1. Introduction

In temperate ectotherms, a species’ occurrence in a given habitat depends on thermal conditions not only during their active season but also during wintering. Ectotherms cope with low temperatures using behavioral and physiological mechanisms (Storey and Storey, 1988; Ullsch, 1989; Tattersall and Boutilier, 1997, 1999; Tattersall and Ullsch, 2008; Costanzo and Lee, 2013; Sinclair, 2015). The most important behavioral response to winter conditions is temperature-dependent microhabitat selection, whereas physiological mechanisms involve freeze thermal tolerance, thermal dependence of metabolic rates, and its seasonal reduction (suppression) patterns. Projections of winter climate change predict the increase in both mean and variation in air temperature (IPCC, 2013), which may accelerate the energy consumption between two newt species, Ichthyosaura alpestris and Lissotriton vulgaris. Oxygen consumption was measured in both species during their active season (daily temperature range = 12–22 °C) and wintering period (4 °C) at 4 °C and 8 °C. The seasonal reduction in metabolic rates differed between species and experimental temperatures. Wintering newts reduced their metabolic rates at 4 °C and 8 °C in I. alpestris, but only at 8 °C in L. vulgaris. Both species reduced the thermal sensitivity of oxygen consumption during wintering. Theoretical calculations of winter depletion of caloric reserves under various thermal conditions revealed that seasonal metabolic reduction is more effective in I. alpestris than in L. vulgaris, and its effectiveness will increase with the proportion of warmer days during wintering period. The variation in winter metabolic reduction between sympatric newt species potentially contributes to their distribution patterns and population dynamics under climate change.
personal observations) should favor the pronounced metabolic reduction in *I. alpestris*. Finally, we calculated the theoretical energy consumption of wintering newts under varying thermal conditions to evaluate the effectiveness of seasonal metabolic reduction in both species.

### 2. Material and methods

#### 2.1. Ethics

This study was performed in accordance with the laws of the Czech Republic. All experimental procedures were approved by the Expert Committee for Animal Conservation of the Institute of Vertebrate Biology CAS (research protocol no. 14/2013). The Environment Department of the Regional Authority of Vysočina, Czech Republic, issued the permission to capture newts (KUJL 224/2013).

#### 2.2. General maintenance

Adult newts were captured from two populations (air distance 4 km) near Jihlava, Czech Republic, in April 2015. The *I. alpestris* population is found in a forest habitat, whereas *L. vulgaris* inhabits open landscapes. In total, we captured 30 individuals of *I. alpestris* (1:1 sex ratio; snout-vent length [SVL] = 46 ± 4 [SD] mm; body mass [BM] = 2.60 ± 0.84 g) and 28 individuals of *L. vulgaris* (1:1 sex ratio; SVL = 40 ± 4 mm; BM = 1.21 ± 0.30 g). Pairs of newts (one male and one female) were placed in tanks (50 × 30 × 18 cm) filled with 18 L of non-chlorinated water. Tank habitats consisted of aquatic plants (*Egeria densa*) and a piece of Styrofoam, allowing newts to leave water. Tanks were placed in a climatic room with natural light:dark (from 12:12 to 8:16) cycles and a diel temperature fluctuating thermal regime (air: 12–22 °C; water: 13–18 °C). The selected temperature range covers most temperatures, which newts commonly experience in the field (Kristín and Gvoždík, 2012). Terrestrial tanks were equipped with moist filter paper as substrate and ceramic shelter. The paper substrate was replaced twice weekly. During the second half of November, we stopped newt feeding and air temperatures were gradually reduced to 4 ± 1 °C, which was maintained during the wintering period (December–March). During the first week, we reduced diel temperature fluctuations to 12 ± 1 °C. During the second week, temperatures were gradually dropped to 4 °C. Newts starved during the whole wintering period. They were weighed (to 0.01 g) before (1st December) and after wintering period (31st March).

#### 2.3. Metabolic measurements

Standard metabolic rates were measured indirectly as oxygen consumption rates, using intermittent respirometry (Kristín and Gvoždík, 2012). We chose the intermittent over continuous respirometry method, because it allows the detection of very low oxygen consumption of wintering newts (Kristín and Gvoždík, 2014a) and the measurement of more individuals during a trial. Accordingly, metabolic trials were performed with minimal temporal variation among individuals (≤8 days). Details of the respirometry system used and its verification are provided elsewhere (Kristín and Gvoždík, 2012). In short, water vapor- and CO₂-free air was pushed by an air pump (PP-2; Sable Systems, Las Vegas, USA) at 120 ± 1 mL min⁻¹ into the system. Before entering the respirometry chamber (60 mL), air was rehumidified (Nafion tube; ME Series, Perma Pure, Toms River, NJ, USA) to minimize evaporative water loss of measured newts. Eight respirometry chambers were placed in a temperature-regulated water bath to maintain stable temperature during a trial (see below). A programmable multiplexer (RM-8 and BL-1, Sable Systems) automatically switched air flow among nine channels (eight chambers and baseline). Excurrent air passed through a Nafion dryer (MD Series, Perma Pure), CO₂ analyzer (FoxBox-C, Sable Systems), gas scrubber (soda lime-silica gel-Drierite), and O₂ analyzer (FoxBox-C, Sable Systems). Room temperature was maintained at 15 ± 2 °C during trials to avoid water vapor condensation inside the system.

Oxygen consumption rates were measured in October–November and January–February at 4 ± 0.5 °C and 8 ± 0.5 °C repeatedly in each individual. Because information about thermal conditions of wintering newts are lacking, we chose these temperatures according to wintering temperatures in other salamandrid species occurring in Central Europe (Balogová and Uhrin, 2014). The order of individuals and testing temperatures was completely randomized. Except for 4 °C during wintering, newts were introduced to the experimental temperature immediately before a respirometry trial. Observations of newt behavior in thermal gradients showed that newts voluntarily swim across a 20 °C gradient within seconds (Marek and Gvoždík, 2012), and thus the sudden drop in body temperature is ecologically realistic for them. Newts measured during the active season were starved for seven days before the beginning of trials. This period is sufficient to eliminate the effect of specific dynamic action at 12–22 °C (P. Kristín and L. Gvoždík, unpublished data). Because newts are predominantly crepuscular and nocturnal animals, respirometry trials were performed during daytime (8:00–17:00). Newts were weighed (to 0.01 g) before respirometry trials, which lasted 5 h. This time interval is sufficient to measure a minimal oxygen consumption rate in newts (Kristín and Gvoždík, 2012). Each respirometry chamber was flushed twice hourly (enclosure time = 1679 s). Newt locomotor activity was continuously monitored during a trial using webcams connected to a PC motion activity system.

Minimal oxygen consumption (VO₂) values were calculated from peak integrals (areas) of raw O₂ measurements, divided by chamber enclosure time (Lighthon, 2008; Kristín and Gvoždík, 2012). The lowest VO₂ values of non-moving individuals (>95% of enclosure time) from each trial were considered estimates of the standard metabolic rate at a given temperature. In 11% (*I. alpestris*) and 10% (*L. vulgaris*) of trials, motor activity violated standard conditions, and thus their VO₂ values were discarded from further analyses. For each VO₂ value we also calculated the corresponding carbon dioxide production (VCO₂) to calculate the respiratory exchange ratio (RQ = VO₂/VCO₂). Because VCO₂ produce similar but less accurate estimates than VO₂ in newts (Kristín and Gvoždík, 2012, 2014b), we present the results only for VO₂. The thermal sensitivity of VO₂ (Q₁₀) was calculated as Q₁₀ = (VO₂(8 °C)/VO₂(4 °C))²⁻¹, where VO₂(8 °C) and VO₂(4 °C) is the minimum oxygen consumption at 8 °C and 4 °C, respectively.

To calculate theoretical energy drain during wintering (122 days) in both species under varying thermal conditions, we multiplied VO₂ with an oxyjoule equivalent [16 ± 5.164(RQ)] (Lighthon et al., 1987). The theoretical wintering period lasted the same time as in our study, which is a good approximation of the newt wintering period in the field (L. Gvoždík, unpublished observations). Daily mean body temperatures were generated within the 4–8 °C range with varying proportions of the highest temperatures (7 °C and 8 °C). Non-measured VO₂ values were estimated using known Q₁₀ between 4 °C and 8 °C (see Section 3). For each temperature distribution, we calculated energy savings (effectiveness) of the winter metabolic reduction as the absolute difference between energy drain with and without reduced metabolic rates.
2.4. Statistical analyses

We performed exploratory data analysis to check for model assumptions, e.g. normality, homoscedasticity, and the absence of outliers. Because VO\textsubscript{2} was measured repeatedly on the same individuals, we analyzed data using general linear mixed models (GLMM). The full model consisted of three fixed factors, species, season, and experimental temperature, and their interactions. Individual identity within species was included as a random factor and body mass as the covariate including interactions with species and season. We applied the model selection procedure to find the minimum adequate model, i.e., explaining most variation of the examined variable with the minimum number of parameters, using the likelihood ratio test. Variation in Q\textsubscript{10} was analyzed using GLMM with species and season as fixed factors, and individual identity within species as a random factor. Because the number of activity episodes and Q\textsubscript{10} violated the normality assumption, we used a permutation approach (9999 permutations) to calculate P-values. For VO\textsubscript{2} analyses, both parametric and randomization approaches produced qualitatively similar results, and thus we used the latter more robust approach only. Mean values are provided with 95% CIs calculated using the nonparametric bootstrap (’bca’ method; 9999 randomizations). Statistical analyses were performed in R 3.0.3 (R Development Core Team, 2014) using ’boot’ and ’lmerTest’ packages, and Primer 6 (PRIMER-E Ltd., Lutton, UK) using the PERMANOVA package.

3. Results

Minimum oxygen consumption was affected by the interaction between experimental temperature and species (F\textsubscript{1,143} = 5.91, P = 0.01), and thus the influence of season and species was analyzed for each temperature separately. At 4 °C, the body mass-corrected seasonal shift in VO\textsubscript{2} varied between species (F\textsubscript{1,37} = 4.18, P = 0.047). Wintering alpine newts, on average, reduced their oxygen demands to 70.5% of autumn values, while smooth newts consumed oxygen at similar rates during autumn and winter trials (Fig. 1a). At 8 °C, the body mass-corrected VO\textsubscript{2} seasonally decreased in both species (F\textsubscript{1,49} = 24.11, P < 0.001; Fig. 1b). The mean magnitude of the seasonal metabolic reduction was 35.4% and 22.8% in I. alpestris and L. vulgaris, respectively. The thermal sensitivity of VO\textsubscript{2} between 4 °C and 8 °C was similar in both species (F\textsubscript{1,64} = 0.06, P = 0.81) and decreased in wintering individuals (F\textsubscript{1,31} = 10.97, P = 0.003; Fig. 1c). Body mass decreased in both species between autumn and winter trials (F\textsubscript{1,46} = 8.42, P = 0.006), but the body mass reduction was relatively small (I. alpestris: from 2.66 ± 0.17 [SE] g to 2.54 ± 0.17 g; L. vulgaris: from 1.34 ± 0.06 g to 1.21 ± 0.05 g).

Species, season, and temperature did not significantly influence RQ (species: F\textsubscript{1,47} = 1.98, P = 0.17; season: F\textsubscript{1,146} = 0.12, P = 0.73; temperature: F\textsubscript{1,145} = 2.07, P = 0.15), and thus we used its overall mean value (0.94 ± 0.01 [SE]) for calculating energy equivalents of VO\textsubscript{2}. Under the equal proportion of daily mean temperatures (4–8 °C), wintering I. alpestris theoretically spend about 33% less energy for maintenance than they would without the metabolic reduction. Lissotriton vulgaris attains only 12% energy savings of winter metabolic reduction. Theoretical energy savings of mass-corrected seasonal metabolic adjustments were two to four times higher in I. alpestris than in L. vulgaris across various thermal conditions (Fig. 2). The magnitude of energy savings increased with the proportion of high daily mean temperatures (7 °C and 8 °C) during the wintering period.

Fig. 1. Body mass-corrected minimal oxygen consumption at (a) 4 °C and (b) 8 °C, and (c) thermal sensitivity (Q\textsubscript{10}) of oxygen consumption in two newt species, Ichthyosaura alpestris and Lissotriton vulgaris, during autumn and winter measurements. Values are means ± 95% CIs. P-values show the results of paired comparisons denoted by horizontal brackets.
the more specialized, sympatric newt species, we predicted that (1) wintering metabolic reserves during wintering, and the effectiveness of energy savings increases with warming winters.

4. Discussion

According to information about the distribution and ecology of sympatric newt species, we predicted that (1) wintering metabolic rates are more sensitive in the habitat generalist, \textit{L. vulgaris}, than in the more specialized, \textit{I. alpestris}, and (2) the magnitude of the winter metabolic reduction is higher in \textit{I. alpestris} than in \textit{L. vulgaris}. Our results partially corroborated these predictions. The thermal sensitivity of oxygen consumption rates was similar in both species during wintering. The magnitude of seasonal metabolic reduction was higher in \textit{I. alpestris} than in \textit{L. vulgaris}, but only at 4 °C. Accordingly, seasonal metabolic adjustments in \textit{I. alpestris} have greater potential to save caloric reserves than in \textit{L. vulgaris}, especially under elevated wintering temperatures.

Newts reduced their oxygen consumption during the wintering period. In amphibians, this phenomenon has been known in aquatic frogs, which depress their metabolic rate under prolonged exposure to normoxic or hypoxic conditions (Donohoe and Boutiliere, 1998; Tattersall and Ulltsch, 2008). Our results demonstrated that metabolic depression occurs also in amphibians wintering on land. Previous studies on thermal acclimation demonstrated that many temperate salamander species reduce their metabolic rate after exposure to low temperatures during their active season (Fitterpatrick et al., 1971, 1972; Feder, 1978, 1985). Unfortunately, our experimental design prevents us from evaluating the contribution of thermal acclimation to winter metabolic reduction. The seasonal shift involves not only temperature, but also photoperiod, food availability, and/or hormonal activity, which affect the variation in metabolic rates (Whitford and Hutchison, 1965; Lagerspetz, 1977; Auer et al., 2015). To what extent thermal acclimation contributes to the seasonal metabolic shift in newts remains to be examined.

Wintering newts lowered their oxygen consumption, on average 23–35% below the minimum oxygen requirements during their active season. Previously, it has been speculated that the winter metabolic reduction would be negligible in comparison with the Q_{10} effect on metabolic rates (Pinder et al., 1992; Hillman et al., 2009). Alpine newts reduce their oxygen consumption about 56% during the shift in body temperatures from 18 °C (mean preferred body temperature; Hadamová and Gvoždík, 2011) to 4 °C (Kristin and Gvoždík, 2014b; present study). This indicates that the magnitude of winter metabolic depression is fairly high in newts, and falls well within the range reported for other taxa (Storey and Storey, 1990; Guppy and Withers, 1995).

Therefore, the prevailing view on the negligible metabolic depression in wintering amphibians needs reconsideration.

Newts decreased their thermal sensitivity of oxygen consumption during the wintering period. During their active season, the thermal acclimation response in other amphibious taxa rather showed the opposite pattern (Dunlap, 1971; Fitzpatrick et al., 1971, 1972 but see Burggren and Wood, 1981). The winter shift in Q_{10} has rarely been reported. The lowered thermal sensitivity of metabolic rates should reduce maintenance costs under thermally varying conditions during wintering (Sinclair, 2015). Indeed, our theoretical calculations show that the decreased thermal sensitivity should save caloric reserves as the proportion of “warm” days increases. On the other hand, more thermally sensitive metabolic machinery should improve feeding and digestion performance during warm episodes (Sinclair et al., 2003). Although salamanders are capable of feeding and digesting food at low temperatures during their active season (Marvin et al., 2016), it may be a risky strategy during winter, because a sudden drop in temperature could stop digestion and putrefaction processes might prevail (Dawson, 1975). The physiological adjustments of wintering newts suggest that an energy saving strategy seems more beneficial for them than risky energy acquisition.

Newt capacity to seasonally reduce their oxygen consumption varied between species and experimental temperatures. Given the limitation of a two species study, we cannot decide whether the observed variation results from adaptation to current environment or phylogegetic distance between species (Garland and Adolph, 1994). However, our aim was to reveal trait variation, which potentially contributes to a species’ success in a given habitat. From this view, the seasonal reduction of oxygen consumption at 4 °C seems advantageous in \textit{I. alpestris}, because it saves energy during the prolonged wintering period, or increases survival under hypoxic conditions (Tattersall and Ulltsch, 2008) during their facultative wintering in water (Fasola and Canova, 1992). The metabolic reduction recalculated to energy savings clearly demonstrated that the seasonal physiological adjustments in \textit{I. alpestris} were two to four times more effective than in \textit{L. vulgaris}. This suggests that \textit{L. vulgaris} has to compensate for the inefficient metabolic response with an increase in caloric reserves and/or by careful microhabitat selection. Further research in this area would be highly informative.

In conclusion, results of this study demonstrated variation in seasonal metabolic reduction in two newt species. Contrary to general opinion, seasonal metabolic adjustments occur in amphibians wintering on land, and the resulting effectiveness of energy savings varies substantially between sympatric newt species. The species variation in winter metabolic reduction potentially contributes not only to their current habitat use, but also to their future climatic change responses. Theoretical calculations showed that the winter metabolic reduction will buffer the impact of an increasing proportion of warm winter days on newt caloric reserves. In addition, species with a better capacity to save caloric reserves at low temperatures, \textit{I. alpestris}, also had lower thermal requirements during the active season (Balogová and Gvoždík, 2015) and vice versa. Hence, winter metabolic adjustments may be linked with thermal ecology during the active season. This clearly supports the recent call (Sinclair, 2015) for more attention on the wintering energetics not only in insects, but also other ectothermic organisms from temperate, arctic, and alpine areas.

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