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IMPROVED SURFACE VISUALIZATION OF LIVING AVIAN BLASTODERM STRUCTURES AND NEIGHBOURING OOPLASMS BY OOCYTAL TRYPAN-BLUE STAINING

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Abstract. By injection(s) of trypan-blue solution during late oogenesis (rapid growth period of the oocytes) into the mother quail we could improve the visibility of the structural details seen from the surface in living quail blastoderms when still *in situ* on their egg yolk balls. This technique of intraoocytal yolk staining by trypan blue allows us to observe, to focus on and to photograph much better the surface morphology of unincubated or shortly incubated blastoderms and their relationship with the neighbouring ooplasms. Indeed the difference in distribution and volume of the trypan-blue-stained yolk granules in the blastoderms and neighbouring ooplasms seems to prevent excessive reflexion by the deep part of the germ disc of the light penetrating through the superficial parts. This diminished scattering of light, greatly increases the contrast between the different regions. By this method we could visualize a higher number of RAUBER's sickles from the surface in living unincubated quail blastoderms.

Key words: trypan-blue-stained yolk, avian blastoderm, RAUBER's sickle, ooplasm, Japanese quail (*Coturnix coturnix japonica*).

INTRODUCTION

It is well known by avian embryologists that it is often difficult or impossible to see from the surface in the living state all the components or the orientation of the unincubated (as represented on Fig. 1) or shortly incubated blastoderm *in situ* on its egg yolk. Thus FARGEIX (1964) and LUTZ (1964) found that in only 30% of the unincubated quail eggs could a KOLLER's (1882) (RAUBER's, 1876) sickle be observed. In the unincubated blastoderms of our laboratory Japanese quails (*Coturnix coturnix japonica*) we found a similar percentage of RAUBER's sickles (CALLEBAUT, 1987). After removal of the unincubated blastoderm from its egg yolk ball f.i. for *in vitro* culture this becomes still more difficult, as well in the chicken as in the quail because often the whole or part of RAUBER's sickle remains fixed to the yolk by the natural tendency of its cells to incorporate underlying ooplasm (CALLEBAUT, 1993a; CALLEBAUT & VAN NUETEN, 1994). In the upper layer in the concavity of RAUBER's sickle (area centralis), the first gastrulation phenomena will take place (CALLEBAUT *et al.*, 1996a) during early incubation. Just like the NIEUWKOOP centre (equatorial vegetal dorsali-

zing cells) described by NIEUWKOOP (1973) in amphibian blastulas, RAUBER's sickle belongs to the equatorial vegetal part of the germ and induces neighbouring upper layer cells of the animal hemisphere (area centralis) to differentiate in endomesoderm. A primitive streak in the living blastoderm, *in situ* on its egg yolk ball, is often also difficult to see during early incubation. The reason for these difficulties (both in unincubated and primitive streak stages) is the lack of colour contrast in the different blastodermal and ooplasmic structures. Moreover, if components of an unstained blastoderm can be made visible under the stereomicroscope, this is usually only possible by a laborious combination of incident and transmitted light. The absence of contrast and the unequal spreading of the light then causes supplementary difficulties for stereomicrography. The obvious difficulties in observation and easy photography of living blastoderms, *in situ* on their egg yolk ball, explains why most studies or experiments are performed *in vitro*. The constituent yolk layers of quail blastoderms are formed when the precursor oocyte is growing from 3 to approximately 19 mm (rapid growth period: CALLEBAUT, 1974; 1983). By injection of solutions of the acid bisazo dye, trypan blue in a laying Japanese quail, it is possible to stain the yolk protein α-livetin in her largest oocytes (D'HERDE & ROELS, 1993). By fluorescence microscopy we have shown that besides the vitelline membrane, only the yolk granules are stained in the blastoderms and egg yolks derived from these oocytes (CALLEBAUT *et al.*, 1981). The method of trypan blue labelling was previously only used for fluorescent studies on sections and not *in situ* for observation of living germ discs from the surface. Seen from the surface these egg yolks present a brown or blue to black staining, depending on the quantity of injected trypan blue. This gives a kind of contrast which permits a much better visualization of the blastodermal structures and of the subgerminal, perigerminal and paragerminal ooplasms in the neighbourhood. In the present study we compare stereomicrographs from surface views after intravital trypan blue staining of the yolk with stereomicrographs taken without staining. The different components of an unincubated quail blastoderm and neigbouring ooplasms and yolk (with current terminology) are represented on a drawing of a mediosagittal section (Fig. 1).

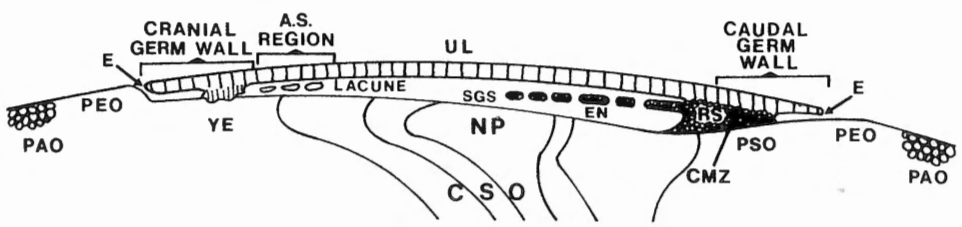

Fig. 1. – Schematic representation of a mediosagittal section through an unincubated quail blastoderm with surrounding ooplasms after fixation *in situ* on their egg yolk ball. UL: upper layer; EN: incomplete endophyll; RS: RAUBER's sickle; CMZ: caudal marginal zone; the caudal marginal zone being a more or less transparent part of the caudal germ wall adherent to the caudal peripheral subgerminal ooplasm (PSO); SGS: subgerminal space; E: edge of the blastoderm; YE: early development of yolk endoblast, growing into the peripheral subgerminal ooplasm; PEO: perigerminal ooplasm; PAO: paragerminal ooplasm; CSO: central subgerminal ooplasm in which the central nucleus of PANDER (NP) is seen.

MATERIAL AND METHODS

Thirty female adult laying Japanese quails were used for injection with unfiltered trypan blue O (SERVA, HEIDELBERG) solution in Ringer. The trypan blue solution was not filtered because it is a colloidal solution, which remains fixed to the filter paper during filtering (personal observation). Fifteen female quails were injected i.m. with 1 ml of 1% trypan blue solution every 2 days, during 1 month. This corresponds to the dose used in a previous study (CALLEBAUT & SYENS, 1985). Fifteen other quails were injected once with 1 ml of 0.25% trypan blue. This is a dose which is lower than the lowest dose (0.3 ml of 1% solution trypan blue) used by D'HERDE & ROELS (1993). The latter dose still gave a temporary slight modification of the yolk morphology exterior to the germinal disc region but had no influence on the yolk in the germinal disc region. Some of the freshly laid eggs were opened for observation and photography of their blastoderm. Special attention was given to the visibility of the morphological details present in unincubated blastoderms (as represented on Fig. 1) f.i. RAUBER's sickle (1876), the localization and eventual eccentricity of the nucleus of PANDER (1817), the caudal marginal zone, the germ wall and its borders. Other eggs were incubated for 10h at 39°C in an incubator, to observe the first signs of gastrulation from the surface, on the living egg yolk ball. The photographs were taken with a Leica camera (R3, electronic) adapted to a Wild (M5) stereomicroscope ocular. For comparison, unstained control blastoderms were photographed through the opening in a black screen placed over the egg yolk ball. This screen functions as a non-reflecting background, preventing intense light reflexion by the surface of the egg yolk. Some of the blastoderms were fixed in Susa without sublimate (ROMEIS, 1948) and after dehydration, embedded in paraffin. Mediosagittal and parasagittal sectioning was performed at 8-10 μm thickness. To demonstrate the relationship of the different components of the avian blastoderm with the subgerminal perigerminal or paragerminal ooplasm after that fixation, some sections were stained with iron hematoxylin and eosin. To compare the stereomicroscopic aspect of the living structures seen from the surface after trypan-blue labelling with the localization of the trypan blue labelled yolk in the germ disc, fluorescence microscopy was used on the sections. To do this, sections were examined under a Leitz Orthoplan microscope equipped with a HBO 100 high-pressure mercury lamp and an incident-light Ploem Opak illuminator. A 4-nm BG 38 red suppression filter and a 2-mm KG1 heat absorption filter were placed in the lamp housing. Blue excitation filters 2 x SP 490 in combination with a chromatic beam splitter (CBS) with a cut-off at 510 nm (filter setting No 3) were used (HARRISSON *et al.*, 1981). The chemical interaction between trypan blue and the yolk proteins results in a red fluorescence and permits the detection of trypan blue-marked yolk with high sensitivity. Fluorescence microscopy was only used on sections and not for surface observations. Other eggs (containing trypan-blue-labelled yolk) remained unopened and were incubated until hatching occurred.

RESULTS

Fig. 2 represents a surface stereomicrograph (without the use of fluorescent methods) from a living unincubated quail blastoderm after oocytal yolk staining with trypan blue.

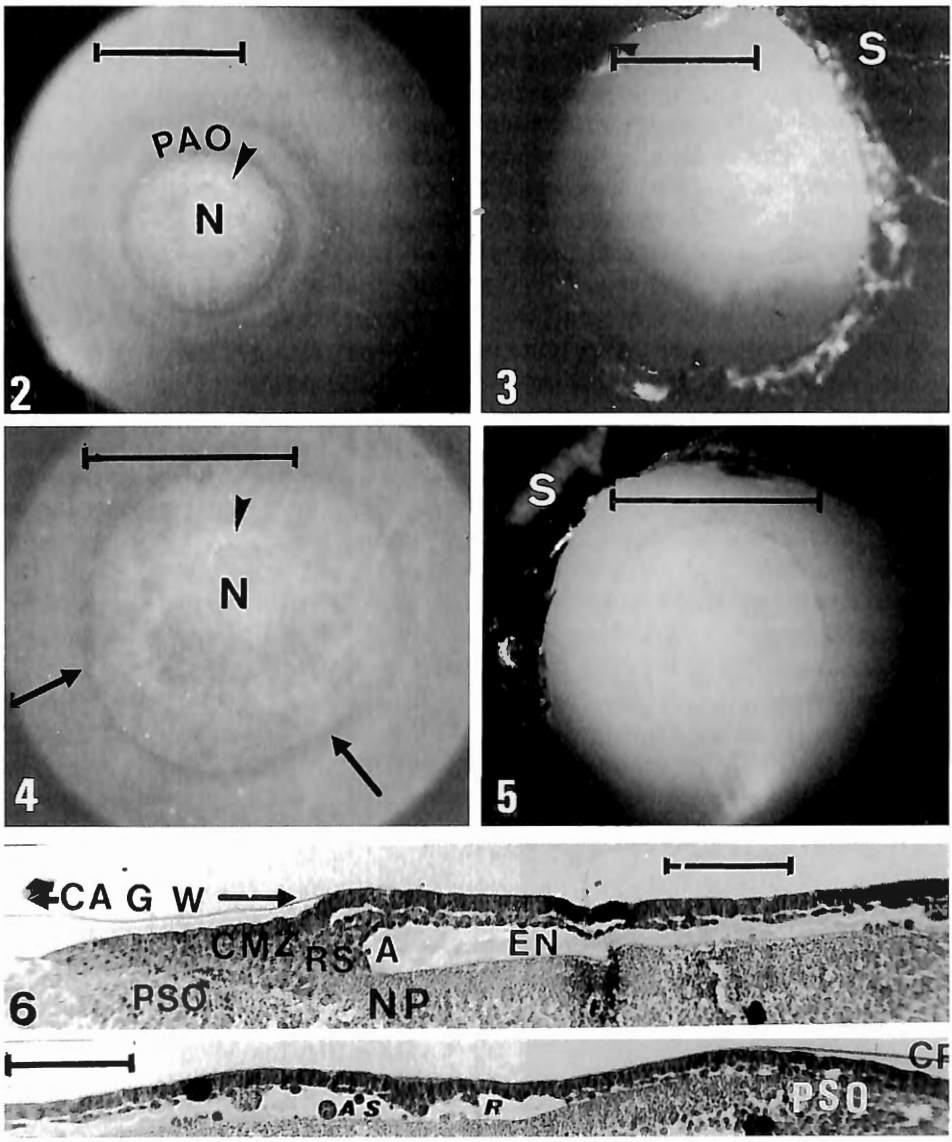

Fig. 2. – Stereomicrograph of a living unincubated quail blastoderm *in situ* on its egg yolk ball after intraoocytal staining with trypan blue (4 injections of 1 ml); N: eccentric nucleus of PANDER; arrow head indicates RAUBER's sickle, very close to the narrow caudal germ wall; PAO: white paragerminal ooplasm; bar: 3 mm.

Fig. 3. – Similar unstained living unincubated quail blastoderm *in situ* on its egg yolk ball; the stereomicrograph is taken through the opening in a black screen (S); the borders of the structures are not so sharply delineated as those of Fig. 2; bar: 3 mm.

Fig. 4. – Stereomicrograph of a living unincubated quail blastoderm *in situ* on its egg yolk ball after a single injection of 1 ml of 0.25% trypan blue solution during oogenesis, 4 days before

On Fig. 3 we see a similar quail germ without trypan-blue staining. The improvement of the visibility of the structural components after trypan blue yolk staining is very obvious. In Fig. 2 the RAUBER's sickle and the nucleus of PANDER are sharply visible in full contrast with the neighbourhood. So their eccentric localization indicates an early stage of primary (paradoxical) eccentricity, visible at the end of the intrauterine period and sometimes still found in just laid eggs (CALLEBAUT, 1993b). The extent of the narrow future caudal germ wall (behind RAUBER's sickle) and of the broader diametrically opposite cranial germ wall are also easily seen and also indicate a primary eccentricity. Most obvious in the trypan-blue-stained egg is the perigerminal ooplasm forming a dark halo around the sharply-defined blastoderm edge. The paragerminal ooplasm (rich in tubulin: CALLEBAUT *et al.*, 1996b) surrounds the perigerminal ooplasm forming a contrasting white halo. The latter peripheral structures are barely visible on the micrographs of unstained eggs (even after the use of a black screen). Fig. 4 represents an unincubated blastoderm *in situ* on the egg yolk ball of an egg laid 3 days after a single injection of 1 ml of 0.25% trypan blue solution to the mother. The staining is less pronounced and has a brown aspect, however it gives sufficient contrast for clear observation and photographing (compare with the unstained, unincubated quail blastoderm of Fig. 5). This intravital yolk staining with trypan blue permits us to localize and visualize much better small and narrow RAUBERIS sickles in unincubated blastoderms. Thus in 50 unincubated trypan-blue-stained blastoderms we could localize 30 RAUBER's sickles. This is a higher percentage (60 %) than could be observed in unstained blastoderms (30 % according to FARGEIX, 1964). When no RAUBER's sickle is visible, even after trypan-blue staining, usually a clear cut eccentricity can be seen. On sections of such blastoderms (stained or not) where only an eccentricity is visible from the surface, still the presence of a RAUBER's sickle as the cranial boundary of the caudal marginal zone (Fig. 1) can be demonstrated when sectioning is performed through regions where contrast is most pronounced (Figs 6, 7). Small dark zones in the germ walls seem to correspond, when sectioned, to voluminous yolk masses or «yolk islands» as described in the caudal marginal zone in the neighbourhood of RAUBER's sick-

laying; N: eccentric nucleus of PANDER; arrow head indicates narrow RAUBER's sickle, close to the caudal germ wall; arrows indicate the perigerminal ooplasm; bar: 2 mm.

Fig. 5. – Stereomicrograph of unstained living unincubated blastoderm *in situ* on its egg yolk ball; the micrograph is taken through the opening of a black screen (S): the different structures are not clearly visible through lack of contrast; bar: 2 mm.

Fig. 6. – Mediosagittal section through the caudal part of an unincubated quail blastoderm that, when alive, presented an eccentricity but no visible RAUBER's sickle (even after trypan-blue staining); RS: RAUBER's sickle, only visible on sections; CMZ: caudal marginal zone; A: axilla-shaped pocket of the subgerminal space; EN: endophyll sheet; note the localization of the nucleus of PANDER (NP), partially below RAUBER's sickle; CAGW: small caudal germ wall in intimate contact with the caudal peripheral subgerminal ooplasm (PSO); iron hematoxylin and eosin staining; bar: 100 µm.

Fig. 7. – The cranial part of the mediosagittal section through the same unincubated quail blastoderm as in Fig. 6; AS: anti-sickle in the cranial recessus (R) of the subgerminal space; CR: cranial germ wall loosely or not bound to the cranial peripheral subgerminal ooplasm (PSO); bar: 100 µm

le (CALLEBAUT, 1993a). The trypan blue *in ovo* staining of unincubated quail blastoderms also permits observations on the frequent individual variability in the volume or localization of the different structures. The nucleus of PANDER (1817) f.i. is often not found below the centre of the blastoderm but is localized more caudally (never cranially), sometimes very close or even partially below RAUBER's sickle (Fig. 6). This is obviously the result of the permanent eccentricity provoked by the inclination of the blastoderm on its egg yolk during the rotation in utero (CALLEBAUT, 1994). After a few hours of incubation the formation of the embryonic shield (first Anlage of the embryo) becomes visible (Fig. 8) in the blastoderms of trypan-blue-stained egg yolks. Often the first ingrowth of sickle endoblast, derived from RAUBER's sickle, into the endophyll of the area centralis (preceding the appearance of the primitive streak: CALLEBAUT & VAN NUETEN, 1994; CALLEBAUT *et al.*, 1997a,b) becomes visible. The initial appearance of the white-stained massive yolk endoblast forming part of a peripheral circle in the area opaca is, however, most prominent (Fig. 8). After approximately 12h of incubation the primitive streak becomes clearly visible (Fig. 9) by contrast with the dark background of the subgerminal ooplasm. In the blastoderms of unstained eggs the primitive streak is usually only faintly visible. On the sections through the germ disc, one sees clearly after fluorescence microscopy, the difference in distribution, volume and intensity of the fluorescence of the trypan-blue-stained yolk granules (Fig. 10). Seen from the surface in the living state this trypan-blue-stained yolk forms a brown, blue or black background from which no or less light is reflected. The more superficial parts of the blastoderm are less labelled and still transparent and so their structure can be better seen.

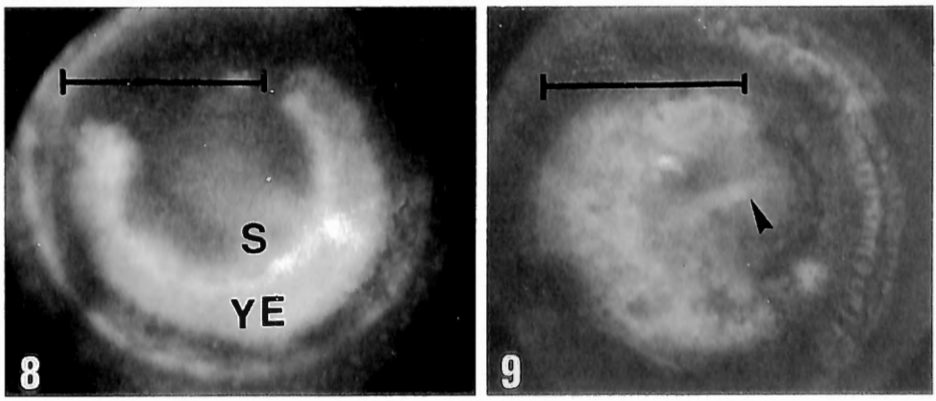

Fig. 8. – Stereomicrograph of a trypan blue stained (6 injections) quail blastoderm after 7h of incubation; an embryonic shield (S) appears in the concavity of RAUBER's sickle; also the white-stained horse-shoe-shaped massive yolk endoblast (YE) surrounding junctional endoblast appears in the area opaca; bar: 2 mm.

Fig. 9. – Stereomicrograph of a trypan-blue-stained quail blastoderm (after 11 injections during oogenesis) *in situ* on its egg yolk ball after 12h of incubation; the primitive streak (arrowhead) is clearly seen in contrast with the dark subgerminal background; bar: 2 mm.

Not withstanding the repeated i.m. injections of 1 ml (1% trypan blue in Ringer), the quails continued to lay eggs. The rate of egg laying somewhat decreased: instead of laying one egg every day, 2 eggs were usually laid every 3 days. However egg laying did not stop even after 1 month of treatment. The baby quails that hatched after the prolonged treatment with trypan blue were apparently normal.

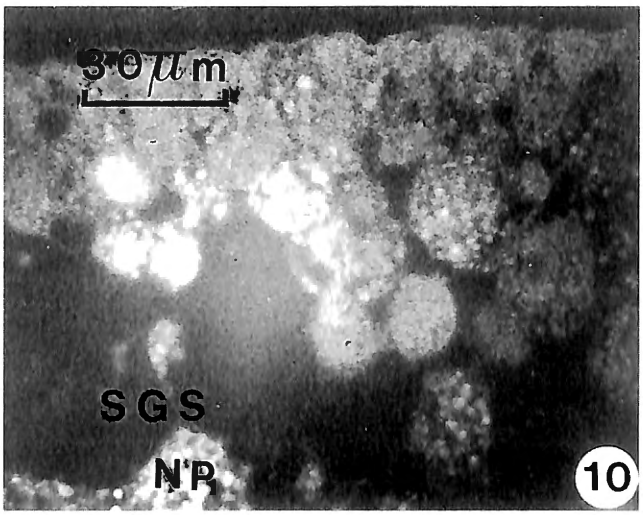

Fig. 10. – Fluorescence micrograph of a sagittal section through a quail germ, after oocytal labelling with trypan blue (6 days after an injection to the mother). The deeper layer of cells is strongly labelled (white aspect on the photograph), whilst the superficial layer contains less-labelled yolk granules. Below the subgerminal space (SGS) also part of the nucleus of PANDER (NP) contains numerous trypan-blue-labelled yolk granules. Seen from the surface in the living state the strongly labelled parts appear brown, blue or black and reflect less or no penetrating light. Thus the more superficial, more or less transparent parts become sharply visible; bar: 30 µm.

DISCUSSION

The technique described herein of intraoocytal trypan-blue staining allows us to more readily and accurately observe, focus on, and photograph the morphological features of living unincubated (represented on Fig. 1) and briefly incubated quail blastoderms. The trypan blue staining gives an impression of transparency to the blastoderm by forming a dark-field-like background in the subgerminal ooplasm. Since only the yolk is stained by trypan blue (CALLEBAUT & VAKAET, 1981; CALLEBAUT *et al.*, 1981; CALLEBAUT, 1983, 1987), the higher contrast between the blastodermal regions seems to be produced by the difference in content and volume of the trypan-blue-stained yolk granules (Fig. 10). This is also the case for the subgerminal, perigerminal and paragerminal ooplasm. Most ob-

vious in trypan-blue-stained blastoderms is the possibility to localize a higher percentage (both a quantitative as a qualitative improvement) of RAUBER's sickles than in unstained blastoderms. Our study suggests that RAUBER's sickle is nearly always present in a normal unincubated blastoderm but that its visibility seen from the surface in the living state, is sometimes hidden by lack of contrast with surrounding structures. Also the visual superposition of RAUBER's sickle sometimes localized above part of the nucleus of PANDER (Fig. 6) can have the same effect. Therefore the trypan-blue staining can be useful in expensive experimental studies to predict and to orient more precisely the future caudocephalic axis in order to make perfect mediosagittal and parasagittal sections of unincubated blastoderms. In the central subgerminal ooplasm, the nucleus of PANDER and its eventual eccentricity becomes clearly visible in contrast to the surrounding (Fig. 2) darker-staining bottom of the subgerminal cavity. The directly visible relationship between the living blastoderm and underlying subgerminal ooplasm is also one of the advantages of the trypan-blue staining. Very obvious in trypan-blue-stained egg yolks is the contrast between the perigerminal acellular tubulin-poor ooplasm (dark) and the rim of the blastoderm. On the other hand the acellular paragerminal ooplasm forms a broad white halo around the perigerminal ooplasm. This is probably due to the fact that in the paragerminal ooplasm numerous tubulin threads surround the local yolk granules (CALLEBAUT *et al.*, 1996b). The intravital trypan-blue staining described here also allows sequential observations on the evolution of the different components of the avian blastoderm and ooplasms *in situ* on their egg yolk ball by culture *in vitro* according to ROMANOFF (1943).

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REFERENCES

CALLEBAUT, M. (1974) – La formation de l'oocyte d'oiseau. *Arch. Biol.(Brux)*, **85**: 201-233.

CALLEBAUT, M. & L. VAKAET (1981) – Fluorescent yolk marking of the primary gonocytes in quail blastoderms by administration of trypan blue during late oogenesis. *I.R.C.S. Med. Sci.*, **9**: 458.

CALLEBAUT, M., F. HARRISSON & L. VAKAET (1981) – Peripheral avian yolk assemblage and its persistence in the blastoderm, studied by trypan blue-induced fluorescence. *Anat. Embryol.*, **163**: 55-69.

CALLEBAUT, M. (1983) – The constituent oocytal layers of the avian germ and the origin of the primordial germ cell yolk. *Arch. Anat. Micros.*, **72**(3): 199-214.

CALLEBAUT, M. & R.J. SIJENS (1985) – Trypan blue induced fluorescence in adult quail oocytes during the prelampbrush and early lampbrush stage. *I.R.C.S. Med. Sci.*, **13**: 920-921.

CALLEBAUT, M. (1987) – Ooplasmic localization and segregation in quail germs: fate of the four ooplasms. *Arch. Biol. (Brux.)*, **98**: 441-473.

CALLEBAUT, M. (1993 a) – Unequal caudocephalic ooplasmic uptake and eccentric formation of the subgerminal space below unincubated quail blastoderms presenting a Koller's sickle. *Belg. J. Zool.*, **123**(2): 107-112.

CALLEBAUT, M. (1993 b) – Early eccentricity in gravitationally oriented quail germs. *Eur. J. Morph.*, 31: 5-8.

CALLEBAUT, M. (1994) – Relationship between the avian blastoderm and the subgerminal ooplasm. *Eur. Arch. Biol.*, **105**: 111-123.

CALLEBAUT, M. & E. VAN NUETEN (1994) – Rauber's (Koller's) sickle: the early gastrulation organizer of the avian blastoderm. *Eur. J. Morph.*, **32**: 35-48.

CALLEBAUT, M., E. VAN NUETEN, H. BORTIER, F. HARRISSON & L. VAN NASSAUW (1996 a) – Map of the Anlage fields in the avian unincubated blastoderm. *Eur. J. Morph.*, **34**: 347-361.

CALLEBAUT, M., L. VAN NASSAUW, F. HARRISSON & A. SCHREVENS (1996 b) – Immunohistochemical localization of β-tubulin in the unincubated avian germ and in peri-, para- and subgerminal ooplasm: homology with meroblastic teleost embryos. *Belg. J. Zool.*, **129**: 169-176.

CALLEBAUT, M., E. VAN NUETEN, H. BORTIER, F. HARRISSON, L. VAN NASSAUW & A. SCHREVENS (1997 a) – Spatial relationship between endophyll, primordial germ cells, sickle endoblast and upper layer in cultured avian blastoderms. *Reprod. Nutr. Dev.*, **37**: 293-304.

CALLEBAUT, M., E. VAN NUETEN, F. HARRISSON, L. VAN NASSAUW, A. SCHREVENS & H. BORTIER (1997 b) – Avian gastrulation and neurulation are not impaired by the removal of the marginal zone at the unincubated blastoderm stage. *Eur. J. Morph.*, **35** (2): 69-77.

D'HERDE, K. & F. ROELS (1993) – Experimental modification of vitellogenesis in Japanese quail by trypan blue *in vivo*. *Reprod. Nutr. Dev.*, **33**: 597-608.

FARGEIX, N. (1964) – La caille domestique (*Coturnix coturnix japonica*). Ponte et étude de l'œuf au stade non incubé. *Arch. Anat. Histol. Embryol. et exp.*, **1**(4): 275-281.

HARRISSON, F., M. CALLEBAUT & L. VAKAET (1981) – Microspectrographic analysis of trypan blue-induced fluoresence in oocytes of the Japanese quail. *Histochemistry.*, **72**: 563-578.

KOLLER, C. (1882) – Untersuchungen über die Blätterbildung im Hühnerkeim. *Arch Mikrosk. Anat.*, **20**: 171-211.

LUTZ, H. (1964) – Le stade non incubé du blastoderme d'oiseau. *Arch. Anat. Histol. Embryol.*, **47**: 531-537.

NIEUWKOOP, N. (1973) – The organization center of the amphibian embryo; its spatial organization and morphogenetic action. *Adv. Morphogen.*, **10**: 1-39.

PANDER, C. (1817) – Historiam metamorphoseos quam ovum incubatio prioribus quinque diebus subit. *F. E. Nitribitt.* Ed. Wirceburgi (69 pp).

RAUBER, A. (1876) – *Uber die Stellung des Hühnchens im Entwicklungsplan*. W. Engelmann, Leipzig (28 pp).

ROMANOFF, A. (1943) – Cultivation of the early chick embryo *in vitro*. *Anat. Rec.*, **87**: 365-369.

ROMEIS, W. (1948) – *Mikroskopische Technik*, 15 Aufl. Leibnitz, München (695 pp).