URCHINS AND OCEANS:
EFFECTS OF NATURALLY OCCURRING WATER QUALITY ON FERTILIZATION OF THE NATIVE HAWAIIAN HERBIVORE, *TRIPNEUSTES GRATILLA*

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF

THE UNIVERSITY OF HAWAIʻI AT MĀNOA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

ZOOLOGY

MAY 2014

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Keywords: Water quality, sea urchin, Kāneʻohe Bay, fertilization

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Introduction

Approximately 44% of the world's population lives within 150 kilometers of the ocean (Nganyi et al., 2010). Changes in land use associated with this demand often lead to the degradation of coastal ecosystems (e.g., Foley et al., 2005) due to pollution from freshwater runoff (e.g., Islam and Tanaka, 2004; e.g., Smith et al., 1999; e.g., Galloway et al., 2003; e.g., Rabalais et al., 2009; e.g., Pal et al., 2010). Anthropogenic impacts have affected stenotypic coral reefs worldwide (e.g., Richmond, 1993; e.g., Fabricius, 2005, Packett et al., 2009; e.g., Hughes et al., 2010), leading to phase shifts from ecosystems with high coral cover and biodiversity to areas dominated by macroalgae and filter feeders (e.g., Szmant, 2002; e.g., Pandolfi et al., 2003).

Eutrophication is often cited as a major cause of phase shifts, although numerous studies have shown that a change in herbivory is often necessary and can be the sole cause (e.g., McCook, 1999; e.g., Szmant, 2002; McManus and Polsenberg, 2004; Hughes et al., 2007; Burkepile and Hay, 2009; Vermeij et al., 2009). Changes in herbivory can occur when dominant algal species shift and are no longer palatable to the intact community of herbivores, or with the loss of grazers themselves**.** While overfishing is a major cause for the decline of herbivorous fish species (e.g., Hughes and Connell, 1999; e.g., Jackson et al., 2001**)**, drivers behind declines in populations of another major grazer, sea urchins, are more varied, and often unknown (Uthicke et al., 2009).

Sea urchins are efficient grazers, making them keystone species that structure coastal marine ecosystems around the world, with population changes leading to massive shifts in the abundance and distribution of macroalgae and seagrasses (Hay, 1984; Harrold and Reed, 1985; Scheibling, 1986; Hughes et al., 1987; Steneck, 1993; Estes and Duggins, 1995; Heck and Valentine, 1995; Palacín et al., 1997; Edmunds and Carpenter, 2001; Lessios et al., 2001; Eklöf et al., 2008; Valentine and Edgar, 2010). A well-known case study occurred in 1983, when a massive die-off of the black sea urchin *Diadema antillarum* led to phase shifts on coral reefs throughout the Caribbean (Lessios et al., 1984; Hughes et al., 1987; Carpenter, 1988; Lessios, 1988; Carpenter, 1990**)**. A pathogen is thought to have driven the loss, but the exact cause remains unknown (Lessios et al.,

1984). As critical grazers in coral reef ecosystems, it is important to examine the stressors that may drive changes in sea urchin populations.

Echinoderms are characterized by a high proportion of species that exhibit a "boom and bust" pattern of density variation, with positive feedback loops that lead to rapid increases and declines in their populations (Uthicke et al., 2009). Echinoderms are broadcast spawners and are therefore subject to Allee effects, whereby the density of conspecifics has a great effect on reproduction (Levitan et al., 1992; Franke et al., 2002; Marshall, 2002), with decreasing densities of adults resulting in exponential declines in fertilization success (Pennington, 1985; Levitan, 1991; Levitan et al., 1992; Babcock et al., 1994; Wahle and Peckham, 1999; Levitan, 2002). Therefore, it has been suggested that once a population decline has been initiated, near complete losses are common and recovery is extremely slow (Uthicke et al., 2009), as evidenced by the continued low populations of *D. antillarum* in the Caribbean over twenty years after their decline (Lessios, 2005). Overharvesting sea urchins can initiate a downward cascade in their populations; however, in other cases, the cause of declines is often unknown (Uthicke et al., 2009). Terrestrial inputs may be a trigger by negatively impacting fertilization directly and aggravating effects of low urchin density on reproduction (Hollows et al., 2007).

As broadcast spawners, sea urchin fertilization takes place in the water column where gametes are exposed to environmental contaminants. It is well established that early life history stages in sea urchins are affected by a broad suite of pollutants including, metals (Dinnel et al., 1989; Larrain et al., 1999; Radenac et al., 2001; Novelli et al., 2003; Xu et al., 2011), antibiotics (Hagström, and Lönning, 1973), pesticides (Bresch and Arendt, 1977; Dinnel et al., 1989; Larrain et al., 1999; Pesando et al., 2004; Buznikov et al., 2001), phosphates (Böttger and McClintock, 2001), and organic compounds such as pharmaceuticals (Hagström, and Lönning, 1973; Vazquez, 2013). Further, studies using bioassays have shown that sea urchin fertilization is negatively affected by naturally occurring waters impacted by terrestrial inputs (Kobayashi et al., 1972; Zúñiga et al., 1995; Riveros et al., 1996; Fairey et al., 1998; Beiras et al., 2001; Beiras and Saco-Alvarez, 2006; Marin et al., 2007); therefore coastal water quality may affect urchin populations in the field.

The severity of terrestrial impacts on sea urchin fertilization may be influenced by the physical conditions of the receiving waters, with greater impacts in places where flushing rates are low relative to the rates of pollutant input. It is widely thought that reefs open to the ocean with high flushing rates have high resistance to terrestrial input (e.g., Pastorok and Bilyard, 1985; e.g., Fabricius, 2005; Brodie et al., 2012); however, those found in embayments with restricted circulation often respond quickly (Jickells, 1998; Ringuet and Mackenzie, 2005), and severely to human-derived pressures (Pastorok and Bilyard, 1985; Dollar and Grigg, 2004; e.g., Fabricius, 2005; Wolanski et al., 2009).

Kāneʻohe Bay, located on the eastern shores of Oʻahu, Hawaiʻi, is an enclosed system with a history documenting the decline of a reef due to anthropogenic impacts (Banner, 1974; Smith et al., 1981; Pastorok and Bilyard, 1985; Jokiel, 1991; Hunter and Evans, 1995; Stimson et al., 2001; Conklin and Smith, 2005). Stream inputs, wave action, tides, and winds are primarily responsible for flushing within the bay (Ringuet and Mackenzie, 2005; Ostrander et al., 2008; Lowe et al., 2009a), with retention times ranging from one day in the north to 1-2 months in the south (Lowe et al., 2009a). Thus there is a range of exposure to terrestrial inputs due to these physical characteristics of the bay.

Phase shifts from coral to algal dominance within the bay were followed by the loss of the native Hawaiian collector urchin (*Tripneustes gratilla*) (Thomas, personal communication). This grazer is an ecosystem engineer elsewhere in the world in seagrass beds (Alcoverro and Mariani, 2002; Vonk et al., 2008) as well as coral reefs (Valentine and Edgar, 2010). They are efficient grazers in Hawaiʻi, and urchins outplanted onto degraded reefs in Kāneʻohe Bay show potential as biocontrol agents against invasive algae (Conklin and Smith, 2005; Stimson et al., 2007). Once thought to be the most abundant urchin within the bay (Alender, 1964), their current population is patchy and so low that they presently do not significantly contribute to herbivory (Conklin and Smith, 2005; Stimson and Conklin, 2008).

The cause for the decline of *T. gratilla* is unknown, however, it is possible that water quality in Kāneʻohe Bay may have affected recruitment. Watersheds surrounding Kāneʻohe Bay deliver significant freshwater input (Cox et al., 2006; Ostrander et al., 2008), carrying nutrients (Ringuet and Mackenzie, 2005), metals (Hédouin et al., 2009)

and other contaminants onto the nearby fringing reefs (Hunter et al., 1995). Like other urchin species, terrestrial pollutants impact *T. gratilla* reproduction (Dinnel et al., 1988; Vazquez, 2013), thus it is possible that exposure to runoff may negatively impact their recruitment, contributing to their population decline, and ultimately exacerbating the phase shift within the bay.

These terrestrial inputs can vary through time however, due to differences in the volume and content in freshwater sources as well shifts in factors that impact hydrodynamics in the bay. Stream outputs, nutrient concentrations, and the spatial range of freshwater impact from streams around Kāneʻohe Bay all increase following storm events, and tides that vary daily and monthly heavily influence post-storm recovery periods (Ringuet and Mackenzie, 2005; Cox et al., 2006; Ostrander et al., 2008). Additionally, freshwater inputs are mediated by circulation and retention times, which are dependent on factors that vary temporally such as precipitation, winds, wave action, stream outputs, and tides (Lowe et al., 2009a).

Our experiments sought to examine impacts of water quality in waters within Kāneʻohe Bay on the fertilization success of the native Hawaiian grazer *T. gratilla* on a spatial and temporal scale. To test this, we applied an Environmental Protection Agency (EPA) *Tripneustes gratilla* fertilization bioassay used to detect toxicity in industrial effluents to naturally occurring waters in the bay. The results of our bioassays then not only provide an industry accepted measure of water quality, but also relate water quality to the reproductive success of a grazer in the bay.

Here we ask whether patterns in fertilization success differ: 1) along an on-to-off shore transect reflecting non-point source pollution, 2) based on location relative to a point source of freshwater input and 3) through time at a given location. Results of this study may indicate whether terrestrial input can account for the loss of *Tripneustes gratilla* from Kāneʻohe Bay and inform managers whether there are regions of the bay that will or will not support fertilization success among populations of outplanted urchins.

Our hypotheses are then: 1) Should uniform non-point sources of freshwater exist along the shores of Kāneʻohe Bay, we would expect to see lower fertilization nearshore due to higher influxes of freshwater input. 2) Should freshwater inputs such as streams

carry terrestrial toxins, we would expect to see decreases in fertilization success associated with shorter distances from freshwater sources and farther distances from oceanic input. 3) Due to temporal differences in freshwater outputs as well as retention and circulation times in the bay, we anticipate that water quality and therefore fertilization success would vary through time at a given location.

Methods

Site

Kāneʻohe Bay is located on the northeast coast of the island of Oʻahu, at 21^0 28'N and 157^0 49' W. As the largest bay within the archipelago, it is 12.7 km by 4.3 km, and covers approximately 16 square miles **(**Banner, 1974; Jokiel, 1991**).** It faces the ocean for 8.8 km of its length, with a barrier reef situated across the mouth of the bay. In addition to the barrier reef, Kāneʻohe Bay contains approximately 30 patch reefs within its interior lagoon, as well as fringing reefs along the shoreline **(**Banner, 1974**)**. The bay is fed by an influx of oceanic water, as well as eleven small streams that originate in the adjacent watersheds (Banner, 1974; Ostrander et al., 2008).

Sampling

Water samples were collected in 500ml certified-clean amber glass jars that were rinsed three times in water from the collection site immediately prior to collection. Water samples were collected at a depth of 0.3-0.6m. No sunscreens or lotions were worn prior to or during collection to minimize contamination. Samples were sealed with Parafilm, and then covered, as preliminary studies suggest that certain plastic covers negatively affect fertilization in *T. gratilla* (author, unpublished data). Samples were stored on ice or refrigerated at 4^0C overnight in accordance with EPA standards. All samples were used within 24 hours of collection. Sampling was restricted to waters above hard substrate to reflect the natural habitat for *T. gratilla*.

Spatial variation in fertilization of *Tripneustes gratilla*

To determine if the assay could detect naturally occurring known differences in water quality, we conducted an initial study in June 2012 targeting six sites from different

areas of the bay. Three sites were located along the shoreline, two sites in southern Kāneʻohe Bay which has a long retention time (Bathen, 1968; Cox et al., 2006; Ostrander et al., 2008), and one near the open ocean. One sample was taken per site. Samples were then used for fertilization assays.

To test for influences of terrestrial input on fertilization success, we then expanded our sampling across a broader spatial scale.

Our first question sought to determine whether fertilization varies spatially, specifically in an onshore to offshore gradient. To answer this we divided the bay into three sections: north, central, and south bay. These divisions were based on differing land use and circulation patterns (Bathen, 1968; Cox et al., 2006; Ostrander et al., 2008). The central bay was selected as our study site due to the presence of both agricultural and residential land in the surrounding watershed, as well as significant freshwater and oceanic inputs.

The central bay was further divided into three regions moving from land to open ocean: nearshore, mid, and offshore. Contours for these regions followed the shoreline, extending approximately 750 m, 2000 m, and 3750 m offshore.

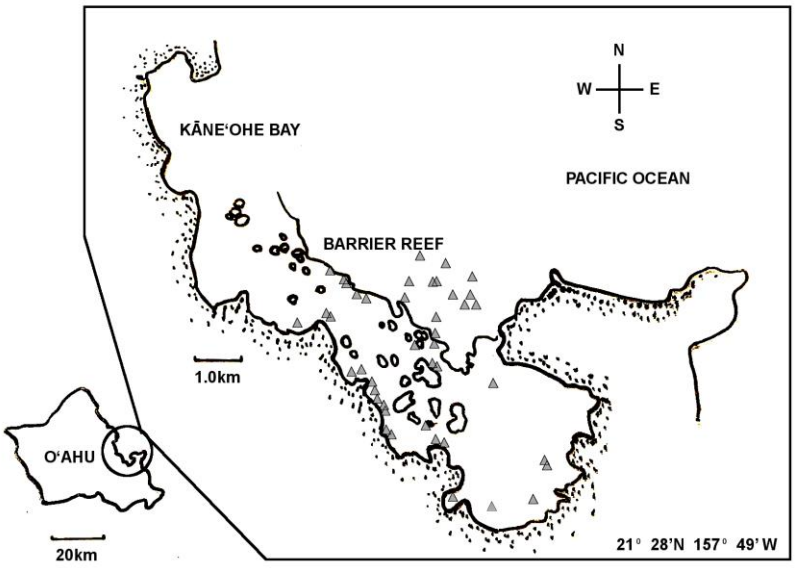

Figure 1. Map of Kāneʻohe Bay showing sites used to determine if fertilization varies spatially. The central bay was divided into three contours extending out from the shoreline: onshore, mid, and offshore. The south bay was also included as a stratum in our sampling scheme. Sites were selected via a stratified random sampling design. 43 sites were sampled in June and December of 2012 and are represented by triangles.

37 sites were randomly selected from these regions and sampled over three days during December 2012. Six of these sites were in the south bay, as we anticipated that water quality would differ due to higher retention and lack of access to the open ocean (Bathen, 1968; Cox et al., 2006; Ostrander et al., 2008).

Temporal variation in fertilization of *Tripneustes gratilla*

To determine if fertilization at a given site varies temporally, due to differences in retention and freshwater input, nine sites were selected to represent a range in fertilization based on results from the spatial study. Three sites followed a transect from shore to open ocean, and six onshore sites were selected to represent a range in fertilization. The six sites consisted of two sites with the lowest fertilization in the previous study, between 40-70% fertilized eggs; two sites with moderate fertilization, approximately 85-90% fertilized eggs; and two sites with high fertilization, above 95% fertilized eggs. Three samples were taken from each site for each assay using 250ml certified-clean amber glass jars. Sampling was conducted during May, mid and late August, and September 2013, with outgoing tides during May and September, and incoming tides in August.

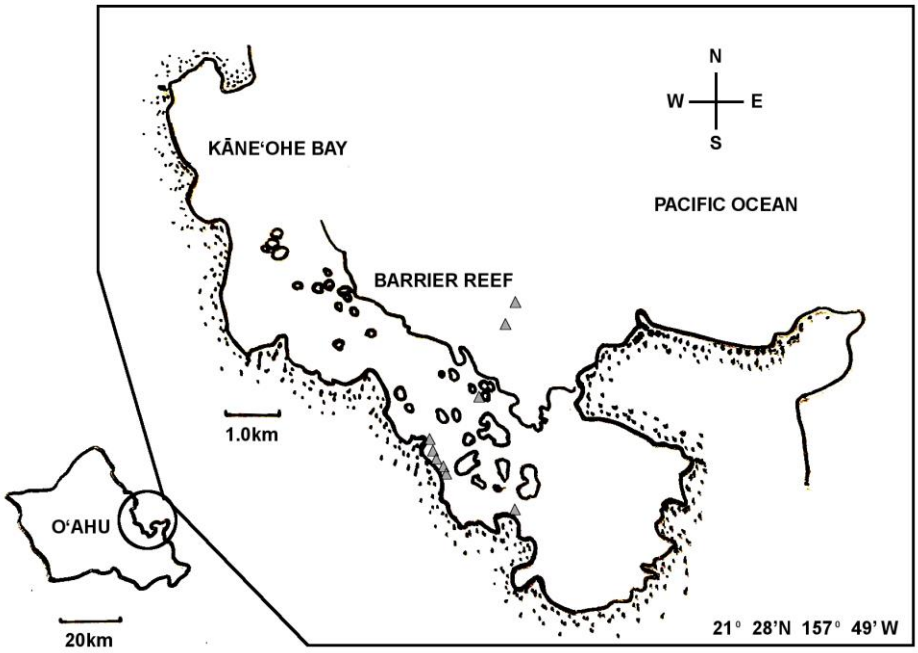

Figure 2. Map of Kāneʻohe Bay showing sites used to determine if fertilization varied temporally. 9 sites were selected to capture a range of fertilization success based on results from the spatial study. Sites were sampled in May, August, and September 2013. Sites are represented by triangles.

Fertilization Assay

Background

The EPA developed the Whole Effluent Toxicity Test to detect and estimate the level of toxicity within industrial effluents that enter the ocean. Sea urchin gametes are exposed to effluent, and fertilization results are then compared to natural seawater that has been filtered (1µm) and UV-treated (FSW). Toxicity is determined by significantly reduced fertilization in effluent samples. 100% effluent is tested when expected toxicity is low and salinity is within 32-36‰. Here we use standard EPA techniques to examine natural water quality conditions.

Glassware preparation

Test chambers consisted of new 100mm x 16mm borosilicate glass test tubes. All pipette tips for solution preparation and disposable glassware used during the bioassay were rinsed three times then soaked for 24 hours in unfiltered seawater followed by three rinses and an overnight soak in Milli-Q water. Test chambers are then rinsed once and filled with FSW. All other glassware were rinsed with Milli-Q and dried on unbleached paper towels, as preliminary studies indicate that bleached paper towels negatively affect fertilization (author, unpublished data). No acids or detergents were used, as they negatively affect fertilization **(**Dinnel et al., 1988; Vazquez, 2003).

Gamete collection

Healthy adult *T. gratilla* were collected from the clean waters of Halona Blowhole and Portlock, O‘ahu prior to each assay. Urchins were kept in flow through aquaria with unfiltered natural seawater and fed *Gracilaria salicornia,* an invasive algal species in the bay. Adults were spawned via gentle shaking. Gametes were collected in designated glass beakers that were cleaned with tap water, rinsed three times with Milli-Q water, and dried. Sperm were collected dry, assessed for motility, and stored on ice. Eggs were collected in FSW, assessed for shape, size, maturity, and sperm contamination, and rinsed twice with FSW. To create sperm stock and egg stock solutions, high quality gametes (highly mobile sperm and round, mature eggs) were pooled from at least two males and two females for each assay to represent a population

and reduce variability due to gamete quality from trial to trial. Stock solutions of each gamete were counted to determine the concentration of sperm and eggs.

Optimization of sperm to egg ratio

Trials were run prior to each bioassay to determine the optimum sperm to egg ratio yielding 90-95% fertilization success in FSW, ensuring sufficient numbers of sperm for fertilization but preventing excess sperm from masking any effects of potential toxicity (Vazquez, 2013). Sperm stock was diluted to seven concentrations ranging from $2x10^5$ to $4x10^6$ sperm/ml. Two technical replicates were used per sperm to egg ratio. 100µl of sperm concentrations were added to test chambers containing 5 ml of FSW. Sperm were exposed to FSW for 45 minutes, upon which 1ml of egg stock solution containing 2000 ± 20 eggs were added, yielding sperm to egg ratios ranging from 100:1 to 2000:1. Eggs were exposed to sperm for 20 minutes, upon which fertilization was terminated via addition of 0.5 ml of 0.02% gluteraldehyde. Results were recorded as a percentage of fertilized eggs after assessment of at least 100 eggs from each test chamber. The optimum sperm to egg ratio was chosen as the ratio resulting in fertilization of 90-95%. Trials were run at 23 ± 1^0C.

Bioassay of samples from bay waters

Test chambers were filled with 5ml of sample waters collected as described above. For our spatial study, four technical replicates were used for each sample; for our temporal study, two technical replicates were used from each sample with FSW as a control. All test chambers were randomized. The bioassay was conducted using the optimum sperm to egg ratio (see above); however, sperm were exposed to samples for 60 minutes prior to addition of eggs. Eggs were also added to test chambers containing only sample waters to ensure that seawater samples did not contain sperm. Salinity, dissolved oxygen, pH, and temperature were measured for each test solution. Salinities were measured using an Atago Master Refractometer, and pH, DO (mg/L), and temperature were measured using a Thermo Scientific Orion 4-Star probe during the assay, thus representing assay conditions and not environmental values.

To provide a toxicity control, gametes were exposed to dilutions of copper in concentrations of 5 mg/L, 10 mg/L, 20 mg/L, 40 mg/L. Copper is a known toxicant and

therefore exposing gametes to increasing concentrations of copper provided a general measure of the sensitivity of the gametes for each assay.

Analysis

Transformation: All fertilization results were normalized to the mean of the FSW control for that day to correct for differences in gamete sensitivity and laboratory conditions. Fertilization results are reported as normalized mean values. For analysis, a logit transformation with a shift of x—0.1 was performed on the mean of the technical replicates of each sample to meet the Analysis of Variance (ANOVA) assumption of normality. Data were analyzed using Minitab 16.

$$logit\ transformed\ fertilization = \ln\left(\frac{sample\ mean\ fertilization - 0.1}{1 - (sample\ mean\ fertilization - 0.1)}\right)$$

Spatial Variation of Fertilization Success of *Tripneustes gratilla*

To determine whether fertilization varies along an on-to-offshore gradient, we used an ANOVA to test for differences between regions. Our model used included site, date, and salinity.

To answer our second question that asked whether fertilization was related to distance to freshwater influences, we regressed the mean normalized fertilization of each sample against an estimate of freshwater influence (FWI). To calculate FWI, we measured the distance of each site in meters from the center of oceanic input (Bathen, 1968; Lowe et al., 2009b) and divided this number by that site's distance to the nearest freshwater source. Current models of circulation within the bay are not at scales fine enough to distinguish between sampling sites, therefore, this non-dimensional value was used as a rough estimate of freshwater influence (FWI). Freshwater sources included streams, harbors associated with streams, and a break the wall of a stream-fed native Hawaiian fishpond.

$$Estimate\ of\ FWI = \frac{distance\ of\ site\ \ in\ meters\ to\ center\ of\ oceanic\ input}{distance\ of\ site\ in\ meters\ to\ nearest\ freshwater\ source}$$

Temporal Variation in Fertilization Success of *Tripneustes gratilla*

A mixed-model ANCOVA was used to determine whether there were differences in fertilization between targeted sites and dates. Site was used as a fixed factor, date as a random factor, with salinity as a covariate. Interactions between salinity and both date and site were also included in our model.

Results

Spatial Variation in Fertilization Success of *Tripneustes gratilla*

Water quality measurements (DO (mg/L), temperature, and pH) of samples fell within EPA accepted ranges, however, salinities of 22 out of 43 samples were 37‰, exceeding the range of 32-36‰. For samples at 37‰, however, fertilization was high, ranging from 0.85±0.07 SD to 0.99±0.03SD. Thus it is not likely that high salinity negatively affected the results.

Using an α of 0.05, fertilization was not significantly different by region (south bay, onshore, mid, offshore), date, or salinity. Fertilization means for the south bay, nearshore, mid and offshore regions were 86.9% ($\pm$0.05SD), 85.8% ($\pm$0.15SD), 93.6% ($\pm$0.04SD), and 92.8% ($\pm$0.05SD), respectively. Fertilization onshore had the highest variation, with average normalized fertilization ranging from 42.7% ($\pm$0.13SD) to 97.9% ($\pm$0.02SD), however, using Levene's test, variances of all regions were equal ($W = 1.49$, $p = 0.232$).

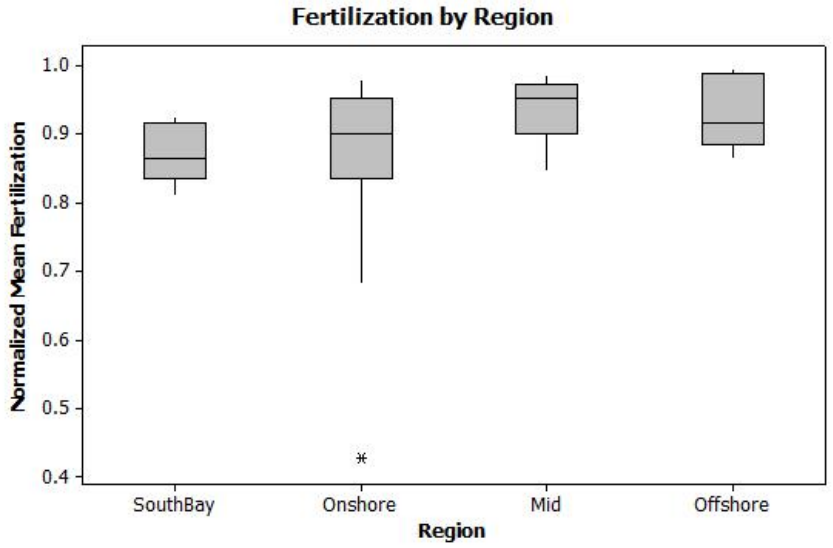

Figure 3. Boxplot of fertilization by region. Fertilization did not differ by region. Results indicate that the bay produces fertilization success that is generally above 80% of the control; however, some sites near shore were as low as 42% of the control (shown as an asterisk).

The lowest observed fertilization of 42.6% (±0.13SD) occurred onshore. To ensure that this outlier was not driving the results, it was removed from the data set. We reanalyzed our data without the outlier using an ANOVA, and found that region, salinity, and date were not significant; however, with salinity removed, region was still not significant, but fertilization did differ by date (df=41, F=3.03, p=0.042).

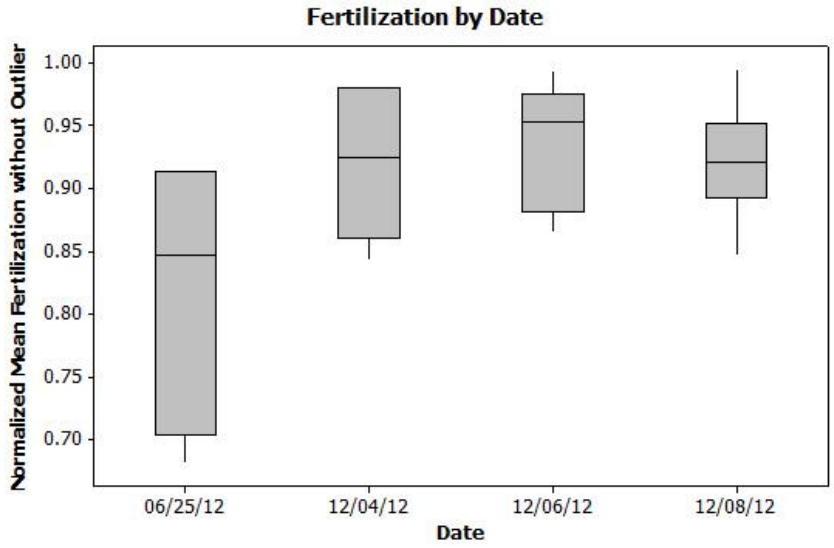

Figure 4. Boxplot of fertilization by date excluding the outlier. With the outlier removed, fertilization is not dependent on region, but it is dependent on date (df=41, F=3.03, p=0.042).

Fertilization was also correlated with the non-dimensional estimate of freshwater influence (r^2=0.48, df=41, n=42, p <0.0001). One site, however, was located very close to a freshwater source, giving it high leverage and influence (FWI = 3350), and was therefore removed prior to regression analysis. Remaining values of FWI ranged from 0.143 to 24.843, with a mean of 3.957(±5.202SD), with smaller values indicating sites closer to the center of oceanic input and farther from freshwater sources.

The site with the lowest observed fertilization of 42.6% (±0.13SD) also had high leverage and influence, so to ensure that it was not driving the significance of the regression, it was also removed. The regression was still significant, however the variation described by FWI was less (r^2=0.16, df=40, n=41, p=0.01).

Additionally, FWI was correlated with salinity (r^2=0.13, df=41, n=42, p=0.019), however it is unlikely that salinity alone is causing the significant results of FWI.

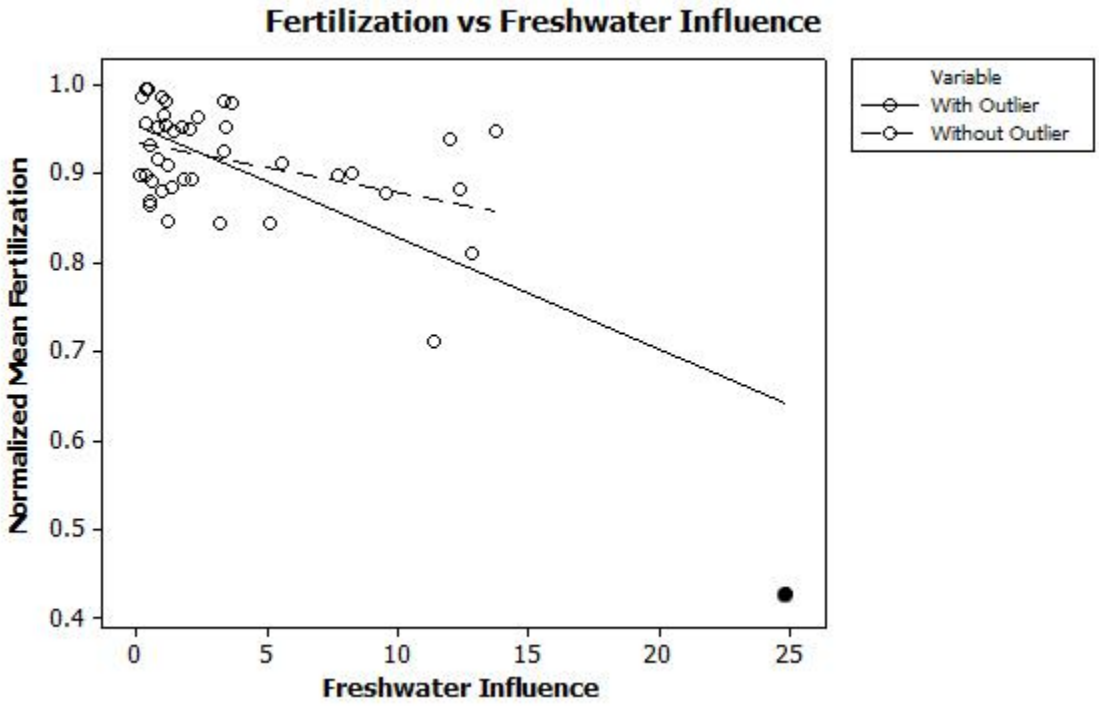

Figure 5. Regression of fertilization against freshwater influence (FWI) with the outlier (r^2=0.48, df=41, n=42, p <0.0001) and without (r^2=0.16, df=40, n=41, p=0.01). The outlier is represented by a closed circle (lower right). Low values of FWI are positively correlated with fertilization, and indicate sites near oceanic input and far from freshwater sources.

Temporal Variation on fertilization success

Water quality measurements for all samples were within acceptable ranges according to EPA standards, except salinity. Salinities of samples ranged from 26-37‰, however, only three sites, a total of nine samples out of 108, were below the accepted 32‰, and four sites, a total of 12 samples, were above the accepted 36‰. Salinities overall were correlated with fertilization sample means (r^2=0.398, df=107, n=108, p<0.001), however, excluding the three sites with salinities below 32‰, fertilization was not correlated with salinity.

Fertilization was varied by date (df=3, F=3.47, p<0.02), but not site, with salinity also contributing to fertilization success (df=1, F=7.24, p=0.009). The interaction between salinity and date was also significant (df=3, F=3.52, p=0.019), indicating that the effects of salinity on fertilization changed with date. All nine samples with salinities below 32‰ occurred on May 31st, the first day of sampling. Salinities of samples taken on the remaining three days were above 32‰. Overall, the mean fertilization was 0.96± 0.09 SD, ranging from 0.73 ± 0.09 SD at one onshore site on May 30, 2013 to a high of 1.05 ± 0.01 SD observed at another onshore site on August 15th, 2013.

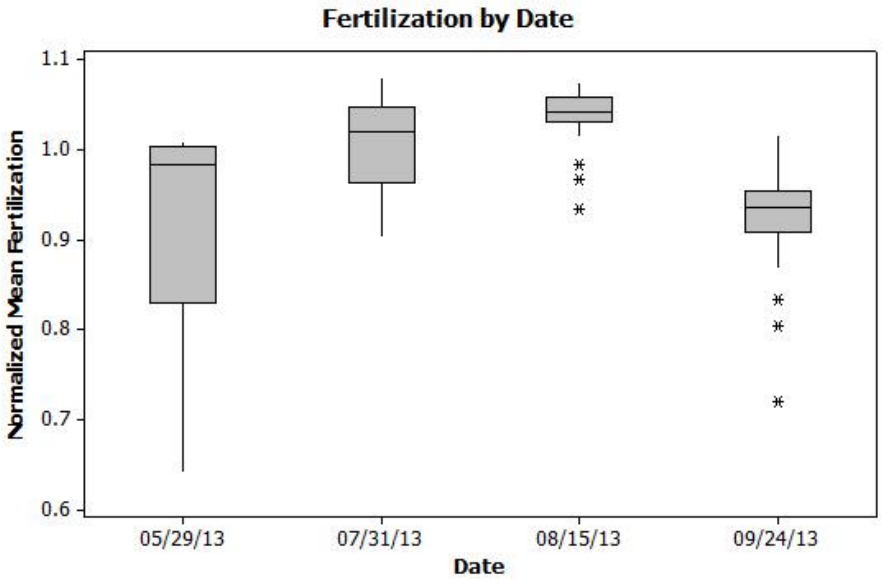

Figure 6. Boxplot of fertilization by date. Fertilization was dependent on date (df=3, F=53.71, p<0.0001). Mean fertilization by chronological date: 0.91 ± 0.11 SD, 1.01 ± 0.06 SD, 1.04 ± 0.04 SD, and 0.92 ± 0.07 SD.

Discussion

The results of this study indicate that fertilization success of *Tripneustes gratilla* is relatively high (generally greater than 80%) in Kāneʻohe Bay and supports our hypothesis that fertilization is correlated with freshwater influence. However, patterns in fertilization success do not support our hypothesis of a uniform non-point source of pollution driving an on-to-offshore gradient in fertilization.

Fertilization success did not differ by region (on, mid, offshore, and south bay); out of a total of 43 sites, we observed only three sites with fertilization below 80% [42.6% ($\pm$0.13SD), 68.2% ($\pm$0.06 SD), and 71.1% ($\pm$0.05SD)]. While these sites were onshore, located near the mouth of a stream, a harbor associated with a stream, and at a break in the wall of a stream-fed ancient Hawaiian fishpond, results were highly localized, as fertilization at the remaining 11 onshore locations was above 87%.

Fertilization was negatively correlated with FWI, a measure of a site's location relative to a freshwater source. Freshwater sources are linked with decreased salinity and terrestrial pollutants (e.g., Islam and Tanaka, 2004) thus this result suggests that water quality may be the main driver of negative impacts on fertilization.

While FWI and salinity were weakly negatively correlated (r^2=-0.13, df=41, n=42, p=0.019), and therefore they may be confounding, salinity was not significant in our ANOVA for our spatial study. EPA standards accept salinities of 32-36‰, and salinities during our spatial study ranged from 32-37‰. While the salinities of many samples were 37‰, other studies have conducted fertilization experiments with *T. gratilla* at salinities above 36‰ with no adverse effects (Byrne et al., 2010). Additionally, high salinity can decrease contaminant uptake and toxicity (Verslycke et al., 2003; Shukla, 2007; Barbieri, 2010), thus we may be observing higher fertilization and therefore be underestimating the effects of contaminants in our high salinity samples.

The significant correlation of fertilization with FWI, therefore, is likely driven by other water quality parameters. All freshwater sources used in our calculations of FWI were associated with streams that the EPA considers impaired due to high levels of nitrogen and turbidity (US EPA, 2006); with turbidity serving as a conduit for various anthropogenic contaminants (e.g., Fabricius, 2005). Previous studies in Kāneʻohe Bay observed that concentrations of nutrients (Ringuet and Mackenzie, 2005), metals and

pesticides (Hunter et al., 1995; Hédouin et al., 2009) were highly variable and site-specific, associated with urbanization in adjacent watersheds and point source pollution from particular streams. This localization of impact may explain the high variation in fertilization observed onshore, from 42.6% (±0.13SD) to 97.9% (±0.02SD), that contributed to the lack of difference between broad-scale regions across the bay.

Submarine groundwater discharge (SGD) may be another source of terrigenous materials (e.g., Paerl, 1997; e.g., Lapworth et al., 2012), and it is prevalent along Hawaiian coasts (Johnson et al., 2008; Kelly et al., 2013**),** including the shores of Kāneʻohe Bay (McGowan, 2004; Dulaiova, unpublished). Atkinson et al. (2003) suggested that groundwater entering Kāneʻohe Bay is contaminated due to high levels of sewage-borne estrogens observed within the bay. Groundwater in Hawaiʻi is known to be contaminated with other various pollutants such as caffeine (Knee et al., 2010), pharmaceuticals (USGS report, 2009), herbicides (Li et al., 2001; State of Hawaiʻi Department of Health , 2006), pesticides (Oki and Giambelluca, 1987; Hawaiʻi Department of Health, 2006; Knee et al., 2010), chemical solvents (State of Hawaiʻi Department of Health, 2006), and high levels of nutrients (Dollar and Atkinson, 1992; Paytan et al., 2006; Johnson et al., 2008; USGS report, 2009). SGD can act as a site-specific point source (Peterson et al., 2009), creating unique environments which increase variability in water quality across larger spatial scales (Burnett and Dulaiova, 2003; Vermeij et al., 2009). This specificity is likely exaggerated in Kāneʻohe Bay due to low flushing rates onshore that prevent mixing and exchange (Bathen, 1968; Lowe et al., 2009b).

Effects of freshwater influence are likely to change over time and our data supports this idea, as date was significant in both our spatial and temporal study. Although FWI was correlated with fertilization in our study, impacts of streams during times of low rain are highly localized and can be affected by tidal state, winds, and rain (Ringuet and Mackenzie, 2005; Cox et al., 2006; Ostrander et al., 2008).

Our results also indicate that date influences fertilization. Date encompasses a wide variety of information, such as seasonality, precipitation, winds, tidal cycles, and wave action. These environmental and physical factors can drive volume and delivery of pollutants in freshwater (Ringuet and Mackenzie, 2005; Cox et al., 2006; Ostrander et al.,

2008), and determine mixing and residence time in the bay (Bathen, 1968; Banner, 1974; Ringuet and Mackenzie, 2005; Cox et al., 2006; Ostrander et al., 2008). Tides drive the volume and timing of SGD (Li et al., 1999; Taniguchi, 2002; Burnett et al., 2003; Burnett and Dulaiova, 2003; Garrison et al., 2003). Thus our study indicates that freshwater influence may be impacting water quality at some locations within Kāneʻohe Bay and that this impact varies with processes associated with date

While we did observe three sites with salinities below 32‰, indicating significant freshwater input, our studies were short in duration, and although we did sample during the wetter winter and drier summer, we did not capture typical precipitation events. Therefore, because concentrations of terrestrial contaminants and the volume of runoff, groundwater, and stream discharge increase during storm events, we may be underestimating the effects of land-based inputs on fertilization success.

Relevance to natural fertilization

Our studies do not represent broadcast spawning behavior in the field, as they were optimized to a single sperm to egg ratio. Allee effects strongly impact marine broadcast spawners, as the density of conspecifics controls sperm availability (Levitan, 1991; Levitan, 1992; Levitan, 1995; Marshall, 2006; Hollows et al., 2007). Currents and turbulence, as well as the viscosity of released gametes can also affect sperm concentrations and fertilization success (Yund and Meidel, 2003; Kregting et al., 2013; Thomas et al., 2013).

Sperm to egg ratios also affect observed levels of toxicity, with more pronounced effects at lower ratios (Marshall, 2006; Hollows et al., 2007; Vazquez, 2003). Our results, while optimized to prevent masking of toxicity, do not describe the full potential of pollutants' effects on fertilization. In areas with low fertilization, actual impacts of pollutants may be underestimated due to our optimized sperm to egg ratios. Likewise, our high salinity samples may also underestimate effects of pollutants, as uptake and toxicity are negatively correlated with salinity in some species (Verslycke et al., 2003; Shukla et al., 2007; Barbieri, 2010).

Further studies should be done to determine the threshold density of conspecifics for fertilization success, if at all possible. At sites with low densities of spawning males,

contaminants entering the bay will likely have a greater effect on reproductive success. Our studies utilized sperm to egg ratios ranging from 220:1 to 900:1, which reflects the diversity of urchin compatibility in haphazardly selected adults with high quality gametes. In natural populations, sperm to egg ratios are dependent on urchin density and local flow conditions, thus our studies may be a best case scenario and impacts might be greater at less than optimal sperm to egg ratios.

Studies utilizing various sperm to egg ratios in situ, however, would shed light on the range of potential effects of contaminants on *T. gratilla* fertilization, simulate a range of urchin densities, and embody naturally occurring pollutant exposure times. Additional studies highlighting known contaminants and their effects on *T. gratilla* fertilization would also shed light on terrestrial impacts on this important herbivore.

Management implications

Degraded reefs throughout Kāneʻohe Bay are becoming home to thousands of hatchery-reared *Tripneustes gratilla* following manual removal of invasive algae by local managers (Department of Land and Natural Resources, 2014), and after algae has been manually removed, these urchins can maintain 3% algal cover for over a year (NOAA, 2011). As a generalist herbivore, *T. gratilla* has proven to be effective against numerous kinds of invasive algae in the bay (Conklin and Smith, 2005; Stimson et al., 2007; Stimson and Conklin, 2008).

Successful reintroduction of *T. gratilla* into Kāneʻohe Bay may reverse the shift seen from coral to algal dominance in the bay. Herbivore-exclusion studies have shown that upon grazer reintroduction, a single species can be responsible for reversing the induced phase shift (Bellwood et al., 2006; Ledlie et al., 2007; Burkepile and Hay, 2008; Hoey and Bellwood, 2009**)**. Additionally, decades after the catastrophic loss of *Diadema antillarum,* recent studies in Jamaica have documented localized recovery of algal dominated coral reefs thanks to increases *D. antillarum* and *Tripneustes ventricosus* populations, and it is thought that *T. ventricosus* led the way (Edmunds and Carpenter, 2001.; Carpenter and Edmunds, 2006)

Results from our study indicate that given adequate density, sexually mature *Tripneustes gratilla* can fertilize successfully within Kāneʻohe Bay. Thus, with thousands of adults being outplanted onto the reefs, repopulation may be possible.

Conclusion

Overall *T. gratilla* exhibits high fertilization potential in central Kāneʻohe Bay, regardless of location. At the times and locations sampled, waters within Kāneʻohe Bay overall do not negatively affect fertilization in *Tripneustes gratilla*, however, we did find that fertilization was dependent on freshwater influence, date, and salinity. This suggests that there is some significant terrestrial impact from surface and/or groundwaters that is affected by date and retention.

As judged by high fertilization, water quality is relatively good throughout waters in Kāneʻohe Bay. While fertilization in this species is highly sensitive to metals (Dinnel et al., 1988), non-polar organic compounds (Vazquez, 2013), chemicals leached from certain plastics, and even bleached paper towels (author, unpublished data); current levels of contaminants within the bay do not severely impact fertilization success. Although numbers of *Tripneustes gratilla* are currently low, there is potential for their future reproductive success, which is encouraging for managers currently utilizing this herbivore to restore the bay. Thus, if *Tripneustes gratilla* is reestablished within the bay, it is likely that we could see a reversal of the current algal dominance on reefs in Kāneʻohe Bay, leading to the recovery of one of the most well-documented reef systems in the world.

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