seminar of the Department of Biology at the College and at the 1985 meeting of the Eastern Colleges Science Conference. She plans to give a presentation at the district meeting of the Tri-Beta Biological Honor Society in May.

Successfully defended for departmental honors, April 24, 1985.

Studies in Pigmentary Pattern Formation
in the Broken-striped Newt,
Notophthalmus viridescens dorsalis

By:
Mary Beth Ludwig

Submitted in Partial Fulfillment
of the Requirements for Graduation
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Introduction

Previous experiments with the red-spotted newt, Notophthalmus viridescens viridescens, by Forbes, Zaccaria, and Dent in 1973, show that the black-ringed red spot is composed of a dermal-epidermal chromatophore unit. This chromatophore unit consists of red-pigmented erythrophores underlaid with more or less colorless reflective dermal iridophores which are surrounded by a ring of dermal melanophores.

The iridophores contain particles of a semi-crystalline material which is regarded as guanine and serve as good reflectors in the superficial part of the dermis. Although they are concentrated in the dermis beneath the erythrophores of the red spots, some are dispersed among the dermal melanophores of the interspot regions. The melanophores contain melanin and may be scattered or accumulated to form a dense and almost continuous dermal layer (Gordon, 1959).

More recent research done by Zaccaria in 1977, and O'Brien and Zaccaria in 1981, has shown that erythrophores autografted to a non-spot region can induce the formation of a totally new spot apparently by attracting scattered iridophores and melanophores from the surrounding dermis. A comparable dermal-epidermal unit has been found by Zaccaria and Forbes (unpublished observation) to be present in another subspecies, the broken-striped newt, Notophthalmus viridescens dorsalis. The present research sought to determine whether the same chromatophore interactions occured in this subspecies. If these interactions did occur, the research would then attempt to determine the following: (1) How many erythrophores are needed to be transplanted to attract surrounding iridophores? (2) Was this attraction of the iridophores to the

erythrophores a form of directed migration? (3) Once the iridophores have underlaid the erythrophores, does this combination attract the dermal melanophores? (4) If this attraction occurs (the dermal melanophores to the erythrophore-iridophore association), what are the dynamics of the melanophore migration?

Another aspect of the present research would also depend on the *in vivo* chromatophore interaction found. If a comparable chromatophore interaction occured in this subspecies, an attempt would be made to ascertain whether the interaction could be demonstrated *in vitro*. This aspect involves finding a sufficient method of tissue culture including developing a nutrient culture medium in which the chromatophores will exist and possibly interact as they do *in vivo*. This *in vitro* analysis would draw upon techniques and culture methods of Yamada (1973), Dunlap (1980), Mescher (1981), and Freshney (1983), who have performed *in vitro* studies using newt tissue.

However, prior to any culture success, another step in the research was added. This step, at the time, was thought to be a step which would facilitate the *in vitro* analysis of the chromatophore interaction by providing some sort of substratum for the cultured tissue. This step would utilize a research method of Donaldson, Smith, and Kang (1982). Their research included observing epidermal migration over a collagen-coated nucleopore filter that had been inserted into a wound made in the hindleg of the newt. Another study performed by Donaldson and Mahan (1983 and 1984) showed that this epidermal migration can occur on fibrinogen and fibronectin coated substrates. The present research sought to determine whether the intended dermal-epidermal chromatophore migration and interaction mentioned above would occur after a fibronectin-coated nucleopore filter was inserted into a wound area between two stripes. If the fibronectin-coated nucleopore filter served as a suitable substratum *in vivo*, it would then be used in the *in vitro* analysis. The

The in vitro analysis of the behavior of chromatophores would attempt to accomplish a simplification of the pigmentary system and would represent a step in the direction of the definitive description of the nature of the interaction.

Methods and Materials

General

The broken-striped newts were obtained from collectors in Wilmington, N.C. The newts were maintained in plastic boxes containing aged tap water. The boxes were kept in the dark at 17-19°C. No more than ten newts were housed in each box. The newts were fed small pellets containing a mixture of brine shrimp and tadpole food or just plain brine shrimp about once a week. The water in the containers was changed within 24 hours after each feeding.

Before any surgical or photographical procedures were performed the newts were anesthetized in 0.2% tricaine methanesulfonate in aged tap water. During the procedures, they were kept at least partially submerged in the same anesthetic but at one-half the concentration.

Autografting and Recording of Migratory Activity

The procedure chosen for autografting was one developed previously in our laboratory. First, in this two step procedure, which is illustrated in Figure 1, donor sites were prepared by surgically removing a piece of skin approximately 2-3 mm in width between two adjacent stripes. The skin was excised in such a way that the erythrophores of each stripe were exposed to the margin of the wound. Also at that time, two recipient sites were prepared on

the dorsolateral area on the body of the same newt in an area remote from the site of the stripes. Each recipient site was prepared by removing a triangular piece of whole skin approximately 1.0 mm on each side (Figure 1.). The excised skin from all three of the areas was discarded. This procedure was performed on 40 newts.

At the donor sites, an epithelium from the surrounding skin began migrating over the wound within several hours. The epidermal erythrophores were carried into the wound with the epithelium.

After sufficient migration of erythrophores, the newts were ready for autografting, which is Step 2. of the procedure illustrated in Figure 1. However, just before autografting was performed, the thin layer of epithelium which had formed at the recipient sites was removed and discarded. For the experimental autograft, the migrated epithelium with erythrophores was carefully removed and placed onto one of the recipient sites, whereas control autografts were completed by transplanting epithelium without erythrophores from the donor site to the remaining recipient site. In the autografting procedure, care was taken not to include any iridophores or dermal melanophores from the surrounding dermis. The Step 2. procedure was performed on the newts that showed significant migration of erythrophores into the wound epithelium. If significant migration of erythrophores had not occurred, the epithelium that had migrated into the original wound or donor site was removed and the wound was extended so that the erythrophores were exposed once again to the edges of the wound.

After the grafting procedures, each newt was placed (with its autografted side up) into a separate culture dish containing only a small amount of aged tap water. The edge of the graft was positioned above the level of the water. The containers were then covered and placed in the dark at 17-19 ^{o}C for approxi-

mately 24 hours. In our laboratory, it was found that this isolation in a cool, dark environment tends to reduce the activity of the newts when they come out of the anesthesia. After 24 hours, the newts were examined to ensure the grafts had not been lost, and if possible, the number of erythrophores that had been autografted was determined. The newts were then kept isolated in these culture dishes and placed into their regular environment.

Migratory activity of chromatophores was recorded by observations under the dissecting microscope and by photomicrography using a Bausch and Lomb stereozoom 7 dissecting microscope with an integrated 35 mm camera system with professional ektachrome film that had an ASA of 50.

Wounding and Filter Implantation

Wounds were made by surgically removing a piece of skin approximately 1-2 mm in width between two adjacent stripes. The skin was excised in such a way that the erythrophores of each stripe were exposed to the margin of the wound. A piece of fibronectin-coated nucleopore filter was then inserted under the wound margins (Figure 2.). The newt was then placed with its injured side up in a culture dish containing a small amount of water in a dark area at 17-19°C for several days. Migratory activity was observed by methods mentioned above.

Coating of Filters

Nucleopore filters (1.0 μm pore size, approximately 1-2 mm in width and 2-3 mm in length) were immersed for 15 minutes in a 0.5% acetic acid solution at 45°C and were then washed with three changes of distilled water. Subsequent-

ly, 10 µl of 0.01 mg/ml of fibronectin was pipetted on the filter and spread evenly. The filters were then allowed to dry overnight.

Tht fibronectin was prepared by first dissolving 1 mg of powdered fibronectin in 1 ml of sterile distilled water. To this dissolved fibronectin, a mixture of phosphate buffered saline (PBS), that consisted of 0.85% NaCl in 0.15 M phosphate buffer, was prepared and added to make a fibronectin concentration of 100 µg/ml.

Tissue Culture

Two different procedures were used for the in vitro analysis. The first procedure involved preparing a donor site as previously described in Step 1. of Figure 1.

At the donor site, after significant migration of erythrophores had occurred, the epithelium and part of the adjacent stripe was removed and placed into culture.

The other procedure for this in vitro study utilized the nucleopore filter technique. First, wounds were made approximately 1-2 mm in width between two adjacent stripes so that erythrophores were exposed to the margin of the wound. A piece of fibronectin-coated filter was inserted under the wound margins (Figure 2.). The newt was then stored as previously described (p. 4). Then, one of two approaches was taken. (1) The nucleopore filter was left in the wound until all the components of the chromatophore unit were present on the filter. After all these components, which included erythrophores, iridophores, and dermal melanophores, were present, the nucleopore filter with the epithelium containing erythrophores, iridophores, and dermal melanophores would then be placed in culture. Or, (2) The nucleopore filter was allowed to re-

main in the in the wound only until sufficient erythrophores had migrated onto the filter. The nucleopore filter containing the epithelium bearing erythrophores was then removed along with part of the adjacent stripe and placed in culture.

Culture Medium

The culture medium was composed of 7.2 ml M_{199}, (ingredients can be found in Paul (1970)), 2 ml distilled water, 2 ml insulin (GIBCO), 0,1 ml Kannamycin (GIBCO), and 0.1 ml antibiotic-mycotic solution (GIBCO). Approximately 1.0 ml of medium was then placed into a small culture dish. Once the tissue was placed in the medium, the apparatus was stored in a petri dish at a temperature between 25-30^{o}C.

Results

Migration at Donor Site

In the preparation of the donor sites, it was observed following the removal of the whole skin that the wound was usually covered with a thin epithelial layer within 24 hours (Figure 3.). However, in larger donor site wounds, a longer period of time was required for this migration. The leading edge advanced at an average rate of approximately 0.10 mm/hr. The newly migrated epidermis at both the donor and recipient sites has the appearance of a delicate membrane and contains a few scattered epidermal melanophores; at the donor sites, erythrophores also are present in this sheet of epidermal cells. Prior to excision, the erythrophores were bright red-orange lying

over the highly reflective dermal iridophores. However, after migration of the erythrophores into the wound area of the donor site (Figure 3.), the erythrophores were no longer underlaid with iridophores and appeared dull orange.

The rate and amount of migration of erythrophores into the wound of the donor site varied among the newts and it often continued over a period of several days.

Of 40 donor sites prepared (one per newt), 30 were judged to have enough erythrophore migration for autografting. Of these 30 experimental autografts performed, 23 became established.

The edges of the autografts (both experimental and control) usually fused with the surrounding dermis within 24 hours.

Iridophore Migration after Autografting

About two weeks after transplantation was completed, iridophores were observed migrating from the surrounding dermis directly toward the accumulation of transplanted erythrophores of the experimental grafts. This directed migration of iridophores was seen in nine newts (Table 1.). Among these newts, however, the rate at which this directed migration occurred seemed to vary. Several newts had their autografted erythrophores completely underlaid with iridophores in four weeks (Figures 8., 9., and 10.). However, other experimental grafts required approximately 6-7 weeks post-grafting to establish iridophores under the erythrophores (Figures 5-7).

Once the iridophores have reached the mass of erythrophores, they have been observed to migrate directly under the erythrophores. The collection of iridophores then takes on a configuration congruent with that of the over-

lying erythrophores (Figures 5-10).

In Table 1., it can be seen that ten of the 23 newts that had established autografts, had erythrophores that completely dispersed or degenerated before any iridophore migration could be seen. The remaining four newts died before sufficient migration of iridophores had occurred.

At the control sites, there was a lack of directed migratory activity of the iridophores.

Melanophore Migration at Site of Autograft

Once the iridophores were essentially under the erythrophores, dermal melanophores began to migrate toward the erythrophore-iridophore association (Figures 6., 8., and 9.). Just as for the the iridophores, the source of these melanophores is the surrounding dermis at the periphery of the site of the experimental graft. This melanophore aggregation that was observed in the experimental autografts may begin before the iridophore migration is complete. However, it did not become apparent before the iridophore migration was well under way. The dermal melanophores have been observed to migrate to the erythrophore-iridophore association in masses (Figures 6., 8., and 9.). However, additional observations are still necessary since complete melanophore borders have not been formed.

Migration at Site of Nucleopore Filter Insertion

Epithelial migration occurred over the fibronectin-coated nucleopore filter if the filter had been inserted so that all four edges of the filter was

"tucked in" under the skin of the edges of the wound (Figure 2.). This epithelial migration occurred at approximately the same rate of 0.10 mm/hr after epidermal migration became apparent as was observed in wounds without inserted filters. The newly-migrated epidermis had the same appearance of a delicate membrane and contained scattered epidermal melanophores and erythrophores.

If, however, all of the edges were not "tucked in" under the wound edges, the filter did not remain in the wound.

In Vitro Analysis

The attempt to perform an *in vitro* study, which involved removing newly-migrated erythrophores in the epithelium of the wound at the donor site with part of the adjacent stripe and then placing them in culture, was not successful. The culture lasted four days until its microscopic appearance showed fungal growth, or the tissue itself showed signs of molting or degeneration in which the erythrophores became very dull and showed signs of sloughing off.

The second attempt to perform an *in vitro* analysis, which utilized the nucleopore filter technique, was also not successful. In each case, as the filter with the erythrophore-bearing epithelium was being removed, the epithelium detached from the filter, Therefore, the epithelium had no attachment to the substratum and no advantage was gained.

The third attempt which involved removing a piece of a stripe and placing it into culture was also not successful. The culture also lasted four days until its microscopic appearance showed fungal growth, or the tissue showed signs of molting or degeneration in which the erythrophores

became very dull and showed signs of sloughing off.

Discussion

In Vivo Dermal-Epidermal Chromatophore Interaction

It was found that autografted erythrophores in the broken-striped newt can induce the formation of a new dermal-epidermal chromatophore unit. The rapidity of this response may be due to the aqueous environment and the temperature in which the newt is kept (Repesh and Oberpriller, 1980). The iridophores have been observed to migrate directly toward the erythrophores as illustrated in Figures 5-10. However, the rate at which these iridophores migrate is observed to vary and seems to depend on the number of erythrophores that are autografted. If an insufficient number of erythrophores are autografted, they appear either to degenerate or disperse into the surrounding dermis without directed migration of any iridophores. These erythrophores appear to be breaking up into smaller components and then fading away (Figure 4.). The erythrophores of autografts that attract the iridophores remain aggregated and become completely underlaid with iridophores. Migration of iridophores then seems to cease after they have completely underlaid the erythrophores. The mass of iridophores seems to take the same shape as the layer of erythrophores, which is illustrated in Figures 5-10, as observed in the red-spotted newt by O'Brien and Zaccaria in 1981. This may suggest that there is a precise interaction between the erythrophores and iridophores in this sub-species comparable to that in the red-spotted newt.

Zaccaria (1977) showed that the epidermal erythrophores play a role in the maintenance of the normal black-ringed red spot and are essential for the reformation of the spot following its partial disruption by surgical means. The erythrophores have also been shown to be capable of inducing a totally new spot in an area devoid of any natural spots (Zaccaria, 1977 and O'Brien and Zaccaria, 1981). The reason for the attraction of the iridophores to the erythrophores has not completely been explained. However, since the stimulus for the migration of the iridophores seems to be the presence of the erythrophores, the migration and aggregation appear to be a clear example of the phenomenon referred to in Collins (1974) as "directed migration." This interaction is also similar to the interaction that has been observed in the natural wound healing process. In the natural wound healing process, the way in which epithelial cells interact with adjacent connective tissue components is of major importance in epithelial function (Donaldson, Smith and Kang, 1982). In fact, Forbes, Zaccaria, and Dent in 1973 have shown that even the iridophores are closely associated with the subepidermal collagen layer and with the fibroblasts of that region. The iridophores therefore are only a result of differentiation from this layer. This differentiation may be due to the collagen itself since collagen substrates can influence the differentiation of certain types of epithelial cells (Michalopoulos and Pitot, 1975; Meier and Hay, 1975; Murray, 1979). These observations further suggest that there is a form of directed migration, perhaps chemotaxis, that occurs during the formation of this dermal-epidermal chromatophore unit. The iridophores usually have a tendency to migrate directionally towards the erythrophores. A similar process is discussed by Senior (1983) in his research that demonstrates fibroblast chemotactic activity. This process is further represented in research by Borak, Karov, and Levin (1983) on human leukocytes. They observed leukocytes

migrating directly toward a chemotactic factor in which clearly defined individual "paths" were carved in the agarose by migrating cells, leaving distinct migration footsteps that could be traced by the stereoscope. The migration and representation of the iridophores in Figures 5-10 tends to resemble these "paths."

Cramer and Gallin (1979) have also found chemotactic influences with submembranous cations in neutrophils. These chemotactic influences may be transmitted through cell to cell contacts and are usually influenced or induced by favorable circumstances - warmth, food, etc. (Durham, 1976). Cooper and Keller (1984) have further shown that a number of cell types have been found to migrate towards the cathode in electrical fields.

The dermal melanophore migration towards the erythrophore-iridophore association has not been as clearly observed as the iridophore migration. However, it has been observed that after sufficient iridophores have underlaid the erythrophores, dermal melanophores seem to migrate in (Figures 6., 8., and 9.). However, additional observations are necessary since complete melanophore borders have not yet been obtained.

Nucleopore Filter Insertion

Another aspect of this research involved inserting a fibronectin-coated nucleopore filter in a wound area between two stripes. It was thought that the fibronectin, which may be substituted with collagen, laminin, or fibrinogen according to Donaldson and Mahan (1981 and 1983), on the nucleopore filter would be useful since untreated nucleopore filters have been found by Donaldson and Dunlap (1981) to be poor substrates for epidermal cells migrating from the

wound area. Further research has shown that as the epithelial sheet advances on the filter, the cells adjacent to the leading edge extend broad, thin, fan-shaped processes onto the substrate in the direction of movement. According to Donaldson and Dunlap (1981), the leading edge of the epithelial sheet has extensively developed flattened lamellipodia that may also extend from the second line cells. These processes or flattened lamellipodia possess specialized attachments on their undersurface that bind the cell to the substrate (Donaldson, Smith, and Kang, 1982). Therefore if the leading cell has no substrate to attach to it can not adhere to it or support migration. This epithelial behavior has also been observed by Croft and Tarin (1970) in the beginning stages of wound healing in the mouse. Anderson and Fejerskov (1974) have also observed this wound healing process in guinea pigs. They have observed distinct projections from epithelial cells that extend into the fibrin network. While the cells move out into a fibrin-filled wound cavity, the epidermal cells migrate between healthy and necrotic tissue. However, the onset of migration has been determined to occur at a significantly slower rate in mammalian wound closure than in amphibian wound closure (Repesh and Oberpriller, 1978).

Repesh and Oberpriller (1980) have shown that in the natural wound the fibrin clot that covers the soft tissues of the amphibian serves as a natural substrate for the advancement of the cells over the wound. In human skin, a fibrin meshwork was described as the defining plane which was followed by epidermal cells migrating over the wound area (Odland and Ross, 1968).

In this aspect of our research, the thin layer of epidermis containing erythrophores was observed to migrate over the fibronectin-coated nucleopore filter at a rate comparable to the natural wound healing process if the filter was inserted under all four edges of the wound. However if the filter became dislodged on one or more of the sides of the wound, the epithelium adhered to the

natural wound area and migrated underneath the filter. As a result, the filter did not adhere to the wound. If successful migration of the epithelial layer over the filter had occured, the filter was to remain in the newt to observe further migration. However, further migration usually did not occur. In most cases, after five days the nucleopore filter had been dislodged from the wound. This could be attributed to the various movements of the newt. Donaldson (1984) experienced similar difficulties with filters implanted on the limb of the newt and overcame the problem by amputating the leg and placing the entire limb in tissue culture. However, in this experiment, a method such as this was not suitable.

In Vitro Study

In the culture attempt which involved removing the filter containing the erythrophore-bearing epithelium and placing it into culture, another unsuccessful result was obtained. The epithelium did not remain attached to the fibronectin-coated nucleopore filter as it was removed. However, according to Donaldson and Mahan (1983), the epithelium should have remained attached since the cells have been observed to reach out and attach to the substrate. This unsuccessful result may be due to the incorrect pore size of the nucleopore filter. At the time this aspect of the research was undertaken, pore size of the filter was chosen on the basis of the "best guess" of the investigators. However, it was found that Donaldson, Smith and Kang (1982) utilized a nucleopore filter with 0.2 μm pore size while we utilized a filter with 1.0 μm pore size. Therefore pore size may have been a factor in the lack of attachment of the cells to the filter.

The first attempt to culture the cells directly in an in vitro study also led to unsuccessful results. These results yielded fungal growth after four days, or the tissue, itself, showed signs of molting or degeneration in which the erythrophores became very dull and showed signs of sloughing off. There are several reasons why this attempt was not successful. (1) There was no substrate in the media for the cells to attach to. (2) The medium wasn't changed after three or four days. According to Sanford (1948) and Conn (1979) and several other researchers who have dealt with in vitro analyses, the medium should be changed every 3-4 days so that nutrient depletion does not occur. However, in other attempts these factors were changed and the same results were obtained. Other explanations for these unsuccessful results may be: (1) The medium; it has been found by Conn (1979) in his experiments that amphibian blastemal cultures in M_{199} yield 1) no morphological change or 2) a number of necrotic cells present in general appearance, epithelium, and mesenchyme of the blastema. Furthermore, there is no mitotic activity found to occur. (2) Sterilization techniques; several unsterile methods were introduced: a) The tissue was not sterilized before placing it into culture because of the liklihood of damaging the cells. b) In recording the results by photography, the petri lid was removed so that clear photographs could be taken thus opening the culture to the air twice a day.

For further reference in culturing newt tissue these factors should be taken into consideration to improve in vitro results. Some suggestions may include altering the medium and increasing the amount or concentration of antibacterial/antifungal reagents.

This entire study involved an in vivo analysis of the chromatophore interaction of the broken-striped newt, Notophthalmus viridescens dorsalis, and its various parameters. Furthermore, an in vitro pilot study was attempted;

however, further investigation needs to be performed to accomplish our goal of obtaining *in vitro* a simplification of the pigmentary system which would represent a step in the direction of the definitive description of the system.

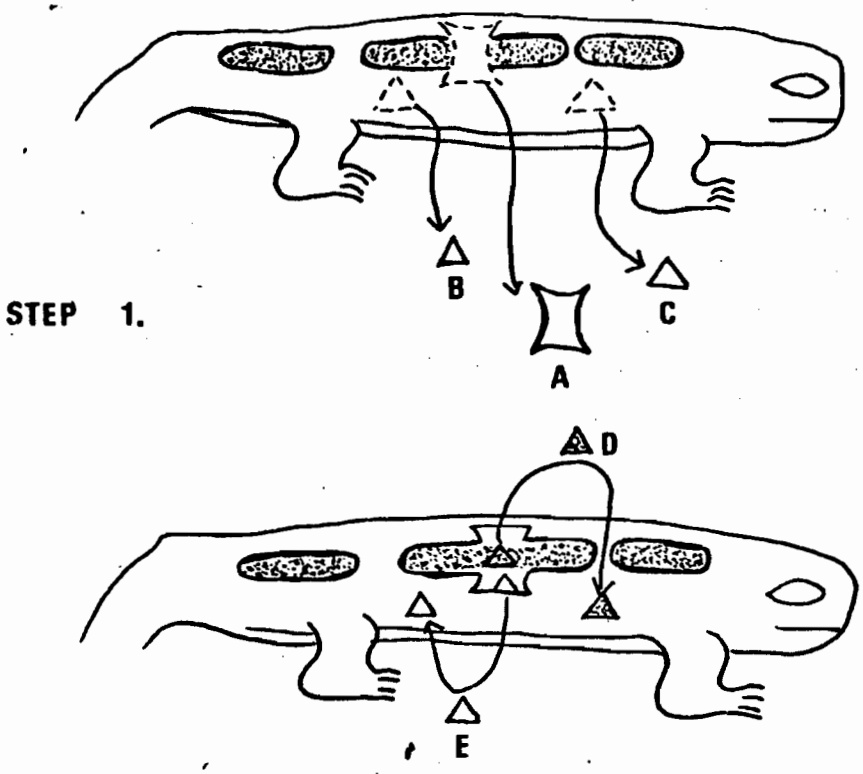


Figure 1. Preparation of donor sites and recipient sites (above) and performance of the experimental and control autografts (below). Step 1: Skin is excised from the donor site (A), the control site (B), and the experimental site (C). All excised skin is discarded. Step 2: Migrated epidermis with erythrophores is grafted to the experimental site (D); migrated epidermis without erythrophores is grafted to the control site (E).

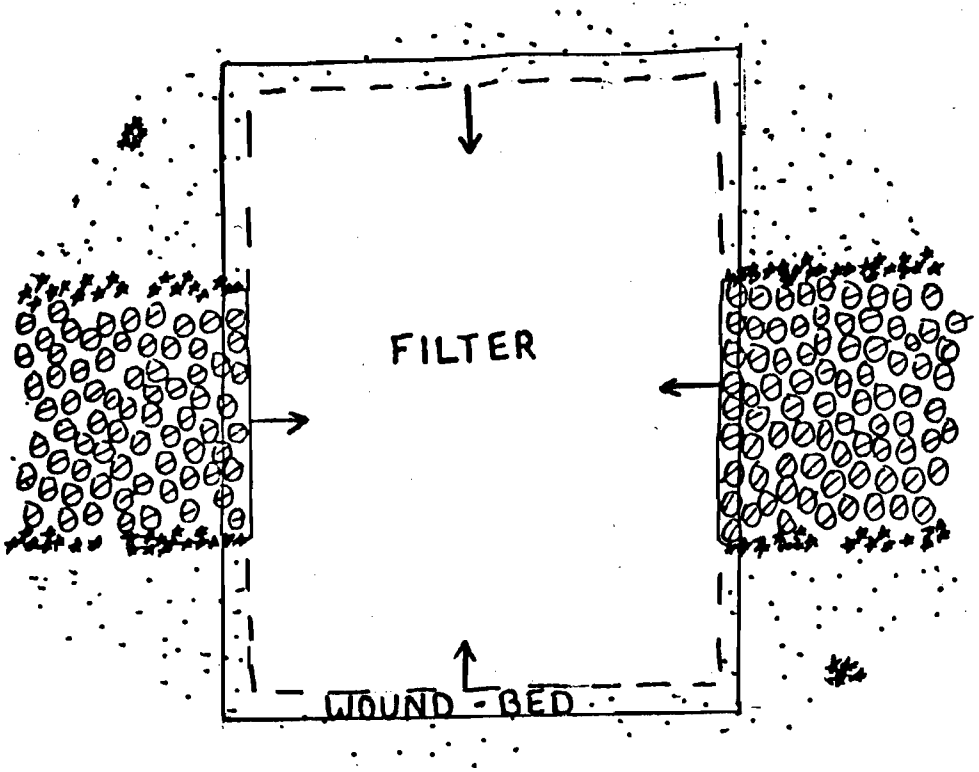


Figure 2. The insertion of the nucleopore filter in an area between two stripes. All four sides of the filter were placed under the edges of the skin. The epithelial migration over the filter is represented by the (→). The (θ) represents erythrophores underlaid with iridophores. Dermal melanophores are represented by (*) and (·) represents epidermal melanophores.

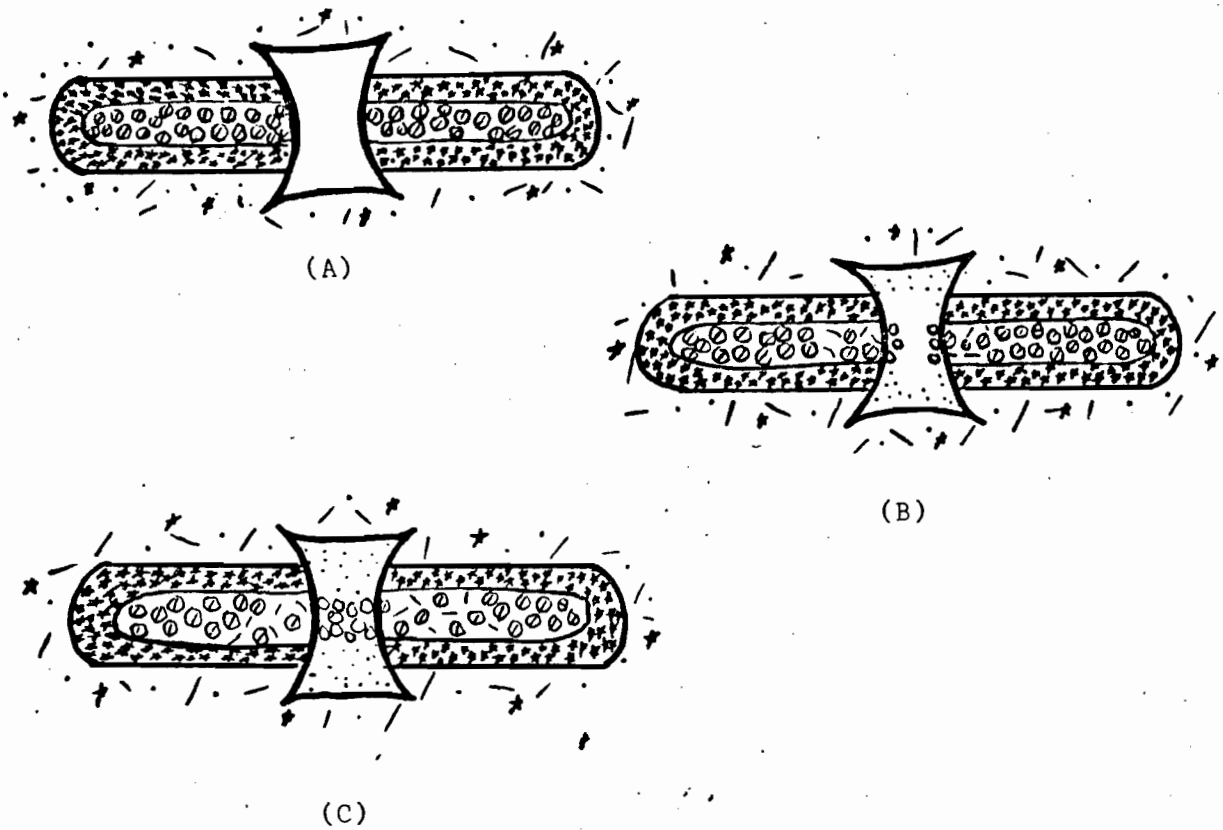


Figure 3. Typical epidermal migratory activity at the donor site. (A), (B), and (C) represent 0, 12, and 48 or more hours, respectively. The hour glass-shaped clear area between the two stripes (A) represents the excised area (2-3 mm^2). (*) = dermal melanophore; (·) = epidermal melanophore; (0) = erythrophore; (-) = iridophore; (θ) = erythrophore underlaid with iridophore(s).

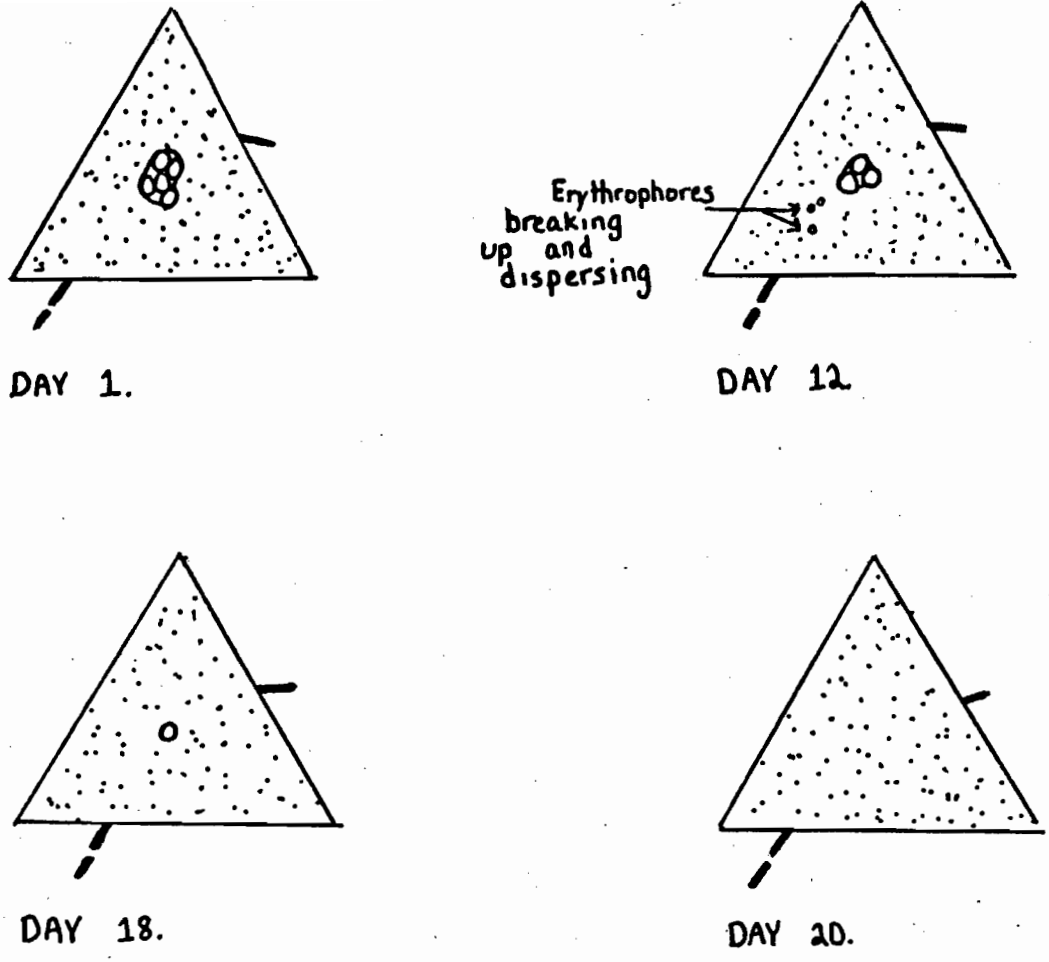


Figure 4. The dispersion and degeneration of erythrophores if enough erythrophores were not autografted. The illustrations represent 1, 12, 18, and 20 days post-grafting. The solid black patch outside the grafted area represents an iridophore while the (·) represent epidermal melanophores. The boundary between the site of the graft and the surrounding whole skin is drawn in the form of a triangle; the other solid line present inside this triangle represents the periphery of the mass of engrafted erythrophores, while each individual erythrophore is represented by (0). In this newt, a total of five erythrophores were autografted.

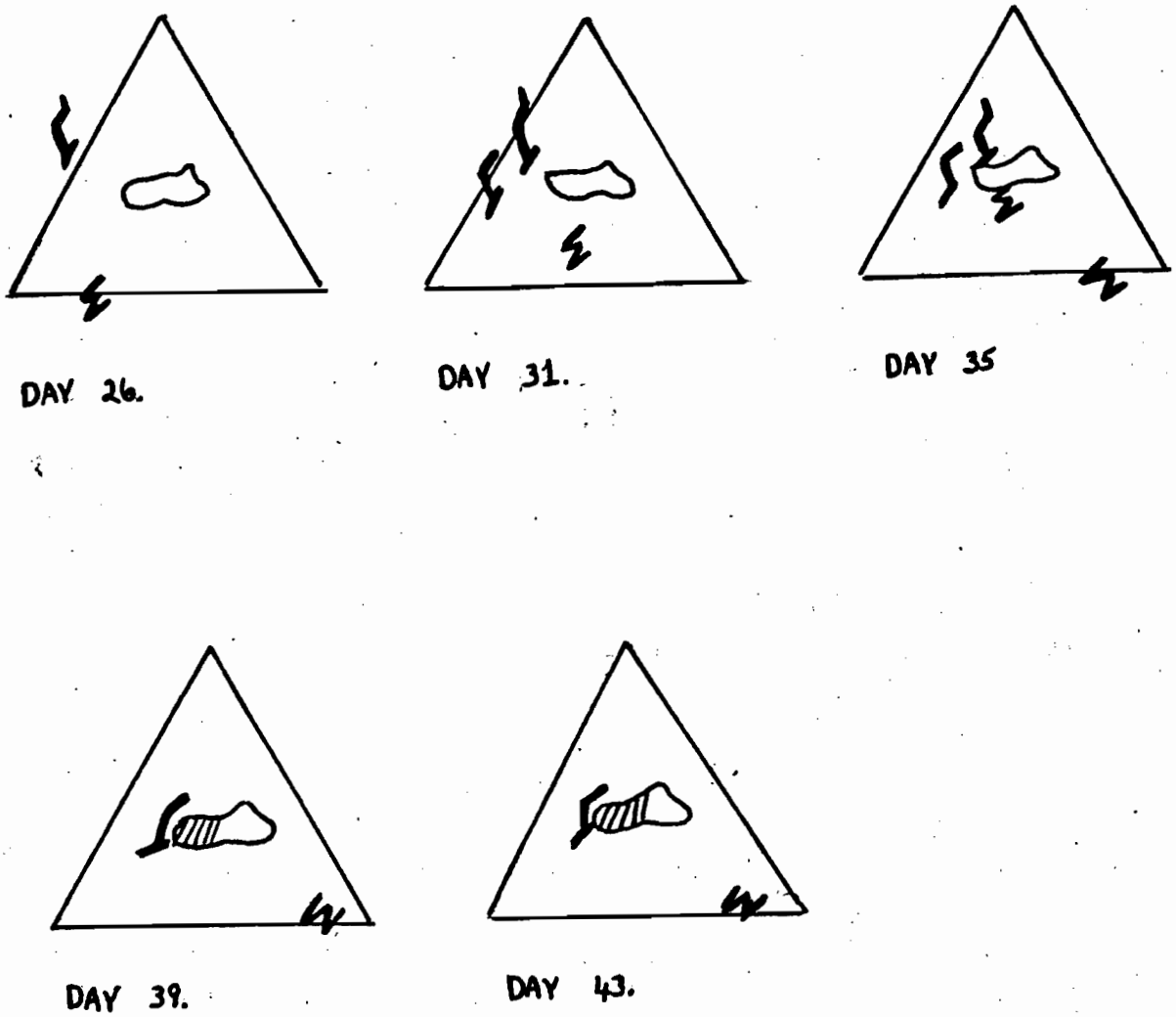


Figure 5. The migration of iridophores from the surrounding dermis toward the autografted erythrophores and their aggregation under the erythrophores. Also the beginning migratory activity of dermal melanophores (Newt 1). Illustrations represent 26, 31, 35, 39, and 43 days post-grafting. The solid black patches represent individual iridophores. The boundary between the site of the graft and the surrounding whole skin is drawn in the form of a triangle; the solid line present inside this triangle represents the periphery of the mass of engrafted erythrophores. The cross-hatching represents erythrophores underlaid with iridophores. Only those iridophores and melanophores actively migrating during the period of observation are illustrated.

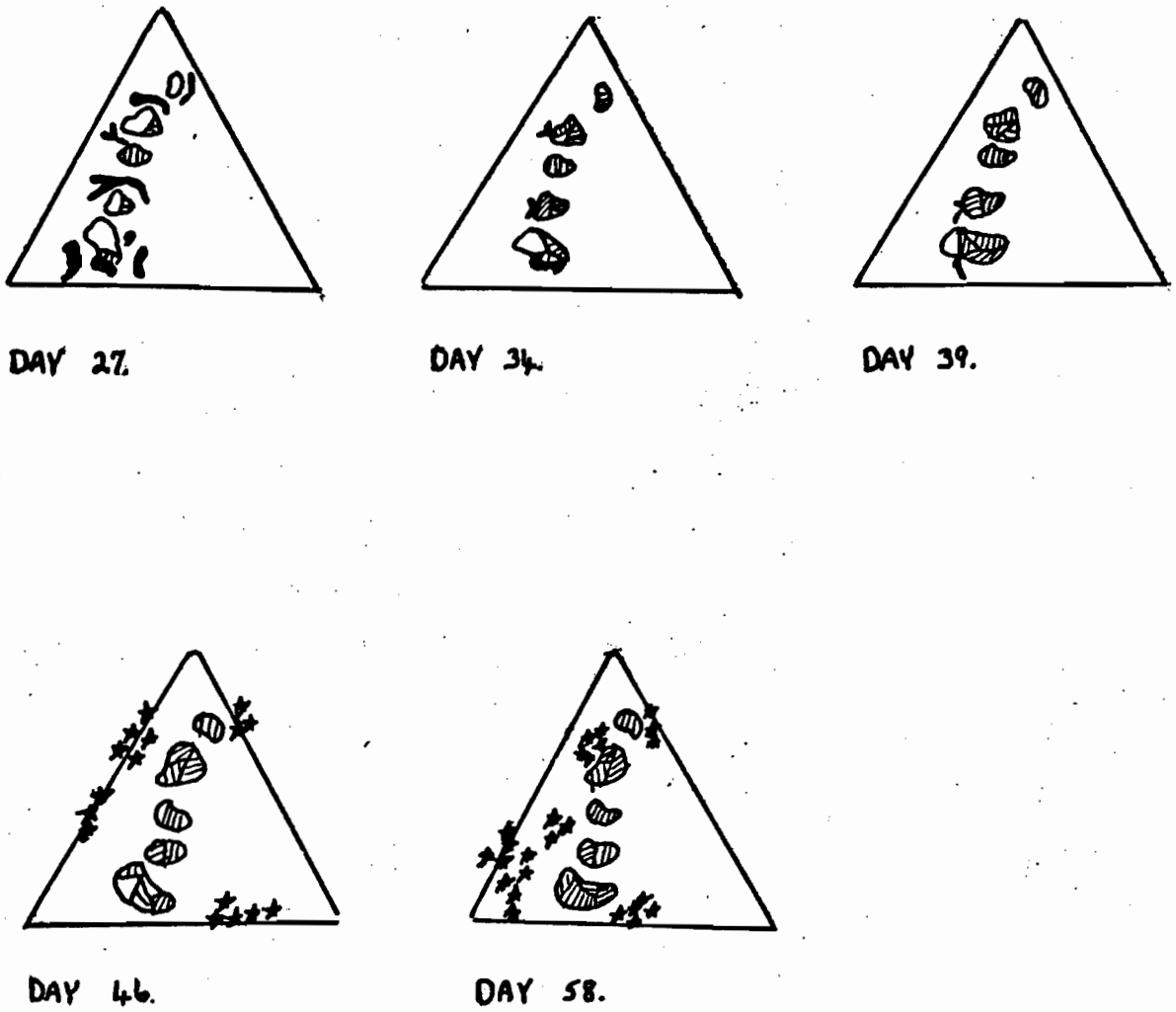


Figure 6. The final stages of the migration of iridophores from the surrounding dermis toward the autografted erythrophores. Migratory activity of the dermal melanophores is also shown (Newt 2). The illustrations represent 27, 34, 39, 46, and 58 days post-grafting. The solid black patches represent individual iridophores, while the (*) represent individual melanophores. The boundary between the site of the graft and the surrounding whole skin is drawn in the form of a triangle; the solid lines present inside this triangle represent the periphery of the masses of engrafted erythrophores. The cross-hatching represents erythrophores underlaid with iridophores. Only those iridophores and melanophores actively migrating during the period of observation are illustrated.

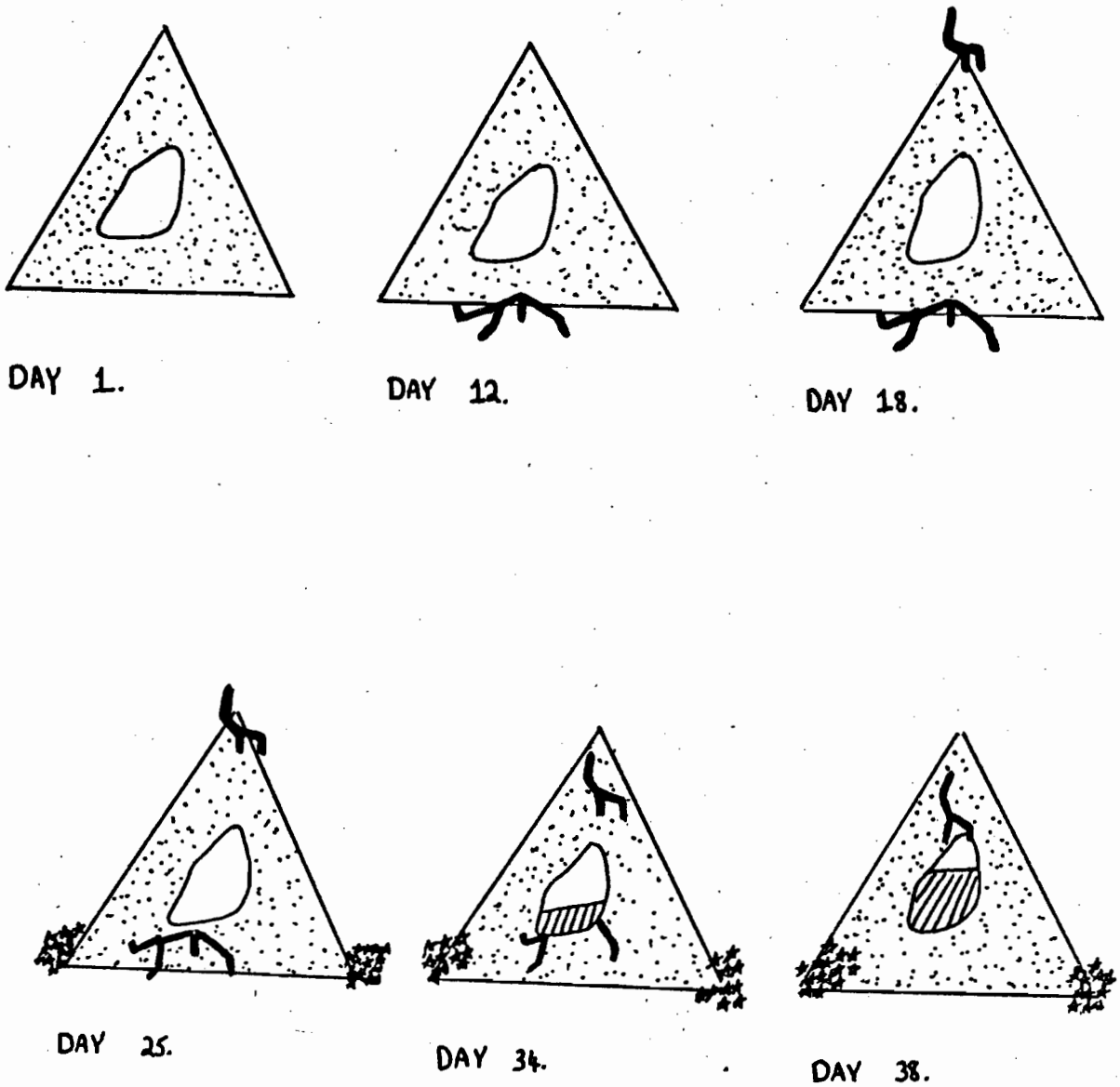
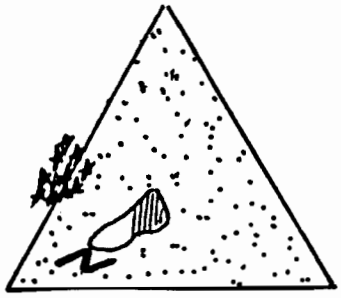
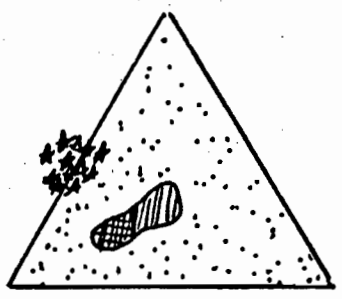


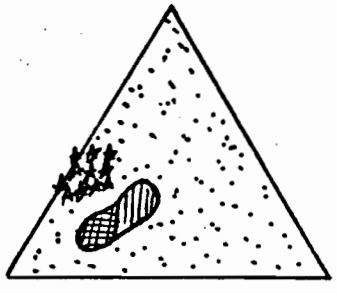
Figure 7. The migration of iridophores from the surrounding dermis toward the autografted erythrophores and their aggregation under the erythrophores. Also the beginning migratory activity of dermal melanophores (Newt 3). Illustrations represent 1, 12, 18, 25, 34, and 38 days post-grafting. The solid black patches represent individual iridophores, while the (*) represents individual dermal melanophores. The (·) represents epidermal melanophores. The boundary between the site of the graft and the surrounding whole skin is drawn in the form of a triangle; the solid line present inside this triangle represents the periphery of the mass of engrafted erythrophores. The cross-hatching represents erythrophores underlaid with iridophores. Only those iridophores and melanophores actively migrating during the period of observation are illustrated.



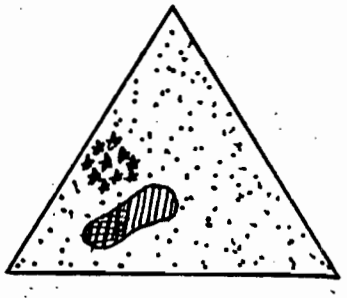
DAY 25.



DAY 32.



DAY 41.



DAY 45.

Figure 8. The final stages of the migration of iridophores from the surrounding dermis toward the autografted erythrophores and their aggregation under the erythrophores. Migratory activity of the dermal melanophores is also shown (Newt 4). The illustrations represent 25, 32, 41, and 45 days post-grafting. The solid black patches represent individual iridophores, while the (*) represent individual dermal melanophores. The (·) represent epidermal melanophores. The boundary between the site of the graft and the surrounding whole skin is drawn in the form of a triangle; the solid line present inside this triangle represents the periphery of the mass of engrafted erythrophores. In this newt, a total of seven erythrophores were autografted. The cross-hatching represents erythrophores underlaid with iridophores. Only those iridophores and melanophores actively migrating during the period of observation are illustrated.

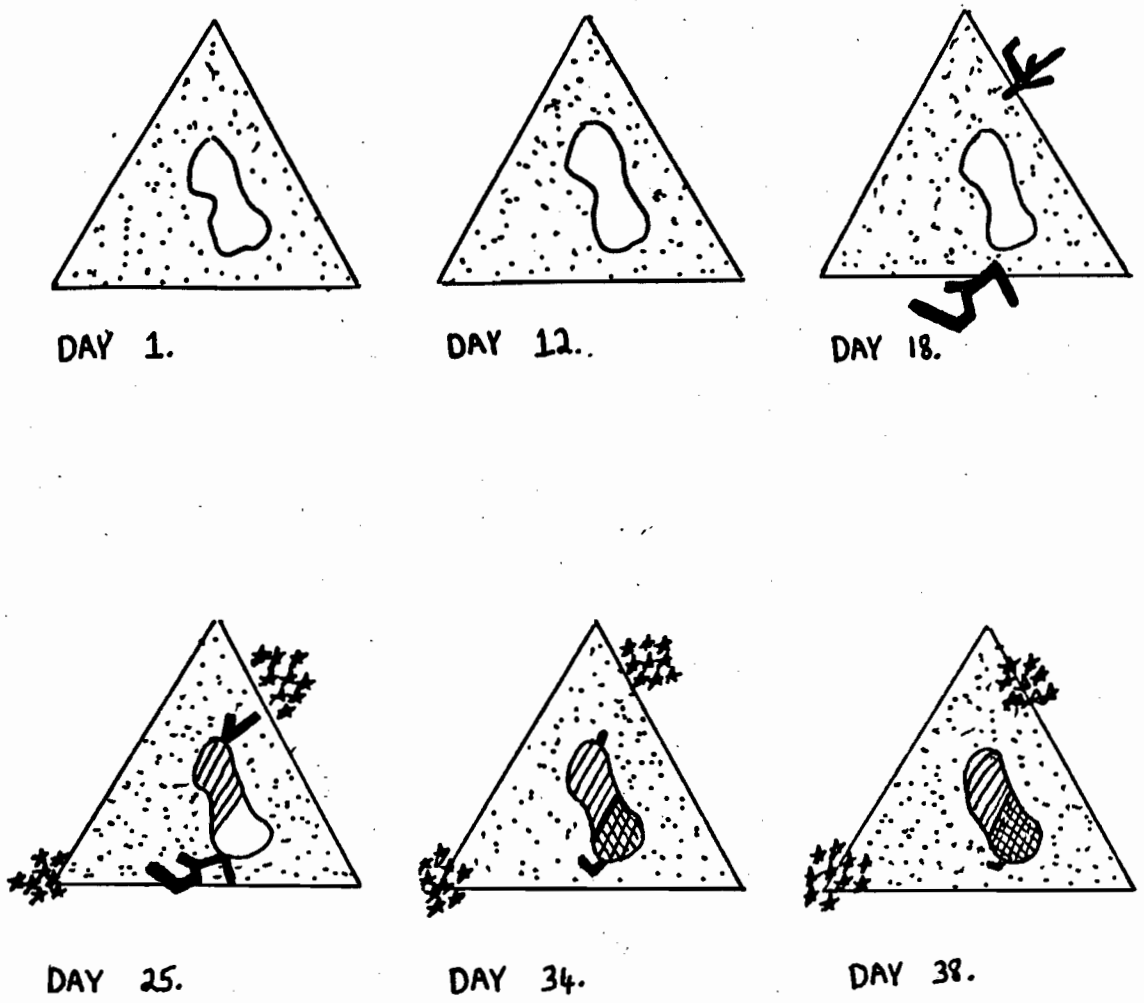


Figure 9. The migration of iridophores from the surrounding dermis toward the autografted erythrophores and their aggregation under the erythrophores. Also the beginning migratory activity of dermal melanophores (Newt 5). Illustrations represent 1, 12, 18, 25, 34, and 38 days post-grafting. The solid black patches represent individual iridophores, while the (*) represent individual dermal melanophores. The (·) represent epidermal melanophores. The boundary between the site of the graft and the surrounding whole skin is drawn in the form of a triangle; the solid line present inside this triangle represents the periphery of the mass of engrafted erythrophores. In this newt, a total of fourteen erythrophores were autografted. The cross-hatching represents erythrophores underlaid with iridophores. Only those iridophores and melanophores actively migrating during the period of observation are illustrated.

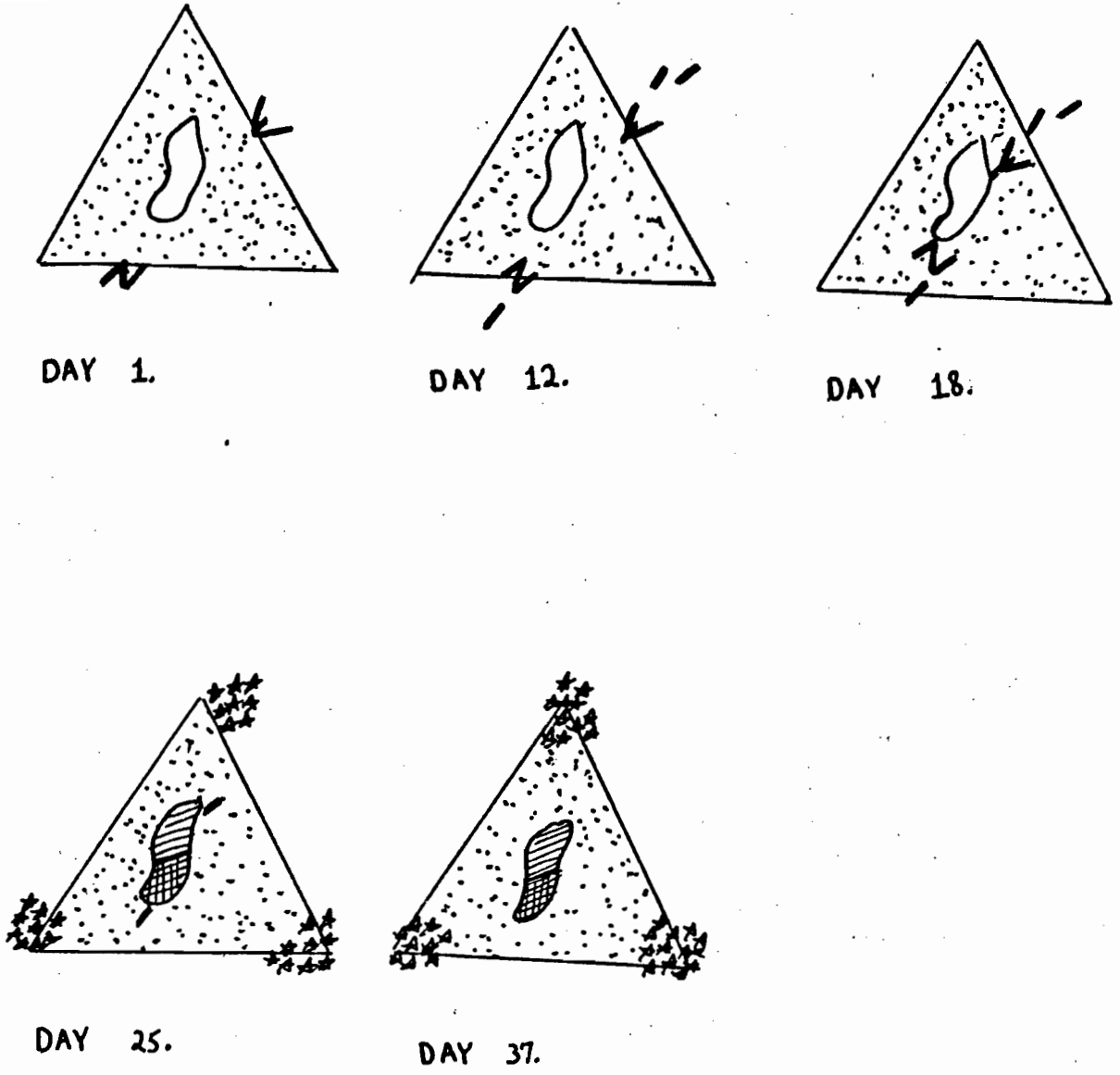


Figure 10. The migration of iridophores from the surrounding dermis toward the autografted erythrophores and their aggregation under the erythrophores. Also the beginning migratory activity of dermal melanophores (Newt 6). Illustrations represent 1, 12, 18, 25, and 37 days post-grafting. The solid black patches represent individual iridophores, while the (*) represent individual dermal melanophores. The (·) represent epidermal melanophores. The boundary between the site of the graft and the surrounding whole skin is drawn in the form of a triangle; the solid line present inside this triangle represents the periphery of the mass of engrafted erythrophores. In this newt, a total of eight erythrophores were autografted. The cross-hatching represents erythrophores underlaid with iridophores. Only those iridophores and melanophores actively migrating during the period of observation are illustrated.

TABLE 1. Migration of iridophores in response to different numbers of erythrophores.

Newt #	# E. Autografted	First I. Migration Noted	I. Migration Completed
1	7	DAY 26	DAY 43/50% **
2	MANY	DAY 18	DAY 52
3	MANY	DAY 18	DAY 43
4	7	DAY 18	DAY 32
5	14	DAY 18	DAY 34
6	8	DAY 12	DAY 25
7	MANY	DAY 18	DAY 35
8	9	DAY 20	DAY 40
9	12	DAY 22	DAY 40
10-19	∠6	NONE	-----
20-23	DIED	-----	-----

** Newt #1 died after 50% of the mass of erythrophores were underlaid with iridophores.

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