JOURNAL OF morphology

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Journal:	*Journal of Morphology*
Manuscript ID	JMOR-19-0213.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Buddle, Alice; The University of Sydney, School of Life and Environmental Sciences Otway, Nicholas; NSW Industry and Investment, Port Stephens Fisheries Institute Van Dyke, James; La Trobe University, College of Science, Health and Engineering Thompson, Michael; The University of Sydney, School of Life and Environmental Sciences Murphy, Christopher; The University of Sydney, School of Medical Sciences (Anatomy and Histology) Dowland, Samson; The University of Sydney, School of Medical Sciences (Anatomy and Histology) Simpfendorfer, Colin; James Cook University, Centre for Sustainable Tropical Fisheries and College of Science and Engineering Ellis, Megan; NSW Industry and Investment, Port Stephens Fisheries Institute Whittington, Camilla; The University of Sydney, School of Life and Environmental Sciences
Keywords:	lecithotrophy, viviparity, reproduction, Chondrichthyes, morphology

Title

Structural changes to the uterus of the dwarf ornate wobbegong shark (*Orectolobus ornatus*) during pregnancy

Authors

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Abstract

Embryos of the viviparous dwarf ornate wobbegong shark (*Orectolobus ornatus)* develop without a placenta, unattached to the uterine wall of their mother. Here we present the first light microscopy study of the uterus of *O. ornatus* throughout pregnancy. At the beginning of pregnancy, the uterine luminal epithelium and underlying connective tissue become folded to form uterine ridges. By mid to late pregnancy, the luminal surface is extensively folded and long luminal uterine villi are abundant. Compared to the non-pregnant uterus, uterine vasculature is drastically increased during pregnancy. Additionally, as pregnancy progresses the uterine epithelium is attenuated so that there is minimal uterine tissue separating large maternal blood vessels from the fluid that surrounds developing embryos. We conclude that the uterus of *O. ornatus* undergoes an extensive morphological transformation during pregnancy. These uterine modifications likely support developing embryos via embryonic respiratory gas exchange, waste removal, water balance, and mineral transfer.

Keywords

Chondrichthyes, lecithotrophy, viviparity, reproduction, morphology

1 Introduction

Viviparity (live-birth) is the most common form of reproduction in sharks (Wourms, 1981). In contrast to oviparous (egg-laying) sharks that oviposit fertilised eggs in the external environment, viviparous sharks retain developing eggs in their uterus until birth (Hamlett *et al.,* 2005). Viviparity has evolved independently from oviparous ancestry at least eight times in chondrichthyans (sharks and rays) (Naylor *et al.,* 2012; Blackburn, 2015; Buddle *et al.,* 2018). Multiple origins of viviparity among sharks have resulted in a variety of reproductive strategies to provide embryos with respiratory gas exchange, waste removal, water, and nutrients (Hamlett *et al.,* 2005; Buddle *et al.,* 2018). Nutrients can be supplied to embryos primarily from the yolk of the egg (lecithotrophy), or these yolk stores can be supplemented by non-yolk derived maternal nutrients during pregnancy (matrotrophy; Wourms *et al.,* 1988; Hamlett *et al.,* 2005). Matrotrophic forms of viviparity are diverse in sharks and likely evolved from lecithotrophic ancestry (Wourms *et al.,* 1988; Blackburn, 2015). Hence, identifying the uterine structures involved in supporting pregnancy in lecithotrophic species can provide information about the early evolution of viviparity (Wourms, 1977; Wourms and Lombardi, 1992; Kormanik, 1993; Blackburn *et al.,* 2010).

Lecithotrophic viviparity is more common than matrotrophic viviparity in sharks: seven of the nine extant shark orders contain lecithotrophic viviparous species, whereas matrotrophic viviparous species are confined to three shark orders (Buddle *et al.,* 2018). Despite the prevalence of lecithotrophic viviparity in sharks, descriptions of uterine structural changes associated with pregnancy are limited to several species of dogfish (order Squaliformes; Ranzi, 1934; Jollie and Jollie, 1967; Hamlett and Hysell, 1998; Hamlett *et al.,* 2005; Braccini *et al.,* 2007; Paiva *et al.,* 2012; Moura, 2011) and the common sawshark (*Pristiophorus cirratus,* order Pristiophoriformes; Stevens, 2002; Hamlett *et al.,* 2005). The dwarf ornate wobbegong (*Orectolobus ornatus)* is a species of carpet shark (Orectolobiformes; Compagno *et al.* 2005). Within the order Orectolobiformes, there are both oviparous and viviparous species (Buddle *et al.,* 2018). Therefore, species of Orectolobiformes provide an important model system for testing hypotheses about the transition from oviparity to viviparity in sharks (Blackburn, 2015; Buddle *et al.,* 2018).

Orectolobus ornatus has been classified as a viviparous lecithotrophic species because the total organic mass of full-term embryos is 32-33 % lower than the total organic mass of uterine eggs (Huveneers *et al.,* 2011). The decrease in total organic mass between uterine eggs and full-term embryos is attributed to the metabolic costs associated with embryonic growth (Wourms, 1981; Van Dyke and Beaupre, 2011; Huveneers *et al.,* 2011). At the same time, there is a 44-89 % increase in wet mass between uterine eggs and full-term embryos, which is attributed to embryonic uptake of water and inorganic matter (ash) (Huveneers *et al.,* 2011). Water and inorganic matter, as well as gas exchange, are provided to developing embryos by the fluid that surrounds embryos during pregnancy (Ellis and Otway, 2011; Otway and Ellis, 2012). This fluid is secreted by the uterine wall in early pregnant *O. ornatus* (Ellis and Otway, 2011). During the early stages of embryonic development, *O. ornatus* are encapsulated by an egg case (Ellis and Otway, 2011). As pregnancy progresses, the egg capsule disintegrates, and embryos develop freely in uterine fluid that is derived from seawater (Ellis and Otway, 2011). This uterine environment is created by females periodically exchanging the uterine fluid with the external seawater (Kormanik, 1993; Ellis and Otway, 2011; Tomita *et al.,* 2016).

The process of introducing external seawater into the uterus during pregnancy occurs in other lecithotrophic sharks and is termed uterine flushing (Kormanik, 1993; Ellis and Otway, 2011; Tomita *et al.,* 2016). Uterine flushing likely removes embryonic wastes and may allow embryos to access oxygen and inorganic ions from the seawater (Ellis and Otway, 2011; Tomita *et al.,* 2016). Additionally, *O. ornatus* embryos can complete embryonic development in an artificial uterus containing filtered, autoclaved seawater (Otway and Ellis, 2012). Our aim was to use light microscopy to identify uterine structures that could support embryonic development in *O. ornatus*. This is the first morphological study on the uterine structures that are involved in supporting embryonic development through pregnancy in any Orectolobiform. Given that dry mass data suggest that *O. ornatus* embryos are lecithotrophic, we tested the hypothesis that the uterus of *O. ornatus* lacks the structural specialisations for the allocation of nutrients to developing embryos.

2 Methods

2.1 Sample collection

Eighteen sexually mature females were used in this study (five non-pregnant and thirteen pregnant). All tissue samples in this study were from sharks used by Ellis and Otway (2011), collected opportunistically from commercial fishing operations off Coffs Harbour and Nambucca Heads, Australia. The sharks were collected under an Animal Research Authority (ACEC 03/04 – Port Stephens) from the NSW Department of Primary Industries (Fisheries NSW) Animal Care and Ethics Committee issued in accordance with the National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes. The research project and its associated sampling protocols were done under a scientific research permit (Permit No. P01/0059[A]) issued by the NSW Department of Primary Industries (Fisheries NSW).

The ovaries of non-pregnant females had pre-vitellogenic white follicles (diameter equal to or greater than 5 mm) or pre-ovulatory vitellogenic follicles (diameter equal to or greater than 30 mm) but no eggs were present in the uterus (Huveneers *et al.,* 2007). Females were classified as pregnant if eggs, or macroscopically visible embryos, were present in their uterus (Huveneers *et al.,* 2007; Ellis and Otway, 2011). We split the thirteen pregnant females into three groups based on the month the female was collected, and the developmental stage of her embryos (**Fig. 1**). The month the female was collected is an accurate predictor of pregnancy because *O. ornatus* has a synchronous reproductive cycle with a 10-11-month gestation period; uterine eggs (mean diameter of 48.50 mm) are present in pregnant females in November to December and parturition occurs in September to October (**Fig. 1**; Huveneers *et al.,* 2007; Ellis and Otway, 2011).

Early pregnant females (n=6) were collected between November and early February and were defined by the presence of uterine eggs or embryos with external yolk sacs (1 to 4 months into pregnancy; **Fig. 1**). Mid pregnant females (n = 5) were collected in late February to the end of April and contained embryos with both an external yolk sac and an internal yolk sac (5 to 6 months into pregnancy; **Fig. 1**). Late pregnant females (n = 2) were collected in August to September and contained embryos that had an internal yolk sac but no external yolk sac (8 to 10 months into pregnancy; **Fig. 1**).

2.2 Light Microscopy

Small (1 cm^3) tissue samples were dissected from the paired uteri of each female and immediately fixed in 10 % neutral buffered formalin (NBF) for 24 h. Samples were then rinsed and stored in 70 % ethanol prior to paraffin embedding. Uterine tissues were then dehydrated through a series of alcohol concentrations (70 %-100 %) and infiltrated with paraffin. Paraffin blocks were sectioned at ~4 um thick on a Tissue-Tek Accu-Cut™ microtome (Sakura, Tokyo, Japan). Sections were mounted onto glass slides and dried at 37 °C for a minimum of 12 h. Slides were stained with either Harris' hematoxylin and Putt's eosin or the combined Alcian blue (AB)/Periodic acid-Schiff (PAS) procedure (pH of Alcian blue = 2.5; Kiernan, 2015). Sections were mounted with BDH DPX mounting medium (Fronine Laboratory Supplies, NSW, Australia) and covered with glass coverslips. Slides were viewed with a Zeiss deconvolution microscope (Carl Zeiss Pty. Ltd., Australasia) in brightfield mode and imaged using a Zeiss AxioCam HR digital colour CCD camera and Axio Scan Slide Scanner (Carl Zeiss Pty. Ltd., Australasia).

3 Results

3.1 Uterus of non-pregnant *O. ornatus*

The luminal uterine epithelium of non-pregnant *O. ornatus* consists of stratified squamous epithelial cells (**Fig. 2 A, B, C**). The basement membrane separates the stratified luminal epithelium from the connective tissue layer (**Fig. 2 B, C**). The outer muscular wall is composed of smooth muscle and is enclosed by a serosal layer with a simple squamous mesothelium (**Fig. 2 A**). A network of coiled blood vessels in the uterine wall penetrate the basement membrane and the luminal epithelium (**Fig. 2 B, C**). Blue staining on the surface of the luminal epithelial cells indicates the presence of acidic mucosubstances (Alcian Blue+ material; **Fig. 2 C**). The underlying luminal epithelial cell layers and the basement membrane stain purple, indicating the presence of neutral mucosubstances (PAS+ material; **Fig 2 C**). In some areas of the uterine wall, surface squamous luminal epithelial cells are ciliated (**Fig. 2 D**).

3.2 Early pregnant

The luminal epithelium and lamina propria are longitudinally folded in early pregnant *O. ornatus,* forming uniform uterine ridges that protrude into the lumen (**Fig. 3 A, B, C, D**). Some of the folds project further into the lumen resulting in the formation of uterine villi that are thinner than the uterine ridges (**Fig. 3 B**). Uterine ridges consist of cuboidal epithelial cells and a connective tissue core that contains capillaries (**Fig. 3 B, C, D**). The epithelium differs along the uterine villi; bases of the villi are covered by a stratified cuboidal epithelium, which transitions to a simple squamous epithelium enclosing the tips of the villi (**Fig. 3 B**). There is minimal loose connective tissue between the endothelium of maternal capillaries and the luminal epithelium of the uterine villi

(**Fig. 3 B, D**). Acidic mucosubstances (AB+ material) are present on the surface of the cuboidal epithelial cells (**Fig. 3 C**). Neutral mucosubstances (PAS+ material) are present in the cuboidal epithelial cell layers (**Fig. 3 C**).

3.3 Mid pregnant

The uterine villi in mid pregnant *O. ornatus* are longer and more numerous than the uterine villi in early pregnant females (**Fig. 4 A, B**). Each of the uterine villi contains numerous large blood vessels and smaller capillaries that are closely associated with the enclosing simple squamous luminal epithelium (**Fig. 4 A, B, C, D**). The uterine ridges contain a large loose connective tissue core with capillaries (**Fig. 4 A, B, C**). The blood vessels in the uterine muscle layers of mid pregnant *O. ornatus* (**Fig. 4 A**) are much larger than the blood vessels in the muscular layers of early pregnant *O. ornatus* (**Fig. 3 A**). Neutral mucosubstances (PAS+ material) occur between the cuboidal luminal epithelial cells and in the blood vessels (**Fig. 4 C, D**). Acidic mucosubstances (AB+ material) dominate at the apical surface of the luminal epithelial cells (**Fig. 4 C, D**). Alcian blue staining is more prominent on the surface of the cuboidal epithelial cells than the squamous epithelial cells that enclose the tips of the uterine villi (**Fig. 4 C**).

3.4 Late pregnant

The structure of the uterine ridges and uterine villi of late pregnant *O. ornatus* females (**Fig. 5 A, B, C, D**) is similar to the uterine ridges and uterine villi in mid pregnant *O. ornatus* females. Large blood vessels and capillaries are abundant in each of the villi (**Fig. 5 A, C).** The luminal epithelium covering the capillaries in the uterine ridges consists of cuboidal epithelial cells (**Fig. 5 B, D**). Blue staining at the apical surface of the cuboidal epithelium indicates the presence of acidic mucosubstances (AB+ material), and neutral mucosubstances are shown by the faint purple staining in the cuboidal cells (PAS+ material **Fig. 5 C, D**). The Alcian blue staining is stronger on the luminal surface of the cuboidal epithelial cells than the squamous epithelial cells of the uterine villi (**Fig 5 C**)

4 Discussion

Morphological changes in the uterus of *Orectolobus ornatus* during pregnancy suggest that the uterine wall facilitates embryonic development. Major modifications to the structure of the uterine wall during pregnancy include an increase in uterine surface area via the development of uterine ridges and uterine villi, an increase in uterine vascularity, and a reduction in the uterine tissue that separates the maternal blood vessels from the uterine fluid. Similar uterine changes also occur in other lecithotrophic sharks including spiny dogfish (*Squalus acanthias*) (Jollie and Jollie 1967 Hamlett & Hysell 1998); birdbeak dogfish (*Deania calcea*) (Paiva *et al.,* 2012); Portugese dogfish (*Centroscymnus coelolepis*) (Moura 2011); piked spurdog (*Squalus megalops*) (Braccini *et al.* 2007) and the lecithotrophic common sawshark (*Pristiophorus cirratus*) (Stevens 2002; Hamlett *et al.* 2005). Common changes to the uterus during pregnancy in diverse lecithotrophic shark clades suggests that structural uterine modifications are homoplasies that support shared embryonic demands across these taxa. These likely include mechanisms for embryonic respiration, and potentially waste removal, and transfer of water and minerals. Comparisons with the uterus of gravid oviparous sharks are, however, required to confirm that these uterine changes are not a pleisomorphy that lecithotrophic viviparous sharks have retained from their oviparous ancestors. Currently, the only published data on the structure of the uterus of any oviparous shark are a light micrograph and an electron-micrograph of the stratified uterine epithelium of the blotchy swell shark (*Cephaloscyllium umbratile*) (Otake 1990; Hamlett *et al.,* 2005). Long vascularised uterine villi are absent in *C. umbratile*, and other oviparous chondrichthyans, such as the little skate (*Raja erinacea*) and the clearnose skate (*Raja eglanteria*) (Hamlett and Hysell, 1998; Hamlett *et al.,* 2005). The lack of extensive uterine villus formation in the oviparous chondrichthyans investigated so far supports the hypothesis that vascularised uterine villi are a synapomorphy for viviparity.

4.1 The formation of uterine ridges and uterine villi – site for respiratory gas exchange?

The development of uterine ridges and uterine villi is the most striking difference between the uterus of non-pregnant and pregnant *O. ornatus.* Folding of the uterine luminal surface increases the surface area available for potential exchange between mothers and embryos in lecithotrophic viviparous sharks (Jollie and Jollie 1967; Hamlett & Hysell 1998; Hamlett *et al.* 2005). As pregnancy progresses in *O. ornatus*, sections of the epithelium covering the uterine ridges project into the lumen, and long uterine villi form. These long uterine villi cover the luminal surface of mid and late pregnant *O. ornatus*. Uterine villi that appear to increase in length and density as embryos grow and develop are associated with pregnancy in other lecithotrophic sharks (Needham 1942; Yano, 1995; Hamlett and Hysell, 1998; Girard and Buit 1999;Moura *et al.,* 2011; Paiva *et al.,*2012; Cotton *et al.,* 2015). As in other lecithotrophic sharks, the uterine villi of pregnant *O. ornatus* include capillaries and large blood vessels that distort the luminal uterine epithelium to a thin layer of cells (Jollie and Jollie, 1967; Hamlett *et al.,* 2005). Efficient gas exchange requires an increased surface area, enhanced vascularisation, and a reduction in diffusion distances (Tomita *et al.,* 2016). The formation of vascularised uterine villi during pregnancy achieve all of these requirements and hence likely functions to facilitate respiratory gas exchange (Jollie and Jollie, 1967; Hamlett and Hysell, 1998; Hamlett *et al.,* 2005). At mid-pregnancy, *O. ornatus* females begin to flush the uterus with the external seawater, which may also contribute to embryonic gas exchange (**Fig 1.,** Ellis and Otway, 2011; Tomita *et al.,* 2016).

Since embryonic oxygen demand increases with increasing size of the embryo during pregnancy (Tullis & Peterson, 2000; Tomita *et al.,* 2016), long uterine villi and uterine flushing may both be necessary to meet the respiratory demands of the developing embryos. Additionally, urea concentrations in the uterine fluid significantly decrease when uterine flushing begins in *O. ornatus,* which suggests that exchanging the uterine fluid with the external seawater removes embryonic wastes (Ellis and Otway, 2011). Uterine flushing occurs intermittently during pregnancy in *O. ornatus* (Ellis and Otway, 2011). Hence, the long uterine villi of mid and late pregnant *O. ornatus* may be required to support embryonic respiration and waste removal in the intervals between the introduction of external seawater. Future work should investigate the contribution of the uterine villi to embryonic gas exchange during pregnancy in *O. ornatus* by determining the frequency of uterine flushing, and the oxygen diffusing capacity of the uterine wall (Tomita *et al.,* 2016; Tomita *et al.,* 2017).

4.2 The cuboidal and squamous uterine luminal epithelium

The luminal epithelium of *O. ornatus* has distinct epithelial cell types at different stages of the reproductive cycle. Ciliated epithelial cells are present in some uterine regions of non-pregnant females, but not in any uterine region of pregnant *O. ornatus*. Cilia may contribute to sperm transportation after copulation, or facilitate sperm storage in the female reproductive tract (Hamlett *et al.* 2002; Storrie 2008). In pregnant females, the luminal epithelium consists of either stratified cuboidal cells or a single layer of squamous cells. The squamous epithelial cells overlay capillaries in the luminal tips of the uterine villi, which suggests exchange of respiratory gases via passive diffusion (Jollie and Jollie 1967; Storrie *et al.* 2009). The function of the cuboidal cells that cover the uterine ridges is less clear. Intercellular spaces in the cuboidal epithelial layers may be involved in water and mineral transfer (Storrie *et al.* 2009). In lecithotrophic *S. acanthias,* the uterine epithelium contains carbonic anhydrase, which is an enzyme involved in the interconversion of carbon dioxide and bicarbonate ions (Flugel and Lutjen-Drecoll 1991; Hamlett *et al.* 2005). The activity of carbonic anhydrase in the uterine epithelium acidifies the uterine fluid to a pH of ~6 and removes carbon dioxide from the uterine fluid (Kormanik 1988; Hamlett *et al.,* 2005). Acidification of the uterine environment may function to remove embryonic wastes by converting ammonia (NH_3) to the less toxic ammonium (NH_4^+; Kormanik 1998; Hamlett *et al.,* 2005). Indentations of the luminal surface of the cuboidal uterine epithelium (**Fig. 5 B, D**) suggest an absorptive function, potentially of embryonic wastes during pregnancy. To test the hypothesis that the cuboidal epithelium of pregnant *O. ornatus* is involved in embryonic waste removal, future work should determine if carbonic anhydrases (enzymes that facilitate carbon dioxide exchange) are present in these cuboidal epithelial cells. In *S. acanthias,* the uterine epithelial cells may also have an osmoregulatory function, but direct evidence for this function is lacking (Jollie and Jollie, 1967; Hamlett *et al.,* 2005). Like *O. ornatus, S. acanthias* flushes the uterus with the external seawater throughout the majority of pregnancy (Kormanik 1993; Ellis and Otway 2011). Therefore, an osmoregulatory function is likely for the uterus of both *O. ornatus* and *S. acanthias,* as the uterine wall is responsible for separating uterine fluid that resembles seawater for the majority of pregnancy, from the maternal blood system (Ellis and Otway, 2011). Future work should use molecular techniques to determine if ion transporters involved in osmoregulation are present in the uterus of pregnant *O. ornatus.* The presence of these transporters would provide evidence for the hypothesis that the uterus of *O. ornatus* has an osmoregulatory function during pregnancy.

4.3 Histological features that suggest uterine secretions

The uterine epithelia of primarily lecithotrophic sharks including *P. cirratus*, *D. calcea,* the gulper shark (*Centrophorus granulosus*) and the great lanternshark (*Etmopterus princeps*) potentially secrete inorganic nutrients and small amounts of organic nutrients (incipient histotrophy; Hamlett *et al.* 2005; Paiva *et al.,* 2012; Cotton *et al.,* 2015). Morphological evidence for maternal nutrient contributions during pregnancy is provided by the presence of mucous secretions or uterine secretory cells (Hamlett *et al.,* 2005; Storrie *et al.,* 2009; Paiva *et al.,* 2012). The cuboidal epithelial cells of the uterine ridges of pregnant *O. ornatus* contain cytoplasmic PAS-positive material, and apical Alcian blue-positive material, which suggests the presence of neutral and acidic mucosubstances. It is possible that these mucosubstances are involved in providing *O. ornatus* embryos with maternal nutrients during pregnancy. It is not clear, however, if the uterus is responsible for secreting these mucosubstances during pregnancy because we did not observe any uterine secretory glands or cells in *O. ornatus*. Additionally, neutral and acidic mucosubstances in the uterus of non-pregnant *O. ornatus* and pregnant *O. ornatus* suggests that these mucosubstances may not be specific to pregnancy. Regardless of whether the mother is responsible for secreting mucosubstances, the tight apposition of the Alcian blue stain to the luminal surface of the cuboidal epithelial cells of pregnant *O. ornatus* indicates that there is an acidic mucus layer covering the uterine epithelium. This mucous layer could be involved in lubrication to prevent individual uterine villi from sticking together, or to protect the thinned uterine tissue of pregnant *O. ornatus* from abrasion by developing embryos (Bouchet *et al.,* 1982;

Hamlett *et al.,* 2005; Ellis and Otway, 2011). Protection of the uterus from abrasion by embryos may be particularly important when *O. ornatus* embryos form dermal denticles during pregnancy (Huveneers *et al.*, 2011). Uterine flushing must occur during pregnancy in angelsharks (Squatinidae) because leeches have been reported on developing embryos (Sunye and Vooren, 1997). Hence, the mucous on the surface of the uterine lining of pregnant *O. ornatus* may also protect the mother from infections by parasites or other pathogens that are introduced during uterine flushing.

There is an increase in inorganic matter content between the uterine eggs and neonates of *O. ornatus*, which suggests that inorganic nutrients are provided to developing embryos from non-yolk sources (Huveneers *et al.,* 2011). Inorganic ions including calcium, magnesium and potassium increase in the uterine fluid, and are at the same concentration as the external seawater when uterine flushing begins during pregnancy (Ellis and Otway, 2011). At the time uterine flushing begins, embryos break out of their egg capsules (Ellis and Otway, 2011). Hence, inorganic nutrients could be obtained by embryos from the external seawater rather than by uterine secretions. Molecular techniques and ultrastructural studies using electron microscopy are required to determine if the uterine epithelium of pregnant *O. ornatus* is potentially involved in synthesis and secretion of mucous or nutrients during pregnancy.

5 Conclusion

The major histological changes associated with pregnancy in *O. ornatus* suggest that the uterus may be specialised for respiratory gas exchange, waste removal, and transfer of water and minerals, as predicted by our hypothesis. Passive diffusion of respiratory gases is likely facilitated by the formation of vascularised uterine villi, while the cuboidal cells of the uterine ridges may be involved in active transport of ions for osmoregulation, waste removal and water transfer. Future research should use molecular techniques to test the physiological hypotheses raised by our results. Uterine flushing during pregnancy in *O. ornatus* likely also facilitates embryonic respiration, waste removal and inorganic nutrient transfer (Ellis and Otway, 2011). The process of flushing the uterus with the external seawater may expose maternal and fetal tissues to bacteria and parasites. Our results show that when uterine flushing occurs during pregnancy, thin uterine tissues separate large maternal blood vessels from the uterine seawater environment. Hence, pregnant *O. ornatus* females may be at risk of infection during uterine flushing. The acidic mucous layer in the uterine epithelium of *O. ornatus* potentially has a role in protecting pregnant females from pathogens. Future research should determine if this acidic mucous layer has antimicrobial properties, and if the mucous layer occurs on the luminal surface of the uterine epithelium of other sharks that flush their uterus with the external seawater during pregnancy. If an antimicrobial mucous layer is common to all sharks that rely on uterine flushing during pregnancy, this would suggest that the mucous layer is required to protect the pregnant female from infection. Additionally, comparing our results to the uterine changes during gravidity in closely related oviparous carpet sharks (Orectolobiformes) would reveal if this mucous layer is specific to viviparity.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

The authors thank Debbie Pepperall for tissue processing and preparing the histological slides used in this study. The authors acknowledge the facilities and technical assistance at the Bosch Institute at the University of Sydney. This research was supported by Australian Research Council Discovery Project Grant DP180103370 to Camilla M. Whittington, Michael B. Thompson, Christopher R. Murphy and Colin A. Simpfendorfer, and a L'Oreal–UNESCO for Women in Science Fellowship to Camilla M. Whittington. Alice L. Buddle is supported by an Australian Research Training Program Scholarship and the Joyce W Vickery Scientific Research Fund.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure legends

Fig. 1 *Orectolobus ornatus* timeline of embryonic development. Three stages of pregnancy were examined in this study: early (November-January), mid (February- May), late (August-September). Uterine flushing begins at the end of early pregnancy, 3-4 months into the gestation period. Grey indicates yolk sac, black indicates embryonic mass.

Fig. 2 Light micrographs of histological sections of the uterus of non-pregnant *Orectolobus ornatus* females. Uterine lumen (L). **A**) The uterine wall consists of a highly stratified luminal epithelium (LE), a connective tissue layer (CT), smooth muscle layers (M) and an outer serosa (S). Blood vessels (arrows) occur in the connective tissue and muscle layers. **B, C**) A network of coiled blood vessels (arrows) in the connective tissue layer penetrate the basement membrane (BM) and the luminal epithelial cell layers (LE). **C**) Acidic mucosubstances (blue) occur on the apical surface of the luminal epithelial cells. Neutral mucosubstances (purple) are concentrated in the basement membrane of the luminal epithelium. **D**) Cilia appear on some squamous luminal epithelial cells (arrowhead). A, B, D sections stained with hematoxylin and eosin; C section stained with Periodic acid-Schiff and Alcian blue.

Fig. 3 Light micrographs of histological sections of the uterus of early pregnant *Orectolobus ornatus* females. Uterine lumen (L). **A**) Longitudinal folds in the luminal epithelium (LE) and underlying lamina propria result in the formation of uterine ridges. Large blood vessels (arrows) occur in the smooth muscle layer (M), connective tissue (CT) layer. **B**) Thin uterine villi (v) contain numerous small capillaries (arrows). **C**) Neutral mucosubstances (purple) are present in the luminal epithelium (LE). Acidic mucosubstances (blue) occur on the apical surface of luminal epithelial cells (LE). **D**) Uterine ridges consist of a stratified cuboidal epithelial cell with a connective tissue core. A, B, D sections stained with hematoxylin and eosin; C section stained with periodic acid-Schiff and Alcian blue.

Fig 4 Light micrographs of histological sections of the uterus of mid pregnant *Orectolobus ornatus* females. Uterine lumen (L). **A**) The luminal epithelium (LE) is irregularly folded and blood vessels in the connective tissue layer are close to the surface of the LE. Long uterine villi (v) with numerous blood vessels (arrows) project into the lumen (L). Large blood vessels are present in the smooth muscle layer (M). **B**) Uterine villi consist of a thin simple squamous luminal epithelium (LE), a connective tissue core and blood vessels (arrows). **C**) Acidic mucosubstances (blue) are more prominent on the luminal surface of the epithelial cells that enclose the base of the uterine villi (v) than the luminal tips. **D**) Neutral mucosubstances (purple) are present in the luminal epithelial cell layer. Acidic mucosubstances (blue) are present on the apical surface of the luminal epithelial cells (arrowhead). A, B sections stained with hematoxylin and eosin; C, D sections stained with periodic acid-Schiff and Alcian blue.

Fig. 5 Light micrographs of histological sections of the uterus of late pregnant *Orectolobus ornatus* females. Uterine lumen (L). **A)** Long uterine villi (v) containing large blood vessels (arrows) project into the lumen. Folding of the luminal epithelium (LE) creates branched uterine ridges that contain many blood vessels (arrows). Blood vessels (arrows) are present in the connective tissue (CT) layer and smooth muscle layer (M). **B**) The cuboidal epithelial cells are indented creating a wavy appearance along the luminal surface (*). The underlying connective tissue layer contains numerous blood vessels (arrows). **C**) Acidic mucosubstances (blue) are prominent on the apical luminal surface of the cuboidal epithelial cells (LE). The squamous epithelial cells enclosing the luminal tips of the uterine villi (V) are lightly stained with blue. **D**) Cuboidal epithelial cells on the luminal surface of the uterine ridges stain positively for acidic mucosubstances (blue, arrowhead). Minimal purple staining in the cytoplasm of the cuboidal epithelial cells indicates the presence of neutral mucosubstances. A, B, sections stained with hematoxylin and eosin; C, D sections stained with periodic acid-Schiff and Alcian blue.

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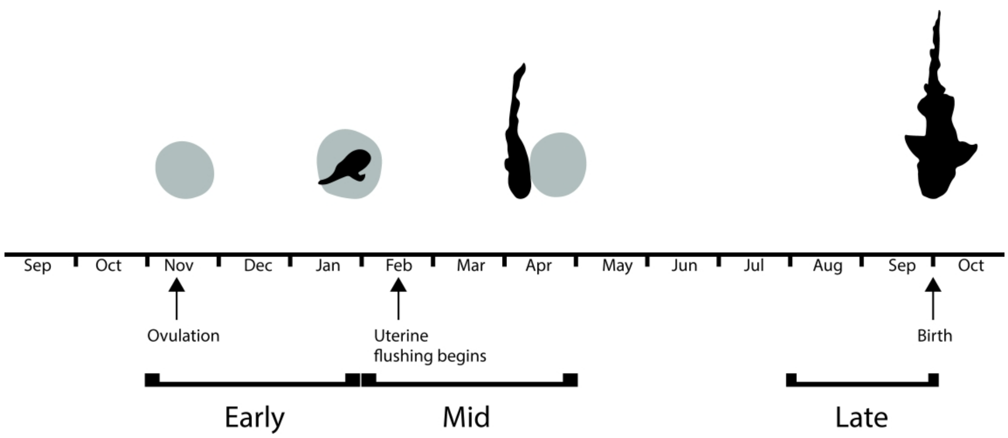

Fig. 1 Orectolobus ornatus timeline of embryonic development. Three stages of pregnancy were examined in this study: early (November-January), mid (February- May), late (August-September). Uterine flushing begins at the end of early pregnancy, 3-4 months into the gestation period. Grey indicates yolk sac, black indicates embryonic mass.

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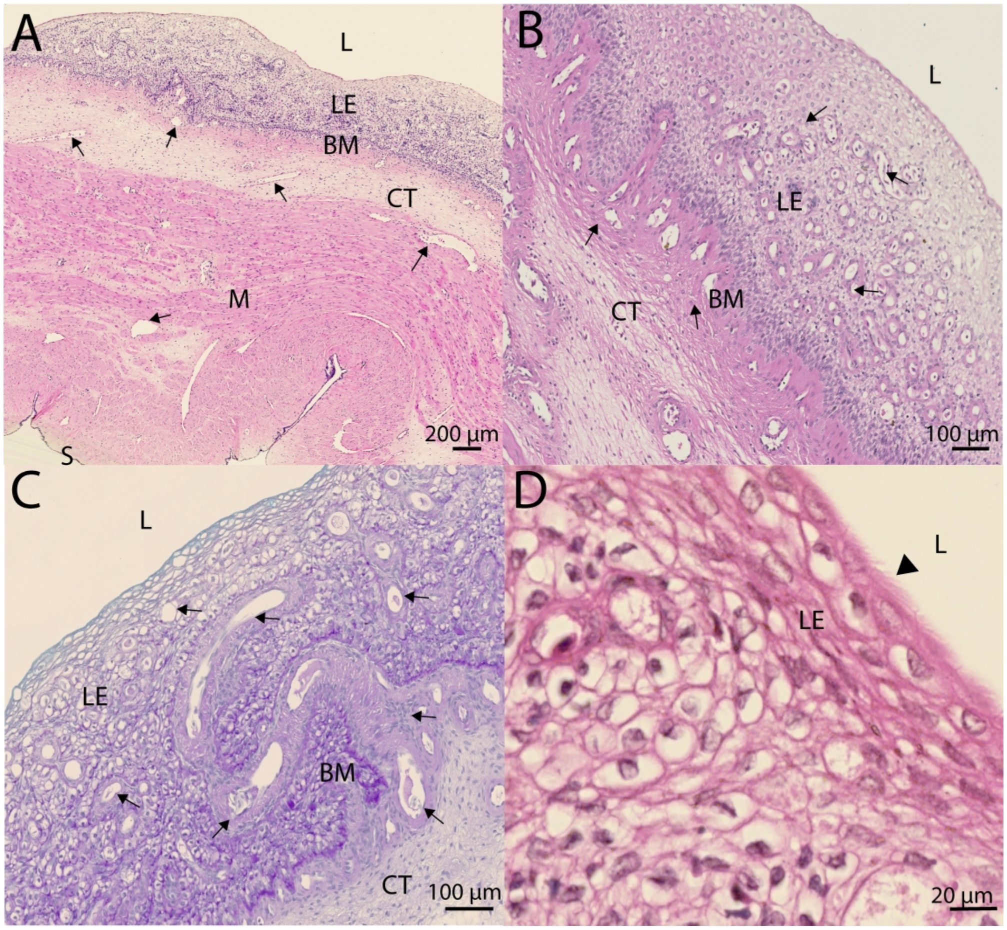

Fig. 2 Light micrographs of histological sections of the uterus of non-pregnant Orectolobus ornatus females. Uterine lumen (L). A) The uterine wall consists of a highly stratified luminal epithelium (LE), a connective tissue layer (CT), smooth muscle layers (M) and an outer serosa (S). Blood vessels (arrows) occur in the connective tissue and muscle layers. B, C) A network of coiled blood vessels (arrows) in the connective tissue layer penetrate the basement membrane (BM) and the luminal epithelial cell layers (LE). C) Acidic mucosubstances (blue) occur on the apical surface of the luminal epithelial cells. Neutral mucosubstances (purple) are concentrated in the basement membrane of the luminal epithelium. D) Cilia appear on some squamous luminal epithelial cells (arrowhead). A, B, D sections stained with hematoxylin and eosin; C section stained with Periodic acid-Schiff and Alcian blue.

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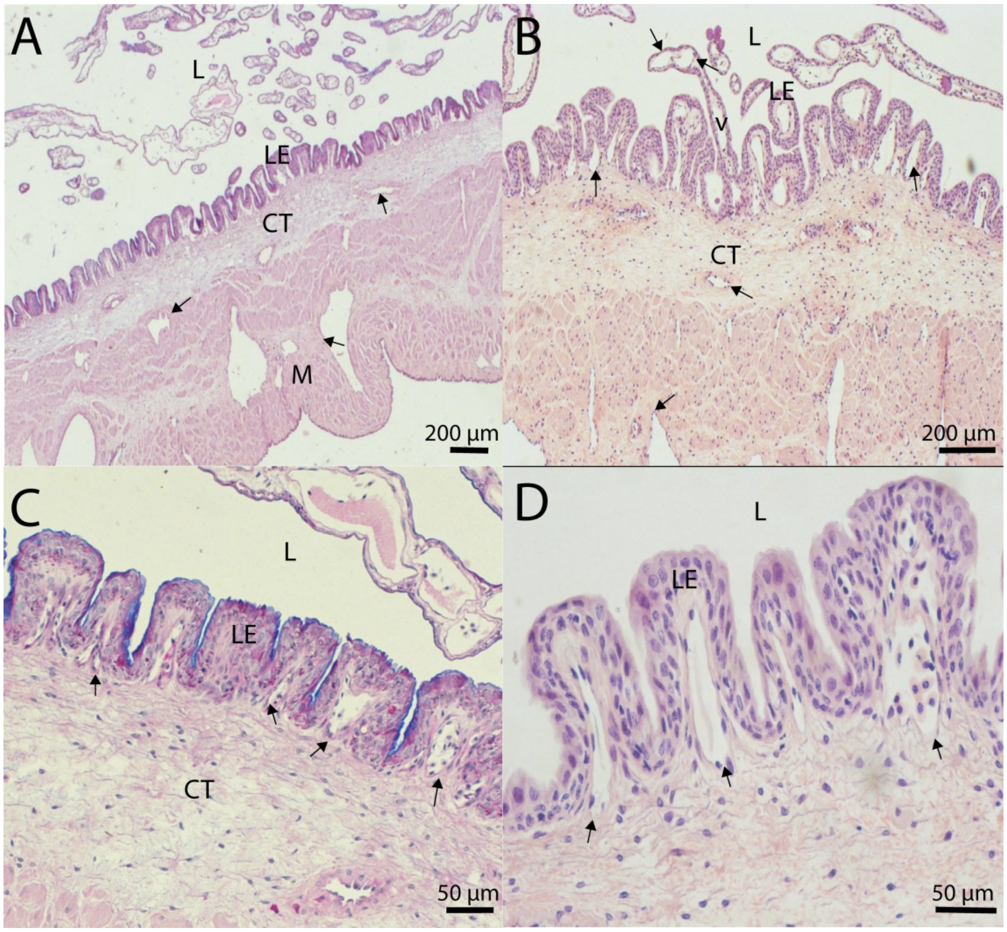

Fig. 3 Light micrographs of histological sections of the uterus of early pregnant Orectolobus ornatus females. Uterine lumen (L). A) Longitudinal folds in the luminal epithelium (LE) and underlying lamina propria result in the formation of uterine ridges. Large blood vessels (arrows) occur in the smooth muscle layer (M), connective tissue (CT) layer. B) Thin uterine villi (v) contain numerous small capillaries (arrows). C) Neutral mucosubstances (purple) are present in the luminal epithelium (LE). Acidic mucosubstances (blue) occur on the apical surface of luminal epithelial cells (LE). D) Uterine ridges consist of a stratified cuboidal epithelial cell with a connective tissue core. A, B, D sections stained with hematoxylin and eosin; C section stained with periodic acid-Schiff and Alcian blue.

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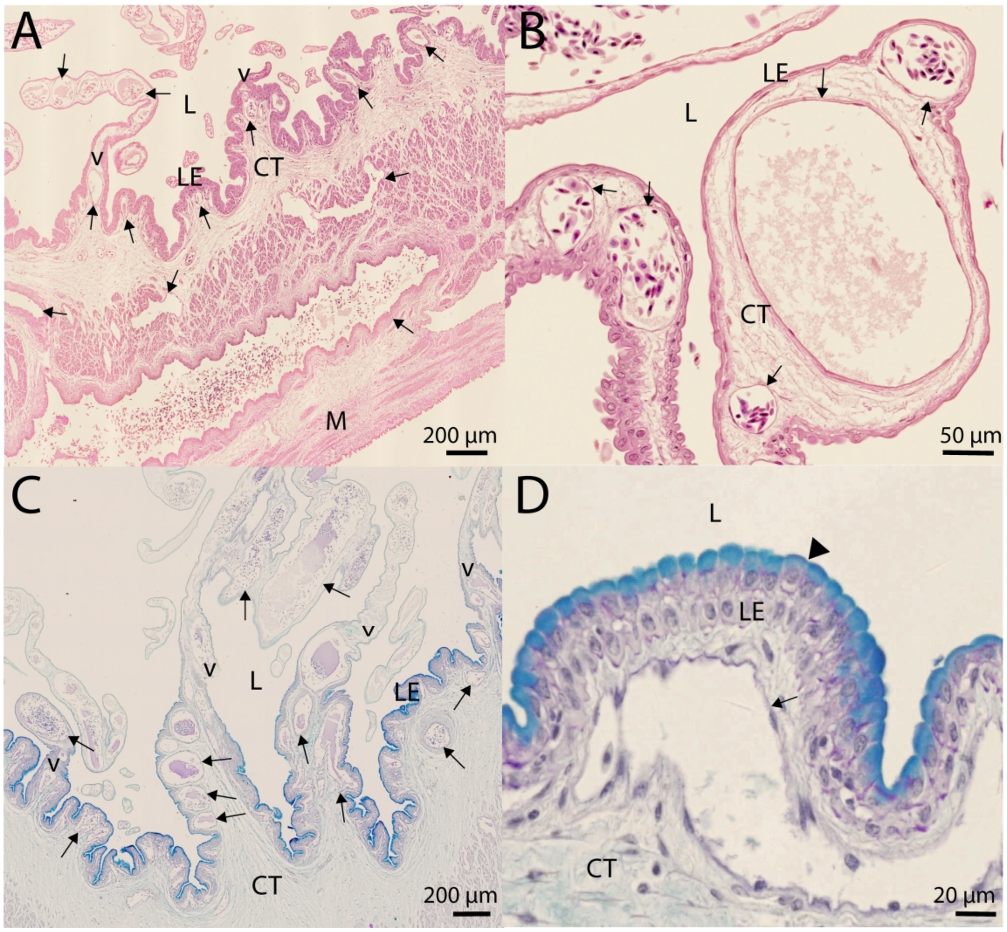

Fig 4 Light micrographs of histological sections of the uterus of mid pregnant Orectolobus ornatus females. Uterine lumen (L). A) The luminal epithelium (LE) is irregularly folded and blood vessels in the connective tissue layer are close to the surface of the LE. Long uterine villi (v) with numerous blood vessels (arrows) project into the lumen (L). Large blood vessels are present in the smooth muscle layer (M). B) Uterine villi consist of a thin simple squamous luminal epithelium (LE), a connective tissue core and blood vessels (arrows). C) Acidic mucosubstances (blue) are more prominent on the luminal surface of the epithelial cells that enclose the base of the uterine villi (v) than the luminal tips. D) Neutral mucosubstances (purple) are present in the luminal epithelial cell layer. Acidic mucosubstances (blue) are present on the apical surface of the luminal epithelial cells (arrowhead). A, B sections stained with hematoxylin and eosin; C, D sections stained with periodic acid-Schiff and Alcian blue.

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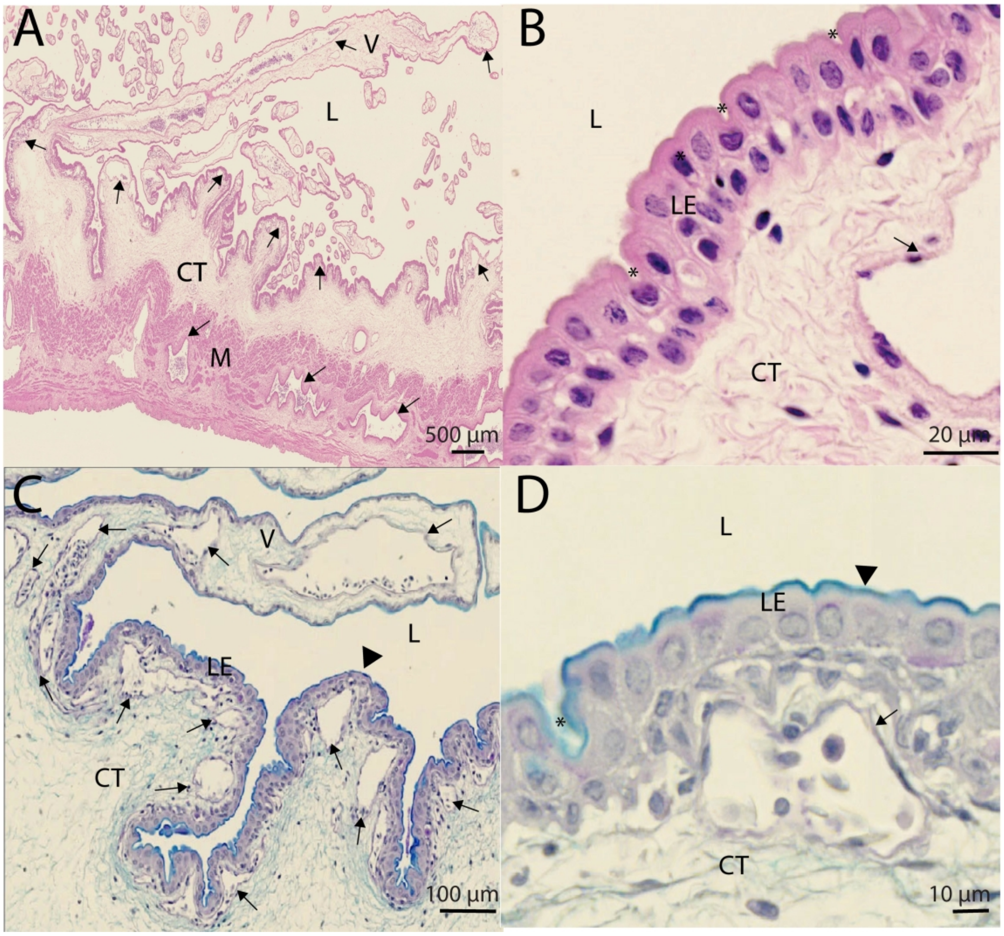

Fig. 5 Light micrographs of histological sections of the uterus of late pregnant Orectolobus ornatus females. Uterine lumen (L). A) Long uterine villi (v) containing large blood vessels (arrows) project into the lumen. Folding of the luminal epithelium (LE) creates branched uterine ridges that contain many blood vessels (arrows). Blood vessels (arrows) are present in the connective tissue (CT) layer and smooth muscle layer (M). B) The cuboidal epithelial cells are indented creating a wavy appearance along the luminal surface (*). The underlying connective tissue layer contains numerous blood vessels (arrows). C) Acidic mucosubstances (blue) are prominent on the apical luminal surface of the cuboidal epithelial cells (LE). The squamous epithelial cells enclosing the luminal tips of the uterine villi (V) are lightly stained with blue. D) Cuboidal epithelial cells on the luminal surface of the uterine ridges stain positively for acidic mucosubstances (blue, arrowhead). Minimal purple staining in the cytoplasm of the cuboidal epithelial cells indicates the presence of neutral mucosubstances. A, B, sections stained with hematoxylin and eosin; C, D sections stained with periodic acid-Schiff and Alcian blue.

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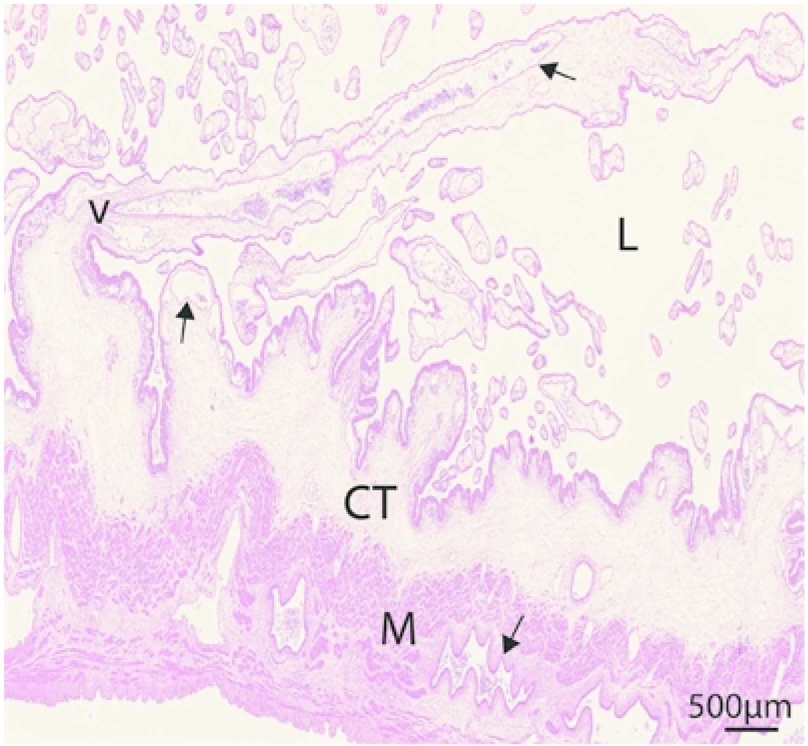
V
L
CT
M
500µm

Graphical abstract text

The uterus of the viviparous dwarf ornate wobbegong (*Orectolobus ornatus*) undergoes a morphological transformation during pregnancy. This image shows the uterine wall of a late pregnant female that contains embryos just prior to birth. The extensive vascularisation by the increase in blood vessels in the long uterine villi (v), uterine connective tissue and muscle layers, likely support embryonic gas exchange, waste removal, water balance and mineral transfer.