Multiple paternity and subsequent fusion-rejection interactions in a kin-structured population

Sheri L. Johnson1,2,* , Philip O. Yund1

1Marine Science Education and Research Center, University of New England, 11 Hills Beach Road, Biddeford, Maine 04005, USA
2Present address: Department of Zoology, PO Box 11-8525, University of Florida, Gainesville, Florida 32611, USA

ABSTRACT: Although numerous studies have explored the ecological and evolutionary consequences of relatedness on interactions among individuals, few have explored how variation in multiple paternity in natural populations affects subsequent interactions at later life history stages. Additional fathers result in a more genetically diverse brood because the ratio of half siblings to full siblings is increased. For sessile, colonial marine invertebrates, which typically have limited larval dispersal, the probability of a juvenile fusing with a neighboring conspecific is likely to be affected by the genetic composition of the brood from which it is derived. Consequently, we explored the relationship between multiple paternity and subsequent fusion-rejection interactions in the colonial ascidian *Botryllus schlosseri*. Microsatellites were used to calculate an effective paternity index for each brood and fusion tests among juveniles collected from recruitment plates adjacent to maternal colonies were used to assay fusion frequency. Results support the hypothesis that fusion frequency among recruits decreases with increasing levels of multiple paternity, indicating that fertilization processes affect interactions among later life stages. By extension, fertilization outcomes that affect levels of multiple paternity should be incorporated into studies of juvenile and adult interactions in other taxa with kin-structured populations.

KEY WORDS: Paternity analysis · Kin interactions · Fertilization processes · Historecognition · Ascidian · *Botryllus schlosseri*

INTRODUCTION

Like most multi-cellular organisms, marine plants and animals typically complete complex life cycles through a series of stages that can differ dramatically in form and function; ecological processes that act on one life stage can potentially alter performance and selection outcomes at later life stages (Wilbur 1980). Numerous studies in diverse taxa have demonstrated functional links among post-fertilization life stages (e.g. marine invertebrates, Emlet & Hoegh-Guldberg 1997; terrestrial plants, Mazer 1989). Few studies, however, have attempted to link mating-system dynamics with subsequent juvenile or adult processes.

Paternity studies have established that female promiscuity is common in both marine and terrestrial systems (Bernasconi et al. 2004), and multiple mating often produces a mixture of full and half siblings. Although studies of the fitness consequences of singly vs. multiply sired progeny are lacking in marine systems, several well-controlled studies in analogous terrestrial plant systems have explored the ecological and evolutionary consequences of relatedness on subsequent interactions among individuals (e.g. Schmitt & Antonovics 1986, Karron & Marshall 1993, Delesalle & Mazer 2002), and some of these studies have used experimentally produced full vs. half siblings as tools to evaluate fitness consequences of singly vs. multiply sired progeny (Schmitt & Antonovics 1986, Karron & Marshall 1993). Yet little effort has been made, in any taxon, to explore whether variation in multiple paternity in natural populations affects subsequent interactions among juveniles.

In copulating animals, multiple mating is associated with both direct (nuptial gifts, acquisition of resources, paternal care) and indirect (good genes, compatibility,
genetic diversity) benefits and thus is subject to an array of selective pressures (reviewed in Yasui 1998, Jennions & Petrie 2000). In contrast, direct benefits to multiple mating are not expected in externally fertilizing species (such as marine broadcast spawners) or in passively mating species with internal fertilization, which includes suspension-feeding marine invertebrates that receive sperm via the water column and terrestrial plants (Bishop & Pemberton 1997), as well as brooding algae (Engel et al. 1999). In these systems, fitness consequences of multiple mating are largely limited to indirect genetic benefits. Yet multiple mating solely to increase genetic diversity is predicted to be advantageous only under rather limited circumstances: (1) during situations of completely unpredictable environmental fluctuation, (2) as a mechanism to prevent inbreeding (Foerster et al. 2003), (3) when there is intense competition among full siblings, or (4) when cooperative or compensatory interaction among half siblings occurs (reviewed in Yasui 1998). In colonial marine organisms and seed plants, passive mating is often associated with limited propagule dispersal, which can lead to inbreeding and competitive or cooperative interactions among kin (Levin & Kerster 1974, Jackson & Coates 1986). Hence, increased genetic diversity from multiple mating could be adaptive in these organisms if the genetic composition of offspring is known or expected to be genetically based (Scofield et al. 1982, Grosberg 1988, Santelices et al. 1999, Hughes et al. 2004). In general, colonies that share sufficient historecognition alleles fuse and form a single, integrated, chimeric colony, while colonies with insufficient match reject one another and either cease growth or actively compete for space (Grosberg 1988). When colonies fuse, genotypes may compete for access to gametes within the resulting chimera, which can have dramatic consequences for fitness (Buss 1982).

Although the genetic mechanism is straightforward, it is not entirely clear whether natural variation in multiple paternity will influence subsequent fusion-rejection interactions among field recruits (but see Hughes et al. 2004 for an example from laboratory crosses). In the field, larval recruitment generally integrates across multiple source broods, so local paternity effects could be swamped by recruits from more distant sources. Consequently, we explored the relationship between multiple paternity and subsequent colony fusion-rejection dynamics in the colonial ascidian *Botryllus schlosseri*. Multiple paternity varies widely in natural populations of this species (Johnson & Yund 2007). Larval dispersal is philopatric, with many larvae recruiting within a few centimeters of the maternal colony, though some disperse much greater distances (Grosberg 1987). Episodes of long-distance dispersal do not appear to completely overwhelm local dynamics because colonies tend to live in kin groups, with colonies genetically similar to one another over small spatial scales (5 m or less), but genetically different over larger scales (10 m or more, Yund & O’Neil 2000). If levels of multiple paternity affect relatedness within a kin group, these relatedness differences should be reflected in fusion-rejection interactions among young colonies.

**MATERIALS AND METHODS**

**Study organism.** *Botryllus schlosseri* colonies are found on hard substrates in shallow waters throughout temperate regions of the world (Van Name 1945). Colonies are composed of asexually produced zooids arranged in systems or clusters around a common exhalent siphon. *B. schlosseri* is a cyclical hermaphrodite in which male and female phases alternate in repetitive sexual cycles linked to an asexual zooid replacement cycle. Over a period of 7 to 14 d the zooids grow and expand, and eventually take over the function of the older zooids, which are quickly reabsorbed. Fertilization is time-integrated, with eggs viable to be fertilized for 24 h after the opening of the siphons of a new generation of zooids (Stewart-Savage et al. 2001). Embryos are brooded until released as tadpole larvae at the end of the cycle. Many larvae settle within a few centimeters of the adult colony, although dispersal is highly leptokurtic; the fate of widely dispersing larvae is unknown (Grosberg 1987). Sperm release commences approximately 16 h after siphon opening and continues for several days, with colonies continuously releasing small volumes of sperm (Stewart-Savage & Yund 1997). Self-fertilization is prevented by the temporal offset of female and male phases, and colonies do not store sperm (Stewart-Savage et al. 2001). Fertilization appears to be very efficient, with long-lived, diluted sperm extracted from the water column (Johnson & Yund 2004). More than 85% of eggs are fertilized in natural populations (Phillippi et al. 2004) and fertilization in the field can occur over distances up to 207 m (Yund et al. 2007). Multiple paternity is highly variable in natural populations of *B. schlosseri* (Johnson & Yund 2007), probably due to ecological factors such as population density and timing of sperm release.
Genetics of historecognition in botryllloid ascidians.

In botryllloid ascidians, fusion-rejection interactions are controlled by a single, highly polymorphic, co-dominantly expressed fusion-histocompatibility (FuHC) locus (Sabbadin 1962, Scofield et al. 1982, De Tomaso et al. 2005). Tens to hundreds of alleles are typically present in a population (Grosberg 1988). Because of this high level of polyorphism, most colonies are heterozygous at the FuHC locus. Fusion occurs when 2 colonies share one or both alleles and retraction occurs in the absence of a shared allele. Fusion is thus more likely among full siblings than among half siblings because shared alleles can be derived from either parent (Scofield et al. 1982). Hence we hypothesized that increased multiple paternity would decrease the frequency of fusion among nearby recruits.

The frequency of fusion among siblings of unknown paternity can be predicted from genetics and is expected to vary within the range of 0.50 to 0.75 (Scofield et al. 1982). Fusion in a brood of full siblings fathered by a single male should occur at a frequency of 0.75, but as the number of fathers increases, the level of fusion should decrease towards 0.50 (if all brood members are half siblings and fathered by different males). These expected frequencies assume that the males and female are unrelated, that all colonies are heterozygous and carry unique historecognition alleles, and that recruitment is entirely local (i.e. interacting recruits are the progeny of a single maternal colony). These assumptions may not be valid in natural populations, so actual fusion frequencies may fall outside the predicted range.

Experimental design. To test for a relationship between paternity and fusion frequency, we sampled brooded embryos and adjacent newly recruited juvenile colonies in a population off an island in Saco Bay, Maine (43° 27' 25.16'' N, 70° 20' 34.42'' W). Recruitment spindles composed of 9.7 cm diameter, round PVC plates (area of 72.1 cm² after subtracting the central mounting hole) were mounted 4 plates per stainless-steel spindle (with plates 2 cm apart) and tethered with the edge of the lower plate within 2 to 4 cm of naturally occurring maternal colonies (see Yund & Stires 2002 for a fuller description of recruitment spindles). Twelve spindles were deployed in August and September of 2005, and July and August of 2006. In a few cases 2 or 3 spindles were deployed simultaneously, but the maternal colonies sampled were separated by at least 2 m, and all nearby colonies (<0.5 m) except the focal maternal colony removed. At the time of spindle deployment we sampled focal colony tissue for determination of the maternal genotype and collected embryos to assay multiple paternity of the brood. Each recruitment spindle was deployed for 3 to 7 d to allow release of larvae from the adult colony and then collected and returned to the laboratory at the University of New England’s Marine Science Center, Biddeford, Maine. Recruits that had settled on the underside of each plate were counted and mapped. Spindles were maintained in flowing seawater until recruits reached the minimum 4 zooid size necessary for successful transplantation. Botryllus schlosseri larvae are thought to preferentially aggregate at recruitment with individuals that share historecognition alleles (Grosberg & Quinn 1986). We specifically avoided conducting fusion tests between nearest neighbors because we wanted to examine the overall effect of multiple paternity on fusion-rejection interactions within a colony’s eventual growth radius. Therefore, fusion-rejection assays were performed on 24 to 36 non-nearest neighbor pairs from each spindle (pairs constituted of recruits from different plates on the same spindle). Tests were conducted by placing 2 recruits 1 to 2 mm apart and allowing their edges to grow into contact. Test colonies were assayed daily for evidence of colony fusion, which was identified solely on the basis of interconnection of the vascular system of the 2 recruits. Results from all the paired tests from each spindle were used to calculate a single fusion frequency for that spindle. We deliberately did not assay parentage in recruits to check whether they were derived from the neighboring maternal colonies.

Paternity assignment of brooded embryos. Genomic DNA was extracted from fresh maternal tissue using the PureGene kit (Gentra Systems) following the marine invertebrate extraction protocol (#00690). DNA was extracted from 17 to 30 offspring per brood by incubating each fresh embryo in 40 µl of distilled, deionized water (ddH₂O) with 30 µg proteinase K (New England Biolabs) for 2 h at 65°C, followed by incubation for 10 min at 90°C. Samples were genotyped at 3 microsatellite loci (Bs811, Bs49, Bs29) using the touch-down PCR protocol detailed in Johnson & Yund (2007). Products were electrophoresed on a LiCor 4200 Global IR² DNA Analyzer and scored with SAGA 2 software (LiCor Biosystems), then confirmed visually. Although we did not conduct population surveys at this particular site, these 3 microsatellite loci have previously been found to provide >95% paternity exclusion in nearby populations (Johnson & Yund 2007).

Comparing the embryo genotypes with the known maternal genotype identified paternal alleles. However, it was impossible to judge which allele was paternal when the mother and embryo shared the same heterozygous genotype. In these cases, we randomly assigned the paternal allele of each heterozygous embryo and repeated this random assignment 5 times for each brood, then took the average as the final estimate of haplotype abundance. To estimate the diversity of sperm haplotypes within each brood we used an...
Effective paternity index \( (K_E) = 1/\Sigma(p_i)^2 \) (Starr 1984, adapted from Simpson’s Diversity Index, Simpson 1949), where \( p_i \) = proportion of offspring fathered by haplotype \( i \), and \( i = 1…k \), the number of paternal haplotypes contributing to the brood (see Johnson & Yund 2007 for further detail). This effective paternity index is superior to a simple minimum number of fathers estimate for questions concerning the relative genetic diversity of a brood, but estimates paternity at the level of the sperm haplotype, rather than the diploid male genotype (Johnson & Yund 2007).

**Data analysis.** Due to heteroscedasticity in the data (higher scatter at low \( K_E \)) we used a non-parametric Spearman correlation to analyze the relationship between the paternity and fusion frequency. Because we analyzed slightly different numbers of offspring for each brood we also used a correlation approach to look for a relationship between the number of embryos sampled and effective paternity. All statistical analyses were conducted using JMP version 4.0.4 (SAS Institute).

**RESULTS**

Effective paternity estimated from 17 to 30 brooded offspring per spindle (20.2 ± 1.09, mean ± SE) ranged from 2.9 to 6.6 haplotypes (Fig. 1). Previous results have indicated that effective paternity is relatively insensitive to variation in sample size within this range (Johnson & Yund 2007). The number of recruits on spindles varied from 0 to 119 per plate (40.5 ± 4.07), and 61 to 382 (162.1 ± 28.7) per spindle. Resulting fusion frequencies estimated from 24 to 36 non-nearest neighbor pairs per spindle (28.9 ± 4.72) ranged from 0.05 to 0.47 per spindle (Fig. 1). We detected a significant negative correlation between \( K_E \) and fusion frequency \( (r = -0.76, \text{Spearman’s correlation coefficient } r_S = -0.80 p < 0.005; \text{ Fig. 1}) \) and this relationship would have been much stronger if one datum was considered to be an outlier \( (r = -0.90; \text{ open symbol in Fig. 1}) \). Effective paternity explained 64\% (\( r_S^2 \)) of the variance in ranks of fusion frequency. We found no correlation between the number of offspring sampled and the effective paternity \( (r = -0.27, r_S = -0.03, p = 0.92) \), suggesting that the slightly different number of offspring analyzed for paternity had no effect on the estimate of paternal diversity.

**DISCUSSION**

Our results support the hypothesis that fusion frequency among recruits decreases with increasing levels of multiple paternity within a brood, measured as effective paternity (Fig. 1). We expected fusion frequency to range from 0.5 to 0.75, but overall frequencies were much lower. Standoff between potentially fusible pairs could explain this discrepancy, but in most cases we observed an area of necrotic tissue, confirming rejection. The presence of chimeric focal brood parents could be another possible explanation, but in no cases did we observe a third allele at any microsatellite locus. A more likely explanation is that a portion of the larval recruitment to our spindles was not derived from the adjacent maternal colony. Most retrieved larvae settle within centimeters of the maternal colony, but fates of the majority of larvae released are unknown (Grosberg 1987). A large proportion of these larvae die in most marine invertebrate taxa, but some fraction do recruit elsewhere, as non-local larval recruitment has been demonstrated in previous studies (Grosberg & Quinn 1986). Overall, the fusion frequencies obtained in this study are comparable to those reported for random samples of natural populations of *Botryllus schlosseri* (Karakashian & Milkman 1967, Grosberg 1988).

The maternal colonies that we assayed were somewhat isolated. Colonies either had no naturally occurring neighbors within 0.5m, or we removed 1 to 2 neighbors within that radius prior to larval release. Population density might well have complex effects on the relationship between multiple paternity and subsequent recruit fusion. Although preliminary data suggest that density does not affect levels of multiple paternity (Johnson & Yund 2007), density is expected to affect recruitment levels (Yund & Stires 2002). If recruits on a surface are drawn from more broods, they will experience lower fusion rates (per above), which
might obscure the relationship that we detected. An exploration of how this relationship changes with population density is merited. Nevertheless, the results we obtained should be relevant to many colonies in natural populations. If non-local recruitment occurred, it did not obscure a relationship between paternity and fusion frequency, suggesting that any non-local recruitment must occur at a relatively constant background level. These new hypotheses could be tested by using genetic markers to estimate local vs. non-local parentage.

Multiple paternity appears only to increase genetic diversity and have no other immediate reproductive benefits in Botryllus schlosseri (or it may simply be a by-product of selection to ensure all eggs are fertilized; Johnson & Yund 2007). Although we have not followed subsequent fusion-rejection dynamics to evaluate ultimate fitness consequences associated with multiple paternity, fusion is expected to have dramatic effects on growth, reproduction, and survival (Sabbadin & Zaniolo 1979, Stoner et al. 1999, Chadwick-Furman & Weissman 2003, Laird et al. 2005), which ultimately influences maternal fitness. Without understanding whether fusion itself is adaptive, we cannot evaluate whether multiple paternity is adaptive with respect to fusion. However, costs and benefits of fusion are complex issues and constitute an area of active research (e.g. Laird et al. 2005, De Tomaso 2006, Nyholm et al. 2006). Hypothesized benefits from fusion include increasing the size of the resulting chimeric colony, which in turn may decrease mortality rates (Grosberg 1988) and lead to earlier sexual reproduction (Buss 1982), which in colonial invertebrates is normally size-rather than age-dependent (Harvell & Grosberg 1988). Fusion may also increase internal genetic diversity, which might enable a chimeric colony to cope with rapidly changing environmental conditions (Rinkevich & Yankelevich 2004). However, potential costs are incurred in chimeric colonies as well. Colonial invertebrates do not sequester a germ line, so stem cells of one colony may be exchanged with the fusion partner. If stem cells of one fusion partner become disproportionately represented in the somatic tissue or in the gametes, one colony has effectively parasitized the other (Buss 1982). Contact between colonies, whether it leads to fusion or rejection, may also result in significantly reduced growth and reproductive output (Chadwick-Furman & Weissman 2003). Regardless of how the net fitness consequences of these costs and benefits are ultimately assessed, fertilization dynamics play an important role by influencing fusion frequencies and thus need to be considered an integral part of the selection regime.

Although the specific relationship between multiple paternity and subsequent fusion-rejection interactions documented here will be limited to colonial marine invertebrates (and perhaps algae that exhibit coalescence; Santelices et al. 1999), the underlying relationship between multiple paternity and subsequent adult interactions should apply to all organisms with limited dispersal, such that kin live out their lives in aggregations. When the outcomes of juvenile or adult interactions are predicted from kin relationships, fertilization processes that determine levels of multiple paternity are inextricably linked to the ecology of subsequent life-history stages. Paternity patterns will affect the impact of subsequent ecological processes, but those ecological processes will in turn determine the fitness consequence of multiple paternity.

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