Temperature Preference During Forelimb Regeneration in the Red-Spotted Newt *Notophthalmus Viridescens*

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Red-spotted newts (*Notophthalmus viridescens*) are model organisms for regenerative research. These animals can regenerate limbs, tails, jaws, spinal cords, as well as the lens of the eye. Newts are small ectotherms that are aquatic as adults; as ectotherms, they naturally conform to the temperature of their surroundings. Environmental temperatures, however, can increase or decrease the red-spotted newt’s metabolic processes, including their rate of tissue regeneration; whether an optimal temperature for this rate of regeneration exists is unknown. However, newts do exhibit behavioral preferences for certain temperatures, and these thermal preferences can change with season or with acclimation. Given this flexibility in behavioral thermoregulation, we hypothesized that the process of tissue regeneration could also affect thermal preference, given the metabolic costs or altered temperature sensitivities of tissue regrowth. It was predicted that regenerating newts would select an environmental temperature that maximized the rate of regeneration, however, this prediction was not fully supported. Thermal preference trials revealed that newts consistently selected temperatures between 24 and 25°C throughout regeneration. This temperature selection was warmer than that of uninjured conspecifics, but was lower than temperatures that would have further augmented the rate of regeneration. Interestingly, regenerating newts maintained a more stable temperature preference than sham newts, suggesting that accuracy in thermoregulation may be more important to regenerating individuals, than to noninjured individuals. *J. Exp. Zool.* 317:248–258, 2012. © 2012 Wiley Periodicals, Inc.

physiological functions such as metabolic rate, muscle activity, blood flow, digestion, and wound healing. Schauble and Nentwig (’74) observed that the rate of forelimb regeneration increased with increasing temperature, with low temperatures (10°C) effectively inhibiting regeneration. These findings are similar to thermal effects on limb regeneration in fiddler crabs (Weis, ’76). Schauble and Nentwig (’74) also explored regeneration by examining both the rate of tissue growth as well as the rate of differentiation. Rate of growth measured the amount of new tissue produced from regenerating forelimbs over time, while the rate of differentiation measured the animal’s limb morphology on a scale from 1 (wound healing) to 13 (completed differentiating). They found that 25°C led to maximum new tissue growth, while 30°C maximized the rate of differentiation. This disparity between measurements of rate of regeneration suggests that although an optimal temperature for both growth and differentiation might exist between 25 and 30°C, that tissue growth and differentiation display different sensitivities to temperature.

Like many ectotherms, however, red-spotted newts use behavioral mechanisms to maintain a preferred body temperature if microhabitats are available; this thermal selection is also season dependent (Berner and Bessay, 2006; Berner and Puckett, 2010). Temperature preference also co-varies with seasonal biochemical changes, suggesting an association between biochemical function and optimal or preferred temperatures (Berner and Puckett, 2010). However, it is unknown whether the biochemical and developmental processes that accompany regeneration from injury are optimized to thermal preference or, indeed whether regeneration influences thermal preference. Although limbs and peripheral structures have been shown to be involved in body heat distribution throughout the body (Tattersall et al., 2009; Darnell and Munguia, 2011), studies on tail autotomy in lizards have found that animals with regenerating tails do not alter temperature preference in a thermal gradient or exhibit different field body temperatures compared to tailed lizards (Chapple and Swain, 2004; Herczeg et al., 2004). However, regenerating lizards alter their basking behavior and microhabitat use in the wild in order to maintain normal thermoregulation (Martin and Salvador, ’93). In all cases, however, body temperature appears to remain constant during tail regeneration, suggesting that behaviors may change to facilitate this constancy in thermal preference.

Nevertheless, forelimb amputation and subsequent wound healing initiates a signal cascade that coordinates immune responses, cell proliferation, cell differentiation, and cell migration (Roy and Levesque, 2006). Since thermoregulation is often altered by different immune states through neuronal or humoral substrates (e.g. TNF-a, IL-6; Bicego et al., 2006), we predict that red-spotted newts will alter their thermal preference while regenerating, selecting temperatures that optimize the rate of regeneration, particularly during the early phases associated with wound healing, cellular dedifferentiation and mitosis resulting in the accumulation of a population of blastema cells. Further, by examining the effects of environmental temperature on regeneration over a narrow range of temperatures (23–29°C), we hypothesize that even small increases in temperature will continue to lead to increases in the rate of regeneration. Additionally, based on the premise that increased metabolic activity itself accompanies local tissue healing, we examined the thermal condition of limbs in the first 2 weeks following amputation.

MATERIALS AND METHODS

Animals, Husbandry, and Forelimb Amputation

Ninety-two adult red-spotted newts (N. viridescens) were obtained from Boreal Labs (St. Catharines, ON, Canada), derived from wild-caught populations in Massachusetts. Animals were identified using individual spot patterns, and mass (ranging from 0.71 to 3.08 g) and snout-vent length (ranging from 30.71 to 44.69 mm) measurements were taken. Newts were housed in rectangular plastic containers with perforated plastic lids (4–5 animals per container). Containers were lined with damp paper towel, half-filled with dechlorinated water and angled to provide newts a choice of being in or out of the water. Newts were fed frozen brine shrimp by hand, which they ate readily, ad libitum three times per week for the duration of the experiments. Tanks were cleaned following feeding. A 12:12 hr light/dark cycle was maintained. Newts were given a minimum of 1-week acclimation prior to forelimb amputation.

Animals were anaesthetized in a bath containing 0.1% MS-222 (Sigma) in dechlorinated water (buffered to pH 7.0 using sodium bicarbonate). Right forelimbs were amputated through the mid-radius/ulna and protruding bones trimmed to the level of the soft tissue. Newts were placed in an ice bath until bleeding significantly slowed or stopped (∼10 min). Animals were then placed in the small, angled plastic containers on damp paper towel and monitored until recovery from anesthetic was evident. All protocols were approved by the Brock University Animal Care and Use Committee.

Series I: Effect of Temperature on Rate of Regeneration

Upon arrival, 40 newts were separated randomly into four temperature groups (23, 25, 27, 29°C) and housed in separate plastic containers. The containers were kept within one of four diurnal growth chambers (ThermoForma) set at 23, 25, 27, and 29°C. Temperature within the growth chambers fluctuated by about ±0.1°C. Each diurnal growth chamber housed 10 newts. Newts were given 1-week acclimation within the diurnal growth chambers prior to limb amputation (see above).

To quantify the extent of limb regeneration, animals were positioned individually with their right forelimbs flat against the bottom of a glass petri dish under a dissecting scope (Leica MS5). Newts were gently held in place by their tails. A grid
(0.5 x 0.5 cm²) was placed under the petri dish as a known measurement. Digital images of regenerating limbs were taken with NIS Element [F 3.0], using a microscope camera (Nikon Digital Sight DS-FiL), between 2 and 70 days post amputation (dpa). Two dimensions of regeneration rate were measured using the photographs of regenerating forelimbs: rate of differentiation and rate of growth (as in Sessions and Larson, ’87). The degree of differentiation of the regenerating forelimb was measured as the external appearance using the staging system of Iten and Bryant (’73) at the intervals of 7, 14, 19, 23, 26, 28, 33, 44, 50, and 70 dpa. This system includes 13 morphological stages. Outgrowth was measured on days 14, 23, 50, and 70 post amputation, using image analysis software (Image J, v1.42), from the amputation plane to the tip of the regenerating limb bud down the midpoint of the limb. These time points should correspond with the formation of early limb buds (14 dpa), moving from palette to early digits (23 dpa), and the completion of outgrowth (70 dpa; Iten and Bryant, ’73). The temperature sensitivity of outgrowth was assessed by calculating the Q₁₀ (across 23–29 °C) for the rates of outgrowth between 0–14, 14–23, 23–50, and 50–70 dpa. Q₁₀,5 were determined by solving for the activation energy from the slope of ln(rate) vs. 1/Temperature, and rearranging the Arrhenius equation as described by Withers (’92).

Series IIa: Thermal Preference of Regenerating Newts
Forty-four newts (separate from the ones in Series I) were housed in an enriched aquarium with dechlorinated water at ~23 °C for 2 weeks following arrival in the lab prior to behavioral trials. Newts were then randomly designated the one of two treatments (sham or regenerating) by coin toss for a total of 22 regenerating and 22 sham individuals. Animals within an individual group were housed in a rectangular plastic container with perforated plastic lid (see description above) 1 day prior to the initiation of behavioral experiments. Water temperature in these small tanks was kept at 23 ± 1 °C throughout the experimental trials. Although all animals were fed three times per week, days of feeding varied to ensure that newts were fed 1 day prior to testing in the thermal gradient to maintain consistent satiation. Limb amputation was performed as described above. Sham animals were anesthetized and placed on ice as above but sustained no injury. An effort was made to begin behavioral experiments at the same time of day, with the majority of experiments starting between 0900 h and 1000 h.

A thermal gradient apparatus with plexiglass walls and a copper floor (27 x 54 cm) was constructed and sealed at the joints to prevent water leakage. The walls of the apparatus were notched to allow the insertion of three opaque plastic dividers, creating four individual lanes (6.75 x 54 cm). Each lane was wide enough to allow a newt to turn around and move without constraint. The apparatus was filled with 1.2-L dechlorinated water, providing a depth of 5-mm water, enough to avoid desiccation while preventing the establishment of a vertical stratification of temperature. Fluid (ethylene glycol) was circulated underneath each end of the gradient apparatus through copper tubing. Cold fluid was pumped through the copper tubing underneath one end of the apparatus while hot fluid was pumped underneath the other. A range of temperatures from about 10 to 40 °C was selected, similar to Berner and Bessay (2006). Average gradient temperatures ranged from 9.1 (±0.03) to 39.0 °C (±0.04) and our preliminary trials found newts staying at 25–27 °C, leaving a considerable temperature range for newts to potentially select warmer or cooler temperatures. The copper floor was covered with white adhesive (contact) paper that was marked with a line every 2.5 cm down the length of the apparatus. The temperature of the gradient in each lane was determined prior to every thermal preference trial with a thermocouple meter (Sable Systems TC1000).

To determine the thermal preference of the newts, individuals were randomly placed into the centre of the gradient within one of the four lanes. Each individual was oriented randomly toward either the hot or cold end of the gradient. A web camera (Microsoft LifeCam), positioned above the gradient captured time-lapsed images using Flix 3.3 (Nimis) every 5 min. Newts were kept in the gradient for a total of 5 hr. The first 2 hr were treated as a habituation period, allowing the newts to investigate their enclosure. Data from this initial period were not included in statistical analyses. The majority of newts settled into one area of the thermal gradient within this time period. Two cohorts of newts were studied, one group (N = 10 sham and N = 10 amputees; also the same newts used in Series III experiments below) that was studied up to 14-day post amputation (dpa), and a second group (N = 12 sham and N = 12 amputees) that was studied weekly up to 5-week post amputation. In total, the newts’ thermal preferences were tested at the following time points: 1 day prior to anaesthetic and amputation and again at 1, 3, 7, 10, and 14 dpa (first group), and at −1, 7, 14, 21, 28 and 35 days for the second group. Sham individuals underwent anaesthetic and thermal preference trials at the same time as regenerating individuals in their group, but sustained no injury. Average temperature, 75th and 25th percentiles, and the coefficient of variation (CV) of the observed selected temperatures (SD/average × 100) were assessed for the final 3 hr of each trial and used in the statistical analysis.

Series IIb: Constant Temperature Behavioral Trials
An additional experiment was designed to test whether regeneration was associated with a lower propensity to move, independent of a thermal gradient. The thermal gradient apparatus was modified so that the temperature across the gradient was consistently 25 °C. All other variables were the same as in the above experiment. If regenerating newts preferred a given temperature, we expected animals to move back and forth in the thermal apparatus throughout the time period, around a distinct temperature level. If regenerating newts were less active,
however, we may expect them to explore the thermal apparatus during the 2 hr habituation period and then randomly select a stationary position, and thus mean selected temperature and precision of thermoregulation would be influenced by the regenerative process. The initial movements of eight uninjured newts across the thermal apparatus were recorded at 5 min intervals for a period of 5 hr as described above. As above, the first 2 hr were considered habituation time and data from this time period were not analyzed statistically. The animals’ right forelimbs were amputated, as above. Seven dpa, the regenerating newts’ movements within the 25°C thermal apparatus were recorded at 5-min intervals for a period of 5 hr, with 1 hr acclimation. The CV for distance along the gradient chamber for each newt under each trial was obtained from the 3-hr measurement period and used in statistical analysis.

Series III: Thermal Imaging of Regenerating Limbs
Regenerating newts (same individuals as in Series IIA) were placed individually in a 12.5-cm diameter plastic container, with 15-cm high walls that prevented newts from climbing out. Since newts are small ectotherms, and body temperature closely matches that of the environment, an infrared thermally contrastive substrate (reflective aluminum duct tape) was adhered to the floor of the container. The difference in infrared emissivity between the animal and the aluminum allowed for ready identification of the animal from the background temperature. A strip of black electrical tape (emissivity similar to tissue) was placed on the bottom of the container as the background thermal reference. The container was located within a temperature-controlled environmental chamber, in which the temperature was controlled (24.6 ± 0.1°C on average) using a water bath connected to an internally mounted heat exchanger/fan assembly. High humidity (mean: 86.7 ± 0.5%) was maintained internally by bubbling water with an air stone in order to reduce evaporative heat loss from newts’ skin. Humidity and temperature were measured using a Type T environmental meter (TC-1000, Sable Systems). A thermal imaging camera (Mikron model 7515) was located at the top of the sealed chamber and a perforation was made through the lid in order for the lens to enter the chamber. Assumptions regarding emissivity and thermal image analysis followed routinely employed techniques in animal thermography (Tattersall and Gerlach, 2005; Tattersall et al., 2009; Tattersall and Cadena, 2010).

Thermal imaging immediately followed the thermal preference trials on days 1, 7, and 14 post amputation. All 10 regenerating newts were imaged at days 1 and 7, but only eight newts were imaged for day 14 post amputation because of thermal camera availability. Excess water was removed from the newts’ skin prior to placement in the chamber by placing animals briefly on dry paper towel. Thermal image data were collected every 10 sec for 20 min immediately following the animal’s placement in the chamber and analyzed using “regions of interest” tools with MikroSpec RT software (Mikron). When both forelimbs were visible in an image frame, a bent line was drawn down the center of each forelimb, avoiding the refraction that occurs at the edge formed by the surface of the limb with the underlying floor. Lines were drawn proximodistally on each arm to the regenerating plane so that both lines measured the same distance. The average temperature of the pixels that made up each line was recorded for each analyzed frame, and the mean regenerating and uninjured forelimb temperatures were compared.

Statistical Analyses
All statistical analyses were performed using SPSS Statistics 17.0. Values are reported as mean ± standard deviation (SD), unless otherwise specified. Rates of regeneration (differentiation and growth) and comparisons of thermal preference between treatments were analyzed using one-way ANOVAs, and individual means compared using post-hoc tests (Holm-Sidak). In cases where normality or equal variance assumptions could not be validated through log transformation, Kruskal–Wallis one-way ANOVAs on ranks were performed. Thermal preferences within treatments (regenerating and sham), were compared using repeated measures ANOVAs, and individual means compared using post-hoc tests (Holm-Sidak). Thermal imaging of regenerating and uninjured limbs was analyzed using pairwise t-tests. Body condition (weight and snout-vent length) between groups and between treatments was compared using one-way ANOVA. All statistical tests were compared against an α = 0.05.

RESULTS
Series I: Effect of Temperature on Rate of Regeneration
It was expected that small increases in temperature would result in increasing rates of forelimb regeneration, affecting both the rate of differentiation and rate of growth. The effects of temperature on differentiation were minimal in the first 2 weeks (Table 1), when limbs were mostly undergoing wound healing (stage 1) and blastema formation (stages 2 and 3; see Fig. 1). Following this period, the effects of temperature on differentiation became increasingly distinct with the rate of differentiation increasing with increasing temperature. This trend continued until about day 30 when limbs at higher temperatures were nearing completion of differentiation (stages 10–13).

The mean rate of differentiation during the rapid phase of regeneration (represented as the rate across the first 0–33 days) increased with temperature, but was only significantly lower at 23°C (Table 1). Housing newts at 29°C produced 100% of newts reaching stage 13 by 70 dpa; animals held at 23°C showed delayed differentiation, with only 20% of the newts reaching stage 13 (i.e., completion) by 70 dpa. Limb stages at 23°C ranged from 9 to 13 at 70 dpa. Animals housed at 23°C had the lowest rate of differentiation of all groups (Table 1). At 25°C, 60% of the newts reached stage 13 by 70 dpa with stages ranging from...
Table 1. Mean rate (±SD) for rate of forelimb differentiation (stages/number of days post amputation) of red-spotted newts housed at four environmental temperatures: 23, 25, 27, and 29°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>0–14 dpa (N)</th>
<th>14–23 dpa (N)</th>
<th>23–50 dpa (N)</th>
<th>50–70 dpa (N)</th>
<th>0–70 dpa</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>0.16 ± 0.054 (10)</td>
<td>0.074 ± 0.093 (10)</td>
<td>0.18 ± 0.11 (6)</td>
<td>0.080 ± 0.050 (5)</td>
<td>0.11 ± 0.039</td>
</tr>
<tr>
<td>25</td>
<td>0.16 ± 0.062 (10)</td>
<td>0.28 ± 0.23 (10)</td>
<td>0.20 ± 0.076 (10)</td>
<td>0.037 ± 0.030 (10)</td>
<td>0.30 ± 0.091</td>
</tr>
<tr>
<td>27</td>
<td>0.20 ± 0.080 (10)</td>
<td>0.42 ± 0.27 (9)</td>
<td>0.15 ± 0.055 (9)</td>
<td>0.13 ± 0.33 (8)</td>
<td>0.32 ± 0.10</td>
</tr>
<tr>
<td>29</td>
<td>0.21 ± 0.066 (10)</td>
<td>0.63 ± 0.36 (10)</td>
<td>0.11 ± 0.073 (8)</td>
<td>0.012 ± 0.023 (8)</td>
<td>0.36 ± 0.13</td>
</tr>
</tbody>
</table>

Statistical differences:
- NS, not significant.
- $F_{3,36} = 1.35$, $P = 0.27$
- $F_{3,36} = 3.36$, $P = 0.024$
- $H_f = 11.7$, $P = 0.006$
- $H_f = 4.3$, $P = 0.228$
- $23 \neq 29$
- $25 \neq 29$
- $23 \neq 25$, $23 \neq 27$

Figure 1. Mean stage of differentiation (±SD) by days post amputation (dpa) for regenerating red-spotted newts housed in four thermal environments: 23°C (filled circles), 25°C (open circles), 27°C (filled triangles), and 29°C (open triangles). Stage 1 refers to wound healing, and stage 13 refers to complete regeneration, and thus the asymptotic approach to completion, according to the staging system of Iten and Bryant (’73). Summary rates of differentiation (and sample sizes) are depicted in Table 1. Lines of sigmoidal fits are depicted in progressive grey-scale spline curves to assist in following the staging data.

9 to 13. At 27°C, 63% of the newts reached stage 13 by 70 dpa with stages ranging from 7 to 13.

Rate of growth (length of total forelimb outgrowth from amputation plane/dpa) was also affected by temperature, although more variably during the early stages of regeneration (Fig. 2). Housing animals at 23°C led to stunted, albeit continuous, forelimb growth; these newts maintained the lowest overall rate of growth (Table 2). Rates of growth at 23°C were generally significantly lower than 27 and 29°C, while overall rates (0–70 days) of growth at 29°C were significantly greater than rates at all other temperatures.

During this particular experiment, nine animals did not survive to the 70th day; five from 23°C, one from 27°C, and two from 29°C. Body condition was examined as a potential contributing factor; there was no significant difference in animal weight or snout-vent length between the four temperature groups (weight: $F_{3,36} = 1.070$, $P = 0.375$; snout-vent length: $F_{3,36} = 1.439$, $P = 0.248$), nor was there a difference in weight or snout-vent length between animals that lived and animals that died (weight: $t_{38} = 0.72$; $P = 0.475$; snout-vent length: $t_{38} = 0.842$; $P = 0.405$). Temperature appeared to be the primary factor driving the successful survival during regeneration; comparing the low survival group (23°C) to the higher survival groups (combined 25–29°C to eliminate 0 scores) yielded a significant effect of temperature on survival (Fisher’s Exact Test, $P = 0.029$).

Series IIa: Thermal Preference of Newts during Early Forelimb Regeneration

The initial temperature selections of both groups of animals prior to amputation were virtually identical (Fig. 3). Over the course of 2-week post amputation, however, regenerating newts consistently maintained their selected temperature of 24.2°C while...
sham animals’ mean temperature selection steadily decreased upon testing, reaching significance from the regenerating newts at day 14 ($P = 0.001$). By day 14 and 21, regenerating newts were selecting environmental temperatures 2°C warmer than sham animals (post-hoc Holm-Sidak $P = 0.007$ and 0.038) even though their selected temperature did not vary over the experimental time period ($P = 0.235$). Body condition did not differ between regenerating and sham animals (weight: $P = 0.981$; snout-vent length: $P = 0.981$).

An examination of newt movements in the thermal gradient apparatus during the preference trials revealed that uninjured animals explored the gradient more each time that they were placed in the apparatus. Regenerating newts tended to explore the apparatus for the initial 2 hr and then settle around a small temperature range. Uninjured newts tended to spend more time exploring the cooler end of the gradient; a visible aversion to high temperatures was observed in both groups as witnessed by the 75th and 25th percentiles (Fig. 3). Often, newts sitting at the warm region of the gradient were observed curling their tail toward the cool end in what appeared to be an avoidance of extreme temperatures. Uninjured newts’ increasing trend to explore the temperature apparatus affected the variability of thermoregulation (CV of selected temperature), such that the CV of sham newts at 7 and 21 dpa was significantly greater than that of regenerating newts on the same day (Fig. 3). Regenerating newts’ CV remained stable throughout the experiment. The variability in thermal preference was driven exclusively by changes in the lower 25th percentile of selected temperatures that were significantly higher in the regenerating newts compared to sham newts at 7, 14, and 21 dpa.

### Series IIIb: Constant Temperature Behavioral Trials

Unlike the thermal gradient trials, regenerating newts moved back and forth across the constant 25°C apparatus for the entire time period. For both pre- (−1 day) and post amputation trials (7 dpa), there was a visible edge effect, where animals showed a preference for the ends of the gradient. This effect was not seen in the thermal gradient trials. Indeed, no animals were observed in the 39°C end of the gradient. Regenerating animals moved frequently across the constant temperature apparatus and

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**Table 2.** Mean rate of outgrowth (±SD) of regenerated tissue from amputation plane/number of days post amputation (dpa) for regenerating forelimbs of red-spotted newts, housed at four environmental temperatures: 23, 25, 27, and 29°C (see Table 1 for sample sizes).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>0–14 dpa</th>
<th>14–23 dpa</th>
<th>23–50 dpa</th>
<th>50–70 dpa</th>
<th>0–70 dpa</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>0.021 ± 0.011</td>
<td>0.045 ± 0.020</td>
<td>0.035 ± 0.023</td>
<td>0.080 ± 0.014</td>
<td>0.032 ± 0.0046</td>
</tr>
<tr>
<td>25</td>
<td>0.027 ± 0.019</td>
<td>0.065 ± 0.047</td>
<td>0.043 ± 0.015</td>
<td>0.11 ± 0.039</td>
<td>0.047 ± 0.013</td>
</tr>
<tr>
<td>27</td>
<td>0.057 ± 0.027</td>
<td>0.054 ± 0.051</td>
<td>0.051 ± 0.024</td>
<td>0.092 ± 0.034</td>
<td>0.048 ± 0.011</td>
</tr>
<tr>
<td>29</td>
<td>0.041 ± 0.020</td>
<td>0.15 ± 0.067</td>
<td>0.052 ± 0.029</td>
<td>0.12 ± 0.037</td>
<td>0.061 ± 0.011</td>
</tr>
</tbody>
</table>

Statistical differences:
- $F_{23,35} = 6.1, \quad P = 0.002$
- $F_{23,29} = 8.6, \quad P < 0.001$
- $F_{23,39} = 0.65, \quad P = 0.58$
- $F_{23,35} = 11.0, \quad P < 0.001$
- $23 \neq 27, 25 \neq 29, 27 \neq 29$
- $23 \neq 25, 25 \neq 27, 27 \neq 29$

**Figure 2.** Mean level of forelimb outgrowth (±SD) by days post amputation (dpa) for regenerating red-spotted newts housed in four thermal environments: 23 (filled circles), 25 (open circles), 27 (filled triangles), and 29°C (open triangles). Outgrowth patterns were mostly linear at each temperature (rates are depicted in Table 2, sample sizes are shown in Table 1). Also shown (plus sign and heavy dotted lines) are $Q_{10}$ values (an assessment of temperature sensitivity of outgrowth) over the 0–14, 14–23, 23–50, and 50–70 days post amputation.
Figure 3. Mean selected temperature parameters (±SEM, for graphical clarity) in sham and regenerating newts. (A) mean selected temperature (°C) of regenerating (open circles) and sham (closed circles) newts tested in a thermal gradient apparatus, at the following time points: pre-amputation (−1), days 1, 3, 7, 10, 14, 21, 28, and 35 days post amputation; (B) coefficient of variation of the selected ambient temperatures of the same newts; (C) 75th percentile of observed selected temperatures in the same newts as above; (D) 25th percentile of observed selected temperatures in the same newts as above. Significant differences (post-hoc Holm-Sidak test) between treatments are indicated by an asterisk (*), while significant differences from the pre-amputation value, but within treatments are indicated by the cross symbol (†). Sample sizes for days −1, 1, 3, 7, 10, and 14 were 22 for each treatment group; for all other days, there were 12 animals per treatment group.
performance, (Pitkin, '77; Else and Bennett, '87; Jiang and Claussen, '93; Denoel, '98; Denoel et al., 2005). Given this temperature sensitivity, this study tested whether the regenerative process is governed by mutual strategies for mitigating the magnitude of the temperature effects: either the temperature sensitivity of regeneration is minimal, thereby minimizing the net effect of altered temperatures, or behavioral control over body temperature is of paramount importance during the regenerative process, and newts could behaviorally select temperatures that best suit regeneration.

The first of these strategies, however, seems not to be supported from the current study. Animals at 23°C had the slowest rate of regeneration with the least regenerated tissue and slowest overall rates of differentiation of any group by day 70 post amputation. Early in regeneration (14 dpa), animals at 27°C had very high rates of growth but no discernible difference in regeneration stage from any other group. The majority of animals at 14 dpa from all temperature groups were at stages 2 and 3 (the dedifferentiation stages; Iten and Bryant, '73). The maximum rate of growth measured in this experiment was achieved at day 23 post amputation by newts at 29°C. At this point in time, animals at 29°C had an extremely wide range of limb stages from 3 to 12, although the mean stage of this group (stage 9; Palette stage) was consistent with Iten and Bryant (’73). By 70 dpa, newts at 29°C had the most new tissue growth and had completed differentiation. Unlike the results of Schauble and Nentwig (’74), forelimb outgrowth did not reach its maximum at 25°C, but instead demonstrated simple temperature sensitivity. However, the rates of differentiation results are consistent with Schauble and Nentwig (’74), in that they increased with higher temperatures, suggesting that the regeneration of the forelimb follows a basic temperature sensitive process.

During the initial 23 days, temperature sensitivity of outgrowth varied with a Q10 of 3–7, substantially higher than normal biochemical Q10 values (1–2 for newts; Berner and Puckett, 2010). Once the forelimb had undergone considerable regeneration (i.e stage 8; late bud), however, the temperature sensitivity of outgrowth returned to a Q10 of ~2. Following the late bud stage, chondrogenesis began to be evident, with digit formation following suit (Iten and Bryant, '73), demarcating the sudden shift in temperature sensitivity with the transition from blastema cell dedifferentiation and migration (stages 2–6) to redifferentiation, and the fixation of cellular fates, as well as eventual digit growth (stages 7–13). While these results cannot pinpoint a common, optimal thermal environment for enhancing regenerative research, they draw attention to an important variable; housing newts in environmental temperatures that vary by only 2°C can significantly alter the progression and rate of regeneration. They also highlight the extreme temperature sensitivity of the early stages of regeneration and thus, emphasize the importance of potential behavioral thermoregulatory mechanisms.

The present study suggests that the behavioral selection or avoidance of temperatures seems to be a stronger potential mechanism of mitigating thermal effects on regeneration. We hypothesized that newts with regenerating forelimbs would select a higher temperature than uninjured (sham) animals, akin to the behavioral selection of elevated temperatures that occurs during infections (i.e behavioral fever). Interestingly, this did not transpire, since the sham animals demonstrated a gradual decline in selected temperature over the period of acclimation to laboratory temperatures, while regenerating newts selected a consistent temperature of ~24.2°C during the first 2 weeks of regeneration. This temperature was the same as the thermal preference prior to amputation, and nearly the same as the thermal preference (24.5 ± 0.3°C) of newts acclimated to summer conditions (Berner and Puckett, 2010). Newts’ thermal preference while regenerating, however, followed the same trend as lizards regenerating autotomized tails, where behaviors are modified in order to maintain similar body temperatures in the field (Martin and Salvador, ’93; Chapple and Swain, 2004).

We examined whether propensity to move within the test chamber contributed to the different thermal preferences between regenerating and sham newts. Regenerating newts were not evidently impaired in their movement, since when tested at a constant 25°C, they exhibited greater variability in their activity compared to pre-amputation trials. Indeed, when kept at 25°C, the variability in newt behavior was much higher than when provided with temperature choices. In light of the high-temperature sensitivity of regeneration during the early stages, the importance of maintaining a narrow range of body temperatures becomes evident. The difference in precision of thermoregulation between regenerating and sham newts may result from the costs of behavioral thermoregulation (Cadena and Tattersall, 2009a,b), which are known to influence temperature regulation. Interestingly, Raffel et al. (2006) showed that increased temperature variability in the environment can lead to deficits in immune system function in red-spotted newts. Given the normally tight association between thermoregulation and immunity (Bicego et al., 2006), it is likely that immune-related processes that influence thermoregulatory control may be at work during tissue regeneration.

It is difficult to conclude that a single optimal temperature exists from our regeneration results alone, because rates changed over time and mainly demonstrated a monotonic increase with temperature (cf., Schauble and Nentwig, ’74) rather than reaching a plateau or decreasing at the highest temperatures tested (Fig. 4A). The precise and stable thermal preference of regenerating newts, however, speaks to a physiological reality. At 14 dpa, newts selected a temperature (~24.2°C) that would have led to a lower rate of regeneration than had they selected warmer temperatures (27–29°C), but corresponded to a temperature that bestowed a moderate rate of regeneration, and was substantially greater than would have occurred at cooler temperatures.
Summary effects of temperature on the average values for forelimb regeneration. The top plot (A) demonstrates the average rate of differentiation (filled circles) and average rate of outgrowth (open circles), along with the 95% confidence limits for preferred temperatures of newts with regenerating forelimbs (mean value is the solid vertical line). The lower plot (B) demonstrates the optimal level of forelimb regeneration (assessed by survival to 70 dpa) as a function of average rate of differentiation and rate of outgrowth. Highest survival occurred at 25°C, even though the rates of differentiation and outgrowth were highest at 29°C.

Figure 4. Summary effects of temperature on the average values for forelimb regeneration. The top plot (A) demonstrates the average rate of differentiation (filled circles) and average rate of outgrowth (open circles), along with the 95% confidence limits for preferred temperatures of newts with regenerating forelimbs (mean value is the solid vertical line). The lower plot (B) demonstrates the optimal level of forelimb regeneration (assessed by survival to 70 dpa) as a function of average rate of differentiation and rate of outgrowth. Highest survival occurred at 25°C, even though the rates of differentiation and outgrowth were highest at 29°C.

This result is not entirely surprising considering regenerating newts must maintain other physiological functions. While optimal temperatures for respiration, digestion, locomotion, and immune system function are unknown, at least some of these daily physiological functions are likely optimum at the newt’s preferred temperature. This is true for amphibian eggs, which are deposited at an environmental temperature optimal for growth and development (Hutchison and Dupré, ’92), as well as swimming velocity in the mudpuppy (Hutchison and Hill, ’76; Miller, ’82).

Similarly, Huey (’92) compared studies of the thermal optima of several physiological processes to the preferred temperature of two species of lizards, Sceloporus occidentalis, and Dipsosaurus dorsalis. Preferred temperature corresponded to a temperature range that supported high performance of physiological processes, despite differences in the absolute optimum temperature of each process. Nevertheless, Hutchison and Dupré (’92) discuss several examples of amphibian physiological function that performs optimally at 1–2°C above preferred temperature. Furthermore, since variation in temperature has been linked to increased susceptibility to infection in red-spotted newts (Raffel et al., 2006), the optimal strategy for regenerating newts may simply be to choose a stable body temperature. Unlike regenerating animals, uninjured newts did not remain at 25°C for the 2 weeks of trials in the thermal gradient. These animals moved more within the gradient, and since extreme warm temperatures (>30°C) are usually avoided, while cooler temperatures are tolerated, the mean selected temperature dropped over the 14 days of assessment. From this, we conclude that accurate thermoregulation around a preferred temperature may be more critical to regenerating individuals.

Behaviorally achieving an ideal temperature is crucial to numerous ectotherms (Hutchison and Dupré, ’92). In the present study, this was manifested in temperature-dependent survivability (Fig. 4B). Low temperatures are known to diminish immune function in amphibians (Maniero and Carey, ’97; Raffel et al., 2006). Since newts in the regeneration trials were held at fixed temperatures, without the opportunity to thermoregulate, newts could have been more susceptible to infection, particularly at 23°C. Wound healing in common garter snakes (Thamnophis sirtalis) is much reduced in snakes held at lower temperatures compared to higher temperatures (Smith et al., ’88). Furthermore, the wound areas in these animals remained open for longer periods, had an increased inflammatory area, and a prolonged inflammatory response (Smith et al., ’88). Similarly, frogs can be cleared of normally fatal chytrid infections by elevating body temperature (Woodhams et al., 2003). In short, temperature, immune function, and ultimately survival are inextricably linked, and thus, appropriate thermal habitat selection may be important in allowing amphibians to cope with pathogens in the environment (see Rojas et al., 2005).
Despite active cell turnover, regenerating forelimbs were not considerably warmer than uninjured forelimbs. First, newts are ectotherms, and the level of metabolism required to raise local temperature is likely to be high. For example, rattlesnake tail shaker muscles contract at up to 90 s⁻¹ (Schaeffer et al., '96), yet only raise tissue temperature by 1–2°C (Tattersall et al., 2004; Tattersall and Cadena, 2010). Second, the thermal camera non-invasively measures radiant heat from the animals’ surfaces; a subcutaneous probe may have revealed larger differences in limb temperature, however, one challenge with assessing amphibians is the high potential for evaporative cooling of the skin (Tattersall et al., 2006; Tattersall and Cadena, 2010), and thus tissue “hyperthermia” in amphibians may simply be difficult to assess. Nevertheless, subcutaneous hyperthermia has been shown to increase wound healing and decrease infections in mammals (Jonsson et al., '88, '91; Ikeda et al., '98), while behavioral fevers are a common host defense mechanism for fighting infections in ectotherms, as well as in mammals (Kluger, '79; Woodhams et al., 2003). Both of these strategies support the commonality of thermal mechanisms associated with healing and immunological responses. Examining regeneration in an animal with more water-impermeable skin (e.g. reptiles with tail autotomy) would allow for the determination of whether postinjury hyperthermia is an exclusively endothermic phenomenon, or initially derived as a supplementary recovery strategy to the behavioral fevers so commonly seen in ectotherms (Kluger, '79; Bicego et al., 2006).

Laboratory measurements of limb regeneration in newts have informed much about the importance of temperature to healing and tissue regrowth. Under natural conditions, as observed in rock lizards [Martin and Salvador, ’93], red-spotted newts may alter behavior to maintain preferred body temperature while regenerating from an injury. This study revealed, however, that behavioral thermoregulation and limb regeneration do not share a common, ideal temperature. Thus, although higher temperatures naturally improve the rate of tissue growth and differentiation, an augmentation in physiological processes is not necessarily the only factor influencing thermal preference. Newts are quite adroit at selecting a narrow range of temperatures that provide a reasonable rate of regeneration at the highest survival rate. This suggests that behavioral thermoregulation represents a compromise of multiple processes. Finally, the results of this study beg the question of whether intraspecific differences in the thermal preference among urodele species could account for at least some of the variation in regenerative ability. Future studies could benefit from examining the importance of temperature, the potential for thermal optima in regeneration, and the thermal preferences of the animals during regenerative processes.

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LITERATURE CITED


