Genetic studies for sustainable aquaculture of the sea urchin, *Tripneustes gratilla*

by

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Declaration

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Summary

The sea urchin, *Tripneustes gratilla*, has been identified as a species with potential for aquaculture production in South Africa, as these animals are distributed along the eastern coast, produce bright orange roe and have a fast growth rate. This study aimed to assess various aspects of *T. gratilla* that could contribute to successful future aquaculture practices, through population genetics, pedigree reconstruction, qualitative assessments, quantitative genetics and metagenomic approaches. Chapter 2 evaluated the genetic diversity and population structure of *T. gratilla* populations along the South African coast, through the application of 22 species-specific microsatellite markers. Geographically representative sampled populations formed a single, interbreeding population, with a moderate degree of genetic diversity. In chapter 3, the markers were applied in two *T. gratilla* cultured cohorts to assess parental contributions, as well as changes in genetic diversity from the progenitor natural population. In these cohorts, the parental skew often associated with broadcast spawning animals was observed, where a single female and male dominated the respective spawning events. This resulted in a decline in genetic diversity, which could have implications for the genetic management of future commercial production. These results suggested that other factors, such as diet, breeding design, gonad and gamete quality could affect reproductive success. Consequently, chapter 4 aimed to assess biological and genetic aspects in *T. gratilla* that could influence reproductive competition, larval growth and juvenile performance. Results illustrated that a factorial breeding design is an effective approach for retaining genetic diversity in cultured populations. Broodstock conditioned on a mixed feeding regime outperformed animals fed the other diets included in this study (formulated feed, *Ecklonia maxima* and *Ulva rigida*). These animals could have had a higher ingestion efficiency, may have been exposed to a broader array of nutrients, displayed improved maternal provisioning or had an improved digestibility promoted by the bacteria introduced through natural feeds. The bacterial communities associated with sea urchin systems play an important role in animal health. In the studied aquaculture environment, bald sea urchin disease has been observed. Chapter 5 explored this disease using a 16S rDNA metagenomics approach, where samples included healthy animals from natural locations along the eastern coast of South Africa, as well as different cultured cohorts: healthy-, diseased- and stressed animals. Results showed that this disease is more likely caused by complex interactions between opportunistic bacteria, rather than by a specific pathogenic agent. Overall, this study showed that the preservation of genetic diversity in cultured *T. gratilla* populations is possible through factorial breeding designs and broodstock conditioning, where precautionary measures and effective animal husbandry practices can contribute to the prevention of diseases associated with opportunistic bacteria. Therefore, an integrated approach should be implemented to maintain genetic diversity, promote reproductive success and manage disease outbreaks in this emerging echinoculture industry.

Opsomming

Die seepampoentjie, *Tripneustes gratilla*, is geïdentifiseer as ‘n spesies met die potensiaal vir akwakultuur produksie in Suid-Afrika, aangesien hierdie diere langs die ooskus voorkom, helder oranje gamete produseer met ‘n kenmerkende vinnige groeitempo tot volwassenheid. Die doel van hierdie studie was om verskeie aspekte van *T. gratilla*, wat kan bydra tot suksesvolle toekomstige akwakultuurprkatyke, te ondersoek. Die navorsing sluit populasiegenetika, heropbou van stambome, kwalitiewe assesserings, kwantitiewe genetika en metagenomiese benaderings in. Hoofstuk 2 evalueer die genetiese diversiteit en genetiese struktuur van *T. gratilla* populasies langs die Suid Afrikaanse kus met behulp van 22 spesie spesifieke mikrosatelliet merkers. Geografies verteenwoordige steekproefpopulasies het ‘n enkele, telende populasie gevorm, met ‘n matige vlak van genetiese diversiteit. In Hoofstuk 3 is die merkers gebruik in twee *T. gratilla* nageslagkohorte om die ouerlike bydra te ondersoek, sowel as om die veranderinge in genetiese diversiteit van die akwakultuur populasie te evalueer. In hierdie nageslagkohorte was die skewe verspreiding van ouerlike bydra waargeneem. Dit word dikwels met hierdie diere geassosieer, waar ‘n enkele wyfie en mannetjie die onderskeie broeigeleenthede oorheers. In hierdie studie het dit gelei tot ‘n afname in genetiese diversiteit, wat direkte gevolge kan hê vir die genetiese bestuur van toekomstige kommersiële produksie. Resultate dui daarop dat ander faktore, insluitend dieët, die teelontwerp, gonade- en gameet kwaliteit, die voortplantingsukses verder kan beïnvloed. Gevolglik was die doel van hoofstuk 4 om biologiese en genetiese aspekte in *T. gratilla* te beoordeel wat betref voorplantingskompetisie, larwegroei en jong seepampoentjie prestasie. Die resultate illustreer dat ‘n faktoriale teelontwerp ‘n effektiewe benadering is om genetiese diversiteit in gekweekte populasies te behou. Teeldiere wat gekondisioneer is met ‘n mengvoedingsregime het beter presteer as diere wat aan die ander diëte blootgestel was (geformuleerde dieet, *Ecklonia maxima* en *Ulva rigida*). Hierdie diere, blootgestel aan ‘n wyer verskeidenheid voedingsstowwe, het waarskynlik ‘n hoër voedsel innamedoeltreffenheid en verteerbaarheid gehad en ook waarskynlik verbeterde moedersvoorsiening vertoon as gevolg van bakterieë wat met die natuurlike voere geassosieer word. Die bakteriese gemeenskappe in noue assosiasie met seepampoentjies speel dus ‘n belangrike rol in dieregesondheid. In die bestudeerde akwakultuuromgewing is “bald sea urchin disease” waargeneem. Hoofstuk 5 het hierdie siekte verder ondersoek, deur gebruik te maak van ‘n 16S rDNA metagenomiese benadering waar monsters van gesonde diere in natuurlike populasies langs die ooskus van Suid-Afrika ingesluit is, sowel as verskillende akwakultuur kohorte: gesonde-, siek- en gestresde diere. Resultate wys daarop dat hierdie siekte meer waarskynlik veroorsaak word deur komplekse interaksies tussen opportunistiese bakterieë, eerder as deur ‘n spesifieke patogene agent. Dit is dus noodsaaklik dat ‘n geïntegreerde benadering geïmplementeer word om genetiese diversiteit te handhaaf, reproduksiesukses te bevorder en siekte-uitbrake in die opkomende echinokultuurbedryf te bestuur.

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Preface

This dissertation is presented as a compilation of six chapters. Below are the scientific contributions directly emanating from the work presented in this dissertation (2017 – 2020):

1. Published papers:

Brink M., Dale Kuys R., Rhode C., Macey B.M., Christison K.W., Roodt-Wilding R., 2018. Genetic diversity and population connectivity of the sea urchin, *Tripneustes gratilla*. *African Journal of Marine Science* 40: 149 – 156 [Chapter 2].

Brink M., Rhode C., Macey B.M., Christison K.W., Roodt-Wilding R. 2019. Metagenomic assessment of body surface bacterial communities of the sea urchin, *Tripneustes gratilla*. *Marine Genomics* 47: 100675 [Chapter 5].

2. Papers in preparation:

Brink M., Cyrus M.D., Macey B.M., Rhode C., Hull K.L., Roodt-Wilding R., in prep. The effects of various diets on the reproductive performance of *Tripneustes gratilla* broodstock. Target journal: *Aquaculture* [Chapter 4].

3. Conference contributions:

Brink M., Dale Kuys R., Rhode C., Macey B.M., Christison K.W., Roodt-Wilding R., 2017. Genetic diversity in the sea urchin, *Tripneustes gratilla*. Poster presented at: World Aquaculture Society, June 26 – 30, Cape Town, South Africa.

Brink M., Rhode C., Macey B.M., Christison K.W., Roodt-Wilding R., 2018. Metagenomic assessment of the body surface microbial communities of the sea urchin, *Tripneustes gratilla*. International Symposium for Genetics in Aquaculture, 15 – 20 July, Cairns, Australia.

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List of Abbreviations

%	Percentage
°C	Degrees Celsius
~	Approximately
≈	Approximately equal
<	Less than
>	More than
∞	Infinity
±	Plus-minus
β	Beta
Δt	Number of days between two points in time
µL	Microlitres
µg	Micrograms
µg/mL	Micrograms per millilitre
µM	Micromolar
µm	Micrometre
σ^2_a	Additive genetic variance
σ^2_e	Error variance
σ^2_d	Dam variance component
σ^2_s	Sire variance component
20U	Formulated feed containing 20% *Ulva*
AFLP	Amplified fragment length polymorphism
A_e	Effective number of alleles
A_n	Number of alleles
A_r	Allelic richness
a*	Redness index
AI-REML	Average information restricted maximum likelihood
AMOVA	Analysis of molecular variance
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
b*	Yellowness index
BLUP	Best linear unbiased prediction
bp	Base pair
BSA	Bovine serum albumin
C	Carbon
CAF	Central Analytical Facility
CFB	Cytophaga-Flexibacter-Bacteroides
CI	Confidence interval
cm	Centimetres
CO1	*Cytochrome oxidase subunit 1*

CO_2	Carbon dioxide
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of variation
DAAD	Deutscher Akademischer Austauschdienst fund
DAFF	Republic of South Africa Department of Agriculture, Forestry and Fisheries
DEFF	Republic of South Africa Department of Agriculture, Environment and Fisheries
df	Degrees of freedom
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	East
EBR1	Egg bindin receptor protein 1
EBV	Estimated breeding value
ECC	Haga Haga, Eastern Cape of South Africa
ECD	Coffee Bay, Eastern Cape of South Africa
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia* (for example)
EMBL-ENA	European Molecular Biology Laboratory - European Nucleotide Archive
et al.	*et alii* (and others)
EtBr	Ethidium bromide
E-value	Expected hits of similar quality
F	Fixation index
FAM	Blue (R100); 5-carboyfluirescein (ABI-fluorescent label)
FAMEs	Fatty acid methyl esters
FDR	False discovery rate
F_{is}	Inbreeding coefficient
FISH	Fluorescent *in situ* hybridisation
$Fr_{(Null)}$	Null allele frequency
F-value	Statistic representing the ratio of variances in ANOVA
F_{st}	Wright's fixation index (subpopulation relative to the total population)
F1	First generation
F2	Second generation
g	Grams
GC-FID	Gas chromatography flame ionisation detector
GC-MS	Gas chromatography mass spectrometry
gDNA	Genomic deoxyribonucleic acid
GOLD	Genomes OnLine Database
GSI	Gonad somatic index
h	Hour
h^2	Heritability (narrow-sense)
h^2_d	Heritability estimate based on dam covariance components

h^2_s	Heritability estimates based on sire covariance components
H_e	Expected heterozygosity
H_o	Observed heterozygosity
HSD	Honestly significant difference
HUFAs	Highly unsaturated fatty acids
HWE	Hardy-Weinberg equilibrium
I	Shannon's information index
IAM	Infinite allele model
i.e.	*id est* (that is to say)
IBD	Isolation by distance
ID	Identification
K	Number of genetic clusters
kb	Kilobase
KD	Dam fed kelp diet
KS	Sire fed kelp diet
km	Kilometre
KZNA	Hibberdene, KwaZulu-Natal
KZNB	Ballito Bay, KwaZulu-Natal
KZNE	Sodwana Bay, KwaZulu-Natal
L	Litre
L/h	Litres per hour
L*	Lightness index
L x W x H	Length by width by height (dimensions)
LD	Linkage disequilibrium
LDA	Linear discriminant analysis
LefSe	Linear discriminant analysis effect size
Log2FC	Log fold change
M	Molar (moles per litre)
m	Metres
M_1	Measurement at initial time point
M_2	Measurement at final time point
MD	Dam fed a mixed diet
$MgCl_2$	Magnesium chloride
mg/mL	Milligrams per millilitre
mg/µL	Milligrams per microlitre
MHC	Major histocompatibility complex
MJ	Megajoules
mL	Millilitres
mm	Millimetres
mM	Millimolars
MP	Multiplex
MS	Sire fed a mixed diet

MYP	Major yolk protein
N	North
n	Sample number
N_e	Effective population size
N_2	Nitrogen
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
n.d.	Not determined
NED	Yellow (Tamra) (ABI-fluorescent label)
NIS-BR	Nikon imaging systems elements basic research package
ng	Nanograms
NGS	Next-generation sequencing
ng/µL	Nanogram per microlitre
NMDS	Non-metric multidimensional scaling
NRF	National Research Foundation
n.s.	Not significant
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCoA	Principal co-ordinates analysis
PCR	Polymerase chain reaction
PE	Probability of exclusion
PERMANOVA	Permutational multivariate analysis of variance
PERMDISP	Permutational analysis of multivariate dispersions
PET	Red (ABI-fluorescent label)
PI	Probability of inclusion
PIC	Polymorphic information content
pM	Picomolar
ppm	Parts per mole
ppt	Parts per ton
PUFAs	Polyunsaturated fatty acids
P-value	Probability value (as a statistically significant threshold)
PVC	Polyvinyl chloride
qPCR	Quantitative polymerase chain reaction
Q-Q	quantile-quantile
Q20	Phred quality score of 20
R^2	Goodness-of-fit measure for linear regression models
r	Relatedness
r	Pearson correlation coefficient
r^2	Correlation coefficient
R^2	Coefficient of determination
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid

RFLP	Restriction fragment length polymorphism
RLE	Relative log expression
RNA	Ribonucleic acid
s	Seconds
S	South
SD	Standard deviation
SE	Standard error
SEM	Scanning electron microscopy
SGR	Specific growth rate
SMM	Stepwise mutation model
SNP	Single nucleotide polymorphism
spp.	Several species
SSR	Simple sequence repeats
T_a	Annealing temperature
Taq	*Thermus aquaticus* DNA polymerase
TBME	Tertiary butyl methyl ether
TCA	Tricarboxylic acid cycle
TMSH	Trimethylsulfonium hydroxide
TPM	Two-phase model
Tris-HCl	Tris (hydroxymethyl) aminomethane (THAM) hydrochloride
uH_e	Unbiased expected heterozygosity
U	Units (enzyme)
US$	United States of America dollar
UV	Ultraviolet
V	Volume
VIC	Green (ABI-fluorescent label)
VNTRs	Variable number tandem repeats
v/v	Volume per volume
W_g	Gonad wet weight
W_t	Total animal weight
w/w	Weight per weight
ZAR	South African rand

Chapter 1
Literature review

1.1. *Tripneustes gratilla*: an overview

1.1.1. Classification

Tripneustes gratilla (Linnaeus, 1758), also referred to as the collector sea urchin, forms part of the phylum Echinodermata (Toha *et al.* 2014). Fossil records show that echinoids evolved approximately 500 million years ago (Kier 1965), and that these fossil species shared morphological similarities, but had different movement and food gathering capabilities (Kier 1965; Bambach 1985). Echinoids underwent a rapid decline in population numbers during the Permo-Triassic extinction event during the Paleozoic and Mesozoic eras, resulting in evolutionary radiation from a single stem group, *Miocidaris* (Erwin 1994; Smith *et al.* 1995; Kroh and Smith 2010; Steneck 2013). Within echinoids, approximately 70 families and 850 species, as well as approximately 10 000 fossil species have been described. These animals are classified by distinct morphological characteristics, such as spine shape and size, projections (tubercles) on the exoskeleton, mouth and tooth structure, the presence or absence of five segments of the exoskeleton (ambulacral plates), as well as the structure of these segments (Littlewood and Smith 1995; Kroh and Smith 2010). However, only 19 genera, including *Tripneustes*, are considered edible as they are palatable and are considered large enough (at a minimum test diameter of 40 – 50 mm) for commercial purposes (Smith 1984; Kelly 2005; Kroh and Smith 2010).

Tripneustes gratilla, which belongs to the class Echinoidea, are characterised by symmetric, round bodies. This class can be further classified into the superorder Camarodonta and order Temnopleuroidia, which includes four families; Echinidae, Echinometridae, Strongylocentridae and Toxopneustidae (Smith 1984). Within Toxopneustidae, the genus *Tripneustes* forms one of only four genera (Figure 1.1). This genus encompasses three extant species: *Tripneustes gratilla*, found in the Indo-Pacific Ocean, *T. ventricosus* (Lamarck, 1816), found in the western Atlantic Ocean, and *T. depressus* (Agassiz, 1863), found in the eastern Pacific Ocean (Mortensen 1943; Lawrence and Agatsuma 2013). Although these morphologically similar species have non-overlapping distributions, previous studies have suggested that they constitute a single species (Lawrence and Agatsuma 2013), as limited genetic differences, based on mitochondrial *cytochrome oxidase 1* (*CO1*)

data, were detected (Zigler and Lessios 2003). Furthermore, a comparison of these species based on a nuclear gene encoding a sperm-egg attachment protein, bindin, found Indo-Pacific *T. gratilla* to share a high degree of similarity with Pacific *T. depressus* (Zigler and Lessios 2003). However, the Caribbean *T. ventricosus* and eastern Pacific *T. depressus* were distinct (Zigler and Lessios 2003). More recent studies, also using *CO1* and bindin, have suggested that the phylogeny of this genus is still poorly understood (Bronstein *et al.* 2017). The authors proposed that the species previously identified as *T. gratilla* around the Kermadec islands in the South Pacific Ocean represents a fourth *Tripneustes* species, *T. kermadecensis*, as these animals were genetically isolated in this peripheral location (peripatric speciation) (Bronstein *et al.* 2017). It has also been hypothesised that *T. kermadecensis* represents the earliest split in the genus (Bronstein *et al*. 2017). Furthermore, within *T. gratilla*, it has been suggested that cryptic species exist, such as *T. gratilla elantensis* found in the Red Sea (Bronstein *et al.* 2016). Therefore, based on mitochondrial markers (*CO1* and control region) and nuclear markers (microsatellites), as well as morphological similarities, these studies suggested that *T. gratilla* be referred to as a “species complex”, rather than a single species (Bronstein *et al.* 2016, 2017).

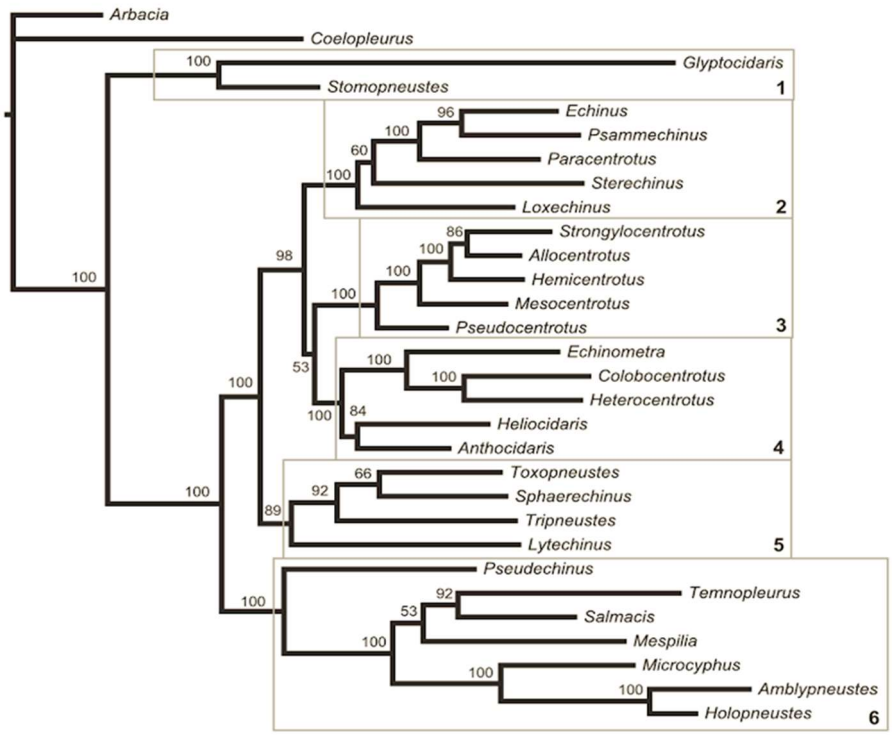


Figure 1.1. Sea urchin phylogeny of 30 genera based on Bayesian analysis of morphological and molecular data, where clades represent the families (1) Stomopneustoidae (2) Echinidae, (3) Strongylocentridae, (4) Echinometridae, (5) Toxopneustidae and (6) Temnopleuridae; numbers represent posterior probabilities for each clade (In: Smith and Kroh 2013).

1.1.2. Distribution and ecology

Tripneustes gratilla are found on both deep and shallow ocean floors in tropical and subtropical regions (Lawrence and Agatsuma 2013). Globally, this species' distribution is limited by the deep stretches separating the eastern and western Atlantic oceans, the Isthmus of Panama, the upwelling at the tip of South Africa, caused by the warm Agulhas and cold Benguela currents, as well as the freshwater outflow from the Orinoco and Amazon rivers (Lessios *et al.* 2003). The collector sea urchin has been reported from South Japan to the Indo-West Pacific, the Torres Strait, the Arafura Sea, the northern coast of Australia, the Gulf of Suez, and the West Indian and Pakistani coasts (Figure 1.2; Mortensen 1943; Clark 1946; James and Pearse 1969; Clark and Rowe 1971). Along the eastern coast of South Africa, *T. gratilla* is distributed northwards from the most southern location, Haga Haga (Marshall *et al.* 1991; Cyrus 2013), where abundance is dependent on environmental disturbances, such as ocean swells and storms, as well as food availability (Steneck 2013). Sea urchins are most abundant approximately 2 – 15 m offshore (Chaot and Schiel 1982; Johnson *et al.* 2005), although they can also be found as deep as 75 m (Mortensen 1943).

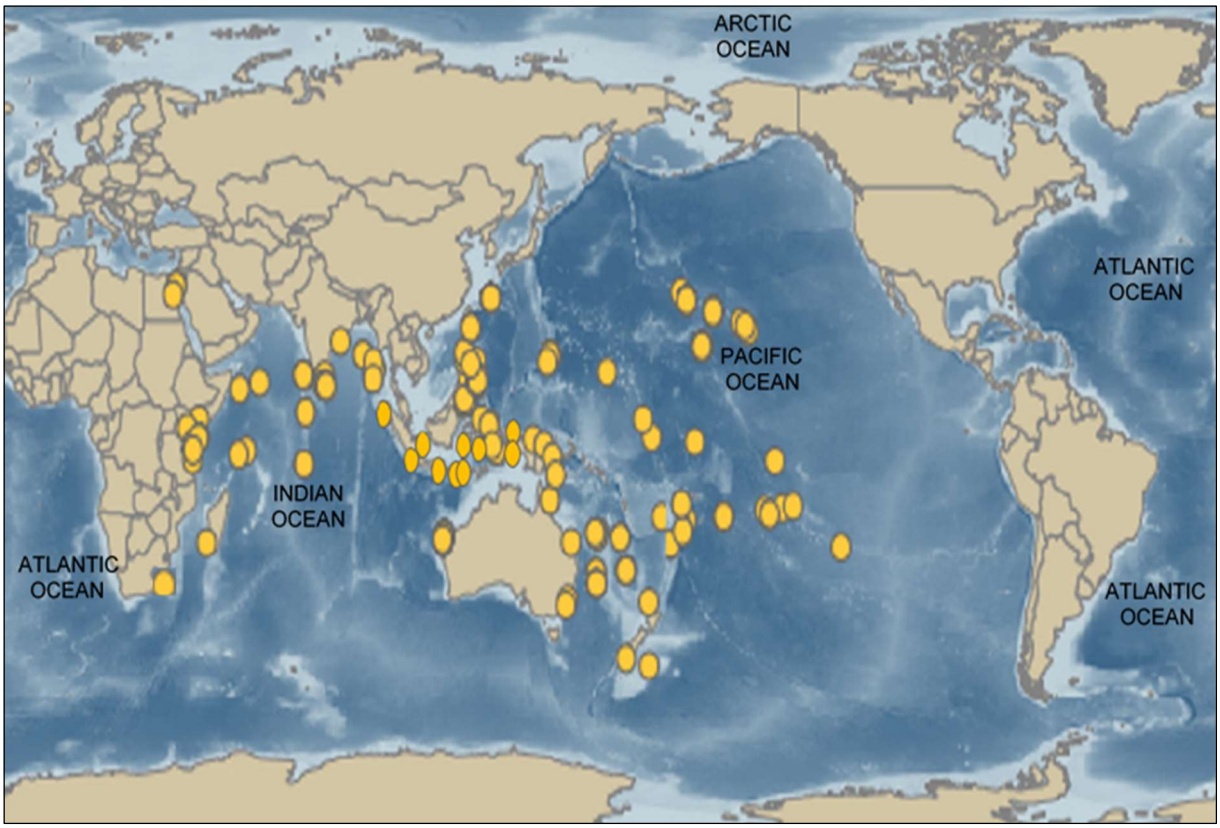


Figure 1.2. Distribution of the sea urchin *Tripneustes gratilla* in tropical and subtropical regions (Adapted from Toha *et al*. 2017).

Sea urchin distribution and ecology is largely dependent on intra- and interspecific competition for resources (Shimabukuro 1991), as was observed when the lagoon sea urchin, *T. ventricosus*, inhabited a reef previously occupied by the long-spined sea urchin, *Diadema antillarum*, after a mass mortality event (Levitan 1988; Moses and Bonem 2001; Trussell *et al.* 2003). Furthermore, sea urchin distribution and abundance is generally also reliant on shelter availability (Hereu 2004), as this is likely to affect their susceptibility to predators (Carpenter 1997; Steneck 2013), such as crabs (Siddon and Witman 2004), rock lobsters (Barker 2007), predatory fish (Vadas and Steneck 1995) and sea otters (Paine 1980; Estes and Duggins 1995). This predation results in a well-documented 'trophic cascade', as the subsequent reduction in sea urchin abundance results in an increase in macroalgae and seagrasses (Estes *et al.* 2010; Steneck 2013), as well as other herbivorous species (Hay and Taylor 1985; Carpenter 1986; Robertson 1991; Steneck 2013). Sea urchins predominantly feeding off seagrasses and macroalgae makes these grazing animals integral to nutrient cycling within marine ecosystems, as well as in protecting native species' abundance and diversity, as they control invasive species (Casilagan *et al.* 2013; Thomsen *et al.* 2013). Sea urchins have been reported to control kelp forests, seagrass beds and coral reef ecosystems and thus play a role in shallow coastal food webs, both as a consumer and as a prey (Steneck 2013).

1.1.3. Biology, reproduction and life history

Tripneustes gratilla are configured pentaradially symmetrically in a round body (Figure 1.3), similar to other sea urchin species. Their internal organs are enclosed in a calcified shell, also known as a test, cast or exoskeleton (Figure 1.3; Toha *et al.* 2017). A maximum exoskeleton diameter of 160 mm has been reported for *T. gratilla* (Rahman *et al.* 2014), but generally, diameter sizes of 77 – 100 mm are observed in nature (Toha *et al.* 2017). This spherical structure has two surfaces, the aboral and oral surfaces, and consists of an internal calcium carbonate mesodermal skeleton, which is covered by a dermal and epidermal layer (Clark and Rowe 1971). The exoskeleton is further separated into fused plates, named ambulacral and interambulacral plates, where tube feet occur mostly on the ambulacral areas and the spines protrude from tubercles on the interambulacral areas (Figure 1.3; Barnes *et al.* 2001). The tube feet, also referred to as globiferous pedicellariae, are used by *T. gratilla* for movement and light detection, initiating the covering behaviour that the collector sea urchin displays (Alender 1964; Ziegenhorn 2016). This covering behaviour, where seagrasses and algae attach to the spines, also acts as a camoflauge strategy

against predators (Agatsuma 2001), a protective mechanism when the urchin has been injured (Ziegenhorn 2016), or to increase the overall weight of the sea urchin to lessen the effect of strong currents (Park and Cruz 1994). In conjunction with protection, spines also play a role in catching drifting food sources, such as macroalgae and seagrasses to subsequently be consumed *via* the mouth, also known as Aristotle's lantern (Barnes *et al.* 2001). This feeding apparatus functions through five teeth-like structures that protrude from the peristome (Figure 1.3; Barnes *et al.* 2001).

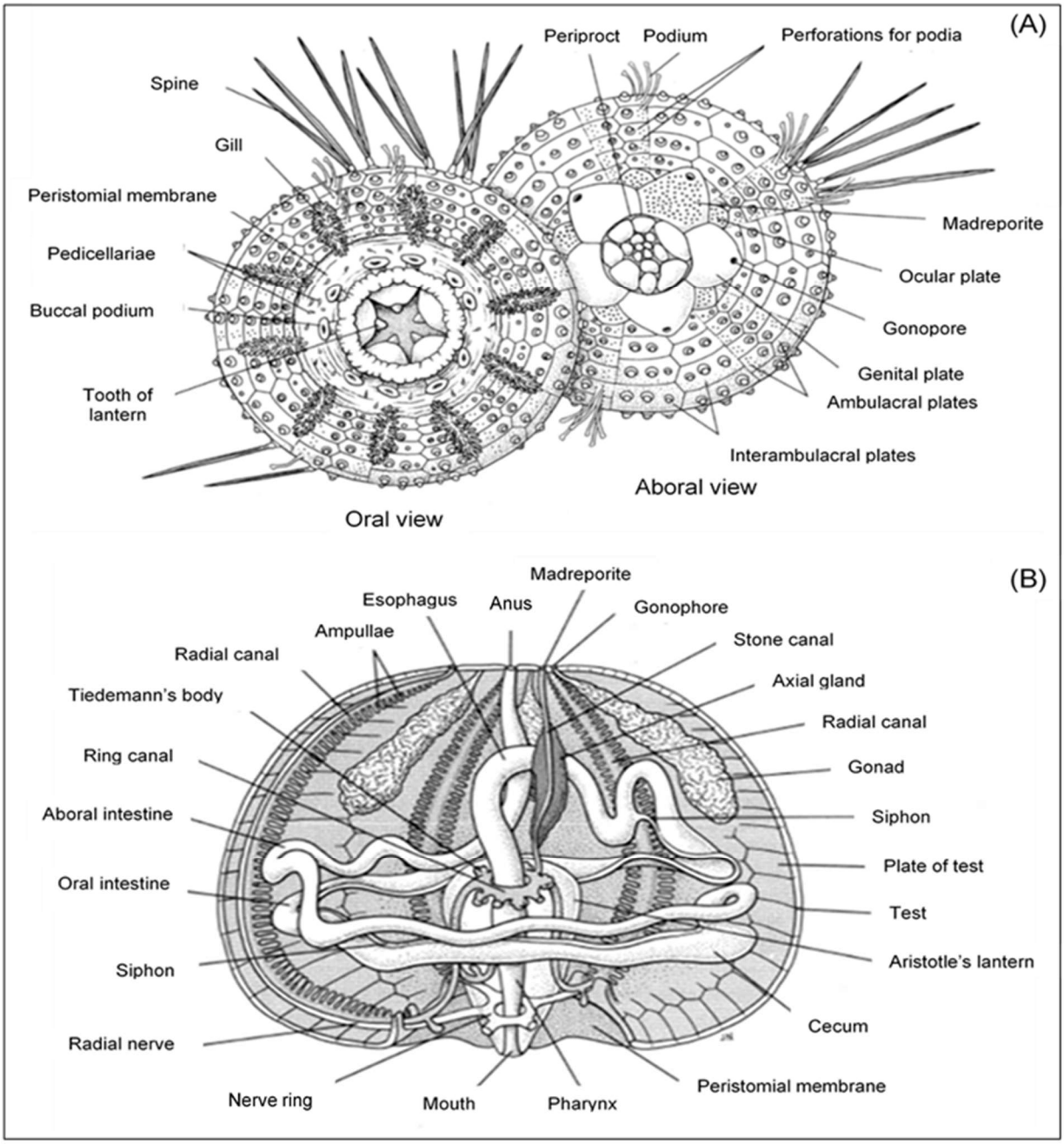


Figure 1.3. Sea urchin external (A) and internal (B) anatomy, where internal organs are enclosed in a hard, calcified shell (Adapted from Ruppert and Barnes 1994).

These animals have a complex open water vascular system, which consists of a perivisceral coelomic system, a water vascular system, a perihemal system and hemal system (Smith 1981). Water enters though the madreporite on the aboral surface and is circulated through the internal canals, to finally be transported to the ampullae and tube feet (Figure 1.3; Pinsino and Matranga 2015). The coelomic fluid, facilitating the translocation, excretion and locomotion of substances, acts as a protection and survival mechanism, as it plays a role in humoral (macromolecule-mediated) immunity and protects internal organs (Chia and Xing 1996).

The economically valuable internal component of sea urchins, the gonads or roe, are enclosed in the exoskeleton and consist of germ cells and somatic nutritive phagocytes that store nutrients (proteins, lipids and carbohydrates) that are required for gametogenesis (Walker 1982; Lawrence and Agatsuma 2013). Germ cells consist of spermatocytes, spermatogonia, spermatozoa and spermatids in males and ovum, oogonia and oocytes in females (Yokota *et al.* 2000). These reproductive structures occupy approximately 25% of the internal cavity, in both males and females, and develop in *T. gratilla* when the exoskeleton diameter reaches roughly 50 mm (Cyrus *et al.* 2014). The reproductive cycle of *T. gratilla* and other sea urchin species is characterised by six gonad maturity stages, namely (1) recovery, (2) growing, (3) premature, (4) mature, (5) partly spawned and (6) spent that are dependent on the interplay between nutritive phagocytes and germinal tissues, as well as female oocyte size and male spermatocyte layer thickness (Byrne 1990; Vaïtilingon *et al.* 2005; Cyrus 2013).

Briefly, as described by Vaïtilingon *et al*. (2005) and Cyrus (2013), the recovery stage is characterised by spermatogonia clusters and primary spermatogonia lining acinal walls in males, where the follicle wall appears contracted (Figure 1.4A). Following this, the growing phase is identified by the presence of rapidly developing spermatogonia and primary spermatocytes forming a layer (thickness of 10 – 30 µm) along the acinal lining of the testes in males, with an abundance of nutritive phagocytes in the lumen (Figure 1.4B). The reduction of these nutritive phagocytes, coupled with an increase in spermatogenesis, is indicative of gonads in a premature stage (Figure 1.4C). This stage is also characterised by spermatozoa detaching from the acinal walls and accumulating in the lumen. Once the lumen is densely packed with spermatozoa and the amount of nutritive phagocytes is largely reduced, gonads are classified as mature (Figure 1.4D). In the partly spawned stage, spermatozoa are less dense in the lumen and vacated areas can be observed along the lumen margin, indicating a partial release of spermatozoa (Figure 1.4E). In contrast, if testes

are in a spent stage, a large reduction in spermatozoa, with corresponding empty spaces will be observed (Figure 1.4F). In this stage, remaining spermatozoa are reabsorbed by nutritive phagocytes.

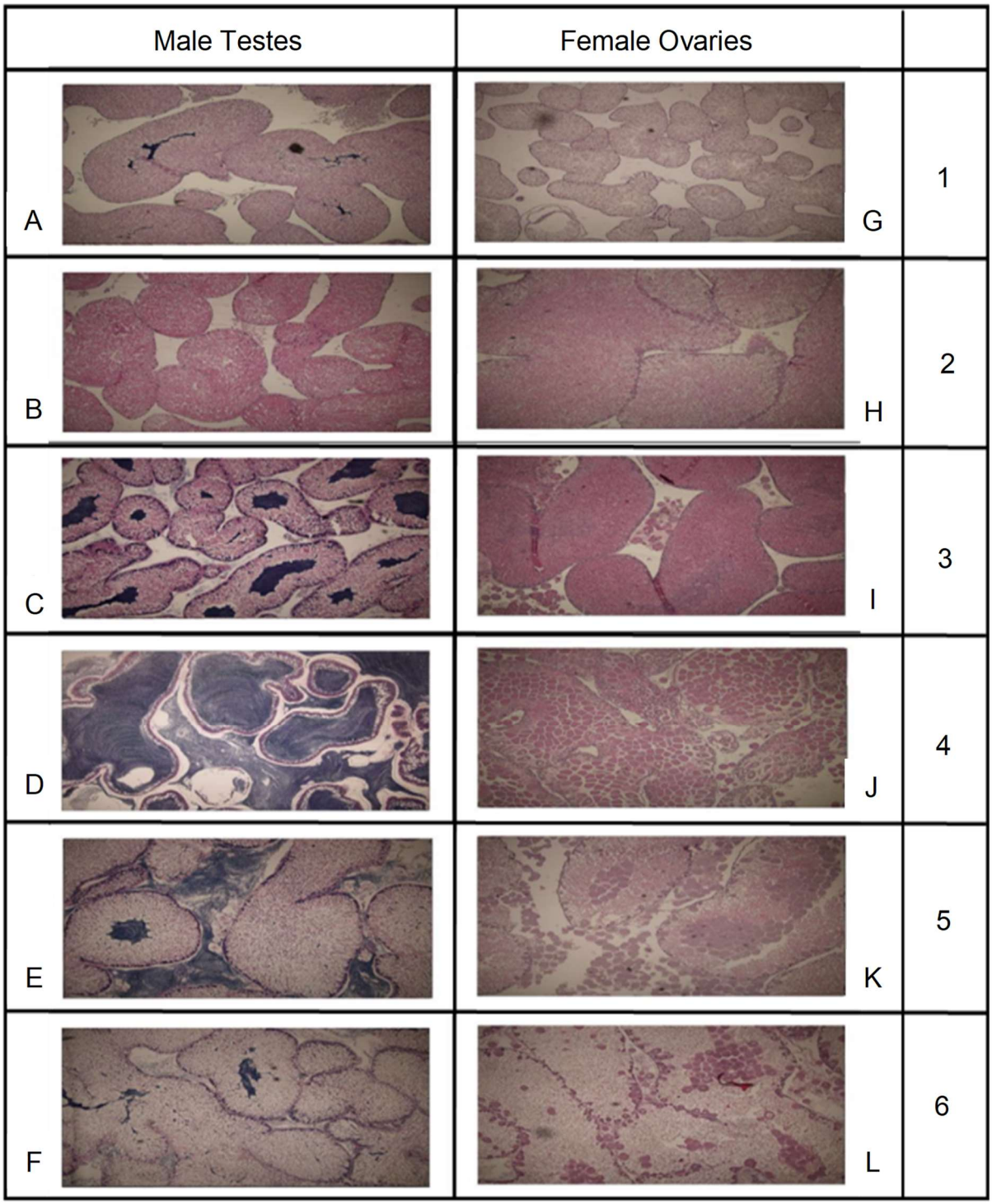


Figure 1.4. *Tripneustes gratilla* gonad histology of a male (A – F) and female (G – L) depicting the respective gonad maturity stages (1 - Recovery; 2 - Growing; 3 - Premature; 4 - Mature; 5 - Partly spawned; 6 - Spent) (Adapted from Cyrus 2013).

In females, ovaries are vacuolated with previtellogenic oocytes (diameter of 10 – 30 µm) along the ovary wall when in the recovery stage (Figure 1.4G). Vitellogenesis starts in the growing stage, where nutritive phagocytes surround vitellogenic oocytes (diameter of 30 – 60 µm) that in some instances have a distinct nucleus (Figure 1.4H). With the onset of oogenesis, the premature stage is characterised by the presence of oocytes with a large range of sizes (diameter of 15 – 90 µm), accompanied by the migration of larger oocytes to the centre of acini (Figure 1.4I). In this stage, oocytes are still surrounded by nutritive phagocytes, as opposed to the mature stage that is characterised by the lumen being filled with mature ova (diameter of 65 – 80 µm) containing a nucleus (Figure 1.4J). Notably, vitellogenic activity continues as vitellogenic oocytes can still be observed in the germinal layer. Partly spawned ovaries are characterised by less compact ova and some vacant spaces (Figure 1.4K), as some ova have been released. Subsequently, these spaces get filled by newly formed primary oocytes as oogenesis continues. In the spent stage, the majority of follicles have an empty space in their centre, however, some vitellogenic oocytes and mature ova can remain (Figure 1.4L). These germinal cells are reabsorbed by nutritive phagocytes throughout the spent stage.

Although limited research has been conducted on the molecular mechanisms for spermatogenesis (Walker *et al*. 2020), studies in the sea urchin *Strongylocentrotus purpuratus* have identified genes involved in oogenesis through whole genome sequencing and qPCR (Song *et al*. 2006). The genes involved in meiosis are largely conserved across animal phyla (Petronczki *et al*. 2003; Page and Hawley 2004) and could probably be extrapolated to males, as well as to other sea urchin species. Briefly, during the formation of the synaptonemal complex, whereby homologous pairs of chromosomes are bound together, the genes; *cohesin*, *STAG3*, *Him-3* and *Rec8* are involved (Song *et al*. 2006). Subsequently, during meiotic recombination, *Spo11* initiates double-strand breaks, *Msh5* and *M1h3* promotes crossing over and *Msh4* is involved in the repair of double-strand breaks (Song *et al*. 2006). Lastly, the genes involved in meiotic division include *Mei-1* and *Mei-2* that play roles in the organisation of the meiotic spindle (Song *et al*. 2006).

Tripneustes reproduce through a broadcast spawning mechanism (Figure 1.5), where fertilisation occurs when eggs and sperm are released into the water column. A sex ratio (male:female) of 1:1 is expected for this genus (Fouda and Hellal 1990; Muthiga 2005), however, sex ratios of 1:1.5 and 1:1.18 have also been reported (McPherson 1965; Vaïtilingon *et al.* 2005). The sexes are difficult to distinguish based on general morphology, but can be identified by assessing the colour of the gametes that are released during

spawning, as males release light yellow sperm and females release bright orange eggs (Cyrus 2013). In some locations, the reproductive cycle seems to be dependent on seasonality (Lawrence and Agatsuma 2013), however, asynchronous reproduction can occur throughout the year, as has been observed in *T. gratilla* in Kenyan lagoons (Muthiga 2005). Spawning cues include various external factors, such as water temperatures, phytoplankton, season and its corresponding day length, as well as food availability, as the nutritive phagocytes provide the nutrients required for gametogenesis (Pearse 1974; Walker 1982; Vaïtilingon *et al.* 2005; Dworjanyn and Pirozzi 2008; Lawrence and Agatsuma 2013; Zhadan *et al*. 2017). Although collector sea urchins are normally evenly distributed across their habitat with individuals preferring being isolated or paired (Shimabukuro 1991), these animals aggregate at higher densities in response to food availability and when spawning (Scheibling and Mladenov 1988; Lauzon-Guay and Scheibling 2007), which could promote reproductive success.

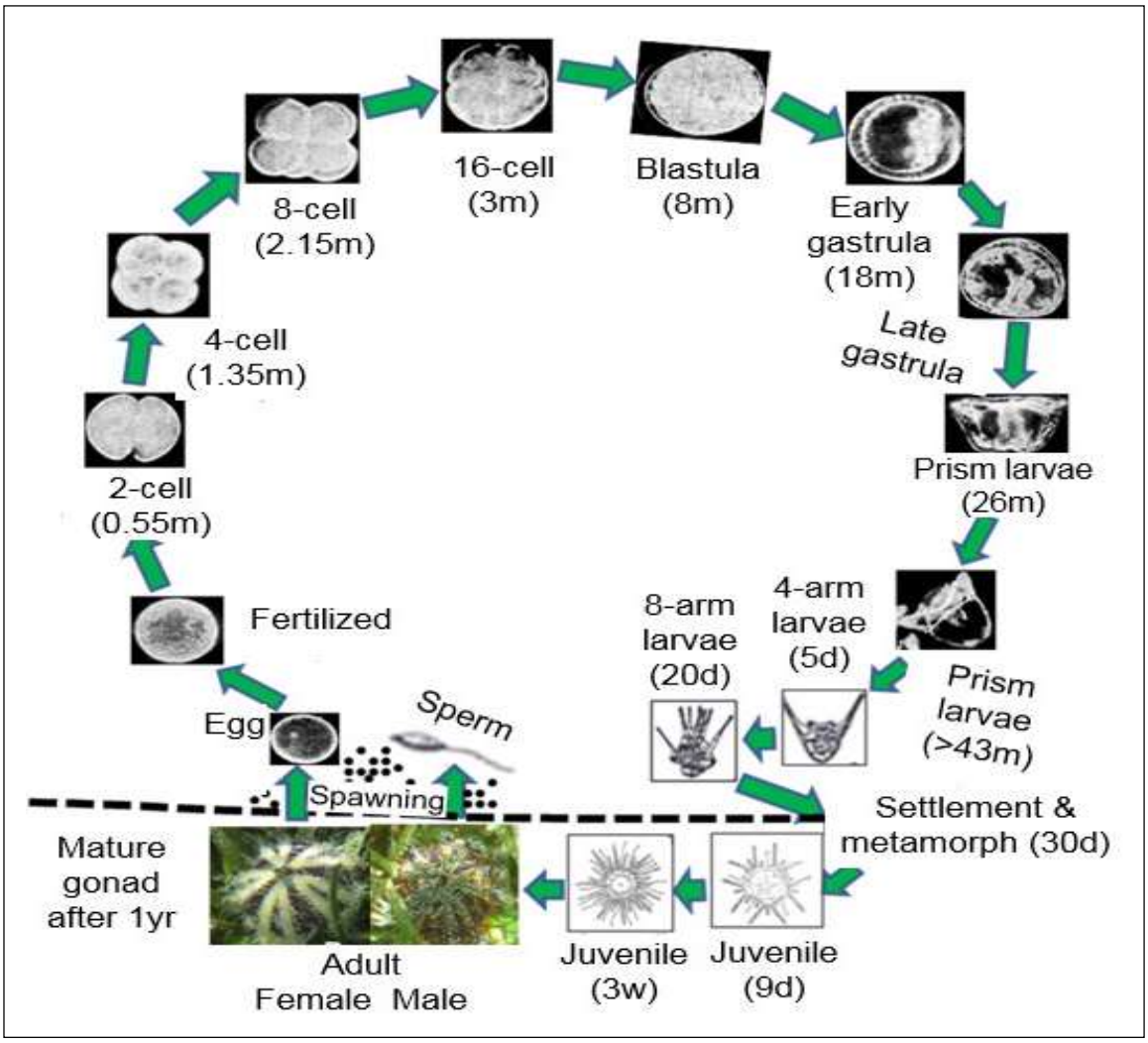


Figure 1.5. *Tripneustes gratilla* life cycle with time (m : minutes; d: days; w: weeks) indicated in brackets (In: Toha *et al.* 2017).

Under aquaculture conditions, spawning can be induced by exposing the animals to thermal, saline or mechanical shocks, conspecific gametes or through the injection of chemicals, such as potassium chloride (KCl) (Osanai 1975; Gago and LuÍs 2011). Subsequently, gametes are collected and eggs are fertilised in seawater. The fertilised eggs develop into larvae through blastular and gastrular phases (Figure 1.5). The larval stage generally last for approximately 30 – 40 days at 25°C under aquaculture conditions (Dworjanyn *et al.* 2007), but can last anything between 15 – 52 days in nature (Juinio-Meñez *et al.* 1998; Rahman *et al.* 2009). In sea urchins, two larval developmental modes have been described, namely; planktotrophic (feeding) and lecithotrophic (non-feeding) (Davidson *et al*. 2019). Although the molecular differences between these developmental modes remains largely unexplored, the evolutionary switch from planktotrophy to lecithotrophy has been well described in many sea urchin species (Wray and Raff 1991; Hart 2002), where sea urchin populations can subsequently be impacted by changes in gene flow, local adaptation and speciation (Jablonski and Lutz 1983; Wray and Raff 1991; Palumbi 1994). A biphasic life cycle is observed across both developmental modes, where a pelagic larval phase in a water column is followed by a benthic adult phase on seafloors after metamorphosis (Davidson *et al*. 2019). *Tripneustes gratilla* is a planktotrophic species and as such, has a greater dispersal potential than lecithotrophic species as a result of a prolonged larval phase. Although larvae from both developmental modes are thought to be influenced by maternal provisioning of nutrients in eggs (Byrne *et al*. 2008a, b; Villinski *et al*. 2002), lecithotrophic species have a greater maternal energy store provided in larger eggs, making them better suited to settle in low-nutrient habitats (Strathmann 1985; Wray and Raff 1991). The growth rate of planktotrophic larvae into juveniles that are capable of settling is dependent on several factors, such as temperature, food availability, pH and genetic factors (Shokita *et al.* 1991; Clark D. *et al.* 2009; Juinio-Meñez and Bangi 2010). In aquaculture environments, juveniles entering the benthic phase of their life-cycle, are reared until they are large enough (40 – 50 mm) to be harvested for commercial purposes (Kelly 2005). Although these animals live for approximately one to five years (Shimabukuro 1991; Regalado *et al.* 2010), their growth rate is fastest during their first year of life (Shimabukuro 1991) and is largely dependent on water temperature (Moore *et al.* 1963) and nutrition (Shimabukuro 1991), as well as genetic factors that influence growth.

1.2. Economic importance of sea urchins

1.2.1. Edible sea urchins

Sea urchin roe, along with tuna, lobster and abalone products, are considered culinary delicacies across Asia and Europe (Andrew *et al.* 2002; Cyrus 2013; Rahman *et al.* 2014), where the increasing demand is largely met by imports (Cilliers 1999; Agatsuma *et al.* 2010). Only certain sea urchin species are considered edible and marketable, possibly as a result of accessibility, palatability, maximum gonad size and cultural traditions (Lawrence and Bazhin 1998; Andrew *et al.* 2002; Cyrus 2013). Although the edible sea urchin industry is dominated by two groups of sea urchins, namely *Loxechinus albus* and various members of the *Strongylocentrotus* genus (Andrew *et al.* 2002), other sea urchin species remain marketable.

Tripneustes gratilla is one of six of the most commercially traded sea urchin species (Wang *et al.* 2013). The market value of sea urchins is dependent on the diet, reproductive phase of the individual, as well as their body size, as this is indicative of the roe size (Agatsuma 2013). Sea urchins with large and firm bright yellow or orange roe, containing few or no gametes, have the highest market value (Robinson *et al.* 2002). Sea urchin roe is most valuable just before gametogenesis, as roe has the greatest palatability when the nutrients stored in gonads have not been mobilised for gametogenesis (Agatsuma 2013). Roe has a higher market value during January and September, as there is low availability during these months (Sonu 2003). When lower quality roe is produced, it is processed by baking, freezing, steaming and salting, however, these products are less valuable (Explorations Unlimited Incorporated 2006). Therefore, there is economic potential in the establishment of aquaculture practices aimed at the production of superior quality roe.

1.2.2. Global echinoculture practices

Historically, there has been a 370% increase in sea urchin trade from 1975 to 1995, where world sea urchin production peaked with a total of 120 306 tonne being harvested in 1995 (Andrew *et al.* 2002; FAO 2006; Agatsuma 2013; Cyrus 2013). In 2009, the contributions of sea urchins and other echinoderms to the world aquaculture sector was valued at US$378 million, with a total of 109 000 tons being produced (FAO 2009). Currently, sea urchin landings are as low as 75 000 – 82 000 tonne (FAO 2014; Rahman 2016), with the lowest estimates of 69 314 tons in 2016 (FAO 2019), meaning that there has been a decrease in

sea urchins harvested from natural populations since 1995, likely as a result of population declines. This has caused a subsequent increase in sea urchin aquaculture (echinoculture) practices and restocking programmes in several countries (Andrew *et al.* 2002; Stefánsson *et al*. 2017).

Sea urchin fisheries and aquaculture have mainly been aimed at the Japanese market, as approximately 80% of sea urchins produced or captured world-wide are consumed in Japan (Hagen 1996; Andrew *et al.* 2002). Commercial sea urchin production occurs mainly in Chile, China and Japan, where Japan mostly produces sea urchins to reseed wild stocks (Andrew *et al.* 2002; Bell *et al*. 2008). Several other countries, including Russia, South Korea, the Philippines, New Zealand, Spain, Fiji, China, Australia, Peru, France and Ireland, also produce sea urchins, but to a lesser extent (Andrew *et al.* 2002). Common sea urchin aquaculture models employed in these countries include suspended cages or containers in the ocean, land-based farming, where sea urchins from natural locations are used to produce larvae in hatcheries, and co-culturing sea urchins with other economically important aquaculture species (Chang 2008; Wang *et al.* 2013). A decline in sea urchin production from the major contributors to this market world-wide (Andrew *et al.* 2002; Williams 2002) has resulted in an increased demand for this high value product, opening the market to other countries to address this deficit (Stefánsson *et al.* 2017).

1.2.3. Potential for sea urchin aquaculture in South Africa

Several countries have had to initiate stock enhancement and restocking programmes as a result of the severe impact that overharvesting has had on natural populations (Andrew *et al.* 2002; McBride 2005; Agatsuma 2013). In South Africa, where sea urchins have not been harvested for commercial purposes, echinoculture can be initiated before the need for restocking or enhancement programmes emerge.

Currently, the South African aquaculture sector is dominated by the mollusc, *Haliotis midae*, although oysters (*Crassostrea gigas*), mussels (*Mytilus galloprovincialis* and *Choromytilus meridionalis*), trout (*Onchorynchus mykiss* and *Salmo trutta*), dusky kob (*Argyrosomas japonicus*), yellowtail (*Seriola lalandi*), tilapia (*Oreochromis mossambicus*, *O. niloticus* and *O. rendalli*), catfish (*Clarias gariepinus*), carp (*Cyprinus carpio*), marron crayfish (*Cherax tenuimanus*) and seaweeds (*Ulva* spp. and *Gracilaria* spp.) are also being produced (DAFF 2012; 2016). The South African aquaculture sector is producing a total of 4000 tons of various aquaculture species, with a total value of ZAR400 million (DAFF 2015). By

establishing aquaculture production of another species, such as the high value, fast-growing collector sea urchin (DAFF 2012; 2016), it will not only be advantageous to the economic growth of South Africa, but can contribute to preventing future overexploitation of this natural resource distributed along the eastern coast of South Africa, as has been observed in other parts of the world.

Although approximately 71 echinoid species have been described along the coast of South Africa (Clark and Courtman-Stock 1976; Clark 1977; Filander 2014), *T. gratilla* has been identified as the most commercially viable (Cyrus *et al*. 2014, 2015a, 2015b), as this species spawns easily in captivity, has a fast growth rate of approximately nine months to marketable size and can produce high-quality gonads that are large enough to be used in the food industry (Juinio-Meñez *et al*. 2008; James and Siikavuopio 2012). Furthermore, this species has a short life span ranging from approximately one year in nature (Elbert 1982), to five years in captivity (Cyrus 2013). This short generation time means that selective breeding programmes could more rapidly result in genetic gains (Kasinathan *et al.* 2015), which could be taken advantage of in this emerging industry. Although *T. gratilla* generally produces high quality gonads and has good market acceptance (Williams 2002; Dworjanyn *et al.* 2007), the value of the product can be further increased by enhancing traits, such as gonad size, which can be controlled in an aquaculture facility by using optimal feeds and controlled tank conditions (Agatsuma 1999, 2013; Cyrus 2013), as well as through genetic management of selective breeding.

1.3. Sea urchin aquaculture, nutrition and genetics

1.3.1. Sea urchin nutrition

Sea urchins are considered generalist, opportunistic omnivores and feeding behaviour largely depends on food availability (Lawrence and Agatsuma 2013). The effects of the environment and diet (exogenous cues) on broodstock gonad physiology could affect gonad regulation of reproduction through endogenous cues (Watts and Wasson 2020). Feeding regimes could potentially be taken advantage of in aquaculture environments, as sea urchins are more likely to invest the nutrients they receive from food into reproductive structures, rather than body maintenance (Lawrence and Bazhin 1998). Therefore, the feeding regime employed could have effects on sea urchin gonad development, subsequently affecting egg quality, reproductive performance, as well as offspring survival through maternal provisioning of nutrients (Byrne *et al.* 2008b; Prowse *et al.* 2008).

Sea urchin diets generally consist of macroalgae and seaweeds that contain the essential nutrients for somatic- and gonadal growth. Major components of sea urchin diets include protein, carbohydrates, lipids, fibre, minerals, vitamins and carotenoids, where the respective nutrients have independent-, as well as interactive effects (Cyrus 2013; Watts *et al.* 2020). Although the exact dietary requirements for sea urchins are not known, the approximate concentrations at which sea urchins produce gonads viable for commercial purposes have been established. Sea urchin diets should contain a moderate amount of protein (approximately 20 – 40%), as proteins are required for growth, body maintenance and reproduction (Akiyama 2001; Cyrus 2013; Powell *et al.* 2020). When dietary protein content exceeds this proposed concentration, it can be detrimental to animal growth rates (Eddy *et al.* 2012), as protein metabolism is energetically costly (Powell *et al.* 2020). However, it should be noted that a protein concentration of 20% is thought to be sufficient for adult sea urchins (de Jong-Westman *et al*. 1995: Pearce *et al*. 2002; Heflin *et al*. 2012). Carbohydrates act as a more efficient energy source, as these are readily processed and stored in nutritive phagocytes to ultimately fuel gametogenesis (Pearse and Cameron 1991; Powell *et al.* 2020; Watts *et al.* 2020). Given the sedentary lifestyle and low respiratory rate of sea urchins (Lawrence and Lane 1982), it is thought that their carbohydrate requirements are low, as studies in the sea urchins, *Strongylocentrotus droebachiensis* (juveniles) and *Lytechinus variegatus* (adults) have found no differences in growth and organ production across a wide range of carbohydrate concentrations (21 – 39% dry weight) (Eddy *et al.* 2012; Heflin *et al.* 2012). It has been suggested that adult sea urchins do not require a concentration of more than 21% for dietary carbohydrates and concentrations exceeding this could diminish an animal's ability to process dietary protein effectively (Heflin *et al*. 2012). Previous *T. gratilla* feeding trials, where feeds had a similar carbohydrate content as proposed by Eddy *et al*. (2012) and Heflin *et al*. (2012), produced commercially acceptable gonads (Cyrus *et al*. 2014).

Four commonly used *T. gratilla* diets include kelp (*Ecklonia maxima*), a green seaweed (*Ulva rigida*), a formulated diet (20U; 20% *Ulva* inclusion) and a mixed feeding regime, where these three feeds are administered interchangeably. *Ecklonia maxima* is a brown seaweed (kelp) that has the greatest moisture (79.39%) and fibre (41.34%) content of these four diets (Table 1.1; Cyrus *et al*. 2014). However, it should be noted that the estimates in Table 1.1 and Table 1.2 can vary across different locations, seasons and samples, as well as across different methods employed for proximate analysis. Nonetheless, the high fibre content of kelp could be beneficial as a sea urchin feed, as the inclusion of fibre could improve gut

functionality (Watts *et al.* 2020), thereby improving feed digestibility and subsequent nutrient uptake. The green seaweed, *Ulva rigida*, has a higher protein content (18.31%) than reported for kelp (11.00%) (Smith 2007; Cyrus *et al*. 2014), and can be even higher (~40% dry weight) when co-culturing marine animals with *Ulva* species (Neori and Sphigel 1999). Though the moisture content of *Ulva* is substantially lower than kelp (Table 1.1), it is more likely similar to that of kelp (Satpati and Pal 2011), as the moisture content reported in Table 1.1 was determined from dried *Ulva* tissue. *Ulva rigida* has been identified as having great potential as a sea urchin feed in aquaculture environments, as they have anti-microbial roles in aquaculture systems (Trigui *et al.* 2013; Bolton *et al.* 2016; Ismail *et al.* 2018), can reduce nutrient output of aquaculture farms when grown in effluent water (Jiménez del Río *et al.* 1996; Mata *et al.* 2010) and can reduce the reliance on naturally occurring seaweeds as sea urchin feeds.

Table 1.1. Nutrient analysis overview for kelp (*Ecklonia maxima*) as described by [a]Smith 2007 and *Ulva rigida* and a formulated feed (20U) as described by [b]Cyrus *et al*. 2014 and [c]Cyrus 2013, where all estimates are expressed as percentage dry weight, unless stated otherwise (n.d. not determined).

Nutrients (% dry weight)	***Ecklonia maxima***	***Ulva rigida***	**Formulated feed (20U)**
Protein	11.00 [a]	18.31 [b]	25.69 [b]
Fat	1.16 [a]	0.38 [b]	2.31 [b]
Moisture	79.39 [a]	15.30 [b]	9.61 [b]
Ash	19.41 [a]	32.66 [b]	13.89 [b]
Gross energy (MJ/kg)	n.d.	9.44 [b]	15.49 [b]
Fibre	41.34 [a]	6.02 [b]	4.75 [b]
Carbon/Carbohydrate	33.82 [a]	27.33 [b]	43.75 [b]
Nitrogen	1.77 [a]	n.d.	n.d.
Phosphorous	0.23 [a]	0.172 [c]	n.d.
Potassium	2.95 [a]	1.897 [c]	n.d.
Calcium	1.17 [a]	1.034 [c]	n.d.
Magnesium	1.46 [a]	4.31 [c]	n.d.
Manganese	0.0006 ppt [a]	0.001 [c]	n.d.
Iron	0.01021 ppt [a]	0.007 [c]	n.d.
Copper	0.0003 ppt [a]	0.001 [c]	n.d.
Zinc	0.0014 ppt [a]	0.001 [c]	n.d.
Bromine	n.d.	0.006 [c]	n.d.
Sodium	2.70 [a]	5.17 [c]	n.d.
Aluminium	n.d.	0.006 [c]	n.d.

[a] *Ecklonia maxima* nutrient analysis from samples collected on the south west coast of South Africa by Smith 2007.
[b] Nutrient analysis of *Ulva rigida* from Irvine & Johnson (I&J) Cape Abalone farm (34°34'0.60'' S; 19°21'00'' E) in the western cape of South Africa, as well as a formulated feed (20U) by Cyrus *et al*. 2014.
[c] *Ulva rigida* from Irvine & Johnson (I&J) Cape Abalone farm (34°34'0.60'' S; 19°21'00'' E) in the western cape of South Africa as described in Cyrus 2013.

It has been suggested that sea urchins respond to toxins released by plants upon ingestion by adjusting metabolically, although this requires further investigation (Watts *et al.* 2020). Furthermore, as feeds are generally emerged in a water system, it is difficult to measure the quantity of feed that the animal ingests *vs* dissolved organic matter in the water (Bamford

1982; Watts *et al.* 2020). Although several minerals and vitamins have been identified throughout the sea urchin body and the mineral requirements of juvenile sea urchins (*Strongylocentrotus droebachiensis*) has been assessed (Kennedy and Robinson 2007), to date, the requirements of these essential building blocks for adult sea urchins are not known (Watts *et al.* 2020). In diets with less or more of these essential building blocks for sea urchins, it is thought that animals are able to adjust their energy source through selective nutrient absorption, however, this could result in reduced gonad growth (Lawrence and Lane 1982; Hammer *et al.* 2006b). Both natural feeds are rich in minerals, however, *Ulva* has greater amounts of magnesium and sodium (Table 1.1). Of these, magnesium plays important roles in the formation of the calcium-magnesium carbonate skeleton of sea urchins (Ebert 2020). Furthermore, *Ulva* spp. have the potential to act as a feeding stimulant and this could promote gonad development by altering sea urchin feeding behaviour (Cyrus *et al*. 2014). This led to the incorporation of *Ulva* into a formulated feed (20U) for *T. gratilla* containing 20% (w/w) dried *Ulva* (Cyrus *et al*. 2014), resulting in improved feed palatability and consumption (Cyrus *et al*. 2014, 2015a, b). This formulated feed has the highest protein- (25.69%), fat- (2.31%) and carbohydrate (43.75%) content of all four feeds (Table 1.1), which could contribute to improved reproductive performance through improved gonad development and nutrient allocation to eggs.

Sea urchin diets need to contain sufficient lipids, that encompass triacylglycerols, phospholipids, sterols, waxes, free fatty acids and carotenoids that act as sources of energy, hormone precursors, emulsifiers and essential fatty acids, where carotenoids influence gonad colour (Watts *et al.* 2020). The natural feeds, *E. maxima* and *U. rigida*, have a rich lipid-, fatty acid- and amino acid composition (Table 1.2). Across lipid classes, *E. maxima* appears to have a higher sterol, triglyceride and free fatty acid content, as opposed to *Ulva*, which has a higher polar lipid content (Table 1.2; Newell 1980; Gordillo *et al*. 2001). Cholesterol acts as a building block for cell membranes and act as precursors for steroid hormones in sea urchins (Giese 1966; D'Abramo 1997). Triglycerides are considered an important source of essential free fatty acids and energy for marine invertebrates (Giese 1966; D'Abramo 1997). Additionally, this lipid class is also the primary energy source that fuels the early developmental stages of *T. gratilla* larvae (Byrne *et al.* 2008a). *Ulva* has an abundance of polar lipids (Table 1.2), which supplies essential nutrients, such as fatty acids, choline, inositol, ethanolamine and phosphorus (Watts *et al.* 2020). Additionally, these lipids are considered emulsifiers in marine invertebrate diets (Teshima 1997; Tocher *et al.* 2008), which suggests that these compounds could improve digestion and nutrient translocation in

sea urchins, however, this requires further investigation. Lastly, the higher free fatty acid content of kelp could be beneficial for growth, as studies in the sea urchin *Strongylocentrotus droebachiensis* found reduced growth when their diets had a low essential fatty acid content (González-Durán *et al.* 2008).

Table 1.2. Kelp (*Ecklonia maxima*) and *Ulva rigida* lipid class-, fatty acid- and amino acid composition as described by [a]Newell 1980, [b]Gordillo *et al*. 2001, [c]Trigui *et al*. 2013 and [d]Shuuluka *et al*. 2013, where lipid composition and fatty acids are expressed as a percentage of the total and amino acid content is expressed as percentage dry weight for kelp and g/100 g protein for *Ulva rigida*.

Compound	*Ecklonia maxima*	*Ulva rigida*
Lipid classes (%)		
Polar lipid	8.30 [a]	n.d.
Acetone-mobile polar lipid	n.d.	45.00 [b]
Monoglyceride	8.30 [a]	n.d.
Cholesterol/sterol	11.00 [a]	2.10 [b]
Alcohol	n.d.	0.30 [b]
Free fatty acid	26.80 [a]	0.50 [b]
Triglyceride	22.60 [a]	16.00 [b]
Wax ester	Trace [a]	0.90 [b]
Hydrocarbons	Trace [a]	n.d.
Phosphoglycerides	n.d.	32.00 [b]
Phosphatidyl	n.d.	3.50 [b]
Fatty acids (%)		
Myristic (C14:0)	3.28 [a]	Trace [c]
Palmitic (C16:0)	17.65 [a]	54.41 [c]
Stearic (C18:0)	6.61 [a]	1.39 [c]
Palmitoleic (C16:1)	6.95 [a]	5.82 [c]
Oleic (C18:1)	23.83 [a]	18.35 [c]
Gadoleic	5.05 [a]	n.d.
Linoleic (C18:2)	6.36 [a]	3.12 [c]
Linolenic (C18:3)	0.43 [a]	7.22 [c]
Arachidic (C20:0)	n.d.	4.01 [c]
Eicosenoic (C20:1)	n.d.	5.67 [c]
Amino acids	% dry weight	g/100 g protein
Isoleucine	0.036 [a]	3.10 [d]
Leucine	0.052 [a]	5.20 [d]
Lysine	0.062 [a]	3.70 [d]
Methionine	0.023 [a]	1.50 [d]
Cysteine	n.d.	1.10 [d]
Phenylalanine	0.017 [a]	3.30 [d]
Tyrosine	0.055 [a]	2.20 [d]
Threonine	0.70 [a]	5.00 [d]
Valine	0.056 [a]	5.60 [d]
Histidine	0.017 [a]	1.40 [d]
Aspartic acid	0.553 [a]	13.00 [d]
Glutamic acid	1.068 [a]	9.40 [d]
Proline	0.102 [a]	4.30 [d]
Serine	0.087 [a]	6.10 [d]
Glycine	0.068 [a]	7.80 [d]
Alanine	0.933 [a]	12.30 [d]
Arginine	0.055 [a]	4.60 [d]
Ammonia	0.150 [a]	1.20 [d]

[a] *Ecklonia maxima* lipid classes, fatty acid and amino acid composition of kelp fronds from Oudekraal on the west coast of the Cape Peninsula in South Africa as described by Newell 1980.

[b] *Ulva rigida* lipid class analysis as described for samples collected from Málaga, Mediterranean Sea, Southern Spain, by Gordillo *et al*. 2001, under normal CO_2 conditions with sufficient N_2.

[c] *Ulva rigida* fatty acid composition as determined by Trigui *et al*. 2013, for samples collected from Sidi Mansour Sfax, Tunisia (35°14'58.36" N, 11°7'17.75" E).

[d] Amino acid composition of wild *Ulva rigida* from Kommetjie (34°09'22" S, 18°19'22" E) on the west coast of the Cape Peninsula, South Africa, as described by Shuuluka *et al*. 2013.

Across fatty acids, *E. maxima* contains greater amounts of oleic (C18:1) and linoleic (C18:2) acids, as opposed to *Ulva* that has a greater palmitic (C16) and linolenic (C18:3) acid content (Table 1.2; Newell 1980; Trigui *et al*. 2013). As these fatty acid classes have been identified as having roles in sea urchin growth and development (Watts *et al.* 2020), these differences in fatty acid composition have the potential to result in differences in reproductive performance in *T. gratilla*, particularly as it has been suggested that the macroalgal diet administered is a driver of sea urchin tissue fatty acids (Schram *et al.* 2018). Specifically, for *T. gratilla* in natural environments in Taiwan, the predominant fatty acids in their gonad tissues were C14, C16, C16:1, C18:1, C20 and C22:1 (Chen *et al.* 2013).

Furthermore, dietary amino acids will likely influence gonad amino acid profiles (Phillips *et al.* 2010), which has a known impact on the sweet, bitter and umami flavour profile of sea urchin gonads. Specifically, the amino acids associated with gonad sweetness are threonine, glycine and alanine, whereas arginine, leucine, isoleucine, lysine, valine and methionine are associated with bitterness, and glutamic acid is associated with the umami flavour of gonads (Osako *et al*. 2007; Phillips *et al*. 2010; Chen *et al*. 2013). Kelp and *Ulva* both contain various amino acids in differing concentrations (Table 1.2; Newell 1980; Shuuluka *et al*. 2013). Among these, glycine has been identified as a dominant free amino acid in several sea urchin species (Lee and Haard 1982; Liyana-Pathirana *et al.* 2002; Osako *et al.* 2007; Phillips *et al.* 2010) and although the amino acids in Table 1.2 are represented in different units, it appears that kelp and *Ulva* contain similar proportions of this amino acid relative to the other amino acids that are present. Additionally, both feeds contain high aspartic acid and glutamic acid relative to the other compounds (Table 1.2). Although the feeds share many similarities, kelp contains higher proportions of tyrosine and *Ulva* contains more phenylalanine (Table 1.2). Therefore, when animals are fed these diets, the incorporation of different amounts of amino acids could subsequently affect gametogenesis and reproductive performance.

Although both the natural feeds have their advantages as singular feeds in aquaculture environments, combination feeding regimes could be advantageous, as the incorporation of a wider range of nutrients would be advantageous for sea urchin reproduction. However, this remains to be investigated for *T. gratilla*. While effective feeding regimes in aquaculture environments remain vital for the improvement of reproductive- and commercial traits, the underlying population structure of natural populations and the degree of genetic diversity present in these could also have important implications for the management of this emerging aquaculture species in South Africa.

1.3.2. Genetics for managing captive populations

Genetics started playing a role in aquaculture environments in the 1980's when molecular techniques were commonly used to advance aquaculture practices (Dunham *et al.* 2001). During aquaculture practices, new selective pressures are introduced (Dunham 1996), which should result in animals better suited for aquaculture environments. However, a loss of genetic diversity in cultured populations is often observed as a result of population bottlenecks caused by a founder event, which can lead to a reduction in effective population size, and subsequent inbreeding, reduced fitness, and poor production output (Rhode *et al.* 2012). Therefore, to ensure sustainability and to maintain long-term genetic fitness, the most genetically diverse populations should be identified and utilised as founder populations for aquaculture practices.

Molecular markers are widely used to study genetic diversity, which occurs as a result of variation in DNA sequences, in both terrestrial and marine animals (Marsjan and Oldenbroek 2007; Abdul-Muneer 2014; Grover and Sharma 2014). These variations result from the substitution of single nucleotides, inversions, and deletions or insertions of DNA fragments of various sizes (Liu and Cordes 2004; Marsjan and Oldenbroek 2007; Abdul-Muneer 2014). Molecular markers can also be used to assess economically important traits, as parental pairs can be assigned to offspring to assess correlations between offspring- and parental phenotypic data. Historically, several nuclear markers have been applied in population genetics. The first of these were allozyme markers, where alleles encode variant forms of an enzyme (Liu and Cordes 2004). Following this, restriction fragment length polymorphisms (RFLPs) were developed, where DNA is digested by restriction enzymes based on variable cutting sites (Lowe *et al.* 2004; Chenuil 2006). Alternatively, randomly amplified polymorphic DNA (RAPD) markers were used. This cost-effective method makes use of short primers (8 – 10 bp) to amplify many random segments of nuclear DNA simultaneously (Liu and Cordes 2004; Agarwal *et al.* 2008). To overcome limitations associated with RFLP and RAPD markers, amplified fragment length polymorphisms (AFLPs) were developed. For this method, DNA is digested and adaptors are ligated to be used as primer binding sites, enabling the amplification of multiple fragments simultaneously in a highly reproducible manner (Liu and Cordes 2004). Currently, microsatellite markers, also known as simple sequence repeats (SSRs) or variable number of tandem repeats (VNTRs), are commonly used in population genetic studies for fisheries management, conservation and aquaculture applications (Abdul-Muneer 2014). More recently, single nucleotide polymorphisms (SNPs) have also been used, as these bi-allelic markers occur at a high frequency (Abdul-Muneer

2014; Grover and Sharma 2014). As microsatellites are the marker of choice for the current study, further focus will be placed on this marker type.

Microsatellites are segments of DNA that consist of tandemly repeated motifs that occur as frequently as once in every 10 kilobases (kb) (Wright 1993; Hoffman and Nichols 2011). These co-dominant markers are found in both coding and non-coding regions, throughout prokaryotic and eukaryotic genomes (Liu and Cordes 2004; Hoffman and Nichols 2011; Abdul-Muneer 2014). The highly polymorphic nature of microsatellite markers, as a result of multiple alleles per locus, and high reproducibility across laboratories enables the evaluation of genetic variation within and among individual organisms, as well as the differentiation between genetically distinct populations (Coupé *et al.* 2011; Casilagan *et al.* 2013; Abdul-Muneer 2014). Studies have successfully used microsatellite markers to genetically characterise organisms to improve management strategies within aquaculture and fisheries practices (Rogers *et al.* 2006; Chauhan *et al.* 2007; Abdul-Muneer *et al.* 2009; Kumar *et al*. 2019; Olubunmi 2019). A total of 30 microsatellite markers have been developed for *T. gratilla* (Carlon and Lippé 2007; Wainwright *et al.* 2012) that can be utilised for several applications in this emerging sea urchin aquaculture industry, as well as to assess population genetic aspects of natural populations.

As sea urchin reproduction occurs by means of broadcast spawning, the widespread dispersal of larvae is expected to facilitate high levels of gene flow, resulting in a state of panmixia (Casilagan *et al.* 2013). Microsatellite markers were used to study population structure, effective population size and demographic history of exploited *T. gratilla* populations in the Philippines (Casilagan *et al.* 2013). The study found little genetic differentiation among populations with a global F_{st} estimate of 0.001 ($P = 0.719$) (Casilagan *et al.* 2013). These results were supported by a study on *CO1* sequences that found that Indian Ocean populations were genetically undifferentiated from Pacific Ocean populations (Lessios *et al.* 2003). Similarly, a study on wild *T. gratilla* populations in Western Luzon found little genetic differentiation between populations ($F_{st} = 0.0122$; $P < 0.01$) by analysing polymorphic allozyme loci and concluded that the populations belong to a single genetically homogenous stock (Malay *et al.* 2000). A study by Coupé *et al.* (2011) used a similar approach where microsatellite markers were amplified to assess conservation and management strategies of the sea urchin, *Paracentrotus lividus*, in France. The study also reported little to moderate genetic differentiation between the populations with an overall F_{st} value of 0.063 ($P < 0.05$), indicative of high levels of gene flow (Coupé *et al.* 2011). Although it could be hypothesised that the natural populations along the South African coast are

panmictic, the effect of current systems along this coast on genetic diversity and population structure of these populations has not been evaluated as yet. Once these animals are translocated to aquaculture facilities, maintaining the genetic diversity present in progenitor natural populations in cultured populations is integral for long-term sustainability of aquaculture practices. This will ensure that there is sufficient genetic variation present in the cultured population to be able to respond to future stressors, as well as selective breeding practices (Frankham *et al*. 2003; Markert *et al*. 2010).

1.3.3. Genetics of complex traits

In recent years, molecular markers have been used as cost- and time efficient methods of assessing complex traits, such as growth rate, reproductive traits, stress tolerance and disease resistance (Gjedrem and Rye 2016), that are dependent on several genes and environmental interactions (Doeschl-Wilson *et al.* 2011). Though these traits, as well as their improvement, will have important implications for future aquaculture of this sea urchin species, this study will place further focus on growth rate and disease resistance. Through the selection of animals or families that display faster growth, as well as moderate to high heritability for this trait, long-term operational costs of aquaculture facilities could be reduced. The feasibility of selective breeding has been assessed in various sea urchin species, including *Strongylocentrotus intermedius*, *S. nudus*, *Anthocidaris crassispina*, *Mesocentrotus nudus*, *Lytechinus variegatus* and *Heliocidarus crassipana*, where heritability estimates indicated that these sea urchins, in their respective locations, could have positive responses to artificial selection (Liu *et al*. 2004, 2005; Ding *et al*. 2007; Chang *et al*. 2012). Previous studies in the sea urchin *S. intermedius* found high heritability estimates (degree of phenotypic variation that is attributed to inherited genetic factors), as well as significant genetic correlations for growth traits, including body weight, exoskeleton diameter and height (Liu *et al*. 2005; Chang *et al*. 2018). In China, a genetically improved strain of *S. intermedius*, that has improved growth and gonad quality when compared to an unselected strain, produced approximately 18 million offspring in 2017 (Lawrence *et al*. 2019). Although this implies that sea urchin populations could be improved through selective breeding practices, a study in the sea urchin *T. gratilla* in the Philippines found limited genetic correlations with growth rates (Pante *et al*. 2007). However, this has not been investigated within a South African context and further investigation is required to inform future selective breeding strategies.

In contrast, disease susceptibility will be more challenging to assess. However, the degree of genetic diversity in a population also plays a role in disease susceptibility and in the ability of these animals to respond to changes in their environment (Silva 2013), such as new predators, competitors, climate change, pollution, as well as diseases (Frankham *et al.* 2003). A decreased genetic diversity and survival rate can occur as a result of inbreeding, especially within small or cultured populations where few broodstock animals were used as founders (Frankham *et al.* 2003). It has also been found that there is a direct relationship between the overall fitness of a population and the level of heterozygosity within the population (Reed and Frankham 2003). Genetic homogeneity has not only been associated with disease susceptibility, but also disease severity and recovery in the case of communicable diseases in animals (Doeschl-Wilson *et al.* 2011). Although selectively neutral markers, such as microsatellites, are not necessarily directly associated with adaptive genes, they are able to give an indication of the overall levels of genetic diversity in a population (Kirk and Freeland 2011). Therefore, these markers could provide insight into the ability of populations to respond to disease and stressors, as the heterozygosity at these microsatellite loci are used as an indication of genome-wide heterozygosity (Kirk and Freeland 2011). Therefore, the assessment of genetic diversity, as well as further investigation of the causative agents of diseases in aquaculture settings is required to address this key limiting factor of future aquaculture practices.

1.4. Sea urchins and disease

1.4.1. Sea urchin balding disease

Sea urchin diseases can be complex and several factors, such as current systems, water temperature and animal density, as well as the relative susceptibility of populations or species, can result in disease outbreaks (Wang *et al.* 2013). Approximately 13 sea urchin diseases have been identified, however, only three have been named; paramoebiasis, vibriosis and bald sea urchin disease (Sweet 2020). The lack of formal naming is likely as a result of similar causal agents with different phenotypic characteristics, or due to overlap in phenotypic characteristics, but different causal agents associated with diseases (Sweet 2020). Although bacteria are most commonly associated with sea urchin diseases, other pathogens include fungi, parasites and algae (Wang *et al.* 2013). Bacterial infections often result in lesions that form on sea urchin body surfaces in both natural and captive sea urchin populations (Maes and Jangoux 1984). The various diseases presenting similar symptoms,

characterised by these lesions, were described as bald sea urchin disease when a disease outbreak occurred in California in 1970 (Maes and Jangoux 1984). This communicable disease has been observed in approximately 19 sea urchin species (Sweet 2020), including *Arbacia lixula*, *Cidaris cidaris*, *Paracentrotus lividus*, *Psammechiinius miliaris*, *Strongylocentrotus droebachiensis*, *S. franciscanus*, *S. purpuratus* and *T. gratilla* (Wang *et al.* 2013).

Bald sea urchin disease is characterised by a loss of spines, tube feet and epidermis resulting in necrotic green lesions on the sea urchin body surface, that turn black as the disease progresses (Figure 1.6; Maes and Jangoux 1984). This disease is likely the oldest disease described in sea urchins, dating back to the Middle Jurassic period, as similar lesions to that of bald sea urchin disease have been identified on fossil records for the holasteroid *Collyrites dorsalis* (Radwańska and Radwański 2005). It has been hypothesised that this disease is caused by opportunistic bacteria when there is a mechanical abrasion on the body surface (Vaïtilingon *et al.* 2004; Becker *et al.* 2007). Sea urchins can recover from bald sea urchin disease by regenerating epidermis tissue, spines and other appendages (Maes and Jangoux 1984). This occurs without difficulty when the affected area is small, as diseased sea urchins develop an inflammatory-like reaction where phagocytic and red-spherule cells isolate the upper layer of necrotic skeleton from bacteria and other disease-causing agents (Maes and Jangoux 1984; Silva 2013). Studies noted that the red sphere cells contain anti-microbial substances, such as echinochrome A (Service and Wardlaw 1984; Smith *et al.* 2006), that could play a role in recovery from bacterial infections. Interestingly, the coelomic fluid of healthy sea urchins is sterile (Wardlaw and Unkles 1978), likely as a result of bactericidal- and phagocytic activity in coelomic fluid, where foreign microorganisms and particles are phagocytosed (Bauer and Young 2000). However, when this fluid becomes infected through an increase in bacterial load, as a result of damage to exoskeletal tissue, sea urchins rapidly succumb to this disease (Sweet *et al.* 2016).

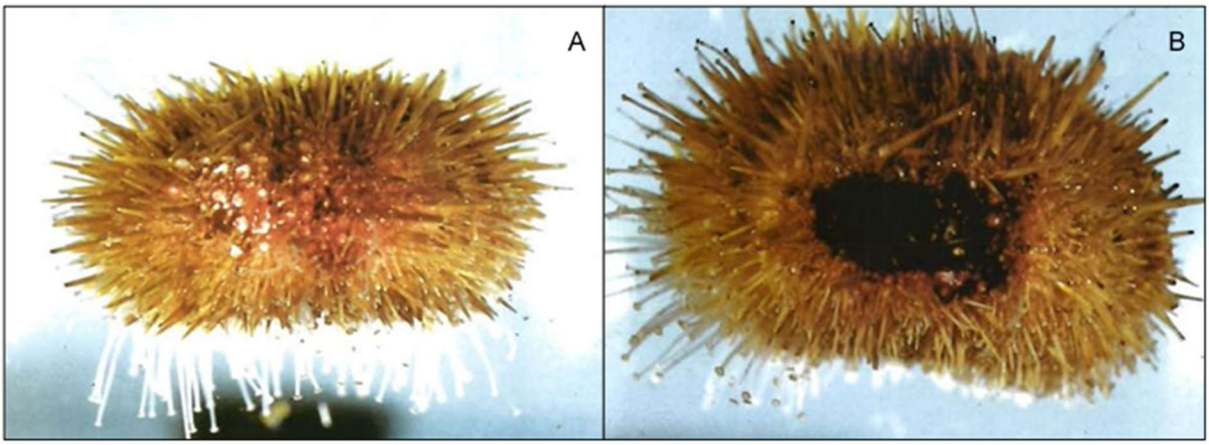


Figure 1.6. Lesions associated with bald sea urchin disease on *Strongylocentrotus droebachiensis*, where (A) a healthy exoskeleton and (B) a loss of spines and appendages, as well as a discolouration of the body surface can be observed (Adapted from Roberts-Regan 1988).

Adaptive (acquired) immune responses in sea urchins remains undescribed (Silva 2013). Although major histocompatibility complex (MHC) gene families that initiate specific immune responses in vertebrates are not found in sea urchins, many gene homologs (related by descent) that are paralogous (evolved a new function) to these have been identified in sea urchins (Rast *et al.* 2000), suggesting some conservation of these important gene complexes. These gene complexes have formed as a result of the competition between pathogens (Smith *et al.* 2006), with short generation times and high mutation rates, and hosts, with comparatively long generation times and low mutation rates (Haldane 1949). The main cellular components of sea urchin immune systems includes four types of coelomocytes, including phagocytic amoebocytes, vibratile cells, red sphere cells and white/colourless sphere cells (Metchnikoff 1968a, b; Johnson 1969; Isaeva and Korenbaun 1990; Larson and Bayne 1994; Mangiaterra and Silva 2001; Silva 2013). These cells predominantly occur in coelomic fluid and are produced by coelomocytes (Silva 2013), where cell numbers may differ across species, individuals, populations and health condition (Smith 1981; Isaeva and Korenbaun 1990).

1.4.2. Aquaculture and disease

In aquaculture environments, this disease has the potential to progress at a faster rate (Maes and Jangoux 1984), as the necrotic tissues of diseased organisms are infectious, meaning that this disease can easily spread. This has been observed in already established aquaculture farms in Japan, where a single disease outbreak resulted in the loss of approximately 800 000 sea urchins (*S. intermedius*) (Tajima *et al*. 1997; Wang *et al.* 2013). However, it is possible that sea urchins display the phenotypic characteristics of bald sea

urchin disease when there is a secondary infection of epidermal lesions as a response mechanism. This presents a further challenge for successful aquaculture practices, as physical injuries to the animals, which easily occur at high animal densities, results in an increased susceptibility to bacterial infections and could result in great economic losses (Tajima *et al.* 1997).

It is also thought that captive populations will have an altered bacterial composition on their body surfaces as these animals will be exposed to fewer bacterial communities, which could affect their ability to combat disease (Loudon *et al.* 2014). Furthermore, Dworjanyn and Pirozzi (2008) found that sea urchin larvae have a greater settlement rate in the presence of a greater abundance of bacteria on algae fed to larvae, suggesting that settlement and metamorphosis is dependent on the chemical cues from bacteria. Bacterial activity also affects various host processes, such as digestion, nutrient absorption, metabolism, immune system development and responses or resistance to pathogens (Turnbaugh *et al.* 2007; Huang *et al.* 2010; Ichata *et al.* 2015). Disease outbreaks are not only a concern within an aquaculture setting, but also affect conservation of natural populations. In a natural environment, a high mortality rate could result in the deterioration of ecosystems, as sea urchins play a role in nutrient cycling and in controlling invasive macroalgae and seagrasses (Feehan and Scheibling 2014).

No method of disease control has been discovered as yet and these bacterial infections are rarely treated successfully (Wang *et al.* 2013). Previous studies have recommended various bacterial control measures, such as water temperature and quality control (Tajima *et al.* 1998), the use of antibiotics that known pathogens are sensitive to (Tajima *et al.* 1998; Li *et al.* 2000; Wang *et al.* 2006), the removal of iron from water (Yamase *et al.* 2006), ultraviolet irradiation, lowering animal density, hydrogen peroxide treatment (Masuda *et al.* 2004), as well as chlorine dioxide treatment (Tajima *et al.* 2000). However, the complex bacterial communities associated with these marine animals and their environments, as well as their interactions, needs to be assessed to contribute to disease management in aquarium settings.

1.5. Metagenomics

1.5.1. Identification of disease-causing bacteria

Bacteria have been identified as the causative agents of bald sea urchin disease through the isolation and culturing of bacteria, scanning electron microscopy (SEM), experimental infections, denaturing gradient gel electrophoreses (DGGE), fluorescent *in situ* hybridisation (FISH), as well as through 16S rRNA gene cloning (Maes and Jangoux 1984; Gilles and Pearse 1986; Tajima *et al.* 1997; Becker *et al.* 2007, 2008, 2009). These studies identified classes Alphaproteobacteria, Cytophaga-Flexibacter-Bacteroides (CFB) bacteria, Fusobacteria and Cytophagaceae as being abundant in the lesions on diseased sea urchins. Abundant genera included *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas*, *Pseudoalteromonas* and *Vibrio*. Specific bacterial species, such as *Aeromonas salmonicida, Exiguobacterium* sp., *Vibrio anguillarum*, *V*. *parahaemolyticus*, and *V*. *nigiripulchritudo* (Gilles and Pearse 1986; Becker *et al.* 2007) were able to cause disease in experimental infections, suggesting their involvement in this opportunistic disease. However, it has been suggested that disease-causing bacteria are not limited to these species, as bacterial composition is dependent on the animal's immediate environment and the lesions are likely caused by opportunistic bacteria in these surroundings (Gilles and Pearse 1986; Becker *et al.* 2008). Further supporting this hypothesis, studies in the sea urchin *S*. *droebachiensis* have found that the bacterial composition of healthy animals were similar to that of diseased animals (Roberts-Regan *et al.* 1988).

A metagenomic approach can be applied to gain a better understanding of this disease, as well as aquaculture environments, by identifying the bacteria present on animals from natural and cultured environments, as well as animals from different health statuses. Metagenomics can be defined as a discipline that enables the genomic study of uncultured microorganisms taken directly from their habitats (Wooley *et al.* 2010). Prior to the use of next-generation sequencing technologies, low resolution fingerprinting technologies (Griffiths *et al.* 2011) or Sanger sequencing was used to assess complex bacterial samples (Rusch *et al.* 2007). However, within aquatic environments, there is an estimated one million microorganisms per millilitre, inevitably resulting in an underestimation of species richness and diversity when complex environments and interactions are being investigated (Handelsman 2004; Glöckner *et al.* 2012). Fortunately, high-throughput next-generation sequencing technologies and bioinformatic strategies have greatly improved in recent years,

resulting in the accurate detection of operational taxonomic units (OTUs), which are groupings of similar sequences (Flynn *et al.* 2015). Two main types of sequencing-based metagenomic approaches have been employed: shotgun sequencing and amplicon sequencing. Shotgun sequencing involves the random shearing of DNA, followed by cloning and sequencing of the fragments (Staden 1979). The less costly alternative, amplicon sequencing, is commonly applied, where a well-characterised 16S rDNA or rRNA gene is sequenced (Woo *et al.* 2008; Kim and Chun 2014; Artur *et al.* 2017) across all taxa that are present in a sample (Odintsova *et al.* 2017). Bacterial 16S rDNA or rRNA genes, used for their superior resolving power (Artur *et al.* 2017), are sequenced to assess taxonomic and functional composition of microbiomes. These genes contain nine hypervariable regions (V1 – V9), which are species-specific regions that can facilitate bacterial species identification (Chakravorty *et al.* 2007).

It should be noted that a next-generation sequencing approach, especially when used for the detection of microorganisms, is not free of limitations. A common limitation addressed by several sequence-based bacterial studies is that it is impossible to differentiate between live, dead or inactive microbes (Oikonomou *et al.* 2012). Additionally, bias can be introduced at several of the experimental stages, as contamination can occur during the sampling process and subsequent transport of samples (Oikonomou *et al.* 2012), as well as during DNA extraction, sample storage and PCR amplification. Despite these limitations, next-generation sequencing and metagenomic analyses remain an important and effective tool for the identification of pathogens, particularly when this can potentially lead to a better understanding of disease progression and the eventual development of a diagnostic tool.

1.5.2. Measures and implications of bacterial diversity

Bacteria can inhabit a wide variety of environments, such as soil, hydrothermal vents, oceans, rivers, glaciers and water bodies with a wide range of salt levels. These organisms play an important role in providing carbon, nitrogen and phosphorus sources through nutrient sequestration and recycling (Whitman *et al.* 1998; Glöckner *et al.* 2012). To further explain differences and similarities between bacterial communities, several diversity measures have been developed, such as alpha (within sample) and beta (between samples) diversity statistics (Legendre and De Cáceres 2013).

Alpha diversity was first described by Whittaker (1960) as “the richness in species of a particular stand or community”. Alpha diversity can be measured through rarefaction curves,

where a fixed number of reads are randomly sampled and the species diversity is assessed for each sampling event (Sudarikov *et al.* 2017), as well as through various diversity indices. These include the Chao1 (Chao 1984), Shannon (Shannon 1948) and Simpson (Simpson 1949) indices, where Chao1 uses the number of rare OTUs (singlets or doublets) to estimate the lowest number of OTUs present within each sample (Oikonomou *et al.* 2012). The Shannon (Shannon 1948) index combines the total number of OTUs (species richness) with the relative abundance of OTUs (species evenness) (Johnson and Burnet 2016), to estimate within sample diversity. The Simpson (Simpson 1949) index describes the probability that randomly drawing two reads from a sample will result in the same OTU (Li *et al.* 2012), where communities with a more even distribution will have a higher index. Beta diversity, also known as differentiation diversity (Jurasinski *et al.* 2009), is described as "the extent of change of community composition" (Whittaker 1960) and is based on pairwise dissimilarity matrices and assessed *via* clustering analysis (Sudarikov *et al.* 2017).

Alpha and beta diversity measures have been successfully used in several ecology, biodiversity and conservation studies (Jurasinski *et al.* 2009). Previous studies investigating sea urchin bacterial communities have been focussed on body surface bacteria (Becker *et al.* 2007, 2008), as previously mentioned, as well as on the gut microbiome of the sea urchins, *Lytechinus variegatus* and *Paracentrotus lividus* (Meziti *et al.* 2007; Hakim *et al.* 2015, 2016), where it was observed that their food source and surrounding environments, such as the sediments that they reside on, harbours a diverse bacterial community. In both natural and cultured sea urchins, the major bacterial genera in the gut included *Arcobacter*, *Vibrio*, *Photobacterium*, *Propiogenium* and *Ferrimonas* (Meziti *et al.* 2007; Hakim *et al.* 2015, 2016). These studies suggest that highly abundant OTUs are likely not harmful to sea urchins as they are consistently predominant across various samples (Hakim *et al*. 2015). However, many bacteria found in aquatic environments are potentially pathogenic and can result in great economic losses in aquaculture environments (Tajima *et al.* 1998). Therefore, by assessing bacterial community diversity, structure and interactions, inferences regarding animal health and potential risk factors can be made.

1.6. Rationale, aims and objectives

1.6.1. Problem statement and rationale

The collector sea urchin, *T. gratilla*, has recently been identified as having potential for aquaculture production in South Africa. Further investigation is required within a South

African context, as it is beneficial to utilise the most genetically diverse populations to establish aquaculture practices. The degree of the genetic diversity present in the progenitor natural population impacts the long-term (response to disease and changes in environmental conditions) and short-term (response to selection) responses of subsequent generations in aquaculture environments. However, a genetic stock assessment of wild *T. gratilla* has not been conducted and could have implications as a founder population for future commercial production. Furthermore, sea urchins reproduce by means of broadcast spawning, which often results in differential parental contributions to subsequent generations in cultured environments. This reproductive competition could cause a decline in genetic diversity when a limited number of the total broodstock successfully contribute to subsequent generations. Once a cultured population is established, the effects of differential parental contributions should be evaluated to ensure that the genetic variation present in the natural locations is being retained in the cultured population, as this could have implications for the genetic management of commercial populations and future selective breeding. There are various biological factors, such as sperm, egg and gonad quality, that could affect this parental skew that could be influenced by broodstock conditioning (feeding regimes), however, this remains to be investigated for *T. gratilla*. Reproductive performance of broodstock could be further evaluated by incorporating gonad and gamete quality information with their parental contributions to subsequent generations, as well as with the performance of offspring in their larval and juvenile life stages. Lastly, in aquaculture farms where stocking densities exceed that of natural environments, an increased risk for infectious diseases has been documented. Economic losses in aquaculture environments have been observed for sea urchins, as various species, including *T. gratilla*, are susceptible to bald sea urchin disease. This disease is thought to be caused by bacteria when there is a mechanical abrasion on the sea urchin body surface, but it is likely that the bacterial species involved in this disease are not limited to those identified by previous studies. This South African aquaculture environment has also experienced bacterial disease outbreaks, where the sea urchins displayed lesions characteristic of bald sea urchin disease on their body surfaces, therefore, the bacterial communities associated with these animals should be further investigated.

1.6.2. Study aim and objectives

The overall aim of this study is to assess genetic aspects of aquaculture practices for *T. gratilla* using a multidisciplinary approach, through the genetic evaluation of natural

populations (chapter 2), parentage and genetic diversity assessment of cultured *T. gratilla* cohorts (chapter 3), the assessment of reproductive- and offspring performance after broodstock conditioning (chapter 4), and a metagenomic analysis of body surface bacterial communities of animals from natural locations along the South African coast, as well as animals of different health statuses from an aquaculture environment (chapter 5).

Chapter 2 aimed to quantify genetic diversity and population structure of *T. gratilla* populations along the eastern coast of South Africa, to test the alternative hypotheses of panmixia versus population stratification in South Africa. Through the application of 30 species-specific microsatellite markers and various software packages, genetic variation was assessed, and the most genetically diverse geographical location could be chosen as a broodstock collection site for future aquaculture practices. In chapter 3, these microsatellite markers were applied in cultured cohorts to assess parental contributions, where parentage assignment was used as a proxy for reproductive success. The aim of this chapter was to test the hypothesis of differential parental contributions and corresponding reductions in genetic variation in a trial aquaculture system, to aid in the eventual implementation of sustainable breeding programmes when sea urchin aquaculture practices are established. Chapter 4 aimed to assess the effects of various diets on the reproductive performance of *T. gratilla* broodstock. Various traits that could influence reproduction, such as body size, gonad- and gamete morphology, as well as egg energetic components and fatty acids, were evaluated. A factorial breeding design was employed to lessen the bottleneck effects associated with establishing cultured populations to preserve the genetic diversity of the progenitor populations. Thereafter, larval growth, parental contribution and offspring phenotypic performance was assessed to aid in the selection of feeding regimes when conditioning sea urchins for reproductive purposes. In chapter 5, bald sea urchin disease was investigated after an outbreak in the studied aquaculture environment. Through next-generation sequencing of hypervariable 16S rDNA regions and bioinformatics approaches, this chapter aimed to characterise the bacterial communities on *T. gratilla* body surfaces of healthy, stressed and diseased animals obtained from an aquaculture environment, as well as animals collected from different natural environments along the east coast of South Africa to provide insight on the bacterial communities associated with this species, as well as with this balding disease.

By assessing various aspects of *T. gratilla* that could contribute to successful future aquaculture practices, such as population- and quantitative genetic aspects, as well as a disease that could result in losses in this emerging industry, this study could contribute to

future economic growth, as well as conservation of this natural resource along the South African coast.

Chapter 2

Genetic diversity and population connectivity of *Tripneustes gratilla* along the eastern coast of South Africa

Abstract

The sea urchin, *Tripneustes gratilla*, has been identified as a species with potential for aquaculture production in South Africa. The species' roe is considered a culinary delicacy in Asia and Europe. However, *T. gratilla* remains genetically uncharacterised in South Africa. Therefore, the purpose of this chapter was to provide baseline genetic information, consisting of estimates of genetic diversity and population stratification that could aid in future sustainable use of the urchin resource. A total of 22 species-specific microsatellite markers were used for the genetic characterisation of *T. gratilla* samples from Haga Haga, Coffee Bay, Hibberdene, Ballito Bay and Sodwana Bay along the South African coast. A moderate level of genetic diversity was observed, with an average number of alleles of 7.89 and an average effective number of alleles of 6.57, as well as an average observed heterozygosity of 0.55. Population differentiation tests showed that the geographically representative samples form part of a single large, interbreeding population with a global F_{st} estimate of 0.02 ($P > 0.05$). This is likely explained by high levels of gene flow between these locations caused by extensive larval dispersal during the planktonic larval stage. The panmixia observed within these natural populations indicate that they could be managed as a single genetic stock.

2.1. Introduction

Tripneustes gratilla (Linnaeus, 1758), also referred to as the collector sea urchin, forms part of the phylum Echinodermata and family Toxopneustidae (Toha *et al*. 2014). These animals are found on both deep and shallow ocean floors, predominantly in the Indo-Pacific Ocean, where they play an integral role in nutrient cycling and in controlling invasive macroalgae and seagrasses within tropical marine ecosystems (Stimson *et al*. 2007; Casilagan *et al*. 2013; Thomsen *et al*. 2013). In recent years, the importance of this species has increased, due to the economic potential of sea urchin production and demand for high quality sea urchin gonads (roe) (Casilagan *et al*. 2013; Cyrus *et al*. 2014, 2015a, b).

Sea urchin roe is considered a culinary delicacy across Asia and Europe (Rahman *et al*. 2014), where the high market value of approximately US$600/kg for high quality gonads (Explorations Unlimited Incorporated 2006), and subsequent over-exploitation of natural populations in several countries, including Chile, Canada, Ireland, Japan and the Philippines, has resulted in a need for sustainable management strategies (Andrew *et al*. 2002; McBride 2005; James *et al*. 2016). In South Africa, *T. gratilla* has been identified as the most commercially viable sea urchin species (Cyrus *et al*. 2014, 2015a, b), as this species spawns easily in captivity, has a fast growth rate of approximately nine months to a marketable size and can produce high-quality gonads that are large enough to be used in the food industry (Juinio-Meñez *et al*. 2008; James and Siikavuopio 2012).

However, many South African marine species, including *T. gratilla*, remain genetically uncharacterised, and an understanding of the genetic diversity and population structure within and between natural populations is necessary to efficiently manage this species within both a conservation and aquaculture context. The degree of genetic diversity not only impacts the short-term fitness of individuals within the population, but also affects the long-term evolutionary potential and adaptability of both natural and cultured populations to changes in environmental conditions (Frankham *et al*. 2003; Markert *et al*. 2010). Populations could be representative of genetically distinct units that represent different evolutionary and adaptive potentials. Introducing gene flow between genetically differentiated stocks can result in reduced fitness, undesirable responses to environmental stresses, mal-adaptations and outbreeding depression (Coupé *et al*. 2011; Rhode *et al*. 2012).

High levels of gene flow are expected between natural sea urchin populations, as these animals reproduce by means of broadcast spawning and the larval stage can last for up to 52 days (Juinio-Meñez *et al*. 1998), resulting in the widespread dispersal of larvae of approximately 116 to as far as 1060 km (Bernardo 2011). Additionally, these animals can have extended larval stages, lasting for up to seven months, as a result of food scarcity or unfavourable environmental conditions that cause growth-related genes to be downregulated (Carrier *et al*. 2015). High levels of gene flow are further supported by previous sea urchin studies in *T. gratilla* and *T. ventricosa* that found little genetic differentiation between populations that were geographically distant, such as those occurring in the Pacific and Indian oceans, respectively (Lessios *et al*. 2003; Juinio-Meñez *et al*. 2008; Coupé *et al*. 2011; Casilagan *et al*. 2013; Toha *et al*. 2014). Similarly, panmixia is expected in *T. gratilla* populations along the South African coast.

Regardless of the high gene flow potential of the species, physical and environmental barriers could prevent gene flow and result in population substructure. Although limited data are available for broadcast spawning animals along the eastern coast of South Africa, barriers to gene flow have been observed for the patellid limpet (*Scutellastra granularis*, previously known as *Patella granularis*) (Ridgway *et al.* 1998), which has a similar reproductive strategy to sea urchins. In this region, larval distribution could be affected by the Agulhas current and its recirculation and retroflection regions (Figure 2.1; Teske *et al*. 2011; Murray 2012). The river systems along the eastern coast could affect *T. gratilla* larval recruitment success, as *Tripneustes* spp. are sensitive to salinity (Cowart *et al.* 2009; Delorme and Sewell 2014; Parvez *et al*. 2018; Metaxas 2020).

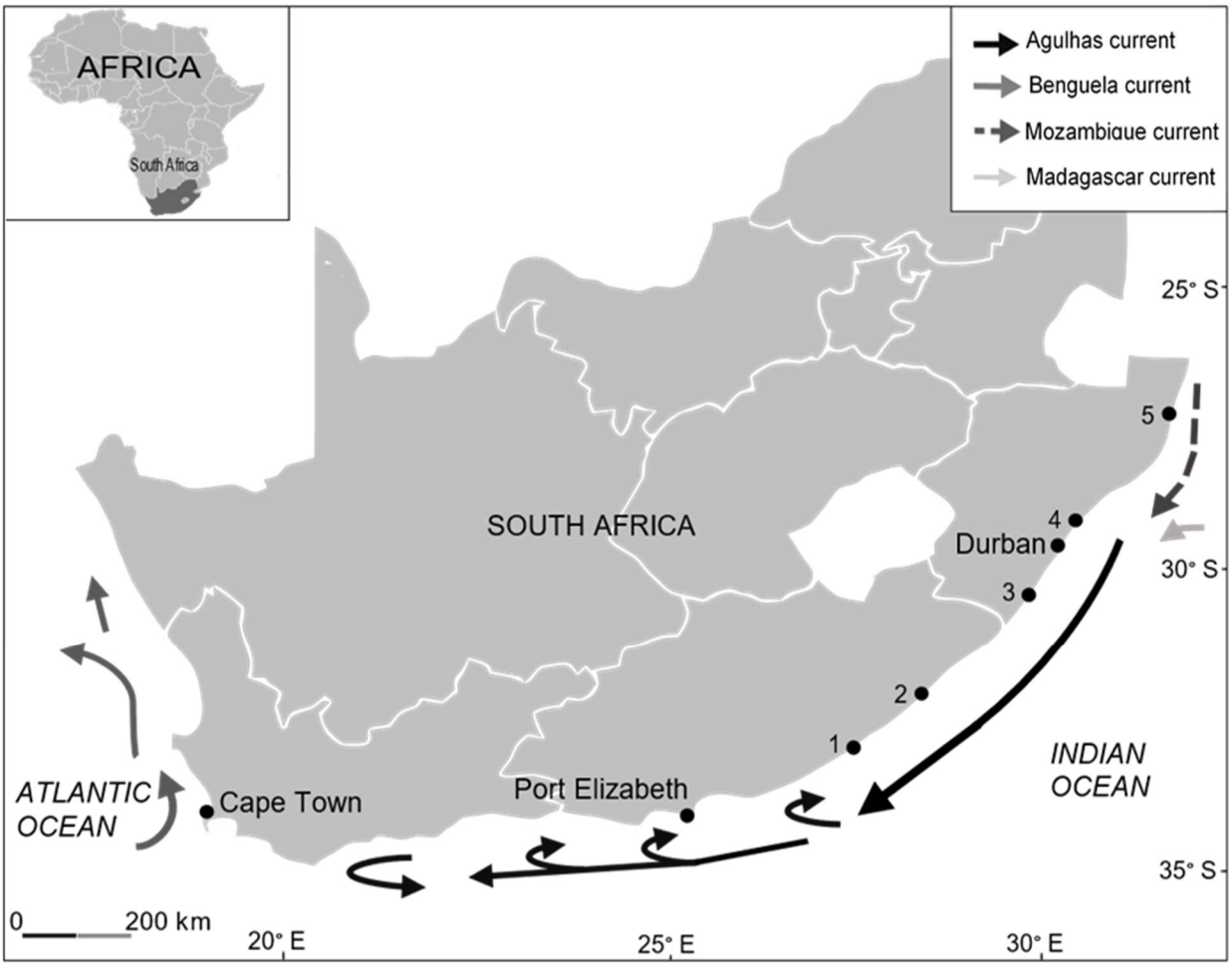


Figure 2.1. Map of South Africa where major currents along the eastern coast (Adapted from Walker 1989) and sampling locations (1 – Haga Haga; 2 – Coffee Bay; 3 – Hibberdene; 4 – Ballito Bay; 5 – Sodwana Bay) are depicted.

Therefore, the aim of this chapter was to characterise and quantify the genetic diversity within and among natural *T. gratilla* populations along the eastern coast of South Africa by means of microsatellite markers and to test the alternative hypotheses of panmixia versus population structuring in the region.

2.2. Materials and methods

2.2.1. Sampling and DNA extraction

Population representative samples from Haga Haga (12) and Coffee Bay (29) in the Eastern Cape Province, and Hibberdene (34), Ballito Bay (19) and Sodwana Bay (29) in the KwaZulu-Natal Province (Figure 2.1), were collected by South African Department of Environment, Forestry and Fisheries (DEFF) personnel using a DEFF permit for the purposes of scientific investigation or practical experimentation in terms of Section 83 of the Marine Living Resource Act (RSA 1998). All gonad tissue samples were placed in 1 mL of 70% ethanol until DNA was extracted from approximately 5 mg of tissue using a modified Cetyl Trimethylammonium Bromide (CTAB) method (Saghai-Maroof *et al*. 1984), and re-suspended in 20 µL MilliQ water. The CTAB method was modified using 300 µL extraction buffer with doubled reagent concentrations [1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 0.2% (v/v) 2-beta-mercaptoethanol], and then adding equal volumes of chloroform:isoamylalchohol (24:1). DNA quantity and quality was evaluated using a NanoDrop 2000 spectrophotometer. Working stock solutions of 20 ng/µL were prepared and stored at -4°C until further use.

2.2.2. Multiplex assay design

A total of 30 species-specific autosomal microsatellite markers were identified from literature (Carlon and Lippé 2007; Wainwright *et al*. 2012) and primers were fluorescently labelled with one of the following dyes: FAM, VIC, PET, or NED. This resulted in a panel of 24 markers being grouped into multiplex reactions according to annealing temperature and fluorescent dye (Appendix A; Table S2.1). Polymerase chain reactions (PCRs) were performed in 10 µL reaction volumes, each containing 20 ng template DNA, 5 µL KAPA2G Multiplex kit (KapaBiosystems) and 10 µM forward and reverse primer, respectively. A negative control with no template DNA was included for each multiplex assay. The PCR conditions were as follows: an initial denaturation step of 3 minutes at 95°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at the respective primer annealing temperatures (Carlon and Lippé 2007; Wainwright *et al*. 2012), and 1 minute at 72°C, followed by a final extension step of 10 minutes at 72°C. Products were size-separated on a 1.5% agarose gel, along with a 1 kilobase (kb) KAPA universal ladder (KapaBiosystems), to verify amplification of the correct size fragments. Where insufficient or no amplification was observed, annealing temperatures were adjusted accordingly (Appendix A; Table S2.1). Following multiplex

optimisation, the resulting six multiplexes (Appendix A; Table S2.1) were used to genotype a total of 123 *T. gratilla* individuals. Successfully amplified PCR products were separated *via* capillary electrophoresis at the Central Analytical Facilities (CAF) DNA sequencing unit, Stellenbosch University, using the LIZ500 internal size standard. Genotyping was performed through fragment length calling and allele binning using Peak Scanner v1 (Applied Biosystems) and Autobin v09 (Salin 2013).

2.2.3. Data analysis

MicroChecker v2.2.3 (Van Oosterhout *et al*. 2004) was used to test for genotyping errors caused by null alleles, allele stuttering or large allele dropout, where null allele frequencies were estimated according to the method of Brookfield (1996). Marker neutrality was assessed by implementing the F_{st} outlier method (50 000 simulations assuming the infinite allele mutation model, with a confidence interval of 0.95 and false discovery rate of 0.1) in Lositan v1.44 (Beaumont and Nichols 1996; Antao *et al*. 2008). Departures from Hardy-Weinberg equilibrium were evaluated using the exact probability test (1000 dememorisations, 100 batches, 1000 iterations per batch) in GenePop v4.2 (Raymond and Rousset 1995; Rousset 2008). Furthermore, an exact test (1000 dememorisations, 100 batches, 1000 iterations per batch) was implemented in GenePop to calculate the probability of linkage disequilibrium between all loci pairs. Lastly, the polymorphic information content (PIC) for each marker was calculated using MsatTools (Park 2001) to determine marker informativeness.

Genetic diversity estimates, including effective number of alleles (A_e), observed (H_o) and expected heterozygosity (uH_e), Shannon's information index (I), and fixation index (F), were calculated in GenAlEx v6.5 (Peakall 2006). To correct for sampling bias caused by the different number of samples for each of the respective population representative groups, HP-rare (Kalinowski 2005) was used to calculate allelic richness (A_r) and the number of private alleles observed. A Kruskal-Wallis test was performed in XL statistics v2016.5 (Carr 2010) to evaluate the significance ($P < 0.05$) of the variation in genetic diversity estimates between any of the sampling sites. To estimate the degree of inbreeding within populations, mean pairwise relatedness (r) for each sampling site was calculated according to the method of Queller and Goodnight (1989) (1000 permutations), as well mean inbreeding coefficients (F_{is}) using the method of Robertson and Hill (1984), in GenePop.

Effective population sizes (N_e) were calculated in NeEstimator v2.01 (Peel *et al*. 2004), implementing the linkage disequilibrium (LD) method (minimum allele frequency of 0.02). Bottleneck v1.2.02 (Cornuet and Luikart 1997) was used to assess whether any of the geographical populations have experienced recent reductions in effective population size by calculating the expected heterozygosity from allele frequencies and comparing this estimate to the expected equilibrium heterozygosity (1000 iterations). Three mutational models were applied to evaluate mutation drift equilibrium: the infinite allele model (IAM), stepwise model (SMM) and two-phase model (TPM) with 95% single-step mutations and 5% multiple-step mutations (multiple-step mutation variance of 12; significance testing: Wilcoxon's test).

Population structure and genetic distance was evaluated by calculating pairwise F_{st} values (Significance testing: 1000 permutations, $P < 0.01$, with Bonferonni correction at the 5% nominal level) and by performing a locus-by-locus hierarchical analysis of molecular variance (AMOVA, significance testing: 1000 permutations, $P < 0.05$), as well as by performing a principal co-ordinates analysis (PCoA) in GenAlEx. Geographical distances between sampling sites were estimated with the 'find path' tool in GoogleEarthPro v7.3.1.4507 (Google Inc.) and a Mantel test (Mantel 1967) was used to test for isolation by distance (IBD) in GenAlEx (999 permutations) by assessing the relationship between geographic distance (km) and genetic distance (F_{st}). Lastly, a Bayesian analysis was performed in the programme Structure v3.2.4 (Pritchard *et al*. 2000) to detect the number of genetic clusters present in the sample set, assuming the admixture model and correlated frequencies (10 replicates across $K = 1$ to $K = 6$, with each run consisting of 500 000 iterations and an initial burn-in phase of 50 000 iterations), as this is considered optimal for detecting population structure in natural populations (Falush *et al.* 2003). The most likely number of clusters (K) was identified using the programme StructureSelector (Li and Liu 2017), implementing the Puechmaille (Puechmaille 2016) and Evanno (ΔK) methods (Evanno *et al*. 2005), and finally, the programme Clumpak (Kopelman *et al*. 2015) was used to visualise the Structure plot.

2.3. Results and discussion

2.3.1. Genetic diversity

Analyses performed on MicroChecker showed no evidence for allele stuttering or allele dropout resulting in genotyping errors, for any of the markers. The Lositan F_{st} outlier test showed that marker *Tgr-B11* was potentially under directional selection and therefore it was

excluded from subsequent analyses. Genotypic patterns associated with null alleles, as well as high fixation indices (-0.29 ± 0.02; 0.86 ± 0.02; Appendix A; Table S2.2) were detected at several loci, with null allele frequencies ranging between -0.01 and 0.35. Among these markers, *Tgr-D128* was excluded from subsequent analyses as frequent PCR amplification failure and significant departure from Hardy-Weinberg equilibrium ($P < 0.001$) was observed. Furthermore, significant departures from Hardy-Weinberg equilibrium ($P < 0.001$) were observed at several loci (10 – 14) within each geographical population representative group (Appendix A; Table S2.2). Significant linkage disequilibrium ($P < 0.001$) was detected at only six of the 276 pairs of loci: *Tgr-C11–Tgr-B11*, *TG07–TG02*, *TG01–Tgr-24*, *Tgr-A11–TG20*, *TG61–Tgr-D134* and *TG55–Tgr-D134*, suggesting that random assortment is occurring at the majority of the loci pairs. However, excluding these markers from further analyses did not affect the outcome, therefore they were included in downstream applications, resulting in a final panel of 22 microsatellite markers being utilised for genetic data analysis.

Estimates of genetic diversity displayed a moderate number of alleles (A_n), when considering the range of average estimates (7.16 ± 0.78; 8.44 ± 0.81), as well as a low to moderate effective number of alleles (A_e) (5.53 ± 0.56; 7.37 ± 0.81) throughout the population representative groups (Figure 2.2; Appendix A; Table S2.2). These results are comparable to previous studies in *T. gratilla*, using the same microsatellite markers, in Hawaii (Oahu and Kauai), the Philippines (West coast of Luzon) and the Indonesian archipelago, where the number of alleles ranged from 5 – 21, 3 – 24 and 5 – 25, respectively (Carlon and Lippé 2007; Wainwright *et al*. 2012). Furthermore, these previous *T. gratilla* studies found high average expected heterozygosity values of 0.82, 0.83 and 0.81, and lower average observed heterozygosity values of 0.63, 0.60 and 0.64, respectively. They attribute this heterozygote deficit and subsequent overall deviation from Hardy-Weinberg expectations to the presence of null alleles, inbreeding or selection (Wainwright *et al*. 2012; Casilagan *et al*. 2013). Similarly, in the current study, the expected heterozygosity was high (uH_e) (0.76 ± 0.04; 0.83 ± 0.03), however a moderate degree of observed heterozygosity (H_o) (0.52 ± 0.04; 0.61 ± 0.03) was found in all sampling groups (Appendix A; Table S2.2).

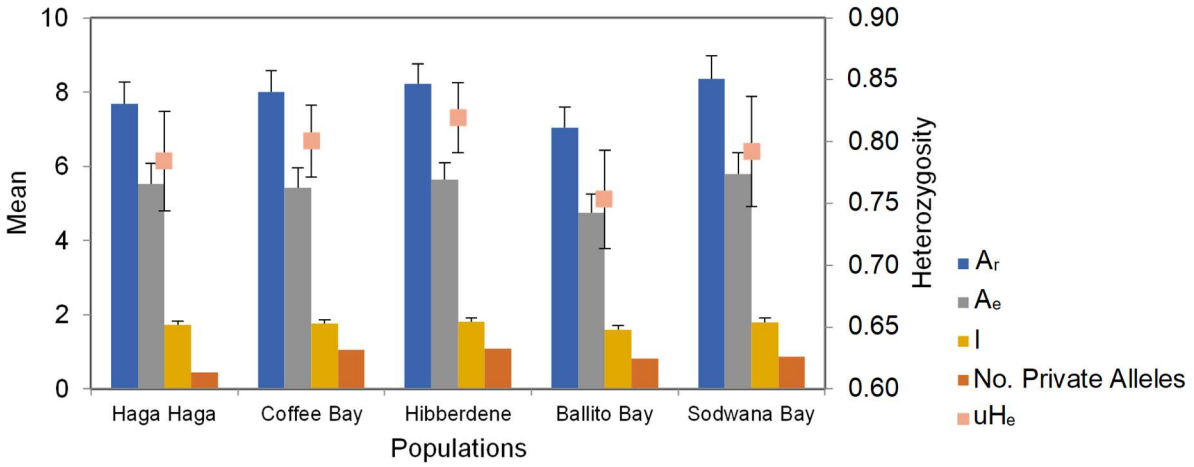


Figure 2.2. Genetic diversity statistics for individuals sampled from different sampling sites along the South African coast (A_r allelic richness, A_e effective number of alleles, I information index, number of private alleles, uH_e unbiased heterozygosity).

This observed heterozygote deficiency is most likely caused by the presence of null alleles. Alternatively, studies suggest that populations that diverged from a genetically homogenous stock during their free-moving larval stage will have a higher homozygosity during their dormant fully-grown stage, which is known as a temporal Wahlund effect (Karlsson and Mork 2005; Suez *et al*. 2013). It has also frequently been observed that broadcast spawners with an extended larval stage will have variable reproductive success and survival during larval and early settlement, resulting in this homozygosity excess (Watts *et al*. 1990; Addison and Hart 2004; Hedgecock *et al*. 2011; Casilagan *et al*. 2013; Lawrence and Agatsuma 2013). In this study, it was found that the only geographical population showing significant differences (Kruskal-Wallis test, $P < 0.05$) in genetic diversity estimates at the respective loci was Ballito Bay (Figure 2.2). Further supporting a similar degree of genetic diversity within these geographically representative populations, are the very low private allele frequencies (0.71 ± 0.17; 1.36 ± 0.39) that were observed (Figure 2.2), indicating that the majority of alleles are shared by at least two population representative groups.

The overall low to moderate genetic diversity could potentially be due to inbreeding, based on the high F_{is} values estimated for each population (Figure 2.3), as well as the significant global F_{is} value of 0.34 ($P < 0.05$, AMOVA, Table 2.1). However, all populations showed a very low degree of relatedness, with relatedness (r) estimates of approximately zero (Figure 2.3). Although a significantly higher degree of relatedness (mean estimates beyond the 95% CI's) was observed in the Haga Haga and Ballito Bay populations, the F_{is} estimates of these

two populations do not significantly differ from the other populations ($P > 0.05$, Figure 2.3), indicating that inbreeding is likely not occurring. A low degree of relatedness is commonly observed in organisms characterised as broadcast spawners and the high F_{is} estimates could therefore more likely be attributed to (i) the overall heterozygote deficit; a consequence of the temporal Wahlund effect, or (ii) to the presence of null alleles.

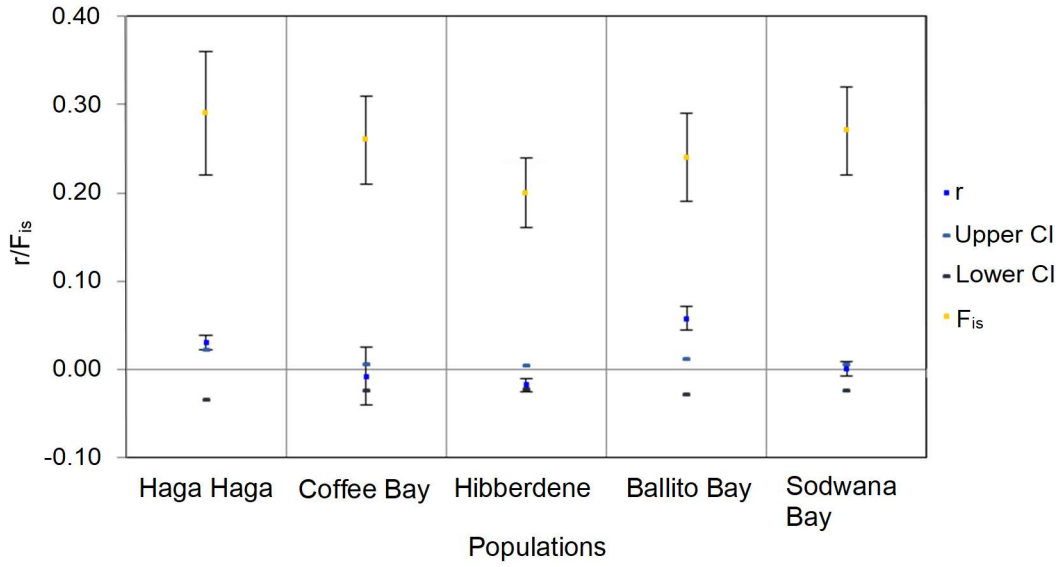


Figure 2.3. Pairwise relatedness (r) for each cohort showing limited relatedness across the respective geographically representative populations, where the corresponding upper and lower bounds of the 95% confidence intervals (CI), as well as inbreeding coefficients (F_{is}) are indicated. Standard error from the mean is indicated for both variables.

Table 2.1. Analysis of molecular variance (AMOVA) across natural *Tripneustes gratilla* populations.

Source of variation	Sum of squares	Estimated variance	% variation	Fixation indices
Among sampling sites	79.57	0.17	2	F_{st} 0.02
Among individuals	1415.49	3.02	33	F_{is} 0.34*
Within individuals	733.00	5.96	65	F_{it} 0.35*
Total	2228.06	9.14		

*Statistically significant at $P < 0.05$

2.3.2. Population differentiation

In the studied *T. gratilla* populations, the effective population sizes can be considered very large, as estimates were above the theoretical critical minimum (1000) (Frankham *et al*. 2003), when treated as separate populations (Table 2.2) or as a single population. The large effective population size estimates could also explain the genetic similarities observed in these populations, as larger population sizes are associated with less variation in allele

frequencies over time (genetic drift), resulting in a similar degree of genetic diversity and responses to long-term evolutionary forces (Wang 2005).

Table 2.2. Bottleneck (Wilcoxon) test under the infinite allele model (IAM), two-phase model (TPM) and stepwise mutation model (SMM), as well as estimates of effective population size (N_e) calculated using the linkage disequilibrium (LD) method, where the 95% confidence intervals (CI) are indicated.

Parameter	Haga Haga	Coffee Bay	Hibberdene	Ballito Bay	Sodwana Bay
Sample size (*n*)	12	29	34	19	29
Wilcoxon test					
IAM	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
TPM	n.s.	n.s.	n.s.	n.s.	n.s.
SMM	n.s.	n.s.	< 0.05	n.s.	n.s.
N_e	2290.60 (89.1 − ∞)	∞ (∞ − ∞)	∞ (∞ − ∞)	∞ (∞ − ∞)	∞ (∞ − ∞)

Additionally, there was little evidence of a recent reduction in effective population sizes across all five population groups under the TPM (Table 2.2), which is most likely the true probability estimate for microsatellite markers. This mutation model incorporates the SMM mutational process where single repeats are added or removed during each mutational event, but tolerates mutations of a larger magnitude and therefore represents an intermediate between the SMM and IAM (Piry *et al*. 1999). However, under the IAM, significant signatures of genetic bottlenecking were detected (Table 2.2). Similarly, this was also found by previous *T. gratilla* studies in over-exploited populations implementing the IAM (Casilagan *et al*. 2013). These studies speculate that the reduction in effective population size is most likely due to undocumented historical anthropogenic or environmental effects, possibly during the late Pleistocene as observed in the barnacle, *Tetraclita serrata*, which could have resulted in patterns of genetic diversity that do not follow mutation-drift equilibrium expectations (Casilagan *et al.* 2013; Reynolds *et al.* 2014).

The *T. gratilla* populations along the South African coast were found to be genetically undifferentiated, based on the AMOVA (Table 2.1) that attributed 65% of the genetic variation to differences within individuals, 33% to differences among individuals and only 2% to differences among the respective sampling sites. Furthermore, the AMOVA showed a low global F_{st} estimate of 0.02 ($P > 0.05$). Similarly, the pairwise F_{st} values (Table 2.3) ranging from 0.00 ($P > 0.05$) to 0.033 ($P = 0.022$), indicated limited population differentiation, with small F_{st} estimates amongst all population pairs. Although many of these pairwise estimates were statistically significant, they are considered to be indicative of high gene flow between the various geographical locations, as the Mantel test, showing no correlation between genetic and geographic distances (Figure 2.4; $r^2 = 0.17$, $P = 0.11$), further supports a high degree of population connectivity.

Table 2.3. Pairwise F_{st} estimates (shaded) and corresponding Bonferroni corrected *P*-values (unshaded).

Population	Haga Haga	Coffee Bay	Hibberdene	Ballito Bay	Sodwana Bay
Haga Haga	-	0.132	0.022	0.088	0.022
Coffee Bay	0.012	-	0.022	0.022	1.000
Hibberdene	0.027	0.019	-	0.022	0.022
Ballito Bay	0.014	0.022	0.033	-	0.022
Sodwana Bay	0.013	0.000	0.023	0.014	-

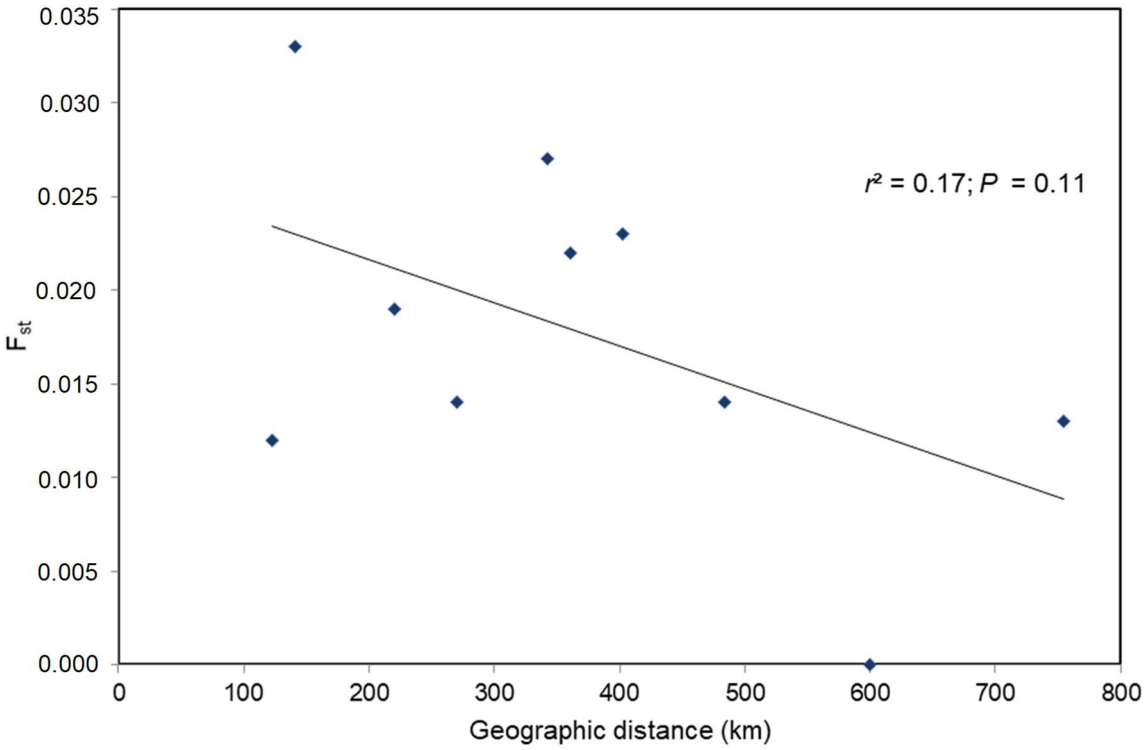


Figure 2.4. Isolation by distance scatterplot showing no correlation (r^2 = 0.17; *P* = 0.11) between geographic (km) and genetic (F_{st}) distances of *Tripneustes gratilla* populations along the eastern coast of South Africa.

When performing a Bayesian analysis in the program Structure, two genetic clusters (*K* = 2) were inferred by placing samples into groups whose members share similar patterns of variation based on variant frequencies (Appendix A; Figure S2.1). The samples from the respective locations had similar genotype membership patterns (Figure 2.5), which shows that samples share similar patterns of variation at the markers included in this study. This suggests that the stock along the eastern coast of South Africa possibly originated from two ancestral populations, but are representative of a single, panmictic genetic stock. This is further supported by the *K* = 3 plot, which was included for comparison, showing similar membership probabilities across the respective populations (Figure 2.5). The overall low degree of genetic differentiation can likely be attributed to high levels of gene flow, probably caused by the strong southward flow of the Agulhas current, as well as the retroflection

regions of the Agulhas current resulting in bidirectional gene flow (see Figure 2.1; Teske *et al.* 2011). Larvae drift in these current systems, where after they settle and become adult sea urchins. Although Structure is widely applied in both conservation and aquaculture genetics, as it is able to differentiate between populations with similar allele frequency distributions, it is possible that *K* is erroneously inferred in panmictic populations with F_{st} estimates below 0.03 (Latch *et al.* 2006; Janes *et al*. 2017). Therefore, given the low genetic differentiation observed from assessments throughout this study, it is likely that these locations form a single, interbreeding population.

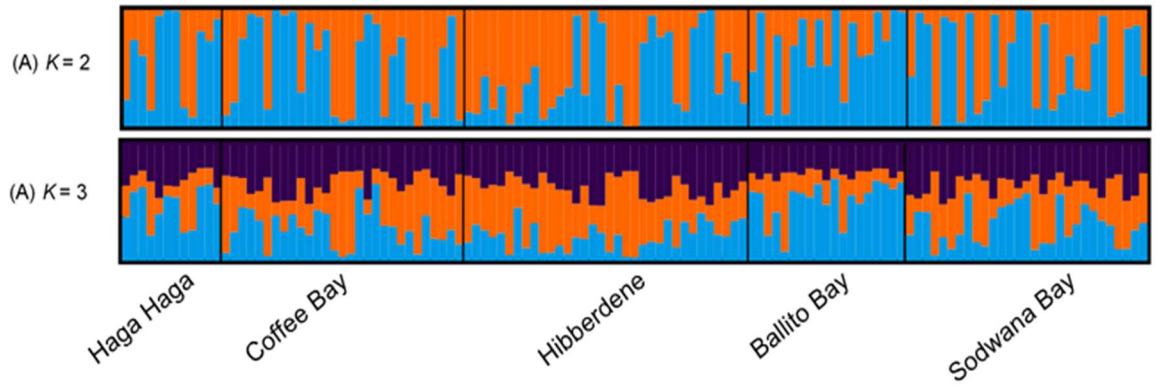


Figure 2.5. Structure bar plots, where (A) *K* = 2 is the most likely number of genetic clusters and (B) *K* = 3 is included for comparison. Each cluster is represented as a different colour and each bar represents an individual within each geographic location.

This lack of population structure is further corroborated by the principal co-ordinate analysis (Figure 2.6), where no distinct clusters could be identified and a large degree of overlap is evident for most of the samples. This absence of genetic differentiation between geographically distinct sampling sites is consistent with previous population genetic studies in *T. gratilla*, which reported F_{st} values ranging from 0.001 ($P = 0.719$) to 0.063 ($P < 0.05$) (Malay *et al*. 2000; Lessios *et al*. 2003; Addison and Hart 2004; Juinio-Meñez *et al*. 2008; Coupé *et al*. 2011; Casilagan *et al*. 2013; Toha *et al*. 2014).

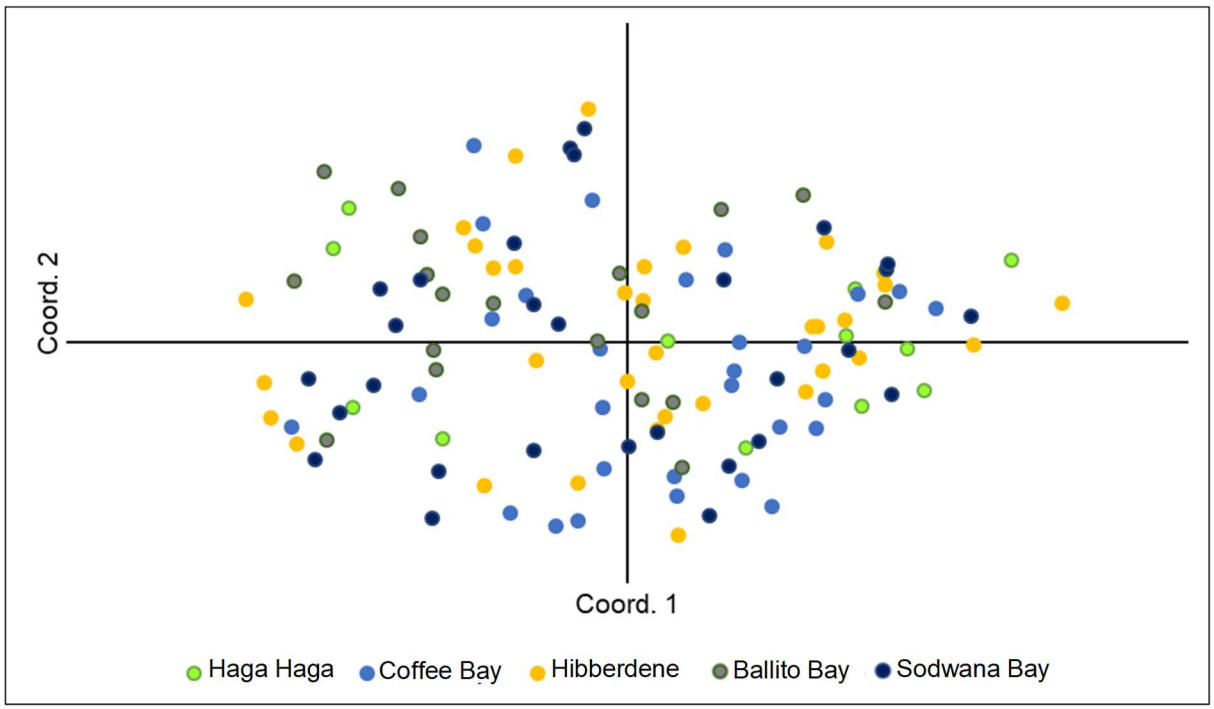


Figure 2.6. Principle co-ordinates analysis (PCoA), indicating little genetic differentiation between *Tripneustes gratilla* individuals from the different geographically representative populations.

Although there have been limited studies along the eastern coast of South Africa in terms of population structure, studies in the sea urchin *Strongylocentrotus purpuratus* in North America have found that local oceanographic features can result in genetic differentiation between populations (Olivares-Bañuelos *et al*. 2008). Interestingly, in the present study, this was observed for populations that were in closer proximity to each other, Hibberdene and Ballito Bay (Figure 2.1, Table 2.3). The effects of the Mozambique and Madagascar currents on the Agulhas current, as well as large river systems that occur in this area, such as the Tugela river (Teske *et al*. 2011), could act as barriers to gene flow and result in larval recruitment success being adversely impacted. Additionally, the Durban harbour activities between these populations could potentially be affecting the population dynamics as it has been shown that sea urchins, such as *T. gratilla*, have a lower survival rate in areas disturbed by anthropogenic processes (Gayashan and Jayakody 2012).

2.3.3. Implications for resource utilisation

Establishing aquaculture production of this species will not only be advantageous to the economic growth of South Africa, but can contribute to preventing future overexploitation of this natural resource, both locally and globally. It is important that genetically distinct populations are managed as separate units, especially within aquaculture practices, as it has been shown that the genetic integrity of the cultured populations can be negatively

affected (Roodt-Wilding *et al*. 2010). This can result in reduced survivability within cultured cohorts, as has been observed in studies in the sea urchin *S*. *purpuratus* (Anderson and Hedgecock 2010), and subsequent economic losses. Conversely, cross-breeding distinct populations could result in heterosis (hybrid vigour), which could potentially enhance desirable traits, such as growth, survival rates, stress tolerance or gonad size or colour; traits which might be useful in the aquaculture context, as well as for future selective breeding programmes (Bryden *et al*. 2004).

Based on the results of this study, the populations along the South African coast appear to form a single genetic unit with large effective population sizes, indicating that farms can be established by collecting broodstock from any of the five sampling sites, without adversely impacting the genetic integrity of this future genetic resource. Furthermore, as effective population size represents how successfully individuals are contributing to subsequent generations, using these populations with very large effective population sizes to establish aquaculture practices potentially lowers the risk of inbreeding and losing genetic diversity in subsequent cultured populations (Frankham *et al*. 2003; Mtileni *et al*. 2016).

However, when a limited number of broodstock animals are collected from natural populations, a loss of genetic diversity is often observed when these are used for aquaculture purposes. This can result in a population bottleneck and a subsequent reduction in effective population size, inbreeding, reduced fitness, poor production output and reduced adaptability to environmental stresses and diseases (Dunham *et al*. 2001; Frankham *et al*. 2003; Rhode *et al*. 2012). Additionally, the response to artificial selection and continued genetic improvement are dependent on the level of genetic diversity present (Dunham *et al*. 2001; Pena *et al*. 2010; Rhode *et al*. 2012). Therefore, a sufficient number of animals should be collected from the locations with the most genetically diverse populations. Within the natural *T. gratilla* populations included in this study, a moderate degree of genetic diversity is present when compared to previous studies (Carlon and Lippé 2007; Wainwright *et al*. 2012), indicating that there is potential for the establishment of aquaculture practices in South Africa. Once aquaculture practices are established, it would be useful to genetically characterise both the broodstock individuals and their offspring, as this can indicate whether the aquaculture practices and breeding programmes are optimal and are retaining genetic diversity (Liu *et al*. 2012). Additionally, as sea urchins are broadcast spawners and pedigree data is challenging to track for such species, there is a higher chance of inbreeding and inbreeding depression when subsequent generations are utilised as broodstock.

2.4. Conclusion

In conclusion, the microsatellite data showed that the *T. gratilla* populations along the South African coast appear to form a single genetic unit, with moderate genetic diversity and large effective population sizes. The Agulhas current and its recirculation and retroflection regions, in combination with the extended planktonic larval phase that sea urchins display, is likely resulting in the high degree of gene flow observed in this study. The moderate genetic diversity along the eastern coast of South Africa indicates that any of these populations can be utilised as broodstock collection sites for future aquaculture practices. Future studies should aim at monitoring inbreeding and genetic variability within cultured populations by utilising the highly polymorphic (PIC > 0.50, Appendix A; Table S2.2) microsatellite markers optimised in this study once aquaculture is established.

Chapter 3

Parentage assignment in first generation cultured *Tripneustes gratilla* cohorts

Abstract

Aquaculture practices can result in declines in the genetic diversity observed in progenitor natural populations, resulting in subsequent poor production output, particularly for broadcast spawning animals that frequently display differential parental contributions. Therefore, this chapter aimed to genetically characterise two first generation (F1) cultured *Tripneustes gratilla* cohorts by assessing pedigree relationships, as well as subsequent changes in genetic diversity after a single generation. Genetic diversity analyses, based on 21 species-specific microsatellite markers, showed an overall decrease ($P < 0.05$) in the average number of alleles and effective number of alleles from the natural cohort to the cultured cohorts. This loss of alleles, accompanied by a reduction in effective population size, is likely a consequence of a genetic bottleneck event caused by using a limited number (six) of broodstock animals to establish these cultured populations. Genetic differentiation analyses detected three distinct clusters, with overlap of the broodstock individuals with the respective cultured cohorts. This moderate degree of genetic differentiation was further supported by a moderate global F_{st} estimate of 0.11 ($P < 0.05$), where differences among cohorts account for 10% of the observed variation (AMOVA). Parentage analysis revealed differential parental contributions, where a single female contributed to 70% of the first F1 cultured cohort and a single male contributed to 92% of the second F1 cultured cohort. Therefore, it is likely that there are other factors that could be influencing reproductive success in *T. gratilla* that should be investigated to aid in the implementation of successful breeding programmes.

3.1. Introduction

Tripneustes gratilla is one of approximately 71 sea urchin species that have been identified along the coast of South Africa (Clark and Courtman-Stock 1976; Filander and Griffiths 2014). This highly marketable species has been identified as having potential for aquaculture production in South Africa (Scholtz *et al.* 2013; Cyrus *et al.* 2014). As this species has not been used commercially in this country (Scholtz *et al.* 2013), commercial

aquaculture production can be implemented to avoid the detrimental effects of overharvesting natural populations (Slabbert *et al.* 2009; Stefánsson *et al.* 2017).

Early echinoculture (sea urchin aquaculture) practices in other parts of the world played a role in the decline of natural stocks, as adults were collected from natural locations, with the animals being maintained or enhanced through dietary approaches to increase gonad quality (McBride 2005; Stefánsson *et al.* 2017). More recently, adults are collected from natural locations, spawning is induced and larvae are reared in an aquaculture facility (McBride 2005; Grant *et al.* 2017; James *et al.* 2017; Stefánsson *et al.* 2017). This strategy has been implemented as a restocking strategy for depleted natural stocks, as well as for the production of sea urchin products, such as gonads (Juinio-Meñez *et al.* 1998; Lawrence and Bazhin 1998; Pante *et al.* 2007; Juinio-Meñez *et al.* 2008a; Grant *et al.* 2017). Additionally, it can be used for the production of superior quality products, as has been implemented for the sea urchin, *Strongylocentrotus intermedius*, in China (Chang *et al.* 2016).

However, as sea urchins are broadcast spawning animals, reproductive success is often highly variable (Levitan 2005), even when held in captivity. This variation in reproductive success, where only a subset of the total number of successfully spawning individuals contribute to the offspring produced, is a result of numerous factors, including the mating scheme implemented (Shuster and Wade 2003), animal densities, sperm and egg quality (Levitan 1995, 2005), as well as sex ratios (Grant *et al.* 2017). Sea urchins are thought to produce approximately one million eggs or 10 – 100 billion sperm during a single spawning event, which further contributes to the differential parental contributions expected from these animals (Levitan 2005; Darszon *et al.* 2006), particularly for males, as a greater number of gametes results in increased reproductive competition amongst animals.

This competition could negatively impact the genetic diversity of subsequent generations, as a result of the population bottleneck caused by a founder event when a limited number of individuals are used to establish cultured populations (Flowers *et al.* 2002; Levitan 2005; Segovia-Viadero *et al.* 2016). This reduction in genetic variation captured in the subsequent cultured populations was observed for the sea urchin *Paracentrotus lividus* after a single generation (Segovia-Viadero *et al.* 2016), as well as for other broadcast spawning animals, such as abalone (Slabbert *et al.* 2009) and oysters (Miller *et al.* 2012). Reduced levels of genetic diversity, lower effective population sizes, as well as an increased probability of inbreeding is often associated with aquaculture practices (Feng *et al.* 2015; Grant *et al.*

2017), particularly when few animals successfully contribute to subsequent generations. This is accompanied by a large variance in family size that can cause micro-evolutionary changes in short periods of time, resulting in genetic differentiation within and between cultured populations, as well as between cultured populations and their progenitor natural population (Grant *et al.* 2017). Over time, this can result in an increase in homozygosity that can affect the overall fitness of organisms, either through the accumulation and expression of deleterious alleles or through the loss of overdominance at homozygous loci (Falconer 1989; Lynch and Walsch 1998). When this occurs, populations have a limited response to environmental changes and diseases, as well as to selection, as a reduced number of unique genetic variants associated with advantageous traits could be present in the cultured population (Grant *et al.* 2017). Although this decline in genetic diversity can be avoided, or at least mitigated by using a greater number of broodstock animals (Slabbert *et al.* 2009), the impact of aquaculture practices has not been investigated in the collector sea urchin, *T. gratilla*. Therefore, parentage assignment, used as a proxy for reproductive success, can be applied to assess the genetic reproductive potential of the broodstock animals and relatedness of offspring (Guerier *et al.* 2012; Liu *et al.* 2012; Yue and Xia 2014).

Furthermore, future selective breeding programmes, likely aimed at the production of superior quality gonads, will benefit from a high degree of genetic diversity (Pante *et al.* 2007). It is hypothesised that the loss of genetic diversity at neutral markers is likely indicative of a decline in the ability to respond to selection, as a corresponding decline in genetic variation at quantitative trait loci will also be expected (Grant *et al.* 2017). Therefore, the capacity for short-term responses to selection is dependent on the additive genetic variance (sum of the effects of numerous alleles on a phenotype) present within a population (Grant *et al.* 2017). International quality standards require the gonads to be a bright yellow or orange colour, palatable, as well as approximately 4 – 5 cm in length (Pante *et al.* 2007; Cyrus 2013; Stefánsson *et al.* 2017). Other important traits for aquaculture species include disease resistance, survival rates, food conversion and growth rates (Elliott 2000; Chang *et al.* 2012; Feng *et al.* 2015; Chang *et al.* 2018). However, the implementation of improvement strategies for these traits can be difficult without any prior knowledge of the degree to which genetic diversity is maintained in an aquaculture environment or the patterns of parental contributions, particularly in high fecundity species, when broodstock are collected from natural populations to establish cultured populations.

Therefore, the aim of this chapter was to test the hypothesis of differential parental contributions and corresponding reductions in genetic variation in a trial aquaculture system, to aid in the eventual implementation of effective/sustainable breeding programmes when sea urchin aquaculture practices are established.

3.2. Materials and methods

3.2.1. Sampling and DNA extraction

Samples were collected by the South African Department of Agriculture, Environment and Fisheries (DEFF) personnel using a DEFF permit for the purposes of scientific investigation or practical experimentation in terms of Section 83 of the Marine Living Resource Act (RSA 1998). A total of 12 broodstock animals that were held at the Marine Research Aquarium in Sea Point, Cape Town, were non-destructively sampled (tube feet). As all broodstock individuals originated from Haga Haga (32°45′4.23″S, 28°16′41.30″E) along the eastern coast of South Africa, individuals collected from this geographical location (n = 38), used in chapter 2, were included in analyses for comparative purposes. Furthermore, two juvenile first generation (F1) cultured cohorts (n = 50, respectively), representative of two independent spawning events, were sampled and measured (diameter in mm) after approximately two months of rearing. Each cultured cohort was produced from a different six broodstock animals, consisting of four females and two males, respectively. Spawning was chemically induced in both sexes by placing animals aboral-side down on Erlenmeyer flasks (filled with 0.2 µM filtered seawater) and injecting animals with 0.5 mL 2 M KCl, where after eggs from all females were collected and combined. Similarly, sperm from all males were collected and combined. Subsequently, eggs and sperm were combined in a 1:100 (egg:sperm) ratio.

Whole juvenile specimens and broodstock tube feet were preserved in 70% ethanol prior to DNA extraction. A modified CTAB DNA extraction method was used, where 300 µL extraction buffer with doubled reagent concentrations [1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 0.2% (v/v) 2-beta-mercaptoethanol], and equal volumes of chloroform:isoamylalchohol (24:1) was added. A NanoDrop 2000 spectrophotometer was used to evaluate DNA quantity and quality. Working stock solutions of 20 ng/µL were prepared and stored at -4°C until further use.

3.2.2. Microsatellite marker amplification and genotyping

A panel of 24 microsatellite markers, labelled with fluorescent dyes (FAM, VIC, PET or NED), were polymerase chain reaction (PCR) amplified in multiplex reactions according to annealing temperature and fluorescent dye (Appendix A; Table S2.1). PCR reactions were performed in 10 µL reaction volumes, each containing 20 ng template DNA, 5 µL KAPA2G Multiplex kit (KapaBiosystems) and 10 µM forward and reverse primer, respectively. A negative control with no template DNA was included for each multiplex assay. The PCR conditions consisted of an initial denaturation step of 3 minutes at 95°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at the respective primer annealing temperatures (Carlon and Lippé 2007; Wainwright *et al*. 2012), and 1 minute at 72°C, followed by a final extension step of 10 minutes at 72°C. Products were then visualised on a 1.5% agarose gel, along with a 1 kilobase (kb) KAPA universal ladder (KapaBiosystems), to verify amplification of the expected size fragments.

PCR products were separated *via* capillary electrophoresis at the Central Analytical Facilities (CAF) DNA sequencing unit, Stellenbosch University, using the LIZ500 internal size standard. A total of 150 *T. gratilla* individuals were genotyped through fragment length calling using GeneMapper v5.0 software (Applied Biosystems).

3.2.3. Data analysis

Genotyping errors caused by null alleles, allele stuttering and allelic dropout at each locus were assessed in Microchecker v2.2.3 (Van Oosterhout *et al.* 2004). Null allele frequencies were calculated implementing the method of Brookfield (1996). An F_{st} outlier test was performed in Arlequin v3.5.1.2 (Excoffier *et al.* 2007) to detect loci deviating from neutrality ($P < 0.05$, 20 000 simulations). Subsequently, an exact test was implemented to assess deviations form Hardy-Weinberg equilibrium expectations (1000 dememorisations, 100 batches, 1000 iterations per batch), as well as to assess linkage disequilibrium between loci pairs (1000 dememorisations, 100 batches, 1000 iterations per batch) in GenePop v4.2 (Raymond and Rousset 1995; Rousset 2008).

Genetic diversity estimates, including the number of alleles (A_n), effective number of alleles (A_e), number of private alleles, observed heterozygosity (H_o) and unbiased expected heterozygosity (uH_e), Shannon's information index (I) and fixation index (F), as well as corresponding standard errors (SE) for each mean, were calculated in GenAlEx v6.5

(Peakall 2006) to assess the changes in genetic variation after a single generation. A Kruskal-Wallis test was performed in XL statistics v2016.5 (Carr 2010) to evaluate the significance ($P < 0.05$) of differences between the diversity statistics of the respective cohorts. Furthermore, the polymorphic information content (PIC) was calculated in MsatTools (Park 2001), and the probability of inclusion (PI) and the probability of exclusion (PE) were calculated in GenAlEx to assess the usefulness of the molecular markers for detecting parental pairs. Furthermore, pairwise relatedness was assessed by calculating mean pairwise relatedness (r) for each cohort using the method of Queller and Goodnight (1989) (1000 permutations) in GenAlEx, as well as mean inbreeding coefficients (F_{is}) using the method of Robertson and Hill (1984) in GenePop, which assesses homozygosity excess as opposed to what is expected under Hardy-Weinberg equilibrium expectations, to estimate the degree of inbreeding within the respective cohorts.

Effective population sizes (N_e) were estimated by means of the linkage disequilibrium (LD) method (minimum allele frequency of 0.02) in NeEstimator v2.01 (Peel *et al.* 2004). Recent reductions or expansions in effective population size were tested for in Bottleneck v1.2.02 (Piry *et al.* 1990), applying the infinite allele model (IAM), the stepwise model (SMM), as well as the two-phase model (TPM) (95% single-step mutations, 5% multiple-step mutations, multi-step mutation variance of 12, significance testing: Wilcoxon's test).

Genetic differentiation between the respective cohorts was assessed by calculating pairwise F_{st} values (1000 permutations, $P < 0.01$, with Bonferroni correction at the 5% nominal level), as well as by performing a locus-by-locus hierarchical analysis of molecular variance (AMOVA, 1000 permutations, $P < 0.05$) and a principal co-ordinates analysis (PCoA) in GenAlEx. A Bayesian clustering analysis was implemented in Structure v3.2.4 (Pritchard *et al.* 2000) to further assess genetic differentiation, where $K = 1$ to $K = 6$ (twice the number of populations) was tested for (10 replicates, 500 000 iterations per run, initial burn-in phase of 50 000 iterations). The most likely number of genetic clusters (K) was identified using the program StructureSelector (Li and Liu 2017), implementing the Puechmaille (Puechmaille 2016) and Evanno (ΔK) methods (Evanno *et al.* 2005). Lastly, the Structure plot was visualised using the program Clumpak (Kopelman *et al.* 2015).

Parentage assignment, as well as the calculation of full-sib proportions, was performed using Colony v2 (Jones and Wang 2010), assuming a polygamous mating scheme, no inbreeding and implementing a full likelihood method (error rate of 0.01). The probability of both the mother and father being amongst the candidate males and females was set at 1

and no candidate male or female was excluded from the dataset. Parental contributions were verified using the exclusion-based method implemented in Vitassign v8.2.1 (Vandeputte and Haffray 2014) with a genotypic error rate 0 – 10%. Size variations of offspring were assessed by calculating the mean diameters (mm) for each family, as well as the standard error for each mean. Statistical significance of size differences between families were assessed in XL statistics by means of a Kruskal-Wallis test.

3.3. Results and discussion

3.3.1. Genetic diversity across cultured cohorts

A total of 150 individuals were genotyped at 24 microsatellite loci, however, markers *Tgr-B11*, *Tgr-D128* and *TG51* were excluded, as the presence of null alleles or allele stuttering was detected. The remaining 21 markers showed no evidence of allele dropout or allele stuttering, although several loci (2 – 14) showed the presence of null alleles in the respective cohorts, possibly causing the overall heterozygote deficiency. However, low null allele frequencies, ranging from -0.12 to 0.32, were obtained (Appendix B; Table S3.1). Furthermore, several loci (6 – 15) deviated from Hardy-Weinberg expectations ($P < 0.05$). Many loci (59 out of 210 locus pairs) were found to be in linkage disequilibrium, which is most likely an artefact of few broodstock animals contributing to subsequent generations. The exact test implemented in Arlequin showed that a single marker, *Tgr-B119*, was potentially under selection ($P < 0.05$). However, removing these markers did not change the outcome of the analyses and therefore, final results are based on a final panel of 21 microsatellite markers (Appendix B; Table S3.1).

The genetic diversity estimates obtained in this study showed a reduction in genetic variation across the majority of the diversity estimates in both cultured cohorts when compared to the natural cohort (Figure 3.1). A significant ($P < 0.05$) reduction was observed for the average number of alleles (A_n) from 13.95 ± 1.41 in the natural cohort to 3.36 ± 0.26 and 3.19 ± 0.26 in the two cultured cohorts, respectively (Figure 3.1). Similarly, the average effective number of alleles (A_e) decreased from 8.15 ± 1.14 in the natural cohort to 1.29 ± 0.08 and 1.21 ± 0.08 in the cultured cohorts, respectively (Figure 3.1). Although no studies evaluating the genetic diversity of cultured populations have been carried out in the sea urchin *T. gratilla*, this pattern is comparable to those found by previous studies in other cultured sea urchin (*Paracentrotus lividus*) populations, where a reduction in the average A_n was observed from

158.50 in the natural cohort to 114.50 in the cultured cohort; and a reduction in A_e was observed from 18.30 in the natural cohort to 13.22 in the cultured cohort (Segovia-Viadero *et al.* 2016). Similarly, studies in cultured abalone (*Haliotis discus hannai* and *Haliotis midae*) populations found reductions in genetic diversity in aquaculture environments (Rhode *et al.* 2014; Chen *et al.* 2017). Given the few broodstock animals used to establish the cultured cohorts in the current study, a reduction in genetic diversity is expected for cultured populations when compared to natural populations.

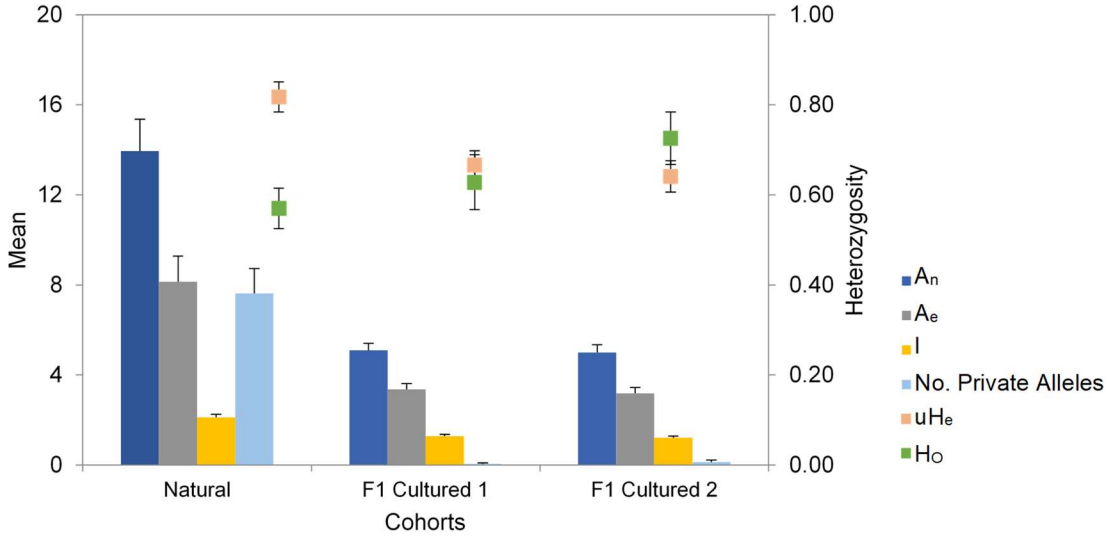


Figure 3.1. Mean genetic diversity statistics for wild versus cultured individuals (A_n number of alleles, A_e effective number of alleles, I information index, number of private alleles, uH_e unbiased heterozygosity, H_o observed heterozygosity).

Furthermore, an overall reduction in the average expected heterozygosity (uH_e) was observed when comparing the natural (0.82 ± 0.03) to the cultured cohorts (0.67 ± 0.03; 0.64 ± 0.04) in the current study. Although the first cultured cohort showed a non-significant ($P > 0.05$) increase in observed heterozygosity (0.63 ± 0.06), there was a statistically significant ($P < 0.05$) increase in observed heterozygosity (H_o) in the second cultured cohort (0.73 ± 0.06) when compared to the natural cohort (0.57 ± 0.05) (Figure 3.1). This transient increase in H_o is generally observed in cultured populations that have experienced a recent genetic bottleneck, as an immediate reduction in heterozygosity is not necessarily expected after a single generation (Araki and Schmid 2010; Segovia-Viadero *et al.* 2016). Further supporting the overall loss of alleles caused by a recent bottleneck event, the natural cohort had a significantly ($P < 0.05$) higher number of private alleles when compared to that of the cultured cohorts (Figure 3.1), which could also be as a result of the small number of broodstock animals used to establish the cultured cohorts.

The Wilcoxon-signed rank test indicated that both cultured cohorts have undergone a recent genetic bottleneck as a significant deviation ($P < 0.05$) from mutation-drift equilibrium was detected under the IAM, as well as the TPM (Table 3.1). The high effective population size of the progenitor natural population (171.10) further corroborates that the natural populations that broodstock animals were collected from to establish these cultured cohorts likely had a high degree of genetic diversity (Table 3.1). Effective population size estimates (Table 3.1) show that the second F1 cultured cohort had a substantially larger effective population size (17.50) than the first cultured cohort (8.10), indicating that a greater amount of genetic variation was captured in this cultured population. Although previous studies have found that a reduction in effective population size is directly correlated to a reduction in genetic diversity, as well as an increased likelihood of inbreeding (Elliott 2000; Slabbert *et al.* 2009), relative to the limited number of broodstock animals used (six) to produce the F1 cohorts in the current study, these effective population size estimates are expected. Nevertheless, the estimates obtained for the cultured cohorts are lower than that of the natural cohort.

Table 3.1. Bottleneck (Wilcoxon) test under the infinite allele model (IAM), two-phase model (TPM) and stepwise mutation model (SMM), as well as estimates of effective population size (N_e) calculated using the linkage disequilibrium (LD) method, where the 95% confidence intervals (CI) are indicated.

Parameter	Natural	F1 Cultured 1	F1 Cultured 2
Sample size (n)	50	50	50
Wilcoxon test			
IAM	< 0.05	< 0.05	< 0.05
TPM	n.s.	< 0.05	< 0.05
SMM	n.s.	n.s.	n.s.
N_e	171.10 (139.70 – 218.80)	8.10 (7.10 – 9.10)	17.50 (12.20 – 19.30)

This proposed minimum effective population size is known as the “50/500” rule, as suggested by Franklin (1980). The author suggests that a short-term effective population size of more than 50 and a long-term effective population size of more than 500, is necessary to maintain genetic diversity in a population. This was suggested as low short-term effective population sizes have been associated with a reduced response to performance and fertility selection, as well as a reduced recovery from inbreeding depression; and low long-term effective population sizes is thought to result in a reduced response to long-term adaptive changes (Franklin 1980). Therefore, in the current study, the short-term effective population size estimate is more applicable, particularly when considering the implementation of future selective breeding strategies. In recent years, this “50/500” rule has been under debate, as studies have suggested that the minimal critical size of 50 be doubled to at least 100 to avoid inbreeding depression (Frankham *et al.* 2014; Grant *et al.* 2017), as offspring from 100

effectively breeding individuals is expected to better represent the genetic diversity of the natural population that animals were collected from.

Therefore, as smaller populations are more susceptible to genetic drift (random fluctuation in allele frequencies) and the deleterious effects thereof (Flowers *et al.* 2002; Segovia-Viadero *et al.* 2016), breeding strategies should incorporate a large number of broodstock animals to increase the effective population size, thereby minimising the effects of genetic drift in cultured populations (Grant *et al.* 2017). This has been successfully implemented in stock enhancement practices for the sea urchin *S. intermedius* in Japan, where approximately 600 broodstock animals were used in seven spawning events to establish the genetically diverse cultured offspring (Agatsuma 2013).

The reduction in genetic diversity could be a consequence of the statistically significant increase in relatedness among cultured individuals (mean estimates beyond the 95% CI's), when compared to the natural cohort that has a relatedness coefficient (r) value of approximately zero (Figure 3.2). This is likely a result of the skewed family sizes, caused by differential parental contributions. In subsequent generations, this could easily result in inbreeding and subsequent inbreeding depression, which is commonly associated with aquaculture environments (Elliott 2000; Grant *et al.* 2017). Although deleterious alleles and the traits affected by inbreeding are often dependent on the species (Lynch and Walsch 1998; Feng *et al.* 2015), studies in the purple sea urchin, *Strongylocentrotus purpuratus*, showed that inbreeding (F = 0.25) resulted in declines in larvae size, as well as reduced larval survival (Leahy *et al.* 1994; Anderson and Hedgecock 2010), due to the accumulation of recessive deleterious mutations. Similarly, inbreeding resulted in inbreeding depression in the sea urchin *S. intermedius*, as gonad protein storage, gonad flavour profiles and gametogenesis were negatively affected (Feng *et al.* 2015), which will most likely affect reproduction. Inbreeding and subsequent reductions in genetic diversity can have an impact on various economically important traits, such as growth, gonad quality and overall survival and therefore, could impact the overall productivity of an aquaculture facility (Macaranas and Fujio 1990; Grant *et al.* 2017). In the current study, neither F1 cultured cohort showed evidence of inbreeding as small F_{is} values of 0.05 and -0.11 (Figure 3.2), respectively, were obtained.

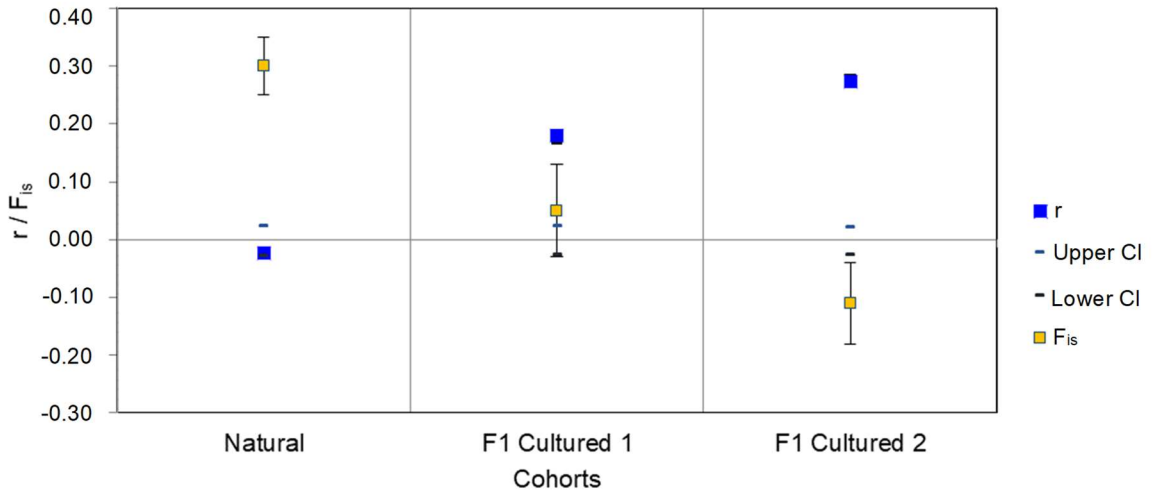


Figure 3.2. Pairwise relatedness (r) for each cohort showing a higher degree of relatedness within the cultured cohorts, where the corresponding upper and lower bounds of the 95% confidence intervals (CI), as well as inbreeding coefficients (F_{is}) are indicated. Standard error from the mean is indicated for both variables.

A lower degree of genetic diversity could possibly affect the short- and long-term adaptive and selective response of these animals. The study aquaculture system investigated in this chapter highlights that F1 individuals should probably not be used as future broodstock replacements, but rather that the geographically representative natural population should be used for broodstock supplementation as it has a moderate to high degree of genetic diversity (Figure 3.1). Furthermore, the success of future selective breeding practices could be impacted by genetic bottleneck events caused by using few broodstock animals to establish cultured populations, as low frequency alleles can be rapidly lost (Lande 1981; Falconer and Mackay 1996). Therefore, in context of the breeder's equation ($R = h^2S$), where R is the response to selection, h^2 is narrow sense heritability (representing the phenotypic variation that can be attributed to the additive effect of genes) and S is the selection differential, it will be beneficial for future sea urchin breeding practices to prevent a loss of genetic diversity by increasing the number of breeders, thereby increasing the genetic diversity and the number of families, as well as the response to selection (Zenger *et al.* 2019). These preliminary results, based on the trial aquaculture system used in this study, indicate that broodstock numbers should be increased, possibly to a similar recommendation as for abalone species of 10 – 13 males and 20 – 25 females (Elliott 2000). It has also been suggested that larvae from several spawning events should be reared together so as to increase the genetic variation of the cultured population (Gaffney *et al.* 1996). Alternatively,

broodstock should be supplemented with additional animals collected from natural locations, that have a similar genetic composition.

3.3.2. Genetic differentiation

The small number of broodstock animals that were used to establish the respective F1 *T. gratilla* cultured cohorts, explains the moderate pairwise F_{st} values ranging from 0.071 to 0.144 ($P < 0.05$) (Table 3.2). The underlying family structure, caused by differential broodstock contributions, is likely contributing to the genetic differentiation between the respective cohorts (Calderón *et al.* 2012).

Table 3.2. Pairwise F_{st} estimates (shaded) and corresponding Bonferroni corrected *P*-values (unshaded), indicating greater genetic differentiation between the cultured cohorts.

Cohort	Natural	F1 Cultured 1	F1 Cultured 2
Natural	-	0.021	0.021
F1 Cultured 1	0.071	-	0.021
F1 Cultured 2	0.101	0.144	-

Comparable to previous studies in cultured sea urchin populations (Segovia-Viadero *et al.* 2016), a moderate global F_{st} value of 0.11 ($P < 0.05$) was obtained from the AMOVA (Table 3.3). Although previous studies in cultured cohorts found that most of the variance was attributed to differences among individuals (Segovia-Viadero *et al.* 2016), in the current study, the genetic differences among individuals does not account for majority of the variance, as most of the genetic variation (78%) was attributed to differences within individuals (Table 3.3). The similarity among cohorts and individuals could be explained by the cultured populations being established using a limited number of broodstock animals, resulting in a small number of families, possibly with skewed family sizes as a result of differential parental contributions.

Table 3.3. Analysis of molecular variance (AMOVA), where a high degree of differentiation can be observed between the respective cohorts.

Source of variation	**Sum of squares**	**Estimated variance**	**% variation**	**Fixation indices**
Among cohorts	195.15	0.89	10	F_{st} 0.11*
Among individuals	1268.33	1.02	12	F_{is} 0.13*
Within individuals	987.50	6.58	78	F_{it} 0.23*
Total	2450.98	8.50		

*Statistically significant at $P < 0.05$

This moderate degree of genetic differentiation is further illustrated by the multivariate principle co-ordinates analysis (Figure 3.3), where three distinct clusters could be identified, representative of the natural-, first F1 cultured- and second F1 cultured cohorts. Although minimal overlap can be observed between the respective cohorts, the slight overlap could be explained by the similarities between the founding broodstock and their corresponding cultured cohorts, as well as by the broodstock animals originally being collected from the natural cohort. Furthermore, the first F1 cultured cohort showed less constricted clustering, suggesting sub-clustering of families within that cohort.

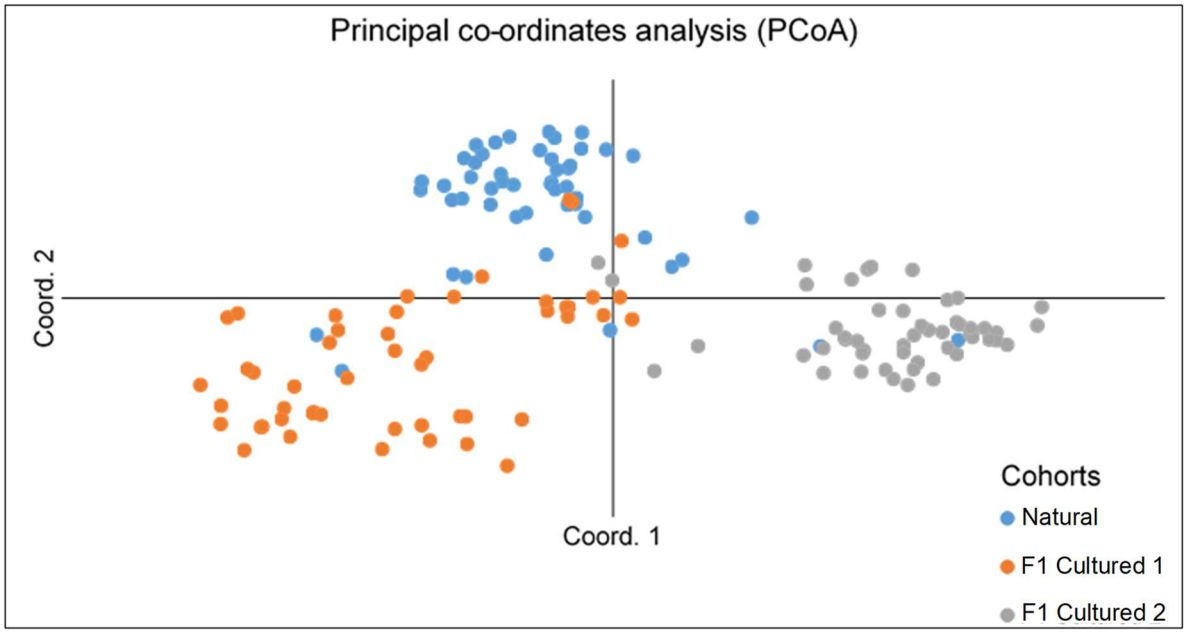


Figure 3.3. Principle co-ordinates analysis (PCoA), showing minimal overlap of three clusters, representative of the natural-, first F1 cultured- and second F1 cultured cohorts.

These results are further supported by the Bayesian clustering analysis performed in Structure, as three genetic clusters (K = 3) were inferred from both the Puechmaille and Evanno methods (Appendix B; Figure S3.1). Although the broodstock and cultured cohorts had similar genotype membership patterns, the natural cohort and the respective cultured cohorts are mostly distinct from each other (Figure 3.4). The Structure plot is also suggestive of variable reproductive success, as the genotype membership patterns of the respective cultured cohorts are predominantly characterised by that of specific broodstock individuals. These results also explain the high degree of relatedness obtained in the cultured cohorts, as the cultured individuals are likely representative of very few families. This large extent of genetic differentiation between natural and cultured cohorts is not uncommon in broadcast spawning animals, as this has been observed for other sea urchins (Natsukari *et al.* 1995), as well as in shellfish aquaculture facilities (Evans *et al.* 2004).

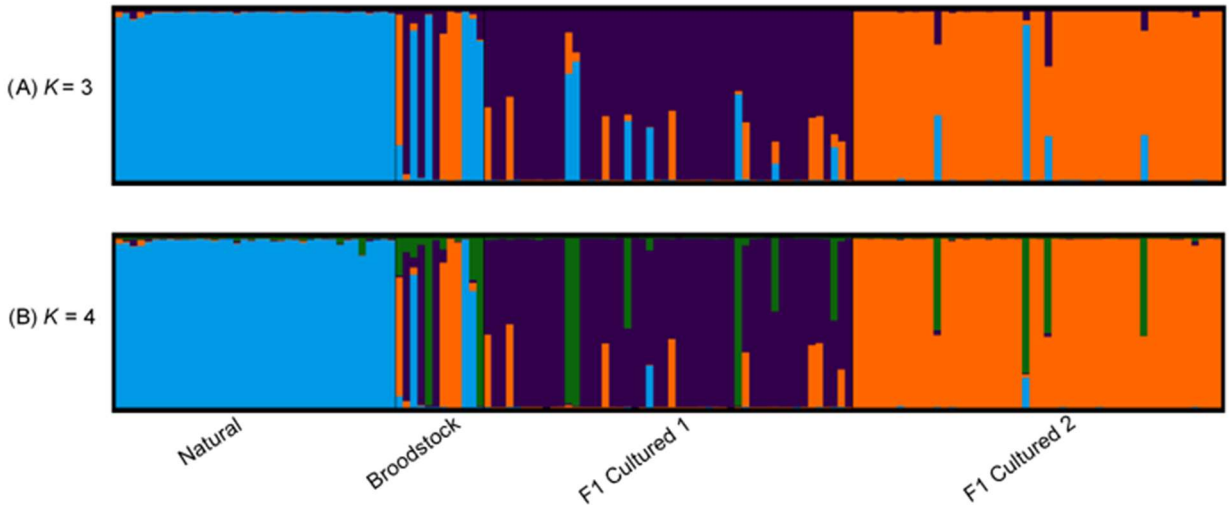


Figure 3.4. Structure bar plots, where (A) *K* = 3 is the most likely number of genetic clusters and (B) *K* = 4 is included for comparison. Each cluster is represented as a different colour and each bar represents an individual within each cohort.

3.3.3. Parentage analysis

The efficiency of microsatellite markers for parentage studies was evaluated by calculating the polymorphic information content (PIC), probability of inclusion (PI) and probability of exclusion (PE) estimates. The high overall average PIC of 0.66 (Appendix B; Table S3.1) indicates that these markers will be useful in the current study, as well as in future *T. gratilla* studies, as a PIC value of above 0.50 for microsatellite markers indicates that the markers are highly informative (Lafarga-de la Cruz *et al.* 2015). Furthermore, the power of a microsatellite maker to include a candidate parent when neither parent is known (PI) ranged from 0.01 – 0.65, with an average PI of 0.15 (Appendix B; Table S3.1). Conversely, the power of a marker to exclude a candidate parent when neither parent is known (PE) ranged from 0.10 – 0.88, with an average PE of 0.50 (Appendix B; Table S3.1). These moderate to high probability values indicate that the species-specific microsatellite markers used in this study are suitable for parentage assignment studies.

In the current study, uneven broodstock contributions were observed in both cultured cohorts (Appendix B; Figure S3.2) when implementing the maximum-likelihood method implemented in Colony, as well as the exclusion-based method implemented in Vitassign. A high proportion of full-sibs was observed in both cultured cohorts (32% and 38%, respectively), which can be explained by only a few of the total broodstock used in each spawning event successfully contributing to the subsequent generation (Figure 3.5).

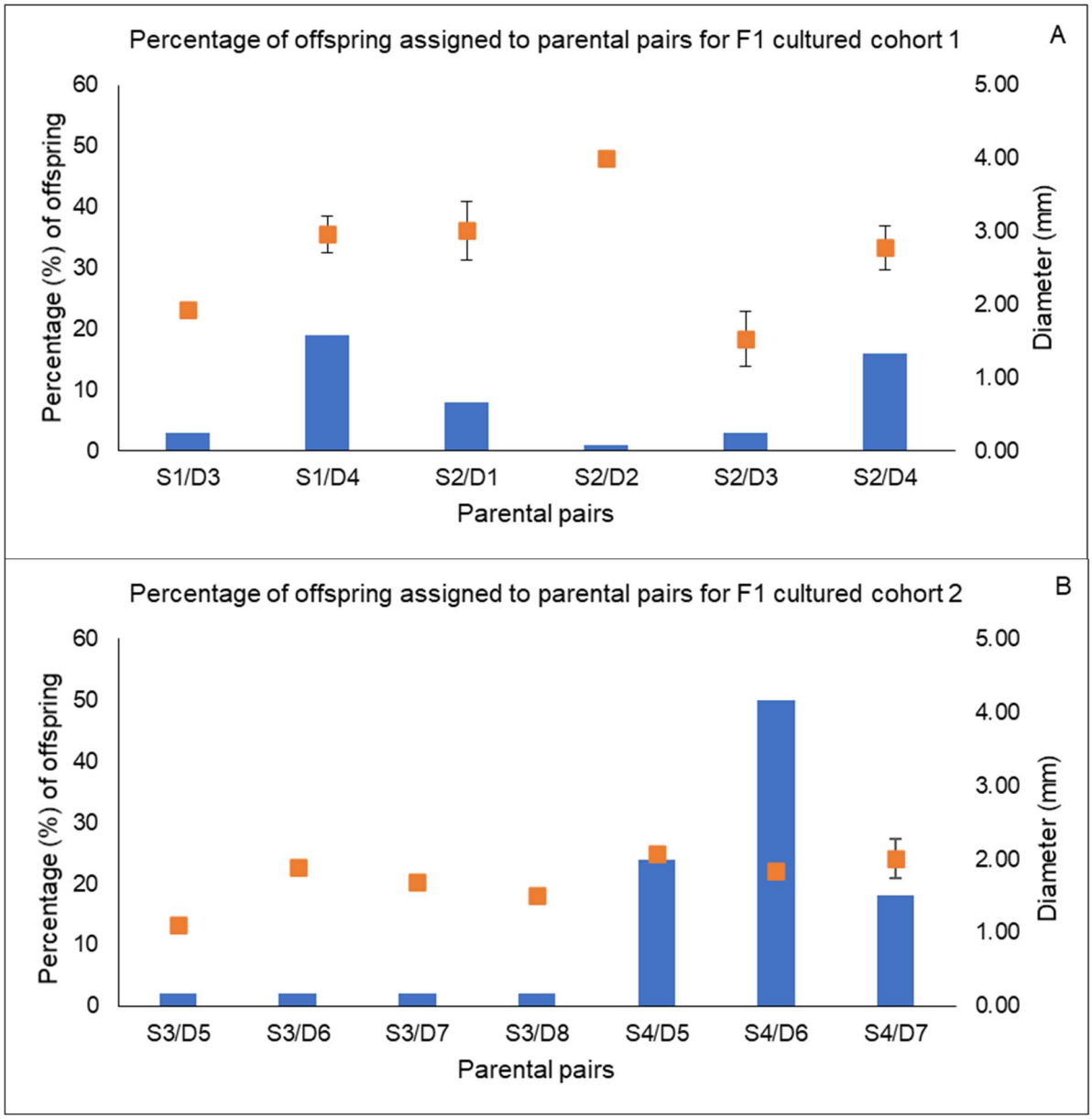


Figure 3.5. Percentage of offspring assigned to parental pairs in the (A) first and (B) second F1 cultured cohort, where six and seven full-sib families were identified, respectively, and mean family diameters (mm) are indicated (S: Sire, D: Dam).

In the first F1 cultured cohort, consisting of 6 full-sib families, four out of six broodstock successfully contributed to the subsequent generation (Figure 3.5A). Although the sires contributed to a similar degree (1:1 ratio), the levels of contribution across the four dams were skewed, as dam D4 contributed to 70% of the offspring that were included in this study. The remaining females, D1, D2 and D3 contributed 12%, 16% and 2%, respectively. In the second F1 cultured cohort, which consisted of seven full-sib families, high variability in male reproductive success was observed as sire S4 contributed to 92% and sire S3 to only 8% of the offspring (Figure 3.5B). This variance in male reproductive success is expected from broadcast spawning animals, as this has been observed in other sea urchin species, where

it is expected that males, with a larger number of gametes, are in higher competition with each other (Levitan 2005). However, some variability in reproductive success was also observed among the females as dams D5, D6, D7 and D8 contributed 26%, 52%, 20% and 2%, respectively (Figure 3.5B). In cultured populations, it is expected that there will be more variation in reproductive success when there is increased reproductive competition through higher densities (Levitan 2005), particularly for males (Levitan 2008), but in the current study, this was observed for the females contributing to the first cultured cohort (Figure 3.5A). However, as this pattern was not observed for the second cohort, this study shows that reproductive competition is not the only factor affecting reproductive success in the collector sea urchin within aquaculture environments.

Parentage studies, where offspring are traced back to specific parental pairs, have limitations when used as a proxy for fertilisation success, as parentage analyses are dependent on the time of sampling and is more likely indicative of post-fertilisation larval survival and not necessarily fertilisation success (Evans *et al.* 2007; Garcia-González and Simmons 2007). Therefore, there are various factors that play a role in reproductive success, as measured by means of parentage assignment. There are several biological explanations for variable reproductive success in sea urchins. In females, egg size likely plays a role in reproductive success, as larger eggs are more likely to make contact with sperm and thus, increases the chance of fertilisation (Marshall *et al.* 2002). Furthermore, overall egg quality and maturity could also play a role in variable reproductive success (Marshall *et al.* 2004), particularly as previous studies have found that *T. gratilla* larvae rely on maternal provisioning of lipids (triglycerides) to fuel their early developmental stages (Byrne *et al.* 2008a), ultimately affecting the overall survival and performance of juveniles. In males, sperm quality in terms of motility, speed and viability, influences reproductive success (Garcia-González and Simmons 2005; Evans *et al.* 2007; Fabbrocini *et al.* 2016). Studies in the abalone, *H. midae*, attributed differential parental contributions to the inability of broodstock animals to produce good quality gametes, gamete competition, or simply as a result of differential survival of larvae (Slabbert *et al.* 2009). Therefore, through the assessment of gamete quality, which can be measured through morphological- (shape, size and colour) and biochemical (carbohydrate-, lipid- and protein content) characteristics, the optimal broodstock animals can be selected to further enhance the subsequent generation.

Reproductive success in echinoids is also thought to be dependent on the interactions between the sperm and egg surface proteins, as this mediates gamete compatibility

(Calderón *et al.* 2009). This hypothesis, known as the genetic compatibility hypothesis, is further supported by a study in the sea urchin *Heliocidaris erythrogramma* where embryo viability, *i.e.* larval survival, was higher for more compatible parental pairs (Evans *et al.* 2007). Furthermore, a recent study in the sea urchin *Strongylocentrotus purpuratus* found that there was significant linkage disequilibrium between loci encoding sperm and egg surface receptor proteins, namely Bindin and egg bindin receptor protein 1 (EBR1), while finding no linkage between neutral loci (Stapper *et al.* 2015). The authors suggest that loci encoding these proteins are likely linked as a result of assortative mating, as the gamete recognition proteins are largely responsible for the patterns of reproductive success through non-random binding of sperm and eggs within species (Stapper *et al.* 2015). Although intraspecific variation at loci encoding these proteins that affect individual reproductive success varies between different species, this remains to be investigated in *T. gratilla*. These results are further supported by studies in abalone (Clark N. *et al.* 2009), sea stars (Hart 2013), oysters (Zhang *et al.* 2010) and the sea urchin *Heliocidaris erythrogramma* that found that genetic compatibility plays a role in fertilisation success and that females respond well to polyandry as a result of this (Evans and Marshall 2005). In the current study, highly skewed parental contributions were observed for both sexes, despite all males having had an equal chance to fertilise eggs. Therefore, the differential parental contributions found in the current study could be further investigated through the evaluation of different mating schemes, such as a factorial breeding design (n sires x n females).

Offspring size differences among/across families were also investigated in the cultured cohorts, and it was observed that sizes varied substantially; however, the differential broodstock contributions observed in the current study limits the inferences that can be made. Future studies could investigate this further, as differences in offspring size for different families was evident for the collector sea urchin. This could be investigated by incorporating other factors that have an impact on sea urchins size in breeding schemes, as studies have found that the types of feeds used significantly ($P < 0.05$) alters *T. gratilla* growth rates (Juinio-Meñez *et al.* 2008a). This is likely as a result of variation in digestibility of different feeds, which subsequently affects nutrient availability, energy uptake and growth (Cyrus *et al.* 2014). Therefore, the effects of broodstock contribution on subsequent generations, as well as various feeds on reproductive success, larval survival and offspring size variation could be evaluated on a larger scale.

3.4. Conclusion

This study showed an overall decline in genetic diversity from the natural progenitor population when compared to cultured cohorts, as a result of the limited number of broodstock animals that were used to establish the cultured populations. This genetic bottleneck resulted in low effective population size estimates, although the estimates are not of concern when considered relative to the total number of broodstock animals used for the respective spawning events. A higher degree of relatedness was observed within the cultured cohorts, but this study found no evidence of inbreeding within the cultured cohorts. However, it remains important to track relatedness in aquaculture environments to avoid the deleterious effects of inbreeding. This study found three distinct genetic clusters, where slight overlap was observed between the natural and cultured cohorts, which was expected. This clustering pattern can be attributed to the similarities among the individuals in each cohort, as unequal parental contributions were observed in both cultured cohorts, where a single dam and a single sire monopolised the respective spawning events. This chapter highlighted the importance of assessing other factors, such as diet, breeding design, as well as gonad and gamete quality that could affect reproductive success in sea urchins. Therefore, in chapter 4, the effects of diet on reproductive performance, as well as the effect of using a larger number of broodstock animals in a spawning event will be assessed.

Chapter 4

The effects of various diets on the reproductive performance of *Tripneustes gratilla*

Abstract

Broadcast spawning animals often display differential parental contributions within aquaculture environments, which can influence offspring performance through the introduction of a genetic bottleneck and subsequent loss of genetic variation in cultured populations, as confirmed for *Tripneustes gratilla* in chapter 3. Therefore, this chapter aimed to assess various biological- and genetic factors for *T. gratilla*, as well as different feeding regimes that could have an impact on reproductive competition, larval growth and juvenile performance. To achieve this, broodstock animals were conditioned on four feeds [formulated feed, kelp (*Ecklonia maxima)*, a green seaweed (*Ulva rigida*) and a mixture of these three diets] for approximately four months, where after a factorial breeding design was implemented. Across broodstock, significant differences ($P < 0.05$; ANOVA) in gonad colour, egg colour and sperm size showed that a formulated feed (20U) should not be fed in isolation for reproductive purposes. Although limited differences in egg energetic components were observed across diets, negative correlations ($P < 0.001$) between these components [proteins ($r = -0.87$), carbohydrates ($r = -0.89$) and lipids ($r = -0.89$)] and egg count were observed. Egg fatty acid profiles show that there are benefits to including a formulated feed, as well as the natural feeds, in a mixed feeding regime. Larvae from broodstock fed kelp and the mixed diet survived for the full duration of larval rearing period (20 days) and displayed similar growth rates throughout. Approximately three months after metamorphosis, 10 species-specific microsatellite markers were PCR amplified across 16 broodstock and 364 offspring. Genetic diversity analyses showed that there were no statistically significant ($P > 0.05$) differences between the broodstock animals and their offspring. Parentage analyses revealed that a total of 26 out of 32 possible parent pairs contributed to the F2 generation. Therefore, the implementation of a factorial breeding design is advantageous in aquaculture practices to preserve genetic diversity present in cultured cohorts. An assessment of offspring phenotypic performance showed low heritability estimates (0.050 ± 0.058) for body diameter, indicating that future studies should assess heritability of growth rates throughout or at the end of juvenile grow-out.

4.1. Introduction

Variable reproductive success is frequently observed for broadcast spawning animals, such as the toxopneustid sea urchin *Tripneustes gratilla*, as observed in chapter 3, where a single male and single female dominated their respective spawning events. This could have occurred as a result of the breeding strategy employed or as a result of individual broodstock gamete quality. In aquaculture environments, feeding regimes and corresponding feed quality used for broodstock conditioning will impact nutrient availability, intake and digestion, and subsequently affect gametogenesis and reproductive performance (Azad *et al.* 2011). Broodstock conditioning can also have important implications for larval growth, development and survival (Quinones-Arreola *et al.* 2015). Prior to the development of the digestive tract, enzyme systems and ciliary feeding structures, larval development is driven by the nutritional content of the egg (Strathmann *et al.* 1992; Byrne *et al.* 2008a, b). This facultative feeding period, where larvae are supported by maternal reserves, is thought to exceed eight days in *T. gratilla*, which is longer than for other sea urchin species with similar egg sizes (Byrne *et al.* 2008b). Studies have suggested that different feeding strategies for adult urchins should be used for different purposes (for example market acceptance *vs* reproductive success), particularly as it is likely that sea urchins have different nutrient requirements during different developmental stages (Heflin *et al.* 2012).

Tripneustes gratilla is primarily an opportunistic grazing omnivore (Lawrence and Agatsuma 2013) that display an affinity towards various diets in the wild and aquaculture environments (Lawrence and Agatsuma 2020), although they prefer certain feeds, such as *Ulva* sp. over others, including *Ecklonia maxima*, *Porphyra capensis* and *Gigartina polycarpa* (Cyrus *et al*. 2014, 2015a). In the aquaculture environment of this study, formulated-, kelp-, *Ulva rigida*- and combination diets are commonly used. *Ecklonia maxima* is a brown seaweed (kelp) that is rich in minerals, bioactive phytochemicals (terpenoids, antioxidants, polyphenols) and fermentable fibres (see chapter 1, Table 1.1, Table 1.2; Mabeau and Fleurence 1993; Lahaye and Kaeffer 1997; Smith 2007; Jiménez-Escrig *et al.* 2012), as well as lipids, fatty acids and amino acids that are essential in sea urchin diets (Newell 1980). The green seaweed, *U. rigida*, has a high nutritive content and is rich in essential amino acids, lipids, proteins and minerals (see chapter 1, Table 1.1, Table 1.2; Gordillo *et al*. 2001; Cyrus 2013; Shuuluka *et al.* 2013; Trigui *et al*. 2013; Cyrus *et al*. 2014; Khairy and El-Sheikh 2015; Garcia *et al.* 2016). *Ulva* spp. are frequently grown in effluent water to reduce the nutrient release from aquaculture farms (del Río *et al.* 1996; Mata *et al.* 2010), as well as to reduce the

reliance on naturally occurring seaweeds required as feed. Furthermore, *Ulva* spp. have known anti-microbial roles (Bolton *et al.* 2016; Ismail *et al.* 2018) and are known feeding stimulants (Cyrus *et al*. 2014), which could contribute to improved gonad development by altering feeding behaviour. This led to the incorporation of *Ulva* into a formulated feed (20U) for *T. gratilla* containing 20% (w/w) dried *Ulva* (Cyrus *et al.* 2014). The inclusion of *Ulva* into formulated feeds or as a supplementary feed for the sea urchin *T. gratilla* has been shown to improve feed palatability, consumption as well as gonad growth and development (Cyrus *et al.* 2014, 2015a). This feed has the highest protein-, carbohydrate- and fat content of all feeds included in this study (see chapter 1, Table 1.1; Cyrus *et al*. 2014). While single feed regimes have advantages in aquaculture, studies suggest that conditioning broodstock on a combination of feeds could be beneficial for somatic growth, gametogenesis and subsequent reproductive performance (Beddingfield and McClintock 1998; Vadas *et al.* 2000).

Ruderal species, such as *T. gratilla*, invest the energy they obtain from their diet in growth and reproductive efforts rather than in body maintenance (Lawrence and Bazhin 1998). However, as observed in chapter 3, as well as in previous studies in other broadcast spawning species (Slabbert *et al.* 2009; Miller *et al.* 2012; Segovia-Viadero *et al.* 2016), there could be differences in broodstock contribution to the genetic pool of the offspring, resulting in a reduction in genetic diversity that could negatively affect future responses to environmental changes and selection (Grant *et al.* 2017). It is possible that broodstock conditioning, combined with the implementation of a factorial breeding design, could improve the chances of reproductive success and subsequently, lessen the bottleneck effect associated with aquaculture practices of broadcast spawning animals. By applying quantitative genetic models, parental effects on offspring performance can be further evaluated through preliminary heritability assessments during the early growth stages of *T. gratilla* juveniles to contribute to future selective breeding programmes for this species.

Therefore, the aim of this chapter was to assess the effect of broodstock conditioning on reproductive performance of *T. gratilla* through the evaluation of various traits that could influence reproduction, such as body size, gonad- and gamete morphology, as well as egg energetic components and fatty acids. A factorial breeding design was employed, and larval growth, parental contribution and offspring phenotypic performance was assessed.

4.2. Materials and Methods

4.2.1. Broodstock conditioning, spawning and fertilisation

Broodstock conditioning trials were conducted at the Department of Environment, Forestry and Fisheries (DEFF) Marine Research Aquarium in Sea Point, Cape Town (GPS coordinates 33°92'05"S, 18°38'11"E). First-generation (F1) sea urchins of approximately 2 years of age were starved for two months prior to conditioning to reduce the size of the gonad (that also functions as a storage organ) and to limit differences in gonad development, as it is common practice to standardise the nutritional condition of the gonads of all sea urchins assigned to the different treatment groups (Spirlet *et al.* 2000). In two independent iterations of broodstock conditioning (for three and four months, respectively), broodstock animals were conditioned on four feeds, namely, 20U [a formulated diet containing 20% dried farmed *Ulva* (w/w)] (Table 4.1), kelp (*Ecklonia maxima*), a farmed green seaweed (*Ulva rigida*) and a diet consisting of a mixture of all feeds, where each feed was administered independently *ab libitum*. A summary of the nutritional profiles of these feeds are in Table 4.2, as well as in Table 1.1 and Table 1.2 in chapter 1.

Table 4.1. Formulated feed composition (in g per kg dry matter) prior to the inclusion of 20% *Ulva* (w/w) as described by Cyrus *et al*. 2015a.

Ingredients	Ratio (g/kg)
Maize (extruded)	321
Wheat bran	321
Fish meal (65)	153
Soybean (44)	153
Di-calcium phosphate	18.40
Lecithin (de-oiled)	13.80
Vitamin and mineral premix	11.00
Oil (fish)	9.63
Total	1000

Table 4.2. Nutrient analysis summary for kelp (*Ecklonia maxima*) as described by [a]Smith 2007 and *Ulva rigida* and a formulated feed (20U) as described by [b]Cyrus *et al*. 2014, where all estimates are expressed as percentage dry weight, unless stated otherwise (n.d. not determined).

Nutrients (% dry weight)	*Ecklonia maxima*	*Ulva rigida*	Formulated feed (20U)
Protein	11.00 [a]	18.31 [b]	25.69 [b]
Fat	1.16 [a]	0.38 [b]	2.31 [b]
Moisture	79.39 [a]	15.30 [b]	9.61 [b]
Ash	19.41 [a]	32.66 [b]	13.89 [b]
Gross energy (MJ/kg)	n.d.	9.44 [b]	15.49 [b]
Fibre	41.34 [a]	6.02 [b]	4.75 [b]
Carbon/Carbohydrate	33.82 [a]	27.33 [b]	43.75 [b]

[a] *Ecklonia maxima* nutrient analysis from samples collected on the south west coast of South Africa by Smith 2007.
[b] Nutrient analysis of *Ulva rigida* from Irvine & Johnson (I&J) Cape Abalone farm (34°34'0.60" S; 19°21'00" E) in the western cape of South Africa, as well as a formulated feed (20U) by Cyrus *et al*. 2014.

The urchins were divided into 12 baskets with a total volume of 40 L each (L x W x H: 42 x 36 x 30 cm) that were fitted with aeration and supplied with heated (to ~25°C), filtered (75 µm) seawater, as well as two outflow holes near the top of each basket for wastewater. A photoperiod of approximately 12 hours per day was maintained during the experiment and the diet replicate baskets were randomly arranged. In this flow-through system, animals were suspended in oyster mesh baskets (L x W x H: 40 x 29 x 16 cm) with 6 mm^2 gaps (mesh) to allow uneaten food and faecal pellets to fall through, aiding in water quality management as baskets could easily be removed for cleaning purposes. Tank cleanliness was assessed daily and siphoned as needed. Diets were administered *ad libitum* and broodstock conditioning was initiated on two separate dates (11 February 2019 and 1 July 2019). Broodstock animals were stocked at 10 animals per basket (12.24 kg/m^2) for the first iteration of broodstock conditioning (in triplicate per diet). These animals (n = 120) were not sexed prior to stocking and had an average weight of 144.63 ± 2.44 g, average diameter of 7.37 ± 0.44 cm and an average height of 3.87 ± 0.05 cm. Feeds were alternated daily (one feed type per day) for the animals under the mixed diet treatment for three months. In contrast, prior to stocking the system for the second iteration of conditioning, animals were spawned to ensure equal sex ratios (four males and four females per diet) and gonad state. The animals included in the second round of conditioning were stocked at eight animals per basket (11.73 kg/m^2), in triplicate per feeding regime, and had an average weight of 170.03 ± 4.35 g, an average height of 7.33 ± 0.07 cm and average diameter of 4.93 ± 0.07 cm (n = 96). Additionally, the mixed diet alternated between feeds on a weekly basis (one feed type per week) for a conditioning period of four months.

Spawning was induced for a total of eight animals (four females and four males) from one replicate of each diet after each broodstock conditioning trial on 13 May 2019 and 4 November 2019, respectively, to establish second-generation (F2) offspring. Gametes were collected in separate 500 mL flasks for each individual and eggs were transferred onto a 60 µm sieve and collected into 15 mL tubes. Eggs were counted using a Bogorov tray under a light microscope and a haemocytometer was used to count sperm. Eggs required for the quantification of energetic components were collected in triplicate per basket for each assay and were stored at -20°C until further use. A factorial breeding design was implemented, where every female was individually crossed with every male within the same feeding regime, resulting in 16 crosses per diet. Gametes were combined with a sperm:egg ratio of 100:1 in individual 500 mL sterile flasks filled with 0.2 µM filtered seawater. Fertilisation was

confirmed by assessing the formation of a fertilisation membrane shortly after combining gametes.

4.2.2. Broodstock phenotypic traits

After each spawning event, the following phenotypic traits were measured for each broodstock animal after briefly blotting excess water (8 animals per diet per spawning event): weight (g), test diameter (cm), test height (cm), gonad weight (g). Sea urchin body volume was calculated as per:

$$V = (\frac{2}{3})\pi r^3 \quad (1)$$

where

$$r = \frac{(horizonta\ \ diameter + vertical\ heigh\ )}{3} \quad (2)$$

Gonad somatic index (GSI; %) was calculated as per:

$$GSI\ (\%) = \frac{W_g}{W_t} \times 100 \quad (3)$$

where W_g represents gonad wet weight and W_t represents the total weight of the sea urchin.

As gonad size or weight does not necessarily indicate the degree to which gametogenesis is occurring, a histological approach was used to assess the reproductive phase of the broodstock at the time of spawning. A single gonad from each animal was transferred to 40 mL Davidson's fixative [per litre: 300 mL 95% ethanol, 200 mL 100% formalin, 100 mL glacial acetic acid and 300 mL distilled water (dh_2O)] in a 50 mL centrifuge tube for 48 hours, before being transferred to 70% ethanol. Following fixation the tissues were processed for routine paraffin histology (Bucke 1989). Briefly, a Shandon Citadel 2000 tissue processor was used to rinse, dehydrate and embed gonad samples in paraffin wax, and samples were sectioned to 7 µm using an LKB 2218 Historange microtome. These sections, stained using Harris' haemotoxylin and eosin, were assessed under a Nikon H600L microscope and images were captured using a Leica digital camera and Nikon Imaging Systems (NIS) Elements Basic Research (BR) software package (version 3.1). Gonad maturity was assessed based on the amount of non-germinal nutritive cells, as well as the thickness of the spermatocyte layer and oocyte presence and size in males and females, respectively. The reproductive phases are: (1) recovery, (2) growing, (3) premature, (4) mature, (5) partly spawned and (6) spent (Vaïtilingon *et al.* 2005; Cyrus 2013), as described in chapter 1. Egg and sperm morphology were assessed at 400X and 1000X magnification, respectively, where egg diameter (µm)

and egg surface area (μm^2), as well as sperm length (µm) were measured in triplicate per individual.

Lastly, the gonad and egg colour of each individual was assessed using a hand-held reflected-light, fibre-optic spectrophotometer (Lovibond) to obtain colour indices, including lightness (L*), yellowness (b*) and redness (a*), which act as a numeric expression of colour profiles, according to the procedures described by Robinson *et al*. (2002) and Cyrus (2013). Gonad colour was assessed by holding the instrument directly on gonad tissue. Egg colour was assessed by briefly centrifuging 1 mL of eggs and transferring 80 µL concentrated eggs to a cavity slide (against a white background). For lightness, positive readings are indicative of a white colour and negative readings indicate blackness. Positive yellowness readings are indicative of yellow and negative readings are associated with blue colour. Lastly, redness readings are positive when colours are red and negative if colours are green. Colour readings were measured in triplicate per basket.

4.2.3. Egg energetic components

The three primary energetic reserves in marine invertebrate eggs, namely proteins, carbohydrates and lipids, were assessed colourimetrically using a microplate reader (Biotek) for eggs of known quantity per mL from each female broodstock animal. Egg proteins were extracted using a modified trichloroacetic acid (TCA) method as described by Imagawa *et al*. (2004). Samples (20 µL) were homogenised in a lysis buffer (200 µL), containing 20 mM Tris-HCl, 130 mM NaCl, 5 mM EDTA and dH_2O, and samples were incubated on ice for 15 minutes. A proteinase inhibitor cocktail (Roche) was added to each sample (5% v/v) prior to lysis to reduce the effect of protein degrading enzymes. After centrifugation at 4°C (at 12000 rpm), 40 µL cold 100% TCA was added to each sample and proteins were precipitated at 4°C for 60 minutes. Samples were centrifuged at 4°C (at 9000 rpm for 15 minutes) and the supernatant (TCA phase) was collected for subsequent carbohydrate quantification. Proteins, resuspended in dh_2O, were assayed using the Micro Bicinchoninic Acid (BCA) Protein Assay Kit (Cat. No. 23235), which is a micro-modification of the Lowry Protein Assay (Lowry *et al.* 1951) and absorbance was read at 562 nm. A dilution series (0 – 200 µg/mL) was prepared from bovine serum albumin (BSA) to provide a standard curve for protein quantification. The TCA phase (40 µL) from the protein extraction protocol was used to quantify total egg carbohydrates using the phenol-sulphuric acid method described by Dubois *et al*. (1956). Briefly, samples (40 µL) were treated with 150 µL 18 M sulphuric acid and 100 µL 5% phenol. Subsequently, samples were incubated at 90°C for 5 minutes before

absorbance was read at 490 nm. A glucose dilution series (0 – 1.25 mg/mL) was prepared to construct a standard curve for carbohydrate quantification. Lastly, egg lipids were extracted using a modified chloroform:methanol method as described by Folsch *et al*. (1957). Egg samples (500 µL) were homogenised and chloroform:methanol (2:1) was added before shaking samples for 15 minutes. Samples were centrifuged at 4°C (at 4000 rpm for 10 minutes) before the liquid phase was recovered and a 0.9% NaCl solution was added. Finally, samples were centrifuged for 5 minutes and the lower chloroform phase, containing lipids, was retained. A Lipid Quantification Kit (Cell Biolabs), which employs a modification of the sulphosphovanillin method, first described by Chabrol and Charonnat (1937), was used. A purified lipid standard was used to prepare a dilution series (0 – 0.025 mg/µL) to quantify total egg lipids. A linear regression was performed to assess whether standard curves (across both spawning events) for protein (R^2 = 0.995; 0.998), carbohydrate (R^2 = 0.994; 0.999) and lipid (R^2 = 0.998; 0.995) quantification could accurately predict the energetic components of eggs given an absorbance reading. Each assay was performed in triplicate per individual.

4.2.4. Egg fatty acid composition

The fatty acid composition of 500 µL total egg lipid extract collected from broodstock of the second spawning event was assessed in triplicate per individual. A 25 µL 1000 ppm heptadecanoic acid (C17) standard was added to the lipid extract that was eluted in chloroform and briefly vortexed prior to completely drying samples under a gentle stream of N_2. Dried lipid samples were reconstituted with 500 µL tertiary butyl methyl ether (TBME). Thereafter, 50 µL trimethylsulfonium hydroxide (TMSH) (derivatising agent) was added to 100 µL of the reconstituted TBME in an insert positioned in a 2 mL gas chromatography (GC) vial. Separation of fatty acid methyl esters (FAMEs) was performed by injecting 1 µL (in a 5:1 split ratio) onto a gas chromatograph (6890N, Agilent Technologies) coupled to a flame ionisation detector (GC-FID) at the Central Analytical Facility, Mass Spectrometry (GC-MS) unit, Stellenbosch University. Separation of the FAMEs was performed on a polar RT-2560 (100 m, 0.25 mm ID, 0.20 µm film thickness) (Restek, USA) capillary column. Helium was used as the carrier gas at a flow rate of 1 mL/minute and the injector temperature was maintained at 240°C. The oven temperature was programmed as follows: 100°C for 4 minutes and increased to 240°C at a rate of 3°C/minute for 10 minutes.

4.2.5. Larval assessments

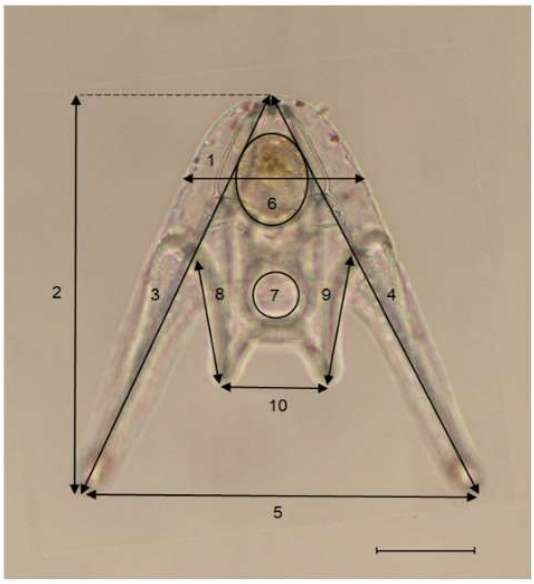
Fertilised eggs (16 crosses per diet) were combined in sterilised 87 L (L x W x H: 80 x 42 x 26 cm) containers (two per broodstock feed) and the temperature was maintained at 25°C. Once eggs hatched (approximately 24 hours post fertilisation), larvae were transferred to larger containers (130 L conical tanks), in triplicate per broodstock diet, where tanks were randomly arranged. Larvae were stocked at a density of approximately 5 larvae/mL. Each tank was supplied with aeration and 0.2 µm filtered seawater, which was processed through a sand filter, bio-filter and protein skimmer and maintained at a temperature of ≈25°C. Temperature was monitored for the duration of the 20 day larval rearing period. Tanks were supplied with fresh water at a rate of 8 L/h on a 12 hour light cycle, resulting in a 70% replacement rate. Each tank was fitted with an overflow outlet with an 80 µm banjo screen in order to retain larvae within the tank during water exchanges. Lastly, tank cleanliness was assessed daily and siphoned every second day to remove uneaten algae and dead larvae that aggregate in the bottom of the tanks.

Larvae were fed the same mixed microalgal diet (*Isochrysis* sp., *Chaetoceros muelleri* and *Rhodomonas* sp.), where all microalgal species have previously been used to feed *T. gratilla* larvae (Scholtz *et al*. 2013; Sonnenholzner-Varas *et al*. 2018). Feeding was initiated with *Isochrysis* sp. and *Chaetoceros muelleri* at a rate of 2000 algal cells (total) per larva. Subsequently, *Rhodomonas* sp. was included in the feeding scheme at 4000 algal cells/larvae when larvae reached their four-armed stage, increasing to a maximum 10 000 cells/larvae when larvae reached their eight-armed stage, as per Scholtz *et al*. 2013.

In addition to the 130 L conical tanks for each treatment, three 500 mL glass flasks per diet were stocked at approximately 5 larvae/mL. Larvae stocked in the flasks were not fed in order to assess growth, development and survival of larvae supported by their maternal reserves. Although fertilisation was successful for the first broodstock conditioning trial, larvae did not survive for longer than 10 days. Therefore, larval growth results from only the second broodstock conditioning will be reported in this chapter.

Throughout the larval rearing process (20 days), a minimum of three larval samples per tank were collected daily using a sterile glass pipette after briefly stirring. Additionally, larvae were sampled and counted (per mL) in triplicate from each of the three tanks per broodstock feed (formulated-, kelp-, *Ulva*- and a mixed diet) from day three post hatching. Larvae from the flasks were also sampled and counted (per mL) daily. Containers where all larvae had died were excluded from average larval counts and corresponding standard errors calculations.

During the course of the study, a total of 550 sampled larvae were placed on a cavity slide under a Nikon H600L microscope and images were captured using a Leica digital camera and NIS-BR software package. Various larval measurements (Figure 4.1; adapted from Scholtz *et al.* 2013; Lenz *et al.* 2019) were recorded using ImageJ software (Bourne and Bourne 2010), where areas were calculated using the elliptical area measurement function.



Larval measurements	
1	Body width
2	Body height
3	Post-oral arm length 1
4	Post-oral arm length 2
5	Length between post-oral arms
6	Stomach area
7	Mouth area
8	Pre-oral arm length 1
9	Pre-oral arm length 2
10	Length between pre-oral arms

Figure 4.1. Image of a *Tripneustes gratilla* pluteus larva depicting the various measurements used to compare growth and morphology of larvae derived from broodstock fed four different conditioning diets. The scale bar (bottom right) represents 50 µm.

Specific growth rates (SGR; %) were calculated across measurements 1 – 6 (Figure 4.1), as well as overall average SGRs for these measurements, for all groups based on the number days that they survived for:

$$SGR\ (\%) = \left(\frac{LNM_2 - LNM_1}{\Delta t}\right) \times 100 \quad (4)$$

where LN is the natural logarithm of M_1 and M_2 that represent the average across the respective larval measurements (as listed in Figure 4.1) at the initial time point and final time point, respectively, and Δt represents the number of days between the first and second time point as implemented by Brown (1975), Siikavuopio *et al*. (2012) and Rahman *et al*. (2016).

4.2.6. Juvenile sampling, DNA extraction, microsatellite amplification and genotyping

Larvae from the kelp- and mixed diet survived for the full duration of larval rearing and were combined across three 87 L settlement containers (L x W x H: 80 x 42 x 26 cm) pre-coated with the settlement substrate *Ulvella lens*. Larvae were deemed competent to settle when

the rudiments were larger than the stomach or when pedicellariae (tube feet) could be observed. *Ulvella lens* was used as a settlement substrate as it is known to promote larval settlement and post-settlement survival (Cyrus *et al.* 2017). Filtered sea water, as described for larval rearing, was supplied for 24 h of the day and outflow pipes were fit with 200 µm banjo screens to avoid the loss of free-swimming larvae before they settle. Water temperature was maintained at 25°C for the duration of juvenile rearing. Juveniles were fed *Ulva rigida* towards the end of the trial when large enough to consume macroalgal diets. A total of 364 juvenile sea urchins were sampled approximately three months after metamorphosis and whole samples were stored in 70% ethanol. DNA extractions were performed on the samples collected from kelp- and mixed diet fed broodstock animals (n = 16) and offspring (n = 364) as per the methods described in chapter 3. A panel of 12 microsatellite markers were polymerase chain reaction (PCR) amplified in multiplex reactions and products were separated *via* capillary electrophoresis, as described in chapter 2 and 3 (MP1 and MP3 in Appendix A, Table S2.1). Peakscanner v1.0 software (Applied Biosystems) was used for fragment length calling and allele binning was performed in Autobin v0.9 (Salin 2013) for a total of 380 individuals.

4.2.7. Genetic data analysis

Data were assessed for genotyping errors, null alleles and markers deviating from neutrality and Hardy-Weinberg equilibrium expectations using the methods outlined in chapter 3. Genetic diversity estimates, including allelic richness (A_r), effective number of alleles (A_e), number of private alleles, observed heterozygosity (H_o) and unbiased expected heterozygosity (uH_e), Shannon's information index (I), fixation index (F), polymorphic information content (PIC), and corresponding standard errors for each mean were calculated as described in chapter 2. The probability of inclusion (PI) and the probability of exclusion (PE), as well as corresponding standard errors (SE) for each mean, were calculated as described in chapter 3. Significant differences ($P < 0.05$) between genetic diversity estimates were evaluated using a Kruskal-Wallis test in JASP software. Effective population size (N_e) and corresponding fluctuations in effective population size was also assessed as per chapter 3.

Parentage assignment was performed in Colony v2 (Jones and Wang 2010) and VitAssign v8.2.1 (Vandeputte and Haffray 2014) as in chapter 3 and size differences between offspring from different progenitor broodstock diets were assessed by performing an ANOVA. The coefficient of variation (CV) for juvenile body diameter was calculated for offspring assigning

to kelp- and mixed diet broodstock, respectively, as well as for offspring treated as a single group:

$$CV = \frac{SD}{Mean} \quad (5)$$

Phenotypic data were assessed for normality (Shapiro-Wlik test) and homogeneity of variance (Levene's test) prior to estimating (co)variance components (additive genetic and residual effects) for body diameter in DMU AI software v6.5.2 (Madsen and Jensen 2008). After correcting for deviations from normal distribution (log transformation), a single trait mixed model was applied to obtain variance estimates using Average Information Restricted Maximum Likelihood (AI-REML) (Jensen *et al.* 1997):

$$y = XB + Zu + e \quad (6)$$

where y represents the phenotype, B, u and e act as the vectors of fixed-, random additive genetic- and residual effects, respectively. For the random additive genetic effects (0, $A\sigma^2_a$), A represents the pedigree derived numerator relationship matrix among animals and σ^2_a is the additive genetic variance. Similarly, for the residual effects (0, σ^2_e), σ^2_e represents the error variance. Incidence matrices relating observations to fixed effects and the additive genetic effect of each individual are represented by X and Z. In the current study, progenitor broodstock diet was fit as a fixed effect (at two levels). Variance components and corresponding standard errors were estimated for each group of broodstock animals (kelp and mixed) separately as these represented independently spawning groups, as well as combined. Poorly represented families that represented less than 2% of each group were excluded, although these families remained included when datasets were combined. Direct heritability (h^2) for a single trait, body diameter, was estimated as per:

$$h^2 = \frac{\sigma^2_a}{(\sigma^2_a + \sigma^2_e)} \quad (7)$$

Additionally, a sire-dam model was fit:

$$y = XB + Zs + Zd + e \quad (8)$$

where s and d represent the sire and dam that each offspring assigned to, respectively, to estimate heritability based on the resulting sire (σ^2_s) and dam (σ^2_d) variance components. Heritability estimates based on the dam covariance component were calculated as per:

$$h^2_d = \frac{\sigma^2_d}{(\sigma^2_d + \sigma^2_s + \sigma^2_e)} \quad (9)$$

and based on sire covariance components as per:

$$h_s^2 = \frac{\sigma_s^2}{(\sigma_s^2+\sigma_d^2+\sigma_e^2)} \quad (10)$$

Standard errors for these heritability estimates were calculated as described in Becker (1984).

Estimated breeding values (EBVs) for body diameter were obtained using Best Linear Unbiased Prediction (BLUP) in DMU AI to assess the influence that specific broodstock animals had on this trait in the offspring cohort. EBVs were calculated based on the combined dataset.

4.2.8. Data analysis to test for differences between treatments

Data analysis was performed using JASP software version 0.9.2 (JASP team 2020) for data collected from both spawning events, where the conditioning trials were treated separately for statistical tests. All data (body size, gonad size and colour, gamete size and counts, egg colour, egg energetic components, as well as egg fatty acid composition) were checked for normality (Shapiro-Wilk test and distribution plotting) and homogeneity of variance (Levene's test and Q-Q plots) before testing for differences between groups, where Brown-Forsythe correction was used prior to performing an analysis of variance (ANOVA), with diet as a fixed factor, if the data were heteroscedastic. A post-hoc Tukey's honestly significant difference (HSD) test was performed to identify the source of significant differences between the four diets if data were normally distributed. Where data were not normally distributed, a Kruskal-Wallis test was applied to assess differences between groups, and Dunn's post-hoc test was used to identify the groups showing differences. Pearson's correlation coefficients (*r*) and corresponding *P*-values were calculated for broodstock phenotypic data to assess the relationship between the respective variables. A correlation network was constructed in JASP (using the pcor estimator at a significance threshold of 0.05 and bootstrapping 999) for variables across all animals, across females and across males for both iterations of broodstock conditioning. Lastly, the R package, *ggplot2* (Wickham 2011), was used to perform a principal component analysis (PCA) of egg fatty acid composition, using log transformed data, to assess differences in fatty acid profiles across diets.

4.3. Results and Discussion

4.3.1. Broodstock phenotypic performance

The reproductive organs of sea urchins, the gonads, act as a nutrient storage organ (James and Siikavuopio 2012). There is an interplay between the primary gonadal cells, nutritive phagocytes and germinal cells that is observed during the six reproductive phases of sea urchins, namely the (1) recovery, (2) growing, (3) premature, (4) mature, (5) partly spawned and (6) spent phases (Vaïtilingon *et al.* 2005; Cyrus *et al.* 2013). The cells responsible for nutrient storage in gonads, the nutritive phagocytes, largely rely on the macro- and micronutrients provided by the diet for growth (Lawrence 2020b). Although there are underlying molecular mechanisms that stimulate gametogenesis, nutrient availability plays an integral role in this process. Therefore, by assessing the reproductive state of gonads, the effect of feed(s) or feeding regimes on reproductive performance can be evaluated.

The histological sections (Appendix C, Figure S4.1) and corresponding gonad reproductive phases (Figure 4.2) of animals from the first spawning event show that many nutritive phagocytes are present across animals from all diets, with females fed *U. rigida* and the mixed diet being in a partly spent state. The gonads sampled from animals in the first spawning event were likely in a growing or immature phase, therefore impeding artificial breeding. In contrast, the growing phase was absent for animals in the second iteration of conditioning (Figure 4.2), showing that the gonads of these animals were more mature (Appendix C, Figure S4.2). In these images, the empty spaces, where gametes used to reside, illustrates that gonads are mostly in a partly spent state (Figure 4.2), with only the formulated feed group having an individual with mature gonads. It is possible that the formulated feed enhanced gonad growth, as observed by previous studies using this formulated feed (Cyrus *et al*. 2015b). Overall, reproductive success was more likely for broodstock animals in the second iteration of broodstock conditioning. Although no notable differences in egg morphology were observed, the sperm morphology of the first spawning event indicated that these animals were likely not ready to spawn, as immature spermatocytes could be observed (Appendix C, Figure S4.3). Contrastingly, mature gametes were observed for animals from the second spawning event (Appendix C, Figure S4.4). Extending the broodstock conditioning period to four months, as opposed to three months for the first iteration of broodstock conditioning, could have resulted in differences in gonad maturity across the respective trials. It should be noted that gamete morphology can

be influenced by animal nutritional state, age, size and number of spawning events (Moran and McAlister 2009), and that the interplay between these factors is poorly understood.

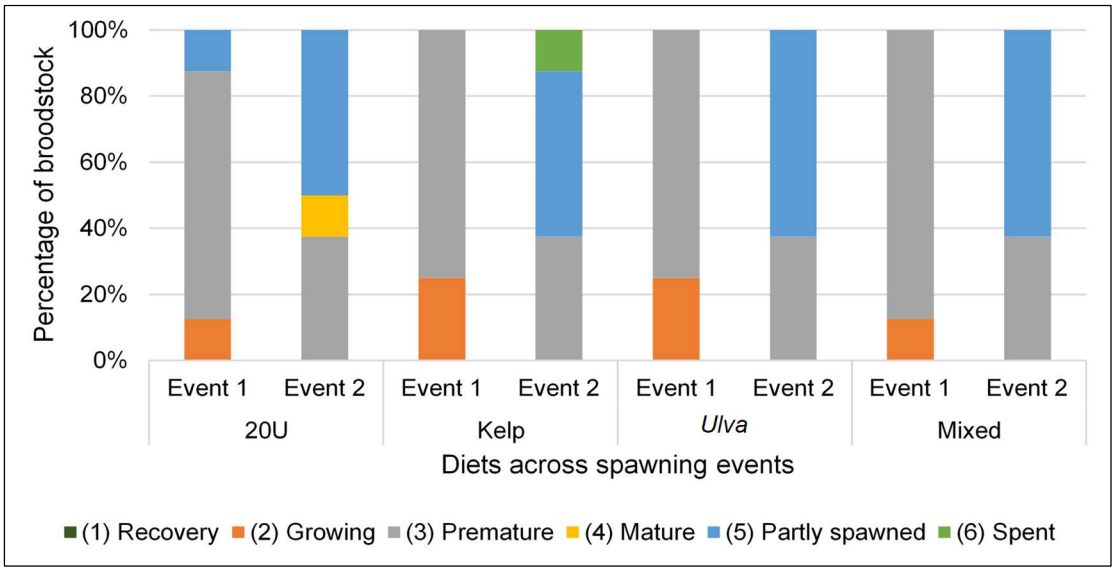


Figure 4.2. Stages of gonad development for *Tripneustes gratilla* broodstock (four males and four females per diet) used in two independent spawning events after conditioning animals on four feeding regimes: a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime.

Broodstock from the first spawning event had an average body weight of 167.72 ± 3.60 g, diameter of 7.57 ± 0.06 cm and a height of 4.54 ± 0.06 cm after the conditioning period. Across broodstock from the second spawning event, an average body weight of 209.59 ± 12.36 g, diameter of 7.91 ± 0.18 cm, and a height of 4.92 ± 0.15 cm was observed, with no statistically significant differences across diets for these size measurements in animals from either spawning event. Gonad size and associated gonad somatic index (GSI) have also been used as indicators of reproductive performance. The GSI of sea urchins can range from 0 – 35% and is likely to be at the upper end of the range when animals are held in a cultured environment with controlled feeding practices (James and Siikavuopio 2012). In the current study, the GSI ranged from 7.75 – 14.71 (average of 11.03 ± 0.96) and 6.36 – 16.14 (average of 11.05 ± 0.99) in the respective spawning events, with no significant differences in GSI between animals fed different diets (Appendix C, Table S4.1). Similarly, in a *T. gratilla* (*elantensis*) feeding trial, where sea urchins (with body diameters ranging from 2.50 – 6.18 cm) were fed a formulated feed, *Gracilaria conferta* (red seaweed) and *Ulva lactuca* (green seaweed) for approximately 36 weeks, maximum gonad indices of 14.40, 13.10 and 13.70 for animals fed the respective diets were found, with no statistically significant ($P > 0.05$) differences across diets (Shpigel *et al.* 2018). Similarly, a previous study on *T. gratilla* found a greater ($P < 0.05$) GSI for 20U fed animals, when compared to animals fed *Ulva*, after a

32 week feeding trial, which can be attributed to the high protein content of the feed (Cyrus *et al*. 2015b). Furthermore, the incorporation of *Ulva* in the formulated diet could have improved consumption and protein digestibility. It should be noted that this study was aimed at somatic growth during early adult stages (Cyrus *et al.* 2015b). In the present study, spawning broodstock prior to weighing and dissecting animals could have resulted in this reduced GSI when compared to previous studies. However, it has been observed that a high gonad index does not always indicate high fecundity (Vaïtilingon *et al.* 2005). Although a high, but decreasing gonad index is generally associated with mature or spent gonads (Vaïtilingon *et al.* 2005), reproductive output does not necessarily indicate reproductive effort (Lawrence 2020a). In the present study, the kelp and mixed diet had the greatest GSI across the first and second spawning events, respectively (Figure 4.3; Figure 4.4).

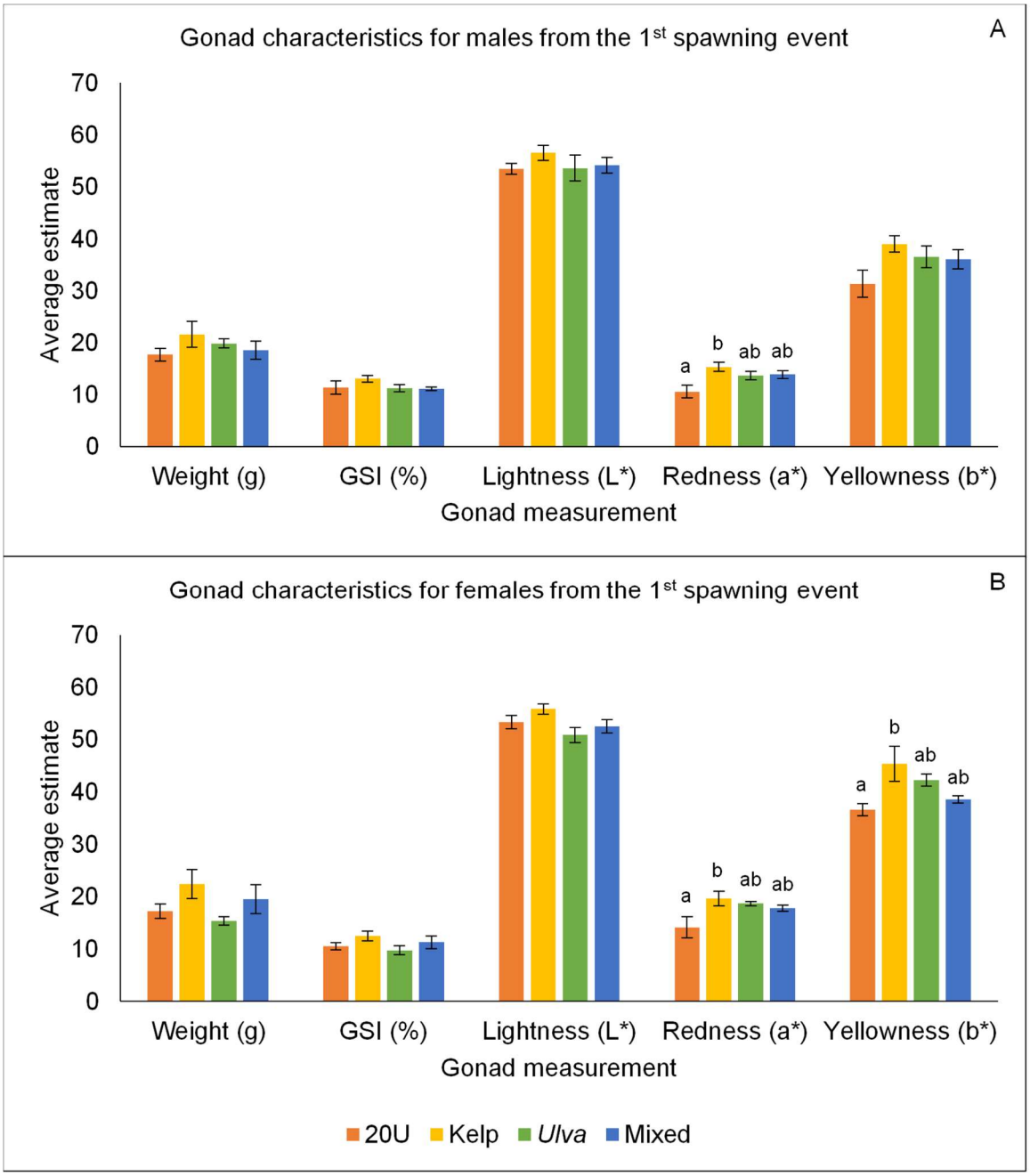


Figure 4.3. Average estimates (n = 4 in triplicate per individual) for *Tripneustes gratilla* gonad characteristics from the first spawning event for (A) males and (B) females conditioned on a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime, where the standard error for each mean is indicated. Different letters indicate statistically significant ($P < 0.05$) differences based on Tukey's post-hoc test, where no letters indicate no statistically significant differences between groups.

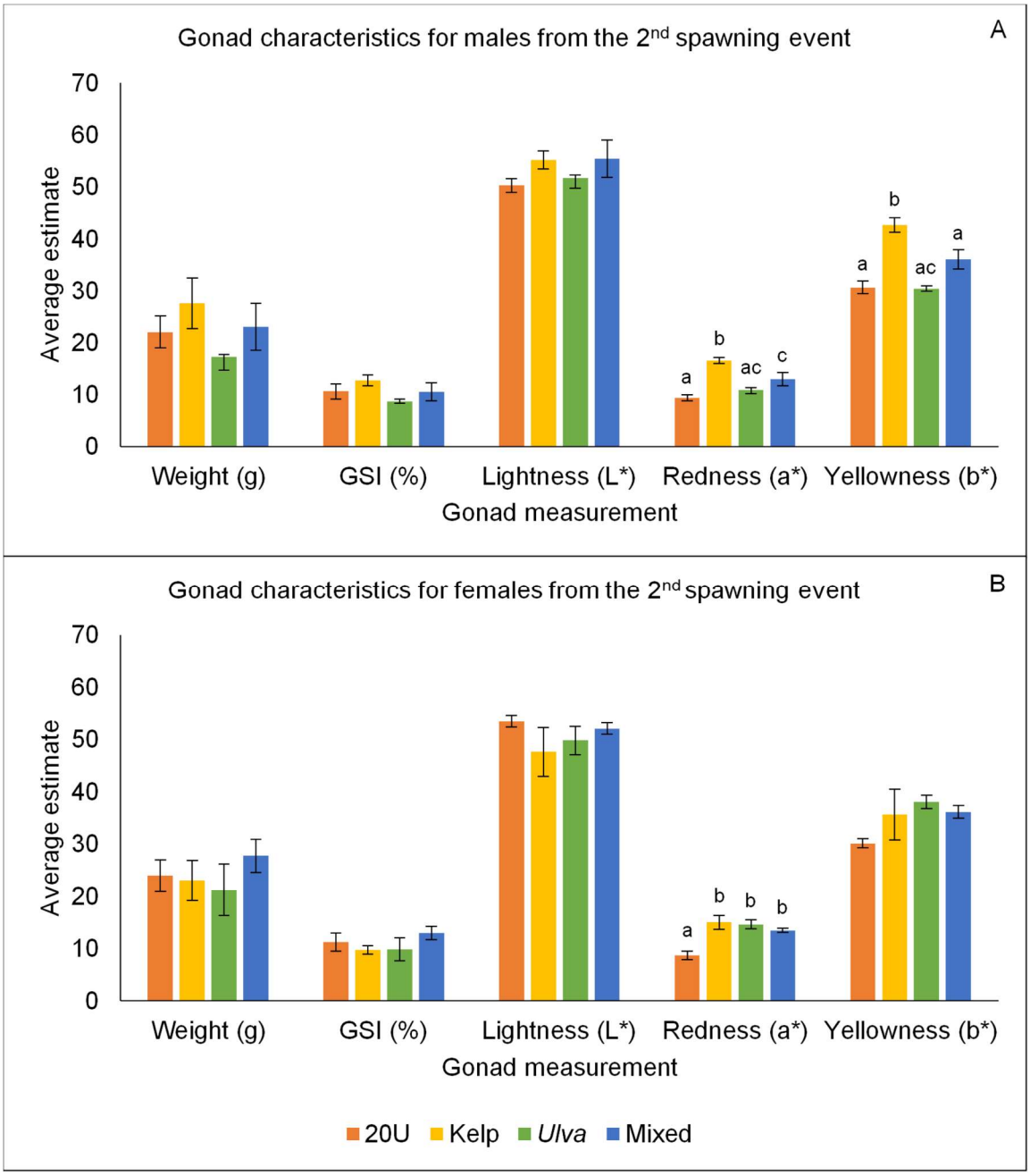


Figure 4.4. Average estimates (n = 4 in triplicate per individual) for *Tripneustes gratilla* gonad characteristics from the second spawning event for (A) males and (B) females conditioned on a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime, where the standard error for each mean is indicated. Different letters indicate statistically significant ($P < 0.05$) differences based on Tukey's post-hoc test, where no letters indicate no statistically significant differences between groups.

Both conditioning iterations resulted in significant gonad colour differences between broodstock fed the formulated feed (20U) and kelp diets, where the gonads of the 20U group consistently had lower redness and yellowness indices (Figure 4.3; Figure 4.4; Appendix C, Table S4.1). Furthermore, in the second iteration of broodstock conditioning, the broodstock fed the formulated feed showed had lower colour indices than all other diets, where gonad

redness was statistically significantly lower ($P < 0.05$) for animals fed a formulated feed (Figure 4.4). These results support the findings of Cyrus *et al*. (2015a), where *T. gratilla* fed fresh seaweed produced gonads with greater redness and yellowness indices than animals fed a formulated feed. It can be challenging for animals to obtain all the essential macro- and micronutrients required for somatic and gonadal growth when a single feed with fixed nutrients is being administered, particularly as the specific nutrient requirements of sea urchins remain understudied (Watts *et al.* 2010; Powell *et al.* 2020).

In sea urchins, gonad colour is influenced by carotenoids, which are naturally occurring red, orange or yellow pigments (Goodwin 1980; Matsuno and Hirao 1989). These pigments are usually of plant origin (Goodwin 1980), which explains the greater incorporation of carotenoids in gonads of animals fed seaweed-based feeds in this study, though the exact carotenoid content of the respective feeds were not assessed in this study. Furthermore, the only carotenoid component of the formulated diet is the inclusion of 20% *Ulva* (w/w) (Table 4.1). Carotenoids also play important anti-oxidant, ant-inflammatory, pro-vitamin A, photoprotection, radical quenching and immunity-related roles (Matsuno 1991; Pozharitskaya *et al.* 2015). A broodstock conditioning trial conducted by Carboni *et al*. (2015) found that gonads of sea urchins fed natural feeds, such as kelp (*Laminaria digitata*), had a higher echinenone content than that of animals fed formulated feeds. Echineone, encompassing 50 – 60% of the total gonad carotenoids, is the dominant carotenoid in sea urchin gonads (Symonds *et al*. 2007) and studies have found that gonadal echineone is correlated to gonad colouration (Pearce *et al*. 2003; Sphigel *et al*. 2005). Gonad echineone content is largely driven by the availability of dietary β-carotene, as well as by the processes that influence the uptake and bioconversion of this echineone precursor (Tsushima 2007; Hagen *et al*. 2008). Furthermore, studies in the sea urchin *Lytechinus variegatus* showed that diet carotenoid content, specifically β-carotene, lutein and zeaxanthin, was associated with greater fecundity (George *et al.* 2001). Therefore, in the current study, the greater gonad redness and yellowness indices for broodstock fed natural feeds suggest the more of these carotenoids were incorporated into their gonads and this could have had an impact on animal health and subsequent reproductive success, as well as roe marketability. However, the specific roles of these pigments in gametogenesis is not yet known and requires further investigation (Powell *et al.* 2020).

4.3.2. Male reproductive performance

Limited studies have assessed the effects of broodstock diet on male reproductive performance in sea urchins and broadcast spawning animals, and in the current study, limited differences in sperm count and length were observed. No statistically significant differences ($P > 0.05$) in sperm count were observed across diets, although the highest average counts were observed for *Ulva* (40 x 10^7 per mL) and kelp (65 x 10^7 per mL) fed broodstock in the first and second spawning events, respectively (Figure 4.5A). In aquaculture environments, sperm availability does not act as a limiting factor, however, sperm availability plays an important and complex role in nature, where various factors, such as seasonality, photoperiods and temperature can affect reproductive success (Albrizio *et al.* 2019).

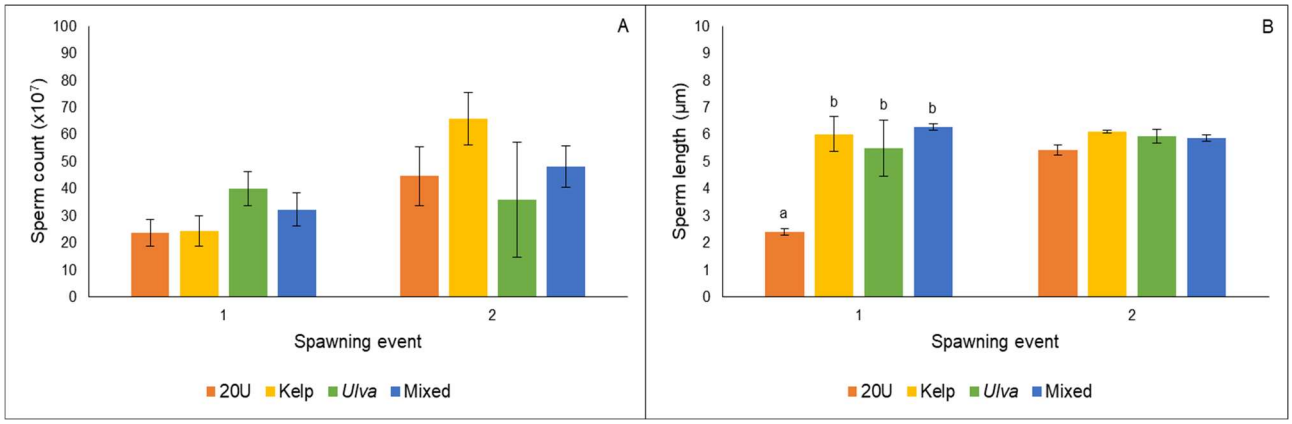


Figure 4.5. Average (A) sperm count (x10^7) and (B) sperm length (µm) across two spawning events for *Tripneustes gratilla* broodstock animals (n = 4 in triplicate per individual) conditioned on a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime, where the standard error for each mean is indicated. Different letters indicate statistically significant ($P < 0.05$) differences based on Tukey's post-hoc test, where no letters indicate no statistically significant differences between groups.

In the current study, sperm length ranged from 2.21 – 6.86 µm and 4.98 – 6.60 µm in the first and second spawning events, respectively (Figure 4.5B). The males fed a formulated diet produced smaller ($P < 0.05$) sperm bodies in the first spawning event (Figure 4.5B; Appendix C, Figure S4.3), suggesting that this feed negatively impacts sea urchin reproductive performance as larvae from this group of broodstock did not survive in either spawning event. However, the sperm produced by animals fed the formulated diet in the first spawning event were likely smaller as a result of the immature reproductive state of the gonads (Figure 4.2; Appendix C, Figure S4.1), as sperm from animals fed a formulated diet were not statistically significantly smaller in the second spawning event (Figure 4.5B). Nonetheless, broodstock fed a formulated feed produced smaller sperm across both

spawning events, highlighting the limitations associated with feeding a formulated diet, generally used for gonad enhancement to increase marketability, in isolation for reproductive purposes. Although no statistically significant differences across the remaining diets were observed, the largest sperm were observed for the males fed a mixed- (average sperm length of 6.28 µm) and kelp (average sperm length of 6.11 µm) diet in the first- and second spawning events, respectively (Figure 4.5). Similarly, for other echinoid species with conical sperm and a similar developmental mode (free-swimming larval phase) to *T. gratilla*, sperm lengths of 3.3 – 9.0 µm have been reported (Chia *et al*. 1975; Raff *et al.* 1990). Future studies could investigate the impact of other sperm functional parameters in *T. gratilla* reproductive success, as studies in the sea urchins *Heliocidaris erythrogamma* and *Lytechinus pictus* have shown that other functional parameters, such as curvilinear velocity, play an important role in fertilisation success (Smith *et al.* 2019).

4.3.3. Female reproductive performance

Across females, no statistically significant ($P > 0.05$) differences were observed for egg count (average of 246800 ± 44400 and 323200 ± 91300, respectively), egg area (average of 5331 ± 150 µm^2 and 5770 ± 276 µm^2, respectively) and egg diameter (average of 82.42 ± 1.18 µm and 84.26 ± 0.98 µm, respectively) across both spawning events (Figure 4.6). It was observed that the egg redness was significantly ($P < 0.05$) lower for the animals fed a formulated diet (average of 3.20) when compared to both the kelp (average of 4.13) and *Ulva* (4.20) diets in the first spawning event (Figure 4.6A). In the second spawning event, *Ulva* fed broodstock produced eggs with a higher redness index (average of 4.80) when compared to the other diets, however, no statistically significant differences were observed (Figure 4.6B). While no significant differences in egg yellowness was observed across diets or spawning events, the colour measurement method for eggs should be reassessed, as an orange or yellow egg colour was observed, but the negative yellowness (b*) indices suggest that the colour tended towards blue (Figure 4.6). It is possible that a white background, as used in the current study, interfered with egg yellowness colour readings.

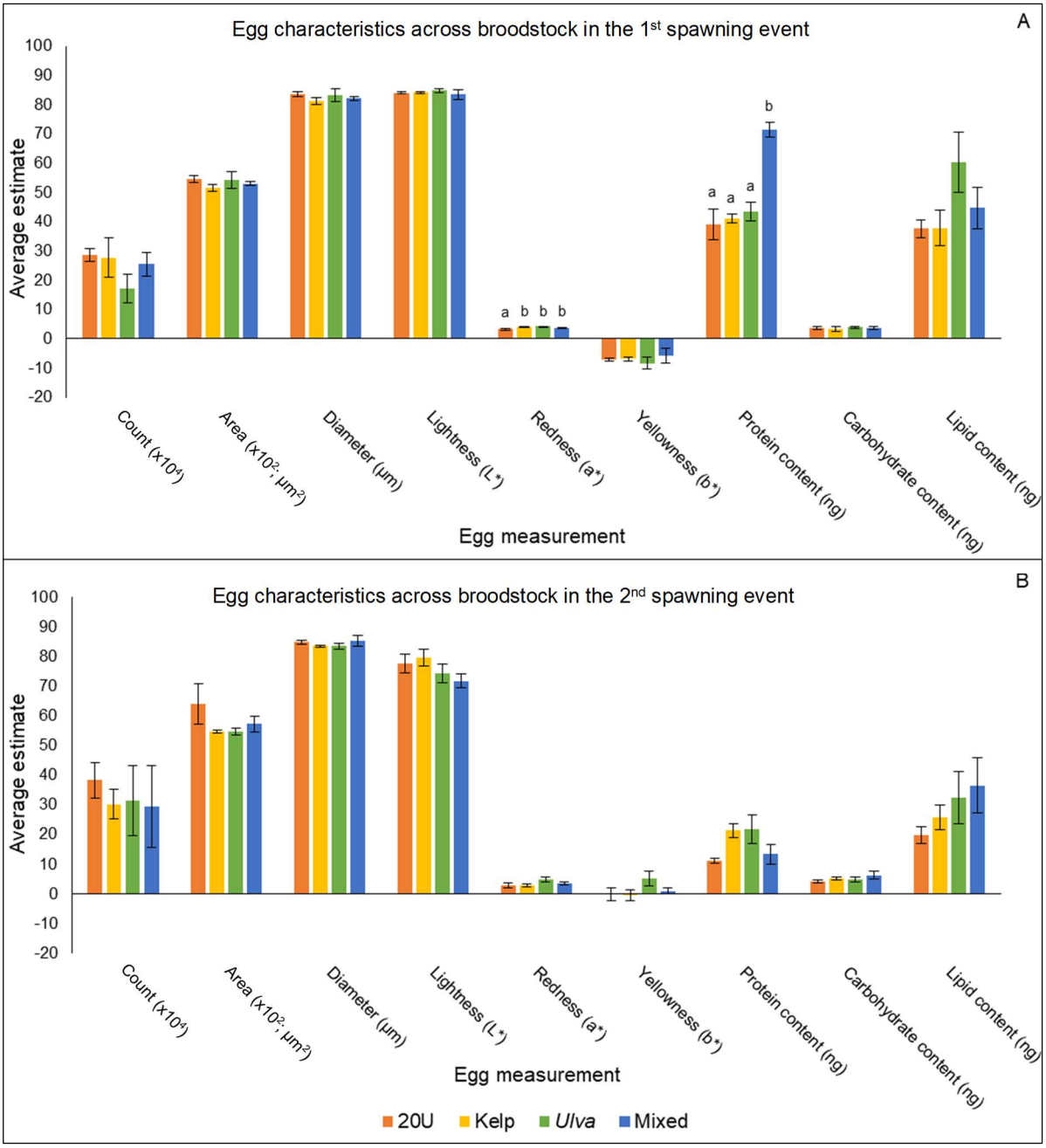


Figure 4.6. Average estimates (n = 4 in triplicate per individual) for egg characteristics for *Tripneustes gratilla* broodstock animals conditioned on a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime from the (A) first and (B) second spawning events, where the standard error for each mean is indicated. Different letters indicate statistically significant ($P < 0.05$) differences based on Tukey's post-hoc test, where no letters indicate no statistically significant differences between groups.

Nonetheless, the difference in colour profile (redness) could be a result of the carotenoids present in the natural feeds, kelp and *Ulva*, though this was not assessed in the current study. The predominant carotenoids in sea urchin eggs, β-carotene and β-echinenone, have been identified as playing a role in egg photoprotection [protection from ultraviolet (UV) radiation], cell division, rate of early larval development, as well as increased egg energy

content (de Jong-Westman *et al.* 1995a; 1995b; Nekvapil *et al.* 2019). Interestingly, across both spawning events, the mixed diet fed broodstock produced eggs with an intermediate redness index of 3.73 and 3.45, respectively (Figure 4.6). These results, coupled with higher gonad redness of broodstock fed natural feeds, suggest that the carotenoids that are provided by the diet are incorporated into gonads and eventually into eggs, where they could play important roles in egg quality. Although the influence of egg colour on reproductive success remains largely unexplored for many echinoderms (Montgomery *et al.* 2017), a study in the sea urchin *Pseudocentrotus depressus* found greater hatching rates and larval survival for broodstock fed diets that were supplemented with carotenoids (Tsushlma *et al.* 1997), suggesting that the carotenoids that influence egg colour profiles could have had an impact on early developmental stages of larvae in the current study, as improved survival was observed for larvae from broodstock with higher redness indices.

The quantification of egg energetic components showed that eggs collected from broodstock fed a mixed diet had a significantly ($P < 0.05$) higher egg protein content than that of the other diets, ranging from 65.18 – 76.28 ng/egg for the first spawning event (Figure 4.6; Appendix C, Table S4.1). These results are comparable to that of a previous study in wild *T. gratilla*, that were feeding on natural food sources, that found an egg protein content of 87.32 ng (Byrne *et al.* 2008a). It would be expected that eggs collected from the females fed a formulated diet, with a high protein content of 25.69% (Cyrus *et al*. 2014), would produce eggs rich in protein. However, the incorporation of fresh feeds in the mixed feeding regime likely increased the sea urchin's ability to assimilate and utilise the protein provided by the formulated feed, as observed for diets supplemented with *Ulva* and kelp in previous studies in *T. gratilla* and abalone (Naidoo *et al.* 2006; Dlaza *et al.* 2008; Cyrus *et al.* 2015a). Furthermore, it has been shown that the enzymatic activity of bacteria introduced by the inclusion of a fresh feed, such as kelp, promotes digestion (Nel *et al.* 2017). The exact dietary protein requirements for sea urchins are unknown, even though this macronutrient plays important roles in reproduction, development, growth and maintenance of body structures (Jaeckle 1995; Watts *et al.* 2020).

Specific to sea urchin reproduction, a major yolk protein (MYP) has been identified (Hammer *et al.* 2006a, b; Watts *et al.* 2020). It has been suggested that free amino acids provided by diet contributes to the production of this protein that is stored in nutritive phagocytes in sea urchin gonads (Prato *et al.* 2018). This protein can be degraded into amino acids that are used for the synthesis of new proteins, nucleic acids, nitrogen-containing substances in eggs and sperm, as well as embryo formation (Unuma 2002; Watts *et al.* 2020). Furthermore, it

serves as a cell-adhesion molecule, through calcium-calcium binding, that is present on the exterior membrane of eggs (Dev and Robinson 2014). Therefore, it has been hypothesised that diets with a similar amino acid composition to MYP should be supplied to reduce the energetic costs of synthesising this protein with important roles in sea urchin reproduction (Prato *et al.* 2018; Powell *et al.* 2020). Feeding trials in the sea urchin *Paracentrotus lividus*, where urchins were fed *Ulva* and a formulated feed, found a greater amount ($P < 0.05$) of free essential amino acids, such as lysine, threonine and tryptophan, in gonad tissues of sea urchins fed a combination of these feeds (Prato *et al.* 2018). The authors also found greater amounts of non-essential amino acids in the gonads of these animals (Prato *et al.* 2018). Therefore, it is possible that the amino acids provided by the diets administered in the current study influenced gonad profiles and the energy required to generate MYP, as both *Ulva* and kelp contain the essential and non-essential amino acids required to produce this protein (see chapter 1, Table 1.2; Newell *et al.* 1980; Shuuluka *et al*. 2013; Prato *et al.* 2018). This could also explain the efficacy of a mixed feeding regime, as a diverse array of amino acids have the potential to be incorporated into the gonads and subsequent gametes. This hypothesis is further supported by the higher, although not statistically significant ($P > 0.05$), egg protein content that was observed for animals fed natural feeds, kelp (21.34 ng/egg) and *Ulva* (21.79 ng/egg), when compared to that of animals fed a formulated diet (11.11 ng/egg), in the second spawning event (Figure 4.6).

Sea urchins have a sedentary lifestyle that requires low energy inputs and thus high protein:carbohydrate ratios in feeds result in greater amounts of growth and production (Hammer *et al.* 2006a; Heflin *et al.* 2012). Egg carbohydrate content ranges from 3.30 to 5.30 ng/egg across various sea urchin species (McAlister and Moran 2012). Similarly, in the current study, average egg carbohydrate contents of 3.63 ± 0.26 and 5.07 ± 0.44 were observed for the respective spawning events across all treatments (Figure 4.6), with no significant differences between the different feeding regimes (Appendix C, Table S4.1). Carbohydrates play a role in protecting eggs from mechanical damages, as polysaccharides form the jelly coat surrounding the egg, as well as the hyaline layer that forms around the embryo during development (Bonnell *et al.* 1994; Cerra 1994). As several carbohydrases have been identified in the sea urchin gut (Lawrence *et al.* 2007), it is thought that sea urchins are readily able to digest carbohydrates, which are subsequently stored in gonads (Marsh and Watts 2007). In previous studies, it was observed that animals fed with low carbohydrate diets had decreased gonad production (Schlosser *et al.* 2005). However, no statistically significant differences in gonad size was observed in the current study.

Therefore, the small carbohydrate component of *T. gratilla* eggs that was estimated in the current study, supports the hypothesis that carbohydrates obtained from diets are not primarily allocated to eggs, but are being used for the cellular processes that support gametogenesis (Zalutskaya *et al.* 1986).

Lastly, egg lipid content, the major source of energy during early larval development, was estimated in this study. An overall average egg lipid content of 45.07 ± 3.95 and 28.58 ± 3.47 was observed across all treatments for the respective spawning events (Figure 4.6). These results are similar to previous studies that found an egg lipid content of 30.82 ng (Byrne *et al.* 2008a) for wild *T. gratilla*. Byrne *et al*. (2008a) found that 55.52% of the total egg lipids are composed of energetic lipids, such as trigylcerides, that fuel early larval development, which was supported by maternally transferred lipids being exhausted after 10 days when larvae were left unfed (Byrne *et al.* 2008a). Although no significant differences between feeding regimes were observed in the current study, eggs from females fed *Ulva* (first spawning event) and a mixed diet (second spawning event) had the greatest amount of lipids (Figure 4.6). The limited differences between feeds in the current study could have occurred as a result of the high quantity/quality of food, as both natural feeds and the formulated feed have high nutrient contents. There is a paradoxical relationship between food quality and assimilation that further complicates the development of sea urchin feeding regimes, as an increase in food quality can result in an increased amount of energy required to incorporate nutrients in their tissues (Mcbride *et al.* 1997). Future studies could investigate the specific lipid classes associated with different feeding regimes, as these could be playing roles in larval performance.

In addition to the effect of different feeding regimes, it is likely that there was a relationship between gonad maturity and egg nutrient profiles. When assessing the correlations between the female phenotypic variables, an interplay between egg count and egg energetic components was observed for the females from both spawning events. It was observed that egg count was negatively correlated with egg lipid content (r = -0.87; P < 0.001) in the first spawning event (Appedix C, Figure S4.5). In the second spawning event, egg protein- (r = -0.66; P < 0.01), carbohydrate- (r = -0.89; P < 0.001) and lipid content (r = =0.89; P < 0.001) was negatively correlated with egg count (Appendix C, Figure S4.5). These results suggest that the energetic cost of producing an egg impacts the number of eggs an individual female can produce (Smith and Fretwell 1974), acting as a driver of fecundity in broadcast spawning animals, such as *T. gratilla*. Additionally, body weight was positively correlated with egg count (r = 0.60; P < 0.05) and negatively correlated with egg size (r = -0.53; P < 0.05) in the

first spawning event cohort (Appendix C. Figure S4.5), indicating that larger animals produce more eggs that are smaller in size. This could be explained by animals from the first spawning event having immature gonads that produced immature eggs. Alternatively, it is possible that larger animals need to allocate more resources to body maintenance, resulting in the formation of smaller eggs. However, previous studies have shown that the same quantity of energy can be allocated to reproductive efforts, regardless of sea urchin size (Hirshfield and Tinkle 1975). In the current study, there were no direct correlations between egg size and egg protein (r = -0.06, P = 0.82; r = -0.30, P = 0.25), lipid (r = 0.03, P = 0.92; r = -0.19, P = 0.48) and carbohydrate (r = -0.13, P = 0.63; r = -0.18, P = 0.51) content across either cohort (Appendix C, Figure S4.5). Therefore, egg size should not be used as a predictor of egg biochemical constituents. It was previously thought that as egg size increases, there would be an increased maternal investment in egg energetic components. This increased energetic investment in larger eggs is thought to improve larval development, increase larval size, increase the larval feeding period and size at the end of the planktonic larval stage, resulting in faster juvenile growth and improved larval survival after metamorphosis (Strathmann 1985; McEdward 1986; Marshall *et al.* 2003; Miner *et al.* 2005; Moran and McAlister 2009). However, in an aquaculture environment, where sperm availability is not a limiting factor in the external fertilisation process, selection for smaller eggs is not necessarily acting against maternal investment in egg energetic components.

4.3.4. Egg fatty acid profiles

Studies have mostly assessed fatty acids in sea urchin gonads in context of commercial applications, as they are thought to impact the taste of the product and the effect of these compounds on sea urchin reproduction remains understudied. However, sea urchin egg fatty acid profiles, influenced by maternal diets, could be involved in reproductive success and larval performance (Carboni *et al*. 2013). In the current study, the least diverse fatty acid profile was observed for animals fed a formulated diet (Figure 4.7). The profile of the formulated feed group showed a large degree of overlap with that of *Ulva* fed animals. This can be explained by this formulated feed containing 20% *Ulva*, thus the fatty acids present in this macroalgae are incorporated into the formulated feed and are subsequently incorporated into the eggs of animals conditioned on this diet. For *Ulva* and kelp fed broodstock, a more diverse egg fatty acid profile was observed across animals (Figure 4.7). These natural feeds likely contain a variety of these compounds. Specifically, the clustering patterns of eggs from animals fed different conditioning diets is driven by differences in three

groups of compounds, namely; C16, C20:1, C20:5n3, C22:1, C20:4n6, C20 and C20:2, C14, C18:2n6, C18:1n9 and C18, C15, C20:3n3, C18:3n3, C16:1, C14:1.

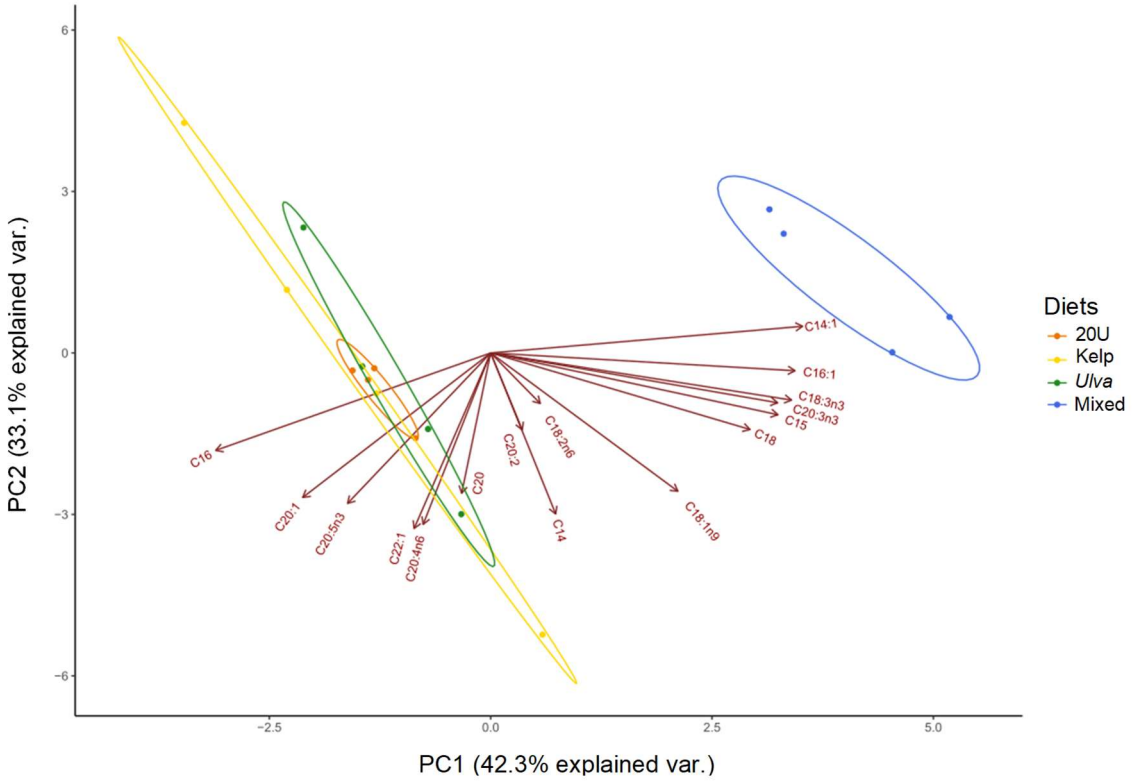


Figure 4.7. Principal component analysis (PCA) of egg fatty acid profiles (log transformed) across *Tripneustes gratilla* broodstock conditioned on a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime (n = 4 per diet), where vectors indicate the effect of each compound on the overall distribution of each plot.

Of these compounds, linoleic acid (C18:2n6), is known to play important roles in sea urchin growth (Watts *et al*. 2020) and was most abundant (%; 17.88 ± 1.60) in eggs from broodstock fed a formulated feed ($P < 0.05$), as opposed to that present in animals fed kelp- (7.79 ± 2.76), *Ulva*- (0.85 ± 0.13) and mixed diet (0.39 ± 0.10) (Table 4.3). Studies have suggested that this compound has negative effects when supplied in excess, as high dietary linoleic acid content is associated with increased arachidonic acid (20:4n6) in tissues, as sea urchins are thought to be capable of *de novo* synthesis of arachidonic acid (C20:4n6) and eicosapentaenoic acid (C20:5n3) (Bell *et al*. 2001; Castell *et al*. 2004; González-Durán *et al*. 2008). Arachidonic acid is an eicosanoid precursor (Funk 2001), promoting inflammatory responses and impeding growth in sea urchins (Castell *et al*. 2004). However, a previous study in *T. gratilla*, where animals were fed green-, brown- and red seaweed diets, suggested that *T. gratilla* is capable of synthesising eicosapentaenoic acid (C20:5n3), but not arachidonic acid (C20:4n6) (Floreto *et al*. 1996). In the current study, this could have

occurred for animals fed a formulated feed. Alternatively, these compounds were present in the diets. Larvae from broodstock fed a formulated feed did not survive for the full duration of larval rearing, which suggests that there could have been a negative dietary effect during broodstock conditioning. In the case of kelp fed animals, with a higher egg linoleic content ($P < 0.05$) than those fed *Ulva* and mixed diets, the linoleic acid content did not impede reproductive success, suggesting that this feed contained appropriate amounts for sea urchin growth, supporting the use of this feed as a broodstock conditioning diet. Interestingly, compounds are not always incorporated in the eggs of animals fed a mixed diet, suggesting that there are interactions between the fatty acids themselves, as well as with other compounds throughout the assimilation process that alter the eventual fatty acid profile of the eggs.

Table 4.3. Average fatty acid composition (%) of *Tripneustes gratilla* eggs collected from the second iteration of broodstock (n = 4 per diet) conditioning on a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime. Statistically significant differences are shown by different letters based on ANOVA or Kruskal-Wallis tests followed by a post hoc Tukey's (t) or Dunn's test (z). No letters indicate that there were no statistically significant differences between groups.

	Broodstock feeding regime			
Compound	**20U**	**Kelp**	***Ulva***	**Mixed**
Myristic acid (C14)	8.70 ± 0.51	9.24 ± 2.16	12.38 ± 1.62	10.60 ± 1.15
Myristoleic acid (C14:1)	0.64 ± 0.08 ^{a}	0.72 ± 0.15 ^{ab}	1.05 ± 0.14 ^{b}	0.97 ± 0.12 ^{c}
Pentadecylic acid (C15)	0.49 ± 0.01 ^{a}	0.53 ± 0.14 ^{a}	0.39 ± 0.04 ^{a}	0.68 ± 0.06 ^{b}
Palmitic acid (C16)	24.05 ± 1.3 ^{a}	29.40 ± 6.66 ^{a}	31.95 ± 3.37 ^{a}	33.54 ± 2.33 ^{b}
Palmitoleic acid (C16:1)	6.62 ± 0.40 ^{a}	8.22 ± 2.26 ^{ab}	12.09 ± 1.57 ^{b}	8.97 ± 1.33 ^{c}
Stearic acid (C18)	5.43 ± 0.24 ^{a}	6.35 ± 0.86 ^{a}	5.96 ± 0.58 ^{ab}	8.88 ± 0.40 ^{b}
Elaidic acid (C18:1n9)	8.30 ± 0.55	6.60 ± 2.05	7.35 ± 0.83	5.60 ± 0.60
Linoleic acid (C18:2n6)	17.88 ± 1.60 ^{a}	7.79 ± 2.76 ^{b}	0.85 ± 0.13 ^{c}	0.39 ± 0.10 ^{b}
Alpha-linolenic acid (C18:3n3)	2.31 ± 0.12 ^{a}	1.62 ± 0.52 ^{a}	1.98 ± 0.23 ^{a}	1.59 ± 0.16 ^{b}
Arachidic acid (C20)	0.97 ± 0.09	0.85 ± 0.34	0.74 ± 0.38	1.62 ± 0.25
Eicosenoic acid (C20:1)	4.74 ± 0.23 ^{a}	5.13 ± 1.48 ^{a}	6.82 ± 0.95 ^{a}	6.32 ± 0.99 ^{b}
Eicosadienoic acid (C20:2)	4.63 ± 0.36 ^{a}	2.78 ± 1.06 ^{ac}	0.47 ± 0.09 ^{b}	0.86 ± 0.17 ^{c}
Eicosatrienoic acid (C20:3n3)	0.59 ± 0.06 ^{a}	1.03 ± 0.32 ^{ab}	1.18 ± 0.19 ^{b}	3.70 ± 0.37 ^{d}
Arachidonic acid (C20:4n6)	5.46 ± 0.68 ^{a}	7.19 ± 2.27 ^{a}	8.87 ± 1.13 ^{a}	3.20 ± 0.46 ^{b}
Eicosapentaenoic acid (C20:5n3)	7.80 ± 0.53 ^{ab}	10.90 ± 2.66 ^{a}	5.85 ± 0.93 ^{b}	10.22 ± 1.11 ^{c}
Erucic acid (C22:1)	1.39 ± 0.07 ^{a}	1.67 ± 0.55 ^{a}	2.08 ± 0.40 ^{a}	2.87 ± 0.51 ^{b}
Saturated fatty acids (SFAs)	39.64 ± 2.16 ^{a}	46.36 ± 10.15 ^{a}	51.41 ± 5.99 ^{a}	55.32 ± 4.20 ^{b}
Monounsaturated fatty acids (MUFAs)	20.31 ± 1.26 ^{a}	20.66 ± 5.94 ^{a}	27.31 ± 3.49 ^{a}	21.85 ± 3.04 ^{b}
Polyunsaturated fatty acids (PUFAs)	40.06 ± 3.42 ^{a}	32.98 ± 10.15 ^{b}	21.28 ± 3.10 ^{b}	22.83 ± 2.89 ^{b}

Alpha-linolenic acid (C18:3n3) has also been identified as an important compound for sea urchins and is known to be abundant in *Ulva* (Trigui *et al*. 2013). In the current study, this compound was present in similar proportions across the eggs of animals fed the respective diets (Table 4.3), and the animals were able to integrate this compound into their eggs effectively. Interestingly, the fatty acid profile of eggs from broodstock fed a mixed diet clustered seperately from the other diets (Figure 4.7). This shows that the mixed diet displayed a unique fatty acid profile in comparison to the other diets, likely as a result of the incorporation of each of the single feeds in gonad tissue and eventually into eggs. Eggs from animals fed a mixed diet also displayed the greatest proportions ($P < 0.05$) of saturated fatty acids (SFAs), with eggs from *Ulva* fed broodstock having a higher monounsaturared fatty acid (MUFA) content (Table 4.3). Eggs from animals fed a formulated diet had the highest polyunsaturated fatty acid content (Table 4.3).

Across SFAs, myristic acid (C14) and palmitic acid (C16) were the most abundant compounds (Table 4.3). Palmitoleic acid (C16:1), eicosenoic acid (C20:1) and arachidonic acid (C20:4n6) were the dominant MUFAs. Across all diets, eicosepentanoic acid (C20:5n3) was the most abundant PUFA (Table 4.3). These results are similar to a previous study that assessed the fatty acid content of wild *T. gratilla* gonads in Vietnam, where SFAs, MUFAs and PUFAs composition was 41.74%, 26.35% and 31.08%, respectively (Huo *et al*. 2018). In the current study, the highest PUFA content was observed for eggs from broodstock fed a formulated feed (Table 4.3), which are predominantly obtained from diets in sea urchins (Watts *et al*. 2020). Studies in the sea urchin *P. lividus* have suggested that sea urchins are able to synthesise PUFAs when they are supplied with a formulated diet (Prato *et al*. 2018). Therefore, these results support that formulated diets supplemented with natural feeds are optimal for sea urchins, as animals from the mixed feeding regime are able to incorporate dietary fatty acids as needed for reproduction. Alternatively, future studies could aim to optimise a formulated feed for reproductive purposes.

Overall, results showed that egg fatty acid profiles could be influenced by dietary fatty acid content, although it remains unclear how these compounds interact. Studies have found correlations between feed- and gonad fatty acid composition (Kelly *et al*. 2008; Schram *et al*. 2018). Furthermore, it has been suggested that digestion of macroalgae is dependent on species and diet, as consistent patterns of retention and depletion of precursor fatty acids was observed for the sea urchins *S. droebachiensis* and *S. purpuratus* fed different diets, possibly as a result of processes performed by the gut microbiome (Schram *et al*. 2018).

Therefore, future studies in *T. gratilla* should aim to assess feed- and gonad fatty acid profiles, in combination with that of eggs, perhaps at various reproductive stages, so as to provide insight on the trophic transfer of fatty acids, their interactions and compositions throughout the ingestion and assimilation process in sea urchins.

4.3.5. Larval growth and survival

Larval development can be influenced by several factors, such as temperature, changes in salinity, presence of minerals or predators in water column, light, UV radiation, density, as well as by the larval feeding practices (Milonas *et al.* 2010; Metaxas 2020). The progenitor maternal diets can also affect larval growth and survival as the energy quotient provided by the eggs are thought to fuel early developmental stages in sea urchin larvae (Byrne *et al.* 2008a). Feeding planktotrophic larvae, such as *T. gratilla* larvae, collect food from their environment to survive their larval phase and successfully undergo metamorphosis (Carrier *et al.* 2018).

Across larvae (established through 16 crosses per diet) that were fed a mixed algal diet for the duration of larval rearing, only those from kelp- and mixed diet fed broodstock survived for the full duration of 20 days (Appendix C, Figure S4.6A). Larvae from broodstock fed a formulated diet and *Ulva* diet survived for 8 and 13 days, respectively (Appendix C, Figure S4.6A). Though not statistically significant ($P > 0.05$), the least amount of growth, across the majority of larval measurements (Appendix C; Table S4.2), with an average SGR across all measurements of 14.28% on day eight, was observed for the formulated diet group (Figure 4.8). These results show that this diet should not be used in isolation for reproductive purposes, as suggested by previous works that utilised formulated diets for broodstock conditioning of *Paracentrotus lividus* (Carboni *et al.* 2015). This is expected as formulated diets are generally used as gonad enhancement diets, rather than for broodstock conditioning for reproductive purposes. Furthermore, these results indicate that egg fatty acid composition could have played a role in larval development, as the eggs from the 20U fed animals displayed the least diverse fatty acid profiles across animals and had some compounds in excess (Table 4.3). Larvae from broodstock fed kelp and a mixed diet displayed similar growth (Appendix C, Table S4.3), as comparable growth rates were observed for these groups throughout the planktonic larval stage across all measurements (day 8 = 17.24%; 17.71%; day 13 = 11.96%; 12.76%, day 20 = 9.81%; 10.03%, respectively) (Figure 4.8). This indicates that kelp is an effective broodstock conditioning feed that is able

to be assimilated for reproductive purposes both when fed in isolation and when fed in a mixed feeding regime.

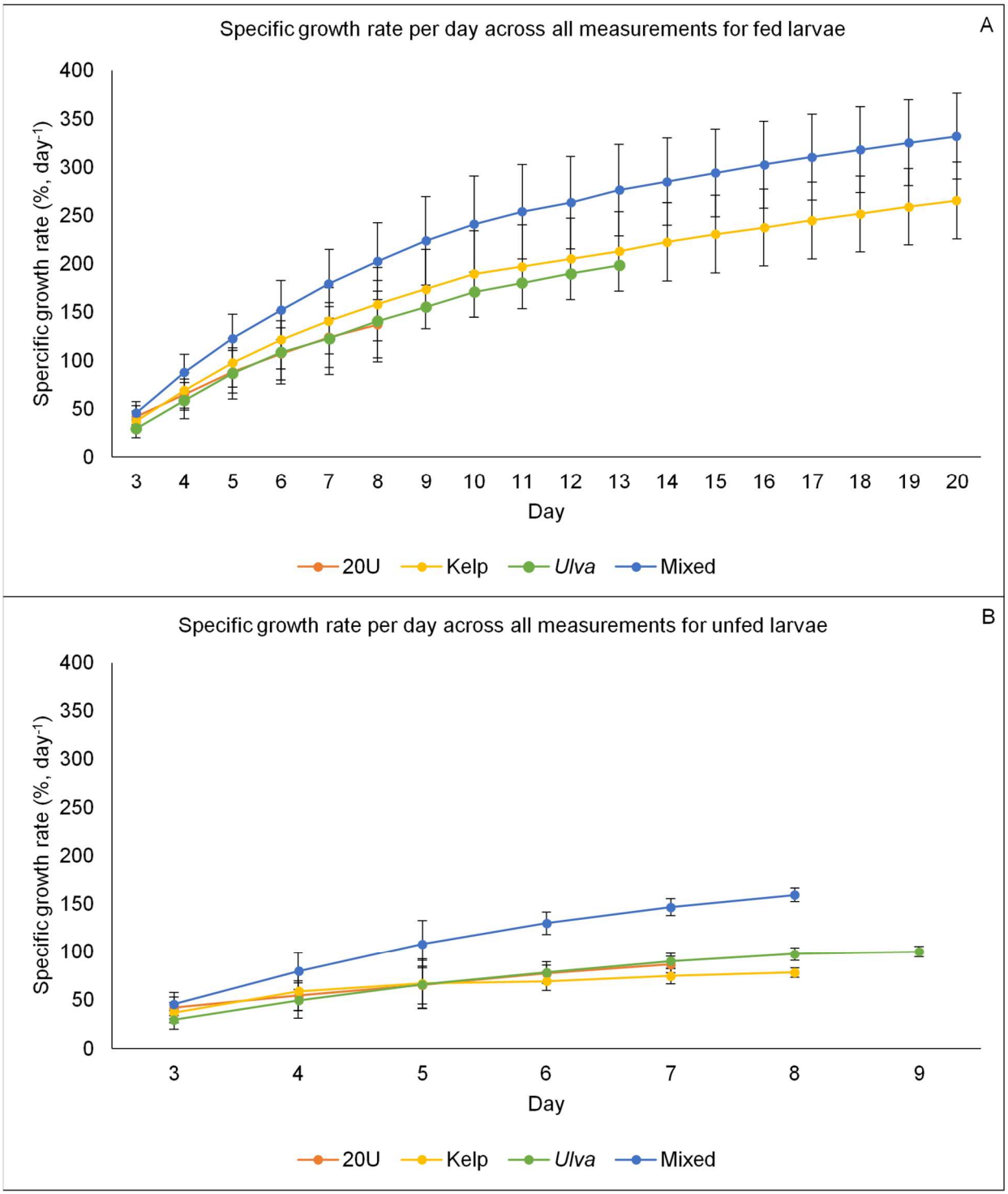


Figure 4.8. Average specific growth rate (µm per day) across larval measurements (body width, body height, post-oral arm length, length between post-oral arms and stomach area) for (A) fed and (B) unfed *Tripneustes gratilla* larvae from broodstock animals conditioned on a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime, where the standard error for each mean is indicated and no statistically significant differences were observed between groups.

Interestingly, larvae from *Ulva* fed broodstock had the greatest overall average SGR for the duration of larval rearing (Figure 4.8; Appendix C, Figure S4.7). These high growth rates could be attributed to the higher energetic reserves that were observed for eggs collected from *Ulva* fed animals (Figure 4.6). It is also possible that the higher β-carotene content of *Ulva* spp. (Shuuluka *et al.* 2012) promoted larval growth, as high gonad- and egg redness indices were observed for *Ulva* fed broodstock in the current study (Figure 4.6). Although fast larval growth is beneficial as it reduces the amount of time larvae spend in this vulnerable life-history phase, there could be an energetic trade-off that affects survival, as larvae from *Ulva* fed broodstock did not survive for the full duration of larval rearing. Alternatively, previous studies in the sea urchin *Paracentrotus lividus* hypothesise that larval mortality is more likely a consequence of larval rearing practices, such as stocking, feeding, microorganisms, temperature, salinity and water exchange, rather than maternal diets (Carboni *et al.* 2015). In the current study, this could explain the mortality of larvae from *Ulva* fed broodstock, as these larvae were growing at a faster rate than other larvae, but were fed (a microalgal diet) at the same rate. Subsequently, this could have resulted in a food deficiency, increased mortalities and corresponding diminishing water quality (not measured in this study), resulting in even more mortalities and eventual death of all larvae in a tank. This is particularly applicable for these larvae, as they had the highest larval counts across both the fed and unfed groups (Appendix C, Figure S4.6), which further exacerbates the competition for food. Therefore, *U. rigida* should not be disregarded as a broodstock conditioning diet in future studies.

The period of time that larvae can survive without consuming exogenous food (facultative feeding period) varies across species and is dependent on maternal egg investments (Miner *et al.* 2005; Byrne *et al.* 2008a). Across the non-fed larvae, the larvae from *Ulva* fed broodstock survived for the longest amount of time (nine days) on their maternal reserves (Appendix C, Figure S4.6B). Although these larvae survived for the longest amount of time without being fed, they did not survive for the full duration of larval rearing when fed, likely due to energetic trade-offs or larval rearing practices as listed above. Nonetheless, a study in *T. gratilla* has found that larvae can survive without feeding for longer than the eight days that was proposed for other echinoids that produce eggs of comparable size (Byrne *et al.* 2008b). In nature, this could be beneficial when food sources are limited, while in aquaculture environments, it could reduce the effect of competition amongst larvae for food as they appear to be more tolerant to changes in nutrient availability (Lawrence and Bazhin 1998; Miner *et al.* 2005; Byrne *et al.* 2008b). Across the non-fed larvae, the *Ulva* and mixed

diet group showed the greatest amount of growth in their non-fed state (Appendix C; Figure S4.8, Table S4.4; Table S4.5), with average SGRs across all measurements of 16.66% and 11.70%, respectively, at day seven (Figure 4.8).

In sea urchins, larval post-oral arm length acts as a function of food availability, as an increased feeding rate is expected for larvae that are able to extend their post-oral arms in response to low food availability, thereby increasing the volume of water and food that gets filtered, particularly during the early larval stages (Byrne *et al.* 2008a, b; García *et al.* 2015). Sea urchin larvae are suspension feeders that propel themselves in an upward or helical direction (McEdward and Young 1995). Food particles are trapped by their arms and the water containing the algal feed gets drawn into their bodies (Hart 1991). Across larvae fed a mixed microalgal diet, statistically significant differences ($P < 0.05$) across progenitor broodstock diets in post-oral arm length were observed in the first seven days of larval rearing (Figure 4.9; Appendix C, Table S4.3), where broodstock fed a mixed diet produced larvae with longer arms, reaching an average post-oral arm length of 231.73 µm by day seven (Figure 4.9). Larvae from broodstock fed a kelp diet had a greater average post-oral arm length (260.07 µm) than those fed a formulated diet (229.47 µm) at day seven (Figure 4.9; Appendix C, Table S4.4). This increase in post-oral arm length was generally accompanied by an increase in the distance between post-oral arms for fed larvae (Appendix C, Figure S4.8), however, this was not observed for larvae from 20U fed broodstock, suggesting that these larvae were not developing normally. Further supporting this, is the lack of growth in body size that was observed for larvae from 20U fed broodstock (Appendix C, Figure S4.8).

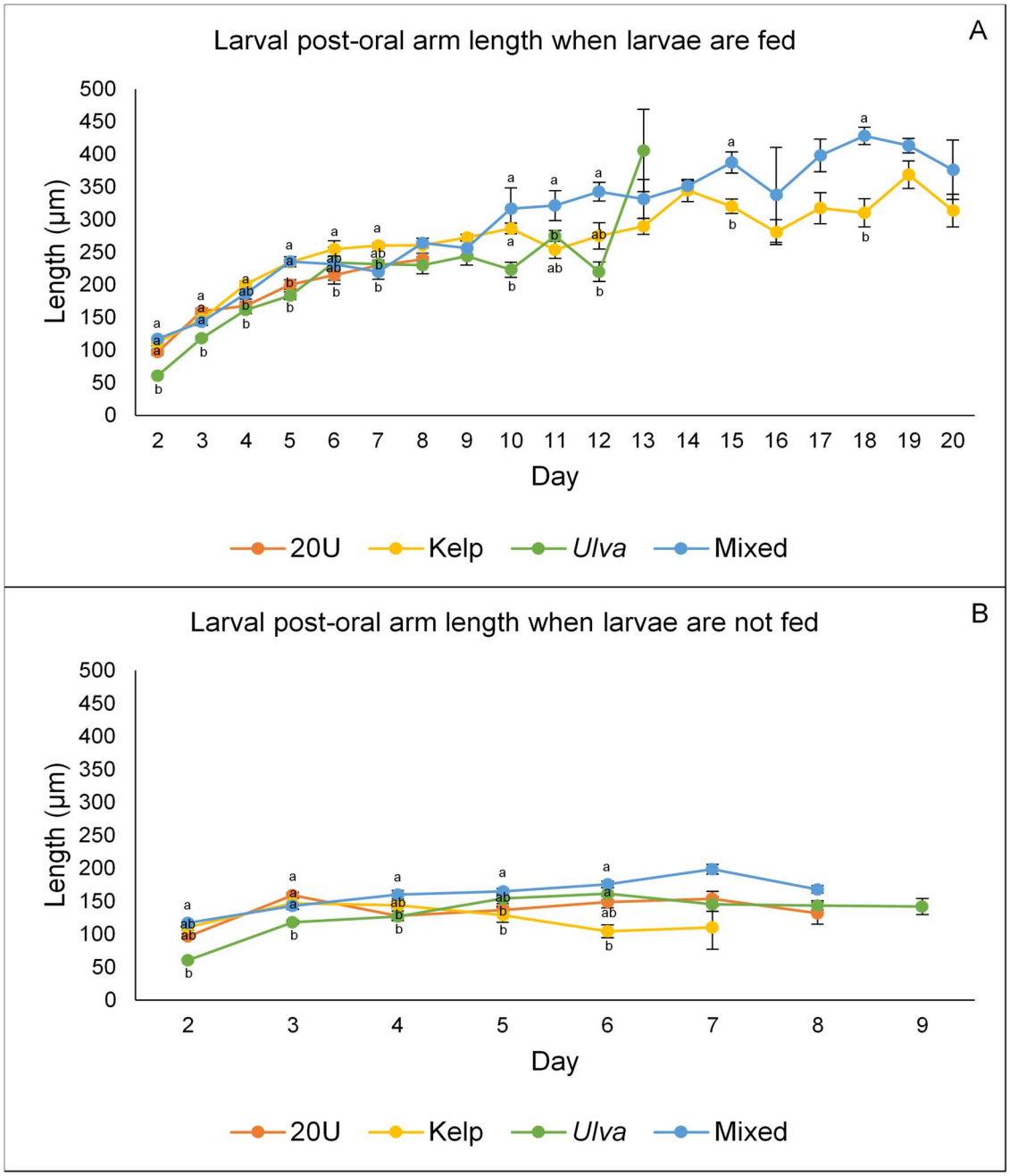


Figure 4.9. Average larval post-oral arm length (µm) for (A) fed (n = 373) and (B) unfed (n = 177) *Tripneustes gratilla* larvae from broodstock animals conditioned on a formulated feed (20U), kelp (*Ecklonia maxima*), *Ulva rigida* and a mixed feeding regime, where the standard error for each mean is indicated. Statistically significant differences are shown by different letters based on ANOVA or Kruskal-Wallis tests followed by a post hoc Tukey's (t) or Dunn's test (z). No letters indicate that there were no statistically significant differences between groups.

The smallest ($P < 0.05$) post-oral arms at day six were observed across non-fed larvae from kelp fed broodstock (104.65 µm), when compared to larvae from broodstock fed *Ulva* (161.51 µm) and mixed diets (175.68 µm) having greater post-oral arm lengths (Figure 4.9). This indicates that kelp larvae are not actively seeking food and that their maternal reserves are better able to support a facultative feeding period. In contrast, larvae from mixed diet fed

broodstock had the longest post-oral arms at day seven, which indicates that they were using their maternal reserves to extend their arms to increase their food capturing abilities (Figure 4.9). Interestingly, non-fed larvae had a similar body width across broodstock diets, ranging from 68.62 – 79.73 µm (Appendix C, Figure S4.8), which shows that larvae from specific diets are extending their arms without growing in body size. Similar results were observed for non-fed *T. gratilla* larvae by a previous study, where the authors found differences in arm length, but not in body size (Byrne *et al.* 2008b). These results could explain the larvae from the mixed diet depleting their maternal reserves and not surviving for long without an exogenous food source, as there would be an energetic cost involved in generating longer post-oral arms (Reitzel and Heyland 2007). However, the energetic cost of arm growth is not known, and this growth may result in the depletion of the triglyceride energy source associated with this early life stage in sea urchins (Reitzel and Heyland 2007; Byrne *et al.* 2008a). Alternatively, the depletion of endogenous energy stores could result in the cue to improve their ability to capture food particles (Herrera *et al.* 1996; Sewell *et al.* 2004). This feedback mechanism is most likely influenced by the stomach, as this serves as the major nutrient storage organ during larval stages (Chia and Burke 1978), although it can be initiated as early as the embryonic phase through the surface epithelial cells that detect food and other dissolved organic compounds (Miner 2007). Therefore, in food limited environments, longer arms are advantageous for larvae as they would have an improved capacity to acquire food particles, subsequently supporting larval growth and larval survival. In the current study, results from both the fed and non-fed larvae suggest that larval development in *T. gratilla*, particularly during the early developmental stages, is supported by maternal reserves provided by the egg, which is influenced by the maternal diet to some extent. It should be noted that these results should be treated with caution, as larval growth could have been influenced by intrinsic genetic factors given the various parental pairs that were used to establish the larval cohorts. Therefore, larval growth should ideally be assessed when larval cohorts are established using a single parent pair (Byrne *et al.* 2008b).

4.3.6. Genetic diversity and parentage analysis

A total of 380 individuals were genotyped at 12 microsatellite loci. Two markers (*Tgr-A11* and *Tgr-D134*) were excluded from analyses, as the presence of null alleles were detected, and these markers frequently failed to amplify. Markers that showed evidence of null alleles being present, with low null allele frequencies, ranging from -0.14 – 0.33 were not excluded (Appendix C, Table S4.6). No evidence of allele dropout or stuttering was detected. No loci

deviating from Hardy-Weinberg expectations were detected, though many loci pairs (33 out of 90) were in linkage disequilibrium, possibly as a result of small sample sizes. No markers under selection were detected, therefore results are based on a panel of 10 microsatellite markers.

Genetic diversity analyses showed that there were no statistically significant (Kruskal-Wallis; $P < 0.05$) differences between the broodstock animals and their offspring (Figure 4.10). The number of alleles averaged at 5.31 ± 0.52 for the broodstock and 5.18 ± 0.42 for the offspring cohort (Appendix C, Table S4.6). The effective number of alleles averaged at 3.62 ± 0.45 for the broodstock and 4.08 ± 0.44 for the offspring cohort. It was observed that the heterozygosity present in the broodstock cohort was largely maintained in the offspring, as the unbiased expected heterozygosity and observed heterozygosity did not significantly decline (Figure 4.10; Appendix C, Table S4.6). Furthermore, a greater degree of genetic diversity was observed across most of the diversity statistics when compared to those found in chapter 3. These results indicate that many of the potential parental pairs contributed to the genetic pool of the offspring. Therefore, the implementation of a factorial breeding design is advantageous in aquaculture practices to preserve genetic diversity present in cultured cohorts.

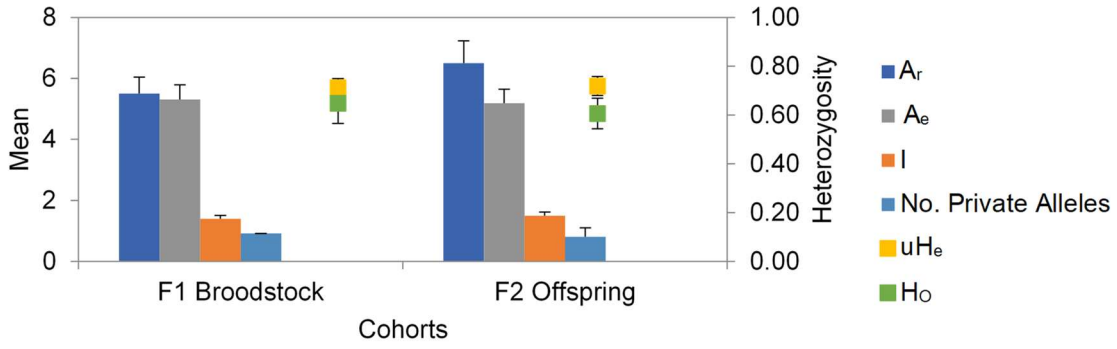


Figure 4.10. Mean genetic diversity statistics for first generation (F1) broodstock and second generation (F2) cultured offspring (A_r allelic richness, A_e effective number of alleles, I information index, number of private alleles, uH_e unbiased heterozygosity, H_o observed heterozygosity).

Although genetic diversity was mostly maintained, the deviation from mutation-drift equilibrium ($P < 0.05$), under the infinite allele- and two-phased model, in the F2 offspring cohort indicated that a genetic bottleneck occurred (Table 4.4). However, effective population size point estimates for the broodstock (N_e = 19.9) and the offspring (N_e = 29.5) indicate that a large amount of genetic variation was preserved in the offspring cohort. These preliminary results indicate that a factorial breeding approach on a larger scale, with more

broodstock animals and potential breeding pairs, could result in a breeding population where genetic diversity can be maintained in the cultured population.

Table 4.4. Bottleneck (Wilcoxon) test under the infinite allele model (IAM), two-phase model (TPM) and stepwise mutation model (SMM), as well as estimates of effective population size (N_e) of F1 broodstock and F2 offspring calculated using the linkage disequilibrium (LD) method, where the 95% confidence intervals (CI) are indicated.

Parameter	F1 Broodstock	F2 Offspring
Sample size (n)	16	364
Wilcoxon test		
IAM	< 0.05	< 0.05
TPM	n.s.	< 0.05
SMM	n.s.	n.s.
N_e	19.9 (9.0 – 124.9)	29.5 (26.4 – 32.9)

Parentage assignment results further support the occurrence of a genetic bottleneck, as majority (79.65%) of the offspring assigned to the kelp fed broodstock (Figure 4.11; Appendix C, Figure S4.9). However, this could be a result of the higher larval numbers of kelp fed broodstock towards the end of larval rearing (Appendix C, Figure S4.6). Nevertheless, within the offspring assigned to the kelp fed broodstock, several (24.73%) assigned to a single parent pair KS2/KD4 (Figure 4.11; Appendix C, Figure S4.9). Broodstock could have dominated the spawning event as a result of the natural variation of feeds within both the feeding regimes, where the composition of food ingested by each individual urchin could differ. There could also be differences in an individual's ability to assimilate nutrients and invest energy. Furthermore, there could be fine-scale differences at an individual animal level, as KD4 consistently produced a large number of offspring that were similar in size (Figure 4.11). Although no notable differences were observed at an individual level for the phenotypic measures included in this study, there could be other factors dependent on individual animal variation, such as egg quality measures (echinenone content or lipid profiles) that had an impact on reproductive success that were not assessed in the current study. Nonetheless, it was observed that a total of 26 out of 32 possible parental pairs contributed to the F2 generation. Within diets, 14 and 12 parent pairs (out of a possible 16) from the kelp and mixed diet fed broodstock, respectively, contributed to the subsequent generation (Figure 4.11). Although all possible parent pairs did not contribute to the subsequent generation, all broodstock animals did, therefore the greater number of contributing parents accounts for the large extent to which genetic diversity was maintained in the F2 generation. This further highlights the benefits associated with the breeding design employed in this study.

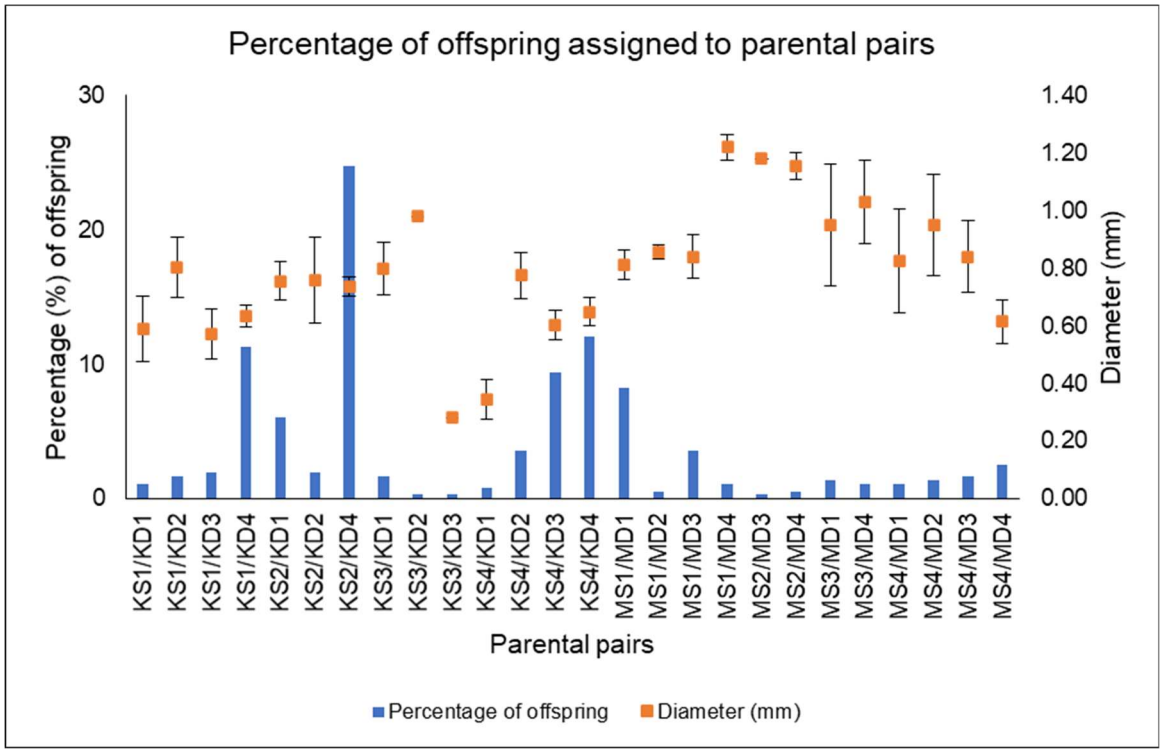


Figure 4.11. Percentage of *Tripneustes gratilla* offspring assigned to parental pairs in F2 cultured cohort, where 26 full-sib families were identified and mean family diameters (mm) are indicated (K: Kelp, M: Mixed, S: Sire, D: Dam).

Across offspring from broodstock conditioned on different diets, it was observed that the juveniles assigning to broodstock that were fed a mixed diet were significantly larger (average offspring size of 0.94 ± 0.10 mm) than those from broodstock fed kelp (average offspring size of 0.66 ± 0.07) (Figure 4.11; ANOVA; $P < 0.05$). Larvae that have access to improved maternal provisioning of nutrients are possibly able to, in turn, sequester more nutrients for development after metamorphosis, as improved larval growth of larvae from broodstock fed a mixed diet was observed across fed and unfed larvae (Figure 4.9). However, it should be noted that there were slightly more larvae from kelp fed broodstock at the end of larval rearing that were transferred to settlement containers (Appendix C, Figure S4.6). Larvae from all treatments were being fed at the same rate, therefore the lower larval numbers from broodstock fed a mixed diet throughout the larval rearing period (Appendix C, Figure S4.6) could have resulted in a larger amount of feed being available to them, subsequently fuelling larval growth and increasing their reserves and size at settlement. Alternatively, it could be hypothesised that offspring are allocating resources differently, as the kelp offspring showed greater survival, but less growth and the mixed diet offspring showed a lower degree of survival, but were larger at the point of sampling. Although it is possible that these results are an artefact of larval numbers and feeding, it is

possible that maternal diets could have effects on juvenile fitness. Additionally, feeding echinoid larvae are thought to require hormones, such as thyroxine, supplied by their eggs, to induce metamorphosis (Carrier *et al.* 2018). Therefore, it is possible that there are fewer non-energetic compounds that aid in metamorphosis, in the eggs provided by the mixed diet, resulting in reduced survival. However, research in this field is limited and this has not been confirmed for *T. gratilla* (Carrier *et al.* 2018; Taylor and Heyland 2018). Nevertheless, this suggests that the egg nutrients that larvae have access to will impact larval size, growth and juvenile performance (Miller and Emlet 1999; Vaïtilingon *et al.* 2001).

4.3.7. Heritability and estimated breeding values

Selective breeding and the improvement of sea urchin stocks could be successfully implemented when economically important traits display a high degree of heritability. In this study, offspring assigning to mixed diet fed broodstock had greater body diameters, but variance of this trait was lower when compared to that of the kelp cohort, as well as the combined dataset (Table 4.5). Overall, a low heritability estimate of approximately zero (0.050 ± 0.058) for body diameter was observed in the current study (Table 4.5). This indicates that if this trait is selected for, a poor response to selection would be observed in this aquaculture environment, as additive genetic effects probably play a limited role in early growth stages of *T. gratilla*. Heritability estimates were higher in the kelp cohort (0.054 ± 0.072), than for the mixed diet cohort (0.033 ± 0.181) when data were treated separately. However, given the high standard error estimates, these results should be treated with caution. The high standard error margin is likely a result of the small number of families each of the cohorts consisted of (kelp = 10, mixed = 9 and combined = 26), or as a result of environmental effects that were not accounted for. In the current study, tank effects could have played a role in this, as larvae from the respective diets (reared in separate tanks) were combined for juvenile rearing. Estimates of heritability based on the sire and dam components of variance were low and approximately equal, given the high standard error (Table 4.5). A previous study in *T. gratilla* found higher dam heritability estimates for body size associated traits (Pante *et al.* 2007), which was attributed to common environmental-, maternal- or non-additive genetic effects. The authors found low to moderate heritability estimates for wet weight (0.027 – 0.063), body diameter (0.033 – 0.286) and body height (0.000 – 0.227) (Pante *et al.* 2007).

Table 4.5. Mean diameter (mm), coefficient of variation and heritability (h^2) estimates for diameter across offspring assigning to kelp- or mixed diet fed broodstock animals, as well as a combined dataset.

	Broodstock diet		
Parameter	**Kelp (n = 270)**	**Mixed (n = 80)**	**Combined (n = 364)**
Mean diameter (mm)	0.686	0.857	0.726
Coefficient of variation	0.455	0.373	0.444
h^2	0.054 ± 0.072	0.033 ± 0.18	0.050 ± 0.058
h^2_{dam}	0.058 ± 0.134	0.000 ± 0.545	0.018 ± 0.075
h^2_{sire}	0.053 ± 0.097	0.031 ± 0.240	0.074 ± 0.102

It should be noted that sea urchins display varying rates of growth during early juvenile stages (0 – 98 days post settlement), as observed for the white sea urchin, *Tripneustes depressus* (Sonnenholzner-Varas *et al.* 2018). The authors found that different sea urchin size classes only started displaying similar fast- and slow growing periods 98 days after metamorphosis. Therefore, in the current study, where juveniles were sampled 79 days after settlement, it is possible that the sampled juvenile sea urchins were not stable in their size class yet, subsequently affecting heritability estimates. In contrast, studies in the green sea urchin, *Lytechinus variegatus*, have found that juveniles with an initial high growth rate continued to grow at a faster rate than those with an initial slow growth rate (Heflin *et al.* 2013). Future studies in *T. gratilla* could assess the effects of size grading juveniles, as it has been suggested that sea urchin growth rates among slow-growers increased when they were separated from the larger, fast-growers, so much so that they reached similar sizes over time (Grosjean *et al.* 1996). In the current study, this could also be a function of access to food, as when animals of different sizes are reared together, larger animals will likely dominate the food source and grow faster as a result of this. Previous studies in various sea urchin species, have obtained moderate to high heritability estimates for body size, height, as well as other traits that are economically beneficial (gonad index, gonad moisture, gonad β-carotene content, gonad sweetness) (Xiaolin *et al.* 2004; Chang *et al.* 2012; Zhao *et al.* 2014). Studies have also found significant phenotypic and genetic correlations between offspring size measurements for the sea urchin, *Strongylocentrotus intermedius* (Liu *et al.* 2005). Therefore, the results of this study do not necessarily indicate that genetic gains are not possible through selective breeding for this species. Future studies should aim to assess other economically important traits throughout or at the end of the grow-out period, when animals reach a marketable size, for more accurate estimates. Alternatively, growth rates could be assessed by evaluating the time it takes animals to reach a marketable size of 50 mm. Furthermore, it has been suggested that a minimum of 50 full-sib families are required

to accurately estimate genetic parameters (Pante *et al.* 2007). Future works should also assess maternal and sire component estimates, as it has previously been observed that maternal component estimates are significantly larger than paternal component estimates for growth traits in *T. gratilla* and other sea urchin species (Liu *et al.* 2004; Pante *et al.* 2007).

Estimated breeding values (EBVs) can be used to quantify the genetic merit of breeding candidates and a moderate to high, positive estimate is associated with improved offspring performance (Gjedrem 2005). In the present study, EBVs were low in both the kelp- and mixed diet fed broodstock. Specifically, the EBVs ranged from -0.040 to 0.041 for the kelp cohort and -0.026 to 0.012 for the mixed diet fed broodstock (Appendix C, Table S4.7). There were no differences between sire and dam EBVs. Limited research in this regard has been conducted for echinoderms in general, but these results support that animals should be ranked based on their full- or half-sibling average phenotypic performance (family selection) (Lush 1947), rather than ranking individual phenotypic performance (individual selection) to select for body size (Pante *et al.* 2007). A combined family selection approach could also be implemented, where individual- and family information is used to accurately describe the potential of artificial selection (Gjedrem 2005; Farias *et al.* 2017). Although the application of quantitative genetic practices is still in its infancy in the aquaculture industry, it remains essential to increase productivity and product value (Farias *et al.* 2017). Therefore, short- and long-term goals for *T. gratilla* breeding should be developed for the improvement of traits important to this emerging echinoid industry in South Africa.

4.3.8. The influence of diet on sea urchin reproductive performance

Sea urchin diets should include the necessary nutrients for optimal gonad and gamete development when broodstock are being conditioned with the purpose of producing a subsequent cultured generation. Establishing these ideal nutrient requirements is a very challenging endeavour. The nutrient profiles of natural feeds can be difficult to establish, particularly when feeds are collected from natural environments where feeds display a degree of chemical and morphological variation (Watts *et al.* 2020).

However, aquaculture establishments could take advantage of the maternal provisioning strategy of sea urchins to benefit future commercial aquaculture production. Animals fed the mixed feeding regime outperformed the other feeds across various measurements taken throughout this study, with relatively equal parental contributions to their offspring. The incorporation of fresh feeds, kelp and *Ulva*, with the high protein formulated feed resulted in

the optimal combination of nutrients to increase their maternal investment in the gametes they were producing during the conditioning period. The results from this study suggest that the natural feeds can act as a source of carotenoids that could influence sea urchin reproductive performance, although dietary carotenoid content was not measured. Furthermore, a formulated feed could act as a dietary source of polyunsaturated fatty acids and that the inclusion of this feed in a mixed diet could be advantageous. Larvae produced from the mixed diet survived for the full duration of larval rearing and this group showed a greater extent of phenotypic plasticity during early larval stages, indicating that these larvae are robust and better suited to adapt to environmental stresses. Furthermore, differences between the egg fatty acid of single feeds and the mixed feeding regime were observed, possibly as a result of the incorporation of a broader array of fatty acids in the gonads and eggs of animals fed a mixed diet, as well as the interactions between these and other compounds in sea urchin diets.

Therefore, studies could assess the order in which the various feeds are administered to the animals, as well as the duration of feeding for each feed. For example, broodstock conditioned on kelp also performed well and therefore, this feed could perhaps be administered for longer periods of time in a mixed feeding regime. A feeding regime aimed at improved reproductive performance should be initiated with the intention to stimulate somatic and gonad growth, with subsequent feeding aimed at gametogenesis. Alternatively, future studies could develop a formulated feed for *T. gratilla* that is aimed at reproductive performance, rather than gonad enhancement for commercial purposes. Studies could also assess the effects of animal stocking density at broodstock- and larval level on feeding behaviour and animal growth. Furthermore, to aid in future selective breeding programmes, studies should assess other reproductive- or commercially important traits through a full factorial design, where they should aim to collect data on a large number of families throughout their development, to increase the statistical power of the subsequent quantitative genetic analyses.

4.4. Conclusion

An improved understanding of the effects of different broodstock conditioning diets on *T. gratilla* reproduction could aid in the development of feeds for aquaculture practices. Feeding regimes for the collector sea urchin aimed at broodstock conditioning for reproductive purposes should contain a balance of the essential nutrients, proteins,

carbohydrates, lipids and fatty acids to promote optimal reproductive development without compromising animal health and water quality. Overall, the broodstock of the mixed and kelp diets outperformed the formulated and *Ulva* diets. However, *Ulva* should not be discredited as a broodstock conditioning diet, as larval stocking and feeding practices could have influenced larval survival in the current study. Furthermore, the inclusion of a formulated feed may still be beneficial during broodstock conditioning, but should not be administered exclusively. Therefore, future studies could further optimise the formulated feed or investigate the diets included in this study further and assess different variations of the mixed feeding regime specifically aimed at improved reproductive performance in *T. gratilla*. The current study also showed that the implementation of a factorial breeding design maximises genetic diversity in subsequent generations by negating unequal parental contributions to some extent. Although low heritability estimates for body diameter were observed in the current study, future studies should assess other traits of interest to the sea urchin aquaculture industry, as these preliminary results do not necessarily mean that genetic gains are not possible through selective breeding for this species.

Chapter 5

Metagenomic assessment of the body surface bacterial communities of *Tripneustes gratilla*

Abstract

Sea urchins, including *Tripneustes gratilla*, are susceptible to a disease known as bald sea urchin disease, which has the potential to lead to economic losses in this emerging aquaculture industry in South Africa. This disease is characterised by lesions that form on sea urchin body surfaces. Therefore, this chapter aimed to characterise the body surface bacterial communities associated with *T. gratilla*, using a 16S rDNA gene metagenomics approach, to provide insight into the bacterial agents associated with this aquaculture species, as well as with this balding disease. Bacterial samples were collected from non-lesioned healthy animals obtained from natural locations along the eastern coast of South Africa, as well as from different cultured cohorts: non-lesioned healthy animals, lesioned diseased animals and non-lesioned stressed animals. A total of 1 067 515 individual bacterial operational taxonomic units (OTUs) were identified, belonging to 133 family-, 123 genus- and 113 species level OTU groups. Alpha diversity analyses, based on Chao1, Shannon and Simpson indices, showed that there were no statistically significant differences (ANOVA; $P > 0.05$) between the respective cohorts. Similarly, beta diversity analyses (non-metric multidimensional scaling) showed a large degree of overlapping OTUs across the four cohorts. Within each cohort, various OTUs commonly associated with marine environments were found, predominantly belonging to the families Vibrionaceae, Saprospiraceae, Flavobacteriaceae and Sphingomonadaceae. Differential abundance analysis (DESeq2) revealed that OTUs that are differentially abundant across cohorts were likely not responsible for this balding disease, suggesting that complex bacterial agents, rather than a specific pathogenic agent, are likely causing this disease. Furthermore, the putative metabolic functions assigned to the bacterial communities showed that heterotrophic bacteria appear to be responsible for tissue lysis of degrading animal matter. The results from this chapter contributes to future management strategies of this emerging aquaculture species by providing insight into the bacterial communities associated with both natural and cultured environments.

5.1. Introduction

Marine microbes comprise a large portion of the total living biomass in the ocean, where they play integral roles in nutrient cycling (Buitenhuis *et al.* 2012; Egan and Gardiner 2016). Although bacteria are most abundant in the ocean water column, the internal and external surfaces of marine plants and animals also provide an ideal habitat for bacterial communities (Dang and Lovell 2016). However, the community structure of surface associated bacteria can be altered by a variety of biotic or abiotic stressors that animals are exposed to, such as changes in temperature, salinity and nutrient availability (Webster *et al.* 2011; Carrier and Reitzel 2017). In some instances, this change in microbial community structure can be detrimental to the health of the host animal.

These diseases are collectively referred to as bald sea urchin disease and are phenotypically characterised by lesions on the sea urchin body surface, accompanied by the separation of tube feet and spines (Wang *et al.* 2013a). The first phase of balding disease progression is characterised by a dis-colouration of the body surface, accompanied by the separation of spines from the exoskeleton, which generally occurs within one to two days after infection (Maes and Jangoux 1984). Subsequently, necrosis of dermal and skeletal tissues occurs, and the body surface progressively turns black. Although sea urchins can recover by regenerating epidermis tissue, spines and other appendages, the disease often results in death of the organism when perforations in the exoskeleton occur or when more than 30% of the body surface is covered in lesions (Maes and Jangoux 1984). Studies have shown that bacteria, such as *Aeromonas salmonicida*, *Exiguobacterium* sp., *Vibrio anguillarum*, *V. parahaemolyticus* and *V. nigiripulchritudo* (Gilles and Pearse 1986; Becker *et al.* 2007), are capable of initiating this disease. However, it is likely that the disease-causing agents are not limited to the bacterial species listed above, as it is hypothesised that this balding disease is caused by opportunistic bacterial agents.

Bacterial disease outbreaks typically occur in natural sea urchin populations when individuals are injured by other marine animals or from physical abrasions on rocks and other surfaces in the ocean (Becker *et al.* 2007). Studies have also shown that increasing ocean temperatures can reduce the immune response of sea urchins, thereby increasing disease susceptibility (Scheibling and Hennigar 1997; Silva 2013). In cultured environments, disease outbreaks are considered a primary constraint for successful aquaculture, where both host resistance and bacterial pathogenicity play a role (Bower *et al.* 1994; Subasinghe *et al.* 2001; Gianasi 2017). Economic losses resulting from sea urchin diseases have been

documented on aquaculture farms in Japan, where a single outbreak resulted in the loss of approximately 800 000 sea urchins (Wang *et al.* 2013a). Cultured environments are particularly susceptible to bacterial diseases, as animals are often stocked at densities that far exceed that which is found in the natural environment. These high stocking densities, when coupled with other stressful farming practices, such as the handling of sea urchins (grading and sorting), poor management practices and poor water or feed quality, can promote bacterial proliferation, increased disease susceptibility and spread of disease agents within the culture system (Elston 1984; Subasinghe *et al.* 2001; Bower and McGladdery 2003; Lafferty 2004; Krkošek 2010; Gianasi 2017). Furthermore, grading or sorting can result in spine damage, where after bacterial infections can easily occur (Roberts-Regan *et al.* 1988; Bower and McGladdery 2003).

Treatment of disease in cultured environments is complex when disease-causing agents are opportunistic, as non-specific treatment strategies often prove ineffective (Subasinghe *et al.* 2001; Bower and McGladdery 2003). Therefore, an improved understanding of the bacterial communities associated with different environments, both natural and cultured, and with healthy and diseased animals, could contribute to the control and prevention of microbial infections, particularly in aquaculture systems that are generally eutrophied, and potentially reduce future economic losses in this developing industry. Many of the natural locations along the eastern coast of South Africa will be used as broodstock collection sites (Brink *et al.* 2018) and therefore, will likely contribute to the bacterial communities subsequently introduced into the cultured environment. Characterisation of the microbiome is therefore required for the detection of possibly unwanted microorganisms at these locations.

This study aimed to identify and characterise the bacterial communities on the body surfaces of healthy and diseased *T. gratilla* obtained from an aquaculture environment, as well as animals collected from different natural environments along the east coast of South Africa to provide insight on the bacterial communities associated with this species and the bacterial agents associated with balding disease. A 16S rDNA gene metagenomics approach was implemented to characterise the microbiome of *T. gratilla* and test whether bald sea urchin disease is associated with specific bacterial species or whether opportunistic bacteria are more likely to be associated with this disease, as previous literature suggests.

5.2. Materials and methods

5.2.1. Sampling and DNA extraction

All samples were collected by the South African Department of Agriculture, Environment and Fisheries (DEFF) personnel using a DEFF "Permit for the purposes of scientific investigation or practical experiment in terms of Section 83 of the Marine Living Resource Act, 1998 (Act NO. 18 of 1998)". A total of 25 sea urchins (*T. gratilla*) were sampled from five natural locations along the eastern coast of South Africa and 21 animals from aquaculture systems in the DEFF National Marine Research Aquarium in Sea Point, Cape Town, South Africa (Table 5.1). All samples, consisting of pieces of urchin exoskeleton and associated epidermal tissue, were fixed in 100% ethanol until further use. Samples obtained from natural locations were collected from non-lesioned healthy sea urchins, as no diseased animals were observed at any of the sampled natural locations at the time of this study. Samples obtained from systems in the Marine Research Aquarium were collected from a first-generation (F1) cultured cohort and included samples of healthy (no lesions) and diseased (lesioned) animals when a disease outbreak occurred in the same recirculating aquaculture system. Tissue samples were also obtained from a 'stressed' cultured cohort of sea urchins in a separate aquaculture system at this facility. The animals in this system were stressed due to a system malfunction (decrease in temperature, with associated spawning event adversely affecting water quality) and animals displayed a loss of spines; but did not display the same necrotic lesions observed in the diseased cohort (Table 5.1). All exoskeleton samples were scraped with a sterile stainless-steel scalpel to obtain DNA from all representative cells. For the diseased animals, tissue was sampled from the lesion, as well as some of the surrounding "healthy" tissue. Subsequently, genomic DNA (gDNA) was extracted using the RTP Bacteria DNA mini kit (Stratec). Before library preparation, DNA concentrations were quantified through fluorometry using a Qubit™ dsDNA HS assay kit (Thermo Fisher Scientific) and the presence of bacterial gDNA was confirmed using the Femto Bacterial DNA Quantification kit (Zymo Research) according to the manufacturer's protocol.

Table 5.1. Sampling information, where the cohort, sample ID, number of individuals pooled per sample, sampling location and sea urchin tissue type that bacterial communities were collected from are indicated.

Cohort	Sample ID	Individuals	Sampling location	Tissue type
Natural	ECC	5	Haga Haga, Eastern Cape (32°45′4″S, 28°16′41″E)	Non-lesioned exoskeleton
	ECD	5	Coffee Bay, Eastern Cape (31°59'15"S, 29°08'58"E)	
	KZNA	5	Hibberdene, KwaZulu-Natal (30°35'3"S, 30°34'29"E)	
	KZNB	5	Ballito Bay, KwaZulu-Natal (29°32'41"S, 31°13'0"E)	
	KZNE	5	Sodwana Bay, KwaZulu-Natal (27°33'27.3"S 32°40'29.9"E)	
Healthy	SP-HB	1	F1 Cultured population at the Marine Research Aquarium, Sea Point, Tank A	Non-lesioned exoskeleton
	SP-HC	1		
	SP-HE	1		
	SP-HF	1		
	SP-HG	1		
	SP-HH	1		
	SP-HI	1		
Diseased	SP-SA	1	F1 Cultured population at the Marine Research Aquarium, Sea Point, Tank A	Lesioned exoskeleton
	SP-SB	1		
	SP-SC	1		
	SP-SD	1		
	SP-SE	1		
	SP-SF	1		
	SP-SG	1		
Stressed	SP-SH	1	F1 Cultured population at the Marine Research Aquarium, Sea Point, Tank B	Devoid of spines, non-lesioned exoskeleton
	SP-SI	1		
	SP-SJ	1		
	SP-SK	1		
	SP-SL	1		
	SP-SM	1		
	SP-SN	1		

5.2.2. 16S PCR amplification

The 16S hypervariable rDNA gene regions were polymerase chain reaction (PCR) amplified using the Ion 16S Metagenomics Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. This kit contains two primer sets (V2,4,8 and V3,6,7,9) that were used in final reaction volumes of 10 µL containing 2 µL gDNA, for 25 cycles, to amplify various 16S hypervariable regions (V2,3,4,6,7,8 and 9). The PCR products were sized and visualised on the LabChip GXII Touch using the DNA Extended Range LabChip (PerkinElmer) system and the Genomic DNA Reagent Kit (PerkinElmer). Thereafter, each sample's PCR products from the two reactions, with the respective primer sets, were

combined, purified using Agencourt AMPure XP reagent (Beckman Coulter Inc) and eluted in 15 µL nuclease-free water. Purified amplicons were quantified and visualised on the LabChip GXII Touch using the DNA NGS 3K LabChip (PerkinElmer) and Reagent Kit (PerkinElmer) according to the manufacturer's protocol. Products were normalised to equimolar concentrations and the amplicons of the bacterial communities obtained from the replicate samples of the natural cohort (n = 5) at each sampling location were pooled, as indicated in Table 5.1. This resulted in a total of 26 metagenomic samples for subsequent library preparation.

5.2.3. Library preparation and next-generation sequencing

Library preparation was performed at the Central Analytical Facility (CAF), Stellenbosch University, where 50 ng of PCR product from each sample was used for library preparation with the Ion Plus Fragment Library Kit (Thermo Fisher Scientific). Amplicons were end-repaired in preparation for blunt-end ligation to the Ion Xpress Barcode Adapters to enable sample identification. Following this, the barcoded libraries were purified using Agencourt AMPure XP reagent (Beckman Coulter Inc) and quantified using the Ion Universal Library Quantification Kit (Thermo Fisher Scientific). The StepOnePlus Real-time PCR system was used for qPCR amplification to determine library concentrations (pM). Libraries were diluted to a concentration of 10 pM and combined in equimolar amounts for template preparation using the Ion 520 & Ion 530 Chef Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequently, next-generation sequencing was performed on the S5 Ion Proton platform using the Ion 530™ Chip Kit according to the manufacturer's instructions. Lastly, flow space calibration and basecaller analysis was performed using default analysis parameters in the Torrent Suite Version 5.4.0 software (Thermo Fisher Scientific).

5.2.4. Raw data processing and normalisation

Ion Reporter™ software (Thermo Fisher Scientific) was utilised to assess the raw data through the 16S Metagenomic Workflow, which makes use of the open-source software QIIME (Caporaso *et al.* 2010). This software detects and removes adapter sequences, as well as low quality reads (< Q20), reads below 150 base pairs (bp) in length and reads with a low abundance (n < 10 counts). Raw data is publicly available on the EMBL-ENA database (study accession number: PRJEB30938). Sequence data was clustered into operational

taxonomic units (OTUs) when sequences matched at 99% when aligned (E-value < 0.01) to the curated MicroSeq 16S and Greengenes reference libraries.

The 16S Metagenomics Workflow was used to perform rarefaction curve analysis to indicate whether coverage was sufficient to capture the bacterial diversity present within each sample. Data was filtered in MicrobiomeAnalyst (Dhariwal *et al.* 2017) for low count reads (at a default of 20% prevalence across all samples), where features (OTUs) containing only or mostly zeros were removed to account for possible sequencing errors. Subsequently, this marginally filtered dataset was used to calculate alpha (within sample) diversity statistics and to quantify overall OTU abundance. Uneven sequencing depth, under-sampling and data sparsity was corrected for through data normalisation using relative log expression (RLE) transformation (Hawinkel 2015) for non-zero cell counts as per:

$$\frac{c_{ij}}{(\Pi_{j=1}^{m} c_{ij})^{1/m}} \quad (1)$$

where a mean (m) across samples is used as a pseudo-reference sample. The median of this measure across all OTUs is used as scaling factor (j) for each sample, where each OTU is denoted as i. This method scales the raw read counts in each sample through a sample-specific factor that is dependent on the median and mean of the number of reads for each sample. The normalised dataset was used for multivariate beta diversity (between sample) tests and univariate differential abundance analysis. Low variance reads (at a default variance of 10%, based on standard deviation), which were most likely uninformative, were removed to reduce the effect of multiple testing in the differential abundance analysis.

5.2.5. Data analysis

Alpha diversity was assessed using the R *phyloseq* (McMurdie and Holmes 2013) and *vegan* (Lixon 2003) packages implemented in MicrobiomeAnalyst. Various alpha diversity measures were calculated, including the Chao1 (Chao 1984), Shannon (Shannon 1948) and Simpson (Simpson 1949) indices. Statistical significance of differences between cohort-wise alpha diversity was assessed through an analysis of variance (ANOVA; statistical significance at $P < 0.05$) for each alpha diversity measure.

Beta diversity analyses were also carried out in MicrobiomeAnalyst using the same R packages, implementing the multivariate ordination between-sample similarity based non-metric multidirectional scaling (NMDS) analysis of Bray-Curtis dissimilarity indices. Corresponding statistical significance ($P < 0.05$) was evaluated using a permutational

multivariate analysis of variance (PERMANOVA; Anderson 2001), permutational analysis of multivariate dispersions (PERMDISP; Anderson 2006) and lastly, through an analysis of similarities (ANOSIM; Clarke 1993).

Overall OTU abundance was evaluated by constructing abundance tables (n < 10 counts are merged and denoted as “Others”) based on relative abundances (%). Differential abundance of OTUs across cohorts was assessed by executing the univariate method, DESeq2 (Love *et al.* 2014). The mean, variance and mean dispersion estimates were calculated for each OTU using the normalised dataset to identify OTUs with means that exceeded the threshold calculated for that OTU (Statistical significance: $P < 0.05$) (Hawinkel 2015). Additionally, the false discovery rate (FDR) was calculated to adjust *P* values for multiple comparisons to minimise the possibility of type I errors (false positives) (Benjamini and Hochberg 1995). Linear discriminant analysis was used to calculate the effect size (LefSe) to test for significant associations between cohorts and bacterial communities, where the 25 OTUs that most likely (Kruskal-Walls rank sum test; Significance: $P < 0.05$; LDA score > 2) explain differences between the cohorts were identified. Following this, the significant OTUs ($P < 0.05$) were retained and a pairwise Wilcoxon test was applied, where these were used in a linear discriminant analysis to assess the effect size of the significant OTUs.

The program, METAGENassist (Arndt *et al.* 2012), was used to assess correlations between bacterial community samples based on the different taxonomies present within each sample and putative metabolic functions. This program makes use of an automated taxonomic-to-phenotype mapping process to a unique microbial phenotype database, which includes NCBI (National Centre for Biotechnology Information) data (Sayers *et al.* 2012), annotations in BacMap (Cruz *et al.* 2012), 1700 annotated protein genomes and GOLD (Genomes OnLine Database) data (Pagani *et al.* 2012). Subsequently, a heatmap was constructed by analysing data for ‘metabolism by phenotype’, based on Pearson (correlation coefficient) distance measures and an average clustering algorithm.

5.3. Results and discussion

5.3.1. Data processing

A total of 13 108 488 reads were generated across the 26 metagenomic libraries created in this study (Appendix D; Table S5.1). A large range in library sizes was observed when

sequences were mapped to family- (66 406 – 492 595), genus- (34 752 – 164 074) or species level (16 513 – 81 804). After filtering and quality trimming, a total of 12 785 911 reads and an average of 494 170 reads per sample remained with an average read length of 236 bp (Appendix D; Table S5.1). After reads were mapped to reference databases, 1 067 515 individual bacterial OTUs were identified, belonging to 257 family-, 411 genus- and 612 species level OTU groups. Sampling depth was sufficient, as rarefaction curves reached a plateau (Appendix D; Figure S5.1), indicating that only rare species remained to be sampled. Subsequent filtering, based on low counts and variance, resulted in the exclusion of 124 family-, 288 genus- and 499 species level OTUs from downstream analyses.

5.3.2. Alpha diversity

This chapter investigated differences within and between the body surface bacterial communities across four *T. gratilla* cohorts, namely natural, healthy, diseased and stressed animals, to contribute to the knowledge of the bacterial communities associated with this species under a variety of conditions/environments and the future management of sea urchin aquaculture practices. Across all alpha diversity measures, the greatest amount of species-level diversity was observed in the natural cohort (Figure 5.1), with an average Chao1 index of 96.29, an average Shannon index of 3.16 and an average Simpson index of 0.90 (Appendix D; Table S5.1). Nevertheless, a similar degree of within sample diversity was observed throughout all four studied cohorts (Figure 5.1). The high degree of diversity in the diseased cohort, across all taxonomic levels, suggests that this balding disease of *T. gratilla* is caused by opportunistic bacteria; as diseases associated with specific bacterial agents are generally characterised by low bacterial diversity in diseased samples (Becker *et al.* 2007; Shi *et al.* 2017). This is supported by previous studies that found comparable levels of within sample microbial diversity in animals with different health statuses, including where diseases were thought to be caused by opportunistic bacteria (Becker *et al.* 2007; Shi *et al.* 2017). In the current study, this observation could also be as a result of the exoskeletal samples from the diseased animals including tissue from both the lesioned area and some surrounding healthy tissue. Future studies should further validate these findings by collecting the bacterial communities present on lesioned and non-lesioned tissue from the same animal.

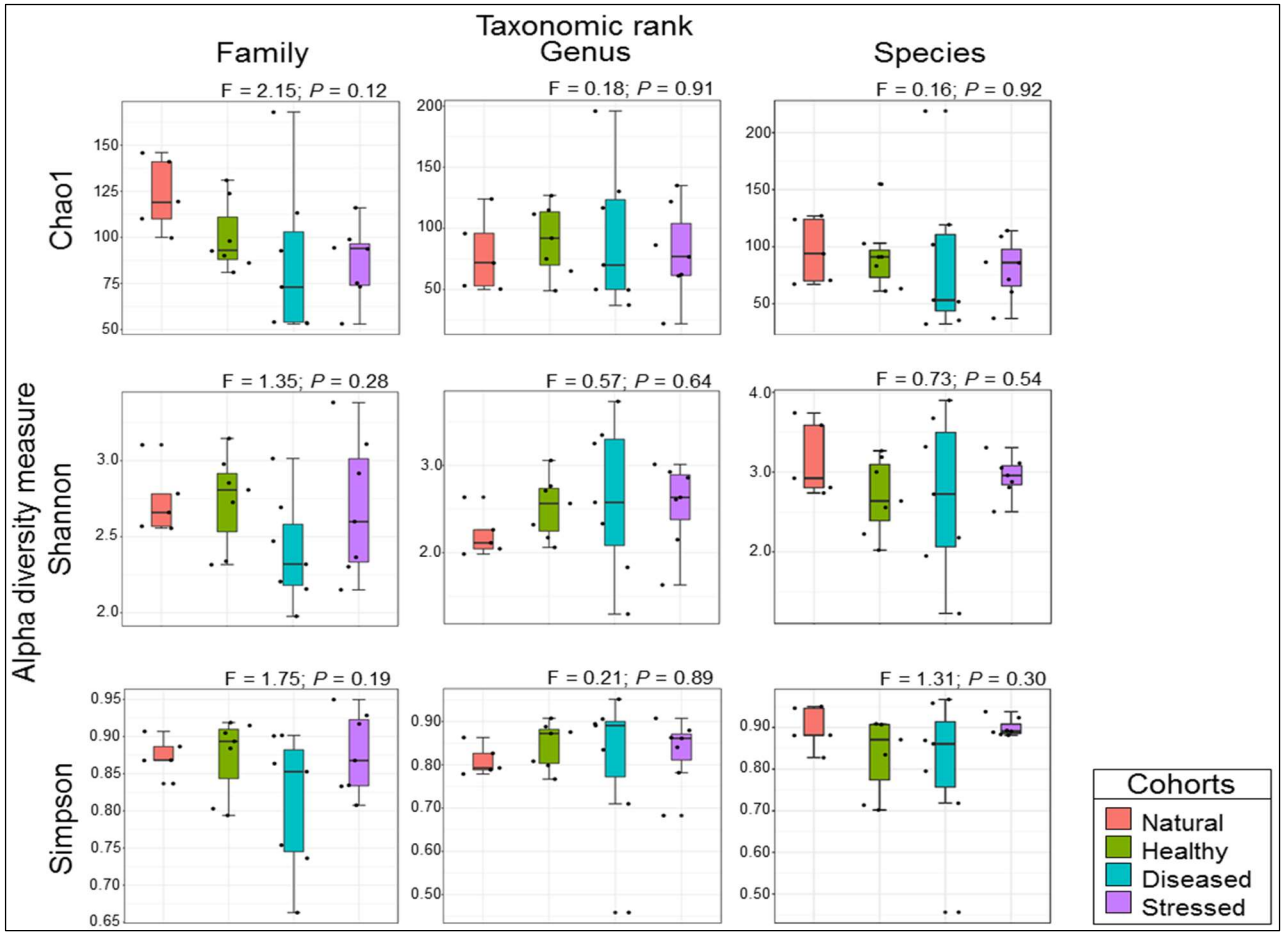


Figure 5.1. Average alpha diversity measures (Chao1, Shannon and Simpson) for each *Tripeustes gratilla* cohort at family-, genus- and species level, where the minimum, maximum and mean, as well as ANOVA F-values and *P*-values are indicated for each cohort.

5.3.3. Beta diversity

The comparisons between body surface metagenomes showed that the natural locations and healthy individuals clustered more closely together across all taxonomic levels in the NMDS plot (Figure 5.2). Similarly, the disease and stressed cohorts clustered more closely together. A large degree of overlap between the cultured samples collected from the aquarium (healthy, diseased and stressed cohorts) was observed (Figure 5.2).

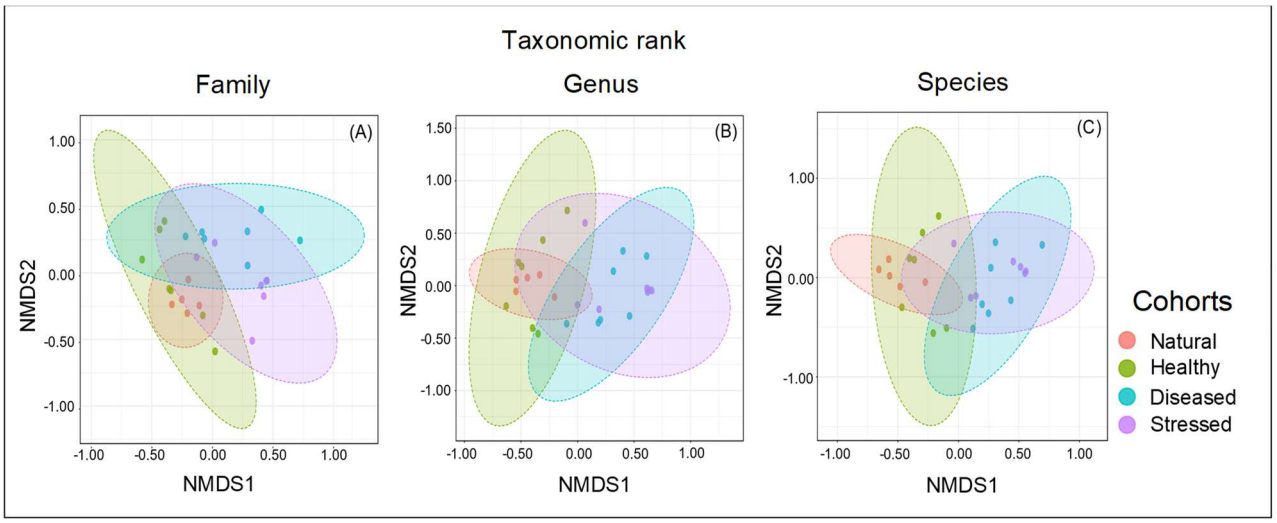


Figure 5.2. Non-metric multidimensional scaling (NMDS) analysis at (A) family-, (B) genus- and (C) species level, indicating little compositional differences across the bacterial communities obtained from the respective *Tripneustes gratilla* cohorts.

Drivers of compositional differences in bacterial communities include both environmental and host factors (Jackson *et al.* 2018), where the microbial communities in the surrounding sediments and water likely influence bacterial colonisation of sea urchin body surfaces. Theoretically, it is expected that diseased animals would have a unique body surface bacterial composition, when compared to that of healthy animals, if the microbes or their specific interactions are the causative agent(s) of the disease in question. In the current study, a large degree of overlap between all four cohorts was observed (Figure 5.2), indicating that there are no major differences in the bacterial compositions of the different cohorts, regardless of health status. These similarities can be attributed to the transfer of bacteria from the natural environments into the aquaculture environment following collection of wild animals from nature to establish the broodstock population for aquaculture purposes. Recent studies have shown that bacteria can be transferred from broodstock to offspring through the unfertilised egg (Carrier and Reitzel 2019), where bacteria likely play roles in lipid metabolism during embryonic development (Riley *et al*. 2008). Throughout sea urchin development, a gradual bacterial compositional shift results in developmental stage-specific bacterial communities that have diverged from environmental microbiota, possibly as a result of host-mediated selection for or against specific bacteria (Carrier and Reitzel 2019). Furthermore, juveniles retain some of the bacterial communities present during larval stages through the absorption of the larval digestive tract during metamorphosis (Chia and Burke 1978). The similarities between the cultured cohorts could be a result of the same natural

water source of the cultured animals or could be attributed to the administration of feeds from the same source.

A large degree of similarity between cohorts is further supported by the statistical analyses conducted in this study, which showed little compositional differences between the respective cohorts (Table 5.2). The PERMDISP analysis (F = 1.69 – 2.66; $P > 0.05$) (Table 5.2) indicated no to little compositional differences between cohorts, whereas the ANOSIM (R = 0.35 – 0.47; $P < 0.001$) and PERMANOVA ($R^2 = 0.33 – 0.39$; $P < 0.001$) results are indicative of low to moderate compositional differences. The discrepancy between the PERMANOVA and PERMDISP results suggests that the cohorts do not differ greatly in their overall OTU composition, but rather that different sets of bacterial communities commonly occurred in the respective cohorts, although there were overlapping OTUs (Azeria *et al.* 2011).

Table 5.2. Between sample similarity tests (PERMANOVA, PERMDISP and ANOSIM), where corresponding *P*-values are indicated in brackets, indicative of low to moderate body surface bacterial community compositional differences.

	Taxonomic rank		
Analysis	**Family**	**Genus**	**Species**
PERMANOVA; R^2	0.39 (< 0.001)	0.33 (< 0.001)	0.33 (< 0.001)
PERMDISP; F	1.69 (> 0.05)	2.24 (> 0.05)	2.66 (> 0.05)
ANOSIM; R	0.47 (< 0.001)	0.35 (< 0.001)	0.42 (< 0.001)

5.3.4. Taxonomic profiling

Taxonomic abundance profiling showed the presence of diverse bacterial communities across all cohorts (Figure 5.3), as 133 family-, 123 genus- and 113 species level OTUs were identified after data filtering (Appendix D; Figures S5.2 – S5.4). The univariate method, DESeq2, identified 25 differentially abundant OTUs at family level, 28 at genus level and 36 at species level (Table 5.4). At the family level, Vibrionaceae had the greatest abundance in the natural (20%), healthy (17%) and stressed cohorts (17%) (Table 5.4). However, this family was not differentially abundant across the respective cohorts (Table 5.3). Corresponding to the family level classification, the genera, *Photobacterium* and *Vibrio*, although not differentially abundant, were highly prevalent across all cohorts (Table 5.4). The same family level OTUs were frequently more abundant in the natural and healthy

cohorts (12 OTUs), as well as in the diseased and stressed cohorts (11 OTUs) (Appendix D; Figure S5.5), with the exception of families, Haloplamataceae and Holosporaceae, that only had a greater abundance in the natural cohort. It was also observed that most OTUs had a substantially greater abundance in the aquarium samples and only the genera *Psychromonas*, *Paramoritella* and *Desulfotalea* had a greater abundance in the natural cohort relative to the other cohorts (Appendix D; Figure S5.6). Similarly, at species level, only four *Vibrio* spp., as well as *Paramoritella sediminis*, *Corynebacterium kroppenstedtii* and *Shewanella japonica* had a statistically significant greater abundance in the natural cohort (Appendix D; Figure S5.7).

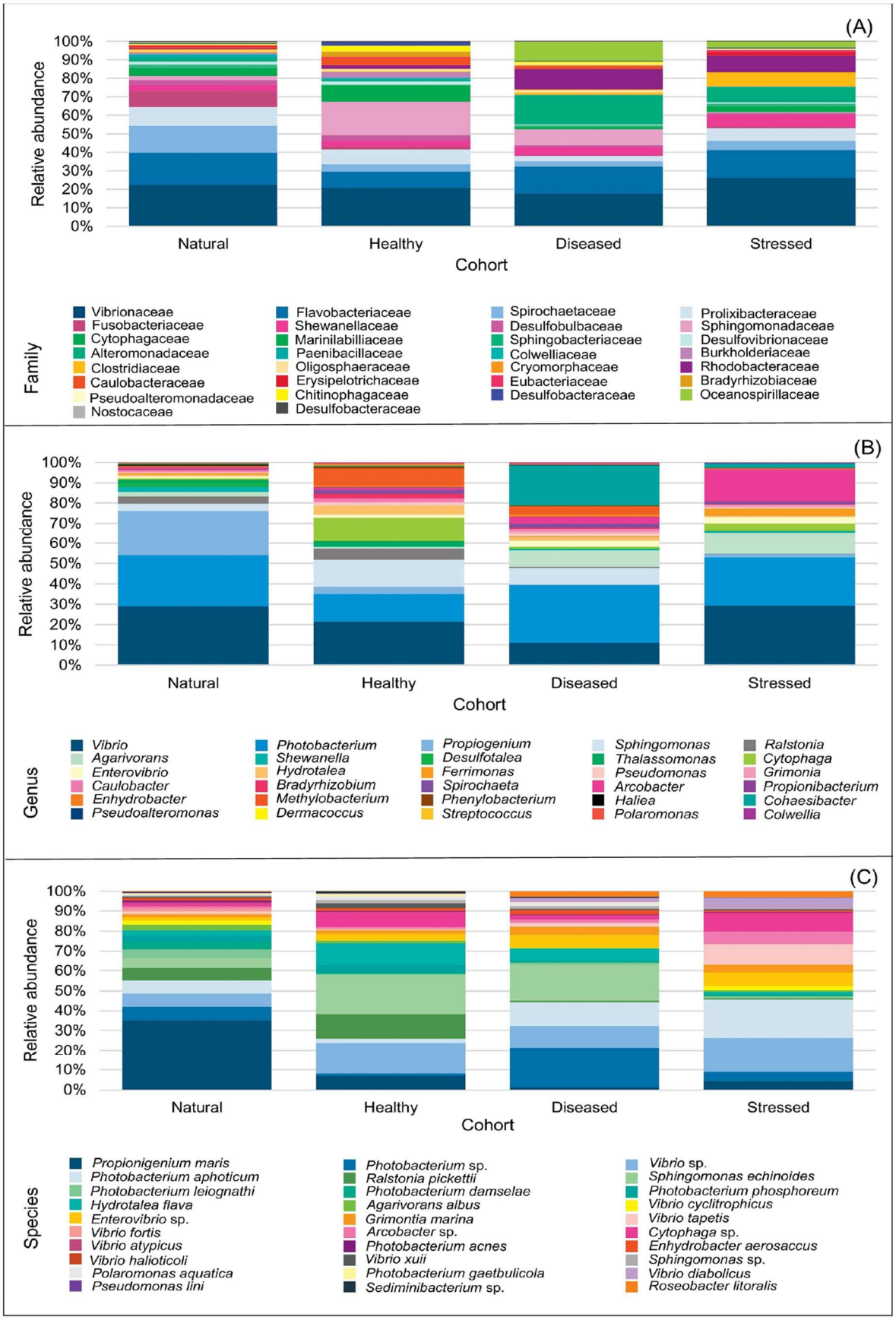


Figure 5.3. Relative (%) OTU abundance at (A) family-, (B) genus- and (C) species level, where cohort-wise OTU abundance is indicated for the 30 most abundant OTUs.

Table 5.3. Statistically significant differentially abundant OTUs at family-, genus- and species level, where the log fold change and standard error (SE), with corresponding *P*-values and false discovery rates (FDR), are indicated.

Family level differential abundance				Genus level differential abundance				Species level differential abundance				
Family	**Log fold change ± SE**	***P*-value**	**FDR**	**Genus**	**Log fold change ± SE**	***P*-value**	**FDR**	**Species**	**Source**	**Log fold change ± SE**	***P*-value**	**FDR**
Psychromonadaceae	-13.57 ± 1.84	<0.001	<0.001	*Psychromonas*	-14.50 ± 2.02	<0.001	<0.001	*Agarivorans gilvus*	Seaweed (Du *et al.* 2011)	13.02 ± 1.44	<0.001	<0.001
Halomonadaceae	11.13 ± 1.60	<0.001	<0.001	*Bacteriovorax*	11.33 ± 2.05	<0.001	<0.001	*Agarivorans sp.*	Marine animals (Kurahashi and Yokota 2004)	9.25 ± 1.48	<0.001	<0.001
Saprospiraceae	6.89 ± 1.14	<0.001	<0.001	*Oleispira*	8.72 ± 1.63	<0.001	<0.001	*Arcobacter bivalviorum*	Shellfish (Levican *et al.* 2012)	7.02 ± 1.18	<0.001	<0.001
Candidatus Brocadiaceae	-7.40 ± 1.44	<0.001	<0.001	*Oceanospirillum*	8.83 ± 1.92	<0.001	<0.001	*Tropicibacter phthalicicus*	Seawater (Iwaki *et al.* 2012)	9.70 ± 1.70	<0.001	<0.001
Simkaniaceae	-8.71 ± 1.78	<0.001	<0.001	*Paramoritella*	-9.94 ± 2.19	<0.001	<0.001	*Ruegeria conchae*	Clam (Lee *et al.* 2012)	9.64 ± 1.84	<0.001	<0.001
Acholeplasmataceae	-9.33 ± 1.92	<0.001	<0.001	*Leisingera*	9.19 ± 2.03	<0.001	<0.001	*Neptuniibacter caesariensis*	Seawater (Arahal *et al.* 2007)	8.95 ± 1.74	<0.001	<0.001
Bacteroidaceae	-8.79 ± 1.88	<0.001	<0.001	*Marinimicrobium*	7.38 ± 1.63	<0.001	<0.001	*Shimia isoporae*	Coral (Chen *et al.* 2011)	7.62 ± 1.49	<0.001	<0.001
Bacteriovoracaceae	6.92 ± 1.54	<0.001	<0.001	*Leucothrix*	8.66 ± 1.94	<0.001	<0.001	*Arcobacter molluscorum*	Shellfish (Fiqueras *et al.* 2011)	9.40 ± 1.84	<0.001	<0.001
Campylobacteraceae	4.46 ± 1.12	<0.001	<0.001	*Marinobacterium*	8.63 ± 1.9	<0.001	<0.001	*Oceanospirillum maris*	Marine environments (Hylemon *et al.* 1973)	9.59 ± 1.99	<0.001	<0.001
Criblamydiaceae	-8.11 ± 2.05	<0.001	<0.001	*Shinella*	8.43 ± 1.90	<0.001	<0.001	*Leisingera aquimarina*	Marine electroactive biofilm (Vandecandelaere *et al.* 2008)	9.94 ± 2.16	<0.001	<0.001
Rhodobacteraceae	3.45 ± 0.88	<0.001	<0.001	*Wenxinia*	8.56 ± 1.94	<0.001	<0.001	*Loktanella litorea*	Seawater (Yoon *et al.* 2013)	8.34 ± 1.92	<0.001	<0.001
Alcanivoracaceae	4.64 ± 1.26	<0.001	<0.001	*Shimia*	6.81 ± 1.55	<0.001	<0.001	*Leucothrix sp.*	Seawater (Zhang *et al.* 2015)	9.20 ± 2.21	<0.001	<0.001
Pelobacteraceae	5.41 ± 1.62	<0.001	<0.001	*Saprospira*	7.02 ± 1.71	<0.001	<0.001	*Paramoritella sediminis*	Marine sediment (Yang *et al.* 2013)	-8.96 ± 2.27	<0.001	<0.001
Rhodospirillaceae	-3.76 ± 1.14	<0.001	<0.001	*Desulfobulbus*	7.72 ± 1.89	<0.001	<0.001	*Cohaesibacter gelatinilyticus*	Seawater (Hwang and Cho 2008)	6.38 ± 1.65	<0.001	<0.001
Clostridiaceae	2.87 ± 0.88	<0.01	<0.05	*Sphingomonas*	-6.52 ± 1.73	<0.001	<0.01	*Ralstonia mannitolilytica*	Clinical material (De Baere *et al.* 2001)	-8.93 ± 2.32	<0.001	<0.001
Streptococcaceae	-3.14 ± 1.01	<0.01	<0.05	*Coccinimonas*	7.72 ± 2.18	<0.001	<0.01	*Vibrio agarivorans*	Seawater (Macián *et al.* 2001)	-9.01 ± 2.41	<0.001	<0.01
Hahellaceae	3.46 ± 1.16	<0.01	<0.05	*Lewinella*	7.17 ± 2.20	<0.01	<0.01	*Vibrio chagasii*	Seawater; marine animals (Thompson *et al.* 2003a)	-8.90 ± 2.43	<0.001	<0.01
Oceanospirillaceae	2.94 ± 0.99	<0.01	<0.05	*Arcobacter*	4.26 ± 1.34	<0.01	<0.05	*Sphingomonas echinoides*	Plate contaminant (Heumann 1960; Denner *et al.* 1999)	-7.43 ± 2.04	<0.001	<0.01
Clostridiales Family XI Incertae Sedis	5.52 ± 1.86	<0.01	<0.05	*Algibacter*	6.73 ± 2.15	<0.01	<0.05	*Polaribacter reichenbachii*	Algae (Nedashkovskaya *et al.* 2013)	8.31 ± 2.38	<0.001	<0.01
Unclassified Burkholderiales	-6.18 ± 2.08	<0.01	<0.05	*Pelagicola*	5.44 ± 1.77	<0.01	<0.05	*Vibrio coralliilyticus*	Diseased coral (Ben-Haim *et al.* 2003)	-8.10 ± 2.48	<0.01	<0.01
Alteromonadaceae	2.19 ± 0.75	<0.01	<0.05	*Neptuniibacter*	3.96 ± 1.35	<0.01	<0.05	*Polaribacter dokdonensis*	Seawater (Yoon *et al.* 2006)	7.21 ± 2.23	<0.01	<0.01
Desulfohalobiaceae	-5.15 ± 1.80	<0.01	<0.05	*Phaeobacter*	6.38 ± 2.31	<0.01	<0.05	*Pelagicola litoralis*	Seawater (Kim *et al.* 2008)	5.93 ± 1.84	<0.01	<0.01
Nostocaceae	-4.86 ± 1.78	<0.01	<0.05	*Aliagarivorans*	5.16 ± 1.89	<0.01	<0.05	*Algibacter pectinivorans*	Seawater (Park *et al.* 2013)	7.28 ± 2.29	<0.01	<0.01
Haloplasmataceae	-5.68 ± 2.11	<0.01	<0.05	*Corallomonas*	6.21 ± 2.28	<0.01	<0.05	*Desulfovibrio dechloracetivorans*	Marine sediment (Sun *et al.* 2000)	6.38 ± 2.07	<0.01	<0.01
Holosporaceae	-5.49 ± 2.06	<0.01	<0.05	*Oceanicola*	6.21 ± 2.33	<0.01	<0.05	*Vibrio fortis*	Marine animals (Thompson *et al.* 2003b)	-5.13 ± 1.67	<0.01	<0.01
				Afipia	-6.27 ± 2.38	<0.01	<0.05	*Corynebacterium kroppenstedtii*	Clinical material (Collins *et al.* 1998)	-7.44 ± 2.45	<0.01	<0.05
				Desulfotalea	-4.02 ± 1.56	<0.01	<0.05	*Lewinella agarilytica*	Marine sediment (Lee 2007)	7.71 ± 2.54	<0.01	<0.05
				Fucophilus	5.79 ± 2.25	<0.05	<0.05	*Ferrimonas kyonanensis*	Clam (Nakagawa *et al.* 2006)	7.93 ± 2.65	<0.01	<0.05
								Fucophilus fucoidanolyticus	Sea cucumbers (Sakai *et al.* 2003)	6.81 ± 2.31	<0.01	<0.05
								Vibrio scophthalmi	Turbot (Cerdà-Cuéllar *et al.* 1997)	6.99 ± 2.41	<0.01	<0.05
								Litorilituus sediminis	Marine sediment (Wang *et al.* 2013b)	6.87 ± 2.51	<0.01	<0.05
								Shewanella japonica	Mussel (Ivanova *et al.* 2001)	-7.04 ± 2.59	<0.01	<0.05
								Vibrio sinaloensis	Fish (Gomez-Gil *et al.* 2008)	-6.69 ± 2.54	<0.01	<0.05
								Phaeobacter sp.	Marine environments (Martens *et al.* 2006)	6.73 ± 2.56	<0.01	<0.05
								Vibrio gallicus	Abalone (Sawabe *et al.* 2004)	-5.98 ± 2.29	<0.01	<0.05
								Desulfovibrio sp.	Marine environments (Postgate and Campbell 1966)	6.34 ± 2.43	<0.01	<0.05

Table 5.4. Most prevalent OTU at family-, genus- and species level in each *Tripneustes gratilla* cohort, where percentage abundance is indicated in brackets.

Classification	Cohort	Most prevalent OTUs		
Family	Natural	Vibrionaceae (20%)	Flavobacteriaceae (15%)	Spirochaetaceae (13%)
	Healthy	Vibrionaceae (17%)	Sphingomonadaceae (15%)	Flavobacteriaceae (7%)
	Diseased	Saprospiraceae (26%)	Vibrionaceae (9%)	Alteromonadaceae (8%)
	Stressed	Vibrionaceae (17%)	Saprospiraceae (11%)	Bacteriovoraceae (11%)
Genus	Natural	*Vibrio* (26%)	*Photobacterium* (22%)	*Propionigenium* (20%)
	Healthy	*Vibrio* (19%)	*Photobacterium* (12%)	*Sphingomonas* (12%)
	Diseased	*Photobacterium* (17%)	*Cohaesibacter* (12%)	*Marinimicrobium* (8%)
	Stressed	*Vibrio* (22%)	*Photobacterium* (17%)	*Arcobacter* (12%)
Species	Natural	*Propiogenium maris* (30%)	*Photobacterium* sp. (6%)	*Vibrio* sp. (6%)
	Healthy	*Spingomonas echinoides* (16%)	*Vibrio* sp. (13%)	*Ralstonia pickettii* (10%)
	Diseased	*Cohaesibacter gelantinilyticus* (13%)	*Agarivorans* sp. (9%)	*Photobacterium* sp. (9%)
	Stressed	*Arcobacter bivalviorum* (13%)	*Agarivorans* sp. (10%)	*Photobacterium aphoticum* (10%)

Although several *Vibrio* spp. were observed in the current study (Figure 5.3), they were observed throughout all four cohorts and did not occur in high abundance in only the diseased cohort, suggesting that the balding disease observed in *T. gratilla* cannot primarily be attributed to the presence of this (frequently pathogenic) family. *Vibrio anguillarum*, *V. cyclitrophicus*, *V. diazotrophicus*, *V. splendidus* and *V. tasmaniensis* have previously been found to be associated with sea urchin diseases, such as bald sea urchin disease and black mouth disease (Gilles and Pearse 1986; Li *et al*. 2000; Wang *et al.* 2005, 2011; Ho *et al.* 2016). However, the *Vibrio* spp. listed above had low prevalence (< 10 counts) across all cohorts in this study, were not differentially abundant or were only present in the natural and healthy cohorts (Figure 5.3).

It is possible that the sea urchin disease investigated in the current study could be the result of non-specific bacterial infectious agent(s), corroborating results from previous studies (Maes and Jangoux 1984; Becker *et al.* 2007). Further supporting the notion that specific microbial agents or communities are not the causative agents of bald sea urchin disease, an experimental infection-based study on *T. gratilla* demonstrated that the bacterial communities associated with lesions from field-collected urchins differed greatly from laboratory-induced lesions (Becker *et al.* 2007). Several bacteria, including *Exiguobacterium* sp., *Spongiobacterium nickelotolerans*, *Vibrio nigripulchritudo* and *V. parahaemolyticus* induced severe infections in the experimentally infected animals, causing the characteristic discolouration associated with bald sea urchin disease (Becker *et al.* 2007). However, these

bacteria were not observed in the diseased or stressed cohort in the current study (Figure 5.3).

Families such as Sphingomonadaceae and Rhodobacteraceae have previously been identified as the major colonising bacteria of lesions on sea urchin body surfaces (Becker *et al.* 2007), as well as in necrotic coral and sponge diseases (Cooney *et al.* 2002; Webster *et al.* 2002). Notably, in the current study, Rhodobacteraceae was significantly more abundant in the diseased and stressed cohorts (Appendix D; Figure 5.5), however, members of this family were present across all four cohorts (Figure 5.3). In the diseased cohort, Saprospiraceae, commonly isolated from aquatic environments (McIlroy and Nielsen 2014), had the highest abundance (26%) (Table 5.4). Additionally, this family had a considerably lower abundance in both the natural and healthy cohorts (Figure 5.3). Members of the family Saprospiraceae have previously been associated with macroalgal diseases, possibly as a result of their protein-hydrolysing function (Xia *et al.* 2008). Furthermore, the diseased cohort in the present study had a high abundance of Altermonadaceae (8%) (Table 5.3), which has also been associated with coral diseases (Sunagawa *et al.* 2009). The consistent abundance of several disease-associated families across all cohorts, further supports the idea of this disease being a polybacterial disease, caused by various opportunistic bacteria that occur naturally in the environment. However, in some cases, species previously identified as pathogenic in various coral species, such as *V. coralliilyticus* (Ben-Haim *et al.* 2003; Ushijima *et al.* 2018), had a greater abundance in the natural and healthy cohorts (Table 5.3). In contrast, species, such as *V. scophthalmi*, previously identified as pathogen to marine animals (Kim 2013), had a statistically significant higher abundance in the cultured populations, potentially acting as a risk factor for disease should this species be cultivated in an integrated aquaculture environment.

In the diseased cohort, *Cohaesibacter gelatinilyticus* was identified as the most prevalent bacterial species (26%) (Table 5.4) and was differentially abundant when compared to the other cohorts (Appendix D; Figure S5.7), suggesting that it could potentially play a role in disease progression or simply proliferate as a result of the necrotic tissue acting as an optimal substrate for this species. The latter is more likely, as this species has been identified as having a symbiotic role in certain marine animals (Herrera *et al.* 2017). Further complicating the prediction of stable bacterial interactions, it has been suggested that some symbiotic bacterial communities can become pathogenic when they occur in high

abundance (Garcias-Bonet *et al.* 2012). Therefore, disease outbreaks can easily occur when these bacteria, that are considered non-pathogenic, proliferate extensively.

The LefSe analysis results showed that the natural cohort had the largest number of family level OTUs associated with this cohort (Figure 5.4). At family level, Fusobacteriaceae and Psychromonadaceae were found to be associated with the natural cohort. Furthermore, species' that commonly occur in aquatic environments, such as *Propiogenium maris* and various *Vibrio* spp. were associated with the natural cohort (Figure 5.4). In the healthy cohort, the LefSe analysis showed that only two families, Simkaniaceae and Planctomycetaceae, a single genus, *Thalassomonas*, and two species, *Photobacterium phosphoreum* and *P. gaetbulicola*, were significantly associated with this cohort (Figure 5.4). In contrast, several families (7), genera (7) and species (4), were associated with the diseased cohort (Figure 5.4). Of these, *Cohaesibacter gelatinilyticus* had the greatest association with this cohort, with an LDA score greater than 3. Similarly, the stressed cohort had several OTUs significantly ($P < 0.05$; LDA > 2) associated with it at family- (3), genus- (8) and species level (7), consisting of various *Arcobacter* and *Agarivorans* species (Figure 5.4).

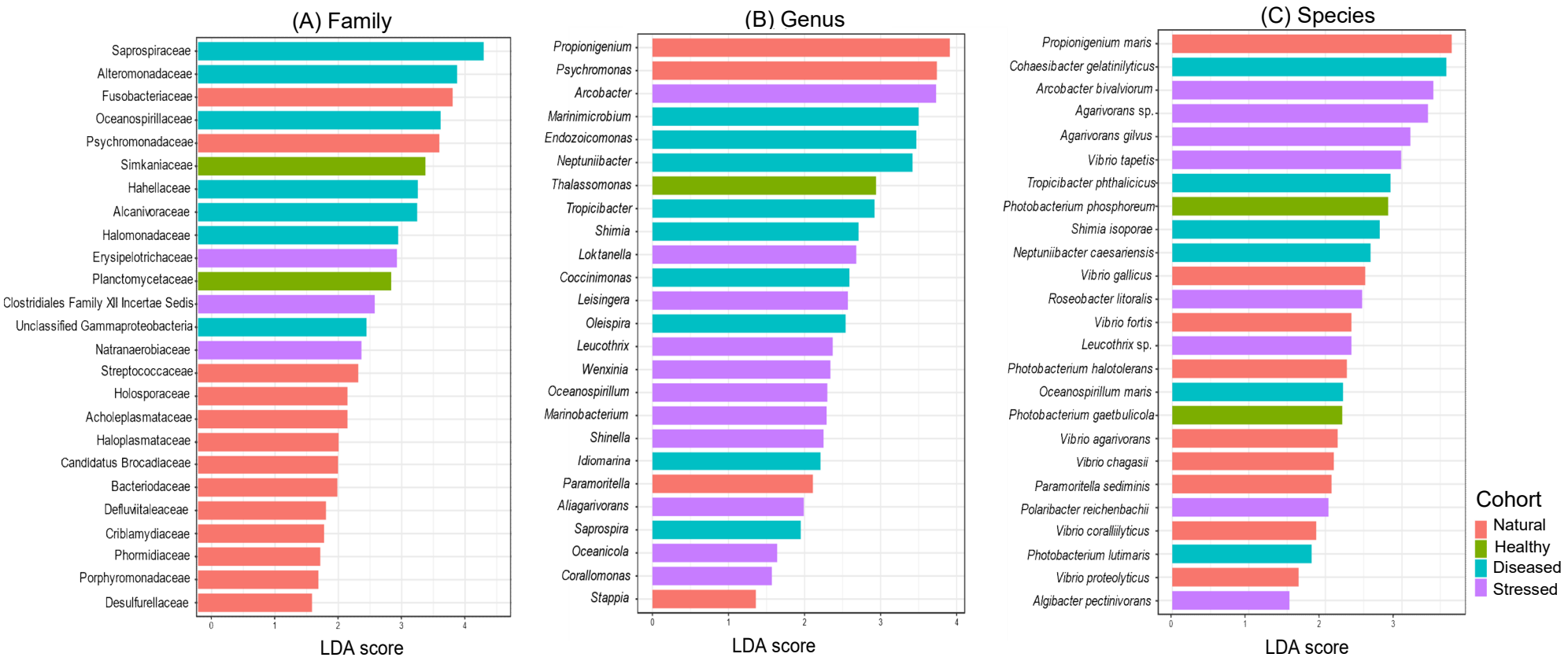


Figure 5.4. Linear discriminant analysis Effect size (LefSe), where linear discriminant analysis was used to calculate effect size for significant associations between cohorts and bacterial communities, where 25 OTUs that most likely ($P < 0.05$) explain differences between cohorts are indicated at (A) family-, (B) genus- and (C) species level.

Studies have found that bacterial communities that do not ordinarily act as disease-causing agents can become pathogenic when animals are exposed to stressful conditions (Schmitt *et al.* 2012). For example, *Arcobacter bivalviorum*, previously found to be associated with healthy sea urchin gut microbiomes (Hakim *et al.* 2016), was the most prevalent species in the stressed cohort in this study (Table 5.4) and occurred in low abundance in the other cohorts. Most notably, the clam pathogen, *V. tapetis* (Borrego *et al.* 1996), was identified as biologically important in the stressed cohort (Figure 5.4), suggesting the involvement of *Vibrio* in compromising animal health under stressful conditions. Although an increase in the primary sea urchin immune system effector cells (phagocytes) has been shown to be associated with stress, studies have found a reduction in phagocytic ability and lytic activity of these cells when sea urchins are subjected to stress (changes in salinity, crowded tanks and animal handling) (Shannon and Mustafa 2015; Fahmi 2016). Therefore, the bacteria that would typically play a mutualistic role, could act as opportunistic pathogens and result in the symptoms associated with bald sea urchin disease when the host immune response is weakened by the stressors associated with aquaculture environments (Shannon and Mustafa 2015). Furthermore, the host environment and inflammatory responses can also induce a bacterial stress response, which can result in the production of toxins (Fang 2004; Fang *et al*. 2016). This has been reported for marine bacteria and could play roles in disease progression, as pathogenic bacteria could produce toxins in reaction to the host immune response (Najafi and Nabipour 2016).

The enriched OTUs on sea urchin body surfaces, particularly in natural environments, likely play a symbiotic role. For example, Spirochaetaceae, the highly prevalent family in the natural cohort (Table 5.4), has been identified as having a mutualistic relationship with various invertebrate animals, including arthropods, molluscs and marine worms (Mayasich and Smucker 1987; Ruehland *et al.* 2008; Brune 2014), where they play important roles in nutrient cycling. Additionally, it has been suggested that bacteria affect larval settlement in echinoderms and that these early life bacteria likely colonise larvae and remain present until adulthood, as was observed for sea stars (Jackson *et al.* 2018). Although the molecular cues for settlement remain poorly understood, the bacterial species *Pseudoalteromonas luteoviolaceae*, as well as genera *Vibrio* and *Shewanella* have been associated with larval settlement in the sea urchin *Heliocidaris erythrogamma* (Huggett *et al.* 2006). In the current study, *Vibrio* and *Shewanella* were observed throughout the cohorts (Figure 5.3), further supporting their roles in echinoderms.

5.3.5. Metabolic functions

A total of 22 putative functional roles were assigned to 113 species level OTUs. When assessing the predominant energy sources used by the bacteria present within each cohort, it was revealed that the diseased and stressed cohorts utilise a limited number of energy sources (Figure 5.5), as these cohorts consist of mostly heterotrophic bacteria. In contrast, the body surface bacterial communities collected from healthy sea urchins utilise a large variety of energy sources (Figure 5.5). The largest amount of versatility can once again be observed in the healthy cohort, where bacteria capable of certain processes, such as nitrogen fixation, are nearly exclusive to this cohort (Figure 5.6). Furthermore, in the diseased cohort, a pronounced up-regulation can be observed for certain metabolic functions, such as propionate metabolism and iron oxidation (Figure 5.6). In the stressed cohort, which has the largest amount of within-cohort metabolic similarity (Figure 5.5), sulphur redox processes, nitrogen cycling through ammonia oxidation, and carbon cycling through xylan (plant cell wall polysaccharide) degradation, was observed (Figure 5.6).

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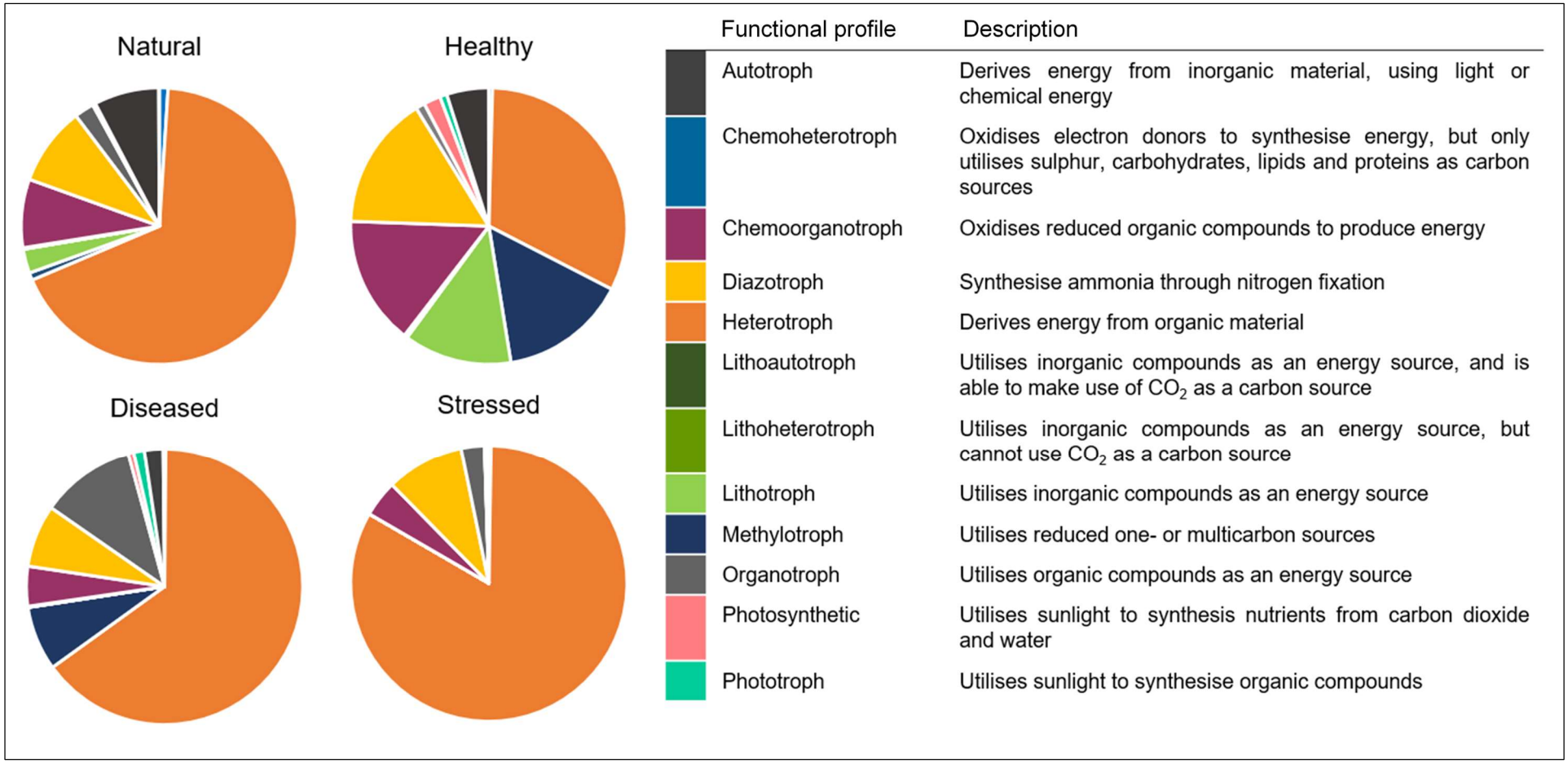

Figure 5.5. Putative energy sources used by the bacterial communities within the respective cohorts, where each functional profile is described.

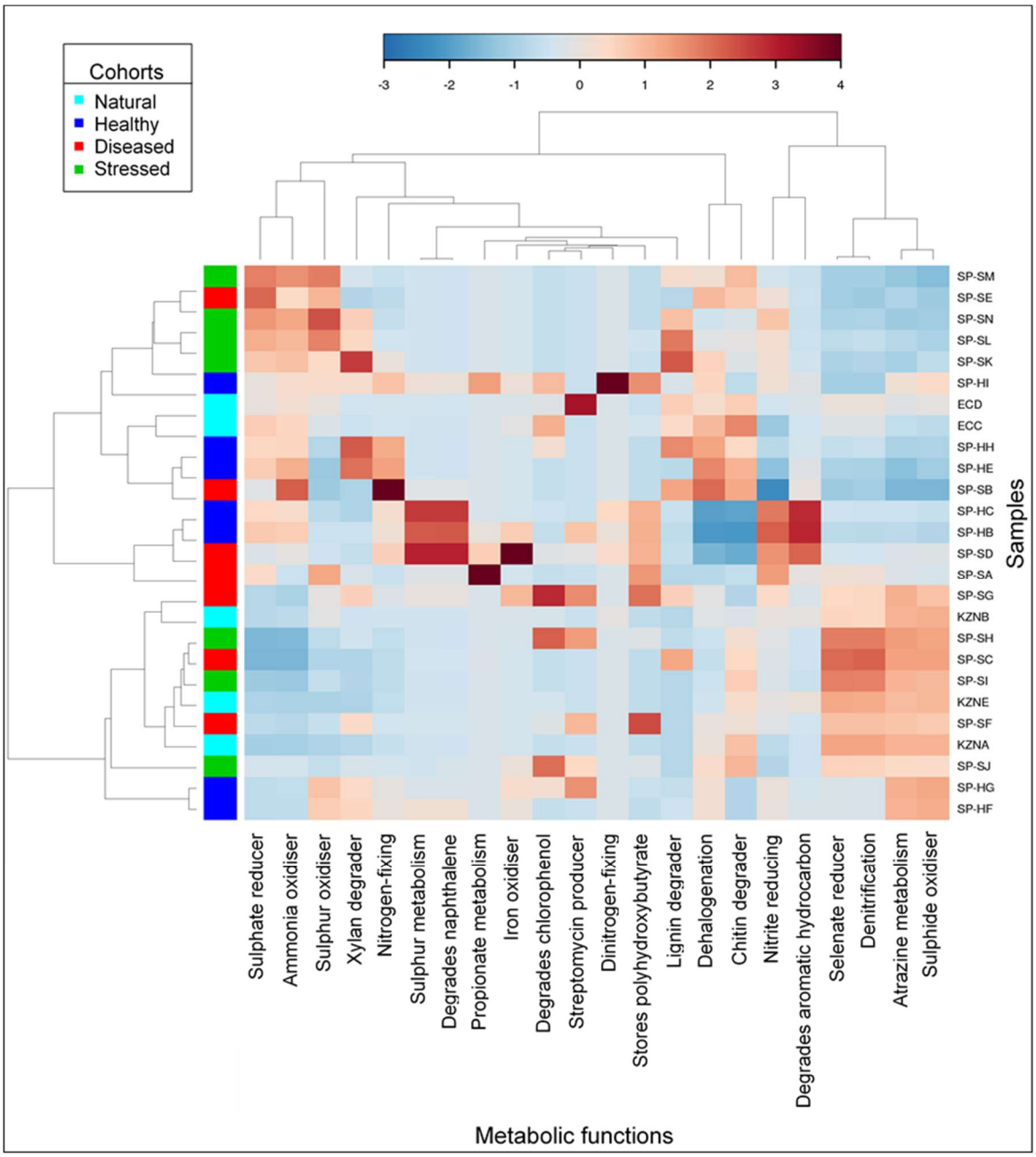

Figure 5.6. Heatmap of putative functional profiles, based on Pearson (correlation coefficient) distance measures and an average clustering algorithm, displaying differences in metabolic functions of different health statuses.

It is well documented that bacterial interactions affect physical, chemical and biological characteristics of their environments, through metabolite exchange, conversion, signalling and chemotaxis (Braga *et al.* 2016). It has been suggested that sea urchins largely rely on these microbial interactions, as they have limited digestive enzymes capable of breaking down the complex polysaccharides present in the macroalgae they consume (Hakim *et al.* 2016). Bacteria associated with feed types (*Ulva rigida* in this study) are ingested with the feed, where they contribute towards the pool of digestive enzymes in the host, thus

contributing to the degradation of complex polysaccharides and uptake of nutrients (Prim and Lawrence 1975; Erasmus *et al.* 1997; Dubilier *et al.* 2008). Some of the ingested bacteria are excreted on the body surface or in close proximity to the animal, and are therefore likely to be similar to the gut bacterial communities (Chao and Chen 2013).

Although several similarities across all four cohorts have been observed throughout this study, the underlying metabolic processes of the bacterial communities often differ, as the OTUs detected in the diseased cohort displayed a down-regulation of metabolic function capabilities (Figure 5.6). This decreased metabolic activity observed in the diseased cohort suggests that microbes do not receive the same provisioning as they would on a healthy animal, such as a stable substrate, previously received from the animal in return for the degradation of complex compounds on the body surface. During immunocompromising situations, such as disease and stress, opportunistic bacteria will thrive, as was observed by the high proportions of heterotrophic bacteria in the diseased and stressed cohorts (Figure 5.5). The high heterotrophic activity indicates that these organisms make use of organic carbon (obtained from plant or animal matter) as a food source. Furthermore, in diseases caused by opportunistic bacteria in marine animals, heterotrophic bacteria appear to be responsible for tissue lysis, and therefore most likely played an integral role in disease progression in this aquaculture system (Barneah *et al.* 2007). Processes such as propionate metabolism, which were observed in the diseased cohort (Figure 5.6) forms part of the central metabolic pathway, the tricarboxylic acid cycle (TCA) (Suvorova *et al.* 2012), whereby propionic acid is produced. This compound has been identified as having anti-bacterial properties (Kumar *et al*. 2017), possibly resulting in the reduction of other advantageous bacteria on the sea urchin body surface in the current study.

The close relationship between sea urchins and the sediments they reside on, likely result in the presence of bacteria capable of sulphur redox processes (Figure 5.6), as these bacteria are known to be responsible for the degradation of organic matter in sediments (Bowles *et al.* 2014). Although influenced by feed type, previous studies have attributed nitrogenase activity in sea urchin intestines, as well as amino acid and protein synthesis pathways to microbial activity, suggesting that these animals rely on bacteria for nitrogen (Fong and Mann 1980; Guerinot and Patriquin 1981). Nitrogen fixation also plays a role in carbon cycling, as bacteria convert fixed carbon at higher rates when excess nitrogen is present (Fuhrman and Azam 1982; Azam *et al*. 1983). Previous studies have shown an association between the genus *Vibrio*, which was highly abundant in the current study, and

the digestion of carbohydrates (Sawabe *et al*. 1995; Hakim *et al*. 2016). Interestingly, bacteria capable of chitin degradation, the process during which exoskeletal tissue is broken down (Berman *et al*. 1990), which is expected during disease progression, was observed throughout all four cohorts (Figure 5.6). Bacteria capable of chitin degradation act as potential risk factors and could be opportunists when an animal is injured.

Overall, the putative metabolic functions identified in this study illustrated that processes involving nitrogen, sulphur and carbon cycling are integral for nutrient cycling in both the natural and cultured environments. Although the interactions between body surface bacterial communities and the host, as well as with each other, are complex and are not only affected by nutrient availability, both oxidative and reductive processes occurred during nitrogen and sulphur cycling, indicating that limited nutrients are not problematic within the respective environments (Röthig *et al*. 2016).

5.4. Conclusion

Disease in an aquaculture environment can result in great economic losses, however disease control is not a simple process and a holistic approach is required for disease management, prevention and treatment. This requires knowledge of the complex bacterial communities associated with marine organisms and aquaculture systems, as well as good management practices. Based on the differential abundance analysis conducted in this study, no specific bacterial OTUs on family-, genus- or species level was found to be exclusively associated with animals in the diseased cohort, which provides further evidence that this disease is likely not caused by any specific bacterium. Therefore, complex bacterial communities likely act as secondary factors in disease progression. This study showed that the diverse body surface bacterial communities likely play an important role in sea urchin health through nutrient cycling. It remains important for future studies to investigate the microbial communities, their stability over time and their interactions within aquaculture practices for future management and prevention of disease outbreaks.

Chapter 6
Study conclusions and future perspectives

6.1. Synopsis of study findings

The collector sea urchin, *Tripneustes gratilla*, has been identified as a commercially viable species for aquaculture production in South Africa, as this species occurs along the eastern coast of South Africa and can produce bright orange roe (gonads) that are considered a culinary delicacy with a high market value of up to $600/kg (Explorations Unlimited Inc. 2005; Rahman *et al.* 2014; Cyrus *et al*. 2014, 2015a, b). Additionally, the collector sea urchin easily spawns in captivity and displays a fast growth rate of nine months to a marketable size (Juinio-Meñez *et al.* 2008a; James and Siikavuopio 2012). Therefore, the aim of this study was to assess various aspects of *T. gratilla*, including population genetics, parental contributions and the impact of feeding regimes on reproductive performance, as well as studying a disease that could result in losses in this emerging industry, through a metagenomics approach.

The degree of the genetic diversity present in the progenitor natural populations impacts the long-term and short-term responses of subsequent generations in aquaculture environments (Dunham *et al.* 2001; Frankham *et al.* 2003; Markert *et al.* 2010; Grant *et al.* 2017). Along the South African coast, *T. gratilla* larval distribution could impact genetic diversity and population connectivity, as this species has a free-swimming larval stage that could last for 52 days (Juinio-Meñez *et al.* 1998), where larvae have the potential to be dispersed as far as 1060 km (Bernardo 2011). Specific to this region, larval distribution could be affected by the Agulhas current and it's retroflection and recirculation regions (Teske *et al.* 2011; Murray 2012), as well as by changes in salinity caused by the river systems, which can affect *T. gratilla* larval recruitment success (Cowart *et al.* 2009; Delorme and Sewell 2014; Metaxas 2020). Therefore, chapter 2 aimed to quantify genetic diversity and population structure of *T. gratilla* populations along the eastern coast of South Africa to assess the hypotheses of panmixia versus population stratification. To achieve this, a total of 22 species-specific microsatellite markers (Carlon and Lippé 2007; Wainwright *et al.* 2012) were used for the genetic characterisation of 123 *T. gratilla* samples from Haga Haga, Coffee Bay, Hibberdene, Ballito Bay and Sodwana Bay. Overall, a moderate level of genetic diversity was observed, comparable to that of previous studies in natural *T. gratilla* populations in Hawaii, the Philippines and the Indonesian archipelago (Carlon and Lippé

2007; Wainwright *et al.* 2012). In the current study, across all geographically representative populations, the lowest degree of genetic diversity (Kruskal-Wallis; $P < 0.05$) was observed for the Ballito Bay population, suggesting that this location is less favourable as a broodstock collection site.

Population differentiation tests, including effective population size estimation, bottleneck (Wilcoxon) tests, analysis of molecular variance (AMOVA), Structure plots and principal co-ordinates of analysis (PCoA) suggested that the geographically representative samples form part of a single large, interbreeding population with a low global F_{st} estimate of 0.02 ($P > 0.05$). Furthermore, no significant correlations ($r^2 = 0.17$; $P = 0.11$) between genetic and geographic distances were observed. This could be explained by extensive larval dispersal during the planktonic larval stage and oceanic currents driving population connectivity between these locations (Juinio-Meñez *et al.* 1998; Teske *et al.* 2011). The high gene flow potential found in this study is supported by studies in *T. gratilla* and *T. ventricosus* that found little genetic differentiation for populations in the Pacific and Indian oceans (Lessios *et al.* 2003; Juinio-Meñez *et al.* 2008b; Coupé *et al.* 2011; Casilagan *et al.* 2013; Toha *et al.* 2014). The panmixia observed within the natural populations included in the current study indicate that they could be managed as a single genetic stock and that these locations could be used as broodstock collection sites without negatively affecting the genetic integrity of this natural resource.

Once a cultured population is established, the effects of differential parental contributions should be evaluated to ensure that the genetic variation present in the natural locations is being retained in the cultured population, ensuring improved production output in the future. Sea urchins reproduce by means of broadcast spawning, which often results in differential parental contributions to subsequent generations in cultured environments. This variable reproductive success could cause a decline in genetic diversity when a limited number of the total broodstock successfully contribute to subsequent generations (Flowers *et al.* 2002), as has been observed for the sea urchin *Paracentrotus lividus* after a single generation (Segovia-Viadero *et al.* 2016). In chapter 3, the species-specific microsatellite markers (21) were applied in cultured cohorts approximately two months after metamorphosis, to genetically characterise two first generation (F1) cultured *T. gratilla* cohorts (n = 50, respectively) by assessing pedigree relationships, as well as changes in genetic diversity after a single generation. Each cultured cohort was established from four females and two males, where eggs from all females were combined and sperm from all males were

combined prior to combining eggs and sperm in a 1:100 ratio. Genetic diversity analyses showed an overall decrease ($P < 0.05$) in the average number of alleles in the natural population that the broodstock were collected from to the cultured cohorts. These results are comparable to that of studies in other sea urchin species (Segovia-Viadero *et al.* 2016) and broadcast spawning animals, such as abalone (Rhode *et al.* 2014; Chen *et al.* 2017), as these studies found significant losses of genetic diversity in cultured environments. In the current study, this loss of alleles, accompanied by a reduction in effective population size from 171.10 in the natural population to 8.10 and 17.50 in the cultured cohorts, is likely a consequence of a genetic bottleneck event caused by using few broodstock animals to establish these cultured populations. These results suggest that the cultured offspring are likely representative of few families and that all broodstock did not successfully contribute to the F1 generations established in this study.

Supporting this hypothesis, a high proportion of full-sibs were observed in both F1 cultured cohorts (32% and 38%, respectively). Parentage analysis showed that a single female contributed to 70% of the first F1 cultured cohort and a single male contributed to 92% of the second F1 cultured cohort. Males generally have a greater reproductive competition amongst themselves, as a large number of gametes are in competition with each other for fertilisation of eggs (Levitan 2005). In the current study, variable reproductive success was observed across females used to establish both cohorts, with females D1, D2, D3 and D4 contributing to 12%, 16%, 2% and 70%, respectively, of the first F1 cultured cohort and females D5, D6, D7, D8 contributing to 26%, 52%, 20% and 2%, respectively, of the second F1 cultured cohort. Furthermore, this study found differences (Kruskal-Wallis test; $P < 0.05$) in average offspring sizes across the respective families. However, parentage studies have limitations when used to assess reproductive success and offspring performance, as they are indicative of post-fertilisation larval survival and larval settlement success, and there are many biological factors that could influence this. It is likely that sea urchin reproduction, their gonad- and gamete characteristics and subsequent offspring performance are influenced by broodstock conditioning (Quinones-Arreola *et al.* 2015), specifically through feeding regimes.

Chapter 4 aimed to assess biological- and genetic aspects, as well as various feeding regimes, that could have an impact on *T. gratilla* reproductive competition, larval growth and juvenile performance. To achieve this, two iterations of broodstock conditioning were performed, where four feeding regimes [formulated feed (20U), kelp (*Ecklonia maxima*),

Ulva rigida and a mixture of these three diets] commonly used in the studied aquaculture environment were tested. After approximately four months of conditioning F1 broodstock animals, a second generation (F2) cultured cohort was established using a factorial breeding design (four males x four females per feed) to promote reproductive success and lessen the bottleneck effect that was observed in chapter 3.

Across males, limited differences in sperm morphology were observed, however, smaller sperm (average length of 2.41 and 5.42 µm, respectively) were observed for broodstock fed a formulated diet across both spawning events, as opposed to average sperm lengths ranging from 5.51 to 6.28 and 5.88 to 6.11 for broodstock fed the other diets included in this study. Discrepancies between the respective spawning events can be attributed to immature gonads of broodstock in the first iteration of broodstock conditioning, likely as a result of a shorter broodstock conditioning period. Across females, a higher egg protein content of 65.18 – 75.28 ng/egg were observed for urchins fed a mixed diet, which could be explained by the incorporation of natural feeds resulting in an improved ability to assimilate and utilise the protein provided by the formulated feed. Similarly, previous studies in abalone and *T. gratilla* found improved digestion of formulated feeds when supplemented with kelp and *Ulva* (Naidoo *et al.* 2006; Dlaza *et al.* 2008; Cyrus *et al.* 2015a). In other sea urchin urchin species, including *Lytechinus variegatus*, *Paracentrotus lividus* and *Strongylocentrotus purpuratus*, improved performance was observed when mixed diets were administered (Beddingfield and McClintock 1998; Fernandez and Pergent 1998; Foster *et al*. 2015). Furthermore, a higher lipid content, though not statistically significant, was observed for broodstock animals fed an *Ulva* and mixed diet in the respective spawning events. Interestingly, no correlations between egg size and egg energetic components were observed in the current study, which means that egg size should not be used as a predictor of egg energetic components. This study also highlighted that carotenoids, the red, orange or yellow pigments that get incorporated into sea urchin gonads and eggs (Goodwin 1980; Matsuno and Hirao 1989), are likely affected by diet as greater gonad- and egg redness were observed for broodstock fed natural diets after both iterations of broodstock conditioning. The incorporation of these pigments could have downstream effects on sea urchin health and reproduction as it has been shown that they have anti-oxidant, anti-inflammatory, pro-vitamin A, photoprotective and immunity-related roles (Matsuno 1991; Pozharitskaya *et al.* 2015; Eggersdorfer and Wyss 2018), as well as improved hatching and larval survival (De Jong-Westman *et al.* 1995a, b; Tsushlma *et al.* 1997; Nekvapil *et al.* 2019).

Egg fatty acid profile assessments showed that the animals fed a formulated feed showed the least diverse fatty acid content across diets and animals, where an excess linoleic acid (C18:2n6) could explain differences in larval performance observed in this study. The fatty acid profile of animals fed a mixed diet clustered seperately (principal component analysis) from the singular feeds, possibly as a result of the various fatty acids that would get incorporated into gonad tissue or the interactions between the compounds obtained from the single feeds. Eggs from animals fed a mixed diet displayed the greatest proportions ($P < 0.05$) of saturated fatty acids (SFAs) and eggs from *Ulva* fed broodstock had a higher monounsaturated fatty acid (MUFA) content. The highest polyunsaturated fatty acid (PUFA) content was observed for eggs from broodstock fed a formulated feed and as sea urchins mainly obtain these compounds from their diet (Watts *et al*. 2020), these results show that there could be benefits to including a formulated feed, kelp and *Ulva* in a mixed feeding regime when conditioning broodstock for reproductive purposes.

Larval growth and survival can be influenced by maternal provisioning of nutrients, as these are thought to fuel early developmental stages (Byrne *et al.* 2008a). Larvae from the first spawning event did not survive for longer than 10 days, likely as the broodstock from the first iteration of broodstock conditioning had immature gametes and gonads, as assessed through histological approaches. In the second spawning event, a fed and non-fed larval cohort was established. Across fed larvae, larvae from broodstock fed kelp and a mixed diet survived for the full duration of larval rearing period (20 days) and displayed similar average growth rates throughout. The least amount of growth was observed for the larvae from broodstock animals fed a formulated diet and these larvae did not survive for longer than eight days, which further supports that a formulated diet should not be used in isolation for reproductive purposes, as suggested by previous studies in the sea urchin *Paracentrotus lividus* (Carboni *et al.* 2015). Non-fed larvae were used to assess the facultative feeding period of *T. gratilla*, which is the amount of time that larvae can survive without consuming exogenous food and are relying on maternal egg investments (Miner *et al.* 2005; Byrne *et al.* 2008a). In the present study, non-fed larvae from *Ulva* fed broodstock survived for the longest amount of time (nine days) and larvae from broodstock fed *Ulva* and a mixed feeding regime displayed the greatest growth rates in this non-fed state. These results indicate that the death of fed larvae from *Ulva* fed broodstock could have been influenced by larval stocking density and feeding practices. Therefore, *Ulva* should not be discredited as a broodstock conditioning diet. Furthermore, larval cohorts were established using several

parental pairs and larval performance could have been affected by intrinsic genetic differences in individual broodstock animals.

Approximately three months after metamorphosis, 10 species-specific microsatellite markers were amplified across 16 F1 broodstock (eight fed kelp and eight fed a mixed diet) and a total of 364 F2 offspring. Genetic diversity analyses showed that there were no statistically significant ($P < 0.05$) differences between the F1 broodstock animals and their F2 offspring. Parentage analyses showed that a total of 26 out of 32 possible parent pairs contributed to the F2 generation. Therefore, the implementation of a factorial breeding design is advantageous in aquaculture practices to preserve genetic diversity present in cultured cohorts by avoiding the parental skew that was observed in chapter 3. The juveniles assigning to broodstock animals that were fed a mixed feeding regime were larger (ANOVA; $P < 0.05$) than that of the kelp fed broodstock, however, a larger number of offspring (79.65%) assigned to kelp fed broodstock. This could be explained by larval survival and the interplay between larval numbers and food availability, or the number of the larvae transferred to settlement tanks. Alternatively, larval settlement success and post-settlement survival could have been influenced by variation in the natural broodstock conditioning feeds, variation in individual ingestion or egg quality parameters that were not assessed in the current study. An assessment of offspring phenotypic performance showed low heritability estimates for body diameter, which suggests that additive genetic effects play limited roles in this trait and that a poor response to selection would be observed if this trait was selected for in this aquaculture environment. Furthermore, low estimated breeding values were observed, suggesting that family- or combined selection, rather than individual selection could be beneficial for future selective breeding purposes.

Overall, results from this chapter show that a mixed feeding regime is recommended when conditioning broodstock for reproductive purposes. The varied nutrient content of the included feeds, the diverse array of essential amino acids and fatty acids supplied by the singular feeds (Newell *et al.* 1980; Cyrus *et al*. 2014; Prato *et al.* 2018) or the improved digestibility through the enzymatic activity of the bacterial communities that are associated with natural feeds (Nel *et al.* 2017) could have contributed to the high reproductive success of the mixed diet fed broodstock that was observed in this study. The microbial consortiums associated with different feeds or diets likely also impact the feeding rates, metabolic processes and digestion efficiency, where a response to diet manifests as a form of phenotypic plasticity (Rendleman *et al.* 2018). For example, Cyrus *et al*. (2015a) found

higher energy values in *T. gratilla* faecal matter of animals fed *Ulva* compared to what was found for *Ulva* itself, which indicates that bacterial communities are playing important roles in sea urchin nutrition. This could also explain why offspring from kelp fed broodstock outperformed broodstock fed *Ulva* and formulated feeds, as bacterial communities associated with kelp have been identified as having key enzymatic roles in digestion in abalone (Nel *et al.* 2017). It is expected that the bacterial communities associated with aquaculture animals, such as sea urchins, and their surroundings play an important role in overall animal health.

The role of bacteria and host development and health is well-reported for many marine species, such as algae (Egan *et al.* 2014), corals (Rädecker *et al.* 2015) and sponges (Webster and Thomas 2016). In nature and aquaculture environments, bacterial communities and their relationships can be influenced by numerous stressors (Webster *et al.* 2011; Carrier and Reitzel 2017). In sea urchins, bald sea urchin disease, where sea urchins have lesions on their body surface, as well as a loss of appendages in affected areas (Maes and Jangoux 1984), has been hypothesised to be caused by bacterial communities when there is an injury on the sea urchin body surface. However, it is likely that the bacteria associated with this disease are not restricted to those identified by previous studies. Chapter 5 aimed to provide insight on the bacterial communities associated with this sea urchin species, as well as with this balding disease by assessing the bacterial communities on *T. gratilla* body surfaces of non-lesioned animals along the eastern coast of South Africa, as well as various health statuses from a cultured environment, including non-lesioned healthy animals, lesioned diseased animals and non-lesioned stressed animals that displayed a sudden loss of spines.

The assessment of bacterial communities associated with the body surfaces of these animals, through next-generation sequencing of hypervariable 16S rDNA regions (V2, 3, 4, 6, 7, 8 and 9), revealed the presence of 133 family-, 123 genus- and 133 species level operational taxonomic unit (OTU) groups. Alpha (within sample) diversity analyses, based on Chao1, Shannon and Simpson indices, showed that all cohorts displayed a similar (ANOVA; $P > 0.05$), high degree of bacterial diversity. These results suggest that this disease is caused by opportunistic bacterial communities, as a lower bacterial diversity is expected for diseased samples if specific causative agents are associated with disease (Becker *et al.* 2007; Shi *et al.* 2017). Furthermore, previous studies in sea urchin and abalone diseases caused by opportunistic bacteria also found comparable diversity across

healthy and diseased cohorts (Becker *et al.* 2007; Shi *et al.* 2017). Beta (between sample) diversity analyses, including non-metric multidimensional scaling (NMDS), showed a large degree of overlapping OTUs across the four cohorts, likely as a result of the transfer of bacteria from the natural environment following collection of wild animals from nature to establish the broodstock population for aquaculture purposes, as well as through the introduction of bacteria into the aquaculture environment through feeds (*Ulva rigida* in this study).

Within each cohort, various OTUs commonly associated with marine environments were found, mainly belonging to the families Vibrionaceae, Saprospiraceae, Flavobacteriaceae and Sphingomonadaceae. Differential abundance analysis (DESeq2) showed that OTUs that are differentially abundant across cohorts were likely not responsible for disease progression. Species previously found to be associated with sea urchin diseases (Gilles and Pearse 1986; Li *et al.* 2000; Wang *et al.* 2005; Becker *et al*. 2007; Wang *et al.* 2011; Ho *et al.* 2016), had a low prevalence across all cohorts, were not differentially abundant or were only present in the natural and healthy cohorts. Notably, *Cohaesibacter gelatinilyticus* was identified as the most abundant (26%) species in the diseased cohort, and was differentially abundant when compared to the other cohorts and had the greatest association with this cohort [linear discriminant analysis (LDA); LDA score > 3]. However, it is possible that the necrotic tissue of diseased animals act as a substrate for this species, particularly as it has been identified as having symbiotic roles in marine animals (Herrera *et al.* 2017). Nevertheless, bacterial species that generally do not act as disease-causing agents can become pathogenic when animals are exposed to stressful conditions, such as the stressors associated with aquaculture practices (Schmitt *et al.* 2012; Shannon and Mustafa 2015).

Putative metabolic functions assigned to the bacterial communities showed that heterotrophic bacteria appear to be responsible for tissue lysis of degrading animal matter, and therefore potentially play a key role in the progression of bald sea urchin disease. Across diseased and stressed cohorts, a lower degree of versatility in putative energy sources and metabolic activities of bacteria was observed, as opposed to the upregulated metabolic capabilities of the bacterial communities on the body surface of natural and healthy cohorts. Sea urchins largely depend on the enzymatic activities of the bacterial communities that they harbour, as they have limited digestive enzymes (Hakim *et al.* 2016). In the current study, several integral metabolic functions, such as nitrogen, sulphur and carbon cycling, were associated with the natural and cultured environments. Interestingly, bacteria capable of

breaking down exoskeletal tissue through chitin degradation (Berman *et al.* 1990), were identified across all cohorts and could act as risk factors for disease when animals are injured in this aquaculture environment. Overall, the results from this chapter suggests that this disease is more likely caused by complex bacterial interactions, rather than by a specific pathogenic agent.

This study showed that the genetic diversity in nature, broodstock feeding regimes, the bacterial communities introduced *via* feeds and surroundings, and breeding designs are largely interconnected, impacting the performance of subsequent generations in aquaculture environments. Therefore, future aquaculture practices should consider the genetic implications of aquaculture practices, as well as the roles of bacterial communities in animal husbandry during the respective stages of echinoculture, from broodstock collection to juvenile grow-out.

6.2. Future prospects

This study showed that the microsatellite markers developed by Carlon and Lippé (2007) and Wainwright *et al*. (2012) are applicable in South African *T. gratilla* populations and are suitable for parentage studies in this species. Therefore, these markers can be applied in future *T. gratilla* studies aiming to assess genetic diversity in natural or cultured populations. In the current study, the moderate to high genetic diversity showed that collector sea urchins from locations along the eastern coast of South Africa can be used to establish cultured populations. This study also showed that once broodstock animals are collected, genetic diversity can be retained by implementing a factorial breeding design, where each female is crossed with each male, preferably with a large number of breeders (Zenger *et al.* 2019). Therefore, future studies can prevent the differential parental contribution generally associated with broadcast spawning animals (Levitan 2005; Darszon *et al.* 2006) and the corresponding genetic bottleneck to improve the response to selection for economically important traits (Zenger *et al.* 2019).

This study illustrated that *T. gratilla* broodstock conditioning prior to spawning animals can have important roles in sea urchin reproduction and that broodstock fed a mixed diet, that included a high protein formulated diet (20U), kelp (*Ecklonia maxima*) and a green seaweed (*Ulva rigida*), outperformed broodstock that were fed single feeds. Therefore, the mixed feeding regime should be further investigated to optimise it for reproductive purposes by initially aiming to stimulate somatic and gonad growth, where after gametogenesis should

be promoted. In the current study, kelp fed broodstock also performed well and therefore, this feed and its associated nutrients and bacteria could be playing important roles in sea urchin health, digestion and reproduction. Future studies could assess the effects of including this feed for a longer period of time in a mixed feeding regime.

Additionally, dietary carotenoid content could account for some of the differences in gonad- and egg colour observed in the current study. Future studies could assess the carotenoid content associated with feeds, as well as the extent to which these are incorporated into gonad tissues. Interestingly, it is thought that some bacterial species are capable of carotenoid synthesis, and a recent study in the golden and brown bivalve, *Chlamys nobilis*, found an association between gut microbiomes and tissue carotenoid content (Liu *et al*. 2020). The authors identified putative functional genes of the gut microbes involved in carotenoid synthesis, as well as fatty acid synthesis, and identified seven bacterial species, belonging to the genera *Brevundimonas*, *Sphingomonas*, *Rhodococcus* and *Acinetobacter*, involved in these processes (Liu *et al*. 2020). As several of these genera were identified on the body surfaces of *T. gratilla* in the current study, the presence and roles of these bacterial species in sea urchin guts could be further explored, as it is possible that bacteria are playing important roles in sea urchin reproductive processes through the synthesis of carotenoids.

The current study assessed limited reproductive traits of males and it has been suggested that the curvilinear velocity of sperm acts as a tool to assess reproductive performance in males (Smith *et al.* 2019). Therefore, future works could assess the effects of feeding regimes on sperm motility in sea urchins. Future studies could aim to assess feed- and gonad fatty acid profiles, in combination with that of eggs, perhaps at various reproductive stages, so as to provide insight on the trophic transfer of fatty acids through their composition and interactions throughout the ingestion and assimilation process in sea urchins. Lastly, studies could assess the inclusion of feed additives, such as prebiotics, probiotics, acidifiers, essential oils and antibiotics in diets for reproductive purposes, as these have been associated with improved immune responses, stress resistance, gut health, enhanced digestion, improved nutrient availability, as well as the optimisation of the gut microbiome in invertebrate animals (Anuta *et al*. 2011; Watts *et al*. 2020), although the mechanisms of this remains to be investigated for sea urchins.

Studies aiming to assess larval performance in response to broodstock feeding regimes should establish larval cohorts from a single parent pair to avoid differences due to intrinsic genetic advantages conferred by different individuals. It should be noted that this approach

has its own limitations, as only one diet will be able to be assessed at a time, which will also introduce environmental differences as larvae will not be reared simultaneously. Studies could also reassess the relationship between larval counts, larval growth and feeding strategies to improve future larval rearing practices of *T. gratilla*. Furthermore, in the present study, juvenile body size assessments and subsequent heritability estimates for this trait could have been influenced by animals not being steady in their size classes at the time of sampling (79 days after settlement) that are thought to stabilise after 98 days, as was observed for the sea urchin *Tripneustes depressus* (Sonnenholzner-Varas *et al.* 2018). Therefore, studies should assess traits later in the juvenile grow-out period. Nonetheless, in the current study, low heritability estimates were observed for body size. Future studies should assess this trait and other economically important traits in *T. gratilla* throughout or at the end of juvenile grow-out, as this could improve the accuracy of heritability estimates. Additionally, studies should aim to include a minimum of 50 full-sib families (Pante *et al.* 2007), as well as to assess the maternal and sire component estimates, as previous works have found significantly larger maternal component estimates in *T. gratilla* (Pante *et al.* 2007), even though this was not observed in the current study. Future selective breeding practices will rely on heritability estimates and estimated breeding values to assist in the assessment of breeding design efficacy. This study supports previous works that suggested that a combined family selection approach should be used for this species (Pante *et al.* 2007). This method incorporates individual-, as well as family phenotypic performance (Gjedrem 2005; Farias *et al.* 2017), which is particularly applicable for the assessment of economically important traits, such as gonad quality, that can only be assessed by sacrificing animals.

Individual selection, a breeding strategy where individual animals are selected for breeding by ranking individual phenotypic performance (Farias *et al.* 2017), requires well-documented phenotypic data for live animals, which means that many of the important traits of sea urchins, economically and for reproduction, such as gonad quality, will not be measured. Individual selection can also result in inbreeding if not managed appropriately, which can reduce genetic variation and the potential for genetic improvement, as well as result in inbreeding depression (Dunham 2011). Lastly, this method requires animals to be held in separate tanks or to be tagged (Gjedrem 2005), which is challenging, but possible for sea urchins (Cirpriano *et al.*, 2014; Rodríguez-Barreras and Sonnenholzner 2014). In contrast, family selection, a breeding strategy where candidates are ranked based on their full- or half-sib counterparts' average phenotypic performance (Lush 1947), could be particularly

beneficial to the sea urchin aquaculture industry, as traits that require animals to be sacrificed can be measured. Furthermore, family-based selection can be effective even when the additive genetic component is small and inbreeding can be managed by crossing individuals from different groups (Gjedrem 2005; Dunham 2011). However, a large number of families (50 – 100), with large family sizes, are required to carry out family-based selection effectively. This requires careful tracking of families, which means that the necessary infrastructure could become a limiting factor (Gjedrem 2005). A combined family selection approach could also be implemented, where individual- and family information is used to accurately describe the potential of artificial selection (Gjedrem 2005; Farias *et al.* 2017). The application of quantitative genetic practices is still in its infancy in the aquaculture industry, but needs to be further explored to improve product value (Farias *et al.* 2017) and short- and long-term goals for *T. gratilla* breeding should be developed for the improvement of important traits.

Feeding regimes can also have an impact on overall animal health, as the microbial communities associated with specific feeds can influence and interact with the bacterial communities present in sea urchin guts. However, the effect of adding compounds as feed additives that stimulate the sea urchin gut microbiome to have improved digestive processes have not been investigated (Watts *et al.* 2020). In sea urchins, their microbiome can have far-reaching effects as bacterial communities associated with aquaculture environments can also affect larval settlement (Jackson *et al.* 2018) and limited studies have investigated this for the collector sea urchin. Furthermore, it has been observed that the early life bacteria could remain until adulthood (Jackson *et al.* 2018) and that sea urchin larvae display a response to their progenitor broodstock diet that can result in improved digestion through an increased metabolic- and feeding rate (Rendleman *et al.* 2018). Therefore, future studies could characterise the bacterial communities associated with the feeds commonly used in sea urchin aquaculture environments to contribute to an improved understanding of the effect of feeds on sea urchin microbiomes and their relationship with host health and digestion.

Although the various bacterial communities on animal body surfaces could result in advantages for the host animals, it remains challenging to identify bacterial communities that are considered advantageous, particularly given the small sample size of this study, as bacteria that are considered beneficial can become pathogenic if they proliferate extensively. Furthermore, the microbial composition in any given environment is very

sensitive to change, therefore the bacterial community structure captured by a metagenomic study is only representative of a specific point in time. Therefore, it remains important for future studies to investigate the bacterial communities and their interactions within aquaculture practices for future prevention of disease outbreaks. Future studies should aim to investigate the potential symbiotic relationship of sea urchins with bacterial communities further by testing whether microbial community structure remains stable over time, as this can contribute to effective management practices of these animals. Once these relationships between bacteria and sea urchins are known, the bacterial diversity of the different natural locations included in this study can also indicate whether these locations are ideal broodstock collection sites. These host-microbe interactions could have effects on growth and immune function, as has been found for larvae of the purple sea urchin, *Strongylocetrotus purpuratus* (Scruh *et al*. 2020). The authors found that in the absence of bacteria, larvae were larger at the four-arm stage when compared to larvae exposed to bacteria. Furthermore, Scruh *et al*. (2020) suggest a trade-off between growth and immunity, as they found that when larvae are held in aquaculture facilities and are exposed to similar amounts of microbes as they would be exposed to in nature, larvae were more resilient to a pathogenic bacterial species. Similarly, Carrier and Reitzel (2018) found that bacterial community structure can influence larval development in the sea urchins *S. purpuratus*, *S. droebachiensis* and *Mesocentrotus franciscanus*. Therefore, results from these studies suggest that phenotypic plasticity in larvae is not only a function of food availability or subsequent gene-environment interactions, but possibly also occurs as a result of the bacteria associated with these feeds and the larval environment. In *T. gratilla*, the host-microbe interactions at their respective life stages remains to be investigated and could provide insight on immunity during larval, juvenile and adult life stages.

Although it is likely that bald sea urchin disease is instigated by opportunistic bacteria, the results from this study should be corroborated by future studies, where samples should be taken from lesioned and non-lesioned tissue of the same animal to assess differences in bacterial communities. Alternatively, it has been suggested that studies should implement the Ecological Koch's postulates, an adaptation of Koch's postulates, to assess diseases that are influenced by opportunistic bacterial communities (Vonaesch *et al.* 2018). The original Koch's postulates, developed in the 1980s, stipulate that for a microorganism to be the causative agent of a disease, that microorganism should be present in all diseased individuals, the microorganism should be isolated from the host and cultured, where after inoculating a healthy host with the microorganism should result in the same disease as the

original host and lastly, the same microorganism needs to be recovered from the newly affected host (Sweet 2020). In contrast, the Ecological Koch's postulates involve the molecular characterisation of a microbiome that results in a diseased state that is termed dysbiosis (Vonaesch *et al.* 2018). The Ecological Koch's postulates require that the dysbiotic microbiome should have a similar composition, with similar characteristics, in all affected individuals, and this dysbiotic bacterial community should be isolated from the affected host. Independent, healthy hosts should be exposed to the cultured microbiota and this should result in similar symptoms as observed in the original host, given that the respective hosts experienced a similar environment in terms of genetics, nutrition and age. Lastly, the dysbiotic microbiota should remain stable in the newly affected host. This adapted version of Koch's postulates is therefore more applicable for the assessment of a disease, such as bald sea urchin disease, that is likely caused by the disruption in a healthy microbiome (Sweet and Bulling 2017; Vonaesch *et al.* 2018). The current study has contributed to the knowledge of the bacterial communities that are associated with *T. gratilla* in healthy and diseased states, but future studies could try to fulfil the proposed postulates to identify causative agents, which are likely opportunistic bacteria.

6.3. Concluding remarks

In summary, this study showed that the natural geographically representative populations can be utilised for the establishment of aquaculture for this sea urchin species. This study also illustrated that the skewed parental contributions results in a decline in genetic diversity. However, this genetic bottleneck can be mitigated through the implementation of a factorial breeding design and reproductive success is influenced by broodstock conditioning diets, as broodstock fed a mixed feeding regime outperformed broodstock fed a single feed for the duration of broodstock conditioning. Lastly, this study contributed to the knowledge of the complex bacterial communities associated with marine organisms in nature and in aquaculture systems. It was found that there are limited differences in bacterial diversity across different health statuses, indicating that bacterial disease control in aquaculture environments requires a holistic approach for disease management throughout the various stages of sea urchin husbandry. This study contributes to the establishment of aquaculture practices of this emerging aquaculture species in South Africa through the assessment of genetic- and bacterial diversity in natural and cultured *T. gratilla* populations, as well as by investigating the effect of breeding designs and broodstock conditioning in a South African context.

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Appendix A

Supplementary information for Chapter 2

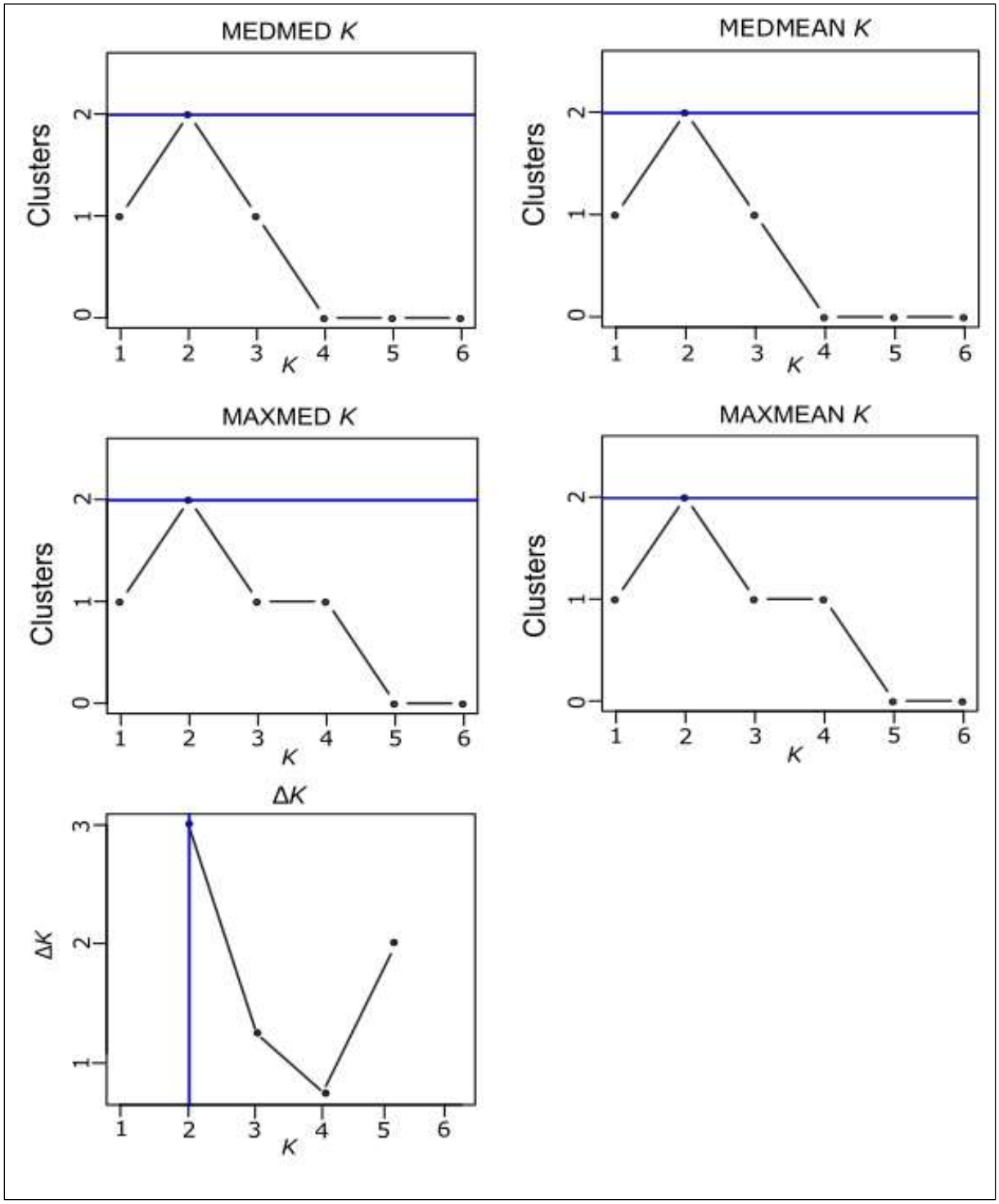

Figure S2.1. Most likely number of genetic clusters, according to Puechmaille (MedMed *K*, MedMean *K*, MaxMed *K* and MaxMean *K*) and Evanno (Δ*K*) methods.

Table S2.1. Markers grouped into six multiplex (MP) assays where repeat motifs, fluorescent dyes, expected fragment sizes in base pairs (bp) and annealing temperatures (T_a) are indicated.

MP	Marker	Repeat motif	Dye	Size range (bp)	T_a	Reference
1	*Tgr-A11*	$(GT)_{18}$	FAM	223-356	55	Carlon and Lippé 2007
	TG01	$(TTGAA)_{10}$	NED	122-172		Wainwright *et al*. 2012
	TG07	$(TCA)_{10}$	PET	72-99		Wainwright *et al*. 2012
	TG66	$(CA)_7$	VIC	174-224		Wainwright *et al*. 2012
	TG52	$(GA)_7$	FAM	107-163		Wainwright *et al*. 2012
	TG11	$(GGT)_7$	VIC	139-169		Wainwright *et al*. 2012
2	*Tgr-C11*	$(CCAT)_3(TCAT)(CCAT)_5$	FAM	254-298	55	Carlon and Lippé 2007
	TG51	$(GA)_8$	PET	112-166		Wainwright *et al*. 2012
	Tgr-B11	$(CT)_4(TCCTCTC)(CT)_6(CTT)_8$	NED	134-196		Carlon and Lippé 2007
	TG02	$(CTATT)_8$	NED	71-116		Wainwright *et al*. 2012
3	*TG20*	$(ACT)_{12}$	FAM	129-180	55	Wainwright *et al*. 2012
	TG26	$(TG)_{12}$	NED	112-186		Wainwright *et al*. 2012
	TG61	$(CA)_{12}$	VIC	137-187		Wainwright *et al*. 2012
	TG60	$(CA)_8$	PET	102-136		Wainwright *et al*. 2012
	TG55	$(CT)_9$	VIC	85-145		Wainwright *et al*. 2012
	Tgr-D134	$(GATA)_7(AATA)(GATA)_6(AATA)_2(GATA)_5$	PET	195-315		Carlon and Lippé 2007
4	*TG28*	$(TG)_{10}$	VIC	97-155	55	Wainwright *et al*. 2012
	TG42	$(GT)_8$	NED	178-220		Wainwright *et al*. 2012
	TG39	$(GT)_{11}$	PET	136-166		Wainwright *et al*. 2012
5	*Tgr-B119*	$(CTTT)_6$	NED	162-202	60	Carlon and Lippé 2007
	Tgr-C117	$(CCAT)_8$	FAM	252-316		Carlon and Lippé 2007
	Tgr-D103	$(TC)_4(TA)(TC)_3(TATC)_6(TATT)(TATC)_{16}$	VIC	220-354		Carlon and Lippé 2007
6	*Tgr-D128*	$(GATA)_9(AGTA)(GATA)_3$	PET	288-332	65	Carlon and Lippé 2007
	Tgr-24	$(GATA)_{15}$	VIC	307-379		Carlon and Lippé 2007

Table S2.2. Basic genetic diversity statistics (per marker) for *Tripneustes gratilla* populations along the eastern coast of South Africa. These include: polymorphic information content (PIC); number of alleles (A_n); effective number of alleles (A_e); observed heterozygosity (H_o); expected heterozygosity (uH_e); Shannon's information index (I); fixation index (F) and null allele frequencies ($Fr_{(Null)}$). An asterisk (*) indicates departure from Hardy-Weinberg equilibrium ($P < 0.001$).

Population	Locus	PIC	A_n	A_e	I	H_o	uH_e	F	$Fr_{(Null)}$
Haga Haga	*Tgr-A11*	0.84	8.00	6.90	2.00	0.30	0.90	0.65*	0.30
	TG01	0.84	7.74	7.02	2.00	0.50	0.89	0.42	0.19
	TG07	0.75	5.20	4.50	1.62	1.00	0.81	-0.29	-0.13
	TG66	0.77	6.64	5.05	1.74	0.50	0.84	0.38*	0.17
	TG52	0.34	3.79	1.56	0.73	0.08	0.37	0.77	0.20
	TG11	0.21	2.81	1.29	0.46	0.25	0.24	-0.11	-0.020
	Tgr-C11	0.79	6.79	5.43	1.80	0.83	0.85	-0.02	-0.01
	TG51	0.89	11.77	9.60	2.41	0.42	0.93	0.53*	0.25
	TG02	0.57	4.64	2.77	1.20	0.58	0.67	0.09	0.03
	Tgr-24	0.65	4.82	3.36	1.34	0.45	0.74	0.35	0.33
	TG20	0.78	6.79	5.14	1.77	0.17	0.84	0.79*	0.15
	TG26	0.89	10.53	9.60	2.33	0.58	0.93	0.35*	0.17
	TG61	0.76	7.31	4.72	1.75	1.00	0.82	-0.27	-0.12
	TG60	0.79	7.62	5.33	1.86	0.67	0.85	0.18*	0.08
	TG55	0.77	8.14	4.88	1.84	0.67	0.83	0.16	0.07
	Tgr-D134	0.84	7.79	7.02	2.00	0.33	0.89	0.61*	0.28
	TG28	0.89	11.22	9.93	2.38	0.42	0.94	0.54*	0.25
	TG42	0.66	5.00	3.33	1.37	0.20	0.74	0.71*	0.29
	TG39	0.87	10.26	8.73	2.27	1.00	0.92	-0.13	-0.06
	Tgr-B119	0.43	4.81	1.82	0.95	0.18	0.47	0.60	0.19
	Tgr-C117	0.78	6.81	5.26	1.77	0.82	0.85	-0.01	-0.01
	Tgr-D103	0.87	11.36	8.34	2.30	0.45	0.92	0.48*	0.23
Mean		0.73	7.27 ± 0.59	5.53 ± 0.56	1.72 ± 0.17	0.52 ± 0.04	0.78 ± 0.04	0.31 ± 0.07	0.13
Coffee Bay	*Tgr-A11*	0.93	12.64	14.25	2.79	0.39	0.95	0.58*	0.28
	TG01	0.83	7.75	6.70	2.05	0.38	0.87	0.55*	0.25
	TG07	0.74	5.20	4.37	1.58	0.72	0.78	0.06	0.03
	TG66	0.72	7.18	3.81	1.78	0.52	0.75	0.30	0.13
	TG52	0.39	4.38	1.70	0.94	0.28	0.42	0.33	0.10
	TG11	0.37	3.62	1.67	0.82	0.45	0.41	-0.12	-0.03
	Tgr-C11	0.78	6.42	5.03	1.78	0.73	0.82	0.09	0.04
	TG51	0.88	9.92	9.33	2.39	0.46	0.91	0.48*	0.23
	TG02	0.80	6.96	5.67	1.89	0.70	0.84	0.15	0.07
	Tgr-24	0.78	7.46	4.93	1.90	0.38	0.81	0.52*	0.32
	TG20	0.85	8.62	7.48	2.16	0.45	0.88	0.48*	0.23
	TG26	0.92	12.56	13.35	2.78	0.48	0.94	0.48*	0.23
	TG61	0.84	8.67	6.76	2.15	0.66	0.87	0.23*	0.11
	TG60	0.82	7.87	6.12	2.02	0.76	0.85	0.09	0.04
	TG55	0.81	7.87	5.96	2.02	0.59	0.85	0.30*	0.13

	Tgr-D134	0.90	11.25	11.14	2.60	0.76	0.93	0.17*	0.08
	TG28	0.92	11.86	13.14	2.68	0.66	0.94	0.29	0.14
	TG42	0.83	8.03	6.40	2.06	0.21	0.86	0.75*	0.35
	TG39	0.85	8.51	7.58	2.18	0.86	0.88	0.01	0.00
	Tgr-B119	0.65	5.68	3.10	1.48	0.24	0.69	0.64*	0.26
	Tgr-C117	0.78	7.12	5.04	1.87	0.76	0.82	0.05	0.02
	Tgr-D103	0.91	12.06	11.52	2.72	0.72	0.93	0.21*	0.10
Mean		0.79	8.26 ± 0.97	7.05 ± 0.78	2.03 ± 0.11	0.55 ± 0.04	0.82 ± 0.03	0.30 ± 0.05	0.14
Hibberdene	*Tgr-A11*	0.92	12.05	13.43	2.73	0.43	0.94	0.53*	0.26
	TG01	0.79	7.26	5.31	1.91	0.44	0.82	0.46*	0.21
	TG07	0.79	7.22	5.40	1.92	0.65	0.83	0.21	0.09
	TG66	0.69	6.55	3.56	1.67	0.85	0.73	-0.19	-0.08
	TG52	0.55	5.08	2.36	1.26	0.42	0.58	0.26*	0.10
	TG11	0.43	4.99	1.81	1.07	0.47	0.45	-0.05	-0.02
	Tgr-C11	0.81	6.84	5.82	1.90	0.75	0.84	0.09	0.04
	TG51	0.88	9.93	8.94	2.39	0.42	0.90	0.53*	0.25
	TG02	0.77	6.51	5.03	1.81	0.73	0.81	0.09	0.04
	Tgr-24	0.85	8.56	7.28	2.19	0.55	0.88	0.37*	0.38
	TG20	0.89	10.14	9.64	2.44	0.64	0.91	0.29*	0.17
	TG26	0.93	12.50	14.73	2.81	0.65	0.95	0.31*	0.15
	TG61	0.85	8.47	7.39	2.16	0.85	0.88	0.01	0.01
	TG60	0.83	8.41	6.64	2.15	0.71	0.86	0.17	0.08
	TG55	0.86	8.74	7.63	2.21	0.65	0.88	0.26	0.12
	Tgr-D134	0.90	10.96	11.22	2.58	0.71	0.92	0.23*	0.11
	TG28	0.89	10.12	10.01	2.45	0.47	0.91	0.48*	0.23
	TG42	0.81	8.55	5.86	2.10	0.45	0.84	0.46*	0.21
	TG39	0.83	9.00	6.45	2.01	0.90	0.89	-0.07	-0.03
	Tgr-B119	0.64	4.73	3.14	1.36	0.34	0.69	0.49*	0.20
	Tgr-C117	0.76	7.03	4.68	1.86	0.69	0.80	0.13	0.06
	Tgr-D103	0.93	12.07	15.75	3.01	0.68	0.95	0.28*	0.13
Mean		0.80	8.44 ± 0.81	7.37 ± 0.81	2.09 ± 0.11	0.61 ± 0.03	0.83 ± 0.03	0.24 ± 0.04	0.12
Ballito Bay	*Tgr-A11*	0.84	9.66	6.92	2.19	0.38	0.88	0.56*	0.26
	TG01	0.83	7.26	6.55	1.95	0.78	0.87	0.08	0.04
	TG07	0.67	5.72	3.48	1.50	0.67	0.73	0.06	0.03
	TG66	0.65	4.83	3.27	1.38	0.50	0.71	0.28	0.12
	TG52	0.21	3.01	1.28	0.48	0.12	0.22	0.46	0.08
	TG11	0.33	4.29	1.52	0.79	0.33	0.35	0.03	0.07
	Tgr-C11	0.78	6.71	5.08	1.80	0.58	0.83	0.28	0.12
	TG51	0.87	9.53	8.17	2.22	0.43	0.91	0.51*	0.24
	TG02	0.68	5.21	3.65	1.47	0.79	0.75	-0.09	-0.04
	Tgr-24	0.87	9.84	8.30	2.30	0.47	0.90	0.46*	0.34
	TG20	0.82	8.07	6.40	2.01	0.38	0.87	0.56*	0.22
	TG26	0.87	9.49	8.20	2.25	0.50	0.90	0.43*	0.20
	TG61	0.84	8.82	6.94	2.14	0.84	0.88	0.02	0.01
	TG60	0.67	5.60	3.41	1.48	0.63	0.73	0.11	0.04

	TG55	0.82	7.73	6.07	1.97	0.68	0.86	0.18	0.08
	Tgr-D134	0.88	10.53	9.26	2.40	0.47	0.92	0.47*	0.22
	TG28	0.82	8.80	6.29	2.09	0.61	0.87	0.27	0.13
	TG42	0.38	2.94	1.73	0.75	0.06	0.43	0.86*	0.26
	TG39	0.72	5.10	4.21	1.53	0.61	0.78	0.20	0.09
	Tgr-B119	0.57	5.10	2.55	1.26	0.50	0.63	0.18	0.07
	Tgr-C117	0.76	5.86	4.81	1.68	0.74	0.81	0.07	0.03
	Tgr-D103	0.93	13.51	14.73	2.78	0.61	0.96	0.34*	0.17
Mean		0.72	7.16 ± 0.78	5.58 ± 0.67	1.75 ± 0.12	0.53 ± 0.04	0.76 ± 0.04	0.29 ± 0.05	0.12
Sodwana Bay	*Tgr-A11*	0.92	12.44	14.13	2.75	0.29	0.95	0.69*	0.33
	TG01	0.88	9.05	9.04	2.27	0.66	0.91	0.26*	0.12
	TG07	0.76	6.11	4.67	1.72	0.83	0.80	-0.05	-0.02
	TG66	0.65	6.18	3.12	1.56	0.45	0.69	0.34	0.14
	TG52	0.27	3.32	1.39	0.63	0.10	0.28	0.63*	0.14
	TG11	0.41	5.04	1.71	1.05	0.45	0.42	-0.08	-0.02
	Tgr-C11	0.79	7.39	5.43	1.93	0.69	0.83	0.15	0.07
	TG51	0.89	10.51	10.25	2.47	0.56	0.92	0.38*	0.18
	TG02	0.75	6.27	4.54	1.71	0.85	0.79	-0.09	-0.04
	Tgr-24	0.81	7.38	5.79	2.01	0.54	0.84	0.35*	0.28
	TG20	0.83	8.13	6.62	2.09	0.38	0.86	0.55*	0.16
	TG26	0.92	12.38	13.56	2.77	0.45	0.94	0.52*	0.25
	TG61	0.87	9.21	8.49	2.28	0.86	0.90	0.02	0.01
	TG60	0.79	7.38	5.43	1.94	0.69	0.83	0.15	0.07
	TG55	0.84	9.09	7.04	2.23	0.69	0.87	0.20	0.09
	Tgr-D134	0.91	11.26	11.44	2.59	0.69	0.93	0.24*	0.12
	TG28	0.92	12.45	14.13	2.77	0.55	0.95	0.41*	0.20
	TG42	0.69	7.15	3.48	1.71	0.21	0.72	0.71*	0.30
	TG39	0.84	7.74	6.98	2.06	0.79	0.87	0.07	0.03
	Tgr-B119	0.61	4.50	2.86	1.28	0.21	0.66	0.68*	0.27
	Tgr-C117	0.82	7.88	6.05	2.04	0.90	0.85	-0.07	-0.03
	Tgr-D103	0.93	12.79	14.50	2.82	0.52	0.95	0.44*	0.21
Mean		0.78	8.35 ± 0.88	7.30 ± 0.92	2.03 ± 0.12	0.56 ± 0.05	0.81 ± 0.04	0.30 ± 0.05	0.13
Overall mean		0.76	7.89 ± 0.43	6.57 ± 0.34	1.92 ± 0.05	0.55 ± 0.02	0.80 ± 0.02	0.29 ± 0.02	0.13

Appendix B

Supplementary information for Chapter 3

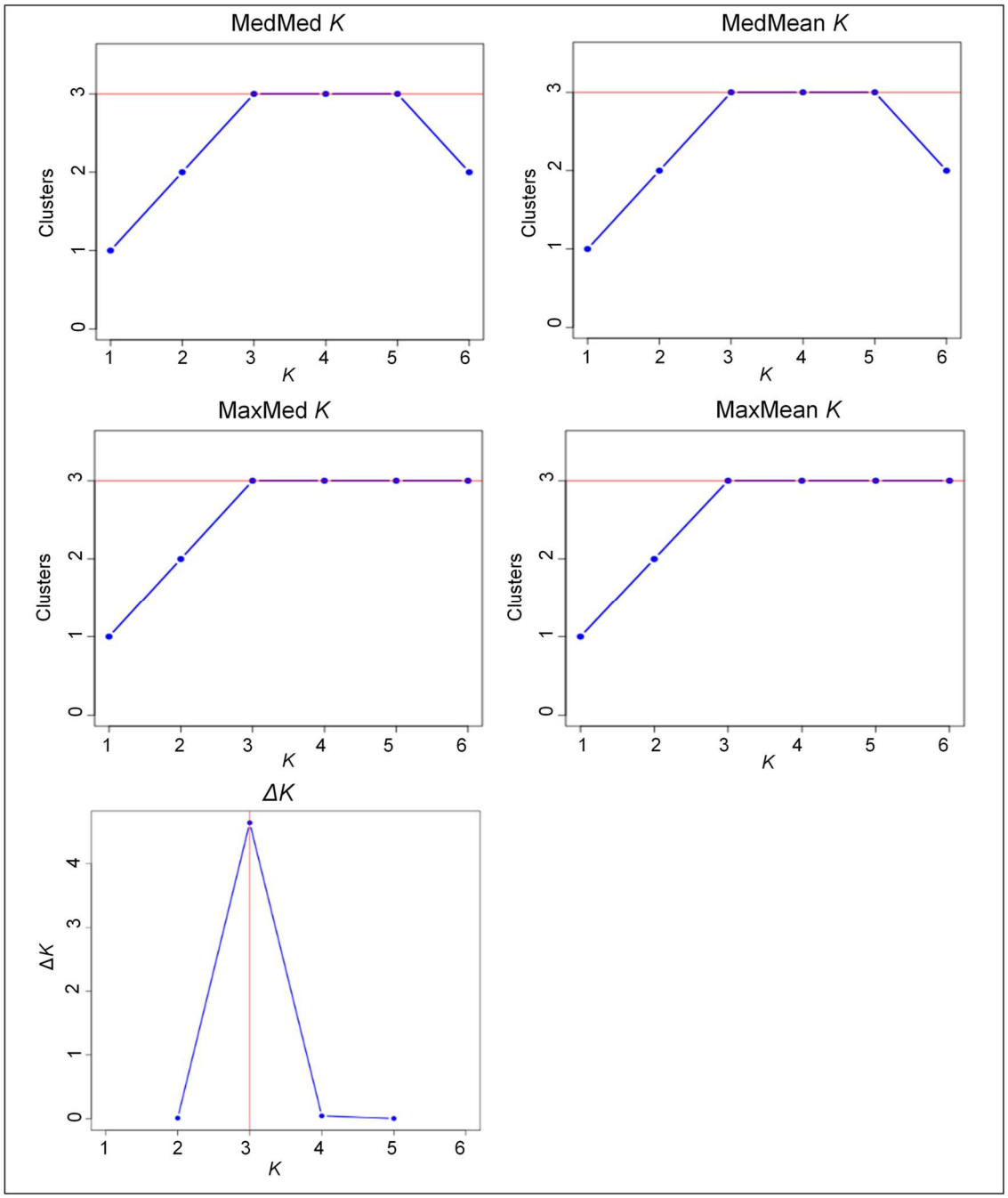

Figure S3.1. Puechmaille (MedMed *K*, MedMean *K*, MaxMed *K* and MaxMean *K*) and Evanno (Δ*K*) methods showing *K* = 3 as the most likely number of genetic clusters.

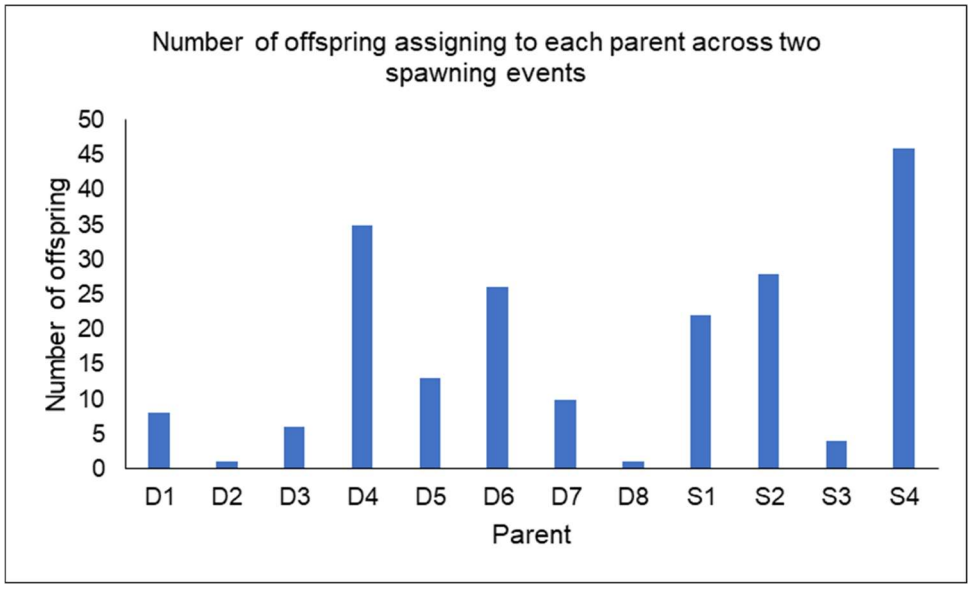

Figure S3.2. Number of offspring assigning to each parent across two independent spawning events, where dams D1 – D4 and sires S1 – S2 contributed to spawning event 1, and dams D5 – D8 and sires S3 – S4 contributed to the second spawning event.

Table S3.1. Basic genetic diversity statistics (per marker) for natural and cultured *Tripneustes gratilla* cohorts. These include: number of alleles (A_n); effective number of alleles (A_e); Shannon's information index (I), observed heterozygosity (H_o); unbiased expected heterozygosity (uH_e); fixation index (F), null allele frequencies ($Fr_{(Null)}$), polymorphic information content (PIC), inclusion (PI) and exclusion probabilities (PE), as well as standard errors for mean estimates. An asterisk (*) indicates departure from Hardy-Weinberg equilibrium ($P < 0.001$).

Cohort	Locus	A_n	A_e	I	H_o	uH_e	F	$Fr_{(Null)}$	PIC	PI	PE
Natural	*Tgr-A11*	23	14.85	2.88	0.35	0.94	0.63*	0.29	0.93	0.01	0.86
	TG01	10	6.87	2.05	0.40	0.86	0.53*	0.24	0.84	0.04	0.71
	TG07	7	4.61	1.66	0.86	0.79	-0.10	-0.04	0.75	0.08	0.58
	TG66	15	4.55	1.98	0.60	0.79	0.23*	0.08	0.76	0.07	0.61
	TG52	9	1.70	0.99	0.26	0.42	0.37*	0.10	0.40	0.36	0.25
	TG11	6	1.64	0.85	0.38	0.40	0.03	0.00	0.37	0.39	0.23
	Tgr-C11	9	5.46	1.86	0.74	0.83	0.09	0.03	0.79	0.06	0.64
	TG02	9	5.28	1.82	0.71	0.82	0.12	0.06	0.78	0.06	0.63
	Tgr-24	12	4.95	1.95	0.37	0.81	0.54*	0.24	0.78	0.06	0.63
	TG20	11	8.03	2.21	0.38	0.88	0.57*	0.28	0.86	0.03	0.75
	TG26	22	17.30	2.97	0.60	0.95	0.36*	0.18	0.94	0.01	0.88
	TG61	13	8.94	2.32	0.72	0.90	0.19*	0.09	0.88	0.02	0.77
	TG60	10	6.13	2.04	0.74	0.85	0.12*	0.05	0.82	0.04	0.69
	TG55	13	7.37	2.22	0.66	0.87	0.24*	0.10	0.85	0.03	0.73
	Tgr-D134	23	13.30	2.79	0.66	0.93	0.29*	0.14	0.92	0.01	0.85
	TG28	22	15.67	2.89	0.64	0.95	0.32*	0.15	0.93	0.01	0.87
	TG39	14	9.67	2.41	0.84	0.91	0.06	0.03	0.89	0.02	0.79
	TG42	12	5.72	2.04	0.23	0.83	0.72*	0.32	0.81	0.05	0.67
	Tgr-B119	10	2.83	1.44	0.29	0.65	0.56*	0.23	0.61	0.16	0.44
	Tgr-C117	12	5.73	1.96	0.80	0.83	0.04	0.03	0.80	0.05	0.66
	Tgr-D103	31	20.45	3.20	0.74	0.96	0.22*	0.12	0.95	0.00	0.90
Mean ± SE		**13.95 ± 1.41**	**8.15 ± 1.14**	**2.12 ± 0.13**	**0.57 ± 0.05**	**0.82 ± 0.03**	**0.30 ± 0.05**	**0.13**	**0.79**	**0.07**	**0.67**
F1 Cultured 1	*Tgr-A11*	4	2.96	1.13	0.18	0.67	0.73*	0.29	0.59	0.18	0.38
	TG01	6	5.41	1.73	0.96	0.82	-0.17	-0.08	0.79	0.06	0.63
	TG07	4	2.81	1.21	0.73	0.65	-0.14	-0.06	0.60	0.17	0.40
	TG66	4	2.00	0.93	0.62	0.50	-0.24	-0.08	0.45	0.30	0.28
	TG52	3	1.25	0.41	0.22	0.20	-0.09	-0.02	0.19	0.65	0.10
	TG11	4	1.85	0.86	0.56	0.46	-0.22	-0.07	0.42	0.33	0.25
	Tgr-C11	5	4.05	1.47	0.83	0.76	-0.10	-0.04	0.71	0.10	0.52
	TG02	5	3.27	1.35	0.73	0.70	-0.06	-0.02	0.65	0.14	0.46
	Tgr-24	7	4.19	1.62	0.33	0.77	0.57*	0.25	0.73	0.09	0.56
	TG20	4	3.66	1.34	0.56	0.73	0.23	0.10	0.68	0.12	0.48
	TG26	7	4.67	1.70	0.90	0.79	-0.15	-0.06	0.76	0.07	0.59
	TG61	5	2.74	1.17	0.78	0.64	-0.23	-0.09	0.57	0.20	0.37
	TG60	6	2.16	1.11	0.50	0.54	0.07	0.03	0.50	0.25	0.33
	TG55	6	3.26	1.36	0.54	0.70	0.22*	0.09	0.64	0.15	0.44

	Tgr-D134	7	5.42	1.76	1.00	0.82	-0.23*	-0.10	0.79	0.06	0.63
	TG28	8	5.07	1.76	1.00	0.81	-0.25	-0.12	0.77	0.07	0.61
	TG39	6	4.46	1.59	0.80	0.78	-0.03	-0.01	0.74	0.08	0.57
	TG42	4	2.59	1.09	0.13	0.62	0.80*	0.30	0.54	0.22	0.34
	Tgr-B119	3	2.88	1.08	0.20	0.66	0.69*	0.27	0.58	0.19	0.36
	Tgr-C117	5	3.00	1.22	0.86	0.67	-0.29	-0.12	0.60	0.17	0.40
	Tgr-D103	4	2.88	1.17	0.75	0.66	-0.15	-0.06	0.59	0.18	0.39
Mean ± SE		**5.10 ± 0.31**	**3.36 ± 0.26**	**1.29 ± 0.08**	**0.63 ± 0.06**	**0.67 ± 0.03**	**0.05 ± 0.08**	**0.02**	**0.61**	**0.18**	**0.43**
F1 Cultured 2	*Tgr-A11*	5	2.60	1.12	0.69	0.62	-0.12	-0.05	0.56	0.21	0.36
	TG01	4	3.74	1.35	0.84	0.74	-0.14	-0.06	0.68	0.12	0.48
	TG07	4	2.11	0.82	0.52	0.53	0.01	0.00	0.42	0.33	0.22
	TG66	5	2.90	1.20	0.98	0.66	-0.50*	0.00	0.59	0.18	0.39
	TG52	5	1.92	0.89	0.63	0.48	-0.32	0.05	0.43	0.32	0.26
	TG11	3	1.90	0.83	0.56	0.48	-0.18	0.00	0.42	0.33	0.25
	Tgr-C11	6	4.33	1.57	0.90	0.78	-0.18*	0.21	0.73	0.09	0.55
	TG02	4	3.98	1.38	0.95	0.76	-0.28*	0.14	0.70	0.11	0.50
	Tgr-24	4	1.55	0.66	0.35	0.36	0.02	0.11	0.32	0.45	0.18
	TG20	5	2.45	1.07	0.20	0.60	0.66*	0.28	0.52	0.24	0.32
	TG26	6	3.98	1.47	0.80	0.76	-0.07*	0.00	0.70	0.11	0.51
	TG61	5	3.94	1.41	0.98	0.75	-0.31*	0.00	0.70	0.11	0.50
	TG60	5	3.93	1.44	0.89	0.75	-0.19*	0.00	0.70	0.11	0.51
	TG55	5	3.42	1.35	0.90	0.71	-0.27	0.00	0.66	0.13	0.46
	Tgr-D134	8	5.05	1.77	0.98	0.81	-0.22*	0.00	0.77	0.07	0.61
	TG28	7	4.85	1.68	0.84	0.80	-0.06*	0.15	0.76	0.07	0.59
	TG39	5	2.67	1.18	0.77	0.63	-0.23*	0.11	0.57	0.20	0.37
	TG42	4	2.93	1.20	0.88	0.67	-0.34*	0.21	0.60	0.17	0.40
	Tgr-B119	2	1.29	0.38	0.04	0.23	0.81*	0.30	0.20	0.63	0.10
	Tgr-C117	4	1.99	0.85	0.59	0.50	-0.19*	0.06	0.42	0.33	0.24
	Tgr-D103	9	5.45	1.82	0.96	0.82	-0.18*	0.00	0.79	0.06	0.64
Mean ± SE		**5.00 ± 0.35**	**3.19 ± 0.26**	**1.21 ± 0.08**	**0.73 ± 0.06**	**0.64 ± 0.035**	**-0.11 ± 0.07**	**0.07**	**0.58**	**0.21**	**0.40**
Overall mean ± SE		**8.02 ± 0.72**	**4.90 ± 0.49**	**1.54 ± 0.08**	**0.64 ± 0.03**	**0.71 ± 0.02**	**0.08 ± 0.04**	**0.07**	**0.66**	**0.15**	**0.50**

Appendix C

Supplementary information for Chapter 4

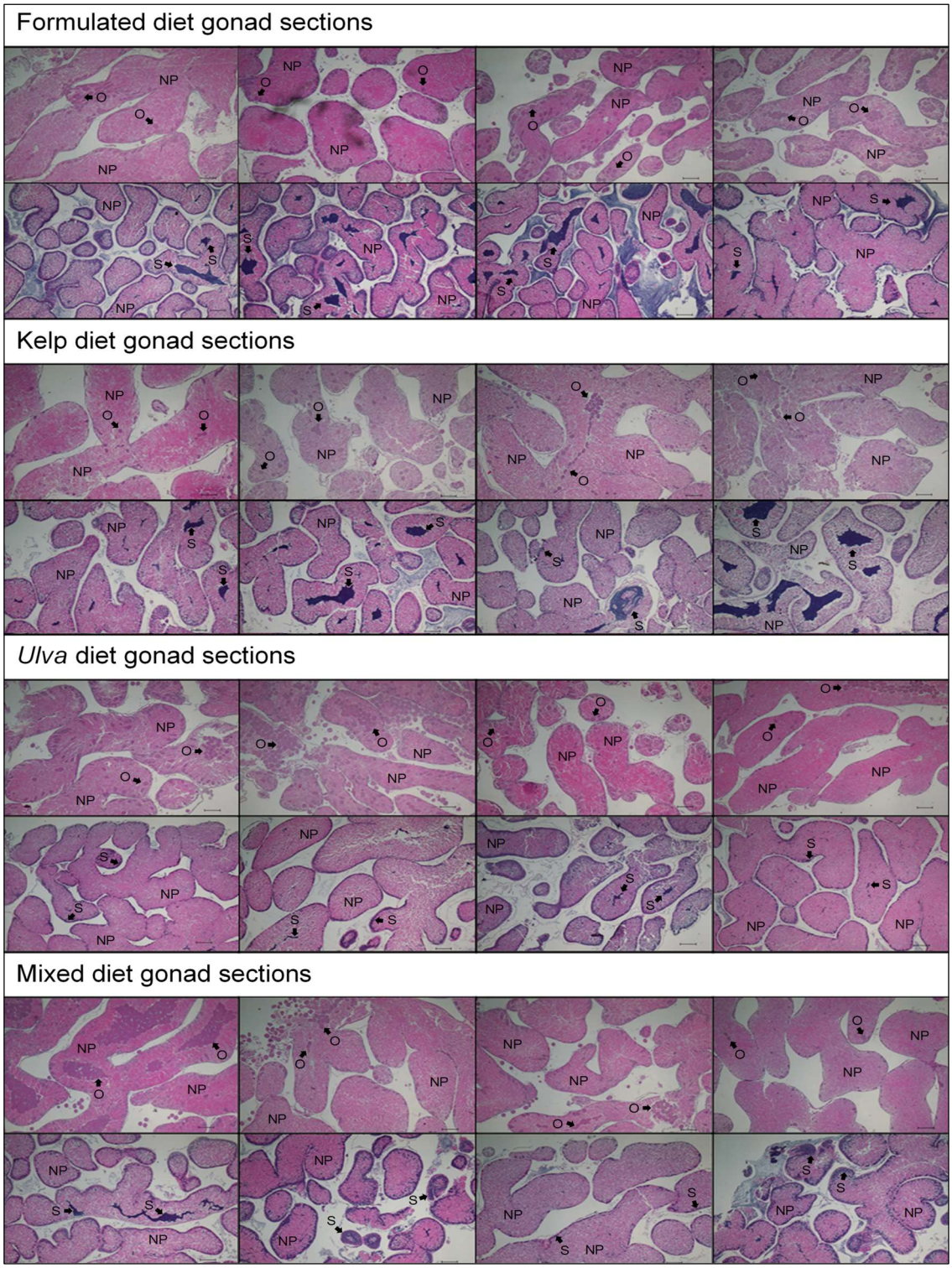

Figure S4.1. *Tripneustes gratilla* gonad histology sections for the first spawning event at 40X animals conditioned on different feeding regimes, where female gonads are shown in the first row and male gonads are in the second row for each diet respectively. NP: Nutritive phagocyte; O: Oocyte; S: Spermatocytes. All scale bars indicate 200 µm.

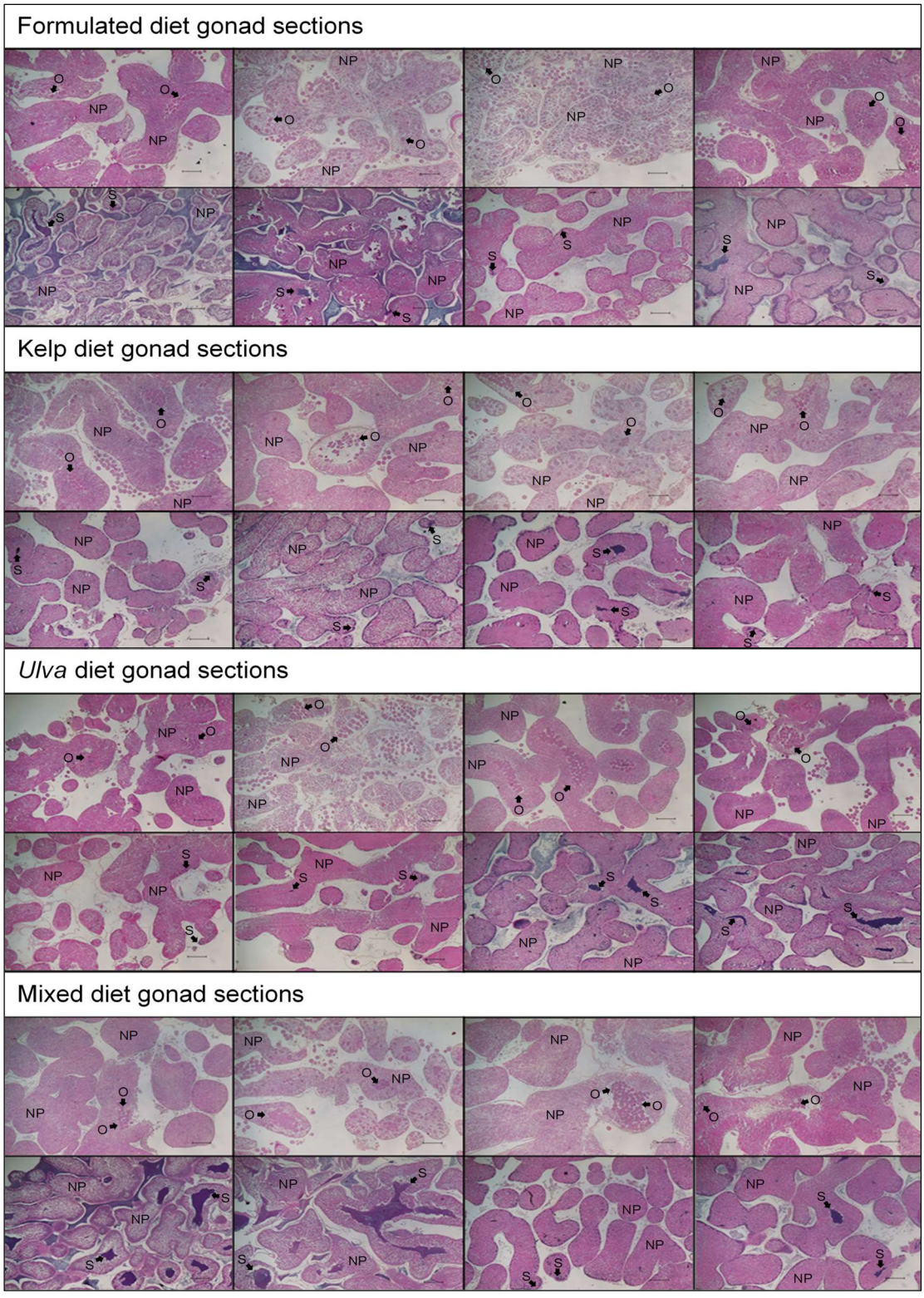

Figure S4.2. *Tripneustes gratilla* gonad histology sections for the second spawning event at 40X animals conditioned on different feeding regimes, where female gonads are shown in the first row and male gonads are in the second row for each diet respectively. NP: Nutritive phagocyte; O: Oocyte; S: Spermatocytes. All scale bars indicate 200 µm.

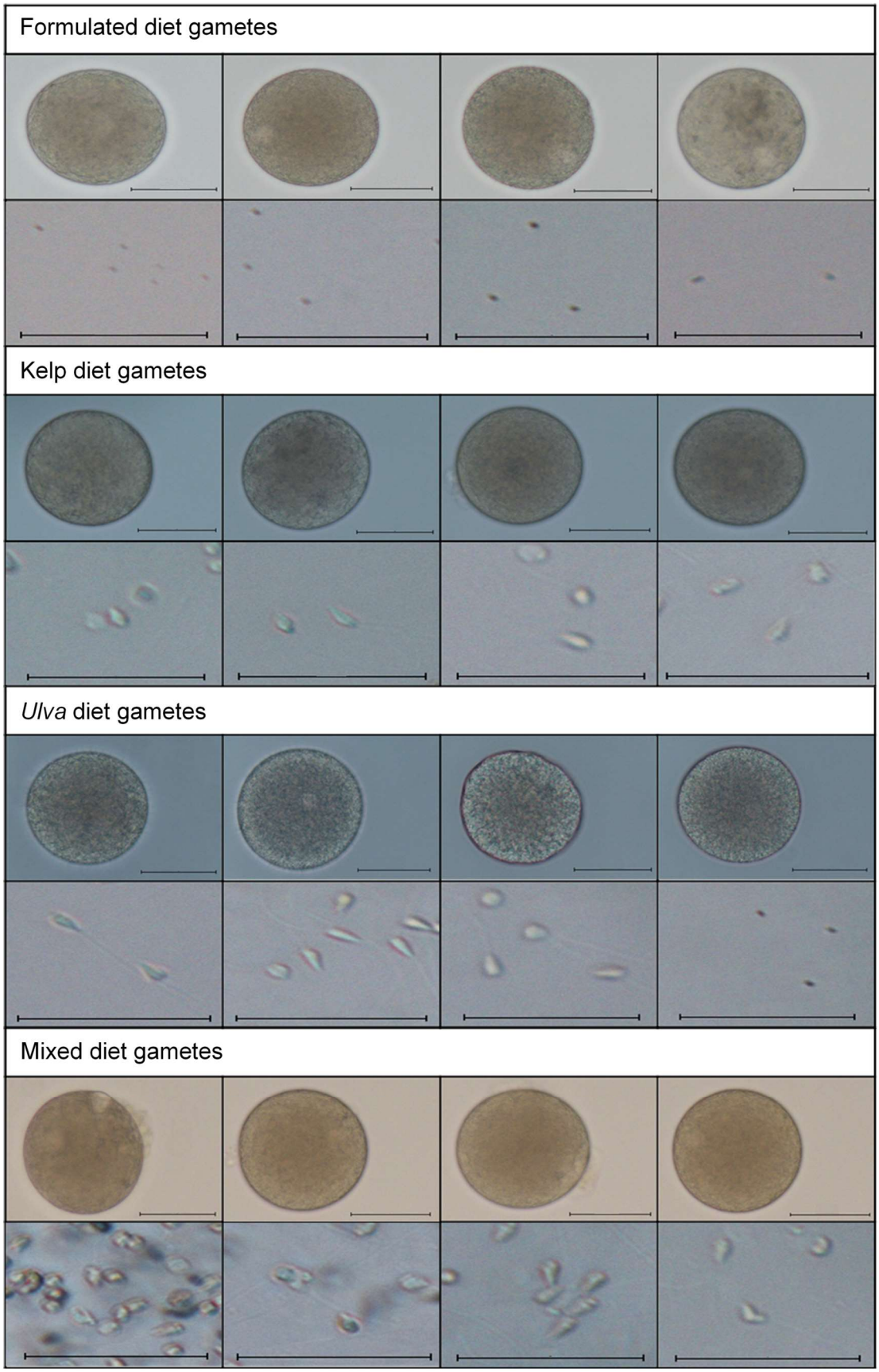

Figure S4.3. *Tripneustes gratilla* egg- (top row at 400X magnification) and sperm (bottom row at 1000X magnification) morphology for gametes collected from the first spawning event, where animals conditioned on different feeding regimes, where all scale bars indicate 50 µm.

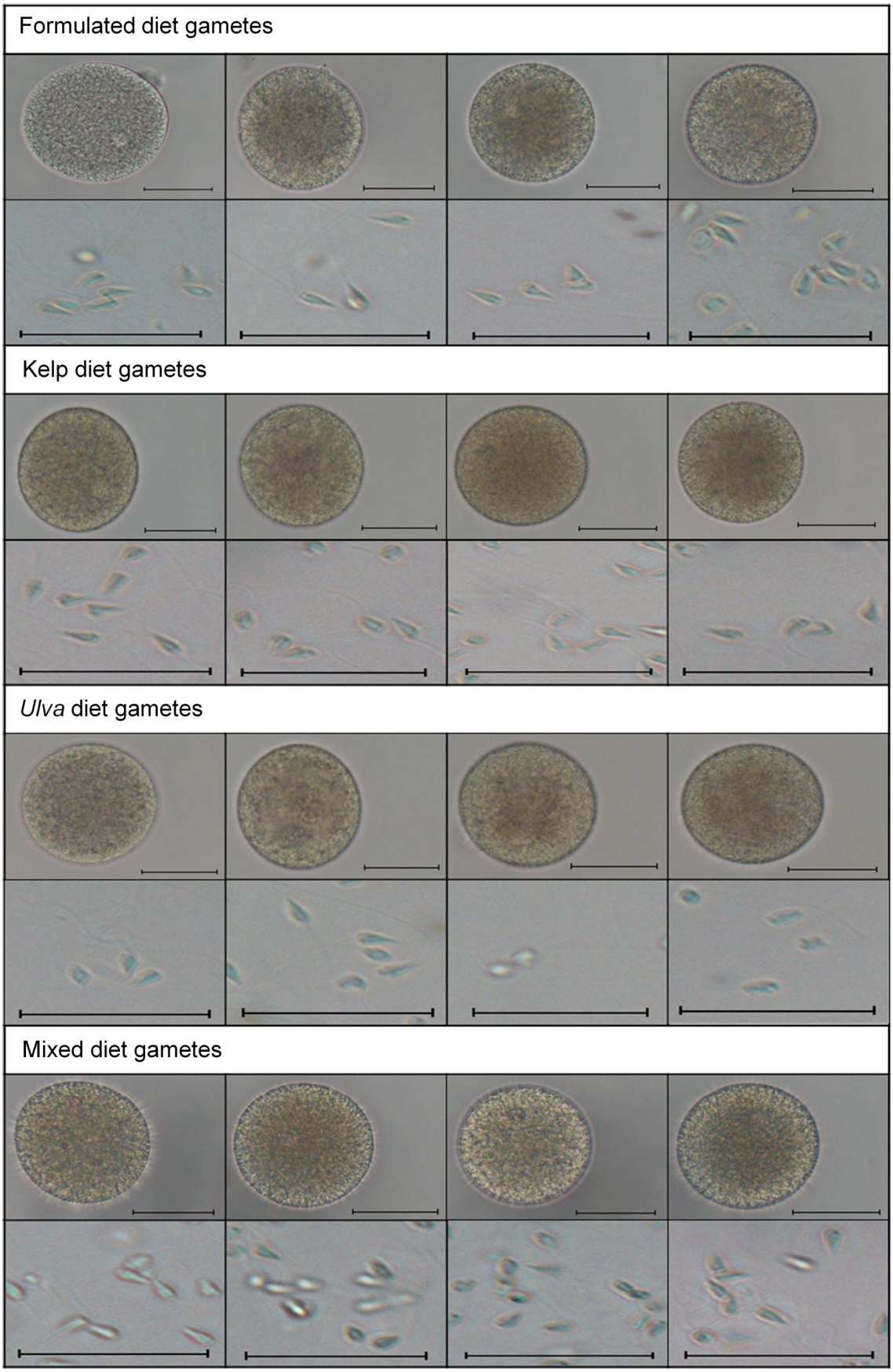

Figure S4.4. *Tripneustes gratilla* egg- (top row at 400X magnification) and sperm (bottom row at 1000X magnification) morphology for gametes collected from the second spawning event, where animals were conditioned on different feeding regimes, where all scale bars indicate 50 µm.

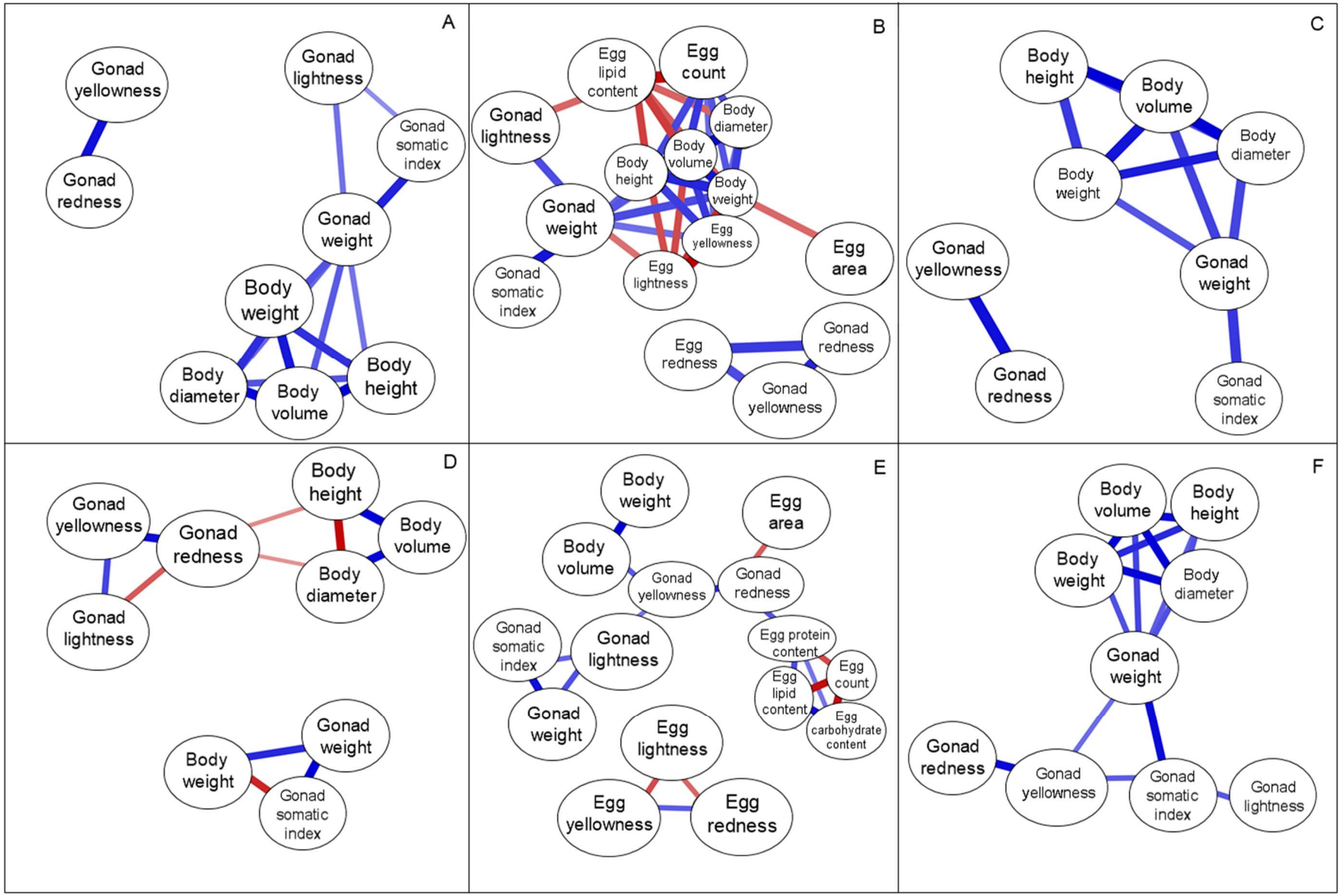

Figure S4.5. Network structures (pcor estimator) of statistically significant ($P < 0.05$) correlations, based on Pearson correlation coefficients (r), (A,D) across all animals (n = 32), (B,E) across females (n = 16) and (C,F) across males (n = 16) when animals from all feeding regimes are grouped together for the first- (A – C) and second (D – F) iteration of broodstock conditioning, respectively. Blue and red lines represent positive and negative correlations, respectively, where line thickness indicates the relative strength of each correlation.

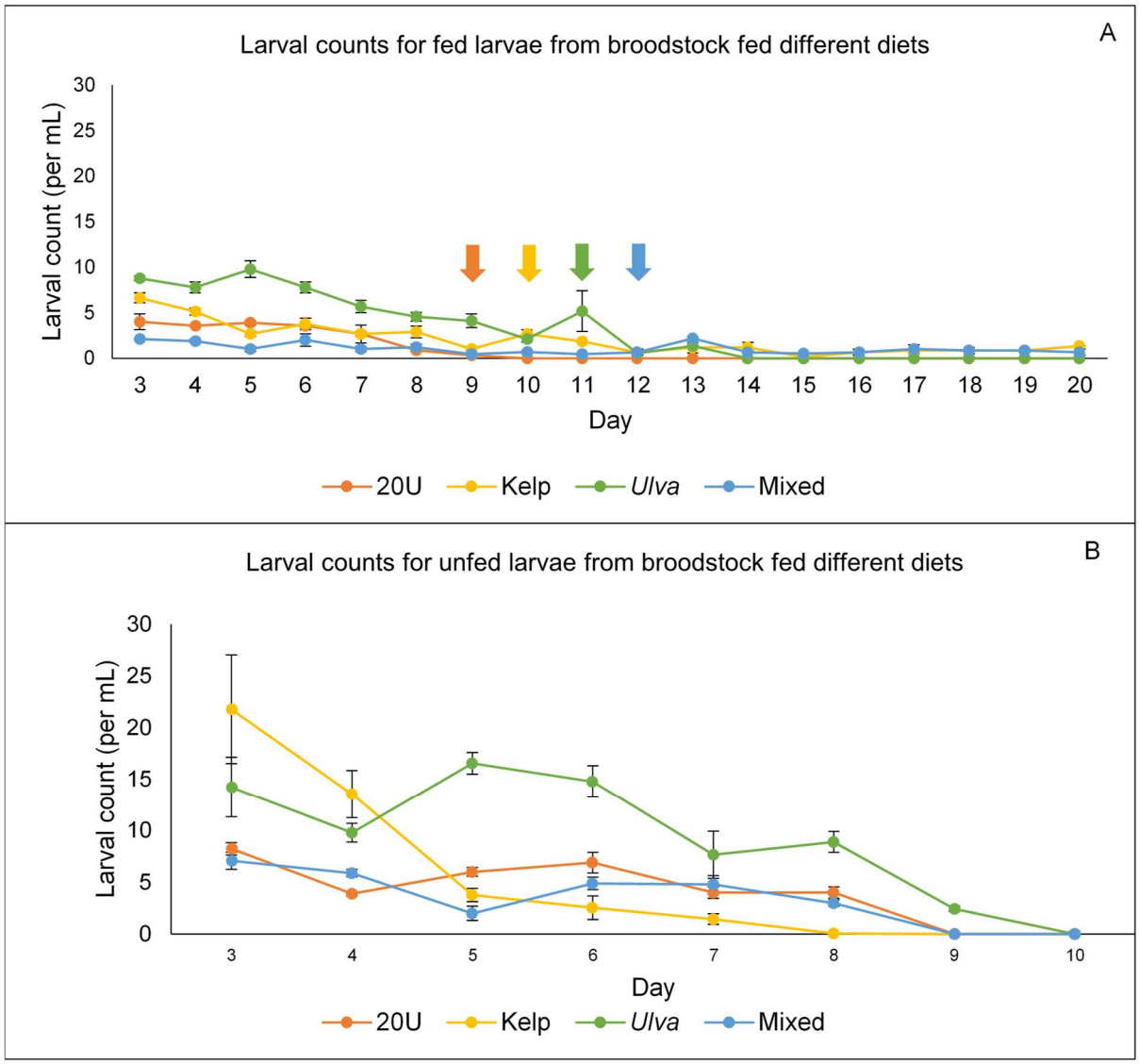

Figure S4.6. Average *T. gratilla* larval counts across three replicate containers per diet, where progenitor broodstock were conditioned on different diets prior to spawning and (A) larvae were fed a microalgal diet and (B) larvae were not fed to assess larval survival without an exogenous food source. Arrows (↓) and corresponding arrow colour indicate where a replicate was excluded due to larval death and average counts, as well as corresponding standard errors are calculated based on remaining replicates.

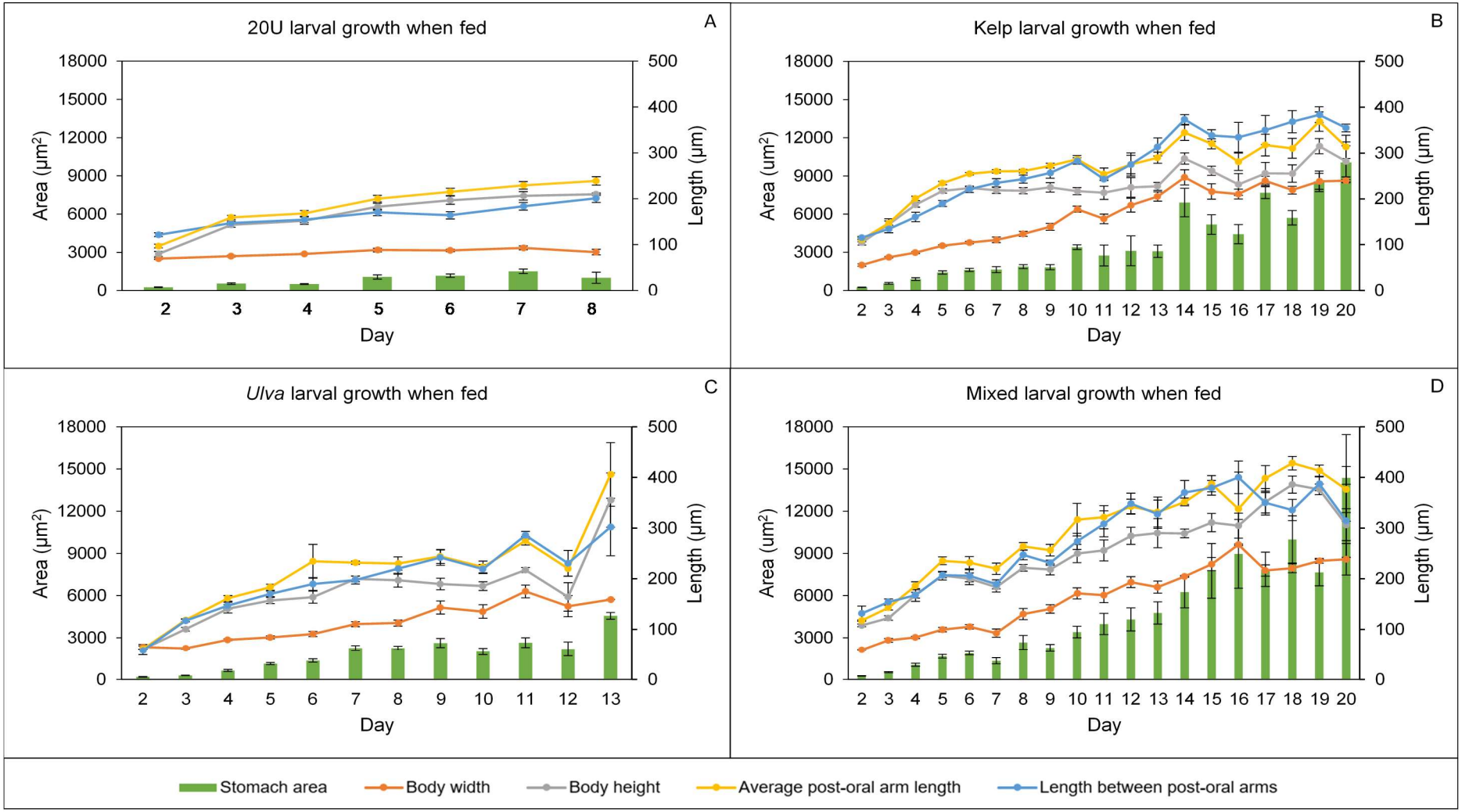

Figure S4.7. Larval growth of offspring from broodstock conditioned on (A) a formulated diet (20U; n = 49), (B) Kelp (*Ecklonia maxima*; n = 119), (C) *Ulva rigida* (n = 84) and (D) a mixture 20U, kelp and *Ulva* (n = 121), that were fed an algal diet. The number of days represent the days of survival of the larvae from the respective groups and standard errors are indicated across measurements. Area is represented in bars and lengths are plotted as lines.

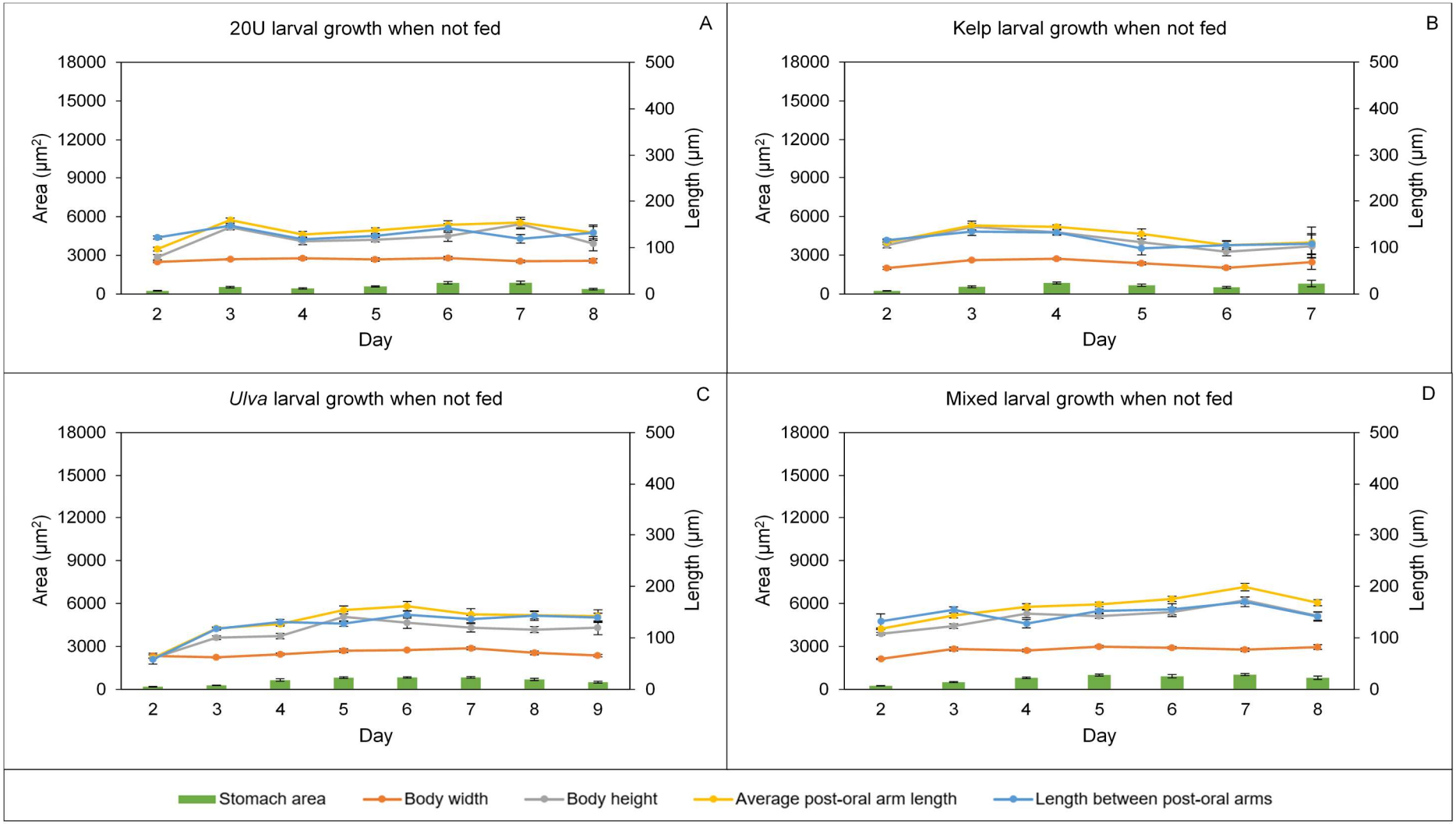

Figure S4.8. Larval growth of offspring from broodstock conditioned on (A) a formulated diet (20U; n = 42), (B) Kelp (*Ecklonia maxima*; n = 37), (C) *Ulva* (n = 50) and (D) a mixture of 20U, kelp and *Ulva* (n = 50) *Ulva rigida* (n = 48), that were not fed. The number of days represent the days of survival of the larvae from the respective groups and standard errors are indicated across measurements. Area is represented in bars and lengths are plotted as lines.

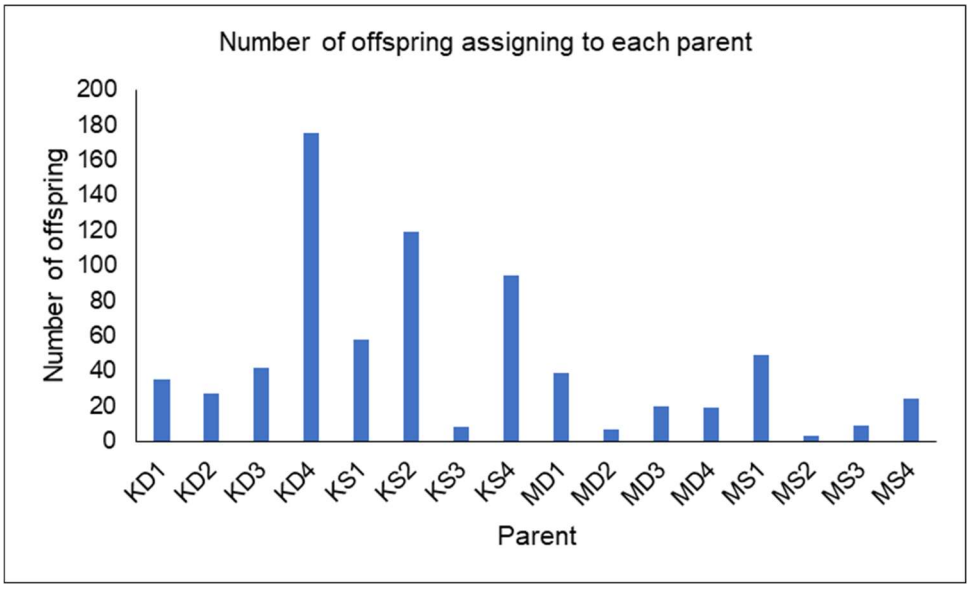

Figure S4.9. Number of offspring assigning to each broodstock animal, where KD1 – KD4 and KS1 – KS4 represent dams and sires, respectively, conditioned on kelp (*Ecklonia maxima*), and MD1 – MD4 and MS1 – MS4 represent dams and sires, respectively, that were conditioned on a mixed feeding regime.

Table S4.1. Analysis of variance (ANOVA) on phenotypic measures after broodstock conditioning on four different feeding regimes, where differences were assessed across all animals, across females and across males, respectively, for both spawning events included in this study.

	Sum of squares	F-value	*P*-value	Sum of squares	F-value	*P*-value
Variable	**First spawning event**			**Second spawning event**		
Across all animals (n = 8 per diet)						
Body weight (g)	584.65	0.43	0.73	905.31	0.06	0.88
Body height (cm)	0.67	1.78	0.17	0.15	0.23	0.87
Body diameter (cm)	0.44	1.44	0.25	0.10	0.11	0.95
Body volume (cm^3)	2408.97	1.97	0.14	303.90	0.06	0.97
Gonad weight (g)	107.79	2.54	0.08	201.24	1.22	0.32
Gonad somatic index (GSI)	23.37	2.59	0.07	27.97	1.14	0.35
Gonad lightness (L*)	68.93	2.72	0.06	39.00	0.45	0.72
Gonad redness (a*)	115.56	4.04	0.02	187.01	16.35	<0.001
Gonad yellowness (b*)	286.74	4.54	0.01	323.58	4.79	<0.01
Across females (n = 4 per diet)						
Body weight (g)	846.05	0.5	0.69	791.64	0.18	0.92
Body height (cm)	0.78	2.31	0.13	0.78	2.13	0.13
Body diameter (cm)	0.54	2.44	0.11	0.54	2.44	0.11
Body volume (cm^3)	2641.17	880.39	0.1	548.28	0.17	0.92
Gonad weight (g)	111.7	2.09	0.16	89.62	0.51	0.68
Gonad somatic index (GSI)	16.02	1.39	0.29	26.39	0.87	0.48
Gonad lightness (L*)	51.84	2.77	0.09	79.50	0.84	0.50
Gonad redness (a*)	69.8	3.49	0.05	102.05	10.72	0.001
Gonad yellowness (b*)	182.1	4.3	0.03	139.45	1.71	0.30
Egg count in 1 mL ($x10^5$)	327.00	1.21	0.35	$2.01x10^{10}$	0.17	0.91
Egg diameter (µM)	13.94	0.64	0.61	10.75	0.71	0.57
Egg area ($µm^2$)	243942.52	0.66	0.59	$2.35x10^6$	1.44	0.35
Egg lightness (L*)	3.65	0.45	0.73	146.85	1.52	0.26
Egg redness (a*)	2.52	8.89	0.002	11.36	2.04	0.16
Egg yellowness (b*)	14.65	0.45	0.72	81.93	1.90	0.18
Egg protein content (ng)	2793.01	20.18	0.001	359.69	3.02	0.07
Egg carbohydrate content (ng)	0.42	0.11	0.95	8.49	0.91	0.46
Egg lipid content (ng)	1362.44	2.28	0.13	654.54	1.15	0.37
Across males (n = 4 per diet)						
Body weight (g)	762.12	0.63	0.61	714.82	0.15	0.93
Body height (cm)	0.43	1.18	0.36	0.04	0.05	0.99
Body diameter (cm)	0.27	0.71	0.57	0.28	0.28	0.84
Body volume (cm^3)	1280.84	0.98	0.44	141.34	0.03	0.99
Gonad weight (g)	34.43	0.99	0.43	217.99	1.22	0.34
Gonad somatic index (GSI)	9.98	1.26	0.33	33.65	1.77	0.23
Gonad lightness (L*)	24.8	0.71	0.57	77.80	1.20	0.35
Gonad redness (a*)	48.15	4.60	0.02	117.67	13.89	<0.001
Gonad yellowness (b*)	122.21	2.40	0.12	401.10	18.48	<0.001
Sperm length (µM)	38.66	10.54	0.001	1.03	2.99	0.07
Sperm count in 1 mL ($x10^5$)	703.00	1.75	0.21	$1.88x10^7$	0.88	0.48

Table S4.2. Analysis of variance (F-values) or Kruskal-Wallis (H-values) test across larval measurements for *Tripneustes gratilla* larvae fed a mixed microalgal diet for the duration of their planktonic larval stage (20 days). Sample numbers per day (n) are shown. Statistical significance is indicated as **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Day	Larval measurements across fed group							
	Body width	**Body height**	**Average post-oral arm length**	**Length between post-oral arms**	**Stomach area**	**Mouth area**	**Average pre-oral arm length**	**Length between pre-oral arms**
2 (n = 12)	F = 2.69	F = 14.03**	H = 7.65*	F = 7.17	F = 1.90	-	-	-
3 (n = 37)	F = 7.65**	F = 12.53***	F = 7.46***	H = 11.72**	F = 6.55**	F = 4.46*	F = 1.08	F = 0.98
4 (n = 36)	F = 1.38	F = 4.58**	F = 7.26***	F = 1.21	F = 7.11***	F = 1.77	F = 7.53***	F = 0.23
5 (n = 37)	F = 4.14**	H = 19.79***	F = 15.65***	F = 8.27***	F = 3.74*	F = 6.20**	F = 7.44***	H = 8.26*
6 (n = 36)	F = 5.05**	H = 10.50**	H = 10.86**	F = 4.29**	F = 5.62**	F = 1.88	F = 2.63	F = 2.65
7 (n = 31)	F = 2.44	F = 2.95*	F = 5.26**	F = 5.77**	F = 2.96*	F = 2.12	F = 2.92*	F = 2.98*
8 (n = 28)	F = 2.90	H = 1.39	H = 5.26	F = 2.25	H = 5.79	F = 5.07**	F = 2.16	F = 2.66
9 (n = 21)	F = 0.04	F = 2.53	F = 1.34	F = 1.10	H = 3.40	H = 3.85	F = 1.18	F = 0.41
10 (n = 20)	F = 3.99*	F = 6.16**	F = 5.44**	F = 8.84**	H = 20.37*	H = 8.03*	H = 13.72**	H = 4.93
11 (n = 14)	F = 0.33	H = 2.33	F = 3.03	F = 2.71	F = 1.28	H = 1.54	F = 1.58	F = 1.40
12 (n = 16)	F = 0.87	F = 2.30	F = 4.91	F = 2.51	F = 0.54	H = 2.96	F = 2.34	F = 0.13
13 (n = 16)	F = 2.03	F = 2.11	F = 1.18	F = 0.12	F = 1.63	F = 0.48	F = 0.31	F = 0.44
14 (n = 8)	H = 5.33*	F = 0.008	F = 0.11	F = 0.01	F = 0.13	F = 20.64**	F = 0.51	H = 5.33*
15 (n = 9)	F = 0.28	F = 2.71	F = 6.02*	F = 2.63	H = 1.67	F = 1.75	F = 2.09	F = 0.67
16 (n = 7)	F = 8.15*	F = 2.32	F = 0.29	F = 1.02	F = 1.64	F = 1.66	F = 2.13	F = 3.11
17 (n = 11)	F = 1.88	F = 6.08*	F = 4.42	F < 0.001	F = 0.02	F = 0.51	F = 0.91	F = 1.34
18 (n = 17)	F = 0.02	F = 23.09***	F = 17.81***	F = 0.92	H = 4.48*	F = 0.38	F = 3.66	H = 2.37
19 (n = 10)	F = 0.02	F = 7.98*	F = 2.84	F = 0.02	F = 0.46	F = 0.01	F = 0.50	F = 0.03
20 (n = 7)	F < 0.001	F = 0.20	F = 1.06	F = 0.17	F = 2.13	H = 2.33	F = 0.42	F < 0.01

Table S4.3. Post hoc Tukey's (t) or Dunn's test (z) results showing the pairwise comparisons of statistically significant ANOVA or Kruskal-Wallis results among *Tripneustes gratilla* larvae that were fed a mixed microalgal diet, where statistical significance (P_{Tukey} or P_{holm}) is indicated as *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Day	Variable	Post-hoc test statistic	Comparisons (across fed larvae)					
			Formulated - Kelp	Formulated - *Ulva*	Formulated - Mixed	Kelp - *Ulva*	Kelp - Mixed	*Ulva* - Mixed
2 (n = 12)	**Body height**	t	-3.30*	2.23	-3.72*	4.57**	-0.42	5.56**
	Average post-oral arm length	z	-1.23	0.94	-1.72	2.04	-0.49	2.48*
3 (n = 37)	**Length between post-oral arms**	z	1.22	2.63*	-0.47	1.38	-1.68	3.11**
	Body width	t	0.62	3.29**	-0.90	2.75	-1.58	4.40***
	Body height	t	-0.11	5.18***	2.57	5.29***	2.68*	2.75*
	Average post-oral arm length	t	1.38	4.60***	1.85	3.22**	0.43	2.87*
	Stomach area	t	0.19	3.81**	0.81	3.74**	0.64	2.98*
	Mouth area	t	0.08	1.14	-3.05*	0.98	-2.77	3.25*
4 (n = 36)	**Body height**	t	-2.61	0.88	-1.18	3.49**	1.43	2.06
	Average post-oral arm length	t	-3.49**	0.70	-1.95	4.20**	1.54	2.66
	Stomach area	t	-3.02*	-0.97	4.13**	2.06	-1.11	3.17*
	Average pre-oral arm length	t	-1.30	3.54**	0.53	4.56***	1.77	2.94*
5 (n = 37)	**Body height**	z	-2.57*	1.42	-1.85	4.03***	0.67	3.27**
	Length between pre-oral arms	z	-0.94	0.46	-2.28	1.35	-1.40	2.59*
	Body width	t	-1.86	0.83	-2.14	2.71	-0.33	2.97*
	Average post-oral arm length	t	-3.74**	1.82	-3.75**	5.62***	-0.10	5.57***
	Length between post-oral arms	t	-2.26	0.02	-4.21**	2.28	-2.06	4.23**
	Stomach area	t	-1.78	-0.30	-2.95*	1.47	-1.24	2.65
	Average pre-oral arm length	t	-2.19	0.79	-3.56**	2.85*	-1.47	4.12**
6 (n = 36)	**Body height**	z	-1.90	1.25	-0.74	3.15**	1.16	1.99
	Average post-oral arm length	z	-2.86**	-0.07	-1.32	2.80**	1.54	1.25
	Body width	t	-2.95*	-0.62	-3.10*	2.33	-0.15	2.48
	Length between post-oral arms	t	-3.43**	-1.55	-2.52	1.94	0.95	1.00
	Stomach area	t	-2.34	-0.94	-3.84**	1.40	-1.50	2.90*
7 (n = 31)	**Body width**	t	-1.06	0.54	1.86	1.57	2.92*	-1.28
	Average post-oral arm length	t	-2.96*	-0.21	0.87	2.65	3.75**	-1.04
	Length between post-oral arms	t	-3.66**	-0.94	-0.37	2.67	3.28**	-0.57
	Stomach area	t	-0.42	-2.30	0.59	-1.96	1.01	-2.80*
	Average pre-oral arm length	t	-2.15	-1.00	0.64	1.04	2.73*	-1.59
	Length between pre-oral arms	t	-2.10	-1.09	0.71	0.91	2.76*	-1.74
8 (n = 28)	**Mouth area**	t	-1.89	-3.26*	-3.23*	-2.18	-2.13	-0.05
10 (n = 20)	**Stomach area**	z	-	-	-	2.53*	-0.01	2.45*
	Mouth area	z	-	-	-	2.76**	1.90	0.75
	Average pre-oral arm length	z	-	-	-	3.57***	0.85	2.58**
11 (n = 14)	**Body width**	t	-	-	-	2.64*	0.38	2.16
	Body height	t	-	-	-	1.79	-1.79	3.51**
	Average post-oral arm length	t	-	-	-	2.25	-1.03	1.48**
	Length between post-oral arms	t	-	-	-	3.91**	0.52	3.24**
12 (n = 16)	**Average post-oral arm length**	t	-	-	-	1.03	-1.77	3.13*
14 (n = 8)	**Body width**	z	-	-	-	-	2.31**	-
	Mouth area	t	-	-	-	-	4.54**	-
	Length between pre-oral arms	z	-	-	-	-	2.31**	-
15 (n = 9)	**Average post-oral arm length**	t	-	-	-	-	-2.45*	-

16 (n = 7)	**Body width**	t	-	-	-	-	-2.85*	-
17 (n = 11)	**Body height**	t	-	-	-	-	-2.47*	-
18 (n = 17)	**Body height**	t	-	-	-	-	-4.81***	-
	Average post-oral arm length	t	-	-	-	-	-4.22***	-
	Stomach area	z	-	-	-	-	-2.12*	-

Table S4.4. Analysis of variance (F-values) or Kruskal-Wallis (H-values) test across larval measurements for *Tripneustes gratilla* larvae that were not fed. Sample numbers per day (n) are shown. Statistical significance is indicated as **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Day	**Larval measurements across non-fed group**							
	Body width	**Body height**	**Average post-oral arm length**	**Length between post-oral arms**	**Stomach area**	**Mouth area**	**Average pre-oral arm length**	**Length between pre-oral arms**
2 (n = 12)	F = 2.69	F = 14.03**	H = 7.65*	F = 7.17	F = 1.9	-	-	-
3 (n = 37)	F = 7.65**	F = 12.53***	F = 7.46***	H = 11.72**	F = 6.55**	F = 4.46*	F = 1.08	F = 0.98
4 (n = 36)	F = 2.31	F = 7.69***	F = 6.92**	F = 1.01	F = 6.47**	F = 1.69	F = 2.11	F = 0.80
5 (n = 29)	H = 17.25***	F = 4.63**	F = 2.89	F = 4.33*	F = 6.72**	F = 1.28	F = 7.54**	F = 1.12
6 (n = 24)	H = 12.51**	F = 4.61**	H = 13.06**	F = 4.52**	F = 3.00	F = 2.00	F = 1.14	F = 0.09
7 (n = 25)	F = 0.39	F = 6.28**	F = 3.27	F = 4.91**	F = 0.43	F = 0.30	F = 2.07	F = 0.80
8 (n = 14)	F = 1.61	F = 3.37	F = 3.24	F = 0.15	F = 1.93	F = 0.88	H = 2.91	F = 4.52

Table S4.5. Post hoc Tukey's (t statistic) or Dunn's test (z statistic) results showing the pairwise comparisons of statistically significant ANOVA or Kruskal-Wallis results among *Tripneustes gratilla* larvae that were not fed, where statistical significance (P_{Tukey} or P_{Holm}) is indicated as *P < 0.05; **P < 0.01; ***P < 0.001.

Day	Variable	Post-hoc test statistic	Comparisons across non-fed larvae					
			Formulated - Kelp	Formulated - *Ulva*	Formulated - Mixed	Kelp - *Ulva*	Kelp - Mixed	*Ulva* - Mixed
2 (n = 12)	**Body height**	t	-3.30*	2.23	-3.72*	4.57**	-0.42	5.56**
	Average post-oral arm length	z	-1.23	0.94	-1.72	2.04	-0.49	2.48*
3 (n = 37)	**Length between post-oral arms**	z	1.22	2.63*	-0.47	1.38	-1.68	3.11**
	Body width	t	0.62	3.29**	-0.90	2.75	-1.58	4.40***
	Body height	t	-0.11	5.18***	2.57	5.29***	2.68*	2.75*
	Average post-oral arm length	t	1.38	4.60***	1.85	3.22**	0.43	2.87*
	Stomach area	t	0.19	3.81**	0.81	3.74**	0.64	2.98*
	Mouth area	t	0.08	1.14	-3.05*	0.98	-2.77	3.25*
4 (n = 36)	**Body height**	t	-1.93	1.03	-1.34**	2.95*	-1.44	;4.39***
	Average post-oral arm length	t	-1.91	0.12	-3.82**	2.03	-1.91	3.94**
	Stomach area	t	-3.94**	-2.01	-3.59**	1.93	0.35	1.58
5 (n = 29)	**Length between post-oral arms**	z	1.33	0.05	-2.84**	-1.08	-3.83***	2.35*
	Body width	t	1.85	-0.11	-1.89	-1.74	-3.72**	1.53
	Average post-oral arm length	t	0.69	-1.43	-1.73*	-2.00	-3.33**	0.91
	Stomach area	t	-0.73	-1.97	-4.22**	1.21	-3.15*	1.67
	Average pre-oral arm length	t	0.29	1.63	-2.77	1.36	-3.09*	4.31
6 (n = 24)	**Body width**	z	2.46*	0.12	-0.78	-2.54*	-3.43**	0.98
	Body height	t	1.96	-0.30	-1.58	-2.42	-3.70**	1.40
	Average post-oral arm length	z	1.52	-0.89	-1.86	-2.53*	-3.50**	1.06
	Length between post-oral arms	t	2.41	-0.21	-0.95	-2.81*	-3.55**	0.81
7 (n = 25)	**Body height**	t	2.45	1.90	-1.34	-0.91	-3.71**	3.42**

Table S4.6. Summary of genetic diversity statistics across 10 species-specific microsatellite markers across *Tripneustes gratilla* F1 broodstock and F2 offspring. These statistics include: number of alleles (A_n); effective number of alleles (A_e); Shannon's information index (I), observed heterozygosity (H_o); unbiased expected heterozygosity (uH_e); fixation index (F), null allele frequencies ($Fr_{(Null)}$), polymorphic information content (PIC), inclusion (PI) and exclusion probabilities (PE), as well as standard errors for mean estimates. An asterisk (*) indicates departure from Hardy-Weinberg equilibrium ($P < 0.001$).

Cohort	Locus	A_n	A_e	I	H_o	uH_e	F	$Fr_{(Null)}$	PIC	PI	PE
F1 Broodstock	*TG01*	5.65	3.21	1.44	0.40	0.71	0.42	0.17	0.66	0.13	0.68
	TG07	4.84	3.28	1.36	0.85	0.72	-0.22	-0.09	0.65	0.14	0.65
	TG66	3.00	2.00	0.87	0.67	0.52	-0.33	-0.11	0.45	0.30	0.41
	TG52	3.85	2.43	1.07	0.23	0.61	0.61	0.23	0.53	0.22	0.50
	TG11	3.96	3.04	1.22	0.79	0.70	-0.17	-0.07	0.62	0.16	0.58
	TG20	4.53	2.28	1.08	0.29	0.58	0.49	0.18	0.51	0.25	0.49
	TG26	9.00	6.91	2.05	0.91	0.90	-0.06	-0.03	0.84	0.04	0.88
	TG61	5.83	4.07	1.57	1.00	0.78	-0.33	-0.14	0.72	0.10	0.73
	TG60	6.87	5.37	1.80	0.86	0.84	-0.05	-0.02	0.79	0.06	0.82
	TG55	5.53	3.63	1.46	0.50	0.75	0.31	0.13	0.68	0.12	0.68
Mean ± SE		**5.31 ± 0.52**	**3.62 ± 0.45**	**1.39 ± 0.11**	**0.65 ± 0.08**	**0.71 ± 0.04**	**0.07 ± 0.11**	**0.03**	**0.64**	**0.15**	**0.64**
F2 Offspring	*TG01*	5.20	4.19	1.57	0.56	0.76	0.27	0.12	0.72	0.10	0.72
	TG07	4.49	3.32	1.35	0.77	0.70	-0.11	-0.04	0.65	0.14	0.64
	TG66	3.29	2.09	0.95	0.50	0.52	0.04	0.01	0.47	0.28	0.45
	TG52	3.90	2.01	1.00	0.55	0.50	-0.08	-0.03	0.47	0.28	0.47
	TG11	3.89	2.95	1.22	0.69	0.66	-0.05	-0.02	0.61	0.17	0.59
	TG20	4.42	3.94	1.44	0.18	0.75	0.76	0.33	0.70	0.11	0.69
	TG26	7.22	5.68	1.96	0.45	0.83	0.46	0.21	0.80	0.05	0.84
	TG61	5.94	5.36	1.76	0.91	0.81	-0.12	-0.05	0.79	0.06	0.81
	TG60	6.89	5.81	1.90	0.67	0.83	0.19	0.08	0.81	0.05	0.84
	TG55	6.61	5.48	1.86	0.78	0.82	0.04	0.02	0.79	0.06	0.82
Mean ± SE		**5.18 ± 0.42**	**4.08 ± 0.44**	**1.50 ± 0.11**	**0.61 ± 0.06**	**0.72 ± 0.04**	**0.14 ± 0.09**	**0.06**	**0.68**	**0.13**	**0.69**
Overall mean ± SE		**5.25 ± 0.33**	**3.85 ± 0.33**	**1.45 ± 0.08**	**0.63 ± 0.05**	**0.72 ± 0.03**	**0.10 ± 0.07**	**0.04**	**0.66**	**0.14**	**0.66**

Table S4.7. Estimated breeding values (EBVS) and standard error (SE) of BLUP predictions (using a univariate model) for *Tripneustes gratilla* broodstock conditioned on kelp and mixed diets, respectively.

Broodstock diet	Broodstock ID	EBV ± SE
Kelp	KS1	-0.011 ± 0.040
	KS2	0.040 ± 0.038
	KS3	0.012 ± 0.046
	KS4	-0.040 ± 0.039
	KD1	-0.008 ± 0.045
	KD2	0.041 ± 0.041
	KD3	-0.034 ± 0.041
	KD4	0.001 ± 0.038
Mixed	MS1	0.002 ± 0.044
	MS2	0.012 ± 0.047
	MS3	0.012 ± 0.046
	MS4	-0.026 ± 0.044
	MD1	-0.013 ± 0.043
	MD2	0.007 ± 0.046
	MD3	0.002 ± 0.044
	MD4	0.003 ± 0.045

Appendix D

Supplementary information for Chapter 5

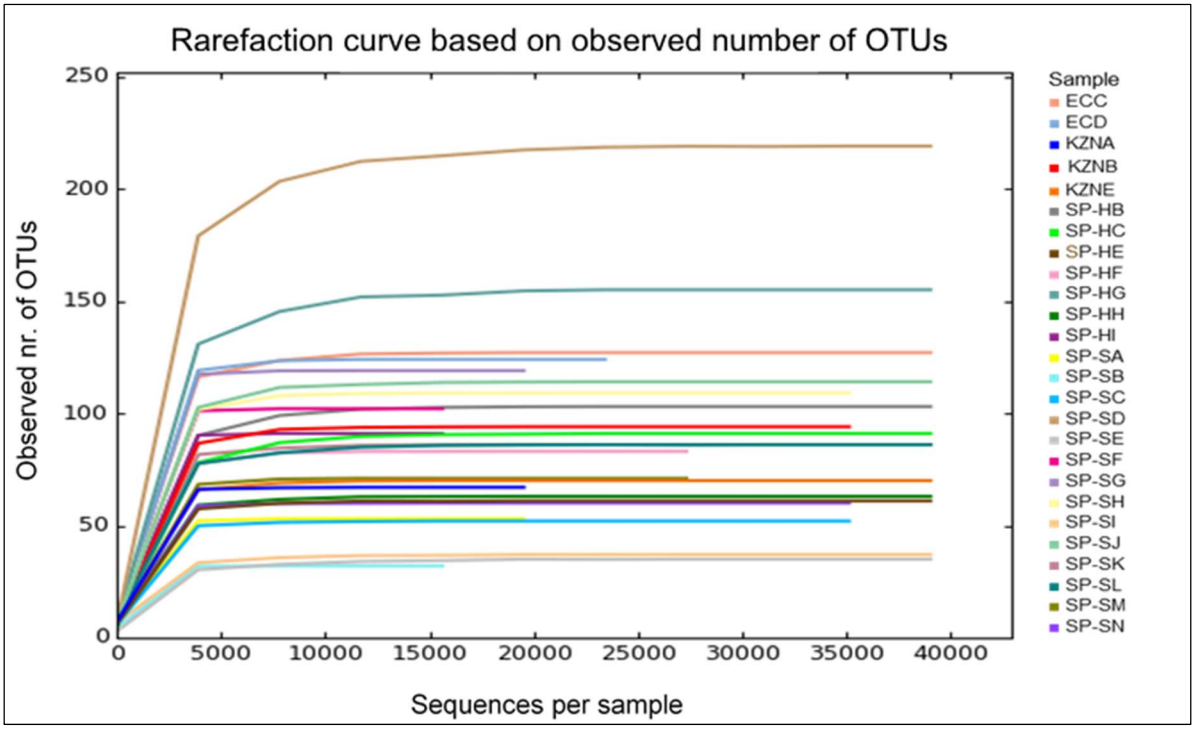

Figure S5.1. Rarefaction curve based on observed number of OTUs showing differences in sequencing depth, where plateaus are reached for each sample.

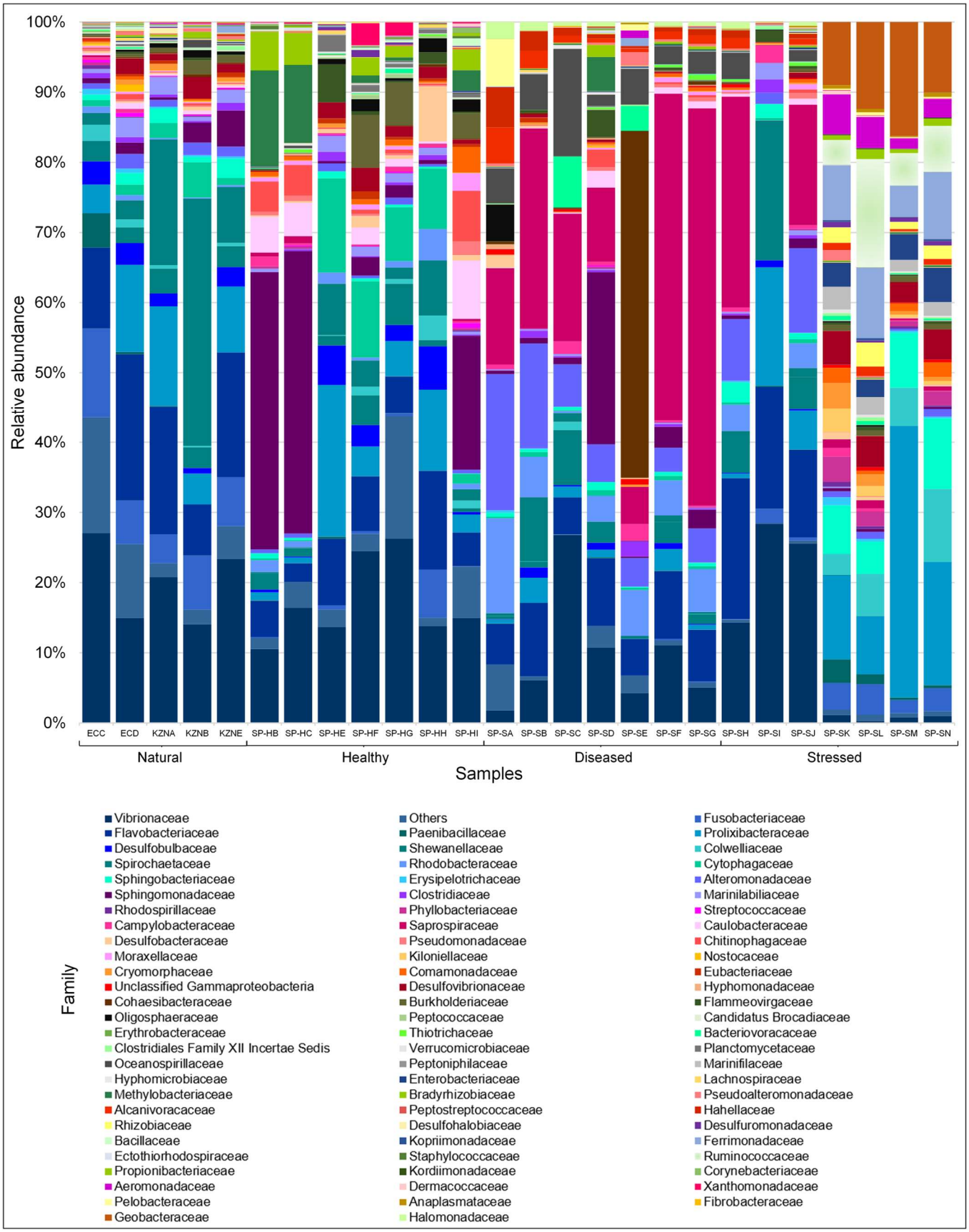

Figure S5.2. Sample-wise relative (%) OTU abundance of *Tripneustes gratilla* body surface bacterial communities at family level.

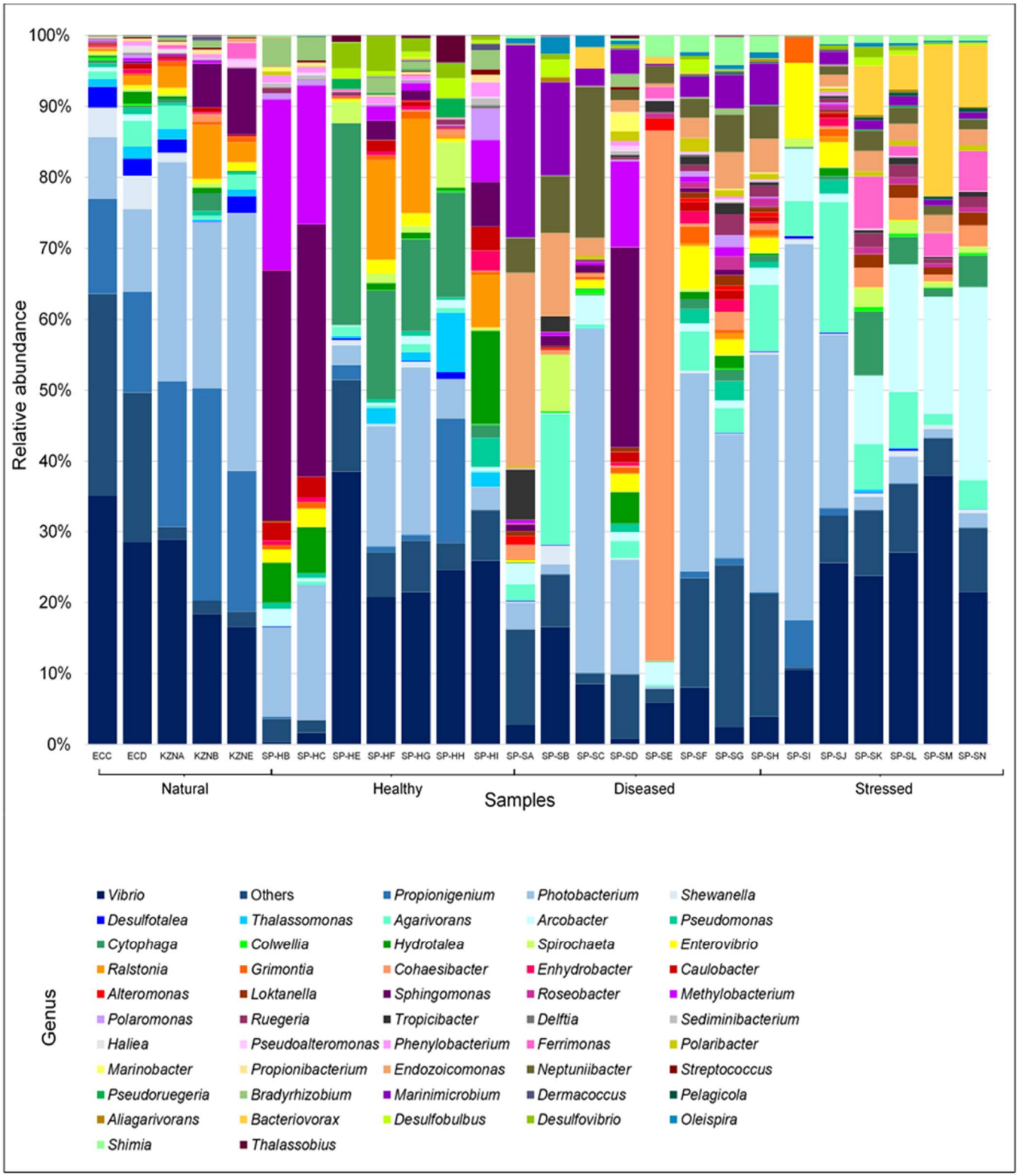

Figure S5.3. Sample-wise relative (%) OTU abundance of *Tripneustes gratilla* body surface bacterial communities at genus level.

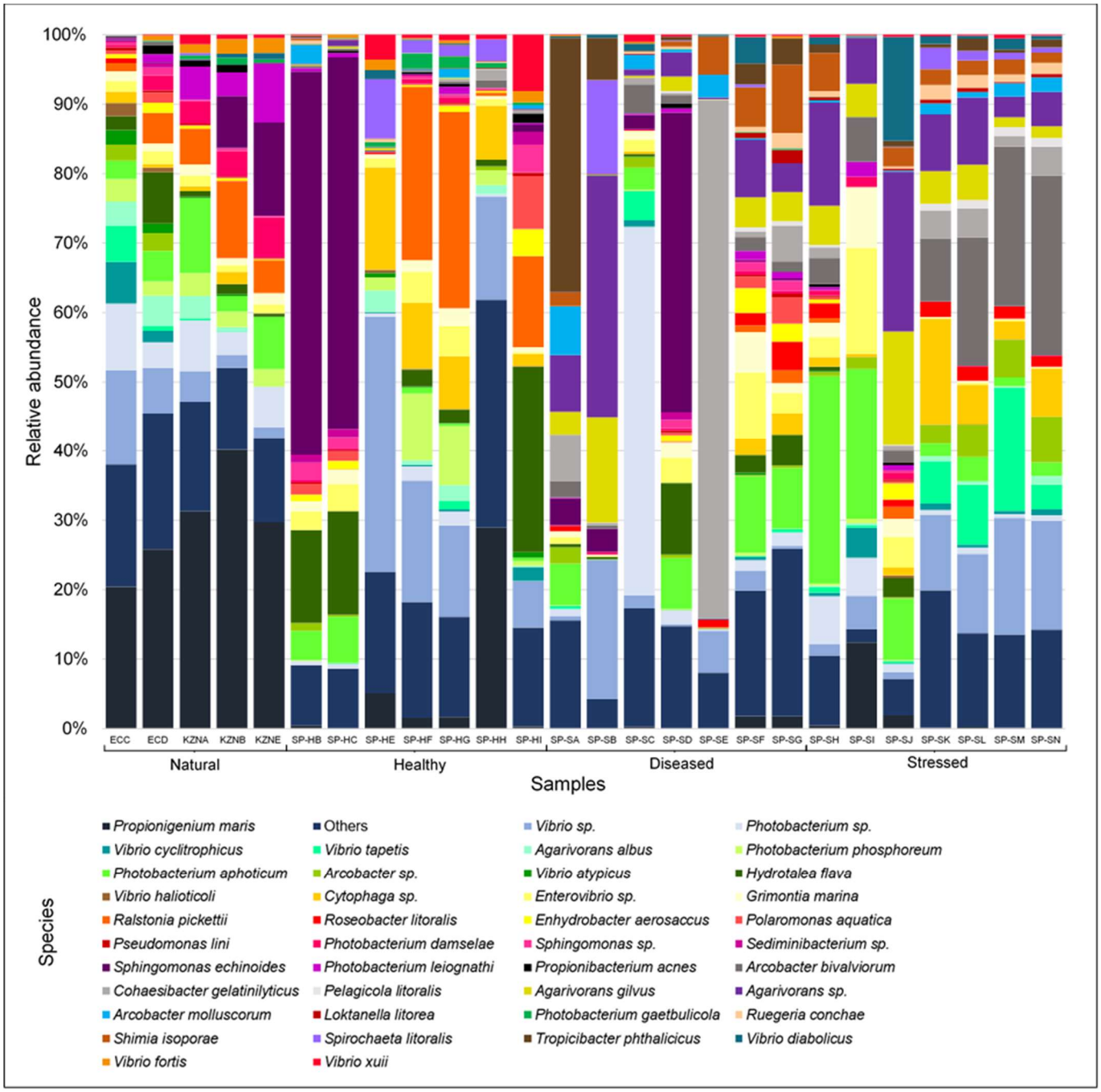

Figure S5.4. Sample-wise relative (%) OTU abundance of *Tripneustes gratilla* body surface bacterial communities at species level.

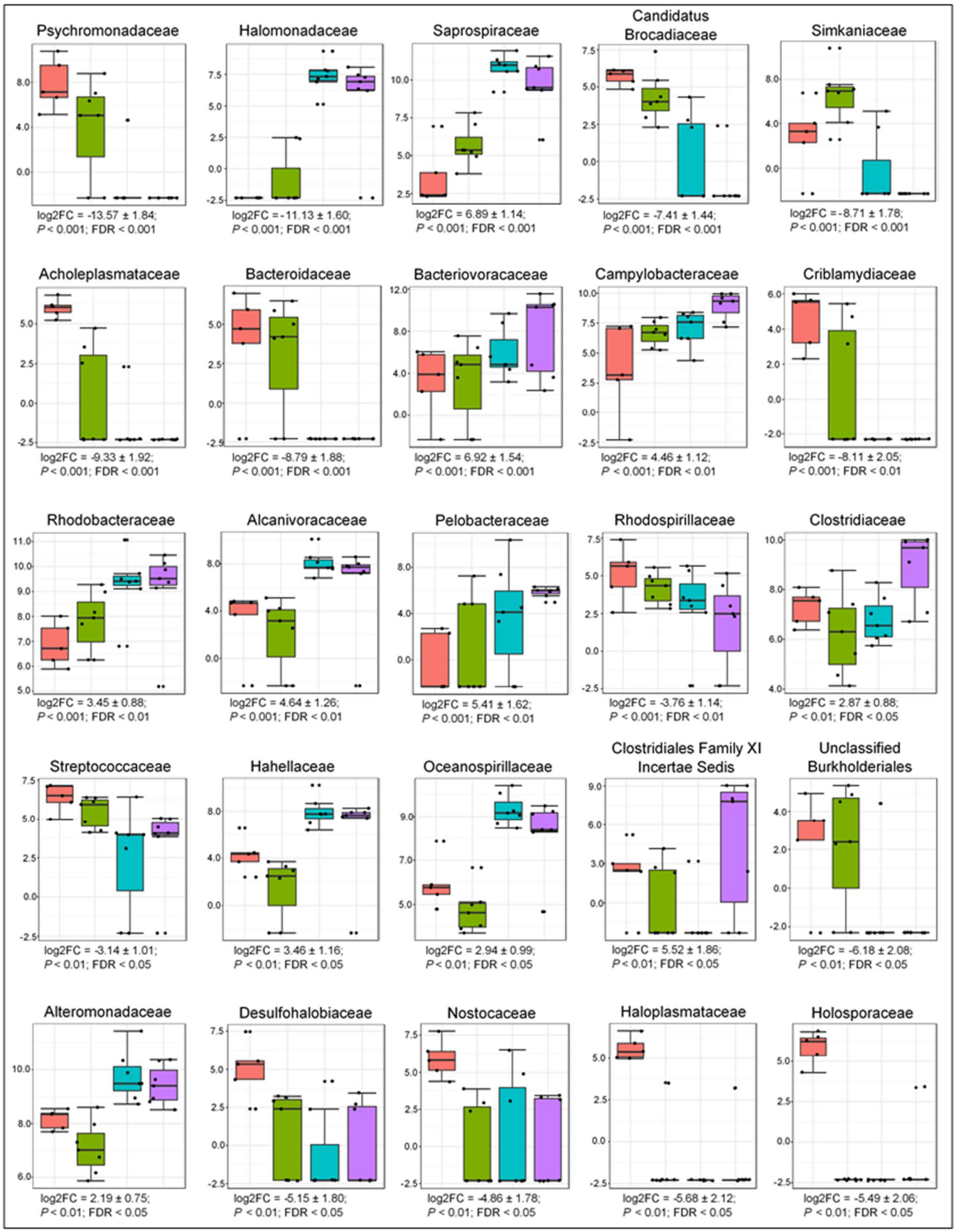

Figure S5.5. Differentially abundant OTUs of *Tripneustes gratilla* body surface bacterial communities at family level, where the log fold change (log2FC), *P*-value and false discovery rate (FDR) is indicated. Cohorts from left to right in each plot: natural, healthy, diseased and stressed.

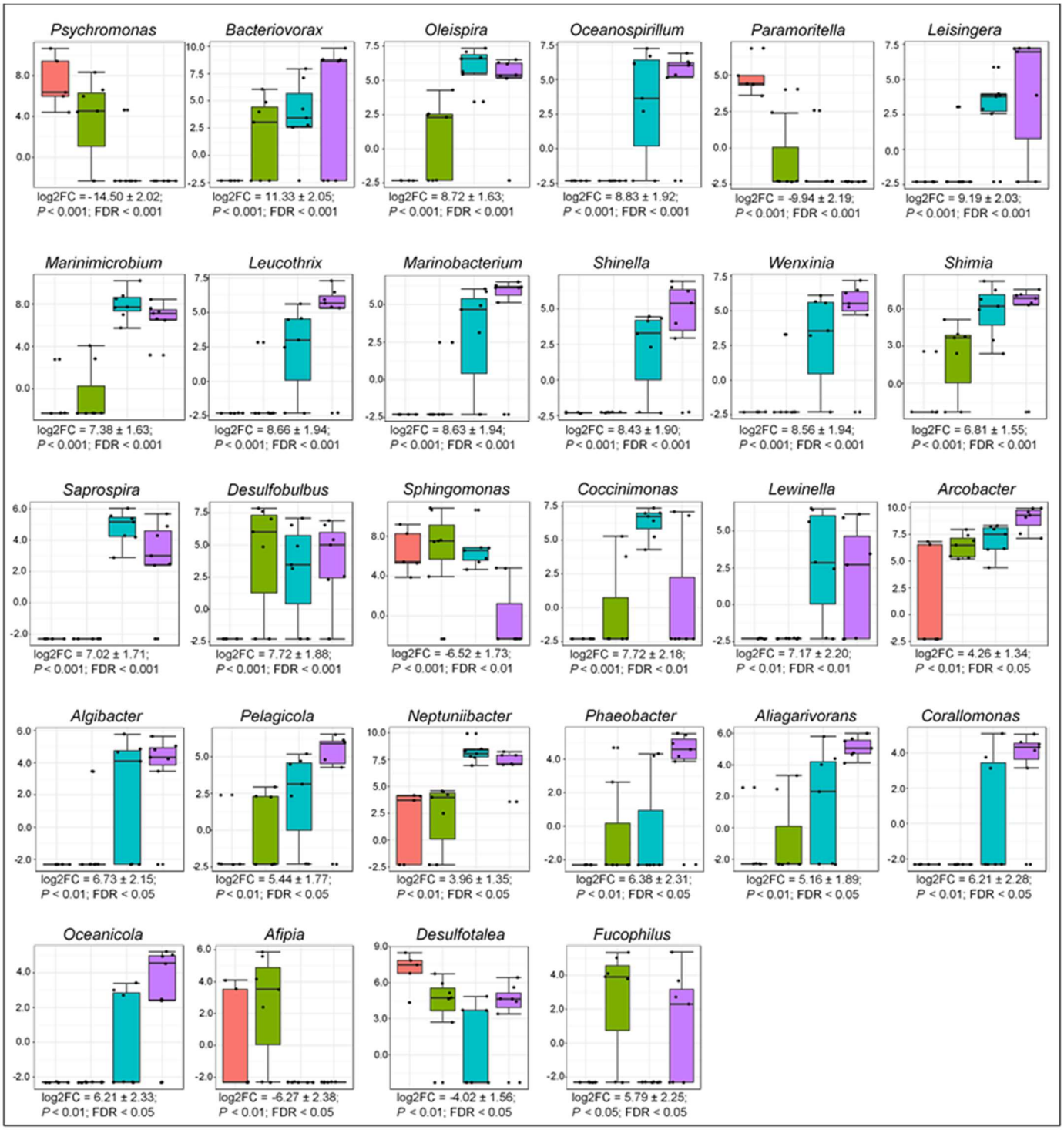

Figure S5.6. Differentially abundant OTUs of *Tripneustes gratilla* body surface bacterial communities at genus level, where the log fold change (log2FC), *P*-value and false discovery rate (FDR) is indicated. Cohorts from left to right in each plot: natural, healthy, diseased and stressed.

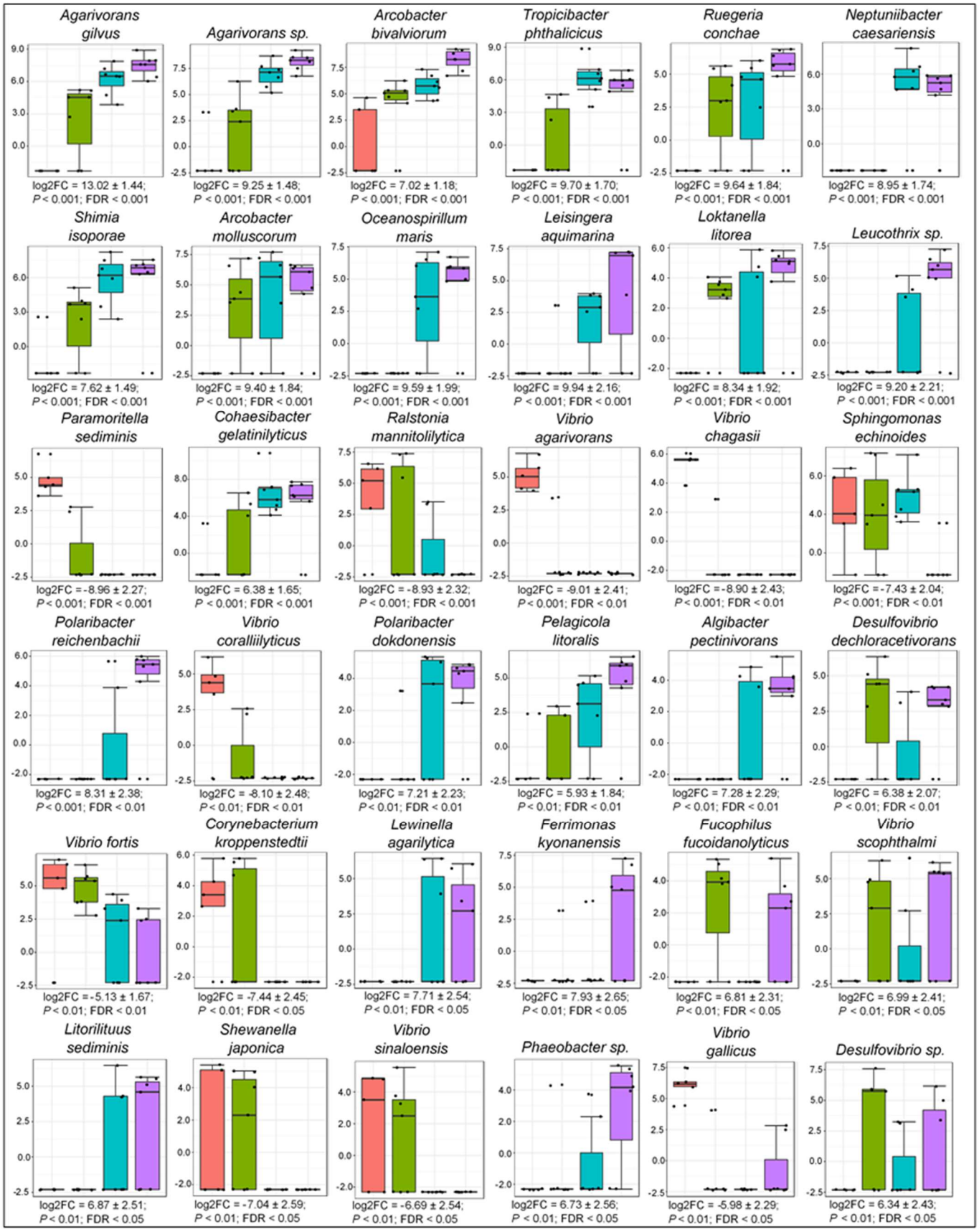

Figure S5.7. Differentially abundant OTUs of *Tripneustes gratilla* body surface bacterial communities at species level, where the log fold change (log2FC), *P*-value and false discovery rate (FDR) is indicated. Cohorts from left to right in each plot: natural, healthy, diseased and stressed.

Table S5.1. Overview of sample-wise alpha diversity statistics, where total number of reads, number of OTU groups, Chao1, Shannon and Simpson indices are indicated on family-, genus- and species level.

			Family level				Genus level				Species level			
Cohort	**Sample ID**	**No. of reads**	**No. of OTUs**	**Chao1**	**Shannon**	**Simpson**	**No. of OTUs**	**Chao1**	**Shannon**	**Simpson**	**No. of OTUs**	**Chao1**	**Shannon**	**Simpson**
Natural	ECC	662 154	141.00	141.00	2.66	0.87	72.00	124.00	2.11	0.79	94.00	126.63	3.59	0.95
	ECD	522 374	146.00	146.00	3.10	0.91	53.00	96.00	2.64	0.86	67.00	123.87	3.74	0.95
	KZNA	434 959	110.00	110.00	2.57	0.87	50.00	53.00	1.98	0.78	70.00	66.93	2.92	0.88
	KZNB	449 702	100.00	100.00	2.56	0.84	65.00	72.00	2.26	0.83	127.00	94.13	2.74	0.83
	KZNE	557 922	119.00	119.00	2.78	0.89	92.00	50.00	2.04	0.79	124.00	69.88	2.81	0.88
Mean		525 422.20	123.20	123.20	2.73	0.87	66.40	79.00	2.21	0.81	96.40	96.29	3.16	0.90
Healthy	SP-HB	387 699	93.00	93.00	2.32	0.80	70.00	112.00	2.32	0.81	103.00	102.95	2.23	0.71
	SP-HC	458 039	131.00	131.00	2.34	0.79	50.00	115.00	2.17	0.80	91.00	91.11	2.02	0.70
	SP-HE	863 177	81.00	81.00	2.73	0.89	75.00	49.00	2.06	0.77	61.00	60.85	2.56	0.83
	SP-HF	278 473	90.00	90.00	2.98	0.90	127.00	75.00	2.71	0.89	83.00	83.11	3.00	0.91
	SP-HG	536 367	124.00	124.00	2.85	0.88	49.00	127.00	2.76	0.87	155.00	154.60	3.27	0.91
	SP-HH	416 963	86.00	86.00	2.81	0.91	112.00	65.00	2.56	0.88	63.00	63.16	2.64	0.87
	SP-HI	150 890	98.00	98.00	3.15	0.92	115.00	92.00	3.06	0.91	91.00	91.05	3.19	0.91
Mean		441 658.00	100.43	100.43	2.74	0.87	85.43	90.71	2.52	0.85	92.43	92.40	2.70	0.83
Diseased	SP-SA	728 093	73.00	73.00	2.69	0.90	124.00	70.00	2.33	0.83	53.00	53.06	2.72	0.86
	SP-SB	338 188	54.00	54.00	2.47	0.86	96.00	50.00	2.58	0.89	32.00	32.11	1.95	0.80
	SP-SC	364 936	54.00	54.00	2.32	0.85	22.00	50.00	1.83	0.71	52.00	51.88	2.18	0.72
	SP-SD	642 587	168.00	168.00	3.01	0.90	135.00	196.00	3.25	0.89	219.00	217.89	3.32	0.87
	SP-SE	314 645	53.00	53.00	2.15	0.74	130.00	37.00	1.30	0.46	35.00	34.54	1.23	0.46
	SP-SF	310 067	93.00	93.00	2.21	0.75	122.00	117.00	3.35	0.91	102.00	102.17	3.67	0.96
	SP-SG	429 570	113.00	113.00	1.98	0.66	37.00	130.00	3.73	0.95	119.00	119.03	3.90	0.97
Mean		446 869.43	86.86	86.86	2.41	0.81	95.14	92.86	2.62	0.81	87.43	87.24	2.71	0.80
Stressed	SP-SH	567 706	99.00	99.00	2.30	0.83	117.00	122.00	2.93	0.86	109.00	109.08	3.05	0.88
	SP-SI	862 717	53.00	53.00	2.15	0.83	50.00	22.00	1.63	0.68	37.00	36.71	2.50	0.89
	SP-SJ	476 600	116.00	116.00	2.60	0.87	196.00	135.00	2.61	0.84	114.00	113.56	2.96	0.89
	SP-SK	582 481	94.00	94.00	3.38	0.95	61.00	86.00	3.01	0.91	86.00	85.85	3.31	0.94
	SP-SL	607 807	94.00	94.00	3.11	0.93	62.00	77.00	2.86	0.88	86.00	85.98	3.11	0.92
	SP-SM	471 735	75.00	75.00	2.36	0.81	86.00	61.00	2.15	0.78	71.00	71.18	2.81	0.88
	SP-SN	370 060	73.00	73.00	2.92	0.92	77.00	62.00	2.63	0.86	60.00	59.89	2.88	0.89
Mean		562 729.43	86.29	86.29	2.69	0.88	92.71	80.71	2.55	0.83	80.43	80.32	2.95	0.90
Overall mean		494 169.84	99.19	99.19	2.64	0.86	84.92	85.82	2.48	0.82	89.17	89.06	2.88	0.86