1 **Paternal nutrient provisioning during male pregnancy in the seahorse *Hippocampus***

2 ***abdominalis***

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ABSTRACT

14   Vertebrates that incubate embryos on or within the body cavity exhibit diverse strategies to

15   provide nutrients to developing embryos, ranging from lecithotrophy (solely yolk-provided

16   nutrition) to substantial matrotrophy (supplemental nutrients from the mother before birth).

17   Syngnathid fishes (seahorses, pipefishes and sea dragons) are the only vertebrates to exhibit

18   male pregnancy. Therefore, they provide a unique opportunity for comparative evolutionary

19   research, in examining pregnancy independent of the female reproductive tract. Here, we

20   tested the hypothesis that the most complex form of syngnathid pregnancy involves nutrient

21   transport from father to offspring. We compared the dry masses of newly-fertilised

22   *Hippocampus abdominalis* eggs with those of fully-developed neonates to derive a

23   patrotrophy index. The patrotrophy index of *H. abdominalis* was 1, indicating paternal

24   nutrient supplementation to embryos during gestation. We also measured the lipid content of

25   newly-fertilised eggs and neonates and found that there was no significant decrease in lipid

26   mass during embryonic development. Since lipids are likely to be the main source of energy

27   during embryonic development, our results suggest that lipid yolk reserves being depleted by

28   embryonic metabolism are replaced by the brooding father. The results of our study support

29   the hypothesis that nutrient transport occurs in the most advanced form of male pregnancy in

30   vertebrates.

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33   **Key Words**: brood pouch, dry mass, embryo incubation, lipid mass, matrotrophy, parental

34   care, paternal investment, patrotrophy, syngnathid

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INTRODUCTION

35   Viviparity (live-bearing reproduction) has independently evolved from oviparity (egg-laying)

36   over 150 times in vertebrates, including at least 13 independent origins in teleost fishes, all of

37   which exhibit embryo incubation inside the female reproductive tract (Blackburn 2015).

38   However, the teleost family Syngnathidae, which includes seahorses, sea dragons, and

39   pipefishes, exhibits a unique form of male pregnancy, in which pregnant males incubate

40   embryos inside a brood pouch (Stölting and Wilson 2007; Whittington and Friesen 2020).

41   Syngnathids are important models for evolutionary research because they allow comparative

42   analyses of pregnancy independent of the female reproductive tract. Such studies can

43   determine whether the pathways regulating female pregnancy are also co-opted in male

44   pregnancy, or whether novel pathways have evolved to support gestation.

45   Pregnant vertebrates exhibit a range of parental care strategies for fetal nourishment during

46   pregnancy (Blackburn 1992). Lecithotrophy is defined as embryos relying solely on the egg

47   yolk for nutrients required for growth and development, whilst matrotrophy describes species

48   in which embryos receive nutritional supplementation from their mother during development,

49   in addition to the yolk. Lecithotrophy and matrotrophy represent two extremes of a fetal

50   nutritional continuum: species can be strictly lecithotrophic, have incipient matrotrophy

51   (primarily reliant on yolk), or substantial matrotrophy (primarily reliant on non-yolk

52   nutrients) (Blackburn 1992). In the case of male pregnancy, nutrient provisioning of embryos

53   during pregnancy is termed patrotrophy.

54   Syngnathid brooding structures are diverse, ranging from a simple unprotected area for egg

55   attachment in some pipefishes, to a fleshy and fully enclosed brood pouch in seahorses

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56   (*Hippocampus* spp.) (Wilson et al. 2003; Wilson and Orr 2011; Whittington and Friesen

57   2020). Consequently, seahorses, with the most complex pouch type, are thought to undergo

58   the most substantial physiological and morphological changes during gestation, which likely

59   facilitate osmoregulation, immunological protection, oxygenation, and waste removal for

60   developing embryos (Stölting and Wilson 2007; Whittington and Friesen 2020). However,

61   the extent to which the seahorse pouch provides additional nutrients to the embryos is not

62   well understood. There is only limited research on the anatomy and physiological regulation

63   of the brood pouch, despite the fact that this information is an important prerequisite for

64   understanding the evolution of male pregnancy (Carcupino et al. 1997; Carcupino et al. 2002;

65   Scobell and MacKenzie 2011; Whittington and Friesen 2020).

66   A major outstanding question in syngnathid reproductive biology is whether fathers transport

67   nutrients to embryos during gestation (Whittington and Friesen 2020). There is direct

68   evidence for nutrient transport from father to offspring in several pipefish species

69   (*Syngnathus fuscus, Syngnathus floridae* and *Syngnathus typhle*) (Haresign and Shumway

70   1981; Kvarnemo et al. 2011; Ripley and Foran 2009; Stölting and Wilson 2007). Since

71   seahorses and *Syngnathus* spp. pipefish are closely related (Hamilton et al. 2017; Wilson et

72   al. 2001), adaptations present in pipefish might also be present in seahorses. *Syngnathus*

73   *fuscus* and *S. floridae* transport stable-isotope labelled lysine to their offspring, especially in

74   late stages of pregnancy, once most embryonic yolk has been consumed (Ripley and Foran

75   2009). A similar study in *S. typhle* tracked two other radioactively labelled amino acids from

76   pregnant males to developing embryos (Kvarnemo et al. 2011), suggesting that patrotrophy

77   occurs through supplementation of amino acids in this genus. However, there has been little

78   research on whether patrotrophy occurs in the most complex syngnathid pouches.

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79   The seahorse brood pouch is lined with modified secretory flame-cone cells, which may be

80   involved in metabolic pathways including protein synthesis (Carcupino et al. 2002). Lipid

81   droplets are also abundant in pouch tissues (Linton and Soloff 1964; Oconer et al. 2003).

82   Although seahorse embryos can survive *in vitro* incubation, suggesting that any nutrients

83   provided by the father are not essential, the neonates suffer developmental costs, including

84   stunted growth, and have increased mortality compared to *in vivo* embryos (Linton and Soloff

85   1964). In *Hippocampus erectus* and *Hippocampus barbouri*, calcium and iron in the brood

86   pouch fluid are absorbed by embryos (Linton and Soloff 1964; Oconer et al. 2003). In

87   addition, genes with putative transport function are highly upregulated during pregnancy in

88   *Hippocampus abdominalis* (Whittington et al. 2015). Most notably, the highest upregulated

89   gene during pregnancy encodes a probable lipid transporter (*apolipoprotein M*), raising the

90   possibility of lipid supplementation to developing embryos (Whittington et al. 2015). These

91   gene expression data provide a range of testable hypotheses around the physiology of

92   reproduction in *H. abdominalis*.

93   Here, we test the hypothesis that male seahorses transport nutrients to developing embryos.

94   Deriving a Matrotrophy Index (MI) is an important technique used to investigate maternal

95   transfer of nutrients in viviparous fish and reptiles (Buddle et al. 2018; e.g. Reznick et al.

96   2002; Stewart 2013; Thompson et al. 2000; Turcotte et al. 2008). This approach compares the

97   dry weight of newly-fertilised eggs and neonates, and thus considers both macro and micro-

98   nutrient transport. Using dry mass is important, because changes in biochemical composition

99   can be masked by changes in water content. Lecithotrophic teleost fish decrease in dry weight

100   by 30-40 % during development, as a result of catabolism (Wourms 1981; Reznick et al.

101   2002).

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102   Thus, in a viviparous animal, less of a decrease or an increase in dry weight both indicate

103   matrotrophy. Since we are dealing with male pregnancy, we calculated a Patrotrophy Index

104   (PI) to examine transport of nutrients from the father to developing embryos. Patrotrophy

105   indices have rarely been calculated for syngnathids, the exceptions being *Hippocampus*

106   *fuscus* [PI 0.72 (Vincent 1990)] and *Syngnathus schlegeli* [PI 0.71, (Watanabe and Watanabe

107   2002)], which suggest either lecithotrophy or very limited patrotrophy.

108   A powerful complement to an MI/PI is the use of a direct method to determine whether

109   nutritional provisioning occurs in the form of transport of a specific nutrient. Here, we

110   specifically examined lipid transport. Lipids are critical to embryonic development: they are

111   the primary source of energy for the embryo, supply lipid components for the biogenesis of

112   cell membranes, and provide the neonate with a source of energy for early life (Faleiro and

113   Narciso 2010; Speake et al. 2003). Thus, our research directly tests whether patrotrophy

114   occurs in male-pregnant pot-bellied seahorses (*H. abdominalis*) by addressing two aims: 1) to

115   calculate the total contribution of paternal nutrient transport to embryonic dry mass during

116   pregnancy, and 2) to determine the extent of lipid transport from male seahorses to embryos

117   during pregnancy. We predicted that, if patrotrophy takes place in the *H. abdominalis* brood

118   pouch: 1) the dry weight of full-term neonates will be the same or significantly greater than

119   the dry weight expected for a lecithotrophic brooder with eggs of the same size, and 2) the

120   lipid content of neonates will be the same or significantly greater than that of the newly-

121   fertilised eggs.

MATERIALS AND METHODS

122 **<u>Animals</u>**

123 *Housing*

124 Reproductively mature male *H. abdominalis* obtained from a captive-bred population

125 (Seahorse Australia, Tasmania, Australia) were maintained under previously described

126 aquarium conditions (Whittington et al. 2013) (University of Sydney Animal Ethics

127 Committee approval number: 2018/1302). Briefly, housing was in 75 L aquaria in a flow-

128 through marine system in an 18 °C controlled temperature room, with animals fed six days

129 per week. We cleaned all tanks twice weekly, monitored and adjusted salinity and

130 temperature six days per week, and tested ammonia, nitrate, nitrite and pH weekly, to

131 maintain water quality parameters within the optimal range. Animals were held under a

132 summer light cycle to encourage breeding, with a 15.5 hour photoperiod including a

133 simulated dawn and dusk period for 1 hour each.

134 *Breeding*

135 We used nineteen female and twenty-one male *H. abdominalis* for this study. We cycled

136 females and non-pregnant males through a 750 L breeding tank deep enough to allow egg

137 transfer (Woods 2000). Mating is rarely observed in captive seahorses (Whittington et al.

138 2013), so we precisely timed the movement of the animals in and out of the breeding tank to

139 ensure the most accurate estimates possible for length of pregnancy. We determined

140 reproductive status based on courtship behaviours, as described by Whittington et al. (2013).

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141 Putatively pregnant males were housed in single-sex tanks until euthanasia (to sample newly-

142 fertilised eggs) or parturition (to sample neonates). To sample newly-fertilised eggs, we used

143 pregnant males that were between three and six days pregnant. To sample neonates, pregnant

144 males were held until parturition in tanks with water outflow pipes covered by mesh to

145 prevent neonates from being drawn into the recirculating water system. We divided each tank

146 down the middle using mesh in order to house two pregnant males in each tank, whilst

147 reducing the risk of non-parental cannibalism of juveniles; however, paternal cannibalism

148 may still have reduced neonate clutch size (Woods 2000). We checked pregnant male tanks

149 for neonates several times on weekdays and twice on weekends to ensure that neonates were

150 collected and processed as quickly as possible.

151 **Sample Collection**

152 Neonate and adult seahorses were removed from their tanks and euthanised via overdose of

153 anaesthetic followed by decapitation and immediate pithing of the brain (Leary et al. 2013).

154 We measured six embryo and seven neonate clutches for dry mass and lipid mass. As the

155 individual embryos and neonates have such a small mass, to accurately determine their dry

156 mass and lipid masses, 5 embryos/neonates from the same clutch were put into each

157 Eppendorf tube and then treated as one sample. If the total number collected for a clutch was

158 not divisible by 5, the remaining 2-4 embryos were placed into an Eppendorf tube and treated

159 as a single sample. The clutch size in this species varies with body size. Reproductively

160 active adults range from ~10–35 cm standard length (head length plus body and tail length)

161 (Woods 2005; Lourie et al. 2004). Larger males produce larger broods (mean ~270 offspring

162 per clutch for males from ~10–25 cm standard length; Woods 2005), but the observed brood

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163 size range in this species is very large [~20–1100 (Foster and Vincent 2004; Woods 2000;

164 Woods 2005; Skalkos pers. obs.; Whittington pers. obs.)]. In our study, released neonates

165 ranged from 5–112 per clutch (however, as noted above, this may be an underrepresentation

166 of actual clutch size, if fathers cannibalised offspring). The total number of samples for

167 embryos was 22, and the total number of samples for neonates was 71 (note that lipid

168 extraction failed for two neonate samples and so the lipid mass sample size is 69 for

169 neonates).

170 *Embryos*

171 We found that newly-fertilised eggs could not be extracted from the male brood pouch in

172 their native state, because they were deeply embedded in pouch epithelium and too delicate to

173 be handled without breaking. Therefore, we performed a pilot study in which the euthanised

174 parent was dissected whilst submerged in a solution of 95 % ethanol and 5 % seawater

175 (salinity: 30 ppm). An incision was made on the ventral surface of the brood pouch from the

176 brood pouch opening towards the tail. We then cut the pouch at the top and bottom in order to

177 fold it open and expose all the embryos embedded in the epithelial tissue of the brood pouch.

178 The pouch was then held open using pins to allow the exposed embryos to harden slightly for

179 40 minutes. We could then extract intact individual embryos from the brood pouch (Figure

180 1). Only intact embryos were used for analysis. We placed these embryos into pre-weighed

181 Eppendorf tubes in groups of five, and dry mass and lipid content were measured (see section

182 below). To ensure that the ethanol fixation did not affect dry mass or lipid content, we

183 validated this method using a dry cat biscuit control, comparing dry mass and lipid

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184   percentage with and without ethanol fixation. The method did not affect either parameter

185   (data not shown).

186   *Neonates*

187   All neonates were euthanised within twenty hours post-parturition (mean and median: 16

188   hours, range: 2 – 20 hours), depending on the time of day at which they were born.

189   Euthanised neonates were individually blotted with kimwipes to remove excess water and

190   placed in pre-weighed Eppendorf tubes in groups of five for measurement of dry mass and

191   lipid content.

192   **Patrotrophy Index**

193   The extent of potential paternal provisioning of nutrients (patrotrophy) in *H. abdominalis* was

194   derived by calculating a PI instead of an MI. The PI equates to the mean dry mass of a

195   newborn neonate at parturition divided by the mean dry mass of an ovum at fertilisation. As

196   seahorse mating is rarely observed, newly-fertilised eggs (i.e. very early embryos from 3 to 6

197   days post fertilisation) were used. If nutrient transport takes place very early in pregnancy,

198   this approach will underestimate rather than overestimate the PI, so our calculated PI is likely

199   to be conservative.

200   In strictly lecithotrophic teleost species, embryos lose 30-40 % of their dry weight during

201   development (i.e. MI or PI = 0.6–0.7) due to metabolic costs associated with embryonic

202   growth and development (Reznick et al. 2002; Wourms 1981;Wourms et al. 1988). If

203   parental provisioning of nutrients occurs, embryos will either lose less dry weight than

204   embryos of lecithotrophic species, or will gain dry weight throughout development (MI or PI

205 > 0.7). To calculate the PI, pre-weighed Eppendorf tubes containing each sample (two to five

206 newly-fertilised eggs or neonates) were weighed on a Mettler H35AR Balance (accurate to

207 0.0001 g) to derive wet mass, and then dehydrated with the lids open in an oven at 60 °C for

208 at least 48 hours. The Eppendorf tubes were then placed in a desiccation jar for a minimum of

209 30 minutes whilst the tubes returned to room temperature. The tubes were then weighed to

210 derive the dry mass of samples. Samples were stored in desiccation chambers prior to lipid

211 extraction.

212 **Lipid Content**

213 Total lipids were extracted from newly-fertilised eggs and neonates by homogenisation in an

214 excess of chloroform-methanol, adapted from a commonly used method used to determine

215 matrotrophy, the Folch Extraction (similar to the Bligh-Dyer method) (Pethybridge et al.

216 2011; Ramirez-Pinilla 2006; Stewart and Castillo 1984; Thompson et al. 1999a; Thompson et

217 al. 2001; Thompson et al. 1999b; Thompson et al. 1999c). In brief, each randomised sample

218 was homogenised in a Dounce Griner in a mixture of chloroform, methanol, and distilled

219 H_2O at a ratio of 2.5 : 2.5 : 1 ml. The homogenate was then filtered through a Buchner Funnel

220 lined with 7 cm filter paper into a Buchner flask with an attached vacuum pump. We rinsed

221 the Dounce grinder with additional chloroform, methanol and distilled H_2O mixture to wash

222 out all remaining homogenate. An additional 5 ml of chloroform was filtered through to

223 collect any lipids that may have remained on the filter paper due to rapid evaporation of

224 chloroform during the process. The filtrate was then poured into a 25 ml graduated cylinder,

225 and the Buchner funnel was rinsed with 10 ml of chloroform into the graduated cylinder.

226 After 10 minutes, the filtrate separated into two phases: the top layer of methanol and water,

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227   containing substances such as proteins, carbohydrates, and minerals, and the lower

228   chloroform layer, containing lipids (Folch et al. 1957). The methanol/water layer was

229   transferred into another graduated cylinder using a transfer pipette, and rinsed with 7 ml of

230   chloroform, again waiting another 10 minutes to allow for the phases to separate. The top

231   layer was then removed and discarded. The chloroform phase from both graduated cylinders

232   was poured into a pre-weighed 25-50 ml flask. Both cylinders were rinsed with 1 ml

233   chloroform and the rinsate was transferred into the same pre-weighed flask. The chloroform

234   was evaporated from the flask (leaving behind the lipids as residue) using a stream of

235   nitrogen gas, whilst sitting the flask in a hot water bath to accelerate the process. After all

236   visible chloroform had evaporated, the flask was placed in the oven at 60 °C for at least 30

237   minutes to remove excess water, then placed into a desiccation jar for at least 30 minutes to

238   return to room temperature, and then weighed. We also performed control lipid extractions

239   with empty vessels, and found that there was often a chloroform residue remaining in the

240   flask. Consequently, the mean of 10 control extractions (0.0008 g) was subtracted from all

241   sample lipid masses to correct for the residue effect.

242   **Statistical Analysis**

243   Measuring 5 embryos/neonates per sample allowed multiple replicates within a clutch to be

244   analysed, and a clutch mean to be calculated. The minimum number of samples per clutch

245   was 1, the maximum was 23, and the mean was 7.15. All results of wet and dry mass

246   comparisons and lipid content calculations are expressed as mean $\pm$ SEM. To remove any

247   potential clutch effects, means were used for each newly-fertilised egg (N = 6) and neonate

248   (N = 7) clutch. A Shapiro-Wilk test was used to ensure that all data met assumptions of

249   normality and heteroscedasticity of variance. The data were analysed using one-way analysis

250   of variance (ANOVA) to determine whether the mean values of groups were significantly

251   different at an α-level of 0.05. All statistical analysis was performed using RStudio 1.1.463.

RESULTS

252   **Patrotrophy Index**

253   Neonates had a significantly greater wet mass than newly-fertilised eggs ($F_{1,11} = 16.880$, p =

254   0.002) (Figure 2). In a lecithotrophic species, we expect a significant decrease in the dry mass

255   of neonates compared to newly-fertilised eggs. In *H. abdominalis* there was no significant

256   difference between the dry mass of newly-fertilised eggs and neonates ($F_{1,11} = 0.461$, p =

257   0.511) (Figure 3). Therefore, the calculated PI is 1.

258   **Lipid Content**

259   There was no significant difference between the lipid mass of newly-fertilised eggs and

260   neonates ($F_{1,11} = 0.105$, p = 0.752) (Figure 4).

261   **Gross Morphological Observations**

262   In the course of this research, we observed morphological changes to the brood pouch during

263   pregnancy. Since the relationship between parental tissue and developing embryos is not well

264   known, we report these observations, as they may be relevant to nutrient transport. Within the

265   first three days of development, newly-fertilised eggs are deeply embedded into the epithelial

266   pouch tissue. Almost all embryos are compartmentalised individually within pits in the tissue

267   lining the pouch wall, and some are entirely enveloped by it (Figure 1 A, B). We thus had to

268 develop a different method to separate embryonic and paternal tissue (see 'Embryos' section

269 of Methods). A male at a later stage of pregnancy (15-20 days) was used for other projects in

270 the course of this study, and we also report our morphological observations here. The pouch

271 contained live embryos that had hatched from their chorions and were still absorbing yolk

272 through their abdomens (Figure 5).

DISCUSSION

273 Our results indicate that the mass of *H. abdominalis* embryos significantly increases during

274 pregnancy, mostly via the uptake of water (Figure 2, 3). In contrast, there was no significant

275 difference between the dry mass of newly-fertilised *H. abdominalis* eggs and neonates

276 (PI=1), meaning that a small amount of dry mass must have been gained during gestation.

277 The MI of lecithotrophic teleosts is approximately 0.6–0.7 due to embryonic catabolism of

278 nutrients during development (Wourms et al. 1988; Wourms 1981; Reznick et al 2002).

279 However, these MI values are based on dry mass losses in embryos of lecithotrophic

280 viviparous poeciliids, and other oviparous teleosts (*Clupea harengus* and *Salmo irideus*). As

281 different fish species vary in rates of embryonic development and yolk conversion efficiency,

282 intraspecies differences in embryonic mass loss make it difficult to assign a precise cut-off

283 value for matrotrophy that is reliable across species (Riesch et al. 2010; Huveneers et al.

284 2011; Frazer et al. 2012). Nevertheless, the *H. abdominalis* PI of 1 calculated here indicates,

285 at a minimum, incipient patrotrophy [PI/MI= 0.7-1 (Riesch et al. 2010)], because some mass

286 must be lost during development via embryonic growth, metabolism, and excretion of

287 nitrogenous wastes (Huveneers et al. 2001). Our data thus suggest that male *H. abdominalis*

288 supply a small amount of nutrients to embryos during pregnancy, which replaces the mass

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289 lost via catabolism during embryogenesis. This study provides the first experimental

290 evidence for paternal provisioning of nutrients to developing embryos during pregnancy in a

291 seahorse, which has the most complex form of male pregnancy.

292 We combined the calculation of a PI with a direct method measuring lipid mass change

293 during embryonic development and found no significant difference in lipid mass between

294 newly-fertilised eggs and neonates. These results indicate that some of the maternal yolk-

295 reserves being consumed by developing embryos are being replaced by paternal lipid

296 provisioning during pregnancy. Whilst reptiles and birds catabolise both yolk proteins and

297 lipids for energy during embryogenesis (Speake et al. 1998; Speake and Thompson 2000), the

298 main source of energy in fish embryogenesis is debated (Finn et al. 1995; Hölttä-Vuori et al.

299 2010; Rosa et al. 2005). However, long-snouted seahorse (*Hippocampus guttulatus*) embryos

300 consume a large amount of lipid as a source of energy during embryogenesis, suggesting that

301 the same may be true of *H. abdominalis* (Faleiro and Narciso 2010). The presence of lipid

302 provisioning in *H. abdominalis* supports brood pouch gene expression data showing several

303 highly expressed lipid transporters including *apoliprotein M*, the most significantly

304 upregulated gene in pregnant *H. abdominalis* (Whittington et al. 2015). In the future,

305 researchers should attempt to confirm whether the mechanism of nutrient transport is via

306 these lipid transporters, and link their localisation and abundance to pouch lipid droplets

307 (Linton and Soloff 1964; Oconer et al. 2003).

308 The only other study of embryonic lipid content in seahorses is in *H. guttulatus* (Faleiro and

309 Narciso 2010). In this species, total lipids contributed roughly 5 % of neonate dry mass,

310 which was a significant reduction from eggs, suggesting that there may be little to no

311 supplementation of lipids during embryogenesis in *H. guttulatus*. In contrast, the results

312 reported here show that total lipids contribute approximately 88 % to *H. abdominalis* neonate

313 dry mass, and that lipid mass is not significantly different between neonates and newly-

314 fertilised eggs. The differences between the results from these two species could be explained

315 by several factors, which are not mutually exclusive. The time of sampling after birth (which

316 was not specified in the *H. guttulatus* study) could have drastically influenced the results, as

317 neonates may have depleted their energy reserves if left to swim in an aquarium. Another

318 possibility is a difference in egg sizes between the two species. If *H. guttulatus* females invest

319 more in eggs than *H. abdominalis* females, additional nutrient supply from *H. guttulatus*

320 males may not be required. In the future, female investment in egg yolk should be compared

321 across species, either directly by microbomb calorimetry, or by using egg volume as a proxy.

322 Finally, male investment may vary according to diet. The *H. guttulatus* were fed *Artemia* spp.

323 (brine shrimp), whilst *H. abdominalis* were fed *Mysis* spp. (mysis shrimp). Brine shrimp have

324 nutritional deficiencies for marine species, particularly in essential omega-3 fatty acids

325 (Conceição et al. 2010; Serrano Jr 2012), which may have affected the availability of lipids

326 for transport by male *H. guttulatus,* if there is plasticity in reproductive allocation in

327 syngnathids as in other taxa (Van Dyke and Griffith 2018). Mysis shrimp are higher in

328 proteins and fats than brine shrimp (Woods and Valentino 2003). Furthermore, if male

329 *Hippocampus reidi* seahorses are fed diets deficient in polyunsaturated fatty acids prior to

330 pregnancy, they produce smaller neonates, further highlighting how parental diet can affect

331 offspring fitness (Otero-Ferrer et al. 2020). The possibility that paternal investment in

332 embryos may be influenced by the father's resource availability is an important area of future

333 research. Since yolk absorption by embryos is still clearly apparent in late-stage pregnancy

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334   (Figure 5), embryos do not diminish their maternal source of lipids until almost birth

335   (Sommer et al. 2012), suggesting that any paternal nutrient supplementation in this study

336   prevented total yolk absorption occurring until very late into pregnancy.

337   The lipid extraction procedures employed here are widely used in studies of other vertebrate

338   embryos, but those samples are often much larger (Ramirez-Pinilla 2006; Thompson et al.

339   1999a; Thompson et al. 1999b; Thompson et al. 1999c; Van Dyke et al. 2014). The samples

340   used in this study were extremely small (mean dry weight ~0.0012 g), and so the amount of

341   lipids in these samples was at the threshold of measurability with the available equipment.

342   Our control lipid extractions indicated that a small amount of chloroform residue is left

343   behind after extraction, which would have increased the measurement of total lipids for all

344   samples. As a result, the lipid content was higher than the dry mass weight for some samples.

345   A comparison of multiple lipid extraction methods found that the Bligh-Dyer method similar

346   to that used here provided significantly higher lipid values for 1 g samples than other non-

347   chloroform methods, but that there was no significant effect on larger 5-10 g samples

348   (Honeycutt et al. 1995). Thus, the small mass of our samples likely contributed to some of the

349   variability in measurements. However, when used in tandem with the calculation of a PI, the

350   lipid extraction results provide strong evidence for nutrient transport to embryos during

351   development.

352   We observed that three-day old embryos are strongly embedded in pits within the epithelial

353   tissue lining the internal surface area of the brood pouch (Figure 1). These observations

354   suggest that nutrient transport may take place across a placental analogue via the close

355   apposition of paternal and fetal tissues (Mossman 1937), although histological work is

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356   required to test this hypothesis. Similar rapid embedding after fertilisation has been shown in

357   other syngnathids, including *Syngnathus abaster* and *Hippocampus kuda* (Carcupino et al.

358   1997; Laksanawimol et al. 2006). Microscopy of the embryo contact region in *H. kuda*

359   revealed a distended inner epithelium and adjacent large blood vessels in the inner loose

360   connective tissue (Laksanawimol et al. 2006). These morphological changes are suspected to

361   facilitate gas exchange and could also contribute to nutritive processes across paternal and

362   embryonic membranes (Laksanawimol et al. 2006). Future transmission electron microscopy

363   studies of *H. abdominalis* characterising the specific changes to the luminal epithelium will

364   elucidate the mechanisms behind the strong attachment of early embryos and may provide

365   insight into the mechanisms of nutrient transport.

366   **<u>Conclusion</u>**

367   This study is the first experiment to indicate that patrotrophy occurs in seahorses. Since we

368   cannot rule out the possibility that nutrients from undeveloped embryos could have been

369   absorbed by siblings [which may occur in *Syngnathus* spp. pipefish (Ripley and Foran

370   2006)], we suggest that further investigations should track the movement of stable isotope-

371   labelled nutrients from parent to embryo, as has been done in matrotrophic vertebrates

372   (Haresign and Shumway 1981; Marsh-Matthews et al. 2005; Swain and Jones 1997; Van

373   Dyke and Beaupre 2012). Furthermore, immunohistochemistry of the genes identified in

374   expression studies will reveal the mechanisms potentially involved in paternal nutrient

375   provisioning.

376   Syngnathids are an essential comparative model species to explore the evolution of

377   pregnancy and fetal nourishment across taxa because they are the only male pregnant

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378 vertebrates. Our research suggests that patrotrophy occurs in *H. abdominalis*, which raises the

379 possibility of sexual and parent-offspring conflict and has implications for flexibility of

380 nutrient provisioning in both sexes. This species also represents a useful comparison to other

381 animal species with non-invasive placentation, where there is gas exchange and nutrient

382 transfer through direct contact between embryonic and maternal tissue but no invasion of the

383 endometrium (Carter and Enders 2013). This work contributes to our knowledge of the

384 fundamental biology of the syngnathid brood pouch and helps in the quest to determine the

385 mechanisms underpinning the evolution of male pregnancy and patrotrophy.

**Acknowledgements**

Conflicts of Interest

392 None to declare.

Ethical Approval

393 All applicable international, national and institutional guidelines for the care and use of

394 animals were followed. All procedures performed in studies involving animals were in

19

395   accordance with the ethical standards of the University of Sydney (University of Sydney

396   Animal Ethics Committee approval number 2018/1302).

Figures
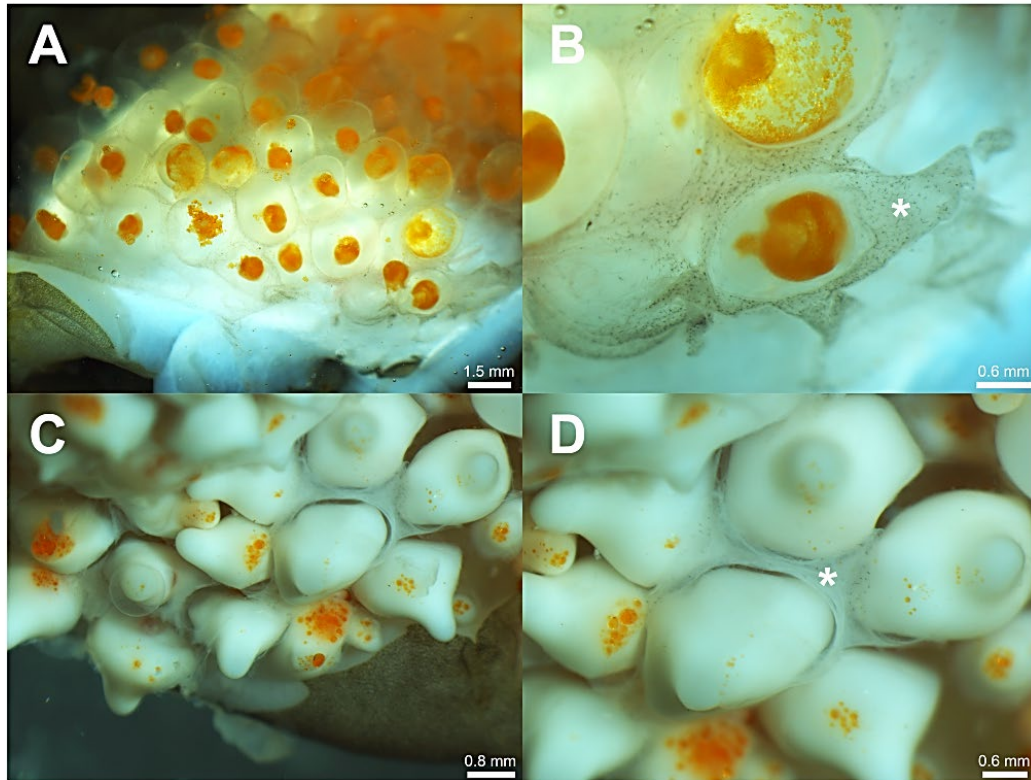
397

398   *Fig. 1 Newly-fertilised eggs (3-6 days of development) in the* Hippocampus abdominalis *brood pouch. A and B show the*

399   *natural state inside the dissected brood pouch, in which the embryos are deeply embedded in the pits of epithelial tissue*

400   *lining the brood pouch wall, and cannot be removed without damage. C and D show the embryos after 40 minutes of*

401   *fixation in 95 % ETOH 5 % seawater (30 ppm). * indicates the paternal epithelial tissue, which covers much of the embryo in*

402   *unfixed pouches, but then shrinks after fixation and partially separates from the embryo, resulting in easier separation of*

403   *paternal and embryonic tissue.*

404

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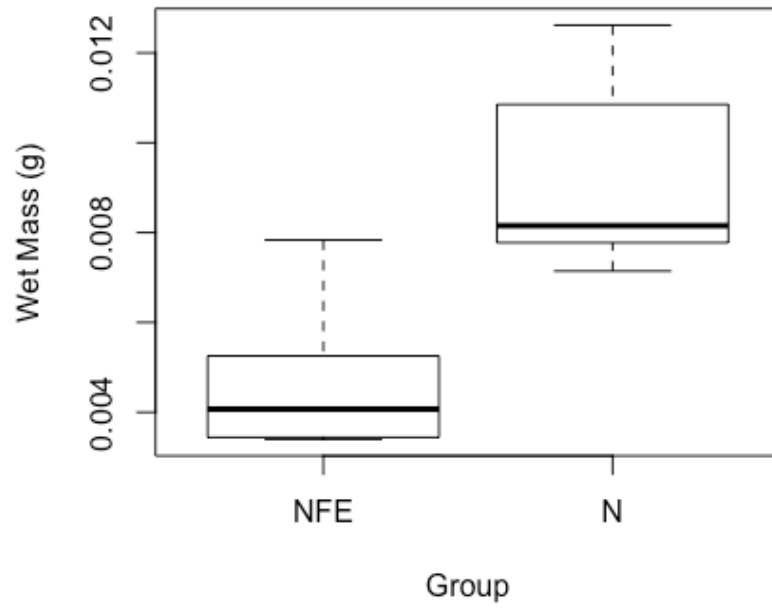
Fig. 2 *Boxplot of wet mass comparison of newly-fertilised* Hippocampus abdominalis *eggs (NFE) and neonates (N). A one-*

407 *way ANOVA revealed a significant difference (P= 0.002). The bold lines indicate the median for each group, and the boxes*

408 *contain 50 % of the data whilst whiskers contain data within 1.5 x the interquartile range.*

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411 **Fig. 3** *Boxplot of dry mass comparison of newly-fertilised* Hippocampus abdominalis *eggs (NFE) and neonates (N). A one-*

412 *way ANOVA revealed no significant difference (P=0.511). The bold lines indicate the median for each group, and the boxes*

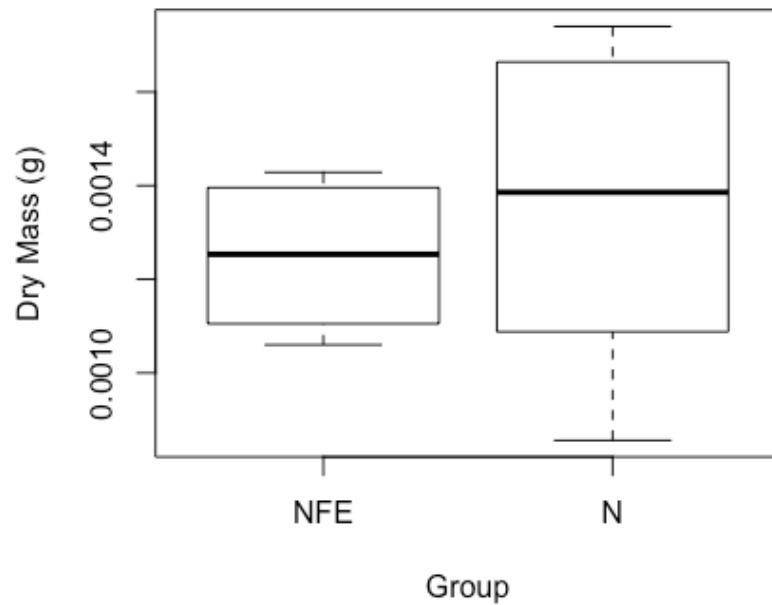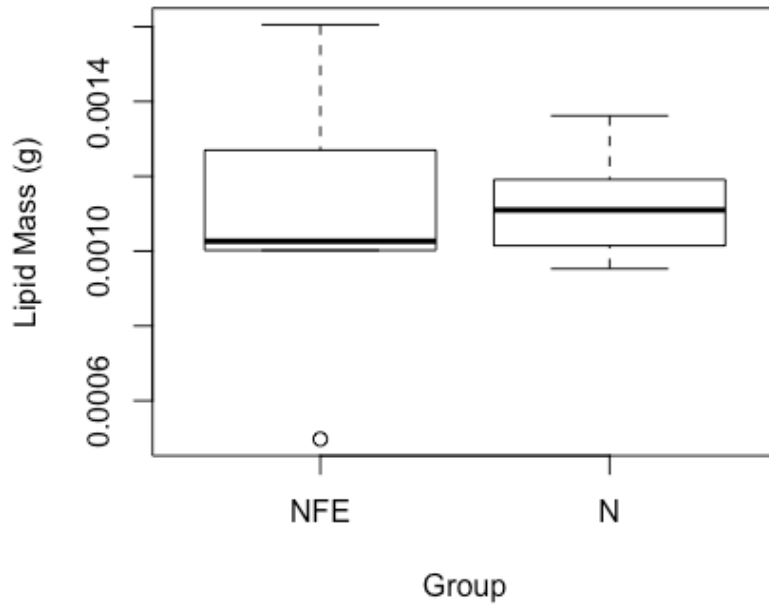413 *contain 50 % of the data whilst whiskers contain data within 1.5x the interquartile range.*

414

415 **Fig. 4** *Boxplot of lipid mass comparison of newly-fertilised* Hippocampus abdominalis *eggs (NFE) and neonates (N). A one-*

416 *way ANOVA revealed no significant difference (P=0.752). The bold lines indicate the median for each group, and the boxes*

417 *contain 50 % of the data whilst whiskers contain data within 1.5x the interquartile range. Outliers are plotted as open*

418 *circles.*

419

22

420

421 **Fig. 5** *Late stage (15-20 day)* Hippocampus abdominalis *embryos, which are still absorbing maternal yolk (arrow) through*

422 *their abdominal epidermis.*

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SUPPLEMENTARY MATERIALS

618   *Table S1. Raw data for* Hippocampus abdominalis *wet weight, dry weight and lipid weight.*

Clutch ID	Replicate ID	No. offspring per Sample	Wet Weight per Sample (g)	Dry weight per Sample (g)	Lipids per Sample (g)
Neonates, clutch 1	1	5	0.0615	0.0078	0.0085
Neonates, clutch 1	2	5	0.0577	0.0084	0.0099
Neonates, clutch 1	3	5	0.0651	0.0094	0.0087
Neonates, clutch 1	4	5	0.0598	0.0089	0.0079
Neonates, clutch 1	5	5	0.0658	0.0090	0.0061
Neonates, clutch 1	6	5	0.0621	0.0094	0.0078
Neonates, clutch 1	7	5	0.0750	0.0097	0.0057
Neonates, clutch 1	8	5	0.0600	0.0055	0.0052
Neonates, clutch 1	9	5	0.0678	0.0096	0.0052
Neonates, clutch 1	10	5	0.0511	0.0024	0.0046
Neonates, clutch 1	11	5	0.0607	0.0098	0.0082
Neonates, clutch 1	12	5	0.0705	0.0101	0.0053
Neonates, clutch 2	1	5	0.0406	0.0081	0.0021
Neonates, clutch 2	2	5	0.0489	0.0091	0.0047
Neonates, clutch 2	3	5	0.0361	0.0082	0.0050
Neonates, clutch 2	4	5	0.0389	0.0083	0.0079
Neonates, clutch 2	5	3	0.0236	0.0047	0.0060
Neonates, clutch 3	1	5	0.0614	0.0087	0.0076
Neonates, clutch 4	1	5	0.0517	0.0072	0.0067
Neonates, clutch 4	2	5	0.0522	0.0074	0.0066
Neonates, clutch 4	3	5	0.0406	0.0053	0.0041
Neonates, clutch 4	4	5	0.0360	0.0055	0.0077
Neonates, clutch 4	5	5	0.0389	0.0051	0.0067
Neonates, clutch 4	6	5	0.0384	0.0052	-
Neonates, clutch 4	7	5	0.0365	0.0051	0.0044
Neonates, clutch 4	8	5	0.0351	0.0057	-
Neonates, clutch 4	9	5	0.0435	0.0055	0.0066
Neonates, clutch 4	10	5	0.0350	0.0055	0.0062
Neonates, clutch 4	11	5	0.0380	0.0058	0.0057
Neonates, clutch 4	12	5	0.0386	0.0060	0.0060
Neonates, clutch 4	13	5	0.0379	0.0054	0.0092
Neonates, clutch 4	14	5	0.0399	0.0056	0.0048
Neonates, clutch 4	15	5	0.0348	0.0058	0.0080

Neonates, clutch 4	16	5	0.0337	0.0057	0.0066
Neonates, clutch 4	17	5	0.0410	0.0059	0.0055
Neonates, clutch 4	18	5	0.0348	0.0057	0.0056
Neonates, clutch 4	19	5	0.0414	0.0054	0.0094
Neonates, clutch 4	20	5	0.0365	0.0056	0.0029
Neonates, clutch 4	21	5	0.0377	0.0061	0.0022
Neonates, clutch 4	22	5	0.0374	0.0059	0.0059
Neonates, clutch 4	23	2	0.0140	0.0019	0.0054
Neonates, clutch 5	1	5	0.0517	0.0081	0.0060
Neonates, clutch 5	2	5	0.0464	0.0068	0.0051
Neonates, clutch 5	3	5	0.0510	0.0081	0.0070
Neonates, clutch 5	4	5	0.0507	0.0081	0.0070
Neonates, clutch 5	5	5	0.0434	0.0062	0.0096
Neonates, clutch 5	6	5	0.0422	0.0057	0.0072
Neonates, clutch 5	7	2	0.0178	0.0022	0.0021
Neonates, clutch 6	1	5	0.0392	0.0045	0.0053
Neonates, clutch 6	2	5	0.0381	0.0047	0.0088
Neonates, clutch 6	3	5	0.0372	0.0047	0.0053
Neonates, clutch 6	4	5	0.0413	0.0052	0.0007
Neonates, clutch 6	5	5	0.0421	0.0053	0.0026
Neonates, clutch 6	6	5	0.0434	0.0055	0.0037
Neonates, clutch 6	7	5	0.0404	0.0051	0.0051
Neonates, clutch 6	8	5	0.0354	0.0050	0.0077
Neonates, clutch 6	9	5	0.0368	0.0051	0.0026
Neonates, clutch 6	10	5	0.0376	0.0055	0.0177
Neonates, clutch 6	11	5	0.0368	0.0055	0.0038
Neonates, clutch 6	12	5	0.0381	0.0060	0.0074
Neonates, clutch 6	13	5	0.0387	0.0052	0.0015
Neonates, clutch 7	1	5	0.0368	0.0048	0.0066
Neonates, clutch 7	2	5	0.0341	0.0044	0.0061
Neonates, clutch 7	3	5	0.0341	0.0043	0.0040
Neonates, clutch 7	4	5	0.0351	0.0046	0.0045
Neonates, clutch 7	5	5	0.0432	0.0046	0.0073
Neonates, clutch 7	6	5	0.0384	0.0047	0.0045
Neonates, clutch 7	7	5	0.0398	0.0045	0.0053
Neonates, clutch 7	8	5	0.0342	0.0045	0.0054
Neonates, clutch 7	9	5	0.0342	0.0047	0.0062
Neonates, clutch 7	10	5	0.0273	0.0017	0.0090
Embryos, clutch 1	1	5	0.0357	0.0055	0.0077
Embryos, clutch 1	2	5	0.0212	0.0073	0.0068

Embryos, clutch 1	3	4	0.0352	0.0054	0.0060
Embryos, clutch 1	4	5	0.0369	0.0077	0.0028
Embryos, clutch 1	5	5	0.0562	0.0073	0.0068
Embryos, clutch 1	6	5	0.0398	0.0075	0.0075
Embryos, clutch 1	7	5	0.0404	0.0068	0.0017
Embryos, clutch 2	1	5	0.0181	0.0071	0.0067
Embryos, clutch 2	2	5	0.0159	0.0063	0.0049
Embryos, clutch 3	1	2	0.0090	0.0022	0.0085
Embryos, clutch 3	2	5	0.0235	0.0056	0.0056
Embryos, clutch 3	3	5	0.0429	0.0053	0.0072
Embryos, clutch 3	4	5	0.0162	0.0048	0.0024
Embryos, clutch 4	1	4	0.0116	0.0045	0.0027
Embryos, clutch 4	2	5	0.0227	0.0049	0.0044
Embryos, clutch 4	3	5	0.0322	0.0064	0.0021
Embryos, clutch 4	4	5	0.0284	0.0063	0.0058
Embryos, clutch 4	5	5	0.0157	0.0044	0.0009
Embryos, clutch 5	1	5	0.0147	0.0072	0.0089
Embryos, clutch 5	2	5	0.0178	0.0071	0.0058
Embryos, clutch 5	3	4	0.0153	0.0057	0.0029
Embryos, clutch 6	1	3	0.0108	0.0035	0.0046

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