

Post-copulatory sexual selection and sexual conflict in the evolution of male pregnancy

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Male pregnancy in seahorses, pipefishes and sea dragons (family Syngnathidae) represents a striking reproductive adaptation that has shaped the evolution of behaviour and morphology in this group of fishes^{1–4}. In many syngnathid species, males brood their offspring in a specialized pouch, which presumably evolved to facilitate male parental care^{5,6}. However, an unexplored possibility is that brood pouch evolution was partly shaped by parent–offspring or sexual conflict, processes that would result in trade-offs between current and future pregnancies. Here we report a controlled breeding experiment using the sexually dimorphic Gulf pipefish, *Syngnathus scovelli*, to test for post-copulatory sexual selection within broods and for trade-offs between successive male pregnancies as functions of female attractiveness. Offspring survivorship within a pregnancy was affected by the size of a male's mate, the number of eggs transferred and the male's sexual responsiveness. Significantly, we also found that embryo survivorship in a current pregnancy was negatively related to survivorship in the prior pregnancy, clearly demonstrating fitness trade-offs between broods. Overall, our data indicate that post-copulatory sexual selection and sexual conflict occur in Gulf pipefishes. The conflict seems to be mediated by a strategy of cryptic choice in which males increase rates of offspring abortion in pregnancies from unattractive mothers to retain resources for future reproductive opportunities. Hence, the male brood pouch of syngnathid fishes, which nurtures offspring^{7–9}, also seems to have an important role as an arbiter of conflict between the sexes.

Male pregnancy, a phenomenon unique to seahorses and their relatives, is facilitated by an elaborate brood pouch into which a female deposits eggs during mating^{10,11}. Far from being a passive bag for offspring (Fig. 1), the male's pouch provides aeration, protection, osmoregulation and nutrition to the developing offspring during a pregnancy that can last several weeks^{7–9,12–14}. The brood pouch is usually viewed as a structure whose main role is to nurture offspring. Recent work has established that nutrients move both from father to offspring^{7–9} and from offspring to father¹⁵ during the pregnancy, raising the hitherto unexplored possibility that the brood pouch may have a role in modulating post-copulatory sexual selection and sexual conflict^{16–18}. Such conflict could occur as a consequence of either males withholding (or taking) resources from some broods to save resources for future pregnancies^{19,20} or females transferring substances during mating that stimulate males to invest more resources in the current pregnancy^{21,22}. Whether sexual conflict is driven by male- or female-mediated effects, the demonstration of a role for the brood pouch in such processes would precipitate a major shift in thought regarding adaptive mechanisms responsible for the evolution of male pregnancy and parent–offspring interactions in this group of fishes.

We focus here on post-copulatory processes in the sexually dimorphic, sex-role-reversed Gulf pipefish, which has the highest

documented opportunity for sexual selection in females of any taxon²³. In nature, males normally mate with a single female per pregnancy^{23,24}, whereas the most successful females can mate with several males, resulting in a limited supply of receptive males. Previous studies of post-copulatory processes in a related pipefish (*Syngnathus typhle*) show that larger eggs experience higher survivorship, but fail to identify male-mediated effects or address between-pregnancy trade-offs^{25,26}. Gulf pipefishes provide an excellent system in which to investigate such trade-offs while eliminating potential complications from multiple mating within a pregnancy.

Our experimental design involved mating each focal male with a single female, allowing the male to carry his first brood to term, mating the male with a second female (Fig. 1) and then monitoring embryo mortality in the second brood. We photographed each brood immediately after egg transfer, near the pregnancy midpoint and just before parturition (Fig. 1a, b). Embryos failing to develop can be distinguished from viable embryos by their diminished size and colour at day seven of the pregnancy (which typically lasts 12–14 days), so we measured brood reduction by dividing the number of surviving embryos by the number of eggs initially received. Offspring survivorship is defined as one minus brood reduction. We controlled the size of the females with which males mated to ensure that males sometimes mated with females that differed from one another in size and sometimes mated with females similar to one another in size. Most of the analyses presented here are based on the second brood for each male

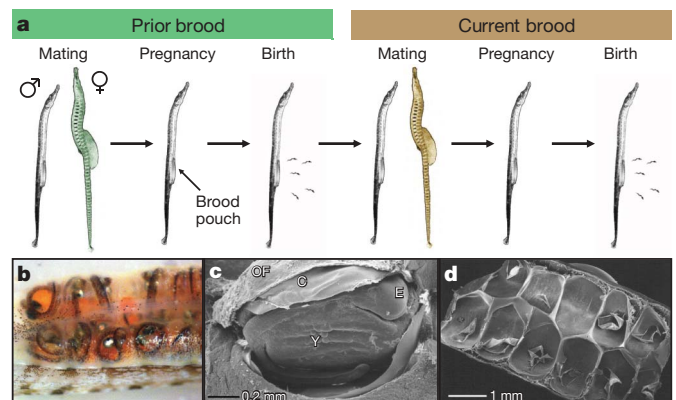


Figure 1 | Experimental design and brood pouch morphology. **a**, Each focal male mated with a single female for each of two successive pregnancies. **b**, We documented the development of offspring during each pregnancy by photographing the brood through the transparent pouch. **c**, Scanning electron micrograph illustrating the close connection between father and offspring; OF, outer pouch flap; C, chorion; E, embryonic tissue; Y, yolk. **d**, A honeycomb of paternal tissue is present in the brood pouch when the embryos are removed at about the half-way point of a pregnancy. (Images, N. Ratterman and C. Partridge.)

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($N = 22$), which we refer to as the ‘current brood’ (Fig. 1). The purpose of the first brood (which we call the ‘prior brood’) was to establish a known mating history for each male. First, we addressed whether or not males practice pre-copulatory mate choice; second, we quantified post-copulatory sexual selection by examining relationships between offspring survivorship and female size; third, we examined factors affecting offspring survivorship within broods, with a particular emphasis on trade-offs between broods; and, finally, we used an individual-based model to investigate the circumstances under which a brood reduction strategy would be adaptive in nature.

With respect to pre-copulatory choice, our results indicate that males prefer to mate with larger females. Each mating trial was a no-choice preference experiment, and we found that males’ reluctance to mate was significantly shorter for larger females (Fig. 2a), a result that was even more pronounced when we considered female size relative to male size (Fig. 2b). Thus, pre-copulatory mate choice favours larger females, and the pattern is very strong, as might be expected for a highly sexually dimorphic species such as Gulf pipefishes²³.

Our second goal was to investigate the nature of post-copulatory sexual selection in Gulf pipefishes. Our results indicated that pregnancies showed substantial variation with respect both to the number of eggs transferred per copulation (range, 4–42; mean, 22.5; variance, 89.8) and to survivorship of eggs in the brood pouch (range, 0–1; mean, 0.71; variance, 0.12), setting the stage for post-copulatory sexual selection. If post-copulatory sexual selection occurs in pipefishes, then one very important question is whether it reinforces or opposes pre-copulatory sexual selection^{27,28}. Other studies of post-copulatory sexual selection in a wide range of taxa have provided mixed results on this issue²⁷. Our results show that males prefer to mate with larger females (who also tend to have more pronounced secondary sexual characters) and that post-copulatory sexual selection acts in the same direction. We observed a strong positive correlation between the number of eggs transferred and female size (Fig. 2c), and saw the same pattern for offspring survivorship (Fig. 2d). In short, larger females transferred more eggs per mating and the resulting embryos experienced a greater probability of surviving to parturition. Thus, post-copulatory sexual selection reinforces pre-copulatory sexual selection in Gulf pipefishes.

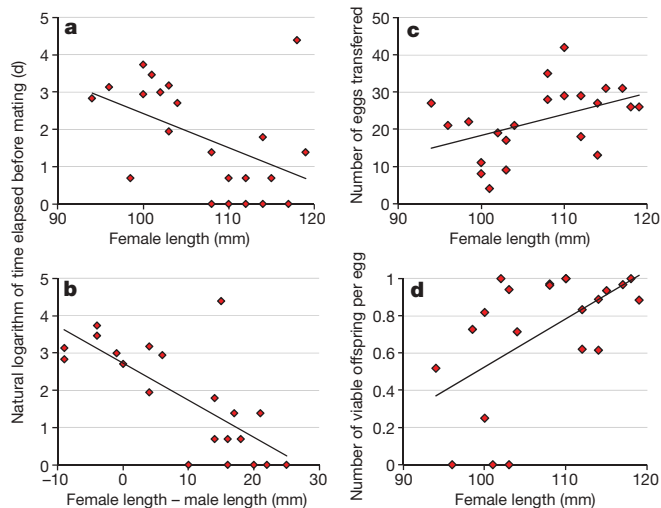


Figure 2 | Evidence for pre-copulatory mate choice and post-copulatory sexual selection by male Gulf pipefishes. **a**, We observed a strong negative correlation between a female’s total length and the time that elapsed before mating took place for male–female pairs (linear regression: $N = 22$, $r^2 = 0.23$, $P = 0.025$). **b**, The pattern was more pronounced when we took into account the female’s length relative to the male’s length ($N = 22$, $r^2 = 0.53$, $P < 0.001$). **c**, We observed a significantly positive relationship between the female’s total length and the number of eggs transferred to the male’s pouch ($N = 22$, $r^2 = 0.20$, $P = 0.038$). **d**, Males also experienced higher levels of offspring survivorship when they mated with larger as opposed to smaller females ($N = 22$, $r^2 = 0.56$, $P = 0.007$).

To ascertain the extent to which interbrood trade-offs are involved in Gulf pipefish reproduction, we examined brood reduction in light of a male’s mating history. If sexual conflict occurs, we expect to see trade-offs in subsequent broods. We addressed this hypothesis by using a stepwise regression and path analysis to study the effects of current and prior brood characteristics on offspring survivorship in the current brood (see Methods for statistical details). Our main result with respect to post-copulatory sexual selection and sexual conflict is that current offspring survivorship is dependent on characteristics of the prior brood (Fig. 3). In particular, current offspring survivorship is negatively correlated with prior female size and prior brood size, suggesting that valuable (and energetically expensive) previous broods hinder a male’s ability to invest in current broods (Fig. 3). Similarly, current offspring survivorship is negatively correlated with prior offspring survivorship (Fig. 3), suggesting that males are capable of reducing investment in some broods to save resources for future reproduction and that investment in a valuable brood decreases survivorship in subsequent broods.

In terms of the post-copulatory process, the simplest explanation for our results is that broods with larger mothers are energetically more costly for males. These costs could be manifested in two ways: either males are selected to invest more resources in embryos from large, attractive mothers as an adaptive strategy of cryptic male choice, or larger females (or their offspring) have evolved a mechanism that induces greater reproductive investment by males (see Supplementary Information for additional data bearing on the second possibility). Overall, the hypothesis of cryptic male choice is most consistent with our data, because all of our observations can be explained by discrimination against smaller females at every phase of pre- and post-copulatory sexual selection. Moreover, the mechanism would be relatively simple, as males could reduce the rate at which resources are transferred from them to the broods from less attractive females, increasing competition among siblings and reducing offspring survivorship.

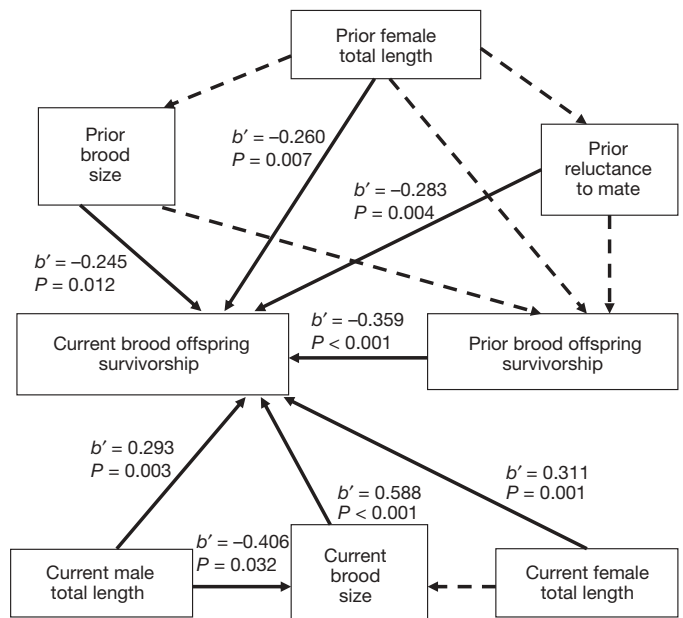


Figure 3 | Results of a path analysis showing the effects of variables from the prior and current broods on current offspring survivorship. Solid arrows represent significant partial regressions; dashed arrows show relationships that were included in the analysis but were not statistically significant. For each significant relationship, we label the corresponding solid arrow with the partial regression coefficient, b' , and the P value. The key result illustrated in this figure is that the survival of embryos in the current brood depends on features of the previous brood; in particular, current offspring survivorship was negatively correlated with prior offspring survivorship and prior female length.

This conclusion is further bolstered by recent observations in a related pipefish, *S. typhle*, which show that amino acids originating in the eggs pass from the embryos through the male's brood pouch to be incorporated into his liver and muscle tissue¹⁵. Thus, male pipefishes possess a mechanism to take resources from their broods, and the male-mediated strategy of brood reduction that our data illustrate is an example of sexual conflict, analogous to infanticide or filial cannibalism¹⁸. Males are partly, or in some cases completely, aborting the offspring from smaller females, at the expense of the reproductive interests (and Darwinian fitness) of those females. This strategy appears to be beneficial to the males, as their future broods, which should have larger mothers on average, have enhanced offspring survivorship. This clash of reproductive interests of the sexes is the crux of sexual conflict¹⁸, so our results raise the hypothesis that females should be selected to somehow resist this male strategy.

If males are capable of manipulating female broods, as our data suggest, then a key question is whether or not such a strategy is likely to be adaptive in natural populations. Intuitively, the best strategy for males would be to mate with large, attractive females every pregnancy. However, several lines of evidence suggest that this strategy may be impossible. For example, the average potential reproductive rates of females are only about twice those of males²⁹ and pipefish sex ratios fluctuate dramatically in nature, often showing a large excess of males³⁰. Thus, attractive females prepared to mate often will be in short supply. Moreover, results from our individual-based model show that under a wide range of circumstances, males are expected to benefit from the cryptic choice strategy observed in our experiments, especially when large females prepared to mate are in short supply (Supplementary Information). The fitness of the cryptic choice strategy increases when the preference threshold is high, as might be expected for a strongly sexually selected species such as the Gulf pipefish, and when the sex ratio is biased towards an excess of males, which is a common occurrence in nature³⁰. In addition, the model predicts that males are reluctant to mate with small females, as we observed. Males that are too eager to mate with small females suffer fitness costs by missing opportunities to mate with females above the preference threshold. Overall, the results of the model indicate that the cryptic choice strategy implied by our empirical results should be adaptive in natural populations of pipefishes.

The results of our study are significant at two levels. First, post-copulatory sexual selection has been virtually unexamined in sex-role-reversed taxa. If cryptic choice is a general mechanism of sexual selection, then we should expect it to evolve in sex-role-reversed species as well as in the more commonly studied species with conventional sex roles. Our results show that such a mechanism has evolved in Gulf pipefishes, despite the fact that this species has effective mate choice before mating, implying that cryptic choice is indeed a process of fundamental significance. Second, our results bear on the adaptive significance of the brood pouch. On casual inspection, the pouch seems to be a structure that evolved to nurture and provide for offspring, and it does serve this function. However, it also may grant the male better control over reproduction. Males seem to be able to adaptively affect their investment in broods as a function of the value of the pregnancy. Perhaps they simply invest fewer resources in broods originating from smaller females (resulting in competition among siblings within the pouch for resources), but males also have the ability to exploit the reproductive contributions of some females by actively absorbing embryos from less valuable broods¹⁵. Thus, the brood pouch serves a more complicated purpose in pipefish reproduction than previously believed, certainly providing parental care but also participating in a conflict between the sexes.

METHODS SUMMARY

We collected Gulf pipefishes from seagrass meadows near Port Aransas, Texas, maintained them in 9.5-l salt-water tanks and fed them *Artemia* nauplii twice daily. Each experimental male mated twice in the lab, producing two successive pregnancies with one mate per pregnancy. For each mating event, the male was randomly assigned to mate with either a large (length, 108–122 mm) or small

(length, 93–106 mm) female. We inspected male brood pouches daily. The brood pouches of pregnant males were photographed under a dissecting microscope on days one, seven and 11 after mating. On these days, embryos were counted and undeveloped eggs were noted. We calculated brood reduction by dividing the number of undeveloped eggs on day seven by the total number of eggs transferred by the female. Offspring survivorship was calculated as one minus brood reduction. Males were paired with their second mate within 24 hours after parturition of the first brood, and this brood also was photographed and scored on days one, seven and 11 after mating. At the end of each trial, we measured male and female body size (total length, snout–vent length, depth and weight). Our statistical analyses focused on the second brood for each male. We examined how both prior and current brood (and mate) characteristics affected mating latency, brood size and offspring survivorship.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 13 October 2009; accepted 22 January 2010.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was supported by grant IOS-0455927 from the US National Science Foundation. We thank S. Scobell and R. Carter for help with animal husbandry. We are also grateful to S. Arnold, A. Berglund, M. Giresi, N. Ratterman, E. Rose, G. Rosenthal, C. Small and D. Zeh for comments on the manuscript.

Author Contributions K.A.P. performed the experiments. A.G.J. and K.A.P. designed the experiments, analysed the data and wrote the manuscript.

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METHODS

Animal collection and maintenance. We collected Gulf pipefishes near Port Aransas, Texas, on three occasions between June and August 2007, transported them to the Texas A&M University campus in College Station and housed them in a flow-through system until they were used in the experiment. Collection date had no significant effect on brood reduction, brood size or time spent in the lab (Tukey–Kramer HSD test; $\alpha \leq 0.05$). Most males were pregnant at the time of collection, so we housed them in groups until they gave birth. Males usually were added into the mating experiment less than 24 hours after releasing their field-conceived brood. Females were housed in female-specific tanks until they were used in the mating experiment.

Mating experiment. We housed males in 9.5-l aquaria equipped with sponge filters and artificial seagrass, and fed them *Artemia* nauplii twice daily. Each male was maintained in the same tank for the duration of the trial, and tanks were thoroughly cleaned between trials. Males were randomly assigned to mate with either a large (length, 108–122 mm) or small (length, 93–106 mm) female. The reproductive status of females was assessed by the presence of the female secondary sexual ornament, which appears as silvery-blue lateral bars on the trunk.

We presented each male with a randomly assigned female of the appropriate size class immediately upon addition to the experiment. Male brood pouches were visually inspected daily. Of the 48 males that started the experiment, 34 accepted eggs from their first mate. The remaining 14 males were excluded on the basis of the following criteria: the death of either individual in the pair, mating outside the intended pair or a failure to mate before the experiment end date (4 November 2007). Two more males were excluded because they died before the brood emerged. Broods were monitored during development (see below). We maintained males and females together until the male was moved into a brooding chamber 11 days after mating. On the day the fry emerged, the female was killed by administration of an overdose of tricaine methanesulphonate (MS222), photographed and preserved in formalin solution. We made measurements of female total length, snout–vent length, weight, standard depth²⁹, maximum depth and dorsal fin area. Up to five eggs were removed from each female, photographed and measured for diameter.

The 30 males still in the experiment after the first pregnancy were each presented with a second mate within 48 hours of giving birth. Males were again randomly assigned to either a large or a small female, and the pairs were monitored as previously described. No females were re-used during the experiment, so the second mates were independent of the first mates. Twenty-three of the 30 males accepted eggs from the second mate and 22 males survived to maturity of the brood. The remaining seven males, which did not mate, were excluded from the experiment. On the day of parturition of the second brood, both the male and female in each pair were killed using MS222, photographed and preserved in formalin. Females were measured as previously described. We also made measurements of male total

length, snout–vent length, weight and standard depth at this time. At the end of the experiment, male total length ranged from 87 to 105 mm. Male and female total lengths were not correlated in this experiment ($N = 22$, $r^2 = 0.13$, $P = 0.107$).

Brood monitoring. On days one, seven and 11 after mating, we counted embryos and assessed the progress of development. The male was transferred to a small amount of water and examined under a dissecting microscope. Embryos are visible through the brood pouch epithelium and can be counted by eye. All broods were counted at least twice to verify accuracy. Unusual-looking eggs were noted, and eggs that showed no obvious development (in comparison with other embryos in the brood) counted towards brood reduction. Undeveloped eggs often shrank and became more transparent during the brooding period. We produced a time series of brood development by photographing the broods on these days using a digital camera (Zeiss AxioCam MRc 5) attached to a stereo microscope (Zeiss Stemi 2000C).

Analysis. We calculated brood reduction by dividing the number of undeveloped eggs on day seven by the total number of eggs transferred by the female. Offspring survivorship was calculated as one minus brood reduction. The photographic time series of the brood was consulted if a question arose as to the fate of a particular egg.

Because of the large number of variables measured in this study, we used stepwise regression as an exploratory tool to evaluate which variables should be included in a path analysis. The response variable was current brood reduction and all included variables had individual P values of less than 0.05 in the stepwise regression analysis. On the basis of these results, we kept variables describing adult total length, reluctance to mate, brood size and prior brood reduction. All variables were transformed to fit a normal distribution before statistical analysis. Univariate and stepwise regressions were performed in JMP 7.

In our path analysis, all variables retained from the stepwise regression analysis were connected to our focal variable, namely current offspring survivorship. Other connections were included to represent all likely biological interactions between the measured variables. We estimated partial regression coefficients, standardized to 1 s.d., by using the maximum-likelihood procedure implemented in the computer program AMOS 5.0.1. The path analysis model resulted in a χ^2 value of 13.05, leading to a P value of 0.522, which implies that the hypothesis of a perfect fit of the model cannot be rejected. This model explained 84% of the variance in current offspring survivorship. The model fit had a root mean squared error of approximation of <0.001 , which is less than the value, 0.05, expected for a model with excellent fit³¹. Overall, the measures of fit for the path analysis suggest that the estimated model is an accurate representation of our data.

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