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The evolutionary puzzle of egg size, oxygenation and parental care in aquatic environments

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Offspring fitness generally improves with increasing egg size. Yet, eggs of most aquatic organisms are small. A common but largely untested assumption is that larger embryos require more oxygen than they can acquire through diffusion via the egg surface, constraining egg size evolution. However, we found no detrimental effects of large egg size on embryo growth and survival under hypoxic conditions. We tested this in the broad-nosed pipefish, *Syngnathus typhle*, whose males provide extensive care (nourishment, osmoregulation and oxygenation) to their young in a brood pouch on their bodies. We took advantage of this species' pronounced variation in egg size, correlating positively with female size, and tested the effect of hypoxia (40% dissolved oxygen) versus fully oxygenated (100%) water on embryo size and survival of large versus small eggs after 18 days of paternal brooding. Egg size did not affect embryo survival, regardless of O_2 treatment. While hypoxia affected embryo size negatively, both large and small eggs showed similar reductions in growth. Males in hypoxia ventilated more and males with large eggs swam more, but neither treatment affected their position in the water column. Overall, our results call into question the most common explanation for constrained egg size evolution in aquatic environments.

1. Introduction

Large juveniles commonly enjoy fitness benefits compared with smaller ones [1], and juvenile and adult size are often positively related to initial egg size [2]. Should we therefore predict evolution of ever increasing egg sizes? Following Smith and Fretwell's [3] influential model, we should not, owing to the trade-off between offspring size and number. Moreover, even though larger offspring have better survival and/or fitness, they do so with diminishing returns [3]. However, this is not the only cost or trade-off associated with increasing egg size in aquatic environments. Because there is much lower O_2 availability in water than in air, oxygenation can be a challenging process in aquatic environments and has commonly been argued to constrain egg size evolution [4–6]. Indeed, most fish eggs are spherical, the shape most favourable for gas exchange in low water velocities [7]. However, given a round shape, an increase in egg size results in a greater increase of volume compared with the surface area. Hence, the relative surface area, where diffusion takes place, decreases as volume increases [4,8]. Assuming that egg volume determines embryonic oxygen (O_2) requirements and egg surface area determines the ability to acquire O_2 by diffusion, limitations in O_2 availability to the embryos may constrain egg size evolution. Yet, while O_2 limitations have been amply demonstrated in egg masses [9–11], they have only been assumed to affect individual embryos in response to egg size. In fact, despite theoretical support

for the principle (reviewed in [8]), a negative effect of egg size on embryo survival due to limited O_2 uptake is yet to be confirmed [4,6,12,13].

The amount of dissolved O_2 in water affects the metabolism and development of embryos [8,14,15] and consequently their survival. A decrease in dissolved oxygen (DO) concentrations in the water can lead to hypoxia (i.e. any level of reduced DO that negatively affects the physiology or behaviour of an organism [16]), which can reduce embryo metabolism [17], decrease yolk conversion efficiency [18], reduce development rates and delay hatching times [19], as well as induce premature hatching [19], cause deformities [20], decrease size at hatching and reduce post-hatching survival [19]. The hypothesis that large eggs do worse than smaller ones assumes that O_2 consumption increases proportionally with egg volume [4–6]. However, if O_2 consumption increases at a lower rate than volume, smaller eggs can be predicted to perform worse [4]. This was demonstrated in Atlantic salmon, *Salmo salar*, where embryo O_2 requirements do not increase in direct proportion to the volume, suggesting that at low DO concentrations, embryos of small eggs, and not of large ones, may have difficulties obtaining sufficient O_2 for their development [4].

In a development of Smith and Fretwells' [3] model, Jorgensen *et al*. [21] showed that optimal offspring size generally decreases with increasing growth rate. However, when the authors included parental care in the model in the form of live bearing, offspring survival became a function of parental survival, with larger parents predicted to produce larger offspring. This prediction is in accordance with interspecific patterns among ectothermic animals, which indicate that parental care and egg size are positively associated [22–24]. In aquatic environments, how and whether parental care and egg size coevolved is complex to disentangle, but parental care often involves the task of oxygenating the developing embryos [6,24]. In giant water bugs (Belostomatidae), selection on egg size and embryo oxygenation are key factors in the evolution of paternal care [25]. Males carry the relatively large eggs on their backs and promote O_2 diffusion by 'pumping' the brood in and out of water or by brushing the eggs with their hind legs [25]. Among fishes, there is substantial variation in egg size [21,26], with larger eggs found in demersally than in pelagically spawning species [26]. Concurrently, parental care is more common in the former than in the latter group [27] and a variety of parental care strategies directly improve the access of O_2 to the embryos. For example, parents may choose nest sites according to O_2 availability [28,29], fan their eggs to improve suboptimal O_2 conditions [30,31], increase nest opening size [32,33] or cannibalize some of the eggs to improve the oxygenation of the remaining embryos [34,35]. In fact, parental care in aquatic species may have evolved, at least partially, to protect developing embryos from low O_2 availability, which in turn could have favoured the evolution of larger egg sizes [6,36].

The aim of this study was to investigate if egg size and O_2 availability interact as expected based on the hypothesis that egg size is evolutionarily constrained by O_2-acquiring ability in aquatic environments. We tested this by examining embryo development and survival in the broad-nosed pipefish, *Syngnathus typhle* L., a species with pronounced variation in egg size, with large females producing eggs significantly larger than those of small females [37]. Together with the fact that it provides extensive parental care and lives in an environment with naturally high variability in O_2 levels [38–41], these characteristics make it a very apt model organism. As is typical for many pipefishes and seahorses (Syngnathidae), males of this genus care for the developing embryos in a sealed brood pouch where they protect, osmoregulate, oxygenate and transfer nutrients to the embryos through the vascularized walls of the pouch [37,42–45]. Moreover, previous work on *S. typhle* has shown that larger embryos have higher respiration rates than smaller ones [46], larger eggs result in larger offspring [47] and that larger juveniles enjoy fitness benefits [48].

We tested whether *S. typhle* embryos are negatively affected by hypoxia during development and whether embryos developing from large eggs are more negatively affected by hypoxia than embryos developing from small eggs. In addition, we collected behavioural data from the brooding males to assess whether activity patterns and opercular movements, indicative of ventilation rates, are affected by the size of the eggs they brood or by ambient DO levels. The position of the brooding males was also recorded as DO levels may vary in the water column. This study provides a valuable contribution to the understanding of how limited access to O_2 affects embryo development and relates to egg size evolution in aquatic environments.

2. Material and methods

(a) Fish collection

This study was conducted at the Swedish west coast at the Sven Lovén Centre for Marine Sciences, Kristineberg (58°15′ N, 11°28′ E). Owing to time and space limitations, the study was done over two consecutive summers (2007 and 2008). *Syngnathus typhle* were collected from shallow eelgrass (*Zostera marina*) meadows in the vicinity of the marine station using a beam trawl pulled behind a boat. For details on fish husbandry, see the electronic supplementary material.

(b) Experimental design

Males were randomly allocated to one of four treatment groups: males brooding small or large eggs in either fully oxygenated or in hypoxic water (large eggs, high oxygen (O_2): $n = 12$; large eggs, low O_2: $n = 11$; small eggs, high O_2: $n = 12$; small eggs, low O_2: $n = 12$). All males and females had their standard body length (SL) measured, to the nearest millimetre, before being introduced into the mating tanks. Only medium-sized males (mean SL $\pm$ s.e.: 170.3 $\pm$ 0.9 mm, range = 155–181 mm, $n = 47$) were used to limit potential size-related differences in male condition, feeding ability, quality of paternal care provided to the embryos and maternal effects resulting from female choice [49]. Male SL did not differ between treatments (one-way ANOVA: $F_{3,43} = 0.41$, $p = 0.75$). As female SL is significantly and positively correlated with egg size [37], females of small (range 160–180 mm) and large (230–260 mm) sizes were chosen to obtain two distinct egg size categories. Males brooded on average 85 $\pm$ 25 eggs (mean $\pm$ s.d., range 25–138) which did not differ between O_2 treatments (*t*-test: $t_{45} = 0.90$, $p = 0.37$). However, males mated with large females received 17% fewer eggs than males mated with small females (*t*-test: $t_{45} = 2.23$, $p = 0.03$, small eggs: 92 $\pm$ 25 eggs, large eggs: 77 $\pm$ 24 eggs, mean $\pm$ s.d.). Given estimates of egg diameter for each female size category [48], and the average number of eggs received in each treatment, the 17% difference in number of eggs brooded by the males in the two egg size treatments translated into only a 3% difference in egg surface area between treatments (see the electronic supplementary material).

Syngnathus typhle is polygynandrous, and in our study population, males typically brood eggs from three to four females per pregnancy [50–53]. Females produce eggs continuously [54] and large females produce enough eggs to fill up almost three similar-sized males during the course of one pregnancy, while small females can fill up little more than one male of similar size during the same period [55,56]. Thus, males were allowed to mate polygynously, by introducing groups of males in large mating aquaria (approx. 70 l) containing groups of either large or small females. Specifically, females and males were introduced in the mating tanks in ratios of 1 : 2 for large females (four females and eight males) and 1 : 1 for small females (eight females and eight males), and individual females were replaced periodically if they looked egg-limited, to ensure that males mated and filled up their brood pouches within a few days. All matings took place in fully oxygenated water.

Each male was kept in the mating tank until his pouch was filled with eggs. Once fully mated, the male was measured and transferred into a smaller (26 × 45 × 40 cm or 26 × 35 × 35 cm) individual tank, where he was kept brooding for 18 days at a high or low level of DO (details below). After 18 days, the embryos have developed eyespots and this period corresponds to between one-third and one-half of the total brooding period at 14–15°C [56].

Brooding aquaria were supplied with either fully oxygenated water (approx. 100% O_2, corresponding to 8.70 mg l^{-1} or 156 mm Hg O_2 at 15°C) or hypoxic water (40% O_2, corresponding to 3.50 mg l^{-1} or 63 mm Hg O_2 at 15°C), referred to as high and low O_2 below. In natural seagrass habitats, O_2 saturation varies substantially with light and temperature, often between 60 and 150% [38–40,57], but 40% is well within the natural range [40]. The hypoxic level of 40% O_2 saturation was chosen based on previous studies on sand gobies [33,58], collected from the same bay as our pipefish, where 30–40% O_2 was used. These levels elicited behavioural changes without causing adult mortality.

DO concentration was decreased by pumping nitrogen into the water with the use of a MiniModule 1.7* 5.5 Membrane Contactor (Liqui-Cel, Celgard, Inc., North Carolina, USA), which removes the O_2 through a counter-current system (i.e. water and nitrogen flow in opposite directions). This unit allowed hypoxic aquaria to have flow-through water like the fully oxygenated aquaria (see the electronic supplementary material for details on maintenance of O_2 treatments).

At day 18 of brooding, males were euthanized by immersion in 1 ml 2-phenoxyethanol/l seawater) solution for 10 min, followed by severing the spinal column immediately posterior to the opercula. Each male was preserved in 70% ethanol for later dissection. One male was removed from all analyses because its eggs were all unfertilized.

(c) Embryo survival and size

Males were dissected to assess relative embryo survival. This was accomplished by calculating the proportion of well-developing embryos out of all eggs in the brood pouch, including unfertilized eggs and substantially underdeveloped embryos.

To obtain estimates of embryo size for each brood at the end of the 18-day brooding period, the average length and weight of embryos were collected as follows: 15 embryos, from separate regions of the brood pouch, were removed from each male. The embryos were separated from the egg membrane and the yolk sac. Photographs of five embryos were taken using a camera (Leica DFC420 A) 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 of the five embryos using Leica Application Suite, v. 2.7.0.RI (Build: 1294). The remaining 10 embryos were placed on a Petri dish in a heating cupboard (60°C) for one week after which the dried embryos were weighed twice on a Sartorius LE26P microbalance (±2 µg). The average of these measurements divided by 10 gave the average embryo weight for each male.

(d) Male behaviour

On days 1, 9 and 18 of brooding, ventilation rates and 10 min video recordings were taken for each male. Ventilation rates were counted during direct observations as number of opercular movements of each individual for 30 s. Videos were analysed using JWatcher v. 1.0. From the video recordings, proportion of time spent above the midline of the water column and proportion of time spent actively swimming were recorded. Six males were removed from these analyses because we did not have data on all days for these males.

(e) Statistical analysis

SPSS v. 22 (IBM SPSS Statistics for Windows, v. 22.0, IBM Corp., Armonk, NY, USA) and PERMANOVA+ for Primer v. 6 (Primer-E, Plymouth, UK) were used to perform all analyses. PERMANOVA+ was chosen because its tests rely on permutations to calculate the distribution of the data, thus avoiding the strict assumptions of parametric tests that are difficult to meet when analysing groups with unequal sample sizes. PERMANOVA has two assumptions: multivariate observations are independent (which they are between males) and identically distributed, i.e. the dispersion clouds are homogenous. To meet the second assumption, some of the data were first transformed as detailed below. Because some of the response variables measured were not independent from each other, e.g. embryo length, dry weight and survival, we chose to analyse our data using multivariate statistics to assess overall effects of our treatments and their interactions on our response variables. Only if the multivariate tests returned a significant effect did we then analysed the response variables separately. PERMANOVA was used to assess: (i) the effects of year (random factor (RF)), egg size treatment (fixed factor (FF): small and large) and O_2 treatment (FF: high and low), on embryo survival, length and dry weight, and (ii) the effects of year (RF), day (FF, repeated-measure: days 1, 9 and 18), O_2 treatment (FF), egg size treatment (FF) and male ID (RF, nested within year, egg size treatment and O_2 treatment) on ventilation rates, proportion of time spent swimming and proportion of time spent in the upper part of the water column. In (i), embryo survival was arcsine-transformed and embryo length was log-transformed and in (ii), proportion of time spent swimming and proportion of time spent in the upper half of the aquarium were arcsine-square root-transformed. Response variables were normalized (each variable had its mean subtracted and was divided by its standard deviation) in order to achieve a common scale between the variables before producing a resemblance matrix based on Euclidean distances [59,60] for each multivariate test. As a rule, interaction terms with p (perm) > 0.2 were sequentially removed from the models, starting with the highest level of interactions, followed by within-level least significance. Owing to its central importance to the questions addressed in this study, the "egg size x oxygen" interaction term was kept in the analyses of embryo survival, length and dry weight. Final models are presented in tables 1 and 2. Whenever the multivariate permutational analyses showed significant terms, permutational ANOVAs were performed on the separate response variables, using the same reduced models and transformed variables as in the MANOVAs. The following options were chosen for all analyses: type III sums of squares, fixed effects sum to zero, model: permutation of residuals under a reduced model, number of permutations: 9999.

As matings were done prior to the experimental O_2 treatments, the effects of O_2 and of egg size treatments on number

Table 1. Permutational MANOVA analyses of the effects of year (2007, 2008), egg size treatment (small versus large) and oxygen treatment (100% versus 40%) on *S. typhle* overall relative embryo survival, average embryo length (mm) and dry weight (mg). ($n = 46$. Analyses were performed on transformed and normalized variables).

source	d.f.	MS	pseudo-*F*	*p* (perm)
year	1	8.92	3.80	0.018[a]
egg size	1	10.16	4.32	0.011[a]
oxygen level	1	16.64	7.08	0.001[a]
egg size × oxygen level	1	0.07	0.03	0.989[a]
residual	41	2.35[b]		

[a]Term mean squares tested against the pooled mean squares of residuals and remaining non-significant interaction terms.
[b]Mean square of pooled residuals and remaining non-significant interaction terms.

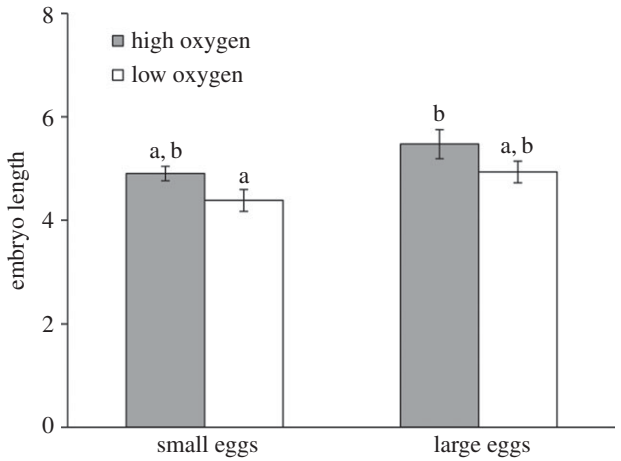

Figure 1. Average embryo length (mm) of *S. typhle* males that received small or large eggs and were kept either in high (100%) or low (40%) oxygen conditions for a brooding period of 18 days. Significant differences are displayed with different letters, based on LSD post hoc tests following one-way ANOVA, using an oxygen level–egg size composite treatment factor with four levels.

of eggs received by the males were analysed separately with *t*-tests. Significance level for all tests, except for interactions key to our study (see the electronic supplementary material), was set at $p < 0.05$.

3. Results

(a) Embryo survival and size

Embryo survival was on average 75% (range: 18–97%) in all males combined. There was a significant overall variability between years in embryo survival and size (table 1). The multivariate tests showed strong effects of egg size and O_2 treatments but not of their interaction (table 1). Post hoc tests show that these effects were due to low O_2 level having a strong negative effect both on embryo length (figure 1) and on embryo weight (electronic supplementary material, figure S1), but not on embryo survival (table 2). Egg size showed a strong positive effect on embryo weight and tended to affect embryo length positively, but neither egg size nor O_2 treatment affected embryo survival (table 2).

(b) Male behaviour

There was a significant year by day interaction in the overall behaviours, but behaviours did not change significantly with day in the brooding cycle (table 3). Both O_2 and egg size treatments affected overall male behaviour (table 3). Individuals differed significantly in their behaviour (table 3), particularly in their ventilation rates (table 4). Ventilation rates were significantly higher in males kept in low O_2 conditions compared with males kept in high O_2 conditions (figure 2 and table 4). Males brooding larger eggs swam significantly more than males brooding smaller eggs, but neither egg size nor O_2 level affected the time they spent in the upper part of the aquaria (table 4).

4. Discussion

(a) Egg size

We found no interactions between egg size and O_2 level on embryo survival or growth, despite the large intraspecific variation in egg size observed in this species [37]. This means that embryos from large eggs did not develop significantly worse than embryos from small eggs in hypoxic conditions, as has been predicted from theory [4,6,13]. If embryos from large eggs have lower metabolic rates per egg volume compared with embryos from small eggs, in similarity to the scaling effect observed in brown trout, *Salmo trutta* [4], such differences in metabolic rates may be large enough to compensate for the less favourable surface area to volume ratio of large eggs. Empirical support for this premise is mixed. For instance, in the mouthbrooding cichlid, *Pseudocrenilabrus multicolour victoriae*, females from populations that experience hypoxic conditions year-round produce smaller yet more numerous eggs than females from populations that consistently experience high O_2 saturation levels [15]. However, a complementary laboratory study showed that F_1 females from both populations produced larger eggs in hypoxic than in fully oxygenated water [15]. This result lends support to the study by Einum *et al*. [4], which showed that brown trout embryos from small eggs had lower survival in hypoxic water compared with embryos from larger eggs. Together, these studies have questioned which egg size is more negatively affected by hypoxia.

In *S. typhle*, embryo respiration increases with increasing embryo dry mass with a slope of 0.44 [59]. Although the relationship between respiration and egg volume is not exactly known, egg diameter and dry mass are strongly and positively correlated [37]. Thus, this relatively low slope (clearly less than 1) indicates that embryos from larger eggs have lower metabolic rates per egg volume compared with embryos from smaller eggs. If so, such difference in metabolic rates could explain why embryos from the small and large egg treatments were similarly affected by our hypoxic treatment. Thus, our study adds to the list of studies questioning whether embryos from larger eggs are indeed more O_2-constrained in aquatic environments.

Our males displayed low swimming activity, consistent with the generally inactive and cryptic behaviour of syngnathids and with previous studies showing that pregnant

Table 2. Permutational ANOVA analyses of the effects of year (2007, 2008), egg size treatment (small versus large) and oxygen treatment (100% versus 40%) on *S. typhle*, relative embryo survival, average embryo length (mm) and dry weight (mg). ($n = 46$. Analyses were performed on transformed and normalized variables).

		survival			length			weight		
source	d.f.	MS	pseudo-*F*	*p* (perm)	MS	pseudo-*F*	*p* (perm)	MS	pseudo-*F*	*p* (perm)
year	1	3.89	4.12	0.049[a]	5.01	7.03	0.011[a]	0.01	0.02	0.892[a]
egg size	1	0.32	0.34	0.565[a]	2.23	3.13	0.081[a]	7.61	11.00	0.002[a]
oxygen level	1	0.26	0.28	0.595[a]	8.40	11.78	0.001[a]	7.98	11.54	0.002[a]
egg size × oxygen level	1	0.06	0.07	0.802[a]	0.00	0.00	0.997[a]	0.01	0.01	0.920[a]
residual	41	0.95[b]			0.71[b]			0.69[b]		

[a]Term mean squares tested against the pooled mean squares of residuals and remaining non-significant interaction terms.
[b]Mean square of pooled residuals and remaining non-significant interaction terms.

Table 3. Permutational MANOVA analyses of the effects of egg size treatment (small versus large), oxygen treatment (100% versus 40%), day (1, 9 and 18) and male ID (nested within egg size, oxygen levels and year) on *S. typhle* overall ventilation rates, proportion of time spent swimming and proportion of time spent in the upper half of the aquarium. ($n = 41$. Analyses were performed on transformed and normalized variables.)

source	d.f.	MS	pseudo-*F*	*p* (perm)
egg size	1	9.29	3.14	0.037[a]
oxygen level	1	44.21	14.94	<0.001[a]
year	1	7.87	2.66	0.054[a]
day	2	14.61	2.71	0.177
year × day	2	5.38	2.68	0.032[a]
male	36	2.94	1.47	0.019[a]
residual	79	2.01[b]		

[a]Term mean square was tested against pooled mean square of residuals and all other interactions.
[b]Mean square of pooled residuals and non-significant interaction terms.

males swim less than females and non-pregnant males [60,61]. However, males brooding larger eggs swam significantly more than males brooding smaller eggs, though they did not differ in the time spent in the upper part of the aquaria. This result is in line with the increased swimming activity found in other species, shown to increase the amount of water that passes through the gills, improving O_2 uptake [62,63]. It also resembles the typical parental behaviour of the giant waterbug, in which fathers improve the access of O_2 to the developing embryos [64]. It is thus possible that swimming in *S. typhle* has similar effects, with the movements of the paternal body facilitating O_2 uptake in the gills, improving gas exchange to meet the higher O_2 requirements of larger embryos.

Males that brooded large eggs received 17% fewer eggs than males brooding small eggs. Thus, our data provide a possibly unique example, in which males that mate with large females pay a cost by caring for fewer offspring. Since large females are preferred as mates in this species [59], this clearly suggests that the fitness benefits males gain from that preference (e.g. larger offspring that survive better; [48,65]) are large enough to override the number's cost. Moreover, caring for fewer embryos, i.e. having a lower embryo density in the pouch, may allow for better oxygenation. However, whether egg size affects O_2 levels in the pouch still remains to be tested.

(b) Oxygenation

The males brooding in hypoxia showed significantly faster opercular movements throughout the experiment. Faster opercular movements result in faster O_2 extraction from the water, increasing O_2 availability for the males' own metabolic needs and, supposedly, for the developing embryos. In species where parents fan the clutches to ensure a steady access of O_2 to the embryos, similar increases in ventilation rates with decreasing O_2 availability have been reported (e.g. [31,33]). Yet, in our study, embryos brooded in hypoxia were shorter and lighter than embryos brooded in normoxia, indicating a clear negative effect of hypoxia on embryo development. A previous study of ours that measured O_2 concentrations in the pouch fluid of brooding *S. typhle* kept in normoxia and hypoxia found that pouch fluid O_2 concentrations were much lower than those of the water surrounding the males in both treatments, and that pouch fluid O_2 concentrations were significantly lower in the males kept in hypoxia compared to the males kept in normoxia [66]. Two important conclusions can be drawn from that study: first, it is noteworthy that pouch brooded embryos naturally develop in much poorer O_2 conditions compared with ambient water (about 40% lower O_2); and second, that males appear to have limited ability to buffer the developing embryos from prolonged environmental hypoxia.

Differences in length and weight of embryos brooded in high and low O_2 conditions can arise from several direct and indirect mechanisms. First, under hypoxia, tissue differentiation rates can retard development, so that embryos from males kept in hypoxia may be at earlier stages of development resulting in smaller sizes [67,68]. Second, development may progress at similar rates but embryos are smaller due to less efficient anaerobic metabolic processes [69,70]. Third, quality of parental care (for instance, nutrients provided) may differ in normal and low O_2 conditions owing to the physiological stress imposed by hypoxia on the caring parent, affecting the

Table 4. Permutational ANOVA analyses of the effects of egg size treatment (small versus large), oxygen treatment (100% versus 40%), day (1, 9 and 18) and male ID (nested within egg size, oxygen levels and year) on *S. typhle* ventilation rates, proportion of time spent swimming and proportion of time spent in the upper half of the aquarium. ($n = 41$. Analyses were performed on transformed and normalized variables.)

	ventilation rate				proportion of time swimming				proportion of time in upper part of tank			
source	d.f.	MS	pseudo-*F*	*p* (perm)	d.f.	MS	pseudo-*F*	*p* (perm)	d.f.	MS	pseudo-*F*	*p* (perm)
egg size	1	0.00	0.00	0.977[a]	1	7.08	7.49	0.010[a]	1	2.21	2.43	0.132[a]
oxygen level	1	43.45	39.39	<0.001[a]	1	0.23	0.24	0.631[a]	1	0.52	0.57	0.450[a]
year	1	1.96	1.78	0.201[a]	1	5.87	6.21	0.017[a]	1	0.04	0.04	0.843[a]
day	2	4.41	13.61	0.105	2	0.43	0.25	0.851	2	9.77	2.91	0.239
year × day	2	0.32	1.13	0.324[a]	2	1.70	1.84	0.164[a]	2	3.36	4.21	0.021[a]
male	36	1.10	3.85	<0.001[a]	36	0.95	1.02	0.452[a]	36	0.91	1.14	0.317[a]
residual	79	0.29[b]			79	0.93[b]			79	0.80[b]		

[a]Term mean square was tested against pooled mean square of residuals and all other interactions.
[b]Mean square of pooled residuals and non-significant interaction terms.

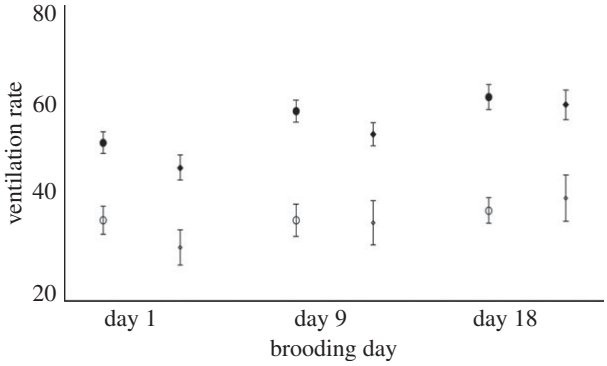

Figure 2. Average (mean ± s.e., ventilations per minute) ventilation rates of *S. typhle* males brooding embryos from large (circles) or small (diamonds) eggs, kept in high (open symbols) or low (filled symbols) oxygen conditions. Ventilation rates were recorded three times during the experiment, on days 1, 9 and 18.

developing young indirectly. Both developmental retardation and smaller sizes at emergence under hypoxia have been recorded in a copepod (*Acartia tonsa* [71]) showing that these mechanisms are not mutually exclusive.

Whether due to anthropogenic effects or natural causes, hypoxia affects aquatic environments worldwide, in particular areas that are important for reproduction such as estuaries, semi-enclosed areas and shallow coastal regions [72–74]. In this study, we kept hypoxic conditions and temperature constant for the duration of 18 days, representing a chronic exposure (more than 4 days; [16]). In our study population, at the onset of the reproductive season, individuals migrate into shallow protected bays with eelgrass, where algal overgrowth is common [75] so that the water may become hyperoxic during the day owing to photosynthesis, but hypoxic at night owing to algal and plant respiration [38–40,57]. Thus, this population may experience hypoxic events that are high in frequency and magnitude but periodical in duration, conditions that are difficult to replicate experimentally. In the paternal egg-guarding plainfin midshipman fish, *Porichthys notatus*, a species that also reproduces in near shore environments with fluctuating O_2 levels, caring males are able to withstand hypoxia for twice as long as females [76]. Whether *S. typhle* males and females differ in their ability to tolerate hypoxic conditions, related to the sexual difference in parental care, is unknown but worth exploring in the future.

(c) Paternal care

Brooding males provide nutrients, osmoregulation and oxygenation to the developing embryos. In addition, inside the brood pouch embryos are safe from external predation so that whole brood mortality depends on paternal survival, whereas individual embryo survival may depend on paternal brooding ability (nutrients, O_2, etc.) or embryo competition [45,77]. In this study, relative embryo survival was around 75% in both treatments; a number comparable to previous studies on this population [48,65] and importantly, embryo survival did not differ between egg size or O_2 treatments. Does this mean males are able to adjust O_2 provision to the embryos in relation to embryonic consumption? Probably not significantly: while we do show that males brooding large eggs swim more than males brooding small eggs, which may facilitate paternal O_2 uptake and allow them to provide relatively more O_2 to larger embryos, we also know that pouch fluid O_2 is considerably lower than that of the tank water [66], both under hypoxia and normoxia. In addition, pouch O_2 drops markedly over the brooding period, that is, when O_2 consumption by the embryos is likely to be higher [66]. Therefore, if males are able to adjust O_2 provisioning to the embryos, such ability appears to be limited.

An alternative explanation for the lack of difference in embryo survival between broods consisting of the large and small egg size, and the lack of interaction between egg size and O_2 levels, is that with smaller egg size, more eggs fit in the pouches. Thus, if there is a benefit of being a small egg in terms of oxygenation, the larger number of embryos developing in close contact, which result in similar total egg surface areas in the two egg size treatments, may mask it. This could be tested in future studies, by keeping egg

numbers rather than pouch fullness constant, while varying egg size.

Why was embryo survival not affected by hypoxia in our study, when it has been documented in other aquatic species (e.g. [78,79])? Our chosen level of 40% DO (3.50 mg O_2 l^{-1}) may not have been low enough to affect embryo survival, even though it clearly affected embryo size negatively and caused a significant increase in ventilation rates of brooding males. A range of O_2 concentrations have been used in other studies and species showing significant variation in embryo sensitivity to hypoxia [4,79]. Yet, given that 40% DO is comparatively low for natural seagrass meadows, and the exposure in our experiment was considerably longer than what is common in nature, this result shows that *S. typhle* is able to cope with brooding also at reduced O_2 levels.

Broadly, our study raises the question as to whether pouch brooding in syngnathids evolved at least partly as an adaptation to hypoxia. It is common in fishes for males that provide parental care during embryonic development to adjust their fanning behaviour in response to ambient O_2 conditions [33,80] as well as to the developmental stage of the embryos [81]. In the seahorse, *Hippocampus zosterae*, males increase O_2 consumption during brooding by up to 50% compared with when not brooding [82]. Interestingly, in our study, day in the brooding period (i.e. embryonic stage) did not influence male ventilation rates or activity patterns. However, since embryonic metabolic demands tend to peak just before hatching [8,46,67,83–85], it is possible that hypoxia impacts embryo survival only towards the end of the brooding period.

(d) The aquatic puzzle: egg size and oxygen under paternal care

Pipefishes and seahorses display substantial variation among genera in where and how brood care is performed [37,43]. Species with embryos attached to the surface of the male body (without a pouch) tend to have smaller eggs than species with enclosed pouches [37]. In the absence of a pouch, embryos access O_2 directly from the ambient water, whereas in pouch brooders embryos depend on paternal oxygenation. In *S. typhle*, egg size is strongly correlated with female body length, with large females producing eggs that are substantially larger than those of small females [37]. Despite this pronounced intraspecific variation in egg size and the knowledge that large embryos respire more than smaller ones [46], we found no support for the hypothesis that large eggs do worse than small eggs in hypoxia due to their lower surface area to volume ratio. We found that under hypoxia, male ventilation rates increased significantly and that embryo length and weight were significantly and negatively affected. However, these effects were similar in both egg size classes and embryo survival at 18 days of age did not differ in relation to ambient O_2 levels. Thus, in contrast to long-established theory, embryos from large eggs were not more constrained in their development than embryos from smaller eggs in males brooding either in hypoxia or normoxia. This may be owing to embryonic metabolic demands not increasing proportionally with egg volume, as discussed above, or to the pregnancy in *S. typhle*, where males are able to provide nutrients and O_2 and to osmoregulate the embryos during brooding. In the theoretical model developed by Jorgensen *et al*. [21], ecological factors favouring the evolution of large offspring size also favour live bearing. In accordance with this, in *S. typhle*, we find females producing large eggs for their body size [37] and males providing long and multifaceted paternal care in the safety of the brood pouch.

In conclusion, our study did not provide support for the surface-to-volume ratio argument for constraints in egg size evolution of aquatic organisms because, although *S. typhle* produces relatively large eggs and has limited ability to oxygenate the developing embryos, the observed negative effects of hypoxia on embryo development were largely independent of egg size. Thus, this hypothesized constraint is unlikely to be universal and the controversy around this issue is far from settled. Our study thus highlights the need for more intra- and interspecific empirical tests, on a greater variety of aquatic organisms, to better understand the evolutionary puzzle of egg size, oxygenation and parental care in aquatic environments.

Ethics. The experiment was done according to Swedish law, with an ethical approval given by the Swedish Animal Welfare Agency (permits no. 196-2005 and 112-2007). This study does not involve any endangered or protected species.

Data accessibility. All data will be made accessible on the Dryad Digital Repository.

Authors' contributions. I.B.G., I.A. and C.K. designed the experiments and wrote the paper. I.B.G. performed the experiment and analysed the data.

Competing interests. We declare we have no competing interests.

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