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EFFECT OF REARING TEMPERATURE ON LARVAL BUFO VIRIDIS VIRIDIS LAURENTI 1768

(Anura, Amphibia)

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ABSTRACT

It is well known that rearing ectotherm animals under low temperatures enhances their growth and decelerates development. In the present study, a group of early prometamorphic tadpoles of the green toad, *Bufo viridis viridis* was reared in the cold room (experimental) and another in the laboratory under room temperature (control) for two months. By the end of the experiment, the experimental group gained a bigger average body weight, and larger dimensions than the control. The latter, on the other hand, achieved more advanced developmental stages. Unlike many of the control animals, none of the experimental tadpoles reached climax by the time the experiment was ended, i.e. after two months. Histological and electron microscopically examination of the epidermis of experimental animals showed gradual erosion of the epidermis and heavy deposition of collagen in the dermis. At the time the experiment was ended, the epidermis was almost completely lost. The study also showed the breakdown of the skeletal muscle fibers into small segments of two or three sarcomeres. Eventually, the tadpoles became incapable of locomotors movements, a result that may recorded for the first time. The present results are in general accordance with previous studies on ectotherms regarding effects of low temperatures on growth and differentiation.

Key words: poikilotherms, amphibian growth, green toad, temperature

1. INTRODUCTION

Amphibian larvae show extreme plasticity of size and age at which they metamorphose (Denver *et al*., 1998). Temperature plays an important role in the lives of ectotherm animals including amphibians. In general, individuals of a population of a particular species living in colder areas attain larger sizes than their relatives living in warmer areas. This observation has long been recorded in both endotherms and ectotherms. It is known as Bergmann's rule (Angilletta and Dunham, 2003; Morrison and Hero, 2003; Angilletta and Sears, 2004). Anuran growth and metamorphosis are affected by intrinsic and extrinsic factors. The most important intrinsic factors are prolactin and thyroid hormones.

Extrinsic factors, on the other hand, include temperature, food availability, predation and some other factors. According to Smith-Gill and Berven (1979) who studied the development of *Rana pipiens*, increasing the temperature of surrounding water 10^{o}C (13^{o}C -23^{o}C) accelerates differentiation ten folds and growth six folds. Low environmental temperatures hinder metamorphosis, while high temperatures accelerate it (Herreid and Kinney, 1967; Michael, 1981). This means that tadpoles growing under low temperature achieve larger sizes than those growing under higher temperature (Atkinson, 1994; 1995). In some cases tadpoles overwinter if it is too late for them to metamorphose.

Bufo viridis viridis Laurenti is a toad whose distribution is almost confined to the Middle East. This study is designed to examine the effect of low

temperature on the growth rate and development of this species. In addition, the study aims at investigating any structural change that might take place due to the low raising temperature.

2. MATERIALS AND METHODS

Materials: Tadpoles of the green toad, *Bufo v. viridis*, at premetamorphic stage (hind limb bud stage) were collected from water bodies in Al Ghore Al Shamali (Northern Jordan) in late February 2008, brought to the laboratory in containers filled with water from the same water bodies. In the laboratory, the animals were divided into two groups; the first of which was reared under room temperature (control), while the second (experimental) was reared in the cold room (at temperature of $6^{o}C$). The tadpoles were fed boiled lettuce and the residues were removed once a day.

Light and Electron Microscopy: The weight, trunk length, tail length and total length were measured for each of the following stages: early prometamorphic (straight, unjointed hind limbs), mid prometamorphic (jointed hind limbs), and late prometamorphic (well developed hind limbs) stages. Samples of skin and skeletal muscle were taken from control and experimental animals, fixed in Bouin's fluid and routinely processed for light microscopy or in gluteraldehyde and postfixed in osmium tetroxide and processed for electron microscopy.

3. RESULTS

The comparison of Measurements of control and experimental tadpoles by T-test analyses showed a significant difference between weight, trunk and tail lengths of control and experimental (reared in the cold room) tadpoles.The comparison of the three larval stages there are observed differences between the means of the measurements of larval stages studied According to Early Pro-meatamorphic, there is a significant difference between the means of the measurements of larval stages studied at (WT1 (control) - WT2 (Experimental) in favor of (WT2).Table1 .

The comparison of three study groups, tadpoles in both early prometamorphic and mid prometamorphic with a significant difference of $p<0.05$. There were also significant differences in the weight, trunk and tail lengths of control and experimental in the late prometamorphic with all ($p<0.05$),according to Mid Prom Cont, there is a significant difference between the means of the measurements of larval stages studied at (WT1 - WT2) in favor of (WT2). While there aren't any significant differences between the means of the measurements of larval stages studied at the other pairs table 2 .

By the end of the first month, experimental tadpoles gained larger bodies and dimensions than control ones, even though both were still in the early prometamorphic stage (Fig.1). On the other hand, control tadpoles showed a more accelerated morphogenesis than experimental ones. A few control animals metamorphosed into froglets by the end of the experiment, while the experimental did not reach the climax stage. Moreover, the latter animals became extremely sluggish and almost showed no locomotor reaction even when stimulated.

The skin in control, early prometamorphic tadpoles was made up of three-cell thick epidermis in the form of non-keratinized, stratified epithelium and a thin, highly vascular dermis (Fig. 2). The superficial cells were low cuboidal with tight junctions and stumpy microvilli. Underlying the epidermis was a thin, highly vascular dermis with a basement membrane separating the two layers. Melanocytes were frequently observed in the superficial dermis, just underneath the basement membrane (Fig. 2).

The epidermis of tadpoles raised in the cold room for one month showed noticeable wearing away of the superficial cells due to loosen intercellular attachments and vacuolation of the cells. Deeper layers looked healthy with normal intercellular attachments (Fig. 3). The basement membrane was exceptionally thick and the dermis became thickened too with heavy deposition of collagen. Electron microscopic examination showed that normal organelles such as rough endoplasmic reticulum, polysomes (Fig. 4) and Golgi apparatus (Fig. 5) were still manifested by the basal cells.

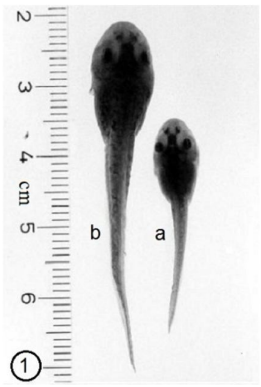
Rearing the tadpoles for another fortnight in the cold room caused further wearing down of the outer two epidermal layers. Electron microscopic examination of the inner cells showed drastic changes from the normal picture of an epithelial cell, most membranous organelles such as endoplasmic reticula and Golgi apparatus had variably disintegrated. Heavy deposition of cytoskeletal elements was evident resulting in alignment of some organelles such as mitochondria (Fig. 6). Some intercellular attachments were still observed (Figs. 6, 7).

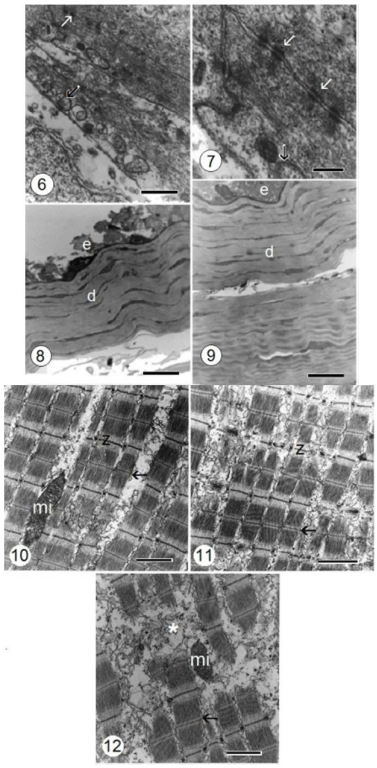
By the end of the second month, the epidermis of the experimental animals had almost worn out leaving an incomplete basal row of cell with disorganized superficial cells (Fig. 8). The basement membrane is clearly thickened. At this stage, the dermis is extremely thickened due to heavy deposition of collagen fibers (Fig. 9).

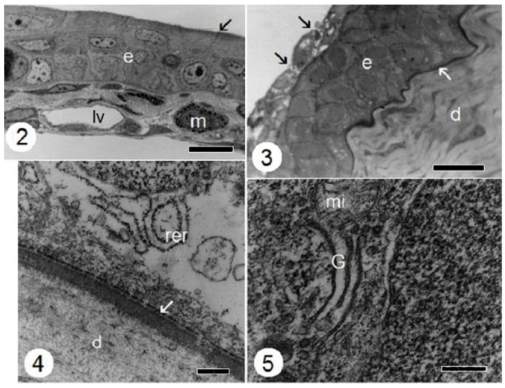
The normal ultrastructural picture of the body musculature of the tadpoles is depicted in figures3A. It showed the classical pattern of the skeletal muscle being made up of parallel, cylindrical fibers that showed the classical sarcomeres and alternating A bands and I bands. The Z lines that link the sequential sarcomeres, alternating A bands and I

bands, H bands, and M lines were all evident (Fig. 10).

The ultrastructural picture of the skeletal muscle of experimental tadpoles raised in the cold room for one month showed early signs of fiber damage represented by breakage of the muscle fibers at the Z lines (Fig. 11). By the end of the experiment, the muscle fibers broke down into segments of a few sarcomeres (Fig. 12).







4. LEGENDS OF FIGURES

Figure 1: Early prometamrphic tadpoles after one month of raising: a control, b experimental.

Figure 2: The histology of control early prometamorphic skin showing three-cell thick epidermis (e) with tight junctions (arrow). The thin dermis is rich with melanocyte (m) and lymph vessels (lv). LM, scale bar: 10µm.

Figures 3-5: Skin of experimental, mid prometamorphic tadpoles raised in the cold room for one month.

Figure 3: The epidermis (e) shows damage of the superficial cells (black arrows). The basement membrane (white arrow) is thick. The dermis (d) is thick and highly collagenous. LM, scale bar: 10µm.

Figure 4: Epidermis-dermis junctional area showing rough endoplasmic reticulum (rer) of a basal epidermal cell and hemidesmosomes (arrow). The dermis (d) is highly collagenous. EM, scale bar: 1µm.

Figure 5: A part of basal epithelial cell. A mitochondrion (mi) and Golgi apparatus (G) are discerned. EM, scale bar: 5µm.

Figures 6 and 7: Histology of the skin of tadpoles raised in the cold room for six weeks.

Figure 6: Cellular organelles such as mitochondria (black arrow), endoplasmic reticulum and intercellular junctions (white arrow) are still evident. EM, scale bar: 0.5µm.

Figure 7: Cytoskeletal elements and intercellular junctions (white arrows) of the basal cells and cytoskeletal elements are discernible. Black arrow: mitochondrion. EM, scale bar: 0.2µm.

Figures 8 and 9: Skin of tadpoles raised in the cold room for two months. Notice the extremely weathered epidermis (e) and heavy collagen deposition in the dermis (d). LM, scale bar: 10 µm.

Figure 10: Skeletal muscle fibers of control tadpole showing normal structure. An elongated mitochondrion (mi), M-lines (arrow) and Z-lines (z) are indicated. TEM, scale bar: 10µm.

Figure 11: Skeletal muscle fibers of experimental animals kept in the cold room for one month. Early fragmentation of the fibers at the Z-line (z) is evident in the upper part of the figure. Arrow: M-line. TEM, scale bar: 10µm.

Figure 12: Skeletal muscle of experimental tadpoles raised in the cold room for two months. Extensive damage of muscle fibers is indicated (rosette). A mitochondrion (mi), M-lines (arrow) and Z-lines are shown. TEM, scale bar: 10µm.

Table (1): Means and Standard Deviations of the measurements of larval stages studied Table (1) shows that there are observed differences between the means of the measurements of larval stages studied. To test the significant of these differences, paired samples (t) test was used as shown in table (1).

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Group	Type	N	Mean	Std. Deviation
Early Promeatamorphic	WT1	22	0.50	1.09
	WT2	22	1.29	0.14
	Head tail	22	21.99	47.81
	Head2	22	20.05	1.56
	Tail	22	31.59	68.68
	Tail2	22	35.64	5.12
	Total1	22	53.58	116.48
	Total2	22	56.18	5.68
Mid Prom Cont	WT1	21	0.64	1.40
	WT2	21	1.65	0.19
	Head tail	21	22.38	48.72
	Head2	21	21.62	1.91
	Tail	21	33.81	73.62
	Tail2	21	36.19	4.21
	Total1	21	56.19	122.34
	Total2	21	57.62	4.96
Late Prom Cont	WT1	20	0.36	0.11
	WT2	20	2.92	0.99
	Head tail	20	11.60	0.82
	Head2	20	25.40	2.11
	Tail	20	17.00	2.47
	Tail2	20	47.05	5.02
	Total1	20	28.60	3.17
	Total2	20	72.45	5.88

Table (2)
Paired samples (t) test results for the differences between the means of the measurements of larval stages studied Table (2) shows that:

1. According to Early Pro-meatamorphic, there is a significant difference between the means of the measurements of larval stages studied at (WT1 (control) - WT2 (Experimental) in favor of (WT2). While there aren't any significant differences between the means of the measurements of larval stages studied at the other pairs.
2. According to Mid Prom Cont, there is a significant difference between the means of the measurements of larval stages studied at (WT1 - WT2) in favor of (WT2). While there aren't any significant differences between the means of the measurements of larval stages studied at the other pairs.
3. According to Late Prom Cont, there are significant differences between the means of the measurements of all larval stages studied pairs in favor of the second reading.

Group	Pairs	Paired Differences		df	(t) Value	Sig.
		Mean	Std. Deviation			
Early Promeatamorphic	WT1 - WT2	0.79	1.07	21	3.448	0.002*
	Head tail – Head2	1.95	47.38	21	0.193	0.849
	Tail – Tail2	4.05	67.18	21	0.283	0.780
	Total1 – Total2	2.60	114.60	21	0.107	0.916
Mid Prom Cont	WT1 - WT2	1.01	1.46	20	3.185	0.005*
	Head tail – Head2	0.76	48.67	20	0.072	0.944
	Tail – Tail2	2.38	74.96	20	0.146	0.886
	Total1 – Total2	1.43	123.99	20	0.053	0.958
Late Prom Cont	WT1 - WT2	2.56	1.02	19	11.259	0.000*
	Head tail – Head2	13.80	2.50	19	24.640	0.000*
	Tail – Tail2	30.05	6.01	19	22.350	0.000*
	Total1 – Total2	43.85	7.36	19	26.653	0.000*

- Significant at ($\propto = 0.05$)

5. DISCUSSION

Results of the present study on the effect of rearing temperature on tadpoles of the green toad *Bufo v. viridis* showed significant differences in weight and measurements between tadpoles reared under room temperature (control) and those reared in the cold room (experimental). The latter clearly gained more weight and larger dimensions than the control, yet they showed lagged morphogenesis. These results are in accordance with observations on ectotherms in general (Partridge and French, 1996; Atkinson *et al*., 2003) and anurans in particular (Warkentin, 1992). According to Alvarez and Nicieza, 2002), temperature and diet had strong effects on the maximum size reached by tadpoles throughout the premetamorphic stages. The latter study on the painted frogs, *Discoglossus galganoi*, showed that a reduction in environmental temperature causes a delayed metamorphosis and increased dry weight (Alvarez and Nicieza, 2002)). Experimental tadpoles in the present study did not metamorphose as is the case with *Pseudacris ornata* larvae (Gail *et al*., 1988). Rearing larvae of the amphibian urodele *Pleurodeles waltl* at a relatively high temperature (30^{o}C) on the other hand resulted into a number of limb abnormalities (Dournon *et al*., 1998). Moreover, tadpoles of ornate chorus frog *Pseudacris ornata* did not metamorphose when raised at 10^{o}C after 111days (Gail *et al*., 1988)), as is the case with the present experiment.

The present observations on the effects of low rearing temperature on the skin (epithelial necrosis and heavy deposition of collagen in the dermis) and body musculature of the experimental tadpoles are, however, unanticipated since the temperature was moderately low. Salahudeen *et al.* (2001) found that human epithelial cells of the renal proximal convoluted tubule undergo necrosis when stored for 48 hrs at 4^{o}C. The situation in the present study is different because frogs are ectotherms while humans are endotherms. The changes observed during the present study are in general agreement with frostbites and cold injuries in animals (Irwin 1996) and humans (Murphy *et al*., 2000). The prime difference between the two phenomena arises from the fact that cold injuries in humans arise from short term, sever cold, whereas the experimental tadpoles in the present study suffered injuries due to prolonged, mild cold.

It is generally accepted that the first reaction of the living vertebrate to cold is vasoconstriction of blood vessels nourishing the cold-subject organ (Britt *et al*., 1991); in the case of the tadpoles the skin. This leads to reduced nourishment supplies to the epidermal cells and, consequently, their death. Additionally, prolonged subject to cold in this experiment induced heavy deposition of collagen in the dermis in order to improve heat insulation. This augments the isolation of the epidermis and deprivation of its cells of nourishments and, consequently, accelerates their death. The pattern of cell death observed during the present study principally falls under necrosis (Kerr, 1971; Salahudeen *et al.* (2001)) rather than apoptosis, even though Majno and Joris (1995) suggest that necrosis

means the changes that follow cell death. The loss of the epidermis (loss of sensory endings) and fragmentation of the skeletal muscle explain the incapability of the experimental animals of locomotion when touched.

6. CONCLUSION

Results of the present study are in general accordance with similar studies performed on ectotherm animals. The results clearly show that tadpoles of the green toad, *Bufo v. viridis*, raised under low temperature achieved a slower differentiation rate and bigger sizes than those raised under higher temperature. The study also shows that low temperature has other negative effects on tadpoles represented by necrosis of the epidermis and break down of skeletal muscle fibers.

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