Colour patterning in the skin of the reed-frog

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A collection of 37 Natal reed-frogs, *Hyperolius marmoratus*, from a single locality was studied for their dorsal colour patterns. Data were assembled on the spectrophotometry, the structure and distribution of the chromatophores by means of light and electron microscopy, and the features of colour patterning. An attempt was made to regard colour patterns as the end result of a definable process of differentiation. A number of possible rules was set out according to which the patterns observed might be generated.

'n Versameling van 37 Natalse rietpaddas, *Hyperolius marmoratus*, afkomstig van 'n enkel gebied, is vir 'n ontleding van die dorsale kleurpatroon bestudeer. Gegewens is oor verskeie aspekte bymekaargemaak — reflekslespektrofotometrie, die bou en verspreiding van die chromatofore met behulp van lig- en elektronmikroskopie, en die eienskappe van die kleurpatrone. 'n Poging is aangewend om die kleurpatrone as die eindresultaat van 'n definieerbare differensiasieproses te beskou. 'n Aantal reëls is geformuleer waarvolgens die patrone, soos dit ontleed was, moontlik tot stand kon gekom het.

During its lifetime the reed-frog, *Hyperolius marmoratus*, develops certain changes of skin patterns. Considerable variation may be seen in the end results; so that specimens comprise a complex, rather than a single stable species (Poynton 1985). These patterns are protective. They also provide an index of maturity and of taxonomic affinity.

Analysis of the pattern could perhaps throw light on the processes by which it is produced. The peripherally established layers of pigment cells are acted upon by stimuli which bring about differentiation to final cell types, making the resulting picture complex and variable. It is these stable ground-patterns, and not the physiological fluctuations, which were examined.

These developmental processes are able to generate patterns which conceal the normal anatomy, mimic the background, and are 'anti-anatomical' in their relationships. For a taxonomic discussion of pattern types and relationships, see Schiotz (1971) and Poynton (1985).

The study comprises two parts — the gross appearances, and the cellular features as related to colour production.

Material

A group of 37 reed-frogs (18 males, 19 females) was collected from a single locality in Natal by Prof. Neville Passmore. These were identified as *Hyperolius marmoratus* Rapp (Amphibia, Anura), and the collection kindly loaned to the writer by Prof. Passmore, whose authority we accepted for the collection, identification and preservation of the material. Further identification of any local races or subspecies lay beyond the needs of the study.

Results

Gross appearance

Patterns and variations in dark stripe formation

The reed-frog displays a striped pattern on its exposed dorsal surface. It lies anteroposteriorly, parallel to the lines of its grassy plant background, while the ventral surfaces and the hidden folds of skin on the flanks are free from pattern markings.

'Primary' longitudinal stripe. In juvenile frogs a single dark lateral stripe is present (Figure 1). It runs from the lower part of the nostril through the lower half of the eye and down the flank, with matching marks on the limbs. It has a cream-coloured upper border. This pattern was seen in 7 male specimens. They displayed a grey to a deep brown background colour over the dorsal skin.

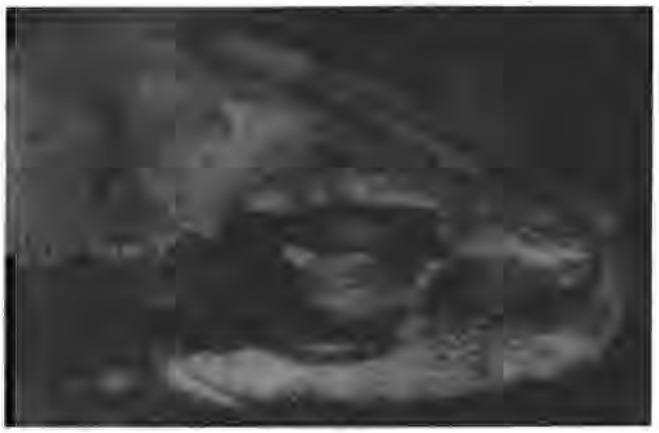

Figure 1 Juvenile pattern. The horizontal pigmented band connects the lower half of the nostril and the eye. A thin white stripe only is present along its upper edge.

Adult longitudinal stripe pattern. In 11 male and 4 female adult specimens, two main black stripes ran anteroposteriorly on either side of the dorsal midline. The stripes were most intensely pigmented anteriorly, and faded posteriorly. The stripe began anteriorly at the upper part of each nostril, splitting into two (Figure 2). Of these the lateral branch passed along the upper eyelid, and the medial branch ran parallel to the dorsal midline on either side.

'Jumping' stripe. When the hind limbs are pressed closely to the sides of the trunk, the dark dorsal stripe may appear to leap across the anatomical gap, continuing sometimes fairly accurately on the other side of the space (Figures 3, 4 and 5).

Departures from formation of dark stripes

Bursts, vortices and islands. In Figure 6 the flow seems to circumvent pale islands, in which pigment inhibition occurs. Occasionally the black colour blows out like a puff of smoke (Figure 7), or it may trickle along a line of skin contact, as

Figure 2 Striate pattern. The two major anteroposterior striae are placed as tangents to the organs of special sense. The medial stria lies at the top of the nostril and the lateral stria along the top of the eye. These are areas not occupied by the juvenile pattern.

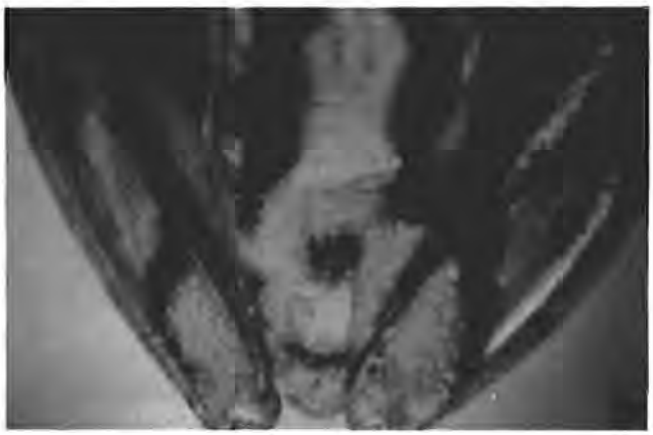

Figure 3 'Coincident disruptive pattern'. The two main dorsal stripes lying on either side of the midline coincide with the stripes on the two tibiae. When the tibiae are pressed against the body the structural discontinuity is obscured by the stripe which runs over directly from the back to the leg.

Figure 4 Close-up of the stripe continuity between the body and leg on another specimen.

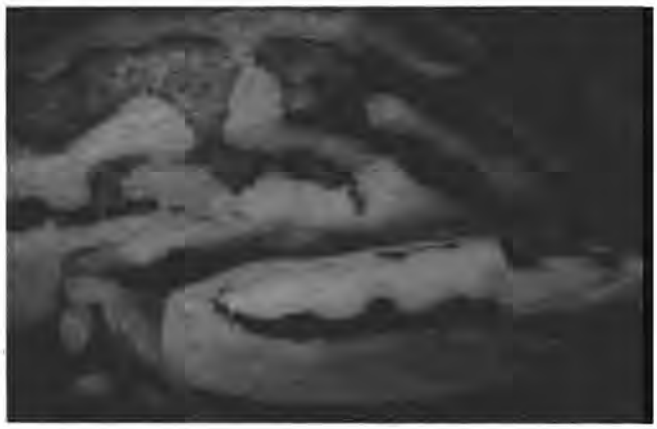

Figure 5 Here a dense, narrow lateral stripe on the trunk seemingly dwindles and disappears. Exactly opposite it on the knee region of the left leg, a similar stripe commences. If one presses the knee against the flank, a close matching of the stripe pattern is evident.

Figure 6 Marbled pattern. An intermediate between striate and vermiculate patterns. Relationships to the nose and eye are evident.

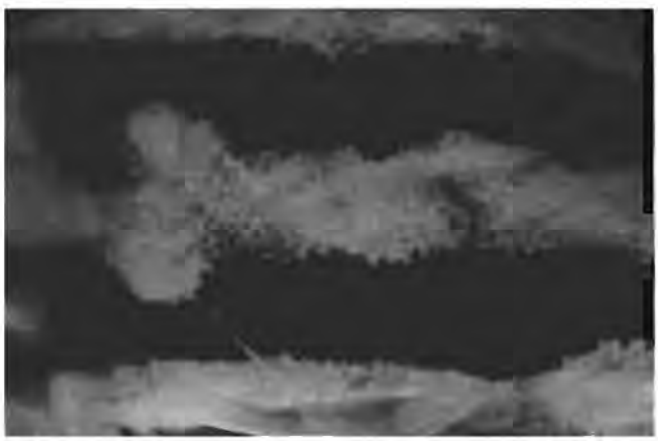

Figure 7 Smoke-like clouds of darkening appear to be bursting from the dark stripes, which contain large numbers of melanophores.

if escaping through a defect. The sides of a stripe are often zig-zag or undulating, justifying the idea of marbling in the species name 'marmoratus'.

Black stripes versus white. The anteroposterior dark stripes varied from 0,6 – 2,5 mm in breadth. This appearance was produced by melanophores, each some 100 – 120 µm across, and where they could be counted, the breadth contained 6 – 20 cells side by side. A feature distinguishing these white bands was that their breadth was rather more constant than that

of the intervening black stripes (Figure 8). No matter whether the white stripes were broad, narrow or sinuous in a given specimen, their edges were strikingly parallel to one another. Thus, if the stripe was broad it could look like a curving roadway, or if narrow it resembled a twisting, vermiculate pattern.

Figure 8 White stripes. Note the 'swerving roadway' effect of the parallel sides of the white bands.

Figure 9 Vermiculate pattern. Note multiple contact points along the upper eyelid.

Figure 10 Vermiculate pattern. These narrower stripes tend to meander, although they keep an anteroposterior trend. Darker melanophores can be seen at the margins of the dark stripes.

Vermiculate patterns. These formed a more reticular and irregularly linear type of marbling. The intervening white zones therefore become vermiculate or spotty. Among 8 examples (4 male; 4 female), the dark bands were about half the width of the broad striate bands of other specimens. The melanophores were larger (up to 200 µm across), and the dark bands were about 3 – 10 cells across (Figures 9 and 10).

Dot patterns. These specimens (1 male; 1 female) fall into an unclassified group of single-cell patterns. Evenly scattered black dots were each caused by the presence of a single large melanophore about 0,2 mm across (Figure 11).

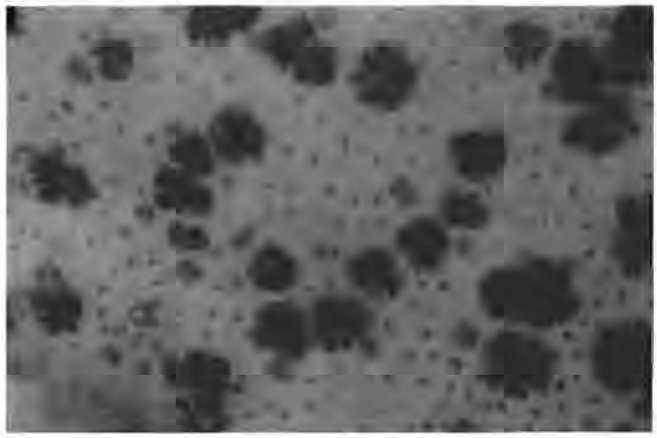

Figure 11 Dot pattern. The black markings comprise single giant melanophores which are ungrouped. The inactive melanophores can just be detected in between.

Cellular features — cells contributing to skin colour

The skin patterns owe their existence to sharp differences between the darker and the lighter cellular elements.

Whole mounts — morphology, optics, cytochemistry

Epidermis. Transillumination of detached sheets, as well as light and electron microscopy of the epidermis, revealed no colour-producing structures (particulate or laminated materials). The epidermal thickness was about 20 µm, of which the stratum corneum made up 1,5 µm.

Fluorescent materials. Under a 253 nm light source, only the ventral skin showed a yellow tint and a yellow-green fluorescence at times. Under illumination at 365 nm there was no fluorescence. Fluorescent materials therefore made no contribution to skin patterning.

Iridophores. Iridophores were best seen in the white areas of the dorsal skin in whole mounts, under incident light with 6 – 200× magnification. As expected from their name, they shimmer somewhat, and appear rounded or angulated in shape. In the seemingly amorphous cell mass one may see tiny reflecting particles by oblique incident light. These particles, in transmitted light, are polarizing, and are located at the cell edges.

Evidence for guanine in iridophores. Heating the frog skin to 180°C for 5 min in silicone oil did not destroy the optical features of the iridophores. This was consistent with the behaviour of guanine. Chromatographically pure amorphous

guanine (BDH) gave the same results. It has also been claimed that guanine, the presumed constituent of iridophore cytoplasm, gives a histochemically positive murexide test (Glick 1949; Lison 1953). We were led to doubt this claim for several reasons.

Applied to whole sheets of iridocyte-rich frog skin, a negative murexide reaction was obtained. Nevertheless a clear xanthoproteic reaction resulted. Using chemically pure uric acid and guanine as spot tests in the murexide reaction, the purple murexide colour was given by uric acid, but guanine became yellow-orange, exactly as in the frog skin.

The white material in the iridocytes was dissolved out in processing for the light and the electron microscope. This unexpected loss of purine crystals from amphibian skin has also been noted by Bagnara (1979).

Fragments of such skin with a surface area of 3 – 7 mm^2 and a moist weight of 90 – 120 µg were extracted by grinding in 0,1N HCl. The supernatant gave an absorption peak at 248 nm identical to guanine, although the remainder of these curves did not fit guanine accurately. Ignoring the effect of impurities, the guanine content of such specimens would be as much as one third to one half the wet weight. The concentrations were nevertheless too low for simple thin-layer chromatography.

In spite of the apparently conflicting test results, the main constituent of the iridophore is evidently therefore guanine.

Melanophores. These cells are plentiful throughout the dorsal patterning, but occur also in the flexible ventral surfaces of the hand, foot, jaw and vocal sac. In the white patterned areas, the melanophores are small and compact. In the dark areas (Figure 12) the melanophores are dendritic, with thick stems and branches. The rounded iridophores are arranged on these dendrites, comprising what has been called the chromatophore unit (Bagnara & Hadley 1973).

Clustered melanophores are often enlarged and specially dark at the edges of dark bands. Scattered melanophores contain less pigment. Where the melanophores are clustered in small numbers (say 3 – 5) the dark lines produced tend to meander. With larger numbers, say 10 – 20 cells across a stripe (Figure 7) the edges are more fixed and straight, and the cells a little smaller.

Xanthophores. These are present in yellow flecks on the flank. In transmitted light they appear brown. The cells have a pseudopod-like outline, and appear to displace the small rounded melanophores. They are not iridescent under incident light.

Figure 12 Junction of black and white areas. In the dark area on the left the melanophores are dendritic with thick branches, and iridophores are caught in clusters around them. This comprises the 'chromatophore unit'. In the pale area on the right, melanophores are small and iridophores are grey and rounded (original × 100).

Gland openings. These are regularly scattered, and appear colourless. Their surface stomata displace melanophores and the region is not iridescent. The stomata are triradiate or cruciform in shape. At least two kinds of gland were seen in the brown juvenile frog — one with acini and the other with dense globules of yolk-like material.

Light microscopy

The stratum laxum of the dermis, or stratum spongiosum (Elkan 1968), is sandwiched in between the epidermis and the deeper, compact collagen on the dorsal skin of the frog. It is a loose layer which houses the optically significant cells and various skin glands. Conventional histological sections are unsuitable for cell topography in this layer because of the enormous size of the cells.

Reflectance spectrophotometry

A colour analysis was made of various regions of the skin, and the results are summarized in Table 1. The International 'CIE' terminology which we employed is set out in many standard sources on visual optics. We relied chiefly on the works of Bouma (1948) and Judd (1933).

Table 1 CIE colour specification of skin

	Appearance and site	No. of specimens	Luminance (ε)	Spectral colour (λ_D)	Purity (p)
1.	Homogeneous dorsal colours; brown to grey-white	5	8 – 31%	573 light grey to 593 in dark brown (i.e. yellow to orange)	0,056 – 0,177
2.	Homogeneous ventral yellow-white skin	3	37 – 46%	580,5 – 582,4 (yellow)	0,168 – 0,215
3.	White skin with minute yellow and red stipples. Behind angle mouth	2	27 – 29%	580,5 – 581,5 (yellow)	0,20 – 0,21
4.	Black skin with brown tinge	1	12%	588,5	0,04
5.	Head dorsum. Black with white speckles or stripes	2	11 – 23%	577,5 – 581,5	0,028 – 0,037
6.	Back. Black and white areas	4	15 – 27%	570,5 – 579,6	0,055 – 0,1

It can be seen that the skin luminance varies between 8 and 46%. It is greatest for the ventral, normally invisible skin. The lowest luminance came from the brown and black areas on the dorsum of the animal. Despite iridescence, the melanophore mixtures probably rendered the white dorsal areas less bright.

The spectral colour of the skin was always a yellow-orange, of low purity.

Discussion

The cellular substrate of pattern formation

The cells responsible for pattern formation in the frog skin are all presumed to derive from multipotent stem cells of the neural crest. Such cells, after migration, and further proliferation in a position under the epidermis, are then subject to further differentiation. Final cell types are thus produced. For this final change to occur, suitable 'cues from the microenvironment' are required (Hall 1983). In their work with other anurans, Ohsugi & Ide (1983) also found that melanoblast differentiation '. . . seems to be controlled by the information which exists in the dermis . . .'.

The final cell types tend thereafter to retain a particular position and shape, and to possess a dominant variety of organelle. Some slight overlap in organelle types has been noted in our material and elsewhere, which tends to confirm the unity of their stem-cell derivation.

Pattern determination

The melanoblasts thus appear to possess the full potential for pattern production, without containing any pre-pattern laid down in the migrating or proliferating cells themselves. Patterns will then be determined by the subsequent 'positional values' affecting the cells at their final site in the periphery. Even if a cell system is committed in this way, it will of course not prevent other patterns from taking the place of the initial ones. The cells may be replaced by others which can be acted on afresh. Transient pattern changes may also come about by neurohormonal influences, acting reversibly on the basic or 'ground-state' pattern.

Evidence from the invertebrate world indicates that this system of peripheral differentiation as a method of pattern production occurs there as well. An example is given by Alfred Kühn (1955) for the butterfly wing of *Ephestia kühniella*. Here the primordial epidermal scales are each capable of developing a number of different colour patterns. At a specific time, after the scale rudiments are already established in the wing epidermis, a developmental determinant flows through the wing in two directions, on either side of a more-or-less equatorial position. Symmetrically balanced, mirror-image patterns arise to the medial and lateral sides of this 'equator', which runs anteroposteriorly down the middle of the wing. At that time, nothing could really be said about the actual sources or the biochemical nature of the stream, such as reacted variously with the epidermal scales on travelling through, to produce the colour differences.

In the reed-frog the main cell which determines the skin pattern is the melanophore. In the black areas the melanophores are large, with extensive dendrites and great amounts of pigment. In the white areas they are shrunken and produce less pigment. These differences are quite extreme, and the transition from one type to the other takes place over microscopic distances. The patterns are consequently very sharply outlined.

Generation of stripe or ribbon patterns

While it is acceptable to refer the origin of a convexly spreading pattern to its centre, the induction of a ribbon or band-like pattern is less simple. These banded patterns are also apt to be repetitive — a feature which must also be accounted for.

Broadly speaking, the induction may have travelled down the length of the band. Its origin will then lie at one or other end of the line. Otherwise, the induction process must cross the line at right-angles, with a linear wave-front and a pre-existing linear source.

Molecular and kinetic requirements for band-like pattern production

As outlined by Hall (1983), the surface of a cell susceptible to a 'microenvironmental cue', reacts to such a cue in the style of an antigen-antibody response. The developmental regulator can be seen as the antibody attaching to appropriate molecules in sufficient numbers on the cell surface to elicit a response. While it is correct to say that 'reaction-diffusion models . . . provide little insight in chemically identifying morphogens' (Pate 1984), it seems likely that peptides are substances with a suitable antibody-mediator character. As Erspamer (1983) points out, 'amphibian skin is an enormous storehouse of biogenic amines and active peptides' which have a mammalian counterpart in organizers of the central nervous system. In the frog it is conceivable that some of these substances could diffuse out into the skin from the spinal cord.

The cell-surface binding of a morphogen, whatever its nature may prove to be, has important consequences. Firstly, the reacting cell must exhibit a threshold of response above a particular concentration of the stimulus. Secondly, this local binding will lead to a local drop in concentration of the diffusible molecular stimulus. Consequently, the wave-front advances beyond, to create a further similar response only when the concentration has built up sufficiently again. Depending on the shape of the wave-front, whether linear or circular, the resulting picture can arise as a series of bands or a set of concentric rings.

Liesegang rings or bands

The classical model system, by which a molecular continuum gives rise to parallel stripes or concentric rings, has long been recognized since Liesegang studied band-like precipitates on early photographic plates. Essential requirements are a fairly stable diffusion medium, the presence of two reactants (at least) — one fixed and one diffusing — a threshold concentration for the visible reaction to occur, and a drop in concentration of the diffusing element to a level below the threshold value after binding.

Striped patterns in the reed-frog

From the considerations given, there seem to be two major possibilities for pattern generation in the material studied:

(i) That the stimulus to differentiation is present only in the black areas, and traverses the length of the ribbon-like strips. (This model is the geometrical equivalent of a pencil-line drawn with a ruler, having a starting point at one specified end of the line.) According to this model, the only site which serves consistently as a suitable focus of origin would be the nostril. With the strong production of a stimulus from the nose, and a strong lateral inhibition, a jet-like stripe production could result. Where the stripe jumps the gap across to the leg, the stimulus would

need to bridge the anatomical discontinuity. With a weaker stimulus the resulting patterns would be more meandering.

(ii) That the stimulus moves laterally from either side of the spine in the style of a Liesegang phenomenon. It would reach the head by a forward movement, while the body and thigh stripes would be made to match by moving corresponding distances down the hind limb as on the flank. No stripe jumps are therefore required. The constant breadth of the white bands would therefore represent the gap needed for building up the stimulus for the black bands. (This model is the rough geometrical equivalent of a roller-printer moving out on either side of the midline.) This interpretation is more comprehensive and versatile than the previous one.

Unknowns

These interpretations of pattern have the advantage of clarifying stepwise responses by the interaction of fixed and moving phases. The source and mode of transmission of the stimulus are still obscure. Is the origin in the spinal cord or the nose? Is the spread by plain diffusion or is there a cell-to-cell transmission, with re-making, amplification, noise etc . . .? Further, the genetic determinants of both the stimuli and response potential are not established.

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