

Multiple paternity and female sperm usage along egg-case strings of the knobbed whelk, *Busycon carica* (Mollusca; Melongenidae)

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Abstract We used genotypic data from three highly polymorphic microsatellite loci (two autosomal and one sex-linked) to examine micro-spatial and temporal arrangements of genetic paternity for more than 1,500 embryos housed along 12 egg-case strings of the knobbed whelk, *Busycon carica*. Multiple paternity proved to be the norm in these single-dam families, with genetic contributions of several sires (at least 3.5 on average) being represented among embryos within individual egg capsules as well as along the string. Two strings were studied in much greater detail; five and seven fathers were identified, none of which was among the several males found in consort with the female at her time of egg-laying. Each deduced sire had fathered roughly constant proportions of embryos along most of the string, but those proportions differed consistently among fathers. A few significant paternity shifts at specifiable positions along an egg-case string were also observed. Although the precise physical mechanisms inside a female whelk's reproductive tract remain unknown, our genetic findings indicate that

successive fertilization events (and/or depositions of zygotes into egg capsules) normally occur as near-random draws from a well-but-not-perfectly blended pool of gametes (or zygotes) stemming from stored ejaculates, perhaps in different titers, of a dam's several mates.

Introduction

As in other *Busycon* species (Gastropoda; Melongenidae), female knobbed whelks (*Busycon carica*; Gmelin, 1791) typically deposit an egg-case string during warm-water periods of the year. Each string, laid over a period of several days, is about 375–730 mm long (mean 511 mm as reported by Power et al. 2002; see also Castagna and Kraeuter 1994) and consists of about 40–160 (mean 89) leathery capsules attached successively to one another by a cordlike structure. Each capsule houses 30–50 (but sometimes up to 100 or more) embryos. After depositing her egg-case string in an intertidal mudflat, the female provides no parental care. During the next several weeks to months, her progeny pass through a veliger stage inside the egg case before hatching as miniature adults to begin a free-living existence. After reaching sexual maturity at about 9 years of age (Castagna and Kraeuter 1994), gravid females can often be observed in consort with several males apparently attempting copulations.

Males are equipped with a large muscular penis that inflates inside the female's mantle cavity, and fertilization is internal. The oviduct contains chambers (the bursa copulatrix and seminal receptacle) for sperm reception and storage, respectively, as well as albumen and capsule glands for egg capsule formation. Sperm

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storage by females is common in prosobranch molluscs, but durations can be highly variable (Paterson et al. 2001). Multiple mating also has been documented in some gastropods (Murray 1964), and, in conjunction with female sperm storage, is likely to increase genetic variability among progeny within a brood.

In previous papers (Avise et al. 2004; Walker et al. 2005), we identified a sex-linked microsatellite polymorphism in *B. carica* and showed how it could be used in conjunction with autosomal markers to document the genetic mode of sex determination, identify the gender of individuals (including embryos), and facilitate paternity analyses in this common marine mollusc of eastern North American shorelines. Here we genetically identify sires and monitor their contributions to collections of embryos along egg-case strings produced by individual females. Our goals were to quantify the frequency of multiple paternity in the wild, to determine whether consort males at the time of egg-laying are the actual sires of progeny in that brood, and to ask whether patterns of sperm use by a female depart significantly from a null model of random successive draws from a well-blended pool of multi-male sperm within her reproductive tract.

Materials and methods

Adults ($n = 139$ genetically assayed) and egg-case strings ($n = 12$) were collected at low tide from mudflats along Wassaw Island, Chatham County, Georgia, in the springs of 2002 and 2003. Fortuitously, on two occasions we encountered a female in the process of depositing her string of capsules. One of these females was accompanied by two males and the other had nine male consorts. All adults in these two groups, together with their embryo-containing capsule strings, were collected as potential families. Ten other egg-case strings that had been abandoned by adults before the time of their collection were also genetically assayed. All of the egg-case strings surveyed were anchored in mud at one end and either attached to the female or floating freely in the water at the other. Egg capsules at the anchored end are easily recognized by their shriveled appearance and lack of embryos. They mark the spatio-temporal starting points for our genetic paternity analyses of embryos housed along the remainder of the egg-case strings.

For the adults, sex was provisionally assessed by presence or absence of a penis (73 males and 66 females). A small piece of tissue was taken from the foot or siphon and the animal was then released. This tissue, stored in 95% ethanol, was later washed in

distilled water, and DNA was extracted using a standard proteinase K phenol:chloroform protocol (Sambrook et al. 1989).

Embryos ($n = 1,513$ assayed at the sex-linked locus) were removed from their egg capsules, washed in distilled water, and stored in 95% ethanol until DNA extraction in 200 μ l of Gloor and Engels (1992) buffer following procedures in Jones and Avise (1997). The large numbers of capsules and embryos ($>1,000$) in most of the strings precluded attempts to genetically assay all individuals, so embryos were sampled from along each string, most often by randomly picking about 20 individuals (or less when fewer were present), either from each successive egg capsule or each fifth egg capsule along the string.

Genomic library construction, microsatellite and primer development, and PCR assay conditions for three microsatellite loci (one sex-linked and two autosomal) are as previously described (Avise et al. 2004). For each individual, 0.8 μ l of PCR product, together with 2.1 μ l deionized formamide, 0.5 μ l loading buffer, and 0.3 μ l ROX 500 Size Standard (Applied Biosystems, Foster City, CA, USA), were electrophoresed through a 4.2% acrylamide gel. Data were collected from runs on an ABI377 automated genotyper and analyzed with the help of GeneScan 3.1 and Genotyper 2.5 software.

Based on the genotypic data from adults (presumably unrelated), the program GENEPOP (Raymond and Rousset 1995) was used to estimate allele frequencies and test for Hardy–Weinberg equilibrium at each locus (Guo and Thompson 1992), and to test for linkage disequilibrium between pairs of loci. Genetic exclusion probabilities were calculated under either of two assumptions: that one parent (the mother) was known and that neither parent was known (Selvin 1980).

For each locus, the genotype of the mother of an egg-case string was determined either directly from her DNA (when she had been collected during egg-laying) or inferred indirectly from the observed ensemble of embryo genotypes in a given brood (when she had not). At each locus, each embryo's paternal allele was then deduced by subtraction of its maternal allele. All procedural details of these analyses (which have some special nuances due to the sex-linked nature of one of the microsatellite loci) are given in Walker et al. (2005). Minimum numbers of sires per brood were determined from direct counts of paternal alleles per locus (following Kellogg et al. 1998), and the program GERUD (Jones 2003) was also used to help estimate paternal genotypes and sire numbers based on multi-locus genotypes in the progeny arrays. To assess whether significant temporal shifts in paternity had

occurred along an egg-case string, heterogeneity G tests (p. 578, Sokal and Rohlf 1969) and runs tests (p. 624, Sokal and Rohlf 1969) were performed.

Results

Population data

Based on data from 139 adults (or $n = 66$ females in the case of the sex-linked locus *bc2.2*), no significant deviations from Hardy–Weinberg equilibrium were detected, nor was linkage disequilibrium at the population level identified between any pair of loci (despite evidence from direct progeny testing that autosomal loci *bc3.4* and *bc3.12* are linked at a chromosomal distance of about 20 cM; Walker et al. 2005). For the three loci considered jointly, genetic exclusion probabilities were 0.99 and 0.97 under the one-parent-known and neither-parent-known models, respectively.

Multiple paternity within broods

Representative embryos from 12 egg-case strings were genetically examined at one or more loci (Table 1). Within all strings except one (family 10), multiple fathers (a mean of at least 3.5 per egg-case string; range 2–7) were evident from the sex-linked locus alone, and these are undoubtedly underestimates of true sire numbers as indicated in part by the multi-locus assessments

(Table 1; see also Walker et al. 2005). Thus, nearly all of the assayed families consisted of mixtures of full-sib and maternal half-sib offspring from several sires.

For the two dams of families 11 and 12 (previously named 523F and 564F; Avise et al. 2004; Walker et al. 2005) collected in the act of depositing egg-case strings, none of the consort males proved to be sire to any of the progeny assayed (data for family 12 are presented in Table 2). Furthermore, all other adult males surveyed ($n = 73$) from the population could likewise be genetically excluded (at one or more loci) as being a sire of these offspring.

Distribution of paternity along egg-case strings

Multiple sires were also typically evident for the collection of embryos housed within each egg capsule. In family 3, for example, 19 of the 22 assayed capsules (86%) demonstrably contained offspring from at least two (and in some cases as many as five) sires (Fig. 1). Similarly in family 12, 100% of 28 assayed capsules displayed multiple paternity, and in 19 of those cases (68%) all five identified sires for the entire brood were involved (Figs. 2, 3).

Due to particular genotypic configurations in the progeny arrays and to large embryo sample sizes, families 3 and 12 also proved to be by far the most favorable for detailed analyses of paternity along the egg-case strings. Daughters in family 3 had at least seven sires unambiguously distinguishable at the sex-linked

Table 1 Background data and estimated sire numbers from genetic analyses of whelk egg-string families

| Family | Number of offspring genetically surveyed | | Number of egg cases surveyed from a string | Minimum number of sires estimated from... | | |
|----------------|--|-----------------------------|--|---|-----------------------------|--------------------|
| | Sex-linked locus | Autosomal loci ^a | | Sex-linked locus | Autosomal loci ^b | GERUD ^c |
| 1 | 113 | 53 | 16 | 2 | 4, 5 | 5 |
| 2 | 27 | – | 5 | 3 | NA ^d | NA |
| 3 ^e | 227 | 236 | 22 | 7 | 4, 8 | NA |
| 4 | 42 | 61 | 8 | 3 | 3, 3 | 3 |
| 5 | 41 | – | 4 | 4 | NA | NA |
| 6 | 45 | 36 | 5 | 3 | 2, 3 | 3 |
| 7 | 57 | – | 6 | 3 | NA | NA |
| 8 | 55 | – | 6 | 6 | NA | NA |
| 9 | 42 | – | 5 | 2 | NA | NA |
| 10 | 40 | – | 7 | 1 | NA | NA |
| 11 | 321 | 310 | 40 | 2 | 3, 4 | 5 |
| 12 | 503 | 459 | 28 | 4 | 3, 5 | 5 |

^a Mean number from the two loci

^b Values listed consecutively for loci *bc3.4*, *bc3.12*

^c Based jointly on loci *bc3.4* and *bc2.12*; program GERUD (adapted from Jones 2003)

^d NA, not applicable, either because autosomal loci were not assayed, showed ambiguity-generating allelic overlaps between possible parents, or exceeded the sire numbers assessable (six) using GERUD

^e This family was labeled U4 in Walker et al. (2005)

Table 2 Genotypes deduced (from paternity analysis) for the sires of family 12, and genotypes of adult male consorts of that family's dam at the time of her capture while laying an egg string

| Locus | | |
|----------------|--------------|---------------|
| <i>bc2.2</i> | <i>bc3.4</i> | <i>bc3.12</i> |
| Deduced sires | | |
| 285 | 215/218 | 168/176 |
| 312 | 211/213 | 129/144 |
| 309 | 211/213 | 120/142 |
| 309 | 213/215 | 146/164 |
| 300 | 211/219 | 127/137 |
| Dam's consorts | | |
| 297 | 212/212 | –/– |
| 306 | 211/211 | 131/133 |
| 300 | 217/222 | 129/129 |
| 309 | 211/211 | 160/170 |
| 294 | 213/217 | 146/176 |
| 309 | 212/213 | 162/170 |
| 309 | 215/217 | 144/146 |
| 309 | 211/217 | 129/170 |
| 309 | 212/224 | 160/178 |

Numbers in the table are allelic designations according to DNA fragment sizes in base-pairs; note that no matches were found between multi-locus genotypes of the deduced sires and those of the consorts

locus, and progeny of both sexes in family 12 proved to have exactly five fathers that could be unambiguously distinguished by the combination of genetic data from all three loci (Walker et al. 2005). For family 3, a total of 147 independent statistical tests (21 capsule comparisons \times 7 sires) could be conducted for possible differences in a father's relative paternity contributions to successive pairs of egg cases. Statistical significance ($P < 0.05$) was reached in seven (4.8%) of these comparisons (see asterisks in Fig. 1), i.e., approximately the expected number due to type I statistical error alone. For family 12, a total of 135 such tests (27 successive capsule comparisons \times 5 sires) were possible, of which 5 (3.7%) were statistically significant. Again, this number is approximately as expected due solely to type I error.

Our inability to reject the null hypothesis of relative paternity constancy along an egg-case string was further evidenced by results from heterogeneity G tests and runs tests. For family 3, only one of the seven identified sires ("309") showed significant overall heterogeneity ($P < 0.001$ in this case) in his relative contributions to different egg cases along the string. In runs tests, three of this family's sires ("297," "306," and "315") showed significant outcomes ($P < 0.05$), but the departures in each case involved an excess of regular alternations of directional sign changes (+ versus –) between successive egg capsules, rather than a consistent upward or downward temporal paternity trend along the egg-case string. Similarly for family 12, only

two of the five sires showed significant overall heterogeneity ($P < 0.01$ for male A, $P < 0.005$ for male B) in their relative contributions to different egg cases along the string; and none of the five runs tests yielded a statistically significant outcome.

A clustered mutation

Spatio-temporal aspects of genetic maternity and egg encapsulation could also be addressed due to our detection of a "clustered" mutation (Woodruff and Thompson 1992; Jones et al. 1999) in family 12. At autosomal locus *bc3.12*, the dam's genotype (directly assayed from her somatic tissue) was 133/151, so each embryo should have carried either the 133 or 151 maternal allele. However, 19 embryos (3.8%) instead displayed copies of a 135 maternal-origin allele (one dinucleotide mutation step removed from 133) that presumably had arisen de novo and pre-meiotically in the mother's germ line. As shown in Fig. 3, copies of this mutation were distributed sporadically from near the beginning to near the end of the capsules examined, thus indicating a thorough micro-temporal and micro-spatial dispersion of this newly arisen mutation along the egg-case string.

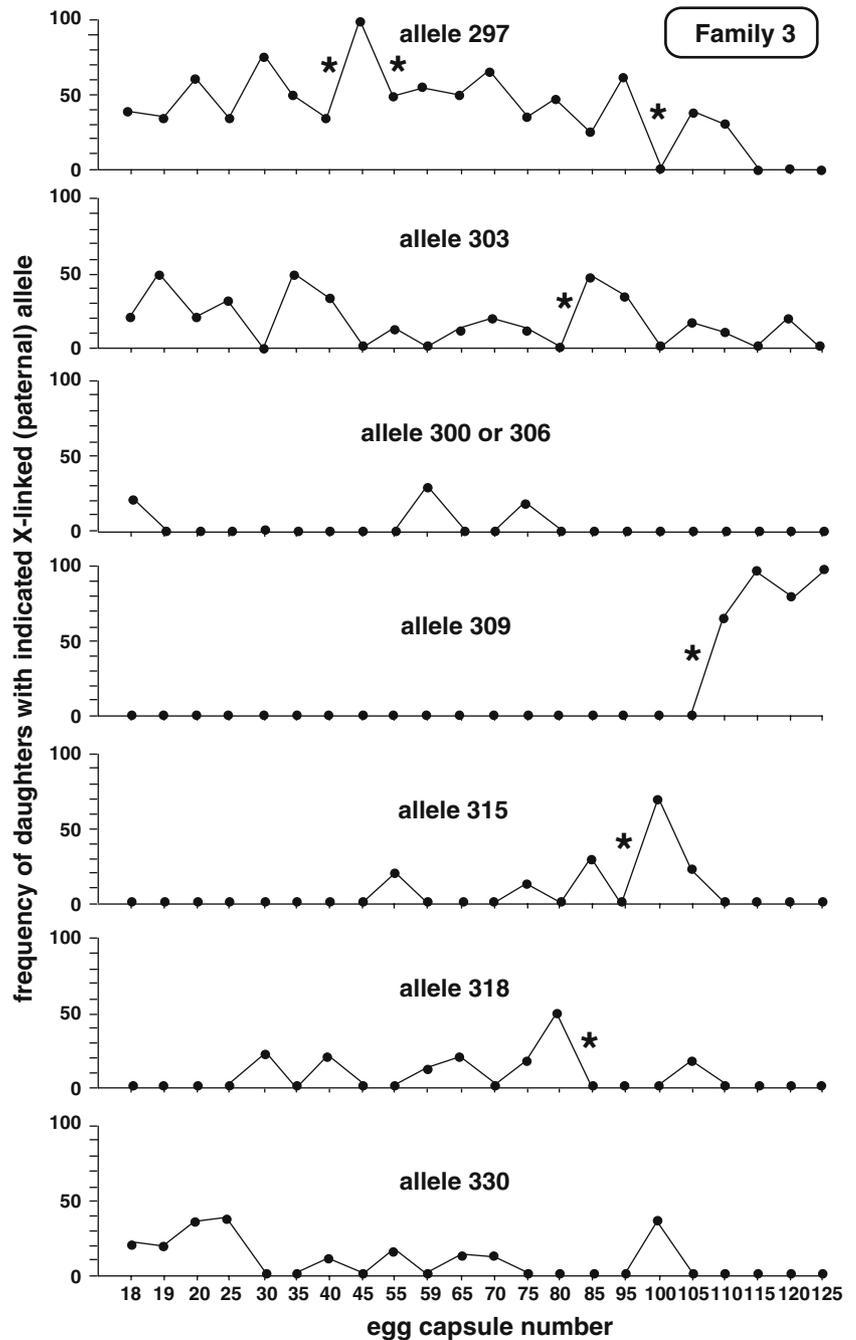
Discussion

With some notable exceptions (Burton 1985; Gaffney and McGee 1992; Urbani et al. 1998; Bishop et al. 2000; Brockmann et al. 2000; Walker et al. 2002; Oppliger et al. 2003), marker-based studies of paternity and genetic mating systems in molluscs, crustaceans, and other major aquatic and marine invertebrate groups have lagged behind those of large vertebrate assemblages such as birds (Westneat et al. 1990; Birkhead and Møller 1992; Avise 1996), "herps" (Avise 2001), and fishes (Avise et al. 2002). Here we have used sex-linked and autosomal microsatellite markers to assess the distribution of multiple paternity along egg-case strings of *B. carica*. Perhaps the closest published analogue to this type of analysis has involved egg-case strings of *Loligo* squid species (*forbesi* and *pealeii*), where high frequencies of multiple paternity within egg cases as well as along a string similarly have been documented using microsatellites (Shaw and Boyle 1997; Buresch et al. 2001; Emery et al. 2001).

Phenomenology: multiple paternity and female sperm usage

Our findings, based on microsatellite assessments of more than 1,500 embryos from 12 *B. carica* families,

Fig. 1 Temporal patterns of paternity, based on the sex-linked microsatellite locus *bc2.2*, for a total of 116 daughter embryos along the egg-case string of family 3. Asterisks indicate statistically significant shifts ($P < 0.05$) in allele frequency (paternity) between successive capsules surveyed



indicate that multiple paternity (including within each egg capsule) is probably the norm for knobbed whelks, and that several males per brood typically are involved. Additionally, intensive examinations of two families that were especially favorable for genetic parentage analysis demonstrated that the proportionate contributions of a given sire remained roughly constant along most or all of an egg-case string, but often differed dramatically and consistently from sire to sire. In family 12, for example, progeny from fathers A, B, and C were common from the beginning to the end of the

egg-case string, whereas progeny from sires D and E were consistently present but rare along virtually the string's entire length (Figs. 2, 3). A similar spatio-temporal pattern of multiple paternity was displayed by the egg-case string of family 3 (Fig. 1).

On the other hand, we also observed significant and even dramatic shifts in paternal contributions between successive egg capsules or portions of an egg-case string. The best example in our data involved male "309" in family 3, who was excluded as the father of all embryos in the first 18 egg capsules surveyed but then

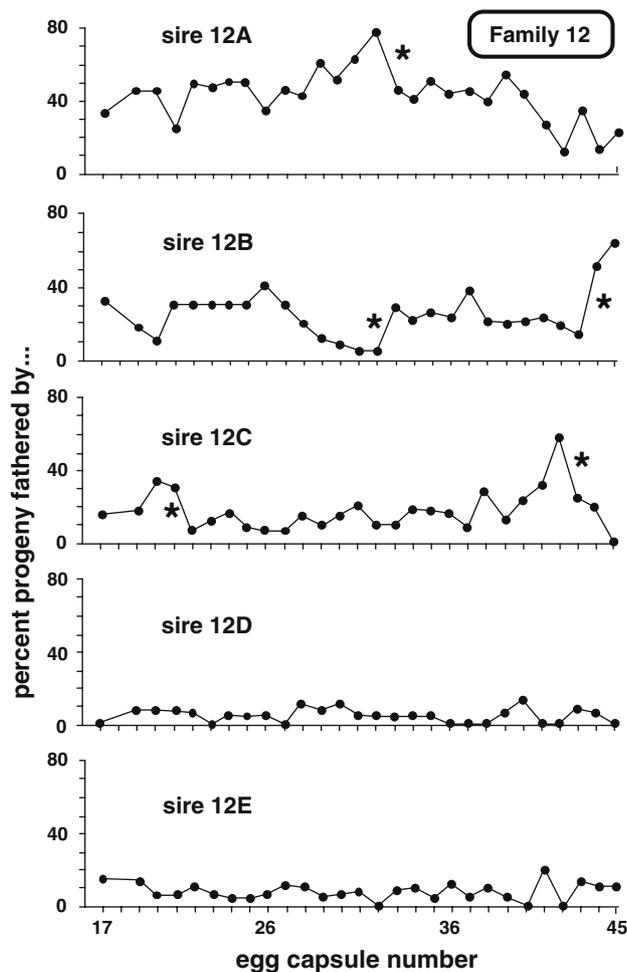


Fig. 2 Temporal patterns of paternity, based on the sex-linked locus *bc2.2* in conjunction with autosomal loci *bc3.4* and *bc3.12*, for a total of 503 sons and daughters along the egg-case string of family 12. Asterisks indicate statistically significant shifts ($P < 0.05$) in a paternity contribution between successive capsules surveyed

suddenly became sire of 11 of 13 embryos (85%) from the last-laid (19th to 22nd) egg cases examined (Fig. 1). Similarly in family 12, sire B was only modestly represented (always $< 40\%$ frequency) in egg cases 17–43 but then quickly became the predominant sire (59% of 27 assayed embryos) in capsules 44 and 45 (Figs. 2, 3). Several less dramatic shifts in sire contributions occurred elsewhere along these two egg strings, but otherwise no significant directional trends in the microspatial or micro-temporal arrangements of multiple paternity were apparent within a brood.

Our genetic findings suggest that fertilization events within the body of a female whelk occur mostly (but not entirely) as if by random draws from a “well-blended sperm pool” comprised of different ejaculate titers by her several mates. A somewhat different scenario that is likewise consistent with current genetic

evidence is that fertilization events by different males are either spatially or temporally clustered within a female’s reproductive tract but that the zygotes then become well mixed before being deposited into successively laid egg capsules. In that situation, the well-blended entities would be pools of eggs fertilized by different sires, rather than their sperm per se. The observed distribution of a clustered mutation in family 12 is compatible with the notion that a female’s eggs are also mixed thoroughly before deposition.

Mechanisms

Last-male sperm precedence is common in many invertebrate species with single “blind-end” sperm storage organs, probably in part because late arriving sperm may interfere with or supercede a female’s usage of previous ejaculates (Smith 1984; Birkhead and Møller 1992; Simmons and Siva-Jothy 1998). Conversely, in some species such as the garden snail (*Helix aspersa*), early sperm donors tend to have the highest paternity scores. In that species, it has been suggested that a unified beating of flagella of sperm residing in the female storage organ generates a resistance to incoming sperm cells such that the probability of new sperm entering a female’s reproductive tract decreases with each successive mating (Rogers and Chase 2002). Our findings for *B. carica* imply that neither first-male nor last-male advantages greatly predominate, but rather that zygote production more closely resembles a “raffle” process in which the probability of egg fertilization is perhaps proportional to the number of sperm deposited by each male.

Another scenario that we cannot exclude with our genetic data is that multiple males all deposit identical sperm titers but that a female preferentially chooses sperm from some over others when fertilizing her eggs. Hypothetically, such post-copulatory mechanisms of female sperm choice could include sperm dumping, sperm digestion in female storage organs, or other means of differential sperm sorting and usage within the female reproductive tract (Paterson et al. 2001). Regardless of mechanisms involved, by pooling the genetic donations of multiple sires, in principle a female gains several fitness advantages such as the promotion of sperm competition, a hedge against genetic abnormalities or inviable sperm from particular males, and high genetic diversity among her progeny (Halliday and Arnold 1987).

Unfortunately, several key physical aspects of sperm storage and utilization are poorly understood in melongenid whelks. In *B. carica*, a female initially receives sperm cells in a bursa copulatrix and subsequently

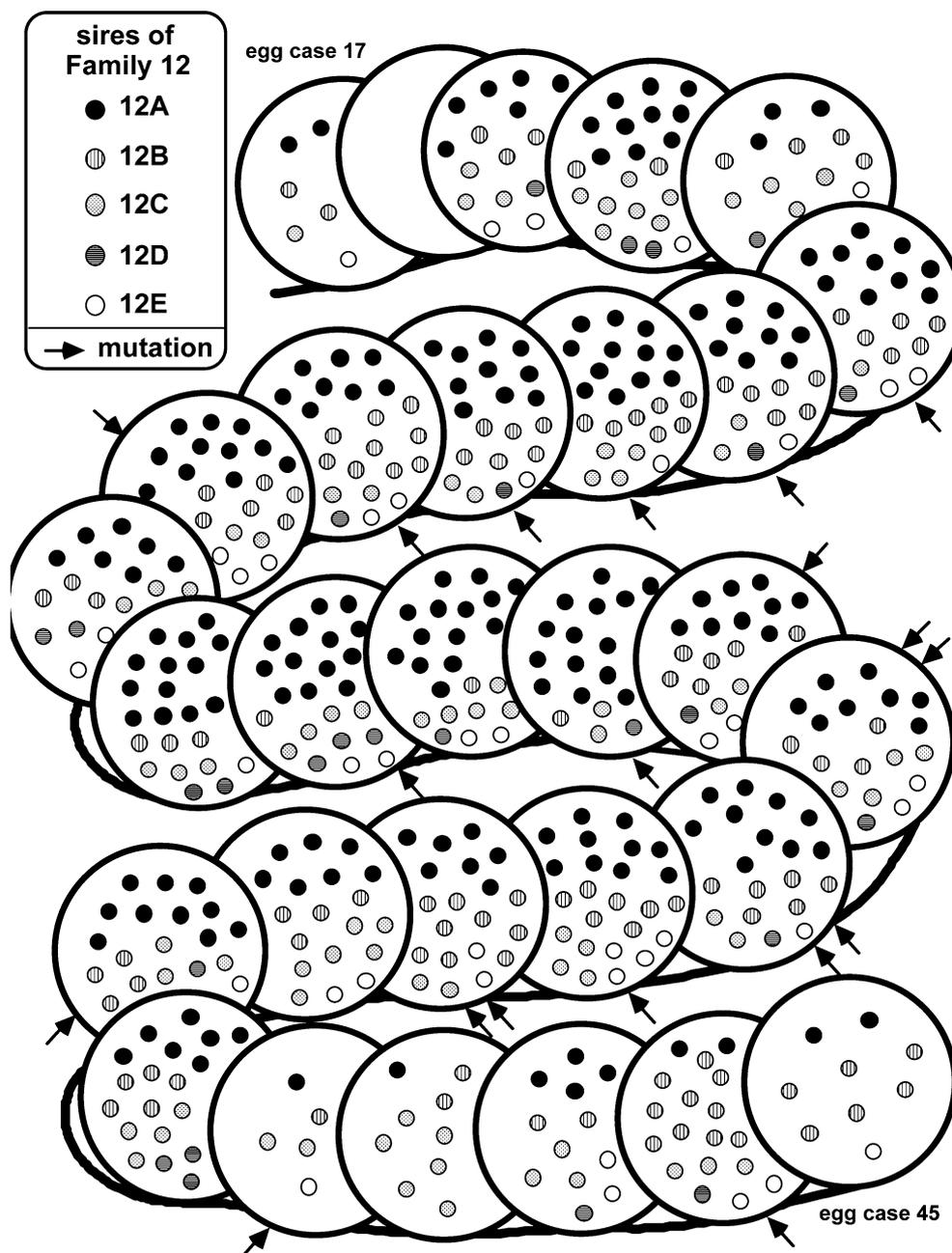


Fig. 3 Diagrammatic representation of paternity for 503 progeny in 28 successive egg capsules from the main portion of the egg-case string of family 12. Small circles represent surveyed embryos, each coded to indicate its genetically deduced sire. Relative positions of embryos within an egg capsule have no meaning (they are

grouped for the sake of simplicity). Each arrow indicates one offspring (within an indicated egg capsule) displaying a copy of a de novo mutation (to allele “135” at locus *bc3.12*) that apparently arose in the maternal germ line

transfers them to a seminal receptacle where they are stored. The time entailed for this transfer is unknown in *Busycon*, but reportedly requires about 3 days in the buccinid whelk *Buccinum undatum* (Martel et al. 1986). It is thought that sperm cells released from the seminal receptacle then meet oocytes in the lumen of the albumin gland or perhaps as they enter the capsule gland prior to encapsulation (Fretter and Graham 1962). If

these ideas are correct, it might seem that a male would increase his likelihood of paternity if he could monopolize his mate for several days or until such time as his sperm has been moved to her seminal receptacle.

Another potential complication is long-term sperm storage by females. Many prosobranch gastropods such as species of *Strombus* (Reed 1995), *Buccinum* (Martel et al. 1986), and *Neptunea* (Power and Keegan 2001)

are known to store viable sperm for weeks, and some species such as *Viviparus* river snails (Ankel 1925) and *Crepidula* slipper limpets (Coe 1942) reportedly can do so for as much as 1 year or more. The duration of viable sperm storage by females is unknown in species of *Busycon*. Castagna and Kraeuter (1994) observed mating in Virginia in June and July, but did not note any egg-laying until mid-August, and they concluded that while copulation was seasonal it did not necessarily coincide with egg-laying. Our genetic data proved that none of the males captured in consort with a female at her time of egg deposition were the sires of her current egg-cased progeny. Thus, these females unquestionably were utilizing sperm stored from earlier matings, and the genetic data further indicate that sperm from those previous mates had for the most part been well mixed by the time of their utilization.

Population considerations

Another point to emerge from our microsatellite investigations is that the effective size of the surveyed *B. carica* population must be large. At least three lines of genetic evidence support this contention. First, the molecular markers themselves were highly polymorphic, with observed numbers of alleles per locus ranging from 12 to 36 across the three assayed loci, and per-locus expected heterozygosities ranging from 0.81 to 0.96 (Avisé et al. 2004). Second, all of the 73 adult males that we genotyped proved to be genetically excludable as plausible sires for any of the families surveyed. Thus, females at this locale must have had available a large pool of potential mates (see the reasoning in Pearse et al. 2001). Third, as already mentioned, none of the consort males captured with a female at the times of egg-case deposition proved to have sired any offspring in the respective broods, thus suggesting that those females were using sperm stored from earlier copulations. This implies that female whelks can be viewed as a sort of temporal “seed bank” into which many males can potentially make gametic deposits and from which successful zygotic withdrawals over time would depend on the temporal length of viable sperm storage by females. Such temporal “seed banks” can elevate the genetic effective size of a local population well above the adult census population size at any single point in time.

Conclusions

We have documented highly polyandrous matings and raffle-like utilizations of stored sperm by female

knobbed whelks. The extent of this polygamous behavior may if anything be under-estimated because the genotypic data for some broods probably failed to distinguish additional sires (because of allelic overlaps and because not all embryos in a family were genetically screened). Apart from correcting these deficiencies, future extensions of our efforts could involve analyzing genetic paternity across successive egg-case strings of particular females, investigating the likelihood of multiple mating by males, and integrating all such information on genetic parentage with further research on the field behaviors and reproductive morphologies and physiologies of this ecologically important mollusc.

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