PHYSIOLOGICAL AND BEHAVIORAL RESPONSES
OF LARVAL SPOTTED SALAMANDERS (AMBYSTOMA
MACULATUM) TO VARIOUS CONCENTRATIONS
OF OXYGEN

LYN C. BRANCH AND DOUGLAS H. TAYLOR
Department of Zoology, Miami University, Oxford, OH 45056, U.S.A.

(Received 2 February 1977)

Abstract—1. Rates of oxygen consumption were determined for developmental stages of Ambystoma maculatum larvae in water ranging from 1.06 to 8.60 ppm dissolved oxygen.
2. Premetamorphic larvae were oxygen conformers at all concentrations, while postmetamorphic animals were oxygen regulators above a critical tension (Pc) of about 5.5 ppm.
3. Tolerance to low concentrations of oxygen decreased progressively during the later stages of larval development.
4. Activity levels varied with developmental stage and oxygen concentration.

INTRODUCTION

Amphibians are the most plastic of all vertebrates in terms of respiratory strategies. Potential surfaces for gas exchange include gills, skin, lungs and buccopharyngeal epithelia. Recently several works have focused on the determination of the relative importance of these surfaces over a range of concentrations of oxygen in species that exhibit various degrees of terrestriality (Whitford & Sherman, 1968; Guimond & Hutchison, 1972, 1973; Bentley & Shield, 1973). In addition to changes in the importance of respiratory surfaces, rapid and complex biochemical alterations in respiratory patterns accompany metamorphosis (Hamada et al., 1964; Wade & Rose, 1972; Broyles & Frieden, 1973).

Previous studies suggest that respiratory patterns in adult amphibians function in determining the ecology and distribution of species (Whitford & Hutchison, 1967; Kenny & Rose, 1974). Because the larval stage ties most amphibians to water, larval respiratory strategies may play an equally important role in species distribution by determining what type of habitat is suitable for development.

The present study investigates oxygen consumption, oxygen tolerance and locomotor activity at various concentrations of dissolved oxygen in premetamorphic, metamorphic and postmetamorphic stages of Ambystoma maculatum.

MATERIALS AND METHODS

Ambystoma maculatum larvae collected at Nokusee Wildlife Refuge, Nokusee Co., MS were maintained in the laboratory on a diet of Artemia, Daphnia and oligochaete worms at 20°C under a light-dark (LD) regime (L:D 12:12; 0600–1800). Feeding ceased 48 hr prior to experimentation so all animals were in a postabsorptive state. In order to avoid light-induced responses, all experiments began at least 1 hr after the onset of the light phase and ceased at least 1 hr before the beginning of the dark phase.

The salamanders were separated into stages (Table 1) based on gill development: (1) premetamorphic (largest prior to gill absorption); (2) metamorphic (gill rami partially reduced); and (3) postmetamorphic (no gills). The ratio of the head width at the widest point and the longest gill ramus was used as an indicator of metamorphic state. Measurements were made with an eyepiece micrometer in a binocular microscope.

Oxygen consumption

Respiration was measured in water over a range of 1.06–8.60 ppm dissolved oxygen (DO). Two 125-ml Erlenmeyer flasks fitted with inflow and outflow tubes were connected to a 3.51 J. jar by a Y-joint and immersed in a constant-temperature water bath at 20 ± 0.5°C. One flask, containing an animal, served as the experimental chamber, while the other flask acted as a control. The jar was filled with dechlorinated water and bubbled with nitrogen to attain a specific oxygen concentration. The jar was then elevated and the flow of water adjusted so that a constant flow through the flasks could be maintained for 30 min. After this acclimation period the oxygen concentration in each flask was determined, and the flasks were stopped. After 1 hr a second reading was taken, and animals were removed and categorized as dead or alive. Oxygen consumption (µl · g⁻¹ · hr⁻¹) was calculated for each animal, and any changes in oxygen concentration in the control flask were noted.

A 10-ml syringe was used to extract the water samples and oxygen concentration was measured by a modified micro-Winkler method (Burke, 1962). The water was fixed in the syringe with Winkler reagents and titrated with sodium thiosulfate from a 0.2 ml micrometer syringe.

Control flasks showed a slight change in oxygen concentration during the tests. A least-squares regression of this change on oxygen concentration gave low predictability (r² = 0.10). An F-test indicated, however, that the slope was significant (F = 13.7, df = 1.89, P < 0.0006). Thus the equation of the regression line, Y = 0.000508 (OT) + 0.00143 (Y = change in oxygen concentration, OT = oxygen concentration), was used to correct the experimental data. Change in oxygen concentration was expressed as the difference in milliliters of sodium thiosulfate used to titrate for oxygen in the control flask at the beginning and end of the experiment.

Experimental data were analyzed by a least-squares regression followed by an F-test. LD (lethal dose) values, the concentrations of oxygen at which 50% of the animals...
died after 1.5 hr exposure, were computed for each stage by a log probit program. LD values were compared within stages and between stages and potency values (ratio of LD values) obtained. Potency values not including the value of 1 within the confidence limits were declared significant at $P < 0.05$ (Finney, 1971; Farrell, personal communication).

**Oxygen tolerance**

Tolerances of the three developmental stages to low concentrations of oxygen were determined by the procedure of Weigmann & Altig (1975a) for determining tolerances of larval amphibians to nitrogen anoxia. The number of bobs (attempted air gulps) per animal during 10 min intervals at specific times during each test was recorded. All tests were conducted at $20 \pm 0.5^\circ C$.

Animals were staged and separated into groups of 10. Each larva was placed in a 2 l glass jug containing water which had been bubbled with nitrogen to either 1.5 or 4.0 ppm oxygen. The jugs were then stoppered to prevent the animals from gulping air. Each group of ten was tested for a specific length of time at a specific oxygen concentration. The animals were then removed from the jugs and placed in oxygenated water. In order to avoid recording quiescence as death, a 30 min recovery period was allowed before tabulations. Each animal was then recorded as alive, ecologically dead, or physiologically dead. Ecological death was defined by Weigmann & Altig (1975a) as the loss of equilibrium, abnormal limb position and erratic swimming. If the animal had not recovered within 12 hr, it was recorded as physiologically dead.

Data were analyzed by a log probit program (Finney, 1971) that computed lethal time LT, which was the length of time required for a given percentage of the animals to die at each oxygen concentration. Potency values and significance level were determined by the same procedure as for LD values.

**RESULTS**

Oxygen consumption plotted as a function of parts per million of oxygen indicated different ontogenetic respiratory patterns in the three larval stages. Combining data for all stages, there was a trend of decreased oxygen consumption with decreased oxygen tension of the water. Premetamorphic animals showed a positive relationship ($V_O_2 = 19.57 + 9.28$ (OT), $r^2 = 0.43$, $F = 22.6$, $P < 0.0001$) between oxygen consumption ($V_O_2$) and oxygen tension (OT) at all concentrations of oxygen (Fig. 1A). A second variable, the ratio of head width/gill length, was significant ($P < 0.02$) in improving the predictability of the linear regression for oxygen consumption. Premetamorphic animals were oxygen conformers, thus critical tension ($P_c$) and standard metabolic rate (SMR) were impossible to determine.

Metamorphic and postmetamorphic animals were oxygen conformers at oxygen concentrations below some critical tension ($P_c$) and oxygen regulators at high oxygen concentrations (Figs 1B and C). Continuous plots of data for each stage were best fit by these quadratic regression equations: Stage 2, $V_O_2 = 18.26 (OT) - 1.19 (OT)^2 - 1.58$, $r^2 = 0.42$, $F = 8.8$, $P < 0.03$; Stage 3, $V_O_2 = 41.30 (OT) - 2.79 (OT)^2 - 47.39$, $r^2 = 0.70$, $F = 32.3$, $P < 0.0001$. Since for stages 2 and 3 the portions of the curves falling below a visually approximated $P_c$ appeared to correspond

<table>
<thead>
<tr>
<th>Stage</th>
<th>N</th>
<th>Weight (g)</th>
<th>Length (mm)</th>
<th>Head width/gill length</th>
<th>SMR (μl/g/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>0.618 ± 0.13</td>
<td>42.7 ± 3.9</td>
<td>2.19 ± 0.43</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>0.559 ± 0.10</td>
<td>42.7 ± 3.1</td>
<td>3.99 ± 2.54</td>
<td>67.09 ± 23.25</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>0.512 ± 0.07</td>
<td>41.2 ± 3.5</td>
<td>ND</td>
<td>101.10 ± 29.64</td>
</tr>
</tbody>
</table>

Fig. 1. Response of three developmental stages of *Ambystoma maculatum* larvae to various concentrations of oxygen. Open circles represent animals which were ecologically or physiologically dead at the end of the experiment. Closed circles represent live animals. The solid line indicates the regression line for all animals combined. The regression line for dead animals is indicated by the dashed line and for live animals, by the hatched line. $P_c$ is the point of intersection of the regression lines for dead and live animals. (A) Stage 1 regression equation: all animals, $V_O_2 = 19.57 + 9.28$ (OT), $r^2 = 0.43$, $OT = 1.19$ (OT) - 1.58, $r^2 = 0.42$; (B) Stage 2 regression equations: live animals, $V_O_2 = 68.65 - 0.22$ (OT), $r^2 = 0.01$; dead animals, $V_O_2 = 14.74 + 8.28$ (OT), $r^2 = 0.25$; all animals, $V_O_2 = 18.26$ (OT) - 1.19 (OT) - 1.58, $r^2 = 0.42$; (C) Stage 3 regression equations: live animals, $V_O_2 = 87.22 + 1.99$ (OT), $r^2 = 0.003$; dead animals, $V_O_2 = 21.00$ (OT) - 16.01, $r^2 = 0.73$; all animals, $V_O_2 = 41.30$ (OT) - 2.79 (OT) - 47.39, $r^2 = 0.70$. 7
Physiological and behavioral responses of *A. maculatum* 271

Table 2. Comparison of lethal time (LT50 in hr) and lethal doses (LD50 in ppm) values for 50% mortality among three developmental stages of *Amblystoma maculatum* at 1.5 and 4.0 ppm DO concentrations

<table>
<thead>
<tr>
<th>Stage and Ecological death</th>
<th>Physiological death</th>
</tr>
</thead>
<tbody>
<tr>
<td>comparisons</td>
<td>LT50 1.5 ppm</td>
</tr>
<tr>
<td>1</td>
<td>4.15</td>
</tr>
<tr>
<td>2</td>
<td>1.02</td>
</tr>
<tr>
<td>3</td>
<td>0.76</td>
</tr>
<tr>
<td>1 × 2</td>
<td>*</td>
</tr>
<tr>
<td>1 × 3</td>
<td>*</td>
</tr>
<tr>
<td>2 × 3</td>
<td>*</td>
</tr>
</tbody>
</table>

*Significantly different at P ≤ 0.05.

NM—Stage 1 exhibited no mortality after 8 hr at which time the experiment was terminated.

NS—Not significantly different at P ≤ 0.05.

to *V*02 for animals dead at the end of the test (hereafter referred to as dead animals) and because these animals and live animals were in different physiological states, separate regression lines were determined for dead and live animals and the intersection of these two lines taken as the *P*<sub*e*>. Because of the variability in oxygen consumption in stage 2, the predictability of the regression line [Y<sub>0</sub> = 14.74 + 0.28 (O2)] for dead animals was low (r<sup>2</sup> = 0.25, F = 2.9, P < 0.12). The *P*<sub>e</sub>, 6.27 ppm, determined from the intersection of the two regression lines is probably high.

During the experimental period, postmetamorphic animals remained quiescent, possibly because of greater physiological stress. This low activity probably contributed to the low variability and thus a higher predictability (Y<sub>0</sub> = 21.00 (O2) - 16.01, r<sup>2</sup> = 0.73, F = 15.6, P < 0.0009) for Y<sub>0</sub> for dead stage 3 animals. Because the *P*<sub>e</sub>, 5.46 ppm, for metamorphosed animals corresponded very closely to that oxygen tension at which animals began dying and because the regression for dead animals was highly predictable, the determined *P*<sub>e</sub> may be a more realistic value in this stage than in stage 2. Therefore, the oxygen tension at which stage 2 animals began dying (5.1 ppm) may more closely approximate a *P*<sub>e</sub> for that stage.

Oxygen regulators maintain a constant level of oxygen consumption across a range of oxygen concentrations. Assuming all other conditions for SMR are met, this level of oxygen consumption should equal the SMR of the larval stage. The regressions for Y<sub>0</sub>, of live animals on oxygen concentration did not have significant slopes in stage 2 (F = 0.16; P < 0.86) and stage 3 (F = 0.20; P < 0.66). Therefore the mean Y<sub>0</sub> for live animals at each stage was taken as the value of SMR (Table 1). The higher metabolic rate of stage 3 animals was significantly different from the metabolic rate of stage 2 animals (t = 4.8, P < 0.001).

A stepwise regression for each stage of Y<sub>0</sub>, oxygen concentration, wet weight, and length indicated that neither wet weight nor length contributed significantly to Y<sub>0</sub> at any stage. Even though the ratio of head width/gill length was more variable in stage 2 than stage 1 it did not significantly increase predictability in stage 2.

LT values were parallel for stages 2 and 3 at 1.5 ppm and 4.0 ppm and stage 1 at 1.5 ppm, thus it was only necessary to compare LT50 values (Table 2). Stage 1 at 4.0 ppm exhibited no mortality after 8 hr and the experiment was terminated. When ecological death was considered, tolerance time significantly decreased with increasing stage. Considering only physiological death, the LT50 values at 1.5 ppm for stages 2 and 3 were not significantly different. When LT50 values were compared within a stage but between 1.5 and 4.0 ppm, stage 1 and 2 larvae were significantly less tolerant to 1.5 ppm. The LT50 values for stage 3 were not significantly different at 1.5 ppm and 4.0 ppm oxygen.

LD40 values for physiological death followed similar trends as the LT50 values; stage 1 animals had a significantly higher tolerance to low oxygen concentrations, but there was no evidence of differences between stages 2 and 3 (Table 2). With ecological death there was a general trend of decreasing tolerance with increasing stage. Stage 1 was tolerant to significantly lower oxygen concentrations while the confidence limits of stages 2 and 3 overlapped. The LD50 for ecological death of stage 3 was very close to the *P*<sub>e</sub> determined for this stage. For stage 2 the LD50 value was considerably lower.

Both stage 1 and stage 2 larvae significantly reduced their activity with increased exposure time to a dissolved oxygen concentration of 1.5 ppm (Fig. 2). Bobbing of stage 2 larvae at 4.0 ppm followed a similar pattern, whereas stage 1 showed little correlation of activity with exposure. At both dissolved oxygen concentrations, stage 3 larvae made only a few unsuccessful attempts at reaching the top of the jug before remaining quietly on the bottom of the test chamber for the remainder of the experimental period.

The overall patterns of activity of stage 1 at 1.5 ppm and stage 2 at 1.5 ppm and 4.0 ppm were not significantly different (Fig. 2), however, the initial activity levels were different. Because most of the stage 2 larvae were dead after 1 hr exposure to 1.5 ppm, stage comparisons of activity levels could only be made for that time period (Table 3). Stage 1 at 1.5 ppm exhibited a significantly higher activity level than stage 1 at 4.0 ppm (X<sup>2</sup> = 48.1, P < 0.0001) and stage 2 at 1.5 ppm (X<sup>2</sup> = 6.14, P < 0.02). At 4.0 ppm stage 1 was significantly (P < 0.001) less active than any other combinations of stage and DO concentration. There was no significant difference in activity levels.
pattern similar to that of other bimodally breathing salamanders emerges. Ultsch (1976) reported increasing $P_e$ values with increasing terrestriality. The $P_e$ values for metamorphic and postmetamorphic *A. maculatum* were not distinctly different which suggests that the biochemical, physiological and anatomical changes necessary for the switch from oxygen conformity to oxygen regulation take place as the larvae lose their gills. Tolerance to low oxygen, SMR and behavior are different among the stages. Therefore it is important to consider the role of such patterns in the process of metamorphosis.

All three stages of larvae used in the tolerance experiments were collected at the same time from ponds which were almost dry. In the laboratory, larvae metamorphosed from stage 1 to stage 3 in less than a week. Stage 1 larvae exhibited no evidence of stress except at very low oxygen concentrations and even then were able to maintain high levels of activity. The tolerance of stage 2 larvae was much lower than stage 1 at intermediate and low levels of oxygen, but the animals remained active for some time at both concentrations. Stage 3 was inactive at either concentration. Although Shrode (1972) could not correlate dissolved oxygen concentration with metamorphic rate in thyroxin-injected *A. tigrinum*, decreased tolerance to low oxygen may still serve as a stimulus for metamorphosing animals to leave the aquatic environment.

Unlike anuran larvae, salamander larvae make only minor adjustments in body proportions and therefore surface area with metamorphosis. Premetamorphic and postmetamorphic animals in the tolerance experiments had the same mean body weight (Table 3) and thus differed only in gill surface area and height of the tail fin. Stage 2 was slightly larger than either stage 1 or stage 3. Because of the increased surface area of the gills and fins, stage 1 may have had a slight advantage in terms of surface/volume ratio. Boell et al. (1963) maintained that the role of gills in respiration in *Ambystoma* larvae is negligible. The growth response of gills to low oxygen (Bond, 1960) suggests that they are serving some function but other mechanisms may be considerably more important in increasing the oxygen tolerance of small larvae.

From his work based on aerial and aquatic respiratory modes in sirensids, Ultsch (1976) proposed that an increase in SMR is a function of increased surface area. The increase in SMR of stage 3 animals over stage 2 larvae cannot be explained solely as a function of increased surface area for gas exchange. Postmetamorphic animals were slightly smaller than metamor-

---

**Table 3.** Wet weight ($\bar{X} \pm$ S.D.), $r^2$ for bobs regressed on time $^{-1}$ and number of bobs (30 animals) calculated for 3 10-min periods at intervals of 10 min, 30 min, and 1 hr are shown for larvae subjected to two DO concentrations in the $O_2$ tolerance experiments. ND = no data

<table>
<thead>
<tr>
<th>Stage</th>
<th>$N$</th>
<th>Weight (g)</th>
<th>DO (ppm)</th>
<th>$r^2$ (bobs vs time $^{-1}$)</th>
<th>Bobs/3 10-min periods</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>0.49 ± 0.11</td>
<td>1.5</td>
<td>0.98 ($P &lt; 0.0001$)</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
<td>0.14 ($P &lt; 0.471$)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>0.51 ± 0.12</td>
<td>1.5</td>
<td>0.99 ($P &lt; 0.048$)</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
<td>0.99 ($P &lt; 0.0001$)</td>
<td>71</td>
</tr>
<tr>
<td>3</td>
<td>102</td>
<td>0.49 ± 0.06</td>
<td>1.5</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.0</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>
Physiological and behavioral responses of *A. maculatum* 273

...phic larvae but a reduction in gills and tail fin probably offset any advantage in surface/volume ratio gained by the smaller size. Animals were forcibly submerged, thus increased exchange area of the lungs did not accrue any advantage to postmetamorphic salamanders. Presumably, increased metabolic rate enables animals to cope with increased energy demands of a terrestrial environment (Gahlenbeck & Bartels, 1970).

The SMR (101.1 μl·g⁻¹·h⁻¹) for postmetamorphic animals was higher than the values (77.02 μl·g⁻¹·h⁻¹ with a 16 hr photoperiod, 55.67 μl·g⁻¹·h⁻¹ with an 8 hr photoperiod) reported by Whitford & Hutchison (1965) for adult *A. maculatum* at 15°C. This higher metabolic rate may be in part a function of higher temperature and a more favorable surface/volume ratio in the postmetamorphic animals. Such metamorphic peaks of metabolism have been reported in other amphibians (Hopkins & Handford, 1945; Wood, 1972).

Modifications in the relative amounts of vascularization of the respiratory surfaces is a second factor which may contribute to levels of oxygen tolerance. Such changes would have to be rapid to account for the differences between stages and may involve a vascular shunting mechanism of the nature proposed by Wassersug & Seibert (1975) for the preferential use of different respiratory surfaces in tadpoles. Biochemical and structural adjustments of the blood may also contribute to the rapid transitions between larval stages. The electrophoretic pattern of hemoglobin is different for larval and transformed *A. maculatum* (Edwards & Justus, 1969). Other metamorphosed amphibians exhibits polycythemia (Wade & Rose, 1972) and increased erythrocyte ATP (Wood, 1971) which results in a decreased affinity of the blood for oxygen.

Patterns of low oxygen tolerance should be examined in light of the oxygen concentrations in the ponds and the field behavior of the larvae. Oxygen concentration in the ponds where larvae were collected measured 2.7 ppm at depths of 4 cm and 1.5 m. This low concentration was primarily because of high organic content and fluctuated little on a diel basis. Tolerance values, therefore, indicate that stage 2 and 3 larvae cannot exist in the ponds without making physiological adjustments. Options include dependence on aerial respiration as a supplement to aquatic respiration, reduction in metabolic rate (Kenny & Rose, 1974), and dependence on anaerobic metabolism (Weigmann & Altit, 1975a). Larvae generally remain hidden under leaf mats during the day and actively feed on plankton in the water column at night (Branch & Altit, unpublished data). They often break the surface of the water to gulp air. During the day larvae do not commonly gulp, but a flurry of such activity is often noticed just at dawn when the larvae are returning to the bottom. These observations suggest that larvae are able to reduce their metabolism or rely on anaerobic energy reserves during periods of inactivity, but during peak activity, they must supplement cutaneous and gill respiration with pulmonary respiration.

**Acknowledgements**—We would like to thank Drs D. L. Clausen and R. Altit for many helpful suggestions throughout the study and for reviewing the manuscript. J. Auburn also critically commented on the manuscript. Dr J. T. Morrow and Mississippi State University provided laboratory space during part of the study. D. Werschulz and Drs K. Dodd and R. Altit aided with the collection of animals. Dr M. P. Farrell is acknowledged for his invaluable help with the statistical analysis. This study was partially supported by a Miami University Faculty Research Grant to Douglas H. Taylor and by NSF Grant 68-NSF-74-01259 to Douglas H. Taylor.

**REFERENCES**


