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Eco-evolutionary mechanisms shaping aggregation and 'piggy-backing' of the endemic Kermadec Islands Giant Limpet, *Scutellastra kermadecensis*

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General abstract

The Kermadec giant limpet, *Scutellastra kermadecensis*, is a broadcast-spawning, protandrous hermaphrodite that is endemic to Rangitāhua, Kermadec archipelago, New Zealand. This limpet is the most abundant intertidal/shallow-subtidal grazer on these islands and lives at very high density, covering most of the available rocky substrate. Nonetheless, the species is extremely range-restricted and therefore vulnerable to extinction. My thesis investigated the socioecology of the Kermadec giant limpet, using surveys as well as genetic analysis of relatedness and spatial structure to better understand the population dynamics of this species. In Chapter 2, I focused on the "piggy-backing" habit of these limpets, whereby small limpets (piggies), predominantly male, piggy-back on the shells of larger, predominantly female, limpets. I investigated whether the ontogenetic habitat shift between piggy-backing and being rock-attached was determined by rock space availability, as a mechanism to avoid bulldozing by larger limpets, to access grazing opportunities on limpet shells, and/or to monopolize breeding with rock-attached females. I found that available rock space did not influence the transitional size from being a piggy to rock-attached, suggesting that social environment motivated piggy-backing behaviour rather than space constraints. As spatial proximity often determines fertilization success in broadcast spawning marine invertebrates, in Chapter 3, I investigated the genetic relationships of piggy-host pairs, and among neighbouring limpets. I sought to understand whether the species had mechanisms to promote genetic diversity, such as kin-avoidance in piggy-host pairings, or among piggies on the same host, or whether there was evidence for limpets moving away from relatives when transitioning from piggy-backing to being rock-attached. I found that paired and neighbouring limpets were no more related to each other than to any other sampled limpet in the subpopulation. However, there was spatial genetic structure among sampled sites and locations, and evidence for adaptive genetic divergence over very small scales (<1 metre). Furthermore, estimated levels of inbreeding were very high (F_{IS} = 1.37-1.43), but in keeping with estimates for other marine invertebrates. Unveiling these eco-evolutionary drivers of piggy-backing behaviour helps us to understand how Kermadec giant limpets have persisted despite their isolation and provides insight into how to conserve them.

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Chapter 1: General Introduction

1.1 Ontogenetic shifts

Ontogenetic shifts are ecological transitions that coincide with a transition between life-history stages, such as between the juvenile and mature stages, during sex change, or pre- and post-metamorphosis (Stamps 1983). Such a shift can include transitions in habitat, nutritional resources, and/or morphology. Often, ontogenetic shifts occur when resource requirements change for an individual, driving them to seek new resources to fulfil their new requirements for growth, survival, and/or reproduction. For instance, the three-spot damselfish (*Stegastes planifrons*) exhibits an ontogenetic shift, where the habitat preference of juveniles' changes from lower quality, largely dead foliose coral heads to the coral reef when they reach maturity, joining the adult population, and defending territories (Lirman 1994). In juvenile habitat, three-spot damselfish juveniles can feed on algal mats defended by resident adults without needing to establish and defend their own territories as their small size allows them to find refuge in tight crevices between rocks when chased (Lirman 1994). This way, energy that would have otherwise been used for territoriality can be allocated to maximise growth. When juveniles grow larger, their size limits them from hiding in small crevices which forces them to abandon to juvenile habitats. Once they reach sexual maturity, the males will return to the adult reef to establish their own territory, and the females will search the adult habitat for potential mates (Lirman 1994). Ontogenetic habitat shifts are generally attributed to such changes in the trade-off between growth potential, mortality risk and reproductive potential that organisms experience as they develop. The shift in habitat in the three-spot damselfish is a strategy that may be selected for, maximising the growth potential to mortality risk ratio in each occupied habitat. Understanding the drivers of ontogenetic shifts is important in predicting the possible trajectories of populations and particularly for species subject to environmental change and anthropogenic pressures.

The timing of ontogenetic shifts can be genetically predetermined or environmentally and/or socially mediated and individuals can vary in the time when they transition. For example, *Lysmate* shrimp males will delay sex change in colder temperatures compared to warmer temperatures (Baldwin & Bauer 2003). Colder temperature is thought to be suboptimal for embryo production and development (Baur 2002, Dickson et al. 2020), hence reproducing in autumn or winter will compromise offspring survival for individuals that have already transitioned and are functioning as hermaphrodites (Baur 2002). Additionally, by delaying sex change until warmer temperatures, resources (i.e., energy and

time) can be allocated to growth instead of reproduction and individuals can transition into a larger hermaphrodite later, which increases the potential to maximise reproductive success as there would be an increased production of gametes (Dickson et al. 2020) and higher offspring survival (Baur 2002). In other species, ontogenetic shifts are mediated by the social environment, in which the shift is influenced by the composition and/or proximity of surrounding conspecifics. For instance, juvenile Caribbean spiny lobsters, (*Panulirus argus*), undergo an ontogenetic shift from habitats rich in algal mats to sheltering in crevices with conspecifics. Juveniles that were experimentally raised with conspecifics underwent accelerated transition at a smaller size than solitary individuals, whereas juveniles raised in the absence of conspecifics delayed the transition and waited until they were larger (Childress & Herrnkind 2001). The presence of conspecifics may indicate that there is a suitable habitat nearby and aggregating earlier may reduce the time to exposed predators as well as enhance the likelihood of securing crevice habitat for themselves given the greater number of competitors in the neighbourhood. As such, the timing of ontogenetic transitions in habitat, sex or morphology may have detrimental effects on an individual's ability to obtain and/or defend resources and thereby the mean fitness of the population, which may be detrimental to the trade-off between growth potential, mortality risk and reproductive potential as it will determine whether the organism will be able to go through an ontogenetic shift to the next stage, whatever that may be, to be able to survive and/or reproduce.

1.2 Dispersal and aggregation of individuals

The dispersal potential of species determines the distribution of genotypes within and among populations, which affects their level of genetic differentiation and genetic structuring. Genetic differentiation is defined as the accumulated allelic frequency differences between (sub)populations and occurs when there is restricted gene flow between them (Pongratz et al. 2002), whereas genetic structure refers to any genetic pattern within a population, such as the number of subpopulations within it, the different allelic frequencies in each subpopulation, and the degree of genetic isolation (Chakraborty 1993, Radosavljevic 2015). Historically, sedentary species were thought to be disadvantaged compared to mobile species due to the assumption that sedentarism hinders gene flow and leaves the resulting genetic diversity of populations vulnerable to decline through genetic drift and directional selection (Grosberg & Cunningham 2001, Kamel & Grosberg 2013). As a result, sedentary species would be expected to have smaller genetic neighbourhoods, be more genetically differentiated, and have a higher chance of inbreeding than more mobile species (Strathmann 1990, Carr et al. 2003, Shanks et al. 2003, , Selkoe et

al. 2006). Nevertheless, several studies have shown that some sedentary marine invertebrates have patterns of genetic structuring more like those observed in mobile species (Ridgway et al. 2001, Tay et al. 2015, 2016, Lourenço et al. 2017). Potential explanations include the broadcast spawning habit of many sessile species (Morrison et al. 2011, Tay et al. 2015, 2016, Lourenço et al. 2017) which spreads gametes into the water column, allowing their production of pelagic larvae capable of dispersing thousands of kilometres (Gaines & Bertness 1992). Oceanic turbulence and/or larval mobility may also enhance the dispersal potential of sedentary species (Gaines & Bertness 1992) and prevent spatial clustering of closely related individuals post-settlement (Victor 1984, Leis 1991). However, dispersal may also be limited by environmental factors such as waves and currents (D'Aloia et al. 2018), and biological factors such as active larval behaviours including kin aggregation (e.g., Ascidians, Grosberg and Quinn 1986), directional orientation, and post-recruitment behaviours (i.e., reassortment or aggression; e.g., *Hydractinia symbiolongicarpus;* Buss & Grosberg 1990, allelopathy/overtopping; e.g., corals; Russ 1982, Baird & Hughes 2000). Despite high dispersal potential, related individuals may recruit to their natal habitats or the same habitats (Selkoe et al. 2006, Velig et al. 2006). Such behaviours can limit the potential enhancement of genetic diversity for the population that is receiving the new recruits and affect the genetic diversity of subsequent generations.

Groups of individuals in some species, cluster and form aggregations deliberately, and sometimes the formation of these clusters is associated with ontogenetic shifts. One common driver of aggregation is to increase survival by cooperating as a group or to avoid predation. For example, pelagic polyps of the sun coral, *Tubastraea coccinea*, form groups of up to eight individuals in the water column. These aggregates have been observed to exhibit longer survival than solitary polyps (Mizrahi et al. 2014). Being in a group may enable increased feeding which meets the higher energy demands of swimming as a group and the larger size may reduce vulnerability to predation (Carr and Hixon 1995; Allen 2008). Another common driver of aggregation is the clustering of mature individuals to maximise fertilization success (Pietsch 1976, Chen et al. 2018). For instance, sexually mature limpets such as *Nacella concinna* and species in the *Crepidula* genus stack on top of each other (Suda et al. 2015, Cledón et al. 2016). Individuals closer to the female often achieve the highest reproductive output as the likelihood of fertilizing a higher number of eggs is enhanced (Picken & Allan 1983, Suda et al. 2015, Chen et al. 2018). Although individuals in aggregations may benefit through reduced predation, increased survival and/or the potential to maximise reproduction success, it may only be short-term, and it increases the intensity of competition for resources. Accordingly, the spatial arrangement of aggregating

individuals associated with ontogenetic shifts could impose adverse effects on the individuals and can have population- and community-level effects, including population regulation (Mittelbach and Osenberg 1993). Furthermore, if kin were aggregated through to sexual maturity, breeding between kin may occur and the genetic diversity of the following generations will be affected (Charlesworth & Charlesworth 1999).

The spatial arrangement of related individuals has important implications for population viability. Even after extensive dispersal and settlement processes, kin are still found in close proximity in wild populations (e.g., Hart & Grosberg 1999, Horne et al. 2016, Adrian et al. 2017). For instance, related individuals of Mouthbrooding tilapia (*Sarotherodon melanotheron*) are found nonrandomly distributed within shoals even after dispersal (Pouyaud et al. 1999). Compared with Hardy-Weinberg equilibrium expectations, these individuals showed significant heterozygote deficiencies which is known to occur as a result of mating between kin, suggesting inbreeding occurs within this population (Pouyaud et al. 1999). Mouthbrooding *tilapia* are known to have fast speciation which could be the result of kin aggregation and preference for kin during the breeding season (Pouyaud et al. 1999). Breeding between related kin can affect individual fitness (Crnokrak & Roff 1999) and increase the risks of inbreeding depression (Pouyaud et al. 1999), producing offspring that may lack a diverse range of genotypes. Low genetic diversity negatively affects a population's ability to survive short-term ecological stresses and their long-term persistence, affecting the evolutionary potential of populations (Frankham et al. 2002).

1.3 The Kermadec giant limpet, *Scutellastra kermadecensis*

The Rangitāhua population of the Kermadec giant limpet (*Scutellastra kermadecensis*) is the last relict population of the world's second-largest limpet species (Fleming 1973). Rangitāhua is an isolated archipelago in the southwest Pacific approximately 750 km Northeast of New Zealand and has had full marine protection since 1990 (Creese et al. 1990). These limpets can grow greater than 130 mm and are the dominant grazers in the low intertidal and shallow subtidal zones of Rangitāhua (Creese et al. 1990, Wood & Gardner 2007). They are protandrous hermaphrodites and are broadcast spawners that produce pelagic larvae which disperse in the ocean currents (Lindberg 1988). The larvae settle on hard substrates, metamorphose and commit to a benthic existence as a largely sedentary or faithfully homing adult, which is evident from the home scars they leave on the rocky substratum (Wood & Gardner, 2007) (Fig. S1). The Kermadec giant limpet has an unusual habit where smaller, presumably male limpets, piggy-back on the shells of larger, presumably female limpets, rather than attaching to, and residing on bare rock surfaces (Creese et al. 1990). Like the home scars on rock surfaces, the shells of large limpets also

have visible scars left by smaller limpets, indicating the long duration of piggy-backing by the smaller individuals. This behaviour could be an example of an ontogenetic habitat shift with several possible drivers. Kermadec giant limpets generally occur in high-density at Rangitāhua (40 individuals m^{-2}; Cole et al. 1992): understanding the determinants of the piggy-backing behaviour and its effects on population dynamics could provide insight into their persistence as an isolated, endemic species.

As limpets of various sizes can be found piggy-backing or rock-attached, it is unlikely that the timing (or size) of this ontogenetic habitat shift is predetermined; rather, the habitat shift appears to be dependent on the environment (i.e., space, food, or the social environment) and the driving forces are likely context-dependent, depending on what the limiting resources are for individuals. For instance, smaller limpets could be piggy-backing to graze the algae that grow on the backs of older and larger limpets – a resource that would otherwise remain ungrazed. Alternatively, they could piggy-back to avoid aggressive interactions with larger conspecifics and/or possibly being bulldozed by them on the rock substrate. Finally, piggy-backing by males may ensure mating opportunities with the larger females. In a previous study on these limpets, Creese et al. (1990) showed that the limpets shift microhabitats from piggy-backing on limpet backs to the rock surface when they reach a size of approximately 55 – 67 mm. The factors that encourage piggy-backing behaviour could also influence the shift away from the behaviour. For instance, piggies may transition from limpet back to the rock surface when food or space on the limpet back becomes limiting (i.e., the piggy itself has consumed all limpet back algae), or, if piggy-backing were to maximise fertilization, the piggy-backing (presumably) male limpets would transition onto the rock surface when going through sex change to females as they would then need piggy-backing males on their own backs for their eggs to be fertilized. Accordingly, individual behaviours may be affected differently due to population characteristics (e.g., genotype, size or sex makeup) or due to different environmental settings (e.g., density, space availability, food availability) which may contribute to the differential timing of this microhabitat transition.

In several species, the genetic similarity (i.e., relatedness) between individuals influences their spatial assortment (Keough 1984, Aguirre et al. 2013), behaviours and rearrangement (Ayre 1982, 1987, Bigger 1980, Brace 1990, Zeh & Zeh 1997, Foster & Briffa 2014, Turner et al. 2003). Similarly, the mechanism behind this aggregative piggy-backing behaviour may be linked to the relatedness between the piggy and the host, which in turn could shape the small scale spatial genetic patterns within the Kermadec giant limpet metapopulation. Depending on the degree of relatedness, limpets could have a preference to aggregate or avoid closely related individuals (i.e., kin). The piggy-backing behaviour

could be kin aggregation to protect young from being bulldozed by other large individuals on the rock surface or it could favour segregation of related individuals to ensure genetically diverse offspring. Such kin-biased behaviour can shape the spatial arrangement of individuals within a population and may have critical effects on the following generation (Arnold 2000, Lehmann & Perrin 2003, Mougeot et al. 2003, Gerlach & Lysiak 2006, Hatchwell 2009, Lehmann & Perrin 2003, Mougeot et al. 2003, De Bona 2019). Specifically, if neighbours are highly related, this may have undesirable impacts on the genetic diversity of the offspring and the general fitness of populations (Charlesworth & Charlesworth 1999), and the long-term persistence and evolutionary potential of species (Frankham et al. 2002). Those impacts can include inbreeding, high genetic differentiation and/or genetic structuring within or between (sub)populations. Or, if neighbours are not kin and are distantly related individuals, this will promote genetic diversity for offspring and following generations which may prolong the species' persistence. Regardless of the mechanisms by which individuals are assorted, the long-term genetic health of Kermadec giant limpets depends upon the degree of relatedness between piggy-backing individuals and neighbouring limpets, and whether the species has mechanisms such as kin-avoidance to promote genetic diversity.

Genetic differentiation and structuring within and among populations are important considerations in the conservation management of vulnerable species. The degree of genetic differentiation and structuring of populations can inform the state of their genetic health, whether they are outbred or inbred, and/or if the population is under some sort of selection. For example, there is an extremely high number of cichlid species in Malawi, presumed to be a result of a historic explosive radiation (Van Oppen et al. 1997). Subpopulations of Cichlids separated by only a few hundred metres show highly significant genetic differentiation, a common pattern often accompanied by fast microallopatric speciation in Cichlid species. Such rapid microallopatric speciation may be explained by the combination of kin shoaling and inbreeding observed within the species (Pouyaud et al. 1999). Similarly, a significant level of genetic differentiation was found among populations of the Kermadec giant limpets separated by <1 km (Wood & Gardner 2007). Limited connectivity and a moderate degree of self-recruitment within limpet subpopulations may have contributed to this pattern. However, populations (or species), especially those that are isolated like the Kermadec giant limpets, reliant on self-recruitment are vulnerable to local extinctions and potentially undesirable genetic consequences such as low genetic diversity, inbreeding depression (indicated by a high level of population differentiation), reduced resilience to disturbances and low adaptive potential which may threaten the recovery of the population in the long-term.

1.4 Thesis outline

This thesis is composed of four chapters, including two empirical data chapters (Chapters two and three) which are written in manuscript format and a general discussion chapter (Chapter four). Chapter two is already published in the journal *Marine Ecology Progress Series*, and I plan to submit chapter three for peer-review as a co-authored manuscript for publication after receiving comments from the thesis examiners. Given that these chapters are written as independent publishable units, there is some repetition of the background information, explanation of the study system and methodological detail among the four chapters presented in this thesis. Furthermore, as the chapters were written as co-authored manuscripts, I use "we" (first-person plural) to acknowledge this; nonetheless, the thesis is my own work, completed under the guidance of my supervisors. Outlines for the subsequent chapters are as follows:

In Chapter two, I examine the influence of the surrounding environment, both biotic and abiotic, on the ontogenetic habitat shifts of the Kermadec giant limpets, i.e., the piggy-backing behaviour. These ontogenetic habitat shifts can allow species to access nutritional resources, avoid conspecific aggression or predation, or secure breeding opportunities when it is advantageous to do so. However, the factors that affect the changes in the piggy-backing behaviour observed in the limpets at the Kermadec Islands, Rangitāhua are not yet understood. First, I test the influence of the social environment (number and size of surrounding limpets) on the relationship between shell length and the probability of an ontogenetic habitat shift from limpet shells to rock surface and on the pigless limpets to become a host limpet. I hypothesized that if access to algal resources was the strongest driver of piggy-backing behaviour, the number of piggy-backing limpets would increase as rock availability decreased. Alternatively, if smaller limpets are piggy-backing to avoid competition, to avoid bulldozing, or to secure breeding opportunities with larger, rock-attached females, I hypothesized that the number of piggies (i.e., piggy-backing limpets) would increase as the number and size of rock-attached limpets increased. Furthermore, I expected that piggies would delay the transition to being rock-attached with increasing host size but would transition to being rock-attached sooner as the number and size of piggy-backing limpets increases, reflecting a situation where limpet shell habitat becomes limiting. Deducing the consequences of population density and size structure in this species is critically important for understanding demographic change in these relict populations of an extremely range-restricted endemic species.

In Chapter three I examine the small-scale neutral and adaptive genetic structure and assortment of individuals within Kermadec giant limpet subpopulations, including

piggy and host pairs. In marine invertebrates with bipartite lifecycles and sedentary adults, the distribution of individuals (genotypes) within and among populations can be influenced by the dispersal potential, larval and post-settlement behaviours. We assess levels of genetic relatedness between piggy-backing limpets on the same host, genetic relatedness of piggies and hosts, and the spatial scales over which limpets are related and exchange geneflow, as well as the impact and scale over which there is divergence in adaptive genetic variance, indicative of divergent selection. If spatial proximity determines fertilzation success, I sought to understand whether the species has mechanisms to promote genetic diversity, such as kin-avoidance among piggies on the same host, piggy-host pairing among less related individuals, and the movement of individuals away from relatives when transitioning from piggy-backing to being rock-attached. I hypothesized that if piggy-backing behaviour is mediated by beneficial kin interactions, I might expect that piggies on the same host are closely related to each other and potentially to their host. Whereas, if the piggy-backing behaviour was to maximise fertilization and reproductive success and individuals had some ability to select for genetic complementarity in their sexual partner, I expected that piggies and hosts might be less related to each other than by chance. To address these hypotheses, and any artefactual role of dispersal limitation, we consider the genetic relationships of piggies and hosts within the wider genetic neighbourhood, including neighbours sampled at set distances, and we replicate this sampling across several locations. The size of their genetic neighbourhood can impact the genetic diversity of offspring, with potentially negative consequences on the population's resilience to short term ecological stresses and evolutionary potential over the longer term.

In Chapter four, I synthesise the results of Chapters two and three. I provide insight into the ontogenetic habitat shifts and the relatedness between different classes (piggy-backers and rock-attached limpets) of the Kermadec giant limpets. I discuss the limitations of my study with reference to data collection and the boundaries of plausible assumptions that can be made from my study. Last, I suggest future directions for continuous monitoring of these limpets to maximise their survival potential in case of any unforeseen events.

This thesis advances our understanding of the potential influence the social environment has on the onset of ontogenetic microhabitat shifts and the current state of the Kermadec giant limpet's genetic composition. Initially, I had planned to test whether piggy-backing behaviour was influenced by rock space available and space only in terms of the number of neighbours in each stratum, to gain an understanding of whether the transition from piggy-backing to attaching to the rock surface was mediated by space limitation on the backs of host limpets. Additionally, I planned to carry out histology on the limpet samples Dr Aguirre and Dr Libby had collected to further investigate the timing of the sex change of

the Kermadec giant limpets as did Creese et al. (1990). Together with my results, I would have been able to test whether sex-change correlates to the shell lengths at which the limpets transitioned the microhabitats depending on their social environment, which we have reported here. However, due to the lockdowns and restricted travel during the COVID-19 pandemic, this plan was unable to advance. Nonetheless, I feel I have progressed an ambitious research plan and have produced two data chapters of publication quality and look forward to the examiner's suggestions for improvement.

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Social environment mediates habitat shifts in a range-restricted giant limpet

2.1 Abstract

The individuals of many species shift habitats at different stages in their lives. Sometimes, these habitat shifts coincide with an ontogenetic transition. These ontogenetic habitat shifts can allow species to access nutritional resources, avoid conspecific aggression or predation, or secure breeding opportunities when it is advantageous to do so. The Kermadec giant Islands limpet, *Scutellastra kermadecensis*, is a protandrous hermaphrodite endemic to Rangitāhua, the Kermadec Islands. These limpets have the unusual habit whereby small limpets (piggies) piggy-back on the shells of larger individuals rather than living on the rocky substrate. We investigated whether the ontogenetic habitat shift between the piggy-backing and rock-attached limpets was determined by the availability of free space on the rock or if the ontogenetic habitat shift was a response to the properties of the surrounding limpet population. We found that the available rock space weakly influenced the size at which an individual transitioned from being a piggy to being rock-attached. Furthermore, larger rock-attached limpets were more likely to have piggies, they had more piggies and the piggies were larger. Overall, our results suggest that Kermadec giant limpets are motivated to piggy-back by the social environment rather than space constraints. The piggy-backing behaviour may be a mechanism to avoid bulldozing by larger limpets, to access grazing opportunities on the shells of larger limpets, and/or to monopolise breeding opportunities with larger rock-attached females. We discuss the repercussions of this life-history strategy for this extremely range-restricted species, with reference to how these populations may be monitored and maintained.

2.2 Introduction

Animals often move between habitats at different stages of their lives. When these habitat shifts coincide with a developmental transition, for example, between juvenile and mature stages, or pre- and post- metamorphosis, they are referred to as ontogenetic habitat shifts (Stamps 1983). Such habitat transitions may occur as resource requirements change with the onset of maturity and/or as individuals grow. For instance, as green turtles (*Chelonia mydas*) mature, they travel to deeper zones along the Pacific coast where adult food is abundant (López-Mendilaharsu et al. 2005). Another reason for moving between habitats may be to avoid or reduce conspecific aggression or predation. For example, juvenile fishes often live in structurally complex nursery habitats, such as mangroves, seagrasses, corals or macroalgae, to avoid predation by larger con- and hetero-specifics (Nagelkerken et al. 2000, Laegdsgaard & Johnson 2001, Unsworth et al. 2009, Evans et al. 2014). In many animals, males and females may also maintain distinct habitats until it is time to reproduce. Anglerfishes (*Lophiiformes*) are potentially one of the most extreme examples, where small males transition from being free-swimming to being permanently attached to larger, female anglerfish to ensure reproductive opportunities when the female spawns (Pietsch 1976). While the mechanisms motivating ontogenetic shifts of individuals are well-understood in some systems, in other systems the drivers are less clear and are likely context-dependent.

The timing of ontogenetic shifts can vary according to the environment experienced by the individual. For instance, in many marine species with bipartite life-histories, individuals will often delay metamorphosis until they have acquired sufficient resources to metamorphose or until they encounter suitable settlement habitats (Marshall & Morgan 2011). In contrast, in sequential hermaphrodites, the timing of a sex change can be determined by the composition of the surrounding social environment (Charnov & Bull 1989, Munday et al. 2006). For instance, in the absence of males, the largest female bluehead wrasse (*Thalassoma bifasciatum*) in the local population will transition into a male (Warner & Swearer 1991). The newly transitioned male will then dominate the harem of females, in turn increasing his reproductive output. The timing of such transitions in morphology, sex, or habitat, can influence the acquisition and defence of resources, and thereby survival and reproductive success (Sergio et al. 2017). Ultimately, by influencing the fitness of individuals, environmentally dependent ontogenetic shifts impact the ecological and evolutionary trajectories of populations.

The Kermadec Islands giant limpet (*Scutellastra kermadecensis*) is endemic to Aotearoa New Zealand, and restricted to Rangitāhua, the Kermadec Islands. These islands are a small, isolated archipelago in the southwest Pacific approximately 750 km northeast

of the North Island of New Zealand. Kermadec giant limpets grow to be the dominant grazers in the low intertidal and shallow subtidal zones of Rangitāhua (Creese et al. 1990, Wood & Gardner 2007). Individuals commonly reach a shell length greater than 130 mm and populations can reach densities of up to 40 individuals m^{-2} (Cole et al. 1992). Despite being locally abundant and resident in a large, old Marine Protected Area (Kermadec Islands Marine Reserve), the Kermadec giant limpet is extremely range-restricted, occupying only these few, isolated islands and outcrops, and therefore there has been much interest in the population ecology and extinction risk of this species (Fleming 1973, Schiel et al. 1986, Creese et al. 1990, Cole et al. 1992, Wood & Gardner 2007).

Like other patellid limpets, Kermadec giant limpets are broadcast spawners with external fertilization and pelagic larvae (Lindberg & Marincovich 1988, Wood & Gardner 2007). The embryos and larvae disperse in the ocean before settling to the substrate and metamorphosing. The adults are largely sedentary, or faithfully homing, which is evident from the home scars they leave on the rocky substrate (Fig. S1; Creese et al. 1990, Wood & Gardner 2007). Kermadec giant limpets are protandrous hermaphrodites, with the unusual habit that smaller individuals, typically male, often piggy-back on the shells of larger, typically female, individuals (Creese et al. 1990). The reasons for the piggy-backing behaviour are unknown, but it may be that male limpets piggy-back on larger females to ensure mating opportunities, to avoid competition and being bulldozed by larger limpets, and/or to graze algae that grows on the back of larger, older limpets – a resource that would otherwise remain ungrazed. Creese et al. (1990) showed that when Kermadec giant limpets reach a size of 55 – 67 mm, they shift microhabitats from predominantly piggy-backing to being mostly attached to the rock surface. As Kermadec giant limpets of various sizes can be found piggy-backing or rock-attached, it is unlikely that the timing of this ontogenetic habitat shift is predetermined, rather the habitat shift appears dependent on environmental factors such as space, food, or the social environment.

While often overlooked, the importance of the social environment in determining individual behaviour, life history outcomes, and population demography of gastropods has been highlighted in numerous studies (e.g., Branch 1975a, b, Schroeder 2011, Le Cam et al. 2014, Martins et al. 2017). Hence, here, we investigate whether the social environment (the number and size of surrounding limpets) influences the shell length – our proxy for age or life stage – at which piggy-backing limpets transition to becoming rock-attached. We hypothesized that if access to algal resources was the strongest driver of piggy-backing behaviour, the number of piggy-backing limpets would increase as rock availability decreased. Alternatively, if smaller limpets are piggy-backing to avoid competition, to avoid bulldozing, or to secure breeding opportunities with larger, rock-attached females, we

hypothesized that the number of piggies (i.e., piggy-backing limpets) would increase as the number and size of rock-attached limpets increased. Furthermore, we expect that piggies would delay the transition to being rock-attached with increasing host size but would transition to being rock-attached sooner as the number and size of piggy-backing limpets increases, reflecting a situation where limpet shell habitat becomes limiting. Deducing the consequences of population density and size structure in this species is critically important for understanding demographic change in these relict populations of an extremely range-restricted endemic species.

2.3 Methods

2.3.1 *Study design and population survey*

The study was conducted during November 2015, at three locations, separated by 100's of metres around islands and islets of the largest island in the Rangitāhua archipelago, Raoul Island (Fig. 1). Nested within each location there were sites separated by 10's of metres (Boat Cove: eight sites, North Meyer Island: seven sites, and South Meyer Island: six sites), and nested within each site there were three 0.25m^2 quadrats (separated by 1-2 metres) placed on the substrate in the low intertidal zone inhabited by Kermadec giant limpets. For each quadrat, a photograph with the camera parallel to the quadrat was taken (Fig. S1). From these photos, we collected information at three scales: quadrat level, subquadrat level (three randomly placed 0.0225m^2 quadrats within each quadrat), and individual (limpet) level. Individual limpets were classified as either: limpets attached to the shells of other limpets, referred to as 'piggies;' or limpets directly attached to the rock surface, referred to as 'rock-attached.' We then further distinguished between rock-attached limpets with piggies attached to their shells ('hosts') and rock-attached limpets without limpets attached to their shells ('non-hosts').

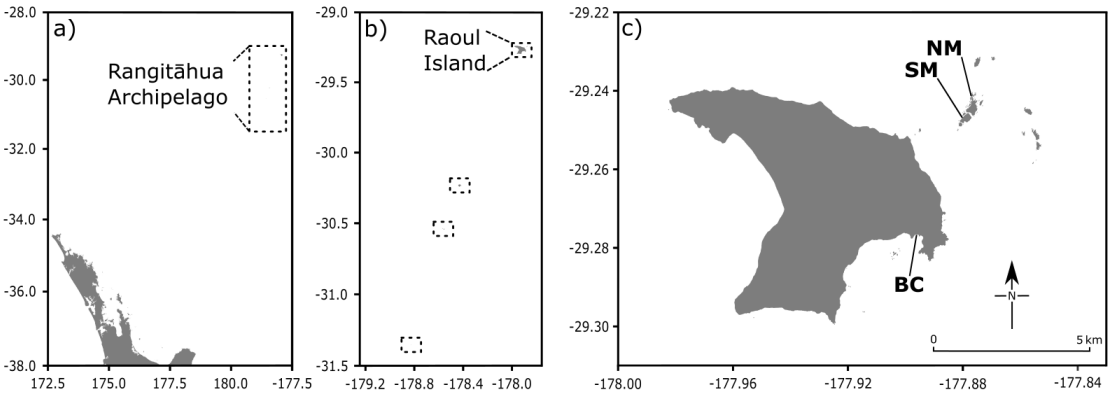

Fig. 1 (**a**) The location of Rangitāhua, the Kermadec archipelago, relative to the North Island of Aotearoa New Zealand is indicated by the dotted rectangle. (**b**) The location of Raoul Island relative to the other islands in the Rangitāhua archipelago. (**c**) The sampling locations distributed around Raoul Island were the western side of North Meyer (NM) and South Meyer (SM) Islets, as well as Boat Cove (BC) on the eastern side of Raoul Island.

2.3.2 Habitat and social environment analysis

Quadrat level data were used to describe the social environment surrounding the subquadrats. To quantify the available rock space, we used the image analysis software 'ImageJ' (Schneider et al. 2012) to randomly place 125 points within the borders of each quadrat (Fig. S1). Points that landed on or outside the quadrat, as well as points that landed on undefined space inside the quadrat (blurriness and/or water), were excluded. The proportion of available rock space was calculated by counting the points that did not land on limpets or home scars (Fig. S1). Then, for each quadrat, we counted the total number of rock-attached limpets (both hosts and non-hosts) including home scars and the number of the piggies on the hosts. We then randomly placed three 0.0225m^2 subquadrats within each quadrat using ImageJ and counted and measured the shell length of each rock-attached (host and non-host) and piggy limpet. For the individual-level data, we measured the size of ten randomly selected hosts in each quadrat, then we counted the number of piggies, and measured the size of all piggies. Although we were able to count all limpets at the subquadrat- and individual-level, we only measured limpets where the shell was oriented parallel, or close to parallel, to the camera and where the individual was clearly visible (i.e., not obscured by blurriness and/or water).

2.3.3 Statistical analysis

To determine if the relationship between shell length and the probability of a piggy transitioning into a rock-attached limpet varied according to the social environment (i.e.,

size and number of rock-attached limpets, as well as piggy size and number), we used binomial generalised linear mixed models (GLMM) with a logit link. In our first analysis, the response variable was binary where 1 indicated the individual was a piggy and 0 indicated the individual was a rock-attached limpet (i.e., host or non-host). The fixed effects were shell length, rock-attached limpet number and size, piggy number and size, available rock space, and the interaction between shell length and rock-attached limpet number and size, piggy number and size, and available rock space. The random effects were location, site nested within location, quadrat nested within site, and subquadrat nested within quadrat (which was treated as an observation level random effect; Elston et al. 2001). In our second analysis, to determine if the relationships between shell length and the probability of a rock-attached limpet becoming a host varied depending on the social environment, we used a binomial GLMM in which the response variable was binary with 1 indicating the individual was a host and 0 indicating the individual was a non-host. The same fixed and random factors were used in the model examining if the social environment influences the shell length at which individuals become hosts as in the previously described model for piggy-backing behaviour.

To examine the relationship between host size and piggy number we used a Poisson GLMM with a log link. The response variable was the number of piggies, the fixed effects were host size, and random effects were location, site nested within location, and quadrat nested within site. Last, to examine the relationship between piggy size and host size, a linear mixed model (LMM) was used with piggy size as the response. The fixed and random effects in the LMM for piggy size were the same as the Poisson GLMM for piggy number.

For both the subquadrat-level data and the individual-level data we used backward model selection using nested log-likelihood ratio-tests to determine the significance of each term (Tables S1, S2, S4 and S5). If none of the random effects was significant, we retained the observation-level random effect (i.e., subquadrat) to account for any possible overdispersion (Elston et al. 2001). To test for overdispersion we used a χ^2 test using the sum of the squared Pearson residuals divided by the residual degrees of freedom as the test statistic and degrees of freedom equal to the residual degrees of freedom (residual degrees of freedom = 548). All analyses were implemented in the lme4 package (Bates 2012) for R version 4.0.2. (R Core Team 2020) using RStudio (RStudio Team 2020). To determine the robustness of our parametre estimates across all optimizers used by lme4, we assessed each model fit for all available optimisers and compared the equivalence of the parametre estimates.

2.4 Results

At the individual-level, piggy number had a positive relationship with host size, and this trend was consistent across all locations (Fig. 2a, Table S5). Most hosts had only one piggy (58%); however, there were hosts with large numbers of piggies and in particular, one relatively small rock-attached individual (48.5mm shell length, SL) that hosted seven piggies (Fig. 2a). When piggies were present, there was also a positive relationship between piggy size and host size (Fig. 2b, Table S5). Overall, bigger hosts were more likely to have piggies, had more piggies (Fig. 2a), and those piggies were larger (Fig. 2b).

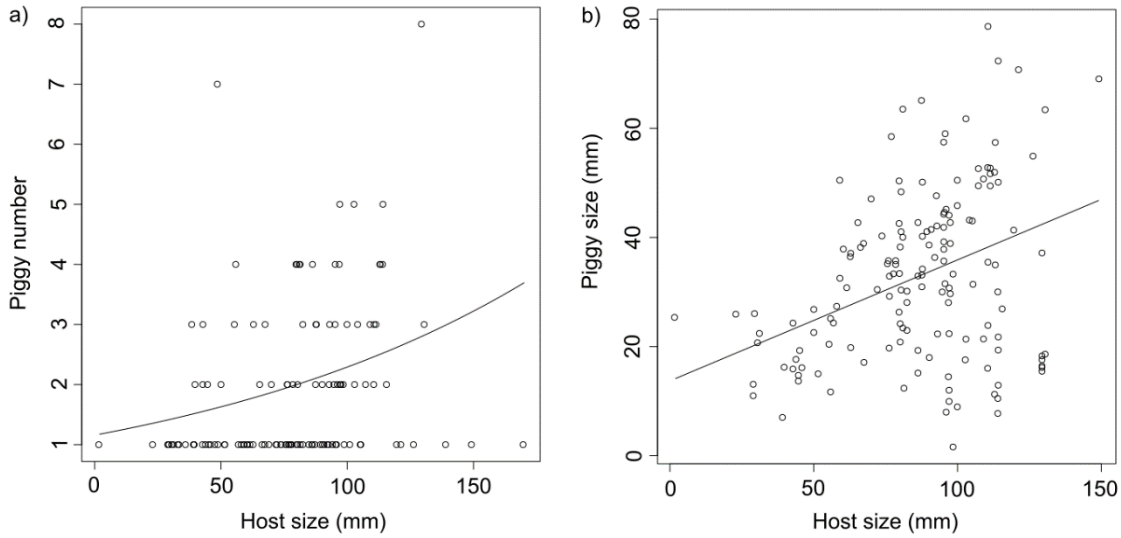

Fig. 2 Relationship between host size and **(a)** total number of piggies and **(b)** piggy size for Kermadec giant limpets at Raoul Island, Rangitāhua. Lines are predicted values from the linear mixed models.

At the sub-quadrat level, the relationship between shell length and the probability of a limpet being a piggy versus rock-attached, varied depending on the rock-attached limpet number, rock-attached limpet mean size, piggy mean size, and rock space availability but not piggy number (Table S2). The shell length at which a piggy transitioned to being rock-attached was smaller when mean piggy size was larger (Fig. 3a). However, when piggy mean size was large (>55mm SL), there was an equal probability of a limpet being a piggy or rock-attached at all shell lengths. When rock-attached limpet number was higher, the shell length at which piggies transitioned into a rock-attached limpet was smaller than when rock-attached limpet number was lower (Fig. 3b). When mean rock-attached limpet size was large, the shell length at which piggies transitioned into rock-attached limpets was also larger. Importantly, there appeared to be a minimum size (indicated by the largest SL where a 0.0 predicted probability of an individual being rock-attached was identified) before piggies transitioned to becoming rock-attached limpets (approximately 32mm SL, lower

left of Fig. 3c). Last, when there was more rock space available, the shell length at which a piggy transitioned into a rock-attached limpet was larger than when rock space availability was lower (Fig. 3d).

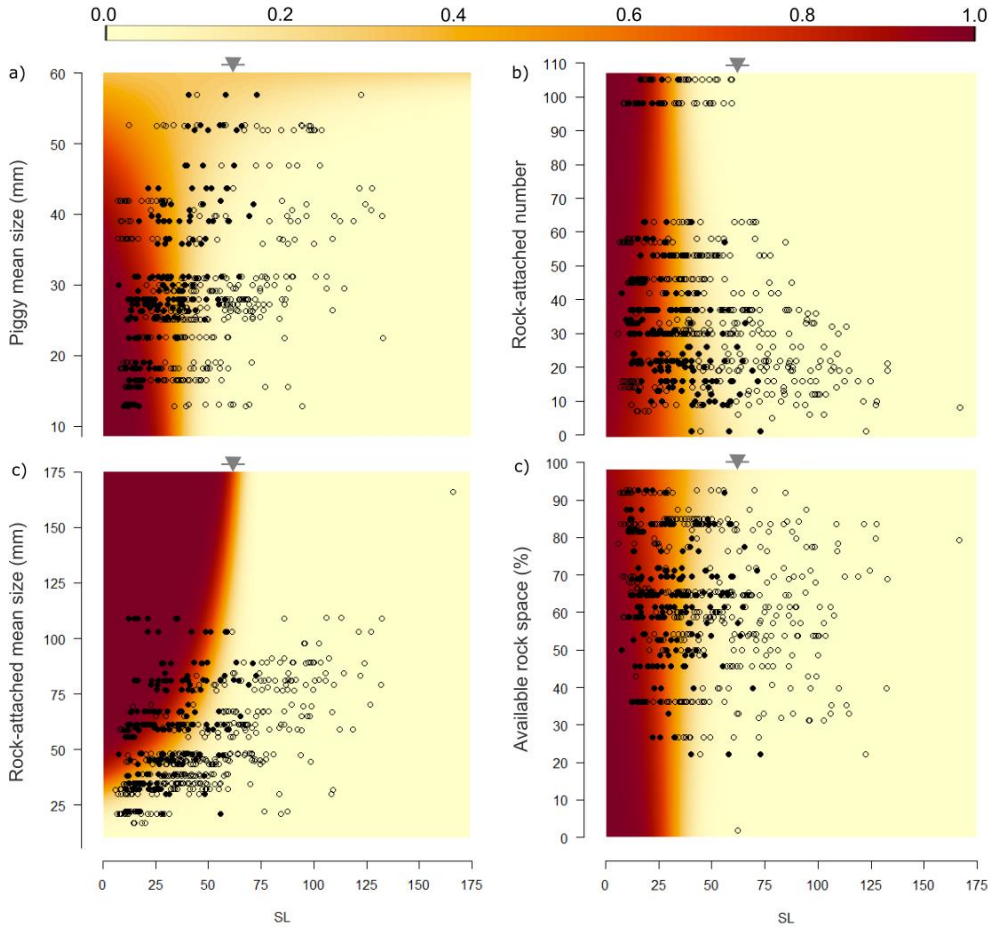

Fig. 3 The probability of a limpet piggy-backing (red) or being rock-attached (pale yellow) for the interaction between shell length and **(a)** the mean size of piggies in the surrounding environment (Piggy mean size); **b)** the number of rock-attached limpets in the surrounding environment (Rock-attached number); **c)** the mean size of rock-attached limpets in the surrounding environment (Rock-attached mean size); and **d)** the available rock space in the surrounding environment. Symbols indicate the observed values for piggies (filled circles) and rock-attached limpets (open circles). The grey triangle and bar above each panel indicates the sizes (55 mm – 67 mm) at which Creese et al. (1990) observed a habitat-shift from piggy-backing to rock-attached.

The shell length at which rock-attached individuals became hosts varied depending on piggy number, host size, and piggy size (Table S2). When piggy number was higher, the shell length at which rock-attached limpets became a host was smaller. However, there was a minimum shell length for a rock-attached limpet to become a host (approximately 47 mm SL, Fig. 4a). When piggies were smaller, the shell length at which a rock-attached limpet became a host was smaller (Fig. 4b). Last, when neighbouring rock-attached limpet mean

size was larger, the shell length at which rock-attached limpets became hosts was also generally larger, although, in the presence of very large rock-attached limpets (approximately 100 mm SL), small rock-attached limpets also had a high probability of being hosts (upper left of Fig.4c). More typically, rock-attached limpets became hosts at approximately 66mm SL (Fig. 4c). Overall, the probability of a piggy transitioning into a rock-attached limpet did not vary significantly among locations, sites, or quadrats (Table S2). However, the probability of a rock-attached limpet transitioning into a host varied among locations and sites (Table S2).

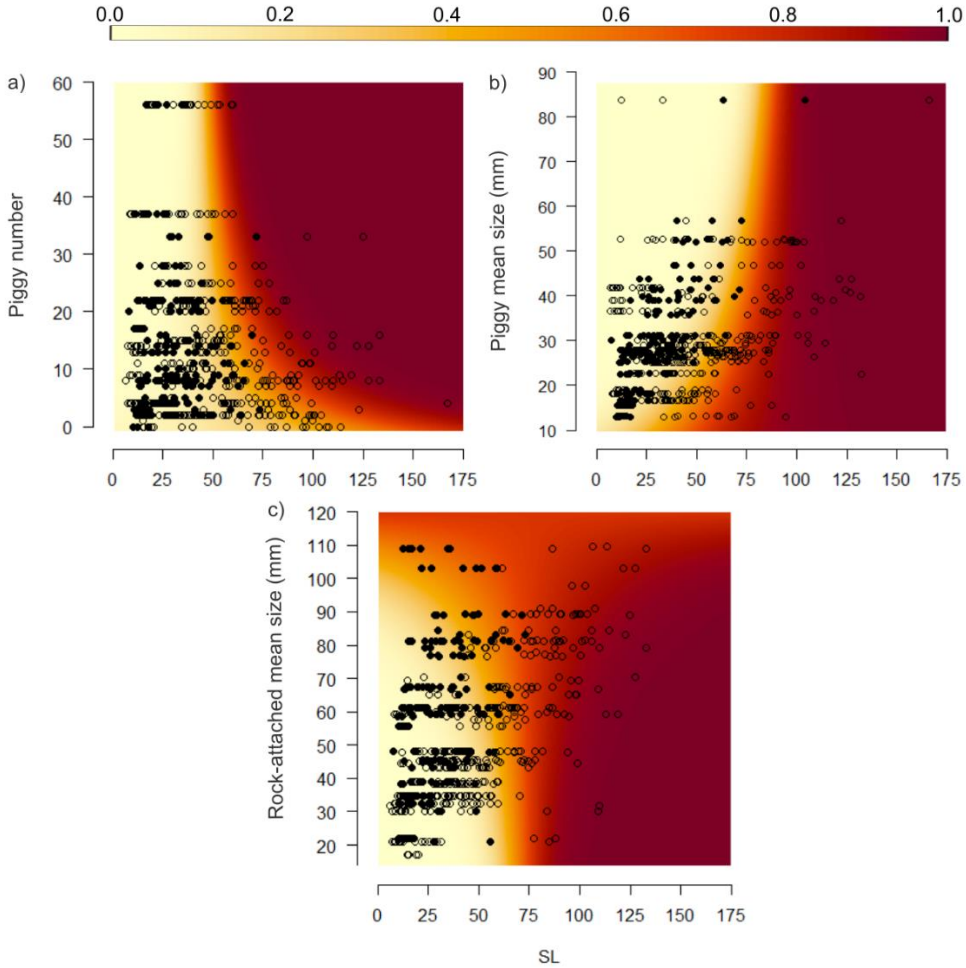

Fig. 4 The probability of a limpet hosting a piggy (red) or being a non-host rock-attached limpet (pale yellow) for the interaction between shell length and **a)** the number of piggies in the surrounding environment (Piggy number); **b)** the mean size of piggies in the surrounding environment (Piggy mean size); and **c)** the mean size of rock-attached limpets in the surrounding environment (Rock-attached mean size). Symbols indicate the observed values for non-hosting rock-attached limpets (filled circles) and host limpets (open circles).

2.5 Discussion

The social environment played an important role in the size at which Kermadec giant limpets transitioned habitats. Although available rock space did influence piggy-backing behaviour, it was not the strongest predictor of habitat transitions (Table S3). Piggies on larger hosts were larger and more densely packed, suggesting that piggies preferentially sought-out these larger hosts or they persisted as piggies for longer when attached to larger hosts. Furthermore, if large hosts were absent and piggy densities were high, rock-attached limpets became hosts at a smaller size, suggesting that these ontogenetic shifts are socioecologically plastic. While it is clear from our results that small Kermadec giant limpets preferentially occupy the back of the shell of host limpets, and that the social environment influences the timing of habitat transitions, there are several possible explanations for these behaviours. For instance, it could be that piggies are utilizing hosts to access grazing opportunities that would otherwise go ungrazed, to avoid bulldozing by larger conspecifics, and/or to secure breeding opportunities with larger, rock-attached females. Although we cannot distinguish among these hypotheses with the data in hand, below we discuss each in turn with regard to our results and the supporting literature.

The Kermadec giant limpet is numerically and spatially dominant in the intertidal zone of Rangitāhua (Schiel et al. 1986, Creese et al. 1990, Cole et al. 1992, Wood & Gardner 2007). In several of our subquadrats/quadrats, more of the area was made up of Kermadec giant limpet backs than limpet-free rocky substrate. Shell-forming invertebrates such as limpets are known to be important secondary-habitat for conspecifics and other organisms (Branch 1975a, b, Thyrring et al. 2013, Martins et al. 2014), including algae which can then be consumed by grazers (van Tamelen 1987, Wernberg 2010). For instance, the large shell of the turban snail, *Turbo torquatus*, is readily colonised by foliose algae, which in turn attracts and supports other grazers such as patellid limpets (Wernberg 2010). In the case of Rangitāhua, an abundance of algae occupies the rocky intertidal, including the backs of the limpets (Fig. S1). Piggy-backing Kermadec giant limpets may simply reside on the back of larger limpets to graze the available resource. Our results indicating that larger limpets have larger, more abundant piggies, support this idea. However, our finding that rock availability, and thereby the availability of algal resources attached to the rock surface, was less important than aspects of the social environment suggests that piggy-backing behaviour is not purely resource-driven.

Given that Kermadec giant limpets are the most abundant macrograzer in the intertidal zone of Rangitāhua (Schiel et al. 1986, Creese et al. 1990, Cole et al. 1992, Wood & Gardner 2007), a large proportion of the biotic interactions for individual Kermadec giant

limpets would be with conspecifics. Mobile macrograzers such as limpets, are known to negatively impact newly settled larvae and recruits of benthic marine invertebrates through bulldozing (Dayton 1971, Ellrich et al. 2020). For example, sessile species such as barnacles are often bulldozed by mobile grazers like periwinkles and limpets (Underwood et al. 1983, Bertness 1984). Barnacles can avoid this bulldozing effect by growing rapidly and achieving a size refuge (Denley & Underwood 1979, Bertness 1984). Mobile species that are also susceptible to bulldozing, have behavioural responses to avoid these disturbances (Dayton 1971, Tegner et al. 1995, Day & Branch 2002). For instance, recruits of the South African abalone (*Haliotis midae*) avoid being bulldozed by hiding beneath larger urchins and moving with their host urchin, thereby remaining concealed and protected from bulldozing as well as predators (Tegner et al. 1995, Day & Branch 2002). The shells of larger Kermadec giant limpets offer a substrate where smaller individuals can avoid antagonistic interactions with the largest individuals in the population, and thereby provide a potential refuge from competition, physical bulldozing, and death. Moreover, we observed cases where small limpets were piggy-backing on piggies, suggesting that size-structured bulldozing among piggies might also occur on the backs of host Kermadec giant limpets.

Resource partitioning and antagonistic interactions are two mechanisms that could underlie piggy-backing behaviours. A third mechanism is males and females aggregating to maximise their reproductive output (Pietsch 1976, Chen et al. 2018). Broadcast spawning marine organisms commonly form mating aggregations to maximise fertilization success by reducing sperm limitation (Stanwell-Smith & Clarke 1998, Yund 2000), with males in closer proximity to broadcast spawning females typically having greater reproductive success (Picken & Allan 1983, Collin et al. 2006, Marshall & Bolton 2007, Henry et al. 2010, Suda et al. 2015, Chen et al. 2018). Creese et al. (1990) used histology to reveal that Kermadec giant limpets are protandrous; small limpets are typically males, transitioning to become females at a larger size and presumably older age. Importantly, none of the limpets that Creese et al. (1990) could confidently identify as female were piggies. Thus, it could be that small, male limpets preferentially piggy-back on larger rock-attached females to ensure they are near eggs when they are released, allowing greater fertilization success.

In accordance with studies demonstrating that limpets will transition to becoming female at a smaller size when females are rare or when male density is high (*Patella ferruginea* [Rivera-Ingraham et al. 2011] and *Crepidula coquimbensi* [Brante et al. 2012]), we found that limpets transitioned to being rock-attached, and potentially transitioning to becoming females, at a smaller size in denser piggy populations. These developmental and habitat transitions may allow individuals to gain reproductive opportunities as a female sooner when there is an abundance of smaller males to sire offspring and to avoid male-

male competition for fertilizations (discussed in Munday et al. 2006). However, although quadrats with larger rock-attached limpets had lower overall numbers of piggies, there were greater numbers of larger piggies on each host. These results appear to support another socially-mediated behaviour – individuals delay the transition to becoming rock-attached, and presumably to become female, when they have access to large, presumably female, hosts (also observed in *Crepidula spp.* by Coe 1938 and Brante et al. 2016, for example). To support the hypothesis that the timing of habitat transitions may be driven by reproductive opportunities, we would need to establish that there is a strong association between the timing of sex change and the timing of the habitat transition in Kermadec giant limpets.

Ontogenetic habitat shifts are often associated with a transition from a life stage focused on growth and survival, to a stage focused on reproduction and survival (Stearns 1989). By avoiding bulldozing and grazing the algae on the shells of host limpets, piggy-backing may allow greater survival and growth of small male limpets while keeping them in the immediate proximity of females should they release their eggs. The positive relationship between female size and fecundity in animals is pervasive (Nobili & Accordi 1997, Chaparro et al. 1999, Chaparro & Flores 2002, Espinosa et al. 2006, Kasamatsu & Abe 2015) and recent studies suggest that larger females get a disproportionately large fecundity advantage over smaller females (Espinosa et al. 2006, Barneche et al. 2018). Thus, piggy-backing in Kermadec giant limpets could be a strategy that maximises growth, survival and ensures reproductive opportunities for males, while simultaneously maximising population growth by allowing the largest individuals to be female, and ensuring females have a reliable supply of sperm to fertilize their eggs when they are spawned.

Here, our focus has been on density-dependent population processes. Of course, populations in small, isolated locations are dependent on the retention of locally produced offspring as there is little or no metapopulation connectivity (Liggins et al. 2014). Some of the variation we observed in the size-structure of these limpet populations may be due to the sporadic recruitment of limpets and the number of recruitment seasons that have contributed to the population. A previous genetic study of the Kermadec giant limpet suggests that populations separated by as little as 400 m can be genetically differentiated and recruiting limpets are predominantly from the local population (Wood & Gardner 2007). The socioecological plasticity we observed in the timing of habitat transitions could be a mechanism to ensure population persistence despite the inherent stochasticity in the recruitment dynamics of Kermadec giant limpet subpopulations.

The Kermadec giant limpet populations at Rangitāhua are the last relict populations of the world's second-largest limpet (Fleming 1973). Although at risk of extinction due to their restricted range, these limpets are common in the lower intertidal zone of these islands, suggesting their life-history strategies and behaviours ensure a relatively large population size and local retention of offspring. For instance, most hosts had only a single piggy, if piggies are not close-kin of their host, these behaviour's may ultimately help to maintain genetic diversity within a small population. Furthermore, if the transition to becoming rock-attached at a smaller size when piggies are densely packed is associated with a sex-change at a smaller size (or younger age), these behaviours would be indicative of a mechanism to ensure population-level reproductive output is maintained (Wright 1989, Warner et al. 1996, Rivera-Ingraham et al. 2011). Nevertheless, while these limpets appear to be well suited to life on small, remote islands and although these islands are one of the least human-modified ecosystems on our planet (Edgar et al. 2005), they are not immune to global climatic changes. External environmental factors (e.g., illegal fishing or natural disasters) that affect population composition, and therefore the social environment, can influence the timing of sex change (Martins et al. 2017) and potentially ontogenetic shifts in limpets. Given that Kermadec giant limpets are protandrous and have a skewed gender ratio, such changes could be detrimental to the population-level reproductive success (Martins et al. 2017) and ultimately the species persistence. Our work has shown that changes to the recruitment dynamics of these limpets or the survival of larger, typically female limpets will trigger a cascade of interactions impacting this already vulnerable species.

Chapter 3:

Relatedness does not predict the aggregation of Kermadec Giant Limpets, but adaptive genetic variance is structured over small spatial scales

3.1 Abstract

In the bipartite lifecycle of marine species with sedentary adults, stochastic events during passive dispersal as well as larval and post-settlement behaviours can influence the distribution of genotypes within and among populations. Subsequently, how individuals are assorted within a population and the size of their genetic neighbourhood can impact the genetic diversity of offspring, with potentially negative consequences on the population's resilience to short term ecological stresses and evolutionary potential over the longer term. The Kermadec giant limpet exists in high density populations on the small, subtropical islands of the Rangitāhua archipelago in New Zealand. Kermadec giant limpets are protandrous hermaphrodites that broadcast spawn, and have an unusual habit where smaller, presumably male, limpets piggy-back on larger, presumably female, limpets that are attached to the rock surface. In previous studies we have shown that small limpets prefer to reside on larger limpets rather than on the rock surface. Here, we investigate if the associations between host and piggies are correlated with their small-scale neutral and adaptive genetic structure. We hypothesized that if piggy-backing behaviours are mediated by beneficial kin interactions, we might expect piggies on the same host to be closely related to each other and potentially to their host. In contrast, if the piggy-backing behaviour is to maximise fertilization and reproductive success, piggies and hosts might be less related to each other than we might expect by chance. To address these hypotheses, we used SNP markers and assessed the levels of genetic relatedness between piggy-backing limpets on the same host, and between piggies and hosts, and the spatial scales over which limpets are related, as well as their divergence in adaptive genetic variance. We found no evidence of kin aggregation or avoidance from genetic relationship comparisons of piggy-piggy pairs, and piggy-host pairs. Although some genetic structuring of piggies was evident among sites (metres), and locations (100s-1000s of metres) for rock-attached limpets, their genetic relatedness did not vary predictably according to their spatial arrangement (up to 2 metres). Nevertheless, based on loci putatively under selection, rock-attached limpets within 1 metre, shared the same adaptive genetic variation indicating a role of spatially divergent selection

in structuring the spatial arrangement of limpets at these scales, and among sites and locations. Although the Kermadec giant limpets seem successful based on their abundance and dominance in the intertidal zones of the Rangitāhua archipelago, the high level of inbreeding may leave them vulnerable to environmental change. Uncovering the drivers of the piggy-backing behaviour would allow us to better understand how this species have persisted despite being extremely restricted to construct an appropriate monitoring and conservation plan for the second largest limpet species in the world.

3.2 Introduction

Patterns of spatial genetic structure are often dependent on a species' dispersal potential, where more vagile species have larger genetic neighbourhoods, less genetic structure, and relatively less genetic differentiation over distance than more sessile species (Burton 1983, Hedgecock 1986, Strathmann 1990, Carr et al. 2003, Shanks et al. 2003). For this reason, in the bipartite lifecycle of marine species where adults are sessile or sedentary, it has been thought that the distribution of genotypes within and among populations is primarily influenced by the dispersal potential of early life stages (Carr et al. 2003, Shanks et al. 2003, Calderón et al. 2007; Blanquer et al. 2009; Ledoux et al. 2010). However, it has also been found that larval behaviours such as kin aggregation (Pineda 1999, Metaxas 2001), and directional orientation of larvae to favoured environments (Griffiths et al. 2003, Dixson et al. 2008, Shanks 1995) can influence the genetic composition of sub-populations and result in genetic structure. For instance, larvae of anemone fish (*Amphiprion percula*) use olfactory cues to preferentially move toward reefs that have rainforest vegetation nearby, or to their natal reef itself (Dixson et al. 2008). Furthermore, the relatedness of three-spot damselfish (*Dascyllus trimaculatus*) on the same or nearby anemones in French Polynesia suggests that related larvae aggregate during their long planktonic dispersal (up to a month approximately), maintaining a non-random distribution of genetic variation in wild populations (Bernardi et al. 2012). These studies suggest that the spatial genetic structure of marine species is also influenced by behaviours of related individuals during their early life history (Leis 1991, Bernardi et al. 2012).

Following dispersal, individuals can also exhibit behavioural reassortment to aggregate with kin or to avoid family groups at recruitment (e.g. Keough 1984, Gamfeldt et al. 2005, Aguirre et al. 2013), sometimes even competing with relatives or non-related conspecifics post-recruitment. For example, juvenile Atlantic salmon (*Salmo salar*) have been found to actively avoid their kin when sheltering in streambed refuges (Griffiths et al. 2003). In contrast, new recruits of the Warratah anemone (*Actinia tenebrosa*), only

aggregate with their clone parents/mates and will attack non-clonemates that differ genotypically (Ayre 1982, 1983). Such relatedness-biased behaviours have been documented in many other marine invertebrates (e.g., , Buss 1979, Ayre 1982, 1983, Buss & Grosberg 1990) - including aggression resulting in death (e.g., *Hydractinia symbiolongicarpus;* Buss & Grosberg 1990) and allelopathy/overtopping (e.g., corals; Russ 1982, Baird & Hughes 2000) - influencing the fitness of individuals, their kin, the surrounding population and ultimately, the spatial genetic structuring of populations.

Regardless of the mechanisms by which individuals are assorted within a population, their neighbours and the size of their genetic neighbourhood can influence their resilience to short term ecological stresses, as well as the adaptive potential of the population. For example, sessile, internally fertilizing barnacles can only mate with neighbours within penile distance (Veliz et al. 2006), and although broadcast spawners can mate with individuals over greater distances, fertilization success is highest when gametes are released in close proximity (Picken & Allan 1983, Suda et al. 2015, Chen et al. 2018). In fact, several gastropod species aggregate, or "stack," during broadcast spawning events thereby enhancing fertilization success (Stanwell-Smith & Clarke 1998, Yund 2000), with the most proximal sire having the highest reproductive success (Picken & Allan 1983, Suda et al. 2015, Chen et al. 2018). Such a reproductive strategy ensures high fertilization success before gametes are diluted in open ocean environment; however, it may have undesirable impacts on the genetic diversity of the offspring, particularly if neighbours are highly related, or there is some mechanism by which kin become aggregated (Frankham et al. 2002).

The Kermadec giant limpet is a relic population of the world's second-largest limpet species (Fleming 1973). Although the remaining populations are high-density (40 individuals m^{-2}; Cole et al. 1992) and reside in a large, old, fully protected, Marine Protected Area (Creese et al. 1990; https://www.doc.govt.nz/parks-and-recreation/places-to-go/northland/places/kermadec-islands/?tab-id=Bird-and-wildlife-watching), they exist only on small, subtropical islands of the Rangitāhua archipelago (Kermadec Islands Marine Reserve), northeast of New Zealand. Kermadec giant limpets are broadcast spawners, whereby the gametes, and then embryos and larvae, disperse in the ocean before settling to the substrate and metamorphose. The adults are largely sedentary, or faithfully homing, which is evident from the home scars they leave on the rocky substrate (Fig. S1; Creese et al. 1990; Wood & Gardner 2007). A previous population genetic study of the Kermadec giant limpet by Woods & Gardner (2007) found significant genetic differentiation and limited connectivity among populations of limpets separated by <1 km. Based on randomly amplified polymorphic DNA markers (RAPDs), the authors concluded that a low to moderate degree of self-recruitment occurs within subpopulations of the limpet, where 26-

64% of individuals were assigned to their population of collection. A high capacity for self-recruitment may be advantageous for the maintenance of populations in isolated locations such as Rangitāhua. However, populations (or species) reliant on self-recruitment are especially vulnerable to local extinction, and potentially undesirable genetic consequences such as low genetic diversity and therefore adaptive potential.

Kermadec giant limpets are protandrous hermaphrodites, with the unusual habit that smaller, presumably male limpets (referred to as 'piggies'), often piggy-back on the shells of larger, presumably female, limpets that are directly attached to the rock surface (referred to as 'rock-attached' or 'host/s' if they have a piggy/piggies on their backs) (Creese et al. 1990). We have shown that these small limpets preferentially piggy-back on the larger conspecifics, rather than on the rock surface (Kim et al. 2021, Chapter 2). This piggy-backing behaviour may be a means to protect kin from bulldozing by larger limpets attached to the rock substrate (i.e., kin aggregation), or may be a mechanism to increase reproductive success, or both. In either case, given that spatial proximity often determines fertilization success in gastropods, the long-term genetic health of Kermadec giant limpet populations, and the species, may be heavily influenced by whether piggy-backing individuals, and neighbouring limpets, are highly related or not.

Here, we investigate the small-scale neutral and adaptive genetic structure and assortment of individuals within Kermadec giant limpet subpopulations, including piggy and host pairs. We assess levels of genetic relatedness between piggy-backing limpets on the same host, genetic relatedness of piggies and hosts, and the spatial scales over which limpets are related and exchange gene flow, as well as the impact and scale over which there is divergence in adaptive genetic variance, indicative of divergent selection. If spatial proximity determines fertilization success, we sought to understand if the species has mechanisms to promote genetic diversity, such as kin-avoidance among piggies on the same host, piggy-host pairings among less related individuals, and the movement of individuals away from relatives when transitioning from piggy-backing to being rock-attached. We hypothesized that if these piggy-backing behaviours were mediated by beneficial kin interactions, we might expect that piggies on the same host are closely related to each other (i.e., aggregated during early life) and potentially to their host (i.e., settled to the protective environment of kin). In contrast, if the behaviour was for the purpose of maximising fertilization and reproductive success, we expected that piggies and hosts might be less related to each other than by chance (hereafter termed 'spawning aggregation'), if the species has some ability to select for genetic complementarity in their sexual partner. To address these hypotheses, and any role of dispersal limitation, we consider the genetic relationships of piggies and hosts within the wider genetic neighbourhood, including

neighbours sampled at increasing distances from the focal host limpet, and we replicate this sampling across several locations.

3.3 Methods

3.3.1 Study design and sample collection

Tissue for genetic analyses was sampled from limpets at multiple sites separated by 10's of metres at three locations around the largest island in the Rangitāhua archipelago, Raoul Island (Boat Cove: seven sites, North Meyer Island: six sites, and South Meyer Island: four sites) (Fig. 1a-c). At each site, rock-attached limpets (i.e., limpets attached to the rock, and not to the backs of other limpets), piggies (i.e., limpets attached to the back of other limpets), and host limpets (i.e., rock-attached limpets that had piggies attached to them) were sampled. First, to test for an association between the genetic relatedness of rock-attached limpets and their spatial arrangement, a focal rock-attached limpet was randomly chosen and sampled, we then sampled rock-attached limpets at each of the following distances ($\pm$2 cm) from the focal limpet: 10 cm, 20 cm, 40 cm, 80 cm, and 160 cm. These non-focal limpets were sampled in alternating directions parallel to the water line away from the focal limpet, such that the limpet sampled 10 cm from the focal limpet, was 30 cm from the limpet sampled 20 cm from the focal limpet, and so on (Fig. 1d). All piggies of focal limpets were sampled. Piggies on non-focal limpets were noted, but these piggies were not sampled. In this design, a total of 166 limpets was sampled across all sites and locations (102 rock-attached, 64 piggies). Second, to test whether the relatedness of piggies on the same host differed to piggies on different hosts, one randomly selected host limpet and its piggies from the same site were sampled where possible. The random host limpets and their piggies (4 hosts and 18 piggies) were retained as voucher specimens and deposited into the Auckland Museum Tāmaki Paenga Hira collection following tissue sampling and measurement. A total of 188 limpets was sampled across all sites and locations across both designs.

The limpets were sampled by sliding a blunt, wide knife between the limpet shell and the rock for rock-attached limpets or between the shell of the host and the piggy for piggies, and gently levering the limpet off the substrate. The shell length at the widest point was measured in millimetres and small tissue samples from the outer margin of the muscular foot removed (<3 mm^3) using sharp surgical scissors to avoid interfering with the individuals' ability to firmly attach to the substrate. Scissors were sterilised between use on different limpets by rinsing them in water and cleaning them with 95% ethanol. Tissue samples were immediately placed in 2 ml tubes containing 95% ethanol. Following tissue

sampling, each limpet was returned to its home scar (on the substrate or on the back of a host limpet) and held in position for approximately 1 minute until it remained attached.

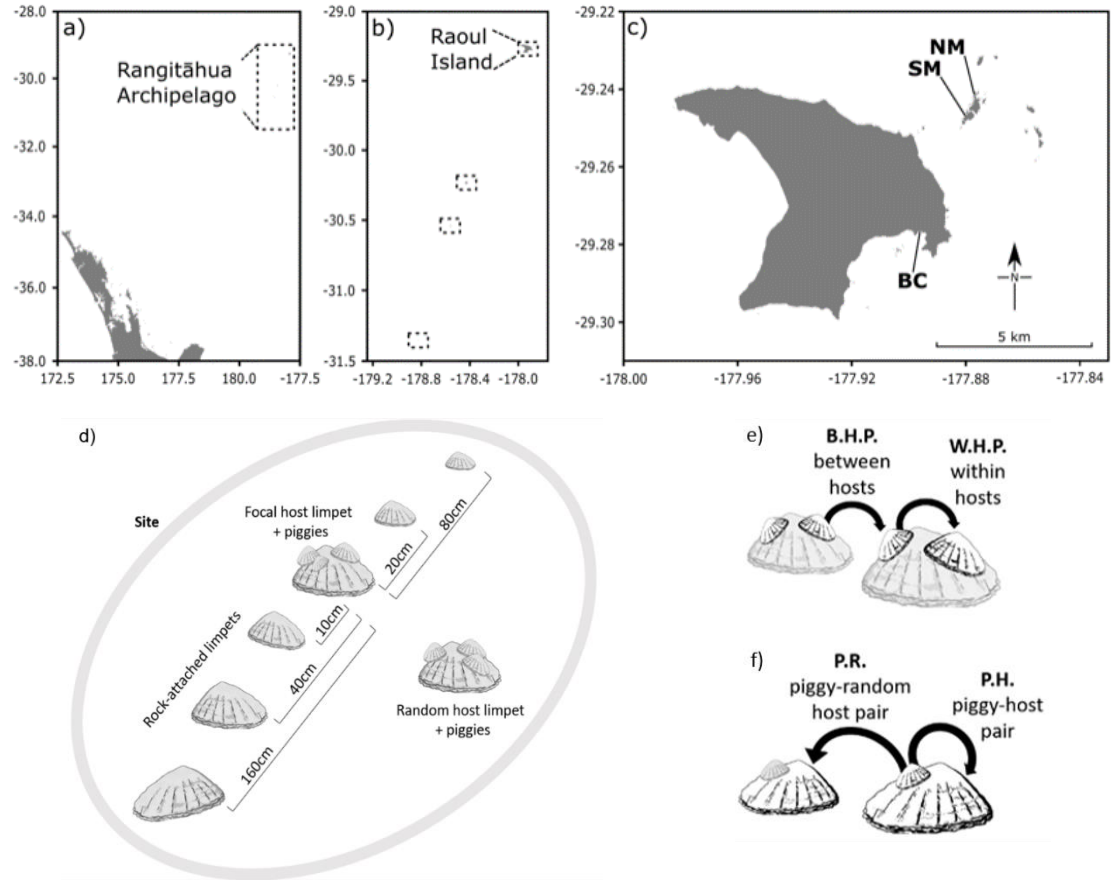

Fig. 1 (a) The location of Rangitāhua, the Kermadec Islands, relative to the Northeast of the North Island of Aotearoa New Zealand is indicated by the dotted rectangle. (b) The location of Raoul Island relative to the other islands in the Rangitāhua archipelago. (c) The sampling locations distributed around Raoul Island were the western side of North Meyer (NM) and South Meyer (SM) Islets, as well as Boat Cove (BC) on the eastern side of Raoul Island. (d) The limpet sampling undertaken at each site within a location, including the Focal host limpet, the Random host limpet (and their piggies), and the spatial sampling of other Rock-attached limpets. (e) The sampling design used to investigate relatedness among piggies residing on the same host (W.H.P.) versus piggies residing on different hosts (B.H.P.) within a site. This grouping of limpets is referred to as a 'bungalow', a collective noun for aggregated limpets. (f) The sampling design used to investigate relatedness among piggy-host pairs (P.H) and piggies with another host within the same site (P.R.).

3.3.2 Laboratory, sequencing, and bioinformatic methods

Genomic DNA was extracted from the tissue samples using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocols for animal tissue with the following modifications: the lysis step was conducted overnight at 56 °C with 20µl Proteinase K added; after digestion, 3µl of RNAse A (Monarch) was added and samples were incubated for 10 minutes at 37 °C; and to maximise DNA yield, the elution step using TE buffer was repeated (each 50µl in volume) providing a DNA extraction of 100µl volume. Extractions were initially assessed for quality by running 2µl of DNA on a 1% agarose gel and then a Qubit 2.0 Fluorometre (Fisher Scientific) was used to quantify the DNA. Samples with DNA concentrations of <10 ng/µl were concentrated where possible or re-extracted from remaining tissue. DNA concentrations were normalized to 50-100 ng/µl for all samples prior to sequencing.

Single Nucleotide Polymorphisms (SNPs) were obtained using a Genotyping-by-Sequencing (GBS) approach, based on a 192-plex, two-plate experimental setup. Enzyme and adapter optimisation, size selection, library preparation, and NGS sequencing was conducted by Elshire Group (Manawatu, New Zealand). Optimisation of the enzyme and adapter concentrations for *Scutellastra kermadecensis* was based on 2µg of pooled DNA from 3 individuals from across the sampling design. Library preparation was conducted for 188 DNA extractions using 100ng of genomic DNA and 1.44ng of adapters. GBS followed the Elshire et al. (2011) method with the following specifications: genomic DNA was fragmented using the *Pst*I restriction enzyme and the library was amplified with 18 PCR cycles, yielding 21.6ng/µl. Samples were sequenced on 1 lane of 2x 150bp Illumina HiSeq.

De novo SNP calling was conducted using the Stacks 2.5 pipeline (http://catchenlab.life.illinois.edu/stacks/). The GBS data were demultiplexed with axe-demux (https://github.com/kdmurray91/axe). Then, the reads were trimmed for adapter and barcode sequences using the batch_trim.pl script (https://github.com/Lanilen/GBS-PreProcess). The reads for both ends of the pair-end data were combined into individual per-sample files. Because *de novo* approaches to GBS SNP calling strongly recommend using reads of identical length, all reads were trimmed to a fixed 64 bp length using skewer (https://github.com/relipmoc/skewer) with no quality filtering thresholds and discarding shorter reads. Next, the trimmed reads were put through the standard Stacks 2.5 pipeline using their denovo_map.pl script. Default parametres were used for the initial run, then the populations program was re-run with the following parametres: /usr/local/bin/populations -P stacks2.5_denovo -M populations.txt -t 12 --min-maf 0.05 -e PstI –vcf.

After SNP calling, the vcf formatted data were filtered using VCFtools (https://vcftools.github.io/index.html). Individuals with >40% missing data were removed. Loci with >70% missing data, average read depths of <8 or greater than 350 reads and minor allele counts of <6 were all removed (See Tables S6 and S7). Two datasets were retained for subsequent analysis: unpruned (used only in relatedness analysis using KGD; Dodds et al. 2015); and pruned (used in all other analysis), where one SNP per locus was retained using the --thin command to ensure that SNPs were not linked.

3.3.3 Detection of loci putatively under selection

We used two methods of outlier loci detection to identify putatively adaptive loci in our pruned data that may be under divergent selection. First, we identified outlier loci using the R package, *pcadapt* version 4.3.3 (Luu et al. 2017) using R version 4.0.3. (R Core Team 2020) and RStudio (RStudio Team 2020). *Pcadapt* implements the method described in Luu et al. (2017), identifying outlier loci by their divergence from the overall population structure within a Principal Components Analysis (PCA). This method does not require a *priori* grouping of individuals and has been shown to perform well under several demographic scenarios when the Mahalanobis distance is used as the test statistic, including where there is hierarchical population structure (Luu et al. 2017). We determined the number of principal components for the PCA from the scree plot using Cattell's rule (i.e., retaining components before the point of plateau is reached on the scree plot where K is ordered by diminishing size on the y-axis, and the x-axis represents the percentage of variance explained by each value of K starting; Cattell 1966), selecting 3 as the K-value, and used the Mahalanobis distance as our test statistic, with a false discovery rate of 10% (q-value <0.1). Because *pcadapt* can suffer from low power if the spatial pattern of selection does not correlate with spatial genetic structure (Capblancq et al. 2018) we also used another R package, *OutFLANK* version 0.2 (Whitlock & Lotterhos, 2015) to increase our confidence in the identification of outliers. *OutFLANK* detects outliers based on the distribution of loci-specific (standardised) F_{ST} values, where loci with values in the centre of the distribution are assumed to be neutral. Loci with the highest (and lowest) F_{ST} values are identified as outliers. Unlike *pcadpapt*, *OutFlank* requires the prior grouping of individuals into (sub)populations and is generally more conservative returning few false positives across many demographic scenarios (Whitlock & Lotterhos, 2015, Luu et al. 2017). Limpets were grouped according to their sampling location and *OutFlank* was run using default parametres and a q-value threshold of 0.03 was used to identify loci with the highest F_{ST} values and are likely under divergent selection. Based on the outlier loci detection, a 'neutral'

pruned dataset was created by excluding loci in the intersection of both methods, and a conservative 'adaptive' pruned dataset only comprising the intersection of both methods.

3.3.4 Spatial genetic analysis

To understand fine scale spatial population genetic structure and relationships among individuals we calculated three individual pairwise genetic measures to be used in subsequent analyses. First, using unpruned dataset the pairwise genomic relatedness of the samples was calculated using KGD package which gives unbiased estimates of relatedness by accounting for read depth (Dodds et al. 2015). We used *calcG* command to calculate the G^5 relatedness matrix using Hardy-Weinberg disequilibrium cut-off at -0.05. Using pruned neutral and adaptive datasets we additionally calculated the absolute genetic distance (i.e. Prevosti's distance, which reflects the number of observed allelic differences between two individuals, relative to the number of possible differences, and can handle missing data) among individuals in R package *poppr* version 2.8.7 (Kamvar et al. 2014, 2015).

These individual pairwise distance measures were then used in Analyses of Molecular Variance (AMOVA, Excoffier, Smouse & Quattro, 1992) using *poppr* (Kamvar et. al 2014, 2015) and spatial autocorrelation analyses using *vegan* version 2.5.7 (Oksanen et al. 2007) to identify the spatial-scale of genetic relatedness and structuring across our sampling design. Analyses were conducted for rock-attached limpets and piggies separately, using both pairwise individual genetic distance matrices calculated from the unpruned (i.e. the inverse of the G^5 pairwise relatedness), and pruned neutral and adaptive datasets (i.e. Prevosti's distance). Analyzing the two life stages separately allowed us to investigate whether ontogenetic habitat shifts (i.e. when transitioning to being rock-attached) result in any reassortment of individuals, influencing the fine scale genetic structuring of populations. Furthermore, examining neutral and adaptive genetic structure separately enabled us to assess the role of selection in determining the spatial genetic structuring of rock-attached limpets during this ontogenetic shift. Using the *poppr* implementation of ade4 AMOVA, the total genetic variance was partitioned among individuals within a site, the variance attributable to the different sites within a location, and the variance among locations; significance at each level assessed using 10,000 permutations. In the spatial autocorrelation analysis, for each site, the Euclidean spatial distance among all individuals within our spatial sampling design (Fig. 1d) was used as a continuous predictor and their pairwise relatedness (G^5) as the response variable.

3.3.5 Relatedness of piggies and hosts

To explore possible drivers (kin aggregation or avoidance) of limpet piggy-backing behaviour, we examined differences in the genetic relatedness of piggies on the same hosts, and the relatedness of piggies to their hosts (Fig. 1f). All analyses used linear mixed effects models in R package *lme4* version 1.1-26 (Bates et al. 2015). First, for each site, we compared the mean relatedness among piggies on the focal host to the mean relatedness of the piggies on the randomly selected host (3 sites from Boat Cove and 2 sites from South Meyer; North Meyer Island was excluded from this analysis as there were no piggies retained following SNP filtering). Second, for each site, we compared the mean relatedness between piggies and their host (both the focal and randomly sampled hosts) to the mean relatedness of the host's piggies to the other host limpet (either the focal or the random host limpet; Fig. 1e) (including 7 sites from Boat Cove, 4 sites from South Meyer, and 1 site from North Meyer).

3.4 Results

Following quality control and filtering, 167 individual limpets from across the sampling design were retained for analysis (61 piggies and 106 rock-attached, of which 22 were hosts). For the unpruned dataset, 6525 loci were used for analysis; the pruned dataset had 4489 loci (see Table S6).

3.4.1 Detection of loci putatively under selection

PCAdapt identified 371 outlier loci and OutFlank identified 215 outlier loci within the pruned dataset. Using the intersection of both outlier detection methods, the 'adaptive' dataset contained 154 loci. Prior to analysis of the adaptive dataset for piggies and rock-attached limpets, for each dataset, loci with >70% missing data across individuals were removed, and individuals with > 40% missing data for remaining loci were removed. After this additional filtering step, we retained 154 loci in adaptive dataset, including 33 piggies (2 from randomly selected rock-attached limpet sampling design), 59 rock-attached limpets (of which 13 were hosts, Table S6). The pruned neutral dataset had a low level of missing data (28.08%) across the 4332 loci and 167 individuals (61 piggies and 106 rock-attached of which 22 were hosts. We retained all 22 random rock-attached limpets, Table S6).

3.4.2 Spatial genetic analysis

Based on the inverse of the G^5 relatedness among individuals, and the individual pairwise Prevosti's distance for the neutral, and putatively adaptive loci, the AMOVAs revealed very small differences in spatial genetic structure across life stages (Table S7). Although much of the genetic variance was found within the sampled sites (97.16% for rock-attached limpets, and 98.35% for piggies), this was significantly less than would be expected by chance ($p = 0.01$), indicating there was spatial genetic structuring of the limpet population. In the case of piggies, both the variation among sites (0.77%, $p = 0.01$) and the variation among locations (0.88%, $p = 0.01$) contributed equally and significantly to the spatial genetic structuring. In contrast, for the rock-attached limpets, the genetic variance attributable to among location differences was greater than among sites (among sites = 0.65%, $p = 0.03$, among locations = 2.19%, $p = 0.01$). Analyses based on Prevosti's distance for the neutral dataset recovered similar patterns of genetic variance partitioning, except that there was no significant contribution of location for either life stage (-0.43% for piggies, $p = 0.86$, and -0.05% for rock-attached limpets, $p = 0.87$), and the amount of variance attributed to among site differences remained slightly higher for piggies (among sites = 0.95%, $p = 0.01$), than for rock-attached limpets (among sites = 0.37%, $p = 0.01$). Based on loci putatively under selection, the partitioning of genetic variance differed from that based on neutral loci, and among life stages. For piggies, the majority of genetic variance was still found among individuals within a site (48.32%), although this remained significantly less than expected by chance ($p = 0.01$). In contrast to neutral loci, more genetic variance for the putatively adaptive loci was partitioned among locations than would be expected by chance for the piggies (36.98%, $p = 0.02$). For rock-attached limpets, most of the putatively adaptive genetic variance was partitioned among locations (61.39%, $p = 0.01$), followed by the variance found among individuals within a site (35.53%, $p = 0.01$). The putatively adaptive genetic variance attributed to among sites differences was less for rock-attached limpets (3.09%, $p = 0.01$) than for piggies (14.70%, $p = 0.02$).

Spatial autocorrelation analyses using G^5 and Prevosti's distance for both the neutral and adaptive datasets indicated small-scale trends (Fig. 2 b-d). However, overall, there was no relationship between genetic relatedness/similarity and the spatial arrangement of rock-attached limpets. The correlograms produced by G^5 and Prevosti's distance for the adaptive dataset revealed a very slight trend of positive spatial autocorrelation over distances <100cm, after which there was a downward trend in the overall slope of the Mantel correlation, indicating no relationship between genetic relatedness/similarity and distance for limpets >100 cm apart. This pattern was more consistently significant for the adaptive loci, indicating that over scales under 100 cm

limpets share alleles at these adaptive loci. In contrast, the correlogram based on the pruned neutral loci had a negative trend over small-scale distance classes (< 100 cm), indicating that individuals closer together shared fewer alleles at neutral loci, than those individuals further spatially separated. Across all sites and locations, there were no predominant patterns that had occurred for sites within one location that could have driven these results (Fig S2).

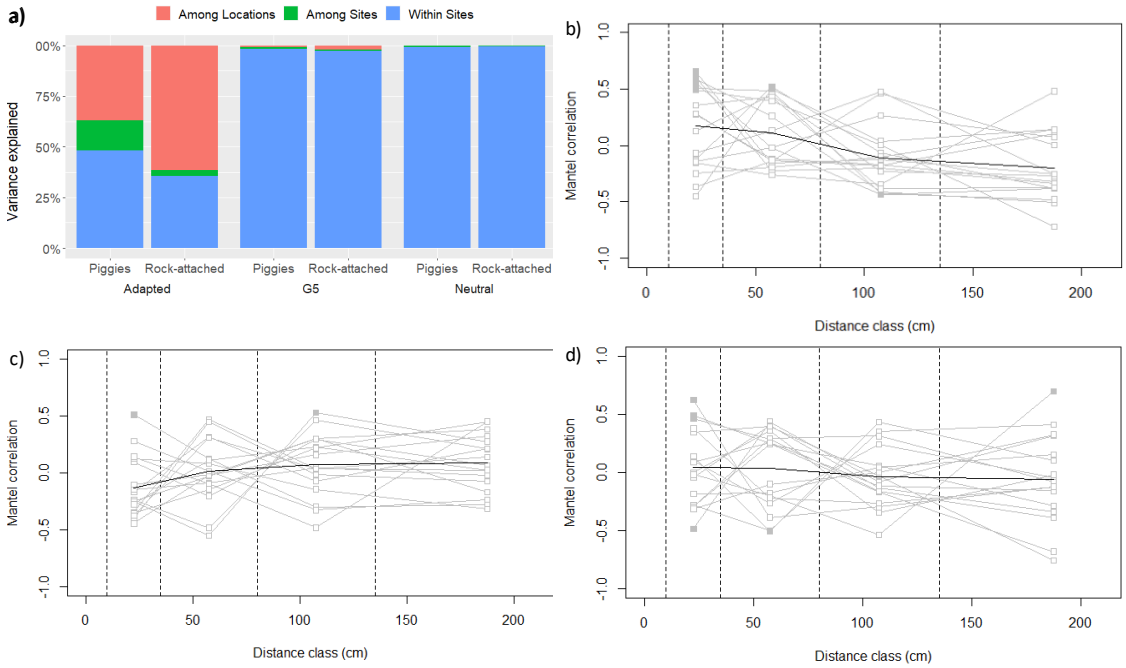

Fig 2 (a) Analysis of Molecular Variance (AMOVA) showing the percentage of variance explained by putatively adaptive loci (A) and neutral loci (N) of piggies (P), rock-attached (RA), and the mean relatedness for within sites, among sites, and among locations. (b) Correlogram of spatial autocorrelation on the mean relatedness of Prevosti's distance based on putatively adaptive loci of rock-attached limpets at each of the sampled distances from the focal limpet (Fig. 1d). (c) Correlogram of spatial autocorrelation on the mean relatedness of Prevosti's distance based on putatively neutral loci of rock-attached limpets at each of the sampled distances from the focal limpet (Fig. 1d). (d) Correlogram of spatial autocorrelation on the overall mean relatedness (pairwise individual genetic distance matrices calculated from the unpruned) of all loci of rock-attached limpets at each of the sampled distances from the focal limpet.

3.4.3 Relatedness of piggies and hosts

The mean relatedness of piggies on the same host and piggies on different hosts revealed no evidence of kin aggregation or strong differences between locations (Fig. 3a). Similarly, the mean relatedness of piggies and their host compared with a randomly selected rock-attached limpet from the same site (e.g., Fig. 1e; Fig. 3b) revealed little evidence for kin or

spawning aggregation as the driver of host-piggy pairings. Overall, the level of inbreeding estimated from the G^5genomic numerator relatedness matrix was very high (1.39 ± 0.004) and showed little variation among locations (Fig. S3).

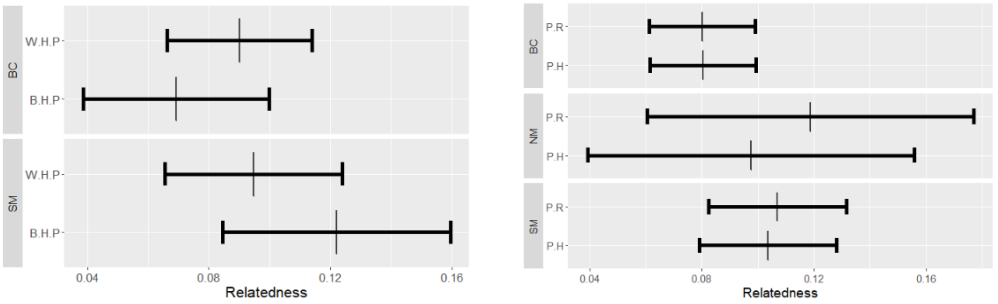

Fig. 3 a) The mean ($\pm$SE) relatedness among piggies on the same host (W.H.P) alongside the mean relatedness among piggies on different hosts (B.H.P) for South Meyer Island (SM) and Boat Cove (BC). b) The mean relatedness between piggies and individuals not in a bungalow (P.R) alongside the mean relatedness between piggies and their host (P.H) for South Meyer Island (SM), North Meyer Island (NM) and Boat Cove (BC).

3.5 Discussion

The piggy-backing behaviour of the Kermadec giant limpet is unusual in that the pairings between a piggy and a host are long-term, with piggies forming home scars on the backs of the host. This longevity of piggy-backing and thereby the resultant pairing of piggy and host, as well as our recent research evidencing the preference of small limpets to inhabit the backs of hosts rather than the substrate (Kim et al. 2021, Chapter 2), suggests there is some benefit to this behaviour. We hypothesized that if piggy-backing behaviour of the Kermadec giant limpet was mediated by beneficial kin interactions, we might expect that piggies are closely related to each other (i.e., aggregated during early life) and potentially to their host (i.e., settled to the protective environment of kin). In contrast, if the piggy-backing behaviour maximises fertilization and reproductive success, we expected that piggies and hosts might be less related to each other than expected by chance to increase the genetic diversity of offspring. There were four major findings from our study. First, there was no evidence of kin aggregation or kin avoidance from comparisons of the genetic relationships of piggy-piggy pairs, and piggy-host pairs. Second, possibly as a consequence, the genetic relatedness (and pairwise genetic distance based on neutral loci) of the Kermadec giant limpets did not vary predictably according to their spatial arrangement (up to 2m), although there was some genetic structuring of piggies evident among sites (separated by tens of metres), and locations (separated by 100s-1000s of metres) for rock-attached limpets. Third, based on loci putatively under selection, rock-attached limpets within 1 metre, shared the same adaptive genetic variation indicating a role of spatially divergent

selection in structuring the spatial arrangement of limpets at these scales, and also among sites and locations (based on the AMOVA, Fig. 2a). Last, overall level of relatedness among individuals was high (Fig. 3), indicating high levels of inbreeding. Below we elaborate on these findings and their relevance to the conservation of these endemic limpets.

The lack of evidence for kin aggregation or avoidance in Kermadec giant limpets indicates there is no evidence for parental care, or that siblings preferentially aggregate to avoid competition. Moreover, we do not see evidence for limpets particularly avoiding related individuals in their pairing for purposes of reproduction and for maximising genetic diversity of offspring. Our estimated mean relatedness values for all types of pairings were no higher than 0.12 (Fig. 3ab), a value indicative of a relationship between cousins or great-grandparents and great-grandchildren (e.g., Huisman 2017, van Kooten 2019). Because our estimates of relatedness are based on identity-by-state, rather than identity-by-descent, the familial relationship among limpets should be inferred with caution. Nonetheless, other studies have found identify-by-state methods, including KGD, perform well when tested using individuals of known pedigree and familial relationships (Galla et al. 2020). Furthermore, high relatedness values are common in hermaphroditic marine invertebrates that broadcast spawn (Olsen et al. 2020). As gametes are broadcast and fertilization occurs externally (Strathmann 1990; Vermeij & Grosberg 2017), adults do not have control over which sperm collide with eggs and depending on the specificity of sperm-egg recognition system in a population, fertilization between gametes of related individuals could occur. Although high levels of relatedness and inbreeding could be a consequence of behaviours such as offspring returning to natal patches (Christie et al. 2010) or kin-aggregation during dispersal (Eldon et al. 2016) or following settlement (Appeldoorn 1995, Pedersen & Guichard 2016), we find no evidence of such behaviours in the Kermadec giant limpet.

The genetic relatedness and genetic differentiation among rock-attached Kermadec giant limpets did not vary predictably according to their spatial arrangement within sites (up to 2m). However, based on the overall trend in the spatial autocorrelation of mean relatedness for Prevosti's distance based on putatively neutral loci (Fig. 2c), neutral genetic distance seemed to correlate negatively with spatial distance (only slightly) up to one metre in distance. If autocorrelation values were significantly negative, this would indicate kin avoidance, possibly to enhance out-crossing and the genetic diversity of offspring. Additionally, the AMOVA revealed that a high percentage of neutral genetic variance is partitioned among individuals within sites, for both life stages (Fig. 3a, Neutral-Piggies and Neutral-Rock-attached; piggies = 99.48%, rock-attached = 99.68%). Our result implies that the level of dissimilarity observed between two limpets from the same site would be similar to the level of dissimilarity between limpets from different sites. The lack of genetic

differentiation, and high proportion of neutral variation partitioned among individuals, may indicate that the subpopulations of the Kermadec giant limpet that we sampled are a highly connected metapopulation. In theory, broadcast spawning species, and those with high-dispersal, planktonic phases should tend toward random mating and have panmictic populations (Palumbi 1994). However, several studies of broadcast spawning species have reported genetic divergence over much smaller spatial scales than those studied here (e.g., *Littoraria flava,* four to a few hundred metres; Andrade & Solferini 2007). In these cases, actual gamete and larval dispersal might be lower than predicted by their dispersal potential allowing genetic differentiation to occur over smaller scales (<1-10km; Barber et al. 2002, Andrade & Solferini 2007). Indeed, a previous genetic study of the Kermadec giant limpet found small-scale genetic structuring of subpopulations over spatial scales of 1 km (Wood & Gardner 2007). The authors suggest that a considerable proportion of recruiting larvae stay close to the natal population and the contributing factors to this may be the geographic distance among populations and/or patterns of connectivity influenced by local hydrographic features. Reconciling the results and interpretations of Wood & Gardner (2007) with our results, we suggest that the genetic structuring detected using RAPD markers may have reflected the impact of selection on the genetic structuring of non-neutral loci or were neutral but linked to regions of the genome under spatially divergent selection.

We observed spatial genetic structuring based on putatively adaptive loci, indicating a role of spatially divergent selection in shaping the genetic composition of Kermadec giant limpet subpopulations. Across both life stages, a considerable amount of adaptive genetic variation was partitioned among-locations (piggies = 36.98%, rock-attached limpets = 61.39%; separated by 100s -1000s m; Fig. 2a, Adapted-Piggies and Adapted-Rock-attached), and for piggies, among-site variation (48.32%; separated by tens of metres; Fig. 2a, Adapted-Piggies) was also important. Additionally, in some cases, adaptive genetic variation was more similar than expected among rock-attached limpets within 1 metre (Fig. 2b), indicating a role of spatially divergent selection in structuring spatial arrangement of limpets over very small scales. Variation between sites and locations (Fig. 2a) and observed differentiation at adaptive loci among locations could arise from differing characteristics of microhabitats within sites and habitat differences among the three locations which favour individuals with specific genotypes, resulting in local adaptation. Several studies have reported significant spatial genetic structuring based on putatively adaptive loci. Genetic structure over small- (metres) to large-scale distances (kilometres) in marine invertebrates (e.g., Great Barrier Reef corals; Riginos et al. 2019, Californian sea cucumbers, *Parastichopus californicus*; Xuereb et al. 2018a, abalone, *Haliotis laevigata*; Sandoval-Castillo et al. 2018). The significance of such results has also been corroborated by experiments in some cases. For instance, three subpopulations of tunicates (*Botrylloides* sp.)

found in southern Gulf of Maine showed local adaptation to local temperature regimes and significant among-site (separated by <60 km) differential survival in response to thermal anomalies (Grosholz 2001). Similarly, offspring of the limpet *Crepidula convexa,* from polluted sites were significantly more copper-tolerant than those from unpolluted sites, suggesting the adaptability of *C. convexa* to locally high metal concentrations. In theory, only a few migrants per generation can maintain genetic homogeneity among (sub)populations (e.g., Dıaz-Viloria et al. 2009, Goldstien et al. 2009, Hellberg 2009), and this dispersal among (sub)populations should dilute local adaptation. However, differentiation at loci under selection can still occur with gene flow, with several studies finding populations that exhibit local adaptation despite homogeneity at neutral loci (Conover et al. 2006).

Differences in the proportion of adaptive variance partitioned at different spatial scales (within site, among sites, and among locations) between piggies and rock-attached limpets potentially implies that the impact of selection varies among life stages, whereby the adaptive genetic variants that are important for surviving within a specific site as a piggy are less important once transitioned to a rock-attached limpet. Such differential selection has been studied in an intertidal acorn barnacle, *Semibalanus balanoides*, where the authors observed differences in Mpi genotype frequencies reflecting habitat-specific patterns of differential mortality among different thermal microhabitats and between the different life stages (Schmidt & Rand 2001, and blue mussel (*Mytilus edulis*); Hilbish & Koehn 2011). In their study, whereas Mpi-FF (F, fast) homozygotes dominated among late juvenile and adult stages in exposed zones of the intertidal, this pattern was not detected in the genotypes of recently settled juveniles. This may reflect a short-term selection that only acts upon early juveniles once they have settled, impacting the genetic patterns of the adults. Likewise, selection that acts on the Kermadec giant limpets could vary in space as well as ontogenetically. The results of our adaptive variance partitioning for piggies versus those of rock-attached Kermadec giant limpet, could reflect that among site habitat differences are more important in determining the distribution of piggies, than rock-attached limpets whereby rock-attached limpets are those with genotypes matched to the location habitat and environmental regime at the piggy stage. However, as our study is only a snapshot of the Kermadec giant limpets' population structure and we did not record any characteristics of microhabitats, we cannot comment on the likely drivers of selection. Nonetheless, it would not be unusual for selection to be occurring within an intertidal zone due to extreme environmental gradients in temperature, salinity, wave action, and irradiation over small spatial scales (e.g., Schmidt & Rand, 2001, Zardi et al. 2011, Hays et al. 2021), particularly in a seascape as dramatic as Rangitāhua.

Patterns of spatial genetic structuring at the site level based on putatively adaptive loci (and neutral loci, to a lesser extent) could also be due to 'chaotic genetic patchiness' (Johnson & Black 1982). Typically, this pattern is referred to as being a result of closely related individuals (possibly as a result of sweepstakes reproduction; Hedgecock 1986, 1994, Hilbish & Koehn 2011) recruiting and settling in close proximity forming a pattern of spatial structure that is patchy (Selwyn et al. 2016, Thia et al. 2021). More recently, it has also been suggested that spatially and temporally varying selection could also contribute to the same pattern (Thia et al. 2021). In our case, even though piggies on the same host, and between hosts within the same site are not highly related (relative to the mean relatedness within locations, Fig. 3), such a pattern of chaotic genetic patchiness could still manifest at the site scale through dispersal and recruitment processes, leading sites separated by 10s of metres to differ in their genetic composition. Alternatively, or synergistically, spatially or temporally varying selection during pelagic dispersal phase, or at recruitment, could also cause sites to vary in their adaptive genetic composition. Distinguishing between neutral and selective processes contributing to this pattern, however, is difficult, given the potential for such non-equilibrium demographic processes to impact the identification of outlier loci, and the potential for neutral loci to be entrained alongside regions of the genome under selection (Smith & Haigh 1974, Barton 1998, and see for examples: Kirk & Freeland 2011; Frichot et al. 2015). Nonetheless, our finding that rock-attached limpets at some sites also shared adaptive genetic variants over a distance up to one metre (Fig. 2b), indicates that the spatial structuring of adaptive genetic variation persists through the life stages, and thus may have some bearing on the survivorship of the rock-attached life stage also.

Our estimated levels of potential inbreeding in the Kermadec giant limpet populations were high across all locations (values of >1.3; Fig. S3), typical of populations that are geographically restricted and consequently have small effective population sizes (Bijlsma et al. 2000, Severns 2003, Frankham 2005, Keever et al. 2013). Although formerly widespread (Fleming 1973), the Kermadec giant limpet is currently isolated to the small rock stacks and islands of the Rangitāhua archipelago. Thus, despite marine invertebrates typically having large effective population sizes (Crow 1986; e.g., Teixeira et al. 2011, Mokhtar-Jamal et al. 2013), and the Kermadec giant limpet having relatively high dispersal potential (i.e., broadcast spawner with feeding larvae), the population may be currently too small, and/or the range of the species too restricted to maintain sufficient outbreeding. Inbreeding can negatively affect the fitness of individuals through a reduction of genetic diversity (Verity et al. 2014, Pongratz et al. 2022), and collectively the population's genetic diversity (Crnokrak and Roff 1999). Consequentially, this can hinder the productivity (Aguirre et al. 2012) and resilience of populations to short-term stresses as well as the evolutionary and adaptive potential of the population, increasing its extinction risk

(Saccheri et al. 1998, Charlesworth & Charlesworth 1999, Frankham 2002). Globally, there are other limpet species that are also threatened or endangered (Espinosa & Rivera-Ingraham, 2017). In particular, the invertebrate at the greatest risk of extinction along the Mediterranean coast is the limpet, *Patella ferrugina* (Casu et al. 2006, Espinosa, 2009; Rivera-Ingraham et al. 2011a,b; Marra et al. 2016). Another giant limpet, *Scutellastra mexicana,* is similar to the Kermadec giant limpets in being a protandrous hermaphrodite that broadcast spawns but is now only found only at low densities due to overexploitation (Valdez-Cibrián 2021). *Scutellastra mexicana*'s low density may impact reproductive success (i.e., a density-dependent response), further exacerbated by strong currents in their habitats that are known to interfere with gamete flow even between individuals that are direct neighbours (Denny & Shibata, 1989; Grosberg, 1991). Such decline in density, as a result of local (e.g., harvesting, volcanism, earthquake, disease) or range wide impacts (e.g., climate change), is similarly of concern for the Kermadec giant limpets whose reproductive success, and ecological and evolutionary resilience, may rely on maintaining high-density populations. Furthermore, the impacts of reduced genetic variance are more severe in stressful habitats (Frankham et al. 2002, Armbruster and Reed 2005, Bell and Okamura 2005), raising the importance in conserving existing genetic diversity in the Kermadec giant limpet that may confer the basis for adaptation to environmental change.

In conclusion, although small limpets preferentially piggy-back, and this behaviour is influenced by the social environment (Kim et al. 2021, Chapter 2), our study reveals which limpet piggy-backs on who, and with whom, is not determined by relatedness. In gastropods, piggy-backing is a behaviour associated with gaining grazing opportunities, avoiding bulldozing, and while it is suggested to also ensure reproductive success, we find no evidence for a behaviour to ensure genetic complementarity between the piggy-host pair to maximise genetic diversity of the next generation for Kermadec giant limpets. Furthermore, we find that the spatial genetic structuring of Kermadec giant limpets over scales as small as 10s of metres may be determined by selection, suggesting microhabitat differences shape the genetic composition of subpopulations. Overall, our study suggests that although the Kermadec giant limpets seem successful based on their abundance and dominance in inter- and shallow sub-tidal zones of the Rangitāhua archipelago, their high level of inbreeding may leave them vulnerable to environmental pressures. Despite being protected within the Kermadec Islands Marine Reserve, the species is extremely range-restricted and therefore vulnerable to extinction caused by global climatic changes. Accordingly, the abundance and genetic composition of the Kermadec giant limpets should be monitored to preserve this range restricted endemic species and the second largest limpet species in the world.

Chapter 4: General Discussion

When Kermadec giant limpets reach a size of 55−67 mm, they shift microhabitats from shells of larger conspecifics to the rock surface. Creese et al. (1990) also showed that the timing of this ontogenetic habitat shift in Kermadec giant limpets coincides with a change in sex from predominantly piggy-backing males to predominantly rock-attached females. However, little was known about the reasons why some limpets piggy-back, what determines their transition from the backs of other limpets to the rock substrate, and the impact of these behaviours on the ecology and evolution of Kermadec giant limpets. In Chapter 2, I investigated whether the social environment (the number and size of surrounding limpets) influences the shell length at which piggy-backing limpets transition to becoming rock-attached. I hypothesized that if access to algal resources was the strongest driver of piggy-backing behaviour, the number of piggies would increase as rock availability decreased. Alternatively, if smaller limpets are piggy-backing to avoid competition, to avoid bulldozing, or to secure breeding opportunities with larger, rock-attached females, I hypothesized that the number of piggies would increase as the number and size of rock-attached limpets increased. I expected that piggies would delay the transition to being rock-attached with increasing host size, but transition to being rock-attached sooner as the number and size of piggy-backing limpets increased, reflecting a situation where limpet shell habitat becomes limiting. In Chapter 3, I investigated the small-scale neutral and adaptive genetic structure within and between Kermadec giant limpet subpopulations. I sought to understand whether the species has mechanisms to promote genetic diversity, such as kin-avoidance among piggies on the same host, forming piggy-host pairings among less related individuals, and the movement of individuals away from relatives when transitioning from piggy-backing to being rock-attached. I hypothesized that if piggy-backing behaviours of the Kermadec giant limpet were mediated by beneficial kin interactions, I might expect that piggies on the same host are closely related to each other and potentially to their host. Whereas, if the species has some ability to select for genetic complementarity in their sexual partner, I expected that piggies and hosts might be less related to each other than by chance to avoid inbreeding and/or maximise outcrossing.

In Chapter 2, I found that social environment plays an important role in the size at which Kermadec giant limpets transitioned habitats, and available rock space did not influence piggy-backing behaviour (Table S3 in the Supplement). This means that piggies willingly choose to live on the shells of conspecifics rather than the substrate (Kim et al. 2021, Chapter 2). The number of piggies increased with the number of hosts (i.e., when there were more limpet backs available) and bigger hosts had more piggies (i.e., when there

was more habitat per limpet back available). Piggies on larger hosts were larger and more densely packed, suggesting that piggies preferentially sought out these larger hosts or they persisted as piggies for longer when attached to larger hosts. Furthermore, if large hosts were absent and piggy densities were high, rock-attached limpets became hosts at a smaller size, suggesting that these ontogenetic shifts are socioecologically plastic. In Chapter 3, I aimed to uncover if limpets piggy-backing behaviour may be mediated by the relatedness of their neighbours, reflecting their preference or avoidance of kin. Here I expected to uncover a pattern in the genetic structure of subpopulations and/or differences in the mean relatedness of a host and its piggies compared with the rest of the piggy population.

My analysis of genetic relatedness and genetic spatial patterns of Kermadec giant limpets showed no evidence for beneficial kin associations within (piggy-piggy pairs) or between (host-piggy pairs) ontogenetic stages. These results do not support the hypothesis that piggy-backing behaviour could be mediated by beneficial kin interactions (i.e., kin aggregation), or aggregation of unrelated individuals (i.e., kin avoidance) to maximise fertilization and reproductive success during spawning, and/or to increase the genetic diversity of offspring. Additionally, genetic relatedness based on neutral loci, did not vary spatially, although some genetic structuring was evident for piggies among sites (separated by 10s of metres), and for rock-attached limpets among locations (separated by 100s-1000s of metres). Overall, it seemed Kermadec giant limpets were not discriminating against their hosts, piggies, or neighbours based on genetic relatedness. Importantly, this may imply that the species does not select for genetic complementarity in their sexual partner, or simply, the Kermadec giant limpets have no preference as to who their neighbours are, resulting in the spatial arrangement of these limpets to be genetically random.

As rock space availability did not influence piggy-backing behaviour, bulldozing or availability of nutritional resources on limpet backs remain potential drivers of piggy-backing behaviour. Discriminating between bulldozing and resource availability as drivers of piggy-backing behaviour based on patterns alone is difficult. For instance, uniform settlement and differential post-settlement survival on rock and limpet back habitats, post-settlement migration to limpet back habitats, and differential settlement preferences for limpet back habitats could all be driven by either bulldozing or resource availability. Snapshot surveys of the population such as those presented here cannot disentangle these two hypotheses and manipulative experiments are required. To test if the piggy-backing behaviour is mediated by resource availability, one could manipulate the amount of algae on the shells of large rock-attached limpets and then survey the abundance and distribution of piggies on hosts with different levels of algal growth. Alternatively, to test if piggy-backing behaviour is mediated by bulldozing, one could remove all but one large limpet with an

empty back and place multiple small limpets on the rock to observe whether they'd relocate onto the large limpets shell to avoid bulldozing and/or flee from the path of the large limpet as seen in Schroeder (2011), who tested territoriality and bulldozing in the owl limpet, *Lottia gigantea.*

External environmental factors (e.g., natural disasters, climate change) that affect population composition, and therefore the social environment, can influence the timing of sex change (Martins et al. 2017) and potentially ontogenetic shifts in limpets. Given that Kermadec giant limpets are protandrous and have a skewed gender ratio, such changes could be detrimental to the population-level reproductive success (Martins et al. 2017) and ultimately the species' persistence. My work has shown that changes to the recruitment dynamics of these limpets or the survival of larger, typically female limpets will trigger a cascade of interactions impacting this already vulnerable species. Although the Kermadec giant limpet is numerically and spatially dominant in the intertidal zone of Rangitāhua (Schiel et al. 1986, Creese et al. 1990, Cole et al. 1992, Wood & Gardner 2007, Kim et al. 2021), the range of the species is so restricted that an environmental shift or natural disaster could significantly increase the likelihood of extinction.

Patterns of genetic connectivity among Kermadec giant limpet subpopulations described by Wood & Gardner (2007) may be explained partly by the geographic distance among populations, but other barriers to gene flow such as the local hydrologic features, or adaptive divergence (such as described here) may have contributed. Currents in this area are not well described, however, it is likely that dispersal of shallow-water marine organisms from the Kermadec Islands to and from other locations is limited (Liggins et al. 2014, Chiswell et al. 2015). This could explain why the species is not found on the mainland currently, despite their presence (or a closely related species) on the main islands of New Zealand being indicated by the fossil record (Fleming 1973) and recent excavations by Auckland Museum in South Auckland in 2020 (Aguirre pers. comm). There are many probable reasons why these limpets, disappeared from the larger geographic regions of New Zealand (i.e., anthropogenic overexploitation, environmental stress, or natural disasters such as volcanic eruptions or earthquakes). Regardless of the reason, the current extent of genetic diversity available to support the species' future is limited to what exists on small rock stacks and islands of the Rangitāhua archipelago.

Impacts of reduced genetic variance due to inbreeding are more severe in stressful habitats (Frankham et al. 2002, Armbruster and Reed 2005, Bell and Okamura 2005). The estimated levels of inbreeding revealed that the Kermadec giant limpets are potentially highly inbred across locations, and, on average, individuals in these populations share a

relationship equivalent to that between cousins, or between great-grandparents and great-grandchildren. Therefore, in addition to changes in sex-ratio (see above), given the reduced genetic diversity. and thereby adaptive potential of Kermadec giant limpet populations, local or global climatic shifts could have severe consequences for the last remaining populations of this species.

High levels of inbreeding in geographically restricted populations are often attributed to small population numbers as a consequence of isolation (Bijlsma et al. 2000, Severns 2003, Frankham 2005, Keever et al. 2013). However, in contrast to other taxonomic groups, inbreeding is not uncommon in marine invertebrates even in well-connected populations with high local abundance (Olsen et al. 2020). The propensity for higher-than-expected levels of inbreeding in marine invertebrates has been suggested as a consequence of their life histories and patterns of dispersal. For instance, a vast number of marine invertebrate species are sessile or sedentary during their adult stages, and in both broadcast-spawning and copulating species fertilization success is often dependent on the proximity between individuals. Furthermore, marine invertebrate populations are often characterized by sweepstakes reproduction where the majority of successfully recruiting individuals are the progeny of a few successful adults. Accordingly, if dispersal is limited, and recruitment is disproportionately dominated by a subset of individuals that occur in close proximity, then inbreeding and reduced genetic diversity may be a common outcome despite high local abundance. This may be particularly evident for species endemic to small, isolated islands like Kermadec giant limpets, but the geographic signature can remain even in widely distributed species. For example, the crown-of-thorns starfish *Acanthaster planci* shows signs that the population inhabiting Rangitāhua is sustained by local retention and has low connectivity to other locations (Liggins et al. 2014).

My research on the population ecology and genetics of the Kermadec giant limpet adds to the limited knowledge that exists for this species. However, these data represent a snapshot of these subpopulations from a single point in time and lack the depth of information that can be achieved by continuous observation or experimental manipulations. In addition, the photographs of the populations used in this research were only of subpopulations in areas that were accessible. Populations on other Islands in the Rangitāhua archipelago and those with different microhabitats (i.e., exposure or volcanism) may have different dynamics, hence more examination is required. Prior to this research, along with Creese et al. (1990) and Wood & Gardner (2007), the early life history, genetic connectivity and behavioural ecology of the Kermadec giant limpet remained completely unknown, and while the species remains understudied, there are numerous avenues for future research.

Future directions

The population and reproductive biology, as well as the behaviour of Kermadec giant limpets, should be investigated to better understand how this species persists in a remote area like Rangitāhua. To do this, more data needs to be collected to address whether piggy-backing behaviour is driven primarily by avoidance of bulldozing, resource acquisition, or to maximise fertilization upon maturity regardless of relatedness. Limpets are the dominant grazers on intertidal and shallow subtidal shores on Rangitāhua (Schiel et al. 1986, Creese et al. 1990),having an immense impact on the ecology of one of the most pristine marine ecosystems on earth (Edgar et al. 2014). If Kermadec giant limpets were to diminish in numbers, algae populations may increase, triggering a cascade of indirect interactions and changes in the ecosystem (Hawkins et al. 1983, 2008, Coleman et al. 1999). By investigating the factors driving the population dynamics in range-restricted species capable of maintaining large population sizes such as the Kermadec giant limpet, I may be able to better understand the reproductive biology and ecology of these animals providing information important for their conservation, and potentially the conservation of other range-restricted endemic species. For instance, we might expect the reproductive strategies and development modes of species that inhabit small, isolated areas to be biased toward those that have mechanisms (behavioural or phenological) to favour local retention and those that have mechanisms to avoid the deleterious effects of inbreeding on individual fitness.

Studying the larval biology of this species may provide further insight into the mechanisms facilitating local retention of the Kermadec giant limpets that may further explain the genetic structuring and differentiation partitioning within and among subpopulations. Observing how long and the distance the larvae of Kermadec giant limpets can disperse, and what behaviours they use to stay in close proximity to the islands to facilitate self-recruitment may reveal more explanations on the genetic structuring and differentiation partitioning found in this study. As discussed in Chapter 3, of the diverse larval behaviours, the Kermadec giant limpet larvae could be orientating directly towards natal islets much like the larvae of anemone fish (*Amphiprion percula*) that uses olfactory cues to move toward preferred reefs (Dixson et al. 2008) or it could be the local current regimes playing a more significant role mixing the gametes and larvae across islets of the archipelago, trapping them within the boundary of the archipelago. Whether the result of self-recruitment within the Kermadec giant limpets is the result of local current patterns or behaviour, studying the properties of the gametes as well as the larvae such as their chemistry and physiology, would be worth considering. The gametes and larvae could be

tracked possibly by dyeing the gametes via injecting non-invasive biological dye directly into the reproductive organs of marked limpets and observing the paths of dyed larvae. In conjunction, the hydrological pattern could be observed by releasing the dye into the water as done in Levitan and Young (1995).

Direct sampling of gonad tissue to test for the presence of protandric sex change as done by Wright and Lindberg (1982) could be carried out, using a syringe to sample gonad tissue from live Kermadec giant limpets. As estimating the sex of piggies in Creese et al. (1990) was limited, direct sampling of gonad tissue in future may enable the timing of sex change to be related to a change in habitat and clarify the timing of sexual maturity for these limpets more precisely. This would allow testing of Creese et al.'s (1990) hypothesis that the sex ratio influences the timing of sex change in the Kermadec giant limpets. Specifically, it may be disadvantageous to produce energy-expensive eggs for young *S. kermadecensis.* At the time of sexual maturity (25 to 40 mm), these limpets restricted to the grazing opportunity of host shells may have a limited supply of energy for reproduction, and so production of sperm would be favoured. By rapidly and inexpensively developing a male gonad, young individual Kermadec giant limpets could participate in a non-trivial way in at least some spawning activity before making the probably hazardous transition from host shell to rock surface.

Finally, my research suggests addressing spatial patterns of local adaptation, and determining microhabitats or environments is critical to advancing our understanding of the potential impacts of climate change, and the spatial management of these Kermadec giant limpets subpopulations. It is crucial to understand that any changes to the recruitment dynamics of these limpets could trigger a cascade of interactions affecting this already vulnerable species. Although the Kermadec giant limpets seem successful based on their abundance and dominance in the intertidal zones of the Rangitāhua archipelago and are protected within the Kermadec Islands Marine Reserve, the high level of inbreeding may leave them vulnerable to environmental change. The Kermadec giant limpets hold great intrinsic value in their uniqueness that is endemic to New Zealand, which would be an immense loss if they were to be extinct from the effects of highly possible climactic effects. Hence, uncovering the drivers of the piggy-backing behaviour would allow us to better understand how this species has persisted despite being extremely restricted to provide insight to construct an appropriate monitoring and conservation plan for this range-restricted endemic species and the second largest limpet species in the world.

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Model no.	Model terms
Model 1: Full model including random effects	Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×RASL + SL×PSL + SL× PN + SL×R + L + S + Q + SQ
Random effects models 2a-d: Does the probability of being a pig or a host vary among: a) locations (L), b) sites nested within locations (S), c) quadrats nested within sites (Q) or d) sub-quadrats nested within quadrats (SQ)	Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×RASL + SL×PSL + SL× PN + SL×R + S + Q + SQ Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×RASL + SL×PSL + SL× PN + SL×R + L + Q + SQ Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×RASL + SL×PSL + SL× PN + SL×R + L + S + SQ Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×RASL + SL×PSL + SL× PN + SL×R + L + S + Q
Fixed effects models 3a-e: Does the relationship between shell length (SL) and the probability of being a pig or the probability of hosting vary depending on: a) availability of rock (SL×R), b) piggy number (SL×PN), c) piggy shell length (SL×PSL), d) host shell length (SL×RASL) or e) host number (SL×RAN)	Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×RASL + SL×PSL + SL× PN + *random effects* Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×RASL + SL×PSL + SL×R + *random effects* Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×RASL + SL× PN + SL×R + *random effects* Y = SL + RAN + RASL + PN + PSL + R + SL×RAN + SL×PSL + SL× PN + SL×R + *random effects* Y = SL + RAN + RASL + PN + PSL + R + SL×RASL + SL×PSL + SL× PN + SL×R + *random effects*
Best supported model for probability of being a piggy-backing limpet	Y = SL + RAN + RASL + PSL + R + SL×RASL + SL×RAN + SL×PSL + SL×R + *subquadrat*
Best supported model for probability of being a host limpet	Y = SL + RASL + PN + PSL + R + SL×RASL + SL×PN + SL×PSL + *subquadrat*

Table S1 Backward model selection procedure for sub-quadrat level analyses. We sequentially removed single terms from the full model until the simplest model that maximised the probability of the observed data was found for the probability of a piggy transitioning to a rock-attached limpet as well as the probability of a rock-attached limpet being a host or non-host. Abbreviations: SL (shell length), RAN (rock-attached number), RASL (rock-attached shell length), PN (piggy number), PSL (piggy shell length), R (availability of rock), L (location), S (site), Q (quadrat), SQ (subquadrat)

	PProb			HProb		
	df	-LL	*P*	*df*	-LL	*P*
Model 1: Full Model	36	-326.57	-	36	-186.85	-
Model 2a: Random effects model: no L	35	-326.57	1	35	-186.85	1
Model 2b: Random effects model: no S	35	-326.57	1	35	-186.85	1
Model 2c: Random effects model: no Q	35	-326.57	1	35	-186.85	1
Model 2d: Random effects model: no SQ	35	-326.57	1	35	-186.89	0.77
Model 3a: (SL×R)	12	-205.29	0.02	12	-142.84	0.76
Model 3b: (SL×PN)	12	-203.17	0.30	12	-148.97	<0.01
Model 3c: (SL×PSL)	12	-211.82	<0.01	12	-145.08	0.032
Model 3d: (SL×RASL)	12	-214.74	<0.01	12	-145.70	0.02
Model 3e: (SL×RAN)	12	-205.66	0.01	12	-142.93	0.60

Table S2. Summary statistics of backward model selection procedure for subquadrat-level analyses for the probability of a rock-attached limpet transitioning into a piggy (PProb) and probability of a rock-attached limpet hosting piggies (HProb). Abbrv.: SL (shell length), RAN (rock-attached number), RASL (rock-attached shell length), PN (piggy number), PSL (piggy shell length), R (availability of rock), L (location), S (site), Q (quadrat), SQ (subquadrat).

Fixed effects	R^2
Full model	0.377
SL	0.770
RASL	0.079
SL×RASL	0.048
SL×PSL	0.043
SL×R	0.022
SL×RAN	0.015
RAN	0.010
R	0.004
PSL	0.001

Table S3. Total and partial R^2 values for the fixed effects included in our best model (see Table S1, Table S2) Abbreviations: SL (shell length), RAN (rock-attached number), RASL (rock-attached shell length), PSL (piggy shell length), R (availability of rock).

Model no.	Model terms
Model 1: Full model including random effects	$Y = HS + L + S + Q$
Random effects models a-c: Does the piggy number/size vary among	
a) locations (L)	$Y = HS + S + Q$
b) sites nested within locaitons (S)	$Y = HS + L + Q$
c) quadrats nested witin sites (Q)	$Y = HS + L + S$
Best-supported model	$Y = HS + L$

Table S4. Backward model selection procedure for individual-level analyses. We sequentially removed single terms from the full model until the simplest model that maximised the probability of the observed data was found for the relationship between piggy number (PN) and host size (HS), and piggy size and host number. Abbreviations: HS (host size (mm), L (location), S (site), Q (quadrat).

		PN			PS	
	df	-LL	*P*	*df*	-LL	*P*
Model 1 Full Model	5	-192.50	-	6	-619.42	-
Model 2a Random effects model× no Q	4	-192.50	1.00	5	-619.48	0.72
Model 2b Random effects model× no S	4	-192.50	1.00	5	-619.42	1.00
Model 2c Random effects model× no L	4	-193.77	0.11	5	-622.09	0.021

Table S5. Summary statistics of the backward model selection procedure for the individual-level analyses. Abbreviations: piggy number (PN), piggy size (PS), L (location), S (site), Q (quadrat).

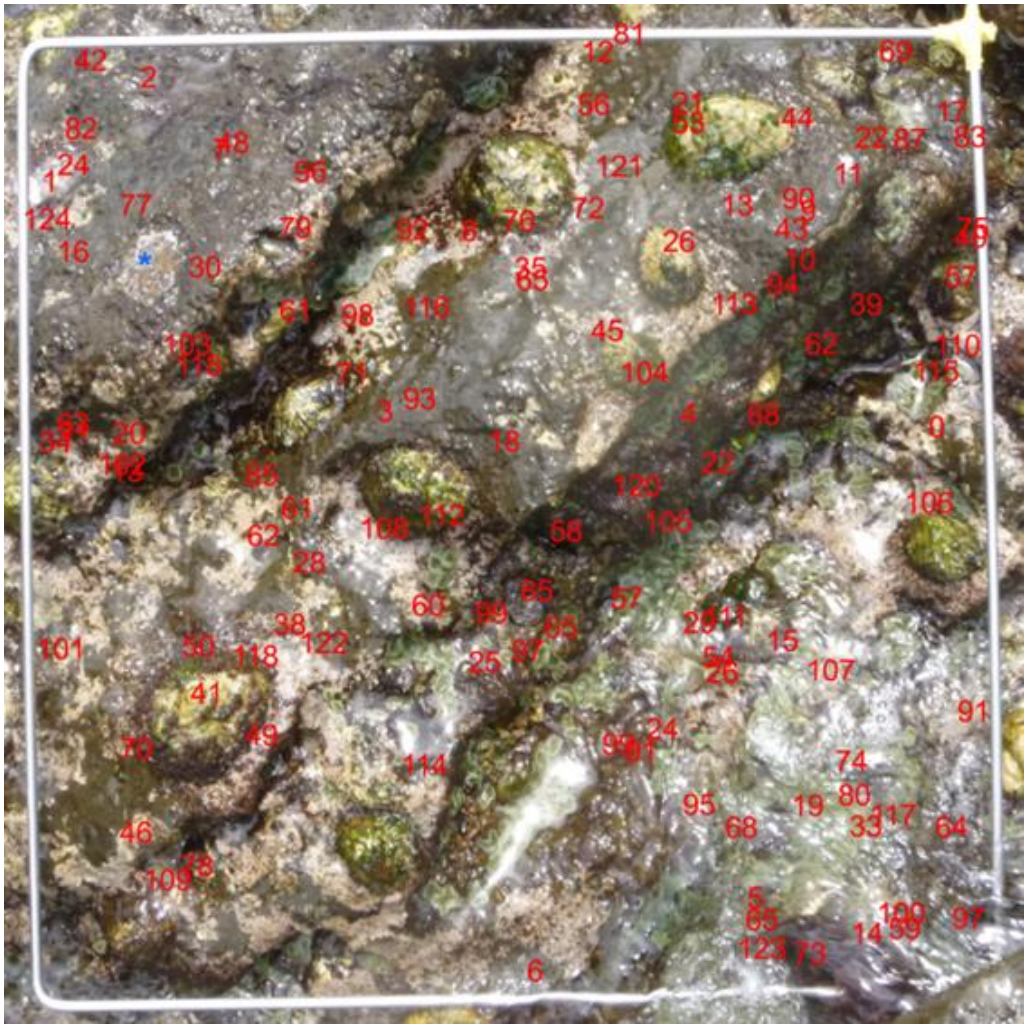

Fig. S1. Example photo of a quadrat with 125 points (red numbers) taken at Boat Cove showing a high density of limpets (the raised bumps on the rock surface) covered in epiphytic algal growth. (Photo shown at lower resolution than those used in data generation). The blue asterisk indicates a home scar for a limpet no longer attached to that site. The reasons underlying why limpets would leave their home scar are unknown, but they must have occupied the same patch for a long period to prevent the growth of algae.

	Parametre	SNPs Post-filter	Samples
START		224786	189
Min/max meanReadDepths	8/350	30850	189
Minor allele count	6	14295	189
max % missing	0.3	7346	189
blacklist individuals removed		7346	167
LD Prune	1snp/locus	4489	167
Neutral loci of individuals after initial filtering		4332	167
Adaptive loci of individuals after initial filtering		154	165
Adaptive loci of rock-attached		154	105
Adaptive loci of piggies		154	60
Adaptive dataset of rock-attached after removing loci of > 70% missing data and individuals with > 40 % missing data		154	59
Adaptive dataset of piggies after removing loci of > 70% missing data and individuals with > 40 % missing data		154	33

Table S6 Summarized filtering of dataset of 224,786 variant sites using VCFTools from initial analysis

Genetic dataset	Piggy/rock-attached	Components of covariance	Variance %	P-value
G^5 inverse (Mean pairwise relatedness)	Piggy	Within sites	98.35	0.01
		Among sites	0.77	0.01
		Among locations	0.88	0.01
	Rock-attached	Within sites	97.16	0.01
		Among sites	0.65	0.03
		Among locations	2.19	0.01
Prevosti's distance - Neutral	Piggy	Within sites	99.48	0.02
		Among sites	0.95	0.01
		Among locations	- 0.43	0.86
	Rock-attached	Within sites	99.68	0.02
		Among sites	0.37	0.01
		Among locations	- 0.05	0.87
Prevosti's distance - Adaptive	Piggy	Within sites	48.32	0.01
		Among sites	14.70	0.02
		Among locations	36.98	0.02
	Rock-attached	Within sites	35.53	0.01
		Among sites	3.09	0.01
		Among locations	61.39	0.01

Table S7 Results of AMOVA analyses. Proportion of variance explained for each type of genetic dataset by within and among sites, and among locations for piggies and rock-attached limpets.

Relationship	Location	Relationship	emmean	SE	df	lower.CL	upper.CL
Between Piggies and its host or a random rock-attached in same site.	Boat Cove	Piggy – Host	0.08	0.01	22.6	0.06	0.10
		Piggy - Random	0.08	0.01	22.6	0.06	0.10
	North Meyer	Piggy – Host	0.10	0.03	27.9	0.04	0.16
		Piggy - Random	0.12	0.03	27.9	0.06	0.18
	South Meyer	Piggy – Host	0.10	0.01	21.7	0.08	0.13
		Piggy - Random	0.11	0.01	21.7	0.08	0.13
Between piggies on same or on different hosts	Boat Cove	BHP	0.07	0.01	10.80	0.04	0.10
		WHP	0.09	0.01	6.17	0.07	0.11
	South Meyer	BHP	0.12	0.02	10.80	0.08	0.16
		WHP	0.09	0.01	6.17	0.07	0.12

Table S8 Relatedness of piggies and rock-attached limpets Between a piggy and its host, or a random rock-attached limpet in the same site. – G^5 comparisons

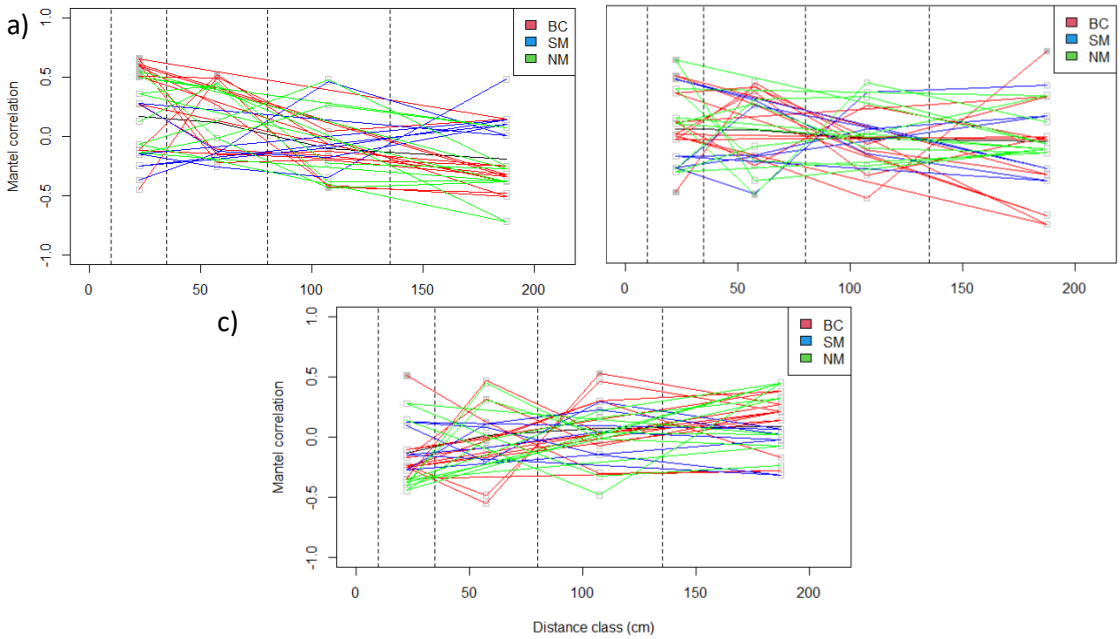

Fig S2 Correlogram of spatial autocorrelation on the mean relatedness of Prevosti's distance based on putatively (a) adaptive loci, (b) neutral loci (c) pairwise individual genetic distance matrices calculated from the unpruned dataset of rock-attached limpets at each of the sampled distances from the focal limpet (Fig. 1d).

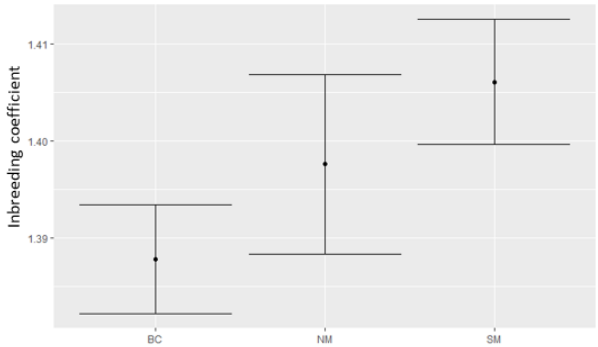

Fig S3 Mean relatedness of individuals of each location Boat Cove (BC), North Meyer Island (NM) and South Meyer Island