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Cover image - Patch reef near the wall off Grotto Beach (photo by Lee Florea).

Evolutionary vs. environmental influences on life history traits in the scaly pearl oyster, *Pinctada longisquamosa*, on San Salvador Island, Bahamas

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1. Abstract

Natural variants in morphology and life history traits were documented for the scaly pearl oyster (*Pinctada longisquamosa*) found living in the inland, anchialine ponds of San Salvador Island in The Bahamas. These traits include pigment variation, presence or absence of a prominent black stripe on the shell and patterns of sex-determination. Oysters in Oyster Pond exhibit classic protandry, starting life as males and switching to female as they grow older. Oysters in Six Pack Pond show precocious feminization. Over 30% are female as soon as they reach sexual maturity and the population rapidly achieves a 50:50 sex ratio that remains stable as oysters age.

Two experiments were conducted to investigate the genetic basis of these phenotypic differences between these two populations, and a third population in Mermaid Pond. First, relocation experiments exchanged juvenile oysters from Six Pack and Oyster Pond in a reciprocal transplant pattern. The pattern of sex-determination within these caged individuals remained unchanged despite juveniles growing up in novel environments. This result supports the hypothesis that observed patterns of sex determination are genetically rather than environmentally determined. Second, partial mitochondrial COI (cytochrome oxidase C subunit 1) sequence data were obtained in an attempt to discern whether oysters from the San Salvador Ponds exhibit signs of genetic isolation. There was very little COI sequence variation, but the analyses support the monophyly of San Salvador oysters, and their genetic isolation from a population in the Florida Keys. Four COI haplotypes were unique to the oysters used in the analysis; though this genetic variation was largely shared among ponds, the relationships

among haplotypes are consistent with emerging pond-specific genetic variation. The general distribution of limited genetic variation within the San Salvador population is consistent with the relatively young age of the ponds.

In summary, our results suggest a paradox. Oysters from neighboring ponds exhibit distinct differences in phenotypic characters despite lack of genetic evidence that these populations are reproductively isolated. This argues for extreme selection pressure driving phenotypic divergence despite a high likelihood of gene flow between populations.

2. Introduction

During the last ice age, sequestration of seawater in polar ice exposed the Bahamian platform as much as 120 meters above sea level. With the end of that ice age about 10,000 years ago, and the consequent rise in sea level due to melting ice, the Bahamian platform was gradually inundated. One of the last places to fill with seawater was the inter-dune collection of "swales" that constitute the great-lakes system on San Salvador Island. Following the last major ice melt, sea levels were likely relatively stable until the mid 1800s. Since then, it is estimated that sea level has risen 6 cm in the 19th century, and 19 cm in the 20th century, totaling 25cm; it is currently estimated to be rising as much as 3 mm/yr (Godfrey and Page 2005). Regardless of the rate of sea level rise, it is clear that the anchialine (salt-water) ponds of San Salvador Island in The Bahamas are of recent geologic origin, and that animal fauna colonizing these salt-water habitats arrived relatively recently. Animals currently residing in the inland ponds probably became established within the past 6,000 years, and in doing so, became isolated from their founding

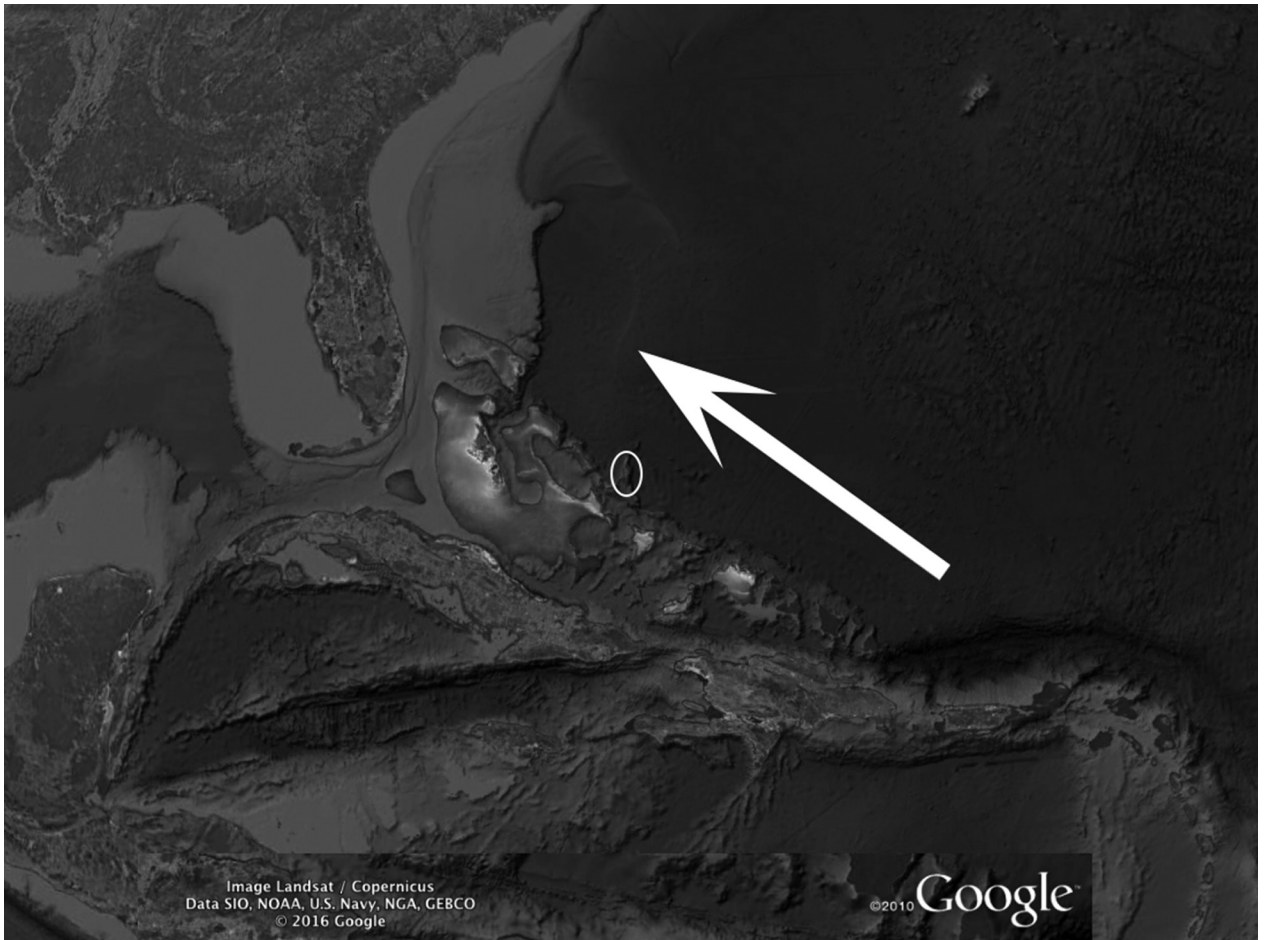


Figure 1. Google Earth visualization showing direction of ocean currents (arrow) affecting San Salvador (circled).

populations (likely to the south and east: Turks & Caicos, Cuba, Dominica, and Puerto Rico, given the prevailing currents; Figure 1).

With such recent colonization, the sub-populations of scaly pearl oysters found within the various anchialine ponds of San Salvador Island have not had much time to accumulate unique genetic and phenotypic differences. However, these ponds do represent a distinctive theater for rapid evolutionary change. They display a range of conditions along several environmental gradients, such as surface area, connectivity to the ocean via conduits, and protection from severe weather events due to surrounding topography and vegetation. Smaller ponds with high connectivity and storm protection exhibit more stability in salinity and water chemistry, even during dry spells or severe storms, while larger ponds lacking

connectivity to the ocean exhibit wild swings in salinity and temperature, and are profoundly disturbed by major storms (pers. obs.).

Many oysters are protandric hermaphrodites, exhibiting testis tissue at sexual maturation and usually switching to exhibit ovary tissue later in the life history; switching back to testis tissue is also observed. Patterns of sex-switching have been studied in the economically important Pacific oyster (*Crassostrea gigas*; Zhang et al. 2014) and various pearl oysters in the genus *Pinctada* (Chavez-Villalba et al. 2011; Teaniniuraitemoana et al. 2014). For *P. longisquamosa* on San Salvador, oysters from Six Pack Pond exhibit a high level of feminization at an early age, and quickly establish a 50:50 sex ratio (Cole et al. 2009, 2011), while oysters from Oyster Pond (and the Florida Keys) exhibit a pattern of sex

determination with young oysters producing only sperm, and older oysters switching over to egg production (Halvorson et al. 2013).

During our most recent expedition we had three goals: quantify morphological traits that distinguish two principal populations, those in Oyster Pond and those in Six Pack Pond; test whether environmental conditions influence the patterns of sex-determination in these ponds; and determine whether neighboring populations are genetically isolated.

3. Methods

3.1. Study locations

Oyster Pond is about 320 meters in diameter and is served by numerous substantial underwater conduits. It is completely surrounded by dense *Rhizophora* mangroves which provide not only protection against wind and wave action but also provide substrate for invertebrate anchorage. The conduit action is significant enough to create a substantial, though delayed, tide cycle which maintains the sea water at nearly perfect marine conditions, even restoring such conditions rapidly following storm events. Mermaid Pond is similar to Oyster Pond in relative size (250 by 400 meters), mangrove margin, and tidal changes via conduits. However, the tidal cycle in Mermaid Pond is even more delayed, so Mermaid recovers slowly from storm-driven changes in salinity. Six Pack Pond is large, at 1.2 km long and 700 meters wide, and devoid of a mangrove prop-root margin. There is zero tidal influence (pers. obs.), and no conspicuous conduit. Six Pack Pond is thus vulnerable to storm-driven changes in salinity and intense wave action.

Six Pack Pond has varied from 20-45 ppt in salinity over the 15 years we have been monitoring it, with a steady rise in salinity during dry years and catastrophic drops in salinity following the great Atlantic storms. Oyster populations within Six Pack have been as high as 250 per square meter (following a storm-induced spawning) and lower than 1 per

100 square meters (or entirely absent) following a sudden, storm-driven drop in salinity. Our 15 years of monitoring Oyster Pond reveal that it is remarkable in its stability regardless of weather conditions.

3.2. Phenotype analysis (shell and soft tissue)

There is distinctive variation in both tissue and shell phenotypes exhibited between the populations in Oyster Pond and Six Pack Pond. Shells range from dark to light, opaque to translucent, and highly to minimally decorated with projections. They also vary in the presence or absence of a prominent black “racing” stripe. The foot-tissues are usually either black or bright orange (very rarely some foot tissue appears more mottled). This was observed in both adult and juvenile oysters. We characterized shell morphology and pigmentation, and foot tissue coloration for 200 oysters per pond Oyster and Six Pack.

3.3. Translocation experiment

In January 2015, the smallest juvenile oysters that could be found in reasonable abundance were collected from Six Pack Pond and Oyster Pond. Hinge lengths were measured using hand-held calipers, measuring along the straight edge of the oyster’s hinge. A von Bertalanffy growth plot was used to convert hinge-length data into age data (Figure 2, reprinted from Carlson et al. 2011). From Six Pack Pond, juveniles averaged 13.1 mm hinge length. From Oyster Pond, juveniles averaged 22.9 mm. These correspond to Six Pack oysters that were 14.6 months in age, and Oyster Pond oysters that were 17.7 months in age. On site, 40 specimens were enclosed in nylon mesh bags and the bags were anchored to the substrate using cinder blocks or iron rebar pounded into the carbonate lake bottom. Styrofoam floats lifted the bags above the pond-sediments. These represented our “control” specimens. Forty additional specimens were collected from each pond and transported to the reciprocal pond for similar anchorage.

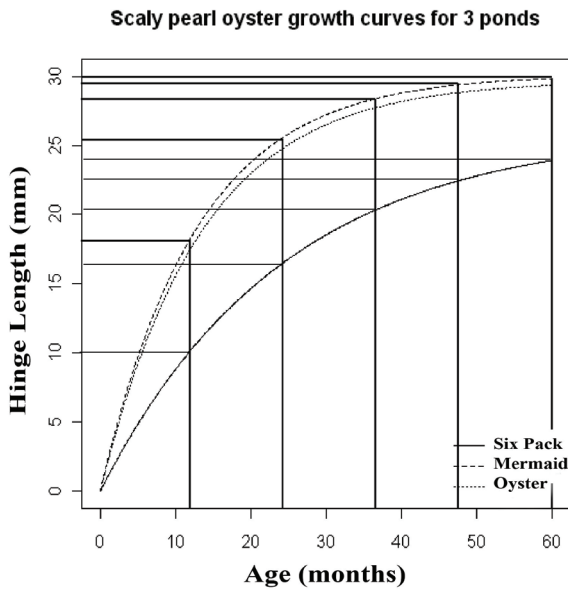


Figure 2. A hinge-length/age conversion graph. Reprinted from Carlson et al. (2011).

Bagged oysters were retrieved in June, 2015. Specimens were dissected, and their visceral masses were fixed in Bouin’s fixative. Specimens were then dehydrated and embedded in paraffin as described by Humason (1979). Eight-nine micron-thick sections were made of each specimen and the slices were stained in Gomori’s trichrome stain (Fisher Inc). Slides were analyzed using an Olympus BH40 microscope using brightfield optics. Photographic images were taken with a DP-71 digital camera.

3.4. Molecular analysis

Pinctada longisquamosa tissue samples were collected from Oyster Pond (N = 37), Six Pack Pond (N = 31), and Mermaid Pond (N = 35). Nine oyster samples were provided from Florida populations (courtesy of I. Temkin, American Museum of Natural History). A small piece of adductor muscle or mantle was removed from each individual and stored in 95% ethanol at 4°C. DNA was isolated from each sample using the Qiagen DNeasy Kit and the manufacturer’s protocol. DNA was eluted with 200 uL of nuclease free water and stored at 4° C.

An 800 bp fragment of the COI gene was amplified using primers designed from published GenBank sequences of the complete *P. fucata* COI gene and from *P. margaritifera* and *P. martensii* COI fragments (5’ end); these primers are PincCOI713F (5’- GGG KGG GGA TCC GGT KTT ATT TCA -3’) and PincCOI1537R (5’- ACT TTC GGT GCT TCC ACT CTA ACG -3’). Reaction conditions were the same as described in Folmer et al. (1994) except with an annealing temperature of 48-50° C. PCR products were purified using the Qiagen QIAquick PCR Purification Kit and commercial sequencing was done at Northwoods DNA (Becida, MN).

Sequences were aligned using MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>) and manually checked against chromatogram images using FinchTV (Geospiza, Inc.; Seattle, WA). The final set of sequences represented 38 individuals: 32 from the San Salvador Ponds and six from Key Largo, Florida. The fragments contained 630 base pairs (bp) of homologous COI gene sequence corresponding to nucleotide positions 736-1366 relative to the complete *Pinctada fucata* COI sequence (GenBank Accession DQ299941). Published COI sequences were also obtained from GenBank for *P. margaritifera* (HM467838), *P. maxima* (GQ452847), and *Pteria loveni* (AB076925) in order to analyze the position of *P. longisquamosa* within the genus *Pinctada* based on 426 bp of homologous COI gene sequence corresponding to nucleotide positions 757-1183 relative to the complete *P. fucata* COI sequence. For both data sets (*P. longisquamosa* only, and species level) phylogenetic tree construction and calculation of pairwise sequence divergences were performed with MEGA (Tamura et al. 2007) using distance, maximum parsimony, and maximum likelihood criteria. The distance (neighbor-joining) tree was evaluated with bootstrap analysis (10,000 replicates).

4. Results

4.1. Phenotype analysis (shell and soft tissue)

Shells of Oyster and Mermaid Pond are typically brown in hue, opaque, and decorated with long projections (Figure 3A). With age, these ornaments are worn off, and shells become more fragile, but they remain opaque. The shells of Six Pack Pond oysters are translucent, and less decorated (Figure 3B-C).

Two easily quantified traits that vary from pond to pond involve pigmentation. Shells exhibit variation in pigmentation, occasionally sporting a prominent black "racing" stripe (Figure 4A-B). Six Pack oysters showed 17% with stripes, while Oyster Pond oysters

exhibited only 8% with stripes. The foot-tissues are usually either black or bright orange (Figure 4C; very rarely some foot tissue appears more mottled). This was observed in both adult and juvenile oysters. In Oyster Pond, 11% of the oysters exhibited the orange-foot phenotype, whereas 38% of 6-Pack Pond oysters showed the orange-foot phenotype.

4.2. *Translocation experiment*

We performed a reciprocal translocation experiment, moving forty Six Pack oysters to

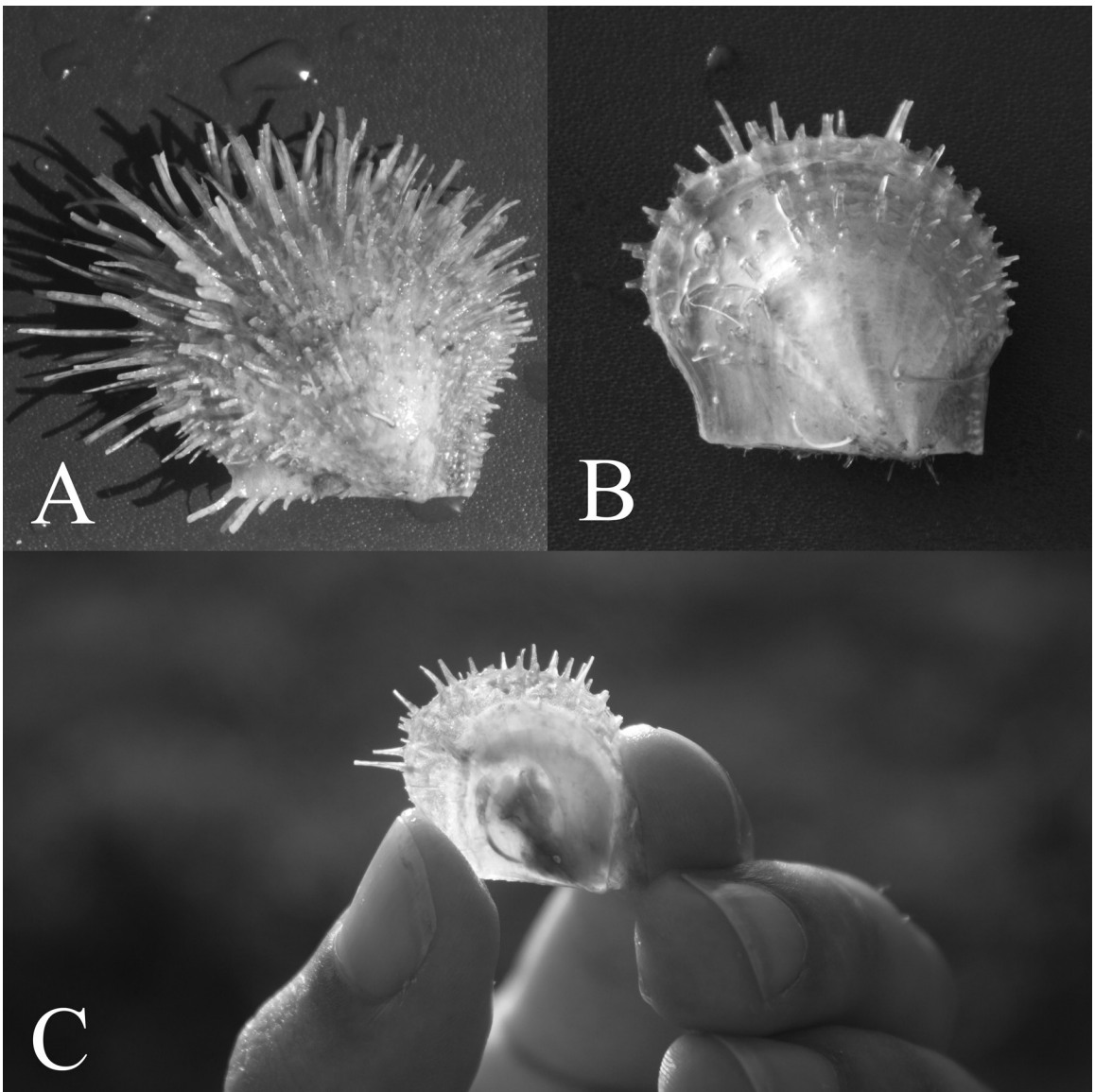


Figure 3. A) Oysters from Oyster Pond and Mermaid Pond exhibit golden, opaque shells with rich ornamentation (when young). B, C) Oysters from Six Pack Pond are translucent with reduced ornamentation.

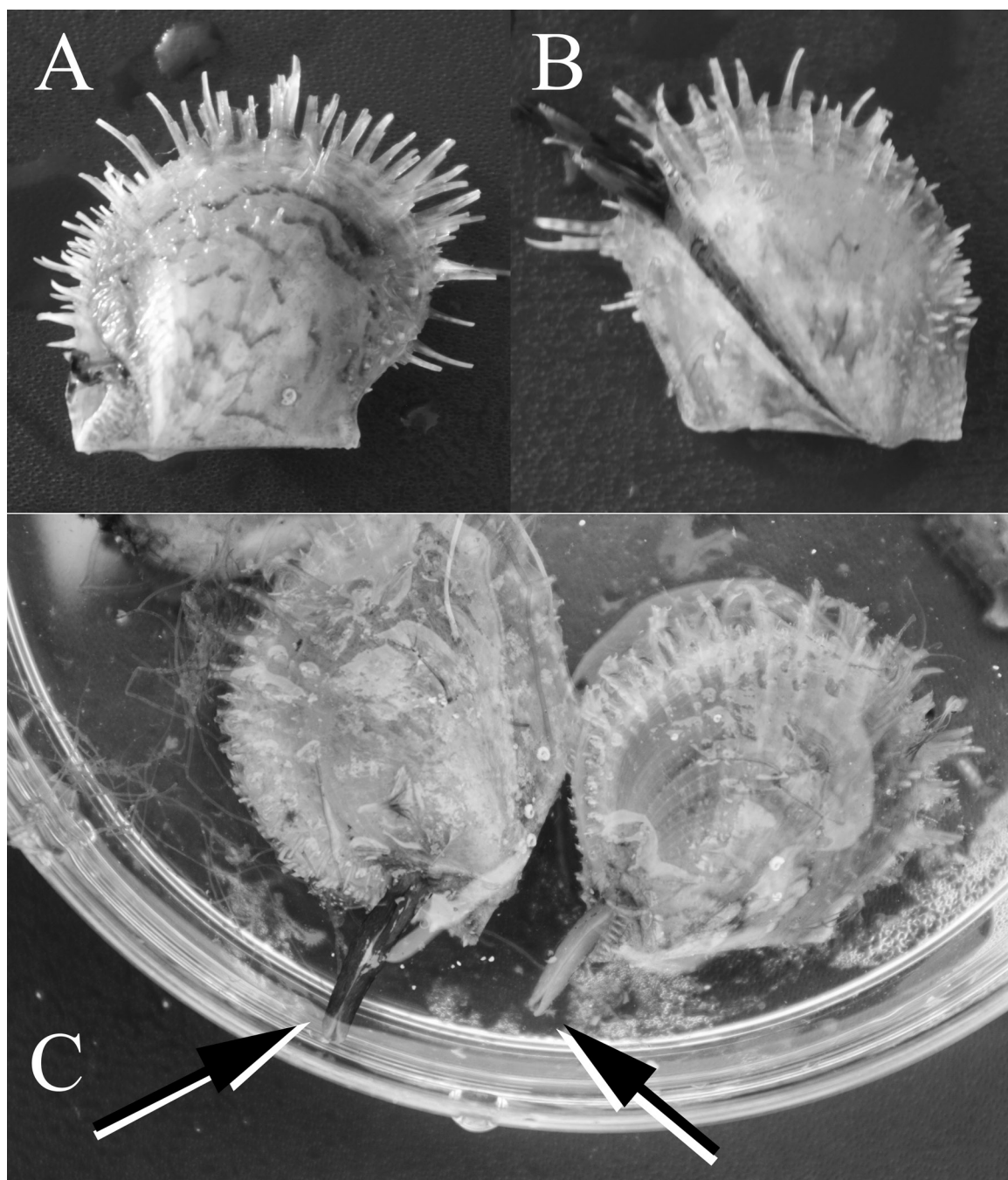


Figure 4. A, B) Six Pack oysters showing variation in shell pigment (note radial stripe in B). C) Oyster Pond oysters showing both black and orange pigmented soft tissues (arrows).

Oyster Pond, and 40 Oyster Pond oysters to Six Pack Pond. Histological analysis four months later showed that both Six Pack and Oyster Pond oysters maintained their sex ratios despite reciprocal transplantation (Figure 5).

4.3. Molecular analysis

The phylogenetic tree (Figure 6) represents the

relationships among *P. longisquamosa* based on COI haplotypes (630 bp partial sequence). It supports the monophyly of both the San Salvador population (haplotypes D-G) and the Florida (Key Largo) population (haplotypes A-C). Pairwise comparisons of the haplotypes further demonstrate that haplotypes within geographic areas (San Salvador versus Florida) are more similar to each other than to haplotypes

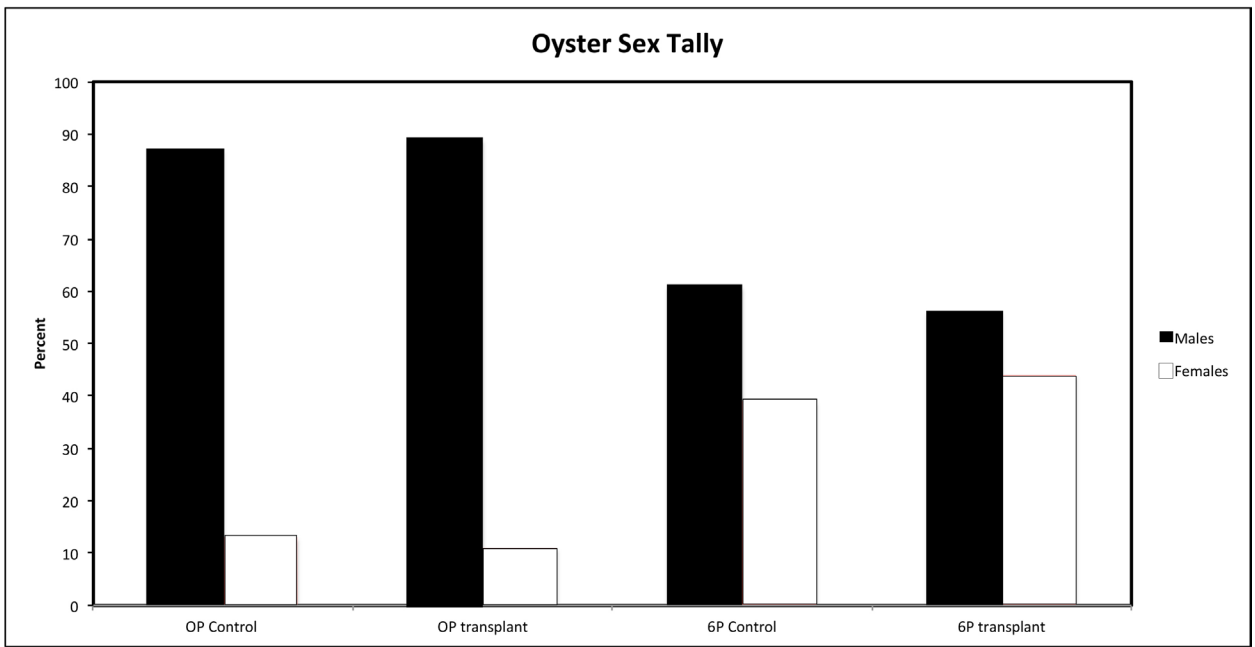


Figure 5. Sex determination among oysters growing in their native pond (control) or after translocation to an experimental pond (transplant). Oysters underwent reciprocal translocation between Six Pack (6-P) and Oyster Pond (OP).

from the other area (Figure 7).

Though there were many more San Salvador than Florida individuals in this analysis, some preliminary observations about genetic diversity can be made. There were three haplotypes found among the six Florida individuals, while 30 of the 32 San Salvador individuals had one of two common haplotypes (Figure 6). Haplotype E was found in all three ponds, and haplotype D was found in the two northern ponds (Oyster Pond and Six Pack Pond). The two rare haplotypes (found in one individual each) were haplotype F (found in Six Pack pond and most closely related to common haplotype D) and haplotype G (found in Mermaid Pond and most closely related to the ubiquitous haplotype E).

Phylogenetic analysis of 426 bp of COI sequence for the *P. longisquamosa* haplotypes and three other *Pinctada* species (using *Pteria loveni* as an outgroup) supports the monophyly of *P. longisquamosa* (Figure 8). The greatest pairwise difference among *Pinctada* sequences was 103 nucleotides, while the *Pteria* sequence differed from all *Pinctada* sequences by 137-142 nucleotides.

5. Discussion

Scaly pearl oysters on San Salvador Island are enigmatic in that they can only be found in the inland anchialine ponds and, despite much searching, not in the surrounding coastal waters. These oysters seem to represent an isolated meta-population with respect to the ocean. Gene flow from other island or coastal populations is likely to be minimal due to the long distances separating them. Consequently, the most significant source of gene flow is likely to be between the ponds themselves. Our data show that both genetic (COI haplotypes) and phenotypic (shell and foot tissue) variation is largely shared between ponds, with some pond-specific differences in phenotype and some unique genetic haplotypes. Data from translocation experiments suggest that pond-specific patterns of sex determination are different in Oyster and Six Pack Ponds, and that these differences remain even when oysters are reared in their non-native ponds. Six Pack and Oyster Ponds are separated by about six overland kilometers, a distance that should allow gene flow between ponds via overland means via

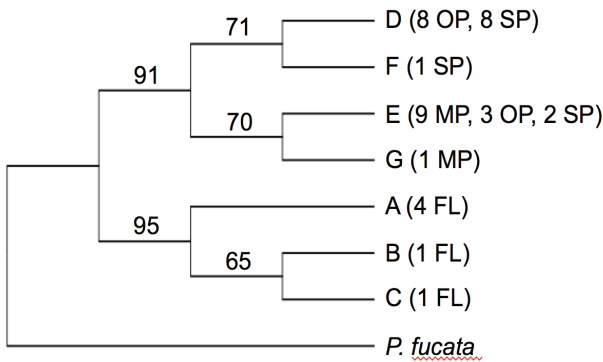


Figure 6. Hypothesized relationships among *P. longisquamosa* haplotypes A-G based on analysis of 630 bp of the mitochondrial cytochrome oxidase 1 locus (COI). The tree was rooted with *P. fucata*. The numbers on the branches are bootstrap values. MP = Mermaid Pond, OP = Oyster Pond, SP = Six Pack Pond, FL = Florida. The numbers in parentheses represent the number of individuals from each pond that exhibit that haplotype.

waterfowl, a process that would be facilitated by establishment of temporary intervening habitat patches that likely form within the Great Lakes during unusually wet years. The Great Lakes are typically hypersaline, and devoid of oysters, though oyster shells do appear in the shell hash lining its shores, and live specimens have been found in the southern-most reaches of the great lakes (R. Ditter, pers. comm.) as well as in adjoining Little Lake. We hypothesize that unusual selection pressures, especially in Six Pack Pond, must be strong enough to drive phenotypic divergence despite the effects of gene flow.

Our molecular data confirm the isolation of San Salvador’s oysters from those of Key Largo, Florida, and a relative paucity of genetic variation across the island. We also found *P. longisquamosa* to be more closely related to other *Pinctada* species than to *Pteria loveni* (Figure 8). This is consistent with the taxonomic switch of *P. longisquamosa* from *Pteria* to *Pinctada* originally based on morphological data (Mikkelsen et al. 2004), as well as with the molecular analyses of different loci found by Tëmkin (2010). The relationship among the four *Pinctada* species in Figure 8 are also

consistent with phylogenetic analyses of Cunha et al. (2011), though *P. longisquamosa* was not included in those analyses (based on COI as well and nuclear 18S sequences).

This paucity of genetic diversity in San Salvador scaly pearl oyster COI sequences is likely due to the recent origin of these anchialine ponds, and the probable small size of the founder population that first colonized the ponds. What genetic diversity does exist is generally shared among pond populations, though there are two monotypic haplotypes. Each of these monotypic haplotypes is most closely related to a different widespread haplotype in a pattern that is consistent with emerging pond-specific genetic variation. This interpretation should be tempered in light of the modesty of our data set. Given the recent origin of these San Salvador pond habitats, a different type of molecular marker that is more likely to reflect quite recent evolution would be more appropriate for identifying pond-specific genetic variation. So far, attempts to use microsatellite loci to investigate scaly pearl oyster population genetics have been unsuccessful, but efforts are ongoing.

Despite the paucity of genetic variation, we found numerous examples of pond-specific phenotype variation. The Six Pack oyster population exhibits a higher frequency of radial striping and orange soft-tissue pigmentation. The oysters of Six-Pack Pond also exhibit thinner, translucent and less ornamented shells. Finally, the Six Pack oysters exhibit a distinctive, alternating pattern of sex-determination in contrast to the straightforward protandry seen in the Oyster Pond population. Finding phenotypic variation between closely neighboring populations that likely experience gene flow raises several problems. First, are the phenotypic differences simply due to developmental plasticity in response to varying environmental conditions between ponds, or are they actually heritable, genetic differences? Second, if any of these traits are driven by genetic variation, what could be driving their selection? More specifically, what would

be the selective advantage for translucent, smooth shells, conspicuous pigment stripes, orange soft tissue pigmentation, and a rapidly alternating pattern of sex-determination within the Six Pack population? Previous work has demonstrated that Six Pack Pond is uniquely subject to intense wave generation during active hurricanes, whereas oysters in Oyster Pond are relatively protected (Cole et al. 2009). The smooth, un-ornamented shells of Six Pack might better resist stronger wave action that could tear more highly ornamented oysters from their byssal thread attachments. The potential adaptive advantage of pigment variation is more difficult to surmise. Perhaps the radial striping observed in some shells might provide visual camouflage in habitats that are dominated by vertical vegetation (I. Temkin, pers. comm.). Oysters in Six Pack pond anchor on vertical strands of *Batophora* algae, whereas Oyster Pond oysters anchor on carbonate outcrops in a visually uniform, sediment-covered bottom. Variation in soft-tissue pigmentation also seems like a potential visual cue.

Hypotheses about shell and soft tissue pigmentation imply the existence of visual predators in Six Pack Pond. This is a bit of a puzzle as the aquatic bird population appears rather minimal and the only fish species in the pond (mosquito fish, *Gambusia hubbsi*, and pupfish, *Cyprinodon variegatus*) are quite small and unlikely to threaten adult bivalves in any meaningful way. Though neither fish species has been found to have bivalves as a significant dietary component in The Bahamas (Martin and Wainwright 2011; Araujo et al. 2014), it is possible that they could consume juvenile oysters. Since the favorite substrates for juvenile oyster settlement are the shells of the resident adult oysters, shell and soft tissue phenotypes may be under selection for juvenile camouflage against settling background, in this case, adult shells. The translucent shell of the Six Pack Pond oyster reveals the pale, orange-pink tissues of their internal organs. The thicker, opaque shells of Oyster Pond oysters

	HapA	HapB	HapC	HapD	HapF	HapE	HapG
HapA	--	--	--	--	--	--	--
HapB	3	--	--	--	--	--	--
HapC	1	2	--	--	--	--	--
HapD	6	8	6	--	--	--	--
HapF	7	9	7	1	--	--	--
HapE	6	7	5	1	2	--	--
HapG	7	8	6	2	3	1	--

Figure 7. Table of pairwise differences (number of nucleotides differing) among the seven *P. longisquamosa* haplotypes. The more darkly shaded boxes represent the comparisons between Florida and San Salvador haplotypes.

are typically darker in color. It seems likely that juvenile oysters whose feet are bright orange in color would be better camouflaged than the intensely black-pigmented variety against the orange-gold background of their parents' shells. Experiments are currently underway to investigate whether or not *Cyprinodon* or *Gambusia* recognize juvenile oysters as food, and whether or not they exhibit color-preferences of their prey against suitable, local background.

The more complex phenotypic difference between Six Pack and Oyster Pond populations is the pattern of sex determination. Scaly pearl oysters in Oyster Pond (and the Florida Keys) exhibit a strict pattern of protandrous sex determination with young oysters producing only sperm, and older oysters switching over to egg production (Temkin 2010). This represents a single, unidirectional sex-change triggered by growth and development. Oysters in Six Pack Pond alternate in a bi-directional pattern, switching back and forth between male and female as frequently as once every three months (Halvorson et al. 2013). This fluctuating pattern of sex-determination appears to result in both precocious feminization (as many as 35% of juvenile oysters begin their reproductive lives as females (Cole et al. 2011; Halvorson et al. 2013)), and a 50:50 adult sex ratio. Our translocation

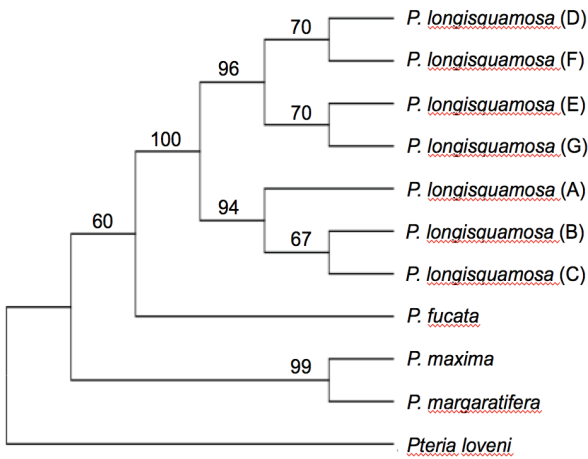


Figure 8. Hypothesized relationships among a subset of *Pinctada* taxa analyzed using 426 bp of the mitochondrial cytochrome oxidase 1 gene (COI). The tree was rooted with *Pteria loveni*. The numbers on the branches are bootstrap values.

experiments demonstrate that these differences in sex determination persist even when oysters are reared in their non-native ponds.

Our hypothesis regarding the adaptive value of alternating sex determination in Six Pack Pond is as follows. On average, San Salvador experiences a hurricane, or major storm event every five years. The oysters of Oyster Pond survive these catastrophes relatively unaffected. The adult population in Six Pack Pond is exterminated, though not before storm-driven changes in salinity and water temperature trigger a mass spawning (Cole et al. 2016). The resulting planktonic veliger larvae survive these storms, possibly finding refuge in deeper water of Six Pack Pond, and re-populate the shallows as the water returns to more normal salinity. As this cohort of “storm-spawn” matures, it would normally consist almost exclusively of males, under the typical protandric trajectory. A mutant exhibiting precocious feminization (or rapid, bidirectional switching that results in precocious feminization) would have an enormous reproductive advantage, being, as it were, the only resident female in the breeding population. Ongoing investigations are exploring the possibility of a storm refuge

for juveniles below a deep-water halocline (the “halocline” hypothesis).

The evidence presented here suggests that some phenotypic variation observed between neighboring populations of *Pinctada longisquamosa* is heritable, and genetically based, and hence subject to natural selection. This is remarkable in that the populations are such close neighbors that gene flow is almost certainly a factor. In order for natural selection to counteract gene flow and drive phenotypic divergence between neighboring populations, that selection must be fierce and extreme (Alleume-Benharira et al. 2006). The incredibly varied inland ponds of San Salvador Island represent an excellent theater for investigations into such forms of extreme selection.

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