AN INVESTIGATION INTO THE TROPHIC ECOLOGY AND INTRODUCTION OF THE WINGED BOX JELLYFISH, *ALATINA ALATA*, IN HAWAI‘I

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ABSTRACT

Alatina alata, the winged box jellyfish, is a stinging cubozoan known for its regular, monthly influxes along O‘ahu’s southern shore occurring consistently between 8 and 12 days after each full moon. In spite of the serious hazard posed by monthly mass sting events, little scientific attention has been focused on the trophic ecology of this species and whether it is native to the region or introduced. The main objectives of this study were to: 1) use stable isotopes to better understand the trophic feeding level of *A. alata*; 2) determine if *A. alata* is the sole species responsible for influx events on O‘ahu; and 3) compare mtDNA sequences to specimens in the Pacific and Atlantic Ocean basins to assess the likelihood of an introduction. Isotope ratios, δ^{15}N and δ^{13}C, from the bell tissue of *A. alata* (n = 32) and well as various taxa within the North Pacific Subtropical Gyre were compared. Isotope ratios between two low trophic level fish, the bigeye scad and the goldspot herring (*Selar crumenophthalumus* and *Herklotsichthys quadrimaculatus*) and *A. alata* were not significantly different (*p*-value = 0.04, 0.10 respectively). Based on the high trophic variance (0.95 for δ^{15}N and 1.10 for δ^{13}C) over the 6 month period, *A. alata* was classified as a generalist. Over the course of 12 monthly influxes, *A. alata* were collected along Waikīkī beach and their mtDNA cytochrome *c* oxidase I (COI) were amplified (n = 108). Hawai‘i sequences were compared to Australia, Saipan and Bonaire. Hawai‘i specimens shared haplotypes with Australia and Saipan specimens and no significant genetic differentiation was exhibited between Hawai‘i and Atlantic (Bonaire) populations (F_{ST} = 0.008). Results from the study suggest *Alatina alata* was introduced to Hawaiian waters, and should be classified as an invasive species in Hawai‘i.

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Chapter 1:

A Review of Box Jellyfish in Hawaiʻi (Cnidaria: Cubozoa)

Ecology, Phylogeography & Implications of Multiple Marine Invasions

Introduction

Jellyfish (Phylum: Cnidaria) comprise an ecologically important and diverse group of primarily pelagic gelatinous marine invertebrates. Cnidarians are often the focus of public attention due to painful stings and interference with power plant, aquaculture, and commercial fishing operations (Purcell 2007; Bayha and Graham 2014). Another issue that has been made popular by the media was the idea that jellyfish "blooms", dramatic population increases, are increasing worldwide (Purcell et al. 2012; Duarte et al. 2013). However, evidence suggests that blooms are mainly driven by climate patterns and decadal-scale climate cycles (Purcell 2005; Condon et al. 2013; Chiaverano et al. 2013).

Box jellyfish (class Cubozoa) are the smallest, most biologically sophisticated and evolutionarily derived class of Cnidarians. The two orders within Cubozoa (Chirodropida and Carybdeida) are characterized by the roughly cube-shaped bell of the medusa phase. They consist of about 50 species, at least three of which, *Alatina alata, Copula sivickisi,* and *Carybdea arborifera,* are well-documented inhabitants of Hawaiian coastal waters (Crow et al. 2015). Box jellyfish share numerous derived synapomorphies that distinguish them from other jellyfish lineages, including possession of complex eyes and sophisticated vision, unique and variable reproductive behavior, strong swimming ability, and powerful venom (Bentlage et al. 2010).

Although the first record of *Alatina alata* at Waikīkī Beach on Oʻahu was in 1948 (Crow et al. 2015) it was not until the 1980's that the regular influx events after each full moon became a well-known phenomenon (Thomas et al. 2001). Influx events, sometimes involving hundreds to several thousand medusae washing ashore in a single two-day event, involve painful stings frequently overwhelming Emergency Medical Services and leading to beach closures (Yoshimoto and Yanagihara 2002). These monthly aggregations of the stinging box jellyfish, currently recognized as a single species *Alatina alata* (Lawley et al. 2016), are associated with the lunar cycle, occurring consistently between 8 and 12 days after each full moon (Thomas et al. 2001; Chiaverano et al. 2013).

Waikīkī Beach is known as the busiest, most heavily used beach in all of Hawai‘i , a state where ocean recreation is an extremely popular year-round activity Waikīkī is considered an economic engine for the state generating 44% of the state's annual revenue from tourism (Lent 2002). However, in spite of the serious nature of the hazard posed by monthly mass sting events, little scientific attention has been focused on the ecology of *Alatina alata*, such as the drivers of the population fluctuations and regular circalunar, a biorhythm that corresponds with the lunar cycle, influx pattern whether the species is native or invasive, and if one or multiple species are responsible for the influxes. While box jellyfish are regularly, periodically conspicuous in Hawai‘i, only a single long-term study exists (Chiaverano et al. 2013) regarding the influx ecology, and little is known about their growth rate, feeding, or distribution. While we do have records regarding the location and timing of the monthly circalunar aggregations, nearly nothing is known regarding their lifespan, reproduction, polyp habitat, and where the medusae occur during the time between monthly influxes. Although in recent years, local beachgoers, surf forecasters, lifeguards, and public safety officials are aware and make efforts to warn the public of box jellyfish presence at the specific intervals following each full moon, a lack of historical documentation has led to questions regarding whether this species is native to Hawaiian waters or has been introduced more recently.

It is difficult to assess the invasive status of this stinging box jellyfish in Hawai‘i. *Alatina alata (A. alata)* may be native to the Hawaiian Islands but simply did not present a hazard to humans prior to anthropogenic changes such as alternations to Waikīkī Beach (i.e., changes in freshwater flow, construction of groins and seawalls) and the dramatic increase in the number of people participating in ocean recreation and increased coastal light pollution with the expansion of beachfront development (Mak 2015). The first documented box jellyfish in Waikīkī occurred on 5 March 1948 and the first mass stinging event 30 June 1951 (Edmondson 1952; Crow et al. 2015), These events were fairly sporadic up until the late 1980s and then in 1994 became regular monthly influx events (Thomas et al. 2001; Chiaverano et al. 2013). Increases in number of beach users beginning in the 1980's coincides with more regular records of stings, but poor documentation before the 1980's lends an amount of uncertainty regarding precise timing of the first mass stinging events. While based on the history of box jellyfish sting records, it is possible that *A. alata* was anthropogenically introduced, the possibility that it has long been present offshore but had not aggregated nearshore until more recently cannot be ruled out. The use of historical data to determine the status of box jellyfish in Hawai‘i has proven inconclusive.

One of the potentially most important changes in terms of implications for influx patterns in Waikīkī is the increase in human population density. Increased density has also been associated with increases in the number of people engaging in ocean recreation. Ocean recreation has developed along with the booming visitor industry, which has reach million people per year in 2018 (HTA 2018 Annual Visitor Survey). The massive number of people engaging in ocean recreation in recent decades could play an important role in both the frequency and severity of mass stinging events. Therefore, a combination of anthropogenic changes to habitat and the coastal light environment, in addition to more people in the water have likely amplified the numbers of box jellyfish stings as well as our awareness of influx events.

Recent phylogenetic studies aimed at elucidation of relationships among the cubozoan families have revealed patterns that suggest the species responsible for the frequent mass stinging events in Waikīkī could be introduced. Evidence is accumulating for a single lineage with a broad anthropogenically driven distribution that includes Hawaiʻi, the Caribbean, Saipan, and Australia (Lawley et al. 2016). Although the influx patterns are not as well documented as in Hawaiʻi, Caribbean and Australian populations appear to follow similar circalunar behavior, arriving along shore 8-12 days after the full moon (Carrette et al. 2014).

Background

I. Anatomy, Venom and Life History

Vision & Eye Structure

Cubozoans have a complex and advanced visual system relative to other classes of Cnidarians such as the Scyphozoa and Hydrozoa (Nilsson et al. 2005). The few species of box jellyfish that have so far been examined have four sensory structures, one on each corner of their bell called rhopalia. Each of the four rhopalia is connected through a nerve ring and contains six eyes, for a total of 24 per individual (Kingsford and Mooney 2014). There are three different morphological eye types, an upper and a lower lensed eye, pit eyes, and slit eyes. In the cubozoan visual systems that have been examined in detail, pit and slit eyes act as photoreceptors with spectral sensitivity peaks in the blue-green region (Garm et al. 2007). Structurally, the upper and lower lens eyes are similar to those of vertebrates and cephalopods (Garm et al. 2011), having evolved independently in each of these three lineages.

Structural studies suggest the upper lens is specialized for looking upwards and in certain species for viewing terrestrial landmarks through the water column to assist in nearshore navigation and habitat recognition. This ability was recognized after experiments by Garm et al. (2011) on *Tripedelia cystophora*, a cubozoan that inhabits shallow mangrove swamps in the Caribbean, was observed orienting towards and swimming back to their preferred habitat when physically moved away from the edges of mangroves. When the jellyfish were transported away from the edges of the mangroves, they swam back to their original habitat as long as the edge of the mangrove was still in their visual field.

The range of vision was found after careful examination of the surface-facing upper lens and the geometry of *T. cystophora's* retina revealed they have an approximately 100° view above them (Garm et al. 2011). This angle corresponds closely to Snell's window, which is 97°. Snell's window is a phenomenon caused by the refraction of light allowing underwater viewers, jellyfish in this case, to compress a 180° view of the surface to a 97° angle of view below water, similar to the point of view of a lensed fish eye or underwater camera, pointed up at the surface. As long as the mangrove edge was included in their line of sight, which was experimentally determined to be up to 8 m away, it appeared the jellyfish could orient themselves relative to the mangrove. Underwater photographs from the water looking up at the mangroves at various distances were processed through an optical model to confirm this hypothesis. This ability implies *T. cystophora's* upper lens eyes evolved to use terrestrial or celestial cues to navigate. While this study focused on *T. cystophora*, the upper lens eye is structurally similar in other cubozoans that do not live in mangroves, and is predicted to have evolved to detect solar positioning (Garm et al. 2011).

Venom

Another characteristic that differentiates the Cubozoa from other cnidarians and perhaps the characteristic they are most known for is their potent venom. Despite their small size, box jellyfish possess powerful nematocysts, or stinging-cells, which they use to capture prey defend themselves from predators, as well as occasionally sting unlucky swimmers. There are two species of Cubozoa that are known to produce fatal stings and several more suspected, *Chironex fleckeri,* of Australia, and *Chironex yamaguchii,* and additional Chirodropid species have caused fatalities in Japan, the Philippines, and Thailand (Brinkman and Burnell 2009; Bentlage et al. 2010; Lee et al. 2018).

The differing toxicities among species results from the surface area of the tentacles. Currently, venom proteins found within Cubozoa are unique and have not been identified in any other species suggesting a new and novel group of bioactive proteins (Nagai et al, 2000; Brinkman and Burnell 2009). In certain cases, the symptoms that follow a Cubomedusae encounter are referred to as "Irukandji syndrome" and can include hours of cramped limbs, headaches, restlessness, and nausea (Kong and Nappe 2020). Considering the severity of symptoms, it is increasingly important for coastal management to document the species of box jellyfish and have a sense of their distribution and population abundance patterns.

Statoliths

Statoliths are unique structures composed of biomineralic crystals (bassanite or calcium sulfate hemihydrate). Statoliths are part of the rhopalium of cubozoans and scyphozoans that support eye orientation within the water column (Sötje 2011). Similar to growth rings in trees, fish otoliths, and amphibian bones, the rings propagating from the center of the statolith can be used to age box jellyfish (Kawamura 2003). First documented in 1995 by Ueno et al. in the species *Carybdea rastonii*, these rings form during the metamorphosis from polyp to medusa. These tiny rings (127 – 732 µm), which can only be observed using a microscope, appear to be daily growth rings as observed Ueno et al. (1995) and further corroborated by Gordon et al. (2004). Kawamura et al. (2003) studied statoliths from the species *Chiropsalmus quadrigatus* from five sites on Okinawa Island. The observed statolith lengths were between the range 127 – 732 µm with anywhere from 15 – 82 rings per statolith.

Careful counting of rings permits box jellyfish to be aged reasonably accurately provided that statoliths are preserved and correctly analyzed. This technique is more reliable than previous methods to estimate age such as measuring medusa length or bell height since species grow rapidly earlier in their life then tend to stay relatively the same size once they reach maturity meaning their bell measurements remain constant after maturity. Using statoliths instead of medusa length can also be useful when comparing populations with different resources and environmental conditions (Gordon et al. 2004). In addition to aging cubozoans, Kingsford and Mooney (2016) found the distinct statolith shapes can also be used to successfully differentiate cubozoans down to species level.

Sötje et al. (2011) compared the structure and organization of the statoliths and rhopalium in both *C. fleckeri* and scyphozoan species, *Periphylla periphylla*. While organizationally different, similar structure suggests statoliths being a homologous structure between scyphozoans and cubozoans. Based on this conclusion, it was hypothesized that cubozoans and scyphozoan share a direct common ancestor (Sötje et al. 2011), a theory that has broad support in other molecular phylogenetic analyses (e.g. Zapata et al. 2015).

Reproductive Behavior

Mating in medusozoans typically involves sexual reproduction during the medusa stage and asexual reproduction in the polyp stage via budding. *Alatina alata* polyps have not been observed in the wild, but are thought to exist in shallow waters since adult medusa come closer to shore to spawn (Lawley et al. 2016). Budding in this species produces motile creeping polyps allowing them to relocate to find suitable substrate (Carrette et al. 2014)

While direct observation of mating in most species of cubozoans is undocumented, there are some species that exhibit unique behavior involving internal fertilization. Distinctive mating and courtship rituals have been reported in certain cubozoans, including *Copula sivickisi* and *Tripedelia cystophora.* The behavior involves the passing of a spermatophore from the male to the female. This was observed in *T. cystophora* by Werner in 1973. Garm et al. (2015) observed *C. sivickisi* males and females entangling their tentacles while the male passed a sperm package into the cavity of the females fertilizing the eggs internally. According to Studebaker (1972), Arneson (1976), and Lewis et al. (2013) species of Alatinidae are predicted to be ovoviparous with males releasing sperm from their manubriums and females taking up sperm from the water column during spawning aggregations.

II. Cubozoa in Hawai‘i

At present there are three established cubozoan species known from Hawaiian waters: *Alatina alata* (Mayer, 1906), *Carybdea arborifera* (Maas, 1897), and *Copula sivickisi* (Stiasny, 1926). Additionally, there has been one sighting of the small Caribbean species *Tripedalia cystophora* (Conant, 1987) on O‘ahu in 2011 (Crow et al. 2015).

Table 1.

Four species of Cubozoans known to occur in Hawai‘i and their distinguishing morphological characteristics

Order	Family	Genus	Species	Rhopalia	Bell Height (mm)
Carybdeida	Alatinidae	*Alatina*	*alata*	T-shaped	67
Carybdeida	Carybdeidae	*Carybdea*	*arborifera*	Heart-shaped	35
Carybdeida	Tripedaliidae	*Copula*	*sivickisi*	Keyhole shaped	14*
Carybdeida	Tripedaliidae	*Tripedalia*	*cystophora*	Frown-shaped	8.3

**Bell diameter*

Carybdea arborifera and Copula sivickisi

Maas (1879) in Honolulu Harbor and later Mayer (1906) recorded *Carybdea arborifera* (as *C. rastonii*) specimens during the 1902 *Albatross* research cruise around Maui, O‘ahu, and Kaua‘i (Crow et al. 2015). This species is known only from the Hawaiian Islands yet seem to have a similar physical characteristics and ecological niche as Australian species *Carybdea rastonii*. In fact, until Bentlage et al. (2010) showed genetic differenciation between the species, it was believed that *C. arborifera* and *C. rastonii* were a single species.

Copula sivickisi (Stiasny, 1926), known to be widespread throughout coastal regions in the Pacific and Indian Oceans, was found on the south shore of O‘ahu in 1996 and the west coast of O‘ahu in 1998 during fishing operations on the surface above 180-550 m deep water

(Matsumoto et al. 2002; Crow et al. 2015). The species has also been spotted in surface waters in Maʻalaea Harbor, Maui (Crow et al. 2006). *Copula sivickisi* is most likely distributed throughout the main Hawaiian Islands and occurs year-round based on capture dates (Matsumoto et al. 2002, Crow et al. 2006). As noted by Lewis and Long (2005), this small Cubozoan undergoes a unique mating ritual in which males attach a tentacle to the female and pass a spermatophore which the female inserts into her manubrium.

Alatina alata and Long-term Influx Trends at Waikīkī

Alatina alata is believed to be the only a deep-sea cubozoan species that has been recorded as far offshore Hawaiʻi as 520 km and at depths up to 1,067 m (Crow et al. 2015, Lawley et al. 2016). The first record for this species at Waikīkī Beach occurred in 1948, where it was reported that "swarms" left many swimmers covered in stings (Crow et al. 2015). Besides the counts along Waikīkī Beach from 1994-2011, reports of this species span additional shorelines of Oʻahu including Hanauma Bay, Sandy Beach, and Nānākuli to Mākua as well as Poʻipū Beach on Kauaʻi (Crow et al. 2015). In 2013, a group of University of Hawaiʻi and Waikīkī Aquarium researchers published a study that used 14 years of Ocean Safety and Lifeguard Services records of box jellyfish influx numbers from a 400 m stretch of Waikīkī. They analyzed the relationships among the number of jellyfish counted on the beach over 173 consecutive full moons, in relation to a number of oceanographic parameters. The objective of this study was to investigate the ecological drivers of variation in influx numbers. One result of this study suggested that *A. alata* come ashore after each full moon to reproduce in nearshore surface waters. In the study the authors also speculated that *A. alata* may be attracted to the bright lights off Oʻahu's southern shores, especially in the area of Waikīkī, and they swim directly towards the lights thus ending up in the shallow waters along the beach (Chiaverano et al. 2013). To test this hypothesis 35 live box jellyfish were placed in a darkened laboratory raceway tank with LED lights of different wavelengths. In general, these box jellyfish exhibited a strong positive phototaxis to various colors of artificial light (Holland et al. pers. comm.).

Prior to 2013, there had been no long-term studies of any cubozoan species. However, the study by Chiaverano et al. (2013) provided important insights regarding the circalunar influx timing, influx size and ecological factors including climate. The study showed that sexually mature *A. alata* were coming ashore 8-12 days after the full moon each month for 1-4 days during an early morning ebbing tide. There was no seasonality or overall increase or decrease in abundances throughout the 14-year sampling period. However, there was significant variation in monthly influx abundance that followed an oscillating pattern over a 3-4 year cycle, where lower than average counts were followed by 3-4 year periods of higher than average counts. Over the 14-year period there were three significant shifts in these counts.

Interestingly, the pattern revealed by the Chiaverano et al. (2013) paper also positively correlated with the North Pacific Gyre Oscillation (NPGO) index, primary production, and macro-zooplankton abundance (>2 mm). This linkage is likely due to the trophic relationships among primary production, herbivorous zooplankton abundance, and tertiary predatory box jellyfish. The NPGO index measures southerly transit of a cooler, nutrient rich water mass to the central Pacific, surrounding the Hawaiian Archipelago. The results suggest that *A. alata* numbers increase due to an abundance of prey, and that they are feeding on macro-zooplankton (zooplankton greater than 2 mm), primarily copepods. The fact that there is also a relationship between box jellyfish and the NPGO Index could indicate that large scale processes in the gyre affect box jellyfish abundance offshore, and this in turn determines influx size at Waikīkī.

As referenced in previous sections, the study by Lawley et al. (2016) showed shared haplotypes among populations of *A. alata* in the Atlantic and Pacific, suggesting this single species has a circumtropical distribution. *A. alata* is known to have strong swimming abilities and specimens have been collected at deeper depths than other medusazoans suggesting *A. alata* may have a wide distribution throughout the water column.

III. Genetics and Evolution

First Phylogeny Study

Bentlage et al. (2010) conducted the first study on evolutionary relationships among cubozoans via molecular phylogenetic reconstruction. Three ribosomal genes were amplified and sequenced including partial mitochondrial 16S fragment, and nuclear 18S (small ribosomal subunit) and 28S (large ribosomal subunit) genes. Based on these genes, two monophyletic clades exist within Cubozoa: Chirodropida and Carybdeida.

Carybdeida contains five families, Carybdeidae, Tripedaliidae, Tamoyidae, Carukiidae, and Alatinidae. Chirodropida consists of three families, Chiropsellidae, Chirodropidae and Chiropsalmidae (Gershwin 2006; Toshino et al. 2015). The fundamental morphological difference between the two orders is the number of tentacles per pedalium (more or less each of the four corners of the bell), where the carybdeids have just one tentacle per pedalium, while chirodropids have multiple tentacles emanating from each pedalium (Bentlage and Lewis 2012).

Genetic Markers

DNA barcoding, taxon identification method using a specific region of DNA, allows for different resolutions depending on the genetic markers used. Bentlage et al. (2010) and Lawley et al. (2016) amplified different genetic loci in their phylogenetic reconstructions. Bentlage et al. (2010), Cartwright et al. (2008) and Collins et al. (2008) used 16S, 18S, and 28S in their phylogenetic studies on various hydrozoan, cubozoan, and scyphozoan species. Bentlage et al. (2010) used genetic markers to successfully resolve relationships among cubozoans and create a new genus, *Copula*, in which the family Tripedaliidae was placed. But since the phylogenetic focus of this thesis will be on investigating the relationships among geographically separated populations of very closely related individuals, slower evolving markers such as nuclear and even mitochondrial rDNA are not as useful.

The primary barcoding locus, mtDNA cytochrome *c* oxidase I (COI), has been extremely useful in separating animal groups at the species level. COI is used to separate closely related invertebrate congeners resulting from relatively recent species radiations (Holland and Hadfield 2004), define evolutionarily significant units (ESUs) within invertebrate species (Holland and Hadfield 2002), and detect geographic sources of invasive species (Rubinoff et al. 2011). Sequencing utilizing COI has been effective in clarifying global molecular systematics and elucidating species boundaries in the scyphozoan genus *Cassiopea* (Holland et al. 2004), as well as species identification medusozoans (Ortman et al. 2010). Zheng et al. (2014) compared 16S and COI genetic markers and determined both COI and 16S are successful biological barcoding tools for identifying and differntiating hydrozoan species.

Systematic Status of Alatina in Hawai'i

Until recently, the most common cubozoan species found in Hawai'i was globally regarded as being one of three separate but closely related species in the genus *Alatina, (A. moseri* (Hawai'i)*, A. mordens* (Australia), *and A. alata* (Caribbean) (Bentlage et al. 2010). Preliminary genetic analysis showed that all three putative species share haplotypes suggesting metapopulation cohesion (Bentlage et al. 2010). Interestingly, when comparing mtDNA sequences from Australian and Hawaiian populations, two different Hawaiian specimens exhibited more genetic differences (average number of nucleotide differences per site (π)), than they did with the Australian specimen (Smith et al. 2012). In addition, Carrette et al. (2014) compared the early life stages for *A. alata* in Hawaiʻi and Australia in a laboratory setting and found that they closely resembled those from the tropical Atlantic.

Lawley et al. (2016) focused on comparative morphology and genetics among distantly separated populations, and showed little genetic divergence between Australian, Caribbean, Northern Mariana Islands (Saipan), and Hawaiian populations. Lawley et al. (2016) used three genetic markers, ORF-PolB, COI, and 16S as well as large ribosomal RNA subunit 28S from the nuclear genome. A maximum likelihood tree showed that specimens from the four locations had no clear geographic structuring. In fact, some Atlantic specimens were more genetically similar to certain Pacific specimens than they were to other Atlantic specimens.

These populations, formerly recognized as three distinct species, are now recognized as a single species. The updated taxonomic status has raised several questions regarding life history as well as the likelihood of human-mediated transport (e.g. where did the species originate, when did they arrive in Hawaiʻi**,** how did they cross ocean basins). Lawley et al. (2016) included a summary of preserved museum specimens collected around the world, including Brazil, Papua New Guinea, Sri Lanka, Bermuda, Virgin Islands, Puerto Rico, Indonesia, and the Northeastern and Northwestern Atlantic, tentatively identified as *A. alata*. While it would be very useful to sequence COI from these museum specimens in order to determine the accuracy of their identification, many have been preserved in formalin for decades making it very challenging to extract and amplify DNA and therefore likely impossible to identify these individuals.

Lawley et al. (2016) proposed changing the name of *A. mordens* (Gershwin 2005*), A. moseri* (Mayer 1906)*,* and *Alatina* sp. from Saipan to *Alatina alata* (Reynaud 1830) because the name *A. alata* has scientific nomenclature priority. While this study was important advancing our understanding of the relationships among *Alatina* isolated populations, the overall sample size was very small.

IV. Provenance and Implications of Marine Invasions

Marine biological introductions include the transport, release and establishment of species outside of their historical range, and are currently recognized as a major global force of ecological and economic disruption (Holland 2000). The introduction of non-native marine species to various areas of the world has increased as larger and faster vessels cross the oceans more frequently. The consequences of invasive species introductions can vary depending numerous contributing to the establishment and success of the introduced species in the new habitat. The natural distributions of over 1,000 marine species have been directly altered by humans. Twenty years ago, Holland (2001) estimated that over 4,500 species had been established non-native in North America alone (Holland 2001).

In commercial ocean-going bulk carriers, ballast water is used to stabilize cargos of varying density, and takes the form of massive volumes of seawater pumped into specialized tanks at the port of origin, often transported across ocean basins, and then discharged at their destination. This mechanism has been identified as directly relocating marine species to areas of the world their natural dispersal would never facilitate. Commercial cargo vessels can carry over 150,000 tons of ballast water per trip. Planktonic organisms and larvae frequently end up in ballast tanks and are often able to survive transit depending on various factors, including their plasticity and nutrient content of the water. Likewise, probability of establishment is dependent on several key factors, including climate and habitat characteristics at the destination and its similarity to conditions in native range of transported species. Numerous marine invasions have resulted in catastrophic impacts to invaded ecosystems. Invasions such as the toxic Japanese dinoflagellate in Australia and the Eurasian zebra mussel in the Great Lakes, have influenced legislations to regulate ballast water movement (Holland 2001). Unfortunately, ballast water regulations are difficult to enforce and implement (Holland 2001).

Like most marine invertebrates, jellyfish pose difficult challenges in terms of detection as bioinvaders. Species crypsis, ability to avoid observation, poor records, and inaccurate identification of species all add up to the under-representation of jellyfish as an invasive species. Additionally, some jellyfish have the ability to rapidly adapt and exhibit morphological plasticity when introduced to a new habitat (Graham and Bahya 2007).

Invasive species in the Hawaiian Islands have caused dramatic disruption of native ecosystems, beginning with the first human arrivals (Kirch 1997). Certain marine species with high plasticity, especially cnidarians, planktonic organisms and larvae, are readily transported across ocean basins in well-documented patterns via commercial shipping, either in ballast tanks or by hull fouling (Graham and Bayha 2007). Whether introduced species become established depends on factors such as habitat suitability, lack of competitors and predators, and availability of food resources. While a majority of introduced species either do not become established or remain inconspicuous without documented impacts for many years after arrival, a small percentage of species that are translocated and released show rapidly appearing, long lasting, devastating impacts on ecosystem integrity and community structure. When a new species is documented, the first few important questions can be challenging to address, including taxonomic identity and geographic source (Carlton and Eldredge 2015). But these two questions are essential to resource management in terms of prioritizing response, predicting impacts and spread, and planning control and management strategies (Graham et al. 2014; Kingsford et al. 2018. However, molecular genetic tools can be informative in providing robust, quantifiable information regarding identification and geographic sources of invasive marine species (Holland 2000) including cryptic or taxonomically challenging taxa such as jellyfish (Holland et al. 2004).

Both *T. cystophora* and *C. sivickisi* are hypothesized to be introduced to Oʻahu from their native ranges in the western Atlantic and in the Philippines respectively, likely transported to Hawaiʻi via shipping (Carlton and Eldredge 2015). Since only one specimen of *T. cystophora* has been observed so far, it is unclear whether the species is established. However, since the specimen was found in Enchanted Lakes, as opposed to a harbor, it is possible the species is established.

If there are multiple individuals of a given species introduced to a new area, the genetic makeup of this founding population can play a role in establishment success. A founding population with a higher genetic diversity is predicted to have an advantage in adapting to the new environment (Allendorf and Lundquist 2003). High genetic diversity in an invasive population precludes a genetic bottleneck and suggests the possibility of multiple introductions rather than a single introduction.

When managing invasive species, it is essential to understand the phylogeny as well as delivery vector(s), to identify and prevent future introductions. Both Bentlage et al. (2010) and Lawley et al. (2016) discuss the possibility of human-mediated transport of *Alatina alata*. Bentlage et al. (2010) reasoned that while an early 1900's introduction to Hawaiʻi is possible, traffic from Australia to Hawaiʻi was very limited in the 19th century and life history information points to natural dispersal as more likely. However, Bentlage et al. (2010) only looked at 16S sequences from 7 specimens in Australia and 19 specimens from Waikīkī which renders accurate estimates of genetic diversity difficult. Additionally, most of the life history information required to determine if natural dispersal is a likely possibility remains unknown. Lawley et al. (2016) discuss the possibility that encysted planulae may be a method for open-ocean dispersal by currents and tides. However, encysted planulae are unlikely to be vectors of long-distance transport due to their predicted short lifecycles.

One major concern in Hawai‘i is that if introductions of marine cubozoans such as *A. alata* from various tropical regions, specifically tropical northeastern Australia where deadly box jellyfish occur, is conceivable that the same transport and release vector could result in introduction of highly venomous, even lethal box jellyfish (e.g. *C. fleckeri*) (Crow et al. 2015). Furthermore, ongoing invasions leading to establishment of novel taxa often impact marine trophic dynamics and can negatively impact marine ecosystems, impacting commercially important fisheries stocks and could have catastrophic economic repercussions for tourism, especially in areas, such as Hawai‘i, that rely heavily on this industry.

The next chapter will categorize the trophic level of *Alatina alata* through stable isotope analyses and isotope ratio comparisons with various taxa throughout the North Pacific Subtropical Gyre. The following chapter will provide robust evidence that *Alatina alata* is the sole species involved in influx events in Waikīkī and that this species was, in fact, anthropogenically introduced.

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Chapter 2

Stable Isotope Analysis of the Trophic Position of the box jellyfish, *Alatina alata*

Main Objectives: To use stable isotope analyses to determine 1) if there are seasonal trophic shifts or trophic shifts with growth and 2) to compare with other species in the North Pacific Subtropical Gyre to categorize trophic level

Introduction

Stable isotope analysis can be used to provide an indirect understanding of exploited prey based on the main taxonomic groups that have been assimilated into the tissue of the consumer during its lifetime (Peterson and Fry 1987). The use of stable isotopes in concert with approaches such as visual or DNA-based gut content analyses stand to provide a robust picture of predatory and trophic dynamics. The challenges in maintaining live specimens in captivity and complications involved with DNA-based diet studies make stable isotope analysis a logical method to determine the trophic position of *A. alata.*

To enhance our understanding of what drives the variation in influx numbers and the consistent timing of *Alatina alata* aggregations in Hawai‘i, it will be necessary to determine some fundamental aspects of their biology. There is a commonly used aphorism in ecology that for any consumer, "diet is destiny". In other words if you want to understand behavior, one of the keys lies in an understanding of diet. Almost nothing is known about the feeding behavior of *A. alata*. One of the first steps in this effort will be to determine the trophic position occupied by this species in the food web, the carbon flow to consumers and diet composition. When *A. alata* aggregate along Waikīkī’s shoreline each month, their bells are completely clear rendering it difficult to determine any information regarding diet. A single study was conducted several years ago, aimed at addressing the diet of *A. alata* in Hawai‘i using a molecular approach and universal PCR primers to amplify partially digested gut contents. Amplified COI sequence fragments were blasted via NCBI Genbank, showing the presence of a variety of vertebrate and invertebrate taxa including ahi tuna, slipper lobster, sponge and various crustaceans (Holland et al. pers comm). Although this is indirect evidence, Chiaverano et al. (2013) found that the observed 3-4 year fluctuations in influx numbers correlated with the North Pacific Subtropical Gyre Oscillation Index (NPGO) and zooplankton biomass of a certain size class (>2mm) While we see the strong correlation between number of jellyfish and zooplankton biomass, the diet composition of *A. alata* remains largely unknown.

Currently, there are very few stable isotope studies on box jellyfish aimed at studying their diet or categorizing trophic level. While cubozoans have been observed *in situ* utilizing their potent venom to subdue and paralyze prey, such observations are uncommon (Kingsford and Mooney 2014). These rare observations, when captured on camera, have led to speculation that these relatively small and fragile invertebrates can attack and consume prey larger than themselves. Junior and Haddad (2008), detected a shift in exploited prey of *Chiropsalmus quadrumanus* with jellyfish development and growth, a very common phenomenon for marine and terrestrial predatory taxa (Pimm and Rice 1987). Smaller individuals consumed mainly brachyuran larvae while larger individuals consumed predominantly pelagic sergestid shrimp, *Peisos petrunkevitchi. Aurelia aurita* and *C. capillata* shifted their trophic position ($\delta^{15}N$) and their energy source ($\delta^{13}C$) as they grew as well (Fleming et al. 2015). Trophic shifts may be associated with development in *A. alata* medusae as well, since size variation in specimen bell height at Waikīkī is commonly observed though this hypothesis has not been tested on any cubozoan.

Stable Isotopes in Diet Studies

The ratios of stable isotopes found within an organism differ depending on diet, tissue type, and the degree of fractionation during metabolic processes. In the marine environment, stable isotope ratios can also be influenced by biological, chemical, and physical oceanographic processes, and are used particularly to see trace carbon and nitrogen sources at the base of the food chain (Peterson and Fry). Stable isotope values are mainly reported as parts per thousand (‰) based on the international standard concentration (atmospheric concentration for nitrogen and the Pee Dee Belemnite for carbon) following the formula below (Peterson and Fry 1987).

$$\delta^{13}C = \left(\frac{(\frac{13C}{12C})\ \text{sample}}{(\frac{13C}{12C})\ \text{PDB}} \right) - 1 \times 1000$$

$$\delta^{15}N = \left(\frac{(\frac{15N}{14N})\ \text{sample}}{(\frac{15N}{14N})\ \text{atmosphere}} \right) - 1 \times 1000$$

The ratio of the isotope N^{15} to the standard atmospheric isotopic ratio has traditionally been used to determine the trophic position at which an organism is feeding. Photosynthetic organisms, such as phytoplankton, preferentially uptake the lighter nitrogen isotope N^{14} over N^{15}. As the nitrogen moves through the food chain, $\delta^{15}N$, or the ratio of N^{15} to N^{14}, increases in each trophic level since animals excrete isotopically "light" or "N^{15} depleted" nitrogen. The lighter isotope (N^{14}) has a lower activation energy than the heavy one, meaning less energy is required to form a new bond (Fry 2006). Typically, enrichment of $\delta^{15}N$ increases 3.4 ± 1 per mil (‰) from diet to consumer (Post 2002; Perkins et al. 2014). Nitrogen enrichment is also dependent on various oceanographic processes. Phytoplankton in very low nutrient water have higher $\delta^{15}N$ because they extract nitrogen from dissolved inorganic nitrogen in the water column.

Farquahar et al. (1982) proposed that in aquatic environments, phytoplankton's carbon isotope ratios are dependent on the discrimination factor of the enzyme responsible for carbon fixation, the isotopic composition of the carbon pool, and the intracellular concentration of carbon dioxide or bicarbonate. Fractionation of carbon isotopes occur during photosynthetic fixation of carbon and causes organic matter to be depleted in C^{13}. Phytoplankton preferentially uptake C^{12} during photosynthesis, once again, due to the lower activation energy of the lighter isotope. Generally, in highly productive areas with rapid phytoplankton growth, $\delta^{13}C$ values are higher thus linked to lower $\delta^{13}C$ values (Goericke and Fry 1994; Popp et al. 1998). The magnitude of fractionation depends on the type of phytoplankton, the growth rate, salinity, and the temperature of the seawater. However, animals fractionate the carbon isotopes less than nitrogen isotopes as they pass carbon up the food chain. Therefore, enrichment of $\delta^{13}C$ between trophic levels is much smaller (0 -1 ‰) but these values can be used to determine the energy source or primary producer baseline for the community since there is very little change between producer and consumers (Peterson and Fry 1987; Rundel et al. 2012). In the marine environment, carbon isotope ratios are reflective of the phytoplankton communities at the base of the food chain and tend to be around 10 ‰ higher for littoral zone algae than for pelagic phytoplankton (France 2006).

Different types of tissue can have varying levels of $\delta^{13}C$ or $\delta^{15}N$. For example, lipids, in general, are more depleted in C^{13} than carbohydrates or proteins and some researchers suggest not including them in analyses (MacKenzie et al. 2017). There is also an equation that can be used to normalize the potential bias from the lipid concentration in cases where the C:N ratio exceeds 3.5 (Post et al. 2007). Towanda and Thuesen (2006) did find differences in the mesoglea tissue of *Aurelia aurita*. However, Pitt et al. (2008) found no enrichment differences between the mesoglea and ectodermal tissue in the blue blubber jellyfish (*Catostylus mosiacus*) indicated more studies are needed for species-specific preparation methods.

Chapter Objectives

The objectives of this chapter are: 1) Use stable isotope C^{13} and N^{15}, to categorize the trophic position of *A. alata* in comparison with other cnidarians, zooplankton, and fish and determine if *A. alata* are ‘Generalists’ or ‘Specialists’, 2) determine if these ratios, and by inference, diet composition, change seasonally or with maturation/size to give insight to likely drivers of high/low influx events. To do this, four sampling events occurred over a six-month period (March, May, July, and September, 2020) to capture the largest range of seasonal variation in the coastal marine environment. Lower trophic level organisms that exhibit faster turnover rates of tissue can vary seasonally so we expected to see the greatest changes between winter (March) and summer (September) months. Isotopic comparisons between various zooplankton taxa occupying the North Pacific Subtropical Gyre taken during Hawaiʻi Ocean Time-Series (HOTS) cruises were used as comparison taxa (Hannides et al. 2009). For further comparative purposes, analyses were conducted with goldspot herring, *Herklotsichthys quadrimaculatus,* Waikīkī Beach, and big eye scad (*Selar crumenophthalumus*) collected at Ala Moana Beach Park, as well as blue button jellyfish (*Porpita porpita*), bluebottle, also known as man o’ war, *(Physalia physalis)*, and the common purple sea snail (*Janthina janthina)* which were collected on the windward side of Oʻahu. Although very little is known regarding the feeding behavior of *A. alata,* this species is known to be a predator, and therefore if its trophic level is similar to these small coastal fishes, this suggests that the stable isotope values for box jellyfish may be similar to those determined for small predatory vertebrates (detailed information about these comparison taxa can be found in Appendix II, Figure 1, 2, 3). Comparisons are made in order to better understand the role of this species in carbon flow through the coastal marine environment in the waters surrounding Oʻahu, as well as the trophic position of *A. alata* relative to baseline values. We selected the outgroup species for this study based on their occurrence in the central Pacific basin, which is strongly influenced by the NPSG and associated seasonal events (mixing, upwelling, temperature changes).

Methods

Sample Preparation

Following the collection protocol described in Appendix I, whole specimens were collected and all bell heights were measured (in mm). For stable isotope analysis of gelatinous marine organisms, a key challenge is elimination of all of the salt in the sample. If dried jellyfish samples contain large salt or metabolic wastes, this will influence the mass and thus alter the ratio calculations since tissue mass calculations are used (Minagawa and Wada 1984; Martínez Del Rio et al. 2009). Whole specimens were rinsed with tap freshwater to remove salt as well as water-soluble waste such as ammonia and excretory compounds which would otherwise skew $\delta^{15}N$. Fleming et al. (2001) found 2-3% enrichment in $\delta^{15}N$ for preserved jellyfish, therefore fresh samples were used and dried on the same day as collection in a desiccator at 80° C for 48 - 72 hours. This approach of using the whole bell tissue worked for most samples. Samples that did not dry fully after more than 72 hours usually turned into a sticky paste when homogenized instead of a dry powder. All sticky paste samples were discarded since a dry powder is required for isotope determination via the elemental analyzer and isotope ratio mass spectrometer.

The goldspot herring (*H. quadrimaculatus*) and bigeye scad (*S. crumenophthalumus*) were caught in shallow (about 1-2 meter deep) water off Waikīkī and Ala Moana Beach Park. Cross sections of the fish were dissected and abdominal-region muscle tissue samples were dried for 48 hours. More information on these two species, including pictures can be found in Appendix II, Figure 1, 2. Specimens from two additional genera of cnidarians, *Porpita* and *Physalia*, and one marine gastropod, *Janthina*, were collected in 2010 and 2012 in Waimanalo, O'ahu by David Hyrenbach (more information on these can be found in Appendix II, Figure 3). *Janthina* preys on cnidarians like *Physalia* and it was predicted that nitrogen isotope ratios might be higher than the cnidarians. Zooplankton isotopic signatures were obtained from Hannides et al. 2009 from seasonal zooplankton tows near station ALOHA from February 1995 to January 2005.

All dried samples were homogenized using a mortar and pestle before being sent to the Biogeochemical Stable Isotope Facility at the University of Hawai'i, Manoa where they were weighed and analyzed using a Thermo Finnigan Delta^{Plus} XP mass spectrometer.

Analysis and Visualization

To determine if isotope ratio values deviated from a normal distribution, a Shapiro-Wilks Test in R was conducted. To determine if there was a correlation between size (bell height) and isotope enrichment/depletion, a correlation test in R was conducted using the Pearson's product-moment correlation (RCore Team 2020).

$\delta^{15}N$-$\delta^{13}C$ scatterplots, bar graphs, and matrices used to compare taxa were created in R and Excel (Excel Version 16.43). A trophic discrimination factor ($\Delta^{15}N$) of 3.4 ± 1‰ (Post 2002; Perkins et al. 2014) was assumed and Kruskal-Wallis tests in R were used to determine seasonal differences between the four different collection times. A Kruskal-Wallis test was used to compare the bell height median bell heights of multiple groups without the assumptions of normality (Kruskal and Wallis 1952). Kruskal-Wallis was also used to determine whether there were significant isotopic differences between *A. alata* and any comparison species ($p < 0.05$). Post-hoc tests in R used the conservative Bonferroni correction to reduce the chances of a type 1 error (false positive). Dunn's post-hoc tests were used to determine which taxa were significantly different from each other and from *A. alata* and the magnitude which they differ (*p*-value of 0.025) (Bonferroni 1936).

Results

Over the course of 6 months of 2020, box jellyfish were collected in March (n=6), May (n=9), July (n=9), and September (n=8) (Table 1). Two specimen of big eye scad (*Selar crumenophthalmus*) and two specimens of goldspot herring (*Herklotsichthys quadrimaculatus*) were collected by fishermen (Table 1, Appendix II, Figure 1, 2). Size ranges for *A. alata* included a maximum bell height of 100 mm and a minimum of 50 mm. No significant correlation was detected between jellyfish size and $\delta^{15}N$ values (Figure 1A). A weak correlation (0.387, *p-value* = 0.029) was detected between $\delta^{13}C$ and jellyfish size (Figure 1B). $\delta^{15}N$ values measured in parts per thousand (ppt) range between 6.5‰ and 10.4‰ with a mean of 7.7‰ ± 0.95‰ (Variance = 0.90). $\delta^{13}C$ values range between -15.6‰ and -19.5‰ with a mean of -18.2‰ ± 1.05‰ (Variance = 1.10).

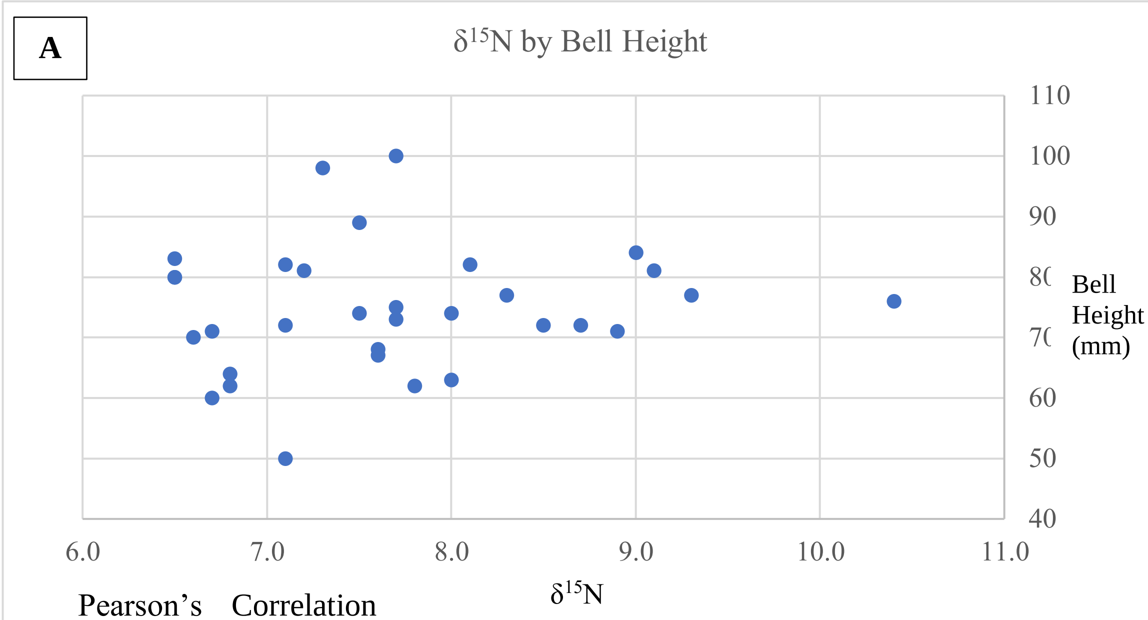
To visualize temporal changes in diet and isotope ratio values, $\delta^{13}C$ and $\delta^{15}N$ values were plotted (Figure 2) and grouped by month sampled (March, May, July, and September 2020). The period between March and September had the maximum sea surface temperature difference of any two sampling periods, 4° F (Price 2019), along with the most extreme thermocline depth changes, 12 m on average (Wang et al. 2000), so the largest difference in carbon and nitrogen isotope signatures was expected between these two months. However, a Kruskal-Wallis test did not show a significant difference in $\delta^{15}N$ values between any seasons with a (Chi square = 2.97, *p*-value = 0.4, df = 3) (Table 2).

The Kruskal-Wallis test revealed a significant difference in $\delta^{13}C$ values over the 6-month period (Chi square = 7.90, *p*-value = 0.05, df = 3). The Dunn's test with a Bonferroni correction revealed it was not September and March that had significant differences but March and May (*p*-value = 0.008) as well as May and July (*p*-value = 0.012) (Table 3). The March median value for $\delta^{13}C$ was 2.41‰ higher than the median in May while the July median was 2.26‰ higher than the May median (Table 3).

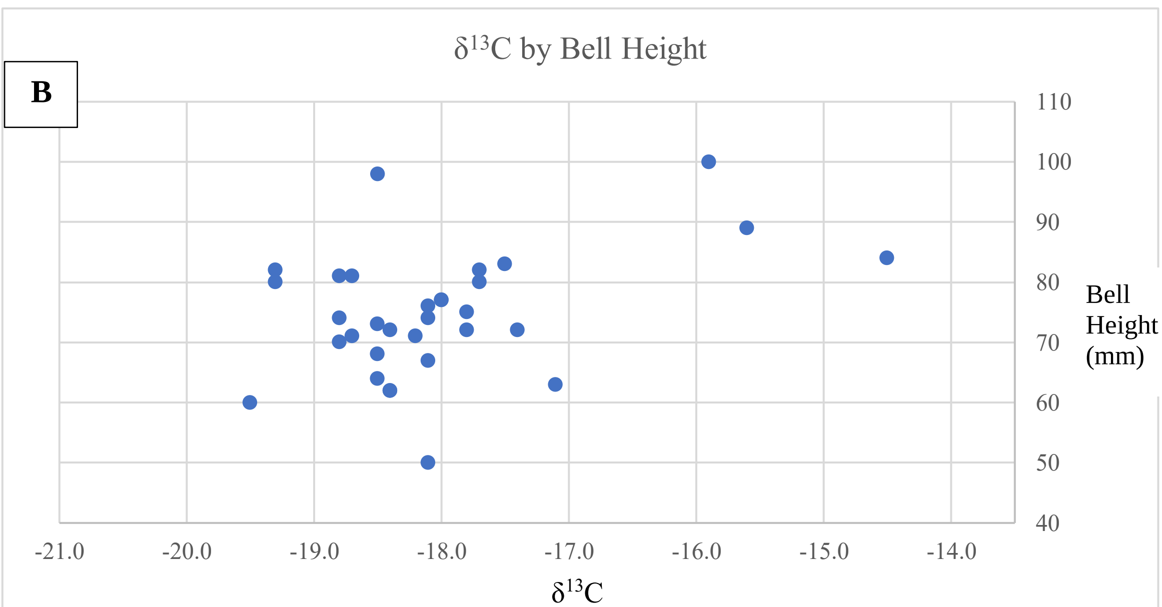
Table 1. Specimens collected for this study, including collection and analysis dates, size (mm), weight (mg), nitrogen and carbon composition (µg), stable isotope ratios, and influx group which were used to make comparisons to test for temporal differences.
1 = March, 2 = May, 3 = July, and 4 = September
n/a not applicable

Species	Date Collected	Specimen #	Bell Height	Date Analyzed	weight mg	µg N	$\delta^{15}N$	µg C	$\delta^{13}C$	Influx Group
A. alata	3/19/20	1	80	4/27/20	2.42	41	6.5	155	-17.7	1
A. alata	3/19/20	2	83	4/27/20	2.54	58	6.5	221	-17.5	1
A. alata	3/19/20	3	82	4/27/20	2.50	71	7.1	295	-17.7	1
A. alata	3/19/20	4	73	4/27/20	2.55	70	7.7	270	-18.5	1
A. alata	3/19/20	5	77	4/27/20	2.50	100	8.3	398	-18.0	1
A. alata	3/19/20	6	75	4/27/20	2.51	91	7.7	368	-17.8	1
A. alata	5/16/20	7	76	6/2/20	2.56	77	10.4	305	-18.1	2
A. alata	5/16/20	8	67	6/2/20	2.56	56	7.6	212	-18.1	2
A. alata	5/16/20	9	74	6/2/20	2.50	52	8.0	202	-18.1	2
A. alata	5/16/20	10	81	6/2/20	2.64	66	9.1	258	-18.8	2
A. alata	5/16/20	11	80	6/2/20	2.49	57	6.5	232	-19.3	2
A. alata	5/16/20	12	64	6/2/20	2.53	71	6.8	282	-18.5	2
A. alata	5/16/20	13	60	6/2/20	2.59	70	6.7	275	-19.5	2
A. alata	5/16/20	14	74	6/2/20	2.52	67	7.5	266	-18.8	2
A. alata	5/16/20	15	72	6/2/20	2.54	53	7.1	207	-18.4	2
A. alata	7/14/20	16	98	7/17/20	0.62	32	7.3	124	-18.5	3
A. alata	7/14/20	17	72	7/17/20	0.67	26	8.7	111	-17.8	3
A. alata	7/14/20	18	100	7/17/20	0.54	10	7.7	42	-15.9	3
A. alata	7/14/20	19	62	7/17/20	0.57	35	7.8	136	-18.4	3
A. alata	7/14/20	20	63	7/17/20	0.56	20	8	84	-17.1	3
A. alata	7/14/20	21	84	7/17/20	0.51	13	9	62	-14.5	3
A. alata	7/14/20	22	68	7/17/20	0.58	24	7.6	96	-18.5	3
A. alata	7/14/20	23	89	7/17/20	0.46	14	7.5	63	-15.6	3
A. alata	7/14/20	24	82	7/17/20	0.59	41	8.1	172	-19.3	3
A. alata	9/11/20	25	62	8/29/20	2.46	93	6.8	384	-18.4	4
A. alata	9/11/20	26	72	8/29/20	2.42	54	8.5	219	-17.4	4
A. alata	9/11/20	27	71	8/29/20	2.58	110	8.9	482	-18.2	4
A. alata	9/11/20	28	71	8/29/20	2.50	74	6.7	301	-18.7	4
A. alata	9/11/20	29	70	8/29/20	2.53	112	6.6	459	-18.8	4
A. alata	9/11/20	30	77	8/29/20	2.43	60	9.3	242	-18.0	4
A. alata	9/11/20	31	81	8/29/20	2.45	69	7.2	301	-18.7	4
A. alata	9/11/20	32	50	8/29/20	2.48	90	7.1	347	-18.1	4
H. quadrimaculatus	7/14/20	F1	SL = 110	7/17/20	0.68	85	10	294	-17.9	n/a
H. quadrimaculatus	7/14/20	F2	SL = 110	7/17/20	0.67	85	9.8	305	-18.2	n/a
S. crumenophthalumus	7/14/20	F3	SL = 138	7/17/20	0.65	81	6.4	296	-18.8	n/a
S. crumenophthalumus	7/14/20	F4	SL = 138	7/17/20	0.49	67	6.4	234	-18.7	n/a

SL = Standard Length



Pearson's	Correlation
p-value	0.497
Correlation	0.125



Pearson's	Correlation
p-value	**0.029***
Correlation	0.387

Figure 1A and 1B: All 32 *Alatina alata* collected for this study with $\delta^{15}N$ values (A) and $\delta^{13}C$ values (B) plotted with bell height (mm) on the y-axis. A Pearson product-moment correlation test was performed in R with significant p-values denoted by * ($\alpha = 0.05$).

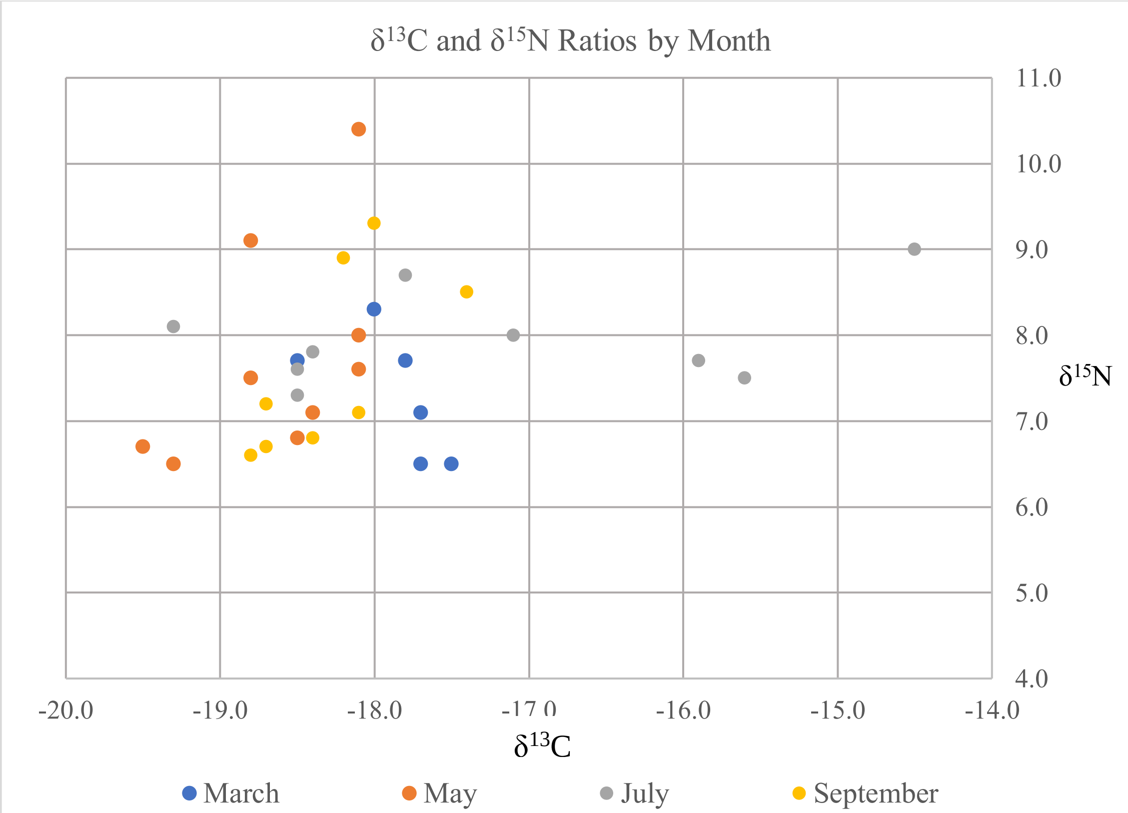


Figure 2. $\delta^{15}N$ values by $\delta^{15}C$ values for 32 *Alatina alata* specimens grouped by month (March (n = 6), May (n = 9), July (n = 9), and September (n = 8)).

$\delta^{15}N$

Table 2. Kruskal-Wallis Rank Sum Test Comparing (top) and $\delta^{13}C$ (bottom) seasonal values to determine if there was a significant change in isotope ratios between months

$\delta^{15}N$	Chi-squared	2.97
	Degrees of Freedom	3
	p-value	0.4
$\delta^{13}C$	Chi-squared	7.90
	Degrees of Freedom	3
	p-value	**0.05**

Table 3. Dunn's Test to determine which specific months had significant differences in $\delta^{15}C$ values. Pairwise *z*-test statistics are based on the difference in mean ranks (top) and *p*-values (bottom). Significant *p*-values (< 0.025) are bolded

	March	May	July
May	**2.406**		
	0.008		
July	0.389	**-2.256**	
	0.349	**0.012**	
September	1.485	-0.960	1.229
	0.069	0.169	0.110

alpha = 0.05
Reject null hypothesis if p <= alpha/2

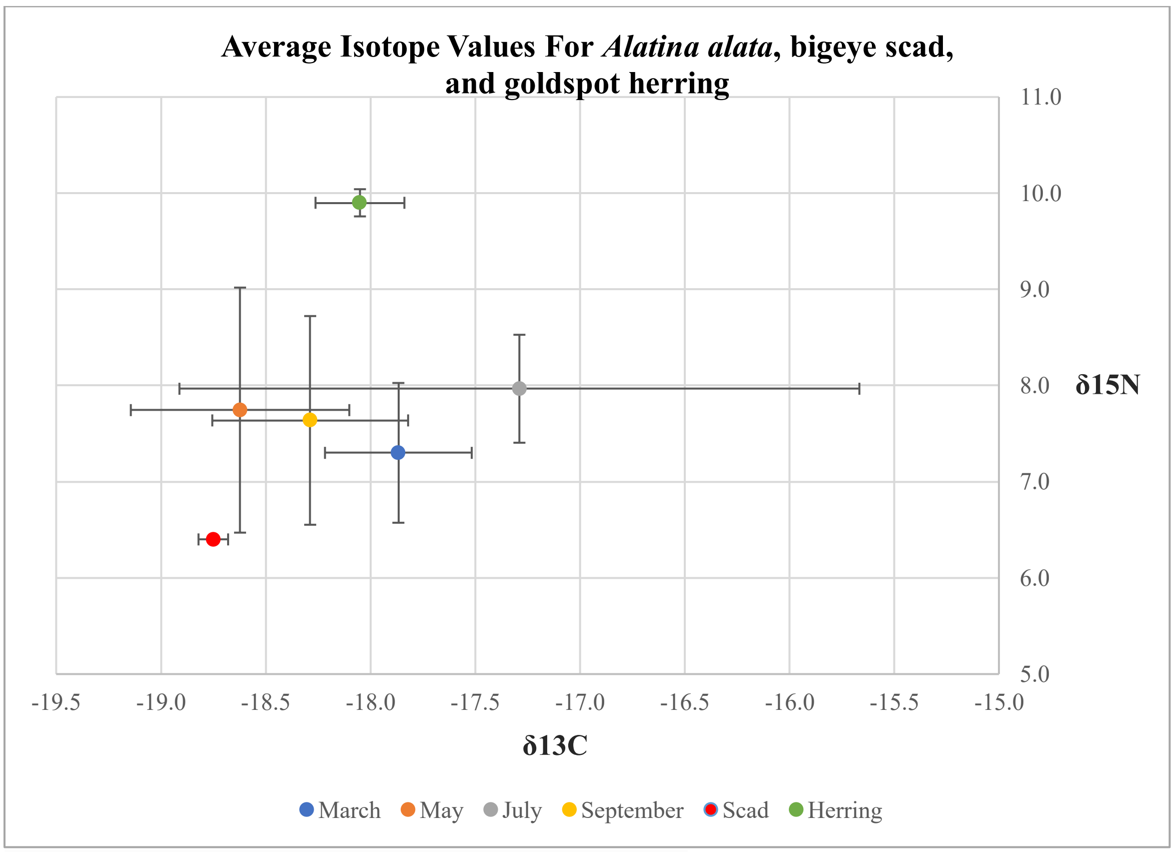


Figure 4. Scatterplot of average and standard deviation $\delta^{15}N$ values by $\delta^{15}C$ values for *Alatina alata* specimens grouped by month (March (n = 6), May (n = 9), July (n = 9), and September (n = 8)) as well as average and standard deviation $\delta^{15}N$ values by $\delta^{15}C$ values for bigeye scad (n = 2) and goldspot herring (n= 2)

Isotopic signatures for zooplankton, fish, gastropods, and cnidarians were obtained to compare with *A. alata* and place in the NPSG trophic web (Figure 4). Raw isotope ratio values for each of these groups, the species included, scientific and common names, and photographs of each species are detailed in Appendix II, Figure 3. Zooplankton values were determined by Hannides et al. (2009) from bulk $\delta^{15}N$ from Station ALOHA Hawai‘i Ocean Time Series (HOTS) tows from various years (1995, 1996, 2000, and 2005). These taxa included four species of copepods, one euphausiid, and a bulk sample of mixed zooplankton in the 1-2 mm size range. There was a large standard deviation in bulk $\delta^{15}N$ for many of these species and mixed assemblages (ranging from 1.1‰ to 3.1‰, with the largest for *Neocalanus robustior* (Figure 5). However, the means did not exceed 4.85‰ or fall below 4.0‰ for any of these groups from the HOTS tows. There was no significant difference detected between copepods when a Dunn's test was conducted (Table 5).

Average $\delta^{15}N$ values for *Janthina, Physalia,* and *Porpita* ranged between 6.6‰ and 7‰ for all three of these groups (Figure 5) and there were no significant differences among these groups and the other taxa, except for *Physalia,* having a significantly higher enrichment in $\delta^{15}N$ than *Euchaeta rimana* (copepod), a secondary consumer. Small sample sizes may account for the lack of significant *p*-values, especially since for *Porpita*, $n = 1$.

Between the two species of fish, there was a 3.5‰ difference in $\delta^{15}N$ means, (9.9‰ for *H. quadramaculatus* and 6.4‰ for the *S. crumenophthalumus*). Average $\delta^{15}N$ values for *A. alata* (7.7‰ ± 0.95) fell between the two species of fish. However, the Dunn's test does not show any statistically significant differences between the three species (Table 5). Figure 4 compares average $\delta^{15}N$ and average $\delta^{13}C$ with standard deviations between the box jellyfish and the two species of fish.

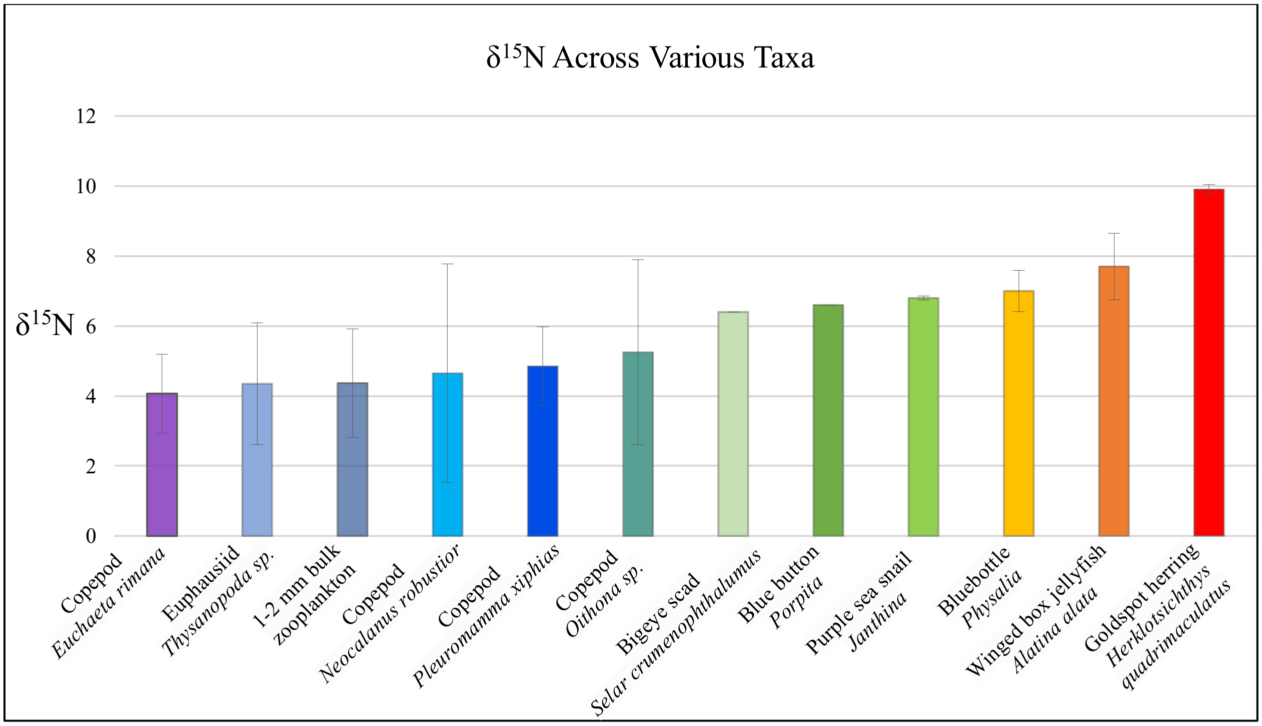


Figure 5. Bulk $\delta^{15}N$ from zooplankton HOTS tows from various years (1995, 1996, 2000, and 2005), big eye scad, and goldspot herring from Waikīkī, cnidarians, marine gastropods, as well as the 6-month average from *A. alatina*. Common names and scientific names are listed on the x-axis. From lowest to highest (left to right): Copepod *Euchaeta rimana,* Euphausiid *Thysanopoda sp.*, 1-2 mm bulk mixed zooplankton, Copepod *Neocalanus robustior*, Copepod *Pleuromamma xiphias,* Copepod *Oithona sp.,* bigeye scad *Selar crumenophthalumus*, blue button jellyfish *Porpita,* Purple sea snail *Janthina,* bluebottle *Physalia,* Winged box jellyfish *Alatina alata, and goldspot herring Herklotsichthys quadrimaculatus*

There were significant differences in N^{15} isotopic signatures between *A. alata* and other taxa (Kruskal and Wallis 1952, Table 4 & 5). For mixed zooplankton 1-2 mm, there is a significant enrichment in $\delta^{15}N$ by 3.17‰ with a ($p = 0.001$). Specimens of *Alatina alata* are also significantly ($p < 0.025$) more enriched in ^{15}N than copepods, one species of primary consumer (*Oithona* spp.), 2 species of secondary consumers (*E. rimana* and *P. xiphias*), 2.89, 4.28, and 3.36‰ respectively (For a full list of all comparisons, see Table 5). Overall, *A. alata* has the second highest mean $\delta^{15}N$ value (7.7‰) of all taxa evaluated just behind the goldspot herring (9.9‰). It must be noted that for accurate estimations of trophic position comparisons, a larger sample size for each comparison taxa is desired. In order to avoid a type 1 error for the

Dunn's Test, due to the small sample sizes of specimen within comparison taxa (n = 1 - 6) a more conservative argument (Bonferroni) was used which adjusts the test so that a *p*-value less than alpha divided by 2 is needed to report statistical significance (0.025) (Bonferroni 1936).

Table 4: Kruskal-Wallis Rank Sum Test comparing $\delta^{15}N$ values among various marine taxa

Chi-squared	47.05
Degrees of Freedom	10
p-value	0

Table 5: Dunn's Test matrix to visualize species – specific comparisons in $\delta^{15}N$ values with pairwise *z*-test statistics based on the difference (x-axis values – y- axis values) in mean ranks (top) and *p*-values (bottom). Significant *p*-values (< 0.025) are bolded

	Mixed Zooplankton	*A. alata*	*E. rimana*	*H. quadrim-aculatus*	*Janthina*	*Neocal.*	*Oithona*	*Physalia*	*P. xiphias.*	*Porpita*	*S. crumenop-hthalumus*
A. alata	**-3.174** **0.001**										
E. rimana	-0.018 0.493	**4.279** **0.000**									
H. quadrim-aculatus	**-3.145** **0.001**	- 1.305 0.096	-3.496 0.000*								
Janthina	-1.551 0.060	1.076 0.141	-1.773 0.038	1.753 0.040							
Neocal.	-1.275 0.101	1.408 0.080	-1.454 0.073	**1.975** **0.024**	0.211 0.417						
Oithona	-0.501 0.308	**2.891** **0.002**	-0.573 0.283	**2.869** **0.002**	1.157 0.124	0.866 0.193					
Physalia	-1.852 0.032	1.364 0.086	**-2.246** **0.012**	1.908 0.028	-0.061 0.476	-0.336 0.368	-1.435 0.076				
P. xiphias.	-0.249 0.402	**3.255** **0.001**	-0.275 0.392	**3.091** **0.001**	1.409 0.079	1.124 0.131	0.272 0.393	1.734 0.042			
Porpita	-0.882 0.189	0.884 0.188	-0.931 0.176	1.510 0.066	0.215 0.415	0.057 0.477	-0.568 0.285	0.269 0.394	-0.741 0.229		
S. crumeno-phthalumus.	-0.694 0.244	1.760 0.039	-0.760 0.224	**2.234** **0.013**	0.694 0.244	0.476 0.317	-0.289 0.386	0.828 0.204	0.511 0.305	0.314 0.377	
Thysanopoda	-0.115 0.454	**3.448** **0.000**	-0.116 0.454	**3.210** **0.001**	1.544 0.061	1.253 0.105	0.418 0.338	1.893 0.029	0.145 0.442	0.832 0.203	0.630 0.264

Discussion

Variations in isotope ratios within Alatina alata

Seasonal variation in sea surface temperatures (SST) off Hawai‘i changes very little throughout the year. In the coldest months, March and February, temperatures average around 23°C while in the warmest months, September and early October, temperatures average around 27° C (Price 2019). The thermocline, at an average depth of around 180 m, shoals during the winter, to around 12 m depth, and can bring nutrient rich, oxygenated, cooler water to the surface, a process that can stimulate higher productivity (Wang et al. 2000). With a local average SST's range of 7° F and only a single six-month period studied, the 5‰ range in carbon isotope ratios between specimens may be a result of the limited sample sizes. However, it is possible this is a biological signal based on seasonal differences in productivity, and or changes in carbon flow in the central Pacific marine ecosystem that influenced carbon isotope values over time. These carbon isotope ratio changes could result from mesoscale eddies upwelling or downwelling nutrients which would influence the fractionation of carbon and nitrogen on seasonal/monthly timescales due to the rapid turnover rate of phytoplankton (Burkhardt et al. 1999) The lack of a seasonal change in $\delta^{15}N$ values can indicate there was no significant changes in diet over the six-month period.

A lack of a significant correlation between the overall body size of the jellyfish based on bell height measurements and $\delta^{15}N$ indicates that the jellyfish that we sampled are feeding at either single trophic level, or at similar levels, despite the fact that bell heights of the largest medusae sampled were double that of the smallest medusae. Previous work has suggested that the medusae arriving at Waikīkī are sexually mature (Chiaverano et al. 2013), and that shoreward aggregation behavior is likely to be related to reproduction. A bell height range of 30 – 70 mm for mature *A. alata* recorded in Bonaire (Lewis et al. 2013) does indicate that specimens collected for this study were all mature. Our inference that despite two-fold variation in specimen body size, they all appear to be feeding at the same trophic level, unlike what was observed in *Aurelia aurita* and *C. capillata* (Fleming et al. 2015), further supports this idea that these are mature adult box jellyfish occupying the same niche.

It is possible that sampling box jellyfish in a different environment, such as offshore at depth, might result in obtaining a larger range, and namely immature specimens could reveal larger differences in stable isotope values and thus indicate differences in exploited prey and diet composition. However, sampling immature box jellyfish has never been accomplished, in part because we simply are not aware of where, or how deep they occur when they are not swimming to Waikīkī Beach in circalunar spawning aggregations, or where they develop and metamorphose from polyp to medusa.

Alatina alata as proxies for Isoscapes

To address isotope variation across temporal time scales in the ocean, generating isoscapes of the area are helpful in creating baselines. An isoscape, a term proposed by West et al. (2008) and refined by Bowen (2010), is a distinct area and the community that occupies it, with predictive isotopic values based on the processes and isotopic fractionation unique to that ecosystem. Isoscapes are especially important when studying higher trophic level migratory species since these taxa can spend time and feed in multiple different isoscapes. However, for *A. alata*, spatial scale may not be an issue since as far as we are aware, this species does not migrate between and feed in different isoscapes, although this remains an intriguing question. Longmore et al. (2014) measured isotopic signatures of the lion's mane jellyfish (*Cyanea capillata*) to test if scyphomeduseae can be used to construct offshore isoscapes and found they were successful in doing so despite their mobile nature. This raises the unique possibility that compound-specific amino acid analyses of *A. alata* tissue can be used to constructing isoscapes offshore Hawaii. Compound-specific amino acid analyses allow for individual amino acids to be analyzed and baselines to be drawn from those. For example, certain amino acids retain the same isotope ratio values as the primary producer at the base of food web in the consumer's ecosystem, so that establishing baselines, is not necessary. However, this method is prohibitively expensive and therefore, a bulk isotope analysis technique was used for this study. Future studies using this compound-specific technique could even be implemented to study offshore isoscape changes over time.

Trophic Comparisons Among Taxa

It was critical to select appropriate taxa for comparison, that feed in and experience similar coastal oceanographic conditions. Some studies of marine ecosystem food webs use particulate organic matter (POM) samples to establish the local trophic baseline. Ideally, POM samples would have been obtained offshore of Waikīkī for the six-month time period *A. alata* were collected in order to establish a baseline. Since this was not possible, the next best approach was to use appropriate comparison taxa to draw broad, trophic level comparisons.

The zooplankton from the HOTS cruises all had very similar mean values of $\delta^{15}N$ ranging from 4 – 5.25 ‰. The accepted trophic discrimination factor is 3.4 ± 1‰ (Post 2002; Perkins et al. 2014) and the trophic discrimination between *A. alata* and these groups was 2.9–4.3‰. If this was a simple food chain, *A. alata* would be one trophic level higher than these zooplankton. Since both of these specimens were collected over multiple months (and years for the HOTS cruise data), it is safe to conclude that *A. alata* is likely to be feeding on these different groups of zooplankton.

While we did see significantly higher $\delta^{15}N$ values in *A. alata* than in primary and secondary zooplankton consumers (Figure 5, Table 5), we did not see significant trophic shifts from the other jellyfish included in the study, *Porpita* and *Physalia*. Perhaps this is because the advanced swimming capabilities and vision of *A. alata* do not necessarily indicate the cubozoan is feeding at a higher trophic level than smaller, less venomous cnidarians from this study.

What is interesting here is that since *Janthina* feeds on *Physalia*, *Janthina* was predicted to occupy a higher trophic level. Perhaps, *Physalia* are not as large as a contributor to their diet or their feeding on *Physalia* varies seasonally. However, the sample sizes for *Porpita* (1) and *Physalia* (6) were small.

Interestingly, the two fish which are similar in size and diet exhibited very different $\delta^{15}N$ values. The bigeye scad feeds on zooplankton like euphausiids nad decapods as well as larval and juvenile fish (Roux and Conand 2000). Similarly, the goldspot herring feeds mainly on teleost larvae and zooplankton (Milton et al. 1994). *A. alata* did not differ significantly from either fish species, but the goldspot herring had significantly higher $\delta^{15}N$ values than the scad.

Overall, these isotopic signatures for the fish were not large as predicted considering their $\delta^{15}N$ values were close to *Porpita, Janthina*, and *Physalia* despite the small size of these species. Since the two fish were caught in the same general location as the *A. alata*, they make a good opportunity for a spatially consistent trophic comparison. The fact that *A. alata* nitrogen values fall between these two fish suggests that they are feeding on very similar species, possibly both large and small zooplankton.

Generalists or Specialists?

Data presented here indicate that *A. alata* consumes zooplankton that occupy multiple trophic levels, including both primary and secondary consumer categories (Hannides et al. 2009), possibly including euphausiids. The trophic position of *A. alata* overlaps with the juvenile schooling fish bigeye scad and the goldspot herring. Box jellyfish are likely consuming and possibly competing for the same types of prey during their medusae stage since there was no evidence for trophic changes with size (bell height). Future stable isotope studies focusing on box jellyfish are warranted, including investigation of long-term and seasonal trends and patterns in the Hawaiian Islands, differences between seasons or years within positive or negative major climate indices such as NPGO and ENSO, as well as for this species from other geographic regions such as tropical Australia, islands in the western Pacific such as the Northern Marianna Islands, and the Caribbean islands. In addition, it would be fascinating to investigate stable isotope values for different box jellyfish species across a gradient of hypothesized life history strategies or venom toxicities. For example, larger, highly venomous taxa such as *Chironex fleckeri*, may be able to consume larger prey than species with less toxic venom. Stable isotope data could inform the hypothesis that the higher the toxicity of the venom, the higher that species is capable of feeding in the food web. Data from this study will be even more valuable once future efforts include baseline data from species at the far ends of the trophic spectrum in central Pacific ecosystems. Stable isotopes comparisons of additional herbivorous grazers, primary producers and apex predators such as large pelagic predatory fishes and sharks can help to further specify trophic positioning of *Alatina alata* and explore their potential as possible isoscape proxies.

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Chapter 3

Population Genetics and Systematic Analyses of *Alatina alata*, a venomous invasive species in Hawai‘i

Main Objectives: To 1) Assess population structure and systematic status of Alatina alata *in the Hawaiian Islands and 2) Assess the likelihood of an introduction vs. a natural circumtropical distribution in the Tropical Atlantic and Pacific.*

Introduction

Background

While box jellyfish are regularly conspicuous and disruptive for ocean recreation at Waikīkī Beach (Crow et al. in review), research to date has been limited, with a few papers aimed at understanding sting and venom biology (Brinkman and Burnell 2009), one study outlining what is known about the history and distribution of box jellyfish in Hawai‘i (Crow et al. 2015), and a single long-term ecological study (Chiaverano et al. 2013) showing that influx numbers are corelated with oceanographic parameters. Little else is known about their taxonomy, growth rate, feeding, reproduction, distribution, or status as an introduced or native species. Without any genetic studies, it is often difficult to assess the invasive status of marine taxa (Holland 2000), and this stinging box jellyfish in Hawai‘i is no exception to this notion. Prior to this study, *Alatina alata* has been suggested to be native to the Hawaiian Islands. Many have assumed that the species simply did not present a hazard to humans prior to several anthropogenic changes in the area, including dramatic increases in number of people participating in ocean recreation and increased coastal light pollution with the expansion of beachfront development. Increases in the number of beach users beginning in the 1980’s coincide with first records of stings, but this phenomenon coupled with poor documentation lends some uncertainty regarding precise timing of the first mass-sting events. The recent nature of box jellyfish sting records, suggests the possibility that *A. alata* was anthropogenically introduced. However, the possibility that it has long been present offshore but had not aggregated nearshore until more recently could not be ruled out. All in all, use of historical data to determine the status of box jellyfish in Hawai‘i has proven inconclusive.

Recent phylogenetic studies aimed at elucidation of relationships among the cubozoan families provide the first evidence for patterns that suggest the species responsible for the frequent mass stinging events in Waikīkī is non-native. This initial evidence comes in the form of matching rRNA gene haplotypes from limited sampling of *A. alata* from distant sampling sites that include Saipan in the western Pacific, tropical Australia, Bonaire in the Caribbean, and a few samples from Waikīkī Beach in Hawai‘i . So evidence had been accumulating for a single lineage with a broad distribution (Lawley et al. 2016). Although the influx patterns are not as well documented as in Hawai‘i , Caribbean and Australian populations appear to follow similar circalunar aggregation behavior, arriving along shore with the same timing as in Hawai‘i, 8-12 days after the full moon (Carrette et al. 2014: Lewis et al. 2013).

This species is capable of delivering a painful, but non-lethal sting and has been known to sting hundreds of beach users in a single day. In fact, one such recent mass sting event in 2019 resulted in about 1,000 envenomations. However, in spite of the threat of mass sting events posed by monthly influxes, plus the fact that local weather forecasts include predictions of timing of arrival of box jellyfish influx each month, scientific attention has only begun to focus on the ecology of *A. alata,* and many questions remain. Local researchers have speculated that there may be more than a single species involved, for example there have been anecdotal reports of box jellyfish on the O‘ahu coastline with unusually large bell heights, raising questions regarding the possibility that more than a single species could be present. There is also some question as to exactly how long these influx events have been taking place, as well as why they seem to only occur with regularity and such high intensity at Waikīkī Beach. In addition, the feeding behavior, diet, trophic position, reproduction, and even where the polyps develop are all unknown. Many basic questions regarding the biology of these intriguing and problematic species remain.

Therefore, the overall objectives of this study seek to address a few fundamental issues pertaining to the provenance, invasive status, population structure, and taxonomic status of this species in Hawai‘i . The focus of this chapter is on the use of neutral molecular markers to test for evidence of population partitioning offshore of O‘ahu. This chapter also addresses whether there is any particular genetic affiliation with published data from three other global locations, which might help us identify the source of introduction to the Hawaiian Islands. Finally, I will generate evidence as to whether the influx consists of multiple lineages by sampling multiple influxes over the course of 12 months (March 2019 – February 2020). While it is assumed that a single species is responsible for the regular Waikīkī influx events, based on behavior and gross morphology, this has yet to be confirmed through genetic surveys.

The Role of Phylogenetics in Management of Invasive Species

Molecular evidence generated via this study strongly suggests that the cubozoan found regularly on O‘ahu's southern shores forms a genetically cohesive lineage with samples from Australia, Saipan, and Bonaire. These findings raise the question, is the species in Hawai‘i native or introduced? And could the species be native to Hawai‘i and introduced to these other areas from here? If the population is found to be introduced and was brought to Hawaiʻi by humans from elsewhere, such as Australia, the possibility arises that additional venomous, dangerous cubozoans could be introduced to Hawaiian waters as well, such as *Chironex fleckeri*, an extremely venomous tropical box jellyfish known for its fatal stings.

In order to address whether *Alatina alata* was introduced to Hawaiʻi, it is critical to understand the taxonomy, provenance, and global distribution. The objective of this chapter will be to 1) elucidate genetic diversity of *A. alata* population(s) in Hawaiʻi, and to 2) compare the results of molecular analysis with populations from other locations including the Caribbean, Australia, and Saipan. Using comparative molecular descriptive statistics, I have addressed these questions and present evidence that this species was introduced to the Hawaiian Islands and originated in the south Atlantic, since this is the type locality for *Alatina alata*.

Geographic Setting

Waikīkī Beach is the most popular and highly visited beach in the state of Hawaiʻi. Of the approximately 9 million visitors to Hawaiʻi in 2016, 5.6 million came to Oʻahu and a majority of these visitors stayed in Waikīkī. However, it was not until after the turn of the twentieth century that tourism rapidly expanded. In the early 1900's Waikīkī would have been unrecognizable relative to today, consisting of rice paddies, duck ponds, and swamps. After the Waikīkī Reclamation Project, which drained the lagoon and dredged the shoreline, the beach became the gateway to all of Hawaiʻi (Mak 2015). Today, the Waikīkī beach front and both small and major streets for several blocks north of the beach, are lined with a dense array of high-rise hotels and apartment buildings, restaurants, and shops, and floodlights which illuminate the beach and adjacent waters each night after sunset. As mentioned, monthly influxes of *A.*

alata did not begin until the 1980's which directly followed the rapid development of Waikīkī in the 1960's and 1970's (Sheldon and Abenoja, 2001). Holland et al. (in prep) have generated strong evidence that *A. alata* exhibits positive phototactic behavior and have been observed swimming directly towards artificial light sources in both the ocean and laboratory experiments. It is likely that *A. alata* occurs offshore of Honolulu, and 10 days after each full moon when there is no moon in the sky, and adult medusae come to the surface to spawn, the point source of light of the illuminated developed beach front at Waikīkī Beach likely acts as a beacon, and the light of Waikīkī is picked up by the sophisticated visual apparatus of *A. alata*, causing them to swim towards Waikīkī Beach.

Methods

Detailed sampling site information found in Appendix I

Figure 1. Waikīkī Beach, O'ahu (Latitude: 21° 16' 23" N, Longitude: 157° 49' 26" W) (Google Earth). Yellow line demarcates the main sampling area along the coastline. The seawall running along the sampling area is visible.

Collection Protocol

Each month, between 8 and 12 days after each full moon, *A. alata* specimens were collected along the beach and from the shallow water within the study area. Depending on the time of the high tide, collections began either late at night or very early in the morning to ensure the specimen were not buried or covered along the shoreline by beachgoers. Preliminary surveys included testing out various days and nights during the influx window in order to optimize sample sizes. These surveys indicated the unpredictable nature of influx events, and suggested that collections should focus on the 9^{th} day after each moon and continue the 10^{th} day if necessary.

Surveys and collections were conducted along the shoreline and in the water up to 1 meter deep. Live specimens were carefully scooped into Ziploc bags along with some seawater. Volunteers were advised to wear gloves and protective gear. The box jellyfish specimens were frozen and stored at the Hawaiian Biodiversity and Conservation Lab at the Oceanic Institute until their tentacles were dissected for DNA extraction.

Tentacle Dissection

To prepare samples for DNA extractions, tentacles were removed from fresh specimens and the jellyfish were then labeled and frozen. To remove tentacles, a combination of forceps and dissecting scissors were used. Dissecting tools were sterilized with a 10% bleach solution to avoid DNA cross-contamination. Removed tentacles were stored in individual vials in 80% ethanol until DNA extraction.

DNA Extraction

Tentacle tissue was removed from the ethanol storage buffer and rinsed in de-ionized autoclaved water. Consistent, equal masses of tentacle tissue (approximately 35 mg per specimen) were excised using a sterile razor blade. Genomic DNA was extracted from the tentacle tissue using the Qiagen NucleoSpin kit (Qiagen, Düren, Germany) according to the manufacturer's protocol (Ref number 740609.250). The DNA was eluted in 200ml of de-ionized autoclaved water and stored at −20°C.

PCR Amplification

A DNA barcoding approach (Hebert et al. 2003) was applied, by sequencing a 460 base pair (bp) fragment of the mtDNA cytochrome *c* oxidase I (COI) gene using PCR primers modified from the widely applicable universal primers (Folmer et al. 1994) shown in Table 2 (Lawley et al. 2016). These primers were used to amplify target fragments using an MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) with *Taq* Plus Master Mix Red and PCR reagents and buffers from Lamda Biotech (Catalog Number D124R). Reaction details and PCR cycle details can be found in Appendix IV. DNA fragments were purified with NucleoSpin spin columns distributed by Takara Bio USA, Inc, according to the manufacturer's protocol (Macherey-Nagel), and visualized via agarose minigel electrophoresis. Amplified mtDNA fragments was cycle-sequenced using forward and reverse PCR primers (Table 1). The DNA was sequenced using Sanger DNA sequencing service at University of Hawai'i (ASGPB).

Table 1. Primers used in this study listed below. The primers used by Lawley et al. (2016) contain a certain level of degeneracy to account for uncertainty in certain segments of the flanking sequence where the symbols used for degenerate bases are as follows: R = A, G ; Y = C, T; H = A,C,T ; N = A, C, G, T. Primers based on Folmer et al. (1994).

Marker	Primer Name	Primer	Reference
COI	med-cox1-F	ACNAAYCAYAAAGATATHGG	Lawley et al. 2016
	med-cox1-R	TGGTGNGCYCANACNATRAANCC	Lawley et al. 2016

DNA Analyses

Mitochondrial DNA sequence fragments were aligned by Muscle (3.7) (Edgar 2004) in CIPRES and trimmed to 460 base pairs using Jalview (2.11) (Waterhouse et al. 2009). Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was conducted using a distance matrix with 25,000 permutations and the Tamura-Nei mutational model (Tamura and Nei 1993) with gamma correction of 0.34. The model (TrN + G) was selected using Akaike's information criterion (AIC) (Guindon and Gascuel 2003) in JModelTest2 (Darriba et al. 2012) on XSEDE in CIPRES. A transition to transversion ratio of 1:1 was used.

Descriptive statistics including number of haplotypes (Nh), nucleotide diversity (π), and haplotype diversity (h) were calculated using Arlequin 3.01 and DnaSP ver. 5.10.1 (Librado and Rozas, 2009). F_{ST} and pairwise distance matrices were calculated in MEGAX (Molecular Evolutionary Genetic Analysis) (Kumar et al. 2018; Stecher et al. 2020) and Arlequin 3.01 (Excoffier et al. 2010) to quantify overall genetic diversity among sampled specimens. Molecular distances between populations based on the number of pairwise differences between haplotypes were determined using the Kimura 2-parameter model for more accurate comparisons with the literature (Kimura 1980). Maximum Likelihood phylogenetic tree was generated using RAxML (in CIPRES) and MEGAX with 500 bootstrap replicates. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value (Tamura and Nei 1993). Haplotype minimum spanning networks were constructed in PopArt (Population Analysis with Reticulate Trees) using statistical parsimony analysis (Leigh and Bryant 2015). Using the specimen record of the public data portal in BarcodingLife.org and GenBank, all existing sequences for *Alatina alata* were compared to sequences generated in this study (Table 1, Appendix III). We also downloaded COI sequences from appropriate outgroups *Carybdea arborifera* (GenBank Accesssion code KM200333.1) and *Chironex indrasaksajiae* (GenBank accession code KU646841.1).

Species Differentiation

Differentiating between relatively distantly related species tends to be a straightforward procedure using a molecular systematic approach based on divergence values at mtDNA loci such as COI, 16S, D-loop, ND2 and ND4 (e.g. Hebert 2003) and this approach has been well tested and accepted for terrestrial and marine taxa (e.g. Morin et al. 2019). However, the differentiation of closely related lineages can be more challenging. Use of a phylogenetic species concept, which relies on the idea of reciprocal monophyly can be informative. A number of studies focusing on marine invertebrate sister taxa have also applied a consensus approach to determine thresholds of genetic divergence. Following a literature review focused on such studies including scyphozoan jellyfish (Holland et al. 2004; Dawson and Jacobs 2001; Bayha et al. 2010), cubozoans (Collins et al., ; Lawley et al. 2016), octocorals (Morin et al. 2019), and various terrestrial invertebrates (Hebert et al. 2003), a COI sequence divergence of ~ 10% has been established as a rule of thumb to differentiate between congeneric species. This arbitrary

10% value was created based on these previous studies to be used as a reference point in this study.

Results

Sample Collection

After collecting 200 specimens over the course of 12 monthly influxes and sequencing the COI gene fragment for each of these specimens, 108 sequences were deemed to be of acceptable quality. Over the course of the sampling period, there were two months where no box jellyfish were found. PCR amplification for jellyfish tentacle tissue proved challenging due to low DNA concentration, which could have been a result of the venom, and resulted in multiple rounds of sequencing for each individual. 41 sequences were obtained from GenBank from Hawaii (n = 19), Australia (n = 11), Bonaire (n = 6), and Saipan (n = 5) for a total of 149 sequences with a total of 52 singletons (Appendix III, Table S1).

Distances and Diversity Indices

There were 96 COI haplotypes and (h= 0.987) for Hawai'i (n = 128) (108 from this study and 19 from GenBank) (Table 4). The other three locations, Australia, Bonaire, and Saipan had no shared haplotypes among them (Nh = 11, h = 1.0; Nh = 6, h=1.0; Nh = 5, h = 1.0; Table 4) but specimens from Australia and Hawai'i shared haplotypes and Saipan, Australia and Hawai'i shared haplotypes as visible in the network (Figure 3). Low nucleotide diversity was found between specimens from each sampling site though Bonaire had the highest nucleotide diversity despite the small sample size (π = 0.015 for Hawaii, 0.012 for Australia, 0.019 for Bonaire, 0.011 for Saipan; Table 4).

An average sequence divergence of 1.6% was calculated using sequences from this study and all available sequences in GenBank (Table 4). Average pairwise distances were calculated for all Waikīkī (0.015 +/- 0.003), Bonaire (0.019 +/- 0.005), Australia (0.013 +/- 0.003), and Saipan (0.011 +/- 0.004) populations (Table 2).

The mean intrapopulation nucleotide diversity is very similar to the mean diversity over all populations (0.01524 and 0.01560) (Table 3). This coupled with the extremely low interpopulation diversity (0.002), indicates there is no evidence of geographic clustering/partitioning between populations across the Pacific or between the Atlantic and Pacific populations (Table 3).

AMOVA and F_{ST}

AMOVA There is significantly higher genetic variance (83.53%) proportioned within populations than between populations (16.47%) (F_{ST} = -0.004, p = 0; Table 8). Very little genetic variation was found between groups, ranging from 0 to 0.008, with non-significant p-values (Table 7). Negative values are considered zero. The highest F_{ST}, 0.008 was between Bonaire and Waikīkī.

Phylogeny

A maximum likelihood tree was constructed with two outgroups, the closely related *Carybdea arborifera* (n = 1) and a more distantly related *Chironex* sp. (n=1) (Figure 2). Utilizing the 10% sequence divergence for distinct species, the 108 specimens sequenced in this study belong to a single species *A. alata*. This tree also shows the lack of any geographic structure with the only exception being the small clade of four Bonaire specimens (bootstrap values for this clade = 75). Australia (n = 11) and Saipan (n = 5) specimens are scattered throughout the tree and do not form any distinct clades. A bootstrap analysis with 500 replicates also confirms that there is no phylogenetic signal associated with the different sampling locations, ingroup bootstrap range from 0 to 96, most falling below 50.

Haplotype Networks

The median joining network has two main clusters with shared haplotypes with the larger top cluster including specimens from Australia, Saipan, and Hawai‘i sharing a single haplotype (Figure 3). Four of the Atlantic haplotypes cluster together and are more similar to each other than any other haplotypes. However, sequences from two Atlantic specimens are more genetically similar to the Hawai‘i population than the four other Atlantic medusae. One haplotype is shared between multiple specimens from Hawai‘i , Saipan, and Australia. Two others are shared between Australia and Hawai‘i .

The second haplotype network shows Hawai‘i specimens from this study color coded based on influx event (month) in which they were collected (Figure 4). A little more detail should be provided to make your case stronger. There is no detectable pattern or genetic structuring among influx events, meaning there is likely as single source of each influx event is representative of the offshore population.

Table 2. Estimates of average distance over sequence pairs within groups. The number of base substitutions per site from averaging over all sequence pairs within each group are shown. Standard error estimate(s) are shown in the second column and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Kimura 2-parameter model. This analysis involved 149 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). Evolutionary analyses were conducted in MEGA X

	Mean	Standard Deviation	Maximum
Waikīkī	0.015	0.003	0.053
Bonaire	0.019	0.005	0.031
Australia	0.013	0.003	0.027
Saipan	0.009	0.002	0.020
Total	0.016	0.002*	0.058

* *standard error*

Table 3. The number of base substitutions per site from mean diversity calculations within subpopulations, over all populations, and inter-population diversity. Standard error estimate(s) are shown in the second column and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Kimura 2-parameter model in MEGAX

Diversity Indices	Diversity	Standard Error
Mean Diversity within each Population	0.014	0.002
Mean Diversity over all Populations	0.016	0.003
Mean Interpopulation Diversity	0.002	0.001

Table 4. Number of individual sequences (N), number of haplotypes (Nh), haplotype diversity (h), and nucleotide diversity (π) calculated in Arlequin 3.01

Indices	Hawai‘i	Australia	Bonaire	Saipan
N	128	11	6	5
Nh	96	11	6	5
h	0.987	1.0	1.0	1.0
π	0.015	0.012	0.019	0.011

Table 5. **a)** F_{ST} (above the diagonal) and **b)** Between group mean distance (below the diagonal) between sampling locations. **a)** None of the F_{ST} *p*-values are significant (>0.05) and negative F_{ST} values

were changed to zero (25000 permutations) Analyses conducted in Arlequin (3.01). **b)** Below the diagonal, the number of base substitutions per site from averaging over all sequence pairs between groups are shown (1000 replicates). Analyses for both were conducted using the Kimura 2-parameter model. This analysis involved 149 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). Evolutionary analyses were conducted in MEGA X.

F_{ST}	Australia	Bonaire	Saipan	Waikīkī
Australia		0.000	0.000	0.000
Bonaire	0.026		0.000	0.008
Saipan	0.011	0.024		0.000
Waikīkī	0.014	0.027	0.012	

Table 6. AMOVA calculated in Arlequin (3.01) including degrees of freedom (df), sum of squares, variance components, and percent variation with significant *p*-value bolded.

Source of Variation	*Df*	*Sum of Squares*	*Variance Components*	*Percentage of Variation*
Among Populations	3	38.81	0.703	16.10 %
Within Populations	146	535.08	3.66	83.90 %
Total	149	573.89	4.37	
p-value	**0.0**			
F_{ST}	-0.004			

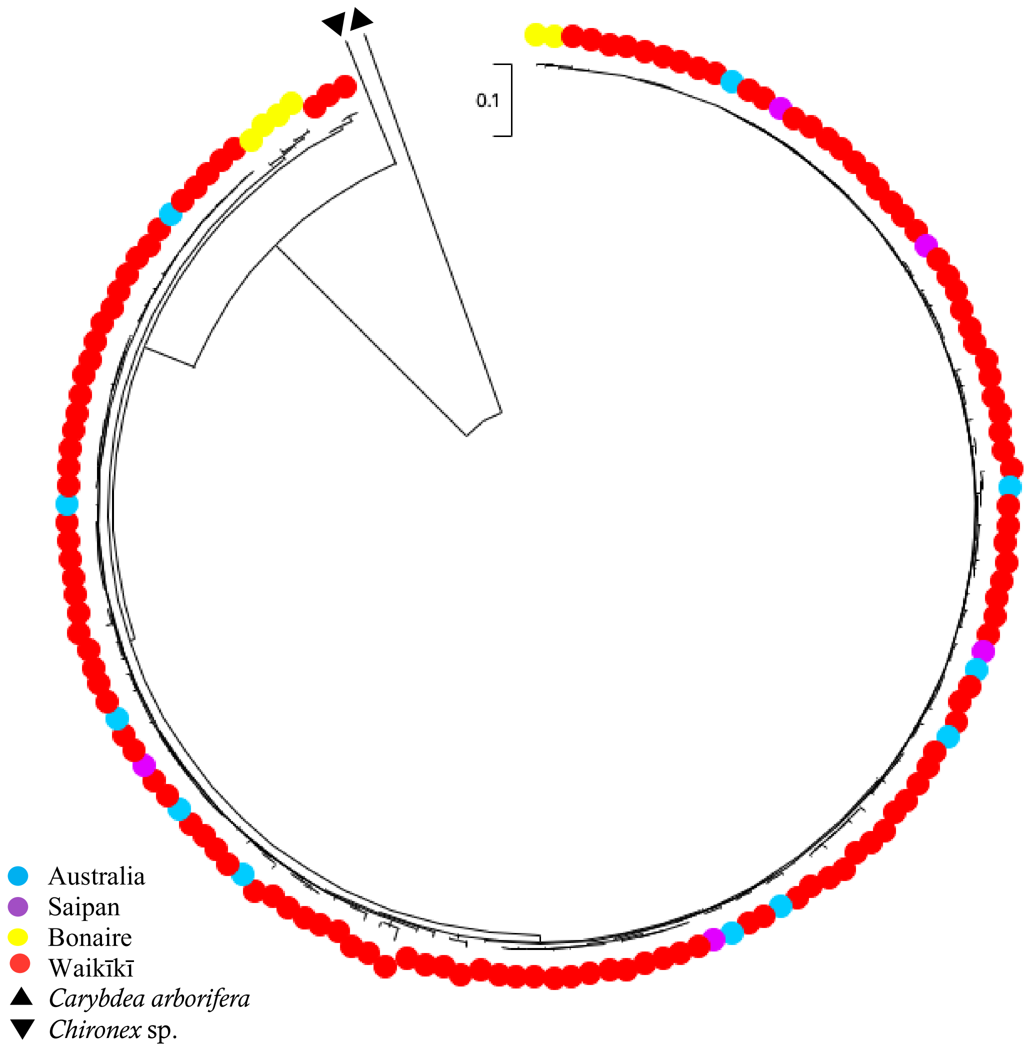

Figure 2. Maximum-likelihood phylogeny of *Alatina alata* specimens collected in this study, including sequencs obtained from GenBank and with the *Chironex sp*. and *Carybdea arborifera* specimens designated as outgroups with 500 bootstrap replicates. The tree with the highest log likelihood (-2493.20) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Analyses conducted in MEGAX.

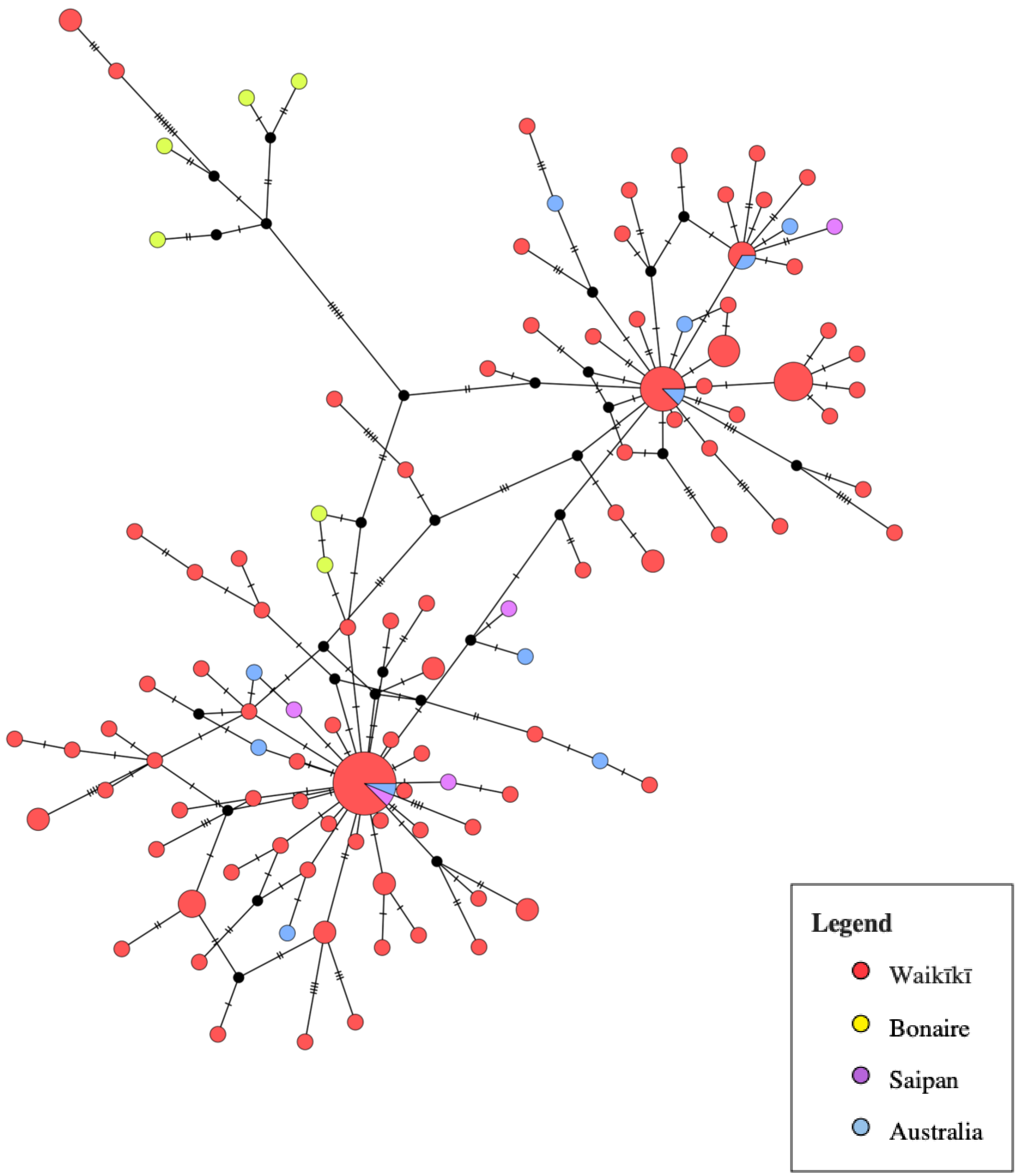

Figure 3. Median joining haplotype network created using POPart of all sequences from this study along with all available GenBank sequences (references listed in Table 1, Appendix III). Each circle represents a single haplotype and each tick mark is a single base pair change. The size of the circles are proportional to frequency of that haplotype. Black circles are inferred haplotypes created by the algorithm to predict haplotypes within the population and reduce the overall length of the branches.

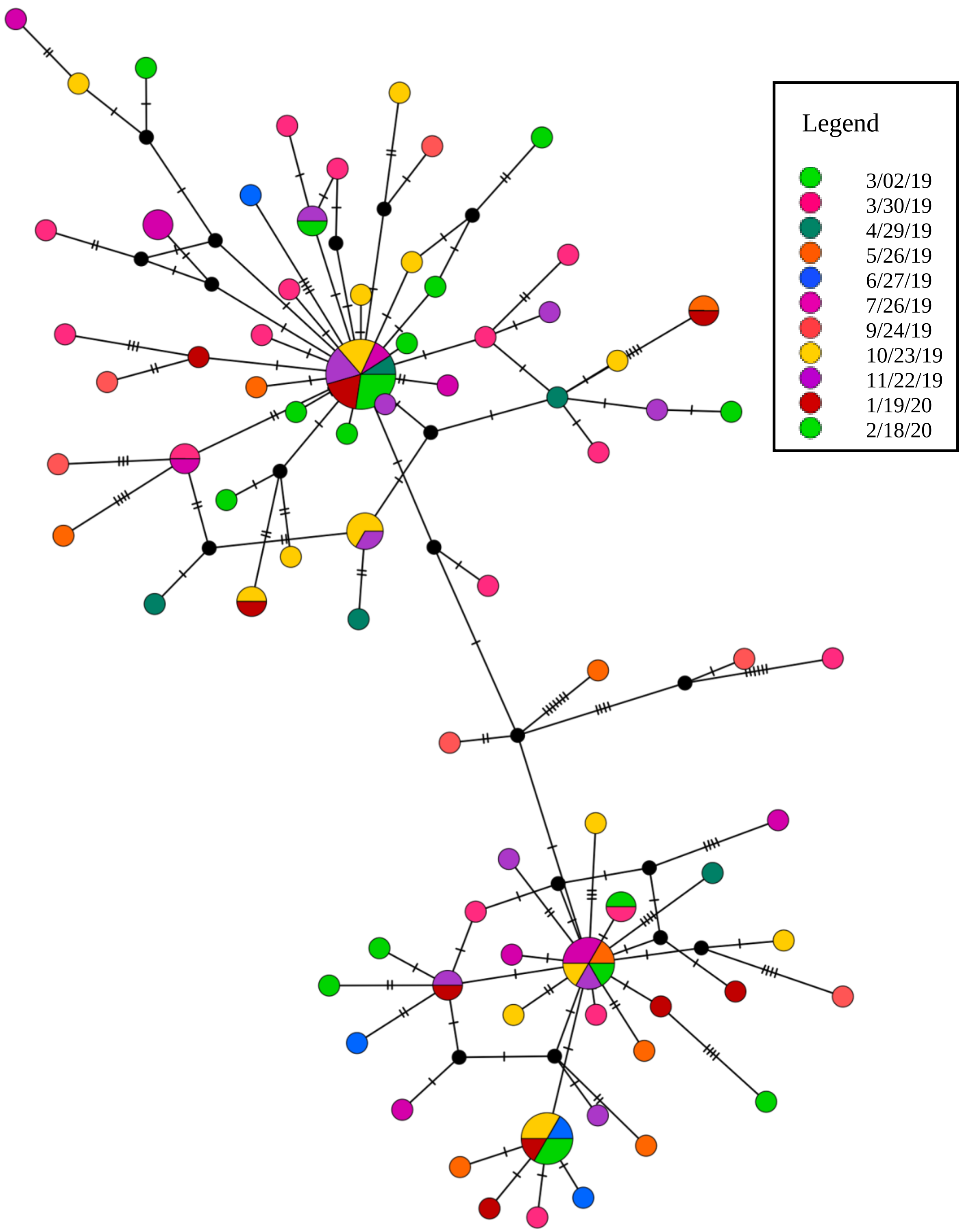

Figure 4. Median Joining haplotype network created using POPart. *A. alata* COI sequences grouped by monthly influx date starting in March of 2019 and ending Feburary of 2020. The the circles are proportional to frequency of that haplotype. Black circles are inferred haplotypes created by the algorithm to predict haplotypes within the population and reduce the overall length of the branches.

Discussion

A single species

Extremely low sequence divergences among Hawai'i specimens (an average of 1.5%) and the lack of topological structure and lack of geographic resolution in the phylogenetic tree (Figure 2) confirm that monthly box jellyfish influxes at Waikīkī consist of a single species. While a Kimura 2-parameter model molecular divergence value of 10% was mentioned earlier as a threshold used to differentiate among species based on COI mtDNA sequences, the divergence values revealed via this study fall substantially below this benchmark, lending confidence to this conclusion. In addition to confirming that this is a single species, over the course of a year (March 2, 2019 – February 18, 2020), there was no detectable pattern of population partitioning or temporal patterns in haplotypes across the 11 different influx events sampled (Figure 4).

Biological Introduction

Lack of a population bottleneck

Lawley et al. (2016) cited high haplotype diversity combined with a lack of population bottleneck in *Alatina alata* as an argument for a natural global distribution. Population bottlenecks occur due to dramatic decreases in population size and thus genetic diversity. If a small group from a large source population is introduced to a new location, there will likely be decreased genetic diversity due to the small population size. However, the absence of a bottleneck, as seen in Hawai'i specimen, is still very possible. Holland (2001) assessed the genetic diversity of six invasive populations and six native populations of the Brown Mussel, *Perna perna.* No significant differences in allelic diversity were detected between the introduced and native populations (recorded F_{ST} values 0.019 for introduced vs. 0.022 for native), and the author concluded this lack of bottleneck was due to either a very large introduction event or multiple introduction events from various populations. Holland (2001) attributed this lack of reduction in genetic diversity to movement of mussel larvae from Venezuela to Texas via bulk carrier ballast water release of thousands of veligers in a mechanism he called "gene pool capture".

The lack of evidence of a bottleneck for *A. alata* in Hawai‘i either indicates that a large enough fraction of the native population was released to avoid a founder effect, or that there have been multiple introduction events over time.

Does life history information support natural dispersal or human introduction?

There are numerous challenges to confidently assessing the provenance and status of *A. alata* in Hawai‘i, as well as its recently revealed circumglobal distribution (Lawley et al. 2016). Often times genetic data combined with timing of historical geographic records provide unambiguous answers to questions pertaining to the native versus introduced status of local taxa such as when there is a genetic match between geographic locations sampled, especially if one of the locations is the type locality of the species in question.

The key facts in this case include the timing of the first record of *A. alata* in the Caribbean and Hawai‘i, (1830 in Caribbean, 1906 offshore Hawai‘i and 1948 on Waikīkī Beach; Crow et al. 2015) and the close genetic affinity of specimens in this study from geographically distant sampling locations. This genetic affinity includes low F_{ST} values (0.008, $p = 0.027$) between Waikīkī to Bonaire in the tropical Atlantic (5,885 mi / 9,471 km from O‘ahu), and a Pacific range that includes shared mtDNA haplotypes that span the Pacific basin including Saipan (5,961 mi / 9,593 km from O‘ahu) and tropical Australia (4,585 mi / 7,379 km – 6,771 mi

/ 10,897 km from O‘ahu). The most realistic way the Pacific range could represent a natural distribution would be via an island-hopping model with ongoing, periodic gene flow and metapopulation structure across a vast area of open ocean. While there are currently no known intermediate populations between these ranges in the Pacific, periodic gene flow between them cannot be completely ruled out.

Natural dispersal of *A. alata* consists of ciliated planula stage carried by currents, a benthic polyp stage, and a swimming medusae form. Reproduction in the Cubozoa entails a demersal polyp phase requiring a shallow water hard substrate. *Alatina alata* collected near Osprey Reef, had a less than 30% survival rate at temperatures less than 14°C, while maximum fecundity occurred in thermal treatments of 21 - 25°C (Courtney and Seymour, 2013). Though the polyp stage for this species (and most cubopolyps) remain inconspicuous, this thermal range restricts polyps to shallow waters.

If the encysted planulae was being dispersed (not the medusae or polyps). A conceivable method in which Hawaiian and Australian *A. alata* are sharing a single gene pool could involve wind and surface water currents dispersing them. While one can argue this may be possible, it would not account for a method in which surface currents can carry planulae from the tropical Atlantic to the tropical Pacific or vice versa.

Planulae larvae only persist for 2 to 6 days (Arneson, 1976; Carrette et al. 2014). The length of the larvae life stage is generally correlated with the dispersal potential of the larvae (Scheltema, 1971; Grantham et al. 2003; Shanks 2009). With such a short larval period, it is unlikely that this life stage is the means of dispersal whether by ocean currents or ballast water. Additionally, other cubozoans, like *Chironex* spp., have adaptations which act to restrict dispersal in the early life stages. Zygotes and blastulae have been found to be negatively buoyant with a sticky coating allowing them to attach to hard substrates (Hartwick 1991). If *A. alata* exhibit similar behavior as *Chironex* spp., this could indicate also restrict their larval dispersal.

Open ocean collections of *A. alata* are as intriguing as they are rare, and we assume that in these unusual instances where box jellyfish occur hundreds of kilometers from Hawaiʻi captured over seamounts, that they are unable to reproduce in such open ocean locations (Crow et al. 2015). Morrissey et al. (2020) used elemental chemistry of statoliths to determine the different temperature in which *A. alata* is living at different life stages. High Sr:Ca values in the center and the outer section of the statoliths correspond with sea surface temperatures in Hawaiʻi. Middle sections of the statoliths had lower Sr:Ca ratios indicating that *A. alata* likely spend the majority of their medusa life stage in depths 200 – 400 m (Morrissey et al. 2020). A shallow water polyp stage followed by a deeper, offshore medusa stage, and then the return to surface waters to spawn and die, does not support the hypothesis that *A. alata* is a naturally distributed species. It does support the need for shallow water habitat during the beginning and end of life. Although long-lived marine vertebrate species such as sea turtles, cetaceans, pinnipeds, and large pelagic fishes travel vast distances over their lifetimes, *A. alata* has been shown to have a short medusa stage of a year or less (Arneson 1976), fragile, delicate, body structure, and likely insufficient swimming ability to cover distances such as those among sampling sites. Average maximum swimming speeds for two cubozoans, *Copula sivickisi and Chironex fleckeri,* were estimated to be around 4.9 cm/s and 11.5 cm/s respectively (Schlaefer et al. 2020; Shorten et al. 2005). Given the distance from the Great Barrier Reef in Australia to Hawaiʻi, 7,400 kilometers, it would take just under 5 years for *Copula sivickisi* to travel this distance maintaining maximum swimming speed and 2 years for the larger cubozoan, *Chironex fleckeri* to cross this distance maintaining their highest recorded swimming velocity.

As far as a marine invertebrate with matching haplotypes from the Pacific (Australia, Saipan, Hawai‘i) to the genetic similarity we see between specimens from the Atlantic (Bonaire) and Pacific, there is no obvious, realistic natural scenario by which this pattern could come about. Gaither et al. (2015) found that in a recent survey of fishes, fewer than 1% have a circumtropical distribution, most of them being pelagic or bathypelagic. Even fewer non-deep sea species of invertebrates are considered truly circumtropical.

When looking at sequence divergence between different ocean basins separated populations of the well-studied scyphozoan, *Aurelia* spp., Dawson and Jacobs (2001) published a range of 13 – 24%, resulting in the taxonomic revision and creation of 7 new species in the genus. Holland et al. (2004) published a similar COI molecular divergence range in the genus *Cassiopea* spp., also revealing evidence of cryptic species, as well as a pattern of introduction to various geographic locations. Marine speciation is often attributed to the presence of geographic barriers, and disruption of gene flow over periods of millions of years (Palumbi 1994). If a similar pattern of deep divergence had been revealed for *A. alata,* our conclusion would be that this is clearly a natural distribution pattern. However, the pattern we are seeing is more similar to that seen in the introductions of *Cassiopea* spp.

The fact that *A. alata* has been collected hundreds of kilometers from Waikīkī, and the museum records of box jellyfish specimens tentatively identified as *A. alata* from locations including the Gulf of Mexico, the Indo-Pacific, the southern tropical Atlantic coast of Brazil, the Mediterranean and the Indian Ocean (Lawley et al. 2016) suggests a range, whether natural or anthropogenic, that greatly exceeds that of our genetic sampling data. Due to the small sample sizes in Saipan and Australia, the allelic diversity cannot be completely resolved in the Pacific to determine if these are differentiated populations. There are a number of unknowns, mainly, the lack of understanding of the global distribution of *Alatina alata* throughout the tropics, that limit the ability to provide a concrete conclusion. However, without any evidence of populations between sampling sites, the most parsimonious explanation for the pattern observed, of shared haplotypes among disjunct sampling locations of a marine invertebrate with a coastal polyp stage and short larval stage, is human-mediated translocation. Increasing genetic sampling on a global scale would certainly be an optimal method to differentiate between the possibilities of a natural versus a human-altered distribution, but the majority of current evidence suggests that this distribution is not natural, and has been altered by human activity, likely commercial cargo vessel traffic. Further implications of our conclusion that *A. alata* is an invasive species will be discussed in the *Conclusions* section.

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Conclusion

A single, introduced species

The first goal of Chapter 3 was to determine whether *Alatina alata* was the only jellyfish responsible for the influxes at Waikīkī and the frequent mass sting events. Our results conclude that this is indeed the case. Over 12 months and about 200 jellyfish collected, 108 of them sequenced, all jellyfish were genetically similar with an average pairwise distance of 0.015 ± 0.003. Additionally, based on monthly observations along the beach, all jellyfish collected and observed were morphologically identical.

Based on the evidence presented in Chapter 3, it is likely that *Alatina alata* was introduced to the Pacific. I suggest that this species be reclassified as an introduced and invasive species. The characteristics shared by successful invasive species include rapid growth, generalist feeding strategy, high dispersal ability, flexibility of physiological tolerance, genetic diversity, and phenotypic plasticity. We have presented evidence of generalist predatory behavior based on stable isotope data in Chapter 2. Since *A. alata* is likely a generalist, consuming many different zooplankton taxa of various size classes, this could have contributed to their successful introduction to the waters surrounding Hawai‘i .

To be considered invasive, the species must be both non-native and harmful. While the ecosystem impacts are largely unknown, this cubozoan does cause harm to human health. The introduction of this stinging cubozoan has also negatively impacted the economy on O‘ahu. Hanauma Bay, a popular tourist destination visited by about 1 million people annually (Hanauma Bay State Park) often closes to avoid mass stinging events due to large box jellyfish influxes. Establishing that *A. alata* is an invasive species is an important step that not only can raise awareness, but also lead to management efforts. Once *A. alata* is reclassified, the Department of

Land and Natural Resource's Invasive Species Council should consider revising their ballast water treatment plan and adopt a plan for hull-fouling mitigation. Additional strategies can include the funding of future studies related to other recently documented introduced box jellyfish on O‘ahu such as *Copula sivickisi* and *Tripedelia cystophora,* small cubozoans from the Pacific and Caribbean (Carlton and Eldredge 2015; Crow et al. 2015).

Future introduction events of more venomous cubozoans may create new and unpredictable patterns of jellyfish which would have drastic socio-economic repercussions. Classifying *Alatina alata* as an invasive species is a crucial first step to start assessing and mitigating anthropogenic introduction of harmful jellyfish to Hawai‘i.

APPENDIX I

Sampling site

The sampling site is a 400 m stretch of Waikīkī Beach which is located on the southern shore of Oʻahu, Hawaiʻi (Figure 1). Specifically, the area sampled began at the Waikīkī Wall to the southeast, and ended by the wall at the Kūhiō Beach Hula Mound to the northwest between Ocean Safety and Lifeguard Services towers 2C and 2D. This site was selected based on its consistent pattern of monthly box jellyfish aggregations (Chiaverano et al. 2013). The illuminated area along the shoreline is sandy and protected from wave action by a rock wall. The protected waters between the wall and shoreline create and easy and safe environment for collecting specimens. The wall also acts as a barrier trapping the box jellyfish after a high tide, allowing for high abundances to collect in this space. This area was also the collection site for the Chiaverano et al. (2013) study, where the beach was sampled monthly for 14 years. Additional lifeguard stations on Oʻahu include Yokohama Bay and beaches along the North Shore were also contacted for sampling but were unable to be sampled due to the rarity of influx events.

Collection Protocol

Each month, between 8 and 12 days after each full moon, *A. alata* specimens were collected along the beach and from the shallow water within the study area. Depending on the time of the high tide, collections began either late at night or very early in the morning to ensure the specimen were not buried or covered along the shoreline by beachgoers. Preliminary surveys included testing out various days and nights during the influx window in order to optimize sample sizes. These surveys indicated the unpredictable nature of influx events, and suggested that collections should focus on the 9th day after each moon and continue the 10th day if necessary.

Surveys and collections were conducted along the shoreline and in the water up to 1 meter deep. Live specimens were carefully scooped into Ziploc bags along with some seawater. Volunteers were advised to wear gloves and protective gear. The box jellyfish specimens were frozen and stored at the Hawaiian Biodiversity and Conservation Lab at the Oceanic Institute until their tentacles were dissected for DNA extraction (Chapter 3). Or, they were immediately rinsed, measured, and dried for stable isotope analysis (Chapter 2).

Each jellyfish specimen was given a unique sample number, consisting of the date followed by a 3-digit identification number. The three-digit code corresponds to an entry in an excel spreadsheet inventory where all the sample information regarding that specific jellyfish have been recorded. In total, over 200 jellyfish were collected for this study.

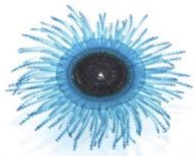
APPENDIX II

Figure S1. Bigeye scad (*Selar crumenophthalumus*)

The bigeye scad (*Selar crumenophthalumus*) is a coastal pelagic species. This species forms schools in bays and feeds on zooplankton at night. Diet ranges from crustacean (euphausiids and decapods) for immature scads to larval and juvenile fish for adults. Observed depth to at least 152 m (499 ft). In Hawaiʻi , juveniles of this species are called halalu and maturity is reached at about 23 cm (9 inches) and live about one year (Roux and Conand 2000). The standard length of the specimen used in this study 14 cm long and caught by fishermen at Ala Moana Beach.

Figure S2. Goldspot herring (*Herklotsichthys quadrimaculatus*)

The goldspot herring (*Herklotsichthys quadrimaculatus*) is a nocturnal zooplanktivore and was first documented in Hawai‘i in 1975 in Kaneohe Bay and likely introduced accidentally in Hawai‘i in the 1970s (Williams and Clark 1983). This species was historically used as live bait in the *Katsuwonus pelamis* (aku or skipjack tuna) fishery They mature at about 5 months and usually live for about one year. A study by Milton et al. found that teleost larvae took up the majority of their diet by weight (1994).



Blue button

Blue bottle

Purple
Sea Snail

Copepods

Krill

Groups	**δ^{15}N**
Porpita	6.6
Physalia	7.9
	7.3
	6.5
	6.5
	6.4
	7.1
Janthina	6.7
	6.8
	6.8
Euchaeta rimnana	3.3
	3.6
	6.0
	2.9
	4.7
	3.9
Pleuromamma xiphias	5.9
	3.6
	5.7
	4.2
Oithona sp	7.5
	3.3
	5.0
	2.6
Thysanopoda sp.	5.5
.	3.7
	6.0
	2.2
1–2-mm mixed zooplankton	5.0
	2.6
	4.4

Figure S3. Scientific and common names listed for comparison taxa as well as bulk δ^{15}N values. *Porpita, Physalia*, and *Janthina* were and collected in Waimanalo and processed by David Hyrenbach while the remaining groups were collected during zooplankton tows at Station Aloha. More information on this collection and analyses process can be found on the HOTS website (see Hannides et al. 2009).

References

Blue button
Photographer: Albert Kang
https://www.jungledragon.com/image/42924/blue_button_-_porpita_porpita.html

Blue bottle
Photo by SeaUnseen
https://seaunseen.com/

The Common Violet Snail
Photo by Queensland Museum
https://www.qm.qld.gov.au/Find+out+about/Animals+of+Queensland/Molluscs/Gastropods/Marine+snails/Common+Violet+Snail

Oithona sp.
Photographer: Peter J. Bryant
http://nathistoc.bio.uci.edu/crustacea/Copepoda/Oithona.htm

Euchaeta sp.
Photographer: Marco Battuello
https://v3.boldsystems.org/index.php/Taxbrowser_Taxonpage?taxid=799586

Pleuromamma
Photographer: D. Steinberg
https://www.vims.edu/newsandevents/topstories/2017/eclipse_dsl.php

Krill
Photographer: J.F. St-Pierre
https://www.dfo-mpo.gc.ca/species-especes/profiles-profils/euphausiids-krill-euphausiaces-eng.html

APPENDIX III

Table S1. All GenBank samples used in this thesis, where they were collected, and which publication they were included in

Accession Number	Location	Publication
KU707303	Kralendijk, Bonaire	Lawley et al. 2016
KU707304	Kralendijk, Bonaire	Lawley et al. 2016
KU707305	Kralendijk, Bonaire	Lawley et al. 2016
KU707306	Kralendijk, Bonaire	Lawley et al. 2016
KU707308	Kralendijk, Bonaire	Lawley et al. 2016
KU707307	Kralendijk, Bonaire	Lawley et al. 2016
KU707292	Osprey Reef, Australia	Lawley et al. 2016
KU707293	Osprey Reef, Australia	Lawley et al. 2016
KU707294	Osprey Reef, Australia	Lawley et al. 2016
KU707295	Osprey Reef, Australia	Lawley et al. 2016
KU707296	Osprey Reef, Australia	Lawley et al. 2016
KU707297	Osprey Reef, Australia	Lawley et al. 2016
KU707298	Osprey Reef, Australia	Lawley et al. 2016
KU707299	Osprey Reef, Australia	Lawley et al. 2016
KU707300	Osprey Reef, Australia	Lawley et al. 2016
KU707301	Osprey Reef, Australia	Lawley et al. 2016
KU707302	Osprey Reef, Australia	Lawley et al. 2016
KU707269	Saipan, Northern Mariana Islands	Lawley et al. 2016
KU707270	Saipan, Northern Mariana Islands	Lawley et al. 2016
KU707271	Saipan, Northern Mariana Islands	Lawley et al. 2016
KU707272	Saipan, Northern Mariana Islands	Lawley et al. 2016
KU707273	Saipan, Northern Mariana Islands	Lawley et al. 2016
KU707276	Waikīkī, Hawaii	Lawley et al. 2016
KU707277	Waikīkī, Hawaii	Lawley et al. 2016
KU707278	Waikīkī, Hawaii	Lawley et al. 2016
KU707279	Waikīkī, Hawaii	Lawley et al. 2016
KU707280	Waikīkī, Hawaii	Lawley et al. 2016
KU707281	Waikīkī, Hawaii	Lawley et al. 2016
KU707282	Waikīkī, Hawaii	Lawley et al. 2016
KU707283	Waikīkī, Hawaii	Lawley et al. 2016
KU707284	Waikīkī, Hawaii	Lawley et al. 2016
KU707285	Waikīkī, Hawaii	Lawley et al. 2016
KU707286	Waikīkī, Hawaii	Lawley et al. 2016
KU707287	Waikīkī, Hawaii	Lawley et al. 2016
KU707288	Waikīkī, Hawaii	Lawley et al. 2016
KU707289	Waikīkī, Hawaii	Lawley et al. 2016
KU707290	Waikīkī, Hawaii	Lawley et al. 2016
KU707291	Waikīkī, Hawaii	Lawley et al. 2016
KU707274	Waikīkī, Hawaii	Lawley et al. 2016
KU707275	Waikīkī, Hawaii	Lawley et al. 2016
KM200330	Waikīkī, Hawaii	Crow et al. 2015

APPENDIX IV

Reaction Components

10 µM forward primer:	1.0 µl
10 µM reverse primer:	1.0 µl
Taq Plus Master Mix Red:	12.0 µl
Water:	8.5 µl
100x Bovine serum albumin (BSA): (4 mg/ml)	0.5 µl
DNA:	2.0 µl
Total Volume:	25.0 µl

PCR Protocol

Initialization	95°C (3-5 min)
Denaturation	95°C (30s) x 40 cycles
Annealing	52°C (30s)
Extension	72°C (2 min)
Final Extension	72°C (5-10 min)
Hold temperature	15°C