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UNIVERSITY OF ALBERTA

ENDOGENOUS LECTINS AND PIGMENT PATTERN DEVELOPMENT IN THE AXOLOTL

BY

GALE N. MARTHA

A THESIS

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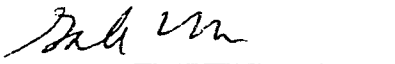

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SUBMITTED BY GALE N. MARTHA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

NADINE MILOS

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DEDICATION

To my husband, Joe, for his encouragement and moral support, without which I could never have done this work.

Thank you, Joleen, for brightening up my longest days at work. All my love, Mom.

ABSTRACT

This investigation was concerned with determining whether endogenous lectin is developmentally regulated in the urodele, Ambystoma mexicanum (axolotl) and whether or not lectin may be involved in the development of the melanophore pattern of dark axolotls. In addition, it was determined whether or not manipulations of endogenous lectin activity in dark embryos could produce phenocopies of the white mutant. The white mutant is characterized by a restricted pigment pattern (Frost et al., 1984b).

Endogenous lectin activity, as measured by a micro-hemagglutination assay, increased significantly and transiently during initial neural crest (NC) migration in embryonic dark axolotl ectoderm tissue but did not change significantly in the remaining carcass. In contrast, white mutant axolotl carcass tissue lectin activity increased transiently during early NC migration but no significant changes in white ectoderm activity occurred.

Because normal ectodermal lectin activity in dark animals coincides temporally with the onset of melanophore migration from the NC, a series of in vivo confrontation experiments were carried out to determine if changes in endogenous lectin activity could result in changes in melanophore migration and subsequent pattern development in the dark axolotl, and produce a phenocopy of the white mutant.

Pre-migratory NC in dark embryos was confronted directly with either a sugar hapten inhibitor, 2-deoxyglucose (2-DG) or a crude NC-stage lectin extract. 2-DG confrontation initially resulted in a pigment pattern that resembled that of older embryos. As development progressed, the pigment pattern became significantly disorganized and melanophore morphologies began to resemble those of more immature animals. In contrast, crude lectin confronted embryos initially exhibited pattern disorganization but later the patterns resembled those of controls. However, melanophore morphologies remained immature in appearance. These results suggest that the treatments may alter lectin activity associated with normal axolotl pigment pattern development and that the timing of transient lectin expression may be important in normal pigment pattern development.

The inhibitory activity of three glycosaminoglycans on NC-stage lectin was determined. Hyaluronic acid (HA) and heparen sulfate, both of which contain significant amounts of N-acetyl-D-glucosamine, inhibited lectin activity. The endogenous lectin was specific for D(+) glucosamine, D(+)

mannose and N-acetyl-D-glucosamine. It is possible that endogenous lectin may interact with HA or heparen sulfate in the ECM *in vivo* to influence melanophore pattern development in the axolotl.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

ANOVA = two-way analysis of variance

BSA = bovine serum albumin

C = carbon

CAM = cell adhesion molecule

D(+) = dextro-rotary

D/- = wild or dark genotype

d/d = white genotype

2-DG = 2-deoxyglucose

ECM = extracellular matrix

ed. = editor

EGF = epidermal growth factor

EM = electron microscopy

FCS = fetal calf serum

FN = fibronectin

GAG = glycosaminoglycan

HA = hyaluronic acid

HU = hemagglutination unit

i.e. = that is

K = kilodalton

LEC-CAM = lectin-like cell adhesion molecule

mM = millimolar

M = molar

MS-222 = methane-tricane-sulphate

NaCl = sodium chloride

$NaKPO_4$ = sodium potassium phosphate

NC = neural crest

PG-LA = HA-aggregating chondroitin sulfate proteoglycan

PMSF = phenylmethylsulfonyl flouride

rpm. = rotations per minute

S.E.M. = standard error of the mean

SEM = scanning electron microscopy

TDG = beta-thiodigalactoside

v/v = volume per volume

= number

% = percent

+/- = plus or minus

X = times multiplied

CHAPTER 1

INTRODUCTION

A. STATEMENT OF THE PROBLEM

Lectins are carbohydrate-binding proteins detected by their ability to agglutinate erythrocytes. Endogenous lectins in developing vertebrate systems have been detected which fluctuate in amount during embryogenesis (Gartner and Podleski, 1976; Kobiler and Barondes, 1977; Cook et al., 1979; Yamagata and Nishiwaki, 1979; Kitamura, 1980; Zalik et al., 1983; reviewed by Barondes, 1981; Zalik and Milos, 1986; Zalik, 1991). These observations along with many others have led to suggestions that these developmentally regulated lectins play important roles in controlling embryonic tissue morphogenesis, for example, in promotion of cell-cell or cell-substratum adhesion in conjunction with their sugar-binding receptors (Beyer et al., 1979; Beyer and Barondes, 1980). An example is the striking increase in lectin activity during myoblast fusion in the chick embryo (Nowak et al., 1976).

The hypothesis I have tested is that endogenous lectins are present during early development and are involved in melanophore pattern formation in the dark axolotl, Ambystoma mexicanum.

Endogenous lectins have also been implicated in the

development of the pigment pattern in the anuran, Xenopus laevis (Milos et al., 1987; Frunchak and Milos, 1990). With the exception of these investigations, no other studies of lectin control of melanophore pattern formation have been conducted to date.

B. SCOPE OF THIS INVESTIGATION

The development of the NC-derived flank melanophore pattern in the axolotl has been studied with regard to changes in endogenous lectin activity. The possibility that endogenous lectin may be involved in the phenotypic expression of the extensively studied white axolotl mutant, characterized by restricted migration of melanophores and xanthophores from the neural crest (Lehman, 1957), was also of interest.

Lectin activity in dark and white mutant embryos has been quantitatively compared followed by a series of in vivo lectin and lectin inhibitor confrontation experiments. Subsequent analysis of the effects of these treatments on different aspects of pigment pattern development was done to test the hypothesis that changes in the titre of endogenous lectin are important in normal melanophore pigment pattern development in the axolotl.

An endogenous lectin specific for D(+) mannose, D(+) glucosamine, N-acetyl-D-glucosamine, and hyaluronic acid has

been found to be developmentally regulated in the axolotl. It differs in amount and distribution between dark and white embryos.

Experimentally imposed in vivo modulation of endogenous lectin activity was accomplished by confronting live embryos with either a non-metabolized lectin inhibitor or a crude endogenous lectin extract. These treatments altered pigment pattern development in dark animals suggesting that endogenous lectin might be involved in pigment pattern development in the axolotl.

C. BACKGROUND

1. THE NEURAL CREST

a) Development of the Neural Crest

The neural crest (NC) is a transient pluripotent cell structure in vertebrates that develops from the lateral ridges of the neural plate as they join during closure of the neural tube (Figure 1). Shortly afterward, the cells of the NC migrate throughout the embryo and give rise to numerous differentiated structures (Barrofio, 1988; Lofberg et al., 1989a).

There are currently several different theories regarding the control of migration and differentiation of NC

cells. One theory suggests that the information controlling NC migration and differentiation is preprogrammed into the cells before they leave the neural tube (Hall and Tremaine, 1979; Wachtler, 1984; Barbu et al., 1986; Frost, 1990). For example, in some species of amphibians, including the axolotl, pigment cells are clearly visible and begin to differentiate prior to their emigration from the neural tube (Epperlein and Lofberg, 1984). In fact, these authors report that xanthophores and melanophores, indentified by the presence of pteridines and tyrosidase, respectively, are present in pre-migratory NC. In the axolotl, the cells appear to be arranged in the NC in distinct mixed chromatophore groups which migrate to populate the skin.

The differentiation of pigment cells prior to migration has already been mentioned above. Other experimental evidence that the NC contains different subpopulations of cells was obtained by Borald (1989) and Maxwell et al., (1990). Using flourescence-activated cell sorting and immunoablation (ie., selectively kill cells based on the types of antigens they express) together with cell culture of NC cells, they found that a series of antigens on the surface of NC cells develop and specialize over time and become different in different areas of the NC tissue prior to migration. Kuritani and Bockman (1991) also suggest a similar organization of NC cells that participate in thymic development based on their work using quail/chick chimeric

NC transplants. By transplanting NC unilaterally from quail donors to chick hosts in the region that NC cells normally migrate to interact with the primordial thymus, they found that different regions of the NC along the longitudinal axis of the embryo contribute differently to thymic development, suggesting that subgroups within the NC exist with varying capacities to interact with different organs "in which they are called upon to participate."

Another theory proposes that the extracellular matrix (ECM) plays an integral role in determining the migratory routes and fates of NC-derived cells (Noden, 1978; Perris et al., 1988; Brönner-Fraser and Fraser, 1989; Doupe et al., 1983). For example, the work of Perris et al. (1990) suggests that local inability of the ECM to support sub-epidermal migration of NC-derived pigment cell precursors is responsible for the white defect in the axolotl. They compared white and dark subepidermal and perineural ECM's immunohistochemically, ultrastructurally, and biochemically and found several subtle ultrastructural discrepancies specific to the white subepidermal ECM which might account for the failure of neural crest cells to enter this region. Also, cultures of quail NC cell clones will differentiate along pigment cell or neural crest pathways depending on the nature of the culture environment (Sieber-Blum, 1989). Based on clonal culture work using a monoclonal antibody against the marker SSEA-1, the commitment of quail NC cells

to the sensory neurone lineage was examined. They found that the NC initially gives rise to sensory, autonomic, and pigment cell progenitors, some of which are tripotent for all three types, some of which are bipotent for autonomic and sensory cell types and some commited to the melanocyte cell line. The commitment of the multipotent progenitors was manipulated by using different growth factors in the culture medium. As the sensory ganglion begins to form, the tripotent cells disappear, giving rise to bipotent and commited cells. Eventually, bipotent cells become commited to either autonomic or sensory cells.

It is most likely that the control of NC migration and localization is due to factors contained both within the NC cells themselves and within their environment, perhaps dependent upon the subpopulation of NC cells in question (Maxwell and Forbes, 1991). They found that catecholamine-containing cells differentiate preferentially from populations of HNK-1+ quail trunk NC cells. These cells were found to bear tyrosine hydroxylase and somatostatin immunoreactive markers, whereas few or no HNK-1- NC cells expressed these phenotypic markers. In contrast, both HNK-1+ and HNK-1- NC cells contained similar numbers of A2B5, E/C8, and NF-160 markers. The HNK-1+ antigen on NC cells correlates with adrenergic NC-derived cells but much less so with other classes of NC-derived cells. In fact, models combining the preprogrammed cell and extracellular matrix

theories have recently become popular, one of which is the hemopoietic-like model reviewed by Anderson (1989). This model suggests that different classes of committed progenitor cells may be derived from the earliest multipotent NC cells, which then respond differently to various environmental cues.

The work of Le Douarin et al. (1991), for example, provides evidence of a hemopoietic-like model regarding NC-derived glial cell differentiation. Quail/chick chimeras were used along with early cell type-specific markers and the analysis of clones of different NC cell progenitors under the same culture conditions to trace the in vivo origin of various peripheral glial cells and non-neuronal peripheral ganglionic cells (different cell types were identified using a battery of different phenotypic markers now available). These experiments suggest that different regions of the NC contain cells with inherently different potentialities. Which differentiation route these cells may take may then be modulated by their local migratory environment. Her results are illustrated in Figure 2. The differentiation of one NC-derived cell, the pigment cell or chromatophore, is discussed below.

b) Differentiation of Pigment Cells From the Neural Crest

There are three types of NC-derived pigment cells in

amphibian embryos: 1) black or brown melanophores containing melanin-rich melanosomes; 2) xanthophores, containing the yellow pigment pteridine in organelles called pterosomes; and 3) iridophores, containing reflective platelets with a pigment based on guanine (Frost et al., 1984b). Together, these cells produce species-specific patterns of larval and adult pigmentation in lower vertebrates.

All of these amphibian pigment cells are derived from the NC. However, in the absence of the NC, the neural plate can also give rise to chromatophores (Niu, 1954). For example, after neural folds were removed from various urodele embryos, melanophores appeared (after a considerable delay) on the head and flank regions but when both the neural folds and remaining neural plate were extirpated, the heads of the experimental animals were always devoid of melanophores (Niu, 1954).

In the dark axolotl, the system I have studied, melanophores are the first chromatophores to differentiate from the NC (Lehman, 1957; Keller et al., 1982). Melanophores are first visible in the subepidermal spaces of the lateral migratory pathway, followed shortly by xanthophores (Keller and Spieth, 1984; Spieth and Keller, 1984). Some melanophores also migrate along the perineural route to populate the dorsolateral walls of the gut and major blood vessels. This perineural route is also taken by NC cells contributing to the peripheral nervous system

(Keller and Spieth, 1984) (Figure 3). The melanophores that populate the gut become visible later than those contributing to the skin pigment.

Dorsal xanthophores and melanophores subsequently form a characteristic pattern consisting of alternating vertical bars over the surfaces of the somites (Lehman, 1957; Epperlein and Lofberg, 1984). How this particular alternating arrangement of cells arises is currently unknown.

There is some specific information about cues that can affect pigment cell differentiation. For example, small molecular weight molecules such as purines and pyrimidines can act as environmental cues and direct the differentiation of pigment cell precursors in the axolotl (reviewed by Frost and Bagnara, 1979). For example, when guanosine is added to the culture medium of melanophores, they will convert into iridophores over a period of time (Ide, 1979). Furthermore, work done by Bagnara et al. (1978) suggests a possible connection between the synthesis of xanthine dehydrogenase, and excessive melanophore differentiation at the expense of other pigment cell types in the melanoid axolotl mutant, which is distinguished by hypermelanized skin. When xanthine dehydrogenase is blocked by allopurinol, synthesis of certain pteridines is blocked. Animals raised in allopurinol solutions exhibit hypermelanization (at the expense of differentiation of xanthophores and iridophores).

The time at which the different cell types become determined in most animals is unknown, although in the case of the axolotl mentioned above some of the first cells appear to differentiate while they are still migrating from the NC. It is currently believed that a primordial pigment cell organelle initially differentiates within a common stem cell. That organelle in then acted on by different environmental cues to give rise to either melanophores, xanthophores, or iridophores (Bagnara et al., 1979).

It is thought that the initiation and rate of melanophore differentiation is linked with the development of surrounding tissues (Weston, 1970; Loring et al., 1982; Stephenson and Hornby, 1985; Perris et al., 1988; Campbell, 1989). For example, NC cells from the axolotl embryo respond <u>in vitro</u> to subepidermal ECM by differentiating into pigment cells and respond to matrix from the site of presumptive dorsal root ganglia differentiation by neurotypic expression (Perris et al., 1988). However, the precise nature of the cues to which the cells are responding is not known.

The continued maintenance of melanophore phenotypes appears to depend, at least in part, on environmental cues. Thus, even differentiated melanophores retain a labile differentiative capacity throughout their lifespan, still responding to environmental cues and changing their pigment cell types accordingly (Ide, 1979). This observation is

further evidenced by the retention of a well developed Golgi apparatus and prepigment organelles in mature melanocytes (Frost et al., 1984a).

For the most part, however, the nature of environmental cues that affect the expression of pigment cells have yet to be fully described.

c) The Extracellular Matrix and the Neural Crest

As mentioned above, one of the important embryonic components involved in pigment pattern formation is believed to be the ECM. Amphibian embryonic ECM is a complicated structure containing chondroitin sulfate proteoglycan, hyaluronic acid (HA), collagen, laminin, tenascin and fibronectin (FN) as well as other proteoglycans, glycosaminoglycans, and glycoproteins (Perris et al., 1990). ECM components bind to one another and to cells via specific cell surface receptors (Lofberg and Ahlfors, 1978; Lofberg et al., 1980; Newgreen et al., 1982; Erickson and Weston, 1983). The ECM is thought to support migration and participate in the guidance of NC cells as they move through the tissue environment (Lofberg et al., 1989a). Some components such as fibronectin are adhesive molecules for NC cells (Dufour et al., 1988; Epperlein et al., 1988), while other components such as tenascin are non-adhesive (Epperlein et al., 1988; Bronner-Fraser, 1988). Different

ECM components may thus promote or impede NC cell migration and act to channel the cells in particular directions. Examples of functional studies on some ECM molecules are given below.

Epperlein et al., (1988) found specific deposition of FN in premigratory stage 25 dark axolotl ECM around the neural tube, somites and the notochord, but no tenascin. Anti-FN stained ECM throughout the embryo during NC migration (stages 28-35) and tenascin was found in the most dorsal aspect of the embryo including the dorsal fin at these stages. The NC cells were adhesive to FN but not to tenascin, suggesting that the differential interactions between NC cells and these ECM molecules may be important in guiding their migration and determining migration pathways.

Other ECM molecules may also be important in NC cell migration. Tucker and Erickson (1986) found evidence that chondroitin/keratan sulfate proteoglycans, which can bind with HA to form large complexes (see Figure 4), are non-permissive as a substrate for avian NC migration when HA is not present <u>in vitro</u> (Tan et al., 1987). They suggest that these proteoglycans may accumulate regionally <u>in vivo</u> to direct NC migration in the embryo (Tucker, 1986; Tucker and Erickson, 1986).

Perris and Johansson (1990) found similar evidence based on their <u>in vitro</u> studies on the involvement of chondroitin sulfate proteoglycan and other HA aggregate

components on NC migration over various substrates. Their work suggests that chondroitin sulfate proteoglycan inhibits NC movement primarily through an interaction with the cell surface rather than by interacting with various motility-promoting molecules such as fibronectin, laminin, laminin-nidogen, vitronectin, and collagen types I, III, IV, and VI. Antibodies against the chondroitin sulfate proteoglycan molecule did not affect its cell migration inhibitory behaviour when pre-incubated with various ECM components and didn't reduce the inhibitory effect of chondroitin sulfate proteoglycans on NC cell migration in vitro when incubated with the NC cells themselves (Perris and Johansson, 1990). However, the addition of hyaluronan fragments or antibodies against the HA-binding region of the proteoglycan reduced the motility-repressing activity of the proteoglycan in a dose-dependent fashion.

These results suggest the following scenerio, illustrated in Figure 5. Molecules of hyaluronic acid may exist bound to the NC cell (Erickson and Turley, 1983) which synthesized them (Pintar, 1978). Chondroitin sulfate proteoglycan molecules secreted either by neighbouring cells or the NC cells themselves (Erickson and Turley, 1987) may then bind to HA attached to the cells and reduce their motility over a cell surface substrate. These HA-proteoglycan interactions could be important in regulating NC migration.

The presence of HA *in vivo* around pre-migratory NC cells also may be critical for initiation of movement from the neural tube, possibly because of its capacity to accumulate water and increase the space through which the NC cells can migrate (Luckenbill-Edds and Carrington, 1988).

The research on HA thus suggests that this molecule may be an important ECM component in directing NC cell migration by potentially creating migratory spaces for NC cells and also by modulating the inhibitory effect of proteoglycans on NC migration. Furthermore, because NC cells can secrete both HA and proteoglycans, they may thus be involved in controlling their own migratory behaviour.

In particular, the ECM has become a popular focus for researchers interested in the white axolotl pigment pattern defect. A brief discussion of work done in this regard is presented in the next section.

2) THE AXOLOTL AS AN EXPERIMENTAL MODEL

a) Introduction

Axolotls hatch about two or three weeks after fertilization and are about 1 cm long. They mature in about 18 months and reach about 12 to 15 cm in length. In captivity, they can live as long as 10 years.

The axolotl has a long history as a laboratory animal.

In fact, the majority of the animals today live in captivity. Their natural habitat which is restricted to two salty lakes in Mexico is currently being endangered by the growth of Mexico City (Brandon et al., 1984).

For researchers interested in pigment pattern development, the axolotl has been, and still is, very popular. Embryos are relatively large compared to other amphibians and are easily accessible to microsurgical techniques. They recover well from experimental manipulation and are easy to raise as larvae. Furthermore, as a result of inbreeding in captivity, several pigment pattern mutants have developed. Furthermore, pigment pattern development can be easily and noninvasively monitored under the dissecting microscope because melanophores are visible beneath the transparent epidermis.

Despite extensive experimentation done with this animal system, surprisingly little is known about how the pigment pattern develops. The white mutant phenotype, in particular, is intriguing.

While melanophores and xanthophores migrate from the neural crest to areas on the body to create a specific pigment pattern in normal or dark larvae, the same cells are restricted to the dorsolateral midline in white larvae (Figure 6).

b) The Axolotl White Mutant: Theories

The axolotl (wild type, DD or Dd) develops a larval pigment pattern that consists of alternating melanin-containing melanophore and pteridine-containing xanthophore bars dorsally, and a broad stripe of melanophores ventrally (Figure 7). The white axolotl pigment pattern mutant is due to a homozygous recessive condition (d/d) (Haecker, 1907) characterized by markedly fewer melanophores than normal. Along with xanthophores, the melanophores line up along the dorsal midline in greatly reduced numbers compared to D/D or D/d animals (Figure 6). Melanophores do not populate the flank in the larval white mutant (Frost et al., 1984b). It is believed that the white phenotype results in part from restricted ventrolateral migration of melanophores from the NC (Dalton, 1950; Bogomolova and Korochkin, 1973; Keller et al., 1982).

All white mutant larvae exhibit the same restricted pigment pattern. However, later on during development, after stage 43, variable amounts of repigmentation can occur, producing some individuals which are indiscernable from normal adults. The mechanisms responsible for the latter repigmentation are unknown (Frost et al., 1984b). The ECM through which pigment cell progenitors move is thought to be very important in the generation of the white defect. In dark embryos, the ECM is thought to become "permissive" just prior to migration (Lofberg and Ahlfors, 1978). Early NC migration is thought to take place in a

highly organized fibrillar network within the ECM. Frequent close contacts between the NC cells and these fibrils suggests that the fibrillar network is an important substratum for the locomotion of these cells and that it may be a regulatory factor for the onset of migration (Lofberg and Ahlfors, 1978).

The possibility that the white pigment cell environment is defective was first studied by Dalton (1950). Differently shaped pieces of ectoderm were grafted between dark and white animals. Dark ectoderm transplanted into white animals became pigmented provided that the ectoderm was oriented so that its dorsal edge was close enough to the host's neural crest to permit pigment cell precursors to enter it. White ectoderm transplanted into dark animals remained unpigmented (Figure 8). These results were interpreted as due to a failure of white ectoderm to support the migration of pigment cell progenitors.

Susequently, using scanning electron microscopy of white and dark embryos during different stages of NC migration, Spieth and Keller (1984) were able to study cells along different migratory routes taken by NC cells in dark and white embryos.

As migration from the NC commences in both embryos, cells pass from the dorsoventral surface of the NC toward the overlying epidermis. These cells are elongate and perpendicular to the cells remaining in the NC. Once cells

reach the apex of the somite, they take one of two pathways: between the epidermis and outer surface of the somites (subepidermal route) or between the somites and neural tube (perineural route). The perineural route appears to be normal in white embryos but there is a lack of NC migration along the subepidermal route (with the exception of an occasional NC cell which sluggishly migrates in a dorsoventral direction down an intersomitic furrow). The first NC cells to migrate into the subepidermal space in dark animals are those that will become melanophores, followed later by cells destined to become xanthophores (Spieth and Keller, 1984). It is believed that heterophobic interactions between the two different pigment cell types together may create the alternating bar pattern characteristic of the axolotl (Lehman, 1957) (Figure 9).

Work done by Tucker and Erickson (1986) on T. torosa, a urodele also exhibiting a striped flank pigment pattern composed of horizontal alternating melanophores and xanthophores, also suggests that inter- and intraspecific cell-cell interactions between the pigment cells as well as their differential adhesions to components of the ECM are responsible for that particular pigment pattern. Alcian Blue staining of paraffin sections and ruthenium red staining of thin sectins were used to identify the composition and distribution of the ECM surrounding pigment cells at various stages of development. Based on these

experiments, they suggested that pigment cells differentiate in regions containing relatively little GAG and that xanthophores can invade the relatively GAG-rich regions of the dorsal fin whereas melanophores cannot. The melanophores that appear in the dorsal fin later during pattern formation are those derived from NC cells that invaded early during development and were delayed in differentiating by the presence of HA.

The ECM ultrastructure was more intensively investigated by Spieth and Keller (1984). They found that the ultrastructure of the subepidermal ECM along migratory routes of pigment cells is different between dark and white embryos. The ECM in white embryos contains fewer spherical bodies among the fibrillar matrix. This work and similar work done on the chick embryo (Mayer et al, 1981) suggest that the arrangement of fibrils and spherical bodies is composed of proteoglycans and fibronectin and may be involved in NC migration by arranging the ECM into specific regions that are more permissive to NC migration, thus creating NC pathways. Spieth and Keller found, however, that perineural ECM ultrastructure appears to be the same in both embryos. NC cell migration through this region is similar in both dark and white embryos. Cells taking this route contribute to melanophores covering the dorsal aspect of the gut as well as to various other structures of the peripheral nervous system (Lofberg et al., 1989b; Perris et

al., 1988). Because white mutant animals are normal phenotypically with the exception of their pigmentary system, the normalcy of this migratory route is expected.

Lofberg et al. (1989b) transplanted age- and region-specific ECM's between dark and white embryos. This was done by cutting microcarriers from Nucleopore membranes and inserting them under the flank epidermis of donor embryos at stages 25-27. The carriers were subsequently removed at a specific stage and inserted into white migratory stage hosts. They found that subepidermal migration of NC cells could be triggered in white host embryos where migration doesn't normally occur by inserting dark stage 30 ECM on an inert microcarrier into the white host's subepidermal space. The stimulation of NC migration seemed to result exclusively from direct contact between the NC cells and the ECM-covered microcarrier, since NC cells were observed only in the specific subepidermal region of the microcarrier. Subepidermal ECM from white stage 30 donors did not stimulate NC migration in white hosts but stage 35 ECM did stimulate migration, suggesting that ECM is only transiently defective and that it is retarded maturation of white ECM that is responsible, at least in part, for the white defect. By the time white subepidermal ECM is competent to support NC cell migration, pigment cells in the white animal may have lost their ability to respond to the ECM and migrate.

Ultrastructural, immunohistochemical and biochemical

characterization of the ECM's of dark and white embryos prior to and during early and late NC migration was subsequently done by Perris et al. (1990) to determine if any specific ECM components are absent in white axolotl embryos.

The composition of the ECM was found to differ between white and dark embryos. There was a significantly higher accumulation of electron-dense granules on collagen fibrils in the dark embryos (Perris et al., 1990). Tucker (1986) had previously suggested that the granules consist of HA microfibrils associated with chondroitin proteoglycan aggregates and that, together, these can interact with both NC cells and collagen fibrils in the ECM.

As well as differences between the distribution of fibronectin, laminin, collagen types I and IV and proteoglycans bearing chondroitin-4-sulfate and keratan sulfate in white and dark embryos, several uncharacterized highly glycosylated proteins were differently distributed in dark and white subepidermal matrices. Finally, chondroitin-6-sulfate-bearing proteoglycans were found to be more abundant in white subepidermal ECM. This class of proteoglycans has been found to inhibit migration of NC cells over fibronectin, vitronectin, laminin and substrates of various collagens (Perris and Johansson, 1987; Perris et al., 1990).

The perineural ECM in both embryos was less granulated

than the subepidermal ECM. However, unlike the results of Spieth and Keller (1984), Perris et al. (1990) revealed that this ECM differed in structural and molecular composition between dark and white embryos though it is permissive to NC cell migration in both embryos. The significance of these findings is unclear.

In conclusion, these results suggest that local differences in the concentration of some specific components of the ECM encountered by moving NC cells may be involved in the abnormal chromatophore migratory behaviour in the white mutant. However, the particular ECM molecules responsible for the white mutant and their mechanisms of action remain unknown. The specific amounts of various components of the ECM do not appear to be critically involved in the white axolotl defect, since there are many differences between dark and white stage 35 ECM yet both can stimulate migration (Lofberg et al., 1989b; Perris et al., 1990). However, it is still possible that the local presence of one or more currently unidentified glycoprotein(s) may be critical for normal subepidermal NC migration in the axolotl.

3) LECTINS

a) A Review of Lectin Research

Lectin research began in 1888 with the discovery of

castor bean hemagglutination ability described by H. Stillmark in his Ph.D. thesis (Stillmark, 1888, discussed in Goldstein and Portez, 1986). Since then, different lectins have been found to be able to discriminate and specifically agglutinate red blood cells based on species (Landsteiner and Raubitschek, 1908, discussed in Olden and Parent, 1987) and different blood cell types (reviewed by Judd, 1980). By the 1950's, it was discovered that simple sugars could inhibit lectin activity and by the 1960's, the molecular biology of lectins was being studied (reviewed by Kocourek, 1986). Lectins exist in all phyla, including vertebrates (Goldstein et al., 1980). Lectins are now popular as mitogens (Oppenheim et al., 1974) and as structural probes for changes in the cell surface during the cell cycle and malignant transformation as well as to separate cell types on the basis of different surface carboydrate markers (reviewed by Bog-Hansen and Freed, 1988).

b) Properties of Lectins

Lectins can agglutinate cells, have no enzymatic function and are often termed sugar-binding proteins, either soluble or membrane-bound (Goldstein et al., 1980).

To assay lectin activity, the hemagglutination assay is most frequently used (Makela, 1957; Lis and Sharon, 1973), but more refined screening procedures are available which

are based on the ability of lectins to precipitate glycoconjugates and polysaccharides in liquid or gel media (Goldstein and Hayes, 1978; Lis and Sharon, 1984; Goldstein and Poretz, 1986). Specific lectin-carbohydrate interactions involving the lectin can be distinguished by sugar specificity assays (Goldstein and Hayes, 1978; Lis and Sharon, 1984; Goldstein and Poretz, 1986). Lectins are most often extracted and purified using affinity column chromatography (Lis and Sharon, 1981; Lis and Sharon, 1984). This work is reviewed by Goldstein and Poretz (1986).

In vivo, lectins most often bind to non-reducing terminal glycosyl groups of polysaccharide and glycoprotein chain termini (Goldstein and Hayes, 1978). In general, lectins tolerate very little variation in C-3 (the third carbon away from the reactive aldehyde group of the hexose) of the sugars they bind (Goldstein and Hayes, 1978). In addition, the C-4 hydroxyl group is probably critical for lectin binding (Goldstein and Hayes, 1978). Based on the hydroxylic (free hexose sugars contain five reactive oxygen-hydrogen or hydroxyl groups) and hydrophobic (lacking an affinity for water, a highly polar molecule) nature of sugars, it is likely that polar interactions like hydrogen bonding as well as dipole interactions also play some role in lectin-carbohydrate binding (Becker, 1975; Hardman and Ainsworth, 1976). Hydrophilic interactions are also probably involved in lectin-carbohydrate binding (see

Goldstein and Poretz, 1986). According to Goldstein and Poretz (1986), the lectin-carbohydrate binding site recognizes "interactions between surfaces [topographical features] and not sugar units per se..."

Interestingly, lectins share few, if any, structural similarities, except that they are all proteins (Liener et al., 1986). Most are glycoproteins with sugar content as high as 55% (potato lectin) but some contain no sugar at all (Concanavalin A, for example) (Lis and Sharon, 1986). Molecular weights and the numbers and types of subunits vary greatly among different lectin molecules. Most, but not all, lectin molecules consist of identical subunits (Goldstein and Hayes, 1978; Lis and Sharon, 1984; Goldstein and Poretz, 1986). Endogenous lectins have been localized on the plasma membrane, endoplasmic reticulum, Golgi apparatus, nucleus and nucleolus in different cells as well as in the ECM (reviewed by Monsigny et al., 1988).

Despite the variability of lectins as a whole, increasing structural evidence suggests that animal lectins can be organized into several categories. For example, Drickammer (1988) has categorized mammalian lectins into three types: C-type lectins are Ca^{++}-dependent and contain a 130 amino acid long domain in which 18 conserved amino acids reside. The C-type category includes a group of lectins called mannose-binding proteins which share a COOH-terminal domain homologous with membraneous lectins and a

series of collagen-like sequences. These lectins are Ca++-dependent and mannose and N-acetyl-glucosamine-specific. Examples are lung surfactant protein, conglutinin and C1q protein (defined as a lectin by some researchers). S-type mammalian lectins also form a distinct group. These lectins are thiol-dependent and contain a frame of about 100 similar amino acids including 39 conserved residues. In contrast to C-type lectins, S-type lectins exhibit carbohydrate-binding activity that is independent of divalent cations but are dependent on the presence of disulfide bonds for activity. These lectins are sometimes called 14-16K lectins because their molecular weights are generally very similar. They have a wide tissue distribution (reviewed by Drickamer, 1988). B-galactose-specific lectins are examples of this group. The third type of mammalian lectins are those that contain neither C- nor S-type conserved residues. The mannose-6-phosphate receptor is an example of this group (Thiel and Reid, 1989).

In addition to collagen-like sequences found in C-type lectins (mentioned above), EGF (epidermal growth factor)-like domains and complement-binding protein-like domains have also been recently revealed in several structural studies on vertebrate C-type lectins, suggesting that lectins, other adhesive proteins, and growth factors may be related. These recent findings are also very interesting because they suggest that lectins may have functions such as

cell adhesion, growth, differentiation, and ECM interactions, etc., in addition to their sugar-binding functions. Examples of these lectins will be discussed in more detail below.

Recently, cell adhesion molecules (CAM's) with lectin-like activity have been identified in mammals (Stoolman, 1989; reviewed by Brandley et al., 1990). These molecules have been called LEC-CAM's or selectins and are implicated in the interactions between leukocytes and platelets or vascular endothelium. Cell-cell adhesion is an early event in this interaction (Stoolmann, 1989). Three LEC-CAM's have been identified thus far and have in common three domains (based on cDNA sequences), one of which is similar to that described for Ca^{++}-dependent lectins (giving rise to the LEC acronymn), an EGF-like domain and several complement-binding protein-like domains (Brandley et al., 1990).

Recently, the core polypeptide of the lymphocyte homing receptor, a cell surface protein, has been found to be composed of a lectin domain (Lasky et al., 1989), an EGF-like domain, and two repeats common with regulatory proteins such as receptors which direct the differentiation and motility of cells (Siegelman et al., 1990). The lectin domain binds specifically with mannose-6-phosphate and appears to influence the binding of lymphocytes to lymph node high endothelial venules but not those of Peyer's patch (Stoolman and Rosen, 1983; Stoolman et al., 1984).

Interestingly, this core polypeptide design (which contains a lectin domain) is homologous in the human lymph node homing receptor (Siegelman and Weissman, 1989).

A 123-amino acid domain homologous with the carbohydrate-binding domain of C-type animal lectins has also been recently identified in Fc receptors (reviewed by Hajela, 1991; Daeron, 1991). These receptors are located in effector cells and recognize the Fc region of IgE antibodies. Interestingly, although IgE is heavily glycosylated, the binding of this receptor is independent of any lectin-like activity. These authors suggests that the carbohydrate lectin-like recognition domain of lectins has evolved as the Fc receptor to recognize the constant region of immunoglobulins.

It isn't surprizing that lectins bind to cells since cells are coated with sugar residues. For agglutination to occur, a lectin must form multiple cross-bridges between opposing cells but there is no simple kinetic relationship between agglutination and the amount of lectin bound because agglutination is complicated by factors such as the number of saccharide-binding sites in the particular lectin, molecular size of the lectin, membrane accessibility of receptor sites, membrane fluidity, metabolic states of the cells and external factors like cell concentration or availability to the lectin and temperature (reviewed by Halina and Sharon, 1986).

c) Lectins During Development

Lectins have been found to have a variety of biological properties in vertebrates, including mitogenic stimulation of lymphocytes, induction of suppressor cells, cytotoxicity, lectin-mediated pinocytosis, insulin-like effects on adipocytes, receptor-mediated endocytosis, and cell adhesion (Lis and Sharon, 1986; Milos and Zalik, 1986; Frunchak and Milos, 1990).

The activity of lectins in some tissues has been found to change dramatically during embryonic development, leading to suggestions that lectins may play specific roles during embryonic tissue organizaton, especially in cellular adhesion (reviewed by Barondes, 1986; Zalik and Milos, 1986; Kobiler, 1987; Zalik, 1991). As discussed below, lectins have been suggested to function as partners in forming adhesive bonds with their appropriate sugar-binding receptors on cell surfaces, or indirectly by modulating the availability of cell surface sugar-binding receptors involved in adhesion. Lectins may be involved in adhesion indirectly by their effects on the organization of the ECM or through the the ECM-like sequences that some of them contain. Developmentally regulated lectins have been detected in a number of differentiating systems (reviewed by Zalik (1991). Examples of these lectins are discussed below.

Changes in endogenous lectin actvity during development have been detected in the slime mold and in chick, frog, and mammalian embryos (Barondes et al., 1983; Den and Malinzak, 1977; Nowak et al., 1977; Harris and Zalik, 1985; Milos et al, 1990; reviewed by Zalik and Milos, 1986; Zalik, 1990; Caron et al., 1990). However, though originally implicated directly in slime mold cell-cell adhesion and in chick muscle adhesion (Rosen et al., 1979; Den and Malinzak, 1977; Nowak et al., 1977), additional studies did not support these interpretations of the data and the main functions of these lectins are still unknown (reviewed by Barondes, 1986; Zalik and Milos, 1986).

In the early gastrulating chick embryo, a galactoside-specific lectin was identified in 1979 (Cook et al., 1979). Evidence for a role in adhesion of extraembryonic endoderm cells from these embryos was subsequently suggested based on experiments in which lectin inhibitors or purified lectin extracts were added to cells in vitro and shown to alter cellular adhesiveness (Cook et al., 1979; Milos and Zalik, 1981 and 1982).

A galactoside-specific lectin in chick skin may be important in embryonic differentiation based on evidence that this lectin photochemically cross-links with a polylactosamine-proteoglycan found in chick embryonic skin (Oda and Kasai, 1989).

The synthesis, degradation, and activation of a

developmentally regulated rat lung lectin was investigated by Clerch et al. (1987). The carbohydrate-binding activity of this lectin increases at day 12 of development due to an increase in activity of the lectin molecule itself (suggesting a structural change in the molecule) as well as increased synthesis of lectin. The decline in lectin activity in lung tissue measured after day 12 is due to a decrease in lectin synthesis and increased degradation.

Another example is syndecan, a developmentally regulated cell surface proteoglycan with lectin-like activity that binds ECM and growth factors (Bernfield and Sanderson, 1990). It behaves as a matrix receptor and changes in quantity, localization, and structure during development (Bernfield and Sanderson, 1990).

A developmentally regulated heparin-specific lectin has been detected in human placenta (Ceri et al., 1990). Based on their results on the localization and time of appearance of this lectin, the researchers have suggested that the lectin may be important in invasion of cytotrophoblasts into maternal tissues during implantation. The lectin is suggested to function in invasion as an adhesive molecule maintaining contact between syncytiotrophoblasts and placental tissue.

Developmentally regulated lectins have also been detected in the mammalian nervous system. Some examples are described below.

Two developmentally regulated endogenous lactose-binding lectins are localized in the dorsal root ganglion and dorsal horn and are expressed during early development of these structures, suggesting that they may contribute to the neuronal-neuronal matching of primary sensory neurones (Regan et al., 1986).

Hynes et al. (1990) have isolated rat brain lectin cDNA. It is homologous to other soluble lectins and its mRNA is localized in the brain and other parts of the rat nervous system such as primary sensory neurones and motorneurones shortly after their differentiation. It is suggested that this lectin may be involved in carbohydrate-mediated interactions contributing to the development of these neurones (or their arrangements).

Finally, the recent discovery of a glycoprotein called Jag-1 which exhibits lectin-like activity may be involved in neuronal development (Farley et al., 1990). It is currently thought that pathfinding by neurones may be mediated by the expression of this glycoprotein on the cell surface of embryonic axons and growth cones because it exhibits neurite outgrowth-promoting activity. Interestingly, this protein also contains six immunoglobulin-like domains, suggesting that lectins and immune system development or function may be related. This is an interesting prospect, considering that cell recognition and communication are essential to immune responses and lectins appear to function in this

capacity.

As discussed in the next section of this thesis, lectins may also have indirect roles to play in directing cell adhesion and migration.

d) Lectins and the Extracellular Matrix

As mentioned above, some lectins have been detected in association with the ECM (Barondes, 1981; Barondes, 1984). These observations have led to suggestions that these lectins may influence the interactions between components of and, therefore, the structure of, the ECM and thus function indirectly in controlling cell adhesion and migration.

Hyaluronic acid is an N-acetylglucosamine-rich glycosaminoglycan (GAG) core to which proteoglycans containing chondroitin sulfate and keratan sulfate bind through a link protein to from large aggregates, for example, during cartilage development (Figure 4). The GAG components of the proteoglycan complexes can interact with other ECM components by noncovalent binding (Cardin and Weintraub, 1989), sugar-specific binding (Lindahl et al., 1980; Atha et al., 1984) or charge-dependent binding (Ruoslahti, 1988a; Ruoslahti, 1988b; Kaesberg et al., 1989; Cole and Akeson, 1989; Gospodarowicz et al., 1987).

However, not all HA interactions are mediated through the GAG components. For example, the protein cores of

chondroitin sulfate and dermatan sulfate proteoglycans contain a lectin-like domain that can bind simple sugars (Doege et al., 1987; Halberg et al., 1988). It is thus possible that a lectin existing extracellularly may interact with the saccharides that constitute HA-containing GAG's and interfere with their aggregation with proteoglycans. Work done by Mitchell and Hardingham (1981) suggests that HA synthesis occurs independently of the synthesis of other GAG's and that HA and proteoglycans are not associated with each other prior to the secretion of proteoglycans into the ECM. Oligosaccharides containing from 16 to 30 residues were found to either slow or prevent HA-proteoglycan assembly. Furthermore, they discovered that there is approximately an 18 hour delay between the completion of proteoglycan synthesis and aggregate formation, suggesting that the proteoglycans may have to mature before they can interact with the HA core. This delay between HA and proteoglycan synthesis and their assembly into aggregates suggests that if a lectin is present at this time in the ECM, it may be possible for it to interact with either HA or the proteoglycans and interfere with the aggregation process.

Interestingly, proteoglycans other than HA-aggregating proteoglycan are known to produce some effects similar to those of lectins <u>in vivo</u>. Cell surface proteoglycans, for example, can affect cell adhesion. An example is syndecan,

an integral membrane proteoglycan with carbohydrate-binding capability (Saunders et al., 1989). Soluble proteoglycans such as chondroitin sulfate and keratan sulfate can affect cell adhesion to FN and collagen possibly through steric hinderence (Rosenberg et al., 1986; Hautenan et al., 1989). Interestingly, laminin also contains lectin-like agglutinating activity. For example, laminin purified from the ECM and soluble fraction of a teratocarcinoma line (COTT6050) can agglutinate trysinized glutaraldehyde fixed rabbit erythrocytes (Ozawa et al., 1985). These investigators also report that heparin and heparan sulfate can also agglutinate the treated erythrocytes.

As discussed previously, proteoglycan-NC interactions are thought to be involved in controlling NC migration and migration pathways (Perris and Johansson, 1987; Perris et al., 1990). The lectin-like domains of proteoglycans may play a role in HA-proteoglycan assembly, possibly by binding to saccharides that constitute HA-containing GAG's and interfering with aggregation. Similar mechanisms utilyzing lectins as regulators of HA-proteoglycan assembly may function in embryonic matrices to control matrix development, maturation, and function.

As mentioned above, some lectins have now been designated as LEC-CAM's. One of the criteria that must be met in a lectin to be called a CAM is the presence of EGF-like sequences in the lectin's structure (Brandley et al.,

1990). Given the importance of growth factors in development as possible controllers of mitosis, adhesion, differentiation, and migration (Greenburg et al., 1980; Gospodarawicz et al., 1978), it is interesting that some lectins have growth factor-like sequences and are also ECM-associated molecules. Thus, some lectins may not just play multiple roles in different tissues, but they may play multiple roles in the same ECM tissue location.

In conclusion, there is no direct evidence that endogenous developmentally regulated lectins can interact with ECM components to affect NC cell adhesion, migration or differentiation. However, if they exist with other ECM components, endogenous lectins and ECM molecules may be able to interact with each other and alter the assembly of ECM-filled NC migratory pathways, and thus the migration of NC cells.

e) The Xenopus laevis Model

The hypothesis that endogenous lectins may be present and that differences in lectin activity may also be involved in pigment pattern differences between dark and white axolotl embryos is based on research regarding the role of endogenous lectin and its sugar hapten inhibitor, B-thiodigalactoside (TDG) in Xenopus laevis pigment pattern formation. As mentioned above, lectins are defined as

carbohydrate-binding proteins of non-immune origin that can agglutinate cells (Goldstein et al., 1980). Frunchak and Milos (1990) and Milos et al. (1987 and 1990) have obtained evidence that endogenous lectins may be important in Xenopus melanophore pigment pattern development.

Briefly, cellular adhesiveness of melanophores differentiating from Xenopus NC explants growing in culture was found to differ in the presence of purified endogenous Xenopus laevis lectin or TDG (Milos et al., 1987). In vivo confrontation experiments were subsequently done by stripping away the dorsal ectoderm of X. laevis embryos and exposing the underlying premigratory trunk NC to purified NC stage lectin. Melanophores populated the dermis but were fewer in number and had altered morphologies and arrangements compared to controls. When confronted with TDG, changes in melanophore morphology primarily resulted (Frunchak and Milos, 1990). Using a monoclonal antibody produced against this lectin, lectin deposition in embryos and tadpoles at various stages of development was determined. It was found that lectin levels change during development in different regions of the embryo, decreasing in migrating cells and increasing in sites where cells become more adhesive and localize (Milos et al., 1990). With respect to melanophores, lectin was found to be deposited in association with the environment of these cells once they reached their final localization sites within the

dermis. The above results suggest that endogenous lectins may be important during melanophore pattern formation in Xenopus.

The possibility that endogenous lectins play a role in the differentiation and localization of other NC-derived structures such as the craniofacial skeleton and heart is currently under investigation. Preliminary research suggests that both structures in X. laevis are altered by in vivo confrontation with endogenous lectin or TDG (P.V. Varma and Y.N. Frunchak, unpublished data).

Research done on the role of endogenous lectins in neural crest cell migration, differentiation and localization in X. laevis therefore suggests that the neural crest contribution to morphological development of the melanophore pigment pattern, craniofacial skeleton and heart is modulated, in part, by endogenous lectin activity.

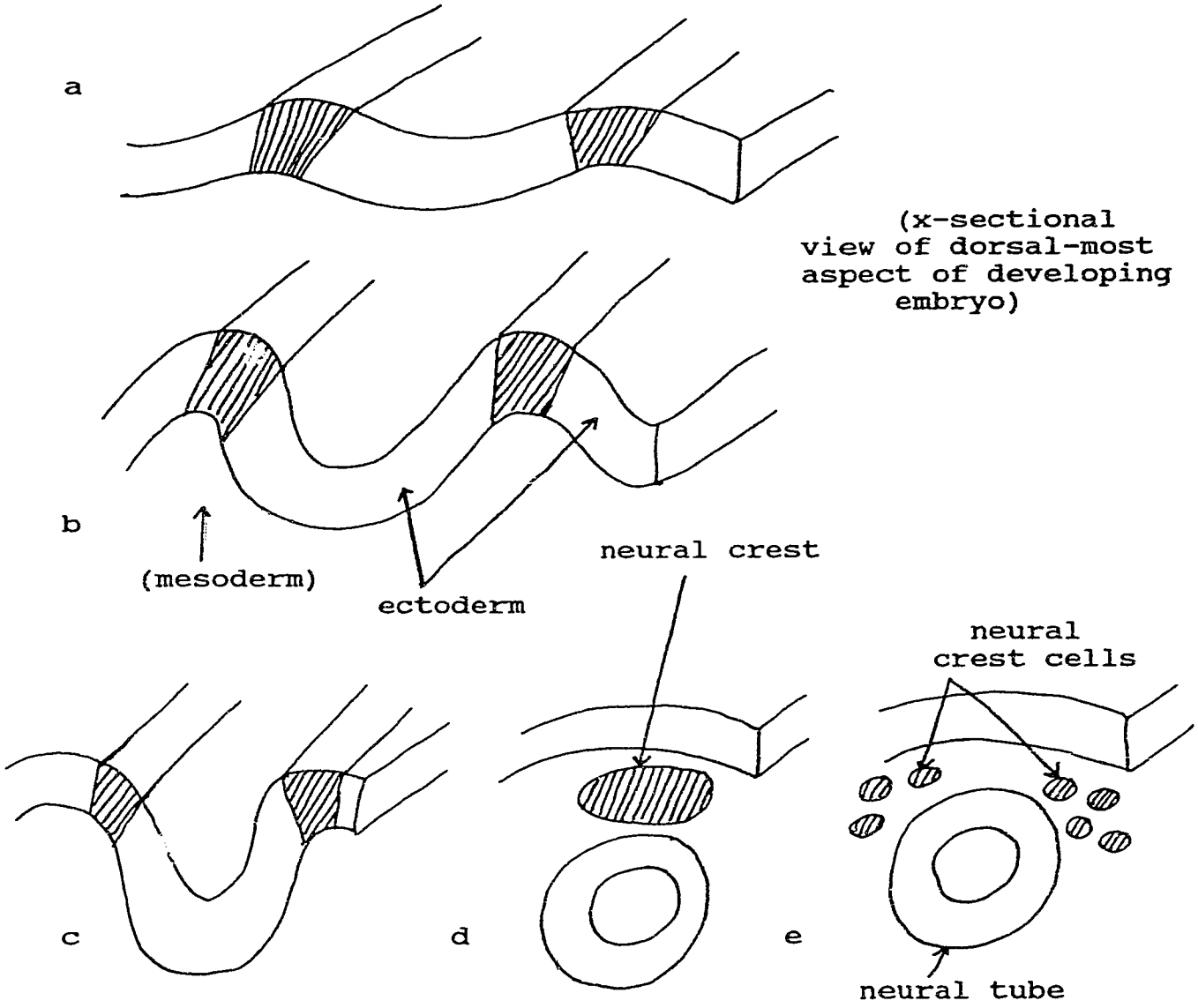
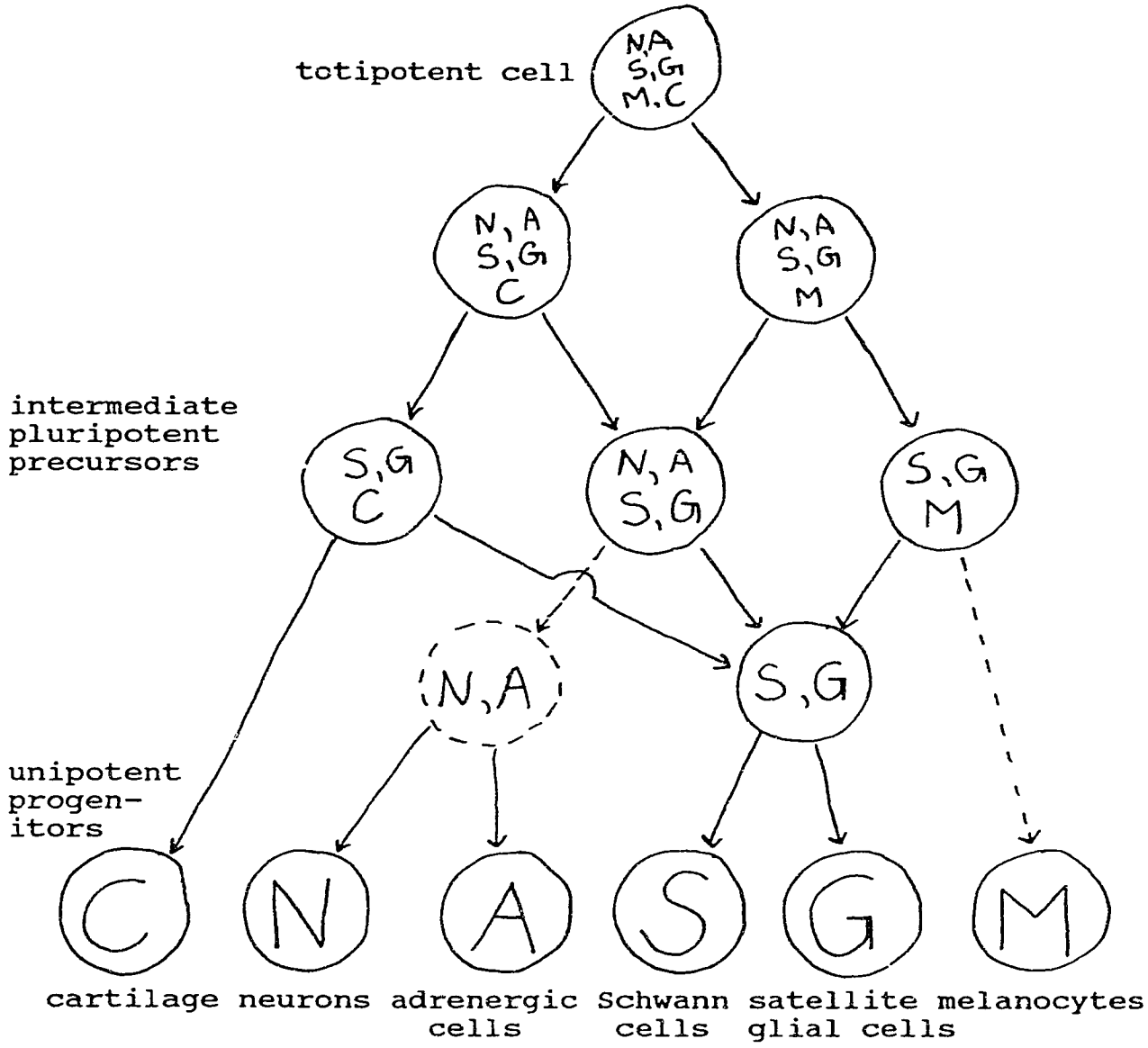


Figure 1. A schematic representation of the localization of the neural crest and neural crest cells from the stage of neural tube closure to initial neural crest cell migration in the amphibian embryo. (a) Neural ridge formation. Presumptive neural crest is illustrated by the two shaded areas of neuroectoderm. (b) and (c) Two lateral neural ridges move toward the dorsal midline of the embryo as the neural tube sinks ventrally into the mesodermal tissue. (d) A separate tissue called neural crest results from the fusion of the lateral neural ridges into a closed neural tube. Neuroectoderm and ectoderm are now physically separate. (e) In the amphibian, neural crest migration commences shortly after the neural tube closes. Modified from "The Neural Crest" Hall, B.K. and Horstadius, S. (eds.) Oxford University Press, London. Chapter 1, p. 16, 1988.



MESOECTODERMAL NEURAL MELANOCYTIC LINEAGES

Figure 2. Hypothetical model of the generation of neural crest-derived cell lineages: each full circle indicates a precursor evidenced by clonal experiments. Letters within a circle define developmental potentialities of this precursor. Arrows show some possible affiliations between different progenitors and are not exhaustive. Dashed circles and arrows indicate possible additional precursors, i.e., others than those demonstrated in the present study. Redrawn from Le Douarin et al. (1991).

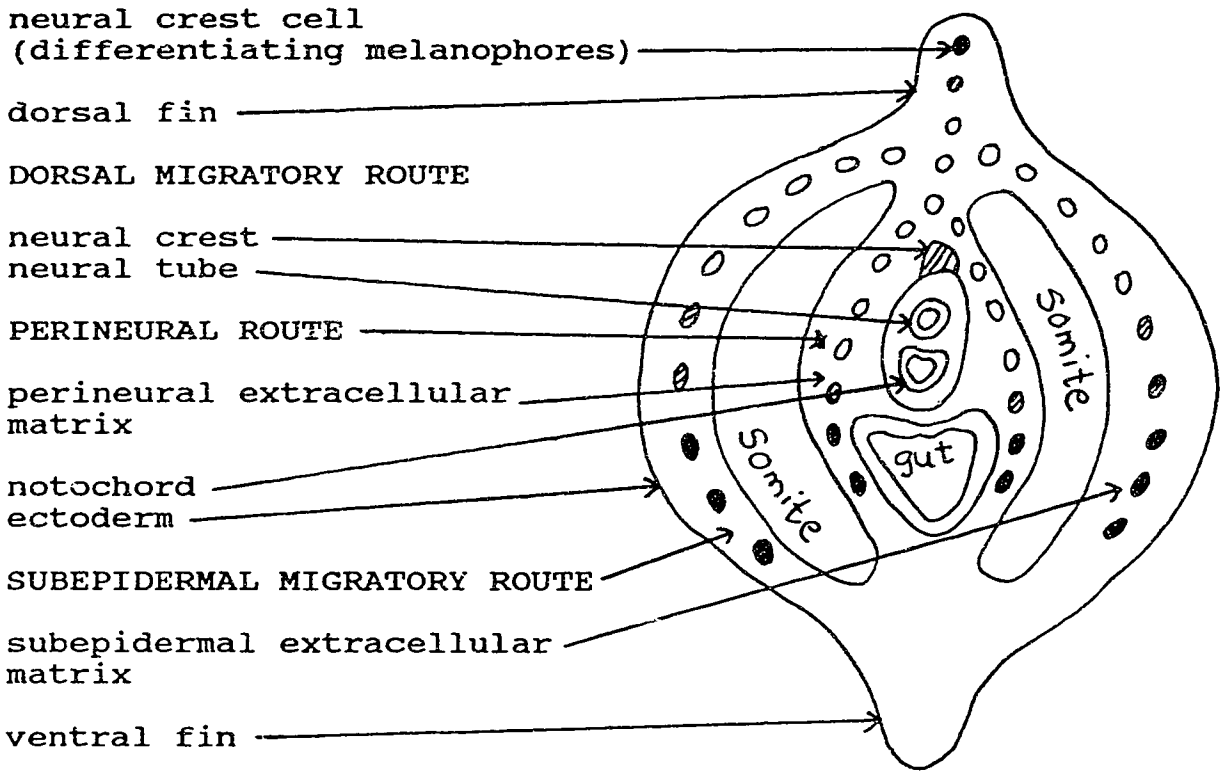


Figure 3. Schematic diagram of neural crest migratory pathways in dark (D/-) stage 37 axolotl embryos. A mid-trunk transverse section is illustrated. Three neural crest migratory routes exist in the dark embryo. The differentiation of melanophores as they migrate is illustrated by their gradual shading.

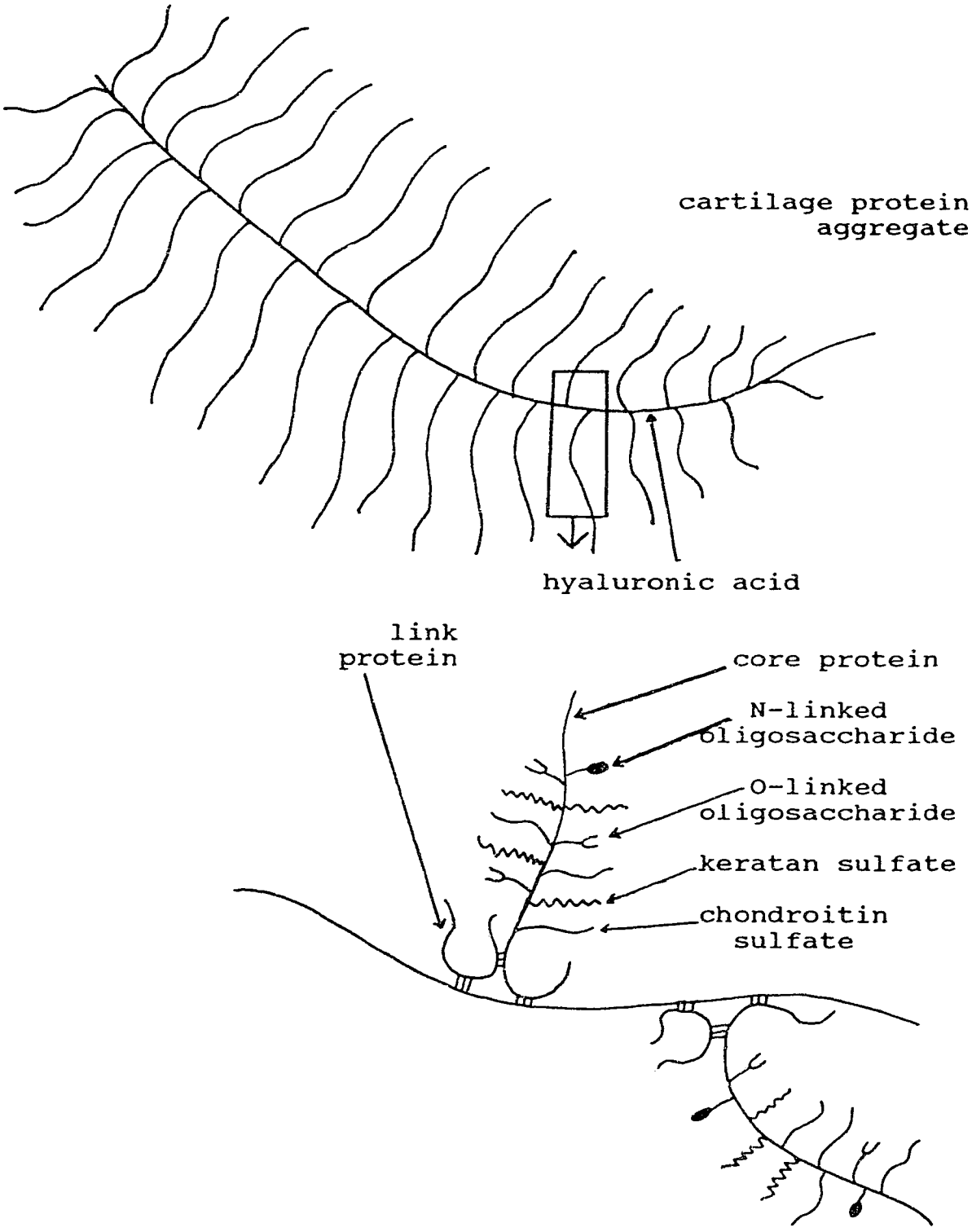


Figure 4. Schematic diagram of typical proteoglycan aggregate structure in cartilage. Hyaluronic acid may exist in unaggregated form in embryonic extracellular matrix. Redrawn from "Cell Biology of the Extracellular Matrix" Hay, E.D. (ed.) Plenum Press, N.Y. Chapter 2, p. 47, 1981.

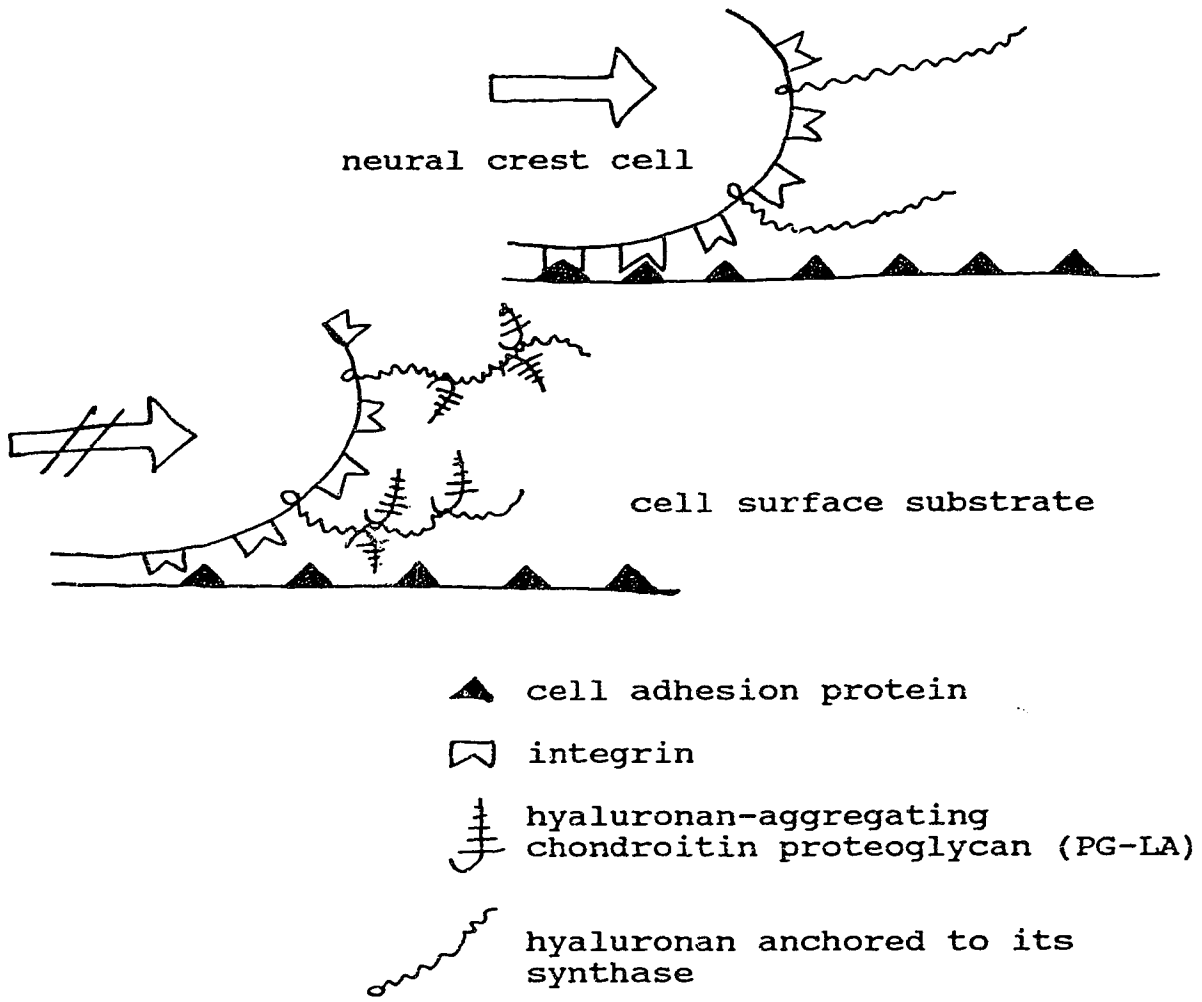


Figure 5. Schematic illustration of how aggregating proteoglycans are proposed to influence neural crest cell migration. (a) A neural crest cell migrating on cell adhesion/motility-promoting proteins by use of corresponding receptors (integrins). (b) Inhibited neural crest cell migration on cell adhesion/motility-promoting proteins in the presence of soluble PG-LA. The proteoglycan, which could be secreted by either the neighbouring cells or the cells themselves (Erickson and Turley, 1987), binds to cell surface hyaluronan anchored to its synthase (Prehm, 1984; Philipson and Schwartz, 1984; Mian, 1986) or specific receptors (Underhill et al., 1987). Accumulation of a large number of proteoglycan molecules on the cell surface would interfere with the function of cell surface receptors involved in cell movement, by either steric hindrance or specific modulatory interaction. From Perris and Johansson (1990).

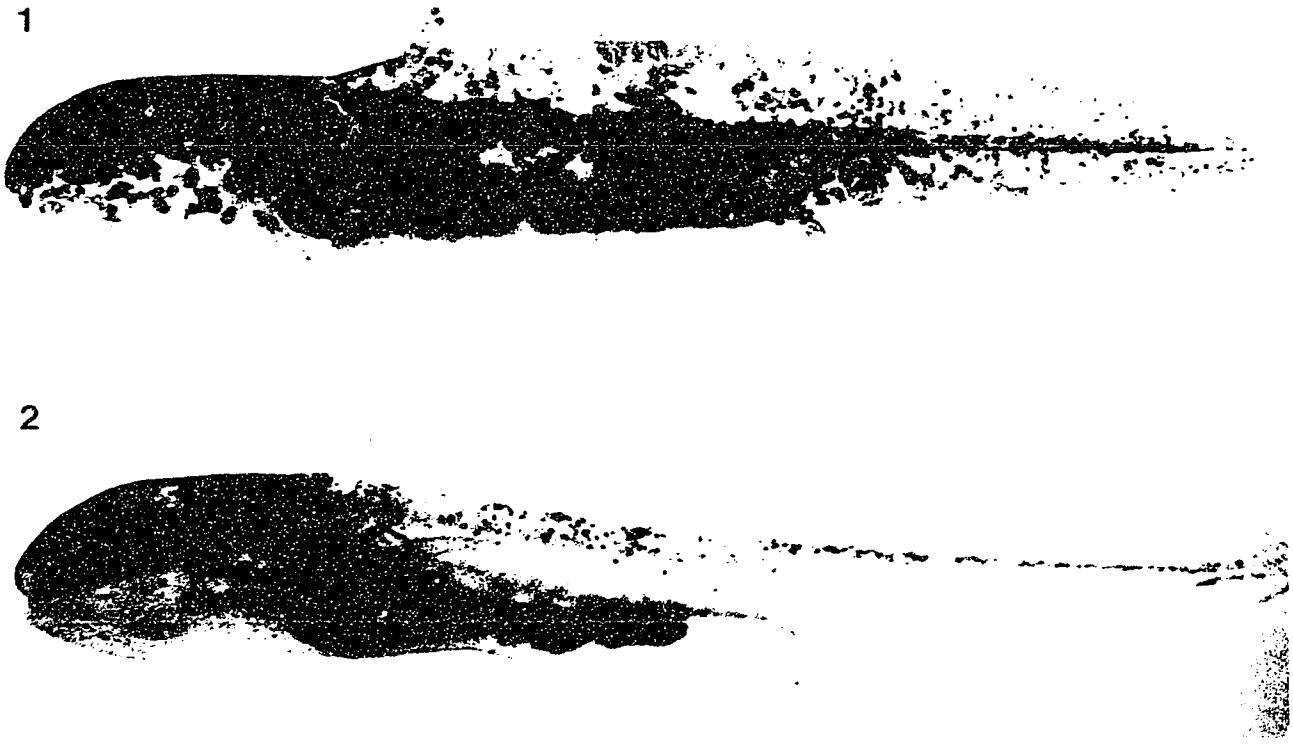


Figure 6. (1) Axolotl larva of the dark phenotype (D/-), stage 42. Pigment cells are widespread over the whole body except for the ventral region. The melanophores are conspicuous, while yellow xanthophores occur in lighter areas between them. (2) Axolotl larva of the white phenotype (d/d), stage 43. Melanophores and xanthophores form an irregular stripe along the dorsal midline of the trunk. The flank as well as the dorsal and ventral fin is unpigmented (shown here larger than actual size). From Lofberg et al. (1989).

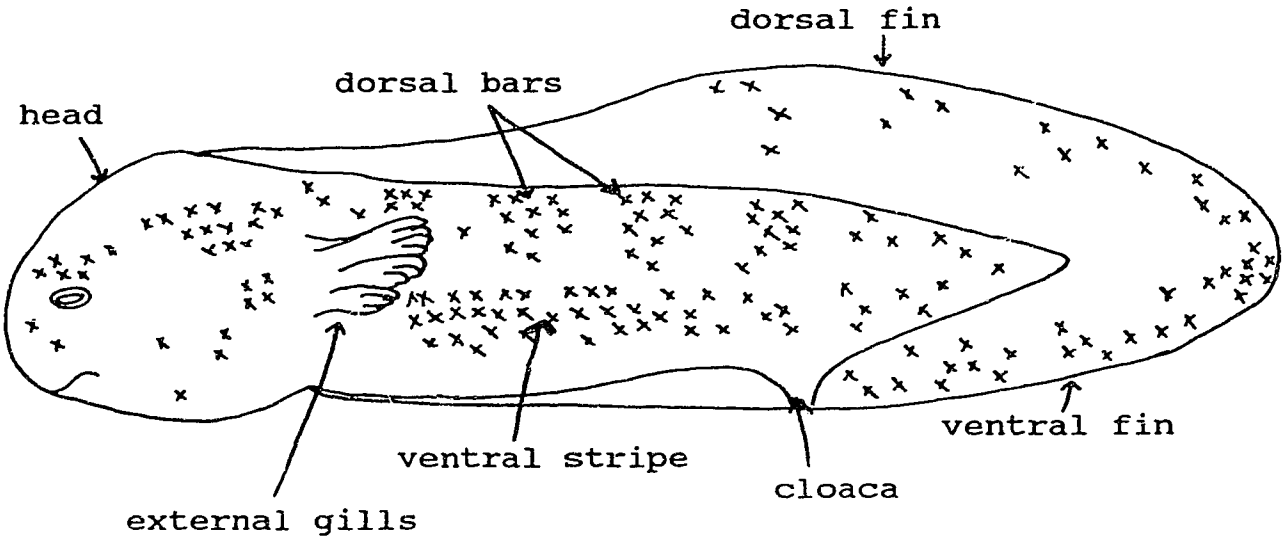


Figure 7. Schematic drawing of a stage 39 axolotl embryo to illustrate the general melanophore pigment pattern. This representation is based on sketches of control D/- axolotl embryos.

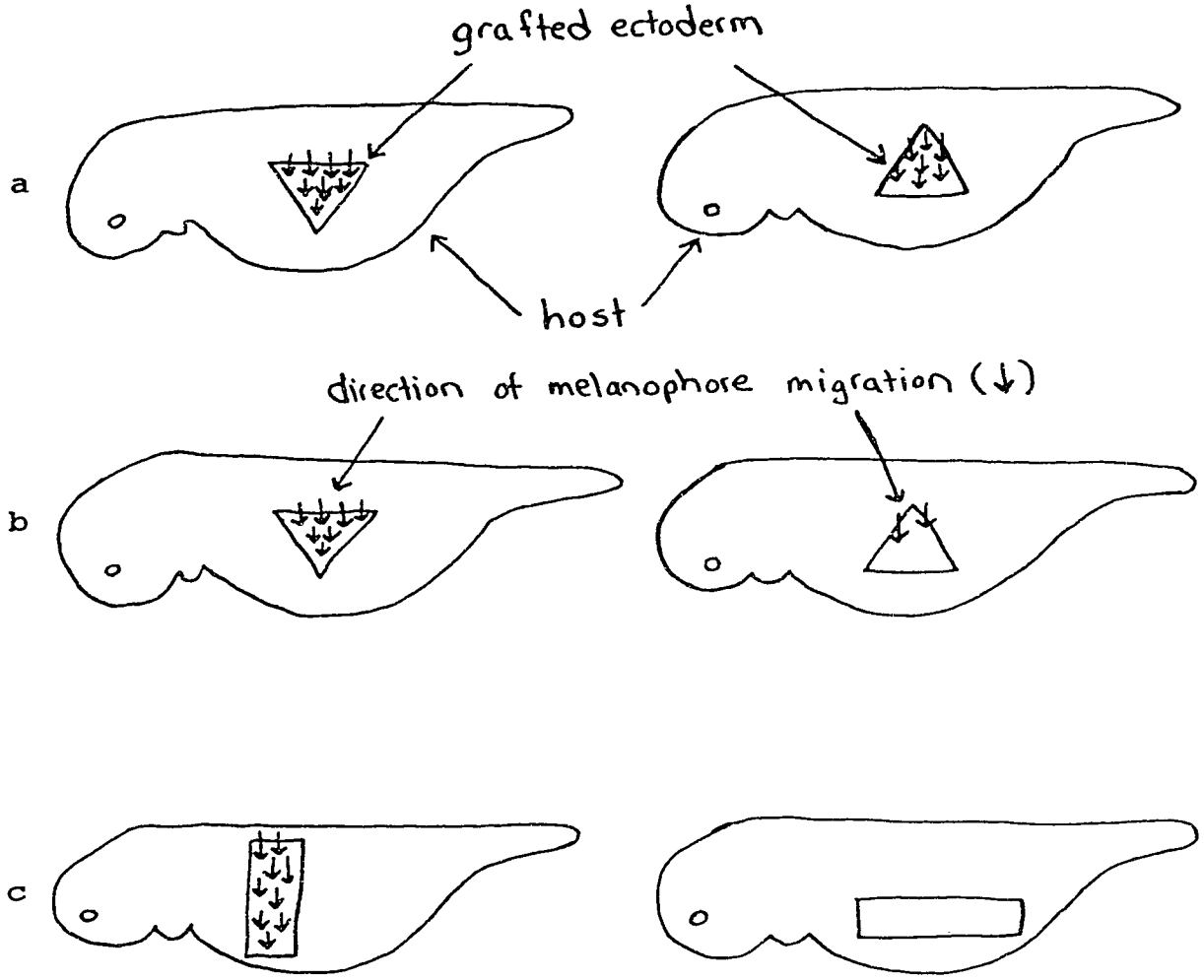


Figure 8. A schematic representation of the experimental results of H.C. Dalton (1950) on dark (D/-) and white (d/d) axolotl embryos. Differently shaped pieces of dark stage 20-23 (premigratory) and dark stage 27-32 (early migratory) ectodermal tissue were grafted to white hosts at corresponding stages of development. Larval stage axolotl shematic diagrams are shown. (a) Pigment cells are expected to migrate equally in a triangular ectodermal graft oriented basal side up or pointed side up if dark ectoderm contains some substance that allows preexisting pigment cell precursors to become pigmented. (b) Expected results if dark ectoderm provides some avenue more suitable for migration than surrounding white host tissues. (a) and (b) are redrawn from Dalton (1950). The results of his experiment resembled those of (b). (c) A vertical dark ectodermal graft elicited pigment cell migration in a white host flank but a horizontal dark ectodermal graft placed ventral to the flank midline did not. Adapted from the text of Dalton (1950).

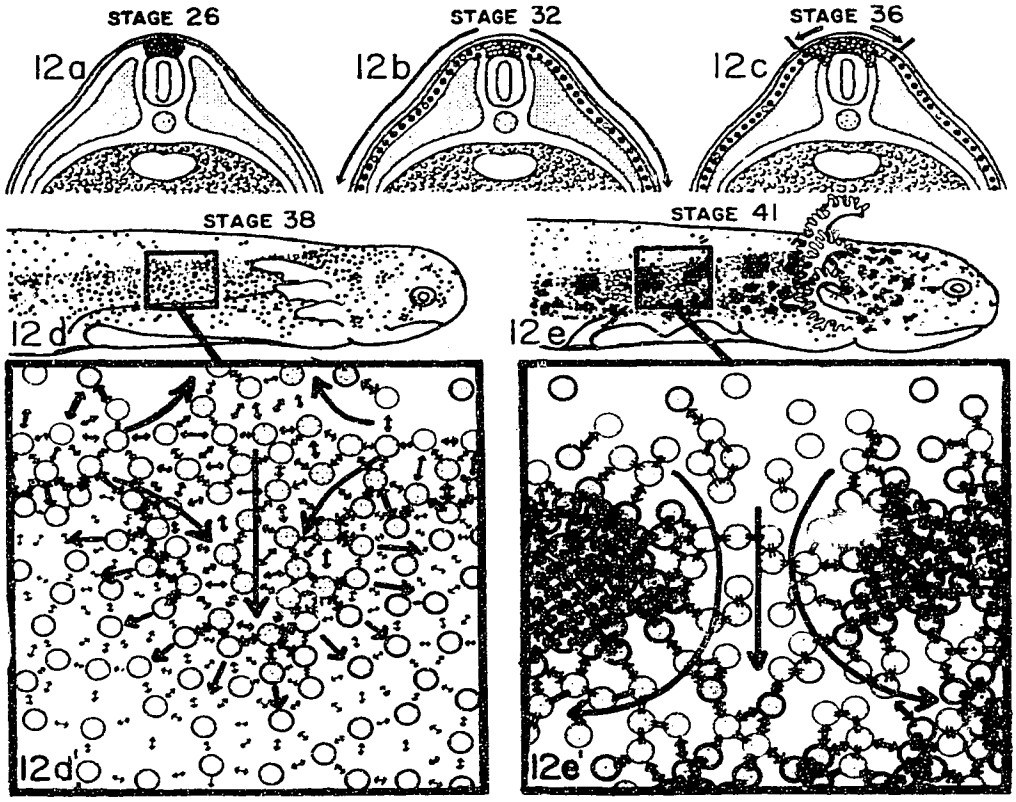


Figure 9. Diagramatic representation of chromatophore interactions during bar formation in the axolotl. Key: dark circles = melanophores; light circles = xanthophores; single arrows = direction of cell movement; double arrows = negative affinities (the darker the arrow, the stronger the repulsion); double bonds = positive affinities between homologous cells. From Lehman, H.E. and Youngs, M. Extrinsic and intrinsic factors influenceing amphibian pigment pattern formation. In: "Pigment Cell Biology" Gordon, M. (ed.) Academic Press Inc. Publishers, N.Y., 1959 p. 23

CHAPTER 2

METHODS AND MATERIALS

A. EXPERIMENTAL DESIGN

To examine the possibility that endogenous lectin is involved in pigment pattern development in the axolotl, four experiments were carried out in the following order:

First, lectin activity was measured over stages 20-24 (pre-migratory), stages 25-28 (early migratory) and stages 29-34 (late migratory) of development in dark (D/-) and white (d/d) axolotl embryos. The sugar specificity of the lectin was determined. These results are published in Martha et al. (1990).

Second, lectin activity was modulated *in vivo* by either exposing endogenous crude lectin extract or its inhibitor, 2-deoxyglucose (2-DG), directly to the neural crest of dark embryos just prior to NC migration. Melanophore morphologies, number and pattern arrangement were systematically compared between test and control larvae at stages 38, 40 and 42 of development.

Third, lectin activity was measured in whole 2-DG confronted and control dark embryos at various stages of development from stage 15/16 (early neural ridge development) until stage 42.

Finally, the lectin was tested for its specificity for

three glycosaminoglycans: chondroitin sulfate, hyaluronic acid and heparan sulfate.

B. QUANTITATION OF LECTIN ACTIVITY IN DARK AND WHITE AXOLOTL EMBRYOS

1. TISSUE PREPARATION

Dark (D/-) and white (d/d) axolotl embryos were obtained from the Indiana University Axolotl Colony. At the University of Kansas, zygotes were placed in 50% Holtfreter's saline (recipe in Appendix I) and were manually dejellied using watchmaker's forceps.

Embryos were raised at room temperature in 50% Holtfreter's saline to the appropriate stage of development according to the stage guides of Bordzilovskaya and Detlaff (1979) and Schreckenberg and Jacobson (1975). Using tungsten microneedles, embryos were manually stripped of ectoderm (except the head) and decapitated. Ectoderm and carcass tissues were collected from stage 20-24 (premigratory NC), stage 25-28 (early migratory NC) and stage 29-34 (late migratory NC) embryos. Each sample consisted of pooled ectoderm or carcass tissue from 6-20 embryos.

The numbers of tissues used in this experiment are as follows:

premigratory stages (St. 20-24):

dark ectoderm : 6 groups containing a total of 50 embryos
white ectoderm: 11 groups containing a total of 110 embryos
dark carcass : 4 groups containing a total of 50 embryos
white carcass : 9 groups containing a total of 79 embryos

early migratory stages (St. 25-28):

dark ectoderm : 6 groups containing a total of 54 embryos
white ectoderm : 10 groups containing a total of 99 embryos
dark carcass : 7 groups containing a total of 64 embryos
white carcass : 5 groups containing a total of 50 embryos

late migratory stages (St. 29-34):

dark ectoderm : 4 groups containing a total of 30 embryos
white ectoderm : 5 groups containing a total of 46 embryos
dark carcass : 4 groups containing a total of 40 embryos
white carcass : 4 groups containing a total of 36 embryos

2. ACETONE PROTEIN EXTRACTION METHOD

Because live axolotl zygotes were not available in Canada at the time the experiment was carried out, acetone protein extracts were made from fresh tissues in the laboratory of S.K. Frost-Mason at the University of Kansas using a procedure I modified from Roberson and Barondes (1982).

Tissues were hand-homogenized 2-3 minutes in 200 microlitres of ice-cold acetone, filtered onto Whatman # 1 filter paper and washed three times (equal v/v) with ice-cold acetone. The tissue residue on the filter paper was air-dried, collected and shipped to the University of Alberta. The tissues were then weighed and rehomogenized in Niu-Twitty saline (recipe in Appendix I). The resulting homogenate was centrifuged for five minutes at setting "5"

on an Eppendorf microcentrifuge. The supernatant was tested for lectin activity, sugar specificity and protein content. These procedures are desrcribed in the following text.

3. HEMAGGLUTINATION ASSAY

Lectin activity was determined by a microhemagglutination assay (Milos and Zalik, 1986). To each well of a V-bottom tissue culture plate (Flow Laboratories; 10 microlitres working volume) was added 4 microlitres of 0.15 M NaCl/0.005 M $NaKPO_4$ saline (pH 7.1). 4 microlitres of embryonic supernatant was added to the first well and serially diluted in the subsequent wells of the row. This was followed by the addition of 2 microlitres of 0.05 % bovine serum albumin (BSA) (dissolved in 0.15/0.005 saline) and 2 microlitres of 4 % trypsinized rabbit erythrocytes (method follows) to each well. The contents of each well were stirred thoroughly with a clean toothpick and left undisturbed for one hour. Hemagglutination activity is assessed in hemagglutination units (HU). 1 HU is the highest dilution at which agglutination still occurs (Cook et al., 1979). The hemagglutination activity is the inverse of this dilution expressed in HU. Agglutination is observed as a solid mat of erythrocytes covering the bottom of the well. Agglutination activity of the supernatants was standardized using a peanut lectin standard (100 micrograms

peanut agglutinin (Sigma Chemical Co.)/ 1 ml 0.15/0.005 saline) and 0.15/0.005 saline "blank" wells.

The 4 % trypsinized rabbit erythrocyte solution was obtained as follows:

i) 5 millilitres blood from healthy rabbits was bled into 5 millilitres Alsever's solution (consists of 20.5 grams dextrose, 8.0 grams sodium citrate dihydrate, 0.55 grams citric acid monohydrate, 4.2 grams sodium chloride into 1 litre double distilled water, sterile, pH = 6.1).

ii) Blood/Alsever's solution was centrifuged at setting "5" for 5 minutes in an IEC clinical centrifuge. Supernatant was discarded.

iii) 5X volume 0.15/0.005 saline was added and the above procedure was repeated three times.

iv) The pellet was resuspended in 25 X volume of 0.05 M NaCl/0.1 M $NaKPO_4$ saline (pH = 7.1) containing 1 milligrams/millilitre trypsin (Sigma Chemical Co.)

v) The solution was incubated for one hour in a 37°C shaking water bath.

vi) The solution was then centrifuged as described above; the supernatant was discarded and replaced with 5 X volume 0.75 M NaCl/0.075 M $NaKPO_4$ saline (pH = 7.1) three times.

vii) The pellet was resuspended in phenylmethylsulfonyl flouride (PMSF) buffer 10 X (v/v) (consists of 4.35 milligrams PMSF (Sigma Chemical Co.), 20 milligrams NaAzide into 100 milligrams 0.75/0.075 saline).

viii) This 10 % solution was diluted to 4 % in 0.15/0.005 saline to obtain a 4 % erythrocyte solution.

4. SUGAR SPECIFICITY ASSAY

Sugar specificity was tested by incorporating different sugars into the agglutination assay as follows:

2 microlitres of supernatant diluted to 8 HU of activity in 0.15/0.005 saline was added to each well of a V-bottom tissue culture plate containing 2 microlitres of a specific concentration of sugar (the addition of 2 microlitres sugar solution results in 4 microlitres of lectin activity to which the BSA and rabbit erythrocytes are added). Two microlitres of BSA solution and 2 microlitres trypsinized rabbit erythrocytes were added and mixed as usual. Sugars to be tested were diluted to 0.01 mM, 0.1 mM, 1 mM, 10 mM and 100 mM concentrations in 0.15/0.005 saline.

The sugars used were D(+) glucosamine, N-acetyl-D-glucosamine, D(+) mannose, D(+) glucose, methyl-alpha-D-mannopyranoside, alpha-D(+) melibiose, lactose, methyl-alpha-D-galactopyranoside, D(+) fucose, methyl-beta-D-galactopyranoside and beta-thiodigalactoside (all obtained from Sigma Chemical Co.).

5. BRADFORD PROTEIN ASSAY

Protein was quantified by the Bradford method (Bradford, 1975). A description of this method follows.

i) In test tubes, 2, 5, 10, 15, 20, 25, and 30 microlitre aliquots of supernatant were diluted in double-distilled water to produce a total volume of 100 microlitre. To each tube was added 3 millilitres Bradford reagent (consists of 100 milligrams Coomasssie Brilliant Blue G-250 (Sigma Chemical Co.) added to 50 millilitres 95 % ethanol and 100 millilitres 85 % phosphoric acid and diluted to 1 litre with double distilled water).

ii) Mixtures were immediately vortexed on high setting for appoximately one minute.

iii) The mixtures were then placed in quartz cuvets and absorbance content was read at 595 nanometre wavelength in a spectrophotometer.

6. STATISTICS

Statistics were carried out on the data just described (quantitation of lectin activity in dark and white axolotl embryos) using the 2-way analysis of variance (ANOVA; alpha = 0.05).

C. IN VIVO CONFRONTATION EXPERIMENTS # 1 AND # 2

1. TECHNICAL CONSIDERATIONS

In order to carry out the in vivo confrontation experiments, several technical problems were solved:

a) 2-deoxyglucose as a Non-metabolized Inhibitor of Endogenous lectin

Because all three of the sugar inhibitors found for the endogenous lectin are known to be metabolic intermediates, they would be expected to be internalized and metabolized by the embryos if placed in the saline in which they develop, and thus affect other biological functions in addition to inhibiting the endogenous lectin activity present. A structurally related non-metabolizable inhibitor had to be used.

Of several structurally related candidate molecules tested in a second sugar specificity assay, both mannan and 2-deoxyglucose (2-DG) strongly inhibited the endogenous lectin (10 uM concentration of the sugars completely inhibited 4 HU of lectin activity) (see Table 4). Because it is possible that mannan could be metabolized in the axolotl embryo by the enzyme mannosidase, if it exists extracellularly, 2-DG was selected as the in vivo inhibitor.

2-deoxyglucose is known to be taken up via facilitated transport as is glucose and is phosphorylated by cells but is not further metabolized (Ciaraldi et al., 1979; Foley et al., 1980; Kreutzberg et al., 1981; Ishibashi et al., 1982). With different mammalian systems, it has been used as a popular tracer molecule used to measure glucose uptake in various tissues under various experimental conditions. This is reviewed by Sokoloff (1981).

Presumably, 2-DG can inhibit an endogenous lectin specific for it but will not affect metabolism or other

cellular processes in the animal. Glucose-confronted embryos were included in confrontation experiment # 2 to rule out any nonspecific glucose-like effects as a result of the 2-DG treatment.

2-deoxyglucose, like glucose, has a small molecular weight so it should not be sterically hindered from being incorporated into the ECM surrounding the NC cells or into the cells themselves and should be available to bind to and inhibit the endogenous lectin present. A control experiment measuring lectin activity in 2-DG confronted and control embryos during development was conducted to help verify that 2-DG can inhibit lectin activity *in vivo*.

Because 2-DG may be removed from embryonic cells and their milieu through mechanisms similar to those used to excrete excess glucose, for example, through facilitated transport, it was important to maintain a continuous supply of 2-DG in the saline. 2-DG was present in the confrontation saline from stage 28 (the stage at which ectodermal stripping was done) to stage 43.

b) Availability of Live Zygotes

Since completing the initial series of experiments using axolotl tissue acetone protein extracts, I subsequently learned that the University of Ottawa Axolotl colony could send live zygotes directly to the University of

Alberta. This made possible a series of *in vivo* experiments to test whether endogenous lectin has any *in vivo* role.

Dark (D/-) axolotl zygotes were obtained from the Ottawa colony (courtesy of Dr. John Armstrong) and arrived at the University of Alberta at approximately stage 13/14. At this stage of development, neurulation has just begun and the yolk plug has almost disappeared within the blastopore.

c) "Stripping" Technique

A series of *in vivo* confrontation experiments were designed to test the effect of an exogenously added lectin sugar inhibitor on pigment pattern development in the dark (D/-) embryo. I decided to expose the lectin inhibitor directly to the neural crest so that 2-DG could bind directly to the endogenous lectin if it exists on the surface of the migrating NC cells or within the ECM surrounding the NC cells. Initially this was done by manually stripping away a section of ectoderm directly adjacent to the neural crest from premigratory embryos.

i) initial stripping technique (confrontation experiment # 1):

Embryos at stages 28-30 were manually dejellied and rinsed twice in sterile 100% Steinberg's saline (recipe in Appendix I). They were then immobilized in 2 % agar wells

containing 20 micrograms/millilitre Gentamycin (Sigma Chemical Co.). This was done by gently placing the embryo dorsal side up snugly into an agar depression cut out with tungsten microneedles and then immersing it in sterile 100% Steinberg's saline containing 1 % antibiotic/antimycotic (v/v; Sigma Chemical Co.) approximately 5 millimetres deep. Some embryos were immersed in sterile 100% Steinberg's saline containing 25 mM 2-DG (Sigma Chemical Co.). Because the central nervous system is not developed at this stage, no anaesthetic was required (by about stage 33, embryos begin to exhibit negative reflex responses to touch stimuli; no coordinated movement is observed until much later in development).

Under the light microscope, a rectangular section of ectoderm was peeled away from the underlying tissue as follows: Sharpened tungsten microneedles were used to make an initial cut through rostral ectoderm just to the left of the neural tube in a caudal direction until approximately half-way down the length of the embryo. The ectoderm was then lifted and peeled away from the embryo in a ventral direction until the section of ectoderm to be removed reached approximately halfway down the embryo dorsoventrally (Figure 10) (I observed that ectoderm could not be successfully removed directly over the neural crest because the two lateral aspects of the closing neural tube always separated from each other producing a wide dorsal gap in the

embryo that didn't heal post-operatively).

The embryo was then left undisturbed in the well for one hour and then transferred to 100 % Holtfreter's saline or 100 % Holtfreter's saline containing 25 mM 2-DG. By this time, the wound was partially covered with ectoderm tissue and healed over completely within another two to three hours. After three hours, operated embryos were transferred to 50 % Holtfreter's saline or 50 % Holtfreter's saline containing 25 mM 2-DG.

Some embryos from the same batch were dejellied but not stripped and raised in either 50 % Holtfreter's saline or 50 % Holtfreter's saline containing 25 mM 2-DG.

Stripped animals recovered well and developed normally except that the stripping procedure itself resulted in significant pigment pattern disruption that was visible by stage 38. Interestingly, I had employed a similar procedure on Xenopus laevis embryos and produced no detectable pigment pattern disruption.

ii) Improved stripping technique (confrontation experiment # 2):

In order to minimize the disruption of the subectodermal tissue (which was thought to be the cause of the pattern disruption seen above, I developed a less invasive stripping technique.

Under conditions similar to those used previously,

stage 28 D/- embryos were immobilized in sterile 100 % Steinberg's saline supplemented with 1 % antibiotic/antimycotic (v/v; Sigma Chemical Co.). A sharp tungsten microneedle was inserted just to the left of the neural tube bulge begining rostrally at the level of the pharyngeal arches and extending two thirds of the way caudally down the embryo to produce a single slit in the ectoderm. This procedure was rapid and easy to perform (Figure 11). Operated embryos were removed immediately to 50 % Holtfreter's saline-containing test solutions.

2. EXPERIMENTAL SET-UP OF CONFRONTATION EXPERIMENTS # 1 AND # 2

Refer to Table 1 for numbers of stripped and unstripped dark embryos which were placed in each experimental solution to develop to stage 43.

3. STATISTICS

Several different statistical tests were done on the data obtained from the <u>in vivo</u> confrontation experiments. A two-sided normal test was used to compare the improved stripping technique to the old technique based on left versus right stripped versus unstripped % top fit data and overall number of flank melanophore counts (the left flank of the embryo was stripped in all stripped embryos). To

statistically interpret the in vivo data from the second (improved) confrontation experiment, the Statistics Department was consulted and the following statistical analyses were carried out by a graduate student, Mr. Samarakoon in consultation with myself. Two statistical tests, the Tukey's Studentized Range (HSD) Test and the Duncan's Multiple Range Test (MEANCT), were carried out to compare the number of melanophores/bar, the total number of flank melanophores, % top fit, and % overall fit between control embryos and embryos confronted with one or more test molecules, at stages 38, 40, and 42 of development (these tests produced identical results). To analyze the effects of the various test molecules on melanophore morphologies, two statistical tests were carried out, a multivariant analysis of variance test and Tukey's studentized range test. See Appendix II for a detailed description of the statistical methods used in analyzing confrontation experiment # 2 data.

4. RAISING AXOLOTL EMBRYOS

Embryos were initially contained in 35 X 10 millimetre covered polystyrene culture dishes which were kept moist inside a large covered petri dish containing a few drops of water. Embryos at approximately stage 35 were transferred to 100 millilitre beakers. At stage 40, they were

transferred again to glass finger bowls. Control and test saline solutions were changed every second day. Animals were raised until stage 43/44. By stage 42/43, the mouth begins to break through the oral epidermis. Animals at this stage were fed live brine shrimp daily (1 gram of shrimp eggs [Murex Aqua Foods Inc.] were added to 1 litre sodium chloride [1.008 specific gravity]. The eggs hatch in 24-48 hours at approximately 30°C. New batches of brine shrimp were hatched every 2-3 days to maintain a constant supply). By stage 44, the diet was supplemented with tiny slivers of fresh beef liver.

5. CRUDE LECTIN EXTRACTION METHOD

275 stage 28/29 D/- axolotl embryos were collected, dejellied and hand-homogenized in minimum volume 100 % Steinberg's saline (without sodium azide or PMSF) for two minutes. The homogenate was stirred for two hours at 4°C and then centrifuged at 15,000 rpm for 20 minutes at 4°C. The resulting homogenate contained three fractions: a top lipid fraction which was discarded, followed by a liquid, then a cellular fraction. Not wanting to lose a potential cell-surface-bound lectin, I recombined the bottom two fractions and rehomogenized them briefly. An acetone protein extract was then made from the resulting homogenate by the same method modified from Roberson and Barondes

(1982) described previously, and redissolved in 50 % Holtfreter's saline. Lectin activity was tested using a microhemagglutination assay as decribed previously in "Materials and Methods". The appropriate amount of crude lectin solution was then added to some stripped and unstripped embryos to produce either 4 or 8 HU activity in the saline containing them.

6. OBSERVATIONS OF THE NORMAL PIGMENT PATTERN

Before confronting embryos, I raised a batch of 15 normal (D/-) axolotls to observe development of the pigment pattern. This type of study was essential as a preliminary step for the in vivo confrontation experiments because development of the normal pigment pattern must be documented for purposes of comparison with control animals. My observations were restricted to dissecting microscopic observations of live embryos. Once the embryos were motile (approximately stage 35), 2-3 millilitres of 2 % MS-222 (methane-tricane-sulphate; Sigma Chemical Co.) was added to the saline containing the embryos (approximately 20 millilitre volume) to temporarily anaesthetize them.

Notes describing the flank pattern of all animal flanks were collected from approximately stage 35 when the first melanophores become visible on the flank (this is approximately the end of NC migration (Lofberg et al.,

1989)) until stage 42/43.

It was difficult to follow melanophore migration on the flank directly because of the coverage of maternal pigment but no dorsoventral melanophore migration was observed after stage 38.

7. SYSTEMATIC ANALYSIS OF PIGMENT PATTERN DEVELOPMENT FOLLOWING CONFRONTATION

To measure the effects of the various treatments on pigment pattern development, photographs were taken of both flanks of all control and experimental animals at specific stages of development. Stages 38/39 and 42/43 were photographed in confrontation experiment # 1 and stages 38, 40 and 42 were photographed in confrontation experiment # 2. All photographs of each particular developmental stage were at the same magnification. Embryos from each experimental group were anaesthetized just prior to being photographed in 50 % Holtfreter's saline containing approximately 0.2 % MS-222.

A rectangular depression was cut out of a 2 % agar plate using microneedles. Each embryo was in turn placed right or left flank side up and gently nudged into the depression and immersed in fresh 50 % Holtfreter's saline (embryos remained immobilized for approximately 10 minutes). Embryos were placed under a microscope, illuminated with a fibre optic lamp and photographed. All confrontation

experiment # 1 photographs were of a 60 X magnification; confrontation experiment # 2 photographs were 120 X (stage 38), 96 X (stage 40) and 60 X (stage 42). The different magnifications described for experiment # 2 were used to compensate for the growth of the larvae so that similar regions of the flank were photographed at all three stages of development. All photographs were 4 X 6 inches in size.

To make reliable comparisons of the pigment patterns between test and control animals, an "ideal pigment pattern" diagram was composed of each photographed stage of development (Figure 12). These were composites based on the notes and diagrams collected from my observations of normal pigment pattern development described previously as well as the photographs of the control embryos obtained from these experiments. The diagrams were transferred to transparencies so they could be flipped over and placed on top of photographs of right or left flanks. They included fin/flank natural demarcations and the position of the external gills and cloaca so that they could be positioned correctly on the photographs.

Photographs were labelled on the reverse side and randomly mixed. Melanophores within all labelled regions as well as those that did not reside within any labelled area were then counted. One melanophore was designated as having an identifiable centre regardless of its size or morphology or whether it contacted other melanophores. A melanophore

was considered to exist within a particular labelled area based on where its centre lay and not on whether its processes extended into an adjacent area. When necessary, the number of melanophores per area was estimated as carefully as possible where individual melanophores were difficult to distinguish. This was done only on photographs where minimal estimation was required, i.e., where approximately one third (or less) melanophores were difficult to distinguish; estimation was done only when counting melanophores in the ventral stripe (the most densely populated region of the larva). This was particularly necessary on the stage 38 photographs where ventral maternal pigment remained. These counts were recorded and used to provide a measurement of the level of pattern organization as well as to compare the mumbers of melanophores within the various regions of igment pattern:

To measure the effects of the treatments on the number of melanophores within the flank pigment pattern, the following calculations were made:

i) the number of melanophores/bar producing an average of 3 bar measurements/photograph

ii) the number of melanophores/ventral stripe labelled area

iii) the total number of melanophores/flank labelled area (includes all labelled areas as well as melanophores residing between labelled areas

To measure melanophore pattern development, "top fit" and "overall fit" were measured:

$$\text{i) \% top fit} = \frac{\text{total number of melanophores within 3 labelled bars}}{\text{number of melanophores in 3 labelled bars plus number of melanophores between labelled bars}}$$

$$\text{ii) \% overall fit} = \frac{\text{number of melanophores in 3 labelled bars plus number of melanophores in ventral stripe}}{\text{number of melanophores/total labelled area}}$$

A second set of diagrams consisted of a labelled dorsoventral area on the mid-flank that included a portion of the dorsal bars and the ventral stripe (these diagrams correspond with those shown in Figure 12 except that the area of interest consists of all of the different labelled regions combined and, therefore, is a rough rectangle covering about two thirds of the flank). These diagrams were used to categorize the melanophores in a representative region of the flank into four arbitrary melanophore morphology types. These morphology categories were composed based on the range of control and test morphologies observed in the photographs. The percentage occurence of each of the four morphology types (see Figure 13) was calculated for each photographed flank. Only clearly distinguishable melanophores were considered and were placed in the category that best fit them. Photographs were observed randomly and those that were improperly focused were omitted.

D. LECTIN ACTIVITY IN 2-DG-CONFRONTED AND CONTROL D/- AXOLOTLS DURING DEVELOPMENT

D/- axolotl embryos from one batch were dejellied at stage 14/15 and placed in either 50 % Holtfreter's saline or 50 % Holtfreter's saline plus 25 mM 2-DG and allowed to develop normally. Equal numbers of embryos from each group were sacrificed at specific stages of development from stage 15 to stage 42 and hand-homogenized in equal amounts of 0.15/0.005 mM saline. Homogenates were immediately frozen at -70°C. Lectin activity was measured with a microhemagglutination assay as previously described in "Materials and Methods".

The number of embryos used are as follows:

stage 15:	10 total
stage 23:	10/group
stage 28/29:	10/group
stage 34/35:	5/group
stage 38:	5/group
stage 40:	4/group
stage 42:	3/group

E. LECTIN GLYCOSAMINOGLYCAN-SPECIFICITY ASSAY

Of five GAG's commonly found in the ECM, three were chosen to be tested because they are most likely to be found in embryonic undifferentiated ECM: chondroitin sulfate, heparan sulfate and hyaluronic acid. Because it is possible

that the endogenous lectin may interact with ECM components containing sugar residues specific for that lectin, these GAG molecules were of particular interest. Chondroitin sulfate contains N-acetyl-galactosamine as part of its repeating dissacharide structure. Both heparan sulfate and hyaluronic acid contain N-acetyl-D-glucosamine as a part of their repeating dissacharide units. Because the last two GAG's contain a sugar specific to the endogenous lectin, they were the most likely candidates to interact with the lectin. Purified powders of the three GAG's (Sigma Chemical Co.; courtesy of Dr. Graeme Hunter) were dissolved in 0.15/0.005 mM saline and diluted to 1 milligram/millilitre, 100 micrograms/millilitre, 10 micrograms/millilitre and 1 microgram/millilitre. Stage 28-30 D/- axolotl homogenate (in 0.15/0.005 saline) was added to the GAG solutions along with trypsinized rabbit erythrocytes and bovine serum albumin (BSA) and assayed using the method previously described in "Methods and Materials".

TABLE 1. Test solutions used in confrontation experiments # 1 and # 2. 8 dark axolotl embryos were either stripped according to the protocol described in the text of Chapter 2 or left unstripped and placed in one of the experimental solutions listed below (concentrations of the additives [or HU activity in the case of crude lectin extract] refer to the concentration to which the embryos were exposed). Numbers of embryos less than 8 reflect embryo death before stage 38 of development when the treatment effects were first assessed.

	# ANIMALS	
TREATMENT	STRIPPED	UNSTRIPPED
EXPERIMENT # 1:		
25 mM 2-DG	5	8
control	4	8
EXPERIMENT # 2:		
4 HU crude lectin extract	8	8
8 HU crude lectin extract	8	8
4 HU crude lectin extract plus 10 mM 2-DG	8	8
10 mM glucose	8	8
10 mM 2-DG	8	8
10 mM 2-DG plus 10 mM glucose	8	8
25 mM 2-DG	8	8
Control	8	8

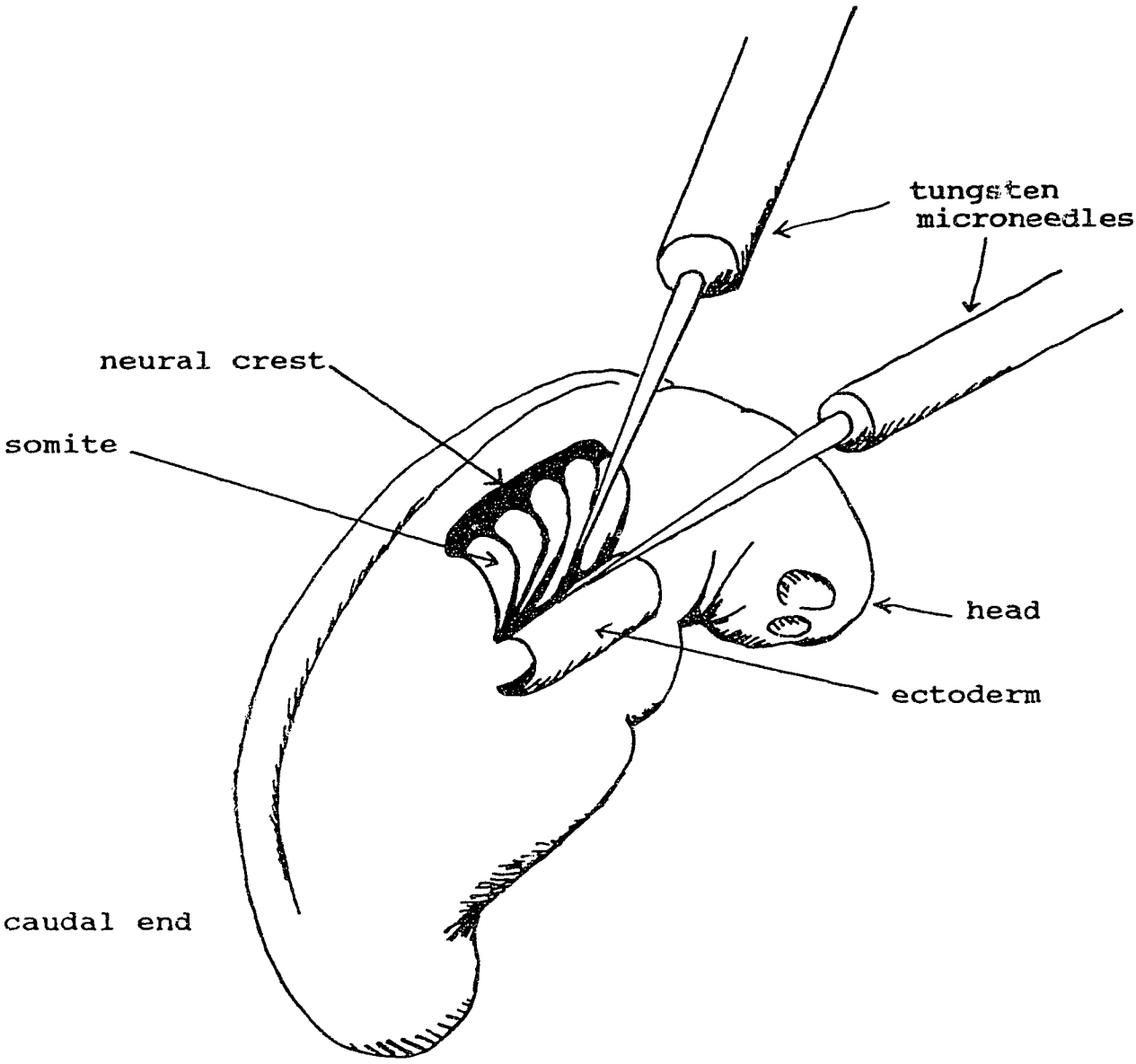


Figure 10. A shematic representation of the ectodermal stripping technique used in confrontation experiment # 1. A stage 29 axolotl embryo is shown.

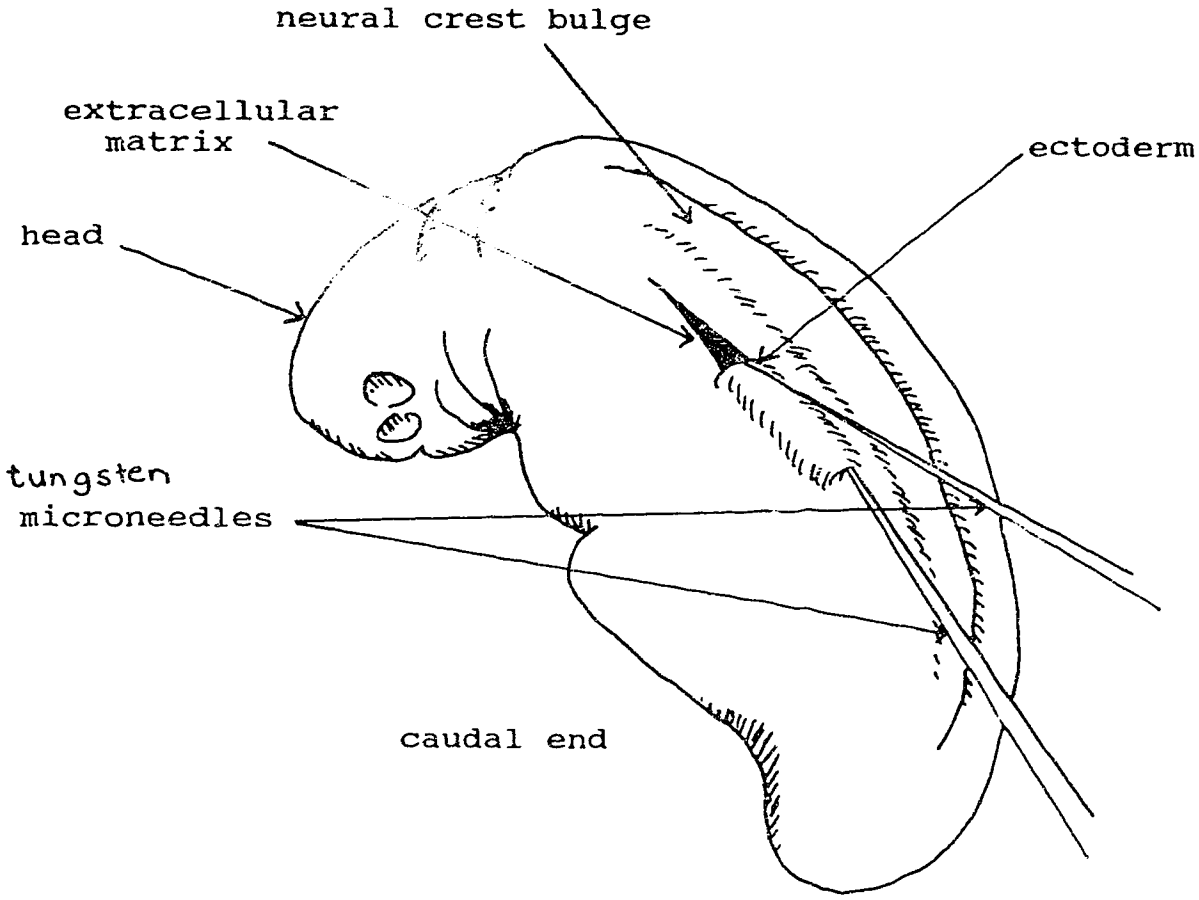


Figure 11. A shematic representation of the ectodermal stripping technique used in confrontation experiment # 2. A stage 29 embryo is shown.

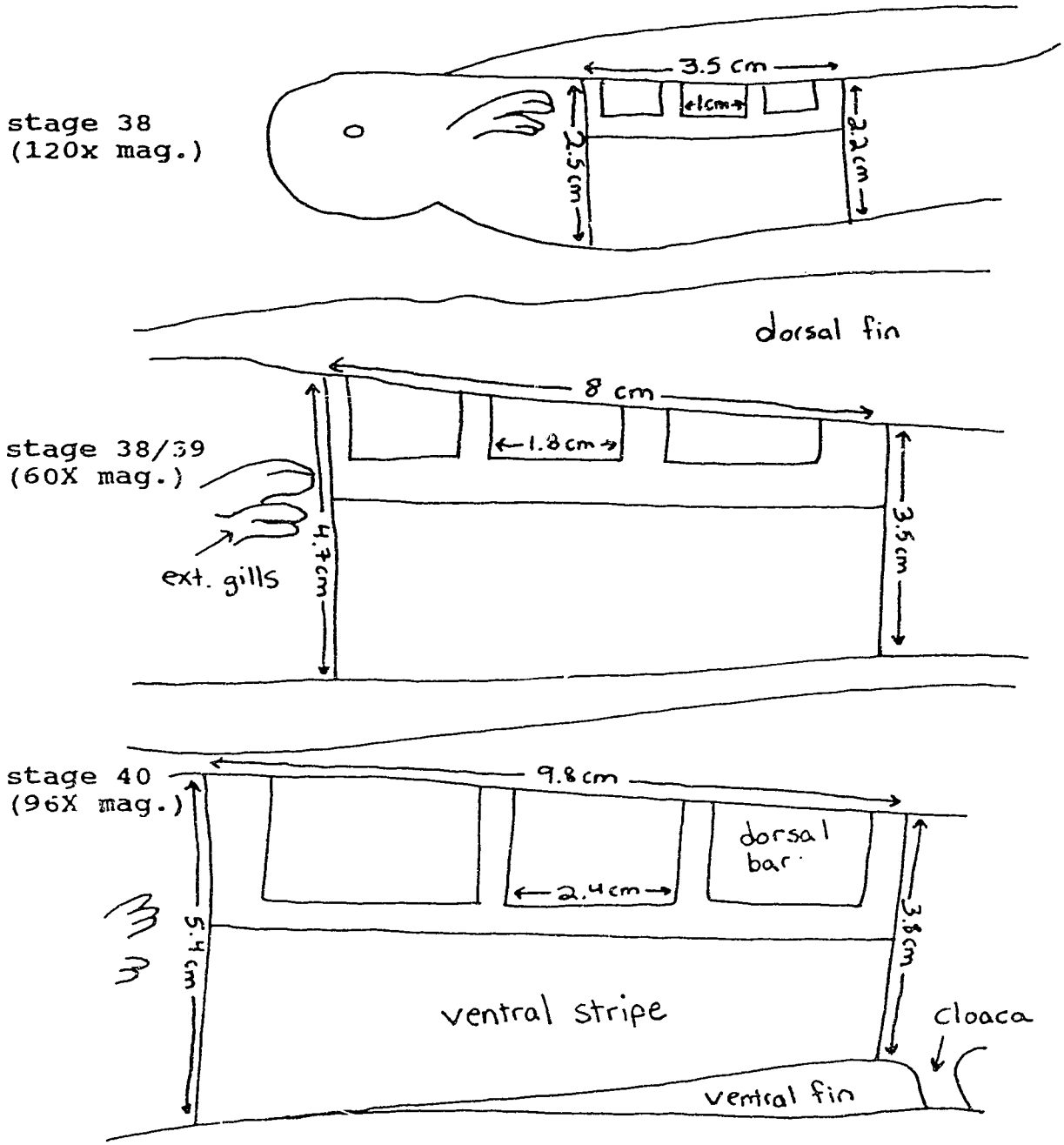


Figure 12. Tracings of "ideal flank pigment patterns" of each photographed developmental stage in control and experimental animals.

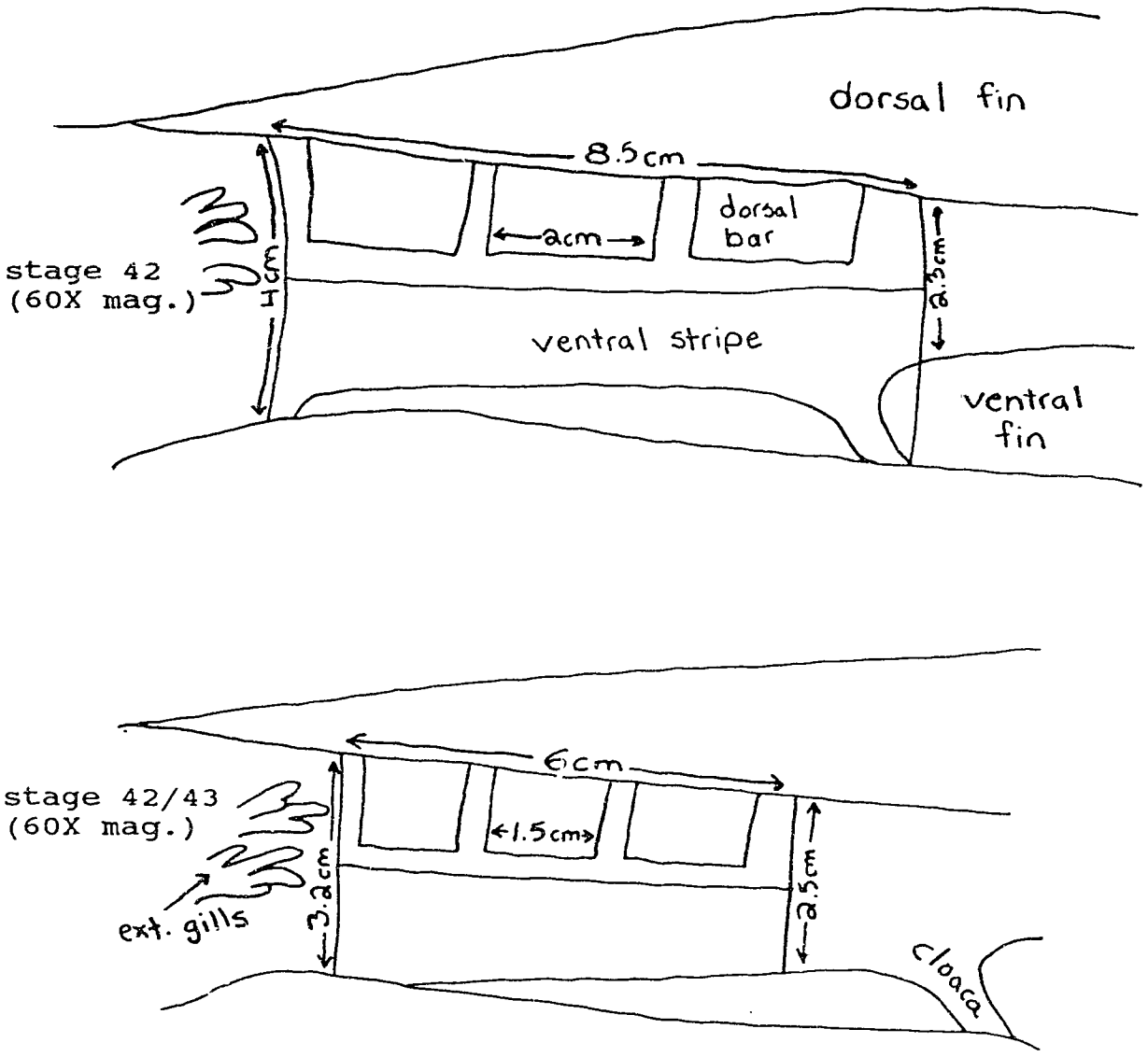


Figure 12 (continued). Tracings made of "ideal flank" patterns of each photographed developmental stage in control and experimental animals.

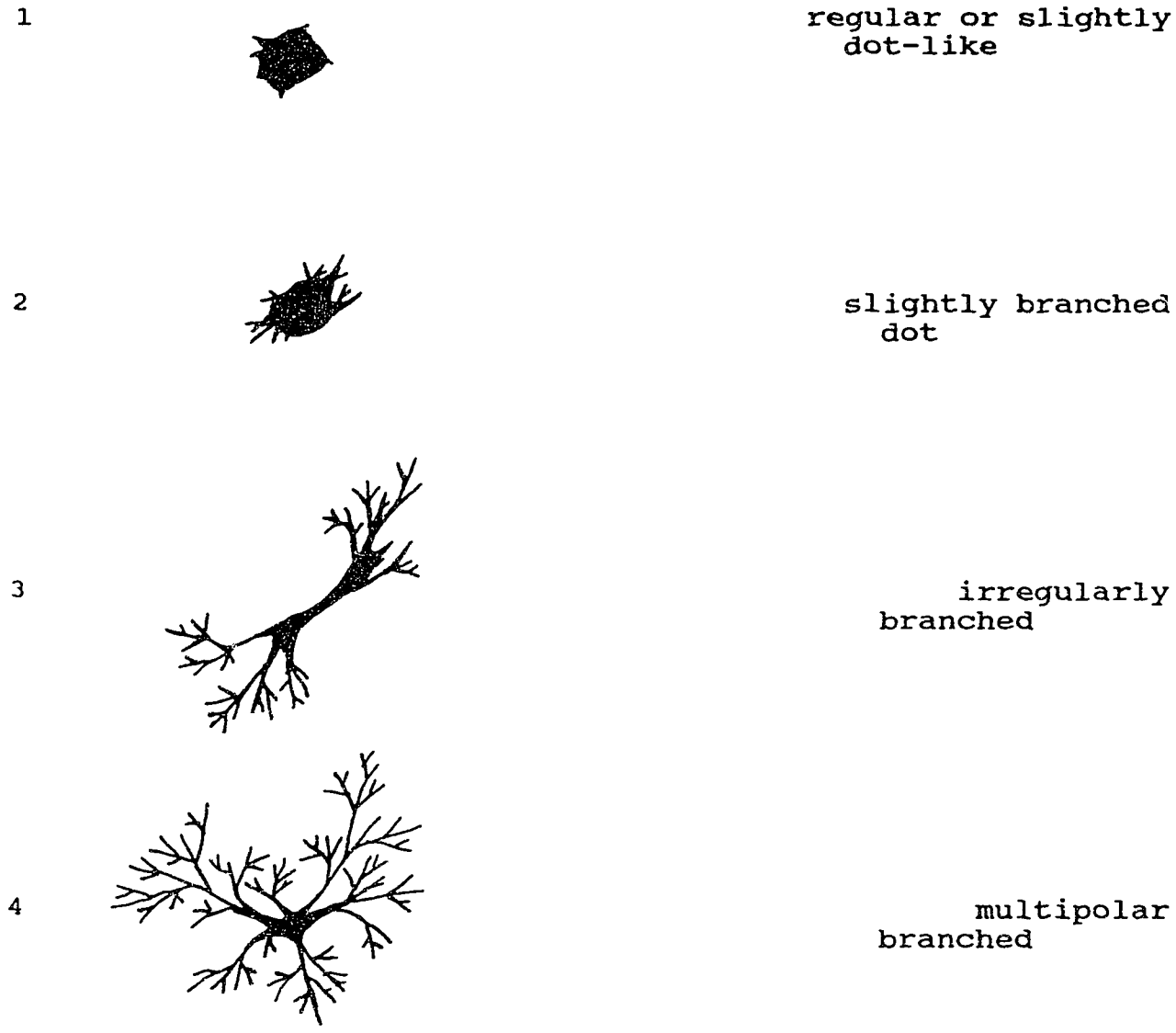


Figure 13. Drawings of four different representative melanophore morphologies most commonly observed in axolotl larvae from stage 38 of development to stage 43 of development (shown here larger than actual size).

CHAPTER 3

RESULTS

A. QUANTITATION OF LECTIN ACTIVITY IN DARK AND WHITE AXOLOTL EMBRYOS

1. LECTIN ACTIVITY IN DARK AND WHITE CARCASS AND ECTODERM

a) Lectin Activity in Dark and White Ectoderm

Before neural crest (NC) migration (stages 20-24), dark ectoderm lectin activity is 40.7 +/- 36 HU/embryo and white ectoderm lectin activity is 28.0 +/- 78 HU/embryo (Figure 14; Table 2). During early migration (stages 25-28), lectin activity increases significantly in dark ectoderm (from 40.7 to 1898 HU/embryo), a 47X increase on average, compared to white ectoderm (from 28.0 to 157 HU/embryo), an average increase of 8X. Late-migratory (stages 29-34) lectin activities are 45.4 +/- 29 HU/embryo for dark embryos and 168 +/- 13 HU/embryo for white embryos (Figure 14 and Table 2).

b) Lectin Activity in Dark and White Carcass

Lectin activity in carcass tissue is 96.1 +/- 72 HU/embryo in dark tissue and 62.2 +/- 42 HU/embryo in white tissue before NC migration (Figure 14 and Table 2). During

early migration, white carcass lectin activity increases significantly (from 62.2 to 635 HU/embryo), an average increase of 10X, whereas dark carcass lectin activity remains closer to pre-migration level (from 96.1 to 135 HU/embryo) (Figure 14 and Table 2).

2. PROTEIN CONTENT OF DARK AND WHITE CARCASS AND ECTODERM

a) Protein Content in Dark and White Ectoderm

Protein content increases significantly between early and late migration in dark ectoderm tissue only (from 2.0 to 9.1 micrograms/embryo). Premigratory protein content is higher in white ectoderm than in dark ectoderm (6.7 micrograms/embryo versus 2.0 micrograms/embryo, respectively) and decreases somewhat by late migratory stages (Figure 15 and Table 3).

b) Protein Content in Dark and White Carcass

The protein contents of dark and white carcass tissue are similar before migration (15.8 +/-2.3 micrograms/dark embryo and 15.9 +/-2.3 micrograms/white embryo). Protein content increases significantly during early migration in dark carcass tissue (from 15.8 to 28.1 micrograms/embryo), then decreases during late migration (to 17.3

micrograms/embryo). White carcass protein content doesn't change significantly during these stages (15.9, 16.9, and 15.9 micrograms/embryo, during pre-, early, and late migration, respectively) (Figure 15 and Table 3).

In conclusion, lectin activity increases in dark ectoderm during early NC migration, while white ectoderm lectin activity increases but not as much during early as well as late NC migratory stages.

With respect to protein content, dark ectoderm protein content increases during late NC migration, while white ectoderm protein content decreases during both early and late NC migratory stages.

In carcass tissues, dark protein content increases during early NC migration and then decreases while in white carcass tissue, protein content remains relatively constant.

3. SUGAR SPECIFICITY OF THE ENDOGENOUS LECTIN

The lectin activity of all early migratory stage (stages 25-28) samples was highly specific for D(+) glucosamine, D(+) mannose, and N-acetyl-D-glucosamine. Concentrations of less than 0.01 mM completely inhibited 4 HU of lectin activity (Table 4). Weaker inhibition was obtained with methyl-alpha-D-mannopyranoside, D-glucose, alpha-D-melibiose, and lactose (Table 4). D(+) fucose, methyl beta-D-galactopyranoside, and beta-thiodigalactose

produced no inhibition of lectin activity even at concentrations of 100 mM (Table 4).

B. *IN VIVO* EFFECTS OF 2-DEOXYGLUCOSE AND CRUDE LECTIN CONFRONTATION ON DARK AXOLOTL PIGMENT PATTERN DEVELOPMENT

1. OBSERVATIONS OF NORMAL PIGMENT PATTERN DEVELOPMENT

The dark axolotl pigment pattern just becomes visible at stage 37. Maternal ectodermal pigment (pigment derived from the unfertilized egg) is continuously sloughed off into the surrounding medium during the early stages of pattern formation, gradually revealing the underlying pigment cells.

The early flank pattern consists mostly of round, irregular dot-like melanophores which, with further differentiation, adopt morphologies ranging from dot-like to highly branched. Xanthophores are visible from about stage 38/39 as diffuse yellow regions between the dorsal melanophore bars and extending approximately half way into the dorsal fin.

The initial pigment cells that appear are quite well organized into the larval pattern consisting of alternating dorsal melanophore and xanthophore clusters, ie., the 'barred pattern'. Beneath the bars there is a more ventral pigment-free area and ventral to this, a broad melanophore stripe extending along the body. At stage 37, this overall

arrangement is ragged and irregular in appearance. The overall pigment pattern becomes sharper and more defined by stage 42. This can be seen by comparing Figure 25 a (A), b (A), and c (A), representative flank photographs of control animals at stages 38, 40, and 42.

a) The Number of Photographs Used to Analyze Data From Confrontation Experiments # 1 and # 2

Table 5 represents the number of photographs obtained from the two experiments conducted which were used to determine the effects of the various experimental treatments on flank melanophore pattern development between stages 38 and 42. Only well-focused and positioned photographs are counted in this list (and used for analysis).

2. EFFECTS OF THE STRIPPING PROCEDURES USED IN CONFRONTATION EXPERIMENTS # 1 AND # 2 ON PIGMENT PATTERN DEVELOPMENT

a) Effects of the Two Stripping Techniques on "Top Fit"

Using stripping technique # 1, percent "top fit" (that is, the number of melanophores that fit into the three "ideal bars" versus the number of melanophores that fit neither into the bars nor ventral stripe) was significantly decreased on the stripped (left) versus the unstripped (right) flanks of stripped control embryos confronted with

saline. Refer to Table 6a. The values were 53% on left (stripped) stage 38/39 and 53% on left (stripped) stage 42/43 flanks of stripped embryos compared to 63% on right (unstripped) stage 38/39 and 64% on right (unstripped) stage 42/43 flanks of stripped embryos, respectively (Figure 16 and Table 6a).

Similar results were obtained from animals incubated in 2-deoxyglucose (2-DG) (Table 6a). The values were 64% on left (stripped) stage 38/39 and 51% on left (stripped) stage 42/43 flanks of stripped embryos compared to 71% on right (unstripped) stage 38/39 and 65% on right (unstripped) stage 42/43 flanks of stripped embryos, respectively (Figure 16 and Table 6a).

Interestingly, 2-DG treatment correlated with significantly higher % top fit values in both stripped and unstripped embryos. Both left and right % top fit values were higher in stage 38/39 2-DG-confronted embryos compared to control embryos (stripped and unstripped) confronted with saline. Refer to Table 6a. The values were 71% (left flank) and 69% (right flank) in unstripped 2-DG-confronted embryos compared to 61% for both left and right unstripped control flanks. For stripped 2-DG-treated animals, the values were 64% (left stripped flank) and 71% (right stripped flank) compared to 53% and 63%, respectively, for (unstripped) right and (stripped) left flanks of stripped control animals raised in saline.

In unstripped animals, 2-DG also affected percent top fit significantly at stage 42/43, decreasing it to 64% (left flank) and 63% (right flank) compared to 70% (left flank) and 71% (right flank) on unstripped control flanks. In stripped animals at stage 42/43, control and test % top fit values were similar on both sides (Figure 16 and Table 6a).

The effect of 2-DG on percent top fit was explored further in the second experiment where the effects of the stripping procedure itself were eliminated.

The improved stripping procedure # 2 did not significantly affect percent top fit. Refer to Table 6b. For example, control left unstripped flank percent top fit values are 58, 65, and 67 % at stages 38, 40, and 42 wheras left stripped values are 59, 65, and 64 % at the same stages (Figure 17 and Table 6b). To compare this data from all of the experimental groups used in confrontation experiment # 2, refer to Table 15).

b) Effects of the Two Techniques on Overall Flank Melanophore Number.

The effects of stripping procedure # 1 on overall flank melanophore number were also tabulated. Refer to Table 7a. At stage 38/39, control unstripped right and left flanks carried 113 versus 114 melanophores, respectively. For stripped control animals, the right (unstripped) melanophore number was 115 versus 108 for the left (stripped) flank.

This latter value was a significant decrease (Figure 18 and Table 7a). The values obtained for 2-DG-treated animals were 128 and 120 melanophores on right and left flanks of unstripped animals and 121 and 116 on right (unstripped) and left (stripped) flanks, respectively, on stripped animals (Figure 18 and Table 7a).

However, by stage 42/43, both the right (unstripped) and left (stripped) flank melanophore numbers of stripped animals (experimental and control animals) differed significantly from those of unstripped test and control animals (Table 7a). The values were 127 and 113 on unstripped control left and right flanks versus 99 and 96 for (stripped) left and (unstripped) right flanks of stripped animals confronted with saline. For 2-DG-treated animals, the respective values are 113 (left) versus 108 (right) for unoperated animals and 82 (left) versus 93 (right) for the left (stripped) versus right (unstripped) sides (Figure 18 and Table 7a).

Interestingly, the above examination revealed that at stage 38, the 2-DG-treated (stripped and unstripped) animals carried significantly more melanophores than did the respective stripped and unstripped controls confronted with saline (Table 7a). For 2-DG-confronted animals, the values were 120 (unstripped left) and 128 (unstripped right) for unstripped animals versus 116 (stripped left) and 121 (unstripped right) for stripped animals. For control

animals, the values were 114 (unstripped left) and 113 (unstripped right) for unstripped animals versus 108 (stripped left) and 115 (unstripped right) for stripped animals.

However, by stage 42/43, the 2-DG-treated unstripped animals carried significantly less melanophores than did unstripped controls (Table 7a). For 2-DG-confronted animals, the values were 113 (unstripped left) and 108 (unstripped right) for unstripped animals versus unstripped control animals with values of 127 (unstripped left) and 113 (unstripped right) melanophores. Interestingly, there were no significant differences in the number of melanophores between stripped controls and 2-DG-confronted animals at stage 42/43 (Figure 18 and Table 7a).

The improved stripping procedure resulted in no significant changes in overall flank melanophore number at stage 38, 40, or 42 in control animals. Refer to Table 7b. For example, left unstripped numbers are 105, 119, and 116 at stages 38, 40, and 42 whereas left stripped numbers are 114, 110, and 117 at the same stages) (Figure 19 and Table 7b). To compare data from all experimental groups, refer to Table 16.

Only unstripped data from confrontation experiment # 1 was included in further analysis of the confrontation experiments. Stripped and unstripped data from confrontation experiment # 2 were combined.

3. THE EFFECTS OF THE TREATMENTS ON THE GENERAL DARK AXOLOTL PIGMENT PATTERN

As the pigment pattern emerges during development in control animals, the dorsal bars and ventral stripe become more well-defined on the flank. Individual melanophores also become larger and more darkly pigmented, giving them a more distinct appearance (refer to photograph A in Figure 25 a, b, and c).

Glucose-confronted animals appear similar to controls at stages 38 to 42 (photograph E in Figure 25 a, b, and c).

At all three stages, 10 mM 2-DG-confronted and 10 mM 2-DG/10 mM glucose-confronted animals appear very similar to each other (compare photograph C [2-DG] and B [2-DG plus glucose] in Figure 25 a, b, and c). Compared to controls at stage 38 (photograph A in Figure 25 a, b, and c), individual bars on these larvae are more clearly distinguishable and the ventral stripe appears wider and more populated. The melanophores also appear darker than those of controls, suggesting that they are synthesizing more melanin than control melanophores. By stage 40, the pigment pattern of these experimental larvae appears drastically different from the stage 38 pattern (compare photographs B and C in Figure 25 b [stage 38] to photographs B and C in Figure 25 a [stage 40]). The flanks are much more sparsely covered. This appears to reflect fewer as well as smaller melanophores. The melanophores on these flanks are much more contracted

and punctate in appearance than controls. They also appear less organized into dorsal bars and the ventral stripe. This could be due to random deaths of flank melanophores.

The effect of 25 mM 2-DG appears even more pronounced than that of 10 mM 2-DG (compare photograph D [25 mM 2-DG] in Figure 25 a, b, and c to photograph C [10 mM 2-DG] in Figure 25 a, b, and c). At stage 38, the dorsal bars look very distinct and well separated from each other, unlike control larvae (compare photograph D to A in Figure 25 a). However, it is not clear whether the confronted individuals contain more flank melanophores. At stage 40, this individual (photograph D; Figure 25 b) appears similar to the 10 mM 2-DG (photograph C; Figure 25 b) and 10 mM 2-DG/10 mM glucose-confronted animals (photograph B; Figure 25 b). At stage 42, however, these individuals (photograph D; Figure 25 c) appear even more sparsely covered than the 10 mM 2-DG animals (photograph C; Figure 25 c). Fewer melanophores and smaller, more punctate melanophores are visible, and the flank pattern is very disrupted (for example, it is very difficult to differentiate the dorsal bars on the individual shown in photograph D; Figure 25 c).

Based on the photographs, 2-DG appears to have a significant and dose-dependent effect on pigment pattern development in dark axolotls.

Crude lectin appears to have little effect on the pigment pattern. At stage 38, 40, and 42, 4 and 8 HU-

confronted larvae appear similar to controls (photographs G and H, respectively, compared to photograph A, in Figure 25 a, b, and c). The only difference observed between these animals and controls is that the horizontal pigment-free stripe that runs along the midline of the flank appears less organized than controls at stages 38 and 40 (compare photographs G and H to photograph A in Figure 25 a and b).

Interestingly, 4 HU crude lectin seems to moderate 10 mM 2-DG's effects on the pigment pattern when both are combined (compare photograph F to photograph C in Figure 25 a, b, and c). Animals at stage 38 look very similar to controls (compare photograph F to photograph A in Figure 25 a). Animals at stage 40 appear somewhat less organized but no other consistent differences between these animals and controls are observed at this stage (compare photograph F to photograph A in Figure 25 b). At stage 42, these animals look similar to controls (compare photograph F to photograph A in Figure 25 c).

In conclusion, based on observations of the overall appearance of the pigment pattern in experimental and control animals, 2-DG appears to have specific consistent and stage-dependent effects on pattern organization, melanophore number, and melanophore morphology (photographs C and D; Figure 25 a, b, and c). Crude lectin correlates with some pattern disorganization at stage 38 and 40 (photographs G and H; Figure 25 a and b) but otherwise no

observable effect on the pigment pattern is observed. When combined with 2-DG, however, crude lectin seems to reverse some of 2-DG's effects (photograph F compared to photograph C; Figure 25 a, b, and c). No pigment pattern effects are observed with glucose confrontation (compare photograph E to photograph A; Figure 25 a, b, and c).

4. THE EFFECTS OF THE TREATMENTS ON MELANOPHORE NUMBER

a) Number of Melanophores per Bar

In control larvae (confronted with saline), the number of melanophores (mels)/bar increased significantly from an average of 11 at stage 38 to 14 at stage 40 (Table 8). The number of mels/bar at stage 42 (13 mels/bar) is not significantly different from the stage 38 or 40 values (Figure 20 and Table 8).

Both 10 and 25 mM concentrations of 2-DG correlated with significantly more mels/bar (15 and 14 mels/bar, respectively) compared to controls (11 mels/bar) at stage 38 (Table 8). By stage 40, both 10 mM and 25 mM 2-DG treatments correlated with significant decreases in the number of mels/bar (from 15 to 11 and from 14 to 11, respectively). At stage 42, both concentrations correlated with counts of 10 mels/bar, not significantly different from a control value of 13 mels/bar (Table 8).

Neither 4 nor 8 HU of crude lectin extract correlated with significant differences in the number of mels/bar at stage 38 (10 and 12 mels/bar, respectively) compared to controls (11 mels/bar) (Figure 20 and Table 8). Similarly, at stage 40, 4 HU lectin-treated animals showed no significant difference from control values. 8 HU lectin treatment, in contrast, correlated with significantly fewer mels/bar than controls (11 compared to 14 mels/bar, respectively) (Table 8). By stage 42, both 8 and 4 HU lectin treatments correlated with no significant differences in the number of mels/bar compared to control values (Table 8).

10 mM glucose treatment did not correlate with any significant differences in the number of mels/bar at stages 38, 40, or 42 compared to controls at the same stages (10, 14, and 12 mels/bar compared to control values of 11, 14, and 13 mels/bar, respectively) (Table 8). When 10 mM glucose was combined with 10 mM 2-DG, the number of mels/bar recorded at all three stages of development resembled mel/bar counts of 10 mM 2-DG data at corresponding stages (14, 13, and 11 mels/bar compared to 10 mM 2-DG values of 15, 11, and 10 mels/bar, respectively) (Table 8).

The addition of 4 HU crude lectin plus 10 mM 2-DG to the saline correlated with no differences between the number of mels/bar in larvae treated with this combination and controls at stages 38, 40, and 42 (experimental values are

13, 16, and 13 mels/bar compared to control values of 11, 14, and 13 mels/bar) (Figure 20 and Table 8).

In summary, both 10 and 25 mM 2-DG concentrations correlated with significantly more mels/bar at stage 38, significantly fewer mels/bar at stage 40, and no significant difference from controls at stage 42. 8 HU crude lectin treatment had a significant effect on the number of mels/bar at stage 40 only, correlating with significantly fewer mels/bar compared to controls at this stage. The 4 HU concentration of crude lectin correlated with no significant differences in the number of mels/bar from controls, at any stage (Table 8; Figure 20).

b) Overall Number of Melanophores per Flank

The number of mels/flank remained constant throughout stages 38 to 42 in controls confronted with saline (Figure 21 and Table 9).

There was no significant difference in the number of mels/flank between 25 mM 2-DG-treated larvae and control larvae at any stage (Table 9). In contrast, 10 mM 2-DG treatment correlated with sigificantly more flank melanophores at all three stages compared to controls (127, 143, and 127 mels/flank compared to 112, 116, and 110 mels/flank, respectively) (Table 9). These results contrast with those of the mel/bar data (Table 8), characterized by

an initial increase in the number of melanophores/bar followed by a decrease in the number of melanophores/bar.

For 4 HU and 8 HU crude lectin-treated larvae, the mel/flank counts somewhat resemble the mel/bar count data (compare Table 9 to Table 8). 4 HU crude lectin treatment correlated with signifcantly more mels/flank at stages 40 and 42 (148, and 132 mels/flank compared to control values of 116 and 110 mels/flank, respectively) but not at stage 38 (Table 9). Eight HU crude lectin treatment correlated with significantly more mels/flank at stage 38 only (124 mels/flank compared to 112 for controls) (Table 9).

10 mM glucose did not correlate with a significant change in the number of mels/flank at stages 38 or 42 but correlated with significantly more mels/flank at stage 40, compared to controls (134 mels/flank compared to a control value of 116 mels/flank) (Table 9). The combination of glucose with 2-DG did not significantly influence the effect of 10 mM 2-DG on the number of mels/flank at any stage (the values were 137, 153, and 129 mels/flank, respectively, compared to 10 mM 2-DG values of 127, 143, and 127 mels/flank) (Table 9).

The combination of 4 HU crude lectin and 10 mM 2-DG correlated with changes in the number of mels/flank that are significantly different at all stages (123, 149, and 137 mels/flank) from controls confronted with saline (112, 116, and 110 mels/flank, respectively) (Figure 21 and Table 9).

In summary, 25 mM 2-DG treatment did not correlate with any significant changes in the number of melanophores in the flank at any stage. In contrast, the 10 mM 2-DG concentration correlated with significantly more mels/flank at stages 38, 40, and 42 (Table 9). These results may reflect an adverse effect of 2-DG on pigment pattern development at the higher 25 mM concentration. 2-DG might be slightly toxic to the melanophores.

The two crude lectin treatment concentrations, in general, correlated with opposite effects, with the 8 HU concentration correlating with an initial significant increase in mels/flank at stage 38, and the 4 HU concentration correlating with significant increases in mels/flank at stages 40 and 42 (Table 9). These results could reflect the greater capacity of the 8 HU concentration to increase the number of flank melanophores earlier during development than the 4 HU concentration.

5. THE EFFECTS OF TREATMENTS ON MELANOPHORE PATTERN ORGANIZATION

a) "Top Fit"

In controls, % top fit increased significantly from 59% at stage 38 to 67% at stages 40 and 42 (Figure 22 and Table 10).

Both 10 and 25 mM 2-DG treatments correlated with significantly greater % top fit at stage 38 (72% and 71%, respectively) but significantly lower % top fit at stage 40 (58% and 57%, respectively) and 42 (53% and 56%, respectively) compared to controls (Table 10).

In contrast, 4 HU crude lectin treatment correlated with significantly lower % top fit at stages 38 (52% compared to 59%) and 40 (60% compared to 67%) but no significant difference in % top fit from controls at stage 42 (62% compared to 67%) (Table 10). 8 HU crude lectin treatment correlated with a significantly reduced % top fit at stage 40 only (58% compared to 67%) (Table 10).

10 mM glucose treatment correlated with no significant differences in % top fit from controls at stage 38, 40, or 42 (58%, 66%, and 62%, respectively, compared to control values of 59%, 67%, and 67%, respectively) (Table 10). In combination with 10 mM 2-DG, the results were consistent with those of 10 mM 2-DG alone (66%, 56%, and 54%, respectively, compared to 10 mM 2-DG values of 72%, 57%, and 56%, respectively) (Table 10).

10 mM 2-DG and 4 HU in combination correlated with results that were intermediate in their effects compared to each treatment alone (Table 10). There was no significant difference in % top fit from controls at stage 38 (57% compared to 59% in controls). Like both 2-DG and crude lectin results, % top fit was significantly lower at stage

40 (62% compared to 67% in controls). At stage 42, % top fit was lower (58%) than that of control (67%) or 4 HU-treated larvae (62%) but greater than that of 10 mM 2-DG alone (53%) (Figure 22 and Table 10).

In summary, both 10 and 25 mM 2-DG concentrations correlated with significant increases in % top fit at stage 38 followed by significant decreases in % top fit at stages 40 and 42. These results may reflect stage-dependent effects of 2-DG on bar pattern organization. The crude lectin treatments correlated with initially lower % top fit at stages 38 and 40 for the 4 HU treatment and at stage 40 for the 8 HU treatment followed by no significant differences form control values at later stages. Crude lectin appears to have an opposite effect on % top fit compared to 2-DG, but these results are not very consistent between the two concentrations used.

b) "Overall Fit"

In controls, % overall fit remained constant at stages 38, 40, and 42 (Figure 23 and Table 11).

Both 10 mM and 25 mM 2-DG treatments correlated with significantly higher % overall fit values at stage 38 compared to controls (85% and 86%, respectively, compared to 82%) (Table 11). By stages 40 and 42, % overall fits were, in general, significantly lower than those of controls (78%

at stage 40 for the 25 mM 2-DG data compared to 82% for controls and 79% for both 10 and 25 mM 2-DG concentrations at stage 42, compared to 83% for controls) (Table 11). These changes resemble those of % top fit (compare Table 11 to Table 10).

At stage 38, % overall fit was significantly lower in 8 HU (75% compared to 79%) but not 4 HU (76% compared to 79%) crude lectin-treated larvae (Table 11). At stages 40 and 42, no significant differences were observed between either 4 (81% and 81%, compared to 82% and 83%, respectively) or 8 HU (79% and 85%, compared to 82% and 83%, respectively) treatments and controls (Table 11).

10 mM glucose treatment correlated with no significant changes in % overall fit at stage 38, 40 and 42 (79%, 82%, and 81%, respectively, compared to control values of 79%, 82%, and 83%) (Table 11). Like the % top fit results, when combined with 10 mM 2-DG, larvae resembled 2-DG-treated larvae (Table 11). Compared to controls confronted with saline, the glucose/2-DG combination correlated with significantly higher % overall fit at stage 38 (84% compared to 79%) compared to % overall fit at stages 40 and 42 (78% and 77% compared to 82% and 83%, respectively) whereas 10 mM 2-DG alone correlated with an initially greater % overall fit compared to controls at stage 38 (85% compared to 79%) and then a significantly lower % overall fit at stage 42 (79% compared to 83%).

When 4 HU lectin and 10 mM 2-DG were combined, no significant differences between these and control larvae were observed at stage 38 (76% compared to 79%) or 40 (82% compared to 82%) but % overall fit was significantly lower than those of controls at stage 42 (79% compared to 83%) (Figure 23 and Table 11).

In summary, 2-DG treatment correlated with greater overall pattern organization during early pigment pattern development (stage 38), but later (stage 40 and 42) pattern organization was lower than that of controls (Table 11).

In contrast, crude lectin treatment correlated with less pattern organization early in development (stage 38 and 40 % top fit was significantly lower with 4 HU treatment and stage 40 % top fit was significantly reduced with 8 HU crude lectin; stage 38 % overall fit was significanlty lower with 8 HU crude lectin treatment). However, by stage 42, there were no significant differences in % top fit or % overall fit in crude lectin treated animals compared to controls.

6. THE EFFECTS OF TREATMENTS ON MELANOPHORE MORPHOLOGY

At stage 38, the majority of melanophores observed in control flanks were of Type 2 morphology (slightly branched dot). At stages 40 and 42, however, four different morphology types were present and fairly equally represented (Figure 24 and Table 12; Note: Different concentrations

used for some of the confrontations are combined in Figure 24 and shown separately in Table 12). The more equal representation of morphologies at stage 42 appears to be due to a significant decrease in the representation of Type 2 morphologies and a significant increase in Type 1 (dot-like) and 4 (multipolar or highly branched) morphology representation.

10 and 25 mM 2-DG treatments initially correlated with significantly higher Type 1 representation at stage 38 compared to controls and a significantly higher Type 4 representation at stages 38 and 40, followed by a significantly lower Type 4 representation at stage 42 compared to controls (Table 12). Type 3 (irregularly branched) morphologies were significantly underepresented at all stages. In general, the morphology patterns of stage 38 2-DG-treated larvae resembled those of older stage 42 control larvae, wheras older stage 42 2-DG-treated larvae resembled younger stage 38 controls (Figure 24).

4 and 8 HU treatments also affected melanophore morphologies. Like controls, Type 2 morphologies were most common at stage 38, but 4 HU treatment correlated with significantly more Type 1 and 4 representation as well, compared to controls whereas 8 HU treatment correlated with significantly more Type 1 shapes than controls (Table 12). By stage 40, significantly fewer Type 3 morphologies were seen with both treatments compared to controls, but Type 2

morphologies continued to be highly represented. By stage 42, most melanophore morphologies were Type 2, closely resembling stage 38 controls (Figure 24).

Glucose treatment correlated with significantly more Type 1 and 4 shapes and less Type 3 morphologies, compared to controls. However, by stage 40, morphology representation was not significantly different from controls (Figure 24 and Table 12).

The combination of 10 mM 2-DG and 4 HU crude lectin correlated quite closely with the effects of 4 HU crude lectin alone (Table 12). Initially, Type 4 morphologies were highly represented at the expense of Type 1 and 3 representations, followed by a high proportion of Type 2 morphologies and a low proportion of Type 3 morphologies by stage 42 of development (Table 12).

For statistical treatment of this data, see Appendix II.

C. LECTIN ACTIVITY IN 2-DEOXYGLUCOSE-CONFRONTED AND CONTROL D/- AXOLOTLS DURING EMBRYONIC AND LARVAL DEVELOPMENT

In control dark embryos, lectin activity increased significantly at stage 28/29 (from 8 to 64 HU) and decreased to pre-migration level by stage 34/35 (8 HU) (Table 13). (These results correlate with those of dark embryos shown in Figure 14). A small transient increase in lectin activity also occured later at stage 40 (16 HU - weak) followed by a

significant reduction in lectin activity below pre-migratory level at stage 42 (2 - 4 HU) (Table 13). In contrast, lectin activity in 25 mM 2-DG-confronted dark embryos was lower than that of controls at stage 28/29 (16 HU compared to 64 HU), producing a much smaller transient increase in activity in stage 28/29 compared to controls (Table 13). Lectin activity of stage 34/35 and older animals was similar in treated and control animals (Table 13).

D. LECTIN-GLYCOSAMINOGLYCAN SPECIFICITY ASSAY

Chondroitin sulfate did not inhibit endogenous dark stage 28-30 lectin activity at concentrations of up to and including 1 milligram/millilitre (Table 14). At concentrations of 100 micrograms/millilitre and higher, heparan sulfate inhibited lectin activity. 1 microgram/millilitre hyaluronic acid solution completely inhibited lectin activity (Table 14).

TABLE 2. Lectin activity in dark (D/-) and white (d/d) axolotl carcass and ectoderm tissue measured as hemagglutination units (HU)/embryo. S.E.M. values are indicated as +/- values.

	HU/EMBRYO			
	DARK ECTODERM	WHITE ECTODERM	DARK CARCASS	WHITE CARCASS
PRE-MIGRATORY (ST. 20-24)	40.7 +/- 36	28.0 +/- 78	96.1 +/- 72	62.2 +/- 42
EARLY MIGRATORY (ST. 25-28)	1898 +/- 748	157 +/- 157	135 +/-442	635 +/-617
LATE MIGRATORY (ST. 29-34)	45.4 +/- 29	168 +/- 13	40.4 +/- 24	10.7 +/- 4

TABLE 3. Protein content in dark (D/-) and white (d/d) axolotl carcass and ectoderm tissue measured in micrograms/embryo. S.E.M. values are indicated as +/- values.

	MICROGRAMS PROTEIN/EMBRYO			
	DARK ECTODERM	WHITE ECTODERM	DARK CARCASS	WHITE CARCASS
PRE-MIGRATORY (ST. 20-24)	2.0 +/- 2.2	6.7 +/- 1.8	15.8 +/- 2.3	15.9 +/- 2.3
EARLY MIGRATORY (ST. 25-28)	2.1 +/- 1.1	5.3 +/- 1.1	28.1 +/- 6.2	16.9 +/- 5.3
LATE MIGRATORY (ST. 29-34)	9.1 +/- 1.6	4.0 +/- 1.4	17.3 +/- 3.7	15.9 +/- 3.3

TABLE 4.

A: Effect of different saccharides on the agglutination of rabbit erythrocytes by stage 25-28 dark (D/-) and white (d/d) axolotl ectoderm and carcass tissue.

SACCHARIDE	LOWEST CONCENTRATION OF SUGAR RESULTING IN INHIBITION OF LECTIN ACTIVITY
D(+) GLUCOSAMINE	<0.01 mM
N-ACETYL-D-GLUCOSAMINE	<0.01 mM
D(+) MANNOSE	<0.01 mM
D(+) GLUCOSE	1 mM
METHYL ALPHA-D-MANNOPYRANOSIDE	1 mM
ALPHA-D(+) MELIBIOSE	10 mM
LACTOSE	10 mM
METHYL ALPHA-D-GALACTOPYRANOSIDE	100 mM
D(+) FUCOSE	>100 mM
METHYL BETA-D-GALACTOPYRANOSIDE	>100 mM
BETA-THIODIGALACTOSIDE	>100 mM

B: The effect of different saccharides on the agglutination of rabbit erythrocytes by stage 25/26 dark (D/-) axolotl embryonic homogenate.

SACCHARIDE	LOWEST CONCENTRATION OF SUGAR RESULTING IN INHIBITON OF LECTIN ACTIVITY
D(+) MANNOSE	<0.01 mM
D(+) GLUCOSAMINE	<0.01 mM
N-ACETYL-D-GLUCOSAMINE	<0.01 mM
2-DEOXYGLUCOSE	0.01 mM
MANNAN	0.01 mM
MANNOPYRANOSYLPHENYLISOTHIOCYANATE	1 mM
N-ACETYL-D-GLUCOSAMINE PHENYLISOTHIOCYANATE	>10 mM
D(+) GLUCOSAMINE-2,6-DISULFATE	>10 mM
D(+) GLUCOSAMINE-2-SULFATE	>10 mM
N-ACETYL-D-GLUCOSAMINE-3-SULFATE	>10 mM

Table 5. The number of photographs used in the confrontation experiments (stripped and unstripped data are combined; unstripped control and unstripped 25 mM 2-DG data from confrontation experiment # 1 was combined with data from confrontation experiment # 2).*

TREATMENT	STAGE	NUMBER OF PHOTOS
CONTROL	38/39	28 (14 ANIMALS)
	40	12 (6 ANIMALS)
	42/43	23 (12 ANIMALS)
25 mM 2-DG	38/39	25 (13 ANIMALS)
	40	16 (8 ANIMALS)
	42/43	27 (14 ANIMALS)
10 mM 2-DG	38	5 (3 ANIMALS)
	40	16 (8 ANIMALS)
	42	12 (6 ANIMALS)
10 mM GLUCOSE	38	11 (6 ANIMALS)
	40	16 (8 ANIMALS)
	42	13 (7 ANIMALS)
10 mM GLUCOSE/ 10 mM 2-DG	38	10 (5 ANIMALS)
	40	15 (8 ANIMALS)
	42	15 (8 ANIMALS)
4 HU LECTIN	38	14 (7 ANIMALS)
	40	15 (8 ANIMALS)
	42	14 (7 ANIMALS)
4 HU LECTIN/ 10 mM 2-DG**	38	4 (2 ANIMALS)
	40	8 (4 ANIMALS)
	42	8 (4 ANIMALS)
8 HU LECTIN**	38	5 (3 ANIMALS)
	40	6 (3 ANIMALS)
	42	6 (3 ANIMALS)
TOTAL NUMBER OF PHOTOGRAPHS ANALYSED:		318 (164 ANIMALS)

* Representative photographs of each treatment at each photographed stage are presented in Figure 25 a, b, and c.

** All stripped embryos from these two experimental groups died before stage 38. Other than these deaths, there were few mortalities (numbers less than 16 (8 animals) reflect mortality and rejected photographs).

TABLE 6a. Left and right flank % top fit of stripped and unstripped control and 25 mM 2-deoxyglucose (2-DG)-confrontated dark (D/-) axolotl larvae at stages 38/39 and 42/43.

	UNSTRIPPED		STRIPPED	
	LEFT	RIGHT	LEFT*	RIGHT
CONTROL ST 38/39:	61+/-2.38	61 +/-2.21**	53+/-3.05s	63+/-3.53
CONTROL ST 42/43:	70+/-4.27	71 +/-2.28	53+/-3.05s	64+/-5.92s
25 mM 2-DG ST 38/39:	71+/-3.83s	69+/- 3.59s	64+/-8.02s	71+/-10.1s
25 mM 2-DG ST 42/43:	64+/-2.40s	63+/-4.73s	51+/-19.6s	65+/-16.9s

(* Only the left flank was stripped on stripped animals; the right flank of stripped animals was left undisturbed)

(** S.E.M. values as calculated using 95 % confidence intervals; differences (s) were significantly different from control right unstripped flank data as assessed using 95 % confidence intervals with alpha = 0.05)

TABLE 6b. Left and right flank % top fit of stage 38, 40, and 42 stripped and unstripped control dark (D/-) axolotl data*.

	UNSTRIPPED		STRIPPED	
STAGE	LEFT	RIGHT	LEFT**	RIGHT
38	58+/-6.72	59+/-9.35***	59+/-1.60	54+/-10.8
40	65+/-5.00	70+/-1.96	65+/-7.90	68+/-2.12
42	67+/-6.55	63+/-10.3	64+/-1.70	64+/-8.22

(* To compare control and experimental results, see Appendix III)

(** Only the left flank was stripped on stripped animals; the right flank was left undisturbed)

(*** S.E.M. values as calculated using 95 % confidence intervals; differences (s) were significantly different from control right unstripped flank data as assessed using 95 % confidence intervals with alpha = 0.05)

TABLE 7a. Overall number of melanophores on right and left stripped and unstripped flanks of control and 25 mM 2-deoxyglucose (2-DG)-confronted dark (D/-) axolotl larvae at stages 38/39 and 42/43.

	UNSTRIPPED		STRIPPED	
	RIGHT	LEFT	RIGHT	LEFT*
CONTROL ST 38/39:	113+/-7.85	114+/-4.57**	115+/-4.04	108+/-1.70s
CONTROL ST 42/43:	113+/-11.6s	127+/-12.1	96+/-6.96s	99+/-13.7s
25 mM 2-DG ST 38/39:	128+/-12.1s	120+/-18.1s	121+/-19.9s	116+/-14.2
25 mM 2-DG ST 42/43:	108+/-9.60s	113+/-5.28s	93+/-2.38s	82+/-11.9s

(* Only the left flank was stripped on stripped animals; the fight flank was left undisturbed)

(** S.E.M. values as calculated using 95% confidence intervals; differences (s) were significantly different from control right unstripped flank data as assessed using 95% confidence intervals with alpha = 0.05)

TABLE 7b. Overall number of melanophores on right and left stripped and unstripped stage 38, 40, and 42 flanks of control larvae*.

	UNSTRIPPED		STRIPPED	
STAGE	RIGHT	LEFT	RIGHT	LEFT**
38	112+/-7.69	105+/-9.05***	107+/-7.98	114+/-19.5
40	109+/-29.1	119+/-31.9	125+/-11.7	110+/-12.4
42	103+/-21.6	116+/-29.6	99+/-10.3	117+/-6.07

(* To compare control and experimental results, see Appendix IV)

(** Only the left flank was stripped on stripped animals; the right flank was left undisturbed)

(*** S.E.M. values as calculated using 95% confidence intervals; differences (s) were significantly different from control right unstripped flank data as assessed using 95% confidence intervals with alpha = 0.05)

TABLE 8. The number of melanophores/bar in control and experimental dark (D/-) axolotl larvae at stages 38, 40, and 42.

TREATMENT	STAGE		
	38	40	42
CONTROL	11+/-2.31	14+/-2.98	13+/-3.21
25 mM 2-DG	14+/-2.44s	11+/-1.10s	10+/-2.88
10 mM 2-DG	15+/-1.91s	11+/-2.12s	10+/-2.50
10 mM GLUCOSE	10+/-2.41	14+/-3.02	12+/-2.99
10 mM GLUCOSE/ 10 mM 2-DG	14+/-1.69s	13+/-2.48	11+/-2.28
4 HU LECTIN	10+/-1.70	16+/-4.08	12+/-3.55
8 HU LECTIN	12+/-3.36	11+/-3.09s	10+/-2.54
4 HU LECTIN/ 10 mM 2-DG	13+/-2.71	16+/-3.02	13+/-2.72

(s = significantly different from control value at the same stage of development as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test)

TABLE 9. The number of melanophores/flank in control and experimental dark (D/-) axolotl larvae at stages 38, 40, and 42.

TREATMENT	STAGE		
	38	40	42
CONTROL	112+/-9.59	116+/-13.57	110+/-16.99
25 mM 2-DG	121+/-14.1	110+/-16.5	96+/-14.4
10 mM 2-DG	127+/-3.30s	143+/-8.85s	127+/-12.0s
10 mM GLUCOSE	110+/-14.6	134+/-13.4s	115+/-14.2
10 mM GLUCOSE/ 10 mM 2-DG	137+/-7.20s	153+/-16.9s	129+/-14.4s
4 HU LECTIN	113+/-7.18	148+/-22.8s	132+/-24.9s
8 HU LECTIN	124+/-13.0s	121+/-19.1	111+/-15.2
4 HU LECTIN/ 10 mM 2-DG	123+/-9.04s	149+/-17.0s	137+/-14.0s

(s = significantly different from control value at the same stage of development as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test)

TABLE 10. % top fit in control and experimental dark (D/-) axolotl larvae at stages 38, 40, and 42.

TREATMENT	STAGE		
	38	40	42
CONTROL	59+/-5.58	67+/-4.95	67+/-6.84
25 mM 2-DG	71+/-3.37s	57+/-7.90s	56+/-7.37s
10 mM 2-DG	72+/-4.79s	58+/-3.91s	53+/-9.36s
10 mM GLUCOSE	58+/-4.40	66+/-6.95	62+/-7.33
10 mM GLUCOSE/ 10 mM 2-DG	66+/-6.51s	56+/-4.50s	54+/-6.96s
4 HU LECTIN	52+/-4.96s	60+/-4.32s	62+/-7.84
8 HU LECTIN	55+/-6.72	58+/-7.77s	64+/-12.6
4 HU LECTIN/ 10 mM 2-DG	57+/-4.57	62+/-6.91s	58+/-6.92s

(s = significantly different from control value at the same stage of development as assessed by Duncan's Multple Range Test and Tukey's Studentized Range (HSD) Test)

TABLE 11. % overall fit in control and experimental dark (D/-) axolotl larvae at stages 38, 40, and 42.

TREATMENT	STAGE		
	38	40	42
CONTROL	79+/-3.54	82+/-3.06	83+/-3.53
25 mM 2-DG	86+/-3.54s	78+/-4.30s	79+/-4.04s
10 mM 2-DG	85+/-2.63s	80+/-2.77	79+/-5.41s
10 mM GLUCOSE	79+/-3.00	82+/-4.46	81+/-3.67
10 mM GLUCOSE/ 10 mM 2-DG	84+/-3.50s	78+/-4.29s	77+/-5.39s
4 HU LECTIN	76+/-2.06	81+/-4.86	81+/-2.68
8 HU LECTIN	75+/-4.41s	79+/-5.00	85+/-5.47
4 HU LECTIN/ 10 mM 2-DG	76+/-1.93	82+/-4.48	79+/-2.43s

(s = significantly different from control value at the same stage of developemnt as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test)

TABLE 12. % melanophores of Type 1, 2, 3, and 4 morphologies in control and experimental dark (D/-) axolotl flanks at stages 38, 40, and 42.

		% MORPHOLOGIES			
TREATMENT	STAGE	1	2	3	4
CONTROL	38	8	66	21	5
	40	X{ 14	X{ 40	33	X{ 14
	42	25	25	20	32
25 mM 2-DG	38	20x	42	5x	33x
	40	19	S{ 35	S{ 5x	S{ 42x
	42	47x	41x	5x	8x
10 mM 2-DG	38	19x	37x	4x	40x
	40	14	S{ 44	S{ 6x	S{ 37x
	42	30	56	7x	8x
10 mM GLUCOSE	38	20x	42	5x	33x
	40	S{ 16	S{ 49	S{ 14	S{ 23x
	42	18	37	20	25
10 mM GLUCOSE/	38	15x	43	13	28x
10 mM 2-DG	40	14	S{ 47	S{ 7x	S{ 32
	42	35	48x	3x	9x
4 HU LECTIN	38	15x	43	13	28x
	40	S{ 9	S{ 57	S{ 8x	S{ 28x
	42	25	61x	1x	13x
8 HU LECTIN	38	22x	64	4x	11
	40	S{ 16	62	S{ 6x	S{ 17
	42	17	70x	9x	5x
4 HU LECTIN/	38	3x	48	6x	43x
10 mM 2-DG	40	7	S{ 44	11x	S{ 41x
	42	22	42x	8x	30

(as assessed by Multivariant Analysis of Variance Test and Tukey's Studentized Range Test; please see Appendix II for a more detailed expanation of these statistical tests)
(x = significant change in % value mean from control value mean at the same stage of development; **X** = change in control value means over the three stages of development is significant; **S** = change in value means over three stages of development is siginificantly different from changes in control mean values in the same morphology category. For example, the % representation of Type 1 morphologies in controls changes significantly between stage 38 and 42, denoted by **X**; this increase is not expressed in 8 HU lectin-treated animals but is expressed in (continued)

(Table 12 continued) 4 HU lectin-treated animals, denoted by **S**; there is significanlty more Type 1 melanophore representation at stages 38 and 42 in 25 mM 2-DG-treated animals compared to control Type 1 representation at these two stages, denoted by x's).

TABLE 13. Lectin activity, expressed in hemagglutination (HU) units, in control and 25 mM 2-deoxyglucose (2-DG)-confronted dark (D/-) axolotl embryos and larvae.

STAGE	# EMBRYOS	TREATMENT	LECTIN ACTIVITY
15	10	N/A	8 HU
23	10	CONTROL	8 HU
23	10	25 mM 2-DG	4 - 8 HU
28/29	10	CONTROL	64 HU*
28/29	10	25 mM 2-DG	16 HU
34/35	5	CONTROL	8 HU
	5	25 mM 2-DG	8 - 16 HU
38	5	CONTROL	8 HU
	5	25 mM 2-DG	8 HU
40	4	CONTROL	(weak) 16 HU
	4	25 mM 2-DG	8 - 16 HU
42	3	CONTROL	2 - 4 HU*
	3	25 mM 2-DG	2 - 4 HU*

(* significantly different from stage 23 control lectin activity, as assessed by ANOVA; $p < 0.05$)

TABLE 14. Effect of different glycosaminoglycans (GAG's) on the agglutination of rabbit erythrocytes by dark (D/-) stage 28-30 axolotl embryonic homogenate.

GAG	LOWEST CONCENTRATION OF GAG RESULTING IN INHIBITION OF LECTIN ACTIVITY
HYALURONIC ACID	<1 micrograms/millilitre
HEPARAN SULFATE	100 micrograms/millilitre
CHONDROITIN SULFATE	>1000 micrograms/millilitre

Table 15. Confrontation Experiment # 2: Left and right flank % top fit of stage 38, 40, and 42 stripped and unstripped control and experimental dark (D/-) axolotl larvae.

TREATMENT	UNSTRIPPED		STRIPPED	
STAGE	LEFT	RIGHT	LEFT	RIGHT
38				
CONTROL	58	59	59	54
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	69	63	68	64
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	57	57
25 mM 2-DEOXYGLUCOSE	70	71	70	68
10 mM 2-DEOXYGLUCOSE	75	NA	71	68
10 mM GLUCOSE	59	57	59	55
4 HU CRUDE LECTIN	52	54	49	53
8 HU CRUDE LECTIN	NA	NA	56	53
40				
CONTROL	65	70	65	68
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	54	54	56	NA
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	66	62
25 mM 2-DEOXYGLUCOSE	55	58	59	54
10 mM 2-DEOXYGLUCOSE	56	65	55	65
10 mM GLUCOSE	62	66	67	62
4 HU CRUDE LECTIN	58	58	59	62
8 HU CRUDE LECTIN	NA	NA	59	62

(Table 15 cont.)

42

CONTROL	67	63	64	64
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	50	48	60	56
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	56	59
25 mM 2-DEOXYGLUCOSE	59	60	57	59
10 mM 2-DEOXYGLUCOSE	51	63	52	53
10 mM GLUCOSE	62	60	67	64
4 HU CRUDE LECTIN	57	57	64	60
8 HU CRUDE LECTIN	NA	NA	68	58

Table 16. Confrontation Experiment # 2: Overall number of melanophores on right and left stripped and unstripped stage 38, 40, and 42 flanks of control and experimental dark (D/-) larvae.

TREATMENT	UNSTRIPPED		STRIPPED	
STAGE	RIGHT	LEFT	RIGHT	LEFT
38				
CONTROL	112	105	107	114
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	132	127	107	114
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	122	124
25 mM 2-DEOXYGLUCOSE	117	118	121	126
10 mM 2-DEOXYGLUCOSE	127	NA	130	125
10 mM GLUCOSE	118	103	107	112
4 HU CRUDE LECTIN	112	115	111	112
8 HU CRUDE LECTIN	NA	NA	124	125
40				
CONTROL	109	119	125	110
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	148	190	NA	NA
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	150	149
25 mM 2-DEOXYGLUCOSE	112	122	107	101
10 mM 2-DEOXYGLUCOSE	140	140	156	144
10 mM GLUCOSE	140	139	149	144
4 HU CRUDE LECTIN	152	143	151	148
8 HU CRUDE LECTIN	NA	NA	121	120

(Table 16 cont.)

42

CONTROL	103	116	99	117
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	127	125	124	140
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	140	133
25 mM 2-DEOXYGLUCOSE	109	102	90	89
10 mM 2-DEOXYGLUCOSE	139	126	122	120
4 HU CRUDE LECTIN	122	121	145	139
8 HU CRUDE LECTIN	NA	NA	118	104

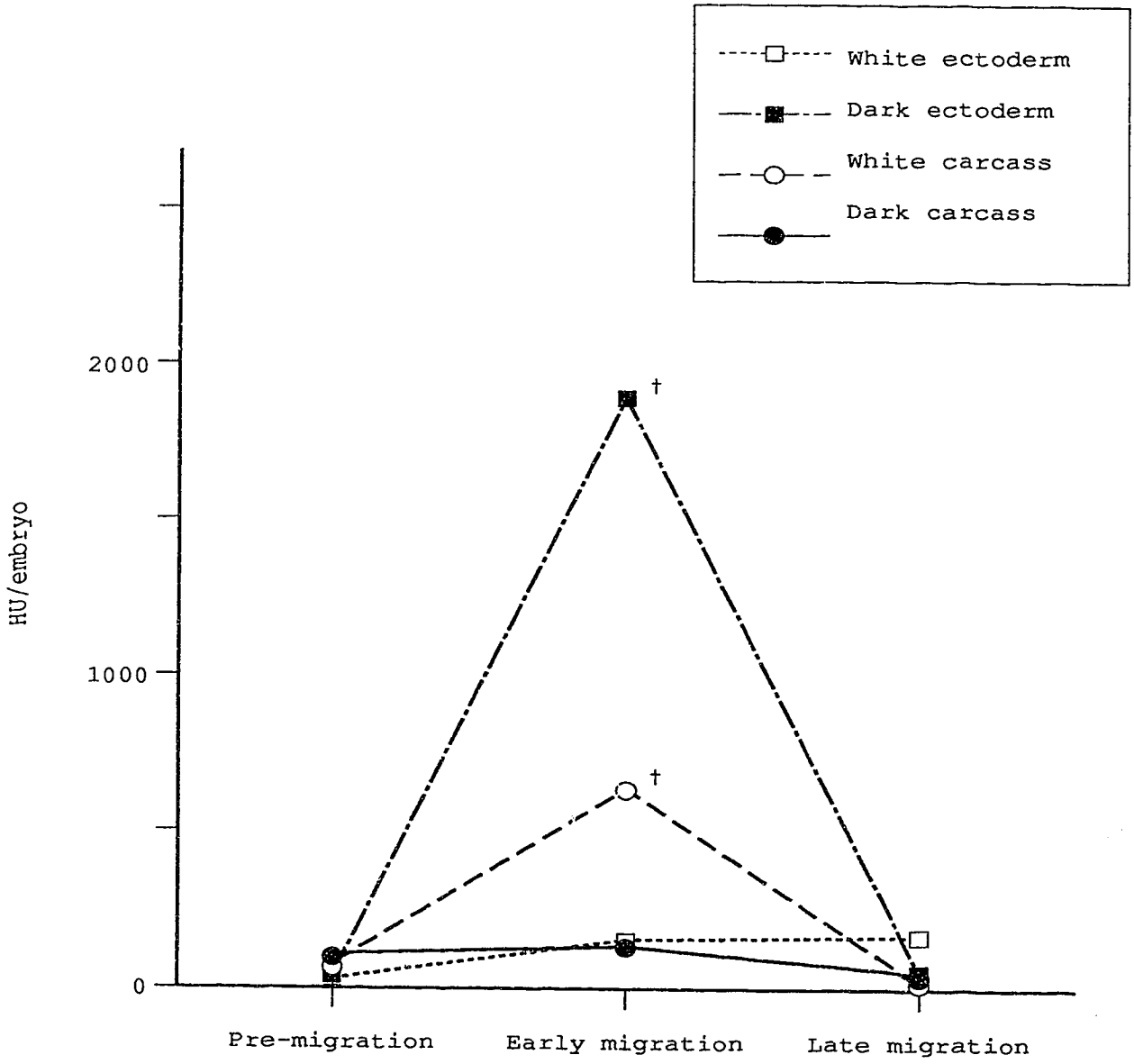


Figure 14. Lectin activity in dark (D/-) and white (d/d) axolotl ectoderm and carcass tissues, expressed in hemagglutination units (HU)/embryo, during pre-migratory, early migratory, and late migratory neural crest stages.

† See Table 2 for actual values and S.E.M.
Significant increase as assessed by ANOVA, $p \leq 0.05$.

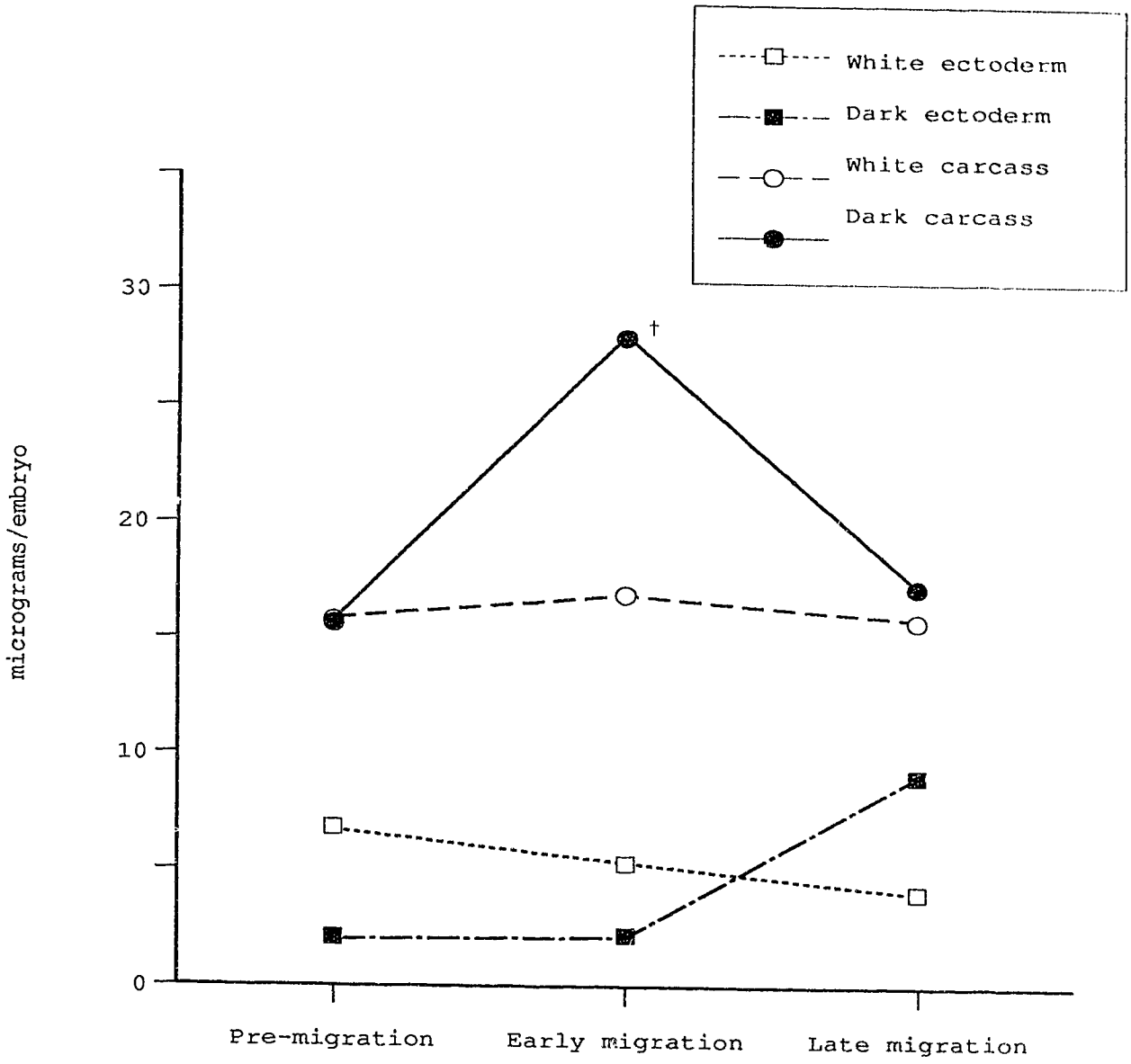


Figure 15. Protein content (micrograms/embryo) in dark (D/-) and white (d/d) axolotal ectoderm and carcass tissues during premigratory, early migratory, and late migratory stages of neural crest development.

† See Table 3 for actual values and S.E.M.
Significant increase as assessed by ANOVA, $p \leq 0.05$.

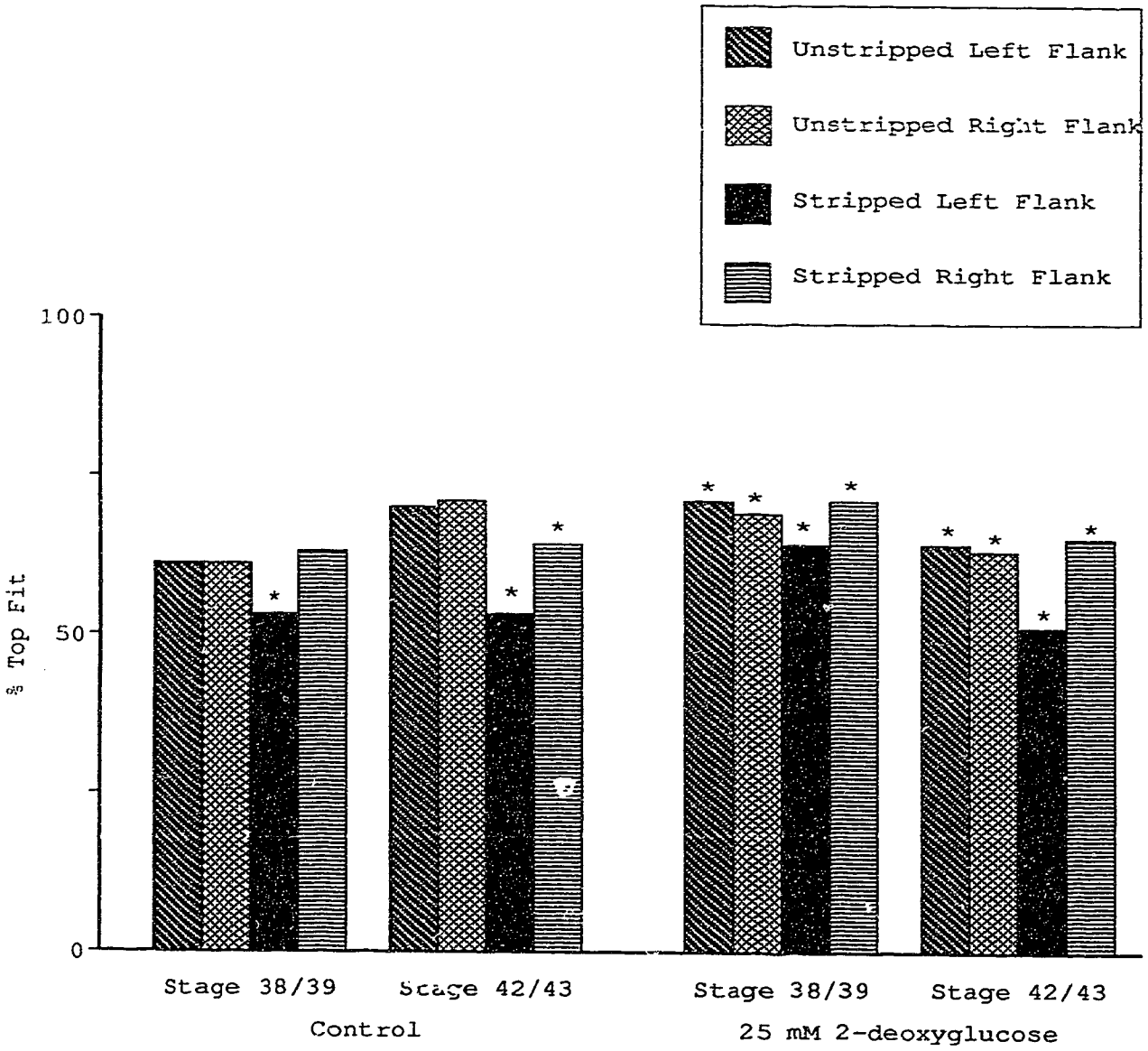


Figure 16. Experiment #1. Left and right percent top fit of stripped and unstripped control and 25 mM 2-deoxyglucose-confronted dark (D/-) axolotl larvae at stages 38/39 and 42/43.

* Significantly different from control right unstripped flank data as assessed by a Two-sided Normal Test.

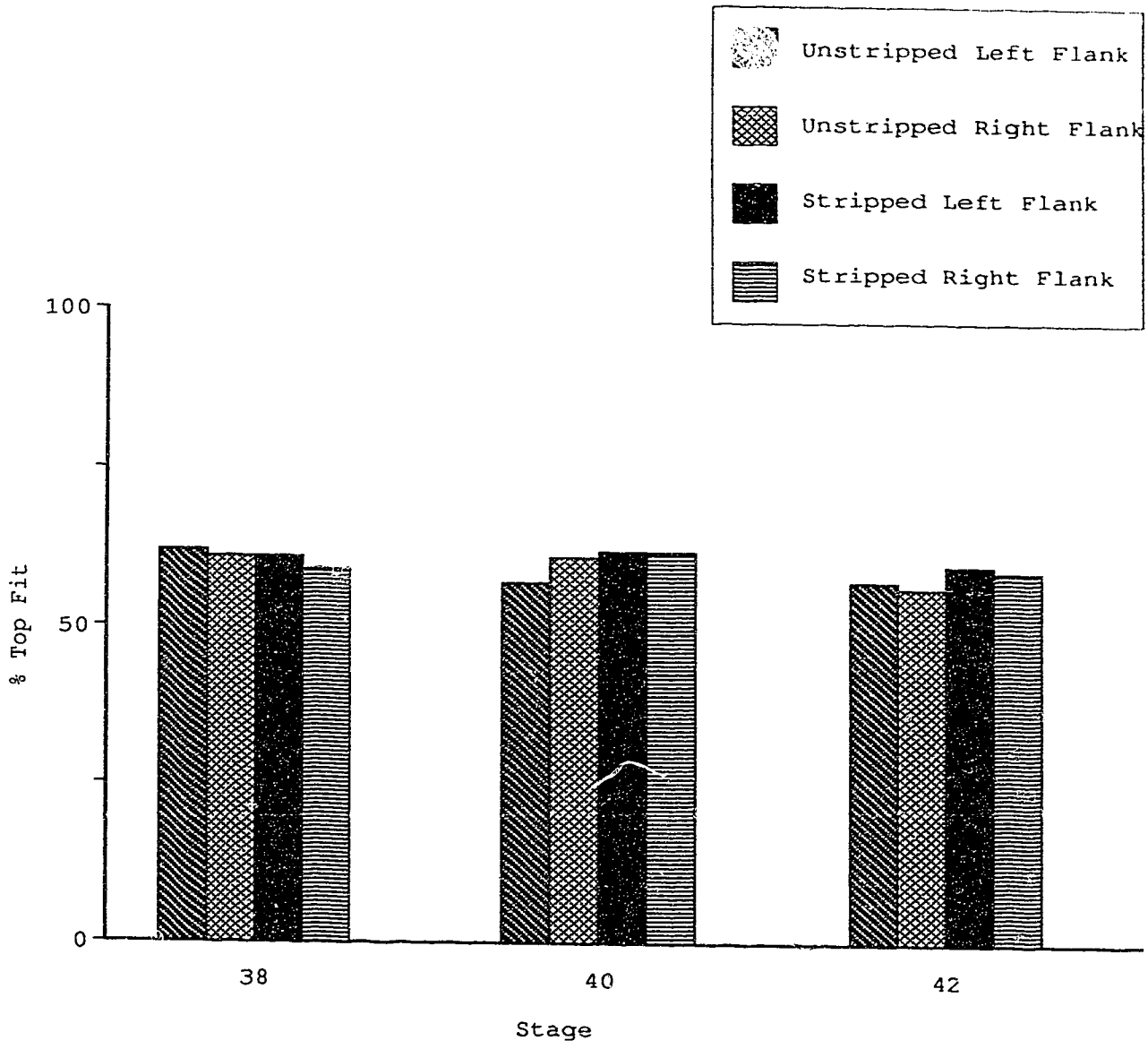


Figure 17. Experiment #2. Left and right percent top fit of stripped and unstripped stage 38, 40, and 42 averaged control and test (dark (D/-) axolotl).

* Significantly different from control right unstripped flank data as assessed by a Two-sided Normal Test.

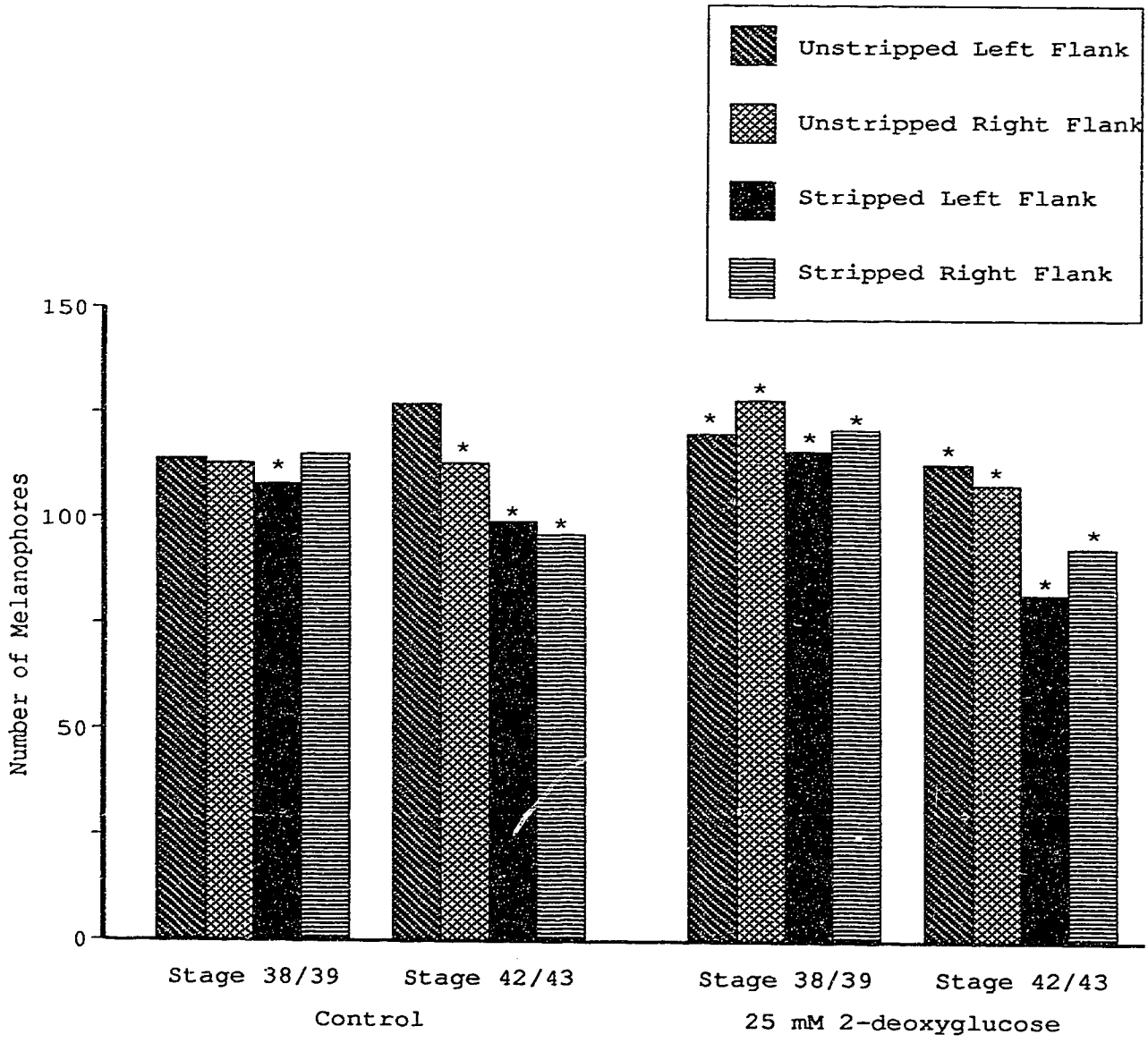


Figure 18. Experiment #1. Overall number of melanophores on right and left stripped and unstripped flanks of control and 25 mM 2-deoxyglucose confronted dark (D/-) axolotl larvae at stages 38/39 and 42/43.

* Significantly different from control right unstripped flank data as assessed by a Two-sided Normal Test.

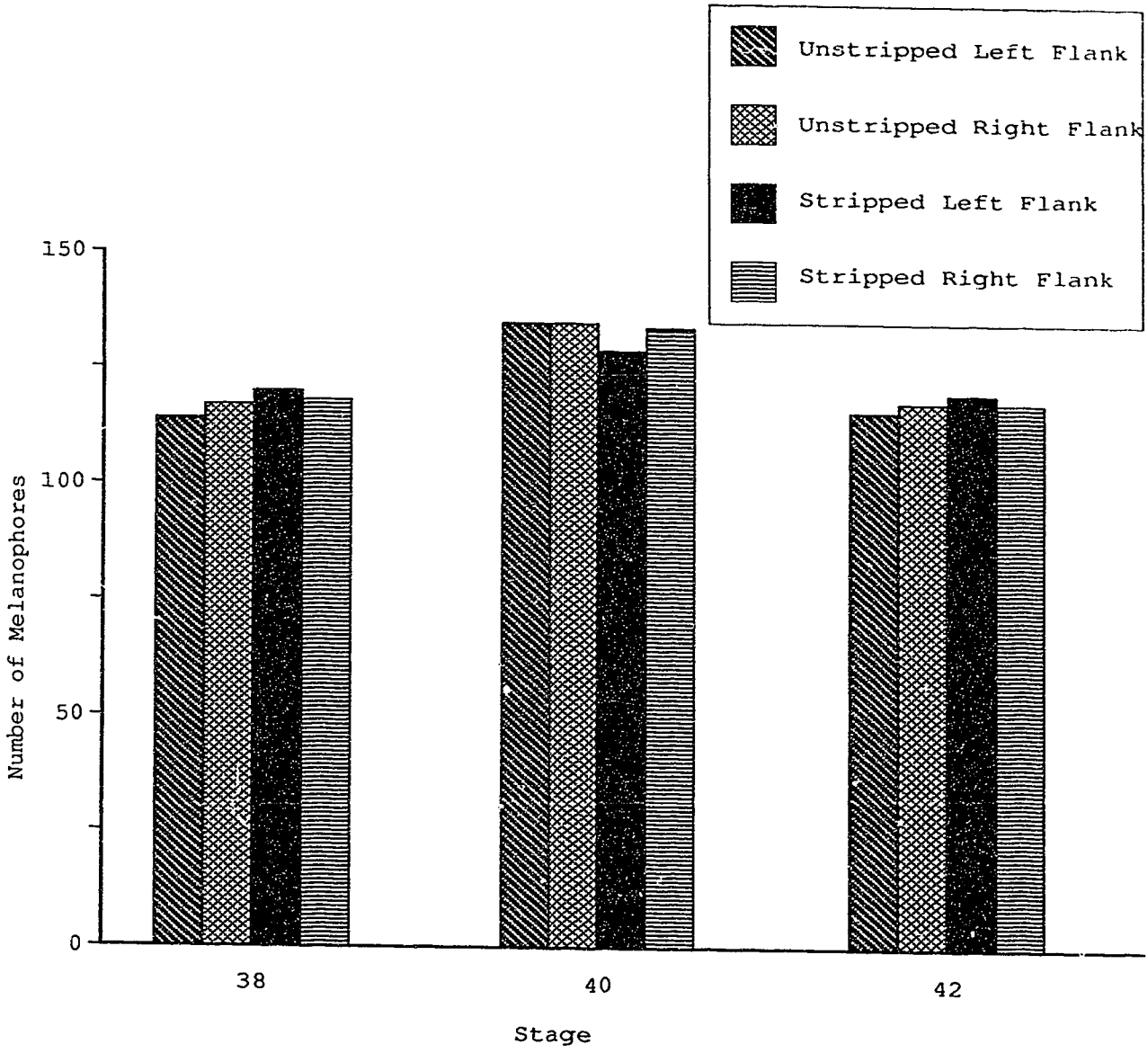


Figure 19. Experiment #2. Overall number of melanophores on right and left stripped and unstripped flanks of stage 38, 40, and 42 averaged control and test (dark (D/-) axolotl) data.

* Significantly different from control right unstripped flank data as assessed by a Two-sided Normal Test.

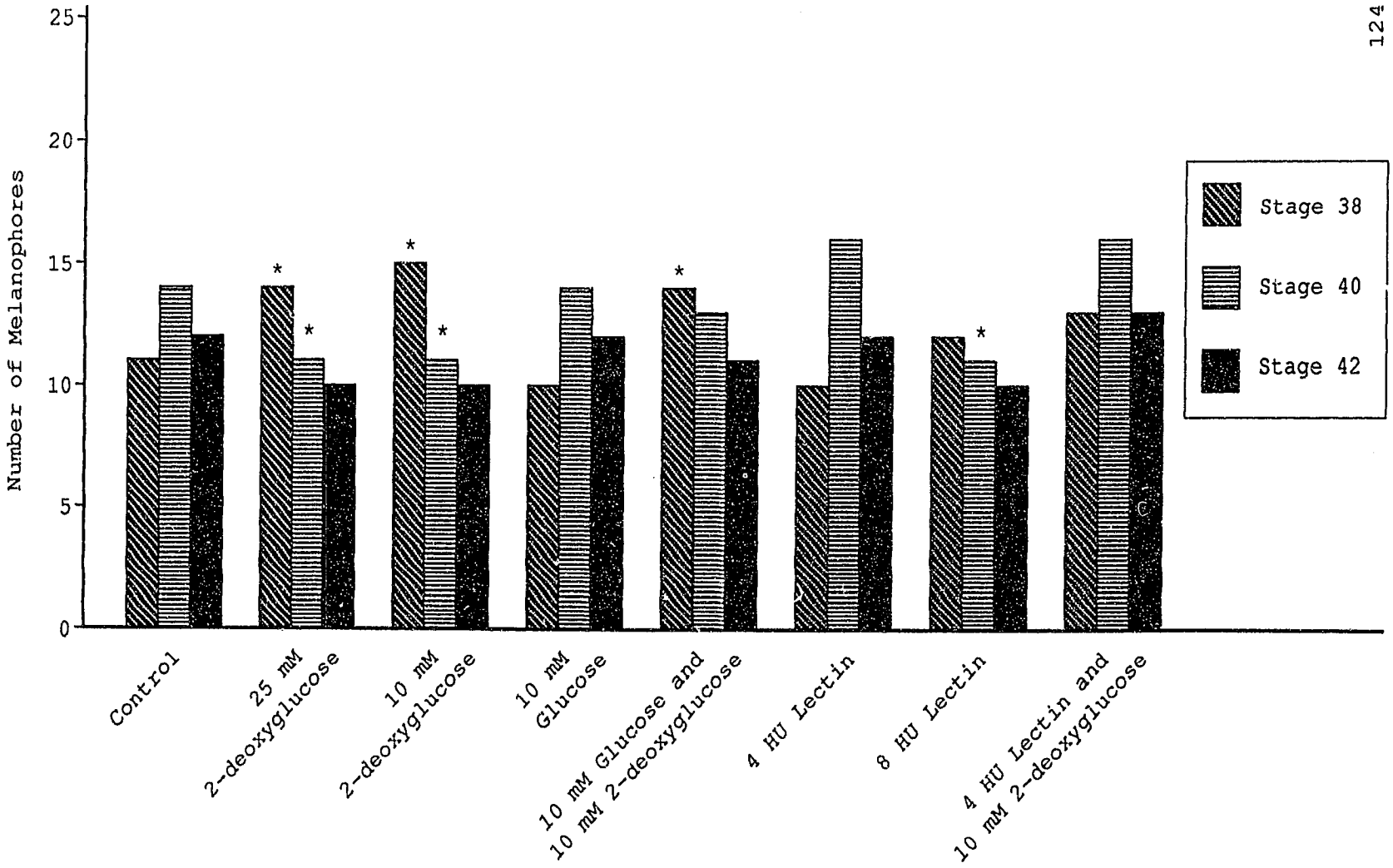


Figure 20. Number of melanophores per bar of control and experimental dark (D/-) axolotl larvae of stages 38, 40, and 42.

* Significantly different from control value at the same developmental stage as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test; α=0.05

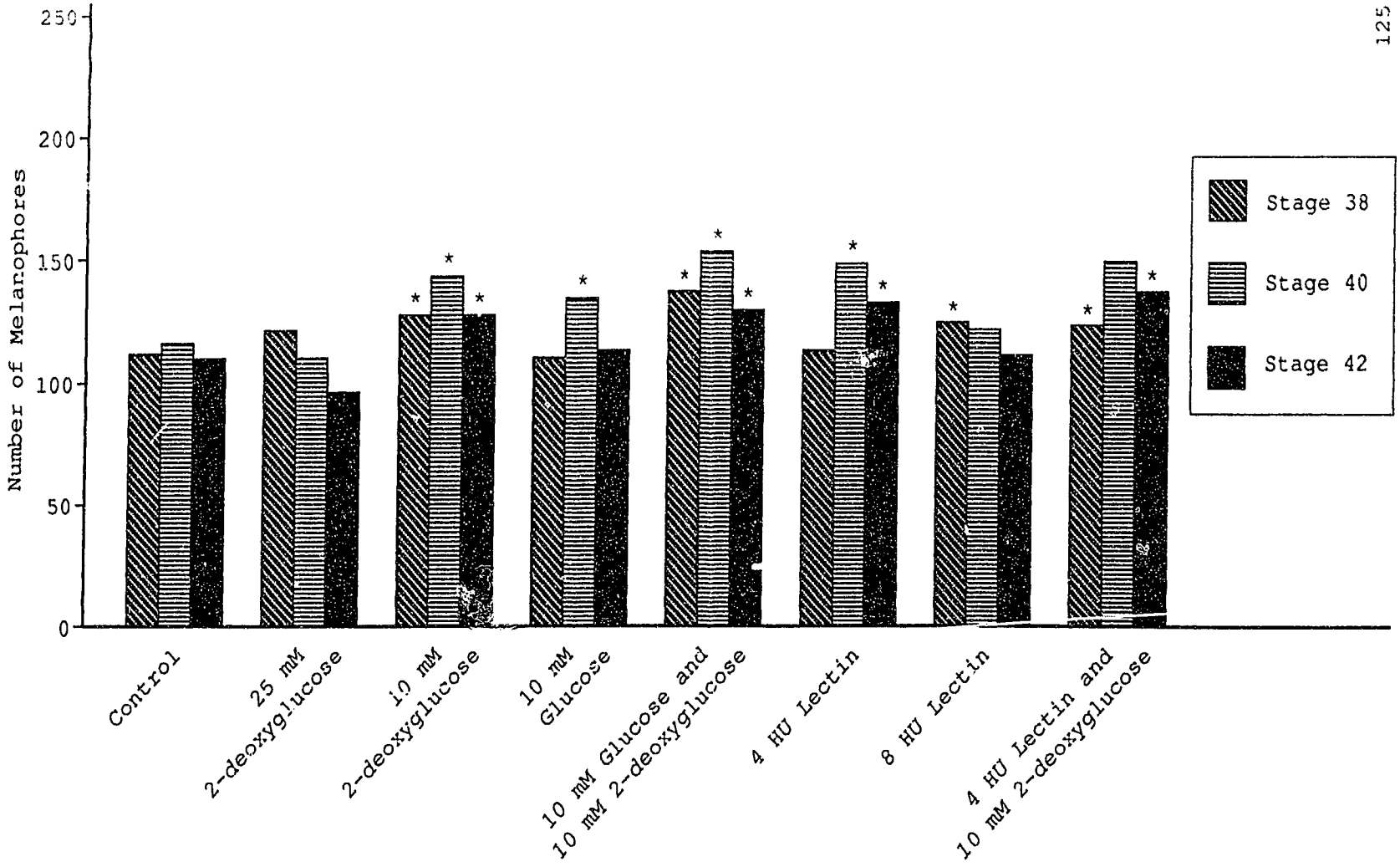


Figure 21. Number of overall flank melanophores of control and experimental dark (D/-) axolotl larvae of stages 38, 40, and 42.

* Significantly different from control value at the same developmental stage as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test; α=0.05

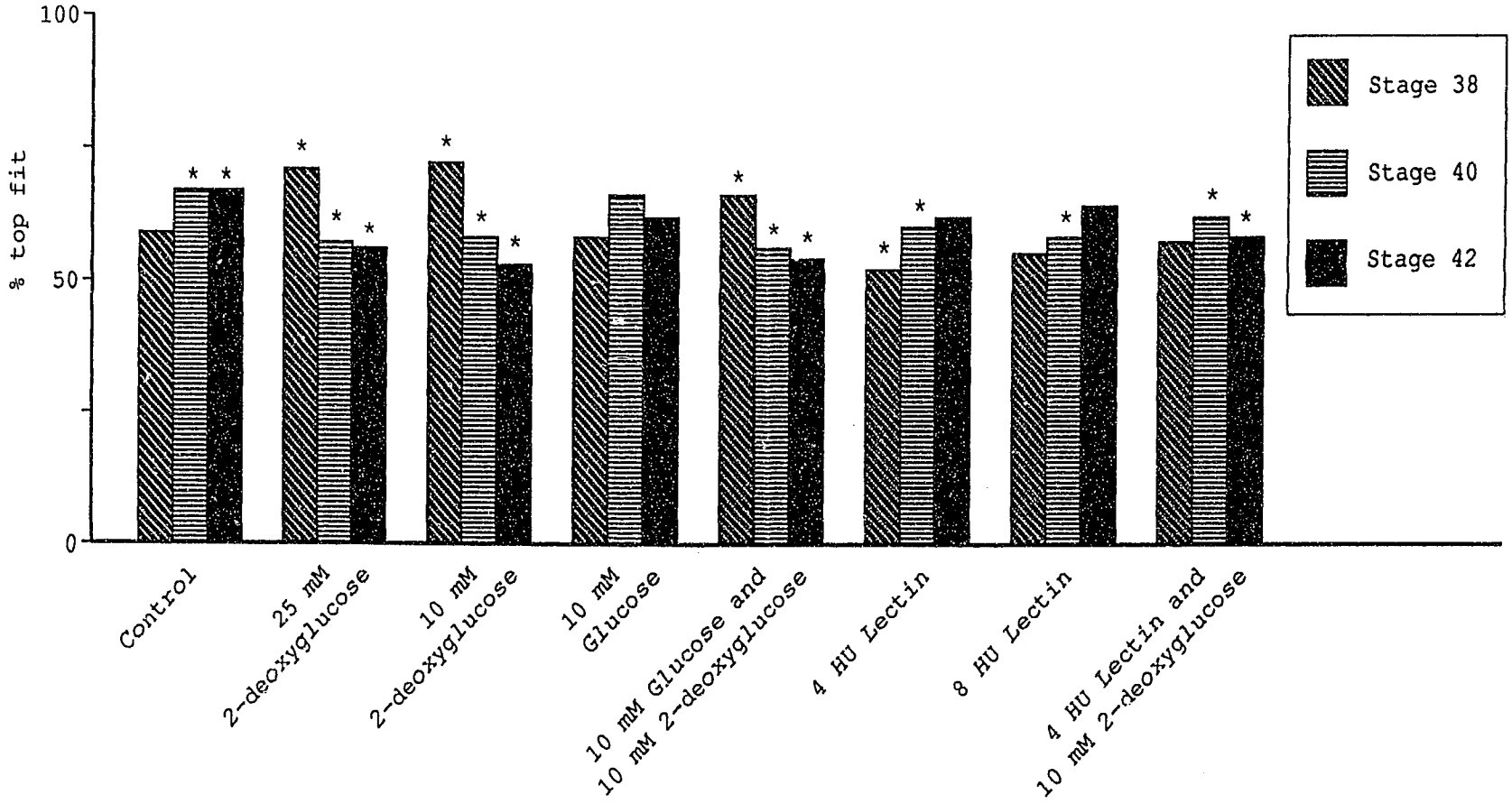


Figure 22. % top fit of control and experimental dark (D/-) axolotl larvae of stages 38, 40, and 42.

* Significantly different from control value at the same developmental stage as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test; $\alpha=0.05$

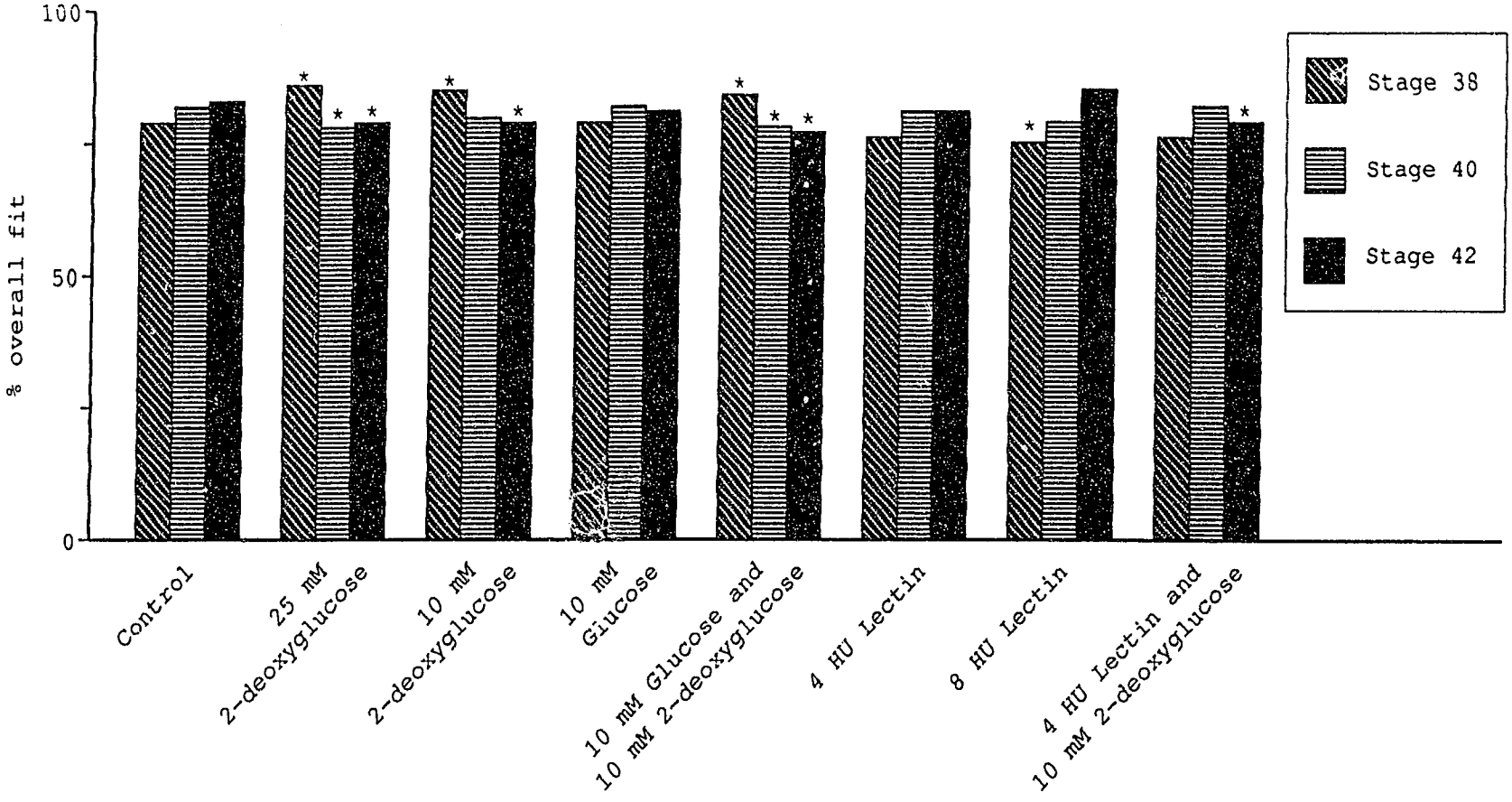


Figure 23. % overall fit of control and experimental dark (D/-) axolotl larvae of stages 38, 40, and 42.

* Significantly different from control value at the same developmental stage as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test; α=0.05

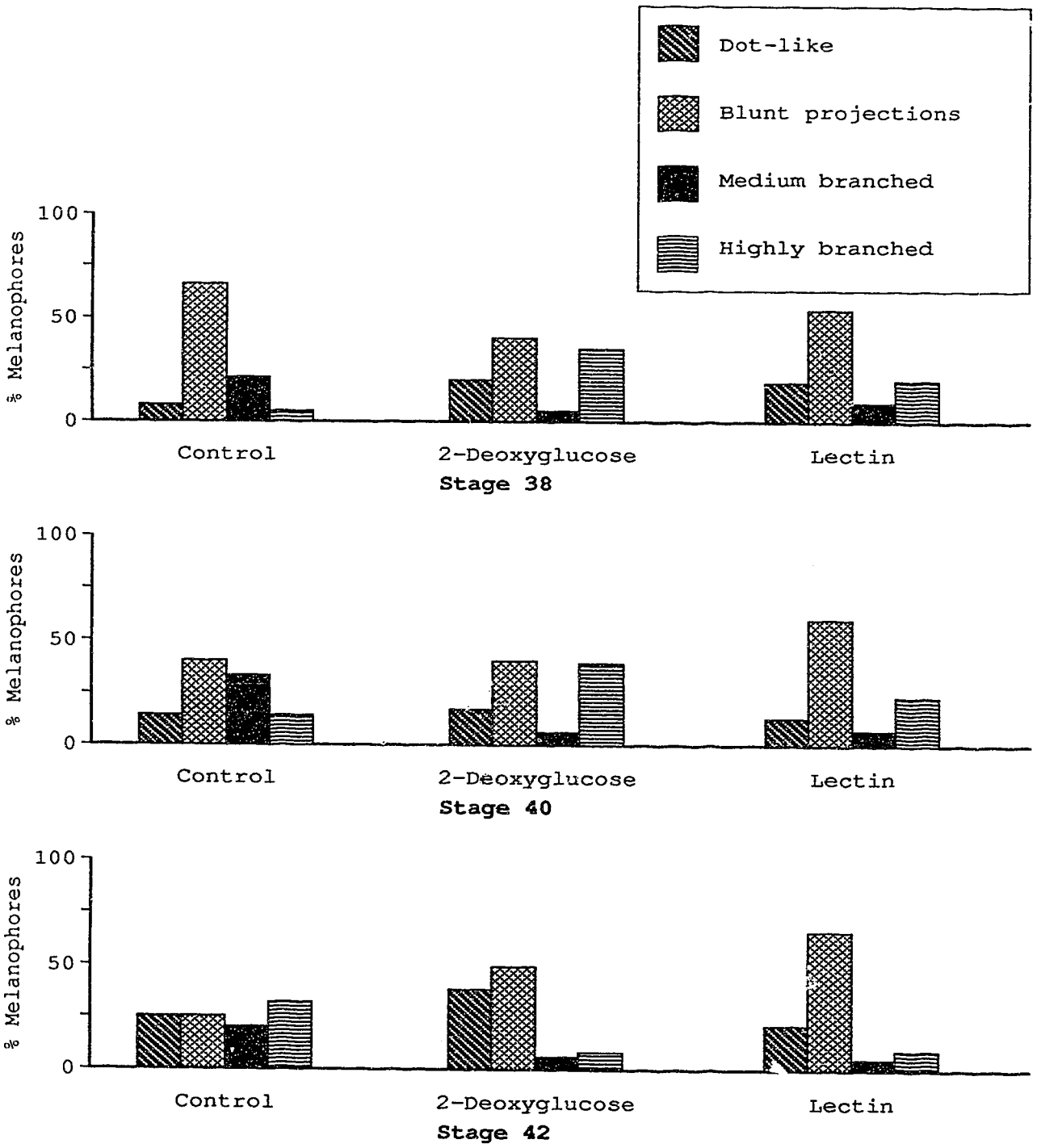


Figure 24. % Melanophores of type 1, 2, 3, and 4 morphologies in control and experimental dark (D/-) axolotl larval flanks at stages 38, 40, and 42.

Figure 25. Representative photographs of experimental and control dark (D/-) axolotl photographs at stages 38, 40, and 42.

Figure 25a. Representative photographs of confrontation experiment # 2 experimental and control stage 38 dark axolotl larval flanks.

A. CONTROL
B. 10 mM GLUCOSE/10 mM 2-DEOXYGLUCOSE
C. 10 mM 2-DEOXYGLUCOSE
D. 25 mM 2-DEOXYGLUCOSE
E. 10 mM GLUCOSE
F. 4 HU CRUDE LECTIN/10 mM 2-DEOXYGLUCOSE
G. 4 HU CRUDE LECTIN
H. 8 HU CRUDE LECTIN

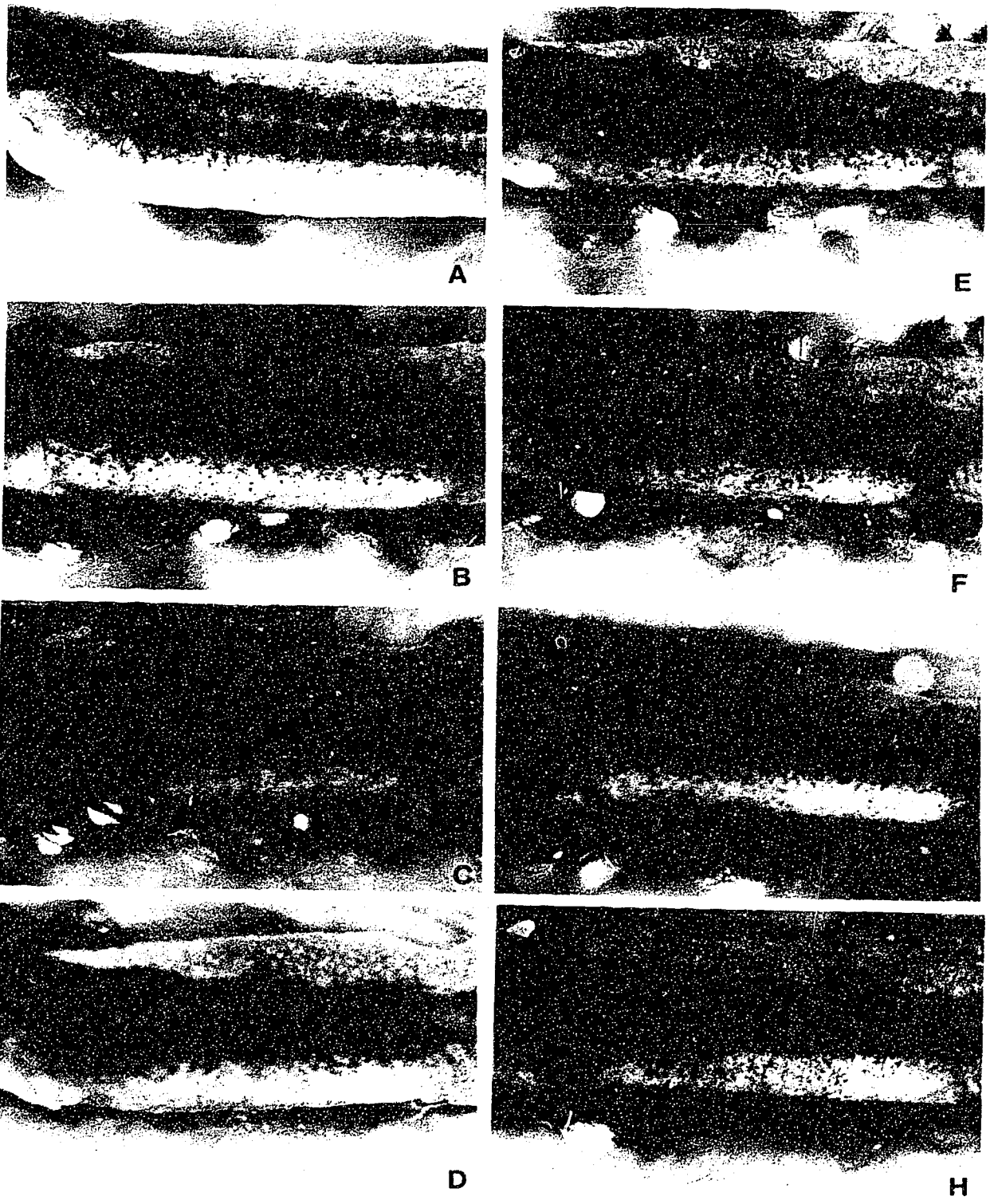

A
E
B
F
C
D
H

Figure 25b. Representative photographs of confrontation experiment # 2 experimental and control stage 40 dark axolotl larval flanks.

A. CONTROL
B. 10 mM GLUCOSE/10 mM 2-DEOXYGLUCOSE
C. 10 mM 2-DEOXYGLUCOSE
D. 25 mM 2-DEOXYGLUCOSE
E. 10 mM GLUCOSE
F. 4 HU CRUDE LECTIN/10 mM 2-DEOXYGLUCOSE
G. 4 HU CRUDE LECTIN
H. 8 HU CRUDE LECTIN

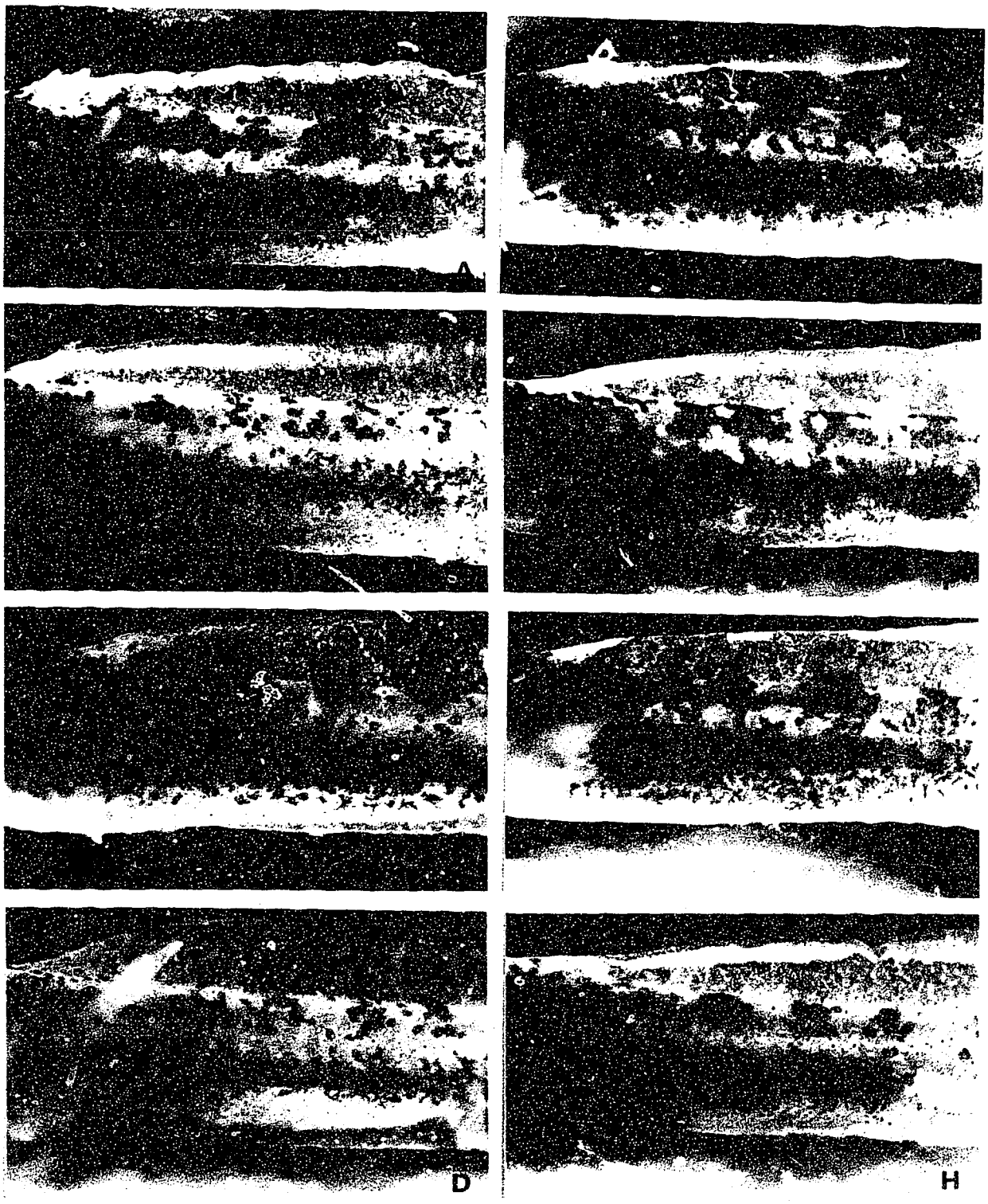
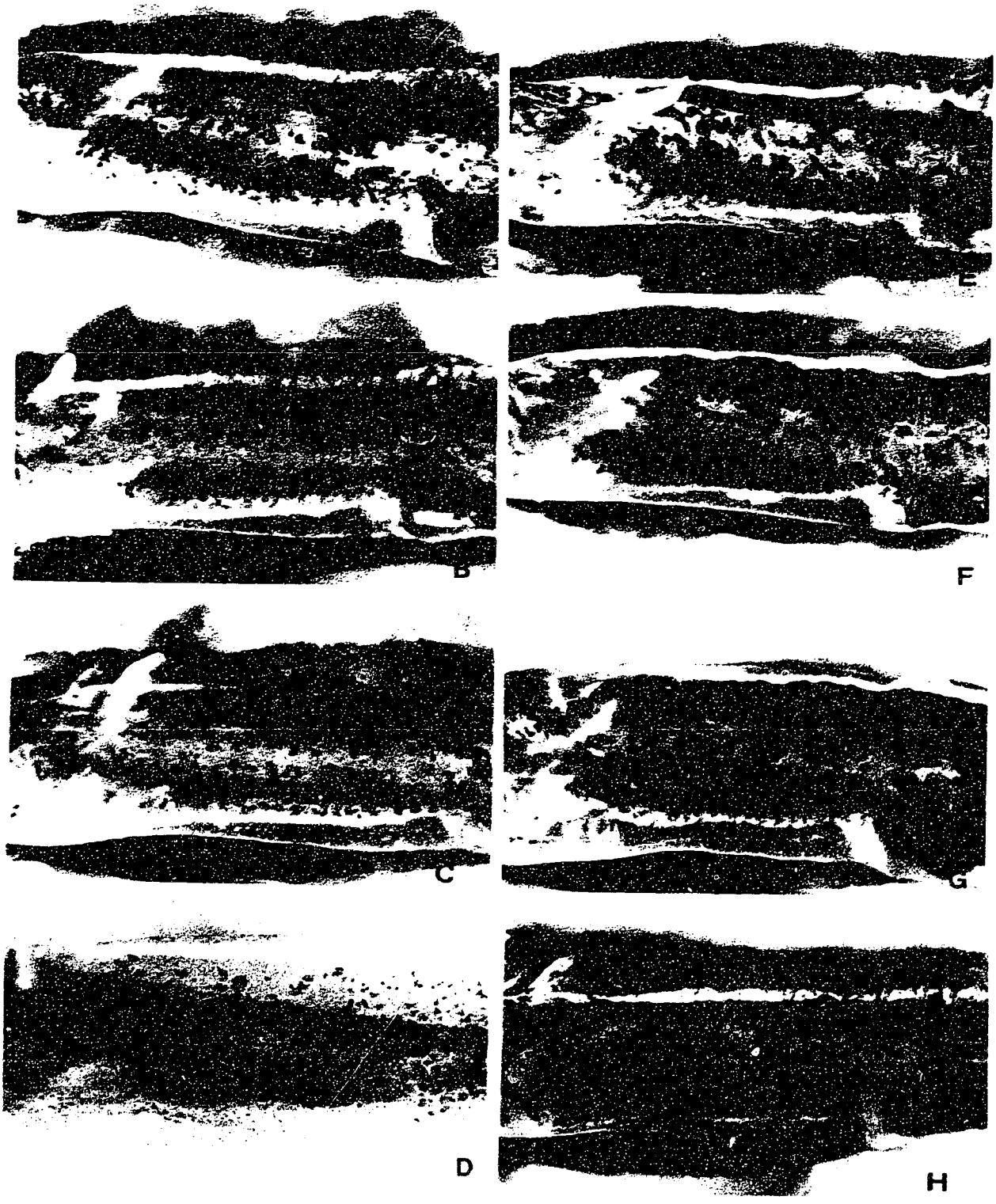

A
D
H

Figure 25c. Representative photographs of confrontation experiment # 2 experimental and control stage 42 dark axolotl larval flanks.

A. CONTROL
B. 10 mM GLUCOSE/10 mM 2-DEOXYGLUCOSE
C. 10 mM 2-DEOXYGLUCOSE
D. 25 mM 2-DEOXYGLUCOSE
E. 10 mM GLUCOSE
F. 4 HU CRUDE LECTIN/10 mM 2-DEOXYGLUCOSE
G. 4 HU CRUDE LECTIN
H. 8 HU CRUDE LECTIN


B
C
D
E
F
G
H

CHAPTER 4

DISCUSSION

A. DIFFERENCES IN THE LOCALIZATION AND TITRE OF LECTIN ACTIVITY IN DARK AND WHITE EMBRYOS

Endogenous lectin activity peaks during early neural crest (NC) migration in dark axolotl ectoderm (embryonic epidermis) (Table 2; Figure 14). An important point is that my investigation has not demonstrated that ectodermal lectin is associated with the ECM. However, the transplantation experiments cited in Chapter 1 implicate an ectodermal defect so it is tempting to speculate that white ectoderm produces less lectin or releases less into the subepidermal matrix and this is involved in the generation of the defect. Lectin activity also increases in white ectoderm but to a much lesser extent (Table 2). This result was very interesting, and suggests that there may be a connection between insufficient lectin present during early NC migration and the inability of white mutant NC cells to invade and migrate through the subepidermal ECM. In this work, a study of the deposition of lectin in the embryo was not done. However, the differences measured between dark and white ectoderm versus carcass tissue (Table 2) suggest that lectin may be localized differently in dark and white embryos. Interestingly, white carcass lectin activity increases significantly during early migration but dark

carcass tissue does not, suggesting that lectin is present in the white embryo during early migration but the majority of it is localized more centrally in the embryo, in contrast to the dark embryos which contain most of their lectin within the ectodermal tissue (Table 2). This result is interesting considering that in white embryos the majority of NC cells appear to migrate more centrally within the embryo.

Also of interest is the finding that even though white embryos contain more carcass lectin activity than dark embryos, the total amount of lectin activity (carcass plus ectoderm activities) is still only approximately one third in white embryos compared to dark embryos (Table 2).

Two conclusions can be made from these measurements. First, lectin activity peaks in both embryos during early NC migration, but white embryos contain significantly less total lectin activity than dark ones. Second, lectin activity peaks in tissues more centrally located in white embryos compared to dark embryos.

It would be interesting to further investigate these results by attempting to determine exactly where the endogenous lectin is located and perhaps which cell type(s) produce it.

The lectin of dark animals is specific for the three simple saccharides, D(+) mannose, D(+) glucosamine, and N-acetyl-D-glucosamine (Table 4). Because all three sugars

have in common identical configurations at C3, C4, C5, and C6 (see Figure 26), this lectin may recognize this specific configuration of the hexose ring.

Based on their configurations, these sugars represent Type III sugars by the classification scheme composed by Makela (1957) and reviewed by Goldstein and Poretz (1986). This classification takes into account the observation that most lectins are tolerant about some variety at C2 in regard to sugar specifity but C3 and C4, particularly, must be specific. Surprizingly, glucose, which is identical at C3, C4, C5, and C6 to the three other sugars (Figure 26), did not interact with this lectin. This suggests that other characteristics of the sugars in addition to their configurations (some of the characteristics of saccharides that affect lectin specificity are described in detail in Chapter 1) may be important in regard to this lectin's activity.

Interestingly, the lectin is also specific for the glycosaminoglycan (GAG), hyaluronic acid (HA) (Table 14). This molecule is a major component of the ECM, and it is tempting to speculate that the endogenous lectin is present in the ECM and binding to HA. HA consists of a repeating saccharide dimer composed of N-acetyl-D-glucosamine and glucuronic acid (see Figure 27), to which proteoglycan aggregates often attach, to form a large aggregate structure. It seems likely that the lectin interacts with

the N-acetyl-D-glucosamine component of this molecule (a possible functional relationship between axolotl lectin and the ECM is discussed later in this Chapter)

Although the calcium requirement has not been tested on this lectin, based on its specificity for mannose and N-acetyl-D-glucosamine, it is tempting to speculate that this lectin is a C-type lectin (C-type lectins have in common a Ca^{++} requirement for sugar binding and are usually extracellular; Drickamer, 1988). This group includes, for example, lectins discovered in ground scorpion epidermal membrane (Mouchamp, 1982), rat liver microsomes (Maynard and Baeziger, 1982), a mannose-binding protein (MBP) in rabbit, rat, and bovine sera (Kawasaki et al., 1989), and a mannose receptor on human placenta and macrophages (Tyler et al., 1990). Lectins in this family appear to have a variety of roles, some of which appear to be immune-related (Kawasaki et al., 1989; Thiel and Ried, 1989). These lectins also appear to have a variety of homologies to other adhesive proteins (the characteristics of this family of lectins is discussed in detail in Chapter 1).

The sugar specificity of dark and white lectin is the same (Table 4a), indicating that the lectin activity measured in the different tissues of dark and white embryos may represent a single lectin.

Thus, this lectin increases in activity simultaneously with the process of NC migration and, based on its sugar

specificity, may interact with HA in the ECM.

To begin to study the lectin's role in pigment pattern development in the axolotl, I then designed experiments to measure the effects of modulating lectin activity on pigment pattern development (ie., to investigate the functional relationship, if any between lectin activity and pigment cell localization in the skin) (Table 5). These experiments are discussed in the following sections.

B. LECTIN'S ROLE *IN VIVO*?

If differences in lectin activity between dark and white embryos are related to restricted pigment cell migration in the white mutant, then an experiment in which the *in vivo* titre of lectin is modulated and the resulting pigment patterns are characterized and compared would be useful.

I reasoned that *in vivo* lectin activity could be modulated by adding either a known inhibitor of the lectin or exogenous lectin to the embryo. If these experiments were successful, then it would be useful in the future to attempt more specific studies, for example, with antibodies against the carbohydrate-binding site of the lectin. First, the added molecules would have to be able to penetrate the embryo and interact with the lectin present within the animal. Second, non-specific effects of these "modulators"

have to be controlled for. Finally, the effects of the modulators on pigment pattern development had to be quantitated. A brief discussion of how these problems were addressed follows.

A variety of saccharides related to the three strongest inhibitors of this lectin were tested for their specificity to this lectin. A potent non-metabolizable inhibitor of the lectin, 2-DG, was discovered (see Chapter 2 discussion; Table 4b). This glucose analogue is widely known to be non-metabolizable in mammalian cells and is used as a tracer molecule in studying glucose metabolism since it is phosphorylated (the first step in this metabolic process) but not further metabolized (Ishibashi et al., 1982). Interestingly, 2-DG has the same molecular configuration as the other three lectin inhibitors (Figure 26).

To control for non-specific effects of this molecule, some embryos were confronted with glucose or glucose plus 2-DG to ensure that 2-DG was not interfering with glucose metabolism. Two different concentrations of 2-DG were used to confront embryos (Table 5).

Two different concentrations of a crude purification of endogenous dark neural crest stage lectin were also used to confront embryos (Table 5). Also, some embryos were confronted with a combination of crude lectin and 2-DG to assess whether their effects in combination would be cancelled out compared to their effects when used alone

(ie., based on differences in lectin activity between dark and white embryos, lectin and 2-DG would be expected to have opposite effects on pattern development) (Table 5). The results of these confrontations on pigment pattern development will be discussed in a later section.

Half of all the experimental and control embryos were stripped of some ectoderm (this procedure is described in Chapter 2 and illustrated in Figure 11) in an attempt to provide an avenue for penetration of the confronting molecules into the embryo and, hopefully, into the vicinity of NC migration. An initial attempt at stripping ectoderm ventrolaterally from the NC (illustrated in Figure 10) produced disruption of the pigment pattern and, therefore, interfered with the analysis of the experimental results. A second much less invasive method was attempted which produced no pattern disruption or any other observable effect on the embryo (Figure 11) and was adopted as the stripping procedure for confrontation experiment 2. I believe that the first stripping procedure extensively damaged the subepidermal ECM, leading to the pigment pattern disruption.

Surprisingly, whether or not the embryos were stripped did not significantly affect the results of any of the confrontations done. This was not surprising in the case of glucose or 2-DG since these are very small molecules (molecular weights are 180 and 164, respectively). If

ectodermal cells in the axolotl embryo are not bound by tight junctions (these are reviewed by Farquhar and Palade (1963), Gumbiner (1987), and Madara (1989), it seems possible that these molecules could leak between cells through the ectodermal barrier. This cannot be confirmed, however, because axolotl ectoderm has never been characterized in this manner and it isn't known what barriers exist against the uptake of certain molecules. Also, instead of diffusing between cells, these molecules might be actively engulfed by ectodermal cells. Simple sugars are known to be actively transported across some epithelia, for example, in the gut. However, it seems unnecessary for the axolotl embryo to have such a mechanism in ectoderm cells since glucose wouldn't likely be present in the jelly coat that normally encapsulates embryos at this stage (however, the composition of the jelly coat hasn't been identified). Furthermore, these embryos rely on internal yolk stores before they are developed enough to feed.

If glucose traverses the ectoderm it would likely be metabolized at once within cells and one might expect some observable effect on the embryo as a result of having more available energy, perhaps faster growth or more activity. Neither of these effects were observed, however. These observations don't rule out the possibility that glucose entered the embryos but had effects that were not readily

noticeable or it had no effects because it was either stored or excreted.

2-DG confrontation had some significant and specific effects on the pigment pattern (these effects are described in Chapter 3; see Tables 8 through 12), and this suggests that the molecule did traverse the ectoderm to interact with NC cells or the matrix. How this might occur is discussed in a later section.

More puzzling are the similar results obtained between unstripped and stripped crude lectin-confronted embryos. The lectin is expected to be either a protein or glycoprotein and therefore, would be a larger molecule than a simple sugar. Proteins cannot passively transverse any epithelia but can be specifically engulfed into the cell (Madden, 1986). This is usually receptor-mediated, so it is unlikely that receptors that recognize the lectin would exist in axolotl ectoderm. However, it may be possible that proteins, in general, are internalized and recycled or stored as an energy source in addition to yolk (this might also occur with glucose, as discussed). Even if lectin is internalized this way, how it could interact with NC migration before being metabolized remains a puzzling question. It's specific effects on pigment pattern development, however (see Tables 8 through 12), suggest that it may to be able to accomplish this.

One cannot rule out the possibility that the crude

purification contained other (smaller) molecules and these affected pigment pattern development in a non-specific manner. However, in this case, one might expect more than the pigment pattern to be specifically altered by crude lectin, and this was not observed at the level that I was working.

Perhaps the ectoderm of axolotl embryos is much more permissive to even large molecules than most epithelia. It appears relatively undifferentiated at the stage of NC migration and is only one cell layer thick. Furthermore, since these embryos are normally surrounded by a controlled environment, the jelly capsule, there may be no reason for the early development of any barrier mechanisms within the ectoderm and free molecular movement across the ectoderm (or "leakiness") might be important for the embryo's homeostasis. This seems to be the most plausible explanation for the similar effects of the confronting molecules on stripped and unstripped embryos.

The most difficult problem in assessing the effects of the various confrontations was establishing a valid and measurable pigment pattern control. To do this, normal embryos were carefully observed during pigment pattern development and compared to each other so that a "typical pattern" diagram at specific developmental stages could be constructed. Normal embryos exhibit some pattern features in common even though there is some variability among

individuals. Specific regions of the flank typically contain clusters of melanophores and other regions contain very few, if any, melanophores (Figure 7; the normal pigment pattern as it changes during development is described in Chapter 3). These characteristics were defined and compared at different developmental stages to produce a schematic representation of a control embryo at the three different stages observed in the experiment. I discovered that the normal pigment pattern consists of three constant parameters which could be measured. These parameters are (1) the number of melanophores on the flank as well as in three of the most consistently appearing bars on the flank, (2) the morphologies of the melanophores that appear on a representative region of the flank, and (3) how well melanophores fit into the "typical pattern" diagram. Both controls and experimental animals were measured by these criteria to give a measurement of normal compared to experimental pigment patterns and to compare the variability among pigment patterns in normal animals and experimental animals. Embryos from one spawning were used in each experiment to avoid the possibility of batch to batch differences in pigment patterns that might exist.

The results of these confrontation experiments designed to modify the <u>in vivo</u> titre of endogeonous lectin and observe the effects of these treatments on subsequent pigment pattern development support the hypothesis that

lectins are involved in pigment pattern formation in the axolotl. A "white" phenotype was not produced when endogenous lectin activity was inhibited by confronting dark animals with 2-DG (see Table 13) but significant changes in pigment pattern development resulted from both lectin inhibition and, as well, from addition of exogenous crude lectin extract.

First, the number of melanophores on glucose-confronted animals was not affected with the exception that stage 40 animals contained significantly more melanophores on their flanks than controls (Table 9). Whole-flank and top pattern fit measurements were not different from controls at any stage (Tables 11 and 10, respectively). Glucose, when combined with 2-DG, did not specifically influence the effects of 2-DG on the pigment patterns of animals at any stage (see Tables 8 through 12).

2-DG significantly affected different parameters of pigment pattern development. Both concentrations of 2-DG correlated with increased melanophores in the bars at stage 38 and decreased mels/bars at stage 40 followed by no significant efffects on this paramenter at stage 42 (Table 8). When overall mels/flank were counted, 10 mM 2-DG-confronted animals exhibited more melanophores on their flanks at all stages and the 25 mM concentration correlated with no differences from controls at any stage (Table 9).

In regard to pattern fit, both 2-DG concentrations

correlated with significantly higher % top fits at stage 38, followed by significantly lower % top fits at stages 40 and 42 (Table 10). These observations correlated very closely with those of overall pattern fit (see Table 11).

Melanophore morphologies were also significantly affected by 2-DG treatment (Table 12). The morphology patterns of stage 38 animals resembled those of older stage 42 controls whereas older 2-DG-confronted animals resembled those of stage 38 controls and, thus, appeared more immature.

In general, 2-DG-treated animals exhibited an advanced pattern in terms of the number of melanophores in their bars (Table 8), better organization of the melanophores into the bars (Table 10) and more developed melanophore morphologies during early pattern development (Table 12) followed by degeneration of the pattern at stage 40 to more closely resemble that of younger control animals.

The question of whether or not the confrontation with 2-DG actually decreased the titre of lectin activity <u>in vivo</u> was investigated by measuring whole embryo lectin activity in 2-DG-confronted and control embryos during development (Table 13). A peak in lectin activity in early migration stage control embyros was expected and observed. This peak also occured in the confronted embryos but was much less, suggesting that 2-DG in the medium decreased endogenous lectin activity in the embryos. These results suggest that

2-DG confrontation specifically affected endogenous lectin activity in the embryos and the resulting changes in lectin activity correlate with changes in pigment pattern development observed in these animals.

Crude lectin treatment produced some effects on the pigment pattern that were opposite to those of 2-DG.

First, the number of melanophores/bar was not significantly affected by either lectin concentration, with the exception of 8 HU crude lectin-treated animals at stage 40, which contained fewer melanophores/bar than controls at this stage (Table 8). The 4 HU concentration correlated with increased melanophores on the flank at stages 40 and 42 whereas the 8 HU concentration correlated with increased melanophores at stage 38 only (Table 9).

Percent top fit was reduced in stage 38 and 40 4 HU-confronted animals and also in stage 40 8 HU-confronted animals (Table 10). There were no differences in this regard between these animals and controls later in development.

The melanophore morphologies of stage 38 crude lectin-confronted animals were, in general, similar compared to controls (Table 12). Later in development, these animals contained less intermediate and more either highly branched or small dot-like types of melanophores than controls. The types of morphologies in stage 42 animals, in general, resembled those of stage 38 controls.

By many measurements, the two crude lectin concentrations correlated with inconsistent effects on pigment pattern development (for example, % top fit at stage 38 (Table 10), % overall fit at stage 38 (Table 11), number of melanophores/bar at stage 40 (Table 8), and number of melanophores/flank at all three stages (Table 9). No clear effect on melanophore number could be found (Tables 8 and 9) but pigment pattern development did not resemble controls in many ways either (Tables 10 and 11). The melanophore morphologies also did not appear to be affected in a consistent way, but somewhat resembled the results obtained with 2-DG confrontation at stage 42, but more opposite to those of 2-DG confrontation at stage 38 (Table 12). The changes in top pattern fit in these animals appears to be opposite to, but less dramatic than, those observed in 2-DG-confronted animals (Table 10).

Although much less conclusive, based on the % top fit data (Table 10), I suggest that crude lectin tended to have opposite effects on **early pigment pattern organization** compared to animals confronted with 2-DG.

It would be useful to repeat this experiment with affinity-purified lectin to determine whether contaminants in the crude extract are responsible for these inconsistencies. The results of these confrontations were much different from what I expected. Based on the data obtained from measuring lectin activity in white versus dark

embryos (Table 2), one would expect that a reduction in lectin activity (by 2-DG) would restrict pigment pattern development and produce white-like larvae. Instead, 2-DG treatment appeared to accelerate and then disrupt pattern development when animals were continuously confronted with the same concentration of 2-DG. **Crude lectin, in some ways, appeared to initially slow pattern development, an effect opposite to that of 2-DG.** Based on these experiments, lectin appears to be involved in pigment pattern development in the axolotl and could be somehow related to the white defect. However, the mechanisms by which lectin may affect dark and white pigment pattern development seem more complicated than just differences in whole embryo titre of lectin during early NC migration.

Like the results obtained with this study, the confrontation of endogenous lectin or its sugar inhibitor, TDG, with live Xenopus embryos (Frunchak and Milos, 1990), did not result in opposite effects on tail melanophore morphologies, numbers, and pattern organization. The addition of endogenous lectin to embryos correlated with a decrease in the number of melanophores on the tail as well as changes in melanophore organization and morphologies. TDG, in contrast, correlated with changes in melanophore morphologies and melanization and, to a lesser extent, changes in melanophore distribution and number. Although the function of endogenous Xenopus lectin in pigment pattern

development appears different in some aspects from axolotl embryonic lectin, these studies suggest that the relationship between lectin and melanophore pattern development is complicated in both species.

Some suggestions about how endogenous lectin might be involved in pigment pattern development in dark and white animals will be discussed in the following sections.

C. TEMPORAL REGULATION OF NEURAL CREST MIGRATION?

Confrontation with the different additives was continuous until stage 42 of development yet the effects of the additives on the pigment pattern were dependent on the stage of development observed. This suggests that the embryos may respond differently to lectin at different stages of pigment pattern development. **2-DG and crude lectin also appeared to alter the normal rate of pigment pattern development in opposite ways, with 2-DG accelerating early pattern development and crude lectin slowing it.** These observations suggest that changes in lectin activity may be involved in the normal sequences of pigment pattern development and when lectin activity is experimentally altered, sequences of pattern formation become disrupted, producing different rates of pattern development.

If the early-migratory peak in lectin activity is responsible for stimulating NC migration, then 2-DG

confrontation would be expected to result in a "white" phenotype, that is, a lack of migration. However, this did not result, although a significant reduction in the number of melanophores on the flank occurred at stages 40 and 42 (Table 11; Figure 21), along with a reduction in melanophore spreading (Table 12; Figure 24). It might be possible that 2-DG confrontation did not sufficiently diminish in vivo lectin activity to produce a "white" phenotype, and that the confrontation produced a partial "white" defect by stages 40 and 42. However, this theory doesn't explain why stage 38 animals exhibited a more mature-appearing pigment pattern (Table 10; Figure 24), including more, rather than less, melanophores on the flank (Figure 21) compared to controls. Therefore, this theory doesn't seem to be valid, in light of the results obtained.

2-DG confrontation produces what appears to be an initial acceleration of pigment pattern development but the pattern is not maintained later on. The results obtained from these experiments suggest that the early peak in lectin activity is not solely responsible for normal pigment pattern development. Perhaps the timing of this peak of activity or the subsequent drop in lectin activity may also be important cues for normal migration and localization of melanophores.

In white embryos, no significant peak in lectin activity occurs with the exception of a smaller peak in

carcass tissue at this time (Table 2; Figure 14). Lectin activity does not significantly decrease in the outer layers of the embryo where migration occurs and such a decrease may be the necessary stimulus for the initiation of NC migration into the subepidermal ECM so, in white animals, a very restricted pigment pattern results.

In 2-DG-confronted dark embryos, the normal peak that occurs in lectin activity during early NC migration may be attenuated by the added 2-DG. The subsequent decrease in lectin activity that normally occurs during early NC migration may be occuring earlier due to the inhibitor, creating the early and perhaps more intense stimulus for NC migration. NC cells migrate earlier and in greater numbers into the subepidermal matrix to produce an advanced and highly populated flank pattern by stage 38 in 2-DG-treated animals (Table 8). Possibly, pattern disruption may follow at stage 40 (Table 10; Table 8) because the NC cells leave the crest before they are competent to maintain the pigment pattern (this might explain why the pigment pattern, though sparsely populated, appears to stabilize by stage 42; see Tables 8 and 10). Alternatively, too many melanophores may exist on the flank initially and contact inhibition (discussed by Lehman and Youngs, 1959) may reduce the viability of melanophores in such close proximity. This theory suggests that either direct contact by cell processes touching eachother or indirect communication through

substances released by a cell can deter adjacent cells of the same type and promote equal spacing between pigment cells, for example, in the skin. The latter possibility seems less likely in light of the observation that 4 HU lectin treatment and glucose treatment also resulted in increases in the number of flank melanophores during stage 40 pattern formation and subsequent melanophore loss was not observed at stage 42 (Table 9).

In crude lectin-confronted dark embryos, the opposite may occur. The decrease in lectin activity after the pre-migratory peak may be less pronounced or more gradual, creating a less intense stimulus for NC migration. Melanophores might begin to migrate later than normal but may migrate over a longer period of time, creating an initially retarded pigment pattern but as pattern organization continues, by stage 40, more melanophores have migrated into the flank and the pigment pattern stabilizes and resembles those of controls by stage 42 (Table 10). Interestingly, however, the effects of the crude lectin and 2-DG treatments on melanophore morphologies seemed to last longer than pattern fit or melanophore number, even though these effects were not constant over time (Table 12; Figure 24). This suggests that the ECM, if modulated by lectin, may program differentiation information differently into migrating melanophores and, therefore, permanently alter their patterns of morphological development.

The experiments of Lofberg et al. (1989), are interesting in regard to the possibility that timing is important in normal NC migration. Their work suggests that white subepidermal ECM matures later during development than dark subepidermal ECM, and that this may be essential in producing the restricted pigment pattern. They transplanted subepidermal ECM absorbed *in vivo* on membrane microcarriers. Dark subepidermal ECM, when inserted subepidermally prior to NC migration, stimulated NC migration in both dark and white hosts. Most importantly, white subepidermal ECM from late-migratory stage 35 white embryos stimulated NC migration in early migratory stage 25-27 white hosts but stage 30 or younger white subepidermal ECM did not, suggesting that the defect existing within white subepidermal ECM is transitory in nature and that by the time the white subepidermal ECM becomes capable of supporting NC migration, NC cells are no longer capable of responding to the stimulus.

There may be a window of time in which NC melanophore precursors are sensitive to a decrease in lectin activity, starting immediately after the peak in lectin activity occurs and ending when lectin activity returns to a pre-migratory level. Their results suggest that a defect exists only within the white subepidermal ECM during the period at which NC cells normally migrate. Perhaps by inserting stage 35 white ECM into stage 30 white hosts, they are artificially creating a instant decrease in lectin activity

in the ECM which could stimulate NC migration. My results cannot confirm that a significant decrease in lectin activity occurs between stage 30 and 35, however, since only stages 25-28 and 29-34 were compared (Table 2). This hypothesis suggests that the post-peak decrease in lectin activity in the ectoderm or subepidermal ECM is a more important stimulus for NC migration than the pre-migratory peak in lectin activity.

It would be interesting to test this hypothesis directly by comparing the onset of NC migration in experimental (2-DG- and lectin-confronted) and control embryos using SEM.

An elaborate confrontation experiment in which confrontation with 2-DG or lectin begins at different stages of NC migration, and continues for different lengths of time during and after migration would also be useful to help determine the different effects of artificially eliminating the changes in lectin activity that normally occur, exaggerating the changes, and changing the timing of those changes to see which, if any, of these manipulations affect pigment pattern development. Perhaps, one could recreate "normal" fluctuations in lectin activity in white embryos to see whether they develop a normal pigment pattern. This might be accomplished by either producing and micro-injecting a monoclonal anti-lectin antibody <u>in vivo</u> or adding purified lectin to the saline of dark embryos.

How does the hypothesis that HA-lectin interaction is important in pigment pattern development agree with that of Lofberg et al. (1989)?

It seems possible that differences in lectin activity may be involved in subepidermal ECM differences between dark and white embryos, given that this lectin is specific for a major constituent of HA (N-acetyl-D-glucosamine) (Table 13) which is a substantial component of the embryonic ECM in axolotls (Perris and Johansson, 1990). Perhaps endogenous lectin modulates the binding activity of HA. Since HA forms large complexes with other ECM constiuents, this could affect the structural organization of the ECM and, therefore, possibly the migration and maturation of NC cells moving through it. It is well known that NC cells interact with the ECM as they migrate (evidence for this is discussed in Chapter 1) so lectin could be an indirect partner in controlling normal NC migration. Several investigations have been conducted on dark and white subepidermal ECM and only minor differences have been reported between them. However, research done by Dalton (1950) and others after him convincingly limits the white defect to the supepidermal ECM. The defect may be subtle and may be expressed for such a short period of time that it has escaped detection using biochemical methods. Perhaps a specific transient NC cell-ECM communication, for example, is essential in controlling NC migration in the ECM and this doesn't occur in white

embryos. Because of the roles of some lectins in cell-cell and cell-ECM recognition, a lectin could be a key factor in promoting normal pigment cell migration.

An extensive examination of dark and white ECM by Perris et al. (1990) revealed that the structural assembly of dark and white subepidermal ECM's were largely comparable but more electron-dense fibrils were observed in dark subepidermal ECM. This might reflect less proteoglycan available to form fibril structures because of recruitment of more proteoglycan aggregates into HA aggregate formation in white embryos. A difference in 30 to 90K protein content between dark and white subepidermal ECM was also found. Whether the differences in protein content between dark and white embryos reflects differences in lectin activity is unknown, but is an interesting possibility.

A hypothetical model for lectin-HA interactions in dark, white, and experimental embryos is suggested in Section E.

D. A ROLE FOR LECTIN IN THE EXTRACELLULAR MATRIX?

How this lectin interacts with components of the ECM or cells, if it in fact does, is unknown. However, if lectin binds to the N-acetyl-D-glucosamine HA backbone <u>in vivo</u>, it may interfere with the assembly of proteoglycan aggregates as both the core protein and link protein associated with

these structures bind directly to HA itself (see Figure 4 for the structure of HA aggregates; it is not known, however, whether the particular arrangement of HA aggregates shown in Figure 4 represents what occurs in axolotl embryonic ECM).

Hyaluronic acid aggregates are an important component of the ECM, imparting particular physical characteristics to the ECM and opportunities for interactions with other components and cells. The communication between cells and components of the ECM is essential to the processes of tissue and cell induction and differentiation in the embryo (Lofberg et al., 1980; Newgreen et al., 1982; Erickson and Weston, 1983). Hyaluronic acid, in particular, appears to be important in regard to NC migration. Work done in this regard suggests that the interaction between HA and proteoglycans may be important in regulating NC migration in vivo.

Two studies are important to note. Work done by Tucker (1986) on two anuran species suggests that chondroitin sulfate proteoglycan in subectodermal ECM restricts the migration of pigment cells by limiting the space available for migration and also by acting as a less adhesive migratory substrate and that HA opens spaces permitting the migration of cells. When chondroitinase ABC is added locally to the matrix in vivo, melanophore migration is promoted along affected pathways. This treatment also

increases the number of HA fibrils locally in the matrix, and the migratory space is enlarged.

In contrast, Tucker and Erickson (1986), based on their work with the California newt (Taricha torosa), report that HA within pigment cell migratory pathways prevents melanophore, but not xanthophore, penetration in vivo. When pigment stem cells enter HA-rich regions in vivo, their differentiation into melanophores is delayed. This may account for the early population of xanthophores into the HA-rich dorsal fin, followed weeks later by the appearance of melanophores in this region.

Differences in the interaction between lectin and HA may also influence NC cell migration, and may be essential for NC migration in dark embryos.

E. SUMMARY: A THEORETICAL ROLE FOR LECTIN IN AXOLOTL PIGMENT PATTERN DEVELOPMENT

The following outline of events is suggested as a mechanism for lectin in melanophore migration in the axolotl: An increase in lectin available occurs at the beginning of NC migration and interacts with HA attached to NC cells themselves or HA existing freely within the ECM and interferes with binding between HA and proteoglycans. This interaction might somehow either sterically inhibit NC migration or provide some signal to NC cells, preventing them from migrating.

The reduction of lectin activity that follows its peak may represent its sequestering by cells. HA either attached to the NC cells or within the ECM becomes free of the inhibitory effect of lectin and is also free of proteoglycan due to the presence of lectin during its production. NC cells are then permitted to migrate.

In white embryos, less lectin is present allowing more HA and proteoglycans to freely interact. HA either attached to the NC cells themselves or in the ECM produces aggregates with the proteoglycans and NC migration is inhibited.

2-DG confrontation, suggested to produce a early sharp decrease in lectin activity following its peak, may free HA earlier than normal, allowing more free HA to exist at one particular time. NC cells may then migrate earlier and may move more easily through the ECM, unencumbered by HA-proteoglycan complexes either attached to them or within the ECM. If the process of migration is important for NC cell maturation, then the early NC cells would also mature earlier. They may be stimulated to differentiate earlier than normal and interact to form a premature pattern.

Lectin confrontation, possibly characterized by a more gradual and less dramatic decrease in lectin activity following its peak, may provide more time for HA and proteoglycans to bind as HA becomes gradually free of lectin. NC cells might then be covered with more HA-proteoglycan complexes than mormal or may have to negotiate

through more of these complexes in the ECM so their migration is slowed and pigment cell maturation is impaired.

For this type of mechanism to work, the production of HA molecules by NC cells or other cells in the ECM must occur at the same time that lectin activity increases in the embryo. It would be useful to test this experimentally perhaps by labelling HA and lectin and quantifying them in dark and white embryos in frequent intervals during early NC migration.

F. CONCLUSION

The work done in this thesis correlates lectin activity with pigment pattern development and with the white defect. Based on the experiments done, the sugar-binding capacity of the endogenous lectin appears to be directly related to pigment pattern development (because 2-DG, its sugar inhibitor, can affect pigment pattern development) and appears to be similar in both dark and white embryos. These results suggest that the timing of lectin's appearance in the embryo is important in pigment pattern development, rather than differences in the lectin molecule itself.

Hyaluronic acid either on migrating pigment cells or in the ECM could be modulated by interacting with endogenous lectin resulting in changes in the aggregation of HA with proteoglycans within the ECM. This modulation, in turn,

might change characteristics of the ECM that are important in regard to migrating pigment cells, perhaps affecting their migration and differentiation. The changes in pigment pattern development observed after confronting embryos with either 2-DG or crude lectin extract suggest that migrating pigment cells are affected when the *in vivo* titre of lectin is modulated. A more direct investigation of the events that happen between changes in lectin activity and changes in pigment pattern development is required to test this hypothesis further.

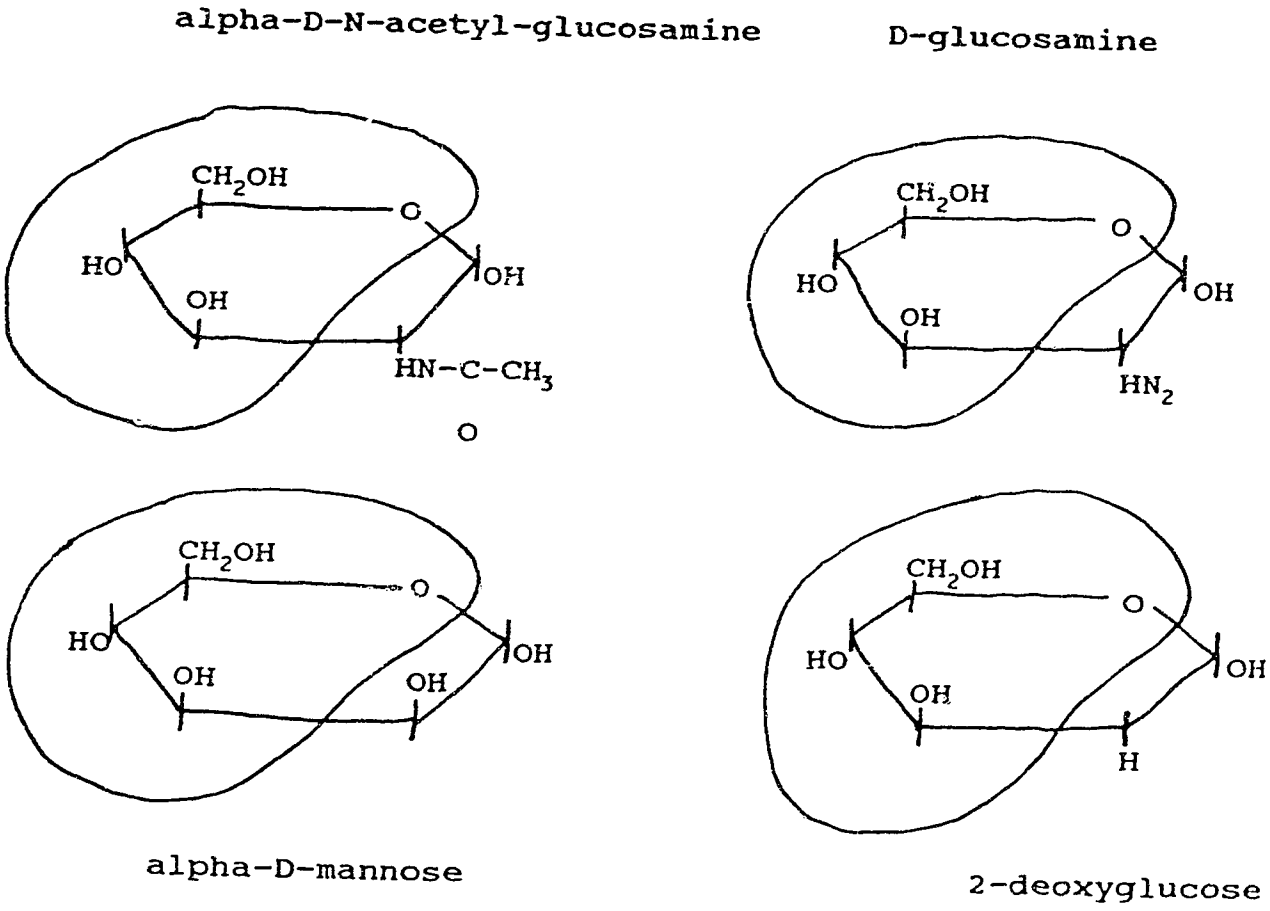
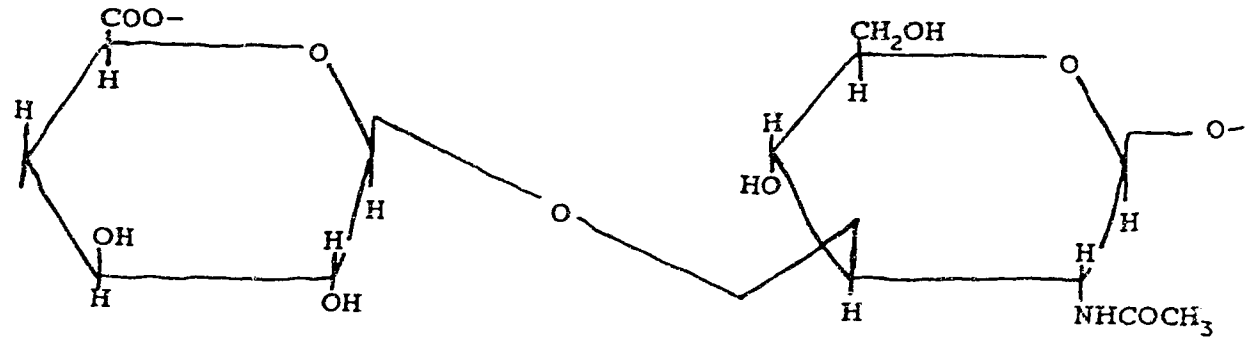


Figure 26. The ring structures of four simple saccharides for which endogenous axolotl embryos are specific. Configurations at C3, C4, C5, and C6 are identical in all four structures (indicated by rings drawn around them).



(glucuronic acid) (N-acetyl-D-glucosamine)

Figure 27. Repeating disaccharide unit of hyaluronic acid.

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produced no inhibition of lectin activity even at concentrations of 100 mM (Table 4).

B. *IN VIVO* EFFECTS OF 2-DEOXYGLUCOSE AND CRUDE LECTIN CONFRONTATION ON DARK AXOLOTL PIGMENT PATTERN DEVELOPMENT

1. OBSERVATIONS OF NORMAL PIGMENT PATTERN DEVELOPMENT

The dark axolotl pigment pattern just becomes visible at stage 37. Maternal ectodermal pigment (pigment derived from the unfertilized egg) is continuously sloughed off into the surrounding medium during the early stages of pattern formation, gradually revealing the underlying pigment cells.

The early flank pattern consists mostly of round, irregular dot-like melanophores which, with further differentiation, adopt morphologies ranging from dot-like to highly branched. Xanthophores are visible from about stage 38/39 as diffuse yellow regions between the dorsal melanophore bars and extending approximately half way into the dorsal fin.

The initial pigment cells that appear are quite well organized into the larval pattern consisting of alternating dorsal melanophore and xanthophore clusters, ie., the 'barred pattern'. Beneath the bars there is a more ventral pigment-free area and ventral to this, a broad melanophore stripe extending along the body. At stage 37, this overall

arrangement is ragged and irregular in appearance. The overall pigment pattern becomes sharper and more defined by stage 42. This can be seen by comparing Figure 25 a (A), b (A), and c (A), representative flank photographs of control animals at stages 38, 40, and 42.

a) The Number of Photographs Used to Analyze Data From Confrontation Experiments # 1 and # 2

Table 5 represents the number of photographs obtained from the two experiments conducted which were used to determine the effects of the various experimental treatments on flank melanophore pattern development between stages 38 and 42. Only well-focused and positioned photographs are counted in this list (and used for analysis).

2. EFFECTS OF THE STRIPPING PROCEDURES USED IN CONFRONTATION EXPERIMENTS # 1 AND # 2 ON PIGMENT PATTERN DEVELOPMENT

a) Effects of the Two Stripping Techniques on "Top Fit"

Using stripping technique # 1, percent "top fit" (that is, the number of melanophores that fit into the three "ideal bars" versus the number of melanophores that fit neither into the bars nor ventral stripe) was significantly decreased on the stripped (left) versus the unstripped (right) flanks of stripped control embryos confronted with

saline. Refer to Table 6a. The values were 53% on left (stripped) stage 38/39 and 53% on left (stripped) stage 42/43 flanks of stripped embryos compared to 63% on right (unstripped) stage 38/39 and 64% on right (unstripped) stage 42/43 flanks of stripped embryos, respectively (Figure 16 and Table 6a).

Similar results were obtained from animals incubated in 2-deoxyglucose (2-DG) (Table 6a). The values were 64% on left (stripped) stage 38/39 and 51% on left (stripped) stage 42/43 flanks of stripped embryos compared to 71% on right (unstripped) stage 38/39 and 65% on right (unstripped) stage 42/43 flanks of stripped embryos, respectively (Figure 16 and Table 6a).

Interestingly, 2-DG treatment correlated with significantly higher % top fit values in both stripped and unstripped embryos. Both left and right % top fit values were higher in stage 38/39 2-DG-confronted embryos compared to control embryos (stripped and unstripped) confronted with saline. Refer to Table 6a. The values were 71% (left flank) and 69% (right flank) in unstripped 2-DG-confronted embryos compared to 61% for both left and right unstripped control flanks. For stripped 2-DG-treated animals, the values were 64% (left stripped flank) and 71% (right stripped flank) compared to 53% and 63%, respectively, for (unstripped) right and (stripped) left flanks of stripped control animals raised in saline.

In unstripped animals, 2-DG also affected percent top fit significantly at stage 42/43, decreasing it to 64% (left flank) and 63% (right flank) compared to 70% (left flank) and 71% (right flank) on unstripped control flanks. In stripped animals at stage 42/43, control and test % top fit values were similar on both sides (Figure 16 and Table 6a).

The effect of 2-DG on percent top fit was explored further in the second experiment where the effects of the stripping procedure itself were eliminated.

The improved stripping procedure # 2 did not significantly affect percent top fit. Refer to Table 6b. For example, control left unstripped flank percent top fit values are 58, 65, and 67 % at stages 38, 40, and 42 wheras left stripped values are 59, 65, and 64 % at the same stages (Figure 17 and Table 6b). To compare this data from all of the experimental groups used in confrontation experiment # 2, refer to Table 15).

b) Effects of the Two Techniques on Overall Flank Melanophore Number.

The effects of stripping procedure # 1 on overall flank melanophore number were also tabulated. Refer to Table 7a. At stage 38/39, control unstripped right and left flanks carried 113 versus 114 melanophores, respectively. For stripped control animals, the right (unstripped) melanophore number was 115 versus 108 for the left (stripped) flank.

This latter value was a significant decrease (Figure 18 and Table 7a). The values obtained for 2-DG-treated animals were 128 and 120 melanophores on right and left flanks of unstripped animals and 121 and 116 on right (unstripped) and left (stripped) flanks, respectively, on stripped animals (Figure 18 and Table 7a).

However, by stage 42/43, both the right (unstripped) and left (stripped) flank melanophore numbers of stripped animals (experimental and control animals) differed significantly from those of unstripped test and control animals (Table 7a). The values were 127 and 113 on unstripped control left and right flanks versus 99 and 96 for (stripped) left and (unstripped) right flanks of stripped animals confronted with saline. For 2-DG-treated animals, the respective values are 113 (left) versus 108 (right) for unoperated animals and 82 (left) versus 93 (right) for the left (stripped) versus right (unstripped) sides (Figure 18 and Table 7a).

Interestingly, the above examination revealed that at stage 38, the 2-DG-treated (stripped and unstripped) animals carried significantly more melanophores than did the respective stripped and unstripped controls confronted with saline (Table 7a). For 2-DG-confronted animals, the values were 120 (unstripped left) and 128 (unstripped right) for unstripped animals versus 116 (stripped left) and 121 (unstripped right) for stripped animals. For control

animals, the values were 114 (unstripped left) and 113 (unstripped right) for unstripped animals versus 108 (stripped left) and 115 (unstripped right) for stripped animals.

However, by stage 42/43, the 2-DG-treated unstripped animals carried significantly less melanophores than did unstripped controls (Table 7a). For 2-DG-confronted animals, the values were 113 (unstripped left) and 108 (unstripped right) for unstripped animals versus unstripped control animals with values of 127 (unstripped left) and 113 (unstripped right) melanophores. Interestingly, there were no significant differences in the number of melanophores between stripped controls and 2-DG-confronted animals at stage 42/43 (Figure 18 and Table 7a).

The improved stripping procedure resulted in no significant changes in overall flank melanophore number at stage 38, 40, or 42 in control animals. Refer to Table 7b. For example, left unstripped numbers are 105, 119, and 116 at stages 38, 40, and 42 whereas left stripped numbers are 114, 110, and 117 at the same stages) (Figure 19 and Table 7b). To compare data from all experimental groups, refer to Table 16.

Only unstripped data from confrontation experiment # 1 was included in further analysis of the confrontation experiments. Stripped and unstripped data from confrontation experiment # 2 were combined.

3. THE EFFECTS OF THE TREATMENTS ON THE GENERAL DARK AXOLOTL PIGMENT PATTERN

As the pigment pattern emerges during development in control animals, the dorsal bars and ventral stripe become more well-defined on the flank. Individual melanophores also become larger and more darkly pigmented, giving them a more distinct appearance (refer to photograph A in Figure 25 a, b, and c).

Glucose-confronted animals appear similar to controls at stages 38 to 42 (photograph E in Figure 25 a, b, and c).

At all three stages, 10 mM 2-DG-confronted and 10 mM 2-DG/10 mM glucose-confronted animals appear very similar to each other (compare photograph C [2-DG] and B [2-DG plus glucose] in Figure 25 a, b, and c). Compared to controls at stage 38 (photograph A in Figure 25 a, b, and c), individual bars on these larvae are more clearly distinguishable and the ventral stripe appears wider and more populated. The melanophores also appear darker than those of controls, suggesting that they are synthesizing more melanin than control melanophores. By stage 40, the pigment pattern of these experimental larvae appears drastically different from the stage 38 pattern (compare photographs B and C in Figure 25 b [stage 38] to photographs B and C in Figure 25 a [stage 40]). The flanks are much more sparsely covered. This appears to reflect fewer as well as smaller melanophores. The melanophores on these flanks are much more contracted

and punctate in appearance than controls. They also appear less organized into dorsal bars and the ventral stripe. This could be due to random deaths of flank melanophores.

The effect of 25 mM 2-DG appears even more pronounced than that of 10 mM 2-DG (compare photograph D [25 mM 2-DG] in Figure 25 a, b, and c to photograph C [10 mM 2-DG] in Figure 25 a, b, and c). At stage 38, the dorsal bars look very distinct and well separated from each other, unlike control larvae (compare photograph D to A in Figure 25 a). However, it is not clear whether the confronted individuals contain more flank melanophores. At stage 40, this individual (photograph D; Figure 25 b) appears similar to the 10 mM 2-DG (photograph C; Figure 25 b) and 10 mM 2-DG/10 mM glucose-confronted animals (photograph B; Figure 25 b). At stage 42, however, these individuals (photograph D; Figure 25 c) appear even more sparsely covered than the 10 mM 2-DG animals (photograph C; Figure 25 c). Fewer melanophores and smaller, more punctate melanophores are visible, and the flank pattern is very disrupted (for example, it is very difficult to differentiate the dorsal bars on the individual shown in photograph D; Figure 25 c).

Based on the photographs, 2-DG appears to have a significant and dose-dependent effect on pigment pattern development in dark axolotls.

Crude lectin appears to have little effect on the pigment pattern. At stage 38, 40, and 42, 4 and 8 HU-

confronted larvae appear similar to controls (photographs G and H, respectively, compared to photograph A, in Figure 25 a, b, and c). The only difference observed between these animals and controls is that the horizontal pigment-free stripe that runs along the midline of the flank appears less organized than controls at stages 38 and 40 (compare photographs G and H to photograph A in Figure 25 a and b).

Interestingly, 4 HU crude lectin seems to moderate 10 mM 2-DG's effects on the pigment pattern when both are combined (compare photograph F to photograph C in Figure 25 a, b, and c). Animals at stage 38 look very similar to controls (compare photograph F to photograph A in Figure 25 a). Animals at stage 40 appear somewhat less organized but no other consistent differences between these animals and controls are observed at this stage (compare photograph F to photograph A in Figure 25 b). At stage 42, these animals look similar to controls (compare photograph F to photograph A in Figure 25 c).

In conclusion, based on observations of the overall appearance of the pigment pattern in experimental and control animals, 2-DG appears to have specific consistent and stage-dependent effects on pattern organization, melanophore number, and melanophore morphology (photographs C and D; Figure 25 a, b, and c). Crude lectin correlates with some pattern disorganization at stage 38 and 40 (photographs G and H; Figure 25 a and b) but otherwise no

observable effect on the pigment pattern is observed. When combined with 2-DG, however, crude lectin seems to reverse some of 2-DG's effects (photograph F compared to photograph C; Figure 25 a, b, and c). No pigment pattern effects are observed with glucose confrontation (compare photograph E to photograph A; Figure 25 a, b, and c).

4. THE EFFECTS OF THE TREATMENTS ON MELANOPHORE NUMBER

a) Number of Melanophores per Bar

In control larvae (confronted with saline), the number of melanophores (mels)/bar increased significantly from an average of 11 at stage 38 to 14 at stage 40 (Table 8). The number of mels/bar at stage 42 (13 mels/bar) is not significantly different from the stage 38 or 40 values (Figure 20 and Table 8).

Both 10 and 25 mM concentrations of 2-DG correlated with significantly more mels/bar (15 and 14 mels/bar, respectively) compared to controls (11 mels/bar) at stage 38 (Table 8). By stage 40, both 10 mM and 25 mM 2-DG treatments correlated with significant decreases in the number of mels/bar (from 15 to 11 and from 14 to 11, respectively). At stage 42, both concentrations correlated with counts of 10 mels/bar, not significantly different from a control value of 13 mels/bar (Table 8).

Neither 4 nor 8 HU of crude lectin extract correlated with significant differences in the number of mels/bar at stage 38 (10 and 12 mels/bar, respectively) compared to controls (11 mels/bar) (Figure 20 and Table 8). Similarly, at stage 40, 4 HU lectin-treated animals showed no significant difference from control values. 8 HU lectin treatment, in contrast, correlated with significantly fewer mels/bar than controls (11 compared to 14 mels/bar, respectively) (Table 8). By stage 42, both 8 and 4 HU lectin treatments correlated with no significant differences in the number of mels/bar compared to control values (Table 8).

10 mM glucose treatment did not correlate with any significant differences in the number of mels/bar at stages 38, 40, or 42 compared to controls at the same stages (10, 14, and 12 mels/bar compared to control values of 11, 14, and 13 mels/bar, respectively) (Table 8). When 10 mM glucose was combined with 10 mM 2-DG, the number of mels/bar recorded at all three stages of development resembled mel/bar counts of 10 mM 2-DG data at corresponding stages (14, 13, and 11 mels/bar compared to 10 mM 2-DG values of 15, 11, and 10 mels/bar, respectively) (Table 8).

The addition of 4 HU crude lectin plus 10 mM 2-DG to the saline correlated with no differences between the number of mels/bar in larvae treated with this combination and controls at stages 38, 40, and 42 (experimental values are

13, 16, and 13 mels/bar compared to control values of 11, 14, and 13 mels/bar) (Figure 20 and Table 8).

In summary, both 10 and 25 mM 2-DG concentrations correlated with significantly more mels/bar at stage 38, significantly fewer mels/bar at stage 40, and no significant difference from controls at stage 42. 8 HU crude lectin treatment had a significant effect on the number of mels/bar at stage 40 only, correlating with significantly fewer mels/bar compared to controls at this stage. The 4 HU concentration of crude lectin correlated with no significant differences in the number of mels/bar from controls, at any stage (Table 8; Figure 20).

b) Overall Number of Melanophores per Flank

The number of mels/flank remained constant throughout stages 38 to 42 in controls confronted with saline (Figure 21 and Table 9).

There was no significant difference in the number of mels/flank between 25 mM 2-DG-treated larvae and control larvae at any stage (Table 9). In contrast, 10 mM 2-DG treatment correlated with sigificantly more flank melanophores at all three stages compared to controls (127, 143, and 127 mels/flank compared to 112, 116, and 110 mels/flank, respectively) (Table 9). These results contrast with those of the mel/bar data (Table 8), characterized by

an initial increase in the number of melanophores/bar followed by a decrease in the number of melanophores/bar.

For 4 HU and 8 HU crude lectin-treated larvae, the mel/flank counts somewhat resemble the mel/bar count data (compare Table 9 to Table 8). 4 HU crude lectin treatment correlated with signifcantly more mels/flank at stages 40 and 42 (148, and 132 mels/flank compared to control values of 116 and 110 mels/flank, respectively) but not at stage 38 (Table 9). Eight HU crude lectin treatment correlated with significantly more mels/flank at stage 38 only (124 mels/flank compared to 112 for controls) (Table 9).

10 mM glucose did not correlate with a significant change in the number of mels/flank at stages 38 or 42 but correlated with significantly more mels/flank at stage 40, compared to controls (134 mels/flank compared to a control value of 116 mels/flank) (Table 9). The combination of glucose with 2-DG did not significantly influence the effect of 10 mM 2-DG on the number of mels/flank at any stage (the values were 137, 153, and 129 mels/flank, respectively, compared to 10 mM 2-DG values of 127, 143, and 127 mels/flank) (Table 9).

The combination of 4 HU crude lectin and 10 mM 2-DG correlated with changes in the number of mels/flank that are significantly different at all stages (123, 149, and 137 mels/flank) from controls confronted with saline (112, 116, and 110 mels/flank, respectively) (Figure 21 and Table 9).

In summary, 25 mM 2-DG treatment did not correlate with any significant changes in the number of melanophores in the flank at any stage. In contrast, the 10 mM 2-DG concentration correlated with significantly more mels/flank at stages 38, 40, and 42 (Table 9). These results may reflect an adverse effect of 2-DG on pigment pattern development at the higher 25 mM concentration. 2-DG might be slightly toxic to the melanophores.

The two crude lectin treatment concentrations, in general, correlated with opposite effects, with the 8 HU concentration correlating with an initial significant increase in mels/flank at stage 38, and the 4 HU concentration correlating with significant increases in mels/flank at stages 40 and 42 (Table 9). These results could reflect the greater capacity of the 8 HU concentration to increase the number of flank melanophores earlier during development than the 4 HU concentration.

5. THE EFFECTS OF TREATMENTS ON MELANOPHORE PATTERN ORGANIZATICN

a) "Top Fit"

In controls, % top fit increased significantly from 59% at stage 38 to 67% at stages 40 and 42 (Figure 22 and Table 10).

Both 10 and 25 mM 2-DG treatments correlated with significantly greater % top fit at stage 38 (72% and 71%, respectively) but significantly lower % top fit at stage 40 (58% and 57%, respectively) and 42 (53% and 56%, respectively) compared to controls (Table 10).

In contrast, 4 HU crude lectin treatment correlated with significantly lower % top fit at stages 38 (52% compared to 59%) and 40 (60% compared to 67%) but no significant difference in % top fit from controls at stage 42 (62% compared to 67%) (Table 10). 8 HU crude lectin treatment correlated with a significantly reduced % top fit at stage 40 only (58% compared to 67%) (Table 10).

10 mM glucose treatment correlated with no significant differences in % top fit from controls at stage 38, 40, or 42 (58%, 66%, and 62%, respectively, compared to control values of 59%, 67%, and 67%, respectively) (Table 10). In combination with 10 mM 2-DG, the results were consistent with those of 10 mM 2-DG alone (66%, 56%, and 54%, respectively, compared to 10 mM 2-DG values of 72%, 57%, and 56%, respectively) (Table 10).

10 mM 2-DG and 4 HU in combination correlated with results that were intermediate in their effects compared to each treatment alone (Table 10). There was no significant difference in % top fit from controls at stage 38 (57% compared to 59% in controls). Like both 2-DG and crude lectin results, % top fit was significantly lower at stage

40 (62% compared to 67% in controls). At stage 42, % top fit was lower (58%) than that of control (67%) or 4 HU-treated larvae (62%) but greater than that of 10 mM 2-DG alone (53%) (Figure 22 and Table 10).

In summary, both 10 and 25 mM 2-DG concentrations correlated with significant increases in % top fit at stage 38 followed by significant decreases in % top fit at stages 40 and 42. These results may reflect stage-dependent effects of 2-DG on bar pattern organization. The crude lectin treatments correlated with initially lower % top fit at stages 38 and 40 for the 4 HU treatment and at stage 40 for the 8 HU treatment followed by no significant differences form control values at later stages. Crude lectin appears to have an opposite effect on % top fit compared to 2-DG, but these results are not very consistent between the two concentrations used.

b) "Overall Fit"

In controls, % overall fit remained constant at stages 38, 40, and 42 (Figure 23 and Table 11).

Both 10 mM and 25 mM 2-DG treatments correlated with significantly higher % overall fit values at stage 38 compared to controls (85% and 86%, respectively, compared to 82%) (Table 11). By stages 40 and 42, % overall fits were, in general, significantly lower than those of controls (78%

at stage 40 for the 25 mM 2-DG data compared to 82% for controls and 79% for both 10 and 25 mM 2-DG concentrations at stage 42, compared to 83% for controls) (Table 11). These changes resemble those of % top fit (compare Table 11 to Table 10).

At stage 38, % overall fit was significantly lower in 8 HU (75% compared to 79%) but not 4 HU (76% compared to 79%) crude lectin-treated larvae (Table 11). At stages 40 and 42, no significant differences were observed between either 4 (81% and 81%, compared to 82% and 83%, respectively) or 8 HU (79% and 85%, compared to 82% and 83%, respectively) treatments and controls (Table 11).

10 mM glucose treatment correlated with no significant changes in % overall fit at stage 38, 40 and 42 (79%, 82%, and 81%, respectively, compared to control values of 79%, 82%, and 83%) (Table 11). Like the % top fit results, when combined with 10 mM 2-DG, larvae resembled 2-DG-treated larvae (Table 11). Compared to controls confronted with saline, the glucose/2-DG combination correlated with significantly higher % overall fit at stage 38 (84% compared to 79%) compared to % overall fit at stages 40 and 42 (78% and 77% compared to 82% and 83%, respectively) whereas 10 mM 2-DG alone correlated with an initially greater % overall fit compared to controls at stage 38 (85% compared to 79%) and then a significantly lower % overall fit at stage 42 (79% compared to 83%).

When 4 HU lectin and 10 mM 2-DG were combined, no significant differences between these and control larvae were observed at stage 38 (76% compared to 79%) or 40 (82% compared to 82%) but % overall fit was significantly lower than those of controls at stage 42 (79% compared to 83%) (Figure 23 and Table 11).

In summary, 2-DG treatment correlated with greater overall pattern organization during early pigment pattern development (stage 38), but later (stage 40 and 42) pattern organization was lower than that of controls (Table 11).

In contrast, crude lectin treatment correlated with less pattern organization early in development (stage 38 and 40 % top fit was significantly lower with 4 HU treatment and stage 40 % top fit was significantly reduced with 8 HU crude lectin; stage 38 % overall fit was significanlty lower with 8 HU crude lectin treatment). However, by stage 42, there were no significant differences in % top fit or % overall fit in crude lectin treated animals compared to controls.

6. THE EFFECTS OF TREATMENTS ON MELANOPHORE MORPHOLOGY

At stage 38, the majority of melanophores observed in control flanks were of Type 2 morphology (slightly branched dot). At stages 40 and 42, however, four different morphology types were present and fairly equally represented (Figure 24 and Table 12; Note: Different concentrations

used for some of the confrontations are combined in Figure 24 and shown separately in Table 12). The more equal representation of morphologies at stage 42 appears to be due to a significant decrease in the representation of Type 2 morphologies and a significant increase in Type 1 (dot-like) and 4 (multipolar or highly branched) morphology representation.

10 and 25 mM 2-DG treatments initially correlated with significantly higher Type 1 representation at stage 38 compared to controls and a significantly higher Type 4 representation at stages 38 and 40, followed by a significantly lower Type 4 representation at stage 42 compared to controls (Table 12). Type 3 (irregularly branched) morphologies were significantly underepresented at all stages. In general, the morphology patterns of stage 38 2-DG-treated larvae resembled those of older stage 42 control larvae, wheras older stage 42 2-DG-treated larvae resembled younger stage 38 controls (Figure 24).

4 and 8 HU treatments also affected melanophore morphologies. Like controls, Type 2 morphologies were most common at stage 38, but 4 HU treatment correlated with significantly more Type 1 and 4 representation as well, compared to controls whereas 8 HU treatment correlated with significantly more Type 1 shapes than controls (Table 12). By stage 40, significantly fewer Type 3 morphologies were seen with both treatments compared to controls, but Type 2

morphologies continued to be highly represented. By stage 42, most melanophore morphologies were Type 2, closely resembling stage 38 controls (Figure 24).

Glucose treatment correlated with significantly more Type 1 and 4 shapes and less Type 3 morphologies, compared to controls. However, by stage 40, morphology representation was not significantly different from controls (Figure 24 and Table 12).

The combination of 10 mM 2-DG and 4 HU crude lectin correlated quite closely with the effects of 4 HU crude lectin alone (Table 12). Initially, Type 4 morphologies were highly represented at the expense of Type 1 and 3 representations, followed by a high proportion of Type 2 morphologies and a low proportion of Type 3 morphologies by stage 42 of development (Table 12).

For statistical treatment of this data, see Appendix II.

C. LECTIN ACTIVITY IN 2-DEOXYGLUCOSE-CONFRONTED AND CONTROL D/- AXOLOTLS DURING EMBRYONIC AND LARVAL DEVELOPMENT

In control dark embryos, lectin activity increased significantly at stage 28/29 (from 8 to 64 HU) and decreased to pre-migration level by stage 34/35 (8 HU) (Table 13). (These results correlate with those of dark embryos shown in Figure 14). A small transient increase in lectin activity also occured later at stage 40 (16 HU - weak) followed by a

significant reduction in lectin activity below pre-migratory level at stage 42 (2 - 4 HU) (Table 13). In contrast, lectin activity in 25 mM 2-DG-confronted dark embryos was lower than that of controls at stage 28/29 (16 HU compared to 64 HU), producing a much smaller transient increase in activity in stage 28/29 compared to controls (Table 13). Lectin activity of stage 34/35 and older animals was similar in treated and control animals (Table 13).

D. LECTIN-GLYCOSAMINOGLYCAN SPECIFICITY ASSAY

Chondroitin sulfate did not inhibit endogenous dark stage 28-30 lectin activity at concentrations of up to and including 1 milligram/millilitre (Table 14). At concentrations of 100 micrograms/millilitre and higher, heparan sulfate inhibited lectin activity. 1 microgram/millilitre hyaluronic acid solution completely inhibited lectin activity (Table 14).

TABLE 2. Lectin activity in dark (D/-) and white (d/d) axolotl carcass and ectoderm tissue measured as hemagglutination units (HU)/embryo. S.E.M. values are indicated as +/- values.

	HU/EMBRYO			
	DARK ECTODERM	WHITE ECTODERM	DARK CARCASS	WHITE CARCASS
PRE-MIGRATORY (ST. 20-24)	40.7 +/- 36	28.0 +/- 78	96.1 +/- 72	62.2 +/- 42
EARLY MIGRATORY (ST. 25-28)	1898 +/- 748	157 +/- 157	135 +/-442	635 +/-617
LATE MIGRATORY (ST. 29-34)	45.4 +/- 29	168 +/- 13	40.4 +/- 24	10.7 +/- 4

TABLE 3. Protein content in dark (D/-) and white (d/d) axolotl carcass and ectoderm tissue measured in micrograms/embryo. S.E.M. values are indicated as +/- values.

	MICROGRAMS PROTEIN/EMBRYO			
	DARK ECTODERM	WHITE ECTODERM	DARK CARCASS	WHITE CARCASS
PRE-MIGRATORY (ST. 20-24)	2.0 +/- 2.2	6.7 +/- 1.8	15.8 +/- 2.3	15.9 +/- 2.3
EARLY MIGRATORY (ST. 25-28)	2.1 +/- 1.1	5.3 +/- 1.1	28.1 +/- 6.2	16.9 +/- 5.3
LATE MIGRATORY (ST. 29-34)	9.1 +/- 1.6	4.0 +/- 1.4	17.3 +/- 3.7	15.9 +/- 3.3

TABLE 4.

A: Effect of different saccharides on the agglutination of rabbit erythrocytes by stage 25-28 dark (D/-) and white (d/d) axolotl ectoderm and carcass tissue.

SACCHARIDE	LOWEST CONCENTRATION OF SUGAR RESULTING IN INHIBITION OF LECTIN ACTIVITY
D(+) GLUCOSAMINE	<0.01 mM
N-ACETYL-D-GLUCOSAMINE	<0.01 mM
D(+) MANNOSE	<0.01 mM
D(+) GLUCOSE	1 mM
METHYL ALPHA-D-MANNOPYRANOSIDE	1 mM
ALPHA-D(+) MELIBIOSE	10 mM
LACTOSE	10 mM
METHYL ALPHA-D-GALACTOPYRANOSIDE	100 mM
D(+) FUCOSE	>100 mM
METHYL BETA-D-GALACTOPYRANOSIDE	>100 mM
BETA-THIODIGALACTOSIDE	>100 mM

B: The effect of different saccharides on the agglutination of rabbit erythrocytes by stage 25/26 dark (D/-) axolotl embryonic homogenate.

SACCHARIDE	LOWEST CONCENTRATION OF SUGAR RESULTING IN INHIBITON OF LECTIN ACTIVITY
D(+) MANNOSE	<0.01 mM
D(+) GLUCOSAMINE	<0.01 mM
N-ACETYL-D-GLUCOSAMINE	<0.01 mM
2-DEOXYGLUCOSE	0.01 mM
MANNAN	0.01 mM
MANNOPYRANOSYLPHENYLISOTHIOCYANATE	1 mM
N-ACETYL-D-GLUCOSAMINE PHENYLISOTHIOCYANATE	>10 mM
D(+) GLUCOSAMINE-2,6-DISULFATE	>10 mM
D(+) GLUCOSAMINE-2-SULFATE	>10 mM
N-ACETYL-D-GLUCOSAMINE-3-SULFATE	>10 mM

Table 5. The number of photographs used in the confrontation experiments (stripped and unstripped data are combined; unstripped control and unstripped 25 mM 2-DG data from confrontation experiment # 1 was combined with data from confrontation experiment # 2).*

TREATMENT	STAGE	NUMBER OF PHOTOS
CONTROL	38/39	28 (14 ANIMALS)
	40	12 (6 ANIMALS)
	42/43	23 (12 ANIMALS)
25 mM 2-DG	38/39	25 (13 ANIMALS)
	40	16 (8 ANIMALS)
	42/43	27 (14 ANIMALS)
10 mM 2-DG	38	5 (3 ANIMALS)
	40	16 (8 ANIMALS)
	42	12 (6 ANIMALS)
10 mM GLUCOSE	38	11 (6 ANIMALS)
	40	16 (8 ANIMALS)
	42	13 (7 ANIMALS)
10 mM GLUCOSE/ 10 mM 2-DG	38	10 (5 ANIMALS)
	40	15 (8 ANIMALS)
	42	15 (8 ANIMALS)
4 HU LECTIN	38	14 (7 ANIMALS)
	40	15 (8 ANIMALS)
	42	14 (7 ANIMALS)
4 HU LECTIN/ 10 mM 2-DG**	38	4 (2 ANIMALS)
	40	8 (4 ANIMALS)
	42	8 (4 ANIMALS)
8 HU LECTIN**	38	5 (3 ANIMALS)
	40	6 (3 ANIMALS)
	42	6 (3 ANIMALS)
TOTAL NUMBER OF PHOTOGRAPHS ANALYSED:		318 (164 ANIMALS)

* Representative photographs of each treatment at each photographed stage are presented in Figure 25 a, b, and c.

** All stripped embryos from these two experimental groups died before stage 38. Other than these deaths, there were few mortalities (numbers less than 16 (8 animals) reflect mortality and rejected photographs).

TABLE 6a. Left and right flank % top fit of stripped and unstripped control and 25 mM 2-deoxyglucose (2-DG)-confrontated dark (D/-) axolotl larvae at stages 38/39 and 42/43.

	UNSTRIPPED		STRIPPED	
	LEFT	RIGHT	LEFT*	RIGHT
CONTROL ST 38/39:	61+/-2.38	61 +/-2.21**	53+/-3.05s	63+/-3.53
CONTROL ST 42/43:	70+/-4.27	71 +/-2.28	53+/-3.05s	64+/-5.92s
25 mM 2-DG ST 38/39:	71+/-3.83s	69+/- 3.59s	64+/-8.02s	71+/-10.1s
25 mM 2-DG ST 42/43:	64+/-2.40s	63+/-4.73s	51+/-19.6s	65+/-16.9s

(* Only the left flank was stripped on stripped animals; the right flank of stripped animals was left undisturbed)

(** S.E.M. values as calculated using 95 % confidence intervals; differences (s) were significantly different from control right unstripped flank data as assessed using 95 % confidence intervals with alpha = 0.05)

TABLE 6b. Left and right flank % top fit of stage 38, 40, and 42 stripped and unstripped control dark (D/-) axolotl data*.

	UNSTRIPPED		STRIPPED	
STAGE	LEFT	RIGHT	LEFT**	RIGHT
38	58+/-6.72	59+/-9.35***	59+/-1.60	54+/-10.8
40	65+/-5.00	70+/-1.96	65+/-7.90	68+/-2.12
42	67+/-6.55	63+/-10.3	64+/-1.70	64+/-8.22

(* To compare control and experimental results, see Appendix III)

(** Only the left flank was stripped on stripped animals; the right flank was left undisturbed)

(*** S.E.M. values as calculated using 95 % confidence intervals; differences (s) were significantly different from control right unstripped flank data as assessed using 95 % confidence intervals with alpha = 0.05)

TABLE 7a. Overall number of melanophores on right and left stripped and unstripped flanks of control and 25 mM 2-deoxyglucose (2-DG)-confronted dark (D/-) axolotl larvae at stages 38/39 and 42/43.

	UNSTRIPPED		STRIPPED	
	RIGHT	LEFT	RIGHT	LEFT*
CONTROL ST 38/39:	113+/-7.85	114+/-4.57**	115+/-4.04	108+/-1.70s
CONTROL ST 42/43:	113+/-11.6s	127+/-12.1	96+/-6.96s	99+/-13.7s
25 mM 2-DG ST 38/39:	128+/-12.1s	120+/-18.1s	121+/-19.9s	116+/-14.2
25 mM 2-DG ST 42/43:	108+/-9.60s	113+/-5.28s	93+/-2.38s	82+/-11.9s

(* Only the left flank was stripped on stripped animals; the fight flank was left undisturbed)

(** S.E.M. values as calculated using 95% confidence intervals; differences (s) were significantly different from control right unstripped flank data as assessed using 95% confidence intervals with alpha = 0.05)

TABLE 7b. Overall number of melanophores on right and left stripped and unstripped stage 38, 40, and 42 flanks of control larvae*.

	UNSTRIPPED		STRIPPED	
STAGE	RIGHT	LEFT	RIGHT	LEFT**
38	112+/-7.69	105+/-9.05***	107+/-7.98	114+/-19.5
40	109+/-29.1	119+/-31.9	125+/-11.7	110+/-12.4
42	103+/-21.6	116+/-29.6	99+/-10.3	117+/-6.07

(* To compare control and experimental results, see Appendix IV)

(** Only the left flank was stripped on stripped animals; the right flank was left undisturbed)

(*** S.E.M. values as calculated using 95% confidence intervals; differences (s) were significantly different from control right unstripped flank data as assessed using 95% confidence intervals with alpha = 0.05)

TABLE 8. The number of melanophores/bar in control and experimental dark (D/-) axolotl larvae at stages 38, 40, and 42.

TREATMENT	STAGE		
	38	40	42
CONTROL	11+/-2.31	14+/-2.98	13+/-3.21
25 mM 2-DG	14+/-2.44s	11+/-1.10s	10+/-2.88
10 mM 2-DG	15+/-1.91s	11+/-2.12s	10+/-2.50
10 mM GLUCOSE	10+/-2.41	14+/-3.02	12+/-2.99
10 mM GLUCOSE/ 10 mM 2-DG	14+/-1.69s	13+/-2.48	11+/-2.28
4 HU LECTIN	10+/-1.70	16+/-4.08	12+/-3.55
8 HU LECTIN	12+/-3.36	11+/-3.09s	10+/-2.54
4 HU LECTIN/ 10 mM 2-DG	13+/-2.71	16+/-3.02	13+/-2.72

(s = significantly different from control value at the same stage of development as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test)

TABLE 9. The number of melanophores/flank in control and experimental dark (D/-) axolotl larvae at stages 38, 40, and 42.

TREATMENT	STAGE		
	38	40	42
CONTROL	112+/-9.59	116+/-13.57	110+/-16.99
25 mM 2-DG	121+/-14.1	110+/-16.5	96+/-14.4
10 mM 2-DG	127+/-3.30s	143+/-8.85s	127+/-12.0s
10 mM GLUCOSE	110+/-14.6	134+/-13.4s	115+/-14.2
10 mM GLUCOSE/ 10 mM 2-DG	137+/-7.20s	153+/-16.9s	129+/-14.4s
4 HU LECTIN	113+/-7.18	148+/-22.8s	132+/-24.9s
8 HU LECTIN	124+/-13.0s	121+/-19.1	111+/-15.2
4 HU LECTIN/ 10 mM 2-DG	123+/-9.04s	149+/-17.0s	137+/-14.0s

(s = significantly different from control value at the same stage of development as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test)

TABLE 10. % top fit in control and experimental dark (D/-) axolotl larvae at stages 38, 40, and 42.

TREATMENT	STAGE		
	38	40	42
CONTROL	59+/-5.58	67+/-4.95	67+/-6.84
25 mM 2-DG	71+/-3.37s	57+/-7.90s	56+/-7.37s
10 mM 2-DG	72+/-4.79s	58+/-3.91s	53+/-9.36s
10 mM GLUCOSE	58+/-4.40	66+/-6.95	62+/-7.33
10 mM GLUCOSE/ 10 mM 2-DG	66+/-6.51s	56+/-4.50s	54+/-6.96s
4 HU LECTIN	52+/-4.96s	60+/-4.32s	62+/-7.84
8 HU LECTIN	55+/-6.72	58+/-7.77s	64+/-12.6
4 HU LECTIN/ 10 mM 2-DG	57+/-4.57	62+/-6.91s	58+/-6.92s

(s = significantly different from control value at the same stage of development as assessed by Duncan's Multple Range Test and Tukey's Studentized Range (HSD) Test)

TABLE 11. % overall fit in control and experimental dark (D/-) axolotl larvae at stages 38, 40, and 42.

TREATMENT	STAGE		
	38	40	42
CONTROL	79+/-3.54	82+/-3.06	83+/-3.53
25 mM 2-DG	86+/-3.54s	78+/-4.30s	79+/-4.04s
10 mM 2-DG	85+/-2.63s	80+/-2.77	79+/-5.41s
10 mM GLUCOSE	79+/-3.00	82+/-4.46	81+/-3.67
10 mM GLUCOSE/ 10 mM 2-DG	84+/-3.50s	78+/-4.29s	77+/-5.39s
4 HU LECTIN	76+/-2.06	81+/-4.86	81+/-2.68
8 HU LECTIN	75+/-4.41s	79+/-5.00	85+/-5.47
4 HU LECTIN/ 10 mM 2-DG	76+/-1.93	82+/-4.48	79+/-2.43s

(s = significantly different from control value at the same stage of developemnt as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test)

TABLE 12. % melanophores of Type 1, 2, 3, and 4 morphologies in control and experimental dark (D/-) axolotl flanks at stages 38, 40, and 42.

		% MORPHOLOGIES			
TREATMENT	STAGE	1	2	3	4
CONTROL	38	8	66	21	5
	40	X{ 14	X{ 40	33	X{ 14
	42	25	25	20	32
25 mM 2-DG	38	20x	42	5x	33x
	40	19	S{ 35	S{ 5x	S{ 42x
	42	47x	41x	5x	8x
10 mM 2-DG	38	19x	37x	4x	40x
	40	14	S{ 44	S{ 6x	S{ 37x
	42	30	56	7x	8x
10 mM GLUCOSE	38	20x	42	5x	33x
	40	S{ 16	S{ 49	S{ 14	S{ 23x
	42	18	37	20	25
10 mM GLUCOSE/	38	15x	43	13	28x
10 mM 2-DG	40	14	S{ 47	S{ 7x	S{ 32
	42	35	48x	3x	9x
4 HU LECTIN	38	15x	43	13	28x
	40	S{ 9	S{ 57	S{ 8x	S{ 28x
	42	25	61x	1x	13x
8 HU LECTIN	38	22x	64	4x	11
	40	S{ 16	62	S{ 6x	S{ 17
	42	17	70x	9x	5x
4 HU LECTIN/	38	3x	48	6x	43x
10 mM 2-DG	40	7	S{ 44	11x	S{ 41x
	42	22	42x	8x	30

(as assessed by Multivariant Analysis of Variance Test and Tukey's Studentized Range Test; please see Appendix II for a more detailed expanation of these statistical tests)
(x = significant change in % value mean from control value mean at the same stage of development; **X** = change in control value means over the three stages of development is significant; **S** = change in value means over three stages of development is siginificantly different from changes in control mean values in the same morphology category. For example, the % representation of Type 1 morphologies in controls changes significantly between stage 38 and 42, denoted by **X**; this increase is not expressed in 8 HU lectin-treated animals but is expressed in (continued)

(Table 12 continued) 4 HU lectin-treated animals, denoted by **s**; there is significanlty more Type 1 melanophore representation at stages 38 and 42 in 25 mM 2-DG-treated animals compared to control Type 1 representation at these two stages, denoted by x's).

TABLE 13. Lectin activity, expressed in hemagglutination (HU) units, in control and 25 mM 2-deoxyglucose (2-DG)-confronted dark (D/-) axolotl embryos and larvae.

STAGE	# EMBRYOS	TREATMENT	LECTIN ACTIVITY
15	10	N/A	8 HU
23	10	CONTROL	8 HU
23	10	25 mM 2-DG	4 - 8 HU
28/29	10	CONTROL	64 HU*
28/29	10	25 mM 2-DG	16 HU
34/35	5	CONTROL	8 HU
	5	25 mM 2-DG	8 - 16 HU
38	5	CONTROL	8 HU
	5	25 mM 2-DG	8 HU
40	4	CONTROL	(weak) 16 HU
	4	25 mM 2-DG	8 - 16 HU
42	3	CONTROL	2 - 4 HU*
	3	25 mM 2-DG	2 - 4 HU*

(* significantly different from stage 23 control lectin activity, as assessed by ANOVA; $p < 0.05$)

TABLE 14. Effect of different glycosaminoglycans (GAG's) on the agglutination of rabbit erythrocytes by dark (D/-) stage 28-30 axolotl embryonic homogenate.

GAG	LOWEST CONCENTRATION OF GAG RESULTING IN INHIBITION OF LECTIN ACTIVITY
HYALURONIC ACID	<1 micrograms/millilitre
HEPARAN SULFATE	100 micrograms/millilitre
CHONDROITIN SULFATE	>1000 micrograms/millilitre

Table 15. Confrontation Experiment # 2: Left and right flank % top fit of stage 38, 40, and 42 stripped and unstripped control and experimental dark (D/-) axolotl larvae.

TREATMENT	UNSTRIPPED		STRIPPED	
STAGE	LEFT	RIGHT	LEFT	RIGHT
38				
CONTROL	58	59	59	54
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	69	63	68	64
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	57	57
25 mM 2-DEOXYGLUCOSE	70	71	70	68
10 mM 2-DEOXYGLUCOSE	75	NA	71	68
10 mM GLUCOSE	59	57	59	55
4 HU CRUDE LECTIN	52	54	49	53
8 HU CRUDE LECTIN	NA	NA	56	53
40				
CONTROL	65	70	65	68
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	54	54	56	NA
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	66	62
25 mM 2-DEOXYGLUCOSE	55	58	59	54
10 mM 2-DEOXYGLUCOSE	56	65	55	65
10 mM GLUCOSE	62	66	67	62
4 HU CRUDE LECTIN	58	58	59	62
8 HU CRUDE LECTIN	NA	NA	59	62

(Table 15 cont.)

	42			
CONTROL	67	63	64	64
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	50	48	60	56
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	56	59
25 mM 2-DEOXYGLUCOSE	59	60	57	59
10 mM 2-DEOXYGLUCOSE	51	63	52	53
10 mM GLUCOSE	62	60	67	64
4 HU CRUDE LECTIN	57	57	64	60
8 HU CRUDE LECTIN	NA	NA	68	58

Table 16. Confrontation Experiment # 2: Overall number of melanophores on right and left stripped and unstripped stage 38, 40, and 42 flanks of control and experimental dark (D/-) larvae.

TREATMENT	UNSTRIPPED		STRIPPED	
STAGE 38	RIGHT	LEFT	RIGHT	LEFT
CONTROL	112	105	107	114
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	132	127	107	114
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	122	124
25 mM 2-DEOXYGLUCOSE	117	118	121	126
10 mM 2-DEOXYGLUCOSE	127	NA	130	125
10 mM GLUCOSE	118	103	107	112
4 HU CRUDE LECTIN	112	115	111	112
8 HU CRUDE LECTIN	NA	NA	124	125
40				
CONTROL	109	119	125	110
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	148	190	NA	NA
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	150	149
25 mM 2-DEOXYGLUCOSE	112	122	107	101
10 mM 2-DEOXYGLUCOSE	140	140	156	144
10 mM GLUCOSE	140	139	149	144
4 HU CRUDE LECTIN	152	143	151	148
8 HU CRUDE LECTIN	NA	NA	121	120

(Table 16 cont.)

42

CONTROL	103	116	99	117
10 mM GLUCOSE/ 10 mM 2-DEOXYGLUCOSE	127	125	124	140
4 HU CRUDE LECTIN/ 10 mM 2-DEOXYGLUCOSE	NA	NA	140	133
25 mM 2-DEOXYGLUCOSE	109	102	90	89
10 mM 2-DEOXYGLUCOSE	139	126	122	120
4 HU CRUDE LECTIN	122	121	145	139
8 HU CRUDE LECTIN	NA	NA	118	104

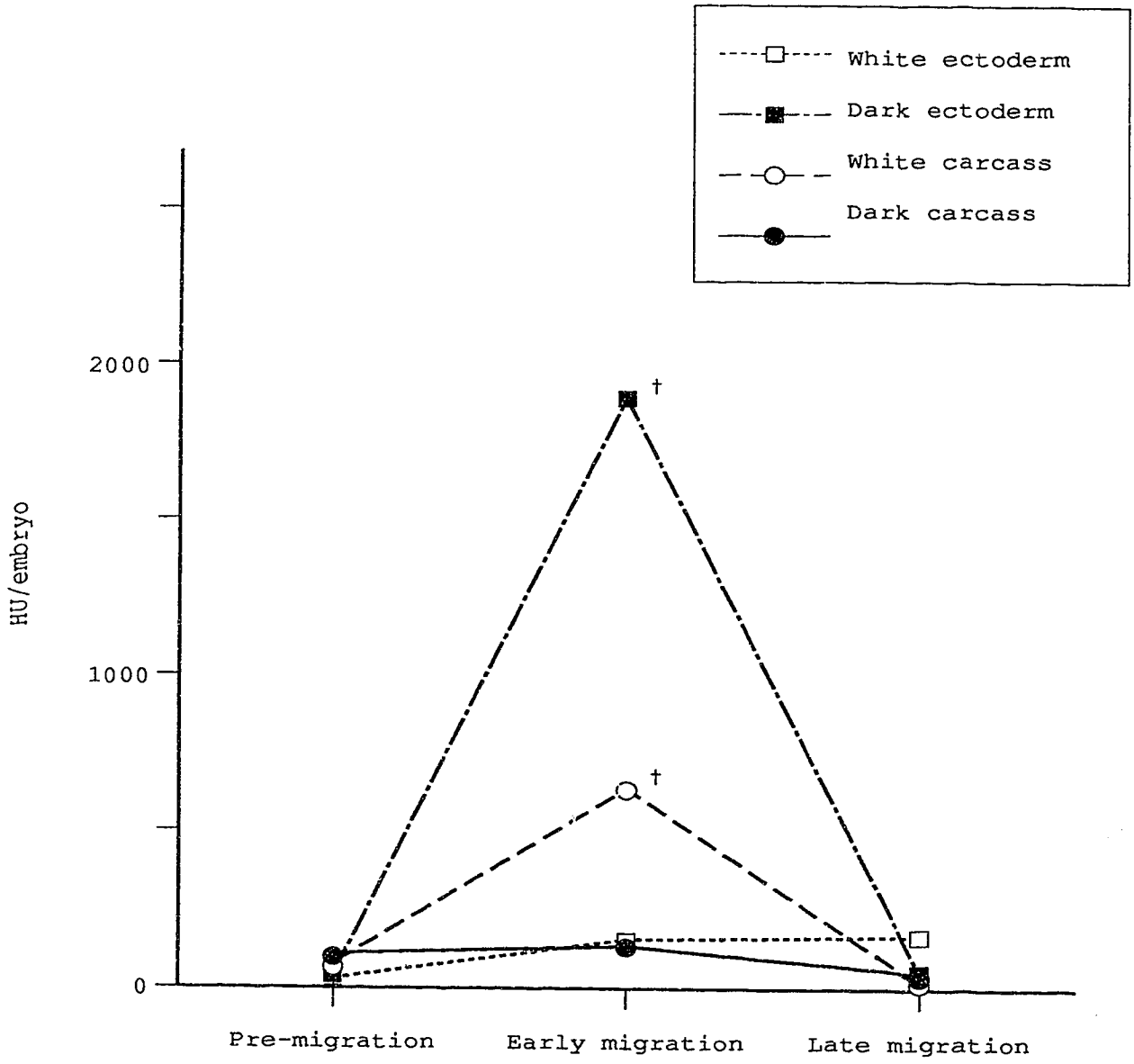


Figure 14. Lectin activity in dark (D/-) and white (d/d) axolotl ectoderm and carcass tissues, expressed in hemagglutination units (HU)/embryo, during pre-migratory, early migratory, and late migratory neural crest stages.

† See Table 2 for actual values and S.E.M.
Significant increase as assessed by ANOVA, $p \leq 0.05$.

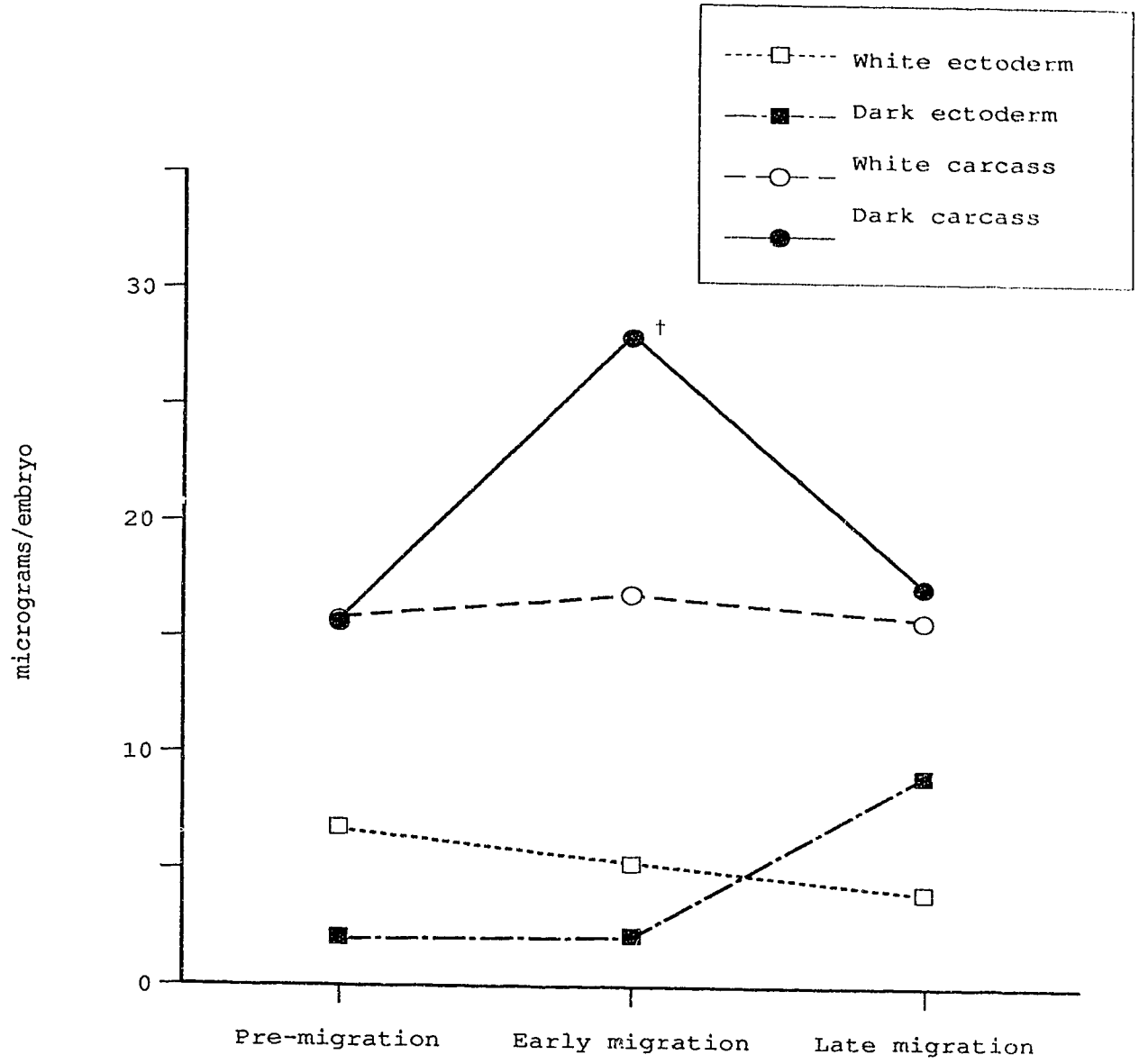


Figure 15. Protein content (micrograms/embryo) in dark (D/-) and white (d/d) axolotal ectoderm and carcass tissues during premigratory, early migratory, and late migratory stages of neural crest development.

† See Table 3 for actual values and S.E.M.
Significant increase as assessed by ANOVA, $p \leq 0.05$.

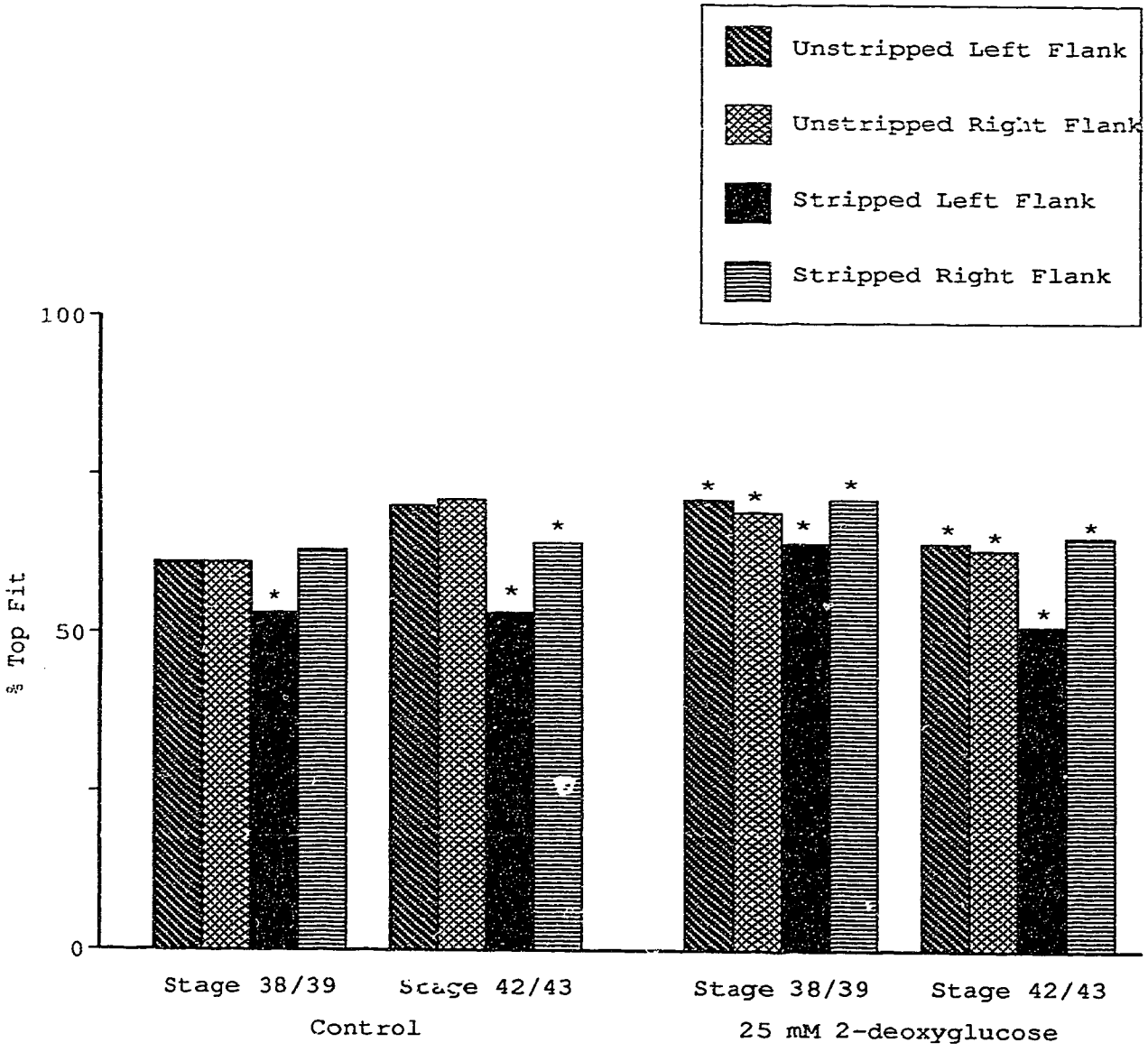


Figure 16. Experiment #1. Left and right percent top fit of stripped and unstripped control and 25 mM 2-deoxyglucose-confronted dark (D/-) axolotl larvae at stages 38/39 and 42/43.

* Significantly different from control right unstripped flank data as assessed by a Two-sided Normal Test.

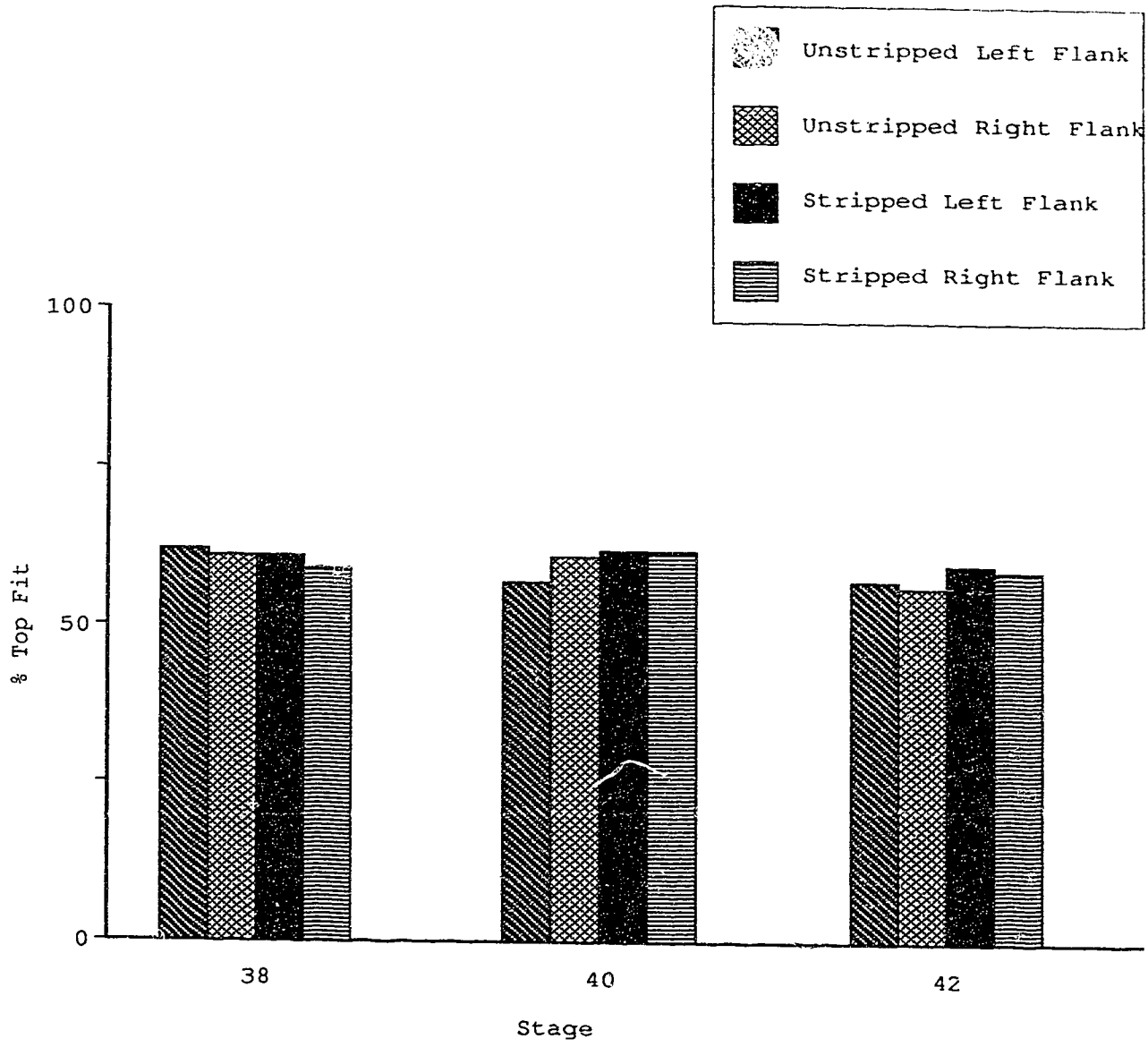


Figure 17. Experiment #2. Left and right percent top fit of stripped and unstripped stage 38, 40, and 42 averaged control and test (dark (D/-) axolotl).

* Significantly different from control right unstripped flank data as assessed by a Two-sided Normal Test.

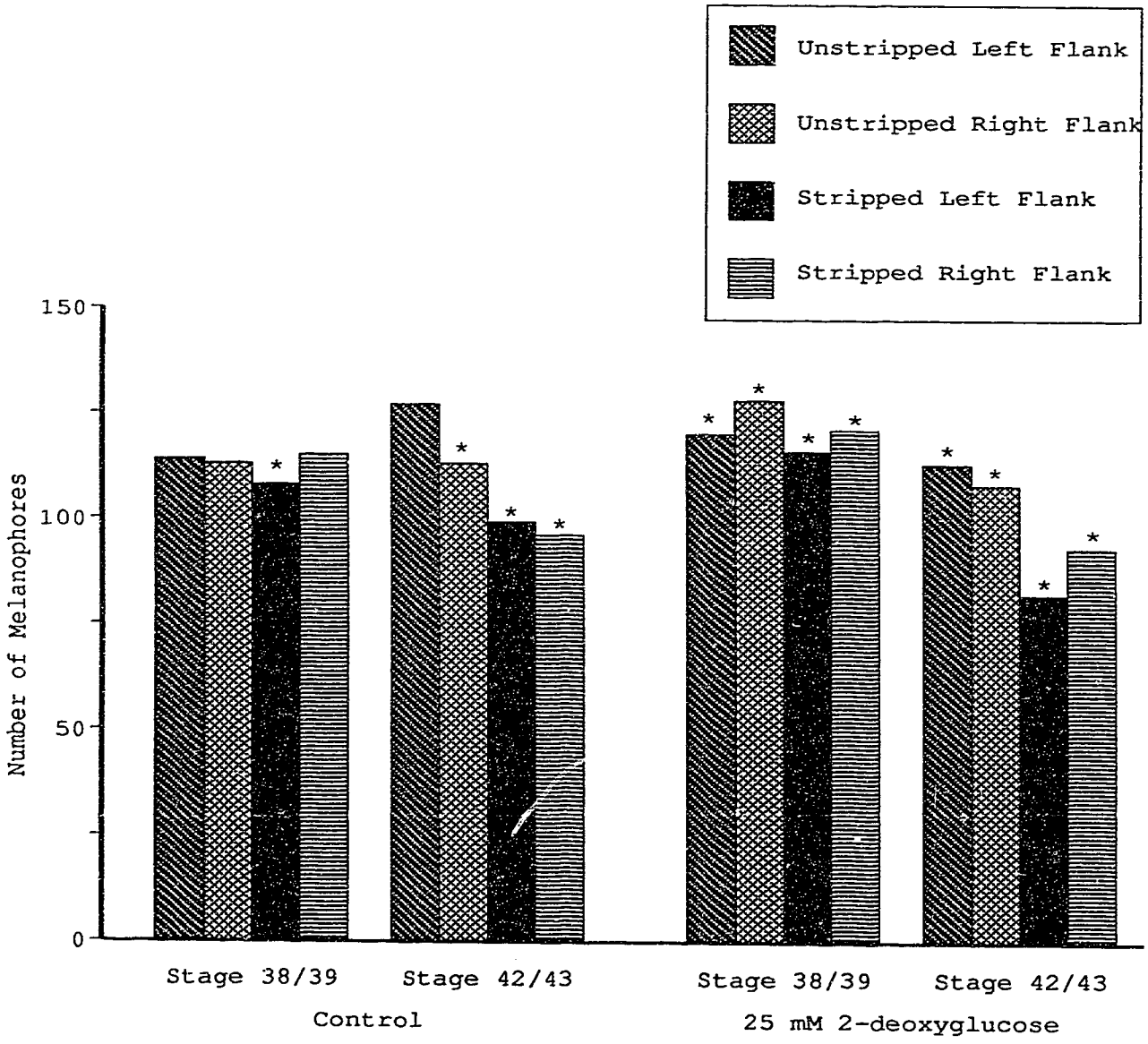


Figure 18. Experiment #1. Overall number of melanophores on right and left stripped and unstripped flanks of control and 25 mM 2-deoxyglucose confronted dark (D/-) axolotl larvae at stages 38/39 and 42/43.

* Significantly different from control right unstripped flank data as assessed by a Two-sided Normal Test.

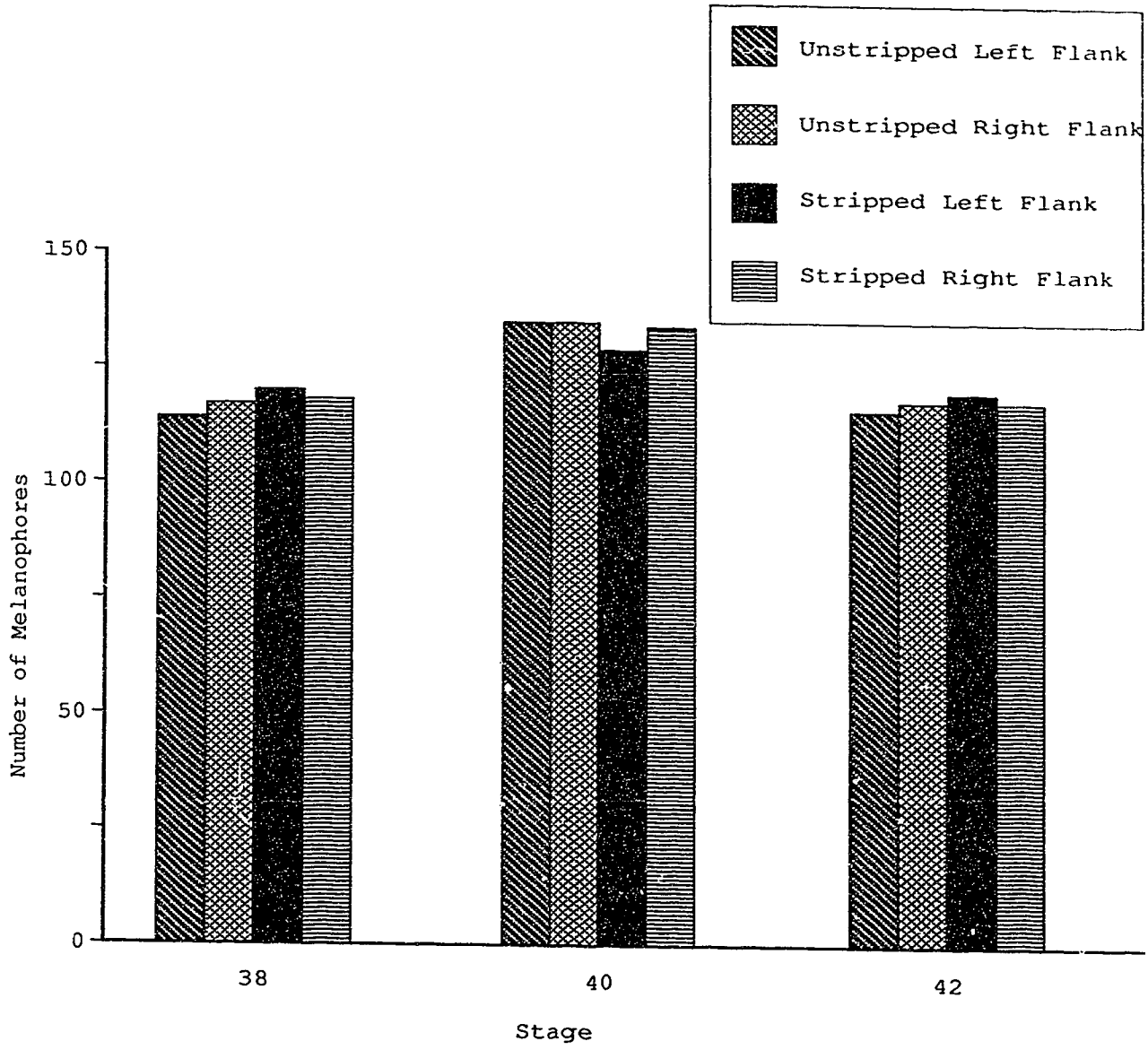


Figure 19. Experiment #2. Overall number of melanophores on right and left stripped and unstripped flanks of stage 38, 40, and 42 averaged control and test (dark (D/-) axolotl) data.

* Significantly different from control right unstripped flank data as assessed by a Two-sided Normal Test.

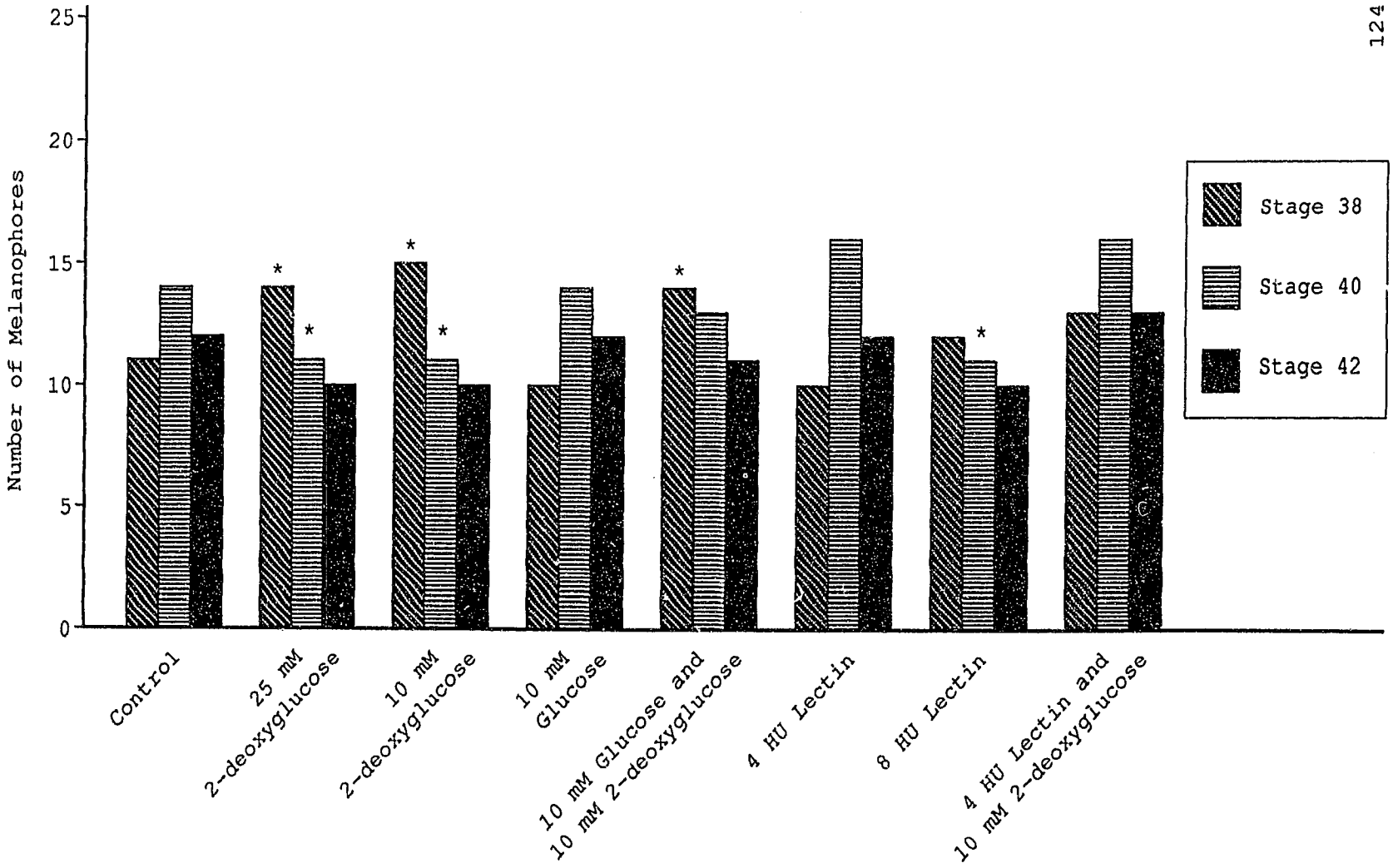


Figure 20. Number of melanophores per bar of control and experimental dark (D/-) axolotl larvae of stages 38, 40, and 42.

* Significantly different from control value at the same developmental stage as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test; α=0.05

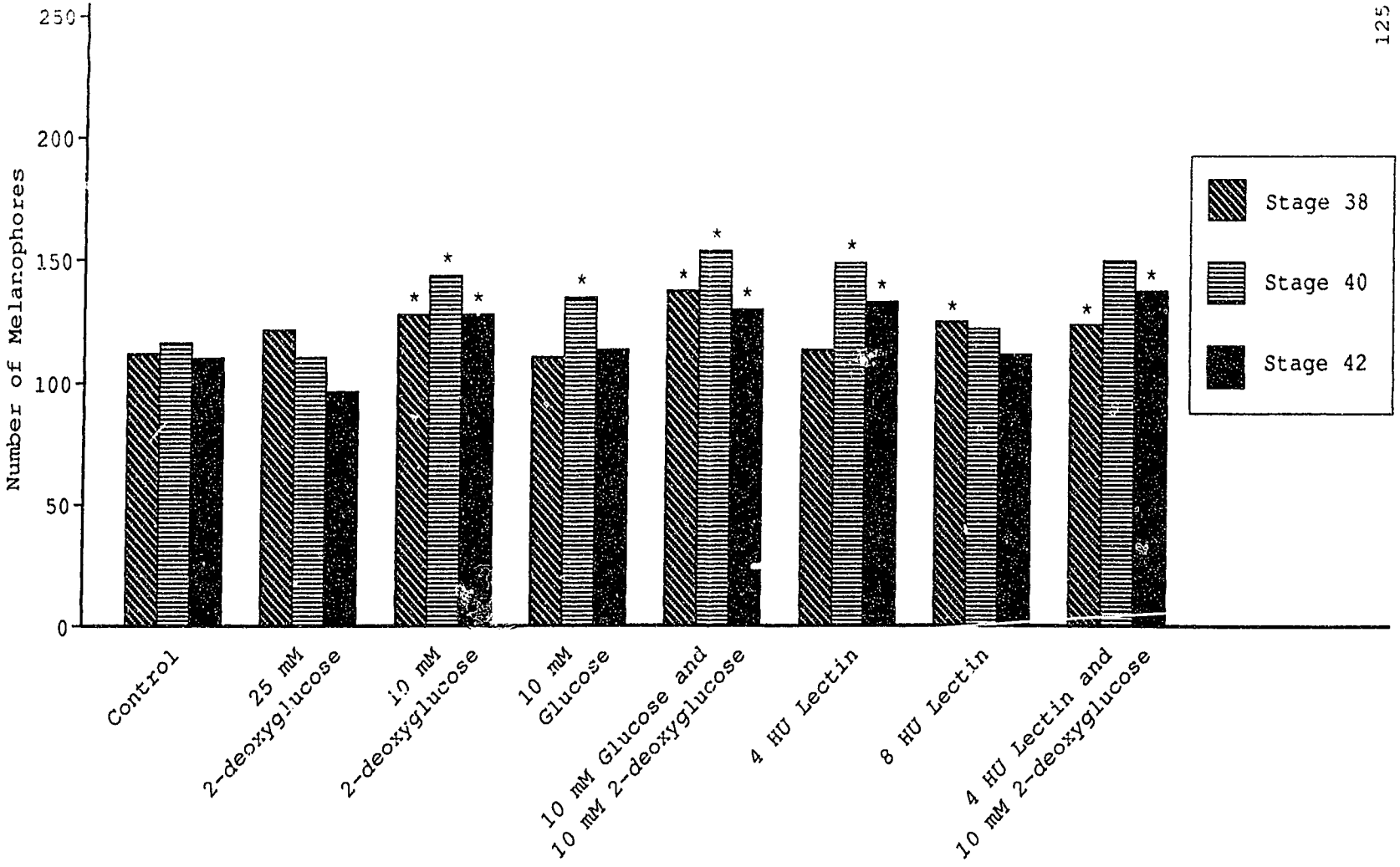


Figure 21. Number of overall flank melanophores of control and experimental dark (D/-) axolotl larvae of stages 38, 40, and 42.

* Significantly different from control value at the same developmental stage as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test; $\alpha=0.05$

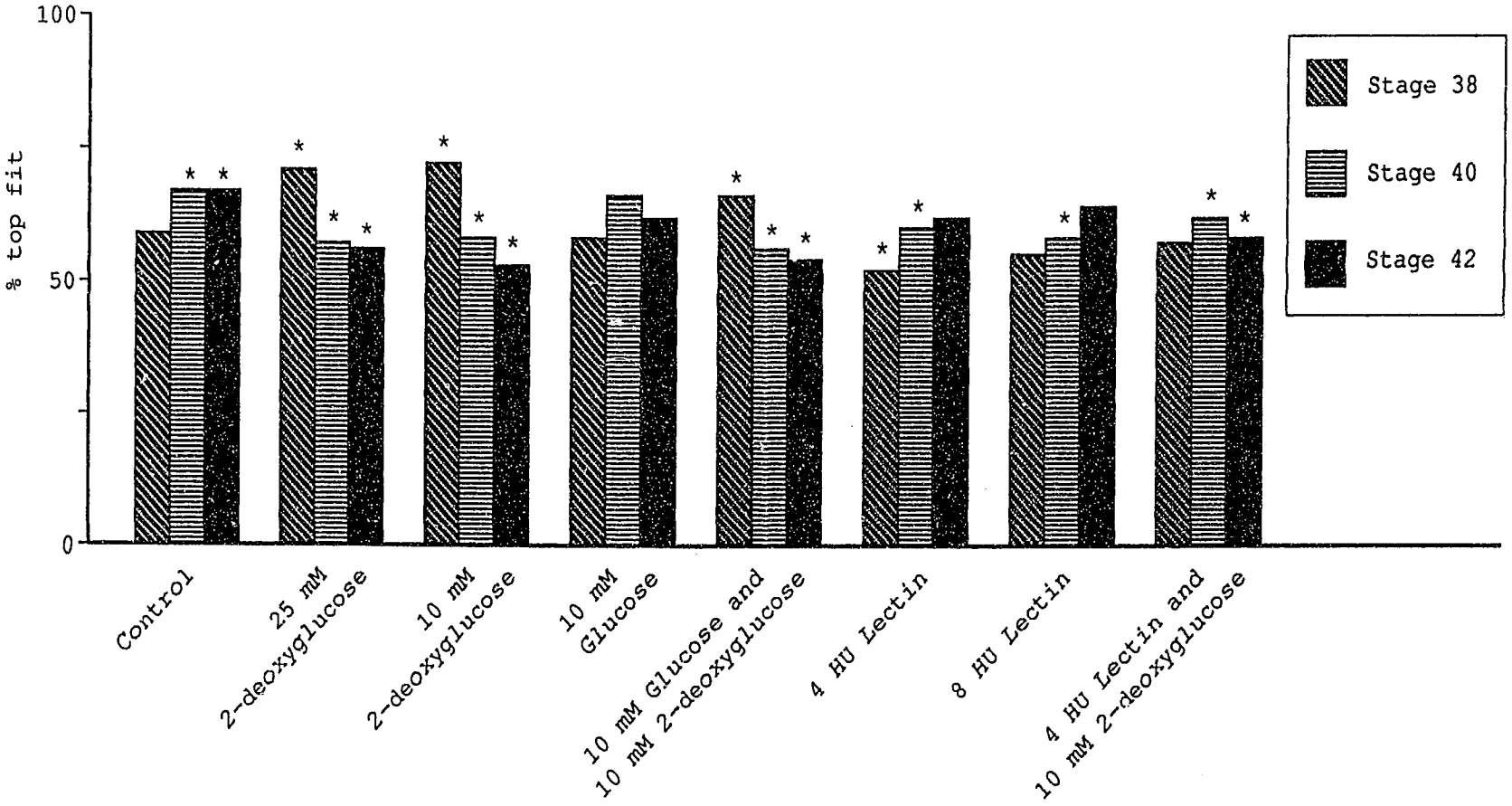


Figure 22. % top fit of control and experimental dark (D/-) axolotl larvae of stages 38, 40, and 42.

* Significantly different from control value at the same developmental stage as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test; α=0.05

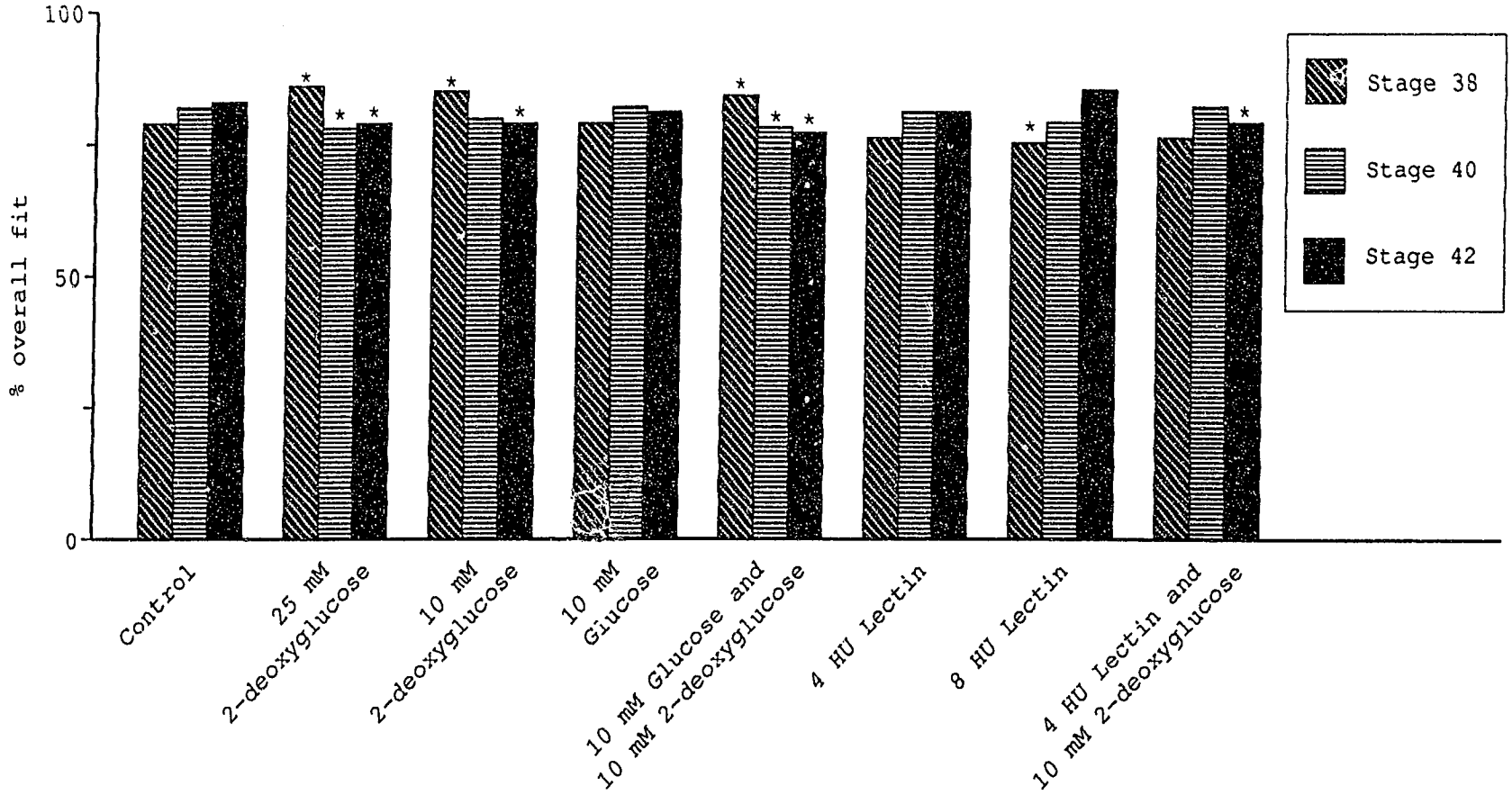


Figure 23. % overall fit of control and experimental dark (D/-) axolotl larvae of stages 38, 40, and 42.

* Significantly different from control value at the same developmental stage as assessed by Duncan's Multiple Range Test and Tukey's Studentized Range (HSD) Test; α=0.05

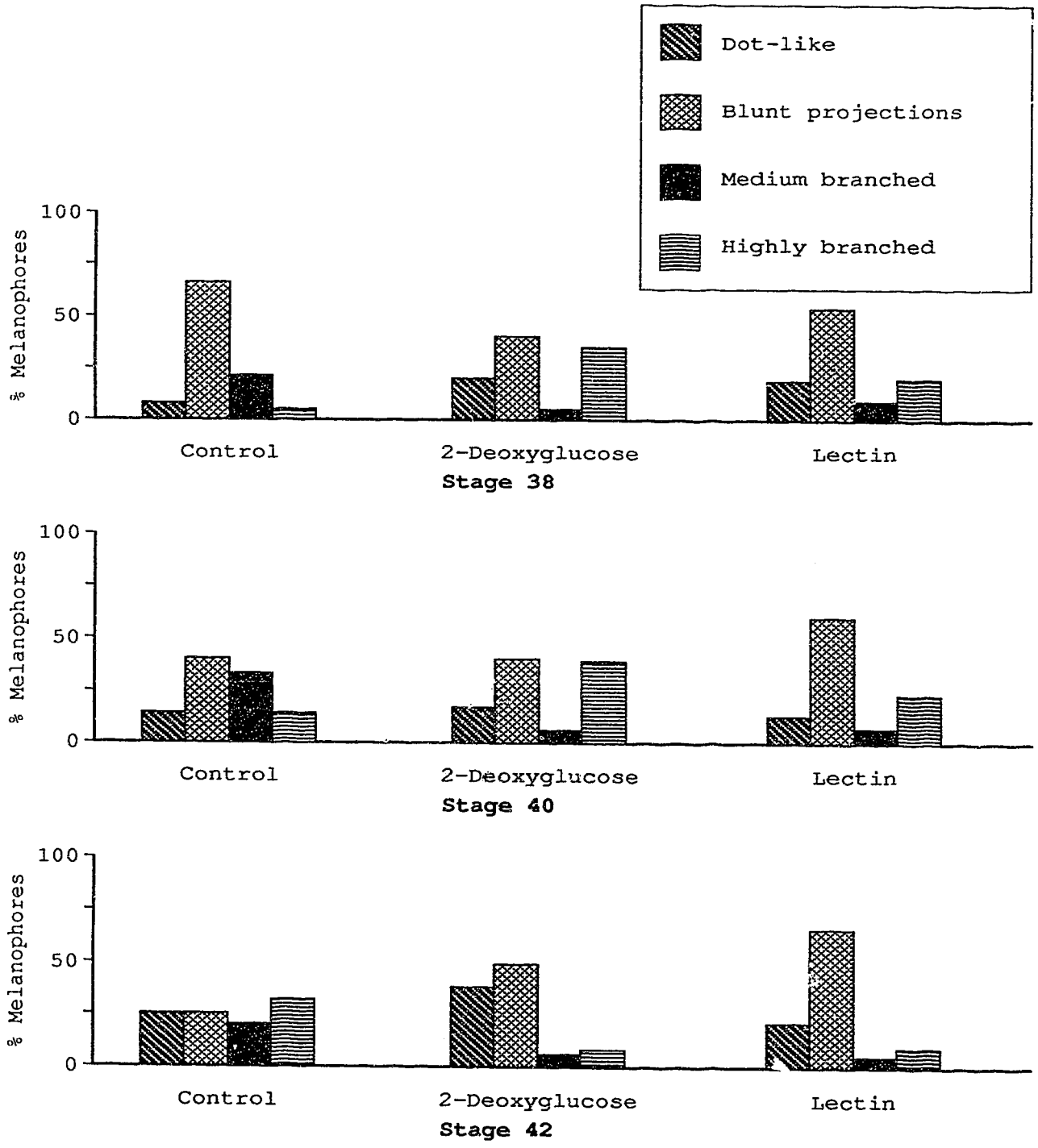


Figure 24. % Melanophores of type 1, 2, 3, and 4 morphologies in control and experimental dark (D/-) axolotl larval flanks at stages 38, 40, and 42.

Figure 25. Representative photographs of experimental and control dark (D/-) axolotl photographs at stages 38, 40, and 42.

Figure 25a. Representative photographs of confrontation experiment # 2 experimental and control stage 38 dark axolotl larval flanks.

A. CONTROL
B. 10 mM GLUCOSE/10 mM 2-DEOXYGLUCOSE
C. 10 mM 2-DEOXYGLUCOSE
D. 25 mM 2-DEOXYGLUCOSE
E. 10 mM GLUCOSE
F. 4 HU CRUDE LECTIN/10 mM 2-DEOXYGLUCOSE
G. 4 HU CRUDE LECTIN
H. 8 HU CRUDE LECTIN

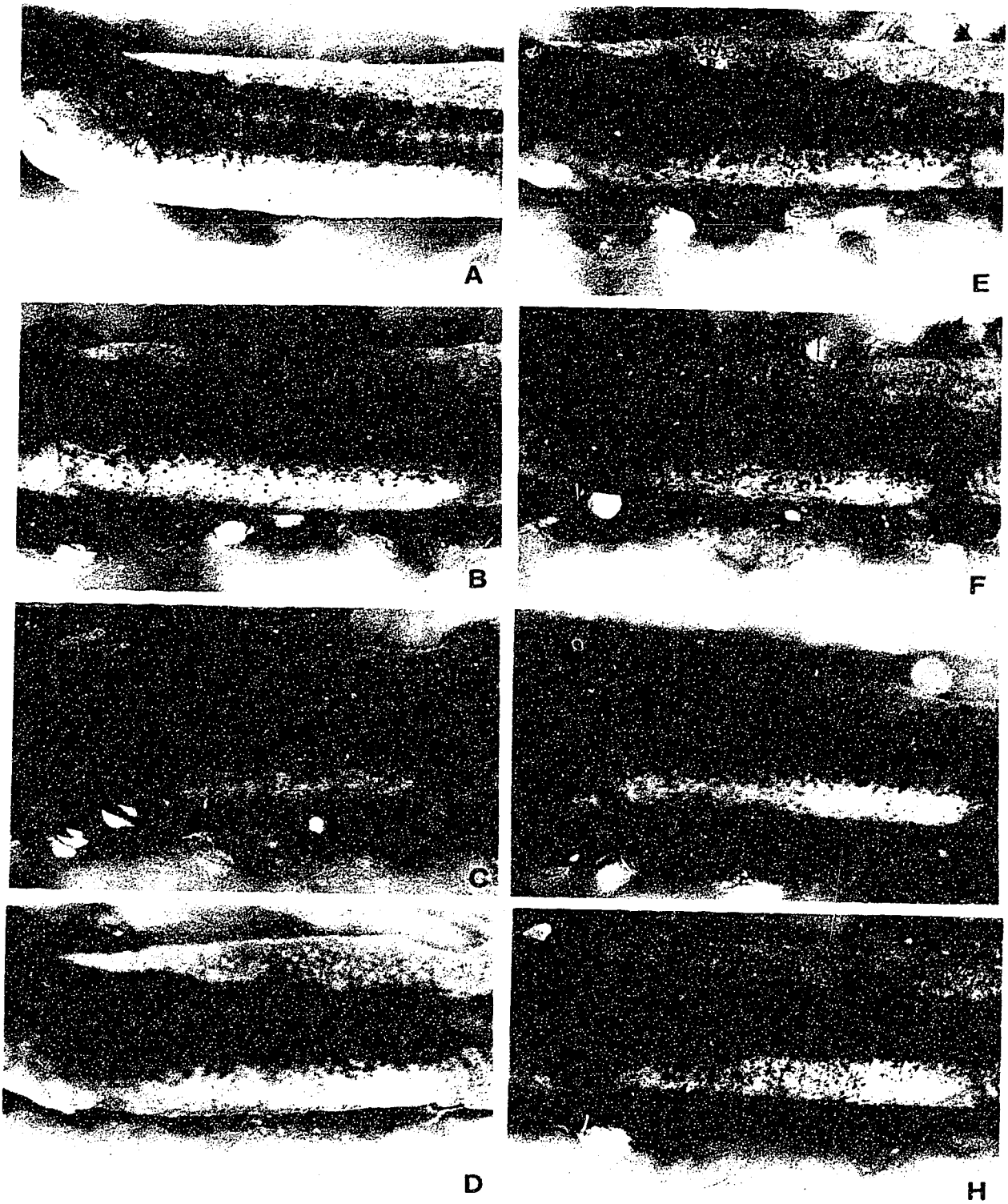

A
E
B
F
C
D
H

Figure 25b. Representative photographs of confrontation experiment # 2 experimental and control stage 40 dark axolotl larval flanks.

A. CONTROL
B. 10 mM GLUCOSE/10 mM 2-DEOXYGLUCOSE
C. 10 mM 2-DEOXYGLUCOSE
D. 25 mM 2-DEOXYGLUCOSE
E. 10 mM GLUCOSE
F. 4 HU CRUDE LECTIN/10 mM 2-DEOXYGLUCOSE
G. 4 HU CRUDE LECTIN
H. 8 HU CRUDE LECTIN

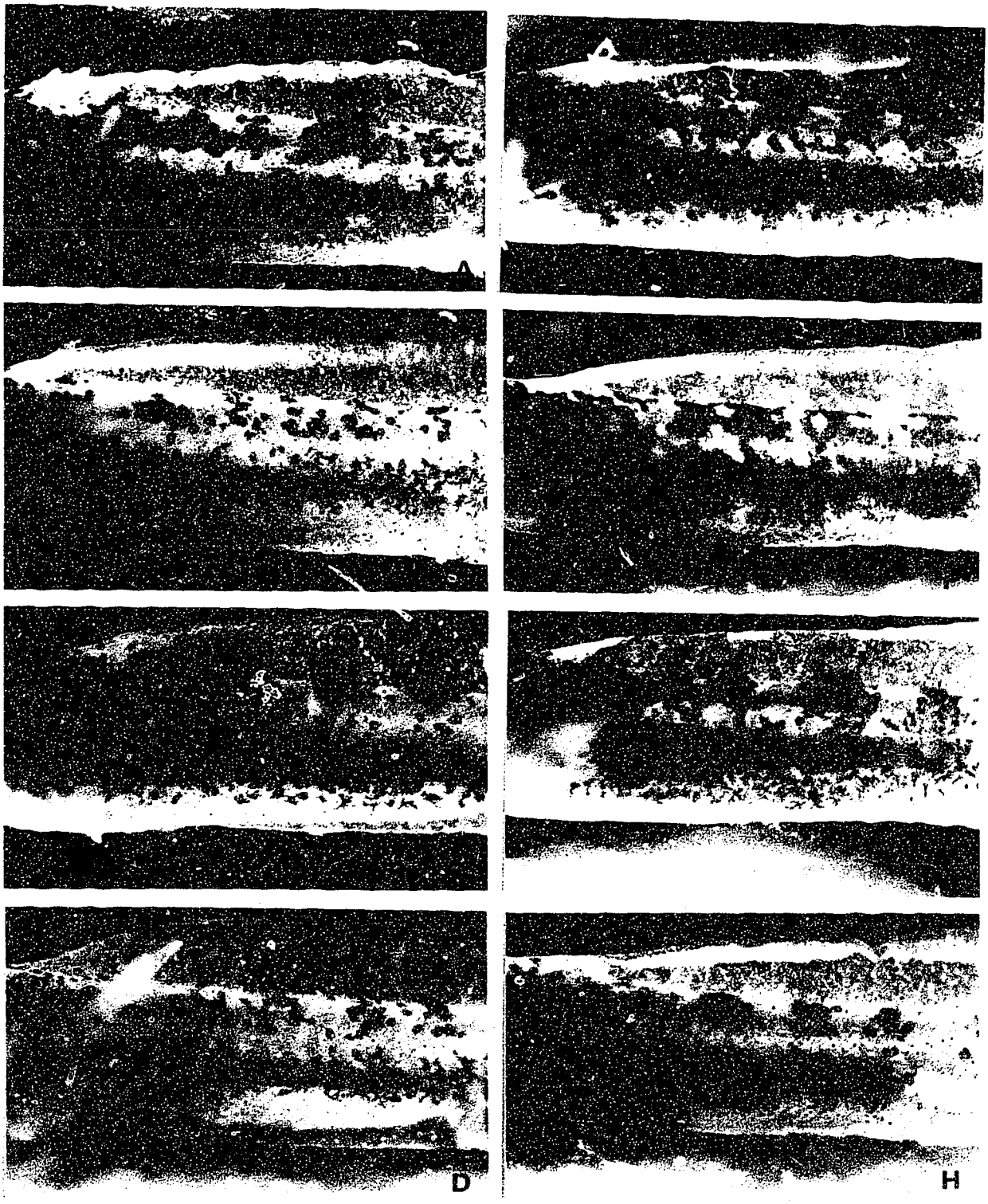
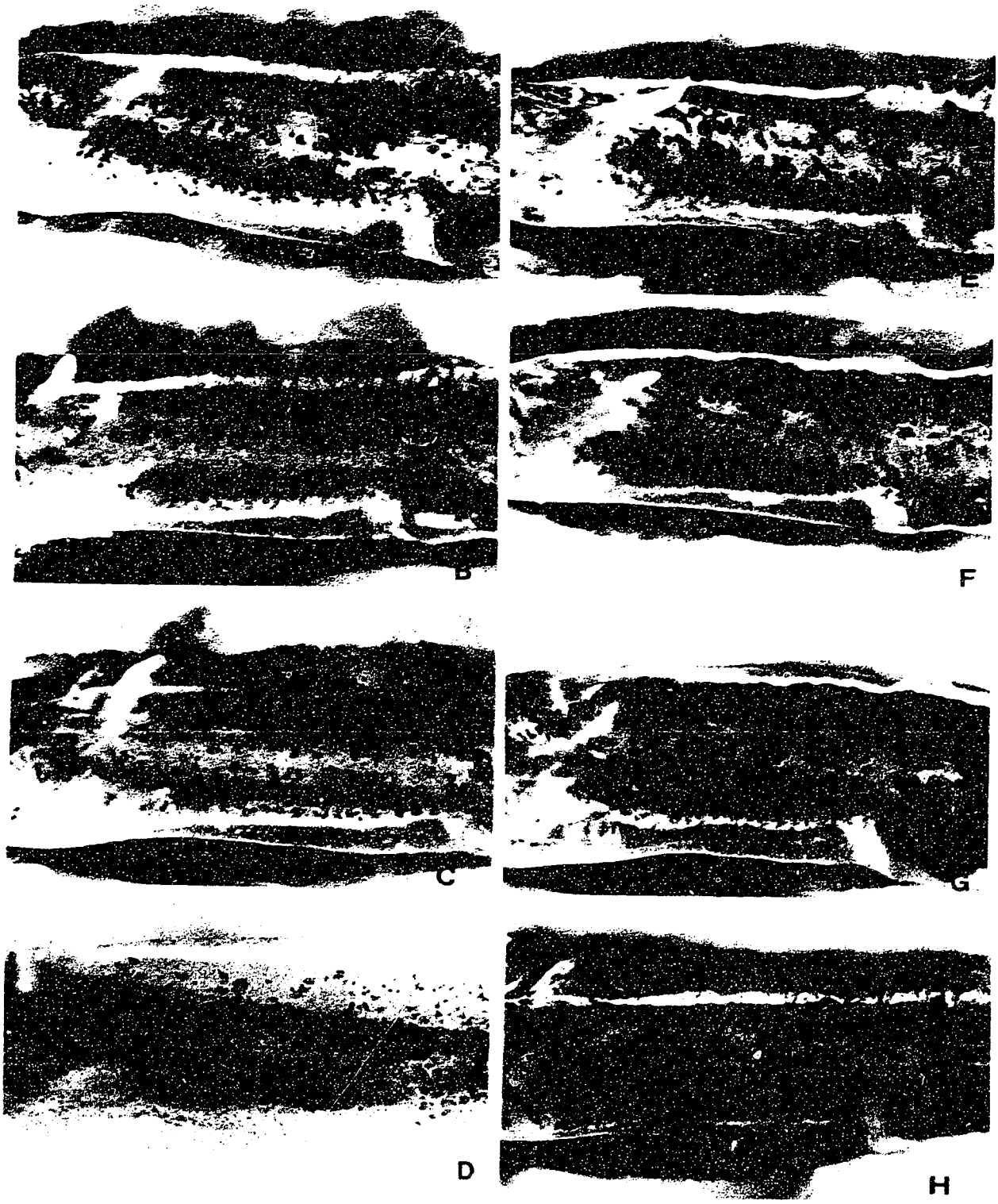

A
D
H

Figure 25c. Representative photographs of confrontation experiment # 2 experimental and control stage 42 dark axolotl larval flanks.

A. CONTROL
B. 10 mM GLUCOSE/10 mM 2-DEOXYGLUCOSE
C. 10 mM 2-DEOXYGLUCOSE
D. 25 mM 2-DEOXYGLUCOSE
E. 10 mM GLUCOSE
F. 4 HU CRUDE LECTIN/10 mM 2-DEOXYGLUCOSE
G. 4 HU CRUDE LECTIN
H. 8 HU CRUDE LECTIN


B
C
D
E
F
G
H

CHAPTER 4

DISCUSSION

A. DIFFERENCES IN THE LOCALIZATION AND TITRE OF LECTIN ACTIVITY IN DARK AND WHITE EMBRYOS

Endogenous lectin activity peaks during early neural crest (NC) migration in dark axolotl ectoderm (embryonic epidermis) (Table 2; Figure 14). An important point is that my investigation has not demonstrated that ectodermal lectin is associated with the ECM. However, the transplantation experiments cited in Chapter 1 implicate an ectodermal defect so it is tempting to speculate that white ectoderm produces less lectin or releases less into the subepidermal matrix and this is involved in the generation of the defect. Lectin activity also increases in white ectoderm but to a much lesser extent (Table 2). This result was very interesting, and suggests that there may be a connection between insufficient lectin present during early NC migration and the inability of white mutant NC cells to invade and migrate through the subepidermal ECM. In this work, a study of the deposition of lectin in the embryo was not done. However, the differences measured between dark and white ectoderm versus carcass tissue (Table 2) suggest that lectin may be localized differently in dark and white embryos. Interestingly, white carcass lectin activity increases significantly during early migration but dark

carcass tissue does not, suggesting that lectin is present in the white embryo during early migration but the majority of it is localized more centrally in the embryo, in contrast to the dark embryos which contain most of their lectin within the ectodermal tissue (Table 2). This result is interesting considering that in white embryos the majority of NC cells appear to migrate more centrally within the embryo.

Also of interest is the finding that even though white embryos contain more carcass lectin activity than dark embryos, the total amount of lectin activity (carcass plus ectoderm activities) is still only approximately one third in white embryos compared to dark embryos (Table 2).

Two conclusions can be made from these measurements. First, lectin activity peaks in both embryos during early NC migration, but white embryos contain significantly less total lectin activity than dark ones. Second, lectin activity peaks in tissues more centrally located in white embryos compared to dark embryos.

It would be interesting to further investigate these results by attempting to determine exactly where the endogenous lectin is located and perhaps which cell type(s) produce it.

The lectin of dark animals is specific for the three simple saccharides, D(+) mannose, D(+) glucosamine, and N-acetyl-D-glucosamine (Table 4). Because all three sugars

have in common identical configurations at C3, C4, C5, and C6 (see Figure 26), this lectin may recognize this specific configuration of the hexose ring.

Based on their configurations, these sugars represent Type III sugars by the classification scheme composed by Makela (1957) and reviewed by Goldstein and Poretz (1986). This classification takes into account the observation that most lectins are tolerant about some variety at C2 in regard to sugar specifity but C3 and C4, particularly, must be specific. Surprizingly, glucose, which is identical at C3, C4, C5, and C6 to the three other sugars (Figure 26), did not interact with this lectin. This suggests that other characteristics of the sugars in addition to their configurations (some of the characteristics of saccharides that affect lectin specificity are described in detail in Chapter 1) may be important in regard to this lectin's activity.

Interestingly, the lectin is also specific for the glycosaminoglycan (GAG), hyaluronic acid (HA) (Table 14). This molecule is a major component of the ECM, and it is tempting to speculate that the endogenous lectin is present in the ECM and binding to HA. HA consists of a repeating saccharide dimer composed of N-acetyl-D-glucosamine and glucuronic acid (see Figure 27), to which proteoglycan aggregates often attach, to form a large aggregate structure. It seems likely that the lectin interacts with

the N-acetyl-D-glucosamine component of this molecule (a possible functional relationship between axolotl lectin and the ECM is discussed later in this Chapter)

Although the calcium requirement has not been tested on this lectin, based on its specificity for mannose and N-acetyl-D-glucosamine, it is tempting to speculate that this lectin is a C-type lectin (C-type lectins have in common a Ca^{++} requirement for sugar binding and are usually extracellular; Drickamer, 1988). This group includes, for example, lectins discovered in ground scorpion epidermal membrane (Mouchamp, 1982), rat liver microsomes (Maynard and Baeziger, 1982), a mannose-binding protein (MBP) in rabbit, rat, and bovine sera (Kawasaki et al., 1989), and a mannose receptor on human placenta and macrophages (Tyler et al., 1990). Lectins in this family appear to have a variety of roles, some of which appear to be immune-related (Kawasaki et al., 1989; Thiel and Ried, 1989). These lectins also appear to have a variety of homologies to other adhesive proteins (the characteristics of this family of lectins is discussed in detail in Chapter 1).

The sugar specificity of dark and white lectin is the same (Table 4a), indicating that the lectin activity measured in the different tissues of dark and white embryos may represent a single lectin.

Thus, this lectin increases in activity simultaneously with the process of NC migration and, based on its sugar

specificity, may interact with HA in the ECM.

To begin to study the lectin's role in pigment pattern development in the axolotl, I then designed experiments to measure the effects of modulating lectin activity on pigment pattern development (ie., to investigate the functional relationship, if any between lectin activity and pigment cell localization in the skin) (Table 5). These experiments are discussed in the following sections.

B. LECTIN'S ROLE *IN VIVO*?

If differences in lectin activity between dark and white embryos are related to restricted pigment cell migration in the white mutant, then an experiment in which the *in vivo* titre of lectin is modulated and the resulting pigment patterns are characterized and compared would be useful.

I reasoned that *in vivo* lectin activity could be modulated by adding either a known inhibitor of the lectin or exogenous lectin to the embryo. If these experiments were successful, then it would be useful in the future to attempt more specific studies, for example, with antibodies against the carbohydrate-binding site of the lectin. First, the added molecules would have to be able to penetrate the embryo and interact with the lectin present within the animal. Second, non-specific effects of these "modulators"

have to be controlled for. Finally, the effects of the modulators on pigment pattern development had to be quantitated. A brief discussion of how these problems were addressed follows.

A variety of saccharides related to the three strongest inhibitors of this lectin were tested for their specificity to this lectin. A potent non-metabolizable inhibitor of the lectin, 2-DG, was discovered (see Chapter 2 discussion; Table 4b). This glucose analogue is widely known to be non-metabolizable in mammalian cells and is used as a tracer molecule in studying glucose metabolism since it is phosphorylated (the first step in this metabolic process) but not further metabolized (Ishibashi et al., 1982). Interestingly, 2-DG has the same molecular configuration as the other three lectin inhibitors (Figure 26).

To control for non-specific effects of this molecule, some embryos were confronted with glucose or glucose plus 2-DG to ensure that 2-DG was not interfering with glucose metabolism. Two different concentrations of 2-DG were used to confront embryos (Table 5).

Two different concentrations of a crude purification of endogenous dark neural crest stage lectin were also used to confront embryos (Table 5). Also, some embryos were confronted with a combination of crude lectin and 2-DG to assess whether their effects in combination would be cancelled out compared to their effects when used alone

(ie., based on differences in lectin activity between dark and white embryos, lectin and 2-DG would be expected to have opposite effects on pattern development) (Table 5). The results of these confrontations on pigment pattern development will be discussed in a later section.

Half of all the experimental and control embryos were stripped of some ectoderm (this procedure is described in Chapter 2 and illustrated in Figure 11) in an attempt to provide an avenue for penetration of the confronting molecules into the embryo and, hopefully, into the vicinity of NC migration. An initial attempt at stripping ectoderm ventrolaterally from the NC (illustrated in Figure 10) produced disruption of the pigment pattern and, therefore, interfered with the analysis of the experimental results. A second much less invasive method was attempted which produced no pattern disruption or any other observable effect on the embryo (Figure 11) and was adopted as the stripping procedure for confrontation experiment 2. I believe that the first stripping procedure extensively damaged the subepidermal ECM, leading to the pigment pattern disruption.

Surprisingly, whether or not the embryos were stripped did not significantly affect the results of any of the confrontations done. This was not surprising in the case of glucose or 2-DG since these are very small molecules (molecular weights are 180 and 164, respectively). If

ectodermal cells in the axolotl embryo are not bound by tight junctions (these are reviewed by Farquhar and Palade (1963), Gumbiner (1987), and Madara (1989), it seems possible that these molecules could leak between cells through the ectodermal barrier. This cannot be confirmed, however, because axolotl ectoderm has never been characterized in this manner and it isn't known what barriers exist against the uptake of certain molecules. Also, instead of diffusing between cells, these molecules might be actively engulfed by ectodermal cells. Simple sugars are known to be actively transported across some epithelia, for example, in the gut. However, it seems unnecessary for the axolotl embryo to have such a mechanism in ectoderm cells since glucose wouldn't likely be present in the jelly coat that normally encapsulates embryos at this stage (however, the composition of the jelly coat hasn't been identified). Furthermore, these embryos rely on internal yolk stores before they are developed enough to feed.

If glucose traverses the ectoderm it would likely be metabolized at once within cells and one might expect some observable effect on the embryo as a result of having more available energy, perhaps faster growth or more activity. Neither of these effects were observed, however. These observations don't rule out the possibility that glucose entered the embryos but had effects that were not readily

noticeable or it had no effects because it was either stored or excreted.

2-DG confrontation had some significant and specific effects on the pigment pattern (these effects are described in Chapter 3; see Tables 8 through 12), and this suggests that the molecule did traverse the ectoderm to interact with NC cells or the matrix. How this might occur is discussed in a later section.

More puzzling are the similar results obtained between unstripped and stripped crude lectin-confronted embryos. The lectin is expected to be either a protein or glycoprotein and therefore, would be a larger molecule than a simple sugar. Proteins cannot passively transverse any epithelia but can be specifically engulfed into the cell (Madden, 1986). This is usually receptor-mediated, so it is unlikely that receptors that recognize the lectin would exist in axolotl ectoderm. However, it may be possible that proteins, in general, are internalized and recycled or stored as an energy source in addition to yolk (this might also occur with glucose, as discussed). Even if lectin is internalized this way, how it could interact with NC migration before being metabolized remains a puzzling question. It's specific effects on pigment pattern development, however (see Tables 8 through 12), suggest that it may to be able to accomplish this.

One cannot rule out the possibility that the crude

purification contained other (smaller) molecules and these affected pigment pattern development in a non-specific manner. However, in this case, one might expect more than the pigment pattern to be specifically altered by crude lectin, and this was not observed at the level that I was working.

Perhaps the ectoderm of axolotl embryos is much more permissive to even large molecules than most epithelia. It appears relatively undifferentiated at the stage of NC migration and is only one cell layer thick. Furthermore, since these embryos are normally surrounded by a controlled environment, the jelly capsule, there may be no reason for the early development of any barrier mechanisms within the ectoderm and free molecular movement across the ectoderm (or "leakiness") might be important for the embryo's homeostasis. This seems to be the most plausible explanation for the similar effects of the confronting molecules on stripped and unstripped embryos.

The most difficult problem in assessing the effects of the various confrontations was establishing a valid and measurable pigment pattern control. To do this, normal embryos were carefully observed during pigment pattern development and compared to each other so that a "typical pattern" diagram at specific developmental stages could be constructed. Normal embryos exhibit some pattern features in common even though there is some variability among

individuals. Specific regions of the flank typically contain clusters of melanophores and other regions contain very few, if any, melanophores (Figure 7; the normal pigment pattern as it changes during development is described in Chapter 3). These characteristics were defined and compared at different developmental stages to produce a schematic representation of a control embryo at the three different stages observed in the experiment. I discovered that the normal pigment pattern consists of three constant parameters which could be measured. These parameters are (1) the number of melanophores on the flank as well as in three of the most consistently appearing bars on the flank, (2) the morphologies of the melanophores that appear on a representative region of the flank, and (3) how well melanophores fit into the "typical pattern" diagram. Both controls and experimental animals were measured by these criteria to give a measurement of normal compared to experimental pigment patterns and to compare the variability among pigment patterns in normal animals and experimental animals. Embryos from one spawning were used in each experiment to avoid the possibility of batch to batch differences in pigment patterns that might exist.

The results of these confrontation experiments designed to modify the <u>in vivo</u> titre of endogeonous lectin and observe the effects of these treatments on subsequent pigment pattern development support the hypothesis that

lectins are involved in pigment pattern formation in the axolotl. A "white" phenotype was not produced when endogenous lectin activity was inhibited by confronting dark animals with 2-DG (see Table 13) but significant changes in pigment pattern development resulted from both lectin inhibition and, as well, from addition of exogenous crude lectin extract.

First, the number of melanophores on glucose-confronted animals was not affected with the exception that stage 40 animals contained significantly more melanophores on their flanks than controls (Table 9). Whole-flank and top pattern fit measurements were not different from controls at any stage (Tables 11 and 10, respectively). Glucose, when combined with 2-DG, did not specifically influence the effects of 2-DG on the pigment patterns of animals at any stage (see Tables 8 through 12).

2-DG significantly affected different parameters of pigment pattern development. Both concentrations of 2-DG correlated with increased melanophores in the bars at stage 38 and decreased mels/bars at stage 40 followed by no significant efffects on this paramenter at stage 42 (Table 8). When overall mels/flank were counted, 10 mM 2-DG-confronted animals exhibited more melanophores on their flanks at all stages and the 25 mM concentration correlated with no differences from controls at any stage (Table 9).

In regard to pattern fit, both 2-DG concentrations

correlated with significantly higher % top fits at stage 38, followed by significantly lower % top fits at stages 40 and 42 (Table 10). These observations correlated very closely with those of overall pattern fit (see Table 11).

Melanophore morphologies were also significantly affected by 2-DG treatment (Table 12). The morphology patterns of stage 38 animals resembled those of older stage 42 controls whereas older 2-DG-confronted animals resembled those of stage 38 controls and, thus, appeared more immature.

In general, 2-DG-treated animals exhibited an advanced pattern in terms of the number of melanophores in their bars (Table 8), better organization of the melanophores into the bars (Table 10) and more developed melanophore morphologies during early pattern development (Table 12) followed by degeneration of the pattern at stage 40 to more closely resemble that of younger control animals.

The question of whether or not the confrontation with 2-DG actually decreased the titre of lectin activity <u>in vivo</u> was investigated by measuring whole embryo lectin activity in 2-DG-confronted and control embryos during development (Table 13). A peak in lectin activity in early migration stage control embyros was expected and observed. This peak also occured in the confronted embryos but was much less, suggesting that 2-DG in the medium decreased endogenous lectin activity in the embryos. These results suggest that

2-DG confrontation specifically affected endogenous lectin activity in the embryos and the resulting changes in lectin activity correlate with changes in pigment pattern development observed in these animals.

Crude lectin treatment produced some effects on the pigment pattern that were opposite to those of 2-DG.

First, the number of melanophores/bar was not significantly affected by either lectin concentration, with the exception of 8 HU crude lectin-treated animals at stage 40, which contained fewer melanophores/bar than controls at this stage (Table 8). The 4 HU concentration correlated with increased melanophores on the flank at stages 40 and 42 whereas the 8 HU concentration correlated with increased melanophores at stage 38 only (Table 9).

Percent top fit was reduced in stage 38 and 40 4 HU-confronted animals and also in stage 40 8 HU-confronted animals (Table 10). There were no differences in this regard between these animals and controls later in development.

The melanophore morphologies of stage 38 crude lectin-confronted animals were, in general, similar compared to controls (Table 12). Later in development, these animals contained less intermediate and more either highly branched or small dot-like types of melanophores than controls. The types of morphologies in stage 42 animals, in general, resembled those of stage 38 controls.

By many measurements, the two crude lectin concentrations correlated with inconsistent effects on pigment pattern development (for example, % top fit at stage 38 (Table 10), % overall fit at stage 38 (Table 11), number of melanophores/bar at stage 40 (Table 8), and number of melanophores/flank at all three stages (Table 9). No clear effect on melanophore number could be found (Tables 8 and 9) but pigment pattern development did not resemble controls in many ways either (Tables 10 and 11). The melanophore morphologies also did not appear to be affected in a consistent way, but somewhat resembled the results obtained with 2-DG confrontation at stage 42, but more opposite to those of 2-DG confrontation at stage 38 (Table 12). The changes in top pattern fit in these animals appears to be opposite to, but less dramatic than, those observed in 2-DG-confronted animals (Table 10).

Although much less conclusive, based on the % top fit data (Table 10), I suggest that crude lectin tended to have opposite effects on **early pigment pattern organization** compared to animals confronted with 2-DG.

It would be useful to repeat this experiment with affinity-purified lectin to determine whether contaminants in the crude extract are responsible for these inconsistencies. The results of these confrontations were much different from what I expected. Based on the data obtained from measuring lectin activity in white versus dark

embryos (Table 2), one would expect that a reduction in lectin activity (by 2-DG) would restrict pigment pattern development and produce white-like larvae. Instead, 2-DG treatment appeared to accelerate and then disrupt pattern development when animals were continuously confronted with the same concentration of 2-DG. **Crude lectin, in some ways, appeared to initially slow pattern development, an effect opposite to that of 2-DG.** Based on these experiments, lectin appears to be involved in pigment pattern development in the axolotl and could be somehow related to the white defect. However, the mechanisms by which lectin may affect dark and white pigment pattern development seem more complicated than just differences in whole embryo titre of lectin during early NC migration.

Like the results obtained with this study, the confrontation of endogenous lectin or its sugar inhibitor, TDG, with live Xenopus embryos (Frunchak and Milos, 1990), did not result in opposite effects on tail melanophore morphologies, numbers, and pattern organization. The addition of endogenous lectin to embryos correlated with a decrease in the number of melanophores on the tail as well as changes in melanophore organization and morphologies. TDG, in contrast, correlated with changes in melanophore morphologies and melanization and, to a lesser extent, changes in melanophore distribution and number. Although the function of endogenous Xenopus lectin in pigment pattern

development appears different in some aspects from axolotl embryonic lectin, these studies suggest that the relationship between lectin and melanophore pattern development is complicated in both species.

Some suggestions about how endogenous lectin might be involved in pigment pattern development in dark and white animals will be discussed in the following sections.

C. TEMPORAL REGULATION OF NEURAL CREST MIGRATION?

Confrontation with the different additives was continuous until stage 42 of development yet the effects of the additives on the pigment pattern were dependent on the stage of development observed. This suggests that the embryos may respond differently to lectin at different stages of pigment pattern development. **2-DG and crude lectin also appeared to alter the normal rate of pigment pattern development in opposite ways, with 2-DG accelerating early pattern development and crude lectin slowing it.** These observations suggest that changes in lectin activity may be involved in the normal sequences of pigment pattern development and when lectin activity is experimentally altered, sequences of pattern formation become disrupted, producing different rates of pattern development.

If the early-migratory peak in lectin activity is responsible for stimulating NC migration, then 2-DG

confrontation would be expected to result in a "white" phenotype, that is, a lack of migration. However, this did not result, although a significant reduction in the number of melanophores on the flank occurred at stages 40 and 42 (Table 11; Figure 21), along with a reduction in melanophore spreading (Table 12; Figure 24). It might be possible that 2-DG confrontation did not sufficiently diminish in vivo lectin activity to produce a "white" phenotype, and that the confrontation produced a partial "white" defect by stages 40 and 42. However, this theory doesn't explain why stage 38 animals exhibited a more mature-appearing pigment pattern (Table 10; Figure 24), including more, rather than less, melanophores on the flank (Figure 21) compared to controls. Therefore, this theory doesn't seem to be valid, in light of the results obtained.

2-DG confrontation produces what appears to be an initial acceleration of pigment pattern development but the pattern is not maintained later on. The results obtained from these experiments suggest that the early peak in lectin activity is not solely responsible for normal pigment pattern development. Perhaps the timing of this peak of activity or the subsequent drop in lectin activity may also be important cues for normal migration and localization of melanophores.

In white embryos, no significant peak in lectin activity occurs with the exception of a smaller peak in

carcass tissue at this time (Table 2; Figure 14). Lectin activity does not significantly decrease in the outer layers of the embryo where migration occurs and such a decrease may be the necessary stimulus for the initiation of NC migration into the subepidermal ECM so, in white animals, a very restricted pigment pattern results.

In 2-DG-confronted dark embryos, the normal peak that occurs in lectin activity during early NC migration may be attenuated by the added 2-DG. The subsequent decrease in lectin activity that normally occurs during early NC migration may be occuring earlier due to the inhibitor, creating the early and perhaps more intense stimulus for NC migration. NC cells migrate earlier and in greater numbers into the subepidermal matrix to produce an advanced and highly populated flank pattern by stage 38 in 2-DG-treated animals (Table 8). Possibly, pattern disruption may follow at stage 40 (Table 10; Table 8) because the NC cells leave the crest before they are competent to maintain the pigment pattern (this might explain why the pigment pattern, though sparsely populated, appears to stabilize by stage 42; see Tables 8 and 10). Alternatively, too many melanophores may exist on the flank initially and contact inhibition (discussed by Lehman and Youngs, 1959) may reduce the viability of melanophores in such close proximity. This theory suggests that either direct contact by cell processes touching eachother or indirect communication through

substances released by a cell can deter adjacent cells of the same type and promote equal spacing between pigment cells, for example, in the skin. The latter possibility seems less likely in light of the observation that 4 HU lectin treatment and glucose treatment also resulted in increases in the number of flank melanophores during stage 40 pattern formation and subsequent melanophore loss was not observed at stage 42 (Table 9).

In crude lectin-confronted dark embryos, the opposite may occur. The decrease in lectin activity after the pre-migratory peak may be less pronounced or more gradual, creating a less intense stimulus for NC migration. Melanophores might begin to migrate later than normal but may migrate over a longer period of time, creating an initially retarded pigment pattern but as pattern organization continues, by stage 40, more melanophores have migrated into the flank and the pigment pattern stabilizes and resembles those of controls by stage 42 (Table 10). Interestingly, however, the effects of the crude lectin and 2-DG treatments on melanophore morphologies seemed to last longer than pattern fit or melanophore number, even though these effects were not constant over time (Table 12; Figure 24). This suggests that the ECM, if modulated by lectin, may program differentiation information differently into migrating melanophores and, therefore, permanently alter their patterns of morphological development.

The experiments of Lofberg et al. (1989), are interesting in regard to the possibility that timing is important in normal NC migration. Their work suggests that white subepidermal ECM matures later during development than dark subepidermal ECM, and that this may be essential in producing the restricted pigment pattern. They transplanted subepidermal ECM absorbed in vivo on membrane microcarriers. Dark subepidermal ECM, when inserted subepidermally prior to NC migration, stimulated NC migration in both dark and white hosts. Most importantly, white subepidermal ECM from late-migratory stage 35 white embryos stimulated NC migration in early migratory stage 25-27 white hosts but stage 30 or younger white subepidermal ECM did not, suggesting that the defect existing within white subepidermal ECM is transitory in nature and that by the time the white subepidermal ECM becomes capable of supporting NC migration, NC cells are no longer capable of responding to the stimulus.

There may be a window of time in which NC melanophore precursors are sensitive to a decrease in lectin activity, starting immediately after the peak in lectin activity occurs and ending when lectin activity returns to a pre-migratory level. Their results suggest that a defect exists only within the white subepidermal ECM during the period at which NC cells normally migrate. Perhaps by inserting stage 35 white ECM into stage 30 white hosts, they are artificially creating a instant decrease in lectin activity

in the ECM which could stimulate NC migration. My results cannot confirm that a significant decrease in lectin activity occurs between stage 30 and 35, however, since only stages 25-28 and 29-34 were compared (Table 2). This hypothesis suggests that the post-peak decrease in lectin activity in the ectoderm or subepidermal ECM is a more important stimulus for NC migration than the pre-migratory peak in lectin activity.

It would be interesting to test this hypothesis directly by comparing the onset of NC migration in experimental (2-DG- and lectin-confronted) and control embryos using SEM.

An elaborate confrontation experiment in which confrontation with 2-DG or lectin begins at different stages of NC migration, and continues for different lengths of time during and after migration would also be useful to help determine the different effects of artificially eliminating the changes in lectin activity that normally occur, exaggerating the changes, and changing the timing of those changes to see which, if any, of these manipulations affect pigment pattern development. Perhaps, one could recreate "normal" fluctuations in lectin activity in white embryos to see whether they develop a normal pigment pattern. This might be accomplished by either producing and micro-injecting a monoclonal anti-lectin antibody *in vivo* or adding purified lectin to the saline of dark embryos.

How does the hypothesis that HA-lectin interaction is important in pigment pattern development agree with that of Lofberg et al. (1989)?

It seems possible that differences in lectin activity may be involved in subepidermal ECM differences between dark and white embryos, given that this lectin is specific for a major constituent of HA (N-acetyl-D-glucosamine) (Table 13) which is a substantial component of the embryonic ECM in axolotls (Perris and Johansson, 1990). Perhaps endogenous lectin modulates the binding activity of HA. Since HA forms large complexes with other ECM constiuents, this could affect the structural organization of the ECM and, therefore, possibly the migration and maturation of NC cells moving through it. It is well known that NC cells interact with the ECM as they migrate (evidence for this is discussed in Chapter 1) so lectin could be an indirect partner in controlling normal NC migration. Several investigations have been conducted on dark and white subepidermal ECM and only minor differences have been reported between them. However, research done by Dalton (1950) and others after him convincingly limits the white defect to the supepidermal ECM. The defect may be subtle and may be expressed for such a short period of time that it has escaped detection using biochemical methods. Perhaps a specific transient NC cell-ECM communication, for example, is essential in controlling NC migration in the ECM and this doesn't occur in white

embryos. Because of the roles of some lectins in cell-cell and cell-ECM recognition, a lectin could be a key factor in promoting normal pigment cell migration.

An extensive examination of dark and white ECM by Perris et al. (1990) revealed that the structural assembly of dark and white subepidermal ECM's were largely comparable but more electron-dense fibrils were observed in dark subepidermal ECM. This might reflect less proteoglycan available to form fibril structures because of recruitment of more proteoglycan aggregates into HA aggregate formation in white embryos. A difference in 30 to 90K protein content between dark and white subepidermal ECM was also found. Whether the differences in protein content between dark and white embryos reflects differences in lectin activity is unknown, but is an interesting possibility.

A hypothetical model for lectin-HA interactions in dark, white, and experimental embryos is suggested in Section E.

D. A ROLE FOR LECTIN IN THE EXTRACELLULAR MATRIX?

How this lectin interacts with components of the ECM or cells, if it in fact does, is unknown. However, if lectin binds to the N-acetyl-D-glucosamine HA backbone *in vivo*, it may interfere with the assembly of proteoglycan aggregates as both the core protein and link protein associated with

these structures bind directly to HA itself (see Figure 4 for the structure of HA aggregates; it is not known, however, whether the particular arrangement of HA aggregates shown in Figure 4 represents what occurs in axolotl embryonic ECM).

Hyaluronic acid aggregates are an important component of the ECM, imparting particular physical characteristics to the ECM and opportunities for interactions with other components and cells. The communication between cells and components of the ECM is essential to the processes of tissue and cell induction and differentiation in the embryo (Lofberg et al., 1980; Newgreen et al., 1982; Erickson and Weston, 1983). Hyaluronic acid, in particular, appears to be important in regard to NC migration. Work done in this regard suggests that the interaction between HA and proteoglycans may be important in regulating NC migration in vivo.

Two studies are important to note. Work done by Tucker (1986) on two anuran species suggests that chondroitin sulfate proteoglycan in subectodermal ECM restricts the migration of pigment cells by limiting the space available for migration and also by acting as a less adhesive migratory substrate and that HA opens spaces permitting the migration of cells. When chondroitinase ABC is added locally to the matrix in vivo, melanophore migration is promoted along affected pathways. This treatment also

increases the number of HA fibrils locally in the matrix, and the migratory space is enlarged.

In contrast, Tucker and Erickson (1986), based on their work with the California newt (Taricha torosa), report that HA within pigment cell migratory pathways prevents melanophore, but not xanthophore, penetration in vivo. When pigment stem cells enter HA-rich regions in vivo, their differentiation into melanophores is delayed. This may account for the early population of xanthophores into the HA-rich dorsal fin, followed weeks later by the appearance of melanophores in this region.

Differences in the interaction between lectin and HA may also influence NC cell migration, and may be essential for NC migration in dark embryos.

E. SUMMARY: A THEORETICAL ROLE FOR LECTIN IN AXOLOTL PIGMENT PATTERN DEVELOPMENT

The following outline of events is suggested as a mechanism for lectin in melanophore migration in the axolotl: An increase in lectin available occurs at the beginning of NC migration and interacts with HA attached to NC cells themselves or HA existing freely within the ECM and interferes with binding between HA and proteoglycans. This interaction might somehow either sterically inhibit NC migration or provide some signal to NC cells, preventing them from migrating.

The reduction of lectin activity that follows its peak may represent its sequestering by cells. HA either attached to the NC cells or within the ECM becomes free of the inhibitory effect of lectin and is also free of proteoglycan due to the presence of lectin during its production. NC cells are then permitted to migrate.

In white embryos, less lectin is present allowing more HA and proteoglycans to freely interact. HA either attached to the NC cells themselves or in the ECM produces aggregates with the proteoglycans and NC migration is inhibited.

2-DG confrontation, suggested to produce a early sharp decrease in lectin activity following its peak, may free HA earlier than normal, allowing more free HA to exist at one particular time. NC cells may then migrate earlier and may move more easily through the ECM, unencumbered by HA-proteoglycan complexes either attached to them or within the ECM. If the process of migration is important for NC cell maturation, then the early NC cells would also mature earlier. They may be stimulated to differentiate earlier than normal and interact to form a premature pattern.

Lectin confrontation, possibly characterized by a more gradual and less dramatic decrease in lectin activity following its peak, may provide more time for HA and proteoglycans to bind as HA becomes gradually free of lectin. NC cells might then be covered with more HA-proteoglycan complexes than mormal or may have to negotiate

through more of these complexes in the ECM so their migration is slowed and pigment cell maturation is impaired.

For this type of mechanism to work, the production of HA molecules by NC cells or other cells in the ECM must occur at the same time that lectin activity increases in the embryo. It would be useful to test this experimentally perhaps by labelling HA and lectin and quantifying them in dark and white embryos in frequent intervals during early NC migration.

F. CONCLUSION

The work done in this thesis correlates lectin activity with pigment pattern development and with the white defect. Based on the experiments done, the sugar-binding capacity of the endogenous lectin appears to be directly related to pigment pattern development (because 2-DG, its sugar inhibitor, can affect pigment pattern development) and appears to be similar in both dark and white embryos. These results suggest that the timing of lectin's appearance in the embryo is important in pigment pattern development, rather than differences in the lectin molecule itself.

Hyaluronic acid either on migrating pigment cells or in the ECM could be modulated by interacting with endogenous lectin resulting in changes in the aggregation of HA with proteoglycans within the ECM. This modulation, in turn,

might change characteristics of the ECM that are important in regard to migrating pigment cells, perhaps affecting their migration and differentiation. The changes in pigment pattern development observed after confronting embryos with either 2-DG or crude lectin extract suggest that migrating pigment cells are affected when the *in vivo* titre of lectin is modulated. A more direct investigation of the events that happen between changes in lectin activity and changes in pigment pattern development is required to test this hypothesis further.

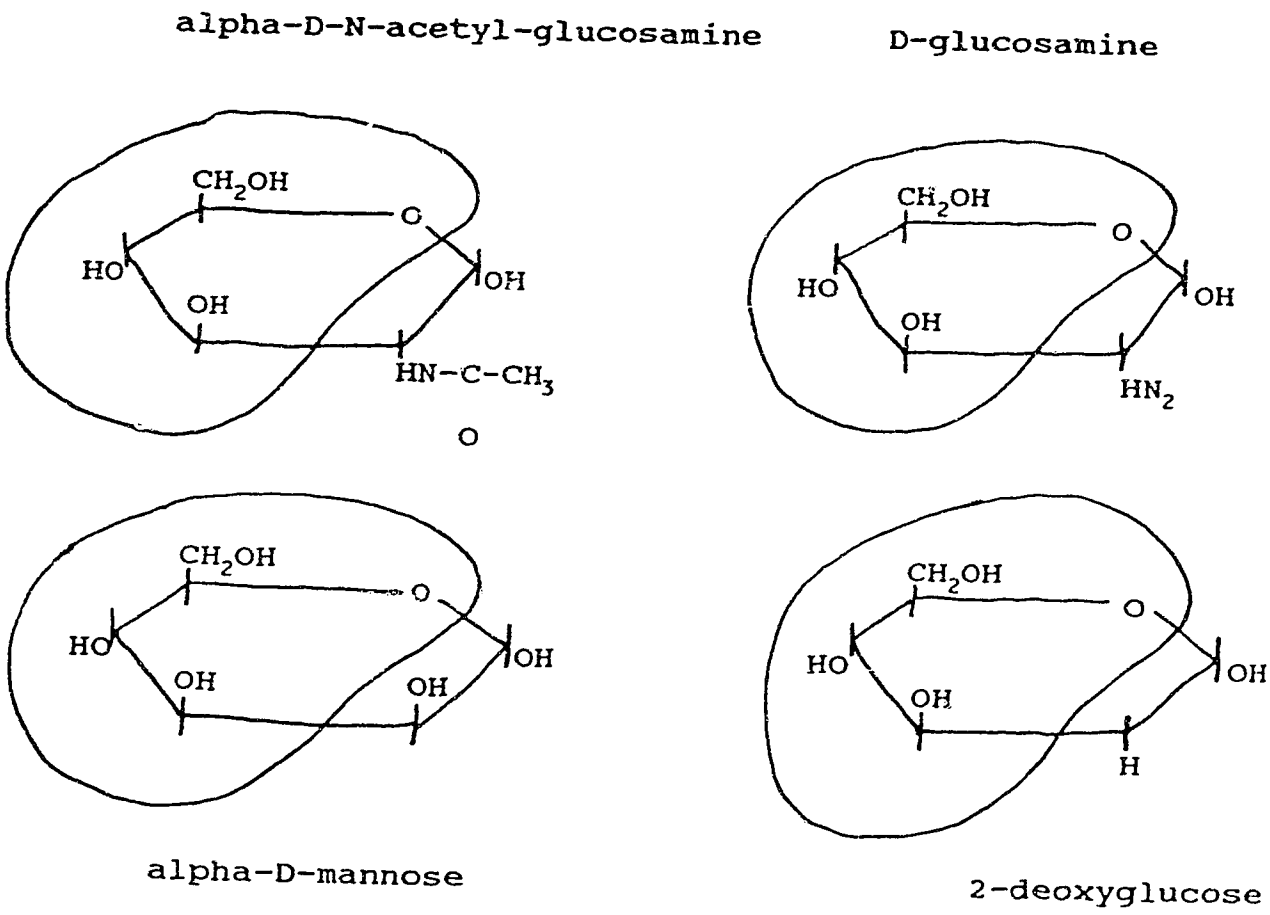
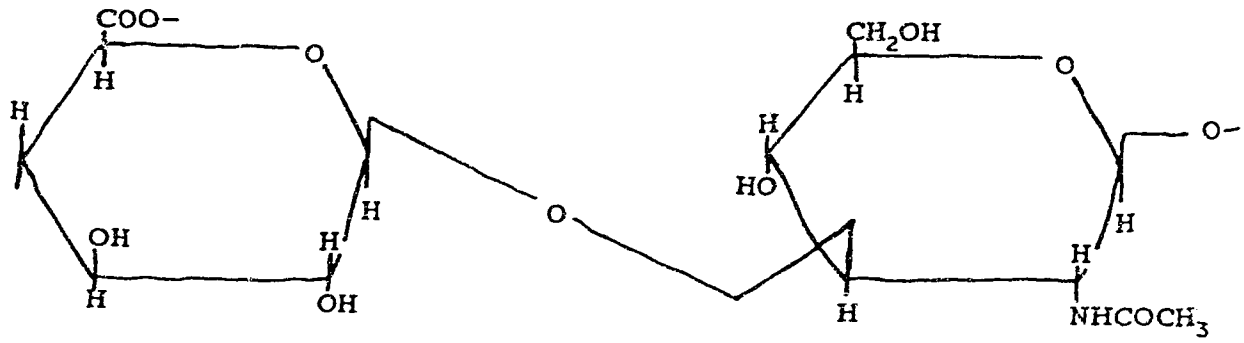


Figure 26. The ring structures of four simple saccharides for which endogenous axolotl embryos are specific. Configurations at C3, C4, C5, and C6 are identical in all four structures (indicated by rings drawn around them).



(glucuronic acid) (N-acetyl-D-glucosamine)

Figure 27. Repeating disaccharide unit of hyaluronic acid.

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APPENDIX I

RECIPIES FOR SALINE SOLUTIONS USED:

HOLTFRETER'S SALINE =

4X STOCK:

NaCl	7 g
KCl	0.1 g
$CaCl_2$	0.2 g
$NaHCO_3$	0.4 g
dH_2O	300 ml

DILUTE 8 X IN dH_2O TO MAKE 50 % SOLUTION

STEINBERG'S SALINE =

NaCl	51 g
KCL	0.75 g
$Ca(NO_3)_2$-$4H_2O$	1.2 g
$MgSO_4$-$7H_2O$	3.075 g
Na_2HPO_4	0.41 g
KH_2PO_4	1.08 g
dH_2O	15 l

NIU-TWITTY SALINE =

NaCl	3.4 g
KCl	50 mg
$Ca(NO_3)_2$-$4H_2O$	0.16 g
Mg SO_4-$7H_2O$	0.21 g
HEPES	3.57 g
GENTAMYCIN SULFATE	8 ml

pH TO 7.5

APPENDIX II

In vivo mel/flank, mel/bar, % total fit, and % top fit data were given to Mr. Samarakoon, a graduate student in the Statistics Department (U. of A.) and analyzed statistically using Tukey's Studentized Range (HSD) Test for variables and Duncan's Multiple Range Test (MEANCT) for variables. These tests were done with modified statistical software packages.

Both tests determined the means, standard deviation and upper and lower confidence limits for each test group at each stage of development with alpha = 0.05 and produced identical results.

The difference between the two tests is that the MEANCT test takes into account only the individual data points entered whereas the HSD test automatically averages the data from each particular group and adds these mean values to construct an identical number of "data points".

Because the different experimental groups consisted of uneven numbers of data points, the second test was theoretically more correct but not as practical because it tends to dilute the actual data. This difference between the two tests was irrelevent for the purposes of this thesis.

Both tests measured the significance of control values over development. As well, all expeimental groups (including controls) were statistically compared to eachother at each stage of development.

A more complicated statistical analysis was done by Mr. Samarakoon to assess the significance of the melanophore morphology data. Type 1, 2, 3, and 4 morphology % representation plots at each stage of development from each group were sorted and normalized using a SAS statistical package and then analyzed using a Multivariant Analysis of Variance Test to obtain error ranges. A Tukey's Studentized Range (HSD) Test was then done on this data to determine which % values were significantly different from eachother.

To simplify, the changes in % representation of each morphology type of test animals were compared statistically to the changes observed in each morphology type of control animals to test if those (test animal) changes form a pattern that is significantly different from that of the control data. In Table 12, the changes in % representation of each morphology type was first tested on controls only. If those changes were significant, then that group was marked with an "X". After this was done, the changes in each test morphology type representation were compared statistically to the changes observed in the controls. If the pattern of change in an experimental group was significantly different from the control pattern, then that group was marked with an "s". Finally, individual value

means (that is, value means at each stage) were statistically compared to control value means at the same stage. These were marked with individual small x's.