

# Nutritional state – a survival kit for brooding pipefish fathers

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A parent's nutritional state may influence its ability to provide care to offspring and ability to handle infections. In the broad-nosed pipefish, *Syngnathus typhle*, males care for their offspring by brooding the developing embryos in a brood pouch, providing nutrients and oxygen, resembling a pregnancy. Here, we demonstrate that the nutritional state of pregnant males covaries with their own survival during a selective event. Brooding males surviving a *Vibrio* sp. infection were in a significantly better nutritional state, as estimated by their hepatosomatic index. Furthermore, a higher nutritional state of the brooding male correlated with a lower embryo mortality, while feeding treatment (low vs. high) had no effect on male survival, nutritional state or embryo mortality. Finally, males brooding heavier embryos also showed a lower embryo mortality. This may reflect a maternal effect (if large eggs result in higher embryo survival), a paternal effect (if higher provisioning of male care promotes both embryo growth and survival), or a combination thereof (males caring more for large embryos). The results demonstrate the importance of a good nutritional state for a caring parent when their immunity is challenged.

ADDITIONAL KEYWORDS: brood reduction – condition – embryo mortality – immunity – parental care – Syngnathidae – *Syngnathus typhle*.

## INTRODUCTION

Parental care promotes offspring development and increases offspring survival (Clutton-Brock, 1991; Klug & Bonsall, 2010; Kvarnemo, 2010), but often at a cost of parental survival and future reproductive success, as a result of reduced body condition or immune defence (Trivers, 1974; Clutton-Brock, 1991; Smith & Wootton, 1995; Zuk & Stoehr 2002). However, during selective events, such as a disease outbreak, a caring parent may adjust its care in order to survive the infection. Hence, life-history trade-offs are expected between resources allocated to the parent's own maintenance, ability to fend off an infection and resources allocated to care (Zuk & Stoehr, 2002). Thus, a selective event may relate to both the survival of the caring parent and the survival and development of the offspring under care. The caring parent may save resources by terminating or reducing care and, furthermore, may gain or regain resources by utilizing the nutrients from the

forfeited offspring (Sargent, 1992; Kvarnemo, Svensson & Forsgren, 1998; Manica, 2002, 2004; Neff, 2003; Klug & Bonsall, 2007; Vallon *et al.*, 2015). Such resources can be used to increase the parent's nutritional state, and be invested into, for example, disease resistance and future reproduction (Zuk & Stoehr, 2002).

An organism's ability to survive and reproduce depends on how its resources are allocated, its physiological state and external circumstances (McNamara & Houston, 1996). The concepts of condition and nutritional state are widely used. Commonly, condition represents the total energy reserves, whereas state refers to what may influence those reserves; however, condition and nutritional state are also used rather synonymously (Clancey & Byers, 2014). As such, the condition/nutritional state of a parent can be manipulated by food provisioning or food deprivation and hence resource allocation and parent–offspring interactions can be affected and studied. For example: (1) in the Gulf pipefish (*Syngnathus scovelli*) pregnant fathers in a high food treatment invested both in current reproduction and in somatic growth (future reproduction), whereas

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low food males sacrificed growth in order to invest in current reproduction (Paczolt & Jones, 2015); (2) in the spiny tropical damselfish *Acanthochromis polyacanthus* well-fed parents (in better condition) had a higher reproductive output and embryo survival (Donelson, McCormick & Munday, 2008); (3) in the maternally mouth-brooding cichlid *Simochromis pleurospilus* food deprivation resulted in smaller young and less brood care (Segers, Gerber & Taborsky, 2011); (4) in the earwig *Forficula auricularia* with facultative care, female condition (manipulated by food) influenced the provisioning of nymphs (Wong & Kölliker, 2012); and (5) in bumblebee (*Bombus terrestris*) workers, an experimental immune challenge resulted in reduced survival compared with controls, but only when the bumblebees were starved, whereas when fed ad libitum there was no change in survival (Moret & Schmid-Hempel, 2000). These studies illustrate interactions between nutritional state and a parent's resource allocations to care and survival.

Here, we investigate if the nutritional state of a caring parent may affect its own survival and embryo development of its brooded young. This was done in pregnant broad-nosed pipefish (*Syngnathus typhle*) males, by manipulating feeding regimes and by analysing indicators of the males' nutritional state after an event of a naturally occurring bacterial infection (*Vibrio* sp.). We predicted that males in a higher nutritional state would have a higher chance of surviving the infection, and to be able to support the development of more and/or heavier embryos. Furthermore, broods consisting of larger embryos were predicted to show lower embryo mortality, either because embryos that originate from larger eggs have more initial resources (maternal effect), or because larger embryos receive more care from the male (paternal effect) and/or because males preferentially support larger embryos due to their higher reproductive value (paternal and maternal effects combined).

## STUDY SPECIES

The broad-nosed pipefish is found in shallow marine areas and exhibits care in the form of a male pregnancy (Kvarnemo *et al.*, 2011) with variable levels of embryo mortality (Ahnesjö, 1992a, b, 1996; Sagebakken *et al.*, 2011). In this species, egg size correlates positively with female body size (Braga Gonçalves, Ahnesjö & Kvarnemo, 2011) and both males and females prefer to mate with larger partners (Berglund, Rosenqvist & Svensson, 1986a; Berglund & Rosenqvist, 2001). The male commonly receives eggs from several females (Jones *et al.*, 1999). The brooding period of the embryos is approximately 5–8 weeks, depending on temperature (Ahnesjö, 1995), and lasts until independent young

are released. Embryos in the paternal brood pouch of *Syngnathus* spp. are oxygenated, osmoregulated and protected (Berglund, Rosenqvist & Svensson, 1986b; Ripley & Foran, 2006; Ripley, 2009). Embryo mortality in the broad-nosed pipefish has been shown to be influenced by the number of embryos in the pouch (Ahnesjö 1992b, 1996; Mobley *et al.*, 2011; Sagebakken *et al.*, 2011, 2016), initial egg size (Ahnesjö, 1996; Mobley *et al.*, 2011) and the number of females contributing to the brood (Sagebakken *et al.*, 2011). Furthermore, using radioactive labelling we have demonstrated that brooding males provide nutrients to the developing embryos (Kvarnemo *et al.*, 2011) and that nutrients originating from embryos can be absorbed by the brooding father's body tissues (Sagebakken *et al.*, 2010). Consequently, this provides opportunities for the male's nutritional state, and potentially also his immune competence, to be influenced by stored resources, by resource intake and by embryonal resource exchange.

## MATERIAL AND METHODS

The study was carried out at Sven Lovén Centre for Marine Sciences – Kristineberg, on the west coast of Sweden (58°15'N, 11°28'E), from April to June 2008. Broad-nosed pipefish were caught from the end of April to the beginning of May. We captured the fish in shallow *Zostera marina* meadows in the Gullmar fjord using a small beam trawl, mesh size 4 mm, pulled by boat or using a handheld beach seine. The fish were separated by sex and size, and kept in 200-litre barrels containing artificial *Zostera*, air stones and continuously renewed seawater at 14 °C, with artificial light from 06:00 to 22:00 h. The fish were fed live, cultivated *Artemia* sp. three times a day and wild caught crustaceans, including *Crangon crangon*, *Praunus flexuosus*, *Neomysis integer* and *Copepoda* sp., ad libitum one to three times a day. Throughout the study, barrels and experimental basins were cleaned daily by careful siphoning, and sick or dead fish were removed during feeding or cleaning.

We randomly assigned males to one of two feeding regimes: the 'low' group was a standard-fed group that was fed cultured *Artemia* sp., and wild-caught *Praunus flexuosus*, *Neomysis integer* and *Copepoda* sp. twice a day. The 'high' group was given the same feeding as the 'low' group but five times a day. Before the males were assigned to a feeding regime, their standard length, trunk width and depth were measured. The males were matched in pairs according to body length and the male with the widest and deepest trunk within each pair was on every second occasion put in the low food treatment. Consequently, males in the two feeding regimes did not differ in length (paired *t*-test,  $t = 0.82$ , d.f. = 49,  $P = 0.42$ ), trunk width (paired *t*-test,  $t = 0.85$ , d.f. = 49,  $P = 0.40$ ), or trunk depth (paired

*t*-test,  $t = 0.10$ , d.f. = 49,  $P = 0.92$ ) at the start of the treatments. After 14 days, males in the high feeding treatment had a significantly deeper trunk (paired *t*-test,  $t = 4.23$ , d.f. = 46,  $P < 0.001$ ), whereas length and trunk width did not differ significantly between the groups (paired *t*-test, length,  $t = 1.76$ , d.f. = 46,  $P = 0.085$ ; trunk width,  $t = 2.65$ , d.f. = 46,  $P = 0.11$ ).

After 14 days of feeding treatment, the males were mated to females. We put 30 females, ranging from 144 to 229 mm in standard length (average  $\pm$  SE = 182.8  $\pm$  2.0 mm), in each of four 350-litre basins. This variation in female length represents the natural demography and consequently also the natural variation in egg size. Males were added to the basins and allowed to mate: 12 males to each basin in two rounds, 48 h apart. Given the excess of females, most males were likely to mate with multiple females, as is typical for this population (Jones *et al.*, 1999). In total, 69 out of 86 males filled their brood pouch to 100%. Thirty of these males came from the 'low' feeding regime and 39 from the 'high' feeding regime. Males that did not fill their brood pouches fully were excluded from the experiment. The mean number of days ( $\pm$  SE) until the males were fully mated was 2.5  $\pm$  1.4 and did not differ between the feeding treatments (multivariate analysis of variance, MANOVA,  $F_{1,67} = 0.70$ ,  $P = 0.41$ ). The fully mated males were then sedated in seawater with 2-phenoxyethanol (100  $\mu$ l L<sup>-1</sup>) and the number of eggs in each pouch was counted through the skinfolds, using a cold light. The treatments did not differ in number of eggs males received in their pouches: low fed males received on average ( $\pm$  SE) 96.1  $\pm$  4.7 eggs at mating, and high fed males received 107.1  $\pm$  4.4 eggs at mating (MANOVA  $F_{1,67} = 2.82$ ,  $P = 0.10$ ). The males were individually colour marked subcutaneously with non-toxic latex paint (Liquitex, NJ, USA), while still sedated. After egg counting and marking, the males were left in seawater to recover (about 10 min) and then returned to their assigned basin. This experiment was carried out in accordance with current Swedish regulations for the use of animals in scientific procedures, and was approved by the Ethical Committee for Animal Research in Gothenburg (Dnr 111–2007).

The aim was to let males brood for 2 weeks, which corresponds to about one-third of a whole brooding period. However, an outbreak of a pathogenic bacterial *Vibrio* infection swept through the fjord and, as we used seawater pumped in straight from the sea, the infection affected our experiment and resulted in a selective event. Consequently, within a period of 5 days, 58 of the males (28 from the 'low' and 30 from the 'high' feeding regime) died or they were in such condition that we killed them due to ethical considerations. These males (non-survivors) had been brooding for 4–14 days. When the experiment was terminated, 11 of the 69 males had survived the infection and

they had been brooding for 9–14 days. All males were killed in seawater with an overdose of 2-phenoxyethanol (1 ml L<sup>-1</sup>) and preserved in 70% ethanol for later dissection.

At dissection of all males, embryos were taken out of the male's brood pouch and counted. Embryo numbers were then compared to the egg number received at mating, and the difference gave us an estimate of embryo mortality (Sagebakken *et al.*, 2011). This estimate sometimes becomes negative, since occasionally some eggs are overlooked when counting number of eggs received at mating. Embryos (with yolk sac) were weighed on a Sartorius LE26P microbalance to the nearest 0.02 mg, after having been dried for 36 h at 70 °C. Mean embryo mass was determined by weighing the total embryo mass and dividing it by the number of embryos in the pouch for each male.

As a proxy for a male's nutritional state after brooding we used the hepatosomatic index (HSI), a common measure of the nutritional state in fish (Hussey *et al.*, 2009). We measured HSI by removing the liver of the males. We dried and weighed the liver and the fish body (without embryos, liver and intestines) using the same procedure as for embryo mass. HSI was then calculated as (liver dry weight  $\times$  100)/body dry weight.

The statistical analyses were done in SPSS 22 (SPSS, Inc., Chicago, IL, USA). As all males (non-survivors and survivors) presumably were affected by the infection, we analysed all 69 brooding males in this study (Supporting information, Table S1). We used a logistic regression analysis with survival (yes or no) of the *Vibrio* infection as dependent variable, number of eggs initially received in the brood pouch, embryo mortality and HSI as covariates, and feeding regime (high or low) as a factor. The effect of feeding treatment on number of days until mating, number of eggs received, number of days brooding, HSI and embryo mortality was tested using MANOVA. In addition, to better understand which variables may be affected by or influence the nutritional state of the male and his ability to provide care, partial correlations were performed pairwise for number of eggs received, embryo mortality, mean embryo mass, male HSI and number of days brooding, while controlling for the effect of the other three variables.

## RESULTS

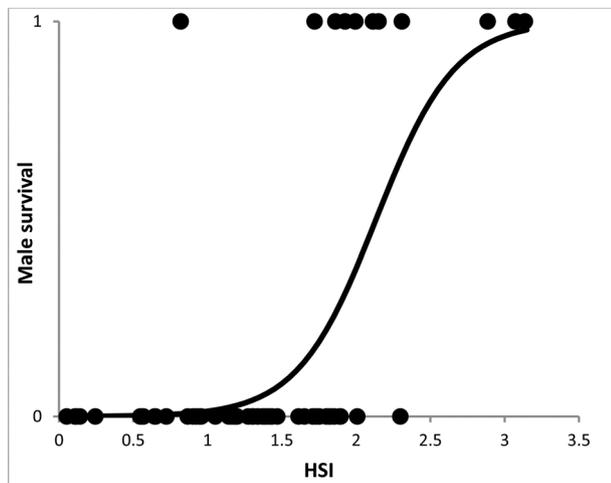
The underlying data for the results presented here are available in Supporting information Table S1. The logistic regression model of whether males survived the infection or not was significant ( $R^2 = 0.59$ ,  $\chi^2 = 28.89$ ,  $N = 69$ ,  $P = 0.001$ , Fig. 1). The only significant covariate was HSI (Wald = 9.94, d.f. = 1,  $P = 0.002$ ). The other covariates (embryo mortality and number of eggs

received at mating) and the factor (feeding regime) were non-significant with  $P \geq 0.14$ . This shows that males that survived had higher HSI than those that did not (Fig. 1), while feeding regime had no effect on male survival. Similarly, feeding regime overall had no significant effect on number of days until mating, number of eggs received, number of days brooding, HSI, or embryo mortality (MANOVA, Wilk's lambda = 0.85,  $F_{6,62} = 1.82$ ,  $P = 0.11$ ).

In the partial correlation matrix (Table 1), we found that higher embryo mortality correlated with lower male HSI ( $P = 0.01$ ). Higher embryo mortality also correlated with lower mean embryo mass ( $P < 0.001$ ) and males with a higher HSI brooded for more days (i.e. survived longer) in the experiment ( $P = 0.01$ ). All other partial correlations were non-significant (Table 1).

## DISCUSSION

In this study of the broad-nosed pipefish, we found that pregnant males that were in a better nutritional state had a higher chance of surviving a selective event



**Figure 1.** Brooding broad-nosed pipefish males that survived a selective *Vibrio* event had higher nutritional state, measured as hepatosomatic index (HSI), than males that did not survive.

caused by a *Vibrio* outbreak. Furthermore, males in better nutritional state showed lower embryo mortality, and males with lower embryo mortality brooded heavier embryos.

Many previous studies on different organisms have found that high and low food treatments affect parental condition and the allocation of resources to care, offspring size and offspring number (Donelson *et al.*, 2008; Segers *et al.*, 2011; Wong & Kölliker, 2012; Paczolt & Jones, 2015). In contrast, in the experimental study presented here, feeding treatment had no effect on the results, except that well-fed males had deeper trunks before brooding. Still, the nutritional state of the males (but not feeding treatment) was of significant importance when their immunity was challenged, as males surviving the infection until the end of the study had higher HSI, and higher male HSI also correlated with lower embryo mortality. The lack of a feeding treatment effect may either demonstrate that inherent differences between individuals are more important than recent resource acquisitions, or that both feeding treatments actually were relatively high. Pipefish are suction feeders (Flammang *et al.* 2009) that feed on live prey and require access to mobile prey. Possibly, in our case, prey availability in the high feeding treatment was more plentiful than the fish were able to catch. It is also common that individuals in an overall good condition or nutritional state do not show trade-offs between allocations (van Noordwijk & de Jong, 1986; Stearns, 1989). For instance, in the Gulf pipefish (*S. scovelli*) pregnant males under high food provisioning invested both in somatic growth and in their current brood (Paczolt & Jones, 2015) and in the seaweed pipefish *S. schlegeli*, males showed positive correlations between expensive organs (relative size of brain, liver and heart) presumably reflecting that individuals in good condition can allocate resources to several traits (Tsuboi *et al.*, 2016).

Vibriosis is a common disease in nature and aquaculture that quickly causes mortality (Hjeltnes & Roberts, 1993), with over 90% mortality within a few days after the first clinical symptoms (Alcaide *et al.*, 2001). When experimentally infected with *Vibrio* sp., males in this specific population of broad-nosed pipefish have been demonstrated to show both a

**Table 1.** Partial correlation coefficients ( $r$ ) and  $P$ -values (significant values in bold type) for pairwise correlations while controlling for the effect of the other variables (d.f.=64)

|                      | No. of eggs received     | Embryo mortality                           | Male HSI                                  | Embryo mass             |
|----------------------|--------------------------|--|---|-------------------------|
| Embryo mortality     | $r = -0.05$ , $P = 0.67$ | –  | –   | –                       |
| Male HSI             | $r = 0.20$ , $P = 0.11$  | $r = -0.32$ , <b><math>P = 0.01</math></b> | –   | –                       |
| Embryo mass          | $r = 0.12$ , $P = 0.32$  | $r = -0.53$ , <b><math>P = 0.01</math></b> | $r = -0.15$ , $P = 0.22$                  | –                       |
| No. of days brooding | $r = 0.16$ , $P = 0.21$  | $r = -0.05$ , $P = 0.67$                   | $r = 0.31$ , <b><math>P = 0.01</math></b> | $r = 0.18$ , $P = 0.14$ |

more active and specific immune defence compared to females (Roth *et al.*, 2011). Thus, the infection by *Vibrio* sp. in this population is likely to be a strong selective agent, in particular, on males. We found that males in a better nutritional state were more likely to survive to the end of the experiment, both in the logistic regression and in the partial correlations where a higher HSI correlated positively to number of days brooding. This means that if a better nutritional state reflects a larger pool of resources it can aid survival of the individual, either by allocating resources to defeat the infection or by having more resources available when the infection directly affects the nutritional state of the individual (Moret & Schmid-Hempel, 2000; Zuk & Stoehr, 2002).

The higher embryo mortality that we found for pipefish males in lower nutritional state (HSI) might be explained by such males having fewer resources available to allocate to brood care, or that the infection affected embryo mortality directly in males of a lower nutritional state. Care in the broad-nosed pipefish (e.g. provisioning of nutrients and oxygen, and immune defence; Berglund *et al.*, 1986b; Svensson, 1988; Braga Gonçalves, Ahnesjö & Kvarnemo, 2015a, b; Roth *et al.*, 2011) is costly to the brooding males and larger broods result in lower paternal survival (Sagebakken, Ahnesjö & Kvarnemo, 2016). Hence, it is possible that males in a lower nutritional state are less able to provide care, leading to higher embryo mortality. Similarly, under experimental food shortage, the growth rate of pregnant males of the Gulf pipefish (*S. scovelli*) was higher when more embryos failed during brooding (Paczolt & Jones, 2015), indicating that males can allocate resources from care to paternal growth under food shortage.

In many nest-tending fish species, males eat some or all of the embryos in their care, a behaviour called filial cannibalism (Manica, 2002). In some species, males are more prone to eat when they are in low condition (Kvarnemo *et al.*, 1998; Neff, 2003), and filial cannibalism has been shown to affect male body condition positively (Mehlis, Bakker & Frommen, 2009). In the broad-nosed pipefish we know that males, via their brood pouch structures, are able to take up nutrients that originate from embryos in the pouch (Sagebakken *et al.*, 2010). The negative correlation between embryo mortality and paternal nutritional state in the current study suggests that while poor paternal nutritional state may have influenced embryo mortality, any uptake of this kind improved the state of the males only marginally.

Males brooding heavier embryos showed lower embryo mortality. This result has many non-exclusive potential explanations. Firstly, it may be a maternal effect, with large eggs giving rise to large embryos (Beacham, Withler & Morley, 1985; Ahnesjö, 1992a;

Donelson *et al.*, 2008), having better survival at the embryo stage (Paczolt & Jones, 2010, 2015). However, previous work has found that embryo survival also depends on the size of the other embryos in the brood pouch (Ahnesjö, 1996), and Mobley *et al.* (2011) found an interaction between female size and number of embryos in the brood pouch, but no effect of initial egg size on embryo survival. Secondly, when high-quality individuals provide high-quality care, this may affect multiple fitness-related traits positively (Qvarnström, Griffith & Gustafsson, 2000; Weladji *et al.*, 2006). Thus, the negative correlation found between embryo mass and mortality could be due to paternal effects, if high-quality male care promotes both embryo size and embryo survival or diminishes negative effects of the infection. A third explanation combines maternal and paternal effects: males might provide more or better care to the initially larger eggs, as they are known to hatch into larger newborn that have a higher chance of survival in the wild (Ahnesjö, 1992b). Hence, the lower mortality of larger embryos may be a result of differential allocation by the male or of paternal progeny choice (Burley, 1988), as males may give extra care to these offspring (Paczolt & Jones, 2010, 2015). The lower mortality of large embryos may also relate to the fact that larger broad-nosed pipefish females (which produce larger eggs, Braga Gonçalves *et al.*, 2011) dominate over smaller females when competing for mating opportunities (Berglund *et al.*, 1986a; Berglund & Rosenqvist, 2001). Hence, in a competitive situation larger females would mate with better quality males, and smaller females would have to mate with lower quality males. This may have been the case in our experimental set-up due to the female-biased sex ratio in the mating basins. Consequently, it would be of future interest to investigate sexual selection and how males and females select for both immune defence and nutritional state of their mating partners.

In conclusion, selective consequences of an infection may depend on the nutritional state of the caring parent, directly or indirectly via its immune system. Here, brooding pipefish males in a higher nutritional state were demonstrated to survive the infection better and to have lower embryo mortality. Furthermore, males with lower embryo mortality also brooded heavier embryos. These results highlight the importance of a good nutritional state for the caring parent.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1.** Data presentation for each brooding broadnosed pipefish male in the experiment: male standard length SL (mm), feeding treatment (high or low), number of days until mating, number of eggs received at mating, number of embryos at dissection, embryo mortality, number of days brooding in the experiment, male body dry mass (mg), male liver dry mass (mg), hepatosomatic index (HSI), mean embryo dry mass (mg/embryo) and male survival (yes or no).