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Brooding fathers, not siblings, take up nutrients from embryos

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It is well known that many animals with placenta-like structures provide their embryos with nutrients and oxygen. However, we demonstrate here that nutrients can pass the other way, from embryos to the parent. The study was done on a pipefish, *Syngnathus typhle*, in which males brood fertilized eggs in a brood pouch for several weeks. Earlier research has found a reduction of embryo numbers during the brooding period, but the fate of the nutrients from these 'reduced' embryos has been unknown. In this study, we considered whether (i) the brooding male absorbs the nutrients, (ii) siblings absorb them, or (iii) a combination of both. Males were mated to two sets of females, one of which had radioactively labelled eggs (using ^{14}C-labelled amino acids), such that approximately half the eggs in the brood pouch were labelled. This allowed us to trace nutrient uptake from these embryos. We detected that ^{14}C-labelled amino acids were transferred to the male brood pouch, liver and muscle tissue. However, we did not detect any significant ^{14}C-labelled amino-acid absorption by the non-labelled half-siblings in the brood pouch. Thus, we show, to our knowledge, for the first time, that males absorb nutrients derived from embryos through their paternal brood pouch.

Keywords: brood reduction; embryo absorption; female competition; post-mating sexual selection; filial cannibalism; nurse eggs

1. INTRODUCTION

Parental care is commonly considered to increase the fitness of the brood being cared for (Clutton-Brock 1991). However, allocation of care among young is often the result of parental trade-offs between current and future reproduction, which may result in conflicts between parents or between parent and offspring, but can also result from conflicts among offspring. In some cases, caring parents may even finish some or all of their offspring, thus seemingly reducing their own fitness (Klug & Bonsall 2007). Yet, since caring males may lose weight and fat reserves while caring, brood reduction may actually enhance parental fitness, if males can use some of their own offspring as a nutritional resource. Such brood reduction in the form of filial cannibalism is well known to occur in fishes with male parental care (Manica 2002). The female providing eggs to the caring male may also benefit, if consuming the brood makes the male a better caregiver for the remaining offspring (Lindström 2000). However, if the male uses the energy for his own use or allocates it to unrelated broods, there may be a conflict between the female and the caring male (Lindström 2000).

When a paternally caring male reduces some of his offspring, he also has to decide which offspring should be reduced. If males favour one female's eggs or offspring over others, or choose to terminate the eggs of certain females, there could be post-copulatory male choice (Haig 1990; Ahnesjö 1996; Lessells 2002; Klug & Bonsall 2007). In general, selection is expected to favour a reduction in brood size if this reduction increases the fitness of the number of young that survive by more than the decreased fitness from the reduced young (Brockelman 1975; Bonabeau *et al.* 1998). Moreover, the reduced young may gain fitness through siblings, i.e. inclusive fitness, if these young contribute to the fitness of their siblings (Brockelman 1975; Bonabeau *et al.* 1998). Yet, unless there is complete parental control of resource allocation, offspring may also compete among themselves to receive a larger share of the parental investment (Parker *et al.* 2002).

Parents are expected to adjust their parental care investment in relation to variation in parental benefits or costs from the different offspring in such a way that it maximizes parental fitness (Trivers 1972; Clutton-Brock 1991; Lessells 2002). For instance, bigger offspring may be of greater value to parents than smaller offspring in the same brood, since larger offspring often have a higher chance of survival. If so, parents might be prepared to take greater risks to ensure the survival of larger, more valuable, offspring than of smaller offspring (Brockelman 1975; Sinervo & McEdward 1988; Nussbaum & Schultz 1989; Clutton-Brock 1991). It is not certain that parents loose fitness when an offspring dies (Mock & Forbes 1995). Parents sometimes overproduce offspring to ensure that extra offspring can be brought up under very favourable conditions or as a replacement for

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offspring that fails to develop, as found in some birds (Mock & Parker 1998). Parents may also place an excess of offspring into an arena (e.g. a nest or a brood pouch) to be able to promote offspring that have better fitness prospects and eliminate others, either by filial infanticide or by allowing offspring to pursue sibling competition (Forbes & Mock 1998).

Viviparous species may transfer nutrients to their embryos in various ways, either through the other embryos (oophagy and adelphophagy), from the parent through placental analogues or through the yolk sac placenta (Wourms 1981). Oophagy and adelphophagy represent forms of sibling competition that may reduce parental fitness (Trivers 1974). However, when parents 'overproduce' offspring, the extra siblings may be expendable as a nutrient source for the other offspring. Examples of overproduction of offspring are nurse eggs (which are embryos consumed by the other, simultaneously reared, embryos; Watanabe *et al.* 1999; Ripley & Foran 2006), as found in sharks (Wourms 1977; Gilmore 1993) and starfish (Byrne 1996), or as in some gastropods where undeveloped eggs serve as extra food source (Cubillos *et al.* 2007). Alternatively, when some offspring suffer from developmental failures or slower development (Forbes & Lamey 1996; Cubillos *et al.* 2007), parents may conserve resources by being able to absorb or feed on the resources from such offspring, thereby recycling resources that otherwise would have been wasted (resource conservation).

In several genera of the family Syngnathidae (pipefishes, seadragons and seahorses) embryos develop in a brood pouch in close association with the brood pouch epithelium (Dawson 1985; Wilson *et al.* 2001; Dzyuba *et al.* 2006; Stölting & Wilson 2007). These structures are thought to protect, aerate, osmotically buffer and nourish the embryos (Quast & Howe 1980; Azzarello 1991; Carcupino *et al.* 1997, 2002; Ripley & Foran 2009). The brood pouch has also been suggested to facilitate transfer of steroids and growth hormones to the embryos, but this function has yet to be fully investigated (Haresign & Shumway 1981; Azzarello 1991; Ripley & Foran 2006). Among syngnathids, the brooding structure is highly variable, from the simple attachment of embryos to the male's body to more complex placenta-like brood pouches and sacs (Vincent *et al.* 1995; Wilson *et al.* 2001, 2003; Carcupino *et al.* 2002; Ripley & Foran 2006, 2009; Stölting & Wilson 2007).

The potential for adaptive brood reduction in syngnathids is high. Ahnesjö (1992*b*, 1996) documented substantial brood reduction in *Syngnathus typhle* and found that large embryos did better than smaller embryos in broods of mixed egg sizes. If larger embryos have a competitive advantage over smaller embryos, we would expect more of the smaller embryos to be reduced and possibly end up as nutrition for the larger embryos (Ahnesjö 1996). In the pipefish *Syngnathus floridae* Ripley & Foran (2006) detected yolk in the pouch fluid. They suggested that these yolk droplets, emanating from reduced embryos, were used by the other embryos as a nutrient source. In this case, they suggested that a brooding male may use nurse eggs as a direct food source for the embryos in the pouch (Ripley & Foran 2006). Here, we define nurse eggs as a subset of eggs or embryos that are absorbed by other embryos, presumably to enhance their growth and survival, as has been implicated in *Syngnathus schlegeli* (Watanabe *et al.* 1999) and *S. floridae* (Ripley & Foran 2006).

Our aim in this study was to trace nutrients that originate from reduced embryos in the paternally brooding pipefish, *S. typhle*. This was done by radioactively labelling eggs in females. The males were then mated to two sets of females, one of which had ^{14}C-labelled eggs, such that approximately half of the eggs in the male's brood pouch were labelled. After about two weeks of brooding, the radioactivity was traced to see if there was (i) paternal uptake, (ii) uptake by unlabelled half-siblings in the pouch (which would indicate a use of nurse eggs), or (iii) a combination of both paternal uptake and sibling uptake.

2. MATERIAL AND METHODS

(a) *Study species*

The broad-nosed pipefish, *S. typhle*, inhabits waters along the coasts of Europe. It is found in shallow eelgrass (*Zostera marina*) meadows during the breeding season, which in our study area is from early May to August. This population is sex-role reversed (i.e. females compete among themselves for mating opportunities with male partners) and polygynandrous (Berglund *et al.* 1989; Jones *et al.* 1999*b*). The fish may live for two or more years and larger females produce larger and more eggs (Berglund *et al.* 1986). The males are known to prefer larger females as mating partners (Berglund *et al.* 1986; Berglund & Rosenqvist 1993), and a male typically broods embryos from several females in his brood pouch (Jones *et al.* 1999*a*,*b*). Brood reduction occurs naturally, and anything from none to all of the embryos in a brood may disappear during the month of brooding (Ahnesjö 1992*a*).

(b) *Husbandry*

Syngnathus typhle were caught in eelgrass meadows nearby Kristineberg Marine Research Station at the Swedish west coast (58°15′ N, 11°28′ E) in May 2006, just before the breeding season started, allowing us to catch unmated males with empty brood pouches. We kept males and females in separate storage tanks (225 l, on average 60 fish per tank). All tanks contained artificial eelgrass and had continuously renewed natural sea water, with temperature maintained at 14°C, and artificial light from 06.00 to 24.00. The fish were fed live brine shrimp (*Artemia* sp.) enriched with Easy DHA Selco (INVE aquaculture) and wild-caught crustaceans (*Crangon crangon*, *Mysidae* and *Copepoda*) ad libitum three times a day.

(c) *Egg labelling method*

To label eggs, we used ^{14}C-labelled amino acids (Amersham Biosciences, [^{14}C(U)]-L-amino-acid mixture, product code CFB25-50UCI), which contained 16 different essential amino acids. The females were first sedated in 2-phenoxy ethanol (100 µl l^{-1}) for 30–50 s and their standard length (SL) was also measured. We filled 20 µl of a mixture of red dye (Ekström food colour) and ^{14}C-amino-acid mixture (in total 1 µCi fish^{-1}), or 20 µl of red dye and water, into a 25 µl Hamilton syringe fitted with a fine, 0.96 mm wide, intramedic polyethylene tube (VWR, PE50). The food colour allowed us to monitor both liquid flow into each fish stomach and to make sure that they did not expel the solution. The tube was inserted from the mouth through the oesophagus and into the middle of the stomach of the fish

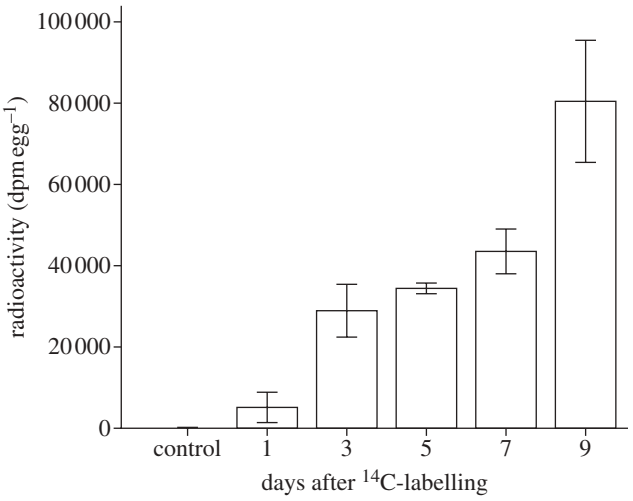

Figure 1. Radioactivity measured as dpm per egg (dpm egg^{-1}, mean ± s.e.) of eggs deposited into the brood pouches of *S. typhle* males 1–9 days after labelling the females ($n = 3$ for control and day 1, $n = 2$ for day 3–9). These data are from the method study done in 2005.

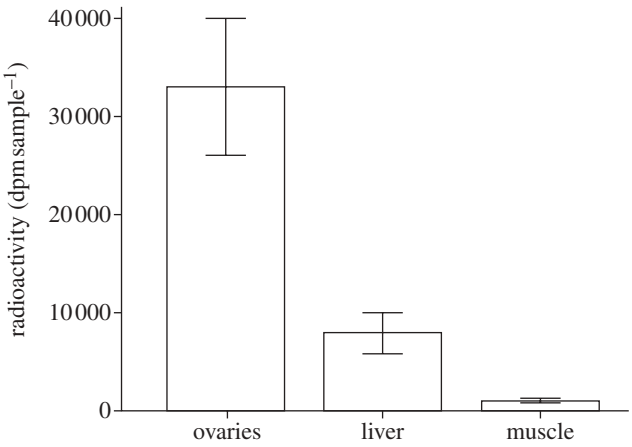

Figure 2. Radioactivity measured as dpm per sample (dpm sample^{-1}, mean ± s.e.) of ovary, liver and body muscle tissue in *S. typhle* females ($n = 12$) 13 days after being tube-fed with the ^{14}C-amino-acid mixture. These data are from the method study done in 2005.

and the content of the syringe was emptied into the stomach of the fish. The females were then left in a bowl of fresh sea water to recover before they were put into their assigned mating aquaria.

In 2005, a method study was performed to find out how long it would take females to incorporate the radioactive amino acids into their eggs. Females ($n = 12$) were tube fed in the same manner as above. These labelled females were then put into a mating tank where two to three males were available for mating from the first day. The mated males were then replaced with new males for mating 3, 5, 7 and 9 days after labelling. The embryos of the mated males were analysed for radioactivity, as described below. The radioactivity in the eggs increased over time from labelling (figure 1) and 7 days was found to be an appropriate time frame for females to be able to provide males with radioactively labelled eggs. Furthermore, the females were sacrificed on day 13 and their ovaries, liver and body muscle tissue were analysed for radioactivity. The ovaries still showed high levels of radioactivity and were most radioactive among these tissues (figure 2).

In the 2006 experiment, we tube fed 20 µl of ^{14}C-labelled amino-acid mixture (in total 1 µCi fish^{-1}) to eight large (207–223 mm SL) and 13 small (153–188 mm SL) females. We also tube fed nine large (205–229 mm SL) and 15 small (154–188 mm SL) females with 20 µl non-labelled red coloured water. The females were left one week after labelling to incorporate the ^{14}C-amino acids into their eggs. Females were then mated to males (147–213 mm SL) according to the following procedure: in the first treatment, males first obtained eggs from large non-labelled females and then from small ^{14}C-labelled females, or in the reversed order. In the second treatment, males first received eggs from large ^{14}C-labelled females and then from small non-labelled females, or in the reversed order. Thus, these four groups of females (small and large, with and without radioactive labelling) were kept in four separate mating tanks and each male was moved from one tank to another, after the pouch was filled to roughly 50 per cent by his first type of females. The division between ^{14}C-labelled and non-labelled eggs, or *vice versa*, in the brood pouch was marked with a single black dot tattooed on the left side of the tail, using a carbon-based, non-toxic ink and a tattoo needle. To prevent females from completely filling up males with eggs on their first mating, we confined males by keeping them in net bags (8 l) inside the aquaria whenever we were unable to monitor them.

All matings took place within a period of 8 days. All females were sacrificed after the last mating in order to record radioactivity in the eggs remaining in the ovaries. Males were left to brood the embryos for up to 16 days, and were thereafter sacrificed, unless prematurely terminated owing to sickness or death ($n = 6$). The fish were euthanized using 2 ml l^{-1} 2-phenoxy ethanol, and then stored in a −20°C freezer, awaiting later dissection.

For the analysis we had 17 experimental males that carried ^{14}C-labelled eggs and three control males. At the beginning of the experiment, we had more males; however, some males did not mate with both types of females ($n = 6$) and some males did not get radioactive eggs from ^{14}C-labelled females ($n = 6$). The reason why some eggs were not radioactive could be that the females spawned eggs that had matured before we labelled them with ^{14}C-amino acids.

(d) *Analysis of radioactivity in tissue samples*

Tissue samples were taken by dissecting each male's pouch for five to seven non-labelled embryos, five to seven ^{14}C-labelled embryos, pouch tissue in close proximity to (i.e. above) non-labelled and labelled embryos, liver and muscle tissue. We also took five to seven mature eggs from the ovaries of each female used in the experiment. Each sample was placed in a glass vial (7 ml, Perkin-Elmer) and freeze-dried for 24 h. The dry samples were weighed to the nearest 0.01 mg, using a Sartorius LE26P microbalance, and then dissolved with 0.3 ml Soluene-350 (Perkin-Elmer) in a 65°C water bath for at least 21 h. When all tissues had dissolved, 5 ml of scintillation fluid (Hionic-Fluor, Perkin-Elmer) was added to each vial and the vials were left to develop in a dark cupboard for at least 24 h. The vials were then analysed using a liquid scintillator (Beckman Coulter LS 5000TD) and disintegrations per minute (dpm) were measured for a maximum of 20 min. To correct for different kinds of tissue blocking the signal to different extents, we prepared three separate quench curves for each of embryo/egg tissue, pouch/muscle tissue and liver tissue.

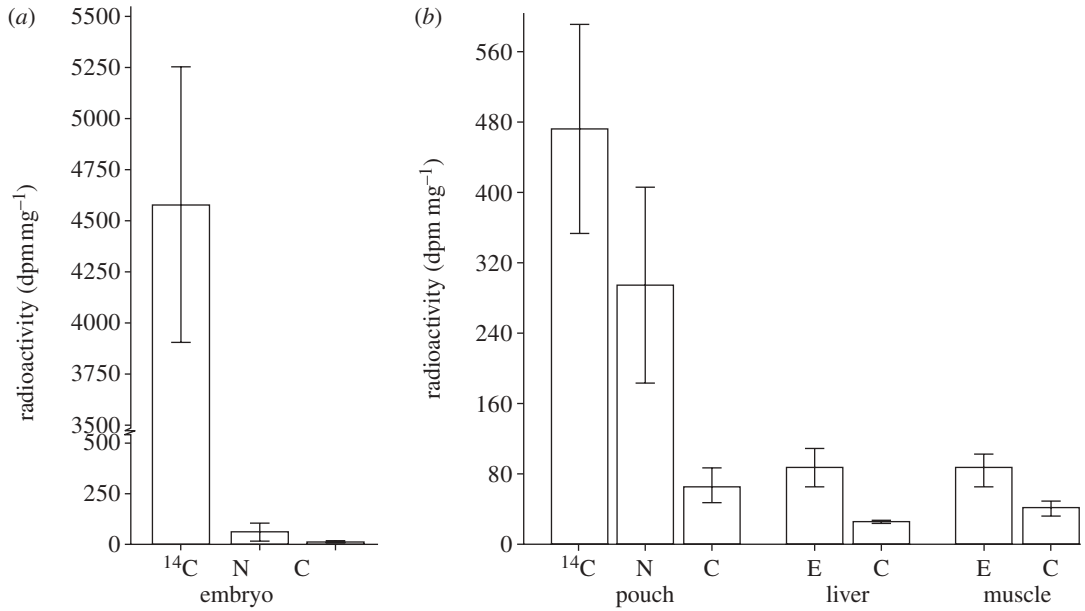

Figure 3. Radioactivity measured in *S. typhle* as disintegrations per minute per milligram tissue (dpm mg^{-1}, mean ± s.e.). The samples were taken from (*a*) ^{14}C-labelled embryos (^{14}C), non-labelled embryos (N) in the experimental males and control embryos (C), and (*b*) different tissues from brooding males. In the experimental males, tissue samples were taken from the pouch above ^{14}C-labelled embryos (^{14}C), pouch above non-labelled embryos (N), and from control males' pouch (C). Liver and muscle tissues were taken from experimental (E) and control (C) males. Note the break in the scale in panel (*a*). These data are from the experimental study done in 2006.

(e) *Statistical analysis*

We calculated radioactivity as dpm per mg dry tissue (dpm mg^{-1}), to correct each measurement for differences in sample tissue mass (not done in the 2005 method study) and $\log(X+2)$ transformed all data to achieve normal distribution. We used the average radioactivity for each of the different tissues in the control males as a base level and tested the radioactivity in the different tissues of the experimental males, using a one-sample *t*-test, to see if these values differed significantly from the base levels. Sequential Bonferroni correction was also applied (Rice 1989). All statistics were carried out using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) and all values were presented in mean ± s.e. unless otherwise specified.

3. RESULTS

In the experimental study, the ^{14}C-labelled eggs taken from the females after the last mating ($n = 18$) had, on average, a radioactivity level of 2861 ± 527 dpm mg^{-1} (range 756–8399 dpm mg^{-1}). The corresponding level for the non-labelled eggs ($n = 22$) taken from control females was 8.59 ± 0.52 dpm mg^{-1} (range 5.59–16.29 dpm mg^{-1}). Thus, the eggs of the ^{14}C-labelled females still contained very high levels of radioactivity at the end of the experiment. The radioactive level in labelled embryos in the experimental males ($n = 17$) was on average 4578 ± 678 dpm mg^{-1} (range 1169–12 807 dpm mg^{-1}). The radioactive level in the embryos of control males was on average 80 ± 31 dpm mg^{-1} (range 50–141 dpm mg^{-1}). Since the range of the labelled embryos and the control embryos never overlap, we conclude that all the 17 experimental males received radioactive embryos. The labelled eggs from small radioactive females ($n = 7$) had on average 6683 ± 1157 dpm mg^{-1} (range 4101–12 807). The labelled eggs from large radioactive females ($n = 10$) had on average 3105 ± 421 dpm mg^{-1} (range 1169–5060).

When testing if the experimental tissue values were significantly different from the control base levels, we found that the non-labelled embryos did not contain larger amounts of radioactivity than control embryos (*t*-test, $t = 1.69$, d.f. = 16, $p = 0.11$; figure 3*a*). However, the brood pouch did have higher levels of radioactivity in the experimental males than in the control males (pouch tissue above labelled embryos: *t*-test, $t = 7.13$, d.f. = 16, $p < 0.001$, pouch tissue above non-labelled embryos: *t*-test, $t = 3.35$, d.f. = 16, $p < 0.01$; figure 3*b*). The liver of experimental males also contained significantly higher radioactivity than that of control males (*t*-test, $t = 5.10$, d.f. = 16, $p < 0.001$; figure 3*b*). Furthermore, the muscle tissue of experimental males also contained higher levels of radioactivity than that of control males (*t*-test, $t = 3.03$, d.f. = 16, $p < 0.01$; figure 3*b*). All significant values remained significant after a sequential Bonferroni correction for $\alpha = 0.025$.

4. DISCUSSION

We have demonstrated that brooding male pipefish do take up radioactivity in the form of ^{14}C-labelled amino acids from labelled embryos. This shows that males are able to take up nutrients that originate from eggs in their brood pouch. We found a significant amount of radioactivity in brood pouch tissue, liver tissue and muscle tissue. The elevated levels of radioactivity can only come from the embryos developing in the brood pouch, as the ^{14}C-amino acids were originally incorporated into the eggs of the females being mated to the males. Brood reduction is a common and well-documented phenomenon in pipefish (Ahnesjö 1992*a*, 1996). Therefore, it is reasonable to assume that the radioactivity

primarily emanates from a paternal uptake of nutrients of radioactive embryos that have been reduced in the pouch, although we cannot exclude that some radioactivity may originate from living, labelled embryos. Next to the ^{14}C-labelled eggs, the highest amount of radioactivity was found in the brood pouch tissue above the ^{14}C-labelled embryos. Therefore, it seems likely that the highly vascularized pouch, rich in blood vessels, allows for the amino acids or their derivates to enter the paternal blood stream and be transported to the liver and muscles of the brooding male (Haresign & Shumway 1981; Carcupino *et al.* 1997, 2002; Watanabe *et al.* 1999).

The fact that there was no nutrient transfer to other embryos in the brood pouch strongly suggests that the uptake was for the male's own use only. Thus, the hypothesis that males may take up and then reallocate the nutrients to the non-labelled embryos did not find any support, as radioactivity in the non-labelled embryos did not differ from the control males' embryos during the time of the experiment. Instead, it seems likely that males allocate the absorbed nutrients to future reproduction or to their own survival, which may benefit both current and future reproduction.

Similarly, we found no support for the hypothesis that other embryos in the pouch may use the amino acids directly from their ^{14}C-labelled half-siblings, based on the same data as above, namely that the radioactivity levels of non-labelled embryos did not differ from embryos of control males. This is an important result as nurse eggs have been suggested to be a possible adaptive explanation for brood reduction in pipefishes (Ahnesjö 1996; Watanabe *et al.* 1999; Ripley & Foran 2006). Still, based on the kin selection theory we might predict nurse eggs to evolve more readily in broods containing only full-siblings rather than half-siblings (Sachs *et al.* 2004). In support of this hypothesis, brood reduction in male *S. typhle* has been indicated to be more pronounced in broods containing only full-siblings as compared to broods including half-siblings (G. Sagebakken, I. Ahnesjö, I. B. Gonçalves & C. Kvarnemo 2007, unpublished data). However, the design of the current experiment does not allow us to trace any transfer of nutrients among full-sibling embryos.

Brood reduction is common in animals in general (Lloyd 1987; Haig 1992; Stanback & Koenig 1992; Lamey *et al.* 1996; Sasvari *et al.* 1999; Manica 2002; Young & Millar 2003; Mock 2004) and it may be affected by many factors. In pipefish, number and size of embryos, number of partners, the quality of eggs, sibling competition and paternal quality may all affect brood reduction. The fact that we found an uptake of proteins from the labelled eggs to the brooding males shows that males can absorb nutrients from their broods. This nutrient uptake resembles filial cannibalism found in many other families of fishes (Manica 2002), but hitherto unheard of in the family Syngnathidae. Similar to many other studies of parental consumption of offspring, we cannot tell whether males kill their offspring or simply use nutrition from dying or impaired embryos. Since two predictions for filial cannibalism are that (i) brooding males are food limited and (ii) the male's own offspring are a food source (Sargent 1992), we would expect males to reduce their embryos more if they are food limited. However, this remains to be investigated. For example, manipulating food availability or condition of brooding males may reveal if males adaptively take up nutrients from embryos to compensate for a reduction in food availability. Additionally, we do not know if and how male absorption of embryos may affect the embryos that are still present in the pouch. Brood reduction may affect such remaining embryos in a positive manner as found in *S. typhle* (Ahnesjö 1992*a*), for instance, by allowing more space or nutrients. On the other hand, brood reduction may be neutral to the fitness of the remaining embryos if the male invests the resources for his own growth or future reproduction. However, based on the current study, we do know that these nutrients are not reallocated to other non-labelled offspring in the brood pouch within the time frame of the study. Yet, since we know that small amounts of amino acids can be transferred from the male to the developing embryos (as shown in two studies in which the males were radioactively labelled but not the embryos; Ripley & Foran 2009; C. Kvarnemo, K. B. Mobley, C. Partridge, A. G. Jones & I. Ahnesjö 2003–2004, unpublished data), it is still possible that a brooding male uses the absorbed nutrients to support the remaining embryos at a later stage in the brood cycle.

In this study, we have found novel and clear evidence of (i) paternal uptake of nutrients from brooded embryos, but (ii) no uptake by non-labelled half-siblings in the brood pouch, and (iii) no reallocation of the paternal uptake to non-labelled half-siblings in the pouch. However, if there is a possible delayed reallocation of nutrients to full- or half-siblings has to be tested further. The possibility for a male to use its embryos as a source of nutrients opens up a scenario that strongly resembles filial cannibalism found in many other paternally caring fishes. Since the male absorbs nutrients that were originally provided by the female, and *S. typhle* do not show long-term pair bonds, such a paternal uptake is likely to generate a strong sexual conflict between the male and female, at least if it benefits future reproduction by the male at the expense of current reproduction. How multiple maternity may interact with paternal nutrient uptake and whether it may be a mechanism for progeny choice remains to be investigated. In conclusion, this study refutes the idea of nurse eggs in this pipefish and improves our understanding of brood reduction, as it demonstrates a previously unknown paternal ability of nutrient uptake from the embryos.

The experiment was approved by the Ethical Committee for Animal Research in Gothenburg (licence number 196-2005).

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