Maternal effects on cloning frequency, larval development, and juvenile size in the seastar Asterias forbesi

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Maternal effects on cloning frequency, larval development, and juvenile size in the seastar Asterias forbesi

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelors of Science in Neuroscience from The College of William and Mary

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Maternal effects on cloning frequency, larval development, and juvenile size in the seastar *Asterias forbesi*

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Abstract

A fundamental life-history trade-off occurs between the size and number of offspring that a female produces. Traditionally, biologists have assumed that there is a species-specific optimal egg size, the value of which can fluctuate with changing environmental parameters. However, in unpredictable environments a bet-hedging strategy resulting in variable offspring sizes may be favored. The seastar *Asterias forbesi* can produce viable eggs that vary more than three-fold in volume within a single clutch (141µm - 212µm diameter). Compared to 12 other echinoderm species with similar modes of development (planktotrophic), *A. forbesi* represents an organism with unusual intra-clutch variation in egg size. In addition, the larvae derived from these eggs have frequently been observed to produce clones. To test for maternal effects on cloning frequency and larval development I reared cultures of large (190µm mean diameter) or small (140µm mean diameter) sibling embryos. Previous studies have shown that exogenous cues can alter the frequency of cloning, but it is unclear whether endogenous reserves might also influence cloning. My results suggest that despite an initial disadvantage in energy reserves, small larvae produced clones at frequencies similar to their larger siblings. Since little is known about the links between maternal investment and juvenile quality in seastars, I continued to follow these larvae and examined the effect of maternal investment on time to and size at metamorphosis. Small larvae took about two additional days to reach metamorphosis compared to large larvae, which was a 6.3% increase in developmental time. Size at metamorphosis did not appear to be affected by maternal investment and varied greatly within size classes. To further examine the
costs of delayed metamorphosis, I set up an additional experiment, which examined the effect of time to settlement on post-metamorphic survivability in juveniles. My results indicate that size at settlement, rather than time to settlement, is correlated with survivability.
Introduction

Developmental biologists, ecologists, and evolutionary biologists all share a long history of interest in why organisms develop the way they do and how development interacts with the environment. In particular, there has been a fascination with the evolution of diverse life histories and life cycles among species. Early life history traits, for example egg size, can have long-lasting effects on survival and reproduction, and therefore the field of life history evolution focuses on identifying and understanding the cause for variation in strategies (Allen and Pizer, 2011; Fabian and Flatt, 2012). Some organisms reproduce once in a lifetime, others do so repeatedly; some release hundreds of thousands of eggs, others invest in a single offspring; some live for only a few years, and yet others live for hundreds of years (Young, 2010; Allen and Pizer, 2011; Fabian and Flatt, 2012). Why has such diversity in life history evolved? One answer may be the environment in which an organism evolved and the environment’s constantly changing factors, which lead to new adaptations and more variation (Shefferson, 2010).

One of the earliest, and most well known, examples of life history studies is David Lack's (1947) work exploring the variation in number of offspring (clutch size) within and across avian species. Lack noticed the potential for environmental factors to alter life histories; he noted that clutch size varied with latitude. At higher latitudes, with longer periods of daylight, parents were able to rear larger clutches than at lower latitudes (Lack, 1947; Allen and Pizer, 2011). There are various potential components of parental investment such as energetic investment in the
content of the egg, genetic investment, and investment beyond the egg (parental care) like foraging for food for offspring and time spent incubating. Lack (1947) also demonstrated that with limited resources, parental investment is likewise limited, and clutches are adjusted to an optimal clutch size based on the available food supply. For example, birds like hawks and owls have asynchronous hatching, which allows for less costly brood reductions by not feeding the smallest, youngest chick (Lack, 1947). Clutch size is expected to decrease when either food abundance or food quality diminishes (Lack, 1947). The idea behind Lack’s work is that when the environment is favorable, a female’s fecundity is increased by producing as many eggs as possible; and when the environment is less favorable, and there may not be enough food for lots of offspring, a female’s fecundity is increased by investing more in a few offspring. Ultimately, Lack hypothesized an optimal clutch size theory in which a species evolves a solution to the trade-off and that optimal clutch size should be most frequently observed in nature (Allen and Pizer, 2011), though after this clutch has been laid, brood reduction may allow for adjustments.

Not only is clutch size affected by limited parental investment, but also offspring size within that brood can be affected by these limits. There exists a fundamental life-history trade off between the size and number of offspring that parents can produce (Smith and Fretwell, 1974). Across the plant and animal kingdoms, organisms have been shown to exhibit a negative correlation between the quantity and size of offspring within taxa (Stevens, 1932; Sinervo, 1990; Guisande et al., 1996; Abell, 1999; Guralnick, 2004; Walker et al., 2008). With limited resources, the strategy of a species may fall along a continuum of which the
extremes are producing a few relatively large offspring or many relatively small offspring.

While tradition has suggested that a species as a whole evolves an optimal egg size-egg number solution, in some species this optimum varies in different populations with environment (Allen and Pizer, 2011). For example, female western fence lizards, Sceloporus occidentalis, living in northern populations tend to produce larger clutches of smaller eggs, while females of more southern populations tend to produce smaller clutches of larger eggs (Sinervo, 1990). In yet another species of the genus Sceloporus, females of a single population can switch between a reproductive mode of few, large offspring and many, small offspring in response to a change in environmental conditions (Abell, 1999). During periods of drought, when food and water are limited, the striped plateau lizard, Sceloporus virgatus, tends to produce small clutches of large offspring. When supplemented with additional food, however, S. virgatus exhibits a change in investment and tends to produce larger clutches of smaller offspring (Abell, 1999).

The environment and ecology of a female directly affects maternal investment, and ultimately egg size, because maternal investment is a joint phenotype of the offspring and the mother (Bernardo, 1996). Because egg size is a non-genetic effect on offspring traits and since maternal investment is greater than the paternal contribution, the effects from differences in egg size are a type of maternal effect (Bernardo, 1991). In the case of Sceloporus, where adults and offspring consume relatively similar food, females sense the quality of the environment and adjust their maternal investment strategy accordingly. However,
in organisms with complex life cycles, in which an individual undergoes a dramatic transition in both habitat and food source, the relationship between parental environment and investment strategy is not as closely linked. For example, adults of the seastar *Leptasterias epichlora* collected from sites with abundant food (i.e. large molluscs) produced larger eggs than adults collected from a less favorable site (George, 1996). Though this seems contrary to what one would predict based on examples in lizards, it is inappropriate to assume that a favorable site for adult seastars (i.e. large molluscs) is correlated with a favorable site for seastar larval development (i.e. abundant phytoplankton).

The trade off between maternal investment per offspring and number of offspring is difficult to examine in organisms like birds or mammals that have an extended period of parental care. In broadcast spawning marine invertebrates, however, no parental care exists beyond energy allocation in the egg. Thus, maternal investment can reasonably be quantified as the energy content within the egg (McEdward and Morgan, 2001).

Though the relationship between egg size and maternal investment seems intuitive, there is controversy in applying it universally to all organisms, or even just to echinoderms. While larger eggs are often considered to have more organic content, in some cases egg size is a poor predictor of maternal investment even within a single female (Turner and Lawrence, 1979; McEdward and Coulter, 1987; Moran and McAlister, 2009; McAlister and Moran, 2012). However, Jaeckle (1995) analyzed 23 echinoderm species and found that total energy content changes in direct proportion to egg volume. More specifically, Jaeckle (1995) noted that
planktotrophic echinoderms (those with feeding larvae, like seastars) show a direct relationship between egg volume and the proportion of organic materials in an egg. Therefore, for the purposes of this study, egg size is an appropriate indicator of energy content and maternal investment.

In planktotrophic echinoderms, the relative amount of energy in the egg can be estimated from the size of the egg, which is measured as diameter and volume (Jaeckle, 1995). Since quantifying maternal investment is so easy in broadcast spawners, marine invertebrates have become organisms of interest for exploring trade offs in ecological and evolutionary developmental biology research (eco-evo-devo; Ledón-Rettig and Pfennig, 2011). More specifically, the radial, indeterminate cleavage in the early deuterostome development of echinoderm embryos allows easy manipulation of egg size/energy content, causing echinoderms to surface as model organisms in this field.

Eco-evo-devo research has begun to examine the environmental and evolutionary basis behind the trade-off in size and number of offspring (Roff, 1992; Walker et al., 2003; Reitzel et al., 2011). Larger offspring fare better in stressful environments than smaller offspring would because offspring fitness in these environments is positively correlated with offspring size (Roff, 1992; Walker et al., 2003). Across closely related species, larger offspring tend to have increased hatching success, increased survival, increased resistance to starvation, and increased overall fitness compared to smaller offspring (Roff, 1992). Despite all of these advantages, small offspring size continues to persist in nature, indicating that large offspring are not always favored (Roff, 1992). For example, in cnidarian
species, the strategy of producing few, large propagules would be favored in environments where development to an adult stage independent of the parent may not be possible (e.g. poor food availability; Reitzel et al., 2011); but the strategy of producing many, small offspring would be favored in stable environments where these smaller individuals can successfully develop independently (Reitzel et al., 2011).

The evolutionary basis of each reproductive strategy (few, large or many, small) is relatively well understood in variable environments for which females may be able to predict the likelihood of offspring survival. Many echinoderms (a model organism for eco-evo-devo research), however, inhabit highly unpredictable nearshore regions, like the shallow subtidal and intertidal. While some fluctuations in these regions can be predictable, like the tides, those caused by sudden weather events are not. For example, one intertidal population of the sand dollar *Echinarachnius parma* on Bailey Island, ME experiences a salinity fluctuation of 11 psu (practical salinity units) over the course of a single one-month spawning season (Allen and Pechenik, 2010; Armstrong et al., 2013). Similarly, over the same time frame, this population of *E. parma* experiences temperature fluctuations of more than 10°C (Allen, J.D., pers. comm.). Such temperature and salinity variation can have dramatic impacts on adult reproductive success through offspring survival; for example, planktotrophic larvae experience decreased growth and decreased developmental rates when exposed to decreased salinity (Roller and Stickle, 1985). Likewise, decreased temperature results in decreased growth rates in planktotrophic larvae (McEdward, 1985). Not only does temperature and salinity
directly affect larval growth and development, but also the fluctuation of these abiotic stressors can then indirectly affect larvae through algal growth and reproduction (Brock, 1975; Kaplan et al., 1986; Chen et al., 2009). For example, as salinity increases, growth and photosynthesis of the green alga *Dunaliella salina* decreases (Brock, 1975; Chen et al., 2009). In variable environments, like the shallow subtidal and intertidal regions, where larval development and access to food are unpredictable, a set egg size-egg number may not be optimal.

When an organism cannot reliably anticipate the fluctuation of biotic and abiotic factors, it may increase a female’s fitness to produce a range of offspring sizes as a bet-hedging strategy (Marshall et al., 2008). In 2009, Olofsson et al. developed a model demonstrating that bet-hedging could be an evolutionarily stable strategy. While bet-hedging may not be the most successful strategy in any given season, it is the most consistently successful over time and can, therefore, be favored by natural selection (Marshall et al., 2008; Olofsson et al., 2009). Take, for instance, the case of two females living in a stochastic environment. Female A produces uniformly sized eggs and Female B hedges her bets and produces a range of egg sizes. During a ‘good’ season, Female A can expect a 50% offspring survival rate, while Female B can expect a 30% offspring survival rate. During a ‘bad’ season, Female A can expect a 10% offspring survival rate and Female B can expect a 20% offspring survival rate. The arithmetic mean for Female A over the two seasons is 30% and that for Female B is 25%; thus, it may appear that a uniform egg size is always better. However, since each spawning season is not independent from that female’s earlier spawning season, the arithmetic mean is not an accurate
representation of the central tendency of these females. Rather the geometric mean should be valued when examining long-term fitness because it is sensitive to large fitness variations (Olofsson et al., 2009). Female A has a geometric mean of 22.4%, while Female B has a geometric mean of 24.5%. Thus, over time, the bet-hedging strategy of Female B has a tendency of being more successful because of a reduction in success variance from year to year (Fig. 1; Roff, 1992).

The overall success of a bet-hedging strategy relies on a variable, changing environment (Einum and Fleming, 2004; Olofsson et al., 2009). Multiple early mathematical models claimed that bet-hedging could only be favored under exceptionally improbable and unrealistic conditions (e.g. McGinley et al., 1987). Rather, these early models set forth the classic view (mentioned above) that there exists an optimal egg size resulting in a clutch of uniformly sized offspring (Smith and Fretwell, 1974; McGinley et al., 1987; Forbes, 1991; Einum and Fleming, 2004). Yet, even these earlier studies admit (McGinley et al., 1987) that observations in nature do not follow the optimal egg size model (Marshall et al., 2008; Turner and Lawrence, 1979). The most classic model of egg size-egg number trade-off from Smith and Fretwell (1974) assumes that organisms experience a constant, heterogeneous environment. It is important to recognize that plants and animals are experiencing the totality of nature, which includes variation and fluctuation. Regaining the ecological aspect in evolutionary developmental biology allows us to more accurately understand the basis of adaptive or maladaptive diversity, and is the goal of the valuable field of eco-evo-devo (Gilbert, 2001; Sultan, 2007).
Of particular importance to marine scientists interested in eco-evo-devo questions is the study of strategies and life histories in organisms currently exposed to the variable nearshore regions. The intertidal seastar *Asterias forbesi* exhibits large variation in egg size within a single clutch (Turner and Lawrence, 1979) and therefore may be a model organism for studies of bet-hedging and maternal investment. For example, a female can produce eggs that vary more than three-fold in volume within a single spawning event (see data in present study). This large variation in volume suggests a considerable difference in per offspring maternal investment, which would lead to drastic impacts on the developing organism.

In echinoid echinoderms, it has been shown that artificially removing 50% of embryonic energy reserves through blastomere separations results in an increased larval period and decreased juvenile quality (Alcorn and Allen, 2009). However, all echinoderm studies examining the effects of egg size reduction on larval and juvenile development result from strictly artificial manipulations of egg size (e.g. Sinervo and McEdward, 1988; Hart, 1995; Emlet and Hoegh-Guldberg, 1997; Alcorn and Allen, 2009). One of the goals of my study was to examine natural, and thus ecologically relevant, reductions in egg or embryo energy content. Based on the results of previous studies that artificially reduced the energy reserves of sea urchin embryos (Sinervo and McEdward, 1988; Hart, 1995; Emlet and Hoegh-Guldberg, 1997; Alcorn and Allen, 2009), the natural energy reduction of *A. forbesi* will likewise have a significant effect on larval and juvenile development. Even more than development, embryonic energy reduction may impact the energetically-costly, non-essential larval behaviors, such as cloning.
During larval development, asexual reproduction or cloning has been widely observed in marine organisms (Eaves and Palmer, 2003; Bosch et al., 1989; Jaeckle, 1994; Vaughn and Strathmann, 2008; Vickery and McClintock, 2000), though elsewhere in the animal kingdom this phenomenon is a rare occurrence. *A. forbesi* has been observed to clone via three main methods: budding, autotomous arm cloning, and autotomous bisections (Fig. 2). During budding, a clone grows directly on the soma of a primary larva (e.g. Bosch et al., 1989) and can develop through early larval stages and even stomach formation before becoming independent (Fig. 2A). Autotomy refers to severing a body part, which subsequently undergoes development (Jaeckle, 1994). Autotomy in *A. forbesi* has been observed to occur at the brachiolaria arms, postoral arms, posterolateral arms (Fig. 2B), or at the preoral lobe through a bisection into anterior and posterior portions (Fig. 2C).

The processes of cloning and subsequent regeneration are highly energetically expensive (Maginnis, 2006; Fleming et al., 2007; Naya et al., 2007; Bely and Nyberg, 2010). For example, after autotomization, individuals suffer developmental trade-offs between the costs of a missing body part (e.g. impaired swimming and food acquisition in larvae; Bely and Nyberg, 2010) and the costs of energy allocation to regeneration (e.g. decreased growth; Maginnis, 2006; Maginnis, 2007; Fleming et al., 2007; Bely and Nyberg, 2010). Despite the costs of asexual reproduction, it can also be advantageous. As larvae clone, their numbers increase and, assuming this increased number leads to increased survival and reproduction, larval cloning can ultimately lead to an increase in genetic representation in future generations (Jaeckle, 1994). Additionally, more recent research has shown that
specific exogenous cues can also induce cloning, suggesting that larval cloning may be an adaptive response to a changing environment (Vaughn and Strathmann, 2008; McDonald and Vaughn, 2010; Vickery and McClintock, 2000). Echinoderm larvae have been observed to clone in response to predator cue (Vaughn and Strathmann, 2008), sudden availability of food (McDonald and Vaughn, 2010), abundant food and optimal temperature (Vickery and McClintock, 2000), and higher quality food/ a multiple algal diet (Vickery and McClintock, 2000). Larval cloning in echinoderms has not been well studied, but most studies examine the effect of exogenous cues and relatively understudied is the potential effect of endogenous factors. In the only study to date on this topic, Vaughn (2009) showed that maternal genotype could play a role in cloning capacity. However, no study has controlled for genetics and examined the effect of maternal investment on the occurrence of cloning.

A larva derived from an egg with reduced maternal investment will presumably have less energy reserves that are available to devote to cloning. Given the same genetic material (i.e. the same instructions for when and how to clone) and the same environmental conditions (i.e. the same epigenetic modifications), a deficit in energy reserves may result in a decreased frequency of clone production. My study is the first to combine the ideas of maternal investment and larval cloning. This may in part due to the difficulty in identifying a model system. A. forbesi exhibits the two rare phenomena of variation in maternal investment and planktotrophic larvae that undergo cloning at relatively high frequencies, making this species the first potential model system for such studies. In regards to maternal investment I wanted to answer the following questions: 1) Is the variation in A.
Asterias forbesi egg sizes a result of bet-hedging? 2) Does maternal investment affect larval development, larval cloning, and metamorphosis? I predicted that smaller embryos would result in smaller larvae with lower cloning rates and longer development periods.

Materials and Methods

Two separate experiments were run in laboratory settings: the first, in Brunswick, Maine, was designed to examine the effect of maternal investment on larval development, larval cloning, and metamorphosis; the second, in Williamsburg, Virginia, looked at the effect of development time on juvenile quality.

Adult collection and spawning methods

In June of 2012, adult seastars (Asterias forbesi, Asterias rubens, and likely hybrids of the two species) were hand collected from intertidal and subtidal habitats at Rockland Breakwater, Rockland, Maine (44°6’34”N, 69°4’47”W) and Alliquippa Landing, Phippsburg, Maine (43°44’35”N, 69°50’46”W). Upon collection, adults were placed in coolers and transported by car to the Bowdoin College Coastal Studies Center on Orr’s Island, ME. For the duration of the experiments seastar adults were kept in a sea table fed by a flow-through seawater system. Animals experienced ambient salinity (29 psu-32 psu) and temperature (10°-16°C). In August of 2012, adults were returned to Aliquippa Landing and new adults for a second experiment were collected and then shipped overnight to the College of
William and Mary. The adults that arrived in Virginia were maintained in recirculating aquaria at 12°C and 32 psu. Adults were identified to the species level using madreporite color and body rigidity as phenotypic markers, although these traits are not completely accurate and species identification is not assured without genetic testing (Harper and Hart, 2007). Oocyte maturation and gamete spawning was induced by intracoelomic injection of 1 mL of 100 μM 1-methyladenine (Strathmann, 1987). Within asteroids, full-grown oocytes are arrested at prophase I in the ovaries (Strathmann, 1987) and 1-methyladenine has been isolated as the naturally produced ovarian factor that stimulates oocytes to resume meiosis, which results in spawning of mature eggs (Kanatani et al., 1969; Strathmann, 1987). In Maine, to collect gametes, adults were placed in bowls containing 5 μM bag-filtered seawater (FSW) at ambient temperature and salinity. In Virginia, gametes were collected in artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, OH) at 21°C and 32 psu. Eggs were rinsed with either FSW or ASW prior to fertilization. A single male-female pair was used in each experiment.

**Experimental setup (first experiment—maternal effects)**

In Maine, spawning was induced and gametes were collected on 21 June 2012. Eggs were fertilized with dilute sperm and placed in 1000 mL glass bowls within sea tables to maintain ambient temperature. The embryos were allowed to develop to the blastula stage (approximately 13 hours) prior to sorting. The largest and smallest healthy embryos were hand sorted using a mouth pipette under a dissecting scope. Selected blastulae were pipetted onto microscope slides then
measured at 100x magnification under a compound microscope fitted with an ocular micrometer. Embryos with diameters greater than or equal to 180 μm were placed into the large size class and those with diameters less than or equal to 140 μm were placed into the small size class. Each replicate consisted of 20 blastulae reared in a 250 mL glass beaker containing 200mL of 0.45 μm FSW. I created seven replicates each of the large and small size classes. Replicates were filled numerically; the first 20 small embryos were places in replicate one, the second 20 in replicate two and so on.

Low levels of cloning began as early as two days post fertilization. On day two a single bisection was observed (both anterior and posterior sections). Cloning continued at a frequency of less than 3% through the first eleven days. Bisected clones were identified by anatomical characteristics as only a portion of a larva. Other clones were identified based on size and developmental stage; clones are much smaller and at an earlier stage compared to the average primary larva for a given day. As these low levels of cloning began, it became difficult to differentiate a clone from an unhealthy or slow-growing larva. To ensure confidence in my cloning data, 12 days post fertilization I removed all individuals under a given size threshold per size class (approximately 1/3 the size of an average larva). The day prior to sorting, large size class averaged 1500.0±8.7 μm in length, thus any individual less than 520 μm was removed. The small size class averaged 1358±16.9 μm in length the day prior to sorting and I set a size threshold of 430 μm. Whereas originally each replicate contained 20 larvae, three days after removal the average number of
larvae per replicate for the large size class was 12.9±1.1 and 13.7±0.9 for small replicates.

*Experimental setup (second experiment—age at settlement effects)*

The second experiment, in Virginia, was originally designed as a replicate of the first experiment and thus a similar protocol and experimental setup was used. Spawning was induced on 14 August 2012. Fertilized eggs were placed in 1000 mL glass bowls and left at room temperature (approximately 21°C) to develop to the blastula stage (approximately 9 hours). Mouth pipetting under a dissecting scope was again used to hand sort embryos; then a compound microscope at 100x magnification was used to measure and assign the blastulae. Again, each replicate contained 20 embryos in a 250 mL beaker containing 200mL of ASW, but this time ten replicates of each size class were created. The remaining embryos were left in a 1000 mL glass bowl at room temperature.

Unfortunately, over the next few days, nearly all of the small embryos stopped developing and replication of the initial experiment became impossible. Data from the first experiment suggest that a decrease in maternal investment results in a longer developmental period before settlement. Since I could no longer examine the effect of maternal investment on settlement, I chose to examine the effect of larval developmental time on juvenile quality. Small larvae take longer to reach settlement; does this increase in developmental time affect juvenile fitness? Six days post fertilization I recombined larvae from the large replicates in a glass bowl and reassigned each to a new replicate. Again, 20 larvae were mouth-pipetted
into each of the ten replicates and only large, healthy-appearing larvae were chosen. Since not all of the 200 original large larvae survived to day six, some replicates were unable to be filled during redistribution. Larvae that were left in the glass bowl at room temperature since fertilization and initial sorting were used to fill these replicates.

*Larval care*

Throughout the first experiment, in Maine, beakers were maintained at ambient temperature in a sea table by a flow-through sea water system. A stirring rack was constructed as described by Strathmann (1987) and used to maintain water circulation. Every other day, glass beakers were drained to about 50 mL by reverse filtration with a 35 μm mesh bottom beaker. Glass beakers were scrubbed clean, rinsed with deionized water and fresh FSW was added to reach 200mL. After each water change larvae were given an equal mixture of *Rhodomonas lens*, *Isochrysis galbana*, and *Dunaliella tertiolecta* at a total concentration of 5000 cells mL⁻¹. There were minor disruptions to this feeding regimen due to unexpected losses of algal cultures. One feeding contained equal portions of only *R. lens* and *D. tertiolecta* and two contained only *I. galbana* and *D. tertiolecta*.

Larval care in Virginia followed a similar protocol. Glass beakers were maintained at 20°C in a chilled water bath, the same stirring rack was used, and the same concentration of all three algae was offered. For the first 20 days post fertilization, ASW was changed, beakers were cleaned and larvae were given algae every Monday, Wednesday, and Friday. Beginning on day 20, beakers were not
scrubbed during water changes to allow a biofilm to build on the beaker, stimulating juvenile settlement (Cameron and Hinegardener, 1974).

**Juvenile care**

In both experiments, the shell of the blue mussel, *Mytilus edulis*, was used to cue settlement. During the first experiment, adult *M. edulis* were collected from Mussel Beach, Orr’s Island, Maine (43°47’29”N, 69°57’29”W) as food for adult *A. forbesi*. Following consumption by adult seastars, empty shells were cut into approximately 2 cm by 2 cm squares of similar masses. The two halves of this bivalve were split such that one half was assigned to a large replicate and the other to a small replicate; this was done to control for a variation in initial settlement cue. When juveniles settled on a shell, the shell was removed to a small glass bowl to allow the larva to complete metamorphosis while keeping track of when each larva settled. The mussel shell in the beaker was replaced with a new square. Juveniles were not fed any further than the nutrients covering the shell upon which they settled.

In Virginia, similar squares of mussel shell were used. These shells were obtained from a recirculating aquaria at 12°C and 32-psu. Juveniles were removed from the shells or wall of the glass beakers two days after settlement. Juveniles were placed in a 250 mL beaker with a complete half of a mussel shell as the only source of food. These beakers were placed in the same water bath as the larvae and water was changed weekly.
Data collection and analysis

To examine the variability in egg volume both across and within females, egg diameter was measured at either 100x or 200x magnification under a compound microscope fitted with an ocular micrometer. The sample size ranged from 15 to 125 eggs. Using these data, the coefficient of variation (CV) of egg volume for a female (within a clutch) was calculated and compared across females of *A. forbesi* as well as other species.

During the first experiment, in Maine, three sets of data were collected: larval growth, larval cloning, and metamorphosis. Larval length and width were measured using a compound microscope fitted with an ocular micrometer. Using a mouth-pipette, five larvae were haphazardly selected from each replicate and placed on a microscope slide, and then small quantities of water were gently removed to constrain a larva from swimming. Width refers to the widest point of a larval body, excluding the postoral arms, posterolateral arms, and oral hood; length was measured as the distance along the midline, again, not including posterolateral arms (Fig. 3). During this time, developmental stage (as described by George, 1999) was also recorded for the five larvae, and then the larvae were returned to their replicate beakers.

Every other day (during water changes) replicate beakers were drained to about 50 mL and poured into Bogorov trays. To determine larval survival, each larva was counted while looking along the Bogorov tray. At this time, I also collected data on cloning frequency by recording each clone and placing it in a 1.5 mL shot glass. Clones were defined as any obviously autotomized segment of a larva (e.g. an oral
hood or posterolateral arm) or an individual that appeared much smaller and at an early developmental stage compared to the larvae of the size class. All clones for a given size class, on a given day were housed together and shot glasses were washed, rinsed, and received food every other day.

To determine the effect of maternal investment on juvenile ecology, time to metamorphosis and size at metamorphosis were recorded for each juvenile. The day post fertilization that a juvenile settled was noted and each juvenile was measured within 24 hours of settlement, though not before larval arms had been reabsorbed and the individual had taken on the juvenile form.

In Virginia, during the second experiment, I collected data of time to metamorphosis, size at metamorphosis, juvenile survivability and juvenile growth rates. Time to metamorphosis was measured in the same manner as before. Initial juvenile measurements, however, were not taken until two days post settlement. In this way, the juvenile body was well formed before data collection began. Juvenile survivability was measured as the days survived post metamorphosis. To determine growth rates, juvenile size was measured every seven days after initial measurements. Juvenile size consisted of four measurements: 1) juvenile width (widest point from arm to arm), 2) spine length, 3) longest arm, and 4) disc/body diameter (Fig. 4). Data for growth was only analyzed from those juveniles that formed an appropriate body shape and survived at least nine days (one week after initial measurements).

All statistical analyses were conducted in SPSS (ver. 20.0). The intra-clutch CV of egg volume for a female was calculated by dividing the standard deviation by
the mean of that sample and multiplying by 100, such that CV describes the standard deviation as a percentage of the mean (Crean and Marshall, 2009). A Kolmogorov-Smirnov procedure was used to test for normality of frequency distributions. A Two-tailed T-test ensured distinct initial size classes. A repeated measures analysis of variance (ANOVA) with a mixed models procedure was used to analyze the effect of egg/embryonic size on larval development, larval growth (length and width), and rate of cloning. A median test was used to examine the timing of cloning and frequency of cloning was analyzed using a Two-tailed T-test. The effect of egg/embryonic size on time to and size at metamorphosis was analyzed with a one-way ANOVA. Where applicable, replicate beakers were modeled as random effects. To further examine trends in my data, I used the curve estimation function in SPSS to fit 11 models to my data: linear, logarithmic, inverse, quadratic, cubic, compound, power, S, growth, exponential, and logistic.

**Results**

*Egg size variability*

In 2012, spawning data from 66 females over four years were compiled to examine the variability of egg diameter within the species *Asterias forbesi*. This compilation contains both the females used during the presently discussed experiments in 2012 as well as previously collected data from 2011, 2010, and 2009. These data were not significantly different from a normal distribution (Fig. 5, Kolmogorov-Smirnov:}
Mean egg diameter across females ranged from 105.8 μm to 170.1 μm and the greatest range within the clutch of a single female was 141.4 μm to 212.1 μm.

The female that produced the largest variation in egg size had a CV of 8.98, which represents a 3.4x difference in egg volume from smallest to largest egg. The minimum CV (2.63) represents a female with a 1.6x difference within a single clutch. The mean CV for *A. forbesi* was found to be 5.68. The female I was able to use for my larval and juvenile experiments was above the average with a CV of 6.27. Going from the largest egg diameter of 135 μm to the smallest of 110 μm in this female, there is a 45.9% reduction in volume.

Because my sample size for *A. forbesi* was relatively large (66 females) I was able to conduct additional analyses in an attempt to correlate the variation I found with other measures. I used NERACOOS data nearest to the site where females were housed (Lower Harpswell sound, Buoy # D02, 10m depth) to understand the environment in which oogenesis had occurred. For each female, I averaged the temperature over the month prior to spawning, and then estimated the curve as done before. An inverse regression showed a statistically significant correlation between past temperature and diameter CV (Fig. 6; adjusted $R^2=0.082, R^2=0.095, \ P=0.009, y=2.07 + 40.8/x$). Though past temperature is a very significant predictor of CV, the $R^2$ value indicates that temperature only explains 9.5% of the variation observed. However, since these females were housed in a flow-through seawater system, they were not exposed to a single, constant environmental cue. Rather, they were exposed to the ambient fluctuations in temperature, salinity, pH, carbon dioxide, oxygen, nitrogen, and even microorganisms; therefore, I would not
anticipate a single factor to explain a large portion of the variation. The trend for temperature is small but significant and suggests that at colder temperatures, small decreases in temperature result in disproportionately large increases in variation. This result may be somewhat intuitive; in more harsh temperature environments, where nutrient availability is not as ensured, females will increase egg size variation.

Additionally, in an attempt to locate the source of variation in females, I compared the diameter CV of each female to her mean egg diameter, maximum egg diameter, and minimum egg diameter. Mean egg diameter had no correlation to diameter CV based on a linear regression ($R^2=0.000$, $P=0.897$). There was a significant positive correlation between diameter CV and the maximum egg size based on a linear regression (Fig. 7A; adjusted $R^2=0.068$, $R^2=0.082$, $P=0.016$, $y=0.0311x + 1.08$). Likewise, a linear regression revealed a significant negative correlation between diameter CV and the minimum egg size (Fig. 7B; adjusted $R^2=0.101$, $R^2=0.114$, $P=0.004$, $y=-0.0443x + 10.8$). More complex models, like the cubic and quadratic functions, fit the data better, but I can find no reason biologically to explain this pattern, so instead I considered the also significant case of a linear relationship. Taken together, these data indicate that females using a relatively uniform investment strategy produce eggs that approximate the mean for the species, rather than uniformly producing the largest eggs or uniformly producing the smallest viable eggs. Females with high CVs, exhibiting a bet-hedging investment strategy, produce eggs that are both larger and smaller than the mean.
for the species. Thus, rather than only reducing the size of eggs to create variation, these high CV females are also investing more in some eggs.

**Comparisons of initial volume**

The initial mean volumes of blastulae for size classes were highly significantly different (Fig. 8; Two tailed T-test: t=-45.3, df=12, P<0.001). The initial mean volume for large embryos was $0.0287 \pm 0.0001 \text{mm}^3$ and the initial mean volume for the small embryos was $0.0117 \pm 0.0004 \text{mm}^3$, which equates to a 2.46x difference in volume.

**Comparisons of growth and development**

Both larvae from large and small size classes reached a developmental stage indicating structural competency to settle. According to a repeated measures ANOVA with mixed models procedure, size class did not have an effect on developmental stage (Fig. 9; F$_{1,4}=2.5$, P=0.188). Maternal investment does not alter larval developmental rate. There was, however, a significant effect of maternal investment on both length (Fig. 10A, repeated measures ANOVA: F$_{1,4}=72.8$, P=0.001) and width (Fig. 10B, repeated measures ANOVA: F$_{1,3}=57.2$, P=0.004). Throughout growth, larvae from the large size class were longer and wider than those from the small size class (Table 1).

**Comparisons of cloning frequency**

Low levels of cloning began as early as two days post fertilization. However, after slow-growing larvae were removed, cloning did not recommence until 25 days post
fertilization. After removal of slow-growing larvae, the average number of larvae in large replicates (12.86±1.12) did not differ significantly from the number (13.71±0.87) of larvae in small replicates (Two tailed T-test: t=0.605, df=12, P=0.556).

Overall, both large and small larvae produced a similar number of clones (Fig. 11). Size class did not have a significant effect on the average cumulative number of clones produced per replicate over time (i.e. rate of cloning; repeated measures ANOVA: F_{1,12}=0.001, P=0.974). Additionally, large and small size classes did not significantly differ on the average total number of clones produced per replicate (i.e. frequency of cloning; Two tailed T-test: t=0.211, df=12, P=0.836). However, there was a four-day delay in initial clone production from the small larvae compared to their larger siblings. On day 25, 12.86% of large larvae had begun to clone, while only 2.14% of small larvae had cloned. Small larvae did not surpass 12% cloning until day 29, at which point both size classes were cloning at a similar frequency (25.00% of large and 28.57% of small). According to a median test, though, the size classes did not significantly differ on the day clones were produced (χ²=0.254, df=1, P=0.615), suggesting that small larvae ultimately compensated for an initial delay. Maternal investment affected the initiation of clone production but not the overall frequency nor rate of cloning.

**Observations of clone development**

Autotomy, both arm clones and bisections, was the primary mode of cloning observed. In the summer of 2011, during a preliminary experiment, both the
anterior and posterior portions of a bisected larva were followed. While in six days the posterior portion (esophagus and below) completely regenerated a larval mouth and oral hood, the anterior portion repaired the larval mouth. The anterior portion, however, did not regenerate lower body parts including a stomach. In a bisected larva from the summer of 2012, I did observe an anterior portion completely regenerate (Fig. 12). The bisection was first observed seven days post fertilization and within five days the anterior portion regenerated an esophagus, stomach, and coeloms. Within 12 days of initial appearance, the larva had grown dramatically, the coeloms were well developed and indicative of a stage-6 larva, and the stomach contained algae.

While only 1.9% of clones were bisections, 98.1% formed through autotomization of the brachiolaria, postoral, and posterolateral arms (diagramed in Fig. 13). I was unable to follow arm clones through development, though I did observe characteristics that would suggest development could proceed. I frequently observed bulges near the origin of cloning, where tissue may be rearranging (Fig. 14A). Additionally, while still attached to the primary larva, the lateral tip of an arm is densely packed with dark red-pigmented cells. Following autotomization, I often observed these pigmented cells migrating from the lateral tip towards the origin of cloning (Fig. 14B). Through this migration these cells resemble mesenchyme cells like those mentioned by Jaeckle (1994) in the cloning arms of other Asterias larvae. Lastly, I observed a potential mechanism through which autotomous arm clones may be able to enter normal development (diagramed in Fig. 15). I hypothesize that the pigmented mesenchyme cells redistribute throughout the individual, then
bulges form where tissue is rearranged until the individual enters a gastrula stage, at which point development could proceed normally.

*Comparisons at metamorphosis (first experiment—maternal effects)*

Juvenile size (widest point arm to arm) at settlement was measured in 34 juveniles from the small size class and 33 from the large size class. The average width of juveniles from the small size class was $872 \pm 19.5 \, \mu m$ and from the large size class was $878 \pm 17.3 \, \mu m$. Thus, juvenile size showed wide variation within size classes and did not significantly differ between the size classes (Fig. 16; one-way ANOVA: $F_{1,12} = 0.386, P = 0.546$).

Time to metamorphosis was noted in 61 juveniles from the small size class and 82 from the large size class. The average age at settlement of small larvae was $36.42 \pm 0.75$ days and $34.25 \pm 0.92$ days for large larva. Though the difference in these means represents a delay of 2.17 days in the small size class, this was not statistically significant (Fig. 17; one-way ANOVA: $F_{1,3} = 2.65, P = 0.127$). This value, however, is very conservative. Because this research was conducted over the summer at Bowdoin College in Brunswick, Maine, there was a time constraint of 42 days for larvae to develop and settle. Of the original 140 larvae per size class, 59% of the large larvae settled while only 44% of the small larvae settled (of those available after slow-growing larvae were removed this represents 64% of small larvae and 91% of large larvae).

The remaining small larvae on day 42 appeared healthy and competent to settle based on the presence of key settlement structures like brachioalaria arms,
adhesive disks, and juvenile rudiments. Since the time constraint biased the data to over-represent early-settling larvae from the small size class compared to average- and late-settling larvae, the average age at settlement does not accurately describe the timing of settlement in the small size class. In an attempt to remove this bias, I plotted the rate of settlement over time and examined the time to reach 50% settlement (Fig. 18).

Of the original 140 larvae per size class, the large size class surpasses 50% settlement (reaching 59%), but the small size class does not (44%). To examine the trend of small larval settlement and predict when this size class should have reached 50% settlement, I estimated the curve and found a simple linear regression to be the best fit based on the R² values. According to this linear regression, the large size class reached 50% settlement 39.48 days post fertilization (Fig. 18, adjusted R²=0.970, R²=0.972, P<0.001, y=0.735x -19.055) and the small size class is predicted to have reached 50% settlement 44.19 days post fertilization (Fig. 18, adjusted R²=0.982, R²=0.983, P<0.001, y=0.664x -19.396). Comparing the time to reach 50% settlement, these data predict an increase of 4.71 days (11.94%) in development time before settlement for small larvae compared to their larger siblings.

When early-settler bias is removed and the assumption is made that small larvae would have continued to settle, size class does have a significant effect on time to metamorphosis (one-way ANCOVA: F₁,₁₇₃=5.2, P=0.023). Additionally, though the slopes representing rate of settlement for these two size classes appear parallel, statistical analysis revealed a highly significant interaction between size class and age at settlement (one-way ANCOVA: F₁,₂₀₈=16.95, P<0.001). Not only did
larvae from the small size class delay settlement, but they also settle at a slower rate than those from the large size class.

**Juvenile morphometrics (second experiment—age at settlement effects)**

As was observed with juvenile size at settlement during the first experiment, the body size and shape of juveniles at settlement can be variable and thus not very informative beyond a rough comparison. In this experiment, since I desired more refinement for my comparisons, I chose to analyze the maximum size reached for any given morphometric. Maximum size refers to the largest juveniles reached before dying, thus maximum size was reached between 2 and 51 days post metamorphosis. In general, for each morphometric (juvenile width, arm length, spine length, and disc diameter), there was a trend for early-settlers to reach larger sizes compared to those settling later (Table 2). The data for each morphometric were analyzed through a curve estimation and a linear regression was found to be the best fit.

The maximum width of juveniles was significantly negatively correlated with age at settlement (Fig. 19A; adjusted $R^2=0.271$, $R^2=0.337$, $P=0.048$, $y=-6.0952x+1048.9$). Late-settling juveniles were unable to reach the sizes attained by earlier-settling juveniles. There also appeared to be a negative correlation between maximum spine length and age at settlement, though this was marginally non-significant (Fig. 19B; adjusted $R^2=0.236$, $R^2=0.305$, $P=0.062$). Early-settling juveniles also tended to reach longer maximum arm lengths compared to those settling later, but again these data were not significant (Fig. 19C; adjusted $R^2=0.217$, $R^2=0.288$,
P=0.072). Lastly, age at settlement had no effect on the maximum disc diameter reached (Fig. 19D; adjusted $R^2=0.050$, $R^2=0.136$, $P=0.238$).

Though statistical analysis shows that age at settlement does not have a significant effect on many of these morphometrics, the trends should not be ignored. Both spine length and arm length are only marginally non significant (P=0.062 and P=0.072 respectively) and the likely cause of this is a small sample size. Though I began with 200 larvae, only 12 larvae (6%) settled and grew as healthy juveniles. With a larger sample size, these trends may become significant. With such robust settlement from the first experiment (56% of large larvae and 44% of small larvae), I had not anticipated an issue in settlement. However, the females used for the second experiment were spawned almost two months later than those spawned for the first experiment and because it was later in the reproductive season, the eggs and larvae from those eggs may have been of lower quality. Many females injected did not spawn, and many of the eggs that were collected did not fertilize or did not develop.

**Juvenile survivorship**

Given that small larvae from the first experiment were older at metamorphosis but settled at the same size compared to larger siblings, I used statistical analyses to test the relationship between age at settlement and size at settlement on juvenile fitness. I estimated the curve of both age at settlement and size at settlement versus survivability. The best fit for age at settlement was a linear regression, which showed that there was no significant effect of age at settlement on survival time.
Those larvae settling earlier were neither at an advantage nor disadvantage for surviving as juveniles compared to those settling later.

The best fit for size at settlement (juvenile width at two days post settlement) was an inverse function, which revealed a statistically significant correlation between size at settlement and survival time (Fig. 20B; adjusted $R^2=0.246, R^2=0.304, P=0.041, y=68.380 -27100.931/x$). According to the fit of an inverse function, for relatively small juveniles, minor increases in juvenile size have a disproportionately large effect on survivorship. At greater sizes at settlement the curve begins to plateau, such that for larger juveniles, the advantage of increasing size is not as effective. These data are congruous with my earlier results from the first experiment. Since age at settlement does not affect survivability and size at settlement does, a small larva should invest its energy in increasing in size without regard for an increased larval period.

**Discussion**

This study is the first to examine maternal effects on cloning frequency in any marine invertebrate and the first, within the phylum Echinodermata, to examine the costs to larvae and juveniles of naturally rather than artificially reduced energy reserves. Additionally, this work is also the first to describe the relationship between maternal investment and larval and juvenile development in sea stars. Lastly, these are the first data demonstrating, rather than predicting, the use of a
bet-hedging strategy in *A. forbesi*; both the largest and the smallest eggs within a clutch are viable.

Specifically, my results indicate that maternal investment did not affect larval cloning, such that small and large larvae cloned at similar frequencies. Likewise, maternal investment did not have an effect on developmental rate. However, there was a strong effect of maternal investment on size (length and width) throughout the larval period, such that small embryos produce smaller larvae. This effect of maternal investment on size was no longer present following metamorphosis, at which point juveniles derived from large and small larvae were similar in size.

Maternal investment also did not have an effect on time to metamorphosis, though analysis of the pattern of settlement suggests a significant delay in time to metamorphosis by small larvae. When controlling for maternal investment, the time to metamorphosis does not affect the quality of a juvenile (survivability); early and late settlers showed similar survival. More important in juvenile quality is the size at settlement of a juvenile, which is positively correlated with survival, thus larvae that settled as larger juveniles tended to survive longer.

*The relationship between maternal investment and larval cloning*

Though my results indicated that there was no relationship between maternal investment and larval cloning frequency, I suspect an effect exists but was masked in my study due to the experimental design. In an attempt to create conditions optimal for survival and growth, I may have unintentionally provided enough high-quality exogenous energy in the form of particulate food to overcome the role of
maternal investment in larval cloning. Vickery and McClintock (2000) showed that, in comparison to single alga diets, larvae of the seastar *Pisaster ochraceus* clone at higher frequencies when fed a mixture of both a brown and green microalga. Additionally, McDonald and Vaughn (2010) found that pulses of high levels of food induced cloning events in the larvae of the sand dollar *Dendraster excentricus*. With the hopes of replicating the high levels of cloning seen in the above two studies, larvae were fed abundantly and given a variety of algae. I suspect that energy reserves affect a larva’s ability to clone; however, the constant presence of a rich diet in my study may have allowed small larvae to overcome an initial energy deficit through externally acquired energy. The role of maternal investment in larval cloning may become more pronounced if larvae are reared in low food treatments, though sufficient to reach metamorphosis, such that they experience mild nutritional stress and are forced to rely on endogenous reserves for cloning behaviors.

Exogenous cues are easy to manipulate and are thus often examined in the literature (Brock, 1975; McEdward, 1985; Roller and Stickle, 1985; Guisande et al., 1996; Phillips, 2002; Chen et al., 2009; Allen and Pechenik, 2010; Armstrong et al., 2013). In addition to the food cues discussed above, Vaughn and Strathmann (2008) found that another inducer of cloning in *D. excentricus* larvae is the presence of fish mucus as a larval predator cue. Four-day old larvae were isolated in shot glasses, where they were either exposed to a control treatment of only seawater or seawater with dilute fish mucus. While 0% of control larvae cloned, 10-40% of individuals exposed to predator cue cloned within 24 hours. Vaughn and Strathmann (2008)
hypothesize that the reduction in size associated with cloning can be an advantageous response to visual predators, like post-larval fish.

While larval cloning in echinoderms, generally, is not well studied nor understood, only one study has ever examined the potential of an endogenous, rather than exogenous, factor. In 2009, Dawn Vaughn explored the effect of intraspecific variation on larval cloning frequency in the sand dollar *D. excentricus*. Vaughn (2009) created 'half sibling' larvae by fertilizing three separate females with a single male and then examined the larval response to a known exogenous cue (i.e. fish mucus). The results suggest there is intraspecific variation in the timing, incidence, and success of cloning (Vaughn, 2009). However, no previous study has explored the possibility of a non-genetic cue within the egg— one that can be altered naturally— such as energy reserves. While an effect of maternal investment on overall cloning frequency and rate was not observed in my study due to masking by exogenous energy, the remnants of such an effect manifested in a delayed initiation of clone production by small larvae. Thus, there is evidence for a non-genetic endogenous cue that can affect the frequency of cloning and further studies should be conducted to reveal this.

*The relationship between egg size and development*

In marine invertebrates, the relationship between egg size, development time, and juvenile size has been studied exclusively in echinoid echinoderms. Prior to this study, similar data for asteroid echinoderms did not exist and, therefore, it was unknown whether the extensive work with echinoids would predict the relationship
in asteroids. In echinoid echinoderms, the costs of egg size reduction, such as increased larval period, decreased juvenile quality, decreased feeding rates, and in some species decreased juvenile size, have been well documented in the literature (Hart, 1995; Sinervo and McEdward, 1988; Emlet and Hoegh-Guldberg, 1997; Alcorn and Allen, 2009; Allen, 2012). In particular, eggs artificially reduced by 50% in volume have been shown to experience increased development time (Allen, 2012). When blastomeres of the sand dollar *Dendraster excentricus* (124.2 μm mean egg diameter), are separated at the two-cell stage, the result is a 12.27% increase in the time taken to reach metamorphosis (Allen, 2012). The mean egg diameter from the female *A. forbesi* used in the present study was 123.8 μm, which is similar in size to *D. excentricus*. According to the calculations described by Allen (2012), if asteroids follow the egg size-development time trend of echinoids, it is predicted that the small larvae in the present study should experience a 12.26% increase in time to reach metamorphosis. And similar to prediction, in *A. forbesi*, I found an increase of 11.95% in development time. Though more work is needed, asteroids might conform closely to the echinoid trend in regards to development time and thus there may be a potential for the relationship in echinoids to apply more broadly to other echinoderm families.

*The latent effects of maternal investment*

Hart (1995) has suggested that small eggs may be at a disadvantage for more than merely an increased larval period. Hart (1995) describes five potential costs for development from small eggs, including decreased fertilization rates, decreased
feeding rates, increased predation rates, decreased juvenile size, and unsuitability for development in heterogeneous environments. In regards to juvenile size, based on the echinoid trend, *A. forbesi* is predicted to decrease by 9.81% (Allen, 2012).

Interestingly, though, my work found no relation between maternal investment and size at settlement. Asteroids resemble echinoids in the relationship between egg size and larval development, but not juvenile size. While echinoid larvae derived from 50% reduced embryos extend their larval period by 12%, this is not enough to completely overcome the initial deficit in endogenous reserves, and the resulting juveniles are 9% smaller. Asteroid larvae derived from naturally reduced embryos, however, extend their larval period by 12% in an efficient way, such that they do overcome the initial deficit and produce equal sized juveniles.

Larval experiences, specifically the amount of energy incorporated in the body and duration of larval period, have direct implications for juvenile performance (Pechenik et al., 1998) and thus there may exist latent effects of maternal investment on juvenile quality. The small larvae in this study extended the larval period and produced juveniles of comparable sizes to their larger siblings, but it is unclear what the costs/benefits of that strategy are. By creating size classes of only large larvae, I removed maternal investment as a variable and was able to examine the effects of only size at metamorphosis and time to metamorphosis on the quality (namely survivorship) of a juvenile.

While, in some species, juvenile size is an appropriate proxy for juvenile quality (Gosselin, 1997; Phillips, 2002), it has been noted in echinoid echinoderms that we do not know enough about juvenile ecology to label predictors of juvenile
quality (Allen, 2012). Similarly, the relationship between juvenile size and quality in asteroids is not well studied; though one study of the brooding seastar Leptasterias aequalis found no significant correlation between juvenile size and survivability (Gehman and Bingham, 2010). In my work, I found that size at settlement is, in fact, a significant measure of juvenile quality. Size at settlement was positively correlated with survivorship as described by an inverse function, therefore, minor increases in the size of a small juvenile have disproportionately large effects on survivorship.

One way to increase in size before settlement is by remaining in the plankton for additional time to feed, but an extended larval period could, in itself, be a deterrent from increasing size. For example, the larvae of the sand dollar Mellita quinquesperforata have been shown to delay metamorphosis for two weeks in the absence of a suitable substrate, but the percent of larvae that can complete metamorphosis (i.e. the quality of the larvae) declines in that time (Caldwell, 1972; Strathmann, 1977). In the present study, time to metamorphosis ranged from 22 to 68 days but was not correlated with survivorship; thus, there was no disadvantage to a seastar larva in settling up to six and a half weeks later than the first juvenile. The results that size at settlement is correlated with post-metamorphic fitness and that age at settlement is not support the findings in my original experiment on maternal effects. Individuals derived from smaller eggs extended the larval period and reached a size at settlement comparable to individuals derived from larger eggs. Combining the results from both of my experiments, it makes biological sense that a larva would delay metamorphosis (i.e. disregard age at settlement) until it was able to produce a larger (higher quality) juvenile.
However, these results were strictly from a lab setting and, as I mentioned earlier, a strong focus of eco-evo-devo research is analyzing strategies in ecologically relevant settings. In the field, where larvae would experience additional environmental cues, I would anticipate a more variable response. One possible trade-off between waiting to increase in size rather than settling early is the likelihood of increased predation risk. Therefore, while the extended larval period is not a deterrent from increasing size, the threat of predation might be. For example, studies in amphibians have shown that larval predator cue induces larvae to metamorphose at a smaller size (Relyea and Hooverman, 2003; Vonesh and Warkentin, 2006), suggesting that these larvae may metamorphose earlier as a way to escape larval predation. It would be interesting to see if the addition of planktonic predator cue to the environments of small larvae could override the advantage of settling at a larger size and push larvae to settle earlier. It has already been shown that echinoderm larvae can respond to predator cue with drastic body changes (Vaughn and Strathmann, 2008). As mentioned earlier, young sand dollar (*D. excentricus*) larvae exposed to a planktonic predator cue may respond by cloning and thus greatly decreasing in size (Vaughn and Strathmann, 2008). However, these larvae were exposed to predator cue at four days old and they require 35 to 49 days of development before *D. excentricus* larvae become competent to settle (Chia and Burke, 1977). Therefore, the same planktonic predator cue may induce metamorphosis, rather than cloning, in later stage echinoderm larvae.

The experiences and life history strategies during the larval period are not erased at metamorphosis, but rather Pechenik et al. (1998) demonstrated that the
experiences and strategies exist as latent effects on juvenile quality. For example, larvae of the slipper shell, *Crepidula fornicata*, that are starved for the first three days of their larval stage have slower growth rates later in life as juveniles (Pechenik et al., 1998). Thus, while it remains true that “metamorphosis is not a new beginning” in an individual’s life cycle (Pechenik et al., 1998), my data suggest that individuals may have evolved plasticity during larval development (e.g. delay metamorphosis to increase juvenile size) that can minimize earlier disadvantages (e.g. reduced maternal investment) upon metamorphosis and produce juveniles of equal fitness.

*Bet-hedging strategies*

Prior to this study, it had not been shown that small eggs from the variable clutches of *A. forbesi* were viable rather than immature or poor quality oocytes. Small eggs may have lacked the capacity to fertilize, develop into larvae, form functional feeding and digestive structures, form settlement structures, initiate metamorphosis, or metamorphose successfully (i.e. into the correct juvenile body form). To begin, however, I designed and conducted my study based upon the assumption that females with variable clutches employ a bet-hedging strategy and that development of small eggs would proceed. A particularly valuable result from my work is that the variable eggs from *A. forbesi* are viable. These observations and the extension that *A. forbesi* does employ a bet-hedging strategy lends empirical credit to the models proposed by Olofsson et al. (2009), in which bet-hedging was shown to be an evolutionarily stable strategy. However, more work is needed to the
show the heritability of egg size variation. Very few studies in only one species (the serpulid polychaete *Hydroides elegans*) have shown that egg size can be heritable and selectable (Miles et al., 2007; Miles and Wayne, 2009), but of those, none have examined the potential heritability of egg size variation rather than mean egg size.

Unlike my study, however, numerous studies report bet-hedging and egg size variability data without assessing the viability of the eggs (Turner and Lawrence, 1979; Marshall et al., 2000; Marshall et al., 2008; Crean and Marshall, 2009). For example, Plaistow et al. (2007) tested the effect of maternal environment on the size of naturally spawned eggs in the soil mite *Sancassania berlesei*. Using the raw data from Plaistow et al. (2007), Crean and Marshall (2009) analyzed the effect of maternal environment on the variation in size of the naturally spawned eggs. The results suggest that mothers in a changing environment produce more intra-clutch variation over their lifetime compared to mothers in a constant environment (Crean and Marshall, 2009); however, beyond diameter measurements, these eggs were not followed. Without data demonstrating the viability of the eggs throughout the size range (i.e. fertilization, growth, development), it is inaccurate to assume that the female mites in a changing environment are using a bet-hedging strategy, or even producing egg size variation rather than poor quality oocytes.

More so than natural spawning, artificial spawning can bias a study towards more variable clutches and more small ‘eggs’ (i.e. immature oocytes). One of the most common spawning techniques in echinoid echinoderms is the injection of a muscle stimulant (KCl; Strathmann, 1987). However, such a crude method of gamete release does not discriminate between eggs that are mature within a female and the
smaller immature oocytes. Similarly, the use of 1-methyladenine in asteroids and strip spawning in any organism can be sources of unnatural variation. For studies examining differences in egg size, such as my own, it is important to acknowledge the potential for smaller eggs and egg size variation to be an artifact of the collection methods. Thus, to report data in the context of bet-hedging or egg size variation, studies must show the viability and development of offspring.

Variation in egg sizes and the strategy of bet-hedging are not exclusive to A. forbesi. Marshall et al. (2008) compared the intra- and inter-clutch variation in offspring size across 23 species of marine invertebrates. They found that bet-hedging tends to occur more in females with planktotrophic (feeding) larvae, which are more likely to be exposed to unpredictable environments, than in females with lecithotrophic (non-feeding) larvae, which do not rely on the environment for nutrition. In the course of conducting my thesis work, I compiled comparative data that suggest the opposite is true. The results of Marshall et al. (2008) may not be accurate because they draw conclusions regarding egg size variation in relation to developmental mode based on inappropriate calculations.

Marshall et al. (2008) report different measures of variation across species (i.e. coefficient of variation of diameters and coefficient of variation of volumes) and it is, therefore, impossible to use these data for a direct comparison. A coefficient of variation calculated from volume will have a far larger value compared to that calculated from the diameters of the same set of data. For instance the average CV for diameter of the 66 Asterias forbesi females for which I compiled data was 5.68, while the average CV for volume was 17.08. It is therefore not accurate to claim that
a species with a CV for volume of 6.00 has greater variation in egg size than a species with a CV for diameter of 5.68. If a developmental mode category is ‘averaged’ with values of volume CV and diameter CV (as is the case in Marshall et al., 2008), the results will be biased towards a false report of greater variation in species with that developmental mode. It is only accurate to obtain an average based on either diameter CV alone or volume CV alone. Because the CV for diameter and the CV for volume ultimately are measures of different units, the two cannot be averaged.

To examine the relationship between CV and developmental mode and to make a more direct comparative study of intra-clutch variation across species, I compiled my own egg size data and contacted the authors of multiple papers referenced in Marshall et al. (2008). From these authors I retrieved raw egg size data so that I could analyze and calculate CV appropriately. I chose to compare diameter CV rather than volume CV because more data was available in this format. Similar to the requirements laid out in Marshall et al. (2008), I only included CV data for species for which I had at least two representative females to average. My compilation also includes only species with spherical eggs because the diameter CV and volume CV of other shaped eggs does not conform to that observed in spherical eggs. For example, I examined data from 5 females of the seastar *Echinaster spinulosus*, which has a prolate spheroid shaped egg and volume described by $V = \frac{4}{3}\pi(L/2)(W/2)^2$, where $V$ is volume, $L$ is length, and $W$ is width. According to the diameter CV (6.75), *E. spinulosus* is far more variable than *A. forbesi* (diameter CV=5.68). However, according to the volume CV of *E. spinulosus* (11.89), this species
is far less variable than *A. forbesi* (volume CV=17.08). Because of this incongruity, I chose not to include data from species with non-spherical eggs.

When comparing across 12 species of planktotrophic echinoderms, I found the average diameter CV for intra-clutch variation to be 4.12±0.40 (Fig. 21A). Recall the diameter CV for *A. forbesi* is 5.68, which is well above the average variation observed in other planktotrophic echinoderms. In fact, the only species that was found to have greater variation was another member of the genus *Asterias*, *A. rubens*. *A. forbesi* and *A. rubens* are such close relatives that they readily form hybrids in the lab (Clark and Downy, 1992) and the field (Harper and Hart, 2005). By some definitions of a biological species, it is inappropriate to separate these into two species, and thus *A. forbesi* would be more variable than every other planktotrophic echinoderm examined.

Comparing across seven species of lecithotrophic echinoderms, the average diameter CV was found to be 5.57±1.20 (Fig. 21B). I found no statistical difference between the mean CV for planktotrophic versus lecithotrophic species (Two tailed T test: t=-1.45, df=17, P=0.165), whereas Marshall et al. (2008) had used most of the same data and found planktotrophic species to have significantly higher CVs (likely, an artifact of inappropriate calculations). According to my data, if there is any trend, it is for lecithotrophic to be more variable (though the low sample size of 6 may have affected these data). Comparing across all echinoderms, *A. forbesi* (diameter CV=5.68) is more variable than the average echinoderm with a CV of 4.69±0.53. Lastly, when comparing across all the 30 marine invertebrate species available in my comparative study (Table 3), which includes feeding and non-feeding of both
echinoderms and other phyla, the average diameter CV is 5.32±0.51. So, again, *A. forbesi* (diameter CV=5.68) represents a species that has more intra-clutch variation than the average marine invertebrate (Fig. 21C). Overall, *A. forbesi* is more variable than 69.7% of marine invertebrates, 77.8% of echinoderms, and 90.9% of planktotrophic echinoderms. These data combined with the ease of adult collection from the field, ease of gamete collection from the adult, and well-documented development make *A. forbesi* a model system for future studies of bet-hedging and maternal investment.

Though I have discussed bet-hedging mostly in the context of an adult investment strategy, it is incorrect to restrict bet-hedging to adult females. For example, cloning is a form of larval bet-hedging. As earlier work has suggested, larvae may clone in response to high predator density (Vaughn and Strathmann, 2008), high food quality (Vickery and McClintock, 2000), and food abundance (McDonald and Vaughn, 2010). Such cued cloning may be a strategy to navigate the tradeoff between expending energy in a clone and increasing the likelihood of one’s genetics survival. Thus, rather than placing bet-hedging solely within the context of an adult strategy for eggs, we should acknowledge its potential employment throughout the complex life cycle of an organism.
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I would like to first thank my advisor, Jonathan Allen, to whom I owe a great debt for taking me into his lab and seeing potential in me. Without his mentoring, which pushed me to pursue research more extensively in my undergraduate career, none of my successes or accomplishments in research would have been possible. I am deeply grateful to him for three years of endless dedication, guidance, support, and, most of all, encouragement. My research experiences have been priceless and I sincerely thank you for everything, Jon.

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References


Figures

Figure 1: Theoretical relation between the coefficient of variation in maternal fitness across generations (e.g. higher coefficient implies more uncertainty in maternal success, mean ± 95% confidence limits) and the coefficient of environmental variation for females employing a uniform reproductive strategy (closed squares, i.e. female A) and females employing a bet-hedging strategy (open squares, i.e. female B). Dotted line represents the lowest environmental variation at which bet-hedging is advantageous within a generation. Reproduced from Marshall et al., 2008.
Figure 2: *Asterias forbesi* larvae undergo asexual reproduction through A) budding, B) autotomous arm cloning, and C) autotomous bisections. The larva in A is a late stage bipinnaria larva, which will later develop into an early stage brachiolaria larva like that pictured in B. Arrowheads indicate larval mouth, arrows indicate esophagus, and asterisk denotes a budding propagule. Scale bars = 100 μm. Fig. 2A reproduced by permission from Ian Haight and Jon Allen, unpublished data.
Figure 3: Diagram of larval measurements for width (W) and length (L). Scale bar = 250 μm.
Figure 4: Diagram of juvenile measurements for juvenile width (1), spine length (2), longest arm (3), and body/disc diameter (4). Scale bar = 500 μm.
Figure 5: Distribution of coefficient of variation from egg diameter (CV) across *A. forbesi* females. The data do not significantly differ from a normal distribution (Kolmogorov-Smirnov; P=0.200). The minimum sample size used to calculate CV was 15 eggs. The mean CV is 0.0562.
Figure 6: Coefficient of variation in *Asterias forbesi* egg size in relation to water temperature over one month prior to spawning. An inverse regression describes a significant effect of temperature on CV ($y = 2.07 + 40.8/x$, adjusted $R^2 = 0.082$, $R^2 = 0.095$, $P = 0.009$). Each data point represents a single female ($n = 66$).
Figure 7: Coefficient in variation in *Asterias forbesi* egg size in relation to maximum egg size produced (A) and minimum egg size produced (B). According to linear regressions, CV was significantly correlated with both maximum egg size ($y=0.0311x + 1.08$, adjusted $R^2=0.068$, $R^2=0.082$, $P=0.016$) and minimum egg size ($y=-0.0443x + 10.8$, adjusted $R^2=0.101$, $R^2=0.114$, $P=0.004$). Each data point represents a single female.
Figure 8: Initial mean volume of blastulae for large (gray, n=140) and small (white, n=140) size classes. The large size class blastulae are significantly larger than the small size class blastulae (Two tailed T-test: t=-45.3, df=12, P<0.001). Bars are mean ± SE.
Figure 9: Average larval developmental stage over time for large (closed circle) and small (open circle) size classes. Size class does not affect developmental stage (Repeated measures ANOVA: $F_{1,4}=2.5$, $P=0.188$). Staging as described by George, 1999. A developmental stage of 15 indicates competency to undergo metamorphosis. Data points represent mean ± SE.
Figure 10: Average larval length (A) and width (B) over time for large (closed circle) and small (open circle) size classes. Large larvae were significantly longer (Repeated measures ANOVA: $F_{1,4}=72.8, P=0.001$) and wider (Repeated measures ANOVA: $F_{1,3}=57.2, P=0.004$) than small larvae. See Fig. 3 for diagram of measurements. Data points represent mean ± SE.
Figure 11: Cumulative clone production in large (closed circle) and small (open circle) size classes. Size class had no effect on rate of cloning (Repeated measure ANOVA: \( F_{1,12}=0.001, P=0.974 \)), nor frequency of cloning (Two tailed T-test: \( t=0.605, df=12, P=0.556 \)). Average cumulative number of clones refers to the average number of clones identified and removed per 20-larvae replicate. Small larvae delayed initiation of clone production from 25 days post fertilization in the large size class to 29 days post fertilization. Data points represent mean ± SE.
Figure 12: Development of the anterior portion of a bisected larva over 12 days. Oral hood was first observed on day 7. Arrowhead indicates larval mouth. Arrow indicates the reappearance of the coeloms. Notice the dark color of the stomach on day 19, indicating the presence of algal food. All images are at the same scale. Scale bar = 100 μm.
Figure 13: Diagram of a late stage brachiolaria larva. Across larvae, autotomous cloning has been observed at the brachiolaria arm (1), preoral lobe (2), postoral arm (3), and posterolateral arm (4). Notice the relatively dense aggregation of pigmented cells in the lateral tip of all arms. Scale bar = 500 μm.
Figure 14: Signs of continued development in autotomized posterolateral arms. Tissue rearrangement (A) likely occurred within the frequently observed bulges (arrow). These bulges were located near the origin of cloning (asterisk). Pigmented (mesenchyme) cell migration (B) occurred in the direction from the lateral tip towards the origin of cloning (denoted by direction of arrow). Arms were released from the primary larva with a dense aggregation of pigmented cells (arrowhead) in the lateral tip. Scale bar = 100 μm.
Figure 15: Diagram of the hypothesized mechanism through which arm clones enter normal development. An autotomized arm will first undergo pigmented (mesenchyme) cell migration and redistribution (A). Then, a bulge forms near the origin of cloning (B). As tissue rearranges, the arm becomes more rounded and oval (C). The arm continues to rearrange and begins to form a blastopore (D) and lastly, the arm enters a gastrula stage (E). Each image is an individual clone that was observed. Arrow indicates a presumptive blastopore. Scale bar = 100 μm.
Figure 16: Juvenile width at settlement for large (gray, n=33) and small (white, n=34) size classes. There was no significant difference between the width of juveniles from small compared to large size classes (One-way ANOVA: F_{1,12}=0.386, P=0.546). Bars are mean ± SE.
Figure 17: Average age at settlement for large (gray, n=82) and small (white, n=61) size classes. Though there appears to be a difference of 2.17 days, this is not statistically significant (One-way ANOVA: $F_{1,3}=2.65$, $P=0.127$). Bars are mean ± SE.
Figure 18: Cumulative juvenile settlement from large (closed circle) and small (open circle) size classes. The dashed line indicates 50% settlement. The large size class reaches an average maximum of 59% of larvae settled per replicate and the small size class reaches an average maximum of 44% of larvae settled. The data were fit with a linear regression to analyze the time to reach 50% settlement in the large size class and predict the time to reach 50% settlement in the small size class. The large size class reaches 50% settlement at day 39.48 (Linear regression: $y=0.746x-19.451$, adjusted $R^2=0.969$, $R^2=0.971$, $P<0.001$) and the small size class is predicted to reach 50% settlement at day 44.19 (Linear regression: $y=0.671x-19.667$, adjusted $R^2=0.982$, $R^2=0.983$, $P<0.001$). There is a significant effect of size class on time to metamorphosis (One-way ANCOVA: $F_{1,173}=5.2$, $P=0.023$) as well as a significant interaction between size class and age (One-way ANCOVA: $F_{1,208}=16.95$, $P<0.001$). Data points represent mean ± SE.
Figure 19: Trends of the maximum size reached for various juvenile morphometrics compared with age at settlement. Juvenile width (A) is significantly negatively correlated with age at settlement (Linear regression: \( y = -6.0952x + 1048.9 \), adjusted \( R^2 = 0.271 \), \( R^2 = 0.337 \), \( P = 0.048 \)). The effect of age at settlement on spine length (B) and arm length (C) is non significant (\( P = 0.062 \) and \( P = 0.072 \) respectively), though both data sets show a trend for a negative correlation. Age at settlement also has no significant effect on disc diameter (\( P = 0.238 \)). Each data point represents an individual juvenile.
Figure 20: Juvenile survivability in relation to age at settlement (A) and size at settlement (B). Age at settlement had no effect on survival time ($P=0.630$). Size at settlement (juvenile width two days post settlement) is best fit with an inverse function and has a significant effect on survival time (adjusted $R^2=0.246$, $R^2=0.304$, $P=0.041$, $y=68.380 - 27100.931/x$). Each data point represents an individual juvenile.
Figure 21: Distribution of coefficient of variation for diameter across species and developmental modes. Comparison across planktotrophic echinoderms (A), all echinoderms (B), and all marine invertebrates (C). Each data point represents the average of at least two females. In all panels: Black bars represent planktotrophic species. White bars represent lecithotrophic species. Gray bar indicates *Asterias forbesi*. Black arrowhead denotes average planktotrophic echinoderm CV (4.12). White arrowhead indicates average lecithotrophic echinoderm CV (5.57). Arrow indicates average CV for all marine invertebrates (4.69).
Tables

Table 1: Repeated measures ANOVA with mixed models procedure results table for the effect of initial egg/embryonic size class (small or large) on the dependent variables length and width. Replicate was included as a random effect.

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<td>Width</td>
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Table 2: Linear regression results table for the effects of age at settlement on juvenile morphometrics.

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<th>Subjects</th>
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<td>Disc diameter</td>
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Table 3: Summary table of the 30 species of marine invertebrates used in the intra-clutch variation study. P = planktotrophic/feeding larval stage, L = lecithotrophic/non-feeding larval stage.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Species</th>
<th>Development mode</th>
<th>Diameter CV within a clutch</th>
<th>Study</th>
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<td>Reference</td>
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