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Adaptations of the reed frog *Hyperolius viridiflavus* (Amphibia: Anura: Hyperoliidae) to its arid environment

VI. The iridophores in the skin as radiation reflectors

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Summary. *Hyperolius viridiflavus* possesses one complete layer of iridophores in the stratum spongiosum of its skin at about 8 days after metamorphosis. The high reflectance of this thin layer is almost certainly the result of multilayer interference reflection. In order to reflect a mean of about 35% of the incident radiation across a spectrum of 300–2900 nm only 30 layers of well-arranged crystals are required, resulting in a layer 10.5 μm thick. These theoretical values are in good agreement with the actual mean diameter of single iridophores (15.0 ± 3.0 μm), the number of stacked platelets (40–100) and the measured reflectance of one complete layer of these cells (32.2 ± 2.3%). Iridescence colours typical of multilayer interference reflectors were seen after severe dehydration. The skin colour turned from white (0–10% weight loss) through a copper-like iridescence (10–25% weight loss) to green iridescence (25–42%). In dry season state, *H. viridiflavus* needs a much higher reflectance to cope with the problems of high solar radiation load during long periods with severe dehydration stress. Dry-adapted skin contains about 4–6 layers of iridophores. The measured reflectance (up to 60% across the solar spectrum) of this thick layer (over 60 μm) is not in keeping with the results obtained by applying the multilayer interference theory. Light, scattered independently of wavelength from disordered crystals, superimposes on the multilayer-induced spectral reflectance. The initial parallel shift of the multilayer curves with increasing thickness and the almost constant ("white") reflectance of layers exceeding 60 μm clearly point to a changing physical basis with increasing layer thickness.

Key words: Arid environment – Aestivation – Iridophores – Skin reflectance – Frog, *Hyperolius viridiflavus taeniatus*

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Introduction

Members of the "super" species *Hyperolius viridiflavus* (Schiøtz 1971) inhabit seasonally very hot and dry African savannas. During dry seasons, these small reed frogs aestivate unhidden mostly on dry plants. Some aspects of their water and energy economy and of nitrogen metabolism have been reported in Geise and Linsenmair (1986, 1988), Schmuck and Linsenmair (1988) and Schmuck et al. (1988).

As indicated by the "climate space" parameters (Gates 1980), in dry seasons a skin reflectance higher than 0.7 is vital during the hottest hours around noon; frogs are exposed to a heavy solar radiation load and air temperatures between 38 °C and 43 °C (Kobelt and Linsenmair 1986). During acclimatization to dry season conditions a very conspicuous change in the wet-adapted, yellow-brownish colour pattern of juvenile frogs can be seen. Skin colour is brownish-white or grey at air temperatures below 35 °C and becomes much paler at air temperatures over 35–37 °C. As acclimatization progresses this colour approaches brilliant white. These changes are accompanied by a thickening of the layer of light-reflecting iridophores (Bagnara 1976) in most parts of the skin from 15 μm up to 80 μm (Kobelt and Linsenmair 1986) and a corresponding increase in the amount of skin-deposited purines (Schmuck et al. 1988).

Even as long as a century ago it was assumed that light interference was involved in the reflectance of the iridophores ["Interferenzzellen": Biedermann (1892); van Rynberk (1906)]. Denton (1970), in work on the iridescence colours of fish scales, and Land (1966, 1972) in studies on the eyes of the scallop *Pecten maximus*, came to the conclusion that iridophores in general might be multilayer interference reflectors [see also Rohrlich and Porter (1972), Menter et al. (1979) and Frost and Robinson (1984)]. Huxley (1966) developed a method to calculate the reflectance of these biological structures. Some morphological aspects of dermal anuran iridophores resemble the structure of Huxley's reflectors and hence

suggest that the reflectance of the anuran skin might be explained by this theory.

Many platelets in the iridophores are arranged in stacks of 3–20 crystals. In recently metamorphosed wet-adapted *H. viridiflavus* these stacks are distributed in an irregular mode. In dry-adapted *H. viridiflavus*, however, a significant alignment is evident, many stacks being arranged more or less parallel to the surface (Kobelt and Linsenmair 1986). However, the reflectance of multilayer structures is wavelength selective, and if iridophores are pure multilayer interference reflectors their reflected light should be coloured and not white.

Some investigators described three groups of iridophores in fishes (Ballowitz 1912; Connolly 1925; Becher 1929; Foster 1933, 1937; Odiorne 1933; Shanes and Nigrelli 1941; Fries 1942, 1958). One group of cells, leucophores, is situated in the dermis above the bony scale and reflects white light due to scattering of poorly ordered platelets. Two other groups, iridophores, one in the dermis beneath the bony scale and another one still deeper and above the muscles, are iridescent due to parallel-ordered platelets (Denton 1970). These findings suggest that two possible physical mechanisms are involved in the high reflectance of light via iridophores: light scattering and multilayer interference.

Both physical mechanisms of reflectance provide advantages and disadvantages: (1) Multilayer interference structures can be manufactured very fast and at low energy and material costs. Layers of not more than 10 µm are highly efficient light reflectors in a given spectral range but are not very efficient across the whole solar spectrum. (2) Structures that scatter light independent of wavelength are much more effective reflectors across the whole solar spectrum but need much more energy and material. This investigation was performed to decide which physical model best explains the high skin reflectance in fully dry-adapted *H. viridiflavus*.

The Kubelka-Munk-Theory

Scattering of white light can be calculated according to the Kubelka-Munk-Theory. A summary of theories dealing with the optical characteristics of powder layers, in which the layer is considered a continuum, has been given by Kortüm (1966). (A detailed description of the model and definitions of symbols are given in Appendix A). When S is estimated from animal data and R_0 is measured, the thickness d of a scattering layer in the skin can be calculated from:

$$S \cdot d \cdot \left(\frac{1}{R_\infty} - R_\infty\right) = \ln \frac{1 - R_0 \cdot R_\infty}{1 - \frac{R_0}{R_\infty}} \qquad (4)$$

The calculated data can then be compared with the morphological thickness in order to see to what extent the Kubelka-Munk theory adequately describes the measured reflectance of the skin.

The multilayer interference theory

The multilayer interference theory for biological systems has been developed by Huxley (1966) and Land (1966). A detailed description of this theory is given in Appendix B. The reflectance (R) of a multilayer interference structure is given by Eqs. 9a and 9b in Appendix B. From this theory the spectral distribution of the reflected light can be calculated. Comparing them with the measured spectral distribution of the light reflected from the skin may provide clues to whether the reflecting layer has the physical properties of a multilayer interference structure.

Materials and methods

Specimen treatment. Frogs were kept in terraria at 28/24 °C, 70/100% relative humidity (day/night) and a light regime of 11L : 13D to simulate "wet season conditions" (spraying of water and feeding daily) and "transitional conditions" (spraying and feeding three times a week). "Dry season conditons" were simulated in an incubator at 30/20 °C, 30/70% relative humidity and 11L : 13D without feeding. Detailed descriptions of specimen treatment of *H. v. taeniatus* for inducing wet and dry season physiological states in the laboratory have been presented elsewhere (Geise and Linsenmair 1988; Schmuck and Linsenmair 1988; Schmuck et al. 1988).

White reflectance measurements. For these measurements freshly transformed froglets were maintained under wet season conditions for 2 days and divided into two experimental groups. The amount of guanine in the skin of these froglets is less variable than in the juvenile frogs of group 3 which may have access to conditions inducing dry season acclimatization.

Group 1: 14 froglets were kept wet-acclimatized by hydrating them every 24 h for 60 min with 500 µl double-distilled water.

Group 2: 20 froglets were dry-acclimatized. 500 µl of double-distilled water was supplied for 15 min only when water losses exceeded 30–35% of body weight. All froglets needed a first water supply between day 10 and 12. A second supply had to be given with great individual differences mostly between day 30 and day 50 (90% of the froglets). Only about 10% received a second supply before day 30.

Spectral reflectance measurements. For these measurements wet-acclimatized juvenile *H. v. taeniatus* were used (age 2–4 months after metamorphosis). These frogs possess 1–2 complete reflecting layers of dermal iridophores. Thus, errors due to iridophore-independent absorption of light deep in the body of the animal, resulting from gaps in the reflecting layer which may be present in freshly transformed froglets, are negligible.

Group 3: 16 juvenile frogs were maintained under transition state conditions for 2 weeks and kept under dry season conditions afterwards. Measurement started at the beginning of the dry season treatment.

Group 4: Ten juvenile frogs were acclimatized and kept for 2–3 months under dry season conditions before measurement. During this period they developed 4–6 iridophore layers, thereby matching animals which had been collected in the field after spending a dry season of comparable duration. All frogs of groups 1–4 were measured every 4 days to determine changes in spectral skin reflectance. They received about 50 µl of double-distilled water after every measurement to replenish water losses which were unavoidably caused by experimental handling during measuring.

Green iridescence measurements. With increasing dehydration in all groups a copper-coloured iridescence was observed, which changed

with further dehydration to a whitish-green. As described above, coloured iridescence is typical of multilayer interference reflectors (Land 1966). This phenomenon could therefore give further clues to the nature of the high skin reflectance.

Group 5: Iridescence was investigated in 13 freshly transformed froglets (age and treatment identical with group 2); green iridescence is much more pronounced in this developmental phase than in other age classes and usually occurred even after 1 week of dehydration. Frogs were weighed daily to determine their dehydration state. As long as their skin was white, spectral reflectance was measured every 2nd day. When the copper-like colour appeared measurements were taken daily. In contrast to the other groups, froglets were given no water after measurement until green iridescence was visible or loss of body weight exceeded 45%. Then, after measurement of spectral reflectance, the frogs received 500 µl of double-distilled water for 60 min and their spectral reflectance was measured again 2 h later. In this experiment colours were classified as follows: *w:* white; *wc:* white and spotted copper-like iridescence; *C:* copper-like iridescence across entire dorsal skin; *Cg:* copper-like and spotted green iridescence; *G:* green iridescence across whole dorsal skin.

Reflectance measurements. White skin reflectance was measured using the apparatus described in Fig. 1a, and spectral reflectance by that shown in Fig. 1b. The physiological colour change of *H. v. taeniatus* is temperature dependent (Fig. 2a). To measure reflectance in the upper temperature range of their habitat, froglets were placed under a Perspex cover (see Fig. 1) on black metal plates on a thermal conductor heated to 40 °C by a thermostat. At air temperatures of about 24 °C their core temperature stabilized at about 39 °C and nearly all froglets became brilliant white displaying their maximum reflectance. They were usually sitting motionless on their metal plates. Thus, when the froglets were handled carefully, no anaesthesia was required for any reflectance measurement. After 45 min of temperature and colour adaptation, the whitened frog together with its metal plate was transferred onto a small adjustable heat conductor which was heated to a constant temperature of 40 °C. The focused beam of light was adjusted to the back of the frog and the animal placed close to the measuring hole of the sphere. The skin reflectances were referred to a white standard of frog-shaped plaster cast covered with a 2-mm-thick layer of Eastman 6080 (Kodak) by a spray gun.

The physiological colour change is very sensitive to different kinds of stress. Thus, animals that did not whiten under these conditions were not subjected to measurements. To investigate the effects of physiological colour change on reflectance, frogs of group 4 were dark-adapted and measured at 20 °C.

Light microscopy. After reflectance measurements some froglets were precooled and double-pithed. The whole dorsal or ventral skin was fixed in 6.25% glutaraldehyde in 0.1 *M* monophospate buffer (pH 7.2) for 30 min, then briefly washed in phosphate buffer and postfixed for 1 h in 1% OsO_4 solution (pH 7.3) (Wohlfahrt 1957). The tissues were dehydrated in a graded series of acetone and flat-embedded in Durcupan (Fluka) plastic resin. For light microscopy, semi-thin sections (1.5 µm) were made across the whole breadth of the skin and stained with methylene blue/azure II (Richardson et al. 1960).

The thicknesses of the reflecting layers was measured with a calibrated ocular micrometer. The mean of 10 measurements at different parts of each cross section was taken as the thickness (*d*) of the reflecting layer.

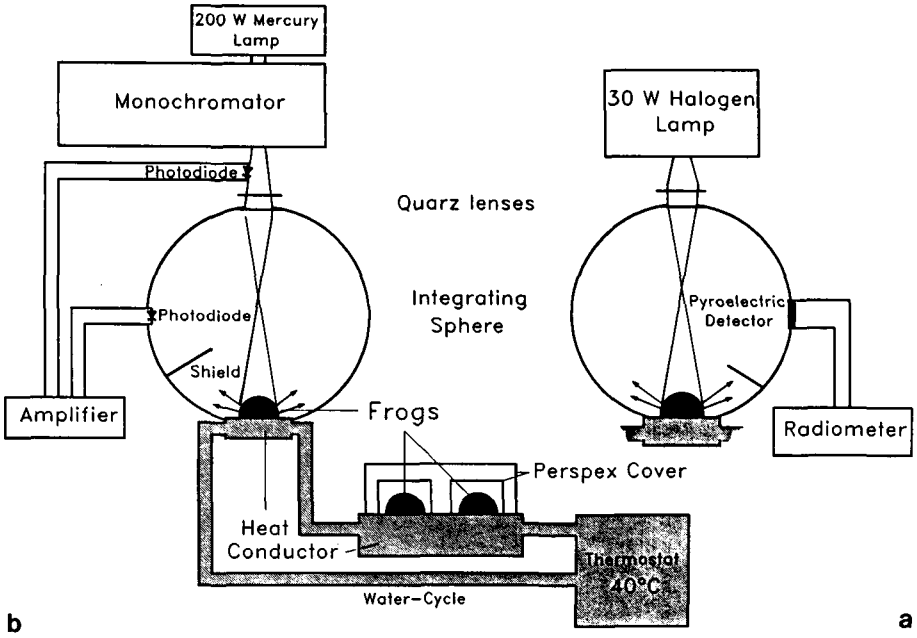
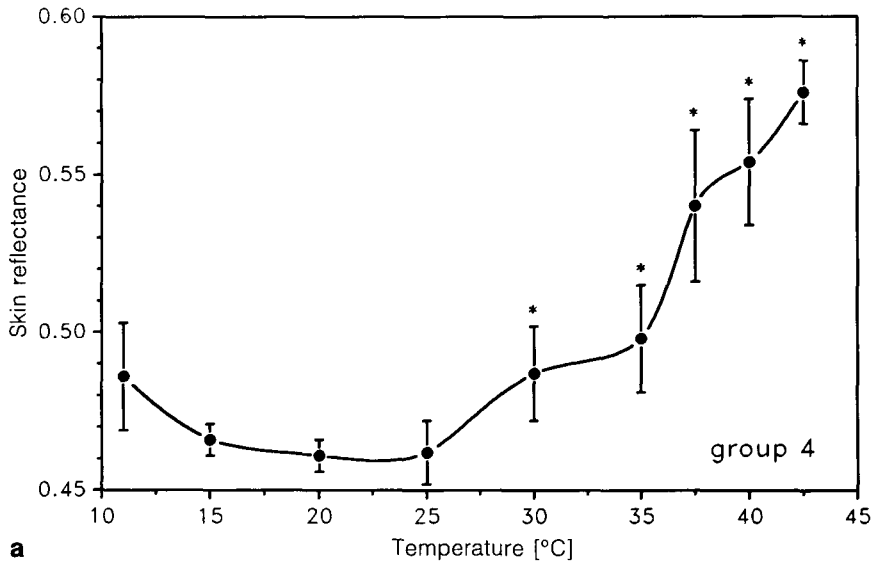


Fig. 1a, b. Schematic diagram of the reflectometer: **a** White skin reflectance was measured with a radiometer (Molectron) mounted on an integrating sphere (Earling). To simulate a more sun-like spectrum, a 30-W tungsten quartz halogen lamp (light colour 3150 K) was run at about 10% higher voltage (Willmer and Unwin 1981). The light from the lamp was focused to a small spot by quartz lenses. Accuracy tested with Eastman 6080 standards was within ±1% of the measured values. **b** Spectral measurements were performed by mounting a 200-W mercury lamp and a monochromator (Zeiss) on the integrating sphere (Earling) instead of the tungsten lamp. Light was measured by Si photodiodes (Hamamatsu). One photodiode replaced the radiometer; a second photodiode, adjusted to the outlet of the monochromator, served as reference to minimize errors due to fluctuations of the lamp. Spectral reflectance (R_S) was calculated from the measured pair of animal data of the two photodiodes (I, I_R) and from the pair of data for the Eastman 6080 standard (I_S, I_{RS}) to give: $R_S = \frac{I \cdot I_{RS}}{I_R \cdot I_S}$. Accuracy was within ±5% of the measured values



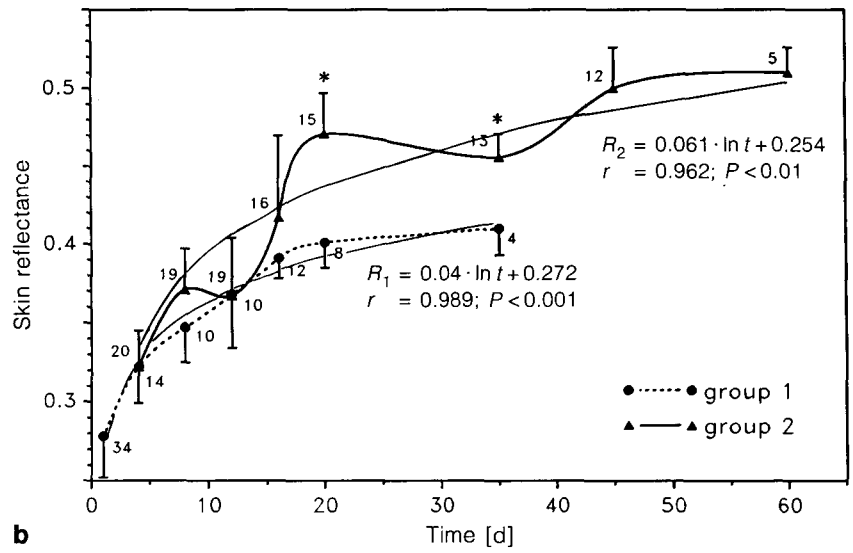


Fig. 2. a Correlation between skin reflectance and core temperature. Frogs of all age classes exhibit a correlation between core temperature and skin colour. This is pronounced in dry-adapted frogs of group 4 which try to match the black colour of their seats between 20 and 30 °C. Note the significant rise of skin reflectance above 30 °C. **b** Increase of skin reflectance under prolonged dry season conditions. Skin reflectance increased in a logarithmic pattern. Note the plateau between day 8 and day 12 in group 2. The formulas represent the relationships of the skin reflectance (R) against the natural logarithm of the experimental time (t) in days derived by regression. In all figures *bars* indicate standard deviation and *numerals* animal numbers. Differences in numbers result from animals which were not counted because of either atypical skin colour at 40 °C or weight loss exceeding 30%. * indicates significant differences from the mean at 25 °C and between corresponding means of groups 1 and group 2 (Wilcoxon test, $P<0.05$)

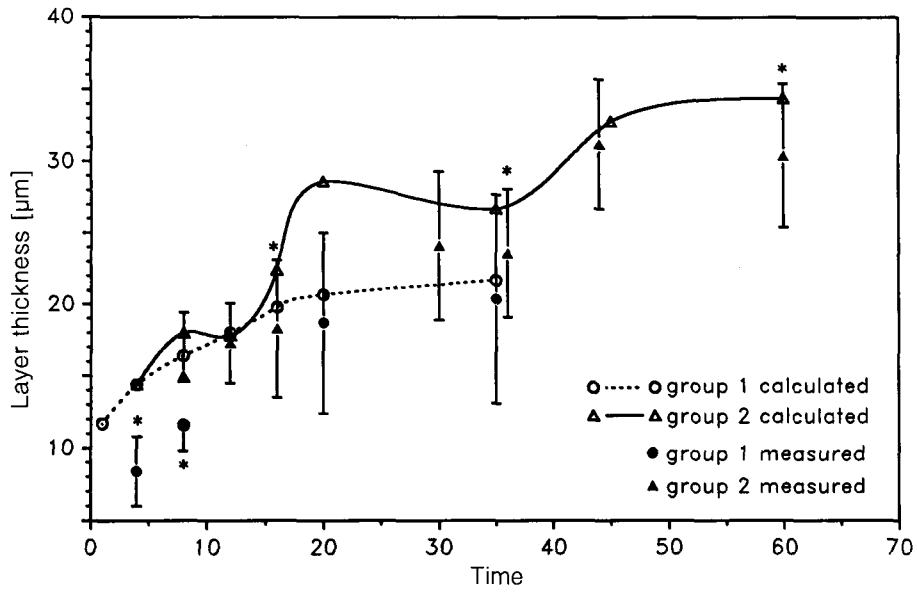


Fig. 3. Thicknesses of the reflecting layer calculated from the Kubelka-Munk theory and measured from skin sections. All measured thicknesses of the reflecting layer are below the calculated curves. Differences are especially high at the beginning of the experiment. * Significant difference between measured and calculated thicknesses (t-test, measured mean against the corresponding theoretical value, $P<0.05$)

Results

White reflectance

At air temperatures between 20 and 30 °C, the froglets show a colour matching that of the background. This colour changes to a brilliant white if the air temperature exceeds 35 °C (Kobelt and Linsenmair 1986). The correlation of skin colour and core temperature is given in Fig. 2a. A detailed description of these results will be presented elsewhere (F. Kobelt and K.E. Linsenmair, in preparation).

Usually the iridophores are shaped like globes with a diameter of 15 ± 3 µm. When the number of iridophore layers exceeds three, these cells become elliptically shaped and the diameter is highly variable (Kobelt and Linsenmair 1986). Skin reflectance data of groups 1 and 2 were presented in detail by Schmuck et al. (1988). A brief summary of these data is given in Fig. 2b as an aid to understanding the following results.

All thicknesses derived from light microscopy are smaller than the values calculated from Eq. 4. Differences are especially high at day 4 (70.6%) and day 8 (43.5%). The mean differences (± standard deviation) are (Fig. 3):
Group 1: $21.3 \pm 17.3\%$, $n=4$;
Group 2: $12.0 \pm 8.0\%$, $n=7$.

Spectral reflectance

The spectral reflectance of group 3 has a broad maximum between 800 and 1100 nm, and two smaller maxima at 360 nm and 520 nm. It increases steeply below 340 nm. After 20 days under dry season conditions skin reflectance is shifted about 0.12, but the shape of the curve is almost identical. The smaller maxima remain at the initially measured wavelengths (Fig. 4a).

Group 4 has a spectral reflectance which is almost constant across the whole measured spectrum. A steep absorption band is seen at 320 nm. From the differences in spectral reflectance between white- and brownish-

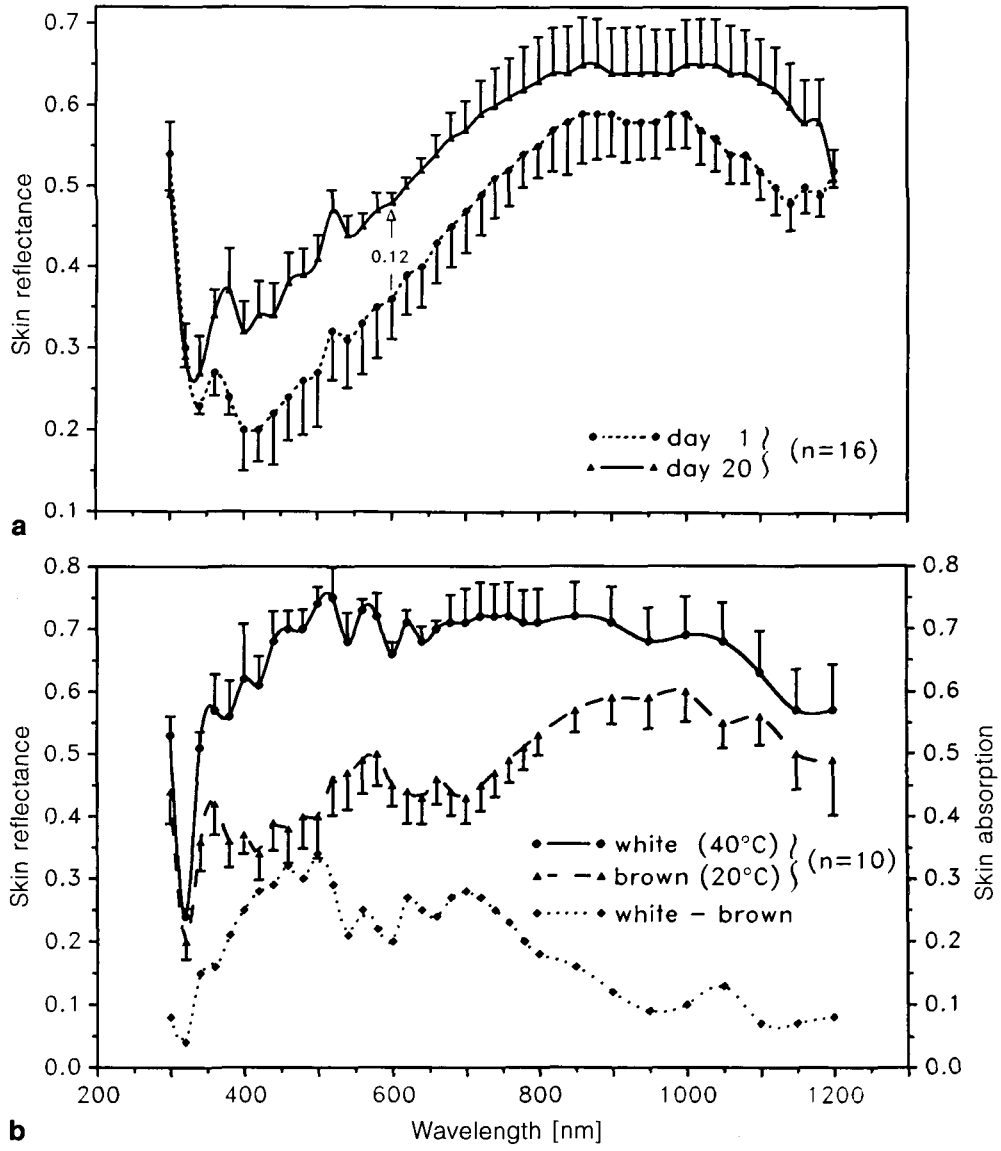


Fig. 4. a Spectral skin reflectance of group 3 frogs before and after 20 days under dry season conditions. The shape of the curve did not change significantly; it is shifted about 0.12 almost parallel to the curve for day 1, but the IR-maximum becomes distinctly broader. **b** Spectral skin reflectance and absorption of group 4 frogs at 20 °C and 40 °C. Frogs with a reflecting layer of a thickness exceeding 60 µm had a high reflectance across the whole spectrum; melanin absorption was calculated by subtracting the curve at 40 °C from that at 20 °C

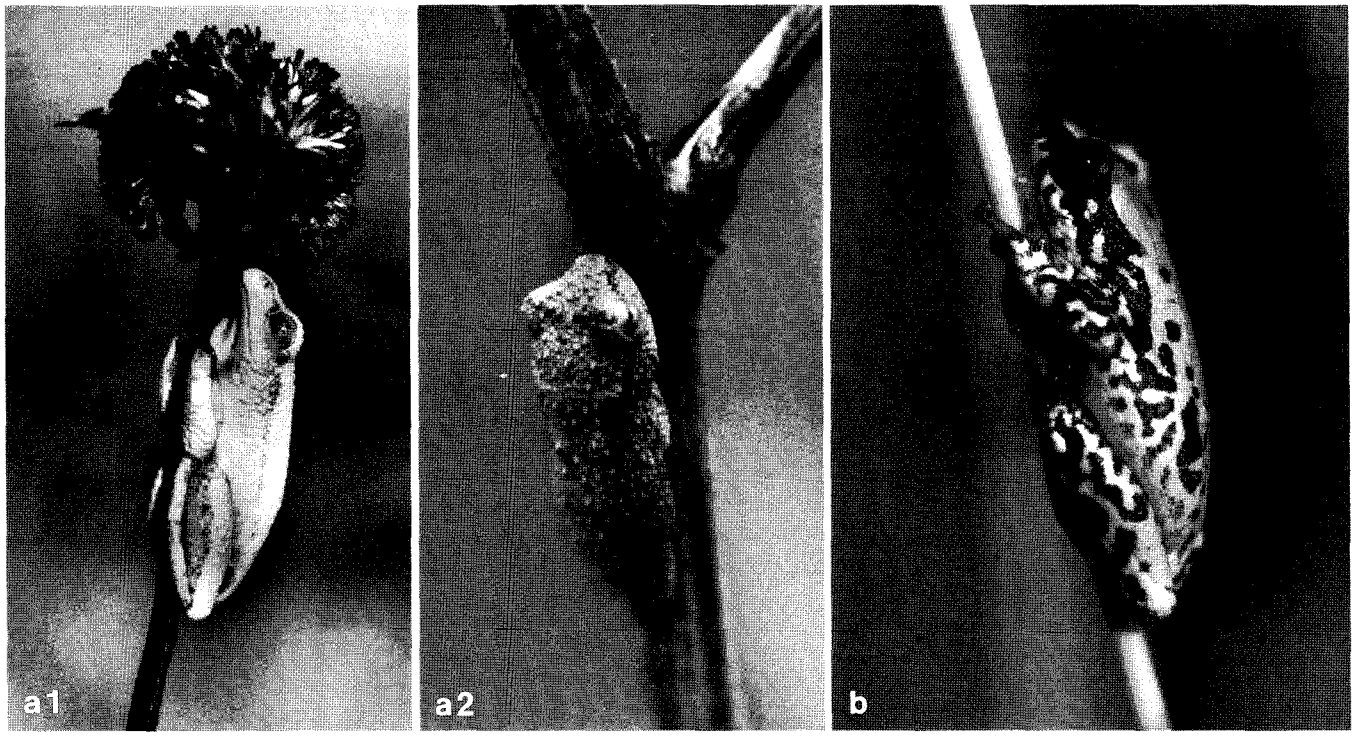


Fig. 5a, b. Skin colour of **a** dry-adapted juvenile and **b** adult *H. viridiflavus*. **a** Juvenile frogs aestivating in a typical position and exhibiting light-adapted skin colour at air temperatures above 35 °C (**a1**) and dark-adapted skin colour below 35 °C (**a2**). Pictures were taken in Comoé National Park. Ivory Coast. **b** Skin colouration of adult frogs is entirely different from that of juvenile frogs, with a variable black and yellow-whitish pattern and red inner sides of limbs and toes

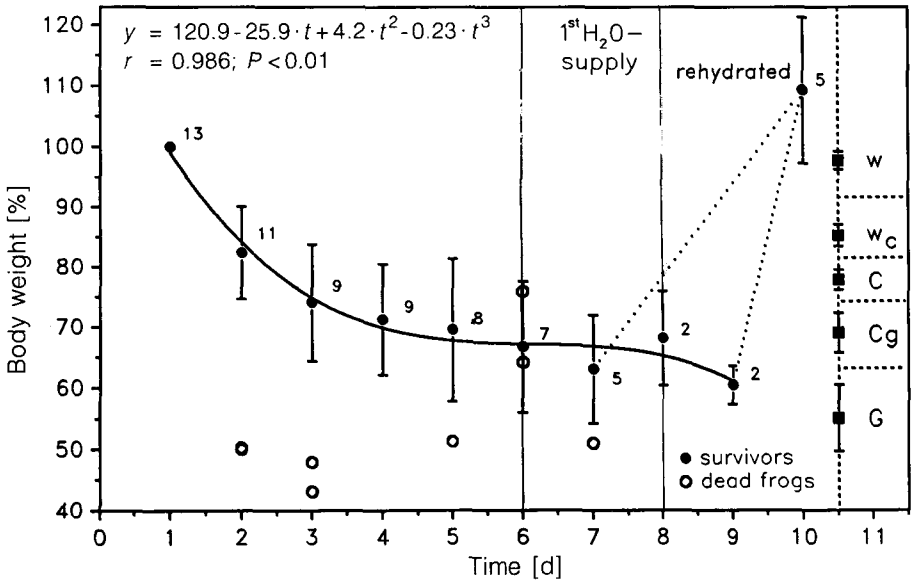


Fig. 6. Body weight changes of group 5 frogs during dehydration. *Open symbols* represent body weight of frogs which died before the next measurement. The colour classification in relation to dehydration state is depicted on the *right side*. The body weight of five specimens 2 h after rehydration is also shown

coloured animals the absorption due to the physiological colour change can be calculated. Dark skin absorbs mainly light of the visible part of the spectrum, IR-radiation only between 0.08 and 0.1 (Fig. 4b). These laboratory frogs exhibit skin colouration and skin reflectance comparable to that of field frogs of the same age (2–4 months after metamorphosis; Fig. 5a).

Skin colouration of adult frogs is entirely different from juvenile frogs with a variable black and yellow-whitish pattern and red inner sides of limbs and toes (Fig. 5b). Their skin reflectance also increases with rising temperature, but the reflectance range (0.38–0.53) is significantly lower than in dry-adapted juvenile frogs.

Green iridescence

Iridescence started mainly at the lower hind legs of group 5 froglets and progressed with increasing dehydration to

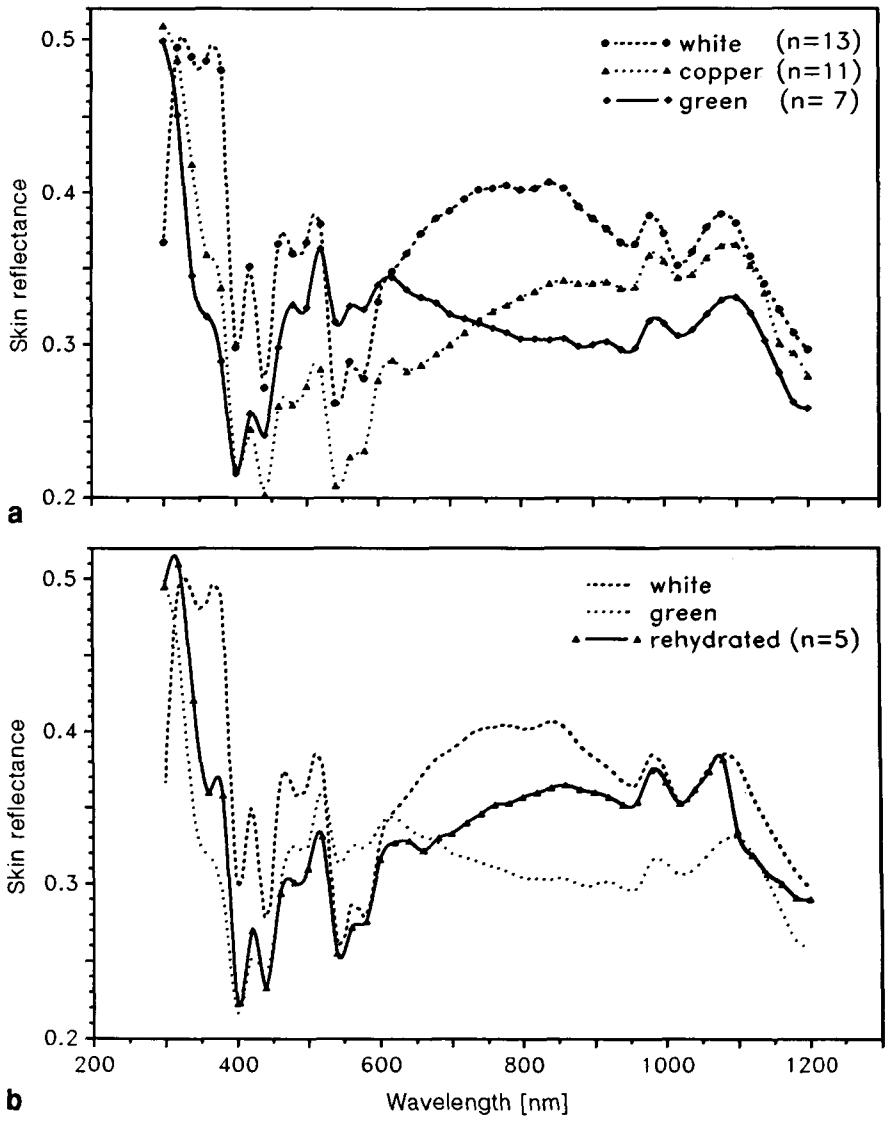


Fig. 7a, b. Changes of skin reflectance of group 5 frogs: **a** during dehydration and **b** after rehydration. With increasing dehydration the broad maximum between 750 nm and 850 nm diminishes and the green maximum at 520 nm becomes more pronounced (**a**); 2 h after rehydration the pronounced green maximum at 520 mm was lowered (**b**) and the spectrum approximately returned to the copper colour depicted in (**a**)

the back of the frog. The change of the iridescence colour is closely related to weight loss due to evaporation and energy depletion (Fig. 6). After a weight loss of about 15%, copper-coloured iridescence is at first weak but is clearly visible when weight loss reaches 22%. A weak whitish-green iridescence is seen after a weight loss of about 31%. Frogs with a weight loss of more than 40%, which is close to the lethal limit of this age class, have a clear whitish-green iridescence. At the end of the experiment more and more frogs come close to their individual dehydration limit, and despite rehydration three of the five whitish-green iridescent frogs with a weight loss of more than 40% died within a week after rehydration (Fig. 6).

In the spectrum of the whitish froglets a high maximum in the UV at 320 nm is seen. Smaller maxima are found at 420, 460, 520 and 560 nm followed by a broad maximum between 750 and 850 nm and two smaller maxima at 980 and 1080 nm. In the spectrum reflected by the copper-coloured frogs the UV maximum is shifted to the UV range of the spectrum and the maxima of the visible spectrum are much lower and less pronounced. A new maximum at 620 nm is found. The broad IR maximum around 800 nm has almost diminished. With increasing dehydration, the UV maximum is shifted further to the UV and the small maximum at 520 nm becomes clearly pronounced. The broad maximum around 800 nm has diminished and the two IR maxima at 980 and 1080 nm are lowered (Fig. 7a). The reflectance of green iridescing froglets recovered to a copper or copper-whitish skin colour within 2 h after rehydration (Fig. 7b). Survivors showed the typical white skin colour the day following rehydration.

Discussion

In freshly transformed froglets purines are synthesized regardless of the environmental conditions. Froglets exposed to dry season conditions almost doubled the rate of purine synthesis after a 10% loss of the initial body weight during the first 4 days of the transition state

(Schmuck and Linsenmair 1988). The plateau between day 8 and day 12 coincides with a decrease of the respiratory quotient (Geise and Linsenmair, in prep.). These phenomena seem to mark the point where the physiology of the frog changes from transition state to dry-adapted state, which is further discussed in Schmuck et al. (1988).

Three different reasons for the tremendous build-up of purines in the skin of some Hylidae and Hyperoliidae during aestivation periods are discussed in the literature:

(1) Skin permeability. Some authors speculate from their results that an unequal distribution of iridophores in dorsal and ventral skin corresponds to differences in the evaporative water loss of these types of skin (Drewes et al. 1977; Yorio and Bentley 1977; Withers et al. 1982). This conclusion is in agreement with the results of Kutchai and Steen (1971) who found that the permeability of the swimbladder of the conger eel *(Conger conger)* is only 10% that of connective tissue. The silvery layer producing this effect contains 13% guanine (dry tissue).

(2) Nitrogen metabolism. Some anurans aestivating above ground use purines to redress osmotic problems resulting from urea accumulation in the body fluids by internal deposition of nitrogenous waste products without fluid loss (Shoemaker et al. 1972; Balinsky et al. 1976; Schmuck and Linsenmair 1988; Schmuck et al. 1988).

(3) Optical properties. Besides physiological colour change (Bagnara 1976), purines seem to be used in anurans aestivating above ground for thermoregulation by protecting the animals against the harmful solar radiation load (Kobelt and Linsenmair 1986; F. Kobelt and K.E. Linsenmair, in preparation). This paper proposes a possible physical basis for the high skin reflectance, and this is discussed in detail in the following sections.

Spectral reflectance. Many anurans have one complete layer of iridophores in the stratum spongiosum. This layer, together with melano- and lipophores, is the substrate of physiological colour change (Fox 1953; Cott 1957; Fox and Vevers 1960; Nopp 1964; Fingerman 1965; Bagnara 1966, 1976; Bagnara and Hadley 1973; Bagnara et al. 1968, 1978; Nielsen 1978). The high reflectance of the thin reflecting layer in the skin of recently metamorphosed *H. viridiflavus* (groups 1 and 2) is almost certainly the result of multilayer interference reflection (Frost and Robinson 1984; Kobelt and Linsenmair 1986).

The mean thickness of one iridophore is 15.0 ± 3.0 µm ($n = 20$ frogs), the measured reflectance of one complete reflecting layer is 0.322 ± 0.023 ($n = 20$), and the measured spectral maxima are in the range 800–1100 nm (see Fig. 4). Calculation of comparable data from our model (Eqs. 9–16) revealed that in order to reflect a mean of about 0.35 across a spectrum of 300–2900 nm at $\lambda_0 = 817$ only 30 layers of well-arranged crystals are needed, resulting in a reflecting layer thickness of 10.5 µm. These values are in good agreement with the measured data. The small differences between calculated and measured values are most probably caused by discontinuities in the reflecting layer (e.g. morphological structures such as gland ducts), leading to absorption of light in the iridophore-free gaps in the skin. Spectral curves with shapes closely comparable to that of *H. viridiflavus* are found elsewhere in the literature (Tracy 1972; Carey 1978; Gates 1980), but the reflectance is much lower because a much greater amount of light is absorbed by melanophores in the investigated species *(Rana pipiens, Bufo boreas)*.

From the multilayer interference theory spectral curves can be calculated that conform in some characteristic traits with the curves derived by averaging the measured spectral data of group 3 (Fig. 8). One characteristic of the theoretical curves is that the addition of crystal layers gradually influences the shape of the theoretical curves only in the range 1–30 layers. Above

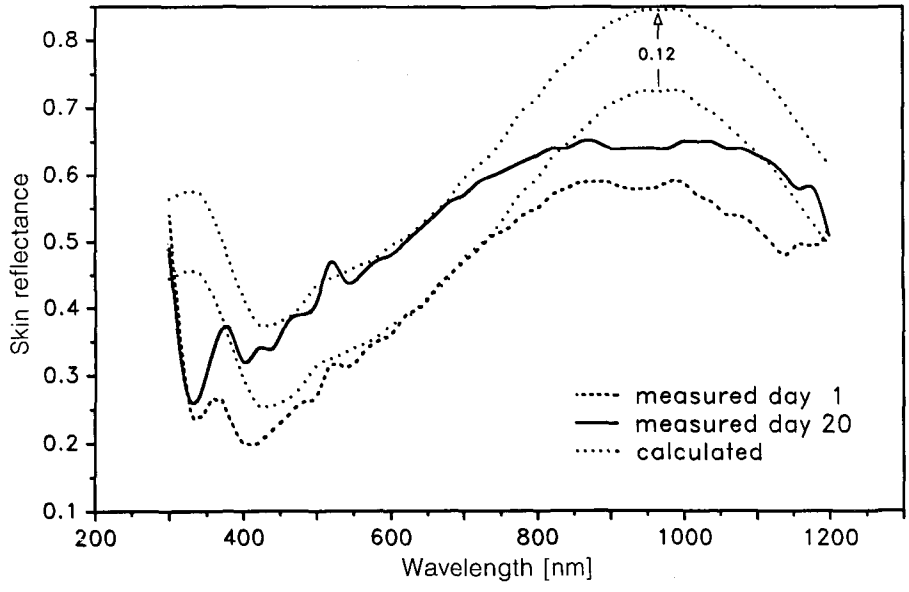


Fig. 8. Comparisons between skin reflectance of group 3 frogs at day 1 and day 20 with the curve calculated from multilayer interference theory. The visible part of the day 20 curve conforms to the calculated curve after shifting the latter up 0.12. The non-correspondence in the UV region is probably caused by absorption, and in the IR region by a decrease of the refractive index which had not been taken into consideration

30 layers the influence of a new layer is almost negligible. The same characteristic holds true for the measured curves; their spectral shape remains unchanged when the reflecting layer is increased from 15 µm to 45 µm. However, the fraction of "white" light scattered independently of wavelength by the thickened layer increases and at a given diameter adds a constant fraction of light to the multilayer-induced reflectance. As a result the reflectance is shifted up with only very small changes of its spectral composition (Fig. 4a). Such a shift is seen in the spectral reflectance at day 1 and day 20 in group 3. Both curves have almost the same shape and differ by about 0.12 from each other (Fig. 8). This is comparable with the 0.15 increase of the white reflectance of group 2 during the same period in our dry season conditions (Fig. 2).

The measured and the calculated curves show similar characteristics, i.e. both rise in the UV and have a broad maximum in the IR. The differences might be brought about by absorption in the skin. Besides xanthophore pigments, many other organic substances strongly absorb light in UV below 320 nm [absorption maxima of purines are between 240 and 290 nm (Stahl 1967)] and the differences in the UV part of the curves can possibly be explained by absorption. Whether absorption is also responsible for the differences in the IR part of the spectrum remains unclear. Possible alternative factors might be: (1) Water absorbs radiation weakly above 700 nm and strongly above 1100 nm. However, a water layer of 60 µm thickness (thickness of the iridophore layer plus epidermis in group 3) alone cannot cause these differences. (2) Porter (1967) found that a shed skin of *Uta stansburiana* about 20 µm thick absorbed about 0.27 of the incident radiation between 700 nm and 2600 nm, which was mainly a result of absorption by keratin. However, a cell layer of comparable keratinization in the skin of *H. v. taeniatus,* the stratum corneum, is only about 2 µm thick and its IR absorption amounts to not more than 0.012. (3) The refractive index of many substances significantly decreases with increasing wavelengths from about 700 nm to 2500 nm (Gates 1980). This decrease might be the main reason for the observed differences above 700 nm because it is accompanied by a displacement of λ_0 to smaller wavelengths, resulting in a lower reflectance at the calculated wavelength. Unfortunately, no refractive index data above 700 nm for purines were available. Therefore, this error could not be corrected in the calculations.

In electron microscope studies Kobelt and Linsenmair (1986) found a highly ordered arrangement of crystals parallel to the skin surface in dry-adapted frogs. The calculations were made under the assumption of parallel layers of crystals, and the relatively good fit of theoretical and measured curves indicates that this assumption is not seriously at fault. Since the reflectance curves of day 1 can also be calculated from the multilayer theory, wet-adapted frogs may also temporarily exhibit a high parallel orientation of crystals in their iridophores. Nielsen (1978), working in light-adapted skin of *Hyla arborea,* reported a parallel orientation of the crystals to the skin surface, whereas in dark-adapted skin the crystals were mainly randomly distributed. He found iridophores with more or less parallel crystals predominantly in connection with light-coloured backgrounds. The orientation of the crystals is presumably performed by a network of filaments between the crystals (Rohrlich and Porter 1972). These results give rise to the assumption that changing the arrangement of the crystals is a fast process allowing quick responses to short-term changes in environmental conditions. Very likely the iridophores of *H. viridiflavus* also have this ability. This problem is currently under investigation.

A system of highly parallel oriented crystals should reflect coloured light, but the reflected light of the iridophore layer in dry-season frogs is yellow-white or brilliant white, indicating a broad-band spectral reflectance. These differences of the spectral reflectance might be

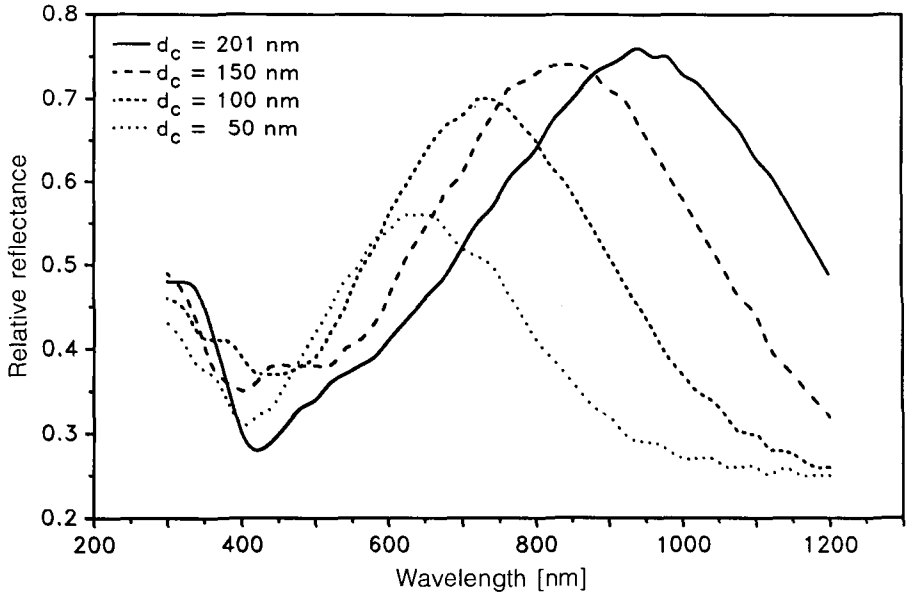


Fig. 9. Theoretical displacement of λ_0. With decreasing d_C the IR maximum is lowered and displaced more and more to the visible spectrum. The displaced maximum shifts into the visible spectral range after a more than 40% decrease of d_C. The smaller maximum at 350 nm is also shifted into the UV region. All curves were integrated in the range of $d_C \pm 0.25 \cdot d_C$

explained by irregularities of the parallel arrangement of the crystals in the iridophores. Usually dry-season frogs show a good orientation of crystals in light-adapted iridophores; however, not all crystals are arranged in stacks and not all stacks are parallel to the skin surface (Kobelt and Linsenmair 1986). These discontinuities enhance scattering of the incident light within the reflecting layer, and the light becomes more diffuse. In addition, the cytoplasm interspaces are very variable, leading to more than one λ_0, and the properties of the layer come close to the interference mirrors of Heavens (1960). This can be seen by examining the skin under a microscope. In light-adapted skin tiny spots of red, yellow or green iridescence of similar diameter to that of a dorsal iridophore can be seen. These iridescent spots are probably the result of multilayer interference within iridophores with highly ordered crystal systems at the top of the reflecting layer. The different colours are probably caused by the different angles of incidence and by differences in the cytoplasm interspace thickness. As a result, "white" light may also be reflected from a multilayer when the incident light becomes diffuse and the crystals are not perfectly spaced quarter-wavelength reflectors (Heavens 1960).

Under certain conditions the reflected light of the whole skin of *H. viridiflavus* is iridescent in colours typical of multilayer interference reflectors. Iridescence colours are seen in the skin of dehydrated frogs after more than about 22% weight loss. A result of dehydration might be a shrinking of the cytoplasm, leading to reduced cell volume and decreased mean thickness of the cytoplasm interspaces. This probably leads to a parallel orientation of the crystals within the shrunken cell resulting in a highly ordered structure with even better multilayer interference properties than the original cell. Additionally, the reduced thickness of the cytoplasm interspaces seems to be responsible for the overall displacement of λ_0 from the IR to the visible spectrum. This hypothesis is supported by Land (1966), who found a shift from the initial blue-green to blue after suspending the eye of *Pecten maximus* in hyperosmotic sucrose, and a shift through yellow to orange in distilled water. These colour shifts were reversible after replacement of the eye in seawater. Such a displacement can be calculated from the multilayer theory, but the measured curves (Fig. 7) do not agree very well with the calculations (Fig. 9).

White reflectance. In *H. viridiflavus* a skin reflectance of more than 0.7 is needed to meet the problems of high solar radiation load in the field (F. Kobelt and K.E. Linsenmair, in preparation). The reflectance of thick iridophore layers (Fig. 4b) does not conform to the results obtained by applying the multilayer interference theory (Fig. 8). Light which is scattered independent of wavelength from disordered crystals superimposes on the multilayer-induced spectral reflectance. The initial parallel shift of the multilayer curves with increasing thickness (Fig. 8) and the almost constant ("white") reflectance of a layer thicker than 60 μm (Fig. 4b) clearly points to changing physical properties with increasing layer thickness.

Below a thickness of 15 μm the difference between the measured and the calculated thickness from the Kubelka-Munk theory averages 21.3%. Just after metamorphosis the froglets possess an incomplete iridophore layer covering 50–80% of the dorsal stratum spongiosum (Kobelt and Linsenmair 1986). Through the uncovered areas a considerable amount of the incident radiation can penetrate deep into the body where it is absorbed. According to the Kubelka-Munk theory absorption of the reflecting material is not taken into consideration. Therefore, skin reflectance at this stage is much lower than calculated from this theory and may differ from the calculated values by up to 70.6% at day 4 and 43.5% at day 8 (Fig. 3). In group 1 and group 2 after about day 12 the reflecting layer is complete and its thickness is

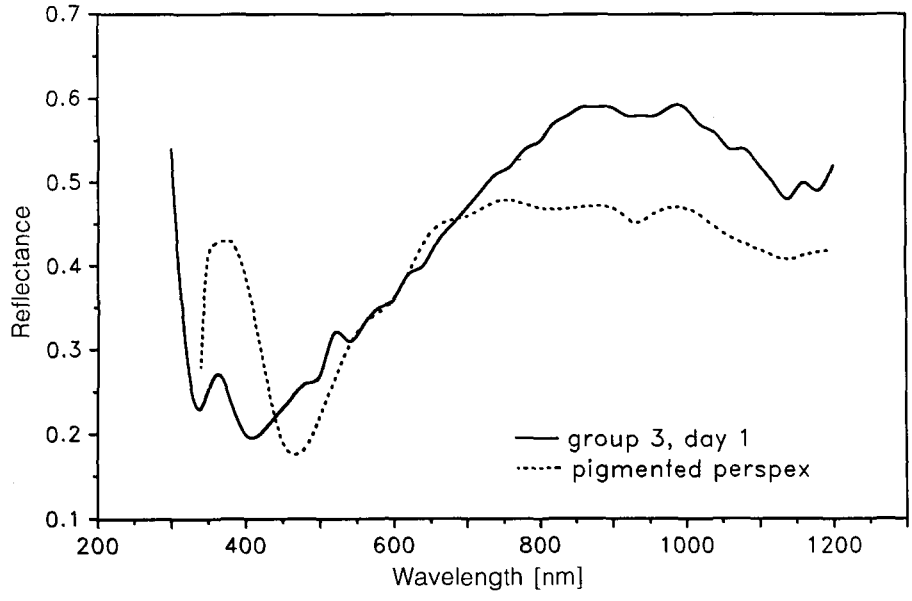


Fig. 10. Comparison between skin reflectance of group 3 frogs and IR-reflecting Perspex. Despite different optical constants, the spectral curves of the iridophore layer and pigment-containing Perspex show some similarities

above 18 μm, the maximum diameter of one iridophore. The accuracy of the results of the Kubelka-Munk theory is within 12% of the measured values, but almost all calculated thicknesses are higher than measured (Fig. 3). Bone and Denton (1971) found that fixation with OsO_4 solutions alters the colour of fish scales, presumably due to volume changes of the cytoplasm. Romeis (1968) reported that the volume of cytoplasm will shrink up to 25% when treated with formaldehyde fixatives. Thus, a reduction in the volume of cytoplasm is probably responsible for the significant differences between theory and measurement (see Fig. 3) of an iridophore layer over 20 μm in thickness. Therefore, the mean thickness of the reflecting layer of the dorsal skin can be calculated by applying the Kubelka-Munk theory after a complete iridophore layer with a thickness over 20 μm has been synthesized.

A similar way of reducing solar radiation load is used to manufacture "IR-reflecting Perspex" (Fischer 1980). A 3 mm thick plane of Perspex with embedded randomly distributed pigment platelets reflects about 0.69 of the incident radiation almost independent of wavelength in a spectral range between 300 and 3000 nm (Fischer 1980). The colour of such a Perspex plate is milky white and the reflectance can almost certainly be described by the Kubelka-Munk theory. When the embedded platelets are arranged parallel to the surface of the plate light is mainly reflected in the spectral range between 600 and 3000 nm and the reflectance is about 0.50. Such a Perspex plate with parallel arranged pigment platelets reflects reddish-gold-coloured light, a colour which is similar to the copper colour of the skin in group 5. Despite different optical constants, the spectral curves of the iridophore layer and of the reddish-gold-coloured Perspex plate show very similar tendencies (Fig. 10). Because of these similarities in spectral reflectance, a Perspex plate with parallel arranged pigment platelets seems to represent a good model to describe the physics of the thin reflecting layer (below 15 μm) in the skin of *H. viridiflavus*.

Conclusions

In the field even freshly metamorphosed froglets have to withstand extreme climatic conditions. The results of this study show that multilayer interference structures of not more than about 10 μm are highly efficient light reflectors within a given spectral range and can be manufactured very fast and at low energy and material costs. As a result of the colouration, this reflectance (0.32) is not very effective across the whole spectrum of sunlight but may suffice during the remaining wet and transitional season. The required high reflectance (>0.7) for aestivation, costing much more in material and energy than a multilayer structure, is not needed before the periods of complete dryness exceed about 5–7 days. In the transitional period the froglets are able to get enough energy by feeding and to replenish water lost by evaporation or used for evaporative cooling. Usually the froglets have sufficient time, material and energy to establish the required high skin reflectance in this period.

Appendix A

The Kubelka-Munk theory

In a powder layer whose dimensions in y- and z-directions are very large compared with its thickness d in the x-direction, a cross section perpendicular to the x-direction of thickness, dx, is struck by a light flux I from the direction of incidence and also by a reflected light flux J from the opposite direction. A fraction Kdx of the incident light I is absorbed by the layer dx and a fraction Sdx is scattered back. By assuming diffuse incident light and restricting the theory to only two constants [coefficient of absorption (K) and light scattering (S)], two differential equations can be derived:

$$-\frac{dI}{dx} = S \cdot J - (K+S) \cdot I \tag{1}$$

$$\frac{dJ}{dx} = S \cdot I - (K+S) \cdot J \tag{2}$$

Using the notation of Kortüm (1966), the solution of these equations is:

$$S \cdot d \cdot \left(\frac{1}{R_\infty} - R_\infty\right) = \ln \frac{\left(R - \frac{1}{R_\infty}\right) \cdot (R_G - R_\infty)}{\left(R_G - \frac{1}{R_\infty}\right) \cdot (R - R_\infty)} \tag{3}$$

If the reflecting layer is placed on an ideal non-reflecting blackground of reflectance $R_G = 0$, and renaming the reflectance of the layer R as R_0, the equation simplifies to:

$$S \cdot d \cdot \left(\frac{1}{R_\infty} - R_\infty\right) = \ln \frac{1 - R_0 \cdot R_\infty}{1 - \frac{R_0}{R_\infty}} \tag{4}$$

where R_∞ is the reflectance R of the layer at infinite thickness in the positive x-direction. The formula can be used to calculate the reflection S of a finite layer above a real background if $R_G \leq 0.1$ (Kortüm 1966). S is not constant at different d and wavelengths, but a mean S can be calculated from our animal data [$d = 81$ μm (Kobelt and Linsenmair 1986), $R_0 = 0.65$ (measured reflectance at $d = 81$ μm, Kobelt unpublished), and $R_\infty = 0.7$ (estimated, see Kortüm (1966)]: $S = 0.0344\ \mu m^{-1}$.

Appendix B

The Multilayer Interference Theory

Light reflected from a single platelet is coloured because of constructive interference. The wavelength of the colour which is best reflected (λ_0) is given by:

$$\lambda_0 = \frac{4}{2 \cdot z - 1} \cdot d \cdot \sqrt{n^2 - \sin^2 \alpha} \tag{5}$$

where $z = 1, 2, 3, \ldots m$.

Taking the first order ($z=1$) and for light incident normally on the platelet ($\alpha=0$) one obtains for the optical thickness (Δ) of the platelet imbedded in cytoplasm [n=refractive index, d=platelet thickness, $_K$=crystal, $_C$=cytoplasm; Land (1966)]:

$$\Delta = n \cdot d_K = \frac{\lambda_0}{4} \qquad n = \frac{n_K}{n_C} \tag{6}$$

A multilayer interference reflector is manufactured by using films of alternating high and low refractive index. If a system of crystals is to function in the manner of a multilayer interference reflector, both the crystals and the spaces between them should have optical thicknesses of $\lambda_0/4$. Huxley (1966) has derived a method to calculate the reflectance of quarter-wavelength systems. The reflectance of one platelet is given by Fresnel's formula:

$$r = \frac{I}{I_0} = \frac{\sin^2(\alpha-\varepsilon)}{\sin^2(\alpha+\varepsilon)} + \frac{\tan^2(\alpha-\varepsilon)}{\tan^2(\alpha+\varepsilon)} \tag{7}$$

where r = reflectance of one platelet, I= reflected light, I_0 = incident light and ε = angle of I. For light incident normally on the platelet ($\alpha=0$):

$$r = \left(\frac{1-n}{1+n}\right)^2 \tag{8}$$

The reflectance (R) of the quarter-wavelength system with p crystal layers for real and complex numbers of μ (see Eq. 13) is:

$$\text{a) } \mu \textit{ real } R = \frac{1}{1+4m^2 \cdot \dfrac{1-p^2}{(1-m)^2}} \tag{9a}$$

$$\text{b) } \mu \textit{ complex } R = \frac{1}{1+(p^2-1)\cdot \operatorname{cosec}^2(2p\Phi)} \tag{9b}$$

where:

$$m^2 = \left(\frac{\mu_1}{\mu_2}\right)^2 \tag{10}$$

$$\cos\Phi = \frac{\cos(\Phi_C+\Phi_K) - r^2 \cdot \sin(\Phi_C-\Phi_K)}{1-r^2} \tag{11}$$

$$p = \frac{\sin(\Phi_C+\Phi_K) - r^2 \cdot \sin(\Phi_C-\Phi_K)}{r \cdot \sin\Phi_K} \tag{12}$$

μ_1 and μ_2 are the solutions of the following squared equation (solve according to: $\mu^2+2\cdot q\cdot\mu+1=0 \geq \mu_{1,2} = -q\pm\sqrt{q^2-1}$):

$$\mu^2 - 2\mu\frac{\cos(\Phi_C+\Phi_K) - r^2\cdot\sin(\Phi_C-\Phi_K)}{1-r^2} + 1 = 0 \tag{13}$$

For light incident other than normally one obtains:

$$\Phi_c = \frac{2\pi}{\lambda}\cdot n_C \cdot d_C \cdot \cos\alpha \tag{14}$$

$$\Phi_k = \frac{2\pi}{\lambda}\cdot n_K \cdot d_K \cdot \cos\varepsilon \tag{15}$$

$$\frac{n_k}{n_c} = \frac{\sin\alpha}{\sin\varepsilon} \tag{16}$$

A single quarter-wavelength crystal reflects between 6% and 9% of the incident light over the whole visible spectrum. However, in a multilayer system the variation of reflectance with wavelength becomes more pronounced with increasing number of layers, the bandwidth becomes narrower and more sharply defined, and the initially unsaturated colour becomes correspondingly more intense. With 30 ideally structured layers the reflectance approaches 100% around λ_0. The wavelength dependency can be reduced by varying the thicknesses of the constituent layers. Interference reflectors with about 95% reflectance over the whole visible spectrum have been manufactured by applying this technique (Heavens 1960).

In a biological system of platelets the incident light is scattered in the epidermis and at the sides of the crystals which are not ideally parallel to each other. Therefore, the light within the multilayer system can be considered as being diffuse. The crystal thickness (d_K) ranged from 0.132 to 0.182 µm and the cytoplasm interspaces (d_C) from 0.153 to 0.253 µm (Kobelt and Linsenmair 1986), resulting in variations of the optical thickness of the crystals ($n_K d_K$) and the intermittent cytoplasm ($n_C d_C$) around the ideal optical thickness ($n_0 d_0$) for both systems. This was taken into consideration by numerical integration of Eq. 9 over α, d_K and d_C with a computer (Fig. 8). λ_0 was calculated to be 817 nm from Eq. 6 with $n_C=1.33$ (Land 1968), $n_K=1.81$ and $d_K=0.150$ µm (Kobelt and Linsenmair 1986).

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