

Effect of parental age and associated size on fecundity, growth and survival in the yellow seahorse *Hippocampus kuda*

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Summary

Seahorses, together with the pipefishes (Family Syngnathidae), are the only vertebrates in which embryonic development takes place within a specialised body compartment, the brood pouch, of the male instead of the female. Embryos develop in close association with the brood pouch epithelium in a manner that bears some resemblance to embryo–placental relationships in mammals. We have explored the hypothesis that parental body size and age should affect offspring postnatal growth and survival if brood pouch quality impacts upon prenatal embryonic nutrition or respiration. Using an aquarium population of the yellow seahorse, *Hippocampus kuda*, we show here that large parents produce offspring whose initial postnatal growth rates (weeks one to three) were significantly higher than those of the offspring of younger and smaller parents. Whereas 90% of offspring from the larger parents survived for the duration of the study (7

weeks), less than 50% of offspring from smaller parents survived for the same period. For the offspring of large parents, growth rates from individual males were negatively correlated with the number of offspring in the cohort ($r=-0.82$; $P<0.05$); this was not the case for offspring from small parents ($r=0.048$; $P>0.9$). Observations of embryos within the pouch suggested that when relatively few embryos are present they may attach to functionally advantageous sites and thus gain physiological support during gestation. These results suggest that male body size, and pouch size and function, may influence the future fitness and survival of their offspring.

Key words: yellow seahorse, *Hippocampus kuda*, post-natal growth, attachment site, pouch, embryo, Barker hypothesis, foetal origins hypothesis.

Introduction

In mammals, inadequate nutrition *in utero*, whether due to poor maternal resources before or during pregnancy, not only affects the birth weight of offspring, but also their future growth and development (Barker, 1995). Much current data support the hypothesis that conditions in the uterus effectively 'programme' postnatal growth dynamics and the efficiency of fundamentally important physiological systems such as cardiovascular and pancreatic function (Barker, 1999; Kwong et al., 2000). Research in humans and experimental mammalian species has linked the prenatal and postnatal effects within a theoretical framework known as the 'foetal origins' or Barker's hypothesis. Here we present a preliminary investigation of a radically different, but analogous, reproductive system to see whether any parallels are discernible. We consider the possibility that similar principles might apply to seahorses that are famous for their elaborate paternal care system, in which embryonic growth and

development occurs exclusively within the brood pouch of the male. In this study we show that there is an effect of age and associated size on fecundity in the yellow seahorse, *Hippocampus kuda* and suggest that this effect is related to aspects of embryonic development.

Even though Syngnathidae (seahorses and pipefishes) embryos possess a yolk sac and are nutritionally independent of the male (Azzarello, 1991), aspects of pouch physiology and size may still influence the future growth and development of offspring. In seahorses the female deposits eggs inside the brood pouch during mating, the pouch opening closes immediately after mating and the fertilised eggs develop to term within an enclosed environment. Pouch physiology and its interaction with embryos bear some resemblance to placental function in mammals; the embryos become embedded within depressions of the interior lining of the brood pouch (Carcupino et al., 1997). Given that the pouch is sealed during gestation, it has been suggested that embryos depend

upon pouch function for gaseous exchange, removal of waste products (Carcupino et al., 1997) and osmoregulation (Linton and Soloff, 1964; Quast and Howe, 1980).

In this study we examine the effects of several sources of variation on the early postnatal growth characteristics of seahorses and experimentally investigate the hypothesis that age-associated size of parents is a significant determinant of offspring growth and viability. We propose that variability in the efficacy of the embryo–pouch interactions may lead to differences that affect future growth and development, thereby causing embryonic heterogeneity within single broods. This is in addition to other sources of variation in embryonic size that can arise from differences in the size of eggs [usually positively correlated with female size (Berglund et al., 1986b) and pouch size, which is related to the density of competing embryos (Ahnesjo, 1992; Ahnesjo, 1996; Watanabe and Watanabe, 2002)].

Seahorses present several experimental limitations that unavoidably confound studies of this type. We were unable to disentangle male size from the effects of female size because the seahorses prefer to mate with individuals of their own size (Foster and Vincent, 2004) and because age and size are strongly correlated, it is also difficult to separate size effects from age effect. Nevertheless, there are also some advantages: as seahorses produce large broods it is also possible to examine heterogeneity of growth within, as well as between, broods. While heterogeneity may reflect differences in egg quality, we also consider the possibility that it may also arise through differences in the positions in the pouch.

Materials and methods

Fish

The model species used for the present study was the Indo-Pacific seahorse, *Hippocampus kuda* (Bleeker 1952), often known as the yellow or spotted seahorse. The animals were obtained from a captive-bred, and therefore inbred, aquarium population that was established several years ago at Chester Zoo, UK. The exact origins and taxonomy of this population are difficult to ascertain, although it is generally regarded as *H. kuda*. Nevertheless, the 2002 *Manual of Seahorse Husbandry in Public Aquaria* (Bull and Mitchell, 2002) suggests that the name *H. kuda* has often been used to describe any Indo-Pacific seahorse that could not readily be identified. For the purpose of this *ex situ* study, however, the use of this population offered a convenient means of generating animals of known ages and sizes.

Culture and breeding of seahorses

Yellow seahorses, *H. kuda*, were housed in two 560 l seawater aquaria (to keep the two age groups separate; see below); each tank was separated into compartments by fine mesh dividers. Water was maintained at a constant temperature (26°C) and tanks were maintained under a 12 h:12 h L:D photoperiod. This photoperiod regime was adopted because this population of *H. kuda* has been shown to breed throughout

the year; this indicated that photoperiod is not an important determinant of breeding activity. Furthermore, the *Seahorse Manual* (Bull and Mitchell, 2002) recommends this photoperiod for six other seahorse species. Adults were fed four times per day with live and frozen food (*Artemia* and *Mysis*). Although embryonic seahorses possess a yolk sac while in the pouch, this normally disappears within 1 day of birth and juveniles are dependent on external nutritional sources. Juveniles were therefore fed four times per day with *Artemia* nauplii enriched with algae (*Nannachloropsis* and *Spirulina*). Normal feeding behaviour was observed in all groups of offspring from 1 day after birth.

To prevent overcrowding in the tanks, the number offspring populations within any single tank had to be reduced to a maximum of 50 individuals at about 4 weeks of age. This population reduction step only affected the old-couples cohort; the young-couples cohort never exceeded 50 individuals after 4 weeks.

Experimental design

Two groups of breeding pairs were established using a captive-bred line of *H. kuda*. One group consisted of adults that had already bred more than once and were over 8 months old [$N=6$ ‘old couples’ (OC)], the other group [$N=6$ ‘young couples’ (YC)] consisted of 3- to 4-month-old animals that had never previously bred. Mass measurements of the adults were made immediately after the birth of each cohort; postnatal body mass (mean \pm s.d.) of OC males and females was 8.43 ± 2.62 g and 6.00 ± 1.85 g, respectively, and of YC males and females, 3.76 ± 1.10 g and 2.46 ± 0.87 g, respectively. The Zoological Society of London Ethics Committee approved the experimental design, handling and measurement techniques.

Measurement techniques

The number of offspring produced by each male was recorded, and the numbers surviving were recorded daily for 7 weeks thereafter. Offspring taken out at the population reduction step were counted and included in the statistical analysis as ‘censored’ cases. For 7 weeks, body length and height of all surviving offspring were measured from standardized video recordings of free-swimming individuals.

Individual newborn and juvenile seahorses were captured in a wide-ended clear plastic pipette. The pipette was placed vertically near the inside front of the tank and image sequences of the captured seahorse, which could move freely within the pipette, were recorded using a video camcorder (Sony, Hi-8). Measurements of height (tip of coronet to tip of tail, following the contours of the dorsal surface) and body length (tip of coronet to base of the dorsal fin) were made manually from the still images (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA). These measurements differed slightly from standard length or height (Lourie, 2003) as they were adapted to provide a non-invasive method of measuring the length of very small seahorse fry. Although body mass is usually preferred for this type of study (Koops et al., 2004) it was impossible to handle these tiny offspring for direct length and

mass measurement without causing damage and stress. Mass of the adults (parents) was measured by placing them in a container of water on a balance.

The relationship between body mass and height was studied in a subsample of newborn seahorses that were sacrificed for management purposes and did not form part of the experiment. Body mass and height of newborn and juvenile seahorses were highly correlated ($N=89$; $r=0.96$; $P<0.0001$) but the relationship was non-linear. Logarithmic transformation of both variables produced a linear ($r=0.98$; $P<0.0001$) scatterplot (Fig. 1), thereby revealing that the variables were related by a power dependency of the form $\log M_b = a + k(\log H)$, where M_b =body mass, H =height; 'a' and 'k' are intercept and coefficient, respectively. This calibration showed that height is a good surrogate for mass; however, for the sake of clarity in presentation within this paper the height data has not been transformed.

Magnetic resonance imaging

Two pregnant male seahorses that had previously been killed and fixed whole in 10% neutral buffered formalin were examined by magnetic resonance imaging (MRI) at King's College Hospital Medical School, Denmark Hill, London.

Statistical analysis

Data were analysed using Statistica V6.1 (Statsoft UK, Letchworth, Herts, UK). Height and body length data from juveniles were used for calculating cohort means from each breeding couple; these values were log-transformed (Zar, 1984) and used to represent the OC or YC replicate results in ANOVAs. Data were treated in this way to avoid pseudoreplication; this would have artificially increased the sensitivity of the experiment if individual values for juvenile height and body length had been used as basic statistical units. Specific contrasts in the ANOVAs were examined using independent orthogonal contrasts. Survival of newborns from OC and YC was compared using the Cox-

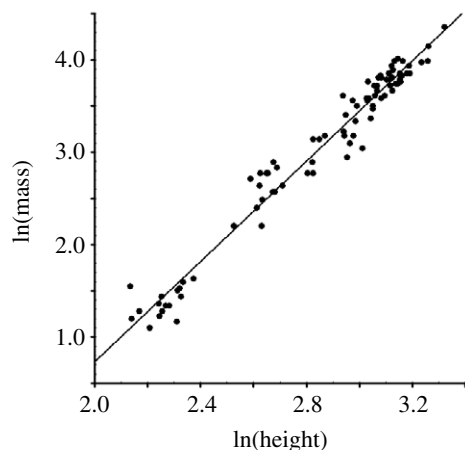


Fig. 1. Graph showing the relationship between height (mm) and body mass (g) for juvenile seahorses ($N=89$; $r=0.98$; $P<0.0001$).

Mantel test, taking account of artificially removed (censored) individuals. Homogeneity of variances was analysed using Levene's test.

Results

Effects of parental size and age on early postnatal growth and mortality

Most seahorses exhibited mating behaviour within a few days of being paired and males produced broods approximately 14–15 days later. Total numbers of 508 and 263 newborns were obtained from the OC ($N=6$) and YC ($N=6$), respectively. Average brood size of the OC was significantly larger than that of the YC (mean \pm s.e.m.; 84.6 ± 14.25 vs 43.8 ± 7.4 ; $F_{1,10}=6.45$, $P=0.029$; Fig. 2A). Nearly 90% of juveniles from OC survived throughout the 7-week experimental period, whereas significantly fewer than 50% of those from YC survived this initial growth period ($P<0.0001$; Fig. 2B). The statistical technique of 'failure analysis' that was used for the analysis of these data takes account of individuals that were removed deliberately. However, the small numbers of offspring obtained from the YC group eliminated the need to reduce the resultant offspring populations; mortality data for this group were therefore not affected by the population control step.

The higher mortality was not attributable to deficient feeding ability, as might be expected if the offspring were significantly smaller than their counterparts. No significant differences in height and body length measurements were detectable in the first week after birth. The overall heights calculated using mean data from each brood were (\pm s.e.m.): OC vs YC, 12.92 ± 0.47 mm and 12.38 ± 0.46 mm, respectively; $P=0.343$. However, juveniles produced by OC showed significantly

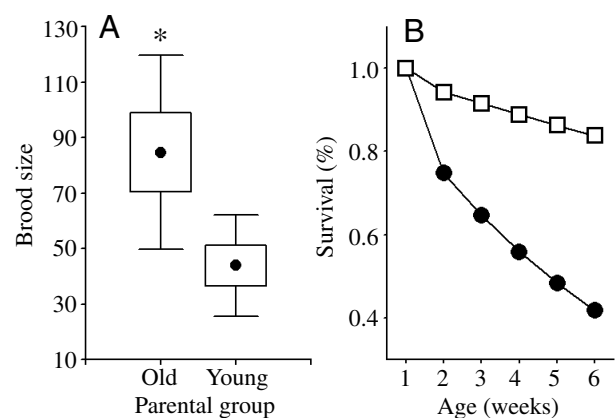


Fig. 2. (A) Brood size comparison between old (OC) and young (YC) couples; box-whisker plot showing the mean \pm s.e.m. (boxes) and s.d. (whiskers) ($F_{1,10}=6.45$, $*P=0.029$). (B) Comparison of offspring survival curves from old (OC; open squares) and young (YC; solid circles) couples. Least-squares survival functions (fitted lines) for offspring from the OC ($N=508$, offspring from six couples) and YC ($N=263$, offspring from six couples). Survival of offspring from YC is significantly poorer than from OC ($P<0.0001$).

higher average growth rate over the first 3 weeks of life ($P<0.001$, $F_{2,32}=133.6$; Fig. 3). Newborn juveniles from the YC group also exhibited significantly greater variance in height than the OC ($P<0.005$). Mean height of the OC group was approximately 10% higher than the YC group by the seventh week (Fig. 3; OC vs YC group mean (\pm s.e.m.) heights were 31.22 ± 1.29 and 27.92 ± 1.05 mm, respectively; $P=0.028$, $F_{1,39}=6.45$).

Effects of parental size on newborn height distributions

When considered together, the heights of newborn seahorses from all 12 males were negatively skewed (coefficient of skew= -0.203) and were therefore not normally distributed ($N=551$; Shapiro–Wilks test; $P=0.0013$), demonstrating that a larger than expected frequency of small newborns were being produced. This result was examined in greater detail by comparing frequency distributions of newborn heights from large and small males separately. Heights of newborns from the six large males showed a pronounced negative skew (coefficient of skew= -0.446 , $N=289$; Shapiro–Wilks test; $P=0.0018$; Fig. 4A) but the 262 newborns from small males did not show this skew (Fig. 4B). Although these were not normally distributed (Shapiro–Wilks test; $P=0.003$), they showed a flattened distribution instead of a left skew. Nonparametric comparison of median heights between the two groups, using data from individuals instead of broods, revealed that the large males tended to produce significantly larger newborn offspring (median for large males= 13.09 mm and median for small males= 12.43 mm; Mann–Whitney U test; $P<0.0001$).

The population derived from the OC showed no statistical skew after week 1 (Fig. 4C), but conversely the population derived from the YC showed significant left-skewing

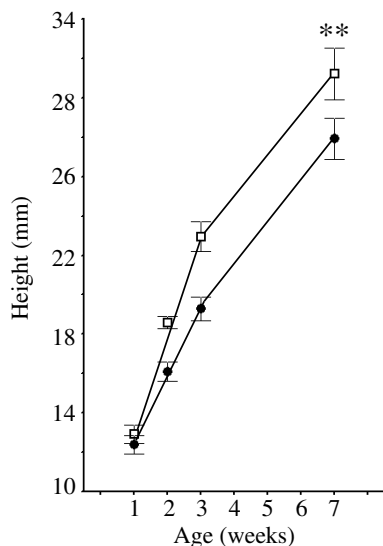


Fig. 3. Comparison of group mean (\pm s.e.m.) heights of offspring from the old couples (OC, open squares; $N=6$) and young couples (YC, solid circles; $N=6$) vs age (weeks). $**P<0.001$, $F_{1,39}=6.45$.

between weeks 2 and 4 (Fig. 4D). This emphasises the unexpectedly high frequency of slower growing individuals from the YC during the first few weeks after release from the pouch.

Effects of parental size on heterogeneity of early postnatal growth rates

When heights of individual newborn seahorses within any single cohort were plotted against age, the average growth curves could be modelled by a logarithmic relationship of the form ($y=q[\log(x)]+b$) where x was age in weeks, q was a coefficient of growth rate and b was the intercept. Two examples of such curves, which represent offspring from one large and one small male, are shown in Fig. 5A; scatter around the two fitted lines is also shown. In these two examples there

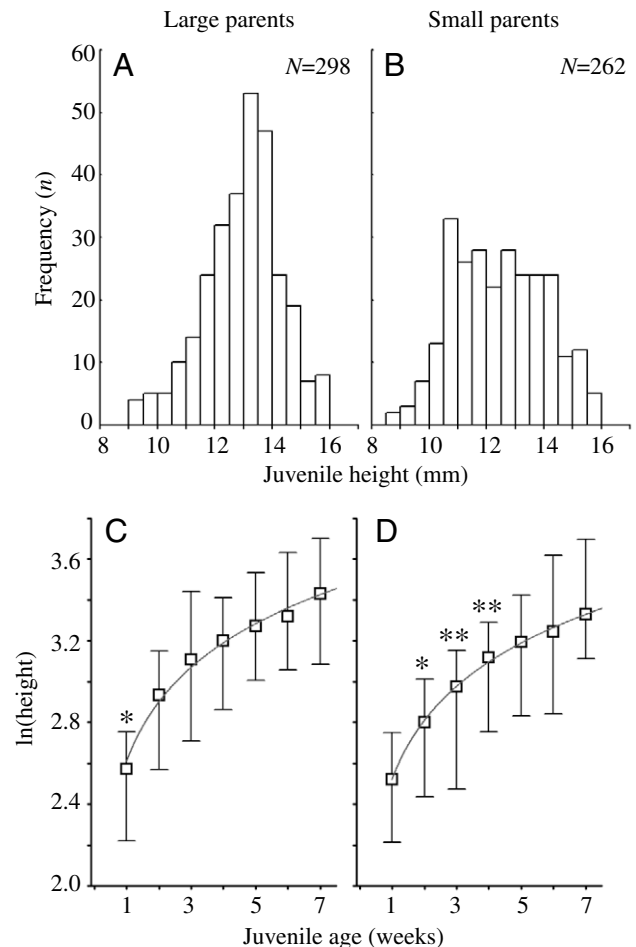


Fig. 4. (A,B) Distribution histograms for heights of newborn seahorses from large parents (A) and small parents (B). Heights of newborns from the six large males showed a pronounced negative skew (coefficient of skew= -0.446 ; $N=298$; Shapiro–Wilks test; $P=0.0018$) but the newborns from small males did not show this skew ($N=262$). (C,D) Medians and 25th percentiles for the height distributions (mm) of newborn seahorse cohorts from large parents (C) and small parents (D) between weeks 1 and 7. Distributions with significant negative skews are indicated ($*P<0.05$ and $**P<0.01$).

is considerable separation of individual heights during weeks 1–3; by week 4 the points overlap, an effect likely to be caused by selective mortality of the smaller individuals.

Growth coefficients and intercepts were calculated separately for broods from each of the couples. To examine the possibility that early growth rate is affected by the number of developing embryos, correlations between growth coefficients and number of newborns were calculated. As a whole group (OC and YC considered together) these values were not significantly correlated. However, when the OC and YC were considered separately, a strong and significant negative correlation (Fig. 5B; $r=-0.82$, $P<0.05$) was apparent for the larger or older couples whereas no correlation was apparent ($r=0.048$, $P>0.9$) for the smaller or younger couples (Fig. 5C).

Magnetic resonance imaging of pregnant males and examination of embryo attachment sites in pouch

Transverse scans through different levels of the pouch (Fig. 6A,B) revealed that embryos were distributed around the periphery and that the pouch lumen was devoid of embryos. This suggested that embryos were directly attached to the walls of the pouch, a finding confirmed by direct examination of embryos in the pouch after dissection. However, the nature of the embryonic attachment differed considerably between different regions of the pouch. Although not formally quantified, it was apparent that whereas dorsally located embryos were deeply embedded within individual compartments of the pouch wall (Fig. 6C), those located ventrally were merely attached to the wall in shallow depressions (Fig. 6D).

Discussion

Here we provide empirical support for the prediction that older (larger) seahorses produce offspring that initially grow faster than those born from younger (smaller) parents. The effect was attributable to differential growth rates over the first 3 weeks of life; between 3 and 7 weeks the comparative growth rates were not significantly different. In this experiment environmental influences on reproductive output were controlled and can therefore be excluded as influential factors.

Effects of parental age on growth rates and mortality

Egg size, concentration of eggs within the pouch, and embryonic mass have previously been shown to depend on male and female size in syngnathids, especially in pipefishes (Ahnesjö, 1992; Ahnesjö, 1996; Berglund et al., 1986a; Watanabe and Watanabe, 2002). Here we present evidence of the relationship between age-associated size of parents and newborn postnatal growth. Our results, in combination with existing knowledge of syngnathid reproduction, support the speculative argument that relationships between pre- and post-natal development in seahorses share some features with those of mammalian development (Barker, 1995).

The slower initial embryonic growth rates from younger (smaller) parents were accompanied by significantly higher

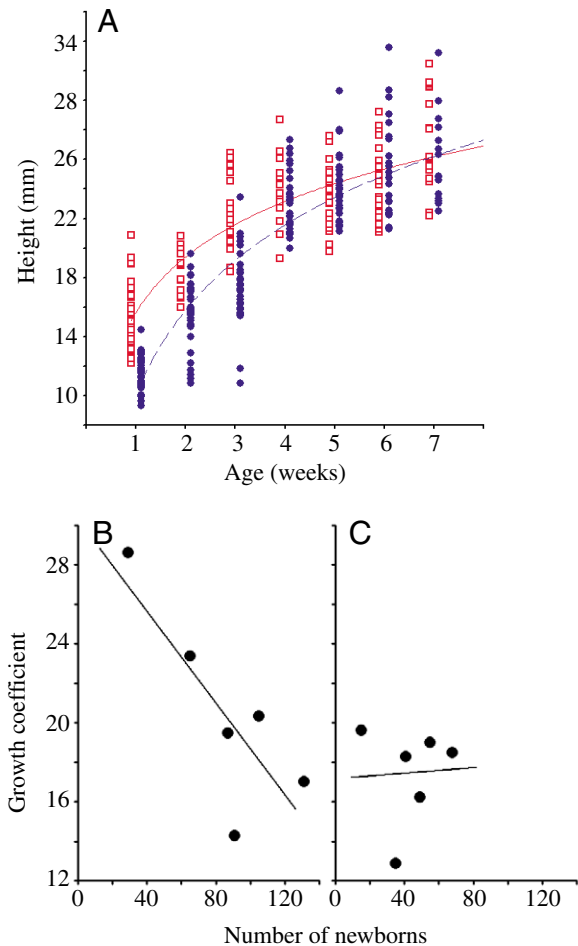


Fig. 5. (A) Exemplar growth curves between weeks 1 and 7 for offspring from one old couple (OC; red open squares) and one young couple (YC; blue circles) male and their associated scatter of individual data points. (B,C) Scatterplots and linear regression lines for the relationships between cohort growth coefficients (q) and number of newborns in the cohorts, for large parents (OC; B) and small parents (YC; C). Significant and negative correlation and regressions were observed for the OC group (correlation: $r=-0.82$, $P<0.05$; regression: growth coefficient= $30.456-0.1173\times$ number of newborns; $F_{1,4}=7.94$, $P=0.047$; $N=6$) but no corresponding correlation was observed for the YC group ($r=0.048$; $P>0.9$; $N=6$).

embryo mortality during the first 7 weeks of life. Statistical techniques for mortality analysis, or failure analysis, take account of individuals that were deliberately removed from the experimental groups ('censored') to prevent overcrowding. The high mortality rate is therefore not a statistical artefact, but neither is it caused by a feeding inability of embryos from the YC group. In our study there was no evidence that newborns from the two groups differed in average size at birth and therefore their respective abilities to catch and consume *Artemia* nauplii are likely to have been similar. The younger, or smaller, parents produced broods with higher than expected frequencies of small newborns and greater variance within the newborn cohort. These features are apparent from the flat-

topped or left-skewed body height histograms that were a feature of the YC offspring during the first 4 weeks of life. Inspection of data from within individual broods showed that the unusually small individuals had mostly disappeared by the fourth week after birth. Although we were unable to trace these newborns individually, the combination of mortality and size data strongly suggests that the high mortality was mainly due to their poor growth and survival. In this experiment the main factors determining offspring survival and growth rates were the age and size of both parents.

The observed negative correlation between number of newborns and growth coefficients for cohorts produced by the OC, and the absence of this correlation for YC, may be explained by differences in embryonic development, which might arise either from differences in egg size, or from topological heterogeneity within the pouch, and possibly both.

Effects of embryo-pouch interactions on growth

Although the reproductive biology of seahorses involves a complex combination of male and female influences on postnatal growth, we have tried to consider separately the extent to which the males and females contribute to the significant effects detected. In agreement with other studies of syngnathid embryo development (Carcupino et al., 2002) our observations showed that, during pregnancy in the male, embryos were distributed around the inner walls of the pouch and individually located within specialised attachment sites. Within an individual pouch it was also apparent that the size and complexity of individual attachment sites was variable, and ranged from simple depressions in the pouch surface to deep cavities into which the embryos were inserted. The hypothesis that these sites are important for the nutrition of embryos has been shown to be unsustainable (Azzarello, 1991), but it is plausible that they control other functions. Histological and ultrastructural characteristics of the brood pouch attachment sites in seahorses (Carcupino et al., 2002), and in pipefishes (Carcupino et al., 1997; Carcupino et al., 2002) that are closely related to the seahorses, were interpreted as indicative of osmoregulatory and gaseous exchange functions. The interfaces between pouch and embryo are therefore important for the control of respiration, as had previously been suggested (Nikolsky, 1963). Moreover, the increased metabolic rate of gravid males (Berglund et al., 1986a) has also been considered indicative of paternal energy investment in embryogenesis.

These observations suggest that, within the pouch of the older and more mature males, the location of embryonic attachment, namely whether within a shallow or deep site, may influence and possibly limit the quality of respiratory support obtained by individual embryos. Within the OC

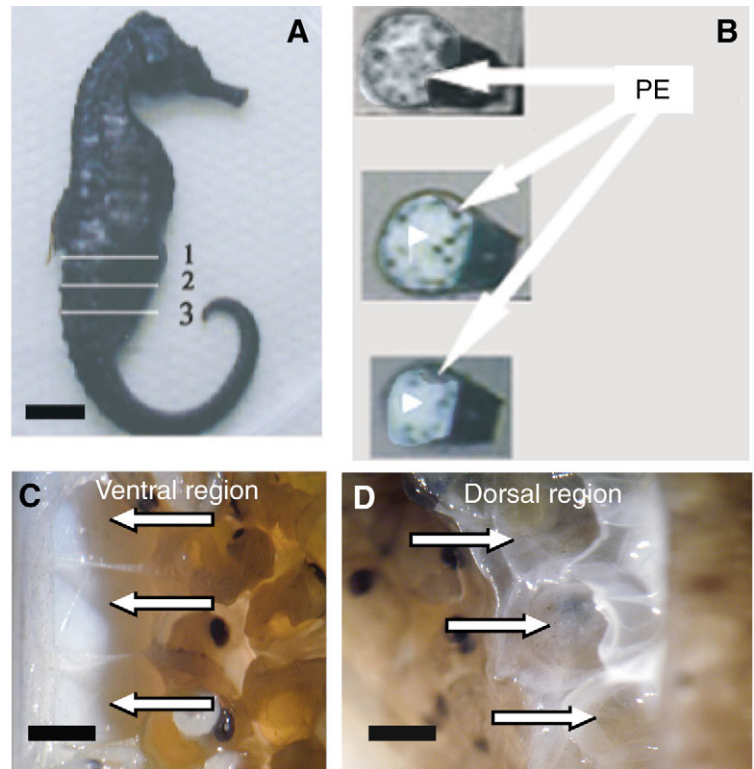


Fig. 6. (A) A photograph of a pregnant male seahorse with three transverse planes through the brood pouch indicated by lines 1–3. (B) MRI scans of transverse planes 1–3 (top to bottom). The positions of individual pouch embryos (PE) around the periphery of the brood pouch are indicated by arrows and the empty interior of the brood pouch is indicated by arrowheads. (C,D) Photographs of ventrally and dorsally located embryo attachment sites within the brood pouch (arrows). Ventrally located attachment sites are very shallow in comparison to those located dorsally. Scale bars, (A) 1.3 cm; (C,D) 3 mm.

group, the significant negative correlation between the number of embryos within a brood and the subsequent growth rate may therefore be interpreted in terms of competition for the highest quality attachment sites within the pouch. When small numbers of embryos are present, they are able to occupy the most functionally advantageous sites; conversely, however, when embryos are present in large numbers they are forced to occupy all available sites. This result is similar to previous observations in pipefish (Ahnesjö, 1992; Ahnesjö, 1996) that superior juveniles are produced when fewer newborns are present in the pouch. Other observations in pipefishes, that larger males with decreased density of embryos in the pouch are associated with better embryonic growth, also support the belief that paternal influence on embryonic growth is exerted through pouch function. The same principle of a parental effect on offspring growth has also been demonstrated recently in the oviparous eelpout, *Zoarces viviparus*, in which embryos grow within ovarian follicles (Vetemaa et al., 2006). In this species, females with higher relative fecundity had significantly smaller average

length, suggesting that 'growth is limited by competition for maternal energy supplies'. In Ahnesjö's investigations (Ahnesjö, 1992; Ahnesjö, 1996) only the dry mass of newborns was studied and the question about future growth was never investigated. In our study it is notable that no evidence of a significant negative correlation between paternal size and embryonic growth rate was detected when the smaller (YC) seahorse group was considered. We speculate that the pouches of the smaller males used in the present study, although obviously functional, were small and still insufficiently mature for the effects of attachment site heterogeneity to confer significant advantage upon any cohort of embryos.

The data presented here underline the importance of the prenatal conditions experienced by embryos in the pouch upon their future development and survival. This parallels the situation in mammals where the concept of foetal programming is widely accepted, although still controversial. The phenomenon has been attributed to poor body condition and nutrition, prior to, and during pregnancy, and epidemiological studies have reported effects that persist into adult life. The evidence presented here mimics the effects of foetal programming in two different ways. Individual seahorse embryos within a pouch may experience different degrees of respiratory and osmoregulatory support. Embryos embedded in deep pouch cavities are likely to be immersed in their own individual microenvironment, whereas others in the shallow depressions may receive less optimal support. The present data also suggest that the reproductive success of individual male seahorses is likely to be correlated with their own body size as this is reflected in their pouch size and physiological condition. As the young male seahorses produced a higher proportion of newborns with poor growth and life expectancy, this mimics the situation in which poor embryonic support results in poor fecundity. As our study considered only the first 7 weeks of life we are, as yet, unable to determine whether the effects persist into adult life, as seen in mammals.

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