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SHORT COMMUNICATION

The presence of vitamin D_3 metabolic system in the ovary of Prussian carp (*Carassius gibelio* Bloch) – preliminary study on the potential role in the final oocyte maturation process

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Abstract. This study aimed to immunolocalize vitamin D_3 (VD) metabolic enzymes (CYP27B1 and CYP24A1) and receptors (VDR and PDIA3) in the ovary of Prussian carp during the spawning season. Furthermore, the effect of VD on the final oocyte maturation measured as the percentage of germinal vesicle breakdown (GVBD) after 24 h of incubation, was investigated. We have shown, for the first time, the cytoplasmic CYP27B1, CYP24A1, and PDIA3 protein immunolocalization in the ovarian follicular cells. We have also confirmed the presence of VDR in the nuclei of follicular cells. Furthermore, we have shown the stimulating effect of VD on the final oocyte maturation in fish. Therefore, VD may contribute to the regulation of ovarian functions by activation of its receptors (VDR and/or PDIA3) and/or *via* the paracrine/autocrine action ensured by important metabolic enzymes, namely CYP27B1 and CYP24A1.

Keywords: CYP24A1, CYP27B1, final oocyte maturation, fish ovary, PDIA3, VDR, vitamin D

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Introduction

Vitamin D_3 (VD) is an important regulator of ovarian function in mammals (Merhi et al. 2014, Grzesiak 2020, Xu et al. 2021, Grzesiak et al. 2022). The biologically active form, 1α,25-dihydroxyvitamin D_3, improved ovarian follicle survival and growth *in vitro* (Xu et al. 2021, Li et al. 2024). VD regulates cellular function mainly through nuclear vitamin D receptor (VDR) (Bikle 2014) or by activating a rapid nongenomic pathway possible through protein disulphide isomerase family A member 3 (PDIA3), which expression has been confirmed in avian and mammalian reproductive organs (Grzesiak et al. 2022, Hrabia et al. 2023). There are data indicating the presence of VDR protein in the ovary of fish such as Atlantic salmon (*Salmo salar*) and zebrafish (*Danio rerio*) (Lock et al. 2007, Craig et al. 2008), which may suggest the direct local action of VD at the gonadal level also in this aquatic vertebrate.

In fish, the final oocyte maturation (FOM) controlled by hormones and other factors, *i.e.* luteinizing hormone (LH) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) is visible as germinal vesicle migration toward the micropyle, then germinal vesicle breakdown

(GVBD) and ovulation (Lubzens et al. 2017, Zohar 2021). We hypothesize that this important stage of oocyte maturation, responsible for reproductive success, is also affected by VD. Fish obtain VD mostly from diets, and have much higher concentrations of biologically active form of VD compared to mammals, which is probably connected with some differences in vitamin D_3 metabolism carried/regulated by important enzymes such as 1α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1) (Bikle 2014, Grzesiak et al. 2022, Cheng et al. 2023).

To date, there is limited information about the role of VD in the cyprinid's ovarian function. Therefore, the aim of this study was to immunolocalize VD metabolic enzymes (CYP27B1 and CYP24A1) and receptors (VDR and PDIA3) in the ovary of Prussian carp during the spawning season. Furthermore, the effect of VD on the final oocyte maturation, measured as the percentage of germinal vesicle breakdown (GVBD) after 24 h of incubation, was investigated.

Materials and Methods

In the present study, tissues (ovaries and liver) were collected at spawning season (June) from five sexually matured Prussian carp females (3 years old, the mean body weight 153 ± 0.24 g and gonadosomatic index GSI about 17%) obtained from the Fishery Station belonging to the Department of Animal Nutrition and Biotechnology, and Fisheries, the University of Agriculture in Kraków, Poland. The use of animals was in accordance with the Act of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes and Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. Collected ovaries were placed in chilled Cortland's medium and used for follicle incubation with vitamin D_3 (Sigma-Aldrich). Other ovarian and liver fragments were fixed in 10% buffered formaldehyde (Sigma-Aldrich), dehydrated through graded ethanol solutions, cleared in xylene, embedded in paraplast (Sigma-Aldrich, St. Louis, MO, USA), and cut into 5 μm thick sections for subsequent immunohistochemical and immunofluorescent analysis.

To examine the *in vitro* effect of VD on the final oocyte maturation, follicles were separated, washed with medium, and transferred into three 24-well microplates, coated with Cortland's medium with 1% BSA (Sigma-Aldrich). Each well contained 0.5 ml of follicles and 1.5 ml medium, including the tested concentration of VD: 0 (control), 1, 10, 50, and 100 ng ml^{-1}. The most of the obtained oocytes had accumulated yolk, and the relative high GSI (17%) may suggest that females were before spawning (Boroń et al. 2011). There were 5 experimental groups, each group consisted of 10 wells (the follicles of each female were in duplicate (n = 5/treatment group/duplicates). The plates were then covered and incubated for 24 h at 22°C with periodical shaking. At the end of the experiment the oocytes were fixed in Serr's fluid (acetic acid–ethanol–formalin mixture 1:6:3 v/v), then gradually dehydrated, with the mixture of ethanol and turpentine oil, and finally, they were immersed in turpentine oil for transparency to visualize cell nuclei. Then the stage of oocyte maturity was determined by examination of the position of germinal vesicle (GV): in the centre (I), shifted but not crossing the half of the oocyte radius (II), located peripherally, near micropyle (III), invisible, after GVBD (IV) (Szczerbik et al. 2008). The results (% of I, II, III and GVBD) were analysed using GraphPad Prism statistical software (version 5, 2007, GraphPad Software, USA).

Immunohistochemistry used for VDR localization was performed as described previously (Grzesiak et al. 2019). Briefly, non-specific binding of IgGs was prevented by 5% normal goat serum (Sigma-Aldrich) before overnight incubation at 4°C with rat anti-VDR antibody (1:50, cat. no. MA1-710; Thermo Fisher Scientific, Rockford, IL, USA), and then 1.5 h with biotinylated goat anti-rat secondary antibody (1:300; Vector Laboratories, Burlingame, CA, USA). Next, avidin–biotin–horseradish peroxidase complex (1:100, 40 min; Vector Laboratories) was applied. The staining was developed using 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) as

chromogen staining substrate. Sections were examined with a Nikon Eclipse Ni-U microscope and a Nikon Digital DS-Fi1-U3 camera with corresponding software (Nikon, Tokyo, Japan).

Immunofluorescence for CYP27B1, CYP24A1, and PDIA3 was performed exactly as described by Hrabia et al. (Hrabia et al. 2023). After deparaffinization, rehydration, microwaving in citric buffer to epitope retrieval, and incubation with 5% normal goat serum, tissue sections were incubated overnight in the presence of rabbit anti-1α-hydroxylase (1:200, cat. no. PA5-79128; Invitrogen, Carlsbad, CA, USA), anti-24-hydroxylase (1:150, PA5-79127) or anti-PDIA3 (1:200) primary antibodies. Sections were then incubated with goat DyLight 594-anti-rabbit secondary antibody (Vector Laboratories) and mounted with VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Laboratories). Negative controls in the absence of primary antibodies were performed for each immunostaining. Sections were examined with an Axio Scope.A1 fluorescent microscope and photographed with an Axiocam 503 color camera and the ZEN 2.3 pro software (Carl Zeiss, Jena, Germany).

All data are presented as means ± SEM. A nonparametric two-tailed Mann-Whitney U-test was performed. The differences between the means were determined as significant for $P \leq 0.05$.

Results

After 24 h incubation with VD, the significant decrease, in the percentages of oocytes with centrally situated germinal vesicle (I), was observed in the groups treated with VD at the concentrations: 10, 50, and 100 ng ml^{-1}. It was statistically different as compared to the control group. The observation of percentages of oocytes with shifted GV, but not crossing half of the oocyte radius (II), and oocytes with peripherally situated germinal vesicle (III) shoved no significant differences between the experimental groups and the control one (Fig. 1). In the case of percentage of oocytes with GVBD the significant increase, over 2.5-fold, was observed in the groups treated with VD at the concentrations: 10, 50, and 100 ng ml^{-1} (Fig. 1).

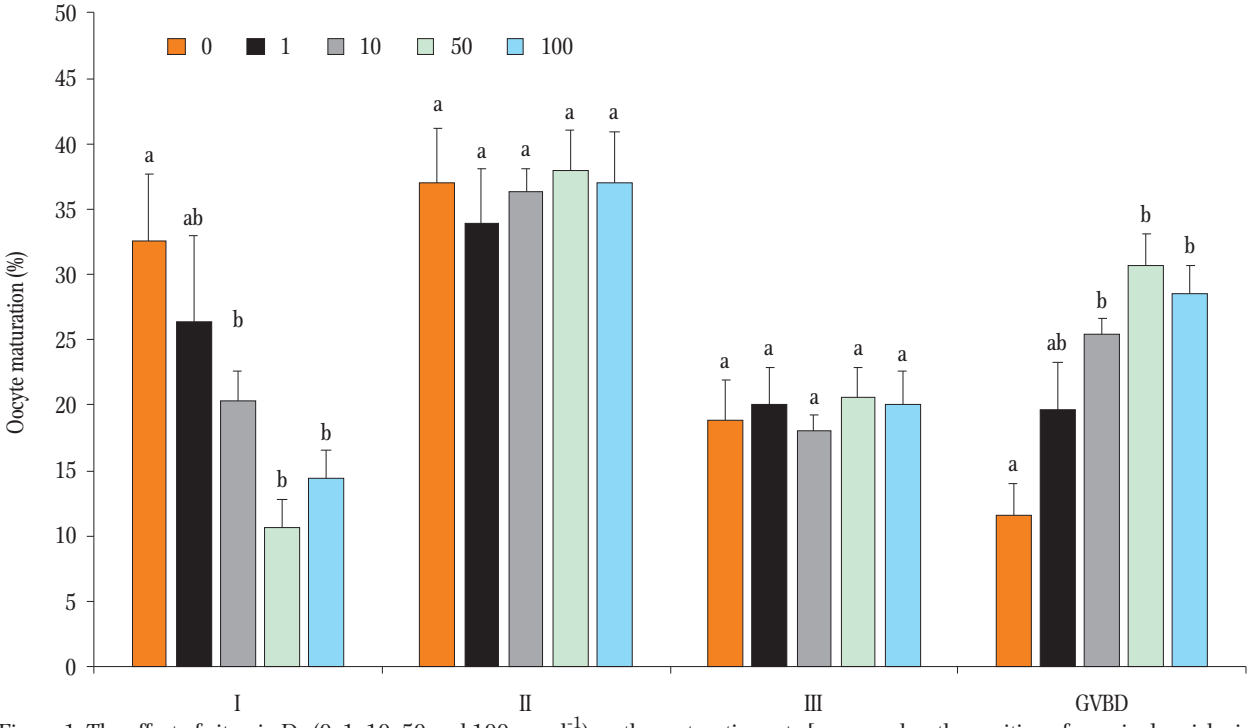

Figure 1. The effect of vitamin D_3 (0, 1, 10, 50 and 100 ng ml^{-1}) on the maturation rate [measured as the position of germinal vesicle: in the centre (I), shifted but not crossing the half of the oocyte radius (II), located peripherally (III), invisible, after germinal vesicle breakdown-GVBD] of Prussian carp (*Carassius gibelio* Bloch) oocytes after 24 h incubation. Data are expressed as means ± SEM (n = 5).

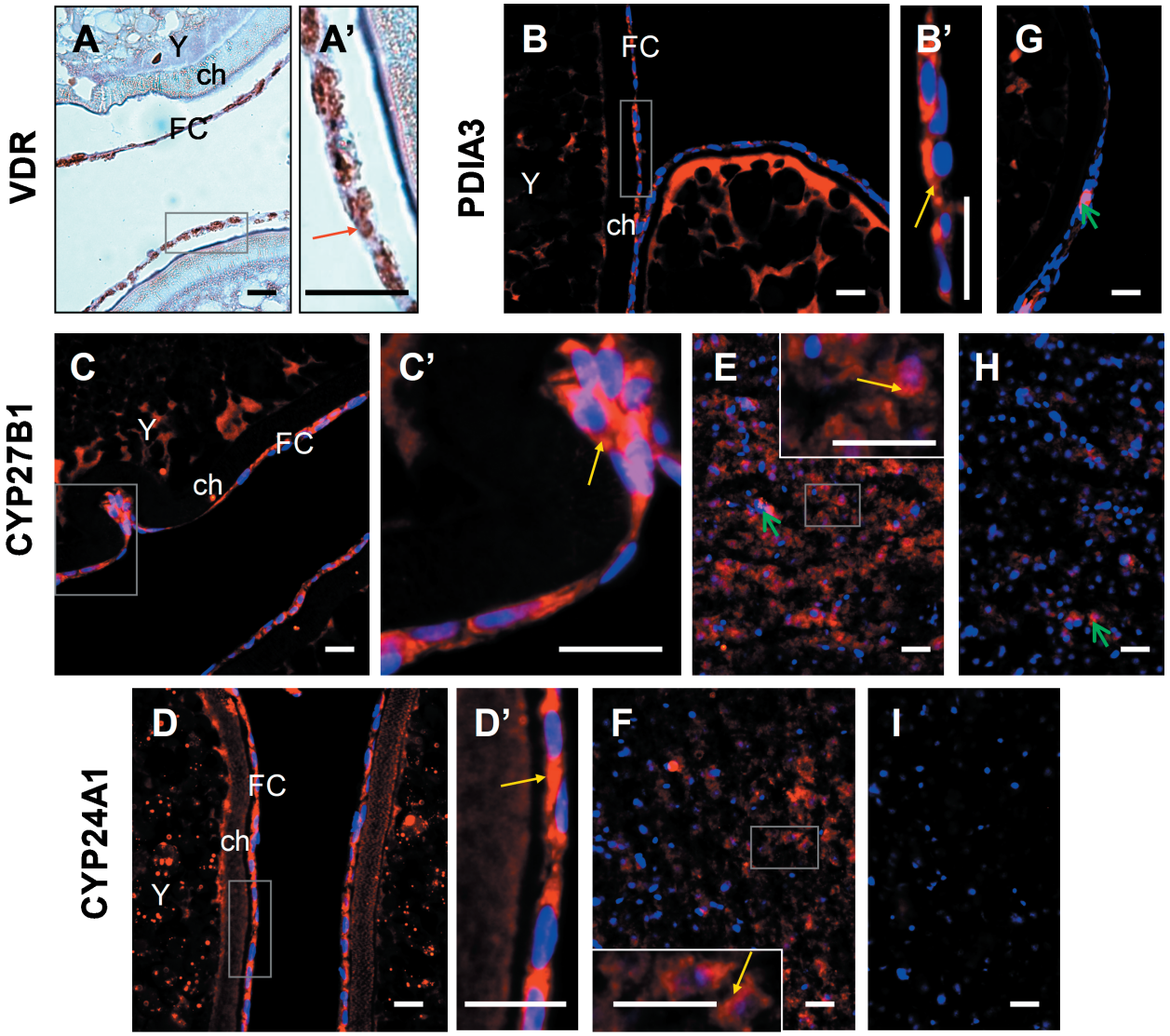

Figure 2. Representative micrographs of VDR, PDIA3, CYP27B1, and CYP24A1 protein immunohistochemical localization in the Prussian carp (*Carassius gibelio* Bloch) ovarian follicles (A-D') and liver (E, F). Immunoreactivity was visualized using an avidin-biotin-horseradish peroxidase complex + diaminobenzidine (A, A') or DyLight 594 detection system (red). Nuclei were counterstained with hematoxylin (A, A') or DAPI (B-I; blue). Positive staining (brown) for VDR (red arrow) was found in the nuclei of follicular cells. Positive signals for PDIA3, CYP27B1, and CYP24A1 (yellow arrows) were found in the cytoplasm of follicular cells. Moreover, immunoreactivity for CYP27B1 and CYP24A1 was observed in the cytoplasm of liver cells. Representative negative control sections (follicle wall in G, and liver in H and I) incubated without primary antibodies do not exhibit positive staining, excluding red blood cells which show nonspecific fluorescence (green arrows). Frames indicate the location of the higher magnification view (insert in E and F; photos A', B', C', and D'). FC – follicular cells, ch – chorion, Y – yolk. Scale bars = 20 μm.

It was statistically different compared with the control group.

Positive nuclear VDR staining was found in Prussian carp ovarian follicular cells (Figs. 2A and 2A'). Positive red immunofluorescence for PDIA3 protein (Figs. 2B and 2B'), as well as for CYP27B1 (Figs. 2C and C') and CYP24A1 (Figs. 2D and 2D') was localized in the cytoplasm of ovarian follicular cells. Immunoreactivity, for both metabolizing enzymes, CYP27B1 and CYP24A1, was also observed in the cytoplasm of Prussian carp liver cells (Figs. 2E and 2F). There was no immunoreactivity when sections of

ovarian follicles (Fig. 2G) and liver (Fig. 2H and 2I) were incubated with normal rabbit serum instead of a primary antibody.

Discussion

To investigate whether VD has any direct role in Prussian carp oocyte maturation, we incubated post-vitellogenic follicles *in vitro* in a presence of VD. Our results showed a stimulatory effect of VD on the final oocyte maturation, measured as increased percentage of observed GVBD in experimental groups as compared to control one. These results confirm the possibility of direct action of VD at the ovary level in fish, similarly as it was identified in mammals and birds (Yao et al. 2017, Xu et al. 2021, Grzesiak et al. 2022, Hrabia et al. 2023). Especially since, in the current study, we confirmed VDR protein immunoreactivity in the nuclei of follicular cells and, for the first time, protein localization of another VD receptor - PDIA3 in the cytoplasm of follicular cells. These results show both options of VD influence on the fish ovarian follicle by triggering a classical nuclear VDR, regulating gene expression and/or a rapid nongenomic action *via* PDIA3. In the present study, the observed stimulation of GVBD in VD-treated ovarian follicles might be caused by the influence on the key factors essential for final oocyte maturation in fish: the maturation-inducing steroid - 17,20β-P and/or on the maturation-promoting factor, a complex consisting of cdc2-kinase and cyclin B (Lubzens et al. 2017, Zohar 2021). Further studies are needed to clarify the mechanism of VD action in fish ovaries which resulted in induction of resumption of meiosis and the final follicular maturation. It is worth mentioning that *in vitro* study on goat follicles showed that supplementation of the incubation medium with VD promotes progesterone synthesis by increasing the expression of steroidogenic enzyme-related genes as well as increases the transcript abundance of Cyclin-dependent kinase 4 and Cyclin D1 (Yao et al. 2017), what may suggest that VD affects follicular development by regulating the cell cycle. Furthermore, the study by Yang et al. (2024) presented that *in vivo* long-term supplementation with VD can promote ovarian development in zebrafish, probably by affecting genes involved in sex steroid hormone synthesis.

The next novel finding of this study, shown for the first time in fish, is the localization of VD metabolic enzymes, CYP27B1 and CYP24A1, in the ovarian follicular cells of Prussian carp, which suggests the possibility of VD synthesis and catabolism within the ovary of cyprinids fish and that VD may be a locally (autocrine/paracrine) factor affecting follicle development and oocyte maturation similarly as it was previously demonstrated in other vertebrates (Grzesiak et al. 2022, Hrabia et al. 2023, Li et al. 2024).

Summing up, it is possible that VD in cyprinids contributes to the regulation of ovary function/final oocyte maturation by activation of its receptors (VDR and/or PDIA3) and/or *via* the paracrine/autocrine action ensured by important enzymes, namely CYP27B1 and CYP24A1.

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