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Serial monogamy in the European long-snouted seahorse *Hippocampus guttulatus*

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Abstract Seahorses (*Hippocampus* spp.) are non-sex-role-reversed members of the Syngnathidae family that provide extensive brood care. Previous studies of seahorses have revealed monogamy within a single brood, but their longer term mating system had not been comprehensively evaluated. The parental contribution to 29 wild-born broods of *Hippocampus guttulatus*, sampled from six Portuguese populations with differing seahorse densities and sex ratios, was assessed using microsatellite DNA markers. To assess the longer term genetic mating system of this species parentage was determined in eleven broods sampled from a captive population over two breeding seasons. Genetic data suggest that this socially polygamous seahorse is serially monogamous across breeding seasons, i.e. monogamous within a season but may switch mates between seasons, and that differing population densities and sex ratios do not affect the mating system.

Keywords Seahorse · *Hippocampus guttulatus* · Serial monogamy · Mating system

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Introduction

Social monogamy is employed as a mating strategy across many taxa, including fish (Herold and Clark 1993; Matsumoto and Yanagisawa 2001; Egger et al. 2006). Social monogamy is not, however, necessarily an accurate indicator of a genetically monogamous mating system (Wilson and Martin-Smith 2007). It is common for species to be socially monogamous but have a genetically polygamous mating system (Withler et al. 2004), or have mating strategies that fluctuate depending on environmental factors (Kamler et al. 2004; Carrete et al. 2006).

Seahorses (family Syngnathidae) are well known examples of fish where the males brood embryos and juveniles in a specialized brood pouch. Many studies indicate social monogamy in seahorses, with the formation of stable pair bonds, and suggest that seahorses are likely to be genetically monogamous (e.g. Vincent and Sadler 1995; Van Look et al. 2007). However social polygamy has been observed in some species (Woods 2000; Wilson and Martin-Smith 2007; Naud et al. 2009). Variation in population demographics (density, sex ratios) has been suggested to influence mating systems in syngnathids (Mobley and Jones 2007, 2009), increased individual densities or highly skewed sex ratio may provide a greater opportunity for mating to occur, and thus increases the chance of polygamy.

Globally many seahorse populations are declining (Foster and Vincent 2004), necessitating management and conservation measures. Knowledge of a species' mating strategy is useful in the design of the most effective conservation mechanism. To date the mating strategies of just two species of seahorse, both native to Australia, have been genetically assessed. *H. subelongatus* was genetically monogamous within a brood cycle (Jones et al. 1998) and males were faithful to a single female over two successive

broods (Jones et al. 2003), although low frequency of polygamy in successive broods has also been observed (Kvarnemo et al. 2000). *H. abdominalis* was also found to be genetically monogamous in both low density wild populations and high density captive populations, despite the observation of social polygamy in wild populations (Wilson and Martin-Smith 2007).

The European long-snouted seahorse, *H. guttulatus* (Cuvier 1829), inhabits temperate European coastal waters. This species can occupy areas at densities much greater than other seahorse species (Curtis 2004; Woodall 2009), and with equal or skewed sex ratios (Curtis and Vincent 2006; Woodall 2009). *H. guttulatus* lives in large socially polygamous groups, with each male displaying to a number of females concurrently and successively (Naud et al. 2009). Given the differences (high density and sex ratios) between *H. guttulatus* and other seahorse species, and the observed social polygamy, *H. guttulatus* is an interesting species on which to investigate seahorse mating strategies. This study comprised two parts: (i) genetic testing for single/multiple maternity within broods of wild-caught males, to determine if *H. guttulatus* display a polygamous mating strategy when present at high densities and/or at different sex ratios in the wild; and (ii) testing for single/multiple maternity in successive male broods *ex-situ*, to investigate longer term mating strategies.

Materials and methods

Sample collection

Wild-caught samples. Pregnant male *H. guttulatus* were collected from six sites in Portugal, where populations were known to differ in abundance (3.0–39.3 seahorses seen per diver hour) and sex ratio (0.81:1–1.76:1 males:females) (Table 1). Abundance was measured crudely as total number of seahorses per total number of hours spent diving, having previously been assessed by surveying and revealed as a reliable relative measure (Woodall 2009).

Table 1 Wild-caught *Hippocampus guttulatus* broods: site, number of broods screened, population relative density (individuals observed per diver hour) and sex ratio (male:female)

Site	Number of broods	Density of seahorses	Sex ratio
RPOa	4	4.4	1.17:1
RPOb	6	5.1	0.81:1
RPOc	1	6.4	N/A
RPOd	12	39.3	1.76:1
PPO	3	6.0	0.85:1
TPO	3	3.0	1:1

Sites RPOa-d were located within the Ria Formosa lagoon system. Sites at Portimao (PPO) and Troia (TPO) were located within river estuaries, 55 km and 220 km from the lagoon respectively. Males were placed into aquaria (with 100% fresh seawater change every 8 h) until they gave birth. Twenty newborns were sampled from each brood (typical brood size 180–567, Curtis and Vincent 2006) and a fin clip of the male was taken, with all material stored in 95% ethanol. The remaining brood and male were then returned to their original collection location.

Ex-situ mating trials. Sixteen adult *H. guttulatus* (8 male:8 female) were captured under collection permit (DR/DRI-565) from the Ria Formosa (site RPOd), and shipped via a local aquarium facility (Oceanario Lisboa) to the Zoological Society of London (ZSL) London Zoo Aquarium under CITES permit (PT/C-1189/20050). The seahorses were housed in a single aquarium (50 × 70 × 175 cm) for the duration of the study. From each of eleven broods produced by the captive population during the study period 10–25 individual newborns were collected and euthanized humanely using MS222, then fixed and stored in 95% ethanol. Following completion of the study, tissue from remaining adults (5 males:4 females) was sampled by fin clipping (Woodall 2009). It was not possible to sample all adults as some died in the early phase of the study.

DNA extraction and amplification of microsatellites

Genomic DNA was extracted using a CTAB protocol (Winnepeninckx et al. 1993) from 2 mm² of fin clip or half the newborn body. Microsatellite loci (Galbusera et al. 2007; Pardo et al. 2007) (*Hcaμ11*, *Hcaμ37* and *Hgu4* for wild specimens; *Hcaμ11*, *Hcaμ37*, *Hgu4* plus *Hcaμ27* for captive specimens) were amplified in 10 μl reactions containing 2 μl template DNA (10–50 ng), 1 μl 10× manufacturer-provided buffer, 0.6 μl MgCl₂ (50 mM-Bioline), 1 μl deoxynucleotide triphosphate mix (dNTP, 1.25 mM), 0.25 μl of each primer (10 μM, one being Cy5 labelled), 0.05 μl Taq polymerase (5U/μl-Bioline). The thermocycle profile comprised an initial denaturing step (3 min at 95°C), followed by 35 cycles of denaturing (30 s at 95°C), annealing (30 s at either 53°C (*Hcaμ11* and *Hcaμ37*) or 56°C (*Hgu4* and *Hcaμ27*)), and extension (30 s at 72°C), and a final extension step (3 min at 72°C). PCR products were run on 6% denaturing polyacrylamide gels in an ALFexpressII automated DNA sequencer (Amersham Pharmacia). Allele sizes were scored using Fragment Manager v2.9 (Amersham Pharmacia).

Data analysis

Departure from Hardy–Weinberg equilibrium within loci and linkage disequilibrium between loci; (GENEPOP 4.0;

Raymond and Rousset 1995), and the potential presence of null alleles (MICROCHECKER; Van Oosterhout et al. 2004) were tested. A multi-locus approach (DeWoody et al. 2000) was used to reconstruct maternal (and paternal, in the case of captive broods) genotypes, and thus assess the number of females contributing eggs to a brood. To confirm correct parental assignments for wild-caught and captive broods, maternal (and paternal, in the case of wild broods) genotypes and offspring assignments were generated using GERUD2.0 (Jones 2005).

Our aim was to minimize impact of sampling (sacrifice of offspring) on populations whilst maximizing statistical power of tests to detect multiple maternity, so a compromise sample size of 20 offspring per brood was selected. Tests using the multiple mating simulation programme PrDM (Neff and Pitcher 2002) indicated that with the loci screened the power to detect multiple maternity within wild broods (one parent known, equal 50:50 or skewed 90:10 maternal contributions) was high (100 and 98% ,respectively), and similarly the power to provide high parental exclusion probabilities (captive broods) was equally high (Table 2). Likewise, simulation studies using GERUDsim 2.0 (Jones 2005) indicated that with sample size of 20 offspring per brood multiple maternity, if present, would be detected in 98% of cases with 50:50 maternal contributions and 93.8% of cases with 90:10 skewed contributions: doubling sample size to 40 offspring resulted in only a 4.2% increase in likelihood of detection (skewed contributions).

Results and discussion

Marker loci characterization

All four microsatellite loci screened were polymorphic, with 3–14 alleles, and expected heterozygosity from 0.34 to 0.79 (Table 2). No loci exhibited significant departure

from Hardy–Weinberg equilibrium ($P > 0.05$) and no linkage between loci was detected ($P > 0.5$). Null alleles, or other scoring errors, were not indicated by MICROCHECKER.

Maternity within single wild broods, and the effect of population density and sex ratio

In wild broods paternal genotype was directly assessed from male tissue, and therefore could be unambiguously stated. Maternal alleles were inferred from brood genotypes once paternal alleles were identified: in all cases GERUD2.0 analysis confirmed reconstructed maternal genotypes inferred by multilocus parsimony methods. For all 29 broods tested, a single maternal genotype was unambiguously assigned within each brood. No reconstructed female genotypes occurred in more than one brood, providing no evidence for multiple mating in wild females.

All wild broods tested were consistent with production from a single female–male mating, providing no evidence of departure from short-term (within a brood cycle) genetic monogamy in *H. guttulatus*, consistent with the breeding system previously observed for other seahorse species (Jones et al. 1998, 2003; Wilson and Martin-Smith 2007). Although *H. guttulatus* occurs at higher densities than most other seahorse species (Foster and Vincent 2004), it has been observed to be socially polygamous (Naud et al. 2009) and may have skewed sex ratios in some populations (Woodall 2009): none of these factors appear to result in deviation from a genetically monogamous breeding system.

Longer term mating system in *H. guttulatus*

Genetic screening of the captive population, to test for longer term monogamy and mate fidelity, confirmed that all 11 broods exhibited a maximum of four alleles at any one locus consistent with each brood being the product of a single female–male mating (i.e. within-brood monogamy). In all cases brood genotypes excluded all but a single male–female parental combination (see Table 3), except for brood ZSL1 for which only the father (M4) could be ascertained. For this brood it is assumed that the mother was an individual that died during the trial and was not genetically sampled. Two broods, ZSL9 and ZSL10, had the same parents assigned to them (M5 and F2—Table 3), but as ZSL10 juveniles were sampled only 10 days after ZSL9 juveniles and were correspondingly larger (Woodall 2009), these two broods were concluded to be part of a single brood. Two unique male–female pairings led to just one brood each: M1 and F1 (ZSL5); M2 and F4 (ZSL8). In two cases multiple broods resulted from the same male–female pairing: three broods (ZSL3, ZSL6 and ZSL9/10)

Table 2 Summary statistics for four microsatellite loci screened for *Hippocampus guttulatus* wild and captive broods

Locus	Number of alleles	H _E	Probability of determining multiple mating	Exclusion probability	
				Neither parent known	One parent known
<i>Hcaμ11</i>	14	0.79	1.00	0.60	0.75
<i>Hcaμ37</i>	14	0.68	0.99	0.57	0.73
<i>Hgu4</i>	3	0.36	0.21	0.10	0.22
Combined			1.00	1.00	1.00
<i>Hcaμ27</i>	3	0.34	0.22	0.13	0.23

Table 3 Individual candidate parents not excluded by analysis of four microsatellite loci for each of 11 captive broods produced over two breeding seasons (2007 and 2008)

Brood	ZSL1	ZSL2	ZSL3	ZSL4	ZSL5	ZSL6	ZSL8	ZSL9	ZSL10	ZSL11
M	4	3	5	3	1	5	2	5	5	5
F	?	3	2	3	1	2	4	2	2	1
Date of brood	01/08/2007	16/08/2007	21/08/2007	10/09/2007	17/10/2007	23/10/2007	07/11/2007	19/11/2007	29/11/2007	10/07/2008

M and F: male (1–5) and female (1–4) individuals (genotypes) not excluded. ? = reconstructed parental genotype does not match any available adult genotype

produced over a 3 month period consistent with M5 and F2; and two broods (ZSL2 and ZSL4) produced over a 1 month period consistent with M3 and F3. Genotypes in the final brood (ZSL11—the only brood produced in the second season) were consistent with F1 and M5, which both previously produced broods with different partners (M1 and F2, respectively).

The analysis of successive captive broods supports the conclusion from the wild samples of within-brood monogamy, and also that there is longer term fidelity/monogamy between male–female pairings (pairs M5 + F2 and M3 + F3) throughout a breeding season. Just one male (M5) produced broods in successive years and did so with eggs from different females (F2 then F1), even though the previous partners of M5 and F1 were still available. This final result suggests that pair fidelity may not be maintained across years and longer term polygamy (although still monogamous within seasons—i.e. serially monogamous) may be present in the breeding system of this species. No previous studies have tested genetic mating systems over successive breeding seasons, for seahorses or any other Syngnathid. Kvarnemo et al. (2000) found mate switching between broods within a season in *H. subelongatus*, in contrast to another study of the same species (Jones et al. 2003), and other Syngnathid (pipefish) mating systems have been found to be variable (Mobley and Jones 2007, 2009). Further investigation is required to assess under what conditions mate switching occurs in Syngnathids, and to confirm whether the mate switching between breeding seasons (serial monogamy) observed in this study in captive seahorses is a regular mating strategy in the wild.

A strict monogamous mating system has the potential to limit effective population size (N_e) where sex ratios are skewed, when the rarer sex limits number of mating pairs, although polygamy may also lower N_e where individual reproductive success is highly skewed. Monogamy usually results when mates are scarce or dispersed and the operational sex ratio is close to equal (Emlen and Oring (1977). In the populations of *H. guttulatus* studied individual densities are high (for seahorses—Curtis and Vincent 2006) and sex ratios skewed (Table 1), and there is evidence for social polygamy and distinct mate preference (Naud et al.

2009), all factors that might not predict strict monogamy. Physical limits of seahorse reproductive biology may, however, explain why monogamy is adhered to: after mating females require a time period, similar to that of male brooding, to produce their next egg batch, at which point they hydrate the eggs prior to re-mating but can carry the hydrated eggs only for a few days (Curtis 2007). The female therefore requires a nearby male ready to mate, so a cycle synchronised with a monogamous mate is optimal for reproductive success. These opposing and interacting factors appear to result in a mating system that is monogamous within a breeding season to optimize offspring production, but in which individuals (predominately males) may select a different ‘best’ (largest) mate each breeding season.

Despite social polygamy in *H. guttulatus*, there is no evidence for genetic polygamy within a brood or within a breeding season. If genetic polygamy does occur, it does so at very low levels and would make very little impact on N_e of a population. For species management purposes, therefore, *H. guttulatus* should be considered as serially monogamous. However if future research provides evidence for more regular mate swapping and a more polygamous mating system then this should be reflected in management practices.

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