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3 **Structural changes to the brood pouch of male pregnant seahorses (*Hippocampus***
4 ***abdominalis*) facilitate exchange between father and embryos**

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15

16 **Abstract**

17 Embryonic growth and development require efficient respiratory gas exchange. Internal
18 incubation of developing young thus presents a significant physiological challenge, because
19 respiratory gas diffusion to embryos is impeded by the additional barrier of parental tissue
20 between the embryo and the environment. Therefore, live-bearing species exhibit a variety of
21 adaptations facilitating respiratory gas exchange between the parent (usually the mother) and
22 embryos. Syngnathid fishes are the only vertebrates to exhibit male pregnancy, allowing
23 comparative studies of the biology and evolution of internal incubation of embryos,
24 independent of the female reproductive tract. Here, we examine the fleshy, sealed, seahorse
25 brood pouch, and provide the first quantification of structural changes to this gestational
26 organ across pregnancy. We describe dramatic remodelling of gestational tissues during
27 seahorse pregnancy, which produces an increased surface area and expansion of the vascular
28 bed of the brood pouch that likely facilitates respiratory gas exchange. These changes mirror
29 the remodelling of gestational tissue in viviparous amniotes and elasmobranchs, and provide
30 further evidence of the convergence of adaptations to support pregnancy in live-bearing
31 animals.

32 **Key words**

33 Angiogenesis, embryo incubation, parental care, respiratory gas exchange, vascularity,
34 syngnathid

35 **Introduction**

36 Oviparity (egg-laying) is the ancestral reproductive mode from which viviparity (live birth)
37 has evolved; viviparity has originated at least 150 times in vertebrates [1], and many more
38 times in invertebrates [2]. While all oviparous species deposit incompletely developed eggs
39 that complete development outside the female reproductive tract, some oviparous parents also
40 provide parental care, which can include incubation of embryos on or in the body of the
41 parent (brooding) [2]. In contrast, viviparous animals incubate developing embryos within the
42 reproductive tract of the mother until development is complete, whereupon live young are
43 born [reviewed in 1]. Internal incubation of embryos via either oviparous brooding or
44 viviparity results in physiological challenges compared to allowing eggs to develop
45 externally, particularly in ensuring a route of respiratory gas exchange to developing embryos

46 [3, 4]. Here we examine potential mechanisms of respiratory gas supply to developing
47 embryos in a male brooding species, the pot-bellied seahorse *Hippocampus abdominalis*, and
48 compare these with the mechanisms supplying respiratory gases to embryos in female-
49 pregnant vertebrates.

50 Seahorses and their relatives (syngnathids, which includes pipefishes and seadragons) are the
51 only vertebrates to exhibit male pregnancy; these fishes are emerging models for comparative
52 studies of pregnancy because they incubate embryos internally in structures other than the
53 female reproductive tract [4, 5]. Male syngnathids gestate embryos in a vascularised ventral
54 brood pouch [6, 7], which varies in complexity from simple embryo attachment sites (e.g.
55 Type 1 pouches in *Nerophis* spp.) to open membranous egg compartments (Type 2), semi-
56 enclosed pouches (Type 3), pouches enclosed by thin folds (Type 4), and pouches opening
57 via a pore (Type 5) [4, 8-11]. Pouch types 1-3 are fully or partially open to the external
58 environment, while pouch types 4 and 5 brood embryos internally. We thus predict different
59 physiological and structural modifications in open versus closed brood pouches because of
60 the physiological challenges in incubating embryos internally [4]. In this study, we focus on
61 the thick, fleshy seahorse (*Hippocampus* spp.) brood pouch, which is the most complex of all
62 syngnathid pouches. This pouch develops in juvenile seahorses via ventrolateral projections
63 of the dermis at the base of the tail [12], and has a single pore-like opening that is closed
64 during pregnancy.

65 Embryos require constant exchange of respiratory gases, the demand for which increases as
66 they develop [e.g. 13, 14]. In species depositing eggs externally, respiratory gases are
67 exchanged between the embryo and the external environment through the permeable eggshell
68 or capsule [reviewed in 15]. In contrast, internal incubation, as in seahorses, presents a
69 physiological challenge to respiratory gas supply, because parental tissues present a barrier to
70 gas exchange and gases diffuse slowly through animal tissues [16]. In particular, because
71 oxygen is less soluble in aqueous solutions than carbon dioxide and thus diffuses at a slower
72 rate, hypoxia is likely to be a major limiting factor in internal embryonic development [17].
73 Thus, one of the major constraints on the evolution of internal embryo brooding and
74 viviparity is the capacity of the parent to meet the increasing demand for embryonic
75 respiratory gas exchange during development [17, 18]. The fleshy, fully enclosed pouch of
76 seahorses presents a significant barrier to respiratory gas exchange, and we predict
77 adaptations to overcome this physiological challenge [4].

78 A variety of mechanisms mediate increased respiratory gas exchange between gestational
79 tissues and embryos in other internal brooders and viviparous species. In fish, these
80 adaptations include increased surface area of gestational tissues [e.g. ornate wobbegong
81 [19]], increased oxygen carrying capacity of embryonic hemoglobin [e.g. surfperch [20]],
82 close association between embryonic and maternal tissues [e.g. eelpout [21]], flushing of
83 uterine fluid over embryonic gills via buccal pumping [e.g., manta ray [22]], and placentae
84 [which also facilitate nutrient transport in some species [23-26]]. A placenta is defined as
85 “any intimate apposition or fusion of the fetal organs to the maternal (or paternal) tissues for
86 physiological exchange” [27]. Placentae develop via remodelling of the gestational
87 epithelium and surrounding tissues during pregnancy, resulting in a large surface area for
88 exchange, and dense capillary networks. In amniotes, these aspects of placental development
89 in the uterus appear to be mediated by angiogenesis (proliferation of the vascular bed) and
90 tissue remodelling genes [28-35].

91 Vascularised placentae are present in the pouch of pregnant male syngnathids [6, 7, 36-38],
92 and expression of vasculogenic and remodelling genes increase in the placental tissue during
93 pregnancy [39]. These facts lead us to speculate that seahorses experience an expansion of
94 the vascular bed of the brood pouch during pregnancy to facilitate exchange between father
95 and embryos [4]. Therefore, we aimed to determine whether structural changes to the
96 seahorse placenta during pregnancy might contribute to increased respiratory gas exchange
97 between father and embryos. We tested the following hypotheses: 1) the surface area of
98 gestational tissues contacting the pouch lumen increases during pregnancy; 2) the diffusion
99 distance from paternal capillaries to the pouch lumen decreases during pregnancy; and 3) the
100 density of the vascular bed of the brood pouch increases during pregnancy.

101 **Methods**

102 **Animal husbandry**

103 Reproductively mature male and female pot-bellied seahorses (*H. abdominalis*) were sourced
104 from a captive bred population (Seahorse Australia, Tasmania, Australia) and maintained
105 under previously described laboratory conditions [40]. All animal experimentation was
106 undertaken with permission from the Animal Ethics Committee of the University of Sydney
107 (2018/1302). Animals mated freely in a mixed-sex breeding tank for up to 72 h before being

108 returned to single-sex tanks. Pregnancy was assessed on the basis of courtship behaviours
109 [41].

110 **Tissue collection**

111 Animals of known reproductive stage were euthanized by decapitation and immediate
112 pithing. The targeted reproductive time points were non-pregnant, early pregnancy [embryos
113 up to ~25 % of the way through development, i.e. eye development stages *sensu* [42]], mid-
114 pregnancy (embryos at frontal jaw stages; 65% of the way through development), and late
115 pregnancy (embryos with protruding snouts in the last third of development). Sample sizes
116 were seven non-pregnant, two early pregnant, three mid-pregnant, five late pregnant and one
117 post-partum adult (sampled within 24 h of birth). The brood pouch was excised from the
118 body in a single piece before being cut longitudinally along the midline from the opening of
119 the pouch to the caudal extremity, and then each longitudinal section was cut approximately
120 in half in transverse orientation. This procedure yielded four quadrants: anterior left, anterior
121 right, posterior left, and posterior right. Measurements were taken from every quadrant to
122 ensure that the results were reflective of the morphology of the entire pouch. Some embryos
123 remained adhering to the pouch tissue during dissection; to avoid distorting the tissues, these
124 embryos were not removed. Each quadrant was fixed in 10 % neutral buffered formalin for
125 24 h, rinsed three times for 10 min each in 70 % ethanol, and then stored in 70 % ethanol.

126 **Histology**

127 The quadrants of brood pouch tissue were dehydrated to 100 % ethanol and embedded in
128 paraffin. The paraffin blocks were serially sectioned at 7 μm using a HM 325 Rotary
129 Microtome (ThermoFisher Scientific). For each quadrant, at least 12 sections of tissue were
130 cut. Sections were mounted on gelatin-coated glass slides and stained with haematoxylin and
131 eosin (H&E) using a standard protocol [43]. Each section was imaged in its entirety using a
132 Zeiss AxioScan.Z1 microscope (Carl Zeiss Australasia). All measurements and image
133 analyses were done by an operator who was blind to pregnancy stage (JSD).

134 **Image analysis**

135 The brood pouch consists of several layers [12]: a single luminal epithelial layer (which faces
136 the lumen of the pouch where the embryos are located), a diffuse inner layer [which stains
137 very pale pink with H&E; this is sometimes referred to as a “pseudeoplacenta” but is actually
138 a functional placenta [4]], a more compact dermis (a dark pink *stratum compactum* layer and

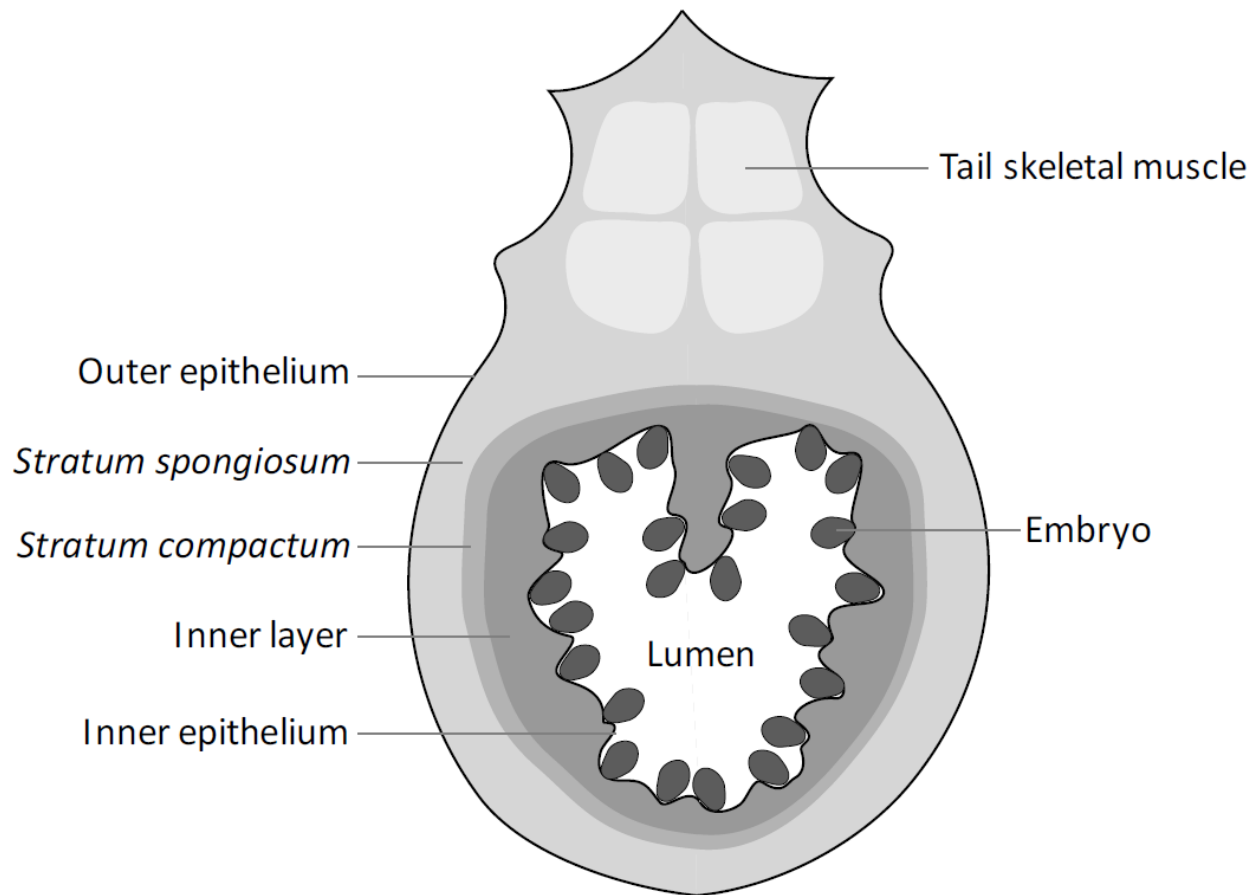
139 a mid-pink *stratum spongiosum* layer when stained with H&E), and an outer epithelial layer
140 which faces the external seawater (Figure 1). To address Hypothesis 1 (that the surface area
141 of gestational tissues contacting the pouch lumen increases during pregnancy), we measured
142 the tortuosity ratio of the luminal epithelial layer. This ratio describes the degree of bends,
143 twists and turns of the inner epithelial layer, and was measured by calculating the ratio of the
144 length of the inner epithelial layer in a single histological section of brood pouch to the
145 straight-line distance between the ‘start’ and the ‘end’ of the epithelial layer in that section
146 (i.e. tortuosity ratio has no unit). Tortuosity ratio was measured in entire sections from each
147 quadrant. We measured at least ten sections per animal, spread across the four quadrants. We
148 have a range of 10-16 sections per animal, all with the entire luminal epithelium intact,
149 allowing tortuosity measurements to be calculated. These sections were imaged and
150 measured at 20 x magnification using Image J Fiji image analysis software (Madison, USA).

151 To address Hypotheses 2-3, we used subsets of the brood pouch sections at 10 x
152 magnification to measure characteristics of the capillaries in the inner layer of the brood
153 pouch. For each animal, ten images (field of view of 200 mm^2) of the inner layer of the brood
154 pouch, including the luminal epithelium, were captured randomly from across the four
155 quadrants. In each field of view, capillaries were counted and measurements were taken from
156 every capillary. Analyses were done using Image J Fiji image analysis software (Madison,
157 USA). Capillaries were distinguished from veins and arteries by morphological differences:
158 capillaries have a single layer of thin endothelium lining the vessel, whereas arteries have
159 thick walls composed of three layers, and veins have thin walls composed of three layers
160 [44].

161 We measured the minimum diffusion distance from each capillary in the inner layer of the
162 brood pouch to the pouch lumen from these images to address Hypothesis 2 (that the
163 diffusion distance from paternal capillaries to the pouch lumen decreases during pregnancy).
164 The minimum diffusion distance (μm) was determined as the shortest distance from the
165 apical surface of the endothelial cells of the capillary to the apical surface of the inner
166 epithelial layer of the pouch, which represents the total distance over which respiratory gases
167 must diffuse.

168 We measured the capillary density in the inner layer of the brood pouch to address
169 Hypothesis 3 (that the density of the vascular bed of the brood pouch increases during
170 pregnancy). Capillary density was calculated as the number of capillaries per mm^2 of brood

171 pouch inner layer. Capillaries were marked and counted using the cell counter plugin feature
172 of Fiji image analysis software (Madison, USA).



173

174 *Figure 1. Schematic of a transverse section (not to scale) through the tail and brood pouch of a pregnant male seahorse*
175 *(Hippocampus spp.) (dorsal surface at top of figure). Bony plates and vertebrae not shown. Modified from Whittington and*
176 *Friesen [4], used with permission.*

177 **Statistical analysis**

178 We compared luminal epithelial tortuosity ratio, capillary diffusion distance to the pouch
179 lumen, and pouch capillary density across pregnancy stages using separate analyses of
180 variance (ANOVA) in PROC MIXED in SAS (University Edition, Cary, NC). Each analysis
181 included multiple datapoints from every seahorse in the study, so we included paternal ID in
182 the models as a random effect to account for pseudoreplication. Pregnancy stage was the only
183 independent variable in every model, and was set as a fixed effect. We followed these
184 analyses with post-hoc Tukey pairwise tests between each pair of pregnancy stages. All
185 means are reported as ± 1 SE and we used an α of 0.05 for all statistical interpretation.

186 **Results**

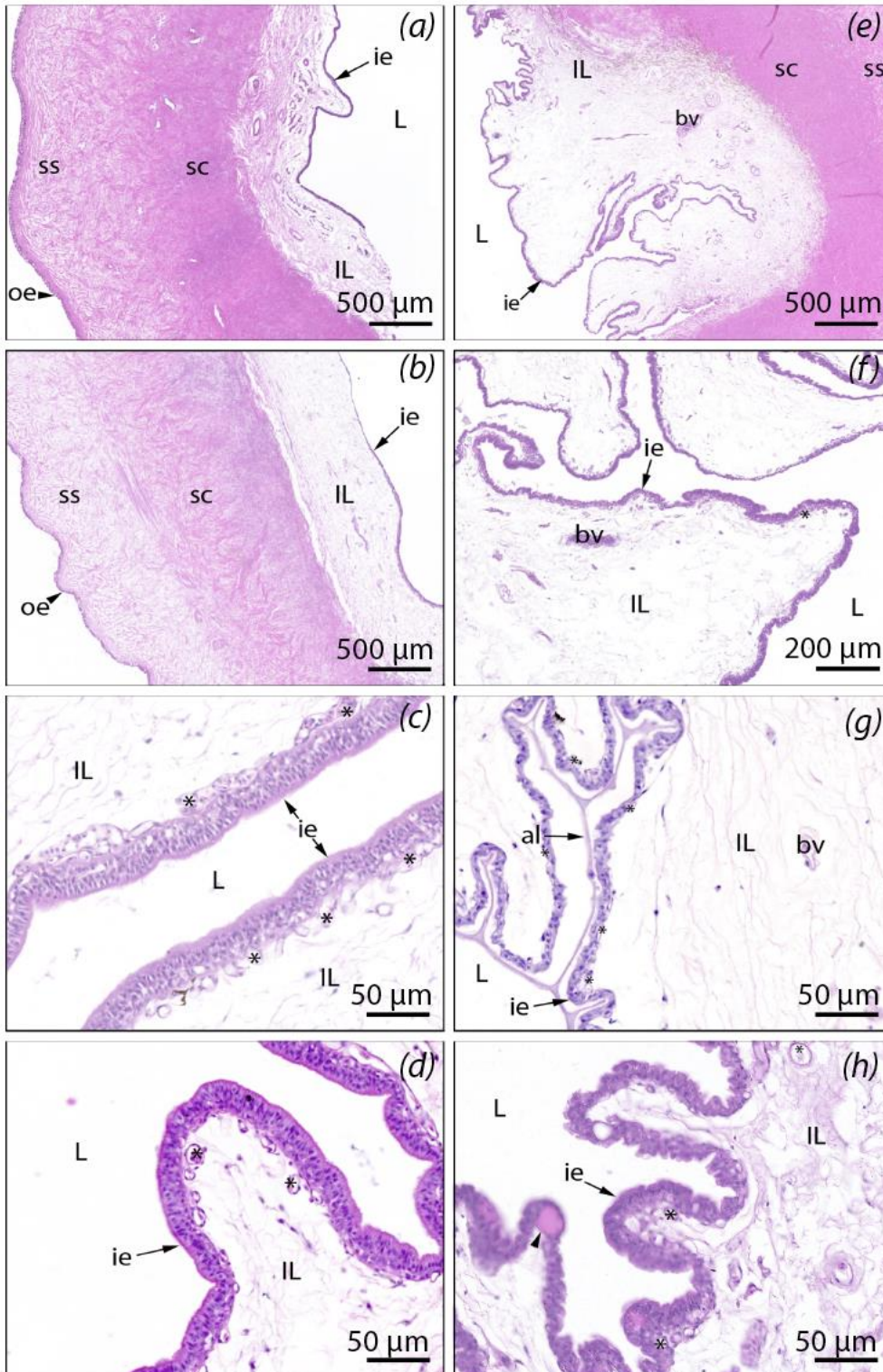
187 **Gross morphology of brood pouch tissues**

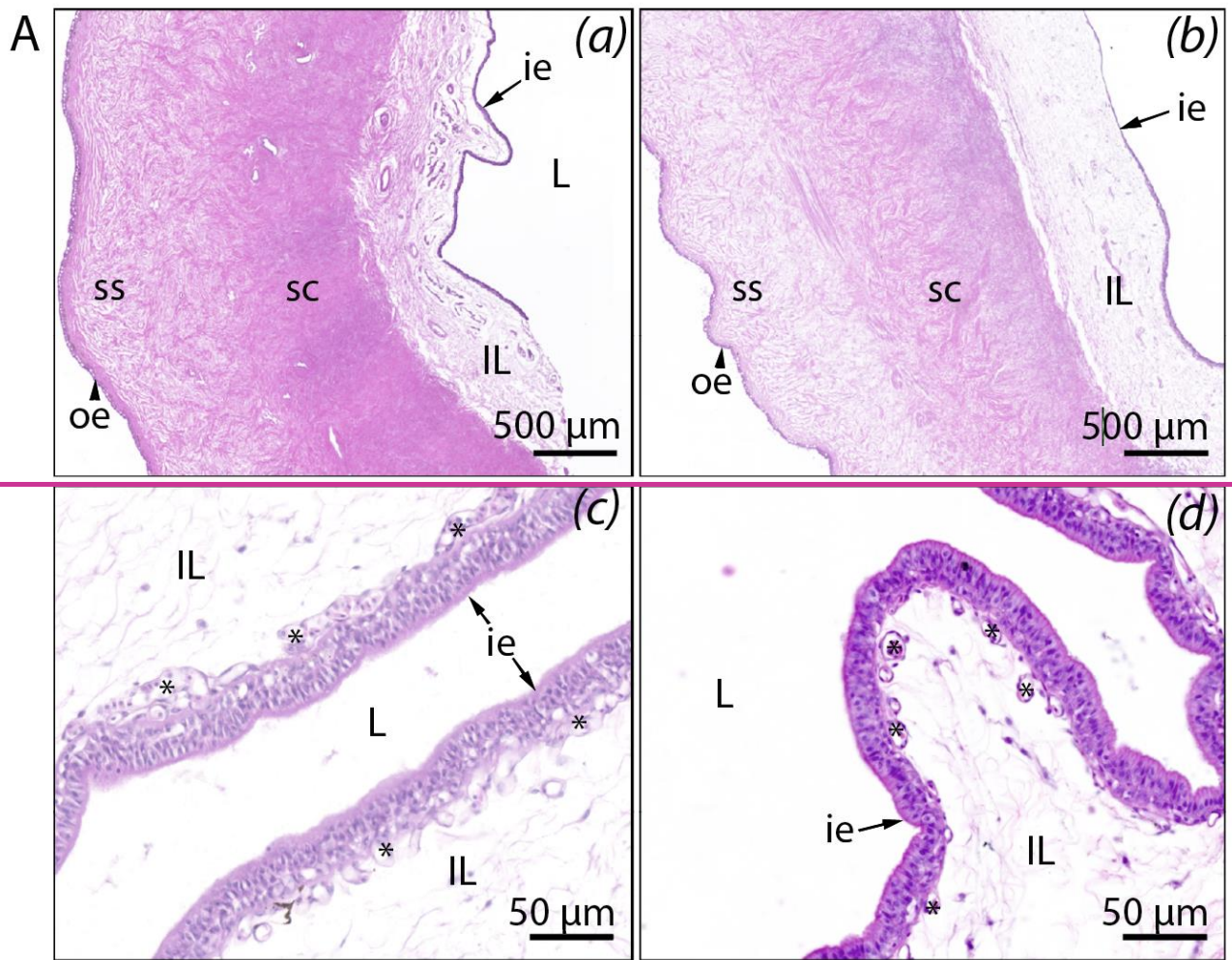
188 In non-pregnant male pouches, the luminal epithelium is composed of pseudostratified
189 columnar cells. The inner layer and the dermis of the brood pouch are more compact than
190 during pregnancy with fewer capillaries present underlying the epithelium (Figure 2A a-d). In
191 early pregnancy, the inner epithelium of the brood pouch is folded with capillaries and larger
192 blood vessels present in the inner layer. The dermis layer is smaller in proportion to the inner
193 layer. There is a thin acellular layer-like structure, probably mucus, present during pregnancy
194 that is closely apposed to the luminal epithelium and embryos. There are no visible cellular
195 components present in this layer (Figure 2 c-h B a-d). During mid-pregnancy the inner
196 epithelium is tortuous, with capillaries present throughout the underlying inner layer (Figure
197 32C a-d). In late pregnancy, the luminal epithelium is very tortuous, often folding back on
198 itself. Both the inner layer and the dermis are more diffuse with a greater number of
199 capillaries present throughout than during earlier stages of pregnancy. Many of these
200 capillaries are located directly underlying the luminal epithelium with blood plasma (dark
201 pink stain) filled luminaens (Figure 32D e-h a-d). During early, mid and late pregnancy there
202 are large eosinophilic cells occasionally underlying the epithelium. After parturition, the
203 inner epithelium is made up of several layers of cells with the luminal cells having rounded
204 apices, characteristic of epithelium that may be degrading and sloughing off. There are
205 capillaries and blood vessels throughout the inner tissue layer (Figure 42E a-d).

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Non-pregnant

Early pregnant





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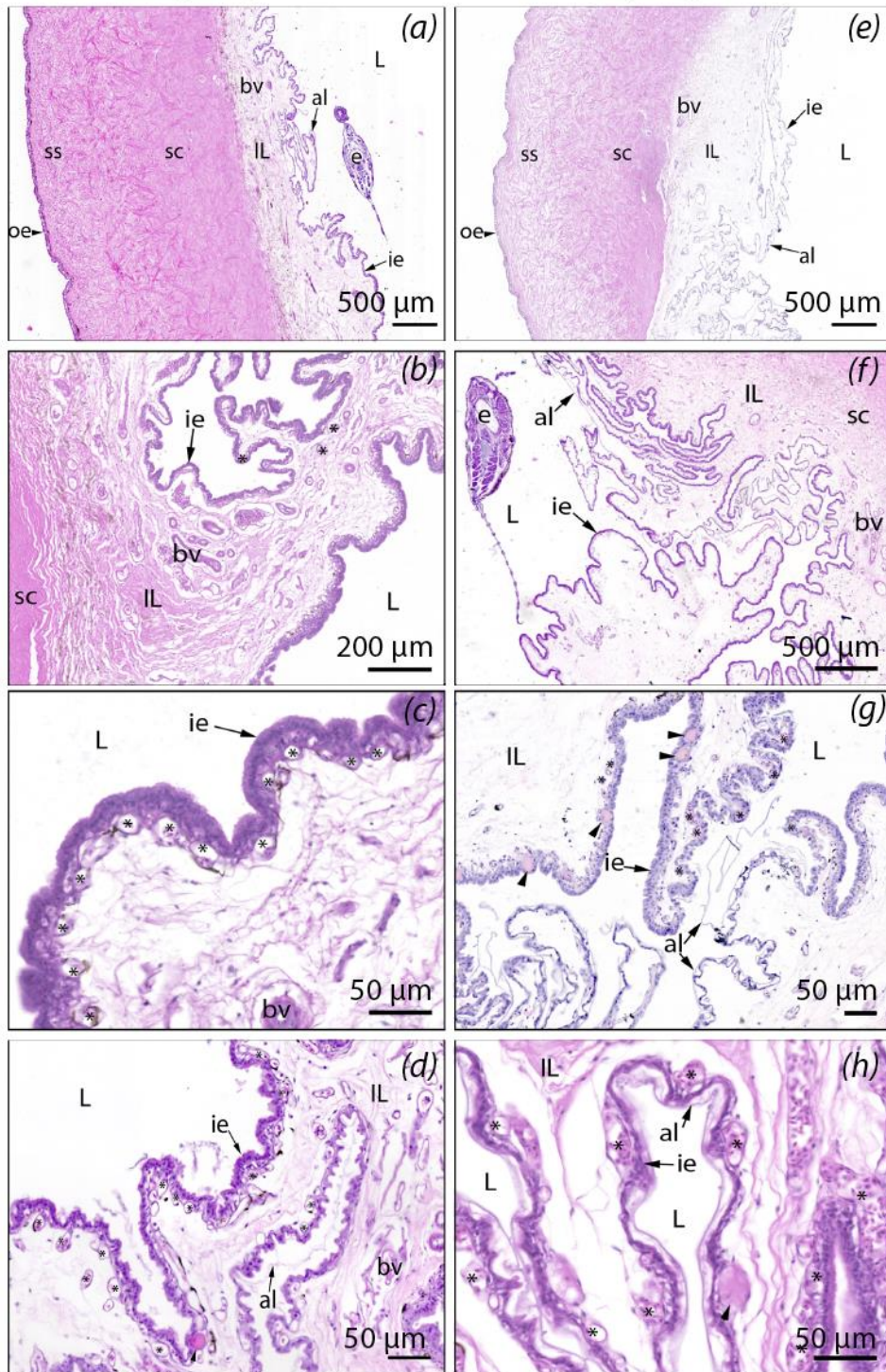
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Figure 22. Representative transverse sections of the brood pouch of male Hippocampus abdominalis during the non-pregnant and ~~early pregnant~~, ~~mid-pregnant~~, ~~late pregnant~~ and ~~post-parturition~~-states, stained with Haematoxylin and Eosin. 2A-(a-d) Cross-sections of a quadrant of brood pouch from non-pregnant males. The inner epithelium (ie) is pseudostratified columnar and there are blood vessels (bv) present. 2B-(e-h) Brood pouch during early pregnancy. Compared to the non-pregnant state, the inner epithelium is thinner and the inner tissue layer (IL) is more abundant. There are more capillaries () underlying the inner epithelium. An acellular structure (al) is present closely apposed to the inner epithelium (ie). Large eosinophilic cells are present underlying the epithelium (arrow head). Abbreviations: Acellular structure (al), Blood vessels (bv), Inner luminal epithelium (ie), Inner tissue layer (IL), Lumen (L), Outer epithelium (oe), Stratum compactum (sc), Stratum spongiosum (ss), Capillaries (*), Large, eosinophilic cells (arrow head).*

Mid-pregnant

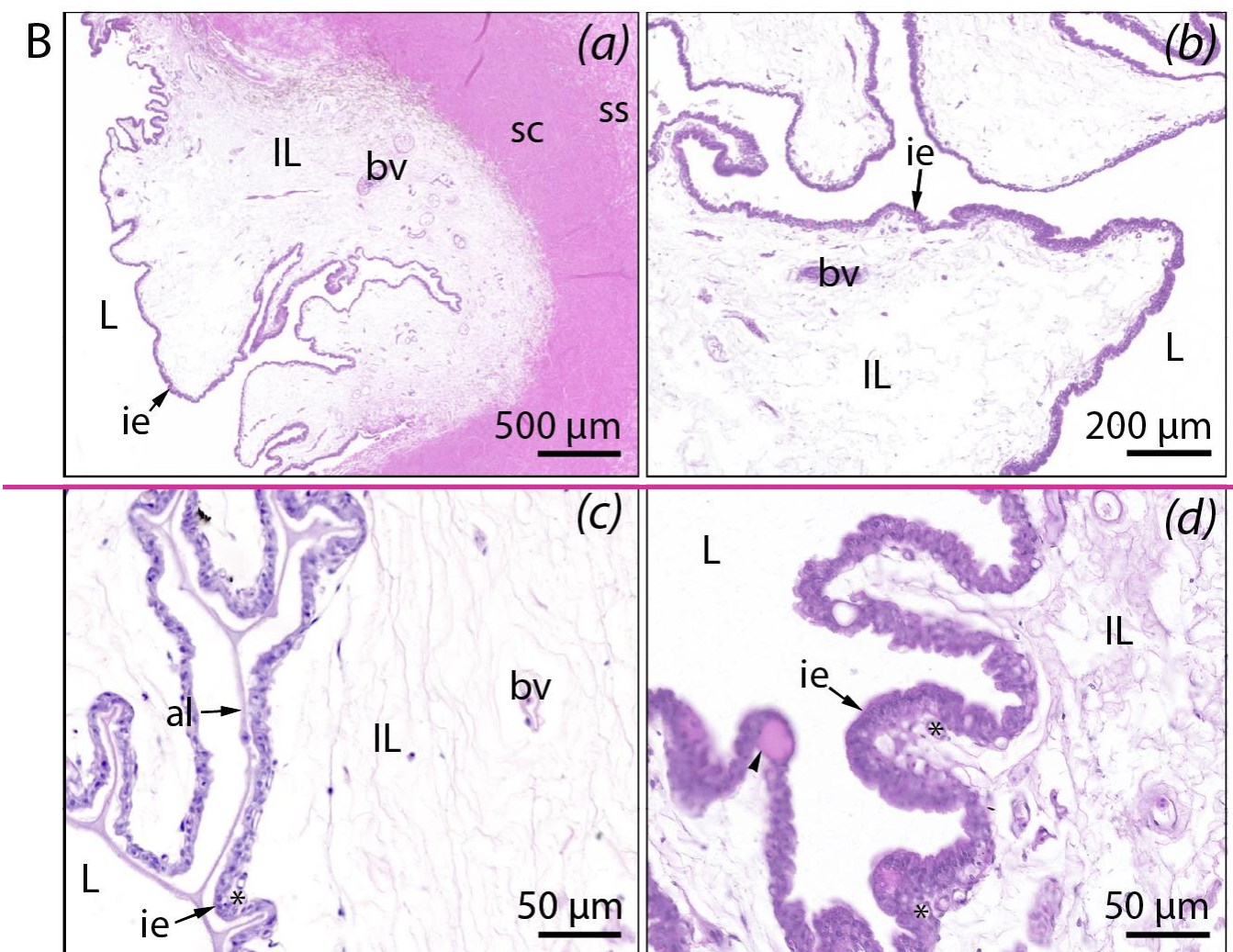
Late pregnant



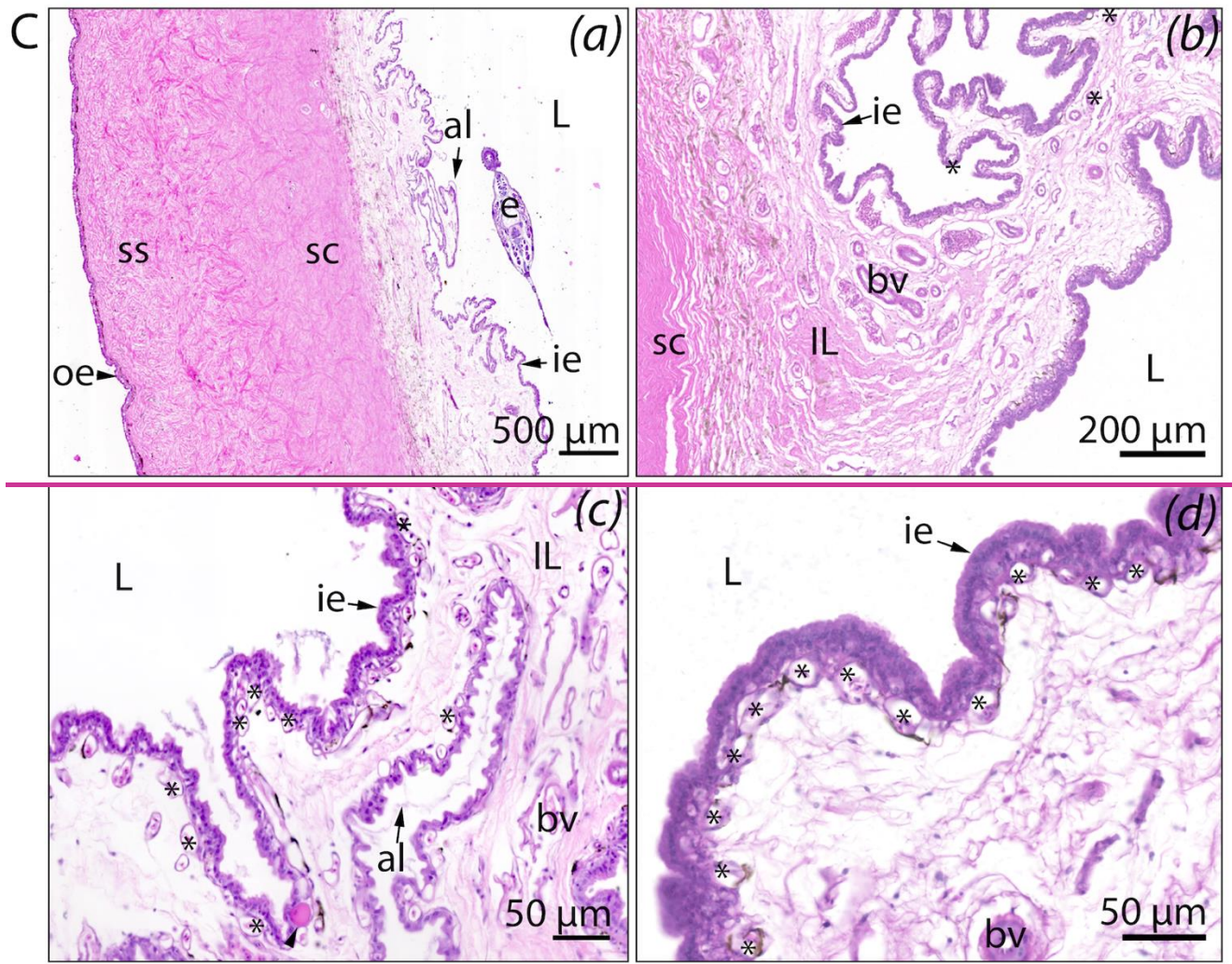
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224 **Figure 3: Representative transverse sections of the brood pouch of male *Hippocampus***
 225 ***abdominalis* during the mid and late pregnant states, stained with Haematoxylin and Eosin.**
 226 **(a-d) Brood pouch during the middle stage of pregnancy. The inner epithelium is tortuous.**
 227 **An acellular structure is apposed to the inner epithelium. (e-h) Brood pouch from**
 228 ***H. abdominalis* late in pregnancy. The inner epithelium is very tortuous with many**
 229 **capillaries within the loose connective tissue of the inner tissue layer. Abbreviations:**
 230 **Acellular structure (al), Blood vessels (bv), Embryo (e), Inner luminal epithelium (ie), Inner**
 231 **tissue layer (IL), Lumen (L), Outer epithelium (oe), Stratum compactum (sc), Stratum**
 232 **spongiosum (ss), Capillaries (*), Large, eosinophilic cells (arrow head).**

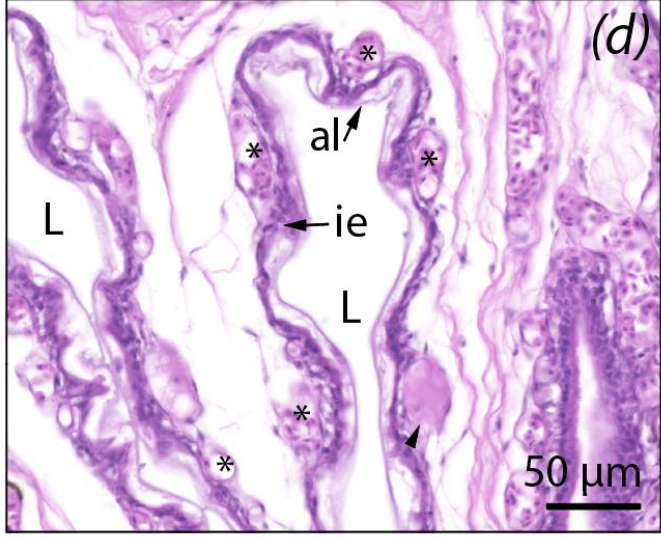
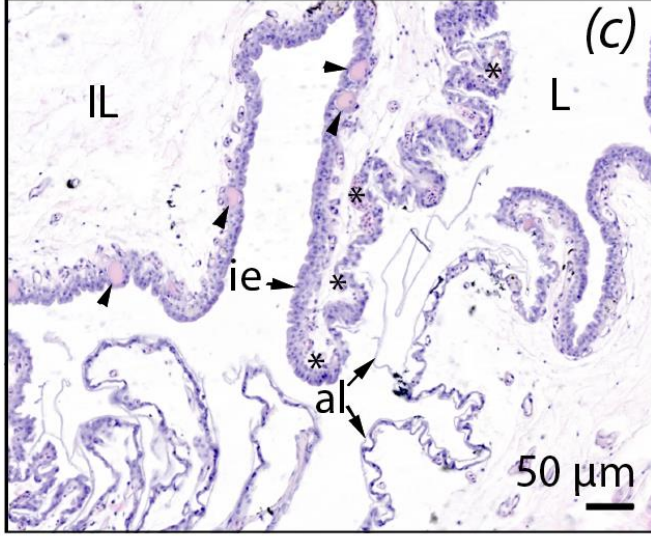
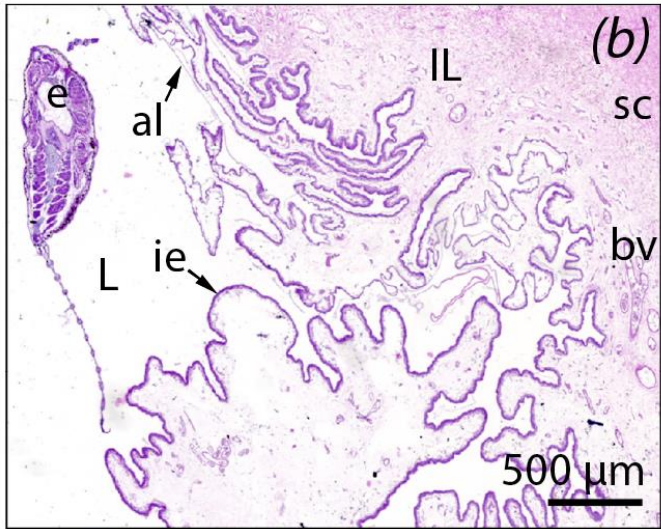
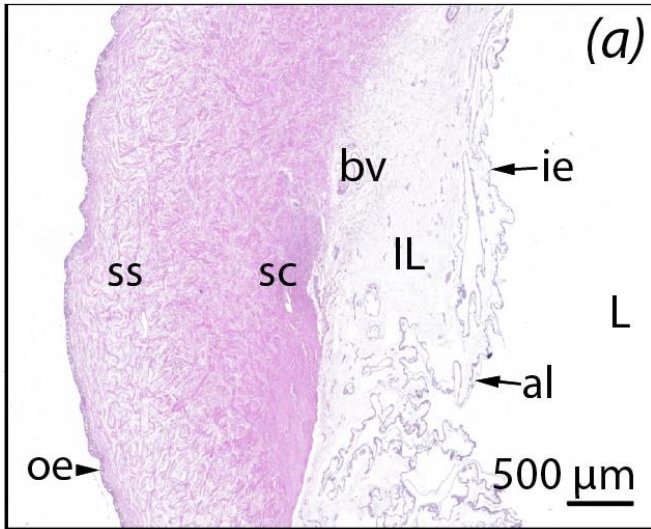


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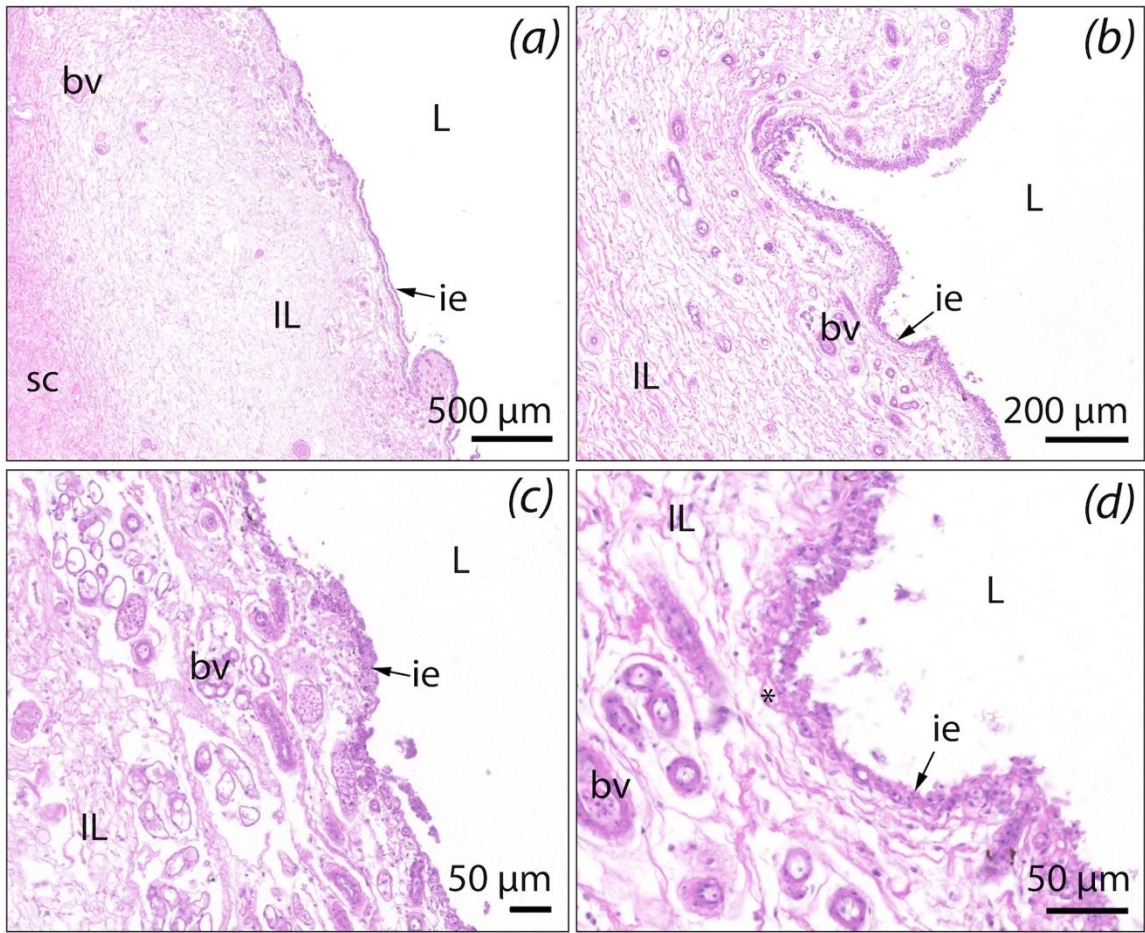


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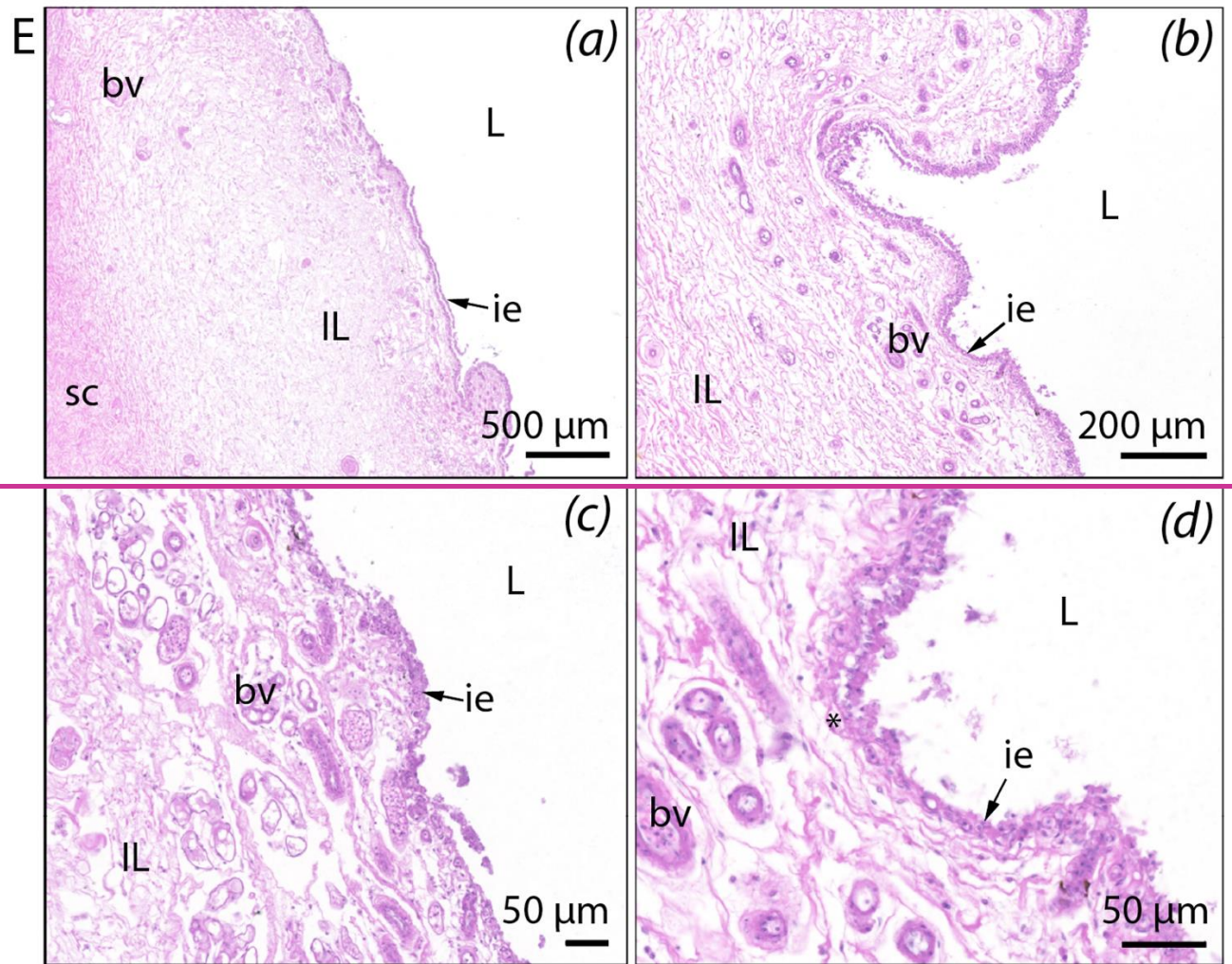
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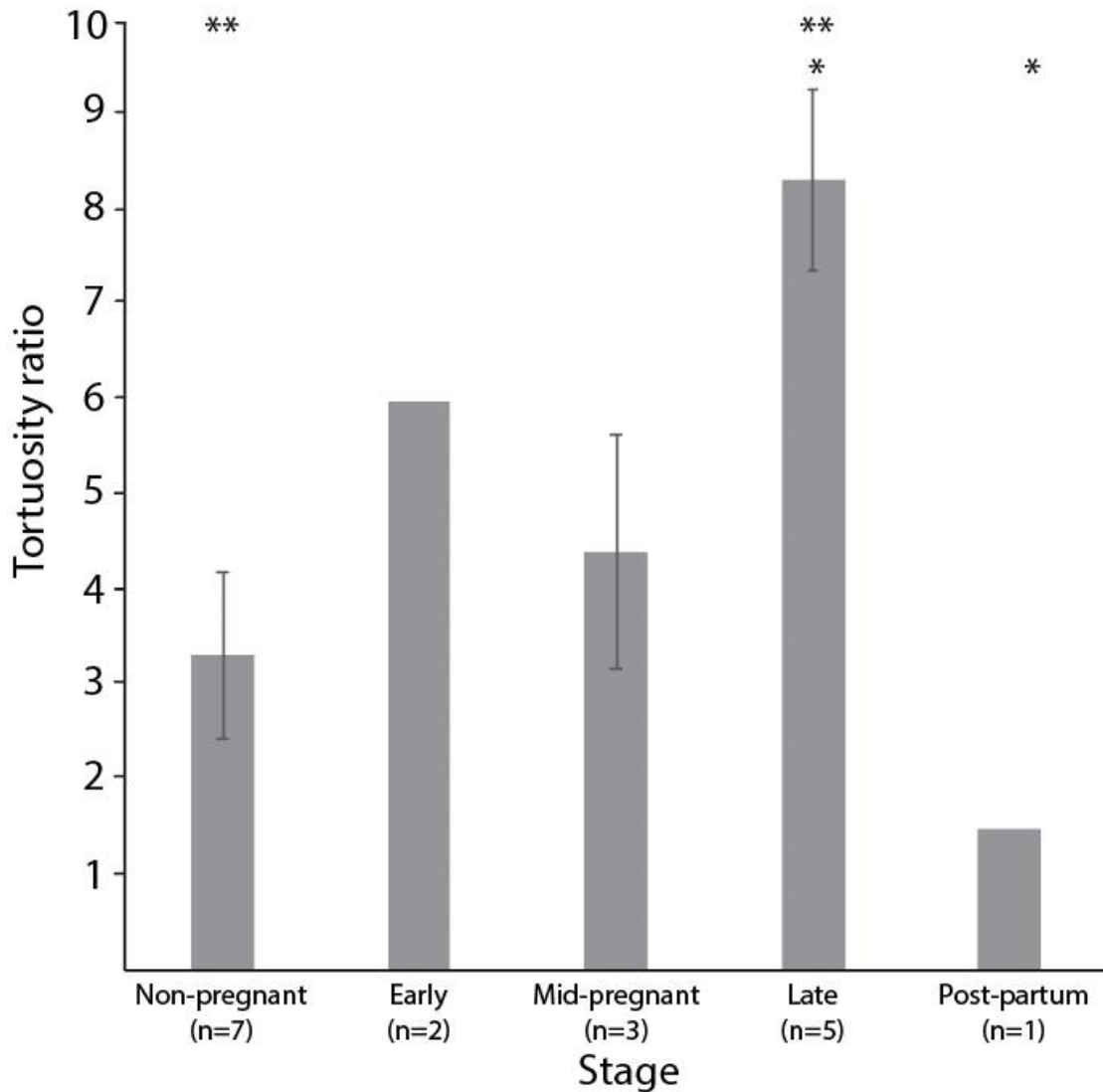
238 **Figure 2. Representative transverse sections of the brood pouch of male *Hippocampus abdominalis* during the non-**
 239 **pregnant, early pregnant, mid pregnant, late pregnant and post-parturition states, stained with Haematoxylin and Eosin. 2A**
 240 **(a-d) Cross sections of a quadrant of brood pouch from non pregnant males. The inner epithelium (ie) is pseudostratified**
 241 **columnar and there are blood vessels (bv) present. 2B (a-d) Brood pouch during early pregnancy. Compared to the non**
 242 **pregnant state, the inner epithelium is thinner and the inner tissue layer (IL) is more abundant. There are more capillaries**
 243 **(*) underlying the inner epithelium. An acellular structure (al) is present closely apposed to the inner epithelium (ie). Large**
 244 **eosinophilic cells are present underlying the epithelium (arrow head). 2C (a-d) Brood pouch during the middle stage of**
 245 **pregnancy. The inner epithelium is tortuous. An acellular structure is apposed to the inner epithelium. 2D (a-d) Brood**
 246 **pouch from *H. abdominalis* late in pregnancy. The inner epithelium is very tortuous with many capillaries within the loose**
 247 **connective tissue of the inner tissue layer. Figure 4: 2E (a-d) Representative transverse sections of the brood pouch of male**
 248 ***Hippocampus abdominalis* post-parturition, stained with Haematoxylin and Eosin. The inner epithelium appears to be**
 249 **breaking down with several layers of cells with the luminal cells having rounded apices. There are capillaries and blood**
 250 **vessels throughout the inner tissue layer. Abbreviations: Acellular structure (al), Blood vessels (bv), Embryo (e), Inner**
 251 **luminal epithelium (ie), Inner tissue layer (IL), Lumen (L), Outer epithelium (oe), Stratum compactum (sc), Stratum**
 252 **spongiosum (ss), Capillaries (*), Large eosinophilic cells (arrow head).**

253 **Quantification of histological features**

254 *Tortuosity ratio*

255 There is a significant difference in tortuosity ratio with stage of pregnancy ($F_{4,247} = 4.73, p =$
 256 0.001). Post-hoc tests show that significant difference is driven by a significant difference

257 between late pregnant and non-pregnant animals ($p = 0.001$) and between late pregnant and
 258 post-partum animals ($p = 0.032$) (Figure 3Figure 4).



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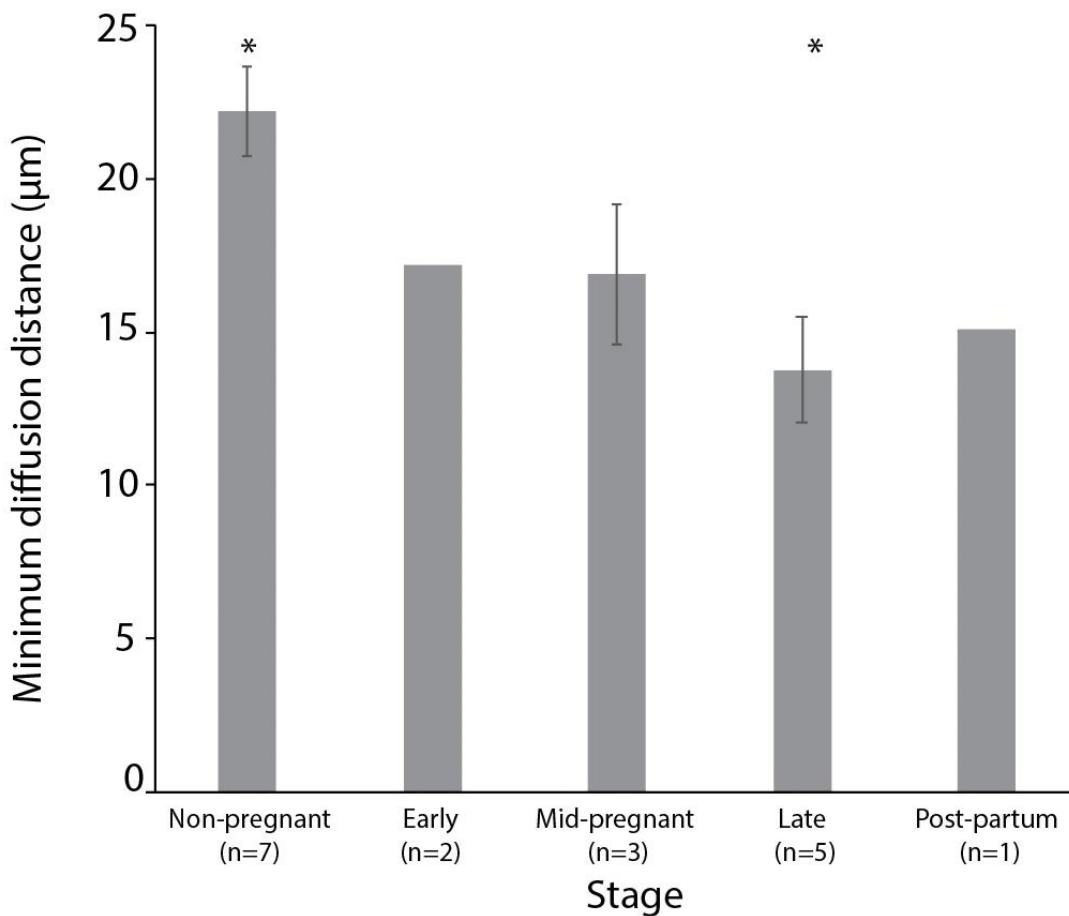
260 **Figure 34.** Tortuosity ratio (describing the degree of bends, twists, and turns) of the inner epithelium of the male
 261 Hippocampus abdominalis brood pouch varies across the reproductive cycle. Error bars: SE. * indicates significant
 262 difference ($p < 0.05$); ** indicates significant difference ($p < 0.005$).

263

264 *Minimum diffusion distance*

265 The diffusion distance between capillaries in the inner layer of the brood pouch and the
 266 lumen is significantly lower in pregnant compared to non-pregnant animals ($F_{4,158} = 3.7$, $p =$

267 0.007). Post-hoc analyses reveal that this difference is being driven by a significant difference
268 between non-pregnant and late-pregnant animals ($p = 0.003$) (Figure 4Figure 5).



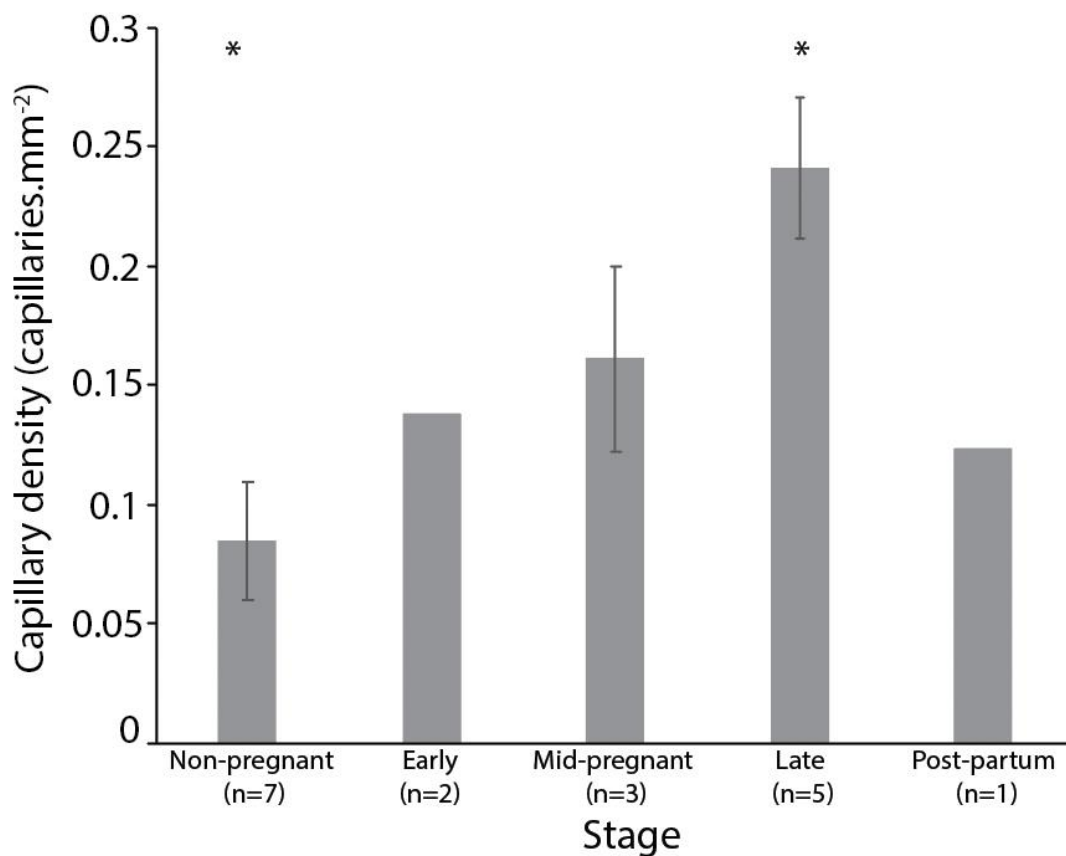
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270 *Figure 45. Mean diffusion distance between capillaries of the inner layer of the male Hippocampus abdominalis brood*
271 *pouch and the pouch lumen varies across the reproductive cycle. Error bars: SE. * indicates significant difference (p*
272 *<0.005).*

273

274 *Capillary density*

275 Capillary density in the inner layer of the brood pouch is higher in pregnant animals than
276 non-pregnant animals ($F_{4,161} = 4.09$, $p = 0.004$). Post-hoc pairwise comparisons revealed that
277 late pregnant animals have significantly higher capillary density than non-pregnant animals
278 ($p < 0.001$) (Figure 5Figure 6).



280

281 *Figure 5-6. Capillary density in the inner layer of the male Hippocampus abdominalis brood pouch varies across the*
 282 *reproductive cycle. Error bars: SE. * indicates significant difference (p < 0.001).*

283 Discussion

284 Our histological examination of seahorse brood pouch indicates remodelling of gestational
 285 tissues during male pregnancy. Tissue proliferation results in the inner layer of the pouch
 286 becoming thicker and more diffuse as it surrounds and partially encloses developing
 287 embryos. Some of these changes are initiated soon after fertilisation, because *H. abdominalis*
 288 embryos become deeply embedded into the epithelial lining of the pouch within the first three
 289 days of pregnancy, to the point where some are completely enveloped by pouch tissue [7,
 290 40]. During pregnancy, large dark staining cells are present underlying the pouch epithelium.
 291 These may be similar to the mitochondria rich cells seen underlying the pouch epithelium of
 292 *Syngnathus abaster* and *Syngnathus schlegeli* [6, 38]. This cell type is also found in fish gill
 293 filaments, where they assist with water and ion balance in response to osmotic challenges

294 [45]. While our examination of post-parturition brood pouch was limited to a single animal,
295 our results support previous suggestions that the seahorse brood pouch rapidly regresses to
296 the non-pregnant state following birth. Male *H. abdominalis* can resume courtship on the day
297 of parturition [46], have short interbrood intervals [47, 48], and remodelling and apoptotic
298 genes are upregulated in the brood pouch after birth [39], which may explain the post-
299 parturition cellular breakdown (Figure 42E).

300 As embryonic development progresses and embryos grow in size, demand for respiratory
301 gases increases. The oxygen consumption of pipefish *Syngnathus typhle* and *Nerophis*
302 *ophidion* embryos increases with developmental stage, particularly in the second half of
303 pregnancy [13]. While embryonic metabolic rate has not been measured in seahorses, oxygen
304 consumption generally increases as development progresses and embryos grow in size [e.g.
305 49, 50]. We posit that this increased embryonic demand for respiratory gas exchange across
306 pregnancy is met by structural adaptations in the father's brood pouch. In support of all three
307 of our hypotheses, we found that during pregnancy, especially late pregnancy, the epithelial
308 layer of the brood pouch becomes highly folded, the distance between capillaries and the
309 pouch lumen decreases, and the capillary density in the inner layer of the pouch increases,
310 which indicates an increase in epithelial surface area contacting the brood pouch lumen, a
311 decrease in the distance over which substances must diffuse to reach developing embryos,
312 and a significant expansion of the pouch vascular bed during pregnancy. We cannot rule out
313 the contribution of additional processes to respiratory gas exchange in the pouch, including
314 pouch flushing or differences in oxygen affinity between embryonic and paternal blood.
315 Future studies should use respirometry to measure the oxygen consumption of seahorse
316 embryos during development to confirm the functional significance of the seahorse brood
317 pouch vascular bed. Comparing oxygen consumption with the capacity for oxygen diffusion
318 across the entire surface area of the pouch (as has been done in elasmobranchs [51, 52])
319 would determine whether other mechanisms contribute to respiratory gas exchange for
320 embryos.

321 The increased surface area and vascularity of the brood pouch during pregnancy probably
322 evolved in tandem with increasing brood pouch complexity, because more complex brood
323 pouches present more significant barriers to embryonic respiratory gas exchange with the
324 external environment, and hypoxia stunts the growth and development of syngnathid
325 embryos [53, 54]. Quantification of brood pouch vascularisation between syngnathids with

326 pouches of differing complexity, including in species where embryos are partially exposed to
327 seawater, is required to test this hypothesis. Vascular quantification of the pouch of
328 *Syngnathus floridae* [36] used vascular casting, so the results are not directly comparable to
329 our quantification of *H. abdominalis* pouch vasculature. *Syngnathus floridae* has a thin-
330 walled Type 4 pouch sealed with a ‘zip’ along the midline. Pouch vasculature develops early
331 in pregnancy, but degenerates prior to parturition [36], which is puzzling given that
332 embryonic oxygen demand in a close relative with Type 4 pouch *S. typhle* is highest late in
333 pregnancy [13]. It is therefore possible that in the Type 4 pouches, some respiratory gases
334 diffuse through the pouch wall, which is so thin that embryos are clearly visible from the
335 outside [53]; males with this ‘zip-front’ pouch type are also apparently able to partially open
336 the pouch to release a portion of the clutch [6], which would presumably allow freshly
337 oxygenated water to enter. Perhaps Type 4 pouch vasculature facilitates exchange of
338 materials other than respiratory gases. Indeed, the *S. typhle* pouch is not well-oxygenated
339 compared to the surrounding water [53]. The expanded vascular bed for part of *S. floridae*
340 pregnancy could facilitate parent–offspring exchange of embryonic wastes, nutrients, and
341 hormones [36]. In *H. abdominalis* examined in this study, respiratory gas exchange is likely
342 to be a more significant function of the pouch vasculature, because the pouch wall is very
343 thick (>5 mm), contains a layer of dense connective tissue (Figure 2), and is therefore likely
344 to provide a significant enough barrier to gas exchange that the required oxygen and carbon
345 dioxide exchange cannot be met by simple diffusion across the pouch wall.

346 Pregnant parents of any species are faced with the physiological challenge of ensuring
347 respiratory gas exchange with embryos being incubated in an internal environment.
348 Viviparous female-pregnant vertebrates exhibit at least three major adaptations in response to
349 this physiological challenge: 1) thinning or loss of eggshell membranes compared to
350 oviparous congeners, which reduces the barrier to diffusion between mother and embryos
351 [e.g. 15, 23]; 2) thinning of tissues at the site of parental and embryonic contact, further
352 decreasing the diffusion distances for exchange, which is taken to an extreme in mammals
353 with invasive haemochorial placentation where maternal tissues are eroded [reviewed in 55];
354 3) increased surface area for exchange between mother and embryos [e.g. 19, 51]; and 4)
355 increased vascularisation of gestational tissues during pregnancy [e.g. 56, 57, 58, 59, 60]. All
356 of these changes are also present in seahorses: 1) egg-coverings [termed the chorion in
357 teleosts [61, 59]] are thinner in seahorses with closed pouches than syngnathids with open

358 pouches: *H. abdominalis* (thick, closed pouch) embryos have chorions less than one-third of
359 the thickness of alligator pipefish (*Syngnathoides biaculeatus*, open pouch) egg-coverings
360 [629]. We have also shown here 2) thinning of paternal tissues, 3) increased surface area of
361 paternal gestational tissues, and 4) an expansion of the vascular bed of gestational tissues.
362 The structural similarities between male pregnancy in seahorses and female pregnancy in
363 other vertebrates support the assertion that common physiological challenges of internal
364 embryo incubation have led to convergent adaptations in live-bearing species [4, 39, 634].
365 These results highlight the utility of syngnathids as models for understanding the biology and
366 evolution of vertebrate pregnancy.

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376 **Data Availability (Electronic Supplementary Material)**

377 The datasets supporting this article have been uploaded supplementary material.
378 Supplementary File 1: Morphological data for tortuosity ratio, minimum diffusion distance,
379 and capillary density in the male *Hippocampus abdominalis* brood pouch.

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