Thermoregulatory strategies in an aquatic ectotherm from thermally-constrained habitats: An evaluation of current approaches

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Abstract

Many ectotherms employ diverse behavioral adjustments to effectively buffer the spatio-temporal variation in environmental temperatures, whereas others remain passive to thermal heterogeneity. Thermoregulatory studies are frequently performed on species living in thermally benign habitats, which complicate understanding of the thermoregulation–thermoconformity continuum. The need for new empirical data from ectotherms exposed to thermally challenging conditions requires the evaluation of available methods for quantifying thermoregulatory strategies. We evaluated the applicability of various thermoregulatory indices using fire salamander larvae, Salamandra salamandra, in two aquatic habitats, a forest pool and well, as examples of disparate thermally-constrained environments. Water temperatures in the well were lower and less variable than in the pool. Thermal conditions prevented larvae from reaching their preferred body temperature range in both water bodies. In contrast to their thermoregulatory abilities examined in a laboratory thermal gradient, field body temperatures only matched the mean and range of operative temperatures, showing thermal passivity of larvae in both habitats. Despite apparent thermoconformity, thermoregulatory indices indicated various strategies from active thermoregulation, to thermoconformity, and even thermal evasion, which revealed their limited applicability under thermally-constrained conditions. Salamander larvae abandoned behavioral thermoregulation despite varying opportunities to increase their body temperature above average water temperatures. Thermoconformity represents a favored strategy in these ectotherms living in more thermally-constrained environments than those examined in previous thermoregulatory studies. To understand thermal ecology and its impact on population dynamics, the quantification of thermoregulatory strategies of ectotherms in thermally-constrained habitats requires the careful choice of an appropriate method to avoid misleading results.

1. Introduction

The body temperature of ectothermic organisms is primarily determined by the heat obtained from the surrounding habitat, and thus many ectotherms are not passive to the thermal heterogeneity of environment. They maintain their body temperatures (Tb) higher than the mean and within a narrower range than operative temperatures (Te) by behavioral means (Adolph, 1990; Hertz, 1992; Bauwens et al., 1996), providing them with a substantial fitness advantage (Gilchrist, 1995). Recently, behavioral thermoregulation is receiving renewed attention, because it allows ectotherms to cope with the pitfalls of climate change (Kearney et al., 2009; Huey et al., 2012; Sunday et al., 2014). The thermoregulatory effort varies substantially among species however, within a continuum between thermoconformity and effective thermoregulation (Hertz et al., 1993; Christian and Weavers, 1996), which complicates predictions about the impact of climate change on their populations (Sunday et al., 2014). Despite its obvious ecological relevance, determinants of thermoregulatory strategies are not fully understood.

The source of variation in thermoregulatory strategies among species may stem not only from ecological factors but also from methods of their quantification. Since 1993, thermoregulatory strategies of ectotherms have become quantified using thermoregulatory indices (Hertz et al., 1993). However, if the thermal conditions of the habitat prevent the attainment of the

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ecototherms’ target temperatures, the index values reflect not only thermoregulatory effort but also the presence of thermal constraints. Following this reasoning, Herczeg et al. (2006) proposed calculations of thermoregulatory indices as deviations from the maximum attainable $T_e$ rather than from the $T_p$ range. Despite sound rationale for its use (Angilletta, 2009), the field evaluation of this approach is missing.

In this study, we examined the applicability of various thermoregulatory indices using behavioral thermoregulation in salamander larvae as a suitable model system. We found a population of fire salamanders with their larvae developing in two water bodies with contrasting thermal conditions, i.e. a forest pool and well, located only 20 m apart. Because water is mixed by the bottom spring in the well, its temperatures are lower and less heterogeneous than in the pool (K. Piasečná and L. Gvoždík, unpublished data). Both habitats lacked amphibian competitors and predaeous insects, which suggests that their potential thermoregulatory behavior was little affected by biotic interactions (Gvoždík et al., 2013). Accordingly, we predicted that if thermal conditions permit larvae to attain their $T_p$, the larvae would thermoregulate. However, if thermal conditions prevent them attaining their target range (i.e., if there are thermal constraints) then the cost(s) of thermoregulation could be too high relative to the benefit(s) for growing larvae, and thus they would become thermoconformers. Hence, this system provides an excellent opportunity to evaluate the applicability of thermoregulatory indices in ectotherms exposed to thermally-constrained conditions.

2. Materials and methods

2.1. Study organisms

Fire salamanders, Salamandra salamandra, are approximately 20 cm long tailed amphibians distributed across Western, Central, and Southern Europe. In Central Europe, larviparous females lay up to 40 larvae into water in May (Kopp and Baur, 2000). They usually prefer clean flowing waters such as creeks and springs for laying, but occasionally use still water bodies (Thiesmeier and Grossenbacher, 2004). Bottom-dwelling larvae prey on various aquatic invertebrates. Larval development lasts 6–12 months, which largely depends on thermal conditions in a given habitat (Zakrzewski, 1987). In Central Europe, larvae often overwinter in water and finish their metamorphosis during the next spring.

2.2. Study site

The study was carried out in two water bodies located on a birch-forested slope near Hukvaldy, Czech Republic (49°37′44″N, 18°13′25″E; 350 m). The forest pool (7.4 × 1.7 m²) and the well (1.2 × 0.8 m²) were oval-shaped and had the same maximum depth of 20 cm. Both water bodies were equally shaded by surrounding trees. In addition to the salamander larvae, two invertebrate taxa were abundant in both water bodies, amphipod crustaceans (Gammarus sp.) and caddisfly larvae (Chaetopteryx sp.).

2.3. Thermal preferences

To test thermal preferences and to estimate preferred body temperatures, 36 salamander larvae (total length [mean ± SD] = 43.9 ± 2.7 mm from pool and 40.4 ± 5.2 mm from well) from each habitat were captured and transported to the laboratory in May 2011. Larvae were transported in plastic bags (two larvae per 1 l water) inside Styrofoam boxes (10–15 °C) for a five hour drive. In the laboratory, larvae were placed individually in aquaria (40 × 26 × 18 cm³) with 5 l of well (non-chlorinated) water at 9–11 °C, which were the most frequent water temperatures in their natural habitat in May 2010 (K. Piasečná and L. Gvoždík, unpublished data). Aquaria were equally equipped with five dry birch leaves, some water vegetation (Egeria densa), and an aeration stone. Larvae were fed with live Chironomus larvae and Tubifex worms at three day intervals. Water (50% of total volume) was regularly changed at three-day intervals. Larvae were subjected to laboratory conditions at least one week prior to the beginning of thermal preference trials.

Thermal preferences were tested in a stainless steel tank (240 × 60 × 60 cm³ high) divided into nine longitudinal lanes (7 × 240 cm²) using solid partitions. The tank bottom was equipped with computer-controlled Peltier modules and a heat-exchange unit to maintain various thermal profiles along the tank (see Gvoždík (2003) for further details). The tank was filled with water to a depth of 2 cm. To test thermal preferences, either a thermal gradient (7–28 °C) or constant temperature (10 °C) was maintained in the tank. The tank was located in a room at 18 ± 2 °C. Illumination was provided with fluorescent tubes (300 lx) during the day (0600–1800). The water was completely changed after each trial.

Because wild-caught larvae contained food in their stomachs, examined larvae were fed 24 h prior to trials. For a thermal preference trial of the experimental group in a thermal gradient (7–28 °C), we randomly selected (without replacement) nine salamander larvae and placed them individually into the 10 °C section of the lane 14 h before the trial (1800). To estimate thermal preferences and preferred body temperatures, every two hours, we carefully (to not disturb the animals) measured the larval horizontal position along the length of the tank (to 5 cm) and water temperature (to 0.1 °C) exactly where it was positioned using a thermocouple probe connected to a digital thermometer (HH 22; Omega Engineering, Stamford, USA). Given the small size of experimental animals and high thermal conductivity and heat capacity of water, we assumed that body temperatures of larvae matched the temperature of the surrounding water (Lutterschmidt and Hutchison, 1997). According to preliminary observations, the presence of experimenter during temperature measurements has a negligible influence on larval motor activity patterns. For each individual, we used the mean and boundaries (minimum and maximum) of preferred body temperatures ($T_p$) measured over 10 h (0800–1800), i.e. the same time period as in the field (see below). Previous analyses (Smolínský and Gvoždík, 2009)

Table 1

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>$T_e$</td>
<td>Field body temperatures.</td>
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<tr>
<td>$T_p$</td>
<td>Operative temperatures; temperatures of a physical model with the same thermal characteristics as a study organism.</td>
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<tr>
<td>$T_a$</td>
<td>Preferred body temperatures; body temperatures that an ectotherm maintains in the laboratory thermal gradient under the absence of abiotic and biotic limitations.</td>
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<tr>
<td>$d_e$</td>
<td>Index of thermal quality of habitat; the mean absolute deviation of $T_e$ from the $T_p$ range.</td>
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<tr>
<td>$d_v$</td>
<td>Thermally-constrained version of $d_e$: the mean deviation of $T_e$ from the maximum $T_e$ at a given time.</td>
</tr>
<tr>
<td>$d_b$</td>
<td>Index of accuracy of thermoregulation (originally the accuracy of $T_b$): the mean absolute deviation of $T_b$ from the $T_p$ range.</td>
</tr>
<tr>
<td>$E$</td>
<td>Thermally-constrained version of $d_b$: the mean deviation of $T_b$ from the maximum $T_b$ at a given time.</td>
</tr>
<tr>
<td>$E_{diff}$</td>
<td>Index of effectiveness of thermoregulation; $E=1−(d_b/d_e)$.</td>
</tr>
<tr>
<td>$E'$</td>
<td>Thermally-constrained version of $E$; $E=1−(d_b/d_e')$.</td>
</tr>
<tr>
<td>$E_{diff}$</td>
<td>Alternative measure of $E$; $E_{diff}=d_b−d_e$.</td>
</tr>
<tr>
<td>$E_{diff}'$</td>
<td>Thermally-constrained version of $E_{diff}$; $E_{diff}'=d_b−d_e'$.</td>
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</table>
confirmed that these estimates were close to commonly used estimates of thermoregulatory bounds, i.e., 10% and 90% of individual $T_{ps}$ (Angilletta, 2009). For the control group, we monitored the behavior of individuals at a constant 10°C. Because salamander larvae may expose part of their bodies to air temperatures (A. Pončová, unpublished observations), we also recorded temperatures inside agar models of salamander larvae (Navas and Araujo, 2000) located at the edge of water, i.e. one half in water and the second half on land, in both habitats (two dataloggers in the pool, one in the well) using thermistor probes connected to dataloggers (resolution 0.1°C; HOBO, Onset Computer, Bourne, USA). Dataloggers recorded water temperatures at 30 min intervals during the measurement period. All dataloggers and the digital thermometer were calibrated to the same reference mercury thermometer (resolution 0.2°C) before their use.

Body temperature of each visible individual was inferred from the water temperature that was measured within 2.5 cm of the individual’s depth in the water column. The vertical position of larvae in the water column was recorded at two-hour intervals (0800–1800) on randomly selected days ($n=10$) during June and July 2011. Vertical positions were estimated using a network of sticks with a known depth (5, 10, 15, and 20 cm of water column). In the pool, sticks were arranged into seven transverse rows located 1 m apart along its length. In the well, two perpendicular rows were used for mapping the bottom. Pilot measurements confirmed that this method provided repeatable estimates ($\pm 3$ cm) of vertical positions without the disturbance of focal animals. At each observing occasion, one person (KP) walked slowly along the edge of water body and recorded vertical positions of all visible larvae. Because motor activity of salamander larvae is relatively low, transect measurements were minimally biased by changing larval positions. The order and direction of transects were randomly chosen. Mean values of operative and body temperatures per sampling interval (two hours) were used for further analyses.

2.5. Thermoregulatory indices

To evaluate thermoregulatory strategies, we first followed the protocol reported by Hertz et al. (1993). Using $T_o$, $T_e$ and the $T_{ps}$ boundaries, we calculated three thermoregulatory indices: (i) The thermal quality of the habitat ($d_e$; see Table 1 for all acronym definitions), the mean absolute deviation of operative

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2.4. Operative and body temperatures

To obtain a thermal map of both natural habitats we placed a series of waterproofed dataloggers (resolution 0.5°C; DS1921G-F5, Maxim Integrated Products, Sunnyvale, CA, USA) to cover the whole depth of each body of water at 5 cm intervals, i.e. five dataloggers per series. Because of their disparate sizes, we used two datalogger series (i.e. across the water column) in the pool and one series in the well. Because salamander larvae may expose part of their bodies to air temperatures (A. Pončová, unpublished observations), we also recorded temperatures inside agar models of salamander larvae (Navas and Araujo, 2000) located at the edge of water, i.e. one half in water and the second half on land, in both habitats (two dataloggers in the pool, one in the well) using thermistor probes connected to dataloggers (resolution 0.1°C; HOBO, Onset Computer, Bourne, USA). Dataloggers recorded water temperatures at 30 min intervals during the measurement period. All dataloggers and the digital thermometer were calibrated to the same reference mercury thermometer (resolution 0.2°C) before their use.

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Fig. 1. Horizontal positions of *Salamandra salamandra* larvae from two aquatic habitats, (a) pool and (b) well, in the experimental tank (240 cm long) under either constant temperature (10°C) for control group ($n=18$) or a thermal gradient (7–28°C) for experimental group ($n=18$). The range of horizontal positions is characterized by the minimum and maximum of individual distribution. Data are jittered horizontally to reduce overlap. Population means are presented with 95% CI. Legend in (a) refers to both graphs.

Fig. 2. Preferred body temperatures of *Salamandra salamandra* larvae from two aquatic habitats. The range of preferred body temperatures is characterized by the minimum and maximum of individual distribution. Data are jittered horizontally to reduce overlap. Population means are presented with 95% CI.
temperatures from the $T_p$ range. If $T_e$ falls within the $T_p$ range, the index equals zero. The lower the index value, the better the thermal quality of the habitat and vice versa. (ii) The accuracy of thermoregulation (originally the accuracy of $T_b$; $d_b$), the mean absolute deviation of body temperatures from the $T_p$ range. A lower index value indicates that an individual maintains its body temperature close to the $T_p$ range and vice versa. (iii) The effectiveness of thermoregulation has been calculated as the ratio \( E = \frac{1}{C_0} \frac{d_b}{d_e} \), or as the difference (Blouin-Demers and Weatherhead, 2001), \( E_{\text{diff}} = d_e - d_b \), and thus we used both versions to allow easy comparison with published results. In the case of the ratio, \( E \approx 1 \) indicates effective thermoregulation, \( E \approx 0 \) thermoconformity, and negative values mean active avoidance of target temperatures. Unlike \( E \), positive values of \( E_{\text{diff}} \) are not constrained by one. A higher value simply indicates more effective thermoregulation. Because all body and operative temperatures were below the $T_p$ range, the index calculations were greatly simplified. It also eliminates the bias resulting from merging values from times of day with the various availability of $T_p$ (Christian and Weavers, 1996).

When thermal conditions prevent ectotherms from attaining their target body temperatures, it has been proposed to calculate thermoregulatory indices from the highest $T_e$ rather than from the $T_p$ range (Herczeg et al., 2006). Accordingly, we calculated the thermally-constrained versions of thermoregulatory indices ($d'_b$, $d'_e$, $E'$, and $E_{\text{diff}}'$; Table 1). Because operative temperatures vary during the day in shallow aquatic habitats (Dvořák and Gvoždík, 2010), thermoregulatory indices were calculated separately for each observing interval.

2.6. Statistical analyses

All data were checked visually for their distribution, homogeneity, and the presence of outliers prior to further analyses. Exploratory analysis revealed that $T_p$ boundaries and horizontal positions contained several outliers. Because we had no rationale for deleting the extreme values, we applied the permutation Monte Carlo test (9999 permutations) instead of the parametric $t$-test for comparisons between the experimental group and the control group. Estimated $P$-values are provided with their 99% confidence intervals.

Hourly mean $T_b$, $T_e$, and thermoregulatory indices violated the independence of data assumption, and thus they were analyzed using the mixed-effect model for short time series (Zuur et al., 2009). First, a graphical exploratory analysis identified the candidate fixed factors, i.e., time of day (linear and quadratic), habitat,
and month. Because residuals of the fitted linear model violated the homogeneity assumption, we chose an appropriate variation and optimal random structure (i.e., random intercept or intercept and slope) of the model using the reduced maximum likelihood approach. Given the unique combination of environmental factors, we used the day of measurement as a random factor. In influential fixed factors and their interactions were identified using a maximum likelihood ratio test ($\alpha = 0.05$). Residuals of the best fitted model were checked for normality and homogeneity. All fits were provided with 95% confidence intervals. The confidence intervals (95%) for means were estimated using a bias-corrected and accelerated bootstrapping procedure (9999 resamples). All analyses were performed in R (R Development Core Team, 2014) using ‘MASS’ (Venables and Ripley, 2002), ‘perm’ (Fay and Shaw, 2010), and ‘nlme’ (Pinheiro et al., 2014) libraries.

3. Results

3.1. Thermal preferences

For the control group at constant 10 °C, salamander larvae from both habitats moved on average across 130 cm, 95% CI [116, 147], of the tank length. For the experimental group in the thermal

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pool</th>
<th>Well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>June</td>
<td>July</td>
</tr>
<tr>
<td>Operative</td>
<td>10.8</td>
<td>12.7</td>
</tr>
<tr>
<td>temperatures</td>
<td>[10.6, 11.1]</td>
<td>[12.4, 13.0]</td>
</tr>
<tr>
<td>(°C)</td>
<td>9.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Body temperatures</td>
<td>10.7</td>
<td>12.8</td>
</tr>
<tr>
<td>(°C)</td>
<td>[10.5, 10.9]</td>
<td>[12.5, 13.2]</td>
</tr>
<tr>
<td>Thermal quality of habitat (°C)</td>
<td>5.6</td>
<td>3.4</td>
</tr>
<tr>
<td>$d_e$</td>
<td>[5.4, 5.9]</td>
<td>[3.0, 3.7]</td>
</tr>
<tr>
<td></td>
<td>4.2, 6.7</td>
<td>1.8, 5.8</td>
</tr>
<tr>
<td>$d_e'$</td>
<td>0.7</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>[0.5, 0.9]</td>
<td>[1.4, 2.0]</td>
</tr>
<tr>
<td></td>
<td>0.4, 1.0</td>
<td>1.2, 2.0</td>
</tr>
<tr>
<td>Accuracy of thermoregulation (°C)</td>
<td>5.8</td>
<td>3.7</td>
</tr>
<tr>
<td>$d_b$</td>
<td>[5.6, 6.0]</td>
<td>[3.4, 4.0]</td>
</tr>
<tr>
<td></td>
<td>4.6, 6.9</td>
<td>2.2, 3.9</td>
</tr>
<tr>
<td>$d_b'$</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>[0.8, 1.4]</td>
<td>[1.6, 2.2]</td>
</tr>
<tr>
<td></td>
<td>0.6, 1.6</td>
<td>1.4, 2.4</td>
</tr>
<tr>
<td>Effectiveness of thermoregulation I $E$</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>[0.04, 0.02]</td>
<td>[0.19, 0.05]</td>
</tr>
<tr>
<td></td>
<td>0.12, 0.01</td>
<td>0.76, 0.10</td>
</tr>
<tr>
<td>$E'$</td>
<td>-0.57</td>
<td>-0.12</td>
</tr>
<tr>
<td></td>
<td>[-0.66, -0.45]</td>
<td>[-0.18, -0.03]</td>
</tr>
<tr>
<td></td>
<td>-0.69, -0.37</td>
<td>-0.21, 0.02</td>
</tr>
<tr>
<td>Effectiveness of thermoregulation II (°C)</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>$E_{diff}$</td>
<td>0.2</td>
<td>[0.0, 0.1]</td>
</tr>
<tr>
<td></td>
<td>0.5, 0</td>
<td>1.4, 0.3</td>
</tr>
<tr>
<td>$E_{diff}'$</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>[0.5, 0]</td>
<td>[0.3, 0]</td>
</tr>
<tr>
<td></td>
<td>-0.6, 0.2</td>
<td>0.4, 0.1</td>
</tr>
</tbody>
</table>
gradient, the lower 10th percentile of the horizontal position was higher than for the control group from both habitats (pool: $P=0.0004$, 99% CI [0.000001, 0.001]; well: $P=0.001$, 99% CI [0.0003, 0.003]; Fig. 1). Accordingly, the horizontal distribution range for the experimental group reduced to 56 cm for individuals from the well, 95% CI [40, 83], and 76 cm for individuals from the pool, 95% CI [61, 100], indicating that they preferred temperatures within a narrower range than that provided by the thermal gradient. Larvae from both habitats maintained their body temperatures within similar boundaries (lower boundary: $P=0.29$, 99% CI [0.27, 0.31]; upper boundary: $P=0.79$, 99% CI [0.76, 0.81]; Fig. 2), and thus their joint values were used for further analyses (lower boundary: 16.5 $^\circ$C, 95% CI [15.1, 17.9]; upper boundary: 22.4 $^\circ$C, 95% CI [21.9, 22.8]).

3.2. Operative and body temperatures

Two-hour means of $T_e$ and $T_b$ from each habitat were calculated from 960 records of water temperatures and 8680 records of larval vertical positions. Temperatures were affected by the interaction between habitat and the month, and thus June and July data were analyzed separately. In both months, $T_e$ and $T_b$ increased during daytime ($P<0.002$ in all cases) in a linear (June body temperatures) or curvilinear fashion (all others; Fig. 3; see Table S1 for model parameters). All $T_e$ were below the lower $T_p$ boundary, though the maximum $T_e$ approached $T_p$ more closely in the pool than in the well (Fig. 3a and b). Larvae in the pool were exposed to a higher $T_e$ than in the well (June: $\chi^2=77.08$, $P<0.001$; July: $\chi^2=146.58$, $P<0.001$), though the difference was more pronounced in July. Body temperatures followed the same trend (June: $\chi^2=111.93$, $P<0.001$; July: $\chi^2=171.17$, $P<0.001$; Fig. 3c and d). Monthly means of $T_e$ and $T_b$ were almost the same in both habitats (Table 2).

3.3. Thermal quality of habitat

Because of the interaction between habitat and month, all thermoregulatory indices were analyzed for each month separately. In June, the variation in $d_e$ was best explained by habitat and time of day. The index decreased during daytime (linear effect of time: $\chi^2=8.97$, $P=0.003$; quadratic effect of time: $\chi^2=4.12$, $P=0.040$) and it was consistently lower in the pool than in the well ($\chi^2=68.50$, $P<0.001$; Fig. 4a and b; see Table S1 for model parameters). In July, $d_e$ variation followed the June pattern though differences between habitats were more pronounced (linear effect of time: $\chi^2=26.39$, $P<0.001$; quadratic effect of time: $\chi^2=13.89$, $P=0.001$; Fig. 4c and d).
3.4. Accuracy of thermoregulation

The diel course of the index varied between habitats (habitat: \(\chi^2 = 13.17, P < 0.001\); Fig. 5a) and between months (month: \(\chi^2 = 5.56, P < 0.02\)). In June, the diel course of the index varied between habitats (habitat × hour: \(\chi^2 = 13.17, P < 0.001\); Fig. 5d). The diel course of the index varied between habitats during both months.

The diel course of the index varied during daytime in a quadratic fashion with a lower boundary in the well than in the pool (\(\chi^2 = 22.21, P < 0.001\); quadratic effect: \(\chi^2 = 17.10, P < 0.001\); Fig. 5d). Larvae consistently had their body temperature on average 2.6 °C closer to the lower \(T_p\) boundary in the well than in the pool (\(\chi^2 = 177.77, P < 0.001\); Table 2).

The diel course of the index varied during daytime in a quadratic fashion with a lower boundary in the well than in the pool (\(\chi^2 = 22.21, P < 0.001\); quadratic effect: \(\chi^2 = 17.10, P < 0.001\); Fig. 5d). Larvae consistently had their body temperatures 0.8–12 °C closer to the lower \(T_p\) boundary in the well than in the pool (June: \(\chi^2 = 44.13, P < 0.001\); July: \(\chi^2 = 97.95, P < 0.001\); Fig. 5c and d).

3.5. Effectiveness of thermoregulation

The effectiveness of thermoregulation (\(E\)) decreased during the day (June: \(\chi^2 = 8.09, P = 0.005\); July: \(\chi^2 = 20.19, P < 0.001\); Fig. 6a and b; see Table S1 for model parameters). Habitat had no significant influence on \(E\) variation (June: \(\chi^2 = 1.35, P = 0.25\); July: \(\chi^2 = 0.97, P = 0.32\)). Overall means of the index were close to zero (Table 2), which indicates thermoconformity of larvae in both habitats. The alternative index, \(E_{diff}\), showed similar results as for \(E\) in June (time: \(\chi^2 = 4.96, P = 0.03\); habitat: \(\chi^2 = 0.03, P = 0.85\); Fig. 7a). In July, the negative \(E_{diff}\) values of the index in larvae from the well were lower than from the pool (\(\chi^2 = 21.40, P < 0.001\); Fig. 7b). The magnitude of this difference (0.3 °C) was fairly low to
be considered ecologically relevant. The thermally-constrained effectiveness of thermoregulation \( (E') \) remained stable throughout the day in June \( (\chi^2 = 0.96, P = 0.33) \). The index markedly varied between habitats \( (\chi^2 = 32.47, P < 0.001) \). Their disparate values indicated that larvae moderately thermoregulated in the well, while on average avoided the highest attainable temperatures in the pool (Fig. 6c). In July, the daily pattern of \( E' \) varied between habitats \( (\text{habitat} \times \text{time}; \chi^2 = 20.95, P < 0.001) \). The index decreased during the day in larvae from the well, whereas in the pool, stable index values close to zero suggest that larvae thermoconformed during the day (Fig. 6d). The alternative index, \( E_{\text{diff}}' \), showed similar but less pronounced variation between habitats \( (\text{June}; \chi^2 = 13.86, P < 0.001; \text{July}; \chi^2 = 37.01, P < 0.001) \). Their positive values still indicate some degree of thermoregulation, at least for a few hours per day, in larvae from the well, while values with 95% CI including zero suggests thermoconformity for larvae in the pool (Fig. 7c and d; Table 2).

4. Discussion

4.1. Thermoregulatory strategies

Although salamander larvae exhibited thermoregulatory behavior in the laboratory, individuals were passive to thermal conditions in two natural habitats. Similarly, adult terrestrial salamanders showing temperature preferences in the thermal gradient are constrained in their ability to thermoregulate (Feder and Pough, 1975; Feder, 1983; Sievert and Andreadis, 2002; but see Camp et al., 2013). These findings corroborate the prediction that if costs of thermoregulation are high relative to its benefits or the thermal environment constrains thermoregulation, ectotherms should abandon thermoregulatory behavior (Huey and Slatkin, 1976; Angilletta et al., 2006). Exact cost and benefit curves of behavioral thermoregulation are lacking for salamander larvae. However, we propose that under lower, less-variable operative temperatures, the possible benefits of increasing body temperature 0–3 °C above the average operative temperature may be too low to outweigh the possible costs associated with the permanent occurrence at the warmest section of the water column. Such costs may include the missed opportunity to capture prey, because important prey categories of salamander larvae, such as oligochaetes and chironomid larvae (Thiesmeier and

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**Fig. 6.** Index of effectiveness of thermoregulation as a function of time of day of *S. salamandra* larvae in two aquatic habitats, pool and well, during June and July. Graphs (a, b) and (c, d) show the original \((E)\) and thermally-constrained \((E')\) versions of the index, respectively. Note that operative and body temperatures (Fig. 2) indicate thermoconformity in both habitats, i.e. \(E \equiv 0\). See Table 1 for definitions. Data in (c) and (d) are shifted horizontally to reduce overlap. Data are fitted with the general linear mixed model (95% CI). See Table S1 for model parameters. Legend in (a) refers to all graphs.
Grossenbacher, 2004), largely occur on the bottom of water bodies. In addition, staying close to the water surface poses high predation risk to aquatic organisms (Kramer et al., 1983; Rodriguez-Prieto et al., 2006).

Although results agreed with the theory, they are in contrast with a number of field studies showing that ectotherms carefully thermoregulate despite increasing costs in thermally challenging habitats (Blouin-Demers and Weatherhead, 2001; Gvoždík, 2002; Blouin-Demers and Nadeau, 2005). In previous studies, although representing different ectothermic taxa, all were able to attain their preferred body temperatures at least during a part of the day or season. However, salamander larvae from these populations had very limited thermoregulatory opportunities even during the warmest months. This may be the rule for this population because previous thermal records for these locations revealed similarly cold thermal regimes (K. Piasečná and L. Gvoždík, unpublished data). The disparate results between previous studies and our field-based study indicate that current theoretical predictions require additional testing. Furthermore, the limited replication of thermal habitats in our study further data on thermoregulatory behavior in ectotherms that inhabit more-thermally-challenging habitats, which includes specific information on cost–benefit curves for thermoregulation, may help to explain the thermoregulatory strategy by salamanders in cold environments.

The most puzzling finding is that salamander larvae thrive in habitats in which they could not attain their preferred temperatures. Theory assumes that ectotherms perform optimally within the $T_p$ range (Angilletta et al., 2002). According to this, salamander larvae either mismatched their thermal optima for physiological functions and the $T_p$ range (Gvoždík, 2015) or they are thermal generalists with slowly decreasing performance at lower temperatures. Unfortunately, information about the thermal dependence of larval performance traits is lacking from our study population. Available results for other fire salamander populations showed that the fastest growth and development of fire salamander larvae occurred within a relatively wide range (10–20 °C; Zakrzewski, 1987; M. Tejedo and I.C. Pereira, unpublished results), which corroborates the latter option.

Although salamander larvae from the population studied live in a habitat that prevents them from thermoregulating, they still possess the ability. At first, this observation is a rather peculiar result because salamander larvae mostly develop in flowing water, such as springs and streams, where water temperatures are nearly constant throughout the water column (Thiesmeier and Grossenbacher, 2004). However, in some populations fire salamanders reproduce in still water, such as pools, puddles, and ponds (Zakrzewski, 1987; Weitere et al., 2004; this study), where the vertical thermal gradient provides some opportunity for behavioral thermoregulation (Hadamová and Gvoždík, 2011). Even in flowing waters larvae may occasionally thermoregulate, if dry weather

Fig. 7. Alternative index of effectiveness of thermoregulation as a function of time of day of S. salamandra larvae in two aquatic habitats, pool and well, during June and July. Graphs (a, b) and (c, d) show the original ($E_{diff}$) and thermally-constrained ($E_{diff}'$) versions of the index, respectively. See Table 1 for definitions. Note that operative and body temperatures (Fig. 2) indicate thermoconformity in both habitats, i.e. $E_{diff} = 0$. Data in (c) and (d) are shifted horizontally to reduce overlap. Data are fitted with the general linear mixed model (95% CI). See Table S1 for model parameters. Legend in (a) refers to all graphs.
interrupts the stream into a series of separated pools (Jablonski et al., 2013). Given the spatiotemporal variation in thermal conditions for larval development, it seems likely that behavioral thermoregulation can be advantageous over thermoconformity in some habitats, and thus the thermoregulatory ability of larvae is still maintained within salamander populations. Unfortunately, the limited replication of thermal habitats in our study prevents us to examine this issue in further detail.

Compared to cold conditions in their natural habitats, salamander larvae preferred relatively high body temperatures in the laboratory thermal gradient. Given the rather low evolutionary rates of this trait in a related salamandrid clade (Gvoždík, 2015), high $T_p$ (see also Degani, 1984) might be inherited from the ancestor of fire salamanders living in a warmer climate (Vences et al., 2014). Alternatively, because their values are close to $T_p$ of newt larvae living in the same area but developing in warmer pools (Smolinský and Gvoždík, 2012), it suggests that larvae originally developed in warmer still-waters allowing behavioral thermoregulation, while the ecological shift to habitats with low and nearly constant thermal conditions occurred relatively recently in Western and Central European populations (Weitere et al., 2004). Another possible explanation is that larval $T_p$ match the thermal requirements of adults due to weak selection on $T_p$ during larval development. A similar mean $T_p$ in adults (Strübing, 1954) provides some support for this explanation.

4.2. Thermoregulatory indices

The comparison of results obtained using original and thermally-constrained versions of thermoregulatory indices with $T_e$ and $T_b$ revealed large discrepancies. While the original index ($E$) suggests thermoconformity in both habitats, the thermally-constrained version ($E'$) indicated significant spatiotemporal variation, i.e., between habitats and months, in thermally-driven behavior ranging from moderate thermoregulation to thermoconformity and thermal evasion. In contrast, the same mean and range of $T_e$ and $T_b$ indicate ineffective thermoregulation or thermoconformity. This clearly shows that the variation in thermally-constrained thermoregulatory indices results from a methodological artifact without ecological relevance.

The calculation of thermoregulatory indices from the maximum attainable temperatures ($d_e$, $d_b$) rather than from the $T_p$ boundary ($d_e$, $d_b$) could bias their interpretation in two ways. First, according to $d_e$, the thermal habitat for larval thermoregulation had better quality in the well than in the pool. In fact, $d_e$ values suggest exactly the opposite. In the well, larvae experienced nearly constant water temperatures that were far below the lower $T_p$ boundary. In contrast, mean water temperatures in the pool were much higher and variable, both on a daily and seasonal basis, with temperature maxima occasionally approaching the lower $T_p$ boundary (Fig. 2). Simply, the low $d_e$ values resulted only from the minor variation in water temperatures irrespective of their distance from the $T_p$ range, which provides misleading information about the quality of thermal conditions for thermoregulation and prevents its comparison among habitats.

Second, $d_b$ suggest that larvae thermoregulated more accurately in the well than in the pool. This is also misleading, because a non-thermoregulating ectotherm will always have lower $d_b$, i.e., the mean deviation of $T_b$ from the maximum $T_e$ in a thermally nearly homogeneous habitat than in a thermally heterogeneous habitat. On the other hand, it can be argued that the original index ($d_b$), is also misleading because it ignores the confounding effect of thermal constraints. However, this issue can be solved for example by quantifying the magnitude of thermal constraints (lower $T_p$ boundary-maximum $T_e$) and using this variable as the covariate in statistical models.

The thermally-constrained effectiveness of thermoregulation ($E'$) produced values that indicate a notable variation in thermoregulatory strategies. However, because the index was calculated using dubious $d_e$ and $d_b$ values, results are hard to interpret. In addition, the relatively high variation in $E'$ resulted from rather small 0.1–0.2 °C differences in $d_e$ and $d_b$ corroborating earlier suggestions for abandoning the use of $E$ in favor of its alternative based on differences between indices ($E_{diff}$ Blouin-Demers and Weatherhead, 2001). A further advantage of $E_{diff}$ is that it provides a measure of effect size. This is especially useful in thermally extreme habitats as in our case, because it clearly shows that the differences between $d_e$ and $d_b$ were too small to be ecologically relevant.

4.3. Conclusions

Our study demonstrated that despite substantial methodological and conceptual advances in this field (Hertz et al., 1993; Christian and Weavers, 1996; Blouin-Demers and Weatherhead, 2001), the quantification of thermoregulatory strategies under thermally constrained conditions still remains problematic. Surprisingly, the application of the originally-proposed thermoregulatory indices (Hertz et al., 1993) with the additional alternative calculation of thermoregulatory effectiveness (Blouin-Demers and Weatherhead, 2001), produced better results than the thermally-constrained alternatives (Herczeg et al., 2006), which should be used with extreme caution, i.e. under controlled laboratory settings, or abandoned altogether.

The correct quantification of thermoregulatory strategies revealed that salamander larvae are thermally passive in their native habitats providing a rare reported case of thermoconformity in temperate aquatic amphibians. Although our findings are based on aquatic organisms that are exposed to less thermally heterogeneous conditions than their terrestrial counterparts, such data illuminate a limitation of current research on thermal ecology. The thermal conditions vary greatly among water bodies (Hadamová and Gvoždík, 2011; this study), which, together with biotic factors, produce various costs of thermoregulation for amphibian larvae with proven thermoregulatory abilities (Smolinský and Gvoždík, 2012; this study). The vertical thermal stratification in still waters allows replicable and easily measurable thermal conditions for experimental thermoregulatory studies (Gvoždík et al., 2013; Balogová and Gvoždík, 2015). Finally, the less effective thermoregulation in aquatic than in terrestrial taxa allows tests of predictions about the complex thermal strategies in ectotherms (Angilletta et al., 2006).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in
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References


