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A rare case of intra-ovarian oocyte maturation

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Short Communication

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Summary

The intra-ovarian presence of ootids, i.e. female gametes that have completed meiosis, is considered exceptional in the animal kingdom. The present study explores the first such case to be reported in a sea cucumber (Echinodermata: Holothuroidea). In the overwhelming majority of animals, including holothuroids, oocytes (i.e. immature female gametes) that are developing in the ovary undergo a primary arrest at the prophase stage of meiosis, which may last from days to decades. In free-spawning taxa, this arrest is normally lifted only during or shortly before transit in the gonoduct, when gamete release (spawning) is imminent. However, oocytes of the holothuroid *Chiridota laevis* were discovered to have resumed the second meiotic division including the completion of germinal vesicle breakdown and polar-body expulsion inside the ovary, effectively reaching the ootid stage concomitantly with ovulation (i.e. escape from follicle cells) prior to spawning. The potential drivers and significance of this exceptionally rare case of full intra-ovarian oogenic maturation are discussed.

Introduction

The term oocyte refers to an immature female reproductive cell. Oocytes are generally embedded in follicle cells (FCs; which modulate both the hormonal control of meiotic maturation and nutrient requirements of growing oocytes), and they possess a germinal vesicle (GV), also called nucleus, surrounded by a nuclear envelope. The GV is large and spherical, containing chromatin (DNA) and a nucleolus (or several nucleoli). Primary oocytes are stored in the ovary while arrested in the first meiotic prophase in almost all animal species (Iwashita *et al.*, 1998). They can maintain this primary meiotic arrest for a period of days in insects to decades in humans while they grow and accumulate the necessary maternal products to support the eventual completion of meiosis, fertilization, and early development. Following this first pause in meiotic division, there is generally a subsequent arrest, which coordinates the completion of meiosis with fertilization, sometimes referred to as "egg activation". Although the meiotic stage of this secondary arrest and the signalling pathways behind it vary among animal species (Delroisse *et al.*, 2021; Jessus *et al.*, 2020; Russo *et al.*, 2009; Von Stetina and Orr-Weaver, 2011), it is normally lifted by cues that are dependent on the presence of spermatozoa or fertilization itself (Nishiyama *et al.*, 2010). Hence, the universal pathway entails a long pause in the ovary where the oocyte grows, followed by a succession of quick maturation steps that unfold in a matter of minutes or hours when fertilization is imminent, whereby the oocyte is released from its matrix into the oviduct (ovulation) and final maturation halts again, briefly, until fertilization is completed (Figure 1A).

Unsurprisingly, the complex successive cellular and molecular mechanisms responsible for keeping meiotic progression on hold in females of most animals have fuelled prolific research (e.g. Filatov *et al.*, 2018; Grossman *et al.*, 2017; Jessus *et al.*, 2020; Sen and Caiazza, 2013; Von Stetina and Orr-Weaver, 2011). While variants abound, true exceptions to this broadly defined oogenic pattern have only rarely been evidenced so far, such as the absence of secondary arrest in a terrestrial nematode (Von Stetina and Orr-Weaver, 2011). The most fundamental known exception, which occurs in the echinoderm class Echinoidea (sea urchins), pertains to the timing of full oogenic maturation, i.e. the stage at which the female gametes are stored in the ovary prior to their release in the oviduct (Figure 1). Rather than primary oocytes with a prominent GV, the ripe ovary of females holds fully mature oocytes (i.e. ootids; Burke and Bouland, 1989) displaying the characteristic GV breakdown (GVBD). This characteristic allows investigators access to competent ootids that can be used in fertilization assays, which has made sea urchins easy to rear for studies in developmental biology (Mercier and Hamel, 2009).

In the class Holothuroidea, as in most animal species, the primary oocytes are arrested naturally in the first meiotic prophase (Iwashita *et al.*, 1998), and resume maturation in response to mechanical (Hamel and Mercier, 2007) and hormonal stimuli (Burke and Bouland, 1989; Masui, 1985; Sagata, 1996) associated with the onset of a natural spawning event. Sequential *in vivo* study of the reproductive tract of a model species (*Holothuria leucospilota*) during natural spawning showed that ovulation occurred in the ovarian tubules at the onset of spawning and that the first sign of GVBD appeared during transit in the oviduct. The oocytes

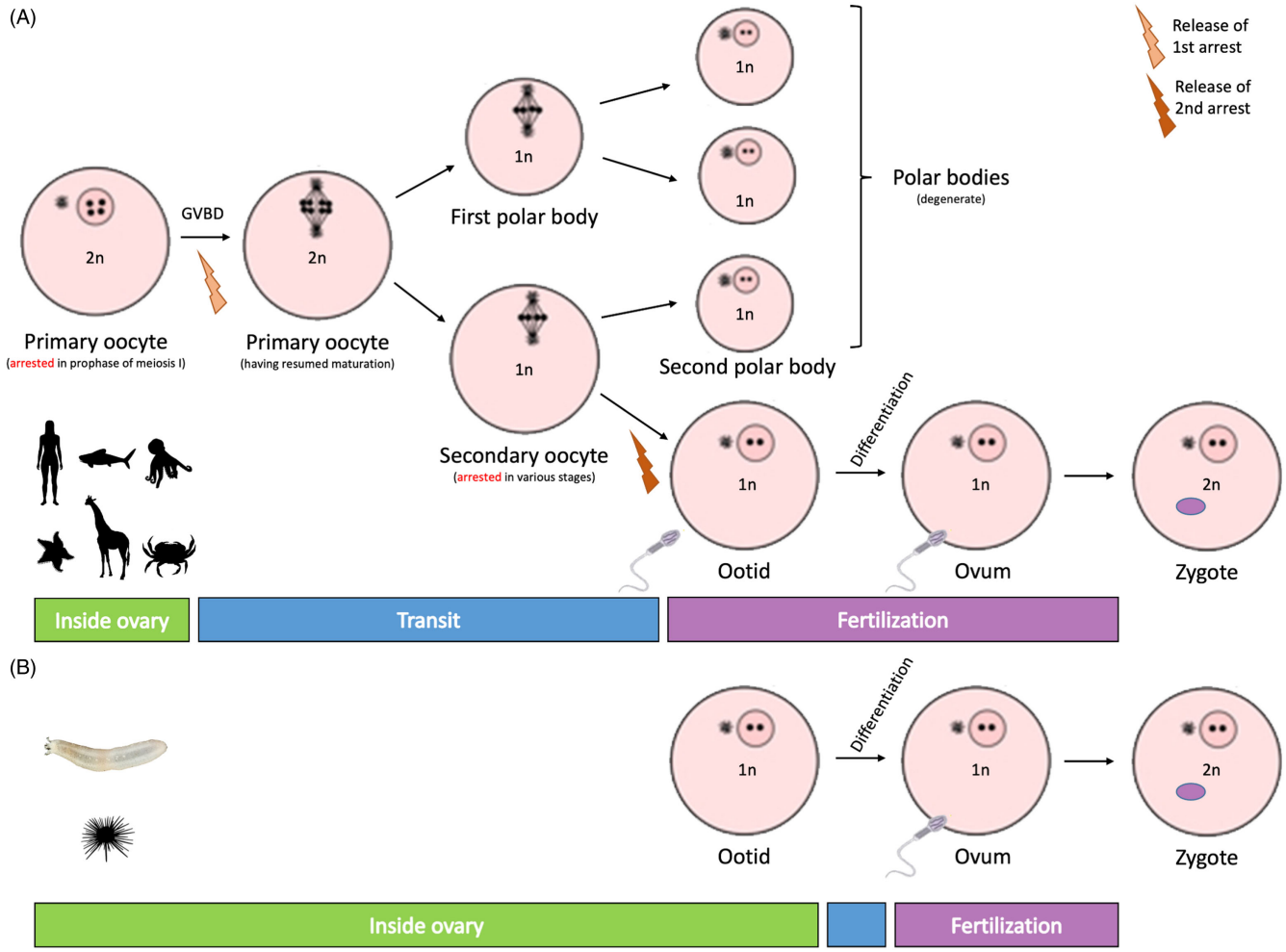

Figure 1. (A) Schematic illustration of the phases of oogenic maturation that are universal to most animals: primary oocytes are stored in the ovary where they grow (green rectangle); the onset of gamete release brings on cues that lift the first meiotic arrest, triggering GV breakdown (GVBD) and ovulation (transit into the oviduct; blue rectangle); a brief second meiotic arrest (occurring at various stages depending on the species, but commonly in metaphase of meiosis II) is lifted by presence of sperm or fertilization (purple rectangle). (B) Illustration of how the timing of oogenic maturation differs in echinoids, and now in *C. laevis*: the first and second meiotic arrests are lifted and GVBD occurs independently of spawning so that ovulated ootids are stored inside the ovary (green rectangle) long before release into the oviduct (blue rectangle) and fertilization (purple rectangle).

accumulated just underneath the genital papilla, in a bulge, and only became fully mature (fertilizable) after exposure to seawater (Hamel and Mercier, 2007). In the context of sea cucumber aquaculture programmes, the need to bypass the vagaries of natural spawning to obtain ootids has emerged (Eriksson *et al.*, 2012; Han *et al.*, 2016; Mercier and Hamel, 2009; Purcell *et al.*, 2012). Several factors, including temperature shocks, and exposure to phytoplankton, or air, have more or less effectively stimulated a proportion of exposed individuals (both males and females) to release their gametes, allowing researchers to have access to competent ootids to begin cultures (Battaglene *et al.*, 2002; Huang *et al.*, 2018; Mercier and Hamel, 2009; Morgan, 2000). Some investigators have tested various natural and synthetic compounds to induce the *in vitro* maturation of oocytes, replacing the necessity to wait for spawning altogether to obtain pools of fertilizable ootids (Burke and Bouland, 1989; Guerrier and Néant, 1986; Hodin *et al.*, 2019; Iwashita *et al.*, 1998; Mercier and Hamel, 2009; Néant *et al.*, 1989).

Meanwhile, the occurrence of internally brooding echinoderms has raised questions related to the timing of GVBD and maturation to the ootid stage in these species, especially in intra-ovarian brooders of the Apodida order. For instance, Sewell (1994) mentioned that unless fertilization in the brooding apodid *Leptosynapta clarki* was explored, its complete life-history strategy would not be understood. Turner (1973) had already suggested that fertilization in *Synaptula hydriformis* could either occur in the gonad or in the perivisceral cavity. Estabrooks (1984) occasionally noted developing young in the gonad tubules, suggesting that fertilizable ootids were held in the same tubule as developing oocytes. But where and when GVBD occurs naturally, leading to the development of ootids, has remained unconfirmed. The present study provides evidence of complete oogenic maturation inside the ovary prior to spawning in the free-spawning apodid holothuroid *Chiridota laevis*. This report documents the first case of intra-ovarian oocyte maturation outside the echinoid class. The possible drivers and implications of this currently unique pathway are explored.

Materials and methods

Collection and holding

Adults of *Chiridota laevis* (Figure 2A) were collected in fall 2021–2023 in Tappers Cove, Newfoundland and Labrador, eastern Canada (47°38'59.61°N, 52°42'53.09°W). Individuals were

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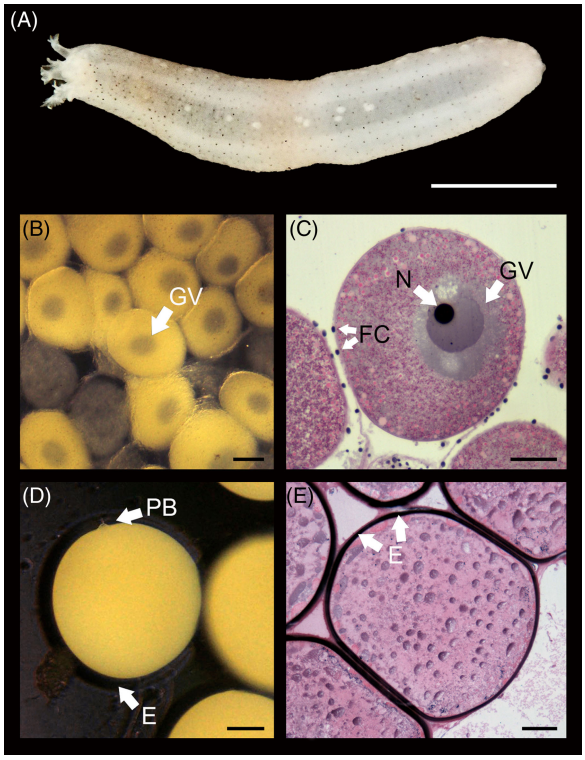

Figure 2. (A) The sea cucumber *Chiridota laevis*. (B-C) Primary vitellogenic oocytes still in the ovarian tubules of *C. laevis* showing the germinal vesicle (*GV*) and nucleoli (*N*) as well as the follicle cells (*FC*). (D) Ootids in the ovarian tubules with completed ovulation, GV breakdown and expulsion of polar bodies. These ootids also demonstrate a clearly developed embryonic coating *(E)*. (E) Ootids in the ovarian tubule, with the surrounding embryonic coating clearly visible (*E)* and no FCs. The scale bar represents 9 mm in A, 100 µm in B and 50 µm in C–E.

hand-collected by divers at subtidal depths (5–7 m) and to minimize stress were transported at low densities inside large coolers filled with seawater. Individuals of *C. leavis* were held in 20 litre tanks (267 X 394 X 216 mm) covered with about 3 cm of mud to mimic the natural environment. Flow rate in the tanks was set to approximately 42 L h^{-1} allowing the water temperature to fluctuate naturally over the annual cycle between 0 and 9 °C, at a salinity around 35 psu, under natural photoperiod. Light intensity was kept below ≤200 lux and was provided through large windows. All individuals fed on naturally deposited particulate organic matter provided by the ambient unfiltered seawater.

Dissection and microscopy

To assess their reproductive status, individuals of *C. laevis* ($n = 25$) were dissected in each season throughout the year with a particular focus during their suspected spawning season of late winter. Individuals were opened longitudinally, and the gonad tufts were removed at the base. To visualize gametes, several subsamples of gonad ($n = 3–5$) measuring ~10 mm in length were placed on a microscope slide and examined under a stereoscope (Leica M205 using LASX software and a Leica DFC 7000T camera) and a compound light microscope (Nikon Eclipse 80i coupled to a digital Olympus DP73 camera).

Histology

To further assess the stage of gamete maturity, the rest of the gonad (not used for microscopy) was preserved in neutrally buffered formalin (4%) and processed for histology. Preserved tissues were washed in ethanol at three successive concentrations (70, 80 and 90%) and embedded in methacrylate resin. The methacrylate was left to polymerize for 12 h at 4°C. The methacrylate-embedded tissues were then sectioned (3 µm) using a Leica RM2165 automated microtome. Seven tissue sections were placed on each slide and stained with celestine-blue and eosin-phloxine. This resulted in the nuclei being stained blue and cytoplasmic inclusions being stained pink. To identify various stages of gametogenesis the slides were then viewed and photographed under a light microscope (Nikon Eclipse 80i) coupled to a digital camera (Olympus DP73).

Results and discussion

Sequential sampling of female gametes in the ovarian tubules of the apodid holothuroid *Chiridota laevis* revealed that ovulation, GVBD (Figure 1B; Figure 2B–E) and polar-body expulsions (Figure 2D) were all completed before the onset of spawning in this species. Immature oocytes were found in some individuals all year round, predominantly from March to July. Mature vitellogenic oocytes surrounded by FCs and with GV were visible in the ovary from July to January, whereas ovulation had occurred and ootids were present in February (Figure 2D–E). Residual unspawned ootids at different stages of degradation remained between March and September. Together, these findings indicate that, uncharacteristically, oocytes can reach full maturity and competency inside the ovary in the days to weeks prior to spawning. This intra-ovarian oogenic maturation strategy may have developed to allow fertilization to occur before the thick protective sticky coating solidifies just minutes after ootid expulsion (Figure 2D–E), following which external fertilization likely becomes impossible.

The present study suggests that the long arrest of oocyte maturation at the prophase stage of meiosis inside the ovary, followed by shorter secondary arrest in the oviduct or outside the body, is not as universal as previously documented. Intra-ovarian final maturation, despite not having been described outside Echinoidea, may in fact be more common than expected and deserves further investigation. No evidence supporting GVBD and polar-body expulsion had previously been presented in brooding holothuroids, or in free-spawning species like *C. laevis*. It would be valuable to verify whether the same occurs in some species of asteroids (sea stars) known to undergo fertilization directly in the ovary (Byrne and Cerra, 1996) and in more species where female gametes develop a protective coating at the moment they are released into the environment.

Since the final meiotic maturation of oocytes in both echinoids and *C. leavis* occurs in the ovary, the primary triggers are likely internal factors (i.e. hormones or proteins; Hunt, 1989, Kanatani and Hiramoto, 1970, Kishimoto, 2011, Mayes, 2002). In contrast, other taxa of marine invertebrates (like most animals) have been shown to require the additional presence of external stimuli like spermatozoa or seawater (Goudeau and Goudeau, 2002, Miller *et al.*, 2001). There is also little known about the time frame of meiotic maturation in marine invertebrates, especially broadcast spawners like *C. laevis* and many echinoids. In broadcast-spawning species that rely on external cues, the final phase of oocyte maturation seemingly occurs in a relatively short time (e.g. 1–2 h in some asteroid species; Hunt, 1989, Voronina, 2003). In sea urchins that store pools of ootids, the delay between the lifting of meiotic arrest and the ootid has been shown to be 8 or more hours (Voronina, 2003). Here, the retrieval of gonad samples from individuals of *C. laevis* over several weeks leading up to the

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spawning season revealed ovarian tubules containing both mature oocytes with a prominent GV and ootids having completed meiotic maturation. This suggests that the final oocyte maturation process takes place over the span of weeks in the ovary, and that it may thus be more asynchronous than in echinoids, though the duration of an individual oocyte-ootid transition in *C. laevis* remains undetermined. In addition, the evolutionary implications of this asynchronous meiotic maturation strategy currently remain unclear and will require further research.

In closing, it should be noted that the uniqueness of the oogenic pathway described herein may not be immediately apparent due to inconsistent terminology used in reproductive biology, especially the (mis)use of the word egg/ovum. Case in point, reproductive research on humans and other mammals (chiefly the mouse model) commonly uses a different nomenclature where "egg" simply describes a cell found in the oviduct that can be fertilized to produce an embryo. This functional definition is deemed necessary to circumvent the fact that female mammals would otherwise not possess eggs (1N 1C cells), since fertilization is necessary to complete meiosis, i.e. the female haploid cell already contains the sperm genome (Duncan *et al.*, 2020). It has been suggested that the lack of a truly haploid phase in female mammals and the tight coupling between completion of oocyte meiosis and fertilization may be advantageous from an evolutionary perspective to reduce parthenogenesis (Duncan *et al.*, 2020). This may be equally true in most sexually reproducing animals. Thus, one interesting avenue of research for a unique pathway like the one seen in *C. laevis*, where true female haploid cells are produced long before fertilization, may be to explore its possible evolutionary link with self-fertilization in hermaphrodites and with modes of asexual reproduction like parthenogenesis.

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Ethical standards. None.

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