Balls 1973). The most common explanation for this phenomenon is that the relatively higher membrane surface–to–cell volume ratio in smaller cells requires them to use more ATP to turn over phospholipids and to use more energy to maintain the cell membrane ionic gradients (Rolfe and Brown 1997; Konarzewski and Książek 2013). On the other hand, cell membranes serve as points of exchange between the cytoplasm and its surroundings. This is particularly important in the context of the oxygen supply required to maintain the metabolism of the cell. Hence, the larger area of exchange in smaller cells combined with the shorter distance of diffusion could increase the speed of transport and enhance metabolic processes (Szarski 1983; Czarnoleski et al. 2013, 2015).

At the organismal level, individuals built of smaller cells (higher total membrane surface) should have a higher MR than individuals of a comparable body size but composed of larger cells. However, an interspecific comparison within different vertebrate classes does not always expose the link between cell size and MR. In birds, mammals, and reptiles, there is a significant negative correlation between mass-specific MR and cell size (Vinogradov 1995; Gregory 2002; Kozłowski et al. 2003; Starostová et al. 2009). In amphibians, the effect of cell size on MR is much more complex. Among salamanders, there was a significant correlation between cell area and MR (both corrected for body mass), but this result was mostly dependent on the inclusion of two genera with the largest cells. Within anurans, the relationship was not significant at any taxonomic level (Gregory 2003).

Large variations in cell size are also observed within species and complexes of closely related species that include both diploid and polyploid forms, and such variations are most likely associated with the positive correlation between genome size and cell size (Gregory 2001; Choleva and Janko 2013). Hence, di- and polyploid relatives within a species or a complex may be

an excellent model for investigating the link between cell size and MR at the organismal level; intraspecific comparisons are not affected by unpredictable variables, for example, an unknown phylogenetic history of different species that are analyzed. Although it is well documented that polyploids are composed of larger cells, numerous studies failed to find appreciable differences in the MR between di- and polyploid forms (see our review of the literature in "Discussion").

In this study, we measured the standard metabolic rate (SMR) in diploid and triploid tadpoles and in froglets of the edible frog *Pelophylax esculentus* (Linnaeus 1758). The edible frog 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, it occurs as a diploid form (LR) and reproduces by hybridogenesis with one of the parental species (Graf and Polls Pelaz 1989). Mostly in the northern populations, diploid individuals coexist with triploids (LLR and/or LRR) and often form all-hybrid populations (see Plötner 2005 for a review). Triploid *P. esculentus* tadpoles and frogs have significantly larger erythrocytes and cells in other tissues that may be, at least in part, a consequence of the larger genome (amphibian erythrocytes are nucleated; Ogielska et al. 2004; Kierzkowski et al. 2011; Hermaniuk et al. 2016; see "Discussion" for details).

For our measurements, we used LLR triploids and LR diploids to test the hypothesis that triploid tadpoles and frogs composed of larger cells will have a lower SMR than diploid relatives composed of smaller cells. We expected that the effect of cell size could be potentially strengthened in LLR triploids by the effect of the genome dosage (the relative excess of the L genotype) because *P. lessonae* (LL) has lower MR than *P. ridibundus* (RR; Plenet et al. 2000).

To test whether the gene dosage was present in the morphology of our LLR frogs, we took linear measurements of hind legs in frogs, calculated morphological indexes, and compared the indexes in LLR with those in LL and RR (from the literature; the indexes differ between LL and RR and are used for their taxonomical identification; Kierzkowski et al. 2011). We also recorded mass of energetically expensive internal organs—the liver, kidneys, intestine, and heart—in our frogs because their size may determine the SMR level (Monnickendam and Balls 1973; Garland 1984; Steyermark et al. 2005).

The measurements of SMR in frogs were performed in individuals that were used in another study that investigated the impact of water temperature on cell size and growth rate in diploid and triploid tadpoles (Hermaniuk et al. 2016). The tadpoles that gave rise to our frogs were kept at one of two temperatures: 19° and 24°C. This created the opportunity to determine whether differences in the thermal history affected the SMR of frogs after metamorphosis, the size of organs, and morphology. Because temperature of larval development did not affect the size of cells in diploid and triploid froglets (Hermaniuk et al. 2016), we hypothesized that there would be no effect of this temperature on the SMR in frogs. The results of our study are discussed making use of our review of published articles that compared the MR between di- and polyploid relatives in different groups of animals.

## Material and Methods

### *Study Animals and Maintenance*

By artificially crossing 19 *Pelophylax lessonae* males (LL) and 19 *Pelophylax esculentus* females (LR and LRR), we produced a total of 9 diploid (LR) and 10 triploid (LLR) crosses of *P. esculentus* offspring in 2009 and 2010. The population sources of parental frogs and crossing procedures were described in detail by Hermaniuk et al. (2016). After fertilization, the eggs and hatchlings were raised in plastic litter trays filled with aerated tap water at room temperature, with each cross kept separately until the tadpoles reached developmental stage 25 according to Gosner (1960; the stage of free swimming and independent feeding). Starting with stage 25, groups of randomly selected tadpoles from each cross were raised in plastic tanks (10 L) under a natural (outdoor) photoperiod and controlled temperature of 18°C. The water was aerated with air stones, and half of the water in each tank was renewed three times each week. Tadpoles were fed ad lib. two times a day. Metabolic measurements in diploid and triploid tadpoles were carried out at Gosner's developmental stages 27–31, when the tadpoles depended mostly on oxygen dissolved in water (Czopek 1965). These measurements were performed in 2010.

Froglets used for the metabolic measurements had developed as tadpoles at different temperatures after Gosner's stage 25. The maintenance protocol is described in detail in a previous report (Hermaniuk et al. 2016) on the effects of temperature on cell and body sizes in diploid and triploid *P. esculentus*. Briefly, when tadpoles reached stage 25, randomly selected individuals from each cross were assigned to two groups and raised at 19° and 24°C until metamorphosis. All froglets were kept at the same air temperature of 23°C and were measured 11–30 wk after metamorphosis. The genome compositions for tadpoles and froglets were determined according to the fluorescent pericentromeric regions of chromosomes, as described in Hermaniuk et al. (2013).

### *Metabolic Measurements*

*Tadpoles.* Tadpoles were starved for 2 d at 20°C before undergoing measurements. Three individuals were measured simultaneously during each respirometry trial. Tadpoles were placed in 285-mL glass metabolic chambers filled with normoxic dechlorinated tap water, submerged in a water bath fitted with a cooling/heating system, which allowed for temperature stabilization within 20° ± 0.1°C. The fourth identical metabolic chamber was left without a tadpole and was used as a reference to measure any bacterial respiration. The concentration of dissolved oxygen (accuracy ±0.1%) was recorded in each chamber with an oxygen electrode (Yellow Spring Instruments, Yellow Spring, OH) equipped with a temperature sensor (accuracy ±0.1°C). The electrode was connected to a CyberScan PCD 6500 meter (Eutech Instruments, Singapore). A small automatic stirrer, which was determined not to disturb the tadpoles, ensured sufficient water mixing. Data were recorded every 5 min with automatic temperature and pressure correction. The entire respi-

rometry trial lasted 9 h (between 0800 and 1700 hours). The SMR was calculated according to the following formula:

$$\mathrm{SMR} = aV,$$

where $a$ is the slope of the linear regression of dissolved oxygen concentration with time (mg $O_2$/h) and $V$ is the volume of the metabolic chamber (L). We considered the SMR measurement as successful when the oxygen concentration decreased strictly linearly during a 3-h time period, as indicated by the nonsignificance (at $P = 0.05$) of the quadratic term of the second-order polynomial fitted to the changes in oxygen concentration with time. The visual inspection of tadpoles showed that linear decrease of oxygen concentration coincided with periods of their inactivity. The tadpoles were acclimated in the metabolic chamber for the first 2 h, which was discarded from the data analysis. To exclude bacterial respiration, the oxygen consumption of each tadpole was adjusted for the oxygen consumption in the reference metabolic chamber. After each experimental run, the chambers were sterilized using 70% EtOH to prevent accumulation of bacteria. After the measurements, the tadpoles were placed on blotting paper to remove water that covered the body, and they were weighed to within 0.1 mg (with the WPA 71 scale, Radwag, Poland), and their body length was measured ($\pm 0.1$ mm). In total, 20 individuals were successfully measured; they originated from five diploid and five triploid crosses, two individuals from each cross.

*Froglets.* For measurements of SMR in froglets, we used a push-mode stop-flow respirometry system (Lighton 2008) with a two-channel oxygen analyzer. Because food digestion and assimilation increase the MR in anurans (Secor et al. 2007), the froglets were starved for 5 d before measurements. Four froglets were measured simultaneously during each respirometry trial. They were placed individually in Plexiglas metabolic chambers with volumes ranging from 180 to 240 mL, depending on the animal's body size, that were submerged in the temperature-controlled water bath (23° ± 0.1°C). The fifth metabolic chamber was left empty as the control. The froglets in the chambers could not see each other during the trials, and the chambers were inspected periodically to ensure that the froglets were resting. To minimize the evaporative water loss from the animals, 7–10 mL of water was added to the bottom of a chamber. Flow of incurrent outdoor air, from which water was removed by passing it through a Drierite column, was maintained by a pump and controlled by a precalibrated mass-flow meter ($\beta$-ERG 1000N2 Warsaw, Poland) at 600 mL $min^{-1}$. The air was directed to the preprogrammed multiplexer (RM-8, Sable Systems, Las Vegas, NV), which was used to automatically switch the air flow between metabolic chambers. The air flow was passed through only one chamber at a time, while the remaining chambers remained sealed. The chambers with froglets were flushed for 8 min in a sequence and separated by an 8-min interval for flushing the empty control chamber. The whole cycle lasted 64 min, and each chamber with a froglet was sealed for 56 min. The cycle was repeated 40 times; therefore, the entire respirometry trial lasted 42 h. Excurrent air from the chamber was subsampled at a rate of 100 mL $min^{-1}$, redried (Drierite), scrubbed of $CO_2$ (Carbosorb AS, BDH Laboratory Supplies, Poole, England), and finally directed to the sensor of the two-channel oxygen analyzer (S-3A/II N 37 M, Ametek, Pittsburgh, PA). The differential output of the $O_2$ analyzer (outside dry air scrubbed of $CO_2$ vs. the excurrent air) was recorded by a computer. The oxygen consumption of each froglet was calculated from the integral of the sample rate of $O_2$ consumption divided by the chamber enclosure time, using equation (4) of Hill (1972). A 1-h period of the lowest oxygen consumption was used for the calculation (using the average of the two lowest 1-h records did not change the results).

Froglets were weighed to the nearest 0.01 g before and after the respirometry trial. On average, they lost 0.9% ± 0.5% (mean ± SE) of their initial body mass during the metabolic measurement, which indicated no dehydration at the end of the experiment. A total of 119 successful measurements was obtained. The froglets were derived from 9 crosses in 2009 (68 individuals) and 10 crosses in 2010 (51 individuals). SMR was also measured in one pentaploid specimen that was described in detail elsewhere (Hermaniuk et al. 2013).

After completing the metabolic trials, the lengths of the tibia (T), first toe (*digitus primus* [DP]), and metatarsal tubercle (*callus internus* [CI]) were measured in each froglet with an electronic caliper (accuracy 0.01 mm). Thereafter, the froglets, held by the hind legs, were stunned by a strong blow to the top of the head and immediately killed by decapitation. Liver, kidneys, intestine, and heart (ventricle without atriums) were dissected from the froglets (in 2010) and weighed to within 0.1 mg (WPA 71 scale). The same animals were used for measurements of the size of erythrocytes and hepatocytes, reported in Hermaniuk et al. (2016). All 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).

### *Statistics*

The SMR of tadpoles was analyzed using a nested ANCOVA model with ploidy (2n, 3n) as a fixed factor, cross nested in ploidy as a random factor, and body mass as a covariate. For the analysis of froglet morphological measurements (DP and T), mass of internal organs (heart, intestine, kidneys, and liver), and MR in froglets, the nested ANCOVA model was used that included ploidy, the temperature in which froglets were reared as tadpoles (19°, 24°C), and their interaction as fixed factors. The cross nested in ploidy and the interaction between temperature and cross nested in ploidy were random factors. Depending on the variable analyzed, different covariates were used in the above model. Measurements of DP and T were adjusted for the variation in CI that was used as a covariate. To adjust the mass of an internal organ for the variation in body mass, the difference between the body mass and the mass of the organ was added to the model as a covariate. The difference was used to avoid autocorrelation between these two masses. Total body mass was used as a covariate in the analysis of froglet SMR.

To test the relationship between froglet SMR and mass of internal organs, mass of organ was used instead of body mass (it is very difficult to separate the impact of organ mass from body mass, and we resigned from controlling for the latter using residuals to avoid statistical and logical problems caused by such an analysis; Freckleton 2002). An ANCOVA was used to analyze DP and T, which were corrected for CI, instead of analyzing DP/CI and T/CI, because using ratios can introduce bias into statistical analyses (Packard and Boardman 1999). Nevertheless, we also reported the values of these indexes, as they are commonly used in the literature (Berger 2008; Kierzkowski et al. 2011).

The assumption of homogeneity of the slopes was always checked before performing an ANCOVA with a common slope. In order to meet the parametric assumptions, the SMR of froglets and tadpoles, as well as their body mass in the analyses of SMR, was transformed logarithmically. Also DP, T, and CI were log transformed. All statistical analyses were performed in SAS, version 9.3 (SAS Institute, Cary, NC), using the MIXED procedure with restricted maximum likelihood method.

## Results

### *Metabolic Rates in Tadpoles*

Diploid and triploid tadpoles with a mean Gosner development stage of 30 did not differ in body size or body mass (table 1). However, their MR (mL $O_2$ $h^{-1}$) was significantly different (ANCOVA; ploidy: $F_{1,8} = 6.30$, $P = 0.036$; body mass as a covariate: $F_{1,9} = 18.02$, $P = 0.0022$), with the SMR in diploid tadpoles 26.6% higher than that in triploid tadpoles (table 1; fig. 1).

### *Morphological Indexes of DP/CI and T/CI in Froglets*

DP and T values corrected for CI were significantly higher in diploid froglets than in triploid froglets, and the effect of the water temperature in which the froglets developed as tadpoles was also significant—both values were higher in froglets derived from tadpoles reared at a higher water temperature. An ANCOVA of DP revealed a significant effect of ploidy ($F_{1,14} = 7.35$, $P = 0.0160$), temperature ($F_{1,14} = 10.87$, $P = 0.0053$), and CI as a covariate ($F_{1,82} = 83.91$, $P < 0.0001$), with a nonsignificant interaction between ploidy and temperature ($F_{1,14} = 0.01$, $P = 0.940$). Similar results were revealed by the ANCOVA of T for ploidy, temperature, CI, and the ploidy × temperature interaction ($F_{1,14} = 21.86$, $P = 0.0004$; $F_{1,14} = 11.29$, $P = 0.0047$; $F_{1,82} = 225.9$, $P < 0.0001$; and $F_{1,14} = 0.03$, $P < 0.862$, respectively). One individual with an abnormally high DP and T versus CI was excluded from these analyses. Therefore, the sample size was 118 froglets.

The values of the DP/CI and T/CI indexes reflected the above effects (table 2). Both indexes were higher in diploid than in triploid froglets, and within each ploidy they were higher in froglets derived from tadpoles reared at 24°C.

### *Mass of Internal Organs in Froglets*

Diploid and triploid froglets differed significantly in their heart and kidney masses, as revealed by an ANCOVA that included, besides ploidy, the temperature at which froglets were reared as tadpoles and their body mass (see "Statistics"). The kidneys and hearts in triploid froglets, when corrected for body mass, were larger than those in diploid individuals by 24.7% and 13.3%, respectively (table 3). Both organs made up a small fraction of the whole-body mass in triploid and diploid froglets: heart, 0.21% and 0.20%, and kidneys, 0.75% and 0.62%, respectively.

The body mass–corrected liver mass, although not dependent on ploidy, was 60.2% larger in froglets that had been reared as tadpoles at 24°C compared with those reared at 19°C (table 3). The liver mass equaled 7.98% and 3.80% of total body mass at 24° and 19°C, respectively. There was no significant relationship between the mass of the intestines and ploidy or temperature (table 3).

### *Metabolic Rates in Froglets*

There was no difference in the SMR (mL $O_2$ $h^{-1}$) between diploid and triploid froglets ($F_{1,15} = 1.40$, $P = 0.255$), as revealed by the ANCOVA model (fig. 2). The impact of the temperature in which froglets had been developing as tadpoles and the interaction between temperature and ploidy were also nonsignificant ($F_{1,15} = 0.00$, $P = 0.958$ and $F_{1,15} = 0.39$, $P = 0.543$, respectively). However, covariate body mass was highly significant ($F_{1,82} = 234.1$, $P < 0.0001$). The slope of the regression of the logarithm of SMR on the logarithm of body mass, calculated from the ANCOVA model, equaled 0.863 (0.751–0.976; 95% confidence interval). The effect of ploidy remained nonsignificant ($F_{1,17} = 1.31$, $P = 0.268$) when temperature was removed from the model.

Because diploid and triploid froglets differed in the mass of heart and kidneys (table 3), we also tested the main ANCOVA

Table 1: Body size, mass, and metabolic rates in diploid and triploid *Pelophylax esculentus* tadpoles with mean Gosner stage 30 development score

| Ploidy | *N* | Body length (mm) | Body mass (g) | Metabolic rate ($\mu$g $O_2$ $h^{-1}$) |
|---|---|---|---|---|
| Diploids | 10 | 28.1 (26.6–29.6) | .188 (.157–.219) | 33.3 (28.6–38.7) |
| Triploids | 10 | 28.3 (26.8–29.7) | .199 (.168–.230) | 26.3 (22.6–30.6) |
| Difference | | $F_{1,8} = .03$, $P = .872$ | $F_{1,8} = .36$, $P = .563$ | $F_{1,8} = 6.30$, $\mathbf{P = .0364}$ |

Note. The metabolic rates are the adjusted means from the ANCOVA with body mass as a covariate. The 95% confidence intervals are shown in parentheses. The *P* value for the statistically significant effect is shown in bold. *N* = number of tadpoles.

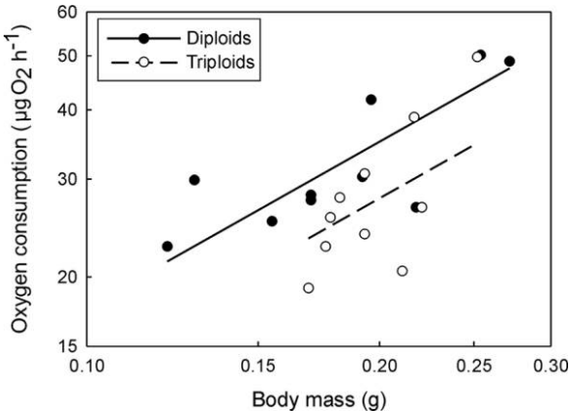


Figure 1. Relationship between oxygen consumption, measured at water temperature of 20°C, and body mass in diploid and triploid tadpoles *Pelophylax esculentus*.

model for SMR in which body mass was replaced with the total mass of these organs. The effect of ploidy was still nonsignificant ($F_{1,3} = 2.08$, $P = 0.245$), as was the temperature ($F_{1,3} = 4.66$, $P = 0.120$) and the ploidy × temperature interaction ($F_{1,3} = 0.05$, $P = 0.839$) but not the combined mass of the heart and kidneys ($F_{1,18} = 66.7$, $P < 0.0001$). The SMR in the pentaploid individual (0.231 mL $O_2$ $h^{-1}$; body mass 3.35 g) was well within the range of variation of diploid and triploid froglets (fig. 2).

## Discussion

In our previous study (Hermaniuk et al. 2016), we found that triploid *Pelophylax esculentus* tadpoles and froglets were composed of larger cells than those of diploid individuals. The areas of erythrocytes and the cross-sectional areas of epidermal cells in triploid tadpoles were 36% and 41% larger, respectively, than those in their diploid counterparts reared at 19°C. The areas of erythrocytes and cross-sectional areas of hepatocytes in triploid froglets were 43% and 80% greater, respectively, than those in diploid froglets. Based on these findings, we expected a lower SMR in triploids. In this study, we found two different effects of polyploidy and cell size on SMR. SMR was higher in diploid than in triploid tadpoles (fig. 1), which was consistent with our hypothesis, but there was no difference in SMR between the two ploidies in froglets. Also, SMR of the pentaploid froglet did not differ from that of other individuals (fig. 2). Our diploid and triploid tadpoles did not differ in body mass. It may be concluded therefore that triploid tadpoles, with larger cells, were composed of fewer cells.

The lack of difference in SMR between LLR and LR froglets cannot be explained by the potential effect of the gene dosage. An excess of L over R genomes in triploids should decrease rather than increase SMR because *Pelophylax lessonae* (LL) is characterized by a lower MR than *Pelophylax ridibundus* (RR; Plenet et al. 2000). This argument may be strengthened by the finding of a clear effect of the genome dosage toward LL in the morphological indexes for our froglets. The DP/CI and T/CI indexes in LLR were lower than those in LR (table 2), which makes them more similar to LL (which have low indexes) than to RR (which have high indexes; Kierzkowski at al. 2011). Interestingly, our study showed a significant effect of the water temperature in which froglets were reared as tadpoles on both indexes in froglets. This makes the usefulness of the DP/CI and T/CI ratios uncertain for taxonomical identification of frogs in the *P. esculentus* complex. On the other hand, an important finding was that the thermal history of tadpoles had no effect on the SMR in froglets. We are not aware of studies that have tested this effect.

The lack of a difference in SMR between the two ploidies in froglets cannot be attributed to differences in the sizes of internal organs. Triploids had significantly larger kidneys and hearts, after controlling for body mass (table 3). These organs are the most metabolically active in ectothermic vertebrates (Monnickendam and Balls 1973; Garland 1984; Steyermark et al. 2005), which might increase the organismal SMR in triploids, thus decreasing a potential difference between ploidies. However, this is very unlikely because these organs account for a very small fraction of total body mass. When the combined mass of the heart and kidneys was included in the ANCOVA model for SMR (instead of body mass), the effect of ploidy was still nonsignificant. The finding that the mass of the internal organs had no effect on SMR in *P. esculentus* is supported by a large mass of

Table 2: Influence of the genome dosage effect and water temperature of tadpole development on the morphological indexes DP/CI and T/CI in *Pelophylax esculentus* froglets

| Temperature of tadpoles development (°C) and genome | *N* | DP/CI | T/CI |
|---|---|---|---|
| 19: | | | |
| LR | 26 | 1.71 (1.63–1.79) | 6.49 (6.30–6.69) |
| LLR | 23 | 1.58 (1.53–1.64) | 5.97 (5.79–6.16) |
| 24: | | | |
| LR | 38 | 1.81 (1.74–1.88) | 6.85 (6.66–7.04) |
| LLR | 31 | 1.73 (1.65–1.80) | 6.33 (6.15–6.51) |
| LLLRR | 1 | 2.10 | 8.05 |

Note. DP = first toe (*digitus primus*) length; T = length of tibia; CI = length of metatarsal tubercle (*callus internus*). Means are presented with 95% confidence intervals in parentheses. *N* = number of individuals.

Table 3: Mass of four internal organs of froglets *Pelophylax esculentus* in relation to ploidy, temperature at which froglets were reared as tadpoles, and body mass diminished by the mass of the given organ

| | Heart | Intestine | Kidneys | Liver[a] | Liver[a] |
|---|---|---|---|---|---|
| A: | | | | | |
| Ploidy | $F_{1,6} = 7.00$, **$P = .038$** | $F_{1,6} = .54$, $P = .491$ | $F_{1,6} = 16.04$, **$P = .0071$** | $F_{1,8} = .00$, $P = .955$ | ... |
| Temperature | $F_{1,6} = 1.09$, $P = .337$ | $F_{1,6} = .22$, $P = .653$ | $F_{1,6} = .97$, $P = .363$ | ... | $F_{1,39} = 14.44$, **$P = .0005$** |
| Ploidy × temperature | $F_{1,6} = .99$, $P = .358$ | $F_{1,6} = .30$, $P = .601$ | $F_{1,6} = .52$, $P = .497$ | ... | ... |
| Body mass | $F_{1,32} = 626.7$, **$P < .0001$** | $F_{1,32} = 75.0$, **$P < .0001$** | $F_{1,32} = 317.8$, **$P < .0001$** | $F_{1,40} = 103.1$, **$P < .0001$** | $F_{1,39} = 93.46$, **$P < .0001$** |
| | Adjusted means (mg) | | | | |
| B: | | | | | |
| Diploids | 9.8 (8.9–10.7) | 73.2 (58.7–87.6) | 30.0 (26.8–33.3) | 352.6 (.263–.442) | ... |
| Triploids | 11.1 (10.3–12.0) | 79.1 (65.6–92.6) | 37.4 (34.3–40.4) | 349.3 (.256–.442) | ... |
| 19°C | 10.2 (9.2–11.1) | 74.6 (60.6–88.7) | 32.8 (29.0–36.5) | ... | 253.0 (186.0–320.0) |
| 24°C | 10.7 (10.0–11.4) | 77.6 (66.8–88.5) | 34.6 (32.0–37.3) | ... | 405.4 (354.5–456.3) |

Note. The results are from the ANCOVA (pt. A; see "Statistics"), and the adjusted means are from an ANCOVA with 95% confidence intervals (pt. B). Sample size = 51 for all organs. The *P* values for the statistically significant effects in part A are shown in bold.

[a]The relationships between the mass of the liver and ploidy and between the mass of the liver and temperature were analyzed in two separate ANCOVA models because of a significant interaction of ploidy × temperature × body mass ($F_{1,29} = 5.94$, $P = 0.0212$).

the liver, which is also an energetically expensive organ that differed dramatically in mass between froglets that were reared as tadpoles at 19°C and those reared as tadpoles at 24°C (table 3) with no effect on SMR (the effect of temperature was nonsignificant).

In summary, our previous study (Hermaniuk et al. 2016) found a significant effect of water temperature on cell size in tadpoles; both triploid and diploid tadpoles reared at 19°C were composed of larger cells than were the triploids and diploids reared at 24°C. This effect did not last beyond the metamorphosis; the effect of the temperature in which froglets developed as larvae was nonsignificant for the size of their cells. This study shows that the temperature of larval development did not affect the SMR in froglets, but it did affect the morphological indexes and the liver mass.

The results of our measurements of the SMR in triploid and diploid *P. esculentus* froglets support a general pattern observed in terrestrial animals (table 4). No difference between MR in diploid and polyploid individuals was found in *Drosophila melanogaster*, salamanders of the *Ambystoma* hybrid complex, or the gray tree frog (*Hyla chrysoscelis*/*Hyla versicolor*) complex. The only example of a significant effect of ploidy on the SMR in an air-breathing animal comes from the Australian gekkonid lizard *Heteronotia binoei*. SMR in juveniles and maximum MR during forced activity in adults were higher in triploids (parthenogenetic line) than in diploids (sexual line; table 4), contrary to our prediction based on cell size.

Although numerous studies have shown that oxygen consumption rates are similar in diploid and polyploid organisms of aquatic environments, significant differences in MR between individuals with different ploidy levels have been reported in certain species. In general, these differences are consistent with our results from tadpoles (table 4). In addition, the water temperatures in which measurements of MR were conducted in various species are important for the interspecific comparison. Our tadpoles were measured at 20°C, which is higher than the average water temperature of 16°C in ponds when tadpoles reach Gosner stages 27–31 (A. Hermaniuk, unpublished data). Similar to our results using tadpoles, it was found that triploid salmon (*Salmo salar*) had a lower oxygen consumption than that of diploid salmon at a relatively high (for salmonids) temperature of 18°C. Similar differences were also observed in diploid and triploid brook trout (*Salvelinus fontinalis*), both in their MR and in their oxygen consumption after exhaustive

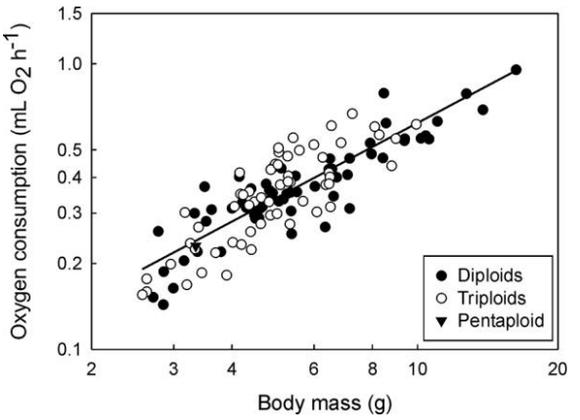


Figure 2. Relationship between oxygen consumption at air temperature of 23°C and body mass in diploid and triploid froglets *Pelophylax esculentus*. Measurement in one pentaploid froglet is also shown.

Table 4: Effect of polyploidy on the metabolic rate in various animal species

| Species | Ploidy | Polyploidy origin | Medium | Temperature (°C) | SMR/RMR | AMR | Source |
|---|---|---|---|---|---|---|---|
| Fish:[a] | | | | | | | |
| *Carassius auratus langsdorfi* | 2n, 3n | Auto | Water | 24, 30 (RMR), 24 (AMR) | NS | NS | Sezaki et al. 1991 |
| *Cobitis taenia* hybrid complex | 2n, 3n | Allo | Water | 25 | 3n < 2n | | Maciak et al. 2011 |
| *Gasterosteus aculeatus* | 2n, 3n | Auto | Water | Room temperature | NS | | Swarup 1959 |
| *Plecoglossus altivelis* | 2n, 3n | Auto | Water | 19 | NS | | Aliah et al. 1991 |
| *Promoxis annularis* | 2n, 3n | Auto | Water | 25 | NS | NS | Parsons 1993 |
| *Salmo gairdneri* | 2n, 3n, 4n | Auto | Water | 20 | 3n, 4n > 2n, NS[b] | | Oliva-Teles and Kaushik 1987*a* |
| *Salmo salar* | 2n, 3n | Auto | Water | 12, 18 | 3n > 2n, 3n < 2n[c] | | Atkins and Benfey 2008 |
| *Salvelinus fontinalis* | 2n, 3n | Auto | Water | 9, 15 | 3n > 2n, 3n < 2n[c] | | Atkins and Benfey 2008 |
| | 2n, 3n | Auto | Water | 16 | NS | 2n > 3n[d] | Hyndman et al. 2003 |
| | 2n, 3n | Auto | Water | 14 | 3n < 2n[e] | | Stillwell and Benfey 1996 |
| Amphibians: | | | | | | | |
| *Ambystoma laterale–Ambystoma texanum* hybrid complex | 2n, 3n, 4n | Allo | Air | 20 | NS | | Licht and Bogart 1990 |
| *Hyla chrysoscelis, Hyla versicolor* | 2n, 4n | Auto | Air | 20 | NS | NS | Kamel et al. 1985 |
| | 2n, 4n | Auto | Air | 15, 21, 26 | NS | | Lukose and Reinert 1998 |
| *Pelophylax esculentus* hybrid complex | 2n, 3n | Allo | Water | 20 | 3n < 2n | | This study |
| | 2n, 3n | Allo | Air | 23 | NS | | This study |
| Reptiles: | | | | | | | |
| *Heteronotia binoei* | 2n, 3n | Allo | Air | 30 (SMR), 25 (AMR) | 3n > 2n[f] | 3n > 2n | Kearney and Shine 2004; Kearney et al. 2005 |
| | 2n, 3n | Allo | Air | 30 | NS | | Roberts et al. 2012 |
| Invertebrates: | | | | | | | |
| *Crassostrea gigas* | 2n, 3n | Auto | Water | 8, 8–15, 8–30[g] | NS | | Shpigel et al. 1992 |
| *Drosophila melanogaster* | 2n, 3n | Auto | Air | 26 | NS | | Ellenby 1953 |
| *Mercenaria mercenaria* | 2n, 3n | Auto | Water | 20, 25, 27, 30, 32 | NS | | Weber 2008 |
| *Mya arenaria* | 2n, 3n | Auto | Water | 18, 20 | NS | | Mason et al. 1988 |

Note. SMR = standard metabolic rate measured in resting (or close to resting) and fasting (in most cases) individuals. In fish, the routine metabolic rate (RMR) is reported, which includes spontaneous activity. AMR = active metabolic rate; maximal or submaximal metabolic rate during forced exercise. Autopolyploidy (auto) originates from the same or a closely related individual; allopolyploidy (allo) originates from the hybridization of two different species. NS = nonsignificant difference between ploidies.

[a]No differences in the RMR between diploid and triploid salmonids were reported by Benfey and Sutterlin (1984), Oliva-Teles and Kaushik (1987*b*, 1990*a*, 1990*b*), and Yamamoto and Iida (1994).

[b]Only triploids obtained by heat shock, and not those originating from diploid-tetraploid crosses, had metabolic rates significantly higher than those in diploid individuals.

[c]There was a significant ploidy × temperature interaction; during three different trials, triploids had consistently higher SMR at the lower temperature and lower SMR at the higher temperature compared to diploids.

[d]Five minutes following exhaustive exercise.

[e]SMR of diploids was significantly higher than those in triploids in one strain ($P = 0.004$) and marginally significant in the other strain ($P = 0.076$).

[f]A significant difference in juveniles but not in adults.

[g]Two groups (one 2n and one 3n) were maintained at a temperature of 8°C. In the other two groups, the temperature increased from 8° to 15°C over a 35-d period, and in the other two groups, the temperature increased from 8° to 30°C over several days.

exercise (table 4). A lower MR in triploid than diploid *S. fontinalis* that was reported at 14°C is also consistent with this pattern. Also consistent is a low SMR found in the triploid spined loach *Cobitis taenia* measured at a relatively high temperature of 25°C (this species prefers cool water below 18°C; Lelek 1987). The only exceptions were triploid and tetraploid rainbow trout *Salmo gairdneri*, which were reported to have higher MR than those of diploid individuals at 20°C (table 4). Interestingly, at relatively low temperatures, *S. salar* and *S. fontinalis* exhibited an opposite pattern from that at higher temperatures: triploids had higher MR than diploids (table 4).

Why do triploid *P. esculentus* tadpoles and triploid individuals of other species have, in general, lower SMR at relatively higher temperatures than their diploid counterparts? In aquatic ecosystems, compared with terrestrial ones, water contains 33 times less oxygen, and the diffusion rate of oxygen in water is approximately $3 \times 10^5$ times lower (Woods 1999; Verberk et al. 2011). It has been long emphasized by ecologists that oxygen concentration and its availability to organisms decreases with higher temperatures, which would put organisms composed of larger cells, such as polyploids, and with less favorable surface-to-volume ratios at a disadvantage (Chapelle and Peck 1999). In contrast, physiologists emphasize the importance of oxygen partial pressure because it determines how oxygen dissolves and diffuses. Verberk et al. (2011) derived an oxygen supply index that integrates diffusion rate, partial pressure, and oxygen solubility (concentration). In this approach, the oxygen supply increases linearly with increasing temperatures; however, it does not increase sufficiently to match the exponential rise in MR, and thus the oxygen demand may exceed the supply (Verberk et al. 2011). Therefore, at relatively high temperatures, polyploid organisms composed of larger cells (with a less favorable surface-to-volume ratio) should be more vulnerable to oxygen limitations and display lower MR.

It is difficult to distinguish between the effect of cell size and the effect of the genome dosage in our triploid tadpoles. As we have mentioned, the relative excess of the L genome in LLR triploid tadpoles might contribute to their lower SMR because LL has lower SMR than RR. However, the lower SMR in LL is associated with the larger size of their cells as compared with RR (Kozłowski et al. 2010; A. Hermaniuk, unpublished data). This suggests that cell size is a major factor that determines SMR in the *P. esculentus* complex. The study on the spined loach, which included several forms of diploids and triploids with different combinations of parental genomes, showed that cell size explained a larger amount of variation in SMR than genomic affiliation. All types of triploids, regardless of their genomic composition, had lower SMR than all types of diploids (Maciak et al. 2011).

Our article demonstrates for the first time two distinct effects of polyploidy on the SMR in one species that develops in two different habitats—first in the aquatic habitat and then in the terrestrial habitat. Although it is difficult to explain why air-breathing diploids and polyploids do not differ in MR despite differences in cell size, our results and those presented in table 4 suggest that the effect of ploidy on MR in water-inhabiting organisms may arise from the combined effect of the cell size and oxygen supply in water that is not a limiting factor in the air. An interesting question is whether different water temperatures may have a different impact on SMR of diploid and triploid *P. esculentus* tadpoles, and this area of research requires further study.

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# General discussion

It has been well documented in various groups of vertebrates that genome size is strongly and positively correlated with cell size (Gregory, 2001; Dufresne and Jeffery 2011), and polyploids are the clearest example of this positive correlation (Gregory, 2003; Mable et al., 2011). My study provides confirmation of this pattern in triploid amphibians, not only in erythrocytes, as in most previous papers, but also in hepatocytes and epidermal cells (**Chapter I**). This suggests that the entire body of triploid *P. esculentus* is composed of larger cells. A notable exception to the aforementioned findings was the pentaploid specimen that was described in my study for the first time within the *P. esculentus* hybrid complex. The mean erythrocyte area of this unusual frog was not proportionally larger than in triploids, despite a proportionally higher DNA content. Only a small fraction of its erythrocytes were distinctly large, whereas others varied markedly in shape and size. The possible mechanism for producing these abnormal cells might be purely mechanical and might have appeared in response to physiological constraint in blood circulation, as discussed in **Chapter II**.

My study showed that water temperature (low and high) strongly affected the size of cells in tadpoles (**Chapter I**). Both diploids and triploids had larger cells at the lower temperature; the same effect of low temperature on cell size has been reported in various diploid ectotherms but not in amphibians (van Voorhies 1996; Blanckenhorn and Llaurens 2005; Arendt 2007). Recent experiments that have been conducted using *Daphnia* and *Drosophila* demonstrate that cell size increase could be partly attributed to the nucleus enlargement and that changed chromatin packing is the most likely cause of the low-temperature response (Jalal et al. 2013, 2015).

It is interesting that the temperature effect on cell size observed during larval development in the aquatic environment was not continued in frogs after several months of living in a terrestrial habitat in the common temperature (**Chapter I**). Moreover, as in the case of cell size, the effect of water temperature in which froglets developed as larvae was non-significant for their SMR corrected for body mass variation (**Chapter III**).

In **Chapter III**, I demonstrated that the SMR was higher in diploid tadpoles (comprised of smaller cells) than in triploid tadpoles, which was consistent with the hypothesis that at the organismal level, individuals composed of smaller cells should have a higher MR than individuals of a comparable body size but composed of larger

cells. Surprisingly, despite the differences in cell size, there was no difference in the SMR between ploidies in froglets (**Chapter III**). I excluded the possibility that the lack of difference in the SMR between the LLR and LR froglets was caused by genome dosage effect because the SMR in *P. lessonae* (LL) is lower than that in *P. ridibundus* (RR) (Plenet et al. 2000). This effect was indicated, however, in the morphological indices of the froglets (**Chapter III**). Furthermore, I also demonstrated that the lack of a difference in the SMR between the two ploidies in froglets cannot be attributed to differences in the sizes of metabolically active internal organs (**Chapter III**). Triploids had proportionally larger kidneys and hearts, which might increase their whole-body SMR and thus potentially decrease the difference between ploidies. An extensive review of the literature, presented in **Chapter III**, shows that the lack of difference in the SMR between diploid and triploid froglets supports a general pattern observed in terrestrial animals. In contrast to terrestrial environments, in aquatic environments, significant differences in the SMR between individuals with different ploidy levels have been reported in some studies. In general, these differences appear in relatively high water temperatures, consistent with our results from tadpoles (**Chapter III**). According to a recent model, oxygen supply (which depends on diffusion rate, partial pressure and oxygen solubility) increases linearly with increasing temperatures but not sufficiently to match the exponential rise in metabolic rate. Consequently, the oxygen demand may exceed the supply (Verberk et al. 2011). I postulate that at relatively high temperatures, polyploid organisms composed of larger cells (with a less favorable surface-to-volume ratio) should be more vulnerable to oxygen limitations and display lower MRs. As I already mentioned, the relative excess of L genome in LLR triploid tadpoles (genome dosage effect) might contribute to their SMR because LL individuals have lower SMRs than do RR individuals (Plenet et al. 2000). Therefore, it is difficult to distinguish between the effect of cell size and the effect of the genome dosage in our LLR tadpoles. Nevertheless, the lower SMR in LL is also associated with their larger cells than in RR (Kozłowski et al. 2010). This emphasizes that cell size is a major factor determining metabolic rates in the *P. esculentus* complex. Similarly, in the *Cobitis taenia* hybrid complex, genome affiliation explained less of the variation in SMR than did cell size (Maciak et al. 2011).

In **Chapter I**, I was able to show for the first time that the low temperature increases the size not only of diploid but also of triploid somatic cells. I also showed that cell size (affected by both temperature and ploidy) affects body size of tadpoles. As

I expected, diploid and triploid tadpoles were larger and developed over a longer time period at the lower temperature, which was associated with larger cells. The direct contribution of larger cell size to larger body size, which was revealed for the first time in amphibians in my study, indicates that variation in cell size may be important in explaining TSR in ectothermic vertebrates. In contrast to the well-documented effect of polyploidy on cell size, its effect on body size in animals is much more elusive. Body size at maturity has been shown to be larger in polyploid invertebrates than in diploids, but there are only a few such examples (Weider 1987; Walsh and Zhang 1992). In vertebrates, the effect of polyploidy on body size has not been reported before the present study, suggesting that polyploids equipped with larger cells regulate their size through changes in cell number (Mable et al. 2011; Choleva and Janko 2013).

My study demonstrated that at the higher temperature, there was no clear difference in growth rate between diploid and triploid tadpoles. In contrast, at the lower temperature, triploids grew faster at the initial stages of development and had larger body size than diploids at the same developmental stages, including metamorphosis (**Chapter I**). This suggests that the larger cells of triploids are not a limiting factor for their growth at lower water temperatures. Thus, my hypothesis that triploids will grow more slowly and develop over a longer time period at a given temperature was not confirmed. In general, my observations of fast growth and large body size of polyploids at the lower temperature suggest that polyploids may possess selective advantages in colder areas that may explain the prevalence of LLR individuals in the northern part of the geographic range of *P. esculentus* (**Chapter I**). It should be noted that the large size of our *P. esculentus* triploid (LLR) tadpoles was not caused by the genome dosage effect, since the parental *P. lessonae* (LL) is smaller than *P. ridibundus* (RR) (Berger 2008).

In conclusion, my thesis demonstrates that the variation in cell size associated with polyploidy and induced by temperature may play a significant role in the physiology of ectothermic vertebrates as a determinant of MR, growth rate and body size. However, the effect of cell size differs substantially between aquatic and terrestrial habitats. I found that SMR corrected for body mass differed between ploidies in tadpoles but not in froglets. This suggests that cell size may have greater consequences for whole-body metabolic rates in aquatic habitats than in terrestrial habitats, likely the result of constriction in the oxygen supply. My study is also the first to report that growth rate of amphibian larvae was enhanced by low water temperature and

polyploidy and that this effect was related to the increase in cell size. Although my research suggests that temperature-related oxygen supply may be a regulator of cell size and metabolic rate, further studies are needed to resolve this puzzle. An important issue that should be addressed in future experiments is whether different water temperatures may have different impacts on the SMR in diploid and triploid tadpoles of *P. esculentus*, given that they show different effects on growth rate.

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# Co-authors's statements

Illustration by J.J. Grandville, from Jean de La Fontaine *Bajki*, PIW, 1986

**UNIVERSITY OF BIAŁYSTOK**
**Institute of Biology**

ul. Ciołkowskiego 1 J, 15-245 Białystok
tel./fax: 48 (085) 738 84 14

Białystok, 3rd November 2016

**To whom it may concern**

This concerns my contribution to the following scientific papers with Adam Hermaniuk where I was one of the co-authors:

**Hermaniuk A., Rybacki M., Taylor J.R.E. 2016. Low temperature and polyploidy result in larger cell and body size in an ectothermic vertebrate. Physiological and Biochemical Zoology 89: 118-129**

**Hermaniuk A., Rybacki M., Taylor J.R.E. 2017. Metabolic rate of diploid and triploid edible frog, *Pelophylax esculentus*, correlates inversely with cell size in tadpoles but not in frogs. Physiological and Biochemical Zoology, in print**.

My contribution to the above papers consisted of developing the concept of the study, research methods selection, participation in collecting water frogs in the field, supervising collection of the data, editing and commenting on the manuscripts at all stages.

Prof. dr hab. Jan R.E. Taylor
Department of Animal Ecology

UNIWERSYTET KAZIMIERZA WIELKIEGO
W BYDGOSZCZY

WYDZIAŁ NAUK PRZYRODNICZYCH

**Katedra Biologii Ewolucyjnej**
Al. Ossolińskich 12, 85-093 Bydgoszcz, tel./fax (52) 32 23 330
e-mail: kbe@ukw.edu.pl

Bydgoszcz, 25-09-2016

*To whom it may concern*

**STATEMENT**

I, undersigned Mariusz Rybacki Ph. D., declare that my part:

in the paper:
**Hermaniuk A., Rybacki M., Taylor J.R.E. 2016. Low Temperature and Polyploidy Result in Larger Cell and Body Size in an Ectothermic Vertebrate. Physiological and Biochemical Zoology 89(2):118-129**,
included collecting of water frogs in the field from populations with special genetic structure, preparation of cross experiments between various types of frogs, participation in literature analysis.

in the paper:
**Hermaniuk A., Rybacki M., Taylor J.R.E. 2017. Metabolic rate of diploid and triploid edible frog, Pelophylax esculentus, correlates inversely with cell size in tadpoles but not in frogs. Physiological and Biochemical Zoology, in print**,
included collecting of water frogs in the field from populations with special genetic structure, preparation of cross experiments between various types of frogs.

Mariusz Rybacki

Uniwersytet Wrocławski

**WYDZIAŁ NAUK BIOLOGICZNYCH**

INSTYTUT BIOLOGII ŚRODOWISKOWEJ
ul. Sienkiewicza 21
50-335 Wrocław

tel. +48 71 375 40 02
fax +48 71 322 28 17

ibs@uwr.edu.pl | www.uni.wroc.pl

Wrocław, 8 July 2016

To whom it may concern:

I, underdesigned Maria Ogielska PhD, professor, declare that my part in the article: **Hermaniuk A, Pruvost NBM, Kierzkowski P, Ogielska M. 2013. Genetic and cytogenetic characteristics of pentaploidy in water frogs. Herpetolgica 69: 36-45** included assistance and advise in selection of various types of erythrocytes in pentaploid individuals for DNA image cytometry.

Maria Ogielska PhD
Professor, head of the
Department of Evolutionary Biology and Conservation of Vertebrates
Institute of Environmental Biology, University of Wrocław
ul. Sienkiewicza 21, 50-335 Wrocław, Poland
phone: (+48) 71 375 4022
fax: (+48) 71 322 2817
http://zbok.biol.uni.wroc.pl/
maria.ogielska@uwr.edu.pl

Universität Zürich[UZH]

**Institut für Evolutionsbiologie und Umweltwissenschaften**

Universität Zürich
Institut für Evolutionsbiologie und Umweltwissenschaften
Winterthurerstr. 190
CH-8057 Zürich
Telefon +41 44 635 49 99
Telefax +41 44 635 57 11
www.ieu.uzh.ch

**Dr. Nicolas Pruvost**
Telefon +41 44 635 49 75
Telefax +41 44 635 68 21
nicolas.pruvost@ieu.uzh.ch

To whom it may concern

Zürich, 3. August 2016

**Statement**

I, undersigned Nicolas Pruvost, declare that my part in the paper :

Hermaniuk, A., Pruvost, N.B.M., Kierzkowski, P. and Ogielska, M. (2013). Genetic and cytogenetic characteristics of pentaploidy in water frogs. Herpetologica 69(1): 36-45.
included

(i) DNA isolation of the studied frogs
(ii) PCR and microsatellite DNA analysis of the studied frogs

Dr. Nicolas Pruvost

Uniwersytet Wrocławski

Wydział Nauk Biologicznych
Instytut Biologii Środowiskowej

ul. Henryka Sienkiewicza 21
50-335 Wrocław
tel. +48 71 375 40 02
fax +48 71 322 28 17
e-mail: sekrzool@biol.uni.wroc.pl
www.biol.uni.wroc.pl

Wrocław, 11.07.2016

*To whom it may concern*

**STATEMENT**

I, undersigned PhD Piotr KIERZKOWSKI, declare that my part in the paper:

**Hermaniuk A., Pruvost N. B. M., Kierzkowski P., Ogielska M., 2013: „Genetic and cytogenetic characteristics of pentaploidy in water frogs.". Herpetologica 69(1):36-45.**

included measuring DNA content in erythrocyte nuclei stained in Feulgen reaction, using KS400 image analysis software.

Piotr Kierzkowski

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Piotr Kierzkowski