© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 1639-1646 doi:10.1242/jeb.120907

RESEARCH ARTICLE

Embryo oxygenation in pipefish brood pouches: novel insights

Ines Braga Goncalves[1,2,*], Ingrid Ahnesjö[3] and Charlotta Kvarnemo[2]

ABSTRACT

The pipefish brood pouch presents a unique mode of parental care that enables males to protect, osmoregulate, nourish and oxygenate the developing young. Using a very fine O_2 probe, we assessed the extent to which males of the broad-nosed pipefish (*Syngnathus typhle*) oxygenate the developing embryos and are able to maintain pouch fluid O_2 levels when brooding in normoxia (100% O_2 saturation) and hypoxia (40% O_2 saturation) for 24 days. In both treatments, pouch fluid O_2 saturation levels were lower compared with the surrounding water and decreased throughout the brooding period, reflecting greater offspring demand for O_2 during development and/or decreasing paternal ability to provide O_2 to the embryos. Male condition (hepatosomatic index) was negatively affected by hypoxia. Larger males had higher pouch fluid O_2 saturation levels compared with smaller males, and levels were higher in the bottom section of the pouch compared with other sections. Embryo size was positively correlated with O_2 availability, irrespective of their position in the pouch. Two important conclusions can be drawn from our findings. First, our results highlight a potential limitation to brooding within the pouch and dismiss the notion of closed brood pouches as well-oxygenated structures promoting the evolution of larger eggs in syngnathids. Second, we provide direct evidence that paternal care improves with male size in this species. This finding offers an explanation for the documented strong female preference for larger partners because, in terms of oxygenation, the brood pouch can restrict embryo growth.

KEY WORDS: Embryo development, Fish, Hypoxia, Male size, Normoxia, Paternal care, Syngnathidae

INTRODUCTION

Parental care is a phylogenetically widespread phenomenon (Clutton-Brock, 1991; Reynolds et al., 2002). In fish, lack of parental care is the norm, but in species that do provide parental care, paternal care is more common than maternal or biparental care (Gross and Sargent, 1985; Reynolds et al., 2002). Parental care, which in fish is largely expressed as fanning behaviour and brood protection (Blumer, 1979, 1982; Clutton-Brock, 1991; Lindström and St Mary, 2008), has generally been linked to greater offspring survival and to the production of larger eggs (Shine, 1978; Sargent et al., 1987; Kolm and Ahnesjö, 2005).

Aquatic environments have much lower O_2 availability compared with air (Keister et al., 2000). This fact has led to the assumption that egg size evolution is constrained in aquatic environments because of the limited O_2 availability (Rombough, 1988). Because O_2 travels by diffusion through the pores in the egg membrane, it is thought that embryos from larger eggs have greater difficulties acquiring sufficient O_2 for successful development, as a result of the unfavourable surface to volume ratio characteristic of sphere-shaped eggs. This assumption has lingered in the literature for decades despite remaining largely untested (van den Berghe and Gross, 1989; Quinn et al., 1995; Einum et al., 2002). At the same time, the evolution of parental care in fishes has been suggested to have facilitated the evolution of larger egg sizes (reviewed in Kolm and Ahnesjö, 2005), because an important parental task is the provision of O_2 to the developing embryos, through fanning behaviour or choice of well-oxygenated nest sites (Lukas and Orth, 1995; Takegaki and Nakazono, 1999; Takegaki, 2001; Wisenden et al., 2009). In this way, the parental care period can be viewed as a 'safe harbour' to the embryos and, with larger egg sizes, this phase can be prolonged (Shine, 1978; Kolm and Ahnesjö, 2005). Can the syngnathid male pregnancy have a similar function?

Because of their unique modes of parental care and their diverse mating systems, pipefishes and seahorses (family Syngnathidae) have been extensively studied over the past decades. All syngnathids show paternal care by carrying the offspring on their body throughout embryo development. Depending on genus, the eggs may be attached to the skin of the male or nurtured inside a brood pouch, consisting of two skin flaps common among pipefishes, or in a sack-like structure typical of seahorses, placed ventrally on the male's tail or upper body (Herald, 1959; Dawson, 1985; Wilson et al., 2001, 2003). During mating, the female transfers her eggs to the male, by inserting her ovipositor through an opening at the top of the brood pouch or sliding it along the male's body. More complex brood pouches enable males to physically protect the embryos from external factors such as predators, and to provision them with nutrients, osmoregulation and oxygenation during brooding (Wilson et al., 2001). Male provisioning of nutrients to the developing young has been shown in a number of pipefish species (Haresign and Shumway, 1981; Ripley and Foran, 2006, 2009; Kvarnemo et al., 2011). Also, the function of the brood pouch in osmoregulation is well established (Quast and Howe, 1980; Carcupino et al., 1997; Partridge et al., 2007; Ripley, 2009). Oxygenation during brooding, however, has remained surprisingly less explored (Carcupino et al., 2002; Monteiro et al., 2005; Stölting and Wilson, 2007), although the presence of a vascularised dermis in all pouch types studied so far suggests a common respiratory function to all species, independent of other types of care provided during brooding (Carcupino et al., 2002).

Here, we used the broad-nosed pipefish, *Syngnathus typhle* Linnaeus, to explore paternal ability to actively provide O_2 for successful embryo development. Females mature large, nutritious eggs (egg diameter range 1.5–2.2 mm; Braga Goncalves et al., 2010, 2011) continuously, allowing them to mate and transfer multiple batches of eggs within a short period of time (Sogabe and

[1]Institute of Evolutionary Biology and Environmental Studies, Animal Behaviour, University of Zurich, Winterthurerstrasse 190, Zürich 8057, Switzerland. [2]Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, Gothenburg SE-40530, Sweden. [3]Department of Ecology and Genetics/Animal Ecology, Uppsala University, Norbyvägen 18D, Uppsala SE-75236, Sweden.

*Author for correspondence (ines.goncalves@ieu.uzh.ch)

Received 10 February 2015; Accepted 23 March 2015

The Journal of Experimental Biology (2015) 218, 1639-1646 doi:10.1242/jeb.120907

Ahnesjö, 2011). Males typically mate with one or several females, until their pouches are filled, at which point they commence brooding (Berglund et al., 1988, 1989; Jones et al., 1999). The number of embryos a fully mated male can brood at a time can vary substantially (between 30 and 180 embryos) because, as males grow, the brood pouch becomes longer and wider, allowing males to accommodate more and longer rows of embryos (I.B.G., I.A. and C.K., personal observation). Similar to *Syngnathus floridae* (Ripley and Foran, 2006), *S. typhle* males have two ventral pouch flaps along the tail that seal in the middle at the onset of brooding, isolating the embryos from the external environment (Fig. 1). At parturition, the seal is broken, allowing the pouch flaps to separate, and the free-living juveniles are expelled from the pouch by paternal shakes and body contractions. Because *S. typhle* males provide extensive care to the developing offspring during a prolonged brooding period (Berglund et al., 1986b), this is an ideal species in which to investigate paternal ability to oxygenate the embryos and to maintain O_2 levels during a significant proportion of the brooding period, in both high and low ambient O_2 conditions. By exploring brooding males' ability to provide O_2 to the developing embryos, we provide insight into the hypothesis that paternal embryo oxygenation may have promoted the evolution of larger eggs in the syngnathid species that have enclosed brood pouches (reviewed in Braga Goncalves et al., 2010; Braga Goncalves et al., 2011).

The aims of this study were to measure pouch fluid O_2 saturation levels at regular intervals (every 6 days) and in different sections of the pouch during 24 days of brooding, and assess whether: (1) males are able to maintain O_2 saturation levels in the pouch fluid that are high enough to support embryo development, even in low ambient O_2, (2) pouch fluid O_2 saturation levels are uniform throughout the brood pouch, (3) embryo size and survival are affected by long-lasting low ambient O_2, and (4) male body size affects paternal ability to oxygenate the embryos. Under normoxia, we expected brooding males to be able to provide O_2 to sustain normal embryo development, and to be able to keep pouch fluid O_2 saturation levels similar to the surrounding water. Under hypoxia, however, male condition should be negatively affected, as O_2 demands of both the male and the offspring are expected to increase while metabolic efficiency is decreased (Kramer, 1987). For that reason, we expected a reduced ability of males brooding in hypoxia to oxygenate the developing embryos and this impact was expected to be more noticeable at more advanced stages of development, as embryos then are larger and require more O_2 (Berglund et al., 1986b). Like other fishes, *S. typhle* individuals grow throughout their lives and it is well documented that larger males are preferred partners and have greater reproductive success than smaller males (Berglund and Rosenqvist, 1990, 2003; Ahnesjö, 1992b). Thus, as small males brood fewer embryos (lower reproductive value) because of their own smaller size, they may gain more from directing their resources into self-maintenance and growth to improve their future reproductive success than from providing excellent care. In other words, we might expect small males to provide lower quality of care to the developing embryos, here measured as lower pouch fluid O_2 saturation levels.

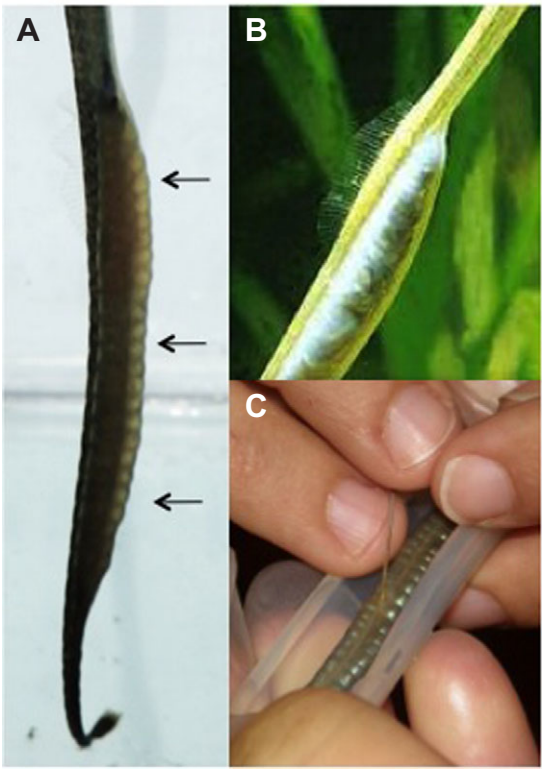

Fig. 1. The *Syngnathus typhle* male brood pouch. (A) Side view of the brood pouch of a newly mated male with arrows depicting the top, middle and bottom entrance points where pouch fluid O_2 saturation levels were measured (photo credit: Gry Sagebakken). (B) Close up of the brood pouch of a male at an advanced stage of pregnancy (photo credit: Ola Jennersten). (C) Measuring O_2 saturation levels in the middle section of the brood pouch of a pregnant male (photo credit: Linus Hammar).

RESULTS

Pouch fluid O_2 saturation levels

Analysis of pouch fluid O_2 saturation levels revealed several significant effects (Table 1). Male standard body length (SL) had a significant effect on O_2 saturation levels (Table 1), with larger males having, on average, higher pouch fluid O_2 levels (Table 1, Fig. 2). Despite high individual variation and significant male × day variation, males brooding in hypoxia showed significantly lower

Table 1. Permutational nested ANCOVA on pouch fluid O_2 saturation levels in *Syngnathus typhle*

Source	d.f.	MS	Pseudo-*F*	P_{perm}
Length	1	0.50	4.50	0.04[1]
O_2 treatment	1	4.38	57.96	<0.01[1]
Pouch section	2	0.15	5.86	<0.01[3]
Day	3	0.85	8.04	<0.01[2]
Tank (treatment)	4	0.06	0.53	0.71
Treatment × pouch section	2	0.04	1.46	0.24
Treatment × day	3	0.08	0.75	0.58
Pouch section × day	6	0.04	1.14	0.38
Male [tank (treatment)]	27	0.12	4.57	<0.001
Tank (treatment) × pouch	8	0.03	1.39	0.22
Tank (treatment) × day	12	0.10	1.65	0.10
Treatment × pouch section × day	6	0.03	0.83	0.55
Male [tank (treatment)] × pouch section	56	0.02	0.80	0.83
Male [tank (treatment)] × day	84	0.06	2.25	<0.001
Tank (treatment) × pouch section × day	24	0.04	1.29	0.17
Residual	168	0.03		
Total	407			

Analyses were done on arcsine-transformed values. Pooled terms had P_{perm}>0.24.
[1]Term mean square tested against the pooled mean squares of male and tank.
[2]Term mean square tested against the pooled mean squares of male×day, tank×day and treatment×day.
[3]Term mean square tested against the pooled mean squares of the residuals, treatment×pouch section, pouch section×day, tank×pouch section, treatment×pouch section×day, male×pouch section and tank×pouch section×day.

The Journal of Experimental Biology

The Journal of Experimental Biology (2015) 218, 1639-1646 doi:10.1242/jeb.120907

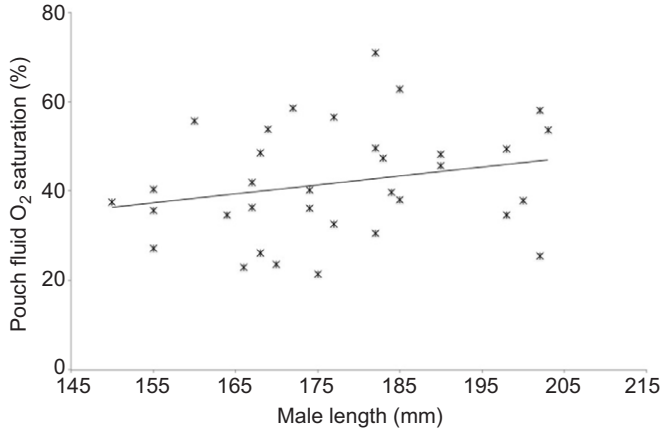

Fig. 2. The overall relationship between *S. typhle* male standard length and pouch fluid O_2 saturation. Data are for normoxic and hypoxic oxygen treatments combined and are averaged across pouch sections and brooding days (*N*=34). See Table 1 for analyses.

pouch fluid O_2 saturation levels (mean±s.e. of all days and pouch sections combined for each male, normoxia: 51.3±2.4%, hypoxia: 32.5±1.7%; Table 1). In both treatments combined, O_2 saturation levels in the bottom section of the pouch (45.1±2.2% O_2) were significantly higher than in the middle section (39.1±2.0% O_2, t=3.75, d.f.=135, permutational probability P_{perm}<0.001) and tended to be higher than in the top section of the pouch (41.2 ±2.6% O_2, t=1.85, d.f.=135, P_{perm}=0.071), whereas top and middle sections did not differ significantly (t=1.34, d.f.=135, P_{perm}=0.183). Furthermore, average O_2 saturation levels decreased over the course of the brooding period (Table 1, Fig. 3). Pair-wise *post hoc* comparisons showed that, irrespective of treatment, O_2 levels were significantly higher on day 6 than on any other day (day 6–day 12: t=3.51, d.f.=33, P_{perm}=0.002; day 6–day 18: t=4.26, d.f.=33, P_{perm}<0.001; day 6–day 24: t=5.36, d.f.=33, P_{perm}<0.001). O_2 levels were also significantly higher on day 12 than on day 24 (t=2.21, d.f.=33, P_{perm}=0.031) but not on day 18 compared with day 24 (t=1.68, d.f.=33, P_{perm}=0.096; Fig. 3).

Embryo survival and embryo dry mass

Despite significant variation between brooding aquaria, reduced O_2 treatment affected overall embryo survival and dry mass negatively

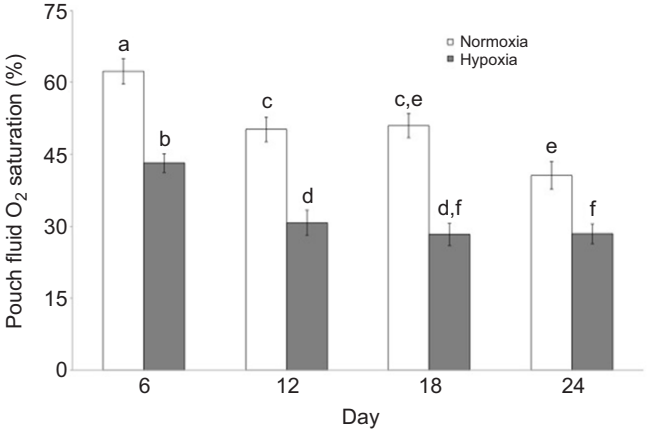

Fig. 3. Mean (±s.e.) pouch fluid O_2 saturation in *S. typhle* males brooding in normoxic or hypoxic conditions. Normoxia: 100% O_2 (*N*=17), hypoxia: 40% O_2 (*N*=17). Data were recorded on days 6, 12, 18 and 24 of the brooding period. Significant differences are indicated by different letters.

Table 2. Permutational nested-MANOVA showing the overall effects of O_2 treatment and tank (nested within O_2 treatment) on *S. typhle* embryo survival and dry mass after 24 days of brooding

Source	d.f.	MS	Pseudo-*F*	P_{perm}
O_2 treatment	1	15.45	4.70	0.02
Tank (treatment)	4	3.60	2.44	0.03
Residual	28	1.48		
Total	33			

O_2 treatment: normoxia (100% O_2) versus hypoxia (40% O_2).
Analyses (multivariate test) were performed on transformed and normalised variables.

(Table 2). Separately, we detected no effects of treatment or tank on relative embryo survival, but embryo mass was significantly lower in the hypoxia treatment (Fig. 4A) and varied significantly across brooding aquaria (Table 3).

Embryo length

Embryos brooded in hypoxia were significantly shorter in length than those brooded in normoxia (Table 4). In addition, embryo length was significantly affected by the section of the pouch in which the embryos had developed (Table 4), such that embryos brooded in the bottom section were significantly longer than those brooded at the top (t=2.42, d.f.=30, P_{perm}=0.018), with the embryos

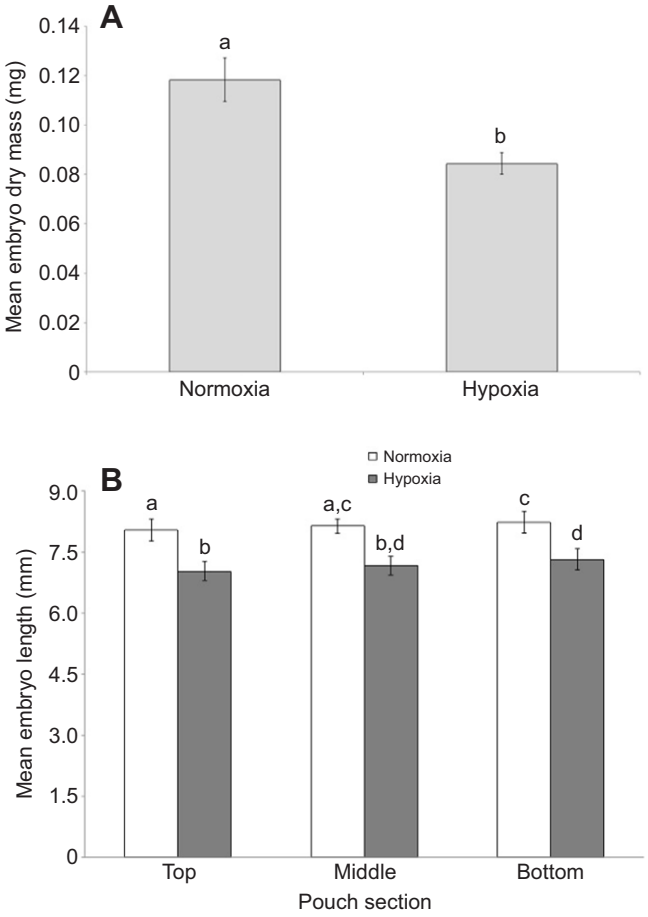

Fig. 4. Effects of O_2 treatment on mean embryo dry mass and mean embryo length. (A) Effect on mean embryo dry mass. (B) Effect on mean embryo length as a function of pouch section. Data were obtained after a brooding period of 24 days (*N*=34). Significant differences are indicated by different letters.

The Journal of Experimental Biology (2015) 218, 1639-1646 doi:10.1242/jeb.120907

Table 3. Permutational nested ANOVA analyses showing the effects of O_2 treatment and tank (nested within treatment) on separate mean embryo survival and dry mass

	Mean embryo survival				Mean embryo dry mass			
Source	d.f.	MS	Pseudo-*F*	P_{perm}	d.f.	MS	Pseudo-*F*	P_{perm}
O_2 treatment	1	0.59	1.55	0.29	1	14.86	5.12	0.03
Tank (treatment)	4	0.26	0.24	0.91	4	3.33	8.64	<0.001
Residual	28	1.09			28	0.39		
Total	33				33			

O_2 treatment: normoxia (100% O_2) versus hypoxia (40% O_2).
Analyses (univariate test) were performed on transformed and normalised variables.

from the middle section presenting intermediate lengths, not significantly different from either of the other sections (top–middle: t=1.08, d.f.=30, P_{perm}=0.284; middle–bottom: t=1.68, d.f.=30, P_{perm}=0.104; Fig. 4B).

Male condition

Male condition was significantly affected by O_2 treament, with males that brooded in hypoxic conditions presenting lower hepatosomatic indices (HSI; mean±s.e: 1.98± 0.09%) than males that had brooded in normoxia (mean±s.e.: 2.81±0.17%, $F_{1,5.27}$=15.26, P_{perm}<0.001).

Probe effects

Overall, mean embryo survival in the two pouch areas between the three insertion points was significantly correlated to the mean survival of embryos in the areas where the O_2 probe was introduced in the pouch (Pearson correlation: r=0.71, P<0.001, N=34). However, in a paired comparison, the insertion of the probe in the pouch had a negative effect on local embryo survival, as embryos from the target areas presented significantly lower survival (mean±s.e.: 71.5±2.9%) than embryos in the areas between the insertion points (mean±s.e.: 80.8±3.1%, paired t-test: t=−4.89, P<0.001, d.f.=33).

DISCUSSION

Using a very fine oxygen probe, this study is the first to measure O_2 saturation levels inside the brood pouches of pregnant pipefish males. We investigated the ability of brooding broad-nosed pipefish (*S. typhle*) males to oxygenate the developing embryos in high and low O_2 conditions during the first 24 days of brooding and we found that pouch fluid O_2 saturation levels were clearly affected by the ambient O_2 conditions experienced by the males. Pouch fluid levels were significantly lower in males brooding in hypoxic water than in males brooding in normoxic water and, importantly, we found that in both normoxia and hypoxia, O_2 saturation levels inside the brood pouches were much lower than those of the surrounding environment. Thus, in accordance with our predictions, hypoxia negatively affected the male's ability to oxygenate the developing brood, resulting in smaller, though not fewer, embryos. In addition, smaller males demonstrated a lower ability to oxygenate the embryos compared with larger males. This finding confirms our expectations and provides an additional explanation for the strong female preference for large partners reported in this population (Berglund and Rosenqvist, 1990, 2003).

Pregnancy, although ubiquitous in therian mammals and common in squamate reptiles, also occurs in fishes (Mank and Avise, 2006; Stölting and Wilson, 2007). The pipefish species investigated here has a completely closed pseudo-placental structure (the brood pouch) with blood vessels and capillaries through which O_2 is transported to the embryos during the prolonged brooding period (Ripley et al., 2010). Our findings, which are based on repeated measurements of O_2 saturation levels in the pouch fluid during the first half of embryo development, provide important new insights into the evolution of male pregnancy in syngnathids, by unveiling an important limitation in the brooding ability of this brood pouch-adorned species: at any ambient O_2 saturation, embryos of *S. typhle* have less O_2 available for their development than embryos that are in direct contact with the surrounding water. Thus, our results provide evidence against the notion that brood pouches should be viewed as well-oxygenated structures that promote the evolution of larger eggs in syngnathids (Shine, 1978; Kolm and Ahnesjö, 2005). They also put into question the generally accepted hypothesis that more complex types of brood pouches, like that of *S. typhle*, equal better paternal care (Wilson et al., 2001).

Interestingly, we found differences in O_2 saturation levels between the different sections of the pouch. O_2 levels were lowest in the middle section, which may result from a greater number of embryos in this section, where the pouch is widest and embryos in multiple layers are most commonly observed (I.B.G., I.A. and C.K., personal observation). The highest levels of O_2 were found in the bottom section of the pouch, coupled with the presence of significantly larger embryos. Because of the way eggs are transferred into a male's pouch and the possibility for males to mate with multiple females before the pouch is completely filled (Berglund et al., 1988; Jones et al., 1999), the eggs at the bottom of the pouch are the first to be received and fertilised by the male, and, arguably, are the first ones to begin to develop. This potential difference in the timing of initiation of development, in combination with a usually smaller number of embryos in this area, may explain why embryos at the bottom of the pouch were better oxygenated and larger than those throughout the rest of the pouch. Indirect negative effects of egg density on embryo development have been shown in

Table 4. Permutational nested-ANOVA assessing the effects of O_2 treatment, brooding tank (nested within O_2 treatment), male (nested within tank) and pouch section on mean *S. typhle* embryo length

Source	d.f.	MS	Pseudo-*F*	P_{perm}
O_2 treatment	1	19.81	7.56	0.01[1]
Pouch section	2	0.19	3.40	0.04[2]
Tank (treatment)	4	4.51	1.94	0.14
Treatment × pouch section	2	0.03	0.13	0.87
Male [tank (treatment)]	25	2.32	15.53	<0.001
Tank (treatment) × pouch section	8	0.20	1.34	0.24
Residuals	50	0.15		
Total	92			

O_2 treatment: normoxia (100% O_2) versus hypoxia (40% O_2); pouch section: top, middle and bottom.
Pooled terms had P_{perm}>0.15.
[1]Term mean square tested against the pooled mean squares of male and tank.
[2]Term mean square tested against the pooled mean squares of residuals, treatment×pouch section and tank (treatment)×pouch section.

The Journal of Experimental Biology (2015) 218, 1639-1646 doi:10.1242/jeb.120907

other species, including the rainbow trout, *Onchorhynchus mykiss* (Dhiyebi et al., 2013), due to changes in water flow in the vicinity of the eggs.

Pouch fluid O_2 saturation levels decreased throughout the brooding period, presenting a sharp decrease between days 6 and 12, irrespective of O_2 treatment. This decrease in O_2 availability in the pouch is likely to be a result of increased respiration by the embryos as they grow (Berglund et al., 1986b). Greater O_2 consumption by embryos with the progression of development is well known and has been recorded, for instance, in rainbow trout (Rombough, 1988). Moreover, in a tropical clown fish, *Amphiprion melanopus*, caring males have been reported to increase fanning effort as the embryos develop and O_2 demands increase (Green, 2004; Green and McCormick, 2005). However, it is also possible that brooding *S. typhle* males struggle to maintain O_2 levels over the brooding period. In our study, it was not possible to determine to what extent the lower within-pouch O_2 saturation levels later in the pregnancy are the result of reduced paternal ability to supply O_2, increased O_2 consumption by the embryos, or a combination of the two.

Embryo survival did not differ between the O_2 treatments, but embryos were significantly smaller in the hypoxic treatment at the termination of the experiment. Body size is a key fitness correlate in *S. typhle*, both in adults and in juveniles. Because by parturition the yolk sacs have usually been fully resorbed (Sommer et al., 2012), juveniles switch to external feeding once released from the paternal brood pouch. A previous study has shown that larger juvenile size at birth is associated both with subsequent faster growth rates, presumably due to higher foraging success, and with higher survival, due to greater success at escaping predators (Ahnesjö, 1992a). Thus, the significant reduction in embryo size we found in just under half the length of a full brooding period, due to ambient hypoxia, is indicative of larger size differences by parturition and suggestive of important detrimental fitness consequences.

Low ambient O_2 conditions lead to costly increases in parental effort. For instance, in common gobies, *Pomatoschistus microps*, males caring in hypoxic conditions fan more and lose more weight, but maintain similar hatching success to males caring in normoxic conditions (Jones and Reynolds, 1999). In our study, brooding in hypoxic conditions significantly lowered male condition (measured as HSI), showing that brooding in poorly oxygenated waters is energetically costly to the males. However, male condition was not related to male length. This result that small and large males showed similar condition within O_2 treatment is interesting, because large males maintained significantly higher within-pouch O_2 saturation levels compared with smaller males. This difference in quality of care is likely to be important and may further explain the strong preference shown by females of all sizes for large mates (Berglund et al., 1986a; Berglund and Rosenqvist, 2003). As sexual selection acts on body size in both sexes, small males may be less willing to invest in current reproductive events if future reproductive opportunities are likely to yield higher fitness pay-offs (Magnhagen, 1990).

We used a technique that allowed us to directly assess O_2 saturation levels in the pouches of brooding pipefish males. Specifically, this technique enabled us to assess pouch fluid O_2 saturation levels throughout the pouch and saturation changes over a long period of time, within the same individuals, i.e. minimising the number of experimental subjects. The technique worked well and measurements were repeatable and reliable. However, the use of the O_2 probe, which is an invasive technique, affected embryo survival negatively, with a slightly higher number of unviable embryos occurring around the insertion points than in the sections between the insertion points. This effect suggests that, even though unique and valuable information is obtained, this technique should be used with caution in future studies, in particular for repeated measurements in the same points.

To conclude, we report two important findings. Firstly, we found that pouch fluid O_2 saturation levels are generally lower than those of the surrounding water, vary throughout the brood pouch and decrease over time. These results highlight a potential O_2 limitation to brooding within the closed environment of the brood pouch. The evolution of the brood pouch among syngnathids has been conceptually linked to more advanced types of care because of the potential to osmoregulate (Quast and Howe, 1980; Carcupino et al., 1997, 2002; Partridge et al., 2007; Ripley, 2009) and to provide nutrients (Haresign and Shumway, 1981; Ripley and Foran, 2006, 2009; Kvarnemo et al., 2011) to the developing young – abilities that are limited in species that do not have brood pouches (Berglund et al., 1986b; Carcupino et al., 2002). Therefore, it is important to note that, in terms of oxygenation, the presence of a brood pouch is here shown to limit, not ease, embryo access to O_2 and thus limit their development.

Secondly, within-pouch O_2 saturation levels were positively correlated with male length. We know that *S. typhle* females strongly prefer to mate with larger partners compared with smaller ones (Berglund et al., 1986a; Berglund and Rosenqvist, 2003) but direct evidence of differences in parenting quality of small and large males is still lacking (Braga Goncalves et al., 2010, 2014). Here, we provide direct evidence that, in terms of oxygenation, large males provide better care to the developing offspring than small males do, which is particularly important when embryo development is likely to be restricted by O_2 availability, as shown by the negative effects on embryo length and mass. This study, thus, provides important contributions to the understanding of the function of the brood pouch during male pregnancy in syngnathids in general (Stölting and Wilson, 2007), and improves the knowledge of factors guiding mate choice in this model species in particular.

MATERIALS AND METHODS

Collection and husbandry

The study was carried out on the Swedish west coast at the Sven Lovén Centre for Marine Sciences, Kristineberg (58°15′N, 11°28′E) between May and July 2009. *Syngnathus typhle* pipefish were collected from eelgrass (*Zostera marina*) meadows with a beam trawl pulled behind a boat in the vicinity of the marine station.

Fish were brought into the lab, separated by sex and kept in storage barrels (225 l). Both barrels and brooding aquaria were provided with artificial eelgrass, a flow-through system of continuously renewed natural seawater providing natural salinity conditions, and light 16 h per day. Air and water temperatures were kept constant at 15°C. Fish were fed three times per day with *Artemia salina* and supplemented with wild-caught shrimps (*Praunus flexuosus* and *Crangon crangon*) and copepod species. Storage barrels were cleaned daily and brooding aquaria every third day.

Experimental design

Matings were performed in barrels by introducing groups of males and females together. Because a large *S. typhle* female produces enough eggs to fill up almost three males of her size during the course of one male pregnancy (Berglund and Rosenqvist, 1990; Ahnesjö, 1995), females and males were put together in a ratio of 1:2 (eight females and 16 males). This ratio ensured that males mated and had their brood pouches filled up quickly. Males were kept in the barrels until their pouches were filled (24–72 h). One day after being fully mated, males were measured and individually identified before being transferred into brooding aquaria (approximately 140 l), where they were kept in groups of six or seven individuals for a period of 24 days. Brooding aquaria had either fully

The Journal of Experimental Biology (2015) 218, 1639-1646 doi:10.1242/jeb.120907

oxygenated water (100% O_2 saturation) or hypoxic water (40% O_2 saturation). The concentration of dissolved O_2 was decreased with the use of a MiniModule 1.7×5.5 Membrane Contactor (Liqui-Cel, Celgard, Inc., Charlotte, NC, USA). This unit enables the flow of nitrogen in a counter-current system in relation to the direction of the water flow, continuously removing O_2 from the water. Hence, we were able to provide a flow-through seawater system in the hypoxic aquaria, similar to the conditions in the normoxic aquaria. O_2 levels in the treatment aquaria were checked daily using a portable O_2 meter (Handy Delta, OxyGuard, Tekno Trading AB, Säffle, Sweden) and we adjusted water and nitrogen flow when necessary. In the normoxic brooding aquaria, flowing seawater was supplemented with air flowing through air stones to maintain high ambient water O_2 saturation conditions. Males were allocated to treatments at random, only ensuring that treatments had similar sample sizes and body size ranges.

The brooding period was set to 24 days, which amounts to little over half of the total brooding period of *S. typhle* at our experimental temperature. At experiment termination, males were killed with an overdose of anaesthetic (10 min in 1 ml 2-phenoxyethanol/litre seawater) followed by severing the spinal column posterior to the operculum. Each male was preserved in 70% ethanol in individual vials for posterior assessment of embryo survival and size. Ethical approval for this study was given by the Swedish Animal Welfare Agency (dnr. 111-2007).

Experimental subjects

The SL and body depth of all females, to the nearest mm, were taken before they were introduced into the mating barrels. The SL of males was similarly measured before they were introduced into the mating barrels and again after mating for identification purposes. A range of male sizes (SL range: 150–203 mm, mean±s.e.: 177±2.5 mm, N=34) was used to assess differences in paternal care in relation to body size. Because egg size correlates positively with female SL (Berglund et al., 1988), only large females (SL range: 231–276 mm, mean±s.e.: 249±1.87 mm; body depth range: 9.6–13.7 mm, mean ±s.e.: 11.3±0.15 mm) were chosen to attempt to standardise egg size among all males and, thus, minimise confounding effects of varying egg sizes on embryo length and mass. Within each brooding aquarium, males could be recognised by their SL and colour, as individuals' body coloration may range from silvery black to brown and pale green. After mating, all females were released close to the site of capture.

O_2 probe calibration

The barometric pressure at the study site ranged between 747.80 and 777.82 mmHg, with an average of 759.99 mmHg during the course of the experiment. Hence, we assumed an atmospheric pressure of 760 mmHg (or 1 atm, as expected at sea level). At a temperature of 15°C, O_2 solubility in seawater at 24 ppt salinity is 8.70 mg l^{-1} O_2. Thus, at 100% O_2 saturation, seawater had 8.70 mg l^{-1} O_2, corresponding to a partial pressure of 156 mmHg; at 40% O_2 saturation, O_2 concentration was 3.5 mg l^{-1} O_2 and the respective partial pressure was 63 mmHg. O_2 measurements were taken with a Foxy-AL300-AF probe, connected to a laptop through a MFPF100-1 O_2 sensor (OOISENSORS, Ocean Optics, B.V., Duiven, The Netherlands). The O_2 sensor was calibrated weekly, in the same temperature-controlled room where the experiments were carried out and measurements taken. Two standards were used for calibration. The first standard, at 0% O_2 saturation, was obtained by bubbling nitrogen into the sample until O_2 saturation stabilised at 0%. The second standard, at 100% O_2 saturation, was obtained by continuously bubbling air into the sample. Both standards were made of seawater collected from the same taps used to feed water into the experimental tanks, and thus had similar salinity levels. The O_2 sensor was set to take measurements every 5 s.

Data collection

Pouch fluid O_2 saturation levels

On days 6, 12, 18 and 24 of the brooding period, O_2 saturation levels (%) were measured from three different sections inside the brood pouch (top, middle and bottom; Fig. 1A). Each male was briefly removed from the experimental aquarium and placed into a silicon tube that had a middle section cut out (Fig. 1C). Because the fish could turn around in the tube, an assistant helped to hold the males in place while measurements were recorded by gently squeezing the sides of the tube at head and tail points. The tube was filled with water from the experimental aquaria in order to keep the surrounding O_2 saturation levels similar to experimental conditions. For each section of the pouch, the needle of the probe was inserted into the brood pouch along the sealed line where the two pouch flaps meet and kept in place for 1–2 min, until three measurements were recorded within 1% of O_2 saturation. The average value of these three recordings was used in the analyses. In between consecutive measurements at the same site, the needle was kept in place. Once the measurements in one section were concluded, the probe was rinsed and put back in the 100% O_2 saturation standard to confirm the quality of the readings, before it was inserted in the next section. For each male, the whole procedure took less than 5–10 min, from the moment the male was removed from the experimental tank, until it was returned. If the male was kept in the tube for more than 5 min, the water was replaced with new water. Measurements were recorded from the same entrance points in the pouch throughout the experiment. A small scar that closed up between collection days (i.e. in between day 6 and day 12, day 12 and day 18, etc.) formed at each of the entrance points. The scar thus marked the exact point of the previous recording and ensured that we took our readings from the same points in the pouch each time.

Embryo survival

The brood pouches of all males were dissected and overall embryo survival was assessed for each male by dividing the number of developing embryos by the total number of eggs transferred by the females (sum of undeveloped and developing embryos, and unviable eggs in the pouch). The eggs in the pouch were further separated into the three measurement sections and the two sections in between to assess relative embryo survival in each section and to compare average embryo survival between the two sections: with or without an entrance point for the O_2 probe.

Embryo length

Five embryos each from the top, middle and bottom of the pouch of each male were separated from the egg membrane and yolk sac. Pictures of the embryos were taken using a camera (Leica DFC420 C) attached to a stereo microscope (Leica MZ16 A). The total length (tip of rostrum to tip of tail) of each embryo (±0.01 mm) was measured from the photographs using Leica Application Suite, version 2.7.0.RI (Build: 1294, Heerbrugg, Switzerland). For each male, the average embryo length was calculated for each of the three pouch sections.

Embryo dry mass

Ten embryos, selected at random from the brood pouch, were separated from the egg membrane and yolk sac, and dried in a heating cupboard (60°C) for 1 week then weighed twice on a Sartorius LE26P microbalance (±2 µg). To calculate the average dry mass per embryo for each male, we divided the average of these values by 10.

Male condition

We dissected the males and removed their livers. Both males and livers were dried in a heating cupboard (60°C) for 1 week and weighed on a Sartorius LE26P microbalance (±2 µg) twice each. The average liver mass was divided by the total body mass (excluding the liver mass; Tomkins and Simmons, 2002) to calculate the HSI for each male, reported as percentages.

Statistical analysis

We used PERMANOVA+ for PRIMER v6 (PRIMER-E, Plymouth, UK) and SPSS 17 (SPSS Inc., Chicago, IL, USA) to perform all analyses. The Permdisp routine was used to assess the spread of the data and ensure that differences detected were due to differences in means rather than differences in dispersion of the data. Data were analysed with the Permanova routine in PERMANOVA+ because of the small and unequal sample sizes between brooding aquaria, which reduced the reliability of parametric tests.

Before conducting the analyses, O_2 readings and relative embryo survival were arcsine transformed. Variables were also normalised (each variable had its mean subtracted and was divided by its standard deviation) before

multivariate analyses, in order to achieve a common scale. Resemblance matrices based on Euclidean distances (Clarke and Gorley, 2006; Anderson et al., 2008) were produced for each statistical test.

We used a nested-ANCOVA design to explore the effects of male SL (covariate), O_2 treatment [fixed factor (FF): 100% and 40% O_2], tank [random factor (RF), nested within O_2 treatment, three aquaria per treatment], male (RF, nested within tank, 34 individuals), pouch section (FF: top, middle and bottom) and day (FF: days 6, 12, 18 and 24) on pouch fluid O_2 saturation levels. The effects of male SL, O_2 treatment and brooding tank on overall embryo dry mass and relative survival were determined with a nested-MANCOVA design, followed by separate nested ANOVA on the individual response variables. The effects of male SL, O_2 treatment, brooding tank and pouch section on embryo length were analysed using a nested ANCOVA design. A nested ANCOVA design was also used to explore the effects of male SL, O_2 treatment and tank on male condition (HSI). Male SL was removed from the analyses of embryo length, dry mass and relative survival, and of male HSI, because it did not affect the response variables significantly (all $P_{perm}>0.65$). Models were sequentially reduced by pooling non-significant factors (interactions and main factors, when $P_{perm}>0.15$), one at a time, starting with highest degree interactions followed by within-level lowest significance factors, to improve the power of the remaining factors. ANCOVA models were tested using type I sums of squares; ANOVA-type models were tested using type III sums of squares. The following options were chosen for all analyses: fixed effects sum to zero, model: permutation of residuals under a reduced model, number of permutations: 9999. Significance level for all tests was set at $P<0.05$, except for removed terms as explained above.

Acknowledgements
We thank M. Hällbom, K. Olsson, I. Duranovic and G. Sagebakken for assistance in the field, A. Berglund and G. Rosenqvist for equipment, M. J. Anderson for statistical advice and G. Sagebakken, O. Jennersten and L. Hammar for photographs. We are grateful for the facilities and support provided at Sven Lovén Centre for Marine Sciences – Kristineberg, University of Gothenburg.

Competing interests
The authors declare no competing or financial interests.

Author contributions
I.B.G., I.A. and C.K. contributed substantially to the conception and design of the study, the interpretation of the data and critical revisions of intellectual content of the paper. I.B.G. performed the experiments, analysed the data and drafted the paper.

Funding
Research funding was provided by Fundação para a Ciência e Tecnologia-Portugal (SFRH/BD/23185/2005), Fundo Social Europeu (POPH – QREN), Helge Ax:son Johnsons Stiftelse, Wilhelm och Martina Lundgrens Vetenskapsfond and KVVS (to I.B.G.), the Inez Johansson's foundation and the Royal Swedish Academy of Sciences (to I.A.) and the Swedish Research Council (to C.K.).

References
Ahnesjö, I. (1992a). Fewer newborn result in superior juveniles in the paternally brooding pipefish *Syngnathus typhle* L. *J. Fish Biol.* **41**, 53-63.
Ahnesjö, I. (1992b). Consequences of male brood care; weight and number of newborn in a sex-role reversed pipefish. *Funct. Ecol.* **6**, 274-281.
Ahnesjö, I. (1995). Temperature affects male and female potential reproductive rates differently in the sex-role reversed pipefish, *Syngnathus typhle*. *Behav. Ecol.* **6**, 229-233.
Anderson, M. J., Gorley, R. N. and Clarke, K. R. (2008). *PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods*. Plymouth: PRIMER-E Ltd.
Berglund, A. and Rosenqvist, G. (1990). Male limitation of female reproductive success in a pipefish: effects of body-size differences. *Behav. Ecol. Sociobiol.* **27**, 129-133.
Berglund, A. and Rosenqvist, G. (2003). Sex role reversal in pipefish. *Adv. Study Behav.* **32**, 131-167.
Berglund, A., Rosenqvist, G. and Svensson, I. (1986a). Mate choice, fecundity and sexual dimorphism in two pipefish species (Syngnathidae). *Behav. Ecol. Sociobiol.* **19**, 301-307.
Berglund, A., Rosenqvist, G. and Svensson, I. (1986b). Reversed sex roles and parental energy investment in zygotes of two pipefish (Syngnathidae) species. *Mar. Ecol. Prog. Ser.* **29**, 209-215.
Berglund, A., Rosenqvist, G. and Svensson, I. (1988). Multiple matings and paternal brood care in the pipefish *Syngnathus typhle*. *Oikos* **51**, 184-188.
Berglund, A., Rosenqvist, G. and Svensson, I. (1989). Reproductive success of females limited by males in two pipefish species. *Am. Nat.* **133**, 506-516.
Blumer, L. S. (1979). Male parental care in the bony fishes. *Q. Rev. Biol.* **54**, 149-161.
Blumer, L. S. (1982). A bibliography and categorization of bony fishes exhibiting parental care. *Zool. J. Linn. Soc.* **75**, 1-22.
Braga Goncalves, I. (2010). Egg size evolution and paternal care in pipefishes. PhD thesis, University of Gothenburg.
Braga Goncalves, I., Mobley, K. B., Ahnesjö, I., Sagebakken, G., Jones, A. G. and Kvarnemo, C. (2010). Reproductive compensation in broad-nosed pipefish females. *Proc. R. Soc. B Biol. Sci.* **277**, 1581-1587.
Braga Goncalves, I., Ahnesjö, I. and Kvarnemo, C. (2011). The relationship between female body size and egg size in pipefishes. *J. Fish Biol.* **78**, 1847-1854.
Braga Goncalves, I., Mobley, K. B., Ahnesjö, I., Sagebakken, G., Jones, A. G. and Kvarnemo, C. (2014). Effects of mating order and male size on embryo survival in a pipefish. *Biol. J. Linn. Soc.* **114**, 639-645.
Carcupino, M., Baldacci, A., Mazzini, M. and Franzoi, P. (1997). Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* Risso (Teleostea, Syngnathidae) before, during, and after egg incubation. *Tissue Cell* **29**, 21-30.
Carcupino, M., Baldacci, A., Mazzini, M. and Franzoi, P. (2002). Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *J. Fish Biol.* **61**, 1465-1480.
Clarke, K. R. and Gorley, R. N. (2006). *PRIMER v6: User Manual/Tutorial*. Plymouth: PRIMER-E Ltd.
Clutton-Brock, T. (1991). *The Evolution of Parental Care*. Princeton: Princeton University Press.
Dawson, C. E. (1985). *Indo-Pacific pipefishes (Red Sea to the Americas)*. Ocean Springs, MS, USA: The Gulf Coast Research Laboratory.
Dhiyebi, H. A., O'Donnell, M. J. and Wright, P. A. (2013). Water chemistry in the microenvironment of rainbow trout *Oncorhynchus mykiss* embryos is affected by development, the egg capsule and crowding. *J. Fish Biol.* **82**, 444-457.
Einum, S., Hendry, A. P. and Fleming, I. A. (2002). Egg-size evolution in aquatic environments: does oxygen availability constrain size? *Proc. R. Soc. B Biol. Sci.* **269**, 2325-2330.
Green, B. S. (2004). Embryogenesis and oxygen consumption in benthic egg clutches of a tropical clownfish, *Amphiprion melanopus* (Pomacentridae). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **138**, 33-38.
Green, B. S. and McCormick, M. I. (2005). O_2 replenishment to fish nests: males adjust brood care to ambient conditions and brood development. *Behav. Ecol.* **16**, 389-397.
Gross, M. R. and Sargent, R. C. (1985). The evolution of male and female parental care in fishes. *Am. Zool.* **25**, 807-822.
Haresign, T. W. and Shumway, S. E. (1981). Permeability of the marsupium of the pipefish, *Syngnathus fuscus* to (14C)-alpha amino isobutyris acid. *Comp. Biochem. Physiol. A Physiol.* **69**, 603-604.
Herald, E. S. (1959). From pipefsih to seahorse - a study of phylogenetic relationships. *Proc. Calif. Acad. Sci.* **29**, 465-473.
Jones, J. C. and Reynolds, J. D. (1999). Costs of egg ventilation for male common gobies breeding in conditions of low dissolved oxygen. *Anim. Behav.* **57**, 181-188.
Jones, A. G., Rosenqvist, G., Berglund, A. and Avise, J. C. (1999). The genetic mating system of a sex-role-reversed pipefish (*Syngnathus typhle*): a molecular inquiry. *Behav. Ecol. Sociobiol.* **46**, 357-365.
Keister, J. E., Houde, E. D. and Breitburg, D. L. (2000). Effects of bottom-layer hypoxia on abundances and depth distributions of organisms in Patuxent river, Chesapeake Bay. *Mar. Ecol. Prog. Ser.* **205**, 43-59.
Kolm, N. and Ahnesjö, I. (2005). Do egg size and parental care coevolve in fishes? *J. Fish Biol.* **66**, 1499-1515.
Kramer, D. L. (1987). Dissolved oxygen and fish behavior. *Environ. Biol. Fish.* **18**, 81-92.
Kvarnemo, C., Mobley, K. B., Partridge, C., Jones, A. G. and Ahnesjö, I. (2011). Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish *Syngnathus typhle*. *J. Fish Biol.* **78**, 1725-1737.
Lindström, K. and St Mary, C. M. (2008). Parental care and sexual selection. In *Fish Behaviour* (ed. C. Magnhagen, V. A. Braithwaite, E. Forsgren and B. G. Kapoor), pp. 377-409. Enfield, NH, USA: Science Publishers.
Lukas, J. A. and Orth, D. J. (1995). Factors affecting nesting success of smallmouth bass in a regulated Virginia stream. *Trans. Am. Fish. Soc.* **124**, 726-735.
Magnhagen, C. (1990). Reproduction under predation risk in the sand goby, *Pomatoschistus minutus*, and the Black Goby, *Gobius niger*: the effect of age and longevity. *Behav. Ecol. Sociobiol.* **26**, 331-335.
Mank, J. E. and Avise, J. C. (2006). Supertree analyses of the roles of viviparity and habitat in the evolution of atherinomorph fishes. *J. Evol. Biol.* **19**, 734-740.
Monteiro, N. M., Almada, V. C. and Vieira, M. N. (2005). Implications of different brood pouch structures in syngnathid reproduction. *J. Mar. Biol. Assoc. UK* **85**, 1235-1241.

Partridge, C., Shardo, J. and Boettcher, A. (2007). Osmoregulatory role of the brood pouch in the euryhaline Gulf pipefish, *Syngnathus scovelli*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **147**, 556-561.
Quast, W. D. and Howe, N. R. (1980). The osmotic role of the brood pouch in the pipefish *Syngnathus scovelli*. *Comp. Biochem. Physiol. A Physiol.* **67**, 675-678.
Quinn, T. P., Hendry, A. P. and Wetzel, L. A. (1995). The influence of life history trade-offs and the size of incubation gravels on egg size variation in sockeye salmon (*Oncorhynchus nerka*). *Oikos* **74**, 425-438.
Reynolds, J. D., Goodwin, N. B. and Freckleton, R. P. (2002). Evolutionary transitions in parental care and live bearing in vertebrates. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357**, 269-281.
Ripley, J. L. (2009). Osmoregulatory role of the paternal brood pouch for two *Syngnathus* species. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **154**, 98-104.
Ripley, J. L. and Foran, C. M. (2006). Differential parental nutrient allocation in two congeneric pipefish species (Syngnathidae: *Syngnathus* spp.). *J. Exp. Biol.* **209**, 1112-1121.
Ripley, J. L. and Foran, C. M. (2009). Direct evidence for embryonic uptake of paternally-derived nutrients in two pipefishes (Syngnathidae: *Syngnathus* spp.). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **179**, 325-333.
Ripley, J. L., Williams, P. S. and Foran, C. M. (2010). Morphological and quantitative changes in paternal brood-pouch vasculature during embryonic development in two *Syngnathus* pipefishes. *J. Fish Biol.* **77**, 67-79.
Rombough, P. J. (1988). Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. In *Fish Physiology - The Physiology of Developing Fish. Part A. Eggs and Larvae*, Vol. XI (ed. W. S. Hoar and D. J. Randall), pp. 59-161. London: Academic Press.
Sargent, R. C., Taylor, P. D. and Gross, M. R. (1987). Parental care and the evolution of egg size in fishes. *Am. Nat.* **129**, 32-46.
Shine, R. (1978). Propagule size and parental care: the “Safe Harbor” hypothesis. *J. Theor. Biol.* **75**, 417-424.
Sogabe, A. and Ahnesjö, I. (2011). The ovarian structure and mode of egg production in two polygamous pipefishes: a link to mating pattern. *J. Fish Biol.* **78**, 1833-1846.
Sommer, S., Whittington, C. M. and Wilson, A. B. (2012). Standardised classification of pre-release development in male-brooding pipefish, seahorses, and seadragons (Family Syngnathidae). *BMC Dev. Biol.* **12**, 39.
Stölting, K. N. and Wilson, A. B. (2007). Male pregnancy in seahorses and pipefish: beyond the mammalian model. *Bioessays* **29**, 884-896.
Takegaki, T. (2001). Environmental factors affecting the spawning burrow selection by the gobiid *Valenciennea longipinnis*. *J. Fish Biol.* **58**, 222-229.
Takegaki, T. and Nakazono, A. (1999). Responses of the egg-tending gobiid fish *Valenciennea longipinnis* to the fluctuation of dissolved oxygen in the burrow. *Bull. Mar. Sci.* **65**, 815-823.
Tomkins, J. L. and Simmons, L. W. (2002). Measuring relative investment: a case study of testes investment in species with alternative male reproductive tactics. *Anim. Behav.* **63**, 1009-1016.
van den Berghe, E. P. and Gross, M. R. (1989). Natural selection resulting from female breeding competition in a Pacific salmon (coho: *Oncorhynchus kisutch*). *Evolution* **43**, 125-140.
Wilson, A. B., Vincent, A., Ahnesjö, I. and Meyer, A. (2001). Male pregnancy in seahorses and pipefishes (Family Syngnathidae): rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. *J. Hered.* **92**, 159-166.
Wilson, A. B., Ahnesjö, I., Vincent, A. C. J. and Meyer, A. (2003). The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). *Evolution* **57**, 1374-1386.
Wisenden, B. D., Unruh, A., Morantes, A., Bury, S., Curry, B., Driscoll, R., Hussein, M. and Markegard, S. (2009). Functional constraints on nest characteristics of pebble mounds of breeding male hornyhead chub *Nocomis biguttatus*. *J. Fish Biol.* **75**, 1577-1585.

The Journal of Experimental Biology