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Western Washington University

Universidad Autónoma de Baja California Sur

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Causes of Balloonfish (*Diodon holocanthus*) stranding in the Gulf of California

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Abstract

Strandings of aquatic organisms can play an important role in the habitat structure of beaches and intertidal regions by affecting population dynamics and species composition within an area. Strandings can result from natural or anthropogenic conditions. Although cetacean strandings are relatively well understood, researchers are still sometimes unable to pinpoint the cause of a stranding. The causes and effects of fish strandings are currently not well understood. We observed several *Diodon holocanthus* populations at multiple beaches around La Paz, B.C.S Mexico, but an uneven distribution of washed up *D. holocanthus* carcasses. We hypothesized that healthy *D. holocanthus* individuals died as a result of stranding, as opposed to dying in the water and washing up at a similar rate as other passive debris. We also predicted that beach slope had an effect on the likelihood of a *D. holocanthus* stranding. We hypothesized that fish body size would effect the likelihood of stranding. We recorded the quantity of garbage and dead fish in transects at four beaches around La Paz, B.C.S and then performed an experiment on live *D. holocanthus.* We exposed 26 individuals to varying slope conditions and recorded strandings and body size. We found no relationship between the slope of the beach and the susceptibility of *D. holocanthus* to stranding. There was also no relationship between body size and likelihood of stranding. We were unable to correlate dead fish with passive debris because no *D. holocanthus* carcasses were found in our transects. We also made some observations while in the field that implicates predator evasion behavior of *D. holocanthus* and large waves created by boats as potential causes of *D. holocanthus* stranding.

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Introduction

Strandings of aquatic organisms can play an important role in the habitat

structure of beaches and intertidal regions by affecting population dynamics within an

area (Nagrodski et al 2012, Hernández-Miranda 2010). They can offer an insight to the

anatomical structures of rare species, and provide information for detailed descriptions

that are otherwise not available due to the lifestyles of the organism (Pyenson 2010).

Occasionally, mass strandings of dozens, or hundreds, of individuals can occur within a

small area and can help provide estimates of population size and species composition

(Balcmob III, 2000, Hohn 2005, Pyenson 2010).

Strandings can result from sudden and unexpected changes in the surrounding

environment, due to natural or anthropogenic causes, like oil spills, hypoxic events,

disease, dramatic drops in water level, or predator evasion behaviors (Hernández-

Miranda 2010, Nagrodski et al. 2012). Strandings can also be triggered by anatomical

causes like malnutrition, secenense, disease or infection (Pyenson 2010). Additional

triggers may be large-scale changes in the patterns of climate, complexities of nearshore

geomorphology or geomagnetic interference of navigation, however this causes are not

exlusive and may transpire simultaneously (Pyenson 2010).

A stranding occurs when an aquatic organism is restricted to a habitat with

unsuitable water depth due to physical separation from a main body of water

(Nagrodski et al. 2012). In 2000, Balcomb and Claridge documented a stranding of two

beaked whales species that washed up on shore (*Ziphius cavirostris* and *Mesoplodon*

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europaeus) in the Bahamas, and determined the cause was naval sonar. However the causes of strandings are not always known. In 2005, NOAA documented a mass stranding of three whales species (*Globicephala* macrorhynchus, *Balaenoptera acutorostrata* and *Kogia sima*). Interestingly researchers were not able to identy a link between all of the stranded individuals and a single anthropogenic or environmental condition (Hohn 2005). Despite the fact that fish stranding may be an important issue, especially when relating to anthropogenic environmental disruptions, there is currently little knowledge related to strandings of fish. Research on this subject is important in order to improve the accuracy when assessing threats to biodiversity or, if need be, to establish effective mitigation strategies.

Despite the importance of strandings for intertidal and beach ecosystems, very little is known about the causes or impacts of fish strandings. This study focused on balloonfish (*Diodon holocanthus*), which is part of the spiny puffer fish family *Diodontidae. Diodontidae* are common in tropical and temperate waters all over the world and *Diodon holocanthus* are common in shallow waters in the Gulf of California and along the Pacific Coast of Baja to Panama (Fujita et al. 1997, Humann and Deloach 2004). They are identifiable by a square shaped body with large straight spines that lie flat (Wiktorowicz et al. 2007). *Diodontidae* are rigid-bodied, undulatory median and paired-fin swimmers (UMPF), which means that they are generally smooth swimmers with maneuverability and stability at slow speeds, but have little power output and slow acceleration (Wiktorowicz et al. 2007, Blake et al. 2011, Korsmeyer et al. 2002). *Diodontidae* are unique in their anti-predator defense of inflation behavior, called

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puffing (Greenwood 2009). During a puffing event, the fish swallows water and uses its stomach to inflate up to three times its relaxed state, which deters predators (Brainerd 1994). However, the anti-predator response of inflating also has its dangers. When a pufferfish inflates, it's ability to maneuver decreases. This increases their risk of drifting into an undesirable environment, like shallow water along a beach and making them more venerable to stranding then other species.

This led us to ask the questions: what causes fish to wash up on some beaches and not others? Do *D. holocanthus* die in the water and wash up on the beach at similar rates as other passive debris, such as plastic and glass? Or are *D. holocanthus* healthy when they become stranded and die as a result?

We conducted our beach surveys and experiments at four sandy beaches near La Paz, B.C.S, Mexico, that varied in beach slope, wave speed, and amount of debris present. To see how the waves influenced the likelihood of a fish stranding, we placed the fish in a holding area that spanned from the shallow water to some exposed sand. We predicted that wave speed would positively correlate with number of dead fish. We also expected beach gradient would be negatively correlated with number of fish stranded in our natural experiment. We hypothesized stranded *D. holocanthus* died due to stranding, as opposed to dying in the water and passively washing up on the shore and therefore expected no relationship between amount of passive debris and number of dead fish. Measurements for body size were also taken because we anticipated that body size would affect the likelihood of a fish stranding in our natural experiment.

Methods

We observed many dead, and one dying, *D. holocanthus* at Balandra, a beach
near La Paz, BCS. Despite the presence of *D. holocanthus* at other nearby beaches, no
carcasses were observed. We were curious about the susceptibility of *D. holocanthus* to
stranding and what aspects of Balandra caused the increase in strandings seen. Our
study had two objectives. The first was to perform an experiment in which *D.
holocanthus* was placed in a testing corral where beach slope and wave speed mimicked
conditions hypothesized to be optimal for stranding to occur. The second was to
correlate stranded fish carcasses with beach slope, wave speed and abundance of non-
organic passive debris, such as plastic and glass. However, we found no dead fish during
our study.

For our study we selected beaches based on previous observations of fish
strandings. We sampled four beaches; Cantamar, Balandra, La Paz and Calerita, which
are all located near La Paz, Baja California Sur. Personal observations of dead *D.
holocanthus* at Balandra and La Paz designated them as possible stranding beaches. The
other two beaches were chosen because no strandings of *D. holocanthus* had been
previously noted. Cantamar was a small beach and was only sampled at one location.
Balandra was a large beach, which has four major coves, so we sampled two coves. We
eliminated the main beach because it is a very popular beach, which is routinely

cleaned. The cove on the left of the main cove was not accessible. At La Paz, we walked

along the main stretch of the beach, and numbered each beach with distinctive edges.

Out of the fifteen beaches, we chose one at random to test. At Calerita, the beach was

extremely long, so we used natural rock barriers to establish individual beaches to test,

and found two sandy beaches with different slopes.

At each beach, we documented trash and fish found within a 30 m by 2.05 m

transect. To locate a transect, we estimated the middle of the beach and randomly

choose a direction, left or right, and the starting distance from the middle. We placed

the meter tape along the edge of high tide mark, placed the of the transect along the

tape and went down beach from there. The trash we found was counted by item. Any

stranded or dead fish in the transect were noted; however no fish were found during

the study. After the transect was complete, we measured the slope of the beach and the

speed of the waves. To measure the slope, we used a straight PVC pipe, a measuring

tape and a level. To measure the wave speed, we dropped a lime into the water and

timed how long it took to become stuck on the beach.

To test our hypothesis that beach slope influenced the likelihood of a *D.*

holocanthus stranding we set up a testing corral which measured 4 meters by 1 meter

by 0.75 meters and was made from mesh and PVC piping at beaches with various slopes.

The testing corral was set up in the middle of the transect, with one end in the water

and the rest of the transect on the beach. We placed the end of the testing corral in the

water at a depth of 20 cm to 30 cm, to account for the change in height when waves

came in. The holding area measured 45 cm x 39 cm x 63 cm was set up before collection

and was used to house fish that had been tested to prevent recapture and retesting.

Once the testing corral was set up, we hand captured *D. holocanthus* by using a

small aquarium net and a mesh PVC net while snorkeling. This allowed a person to

corral the fish before scooping it into the net. The fish was then swam to shore, and

placed into a large bucket. Once the testing corral was double checked for holes, the fish

was introduced to the corral by submerging the bucket. Once the fish was securely in

the testing corral, a test began. If natural waves were not sufficient, a researcher

created artificial waves for one minute using the lid to the plastic tub, which was 45 cm

by 65 cm. If, at the end of a minute, the fish was not stranded, it was placed into the

holding area. If however, the fish became stranded within the time limit, for longer than

15 seconds, it was considered a stranding. We then returned the fish to the water. If the

fish did not strand for the full 15 seconds, and returned to the water by itself, then no

stranding was recorded. After each test, we took the fish's length, width and height

measurements**.** If, before the test started, the *D. holocanthus* puffed, we allowed the

fish to return to its normal state before testing. If before testing started, a fish became

stranded within the testing corral due to natural waves, we timed the stranding to 15

seconds and returned the fish to the water. Also, if during the test, waves from a boat or

another artificial source besides the experimental waves, caused the fish to strike the

side of the enclosure, then we paused the test and removed the fish to ensure no injury.

Our data analysis included calculations of fish volume and two statistical tests. For simplicity, we assumed a rectangular shape to estimate the volume of the *D. holocanthus*, acknowledging that it is an over-approximation. Correlation and chi-squared tests were run on five variables testing the strength of the relationships. We analyzed our data using Pearsons's product-moment correlation and Pearson's Chi-squared tests. We ran a correlation test on the number of strandings versus the angle of the beach. We ran a chi-squared test on the size of the fish versus stranding occurrence. We ran another chi-squared test on the number of times an individual fish puffed prior to the experiment and whether or not they stranded. This was to ensure the stress induced by capture was not affecting the likelihood of a stranding. All tests were run using the statistical program R.

Results

Every location had at least two strandings occur, except for La Paz where the one fish tested did not strand. 40% of fish stranded at Cantamar, 20% stranded at Balandra, and 30% stranded at Calerita. Of the 26 total fish we tested, 7 stranded, and several more fish struggled to swim against waves or even became stuck on the sand. Often times, the fish that were stranded for less then 15 seconds were unable to free themselves from the beach but they were pulled back into the deeper part of the testing corral by a large wave. Those that stranded for less then 15 seconds were not recorded as a stranding. We also observed a stranding outside of our experiment while pursuing a

D. holocanthus. One individual swam into very shallow water while attempting to evade capture, was stranded by a natural wave and had to be removed from the beach and placed into our holding area.

Beach slope was greatest at the first sandy cove at Balandra, with an angle of 12.33 degrees. The beach with the lowest gradient was the second beach at Calerita, with an angle of 3.52 degrees. The other angles ranged from 6.09 to 9.28 degrees. The highest number of strandings occurred at the transect with the lowest angle, however we found no significant correlation between the number of fish that became stranded and the slope of the beach (Figure 1, r=0.464, p=0.0354, df=4).

The volumes of the fish ranged from 14.9 cm^3 to 39.1 cm^3, however there was no relationship between the volume of a fish and stranding. 13 fish puffed a total of 26, though there was no relationship between the number of times a fish puffed and the likelihood of a stranding occurring. The natural wave speed ranged from 30 seconds per meter to 122 seconds per meter. However, we were unable to test the relationship between wave speed and number of fish stranded because the wave speed of the artificial waves was never measured.

Discussion

Contrary to our hypothesis, our data suggests that beach slope has no effect on strandings of *Diodon holocanthus*. We also predicted there would be a relationship

between fish volume and likelihood of a stranding but we found no significant

relationship. Although our methods of capturing and transporting *D. holocanthus* did

cause stress, we found no relationship between the number of puffing events and the

likelihood of them being stranded. In other studies, the water level played a part in the

strandings of live fish, however currently there is no evidence that wave action, or water

level, effects *D. holocanthus* standings (Nagrodski 2012). Unfortunately, due to the lack

of data on garbage and dead fish we were not able to determine if the *D. holocanthus*

were washing up dead, and passively along with garbage, or whether they washed up

alive and then died.

We made several important observations during data collection that warrants

provide alternative explanations besides beach morphology. During one trial a *D.*

holocanthus was in the testing corral on a calm day, the individual the beach was struck

by waves created by a large boat. These boat waves were powerful enough to cause

unnecessary stress and the experimental was halted. Had we left the fish in the testing

corral, it was likely the fish would have been stranded and possibly injured. This leads to

the question about whether unexpected boat waves are causing strandings, and that

beach morphology does not play a role. We also observed a stranding while a *D.*

holocanthus was attempting to evade our capture. This individual swam into shallow

water and was stranded by a naturally occurring wave. The predator evasion technique

this fish displayed may indicate a behavioral aspect to fish strandings. Based on these

observations, boat waves and predator evasion behaviors may be two of the causes of

D. holocanthus strandings.

Despite not finding dead fish within our transects, there were carcasses on the beach. A future study including larger transects and necropsies of freshly stranded *D. holocanthus* might be able to address the question as to whether the fish are healthy when washing up on the beach or if they are dying or dead. Though we tried limiting confounding variables, most beaches we tested were cleaned on a regular basis, thus eliminating the possibility of finding carcasses. Also, testing a wider range of body sizes might draw out a correlation between size and stranding. There is still much more investigation needed regarding the causes of fish stranding.

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Figure Caption

Fig 1. A correlation between angle of beach slope and the number of strandings per

beach transect.

Figure 1.
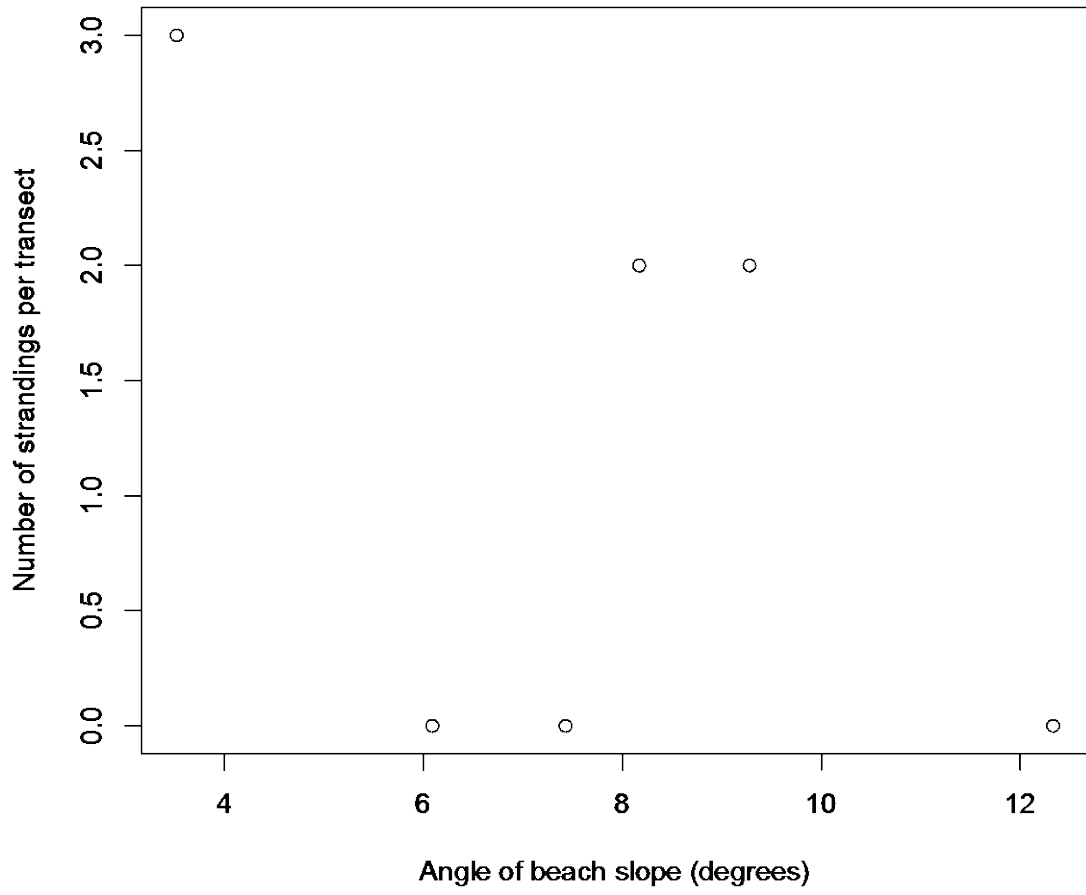
Gyovani Castaneda

Thursday, July 31st 2014

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<u>Genetic diversity comparison between the bottlenose dolphins *Tursiops truncatus* inhabiting the Gulf of California and those inhabiting the Atlantic Ocean</u>

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Abstract

Data obtained from Natoli *et al*, 2004 and Iris-Segura *et al*, 2006 was re-analyzed in order to compare the different populations of bottlenose dolphins belonging to the type *Tursiops spp.* Found within the gulf of California, the eastern north Pacific (ENP), the north western Atlantic pelTagic (WNAP), north western Atlantic coast (NWAC), south Africa (SA) and Bahamas (BAH), the mtDNA sequences were processed with CLUSTALX, a multiple sequence alignment (MSA) software that enabled us to compare the sequences yet with another software, ARLEQUIN. Results showed the molecular divergence (Fst = 0.18) along with their significance (P) values and also the migration index within the populations (M). The results obtained supported our hypotheses that had been formulated prior to the experiment, which stated that genetic divergence between bottlenose dolphin populations would increase with geographic distance and significant geographic barriers (i.e. continents). Our discussion focuses on how is it that genetic differentiation is low within bottlenose dolphins within the gulf and high compared to the populations from other parts of the world according to geographical barriers. Although dolphins are highly mobile individuals, there are ecotypes that will be isolated from other populations due to extensive risk when it comes to migration activity.

Introduction

Bottlenose dolphins are known to inhabit both hemispheres, ranging from cold-temperate to tropical waters. Although geographically close to each other,

differentiation among morphotypes is highly noticed. Ecological and environmental pressures affect the evolutional trajectories of morphological traits within bottlenose dolphin populations, thus promoting differentiation of ecotypes (diet, morphology and spatial distribution) among these groups. It is believed that founder events followed by parallel adaptations marked the distinct ecological niches with their "signature" and ever since different groups have struggled against natural selection and thus developed maintained specific morphotypes that fit into that specific ecology (Louis *et al.,* 2014). The pressure for reproduction in dolphins in high, due to the fact that females only become fertile once every four years, making them a highly precious resource for males. Interbreeding does take place within populations to maintain habitat specialization, but this is also double edged sword, since a lot of inbreeding could cause a genetic disorder within populations. Groups of males are formed in order to venture into different areas and look for females in order to spread their genes, thus homogenizing populations and making them more similar. Offshore male bottlenose dolphins are thought to be the primary diversity vectors when it comes to species gene flow, since males tend to disperse as their reproductive success is constrained by access to mates (Emlen & Oring, 1977; Greenwood, 1980), these males can travel long distances in order to breed with coastal populations from different locations. It is well documented that offshore bottlenose dolphin populations are more genetically diverse than costal ones, thus becoming the major influence when it comes to homogenizing worldwide populations. In the Pacific, mitochondrial DNA (mtDNA) genetic differentiation between coastal and pelagic bottlenose dolphins is significant, but there is no complete lineage sorting (Segura et al. 2006). Tezanos-Pinto et al. (2009) suggested that ecotype differentiation in the NWA may not be representative of genetic structuring of bottlenose dolphins worldwide.

Materials & Methods

Data-mining of the results found in two literature articles from Iris-Segura *et al.* (2006) and Natoli *et al.* (2004), which show the mtDNA sequences they obtained

through the sampling of different bottlenose dolphins. The 83 skin tissue samples from Iris' paper were obtained at different latitudinal locations throughout the Gulf of California: in the northern region they sampled at the Rio Colorado delta, south of the Midriff islands, off Bahia Concepcion, around Loreto, around San Jose and Espiritu Santo islands, in Bahia de La Paz and the southern GC. In Natoli's paper, the 269 *Tursiops* samples used were obtained from seven geographic regions, but we only included five of them, leaving out the data they obtained from the Mediterranean Sea and Gulf of Mexico. Samples from SA were collected from a coastal population described as *T.Aduncus,* while all others samples were from individuals described as *T. truncates*. Samples from WNAC and WNAP are from Hoelzel *et al.* (1988). ENP samples were from California (strandings and probably coastal habitat, but this is not known). Both sampling collections differ in that, in Natoli's investigation they got their samples from stranding and byfishing individuals, whilst samples obtained in Iris' investigation were acquired with the use of a crossbow and darts with steel collector tips. Both authors preserved their samples in a saline buffer (saturated NaCl, 20% DMSO) but only in the Iris' paper they mention 250 mM EDTA. Both authors extracted the DNA from their samples, differing in their methods, Natoli used a standard phenol/chloroform extraction method and Iris used proteinase K digestion, LiCl protein salting-out organic extraction, and ethanol precipitation (Aljanabi and Martinez, 1997) and with the DNA-easy tissue kit. Authors used different primers for amplifying their DNA, Iris used the L15812 (Escorza-Trevino et al., 2005) (50 CCT CCC TAA GAC TCA AGG 30) and H16343 (Rosel et al., 1994) (50 CCT GAA GTA AGA ACC AGA TG 30) in 25 IL PCR reactions (150 IM each dNTP, 1.5 mM MgCl2, 10mM Tris, 50 mM KCl, 0.3 IM each primer and 0.5 U Taq polymerase). Natoli used Primers KWM1b, KWM2a, KWM2b, KWM9b and KWM12a that derived from *O.orca* (Hoelzel *et al.*, 1998b), EV37Mn from *Megaptera novaengliae* (Valsecchi & Amos, 1996), TexVet7 and D08 from *T.truncatu*s (Shinohara *et al.*, 1997; Rooney *et al.*, 1999). Each author then went through the process of PCR cycling with different time lengths and annealing temperatures and consequently obtained the allele sizes with biosystem and

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gene analyzer software. Microsatellite loci were used to screen samples from different geographical areas as well as different software for multiple sequence alignments. Natoli used FSTAT 2.9.3 (Goudet, 2001) to calculate allelic richness controls for variation in sample size by a refraction method and Iris used MODELTEST 3.6 to determine the optimal model of nucleotide evolution, which was employed in the rest of molecular analyses. Iris also used ARLEQUIN to compute genetic diversity indices and phylogenetic relationships among mtDNA haplotypes were reconstructed with the program PAUP* 4.0b10.

Our methods consisted in performing the MSA with the data obtained from both authors' results and cutting the strands that were not able to aligned, "leftover strands". Then the strands were copied to a word document, so they could be arranged in a FASTA format previous to be introduced into the ARLEQUIN software for genetic differentiation testing. Population names for the groups of sequences were assigned separated into four divisions: 1: GN, 2: GI, GC, GS, SIN, BB and ENP; 3: WNAP, WNAC and BAH; 4: sAFR. In the AMOVA section, standard AMOVA computations (haplotypic format) option was selected, with a No. of 1000 permutations and conventional F-statistics were used. In the Population structure section, "Compute pairwise FST was selected and for the genetic distances, the Slatkins and Reynolds distances were selected. Compute pairwise differences (pi) was also selected as a population comparison setting with a number of 1000 permutations and a significance level of 0.05, and then the option for using conventional F-statistics was selected as well. In the population differentiation section, Exact test of population differentiation was selected, with a 1000 steps in the Markov chain as well for No. of dememorization steps. The program was set to run and the results sheet was shown. Once results were obtained, matrixes obtained from FST and M values (see table 1 and 2) were analyzed and significance values marked thick (denoted), the highest and lowest values for both Gulf of California and rest of the world were marked with different colors. Graphs were made in excel to show

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the correlation between both genetic divergence and migration indexes with distance (see fig 1 and 2).

Results

Computing conventional F-Statistics from haplotype frequencies. There is a pattern observed between the different groups, when comparing the Gulf populations within each other and then comparing them with the other populations farther away. Below the line that separates the gulf populations from the rest, FST values are significant different between gulf populations and the rest of the world (P<0.05, t= 2.82, D.F=8).

```
            N.Gulf   Gulf I.   C. Gulf   S.Gulf    Sinaloa    B.B.      E.N.P.    W.N.A.P.  W.N.A.C.  S.AFR
Bahamas
    N.Gulf      *
   Gulf I.  0.11240      *
    C.Gulf  0.09409   0.05849      *
    S.Gulf  0.09485   0.06024   0.05038      *
   Sinaloa  0.16847   0.13929   0.11442   0.11501      *
       B.B. 0.08219   0.03648   0.02776   0.02985   0.10762      *
      E.N.P.0.35812   0.37223   0.29146   0.28939   0.42609   0.34375      *
    W.N.A.P.0.13872   0.10694   0.08955   0.09044   0.16351   0.07652   0.35354      *
    W.N.A.C.0.36543   0.39655   0.29377   0.28323   0.43130   0.37687   0.64455   0.36282      *
     S.Afr. 0.19032   0.16757   0.13672   0.13634   0.22297   0.13813   0.43180   0.18576   0.43004      *
    Bahamas 0.27739   0.27974   0.21571   0.21304   0.33016   0.25311   0.60396   0.27321   0.53673   0.34081      *
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Table 1. Haplotypic tree of the 11 different populations sampled, horizontal line separates Gulf of California populations from Pacific and Atlantic populations. Data marked with yellow marker represent the highest values whilst data marked with blue marker represent the lowest. Significant values are thick black marked.

The matrix for the migration indexes correlates with the FST values from Table 1. The lower the FST value between two populations, the higher the migration index between two populations. Black line delimits Gulf of Calfornia populations and Atlantic Ocean populations. M values for Gulf of California and Atlantic Ocean are significantly different (P<0.05, t=-3.5, D.F.=8).

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-----------------------------------------------------------------
Matrix of M values (M=Nm for haploid data, M=2Nm for diploid data)
-----------------------------------------------------------------

            N.Gulf    Gulf I.   C. Gulf   S.Gulf    Sinaloa    B.B.      E.N.P.    W.N.AP.   W.N.A.C.  S.Afr.
    N.Gulf
   Gulf I.  3.94852
    C.Gulf  4.81419   8.04793
    S.Gulf  4.77137   7.80065   9.42536
   Sinaloa  2.46795   3.08959   3.86991   3.84743
       B.B. 5.58332  13.20755  17.50989  16.24938   4.14615
      E.N.P. 0.89618   0.84324   1.21549   1.22774   0.67347   0.95455
    W.N.A.P. 3.10448   5.08352   5.02831   2.55799   6.03465   0.91425
    W.N.A.C. 0.86824   0.76087   1.20201   1.26536   0.65930   0.82673   0.27574   0.87808
     S.Afr.  2.12710   2.48379   3.15722   3.16723   1.74243   3.11985   0.65794   2.19158   0.66267
    Bahamas            1.28738   1.81798   1.84693   1.01441   1.47541   0.32787   1.33007   0.43157
0.96711
```

Table 2. Matrix value of migration indexes showing how much migratory activity there is between two populations. Data marked with yellow marker represent the highest values whilst data marked with blue marker represent the lowest. Black line delimits Pacific populations and Atlantic populations. Significant values are thick black marked.

The highest value that the genetic differentiation test showed between populations that within the Gulf of California is 0.16847, which is significant P<0.0001 between the Northern Gulf and Sinaloa and the lowest being 0.05038 with a significance P value of <0.001 between the Central Gulf and Southern

Gulf. As for the Gulf of California being compared to worldwide populations, the highest value observed is 0.64455 between the W.N.A.C. and E.N.P. with a significance of P<0.001 whilst the lowest value observed is found between W.N.A.P. and C. Gulf with a significance value of P<0.001.
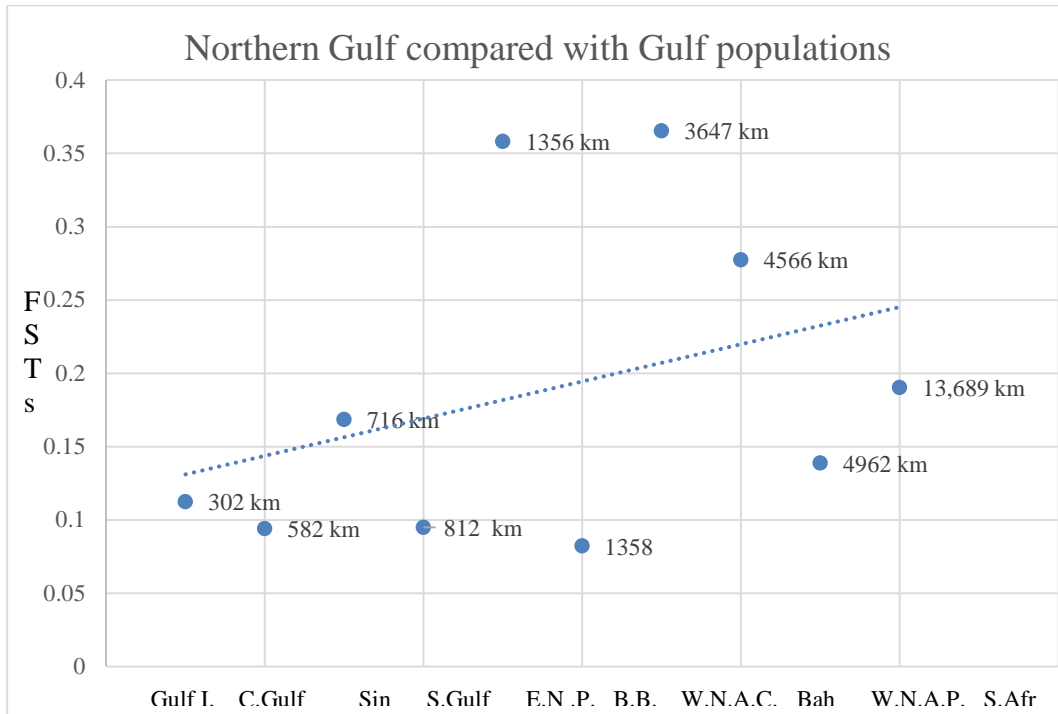

Fig. 1- Northern Gulf populations being compared with different populations (Pacific and Atlantic). Trend line is positive, suggesting that there is a positive correlation between both distance and geographical with genetic divergence.
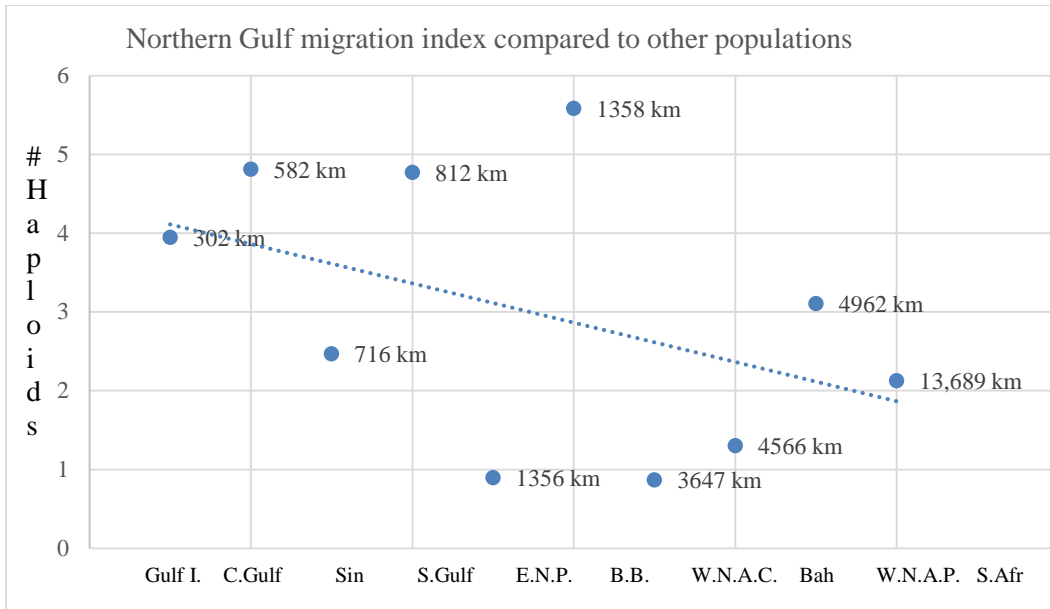
Fig. 2 - Northern Gulf migration indexes compared to other populations. There is a negative correlation of number of migrations and distance, the farther away 2 populations are, the less number of migrations there will be.

Discussion

The northern Gulf was taken as the main reference when comparing the FSTs and M values with one another because that is where Iris-Segura *et al.* (2006) got their most samples from. Geographical distance and barriers do affect the genetic divergence between populations of bottlenose dolphins. There had been thoughts about transient pods of bottlenose dolphins that migrate from coast to coast, often breeding with other populations already settled in that area, in the long run this could've had an enormous gene flow. The fact that some FSTs values are higher than others and there is less distance between those two populations might be due to the fact that populations overlap when because of excursions in response to prey movements, such as those from epipelagic fishes that are known to seek refuge in Bahia de La Paz (Arreola and Gonzales, 1997). This, however does not mean that will be genetic gene flow between populations, philopatry may help preserve gene differentiation, more likely in marine mammals (Dizon *et al.,* 1992). Specialized coastal populations are likely to only roam around defined areas, unlike offshore ones that travel in big pods and are usually unstable when it comes to area coverage. In both the W.N.A.C. and the G.C. genetic diversity was

higher in coastal populations that offshore populations, one of the reasons that could explain this may be the fact that offshore populations travel in bigger pods and so they maintain higher levels of gene diversity. The ecological conditions in the Gulf of California favor the reproductive isolation between ecotypes within it, making it harder for other pelagic populations traveling inside the gulf to breed with local individuals. However, the Gulf of California has a relatively narrow continental shelf and a complex topography of the, that could make it easier for pelagic pods to come and associate with the coast line, where deep habitats can be found close to shore (e.g. deep basins), making it harder to identify coastal boundaries.

The W.N.A.P. populations showed low divergence comparing them to the central G.C. so there could be pelagic populations interacting with coastal ones and somehow reaching as far as going around a whole continent. Some coastal groups are resident but movements were reported between Corsica and France (360 km) (Gnone *et al*. 2011), indicating that individuals crossed pelagic waters. Since female bottlenose dolphins' reproductive success depends mostly on food resources it is most important for them to be familiarized with their natal habitat and won't have to worry about dispersing (philopatry), unlike males whose reproductive success depends on finding fertile females and have to spread around large areas in order to find them. These processes could lead to large dispersal behaviors among males and thus maintaining genetic diversity at a large scale between coastal and offshore populations (Louis *et al*. 2014).

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The ingestion of thermoplastic particulates by coral reef fish *Abudefduf troschelii*

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Abstract

Approximately six and a half million tons of plastic debris ends up in our oceans annually negatively impacting over 267 species of marine organisms. The most common of this debris found is high and low density thermoplastics. Many large mammals and birds have been known to ingest plastic, but little information has been gathered on ingestion by planktivorous fish. In this study we looked at the species *Abudefduf troschelii*: a small pelagic coral reef fish that feeds on zooplankton and other microorganisms. Our goal was to see if micro-plastics would be ingested by this species. We hypothesized that the life-stage of the fish would affect the amount of plastics consumed and that there would be a preference in clear, white, and green plastics associated with their planktonic or micro-algal food sources. We tested in the field the ingestion of two high-density plastics (HDP): polypropylene and polyvinyl chloride (PVC) as well as two low-density plastics (LDP): polystyrene and polyethylene. Both juveniles and adults ingested all four types of plastics, but juveniles consumed more plastic than adults. This was due to the excessive amount of polystyrene consumed by the juveniles. A preference for micro-plastics that were pink, red, and white was observed. These colored plastics could have been confused as certain foods in their diet like mesocrustaceans and polychaetes. We concluded that the ingestion of micro-plastics by *A. troschelii* is a result of their confusion between the plastics and the species they typically consume. The plastics that were used in this experiment are known to absorb and transmit harmful chemicals. The fact that these lower trophic-level fish consumed them poses a threat to the entire food web by being transferred up the trophic pyramid.

Introduction

Plastic pollution is a serious environmental problem in the marine environment, and has only recently been recognized by the scientific and global community (Derraik, 2002). Even in the most remote regions of the world's oceans, plastic pollution is becoming a rising problem (Morris, 1980). Due to the photo-degradation of plastic objects on surface waters, most plastic particulates persist in the ocean as micro-plastics ranging from 1-5mm in diameter (Cozar, et al. 2014). Other forms of plastic breakdown consist of embrittlement and fragmentation by wave action (Cozar, et al. 2014). This material has become the fastest growing segment of the US municipal waste stream (Moore, 2008). Marine litter is now 60–80% plastic in most regions, but can be 90–95% in other parts of the ocean (Moore, 2008). Currently, there are 265 million tons of plastic produced annually, where it is estimated that 6.5 million tons of that plastic waste ends up in the ocean (Hammer et al., 2012). Oceanic plastic waste input is much higher than the quantitative output observed in the water column (Cozar et al., 2014). There are four reasons as to why this might occur: nano-fragmentation, bio-fouling, shore deposition and predation of plastics can all play a role in elimination of plastic from the ocean (Cozar et al., 2014). While the ingestion of macro-plastic debris by turtles, seabirds, and large marine mammals has been a common area of study for many researchers, the research on micro-plastics ingested by lower trophic level and planktivorous fish has not been examined as thoroughly (Boeger, et al., 2010).

As the levels of micro-plastics increase due to the breakdown of macro-plastics (Ivar do Sul and Costa, 2014), researchers are starting to focus on the ability of plastic to absorb and transport toxins to aquatic organisms via ingestion (Moore, 2008). The

general concept understood thus far is that plastic particulates present in the marine environment can carry chemicals of a very small molecular size (MW<1000 g/mol) (Teuten et al., 2009). If ingested, these chemicals can penetrate through the cell membrane and interact with biologically important molecules, which can disrupt the endocrine systems of marine organisms (Teuten et al., 2009). The knowledge on hazards correlated with the composite mixture of plastic and accumulated toxins is not thoroughly developed and is in desperate need of research (Rochman, et al., 2013a).

Our intention was to further the understanding of plastic ingestion by fish that might feed on micro-plastics, such as *Abudefduf troschelii*. Juvenile *A. troschelii* form feeding aggregations of ten to thirty individuals and feed on planktonic organisms swimming in the mid-water column or just under the surface (Hobson, 1965). Some studies reveal diet changes during the growth of damselfishes, although throughout their lifetime they continue to feed on zooplankton (Hobson, 2005). As adults, they are seen over rocky bottoms where the majority of their food consists of material taken from the rock surfaces such as algae, small crustaceans, anthozoans, copepods, tunicates, polychaetes, and fish eggs (Hobson, 1965; Moreno-Sanchez, 2009). There is not much information on this size interval, corresponding to that of zooplankton, and is due to the fact that these data are hard to track (Cozar, et al. 2014).

Because zooplanktivorous fish exemplify a large portion of the trophic pyramid within the ocean, and it is known that accidental ingestion of plastic occurs during their feeding activity, this provides a stimuli for further investigation on the effects of plastic toxins in a species and their predators (Cozar, et al. 2014). Higher trophic level predators prey upon these smaller pelagic fish and once ingested, the toxins absorbed from the

plastics can be transferred through the predator's body and eventually make their way up the food chain. (Cozar et al., 2014). "The reported incidence of plastic in stomachs of epipelagic zooplanktivorous fish ranges from 1 to 29%, and in stomachs of small mesopelagic fish from 9 to 35%" (Cozar et al., 2014). Our study aimed to find out if plastic particulates are being consumed by zooplanktivorous fish. The results from this study could be used in further research to discover if and how these toxins proliferate through the food web.

The question we asked was: does *Abudefduf troschelii* ingest planktonic-sized thermoplastics, and if so, is one type of thermoplastic more commonly ingested than others? The *A. troshelii* that were used for this experiment were collected off the shore of Hotel Cantamar in Pichilingue Bay, B.C.S., Mexico and were exposed to polyethylene, polypropylene, polystyrene, and polyvinyl chloride. We chose these plastics because they are known to be associated with toxins that can proliferate through ecosystems if broken down or unmonitored (Rochman, et al. 2013a). We hypothesized that *A. troschelii* would ingest planktonic-sized thermoplastics because they would confuse floating micro-plastics with their food source. Our second hypothesis was that juveniles would ingest more plastic than adults because juveniles consume more food particles due to their smaller body size and faster metabolic rate. We also hypothesized that *A. troschelii* was more likely to ingest plastics that are similar in color to their planktonic or micro-algal food source. Our predictions were that clear or translucent particles would be ingested the most, followed by green and white, and that there would be preference for these colors over the other colors tested (red, pink, black, yellow, brown, blue, and orange). Other studies have shown that the ingestion of plastics similar in color to those of the area's

plankton could be simultaneously consumed with the primary food sources of surface feeding fish (Boeger, et.al. 2010).

Methods

In order to induce the least amount of stress in our fish, we decided to do field tests by creating submersible cages that were anchored close to their collection site. Cages were constructed out of PVC piping, duck tape, zip ties, and gardening mesh. We attached plastic bottles to the cages for floatation to allow better observation of the fish while recording data. The 3 adult submergible cages were made into 1 x 1 x 1 meter enclosures and the 3 juvenile cages were 0.7 x 0.7 x 1 meters.

We captured our fish at night in the rocky intertidal area off the shore of Hotel Cantamar in Pichilingue Bay, B.C.S., Mexico. We used dive lights and handheld nets to capture fish. Once we had collected forty-five individuals, we divided them into their respected cage. We had five adults (~15-25 cm in length) in each designated adult cage and ten juveniles (~7-15 cm in length) in each designated juvenile cage where we kept them contained until experimentation.

After a day of acclimation, we started our first trial. This consisted of a morning and afternoon plastic feeding session where we tested one HDP and one LDP during each session. We repeated this for three days switching the types of plastics fed in the morning and the evening. Each trial consisted of two sessions where each session tested one high-density and one low-density plastic on all six submersible cages. We offered the fish plastic each day during their natural feeding times: 11:00 a.m. and 4:00 p.m.

To determine whether juveniles and adults consumed or preferred different types of microplastics, we manually fed two low-density plastics (LDP): polyethylene and polystyrene as well as two high-density plastics (HDP): polypropylene and polyvinyl chloride to the test subjects over a three day timespan. Polypropylene (HDP) was prepared by cutting up varying colors of plastic bottle caps (red, blue, and orange) and for the white polypropylene, yogurt containers were cut up and used. The polyethylene (LDP) samples were assembled from tiny pieces of plastic bags colored pink, brown, black, green, blue, yellow, clear and white. Polystyrene (LDP) was pulled apart from a Styrofoam block and polyvinyl chloride (HDP) was cut with a PVC cutter. There was only one color option for polystyrene and polyvinyl chloride, which was white.

During feeding, we released eighty plastic particulates into each cage and observed the behavior of the fish for five minutes. For the plastics that had multiple colors, the eighty pieces were split up evenly between particulates (i.e. polyethylene had ten pieces of each colored plastic and polypropylene had twenty pieces of each colored plastic) and all of the colors were released at the same time. The high-density PVC would sink, so these samples would be dropped from the top of the submersible cage. The low-density polyethylene pieces were small squares of plastic bag that would slowly sink, but would often times float out of the cage if we released them from the top. To ensure the particulates were getting through the testing site we opened the bag in the middle half of the cage and pushed it through horizontally by swishing the water towards the cage. The polystyrene and polypropylene floated to the top so it was released at the bottom of the cage. After the distribution of plastic at each cage, we collected the floating plastic with

handheld nets and discard the remnants in a waste container. We recorded the number of ingestions of plastic particulates for each trial using underwater recording tablets.

Statistical analyses were done using R (R Development Care Team, 2014) where ANOVA tests were used to test for differences among fish life-stage, plastic color and type. A blocked two factor ANOVA was performed to test whether there was a difference in type of plastic ingested, a difference in life stage of the test subjects, and whether or not there was a relationship between the life stage of the fish and the amount of plastic ingested per fish per cage per trial. Another blocked two factor ANOVA was performed and tested the variance within color preference of plastic and the relationship between plastic color and type of plastic. The last test we ran was a blocked one factor ANOVA to see if there were differences in the amount of plastic ingested for each trial.

Ethical Statement

The capture of individuals was performed as cautiously as possible. After we caught the fish, we left them in the submersible cages for one day to acclimate. Throughout the length of the experiment we made sure our test subjects were in good condition prior to running our tests. We would have stopped the experiment immediately if the health of the fish seemed to be effected, however this was never an issue. Following the plastic ingestion trials, we recollected all the plastics from the testing area. After all of our trials, we released the fish where we had collected them.

Results

Plastic particulates were ingested by *A. troschelii*. Polystyrene was ingested at a significantly higher level than the other plastics (Table 1, ANOVA, $F_{3, 48} = 27.8985$, p = < 0.001). The total number of polystyrene particles ingested was four times greater than the total number of polypropylene particles ingested, five times greater than the total number of polyethylene particles ingested, and eleven times greater than the total number of polyvinyl chloride particles ingested (Figure 1). The plastic that had the least overall amount ingested was polyvinyl chloride and had an average ingestion rate of 4.67 particulates/trial (Figure 1). Polypropylene particles had an average ingestion rate of 12 particulates/trial, polyethylene had an average ingestion rate of 10.67 particulates/trial, and polystyrene had an average ingestion rate of 50 particles/trial (Figure 1).

Juvenile *A. troschelii* ingested more plastic particulates than adults (Table 1, ANOVA, $F_{1, 48} = 22.2000$, p = <0.001). The difference lies within the fact that juveniles ingested significantly more polystyrene than the adults (Table 1, $F_{3, 48} = 24.1315$, p = <0.001). While the three other plastics (polyethylene, polypropylene, and polyvinyl chloride) showed no major differences in overall plastic ingested between the two life stages, juveniles ingested 7.33 times more polystyrene than the adults (Figure 2).

There was a significant difference in plastic ingested due to color (Table 2, ANOVA, $F_{10, 143} = 5.3331$, p = < 0.001). The plastics that were ingested the most were pink polyethylene pieces, followed by white polypropylene pieces and red polypropylene pieces (Figure 3). There were no black polyethylene pieces ingested and no blue pieces were ingested of either plastic (Figure 3). There was a significant relationship between

color of plastic and type of plastic ingested (ANOVA, $F_{1, 143} = 5.5000$, $p = 0.0204$). This was because there was a lot more white polypropylene particles ingested than white polyethylene pieces ingested (Figure 3). The average amount of white polypropylene ingested was thirteen times greater than the average amount of white polyethylene ingested (Figure 3).

This experiment lasted three days, or three trials, and we found no significant relationship between plastics ingested and trial number (Table 3, ANOVA, $F_{2, 64} = 0.789$, $p = 0.4623$). However, the overall amount of plastics ingested increased with each trial (Figure 4).

Discussion

Our first hypothesis that *A. troschelii* would ingest planktonic-sized thermoplastics was supported. Due to their planktivorous diet (Kerr, et al. 2014), they were more likely to confuse the plastics we tested on them for prey. Planktivorous organisms have a higher incidence of ingesting plastics than piscivores (Derraik, 2002). In other studies conducted in the North Pacific Central Gyre, large concentrations of micro-plastics are mixing with planktivorous organisms' food sources (Boeger, et al., 2010). About 35% of the mesopelagic planktivorous fishes surveyed after six night trawls had plastic pieces in their guts (Boeger, et al., 2010). Due to the buoyancy of micro-plastics, they are easily suspended within the water column and intermixed with surface food sources making it difficult for fish to distinguish between the plastic and their prey (Morris, 1980; Boeger, et al., 2010; Derraik, 2002).

We found a difference between the amount of plastic ingested by the juveniles and adults, thus supporting our second hypothesis. However, the reason was not proved to be because of their differing metabolic rates. Our results can be attributed to the physical and dietary changes of *A. troschelii* throughout their lifetime (Frederich, 2007). Most reef fish undergo what is called an ontogenetic modification in their diet (Frederich, 2008). This modification is stimulated by their interactions with fluctuating external factors such as habitat, food supply, and predation risk (Grutter, 2000). This in turn affects their internal conditions such as anatomical structures, behavior and physiological demands (Frederich, 2008). During the lifetime of *A. troschelii*, ontogenetic modifications alter their physiological structures, such as the size and the shape of their mouth (Frederich, 2007). Juveniles swim closer to the surface feeding on minute organisms; therefore their mouths are small and simple in structure (Hobson, 1965). As they mature, they begin to forage over the rocky bottom causing the size of their mouth to grow and the shape of their mouth to change (Frederich, 2007).

The juveniles ingested more polystyrene particles than the adults. Panamic sergeant major juveniles are known to deliberately aggregate around and feed on floating objects (Nelson, 2003). The highly buoyant nature of polystyrene and its ability to fragmentize into small pieces creates an ideal situation for ingestion by juvenile *A. troschelli* (Davidson, 2012). This plastic has a high affinity for PAHs (Polycyclic aromatic hydrocarbons) and, if consumed, these toxins are capable of transferring carcinogenic and endocrine disrupting monomers to the organism (Rochman et al., 2013b). Polystyrene is often found floating under docks and is known to expel millions of micro-plastic particulates due to the boring of isopods (Davidson, 2012).

There was a preference in the color of plastic ingested, thus supporting our third and final hypothesis. However, the colors we thought *A. troschelii* more likely to ingest were clear, white, or green and that was inaccurate. The most preferred colors of plastic by both juveniles and adults were pink, followed by white, and red. In the bay of Pichilingue, one of the main food sources of *A. troschelii* is fish eggs, which are known to have different color tones ranging between clear, white and pink (Garcia-Lopez, et al. 2004; Zavala-Leal, 2007). Small planktonic crustaceans from this area of the bay are green and red-orange in color (Aceves-Medina, 2007). This could have been the reason that *A. troschelii* ingested more red and polypropylene. Following the ingestion of pink, red and white, the green colored polyethylene was of interest to the fish. Since polyethylene is a low-density plastic and floats on the surface, this could have resembled the green microalgae found in *A. troschelii*'s diet (Moreno-Sanchez, 2009).

We found that white polypropylene was preferred over white polyethylene. This could have been due to the densities of the plastics, however we found no research on preferences for plastic densities. Another reason could be that with the polyethylene, the fish were given seven other colors to choose from, while with the white polypropylene there were only three other colors, thus increasing the likelihood that they would eat the white polypropylene.

This experiment only lasted three days so we were unable to find any long-term effects of plastic ingestion in the fish. However, hydrophobic organic contaminants have a greater affinity for polyethylene, polypropylene and PVC compared to natural sediments (Teuten et al., 2009). These plastics carry POPs (Persistent Organic Pollutants) and other studies have shown that these toxins can bioaccumulate in an animal if plastics

containing them are ingested and can directly affect the health of that animal (Rios, et al. 2007). Polypropylene in the marine environment can adsorb and transport PCBs, DDE and nonylphenols (Moore, 2008) while polystyrene has been known as a source and sink for PAH's (Polycyclic aromatic hydrocarbons) in the marine environment (Rochman et al., 2013b). There is also the threat of indirect consumption of these chemicals by higher trophic level organisms via predation on animals that initially ingested the plastic. The yellow snapper is a predator of the species *Abudefduf troschelii* (Vazques, *et al*. 2008), and is also a species commonly served in many restaurants in the La Paz area. If the yellow snapper were to eat a toxic fish, then there is a chance that humans would eventually consume the same toxins initially transferred by the consumption of plastics in our ocean. More research conducted on topics relating to plastic ingestion will provide input for conservation management, support the foundation for educational campaigns, and also provide other scientists with better evidence to demand more effort to mitigate the problem from the authorities (Derraik, 2002).

For future experiments, it would increase the well being of the test subjects if a softer type of netting was used. A reason there may have been error in our experiment was due to the collection method. For future experimentation it would be beneficial to have more people observing the cage at the same time and a more efficient form of plastic delivery.

Acknowledgements

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Table 1. A blocked 2 factor ANOVA was performed and tested the variance within life stage of *A. troschelii* ("Stage"), cage number ("Cage"), and type of plastic ingested ("Plastic"). It also tested the relationships between life stage and plastic type ingested ("Stage : Plastic") and cage number and type of plastic ingested ("Cage : Plastic").

	Degrees of Freedom	Sum Squared	Mean Squared	F-Value	P-Value
Stage	1	171.13	171.125	22.2000	<0.001
Cage	4	33.50	8.375	1.0865	0.3738
Plastic	3	645.15	215.051	27.8985	<0.001
Stage : Plastic	3	558.04	186.014	24.1315	<0.001
Cage : Plastic	12	64.06	5.338	0.6925	0.7503
Residuals	48	370.00	7.708		

Table 2. A blocked 2 factor ANOVA was performed and tested the variance within life stage of *A. troschelii* ("Stage"), type of plastic ingested ("Plastic"), cage number ("Cage"), and color of plastic ingested ("Color"). It also tested the relationships between life stage and plastic type ingested ("Stage : Plastic"), cage number and type of plastic ingested ("Plastic : Cage"), life stage and color of plastic ingested ("Stage : Color"), type of plastic ingested and color of plastic ingested ("Plastic : Color"), and cage number and color of plastic ingested ("Cage : Color"). The relationship between life stage, plastic type, and plastic color ("Stage : Plastic : Color") as well as the relationship between plastic type, cage number, and plastic color ("Plastic : Cage : Color") were also tested.

	Degrees of Freedom	Sum Squared	Mean Squared	F-Value	P-Value
Stage	1	0.024	0.02436	0.0670	0.79616
Plastic	1	1.780	1.78041	4.8961	0.02850
Cage	4	0.205	0.05133	0.1412	0.96661
Color	9	14.837	1.64858	4.5336	<0.001
Stage : Plastic	1	0.160	0.15954	0.4387	0.50880
Plastic : Cage	4	0.374	0.09356	0.2573	0.90482
Stage : Color	9	4.211	0.46793	1.2868	0.24895
Plastic : Color	1	2.000	2.00000	5.5000	0.02039
Cage : Color	36	5.862	0.16283	0.4478	0.99693
Stage : Plastic : Color	1	0.222	0.22222	0.6111	0.43566
Plastic : Cage : Color	4	0.444	0.11111	0.3056	0.87388
Residuals	143	52.000	0.36364		

Table 3. A blocked 1 factor ANOVA was performed and tested variance within cage

number ("Cage") and trial number ("Trial").

	Degrees of Freedom	Sum Squared	Mean Squared	F-Value	P-Value
Cage	5	204.62	40.925	1.6388	0.1625
Trial	2	39.00	19.500	0.7809	0.4623
Residuals	64	1598.25	24.973		

Figure Captions

Figure 1. Total ingestion of plastic particulates by *A. troschelii* over a timespan of three days with one trial/day. Plastic particulates were 1mm-5mm in diameter. The four types of plastic that were ingested were two low density plastics: polyethylene and polystyrene and two high density plastics: polypropylene and polyvinyl chloride (PVC). Error bars show standard deviation (n=6).

Figure 2. Total number of plastic particulates ingested by juveniles and adults from all three trials. Plastics ingested were polyethylene, polypropylene, polystyrene, and polyvinyl chloride. Error bars show standard error (n=6).

Figure 3. Average number of colored plastic particulates ingested by both juveniles and adults from all three trials. The thermoplastics ingested were polyethylene: a low density thermoplastic and polypropylene: a high density thermoplastic. There were eight tested colors of polyethylene and four tested colors of polypropylene.
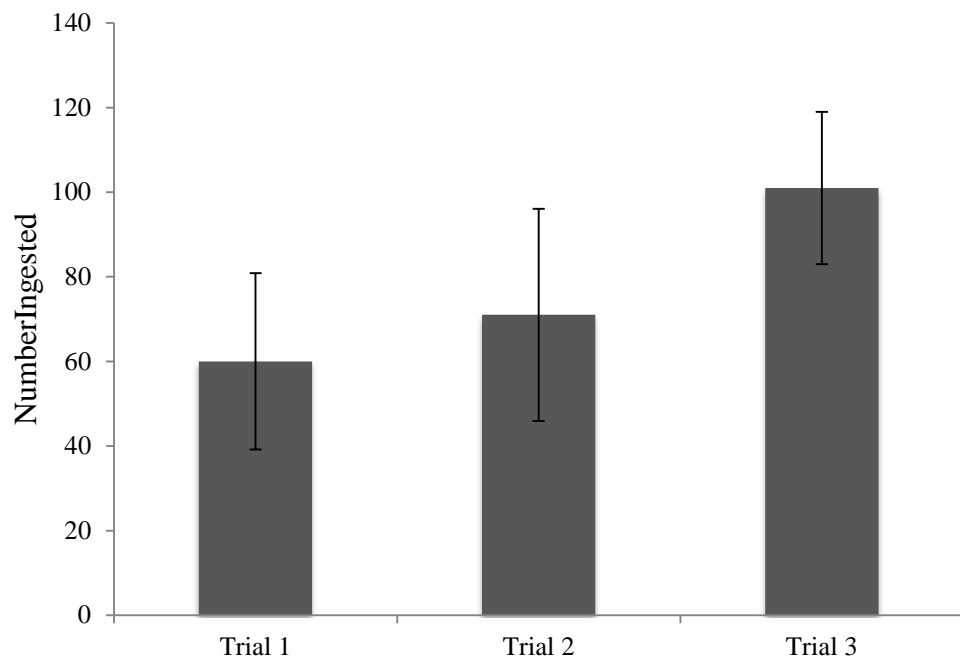Error bars show standard error (n=6).

Figure 4. Total number of plastic particulates ingested by *A. troschelii* during three trials where there was one trial/day. This data incorporates all four plastics: polyethylene, polystyrene, polypropylene, and polyvinyl chloride. Error bars show standard deviation (n=6).
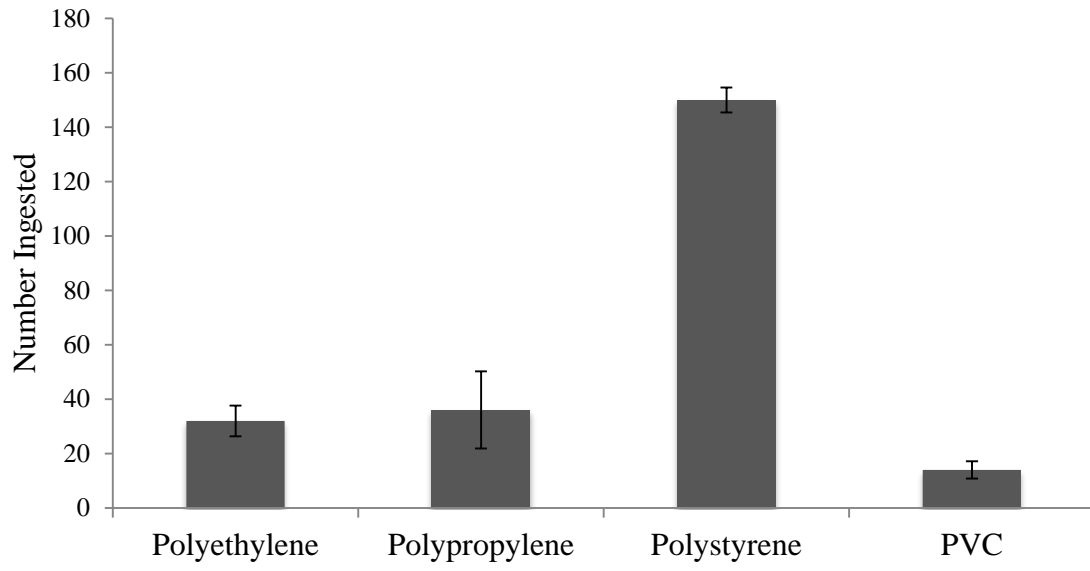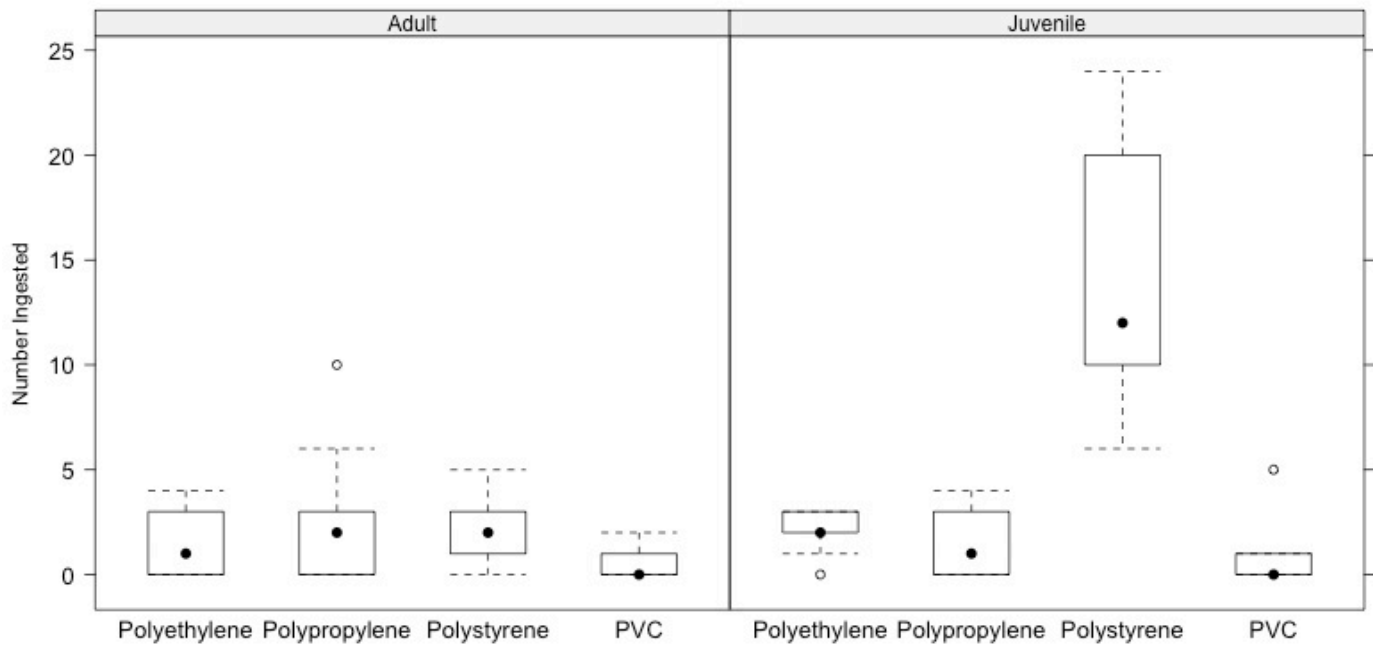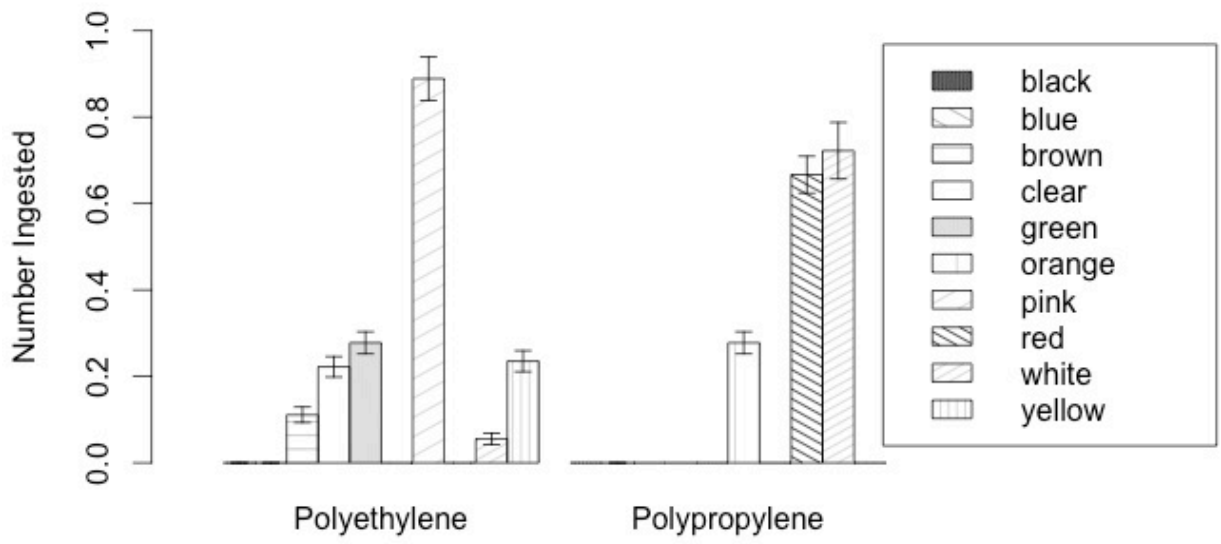
Haplotype frequencies of Green Sea Turtles (*Chelonia mydas*) involved in a mass die-off event along the western coast of Baja California Sur, Mexico.

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Abstract

Green sea turtles (*Chelonia mydas*) are found all over the world, and are an endangered species that has experienced severe population decline. Causes of this decline are often anthropogenic. The green sea turtle populations of Baja California have been subjected to poaching, bycatch, and pollution and their numbers have dropped to mere fractions of what they once were. In this study, we look at a mass mortality event that occurred in Laguna Ojo de Liebre in February 2011. Our question focused on the population structure of the turtles that died in this event, which we analyzed using mitochondrial DNA from tissue samples. We predicted that our sample haplotypes would be found in a variety of different rookeries in the Pacific Ocean. DNA from the tissue was extracted, amplified, and sequenced from 11 individuals found washed up on the beach. The mitochondrial DNA sequences were analyzed and run through BLAST to determine which haplotype and rookery they belonged to. Seven of the individuals had the haplotype CMP5 and every haplotype we had has been found at the Michoacán rookery on the Pacific coast of Baja California. That the Michoacán rookery is

geographically close to the foraging grounds of Ojo de Liebre could be a possible

explanation for this result. Other explanations include a virus or environmental factor at

the nesting grounds that selectively affected the haplotypes found at Michoacán.

Whatever the cause, a considerable number of East Pacific Green Turtles died at Ojo de

Liebre. Conservation efforts need to not only focus on nesting grounds but also on the

foraging grounds of this species.

Introduction

Green Sea Turtles (*Chelonia mydas)* are found worldwide between 30°N and 30°S in tropical and subtropical waters (Bowen et al., 1992). The large-scale population structure of green turtles depends on the natal homing behaviors of females, as they return to their original nesting grounds to lay eggs (Hirth, 1997). This behavior creates a distinct matrilineal structure at each rookery (Formia et al., 2007), and has affected the evolution of worldwide green turtle phylogenic subsets, including the East Pacific (known as the "black turtle",) and the Atlantic-Mediterranean form (Bowen et al., 1992). In this study, we focus on East Pacific Green Turtles (EPGT), which nest in the main rookeries of the French Frigate Shoals, Galapagos Islands, Revillagigedos Archipelago and Michoacán, Mexico (Chassin-Noria et al., 2004).

The World Conservation Union (IUCN) currently lists green sea turtles as endangered throughout their range. Presently, illegal fishing, habitat loss and pollution threaten the global populations of green turtles (Kasparek et al., 2001; Cliffton et al.,1982). Data on the population numbers of rookeries, and rookery trends, such as migration patterns of EPGT, are crucial to properly manage their recovery (Wallace et al, 2010). Throughout the life cycle of EPGT, migration occurs from the nesting grounds to pelagic or neritic foraging habitats (Reich et al., 2007). This movement leads to feeding grounds having a heterogeneous array of green turtle individuals from many different populations (Luke et al., 2004). Therefore, the loss of foraging habitat and mortality of turtles in these areas can negatively affect many different populations of EPGT (Amorocho et al., 2012).

Genetic sampling of haplotype frequencies in foraging grounds has proved successful in quantifying how many turtles in a feeding area are from a specific rookery and how they are being affected by human impacts (Dutton et al. 2008). This technique has proved successful in identifying genetic differences between the foraging and nesting aggregations of turtles in many areas. Dutton et. al (2008) used haplotype analysis to determine the nesting origin of green turtles in foraging grounds in Hawaii. The results of this analysis led them to conclude that the foraging populations were from one genetic stock from French Frigate Shoals nesting grounds, which had implications for management of Hawaiian green turtles. In this study as well as others, mitochondrial DNA (mtDNA) was used instead of nuclear DNA to determine population structure. Mitochondrial DNA is more useful because the populations follow a matrilineal structure and mtDNA is passed to the offspring from the mother (Amorocho et al., 2012). Therefore, the haplotypes of the mtDNA can supply insight into which specific rookeries the turtles hatched at (Dutton et al., 2008).

This technique has also been useful in analyzing the sea turtle population structure in the foraging grounds off the western coast of Baja California Sur, Mexico (Nichols, 2003). The coastal lagoons in this region serve as sheltered feeding grounds for juvenile sea turtles, while exposed coastal areas have higher proportions of adult EPGTs (Lopez-Mendilaharsu et al. 2005). Many conservation groups have identified these areas as critical feeding grounds for EPGT because of the mixed age groups and habitats in this region. However, there has been no specific action taken by the government of Mexico towards protecting these areas and the EPGT populations continue to decline (Lopez-Mendilaharsu et al. 2005). The main causes of mortality in this region have been

attributed to fishing, either poaching or bycatch from gill nets in the region (Koch et al., 2006). It has been estimated that over 3,000 EPGTs are slaughtered each year for the purpose of human consumption and a minimum of about 1,500 are annually killed from bycatch (Koch et al., 2006; Mancini et al., 2012).

In this study, we attempt to distinguish the population identity of green turtles that were found washed up on a beach on the western coast of Baja California Sur. The turtles were found all at once and numbered over 200 individuals, which implied that this was a mass mortality event. The event occurred in February of 2011 in the lagoon foraging grounds of Ojo de Liebre. When researchers examined the turtles they displayed significant signs of decomposition, so the exact day or temporal spread of mortality in this event is unknown. Approximately 30 tissue samples were taken from different sea turtles that were washed up on the beach.

In order to determine which populations these turtles belonged to, we performed haplotype analysis on tissue samples collected from 15 of the deceased turtles. We analyzed the mtDNA of the turtles to determine which rookery they belonged to. We hypothesized that the turtles sampled from this event are from different nesting populations, based on the assumption that the feeding grounds around Ojo de Liebre would display the same heterogeneity of EPGT that is found in other foraging areas. The results of this study increase our understanding of population recovery rates of rookeries in the area, and inform conservationists and managers about the populations that were negatively affected by this event.

Methods

For this study, we extracted DNA, performed PCR on, and received sequences on 17 green sea turtle tissue samples from a mass die off at Ojo de Liebre Lagoon that occurred in February 2011. Two samples were from a stranding that was not during the mass die off, and was tested for a comparison. Each of the 17 original samples was macerated and placed in an 1 ml epitube containing 300 μl of an extraction buffer, which contained 100 mM NaCl, 50 mM Tris-HCl, 15 SDS, and 50 mM EDTA. We added 10 μl of proteinase-K to each sample while stirring and incubated them at 65°C for approximately two hours. Some of the samples were not fully degraded after the incubation period, so they were mashed manually and 5 more microliters of proteinase-K were added. Once the samples were fully degraded, 300 μl of LiCl was added to each of the tubes and were continuously inverted for one minute. Samples 4, 5, 10, 8, and 9 had the chloroform added before the LiCl. We performed three consecutive washes, the first and last using Chloroform: Isoamyl Alcohol (24:1), the second using Phenol: Chloroform: Isoamyl Alcohol (25:24:1). For each wash, the chloroform mixture was added and the tubes were vortexed for 10 seconds. Each tube was then rotated for 30 minutes and placed in a centrifuge for 15 minutes. Once out of the centrifuge, the upper aqueous layer was carefully removed from the lower chloroform layer and placed in a new tube and the process was repeated.

After the third wash, 1000 μl of cold 95% ethanol and 50 μl of Sodium Acetate 3M was added to each tube and inverted several times. The tubes then sat for 12 hours at -20°C, allowing the DNA to precipitate. Each tube was then centrifuged and the ethanol was poured off, leaving the DNA pellet. Seven hundred fifty μl of cold 70% ethanol was

added to each tube and centrifuged again. Excess ethanol was discarded and the DNA

pellet was dried at 55°C. The remaining DNA pellets were then re-suspended in 50 μl of

TE buffer solution. Gel electrophoresis was then performed in an ETBr 1% agarose gel

to verify the quality of the DNA by evaluating the brightness of the DNA lines. Tubes

T10, T12, T19, T24, T26, and T20 still contained chloroform from the washing steps,

which made performing PCR on them unviable as the chloroform encases the DNA, not

allowing the necessary reactions to take place. PCR was not done on these samples, and

they were excluded from the rest of the analysis.

A PCR cocktail was prepared using 402.5 μl water, 57.5 μl buffer, 11.5 μl dNTPs,

23 μl MgSO4, 23 μl Primer R, 23 μl Primer F, and 4.6 μl Taq polymerase. The primers

used were HDCM2 and LTCM2, which are designed to target the 488 bp at the 5' end of

the mitochondrial genome control region (Dutton et al 2008). Twenty-four microliters of

this cocktail was added to 1 μl of DNA from each original samples. There were two PCR

tubes for each sample. The tubes were put through a thermocycler program for 35 cycles

that lasted a total of 2 hours 5 minutes. Another Gel Electrophoresis was done on the

PCR products to verify DNA quality. Those samples with high quality DNA were then

sent to a lab for nucleotide sequencing.

After receiving the nucleotide sequences, we used Clustalx to test the quality of

the DNA and to verify that the forward and reverse sequences were from the same

sample. The forward and reverse sequences of dirty or overly decayed DNA would not

be complimentary. All eleven of the samples had complimentary forward and reverse

sequences. Each sequence was then put through the program BLAST which matched our

sequences to existing ones to determine the haplotype of the turtle. We used data from

Dutton et al. (2008) to determine the rookery locations at which each haplotype is typically found.

Results

Five different haplotypes were identified from the 11 individuals sampled (Table 1). All of the haplotypes found occur in rookeries in the east Pacific. Seven of the ten turtles samples from the Ojo de Liebre die off had the CMP5 haplotype. Dutton et al. (2008) only found this haplotype in individuals at the Michoacán rookery. The other two haplotypes found were CMP7 and CMP12, which are both from the Michoacán rookery. All other haplotypes in our samples from the die off occur in the Michoacán rookery. The sample labeled T3 had the haplotype CMP4 (Table 1). This haplotype is on of the most common in Michoacán rookery, but has also been found in individuals from the rookeries of the Galápagos Islands and Revillagigedos Archipelago (Dutton et al., 2008). Sample 4 was not from the die off event, but stranded further south on the Baja Peninsula. The haplotype from sample 4 was CMP8, which also occurs in the Michoacán rookery, which suggests that many individuals along the entire Baja Peninsula coast are from this rookery.

Discussion

Our hypothesis was not supported by the results of this analysis, as the green turtles sampled all had haplotypes that are found in a single rookery. The only evidence of an individual belonging to another rookery population was found in sample T3, where there was uncertainty in the nesting population it belonged to, although it is very likely that it is from the Michoacán rookery. In the remaining samples, there was variance

within the haplotypes of the individuals even though the haplotypes all corresponded to the Michoacán rookery. In a study by Chassin-Noria et al. (2004), they found that Michoacán has two common haplotypes (CMP4 and CMP5) and three uncommon haplotypes (CMP7, CMP8 and CMP12). However, the population of all the beaches within Michoacán did not show significant genetic sub-structuring when the mtDNA of nesting females was tested. Therefore, it is not unreasonable to conclude that all of the individuals we sampled that had haplotypes from Michoacán belonged to a single population.

Since Michoacán is one of the closest rookeries to Ojo de Liebre, these results may be explained simply by the proximity between the nesting and foraging grounds. Also, Michoacán is the second largest rookery in this area, only surpassed recently by the Galapagos rookeries (Chassin-Noria, 2004). This could justify our results that all the sampled turtles belong to this population. There are no previous studies on haplotype frequency and population structure of green turtles in this region. Therefore, we can't compare our results to a larger sample size and determine if our results mirror the population structure of the foraging turtles that were not impacted by this event.

The source of deviation between our results and predictions in this study may have been due to our assumptions when forming our hypothesis. We determined it likely that the Baja foraging ground population structure would show the same heterogeneity as other regions often show based on previous research. However, in a previous study on population structure in Hawaiian green turtle populations, Dutton, et al. (2008) found there to be less mixing of haplotypes than most studies in other foraging areas. The results of this study indicated that in this foraging ground, there was a large majority of

turtles that belonged to one population, and a significantly lower amount turtles that belonged to different populations (Dutton et al. 2008). With our small sample size, we may have experienced a skew in the turtles sampled that only reflected the major population component of the 200 turtles that were impacted by this event. Also, from our results, we can't exclude the possibility that this event was specific to the haplotypes affected. Mortality causes, such as viruses or factors from the nesting grounds, may have been responsible for the selective death of these green sea turtles from the Michoacán rookery.

Though we can't extrapolate the data from these individuals to the entire mass die-off event, the results clearly indicate that the population of Michoacán was decreased by some unknown degree during this occurrence. These results have implications for the conservation of nesting EPGT in Michoacán, as it indicates mortality of individuals in their foraging grounds. This rookery was heavily overfished in the 1960's and 1970's for consumption of green turtles in the Northern areas of Mexico (Chassin-Noria et al., 2004). This exploitation resulted in the population of nesting females in this area to drop from 25,000 to 1,400 in the 1980's (Chassin-Noria et al. 2004). In 1986, the beaches in Michoacán were declared a natural reserve area in order to protect the EPGT nesting grounds (Alvarado-Diaz et al. 2001). However, this population continued to decline through 1999, and because of the slow-growth rate of individuals, is still considered endangered and vulnerable to extinction (Seminoff et al., 2002). Without proper management of foraging grounds for this population, it is unlikely that the population will recover to its original numbers. Human threats such as bycatch, poaching and habitat destruction of foraging grounds can have great impacts on the recovery rate of this

population, and in order to encourage population recovery, both lawmakers and communities should enforce protection of these areas.

Future studies on large mortality events should be continued in order to analyze the population structure of turtles killed, providing information on the affected populations and therefore rookeries. Emphasis should be placed on collecting adequate sample sizes that can provide broader picture of precisely which populations are being affected by these events. Haplotype sampling in this foraging region would be greatly beneficial to future research in determining a baseline population structure of turtles. Also, research on the causes of mass mortality events should be conducted to determine what steps can be taken to avoid losses of green turtles in the future.

The preservation of green turtle populations worldwide has been a cause of concern for researchers and conservationists. In order to fully manage and continue the protection of this species, efforts have to focus not only on the nesting behavior and breeding areas of green turtles, but also the foraging grounds. The preservation of foraging populations is based in enforcing poaching and bycatch laws, reducing habitat loss and carefully tracing the dynamics and numbers of each population of EPGT. A collaboration of data on the threats to the EPGT in both nesting and foraging habitats is necessary to preserve this charismatic megafauna.

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Table 1 Green sea turtle haplotypes and nesting populations of the 10 individuals sampled from a mass die off event in Ojo de Liebre lagoon, February 6, 2011. Individual with haplotype CMP8 was not from the event, and was found stranded along the west coast of Baja California on a different date.

Haplotype	Score	Identites	Nesting Population
CMP12	733 (812)	406/406	Michoacán
CMP4	733 (812)	406/406	Michoacán/Revillagigedos/Galápagos
CMP8	733 (812)	406/406	Michoacán
CMP7	733 (812)	406/406	Michoacán
CMP5	733 (812)	406/406	Michoacán
CMP5	724 (802)	404/406	Michoacán
CMP5	728 (806)	405/406	Michoacán
CMP5	728 (806)	405/406	Michoacán
CMP5	724 (802)	404/406	Michoacán
CMP5	715 (792)	402/406	Michoacán
CMP5	706 (782)	400/406	Michoacán

Figure 1 A map of Ojo de Liebre, the location of the mass die-off event of green turtle's in Baja California Sur, Mexico.

**Alarm response in *Centrostephanus coronatus* with increasing exposure to the
predatory chemosensory cues of *Heliaster kubiniji***

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Abstract

In marine environments, predators and prey emit and respond to chemosensory cues. In many cases, prey species have evolved the ability to detect these cues which can help them avoid predation by fleeing or use of other behavioral responses. This study focused on the possible acclimation of the Crowned sea urchin, *Centrostephanus coronatus* to cues emitted by the predatory Gulf sun star, *Heliaster kubiniji*. Behavioral alarm responses of *C. coronatus,* were measured in the field when contact was made with *H. kubiniji,* and compared to alarm responses exhibited when contact was made with the non-predatory Tan sea star, *Phataria unifascialis*. Responses to the predatory species were significantly greater than responses to the non-predatory species, supporting that *C. coronatus* detects chemosensory cues produced by its predator, *H. kubiniji*. To determine if the *C. coronatus* acclimated to its predator's presence, urchins were placed in tanks with *H. kubiniji* and separated by a mesh barrier. The behavioral responses of the urchins to contact with the sun star were measured at varying times of exposure, up to 40 minutes. No significant relationship was found between the response of *C. coronatus* and the time of exposure to its predator. This shows that in 40 minutes, *C. coronatus* does not acclimate to the predatory chemosensory cues of *H. kubiniji*. These species are known to live in close proximity with one another. In order to utilize energy efficiently, *C. coronatus* has evolved to expend energy only in the presence of species that are a direct threat and to sense cues for an extended period of time. These both are favorable for the fitness of *C. coronatus.*

Introduction

In any given niche, a complex hierarchical web exists, shaped by the coevolution of predators and prey. Traits involved in predation are subject to strong selection forces because survival is critically dependent on avoidance of predators as well as success in finding food (Abrams 2000). The coevolution of predator and prey occurs in a cyclical fashion where species must adapt to maximize fitness for a given environment so they can successfully forage, reproduce, and escape predation (Abrams 2000). These changes are slow to develop, as they occur through natural selection over evolutionary time periods. However, some species may be able to acclimatize behaviorally within their own lifetimes. Like adaptations over an evolutionary time scale, behavioral changes within an individual's lifetime also involve a risk-reward tradeoff between activities with high death risk and energy expenditure and meeting the necessity to eat and reproduce (Lima and Dill, 1990). Common behavioral modifications include varying feeding time or location, making decisions on when to flee, and living in a group structure (Lima and Dill 1990).

Predator and prey interactions are often dependent on the release and response to chemical cues in marine environments (Wisenden, 2000). Predators and prey give off a variety of cues (Eklöv, 2000). These cues may range from detection cues, sensed while the predator is approaching to attack, capture cues, sensed after the predator finds the prey, and ingestion cues, which can warn other prey of the predator's attack (Wisenden, 2000). The chemosensory cues released from predator species may be a result of diet (Hagen et al. 2002). Hagen et al. (2002) found that prey reacted significantly to predators only after being fed a diet of other organisms that were capable of releasing cues detectable to the prey

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individuals (Hagen et al. 2002). Prey species may have chemical cues that amplify in the digestive tract of predators, thereby creating the "signal" to which prey species react (Hagen et al. 2002).

Chemosensory cues are critical to the understanding of the relationship between predator and prey. Relatively little is known about them, despite their large role in marine interactions (Wisenden, 2000). It is important for the marine science community to attempt to understand these interactions in order to gain greater insight into the distribution of trophic levels as well as the relationships with predators and prey in a marine environment. Some studies have been conducted to increase the knowledge base surrounding chemosensory cues and have found they are highly variable amongst different species and in different communities (Wisenden, 2000). Cues can vary from zooplankton (Harvey et al., 2013), to sun stars (Escobar et al., 2011), to seahares who produce different cues in response to different predators (Kicklighter et al., 2007). These are only a few examples of how these cues are complex and ubiquitous the marine environment.

Urchins, as a prey species, respond to predatory cues in a variety of manners. As with many other species, the flight response is common among urchins (Parker and Shulman 1986) and they might move and forage when predatory species are not active (Nelson and Vance 1979). Urchins demonstrate other adaptive behaviors to avoid predation. Some species attempt to protect their skeletal structure by pulling down spines and erecting pedicellariae (Parker and Shulman 1986). Many urchins, especially relatively small ones, seek shelter in small crevices or holes in rocks (Berstein et al., 1981).

For this experiment we studied the crowned sea urchin *Centrostephanus coronatus*. It is a very common, omnivorous species of sea urchin found throughout the Gulf of California, and ranges from southern California to northern Peru. It feeds mostly on algae, but also consumes sponges, cnidarian, bryozoan, and tunicates (Vance and Schmitt 1979). *C. coronatus* is preyed upon by multiple fish species, as well as sea stars, including *Heliaster kubiniji* (Vance and Schmitt 1979).

The Gulf sun star *H. kubiniji* is a top predator in the intertidal communities, found in the Pacific Ocean ranging from California to Nicaragua. It is also common in the Gulf of California, where it was once the most common sea star, until a rapid die-off drastically reduced its abundance 1978 due to what was believed to be a bacterial infection caused by either temperature stress or pollution (Boyer 1987). Since then, the species has recovered in some areas. *Heliaster kubiniji* feeds primarily on barnacles and mussels, but is also known to consume almost anything edible, including bivalve molluscs, gastropod molluscs, sea anemones, chitons, sea cucumbers, crabs, and urchins (Boyer 1987).

The interaction between *H. kubiniji* and *C. coronatus* is interesting due to the fact that they are often found living in very close proximity. Some species of urchin can detect sea star cues from up to 50 cm away (Manzur and Navarrete, 2011, Urriago et al., 2011). Therefore, they might spend large quantities of time in contact with sun star cues, which could potentially lead to a large amount of energy allocated to defensive behavior. We predict that urchins have evolved over time to acclimatize to this cue after long periods of exposure, as not to waste energy.

The first part of our study focuses on the reaction by the *C. coronatus* to a predatory species, *H. kubiniji*, and a non-predatory species, *P. unifascialis*. We hypothesize that *C. coronatus* will demonstrate a greater defensive response to the predatory species than to the non-predatory species. The second part of our study subjects *C. coronatus* to different lengths of exposure to the predatory cues of *H. kubiniji* to determine whether length of time exposure to the predator affects the responses of *C. coronatus*. We hypothesize that for longer periods of exposure to the predatory cues of *H. kubiniji*, *C. coronatus* will acclimate and thus demonstrate less of a response to contact with *H. kubiniji*.

Methods

Response to a Predatory and Non-predatory Species

The first part of our experiment was performed in the field at Balandra in Southern Baja California to determine if a significant difference existed between response of *C. coronatus* to predatory species *H. kubiniji,* and non-predatory species *Phataria unifascialis*.

While snorkeling, we collected one specimen of both sea star species, *H. kubiniji* and *P. unifascialis*. We selected forty adult *C. coronatus* individuals, with sizes ranging from roughly 10-20 cm in diameter. Twenty individuals were used for each treatment, where contact was made with either a predatory or non-predatory sea star.   We recorded the activity level of *C. coronatus* before and after we held the one of the two sea stars on the edge of the urchin's spines for 5 seconds. We determined the activity level of the urchins based on a scale of 0 to 3 with the following assignments:

0- no movement of spines or tube feet, completely still

1- slight movement of spines and/or tube feet, slowly shifting position

2- moderate movement of spines and/or tube feet

3- rapid movement of spines and/or tube feet, obviously trying to escape

Response to Increasing Predatory Cue Exposure

The second part of the experiment was performed in tanks with individuals collected from Pichilingue Bay, in Southern Baja California to determine if *C. coronatus* acclimated to the predatory chemosensory cues of *H. kubiniji.* In order to accomplish this, we collected two *H. kubiniji* individuals and a total of thirty-five adult *C. coronatus* individuals, ranging from about 10-20cm. For each of the seven trials we performed, we collected five new urchins from the beach at Club Cantamar. Each of the five urchins was randomly assigned to one of the five treatments, including 0, 10, 20, 30 and 40 minutes of exposure to the predatory *H. kubiniji*. After each exposure treatment, the response of *C. coronatus* to contact with its predator was recorded.

For each treatment, four urchins were placed in a 0.2 square meter tank filled with 30 Liters of seawater to adequately cover the species. This tank had mesh divider with the four urchins on one side and one *H kubiniji* on the other side. These urchins were then exposed to this sun star for 10, 20, 30, or 40 minutes. Initially, a control urchin was separated and placed in tank two, of same dimensions and water amount as the first tank. It was touched with a second *H. kubiniji* individual for five seconds. Activity rating was recorded before and during contact with the same scale used in part one of the experiment. Additionally, we

recorded the total distance moved by each urchin in a span of ten minutes. We used this same procedure for each urchin after they had been exposed to the sun star for their designated amount of time.

In order to maintain the density of urchins in tank one, we removed an urchin from tank one for testing and simultaneously replaced it with the urchin whose measurements had been completed. After every trial, we rinsed out the test tanks and replaced them with new seawater.

To determine whether a predatory species invoked a larger response in *C. coronatus* than did a non-predatory species, we ran a t-test using R to test for differences in response between *H. kubiniji* and *P. unifascialis.* For the second part of our experiment we used R to run one-way ANOVAs to test for differences among time exposure treatments for both variables (distance traveled and activity level) used to gauge the response of *C. coronatus* to *H. kubiniji*.

Results

In the field experiment, all observed individuals began with little to no initial activity. When contact was made, all individuals exhibited an increase in activity level. Average behavioral responses of *C. coronatus* to contact with *P. unifascialis*, consisted of slight movement of spines and tube feet (Figure 1). When contact was made with *H. kubiniji,* average behavioral responses consisted of moderate to very rapid movement of spines and tube feet (Figure 1). *C. coronatus* reacted significantly higher in response to contact with the *H. kubiniji* than to *P. unifascialis* (Welch t test: $t_{36.582} = 5.688$, $p<0.001$). The mean activity

level response to the *H. kubiniji* was 87.5% higher than the mean activity level for *P. unifascialis* (Figure 1).

In contrast to part one of our experiment, baseline activity levels of *C. coronatus* individuals following placement in tanks for part two varied greatly. We ran a one-way ANOVA that tested for differences in total distance traveled in ten minutes (Table 1) after different times of exposure. *Centrostephanus coronatus* individuals did not respond differently over any of the various trials (One-way ANOVA: $F_{6,24}=1.211, p=0.335$) or different times of exposure (One-way ANOVA: $F_{4,24}=0.105, p=.980$) (Table 1). Lowest mean distance was 104.1 cm and highest mean distance was 136.0 cm. *Centrostephanus coronatus* neither demonstrated an increasing nor a decreasing sensitivity with increasing time exposed to *H. kubiniji* (Figure 2).

We ran a second ANOVA to test differences in activity levels among the five different time treatments and found *Centrostephanus coronatus* individuals did react significantly differently between trials (One-way ANOVA: $F_{6,24} = 2.616$, $p =.043$) and time exposure treatments (One-way ANOVA: $F_{4,24} = 4.500$, $p=.007$) (Table 2). However, the differences among means did not demonstrate a decreasing trend that we expected based upon the hypothesis that *C. coronatus* would acclimate over time (Figure 3). The activity level increased from 0 to 10 minutes and 20 to 30 minutes, although these increases are not representative of a trend that would help us support or reject our hypothesis.

Discussion

As we hypothesized, *C. coronatus* could differentiate between the two sea stars, and displayed a significantly greater response to contact with its predator *H. kubiniji* than

it did to the non-predator species, *P. unifascialis.* The ability of *C. coronatus* to differentiate between predatory and non-predatory species is an important adaption that has been demonstrated in many prey species, as well as other urchin species (Hagen et al. 2002). To counteract the effects of predation, prey species must use energy and time to defend themselves or to flee from possible attacks. While these responses may decrease the risk of injury or death, being in a constant state of defense utilizes valuable energy needed for foraging, growth, or reproduction (Urriago et al., 2011). Being able to differentiate between predator and non-predator contact allows prey species, including *C. coronatus*, to conserve energy by only reacting when threatened by a predator.

Contrary to our hypothesis, the increasing time of exposure to *H. kubiniji* did not have an effect on the distance *C. coronatus* would flee or the activity levels demonstrated by *C. coronatus* in response to the predator's contact. Therefore, there is strong evidence that *C. coronatus* does not acclimate to the predatory chemical cues of the sea star in a 40-minute time range. These results are consistent with some other studies. Escobar and Navarrete (2011) examined the response of chitons and limpets to *Heliaster helianthus*. They studied three different levels of contact: frequent, occasional, and never. The results showed that the greater contact correlated with greater escape responses from the predator (Escobar and Navarrete, 2011). The prey species did not acclimate after living with the predator. In fact the limpets and chitons that had never had contact with the predator, and presumably the cue also, did not demonstrate escape responses. Because *C. coronatus* can be found in close proximity to *H. kubiniji,* successfully recognizing the cue from *H. kubiniji* may be more likely, than if the two species were to live in different

environments. Therefore, even after 40-minutes of exposure to *H. kubiniji*, *C. coronatus* can still successfully recognize the cue.

C. coronatus utilizes the method of escape in order to avoid predation by *H. kubiniji.* Although *H. kubiniji* is a top predator, it still tends to select prey that are the least energy expensive to catch and consume, and from which it can gain the most energy (Gaymer et al., 2004). Some urchin species have the ability to outrun sun stars, as demonstrated by the study Urriago et al. (2011) where *Tetrapygus niger* was able to flee from the *Heliaster helianthus* on flat surfaces. However, if trapped by a rock or another organism, *T. niger* were more easily caught and consumed (Urriago et al., 2011). Due to the urchins' ability to detect cues and flee, preying upon them is often not energy effective for the *H. kubiniji*. This is contrary to our hypothesis that constant defense would be an inefficient use of energy for the *C. coronatus*. In order to survive it needs to be able to sense the predator, even after long periods of exposure, because its primary tactic against predation is escape.

The movement of urchins from one tank to another was frequent and it is difficult to gauge the amount of stress they incurred. Initial movements when placed in tank two were often high, likely due to stress and moving urchin from an aggregation. In some cases urchins have been known to be more responsive when not in aggregations (Dumont et al., 2006). It is possible there would have been larger responses if the urchin did not have to be moved for each individual test. Therefore, in future experiments, each *c. coronatus* individual should be in an individual tank from which it will not be moved under the course of the experiment to avoid additional stress. Additionally, all data from part 2 had to be recorded in one day because resources did not allow for sun stars to be

kept overnight. Future experiments should be performed under strictly monitored conditions in a lab, allowing for more individuals to be kept for longer so more data could be recorded.

Natural selection favors traits that allow organisms to successfully acquire food, while simultaneously preventing their own predation. In the case of *C. coronatus,* predation is often avoided by the flight response. In this study we found that *C. coronatus* reacted significantly more to a predatory species than a non-predatory one, and that they did not adapt to the cue emitted by *H. kubiniji* over a 40-minute time period. Both of these findings demonstrate a critical function for their overall fitness. By sensing the cues of a predatory species and only utilizing energy to escape species that are an immediate threat, they are able to respond to predation in the most efficient way possible.

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Table 1. Results from one-way ANOVA for five treatments, comparing time exposed to *H. kubiniji cue* with distance traveled by *C. coronatus* in ten minutes.

	Degrees of Freedom	**Sum Squared values**	**Mean Squared Values**	**F value**	**P value**
Trial	6	68689	11448	1.211	0.335
Time	4	3967	992	0.105	0.980
Residuals	24	226971	9457		

Table 2. Results from one-way ANOVA, for five treatments, comparing exposure time with difference in activity rating for *C. coronatus* after contact with *H. kubiniji* and before contact.

|	**Degrees of Freedom**	**Sum Squared values**	**Mean Squared Values**	**F value**	**P value**
Trial	6	14.80	2.467	2.616	.04278
Time	4	16.97	4.243	4.500	.00745
Residuals	24	22.63	.943		

Figure 1: Mean activity ratings of *C. coronatus* in response to contact with *H. kubiniji* and *P. unifascialis*. These responses were tested in the field, using 20 different urchins for each asteroid.

Figure 2: One-way ANOVA comparing the time exposed to *H. kubiniji* cue with the distance the *C. coronatus* traveled in the ten minutes after contact with *H. kubiniji*.

Figure 3: One-way ANOVA comparing the time exposed to *H. kubiniji* cue with the difference in activity rating of *C. coronatus* after contact with *H. kubiniji* and before contact.
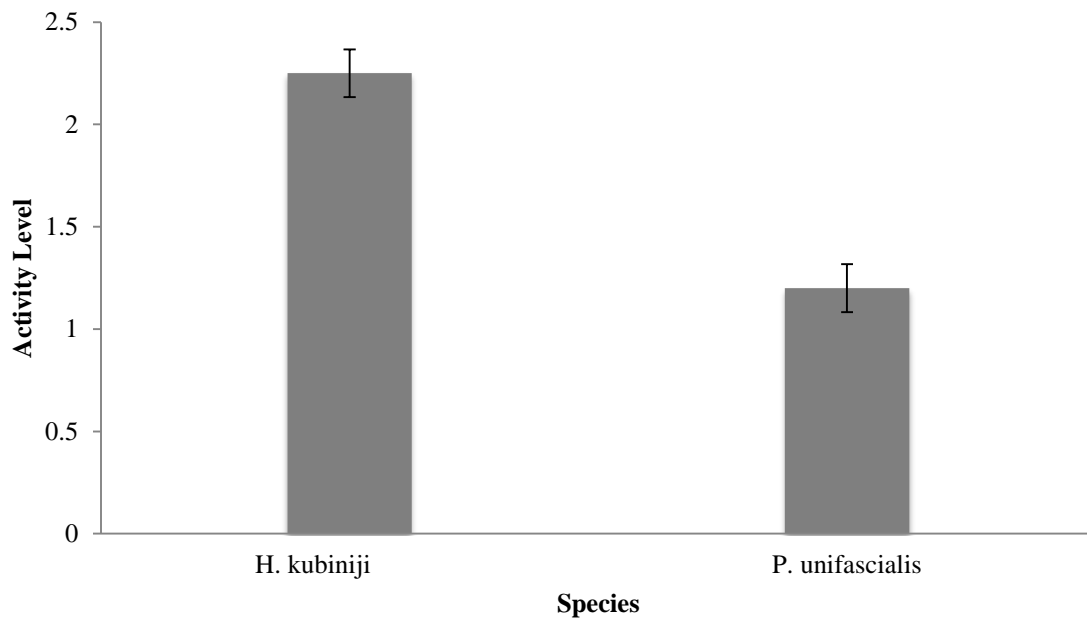
Figure 1.
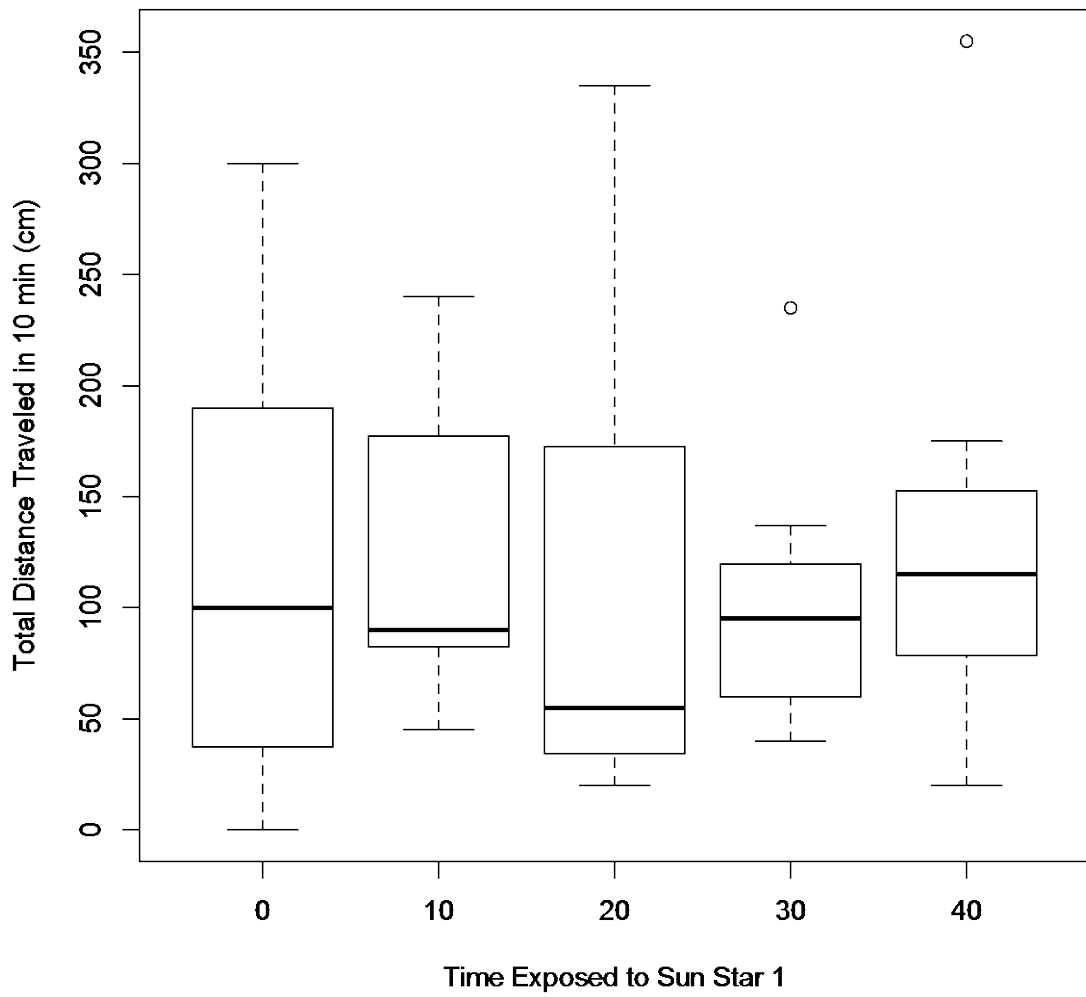
Figure 2.
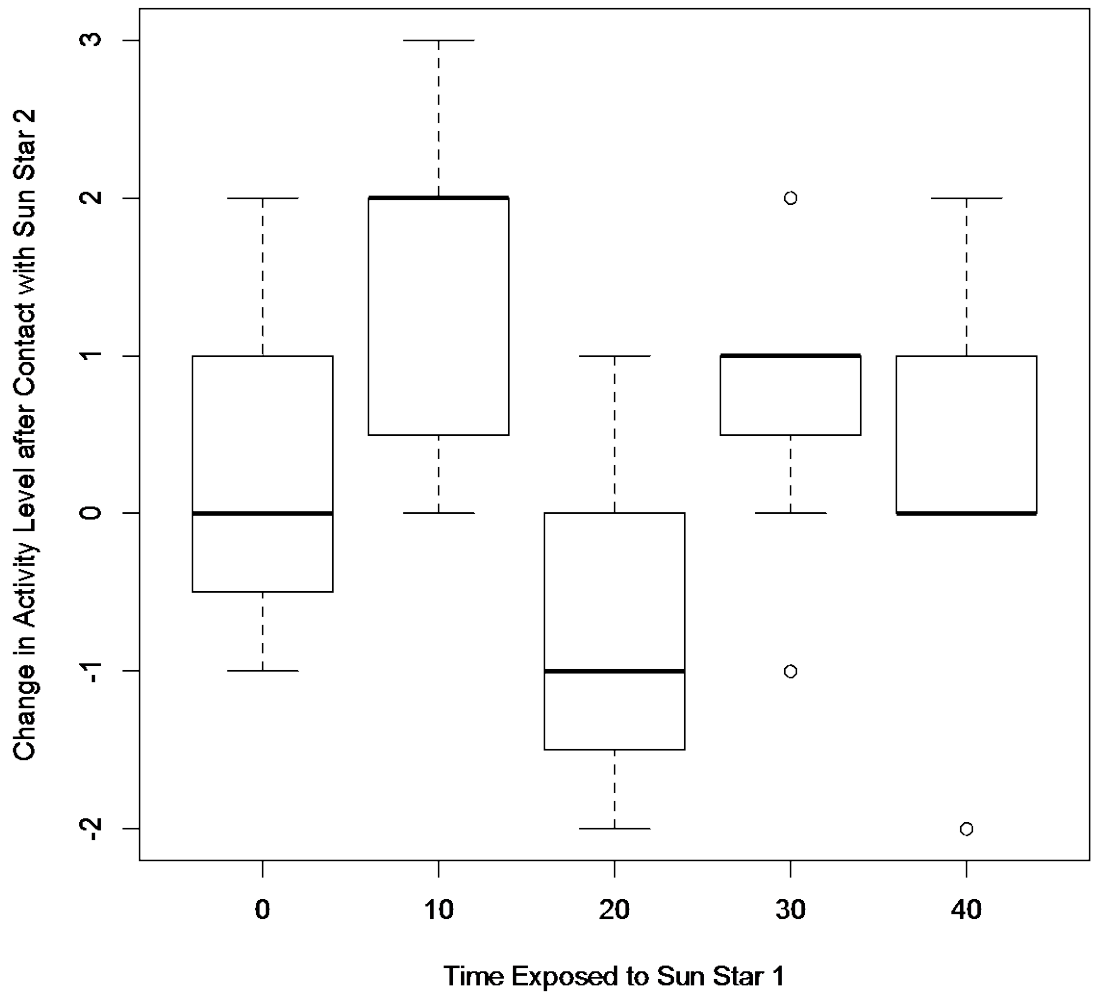
Figure 3.

The effects of temperature, diameter and arm number on the righting response of sea stars in the Gulf of California

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Abstract

Marine environments are experiencing changes in many abiotic factors such as sea temperature, which has significantly increased over the past 30 years. This change has had many implications on marine organisms, such as sea stars. Any stressor that influences the distribution or behavior of sea stars could have major influence over the entire ecosystem because of the sea star's predatory nature as a keystone species. Righting response, or the ability to correct itself after having been flipped over, of sea stars has been used as an indication of their response to environmental changes. The question we were interested in answering was how different temperatures affected the righting response time of sea stars, which could give us a better idea of their temperature tolerance. We also investigated how arm number and diameter of the stars affected righting time. We hypothesized that righting response time would decrease as temperature increased. We also postulated that righting response time would decrease with decreasing diameter as well as with increasing arm number. The three species (*H. kubiniji, P.unifascialis*, and *P. pyramidata*) were collected at two sites along the southern part of the Gulf of California. Their righting response was tested in acute treatments of seawater ranging from 29-36° C. Morphological measurements of diameter and arm number were collected after each individual had righted. There were no significant trends in our results. The data suggest that righting response is independent of 1) the experimental temperatures tested 2) temperature change from treatment to treatment 3) diameter of the star and 4) number of arms a star has in the case of *H. kubiniji*. These species seem to be tolerant of temperatures above their natural tolerance range which suggests their distribution will not change as ocean temperatures rise.

Key Words
Temperature, righting response, Heliaster kubiniji, Pharia unfascialis, Pharia pyramidata, morphological traits.

Introduction

Marine environments are experiencing changes in many abiotic factors such as ocean acidification, sea level, and sea surface temperature. Sea surface temperature has significantly warmed during the past 30 years along more than 70% of the world's

coastlines. The average rate is 0.18 °C per decade (IPCC, 2014). Abiotic stress such as temperature change has been shown to affect feeding and reproductive behavior in echinoderms (Lawrence, 1990). A direct effect on feeding or reproduction could influence the distribution and abundance of species. Temperature has been shown to be the limiting factor of the distribution of a sea star in the Gulf of California (Morgan and Cowles, 1996). Any stressor that influences the distribution or behavior of the sea stars could have major influence over the entire ecosystem because of the sea star's predatory nature as a keystone species (Cintra et al. 2005).

Righting response of sea stars has been used as an indication of their response to environmental changes (Stickle and Rice, 2009). The righting response of sea stars can be defined as the ability when placed on their aboral surface, to turn over onto the normal position with the oral surface down (Polls and Gonor, 1975). Stickle and Rice measured righting response to determine the temperature tolerance of a sea star from the Pacific coast of North America. Righting time can also provide an organismal indication of both biotic and abiotic stress on sea stars (Lawrence and Cowell, 1995).

Two biotic factors that may play a role in a sea star's ability to right are the number of arms it has and its diameter. Although not much research has been conducted on the effect of non-autotomized arm number on righting response or diameter on righting response, we hope to investigate these relationships further.

Three species of sea star were tested in this experiment. The sea star *Phataria unifascialis* has a mostly tropical distribution from the Gulf of California to northern Peru and the Galapagos Islands. It is found in the rocky intertidal as well as depths up to 139 meters (Kerstitch and Bertsch, 108-110). *Pharia pyramidata* has a similar distribution to

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P. unifascialis but can also be found on the west coast of the Baja peninsula. *P. pyramidata* is also found in the rocky intertidal as well as depths up to 139 meters (Kerstitch and Bertsch, 108-110). The sea star *Heliaster kubiniji* is distributed throughout the Gulf of California and as far south as Nicaragua. It lives in the rocky intertidal as well as the subtidal to depths of 37 meters (Kerstitch and Bertsch, 108-110). *H. kubiniji* is different from the *P. unifascialis* and *P. pyramidata* in that it has a range of 20-25 arms (Kerstitch and Bertsch, 108-110).

The three species were collected at two sites along the southern part of the Gulf where surface seawater seasonal fluctuations range from 17°C to 31°C (Morgan and Cowles, 1996). *H. kubiniji* was collected at Balandra beach. The specific site at Balandra where we collected was semi-isolated with frequent disturbance of recreational activity from snorkelers and boats. *P. unifascialis* and *P. pyramidata* were collected at Club Cantamar. The beach at Cantamar had about the same amount of recreational activity as Balandra, except there was much more commercial boat activity. Also at Cantamar, the rocky intertidal was manmade from large stones, whereas at Balandra, the rocky intertidal was naturally made.

The aim of this study was to quantify how a stressor (temperature) affected these sea stars. This was measured through the difference in righting response times over a range of temperatures. We hypothesized that each species would have decreased righting response times when exposed to warmer seawater treatments compared to ambient seawater treatments due to the increased metabolic rate of the organisms at a higher temperature. We also hypothesized that there would not be a change in righting response

due to the stress imposed on the organism by the acute temperature change, negating any metabolic activity increase.

Two morphological characteristics of sea stars were looked into as well. We asked if diameter had an effect on the righting response time. We hypothesized that sea stars with smaller diameters would have shorter righting response times than sea stars with larger diameters because stars with smaller diameters would have less surface area to move. Specific to *H. kubiniji*, we questioned how the number of arms affected righting response. We hypothesized that *H. kubiniji* individuals with more arms would have quicker righting response times than individuals with fewer arms, because more arms would provide more opportunity to make contact with the substrate and flip over.

Methods:
Experimental Protocol

Each species of sea star had slightly different experimental protocols due to a lack of resources and ability to control experimental variables. The stars were tested in both hot and ambient seawater treatments to see if the temperature had any effect on the amount of time they took to right themselves after being flipped over. A star was considered righted when its central disc was parallel to the bottom of the tub. The experiment took place in 60x43x47 centimeter plastic tubs filled with about 10 liters of seawater, all kept in the shade so as to reduce the heating effect of sunlight. The righting time, diameter, and number of arms were recorded for each star.

Eight *H. kubiniji* were collected from the intertidal of Balandra (where surface seawater temperature was 29°C at 10:00 AM) and transported to Club Cantamar. The beach at Club Cantamar had access to a stove which improved the efficiency of heating

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seawater compared to heating it with sunlight. At Cantamar, the eight stars were stored in two of the plastic tubs filled with ambient (31°C) seawater. The tanks did not have water filters so water changes were done every hour to simulate the natural environment and to dispose of any waste. Hot seawater treatments were prepared in a tub using ambient seawater and enough hot seawater (prepared on the stove) to reach the 32°C mark. Each star was placed aboral surface down in the acute treatment of hot (32°C) seawater, and the amount of time they took to correct (right) was recorded in seconds. Each star's diameter was measured in centimeters three times and averaged, and the numbers of arms were counted. After righting in the 32°C treatment, they were returned to a holding tub filled with ambient temperature seawater different from the one the untested stars were held in. After each trial, the water was removed from the acute treatment tub and a new hot treatment was prepared for the next star. One star was tested for each hot treatment and the same tub was used for all hot treatments. After all the trials were completed, the stars were returned to Balandra and allotted a 10 minute resting period in the intertidal before the next trials began, to decrease the stress induced by transportation. The same experimental protocol was followed using the ambient sea water, which was 31°C at 6:30 PM. After each sea star had righted itself, it was released into the area in which it was obtained.

Four days later, ten *H. kubiniji* were tested at Balandra following the same protocol, in ambient 30°C seawater. There is a distinct possibility at least one of the stars on the second day of testing was one of the individuals tested on the first day.

Fourteen *P. unifascialis* and nine *P. pyramidata* were collected from the intertidal and subtidal areas surrounding Club Cantamar over a three day period. Experiments were

conducted on the resulting beach. The stars were held in two tubs containing ambient temperature water, which varied throughout the day. Each star was to be exposed to 3 different temperature treatments, an ambient for the time of day, and 2 randomly assigned heat treatments ranging between 29°C and 36°C. Seawater was heated on the stove and then mixed with ambient seawater to create the random hot treatments. The treatment order was randomly assigned (via coin flip), and the righting time was recorded. Between each treatment, each star was given at least a ten minute resting period in it's own ambient temperature tub. After the three trials were completed, the star's diameter was measured three times in centimeters and averaged, and the number of arms counted. The star was then returned to the area from which it was acquired. Water was changed out of the tubs after each star had completed the experiment, and new water was used for the next star.

Statistical Analyses in R

A correlation test was performed to determine if there was a significant trend in the relationship between *H. kubiniji* arm number and diameter (α=0.05). This test was run because if there was a significant correlation between arm number and diameter, then only one of these factors would be analyzed with righting response time. There was no significant correlation, so arm number and diameter were analyzed independent of each other with righting response through a linear regression. The tests concerning variable arm number were only conducted on the *H. kubiniji*, because they were the only species in our experiment that had a variable arm number (20-25 arms) (Kerstitch and Bertsch, 108-110). Linear regression models were used to determine if there were any significant trends in 1) experimental temperature and righting time, 2) diameter and

righting time, and 3) changes in temperature between treatments and righting time (α=0.05).

Results:

Linear regressions of righting response times over the range of temperatures were consistent across the three species. *P. pyrimidata* experienced no significant change in righting response time over the range of experimental temperatures (p>0.05) (Figure 1). The quickest righting response time of *P. pyramidata* was 80 seconds and the longest 889 seconds, with an average righting response time of 183 seconds. Temperature had no significant effect on the righting response time of *P. unifascialis* (p>0.05) (Figure 1). The quickest righting response time of *P. unifascialis* was 92 seconds and the longest 2009 seconds, with an average time of 359 seconds. *H. kubiniji* also experienced no significant change in righting response time over the range of experimental temperatures (p>0.05) (Figure 1). The quickest righting response time of *H. kubiniji* was 70 seconds and the longest 1198 seconds, with an average time of 190 seconds.

The change in temperature that each sea star experienced from treatment to treatment was also analyzed to see if there was an effect in righting response time. Data from *P. pyramidata* showed no significant effect of change in temperature between experimental treatments on righting response time (p>0.05) (Figure 2). *P. unifascialis* experienced no effect in righting response time as temperature changed from treatment to treatment (p>0.05) (Figure 2). Data from *H. kubiniji* also showed no significant effect of change in temperature between experimental treatments on righting response time (p>0.05 (Figure 2).

A Pearson's product-moment correlation test revealed no significant effect of arm number on diameter in *H. kubiniji* (p>0.05) (Figure 3), so both of these factors were entered into a linear regression to see if either of them had an effect on righting response time. The relationship between righting response time and diameter was consistent over the three species. Data from *H. kubiniji* showed no significant effect of diameter on righting response time (p>0.05) (Figure 4). The average diameter of *H. kubiniji* in our experiment was 17 cm. The data collected from *P. pyramidata* showed no significant effect of diameter on righting response time (p>0.05) (Figure 4). The average diameter of *P. pyramidata* in our experiment was 19 cm. *P. unifascialis* also experienced no effect of diameter on righting response time (p>0.05) (Figure 4). The average diameter of *P. unifascialis* in our experiment was 18 cm. Arm number had no significant effect on righting response time in *H. kubiniji* (p>0.05) (Figure 5). The *H. kubiniji* used in our experiment had an average of 21 arms.

Discussion:

As stated in the results, there were no significant trends in our data. The data suggest that righting response is independent of 1) the experimental temperatures tested 2) temperature change from treatment to treatment 3) diameter of the star and 4) number of arms a star has in the case of *H. kubiniji*. The possible explanations for these results could be based in our methodology or the biology of the organisms.

Our methodology was limited mainly by the challenges of field research. Acquiring and maintaining a set temperature was incredibly difficult at Balandra, causing the move to Club Cantamar. At Club Cantamar, we had the ability to rapidly heat sea

water (on a stove) that allowed quicker trials. The tradeoff for this was that the *H. kubiniji* at Balandra needed to be transported to Cantamar. This transportation may have induced a great deal of physiological stress on the stars, which may have affected the results despite the rest period in the ambient temperature containers. This may have been prevented by allowing time for the organisms to acclimate to the new substrate/environment. Another problem was the assumption that temperature of the habitat was surface temperature. Although this assumption may have been correct for *H. kubiniji*, who were found very close to the surface, it was not for *P. unifascialis* and *P. pyramidata*, who can be both be found at depths up to 139 meters (Kerstich and Bertsch, 108-110). Most asteroid populations are not effected by surface temperature because very few populations live in the intertidal or very shallow subtidal (Cintra et al., 2005).We used surface temperature as our set ambient point and executed the experiment with temperatures greater than it. This may have affected the results because the ambient temperatures that were supposed to simulate the natural environment were not accurate of the natural environment. One aspect of our methodology that proved successful was that randomizing the change in temperature from treatment to treatment did not have an effect on righting response.

The other explanations of these results are based in the biology of these organisms. A study done on the sea star *Pisaster ochraceus* found that their aquatic oxygen consumption (as an assay for metabolic rate) significantly increased over a range of 15-25 °C (Fly et al., 2012). It is possible that the metabolism of the stars in our experiment did increase in the warmer temperature treatments, but we suggest this

increase was negated by the stress of being placed in the acute treatment which is supported by our data. Our stress cancellation hypotheses was supported.

Results from a study done on *Leptasterias spp.* differed from ours. Righting response times of *L. spp* were measured in different water temperatures over a period of 160 hours. Initially, stars in 14°C and 9 °C water had the same righting response time, but over time, the stars in the 14 °C treatment had a significantly quicker righting response time (Stickle and Rice, 2009). This shows that after stars were acclimated to a higher temperature, they were able to right faster. These results suggest that acclimated sea stars do not have as much physiological stress compared to stars in acute treatments. Acclimation reduces the stress on sea stars which allows the effect of metabolic rate to play out more clearly as a result.

A similar study to ours done on *P. unifascialis* showed that righting response times did not significantly differ over a range of 17-27 °C (Morgan and Cowles, 1996). These temperatures fall within the range *P. unifascialis* naturally experience. Although we used experimental temperatures above the natural range our species experience, it is possible the experimental temperatures were in the tolerance range of the three species. We did not hypothesize about this, but it does support our results showing there was no significant effect of temperature on the sea stars. It is possible that the range of experimental temperatures used were too subtle to have any significant influence on the righting times of the stars tested.

The results of the analysis of the morphological measures of the stars did not support our hypotheses that the righting response was affected by diameter and arm number. This suggests that righting response is independent of these morphological

features. Not much is known about the relationship between arm number and righting response time, independent of temperature. Studies have been conducted about the behavior and energetic implication of sea stars with autotomized (shed by the sea star) arms, but not on the natural numbers of arms (Ramsay et al., 2001). Further investigations over a larger range of diameters (with the addition of mass) and arm number may produce valuable information.

Several conclusions can be made from the results. First, righting response time is independent of temperature due to the stress induced by the acute treatment. Second, temperature change from treatment to treatment does not influence righting time. Third, morphological features, including arm number and diameter, have no effect on righting response time of sea stars in the Gulf of California. As ocean temperatures continue to rise, the distribution of the sea stars we tested will not be affected as they seem to be tolerant of temperatures above which they naturally experience, as our data suggest. Further research could include measuring the metabolic rate of these species in different water temperatures. Also, a broader range of temperatures should be tested so as to better determine the tolerance range of these species. Although there were flaws in this experiment, further investigation should be done on this subject to maintain the integrity of ecosystems dependent on the predatory nature of sea stars.

We would also like to extend our gratitude to Denielle Beenken, Jessica Stanley, and Brittany Weiss who helped us during our data collection.

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Figure 1. Righting response times of *P. pyramidata* (a) (n=9), *P. unifascialis* (b) (n=14), and *H. kubiniji* (c) (n=18) subjected to experimental and ambient seawater treatments.
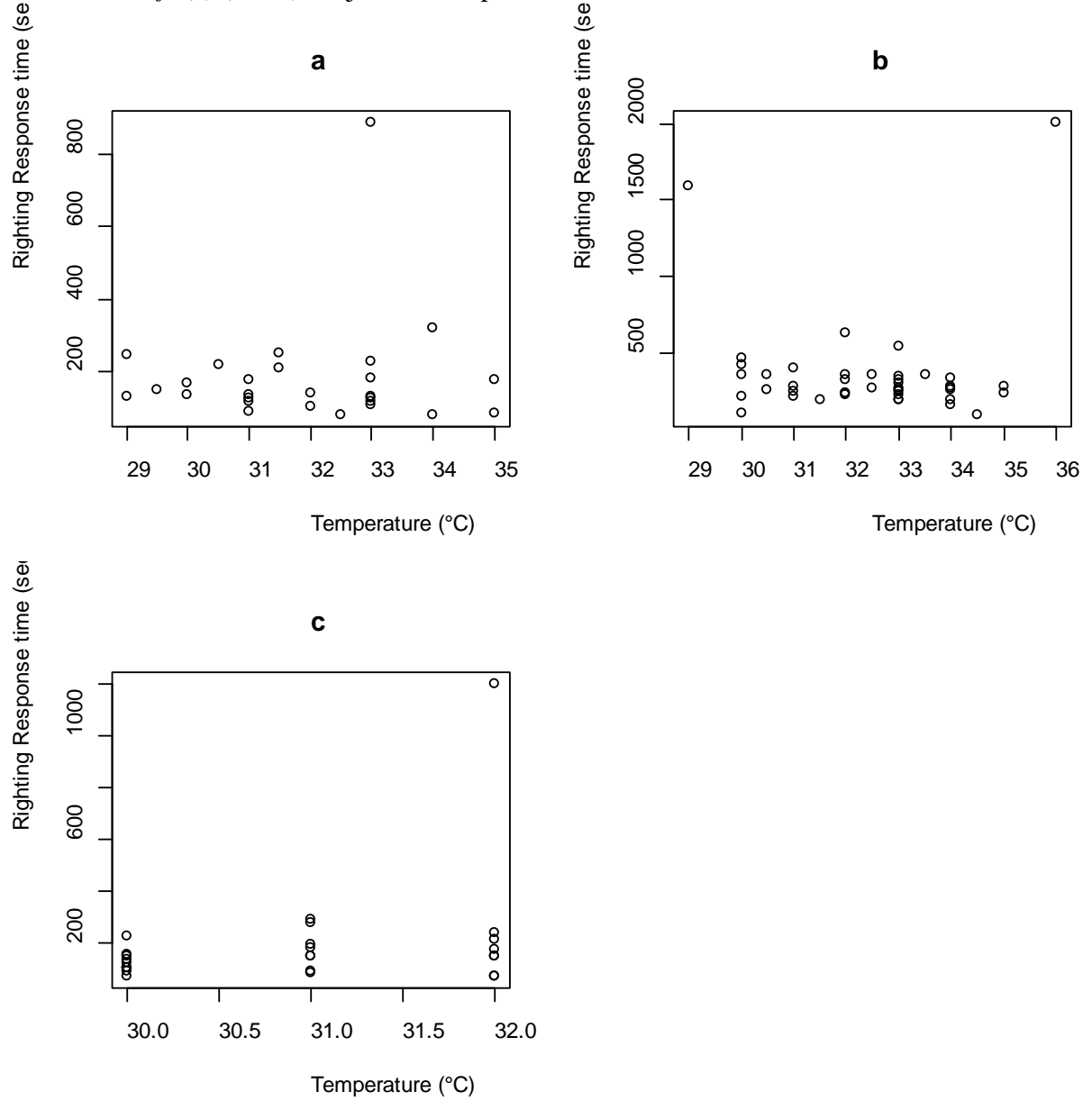
Figure 2. Righting response times of *P. pyramidata* (a) (n=9), *P. unifascialis* (b) (n=14), and *H. kubiniji* (c) (n=18) after a change in temperature. The change was calculated based on the difference between the previous and the current treatment temperatures.
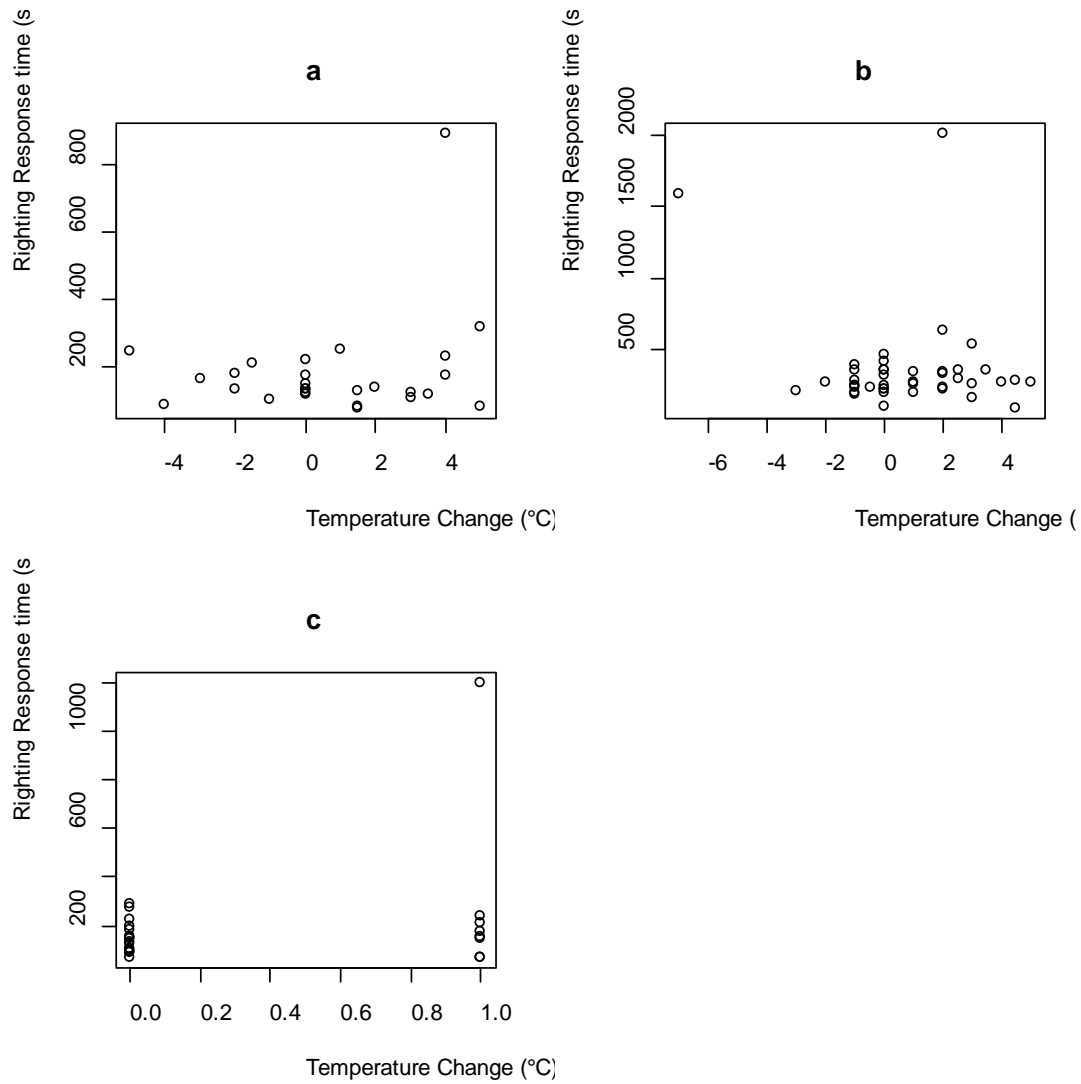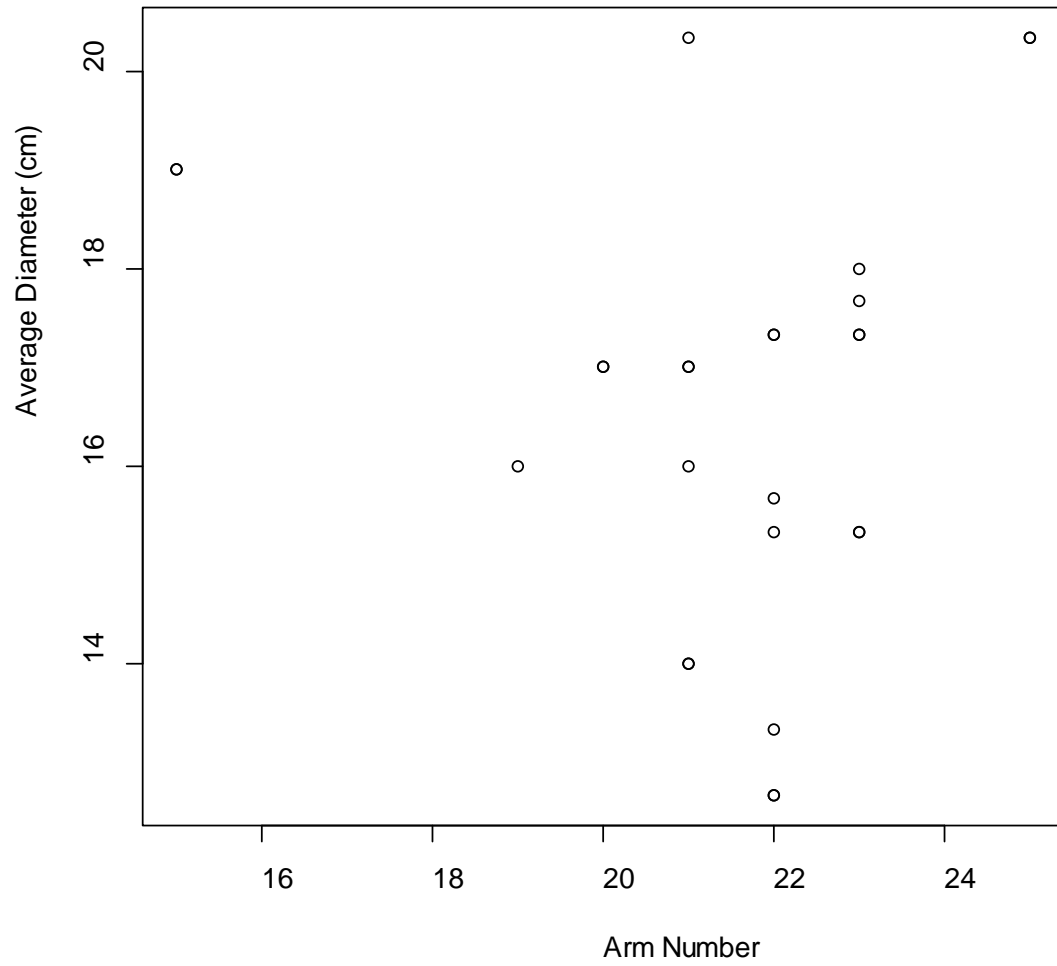
Figure 3. The average diameter of *H. kubiniji* (n=18) plotted against a range of arm numbers.

Figure 4. Righting response times of *P. pyramidata* (a) (n=9), *P. unifascialis* (b) (n=14), and *H. kubiniji (*c) (n=18) having a variety of diameters. Diameter values are the average of three measurements taken.
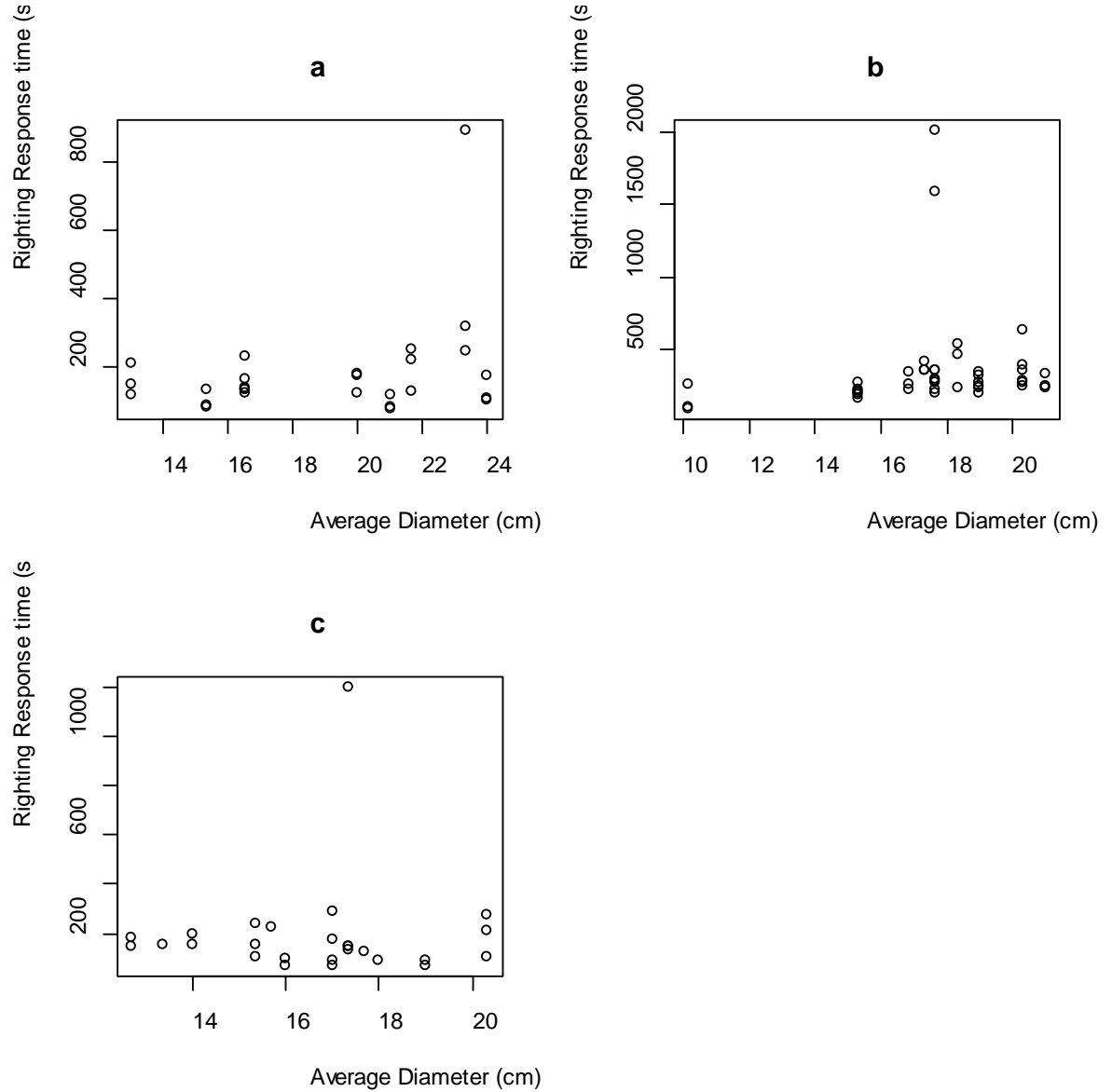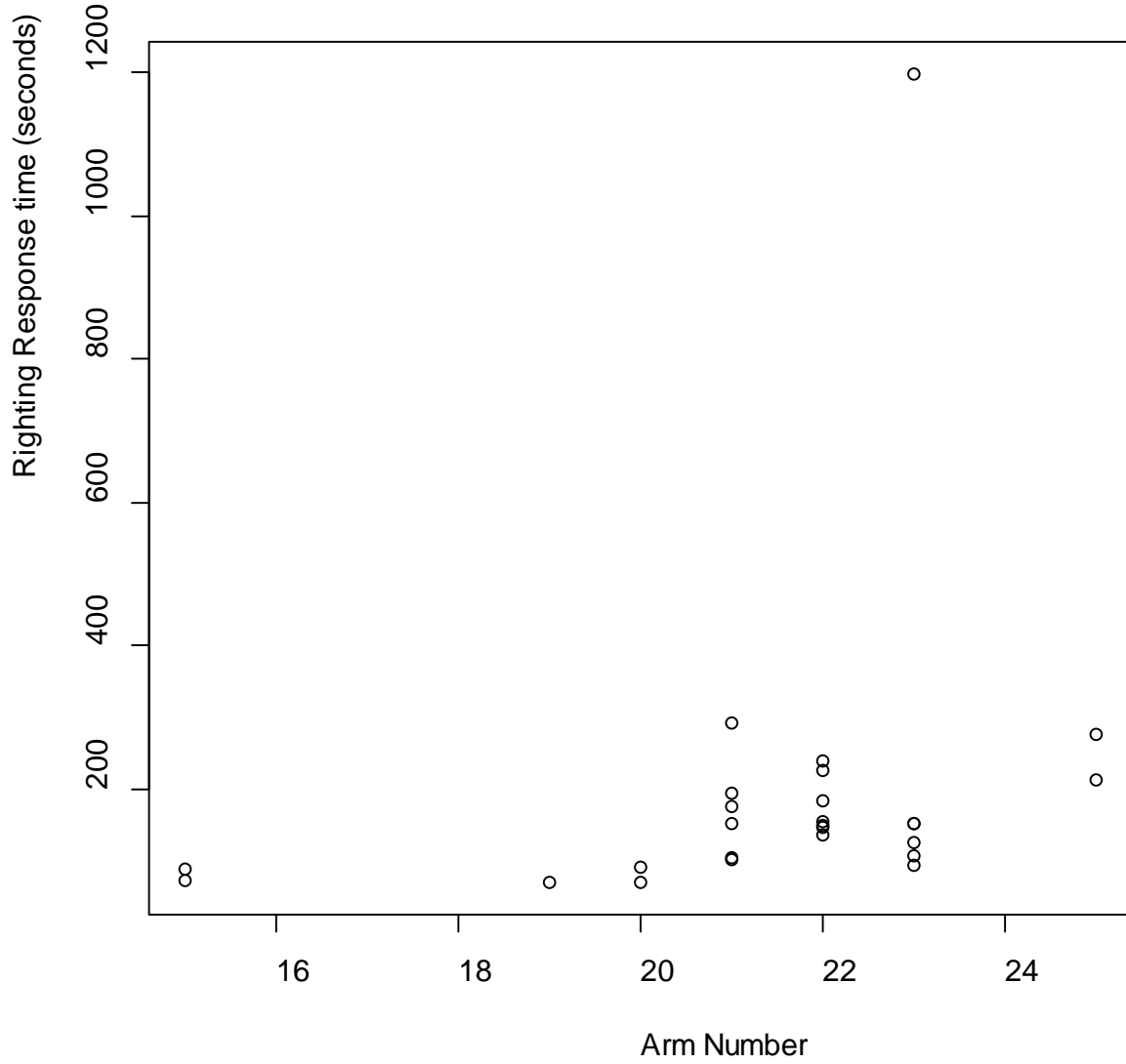
Figure 5. Righting response times of *H. kubiniji* (n=18) having a range of arm numbers at experimental temperatures of 30, 31 and 32°C.

A forensic, genetic analysis of the ribosomal ITS2 region in potential shark samples taken from markets in the Baja Peninsula, México

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Abstract

Sharks are being caught and sold in markets around La Paz and Ensenada, Baja California Peninsula, Mexico. Shark meat is banned for the season beginning May 1 and continuing until July 31 (DOF 2012). Markets in Baja have continued to sell fresh or dried shark throughout the season. The question we examined for our experiment was: Are protected sharks present in samples taken from markets in the Baja California peninsula, Mexico? We hypothesized that there will be protected sharks by international institutions (CITES, IUCN Red List, CMS) present in the samples taken from markets in Baja. Our prediction depended on whether DNA samples, taken from markets in Baja, match universal shark strands in electrophoresis gel. Protected sharks are being opportunistically caught and sold as "cazón" at markets in Baja. The approach taken to examine the question was to extract DNA from several samples of meat taken from local markets in La Paz and Ensenada. We used two different methods of DNA extraction for three different types of samples:

dried, fresh and salty/fresh samples. Once extracted, electrophoresis was completed to look for good quality shark DNA. We recorded which samples showed the most promise in containing shark DNA and began polymerase chain reaction (PCR) using two universal shark primers that amplify the entire ITS2 region. PCR products were sent to sequence to analyze them using BLAST (Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information) looking up the most similar sequences in the genbank. The results from sequences showed the presence of 3 different shark species: Sharpnose Pacific Shark (*Rhizoprionodon longurio*), Shortfin Mako (*Isurus oxyrhinchus*) and Giant Hammerhead Shark (*Sphyrna mokarran*). The conclusion of the experiment was that different species of marine life that some of them are protected are being sold as "cazón" or marlin in Baja.

Key words: Shark fisheries, genetics conservation, DNA samples, universal ITS2 shark primers, endangered species

Introduction

It is well know that pelagic sharks are being caught in fisheries worldwide, however fishery managers often inadequately document shark catches, landings, and discards (Baum *et al*. 2003). Despite that fact that requirements for documenting such information is increasing, compliance remains low. Furthermore, management implementations to reduce shark mortality, such as catch limits, are rare at the national level and nonexistent at the international level (Dulvy *et al.* 2008). Since sharks are highly mobile species that often traverse national borders or occur in international waters, there is not consistent

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protection for them and therefore they do not fully benefit from protective regulations applied at national or local levels (Camhi *et al.* 2009). Demand for shark products (i.e. skin, meat, oil, and fins) as well as the evidence of shark overfishing is on the rise.

Depletion of shark populations as a result of overfishing is a global issue (Clarke *et al.* 2006; Dulvy *et al.* 2008). Many sharks are apex predators in marine ecosystems, which not only makes them vital to the health of the ecosystem, but also means they are less abundant than other commercial fish. Sharks are extremely sensitive to overfishing as a result of their life-history characteristics; recovery from overexploitation is extremely slow due to low fecundity and K selection. Most sharks demonstrate slow growth and long reproductive cycles along with long life spans and must expend energy on actively finding mates, unlike many other fish species. According to data for a few protected shark species, there is an increasing decline in numbers (Castro *et al.* 1999).

The identification of shark species is often difficult because of the morphological similarities present among a variety of species (Abercrombie *et al.* 2005; Clarke *et al.* 2005). Identification is further hindered by the removal of body parts (i.e. flesh, fins, head, and tail) for commercial value in markets, such as the ones found in the Baja California Peninsula. The market for shark fins is rapidly increasing causing post-catch species identification impossible. Shark fins are in high demand in South East Asia and are used in shark fin soup which is an expensive delicacy. The removal of fins inhibits visual inspection and creates a problem in the assessment of catch rates. The catch rates of sharks are helpful

to understand how to protect species for conservation efforts. A main method used for conservation is through the process of genetic analysis of species-specific DNA sequences to allow for species identification from small sample sizes (Briscoe 2005).

To help identify sharks for conservation at a rapid pace, we used a method known as PCR. Two primers were used for identification purposes in our experiment, the universal shark primers from ITS2 region. Shark fisheries have grown immensely over the past two decades, because of the high demand of shark fins and meat. The decline in shark populations is caused by both directed shark fishing and bycatch that occurs in tuna or pelagic fisheries. Sharks are susceptible to population crashes because of overfishing in short periods of time (Shivji *et al.* 2001)

We examined the question: Are protected sharks present in samples taken from markets in Baja California peninsula, Mexico? We hypothesized that protected sharks, such as the Great White would be present in the samples. Our prediction depended on whether DNA samples, taken from markets in Baja, match universal shark strands in electrophoresis gel 1%. We performed DNA analysis on the samples taken and found that protected sharks are being opportunistically caught and sold as "cazón" at markets in Baja.

Material and Methods

Samples collection

17 samples for unknown sharks were collected from different markets in the Baja California Peninsula. We obtained 12 samples from a market in

Ensenada and 5 samples from 4 different markets in La Paz. In the Ensenada market the samples were obtained from the products sold as "marlin" or "swordfish". These samples were conserved in ethyl alcohol 96° and transported in vials to the Molecular Ecology & Genetics Conservation in the Pichilingue Unit of the UABCS. On the other hand, the products obtained in La Paz were obtained from products sold as "cazón". One cazón sample was a fresh sample, another cazón sample was a salty/fresh sample, and the other three cazón samples were dried samples.

DNA extraction and PCR universal shark primers amplification

DNA from Ensenada samples were extracted using a modified NaCl extraction method (Aljanabi & Martinez 1997) (Appendix 2). DNA from La Paz samples were extracted using a modified NaCl extraction method and a modified phenol-chloroform method (Sambrook *et al.* 1989) (Appendix 3). As there were three different types of samples in La Paz (Fresh, salty/fresh and dried), three different variations in each DNA extraction method were assessed to extract high quality DNA. The variations consisted of three different times for the proteinase-k digestion in the samples (2 hours, 6 hours and overnight).

Two universal shark primers (**FISH5.8SF**: 5'-TTA GCG GTG GAT CAC TCG CGT CGT-3'; **FISH28SR**: '5-TCC TCC GCT TAG TAA TAT GCT TAA ATT CAG C-3') were used from the ITS2 region to amplify a positive control with a range of amplification of 860-1500 bp due the different shark species that exist. A

Great White Shark (*Carcharodon carcharias*) sample was used as positive control.

Total amplification reaction volume was 25 µL, and contained 10 X PCR buffer, 10 mM dNTPs, 50 mM MgSO₄, 12.5 pmol of each primer and 1 unit of Platinum ® Taq (Invitrogen Inc.) filled with distilled and sterilized water. The PCR thermal cycling profile employed was: 94 °C initial heating for 15 min in stage I, followed by 35 cycles of 94 °C for 1 min of denaturalization, 65 °C for 1 min of annealing and 72 °C for 2 min of extension in stage II, and a 72 °C for 5 min final step in the final stage.

Data analysis

PCR products were sent to sequence. Genedoc Ver. 2.07 and Clustal X Ver. 2.1 software were used to clean and align the sequences for working and use them properly. BLASTn of the NCBI was used to look up for similar sequences to match on the genbank.

Results

DNA extraction

The DNA from the samples of Ensenada (Table 1) were correctly extracted by following the modified NaCl DNA extraction method. But the samples from La Paz were not correctly extracted and it could not achieve good quality DNA (Fig. 1), except in the sample M3 (Fig. 2).

Amplification of universal shark primers and identification of sharks

PCR assay was successful in 14 of 17 DNA samples collected from the markets in Baja. All of these samples amplified a universal strand.

Of the 14 PCR products 12 sequences were obtained. Three different species of sharks were identified: Giant Hammerhead Shark, *Sphyrna mokarran;* Pacific Sharpnose Shark, *Rhizoprionodon longurio; and* Shortfin Mako, *Isurus oxyrinchus* (Table 2). All three species of sharks were found in Ensenada: the Giant Hammerhead Shark, the Shortfin Mako Shark, and the Pacific Sharpnose Shark (Fig. 3). In La Paz, only the Pacific Sharpnose Shark was present (Fig. 4).

Discussion

The empiric knowledge of shark morphology was used for the identification of sharks being sold as "marlin" or "swordfish" in Ensenada markets. The caudal keel could be seen at the posterior end of the sharks' bodies, which is exclusive to sharks of the Lamnidae Family (Castellanos-Betancourt *et al.* 2014). In La Paz markets, sharks are often identified as "cazón", which includes all of the sharks that are being fished that have a total length (TL) of 1.5 m or less (DOF 2007). This has important implications for conservation because juvenile hammerhead sharks of less than 1.5 m would be classified as cazón even though hammerhead sharks are an endangered and protected species.

According to our results, two of the species we found were endangered: Giant Hammerhead and Shortfin Mako sharks. These species are protected under international law, but not under Mexican law (DOF 2007; DOF 2010) in the period that not correspond to the banned season (DOF 2013). As discussed

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previously, endangered shark species like the hammerhead occur in international waters, but will migrate to national waters where there are different laws protecting them (Camhi *et al.* 2009). If juvenile sharks of endangered species, such as the Giant Hammerhead, are being fished because they are not protected in Mexican waters, this will change the population structure and gene pool of local populations, reducing genetic diversity and potentially adaptability of the species. In order to help conserve endangered species, laws must prohibit the catching, selling, or killing of these species in any capacity, regardless of size or other national and local regulations. Just the White Shark has been permanent banned for fisheries in Mexican waters (DOF 2014), and towards the basking shark and the whale shark, they can't be fished (DOF 2007; DOF 2010).

Genetic methods utilized in this study also successfully identified cryptic species or body parts of species in several other studies, and are considered the most reliable methods for identifying unknown samples to species level (Pank *et al.* 2001, Chapman *et al.* 2003; Shivji *et al.* 2001). The purpose of these PCR assays are to identify a range of tissue types in endangered species (muscle, liver, fins) in order to contribute to worldwide conservation efforts for these species. Assays such as these can help with cases that involve illegal marketing of endangered shark parts, as genetic evidence is considered more substantial than circumstantial evidence (Chapman *et al.* 2003). In addition, international conservation organizations, such as the IUCN and CITES, rely on scientific literature in order to determine the global status of endangered species (Camhi *et al.* 2009). Studies such as this one are vital for these organizations because they

provide important information on the local management of endangered species and allow them to assess the global endangerment level and provide the most informed suggestions regarding international management and conservation.

Although the genetic methods used in this experiment are extremely effective in determining species of shark caught and sold in markets, marine fisheries management and conservation is still rare (Shivji *et al.* 2001). Currently, fishing countries voluntarily submit shark landing data to the Food and Agriculture Organization's (FAO) capture production database. This means that the quality and comprehensiveness of the database is wholly dependent upon the reporting priorities of the world's fishing countries, which at present leaves sharks grossly misrepresented. Furthermore, the FAO's database only includes shark landings, not catches or discards, rendering shark mortality data less accurate than if all possible data were reported. The lack of data regarding shark fishing makes it difficult to understand the conservation and management needs of endangered species (Camhi *et al.* 2009).

Currently, international conservation organizations lack sufficient data on shark populations, catches, landings, and discards; however, this does not mean we should wait to implement management or conservation programs and regulations (Camhi *et al.* 2009). We suggest international laws and regulations that take precedence over all national and local laws should be put into place prohibiting the catching or discarding of any endangered species. Further research on the selling of potential endangered shark species in markets on the Baja California Peninsula should be conducted, as well as similar studies in other

parts of the world. The IUCN asserts that improved monitoring and further research is needed regarding the endangered shark species with priority placed on biology, threats, critical habitats, and conservation needs, particularly in regions with little to no data. In addition, international regulations must require mandatory reports of catches, landings, and discards of all shark species, regardless of endangerment status. Sharks are a vital part of marine ecosystems and action must be taken to counteract the effects of depletion and overexploitation.

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DOF. 2013. ACUERDO por el que se modifica el aviso por el que se da a conocer el establecimiento de épocas y zonas de veda para la pesca de diferentes especies de la fauna acuática en aguas de jurisdicción federal de los Estados Unidos Mexicanos, publicado el 16 de marzo de 1994, con el objetivo de concluir el periodo de veda de tiburones y rayas en el litoral del Océano Pacífico durante el 2013.

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Appendix

Appendix 1

Table 1. Samples collected from the markets in Baja.

Sample	Locality	Date	Species
A	Ensenada	24-sep-12	"Marlin"
B	Ensenada	25-sep-12	"Marlin"
C	Ensenada	10-sep-12	"Marlin"
D	Ensenada	11-sep-12	"Marlin"
E	Ensenada	30-aug-12	"Marlin"
#1	Ensenada	08-nov-12	"Marlin"
#2	Ensenada	08-nov-12	"Marlin"
#3	Ensenada	08-nov-12	"Marlin"
#4	Ensenada	08-nov-12	"Marlin"
#5	Ensenada	08-nov-12	"Marlin"
Y	Ensenada	18-sep-13	"Swordfish"

Sample	Locality	Date	Species
Z	Ensenada	18-sep-1	"Swordfish"
M1	La Paz	14-Jul-14	"Cazón fresco"
M2	La Paz	14-Jul-14	"Cazón seco"
M3	La Paz	14-Jul-14	"Cazón seco"
M4	La Paz	14-Jul-14	"Cazón seco"
M5	La Paz	14-Jul-14	"Cazón salado"

Table 2. PCR sequence matches compared in BLAST (Basic Local Alignment Search Tool) databases for three different shark species with Carcharodon carcharias for control.

Sample	Identities	Strand	Species
Sequencia Prueba	1280/1280 (100%)	Plus/Plus	Carcharodon carcharias
1ENS	1267/1267 (100%)	Plus/Plus	Isurus oxyrinchus
2ENS	1267/1267 (100%)	Plus/Plus	Isurus oxyrinchus
3ENS	1313/1313 (100%)	Plus/Plus	Rhizoprionodon longurio
4ENS	1267/1267 (100%)	Plus/Plus	Isurus oxyrinchus
5ENS	1313/1313 (100%)	Plus/Plus	Rhizoprionodon longurio
6ENS	1267/1267 (100%)	Plus/Plus	Isurus oxyrinchus
7ENS	1265/1267 (99%)	Plus/Plus	Isurus oxyrinchus
8ENS	1264/1267 (99%)	Plus/Plus	Isurus oxyrinchus
9ENS	799/799 (100%)	Plus/Plus	Sphyrna mokarran
10ENS	799/799 (100%)	Plus/Plus	Sphyrna mokarran
1LPZ	1313/1313 (100%)	Plus/Plus	Rhizoprionodon longurio
2LPZ	1310/1313 (99%)	Plus/Plus	Rhizoprionodon longurio

Appendix 2

NaCl modified DNA extraction method

1. Cut tissue in small parts to get ~10 mg.

2. Add 500 µL LiCl buffer extraction and 10 µL proteinase-K [10 mg/mL].

3. Incubate 37-39 °C (Depending on the incubate time).

4. Add 100 µL LiCl [3 M] and 600 µL Chlorofom isoamilic alcohol 24:1

5. Vortex for 10 seconds.

6. Stir at 700 rpm for 30 minutes

7. Centrifuge at 4 °C for 20 minutes at max speed (14,000 rpm).

8. Take 550 µL of the upper layer, taking care to not take from the lower layer. Add the volume extracted to a new vial and add 600 µL of chloroform isoamilic alcohol 24:1

9. Repeat steps 5, 6 and 7.

10. Take 500 µL of the upper layer and add it to a new vial.

11. Add 1000 µL et OH 100° and 50 µL sodium acetate [3 M].

12. Precipitate the samples in a cooler at -20 °C overnight.

13. Centrifuge at max speed at 4 °C for 40 minutes at max speed.

14. Pour the alcohol and add 750 µL et OH 70°.

15. Stir at 700 rpm for 20 minutes.

16. Centrifuge at 4 °C for 20 minutes at max speed.

17. Pour the alcohol.

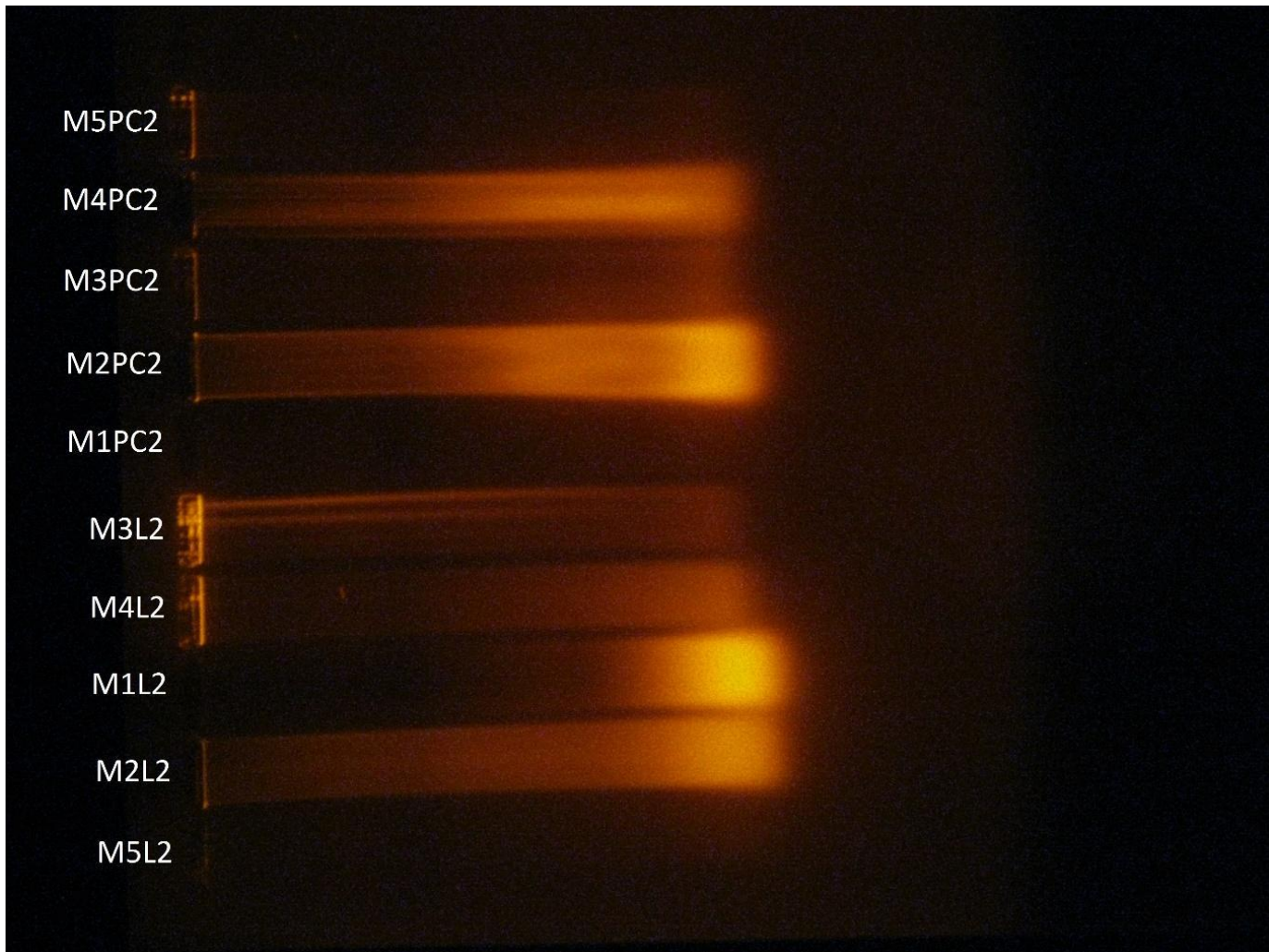18. Dry the alcohol left in a hot plate at 60 °C for 30 minutes.

19. Add 50 µL TE 1X.

Appendix 3

Phenol-chloroform DNA extraction method

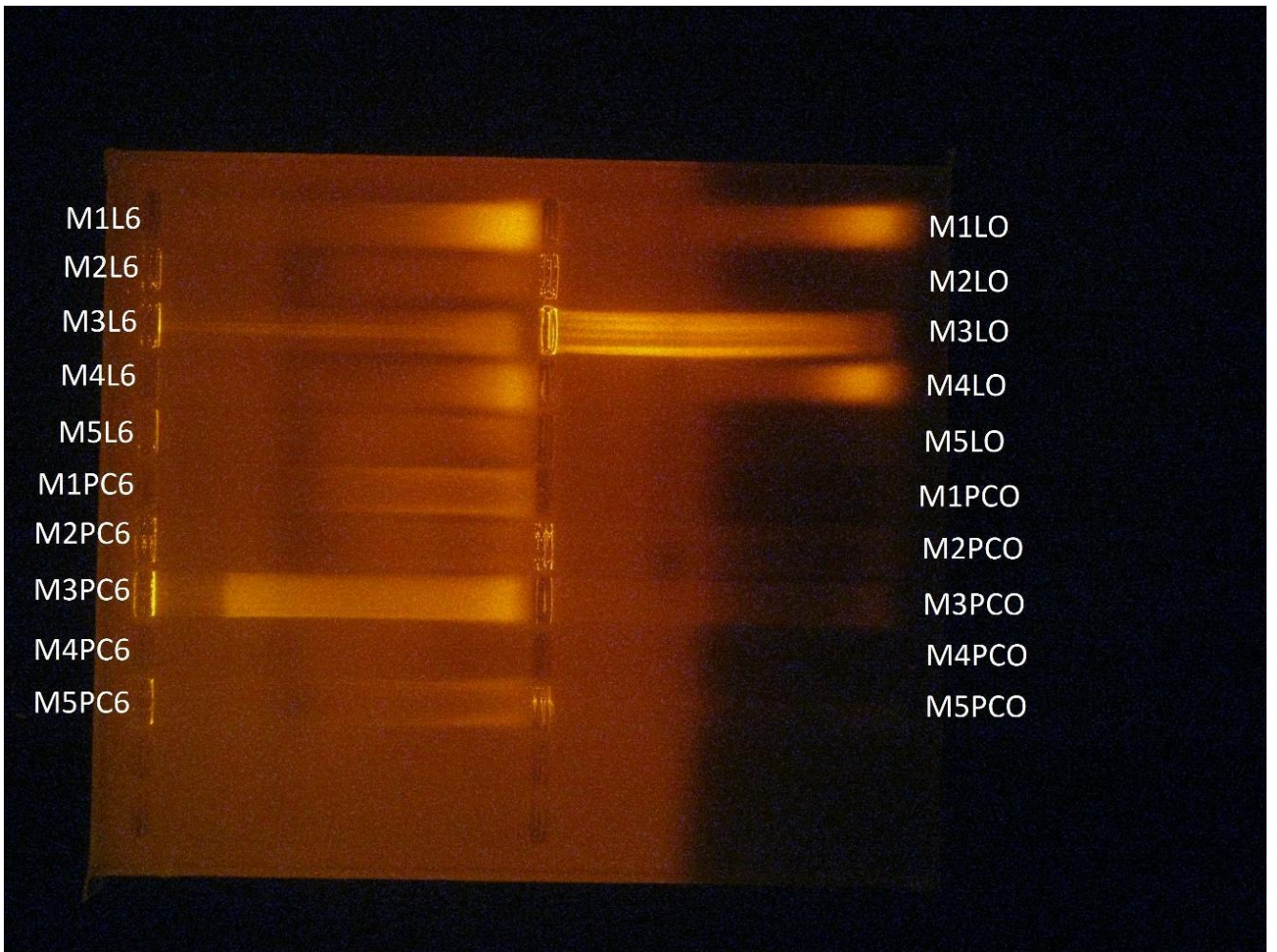1. Cut tissue in small parts to get ~10 mg.

2. Add 500 µL Phenol-Chloroform buffer extraction and 10 µL proteinase-K [10 mg/mL].

3. Incubate 37-39 °C (Depending on the incubate time).

4. Add 500 µL Phenol-Chlorofom Isoamilic alcohol 25:24:1

5. Vortex for 10 seconds.

6. Stir at 700 rpm for 30 minutes.

7. Centrifuge at 4 °C for 20 minutes at max speed (14,000 rpm).

8. Take 550 µL of the upper layer, taking care to not take from the lower layer. Add the volume extracted to a new vial and add 500 µL Chlorofom Isoamilic alcohol 25:24:1

9. Repeat steps 5, 6 and 7.

10. Take 500 µL of the upper layer and add it to a new vial.

11. Add 1000 µL et OH 100° and 50 µL sodium acetate [3 M].

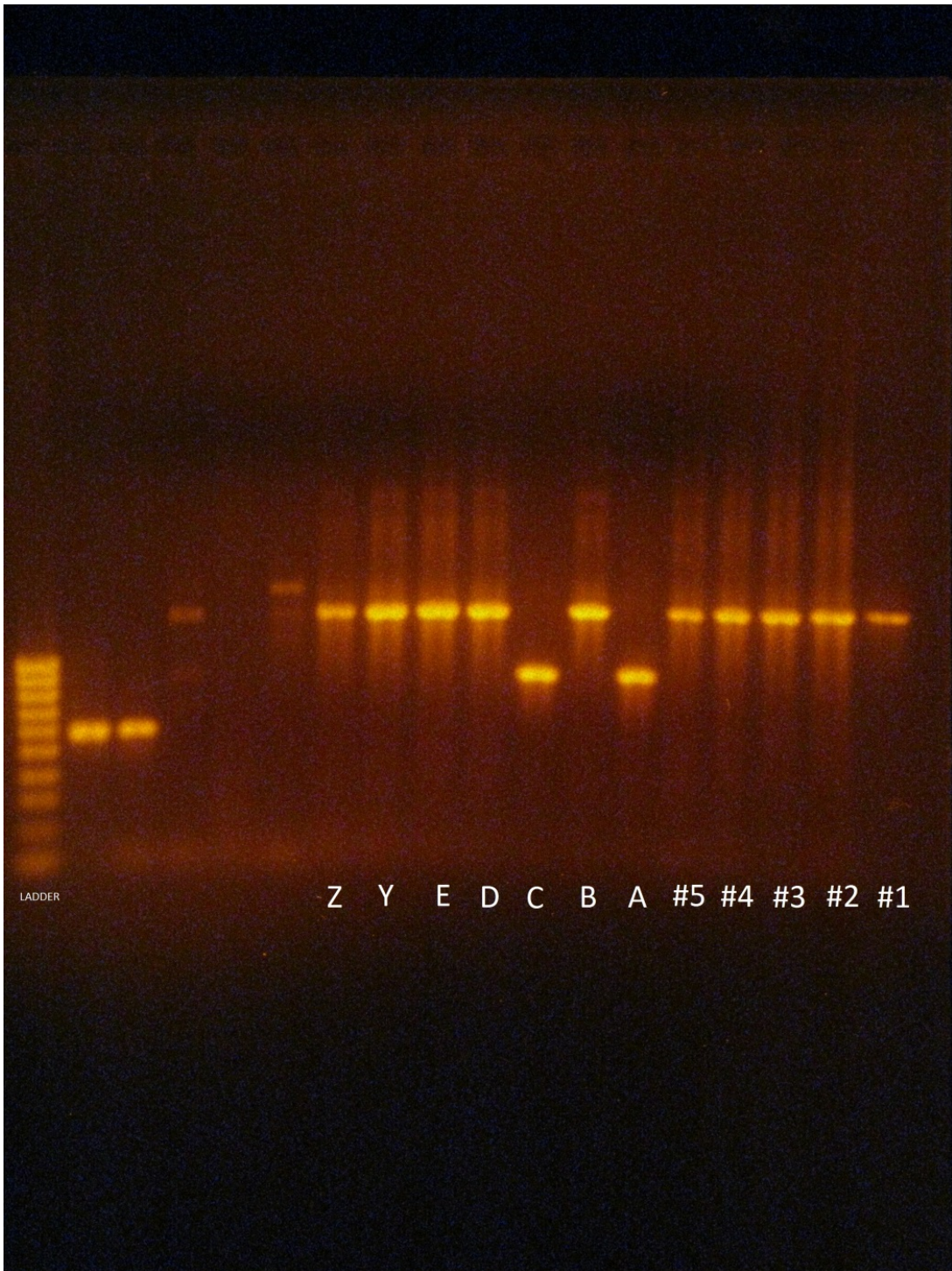12. Precipitate the samples in a cooler at -20 °C.

13. Centrifuge at max speed at 4 °C for 40 minutes at max speed.

14. Pour the alcohol and add 750 µL et OH 70°.

15. Stir at 700 rpm for 20 minutes.

16. Centrifuge at 4 °C for 20 minutes at max speed.

17. Pour the alcohol.

18. Dry the alcohol left in a hot plate at 60 °C for 30 minutes.

19. Add 50 µL TE 1X.

Appendix 4

M5PC2
M4PC2
M3PC2
M2PC2
M1PC2
M3L2
M4L2
M1L2
M2L2
M5L2

LADDER   Z Y E D C B A #5 #4 #3 #2 #1

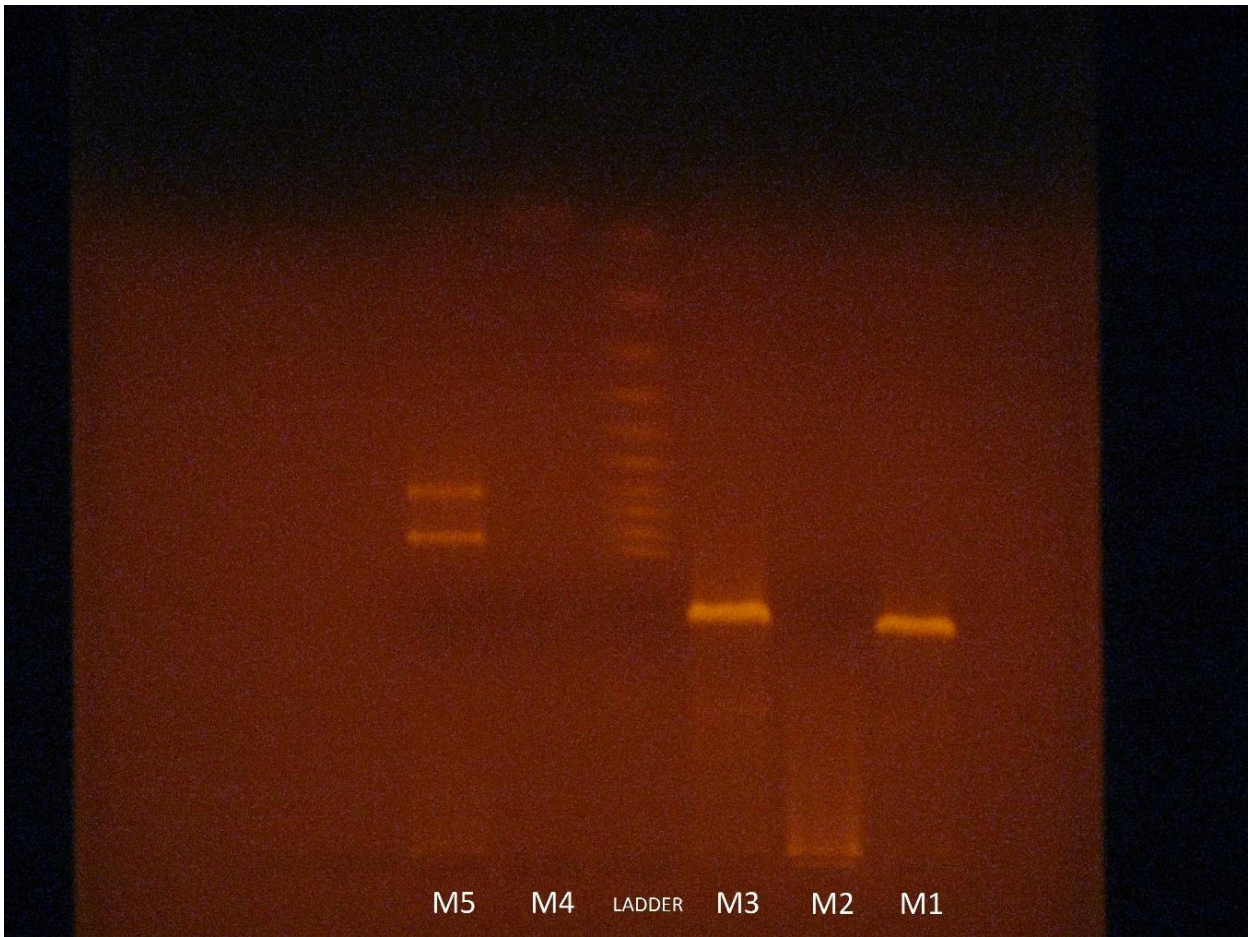M5   M4   LADDER   M3   M2   M1

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Figure 1. DNA verification with two hours in proteinase-k digestion from two different DNA extraction methods.

Figure 2. DNA verification with 6 hours and overnight variations in digestion in proteinase-k from two different DNA extraction methods.

Figure 3. PCR products from Ensenada market with a molecular weight ladder of 1000 bp.

Figure 4. PCR products from La Paz markets with a molecular weight ladder of 1000 bp.