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Original Article

Pituitary-thyroid axis controls the final differentiation of the dorsal skeletal muscle in urodelan amphibians

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ABSTRACT A histoenzymological study of the ATPase activity of myosin in the dorsal axis muscle *(dorsalis trunci)* was carried out on two species of urodelan amphibians: *Pleurodeles waltlii*, a euthyroid species with spontaneous metamorphosis and *Ambystoma mexicanum*, a neotenic hypothyroid species. *P. waltlii* and *A. mexicanum* underwent an operation after which cytological analysis of the remaining pituitary were carried out in parallel. The muscle phenotype of urodelan amphibians varies according to the thyroid status of the species. In euthyroid adults, IIA fibers are dominant whereas in hypothyroid adults, IIC fibers are dominant. The number of type IIB (fast) and type I fibers (slow) are similar in both species. Physiological or experimental modulation of the concentration of circulating thyroid hormones results in a modification of the muscle fiber type profile pertaining to the considered species. We found that pituitary (TSH) plays a dominant role in the maturation of type IIC fibers in both species. Moereover, it seems to modulate the development of IIA fibers in *P. waltlii* and that of IIB fibers in *A. mexicanum*. Its action is thus species specific. Through partial or total hypophysectomy experiments, we have been able to demonstrate the influence of the hypophysothyroidian axis on the appearance of the adult muscle phenotype during metamorphosis.

KEY WORDS: *urodelan amphibians, muscle fibers, pituitary and thyroid hormones*

Introduction

As has been shown by several authors in the past years, the metabolic differentiation of skeletal muscle which leads to the charateristic distribution of fiber types is controlled by a variety of genetic and epigenetic factors (d'Albis and Butler-Browne, 1993). Identification of the different fiber types is frequently established according to the myosin ATPase activity and the different sensitivities of this activity to an acid or alkaline preincubation. Using this technique, Brooke and Kaiser (1969, 1970) were able to define three (type I, IIA and IIB) different fiber types in mature mammalian muscle. A fourth type (IIC) is characteristic of immature muscle. These same fiber types are also present in amphibian muscles (Chanoine *et al.*, 1987). The dorsal muscle of urodelan amphibians is a mixed muscle, containing both fast (Type IIB glycolytic and type IIA oxydative) and slow (type I oxydative) muscle fibers. The distribution of these fiber types varies depending upon the thyroid status and the developmental stage of the animals. (Chanoine *et al.*, 1989).

In the mature muscles of the two species which metamorphose spontaneously, *Pleurodeles waltlii* and *Ambystoma tigrinum* IIA fibers are dominant (67% and 57% respectively) and type IIC muscle fibers were absent. This correlated with the biochemical analysis of these muscle fibers showing the exclusive expression of adult fast and slow myosin isoforms (Chanoine *et al.*, 1987).

In *Ambystoma mexicanum*, which is a naturally hypothyroid neotenic species, and in the perennibranch species *Proteus anguinus*, there are very few type IIA fibers (10% in one case, 6% in the other) and type IIC fibers are dominant (60% in *A. mexicanum*, 52 % in *P. anguinus*) (Chanoine *et al.*, 1987, 1989).

The action of thyroid hormones on the expression of myosin isoforms has been extensively studied in mammals (Leloup and Buscaglia, 1977; Whalen *et al.*, 1985; Izumo *et al.*, 1986; Russel *et al.*, 1988), fish (Zawadowska and Karazinski, 1988) and amphibians (Chanoine *et al.*, 1987, 1990).

A correlation between the increase in level of thyroid hormone and the transition from larval myosin towards adult fast isoforms in the adult has been shown to exist in urodelan amphibians (Chanoine *et al.*, 1987) at the critical stage of metamorphosis as well as in mammals during post-natal development (Gambke *et al.*, 1983; Butler-Browne *et al.*, 1984; d'Albis *et al.*, 1987). It seems therefore that variations in the concentration of circulating thyroid hormones may be one of the key factors involved in the modification of the expression of myosin isoforms and may cause changes in the metabolical properties of the skeletal muscles. It has however been

Abbreviations used in this paper: TSH, thyroid stimulating hormone; STH, somatotrope hormone; GPH, growth hormone; ACTH, adrenocorticotropic hormone; PAS, periodic acid schiff.

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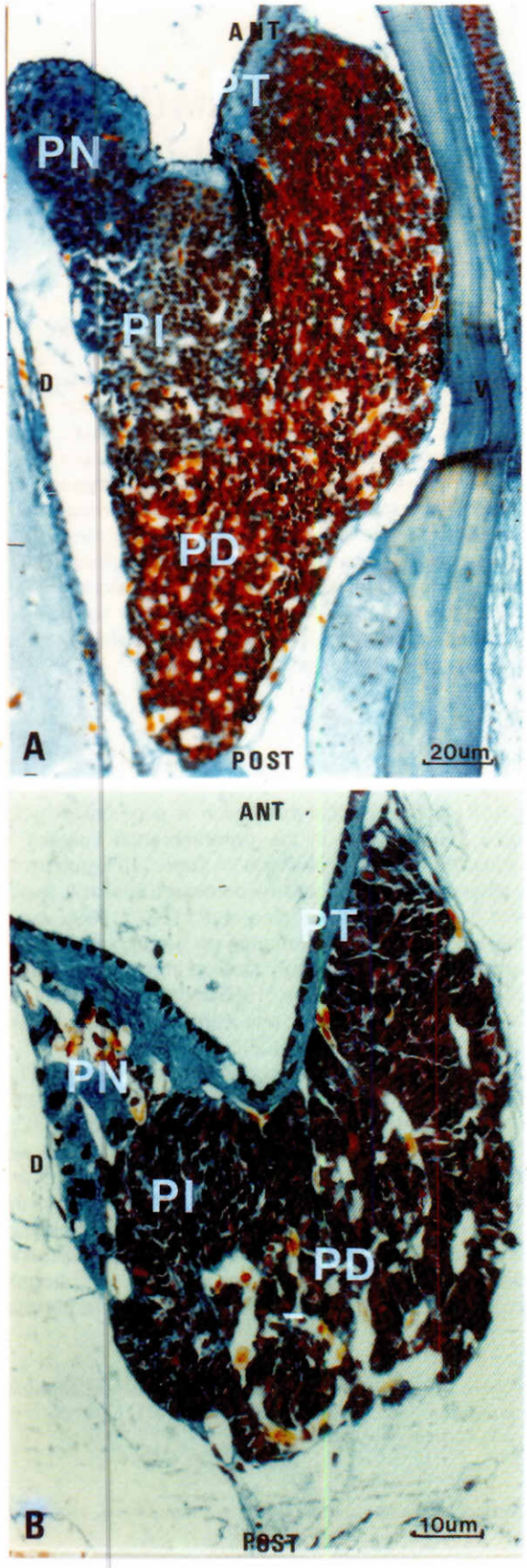


shown that thyroid hormones seem to act differentially on the expression of the various myosin heavy chains depending on the muscle (Izumo *et al.*, 1986).

The pituitary gland

The anterior pituitary gland is a heterogenous tissue containing different cell types. As these cellular groups have different functions and control mechanisms, they can be considered as independant endocrine glands. It can be divided into 3 lobes :

- the dorso-caudal lobe, called *pars intermedia*
- the rostro-ventral lobe, called *pars tuberalis*
- between these two, *the pars distalis*, which is the largest of the three.

The correlation between the cytology and the activity of the pituitary gland during growth has been studied in detail by Cordier (1948, 1953a,b), Cordier and Herlant (1957) and Kerr (1965, 1966) in *Xenopus laevis*, by Pasteels (1954, 1957, 1960) in *P. waltlii*; by Doerr-Schott (1968) and Van Oordt (1961, 1974) in *Rana temporaria* and by Totland (1976) in *A. mexicanum*. Metamorphosis is essentially characterized by the regression of acidophilic cells and the hypertrophy of thyrotrope cells (Cordier, 1953b; Cordier and Herlant, 1957).

The effects of hypophysectomy on growth, pigmentation and metamorphosis of amphibians have been well documented in the works of Allen (1916), Smith (1916, 1920), Gallien, L. (1952), Wingstrand (1966), Etkin (1968), Dodd and Dodd (1976), White and Nicoll (1981) all of whom have stressed the complexity of the hormonal action on metamorphosis.

The main action of the thyroid-stimulating hormone (TSH) on metamorphosis has now been well established. The metamorphosis that can be induced by T3 in the neotenic *A. mexicanum* (Chanoine *et al.*, 1987) can also be induced by TSH (Leloup and Buscaglia, 1977; Kühn and Jacobs, 1989).

However other pituitary hormones also seem to be involved in this process (Hanke, 1974). The somatotrope hormone (STH) and the prolactin-like hormone (named after the hormones that have a similar action in mammals) may well have, in addition to their stimulating action on growth, a global inhibitory effect on the chemical events associated with metamorphosis (Doerr-Schott, 1968). Eddy and Lipner (1975) have injected *Rana castesbiana* tadpoles with anti-prolactin antibodies. The larvae treated with this anti-serum showed inhibited growth and a stimulated thyroid activity. This observation seems to confirm the anti-metamorphic effect of growth hormone in amphibians (GPH endogens).

Prolactin may be antagonistic to thyroid hormone in many chemical reactions (Etkin and Gona, 1967) but seems to act synergistically with thyroxine, particularly in the liver. The pituitary ACTH may also play a role in metamorphosis. Remy and Croiset (1967) have described an accelerated reduction of legs in the hypophysectomized animal *Alytes obstetricans* when ACTH was inoculated simultaneously with thyroxine.

The objective of this study was to investigate the effects of the pituitary hormones and in particularly TSH on the appearance of the adult muscle phenotype in the fibers of the dorsal muscle of two urodelan amphibians: *P. waltlii* and *A. mexicanum*.

Fig. 1. Sagittal sections of control hypophysis stained with Herlant's tetrachrome. (A) *3-year-old P. waltlii;* **(B)** *2-year-old A. mexicanum. PN, Pars Neuralis; PI, Pars Intermedia; PD,Pars Distalis; PT, Pars Tuberalis; D, Dorsal area; V, Ventral area; Ant , Anterior area; Post, Posterior area.*

In hypophysectomy experiments which were partially or totally successful, we evaluated the effect of this operation on the development of the adult muscle phenotype in relation to the animal's development and the cytological appearance of the pituitary gland.

Results

Development of hypophysectomized animals and histological analysis of the pituitary gland

Control animal

The *pars distalis* of *P. waltlii* (Fig. 1A) and *A. mexicanum* (Fig. 1B) adults are composed of two zones which can be distinguished by their different staining properties according to the tinctorial patterns of the different cell types (Table 1).

- In the central and ventral areas, the acidophilic cells are dominant and well developed at this stage, although a few poorly developed type I basophilic cells were isolated in the mass of acidophilic cells.
- In the dorsal area basophilic cells were dominant and the majority were gonadotrope cells, Even the basophilic III cells (corticotropes) localized in the anterior portion of the dorsal area.

The *pars intermedia* was composed of basophilic melanotrope cells. The distribution of these different cell types in the pituitary gland is the same in *A. mexicanum* and *P. waltlii* even though in *A. mexicanum* the pituitary gland is slightly smaller (Table 2A and 2E).

Experimental animals

Two animals metamorphosed and pigmented: n°1 (Fig. 3B) and n°2 (Fig. 3C). Each animal had a histologically normal pituitary gland but these glands were slightly smaller than the control gland (350 to 360 μm diameter versus 460 to 470 μm) (Fig. 3A). The distribution of the cells was less clear. The acidophilic cells, though dominant, shared their principal ventral situation with a few bulky thyrotrop cells.

Two animals did not metamorphose but pigmented: n°3 (Fig. 3E) and n° 4 (Fig. 3G). There had been no redistribution of pigmentation to give animals their characteristic flecked appearance; the pigmentation had remained homogeneously very dark. The pituitary gland of the 8-month-old animals (n°3) was limited to the *pars intermedia* and three grouplets of isolated cells in the conjunctive network of the anteroventral area of *pars distalis* could be distinguished. The *pars neuralis* was very poorly developed and contacts with the hypothalamus were not clearly defined (Fig. 3E). The pituitary gland of a 3-year-old-giant *P. waltlii* larva (n°4) had the appearance of a cell mass separated from its neural connections, and essentially corresponded to a very developed *pars intermedia*. The cells seemed to be or had been hyper-active at one stage, as they appeared to be degenerating: some had picnotic cores in an overabundant but well-delimited cytoplasm; others lacked cores in a split cytoplasm (Fig. 3G).

One animal metamorphosed but did not pigment: n°5 (Fig. 3D). Its pituitary gland was reduced to a small cell mass in the anteroventral area and was composed of a few acidophilic basophilic

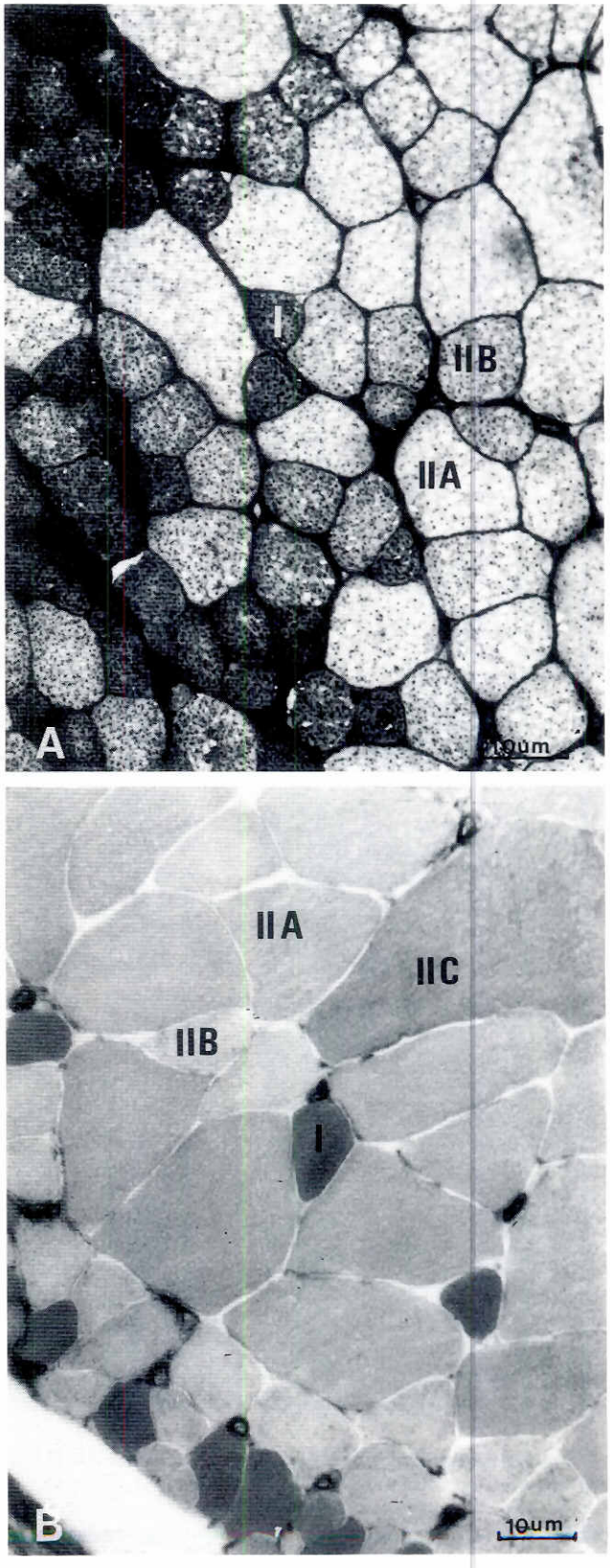


Fig. 2. Transversal sections of axial dorsal muscle of control adult. *Tinction with ATPase reaction, preincubation with pH 4.63.* **(A)** *P. waltlii (IIA, IIB and I fibers);* **(B)** *A. mexicanum (IIA, IIB, IIC and I fibers).*

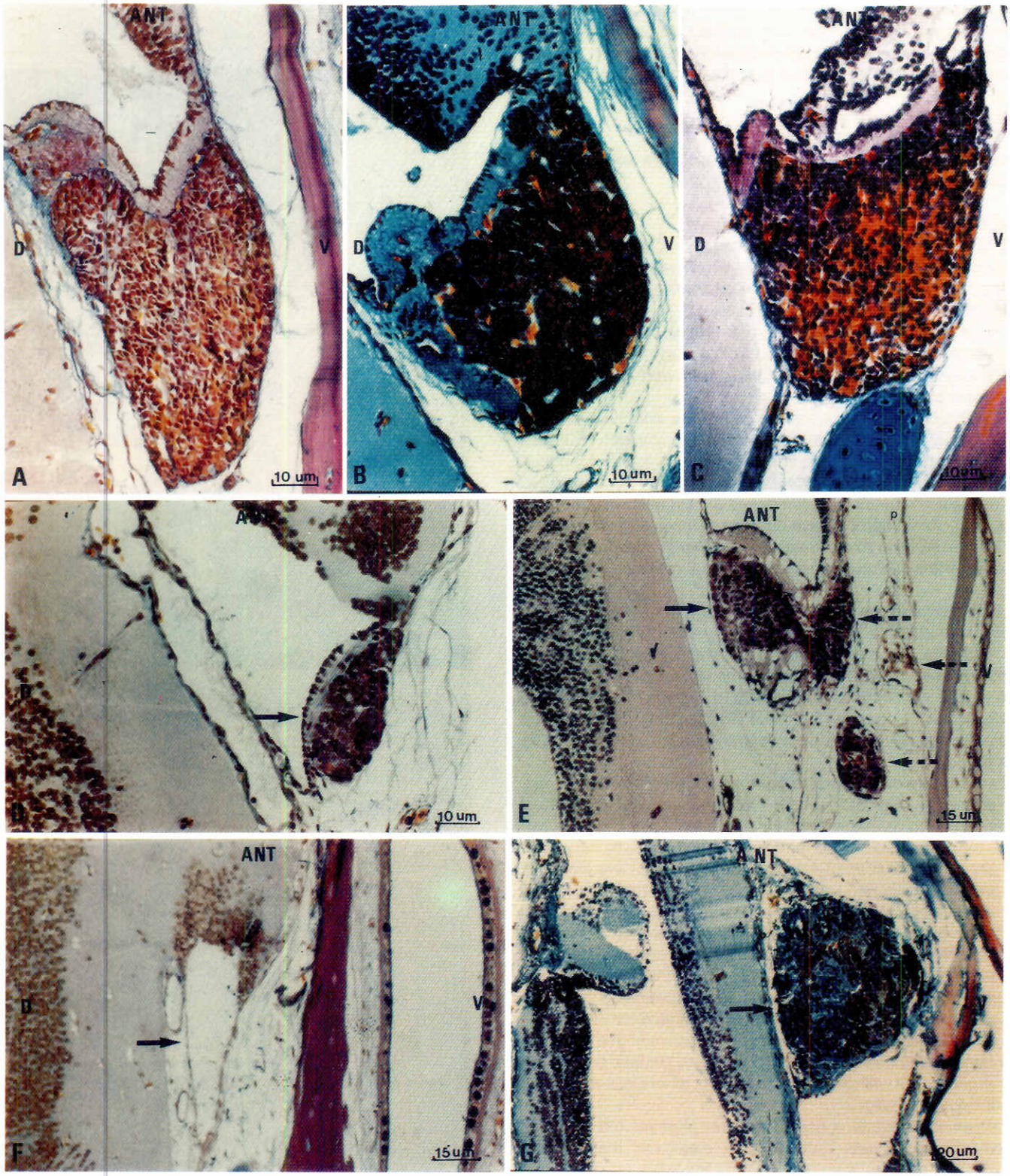


Fig. 3. Sagittal sections of hypophysis of operated *P. waltlii.* **(A)** *8-month-old control specimen. Tinction with alcian blue.* **(B)** *Herlant's tetrachrome. Slightly reduced hypophysis.* **(C)** *Alcian blue. Hypophysis slightly reduced.* **(D)** *Alcian blue. Hypophysis reduced to a anteroventral cell mass.* **(E)** *Staining with alcian blue. Hypophysis reduced to pars intermedia (→) and to 3 grouplets of cells in the anteroventral area (→)* **(F)** *Alcian blue. Lack of hypophysis (→)* **(G)** *Herlant's tetrachrome, 3 years, hypophysis essentially reduced to a hyperdevelopped pars intermedia (→). Ant, Anterior area. D, Dorsal area. V, Ventral area.*

TABLE 1

STAINING REACTIONS OF THE SECRETORY GRANULEs IN THE CELL TYPES OF THE ADENOHYPOPHYSIS IN URODELAN AMPHIBIANS

	Adenohypophysis					
	Pars intermedia	*Pars distalis*				
	Basophils	Basophils			Acidphils	
		I	II	III	I	II
Staining method	Melanotropes	Thyrotropes	Gonadotropes	Corticotropes	Somatotropes	"Prolactin"
Herlant's nomenclature	Light-violet	purple	orange-purple	orange-purple	red	red-brown
Alcian blue-PAS-orange G.	Red-purple	blue-purple	blue-purple	red	red-purple	orange-orange-brown

I and III cells. This area is essentially occupied by basophilic III (ACTH) in control animals of the same age (Fig. 3D).

One animal did not metamorphose or pigment: n°6 (Fig. 3F). One experimental *P. waltlii* was light in color and slightly smaller in size than the adult control animal and had retained its larval features. It was shown to be totally deprived of its hypophysis (Fig. 3F).

One experimental *A. mexicanum* also deprived of its hypophysis followed the same of pattern evolution (light color and small size).

Correlation between histological appearance and ATPase staining

The ATPase profile of both the n°1 and n°2 animals which had developed normally after an aborted hypophysectomy attempt was rather similar to that of the control animals of the same age, after metamorphosis (Fig 3A,B and C). The different fiber types in the control specimen are represented in Fig. 2A. At the dorsal muscle level, it can be noted that IIA fibers were most abundant (50% in one case, 60% in the other case, 67% in the control specimen). Nevertheless, the persistence of type IIC (16% in one case, 4% in the other case) fibers, suggests a delay in the maturation of type II fibers. The proportion of IIB fibers was very close to that found in control animals (21% in one, 25 % in the other) (Table 2A-C).

In the *P. waltlii* which did not undergo metamorphosis and which had a light pigmentation (n°6, Fig. 3F) and no pituitary gland, only three types of fibers could be distinguished following ATPase staining. IIC fibers were abundant (70%). The type IIB (19%) and type I fibers (11%) were close to that of the control specimen however type IIA fibers were totally absent. (Table 2B).

The metamorphosed but not pigmented individual (n°5, Fig. 3D) which had a pituitary gland which was restricted to a mass developed in the antero-ventral area of the anterior lobe and composed of thyrotropic, corticotropic and somatotrophic cells. ATPase profile with 45% IIC fibers had an immature type. The proportion of IIA fibers was rather low (19%) in comparison to that of the control specimen (Table 2C-H.1).

The 8-month-old individual with no metamorphosis but a strong pigmentation, (n°3, Fig. 3E) had a fragmentary pituitary gland with almost a total absence of acidophilic and basophilic thyrotropic cells. Its ATPase profile was also immature. Four types of fibers could be identified, but there were only a few type IIA fibers (6%) compared to the high percentage of IIC (50%) (Table 2D).

The 3 year old individual with no metamorphosis and a strong pigmentation, (n°4, Fig. 3G) had a rudimentary pituitary gland that essentially corresponded to the *pars intermedia*. The histoenzymological analysis of the muscle showed that IIA fibers were reduced in number (25%) though more numerous than in the 8 month old specimen. IIC fibers, which are indicative of a delay in maturation, reached 47% (Table 2D). The individual *A. mexicanum* which had a total absence of the pituitary gland did however also have all four types of fibers as in the skeletal muscle of the control specimen (Fig. 2B). IIA fibers (9%) were normally developed (control: 10%) as well as type I, 13% (control: 14%). IIB fibers (8%) were greatly reduced (control: 16%) (Table 2E and F).

Discussion

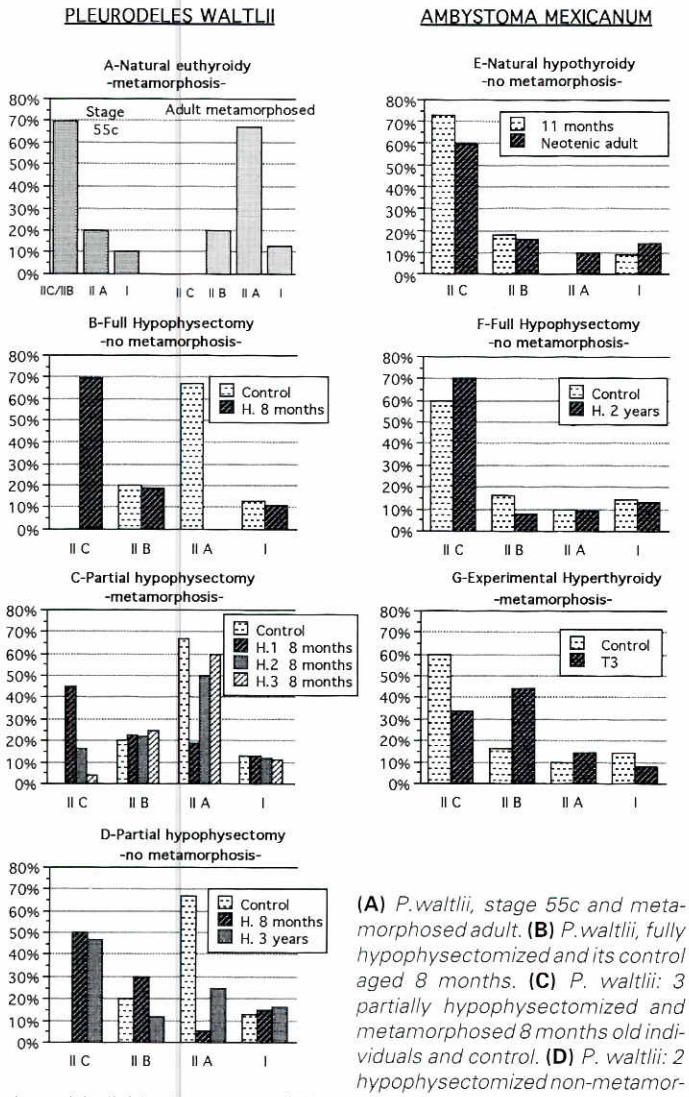
In all of the hypophysectomized animals the muscle differentiation which had already begun did not present the adult muscular phenotype when they were sacrificed: persistence of type IIC fibers was a characteristic feature of all muscles.

The results of our experiments carried out on *P. waltlii* highlight the implication of the pituitary-thyroid axis in the acquisition of the adult muscle phenotype, and more specifically in the maturation of IIA fibers. These fibers, which are the most predominant type in the control specimen, have been identified in both of the metamorphosed animals that had a reduced but harmonious pituitary gland, in close proportions. In the metamorphosed but unpigmented animal, the pituitary gland was reduced to a cell mass in the anteroventral lobe of *pars distalis* where most thyrotropic cells should normally be, and in this animal the proportion of type IIA fibers was lower (19%). From these results we conclude that in *P. waltlii*, the variable proportion of type IIA fibers seems to be directly correlated with the variable numbers of thyrotropic cells. In *P. waltlii* where the ablation was a complete success, IIA fibers were absent and the high number of IIC fibers suggests either a delay or an inhibition in the maturation of IIA fibers. In the absence of a functional pituitary-thyroid axis, IIA fibers only appear in *P. waltlii* after 8 months at which time the control animal has attained its mature adult phenotype.

We have not studied totally hypophysectomized specimens older than eight months old. It is therefore difficult to check whether or not IIA fibers progressively differentiated from the IIC population in a totally hypophysectomized *P. waltlii*. Nevertheless, in the 3 year old animal where the pituitary gland was essentially reduced to the *pars intermedia* and contained only very few thyrotropic cells, a considerable number of IIA fibers (25%) had matured during this time. This observation suggests that IIA fibers probably appear through a mechanism independant from the pituitary TSH. On the contrary, their development seems to depend largely on the

TABLE 2

DISTRIBUTION OF THE DIFFERENT MUSCLE TYPE FIBERS IN THE *DORSALIS TRUNCI*, DETERMINED WITH ATPase MYOFRIBRILLARY REACTION WITH PREINCUBATION pH 4.63



(A) *P.waltlii, stage 55c and metamorphosed adult.* **(B)** *P.waltlii, fully hypophysectomized and its control aged 8 months.* **(C)** *P. waltlii: 3 partially hypophysectomized and metamorphosed 8 months old individuals and control.* **(D)** *P. waltlii: 2 hypophysectomized non-metamorphosed individuals: one aged 8 months, the other aged 3 years and control.* **(E)** *Young A. mexicanum (11 months), and neotenic adult (2 years).* **(F)** *Hypophysectomized adult and control.* **(G)** *A. mexicanum, made hyperthyroidian (T3) at the age of one year, examined one year later and control.*

hormonal level. In conclusion, the skeletal muscle of all of the hypophysectomized animals which we have analysed were characterized by the persistence of immature type IIC fibers. A mature adult phenotype was never observed in any of these animals.

In *P. waltlii*, IIB fibers seem to escape the control of the pituitary-thyroid axis. They differentiate normally during development, independently of whether the animal undergoes metamorphosis or not, whether it has a normal hypophysis or one reduced to a few cells in the anteroventral area or even no hypophysis at all. The proportions vary very little, independently of the results of the operation. *A. mexicanum*, when totally deprived of its pituitary gland, developed an immature ATPase profile but all of the adult fiber types were present. These results show that the appearance of the adult phenotype is independent from the pituitary-thyroid axis. The large quantity (70%) of IIC transition fibers shows delay or total inhibition in the repression of the larval MHC in type IIA and IIB fibers. It has been previously demonstrated that hypothyroidia in adult rats reduces the percentage of fast fibers in several mixed muscles whereas the number of fast fibers could be increased in the hypothyroidian rat by injection of T3 for 6 weeks (Ianuzzo *et al.*, 1977). A similar result has been reported in our laboratory. Experimental hyperthyroidia (T3) results in a large increase in the number of IIB fibers (44% in *A. mexicanum* treated at the age of one year and examined one year later) in neotenic species. Thyroid hormones seem to activate the expression of the isoform with highest ATPase activity.

From these results it can be concluded that pituitary TSH or thyroid hormones act on *A. mexicanum* muscle by promoting the development of IIB fibers whereas IIA fibers seem to escape hypophysarian hormonal control (control *A. mexicanum*, 10%; totally hypophysectomized *A. mexicanum*, 9%; hyperthyroidian *A. mexicanum*, 10%). Yet the response to hormonal stimulation is not immediate, which suggests an indirect mechanism of action (maybe via the nervous system). The way in which thyroid hormones act is rather complex. The expression of an isomyosin may be regulated by thyroxine in many different and even opposite ways, according to the muscle which is concerned (Izumo *et al.*, 1986).

It has been demonstrated in our laboratory (Saadi *et al.*, 1992) that in *A. mexicanum* the transition of isomyosins is incomplete at the level of dorsal muscles whereas it is total in the anterior limb muscles. This muscle-specific result suggests a possible deficiency of functional receptors for thyroid hormones in the dorsal muscle.

In this study we show that the pituitary-thyroid axis modulates the development and maturation of IIA fibers in the dorsal muscle of *P. waltlii* but probably not in the hypothyroid species *A. mexicanum* in which short-term hyperthyroidia experiments or hypophysectomies did not alter the proportion of IIA fibers. IIB fibers do, however, seem to be sensitive to the variations in hypophysarian TSH and of thyroid hormones in *A. mexicanum* but seem to escape hormonal control in *P. waltlii*. Furthermore, the appearance of the adult fiber types is independant from the pituitary-thyroid axis both in *A. mexicanum* and *P. waltlii*. By these hypophysectomy experiments, it has been possible to demonstrate that TSH is the hormone that plays the main role in the final differentiation of the dorsal muscle in urodelan amphibians and that its action is species-specific.

Materials and Methods

Animals

The breeding technique was previously established by Gallien L. (1952). Two species of urodelan amphibians were chosen: *Pleurodeles waltlii* Michah and *Ambystoma mexicanum* Shaw. In order the facilitate the comparison between these two species, the chronology of the development of both species is described with reference to the data established in *P. waltlii* by Gallien and Durocher (1957).

Hypophysectomy of the animals

The hypophysectomized larvae and the corresponding control samples (Stage 45) were anesthetized with MS 222 (Sandoz). With very fine

dissection forceps, the palatal vault was reached through the mouth floor by a lateral cut between the two branchial axes. After the ablation of a part of the sphenoid, the infudibularian region was exposed. The glandular and intermediary lobes were parted from the infundibulum with a stylet. The hypophysis was then pumped up with a pipette. Six pleurodeles and one axolotl were hypophysectomized at stage 45 of their development. The results obtained were varied:

- either the hypophysis was correctly separated; in this case, the operation corresponded to a total hypophysectomy,
- or only a part of the hypophysis was separated and the rest of the gland was left in place without correction,
- or the hypophysis was slightly modified.

Sampling of muscles

Five *P. waltlii* from the same laying were sacrificed at the age of eight months and one at the age of three years. One black *A. mexicanum* was sacrificed at the age of two years. Samples of the dorsal muscle *(dorsalis trunci)* were removed by dissection, immersed in liquid nitrogen cooled isopentane, and mounted in the Bright cryo-M-bad embedding compound (Bright Instrument Company).

Enzyme histochemistry

The muscles were cut into 8 µm serial transverse frozen sections. ATPase enzyme-histochemistry for *P. waltlii* was carried out as described by Padykula and Herman (1955) and modified by Brooke and Kaiser (1969). For *A. mexicanum*, the best results were obtained in the experimental conditions described by Johnston *et al.* (1974) for fish (preincubation with pH 10.4). Fiber types were defined according to the Brooke and Kaiser nomenclature (1970) for mammals. The spacial distribution and the percentage of different fiber types were established based on the total area of the transverse section of the muscle.

Histological analysis of the pituitary gland

The head of the hypophysectomized animals was placed *in toto* in Bouin's fixative for 15 days, dehydrated in ethanol, washed in two baths of toluene and paraffin embedded. The block of paraffin was then cut into 8 µm serial sagittal sections.

Staining of the paraffin sections

Two histochemical techniques were selected: (i) Herlant's tetrachrome (Cordier and Herlant, 1957): the hydrated sections were treated for 5 min in lugol, destained by sodium hyposulfite and stained for 15 min in a solution of erythrosin pH 6.2. The sections were stained for 5 to 10 min in Mallory's mixture (methylene blue, 0.5 g; orange G, 2 g; oxalic acid, 2 g; distilled water 100 ml) then 10 min in alizarin blue (alizarin blue, 1 g; pure ammonium sulfate, 10 g; distilled water, 100 ml), then rinsed in phosphomolibdic acid 5% and differentiated in phosphomolibdic acid 1% in ethanol 70% and then ethanol 95%; and (ii) Alcian blue-PAS-orange G (Gabe, 1968): the sections were oxydized by the Gomori mixture (potassium permanganate 2%, 1V; sulfuric acid 5%, 1V; distilled water, 6V, destained by potassium metabisulfite 2%, stained for 15 min in a 1% alcian blue solution (alcian blue, 1 g; sulfuric acid, 10 ml; pH 0.2). The sections were then oxydized by 1% periodic acid for 10 min, and then treated with Schiff's reagent for 15 min. The cores were stained with Groat's hematoxylin for 3 minutes, and the acidophilic cells with orange G-phosphomolibdic (orange G, 2 g; phosphomolibdic acid, 1 g; distilled water 100 ml) for 3 min.

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