

Fitness consequences of marine larval dispersal: the role of neighbourhood density, arrangement, and genetic relatedness on survival, growth, reproduction, and paternity in a sessile invertebrate

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Abstract

Dispersal can evolve as an adaptation to escape competition with conspecifics or kin. Locations with a low density of conspecifics, however, may also lead to reduced opportunities for mating, especially in sessile marine invertebrates with proximity-dependent mating success. Since there are few experimental investigations, we performed a series of field experiments using an experimentally tractable species (the bryozoan *Bugula neritina*) to test the hypothesis that the density, spatial arrangement, and genetic relatedness of neighbours differentially affect survival, growth, reproduction, paternity, and sperm dispersal. We manipulated the density and relatedness of neighbours and found that increased density reduced survival but not growth rate, and that there was no effect of relatedness on survival, growth, or fecundity, in contrast to previous studies. We also manipulated the distances to the nearest neighbour and used genetic markers to assign paternity within known mother-offspring groups to estimate how proximity affects mating success. Distance to the nearest neighbour did not affect the number of settlers produced, the paternity share, or the degree of multiple paternity. Overall, larger than expected sperm dispersal led to high multiple paternity, regardless of the distance to the nearest neighbour. Our results have important implications for understanding selection on dispersal distance: in this system, there are few disadvantages to the limited larval dispersal that does occur and limited advantages for larvae to disperse further than a few 10s of metres.

Keywords: bryozoan, kin competition, inbreeding, sperm dispersal, marine

Introduction

Dispersal, the movement of individuals away from their parents and subsequent reproduction in another location, affects gene flow, local adaptation, and the dynamics of spatially structured populations (Clobert et al., 2012; Strathmann et al., 2002). As a result, it is important to understand the evolution of dispersal to explain the causes of variation and predict future changes, in dispersal. However, empirical understanding of the evolution of dispersal lags far behind theoretical understanding (Duputié & Massol, 2013), and this is particularly true in marine systems. Therefore, field studies that experimentally assess the fitness consequences of the key factors influencing selection for dispersal are sorely needed.

In models, dispersal is selectively advantageous when there is spatial and temporal variation in expected fitness (Bowler & Benton, 2005; Clobert et al., 2012; Johnson & Gaines, 2003; Palmer & Strathmann, 1981; Pechenik, 1999). Variation in expected fitness can occur through differences in competition and mating with conspecifics or kin, in addition to external environmental factors (Hamilton & May, 1977; McPeck & Holt, 1992; Pechenik, 1999; Strathmann, 1974). In particular, even if environmental factors remain similar over a given space and time scale, changes in population size at smaller

space and time scales can lead to changes in the density and genetic relatedness of neighbours, which in turn leads to the variation in individual fitness necessary to select for increased dispersal (Bitume et al., 2013; McPeck & Holt, 1992; Rousset & G&con, 2002).

The density and genetic relatedness of conspecific neighbours is particularly important in plants and sessile invertebrates where the physical positions of juveniles and adults in the population are fixed (Buss, 1981; File et al., 2012; Harper, 1977; Strathmann, 1974). Especially for sessile marine invertebrates, space has often been viewed as the dominant limiting resource because the available space that an individual can occupy is finite, and an individual must first gain space via larval dispersal and settlement to then gain access to other vital resources (Roughgarden et al., 1985). Larval dispersal can be advantageous when it allows individuals to colonize an empty location where competition for space is less intense than crowded locations. However, competition for food can cause decreases in growth and survival before space itself becomes overcrowded (Buss & Jackson, 1981; Okamura, 1988). Many sessile marine invertebrates actively acquire food particles suspended in a fluid medium (i.e., suspension feeding), and individuals may or may not have

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an unlimited supply of a continually replenished “plankton soup” (Buss & Jackson, 1981; Okamura, 1988; Svensson & Marshall, 2015). Instead, clustered groups of individuals can locally deplete food particles and, therefore, food availability in the surrounding water column (Okamura, 1990; Pratt, 2004; Riisgård & Manríquez, 1997). In these systems, therefore, food can often be a limiting resource, even when there is space available (Svensson & Marshall, 2015), and affect selection for dispersal in addition to, and often prior to, space limitation.

Furthermore, conspecific competition for resources depends not only on the number of neighbours but also on the relatedness of neighbours (File et al., 2012; Kamel & Grosberg, 2013; Mazzei & Rubenstein, 2021). Empirically estimated dispersal kernels in marine invertebrates and fish all indicate that the probability of successful dispersal declines rapidly with distance, with higher-than-expected levels of self-recruitment (e.g., Almany et al., 2017; Buston et al., 2012; D’Aloia et al., 2015; Pinsky et al., 2017; Smith et al., 2023). Furthermore, many sessile marine invertebrate species have larval durations in the order of minutes to hours (Burgess & Marshall, 2011a; Grantham et al., 2003; Olson, 1985; Shanks, 2009), all resulting in a high opportunity for neighbours to be relatives. Neighbours that are genetic relatives can mediate competition for food through niche partitioning (Aguirre & Marshall, 2012; File et al., 2012), when groups of kin have reduced fitness compared to groups of strangers because kin have more similar phenotypes and therefore have greater overlap in their niche requirements (Bolnick et al., 2003). Alternatively, relatives can mediate competition for food through kin selection, where groups of kin have higher fitness compared to groups of strangers because relatives cooperate. In some sessile invertebrates, neighbours that are relatives can also fuse, increasing colony size, and potentially fitness (Blanquer et al., 2009; Puill-Stephan et al., 2012; Raymundo & Maypa, 2004). Note that the strength of competitive interactions among kin need not be greater than among unrelated conspecifics to still provide a kin selection advantage to dispersal (Hamilton & May, 1977; Rousset & Gandon, 2002), but increased competition among kin can increase the selection to avoid kin competition through dispersal and habitat selection (Johnson & Woollacott, 2010). Increased cooperation among kin, as well as larger population sizes and high offspring numbers, would then decrease any kin selection advantage to dispersal (Comins et al., 1980).

While dispersal to low-density locations may provide advantages to dispersers in terms of reduced competition for resources that fuel survival, growth, and egg production, it may also lead to reduced opportunities for egg fertilization, especially in sessile marine invertebrates with proximity-dependent mating success (Grosberg 1987, 1991; Johnson & Yund, 2009; Warner et al., 2016). In sessile marine invertebrates, broadcasted sperm are rapidly diluted and sperm viability typically declines within minutes to hours (Grosberg, 1991; Manríquez et al., 2001; Yund, 1990; Yund & McCartney, 1994), such that isolated females may not acquire enough sperm to fertilize their eggs (i.e., sperm limitation). For example, in a colonial ascidian, sperm concentration in the water column declined rapidly within as little as 50 cm, as evidenced by paternity analysis (Grosberg, 1991; Yund, 1998). The advantages of dispersal may also differ for male versus female fitness, which creates potential conflicts between sexes that may be particularly intense in

hermaphrodites (Campbell, 1989; Charnov, 1979; Kulbaba & Shaw, 2021; Schärer & Schärer, 2009). Female fitness may be positively impacted by density when fertilization is limited by sperm availability and neighbours provide mating assurance in outcrossing species, or negatively impacted by density when there is polyspermy (Levitan et al., 1992). Male fitness may be negatively affected by density due to male–male competition for fertilization, such that the presence of a nearby male reduces the fertilization success of a more distant male (Johnson & Yund, 2009; Pemberton et al., 2003; Yund & McCartney, 1994). In addition, individuals who are far away from other conspecifics may lose out on opportunities to fertilize the eggs of other individuals (Grosberg, 1987; Yund & McCartney, 1994). Therefore, in hermaphroditic species, individuals that are far away from other conspecifics may have greater access to resources due to lack of competition and, therefore, be able to produce many eggs, but may or may not be able to have those eggs fertilized by outcrossed sperm.

Our overall goal was to test how factors known to select for dispersal in theoretical models—density, relatedness, and spatial arrangement of neighbours—affect multiple components of fitness in the field. This is an important step towards empirically understanding the evolution of dispersal because, without effects on fitness, spatial and temporal variation in density, relatedness, and spatial arrangement will not be selected for or against dispersal. We performed three manipulative field experiments using the experimentally tractable marine bryozoan *Bugula neritina*, in which most larvae settle within metres of the maternal colony and exhibit distinct spatial and temporal variation in adult abundance driven by dispersal limitation (Keough & Chernoff, 1987; Burgess et al. 2023). The first experiment tested the hypothesis that the density and relatedness of neighbours affect individual survival and growth throughout adulthood. The second experiment tested the hypothesis that relatedness of neighbours affects survival, growth, and reproductive output. The third experiment tested the hypothesis that the distance to the nearest neighbour affects female reproductive output, paternity within broods, and proximity-based competition among males where the nearest colony dominates the paternity share. At the fine spatial scales of these manipulations, we found that: (a) increased density reduced survival but not growth rate, (b) there was no effect of relatedness on any component of fitness in contrast to previous studies, and (c) colonies produced a similar number of settlers with or without local neighbours, and distance to the nearest neighbour did not affect multiple paternity or the paternity share. These results indicate that if dispersal were to regularly facilitate settlement to vacant locations (without conspecific competitors), it would not result in reduced mating opportunities, but also indicates limited disadvantages to reduced dispersal if it increased kin interactions.

Methods

Study species

Bugula neritina is a sessile, filter-feeding marine bryozoan in the Phylum Bryozoa (Gymnolaemata, Cheilostomata). An individual begins as a sexually produced larva that attaches to the substrate (called a “settler”), metamorphoses into a (ancestrula) zooid (1–2 days), and then grows through the addition of clonal zooids to form an individual colony. Therefore, an individual is referred to as a settler or a colony,

depending on its age. Each zooid contains all of the nutritive, reproductive, and other organs needed to be self-supporting. Individual colonies do not fuse, and colony clones are absent. Colonies typically begin releasing larvae around ~21 days old and, will typically live for up to a few months in the field (Burgess & Bueno, 2021). *Bugula neritina* are simultaneous hermaphrodites, where each zooid produces both sperm and egg (Ostrovsky et al., 2013). Individual spermatozoa are released through a terminal pore in the tips of the lophophore tentacles and eggs are retained. Fertilization occurs inside the maternal zooid from sperm acquired from the water (spermcast mating; Bishop & Pemberton, 2006). The fertilized oocyte is transferred into a brood chamber (called an ovicell) on the outside of the zooid, where it develops into a coronate larva over a period of ~7 days (Woollacott & Zimmer, 1975). Each ovicell broods a single embryo at a time. The larvae are free-swimming and developmentally competent to settle immediately after release and typically settle within minutes to hours (Burgess & Marshall, 2011a; Burgess et al., 2009).

In the northern Gulf of Mexico, *B. neritina* commonly live in shallow, subtidal seagrass meadows attached to seagrass blades or, occasionally, on hard substrates such as polychaete tubes and shells. They are found in a range of densities depending on the location and season, often with multiple individuals on a single seagrass blade (Burgess et al., 2023). Colonies reproduce, and are most abundant, from March to May, and again from October to December (Keough & Chernoff, 1987). Spatial variation in density occurs on small spatial scales (< 100 m) and transplanting juveniles to sites where conspecifics are consistently absent has effects on survival and reproduction ranging from neutral to positive, indicating that dispersal limitation rather than environmental variation determines spatial abundance patterns (Keough & Chernoff, 1987). Direct measurements of larval dispersal have revealed that most larvae settle within ~1 m of the maternal colony (Burgess et al., 2023), yet population genetic surveys have identified limited kinship between adult neighbours, and very few half-sibs (and no full-sibs) in adult populations overall, and it is still unclear why. There is also very little evidence for inbreeding in natural populations (Burgess et al., 2019), possibly because related sperm are rejected or are diluted by high multiple paternity, but this remains to be determined.

Adult collection

Mature *B. neritina* colonies were collected from a shallow seagrass bed on the north side of Dog Island, Florida in the northern Gulf of Mexico. Colonies were collected at least 5 m apart to minimize the possibility of sampling-related colonies, even though previous sampling detected minimal kinship in adults at this scale (Burgess et al., 2023).

Obtaining larvae

Immediately following collection, individual colonies were transported to the laboratory on the Florida State University main campus in Tallahassee, Florida. Individuals were separated into individual glass beakers containing 500 ml of filtered seawater (FSW). Acetate sheets were floated on the surface of the water. The acetate sheets had been previously roughened with sandpaper and soaked in unfiltered seawater from the Florida State University Coastal and Marine Laboratory (FSUCML) for multiple days to encourage the growth of biofilm, which encourages settlement for *B. neritina*. Individuals in the beakers were moved into a dark

incubator set to 23 °C. After 2 days, lights in the incubator were turned on for 8 hr to induce larval release (Burgess et al., 2012). Larvae settled onto the acetate sheets within minutes to hours. The day following larval release and settlement, each sheet was removed from the beakers and individual settlers were removed by cutting a ~5 mm diameter circle of acetate sheet around each focal settler. Settlers were housed individually in separate glass bowls for 3 days until they grew new zooids and began to feed.

For every settler, we kept track of which maternal colony it originated from, so that we could establish groups of related and unrelated individuals. Individuals were considered related if they shared the same mother and, therefore, were full- or half-siblings. Individuals were considered unrelated if they did not share the same mother.

Laboratory rearing

Individual colonies were maintained separately in glass bowls with 250 ml of FSW. Every 2–3 days, water was changed, and the colonies were fed ~100,000 cells/ml of live *Rhodomonas salina* (CCMP1319) algae. Individuals were reared in the lab for 17 days (experiment 1), 20 days (experiment 2), or 26 days (experiment 3) to ensure survival to a minimum size at which interference and mating competition was expected (i.e., when individual colonies ~1 cm apart could touch, and when colonies become reproductively mature), and to minimize size variation among colonies prior to field deployment. This allowed the establishment of treatments at the stage when competition for food and mates was expected to occur, such that inferences were not confounded by prior differences in growth and survival caused by factors other than the experimental treatments. Before transport to the field, the size of the colonies was measured by counting the number of zooids under a dissecting microscope.

Field deployment

Experimental colonies were deployed to a shallow (~1 m below average tide line) seagrass meadow located directly in front of the FSUCML, near Turkey Point, Florida. Individual colonies were attached to polyvinyl chloride (PVC) poles (2.5 cm diameter, 61 cm length). Each pole was driven into the sediment so that colonies were positioned approximately 30 cm above the benthos, which approximates the height colonies grow above the substratum when attached to seagrass blades. The PVC poles had a 2 cm diameter hole drilled into them and the top 5 cm of a 15 ml centrifuge tube was inserted into the hole and secured using hot glue. Petri dishes or pieces of acetate sheets with colonies attached were glued to the screw cap of the centrifuge tube. This setup allowed easy deployment and retrieval of experimental colonies by simply screwing and unscrewing the cap from the centrifuge tube. Colonies were monitored every 4 days and any new *B. neritina* colonies that settled onto the plates or poles were removed to maintain experimental densities.

Experiment 1: the effect of relatedness and density on survival and growth

The first experiment ran from October to December 2021. There were 10 density treatments (ranging from 2 to 20 individuals per dish in increments of 2) crossed with two relatedness treatments (related or unrelated) (Figure 1a). Overall, there were 25 maternal families. In the “related” treatment, 10 of these maternal families were used, where each of the

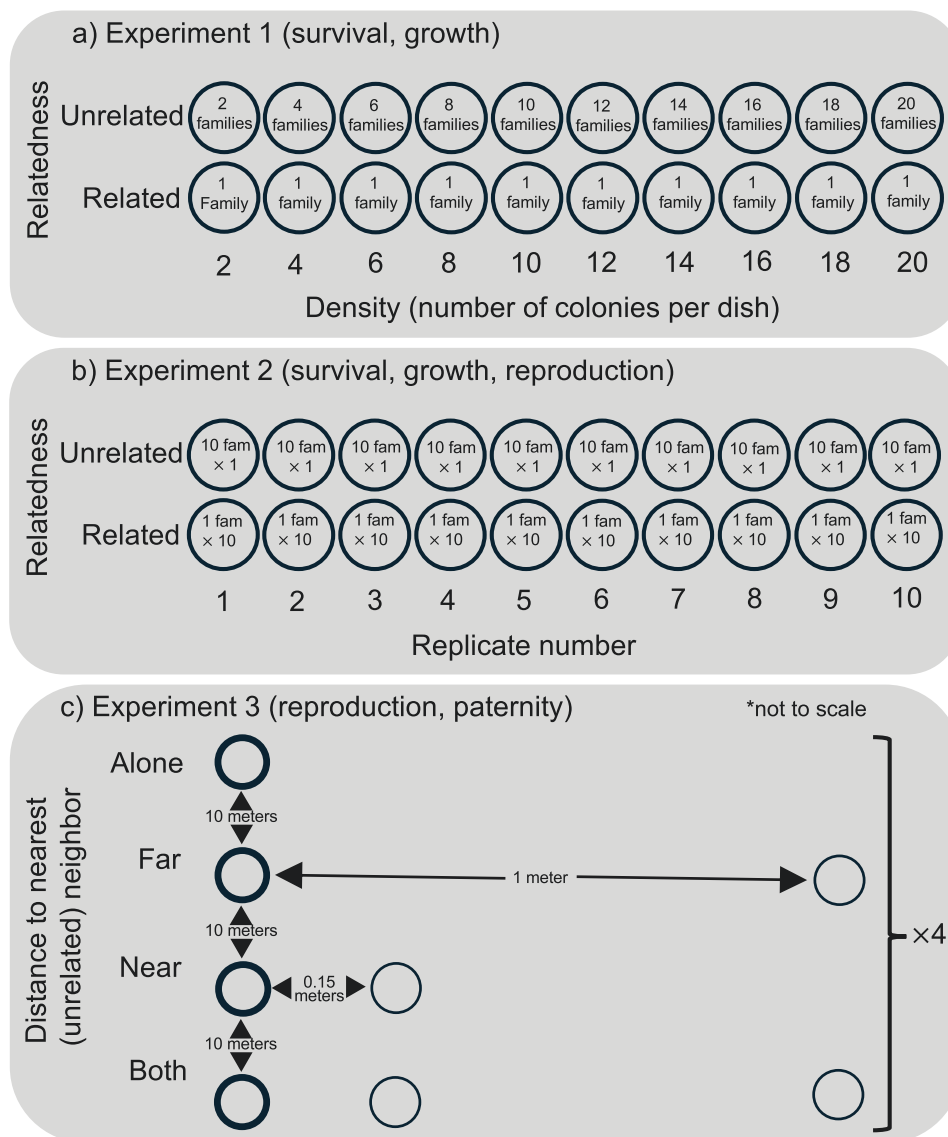


Figure 1. (a) Experiment 1 measured the effect of density on survival and growth in groups (dishes, circles) of related and unrelated individuals. (b) Experiment 2 measured the effect of relatedness (related = 10 individuals from one maternal family; unrelated = 1 individual from 10 maternal families) on survival, growth, and reproduction (number of ovicells). In (a) and (b), related individuals share a mother (maternal half- or full-sibs), and each density (a) or replicate (b) was a different maternal family. Unrelated individuals each come from a different maternal family. (c) Experiment 3 measured the effect of (unrelated) nearest neighbour (alone, far, near, both) on reproduction (number of settlers) and paternity (note scale differences). Circles represent individual colonies, and the darker circles represent focal colonies. The focal colonies were located parallel to the shoreline, and the direction (shoreward or seaward) of the non-focal colonies was randomized. This layout was repeated in four spatial blocks, each separated by 10 m along the shore line.

10 density treatments comprised offspring from a different maternal family (i.e., density = 2 is two offspring from family A, density = 4 is four offspring from family B, and so to where density = 20 is 20 offspring from family J). In the “unrelated” treatment, each of the 10 density treatments comprised one offspring randomly chosen from one of the 25 maternal families, such that the 10 maternal families in the related treatment were also used in the unrelated treatments, plus an additional 15 maternal families to achieve the desired density.

Within each dish, individual colonies were randomly assigned to a position in a 3 × 4 cm grid on a Petri dish lid. The distance between grid points was 1 cm. To secure the individuals in their arrangement, the sheet that the individual had settled on was glued to a Petri dish lid using Loctite Super Glue.

Petri dishes were randomly assigned to PVC poles, spaced 1 m apart and arranged in a 4 × 5 grid. After 7 days in the field, individuals were collected and transported back to the lab, where survival (present or absent) and size (number of zooids) were measured under a dissecting scope. Individuals were returned to the field the following day. Individuals were left in the field for another 7 days, at which point they were collected, transported back to the lab, and measured for survival and size.

Experiment 2: the effect of relatedness on survival, growth, and reproduction

The second experiment ran from March to June of 2022. Individual colonies were arranged into two treatments, related and unrelated, each with 10 replicate Petri dishes

(Figure 1b). On each replicate dish, there were 10 colonies randomly assigned to a position in a 3 × 4 cm grid, reflecting a common density in the field at times when the population is large, and at the middle density used in Experiment 1. Overall, there were 21 maternal families. In the “related” treatment, 10 of these maternal families were used, where each of the 10 replicates comprised offspring from a different maternal family (i.e., replicate 1 had 10 offspring from family X, replicate 2 had 10 offspring from family Y, and so on). In the “unrelated” treatment, each of the 10 replicates comprised one offspring randomly chosen from one of the 21 maternal families, such that the 10 maternal families in the related treatment were also used in the unrelated treatments, plus an additional 11 maternal families to achieve the desired density. Petri dishes were randomly assigned to PVC poles. There were 10 replicate Petri dishes in each treatment at the start of the experiment; however, two dishes were lost after deployment, resulting in eight Petri dishes in the unrelated treatment.

After 20 days in the field, individual colonies were collected and returned to the laboratory. The surviving individuals were preserved in 95% ethanol. Survival (present or absent), size (number of zooids), and reproductive output (total number of ovicells) were recorded for each individual colony under a dissecting microscope. The number of ovicells is considered proportional to the production of sperm and eggs and the potential number of brooded larvae.

Experiment 3: the effect of distance to the nearest neighbour on reproductive output and paternity

The third experiment ran from April to June of 2021. Each acetate sheet containing a single settler was glued directly to the cap of a 15 ml centrifuge tube for deployment in the field. The PVC poles were arranged in a transect that ran parallel to the shore (Figure 1c). There were four treatments:

- (1) alone (one focal colony);
- (2) far (a focal colony with nearest neighbour 1 m away);
- (3) near (a focal colony with nearest neighbour 15 cm away); and
- (4) both (a focal colony with one neighbour 15 cm away and another colony 1 m away).

These distances were chosen specifically to represent ecologically relevant distances for the scale of expected sperm dispersal and male–male competition (Yund & McCartney, 1994) and are commonly observed in natural populations (Keough & Chernoff, 1987).

Each treatment had four replicates, one in each of four spatial blocks (8 colonies × 4 spatial blocks = 32 total colonies). Within each block, the order of the treatment was randomized. Within a treatment, all colonies were unrelated (i.e., offspring of different mother colonies). Each focal colony was placed on a transect line parallel to the shore. The distance between spatial blocks was 10 m. Neighbouring colonies were placed perpendicular from the transect line at their allocated distances, and the direction of those neighbours from the focal colony (either towards or away from the shore) was randomized for each replicate (Figure 1c).

Prior to deployment, the surrounding seagrass was searched to ensure no *B. neritina* colonies were present. During deployment, the poles and surrounding benthos were monitored every 4 days and any non-experimental colonies were

removed to minimize the contribution of sperm from non-experimental colonies. Experimental colonies remained in the field for 14 days, which is sufficient time to exchange sperm, fertilize eggs, for embryos to develop, and for larvae to brood.

All 32 colonies were collected from the field after 14 days and transported back to the lab, where survival and the number of zooids per colony were measured. All colonies were then placed into individual glass bowls (one colony per bowl) with 250 ml of FSW and a roughened acetate sheet was floated on the surface for larvae to settle upon. Each day, the colonies were placed under a hanging LED light for approximately 8 hr to induce larval release. Every 4 days, the sheets were removed and replaced. To measure reproductive output, the number of settlers from each colony was counted every 4 days for 20 days (i.e., six occasions). No new offspring were released after 20 days. Settlers from known maternal colonies were preserved in 95% ethanol for paternity analysis.

Genetic analysis

Out of the 32 colonies in the experiment, 25 produced offspring. From these 25 colonies, 23–27 offspring from each colony were genotyped, resulting in 619 offspring genotypes with known maternity. Four offspring had missing data at three or more loci so they were excluded from downstream analyses, resulting in a total of 615 offspring genotypes.

All 32 colonies in the experimental array were also genotyped, 25 of which were the known mothers and all 32 were potential sires. All 647 samples were genotyped at 13 microsatellite loci, following methods developed by Burgess et al. (2019, 2023). To extract DNA, tissue was placed in a solution of 100 µl of Chelex 100 Resin (Bio-Rad) and placed in a thermocycler at 55 °C for 60 min followed by 99 °C for 15 min and held at 4 °C. Extracted DNA was amplified at 13 microsatellite loci in four multiplex polymerase chain reactions (PCRs). The PCR included four primer pairs (8 total, 0.5 pmol each) which were combined with Qiagen Multiplex PCR Plus Mix (Qiagen, Valencia CA, USA), following the manufacturer’s protocols calculated for 10 µl reaction volumes including 1 µl of DNA template and the addition of 1 µl of 0.1% bovine serum albumin. Each end of the 5’ forward primer contained a fluorescently labeled dye (6-FAM, NED, or PET) (ThermoFisher Scientific, Waltham, MA, USA). Thermocycling conditions for the PCR were: 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s, 57 °C for 90 s, and 72 °C for 60 s; followed by a final extension of 30 min at 68 °C. The PCR product was diluted 1:2 and 2 µl was transferred to a plate containing 9 µl of HiDi Formamide and 0.2 µl of LIZ500. The plate was denatured at 95 °C for 5 min before being loaded on ABI 3730xl DNA Analyzers at the Institute of Biotechnology at Cornell University. Fragment lengths were scored manually using Geneious Prime v2023.0.3 (Biomatters Ltd).

Paternity analysis

Paternity analysis was used to estimate the number of fathers per maternal brood and to test the extent to which an individual colony’s brood is dominated by paternity from the nearest individual colony. To analyze paternity, we used the computer program COLONY v2.0 (Jones & Wang, 2010) to assign paternity of 615 offspring from known mothers based on 13 reliable microsatellite markers (Burgess et al., 2019, 2023). In a previous controlled mating experiment in the laboratory, where both the mother and father were known, these markers

correctly assigned fathers to all 157 offspring from a set of 45 candidate fathers with a probability of 1 (Burgess et al., 2019). The genetic structure between populations from Dog Island and FSUCML is very low ($F_{st} < 0.001$, $p = 0.446$) and there are no population-specific alleles (Burgess et al., 2019).

In COLONY, the paternity analysis determined, for each offspring with a known mother, the probability of observing the offspring's genotype given the proposed relationship of candidate father and offspring, and then compared that likelihood for all candidate fathers to determine the most likely candidate father (Flanagan & Jones, 2019; Jones & Wang, 2010). Candidate fathers were all 32 individual colonies in the experimental array. In addition to paternity analysis, we also used paternal reconstruction to infer paternal genotypes for every offspring. For all offspring, COLONY infers full-sib families (BestFSFamily) and provides a unique father identification for offspring sharing the same paternity (BestCluster). In other words, when offspring were not assigned a father from the set of candidate fathers, COLONY also provides a unique identification for each reconstructed paternal genotype. To do this, COLONY uses the genotypes of offspring and their known mothers to identify the allele in offspring inherited from the mother, then examines the associations of alleles originating from the unknown parent across loci. Multiple paternity was then estimated from the number of full-sib families inferred by COLONY for each known mother.

To assess the strength of evidence for each full-sib family (and therefore the unique paternal identification), we used the inclusion probability, which is the probability that all individuals of a given full-sib family are full-sibs (Jones & Wang, 2010). A higher inclusion probability indicates a lower likelihood that the family can be split into two or more families. We also assessed the exclusion probability, which is the probability that no other individuals are full-sibs with a given full-sib family.

The input parameters were: both sexes were polygamous and monoecious, cloning absent, diploid, three medium runs of the full-likelihood model, high likelihood precision, and updating of allele frequencies to account for pedigree. We had expected colonies in the experiment to sire most offspring, so we set the estimated probability of an offspring having a father in the candidates at 0.9, but also compared results with the estimated probability of the father being in the candidate fathers set to 0.1, to account for the possibility for sperm from outside of the experimental array (i.e., outside of all four spatial blocks). In COLONY, this parameter is treated as a guess only, such that a lower value simply requires stronger information from the markers to assign a father. We estimated the marker error rates and null allele frequencies for each marker individually by manually identifying the frequency of known mismatches within each mother-offspring group (e.g., an allele in an offspring that is missing or not present in the mother). To further assess genotyping errors, fragment analysis and genotype calling were performed twice on five parental colonies. All allele calls were identical, except one sample indicating a null allele at one locus (which is not unusual).

Statistical analyses

To model survival to a given time point, we used a generalized linear mixed effects model (GLMM) with a binomial distribution. To model growth, we first calculated relative growth rate as $(\ln Z_2 - \ln Z_1) / (t_2 - t_1)$, where Z represents the

number of zooids at time 1 or 2 (subscript), and t represents the age at the time 1 or 2 (subscript) measured in days post-settlement (Hoffmann & Poorter, 2002). The relative growth rate is therefore in units of zooids produced per zooid per day. The relative growth rate was modelled using a GLMM with a Gaussian distribution. To model reproductive output, we used a generalized linear mixed effects hurdle model, using a binomial and a truncated negative binomial distribution. In all models for Experiments 1 and 2, "plate" was included as a random effect. In Experiment 1, "density" (continuous) and "relatedness" (categorical, two levels) were fit as fixed effects, and their interactive and additive effects were assessed using log likelihood ratio tests. In Experiment 2, "relatedness" (categorical, two levels) was fit as a fixed effect and was assessed using log likelihood ratio tests.

For Experiment 3, to test for an effect of distance from nearest neighbours on the relative growth rate of colonies and on the total reproductive output (total number of offspring produced), we used a generalized linear mixed effects hurdle model, using a binomial and a truncated negative binomial distribution. The number of unique sires estimated for each focal colony was standardized to the same sample size of 20 using rarefaction, to account for differences in the number of offspring genotyped per mother. Treatment (alone, near, far, or both) was fit as a fixed effect and "block" ($n = 4$) was included as a random effect in these models. All analyses were performed in R v4.4.0 using "glmmTMB" (Brooks et al., 2017) to fit models, "DHARMa" (Hartig, 2022) to assess model fit, "emmeans" (Lenth, 2024) to extract fitted values and 95% confidence intervals, and "vegan" (Oksanen et al., 2024) to perform rarefaction.

Results

Experiment 1: the effect of relatedness and density on survival and growth

The probability of individual colony survival in the field declined with density after 8 days ($\chi^2 = 10.268$, $df = 1$, $p = 0.001$; Figure 2a) and after 15 days ($\chi^2 = 8.346$, $df = 1$, $p = 0.004$; Figure 2b). The odds of survival declined by 10.07% (4.03–15.72, 95% CI) after 8 days, and by 8.46% (2.9–3.71, 95% CI) after 15 days, for every additional neighbour colony. However, there was no statistical evidence that the relatedness of neighbours had additive (8 days: $\chi^2 = 1.626$, $df = 1$, $p = 0.202$; Figure 2a; 15 days: $\chi^2 = 2.363$, $df = 1$, $p = 0.124$; Figure 2b), or interactive effects on individual colony survival (8 days: $\chi^2 = 0.225$, $df = 1$, $p = 0.636$; 15 days: $\chi^2 = 2.551$, $df = 1$, $p = 0.110$).

There was no statistical evidence that density (8 days: $\chi^2 = 1.034$, $df = 1$, $p = 0.309$; 15 days: $\chi^2 = 2.383$, $df = 1$, $p = 0.123$) or relatedness (8 days: $\chi^2 = 0.295$, $df = 1$, $p = 0.587$; 15 days: $\chi^2 = 0.142$, $df = 1$, $p = 0.706$; Figure 2) had additive effects or interactive effects (8 days: $\chi^2 = 0.065$, $df = 1$, $p = 0.799$; 15 days: $\chi^2 = 2.305$, $df = 1$, $p = 0.129$) on the relative growth rate of colonies (Figure 2).

Experiment 2: the effect of relatedness on survival, growth, and reproduction

There was no evidence that neighbour relatedness affected the probability of survival ($\chi^2 = 0.036$, $df = 1$, $p = 0.849$; Figure 3a), relative growth rate ($\chi^2 = 0.042$, $df = 1$, $p = 0.838$; Figure 3b), or the total number of ovicells produced per colony ($\chi^2 = 0.121$, $df = 2$, $p = 0.942$; Figure 3c) after 20 days in the field.

We also assessed whether the position of an individual colony within a group of colonies affected survival and reproduction, hypothesizing that the effects of relatedness would differ for individuals in the centre of an aggregation relative to individuals on the outer edge of an aggregation. From 20 positions arranged in a 5×4 numbered grid, colonies in grid positions 7, 8, 9, 12, 13, and 14 were categorized as “inner” positions, and the remaining grid positions were categorized as “outer” positions. There was no evidence that the position

of an individual had interactive ($\chi^2 = 0.058$, $df = 1$, $p = 0.810$) or additive ($\chi^2 = 0.354$, $df = 1$, $p = 0.552$) effects on the probability of survival after 20 days in the field. Similarly, there was no evidence that the position of a colony had interactive ($\chi^2 = 2.585$, $df = 1$, $p = 0.108$) or additive ($\chi^2 = 0.001$, $df = 1$, $p = 0.993$) effects on relative growth rate. Finally, there was also no evidence that the position of an individual had interactive ($\chi^2 = 1.922$, $df = 2$, $p = 0.382$) or additive ($\chi^2 = 0.314$, $df = 2$, $p = 0.855$) effects on reproductive output.

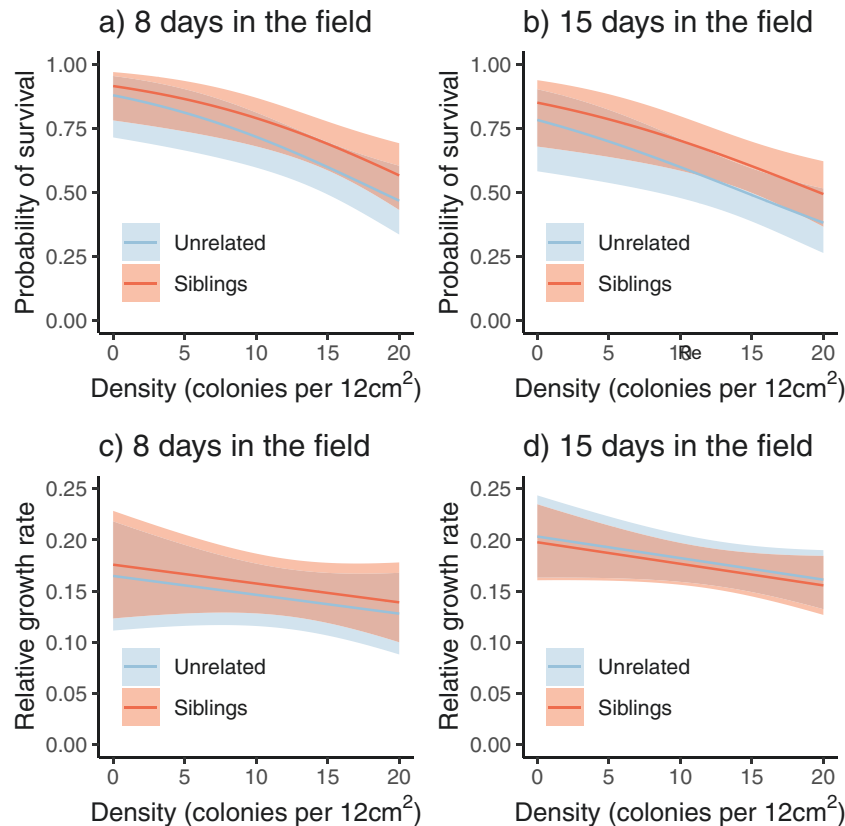


Figure 2. (a) Probability of survival after 8 days in the field, (b) and after 15 days in the field in relation to the density and relatedness of neighbours, estimated from a generalized linear mixed effects model with binomial distribution. (c) The relative growth rate (number of zooids produced per zooid per day) after 8 days in the field, and (d) after 15 days in the field in relation to the density and relatedness of neighbours, estimated from a generalized linear mixed effects model with Gaussian distribution. The bands show the 95% confidence intervals.

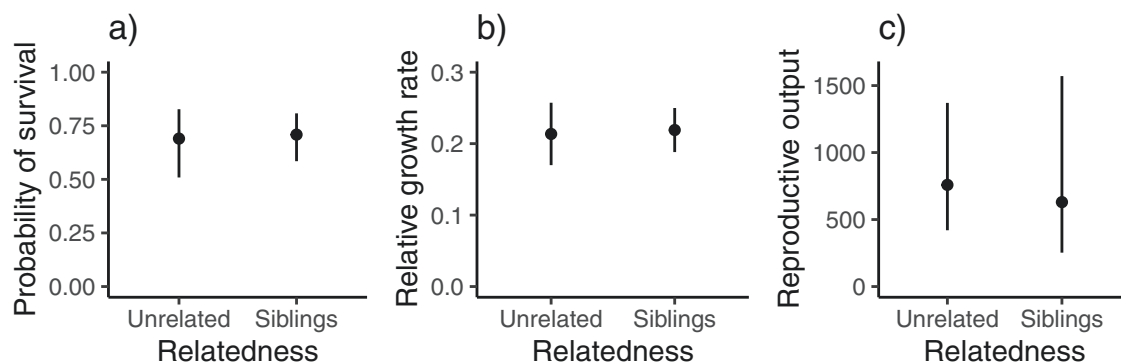


Figure 3. (a) Probability of survival, (b) relative growth rate (zooids zooid⁻¹ day⁻¹), and (c) reproductive output (total number of ovicells per individual colony) of individual colonies in the field when neighbours were unrelated vs related (maternal siblings). Black points are the means estimated from the generalized linear mixed effects model (GLMM) with binomial (a) or Gaussian (b) distributions. Black points in (c) are the conditional means estimated from a GLMM hurdle model. Each plate contained 10 individual colonies and included reproductive output as zero for colonies that did not survive. Error bars show 95% confidence intervals.

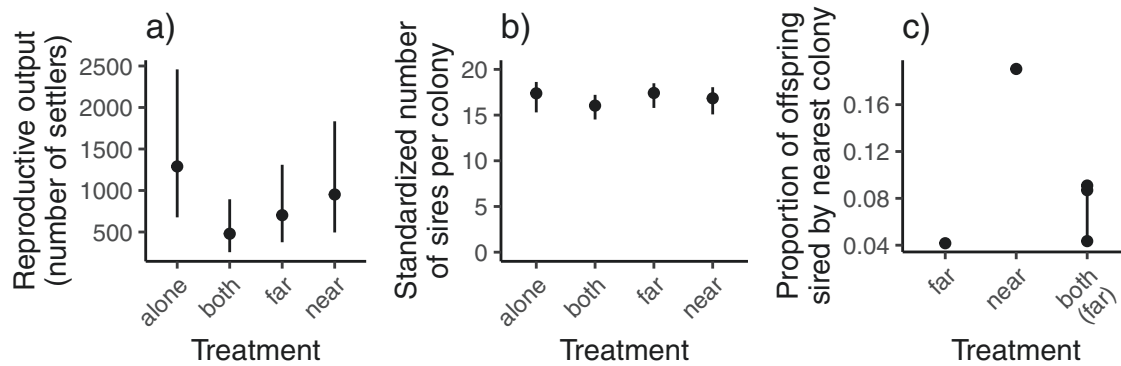


Figure 4. (a) Reproductive output (number of settlers per individual colony), (b) number of unique sires per individual colony (standardized to sample size of 20), and (c) the proportion of offspring sired by the nearest colony after 14 days in the field in each treatment (see Figure 1). In (a) and (b), black points are the means estimated from generalized linear mixed effects models. Error bars show 95% confidence intervals. In (c), each dot is a focal colony that had at least one offspring sired by the nearest colony, and the line extends up to the proportion sired by the most successful father. The absence of a line indicates that the nearest colony sired the most offspring. The “both” treatment is for the far colony (see Figure 1c).

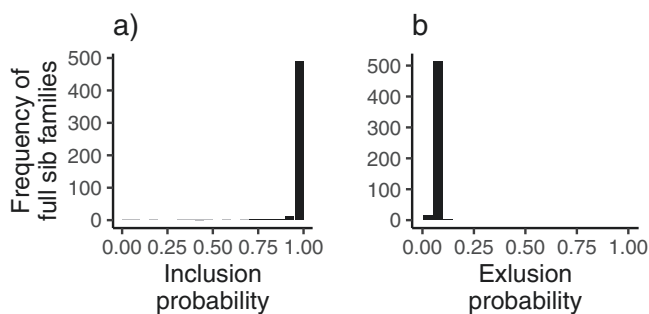


Figure 5 The number of full-sib families (known mother, inferred father) with a given (a) inclusion probability (the probability that all individuals of a given full-sib family are full-sibs), and (b) exclusion probability (the probability that no other individuals are full-sibs with a given full-sib family). Grey bars in (a) indicate the 17 full-sib families (3.3% of the 511 reconstructed full-sib families) with low inclusion probabilities (< 0.7) that were excluded from estimates of paternity.

Experiment 3: the effect of distance to the nearest neighbour on reproductive output and paternity

Although individuals growing alone tended to produce the most settlers (Figure 4), this effect was not statistically significant ($\chi^2 = 8.147$, $df = 6$, $p = 0.228$). Relative growth rate, and therefore size, was similar for individuals in all treatments ($\chi^2 = 0.936$, $df = 3$, $p = 0.817$) (Figure 4).

From 25 maternal broods, there were 528 estimated full-sib families. The probability that all offspring of a given full-sib family were full-sibs (inclusion probability) was > 0.7 for 511 (96.78%) families, and was 1 for 466 (88.25%) families (Figure 5). Within each full-sib family, the probability that no other settlers were full-sibs (exclusion probability) tended to be low (0.095; maximum 0.115). Excluding the 17 full-sib families (34 offspring; each family contained two offspring) with low inclusion probabilities (< 0.7), levels of multiple paternity were quite high (most larvae were half-sibs). From the 511 offspring with inclusion probabilities > 0.7, there were 291 unique sires, 286 (98%) of which were from outside the experimental array. A total of 43% ($n = 126$) of sires sired offspring from one maternal colony, 39% ($n = 114$) of sires sired offspring from two maternal colonies, 16% ($n = 46$) of sires sired offspring from three maternal colonies, 2% ($n = 5$) of sires sired offspring from four maternal colonies. For every 20 offspring produced per colony, there were typically 15–19

sires, though one brood had six sires (Figure 4). The number of standardized unique sires per colony did not differ among treatments ($\chi^2 = 2.502$, $df = 3$, $p = 0.475$; Figure 5).

Out of 511 offspring with full-sib inclusion probabilities > 0.7, 13 (2.5%) offspring were assigned paternity from one of five candidate fathers in the experimental array. All 13 offspring were assigned their father with a probability of 1. Within the experimental array, four paternal individuals sired offspring from one maternal individual each, and one paternal individual sired offspring from three maternal individuals. When the candidate father probability was set to an estimate of 0.1 (thereby requiring stronger information from the markers to assign a sire), fathers from the array were assigned to 11 offspring (probability = 1), which were the same offspring as when the candidate father probability was set to an estimate of 0.9. Scrutiny of the genotypes for the two offspring not assigned a father from the array when the candidate father probability was set to 0.1, revealed that all alleles in the offspring were present in both parents consistent with Mendelian inheritance. There was no evidence for selfing, since no offspring were sired by the maternal colony they originated from. For the 25 mothers, the mean number of alleles per locus was 13 (range: 5–29).

For the 13 offspring assigned paternity from a candidate father anywhere in the experimental array (across all four spatial blocks), we looked at the spatial distance between the known mothers and inferred fathers (i.e., both had known spatial locations) (Figure 6). In total, 77% of offspring ($n = 10$) were sired by a colony 15 cm away from the maternal colony. One offspring was sired by a colony 70 m away (Figure 6). Similarly, when mating occurred within spatial blocks, the nearest colony sired the highest proportion of offspring compared to other sires (“near” treatment), even though they sired a low proportion of offspring overall (Figures 4c and 6).

Discussion

Dispersal can be adaptive if it allows escape from competition with conspecifics or kin, but it can also result in reduced opportunities for mating when there is proximity-dependent mating success (Grosberg, 1987; Yund & McCartney, 1994). Through a series of manipulative experiments in the field, we found no effect of neighbor-relatedness on the fitness of *B. neritina*, and increased density reduced the probability of individual colony

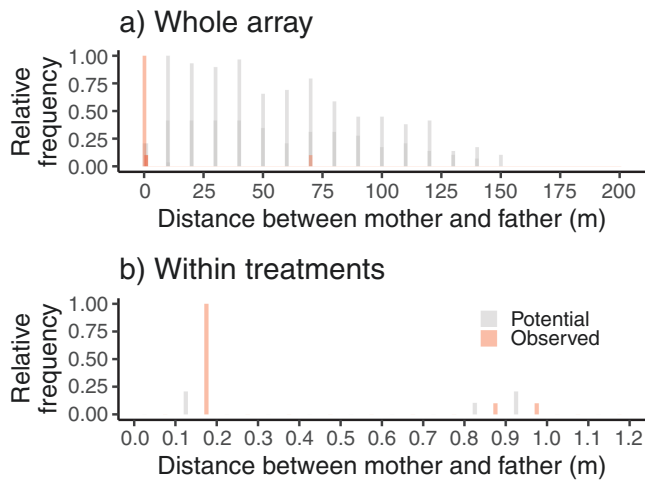


Figure 6 (a) and (b) Estimated distances (observed) between known mothers and assigned fathers for the 13 offspring that were sired by one of 32 potential fathers anywhere in the experimental array. (b) is a zoomed-in view of (a) across distances that reflect dispersal within the nearest neighbour treatment. The grey bars show the frequency of pairwise distances between all 32 colonies in the experimental array, as a guide to indicate the potential for sampling a given sperm dispersal distance (assuming all colonies release the same number of sperm, which have an equal chance of dispersing all distances).

survival but not the growth rate. Additionally, we found that distance to the nearest neighbour did not affect the number of settlers produced or the degree of paternity. Overall, paternity and full-sib families were identified with high confidence, and larger than expected sperm dispersal (e.g., from outside of the array) led to high multiple paternity (i.e., very few full-sibs within known mother–offspring groups) and prevented individuals from dominating the fertilizations of nearby colonies. The important implication arising from our results is that there appear to be no advantages nor disadvantages in terms of reproductive success of dispersing to vacant sites, no disadvantages to survival or growth if reduced dispersal increases kin interactions, and only small advantages for post-settlement survival for larvae that disperse further than a few 10s of metres, if there are no other costs to dispersal (though see Burgess et al., 2012, 2013). When connecting our results to previous work in this system or similar species, it appears there is little disadvantage to the limited dispersal that does occur (Burgess et al., 2019, 2023; Keough, 1989) if sperm remain viable for long enough to disperse beyond the scale of larval dispersal (Johnson & Yund, 2004; Pemberton et al., 2003; Yund et al., 2007), relatively high multiple paternity (Bishop et al., 2000; Johnson & Yund, 2007), the possibility for preferential selection of sperm from unrelated colonies (Bishop et al., 1996), and low relatedness in natural populations (Burgess et al., 2019, 2023; Johnson & Woollacott, 2010) all limit inbreeding and the likelihood of competing with kin (Grosberg, 1987).

Some of our results contrast previous studies on the effects of density and relatedness in similar systems, which may indicate the effects of density and relatedness differ if they are experienced before or after reproductive maturity. For example, Aguirre and Marshall (2012) showed that groups of kin had significantly lower survival, size, and fecundity compared to groups of unrelated colonies. Significant effects on survival and fecundity manifested after 6 weeks in the field, and size

was significant at 2, 4, and 6 weeks in the field (Aguirre & Marshall, 2012). In the same population that was studied here, Burgess et al. (2023) showed that high density increased mortality at 38 days post-settlement and reduced growth by 24 days post-settlement when offspring from each mother was arranged in groups of siblings compared to groups with unrelated conspecifics (Burgess et al., 2023). These prior studies outplanted colonies to the field within days of settlement in the lab, whereas we outplanted colonies after 23 days of growing in the lab. We deliberately delayed outplanting to the field to examine effects beginning around the time of reproduction, reasoning that interference competition, in which access to resources is limited due to obstructions due to another individual blocking access, would be greater between colonies at this life history stage than between colonies of smaller sizes (i.e., fewer zooids) (Birch, 1957; Holdridge et al., 2016; Svensson & Marshall, 2015). However, conditions in juvenile life may also affect how density affects fitness (Gosselin & Qian, 1997). Exploitative competition could occur at smaller stages, in which an individual depletes the resources that others can use, which may be just as likely to occur in juvenile stages (Birch, 1957; Holdridge et al., 2016; Svensson & Marshall, 2015). Additionally, colonies do not have to be in direct contact for them to respond to surrounding conspecifics (Thompson et al., 2015). For example, in the presence of conspecifics, metabolism is suppressed in *B. neritina* regardless of the food availability or abiotic conditions (Lovass et al., 2020). Similarly, density could affect colony fitness at all stages through local oxygen depletion, especially in environments with low water flow (Ferguson et al., 2013). Finally, abiotic conditions, such as temperature, salinity, or pollution, could mediate density effects (Lange & Marshall, 2017).

There are several mechanisms that could explain why density affected survival, but not growth. Predators can be attracted to high densities of prey, and thus higher densities can have higher mortality compared to lower densities (Janzen, 1970; Jones & Comita, 2010; Root, 1973; Wenninger et al., 2016). In our study, the pattern of mortality observed most often was the complete disappearance of a colony, which would occur if benthic-feeding fish (e.g., pinfish, *Lagodon rhomboides*) were feeding on, or biting and rejecting, colonies (Keough, 1986). Sedimentation has been implicated as a source of mortality for juvenile and other epifaunal invertebrates by suffocation or blocking feeding mechanisms (Hunt & Scheibling, 1997; Keough, 1986; Maughan, 2001), where acute exposure would affect survival, and only chronic exposure would affect growth more than survival. Finally, the disease may spread more rapidly under high compared to low density and cause increased mortality in the host (Hochachka & Dhondt, 2000) before affecting growth. However, the potential species-specific pathogens in this species are currently unknown.

The extent to which our results help understand the conditions that are selected for dispersal depends on the strength of dispersal costs. The effects of competition with conspecifics after settlement that we report may be stronger than that reported here for larvae that have accrued physiological costs associated with longer-distance dispersal but failed to locate vacant habitat (Burgess & Marshall, 2011b). Similarly, post-settlement survival benefits of dispersal to vacant habitats are less likely to be realized if larvae have depleted too much energy to disperse there. Larval dispersal often entails costs in the form of larval mortality, transport to unfavourable

habitats, and, in the case of species with non-feeding larvae, physiological costs associated with the depletion of energy reserves (Burgess et al., 2012; Marshall & Morgan, 2011). Non-feeding larvae deplete maternally derived energy sources during dispersal, which can increase the likelihood that larvae accept a poor quality habitat (the “desperate larva hypothesis”; Botello & Krug, 2006; Burgess et al., 2012; Marshall & Keough, 2003), or have reduced post-settlement performance (“latent effects”; Burgess et al., 2013; Marshall & Keough, 2003; Pechenik, 2006).

Another potential cost of dispersal is settlement in a vacant location with limited access to mates in predominantly, or obligately, outcrossing species (Kulbaba & Shaw, 2021; Pannell, 1997; Pannell et al., 2014). However, our results provide no evidence that distance from conspecific neighbours reduces the number of offspring produced, in contrast to what would be hypothesized if females were under sperm limitation. We found that most of the offspring sampled were sired by fathers from outside of the experiment and there was high multiple paternity. Despite our best efforts to remove any *B. neritina* colonies from the surrounding seagrass prior to deployment, and high confidence that the site was cleared of other colonies within at least 10 m of any of the experimental colonies, it remains possible that we may have missed some colonies (colonies are generally small [maximum size a few centimetres] and can be hard to locate in thick seagrass patches when water visibility is < approx. 1 m) or that colonies from further away sired offspring from colonies in the experiment. Regardless, we are confident that we successfully manipulated the nearest neighbours.

Beyond the experimental individuals, it remains unclear where exactly the siring sperm originated from. While the scale at which sperm can travel is still unclear at this location, we found evidence for sperm to disperse at least ~70 m. In a colonial ascidian in an advective river estuary, Yund et al. (2007) found evidence for sperm dispersal over 10s to 100s of metres, in contrast to typical ranges in the order of 10s–100s of centimetres (Grosberg, 1987). Furthermore, the high estimates of multiple paternity (estimated through reconstructing expected paternal genotypes, where nearly every offspring had a unique sire), could occur if sperm are efficiently filtered and utilized even if they are rapidly diluted (Pemberton et al., 2003). Another hypothesis is that sperm from *B. neritina* were already in the water column prior to the deployment of the experiment. Sperm can postpone activation and swimming until they sense chemical cues from conspecific eggs (Silén, 1966; Temkin, 1994), which could increase longevity and dispersal distance via currents. Therefore, colonies that were removed prior to the experiment could have already released sperm, which persisted in the water column by being inactive, and becoming activated when the experimental colonies were outplanted, and prior to the experimental colonies releasing sperm. The longevity of *B. neritina* sperm is unknown, but in an ascidian (*Botryllus schlosseri*), sperm half-life was ~16–26 hr (Johnson & Yund, 2004), and in a bryozoan (*Celleporella hyalina*), sperm half-life was ~1 hr (and perhaps up to ~4 hr at higher sperm concentrations; Manríquez et al., 2001). Therefore, if experimental colonies were fertilized by sperm already in the water column, it would likely be from sperm released on the day or prior day of deployment, and overall we consider this scenario an unlikely explanation for where the siring sperm originated from. In spermcast species, there is also an opportunity for individuals to enact safeguards

against unwanted sperm (Bishop & Pemberton, 2006; Firman et al., 2017; Levitan, 2018), such that even if the sperm from the nearest neighbour in the experiment had reached the focal colonies first, the colony instead chose sperm that was perceived as higher “quality” than the nearest experimental neighbour. In any case, such high multiple paternity would also provide a means to dilute any negative consequences of inbreeding (Bocedi 2021).

Overall, we show that if larvae were to disperse and select vacant locations to settle, they could avoid the competition that results in mortality without losing opportunities to fertilize eggs and produce viable offspring. These results suggest that larger scales of sperm dispersal may offset any genetic costs of the limited larval dispersal of larvae (Grosberg, 1987), though this would need to be estimated directly. In this system, there may, therefore, be little disadvantage to limited larval dispersal because the scale of sperm dispersal, relatively high multiple paternity, the possibility for preferential selection of sperm from unrelated colonies, and low relatedness in natural populations, all limit inbreeding and the likelihood of competing with kin (Burgess et al., 2023; Grosberg, 1987; Johnson & Woollacott, 2010; Olsen et al., 2020).

Data availability

Data and R code to reproduce the analyses and plots are available at: <https://zenodo.org/records/13821159>

Author contributions

Danielle Barnes (Data curation [lead], Formal analysis [equal], Investigation, Methodology, Writing—original draft [lead]) and Scott Burgess (Conceptualization [lead], Data curation [supporting], Formal analysis [equal], Funding acquisition [lead], Methodology [equal], Project administration, Resources, Supervision, Writing—review & editing [lead])

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Conflicts of interest

None declared.

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