Changes of the external and internal pigment pattern upon fertilization in the egg of *Xenopus laevis*

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SUMMARY

External and internal pigment shifts in *Xenopus laevis* eggs were studied between fertilization and first cleavage.

Externally visible, constant features are: (1) the 'activation contraction', a pigment shift towards the animal side taking place between 5 and 15 min post fertilization (p.f.) and (2) the concentration of the pigment around the sperm entrance point leading to the formation of the grey crescent at the opposite side of the egg. Hence, in *Xenopus* the grey crescent is not formed by rotation of the pigmented cortical layer with respect to the internal egg mass.

Histological examination reveals that during the activation contraction the pigment is mainly concentrated in the cortical cytoplasm. Except in the region around the sperm entrance point, from 15 min p.f. onwards, the pigment progressively disperses through the subcortical layer and part of it even moves more deeply into the egg. After fusion of the pronuclei (45–60 min p.f.) the pigment in the subcortical layer forms aggregates.

During the pigment shift the yolk-free cytoplasm is displaced dorsally and is ultimately found opposite the sperm entrance point. Thin fibrillar structures in the yolk-free cytoplasm progressively orient themselves parallel to the dorso-ventral plane, and from 40 min p.f. onwards towards the pronuclei.

These observations are discussed in connexion with cinematographic observations by Hara, Tydeman & Hengst (1977).

INTRODUCTION

In the majority of anuran species the appearance of the grey crescent is the first sign of bilaterality in the uncleaved egg. The location of the grey crescent is determined by the sperm entry (Roux, 1887). The first cleavage plane passes through the sperm entrance point and the centre of the grey crescent area, where later the dorsal blastoporal lip will appear (Newport, 1854; Roux, 1887; Brachet, 1905; Ancel & Vintemberger, 1948; Pasteels, 1964; Clavert, 1973), which in normal development forms the axial mesoderm of the embryo (Spermann, 1936; Nieuwkoop, 1973).

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In the newly laid egg of *Xenopus laevis* the animal–vegetal polarity is externally expressed in the distribution of pigment, which is mainly concentrated in the superficial layer of the cytoplasm of the animal moiety of the egg (Nieuwkoop, 1956). The animal pole is marked by a light 'polar spot' indicating the place of extrusion of the first polar body (Rzehak, 1972), the vegetal pole by a region slightly more pigmented than the nearly unpigmented vegetal moiety (Nieuwkoop & Faber, 1975). The pigmented 'animal cap', just as in *Rana temporaria* (Wittek, 1952) and *R. esculenta* (Pasteels, 1937), may be asymmetrical with respect to the egg axis (Nieuwkoop & Faber, 1975). In *R. esculenta* such an asymmetrical pigment cap is observed in the ovarial oocytes and foreshadows the ultimate bilaterality of the gastrula (Pasteels, 1937). However, such a relationship has not been found for *Xenopus laevis*, where external bilaterality is first expressed by the appearance of the grey crescent.

The yolk granules in the *Xenopus* egg are unequal in size. In the unfertilized egg they are arranged in a radially symmetrical fashion in all planes perpendicular to the animal–vegetal axis. As found by Ubbels & Hengst, 1974, this typical axial symmetry is disturbed by the penetration of the sperm: this evokes cytoplasmic segregation, which in its turn leads to internal bilateral symmetrization. They found that a yolk-free region of the cytoplasm, situated in the centre of the newly laid egg, progressively increases in size and is displaced towards the future dorso-animal side, where it can be found from 45 min post fertilization (p.f.) onwards. It is then called 'dorsal cytoplasm'. A similar cytoplasmic segregation and dorsad movement of the segregated cytoplasm has been observed in *Discoglossus pictus* (Klag & Ubbels, 1975). However, a causal relation between grey crescent formation on the one hand and cytoplasmic segregation and dorsad movement on the other has not been demonstrated in either of the anuran species, although the two processes occur more or less simultaneously.

The present study shows convincingly that in well-oriented serial sections through the animal–vegetal axis and the sperm entrance point the 'dorsal cytoplasm' is always found opposite the place of sperm entrance. It is also concluded that in *X. laevis* the grey crescent is formed as a result of the pigment shifting towards and concentrating around the sperm entrance point. This is different from the situation in various other anuran species, where grey crescent formation involves rotation of the entire pigment cap with respect to the internal cytoplasm (Ancel & Vintemberger, 1948).

**MATERIAL AND METHODS**

Fertilized eggs of *Xenopus laevis* were obtained either by natural mating (Verhoeff-de Fremery, Gorlee & Tydeman, 1977) or by artificial insemination according to a procedure modified after that of Wolf & Hedrick (1971). The eggs were stripped into dry Petri dishes, flooded for 5 min with a 1:3 diluted
Pigment shifts upon fertilization in *Xenopus* egg

sperm suspension and washed three times with De Boer's solution diluted 1:20. The jelly was removed by a 2–3 min treatment with 2 % thioglycolic acid in distilled water, pH 7.5–8.0 (Gusseck & Hedrick, 1971).

Some variation in pigment pattern may occur among batches from different females and also among eggs from the same batch. In order to correlate external and internal (histological) observations, only those eggs were selected which showed approximately the same regular pigment pattern. The eggs were constantly observed during development at 21 °C and photographed every 2–3 min. Parallel to these observations, groups of five eggs were fixed at 5 min intervals, covering the period from the addition of the sperm suspension (time 0) until first cleavage. The age of the eggs is indicated in min p.f. In the case of natural mating, time 0 was the time of oviposition.

Fixation for 48 h in San felice (Romeis, 1968) was followed by a 24 h wash in running tap water, alcoholic dehydration, and embedding in paraffin wax (m.p. 58–60 °C) via amyl acetate; serial 5 μm sections were cut on an AO Spencer Precision Rotary Microtome and carefully flattened on a 90 % alcohol bath at exactly 40 °C. Azan-stained serial sections of 8–10 eggs of each stage were analysed.

**RESULTS**

(1) *External appearance*

All observations started at 10 min p.f. As in other anurans, the newly deposited *Xenopus* egg usually lies with its pigmented cap in an arbitrary orientation. After activation by sperm penetration the egg rotates inside the newly formed perivitelline space. In this way the animal–vegetal axis is brought into a vertical position. The slightly greyish or white polar spot (0.1–0.2 mm in diameter) is now pointing upwards, the first polar body being sometimes visible in its centre. The second polar body appears at 20 min p.f. The polar spot is surrounded by a narrow dark-brown ring (Fig. 1), itself surrounded by a ring-shaped or horse-shoe-shaped light-brown region.

In the newly fertilized egg the pigment moves more and more towards the animal pole region (Fig. 1; see also Figs. 2 and 3). This pigment movement, known as ‘activation contraction’, starts before the rotation of the egg and continues until 15 min p.f. Fig. 1 shows that this process is followed by a period of pigment dispersion leading to repigmentation of the entire animal hemisphere. However, as soon as pigment dispersion starts part of the pigment moves towards a point situated somewhere in the animal hemisphere, only exceptionally very close to the animal pole. Lines of pigment granules converge upon this point, which darkens progressively. This concentration of pigment was most conspicuous in lightly pigmented eggs and sometimes was so strong that the egg cortex adjacent to this region (on the future ventral side) became almost unpigmented. Histological sections show unambiguously that the point where the pigment concentrates represents the point of sperm entrance (Fig. 6).
Fig. 1. Changes in the pigment pattern of an artificially fertilized egg of *Xenopus laevis* photographed at 3 min intervals starting at 10 min p.f. The numbers indicate time after fertilization in min. *sp*, Sperm entrance point; *ge*, incipient grey crescent; *ps*, polar spot; *r*, dark-brown ring.
Pigment shifts upon fertilization in Xenopus egg

From 20 min p.f. onwards the ring-shaped or horse-shoe-shaped light-brown region adjacent to the ring surrounding the polar spot extends progressively. This extension is most pronounced on the side opposite the sperm entrance point (Fig. 1). As a consequence of these pigment shifts, about 30 min p.f. a less pigmented (= partly depigmented) area becomes visible on the dorsal side of the egg, in which a coarser granulation develops (Fig. 1). Histological sections show that during this period the pigment, after having been concentrated in the egg cortex, partially descends into the subcortical layer (Fig. 4). Our observations strongly suggest that all these pigment shifts finally lead to the formation of the grey crescent, which is most conspicuous at 45–50 min p.f. It appears therefore that in *Xenopus* the grey crescent is formed as a result of the shift of the pigment towards the sperm entrance point, not by a rotation of the entire pigment cap around the internal cytoplasm.

Although in several batches the position and extent of the grey crescent can be determined unambiguously, its demarcation is very often unclear. This is due to the comparatively light pigmentation of the *Xenopus* egg.

The polar spot disappears between 70 and 80 min p.f. and further pigment movements in the animal region are connected with the formation of the first cleavage furrow, which becomes visible 80–90 min p.f. It is foreshadowed by a single line of darker pigmentation, followed by a shallow groove and small stress marks perpendicular to it (Bluemink, 1971). In all cases studied the first cleavage plane passed through the sperm entrance point (Fig. 1), the animal pole (polar spot), and the approximate centre of the grey crescent. It should however be remembered that the centre of the grey crescent is difficult to determine and therefore the relation between the cleavage plane and the centre of the grey crescent is uncertain.

(2) Internal appearance

In histological sections of newly laid, unfertilized eggs the pigment is diffusely distributed in the peripheral (cortical and sub-cortical) regions of the cytoplasm. The entering sperm drags some of the cortical pigment along, thus marking its rather irregular pathway, which is variable in length (Fig. 6). The egg nucleus also, when moving inwards after completion of the second maturation division, contributes to a displacement of cortical pigment. During the ‘activation contraction’ the pigment granules concentrate in the egg cortex (Fig. 3), but from about 15 min p.f. onwards they progressively disperse through the subcortical layer (Fig. 4) and even more deeply. After syngamy at ca. 45–60 min p.f. the pigment granules in the subcortical layer form aggregates (Fig. 5), giving a spotted appearance (Rzehak, 1972) to the pigmented side of the egg (Fig. 1). Shortly before and during the formation of the first cleavage furrow these aggregates disappear or are passively displaced inwards.

Sections through the sperm entrance point and the animal–vegetal axis conclusively show that at 60 min p.f. the yolk-free area – the ‘dorsal cytoplasm’ –
Pigment shifts upon fertilization in Xenopus egg

of Ubbels & Hengst (1974) is located opposite the sperm entrance point, i.e. dorsally.

Azan-stained sections show that the yolk-free cytoplasm is characterized by irregularly arranged acidophilic fibrillar structures (Fig. 7). From 30 min p.f. onwards these structures progressively orient themselves parallel to the dorso-ventral plane, and from 40 min p.f. onwards they bend towards the pronuclei (Figs. 8 and 9). About 60 min p.f. the pronuclei fuse in the neighbourhood of the dorsal cytoplasm and form the rather large zygote nucleus. The first cleavage division starts at about 90 min p.f.

DISCUSSION

Animal-vegetal polarity and dorso-ventrality have been related to morphogenetic fields located in the egg cortex (Dalcq & Pasteels, 1937; Dalcq & Pasteels, 1938; Pasteels, 1938; Dalcq, 1941; Raven, 1961; Pasteels, 1964; Nieuwkoop, 1967), the cortex usually being defined as the plasmalemma plus some pigment-containing underlying cytoplasm (Pasteels, 1964; Hebard & Herold, 1967). Changes in the position and composition of the egg cortex may affect the underlying structures and thus influence subsequent development. Conversely, changes in the superficial layers of the egg may reflect changes in the egg interior.

Between fertilization and first cleavage the egg acquires an externally visible bilateral symmetry as a result of grey crescent formation. At the same time cytoplasmic displacements render the egg of X. laevis internally bilaterally symmetrical.

FIGURES 2-5

Azan-stained 5 μm paramedian paraffin sections of artificially fertilized eggs of Xenopus laevis (animal most part of the eggs) (Fig. 5, courtesy R. T. M. Hengst).

Fig. 2. Fixed immediately after artificial fertilization. Pigment granules (p) mainly concentrated in the peripheral layer; a thin layer (c) of blue-stained cytoplasm covers the pigmented region.

Fig. 3. 15 min p.f.: ‘activation contraction’.

Fig. 4. 40 min p.f.: pigment dispersion.

Fig. 5. 70 min p.f.: aggregates of pigment granules in the future furrow region (arrows).

Figs. 6-9, Azan-stained 5 μm paramedian paraffin sections of artificially fertilized eggs of Xenopus laevis.

Fig. 6. 25 min p.f.: section through the black spot in the animal hemisphere. ♀, sperm head; pt, pigment trail.

Fig. 7. 30 min p.f.: irregularly arranged acidophilic fibrillar structures in the yolk-free cytoplasm (yfc), starting to orient parallel to the D/V plane.

Fig. 8. 40 min p.f.: ♀ and ♂ pronucleus, situated closely together in the vicinity of the dorsal cytoplasm (yfc), in which fibrillar structures arranged in the D/V plane bend towards the pronuclei.

Fig. 9. 40 min p.f.: fibrillar structures in the yolk-free cytoplasm (yfc), larger magnification.
symmetrical (Ubbels & Hengst, 1974). Similar events have been described for *Discoglossus pictus* (Klag & Ubbels, 1975). Although the external and internal bilateral symmetry develop almost simultaneously, no causal relation between the two processes has yet been demonstrated. Therefore, a combined cytological, cytochemical and cinematographical analysis has been initiated in this Laboratory, of which the present study constitutes the first step. In the course of these studies Hara (unpublished observations developed a method of artificial insemination which allows cinematography immediately upon fertilization. So far only Rzehak (1972) has made a cinematographic study of pigment shifts in the fertilized egg, although *Xenopus* is among the most frequently used species in experimental embryology. The present observations agree in many respects with those described in Rzehak’s paper.

After penetration of the sperm, the first externally visible pigment movement is the shift towards the animal pole region (5–15 min p.f.). This activation contraction is immediately followed by the fertilization rotation (Ancel & Vintemberger, 1948; Pasteels, 1964; Clavert, 1973). This observation is substantiated by cinematographic analysis by Hara (1977). The activation contraction probably reflects a real contraction of the cortex in the animal hemisphere. The activation contraction, together with cortical granule breakdown (Balinsky, 1966; Grey, Wolf & Hedrick, 1974), may widen the perivitelline space, thus facilitating egg rotation and gravitational orientation (Ancel & Vintemberger, 1948). Such a contraction has also been described for *Rana temporaria* (Ancel & Vintemberger, 1948; Lovtrup, 1962), *Rana nigromaculata* (Kubota, 1967), *R. pipiens* (Elinson, 1975), *Xenopus laevis* (Ortolani & Vanderhaeghe, 1965; Rzehak, 1972), and *Bufo regularis* (Balinsky, 1966), and therefore probably is a regular feature of anuran development. Using the technique of local insemination, Elinson (1975) demonstrated that in *R. pipiens* the sperm may enter the egg anywhere in the animal hemisphere but that it only exceptionally does so in the vegetal hemisphere. Since, however, the entrance point is later always found in the animal pole region, within 60° from the pole, Elinson suggests that the cortical contraction carries the sperm towards the animal pole.

Hara et al. (1977), in a cinematographic study of *Xenopus* eggs, showed that two dark zones, designated as ‘post-fertilization waves’, consecutively originate at and travel away from the pigment spot marking the sperm entrance point shortly after egg rotation, i.e. well before the ‘surface contraction waves’ (Hara, 1971) connected with cleavage furrow formation start. Unpublished data of these authors suggest that another wave originates from the sperm entrance point before egg rotation. We therefore presume that this very first wave, called ‘activation wave’ by Hara, is connected with the extrusion of the cortical granules and that the two ‘post-fertilization waves’ are related with the internal cytoplasmic displacements observed by Ubbels & Hengst (1974), or grey crescent formation, or with both. A few additional observations seem to be in favour of the very first alternative (Ubbels, 1978).
Pigment shifts upon fertilization in Xenopus egg

Kas'yanov, Svyatogor & Drozdov (1971) studied endocoelomic eggs of Rana temporaria and R. ridibunda after activation by pricking. Under reflected light they observed a propagating 'ring of ripples', which they consider to be connected with the extrusion of the cortical granules. In Bufo vulgaris formosus cortical granule breakdown after pricking is preceded by a change in membrane potential, which cannot be evoked in either unovulated or already activated eggs (Maeno, 1959).

As soon as the pigment starts to disperse after the initial 'activation contraction' (ca. 15 min p.f.) a local pigment accumulation is observed at a point somewhere in the animal hemisphere. This is in accordance with observations of Rzehak (1972) in Xenopus laevis, of Ancel & Vintemberger (1948) in Rana fusca (= R. temporaria), and of Elinson (1975) in R. puienus. Rzehak suggests that the variable location of this point in different eggs may well point to a relation between it and the sperm entrance point. Our observations definitely prove the identity of the two points.

In sections passing exactly through the animal–vegetal axis and the sperm entrance point the latter is always found on the side opposite the dorsal yolk-free cytoplasm. Ubbels & Rzehak (1976) have suggested that sperm penetration in the anuran egg evokes a kinetic system that is involved in cytoplasmic segregation and further movement of the segregated cytoplasm. Preliminary observations revealed that in newly laid, unfertilized Xenopus eggs activated by tap water (Ortolani & Vanderhaeghe, 1965) cytoplasmic segregation and further dorsad movement do not occur, although pigment movements leading to grey crescent formation do take place (Rzehak & Ubbels, unpublished). Therefore, cytoplasmic movement and pigment shifts in X. laevis may be independent.

Grey crescent formation after artificial activation by pricking had been described earlier in Rana fusca (= R. temporaria) (Brachet, 1911).

The region situated immediately adjacent and ventral to the sperm entrance point is often much more depigmented than the future dorsal region, which progressively attains a coarsely granulated appearance. In such cases the former region looks nearly white with only a few very fine vertical pigment stripes, and is therefore much more conspicuous than the grey crescent proper. In sections of eggs fixed ca. 60 min p.f. the two pronuclei – still unfused – are found somewhere beneath the coarsely granulated part of the egg surface, i.e. adjacent to the dorsal cytoplasm. This agrees with observations of Ubbels & Hengst (unpublished).

In various anuran and urodelan species the grey crescent is believed to be formed by a shift of the whole pigment cap with respect to the internal cytoplasm (Banki, 1929; Ancel & Vintemberger, 1948; Pasteels, 1964; Klag & Ubbels, 1975). In contrast, the present observations suggest that in X. laevis the formation of the grey crescent is the consequence of a pigment concentration around the point of sperm entrance. Such a mechanism has also been suggested by Løvtrup (1962, 1965). Rzehak (1972) considers it probable that the local
concentration of pigment leading to partial depigmentation of adjacent animal regions and to the formation of the grey crescent is the result of a contraction process which spreads from the point of sperm entrance. Consequently, in anurans at least two different modes of grey crescent formation occur. In *Discoglossus pictus*, where the sperm definitely enters very close to the animal pole (Pasteels, 1937; Campanella, 1975), the grey crescent is formed by a shift of the entire cortical layer around the internal cytoplasm (Klag & Ubbels, 1975). It is still unknown whether in anurans a specific relation exists between the place of sperm penetration on the one hand and the two modes of grey crescent formation on the other. In urodelan eggs, which are polyspermically fertilized, grey crescent formation takes place as in *Discoglossus*.

In *X. laevis* irregularly arranged acidophilic fibrillar structures are seen in the yolk-free cytoplasm; they progressively orient themselves parallel to the dorsoventral plane and bend towards the pronuclei from 40 min p.f. onwards, which agrees with other observations by Ubbels & Rzehak (unpublished). However, a preliminary EM study failed to reveal fibrils in the dorsal cytoplasm at 60 min p.f. (Herkovits & Ubbels, unpublished). A cytochemical and submicroscopical analysis of the dorsal cytoplasm and the kinetic system involved in the cytoplasmic displacements is in progress.

The authors thank Professor Dr P. D. Nieuwkoop and Dr K. Hara for their continuous interest and critical reading of the manuscript, Dr Faber for discussions and editorial assistance. They are also indebted to Miss C. Koning, for technical assistance, and Mrs E. Wolters, Mrs I. Aleven and Mr L. Boom for preparing the illustrations.

J. Paleček stayed at the Hubrecht Laboratory from January till July 1976 as a member of the 7th International Research Group in Developmental Biology.

K. Rzehak stayed at the Hubrecht Laboratory from June till November 1975 and took part in the preliminary cinematographic studies of pigment distribution.

REFERENCES


Pigment shifts upon fertilization in Xenopus egg


NEWPORT, G. (1854). Researches on the impregnation of the ovum in the amphibia; and on the early stages of development of the embryo (third series). *Phil. Trans. R. Soc. Lond.*, 144, 229–244.


(Received 7 November 1977, revised 18 January 1978)