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GROWTH AND METAMORPHOSIS IN TADPOLES OF *RANA PRETIOSA PRETIOSA*, TREATED WITH RECOMBINANT PORCINE GROWTH HORMONE, PROLACTIN, SOMATOSTATIN (SRIF) AND ANTI-SRIF.

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Summary

The effect of prolactin (PRL), growth hormone (GH), somatostatin (SRIF) and anti-SRIF (αSRIF) treatment upon growth and metamorphosis of western spotted frog tadpoles, Taylor and Kollros (1946) stage (TK st.) XVI (n = 18/group) was examined. Treated animals received 2.5 g protein/g body weight every 5 days until one group exhibited ≥ TK st. XXIV and complete terrestrialization. Administration of GH and PRL resulted in 18/18 and 15/18 animals respectively expressing complete terrestrialization. 12/18 SRIF injected tadpoles achieved ≥ TK st. XXIV. In contrast, only 5 each of control and SRIF treated tadpoles transformed to ≥ st. XXIV. Significantly higher ($P<0.05$) length: weight ratios were recorded for control versus all other treatment groups. PRL and αSRIF treated animals exhibited reduced body length: fore limb length ratios ($P<0.05$) when compared to controls. Greater hind-limb length was noted in animals treated with GH, SRIF and PRL. PRL treatment resulted in significantly increased length and weight ($P<0.05$) when compared

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to controls, and highly significant (P<0.01) reduction in condition factor compared against all other treatment groups.

Key words: Rana pretiosa pretiosa, metamorphosis, anti–somatostatin, somatostatin, prolactin, growth hormone, growth

INTRODUCTION

The changes that occur during amphibian morphogenesis are dependent upon a complex and coordinated interplay between environmental and hormonal milieux. Central to the conclusion of anuran transformation are the thyroid hormones and prolactin (PRL), the funcional significance of which have been extensively reviewed (Fox, 1983; Rosenkilde and Ussing, 1990). The actions of PRL are pivotal in the maintenance and growth of larval structures. As such this hormone has been assigned an antimetamorphic role, although the precise mode of action of PRL remains to be more fully characterized. The interactions and importance of the adrenocortical hormones to metamorphic climax have also been considered in detail (reviews: Rosenkilde, 1985; Leloup–Hatey et al., 1990). These hormones are believed to promote metamorphosis, but their precise actions require more complete characterisation. In contrast to PRL, the thyroid hormones are thought to drive those morphophysiological modifications that conclude with transformation (Rosenkilde, 1985; Puzianowska–Kuznicka et al., 1996).

Prominent by its absence from the above scheme is growth hormone (GH). Yet, previous studies with ranid tadpoles have shown that treatment with pituitary–derived GH increases body weight and rate of limb development (review: McLean and Donaldson, 1993). Moreover, in bullfrog *Rana catesbeiana* tadpoles, circulating and pituitary GH concentrations increase during later stages of development (Clemons, 1977; Kobayashi and Kikuyama, 1991). These observations suggest that GH may have a more prominent role in larval amphibian morphogenesis than has been proposed. Notwithstanding the presence of comparatively high levels of circulating GH in tadpole plasma, the influence of exogenous GH therapy on metamorphosis has generally been attributed to contamination of pituitary–derived preparations with thyrotropin (TSH), or as a consequence of GHs inherent thyrotropic effect (Delidow, 1989). Approaches to examining, and interpreting the specific role of GH in amphibian development have thus been frustrated. With the advent of recombinant(r) DNA technologies however, interpretative problems associated with GH contamination may be eradicated. But, the use of rGH does not exclude GHs possible thyrotropic effect(s).

Several studies have indicated that GH secretion in amphibians is under the control of somatostatin (SRIF) and somatocrinin or GH–releasing factor (GRF; review: Harvey 1993). An alternative strategy to examine the potential significance of GH to amphibian morphogenesis may be intervention at the SRIF–GRF level. Such an approach, when combined with the application of rGH therapy, might provide for greater confidence in evaluating the effect(s) of GH on anuran transformation. Accordingly, a series of experiments were undertaken in which ovine PRL, r porcine(p) GH (rpGH), SRIF and anti–SRIF (αSRIF) were administered to Western spotted frog tadpoles. The effects of each treatment on growth and morphogenesis was examined with reference to a control, untreated group of animals.

MATERIALS AND METHODS

Animals and Husbandry

One hundred and fifty tadpoles of the western spotted frog *R. pretiosa pretiosa*, were collected from Alice Lake Provincial Park, Brackendale, British Columbia by netting. At collection, water temperature and pH were measured. Animals were transferred to the West Vancouver Laboratory in black plastic bags containing aerated lake water. For transportation, bags were placed in coolers and surrounded by ice. Tadpoles were subsequently maintained in 20 *l* tanks containing spring water. Both temperature and pH mimicked that measured at the collection site (16 °C, pH 6.8). Water was changed every other day. After a 24 h acclimation period, tadpoles were fed cooked spinach *ad libitum*.

Table 1. Mean starting weights and lengtsh, and treatment protocols of experimental groups. Control animals were injected with 50μl distilled water. Bioactive materials were delivered in an identical quantity of distilled water. All treatments were administered at five day intervals over a 25 day period. There were no significant differences between groups with respect to either weight or length at trial start.

Tablica 1. Srednje vrijednosti težina i dužina na početku pokusa, te protokol tretmana pokusnih grupa. Kontrola je obrađena s 50μl destilirane vode. Bioaktivne tvari dane su u istoj količini destilirane vode. Svi tretmani davani su svakih pet dana u trajanju od 25 dana. Nije bilo značajnih razlika između grupa s obzirom na težinu i dužinu tijela u odnosu na početne vrijednosti.

Treatment group	Treatment protocol	Weight (mg±SEM)	Lengt (mm±SD)
Control	untreated	628.9±14.5	34.25±1.74
GH	2.5g/g/5 days	645.6±12.9	34.17±1.31
αSRIF	2.5g/g/5 days	641.4±18.1	34.09±1.16
PRL	2.5g/g/5 days	639.3±18.3	34.19±1.13
SRIF	2.5g/g/5 days	640.8±31.6	34.22±1.08

Developmental stage for each animal was assessed using the scheme of Taylor & Kollros (TK; 1946). Two weeks post–collection, tadpoles at TK st. XVI were sorted into six groups of 18 animals, and the remainder were released at their point of collection. Groups were maintained in identical plastic see–through containers of 21 cm x 34 cm x 9 cm. Water, derived from the holding source, was added to each container and changed every other day. During handling, animals were sedated with metomidate hydrochloride (20 mg/*l*; Wildlife Laboratories Inc., Fort Collins, CO, USA). Prior to weighing, measuring and staging, animals were blotted dry on absorbent cloth. Mean starting weights and lengths, together with treatment regimes are presented in Table 1. RpGH (Amgen Inc., Thousand Oaks, CA., USA), ovine PRL, SRIF–14 (Sigma Chemical CO., St. Louis, MO., USA) and αSRIF (SOMA 10, MRC Regulatory Peptide Group, Department of Physiology, University of British Columbia, Vancouver, B. C., Canada) were injected in 50 μ*l* distilled water, using a 1 m*l* tuberculin syringe attached to a 28 gauge needle, once every 5 days for a study period of 25 days. Controls received distilled water alone. Injections were made at a point immediately posterior to the fore–limb bud region. The investigation was terminated once a single group had become terrestrial (*i. e.,* had undergone metamorphosis to ≥ Stage XXIX). This cut–off point was selected to reduce problems with froglet feeding. Stage XXV animals were used to examine the effect of each treatment on various growth–related morphological parameters. Throughout experimentation, animals were fed *ad libitum* as above, and were subjected to a natural photoperiod. All results were analysed for significance using one–way ANOVA.

RESULTS

There were no differences in either weight, length or stage of development between treatment groups at the start of the experiment. The scheme of Taylor and Kollros (1946), used to establish specific stages in anuran development was deemed appropriate for the species under investigation. All animals readily accepted frozen and canned spinach as their primary food source. Tranquillization of tadpole and froglet stages with metomidate hydrochloride was effective, and facilitated handling and treatment procedures. No mortalities were recorded during ransportation or holding.

Table 2 outlines the stages of development attained by each of the treatment groups at the termination of the study. In control animals, a spread over 5 distinct stages was exhibited, with only 28% achieving complete metamorphosis (TK st. XXV). In contrast, 83% of GH treated animals expressed complete terrestrialization. In αSRIF injected tadpoles, 33% of treated animals reached TK st. XXV (Table 2). Tadpoles receiving PRL were dispersed over 5 stages, with 13 (72%) attaining terrestrialization. In SRIF injected animals only one individual (6%) achieved the froglet stage. Analysis of resultant TK st. for each groupusing a »developmental score«, where TK st. XVIII = 0 trough to TK st. XXV = 7 revealed the following progression: GH 123 > PRL 115 > αSRIF 93 > control 80 > SRIF 63 (Table 2).

Table 2. Developmental stages attained by TK st. XVI western spotted frog tadpoles following 25 day treatment with distilled water, PRL, rpGH, αSRIF and SRIF. The study was terminated when one group achieved metamorphosis to > TK st. XXIV (terrestrialization). The higher the developmental score achieved, the greater the stage of development of individuals within a group.

Tablica 2. Razvojni stadij punoglavaca žaba Rana pretiosa pretiosa TK st. XVI postignuti nakon 25 dana obrade destiliranom vodom, PRL, rpGH, αSRIF i SRIF. Istraživanje je završeno kada je jedna grupa postigla preobrazbu do TK st. XXIV (potpuna preobrazba tijela u kopneni oblik — terestrijalizacija). Što je viši postignuti razvojni rezultat, to je veći razvojni stadij jedinki unutar jedne grupe.

Treatment	XVIII	XIX	XX	XXI	XXII	XXIII	XXIV	XXV	Developmental score*
Control	—	1	4	—	4	4	—	5	80
GH	—	—	—	—	—	—	3	15	123
SRIF	—	3	—	—	3	—	6	6	93
PRL	—	—	—	1	1	1	2	13	115
SRF	4	—	3	—	4	2	4	1	63

* Where TK st. XVIII=0 TK st. XXV=7
* gdje TK st. XVII=0 TK st. XXV=7

Throughout the 25 day period of measurement, all groups lost weight, with overall percent decrease being of the order: GH > αSRIF > control > PRL > SRIF. The declines in mean weight over the 25 days of study, were matched by a concomitant decrease in body length. Final mean weights and lengths of experimental froglets at TK st. XXV are presented in Table 3. Fully metamorphosed αSRIF treated froglets expressed significantly lower mean weights when compared to control and GH treated animals (P<0.05). PRL treated animals were significantly longer than all other treated and control groups. Various morphometric parameters for control and treated groups at the end of experimentation are also noted in Table 3. In all cases, the ratio of body length to hind–limb length, for fully metamorphosed froglets approached 1. PRL and αSRIF treated animals expressed significantly reduced body length: fore–limb length ratios when compared to control froglets (P<d0.05). Greater hind–limb length was noted in GH— (1.31±0.07 cm), αSRIF— (1.35±0.13) and PRL–treated (1.37±0.05) animals when compared to controls (1.27±0.11). Similar trends were observed for fore–limb length (0.81±0.06 (GH); 0.84±0.09 (αSRIF); 0.87±0.05 (PRL); 0.77±0.09 (controls)). However, consideration of body length: hind limb length ratios revealed no significant differences (P>

Table 3. Relationships between fore–, and hind–limb length and body length, condition factor, length: weight ratio and final weights of TK st. XXV. western spotted froglets subjected to various experimental treatments. Alike supercripts in a column signfy statistical parity between groups (P > 0.05).*

Tablica 3. Odnosi između dužina prednjih i stražnjih udova i dužine tijela, faktor kondicije, omjer dužina: težina i konačne težine TK st. XXV žaba podvrgnutih različitim eksperimentalnim postupcima. Jednaki superskripti u stupcu označuju statistički paritet između grupa (P > 0,05).*

Treatment	Number α TK st. XXV	Body lenght : hind-limb length	Body lenght : fore-limb lenght	Final-length æSD	Final WTæSEM	Length : weight	Condition factor
Control	5	1.12±0.108^{a}	1.85±0.24^{a}	1.36æ0.10^{a}	198.0æ18.06^{a}	6,89æ0.50^{a}	8.35æ1.32^{a}
GH	15	1.08±0.80^{a}	1.72±0.15^{ab}	1.40æ0.21^{a}	259.8æ40.90^{b,c}	5.45æ0.62^{b}	8.63æ1.37^{a}
αSRIF	6	1.03±0.075^{a}	1.70±0.12^{b}	1.33æ0.08^{a}	224.0æ36.15^{a,b}	5,85æ0.62^{b}	9.44æ1.05^{a}
PRL	13	1.01±0.040^{a}	1/59æ0.09^{b}	1.70æ0.06^{b}	245,0æ20.22^{b,c}	5.62æ0.46^{b}	5.03æ0.72^{b}
SRIF	1	1	2	1.4	173	8.09	6.31

* condition factor was calculated using the expression weight/length^{3}x100
* faktor kondicije izračunan je po formuli težina/dužina^{3}x100

0.05) between groups. Significantly greater ratios for length: weight were recorded in control versus all other treatment groups. Changes in condition factor was highly significant ($P<0.01$) in PRL treated animals, when compared to all other groups (Table 3).

DISCUSSION

Emerging evidence implies that GH secretion in anura is under the control of the antagonistic hypothalamic neuropolypeptides SRIF and GRP. Support for the existence of such a relationship is based upon the demonstrated GH-releasing activity of crude hypothalamic preparations (e. g., Hall and Chadwick, 1984); and SRIF-, (King and Millar, 1981) and GRF-like (Mariovet et al., 1988) immunoreactivity in the anuran hypothalamo-hypophysial system. It is generally accepted that SRIF acts to supress GH release in vertebrates (Harvey, 1993). Thus, it may be postulated that supplementation to the natural SRIF secretion should result in augmented action, while inhibiton of, or reduction in SRIF activity, would nulify or reduce this effect. Injection of Wester spotted frog tadpoles with SRIF increased the time required for complete transormation to occur, as depicted by the lower developmental score attained (Table 2). This may have resulted due to SRIFs inhibitory action on GH secretion. However, SRIF may have disrupted the hormonal milieu in general, since it is unlikely that the median eminence was formed at the ecamined stages. In mammals, SRIF has been demonstrated to inhibit the release of TSH and PRL both *in vitro* and *in vivo* (Vale et al., 1974); while Cheung et al. (1988) discerned an increase in circulating corticosteroids in cockerels following αSRIF treatment. Inhibition or disturbance in PRL, TSH and adrenocorticoid secretion due to exogenous SRIF would be expected to interfere with metamorphosis. In the present study, injected SRIF resulted in the widest developmental distribution pattern among all groups (Table 2). This effect conceivably resulted from the unfolding of atypical hormone profiles. Further support for a functional role for SRIF in anuran metamorphosis was provided by results pertaining to immunoneutralization of SRIF. Theoretically, therapy with αSRIF should result in the supression or modification of SRIF-mediated regulation of hormone release, as suggested in studies with teleosts (Mayer et al., 1994). If operative in anura, this might result in hypersecretion of an array of hormones, which in turn would activate premature transformation. In αSRIF treated tadpoles, the characteristic series of events which culminates in complete transformation was accelerated when compared to SRIF injected and control tadpoles, such that 66% αSRIF achieved TK st. XXIV-XXV compared to 27% in SRIF and control groups. A variety of studies have shown that animals receiving αSRIF express increased plasma GH, TSH and or, somatomedin levels (e. g., Tannenbaum et al., 1978; Vale et al., 1974; Diez et al., 1992). If this occured in like–treated anuran tadpoles, the resultant increased plasma TSH could stimulate PRL release (Clemons et al., 1979). Such abnormal hormonal oscillations, might drive a more rapid development of specialized organs (e. g., gastrointestinal tract, limbs), which, together with increased corticosteroid levels, would have the effect of accelerating climax.

Various studies have commented upon the growth–promoting and metamorphosis inhibiting effect of mammalian PRL in anura (e. g., Enemar et al., 1968). The antimetamorphic effect of PRL is considered to occur as a result of antagonism between PRL and thyroid hormone up to the point of metamorphic climax. However, rather than plasma PRL declining as transition approaches, as might be anticipated, PRL levels are now known to increase (review: Rosenkilde and Ussing, 1990). This has led to the suggestion that PRL is somehow involved during final transformation. Such a supposition is provisionally supported by the present study, since metamorphic progression and terrestrialization was accelerated in PRL injected tadpoles compared to controls. However, considerable care should be taken in evaluating PRL responses in tadpoles, since this hormone is known to be promiscuous, being influenced by thyroid and other hormones. For example, supraphysiologic PRL therapy of bullfrog tadpoles results in down–regulation of PRL receptors, and inhibition of thyroxine induced PRL recoptor induction (White et al., 1981). Several studies have identified GH–like actions for PRL (see Nicoll, 1993). While the structural components essential for PRL and GH binding activity differ slightly, it is possible that in the presence of supraphysiologic concentrations of PRL, this hormone may bind to GH receptors. But in the absence of data relating to PRL–GH competitive binding in amphibians, it is only possible to speculate as to the potential for such an occurence. Moreover, differential species–specific responses to PRL therapy may result at distinct developmental stages. It is of interest to note that PRL therapy resulted in proportionatly longer TK st. XXV Western spotted frogs, with the consequence of a highly significant reduction in condition factor when compared to all other froglets. PRL is generally designated as a growth promotant for larval amphibians (White and Nicoll, 1981). It is possible therefore, that PRL induced increases in larval body weight and length, which were subsequently translated into a larger froglet.

Our observation of decreased body weight in tadpoles receiving recombinant porcine GH differ to the reports of others. For example, Clarke et al. (1973) and Enemar et al. (1968(, described growth stimulation of bovine GH treated bullfrog and brown frog (*R. temporaria*) tadpoles respectively; while Delidow (1989), observed weight reduction in GH treated bullfrog tadpoles and a null effect in the grass frog (*R. pipiens*). However, these differences may relate to the developmental stage at which treatment was provided. Delidow (1989), employed stage VIII–XII animals, and Clarke et al. (1973). Ist year bullfrog tadpoles. Alternatively, the reported responses may reflect husbandry–, temperature–, species–, or dosage–related phenomena.

The type of GH preparation employed (i. e., porcine recombinant versus pituitary-derived bovine) might also account for these contrasting reactions, since recombinant GHs would not be contaminated by, for example, TRH. Alternatively, it has been suggested that one of the triggers for metamorphosis is attainment of critical body size (Fox, 1983). If treated animals, such as with SRIF, failed to grow well, then this may inhibit or retard final transformation.

Delidow (1989), suggested that GH plays a specific role in larval development with respect to limb growth. She argued that the effects of GH on limb growth in grass frog tadpoles could not be explained by a thyrotropic effect, since GH treatment failed to produce tail regression or loss of body weight. In the present studies, analysis of hind-limb length to body length indicated parity between control and all other treatment groups. However, significant differences were seen in the relationship between body length and weight, where GH treated animals consistently returned greater body weights, but not lengths, than observed in controls. Thus, the hind limbs of GH-, and αSRIF-treated tadpoles were proportionately longer than that observed in control animals. These results, together with observations relating to αSRIF therapy, provide further evidence for a more conspicuous role for TH in anuran transformation. However, it is recognized that hormonal factors which induce specific effects in one species may have different influences in others.

Sažetak

RAST I PREOBRAZBA PUNOGLAVACA ŽABE *RANA PRETIOSA PRETIOSA* TRETIRANIH REKOMBINIRANIM HORMONOM RASTA, PROLAKTINOM, SAMOTOSTATINOM (SRIF) I ANTISOMATOSTATINOM (ANTI-SRIF)

U radu je ispitivan učinak tretiranja prolaktinom (PRL), hormonom rasta (GH), somatostatinom (SRIF) i anti-SRIF-om (αSRIF) na rast i metamorfozu punoglavaca žabe *Rana pretiosa pretiosa* Taylor-Kollros (1946) stadij (TK st.) XVI. Tretirane životinje, kojih je bilo 18 komada/grupa primile su 2,5 g proteina/g tjelesne težine svakih pet dana sve dok jedna grupa nije poprimila ≥TK st. XXIV i kompletni oblik tijela kopnenog organizma (terestrijalizaciju). Dodavanje GH i PRL rezultiralo je u 18/18 odnosno 15/18 životinja koje su postigle kompletnu preobrazbu tijela. 12/18 punoglavaca kojima je bio injiciran SRIF poprimili su ≥TK st. XXIV. Nasuprot tome, samo je 5 punoglavaca iz kontrolne grupe i SRIF-om obrađenih punoglavaca transformirano u ≥st. XXIV. Značajno veći omjer dužina: težina ($P<0,05$) bio je zabilježen u kontrolnoj grupi za razliku od ostalih tretiranih grupa. Životinje obrađene PRL-om i SRIF-om pokazale su smanjeni omjer dužina tijela: dužina prednjih udova (P<0,05) u usporedbi s kontrolnim grupama. Veća dužina stražnjih udova bila je zapažena u životinja obrađenih GH–om, SRIF–om i PRL–om. Obrada PRL–om rezultirala je znatno povećanom dužinom i težinom (P<0,05) u usporedbi s kontrolnim grupama i veoma značajnim smanjenjem faktora kondicije (P<0,01) u usporedbi sa svim ostalim tretiranim grupama.

Ključne riječi: Rana pretiosa pretiosa, preobrazba, antisomatostatin, somatostatin, prolaktin, hormon rasta, rast

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