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Arrested development in *Xenopus laevis* tadpoles: how size constrains metamorphosis

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Summary

***Xenopus laevis* tadpoles that arrest development and remain as larvae for several years sometimes occur spontaneously in laboratory populations. These tadpoles cease development at an early hindlimb stage, but continue to grow and develop into grossly deformed giants. Giant tadpoles lack thyroid glands, and differ in morphology and behaviour from normal larvae. They are negatively buoyant, typically with small and partially solidified lungs, and have greatly enlarged fat bodies. Giant tadpoles have mature gonads with eggs and sperm, whereas normal tadpoles of the same stage have undifferentiated gonads. Larval reproduction has never been reported in anurans, but gonadal development decoupled from metamorphosis brings these giants the closest of any anurans to being truly neotenic. We discuss behavioural and morphological factors that may hinder both reproduction in giant *Xenopus* larvae and the evolution of neoteny in anurans in general. Experimental treatment with exogenous thyroid hormone induces some, but not complete, metamorphic changes in these giants. The limbs and head progress through metamorphosis; however, all tadpoles die at the stage when the tail would normally be resorbed. The disproportionate growth of tissues and organs in giant tadpoles may preclude complete metamorphosis, even under exogenous thyroid hormone induction.**

Key words: *Xenopus laevis*, giantism, thyroid gland, growth, development, neoteny.

Introduction

In anurans metamorphosis is obligatory; neoteny, i.e. reproduction in the larval form (Gould, 1977), has never been reported. Neoteny, however, is common in urodeles, implying fundamental differences in developmental timing for the germ and somatic cell lines in these two groups of amphibians (Wakahara, 1996).

The African clawed frog *Xenopus laevis* is the only anuran species in which a spontaneous lack of metamorphosis has been documented; on rare occasions, *X. laevis* tadpoles with arrested development occur alongside normal siblings. Although these tadpoles cease developing at approximately Nieuwkoop–Faber (NF) developmental stages 53–55, i.e. at early hindlimb bud stages (Nieuwkoop and Faber, 1956), their growth continues, and they develop into grossly deformed giants (Fig. 1). These giant tadpoles can remain at this stage for several years (Jurand, 1955; Dodd and Dodd, 1976), without developing further.

Giant, non-metamorphosing, tadpoles have occasionally been reported in a number of European populations within the *Rana esculenta* complex (see review in Borkin et al., 1982). However, this giantism is usually attributed to the fact that *R. esculenta* is a hybrid; triploids frequently occur among individuals of that phenotype, and reach much larger body size than diploids (Berger and Uzzell, 1977).

Arrested development in *X. laevis* has been reported in the literature at least four times (Toivonen, 1952; Jurand, 1955; Srebro, 1970; Dodd and Dodd, 1976). Our laboratory *X. laevis* colony has spontaneously produced two such giants in the last three decades. In surveying other laboratories that raise *X. laevis* (cf. Major and Wassersug, 1998), we confirmed that the spontaneous appearance of these giant tadpoles is a very rare event. However, Xenopus One (Dexter, MI, USA), a commercial supplier of *X. laevis* in North America, had 45 live giant tadpoles collected over a period of 8 years and made these specimens available to us.

Jurand (1955) found that giant non-metamorphosing *X. laevis* larvae lack thyroid glands. As a result, they do not produce the thyroid hormones (TH) that are necessary for the initiation and completion of metamorphic transformation (Shi, 2000). Developmental stages of all giant *X. laevis* tadpoles, both in this study and reported in the literature, range from NF 53 to 56. At these stages there is little or no TH present in a tadpole's circulation, and differentiation proceeds independent of hormone concentration.

Non-metamorphosing *X. laevis* tadpoles show obvious morphological and behavioural differences compared to normal *X. laevis* tadpoles of a corresponding stage (Rot-

Fig. 1. A giant athyroid *X. laevis* tadpole (below) compared to a normal *X. laevis* larva (above) at the same developmental stage (NF 54). Note the massive, hunchback morphology of the giant tadpole compared to the normal specimen. Scale bar, 1 cm.

Nikcevic and Wassersug, 2003). Giants are on average 4 times longer and up to 50 times more massive than normal tadpoles, and they have scoliosis, with C- or S-shaped back curvature. Their axial and tail muscle masses are disproportionately large relative to their body mass. They have difficulty swimming and holding their position in the water column; instead they frequently rest on the bottom. In this paper we explore how excessive somatic growth in giants may affect their subsequent development, and eventually preclude normal metamorphosis.

Allen (1916, 1918) showed that removing thyroid glands in *Rana pipiens* tadpoles at early stages resulted in giant, non-metamorphosing larvae, similar to our naturally occurring *X. laevis* tadpoles, and the ones described by Jurand (1955). Development in thyroidectomized *R. pipiens* tadpoles proceeded normally until the hindlimbs began to form. At this stage tadpoles arrested their development, their forelimbs never erupted through the skin, and their tails retained larval proportions. Tadpoles continued to grow into giants. In addition, Allen (1918) noted that thyroidless tadpoles retained the head and intestinal features of larvae, but their gonads proceeded to differentiate and mature. Gonadal development in thyroidectomized *R. pipiens* larvae confirmed that germ cell differentiation is not under the influence of the thyroid gland, while somatic differentiation and metamorphosis require thyroid gland induction.

In a previous study we showed that, although naturally occurring giant *X. laevis* tadpoles lack endogenous TH, their tail tissue retains its sensitivity to TH, and triiodotironine (T_3)-mediated tailtip resorption could be induced *in vitro* (Rot-Nikcevic and Wassersug, 2003). This suggests that treatment with exogenous T_3 in whole animals might induce giants to resume development and eventually metamorphose. Alternatively, giantism *per se* could physically inhibit normal development and metamorphosis in tadpoles. The upper size limit for a tadpole that can still metamorphose is likely to be species-specific though, as the largest of all tadpoles, *Pseudis paradoxa* larvae, for example, can reach body lengths up to 22 cm (Emerson, 1988), and still have normal obligatory metamorphosis.

In the present study we explore how the disrupted ratio between development and growth in giant *X. laevis* tadpoles limits their normal development. We examine whether growth in giants is a simple allometric extension of normal growth, and whether increase in visceral size, for example, follows the excessive increase in body length and mass. The fact that giant tadpoles have difficulty swimming, and frequently rest on the bottom, leads us to hypothesize that visceral growth is not proportional to total body growth – specifically, the lungs of these giants are probably not large enough to provide buoyancy.

We also hypothesize that, even if treatment with exogenous TH can induce metamorphosis in *X. laevis* giant larvae, the resorption of their massive tail muscle will take disproportionately longer than the transformation of other parts of their body, particularly the viscera. This might pose a barrier for normal development and metamorphosis of these animals.

However, the fact that *X. laevis* can live for several years as larvae, without metamorphosing, raises the question of whether larval reproduction is possible in tadpoles, and if so, why neoteny, although common in urodeles, has never evolved in anurans. Hayes (1997b) suggested that decoupling of the hormonal requirements for metamorphosis and reproduction allowed neoteny to evolve several times in urodeles. The fact that thyroidectomized *R. pipiens* larvae and naturally thyroidless *X. laevis* tadpoles show advanced gonadal development without metamorphosis (Allen, 1918) suggests that larval reproduction – at least in terms of maturation of sperm and egg production – may be possible in anurans. Here, we investigate limitations other than hormonal requirements that may hinder the evolution of neoteny in anurans.

Materials and methods

Animals

Forty-five giant *Xenopus laevis* Daudin tadpoles with arrested development, ranging in age from 1 to 8 years, were obtained from Xenopus One (Dexter, MI, USA). Twenty-five were used to study external and internal growth and differentiation, and ten for hormonal induction of metamorphosis. Tadpoles originated from different clutches and, although we lack detailed information on their genetic relationship, they were not full sibs. Normal *X. laevis* tadpoles were obtained from our in-house breeding colony, and all were from the same clutch.

Morphology – Dissection and measurements

Animals were killed using MS 222, and staged (Nieuwkoop–Faber, 1956) prior to dissection. Total body length, tail length, pleuroperitoneal cavity length, maximum head width and height, maximum tail fin height and tail muscle height, were measured on intact animals using a digital caliper. The volume of intact animals was measured by water displacement in tubes of precisely known volumes.

Transverse cuts were made at the vent and throat, and along the midline, and the skin flaps pulled to the sides to expose the viscera. The alimentary tract was dissected away, and its intestinal volume measured by displacement of water in

notched pipette tips using Hamilton gas syringes graduated to 0.01 ml. Maximum length and width of the liver, heart, spleen, gallbladder, kidneys and gonads were measured *in situ*, using an ocular micrometer. Fat bodies were dissected out and their volume measured by water displacement. The gallbladder was too small to measure its volume reliably, therefore we used the equation for the volume of a spheroid, $V=4/3\pi[(L+W)/4]^3$, where L and W represented gallbladder length and width, respectively (Frankenberg and Werner, 1992).

The lungs were first outlined *in situ* using a camera lucida, and their area measured in NIH Image (NIH Image for Macintosh 1999, Version 1.62, National Institute for Mental Health). Both the lungs and the gonads were then dissected out and embedded in paraffin, cut in 4 μm cross-sections, and stained with Hematoxylin and Eosin or Masson's Trichrome. After all the viscera were dissected away, the volume of eviscerated animals was measured.

A cross-section of axial muscle from one giant and one normal *X. laevis* tadpole was traced using a camera lucida and area was measured in NIH Image.

Animals were preserved in 10% formalin. For all giant tadpoles, lungs, intestine and gonads (if present) were preserved.

One normal tadpole, two giant tadpoles and one giant tadpole exposed to T_3-treatment (see below) were cleared and double-stained for bone and cartilage using Alizarine and Alcian Blue, following the protocol of Hanken and Wassersug (1981).

Sample sizes for the gallbladder volume and the lung area in giant tadpoles are smaller than the full sample size, due to the destruction during dissection of these organs in a few specimens.

Hormonal treatment

Five normal and five giant *X. laevis* tadpoles of the same stage were reared in two separate 10 l tanks, with exogenous T_3 added to a 3 nmol l^{-1} concentration. Five normal tadpoles reared in a separate tank without added hormone were used as a control group, as well as 25 giant tadpoles.

Prior to hormonal treatment animals were staged, and their body mass, volume, total body length, body width, tail length, tail fin and muscle height measured. Every 2 weeks the animals were anaesthetised in MS 222, staged and measured. During the first 2 weeks of treatment, water samples from both experimental tanks were drawn and T_3 concentrations assayed every other day, at the IWK Health Center in Halifax, NS, USA. Once a week half of the water in the tanks was changed, and fresh hormone added. This proved to be frequent enough to maintain a constant T_3 concentration.

The same experiment was repeated with another set of five giant tadpoles exposed to 1 nmol l^{-1} exogenous T_3, to test for the possible effects of hormone dosage.

Statistical analyses

The analyses were run in STATISTICA (STATISTICA for Windows 1997, Version 5.0, StatSoft, Inc.). All body measurements were log-transformed. To control for the overall effect of body size, measurements were regressed to total body length, snout–vent length (*SVL*) or total body volume. An analysis of covariance (ANCOVA) was used to test for differences in relative body measurements among normal and giant tadpoles. Differences in the slopes of the regression lines were analysed using the Test for Parallelism (STATISTICA), to determine whether growth in giants is a simple allometric extension of normal growth.

Differences in external body measurements in animals before and after T_3 treatment were tested using analyses of variance (ANOVA). Visceral measurements in the 10 giant tadpoles exposed to hormonal treatment were compared to corresponding measurements in 25 control giants not exposed to T_3.

Results

General description

External morphology

Developmental stages of all giant tadpoles ranged from NF stage 53 to 56, i.e. early hindlimb bud stages. Body mass and eight external morphological dimensions of giant and normal

Table 1. *External morphological traits in normal and giant tadpoles*

Trait	Mean value (Range)	
	Normal	Giant
NF developmental stage	54.2±0.2 (53–56)	54.3±0.2 (53–56)
Total body length (mm)	33.1±0.6 (23.86–38.57)	84±4 (55.06–113.29)
Tail length (mm)	21.4±0.4 (14.57–24.82)	57±3 (32.61–76.39)
Snout–vent length (mm)	11.7±0.2 (9.29–14.32)	27±1 (18.69–37.70)
Pleuroperitoneal cavity length (mm)	4.9±0.1 (3.85–6.48)	15.4±0.9 (9.80–26.21)
Body width (mm)	7.0±0.1 (5.13–7.92)	12.4±0.4 (9.25–16.57)
Body height (mm)	5.0±0.1 (3.01–5.59)	8.0±0.3 (5.19–11.45)
Tail fin height (mm)	3.7±0.1 (2.87–6.29)	10.6±0.7 (5.99–18.38)
Tail muscle height (mm)	2.34±0.06 (1.44–2.80)	7.2±0.6 (2.70–12.08)
Body mass (g)	0.21±0.01 (0.12–0.26)	3.3±0.4 (1.08–10.31)

Values are means ± S.E.M. (N=25 in both groups).

tadpoles of the same stage are presented in Table 1. Total body length in giant *X. laevis* tadpoles was 2.5–5 times larger than that of normal ones. However, giants' body mass was, on average, 15 times greater, with the largest specimens up to 50 times heavier than the average normal tadpole.

Visceral morphology

The most obvious qualitative differences between giant and normal tadpoles were in the lungs, fat bodies, gonads and the coiling of the intestine.

All normal *X. laevis* tadpoles had functional, inflated lungs. However, the majority of giants, i.e. 17 out of 25 tadpoles (68%), had small and, to varying degrees, solidified lungs (Fig. 2A). Only eight giant tadpoles (32%) had well-developed and inflated lungs (see Fig. 2B). A significant relationship between the total body mass, or volume, and the lung area was not found.

The lungs of normal tadpoles were not septate (Fig. 3A). The lung structure in giant tadpoles with inflated lungs (Fig. 3B) resembled that of normal tadpoles, i.e. most of the lung was air-filled space, but had more septa. Solid lungs, however, showed a large number of septa with thick layers of smooth muscle tissue, and little space left in the lung sacs to be filled with air (Fig. 3C). The wall of solid lungs consists of a thin lung epithelium, a thick smooth muscle layer with abundant collagen, and numerous melanocytes. At higher magnification, thick bands of collagen were visible within the septa of solidified lungs in giants (Fig. 3D). In our years of experience with giant *X. laevis* tadpoles we have never seen individuals that were negatively buoyant and resting on the bottom swim to surface and gulp air.

In normal tadpoles the alimentary tract is arranged in a spiral, and fills most of the pleuroperitoneal cavity. In giant tadpoles the classic coiling pattern was not present; i.e. the intestine made random left and right loops, with few coils and no consistent pattern for intestinal packing.

Fat bodies were present in 15 giant tadpoles (60%), and in most cases (80%) the fat bodies were large and occupied most of the visceral cavity. They were bright yellow in colour, with numerous finger-like projections entwined among the other viscera (Fig. 4). Fat bodies were absent in all normal tadpoles of the same stage.

None of the normal tadpoles had developed gonads. However, many giants had advanced gonadal differentiation. Out of 25 giant tadpoles, 13 had well-developed gonads. Out of these 13 tadpoles, 12 were females and their ovaries were full of eggs (Fig. 5A). The largest oocytes in giant tadpoles were at stage IV (Dumont, 1972); the animal and vegetal hemispheres were differentiated, oocytes had yolk, and oocyte size was on average 600 μm. Consistent left/right asymmetry (handedness) typical of anurans (Kendal 1938; cited in Viertel and Richter, 1999) was not found in the size of ovaries (see also Malashichev and Wassersug, 2004). One giant tadpole was male, with well-developed testes. Histological analyses

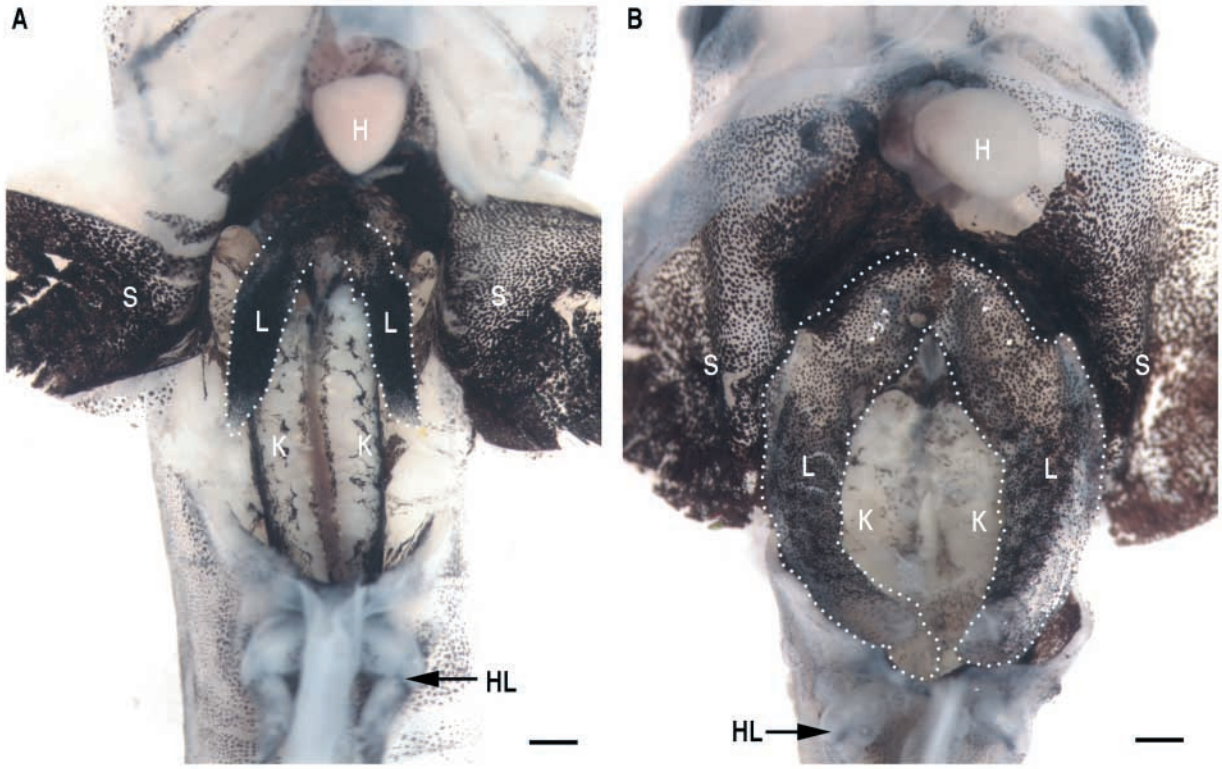

Fig. 2. Partially solidified (A) and inflated (B) lungs in two giant *X. laevis* tadpoles seen *in situ* in ventral view. The majority (68%) of giant tadpoles have small and partially solidified lungs as shown in A. The alimentary tract and associated organs have been removed. Lung sacs are outlined with a white dotted line. L, lung sac; H, heart; K, kidney; S, skin flap; HL, hind limbs. Scale bars, 0.2 cm.

showed that sperm was present in its testes (Fig. 5B). Interestingly, this only male giant came from our *Xenopus* colony almost three decades ago. The 12 females all originated from the Xenopus One breeding colony.

Morphometric analyses

The volume of intact and eviscerated tadpoles and the volume of their internal organs are presented in Table 2. Giant tadpoles had greater variance in all measured morphological traits than did normal tadpoles.

External morphology

Differences in tail length between giant and normal tadpoles, when total body length was controlled for, were not significant (ANCOVA, P=0.108). Also, there were no significant differences in the slopes of the regression lines (P=0.942). Therefore, the relationship between the increase in the tail length with increase in total body length was similar in the two groups (see Fig. 6). The test for parallelism for head length, head width, and tail fin and tail muscle height, revealed homogeneity of slopes (P>0.05) as well, suggesting that all these traits retain their normal allometric relationships. Similar results were obtained when the head length and width were regressed to *SVL* instead of total body length. Although differences between giant and normal tadpoles in the length of the pleuroperitoneal cavity relative to total body length were significant (ANCOVA, P=0.020), with giant tadpoles having a longer pleuroperitoneal cavity than normal tadpoles, the regression line slopes were the same (Fig. 6).

When total body length was accounted for, giant tadpoles had significantly larger body mass than normal tadpoles (ANCOVA, P=0.004). However, the regression of body mass on total body length revealed no differences in slopes (P=0.103), indicating that the increase in body mass with the increase in total body length had the same growth trajectory in both groups (Fig. 6).

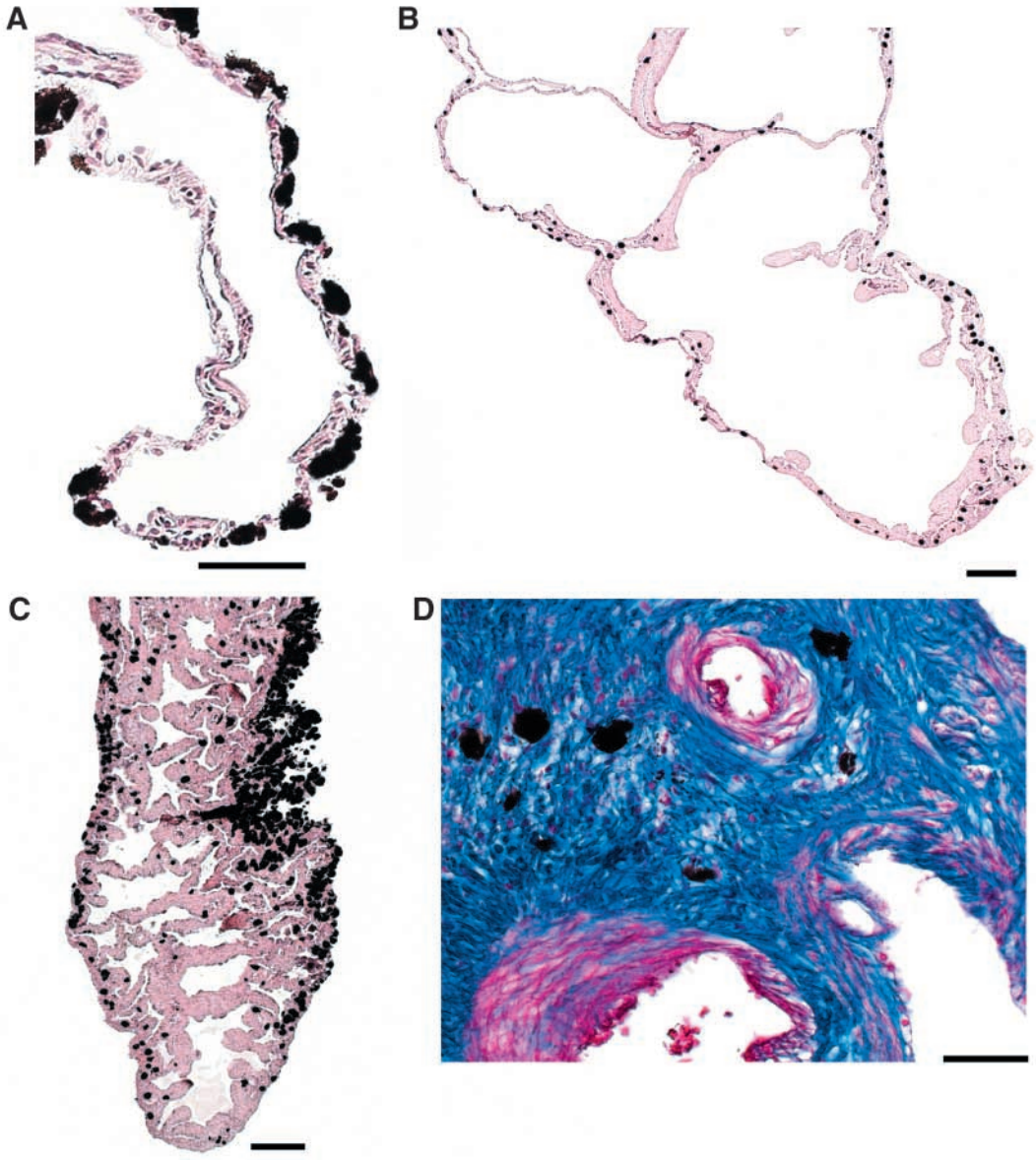

Fig. 3. Cross-section of a lung sac in (A) normal *X. laevis* tadpole, (B) giant *X. laevis* tadpole with inflated lungs and (C) giant *X. laevis* tadpole with solidified lungs. Sections are stained with Hematoxylin and Eosin. Inflated lungs in giants are functional, but more septa are present than in normal tadpoles' lungs. Solidified lungs in giants show numerous septa and little air space. Dark melanocytes are present in all lungs. (D) Cross-section through a septum of a solidified lung, stained with Masson's Trichrome. Abundant collagen (blue) is present in the walls and septa of solidified lungs. Smooth muscle tissue (red) is lining a blood vessel. Scale bars, 200 μm (A–C); 50 μm (D).

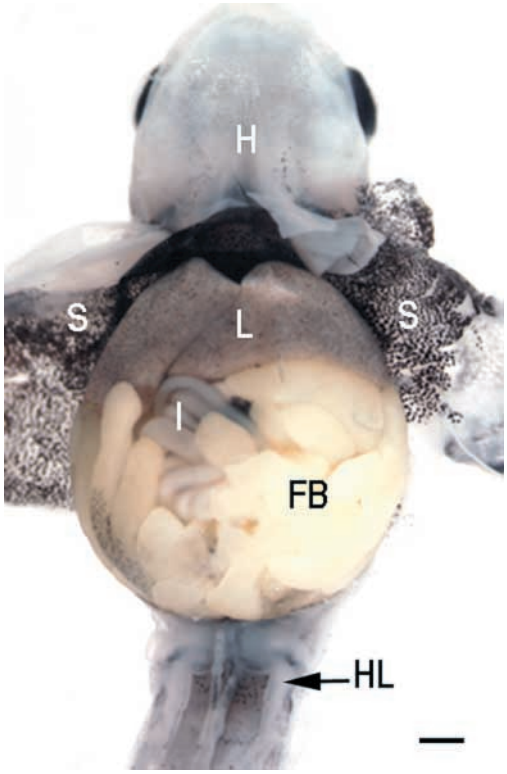

Fig. 4. Ventral view of a giant *X. laevis* tadpole with the skin of the abdominal wall reflected. Large fat bodies (FB) occupy most of the visceral cavity in giants. L, liver; I, intestine; S, skin flap; H, head; HL, hind limbs. Scale bar, 0.2 cm.

Eviscerated body volume relative to intact body volume was significantly greater for giant tadpoles than normal ones (ANCOVA, P=0.047). In other words, the somatic (non-visceral) tissue in giant *X. laevis* tadpoles represents a significantly larger portion of the total body volume than in normal tadpoles. The axial muscle mass in our giant tadpoles was disproportionately enlarged. The cross-sectional area of axial muscle tissue was 16 times larger in a representative giant than in a normal tadpole of the same stage (Fig. 7), whereas body length was only 2.5 times greater. Therefore, the axial muscle mass was approximately 2.5 times larger than would be expected if it scaled proportionately as the square of body length.

Visceral morphology

Although the alimentary tract in giant tadpoles was not coiled as in normal *X. laevis* tadpoles, there were no significant differences between the groups in intestinal volume relative to total body volume (ANCOVA, P=0.461).

Giant tadpoles had longer livers relative to their total body length than normal tadpoles (ANCOVA, P=0.0001). Also, the difference between the regression line slopes was significant (P=0.022), showing a slower increase in liver length with the increase in total body length in giant compared to normal tadpoles (Fig. 6). The same results were obtained for liver length relative to *SVL*. No significant differences were found in liver width relative to total body length between groups.

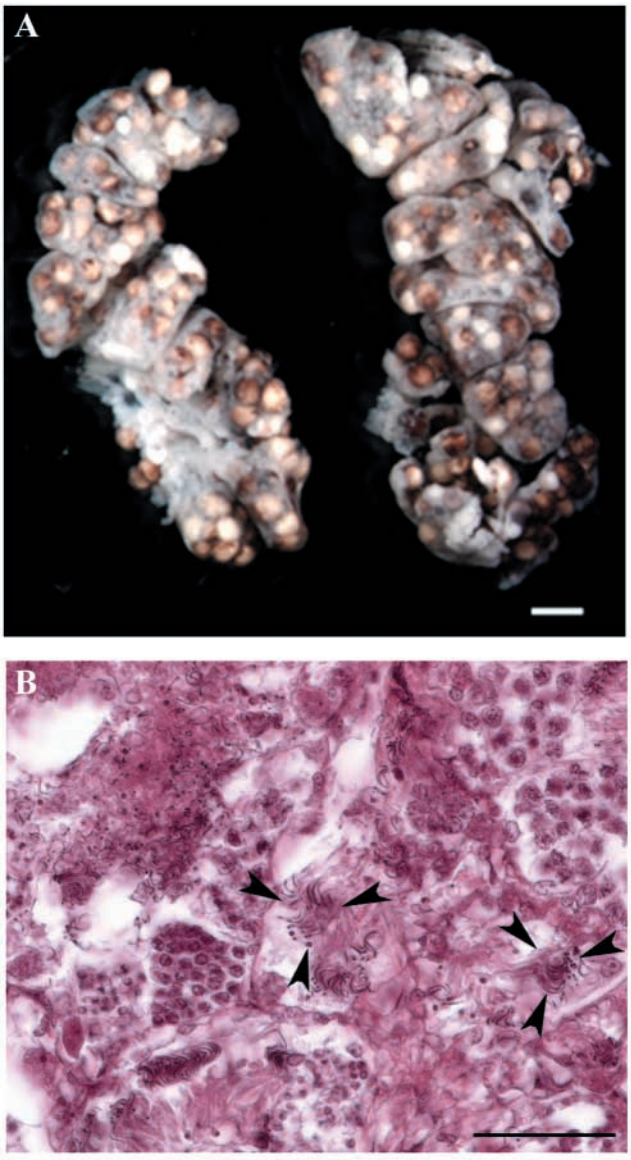

Fig. 5. Gonads in giant *X. laevis* tadpoles. (A) Left and right ovaries in the female giant, NF stage 54. The largest oocytes are at Dumont stage IV, the animal and vegetal hemispheres are differentiated, and yolk is present. (B) Cross-section through the testis of the male giant. The presence of sperm in the testis (arrowheads) shows that the gonads are mature; however, the tadpole's external morphology corresponds to NF stage 53. Scale bars, 1 mm (A); 50 μm (B).

We compared lung area against total body volume in three groups: normal tadpoles (with inflated lungs), giant tadpoles with inflated lungs, and giant tadpoles with solidified lungs. Relative lung area was significantly different in all groups (ANCOVA, P<0.001). Giants with inflated lungs had the largest lung area relative to body volume, followed by normal tadpoles, and lastly by giants with solidified lungs. Slopes of the regression lines showing the relationship between lung area and total body volume (Fig. 8), significantly differed only between normal tadpoles and giants with solid lungs (P=0.047), but were homogenous in normal tadpoles and

Table 2. *Visceral measurements in normal and giant tadpoles*

Trait	Normal		Giants	
	N	Mean value (Range)	*N*	Mean value (Range)
Volume (intact) (mm^3)	25	0.30±0.05 (0.08–1.50)	21	3.0±0.4 (1.00–9.80)
Volume (eviscerated) (mm^3)	25	0.22±0.04 (0.07–1.00)	20	2.4±0.3 (0.60–7.00)
Intestine volume (mm^3)	25	0.020±0.001 (0.002–0.04)	21	0.07±0.01 (0.10–0.17)
Liver length (mm)	25	1.91±0.06 (1.13–2.86)	21	5.7±0.3 (3.13–8.13)
Liver width (mm)	25	1.73±0.04 (1.25–2.00)	21	7.1±0.5 (3.50–10.75)
Heart length (mm)	25	0.99±0.04 (0.63–1.50)	21	2.5±0.1 (1.63–3.25)
Heart width (mm)	25	0.92±0.02 (0.63–1.13)	21	2.5±0.1 (1.50–3.13)
Spleen length (mm)	13	0.35±0.02 (0.25–0.50)	20	1.6±0.1 (0.75–2.25)
Spleen width (mm)	13	0.27±0.02 (0.25–0.50)	20	1.34±0.09 (0.63–2.13)
Gallbladder volume (mm^3)	25	0.93±0.08 (0.01–1.55)	20	12±2 (2.25–35.09)
Lung area (mm^2)	23	2.7±0.2 (1.02–5.57)	21	31±4 (6.64–75.89)
Kidney length (mm)	48	2.98±0.07 (2.50–4.13)	42	9.9±0.3 (7.00–14.63)
Kidney width (mm)	48	0.50±0.01 (0.38–0.75)	40	1.78±0.06 (0.88–2.75)
Fat bodies volume (mm^3)	–	Absent	10	0.05±0.01 (0.003–0.09)
Ovary length (mm)	–	Absent	24	9.6±0.7 (5.63–15.13)
Ovary width (mm)	–	Absent	24	3.2±0.3 (2.00–4.88)

N=sample size.
Data for ovaries and kidneys were added together for left and right side.

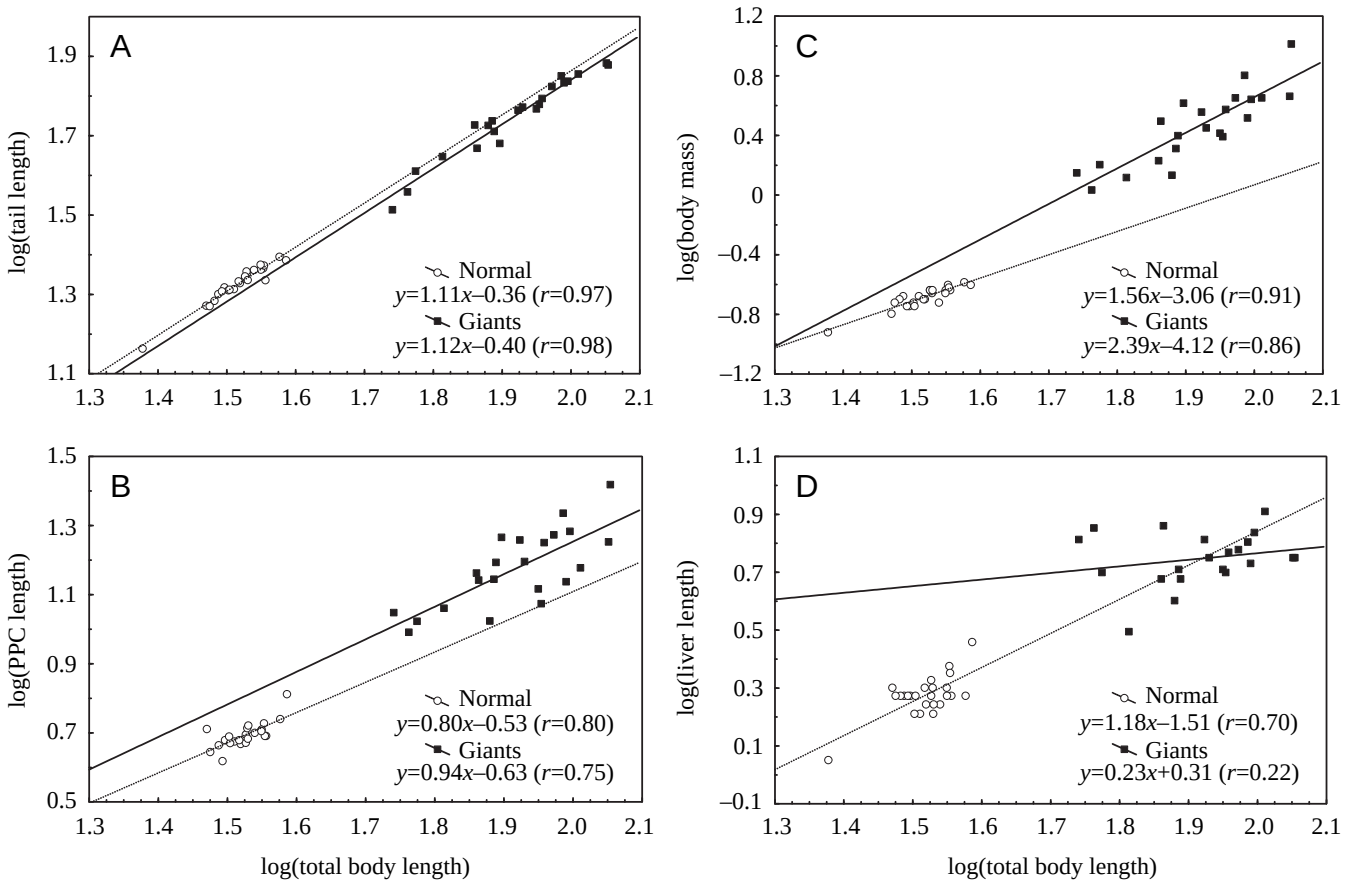

Fig. 6. Regression lines for the tail length (A), pleuroperitoneal cavity (PPC) length (B), body mass (C) and liver length (D) against total body length in normal and giant *X. laevis* tadpoles. The tail in giant tadpoles retains normal allometric proportions. Giants had significantly longer relative visceral cavities, and were relatively more massive than normal tadpoles. The liver is the only organ in giants that shows a slower increase in length with the increase in the total body length, compared to normal tadpoles. All regressions are significant ($P<0.05$) except for the liver length in giants ($P=0.49$).

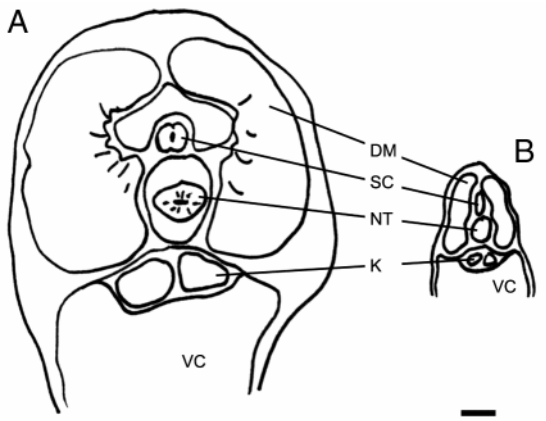

Fig. 7. Cross-section through the body of a giant (A) and a normal (B) *X. laevis* tadpole (NF stage 53), taken at the point of maximum body height. The cross-sectional area of axial muscle tissue was 16 times larger in the giant than in the normal tadpole, i.e. 51.28 mm^2 *vs*. 3.14 mm^2. DM, dorsal muscle; SC, spinal cord; NT, notochord; K, kidney; VC, visceral cavity. Scale bar, 1 mm.

giants with inflated lungs (P=0.832), as well as in giants with solid lungs and giants with inflated lungs (P=0.147). In giant and normal tadpoles with inflated lungs, the lung area increased with an increase in body volume, following the same pattern. However, in giant tadpoles with solid lungs, the regression line's slope suggests a faster increase in lung area with body volume, when compared to normal and giant tadpoles with inflated lungs.

Heart length and width relative to total body length were both significantly larger in giant tadpoles (ANCOVA; P=0.02 and P<0.001, respectively) than normal ones. Similar results were obtained when heart length and width were regressed to *SVL*. However, differences in the regression lines' slopes in the two groups were not significant for either heart length or width (length, P=0.139; width, P=0.614). Thus, giants have relatively larger hearts, but the increase in heart size with increasing body size follows the same pattern in giant and normal tadpoles.

Both spleen length and width relative to total body length were greater in giant tadpoles than normal ones (ANCOVA; length, P=0.006; width, P=0.001). The relationship between the increase in spleen size and increase in total body length was similar in giant and normal tadpoles, since the regression slopes did not differ significantly (length, P=0.681; width, P=0.260). Similar results were obtained when spleen measurements were regressed to *SVL*.

Gallbladders in both normal and giant tadpoles were transparent spherical sacs. Giant tadpoles had larger gallbladders than normal tadpoles relative to total body volume (ANCOVA, P=0.047), but the regression lines' slopes did not differ (P=0.149).

Relative kidney length and width (left and right) were larger in giants than in normal tadpoles, with no significant differences in regression slopes. Left/right asymmetry in the size of kidneys was not present in either giants (ANOVA, length, P=0.619; width, P=0.646), or in normal tadpoles (ANOVA, length, P=0.875; width, P=0.464).

T_3-induced metamorphosis in giants

Adding T_3 to the water housing the tadpoles induced metamorphic changes in both normal and giant *X. laevis* tadpoles. Hormone-induced metamorphosis proceeded faster in normal tadpoles than in giants, and after a 3-week exposure to 3 nmol l^{-1} T_3, all normal tadpoles had transformed into froglets. During the same period of time, giant tadpoles developed only to the stage when forelimbs emerge (stage 58). After an additional week of T_3 treatment, all giant tadpoles reached stage 62, when both fore- and hindlimbs are developed, and the tail starts resorbing. The developmental and morphological response in giants was the same in 1 nmol l^{-1} T_3. In water containing both 1 and 3 nmol l^{-1} T_3, all giant

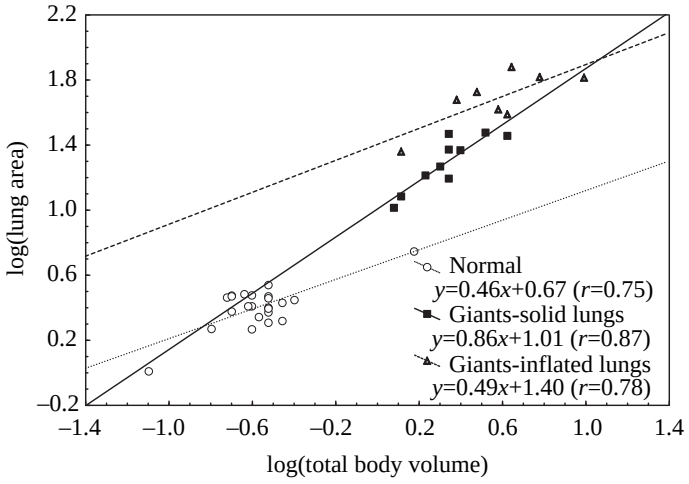

Fig. 8. Regression lines for the lung area against total body volume in normal *X. laevis* tadpoles, giant tadpoles with solid lungs and giant tadpoles with inflated lungs. Giants with inflated lungs had the largest relative lung area, followed by normal tadpoles, and lastly by giants with solidified lungs. However, the increase in lung area relative to the body volume was the fastest in giant tadpoles with solidified lungs. All regressions are significant (P<0.001).

Fig. 9. A giant *X. laevis* tadpole (stage 60) following exposure to exogenous 3 nmol l^{-1} thyroid hormone for 21 days. Note the developed hindlimbs, the emergence of the forelimbs and the narrowed head. The tail fin has started to resorb; however, total body length and tail length did not change. Scale bar, 1 cm.

Table 3. *Thyroid hormone treatment in ten giant* Xenopus laevis *tadpoles: developmental stage (NF) and body measurements prior to (Day 0) and after 28 days of 3 nmol l^{-1} and 1 nmol l^{-1} T_3-treatment*

	3 nmol l^{-1} T_3			1 nmol l^{-1} T_3		
Trait	Day 0	Day 28	ANOVA	Day 0	Day 28	ANOVA
NF stage	54.4±0.5	62.0±0.0	***P*<0.001**	54.0±0.0	62.0±0.0	–
Total body length (mm)	94±4	90±5	*P*=0.563	88±6	73±6	*P*=0.122
Tail length (mm)	66±3	63±4	*P*=0.659	60±5	50±5	*P*=0.224
Tail fin height (mm)	13.4±0.8	10.4±0.9	***P*=0.035**	12.4±0.6	7.1±0.3	***P*<0.001**
Tail muscle height (mm)	10.3±0.7	7.3±0.5	***P*=0.006**	7.6±0.4	6.4±0.3	***P*=0.030**
Body width (mm)	13.4±0.3	10.4±0.2	***P*<0.001**	13.4±0.7	9.2±0.4	***P*=0.001**
Body mass (g)	3.7±0.4	2.5±0.3	***P*=0.043**	3.7±0.3	1.9±0.2	***P*=0.001**

All tadpoles in the 1 nmol l^{-1} T_3 treatment group were the same stage, therefore ANOVA cannot be computed.
Significant differences are given in bold.

tadpoles died during the fifth or sixth week, upon reaching stage 63 (determined by limb and cranial morphology). During transformation the head of giants significantly narrowed, and their body mass decreased. However, the tails did not reach stage 63, since they had not started resorbing at that time. Total body length, as well as tail length, did not change. The only change in the tail induced by T_3 treatment was a significant decrease in tail fin and tail muscle height. A giant tadpole that underwent T_3 treatment, and subsequently developed forelimbs, is shown in Fig. 9. Developmental stages and external body measurements of giants at the beginning (Day 0) and after 4 weeks (Day 28) of treatment are given in Table 3, for both 1 nmol l^{-1} and 3 nmol l^{-1} T_3 concentrations.

While some external morphological characters showed metamorphic changes under T_3 treatment, visceral morphology did not change significantly. The only significant difference found between groups of giant tadpoles before and after T_3 treatment was in gallbladder volume (ANCOVA, P=0.0004). The average gallbladder volume in giant tadpoles not exposed to T_3, was 11.87±1.72 mm^3 (range 2.25–35.09). In giant tadpoles that had been exposed to exogenous T_3 for 4 weeks, gallbladder volume was 1.23±0.19 mm^3 (range 0.24–1.94), i.e. reduced to a tenth its former size.

Double-staining for bone and cartilage revealed substantial metamorphic changes in the head of T_3-treated giant tadpoles after 4 weeks. These include the normal resorption of the branchial baskets, shortening and reorientation of the palatoquadrate, and extensive elongation of Meckel's cartilage. Many of the dermal bones have started to ossify. The anlage of the adult teeth are now present in the upper jaw. The changes we observed in the head are completely consistent with the normal metamorphic processes for *X. laevis* at NF stage 63.

Discussion

In the absence of thyroid glands, and therefore TH, *X. laevis* tadpoles arrest their development around NF stage 54 and fail to metamorphose. Instead they continue to grow and develop into deformed giant larvae. The precise hormonal pathways that lead to this giantism are not known.

All external morphological characters that we measured in giants retained normal allometric relationships, except for pleuroperitoneal cavity length. Disproportionate increase in pleuroperitoneal cavity size is an apparent adjustment to the increase in visceral volume, primarily associated with advanced development of the gonads and hypertrophy of fat bodies. The increase in total body volume, which is due to an enlarged axial muscle mass, has a strong effect on giants' morphology and behaviour.

Huang and Brown (2000a) noted that transgenic frogs overexpressing growth hormone (GH) typically develop skeletal abnormalities, such as disproportionately large heads and feet, as well as deformed vertebral columns. This suggests that scoliosis in our giant *X. laevis* tadpoles could result from the excessive growth of the tadpole's body; i.e. enlarged axial muscles could exert excessive and unbalanced loads on the vertebral column and notochord, forcing them to curve.

The increased axial muscle of giants would also contribute to the tadpoles' negative buoyancy. In addition, the majority of giants had solid lungs, with a relative lung area smaller than in giants with inflated lungs. We suspected that the enlarged fat bodies of giants, by occupying most of the pleuroperitoneal cavity space, might prohibit proper lung development. However, such a relationship was not found, as small, solid lungs were present in tadpoles both with and without fat bodies. The negative buoyancy in giants, caused primarily by their excessive mass, likely limits proper lung ventilation and leads to their partial solidification. Pronych and Wassersug (1994) showed that the lungs of *X. laevis* tadpoles that have been denied access to air were substantially smaller and frequently showed anomalies, such as partial solidification.

Atkinson and Just (1975) showed that during later metamorphic stages of normal tadpoles there is an increase in the number and size of septa, which potentially increases the surface area of respiratory epithelium. The increased number of septa in the inflated lungs of giant tadpoles in our study compared to normal tadpoles' lungs suggests that the lungs of these giants underwent metamorphic transformation far beyond the development of the tadpole as a whole. However, in the solidified lungs of giants the tissue proliferation is so

abundant, and the number of septa extending into the lumen of the lung so extensive, that the luminal volume is compromised. The faster increase in lung area with increasing body volume that we observed in giant tadpoles with solid lungs, compared to normal tadpoles and giants with inflated lungs, may be compensatory growth in response to the reduced functionality of the solidified lungs.

In contrast to the growth of the lungs, the growth of most of the viscera in giants keeps up with the increase in total body size. However, the fact that the biliary system and intestine in giants do not follow the same growth trajectory could be a limitation to the tadpoles' normal transformation and viability. Interestingly, in giants the typical coiling pattern in the intestine was absent, and only irregular loops were present. Kemp (1951) concluded that intestinal coiling in anuran larvae results from elongation of the intestine within a visceral cavity that is not spacious enough to permit development as a straight tube or a single loop. By experimentally increasing the coelomic cavity in *Hyla regilla* embryos, Kemp (1946) induced the development of an intestine that was nearly straight for most of its length, terminating in a short, irregularly coiled region. The giant larvae in our study had significantly larger pleuroperitoneal cavities compared to normal tadpoles, but the intestine volume relative to total body volume did not differ, which might explain the absence of typical gut coiling in giants.

A left-right asymmetry in the size of the kidneys was not found, although generally, larval kidneys are asymmetrical in size and shape (Nodzenski et al., 1989; Malashichev and Wassersug, 2004).

Of special interest to us were the hypertrophied fat bodies in the giants, and the complete absence of fat bodies in normal tadpoles. It has been shown that hypophysectomy of normal *X. laevis* tadpoles augments the total body lipid content, and over the long term fat bodies may become so enlarged that they occupy most of the abdominal cavity (Dodd and Dodd, 1976). Treatment of tadpoles with adrenocorticotropin (ACTH, secreted by the pituitary gland), lowers total body lipids, especially those of the fat bodies, and also increases lipase activity in the fat bodies (Dodd and Dodd, 1976). It remains unclear if the lack of a thyroid gland in giant tadpoles and therefore production of TH, possibly affects the pituitary production of ACTH by abolishing negative feedback to the pituitary gland.

Our most remarkable finding was the advanced gonadal development in giant tadpoles. It is still an ongoing debate as to how TH affects gonadal differentiation. Some researchers claim that gonadal differentiation is not under TH control (see review by Hayes, 1997b). However, Hayes (1997a) suggested that TH might be required for testicular development in *X. laevis*, since the administration of goitrogen thiourea to *X. laevis* larvae blocked TH production and resulted in a skewed sex ratio, i.e. 100% females. Our results show that gonadal development is not affected by the absence of TH the way the rest of the body is. Moreover, both male and female gonads were developed in our athyroid *X. laevis* tadpoles. In both sexes the gonads were mature, with sperm present in testes, and eggs in ovaries. Oocytes in giant tadpoles have yolk, which is normally produced in the liver (Duellman and Trueb, 1986), thus proving that the giants' reproductive systems are mature (if not functional). This is in contrast to previous findings (Wangh and Schneider, 1982) that without TH *X. laevis* is unable to synthesize vitellogenin and produce eggs. It is thought that estrogens from the ovary induce vitellogenin synthesis and secretion by the liver only after the liver has been exposed to TH (Hayes, 1997b). There is a slim possibility that our giant tadpoles had ectopic thyroid tissue that we failed to find, and thus may have produced some hormone that influenced liver function. However, if this were true, we would expect to see more metamorphic changes in giant tadpoles, and their development to proceed beyond NF stage 56.

Allen's work on thyroidectomized *Rana pipiens* larvae (Allen, 1916, 1918), also showed that gonadal development is advanced in both sexes in the absence of TH. We propose that TH are not needed for gonadal differentiation in *X. laevis*.

However, the fact that the only male in our study was from a different population than all the females, suggests that other factors may be influencing sex determination. For example, Hayes et al. (2002) have reported that extremely low concentrations of the commonly used pesticide atrazine demasculinizes *X. laevis* tadpoles. Some antioxidants, such as bisphenol A, have also been shown to induce feminisation in *X. laevis* tadpoles as well (Levy et al., 2004). We do not have data, though, showing high levels of these pesticides in the water used to raise tadpoles at Xenopus One.

Ogielska and Kotusz (2004) pointed out that the somatic stage of a tadpole often does not reflect its actual age; in fact age, rather than somatic stage, seems to be crucial for gonadal development. Chang and Hsu (1987) compared two groups of *Rana catesbeiana* tadpoles; the tadpoles did not differ in somatic stage, but did differ in age and size (older tadpoles were larger). Older tadpoles had more advanced differentiation of ovaries. The giant tadpoles in our study were up to 8 years old, which may have provided the time necessary for gonadal development beyond what would be expected for their NF developmental stage.

The advanced gonadal differentiation in our larval *X. laevis* raises the possibility of neoteny in anurans. In one sense, Allen (1918) had artificially produced neoteny in an anuran; i.e. although his tadpoles could not reproduce, they had differentiated gonads in the larval body. However, neotenic tadpoles have never been found, and several researchers have hypothesised why. Wassersug (1975), for example, speculated that space for adult genitalia, and particularly for storage of eggs in the female, is only made at metamorphosis by the extension of the body cavity, when the pelvic girdle and urostyle elongate. Our results show, however, that giant tadpoles have longer pleuroperitoneal cavities relative to body size than normal tadpoles, which may allow for enlarged viscera, including gonads, without full metamorphosis of the pelvis. Indeed, the giant tadpoles in our study were able to develop large ovaries full of eggs within their coelomic cavities.

However, typical anuran amplexus is not possible in tadpoles since their forelimbs are covered by the operculum, or, if already emerged at later stages, not large enough to embrace another individual. Tadpoles would need a way of proximating their sperm and eggs to ensure fertilisation, which would require major changes in their behaviour. Alternatives to amplexus do exist in anurans. For example, in the family Dendrobatidae adult frogs use cloacal apposition to transfer sperm (*Dendrobates granuliferus*, Crump, 1972; *Dendrobates pumilis*, Limerick, 1980). But, extrusion of eggs and sperm may still not be possible in tadpoles, simply because premetamorphic anurans lack the musculoskeletal features necessary to raise the pleuroperitoneal pressure to forcefully eject materials from their body cavity (see Naitoh et al., 1989; Wassersug, 1996, 1997). Obviously, for neoteny to occur in anuran larvae, some major behavioural and morphological changes beyond gonadal differentiation are necessary. In sum, the gonadal differentiation seen in our giant *X. laevis* tadpoles is necessary, but not sufficient, for neoteny.

The fact that tadpoles fail to metamorphose due to a lack of TH raises the question of whether athyroid giants can resume their development and eventually transform into normal frogs if given TH. Our treatment of giants with exogenous T_3 indicates that the arrested development of giant tadpoles is not due to an absence of thyroid hormone receptors (TR).

Xenopus laevis has at least two types of receptor isoforms, TRα and TRβ (Yaoita et al., 1990). TRα in tadpoles is expressed constitutively and is present before the appearance of TH (Eliceiri and Brown, 1999). TRβ responds to TH and its expression follows the rise of TH concentrations in cells (Yaoita et al., 1990; Wang and Brown, 1993; Eliceiri and Brown, 1999). During metamorphosis growing limbs have high TRα and low TRβ levels. However, during the climax of spontaneous metamorphosis TRβ is upregulated in tails and the TRβ protein is more abundant than TRα (Eliceiri and Brown, 1999). Our treatment with exogenous T_3 resulted in further growth and development of hindlimbs, as well as the eruption of forelimbs and loss of tail fin in giant *Xenopus* tadpoles, which suggests that, most probably, both TRα and TRβ are present in our giants. Significant decreases in tail fin and muscle height, a decrease in body mass, narrowing of the head, and a change in the shape of the mouth were all observed. These are features associated with normal metamorphosis in tadpoles. Total body length and tail length, however, remained unchanged, and all giant tadpoles died at NF stage 63, which is the stage when tail shrinkage occurs in normal tadpoles (Nieuwkoop and Faber, 1956).

X. laevis tadpoles that retain their tails have been observed several times before (Kinoshita and Watanabe, 1987; Elinson et al., 1999; Huang and Brown, 2000b). The ‘tailed frogs’ produced by Huang and Brown (2000b), were transgenic *X. laevis* tadpoles overexpressing PRL. These animals developed to NF stage 63 with a long thin tail. The muscle resorbed and the notochord collapsed, but the fins did not resorb. In fact the tadpoles continued to grow in the PRL-overexpressing tailed frog. Tailed frogs were also produced by treatment with goitrogen methimazole (Elinson et al., 1999), and in transgenics overexpressing type III deiodinase that encodes a T_3-inactivating enzyme (Huang et al., 1999). In all these cases though, the tails are small compared to ours and lack the typical chevron-shaped blocks of fast muscle. They do, however, retain the longitudinal slow muscle cords. It should be noted that those tails look very different from the ones found in our tadpoles. Ours are the opposite: the excessive muscle mass was retained, although the fins are lost with T_3 induced metamorphosis.

None of these ‘tailed frogs’, however, have a tail like that of *Ascaphus truei*, which is a copulatory organ only present in the males (Stephenson and Verrell, 2003). Our giant *X. laevis* tadpoles, in contrast, still maintained fast muscle chevrons in their tails after T_3 treatment, suggesting that there are several different scenarios for tail retention in *Xenopus* after metamorphosis. Huang and Brown (2000b) showed that transgenic tadpoles overexpressing PRL develop into frogs whose tails are filled with collagen and not muscle. Interestingly, their tails are resistant to T_3 treatment. Since our giant tadpoles lack TH that would counteract the effects of PRL, it is possible that their tails have excessive collagen; that, in turn, might make them resistant to shrinkage under T_3 treatment.

The retention of a tail of any sort in a post-metamorphic anuran may reflect the atavistic fact that the amphibian ancestor of modern anurans surely had a tail. The fact that these tails show up in *Xenopus* may reflect the more generalized nature of this pipoid frog compared to other anurans. Alternatively it may simply be due to the fact that scientists work more with *Xenopus* than other anuran species.

No substantial metamorphic changes in viscera were detected in T_3-treated giant tadpoles, except for the drastic decrease in gallbladder volume. (The reason for this extreme reduction in gallbladder size with metamorphosis is unclear. In fact, the function of the large gallbladders typical of tadpoles remains unexplored.)

We propose that giant tadpoles might be incapable of resorbing the massive axial musculature in their tails and backs. As well, they may not be capable of metamorphosing their viscera at the same rate as the rest of their body. All this, as well as small and solidified lungs, may preclude normal metamorphosis in giant *X. laevis* tadpoles.

The increased somatic growth in giants has interesting implications to phenotypic plasticity in tadpoles. It has been demonstrated that tadpoles alter their morphology when exposed to increased competition, i.e. competition induces tadpoles to have larger bodies and smaller tails (Relyea, 2002a,b). Larger tadpoles have a larger feeding apparatus (i.e. branchial baskets) which, in turn, makes them better competitors. Huang and Brown (2000a) noted that *X. laevis* tadpoles overexpressing GH show a disproportionate increase in head size due to the increase in gill arches, i.e., their branchial baskets. It is, thus, possible that stress from competition raises levels of GH, which could produce larger heads and specifically larger branchial baskets in tadpoles. So far, tadpoles subjected

to stressful environments have been shown to exhibit elevated hypothalamic corticotropin-releasing hormone content, which, by stimulating the production of pituitary hormones, leads to the increase in TH and corticosteroid levels. The pituitary production of GH could be under similar control. However, this has not yet been investigated.

Conclusions

The growth in giant *X. laevis* tadpoles represents an allometric extension of normal development for most traits. However, the lung development does not keep up with the increase in the total body volume, which evidently contributes to negative buoyancy, and results in the development of lung anomalies, such as partial solidification. At the same time, the pleuroperitoneal cavity is significantly enlarged in giant tadpoles, allowing for the storage of enlarged viscera and advanced gonadal differentiation.

The advanced gonadal differentiation associated with giantism would in principle allow neoteny; however, giantism constrains development of other organ systems in anuran larvae. Giants may mature reproductively, but are not capable of reproducing. Major behavioural and morphological changes would be necessary for making larval reproduction possible. Urodele larvae, in contrast, are similar to adults, and fewer morphological changes are necessary during maturation and metamorphosis, allowing for multiple evolution of neoteny in urodeles.

Moreover, tadpoles usually inhabit temporary, uncertain habitats with large fluctuations in resources, in which neoteny would not be adaptive (Wassersug, 1975). Clearly, given the collective morphological, behavioural and ecological limitations on larval reproduction in anurans, neotenic anurans have not and are not likely to evolve.

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