Sexual selection in the dwarf seahorse, *Hippocampus zosterae* (Syngnathidae): An investigation into the mechanisms determining the degree of male vs. female intrasexual competition and intersexual choice

A dissertation

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ABSTRACT

I used dwarf seahorses (*Hippocampus zosterae*) as a model system to examine the relationship between the observed courtship-roles of males and females and both the relative parental investment and potential reproductive rates of males and females. It has been hypothesized that both the relative investment parents make in offspring and their potential reproductive rates influence the relative intensity of selection on males and females due to mate choice or mate competition. Seahorses make an excellent model system for this type of investigation because they are distinguished by an extreme degree of morphological specialization for paternal care in addition to the formation of monogamous pair bonds.

The relative intensity of mate competition and choice was quantified by exposing fish to either male-biased (2M:1F) or female-biased (1M:2F) sex-ratios. Male *H. zosterae* were found to display competitive behaviors that were not observed between females, as well as a significantly higher frequency of the competitive behaviors that were observed in both sexes. Both studies suggested that male and female mating success may be related to body size, with pairs possibly size-assorting, but the results of the mate choice experiments did not clearly indicate criteria which fish may be using to select mates.

Relative parental investment was estimated by measuring the biochemical composition of eggs and juveniles and quantifying the respiration rates of adult *H. zosterae* and their developing embryos. Male respiration rate throughout gestation was substantially higher than when not brooding embryos. In addition, the increase in respiration rate of males during gestation was significantly higher than total embryo respiration rate, indicating that males did invest energy in their offspring while brooding. When compared directly to female caloric investment per egg,
however, males were determined to invest almost 50% less per offspring than did females. The potential reproductive rates of males and females were determined by providing sexually-isolated males and females with sexually-receptive partners, measuring the time it took each sex to prepare to mate, and converting the time difference to the number of offspring that males and females could produce per unit time. Sexually-isolated males mated two days sooner than sexually-isolated females; thus males could potentially sire 17% more offspring than females over the course of a breeding season assuming continuous availability of mates.

Because female *H. zosterae* were found to invest more energy in each offspring and have a lower potential reproductive rate than did males, the predictions of both parental investment theory and potential reproductive rate are consistent with the observed courtship-roles, with males competing amongst themselves for access to females, a limiting resource. In addition, the results of this study have important implications for the evolution of the fish within the Syngnathidae (pipefish and seahorses) family.
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Sexual selection in the dwarf seahorse, *Hippocampus zosterae* (Syngnathidae): An investigation into the mechanisms determining the degree of male vs. female intrasexual competition and intersexual choice
CHAPTER I: Introduction

Darwin (1871) originally defined sexual selection as "the advantage which certain individuals have over others of the same sex and species solely in respect to reproduction". According to Darwin, reproductive advantage may arise in sexually reproducing species through two distinct processes: 1) intrasexual competition, in which some members of a sex are better able to compete for access to mates of the opposite sex than others, and 2) intersexual mate choice, the process by which one sex preferentially chooses mates of the opposite sex based on certain criteria. Both of these components of sexual selection can contribute to differential mating success among members of a population.

Sexual selection has also been defined in terms of the concept of a limiting sex. When one sex becomes limiting to the other for whatever reason, the abundant sex is expected to compete for access to the limiting sex and the limiting sex is predicted to be more discriminating in their choice of mates (Emlen & Oring, 1977). The strength of sexual selection is anticipated to increase as that sex becomes yet more limiting. The question then arises of what may cause the limitation of one sex over another, and thus influence the operation of sexual selection in a species or various species. Three mechanisms that may influence which sex limits reproduction have been suggested in the literature: 1) Relative parental investment (RPI), 2) Operational sex ratio (OSR), and 3) Potential reproductive rate (PRR).

The first mechanism described was relative parental investment, in which the sex that invests the greatest amount of energy in their offspring is predicted to be the most discriminating and the sex that invests the least is expected to compete for access to members of the limiting sex (Trivers, 1972). Trivers defined RPI in terms of individual offspring; so
that parental investment is "any investment by the parent in an individual offspring that increases the offspring's chance of surviving (and hence reproductive success) at the cost of the parent's ability to invest in other offspring." The concept of parental investment is an extension of the theory developed by Bateman (1948), who suggested that the relative investments of the sexes in their gametes is what determines which sex will be limiting. Trivers extended the idea to include both gametic investment as well as post-gametic investment. Post-gametic investment (or zygotic investment, see Baylis, 1981), also termed parental care, includes all of the energy invested in offspring after the gametes are formed, such as embryo incubation and/or aeration, nest defense, feeding and protection of juveniles and increased predation on adults caring for offspring (reviewed in Clutton-Brock, 1991). Across taxa displaying parental care, females care in the majority of species, although there are many examples of biparental care (over 90% of bird species, 25% of fish), and paternal care (58% of fish, about 20% of amphibians).

There have been few studies that estimate the relative parental investments of males and females in a comprehensive manner (but see Simmons, 1992). In this study, an unnamed genus and species of zaphrochilone katydid (Orthoptera: Tettigoniidae) was used to determine relative parental investment patterns and in a separate study experimentally manipulate courtship-roles (Gwynne & Simmons, 1990). Male parental investment is provided in this species in the form of a large spermatophore passed to the female during copulation, a portion of which is consumed by the female and used to provision eggs (Simmons, 1990). By exposing katydids to either abundant food conditions or limiting their food intake, Gwynne and Simmons (1990) observed that when well fed, females were more selective of their mates and competition was not observed between males or females, but when starved, males were
more choosy and females competed more intensely for access to males. The authors hypothesized that selection on males and females changed with varying food availability because when food was limiting, all males produced spermatophores at a slower rate and females mated more often than when food was abundant, thus biasing the ratio of sexually available mates towards females. These results led Simmons (1992) to use this species to quantify the relative parental investments of males and females under high and low food availability to determine if the relative levels of parental investment of the sexes matched the observed patterns of courtship behavior as predicted by parental investment theory. An energy budget was created for males and females through a bomb calorimetric analysis of food ingested, faeces, eggs, spermatophores and the bodies of males and females. In abundant food conditions, females invested more energy per zygote than males, but in food-limited conditions, males invested relatively more per zygote than did females when female food intake was below a threshold level. Thus, in this species the relative parental investment of males and females correctly predicted courtship behavior patterns.

The second mechanism proposed to determine the limiting sex was the operational sex ratio (OSR), first defined explicitly by Emlen and Oring (1977), but discussed by Darwin (1871) and Trivers (1972) in some detail. The OSR is defined as the ratio of mature, breeding males to fertilizable females (Emlen & Oring, 1977), and it is this ratio in a population that is hypothesized to directly influence which sex is limiting at any given time. There are many biological and ecological factors that will determine the OSR for each species, including the disparity in parental investment between the sexes and the distribution of mates and resources in time and space. For example, in some populations of *Bufo calamita*, the natterjack toad, Tejedo (1988) has found that females are only sexually receptive for a very brief period of
time and arrive at the breeding site asynchronously, causing the OSR to be highly biased towards males. In these populations, competition was intense among males for access to females with which to mate even though the adult sex ratio appeared balanced. Similarly, in an undescribed species of zaprochiline katydid (the same species as above), Simmons and Bailey (1990) found that the seasonal distribution of resources dramatically altered the OSR. In food-rich seasons, females mated once, thus receiving only one spermatophore from a male. In these conditions, females were limiting, the OSR was biased towards males, and males competed for access to females. In food-poor seasons, however, females mated more often to receive nutritious spermatophores from males, but males produced them at a slower rate. Under these conditions, males were limiting, the OSR was female biased, and females were observed to compete for access to males and males were more discriminating.

A third mechanism, differences in the potential reproductive rates (PRR) of males and females, has been proposed more recently (Clutton-Brock & Vincent, 1991; Clutton-Brock & Parker, 1992) as an alternative mechanism to determine which sex will be limiting. PRR is defined as the “maximum number of independent offspring that parents can produce per unit time” (Clutton-Brock & Vincent, 1991). By this definition, the sex with the lower potential reproductive rate would limit the sex with the higher reproductive rate, resulting in a prediction of greater competition among members of the limited sex. Kvarnemo (1994) was one of the first researchers to describe a method by which to empirically determine the PRR. Although in any species the realized reproductive rate of males and females must be equal, Kvarnemo determined that the potential reproductive rates of each sex in a population could be quantified by providing an unlimited supply of mating partners while holding other environmental variables constant, such as temperature, food availability and nutritional
content, and nesting sites. In sand gobies (*Pomatoschistus minutus*), males care for developing embryos in a nest by aerating them and defending them from egg predators. Kvarnemo estimated the potential reproductive rates of sand gobies and illustrated the importance of maintaining a constant temperature when measuring reproductive rate by quantifying the number of offspring produced per day by males and females. She determined that at temperatures below 11°C (early season) males and females had equal potential reproductive rates, but above 11°C (mid-late season) male reproductive rate exceeded female reproductive rate.

Although these mechanisms hypothesized to determine courtship-roles appear independent of one another, the relative parental investment of males and females ultimately dictates their potential reproductive rates and directly influences the OSR (Figure 1). Because in many systems resources needed for reproduction are limiting, the sex investing the least per offspring will have more resources to channel into obtaining mates and increasing its reproductive success (Andersson, 1994). Potential reproductive rates will in turn influence the OSR, causing it to become biased towards the “faster” sex (Clutton-Brock, 1991). RPI and PRR can affect the OSR in other ways as well, through mitigating factors such as life
history characteristics, the distribution of resources, other aspects of the ecology of a species, and environmental variables such as temperature (Grant et al., 1995, Wootton et. al., 1995; Kvarnemo, 1996). Simmons (1992) suggested that relative parental investment in offspring determines the potential reproductive rates of males and females and thus directly affects the operational sex ratio. For example, in his paper on the relative parental investments of male and female katydids, he determined that in food-poor environments, females rely more on spermatophores than food for reproductive resources and thus mate more often than when food is abundant. Males, however, cannot prepare spermatophores as quickly when food is limited, causing males to mate less often than when food is plentiful. In this example, a male’s parental investment causes a decrease in his reproductive rate relative to females, thus biasing the operational sex ratio, and therefore causing females to compete for males and males to be discriminating about the females with which they mate.

Potential reproductive rate was developed as a concept primarily to explain patterns of courtship in species in which both parents may have a substantial investment in offspring, and more specifically, those species that demonstrate uniparental male care. Because relative parental investment can be difficult to measure, Clutton-Brock and Vincent (1991) suggested that measuring the potential reproductive rates of males and females is an better way to determine the limiting sex because it is more amenable to quantification. Moreover, Clutton-Brock and Parker (1992) claimed that Trivers’ (1972) definition of parental investment and its predictions for mating competition are too restrictive because he did not include other factors which may affect the reproductive rates of males and females, and thus the OSR, such as the distribution of the limiting sex in time and space. As examples, they cited a number of species in which males are the predominant caretakers of offspring, yet still compete more
intensely for mates than do females, thus indicating a poor fit between parental care patterns and courtship-roles.

The main problem, however, with assuming that the parent who cares for offspring is the parent with the highest investment per offspring, is that there are other substantial sources of investment (eg. the provisioning of eggs by females, nest preparation, etc.) that are not considered. The proponents of PRR (Clutton-Brock & Vincent, 1991; Clutton-Brock & Parker, 1992; Andersson, 1994) have suggested it as a better predictor of the limiting sex than relative parental investment, but this is because they have equated parental care with the parental investment made by a given sex. In Trivers’ (1972) original definition of parental investment he was careful to state that the investment should be calculated on a per offspring basis, and should include the original investments made by parents in their gametes. The threespined stickleback, Gasterosteus aculeatus, is cited as an example where males care for young, but are still the primary competitors for mates (Craig & Fitzgerald, 1982). Clutton-Brock (1991) and Andersson (1994) claim that patterns of sexual behavior in sticklebacks are contrary to that predicted by relative parental investment, since nest defense by males is assumed to represent the greater investment in offspring. However, these researchers failed to determine paternal investment per offspring; males can defend up to 10 clutches of eggs during a 2-3 week incubation period (Kynard, 1978), compared with females who can produce only 3-6 clutches during the same period of time. Female gametic investment in offspring is substantial (7-9 Joules; Woottton, 1994), because of the size and yolk content of stickleback eggs (Wallace & Selman, 1979). Recent evidence suggests that in G. aculeatus, maternal investment per offspring exceeds that of males’ and the observation that males are more
competitive in this species would match the predictions based on relative parental investment (Wootton et al., 1995).

Potential reproductive rate has also been suggested to be an easier characteristic of a species to quantify than the relative parental investments made in offspring by males and females (Clutton-Brock & Vincent, 1991; Clutton-Brock & Parker, 1992). In many cases, this may be true. For example, in threospined sticklebacks, males and females breed continuously throughout the reproductive season and are polygynandrous (Dufresne et al., 1990). In this species, the mating system does not limit the rate at which each sex reproduces and the potential reproductive rate can thus be measured. Parental investment of females, limited to gametic investment, is fairly easy to measure, but it is difficult to quantify parental investment for males. Attempts to measure male parental investment in sticklebacks have focused on quantifying male aggression level to potential predators of eggs and fry (Fitzgerald & Caza, 1993), but the energetic cost to males of egg ventilation and the potential cost of increased mortality to males due to predation are more difficult to estimate. A technique has been used in male sand gobies (Pomatoschistus minutus) to measure the costs of egg ventilation using weight loss over incubation, but depriving males of food over this period can dramatically affect behavior and thus estimates of energy expenditure (Lindström & Hellström, 1993).

For some species, such as sticklebacks, comprehensively measuring parental investment is difficult (Andersson, 1994); however there exist species that are amenable to its quantification. Unfortunately, few researchers have attempted to measure RPI in natural systems. Simply assuming RPI based on the behavior of males and females has proven problematic for species that have biparental or paternal care, where the relative energy investments of the parents are not obvious. In the majority of cases where parental investment
has been quantified, it has been a measure of either 1) the investment of only one parent (Fitzgerald & Caza, 1993; Lindström & Hellström, 1993) or 2) only a portion of parental investment has been measured, and the estimate is not likely to reflect the total parental investment levels (Sherley, 1993; Wisenden et al., 1995). In addition, even when all aspects of parental investment can be measured, many researchers have been faced with the problem of comparable currency, in which the investments of males (such as increased mortality causing lower lifetime fitness) and females (energy contained in eggs in Joules) are in different forms and are thus not additive (Knapton, 1984).

Given the problems associated with quantifying the investments males and females make in offspring, it would be useful to empirically define the relationship between RPI and PRR to determine if they vary in a consistent and predictable manner. To answer this question, both RPI and PRR must be estimated in a number of species to clarify their relationship to one another. The current dearth of empirical evidence to support or refute the hypotheses that either relative parental investment or potential reproductive rate determine courtship-roles, let alone experimental data relating them in the same species, emphasizes the importance that studies be conducted in this area. In the present study, I have focused on one species that exhibits male care to quantify the relative parental investment of males and females and their potential reproductive rates, in conjunction with behavioral studies to describe courtship roles, to determine how well each hypothesized mechanism predicts which sex will be limiting.
Background

Species that display male parental care are known in the literature as being *parentally role-reversed* (Gwynne, 1991). Parentally role-reversed organisms have been suggested as ideal model systems for testing sexual selection theory (Williams, 1966; Trivers 1985). It has been assumed that parental role-reversal signifies greater male parental investment, and these species have been predicted to display *courtship-role reversal* (Vincent et al., 1992), in which females compete for access to males and males exhibit choice. In fact, courtship-role reversal should only be observed if: 1) male parental investment exceeds female investment, 2) the operational sex ratio is female biased and/or 3) the potential reproductive rate of females exceeds that of males.

Most of the examples of sole paternal care come from fish, where paternal care occurs in over 60% of the species that display any care (Ridley, 1978; Blumer, 1979; Blumer, 1982). A great deal of speculation has surrounded the existence of a disproportionate number of caring fathers in fish, when compared to both other vertebrate and invertebrate taxa (Sargent & Gross, 1986). Blumer (1979) suggested that the main factors in the evolution and persistence of paternal care in fish are the male’s probability of genetic relatedness to a females offspring and the increased number of offspring a male could produce if he remains site-attached and provides parental care while continuing to pursue additional matings. Because fecundity in many fish increases at a greater rate in females than in males in relation to body size, the costs due to decreased foraging because of nest maintenance and defense are lower for males than for females (Gross & Sargent, 1985). In these species, parental males may increase their reproductive success over time because females are free to forage.
Embryo-bearing in male fish may be selected for in males that display nesting behavior when the environment is highly variable and when predation pressure on embryos is high (Baylis, 1981). Although male care of offspring is the dominant parental care pattern in fish, little evidence exists to determine whether these males actually invest more per offspring than do females.

**Syngnathid biology and ecology:** The dwarf seahorse (*Hippocampus zosterae*, family Syngnathidae), is the model system used in this thesis to test hypotheses in sexual selection theory. Seahorses are teleost fish found in the order Syngnathiformes, which contains seven distinct families (Orr & Pietsch, 1995). Related fish include sticklebacks and tubersnouts (order Gasterosteiformes, families Gasterosteidae and Aulorhynchidae), and the more closely related ghost pipefishes and seamoths (order Syngnathiformes, families Solenostomidae and Pegasidae).

Fishes in the family Syngnathidae exhibit a range of anatomical and physiological adaptations which represent varying degrees of specialization for paternal care (Herald, 1959; Vincent et al., 1992). Male care of offspring ranges from the pipefish subfamily Nerophinae in which eggs are loosely attached to the ventral surface of the male, to seahorses (subfamily Hippocampinae), in which eggs are deposited into a highly vascularized brood pouch on the ventral surface of the male that is sealed from the external environment until the end of a 10-30 d incubation period (Herald, 1959; Breder & Rosen, 1966; Vincent et al., 1992).

**Pipefish:** Pipefish, specifically *Syngnathus typhle* and *Nerophis ophidion*, have been the focus of a great deal of behavioral and physiological work pertaining to sexual selection theory. In *N. ophidion*, eggs are glued to the ventral surface of the male, with no external covering (Breder & Rosen, 1966; Berglund et al., 1986a). *N. ophidion* is highly sexually
dimorphic, with females larger and more conspicuous than males, possessing a blue sexual coloration and dorsoventral skin folds (Fiedler, 1954; Berglund et al., 1986a). This reversal in sexual dimorphism is also reflected in the reversal of courtship-roles displayed by males and females. Male *N. ophidion* prefer females with a larger blue nuptial patch and larger skin folds, associated with larger body size and a higher fecundity (Berglund et al., 1986a; Rosenqvist, 1990). Female *N. ophidion* were not found to choose mates on the basis of size or any other criteria (Berglund & Rosenqvist, 1993). Indirect competition between females has also been demonstrated in *N. ophidion*, with larger females suppressing the development of the blue patch and skin folds of smaller females, correlated with a decrease in egg production (Berglund, 1991). In *Syngnathus typhle*, males brood embryos enclosed in two flaps that completely seal them from the external environment (Berglund et al., 1986a). There is little sexual dimorphism in this species, with females being slightly larger than males (Berglund et al., 1986a), although this size difference increases as the age of cohorts increases (Berglund & Rosenqvist, 1990). In *S. typhle*, both sexes preferred larger mates (Berglund et al., 1986a), although females were found to be less selective in their choice of mates than males (Berglund & Rosenqvist, 1993).

Because true courtship-role reversal is so unusual, its appearance in one of these two species led to research investigating the mechanisms patterning their courtship behaviors. Early work in this area focused on quantifying the relative parental investment of males and females, specifically to determine the caloric investment made by each parent in offspring. Haresign & Shumway (1981) demonstrated that a non-metabolizable amino acid, [*14C*-alpha amino isobutyric acid, injected intra-peritoneally into male *Syngnathus fuscus* (pipefish) was transferred to developing embryos over the course of gestation. [*14C*-alpha amino isobutyric
acid has a low molecular weight, but because of its negative charge at neutral pH (Windholtz, 1983) most likely depends on facilitated or active mechanisms of transport from the male's system to embryos (Lehninger et al., 1993). Thus, using this compound as a marker indicates that substances are transported by active mechanisms from the paternal system to developing embryos, and suggests that males may be provisioning developing embryos with carbon in some form.

Berglund et al. (1986b) determined relative energy investments by male and female S. typhle and N. ophidion more directly, by measuring the caloric content of eggs and newly born fry and the respiration rates of embryos across gestation. Because of the observed courtship role-reversals in these species, they hypothesized that males must invest more per offspring than females. By measuring the respiration rates of embryos across different stages of gestation, they calculated the energy required for an embryo to complete development. By calculating the energy change from egg to juvenile and comparing this to the estimated energy requirement, they hoped to indirectly determine the amount of energy that males provide to their offspring. Their results indicated that energy required by developing embryos (calculated from their metabolic rates) exceeds that which is lost over the course of gestation, suggesting that males of both species may provision their developing young. Through this technique of measuring paternal investment, Berglund et al. (1986b) found that male N. ophidion, however, provided a much lower level of investment to their offspring than conspecific females, contrary to their predictions. Also male S. typhle provided more energy per zygote than N. ophidion males, and provided equal investment to their offspring as S. typhle females. The authors suggested that male parental investment in offspring may limit
female reproduction and thus cause the high level of sexual dimorphism and reversal of
courtship roles in *N. ophidion*.

Unfortunately, there were two major problems with this study: 1) incomplete
estimation of paternal investment, and 2) possible elevation of embryo respiration rates due to
methodological complications. Berglund et al. (1986b) made the assumption that paternal
investment consisted solely of the caloric provisioning made to offspring, and as a result
underestimated male parental investment. In actuality, there are other metabolic costs
associated with brooding embryos, including waste removal, osmoregulation and gas
exchange which could be estimated by measuring male respiration rate before and during
gestation. By subtracting the metabolic rate of the embryos from the increase in metabolic
rate in the gestating father, the result would provide a reasonable estimate of the total
energetic investment made by the male.

The second problem with this study was methodological in nature. To measure the
respiration rates of developing embryos, Berglund et al. (1986b) cited Linton and Soloff
(1964) as support for the fact that embryos could survive outside of the male’s pouch
environment. This is only partly true. Linton & Soloff showed that embryo mortality was
high (25% after 6 hours) in normal seawater (salinity = 32%o), but that mortality of embryos
could be reduced to 10% after 6 hours if embryos were placed in 40% seawater (salinity =
13%o). Berglund et al. (1986b) used normal seawater (salinity = 32 to 33.8%o) which was
probably sufficient for *N. ophidion* because the eggs are exposed, but in *S. typhle* may have
caused them to be severely stressed because eggs are usually protected from changes in
salinity by sealed brood flaps. Berglund et al. (1986b) also claimed that their use of embryos
removed from the pouch and placed into ambient seawater was justified because of the
similarity of the curves and small standard deviations of the respiration rates obtained across gestation for the two species. However, curves and standard deviations would also be similar if the respiration rates measured were from embryos respiring maximally due to severe environmental stresses. Measurement of the respiration rates of the fathers of these embryos before and during gestation would have directly measured total paternal investment, and also served to check if the respiration rates of the embryos were realistic (e.g. in *S. typhle* the respiration rates of embryos should not exceed the difference in respiration rate of their father before and during gestation).

Although Berglund et al. (1986b) considered only direct caloric investment in embryos, later studies identified other aspects that may play a role in male parental investment limiting female reproductive success and thus causing an increase in sexual selection on females (Svensson, 1988). In *S. typhle*, brooding males had a lower food intake than reproducing females. Brooding males also grew more slowly than females, which indicates a tradeoff of investment in present reproduction at the expense of future reproduction, as larger males of this species have been shown to brood more offspring (Berglund et al., 1988). Males also had a lower overwintering survival than females, possibly caused by a decrease in fat deposition because of their energy investment in brooding. In *N. ophidion*, evidence for reproductive costs such as decreased growth and lower food intake was not found, but brooding males had a higher predation risk than females (Svensson, 1988). Thus, reproductive costs other than provisioning of offspring are associated with male parental investment in these two pipefish species.

Recent work has also examined the effect of the operational sex ratio on the action of sexual selection in these two pipefish species. Although adult sex ratios of both species have
been observed to be equal (Berglund, 1986a), operational sex ratios in the field, the number of females with ripe eggs to the number of males ready to receive eggs, were significantly skewed towards females (Berglund & Rosenqvist, 1993). Further, when the field data from *S. typhle* was examined more closely, it revealed that the OSR early in the season when fish arrived at the breeding grounds was equal or slightly male-biased, and males were less selective about their choice of mates under these conditions. As the season went on, however, the OSR became extremely female-biased and males preferred larger females (Vincent et al., 1994). This may be because larger females provide larger eggs to males (Berglund et al., 1986a,b), and larger eggs appear to result in heavier offspring (Ahnesjö, 1992). Laboratory data support the claim that male choosiness based on female mass is influenced by the perceived OSR, under male-biased conditions males mated randomly and more quickly than under female-biased conditions, where males mated with the largest female provided (Berglund, 1993).

The observed patterns of male choosiness under a female-biased OSR may be explained by the observation that in both species, females can produce eggs at a faster rate than males can brood them; *N. ophidion* females could fill 1.8 males during the average course of one incubation period, and *S. typhle* females 1.9 males during the same period (Berglund et al., 1989). This difference in the reproductive rates of male and female *S. typhle* increases as the fish age because males grow more slowly than females (Svensson, 1988), but female fecundity increases over 2.5 times from small females (1-year old) to large females (2-years old) (Berglund & Rosenqvist, 1990). This difference in the reproductive rates of *S. typhle* males and females accurately reflects their potential reproductive rates, since work investigating the relationship between temperature and potential reproductive rate in this
species rate also suggests that males have a lower rate than do females (Ahnesjö, 1995). In addition, temperature was found to be an important environmental factor, since at colder temperatures, gestation length was longer than at warmer temperatures, with almost no differences between temperatures in female egg preparation time. Thus, in this species temperature differences were found to influence the operational sex ratio because of dramatic differences in the potential reproductive rates of males and females (Ahnesjö, 1995).

Although there has been recent work on S. typhle pipefish to determine how the OSR and PRR of males and females influence the limiting sex, more studies quantifying the RPI of males and females remain to be completed. To date, it has been established that pipefish males may provide caloric input to embryos (Haresign & Shumway, 1981; Berglund et al., 1986b), but this link needs additional study to identify the materials passed to embryos before conclusions can be drawn. Male parental investment may exceed female investment in both species, and the difference may be causing a difference in the reproductive rates of males and females, thus skewing the operational sex ratio towards females and causing the courtship-role reversal that has been observed. A more comprehensive quantification of the relative parental investment in these species must be completed, however, before a clear relationship between potential reproductive rate and relative parental investment can be determined.

**Seahorses:** If species with substantial male care make the best models for testing sexual selection theory, then seahorses should present the ideal case, given their high degree of morphological specialization for paternal care. Though there has been a good deal of speculation regarding courtship-role reversal in seahorses (Williams, 1966; Trivers, 1985), much of the speculation has unfortunately been based on anecdotal evidence (Gill, 1905; Fiedler, 1954; Breder & Rosen, 1966). Of the 35-40 extant species of seahorses (Vincent,
1990), only a few have had aspects of their courtship behavior or reproductive physiology investigated.

*Hippocampus* species studied to date have all shown highly complex and ritualized courtship behaviors (*H. guttulatus, H. brevirostris*, Fiedler, 1954; *H. fuscus*, Vincent, 1990; *H. whitei*, Vincent & Sadler, 1995; *H. zosterae*, Masonjones & Lewis, 1996). These behaviors are organized into two distinct types of courtship: Initial courtship/daily greetings, and courtship culminating in copulation. Seahorses have been shown to be monogamous and tightly pair-bonded (Vincent & Sadler, 1995), and the daily greeting courtship may be important in establishing and maintaining pair bonds (Vincent, 1995). Very little research has been done to determine the limiting sex in hippocampids. Competition has been found to be more intense between males than between females in *H. fuscus* (Vincent, 1994a), contrary to predictions made based on the assumed parental investment patterns. Because of the relationship between body size and fecundity in both males and females established in *H. fuscus* (Vincent, 1990), larger mates should be preferred, but no research on mate choice in hippocampids has been conducted.

Study of the reproductive morphology of female seahorses has indicated that they have an ovarian structure which is similar for several syngnathid species (Boisseau, 1967; Anderson, 1967; Wallace & Selman, 1981; Begovac & Wallace, 1987; Selman et al., 1991), but highly unique when compared to other organisms. In most teleosts, follicles arise either singly or in groups throughout the ovary, in a less ordered fashion than has been observed in syngnathids (Wallace & Selman, 1981; Begovac & Wallace, 1987). In addition, syngnathids spawn only a fraction of their post-vitellogenic oocytes at a time, as compared to other teleosts which begin again with primary oocytes after each spawning (Wallace & Selman,
1981). *H. erectus* females have paired ovaries which are connected by a single oviduct (Selman et al., 1991). Each ovary has a set of two dorsally-located germinal ridges containing oogonia and early oocytes, from which follicles are produced of sequential developmental ages spiraling from each germinal ridge outward (Figure 2). The final stages of oocyte hydration occur visibly on the day of copulation (Boisseau, 1967; Vincent, 1990); ovulation occurs and eggs are transferred from the female to the male’s pouch through her genital papilla in a single copulation (Fiedler, 1954; Boisseau, 1967). Eggs are fertilized as they are placed into the pouch or immediately afterward (Fiedler, 1954). Evidence from both Fiedler’s work (1954) and Boisseau’s work (1967) suggests that fewer sperm are produced in seahorses (*H. guttulatus, H. brevirostris*) than in other teleosts, and that sperm live for only a short time in the male’s pouch.

![Diagram of an ovary from *Hippocampus erectus*, from Selman et al. (1991).](image)

*Figure 2: Diagram of an ovary from *Hippocampus erectus*, from Selman et al. (1991). Two germinal ridges (GR) per ovary contain oogonia, early oocytes and pre-follicle cells from which follicles develop. Follicles are organized in order of developmental age, with the most mature follicles on the ventral side of the ovary farthest away from the germinal ridges. The foreground picture depicts an unrolled ovary to indicate more clearly its organization.*

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Despite the debate surrounding parental investment patterns in seahorses, no quantification of relative parental investments of the sexes has been done. Early research on *H. erectus* characterizing the environment of the male’s pouch indicated that the pouch is completely sealed from the external environment, and that sodium and calcium levels of pouch fluid change predictably over time (Linton & Soloff, 1964). Sodium concentrations decrease dramatically below that of serum just after fertilization and increase during gestation to that of seawater just before parturition. Calcium levels decrease from 6.8 Meq/L to 5.6 Meq/L over the course of gestation, while radiolabeled calcium provided to the male is taken up by embryos during that time. The pouch epithelium, which surrounds the embryos during gestation, was described as a highly vascular environment, containing many capillaries. The pouch epithelium in mid- to late-stage brooding males also has many lipid pockets scattered throughout, although it was unclear whether these lipid deposits were produced by the male, or absorbed by the male from the yolk of the developing embryos (Linton & Soloff, 1964).

Boisseau (1967) studied the relationship between the developing embryos and the paternal pouch tissue in *H. brevirostris* and *H. guttulatus*. During early development (just before copulation and during the two days following) there is an intense development of blood vessels just under the pouch epithelium, which creates the highly vascularized environment of the pouch. Soon after copulation and fertilization, the pouch epithelium and connective tissue proliferate around the embryos until only a small portion of the embryo surface is exposed to the pouch lumen and bathed in the “marsupial fluid” that fills the pouch. Early work suggests that all proteins identified in the pouch fluid are of maternal (egg) origin (Boisseau & LeMenn, 1967). The epithelial cells become highly secretory during early gestation, and this secretory effect appears to be controlled by the hormone prolactin released from the pituitary.
(Boisseau, 1967). The role of the epithelial secretions in provisioning embryos with nutritive substances other than proteins (e.g. lipids, carbohydrates, or minerals) is unknown. Prolactin has also been suggested to be responsible for the maintenance of the male pregnancy, since both removal of the pituitary and the addition of substances to specifically block the action of prolactin result in the expulsion of all embryos (Boisseau, 1967). The intimate relationship between developing embryos and paternal epithelium, coupled with the secretory nature of the epithelium, strongly suggest that paternal investment in this species may be substantial. In addition, there may be some costs to the male associated with maintaining the optimal ionic and osmotic conditions for the developing embryos.

Research into the possible mechanisms of the operational sex ratio and potential reproductive rates driving the action of sexual selection in seahorses is also sparse. Beam-trawls of field populations of *H. brevirostris* and *H. guttulatus* indicate that adult sex ratios are equal (Boisseau, 1967), although similar data gathered on *H. zosterae* using push net collection indicates that females outnumber males throughout the breeding season (Strawn, 1958). Of the male *H. zosterae* collected during peak breeding season, 32% had empty pouches ready to receive eggs, but the percentage of females with mature eggs (fertilizable females) in the population was unknown (Strawn, 1958). The operational sex ratios and realized reproductive rates of paired seahorses are equal, given their monogamous mating system. However, among unmated *H. fuscus*, the OSR may be male-biased because males appear to be able to prepare to mate faster than females (Vincent, 1994b), and thus may have a higher potential reproductive rate. This was determined by measuring the time it took either isolated males or isolated females to mate once placed with a ready-to-mate opposite sexed partner. Isolated *H. fuscus* females took about 2 days longer to mate than isolated males, but
in this study the differences in mating latency between males and females was not placed into the context of potential differences in their reproductive rates.

**Study organism:** I used the dwarf seahorse, *Hippocampus zosterae*, to test mechanisms hypothesized to influence courtship-roles. *H. zosterae* occurs in shallow seagrass beds from the Gulf of Mexico east through the Bahamas, Bermuda and Cuba (Ginsburg, 1937; Böhke & Chaplin, 1966), and adult size ranges from 16-38 mm (measured as the distance from the top of the coronet to the end of the tail; Strawn, 1958). The monogamous mating system exhibited by *H. fuscus* (Vincent, 1995) and *H. whitei* (Vincent & Sadler, 1995) has also been found in laboratory studies of this species, with a male and a female remaining together and mating repeatedly over the course of the breeding season (Masonjones, unpublished data). The breeding season typically lasts from early-mid February to late October or early November in the field (Strawn, 1958), but can be induced in the laboratory year-round by providing 12 or more hours of daylight. During copulation the female transfers her entire clutch of eggs to a single male in both this species ($\bar{X}$ (1 SE) = 12.5 (1.32) eggs, n=14; Chap. 2) and in *H. whitei* (Vincent & Sadler, 1995). Five to 25 ($\bar{X}$ = 11.4 (1.58), n=18) fully independent young are born following a male gestation period of approximately 10 days, and pairs remate within 4-20 hours of a male’s giving birth.

**Summary of Research:**

The present study used the dwarf seahorse, *Hippocampus zosterae*, as a model system to evaluate the relative importance of the mechanisms underlying courtship behavior patterns. Chapter II describes the measurement of relative parental investment in male and female *H. zosterae*, using techniques to determine both metabolic and material contributions to
offspring. Chapter III quantifies the potential reproductive rates of males and females by presenting each sex with a sexually-receptive partner, measuring the time it takes each sex to prepare to mate, and using this difference in preparation time to calculate the potential number of offspring produced by males and females. Chapter IV describes and quantifies the courtship and mating behaviors of this species. In Chapters V and VI, the courtship roles of male and female seahorses are described using a biased sex-ratio design to identify which sex competes most intensely for access to mates and which sex is most selective of its mates.
CHAPTER II:

Parental investment of male and female dwarf seahorses (*Hippocampus zosterae*, Syngnathidae): Which sex invests more energy in offspring?

Abstract. Seahorses (genus *Hippocampus*) are distinguished by an extreme degree of morphological specialization for paternal care, the formation of monogamous pair bonds, and male competition for mates. Females deposit eggs into a brood pouch on the ventral surface of males, in which embryos develop until their birth as fully independent offspring. This study tested the common assumption based on observations of male brood pouch morphology that seahorse males invest more per offspring than do seahorse females. In the dwarf seahorse (*Hippocampus zosterae*) relative parental investment was estimated by measuring the biochemical composition of eggs and juveniles, and by quantifying the respiration rates of reproductive adults and developing embryos. Male respiration rate throughout gestation was significantly higher than total embryo respiration rate, indicating males did invest energy in their offspring while brooding. When compared directly to female caloric investment per egg, though, males invested significantly less per offspring than did females. It has long been assumed that male seahorses invest more in their offspring because of their specialized brooding structures, but this study suggests otherwise.
INTRODUCTION

Sexual selection refers to differential reproductive success based on intersexual choice and intrasexual competition (Darwin, 1871, Anderson, 1994). In most species, females are more selective of their mates and males compete for access to females (Gwynne, 1991), a pattern that has been hypothesized to depend on the relative parental investment males and females make per offspring (Williams, 1966, 1975; Trivers, 1972, 1985). Parental investment theory predicts that if males have a higher relative energy investment per offspring than females, they should be more selective of their mates, and females should compete for access to males (Trivers, 1972). Two related factors may also influence courtship behavior patterns: 1) the operational sex ratio (OSR), defined as the “ratio of fertilizable females to breeding males in a population” (Emlen & Oring, 1977) and 2) the potential reproductive rates of males and females, defined as the “maximum number of independent offspring that parents can produce per unit time” (Clutton-Brock, 1991; Clutton-Brock & Vincent, 1991). However, the potential reproductive rates of males and females and ultimately the operational sex ratio are primarily determined by the relative energy investments parents make in each offspring.

Parental investment has been broken down into two components (Baylis, 1981): 1) direct (material) investment, such as the materials contributed to eggs and those provided directly to the offspring through the placenta or during lactation, and 2) indirect (metabolic) investment, which is the energy invested in performing tasks such as brooding, egg fanning, feeding juveniles, protecting young from predators, increased predation risk to parents, and delays in producing additional offspring (Trivers, 1972). To assess relative parental investment, both components must be measured to accurately determine which parent donates
more energy to offspring. In the past, it has often been assumed that the parent caring for offspring necessarily has the greater parental investment (Clutton-Brock and Parker, 1992; Anderson, 1994). This difficulty is exacerbated in species which exhibit male care of young, such as some frogs, some birds, and many fish (Ridley, 1978), but which may also have a substantial female investment in eggs. Parental investment by males and females has only been comprehensively quantified (including both material and metabolic parental contributions) in a few species (but see Simmons, 1992), and the relationship between parental investment patterns and courtship roles has yet to be experimentally tested.

Fish from the family Syngnathidae (pipefish and seahorses) vary in anatomical and physiological specialization for paternal care. In some pipefish, such as *Nerophis ophidion*, females attach their eggs to the ventral surface of the male (Berglund et al., 1989; Rosenqvist, 1990). In other pipefish and all seahorses (genus *Hippocampus*), females deposit their eggs into a pouch on the ventral surface of the male where fertilization takes place (Fiedler, 1954) and embryos are sealed from the external environment (Linton & Soloff, 1964). The size of seahorse testes and the number of sperm produced per fertilization event are very small compared to other teleosts (Fiedler, 1954), and as a result male gametic investment can be assumed to be negligible. Male investment is likely to be primarily indirect, as the epithelial lining of the male seahorse brood pouch is highly vascularized, and provides the developing embryos with services such as gas exchange, osmoregulation and waste removal during their 10-30 day gestation (Strawn, 1958; Boisseeau, 1967; Vincent, 1990). There is also limited evidence to suggest that males of some pipefish species provide material contributions to their offspring during gestation (*Syngnathus fuscus*, Haresign & Schumway, 1981; *N. ophidion*, *S. typhle*, Berglund et al., 1986). In female *H. erectus* seahorses, mature follicles appear to be
produced continuously from two germinal ridges in each ovary, with follicles of sequentially increasing developmental stages progressing from each germinal ridge outward (Selman et al., 1991). Mature eggs are large relative to adult female body size compared to other teleosts (Wallace & Selman, 1981) and contain a number of lipid droplets and visible yolk proteins (Selman et al., 1991). Eggs are likely to represent a considerable energy investment on the part of the female.

Based on the observation that seahorse males provide paternal care and have a highly specialized brood pouch in which embryos develop, seahorses have long been assumed to exhibit higher male investment in offspring (Williams, 1966; Trivers, 1985). The purpose of the present study is to estimate the relative parental investments of male and female dwarf seahorses (Hippocampus zosterae), measured as the direct, or material, investments females make in eggs and both the material (non-gametic) and metabolic contributions that males make to embryos developing in the pouch. Material energy investment per offspring made by males and females was determined by analyzing the biochemical composition of eggs and juveniles. Metabolic investment in offspring was determined by first measuring oxygen consumption rates of breeding males at various times across the reproductive cycle. Respiration rates of embryos removed from male pouches at various developmental stages were then quantified, and subtracted from the gestational respiration rate of males to yield an estimate of male metabolic investment. This study represents one of the first comprehensive determinations of relative parental investment in a vertebrate.
METHODS

Study Organism

*Hippocampus zosterae* occurs in shallow seagrass beds from the Gulf of Mexico east through the Bahamas, Cuba and Bermuda (Ginsberg, 1937; Böhlke and Chaplin, 1966). Adult size ranges from 16-38 mm (measured as the linear distance from the top of the coronet to the end of the tail; Strawn, 1958). The monogamous mating system exhibited by *H. fuscus* and *H. whitei* (Vincent, 1995) occurs in *H. zosterae* in the laboratory, with a male and female remaining together and mating repeatedly over the course of the breeding season (HDM, unpubl. data). In the lab, females transfer one entire clutch of eggs to a single male [$\bar{x} (\pm 1 \text{ SE}) = 12.5 (1.32) \text{ eggs, } n=14$]. Five to 25 ($\bar{x}=11.4(1.58); n=18$) fully independent young are born after approximately 12 days of gestation within the male brood pouch at 26°C in the lab, and pairs remate within 4-20 hours of the male’s releasing young.

*Hippocampus zosterae* were collected in early September (1996) and late January (1997) near Key Largo, FL. Fish were maintained in small (5-8 fish) same-sexed groups (in sexual isolation) for 10 days to 8 weeks before use in all experiments in an attempt to standardize reproductive status. Fish were kept in 38 L aquaria prior to and during experiments. Tanks were maintained at an average temperature of 26°C on a 13 hours light/11 hours dark photoperiod with a salinity range of 26-33‰, and two artificial seagrass plants were supplied for attachment sites. Fish were fed daily with recently hatched *Artemia*, and supplemented every other day with Selcon (American Marine), a food additive containing highly unsaturated fatty acids.
Courtship Behavior Patterns

Four discrete phases of courtship in paired dwarf seahorses have been observed (Masonjones and Lewis 1996). Phase 1 courtship occurs during the one to two days preceding the day of copulation, and is characterized by reciprocal quivering, during which fish assume an erect body posture, with pectoral fins extended, and rapidly vibrate their bodies from side to side. Courtship phases 2-4 occur on the day of copulation. During phase 2 of courtship, females first display pointing (a behavior used as an indicator of a female’s readiness to mate and the beginning of the day of copulation), defined as when a female raises her head upward toward the water surface to form an oblique angle with the main body axis and then lowers it again to a horizontal position. Males generally responded to female pointing by quivering. Phase 2 courtship is usually followed by a latency period of 23-220 min, during which it is hypothesized that females undergo the last stages of egg maturation and ovulation (Vincent, 1990). In phase 3 courtship, males begin to display pointing in response to female pointing. Phase 4 courtship is characterized by the male and female rising repeatedly. During this phase, fish release their respective holdfasts and rise up into the water column facing one another. In a copulatory rise, the female genital papilla is placed inside the male brood pouch opening, followed by egg transfer and fertilization.

Wet mass of all fish used in trials was measured by blotting fish dry and weighing them to the nearest 0.01 mg in seawater in a 2ml vial. Experimental fish ranged from 60-245 mg wet mass, and all pairs used were size-matched, with a mass difference of less than 30 mg.

Direct Investment in Offspring

These experiments estimated the material energy investment made by H. zosterae parents in their offspring by determining the energy content of eggs (female investment), and
the energy change across gestation, from eggs to newborn juveniles. Material investment in
eggs was used as the only measurement of female contributions to offspring, due to the
difficulty partitioning female metabolic investment into behavioral and reproductive
components. The material investment of sperm was assumed to be negligible, based on the
observations of Fiedler (1954) and Boisseau (1967) that very few sperm are produced in
seahorses compared to other teleosts.

A *H. zosterae* male and female were selected to be of similar size and placed together
to court and mate. To obtain eggs (n=15 clutches), pairs were observed continuously through
the last stages of courtship. Immediately after copulation (within 10 s) the male was removed
from the tank and anesthetized in a 0.03% solution of cold tricane (MS-222), in 32% seawater
for 3-5 minutes. By removing the male and anesthetizing him within a few seconds, the
pouch sphincter remains open, thus allowing removal of eggs from the pouch with a pipette.
After egg removal, males were put back with the female to recover from the procedure. Eggs
were rinsed quickly in distilled water to remove excess chloride and placed onto weighed
drying boats.

Juveniles (n=20 broods) were obtained by placing a pair together, observing them
through copulation, and then isolating the male and female in a fine-meshed container late on
day 11 of a 12 day gestation. This ensures that all juveniles in a brood are recovered and that
food present in the tank would not be available to newly born juveniles, since feeding by the
newborns could artificially increase their energy content. After harvesting 1-4 hours after
birth, juveniles were killed by freezing, rinsed in distilled water and placed on drying boats.
Both egg and juvenile samples were dried in a desiccator heated to 30°C for 24 hours (drying
procedure modified from Baker, 1994). After drying, samples were weighed on a Mettler
AT20 balance to 0.001 mg and stored in a desiccator at room temperature until their use in either of the two assays to determine energy content. A subset of mothers (n=9) were sacrificed and dried to obtain maternal dry weights and determine the percentage of maternal dry weight that eggs represent.

**Total organic carbon content (TOCC) assay** - Eggs (n=58, from 8 families) and juveniles (n=30, from 7 families) were analyzed for their organic carbon content using a standard acid dichromate technique (McEdward & Carson, 1987). Samples were incubated in phosphoric acid to volatilize residual chloride and acid dichromate to completely oxidize them. Absorbances (inversely related to carbon content) were read in a spectrophotometer at 440nm and converted to μg Carbon using a glucose standard curve constructed from 0-350 μg C (based on the predicted sample range).

Dry mass, carbon content, and mass-adjusted carbon content of eggs and juveniles were compared using independent t-tests when assumptions of equivariance and normality were met, and Mann-Whitney tests, when treatment variances were unequal. To estimate energy content of eggs and the energy change over gestation, mean organic carbon content of eggs and juveniles was multiplied by the average energy contained in the associated carbon bonds (1 μg Carbon = 3.93x10⁻² Joules; Gnaiger & Forstner, 1983).

**Carbon, hydrogen, nitrogen (CHN) analysis** - Eggs (n=11, from 5 families) and juveniles (n=5, from 5 families) were placed into tin capsules and their carbon and nitrogen contents determined using a continuous flow-technique on a Europa Scientific Automated Nitrogen and Carbon Analyzer (ANCA). Dumas combustion (1000°C) was used to convert samples of bulk organic matter to N₂ and CO₂ in an ultra-high purity helium carrier stream (Preston &
Owens, 1983). This system has a limit of detection of nitrogen of 1 μmol, with a precision for elemental nitrogen and carbon composition of less than ±0.2% of the absolute value of the sample.

To calculate the biochemical composition of samples from their carbon and nitrogen components, a number of assumptions were made, regarding: 1) the ash component of samples, 2) the hydrogen composition of samples, 3) residual water, 4) non-protein sources of nitrogen and conversion of organic nitrogen to protein, and 5) inorganic sources of carbon, hydrogen and nitrogen. These are outlined below.

1) Percent ash of eggs and juveniles was obtained for a separate set of dried samples. Eggs contained 10.6 (0.72)% ash (n=7) and juveniles were 36.2 (5.9)% ash (n=6). These results were then used to determine the ash-free mass of experimental samples analyzed by CHN.

2) Because the elemental analyzer used in this study was configured to measure carbon and nitrogen, and hydrogen content is necessary to estimate biochemical composition, the carbon, hydrogen, and nitrogen fractions obtained by Gnaiger and Bitterlich (1984) for various body tissues of Chinese silver carp (*Hypoptalmichthys molitrix*, muscle, fat, liver, and gut) were used to approximate the hydrogen content of eggs and juveniles in the present study. This was accomplished by determining the relationship between carbon (w_c), hydrogen (w_h) and nitrogen (w_n) mass fractions (mass of element in sample/total dry weight of sample) in carp samples using a two variable linear regression. As a result of this regression, hydrogen mass fractions were calculated using

\[ w_h = 0.155(0.02) \times w_c - 0.133(0.05) \times w_n + 0.007(0.01) \]
with an overall \( r^2 = 0.99 \), and carbon (\( w_c \)) and hydrogen (\( w_h \)) with \( r^2 = 0.933 \), and \( r^2 = 0.71 \) for nitrogen (\( w_n \)). Nitrogen content is less strongly related because of the differences in protein composition of eggs and juveniles. When hydrogen mass fractions were predicted for carp samples from the above equation, they differed from observed values by between 1.2 and 5.4\%, indicating a close fit between the model and experimental data. Using this equation, \( w_h \) values were calculated for seahorse eggs and juveniles from measured fractions of carbon and nitrogen.

3) The amount of water remaining in a sample after drying can dramatically alter the estimation biochemical composition. The mass fraction of residual water and chemically bound water was estimated by Gnaiger and Bitterlich (1984) to be between 0.1 and 0.2, thus for these experiments, 0.15 was used.

4) In addition, Gnaiger and Bitterlich (1984) estimated that non-protein sources of nitrogen (\( 1-\chi_{PN} \)) ranged from 2 to 26\% of total nitrogen measured across their samples; because it is unclear what seahorse eggs and juveniles are most like in composition, my model assumes a non-protein nitrogen correction factor of 0.14 (\( \chi_{PN} = 0.86 \)). In the past, organic nitrogen was multiplied by 6.25 for conversion into protein, based on the assumption of a 16\% nitrogen content in protein, \( N_p = 0.16 \) (Brody, 1945). However, Gnaiger and Forstner (1983) determined that for aquatic animals, \( N_p \) averaged 0.173(0.004 sd); therefore, protein sources of nitrogen were converted into the mass of protein in the sample by using this new experimentally-determined conversion factor, where \( P_N = 1/N_p = 5.8 \).
5) Inorganic sources of carbon, hydrogen and nitrogen (in ash) were assumed to be zero in this model, given that Gnaiger and Bitterlich (1984) determined that across most tissue types, these elements in the ash accounted for less than 1% of total measured fractions.

Using the experimentally determined mass of carbon and nitrogen in samples, the above assumptions and estimated fraction of hydrogen and ash, the following method from Gnaiger & Bitterlich (1984) was used to calculate the amount of protein, lipid and carbohydrate in seahorse eggs and juveniles. The organic carbon fraction in ash-free biomass ($w_C$) was calculated as

$$w_C = \frac{(\omega_C w_C - \omega_w w_C) \times w_{\text{ash}}}{1 - w_{\text{ash}}}$$  \hspace{1cm} (1)$$

where $\omega_C w_C$ is equal to the total carbon mass in the total dry biomass, $\omega_w w_C$ is the inorganic carbon fraction in the ash (assumed in my model to be zero), and $w_{\text{ash}}$ is the mass fraction of ash in the dry weight. Total ash-free biomass was partitioned into three organic nutrient groups, $w_K$, gram carbohydrate, $w_L$, gram lipid, and $w_P$, gram protein, that are related through the equation

$$1 - w_{\text{H}_{10}} = w_K + w_L + w_P$$ \hspace{1cm} (2)$$

The mass fraction of protein was calculated as

$$w_p = P_v \times x_{\text{N}} \times w_N$$ \hspace{1cm} (3),$$

where $P_v$ is 5.8, $x_{\text{N}}$ is 0.86, and $w_N$ is the mass fraction of nitrogen in the sample. The mass fraction of lipid can be calculated as

$$w_L = b_L \times (1 - w_{\text{H}_{10}}) + b_{LC} \times w_C + b_{LN} \times x_{\text{N}} \times w_N$$ \hspace{1cm} (4),$$

where $b_{LC} = \frac{C_k}{C_L - C_k}$ \hspace{1cm} (5),$$

$$b_{LN} = \frac{1}{C_L - C_k}$$ \hspace{1cm} (6),$$
and \( b_w = -\frac{C_p - C_k}{N_p \times (C_l - C_k)} \)  \( \text{(7).} \)

\( C_p, C_l, \) and \( C_k \) are constants based on the standard C H N compositions of protein, lipid, carbohydrate, and \( N_p \) is equivalent to \( 1/P_N \). The mass fraction of carbohydrate can be calculated by solving for \( w_k \) in equation (2).

After mass fractions were obtained, they were then multiplied by the ash-free dry weight of samples to obtain the masses of protein, carbohydrate and lipid. The mass of each biochemical component in each sample was then multiplied by standard conversions from mass to energy of 20.1 Joules mg\(^{-1}\) for protein, 17.7 J mg\(^{-1}\) for carbohydrate (glucosyl units), and 39.2 J mg\(^{-1}\) for lipid (from Withers, 1992). Energy contained in each component were then summed for each sample, to obtain the total energy contained in each egg and juvenile. Mean dry weight, carbon and nitrogen content, mass fraction of protein, carbohydrate and lipid, and energy for each sample type were compared using independent t-tests, when assumptions of normality and equal variances were met, and Mann-Whitney tests when they were not.

**Differences in Feeding Rate between Males and Females**

Two methods were employed to estimate feeding rate of female, male, and gestating male \( H. \) zosterae. The first was the supersaturation method in which fish were deprived of food at 24 hour intervals (5 such intervals for each group of fish), provided with excess food, and then feeding rates observed over the period of 1 hour. This is a standard method used to determine the maximum rate at which fish feed (Adams & Breck, 1990). In other species, feeding rates under these conditions have been shown to directly reflect the reproductive status of fish, with maximal feeding rates higher during the breeding season than at other

36
times of the year, even when temperature and experimental conditions were held constant (Wooton, 1994). Because of the possibility of obtaining inflated feeding rates for all groups of fish because of starvation, the second method observed feeding during the morning hours when fish are most active under natural tank conditions with food available *ad libitum*.

*Supersaturation* - To determine if females, males and gestating males had different levels of energy intake, two different assays were used to determine their relative rates of *Artemia* ingestion. In the first, ingestion rates of gestating males were determined in groups of 2-4 at a time, as they became available from other experiments. To determine the feeding rates of breeding males and females, four trials were run containing 4 males and 4 females each (for a total of n=16 males and n=16 females). Individuals were selected based on differences in body size and colors, so that each individual could be identified. To verify that all fish were in a reproductive state, trials were observed each morning for 1-2 h for the appearance of courtship behaviors exchanged between members of specific pairs. Fish were not used in the analysis of feeding rate if they were not observed to court for at least 2 of the five days of a trial.

In this experiment, all fish were isolated for at least 1 week to standardize their reproductive status before the beginning of trials. Fish were weighed (to the nearest 0.01g) on the morning before the first feeding rate measurements and placed in groups of 8 (4 females, 4 males) into 38 L aquaria at 26.5°C with a brine shrimp density of less than or equal to 0.2 nauplii per ml seawater, a very low food density in which usually sedentary seahorses actively search for food. At 20-24 hour intervals for five days (five sampling periods), the aquarium water was supersaturated by adding 1.2±0.01 grams of nauplii for a density of 2.4 nauplii ml⁻¹. 
seawater. Repeated density counts were taken before and after feeding rate measurements, and no differences were detected in the mean density counts between days, between trials or before and after each trial (t-tests and ANOVA, all p>0.05).

After adding the shrimp, individuals were observed for ten minutes each, and their intake scored. Because seahorses have a characteristic feeding posture and only ingest one shrimp at a time, accurate measurements of intake can be made through observation. The order that individuals were observed was randomized on each day of the 5 day trial. One to three individuals could be observed during each 10 minute observation period, depending on proximity. At the end of each trial day, the aquarium water was siphoned through a 50 μm mesh to remove most of the shrimp, until density counts were less than 0.2 per ml seawater.

After each 5 day trial, the feeding rates of each individual were averaged to get an overall feeding rate score. Mean feeding rate (# shrimp ingested min⁻¹) of males, females, and gestating males were first standardized by body weight and then compared using a Kruskal-Wallis test, because treatment variances were unequal.

**Feeding rates in unmanipulated pairs** - The feeding rates of pairs under standard conditions with constant food were also determined. Seven pairs were observed from the day of introduction through the day of copulation, for the first three hours of each day. Food was available *ad libitum* in the water column, at a density of about 1 shrimp ml⁻¹ of seawater. Food intake was monitored continuously for each member of the pair on every observation day. Male and female ingestion rates were compared across days with a repeated-measures ANOVA.
Indirect (Metabolic) Investment in Offspring

*Adult oxygen consumption rates* - To determine oxygen consumption rates of adult *H. zosterae*, a gravity-driven, flow-through respirometer maintained at 26°C was used (Figure 1). Seawater, aerated for at least 24 hours, flowed from the water source through capillary tubing and the chamber containing a single seahorse at an average rate of 1 ml min⁻¹. For 90 minutes, the outflow tubing was connected at approximately 10 minute intervals to a Strath-Kelvin (Model 781, Probe 1302) oxygen meter, equipped with a polarographic oxygen electrode, to determine changes in oxygen concentration over time. Readings were taken precisely 2 minutes after the outflow tubing was attached to the oxygen sensor, and during that time interval the flow rate through the sensor was determined as well as the gill ventilation rate of the subject in the chamber. Gill ventilation rates were monitored both before oxygen measurements (while subjects were in holding tanks) as well as at 5 minute intervals during respiration rate trials, to monitor fish stress levels (Cech, 1990). No significant differences were detected between the gill ventilation rates of fish in holding tanks and fish in the experimental apparatus (paired-samples t-test, t = 0.199, 75 df, p = 0.842), so the procedure did not appear to affect respiration rates. Oxygen consumption rate (μl hr⁻¹) was calculated by subtracting the mean oxygen concentration (μl O₂ ml⁻¹ seawater) during the last 60 minutes of the trial (the first 30 minutes allowed for acclimation to the chamber and for oxygen consumption rate to reach a steady state) from the starting concentration in the empty chamber before the seahorse was placed inside, and multiplying the result by the average flow rate (ml min⁻¹).
Figure 1: Adult Respirometer - Water flow, driven by gravity at an average rate of 1 ml/minute, runs from a source of fully aerated seawater (salinity 28-32‰, ambient temperature) through capillary tubing to the chamber holding an adult seahorse (*H. zosterae*), and out of the system. A water bath around the subject chamber maintains the temperature at 26°C. At approximately 10 minute intervals for 90 minutes, the outflow tubing from the chamber is attached to an oxygen meter (Strath-Kelvin, Model 781, Probe 1302), which detects changes in oxygen concentration over time, and is maintained at 26°C with a water jacket.

Oxygen consumption was measured to determine the resting-routine respiration rates of: 1) fish isolated from the opposite sex (baseline, single timepoint), 2) fish isolated from the opposite sex (multiple timepoints), 3) courting fish (within 5 days of initial baseline measurements), 4) fish immediately prior to copulation, 5) females within 2 days after copulation, 6) courting fish (no baseline measurement) and 7) gestating males at 4 timepoints over the course of gestation. Fish were weighed (as described above), and the baseline respiration rates of 45 females and 48 males were determined after an isolation period from the opposite-sex of at least 1 week. This baseline measurement was repeated 2-3 times over the course of a week for 5 females and 5 males maintained in isolation between measurements. Repeating respiration rate measures in these fish indicated that there was not a significant effect of repeated exposures to the experimental apparatus, with no differences detected between the first ($\bar{x} = 40.8 \ (3.2)\mu l \ O_2 \ hr^{-1}$) and subsequent ($\bar{x} = 37.2 \ (2.11) \mu l \ O_2 \ hr^{-1}$) respiration rate measurements (paired t-test, $t = 1.54$, $p = 0.157$).
To determine respiration rates of reproductive fish, males and females were placed in pairs into aquaria, and observed each morning for an hour, from their introduction through the day of copulation, to determine the beginning of gestation. At two timepoints during this courtship period, the day before copulation (courting, n=15 females, n=14 males) and during latency on the day of copulation (DOC, n=5 females, n=4 males), the respiration rates were again determined for sub-samples of fish. This was because the increased activity level of fish during courtship and their preparations for copulation (males preparing the pouch epithelium to receive zygotes and females maturing eggs) may alter oxygen consumption rates. In a sub-sample of females (post-ovulation, n=10), oxygen consumption rates were also measured once during the three days after copulation.

In the sixth group of fish, courting respiration rates were measured without previously measuring baseline rates. This was done because preliminary analyses of the data indicated that courtship respiration rates in fish previously in the experimental apparatus were substantially lower than their initial rates of respiration. In this group, pairs were formed, observed until they displayed courtship behaviors, and their oxygen consumption rates were then measured (baseline-courting, n=10 females, n=10 males). This provided a method of determining if high baseline measurements were due to the initial effect of the experimental apparatus, or a real phenomenon observed in fish isolated from the opposite sex.

Oxygen consumption rates of gestating males were determined across the 12 day brooding period, measuring each male only once during gestation. These measurements were partitioned into four classes (stages 1-5, n=8 males, stages 6-10, n=7 males, stages 11-15, n=8 males, stages 16-20, n=9 males), determined for each male after measuring respiration rate, by
removing the embryos from his pouch and staging embryos according to Boisseau (1967: see below).

Oxygen consumption rates of developing embryos - After respiration rates of gestating males were measured, males were placed into warm 0.03% tricane (MS-222) in 32% seawater for 3-5 minutes. Anesthetized males were moved to a dissection dish filled with seawater and immobilized with small straps across their upper abdomen and across the base of the tail just below the pouch. Using fine forceps, the pouch seal was broken and slowly dilated by stretching repeatedly in all directions. By gently massaging the outer surface of the pouch with a blunt probe, I was able to break the attachments between the pouch epithelium and the embryos. By carefully squeezing the pouch while using suction from a pasteur pipette, the embryos could be removed intact, after which the male could be returned to an aquarium to be used in other experiments. This technique worked with embryos in stages 1-2 and older than stage 9. Stage 3-9 embryos had to be removed by cutting along one side of the male’s pouch to expose them, using a combination of suction and forceps to detach the embryos manually.

Once embryos were removed, they were placed into seawater of a salinity lower than that in which the adults were housed. Embryos were placed into seawater of salinity 10-15 % (one third to one half that of adults) based on previous work by Linton & Soloff (1964) with H. erectus embryos. In addition, in the genera Syngnathus and Hippocampus, embryos are either intermittently or completely sealed from the outside environment, respectively. Males of these genera provide a controlled osmotic environment for the offspring, and embryos have a much higher mortality after removal from the pouch when placed in normal seawater (32%) compared to their survival in lower salinity seawater (Gudger, 1906; Linton & Soloff, 1964). Previous work by Berglund et al. (1986b) measured the respiration rates of Syngnathus fuscus
embryos collectively in beakers of 32-33% seawater (Winkler method). Because of this it is entirely possible that their estimates are artificially elevated relative the oxygen consumption rates of the embryos measured in my study which were bathed in seawater of salinity 13-20% and measured individually in very small chambers.

Embryos were staged according to a 21 stage protocol established by Boisseau (1967) for *H. guttulatus* with a 21 d gestation period (see Figure 2 for the pattern of *H. zosterae* development). Embryos of these two species were similar, with minor differences in the later stages such as the shorter snout and less-developed dermal spines of *H. zosterae.*

**Figure 2** (following two pages): Development of *Hippocampus zosterae* embryos. Stages of development determined using the protocol established for *H. guttulatus* by Boisseau (1967). (a) Unfertilized egg, (b) fertilized egg, within 10 minutes of fertilization; (c) and (d) Stage 1 embryo, about 5-6 hours after fertilization, with the neural groove just beginning to form; (e) Stage 3 embryo; (f) Stage 5 embryo; (g) Stage 7 embryo, melanophores visible in the eye area; (h) Stage 8 embryo, dorsal fin now visible; (i) Stage 9 embryo, with melanophores developing along the body; (j) Stage 11 embryo, with the embryo still enclosed within the delicate chorion; (k) Stage 13 embryo, in which fin blades are first clearly visible; (l) Stage 15 embryo, with upper jaw beginning to extend out from the eye; (m) Stage 16 embryo; (n) Stage 18 embryo; (o) Stage 20 embryo, with yolk nearly completely resorbed; and (p) Stage 21 embryo, immediately after birth.
Embryos and the gestating males were sorted into four classes based on specific morphological criteria. The first class, stages 1-5, is defined based on the lack of eye pigmentation (see Figures 2c-2f for representative embryos; n=6 broods, n=17 embryos). The second stage class, stages 6-10, begins with the first appearance of melanophores in the eye area and ends just before the jaws are parallel to the frontal plane (see Figures 2g-2i; n=5 broods, n=32 embryos). The third stage class consists of stages 11-15, and begins when the jaws are parallel to the frontal plane and ends when the jaw is between 0.5 and 0.75 times the diameter of the eye (see Figures 2j-2l; n=6 broods, n=41 embryos). Stage class 16-21, begins when jaw length is between 0.75-1.25 times the diameter of the eye and ends when the yolk slit on the anterior of the body is nearly closed, a few hours before birth (see Figures 2m-2o; n=9 broods, n=67 embryos).

After staging, the oxygen consumption rates of embryos were determined, using a micro-respirometer (Embryo Viability Assessment System [EVAS] developed at Tufts Veterinary School). Individual embryos were placed into 10 µl conical wells of a tissue culture plate with fully aerated seawater ranging in salinity from 9-15 %. The respiration rates of *H. zosterae* embryos were measured individually in small wells because in all syngnathids (both those with ventrally attached embryos and fully enclosed brood pouches), embryos are tightly confined either in the original egg chorions or in invaginations in the pouch epithelium. In seahorses, the process of removing embryos from the pouch usually ruptures the egg chorion and allows the embryos to move freely. When oxygen consumption rate is measured in groups of seahorse embryos, their movement substantially increases their respiration rate as compared to when they are measured individually (pers. observation). Past
work by Berglund et al. (1986b) with S. fuscus (pipefish) measured embryos in batches of 20-170, and this may have artificially increased their respiration rates.

After placement of embryos, the plate was placed into the base of the EVAS, and the lid containing the individual polarographic oxygen microelectrodes was closed, causing the oxygen probes to seal with the tops of the wells of the plate and creating a closed environment in which to measure changes in oxygen concentration over time at a constant 26°C. This system directly interfaced with an IBM Thinkpad, to allow real-time monitoring (updated every 15 seconds) of temperature, oxygen concentration, and percent of starting oxygen concentration. The duration of embryo measurements ranged from 5-20 minutes, depending on the age and oxygen requirements of the embryo, with younger embryos monitored for longer periods. Immediately following each trial, embryos were removed from the wells and their status evaluated. Mortality with this system was low, as only 8 of 165 measured embryos died while in the apparatus. Overall, for embryos removed live from the brood pouches of males, embryo mortality for H. zosterae was less than 10% during the first 4 hours. All embryonic oxygen consumption rates were measured within 45 minutes after removal from the pouch.

Because embryos at different stages consumed different amounts of oxygen and some depleted the oxygen available before the end of a trial, oxygen consumption rate readings between 95-55% starting oxygen concentration were averaged, to obtain a mean oxygen consumption rate for each embryo in μl hr⁻¹. For each brood, both an overall mean oxygen consumption rate and a total oxygen consumption rate was determined. In broods where not all embryos were measured, the average rate was multiplied by the number of embryos in the
brood to obtain an estimate of the total respiration rate of the developing brood. To determine the amount of energy that a single embryo would need to complete development, the relationship between mean embryo respiration rate and stage was determined. Because embryo respiration rate increases exponentially over development (see Results), respiration rate was log transformed before regression analysis. The analysis generated the regression:

\[ \log_e (\text{Respiration Rate}) = (-4.84 \pm 0.104) + (0.225 \pm 0.0082) \text{ Stage}. \]

We derived the total oxygen consumed (in \( \mu l \)) per embryo during development, EE, from the regression for respiration rate, \( \log_e (\text{RR in } \mu l \text{ O}_2 \text{ hr}^{-1}) = a + b \text{ (stage)} \) as:

\[ EE = C \int_0^{21} \text{ RR } d(\text{stage}) = C \int_0^{21} e^{a + b(\text{stage})} d(\text{stage}) = C \frac{e^a}{b} (e^{21b} - 1) \]

where \( a = -4.84 \pm 0.104 \), \( b = 0.225 \pm 0.0082 \text{ stage}^{-1} \), and \( C = 14.49 \pm 0.615 \text{ hr stage}^{-1} \) (calculated by dividing the mean gestation length, 289.7 (12.3) hours, by 21 stages).

Using an extension of the pooling technique from Box et al. (1978), error estimates around the mean were derived using the appropriate Taylor polynomial expansions of \( e^x \), \( \log(1+x) \) and \( (1+x)^n \) for small \( x \) as follow:

1. \( e^{y \pm z} = e^y (1 \pm z) \)
2. \( \frac{y \pm z}{w \pm x} = \frac{y}{w} \left(1 \pm \sqrt{\frac{z^2}{y^2} + \frac{x^2}{w^2}}\right) \)
3. \( (y \pm z)(w \pm x) = yw \left(1 \pm \sqrt{\frac{z^2}{y^2} + \frac{x^2}{w^2}}\right) \)
4. \( (y \pm z)^w = y^w \left(1 \pm \frac{wz}{y}\right) \)
5. \( (y \pm z) + (w \pm x) = y + w \pm \sqrt{z^2 + x^2} \)
where \( x \) and \( z \) are standard errors. Total oxygen consumed over the course of development was converted to Joules using a standard relationship between energy and oxygen consumption of 20.08 Joules ml \( O_2 \) consumed (Withers, 1992).

**Analysis of metabolic rate measurements** - Because of the non-linear relationship between body mass and respiration rate \( (Y = aX^b) \), adult oxygen consumption measurements were standardized by body mass by calculating a mass exponent, \( b \), specifically for dwarf seahorses (Cech, 1990, Withers, 1992). Courting fish (20 males and 20 females) were used to determine this relationship because courtship respiration rates were significantly lower than baseline (see Results section), and this was apparently due to actual differences in the physiology of dwarf seahorses rather than an artifact of the experimental apparatus. In addition, fish in the field are continuously paired, and the courtship respiration rates would reflect most accurately their basal metabolic rate. To estimate \( b \), the relationship between \( \log_{10} \) of oxygen consumption rate (\( \mu l \ hr^{-1}; Y \)) and \( \log_{10} \) of body mass (mg, \( X \)) was determined, resulting in the regression equation

\[
\log_{10} Y = (0.35\pm0.2) + (0.54\pm0.1) \log_{10} X \quad \text{(Figure 3)}.
\]

There was a positive and moderate relationship between these two variables \( (r^2 = 0.354, p<0.0001) \), with a slope \( (b) \) of 0.54. This number was used for all comparisons of respiration rates by dividing them by (body mass\(^{0.54} \), to obtain mass-independent respiration rates. The respiration rates of gestating males were normalized by their weight before copulation.
Figure 3: Linear regression of log_{10} body mass (mg) and log_{10} oxygen consumption rate (µl hr^{-1}), calculated to obtain the mass exponent. Fish (Hippocampus zosterae) used in this analysis were 20 males and 20 females that had been courting for at least 1 day, ranging in body mass from 70-245 mg. Respiration rates were measured using a gravity-driven, continuous-flow respirometer maintained at 26°C.

To determine male metabolic investment in each offspring over the course of gestation (I_m, in µl O_2 embryo^{-1}),

\[ \text{IR}_m = \frac{[M_{gest} - M_{basal} - M_{brood}]}{\text{# embryos in brood}} \]

was calculated, where IR_m = µl O_2 hr^{-1} emb-1 by each male, M_{gest} = gestational respiration rate in µl O_2 hr^{-1}, M_{basal} = resting routine respiration rate in µl O_2 hr^{-1}, M_{brood} = µl O_2 hr^{-1} by entire brood. IR_m represents the increase in a male’s oxygen consumption rate during gestation that can be attributed to the individual costs associated with maintaining each embryo. A multiple regression analysis was used to determine the effects of male mass (mg) and embryo stage on the rate of oxygen consumption of each male per embryo, and it indicated that there was an
effect of stage but not of male mass on the rate of oxygen consumed (see Results section). Because there was no relationship between male investment per offspring and his body mass, these calculations were not corrected for body mass. To extrapolate total investment across all stages of gestation from the measurements of male oxygen consumption per offspring at each stage, the regression relationship between stage and male oxygen consumption rate per offspring was determined as,

\[ IR_m = (0.0346 \pm 0.0149) \text{ Stage} + (0.6203 \pm 0.194). \]

The area under the curve from 0 to 21 (number of stages) was then calculated by integration to obtain total oxygen consumed by a male for each embryo (\( I_m \), in \( \mu l \ emb^{-1} \)):

\[ I_m = C \int_0^{21} IR_m \ d(\text{Stage}) = C \left( a \frac{(21)^2}{2} + b \ (21) \right) \]

where \( C = 14.49 \pm 0.615 \ \text{hr stage}^{-1} \), \( a = 0.0346 \pm 0.0149 \), and \( b = 0.6203 \pm 0.194 \text{stage}^{-1} \). The amount of energy expended over the course of gestation by an average male in each offspring was calculated by converting the final number in \( \mu l \ O_2 \ emb^{-1} \) to milliliters, and then multiplying it by 20.08 Joules/ml O\(_2\), the mean amount of energy expended for each milliliter of oxygen consumed (Withers, 1992). Using an extension of the pooling technique from Box et al. (1978), error estimates around the final value in Joules were derived by again using the appropriate Taylor polynomial expansion for small deviations (see above for equations).

**RESULTS**

**Direct Investment in Offspring**

Overall, egg dry mass (n=58) was larger than juvenile dry mass (n=25), although this was not statistically significant (Figure 4; independent t-test, \( t = 1.93, p = 0.057 \)). The total
clutch dry mass was between 8-29% of female dry body mass (15.9(±2.2)%, n=9), but total clutch dry mass was only weakly related to maternal wet mass (Figure 5; F_{1,12} = 1.89, p = 0.194). There was also a weak relationship between mean egg dry mass and maternal wet mass (Figure 6; F_{1,12} = 1.71, p = 0.215), and no relationship between clutch size and maternal mass (Figure 7; F_{1,12} = 0.145, p = 0.710).

Figure 4: Comparison of the dry mass of *H. zosterae* eggs (n=58) and juveniles (n=25). Samples were dried at 30°C over dessicant for 24 hours to a constant weight. Mean ± 1 SE dry weight in milligrams provided.

Figure 5: Relationship between maternal wet mass and total clutch dry mass for *H. zosterae*. Mothers were weighed to the nearest 0.01 mg in seawater. Dry clutch mass (mg) was obtained by multiplying the mean egg dry mass by the original number of eggs in the clutch. Regression line and 95% confidence intervals provided.

Figure 6: The relationship between maternal mass and mean egg mass for *H. zosterae*. Mothers were weighed to the nearest 0.01 mg in seawater. Mean (± 1SE) egg dry mass was obtained after drying at 30°C over dessicant for 24 hours to a constant weight. Regression line and 95% confidence intervals provided.

Figure 7: Relationship between maternal wet mass and clutch size for *H. zosterae*. Mothers were weighed to the nearest 0.01 mg in seawater. Regression line and 95% confidence intervals provided.
**Total organic carbon content (TOCC) assay** - Eggs contained significantly more carbon than juveniles (Figure 8; Mann-Whitney test, U = 1219.00, p << 0.0001), even when adjusted for the difference in mass (U = 1210.00, p << 0.0001). Differences in carbon content independent of mass may indicate changes in biochemical composition. A difference of 74 μg C was observed over the course of gestation, indicating a net loss in the carbon content of embryos. This represents a 36% decrease in organic content from the original egg source during embryo development. When converted to energy, using the average amount of energy in each carbon bond, eggs contained 7.95 (7.2) Joules and juveniles dropped to 5.05 (0.04) Joules.

The total amount of carbon contained in entire clutches varied considerably between families, with the mean carbon content of egg clutches ranging from 135 (14.2) to 248 (8.8) μg C, and in juvenile broods from 77 (2.6) to 182 (2.3) μg C. Clutches had a significantly higher carbon content than broods, although when adjusted for the difference in mass, there was no difference in carbon content (Table 1).

![Figure 8: Mean ± 1 SE carbon content for *H. zosterae* eggs (n = 58) and juveniles (n = 25). Carbon content determined using an acid dichromate oxidation technique, modified from McEdward and Carson (1987).](image)
Table 1: Characteristics of *H. zosterae* clutches and broods. Means ± 1 SE provided.

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<th>CLUTCH</th>
<th>BROOD</th>
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<td>Sample Size</td>
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<td>7</td>
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<tr>
<td>Number of Offspring</td>
<td>12.4 (2.2)</td>
<td>7.9 (1.6)</td>
</tr>
<tr>
<td>Total Dry Weight (mg)</td>
<td>7.0 (1.03)</td>
<td>3.8 (0.975)</td>
</tr>
<tr>
<td>Carbon Content (μg)</td>
<td>1891.4 (325.9)</td>
<td>810.7 (206.7)</td>
</tr>
<tr>
<td>Mass-Adjusted Carbon</td>
<td>279.6 (32.3)</td>
<td>219.2 (8.60)</td>
</tr>
</tbody>
</table>

Elemental carbon, hydrogen, nitrogen (CHN) analysis - CHN analysis revealed that seahorse eggs contained significantly more carbon and nitrogen than seahorse juveniles, even when adjusted for mass (Table 2). However, the atomic ratio of carbon to nitrogen was approximately the same for eggs and juveniles. Converting from elemental fractions to biochemical fractions indicated that *H. zosterae* eggs and juveniles did not differ in biochemical composition, each containing approximately 60% protein, 17% carbohydrate, and 21% lipid. However, using standard energy equivalents to convert from biochemical content to Joules, energy content showed an average decrease of 47% from egg to juvenile, while dry weight only dropped 21%.

Table 2: Biochemical composition of *H. zosterae* eggs and juveniles obtained from elemental carbon, hydrogen, nitrogen (CHN) analysis. Means ± 1 SE provided. Samples compared using independent t-tests, except for (***) categories, which were compared with Mann-Whitney tests, because variances were unequal.

<table>
<thead>
<tr>
<th></th>
<th>EGGS</th>
<th>JUVENILES</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Dry mass (mg)</td>
<td>0.61 (0.03)</td>
<td>0.48 (0.04)</td>
<td>0.026</td>
</tr>
<tr>
<td>Percent ash (%) **</td>
<td>10.6 (0.7)</td>
<td>36.2 (5.9)</td>
<td>0.0027</td>
</tr>
<tr>
<td>Carbon content (μg)</td>
<td>309.7 (15.4)</td>
<td>158.2 (10.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Nitrogen content (μg)</td>
<td>67.4 (3.9)</td>
<td>36.3 (3.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C:N ratio(atomic)</td>
<td>5.39 (0.08)</td>
<td>5.1 (0.2)</td>
<td>0.248</td>
</tr>
<tr>
<td>Body-mass adjusted Carbon (μg C/mg)</td>
<td>507.4 (25.3)</td>
<td>327.2 (22.1)</td>
<td>&lt;&lt;0.0001</td>
</tr>
<tr>
<td>Body-mass adjusted Nitrogen (μg N/mg)</td>
<td>110.5 (6.3)</td>
<td>75.1 (7.2)</td>
<td>&lt;&lt;0.0001</td>
</tr>
<tr>
<td>Percent Protein **</td>
<td>60.7 (0.009)</td>
<td>59.0 (0.04)</td>
<td>0.86</td>
</tr>
<tr>
<td>Percent Carbohydrate</td>
<td>14.4 (0.03)</td>
<td>20.9 (0.06)</td>
<td>0.27</td>
</tr>
<tr>
<td>Percent Lipid</td>
<td>23.3 (0.02)</td>
<td>18.2 (0.04)</td>
<td>0.27</td>
</tr>
<tr>
<td>Energy (Joules)</td>
<td>13.1 (0.68)</td>
<td>6.9 (0.42)</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

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Differences in Feeding Rate between Males and Females

_Supersaturation_ - Breeding males, females and gestating males, had almost identical ingestion rates (Figure 9; 1-way ANOVA, $F_{2,39} = 0.028, p = 0.97$) even when standardized by body mass ($F_{2,39} = 0.803, p = 0.46$).

_Feeding rates in unmanipulated pairs_ - Under normal feeding conditions, there was also no difference between males and females in ingestion rate (Figure 10; repeated-measures ANOVA, male vs. female $F_{1,12} = 0.84, p = 0.38$), but there was a significant effect of day on the rate of feeding (within subjects $F_{1,12} = 4.79, p = 0.049$).

![Figure 9: Brine shrimp (Artemia) ingestion rates (# shrimp min$^{-1}$) of courting H. zosterae females (n=16) and males (n=16) and gestating males (n=10). Ingestion rates were determined over 5 consecutive days, supersaturating the water with shrimp while counting the number of feeding strikes for each individual, then removing the shrimp after each counting replicate, leaving fish without food until the next sampling period (24 hours later).](image1)

![Figure 10: Rates of Artemia ingestion in unmanipulated pairs (n=7) of H. zosterae, compared over the first day of courtship (solid bar), the second day of courtship (hatched bar), and the day of copulation (open bar). Feeding rates were recorded at a food density of 8 shrimp/6 ml seawater for the first 3 hours of light on each sampling day.](image2)

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Indirect (Metabolic) Investment in Offspring

**Adult oxygen consumption rates** - Oxygen consumption rates differed dramatically between fish of varying reproductive status (Figure 11; Kruskal Wallis test, $H = 41.54$, $10$ df, $p << 0.0001$). Baseline respiration rates were almost identical for non-courting males and females, and oxygen consumption rates dropped for both sexes during courtship. This difference was not due to habituation to the experimental apparatus, because a comparison of the respiration rates of fish measured initially during isolation and initially after courtship still showed a decline in respiration rates across groups (Kruskal-Wallis test, $3$ df, $H = 9.4$, $p = 0.024$). Respiration rates determined initially while courting were lower for females (Dunn’s test for multiple comparisons; $Q_1 = 2.5$, $0.05 < p < 0.1$) and significantly lower for males ($Q_4 = 4.58$, $p < 0.001$) than rates measured initially while in isolation (Figure 12).
In females, oxygen consumption rate increased dramatically between courtship and the day of copulation (Figure 11; $Q_{11} = 10.85$, $p < 0.001$). In the three days following copulation, however, female respiration rate dropped back to courtship levels in intact pairs. Males showed a similar pattern, exhibiting a significant increase in oxygen consumption rates between courtship and the day of copulation ($Q_{11} = 12.32$, $p < 0.001$), and a decrease in respiration rate between the day of copulation and stage class 1 of gestation. However, this
decrease remained significantly higher than the male respiration rate observed during courtship \((Q_1 = 3.63, \text{ } 0.01 < \rho < 0.02)\). Respiration rates increased dramatically between stage classes 1 to 4, to almost 55% above courtship values in the Stage Class just before birth (see below for separate analysis of gestational respiration rates in males).

**Oxygen consumption rates of developing embryos** - There was a marked exponential increase in embryonic respiration rates across development (Figure 13). Early in gestation, from stages 1-11, oxygen consumption rate increased almost 13 times, and from stage 11 to stage 20 (just before birth) increased again over 7 times. There was a highly significant relationship between embryo stage and natural log transformed consumption rates \((r^2 = 0.978, F_{1,17} = 756.0, =<0.0001)\).

![Figure 13: Respiration rate (µl/hr) of individual *H. zosterae* embryos plotted against embryo stage. The amount of oxygen that embryos consumed was measured using a micro-respirometer (see text) maintained at 26°C. Mean ± 1 SE embryo respiration rates provided with the number of embryos measured at each stage above each point. The embryos measured at each stage came from between 1-3 families, with a total of 157 embryos measured from 36 males.](image)

To estimate the amount of oxygen that a single embryo consumed over the course of gestation, \(O_2\) consumption rate was integrated across embryo stages from 0 to 21 to obtain µl
O₂·stage hr⁻¹·embryo⁻¹, and converted to energy by multiplying by (14.49±0.6 hr stage⁻¹·20.08X10⁻³ Joules μl O₂⁻¹). This yielded an estimate of 1.16 (0.24) Joules required for each embryo to complete development.

**Male metabolic investment in offspring** - Respiration rate of gestating males increased across gestation, although not significantly (Figure 14; 1-way ANOVA, F₁,₂₇ = 2.48, p = 0.082). Included in the total male respiration rate during gestation was basal respiration rate and the increasing amount of oxygen consumed by developing embryos.

![Figure 14: Mean ± 1 SE mass-independent respiration rates (in μl O₂/hr/mg⁰.₅₄) of gestating male *H. zosterae* (total bars) with brood fractions (in white) and basal respiration fractions (lower hatched section) indicated. Male sample sizes above bars, and brood sample sizes within bars. The upper hatched section of bars represents the increase in male respiration rates due to gestation and not embryo respiration. The low baseline respiration rate in last stage class most likely due to the observation that males in that class tended to be larger.](image)

To determine the amount of the increase in a male’s respiration rate during gestation that was not due to the cumulative respiration rates of his offspring, his basal oxygen consumption rate (in μl O₂ hr⁻¹), in addition to the oxygen consumption rate of his brood (all embryos), were subtracted from his gestational respiration rate. The remaining oxygen
consumed each hour represents the respiration rate of gestating *H. zosterae* males associated with maintaining the developing brood. To determine the amount of respiration per embryo, consumption rate was divided by the total number of embryos in a male’s brood. Direct oxygen consumption rates were used for this calculation instead of mass-independent values, because when the amount of oxygen consumed by each male per embryo was regressed on male body mass, no relationship was found (Figure 15). A multiple regression analysis to determine the effect of embryo stage and male mass on the rate of oxygen consumed by males for each embryo indicated that there was a significant effect of embryo stage (Figure 16; \( t = 2.51, p = 0.02 \)), but that male mass explained very little of the variation in the model (\( t = 0.996, p = 0.33 \)). Larger males did brood more offspring, however, with a positive relationship between male wet mass and the total number of embryos in the pouch (Figure 17; \( r^2 = 0.343, F_{1,27} = 13.75, p = 0.001 \)).

---

**Figure 15:** The relationship between male *H. zosterae* wet mass (mg) and the amount of oxygen consumed per hour by each male per embryo in his pouch (\( \mu l \ O_2/hr/embryo \)). Regression analysis indicated that there was no relationship between these two variables. Regression line and 95% confidence intervals provided.

**Figure 16:** The effect of embryo stage and male mass (mg) on the rate of oxygen consumption by each male per embryo (\( \mu l \ O_2/hr/embryo \)).
To determine the amount of energy males are investing over the course of gestation, the rate of oxygen consumed by each male per embryo was integrated across embryo stages from 0-21 (Figure 18), converted to the total oxygen consumed over gestation and then to energy. Using this technique, males expend 6.00 (1.54) Joules per embryo over the course of gestation. Because females make a direct investment of 13.1 (0.68) Joules in each egg (according to CHN analysis), females invest twice as much per offspring than do males.

Figure 17: The effect of male mass (mg) on the number of embryos (across all stages) in his pouch. As mass increases, the number of offspring in the pouch also increases. Regression line and 95% confidence intervals provided.

Figure 18: The relationship between embryo stage and the amount of oxygen males consumed per embryo ($\mu l$ $O_2$/hr/embryo). Males increased their metabolic rate as embryos developed over gestation. Regression line and 95% confidence intervals provided.
DISCUSSION

Female *Hippocampus zosterae* invest between 8 and 13 Joules in each offspring, estimated as the material contribution to eggs. *H. zosterae* males, however, expend between 5 and 7 Joules per offspring, estimated as the metabolic contributions to each embryo during gestation. These data indicate that the ratio of female parental investment to male parental investment in dwarf seahorses is about 2:1, despite equal food ingestion rates. In addition, *H. zosterae* embryonic respiration rates indicate that each embryo requires only 1.2 J of energy to develop while between 3 and 6 J are lost per embryo across gestation. Because embryos did not require additional energy input from the male, male material investment in offspring appears unlikely.

**Direct Investment in Offspring**

There was little agreement between energy estimates for egg and juvenile *H. zosterae* that were obtained through measurement of total organic carbon compared to elemental CHN analysis, although both indicated that female direct investment per offspring exceeds the metabolic investment made by males. CHN analysis yielded higher estimates of energy content of *H. zosterae* eggs and juveniles that exceeded the TOCC estimates by 5 J and 2 J, respectively. There are a number of possible reasons for this discrepancy. First, the energy conversion used for TOCC analysis is based upon the average energy contained in a carbon bond, and does not take into account the biochemical composition of samples as in CHN analysis. If the average biochemical fractions obtained during CHN were used to calculate energy in the TOCC samples, the energy estimates for both eggs and juveniles increase by over 0.5 J. In addition, because eggs and juveniles were not homogenized before use in the
assay to avoid potential sample loss, there were instances where pieces of tissue remained incompletely combusted at the end of the assay. Care was taken to visually assess each sample tube, but this type of assessment is prone to errors and could have caused mean measures of organic carbon content to be artificially low. While this method has been used successfully in other systems (McEdward and Carson, 1987), it has never been directly compared to CHN analysis.

It is also possible that estimates based on CHN analysis are too high. The model used assumes that the amount of carbon, hydrogen and nitrogen in the inorganic fraction (ash) is zero, based on the results obtained by Gnaiger and Bitterlich (1984) for samples of muscles, liver and fat from Chinese silver carp (Hypopthalmichthys molitrix). These elements in ash were found in quantities less than 1% of total measured amounts for all but carbon in liver, in which 10% of total measured carbon was in ash. If egg and juvenile samples were more like liver in their carbon distribution, then that could account for almost half of the difference between the estimates of these two methods. This is possible, since eggs are composed primarily of vitellogenins (yolk proteins), which in many species are synthesized in the liver and transported to oocytes during oogenesis via the maternal bloodstream (Anderson, 1968; Ho, 1991). Because of possible complications in both of these techniques, the most conservative estimate of the direct energy sequestered in offspring may lie between the results obtained using these two methods.

Regardless of the technique used, energy content of developing embryos declined dramatically, between 3 and 6 Joules, over the course of gestation. This decrease suggests that embryos are dependent upon the energy females sequester in eggs to complete development, and are not receiving organic material from the male. Data from embryo
respiration rates support this observation, because embryos required only 1.2 Joules to complete development. Thus it appears that male investment in offspring is non-nutritive in nature, most likely associated with the costs of osmoregulation, gas exchange, transfer of inorganic nutrients, and waste removal. This finding is contrary to what has been observed in two pipefish species, *Nerophis ophidion* and *Syngnathus typhle* (Berglund et al., 1986b). In both of these species, embryo respiration rates exceeded the loss in energy across gestation, suggesting that males make material contributions to embryos to complete development.

**Indirect (Metabolic) Investment in Offspring**

Metabolic rates of adult *H. zosterae* seahorses varied considerably across their reproductive cycle. One unexpected difference was the significant decrease in respiration rate observed between fish housed in isolation from the opposite sex and fish that had pair-bonded, but not yet mated. This finding is important because it indicates that there may be a physiological change that accompanies pair-bonding. In most studies that have determined the metabolic rates of various fish species, little attention has been paid to gender, let alone reproductive status (Beamish, 1964; Beamish & Mookherjii, 1964; van der Lingen, 1995). This can cause two problems: 1) it can add considerable and unnecessary variability to measurements (Burggren, 1997), and 2) it can lead to an inaccurate determination of the mass exponent, b. In seahorses, because they are almost continually paired in the field (Vincent & Sadler, 1995) and are exceptionally sedentary, it was important to calculate the appropriate mass exponent that reflected these unique aspects of their biology. To do this, a mass exponent of 0.54 was calculated from courting fish that were of both genders and had body masses that were representative of fish from the subject population. This estimate of b is low compared to others in the literature, but this may be because of the prominent differences
between seahorses and other species in their behavior and physiology. Most previous studies used the standard mass exponent, 0.67 (Cech, 1990), which is an average of the mass exponents from a number of teleost species, but this number has varied dramatically in the literature, from 0.65 in many resting fish to 0.97 in sockeye salmon during active metabolism (Brett, 1965).

Another marked pattern in the metabolic rates of males and females was the increase in oxygen consumption in both sexes on the day of copulation. In females, this increase may be due to physiological changes occurring early on the day of copulation. During this time when females are thought to release a large amount of deoxycorticosterone, which in sticklebacks is responsible for egg hydration and the final stages of egg maturation (Wallace & Selman, 1979). The presence of corticoids in many species is correlated with an increase in metabolic rate (Mueller et al., 1994; Pankhurst & van der Kraak, 1997). Physiological changes are also occurring in males at this time. Boisseau (1967) described dramatic changes in the morphology of the male pouch epithelium on the day of copulation, including increased vascularization and secretory activity. Thus it appears that increased oxygen consumption observed in both males and females may be due to increasing physiological demands on the day of copulation that are associated with parental duties. However, because there is a dramatic increase in courtship activity in both sexes on the day of copulation (see Chap. 4), it is also possible that a portion of the increase in metabolic rate is associated with increased activity levels. The increase in respiration rate due to courtship is considered “non-parental” mating effort (Trivers, 1972), and is difficult to tease apart from the energy invested in offspring on the day of copulation. In any case, it appears that the magnitude of the increase in oxygen consumption rate of males and females is similar on the day of copulation,
indicating that omitting this from the determination of relative parental investment would not bias estimates towards one sex or the other.

The most important measurement of metabolic rate across reproductive cycles in this study is from gestating males, since it is the only method available to quantify male metabolic investment in offspring and there is no obvious material contribution made by males. Determining the oxygen consumption rates of gestating males and then removing their embryos and determining embryonic respiration rates allows for a precise determination of the increase in male metabolic rate associated with brooding embryos, independent of the respiration rates of the embryos themselves. This is a rather novel approach to the determination of parental investment, because other studies using similar techniques have compared the respiration rates of gestating adults to the loss in energy content of embryos over gestation, thus only indirectly inferring the metabolic rates of developing offspring (Hopkins et al., 1995).

The investment by males per embryo was considerable (6 Joules) and appeared to be fixed across a range of male masses, since as males got larger investment per offspring remained constant. Larger males did successfully brood more offspring, though, instead of increasing investment in each offspring. It is unclear, however, whether males brought a higher percentage of the eggs in their pouch to term or if females placed more eggs into the pouches of larger males. It is likely that the latter is the case, as in other hippocampids (Vincent, 1990) female fecundity increased with increasing body mass, but this was not observed in H. zosterae. In H. whitei (Vincent & Sadler, 1995) pairs have been observed to size-assortatively mate, thus supporting the possibility that relatively larger males receive more eggs from their relatively larger female partners. In this study, it may be an artifact of

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the experimental design, since males and females used in pairs were size-matched which in
previous studies has been shown to increase mating success.

Given the dramatic difference in the amount of reproductive energy males and females
expend per offspring, there may be important differences in the manner in which males and
females allocate other aspects of their resources, such as somatic growth or survival.

According to bioenergetics theory, where

\[ C = (M_B + M_A + SDA) + (F + U) + (G_s + G_r) \]

\begin{align*}
C & = \text{Consumption} \\
M_B & = \text{Standard metabolic rate} \\
M_A & = \text{Active metabolic rate} \\
SDA & = \text{Metabolic rate due to specific dynamic action} \\
(F + U) & = \text{Waste losses} \\
G_s & = \text{Somatic growth rate} \\
G_r & = \text{Reproductive growth rate},
\end{align*}

either females must consume more than males or there must be differences in the way they
partition other aspects of their metabolism (Adams & Breck, 1990). Since male and female
\textit{H. zosterae} have strikingly similar consumption rates and both resting and active metabolic
rates, and it is likely that metabolism associated with digestion (SDA) and waste processes is
comparable, there may be differences in the amount of energy they allocate to somatic growth.
In some species of seahorses there is evidence to suggest a sexual size dimorphism in
censuses of natural populations (Vincent, 1990), but no controlled studies of age-matched
growth in males and females have been attempted. Because females appear to partition more
of their energy into reproduction, however, the potential exists for long-term costs to females,
such as a decreased opportunity for growth or higher mortality rate (Shine, 1980).
Other Costs Associated with Parental Investment

Many studies quantifying relative parental investment in fish that have paternal care have focused on the non-metabolic costs associated with brooding eggs in a nest, such as reduced opportunity to forage and increased mortality due to predation (Lachance & FitzGerald, 1995; Lavery, 1995; Smith & Wootton, 1995a; Smith & Wootton, 1995b). In the pipefish, *Syngnathus typhle*, no sex differences were found in predation risk but gestating males had a lower food intake than females. In *Nerophis ophidion*, another pipefish species, the opposite pattern was observed (Svensson, 1988), but predation risk in this species was directly related to an increase in conspicuousness, because of bright orange eggs visible along the male’s body during brooding. If there were differences in the predation risk of male and female seahorses, biases should be observed in the ratio of males and females collected in the field. There is little evidence in the literature to suggest that this is occurring (*H. zosterae*, Strawn, 1958; *H. guttulatus*, Boisseau, 1967; *H. whitei*, Vincent & Sadler, 1995). Based on the feeding ecology and predator avoidance mechanisms of seahorses, it is unlikely that there would be differences between males and females. Seahorses are ambush predators (James & Heck, 1994), sitting and waiting for prey items to come into range, suggesting that foraging ability would not differ between gestating males and females. This was supported by the present work, since under food-limited conditions gestating males did not feed at a lower rate than females. In addition, seahorses do not swim to elude predators, but rely on elaborate mechanisms for camouflage, including color changes, growth of dermal appendages, and encrustation with microfauna and algae (Gill, 1905; Bellomy, 1969). Unless males have a decreased ability to implement these mechanisms during gestation, differential predation rates on gestating males is unlikely.
Parental Investment Patterns Across Syngnathid Genera

The pattern of relative parental investment observed in this study of *Hippocampus zosterae* seahorses has important implications when considered in the context of work done with other members of the Syngnathidae family. Two pipefish species, *Nerophis ophidion*, considered one of the least modified of the syngnathids with eggs glued onto the males' ventral surface, and *Syngnathus typhle*, one of the most modified pipefish with brood flaps that seal together along a ventral groove, have been studied in great detail. The work of Berglund et al.' (1986b) quantifying the relative parental investments of these two species suggests that males transfer nutritive substances across the marsupium for the growth and development of embryos. Females of both species, however, appear to invest more energy in each egg than do males in each embryo across gestation. One difficulty with this study is that it did not measure total paternal investment, assuming that male investment was limited to caloric transfer. Because Berglund et al. did not include the energy invested to maintain the appropriate osmolarity of the pouch environment (*S. typhle*) or provide gas exchange and waste removal services to embryos (both species), male investment per offspring may actually be higher than female investment.

Because of the more extreme morphological specialization for paternal care in seahorses, it has been assumed that male seahorses invest more per offspring than do male pipefish. This study suggests that this assumption may be false. There are two possible explanations for observation that seahorse males invest less than do pipefish males. First, the male seahorse pouch may serve primarily to maintain environmental homeostasis. In the seahorse pouch, increased vasculature for gas exchange and waste removal (Linton & Soloff, 1964; Boisseau, 1967) and more constant osmolarity (Linton & Soloff, 1964) may represent a
decrease in the environmental stressors placed on developing seahorses as compared to pipefish embryos. Without the need to adapt to changing salinity and oxygen conditions, seahorse embryos may expend less energy than do pipefish embryos during development. Support for this hypothesis comes from sticklebacks (*Gasterosteus aculeatus*, Gasterosteiformes, a related order to the syngnathids), in which embryos that have been subjected to varying salinity and hypoxia are smaller at hatching (Whoriskey & FitzGerald, 1994), indicating that these embryos may have a higher metabolic rate during gestation.

A second explanation for the observed differences between pipefish and seahorses in their parental investment patterns is that differences may exist in female investment relative to males. Pipefish eggs are substantially smaller (\( \overline{\text{egg diameter}} = 1.12(0.17) \) mm, for 26 species) than seahorse eggs (\( \overline{\text{egg diameter}} = 1.61(0.09) \) mm, for 11 species; Hardy, 1978; Vincent, 1990), and across species there is a strong positive relationship between egg size and energy content (McEdward & Carson, 1987). Large egg size may represent substantially more energy invested per offspring by seahorse females than pipefish females. Over evolutionary time, this increase in female egg size may have allowed males to decrease their material investment in offspring, with the high degree of pouch development serving the primary functions of osmoregulation and gas exchange in seahorses. It is difficult to determine, however, whether the differences in egg size between pipefish and seahorses occurred before the pouch modifications for increased homeostasis. It is entirely possible that the increase in predictability of the pouch environment for developing seahorse embryos as compared to pipefish embryos was directly related to the subsequent increase in egg size and the loss of male material investment in offspring in seahorses. The increase in egg size over evolutionary time could also be related to the observation that larger juveniles are less subject
to predation and have higher survivorship than do smaller juveniles (Shine, 1979; Ahnesjö, 1992a; Kozlowski, 1996). Because female energy investment is often the largest determinant of offspring size (Ahnesjö, 1992b; McEdward, 1996), selection may have favored a modification of egg size over a modification in male investment to produce more viable offspring with a higher probability of surviving to maturity. These theories are highly speculative, but with further research to determine the metabolic costs of development of pipefish and seahorse embryos, coupled with a more complete study of parental investment across syngnathids, we will have a better understanding of the physiological processes that may have driven the evolution of both male and female modifications for parental investment.

**Summary**

Seahorses have long been the topic of speculation on the relationship between relative parental investment in offspring and courtship roles (Williams, 1975; Trivers, 1985). Although both suggested that because of the extreme specialization for paternal care females should be the predominant competitor for mates, Williams (1975) stated that “the behavioral masculinity and femininity will closely parallel variation in parental burdens of male and female” and that the problem could only be solved by a “detailed study of reproduction in these fishes”. The present study indicates that despite the level of specialization for paternal care in dwarf seahorses (*Hippocampus zosterae*), females invest more energy per offspring than do males. Previous work by the author (Chapter 5) with *H. zosterae* and studies of *H. fuscus* by Vincent (1994a), indicate that male seahorses engage in more direct competition for mates than do females. In addition, there is limited evidence to suggest that female seahorses are more selective of their mates (Chapter 6). Thus the findings of the present study are
consistent with the predictions of sexual selection theory, which suggests that it is the relative parental investment of males and females in offspring that is the primary determinant of courtship roles.
CHAPTER III:

Potential reproductive rates of male and female dwarf seahorses
(Hippocampus zosterae, Syngnathidae): Do differences in reproductive rates match observed courtship roles?

Abstract. Dwarf seahorses (Hippocampus zosterae, Syngnathidae) are distinguished by an extreme degree of morphological specialization for paternal care and the formation of monogamous pair bonds. The purpose of this study was to determine the potential reproductive rates of male and female dwarf seahorses, measured as the maximum number of independent offspring produced per unit time when sexually-receptive mates are unlimited. Sexually-isolated, non-prepared males and females were placed with sexually-receptive partners that were behaviorally prepared to mate and observed from the day of introduction through copulation, to determine the length of time it takes for each sex to prepare to mate. Potential reproductive rate was estimated for males and for females by dividing the mean number of offspring per breeding cycle by the time it takes to prepare to mate plus the mean gestation length (breeding cycle duration), to obtain the potential number of offspring per hour of the breeding cycle. In addition, for a small number of trials the time from the first copulation to subsequent copulations was measured for females, given an excess of sexually receptive males. Sexually-isolated females took two days longer to mate, on average, than sexually-isolated males, when paired with sexually-receptive partners. As a result, males could potentially produce 17% more offspring than females over the course of a breeding season. Females were not observed to remate before the end of the gestation period of her mate, despite the presence of additional sexually-receptive males. These results are consistent with previous work indicating that seahorses display traditional courtship roles, indicating that
the sex with the higher potential reproductive rate competes more intensely for access to the opposite sex.

INTRODUCTION

Sexual selection refers to differential reproductive success based on intersexual choice and intrasexual competition (Darwin, 1871, Andersson, 1994). In most species, females are more selective of their mates and males compete for access to females (Gwynne, 1991), a pattern that has been hypothesized to depend on the relative parental investment males and females make per offspring (Williams, 1966; Trivers, 1972, 1985). Two related factors may also influence courtship behavior patterns, 1) the operational sex ratio, defined as the “ratio of fertilizable females to breeding males in a population” (OSR, Emlen & Oring, 1977) and 2) the potential reproductive rates of males and females, defined as the “maximum number of independent offspring that parents can produce per unit time” (Clutton-Brock, 1991; Clutton-Brock & Vincent, 1991). Relative parental investment should determine potential reproductive rates, assuming that resources are limiting and the sex with the lower per-offspring investment will have more resources to put into obtaining mates and increasing their reproductive success (Andersson, 1994). Potential reproductive rates in turn will influence the operational sex ratio, causing it to become biased towards the sex with the higher potential reproductive rate. Other factors can influence the OSR as well, such as life history characteristics, the distribution of resources, other aspects of the ecology of species and environmental variables such as temperature (Grant et al., 1995; Wootton, et al., 1995; Kvarnemo, 1996).
Because females always invest more in their gametes than males, and because in most species, any parental care is usually provided by females (Clutton-Brock, 1991), relative parental investment theory accurately predicts the generally observed patterns of competition and mate choice. Additional mechanisms to predict patterns of courtship behavior have been developed primarily to explain the exceptions to the rule; species in which both parents care for offspring or in which males provide most or all care of offspring. In the latter case, relative parental investment theory predicts that if males have a higher relative energy investment per offspring than females, they should be more selective of their mates and females should compete for access to males. Relative parental investment of males and females has been quantified comprehensively in only a few species with male parental care (but see Simmons, 1992 for estimation of parental investment in zaprochilne katydids). However, if it is assumed that male care of young can be equated with a higher energy investment per offspring by males as compared to females, then it would appear that relative parental investment does not accurately predict observed patterns of mate competition (Clutton-Brock & Vincent, 1991; Clutton-Brock and Parker, 1992). For example, a number of fish species exhibit male care of young, but males are also the more competitive sex (Gasterosteus aculeatus, three-spined stickleback, Kynard, 1978; Chrysiptera cyanea, damselfish, Gronell 1989).

The most extreme example of a system in which males are highly specialized for paternal care but are the predominant competitors for mates are seahorses (Hippocampus fuscus, Vincent, 1994a; H. zosterae, Masonjones, Chapter V; Herald, 1959; Vincent et al., 1992). Within the Syngnathidae family (pipefish and seahorses), there is considerable variation in anatomical and physiological specialization for paternal care. In some pipefish,
such as *Nerophis ophidion*, eggs are attached unprotected onto the ventral surface of the male (Berglund et al., 1989; Rosenqvist, 1990). In other pipefish (genus *Syngnathus*) and all seahorses (genus *Hippocampus*), females deposit their eggs into a pouch on the ventral surface of the male where fertilization takes place (Fiedler, 1954) and embryos are either partially protected (pipefish) or completely sealed (seahorses) from the external environment (Linton & Soloff, 1964). The epithelial lining of the male seahorse brood pouch is highly vascularized, and provides the developing embryos with services such as gas exchange, osmoregulation and waste removal during their 10-30 day gestation (Boisseau, 1967).

The structure of seahorse ovaries suggests that it may be physiologically possible for females to mate again soon after copulation, and more often than the mating interval observed for females in field populations (Vincent, 1990; Vincent & Sadler, 1995). In female *H. erectus* seahorses, mature follicles appear to be produced continuously from two germinal ridges in each ovary, with a range of follicles of sequentially increasing developmental stages arising from each germinal ridge outward (Selman et al., 1991). In other teleosts, follicles arise either singly or in groups throughout the ovary, in a less ordered fashion than has been observed in syngnathids (Wallace & Selman, 1981; Begovac & Wallace, 1987). In addition, syngnathids spawn only a fraction of their post-vitellogenic oocytes at a time, as compared to other teleosts which begin again with primary oocytes after each spawning (Wallace & Selman, 1981).

Because male seahorses are so specialized for paternal care but also compete more intensely for mates than do females, it has been suggested that the potential reproductive rates of males and females may be more useful in predicting patterns of mate competition (Clutton-Brock & Vincent, 1991; Vincent, 1994b). Because all species of seahorses studied both in the
laboratory (*H. fuscus*, Vincent, 1994a, 1994b, 1995; *H. zosterae*, Masonjones & Lewis, 1996) and the field (*H. whitei*, Vincent & Sadler, 1995) have displayed complete sexual monogamy over the course of a breeding season, the *realized* reproductive rate among paired seahorses is equal (Vincent, 1994b, Clutton-Brock & Parker, 1992). Pairs remate within a few hours of the male’s giving birth (HDM, pers. obs., Vincent & Sadler, 1995). Recent laboratory studies (Vincent, 1994b) of *H. fuscus* have suggested that among unpaired seahorses, male *potential* reproductive rate may exceed female potential reproductive rate. This is because males can prepare to mate faster than females, are sexually receptive longer than females, and because neither males nor females remate during the gestation period of the male. This distinction between the *realized* reproductive rate and the *potential* reproductive rate is important (Kvarnemo, 1994), as the latter can only be accurately determined when there is unlimited availability of sexually-receptive partners.

The purpose of the present study was to estimate the potential reproductive rates of male and female dwarf seahorses (*Hippocampus zosterae*), measured as the maximum number of independent offspring produced per unit time when sexually-receptive mates are unlimited. This was accomplished by behaviorally preparing males and females to mate and then pairing them with sexually-isolated, non-prepared partners of the opposite sex and observing pairs from the day of introduction through copulation, to determine the length of time it takes for each sex to prepare to mate. Potential reproductive rate was estimated for males and for females by dividing the mean number of offspring per breeding cycle by the time it takes to prepare to mate plus the mean gestation length (breeding cycle duration), to obtain the potential number of offspring per hour of the breeding cycle. In addition, females were
provided with an excess of sexually receptive males during the gestation period of her mate, to determine if females remate during this period of time.

**METHODS**

*Study Organism—Hippocampus zosterae* occur in shallow seagrass beds from the Gulf of Mexico east through the Bahamas, Bermuda and Cuba (Ginsberg 1937; Böhkle and Chaplin 1966). Adult sizes range from 16-38 mm (measured as the linear distance from the top of the coronet to the end of the tail; Strawn 1958). The monogamous mating system exhibited by *H. fuscus* and *H. whitei* (Vincent 1995) is also observed in laboratory studies of *H. zosterae*, with a male and female remaining together and mating repeatedly over the course of the breeding season (HDM, unpubl. data). In the lab, females transfer one entire clutch of eggs to a single male \( [\bar{x} (\pm 1 \text{ SE}) = 12.5 (\pm 1.32) \text{ eggs, } n=14] \). Five to 25 \( (\bar{x} =11.4(1.58); \ n=18) \) fully independent young are born after approximately 12 days of gestation within the male brood pouch at 26°C in the lab, and pairs remate within 4-20 hours of the male’s releasing young. Brood sizes in males that copulated in the field are larger than those of lab mated males \( (\bar{x} =17.3(1.31); \ n=24; \ \text{independent t-test, } t=2.89; 40 \text{ df, } p=0.0062) \).

*Hippocampus zosterae* were collected near Key Largo, FL from March through September, and maintained in small (5-8 fish) same-sexed groups (in sexual isolation) for 10 days to 8 weeks before trials in an attempt to standardize reproductive status. Fish were kept in 57 L aquaria prior to and during experiments. Tanks were maintained at 26°C on a 13 hours light:11 hours dark photoperiod with a salinity range of 26-33‰, and two plastic seagrass plants were supplied for attachment sites. Fish were fed each day immediately after
observation with recently hatched *Artemia* supplemented every other day with Selcon (American Marine), a food additive containing highly unsaturated fatty acids.

**Courtship Behavior Patterns**— Four discrete phases of courtship in paired dwarf seahorses have been observed (Masonjones and Lewis 1996). Phase 1 courtship occurred during the one to two days preceding the day of copulation, and was characterized by reciprocal *quivering*, during which fish assume an erect body posture, with pectoral fins extended, and rapidly vibrate their bodies from side to side. Courtship phases 2-4 occurred on the day of copulation. During phase 2 of courtship, females first displayed *pointing* (a behavior used as an indicator of a female’s readiness to mate and the beginning of the day of copulation). This behavior is defined as when a female raises her head upward toward the water surface to form an oblique angle with the main body axis and then lowers it again to a horizontal position, to which males generally responded by quivering. Phase 2 courtship is usually followed by a latency period of 23-220 min (median - 111 min), during which it is hypothesized that females undergo the last stages of egg maturation and egg ovulation. In phase 3 courtship, males began to display pointing in response to female pointing. Phase 4 courtship was characterized by the male and female repeatedly *rising*, in which fish release their respective holdfasts and rise up into the water column facing one another. In a *copulatory rise*, the female genital papilla is placed inside the male brood pouch opening, followed by egg transfer.

**Latency to Mate Trials**— This experiment included two treatments: 1) sexually-receptive *H. zosterae* females with sexually-isolated *H. zosterae* males (n=18), and 2) sexually-receptive *H. zosterae* males with sexually-isolated *H. zosterae* females (n=16). Sexually-receptive fish were prepared using two techniques, similar to the techniques used in
Vincent (1994b). Sexually-receptive females were prepared through the first method (half of all successful trials, n=4) by placing two females into a tank with one male. They were then observed continuously during the first three hours after tank lights came on (defined as dawn) on each day before copulation, and from dawn through the copulation of one of the females and the male on the final day. Previous observations have indicated that all seahorse courtship occurs in the morning, except on the day of copulation (Vincent 1994a; HDM pers. obs.). Under these conditions, often both females would court with the male, and on the day of copulation, both females would display the pointing behavior, indicating their readiness to mate. When this occurred, once the male and one of the females mated, the pair was removed, leaving the unmated but sexually-receptive female in the tank. Sexually-receptive males (n=5 trials) were prepared by starting with two males and one female, observing them through early courtship and the copulation between one male and the female. The unmated male was considered ready-to-mate if he displayed pumping, either alone or in response to the female’s points, and engaged in courtship with the female on the day that she exhibited the pointing posture.

The second technique used to prepare sexually receptive males and females (n=5 female trials, n=9 male trials) prepared them simultaneously. One male and one female were placed together and observed each morning through their first bout of courtship. On the day of courtship when the female displayed pointing and the male pumping, pairs were watched continuously through the second and third phases of courtship. At the first indication that pairs were about to rise together (indicating the beginning of phase 4 courtship), the male and female were separated by sliding an opaque barrier down through the center of the tank.
creating two completely separate compartments, one containing the male and one containing the female.

Once a fish was sexually receptive, an opposite-sex, sexually-isolated fish was added to the tank. Experimental pairs were then observed continuously for the next two hours, after which they were checked at 1 hr intervals for courtship behaviors and positive indications of mating in both the male and female until the lights went off for the night. Positive indications of mating included an increase in male pouch volume coupled with change in pouch color from neutral to light orange, and a decrease in female abdomen girth coupled with a change in color from light orange to neutral. Both indicated a transfer of orange-red eggs from the female to the pouch of the male (Vincent and Sadler, 1995). Every morning thereafter pairs were observed through their first courtship bout, until the appearance of pointing in females and pumping in males. On the day that these behaviors were noted, pairs were observed continuously until copulation.

Wet masses for all fish were measured by blotting fish dry and weighing them to the nearest 0.01 mg in seawater in a 2ml plastic vial. Experimental fish ranged from 80-212 mg wet mass, with no mass difference between males and females used in trials [(Mean (1 SE); females $\bar{x} = 134.0$ (4.1) mg, males $\bar{x} = 141.7$ (3.8) mg, t-test, $t = 1.36$, $p=0.18$]. Sexually isolated fish were size-matched to sexually receptive fish, with no more than a 30 mg mass difference.

Eighteen receptive female/isolated male trials and 16 receptive male/isolated female trials were observed daily from the day of introduction until copulation took place, and 15 and 9 trials, respectively, resulted in successful copulations. Duration in hours from pair
introduction to copulation was recorded, as well as the length of time fish were sexually isolated prior to introduction, and the field collection date of each fish used in trials.

**Female and male remating latency**— To determine whether females remate before the end of her previous mating partner's gestation period, females were housed with an additional sexually-receptive male (n=9). Two males and either 1 (n=6) or 2 (n=3) females all of similar size were kept in 38 L aquaria at 28°C and allowed to interact freely. One additional trial where there was 1 male and 2 females was included in the analysis because the male dropped the clutch and was ready to mate before the end of a normal gestation period. Tanks were observed each morning for courtship behaviors and the date and time of all copulations and births were recorded. For each trial, the identity of fish involved in copulations was recorded, and for each male the duration from copulation to birth was recorded and for each male and female the duration from copulation to subsequent copulations was recorded.

**Conversion of Mating Latency to Potential Reproductive Rate and Statistical Analysis**—
The time in hours from pair introduction to mating for both sexually-isolated males and females was added to the average gestation length in the lab at 26°C, 12.3 days (289.7 hours; n=7), to obtain the breeding cycle duration. To calculate the potential reproductive rate per breeding cycle, the mean number of offspring per brood ($\bar{x} = 17.3(1.31)$) was divided by the cycle duration for both males and females to get potential reproductive rate in number of offspring per hour of the breeding cycle. The brood size estimate used was that of field-mated males, because it is higher and more closely estimates the number of offspring produced in the field. The difference in potential reproductive rate between males and females over the
breeding season was estimated by determining how many broods males and females could have over their approximately 200 day (March to October) breeding season, if the number of sexually-receptive mates was unlimited.

Differences in the time from introduction to copulation between the receptive female/isolated male treatment and receptive male/isolated female treatment were compared using the nonparametric Mann-Whitney U-test, because treatment variances were unequal and values were not normally distributed. To determine the effect of gender and collection date on mating latency, an extension of the Kruskal-Wallis test modified for two-factor analysis was used with a correction factor, applied because of unequal but proportional replication within cells (Zar, 1996). The relationship between mating latency and duration of sexual isolation was determined through a regression analysis.

RESULTS

When paired with a fish (male or female) that was sexually-receptive, sexually-isolated males copulated in 7.4(1.1) hours (Mean (1 SE), n=15), which was significantly shorter than for sexually-isolated females (\( \bar{X} = 55.6(3.8) \) hrs, n=9; Mann-Whitney test, \( U = 135.0, p = 0.00006 \)). Thus it took sexually-isolated females an average of 2 days longer to prepare to mate with a sexually-receptive male than it took sexually-isolated males.

Because fish may be more motivated to mate earlier in the breeding season, mating latency was determined as a function of gender and collection date. Sexually-isolated males mated more quickly than sexually-isolated females when placed with a sexually-receptive partner (Kruskal-Wallis test, \( H = 15.75, 1 \) df, \( p < 0.001 \); Figure 1), but there was no effect of
collection date on mating latency (H=1.51, 1 df, 0.1<p<0.25) and no interaction effect between gender and collection date (H=0.270, 1 df, 0.5<p<0.75).

What may also be important is the duration of time fish were kept in the lab prior to experiments. For both males and females, the median time that fish were in the lab and sexually-isolated was 4 weeks, but as the length of time in the lab prior to use in trials increased, males but not females took significantly longer to mate (males: r²=0.569, F₁,₁₂=15.87, p=0.002; females: r²=0.113, F₁,₁₇=0.895, p=0.376).

![Figure 1. Mean (± SE) mating latency (time from sexually-isolated fish placement with a sexually-receptive fish to copulation) for H. zosterae males and females collected and assayed early in the season (February-April) as compared to those collected and assayed later in the season (May-July). Sample sizes are given within each bar. Significant differences were detected using a two-factor extension of the Kruskal-Wallis test, with a correction for unequal but proportional cell replicates; indicating that there was a significant difference due to gender (p<0.001), but no differences were detected due to collection date nor an interaction between gender and collection date.]

At 28°C, the duration from copulation to birth in males was 10.56±0.49 days (n=7). For both males and females, the time from copulation to subsequent copulation was 11.82±0.51 days (n=10), and the time from birth to the next copulation was 17.5±4.4 hours (range 7.5-30 hours, n=7). In the seven trials in which a male and female successfully copulated and the male gave birth, the female was never observed to copulate with any other males and none of the other males were observed to be pregnant during the gestation of her first mating partner. In two additional trials, the male and female either did not copulate successfully and the eggs were dropped outside of the males' pouch or they successfully
copulated and the male dropped the clutch within 5 days. In neither of these trials was the male nor the female observed to mate until the end of a normal gestation period (one mated 12 days and the other 14 days after the first attempted copulation). In the last trial, the male and female successfully copulated, but the male died 7 days later. That female was observed to copulate with a new male 12 days after her previous copulation.

To calculate the potential reproductive rate of males and females when sexually-receptive mates are unlimited, the mating latency was added to the mean gestation length to determine the cycle duration. Because of the longer mating latency in sexually-isolated females than sexually-isolated males, female cycle duration was 48 hours longer than the cycle duration of males (Table 1). As a result, because males and females produce the same number of offspring per cycle, male number of offspring per cycle hour was 1.2 times higher than that of females. Over the course of the breeding season, males could potentially have 40 more offspring than females, if presented with a receptive female at the end of each cycle.

<table>
<thead>
<tr>
<th>Table 1. Calculation of the potential reproductive rates of male and female Hippocampus zosterae. Means (1 SE) provided.</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mating latency (hours)</td>
<td>7.4 (1.1)</td>
<td>55.6 (3.8)</td>
</tr>
<tr>
<td>Gestation duration (hours)</td>
<td>289.7 (12.3)</td>
<td>289.7 (12.3)</td>
</tr>
<tr>
<td>Cycle duration (hours)</td>
<td>297.1 (12.3)</td>
<td>345.3 (12.9)</td>
</tr>
<tr>
<td>Cycle duration (days)</td>
<td>12.4 (0.51)</td>
<td>14.4 (0.56)</td>
</tr>
<tr>
<td>Number of offspring/cycle</td>
<td>17.3 (1.3)</td>
<td>17.3 (1.3)</td>
</tr>
<tr>
<td>Number of offspring/hour</td>
<td>0.0582 (0.005)</td>
<td>0.0502 (0.004)</td>
</tr>
<tr>
<td>Number of offspring/day</td>
<td>1.4 (0.12)</td>
<td>1.2 (0.10)</td>
</tr>
<tr>
<td>Season duration (days, hours)</td>
<td>200, 4800</td>
<td>200, 4800</td>
</tr>
<tr>
<td>Mean number of cycles per season</td>
<td>16.2</td>
<td>13.9</td>
</tr>
<tr>
<td>Mean number of offspring per season</td>
<td>279.5</td>
<td>240.5</td>
</tr>
</tbody>
</table>
DISCUSSION

In this study, female *Hippocampus zosterae* took longer to prepare to transfer a clutch of eggs to a male than did males preparing to accept a clutch. This effect was even more pronounced early in the breeding season, with males preparing to mate in about half the time it took them later in the breeding season whereas in females there was no effect of collection date on the time it took them to prepare to mate. This mating latency translates to a 17% higher potential reproductive rate in sexually-isolated males than in sexually-isolated females, when both were provided with sexually-receptive mates. In addition, females were never observed to remate with another male during the gestation period of her previous partner, even when the previous copulation was unsuccessful, he dropped the clutch, or when that previous mate died before the end of gestation.

In spite of the usefulness of the concept of potential reproductive rate, empirically measuring potential reproductive rates, defined as the maximum number of independent offspring produced by each sex per unit time, has proven difficult. Clutton-Brock and Vincent (1991) first suggested estimating potential reproductive rates of females and males, respectively, by comparing clutch and brood sizes. However, this estimate overestimates the maximum number of independent offspring produced by females because in many species more eggs are produced than are independent offspring born (Clutton-Brock, 1991; Charleston & Wiegand, 1995; including some species of seahorses, Vincent, 1994b). Differences in the number of eggs and juveniles can be due to a number of factors, including: 1) maternal effects, such as genetic defects in eggs and uneven distribution of resources across eggs (eggs vary considerably in dry weight, but it is unknown what the relationship is between egg size and the probability of completing development, Strathmann, 1995), 2) paternal effects such as
low fertilization rate (due to low sperm count or motility, Fiedler, 1954), differential male investment due to embryo positioning in pouch (as in mammals, Clark et al., 1992), selective lysing of eggs to provide for the male and/or other offspring (Jones & Baxter, 1991), or 3) sibling effects such as competition for space and other resources (Nonneman et al., 1992). As a result, the most conservative and appropriate measure of female reproductive rate should use the mean number of independent offspring born and not the mean number of eggs placed into a male's pouch. Kvarnemo (1994) proposed two clarifications of potential reproductive rate: 1) distinguishing between realized and potential reproductive rate, and 2) quantitatively measuring potential reproductive rate by providing males and females of a species with an unlimited supply of sexually-receptive mates while holding temperature, food and other resources needed for reproduction constant.

This study demonstrates that male *H. zosterae* seahorses have a higher potential reproductive rate than do females, with males exhibiting a shorter time to prepare to mate and females behaviorally limited to mating with their previous partner at an interval longer than that of a typical male's gestation length. Thus, these results tightly support Clutton-Brock and Vincent's (1991) argument that the sex with the higher potential reproductive rate should also compete more intensely for access to mates. Their model originally suggested seahorses as an example of a system that did not fit this pattern, because seahorse males compete more intensely for mates but produce fewer juveniles than seahorse females produce eggs (Vincent, 1990). However, more recent work by Vincent (1994b) indicates that although *H. fuscus* females produce more eggs than do males, because males prepare to mate faster and are sexually receptive longer than females, the potential reproductive rate of males actually exceeds that of females. As a result, all of the recent studies on seahorses support the original
hypothesis of Clutton-Brock and Vincent (1991) that the sex with the higher potential reproductive rate competes more intensely for mates than does the sex with the lower potential reproductive rate.

Two main questions arise from this study and previous studies of seahorse reproductive ecology (Vincent, 1990; Vincent, 1994a, b; Masonjones & Lewis, 1996). First, why does this differential in potential reproductive rates exist, and why don't females mate polyandrously? The unique structure of female ovaries with continuous production of mature follicles (Selman et al., 1991) suggests that it may be physiologically possible for females to mate much sooner after being placed with a sexually-receptive male and for females to remate before the end of the average male gestational cycle. Vincent (1994b) suggests that it is possible that females need three days to fully mature a clutch of eggs, but that it is more likely that females do not mate sooner because the loss of a clutch is so costly. In dwarf seahorses, females sequester a substantial amount of energy in each egg (Chapter 2) and egg clutches are between 8-29% ($\bar{x} = 15.9$ (2.2)%, $n = 9$, by dry weight) of a female's body weight. Thus the loss of even one clutch of eggs represents a significant investment in reproduction, and this may be one of the constraints that imposes the mating latency on females. The first interaction with a male may not be sufficient indication that he is unmated and available, but after courting with him for 2-3 days a female can evaluate his reproductive status and hydrate a clutch of eggs being assured that there will be a male to receive them.

The next question to be asked is, with the greater potential reproductive rate in males than females, why do seahorses exhibit complete sexual monogamy? Sexual monogamy is a behavior pattern that is rare across taxa (Wittenberger & Tilson, 1980; Mock & Fujioka, 1990; Barlow, 1992), and highly uncommon among marine fish. For both males and females,
monogamy appears to maximize long term reproductive success in a number of ways. Vincent and Sadler (1995) found that the field distribution of seahorses (*H. whitei*) is patchy and their abundance is relatively low. In addition, of 42 pairs observed over the course of a breeding season, 11 ended in the death or disappearance from the population of a mate before the end of the breeding season (26.2%). Of those that lost a mate, 64% remated, but it took up to 30 days to find a replacement. Thus, in wild populations, polygamous mating would likely not be as productive as long term monogamy, because of the difficulty of locating available mates, due to the low motility and low abundance of seahorses. These factors could result in high mate search costs (Bateson, 1983).

In addition to the expensive search costs, monogamy insures that females do not hydrate eggs that will be later dropped because of the lack of a mate. The 2 day mating latency imposed by females is only observed in newly mated pairs. In subsequent matings of the same pair, copulation can occur within about 4-6 hours of the birth of the previous brood. In both *H. fuscus* (Vincent, 1994b) and *H. zosterae* (HDM, unpub. data), reproductive success of mated pairs is increased by the ability to copulate almost immediately after birth, insuring that the male is almost continuously brooding embryos. Long-term reproductive success is also increased by an increase in brood size. Vincent (1994b) found that *H. fuscus* pairs that are together longer than 7 days before mating have significantly larger broods than pairs together less than seven days. She suggests that it is the interaction of the male and female that causes the increase in the number of eggs that a male can successfully brood. Lab data from *H. zosterae* indicate that the longer a pair is together, broods increase in size because the female places more eggs into the pouch of the male, not because the male increases in his ability to rear young (HDM, unpub.). In addition, pairs that are kept together
over successive reproductive cycles produce more eggs and ultimately more juveniles per brood. This increase in the number of eggs that a female prepares for her mate with increasing time together is consistent with the explanations that Vincent (1994b) provides for the existence of the 2 day mating latency imposed by females. With increasing time together, female assurance of male fidelity should increase, and as a result her number of eggs produced per clutch would increase to a physiologically determined maximum, most likely related to body size.

The relative parental investment that males and females invest per offspring has been suggested to be the primary determinant of the potential reproductive rates of males and females, and ultimately the dominant factor dictating operational sex ratios (Simmons, 1992; Anderson, 1994). Recent work suggests a strong link between potential reproductive rate, operational sex ratios, and courtship-roles (Ahnesjö, 1995; Balshine-Earn, 1996), but little direct information exists to determine how parental investment patterns may influence the expression of these other factors. An important research focus in future studies of the factors influencing courtship roles should be to determine relative parental investment and potential reproductive rates of males and females in a number of species to examine their relationship in natural systems. By carefully quantifying both, we can determine if there is a predictable relationship between them or if there are mediating factors that might cause them to vary independently.
CHAPTER IV:

Courtship behavior in the dwarf seahorse, *Hippocampus zosterae*


**Abstract:** The seahorse genus *Hippocampus* (Syngnathidae) exhibits extreme morphological specialization for paternal care, with males incubating eggs within a highly vascularized brood pouch. Dwarf seahorses, *H. zosterae*, form monogamous pairs that court early each morning until copulation takes place. Daily behavioral observations of seahorse pairs (n=15) were made from the day of introduction through the day of copulation. Four distinct phases of seahorse courtship are marked by prominent behavioral changes, as well as by differences in the intensity of courtship. The first courtship phase occurs for one or two mornings preceding the day of copulation, and is characterized by reciprocal quivering, consisting of rapid side-to-side body vibrations displayed alternately by males and females. The remaining courtship phases are restricted to the day of copulation, with the second courtship phase distinguished by females pointing, during which the head is raised upward. In the third courtship phase males begin to point in response to female pointing. During the final phase of courtship, seahorse pairs repeatedly rise together in the water column, eventually leading to females transferring their eggs directly into the male brood pouch during a brief mid-water copulation. Courtship activity level (representing the percentage of time spent in courtship) increased from relatively low levels during the first courtship phase to highly active courtship on the day of copulation. Males more actively initiated courtship on the days preceding copulation,
indicating that these seahorses are not courtship-role reversed, as has previously been assumed.

**INTRODUCTION**

Sexual selection theory predicts that the relative investment made by males and females in their offspring is a primary determinant of sexual selection intensity and patterns of courtship behavior within species (Trivers, 1972; Williams, 1975; Thornhill and Gwynne, 1986). Across the animal kingdom, female investment in offspring typically exceeds that of males (reviewed by Clutton-Brock, 1991), and traditional sex roles during courtship involve males playing a more active role in initiating courtship, males competing for access to females, and females being selective about mates (Trivers, 1972, 1985; Williams, 1975; Gwynne, 1991). In numerous groups, however, males provide considerable paternal care (Ridley, 1978), which may take the form of egg incubation or aeration, protection, or provisioning of juveniles. In species where male investment exceeds that of females, sexual selection theory predicts that these traditional courtship patterns should be reversed.

Fishes in the family Syngnathidae (pipefishes and seahorses) show wide variation in the degree of male anatomical and physiological specialization for paternal care (Herald, 1959; Vincent et al., 1992). In some pipefishes such as *Nerophis ophidion*, females attach their eggs onto the ventral surface of the male (Berglund et al., 1989; Rosenqvist, 1990). Seahorses (genus *Hippocampus*) possess the greatest specialization for paternal care, with females depositing their eggs directly into an abdominal brood pouch on the male where fertilization takes place (Fiedler, 1954). In *Hippocampus*, the male brood pouch epithelium is highly vascularized, and provides gas exchange and osmoregulation for developing embryos.
throughout gestation (Boisseau, 1967; Linton and Soloff, 1964).

Due to their high degree of morphological specialization for paternal care, seahorses are an important group for testing the prediction that relative parental investment determines sex roles during courtship. However, previous descriptions of seahorse mating behavior have been largely anecdotal (Gill, 1905; Fiedler, 1954; Breder and Rosen, 1966). Recent laboratory studies on *Hippocampus fuscus* (Vincent, 1994a, b, 1995) and field studies on *H. whitei* (Vincent and Sadler, 1995) have revealed both of these species to be monogamous, with a single male and female mating repeatedly and exclusively over the course of the reproductive season. Studies on *H. fuscus* have shown that males compete more intensely for access to mates than do females (Vincent, 1994a), indicating that extreme male care is not associated with courtship-role reversal, as has previously been assumed (Trivers 1985).

Here we present a quantitative analysis of courtship and copulatory behavior in the dwarf seahorse, *Hippocampus zosterae* Jordan and Gilbert, a species exhibiting an extreme degree of paternal care. This study is based on daily observations of paired seahorses maintained under standardized conditions in the laboratory from the day of introduction until copulation occurred. *Hippocampus zosterae* courtship is partitioned into four discrete phases, distinguished by marked behavioral changes as well as by differences in courtship intensity.

**METHODS**

*Study Organism.*--- *Hippocampus zosterae* occurs in shallow seagrass beds from the Gulf of Mexico east through the Bahamas and to Bermuda (Ginsburg, 1937; Böhlke and Chaplin, 1966), and adult size ranges from 16-38 mm (measured as the linear distance from the top of coronet to end of tail; Strawn, 1958). The monogamous mating system exhibited by *H. fuscus*
and *H. whitei* (Vincent, 1995) is also observed in laboratory studies of *H. zosterae*, with a male and female remaining together and mating repeatedly over the course of the breeding season (Masonjones, unpublished data). Females transfer one entire clutch of eggs to a single male (\[X \pm 1SE\] = 12.4 [\pm 2.2] eggs, n=9). Three to 16 fully independent young are born after approximately 10 d of gestation within the male brood pouch, and pairs remate within 4-20 h of the male's releasing young.

*Hippocampus zosterae* were collected near Key Largo, Florida and maintained in same-sex groups for 1-8 weeks before trials in an attempt to standardize reproductive states. Tanks were maintained at 25.5°C on a 13L:11D photoperiod, and two plastic seagrass plants were supplied for attachment sites. Fish were fed recently-hatched *Artemia* supplemented with calcium (Kalkwasser, Thiel Aquatech) daily.

*Courtship Behavior Trials.*— On the morning before each trial began, wet mass of adult male and female seahorses was measured by blotting fish dry and weighing them to the nearest 10 mg in seawater in a cuvette. Experimental fish ranged from 90-280 mg wet mass, and pairs were selected for similarity of body size, with a mean difference between paired individuals of 27 (± 4) mg. Pairs were placed singly in 57 l aquaria, and each seahorse was used in only one trial.

We began daily behavioral observations for each pair starting on the day of introduction into tanks, and continuing until copulation occurred. Observations were made continuously during the first 3 h after tank lights came on (defined as dawn) on each day before copulation, and from dawn until copulation occurred on the last day. Previous observations have indicated that all seahorse courtship occurs in the morning, except on the
day of copulation (Vincent, 1994a; personal observation). Our analyses were based on a total of approximately 320 h of observation.

Beginning at dawn each day, the location of both fish and their proximity was recorded. Proximity was classified as either far apart (distance > 4 cm), close (4 cm), or touching (in direct physical contact). When transitions between these states occurred, the individual approaching or departing was identified. Behavior was recorded continuously using the following definitions of behavior patterns (modified from Fiedler, 1954, Vincent 1990, 1994a): brightening refers to a rapid change in coloration from normal body color (varying from black to white) to lighter body color over most of the body, excluding portions of the head as well as the dorsal midline that remain dark. Brightening characterizes social interaction in seahorses, and is not restricted to courtship (Masonjones, personal observation). In quivering fish assume an erect posture with pectoral fins expanded, and rapidly vibrate their body from side-to-side at a rate of approximately 12 cycles per sec. In pointing a fish raises its head upward toward the water surface to form an oblique angle with main body axis, then lowers it again to a horizontal position. Pumping is shown exclusively by males in H. zosterae, and consists of males opening their brood pouch and repeatedly flexing the tail in a motion similar to that displayed during the release of young. A single pumping motion lasts from 1-32 sec, with a mode of 8 sec. During rising, males and females release hold of their respective holdfasts and rise up into the water column facing one another. In a copulatory rise, the female genital papilla is placed inside the male brood pouch opening, followed by egg transfer. Additional behaviors not involved in courtship, including feeding and swimming, were also recorded during each observation period.

We defined courtship interactions as occurring when a male and female were within 4
cm of one another and both fish exhibited brightened coloration. During courtship interactions, both sexes exhibited a characteristic posture consisting of an erect body with head inclined downward. During courtship fish were also generally positioned side-by-side facing in the same direction, except during phase 4 when fish faced one another in preparation for rising. Courtship took place in discrete bouts, which were defined as beginning with the first display of any courtship behavior (quivering, pointing, or rising) by either fish, and ending when one or both individuals stopped courting and moved out of the side-by-side position. Pumping by males that occurred following phases 1 and 2 was not included as courtship interaction because males and females were not in the side-by-side position, and females were no longer bright. Courtship activity levels (representing the percentage of time spent in courtship) were calculated separately for each courtship phase by dividing the total time that pairs spent courting (summed across all bouts) by either the total observation time each day (for phase 1) or the total time spent in that phase (2-4).

Nineteen seahorse pairs were observed each day from the day of introduction until copulation took place, and 15 of these trials resulted in successful copulations. Courtship duration and number of bouts, as well as initiators of each approach, brightening, and courtship behavior within each bout were recorded for all 15 pairs. The frequency and duration of specific courtship behaviors were recorded for seven of these pairs. Pairs that failed to mate within 7 days were analyzed separately (n=4 pairs).

**Statistical analysis**—To examine which sex was more likely to initiate courtship interactions, we conducted exact binomial tests against a null hypothesis of equal probability of either sex being the initiator. Changes in courtship duration and activity level across phases were examined using nonparametric procedures, as within-phase variances were
unequal. To examine changes in courtship duration and time from dawn to the beginning of courtship within pairs between the first and second courtship days, we used Wilcoxon paired-sample tests. Changes in courtship activity level over time within pairs were examined using the Kruskal-Wallis test procedure.

**RESULTS**

*Hippocampus zosterae* exhibited four distinct phases of courtship marked by prominent behavioral changes, as well as by differences in the intensity of courtship. An initial courtship phase, designated as phase 1, took place early each morning for the one or two days preceding copulation. The primary courtship behavior characterizing this courtship phase was reciprocal quivering, rapid side-to-side body vibrations exhibited alternately by males and females. The remaining phases all occurred sequentially on the day of copulation, each distinguished by the appearance of novel courtship behaviors. Phase 2 on the day of copulation was marked by female pointing, in which females raised their heads to form an oblique angle with the main body axis. In phase 3 males began to point in response to pointing by females. During the final phase of courtship, seahorse pairs repeatedly rose upward together in the water column, ending in mid-water copulation and the transfer of eggs from females directly into the male brood pouch. These four courtship phases are described below.

**Phase 1: Initial Courtship (Reciprocal Quivering).---** Initial courtship took place in all seahorse pairs about 30 min after dawn on each courtship day until the day of copulation. During the night, fish remained immobile on separate holdfasts, but after dawn males and females assumed adjacent side-by-side positions, brightened, and engaged in 2-38 min of
courtship. Courtship in this phase was characterized by repeated occurrences of reciprocal quivering, in which one fish quivered and the other responded by quivering within 5 sec. During reciprocal quivering tails of the two seahorses were positioned within 1 cm on the same holdfast, and their bodies were angled outward slightly from the point of attachment. As a male quivered he rotated his body toward the female, who generally rotated her body away before responding. Females periodically shifted their tail attachment site, which led to some pairs circling around their common holdfast.

On the first courtship day, males approached females to initiate courtship significantly more often than expected by chance (Table 1: exact binomial, p=0.011). During this initial approach, males also brightened before females (p=0.029). After a male approached and brightened, females responded by brightening. Once both fish had brightened, males generally showed the first courtship behavior by quivering, although the prevalence over females quivering first was not significant (p=0.212).

During the first bout on the first day of courtship, females always responded to males' quivering by quivering. This reciprocal quivering behavior was repeated 36 (± 8.2) times during the first courtship bout. The first bout was terminated when either fish, often the female, moved away (p=0.133). Following the first courtship bout, males in 4 out of 15 pairs (27%) displayed pumping behavior. In 8 of 15 pairs, 2-3 additional courtship bouts ensued 1-23 min later in the morning on the first day. Identical behaviors occurred during all subsequent bouts, except for one pair in which the female did not respond to the male's approach, brightening and quivering.

Courtship occurred in the morning for either 1 day (7 pairs) or 2 days (8 pairs) preceding the day of copulation. Courtship duration was significantly longer on the first day
(15.2 ± 4.2 min) than on subsequent days of courtship (5.3 ± 0.8 min; Wilcoxon T=3, n=8 pairs, p=0.05). Males continued to initiate courtship interactions on each day before copulation, with males approaching females, brightening before females, and generally exhibiting quivering behavior first.

Phase 2: Day of Copulation (Pointing-Pumping).— On the day of copulation, *H. zosterae* pairs exhibited a series of dramatic changes in behavior during the prolonged and active courtship that began shortly after dawn and lasted until copulation had occurred, an average of 3.9 (± 0.4) h after dawn. This second courtship phase was distinguished by females exhibiting the pointing posture. As a female pointed, she leaned her body toward the male, who simultaneously leaned away. Males generally responded to female pointing by leaning back toward the female and quivering. Females leaned away as the male quivered, but in contrast to reciprocal quivering, females maintained their position on the common holdfast. Pumping behavior was displayed by males in all seahorse pairs following the first courtship bout in this phase.

During phase 2 of courtship, males continued to initiate approach significantly more often than did females (Table 1: p=0.006) and males brightened before females did (p=0.011). However, in contrast to previous courtship interactions, females rather than males almost always (p=0.018) displayed the first courtship behavior, pointing, which occurred an average of 28 times during this courtship phase. In response to female pointing, males most often quivered (55 ± 6.1% of female points responded to by male quivering), although males did not respond to 45% (± 6%) of female points. Pointing remained the predominant female behavior throughout this phase of courtship, but females also quivered in 6 of the 15 pairs. Courtship bouts were equally likely to be terminated by either sex (p=0.387).
Courtship began earlier on the day of copulation than on previous days: time from dawn to first courtship behavior on the day of copulation was 15.7 (±3.3) min, compared to 31.9 (± 6.8) min on the first day of courtship (Wilcoxon T=20, n=15 pairs, p<0.05). The second phase of courtship lasted 54.3 (± 12.6) min, with a total courtship duration of 10.9 (±1.9) min during 1 to 6 bouts. Phase 2 was followed by a latency period lasting 23-220 min (median=111 min), during which no courtship behaviors were observed and females were not bright. During this latency period males in every seahorse pair displayed pumping behavior, with frequencies varying from 8-30 pumps/h.

Phase 3: Day of Copulation (Pointing-Pointing).— The third phase of courtship occurred on the day of copulation, and was distinguished by males assuming the pointing posture in response to female pointing.

During this phase, males and females initiated courtship by approaching equally often (Table 1), and they tended to brighten simultaneously (79% of pairs). Males and females also exhibited the first courtship behavior, which was pointing in both sexes, equally often (p=0.395). During the first courtship bout, males generally responded to female points by pointing (38 ± 8.8 % of female points responded to by male point), by quivering (34 ± 11.2%), or rarely, by pumping (8 ± 3.9%). Males did not respond at all to 20 (± 6)% of the female's points. During the final courtship bout of phase 3, males showed an increased tendency to respond to female points by pointing (57 ± 21.6%), rather than by quivering or pumping. Phase 3 typically ended with the male departing (p=0.073). This courtship phase had a total duration of 48.0 (± 13.8) min with courtship lasting 9.1 (± 1.8) min during 1 to 6 bouts. A single seahorse pair did not show phase 3, but rather proceeded directly from phase 2 to phase 4.
**Phase 4: Day of Copulation (Rising and Copulation).**— The final courtship phase on the day of copulation included a series of 5-8 courtship bouts. Each bout began with the male and female on the same plant about 3 cm apart, usually facing one another. Bouts during this phase were distinguished by rising, in which both fish rose upward together 2-13 cm in the water column.

At the start of this phase, pairs were usually already close, and both fish retained their brightened color from the previous phase. Males and females were equally likely to display the first courtship behavior (Table 1: p=0.500), which as in phase 3 was predominantly pointing. Males responded to a female's point by either pointing (79 ± 10.6%), pumping (12 ± 3.5%), or more rarely, quivering (1 ± 1.4 %). The overall rate of male response to female points increased in this courtship phase to 92%. Interspersed with these courtship behaviors, pairs engaged in 5-44 rises/h during this phase.

**Table 1:** *Hippocampus zosterae* courtship phases with the number of pairs in which approach, brightening, courtship, and courtship termination were initiated by each sex and the most frequent courtship behaviors displayed by each sex. Seahorse pairs (n = 15) were observed from the day of introduction through the day of copulation (pairs in which both fish exhibited the behavior simultaneously are excluded).

<table>
<thead>
<tr>
<th>Courtship Phase</th>
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<td>Female</td>
<td>Quiver</td>
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<td>Point/Rise</td>
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101
During this phase females became increasingly active in courtship. The first rise, consisting of a male and female moving upward in the water column together, was initiated by females significantly more often than by males (p=0.018). During the final copulatory rise, the female inserted her ovipositor and transferred eggs through the opening into the male's brood pouch. The copulatory rise was also initiated by the female (p=0.018), and copulations lasted 13.3 (± 0.8) sec. This phase ended after copulation, and the male and female generally swam down to separate plants to feed. For a few minutes after copulation, males remained bright and swayed back and forth. The total duration of this phase was 41.5 (± 3.9) min, which included 20.4 (± 3.1) min of courtship.

Courtship activity (time pairs spent courting as a percentage of the total time spent in that phase) changed significantly across courtship phases (Fig. 1: Kruskal-Wallis H=36.5, 4 df, p<0.0001). Courtship increased from relatively low levels on the first and second days of courtship to increasingly active courtship during the three consecutive courtship phases on the day of copulation.
Courtship Interactions in Pairs that Failed to Mate.--- Four of 19 seahorse pairs failed to court or mate within 7 days. In successfully mating pairs, males generally initiated courtship by approaching the female and brightening first. After females brightened, the male quivered and females then responded by quivering within 3-5 sec. In two pairs that failed to mate, the initial approach and brightening was made by the male, but both females failed to brighten in response. In both pairs males then proceeded to quiver, but again neither female responded. In the remaining two pairs that failed to mate, females made the initial approach and brightened first, and the male responded by brightening and then quivering. However, neither female responded further by quivering after this initial contact, even though the males remained bright and continued to quiver. These unsuccessful attempts at courtship were usually repeated in the morning on the first 2-3 days that the pairs were together, but the fish were rarely close for the remaining 4-5 days.
DISCUSSION

This study provides the first quantitative description of courtship behavior in the dwarf seahorse, *Hippocampus zosterae*, and reveals several major behavioral changes occurring among four distinct phases of seahorse courtship. Reciprocal quivering characterizes the first phase of courtship that occurs in early morning for one or two days preceding copulation. On the day of copulation there are three distinct phases of courtship. Phase 2 is marked by the novel appearance of female pointing and male pumping. During phase 3 on the day of copulation, males exhibit an increasing tendency to point, rather than to quiver, in response to female points. In the final phase of courtship, seahorse pairs repeatedly rise together in the water column, ending in mid-water copulation during which eggs are deposited by females directly into the male brood pouch.

Initial courtship behavior during phase 1 in *H. zosterae* is similar to the elaborate sunrise greeting rituals that have been described for field populations of the pipefish *Corythoichthys intestinalis* (Gronell, 1984) and *Hippocampus whitei* (Vincent, 1990), both of which exhibit monogamous pair-bonding throughout a reproductive season. It has been suggested that daily repetition of this elaborate courtship display serves to facilitate reproductive synchronization of males and females (Gronell, 1984; Vincent 1995). Laboratory experiments on *H. fuscus* indicate that this daily greeting plays a role in the establishment and maintenance of pair bonds (Vincent, 1995), since females were found to mate preferentially with their greeting partner rather than with their previous mate.

There are distinct changes across the different phases of seahorse courtship in whether males or females are more likely to initiate courtship interactions. Males initiate courtship interactions on each day before copulation, with males approaching females, males exhibiting
brightened coloration before females, and males generally displaying the first courtship behavior. During phase 2 on the day of copulation, males continue to approach and brighten first, but females exhibit the first courtship behavior by pointing. Later on the day of copulation, females become increasingly active in the initiation and continuation of courtship bouts, initiating courtship approaches as often as males, and females consistently initiate the rises that eventually lead to copulation. The four *H. zosterae* pairs that never courted or mated also support a more active courtship role for males, since courtship apparently fails to progress when females do not respond to repeated brightening and quivering by males.

The latency period following phase 2 on the day of copulation appears to coincide with the final stage of oocyte maturation during which eggs are hydrated prior to ovulation (Wallace and Selman, 1981; Vincent, 1990; Selman et al. 1991). Toward the end of this latency period, distinctive orange coloration of the eggs becomes visible through the abdominal wall of the female. Ovulation seems to represent an irreversible commitment by the female to transferring eggs, since if pairs are separated after this point the female will eventually release the unfertilized clutch into the water (Masonjones, personal observation; Vincent, 1990). The increasingly active role played by females in initiating pointing and rising in the final two courtship phases following ovulation may reflect the exigency for a female to find a male to fertilize and incubate her eggs. In addition to the large material investment in eggs, loss of a clutch is also costly because these females generally do not mate again until after the normal gestation period would have elapsed (Masonjones, personal observation; Vincent, 1990, 1994b).

In spite of their extreme male care, seahorses do not appear to show a reversal of traditional courtship roles as has been predicted by sexual selection theory. The present study
indicates an active role by *H. zosterae* males in initiating courtship interactions up until the day of copulation. This finding is consistent with previous studies on *H. fuscus* (Vincent, 1994a), in which males were found to compete more intensely than females for access to mates. These results contradict some influential summaries (Williams, 1975; Trivers, 1985) that have suggested that seahorses are courtship-role reversed. In the seahorse species studied to date, the presence of traditional courtship roles in terms of males being more active in courtship and males competing for access to females has demonstrated that courtship roles are not necessarily reversed in species with a high degree of paternal care.

**ACKNOWLEDGMENTS**

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CHAPTER V:

Mate competition in the dwarf seahorse, *Hippocampus zosterae* (Syngnathidae)

Abstract. Dwarf seahorses (*Hippocampus zosterae*, Syngnathidae) are distinguished by an extreme degree of morphological specialization for paternal care and the formation of monogamous pair bonds. The purpose of this study was to determine the degree of male vs. female competition in this species. Daily behavioral observations of male-biased (2 males, 1 female) and female-biased (1 male, 2 females) treatments were made beginning when seahorses were introduced to tanks continuing through the day of copulation. In male-biased treatments, males displayed a competitive behavior known as snapping, during which the initiating male oriented his snout towards the operculum of the receiving male, rapidly elevating and extending the head in the direction of the receiver. This behavior was never observed between females. Males also displayed a significantly higher frequency of two additional competitive behaviors, holding and intruding, than did females. The frequency of competitive behaviors in both male-biased and female-biased treatments increased linearly with increasing duration of isolation from the opposite sex. Absolute body mass in males was not related to mating success, but larger females mated more often than smaller ones, and there was some evidence to suggest that pairs were size-assortatively mating. Behavioral correlates with mating success included early trial interactions between the mating and focal fish and a longer total duration of courtship between the mating pair than the nonmating pair.
INTRODUCTION

Sexual selection, originally defined by Darwin (1871), consists of two processes which can cause differential mating success: 1) intersexual choice, and 2) intrasexual competition. Typical courtship-roles in most species include females being more selective of their mates and males competing for access to females (reviewed by Gwynne, 1991; Anderson, 1994). This pattern of courtship behavior has been hypothesized to depend on the relatively greater parental investment made by females to offspring (Williams, 1966; Trivers, 1972, 1985). In a number of groups, however, males provide considerable parental care (reviewed by Ridley, 1978; Clutton-Brock, 1991), during which males may incubate, aerate, protect, and/or provision developing embryos and juveniles. Parental investment theory predicts that in species where male parental investment exceeds that of females, males should be more selective of their mates and females should compete more intensely for access to males (Trivers, 1972).

One extreme example of male specialization for paternal care comes from seahorses (Herald, 1959; Vincent et al., 1992). Within the Syngnathidae (pipefish and seahorses), there is considerable variation in the degree of anatomical and physiological specialization for paternal care. In some pipefish, such as *Nerophis ophidion*, females attach their eggs to the ventral surface of the male (Berglund et al., 1989; Rosenqvist, 1990). In seahorses (genus *Hippocampus*), females deposit their eggs into a pouch on the ventral surface of the male where fertilization takes place (Fiedler, 1954) and embryos are sealed from the external environment (Linton & Soloff, 1964). The epithelial lining of the male seahorse brood pouch
is highly vascularized, and provides the developing embryos with services such as gas exchange, osmoregulation and waste removal during their 10-30 d gestation (Boisseau, 1967).

Until recently, descriptions of seahorse courtship behavior was largely anecdotal (Gill, 1905; Fiedler, 1954; Breder & Rosen, 1966). In the last few years, however, laboratory studies on *Hippocampus fuscus* (Vincent, 1994a, 1994b, 1995) and field studies of *H. whitei* (Vincent & Sadler, 1995) have indicated that both species display complete sexual monogamy, with a single male and female mating repeatedly and exclusively over the course of a reproductive season. *H. fuscus* males have also been observed to compete more intensely for mates that females (Vincent, 1994a), contrary to the predictions of some influential summaries of seahorse behavior (Trivers, 1985).

The purpose of the present study is to determine whether competition over mates is more intense in male or female *Hippocampus zosterae* Jordan and Gilbert. Previous work describing courtship behaviors of pairs of *H. zosterae* has indicated that males more actively initiate courtship interactions before copulation (Masonjones & Lewis, 1996), but competitive behaviors for *H. zosterae* have not previously been described. To induce mating competition, biased sex-ratio conditions were established in the laboratory (either 2 males:1 female or 1M:2F) and courtship behavior observed daily.

**METHODS**

**Study Organism**

*Hippocampus zosterae* occurs in shallow seagrass beds from the Gulf of Mexico east through the Bahamas, Cuba and Bermuda (Ginsberg, 1937; Böhlke and Chaplin, 1966). Adult size ranges from 16-38 mm (measured as the linear distance from the top of the coronet to the end of the tail; Strawn, 1958). The monogamous mating system exhibited by *H. fuscus*
and *H. whitei* (Vincent, 1995) occurs in *H. zosterae* in the laboratory, with a male and female remaining together and mating repeatedly over the course of the breeding season (HDM, unpubl. data). In the lab, females transfer one entire clutch of eggs to a single male [\( \bar{x} (\pm 1 \text{ SE}) = 12.5 (1.32) \text{ eggs, } n=14 \)]. Five to 25 (\( \bar{x} = 11.4 (1.58); \ n=18 \)) fully independent young are born after approximately 12 days of gestation within the male brood pouch at 26°C in the lab, and pairs remate within 4-20 hours of the male’s releasing young.

*Hippocampus zosterae* were collected periodically from early March through September near Key Largo, FL. Fish were maintained in small (5-8 fish) same-sexed groups (in sexual isolation) for 10 days to 8 weeks before use in all experiments in an attempt to standardize reproductive status. Fish were kept in 38 L aquaria prior to and during experiments. Tanks were maintained at an average temperature of 26°C on a 13 hours light/11 hours dark photoperiod with a salinity range of 26-33‰, and two artificial seagrass plants were supplied for attachment sites. Fish were fed recently hatched *Artemia* each day immediately after observation, and supplemented every other day with Selcon (American Marine), a food additive containing highly unsaturated fatty acids.

**Competition Trials**

Daily observations of male-biased (2M:1F) and female-biased (1M:2F) experimental treatments were conducted to determine whether mating competition is more intense in male or female *H. zosterae*. On the day before each trial began, wet masses for the three fish were measured by first blotting fish dry and weighing them to the nearest 0.01 mg in seawater in a 2ml plastic vial. Experimental fish ranged from 80-212 mg wet mass, with no mass difference between males and females used in trials [(Mean (1 SE); females \( \bar{x} = 134.0 (4.1) \),
n=44, males $\bar{x} = 141.7 (3.8)$, n=44, $t$-test, $t = 1.36$, $p=0.18]$. Same-sexed fish selected for each trial differed in mass by at least 5 mg (range of mass difference 5-80 mg), with a mean difference of 28.7(4.30) mg, while the focal fish was selected to be near the midpoint of this range. Each fish was used in only one trial.

Fish were placed singly into 38 L aquaria, with the two same-sexed fish placed first in random order with respect to body mass and then the opposite-sexed fish. I began behavioral observations for each trial starting immediately after all fish were placed into tanks and continuing until copulation occurred. Observations were made continuously during the first three hours after tank lights came on (defined as dawn) on each day before copulation, and from dawn through copulation on the final day. Previous observations have indicated that all seahorse courtship occurs in the morning, except on the day of copulation (Vincent 1994a; HDM pers. obs.). Approximately 540 hours of observation were conducted, with statistical analyses based on a total of 330 hours. Behavioral observations were recorded on an IBM Thinkpad using a behavior recorder program written in Visual Basic specifically for recording seahorse behavior patterns (I designed the program and M.C. Masonjones wrote the computer code; see Appendix II for description of computer program).

The location of all three fish and their relative proximity were recorded beginning at dawn each day. Proximity was classified as either far apart (distance $> 4$ cm) or close ($\leq 4$ cm). When transitions between these states occurred, the individual approaching or departing was identified. Courtship and competitive behaviors were recorded continuously for all fish.
Seahorse Behavior Patterns

Courtship behaviors—Four discrete phases of courtship in paired dwarf seahorses have been observed (Masonjones and Lewis 1996), including a number of unique courtship behaviors (Fiedler 1954; Vincent 1990, 1994a). All courtship in seahorses is accompanied by a rapid and sustained change in coloration (brightening) from normal body color (varying from black to white) to a lighter body color over most of the body, excluding portions of the head and dorsal midline which remain dark. Phase 1 courtship occurs during the one to two days preceding the day of copulation, and is characterized by reciprocal quivering, during which fish assume an erect body posture, with pectoral fins extended, and rapidly vibrate their bodies from side to side. Pumping is a behavior shown exclusively by male seahorses, and consists of a male opening his brood pouch and repeatedly flexing his tail in a motion similar to that displayed during the expulsion of young. Pumping is observed infrequently on the days before copulation, but is used at an indicator of a male’s readiness to mate on the day of copulation.

Courtship phases 2-4 occur on the day of copulation. During phase 2 courtship, females first display pointing, (a behavior used as an indicator of a female’s readiness to mate and the beginning of the day of copulation), defined as when a female raises its head upward toward the water surface to form an oblique angle with the main body axis and then lowers it again to a horizontal position. Males generally responded to female pointing by quivering. Phase 2 courtship is usually followed by a latency period of 23-220 min (median - 111 min), during which it is hypothesized that females undergo the last stages of egg maturation and ovulation (Vincent, 1990). In phase 3 courtship, males begin to display pointing in response to female pointing. Phase 4 courtship is characterized by the male and female rising
repeatedly. During this phase, fish release their respective holdfasts and rise up into the water column facing one another. In a *copulatory rise*, the female genital papilla is placed inside the male brood pouch opening, followed by egg transfer. Although the above listed courtship behaviors are described between males and females, the behaviors were also recorded if they occurred between same-sexed pairs or if all three fish were engaged in these behaviors simultaneously.

*Competitive behaviors*—Three competitive behaviors were also continuously recorded during observations (modified from Fiedler 1954; Vincent 1990, 1994a). *Snapping* was an instantaneous behavior during which the initiating fish oriented its snout towards the operculum of the receiving fish, rapidly elevating and extending the head in the direction of the receiver. This behavior has been observed to be associated with a snapping noise (audible with a hydrophone; Colson et al., in press). The receiving fish often responded to this behavior by darkening its coloration and flattening its body parallel to the bottom of the tank.

During *holding*, the initiating seahorse used its tail to hold onto a portion of the body of the receiving seahorse, including the head. *Intruding* occurred when the initiating seahorse placed its body between the bodies of a male and female courting pair. Both snapping and holding were scored as behaviors whether observed between same-sexed or opposite-sexed pairs, but intruding was specifically defined as being initiated between a courting male-female pair. Additional behaviors not involved in courtship or competition, including feeding and swimming, were also recorded during each observation period.

Courtship interactions occurred when at least two fish were within 4 cm of one another (interacting proximity) and both fish exhibited brightened coloration. During courtship interactions, both sexes exhibited a characteristic posture consisting of an erect body with
head inclined downward and were generally positioned side-by-side facing the same direction. Courtship occurred in discrete bouts, which were defined as beginning with the first display of any courtship behavior (quivering, pointing, or rising) by any one of the three fish and ending when individuals stopped courting and moved out of the side-by-side position. When two fish started a courtship bout and then the third entered into the interaction, it was scored two ways. The first pair was scored as continuing courting until one of them terminated contact, but a new courtship bout between all three was also recorded to determine the time that all three fish were interacting. In the final determination of the total duration of courtship for any individual, pair or triad, courtship durations were verified to ensure they were accurate and not inflated in any given category. Courtship activity levels (representing the percentage of time spent in courtship) were calculated separately for each courtship phase by dividing the total time that a given pair (focal/mating, focal/non-mating, mating/non-mating) spent courting (summed across all bouts) by either the total observation time each day (for phase 1) or the total time spent in that phase (phases 2-4).

Nineteen male-biased treatments (2M:1F) and 28 female-biased treatments (1M:2F) were observed each day from the day of introduction until copulation took place, and 15 male-biased treatments and 14 female-biased treatments resulted in successful copulations. Courtship duration and number of bouts, as well as initiators of each approach, brightening, courtship and competitive behavior were recorded for all 29 successful trials. The frequency and duration of specific courtship and competitive behaviors were also recorded for all trials.

Statistical Analysis

Differences in the frequency of competitive behaviors exchanged between males as opposed to between females were examined using nonparametric procedures (Mann-Whitney
U-test), because treatment variances were unequal and variables were not normally
distributed. Body mass differences between mating and non-mating fish in trials were
analyzed using paired t-tests. Courtship activity differences between phases for male-biased
and female-biased courting pairs (focal/mating, focal/nonmating), were determined by
repeated measures analysis of variance on natural log transformed data, because variances
were unequal across phases. Total courtship interaction time differences between pairs
(focal/mating, focal/non-mating, mating/non-mating) for both treatments and changes in
phase durations and courtship activity levels were analyzed using Kruskal-Wallis tests, with
differences between groups detected through the Nemenyi test, a nonparametric multiple
comparisons test (Zar, 1996). Differences in initial proximity between mating and nonmating
fish were determined using exact binomial tests, against a null hypothesis of equal probability
of either the mating or nonmating fish coming into contact with the focal fish first.

RESULTS

Sex Differences in Competitive Behaviors

Male H. zosterae exhibited a higher frequency of three competitive behaviors than did
females (snapping, holding, and intruding). Males displayed snapping (Fig. 1; n=15 males,14
females, Mann-Whitney U = 133.0, p = 0.049) and holding (U = 135.5, p = 0.041) at a
significantly higher rate than females on the first day of courtship, and all three behaviors at a
significantly higher frequency than females on the day of copulation (snapping, U = 149.5, p
= 0.003; holding, U = 148.0, p = 0.011; intruding, U = 134.5, p = 0.047). On both the first
day of courtship and the day of copulation, snapping was the most frequent competitive
behavior displayed by male *H. zosterae*, followed by holding and then intruding. The frequency of these behaviors as displayed by males increased from the first day of courtship to the day of copulation, although these differences were not significant (Wilcoxon rank-sum tests: snapping, $W = 1.54, p = 0.123$; holding, $W = 1.78, p = 0.074$; intruding, $W = 1.68, p = 0.093$). Females were not observed to display snapping on either the first day of courtship or the day of copulation, and holding and intruding remained at a constant low frequency for females the first day of courtship and the day of copulation.

The duration for which fish were isolated from the opposite sex (varying from 7-56d) also influenced the frequency of competitive behaviors. Isolation duration was significantly correlated with the frequency of competitive behaviors exhibited over the course of a trial for both male- (Fig. 2: $r^2 = 0.37, n = 14, p = 0.021$) and female-biased treatments ($r^2 = 0.69, n = 13, p = 0.0002$). The longer the same-sexed fish were isolated from the opposite-sex, the more likely they were to engage in competitive behaviors, with all of the 10 trials in which
individuals were isolated for longer than 25 days displaying competitive behaviors in both male- and female-biased trials.

Figure 2: Relationship between the duration dwarf seahorses (H. zosterae) were isolated from the opposite sex and the frequency of competitive behaviors calculated for the entire duration of trials. Female-biased trials (1M:2F) are shown by open symbols and male-biased trials (2M:1F) are shown by filled symbols. Least-squares regression lines provided.

Differences in Courtship Behavior

Fish in male-biased and female-biased treatments exhibited marked differences in courtship behavior. Courtship activity between the mating and focal fish dramatically increased across four courtship phases (Fig. 3A: repeated-measures ANOVA, within trial effects, $F_{3,69} = 55.0$, $p < 0.0001$) with no differences detected between male- and female-biased treatments (between subjects effects, $F_{1,23} = 2.93$, $p = 0.10$). In addition, courtship activity between the nonmating and focal fish in the first three courtship phases was slightly higher in female-biased trials than in male-biased trials, although this difference was not significant (Fig. 3B: between-subjects effects on natural log transformed data, $F_{1,23} = 1.97$, $p = 0.17$). No differences were detected between phases in the courtship activity of the nonmating pair (within-subjects effects, $F_{3,69} = 1.13$, $p = 0.342$).
Figure 3: Courtship activity levels (representing the percentage of time spent in courtship) observed across different courtship phases in *Hippocampus zosterae*, for courtship activity between the focal fish and the mating fish, and courtship activity between the focal fish and the nonmating fish. Female-biased trials (1M:2F) are shown by dark bars and male-biased trials (2M:1F) are shown by hatched bars. (X ± 1 SE).

Two courtship behaviors on the day of copulation, pointing in females and pumping in males, indicate readiness to mate (Vincent, 1994a; pers. observation). In 9 of 15 (60%) male-biased trials, both males displayed pumping during phase two on the day of copulation, and in 6 of 14 (43%) female-biased trials, both females displayed pointing during this phase. This indicates that both of the same-sex fish were ready to mate, with no significant differences between treatments (G = 0.857, 1 df, 0.25 < p < 0.5).

Male- and female-biased treatments also differed in the distribution and frequency of female initiation of rising, a behavior marking the transition to the final phase of courtship. In all 14 female-biased trials, the only rises that occurred were between the focal male and mating female. Of the 58 rises observed between the focal male and mating female, no sex
difference was observed in the frequency of initiation, with 47% (27 of 58 rises) initiated by the mating female (G=0.258, 1 df, p>0.05). In male-biased trials, however, females initiated rising significantly more often than males, with 79% (45 of 57) of all rises observed between the focal female and mating male initiated by the female (G=20.26, 1 df, p<<0.001). In addition, in 2 of 15 male-biased trials, rises were observed to occur between the focal female and nonmating male (9 rises total, 8 (89%) female initiated) and between all three fish (27 rises total, 25 (92%) female initiated; although 25 of these rises came from one trial). Rises were not observed between females in female-biased trials, but in 4 of 15 male-biased trials, males rose together in the water column (9 rises total, 7 (78%) initiated by the mating male). However, this behavior occurred predominantly during late phase 2 (2 trials; the only trials that did not transition through phase 3) or phase 3 (2 trials).

Correlates with Mating Success

Male competition trials - Male H. zosterae that successfully mated showed no difference in mass compared to nonmating males (mating male: \( \bar{x} = 143.6 \) (7.2) mg, nonmating male: \( \bar{x} = 144.2 \) (7.3) mg; paired t-test, \( t = 0.064, \) 14 df, \( p = 0.95 \)). Mating males appeared closer in mass to the female than nonmating males, although this difference was not significant (mean mass difference between female and mating male - \( \bar{x} = 18.4 \) (3.7), mean mass difference between female and nonmating male - \( \bar{x} = 24.2 \) (5.0); paired t-test, \( t = 1.3, \) 14 df, \( p = 0.22 \)).

Mating and nonmating males also exhibited behavioral differences. Mating-males engaged in longer courtship interactions with females than did nonmating males, both on the first day of courtship and the day of copulation (Fig. 4A; first day of courtship: Kruskal-Wallis \( H = 29.3, \) 2 df, \( p < 0.0005 \), Nemenyi nonparametric multiple comparisons test, \( q = \)
5.32, p<0.05; day of copulation: $H = 22.8$, 2 df, $p = 0.0001$, Nemenyi test, $q = 5.72$, p<0.05).

Males were observed to exchange courtship behaviors with each other, but these same-sex courtships were shorter in duration than either of the male-female courtships on both the first day of courtship and the day of copulation. Mating and nonmating males also differed in the frequency of competitive behaviors (Fig. 5). On both the first day of courtship and the day of copulation, mating males employed snapping and holding more frequently than nonmating males, while nonmating males intruded more often than mating males, although these differences were not significant (Wilcoxon signed ranks test, all p>0.05).

![Figure 4](image)

**Figure 4:** Duration of time in minutes *H. zosterae* spent interacting [close (<4 cm), bright, and exchanging courtship behaviors] during male-biased and female-biased treatments on the (A) first day of courtship and (B) the day of copulation. Mean (+ 1 SE) interaction times provided for the focal fish and mating fish (dark bars), focal fish and nonmating fish (hatched bars), and mating and nonmating fish (open bars).

![Figure 5](image)

**Figure 5:** Frequency of three competitive behaviors observed in *H. zosterae* males (Snapping, Holding, and Intruding) in counts per minute multiplied by 10³, given for the (A) first day of courtship and the (B) day of copulation for mating males (dark bars) and nonmating males (hatched bars). Means $\bar{X}$ (+ 1 SE).

Female competition trials - Female *H. zosterae* that mated at the end of a trial were slightly but not significantly larger than females that did not mate (mating female - $\bar{x} = 140.1$ (25.4),

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nonmating female - $\bar{x} = 122.3$ (25.3); paired t-test, $t = 1.83, 13$ df, $p = 0.09$. Mating females appeared closer in mass to the male than nonmating females, although this difference was not significant (mean mass difference between male and mating female - $\bar{x} = 16.8$ (2.1), mean mass difference between male and nonmating female - $\bar{x} = 21.7$ (4.6); paired t-test, $t = 0.97, 13$ df, $p = 0.35$).

Few behavioral differences were observed between mating and nonmating females. On the first day of courtship, courtship interactions were longer between mating females and focal males than between nonmating females and focal males or between the two females (Fig. 4B; Kruskal-Wallis $H = 14.8$, 2 df, $p = 0.0006$). However, the difference in duration of courtship between the mating pair and the pair that did not mate was not significant (Nemenyi test, $q = 2.38$, $p > 0.05$). On the day of copulation, the mating pair courted significantly longer than either of the other two groups ($H = 23.6$, 2 df, $p < 0.0001$; Nemenyi test between mating and nonmating pairs, $q = 4.20$, $p < 0.05$; Nemenyi test between mating and same-sexed pairs, $q = 5.86$, $p < 0.05$).

In both male- and female-biased treatments, mating success appears to be related to initial proximity to the focal fish at the start of a trial. Mating males and females from both sex-biased treatments were usually the first of the same-sex fish to approach the focal fish to within interacting proximity, although this difference was not significant (exact binomial: male-biased, $p = 0.056$ [one-tailed]; female-biased, $p = 0.18$ [one-tailed]). This indicates that focal fish may be mating with the first same-sexed fish they encounter.

**Courtship behavioral differences between biased sex-ratios and pairs**

Biased sex-ratio treatments used in this study caused substantial shifts in the courtship patterns compared to those observed between pairs (Masonjones & Lewis 1996; Table 1).
Pairs, male-biased, and female-biased treatments differed in durations of each courtship phase on the day of copulation. However, both the duration of each trial from introduction to copulation (Table 1; Kruskal-Wallis test, $H = 0.168$, 2 df, $p = 0.92$) and the time from the first observed courtship to copulation on the final day of each trial ($H = 0.248$, 2 df, $p = 0.88$) were similar. Over all three groups, there was a significant decrease in the phase duration from phase 2 through phase 4 (repeated-measures, $F_{2,10} = 77.2$, $p < 0.00005$). Courtship phase 2 was longest in male-biased treatments and shortest in female-biased treatments (Kruskal-Wallis, $H = 5.32$, 2 df, $p = 0.07$, Nemenyi test between female- and male-biased treatments, $q = 4.29$, $p < 0.05$). The latency period, which occurred at the end of phase 2, was almost 30% longer in male-biased trials than female-biased trials, although this difference was not statistically significant (ANOVA, $F_{2,10} = 0.51$, $p = 0.61$). Phase 3 was longer in female-biased treatments than male biased treatments, indicating that it took fish longer to begin to rise together in the water column. This is important because rising is the transition behavior indicating the end of phase 3 and the beginning of phase 4 (Kruskal-Wallis, $H = 6.80$, 2 df, $p = 0.03$, Nemenyi test between female- and male-biased treatments, $q = 2.81$, $p > 0.05$). Courting fish in female- and male-biased treatments spent equal time in phase 4, but it took pairs significantly longer than either of the other two groups to transition from the initiation of rising to actual copulation (Kruskal-Wallis, $H = 7.29$, 2 df, $p = 0.026$, Nemenyi test between male-biased treatment and pairs, $q = 4.02$, $p < 0.05$; between female-biased and pairs, $q = 3.51$, $p < 0.05$).
Table 1. *Hippocampus zosterae* courtship phase characteristics under three different conditions: in pairs (1 male, 1 female), in male-biased trials (2M:1F), and in female-biased trials (1M:2F). [X (1 SE)].

<table>
<thead>
<tr>
<th></th>
<th>PAIRED TRIALS</th>
<th>MALE-BIASED TRIALS</th>
<th>FEMALE-BIASED TRIALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Size</td>
<td>5</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Trial Duration (hrs)</td>
<td>61.4 (±4.0)</td>
<td>56.8 (±2.6)</td>
<td>57.5 (±6.2)</td>
</tr>
<tr>
<td>First copulation to copulation on final day (min)</td>
<td>223.8 (±22.6)</td>
<td>211.1 (±13.8)</td>
<td>231.7 (±17.3)</td>
</tr>
<tr>
<td>Phase 2 duration (min)</td>
<td>134.3 (±16.1)</td>
<td>162.6 (±8.5)</td>
<td>127.4 (±14.2)</td>
</tr>
<tr>
<td>Latency duration (min)</td>
<td>81.3 (±15.9)</td>
<td>79.4 (±15.4)</td>
<td>61.6 (±12.5)</td>
</tr>
<tr>
<td>Phase 3 duration (min)</td>
<td>48.0 (±13.8)</td>
<td>25.6 (±5.1)</td>
<td>79.2 (±17.5)</td>
</tr>
<tr>
<td>Phase 4 duration (min)</td>
<td>41.5 (±5.9)</td>
<td>23.0 (±8.4)</td>
<td>25.1 (±5.2)</td>
</tr>
<tr>
<td>Copulation duration (s)</td>
<td>12.5 (±0.74)</td>
<td>14.1 (±1.7)</td>
<td>14 (±1.1)</td>
</tr>
</tbody>
</table>

The duration of courtship between the mating and the focal fish (Fig. 6; Kruskal-Wallis, H = 0.21, 2 df, p = 0.91) as well as the total duration of courtship (duration of courtship between mating and focal fish plus the duration of courtship between the nonmating and focal fish, H = 1.06, 2 df, 0.59) was similar between pairs, male-biased, and female-biased treatments on the first day of courtship (early Phase 1 courtship). However, in both male- and female-biased treatments total courtship increased on the second day of courtship (late Phase 1 courtship), although not significantly ($F_{1,18} = 1.31$, p = 0.294). All courtship durations were normalized by phase durations to determine the courtship activity of each phase. Significant differences were not detected between pairs, male-biased, or female-biased trials in the courtship activity in any phase (Kruskal-Wallis tests, all p>0.05), indicating that apparent differences in courtship duration between pairs and sex-biased treatments in phases 3 and 4 are due to the differences in phase duration and not actual differences in the amount or duration of courtship.

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Differences were also observed in the occurrence of courtship behaviors between pairs and biased sex-ratio treatments. In pairs, pointing is observed only on the day of copulation (Masonjones & Lewis, 1996). However, in 24% (7 of the 29 total) of the biased sex-ratio treatments, females displayed pointing on the first day of courtship. Four of these trials were female-biased (including 2 cases of pointing in the mating female and 2 in the nonmating female), and three were male-biased trials in which pointing was exhibited by the focal female.

DISCUSSION

Sex Differences in Competitive Behaviors

Male *Hippocampus zosterae* engaged in a higher frequency of all three competitive behaviors (snapping, holding, and intruding) than *H. zosterae* females. Females were never observed to snap at one another, but did display intruding and holding with the frequency of these behaviors unchanged between initial courtship and courtship on the day of copulation.
Males exhibited snapping in addition to holding and intruding, and the frequency of all three behaviors increased on the day of copulation. The increase in male competitive activity on the day of copulation may be related to the female’s display of pointing, indicating her readiness to mate. Competition during early courtship may serve to aid in the establishment of a pair bond with the female, which would later lead to copulation if the pair bond successfully forms. Once females pointed on the day of copulation, indicating their availability to mate, male competition escalated, perhaps because males were competing directly for copulation.

An increased frequency of competitive behaviors with increasing duration of isolation from the opposite sex may indicate of the importance for fish to secure mates. Longer isolation times may result in the assessment of reduced mate availability. With fewer potential mates available, it becomes more important to establish a pair bond with the first available unmated fish to prevent having to search further and possibly failing to reproduce. A lower probability of encountering unmated fish would also increase the costs associated with rejecting a current prospect in favor of searching further for an acceptable mate (Bateson 1983). Increasing costs associated with mate choice have been shown in other systems to reduce the “choosiness” of both males and females (Forsgren, 1992; Berglund, 1993; Backwell & Passmore, 1996).

Courtship differences also existed between sex-biased treatments, with the nonmating female and focal male from female-biased treatments courting longer and more frequently than the nonmating male and focal female from male-biased treatments. The differences observed between male- and female-biased treatments may have resulted from the differing levels of competition. Competition was more prevalent between males and this may have
prevented courtship between nonmating males and females in male-biased trials. In contrast, female-biased trials showed less competition between females and the duration of courtship between the nonmating female and male was shorter than between the mating pair, although not significantly shorter. These differences in courtship duration were not due to differences in the readiness to mate of nonmating individuals between male and female biased treatments. This is because in both treatments about half of the trials consisted of both the mating and nonmating fish displaying precopulatory behaviors, pumping in males and pointing in females on the day of copulation.

Another difference between male- and female-biased treatments was in the frequency of the female initiation of rising, the behavior signaling the transition from phase 3 to phase 4 culminating in a final copulatory rise during which egg transfer occurs. In male-biased trials, females initiated almost 80% of rises occurring with the mating male, but in female-biased trials, the mating female only initiated 47% of all rises. In addition, no rises were observed between the male and nonmating female, despite the observation that in 6/14 trials nonmating females were observed to point, indicating their ability to mate. If female initiation of rising is indicative of her active involvement in courtship, then in the presence of a competitor (female-biased trials), a female should increase the number of rises she initiates, and in the absence of a competitor (male-biased trials), females should decrease initiation rate. The exact opposite pattern was observed in dwarf seahorse females.

This pattern may be related to the differences in female behavior between trials in which both females pointed and those in which only the mating female prepared to mate, since it is only in the former case that a potential competitor exists (seahorse females have never been observed to mate without first exhibiting pointing, Vincent, 1990; 1994a; personal
observation). In trials in which only the mating female exhibited pointing, there was an average of 5.3 rises per trial, but when both females pointed, rising rate per trial decreased by half. This may indicate that in the presence of a competitor, females put more effort into coordinating rises with the male, to be able to transfer eggs sooner. This may represent an indirect form of female competition, in which it is the female who is ready to copulate sooner that successfully mates.

Differences observed in the initiation of rising between treatments may also be related to differences in mate selectivity. If the initiation of rising is related to active mate choice on the part of males and females, the fact that males initiate a larger proportion of rises in the presence of two females may indicate their preference for one female over the other. This may also explain the dramatic increase in female initiation of rising in male-biased treatments. If presented with two males, a female may indicate her preferred mate by initiating rises with him. In *H. zosterae* pairs, females initiated the first rise significantly more often than did males (Mason-jones & Lewis, 1996), indicating that in the absence of a competitor females may dictate the pattern of rising.

**Correlates with Mating Success**

In *H. zosterae*, there were a number of morphological and behavioral correlates with mating success. Absolute body mass was not related to the mating success of males, but larger females mated more often than smaller females, although not significantly. In *H. fuscus*, fecundity was more directly related to body size (girth) in females than in males (Vincent 1990). This may be why larger female *H. zosterae* were more successful in mating. For both male- and female-biased treatments, chosen mates were more closely matched to the focal fish in body mass than non-mating fish, although these differences were not significant.

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This may indicate a trend towards size-assortative mating in this species, possibly because reproductive success for both partners over time is optimized by pairing a larger mate, causing fish to assort positively with size. Evidence of assortative mating is common in fish (midas cichlid, Barlow et al., 1977; threespine stickleback, Whoriskey & FitzGerald, 1994), and in many species, size matched pairs are more successful in rearing young and protecting broods from predation (Kuwamura et al., 1993). In addition, the pipefish _Syngnathus týphle_ also exhibits size-assortative mating, with both males and females preferring larger mates (Berglund et al., 1986a; Berglund & Rosenqvist, 1993).

There is strong evidence that seahorse pairs are size-assorting in the field (Vincent & Sadler, 1995). During a field study of _H. whitei_, Vincent & Sadler (1995) observed that pairs formed at the beginning of the breeding season were closely matched in size, and that fish who lost their mates form new pairs later in the season were not size-matched. Thus there is evidence for size-assortative mating in seahorses, but we may not have observed a strong trend because the _H. zosterae_ used in this study were collected during the breeding season. This apparent reduction in mate selectivity after the beginning of the breeding season in _H. whitei_ may explain our observation in _H. zosterae_ that successfully mating fish were usually the first within interacting proximity of the focal fish. Because unpaired seahorses are uncommon during the breeding season (Vincent & Sadler 1995), it is possible that after initially pairing for a season seahorses will accept the first available mate.

Between males, mating males displayed competitive behaviors at a higher frequency than non-mating males, although these differences were not significant. Mating males displayed snapping, the competitive behavior that often elicits the strongest reaction from the recipient, considered for this reason as a more direct competitive behavior than holding and
intruding, behaviors more commonly displayed by nonmating males. Because of the relatively low rate of these competitive behaviors in trials, a larger sample size would be needed to more clearly establish the differences between mating and non-mating males.

**Differences between biased sex-ratios and pairs**

Although no differences were observed between pairs, male-biased, and female-biased trials in either the total length of a trial or in the duration of the day of copulation, biasing the sex ratio of trials either towards males or towards females caused marked changes in the progression of courtship phases on the day of copulation. Courtship phase 2 was observed to be longest in male-biased treatments and shorter in pair and female-biased trials. In trials where males were exhibiting competitive behaviors, males often spent a large portion of phase 2 directly interacting, exchanging both courtship and competitive behaviors. This interaction between males may have slowed down their transition to phase 3 courtship, in addition to possibly changing the quality of the males' interaction with the female, prolonging her preparation of eggs during latency and delaying the beginning of phase 3. Female-biased treatments may have the shortest phase 2 because females are indirectly competing to secure the male as a mate. Latency is a period of inactivity that occurs at the end of phase 2, which is most likely when the final stage of oocyte maturation occurs during which the eggs are hydrated prior to ovulation (Wallace & Selman 1981; Vincent 1990; Selman et al. 1991). At the end of latency, the characteristic orange coloration of the eggs becomes visible through the body wall of the female, and the abdomen is visually distended (Vincent, 1990; Masonjones & Lewis, 1996). Latency is shorter in female-biased treatments than in male-biased treatments, which may indicate that females prepare eggs more quickly in an attempt to be the first to copulate with the male.
Phase 3 was longest in female-biased trials than in male-biased trials or pairs. Male-biased trials may have had the shortest phase 3 because this is the phase during which male-female rises are first observed, another form of male competition. The transition from phase 3 to phase 4 is defined by the first observance of a male and female rising together in the water column. Male rises may trigger the behavioral change to rising in females more quickly than in the other two treatments, thus shortening or completely eliminating phase 3. Since females across most treatments initiated the first rise more often than did males, the transition from phase 3 to phase 4 may be under female control. If females do have control over the beginning of phase 4, then it is possible that Phase 3 is longest in female-biased treatments because females are delaying the beginning of phase 4 until they are ready to copulate with the male. This is supported by the observation that in all trials in which both females indicated their readiness to mate, phase 3 was longer and phase 4 was shorter than in trials in which only the mating female exhibited pointing. In addition, phase 4 may be shorter in sex-biased treatments than in pairs because with both the mating and nonmating fish physiologically able to copulate, both the mating male and female will secure the focal fish as soon as possible. This is most likely done to protect their investment in either the preparation of eggs in female-biased treatments or the time invested in the interaction by males.

Summary

Contrary to a number of influential summaries (Williams, 1975; Trivers, 1985), seahorses are not courtship-role reversed. Male H. zosterae display a greater variety and higher frequency of competitive behaviors than do females, indicating that males are the more competitive sex in this species. This is consistent with earlier studies of H. fuscus, which indicated that males compete more intensely for mates than do females (Vincent, 1994a). In
addition, evidence exists to suggest that mate choice may be occurring in *H. zosterae*, with trends towards males and females assorting positively with respect to size and larger females exhibiting higher mating success. Unfortunately, little information exists regarding pair formation in the field early in the breeding season, thus making it difficult to interpret the relative roles of competition and choice in determining mating success. Future studies should focus field investigations on this crucial period, to determine the importance of mate selectivity and competition patterns in the formation of monogamous pair-bonds in seahorses.
CHAPTER VI:

Mate choice in the dwarf seahorse,
_Hippocampus zosterae_

**Abstract:** Dwarf seahorses (_Hippocampus zosterae_, Syngnathidae) are distinguished by an extreme degree of morphological specialization for paternal care and the formation of monogamous pair bonds. The purpose of this study was to determine the degree of male vs. female mate choice in this species. Daily behavioral observations of male-biased (2 males, 1 female) and female-biased (1 male, 2 females) treatments were made from the seahorses' introduction to tanks through the day of copulation. Experimental replicates were designed so that two same-sex individuals, selected based on differences in body mass and side area, were separated from each other by an opaque partition, and from an opposite-sexed focal individual by mesh. The focal individual could interact with either of the two same-sexed individuals, they could not interact with each other. Males apparently select their partner in mating, as 9 of 14 males were able to choose between two healthy females, but only 4 of 14 females were able to choose between two males. Focal-males also spent significantly more time than did focal-females interacting with their selected mate partner. Male-focal replicates also mated 3 days sooner, on average, than did female-focal replicates. _Hippocampus zosterae_ do not select their mate partner based on the body size parameters of wet mass, girth, width, side area, or trunk length. These data and behavioral observations suggest that males and females actively choose their mates, but at different rates and stages of courtship. The implications of mutual choice are discussed.
INTRODUCTION:

Sexual selection occurs in populations primarily through two mechanisms: 1) mate choice, the process by which one sex chooses mates of the opposite sex based on certain criteria, and 2) mate competition, or the process by which members of one sex compete for access to members of the opposite sex (Bateson, 1983; Bradbury & Andersson, 1987; Maynard Smith, 1991). In most species, it is the males that compete for access to mates and the females who exhibit choice (Balmford & Read, 1991), a pattern that has been hypothesized to depend on the relatively greater parental investment females make in offspring (Williams, 1966; Trivers, 1972, 1985). In a number of groups, however, males provide considerable parental care (Ridley, 1978; Clutton-Brock, 1991), during which males may incubate, aerate, protect, or provision developing embryos and juveniles (Sargent & Gebler, 1980; Potts, 1984.)

According to parental investment theory, in species where male parental investment exceeds that of females, males are predicted to be more selective of their mates and females are expected to compete more intensely for access to males (Trivers, 1972). However, recent theoretical and empirical work suggests that some aspects of mate choice may be independent of parental investment, influenced more directly by the costs and benefits of choice such as variation in mate quality, time constraints in searching for mates and other ecological factors (Gronell, 1989; Kodric-Brown, 1989; Johnstone et al., 1996; Johnstone, 1997). In addition, although past work has focused on mate choice as occurring in either females or males of a species (Janetos, 1980; Seger, 1985), when both sexes potentially vary in quality due to biparental care or substantial investment in gametes, it is likely that mutual mate choice will occur, with males and females both being selective of their mates. Mutual mate choice has
been demonstrated in many organisms such as the mormon cricket (Gwynne, 1981), bush cricket (Simmons, 1992), moorhens (Petrie, 1983), threespine sticklebacks (Sargent et al., 1986), coho salmon (Sargent et al., 1986), and the pipefish *Syngnathus typhle* (Berglund & Rosenqvist, 1993).

One extreme example of male specialization for paternal care comes from seahorses (genus *Hippocampus*; Herald, 1959; Vincent et al., 1992). Fish from the Syngnathidae family (pipefish and seahorses) vary in anatomical and physiological specialization for paternal care from pipefish such as *Nerophis ophidion*, in which females attach eggs onto the ventral surface of the male (Berglund et al., 1989; Rosenqvist, 1990) to seahorses, in which females deposit their eggs into a pouch on the ventral surface of the male where fertilization takes place (Fiedler, 1954) and embryos are sealed from the external environment (Linton & Soloff, 1964). The epithelial lining of the seahorse brood pouch is highly vascularized, and provides the developing embryos with services such as gas exchange, osmoregulation and waste removal during their 10-30 day gestation (Boisseau, 1967).

In female *H. erectus* seahorses, mature follicles appear to be produced continuously from two germinal ridges in each ovary, with a range of follicles of sequentially increasing developmental stages from each germinal ridge outward (Selman et al., 1991). Past work with *H. fuscus* and *H. guttulatus* has shown a positive relationship between female body size and clutch size, and a weaker, but positive relationship between male pouch volume and size and number of young produced (Movchan, 1989; Vincent, 1990). Recent evidence suggests that although male seahorses do invest a substantial amount of energy in each offspring, female seahorses invest far more energy than do males (Chapter II).
Until recently, information describing seahorse courtship behavior was largely anecdotal (Gill, 1905; Fiedler, 1954; Breder & Rosen, 1966). In the last few years, however, laboratory studies on *Hippocampus fuscus* (Vincent, 1994a, 1994b, 1995) and field studies of *H. whitei* (Vincent & Sadler, 1995) indicated that both species display complete sexual monogamy, with a single male and female mating repeatedly and exclusively over the course of a reproductive season. Both *H. fuscus* (Vincent, 1994a) and *H. zosterae* (Chapter 5) males have also been observed to compete more intensely for mates that females, contrary to the predictions of some influential summaries of seahorse behavior (Trivers, 1985). Little is known about the role that mate choice plays in determining mating success in syngnathids. In pipefish, males and females have been shown to prefer larger mates (Berglund et al. 1986; Berglund et al. 1989; Berglund & Rosenqvist, 1990).

The purpose of the present study is to determine the degree of male and female mate choice present in the dwarf seahorse, *Hippocampus zosterae*, and to identify possible criteria for those choices. Because of the relationship between body size and fecundity established in other species of seahorses, my hypothesis in this study is that larger individuals will be preferred when males or females are given a choice of large and small individuals.

**METHODS**

*Study Organism*— *Hippocampus zosterae* occurs in shallow seagrass beds from the Gulf of Mexico east through the Bahamas, Cuba and Bermuda (Ginsberg, 1937; Böhlke and Chaplin, 1966). Adult size ranges from 16-38 mm (measured as the linear distance from the top of the coronet to the end of the tail; Strawn, 1958). The monogamous mating system exhibited by *H. fuscus* and *H. whitei* (Vincent, 1995) occurs in *H. zosterae* in the laboratory,
with a male and female remaining together and mating repeatedly over the course of the breeding season (HDM, unpubl. data). In the lab, females transfer one entire clutch of eggs to a single male \[ \bar{x} (\pm 1 \text{ SE}) = 12.5 \ (1.32) \text{ eggs, n=14}. \] Five to 25 \( (\bar{x}=11.4(1.58); \ n=18) \) fully independent young are born after approximately 12 days of gestation within the male brood pouch at 26°C in the lab, and pairs remate within 4-20 hours of the male’s releasing young.

*Hippocampus zostereae* were collected periodically from early March through September near Key Largo, FL. Fish were maintained in small (5-8 fish) same-sexed groups (in sexual isolation) for 10 days to 8 weeks before use in all experiments in an attempt to standardize reproductive status. Fish were kept in 57 L aquaria prior to and during experiments. Tanks were maintained at an average temperature of 26.5°C on a 14 hours light/10 hours dark photoperiod with a salinity range of 30-33‰, and two plastic seagrass plants were supplied for attachment sites. Fish were fed recently-hatched *Artemia* supplemented with calcium (Kalkwasser, Thiel Aquatech) daily. Water was tested weekly to ensure that pH, ammonia, and nitrite did not exceed acceptable levels. If water quality was poor (high levels of ammonia or nitrite) a partial water change was performed in the affected tank. Erythromycin (100mg at first, increasing to 200mg) was added weekly to retard algae growth.

*Seahorse Courtship Behaviors*— Four discrete phases of courtship in paired dwarf seahorses have been observed (Masonjones and Lewis 1996). Phase 1 courtship occurs during the one to two days preceding the day of copulation, and is characterized by reciprocal *quivering*, during which fish assume an erect body posture, with pectoral fins extended, and rapidly vibrate their bodies from side to side. Courtship phases 2-4 occur on the day of
copulation. During phase 2 courtship, females first display *pointing* (a behavior used as an indicator of a female's readiness to mate and the beginning of the day of copulation), defined as when a female raises its head upward toward the water surface to form an oblique angle with the main body axis and then lowers it again to a horizontal position, to which males generally responded by quivering. Phase 2 courtship is usually followed by a latency period of 23-220 min (median - 111 min), during which it is hypothesized that females undergo the last stages of oocyte maturation and egg ovulation. In phase 3 courtship, males begin to display pointing in response to female pointing. Phase 4 courtship is characterized by the male and female repeatedly *rising*, in which fish release their respective holdfasts and rise up into the water column facing one another. In a *copulatory rise*, the female genital papilla is placed inside the male brood pouch opening, followed by egg transfer.

*Body Size Determination*--- On the morning before each trial began, wet mass of adult male and female seahorses was measured by blotting fish dry and weighing them to the nearest 10 mg in seawater in a plastic cuvette. Additional body size measurements were made on digitized images recorded with a Sony Hi 8mm camcorder and captured on a Videospigot digitizing board in a MacIIsi. Lateral and frontal views of each individual were taken. NIH Image (version 1.40) was used to measure various body dimensions. Frontal views were used to measure width, taken at the widest part of the body. Lateral views were used to measure girth, taken at the widest part of the body measured perpendicularly to the dorsal fin, trunk length, measured as the distance from the posterior edge of the coronet to operculum to anal pore, and side area.
Experimental Enclosures--- Each replicate consisted of a 38 L enclosure (37x30x25 cm), divided into three separate sections by plexiglass and mesh (hole size 2 mm²) partitions (Fig. 1). A mesh barrier bisected the enclosure, and black plexiglass perpendicular to the mesh further divided the back section into two smaller compartments (territories 1 & 2). This resulted in one 20x37cm compartment and two 10x18cm compartments. This experimental setup allowed us to investigate mate choice independent of the effects of competition. One 4" artificial seagrass plant was placed in each of the smaller compartments 2.5 cm from the mesh. The plant and the mesh provided the only holdfasts in these smaller compartments. Three plants were placed in the larger compartment: one 2.5 cm in front of the mesh of territory 1, one 2.5 cm in front of the mesh of territory 2, and 1 centered 8 cm back from the mesh opposite the black plexiglass divider. Other neutral holdfasts were provided at the front of the larger enclosure by heater wires, airline tubing and filter plates.

Male-focal replicates consisted of a single male placed in the large front compartment, designated the "focal" individual, presented with a choice between two females (one in each of the smaller compartments), known as the mating female and nonmating female, depending on the mating outcome of each trial. Female-focal replicates consisted of a single female
placed in the large front compartment presented with a choice between two males in the rear compartments. Mating and nonmating fish were selected based on a combination of area and mass. Experimental fish ranged from 90-290 mg wet mass, with a mean difference between the two females from male-focal replicates of 26.9 mm$^2$ in area and of 76 mg in mass, and between males from female-focal replicates of 28.6 mm$^2$ in area and 75 mg in mass.

Observation Periods—Same-sexed fish were weighed, videoimaged and then placed into the smaller compartments of the tank and allowed to acclimate for one day before focal individuals were introduced. Before lights on (0900 EST), on the first day focal individuals were introduced to the tanks, they were weighed, video imaged, and placed on neutral plants in darkness. Observations were made continuously during the first 3 h after tank lights came on (defined as dawn) on each day before copulation, and from dawn until copulation was attempted on the last day. Previous observations have indicated that all seahorse courtship occurs in the morning, except on the day of copulation (Vincent, 1994a; Masonjones & Lewis, 1996). During observation periods, focal individual position in the tank (territory 1, territory 2 or neutral) was continuously recorded and all movement between territories was noted. This allowed for detailed estimates of the amount of time each focal fish spent near the mating and nonmating fish, as well as how much time the focal individual spent in neutral territory. Specific behaviors associated with courtship and mating were also recorded during each observation period. Observations continued until mating was imminent, eight days of observation had passed and no courtship was evident, or until an individual died (in this study there was a 23% mortality rate overall, with no sex difference).

The total amount of time spent by the focal fish in each territory was calculated for each observation period. The number of days from the beginning of the trial until the day of
mating, determined as the day the female of the pair began to point and copulation attempts occurred, was recorded for each replicate. This days to mate measure was further broken down into a choice phase and a preparation to mate phase. The choice phase continued as long as the focal fish spent any time with each same-sex fish. Once the focal individual spent time exclusively with one same-sex fish, the preparation to mate phase commenced. The preparation to mate phase ended with attempted copulation, indicated by fish rising on either side of the mesh partition. Fourteen male-focal and 14 female-focal replicates were observed.

Statistical Analysis—Behavioral differences between male-focal and female-focal replicates in terms of the length of the choice phase, length of preparation to mate phase and proportion of each observation day spent with mating and nonmating fish or in neutral area were examined using Mann-Whitney tests, because between treatment variances were unequal. Contingency table analyses were conducted using log likelihood ratio (G) tests to examine differences in the number of male- and female-focal replicates that mated with the initial same-sex fish they contacted. Differences between mating and nonmating fish were examined using paired t-tests.

RESULTS

Male-focal individuals showed a pattern of high fidelity to chosen females, spending an average of about 70% of each day's observation time with the chosen female (Figure 2). Focal-males spent increasing percentages of their day with the mating female culminating on fourth day with attempted copulation. On the second day of observation, the male usually spent part of his time with the nonmating female. The amount of time males spent in neutral territory declined steadily as the trial progressed. Focal-females spent more time alternating
between the mating and nonmating males and had longer choice and preparation to mate phases. Females also spent between 8 and 44% of their time in neutral territory, depending on the trial day (Figure 3).

![Figure 2: Mean percentage of observation time spent by male focal *H. zosterae* (n=9). Dark hatching in time spent with mating female, medium hatching is time spent with nonmating female and light hatching is time spent in neutral areas. Means based only on replicates in which attempted copulations occurred. Numbers directly below each chart indicate the number of days before copulation with 0 indicating the copulation day, and numbers in parentheses indicate the number of replicates used in the analysis.](image)

![Figure 3: Mean percentage of observation time spent by female focal *H. zosterae* (n=4). Dark hatching in time spent with mating male, medium hatching is time spent with nonmating male and light hatching is time spent in neutral areas. Means based only on replicates in which attempted copulations occurred. Numbers directly below each chart indicate the number of days before copulation with 0 indicating the copulation day.](image)

Males and females differed dramatically in several aspects of their courtship behavior. Although 14 replicates in each treatment were observed, female-focal trials were significantly less likely to result in an attempted copulation (Table 1; G=5.952, 3 df, 0.01<p<0.025), with 68.3% of male trials attempting copulation, but only 28.6% of female trials. Focal-females spent significantly more of their time neutral than did males (Mann-Whitney U test, U<0.001, p=0.005). In addition, focal-males spent a greater amount of time with their chosen mate than did females (Mann-Whitney U test, U=3.1, p=0.045). Between treatments, there was a
substantial difference in the number of days it took them to initiate copulation, with focal-males mating significantly sooner with their chosen mate than did focal-females (Mann-Whitney test, U=2.0, p=0.011). This difference was due in part to a disparity in the duration of the mate-choice phase, in which females took longer to make a choice than males (although not statistically significant, Mann-Whitney test, p=0.267), but did not take longer to prepare to mate (Mann-Whitney test, p=0.758).

Table 1: Summary of the differences in the characteristics of courtship (Mean + 1 SE) exhibited by focal-males versus focal-females. Replicates used in the analysis were restricted to those in which copulation was attempted within 8 days of observation.

<table>
<thead>
<tr>
<th></th>
<th>Focal Males</th>
<th>Focal Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of replicates observed</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Number of trials that attempted copulation</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Percent time spent neutral on days before copulation</td>
<td>6.6 (1.3)</td>
<td>24.0 (4.0)</td>
</tr>
<tr>
<td>Percent time spent with mating fish on days before copulation</td>
<td>79.4 (5.0)</td>
<td>59.9 (5.9)</td>
</tr>
<tr>
<td>Days from introduction to attempted copulation</td>
<td>3.9 (0.26)</td>
<td>7.3 (1.4)</td>
</tr>
<tr>
<td>Duration of choice phase (hrs)</td>
<td>27.2 (9.4)</td>
<td>102.3 (48.0)</td>
</tr>
<tr>
<td>Duration of preparation to mate phase (hrs)</td>
<td>48.7 (11.0)</td>
<td>50.2 (9.1)</td>
</tr>
</tbody>
</table>

No significant size differences were found between mating and nonmating fish for either male- or female-focal replicates. Spearman rank correlations indicated that both body mass and girth were strongly related to other body size variables (all p<0.005; trunk length, body width, and side area), but not as strongly related to each other (p<0.08). In male-focal replicates, both mass (Fig. 4; paired t-test, t=0.033, df=8, p=0.974) and girth (t=1.159, df=8, p=0.280) were similar in the mating and nonmating females. The same pattern was found in female replicates, with no significant differences detected between the masses (t=0.831, df=3, p=0.467) and girths (t=0.453, df=3, p=0.681) of mating and nonmating males, although mating males were slightly larger than nonmating males. In addition, there was no evidence in either treatment for size assortative mating on the basis of any of the size variables measured.
Although not statistically significant, there appeared to be a relationship between the mating partner and the individual first contacted in males but not in females. In 7 out of 9 male-focal trials that attempted copulation, the first female that the male approached was the eventual mating partner (Contingency table analysis: G=2.942, 1 df, 0.10<p<0.25). In focal-female replicates, half of the trials mated with the first male contacted and the other half did not.

**DISCUSSION**

In *Hippocampus zosterae*, focal-males 1) spent significantly more time interacting with their chosen mate, 2) mated sooner, and 3) were more likely to mate than were focal-females. These results suggest that male choice may be important in the establishment of the pair bond, however may also indicate that males are less discriminating than females. Behavioral observations indicate, though, that the female response is crucial to the continuation of courtship during the choice phase. It appears that although it is the male that
actively pursues a particular female, that female can choose not to respond at any stage of courtship. This female behavior is evidence that females also select their mates. Thus, our hypothesis that both male and female dwarf seahorses exhibit choice was supported, although body size did not appear to be an important criterion for those choices in either males or females.

The observation that focal-males mated more often with the female they came into contact with first suggests two opposing hypotheses: 1) either males are not being selective of their mates and are simply mating with the female that was first detected, or 2) males are selecting females based on cues that can be discerned from a distance. Despite the observation that focal males did not display a preference for females with larger mass, there did seem to be a slight, but insignificant, trend towards a preference for females with larger girths. Seahorses make clicking sounds during feeding (Colson et al., in press), and feeding is a behavior that is observed almost continuously through the daylight hours in males and females. The peak frequencies of these feeding clicks have been shown to decrease significantly with increasing body mass in H. zosterae (Colson et al., in press), indicating that information could be discerned about body size from characteristics of the feeding clicks. Another cue that could be used by fish to discern between potential mates is pheromonal, with information regarding hormonal status passed to potential mates through the water column (Stacey & Cardwell, 1995; Vermeirssen, 1995). Because males were observed to interact for a short time with the nonmating female on the first two days of trials, it appears that males may be distinguishing between females and preferring one over the other.

Although focal-females spent the majority of their time across all days of observation in close proximity to their eventual mate, they spent more time than did focal-males close to
the fish with which they did not attempt copulation. This could indicate that females take
longer to assess each male and make a final choice of a mate. In other species, female
reluctance to mate has been attributed to her increased choosiness as compared to males
(Berglund & Rosenqvist, 1993). Females also spent a greater amount of time in the neutral
areas of the tank, far away from either male. I believe, however, that these observations shed
more light on the necessity for contact between males and females during courtship than on
the process of mate choice in females. Behavioral observations suggest that male approach
and initiation of courtship might be important to the establishment of a pair bond and eventual
mating of a pair. Male *H. zosterae* were shown to be more active in courtship in seahorse
pairs on the days before copulation, and females usually dictated the termination of courtship
contact by moving away from males (Masonjones & Lewis, 1996). The experimental
enclosures may have inadvertently exaggerated these existing sex biases in courtship.
Because the smaller rear enclosures forced fish to stay in close proximity to the mesh, focal-
males could stay close to females and females could not move away. Focal-females, however,
could stay in neutral areas and males could not reach them to initiate courtship interactions.

As a result, without males to instigate courtship with focal-females, these trials took longer to
attempt copulation and fewer resulted in copulation than did focal-male trials because females
were forced into the uncommon role of approach and initiation of courtship. The lower
success rate (29%) observed in female-focal trials was unusual when compared to other
studies. When pairs and groups of three fish are allowed to interact freely, copulation resulted
in between 60-80% of trials, comparable to the success rate observed in male-focal replicates.

Field studies of *H. whitei* have also indicated that although females have a larger home range
than males and migrate some distance each morning to reach their mate, it is almost always
the male that makes the final approach to initiate courtship by brightening and quivering (Vincent & Sadler, 1995).

Females were observed to initiate courtship interactions during these experiments, but quite infrequently. In three of the female focal replicates that did not result in mating, the females clearly initiated courtship bouts with the stationary males. Because some females initiated courtship in this experiment, there is evidence to suggest that females also exhibit active choice. The appearance of this behavior in relatively few of our replicates may depend on the reproductive history of the females involved. Age, number of previous mating encounters and reproductive latency have been shown to affect willingness to court or mate (Shelly & Bailey, 1992; Svensson et al. 1989). We could not control for these factors because we obtained our seahorses from the field, but in future work we are hoping to use seahorses that have been raised in the lab in single sex conditions. Thus, male mate choice and female choice or rejection is the common system, but this may change depending on the history of the individuals involved.

There was only minimal evidence in this study to suggest that mates are chosen based on body size, with females preferring males of greater mass and larger girth and males preferring females with larger girths, although none of these preferences were significant. This may be an artifact of the time of year during which fish used in the study were collected. In H. whitei, the body masses of pairs formed at the beginning of the season are highly similar, but at the season progresses, later-formed pairs are much more unlike in mass (Vincent & Sadler, 1995). Because the frequency of unpaired fish on the breeding grounds decreases dramatically as the season progresses, pairs formed later may not have the opportunity to be selective of their mates. Thus fish collected later in the season may simply
mate with the first fish with which they interact, since hesitation may mean a lost opportunity to reproduce.

In species that mate monogamously for a breeding season or longer, both male and female mate choice, or mutual mate choice, should be adaptive, since the reproductive success of each individual in the pair depends solely on the other (Wittenberger & Tilson, 1980; Mock & Fujioka, 1990). In seahorses, this would be especially true considering that complete sexual monogamy has been observed, with pairs continuously producing broods over the course of a 6-8 month breeding season. Considerable variability exists in the number of young born to male H. zosterae of similar sizes, thus the opportunity exists for variation in mate quality (Chapter 2). This variation is in addition to the strong and positive relationship between male size and brood size, and the weaker but positive relationship between female mass and characteristics of egg clutches.

Species-specific patterns of mate choice depend on a balance of the benefits of choice and the costs of choice to each sex (Janetos, 1980; Bateson, 1983; Johnstone et al., 1996). Because there is a greater potential for variation in the quality of the sex that provides most of the care of offspring, there is often a greater benefit of choice to the opposite sex. This is counterbalanced by the observation that the sex that does not care for offspring also has the greater potential reproductive rate, thus biasing the operational sex ratio towards them (Clutton-Brock & Parker, 1992). As a result, refusing potential mates represents a greater cost to the sex that does not care for young, since available mates would be scarce. Johnstone et al. (1996) developed a model to predict species-specific patterns of choice incorporating male and female interbrood/interclutch intervals, population density and encounter rate, and the variation in quality of potential mates. By applying their model to seahorses, assuming that 1)
male and female processing time is similar among paired seahorses, 2) a substantial difference exists in the time it takes for each sex to initially prepare to mate (Chapter 3), and that 3) variation exists in the ability of both sexes to provision offspring (Chapter 2), it is expected that there should be components of mate choice in both sexes. Sexual selection on males due to female mate choice is predicted to be stronger, however, given the greater investment females make in offspring (Chapter 2).

In summary, there exists the opportunity for mate choice to occur in both male and female dwarf seahorses. Unfortunately, due to the design of experimental enclosures, the results of the mate choice assays were inconclusive, with only minimal evidence to suggest a preference for larger mates and no evidence to support size assortative mating. This study did successfully determine that male approach and initiation of courtship is crucial to the normal process of courtship and mating in this species. This is consistent with other work by the authors which suggests that it is the male seahorse that is the most active in initiating courtship (Masonjones & Lewis, 1996) and competes more aggressively for mates than do females.
Chapter VII: DISCUSSION

The main goal of this thesis was to use dwarf seahorses (*Hippocampus zosterae*), a genus distinguished by extreme morphological specialization for paternal care, to relate the observed courtship-roles of males and females to the relative parental investment and potential reproductive rates of males and females, for the purpose of evaluating the predictions made by each of these theories regarding courtship behavior patterns. Contrary to earlier assumptions made regarding seahorse courtship behaviors (Trivers, 1985), seahorses are not courtship role-reversed. Male *H. zosterae* are more active in courtship and compete more intensely for mates than do females. No clear evidence exists for either female or male mate choice in this species based on body size or differences in courtship behaviors, but it is possible that either there are no benefits to mate choice in this species or that females and/or males become less discriminating after the beginning of the breeding season.

Seahorses are also not parentally role-reversed. Although males are highly modified for brooding offspring and make a metabolic investment of 6 J in each offspring, females make a direct investment of 13.1 J in each egg. As a result, the ratio of female parental investment to male parental investment is 2:1, with each egg containing twice as much energy as males invest over the course of gestation. In addition, male seahorses also exhibit a higher potential reproductive rate than do females, with the opportunity to produce almost 17% more offspring over the course of a breeding season. As a result, both the observed patterns of parental investment and the potential reproductive rates of male and female dwarf seahorses correctly predict courtship roles. Since female *H. zosterae* invest more per offspring and have a lower
potential reproductive rate than do males they are a limiting resource, thus inducing males to compete for access to them.

It is unclear, however, whether the investment that females make in offspring physiologically limits their potential reproductive rate. The ovarian structure of seahorses indicates that mature follicles are continuously prepared (Selman et al., 1991), suggesting the potential for females to mate more often. It is possible that the investment females make in offspring may indirectly influence their potential reproductive rate, through the mediating forces of behavior rather than physiology. The 2-day mating latency imposed by both female *H. zosterae* and *H. fuscus* (Vincent, 1994b) may have evolved as a method by which a female could protect her significant investment in each clutch by insuring that a male would be available to transfer eggs to after ovulation. By courting a male for two days before egg hydration and ovulation, a female could adequately assess his reproductive state and increase the likelihood that he would be available to mate with on the third day. Vincent et al. (1992) have suggested, however, that there are other ecological, behavioral and physiological factors that may influence the potential reproductive rates of the males and females of a species, including anatomical restrictions of egg production/embryo care and risks associated with reproduction. It is unlikely that there are differential risks associated with paternal seahorses, given the slow speed with which seahorses swim in general and their dependence on crypsis for predator avoidance. However, there may be constraints on the number of eggs females can produce at a time or over time and a limit to the number of embryos a male can brood in one reproductive cycle. As a result, although both the potential reproductive rates and relative parental investments of male and female dwarf seahorses are consistent in their predictions
regarding courtship-roles, the present study does not indicate how these mechanisms are related.

The patterns of courtship behavior, relative parental investment and potential reproductive rates observed in seahorses are not characteristic of all members of the Syngnathidae family. Two pipefish species, *Nerophis ophidion*, considered one of the least modified of the syngnathids with eggs glued onto the ventral surface, and *Syngnathus typhle*, one of the most modified pipefish with brood flaps that seal together along a ventral groove, have been studied in detail. In both species, females compete more intensely than do males for mates (Rosenqvist, 1990), and males are more selective of their mates than are females (Berglund et al., 1986a; Berglund & Rosenqvist, 1993; Berglund, 1994). In addition, female reproductive rate is limited by the rate of reproduction of males in both species (Berglund et al., 1989; Ahnesjö, 1995). Berglund and colleagues’ (1986b) work quantifying the relative parental investments of *N. ophidion* and *S. typhle*, in addition to Haesign & Schumway’s (1981) work with *S. fuscus*, suggests that males transfer nutritive substances across the marsupium for the growth and development of embryos. Females of both *N. ophidion* and *S. typhle* were found to invest more energy in each egg than do males in each embryo across gestation (Berglund et al, 1986a). However, since Berglund et al. (1986a) did not measure total paternal investment, assuming that male investment was limited to caloric transfer, due to substantial methodological problems (see Chapter 2) and because they did not include the risks associated with increased predation on parental males (Svensson, 1992), it is possible that males of these species do invest more per offspring than females. Until a more complete analysis of the relative parental investment patterns of these pipefish is attempted, conclusions
regarding the merit of parental investment theory as a predictor of courtship-roles should not be drawn. As it stands, these pipefish have been cited as examples of species in which parental investment theory is not predictive (Clutton-Brock & Parker, 1992) and is not the most fundamental determinant of the potential reproductive rates of males and females. In both species, anatomical constraints on the number of eggs that a male can successfully brood have been suggested to be more important in limiting male reproductive rate than the investment males make in offspring (Berglund et al., 1989).

The present work suggests that the common assumption that seahorse males invest more energy per offspring than pipefish males is false. This assumption is based on the extreme morphological specialization of the male brood pouch in seahorses as compared to pipefish, but the present work indicates that one reason that seahorse pouches may be so specialized is to provide environmental homeostasis to developing embryos and not material investment. In the seahorse pouch, increased vasculature for gas exchange and waste removal (Linton & Soloff, 1964; Boisseau, 1967) and more constant osmolarity (Linton & Soloff, 1964) as compared to pipefish brooding structures may represent a decrease in the environmental stressors placed on developing seahorses. Without the need to adapt to changing salinity and oxygen conditions, seahorse embryos may expend less energy than do pipefish embryos during development. Support for this theory comes from sticklebacks (Gasterosteus aculeatus, Gasterosteiformes, a related order to the syngnathids), in which embryos that have been subjected to varying salinity and hypoxia are smaller at hatching (Whoriskey & FitzGerald, 1994), indicating that these embryos may have a higher metabolic rate during gestation.
A second explanation for the observed differences between pipefish and seahorses in their parental investment patterns is that differences may exist in female investment relative to males. Pipefish eggs are substantially smaller than seahorse eggs (Pipefish: \( \bar{x} \) diameter of mature eggs = 1.12(0.17) mm, range 0.5-2.0 mm, 26 species; clutch size [number of eggs transferred at each spawning event], range = 10-80; female body size, range = 0.28-8.5 g; Seahorses: \( \bar{x} \) diameter of eggs = 1.61(0.09) mm, range 1-3 mm, 11 species; clutch size range = 10-945; female body size, range = 0.065-25.0 g; Hardy, 1978; Movchan, 1988; Berglund et al., 1989; Vincent, 1990), and in many organisms there is a strong positive relationship between egg size and energy content (McEdward & Carson, 1987). Large egg size may represent substantially more energy invested per offspring by seahorse females than pipefish females. This could be related to the observation that male seahorses have a lower material investment in offspring than male pipefish. Over evolutionary time egg size from pipefish to seahorses may have increased, possibly allowing for the increase in pouch specialization for other functions, such as osmoregulation and gas exchange. Embryos with stable osmotic environments would expend much less energy during development than embryos subject to periods of high salinity, and as a result even if eggs size from pipefish to seahorses remained unchanged juveniles would be larger at birth. The increase in egg size over time could be related to the observation that larger juveniles are less subject to predation and have higher survivorship than do smaller juveniles (Ahnésjö, 1992a; Kozlowski, 1996). Because female energy investment is the largest determinant of offspring size (Shine, 1980; Ahnésjö, 1992b), selection may have favored a modification of egg size over a modification in male investment to produce more viable offspring with a higher probability of surviving to maturity.
It is difficult to determine, however, whether the differences in egg size between pipefish and seahorses occurred before or after the pouch modifications for increased homeostasis. It is possible that the increase in the predictability of the pouch environment for developing seahorse embryos as compared to pipefish embryos was directly related to the subsequent increase in egg size and the loss of male material investment in offspring in seahorses. These theories are highly speculative, but with further research, we will have a better understanding of the physiological processes that may have driven the evolution of both male and female modifications for parental investment.

**Future Directions**

This research has highlighted a number of avenues for future work. To increase our understanding of the relationship between the investment males and females make in offspring and their potential reproductive rates, future studies need to be done investigating these mechanisms in a broad array of species. The present study suggests that the predictions of each mechanism regarding courtship-roles are consistent, but does not explain the factors that may cause this pattern. Only more studies on a diverse suite of species can begin to address the complex interactions that may exist between relative parental investment and the potential reproductive rates of males and females.

Although males were conclusively the predominant competitors for mates in *H. zosterae*, it was unclear whether sexual selection due to male choice was occurring in this species. In addition, the relevance of these sexual selection patterns in the context of their completely monogamous mating system remains to be addressed. Because permanent pairs are
formed at the beginning of the breeding season, that is the only time during the season where competition and mate choice will be important (O'Donald, 1980a, b). Later in the season the probability of encountering an unpaired fish is low (Vincent & Sadler, 1995) and pairs formed during this time not have little choice of mates or any need to compete. Although field observations of *H. whitei* have provided us with very valuable information about the population dynamics of seahorses later in the season, this work did not address the crucial pair formation period.

This study, when considered in the context of recent work with other syngnathids, highlights the need for a more synthetic approach to parental investment to determine how patterns of investment and embryo energetic constraints differ across syngnathids. In addition, once more detailed molecular evidence is available to determine the phylogenetic relationships between members of this family and related groups (e.g. sticklebacks), it may become possible to infer the evolutionary development of body shape, forms of parental care (pouch morphology and egg size) and mating systems across the family. This group provides an excellent model system for this type of analysis, because there is an obvious progression of changes in brooding techniques by males, and through mapping these morphological and behavioral traits onto the phylogenetic relationships, we may be able to determine how this suite of unique characters coevolved in syngnathids and related orders.
APPENDIX I:

Aquaculture conditions for *H. zosterae* seahorses

Seahorses are notoriously difficult to keep in captivity. Aquarium hobbists have had almost no success with the larger species (*H. erectus, H. guttulatus, H. fuscus, H. ingens*, others) and only mixed results with maintaining breeding groups of dwarf seahorses, *H. zosterae*. Even professional aquarists from organizations like the New England Aquarium and the Mystic Aquarium have not been able to maintain large seahorses in captivity through successive generations. There are four main problems with maintaining breeding populations of seahorses: 1) they are very prone to disease and parasitic infestations, 2) they require a highly varied diet of live prey items, 3) they do not court and breed easily in tanks and are very sensitive to changes in their environment, and 4) juveniles suffer extremely high mortality and are exceptionally susceptible to disease and changes in water chemistry, temperature, and food nutritional content.

Our lab has been successful in breeding and culturing dwarf seahorses, but only after a number of years of trial and error. Although we work primarily with the smaller species, I believe that the laboratory conditions we have developed should aid in the captive propagation of larger species.

*Aquarium conditions*— Dwarf seahorses (*Hippocampus zosterae*) used in these experiments were housed in tanks from 38-72 L, provided with undergravel biological filtration and activated charcoal for chemical filtration (in the gravel bed). Fish were kept in groups of no more than 10, and frequently in pairs, to prevent crowding which can cause problems with filtration and inhibit breeding (Spotte, 1973; Coward & Bromage, 1995). Artificial seawater (Coralife) was mixed with distilled water to a specific gravity of between 1.017 and 1.025, and
plastic seagrass plants were provided for attachment sites. Lighting was provided by double-ballast 20W fluorescent bulbs and overhead room lights. Photoperiod varied from 14L:10D to 13L:11D, with 30 minute half light periods to simulate dawn and dusk. Tanks were maintained at 26°C(@76°F) using submersible heaters. Higher temperatures were used initially, but because parasitic infestations appeared to be related to increasing temperatures, colder temperatures were used for most of the experiments in this thesis.

Tanks were cleaned every 10-18 days by removing algae from the inner surfaces with a sponge and then agitating the gravel bed while siphoning off 1/3 of the tank water. Water was replaced with artificial seawater mixed to adjust the specific gravity to 1.020. Water chemistry (nitrites, nitrates, pH, and ammonia) was tested frequently during tank cycling and weekly after nitrites dropped to zero, indicating the completion of the nitrogen cycle of the bacterial filter. To encourage bacterial growth, Nitrobacter starters such as Sure Start were used when tanks were first set up.

Fish were initially housed in a common aquarium room in close association with refrigeration tanks. Mortality under these conditions was high, despite favorable water conditions. In addition, fish did not appear to be sick or parasitized, but did exhibit behaviors such as excessive swimming (often in circles) and banging themselves against the glass. These behaviors and others have been observed in fish that have been subjected to some type of stressor (Myrberg, 1990; Wedemeyer et al., 1990). Interest in courtship sounds produced by seahorses caused us to use a hydrophone to record sound in tanks, resulting in the observation that the noise levels in tanks were excessive. Controlled studies of the effects of low frequency boat noise on H. zosterae indicated that courtship patterns in adults were disrupted and juvenile
growth was significantly reduced under high noise levels (I. Dodge & S.M. Lewis, unpublished). In addition, anecdotal evidence suggests that gestating males are not able to bring embryos to term when subjected to high noise levels. These studies indicated that noise stress can have severe behavioral and physiological effects on seahorses, but that these problems can be mitigated by placing tanks on 3.75 cm thick flextube (a closed cell foam that acts as a sound barrier).

After these initial experiments, all experiments used tanks that were placed on foam to decrease noise stress to seahorses. In addition, fish were moved to an independent aquarium room with a central forced air system housed in an adjacent room, to further decrease pump noise.

*Seahorse nutrition*— During the initial phase of experiments, all fish were fed recently hatched *Artemia* nauplii. This is a convenient source of live food because it can be cultured easily in the lab. However, recent work with seahorses and other fish species has indicated that this is not sufficient to maintain populations over time because of the relatively low lipid content of brine shrimp (specifically low level of highly unsaturated fatty acids (HUFA); Navarro et al., 1993a) compared to other food items naturally available (Navarro et al., 1993b; Rodriguez et al., 1994; Furuita et al., 1996; Giwojna, 1996). Low lipid content of the diet has been shown to affect circulating testosterone, estradiol, and GTHII levels (similar to lutenizing hormone in tetrapods), causing changes in reproductive function and behavior and decreased ovarian output (Navas et al., 1995). Supplementing *Artemia* with products with enriched HUFA (such as Selco) has been shown to increase the HUFA content of brine shrimp nauplii (Ozkizilcik & Chu, 1994; Southgate & Lou, 1995), and use of enriched *Artemia* as food for larval and adult fish has been shown to increase growth rates and survivorship, as well as maintain breeding (Clawson &
Lovell, 1992; Navarro et al., 1993b; Rodriguez et al., 1994; Navas et al., 1995; Furuita et al., 1996; Giwojna, 1996). *Artemia* enrichment with antibiotics and other medications is a promising new technique that is in the process of being developed for the treatment of juvenile and adult fish diseases (Hontoria et al., 1994; Nelis et al., 1996). In the future, this may be a method that will help increase the survivorship of juvenile seahorses.

In the last two years of this project, *Artemia* nauplii have been supplemented with Selcon (American Marine) every other day by siphoning off nauplii from the culture and aerating with 0.5 ml Selcon in 10 ml seawater for 30-40 minutes.

*Parasitic infestations*— Three main parasites were problematic in maintaining seahorses in culture: 1) *Argulus*, a shield-shaped, flattened crustacean which is a common fish parasite (Mills, 1983) was found attached to the head and gill coverings of seahorses, causing fish to rub themselves against tank surfaces and fail to thrive in favorable tank conditions, 2) nematodes, very small, rod-shaped organisms with pointed ends (Bellomy, 1969; Pechenik, 1991) which were found associated with the skin of seahorses (specifically of the tail) causing swelling, red lesions to appear just under the skin surface, and fish to rub against holdfasts, and 3) turbellarians, flatworms with triangularly shaped heads with two eyespots (Mills, 1983; Pechenik, 1991), causing swelling, bubbles to attach to the skin surface, and fish to rub against holdfasts. *Argulus* can be detected visually and removed with fine forceps, but the nematode and turbellarian infestations must be diagnosed with a microscope. All three of these infestations caused substantial mortality in seahorses if left untreated. Treatment of the latter two parasites consisted of mixing a solution of 1 ml 10% formalin with approximately 3500 ml of seawater, placing seahorses into it (with care taken to not shock already sick fish by dramatic changes in temperature and salinity) and gently aerating the solution with an airstone for 10 minutes. Fish
were treated three times over the course of a week to kill parasites, and immediately after the last
treatment they were placed into newly-started tanks free of parasites. Parasites were associated
with temperatures above 80°F, and as a result, fish are now cultured at a constant 76°F. As a
preventative measure, tanks are treated monthly with a copper preparations (CopperSafe) to
avoid problems with common diseases such as Oodinum (ich) and velvet disease.
APPENDIX II: Seahorse Behavior Recorder

The behavior recorder was written in Visual Basic (4.0) to record the courtship and competitive behaviors of 2 or 3 seahorses at a time. In addition to the recorder, the program includes a statistical analysis module which uses the original data files to generate output files containing the calculated duration, frequency, and response patterns of behaviors.

Behavior Recorder:

The behavior recorder was designed to be used on an IBM Thinkpad to record the behaviors of seahorses while observing in front of aquaria. Courtship behaviors, competitive behaviors, and feeding and swimming are all behaviors that can be recorded. In addition, approaching, departing and general proximity can be indicated through short series of letters and numbers. Up to two tanks at a time can be observed using the recorder, with a toggle switch to alternate between tank records.

To use the program to record behaviors, choose the Record New option from the initial file menu. The next series of windows prompts the user for the name of the file, identities of the fish being observed (1 is always the opposite-sexed fish and 2 and 3 are the same-sex fish), and the day of courtship. Once entered, this information is placed at the beginning of the data file, visible once the recorder window opens. An independent timer records the duration of the observation period and must be started using the mouse. In addition, brightening in all fish must be triggered by the mouse, unless it is associated with courtship in which it starts automatically. All other behaviors (listed below) can be turned on or off with one letter codes and the fish number or the mouse. Once keyed in, all behaviors and commands are entered into the record with the current system time. In addition to the specific
behaviors recorded, comments can be placed into the data record with time signatures to explain observations. Both specific behavior entries and comments can be edited during observations using a line editor provided in the recorder window, or after observations using a standard word processing system, since data files are saved as simple text files.

Output Files

After an observation period, data files (*.dat) can be processed using the statistical module to calculate the durations of behaviors. In addition, this module has been programmed to organize

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightening</td>
<td>none</td>
<td>mouse driven unless associated with a specific courtship behavior, in which it triggers automatically</td>
</tr>
<tr>
<td>Courtship</td>
<td>none</td>
<td>mouse driven unless associated with a specific courtship behavior, in which it triggers automatically</td>
</tr>
<tr>
<td>Pumping</td>
<td>u</td>
<td>start and stop by letter and number of fish pumping</td>
</tr>
<tr>
<td>Holding</td>
<td>h - 1°</td>
<td>start and stop by letter, number of fish initiating hold, and number of fish receiving hold in that order.</td>
</tr>
<tr>
<td>Rising</td>
<td>r</td>
<td>start and stop by letter and number of fish rising; if two fish rising in sequence, the first will be indicated in the report as the initiator, the second the receiver</td>
</tr>
<tr>
<td>Copulating</td>
<td>o</td>
<td>start and stop by letter and then the two numbers of the fish copulating</td>
</tr>
<tr>
<td>Quiver</td>
<td>q</td>
<td>instantaneous behavior, indicate by letter and number of fish quivering</td>
</tr>
<tr>
<td>Point</td>
<td>p</td>
<td>instantaneous behavior, indicate by letter and number of fish pointing</td>
</tr>
<tr>
<td>Intruding</td>
<td>l</td>
<td>start and stop by letter and number of intruder</td>
</tr>
<tr>
<td>Wrestling</td>
<td>w</td>
<td>start and stop by letter, number of fish initiating wrestle, and number of fish receiving wrestle</td>
</tr>
<tr>
<td>Snap</td>
<td>s</td>
<td>instantaneous behavior; indicate by letter, number of fish snapping and number of fish at which snap was directed</td>
</tr>
<tr>
<td>Swimming</td>
<td>m</td>
<td>start and stop by letter and number of fishing swimming</td>
</tr>
<tr>
<td>Feeding</td>
<td>e</td>
<td>instantaneous behavior; indicate by letter and number of fish that fed</td>
</tr>
<tr>
<td>Territory</td>
<td>y</td>
<td>Indicates region of tank that fish is in (1,2 or N [neutral]). Indicate by letter and number of fish</td>
</tr>
<tr>
<td>Comment</td>
<td>x</td>
<td>use letter to immediately jump to the comment window to record text in data file</td>
</tr>
</tbody>
</table>

the courtship behaviors into the phases of seahorse courtship (see Chapter 4 for full description of seahorse courtship phases). Output files have two sections: 1) the durations, frequency and initiators and receivers of specific behaviors, including added comments, all in

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order of their associated time signatures, and 2) the phase analysis section, which summarizes all behavioral frequencies and durations by courtship phase (see attached for sample output file).

To use the analysis program to summarize a data file, highlight a data file (*.dat) in the opening window and select Run Statistics from the file menu. This will allow you to choose to analyze your data for either 2 males and 1 female or 1 male and 2 females. There are differences in the analysis of these two types of observations because phases are determined by specific sexes performing specific behaviors, and the program needs to be able to determine which fish is male and which fish is female for this purpose. If files are not in the proper format (all behaviors with durations must have start and stop codes, spacing must be correct, etc.), the analysis program will prompt the user for the appropriate changes and jump the editing window at the spot in the file in which the error occurred. When all errors are corrected, the analysis program will run for a few minutes and the user will get a message to indicate that it was successfully completed. After the analysis, push the button on the opening window to toggle between *.dat files and *.out files to locate the file that was analyzed. These files can be printed and edited from any word processing program.
SAMPLE OUTPUT FILE FROM BEHAVIOR RECORDER

Seahorse Behavior Recorder Version 2.04 (6/12/96)
Data File: c476061.dar
6/6/96 Tank 1, Day of Courtship = 3
Fish1 = f133, Fish2 = m161, Fish3 = m125
Female Choice = 1F:2M
8:21:16 AM Latency to Court = 0:07:11 (7.183 mins)
8:28:26 AM 1 Approaches 3 Close
8:28:27 AM Phase 1 Starts
8:28:27 AM Court Bout 1, Fish 1 = 0:01:02 (1.033 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0,
   Points = 6
8:28:27 AM Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 3, Resp beh: Quiver, # repeats: 1, time
to resp: 0:00:02 (0.033 mins)
8:28:27 AM Court Bout Transition Matrix: No Response: 0 (0.00%), Points: f2 = 0 (0.00%), f3 = 0 (0.00%), Pumps:
f2 = 0 (0.00%), f3 = 0 (0.00%), Quivers: f2 = 0 (0.00%), f3 = 6 (100.00%), Rises: f2 = 0 (0.00%), f3 = 0 (0.00%)
8:28:27 AM Bright Bout 1, Fish 1 = 0:05:34 (5.567 mins)
8:28:29 AM Court Bout 1, Fish 3 = 0:01:00 (1.000 mins), Rece, Totals: Pumps = 1, Pumps Prior = 0, Quivers = 6,
   Points = 1
8:28:29 AM Bright Bout 1, Fish 3 = 1:53:17 (113.283 mins)
8:28:31 AM Swim Bout 1, Fish 2 = 0:02:02 (2.033 mins)
8:28:47 AM Hold Bout 1: Fish 1, 3 = 0:00:03 (0.050 mins)
8:28:49 AM Comment: at tail
8:28:52 AM Swim Bout 1, Fish 1 = 0:00:08 (0.133 mins)
8:28:54 AM 1 Departs 3 Close
8:28:57 AM Pump Bout 1, Fish 3 = 0:00:03 (0.050 mins)
8:29:12 AM 3 Approaches 1 Close
8:29:28 AM Swim Bout 2, Fish 1 = 0:01:05 (1.083 mins)
8:29:29 AM 1 Departs 3 Far
8:30:28 AM Bright Bout 1, Fish 2 = 1:48:59 (108.983 mins)
8:30:31 AM 2 Approaches 1 Close
8:30:38 AM Comment: on airline mid
8:30:49 AM Swim Bout 2, Fish 2 = 0:00:21 (0.350 mins)
8:30:49 AM Court Bout 2, Fish 1 = 0:02:25 (2.417 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 1,
   Points = 6
8:30:49 AM Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 2, Resp beh: Quiver, # repeats: 3, time
to resp: 0:00:31 (0.517 mins)
8:30:49 AM Court Bout Transition Matrix: No Response: 3 (50.00%), Points: f2 = 0 (0.00%), f3 = 0 (0.00%), Pumps:
f2 = 0 (0.00%), f3 = 0 (0.00%), Quivers: f2 = 3 (50.00%), f3 = 0 (0.00%), Rises: f2 = 0 (0.00%), f3 = 0 (0.00%)
8:31:20 AM Court Bout 1, Fish 2 = 0:01:52 (1.867 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 3,
   Points = 0
8:33:10 AM 2 Departs 1 Far
8:33:14 AM Swim Bout 3, Fish 2 = 0:00:17 (0.283 mins)
8:33:14 AM Swim Bout 1, Fish 3 = 0:00:17 (0.283 mins)
8:33:18 AM 3 Approaches 1 Close
8:33:26 AM 3 Departs 1 Far
8:33:36 AM Comment: 2 on wire, 3 on rt p
8:33:45 AM Comment: 3 pouch a 2-3
8:33:46 AM Pump Bout 1, Fish 3 = 0:00:02 (0.033 mins)
8:34:25 AM Swim Bout 4, Fish 2 = 0:00:09 (0.150 mins)
8:34:27 AM 2 Approaches 3 Close
8:34:43 AM Pump Bout 2, Fish 3 = 0:00:03 (0.050 mins)
8:34:48 AM 3 Departs 2 Far
8:34:51 AM 3 Approaches 1 Close
8:35:09 AM Swim Bout 2, Fish 3 = 0:00:02 (0.033 mins)
8:35:09 AM Swim Bout 5, Fish 2 = 0:00:52 (0.867 mins)
8:35:09 AM Court Bout 3, Fish 1 = 0:01:45 (1.750 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 8
8:35:09 AM Court Bout Transition Matrix: No Response: 0 (0.00%), Points: f2= 0 (0.00%), f3= 0 (0.00%), Pumps: f2= 0 (0.00%), f3= 1 (12.50%), Quivers: f2= 4 (50.00%), f3= 3 (37.50%), Rises: f2= 0 (0.00%), f3= 0 (0.00%)
8:35:09 AM Bright Bout 2, Fish 1 = 0:07:44 (7.733 mins)
8:35:13 AM Court Bout 2, Fish 3 = 0:00:23 (0.383 mins), Rece, Totals: Pumps = 0, Pumps Prior = 2, Quivers = 5, Points = 0
8:35:27 AM 2 Approaches 13 Close
8:35:35 AM 1 Departs 23 Far
8:35:37 AM Swim Bout 3, Fish 1 = 0:00:35 (0.583 mins)
8:35:44 AM 1 Approaches 23 Close
8:35:44 AM Hold Bout 2: Fish 2, 1 = 0:00:39 (0.650 mins)
8:35:48 AM Comment: at tail
8:35:54 AM Pump Bout 1, Fish 3 = 0:00:02 (0.033 mins)
8:35:58 AM Court Bout 2, Fish 2 = 0:00:57 (0.950 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 6, Points = 0
8:35:58 AM Court Bout First Behaviors: 1st fish: 1, 1st.beh: Point, Resp fish: 3, Resp beh: Quiver, # repeats: 1, time to resp: 00:00:04 (0.067 mins)
8:36:02 AM 3 Departs 12 Far
8:36:16 AM Comment: 1 2 at rt p
8:36:36 AM Pump Bout 2, Fish 3 = 0:00:02 (0.033 mins)
8:36:54 AM Swim Bout 3, Fish 3 = 0:01:09 (1.150 mins)
8:37:04 AM Hold Bout 3: Fish 1, 2 = 0:00:07 (0.117 mins)
8:37:06 AM Comment: at body
8:37:14 AM Swim Bout 6, Fish 2 = 0:00:07 (0.117 mins)
8:37:21 AM Hold Bout 4: Fish 2, 1 = 0:00:24 (0.400 mins)
8:37:23 AM Comment: at head
8:37:46 AM Swim Bout 7, Fish 2 = 0:01:01 (1.017 mins)
8:37:49 AM 2 Departs 1 Far
8:37:52 AM 2 Approaches 1 Close
8:38:00 AM Comment: 1 hanging upside down
8:38:06 AM Comment: high airline
8:38:11 AM 2 Departs 1 Far
8:38:15 AM Pump Bout 1, Fish 2 = 0:00:04 (0.067 mins)
8:38:35 AM 2 Approaches 1 Close
8:38:43 AM Comment: 1 upside down
8:38:51 AM 2 Departs 1 Far
8:39:12 AM Swim Bout 4, Fish 3 = 0:00:38 (0.633 mins)
8:39:14 AM Swim Bout 8, Fish 2 = 0:00:29 (0.483 mins)
8:39:46 AM Comment: 2 on lift plt
8:39:48 AM Swim Bout 9, Fish 2 = 0:00:09 (0.150 mins)
8:39:53 AM Comment: 3 on wire
8:39:57 AM 2 Approaches 1 Close
8:39:59 AM Court Bout 3, Fish 2 = 0:00:27 (0.450 mins), Init, Totals: Pumps = 0, Pumps Prior = 1, Quivers = 3, Points = 0
8:39:59 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 0, Resp beh: , # repeats: 3, time to resp: 00:00:00 (0.000 mins)
8:40:21 AM Comment: 1 fed and 2 jumped and departed
8:40:23 AM Swim Bout 10, Fish 2 = 0:00:18 (0.300 mins)
8:40:24 AM 2 Departs 1 Far

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8:40:29 AM Pump Bout 3, Fish 3 = 0:00:07 (0.117 mins)
8:40:38 AM 2 Approaches 1 Close
8:40:48 AM Comment: 3 shift down to low airline
8:40:55 AM Court Bout 4, Fish 2 = 0:00:10 (0.167 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 2, Points = 0
8:40:55 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 1, Resp beh: Point, # repeats: 1, time to resp: 0:00:01 (0.017 mins)
8:40:56 AM Warning: Potential phase change while fish 2 courting
8:40:56 AM Court Bout 4, Fish 1 = 0:00:06 (0.100 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 1
8:40:56 AM Court Bout Transition Matrix: No Response: 0 (0.00%), Points: f2= 0 (0.00%), f3= 0 (0.00%), Pumps: f2= 0 (0.00%), f3= 0 (0.00%), Quivers: f2= 1 (100.00%), f3= 0 (0.00%), Rises: f2= 0 (0.00%), f3= 0 (0.00%)
8:41:05 AM Swim Bout 11, Fish 2 = 0:01:09 (1.150 mins)
8:41:10 AM Swim Bout 5, Fish 3 = 0:00:02 (0.033 mins)
8:41:16 AM Comment: back at low airline
8:41:28 AM Pump Bout 4, Fish 3 = 0:00:06 (0.160 mins)
8:42:11 AM 2 Approaches 1 Close
8:42:18 AM Swim Bout 6, Fish 3 = 0:00:15 (0.250 mins)
8:42:39 AM Comment: 3 on wire
8:42:42 AM Swim Bout 12, Fish 2 = 0:00:19 (0.317 mins)
8:42:57 AM Pump Bout 1, Fish 2 = 0:00:03 (0.050 mins)
8:43:03 AM Comment: 2 on lift plt
8:43:05 AM Swim Bout 13, Fish 2 = 0:00:14 (0.233 mins)
8:43:08 AM Pump Bout 5, Fish 2 = 0:00:05 (0.083 mins)
8:43:14 AM 2 Approaches 1 Close
8:43:43 AM Court Bout 5, Fish 2 = 0:00:03 (0.050 mins), Init, Totals: Pumps = 0, Pumps Prior = 1, Quivers = 0, Points = 1
8:43:43 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Point, Resp fish: 0, Resp beh: , # repeats: 1, time to resp: 0:00:00 (0.000 mins)
8:43:54 AM Comment: was real
8:44:15 AM Swim Bout 14, Fish 2 = 0:01:01 (1.017 mins)
8:44:19 AM 2 Departs 1 Far
8:44:27 AM Pump Bout 6, Fish 3 = 0:00:03 (0.050 mins)
8:44:40 AM Comment: pouch is getting big, but 2's is not
8:44:45 AM Swim Bout 4, Fish 1 = 0:00:20 (0.333 mins)
8:45:09 AM Comment: 1 on lift plt
8:45:15 AM 2 Approaches 1 Close
8:45:43 AM Swim Bout 15, Fish 2 = 0:04:17 (4.283 mins)
8:49:02 AM 2 Departs 1 Far
8:49:05 AM 2 Approaches 3 Close
8:49:46 AM Pump Bout 7, Fish 3 = 0:00:02 (0.033 mins)
8:49:50 AM 2 Departs 3 Far
8:49:56 AM 2 Approaches 1 Close
8:52:03 AM Comment: 2 trying to interact, but 1 upside down
8:52:43 AM 2 Departs 1 Far
8:52:46 AM Swim Bout 7, Fish 3 = 0:00:01 (0.017 mins)
8:52:50 AM Comment: 3 on rt p
8:52:53 AM 2 Approaches 3 Close
8:53:05 AM Pump Bout 8, Fish 3 = 0:00:02 (0.033 mins)
8:53:31 AM 2 Approaches 1 Close
8:53:33 AM Hold Bout 5: Fish 1, 2 = 0:00:13 (0.217 mins)
8:53:36 AM Comment: at tail
8:53:55 AM Court Bout 5, Fish 1 = 0:00:25 (0.417 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 2
Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 0, Resp beh: , # repeats: 2, time to resp: 0:00:00 (0.000 mins)

Court Bout Transition Matrix: No Response: 2 (100.00%), Points: 2=0 (0.00%), 3=0 (0.00%), Pumps: 2=0 (0.00%), 3=0 (0.00%), Quivers: 2=0 (0.00%), 3=0 (0.00%), Rises: 2=0 (0.00%), 3=0 (0.00%)

Bright Bout 3, Fish 1 = 0:33:59 (33.983 mins)

Comment: promenading

Swim Bout 5, Fish 1 = 0:00:15 (0.250 mins)

Swim Bout 16, Fish 2 = 0:00:16 (0.267 mins)

Comment: lft plt

Comment: 2 kept trying to grab 1’s tail, but she kept leaning into him, thus moving her tail away

Swim Bout 17, Fish 2 = 0:01:16 (1.267 mins)

2 Departs 1 Far

2 Approaches 1 Close

Comment: 1 turned upside down on 2’s approach

Swim Bout 18, Fish 2 = 0:09:38 (9.633 mins)

2 Departs 1 Far

2 Approaches 1 Close

Comment: 1 upside down again - as he leaves, she rights, as he returns, she goes upside down

Pump Bout 9, Fish 3 = 0:00:03 (0.050 mins)

Swim Bout 8, Fish 3 = 0:00:45 (0.750 mins)

Swim Bout 6, Fish 1 = 0:01:07 (1.117 mins)

Comment: 3 on wire

1 Approaches 3 Close

Comment: 1 upside down

2 Approaches 13 Close

2 Departs 13 Far

2 Departs 9 Far

2 Departs 1 Far

3 Departs 1 Far

Comment: 3 on airline

2 on lft plt

Comment: 2 3 "arm wrestling" on wire near female - 3 btn 12 and 2 pushing against 3

Swim Bout 10, Fish 3 = 0:00:07 (0.117 mins)

Swim Bout 19, Fish 2 = 0:02:36 (2.600 mins)

2 Departs 13 Far

3 Departs 1 Far

Comment: 3 on airline

Swim Bout 11, Fish 3 = 0:00:06 (0.100 mins)

3 Approaches 1 Close

1 upside down and 3 at same tail attachment on top

bright and erect

Swim Bout 12, Fish 3 = 0:00:29 (0.483 mins)

3 Departs 1 Far

Comment: 3 on r p

2 Approaches 3 Close

Swim Bout 20, Fish 2 = 0:03:55 (3.917 mins)

2 Departs 3 Far

Pump Bout 10, Fish 3 = 0:00:07 (0.117 mins)

2 Approaches 1 Close

Comment: 2 swimming in ft of 1 - she upside down on arrival

2 Departs 1 Far

Swim Bout 7, Fish 1 = 0:00:28 (0.467 mins)

2 Approaches 1 Close

Swim Bout 13, Fish 3 = 0:00:38 (0.633 mins)
9:32:02 AM 3 Approaches 12 Close
9:32:16 AM Comment: 1 hanging on underside of heater upside down
9:32:23 AM Comment: 2 3 swimming in ft of her
9:32:37 AM 3 Departs 12 Far
9:32:46 AM Comment: 3 wire high
9:32:52 AM Swim Bout 8, Fish 1 = 0:01:18 (1.300 mins)
9:32:53 AM Hold Bout 6: Fish 2, 1 = 0:01:00 (1.000 mins)
9:32:58 AM Comment: at tail
9:33:40 AM Comment: 1 leaning toward 2 - a posture that seems to be avoidance of interaction
9:33:46 AM Comment: like a flatten
9:34:20 AM Comment: at lift pt
9:34:22 AM Court Bout 6, Fish 2 = 0:01:13 (1.217 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 1, Points = 0
9:34:22 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 0, Resp beh: , # repeats: 1, time to resp: 0:00:00 (0.000 mins)
9:34:36 AM Hold Bout 7: Fish 2, 1 = 0:01:44 (1.733 mins)
9:34:38 AM Comment: at head
9:37:16 AM Court Bout 7, Fish 2 = 0:00:04 (0.067 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 2, Points = 0
9:37:16 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 0, Resp beh: , # repeats: 2, time to resp: 0:00:00 (0.000 mins)
9:37:23 AM Comment: 1 upside down
9:37:55 AM Pump Bout 11, Fish 3 = 0:00:09 (0.150 mins)
9:38:10 AM Court Bout 8, Fish 2 = 0:03:03 (3.050 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 6, Points = 0
9:38:10 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 3, Resp beh: Pump, # repeats: 4, time to resp: 0:02:36 (2.600 mins)
9:40:03 AM Swim Bout 14, Fish 3 = 0:00:31 (0.517 mins)
9:40:46 AM Pump Bout 12, Fish 3 = 0:00:03 (0.050 mins)
9:40:52 AM Comment: 3 on r p
9:41:01 AM Comment: 1 still upside down
9:41:13 AM Hold Bout 8: Fish 2, 1 = 0:00:24 (0.400 mins)
9:41:17 AM Comment: at tail
9:41:20 AM Swim Bout 9, Fish 1 = 0:00:14 (0.233 mins)
9:41:23 AM 1 Departs 2 Close
9:41:25 AM 2 Approaches 1 Close
9:42:39 AM Hold Bout 9: Fish 2, 1 = 0:00:23 (0.383 mins)
9:42:41 AM Comment: at body
9:42:57 AM Court Bout 9, Fish 2 = 0:00:26 (0.433 mins), Init, Totals: Pumps = 1, Pumps Prior = 0, Quivers = 2, Points = 0
9:42:57 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 0, Resp beh: , # repeats: 3, time to resp: 0:00:00 (0.000 mins)
9:43:12 AM 2 Departs 1 Far
9:43:14 AM Pump Bout 1, Fish 2 = 0:00:01 (0.017 mins)
9:45:56 AM Swim Bout 10, Fish 1 = 0:00:36 (0.600 mins)
9:45:56 AM Swim Bout 21, Fish 2 = 0:00:36 (0.600 mins)
9:45:56 AM Court Bout 6, Fish 1 = 0:00:44 (0.733 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 1
9:45:56 AM Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 2, Resp beh: Quiver, # repeats: 1, time to resp: 0:00:32 (0.533 mins)
9:45:56 AM Court Bout Transition Matrix: No Response: 0 (0.00%), Points: 0 (0.00%), 0 (0.00%), Pumps: 0 (0.00%), Quivers: 0 (100.00%), Rises: 0 (0.00%), 0 (0.00%)
9:45:56 AM Bright Bout 4, Fish 1 = 0:33:29 (33.483 mins)
9:46:24 AM Comment: 1 2 interacting while swimming
9:46:28 AM Court Bout 10, Fish 2 = 0:00:11 (0.183 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 1, Points = 0
9:46:53 AM Comment: 1 hanging upside
9:48:16 AM Court Bout 11, Fish 2 = 0:00:31 (0.517 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 4, Points = 0
9:48:16 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 0, Resp beh: , # repeats: 4, time to resp: 0:00:00 (0.000 mins)
9:48:32 AM Comment: 3 on lower airline
9:49:10 AM Swim Bout 22, Fish 2 = 0:00:04 (0.067 mins)
9:49:11 AM 2 Departs 1 Far
9:49:23 AM Comment: 2 on rt p
9:49:31 AM Swim Bout 11, Fish 1 = 0:00:03 (0.050 mins)
9:49:33 AM 1 Approaches 2 Close
9:49:37 AM Comment: 1 2 on rt p
9:49:46 AM Comment: 1 trying to engage 2
9:50:48 AM Pump Bout 1, Fish 2 = 0:00:02 (0.033 mins)
9:53:18 AM Comment: 2 trying to engage 1
9:53:24 AM Pump Bout 13, Fish 3 = 0:00:04 (0.067 mins)
9:53:28 AM Pump Bout 14, Fish 3 = 0:00:09 (0.150 mins)
9:53:41 AM Comment: 1 righted
9:54:20 AM Hold Bout 10: Fish 2, 1 = 0:00:05 (0.083 mins)
9:54:22 AM Comment: at head
9:54:26 AM Court Bout 12, Fish 2 = 0:00:55 (0.917 mins), Init, Totals: Pumps = 0, Pumps Prior = 1, Quivers = 5, Points = 0
9:54:49 AM Pump Bout 15, Fish 3 = 0:00:05 (0.083 mins)
9:55:11 AM Warning: Potential phase change while fish 2 courting
9:55:11 AM Court Bout 7, Fish 1 = 0:00:11 (0.183 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 1, Points = 1
9:55:11 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 3, Resp beh: Pump, # repeats: 2, time to resp: 0:00:23 (0.383 mins)
9:55:11 AM Court Bout Transition Matrix: No Response: 1 (100.00%), Points: 0 (0.00%), 0 (0.00%), Pumps: 0 (0.00%), 0 (0.00%), Quivers: 0 (0.00%), 0 (0.00%), Risers: 0 (0.00%), 0 (0.00%), 0 (0.00%)
9:55:16 AM 2 Departs 1 Far
9:55:22 AM Swim Bout 23, Fish 2 = 0:01:12 (1.200 mins)
9:55:31 AM Swim Bout 12, Fish 1 = 0:00:18 (0.300 mins)
9:55:31 AM Latency Period = 1:24:24 (84.400 mins): Pumps: fish1 = 0, freq= 0; fish2 = 7, freq= 4.97; fish3 = 27, freq= 19.1
9:55:38 AM Swim Bout 15, Fish 3 = 0:00:42 (0.700 mins)
9:55:50 AM Comment: airline
9:56:33 AM 2 Departs 13 Far
9:56:36 AM Comment: rt p
9:56:49 AM Comment: 1 upside down
9:56:58 AM Swim Bout 16, Fish 3 = 0:00:45 (0.750 mins)
9:56:59 AM 3 Departs 1 Far
9:57:07 AM 3 Approaches 2 Close
9:57:08 AM Swim Bout 24, Fish 2 = 0:00:34 (0.567 mins)
9:57:09 AM 2 Departs 1 Far
9:57:44 AM Swim Bout 13, Fish 1 = 0:00:41 (0.683 mins)
9:58:25 AM Swim Bout 17, Fish 3 = 0:00:05 (0.083 mins)
9:58:27 AM 3 Approaches 1 Close
9:58:30 AM Hold Bout 11: Fish 3, 1 = 0:02:02 (2.033 mins)
9:58:34 AM Comment: at body
10:00:17 AM Swim Bout 25, Fish 2 = 0:00:19 (0.317 mins)
10:00:32 AM Swim Bout 18, Fish 3 = 0:00:50 (0.833 mins)
10:00:33 AM 3 Departs 1 Far
10:00:38 AM Comment: low wire
10:01:11 AM Pump Bout 1, Fish 2 = 0:00:02 (0.033 mins)
10:01:25 AM Comment: 3 on rt p
10:01:36 AM Swim Bout 19, Fish 3 = 0:00:05 (0.083 mins)
10:01:40 AM 3 Approaches 2 Close
10:02:35 AM Pump Bout 16, Fish 3 = 0:00:04 (0.067 mins)
10:03:11 AM Swim Bout 20, Fish 3 = 0:03:04 (3.067 mins)
10:03:19 AM 3 Approaches 1 Close
10:05:18 AM Comment: 3 trying to engage 1
10:05:25 AM 3 Departs 1 Far
10:06:11 AM Pump Bout 2, Fish 2 = 0:00:02 (0.033 mins)
10:06:14 AM Swim Bout 26, Fish 2 = 0:03:54 (3.900 mins)
10:06:21 AM Comment: 3 just below 1
10:06:23 AM Court Bout 3, Fish 3 = 0:00:15 (0.250 mins), Init, Totals: Pumps = 0, Pumps Prior = 16, Quivers = 3, Points = 0
10:06:23 AM Court Bout First Behaviors: 1st fish: 3, 1st beh: Quiver, Resp fish: 0, Resp beh: , # repeats: 3, time to resp: 0:00:00 (0.000 mins)
10:07:16 AM Pump Bout 1, Fish 3 = 0:00:09 (0.150 mins)
10:08:17 AM Pump Bout 2, Fish 3 = 0:00:02 (0.033 mins)
10:08:40 AM Comment: 3 hanging upside down - 1 upright
10:10:01 AM 3 Departs 1 Far
10:10:06 AM Comment: 3 shift
10:10:11 AM Comment: 2 on rt p
10:10:16 AM Swim Bout 27, Fish 2 = 0:09:54 (9.900 mins)
10:10:37 AM 2 Approaches 3 Close
10:10:55 AM Swim Bout 21, Fish 3 = 0:00:08 (0.133 mins)
10:11:07 AM Comment: 3 on wire mid
10:11:11 AM 2 Departs 3 Far
10:11:24 AM Swim Bout 22, Fish 3 = 0:00:10 (0.167 mins)
10:11:28 AM 3 Approaches 1 Close
10:11:45 AM Comment: wire mid
10:12:11 AM 2 Approaches 1 Close
10:13:22 AM Pump Bout 3, Fish 3 = 0:00:05 (0.083 mins)
10:13:50 AM Swim Bout 23, Fish 3 = 0:00:02 (0.033 mins)
10:14:07 AM Comment: 3 on airline 5 cm below 1
10:14:08 AM Pump Bout 4, Fish 3 = 0:00:07 (0.117 mins)
10:19:19 AM Pump Bout 5, Fish 3 = 0:00:03 (0.050 mins)
10:20:17 AM Comment: 2 on 1ft plt
10:20:19 AM Pump Bout 6, Fish 3 = 0:00:03 (0.050 mins)
10:20:36 AM Comment: 2 rubbing against hold - 3 rubbing too
10:21:37 AM Pump Bout 7, Fish 3 = 0:00:04 (0.067 mins)
10:21:38 AM Swim Bout 28, Fish 2 = 0:00:36 (0.600 mins)
10:21:57 AM Comment: 1 rubbing too
10:22:16 AM Comment: rt p
10:22:17 AM Swim Bout 24, Fish 3 = 0:00:50 (0.833 mins)
10:22:20 AM 3 Approaches 1 Close
10:22:23 AM Bright Bout 2, Fish 3 = 2:12:14 (132.233 mins)
10:22:33 AM Comment: 1 turns upside down
10:22:35 AM Pump Bout 8, Fish 3 = 0:00:05 (0.083 mins)
10:22:49 AM Comment: 3 swimming and pumping
10:23:11 AM Comment: 3 on mid airline
10:23:14 AM 3 Departs 1 Far

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10:30:05 AM Swim Bout 29, Fish 2 = 0:00:08 (0.133 mins)
10:30:12 AM Swim Bout 25, Fish 3 = 0:00:02 (0.033 mins)
10:30:19 AM Comment: both on low airline
10:30:21 AM 2 Approaches 3 Close
10:30:25 AM Pump Bout 9, Fish 3 = 0:00:04 (0.067 mins)
10:32:25 AM 2 Departs 3 Far
10:32:25 AM Swim Bout 30, Fish 2 = 0:05:29 (5.483 mins)
10:33:06 AM Comment: as 2 left, made a snapping gesture, but not at 3 directly - was not a feed, though
10:34:18 AM Pump Bout 10, Fish 3 = 0:00:02 (0.033 mins)
10:34:21 AM Pump Bout 3, Fish 2 = 0:00:00 (0.000 mins)
10:34:22 AM Swim Bout 26, Fish 3 = 0:00:32 (0.533 mins)
10:34:25 AM 3 Approaches 1 Close
10:34:27 AM 2 Approaches 13 Close
10:34:35 AM Hold Bout 12: Fish 3, 2 = 0:00:14 (0.233 mins)
10:34:36 AM Comment: at tail
10:34:44 AM Comment: 3 pulling 2 away from female
10:34:49 AM 2 Departs 1 Far
10:34:50 AM Pump Bout 11, Fish 3 = 0:00:02 (0.033 mins)
10:34:57 AM Comment: 3 on mid airline
10:35:00 AM 2 Approaches 3 Close
10:35:02 AM Comment: 2 swimming near him
10:35:31 AM 2 Departs 3 Far
10:35:34 AM Pump Bout 12, Fish 3 = 0:00:05 (0.083 mins)
10:36:01 AM Pump Bout 13, Fish 3 = 0:00:07 (0.117 mins)
10:36:25 AM Swim Bout 27, Fish 3 = 0:00:11 (0.183 mins)
10:36:34 AM 3 Approaches 2 Close
10:36:40 AM Comment: 3 on wire
10:36:47 AM Comment: 2 swimming in ft
10:37:01 AM 2 Approaches 1 Close
10:37:07 AM Swim Bout 28, Fish 3 = 0:00:24 (0.400 mins)
10:37:09 AM 3 Approaches 12 Close
10:37:18 AM Comment: 1 shift upside down
10:37:35 AM Comment: 2 on wire high
10:37:37 AM 3 Departs 1 Far
10:37:41 AM 2 Departs 1 Far
10:37:44 AM 2 Approaches 3 Close
10:37:58 AM Comment: 2 just above 3 on wire
10:38:02 AM Pump Bout 14, Fish 3 = 0:00:09 (0.150 mins)
10:38:19 AM Comment: 1 righted again
10:39:49 AM Comment: 3 erect and starting to interact w 2
10:40:10 AM Comment: 3 upright and 2 upside down
10:43:11 AM Swim Bout 29, Fish 3 = 0:00:05 (0.083 mins)
10:43:12 AM 3 Departs 2 Far
10:43:15 AM 3 Approaches 1 Close
10:43:27 AM Comment: 1 in ct position
10:43:36 AM Comment: 1 upright
10:43:40 AM Bright Bout 5, Fish 1 = 0:07:07 (7.117 mins)
10:44:07 AM Swim Bout 31, Fish 2 = 0:00:34 (0.567 mins)
10:44:11 AM 2 Approaches 13 Close
10:44:12 AM Pump Bout 15, Fish 3 = 0:00:07 (0.117 mins)
10:44:20 AM Swim Bout 30, Fish 3 = 0:00:25 (0.417 mins)
10:44:22 AM 3 Departs 1 Far
10:44:26 AM 2 Departs 1 Far
10:44:30 AM Hold Bout 13: Fish 3, 2 = 0:00:06 (0.100 mins)
10:44:32 AM Comment: at tail
10:44:36 AM 2 Departs 3 Far
10:44:42 AM Comment: rt p
10:44:57 AM Comment: low airline
10:45:01 AM Swim Bout 32, Fish 2 = 0:04:47 (4.783 mins)
10:45:02 AM Pump Bout 4, Fish 2 = 0:00:03 (0.050 mins)
10:45:17 AM 2 Approaches 3 Close
10:45:32 AM Pump Bout 16, Fish 3 = 0:00:03 (0.050 mins)
10:46:30 AM 2 Departs 3 Far
10:46:35 AM 2 Approaches 3 Close
10:49:51 AM Comment: on heater
10:50:00 AM Pump Bout 17, Fish 3 = 0:00:09 (0.150 mins)
10:50:29 AM Swim Bout 31, Fish 3 = 0:00:05 (0.083 mins)
10:50:39 AM Comment: 5 cm below 1 on airline
10:55:13 AM Swim Bout 33, Fish 2 = 0:00:22 (0.367 mins)
10:55:16 AM 2 Approaches 1 Close
10:55:22 AM Hold Bout 14: Fish 2, l = 0:00:10 (0.167 mins)
10:55:25 AM Comment: at neck
10:55:31 AM Comment: 1 upside down on 2's app
10:55:43 AM Comment: just below upside down female
10:55:56 AM Comment: 3 on lower plant rt
10:56:00 AM Swim Bout 34, Fish 2 = 0:01:00 (1.000 mins)
10:56:01 AM 2 Departs 1 Far
10:56:35 AM 2 Approaches 1 Close
10:56:35 AM Swim Bout 14, Fish 1 = 0:00:03 (0.050 mins)
10:56:43 AM Comment: 1 shift down airline
10:56:52 AM Comment: upside down again
10:56:58 AM Comment: 2 swimming around her
10:57:05 AM Comment: just below
10:57:34 AM Pump Bout 5, Fish 2 = 0:00:02 (0.033 mins)
10:57:37 AM Swim Bout 35, Fish 2 = 0:00:02 (0.033 mins)
10:57:42 AM Comment: 2 on high airline
11:00:37 AM Swim Bout 36, Fish 2 = 0:00:03 (0.050 mins)
11:00:39 AM 2 Approaches 1 Close
11:00:43 AM Comment: just below 2
11:03:04 AM Pump Bout 18, Fish 3 = 0:00:03 (0.050 mins)
11:03:23 AM Comment: 1 slide down to 2
11:03:27 AM 1 Approaches 2 Close
11:03:31 AM Swim Bout 32, Fish 3 = 0:00:01 (0.017 mins)
11:03:35 AM Comment: 3 on high wire
11:04:05 AM Swim Bout 37, Fish 2 = 0:00:26 (0.433 mins)
11:04:07 AM 2 Departs 1 Far
11:04:12 AM Swim Bout 33, Fish 3 = 0:00:19 (0.317 mins)
11:04:30 AM 2 Approaches 3 Close
11:04:34 AM Comment: high wire
11:04:49 AM Comment: wrestling - pushing against each other with sides
11:04:50 AM Pump Bout 19, Fish 3 = 0:00:04 (0.067 mins)
11:05:03 AM Comment: 3 hanging upside down - no longer wrestling
11:05:23 AM Swim Bout 34, Fish 3 = 0:00:03 (0.050 mins)
11:05:26 AM 3 Departs 2 Far
11:05:35 AM Comment: 3 on high airline
11:06:04 AM Pump Bout 20, Fish 3 = 0:00:03 (0.050 mins)
11:06:12 AM Comment: 3 shift down to mid airline
11:06:51 AM Pump Bout 6, Fish 2 = 0:00:02 (0.033 mins)

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11:06:53 AM  Pump Bout 21, Fish 3 = 0:00:02 (0.033 mins)
11:07:09 AM  Pump Bout 7, Fish 2 = 0:00:01 (0.017 mins)
11:07:13 AM  3 Approaches 1 Close
11:07:46 AM  3 Approaches 1 Close
11:07:54 AM  Comment: 3 shifts into court position
11:08:05 AM  Comment: 3 shift below 1
11:08:10 AM  3 Departs 1 Close
11:09:02 AM  Comment: 1 hanging upside down, but seems to be trying to get closer to 3, not avoid interaction
11:09:24 AM  Comment: 2 pouch still small - barely a 1
11:10:11 AM  Swim Bout 38, Fish 2 = 0:01:09 (1.150 mins)
11:10:19 AM  2 Approaches 13 Close
11:10:24 AM  2 Departs 13 Far
11:11:24 AM  Comment: 2 on lft plt
11:11:31 AM  Comment: 3 righted himself
11:11:36 AM  Comment: very bright
11:11:44 AM  Pump Bout 22, Fish 3 = 0:00:06 (0.100 mins)
11:11:48 AM  Swim Bout 39, Fish 2 = 0:00:21 (0.350 mins)
11:11:52 AM  Swim Bout 35, Fish 3 = 0:00:07 (0.117 mins)
11:11:54 AM  3 Departs 1 Far
11:11:57 AM  3 Approaches 1 Close
11:12:02 AM  Comment: just above her
11:12:11 AM  Comment: 2 on rt plt
11:12:39 AM  Comment: 3 hanging down next to 1, trying to engage
11:12:44 AM  Pump Bout 23, Fish 3 = 0:00:07 (0.117 mins)
11:13:13 AM  Swim Bout 40, Fish 2 = 0:00:05 (0.083 mins)
11:13:22 AM  Comment: on top of filter
11:13:48 AM  Swim Bout 36, Fish 3 = 0:00:22 (0.367 mins)
11:13:53 AM  3 Departs 1 Far
11:14:05 AM  3 Approaches 1 Close
11:14:18 AM  Comment: 3 in ct position, 1 upside down
11:15:42 AM  Swim Bout 37, Fish 3 = 0:00:31 (0.517 mins)
11:15:45 AM  3 Departs 1 Far
11:16:23 AM  Comment: 3 on low wire
11:16:49 AM  Comment: 1 upright again
11:17:10 AM  Pump Bout 24, Fish 3 = 0:00:08 (0.133 mins)
11:17:15 AM  Swim Bout 15, Fish 1 = 0:00:22 (0.367 mins)
11:17:21 AM  Swim Bout 38, Fish 3 = 0:00:07 (0.117 mins)
11:17:31 AM  Comment: 3 on high airline
11:17:40 AM  Comment: 1 on low wire
11:18:00 AM  Pump Bout 25, Fish 3 = 0:00:09 (0.150 mins)
11:18:44 AM  Pump Bout 26, Fish 3 = 0:00:06 (0.100 mins)
11:19:30 AM  Comment: 1 upright on wire
11:19:32 AM  Swim Bout 39, Fish 3 = 0:00:22 (0.367 mins)
11:19:34 AM  3 Approaches 1 Close
11:19:46 AM  Comment: 3 very slowly approaching
11:19:55 AM  Phase 2 Missing
11:19:55 AM  Phase 3 Starts
11:19:55 AM  Court Bout 8, Fish 1 = 0:01:32 (1.533 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 12
11:19:55 AM  Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 3, Resp beh: Quiver, # repeats: 2, time to resp: 0:00:08 (0.133 mins)
11:19:55 AM  Court Bout Transition Matrix: No Response: 10 (83.33%), Points: f2 = 0 (0.00%), f3 = 0 (0.00%), Pumps: f2 = 0 (0.00%), f3 = 0 (0.00%), Quivers: f2 = 0 (0.00%), f3 = 1 (8.33%), Rises: f2 = 0 (0.00%), f3 = 1 (8.33%)
11:19:55 AM Bright Bout 6, Fish 1 = 1:14:23 (74.383 mins)
11:19:58 AM Hold Bout 15: Fish 3, 1 = 0:00:25 (0.417 mins)
11:20:00 AM Comment: at tail
11:20:03 AM Swim Bout 16, Fish 1 = 0:00:22 (0.367 mins)
11:20:03 AM Court Bout 4, Fish 3 = 0:00:30 (0.500 mins), Rece, Totals: Pumps = 0, Pumps Prior = 26, Quivers = 1, Points = 0
11:20:04 AM Swim Bout 40, Fish 3 = 0:00:23 (0.383 mins)
11:20:06 AM Comment: promenading
11:20:30 AM Comment: at airline
11:20:41 AM 2 Approaches 13 Close
11:20:45 AM Rise Bout 1, Fish 1 = 0:00:04 (0.067 mins), Init
11:20:46 AM Rise Bout 1, Fish 3 = 0:00:03 (0.050 mins), Rece
11:20:46 AM Court Bout 5, Fish 3 = 0:00:07 (0.117 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 0
11:20:46 AM Swim Bout 41, Fish 2 = 0:01:08 (1.133 mins)
11:20:46 AM Swim Bout 41, Fish 3 = 0:01:09 (1.150 mins)
11:21:18 AM Comment: 2 swimming around pointing female
11:21:30 AM Comment: 3 pouch an 8, 2's a 2
11:21:44 AM Comment: 1 upside down now
11:22:02 AM Comment: 2 3 just below upside down female
11:22:21 AM Swim Bout 42, Fish 3 = 0:00:13 (0.217 mins)
11:22:30 AM Comment: 3 swimming around 1 2
11:22:38 AM Hold Bout 16: Fish 3, 2 = 0:00:25 (0.417 mins)
11:22:40 AM Comment: at body
11:22:43 AM Court Bout 6, Fish 3 = 0:02:08 (2.133 mins), Init, Totals: Pumps = 2, Pumps Prior = 0, Quivers = 4, Points = 0
11:22:43 AM Court Bout First Behaviors: 1st fish: 3, 1st beh: Quiver, Resp fish: 0, Resp beh:, # repeats: 6, time to resp: 0:00:00 (0.000 mins)
11:22:54 AM Comment: 2 3 in court position
11:22:59 AM Pump Bout 1, Fish 3 = 0:00:03 (0.050 mins)
11:23:27 AM Swim Bout 43, Fish 3 = 0:00:07 (0.117 mins)
11:23:30 AM 3 Departs 12 Close
11:23:32 AM 3 Approaches 12 Close
11:23:40 AM Intrusion Bout 1: Fish 2 = 0:00:32 (0.533 mins)
11:24:02 AM Comment: 2 pushing 3 away with his head
11:24:14 AM Comment: 2 shift down
11:24:16 AM Pump Bout 2, Fish 3 = 0:00:05 (0.083 mins)
11:24:44 AM Comment: 3 trying to engage 1
11:24:54 AM Pump Bout 1, Fish 3 = 0:00:03 (0.050 mins)
11:24:54 AM Swim Bout 44, Fish 3 = 0:00:08 (0.133 mins)
11:25:01 AM 3 Departs 1 Close
11:25:07 AM Comment: 3 4 cm above
11:25:12 AM 3 Departs 2 Far
11:25:13 AM Swim Bout 17, Fish 1 = 0:00:05 (0.083 mins)
11:25:21 AM Comment: 1 on rt plt
11:25:22 AM Swim Bout 45, Fish 3 = 0:00:08 (0.133 mins)
11:25:22 AM Swim Bout 42, Fish 2 = 0:00:08 (0.133 mins)
11:25:25 AM 3 Approaches 1 Close
11:25:26 AM 2 Approaches 13 Close
11:25:33 AM Court Bout 13, Fish 2 = 0:00:38 (0.633 mins), Init, Totals: Pumps = 0, Pumps Prior = 7, Quivers = 4, Points = 0
11:25:33 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 3, Resp beh: Pump, # repeats: 3, time to resp: 0:00:19 (0.317 mins)
11:25:33 AM Bright Bout 2, Fish 2 = 1:09:03 (69.050 mins)
11:25:39 AM Comment: 1 upside down again
11:25:50 AM Comment: 2 in ct position next to 1 - trying to engage
11:25:52 AM Pump Bout 2, Fish 3 = 0:00:07 (0.117 mins)
11:26:08 AM Swim Bout 43, Fish 2 = 0:00:06 (0.100 mins)
11:26:20 AM Comment: 2 shift up slightly
11:26:25 AM Comment: 2 3 in ct position
11:26:33 AM Comment: 1 beginning to right herself
11:26:34 AM Pump Bout 3, Fish 3 = 0:00:05 (0.083 mins)
11:26:51 AM Comment: can see red spot, middle bright
11:26:55 AM Pump Bout 4, Fish 3 = 0:00:02 (0.033 mins)
11:26:55 AM Swim Bout 46, Fish 3 = 0:00:32 (0.533 mins)
11:27:00 AM 3 Departs 12 Far
11:27:04 AM Comment: 3 shift to top of plt
11:27:06 AM Swim Bout 44, Fish 2 = 0:00:05 (0.083 mins)
11:27:08 AM 2 Departs 13 Far
11:27:16 AM Comment: 2 on edge of filter
11:27:18 AM Swim Bout 45, Fish 2 = 0:01:07 (1.117 mins)
11:27:20 AM 2 Approaches 13 Close
11:27:24 AM 3 Approaches 12 Close
11:27:26 AM Comment: 3 shift down
11:27:31 AM 2 Departs 13 Far
11:27:34 AM Pump Bout 1, Fish 2 = 0:00:04 (0.067 mins)
11:28:02 AM Swim Bout 47, Fish 3 = 0:00:05 (0.083 mins)
11:28:04 AM 3 Departs 1 Far
11:28:12 AM Comment: 3 on mid airline
11:28:17 AM 2 Approaches 1 Close
11:28:39 AM Comment: 1 upside down on approach - was fully upright
11:28:59 AM Comment: 1 upright again
11:29:02 AM Court Bout 14, Fish 2 = 0:00:33 (0.550 mins), Init, Totals: Pumps = 0, Pumps Prior = 1, Quivers = 3, Points = 0
11:29:02 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Quiver, Resp fish: 0, Resp beh: , # repeats: 3, time to resp: 0:00:00 (0.000 mins)
11:29:29 AM 1 Departs 2 Far
11:29:38 AM 3 Approaches 1 Close
11:30:08 AM Comment: 1 3 swimming together, almost rising
11:30:10 AM Swim Bout 18, Fish 1 = 0:00:36 (0.600 mins)
11:30:10 AM Swim Bout 48, Fish 3 = 0:00:37 (0.617 mins)
11:30:10 AM Court Bout 9, Fish 1 = 0:00:44 (0.733 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 2
11:30:10 AM Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 0, Resp beh: , # repeats: 2, time to resp: 0:00:00 (0.000 mins)
11:30:10 AM Court Bout Transition Matrix: No Response: 2 (100.00%), Points: f2= 0 (0.00%), f3= 0 (0.00%), Quivers: f2= 0 (0.00%), f3= 0 (0.00%), Rises: f2= 0 (0.00%), f3= 0 (0.00%)
11:30:15 AM Comment: pouch a 10
11:30:52 AM Comment: lift plt
11:30:54 AM Swim Bout 46, Fish 2 = 0:02:02 (2.033 mins)
11:31:01 AM Comment: on arrival, 1 upside down
11:31:03 AM Swim Bout 49, Fish 3 = 0:04:58 (4.967 mins)
11:31:04 AM 3 Departs 1 Far
11:31:06 AM Pump Bout 5, Fish 3 = 0:00:07 (0.117 mins)
11:32:36 AM Pump Bout 6, Fish 3 = 0:00:03 (0.050 mins)
11:33:00 AM Comment: 2 on mid airline
11:33:05 AM Comment: 1 upright again
11:33:14 AM Swim Bout 47, Fish 2 = 0:02:41 (2.683 mins)
11:36:00 AM Comment: 2 high airline
11:36:10 AM Comment: high wire
11:37:10 AM Swim Bout 50, Fish 3 = 0:00:04 (0.067 mins)
11:37:12 AM 3 Approaches 2 Close
11:37:34 AM Pump Bout 7, Fish 3 = 0:00:04 (0.067 mins)
11:39:07 AM Hold Bout 17: Fish 2, 3 = 0:00:11 (0.183 mins)
11:39:14 AM Snap Bout 1: Fish 3, 2
11:39:21 AM Swim Bout 48, Fish 2 = 0:00:43 (0.717 mins)
11:39:24 AM 2 Departs 3 Far
11:39:27 AM Pump Bout 8, Fish 3 = 0:00:02 (0.033 mins)
11:39:33 AM Comment: 3 rubbing again
11:39:55 AM 2 Approaches 3 Close
11:39:57 AM Swim Bout 51, Fish 3 = 0:00:09 (0.150 mins)
11:40:01 AM 3 Departs 2 Far
11:40:06 AM Comment: wire
11:40:08 AM Comment: rt plt
11:40:29 AM Pump Bout 9, Fish 3 = 0:00:03 (0.050 mins)
11:40:33 AM Pump Bout 1, Fish 2 = 0:00:03 (0.050 mins)
11:40:39 AM Swim Bout 52, Fish 3 = 0:00:27 (0.450 mins)
11:41:09 AM Comment: 3 on low airline
11:41:12 AM 1 Approaches 2 Close
11:41:18 AM 2 Approaches 1 Close
11:41:21 AM Swim Bout 19, Fish 1 = 0:00:24 (0.400 mins)
11:41:21 AM Court Bout 10, Fish 1 = 0:01:01 (1.017 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 9
11:41:21 AM Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 0, Resp beh: , # repeats: 9, time to resp: 0:00:00 (0.000 mins)
11:41:21 AM Court Bout Transition Matrix: No Response: 9 (100.00%), Points: f2= 0 (0.00%), f3= 0 (0.00%), Pumps: f2= 0 (0.00%), f3= 0 (0.00%), Quivers: f2= 0 (0.00%), f3= 0 (0.00%), Rises: f2= 0 (0.00%), f3= 0 (0.00%)  
11:41:28 AM Comment: 1 2 swimming and courting
11:41:42 AM Comment: twirling, but not holding
11:41:47 AM Comment: wire
11:41:56 AM 3 Approaches 12 Close
11:42:02 AM Hold Bout 18: Fish 2, 1 = 0:00:20 (0.333 mins)
11:42:03 AM Comment: at body
11:42:22 AM Swim Bout 20, Fish 1 = 0:00:02 (0.033 mins)
11:42:22 AM Swim Bout 49, Fish 2 = 0:00:20 (0.333 mins)
11:42:22 AM Swim Bout 53, Fish 3 = 0:05:05 (5.083 mins)
11:42:27 AM Comment: 1 on mid wire
11:42:38 AM Comment: 2 3 swimming around pointing female
11:42:41 AM 2 Approaches 1 Close
11:42:50 AM Comment: 1 upside down, 2 just below her
11:43:22 AM Comment: while swimming, males were pushing against each other - could not tell a winner, though
11:43:24 AM Pump Bout 10, Fish 3 = 0:00:01 (0.017 mins)
11:43:29 AM Comment: 2 pouch a 2-3
11:43:41 AM 3 Approaches 12 Close
11:44:00 AM Comment: pouch large and bright (3)
11:44:03 AM Pump Bout 11, Fish 3 = 0:00:04 (0.067 mins)
11:44:48 AM Comment: 3 just swimming around very close to 1, 2 keeps warning him away with his head
11:44:49 AM Pump Bout 2, Fish 2 = 0:00:05 (0.083 mins)
11:45:17 AM 3 Departs 12 Far
11:45:24 AM 3 Approaches 12 Close
11:45:43 AM  3 Departs 12 Far
11:45:58 AM  Comment: 3 keeps swimming in ft of female - maybe to get her to rise?
11:46:13 AM  Pump Bout 3, Fish 2 = 0:00:02 (0.033 mins)
11:46:20 AM  1 Departs 2 Far
11:46:26 AM  Comment: 1 shift to bottom airline
11:46:27 AM  Swim Bout 50, Fish 2 = 0:00:04 (0.067 mins)
11:46:28 AM  2 Approaches 1 Close
11:46:36 AM  Comment: 2 just below 1 on airline
11:47:03 AM  Pump Bout 4, Fish 2 = 0:00:03 (0.050 mins)
11:47:19 AM  Comment: 2 pumps are larger and pouch is opening now
11:47:21 AM  Swim Bout 51, Fish 2 = 0:00:20 (0.333 mins)
11:47:22 AM  2 Departs 1 Far
11:47:30 AM  Comment: 3 mid airline
11:47:39 AM  Comment: 2 pouch a 4
11:47:46 AM  Comment: 2 on top of rt plt
11:47:49 AM  Pump Bout 12, Fish 3 = 0:00:10 (0.167 mins)
11:47:52 AM  Pump Bout 5, Fish 2 = 0:00:05 (0.083 mins)
11:47:55 AM  Swim Bout 52, Fish 2 = 0:00:10 (0.167 mins)
11:48:02 AM  2 Approaches 1 Close
11:48:05 AM  Court Bout 11, Fish 1 = 0:00:38 (0.633 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 2
11:48:05 AM  Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 3, Resp beh: Pump, # repeats: 1, time to resp: 0:00:29 (0.483 mins)
11:48:05 AM  Court Bout Transition Matrix: No Response: 1 (50.00%), Points: \( \mathcal{O}_2 = 0 \) (0.00%), \( \mathcal{O}_3 = 0 \) (0.00%), Pumps: \( \mathcal{O}_2 = 0 \) (0.00%), \( \mathcal{O}_3 = 1 \) (50.00%), Quivers: \( \mathcal{O}_2 = 0 \) (0.00%), \( \mathcal{O}_3 = 0 \) (0.00%), Rises: \( \mathcal{O}_2 = 0 \) (0.00%), \( \mathcal{O}_3 = 0 \) (0.00%)
11:48:16 AM  3 Approaches 12 Close
11:48:27 AM  Comment: 1 upside down
11:48:32 AM  3 Departs 12 Far
11:48:34 AM  Pump Bout 13, Fish 3 = 0:00:02 (0.033 mins)
11:48:36 AM  Pump Bout 6, Fish 2 = 0:00:03 (0.050 mins)
11:48:43 AM  Swim Bout 54, Fish 3 = 0:00:04 (0.067 mins)
11:48:46 AM  Swim Bout 53, Fish 2 = 0:01:13 (1.217 mins)
11:48:50 AM  Comment: low wire
11:49:03 AM  Comment: 2 swimming in front of 1 trying to get her to rise
11:49:12 AM  Pump Bout 14, Fish 3 = 0:00:04 (0.067 mins)
11:49:17 AM  Pump Bout 7, Fish 2 = 0:00:00 (0.000 mins)
11:49:32 AM  Swim Bout 55, Fish 3 = 0:00:24 (0.400 mins)
11:49:52 AM  2 Departs 1 Far
11:49:57 AM  Comment: rt plt
11:50:01 AM  Comment: same
11:50:15 AM  Swim Bout 54, Fish 2 = 0:00:24 (0.400 mins)
11:50:18 AM  2 Departs 3 Far
11:50:20 AM  2 Approaches 1 Close
11:50:27 AM  Pump Bout 8, Fish 2 = 0:00:03 (0.050 mins)
11:50:35 AM  2 Departs 1 Far
11:50:42 AM  Comment: 2 mid air
11:50:48 AM  3 Approaches 1 Close
11:51:00 AM  Swim Bout 56, Fish 3 = 0:01:19 (1.317 mins)
11:51:00 AM  Court Bout 12, Fish 1 = 0:01:45 (1.750 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 6
11:51:00 AM  Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 3, Resp beh: Quiver, # repeats: 4, time to resp: 0:00:17 (0.283 mins)
11:51:00 AM  Court Bout Transition Matrix: No Response: 3 (50.00%), Points: \( \mathcal{O}_2 = 0 \) (0.00%), \( \mathcal{O}_3 = 0 \) (0.00%), Pumps: \( \mathcal{O}_2 = 0 \) (0.00%), \( \mathcal{O}_3 = 0 \) (0.00%), Quivers: \( \mathcal{O}_2 = 0 \) (0.00%), \( \mathcal{O}_3 = 1 \) (16.67%), Rises: \( \mathcal{O}_2 = 0 \) (0.00%), \( \mathcal{O}_3 = 2 \) (33.33%)

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11:51:09 AM Swim Bout 21, Fish 1 = 0:01:02 (1.033 mins)
11:51:10 AM Rise Bout 2, Fish 1 = 0:00:01 (0.017 mins), Init
11:51:17 AM Court Bout 7, Fish 3 = 0:00:39 (0.650 mins), Rece, Totals: Pumps = 0, Pumps Prior = 14, Quivers = 1, Points = 0
11:51:33 AM Hold Bout 19: Fish 3, 1 = 0:00:03 (0.050 mins)
11:51:33 AM Swim Bout 55, Fish 2 = 0:00:46 (0.767 mins)
11:51:35 AM Comment: at tail
11:51:37 AM Rise Bout 3, Fish 1 = 0:00:04 (0.067 mins), Init
11:51:38 AM 2 Approaches 13 Close
11:51:38 AM Rise Bout 2, Fish 3 = 0:00:03 (0.050 mins), Rece
11:51:40 AM Rise Bout 1, Fish 2 = 0:00:04 (0.067 mins), Rece
11:51:40 AM Court Bout 15, Fish 2 = 0:01:05 (1.083 mins), Rece, Totals: Pumps = 0, Pumps Prior = 8, Quivers = 0, Points = 0
11:51:53 AM Rise Bout 4, Fish 1 = 0:00:02 (0.033 mins), Init
11:51:54 AM Rise Bout 3, Fish 3 = 0:00:01 (0.017 mins), Rece
11:51:54 AM Rise Bout 2, Fish 2 = 0:00:02 (0.033 mins), Rece
11:52:09 AM Comment: all chasing 1
11:52:12 AM Comment: r pILT
11:52:18 AM Comment: 1 upside down
11:52:21 AM Comment: r pILT
11:52:31 AM Pump Bout 1, Fish 3 = 0:00:03 (0.050 mins)
11:52:31 AM Swim Bout 57, Fish 3 = 0:00:04 (0.067 mins)
11:52:38 AM Comment: top of pILT
11:52:41 AM Rise Bout 5, Fish 1 = 0:00:03 (0.050 mins), Init
11:52:43 AM Rise Bout 3, Fish 2 = 0:00:02 (0.033 mins), Rece
11:52:51 AM Comment: 1 upside down
11:52:53 AM Pump Bout 1, Fish 2 = 0:00:01 (0.017 mins)
11:53:02 AM Comment: 23 in court position
11:53:03 AM Swim Bout 58, Fish 3 = 0:00:27 (0.450 mins)
11:53:04 AM 3 Departs 12 Far
11:53:09 AM 2 Departs 1 Far
11:53:19 AM Rise Bout 4, Fish 2 = 0:00:05 (0.083 mins), Rece
11:53:19 AM Rise Bout 4, Fish 3 = 0:00:06 (0.100 mins), Init
11:53:19 AM Court Bout 16, Fish 2 = 0:00:57 (0.950 mins), Rece, Totals: Pumps = 1, Pumps Prior = 1, Quivers = 0, Points = 0
11:53:19 AM Court Bout 8, Fish 3 = 0:00:57 (0.950 mins), Init, Totals: Pumps = 1, Pumps Prior = 1, Quivers = 0, Points = 0
11:53:19 AM Court Bout First Behaviors: 1st fish: 3, 1st beh: Rise, Resp fish: 2, Resp beh: Rise, # repeats: 1, time to resp: 00:00:00 (0.000 mins)
11:53:27 AM Comment: no winner
11:53:33 AM Comment: on high airline
11:53:44 AM Comment: 2 3 chasing each other
11:53:45 AM Pump Bout 1, Fish 2 = 0:00:02 (0.033 mins)
11:53:47 AM Pump Bout 1, Fish 3 = 0:00:00 (0.000 mins)
11:53:59 AM Warning: Potential phase change while fish 2 courting
11:53:59 AM Warning: Potential phase change while fish 3 courting
11:53:59 AM Rise Bout 6, Fish 1 = 0:00:16 (0.267 mins), Init
11:53:59 AM Court Bout 13, Fish 1 = 0:00:16 (0.267 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 0
11:53:59 AM Court Bout Transition Matrix: No Response: 0 (0.000%), Points: f2= 0 (0.000%), f3= 0 (0.000%), Pumps: f2= 0 (0.000%), f3= 0 (0.000%), Quivers: f2= 0 (0.000%), f3= 0 (0.000%), Rises: f2= 0 (0.000%), f3= 0 (0.000%)
11:54:02 AM Rise Bout 5, Fish 2 = 0:00:14 (0.233 mins), Rece
11:54:05 AM Rise Bout 5, Fish 3 = 0:00:11 (0.183 mins), Rece
11:54:15 AM Swim Bout 56, Fish 2 = 0:00:15 (0.250 mins)
11:54:15 AM Swim Bout 59, Fish 3 = 0:00:16 (0.267 mins)
11:54:27 AM Comment: 1 2 in closest to cop, 3 just following
11:54:37 AM Comment: all on rt plt
11:54:45 AM Comment: 1 was swimming during rising
11:54:48 AM Swim Bout 60, Fish 3 = 0:00:14 (0.233 mins)
11:54:48 AM Swim Bout 57, Fish 2 = 0:00:21 (0.350 mins)
11:54:50 AM 2 Departs 1 Far
11:54:52 AM 3 Departs 1 Far
11:54:56 AM 2 Departs 3 Far
11:54:59 AM Pump Bout 1, Fish 2 = 0:00:01 (0.017 mins)
11:55:06 AM Comment: rt plt top
11:55:09 AM 2 Approaches 1 Close
11:55:10 AM Swim Bout 61, Fish 3 = 0:00:14 (0.233 mins)
11:55:11 AM 3 Departs 1 Far
11:55:14 AM Pump Bout 1, Fish 3 = 0:00:02 (0.033 mins)
11:55:22 AM Comment: eggs look like falling out
11:55:26 AM Comment: airline
11:55:39 AM Swim Bout 62, Fish 3 = 0:00:01 (0.017 mins)
11:55:42 AM 3 Approaches 12 Close
11:55:49 AM Court Bout 17, Fish 2 = 0:00:06 (0.100 mins), Init, Totals: Pumps = 0, Pumps Prior = 1, Quivers = 0, Points = 2
11:55:49 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Point, Resp fish: 0, Resp beh: , # repeats: 2, time to resp: 0:00:00 (0.000 mins)
11:55:55 AM Pump Bout 2, Fish 3 = 0:00:04 (0.067 mins)
11:55:59 AM Rise Bout 7, Fish 1 = 0:00:11 (0.183 mins), Init
11:55:59 AM Rise Bout 6, Fish 3 = 0:00:11 (0.183 mins), Rece
11:55:59 AM Court Bout 14, Fish 1 = 0:00:15 (0.250 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 0
11:55:59 AM Court Bout Transition Matrix: No Response: 0 (0.00%), Points: f2 = 0 (0.00%), f3 = 0 (0.00%), Pumps: f2 = 0 (0.00%), f3 = 0 (0.00%), Quivers: f2 = 0 (0.00%), f3 = 0 (0.00%), Rises: f2 = 0 (0.00%), f3 = 0 (0.00%)
11:55:59 AM Court Bout 9, Fish 3 = 0:00:15 (0.250 mins), Rece, Totals: Pumps = 0, Pumps Prior = 2, Quivers = 0, Points = 0
11:55:59 AM Court Bout First Behaviors: 1st fish: 1, 1st beh: Rise, Resp fish: 3, Resp beh: Rise, # repeats: 1, time to resp: 0:00:00 (0.000 mins)
11:56:14 AM Swim Bout 63, Fish 3 = 0:00:13 (0.217 mins)
11:56:15 AM Comment: 1 3 almost copulated
11:56:42 AM Swim Bout 22, Fish 1 = 0:00:08 (0.133 mins)
11:56:42 AM Swim Bout 58, Fish 2 = 0:00:09 (0.150 mins)
11:56:43 AM Swim Bout 64, Fish 3 = 0:00:08 (0.133 mins)
11:56:53 AM Comment: lft plt
11:57:03 AM Rise Bout 7, Fish 3 = 0:00:16 (0.267 mins), Rece
11:57:03 AM Rise Bout 6, Fish 2 = 0:00:16 (0.267 mins), Init
11:57:03 AM Court Bout 10, Fish 3 = 0:01:20 (1.333 mins), Rece, Totals: Pumps = 1, Pumps Prior = 0, Quivers = 0, Points = 0
11:57:03 AM Court Bout 18, Fish 2 = 0:01:21 (1.350 mins), Init, Totals: Pumps = 3, Pumps Prior = 0, Quivers = 0, Points = 0
11:57:03 AM Court Bout First Behaviors: 1st fish: 2, 1st beh: Rise, Resp fish: 3, Resp beh: Rise, # repeats: 1, time to resp: 0:00:00 (0.000 mins)
11:57:14 AM Pump Bout 1, Fish 2 = 0:00:03 (0.050 mins)
11:57:22 AM Pump Bout 1, Fish 3 = 0:00:04 (0.067 mins)
11:57:23 AM Pump Bout 2, Fish 2 = 0:00:03 (0.050 mins)
11:57:35 AM 2 Departs 1 Far
11:57:36 AM 3 Departs 1 Far
11:57:59 AM Pump Bout 3, Fish 2 = 0:00:02 (0.033 mins)
11:58:20 AM  Comment: 2 just chasing each other
11:58:24 AM  Swim Bout 65, Fish 3 = 0:00:33 (0.550 mins)
11:58:26 AM  Swim Bout 59, Fish 2 = 0:00:06 (0.100 mins)
11:58:29 AM  Comment: on mid air line
11:58:34 AM  2 Departs 3 Far
11:58:34 AM  Swim Bout 60, Fish 2 = 0:00:42 (0.700 mins)
11:58:36 AM  Pump Bout 1, Fish 2 = 0:00:02 (0.033 mins)
11:58:58 AM  Swim Bout 66, Fish 3 = 0:00:06 (0.100 mins)
11:59:03 AM  Comment: 3 stopped at airline
11:59:12 AM  2 Approaches 3 Close
11:59:14 AM  Swim Bout 67, Fish 3 = 0:00:17 (0.283 mins)
11:59:19 AM  Comment: rt plt
11:59:27 AM  Comment: 3 swimming around 2
11:59:28 AM  Pump Bout 2, Fish 2 = 0:00:02 (0.033 mins)
11:59:33 AM  Comment: rt plt
11:59:43 AM  Comment: 1 upright on lft plt bottom
11:59:46 AM  2 Approaches 1 Close
11:59:50 AM  Rise Bout 8, Fish 1 = 0:00:10 (0.167 mins), Init
11:59:50 AM  Swim Bout 61, Fish 2 = 0:00:17 (0.283 mins)
11:59:50 AM  Court Bout 15, Fish 1 = 0:03:08 (3.133 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 8
11:59:50 AM  Court Bout Transition Matrix: No Response: 3 (37.50%), Points: f2 = 0 (0.00%), f3 = 0 (0.00%), Pumps: f2 = 1 (12.50%), f3 = 0 (0.00%), Quivers: f2 = 1 (12.50%), f3 = 0 (0.00%), Rises: f2 = 3 (37.50%), f3 = 0 (0.00%)
11:59:51 AM  Rise Bout 7, Fish 2 = 0:00:10 (0.167 mins), Rece
11:59:51 AM  Court Bout 19, Fish 2 = 0:03:09 (3.150 mins), Rece, Totals: Pumps = 2, Pumps Prior = 2, Quivers = 4, Points = 0
11:59:51 AM  Court Bout First Behaviors: 1st fish: 1, 1st beh: Rise, Resp fish: 2, Resp beh: Rise, # repeats: 1, time to resp: 0:00:01 (0.017 mins)
12:00:09 PM  Hold Bout 20: Fish 2, 1 = 0:00:05 (0.083 mins)
12:00:11 PM  Comment: at tail
12:00:13 PM  Swim Bout 23, Fish 1 = 0:00:14 (0.233 mins)
12:00:14 PM  Rise Bout 9, Fish 1 = 0:00:04 (0.067 mins), Init
12:00:14 PM  Rise Bout 8, Fish 2 = 0:00:05 (0.083 mins), Rece
12:00:14 PM  Swim Bout 62, Fish 2 = 0:00:33 (0.550 mins)
12:00:21 PM  Comment: promenading
12:00:50 PM  Hold Bout 21: Fish 1, 2 = 0:00:09 (0.150 mins)
12:00:52 PM  Comment: at tail
12:01:06 PM  Rise Bout 10, Fish 1 = 0:00:04 (0.067 mins), Init
12:01:07 PM  Rise Bout 9, Fish 2 = 0:00:03 (0.050 mins), Rece
12:01:13 PM  Pump Bout 1, Fish 2 = 0:00:01 (0.017 mins)
12:01:16 PM  Rise Bout 11, Fish 1 = 0:00:08 (0.133 mins), Init
12:01:16 PM  Rise Bout 10, Fish 2 = 0:00:09 (0.150 mins), Rece
12:01:45 PM  Comment: 1 at lft plt w 2
12:01:50 PM  Swim Bout 63, Fish 2 = 0:00:18 (0.300 mins)
12:01:55 PM  Pump Bout 2, Fish 2 = 0:00:03 (0.050 mins)
12:02:05 PM  Swim Bout 68, Fish 3 = 0:00:13 (0.217 mins)
12:02:24 PM  Comment: low wire
12:02:30 PM  Swim Bout 24, Fish 1 = 0:00:06 (0.100 mins)
12:02:41 PM  Comment: upside down
12:02:50 PM  Comment: really looking like falling out
12:02:51 PM  Rise Bout 12, Fish 1 = 0:00:06 (0.100 mins), Init
12:02:52 PM  Rise Bout 11, Fish 2 = 0:00:06 (0.100 mins), Rece
12:02:58 PM  Swim Bout 64, Fish 2 = 0:00:26 (0.433 mins)
12:03:18 PM  Comment: 2 pouch doesn't look open wide enough for cop - may be why 1 keeps turning away

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12:03:37 PM Comment: 12 were swimming, now on low plt lft
12:03:56 PM Rise Bout 13, Fish 1 = 0:00:05 (0.083 mins), Init
12:03:56 PM Rise Bout 12, Fish 2 = 0:00:06 (0.100 mins), Rece
12:03:56 PM Court Bout 16, Fish 1 = 0:00:37 (0.617 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 0
12:03:56 PM Court Bout Transition Matrix: No Response: 0 (0.00%), Points: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%), Pumps: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%), Quivers: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%), Rises: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%)
12:03:56 PM Court Bout 20, Fish 2 = 0:00:39 (0.650 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 0
12:03:56 PM Court Bout First Behaviors: 1st fish: 1, 1st beh: Rise, Resp fish: 2, Resp beh: Rise, # repeats: 1, time to resp: 0:00:00 (0.000 mins)
12:04:13 PM Comment: now just swimminh
12:04:22 PM Rise Bout 14, Fish 1 = 0:00:08 (0.133 mins), Init
12:04:23 PM Rise Bout 13, Fish 2 = 0:00:10 (0.167 mins), Rece
12:04:33 PM Swim Bout 25, Fish 1 = 0:00:26 (0.433 mins)
12:04:33 PM Swim Bout 65, Fish 2 = 0:00:37 (0.617 mins)
12:04:33 PM Swim Bout 69, Fish 3 = 0:00:59 (0.983 mins)
12:04:50 PM Comment: 2 pouch just not open far enough yer
12:04:56 PM Comment: now just swimming
12:05:30 PM Comment: at lft plt l2
12:05:34 PM Comment: low airline
12:06:03 PM Court Bout 21, Fish 2 = 0:00:35 (0.583 mins), Init, Totals: Pumps = 1, Pumps Prior = 0, Quivers = 1, Points = 3
12:06:03 PM Court Bout First Behaviors: 1st fish: 2, 1st beh: Point, Resp fish: 0, Resp beh: , # repeats: 5, time to resp: 0:00:00 (0.000 mins)
12:06:19 PM Pump Bout 1, Fish 2 = 0:00:04 (0.067 mins)
12:06:33 PM 1 Departs 2 Close
12:06:36 PM Comment: 1 shift down
12:06:55 PM Swim Bout 70, Fish 3 = 0:00:12 (0.200 mins)
12:06:55 PM Court Bout 17, Fish 1 = 0:00:19 (0.317 mins), Init, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 0, Points = 1
12:06:55 PM Court Bout First Behaviors: 1st fish: 1, 1st beh: Point, Resp fish: 2, Resp beh: Pump, # repeats: 1, time to resp: 0:00:06 (0.100 mins)
12:06:55 PM Court Bout Transition Matrix: No Response: 0 (0.00%), Points: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%), Pumps: $f_2$ = 1 (100.00%), $f_3$ = 0 (0.00%), Quivers: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%), Rises: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%)
12:06:58 PM Swim Bout 66, Fish 2 = 0:00:07 (0.117 mins)
12:07:01 PM Pump Bout 1, Fish 2 = 0:00:03 (0.050 mins)
12:07:10 PM Comment: airline
12:07:20 PM 2 Departs 1 Close
12:07:26 PM Comment: mid plt she low
12:07:49 PM Court Bout 22, Fish 2 = 0:26:28 (26.467 mins), Init, Totals: Pumps = 3, Pumps Prior = 1, Quivers = 2, Points = 6
12:07:52 PM Swim Bout 71, Fish 3 = 0:00:10 (0.167 mins)
12:07:56 PM Pump Bout 1, Fish 2 = 0:00:05 (0.083 mins)
12:08:04 PM Comment: rt plt
12:08:14 PM Warning: Potential phase change while fish 2 courting
12:08:14 PM Court Bout 18, Fish 1 = 0:26:04 (26.067 mins), Rece, Totals: Pumps = 0, Pumps Prior = 0, Quivers = 1, Points = 3
12:08:14 PM Court Bout First Behaviors: 1st fish: 2, 1st beh: Point, Resp fish: 1, Resp beh: Quiver, # repeats: 4, time to resp: 0:00:25 (0.417 mins)
12:08:14 PM Court Bout Transition Matrix: No Response: 2 (66.67%), Points: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%), Pumps: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%), Quivers: $f_2$ = 0 (0.00%), $f_3$ = 0 (0.00%), Rises: $f_2$ = 1 (33.33%), $f_3$ = 0 (0.00%)
12:08:23 PM 2 Approaches 1 Close
12:08:25 PM Swim Bout 67, Fish 2 = 0:00:09 (0.150 mins)

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12:08:30 PM  Swim Bout 26, Fish 1 = 0:00:02 (0.033 mins)
12:08:38 PM  Comment: top of pit
12:08:42 PM  Swim Bout 68, Fish 2 = 0:00:26 (0.433 mins)
12:08:47 PM  Swim Bout 27, Fish 1 = 0:00:20 (0.333 mins)
12:08:48 PM  Rise Bout 15, Fish 1 = 0:00:06 (0.100 mins), Init
12:08:48 PM  Rise Bout 14, Fish 2 = 0:00:07 (0.117 mins), Rece
12:08:57 PM  Swim Bout 72, Fish 3 = 0:00:11 (0.183 mins)
12:08:59 PM  3 Approaches 12 Close
12:09:06 PM  Comment: 3 pouch is bigger
12:09:13 PM  Rise Bout 16, Fish 1 = 0:00:06 (0.100 mins), Init
12:09:14 PM  Rise Bout 15, Fish 2 = 0:00:06 (0.100 mins), Rece
12:09:14 PM  Rise Bout 8, Fish 3 = 0:00:07 (0.117 mins), Rece
12:09:14 PM  Court Bout 11, Fish 3 = 0:25:02 (25.033 mins), Rece, Totals: Pumps = 7, Pumps Prior = 0, Quivers = 0, Points = 0
12:09:29 PM  Comment: 1 2 almost copulated
12:09:45 PM  Comment: back at pit
12:09:52 PM  Swim Bout 28, Fish 1 = 0:00:31 (0.517 mins)
12:09:52 PM  Swim Bout 69, Fish 2 = 0:00:31 (0.517 mins)
12:09:53 PM  Swim Bout 73, Fish 3 = 0:00:31 (0.517 mins)
12:09:55 PM  Rise Bout 17, Fish 1 = 0:00:14 (0.233 mins), Init
12:09:56 PM  Rise Bout 16, Fish 2 = 0:00:13 (0.217 mins), Rece
12:09:56 PM  Rise Bout 9, Fish 3 = 0:00:14 (0.233 mins), Rece
12:10:04 PM  Snap Bout 2: Fish 2, 1
12:10:22 PM  Comment: 2 3 swimming around and around 1 to get into cop alignment
12:10:35 PM  Snap Bout 3: Fish 3, 2
12:10:36 PM  Snap Bout 4: Fish 3, 2
12:10:37 PM  Snap Bout 5: Fish 3, 2
12:10:39 PM  Swim Bout 74, Fish 3 = 0:00:23 (0.383 mins)
12:10:40 PM  Swim Bout 70, Fish 2 = 0:00:21 (0.350 mins)
12:10:43 PM  3 Departs 12 Far
12:10:44 PM  2 Departs 1 Far
12:10:49 PM  2 Departs 3 Far
12:10:51 PM  2 Approaches 1 Close
12:10:53 PM  Pump Bout 1, Fish 3 = 0:00:01 (0.017 mins)
12:10:54 PM  Pump Bout 2, Fish 2 = 0:00:15 (0.250 mins)
12:10:55 PM  Pump Bout 2, Fish 3 = 0:00:15 (0.250 mins)
12:11:07 PM  3 Approaches 12 Close
12:11:18 PM  Swim Bout 29, Fish 1 = 0:00:03 (0.050 mins)
12:11:19 PM  Swim Bout 71, Fish 2 = 0:00:02 (0.033 mins)
12:11:22 PM  Hold Bout 22: Fish 2, 1 = 0:00:06 (0.100 mins)
12:11:25 PM  Comment: at neck
12:11:40 PM  Hold Bout 23: Fish 2, 3 = 0:00:16 (0.267 mins)
12:11:43 PM  Comment: at neck
12:11:49 PM  Wrestle Bout 1: Fish 3, 2 = 0:00:03 (0.050 mins)
12:11:59 PM  Swim Bout 75, Fish 3 = 0:00:05 (0.083 mins)
12:12:00 PM  3 Departs 12 Close
12:12:03 PM  3 Approaches 12 Close
12:12:05 PM  Swim Bout 72, Fish 2 = 0:00:07 (0.117 mins)
12:12:07 PM  2 Departs 13 Far
12:12:15 PM  Comment: top of pit
12:12:19 PM  Swim Bout 76, Fish 3 = 0:00:37 (0.617 mins)
12:12:20 PM  Swim Bout 30, Fish 1 = 0:00:27 (0.450 mins)
12:12:21 PM  Swim Bout 73, Fish 2 = 0:00:27 (0.450 mins)
12:12:22 PM  Rise Bout 18, Fish 1 = 0:00:17 (0.283 mins), Init
12:12:22 PM  Rise Bout 10, Fish 3 = 0:00:18 (0.300 mins), Rece
12:12:25 PM  Rise Bout 17, Fish 2 = 0:00:15 (0.250 mins), Rece
12:12:45 PM  Comment: now just chasing l
12:12:53 PM  Comment: rt pkt
12:13:02 PM  Swim Bout 77, Fish 3 = 0:00:39 (0.650 mins)
12:13:04 PM  Swim Bout 31, Fish 1 = 0:00:25 (0.417 mins)
12:13:04 PM  Swim Bout 74, Fish 2 = 0:00:36 (0.600 mins)
12:13:05 PM  Rise Bout 18, Fish 2 = 0:00:13 (0.217 mins), Init
12:13:07 PM  Rise Bout 19, Fish 1 = 0:00:10 (0.167 mins), Rece
12:13:09 PM  Rise Bout 11, Fish 3 = 0:00:10 (0.167 mins), Rece
12:13:24 PM  Comment: now just chasing her
12:13:33 PM  Comment: rt pkt
12:13:46 PM  Comment: all close at rt pkt
12:13:49 PM  Pump Bout 3, Fish 3 = 0:00:02 (0.033 mins)
12:13:50 PM  Swim Bout 78, Fish 3 = 0:00:04 (0.067 mins)
12:13:53 PM  Swim Bout 75, Fish 2 = 0:01:12 (1.200 mins)
12:14:00 PM  2 Approaches 1 Close
12:14:03 PM  Swim Bout 32, Fish 1 = 0:01:01 (1.017 mins)
12:14:04 PM  Rise Bout 20, Fish 1 = 0:00:22 (0.367 mins), Init
12:14:05 PM  Rise Bout 19, Fish 2 = 0:00:22 (0.367 mins), Rece
12:14:11 PM  Swim Bout 79, Fish 3 = 0:00:54 (0.900 mins)
12:14:20 PM  Rise Bout 12, Fish 3 = 0:00:07 (0.117 mins), Rece
12:14:23 PM  Hold Bout 24: Fish 2, l = 0:00:19 (0.317 mins)
12:14:24 PM  Comment: at tail
12:14:40 PM  Comment: 23 rising together, and 2 dragging 1 along
12:14:46 PM  Rise Bout 13, Fish 3 = 0:00:07 (0.117 mins), Init
12:14:46 PM  Rise Bout 20, Fish 2 = 0:00:08 (0.133 mins), Rece
12:14:49 PM  Rise Bout 21, Fish 1 = 0:00:03 (0.050 mins), Rece
12:15:07 PM  Comment: at rt pkt
12:15:10 PM  Hold Bout 25: Fish 2, l = 0:00:12 (0.200 mins)
12:15:12 PM  Comment: at body
12:15:15 PM  Swim Bout 80, Fish 3 = 0:00:37 (0.617 mins)
12:15:17 PM  2 Departs 12 Far
12:15:22 PM  Swim Bout 76, Fish 2 = 0:00:33 (0.550 mins)
12:15:24 PM  Rise Bout 22, Fish 1 = 0:00:17 (0.283 mins), Init
12:15:24 PM  Swim Bout 33, Fish 1 = 0:00:27 (0.450 mins)
12:15:25 PM  Rise Bout 21, Fish 2 = 0:00:17 (0.283 mins), Rece
12:15:25 PM  Rise Bout 14, Fish 3 = 0:00:17 (0.283 mins), Rece
12:15:58 PM  Comment: rt pkt
12:16:00 PM  Swim Bout 77, Fish 2 = 0:00:08 (0.133 mins)
12:16:01 PM  2 Departs 13 Far
12:16:03 PM  Swim Bout 81, Fish 3 = 0:02:06 (2.100 mins)
12:16:04 PM  Pump Bout 4, Fish 3 = 0:00:01 (0.017 mins)
12:16:07 PM  2 Approaches 1 Close
12:16:10 PM  Swim Bout 34, Fish 1 = 0:00:22 (0.367 mins)
12:16:10 PM  Swim Bout 78, Fish 2 = 0:01:59 (1.983 mins)
12:16:11 PM  Rise Bout 23, Fish 1 = 0:00:17 (0.283 mins), Init
12:16:12 PM  Rise Bout 22, Fish 2 = 0:00:17 (0.283 mins), Rece
12:16:22 PM  Rise Bout 15, Fish 3 = 0:00:07 (0.117 mins), Rece
12:16:39 PM  Hold Bout 26: Fish 2, l = 0:00:05 (0.083 mins)
12:16:42 PM  Comment: at tail
12:16:45 PM  Swim Bout 35, Fish 1 = 0:01:23 (1.383 mins)
12:16:48 PM  Rise Bout 24, Fish 1 = 0:00:11 (0.183 mins), Init
12:16:48 PM  Rise Bout 23, Fish 2 = 0:00:12 (0.200 mins), Rece
Rise Bout 16, Fish 3 = 0:00:10 (0.167 mins), Rece
Comment: just pulled her off plan be fore rise
Rise Bout 25, Fish 1 = 0:00:18 (0.300 mins), Init
Rise Bout 24, Fish 2 = 0:00:18 (0.300 mins), Rece
Rise Bout 17, Fish 3 = 0:00:18 (0.300 mins), Rece
Comment: 1 2 trying to get lined up, but 3 int he way
just chasing her now
Comment: 2 has to flex so hard to get pouch open that his tailgets in the way
Pump Bout 5, Fish 3 = 0:00:01 (0.017 mins)
Comment: lft plt
Swim Bout 36, Fish 1 = 0:00:30 (0.500 mins)
Swim Bout 79, Fish 2 = 0:02:44 (2.733 mins)
Swim Bout 82, Fish 3 = 0:02:40 (2.667 mins)
Comment: 1 2 almost had it but hit water surface with a bunkp
2 Departs 13 Far
Comment: lft plt
3 Departs 12 Far
2 Departs 1 Far
2 Approaches 1 Close
Swim Bout 37, Fish 1 = 0:01:56 (1.933 mins)
Rise Bout 26, Fish 1 = 0:00:10 (0.167 mins), Init
Rise Bout 25, Fish 2 = 0:00:10 (0.167 mins), Rece
Comment: tail in way
Rise Bout 27, Fish 1 = 0:00:08 (0.133 mins), Init
Rise Bout 26, Fish 2 = 0:00:07 (0.117 mins), Rece
Comment: tail in way
Comment: 2 3 chasing 1
2 Departs 13 Far
3 Approaches 1 Close
1 Departs 3 Far
Rise Bout 28, Fish 1 = 0:00:09 (0.150 mins), Init
Rise Bout 18, Fish 3 = 0:00:13 (0.217 mins), Rece
Rise Bout 27, Fish 2 = 0:00:10 (0.167 mins), Rece
Comment: twirling and spinning trying to get in alignment
Comment: rt plt
Swim Bout 80, Fish 2 = 0:00:03 (0.050 mins)
Swim Bout 83, Fish 3 = 0:00:08 (0.133 mins)
Pump Bout 6, Fish 3 = 0:00:01 (0.017 mins)
Comment: all close on rt plt
Swim Bout 38, Fish 1 = 0:01:08 (1.133 mins)
Swim Bout 81, Fish 2 = 0:01:08 (1.133 mins)
Rise Bout 29, Fish 1 = 0:00:10 (0.167 mins), Init
Rise Bout 28, Fish 2 = 0:00:10 (0.167 mins), Rece
Swim Bout 84, Fish 3 = 0:01:06 (1.100 mins)
Rise Bout 30, Fish 1 = 0:00:09 (0.150 mins), Init
Rise Bout 29, Fish 2 = 0:00:08 (0.133 mins), Rece
Rise Bout 19, Fish 3 = 0:00:04 (0.067 mins), Rece
1 Departs 23 Close
Rise Bout 31, Fish 1 = 0:00:06 (0.100 mins), Init
Rise Bout 20, Fish 3 = 0:00:05 (0.083 mins), Rece
Rise Bout 30, Fish 2 = 0:00:03 (0.050 mins), Rece
2 2 33 Comment: rt plt
Rise Bout 32, Fish 1 = 0:00:05 (0.083 mins), Init
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12:22:37 PM Rise Bout 31, Fish 2 = 0:00:04 (0.067 mins), Rece
12:22:48 PM Intrusion Bout 2: Fish 2 = 0:00:07 (0.117 mins)
12:23:16 PM Rise Bout 33, Fish 1 = 0:00:10 (0.167 mins), Init
12:23:16 PM Swim Bout 39, Fish 1 = 0:01:44 (1.733 mins)
12:23:16 PM Swim Bout 82, Fish 2 = 0:01:45 (1.750 mins)
12:23:17 PM Rise Bout 32, Fish 2 = 0:00:11 (0.183 mins), Rece
12:23:41 PM Comment: 1 3 were rising - almost copulated and 2 intruded
12:23:46 PM Swim Bout 85, Fish 3 = 0:01:48 (1.800 mins)
12:23:50 PM Comment: all were swimming
12:23:55 PM Rise Bout 34, Fish 1 = 0:00:12 (0.200 mins), Init
12:23:55 PM Rise Bout 21, Fish 3 = 0:00:13 (0.217 mins), Rece
12:23:56 PM Rise Bout 33, Fish 2 = 0:00:11 (0.183 mins), Rece
12:23:57 PM Intrusion Bout 3: Fish 2 = 0:00:06 (0.100 mins)
12:23:59 PM Snap Bout 6: Fish 3, 2
12:24:22 PM Intrusion Bout 4: Fish 2 = 0:00:05 (0.083 mins)
12:24:33 PM Intrusion Bout 5: Fish 2 = 0:00:04 (0.067 mins)
12:24:51 PM Comment: 1 figured out that 3 bete than 2, but 2 keeps intruding
12:24:58 PM 3 Departs 12 Far
12:25:02 PM Comment: rt plt
12:25:12 PM Rise Bout 35, Fish 1 = 0:00:13 (0.217 mins), Init
12:25:12 PM Rise Bout 34, Fish 2 = 0:00:14 (0.233 mins), Rece
12:25:20 PM Rise Bout 22, Fish 3 = 0:00:07 (0.117 mins), Rece
12:26:05 PM Comment: rt plt
12:26:13 PM Comment: was 1 more rise there for both
12:26:17 PM Rise Bout 36, Fish 1 = 0:00:09 (0.150 mins), Init
12:26:18 PM Rise Bout 35, Fish 2 = 0:00:08 (0.133 mins), Rece
12:26:19 PM Swim Bout 40, Fish 1 = 0:00:57 (0.950 mins)
12:26:19 PM Swim Bout 83, Fish 2 = 0:01:33 (1.550 mins)
12:26:20 PM Swim Bout 86, Fish 3 = 0:01:33 (1.550 mins)
12:26:25 PM Rise Bout 23, Fish 3 = 0:00:02 (0.033 mins), Rece
12:26:33 PM Rise Bout 24, Fish 3 = 0:00:08 (0.133 mins), Rece
12:26:33 PM Rise Bout 37, Fish 1 = 0:00:10 (0.167 mins), Init
12:26:34 PM Intrusion Bout 6: Fish 2 = 0:00:13 (0.217 mins)
12:26:40 PM Rise Bout 36, Fish 2 = 0:00:05 (0.083 mins), Rece
12:27:07 PM Rise Bout 38, Fish 1 = 0:00:05 (0.083 mins), Init
12:27:08 PM Intrusion Bout 7: Fish 3 = 0:00:03 (0.050 mins)
12:27:08 PM Rise Bout 37, Fish 2 = 0:00:04 (0.067 mins), Rece
12:27:23 PM Swim Bout 41, Fish 1 = 0:00:28 (0.467 mins)
12:27:24 PM Rise Bout 38, Fish 2 = 0:00:05 (0.083 mins), Rece
12:27:24 PM Rise Bout 39, Fish 1 = 0:00:13 (0.217 mins), Init
12:27:26 PM Rise Bout 25, Fish 3 = 0:00:12 (0.200 mins), Rece
12:27:30 PM Intrusion Bout 8: Fish 2 = 0:00:10 (0.167 mins)
12:27:43 PM Rise Bout 40, Fish 1 = 0:00:06 (0.100 mins), Init
12:27:44 PM Rise Bout 39, Fish 2 = 0:00:05 (0.083 mins), Rece
12:27:45 PM Intrusion Bout 9: Fish 3 = 0:00:03 (0.050 mins)
12:27:56 PM Comment: rt plt
12:28:04 PM Pump Bout 7, Fish 3 = 0:00:05 (0.083 mins)
12:28:09 PM Swim Bout 87, Fish 3 = 0:00:05 (0.083 mins)
12:28:13 PM 3 Departs 12 Far
12:28:16 PM Swim Bout 88, Fish 3 = 0:01:22 (1.367 mins)
12:28:25 PM Rise Bout 41, Fish 1 = 0:00:15 (0.250 mins), Init
12:28:25 PM Rise Bout 40, Fish 2 = 0:00:15 (0.250 mins), Rece
12:28:25 PM Swim Bout 42, Fish 1 = 0:00:27 (0.450 mins)
12:28:25 PM Swim Bout 84, Fish 2 = 0:01:12 (1.200 mins)
12:28:37 PM  Rise Bout 26, Fish 3 = 0:00:04 (0.067 mins), Rece
12:28:48 PM 2 Departs 1 Far
12:28:49 PM 2 Approaches 3 Close
12:28:54 PM  Comment: lift plt
12:28:57 PM 1 Departs 23 Far
12:29:10 PM 2 Approaches 1 Close
12:29:14 PM  Comment: zoomed over to her
12:29:16 PM  Rise Bout 42, Fish 1 = 0:00:06 (0.100 mins), Init
12:29:17 PM  Rise Bout 41, Fish 2 = 0:00:05 (0.083 mins), Rece
12:29:20 PM  Rise Bout 27, Fish 3 = 0:00:03 (0.050 mins), Rece
12:29:42 PM  Comment: rt plt
12:29:44 PM  Comment: lift plt
12:29:46 PM  Swim Bout 89, Fish 3 = 0:01:04 (1.067 mins)
12:29:47 PM 3 Departs 12 Far
12:29:50 PM 3 Approaches 12 Close
12:29:51 PM  Swim Bout 43, Fish 1 = 0:00:51 (0.850 mins)
12:29:51 PM  Swim Bout 85, Fish 2 = 0:00:56 (0.933 mins)
12:29:53 PM  Rise Bout 42, Fish 2 = 0:00:37 (0.617 mins), Rece
12:29:53 PM  Rise Bout 43, Fish 1 = 0:00:39 (0.650 mins), Init
12:29:54 PM  Rise Bout 28, Fish 3 = 0:00:39 (0.650 mins), Rece
12:30:54 PM  Comment: lift plt
12:30:57 PM  Rise Bout 44, Fish 1 = 0:00:13 (0.217 mins), Init
12:30:57 PM  Swim Bout 44, Fish 1 = 0:00:28 (0.467 mins)
12:30:57 PM  Swim Bout 86, Fish 2 = 0:00:28 (0.467 mins)
12:30:58 PM  Rise Bout 43, Fish 2 = 0:00:10 (0.167 mins), Rece
12:31:01 PM  Swim Bout 90, Fish 3 = 0:00:25 (0.417 mins)
12:31:05 PM  Rise Bout 29, Fish 3 = 0:00:07 (0.117 mins), Rece
12:31:31 PM  Swim Bout 87, Fish 2 = 0:01:22 (1.367 mins)
12:31:33 PM  Swim Bout 45, Fish 1 = 0:00:47 (0.783 mins)
12:31:33 PM  Swim Bout 91, Fish 3 = 0:01:21 (1.350 mins)
12:31:37 PM  Rise Bout 44, Fish 2 = 0:00:08 (0.133 mins), Rece
12:31:37 PM  Rise Bout 45, Fish 1 = 0:00:24 (0.400 mins), Init
12:31:38 PM  Rise Bout 30, Fish 3 = 0:00:23 (0.383 mins), Rece
12:31:46 PM  Intrusion Bout 10: Fish 2 = 0:00:06 (0.100 mins)
12:32:19 PM 1 Departs 23 Far
12:32:23 PM  Comment: lift plt
12:32:25 PM 3 Approaches 1 Close
12:32:28 PM 2 Approaches 13 Close
12:32:30 PM  Swim Bout 46, Fish 1 = 0:00:23 (0.383 mins)
12:32:35 PM  Rise Bout 45, Fish 2 = 0:00:04 (0.067 mins), Rece
12:32:35 PM  Rise Bout 46, Fish 1 = 0:00:06 (0.100 mins), Init
12:32:39 PM  Rise Bout 31, Fish 3 = 0:00:03 (0.050 mins), Rece
12:32:45 PM  Hold Bout 27: Fish 3, 1 = 0:00:03 (0.050 mins)
12:32:46 PM  Comment: at tail
12:32:55 PM  Swim Bout 47, Fish 1 = 0:01:09 (1.150 mins)
12:32:56 PM  Swim Bout 88, Fish 2 = 0:01:15 (1.250 mins)
12:32:56 PM  Swim Bout 92, Fish 3 = 0:01:16 (1.267 mins)
12:32:57 PM  Rise Bout 47, Fish 1 = 0:00:20 (0.333 mins), Init
12:32:58 PM  Rise Bout 46, Fish 2 = 0:00:19 (0.317 mins), Rece
12:33:03 PM  Copulation Bout 1: Fish 1, 2 = 0:00:11 (0.183 mins)
12:33:25 PM  Comment: eggs falling out of 2
12:34:03 PM  Comment: during transfer, 3 trying to put his abdomen into between 1 2, occurred at water surface
12:34:18 PM  Elapsed time Phase 3 = 1:14:23 (74.383 mins)
12:34:31 PM  Comment: eggs spilling out of 2

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12:34:43 PM Comment: I think female still has eggs, because she is trying to rise again with 3
12:34:44 PM Comment: she was also rubbing against the plant again

Total time in Territory neutral = 0:00:00 (0.000 mins)
Total time in Territory 1 = 0:00:00 (0.000 mins)
Total time in Territory 2 = 0:00:00 (0.000 mins)

Totals:
Latency to Court = 0:07:11 (7.183 mins)

Phase 1: 2:51:28 (171.467 mins)
- Holds: 1 -> 2: 2, 0:00:20 (0.333 mins), avg =10.000 secs +3.000
- Holds: 1 -> 3: 1, 0:00:03 (0.050 mins), avg =3.000 secs
- Holds: 2 -> 1: 8, 0:04:49 (4.817 mins), avg =36.125 secs +11.420
- Holds: 2 -> 3: 0
- Holds: 3 -> 1: 1, 0:02:02 (2.033 mins), avg =122.000 secs
- Holds: 3 -> 2: 2, 0:00:20 (0.333 mins), avg =10.000 secs +4.000
Pumps, fish 1: 0
- Pumps, fish 2: 11, 0:00:22 (0.367 mins), avg =2.000 secs +0.330
- Pumps, fish 3: 45, 0:00:35 (3.583 mins), avg =4.778 secs +0.359

First half of phase:
Pumps, fish 1: 0
- Pumps, fish 2: 4, 0:00:10 (0.167 mins), avg =2.500 secs +0.645
- Pumps, fish 3: 17, 0:01:12 (1.200 mins), avg =4.235 secs +0.597

Second half of phase:
Pumps, fish 1: 0
- Pumps, fish 2: 7, 0:00:12 (0.200 mins), avg =1.714 secs +0.360
- Pumps, fish 3: 28, 0:02:23 (2.383 mins), avg =5.107 secs +0.446

Snaps: 1 -> 2: 0
- Snaps: 1 -> 3: 0
- Snaps: 2 -> 1: 0
- Snaps: 2 -> 3: 0
- Snaps: 3 -> 1: 0
- Snaps: 3 -> 2: 0

Wrestles: 1 -> 2: 0
- Wrestles: 1 -> 3: 0
- Wrestles: 2 -> 1: 0
- Wrestles: 2 -> 3: 0
- Wrestles: 3 -> 1: 0
- Wrestles: 3 -> 2: 0

Intrudes, fish 1: 0, 0:00:00 (0.000 mins)
- Intrudes, fish 2: 0, 0:00:00 (0.000 mins)
- Intrudes, fish 3: 0, 0:00:00 (0.000 mins)

Swims, fish 1: 15, 0:08:52 (8.867 mins)
- Swims, fish 2: 40, 1:07:06 (67.100 mins)
- Swims, fish 3: 39, 0:16:57 (16.950 mins)

Shrimp injected, fish 1: 3
- Shrimp injected, fish 2: 2
- Shrimp injected, fish 3: 5

Courts, 1-2: 5, 0:03:15 (3.250 mins)
- Courts, 1-3: 2, 0:01:23 (1.383 mins)
- Courts, 2-3: 0, 0:00:00 (0.000 mins)
- Courts, 1-2-3: 0, 0:00:00 (0.000 mins)

Close, 1-2: 0:51:26 (51.433 mins)
- Close, 1-3: 0:17:30 (17.500 mins)
- Close, 2-3: 0:34:38 (34.633 mins)
- Close, 1-2-3: 0:47:31 (47.517 mins)
9:55:31 AM  Latency Period = 1:24:24 (84.400 mins); Pumps: fish1= 0, freq= 0; fish2= 7, freq= 4.97; fish3= 27, freq= 19.1

Phase 2: 0:00:00 (0.000 mins)
- Holds: 1 -> 2: 0
- Holds: 1 -> 3: 0
- Holds: 2 -> 1: 0
- Holds: 2 -> 3: 0
- Holds: 3 -> 1: 0
- Holds: 3 -> 2: 0
- Pumps, fish 1: 0
- Pumps, fish 2: 0
- Pumps, fish 3: 0
- Snaps: 1 -> 2: 0
- Snaps: 1 -> 3: 0
- Snaps: 2 -> 1: 0
- Snaps: 2 -> 3: 0
- Snaps: 3 -> 1: 0
- Snaps: 3 -> 2: 0
- Wrestles: 1 -> 2: 0
- Wrestles: 1 -> 3: 0
- Wrestles: 2 -> 1: 0
- Wrestles: 2 -> 3: 0
- Wrestles: 3 -> 1: 0
- Wrestles: 3 -> 2: 0
- Intrudes, fish 1: 0, 0:00:00 (0.000 mins)
- Intrudes, fish 2: 0, 0:00:00 (0.000 mins)
- Intrudes, fish 3: 0, 0:00:00 (0.000 mins)
- Swims, fish 1: 0, 0:00:00 (0.000 mins)
- Swims, fish 2: 0, 0:00:00 (0.000 mins)
- Swims, fish 3: 0, 0:00:00 (0.000 mins)
- Shrimp injected, fish 1: 0
- Shrimp injected, fish 2: 0
- Shrimp injected, fish 3: 0
- Courts, 1-2: 0, 0:00:00 (0.000 mins)
- Courts, 1-3: 0, 0:00:00 (0.000 mins)
- Courts, 2-3: 0, 0:00:00 (0.000 mins)
- Courts, 1-2-3: 0, 0:00:00 (0.000 mins)
- Close, 1-2: 0:00:00 (0.000 mins)
- Close, 1-3: 0:00:00 (0.000 mins)
- Close, 2-3: 0:00:00 (0.000 mins)
- Close, 1-2-3: 0:00:00 (0.000 mins)

Phase 3: 1:14:23 (74.383 mins)
- Holds: 1 -> 2: 1, 0:00:09 (0.150 mins), avg =9.000 secs
- Holds: 1 -> 3: 0
- Holds: 2 -> 1: 6, 0:01:07 (1.117 mins), avg =11.167 secs +2.845
- Holds: 2 -> 3: 2, 0:00:27 (0.450 mins), avg =13.500 secs +2.500
- Holds: 3 -> 1: 3, 0:00:31 (0.517 mins), avg =10.333 secs +/-3.333
- Holds: 3 -> 2: 1, 0:00:25 (0.417 mins), avg =25.000 secs
- Pumps, fish 1: 0
- Pumps, fish 2: 24, 0:01:15 (1.250 mins), avg =3.125 secs +0.591
- Pumps, fish 3: 28, 0:01:44 (1.733 mins), avg =3.714 secs +0.588
- Snaps: 1 -> 2: 0
- Snaps: 1 -> 3: 0
- Snaps: 2 -> 1: 1
Snaps: 2 -> 3: 0  
Snaps: 3 -> 1: 0  
Snaps: 3 -> 2: 5  
Wrestles: 1 -> 2: 0  
Wrestles: 1 -> 3: 0  
Wrestles: 2 -> 1: 0  
Wrestles: 2 -> 3: 0  
Wrestles: 3 -> 1: 0  
Wrestles: 3 -> 2: 1  
Intrudes, fish 1: 0, 0:00:00 (0.000 mins)  
Intrudes, fish 2: 8, 0:01:23 (1.383 mins)  
Intrudes, fish 3: 2, 0:00:06 (0.100 mins)  
Swims, fish 1: 32, 0:22:14 (22.233 mins)  
Swims, fish 2: 48, 0:38:14 (38.233 mins)  
Swims, fish 3: 53, 0:43:28 (43.467 mins)  
Shrimp injected, fish 1: 1  
Shrimp injected, fish 2: 1  
Shrimp injected, fish 3: 0  
Courts, 1-2 : 2, 0:05:34 (5.567 mins)  
Courts, 1-3 : 3, 0:01:15 (1.250 mins)  
Courts, 2-3 : 1, 0:02:01 (2.017 mins)  
Courts, 1-2-3: 3, 0:25:34 (25.567 mins)  
Close, 1-2 : 0:14:52 (14.867 mins)  
Close, 1-3 : 0:05:48 (5.800 mins)  
Close, 2-3 : 0:04:18 (4.300 mins)  
Close, 1-2-3: 0:34:49 (34.817 mins)  
Rises, 1-2 : 18, 0:03:22 (3.367 mins)  
Rises, 1-2 : Initiator = Fish 1: 18, Fish 2: 0  
Rises, 1-3 : 3, 0:01:02 (1.033 mins)  
Rises, 1-3 : Initiator = Fish 1: 3, Fish 3: 0  
Rises, 2-3 : 2, 0:00:37 (0.617 mins)  
Rises, 2-3 : Initiator = Fish 2: 1, Fish 3: 1  
Rises, 1-2-3: 26, 0:03:11 (3.183 mins)  
Rises, 1-2-3: Initiator = Fish 1: 24, Fish 2: 1, Fish 3: 1  
Total Day: 4:13:31 (253.517 mins)  
Time spent courting individually:  
Fish 1: 0:05:56 (5.933 mins)  
Fish 2: 0:08:59 (8.983 mins)  
Fish 3: 0:02:23 (2.383 mins)  
Brights, fish 1: 6, 2:42:16 (162.267 mins)  
Brights, fish 2: 2, 2:58:02 (178.033 mins)  
Brights, fish 3: 2, 4:05:31 (245.517 mins)  
Holds: 1 -> 2: 3, 0:00:29 (0.483 mins), avg =9.667 secs +1.764  
Holds: 1 -> 3: 1, 0:00:03 (0.050 mins), avg =3.000 secs  
Holds: 2 -> 1: 14, 0:05:56 (5.933 mins), avg =25.429 secs +7.293  
Holds: 2 -> 3: 2, 0:00:27 (0.450 mins), avg =13.500 secs +2.500  
Holds: 3 -> 1: 4, 0:02:33 (2.550 mins), avg =38.250 secs +28.394  
Holds: 3 -> 2: 3, 0:00:45 (0.750 mins), avg =15.000 secs +5.508  
Pumps, fish 1: 0  
Pumps, fish 2: 35, 0:01:37 (1.617 mins), avg =2.771 secs +0.424  
Pumps, fish 3: 73, 0:05:19 (5.317 mins), avg =4.370 secs +0.320  
Snaps: 1 -> 2: 0  
Snaps: 1 -> 3: 0  
Snaps: 2 -> 1: 1
Snaps: 2 -> 3: 0
Snaps: 3 -> 1: 0
Snaps: 3 -> 2: 5
Wrestles: 1 -> 2: 0
Wrestles: 1 -> 3: 0
Wrestles: 2 -> 1: 0
Wrestles: 2 -> 3: 0
Wrestles: 3 -> 1: 0
Wrestles: 3 -> 2: 1
Intrudes, fish 1: 0, 0:00:00 (0.000 mins)
Intrudes, fish 2: 8, 0:01:23 (1.383 mins)
Intrudes, fish 3: 2, 0:00:06 (0.100 mins)
Swims, fish 1: 47, 0:31:06 (31.100 mins)
Swims, fish 2: 88, 1:45:20 (105.333 mins)
Swims, fish 3: 92, 1:00:25 (60.417 mins)
Shrimp injected, fish 1: 4
Shrimp injected, fish 2: 3
Shrimp injected, fish 3: 5
Courts, 1-2: 7, 0:08:49 (8.817 mins)
Courts, 1-3: 5, 0:02:38 (2.633 mins)
Courts, 2-3: 1, 0:02:01 (2.017 mins)
Courts, 1-2-3: 3, 0:25:34 (25.567 mins)
Close, 1-2: 1:06:18 (66.300 mins)
Close, 1-3: 0:23:19 (23.317 mins)
Close, 2-3: 0:38:56 (38.933 mins)
Close, 1-2-3: 1:22:49 (82.817 mins)
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