Title

Vitellogenin uptake activity in the intestinal ducts of intraovarian embryos in a viviparous teleost *Xenotoca eiseni*

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Keywords

Goodeidae, histotrophy, intestine, nutrient absorption, viviparity

Highlights

- ✓ Intestinal ducts can take up vitellogenin in *Xenotoca eiseni* embryos.
- ✓ Endocytosis-related genes are expressed in the embryonic intestine.
- ✓ Lipid transporter genes undetectable in trophotaenia are expressed in intestine.

Abstract

In the viviparous teleost species belonging to the family Goodeidae, intraovarian embryos absorb maternal supplements while they grow during the gestation period. They take up the components via trophotaeniae, a hindgut-derived placental structure. Our previous study using a goodeid species *Xenotoca eiseni* revealed that intraovarian embryos absorb the yolk protein vitellogenin (Vtg) via the trophotaenia. However, another group indicated yolk components accumulate in the intestinal lumen of *X. eiseni* embryos. Here, we investigated whether the intestinal duct is capable of protein uptake, as is the trophotaenia. Immunohistochemical studies indicated that endogenous vitellogenin is detected in the intestinal epithelial cells of the intraovarian embryo. Tracer analysis using FITC-Vtg also indicated that intestinal tissues can take up protein. The endocytosis-related genes expressed in trophotaenia were also detected in the intestinal tissues of the embryo. Lipid transporter genes which are not expressed in the trophotaenia were detected in the embryonic intestine. This evidence suggests that the intraovarian embryo of *X. eiseni* possesses two distinct sites for uptake of the maternal proteins. However, the presumed functions of the embryonic intestine and trophotaenia might be not identical. The study provides a new perspective on how mother-to-embryo matrotrophic interactions have changed in the evolution of viviparous teleosts.

Introduction

Viviparity is a reproduction system of animals in which the embryo hatches and grows in the mother's body until delivery. The most familiar viviparous system in vertebrates is that of eutherians, which is based on the placenta and an umbilical cord, but other taxa, including fish, amphibians, and reptiles have different types of viviparity [1-3]. In these species, the viviparity system can be classified into lecithotrophy or matrotrophy, according to the nutrient supply into the embryo. In lecithotrophic species, embryos in the female body are dependent solely on their own yolk nutrients for growth, without receiving any additional maternal supplements. In fish, the dry weight of the delivered fry is not grossly increased compared to that of the unfertilized oocyte [4, 5]. In matrotrophic species, embryos are thought to receive additional nutrients from the mother's tissues because their body mass dramatically increases during gestation periods, which consists not only of placentotrophy, but also histotrophy, including oophagy, embryophagy or matrophagy. Placentotrophic species possess specific structures to receive maternal supplements, as exemplified by the mammalian placenta [3]. In contrast, histotrophic species take up the components orally. We consider the two traits do not exclude each other; they could coexist in the embryonic development of viviparous animals.

The extant teleost fishes include approximately 500 viviparous species in which viviparity has been independently acquired in 14 families [4]. Viviparous teleosts belonging to the family Goodeidae are known to be placentotrophic species. Their embryos grow in the female ovary during gestation and most possess trophotaeniae, hindgut-derived placental structures elongated from around the perianal region [6]. Since the mid-1900s, the trophotaenia has been considered an absorptive tissue that

takes up maternal supplements such as proteins, lipids, or other components secreted in the ovarian fluids [7]. We are investigating molecular mechanisms of the mother-to-embryo nutrient transfer using a goodeid species *Xenotoca eiseni*. Our previous publications indicated that the trophotaenia of *X. eiseni* takes up vitellogenin via endocytic absorption [8, 9]. The epithelial layer cells of trophotaenia possess proteolytic and lipolytic activities for the endocytic nutrients. Endocytosis-mediated nutrient absorption has also been reported in stomachless fishes and preweaning mammals [10, 11]. However, gene expression analysis suggested that trophotaenia has no activities for the uptake of fatty acids or cholesterols via membrane transporters such as are expressed by the adult intestinal duct [9]. Therefore, we consider that the trophotaenia is a similar absorptive tissue to the intestine, but the function is not completely identical.

The question arises: is the trophotaenia the only channel to take up nutrients from the mother for the embryo? *Ataeniobius toweri* is a viviparous teleost belonging to the family Goodeidae, and is the only goodeid livebearer that possesses no developed trophotaenia [12]. *A. toweri* is considered a matrotrophic species because the female fish delivers well-grown fry [13]. Furthermore, morphological analysis suggests that *A. toweri* is a histotrophic species that exhibits oophagy because yolk contents derived from the unfertilized oocytes were observed in the intestinal lumen of the intraovarian embryo [14]. Phylogenetic analysis predicted that *A. toweri* was derived from ancestral species which possessed trophotaenia [15]. These results suggest that the placentotrophic ancestor and extant goodeid livebearers include both placentotrophic and histotrophic activities to support their intraovarian growth. Histotrophic yolk contents were also observed in the embryonic intestine of an extant goodeid livebearer

X. eiseni [16]. This result was based on a histological observation, and the substances being absorbed or the molecular machinery responsible have not yet been experimentally determined.

Using *X. eiseni* as our model system, we investigate here the absorption of vitellogenin in the intestinal duct of the embryo and explore the mechanisms involved. We use a molecular genetic approach to compare absorptive functions between the embryonic intestine and the trophotaenia.

Methods

Animal Experiments

This study was approved by the ethics review board for animal experiments of Nagoya University (A210264-001). We euthanized live animals in minimal numbers under anesthesia according to the institutional guidelines.

Fish

Xenotoca eiseni were purchased from Meito Suien Co., Ltd (Nagoya, Japan). Adult fish were maintained in fresh water at 27℃ under a 14:10 L:D photoperiod cycle. The adult fish breed in mass-mating. The developmental stages of the embryos obtained were presumed based upon morphological observation according to our previous study [17]. In this study, approximately 4-week post-fertilization embryos were used for the experiments. Fish were anesthetized using tricaine on ice before the surgical extraction of embryos. The embryos obtained were stored on ice until subsequent experiments.

Histology

Fish samples were fixed with Davidson's fixative solution (33% ethanol, 8% formaldehyde and 11% acetic acid) at room temperature. The fixed samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution for 1 week. The decalcified samples were dehydrated with ethanol, embedded in paraffin and sectioned into sagittal or transverse serial sections every 5 μm using a sliding microtome. The paraffin-fixed sections were dewaxed using xylene and ethanol, and subsequently stained with hematoxylin and eosin (HE). Microscopic observation was performed using an Olympus BX53 microscope and photographed using a DP25 digital camera

(Olympus, Shinjuku, Japan).

RT-PCR

Total RNA samples were extracted from adult intestines, embryonic intestines and trophotaeniae using the RNeasy Plus Mini kit (Qiagen). Reverse-transcription was performed using SuperScript IV reverse transcriptase (Thermo Fisher Scientific). PCR was performed using KOD-FX-Neo (Toyobo, Osaka, Japan) under the following conditions: 94 ℃ for 100 s, followed by 35 or 40 cycles of 94 ℃ for 20 s, 55 ℃ for 10 s, 72 ℃ for 10 s and 20 s at 72 ℃. The target genes and primer sequences are listed in Table S1.

Tracing of Vitellogenin absorption

FITC-conjugated goldfish Vtg protein was prepared according to a previous study [18]. The embryos obtained were incubated in 250 μg/mL Vtg-FITC solution for 1 h. After the treatment, embryos were washed with PBS and fixed with 10% formalin. The control embryos were fixed without Vtg-FITC treatment. The fixed samples were used for paraffin sectioning and immunohistochemistry (IHC).

Immunohistochemistry

Deparaffinized section samples were treated with 3.0% hydrogen peroxide in PBS for 10 min to inactivate endogenous peroxidases. To detect the endogenous proteins (Vtg, Cubn and Amn), the samples were autoclaved in citric buffer to activate the antigens. The samples were treated with Blocking-One solution (Nacalai) at room temperature for 15 min. Primary antibodies (anti-FITC, anti-Cubn) were used at 1:500 dilution.

Antisera (anti-Amn, anti-Vtg) were used at 1:5000 dilution. The samples were incubated with primary antibody or the antiserum at 4℃ overnight (anti-FITC) or for 1 h (the others). Secondary antibody was used at 1:500 dilution with 0.1% Tween-20/PBS. The samples were incubated with secondary antibody solution at 4℃ for 1 h. After incubation, 3,3'-diaminobenzidine tetrahydrochloride (DAB) color development was performed using the DAB Peroxidase Substrate Kit, ImmPACT (Vector Laboratories, Inc., Burlingame, CA, USA), as per manufacturer instructions. The antibodies used in this study are listed in Table S2.

Quantitative reverse transcription-polymerase chain reaction (qPCR)

qPCR was performed using the Roche LightCycler 96 system (Roche, Mannheim, Germany) with Thunderbird SYBR qPCR Mix (Toyobo) under the following conditions: preincubation (90℃ for 30 s), three step amplification (50 cycles of 94 ℃ for 15 s, 58 ℃ for 30 ℃ and 72 ℃ for 30 s) and melting (95 ℃ for 10 s, 65 ℃ for 60 s and 97 ℃ for 1 s). After normalization using Ct values for β-actin, the relative expression values for the intestines and trophotaeniae were compared. The primer sequences are listed in Table S3.

Results

Vitellogenin absorption into absorptive cells in intestine and trophotaenia

X. eiseni embryos at the 4th-week post-fertilization stage and with well-developed trophotaeniae were obtained from pregnant females and were used in the experiments (Figure 1A). In this type of embryo, two pathways for uptake of maternal supplements can be considered. One is via oral intake; the other is via specific structures for nutrient transfer like the placental structure (Figure 1B). The absorptive epithelial cells in the intestinal duct and trophotaenia of the embryo are regarded as the uptake channels for vitellogenin during the embryonic growth of *X. eiseni*. Endocytic vesicles were observed in the cytosol of the epithelial layer cells (Figure 1C). In the ovarian lumen of the mother fish, the intraovarian embryos are exposed to the mother-derived vitellogenin. In this study, to validate whether vitellogenin absorption occurs in these tissues, we developed methods for *ex vivo* culture and exposure of fluorescence-tagged vitellogenin using the extracted *X. eiseni* embryos (Figure 1D). Immunohistochemistry for *X. eiseni* vitellogenin indicated the endogenous vitellogenin is distributed in the intestinal duct and epithelial layer cell of the trophotaenia (Figure 1E). The tracer analysis conducted with a short-time exposure of FITC-Vtg molecule to the extracted embryo indicated that FITC is accumulated in the epithelial layer cells in both the intestinal duct and the trophotaenia (Figure 1F).

Endocytic gene expression in intestine and trophotaenia

Our previous publication indicated that endocytosis-related genes are highly expressed in trophotaenia [9, 19]. We proposed a model in which a membrane-bound receptor binds vitellogenin and internalizes it via endocytosis driven by vesicle proteins,

and then undergoes degradation by proteases and lipases in the intracellular vesicle. Molecular mechanisms operating in the embryonic intestine remained unknown (Figure 2A). The results of the RT-PCR analysis indicated that the endocytic genes, *cubn*, *amn*, *clta*, *cltc*, *ctsl.1* and *lipa* were expressed in the intestine of *X. eiseni* embryos (Figure 2B). All the bands were detected in the correct size predicted from the design of the primers (Table S1). Immunohistochemistry for *X. eiseni* Cubn and Amn indicated both proteins were distributed in the epithelial layer of the intestine and the trophotaenia (Figure 2C). In the intestine, the signal for Cubn exhibited mosaic distribution in the epithelial layer cells, whereas the signal for Amn was equally distributed in the monolayer. In the trophotaenia, the Cubn distribution in the epithelial layer was broader than that observed in the intestine.

Comparison of absorption functions between intestine and trophotaenia

Our qualitative RT-PCR indicated endocytosis and intracellular digestion are active in both the intestine and trophotaenia. To compare gene expression values quantitatively, qPCR was performed for *clta* and *cltc*, which encode vesicle coat proteins that help drive internalization of the substrate, and for *ctsl.1* and *lipa*, which encode digestive enzymes that directly regulate proteolysis and lipolysis in endocytic vesicles. The relative expression value of *lipa* in the intestine was statistically significantly lower than that in the trophotaenia. The values of the other genes did not exhibit significant differences between the tissues according to the student *t*-test (Figure 3A). In addition, we validated channels other than the endocytic pathway for lipid absorption, in particular, membrane transporter-mediated uptake of cholesterol or fatty acids. A cholesterol transporter gene *npc1l1* and a fatty acid transporter gene *cd36* were

219 expressed in the embryonic intestine like the adult intestine, whereas these were
220 absent in the trophotaenia (Figure 3B). By comparison, the cholesterol mediator genes
221 *npc1* and *npc2,* which are related to vesicle trafficking following vesicle degradation,
222 were expressed in all three tissues: the embryonic intestine, the adult intestine and the
223 trophotaenia.
224

Discussion

Our investigations indicated that the embryonic intestine of *X. eiseni* can absorb the vitellogenin that is secreted in the ovarian lumen of the mother. This is an identical trait to what was seen previously in the trophotaenia [9]. Furthermore, the expression profile of the endocytosis-related genes in the intestine resembled that of the trophotaenia. In contrast, there was a different pattern in gene expression of the lipid transporters between the intestine and the trophotaenia, suggesting that although the intestine and the trophotaenia in the intraovarian embryo of *X. eiseni* are presumed to be homologous tissues, their functions are not completely identical (Figure 4).

In goodeid species, trophotaeniae are well known as absorption channels for the maternal supplement during gestation [20]. The role of the embryonic intestine in absorption is less clear. A recent publication found that the yolk component derived from the unfertilized oocytes fills the intestinal lumen of *X. eiseni* intraovarian embryos [16]. Vitellogenin is one of the major yolk component proteins, thus the current approach focused on vitellogenin does not conflict with the previous observation [8]. The results in this study suggest the possibility that the intraovarian embryo takes up the vitellogenin derived from the ovarian luminal fluids via oral intake and then from the intestinal duct. However, we are not sure that the embryo possesses a swallowing activity in the ovarian lumen. We have not excluded the possibility that the vitellogenin in the intestinal lumen is just a result of passive penetration of the liquid component. This concern affects the ratio of the contributions that the intestine and the trophotaenia each make to embryonic growth during gestation. We hypothesize that the trophotaenia contributes more nutrition than the intestine, because the trophotaenia faces the ovarian luminal fluid directly and its surface area is bigger than that of the

intestine. To test this, we need to assess swallowing abilities of the intraovarian embryo and calculate the exact absorption ratio for the maternal supplements per area of the epithelium in both the intestine and the trophotaenia.

In the family Goodeidae, *A. toweri* is the only known species of matrotrophic viviparous teleost without trophotaenia. Histological analysis indicated that the intraovarian embryo of *A. toweri* takes up the maternal supplement via oral intake [14]. The intraovarian embryo is thought to absorb the nutrients required for growth during gestation via the intestinal duct. Phylogenetic analysis suggested that the absence of the trophotaenia in *A. toweri* is not an ancestral trait of the Goodeidae; rather, they lost the placental structure quadratically during evolution [15]. Trophotaeniae are helpful for efficient nutrient absorption in the mother body or to aid in competition between the littermates, but they might not be indispensable to reproduction. We hypothesize that *X. eiseni* and the other extant viviparous species belonging to the family Goodeidae have a possibility of losing their trophotaeniae in the future, similarly to *A. toweri*, provided the absorption activity of the embryonic intestine is maintained. We do not exclude the possibility that intestinal absorption is not a specific trait for intraovarian embryonic growth. A previous study reported that Cubn-mediated endocytosis contributes to macromolecule absorption into the lysosome-rich enterocytes in the intestine of zebrafish fry [10]. Stomachless fish, including zebrafish, maintain endocytosis-mediated absorption their whole life [21,22]. These observations could suggest that embryonic intestinal absorptive activity is merely preparation for life outside of the mother, and for oral feeding after birth. In that case, the histotrophy-related trait must be retained in the embryonic stages and coexists with the placentotrophic trait, which consists of trophotaenia.

Our previous studies of gene expression in *X. eiseni* suggested that trophotaenia do not possesses transporter-mediated cholesterol or fatty acid absorptive pathways [9]. We proposed that the intraovarian fluids do not include digestive enzymes to avoid autolysis of the ovarian wall and embryo. This would suggest that the maternal supplements contained in the ovarian fluids contain few small-molecule lipids like free cholesterol or fatty acids as the nutrients for the offspring. However, the lipid transporter genes *npc1l1* and *cd36* were expressed in the embryonic intestine of *X. eiseni*. We describe two possible hypotheses to explain this gap in the gene expression between the intestine and trophotaenia. The first is that the intestinal duct contributes to embryonic growth via luminal digestion of lipid complexes, which is a function that trophotaenia do not possess. The other possibility is that gene expression of the lipid transporters is due to the embryo preparing for life after birth and does not contribute to the intraovarian growth of the embryo. To validate this hypothesis, the development of methodologies for molecular tracing and microscopic observation of cholesterol or fatty acid is required [23, 24].

In conclusion, this study demonstrated experimentally that vitellogenin absorptive activity exists in the embryonic intestine using a placentotrophic viviparous species *X. eiseni*. This finding contributes to a broader understanding of the evolutionary modification of viviparity, not only in the family Goodeidae, but also in other viviparous vertebrates, including other fish species, amphibians, and reptiles.

Author contributions

A.I. designed the study. J.N. and A.I. carried out the experiments and analysis. H.Y. contributed to the sample preparation and experimental procedure. E.H. contributed to the data analysis and discussion. J.N. wrote the draft manuscript. A.I. edited the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Figure legends

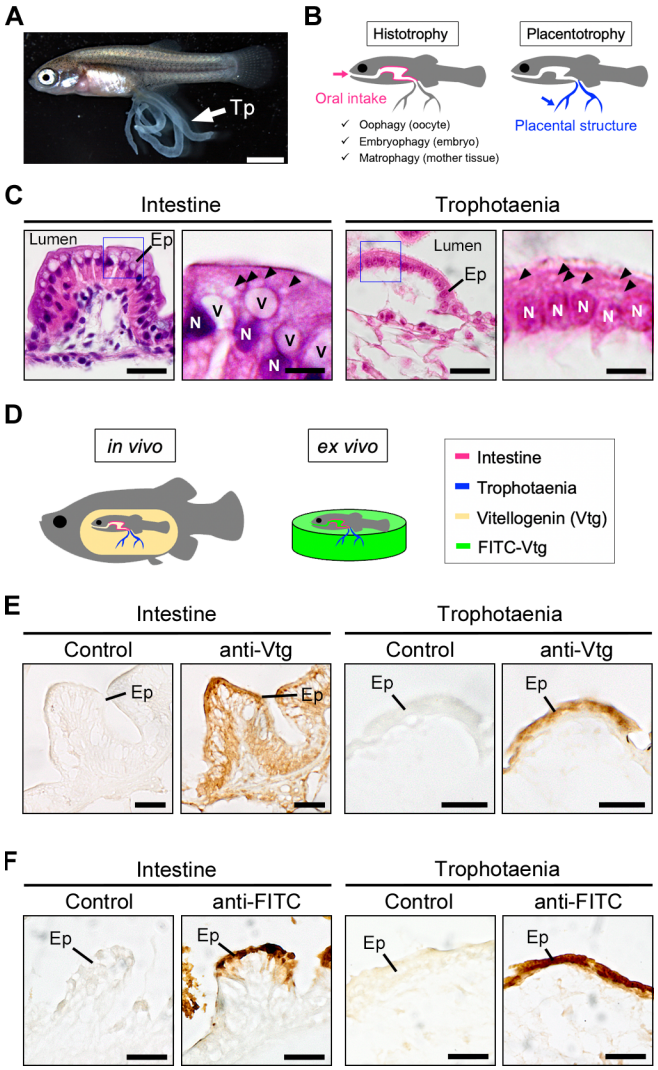


Figure 1. Vitellogenin absorption activity in intestine of *X. eiseni* embryo

A. Typical image for a 4th week post-fertilization embryo of *X. eiseni*. Well-developed trophotaeniae (Tp) are elongated from around the perianal region. **B**. Pathways for maternal supplements in intraovarian embryos of goodeid species. Histotrophic pathway involves oral intake of the components and absorption that in the intestinal duct (magenta). Placentotrophic pathway involves direct absorption from the trophotaeniae (blue). **C**. Histological structure of epithelial layer cells of the intestine and the trophotaenia in an *X. eiseni* embryo. Endocytic vesicles are observed in the

cytosol of these cells (arrowheads). V, vacuole. N, nucleus. Scale bar, 20 μm (wide) and 5 μm (enlarged). **D**. Working model of vitellogenin absorption *in vivo* and *ex vivo*. In the pregnant female, the intraovarian embryo is exposed to maternal vitellogenin secreted in the ovarian fluids. In our tracer assay, the extracted embryo is exposed to the FITC-labelled vitellogenin. After the treatment, the absorption of the tracer can be detected by immunohistochemistry. **E**. Immunohistochemistry for endogenous vitellogenin. Pre-immune serum was used as the primary antibody for a negative control. The signals were visualized by DAB staining. Ep, epithelial cell layer. Scale bar, 20 μm. **F**. Immunohistochemistry for FITC, which is fused to the vitellogenin as a tag. Normal rabbit IgG was used as primary antibody for negative control. The signals were visualized by DAB staining. Ep, epithelial cell layer. Scale bar, 20 μm.

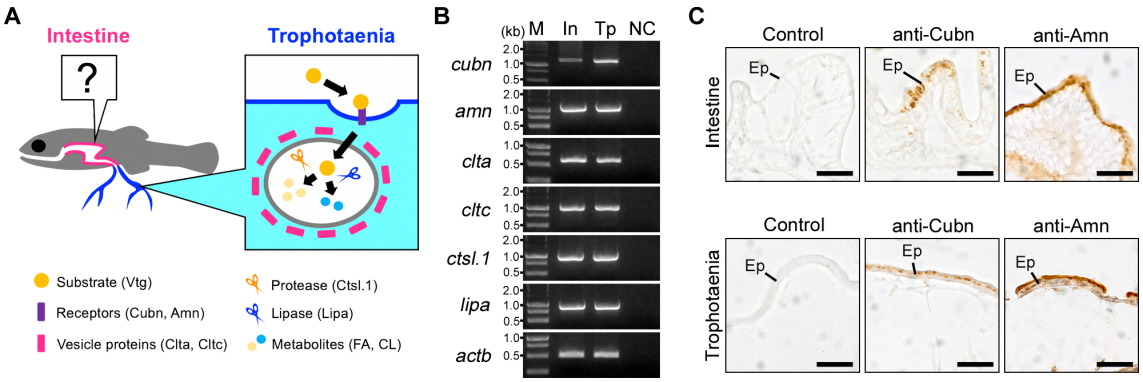


Figure 2. Presence of endocytic factors in intestine of *X. eiseni* embryo

A. Working model for endocytic vitellogenin absorption in trophotaenia (Iida et al., 2022). FA, fatty acid. CL, cholesterol. **B**. RT-PCR for the endocytic genes expressed in trophotaenia. The genes were also all detected in the embryonic intestine. β-actin (*actb*) was used for a positive control. M, size marker. In, embryonic intestine. Tp, trophotaenia. NC, negative control (no template DNA). **C**. Immunohistochemistry for endogenous Cubn and Amn proteins. Pre-immune serum was used as primary antibody for negative control. The signals were visualized by DAB staining. Ep, epithelial cell layer. Scale bar, 20 μm.

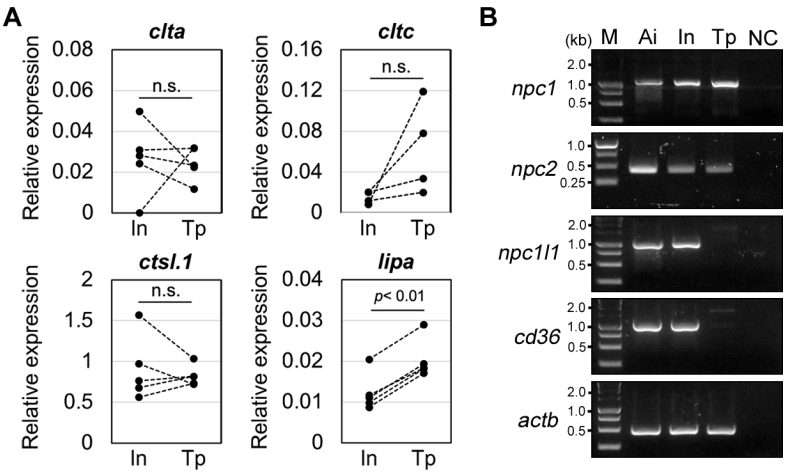
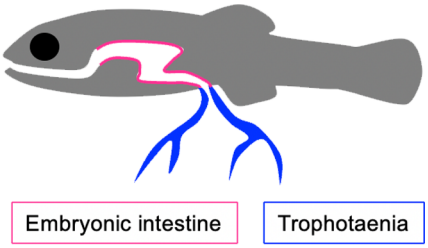


Figure 3. Comparison of gene expression in intestine and trophotaenia of *X. eiseni* embryos

A. Comparison of relative gene expression values of the endocytic genes *clta*, *cltc*, *ctsl.1*, and *lipa* by qPCR. The expression value of each sample was normalized to that of β-actin (*actb*). The vertical axis indicates the ratio of the target gene to *actb*. The dotted lines indicate the tissue sample derived from the same specimen. The Ct values are listed in Table S4 (*clta*, *cltc* and *actb*) and Table S5 (*ctsl.1*, *lipa* and *actb*). Student's *t*-test was used for statistical analyses. In, embryonic intestine. Tp, trophotaenia. **B**. RT-PCR for the genes related to lipid transport. The proteins encoded by *npc1* and *npc2* play roles in endocytosis-mediated lipid absorption. The proteins encoded by *npcl1l* and *cd36* regulate membrane transport of cholesterol or fatty acids. The embryonic intestines express lipid transporter genes, which are lacking in the trophotaeniae. M, size marker. Ai, adult intestine. In, embryonic intestine. Tp, trophotaenia. NC, negative control.



Vitellogenin uptake	Yes	Yes
Endocytosis	++	++
Proteolysis	++	++
Lipolysis	+	++
Lipid transport	++	−

Figure 4. Absorptive activities of the intestine and the trophotaenia in the *X. eiseni* embryo

Both intestines and trophotaeniae possess endocytic activities for vitellogenin uptake. By our assay, the intracellular lipolysis activity in the intestine might be lower than in the trophotaenia. Intestines also possess the ability to transport cholesterol and fatty acid, unlike trophotaeniae.