Annls Soc. r. zool. Belg. — T. 113 (1983) — suppl. 1 — pp. 327-334 — Bruxelles 1983

(*Manuscript received on 02.03.1983*)

VITELLOGENINS IN *GASTEROSTEUS ACULEATUS*

by

F. OLLEVIER and M. COVENS
Zoological Institute,
Naamsestraat 59, B - 3000 Louvain (Belgium)

SUMMARY

Oestradiol treatment of sexually immature three-spined sticklebacks induced four specific lipoproteins in the plasma. This vitellogenin induction was dose dependent. An antibody prepared against the most concentrated vitellogenic protein reacted positively with two proteins from ovarian homogenates. Immunocytochemical treatment of ovarian sections with this antibody revealed the presence of immunopositively staining granules. This positive reaction was limited to the oestradiol treated fishes and appeared only in oocytes from stage 5 on. These findings are discussed.

INTRODUCTION

In teleosts oocyte growth goes through four periods : primary growth, vitellogenesis by endogenous yolk synthesis, vitellogenesis by uptake of exogenous yolk precursors and maturation (Reinboth, 1972; Clemens, 1974; Van Bohemen, 1981).

Gonadotropin is a major control factor in all stages except in primary growth (Balinsky, 1975; Khoo, 1979). Periodic acid Schiff positive yolk vesicles produced by the follicles themselves, appear during the first gonadotropin dependent period, corresponding to stage 4 according to Tromp-Blom (1959). These vesicles give rise to cortical alveoli (Wallace and Selman, 1981; Van Bohemen, 1981).

Oestrogens are essential for the induction of synthesis in the liver of vitellogenins. These proteins are female specific lipophosphoglycoproteins. They are secreted into the blood, transported to the ovaries and there selectively taken up by the follicles during the third growth phase or the phase of true vitellogenesis as referred to by Wallace and Selman (1981) or stage 5 as referred to by Tromp-Blom (1959).

During maturation the oocyte further increases in size due to hydratation (stage 6 in Tromp-Blom, 1959).

We prepared an antibody against purified stickleback vitellogenin and used immunocytochemical methods to establish the site and moment of vitellogenin uptake in the oocytes of untreated and oestradiol-treatment animals.

METHODS

Animals

Fully-plated three-spined sticklebacks, *Gasterosteus aculeatus* forma *trachurus*, were kept in the laboratory in dechlorinated running tap water at 11 $\pm$ 1° C under normal daylight conditions. The experiments were performed from October to

February. Sexually immature ♂♂ and ♀♀ sticklebacks were injected 6 times, once every two days. Three experimental groups were treated with 6.6 ng, 66 ng or 660 ng oestradiol-3-benzoate per g body weight respectively. Oestradiol was dissolved in peanut oil and the controls received only peanut oil. Blood obtained by tail severance was collected in heparinised micro hematocrit tubes and centrifuged immediately.

Electrophoresis

Plasma proteins were separated electrophoretically in polyacrylamide (PAA) gradient gels (5-15 %) or in agarose gels (1 % Corning universal electrophoresis film). Proteins were stained with Coomassie brilliant blue R 250 (0.01 % SERVA) and lipids with Sudan black B (1 % MERCK).

Immunology

After PAA gradient gel electrophoresis and staining with Coomassie brilliant blue for 15 min, the central part of the most concentrated vitellogenin band was collected and stored at — 20° C. Prior to injection, the vitellogenin containing gel pieces were homogenised in Ringer and then mixed with an equal amount of Freund complete adjuvans in an ultrasone apparatus (MSE, soniprep 150).

A rabbit was injected 5 times over a 4 month period : a first intradermal injection was followed by 4 subcutanous booster injections.

After collecting blood the serum fraction was treated with ammoniumsulphate in order to purify the Ig G fraction. The presence of antibodies was verified by immunodiffusion in agarose. Tissues were embedded in paraplast and 5 µm sections were prepared. The immunocytochemical peroxidase-anti-peroxidase technique (PAP) as described by VANDESANDE (1978) was used. A range of antibody dilutions 1/100 to 1/3000 was used.

RESULTS

Electrophoresis

Upon treatment of sexually immature sticklebacks with doses $\geqslant$ 6.6 ng oestradiol-3-benzoate, four lipoproteins, which are absent in immature and untreated ♂♂ and ♀♀, appeared in the plasma. The most intensively stained lipoprotein band is called Vg 1 and the molecular weight is 640,000 $\pm$ 20,000 Dalton (Pl. I : 1). The

---

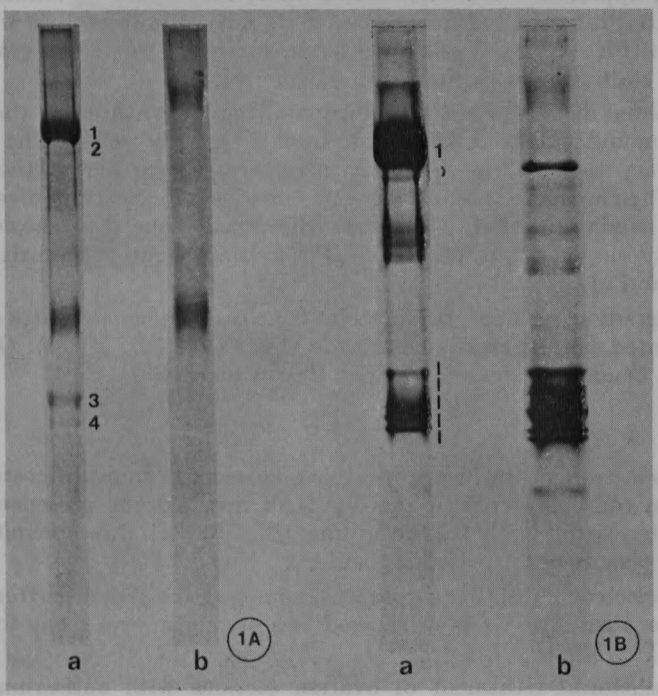
PLATE I

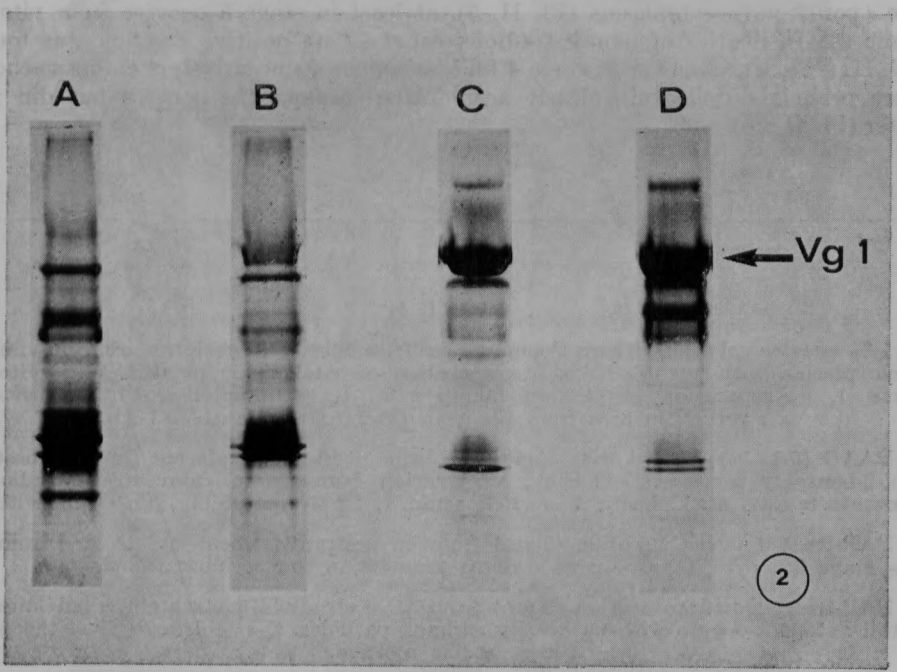
1 A. PAAG (5-15 %) stained with Sudan black B. *a :* plasma from an oestradiol-3-benzoate treated stickleback, *b :* plasma from a control animal; 1 = vitellogenin 1, 2 = vitellogenin 2, 3 = vitellogenin 3, 4 = vitellogenin 4.

1 B. PAAG (5-15 %) stained with Coomassie brilliant blue. *a :* plasma from an oestradiol-3-benzoate treated stickleback, *b :* plasma from a control animal; 1 = vitellogenin 1, 2 = vitellogenin 2; - - - - = region where Vg 3 and Vg 4 are situated.

2. PAAG (5-15 %) stained with Coomassie brilliant blue. Induction of vitellogenesis in non-vitellogenic ♀♀ by injections of oestradiol-3-benzoate (6 injections). A : control animal, B : 6.6 ng oestradiol per g body weight, C : 66 ng oestradiol per g body weight, D : 660 ng oestradiol per g body weight.

PLATE I





three other proteins (Vg 2, 3 and 4) were much less concentrated. Two of them (Vg 3 and 4) apparently contained much lipid; they could be stained easily with Sudan black B (Pl. I : 1A), although they could not easily be stained with Coomassie brilliant blue (Pl. I : 1B). Vg 2 lipoprotein, however, was more readily detected with protein stain than with lipid stain (Pl. I : 1).

The minimal dose required to induce vitellogenin synthesis in the three-spined stickleback amounts to about 6.6 ng oestradiol per g body weight. The 66 ng dose always gave very clear results. The response increased further at the 660 ng dose (Pl. I : 2). When homogenates of vitellogenic ovaries were electrophoresed in agarose, two protein bands appeared. The most concentrated one (Lp) showed nearly the same mobility as Vg 1, the other one (Ph) moved slightly towards the cathode (Pl. II : 3b and c).

In PAA gradient gel these two proteins from ovarian homogenates of vitellogenic ovaries migrated more towards the anode than Vg 1 (Pl. II : 4). Only the most concentrated band could be stained with Sudan black B.

Immunology

Immunoelectrophoresis in agarose gels of plasma proteins of oestradiol treated ♂♂ and ♀♀ in combination with anti-Vg 1-Ab revealed the presence of a plasma protein which reacted with the antiserum (Pl. II : 3a). No precipitin arcs were found in the plasma of untreated ♂♂ and ♀♀.

Immunoelectrophoresis in agarose with homogenates of ovaries from vitellogenic sticklebacks against anti-Vg 1-Ab showed two precipitin arcs : one for both major proteins (Pl. II : 3d).

Immunochemical treatment of ovarian sections with antiserum dilutions of 1/1200 to 1/2000 revealed that immuno positive staining granules were present in the « peripheral » cytoplasma (Pl. II, 5), at least in stage 5 oocytes from vitellogenic ♀♀. In ovaries of non-oestradiol treated ♀♀ no positive reaction was found (Pl. III : 7). In ovaries with stage 4 follicles numerous negatively staining vacuoles were present. Vitellogenin clearly accumulated around the oocytes but did not enter (Pl. II : 6).

PLATE II

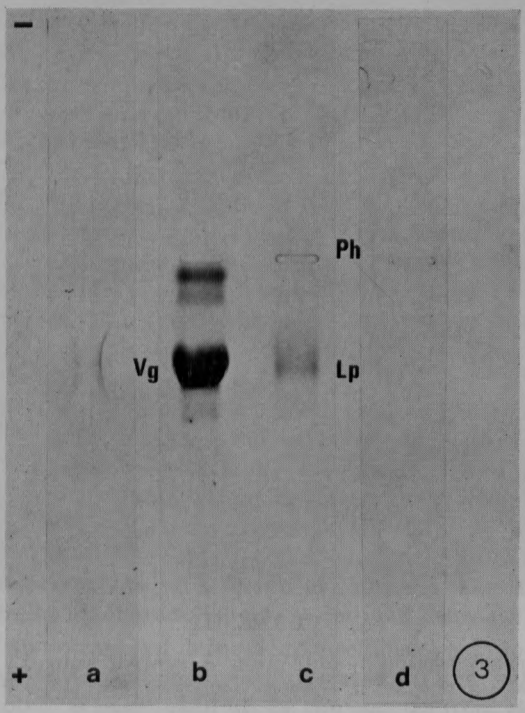
3. 1 % agarose gel stained with Coomassie brilliant blue. *a :* precipitin arc from vitellogenic plasma with anti-Vg 1-Ab, *b :* separation of vitellogenic plasma, Vg = vitellogenin 1, *c :* separation of ovarian homogenate, Lp = lipovitellin, Ph = phosvitin, *d :* precipitin arcs from the yolk proteins with anti-Vg 1-Ab.

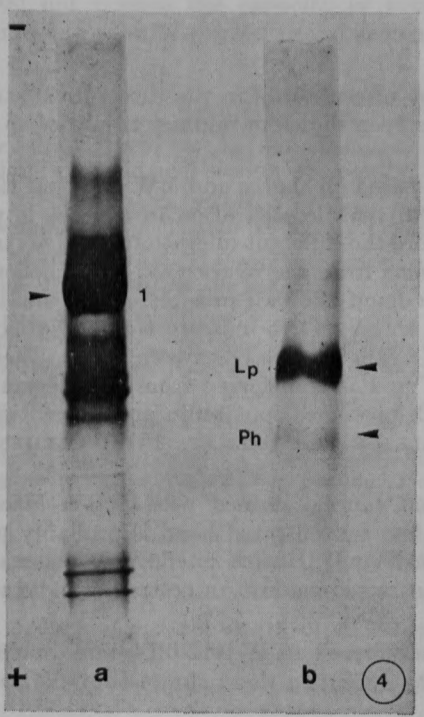
4. PAAG (5-15 %) stained with Coomassie brilliant blue. *a :* plasma from an oestradiol-3-benzoate treated stickleback, *b :* ovarian homogenate from an oestradiol-3-benzoate treated stickleback; 1 = vitellogenin 1, Lp = lipovitellin, Ph = phosvitin.

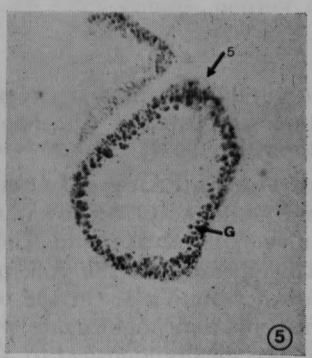
5. PAP-treated ovarian section (5 μm) from an oestradiol-3-benzoate treated animal. 5 = stage 5 oocyte, G = positive staining granules in the peripheral ooplasma.

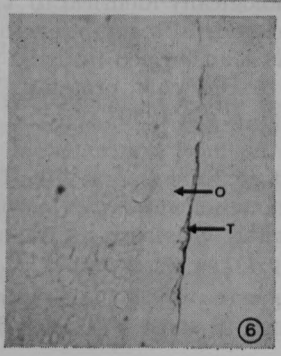
6. PAP-treated ovarian section (5 μm) from an oestradiol-3-benzoate treated animal, detail : stage 4 oocyte with negatively staining vacuoles. O = oolemma, T = thecacell layer stains positive.

PLATE II









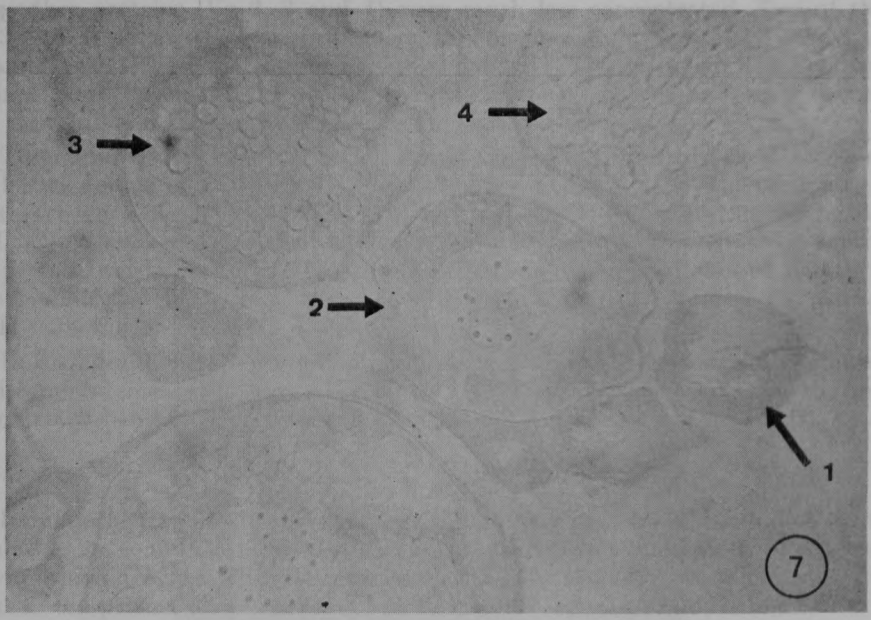


PLATE III

7. PAP-treated ovarian section (5 μm) from a control animal. 1 = stage 1 oocyte, 2 = stage 2 oocyte, 3 = stage 3 oocyte, 4 = stage 4 oocyte.

DISCUSSION

The vitellogenin inducing capacity of oestradiol in the threespined stickleback is clearly dose dependent. This has also been shown in rainbow trout *Salmo gairdneri* (Van Bohemen, 1981).

Separation in PAA gradient gel is based on charge and MW (Mauer, 1971; Sargent and George, 1975; Tanaka, 1981) while separation in agarose is primarily based on charge of the protein. Therefore the different migration positions in agarose and PAA indicate that the yolk proteins from the ovaries — of vitellogenic ♀♀ — are different from Vg 1. On the other hand the two precipitin arcs caused by the reaction of ovarian homogenate with anti-Vg 1-Ab indicate that the yolk proteins are immunologically identical to Vg 1 in the plasma (Barrett, 1978). These results support the general assumption that Vg 1 is transferred from the plasma into the oocytes and transformed into the yolk proteins, lipovitellin and phosvitin : it is a precursor to the yolk proteins (Wallace and Selman, 1979; Clemens, 1974; De Vlaming *et al.*, 1980).

Only the most concentrated band can be stained with Sudan black B and therefore it contains lipovitellin. The less concentrated band is probably phosvitin.

The immunological reaction of anti-Vg 1-Ab with vitellogenic plasma proteins shows that the antiserum was indeed directed against an oestradiol induced plasma protein (Vg 1), as we expected.

The oocytes in the sticklebacks increase from 0.12-0.14 mm in stage 1 to 0.35-0.56 mm in stage 4 (Tromp-Blom, 1959). In these stages they do not contain

proteins which react with the anti-Vg 1-Ab. We may therefore conclude that the growth during these stages is not due to accumulation of substances that are immunologically related to Vg 1 or to the yolk proteins.

The positively staining granules in stage 5 are peripherally situated in the oocytes and this is in agreement with autoradiographic results obtained in ovaries of the zebrafish (KORFSMEIER, 1966).

Our results lead to the conclusion that sticklebacks, in which vitellogenesis is induced by oestradiol, have the capacity to transfer vitellogenin from the plasma into the oocytes, at least during stage 5. We cannot exclude that the injected oestradiol also stimulates the uptake either directly or indirectly. It is however generally accepted that teleost pituitary gonadotropins enhace the transfer of vitellogenin from the blood into the vitellogenic oocytes (WAHLI *et al.*, 1981), apparently by stimulating extensive micropinocytotic activity at the oocyte surface (WALLACE and SELMAN, 1981, 1982).

Since the oocytes in stage 4 show an accumulation of vitellogenin outside the oocyte, it seems as if in these follicles the uptake mechanism is not yet activated. These results confirm a selective activation of oocytes.

CONCLUSION

The minimal dose to induce vitellogenin synthesis in male as well as in female three-spined sticklebacks amounts to 6.6 ng oestradiol-3-benzoate per g body weight.

At least one of the oestradiol induced lipoproteins (Vg 1) in the plasma of the three-spined stickleback is immunologically related to the yolk proteins in the oocytes. This lipoprotein is only taken up by the oocytes from stage 5 on. Younger oocytes do not possess the capacity of taking up vitellogenins, neither do they contain immunologically related proteins.

REFERENCES

BALINSKY, B. I. (1975) — *An introduction to embryology*. W. B. Saunders Company, Philadelphia, London, Toronto, 648 pp.

BARRETT, J. T. (1978) — *Textbook of immunology. An introduction to immunochemistry and immunobiology*. The C. V. Mosby Company Saint Louis, 3rd ed., 505 pp.

CLEMENS, M. J. (1974) — The synthesis of egg yolk protein by steroid hormones. *Progr. Biophys. Mol. Biol.*, **28**, 69-107.

DE VLAMING, V., H. S. WILEY, G. DELAHUNTY and R. A. WALLACE (1980) — Goldfish (*Carassius auratus*) vitellogenin induction, isolation, properties and relationship to yolk proteins. *Comp. Biochem. Physiol.*, **67** B, 613-623.

KHOO, K. H. (1979) — The histochemistry and endocrine control of vitellogenesis in goldfish ovaries. *Can. J. Zool.*, **57**, 617-626.

KORFSMEIER, K. H. (1966) — Zur Genese des Dottersystems in der Oocytes von *Brachydanio rerio*, Autoradiographische Untersuchungen. *Z. Zellforsch. Mikrosk. Anat.*, **71**, 283-296.

MAUER, H. R. (1971) — Basic principles of polyacrylamide gel electrophoresis and some recent advances of the technique. *Ann. Biol. Clin.*, **29**, 205-210.

REINBOTH, R. (1972) — Hormonal control of the teleost ovary. *Am. Zool.*, **12**, 307-324.

SARGENT, J. R. and S. GEORGE (1975) — *Polyacrylamide gel electrophoresis. Methods in zone electrophoresis*. 3rd ed. BHD, Chemicals Ltd. Poole, England, 219 pp.

TANAKA, T. (1981) — Gels. *Scientific American*, **1**, 110-123.

TROMP-BLOM, N. (1959) — The ovaries of *Gasterosterus aculeatus* before, during and after the reproductive period. *Proc. K. Ned. Akad. Wet.*, **62**, 225-237.

VAN BOHEMEN, Ch. G. (1981) — Estrogens and vitellogenin in the female rainbow trout (*Salmo gairdneri* L.). Proefschrift ter verkrijging van de graad van Doctor in de wetenschappen aan de Rijksuniversiteit te Utrecht, 122 pp.

VANDESANDE, F. (1978) — Immunohistochemisch onderzoek van het hypothalamo-hypofysair systeem bij het rund, de rat, de brattleboro rat en de kikker met anti-oxytocine. anti-mesoticine. anti-vasotocine. anti-runderneurofysine I en anti-neurofysine II antiserum. Proefschrift tot het verkrijgen van de graad van geaggregeerde van het hoger onderwijs, Rijksuniversiteit Gent, 228 pp.

WAHLI, W., I. DAVID, G. U. RUFFEL and R. WEBER (1981) — Vitellogenin and the vitellogenin gene family. *Science*, **212**, 298-304.

WALLACE, R. A. and K. SELMAN (1979) — Physiological aspect of oogenesis in two species of sticklebacks, *Gasterosteus aculeatus* L. and *Apeltes quadratus* (Mitchell). *J. Fish. Biol.*, **14**, 551-564.

WALLACE. R. A. and K. SELMAN (1981) — Cellular and dynamic aspects of oocyt growth in teleosts. *Am. Zool.*, **21**, 325-434.

WALLACE, R. A. and K. SELMAN (1982) — Oocyte growth in the sheephead minnow : uptake of exogenous proteins by vitellogenic oocytes. *Tissue and cell*, **14** (3), 555-571.