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PATTERNS OF SEX DETERMINATION IN THE SCALY PEARL OYSTER IN FOUR ANCHIALINE PONDS ON SAN SALVADOR ISLAND, BAHAMAS

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ABSTRACT

Populations of scaly pearl oyster (*Pinctada longisquamosa*) have been identified in five anchialine ponds on San Salvador Island in the Bahamas. These ponds offer habitat that varies considerably from one pond to another. Conducting histology on oysters from four of these ponds has allowed us to characterize their life history trajectories as they progress from immature, to sexually mature, and as they undergo gender reassignment during aging (Cole, et al., 2007). Demographic data (Swanson, et al., 2007, Carlson, et al., 2009, this symposium), have allowed us to determine the age of sexual maturation and sexual reversal. Other oysters of the *Pinctada* genus have been characterized as protandrous hermaphrodites, beginning life male and switching to female as they age (Southgate & Lucas 2008). Our most recent evidence suggests that sex determination in *P. longisquamosa* follows different trajectories within the different ponds, from pure protandry, to a pattern of alternating sex reversals, and ultimately to a pattern resembling gonochorism. We comment on the possible causes of this variation in life history traits.

INTRODUCTION

Among aquatic invertebrates, and especially among the more sessile, marine invertebrates, breeding occurs through release of gametes into the water column during episodes of group spawning. Consequently there are no dominance contests among adults for breeding privileges, nor much in the way of conspicuous mate selection. The main selective advantage to be gained lies in the ability to express large numbers of fertilizable

gametes during strategically timed spawning events. Since eggs are energetically costly to manufacture, small females may be at an inherent reproductive disadvantage to large females, though males show less size disadvantage (Wright 1988, Ghiselin, 1974). For these reasons, it has been argued, marine bivalves frequently exhibit either protandrous hermaphroditism, in which individuals begin life male and switch to female as they mature, or a pattern of alternating hermaphroditism in which individuals begin male, and undergo multiple episodes of switching between male and female at various times in their life (See Hoagland 1984). The protandrous pattern allows small individuals to optimize their reproductive output by adopting the energy-misering process of spermatogenesis and switching to oogenesis only after attaining sufficient biomass to be competitive with more mature (and larger) egg-producing females. This pattern has been documented in numerous mollusks (See Hoagland 1978, Charnov, 1982, Heller, 2008, Southgate & Lucas, 2008).

Sex determination itself can be either genetically determined or exhibit environmental lability (Bull 1983). Environmental triggers for gender assignment include temperature, intraspecific density, host size (in parasitic nematodes), food availability, substrate quality and conspecific social interactions (Bacci 1965; Bull, 1980). In many cases of environmental sex determination, gender is determined within a critical developmental period, usually during embryogenesis or larval development (Janzen & Phillips, 2006). In such examples, gender assignment is usually a binary decision made only once early in development. In other cases, there can be an initial sex determining event occurring early in develop-

ment, followed by sex reversals occurring later in life and triggered by environmental stimuli such as population density, food abundance, and even the gender composition of the adult population (Coe, 1936, Warner, et al., 1996, Proestou, et al., 2008). The animal world provides examples ranging across a broad scale of stability and lability with genetically determined, inflexible sex-determining mechanisms occurring at one end of the spectrum, and labile, environment-sensing mechanisms driving adaptive sex-reversals at the other extreme.

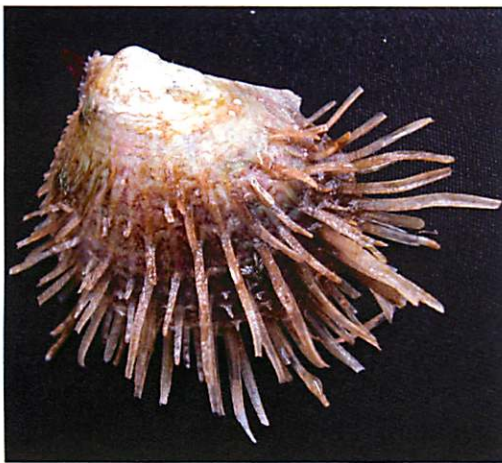


Figure 1. *Pinctada longisquamosa*.

We have been studying life history traits including growth rate, reproductive maturation and sex determination in the scaly pearl oyster *Pinctada longisquamosa* (Figure 1) located in a number of isolated saltwater ponds on the island of San Salvador in the Bahamas. In three of our ponds we've observe three distinctly different patterns of sex determination ranging from protandry to alternating sex reversal, and finally to a condition resembling a gonochoristic (two-sex) state. There is no published data on sex determination in this species other than our own (Cole, et al., 1997), although related, commercially valuable species of *Pincatada* have a substantial literature suggesting either formal protandry, or alternating hermaphroditism are the norms (Saucedo & Southgate 2008). This dramatic variation in life history trajectory offers a rich model for exploring the relative roles of genetics and environment in determining patterns of sex determination.

The Ponds

The three ponds we initially identified on San Salvador Island that host our research organism (Figure 2) represent three points along a spectrum from fully marine to modestly hypersaline. Oyster Pond is shallow (1-1.5 m) and has substantial conduits supporting a 20-30 cm tidal flux with a 50 minute tidal lag. Over 12 years of moni-

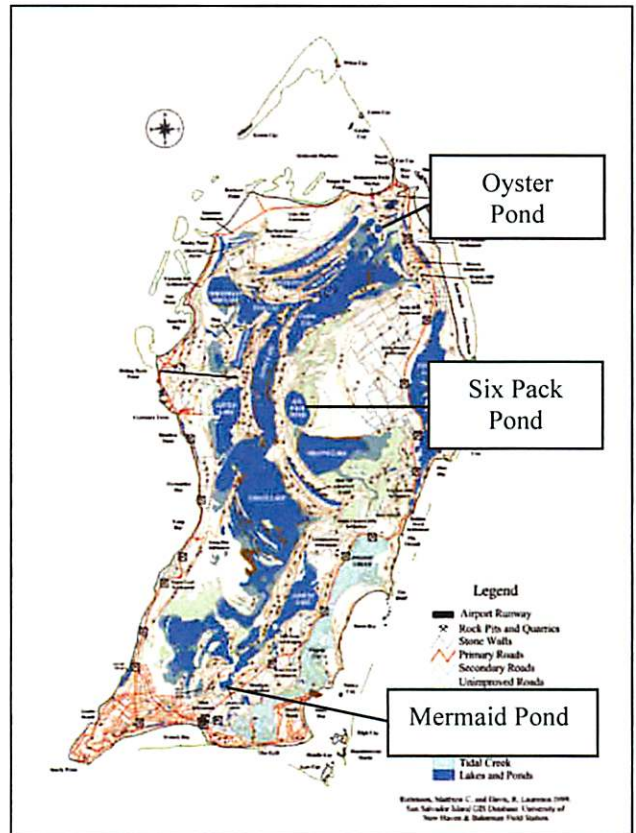


Figure 2. San Salvador Island and study ponds harboring *Pinctada* populations under study.

ing, salinity has varied only slightly from a fully marine condition (35 g/L TDS). In this regard, it is our most stable pond environment restoring its marine salinity quickly from even the most punishing storm events. It is rich in biodiversity, has a small surface area (71,000 sq. meters) and a thick red-mangrove prop-root habitat around its margins. All these features make it an ideal shelter against storm perturbations, and indeed, 4 months following Hurricane Frances, we saw very little damage to its native oyster population.

Mermaid Pond is similarly shallow (1-1.5 m) yet has more congested conduits as evidenced by a nearly three hour tidal lag. Its long-term salinity varies significantly more than Pond 1, though it still maintains a rich biodiversity. It also has a small surface area (104,000 sq meters) and a substantial red mangrove prop-root margin. It seems slightly less protected against hurricane-mediated disturbances than Oyster Pond, in that we see modest population loss, particularly of juvenile oysters, following significant storm events.

Six Pack Pond is the most extreme habitat that our oysters seem able to tolerate. It has a large surface area, (762,000 sq meters) permitting high-energy wave generation during storms, it lacks a protective prop-root habitat, and has no measurable tidal flux. The salinity typically hovers between 45-47 g/L, though following Hurricane Noel we saw it drop below 20 g/L. Its saving grace (from the oysters' perspective) is that it is a deep pond (4 meters in the center with a soft-sediment bottom) though the adult oyster population is confined to algal beds growing in a shell-hash/ carbonate bottom around the shallow perimeter. We suspect that this depth creates a protective halocline during storm-deluges, allowing oysters (or their larvae), refuge from freshwater inundation. We have evidence that particularly severe hurricanes periodically annihilate the adult population while triggering spawning, (an event we refer to as "suicide spawning") yet the planktonic larvae somehow survive extermination of the adult population and repopulate the shallows after evaporative restoration of salinity.

Our initial studies have shown that the conduit-driven tidal ponds (Oyster & Mermaid) have less nutrient and organic carbon suspended in the water column than land-locked 6-Pack Pond (Cole, et al., 2007). 6-Pack pond also supports a richer algal plankton. This makes sense in that the surrounding ocean water that periodically flushes the conduit-driven Ponds is highly oligotrophic, whereas Six-Pack Pond drains a substantial bit of forestland with no visible conduits or outlets connecting it to the sea. This creates conditions that effectively concentrate nutrients.

The Oysters

The Oysters of Oyster Pond show a wide size distribution (Carlson, et al., 2009), exhibit rapid growth, and yet are relatively slow to mature (See below). Over a series of successive non-hurricane years, we observed the population growing increasingly "older", losing juveniles, and showing a progressive upward shift in mean size. This pond harbors the largest (oldest) oysters we have seen. In short, during storm-free years, this population appears to grow increasingly senescent. Curiously, hurricane events rejuvenate this population. This appears to occur in part through an induced, synchronous spawning event that re-establishes a juvenile cohort.

The oysters of Mermaid Pond also show a wide size distribution with relatively fast-growing individuals (Carlson, et al., 2009) that are also relatively fast to mature (see below). Mean-size appears stable over the years suggesting a population that has more regular reproductive activity than Oyster Pond (i.e. no progressive senescence). Hurricanes appear to remove the youngest cohorts in this population while triggering spawning, and delayed mortality in the larger adults. There are several notable differences between populations in Oyster and Mermaid Pond. Mermaid Pond maintains its juvenile cohorts during non-storm intervals (though this juvenile cohort is particularly sensitive to storm-activity), whereas juveniles fail to appear in Oyster Pond except during the months immediately following severe storm-triggered spawning event.

The oysters of Six-Pack Pond are relatively small with considerably less size variation (Carlson, et al., 2009). They also show slower growth than individuals from the other two populations. We postulate that this is due to the relatively hypersaline conditions (pearl oysters typically grow faster under less saline conditions, Lucas & Southgate, 2008). Hurricanes have a devastating impact on this population. We have evidence suggesting that Hurricane Floyd (Sept. 1999) exterminated the adult population even while triggering spawning. Remarkably, the spawn survived (possibly finding refuge below a deep-water halocline) and re-established the

breeding population we observed in 2001 (See Cole, et al, 2005). Hurricanes Frances and Noel showed similar cycles of devastation followed by reproductive rebound. This population exhibits a remarkable sensitivity to storm events coupled with an equally remarkable long-term resilience.

We also located live populations of *P. longisquamosa* in Little Lake (which resembles Six Pack Pond) and South Stout Lake (which resembles adjacent Mermaid Pond). Curiously, we have been unable to find a coastal population of *Pinctada* on San Salvador Island despite an abundance of appropriate seagrass habitat.

The Food Source

Histology and gut-content analysis reveals a diet of diatoms and other planktonic algae in the nano-plankton range. It is worth pointing out that within the more hypersaline ponds on the island, *Dunaliella sp.* may predominate having greater salt-tolerance than other green algae. This is noteworthy in that *Dunaliella* have remarkably high levels of estrogen-like sterols in their lipid membranes (Peeler, et al., 1989, Zelazny, et al., 1995). These facts become important when we consider a hypothesis regarding the influence of environmental estrogens on sex determination in *Pinctada* (see below).

METHODS AND MATERIALS

Beginning in 2001, Oysters were collected from Oyster, Six Pack and Mermaid Ponds over the course of 9 years from randomly placed transect lines. In January 2009, we began collecting oysters from a fourth population in Little Lake. Hinge length for each oyster was measured and recorded with calipers. Oysters were collected from each study site and sorted by size (class "A" = 0 to 5.0 mm hinge length, class "B" = 5.0 to 10.0 mm hinge length, and on in 5mm increments). Hinge lengths were measured using hand-held calipers and measuring along the straight edge of the oyster's hinge. In January of 2006, we began our histological studies. Specimens of each size class were collected, and their visceral masses were dissected and fixed in

Bouin's fixative. Tissue was dehydrated and embedded in paraffin as described by Humason (1979). Eight-micron thick sections were made and slices were stained in Gomori's trichrome stain (Fisher Inc.). Slides were scored using an Olympus BH40 microscope with brightfield optics (See Figure 3). Images were taken using a SPOT-RT digital camera.

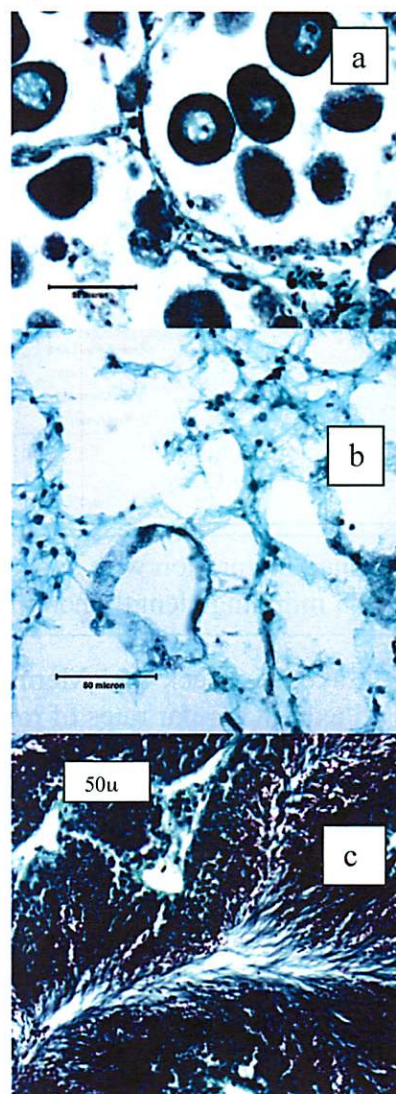


Figure 3. Images of *Pinctada longisquamosa* a) ovarian follicles with oocytes, b) gonad empty of gametes, and c) testis with spermatocytes.

RESULTS

Oyster maturation data (Figure 4) is displayed as the % specimens with recognizable gametes (eggs or sperm) in their gonads using light microscopy and Gomori trichrome-stained

sections. Sex ratios are displayed as the percentage of the mature population comprised of females (Figure 5). Size classes A-G represent 5 mm hinge-length increments (A= 1-5mm hinge length, B= 5-10 mm hinge length etc.). Oyster feminization data from size class A was absent from most ponds due to immaturity of that smallest size class. Size class B data is also absent from Oyster Pond due to relative senescence of that population during sample years.

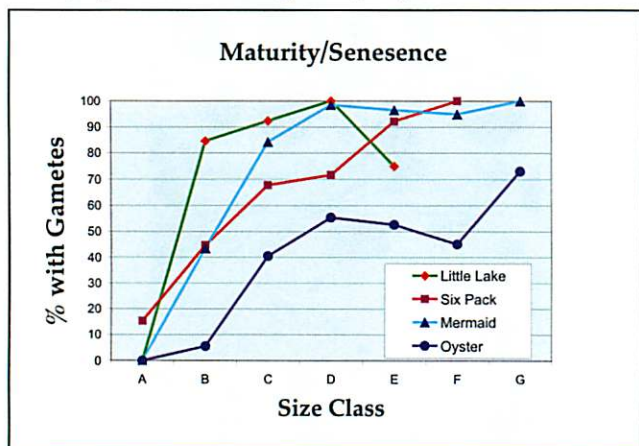


Figure 4. Gonad maturation with age. Size classes are in 5 mm hinge length increments.

Little Lake, Six Pack and Mermaid Pond populations all exhibit similar rates of rapid maturation (Carlson, et al., 2009), while the maturation curve for Oyster Pond is noticeably slower (Figure 4). Curiously, the larger oysters in Oyster Pond frequently have gonads lacking gametes suggesting senescence.

The Oyster Pond population exhibits a classic pattern of protandrous hermaphroditism. (Figure 5). Individuals first mature as males, and turn into females. The largest oysters in Oyster Pond are nearly 100% female. Mermaid Pond oysters also begin uniformly male, but only 50-60% of the mature population appear as females.

Six Pack and Little Lake exhibit a significant female population among even the smallest sexually mature individuals (33% and 45% respectively) suggesting gonochorism rather than protandrous life history trajectories. Curiously, Six Pack oysters establish a crisp 50:50 sex ratio as they achieve full maturity, whereas Little Lake oysters reach about 68% feminization (it should

be noted that for Oyster Pond, Mermaid Pond, and Six Pack, sample sizes are N=50 per size class, whereas for Little Lake, we have only processed 14 specimens per size class, and its trends should be viewed as tentative. This is also true for the largest size class in Mermaid Pond).

Water chemistry analysis (Carlson, et al., 2009, Cole, et al., 2007) shows that the relatively larger Six-Pack Pond and Little Lake are both mildly hypersaline and relatively nutrient rich.

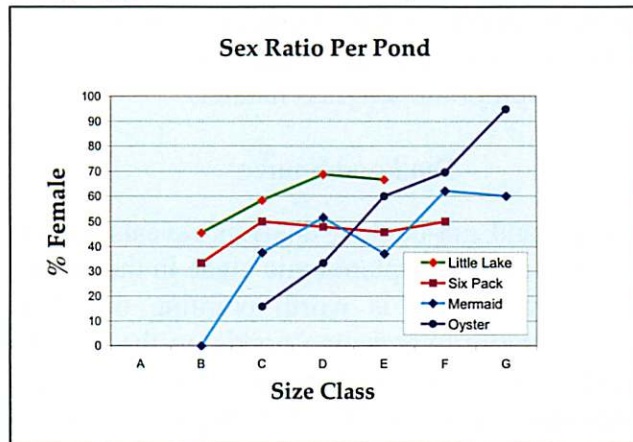


Figure 5. Gender-ratio with age. Size classes are in 5 mm hinge-length increments.

DISCUSSION

Trends

Five different populations of Scaly Pearl Oysters have been identified in five different anchialine ponds on San Salvador Island in the Bahamas. We have studied three of these in detail tracking trends in demography (Carlson et al., this volume) and sex determination, attempting to correlate life history trends with ecological characteristics unique to each pond. What we are left with is a set of intriguing hypotheses as to what is driving the divergence in life history trajectories within these various populations.

1) Oyster Pond: This pond appears most like open-ocean habitat in that salinity remains constant and fully marine despite occasional torrential rainfalls. It differs from open-ocean in that tides are attenuated, due, no doubt, to the labyrinthine

subterranean connections linking the pond to the sea.

The Oyster Pond *Pinctada* population exhibits a wide range of size classes, with a notable abundance of older, larger individuals. The population seems to undergo periods of progressive senescence during weather-stable years, punctuated by hurricane-correlated episodes of spawning/ rejuvenation (Carlson, et al., this volume). Hurricanes exert multiple effects on this population: the youngest individuals undergo preferential mortality, while older individuals survive, spawn successfully, and then die in the subsequent year, an event we refer to as “suicide spawning”. The net impact on this population, immediately following severe hurricane activity, appears to be a transient upward shift in mean size (immediate juvenile mortality), followed by a rejuvenation brought on by hurricane-induced spawning, successful larval recruitment, and delayed mortality of the oldest/ largest individuals. This effect is profound.

We hypothesize that the uniquely sheltered environment offered by Oyster Pond: small size and protective *Rhizophora* habitat restrict wave generation, twice-daily tidal purging of the pond water (including storm-generated freshwater influx) prevents storm-driven changes in salinity, has effectively damped environmental signals that might otherwise synchronize breeding episodes in this population. Being isolated from the Open Ocean, and connected only via restrictive subterranean conduits, these ponds also have highly attenuated tides that might normally serve as environmental triggers of breeding episodes. Without the synchronizing influence of environmental triggers (tidal influences or dramatic fluctuations in temperature and salinity), the result is a population that drifts towards senescence during calm years, relying on particularly powerful storms to overwhelm these buffering agencies to trigger productive, synchronous spawning episodes.

Sexual identity follows a classic pattern of “pure protandry”. Small (younger) individuals are exclusively male, with % female increasing linearly with size measured as hinge length. If Oyster Pond truly mimics an open-ocean habitat, we

might expect that protandry represents the normal or wild-type life history pattern.

In one regard this hypothesis seems problematic. Surely open-ocean populations do not drift towards senescence relying on storm disturbance to synchronize breeding episodes. We hypothesize that open-ocean *Pinctada* populations synchronize breeding with a more regular set of environmental cues, cues that may be damped or attenuated in the highly buffered habitat found in Oyster Pond. This would rule out day-length, or lunar cycles (though those may help define periods of gamete production). The synchronous release of gametes that would ensure high fertility spawning episodes could be a combination of gamete readiness (driven by seasonal cues) and environmental triggers driving synchronous spawning. One possibility is that the more extreme tides (the unusually high “Spring Tides”, or low “Neap tides”) could serve to synchronize spawning episodes in an open-ocean environment, but the attenuation of tidal activities in our ponds might dampen this potentially synchronizing cue resulting in more sporadic, and asynchronous spawning events with a concomitant reduction in fertilization success. This could account for the pattern of senescent drift and hurricane-driven rejuvenation witnessed in Oyster Pond. With attenuated tidal influences damping the potential cues from the more extreme spring tides and neap tides, and conduit driven tidal-exchange buffering our ponds against modest storm-driven changes in temperature and salinity (which can also serve to trigger spawning), Oyster Pond residents may have lost their fecundity as spawning episodes become diffused over time.

2) Mermaid Pond: This pond is nearly marine (a bit hypo-saline, actually), though it shows much less buffering against storm-driven changes in salinity than does Oyster Pond. This is due, no doubt, to less efficient tidal purging through what must be either a more congested or more lengthy conduit network connecting it to the sea.

The Mermaid Pond population resembles the Oyster Pond population in that it exhibits a wide size distribution. Unlike the Oyster Pond population, the presence of juveniles (< 8mm) in

the population during non-hurricane years indicates that the Mermaid Pond population is not growing senescent, but remains reproductively active year after year. We hypothesize, that given the less efficient tidal purging, and more dramatic swings in salinity following even modest storm events, Mermaid Pond oysters may be utilizing not just hurricanes, but regular seasonal storms to synchronize breeding through changes in salinity and temperature. (Again, breeding can be triggered by sudden changes in salinity or temperature, a fact routinely exploited in the aquaculture industry; Southgate & Lucas, 2008).

The adult population of Mermaid Pond survived Hurricane Noel (2007), but the conspicuous absence of juveniles in the months immediately following suggests that the hurricane did lead to differential mortality targeting smaller, younger individuals. From March 2008 to January 2009 there was a large decrease in mean hinge length, suggesting a delayed “suicide-spawning” event that almost exactly parallels the one observed in Oyster Pond over the same interval.

Mermaid Pond oysters enjoy a growth rate nearly identical to those in Oyster Pond (Carlson, et al., this volume), with a rapid, efficient maturation trajectory approaching 100%. Unlike Oyster Pond oysters, Mermaid Pond oysters begin life following a protandrous trajectory, (all juveniles mature initially as males), but fail to approach 100% female with age. Instead, gender distribution levels off between 50-60% female remaining there as oysters continue to grow. This means that 40-50% of the adult population expresses a male identity despite increasing age. (This trend needs corroboration by increasing our sample size for the largest, Mermaid Pond individuals).

If this trend is born out by extended sampling, this is intriguing! Two models could account for this life history pattern. In one model, *Pinctada longisquamosa* may actually be a sexually dimorphic species producing both “true males” and hermaphrodites. True males would remain male as they age, while hermaphrodites would begin life male, and undergo unidirectional, protandrous gender reassignment as they matured. This would yield an all-male juvenile class and a mixed 50:50 sex ratio among older

adults. To account for the “pure protandry” observed in the Oyster Pond population, we would have to propose that Oyster Pond was colonized exclusively by hermaphrodites. (We should note that nothing is known of the genetics of sex determination in this species).

Alternatively, we could be witnessing a population in which bi-directional sex changes are occurring. In this model, there is only one sex (*hermaphrodite*), but conditions favor unidirectional sex changes in Oyster Pond, and bi-directional sex changes in Mermaid Pond. If this is true (a hypothesis we are currently testing by following sex changes in a collection of caged, pond-anchored populations), we predict that Mermaid Pond oysters begin life male, and begin a trajectory of sex changes with random orientation. This would result in a 50:50 sex ratio as male-female conversions would come to equal female-male conversions.

Why might these two populations differ? There could be environmental cues that trigger feminizing sex reversals in Mermaid Pond (cues we cannot guess at this point), or there could have been rapid evolution resulting from mutation and natural selection. As we noted, severe hurricanes (which hit the island roughly every 5-10 years), preferentially damage the juvenile population in Mermaid Pond. Since the juvenile population is typically male, we might predict that successive elimination of the young males would create conditions favoring individuals that could “switch back” from female to male, thereby assuring the persistence of a male breeding cohort even following severe storms. {This begs the question: why wouldn't female-male sex reversals also be favored in Oyster pond where small oysters are rare? A possible response is that oysters in Oyster Pond show a slower sex-change trajectory, achieving the 50:50 ratio more than one size class (roughly 6 months) later than what is seen in Mermaid Pond. Hence Oyster Pond males are better represented amongst the older, larger, and more hurricane-resistant members of the population.}

3) Six Pack Pond: Six Pack Pond represents a dangerous and dynamic world for our oysters.

Salinities typically hover between 40 to 45 g/L (mildly hypersaline), and fluctuate dramatically following large storms or prolonged dry-spells. The lake has a large surface area making wave generation during hurricanes a significant factor, and it has no stabilizing mangrove habitat or tidal flux to purge sudden changes in salinity. We have witnessed three hurricane events that effectively annihilated the adult population, while triggering spawning. These years were followed by “recovery years” as spat (presumably protected beneath a deep-water halocline), settled and re-established themselves in the shallows.

Six Pack oysters grow significantly slower than those of either Oyster or Mermaid Pond, possibly due to the elevated salinity. Sexual maturation is also delayed. This trend appears even more pronounced if one plots maturation vs. age rather than size, given the observed slow growth rates (Carlson, et al. 2009). Sex determination in Six Pack oysters is striking in that the smallest oysters already exhibit significant female expression (32%), that rises quickly to a prominent and stable 50:50 sex ratio. Oysters in Little Lake (a pond with characteristics similar to Six Pack pond), show an even higher initial abundance of feminized individuals.

What could account for these populations exhibiting a near 50:50 ratio from the onset of sexual maturation? We propose several testable hypotheses. First, the difference could be genetic. The hurricane-exposed ponds (Six Pack and Little Lake) exhibit periodic destruction of the adult breeding population (Carlson, et al., 2009). During these severe storms, mass spawning events occur even as the adult population is exterminated. In the absence of adults, (females), selection would favor mutations leading to precocious feminization, which would appear as gonochorism (sexual dimorphism).

Alternatively, differences in life history trajectory could be driven by environmental stimuli and reflect an adaptive developmental response. The richer nutrients of the non-tidal ponds (Six Pack and Little Lake) might trigger precocious feminization as a developmental response to greater nutrient abundance. Without significant tidal exchange, nutrients accumulate in

these ponds as runoff from surrounding forest land. These nutrients support a richer algal biomass in both Six Pack Pond and Little Lake.

Finally, we propose an even more exotic hypothesis: environmental estrogens. We recently learned that hypersaline conditions favor algae of the species *Dunaliella*. These algae (which are the right size to feed our oysters), insert an abundance of cholesterol-derivatives within their membranes to stabilize cell integrity within a hypersaline environment. These derivatives may either exert an estrogen-activity directly, or be metabolized into compounds with estrogen-like activity. The most abundant of these sterols (ergosterol) has been shown to have estrogenic activity (Peeler, et al., 1989; Cook, et al., 1934). It is tempting to propose that, in the more hypersaline ponds, the *Pinctada* diet becomes enriched in a species of algae that acts as a feminizing influence during oyster development.

Experiments are currently underway to explore whether the observed differences in oyster life histories are due to genetic or environmental causes, whether oysters can undergo unidirectional or bi-directional sex change, and whether or not *Dunaliella* is in our hypersaline ponds, and if so, does it indeed exhibit estrogen-like activity.

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