INVESTIGATING INCIPIENT SPECIATION IN THE WIDESPREAD FRESHWATER SHRIMP, *PARATYA AUSTRALIENSIS* (KEMP 1917)

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Asymmetrical Hybridisation, Atyidae, Hybridisation, Hybrid Breakdown, Mate Choice, Mate Recognition, Reproductive Barriers, Reproductive Isolation, *Paratya australiensis*, Prezygotic Barriers, Speciation, Species Concepts, Shrimp, Species, Sympatric Speciation.

Abstract

The current definition of a species is quite ambiguous due to long-lasting disagreements over various species concepts (currently over 26 concepts) within the scientific community. This ambiguity can have a significant impact on the management of populations in a conservation and/or policy development framework. To provide greater clarity to the species definition, a greater understanding of the speciation process is required. Reproductive isolation in the form of: i) sexual isolation – pre-mating barriers; ii) post-mating – pre-zygotic barriers or iii) post-zygotic barriers, is a common theme within most species concepts. These traits could provide the key to determining when it is appropriate to separate populations into different species or conversely combine currently recognised taxa into a single species.

Previous reciprocal translocation studies on two highly genetically divergent lineages of the freshwater shrimp, *Paratya australiensis*, in separate, but connected, creek systems in the upper reaches of the Brisbane River in South East Queensland, found a potential reproductive breakdown between the populations in the form of non-random mating and asymmetrical hybridisation. Through controlled mate choice experiments in the lab, we found significant preferences associated with females from one lineage (L4), in that they occasionally produced hybrid offspring with males from the second lineage (L6), they preferred to reproduce with males of the same lineage. Whereas the females from lineage 6 only reproduced with males from their own lineage. This non-random mating and asymmetrical hybridisation indicates that pre-zygotic reproductive barriers exist between the populations, consistent with the idea that this species has progressed considerably along the speciation continuum. Based on numerous species concepts discussed in greater detail here, these lineages appear to meet criteria for species delimitation. The data generated here coupled with the findings of prior studies show a strong potential for genetic diversity loss in the event of relocation suggesting that a re-classification of the species may be beneficial for the ongoing maintenance of populations throughout its range.

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List of Abbreviations

BC	Branch Creek Translocation Site
BSC	Biological Species Concept
COI	Cytochrome C Oxidase Subunit I
DO	Dissolved Oxygen
dPSC	Diagnosability Phylogenetic Species Concept
EC	Electrical Conductivity
EcSC	Ecological Species Concept
ESC	Evolutionary Species Concept
GSC	Genic Species Concept
HiDi	Highly Deionised Formamide
HMDS	Hexamethyldisilisane
KC	Kilcoy Creek Collection Site
L4	Lineage 4
L6	Lineage 6
mPSC	Monophylic Phylogenetic Species Concept
mtDNA	Mitochondrial DNA
PCR	Polymerase Chain Reaction
PhSC	Phenetic Species Concept
PSCs	Phylogenetic Species Concept
QUT	Queensland University of Technology
RC	Rum Crossing Translocation Site
RO	Reverse Osmosis
RSC	Recognition Species Concept
SC	Stony Creek Collection Site
SFP	Seminal Fluid Protein

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: QUT Verified Signature

Date: 11 March 2021

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Chapter 1: Introduction

1.1   BACKGROUND

Paratya australiensis, a widespread freshwater Atyid shrimp species, inhabits freshwater stream systems throughout eastern Australia (Cook, et al., 2006). First described by Kemp in 1917, this species has been subject to a number of taxonomic reviews based on morphology with Riek (1953) recognising five distinct taxa before Williams and Smith (1979) reviewed the paratypes previously described by Riek (1953) and concluded that there was no significant variation in diagnostic characters. Based on this review, Williams and Smith (1979) re-synonymised all species and subspecies back to the single species known today as *P. australiensis*. Over the past three decades however, several molecular studies have brought the current taxonomic classification back into question with mitochondrial DNA (mtDNA) work performed by Baker et al. (2004) and Cook et al. (2006) identifying nine equally divergent lineages (see Figure 1).

Twenty five years ago, Hughes et al. (1995) used allozyme electrophoresis to assess the genetic population structure of *P. australiensis* in the Conondale Ranges in southeast Queensland, in order to test the relevance of the Stream Hierarchy Model of gene flow (*sensu* Meffe & Vrijenhoek, 1988). Results showed significant genetic differentiation at seven allozyme loci with a greater level of differentiation occurring between subcatchments compared with among catchments, indicating a relatively poor fit to the Stream Hierarchy Model. In a subsequent study, Hancock and Hughes (1999) used these genetic differences (fixed allelic differences at three loci) as markers to measure instream dispersal, reciprocally translocating 10,000 individuals among two headwater locations within the Brisbane River (see Figure 6). Results of subsequent sampling over the following seven years indicated that minimal movement occurred with small numbers of juveniles moving downstream (possibly passive drift) and adults tending towards an upstream movement (positive rheotactic behaviour).

Hurwood et al. (2003) performed further allozyme and mtDNA analyses and found that the two populations used by Hancock and Hughes (1999) formed two reciprocally monophyletic clades that were highly divergent (~6%), suggesting that populations had been isolated from each other for approximately two to three million years based on the COI molecular clock calibrated for Caridean shrimp by Shank, et al. (1999). Fawcett et al. (2010) assessed the

population structure at the Branch Creek translocation site between 2001 and 2002 and found the translocated mtDNA haplotype not only displaced the resident type at the translocation site but also rapidly displaced the resident haplotype in all the sampled sites upstream. The comparison between adult and juvenile populations across years showed the high reproductive success in the translocated genotype coupled with asymmetrical hybridisation between the translocated and resident populations, producing genotypes with reduced fitness for the sites above the original translocation site. Despite reproductive success, the F1 hybrid offspring showed relatively poor adaptation to the local environment resulting in lower recruitment to the subsequent adult population compared to offspring from residents mating among themselves.
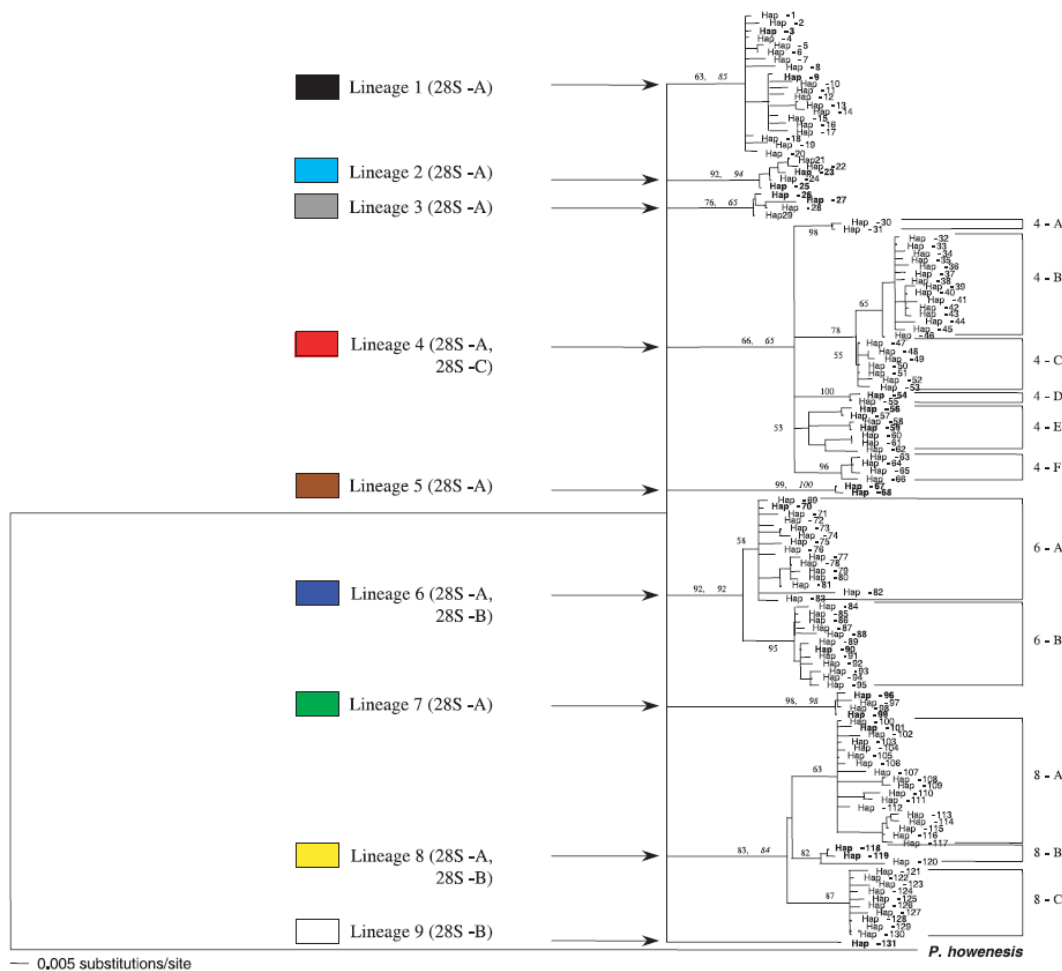
Figure 1: Neighbour-joining (NJ) COI mtDNA gene tree for *Paratya australiensis* including haplotypes and bootstrap values showing 9 equally divergent lineages (Cook, et al., 2006).

Under several species' definitions (e.g. Biological Species Concept (Dobzhansky, 1937; Mayr, 1942), Recognition Species Concept (Paterson, 1985), Evolutionary Species Concept (Simpson, 1961; Wiley, 1978)), non-random mating and poor hybrid fitness seen here are

strong indicators that these two populations of *P. australiensis* do not represent a single 'good' species.

1.2 AIMS

The primary aim of this project was to investigate the nature of the non-random mating between the two divergent lineages identified by Hurwood et al. (2003) under controlled laboratory conditions in order to understand the extent and nature of the breakdown in the reproductive process in *P. australiensis*. A review of the results of mate choice experiments interpreted in the context of various species concepts to assess the suitability of the current synonymised classification for this species is presented here.

Chapter 2: Literature Review

2.1 INTRODUCTION

Ultimately, most species are good (i.e. a house mouse (*Mus musculus*) is genetically, behaviourally, morphologically, and reproductively distinct from an African bush elephant (*Loxodonta Africana*)). In many cases however, the line between species is less well defined. Ring species represent a classic example. Subspecies of *Ensatina* salamanders represent a model "ring species" where up to seven distinct subspecies surround a peninsular style mountain range in western USA (see Figure 2) (Devitt, et al. 2011). While these subspecies vary morphologically throughout their range, each subspecies is connected via gene flow with neighbouring subspecies. However, subspecies at the terminal ends of the expansion do not interbreed making taxonomic assignments difficult (Wake, 1997). In fact, the term "cryptic species" was developed to account for our lack of ability to discern between species easily, which is important not only from a taxonomic and systematics point of view but also for policy making and conservation efforts. Adding to the complexity of species differentiation is that most, if not all populations, both allopatric and sympatric, are in the process of becoming different species (Hey, et al., 2003). Therefore, understanding the processes leading to speciation is critical for accurate species classification.

Speciation often occurs in allopatry where an absence of gene flow because of geographic barriers eventually leads to genetic divergence. Upon secondary contact, reinforcement acts to further separate the two populations genetically through reproductive barriers as specified by the Biological Species Concept (Dobzhansky T. , 1937). Speciation can also occur in sympatry where small genetic mutations form within a population which, through genetic drift and assortative mating, spread throughout the population eventually forming reproductive barriers (Hey, 2006; Butlin, et al., 2012).
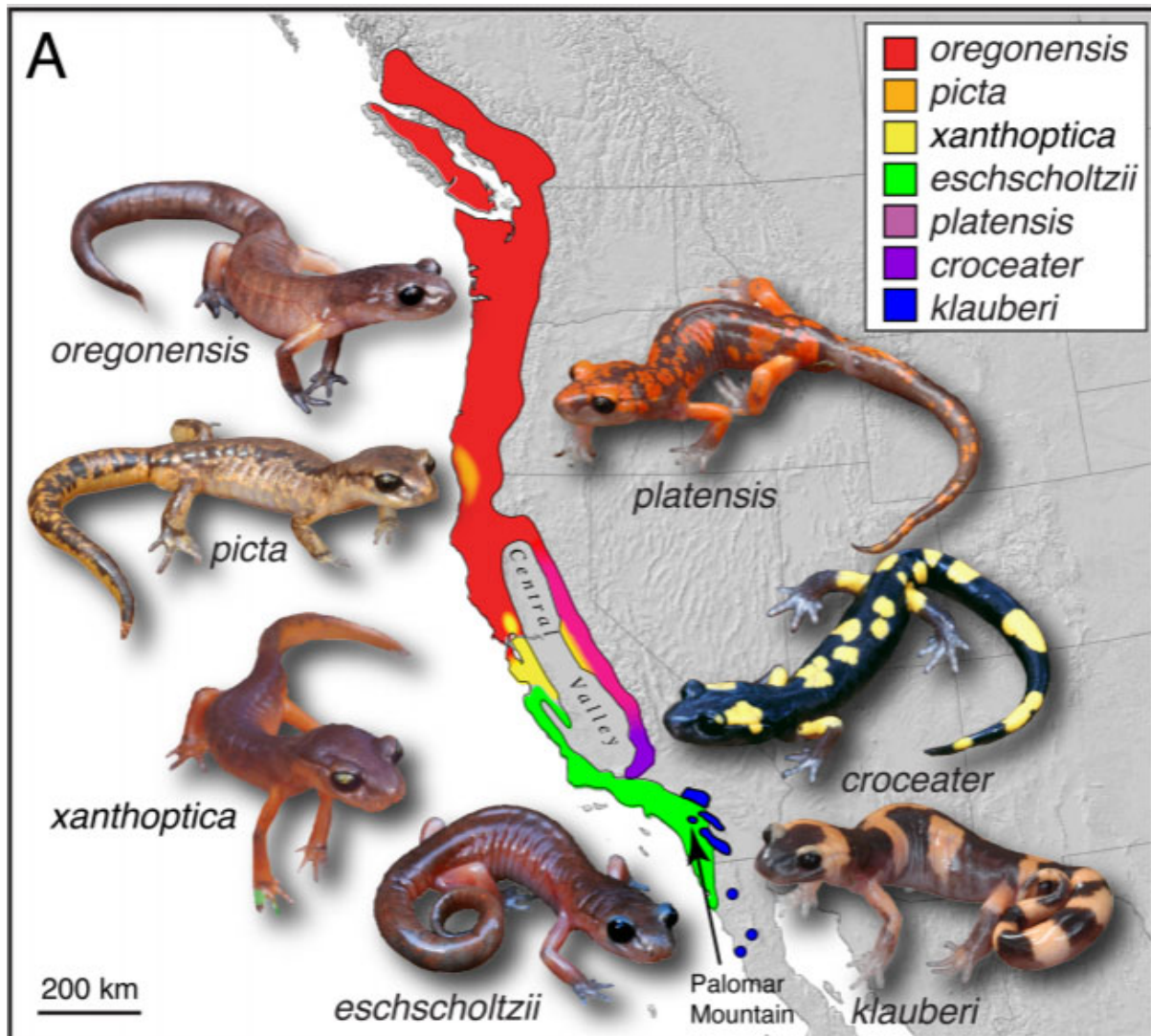
Figure 2: The *Ensatina* complex, showing the distribution of subspecies surrounding a peninsular mountain range in southwest USA (Devitt, et al. 2011). All neighbouring subspecies interbreed except for the subspecies at the terminal ends of the expansion, *E.e.eschscholtzii* and *E.e.klauberi*.

2.2   SPECIES CONCEPTS

Hamilton (2014) explained that the notion of the term "*species*" was born out of a human natural desire to categorise, group and rank things and in doing so allowed for the development of modern phylogenetic systematics which has provided a significant insight into the history and relationships of individuals and populations.

Despite the significant role the term "*species*" has played in our understanding of the natural world, there is still some ambiguity as to what the true definition of a species is (Mayden, 1997; Hausdorf, 2011). Hey et al. (2003), explained that the term "*species*" was commonly used in three different contexts causing what is known as "*the species problem*". They highlight that the term is not only used in relation to a taxonomic rank but also taxon within the rank as well

as a term for an evolving group of organisms and suggest that the ambiguity of the term can cause uncertainty in species assessments that would have significant implications on effective policy and management decision making.

The ability to accurately classify species can impact how we maintain diversity and populations of threatened species (Mayden, 2002). Accurate classification and understanding the relationships between lineages and even between species can assist conservation efforts, for instance, by providing a basis for potential surrogate programs to help strengthen weakened population structures by restoring gene flow (Frankham, et al., 2012; Zachos & Lovari, 2013). In contrast, incorrectly separating two closely related species, that are not intrinsically reproductively isolated, may lead to accelerated extinction by restricting the potential for genetic rescue. Frankham et al. (2012) suggests that species concepts that are too broad in their definitions can lead to outbreeding depression when crossing populations. Concepts that are too specific with the requirements and lead to excessive splitting of species may prevent genetic rescue in small populations where regulatory restrictions prevent interbreeding between species (O'Brien & Mayr, 1991).

Throughout the last century, several species concepts were proposed and debated within the scientific community including, but not limited to, the Biological Species Concept (Dobzhansky, 1937; Mayr, 1942), Recognition Species Concept (Paterson, 1985), Evolutionary Species Concept (Simpson, 1961; Wiley, 1978) and Phenetic Species Concept (Sokal & Crovello, 1970). These concepts mainly focus on defining a species based on reproductive isolation and hybridisation as the drivers for speciation. The criteria for defining a species has been debated strongly and is considered a large grey area in our understanding of the speciation process (De Queiroz & Weins, 2007).

With rapid advances in technology since the turn of the century, the debate over the definition of a species has reignited with several new species concepts being developed including the Genic Species Concept (Wu, 2001) and Speciation with Gene Flow (Hey, 2006). These concepts focus on the isolation of certain "*speciation*" or "*barrier*" genes that enable the speciation process to occur by preventing the flow of genes between diverging lineages. By gaining an understanding of these genes and the role they play in promoting speciation, further clarity over the criteria for defining a species may be achieved.

Frankham et al. (2012) identified at least 26 species concepts in scientific circulation. Brief details on the most recognised species concepts are discussed below.

2.2.1 Biological Species Concept

The Biological Species Concept (BSC) is perhaps the most widely accepted species concept and was first defined by Dobzhansky (1937) as "*species are systems of populations: the gene exchange between these systems is limited or prevented by a reproductive isolating mechanism or perhaps by a combination of several such mechanisms*". Dobzhansky's definition provided that a species could be classified as such if there was a distinctness of individuals of a particular population when compared to another population, interbreeding does or could take place within the population, and the population lacks gene flow from other populations.

The definition was adjusted slightly by Mayr (1942) to "*groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups*".

Both versions identified that speciation is the consequence of developing isolating mechanisms such as physical isolation or reproductive isolation or a combination of both (Dobzhansky & Dobzhansky, 1971; Mayr, 1976), however, BSC adherents are now in general agreement that reproductive isolation occurring in allopatric populations is in fact a result of genetic differentiation (Cracraft, 1994).

Gene flow is not permitted between species under the BSC, which in practical terms can restrict the splitting of well-differentiated species that display limited intrinsic gene flow capabilities (Frankham, et al., 2012, Hausdorf, 2011). For example, blue whales (*Balaenoptera musculus*) and fin whales (*B. physalus*) are considered distinct species, however, hybrid offspring have been encountered in wild populations (Arnason, et al., 1991) and therefore would be considered a single species under a strict interpretation of the BSC. Mallet (2005) estimated that a significant level of hybridisation occurs with at least 25% of plant species and 10% of animal species engaged in various degrees of introgression with other species. The application of the BSC on uniparental organisms is also restricted (Mayden, 1997) despite evidence suggesting these organisms resemble species of biparental organisms (Fontaneto, et al., 2007). Despite these limitations, the BSC is the most widely adopted species concept, largely due to the perceived weaknesses of other concepts (Mayr, 2000) and possibly its intuitive nature to non-evolutionary biologists.

2.2.2 Evolutionary Species Concept

Evolving species do not suddenly become separate species through the immediate ceasing of the ability to produce fertile hybrids (Simpson, 1961). The process is a gradual progression making it difficult to establish a definable point where two speciating populations can be

separated. While Simpson acknowledges that the BSC is, for the most part, a valid concept due to its consistency with evolution, he proposed the Evolutionary Species Concept (ESC) as a theoretical concept to remove its limitations. Simpson states "*An evolutionary species is a lineage (an ancestral-descendant sequence of populations) evolving separately from others and with its own unitary evolutionary role and tendencies*".

Wiley (1978) slightly modifies Simpson's definition of the ESC to state "*a species is a lineage of ancestral descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate*". Wiley explains that the purpose of the modification is to imply that species are thought of as individuals instead of classes that are historical, temporal and spatial entities. This modified concept is based on four key corollaries:

1) "*All organisms, past and present, belong to some evolutionary species*" which implies that organisms cannot be spontaneously generated and must belong to a lineage.

2) "*Separate evolutionary lineages (species) must be reproductively isolated from one another to the extent that this is required for maintaining their separate identities, tendencies, and historical fates*". This corollary supports the BSC but is not as restrictive in relation to gene flow.

3) "*The evolutionary species concept does not demand that there be morphological or phenetic differences between species, nor does it preclude such differences*" which is designed to limit the over- or under-estimation of evolutionary species based on morphological or phenetic differences in response to the Phenetic Species Concept (see below).

4) "*No presumed separate, single, evolutionary lineage may be subdivided into a series of ancestral and descendant species*". This corollary is designed to prevent the separation of species along the sample evolutionary lineage. This practice effectively forms multiple pseudo species throughout key stages of the evolutionary history of a species rather than displaying a single evolving species.

The ESC however, is considered a theoretical concept rather than an operational concept and as a result fails to provide detail for the criteria for differentiation (Mayden, 1997). Mayden (1997) proposes that the ESC is the most consilient with other species concept and is the most suitable primary concept under a hierarchical approach (discussed later in this chapter).

2.2.3 Phenetic Species Concept

Sokal and Crovello (1970) critically evaluated the BSC and mapped out the process of grouping species in detail. They found that behind geographic contiguity, the most important step in assessing differentiation is establishing phenetic similarity. After this process, reproductive isolation is observed which is where, according to Sokal and Crovello (1970), the BSC begins to become ineffective. In order to determine the reproductive behaviour adequately between two populations, a significant portion of the population needs to be tested to remove individual barriers, such as sterility genes, and mating preferences. This is overcome usually through the extrapolation of results from small sub-sample trials. They point out that to extrapolate such results effectively, phenetic assessment is required.

This reliance on phenetic assessment led to Sokal and Crovello (1970) proposing the Phenetic Species Concept (PhSC). Under this concept, phenetic similarities are used to not only narrow down populations (i.e. a field mouse is clearly a different species to a pine tree) but also extrapolate results of studies on interbreeding to decrease the need to assess the ability of every individuals to breed with every other individuals of a population. Due to the difficulty in being able to establish the reproductive behaviour of an entire population, localised biological population samples need to be separated based on phenetic differences and these phenetic differences are then used to infer behaviour across those populations. This concept allows for a more simplified approach for differentiation.

There are limitations to the PhSC acknowledged by Sokal and Crovello (1970) which include polyploidy without phenotypic change, cryptic species and phenetic variation because of environmental factors (e.g. stunted growth as a result of droughts that could also occur as a result of a mutation).

2.2.4 Ecological Species Concept

Following the thought process behind the ESC, Van Valen (1976) argued that species differentiation on the basis of reproductive isolation was invalid and that ecology has a greater bearing on the preservation of a species than reproductive patterns. Van Valen (1976) formed the Ecological Species Concept (EcSC) stating that *"a species is a lineage (or a closely related set of lineages) which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range"*.

Van Valen (1976) highlights that incomplete reproductive isolation provides improved evolutionary adaptation. Such adaptations will either benefit both "*species*", removing the

requirement for separation, or benefit one of the "*species*" effectively keeping the adaptation from the other and providing a basis for differentiation. Reproductive isolation under this concept is considered a product of the speciation process rather than a driver.

Wiley (1978) argues that two species can exist together within the same adaptive zone provided there are sufficient resources to maintain both populations. Where resources are limited, interspecific competition would be the main driver for the extinction of one species as opposed to the swamping of the gene pool. Where this process occurs, the unsuccessful species may not be considered a species under the EcSC.

2.2.5 Phylogenetic Species Concept

The Phylogenetic Species Concepts (PSCs) are separated by two methods of distinction. Both methods take a systematics approach to differentiation and argue that the ability to interbreed is not a criterion for phylogenetic grouping (Mishler & Theriot, 2000) instead apomorphic or diagnosable characters should be used.

Rosen (1978) first proposed the Monophyletic Phylogenetic Species Concept (mPSC) stating "*A species is a unit of taxonomic convenience, and that the population, in the sense of a geographically constrained group of individuals with some unique apomorphous characters, is the unit of evolutionary significance*".

Mishler and Theriot (2000) further simplified the concept by defining a species as "*the least inclusive taxon recognised in a formal phylogenetic classification. As with all hierarchical levels of taxa in such a classification, organisms are grouped into species because of evidence of monophyly.*"

The second method of distinction, proposed by Eldridge and Cracraft (1980), is referred to here as the Diagnosability Phylogenetic Species Concept (dPSC). They state that a species is "*a diagnosable cluster of individuals within which there is a parental pattern of ancestry and descent, beyond which there is not, and which exhibits a pattern of phylogenetic ancestry and descent among units of like kind*". A key feature of this method of distinction is that a phylogenetic analysis is not required for the concept to be applied (Nixon & Wheeler, 1990).

Wheeler and Platnick (2000) modified the concept by stating that a species is "*the smallest aggregation of (sexual) populations or (asexual) lineages diagnosable by a unique combination of character states*". The focus on characteristics, according to Wheeler and Platnick (2000), means that monophyly and apomorphy is irrelevant for species recognition (Zachos, 2016). They further defined the differences between traits and characters with traits

being properties that are variable within a species and characters being properties that are variable between species (Zachos, 2016).

Critics of the PSCs argue that diagnosability and monophyly can occur at every level in the hierarchy of living things and the process of species differentiation based purely on the PSCs could lead to an over statement of species (Hausdorf, 2011; Zachos & Lovari, 2013). Furthermore, species identified under the PSCs may not be reproductively isolated (Frankham, et al., 2012) potentially resulting in two diagnosable species undergoing reverse speciation over time (Zachos & Lovari, 2013).

2.2.6 Recognition Species Concept

In contrast to isolating mechanisms referred to in the BSC, the Recognition Species Concept (RSC) is a function of the presence of a common fertilisation system within populations (Paterson, 1985). According to Paterson (1985), the key isolating factor that distinguishes one species from another is its ability to detect conspecific mates through premating behaviour such as courtship rituals and the use of pheromones. Post mating barriers such as offspring viability does not factor in differentiating species (Coyne, 1993). For speciation to occur under the RSC, the "new species" would develop a different fertilisation system to the existing population. This is observed in allopatric populations where a small subpopulation enters a new environment. The population will either fail to adapt to the new environment and become extinct or it will adapt and grow in population size. During this growth process, a new reproductive system may evolve preventing recombination with the parent colony upon recontact. Paterson (1985) proposed that reproductive isolation was a product of the speciation process, not a driver as suggested in the BSC (Cracraft, 1994).

Critics argue that the RSC is not an alternative to the BSC but instead is considered a narrowed down version, focusing on one isolating factor raised by the BSC (Cracraft, 1994).

2.2.7 Genic Species Concept

Many of the species concepts noted above are based on differentiation on an individual level, effectively comparing the entire genome of an individual to another. Wu (2001) suggests that speciation occurs at a more detailed level insisting that differentiation should be based on single genes or a group of genes called "speciation genes".

The process in which speciation occurs under the Genic Species Concept (GSC) involves a gradual progression from two populations showing a low level of gene differentiation at key loci responsible for functional divergence. As these populations progress through the speciation

process, regardless of being in allopatry or sympatry, additional differentiation occurs on nearby loci causing initial low levels of unviability of F1 offspring. As more loci differentiate, the extent of infertility and unviability increases before both populations demonstrate complete reproductive isolation. Throughout the speciation process gene flow occurs with a decreasing level of success. According to Wu (2001), the point where two species can be separated does not occur at complete reproductive isolation (as is the case under BSC), rather it occurs at the point where a population ceases to lose its divergence regardless of contact with other populations. In other words, differentiation is still present after contact allowing for divergence to continue.

A major criticism of the GSC is the applicability of chromosomal rearrangements in the speciation process (Britton-Davidian, 2001). Wu (2001) allows for gene mutation being the cause for differential adaptation, however, chromosomal changes are considered "*special cases*" in providing reproductive isolation (Hausdorf, 2011).

2.2.8 Speciation with Gene Flow

Generally, the above-mentioned species concepts largely only apply to populations that are living in allopatry usually due to geographic barriers. Under these conditions', speciation can occur relatively unimpeded by gene flow (Hey, 2006). However, in the advent of the genomics era and as genetic technology has improved over the past few decades, researchers are identifying an increasing number of examples where speciation is occurring between sympatric or parapatric populations (Hey, 2006; Nosil, 2008; Papadopulos, et al., 2011). This goes against existing theories where gene flow only works to homogenise populations and restrict divergence (Kopp, et al., 2018). Hey (2006) suggests that the process occurs when a small number of genes begin to diverge within a population while the remaining genes maintain regular levels of gene flow. Over time, additional gene loci begin to diverge and through selection, assortative mating and hybrid back-crossing, levels of gene flow gradually decrease resulting in the development of reproductive isolation.

Another force assisting with the divergence between two sympatric populations could be habitat heterogeneity (Golestani, et al., 2012). Phrased as "*micro*" allopatric barriers, minor biogeographic or geographic barriers, while not adequate to completely prevent gene flow, may provide sufficient selective pressure to facilitate speciation with gene flow.

2.2.9 Modern Approaches to a Universal Concept

One of the concerns with the inability to settle on a universal concept is that frustration will cause biologists to lose interest and apply what they consider to be the "*most favourable*" or "*reasonable*" approach which could lead to inconsistency across the biological community (Mayden, 1997; Mayden, 1999). Mayden (1997) proposes the implementation of a hierarchical system where a non-operational concept that is most consilient with the other species concepts, in this case the EcSC, is considered the primary concept guiding the theory of diversity. The primary theory is then supported by complementary secondary concepts that provide operational guidelines for the differentiation of species depending on the nature of the taxa. For example, uniparental taxa are not factored in the BSC so the BSC would not be considered an appropriate secondary concept for assessing uniparental taxa.

De Quieroz and Weins (2007) raises a similar unifying system based on his assessment that all species concepts share a common element in that species are considered "*separately evolving metapopulation lineages*" and all other properties raised by each individual species concept acts as a method for describing how far the species has progressed through the speciation process (see Figure 3). For example, a species under the BSC would be considered a reproductively isolated species or an ecologically differentiated species under the EcSC. Under this unifying system properties from all species concepts are relevant in species differentiation.
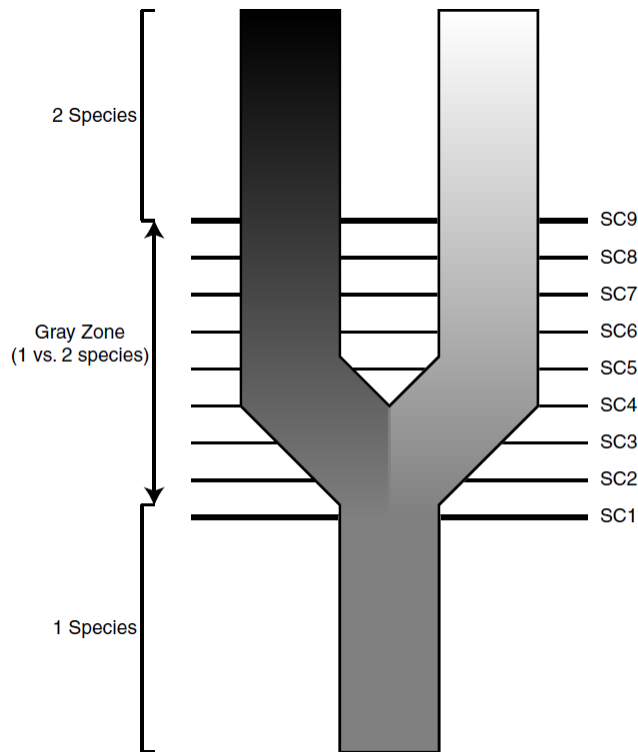
Figure 3: A simplified diagram illustrating where various theoretical species concepts are applied throughout the speciation process (De Queiroz & Weins, 2007)

Both the hierarchical and unifying approaches discussed above emphasise the importance of understanding the process of speciation when attempting to differentiate closely related species. Reproductive isolation appears to be a key factor in many of the above-mentioned concepts. As technology advances and becomes more affordable, our ability to gather information on gene flow, hybridisation, reinforcement, mate choice and other reproductive barriers are greatly enhanced, improving our knowledge of the speciation process.

2.3   REPRODUCTIVE ISOLATION

Reproductive isolation can occur at three main stages of the reproductive process. The earliest stage is referred to as sexual isolation or premating isolation. This occurs prior to copulation and barriers usually form as a result of a lack of mate recognition (*sensu* RSC). Post mating – prezygotic barriers occur in reproductive systems where the individuals recognise each other as reproductive mates, however barriers prevent gamete transfer. The final stage where reproductive isolation occurs is in the post-zygotic stage and usually occurs after fertilisation in the form of inviable offspring or infertile F1 hybrids.

2.3.1 Sexual Isolation – Pre-Mating Barriers

Sexual or behavioural isolation tends to occur prior to other forms of reproductive isolation such as hybrid inviability (Mendelson, 2003) and could be considered an early indicator of speciation. Mendelson (2003) assessed the rates of evolution based on genetic differences and magnitude of reproductive isolation between various species of freshwater darter fish from the genus *Etheostoma*, and found that sexual isolation appeared to occur before other reproductive barriers, particularly in sympatric species with high levels of sexual dimorphism.

Mate choice experiments have been used to identify behavioural isolation between lineages of numerous species. Bay et al. (2017) performed mate choice experiments between two morphologically divergent lineages of stickleback that had adapted to either benthic or limnetic habitats. The results showed that females would select males with similar body shapes to their own and rarely selecting mates from alternative habitats. To further test this, hybrids of the two lineages were bred to observe the behaviour of the F2 hybrids and their mate choice towards each parent's lineage. The results showed non-random mating continued to occur, with F2 females again selecting mates with similar body shapes to their own. Because this positive assortative mating is maintained across generations, there is support for the idea of speciation genes.

In reproductive systems where phenotypic traits are the basis for choice, a single attribute such as the male call of the green-eyed tree frog (*Litoria genimaculata)* (Hoskin, et al., 2005) could be the main driver for choice. Alternatively, a combination of multiple attributes may be responsible for producing a pre-mating barrier. Vortman et al. (2013) manipulated two phenotypic traits, tail elongation and plumage colour, on a population of barn swallows (*Hirundo rustica transitive*) that appeared to be sexually isolated from another nearby population. These two traits were identified as being the key phenotypic differences between the populations. Vortman et al. (2013) found that when one of the traits was manipulated to mimic the neighbouring population's morphology, resident females still recognised the resident male. However, when manipulations were made on both traits together, the paternity rates of the manipulated males decreased significantly indicating that a combination of multiple traits, rather than single traits alone, could form a reproductive barrier.

Mate searching in crustaceans is highly dependent on the detection of chemical cues, often emitted by females after moulting (Diaz & Thiel, 2004). An inability to detect these reproductive chemical cues can result in the development of premating reproductive barriers. Zhang et al. (2009) observed the reproductive behaviour of two closely related *Lysmata* shrimp,

L. boggessi and *L. wurdemanni*, and found that pre-mating barriers as well as post-zygotic barriers existed between the species. The pre-mating barriers identified were the ability for species to detect the reproductive chemical cues of the other species. In heterospecific experiments, *L. wurdemanni* males were able to detect the chemical cues produced by a receptive *L. boggessi* female, however when a male *L. boggessi* was added to the tank, the ability for the male *L. wurdemanni* to detect the *L. boggessi* female appeared suppressed suggesting that the male *L. boggessi* prevented detection. In contrast, *L. boggessi* males did not appear to detect the sexual cues of female *L. wurdemanni* in either heterospecific or mixed experiments indicating a reproductive barrier between *L. boggessi* males and *L. wurdemanni* females. These results also demonstrate that reproductive isolation can occur unidirectionally with the barrier completely preventing gene flow between males of one species and females of the other while the reverse complement shows minimal restrictions.

Pre-mating barriers are not limited to mate choice or detection. Changes in courtship behaviour and male on male competition can also lead to a reduction in the reproductive fitness of a diverging species. Becher and Gumm (2018) conducted mate choice and behavioural experiments on closely related pupfish species. The mate choice experiment found that there was not a preference when it came to female choice over a successful male from either species, however, behavioural studies on the territorial males found that one of the species exuded more effort in interspecific competition than in intraspecific competition in obtaining and maintaining territories without achieving any significantly greater outcome. Becher and Gumm (2018) identified that the male-male competition is the potential reproductive barrier for these species as selection will likely favour the species that expend the lower amount of energy in maintaining a reproductive edge.

2.3.2 Post-Mating – Prezygotic Barriers

For internal fertilisation systems, incompatibility between male ejaculate and the female reproductive tract can cause a reduction in fertilisation success (Garlovsky & Snook, 2018). Mate choice experiments were performed on a species of guppy in Trinidad where multiple lineages have been geographically isolated for ~ two million years and still maintained an ability to breed between those lineages (Magurran, 1998). Although these lineages had the ability to reproduce, non-random mating behaviour occurred suggesting the potential for speciation. Further studies into post-mating - pre-zygote factors in guppies were investigated to see if female ovary fluids provided advantage to native male sperm (Devigili, et al., 2018). Results found that the ovary fluids produced by females from the native lineage were slightly

more viscous than that of the introduced lineage, decreasing the fertilisation success rate of males from the introduced lineage and allowing males of the native lineage the ability to successfully fertilise the eggs. In this instance, although both lineages are capable of successfully breeding, the production of inhibiting ovary fluids is considered to be evidence of speciation in action.

Male production of Seminal Fluid Proteins (SFPs) is common for both internal and external fertilisation systems (Poiani, 2006). SPFs act to increase fertilisation success through providing conditions to facilitate spermatozoa movement, protecting spermatozoa from female immune attack, trigger ovulation etc., as well as influencing sperm competition through the implementation of sperm plugs and allospermicidal properties (Sirot, et al., 2014). Garlovsky et al. (2020) compared the SPFs composition of several closely related *Drosophila* species and found 150 differentially abundant proteins across the populations, thought to be responsible for a reduction in fertilisation rates when crossing species. Variation in the composition of SPFs can lead to reproductive breakdown between populations by not only reducing fecundity but also through reducing the coordination of reproductive tract interactions. An example of this breakdown in coordination was demonstrated when Garlovsky and Snook (2018) found slight variations in the SPFs produced by different populations of the same species of *Drosophila* that led to decreased fertilisation success due to a breakdown in the chemical cues that trigger ovulation.

Many decapod crustaceans have an external fertilisation system. In these systems, SPFs occur in the form of a spermatophore that protects the spermatozoa against environmental conditions as well as acting as an adherent during deposition onto the females thelycum (Farhadi & Harlioglu, 2019). Similar barriers are produced by the female to protect the ovaries upon spawning and to prevent polyspermy (Gallo & Constantini, 2012; Pongtippatee-Taweepreda, et al., 2004). Changes in the chemical compositions of these protective fluids could lead to the development of a fertilisation barrier. For example, in two species of sea urchin, *Arbacia punctulata* and *Strongylocentrotus purpuratus*, the differences in the chemical composition of the protective barrier around both the spermatozoa and ovary prevents interspecies fertilisation as the spermatozoa of one species is unable to bind with the ovary of the other (Glabe & Lennarz, 1979). It has been suggested (Vacquier, et al. 1997) that changes in ovarian protective fluid composition is actively selected for (referred to as sexual antagonism) as a mechanism to avoid polyspermy and influence sperm selection, particularly in broadcast and *in situ*

reproductive systems where the female has a lower degree of mate selection (Johnson, et al., 2020).

2.3.3 Post-Zygotic Barriers

Post-zygotic barriers occur after fertilisation, working to prevent the zygote from developing into a reproductively functional adult (Misamore & Browdy, 1997) and can occur in F1 and F2 generations. Post-zygotic barriers can be separated into three main categories: hybrid inviability, hybrid sterility and hybrid breakdown.

Hybrid inviability occurs after successful fertilisation results in mortality prior to reaching sexual maturity. During the same mate choice experiments used to identify the presence of the pre-mating barriers of two closely related *Lysmata* shrimp described above, Zhang et al. (2009) also identified post-zygotic barriers in the form of hybrid inviability in the same two species. When males of one species were placed in tanks with females of the other species, restricted egg fertilisation occurred. However, in both crosses the fertilised eggs did not develop into fry and were dropped by the female prior to reaching the average embryo development period for non-hybrid embryos.

The second category of post-zygotic barriers is hybrid sterility. This occurs when hybrid offspring mature to reproductive age and demonstrate reduced or complete sterility. Hybrid offspring from various species of laboratory reared freshwater darter fish from the genus *Etheostoma* showed varying levels of sterility (Martin & Mendelson, 2018). Under laboratory conditions, six species pairs were crossed in vitro, and hybrid offspring raised to sexual maturity. Various in vitro hybrid backcross combinations were examined, and in most crosses, high levels of male sterility were observed while female sterility was relatively low. In addition, hybrids produced from pairs with increased genetic divergence showed increased levels of sterility.

The third category of post-zygotic barriers is hybrid breakdown. This breakdown is the reduction of hybrid fitness over a number of generations. An example exists between two closely related crayfish, *Orconectes rusticus* and *O. propinquus* (Arcella, et al., 2014). F1 hybrids produced between these two species demonstrate increased survivorship and growth rates when compared to parental populations, however backcross hybrids lose their vigour and significantly drop in survivorship as further generations are produced. The loss of fitness in these later generations appeared to be greater when Fn hybrids backcross with one of the parental species ultimately leading to a significant decline in the population of that species.

The above literature review clearly demonstrates the difficulty that biologists and systematists face in defining and applying a universal species concept. This report aimed to understand the extent to which *P. australiensis* has progressed through the speciation process and assess the appropriateness of the current taxonomic classification against the various species concepts.

Chapter 3: Research Design

3.1   TANK SET UP

3.1.1 Experimental Tanks

Four x 20L aquariums were used for mate choice experiments (see Figure 4). Tank equipment included drip filtration including a carbon pad and filter floss, LED lighting, air stone and tubing, gravel substrate and 5cm long sections of PCF piping for shelter. Tanks were established four weeks prior to collecting samples to allow water to cycle and underwater surfaces to be populated with bacteria and algae. Where possible, the water used in the aquariums was collected from an accessible section of Stony Creek in the Bellthorpe National Park (see Appendix B for pilot study to determine the most suitable tank conditions for maintaining and breeding the shrimp). During the later stages of the experiments, access to creek water was restricted due to drought and bushfires. When collecting water was restricted, a combination of conditioned tap water and deionised water was used. Changing the water source had no observable impact on the study when used which may be attributable to the shrimp acclimating to living in tank conditions over time allowing for more resilience when the water source was changed.

Figure 4: Experimental tank set up with environmental enrichment and established algal growth.

3.1.2 Isolated Grow-Out Tanks

An additional 23 x 10L aquariums were established as isolated grow-out tanks for berried females collected from the experimental tank as well as holding tanks for additional individuals from both lineages to replenish experimental tanks over time. These tanks were set up with a sponge filter connected to an air pump for filtration, a section of 10mm gutter mesh and a section of PVC pipe for shelter. LED lights were fitted across the tanks to help promote algal growth which is used to supplement the feeding of the shrimp. As with the experimental tanks, water was collected from an accessible section of Stony Creek and tanks were established at least four weeks prior to introducing any shrimp to allow for the water to cycle and beneficial algae and bacteria to establish. During periods where water could not be collected from Stony Creek, a 50/50 mixture of aged tap water and RO water was used. Seachem Prime water ager was added to the tap water to remove chlorine, ammonia and nitrite and the mix was let to sit with an air stone for agitation for at least 24 hours.

3.1.3 Tank Parameters

Similar parameters for all tanks were established and maintained to avoid added stress upon moving samples between tanks. This also decreased acclimation time enabling earlier breeding to take place. Temperature was controlled using a central air-conditioning unit for the entire room. The initial room temperature was set at 16°C to reflect the water temperature at the collection sites. A couple of weeks after samples were allocated to their respective experimental tanks, the room temperature was gradually increased by 1°C every three to four days to 20°C to reflect summer water temperatures and help induce breeding. Other water parameters were maintained as shown in Table 1.

Table 1: Experimental tank water parameters compared to collection sites.

Parameter	Target Range	Stony Creek Collection Site	Kilcoy Creek Collection Site
Temperature (°C)	16 – 22	21.4 (Summer Temp)	21 (Summer Temp)
pH	6.5 – 7.5	7.8	6.92
Ammonia (ppm)	0	N/a	N/a
Nitrite (ppm)	0	N/a	N/a
Nitrate (ppm)	0 – 25	N/a	N/a
Electrical Conductivity (mS)	100 – 300	212	125.9
Dissolved Oxygen (mg/l)	8 – 9	8.2	N/a

Water tests were performed regularly using API freshwater Ammonia, Nitrite and Nitrate kit and a Multi 3430 multimeter to test pH, EC and DO. Other tank conditions, such as excessive algal growth, were monitored as indictors of the above parameters shifting outside the optimum range. Water changes of between 20% - 40% of the tank volume were conducted when parameters started to shift out of range using water collected from Stony Creek (when possible) or the tap water/deionised water mix.

3.2 SHRIMP COLLECTION AND ACCLIMATION

3.2.1 Collection Sites

Shrimp were collected from the Conondale Ranges, approximately 90km northwest of Brisbane, Queensland (see Figure 5). Collection sites were identified based on the location of pure lineages found in previous studies (see Figure 6) (Hurwood, et al., 2003).
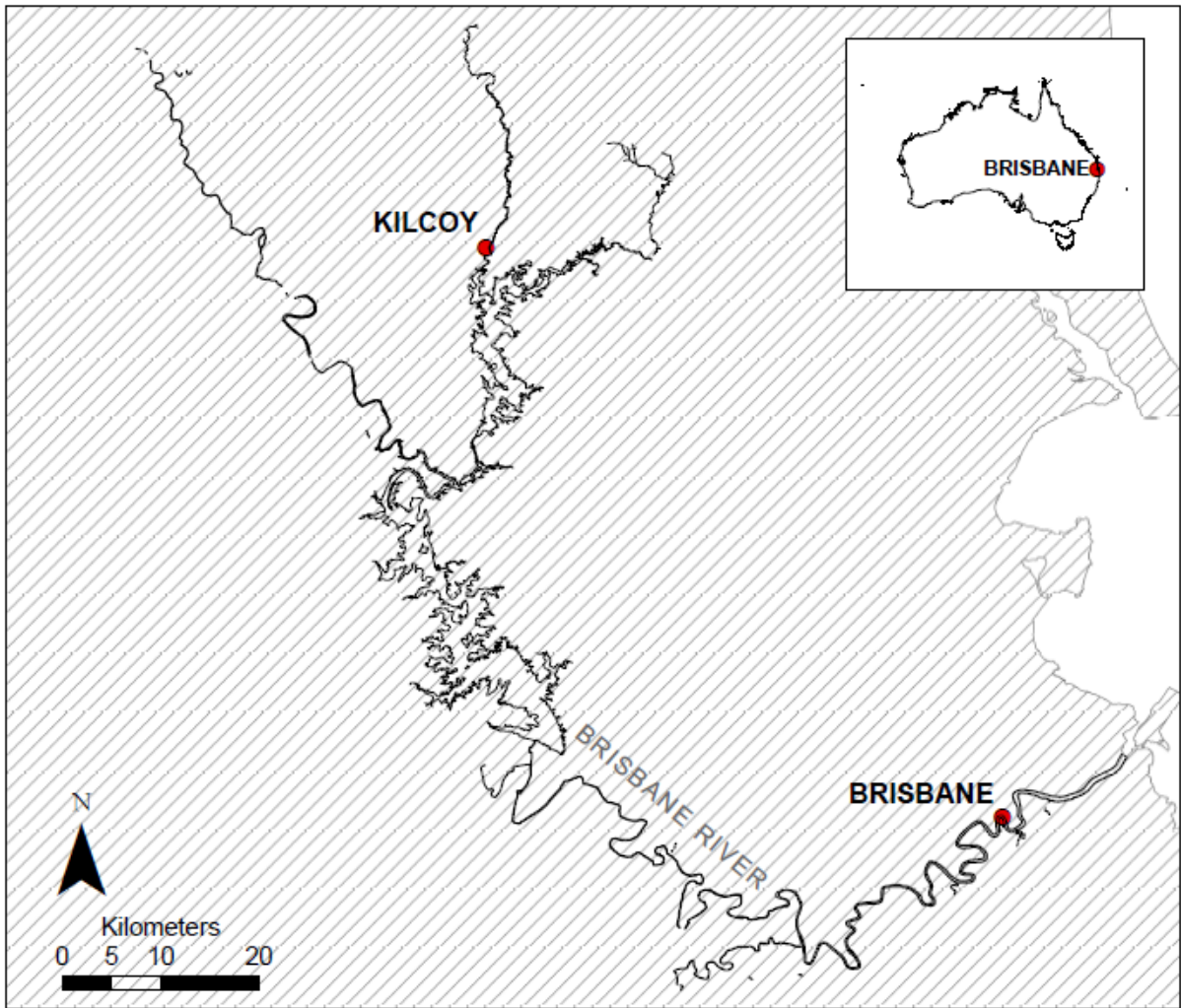
Figure 5: Brisbane River Catchment Area. The shaded grey area indicates land region with the unshaded depicting the ocean.
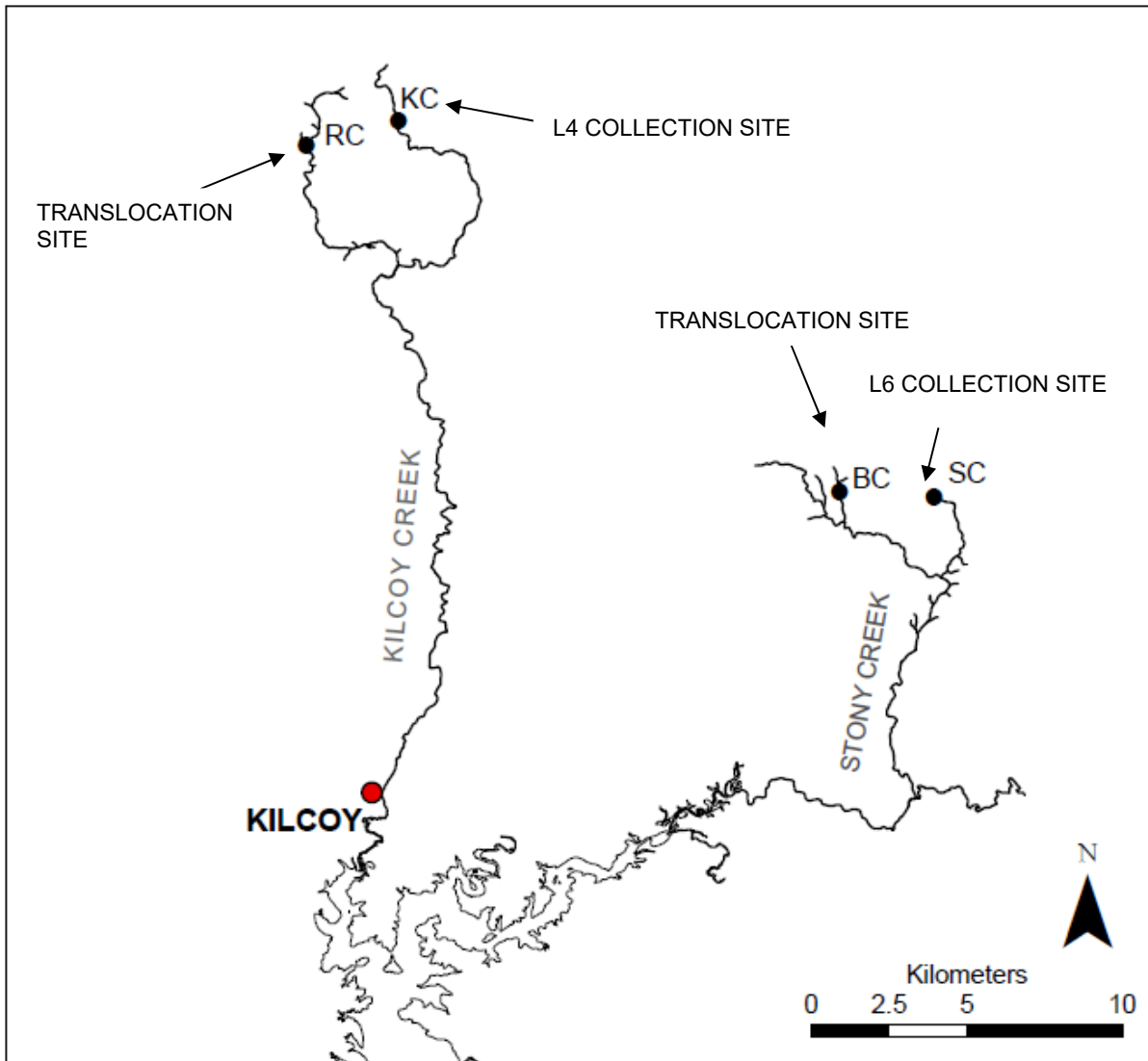
Figure 6: Study area showing four sites. RC – Rum Crossing and BC – Branch Creek represent the two translation sites as per Hughes, et al. (1995). KC – Kilcoy Creek East and SC – Stony Creek represent the collection sites for this study for pure lineages.

Lineage 4 samples (L4, *sensu* Cook, et al., 2006) were collected from Kilcoy Creek East, see Figure 7 and Lineage 6 samples (L6) were collected from Stony Creek, see Figure 8.

Figure 7: Kilcoy Creek East Collection Site

Figure 8: Stony Creek Collection Site

3.2.2 Collection Methods

Prior to collection, pool GPS coordinates and water parameter measurements were taken and recorded using a Garmen eTrex 10 GPS and a Multi 3430 multimeter, respectively. Parameters recorded included pH, temperature, dissolved oxygen, and electrical conductivity. These were measured to provide baseline data with which to set up the tank experiments.

Shrimp were collected using a 2m seine net made from high density shade cloth with a row of heavy chain sewn closely to the bottom edge for anchorage. The net required two people to manoeuvre and was dragged through the pools ensuring the bottom edge of the net was scraping the substrate and the top edge was above the water surface. The movement of the bottom edge of the net along the substrate would prompt the shrimp to flick up into the water column and drift into the centre of the net allowing for effective collection. In most cases, a single pass of the net through the pool would provide 50+ individuals and to avoid excess disturbance to the pools, no more than two passes were conducted in one visit.

After a pass was conducted, the net was placed on the bank of the stream to allow for the samples to be place promptly into an aerated 20L bucket of water containing approximately 10L of water collected from the sampling pool. Any excess shrimp and non-target species collected were returned immediately to the pool at point of capture.

3.2.3 Transport and Acclimation

The samples were transported to the aquaculture facility at QUT's Banyo Pilot Plant Precinct in the buckets containing creek water sampled at point of capture. Buckets were secured in the air-conditioned cab of the vehicle with a TMC battery operated air pump attached the provide oxygenation.

Upon arriving at the aquaculture facility, the samples remained in the buckets for approximately one hour to allow for the bucket water temperature to match the controlled room temperature. During this time small amounts of tank water from the established experimental tanks were gradually added to the bucket to acclimate the shrimp to the tank water conditions. After this time, the shrimp were individually netted using a small aquarium net where they were carefully inspected and sorted into holding tanks. Six holding tanks were established, and shrimp were added based on the observable attributes recorded in Table 2.

Table 2: Sample Holding Tank Allocations

Holding Tank Number	Collection Site	Observable attributes
Holding Tank 1	Stony Creek	Berried Females
Holding Tank 2	Stony Creek	Large un-berried shrimp
Holding Tank 3	Stony Creek	Small to medium sized shrimp
Holding Tank 4	Kilcoy Creek East	Berried Females
Holding Tank 5	Kilcoy Creek East	Large un-berried shrimp
Holding Tank 6	Kilcoy Creek East	Small to medium sized shrimp

The observable attributes used in sorting the shrimp into holding tanks were an attempt to roughly separate the males and females without having to measure each individual which after the collection and transportation process was deemed to be too damaging to the health of the shrimp and may have resulted in unnecessary losses. Instead, the shrimp were monitored closely over the following two-week settling period and moved between tanks if the original assessment was deemed incorrect. The original assessment was based on obvious attributes such as the production of eggs, extended ovaries (as shown in Figure 9) or significant size difference compared with the other shrimp in the tank. Many of the un-berried samples collected were noticeably larger than the remaining samples and were comparable in size with confirmed females (either berried or with extended ovaries). Given the sexual dimorphism in the species (discussed below at 3.3.1), these samples were assessed as being female and placed in the female holding tank. During the two-week settling period, if a female from holding tank 2 or 5 became berried, she was placed into tank 1 or 4 respectively and the smallest shrimp from 2 or 5 was placed in tank 3 or 6 respectively. The assumption was that for a female to become berried in tanks 2 or 5, a male must be present (Bauer & Abdalla, 2001) and removal of the smallest shrimp was an attempt to remove the potential male which are generally smaller than females. By the end of the two-week settling period there were no further berried females appearing in the large un-berried shrimp tanks and throughout the period there were no cases of berried females in either of the small to medium sized shrimp tanks.

3.3   MATE CHOICE EXPERIMENTS

3.3.1 Sexual Dimorphism

Sexual dimorphism is common for Atyidae and can provide an indication of mating behaviour (Christodoulou & Anastasiadou, 2017). Where sexual dimorphism moves beyond differences in reproductive organs, such as colouration or size, inferences can be made of the method in which courtship and mate selection occur. Decapods provide a number of examples where size

and weaponry are indicative of mating behaviour. Bauer et al. (2014) found sexual dimorphism in two *Cinetorhynchus* species of shrimp where the male shrimp are larger than female shrimp and possess enlarged chelae used for mate guarding. In contrast, smaller males could indicate scramble competition where males detect receptive females through random contact (Andersson, 1994). These scramble systems usually occur in very mobile species that have high encounter rates in nature (Bauer & Abdalla, 2001). Populations with smaller males have an advantage of producing sexually mature males earlier than populations with large males (Andersson, 1994). *P. australiensis* have a relatively short lifespan of approximately two years with individuals reaching sexual maturity during the first year (Hancock, 1995). Having a scramble reproductive system will likely maximise the reproductive duration of the shrimp.

Sexual dimorphism within *P. australiensis* is not as obvious as many other decapods. Morphological differences between sexes are subtle except for mature females with fully developed carapace and pleopods suited to brood management (Smith & Williams, 1980). Hancock (1995) collected samples from Stony Creek and Kilcoy Creek over a one to two-year period and found shrimp with a carapace length greater than 6.5mm are likely to be females. However, most confirmed females collected were less than 6.5mm and ranged down to 4mm which overlapped the range of adults that were unable to be sexed in the field (see Table 3). Smith and Williams (1980) recorded statistically significant size differences in other attributes such as antennular peduncle length, however also noted that the range of values overlapped between sexes.
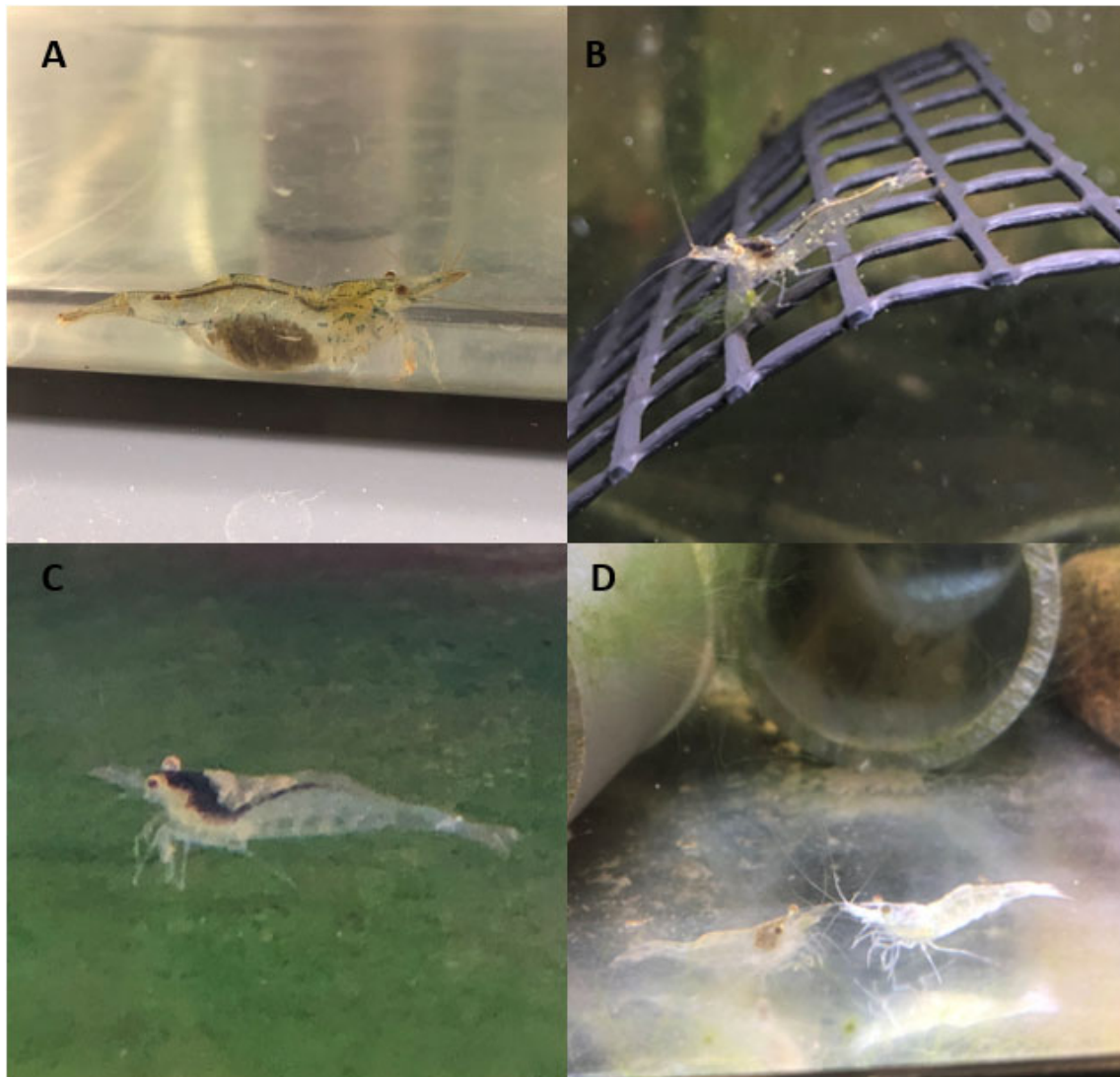
Figure 9: A - Berried female. B - Sub-adult female/ adult male. C - Adult female with extended ovaries. D - Colour variation of two adult shrimp collected from Kilcoy Creek.

Table 3: Summary of findings from population studies performed by Hancock (1995).

Collection Site and Study Year	Max Carapace Length of Berried Female (mm)	Min Carapace Length of Berried Female (mm)	Max Carapace Length of Unsexed Adults (mm)
Stony Creek Year 1	7.5	4.5	6.0
Kilcoy Creek Year 1	7.0	4.5	6.5
Kilcoy Creek Year 2	6.5	4.0	5.5

Other morphological differences such as the presence of the appendix masculina on the second pleopod (see Figure 10) or a pointed sternite between the fifth pereopods can be observed in male *P. australiensis* under microscope (Smith & Williams, 1980; Christodoulou & Anastasiadou, 2017). An inspection of these appendages was undertaken on the collected

shrimp prior to adding to the experimental tanks, however, the marginal differences could not be distinguished on live samples.
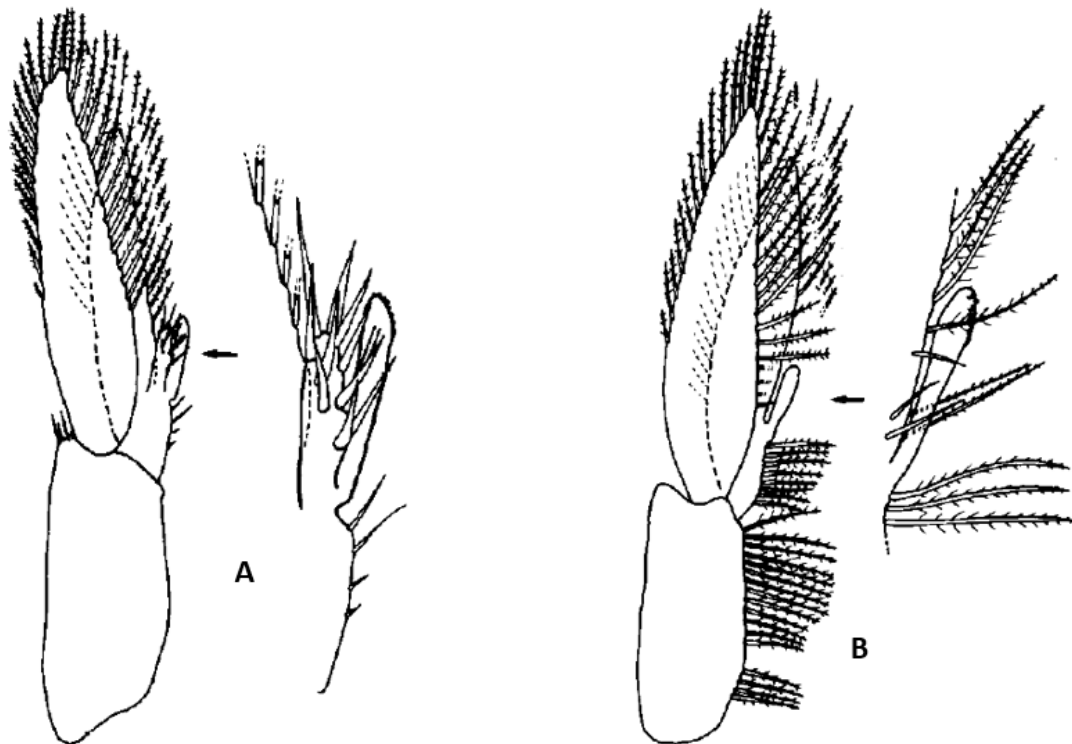
Figure 10: Similarities in morphology on pleopod 2. A – arrow shows the appendix masculina present on male shrimp. B – arrow shows the appendix interna present on female shrimp. (Williams & Smith, 1979)

Pure search mating systems often produce full or partial protandry or hermaphroditic systems (Correa & Thiel, 2003) and which has been observed in other Caridean shrimp including the closely related, *Paratya curvirostris* (Carpenter, 1978) however a life history study conducted by Williams (1977) indicates that this is not the case for *P. australiensis* based on the abundance of male and female samples of the same length.

To overcome the uncertainty in sex determination, experimental tanks contained multiple individuals of each sex. This will be discussed below in the experimental tank composition section.

3.3.2 Reproductive Cycle

P. australiensis appear to have a two year life cycle for both males and females based on studies performed by Williams (1977). Life history studies performed by Hancock (1995) found that although instances of breeding in the first year occasionally occurred, the majority of breeding occurred in a female's second year.

There is minimal information available detailing the reproductive process of *P. australiensis* with more studies having been conducted on other carid decapods. Bauer (1976) observed and recorded the reproductive process for a Caridean species of shrimp, *Heptacarpus pictus*. Bauer (1976) detailed that after moulting, females become receptive for reproduction. Male shrimp identify receptive females by using outstretched antennal flagellum that detect a non-diffusible substance on any part of the female's body. Once a male has detected a responsive female, his behaviour changes abruptly and begins to attempt to grab on to the female using his pereopods and climb onto the dorsal midline of the female. This fortuitously was observed on one occasion during the project (see Figure 11). This step is usually where the female accepts or rejects the male. If accepted, the male will manoeuvre his body to the underside of the female, positioning his abdomen against hers. He then manipulates his thoracoabdominal junction beneath and perpendicular to the female's first abdominal sternite. The male then transfers his spermatophores by beating his pleopods before disengaging the female. The period between spermatophore deposition and the female releasing their eggs for fertilisation varies among Caridean shrimp ranging from immediate release after spermatophore deposition to up to 24 hours (Bauer, 1976; Habashy, 2013; Ganeswaran, 1989).

Figure 11: This image shows a smaller male *P. australiensis* grasping the back of a larger female during the early stages of courtship.

3.3.3 Initial Lineage Confirmation

To test that the sample sites contained pure L4 and L6 shrimp, a sample of six shrimp from each collection site were removed from the collected samples and placed in 90% ethanol for DNA extraction. DNA extraction was conducted using a salt extraction adapted from (Miller, et al., 1988). A small segment of tail tissue was placed in a 1.5mL tube along with 500μL of solution 1 containing 50mM of Tris HCl pH8, 20mM EDTA pH8 and 2% SDS. 5μL of protease K (20mg/mL) was added before being vortexed, centrifuged and incubated at 37°C overnight. The following day, samples were chilled on ice for 10 minutes before adding 250μL of 6M NaCl solution followed by placing the samples on ice for a further five minutes. Samples were placed on a centrifuge and spun at 13,000rpm for 15 minutes before removing 500μL of the clear supernatant and placing into a new 1.5mL centrifuge tube along with 1,000μL of 100% ethanol to precipitate. Samples were placed into a freezer at -20°C for two hours before being spun on a centrifuge for a further 20 minutes at 13,000rpm. The supernatant was removed, and the DNA pellet was rinsed with 500μL of cold 70% ethanol before being spun on a centrifuge at 11,000rpm for five minutes. The supernatant was carefully removed using a pipette and the remaining pellet was dried on a heat block for five minutes at 55°C. The DNA pellet was resuspended in 100μL of TE buffer.

Lineage was confirmed by amplifying the mitochondrial gene, cytochrome c oxidase subunit I (COI) using LCO1490 and HCO2198 primers (Folmer, et al., 1994) and Bioline MyTaq Mix (Meridian Life Science, Inc (Memphis, Tennessee, USA)).

A PCR solution was created with each sample containing 1μL of DNA template, 1μL of COI forward primer (LCO1490), 1μL of COI reverse primer (HCO2198), 12.5μL of Bioline MyTaq Mix and 9.5μL of purified water bringing the total volume per sample to 25μL.

PCR was run on Biorad T100 thermal cycler with the initial denaturation process of 95°C for two minutes followed by 34 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1.5 minutes and a single extension cycle of 72°C for two minutes before holding at 12°C based on the protocol used by Hurwood, et al. (2003).

To determine the success of the PCR, the final PCR products were run on an electrophoresis gel made from 100ml of 1.5% agarose with 7μL of gel red. The samples were loaded into the gel along with a 100bp ladder and run at 100v for 30 minutes. The results showed evidence of non-specific priming in the form of multiple bands forming on the check gels indicating that the primers were ineffective at amplifying the appropriate fragment. A review of the LCO1490

and HCO2198 sites was performed to identify any adjustments that could be made the primers to make them more effective.

The entire *P. australiensis* DNA sequence was extracted from GenBank accession and the COI sites were located based on fragment size and sequence data information provided by Folmer et al. (1994). A direct comparison of each site was made with a number of variances noted for each site. New primers were developed based on the sequences recorded in GenBank as shown in Table 4.

Table 4: COI Primers

Locus	Primer Name	Sequence
COI	LCO1490	5'<GGTCAACAAATCATAAAGATATTGG>3'
COI	HCO2198	5'<TGATTTTTTGGTCACCCTGAAGTTTA>3'
COI	MPaLCO	5'<TGTCAACAAACCACAAGGATATTGG>3'
COI	MPaHCO	5'<AGACTTCTGGGTGTCCGAAAAATCA>3'

The PCR process was run again with the new primers, labelled MPaLCO and MPaHCO, on the Biorad T100 thermal cycler with the initial denaturation process of 94°C for two minutes followed by 34 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 1.5 minutes and a single extension cycle of 15°C for two minutes before holding at 4°C with results indicating sufficient product was created. Samples were then sent to Macrogen Co., Ltd (Seoul, South Korea) for sequencing. Of the 12 samples sent, five were successfully sequenced. These results were loaded into Bioedit Sequence Alignment Editor where they were cleaned up and blasted using the NCBI Standard Nucleotide BLAST database and confirmed against previously contributed sequences submitted by Hurwood, et al. (2003). Further lineage confirmation analysis using the protocols described here was performed on fry and maternal females from successful mate choice experiments, detailed later in the chapter.

3.3.4 Experimental Tank Compositions

Although reasonable confidence was maintained in relation to the ability to accurately separate the different sexes, total confidence of the sex determination process was difficult to achieve on live specimen without causing stress or damage that could ultimately impact breeding. To reduce the impact of potential mis-identification of sexes, tank composition consisted of ten females from a single lineage and either ten males from the other lineage for control experiments or five males from both lineages for mate choice experiments (see Table 5). Under this composition structure, incorrectly sexed females in the mate choice tanks would be identified during the maternal delineation confirmation process. Incorrectly sexed males in

both the mate choice and control tanks would not impact the results unless all males were incorrectly sexed. Having 10 males and 10 females in each tank reduces the likelihood that all males are incorrectly sexed. In addition, two experimental tanks for each mate choice treatment were established.

The control tank was set up to determine if L6 females are capable of breeding with L4 males. Data collected from a translocation event for these two lineages obtained by Hughes et al. (2003) confirmed that L4 females were able to reproduce with L6 males, therefore a control tank was not established for this treatment.

Table 5: Experimental Tank Composition

	Female Lineage	# of Shrimp	Male Lineage	# of Shrimp
Experiment Tank 1 (Stony Creek Control)	L6 - Stony Creek	10	L4 - Kilcoy Creek	10
Experiment Tank 2 (Stony Creek Mate Choice)	L6 - Stony Creek	10	L6 - Stony Creek L4 - Kilcoy Creek	5 5
Experiment Tank 3 (Kilcoy Creek Mate Choice)	L4 - Kilcoy Creek	10	L6 - Stony Creek L4 - Kilcoy Creek	5 5
Experiment Tank 4 (Kilcoy Creek Mate Choice)	L4 - Kilcoy Creek	10	L6 - Stony Creek L4 - Kilcoy Creek	5 5
Experiment Tank 5 (Stony Creek Mate Choice)	L6 - Stony Creek	10	L6 - Stony Creek L4 - Kilcoy Creek	5 5

3.3.5 Berried Females and Collection of Fry/Eggs

Experimental tanks were inspected every two to three days for berried females. A breeding event was recorded when a female from any of the five experimental tanks became berried. Once berried, the female was removed from the experiment tank and placed in isolation (Habashy, 2013). A successful breeding event was recorded when a berried female successfully released fry. Unsuccessful breeding events were recorded when the berried female died or prematurely dropped her eggs. Dropped eggs were not found in any of the unsuccessful breeding events. We expect that they were consumed by the female shortly after releasing them. The females in isolated tanks were inspected and fed regularly until they commenced releasing fry. The process of releasing fry appeared to take several hours and to avoid additional stress on the female, the fry were not collected until after all were released.

Newly released fry appeared planktonic (see Figure 12) which allowed for safe removal using a fine mesh aquarium net. Older fry lost their planktonic behaviour and became more benthic (see Figure 13) which made it more difficult to remove without compromising the structure of the fry.
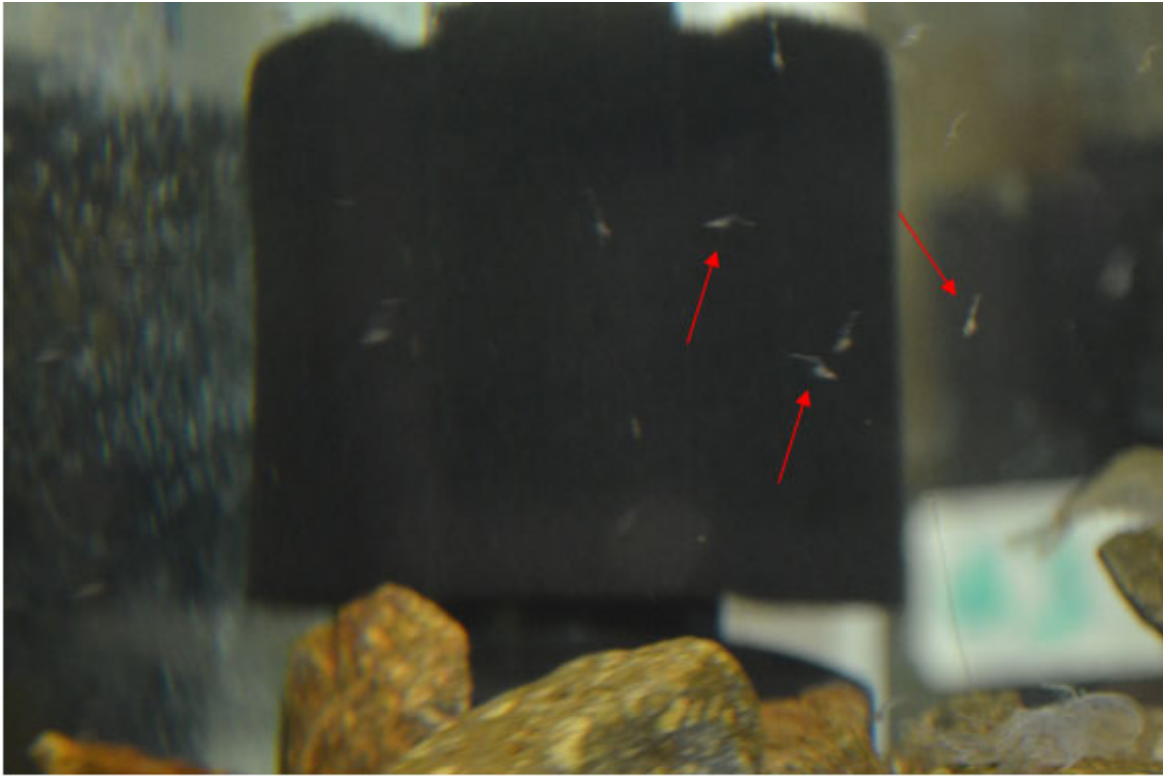
Figure 12: *P. australiensis* fry in planktonic stage
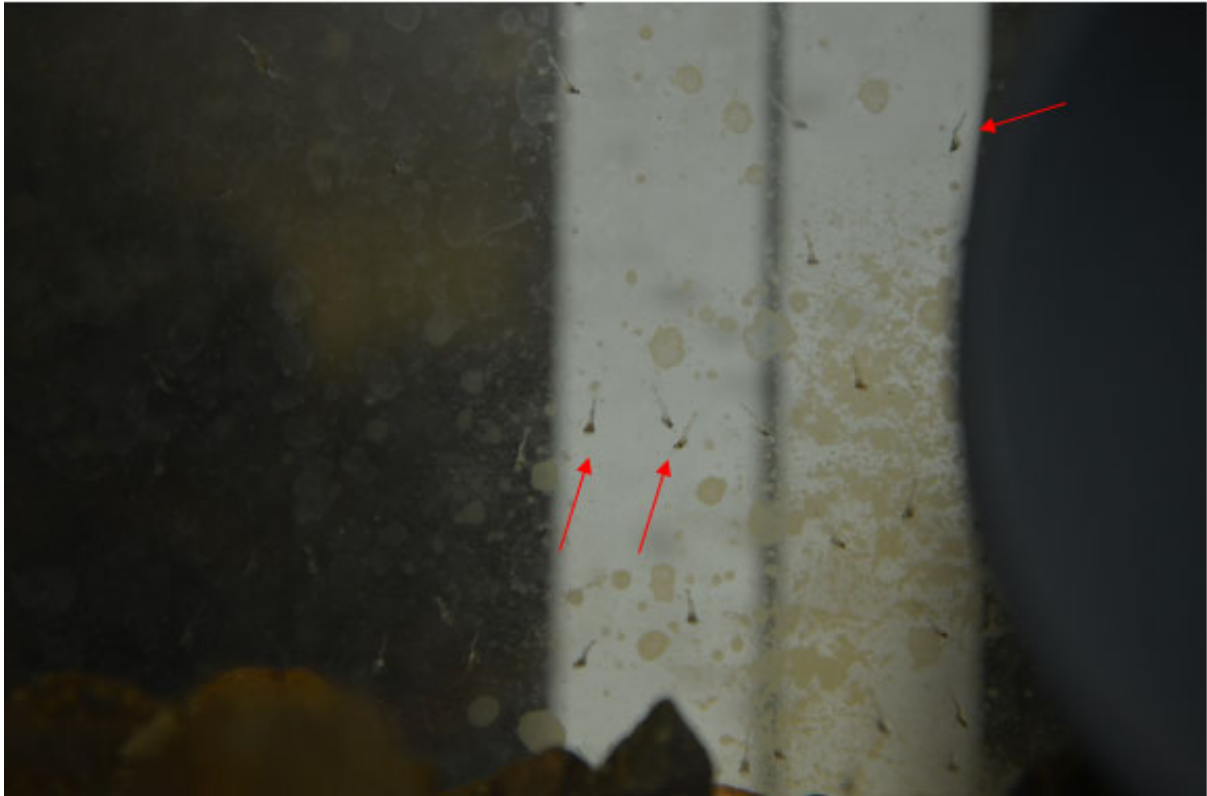
Figure 13: *P. australiensis* fry displaying benthic preference behaviour by attaching onto the aquarium glass.

The filter, female and any décor were removed from the tank and the net was carefully moved through the tank. The net was then dipped into a petri-dish where a small amount of water assisted with detaching the fry from the next material. A disposable pipette was used to transfer the fry from the dish to a 2mL collection tube. Excess water was pipetted out of the tube using a 2-20µL pipette as the fine tip prevented fry from being removed. Euthanised fry were preserved in ethanol before storing in a freezer at -20°C until DNA extraction.

There were multiple instances where a berried female would drop her eggs prior to hatching. Where this occurred, the female was kept in isolation and used as a replacement for any additional berried females from the same experimental tank.

Isolated females would occasionally produce eggs without being in contact with any males. These eggs, referred to here as "phantom eggs", were usually dropped within a couple of days of forming. Where possible, these eggs were collected and fixed in glutaraldehyde before being dehydrated in a series of ethanols and chemically dried in hexamethyldisilisane (HMDS). Samples were then mounted on aluminium stubs and sputter-coated with gold before being viewed in a Jeol Neoscope 5000 tabletop scanning electron microscope at 10kV to determine if they were fertilised.

3.4 GENETIC ANALYSIS

3.4.1 DNA Extraction

Total genomic DNA was extracted using a Qiagen DNeasy Blood and Tissue kit containing spin columns and 2ml centrifuge tubes, lysing buffer (ATL Buffer), Proteinase K, binding solution (AL Buffer), wash solution (AW1 and AW2 Solution) and elution buffer (AE Buffer).

DNA was extracted from a sample of individual fry from each successful batch to determine paternity and to detect potential multiple mating events. DNA was also extracted from females who successfully released fry to confirm maternal lineage.

For extractions involving adult shrimp, a small 3-4mm section of tail segment was used. For extractions involving fry samples, the entire fry was used.

Tissue samples were placed in 1.5ml tubes along with 180µL of buffer ATL and 20µL of Proteinase K. Samples were vortexed and incubated overnight in a water bath at 56°C. 200µL of AL buffer was added and mixed thoroughly using a vortex before adding 200µL of 100% ethanol.

Samples were pipetted into spin columns in 2ml centrifuge tubes and centrifuged at 8,000rpm for one minute. The flow through was discarded and 500µL of AW1 solution added to the spin column in the same 2ml tube and placed back on the centrifuge for one minute at 8,000rpm. The flow through was discarded and 500µL of AW2 buffer was added to the spin column in 2ml centrifuge tube and placed on the centrifuge for three minutes at 14,000rpm. The flow through was discarded and 200µL of AE buffer added to the spin column directly on the membrane before incubating at room temperature for one minute. The spin column was placed in a 1.5ml centrifuge tube and samples were then centrifuged at 8,000rpm for one minute with the flow through forming the final template.

3.4.2 Maternal Lineage Confirmation

Females that had successfully reared and released fry were kept in the isolated tanks for the remainder of the experimental process to observe ongoing behaviour. At the end of the experimental period, the females were removed from the isolation tanks, euthanised and placed into 90% ethanol for transport and storage. DNA was extracted and lineage was confirmed using the same process as the initial lineage confirmation. Where the female was not collected, DNA extracted from the fry was used.

3.4.3 Microsatellite Analysis

Two of the three microsatellite loci identified by Wilson et al. (2016) were selected for optimisation based on having similar annealing temperatures. Both loci were proposed by Wilson et al. (2016) as being accurate at discriminating between the two target lineages and hence would be appropriate for determining paternity. The appropriate primers for the loci are provided in Table 6.

Table 6: Details of microsatellite loci (Wilson, 2016) used to determine paternity.

Locus	Primer Sequence	Repeat Motive	Annealing Temp (°C)	Size Range	Dye
ION09	F: TTCTGCCTTGACTGCACCTT R: GGTGAGCATCGTGTGGACTT	AG	55	220-240	FAM
ION44	F: AGCAGCAATGAGGCACTAGG R: ATCCTGGGCAAAGCAACATA	AC	55	130-150	VIC

A working solution of each primer was created and diluted to a concentration of 10μM. A PCR solution was created for each locus with 1μL of DNA template, 1μL of forward primer, 1μL of reverse primer, 12.5μL of MyTaq mix and 9.5μL of purified water giving a total volume of 25μL for each sample.

PCR for both loci was run on Biorad T100 thermal cycler with the initial denaturation process of 94°C for two minutes followed by 34 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a single extension cycle of 72°C for seven minutes before holding at 4°C based on the protocol used by Wilson et al. (2016).

The final PCR products were run on an electrophoresis gel made from 100ml of 1.5% agarose with 7μL of gel red. The samples were loaded into the gel along with a 100bp ladder and run at 80v for 40 minutes. Results indicated some non-specific priming on ION09 loci but both primers appeared to work on the target range.

Samples were then multiplexed with highly deionised formamide (HiDi)to combine both products before sending to Macrogen for fragment analysis. Each sample contained 1μL of each primer template and 9μL of HiDi bringing to total volume of each sample to 11μL. Instructions were given to Macrogen to add 1μL of the LIZ600 ladder prior to analysis.

The results provided by Macrogen indicate the primers worked effectively and the non-specific priming noted in the ION09 samples did not impact the results of the fragment analysis.

DNA was initially extracted from individual fry from each batch produced and PCR was run using the same process as above. Samples were multiplexed with HiDi and sent to Macrogen for fragment analysis. The results provided were loaded into Geneious Prime 2019.2.1 with the microsatellite plugin installed. Loaded samples were aligned using the Liz600 ladder and locus information provided by Wilson et al. (2016) entered. The range was subsequently expanded for each locus as there were several peaks identified outside the proposed range. Each sample was reviewed to ensure true peaks were marked and false peaks were removed, and the final peak information was summarised in Excel.

3.4.4 Statistical Analysis

Mate choice was summarised by labelling successful breeding events as either hybrid or non-hybrid producing batches. Data was loaded into IBM SPSS Statistics v26 and non-parametric Chi-Square and binomial tests were performed for each lineage type to determine if preferential mating is occurring between lineages.

Chapter 4: Results

4.1   LINEAGE CONFIRMATION

The reproduction process in decapods usually involves a brief window of opportunity for male copulation directly after a female moult. Such a small window often leads to a larger portion of ready males compared with receptive females often resulting in the adaptation of mate guarding by the males to ensure opportunities are not missed (Andersson, 1994). There are examples, however, of decapods that do not show mate guarding tendencies despite copulation occurring within two to three hours of female moult. Bauer and Abdalla (2001) observed pure searching behaviour in a caridean shrimp species, *Palaemonetes pugio*, with low frequency male contact with females in the days leading up to moult compared with the immediate hour prior. Bauer and Abdalla (2001) suggests the lack of mate guarding is a result of the species being a highly active species living in a high population abundance allowing males to encounter up to two or more females per day.

Of the 12 samples processed for lineage confirmation, five were successfully sequenced and blasted against the GenBank database. The initial lineage confirmation results indicate that the collection sites contain the expected lineages with a high query cover and percent identity for all samples completed (see Table 7).

Table 7: Initial Lineage Confirmation Results.

Sample I.D.	Collection Site	Blast Match Haplotype	Location Per Hurwood (2003)	Accession Number	Query Cover	Per. Ident
KC01	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	95%	100%
KC02	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	95%	100%
KC03	Kilcoy Creek	N/a	N/a	N/a	N/a	N/a
KC04	Kilcoy Creek	N/a	N/a	N/a	N/a	N/a
KC05	Kilcoy Creek	N/a	N/a	N/a	N/a	N/a
KC06	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	95%	100%
SC01	Stony Creek	Pa9	Stony Creek	AF534902.1	95%	100%
SC02	Stony Creek	Pa9	Stony Creek	AF534902.1	95%	100%
SC03	Stony Creek	N/a	N/a	N/a	N/a	N/a
SC04	Stony Creek	N/a	N/a	N/a	N/a	N/a
SC05	Stony Creek	N/a	N/a	N/a	N/a	N/a
SC06	Stony Creek	N/a	N/a	N/a	N/a	N/a

All maternal lineage confirmation samples were successfully sequenced and blasted against the GenBank database. Results confirmed that the maternal lineage for all samples were in line with the expected lineages for their relative collection sites in accordance with Hurwood et al. (2003), except for sample 2-1-1. This sample was collected from Stony Creek but produced mtDNA consistent with the Kilcoy Creek lineage. This is in contrast to the nucleic DNA for sample 2-1 which showed two Stony Creek alleles for both the ION09 and ION44 loci (see Appendix A). High query cover and percent identity results for each sample support the lineage matches. Sample 3-6-1 did not produce a signal when sequencing. This is likely due to poor PCR results or insufficient DNA product. Microsatellite results for Female 3-6 and all fry from the 3-6 batch showed Kilcoy Creek types on both loci therefore it was concluded that the female was a true Kilcoy Creek type.

Table 8: Maternal Lineage Confirmation Results

Sample I.D.	Collection Site	Blast Match Haplotype	Location Per Hurwood et al. (2003)	Accession Number	Query Cover	Per. Ident
2-1-1	Stony Creek	Pa6	Kilcoy Creek	AF534899.1	96%	99.84%
3-2-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	99.84%
3-3-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	100%
3-4-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	100%
3-5-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	100%
3-6-1	Kilcoy Creek	N/a	N/a	N/a	N/a	N/a
4-1-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	99.84%
4-2-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	100%
4-3-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	100%
4-4-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	100%
4-5-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	100%
4-6-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	99.84%
4-7-1	Kilcoy Creek	Pa6	Kilcoy Creek	AF534899.1	96%	100%
5-1-1	Stony Creek	Pa9	Stony Creek	AF534902.1	96%	100%

4.2 MATE CHOICE

A total of 23 breeding events were recorded with 14 successfully producing fry (see Table 9) producing a success rate of 61%. This is consistent with the laboratory spawning success rates for another caridean species, *Macrobrachium rosenbergii*, which ranged between 46% and 68% (Wickins & Beard, 1974; Ganeswaran, 1989). For the L6 female X L4 male treatment, two breeding events were recorded, however both batches of eggs were dropped within two to

three days after placing in isolation. The reason the eggs were dropped is unknown. The process of moving the shrimp into another tank could have caused the female to release the eggs due to stress. Alternatively, the eggs may not have been fertilised which has been observed in other decapod tank breeding experiments (Ganeswaran, 1989; Fiedler, 2000). Treatments for L6 female X mixed males produced three breeding events with two successfully producing fry giving a 67% success rate.

A total of 18 breeding events were recorded for the L4 Female X mixed male treatment with 12 females successfully producing fry giving a 67% success rate. The gestation periods recorded for each successful female ranged from 23 to 30 days with an average of 27.75 days.

Table 9: Recorded successful and unsuccessful breeding events per treatment.

	Successful	Unsuccessful	Total
L6 ♀ x L4♂	0	2	**2**
L6♀ x Mixed	2	1	**3**
L4♀ x Mixed	12	6	**18**
Total	**14**	**9**	**23**

The microsatellite analysis on the fry collected from each successful breeding event showed that both the L6 female X mixed male treatment events produced non-hybrid results and two of the 12 L4 female X mixed male treatment events produced hybrid offspring (see Appendix A for allele summary and Figure 14 showing microsatellite peaks for both locations for Female 4-3). These results are consistent with results provided by Hughes, et al. (2003) where L4 type females were able to breed with L6 type males, however there was no evidence of L6 type females breeding with L4 type males.
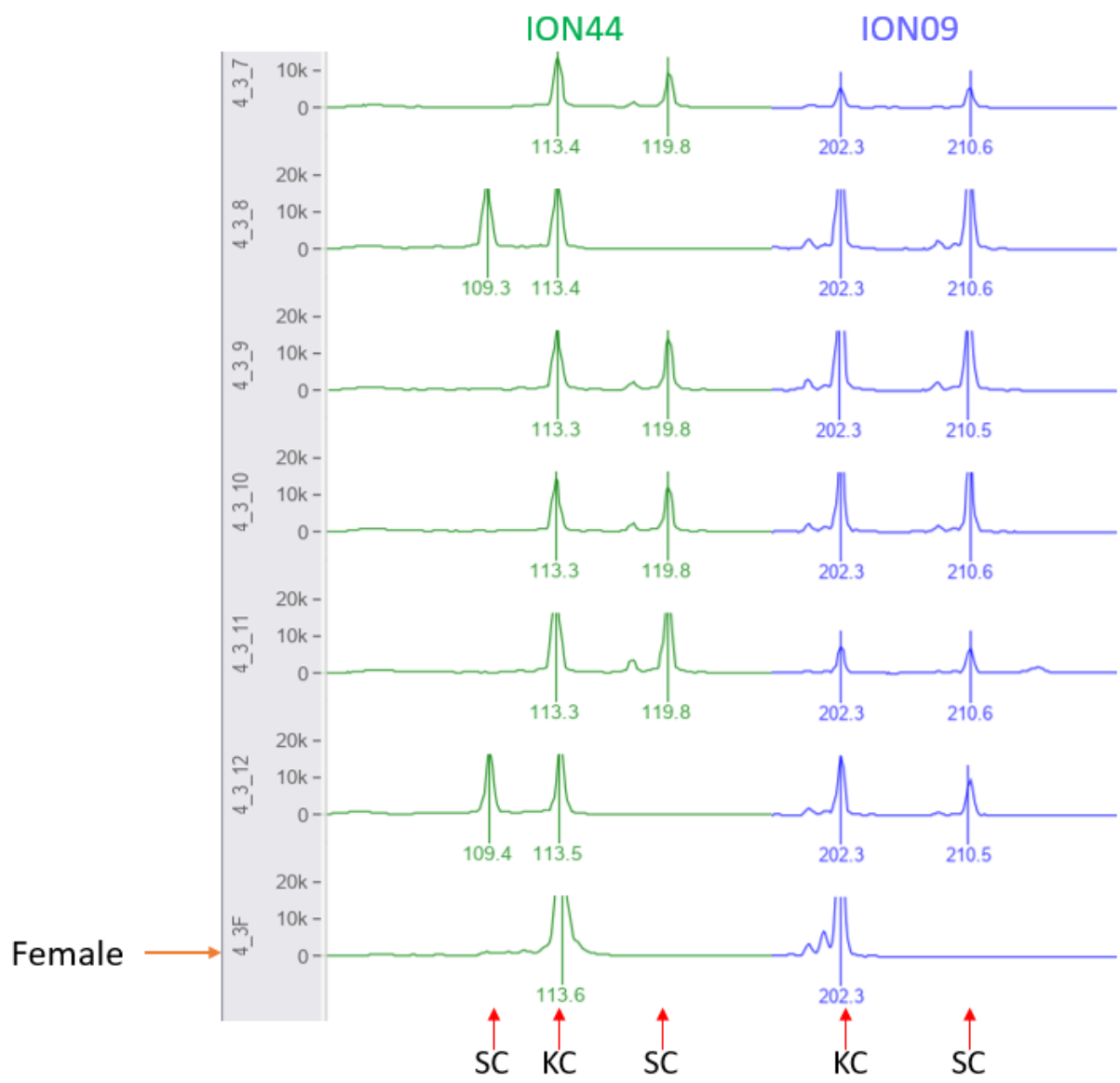
Figure 14: Microsatellite peak results for fry produced by female 4-3 showing hybrid allele combinations for both loci. Lineage types for each location are indicated along the bottom. SC = L6 Stony Creek type. KC = L4 Kilcoy Creek type.

A single fry (4_4_4) from the batch produced by female 4-4 showed a hybrid 202 – 210 genotype on the ION09 location with 202 being a L4 type and 210 being a L6 type (see Figure 15). The ION44 location for this fry sample produced a homozygous 114 genotype with 114 being a L4 specific allele. The other fry analysed from the batch produced by female 4-4 only produced L4 type alleles. This raises the possibility of multiple paternity, however it is difficult to explain why this pattern is seen in only one locus.
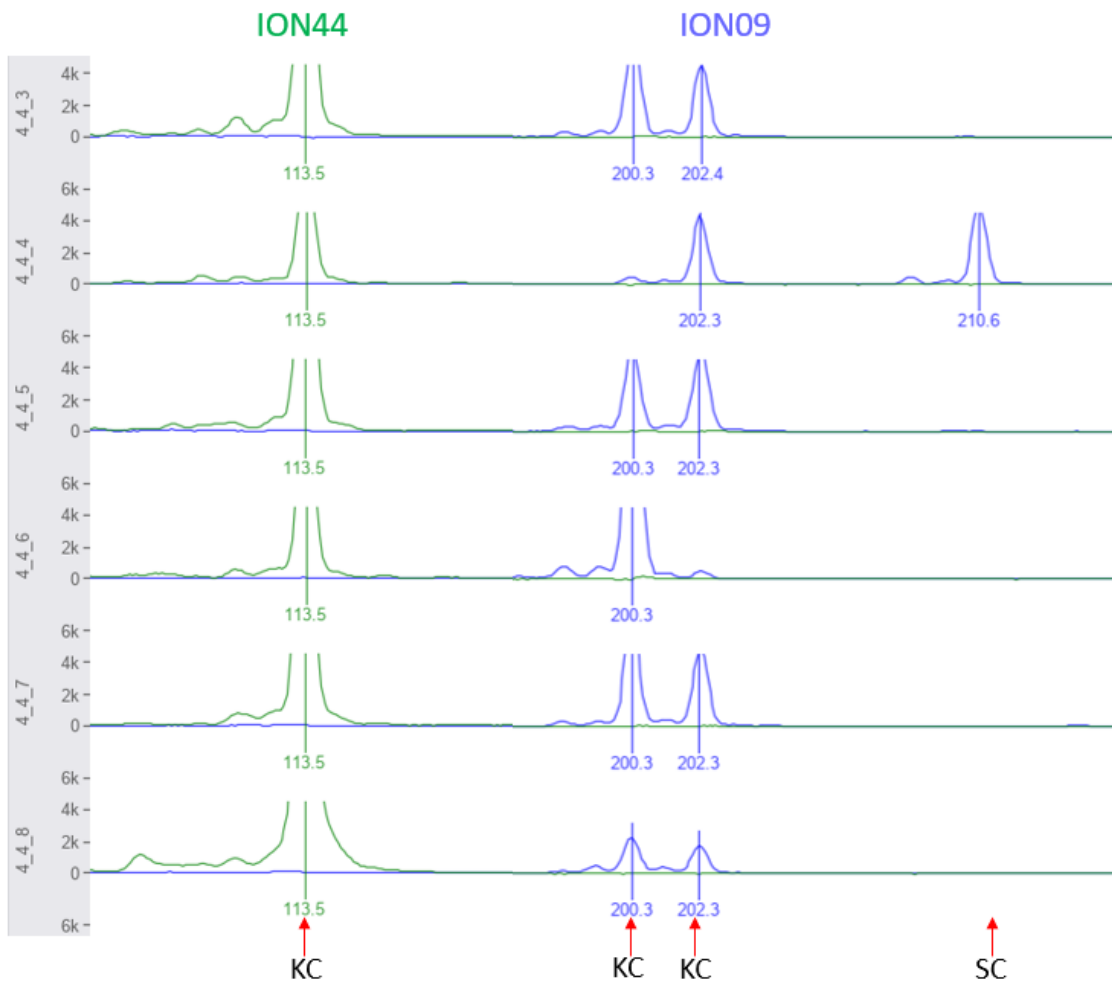
Figure 15: Microsatellite peak results for fry produced by Female 4-4 showing a single fry with 202-210 hybrid peaks on ION09 site. Lineage types for each location are indicated along the bottom. SC = L6 Stony Creek type. KC = L4 Kilcoy Creek type.

The fry produced by Female 4-7 all showed hybrid allele combinations on both sites except for one fry (4_7_17) that produced a L6 type 120-120 combination on the ION44 site (see Figure 16). The allele combinations for Female 4-7 were 114-114 L4 alleles on ION44 and 202-202 L4 alleles of ION09. Once again, we see a cohort of larvae that does not conform to the idea of single male parent. In fact, individual 4_7_17 does not share a single allele with its mother for ION44. This goes beyond a multiple paternity explanation.
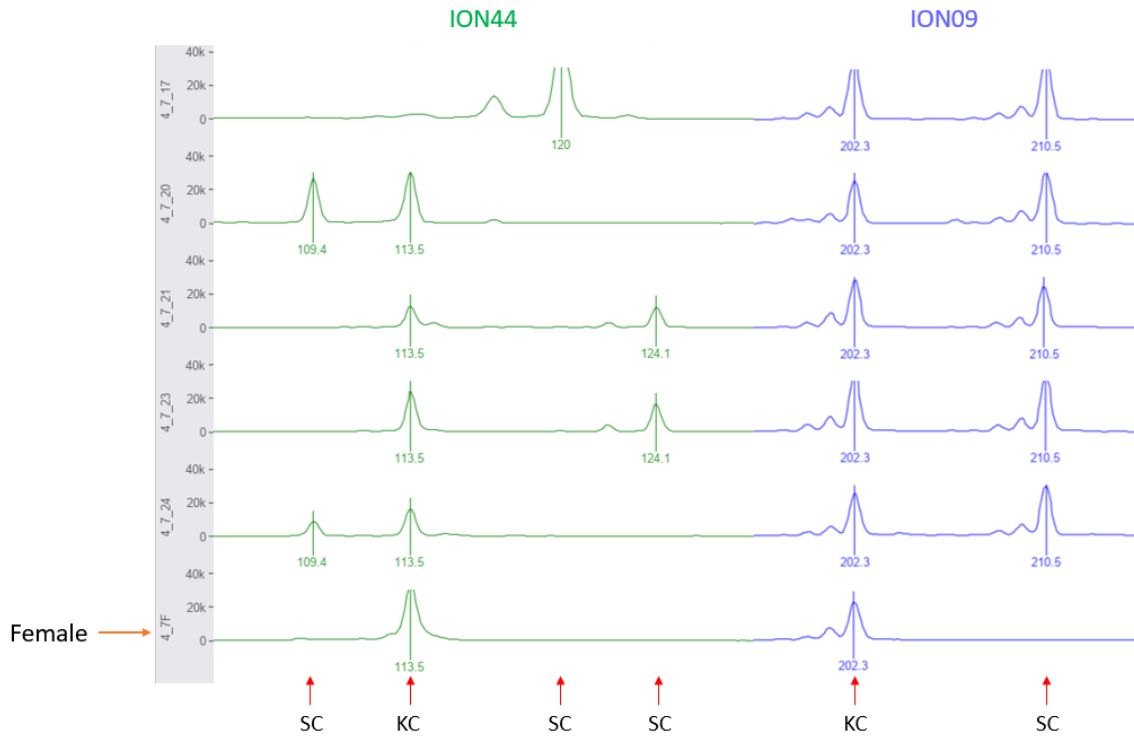
Figure 16: Microsatellite peak results for fry produced by Female 4-7 showing a single fry with 120 homozygous peak on ION44 site with the Female producing a 114 homozygous peak on the same site. Lineage types for each location are indicated along the bottom. SC = L6 Stony Creek type. KC = L4 Kilcoy Creek type.

The null hypothesis that there is no preference of mate choice between lineages can be rejected for the L4 lineage with $\chi^2_{0.05,1} = 5.33$, P = 0.021 and binomial test T = 2, P = 0.039. The Chi-Square analysis could not be performed on the L6 lineage due to the low sample size. The binomial test results for the L6 lineage T = 2, P = 0.5, therefore the null hypothesis could not be rejected.

4.3 PHANTOM EGGS

Females that successfully reared and released fry were kept in their isolation tanks for the remainder of the project. Despite having no exposure to any other shrimp, on eight occasions a female produced a batch of eggs, referred to as "Phantom Eggs" (see Table 10). This behaviour has been observed in other carideans where females that have not been exposed to males within a few days of a reproductive moult, will still produce unfertilised eggs that are usually dropped shortly after (Bauer, 1976; Correa & Thiel, 2003). Female 3-6 produced three of the eight batches of phantom eggs with the second batch being produced 47 days from the first batch and the third batch being produced 49 days from the second batch. Microscopic imaging of these eggs shows no indication of fertilisation in the form of cell division (see Figure

17). Irrespective of the anomalies identified in the previous section, this suggests that there is no retention of viable sperm between egg production events, indicating that multiple paternity within a cohort is unlikely.

Table 10: Phantom Egg events recorded including lineage information. * Female 2.1 produced Kilcoy Creek mDNA and Stony Creek microsatellite alleles. Female was collected from Stony Creek.

Batch #	Female #	Female Lineage	Date Berried
1	K3.6	L4 - Kilcoy Creek	27/12/2019
2	K4.1	L4 - Kilcoy Creek	3/02/2020
3	K3.6	L4 - Kilcoy Creek	12/02/2020
4	K4.2	L4 - Kilcoy Creek	24/02/2020
5	K4.3	L4 - Kilcoy Creek	16/03/2020
6	K4.7	L4 - Kilcoy Creek	16/03/2020
7	K3.6	L4 - Kilcoy Creek	2/04/2020
8	S2.1	L6 - Stony Creek*	11/04/2020
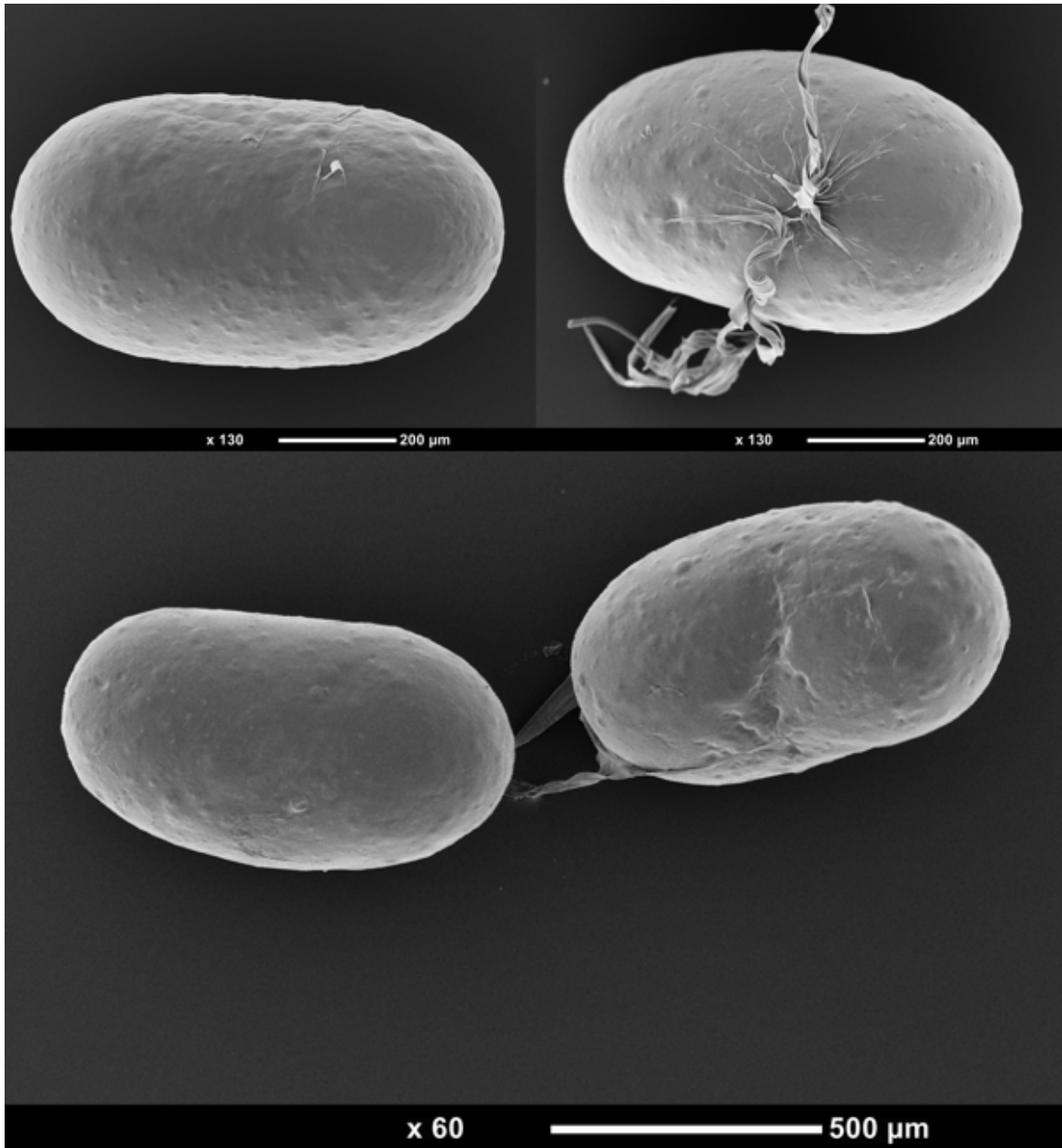
Figure 17: "Phantom Eggs" collected from females not exposed to any males during moult cycle.

Chapter 5: Discussion

5.1 MATE CHOICE AND REPRODUCTIVE BREAKDOWN

The observations following the translocation event for these two populations in 1993 indicate a distinct breakdown in the reproduction between lineages. Hughes et al. (2003) suggests the breakdown is a result of two separate reproductive barriers. The first being a pre-mating barrier in the form of unidirectional mating preference between lineages causing non-random mating, the second being a breakdown in hybrid fitness. An analysis of mtDNA and nuclear genes at three allozyme loci indicated that in the Branch Creek translocation site where L6 populations occur, resident females were suggested to show a preference towards introduced, L4 males resulting in the eventual displacement of the resident mitochondrial genotype within seven generations. In contrast, the opposite translocation event where L6 samples were translocated into a L4 dominated site in Kilcoy Creek, no evidence of the translocated genotype was found in later sampling efforts.

Grazon (2009) observed a change in reproductive timing in the translocation pool when compared with natural ranges. L6 females present in the translocation pool did not reproduce until Autumn with L4 females breeding throughout Spring and Autumn. In their natural ranges, both lineages tend to breed during Spring with minimal breeding occurring in Autumn. He proposed the change in reproductive behaviour was due to the L4 females (adapted to cooler temperatures) becoming reproductively receptive earlier in the season and utilising the male reproductive resources before the L6 females are receptive, resulting in a reduced period of reproduction for the L6 females.

Our results support the ability of L4 females to utilise the gametes of L6 males with the mate choice experiments showing that the L4 females can reproduce with L6 males. The lack of reproductive success in the reverse direction, L6 females crossed with L4 males, in our mate choice experiments could point to an additional pre-mating reproductive barrier not previously raised. A complete or partial breakdown in recognition between L6 females and L4 males would explain the lack of any observed crosses in both the translocation studies and mate choice experiments. The extent of the breakdown in recognition would require further studies, however we can hypothesise that the breakdown could be either a failure of L4 male

recognition by the L6 female or a failure of L6 female recognition by the L4 male (both partial breakdowns), or a combination of both (complete breakdown).

A partial breakdown in L4 male recognition would occur where the L4 male recognises the L6 female, but the L6 female no longer recognises the L4 male. In this event, the L4 male would detect the chemical cues released by the recently moulted L6 female, triggering the "*climb phase*" of the courtship where the male attempts to mount the female followed by the "*straddle phase*". It is at this point where the female would likely reject the male preventing copulation (Bauer, 1976). This form of recognition breakdown not only prevents inter-lineage hybridisation but could also reduce the reproductive success of L6 female and L6 male events through competition, explaining the lower numbers of total reproductive events involving L6 females in our research. *P. australiensis* appears to utilise a scramble mating system that favours smaller, more mobile males (Andersson, 1994). The slightly smaller size of L4 males (Hancock, 1995) may give them an advantage over the L6 males in initiating courtship and "*occupying*" a female, preventing L6 male/female courtship. Bauer (1976) recorded the period in which female *Hippolytid* shrimp are receptive to reproduction as approximately 24 hours. If the receptive period of *P. australiensis* is similar to *Hippolytid* (another member of the Caridean family), the ability for one or more L4 males to prevent reproductive interactions between a L6 female and potential L6 males during such a small time period is plausible. Such interactions would result in the female eventually releasing her unfertilised eggs while the L4 males keep their gametes to use in the next reproductive interaction.

In contrast, partial breakdown in female recognition would occur when the L4 male fails to recognise the L6 female due to a loss in the ability to detect the females post-moult chemical cues. This breakdown in recognition would explain the lack of L6 female/L4 male hybrids found in translocation studies as well as our mate choice experiments, however, the consequential impact on L6 female and male interactions would not be as significant as the breakdown in male recognition. The L4 males in this scenario would not attempt to reproduce with the L6 female, preventing any wasted gametes or resource on an unsuitable partner.

A breakdown in L6 female recognition by L4 males would explain the lack of hybridisation in the mate choice tanks as well as the tank with L6 females and L4 males only, however, it does not explain the overall lower numbers unless the combination of both L4 and L6 males resulted in the suppression of chemical cues produced by the L6 female, preventing detection by L6 males. Suppressed recognition of chemical cues has been observed in other Caridean species. Zhang et al. (2009) noticed females from two closely related *Lysmata* shrimp produced both

dispersive and contact pheromones when becoming reproductively receptive. Through laboratory mate choice experiments, Zhang et al. (2009) found that the dispersive pheromones of the females on one species were suppressed when males from both species were present, resulting a lack of recognition by both males. This suppression of recognition could explain the low number of reproductive events in the experimental tanks containing L6 females in our study, however further studies would need to be performed to understand the extent in which chemical cues are used by *P. australiensis* during courtship. The species studied by Zhang et al. (2009) used two methods of chemical cues, both dispersive and contact, however many other caridean shrimp have been noted as only using contact cues (Bauer, 1976).

In our study, courtship generally occurred in the late evening and premating behaviour was not observed for most mating events. However, such interactions provide a plausible explanation for not only the delayed reproductive timing observed by Grazon (2009) but also the displacement of the resident L6 genotype in the translocation trials performed by (Hancock & Hughes, 1999).

Orr et al. (2004) identified speciation genes as ordinary genes that rapidly evolve to provide a reproductive barrier between individuals. Being ordinary genes that perform normal functions within the species, the development of a speciation gene can impact both pre-mating and post-mating processes from courtship behaviour (Heinrich, et al., 2001) to hybrid inviability (Price & Bouvier, 2002). A recent study conducted by Rogl (2020) identified approximately 50 candidate speciation genes in *P. australiensis*, one of which is thought to be responsible for mate recognition. Using transcriptomic techniques, Rogl (2020) observed a gene called "*takeout*" gene in both L4 and L6. This gene has been found to influence mate choice in *Drosophila*. Observing the expression of this gene in both lineages may provide evidence as to whether pre-mating barriers are present and responsible for the reproductive breakdown between the two lineages.

An alternative hypothesis to the possible recognition barriers described above is the presence of a post-mating – pre-zygotic barrier preventing fertilisation. These barriers are often found in species with an internal fertilisation system in the form of ovarian tract incompatibilities, however, they have also been observed in external fertilisation systems (Glabe & Lennarz, 1979). For external fertilisation systems, the lack of ovarian tract interactions results means that post mating – pre-zygotic barriers occur in the form of a breakdown in chemical cue receptivity preventing ovary release (Garlovsky, et al., 2020) or as a result of slight changes in spermatophore and/or ovary barrier chemical composition inhibiting fertilisation (Gallo &

Constantini, 2012). A substantial number of studies have been conducted on the endocrinology of various species of shrimp (Huberman, 2000), however the extent to which chemical cues are utilised to trigger ovary release post spermatophore deposition have not been documented. However, a study has been performed on the role that male produced hormones play in the development of female ovaries in *Paratya compressa* (Takayanagi, et al., 1986). In the study, ovary development in females with no contact to males was significantly reduced. They suggest that a hormone produced by males induces the development and maturation of the female embryos. Variation in these proposed ovary-stimulating hormones in our study species could produce a reproductive barrier and explain the lack of reproductive events in the L6 female x L4 male experimental tank, however, it wouldn't explain the low number of reproductive events in the L6 female mate choice tank unless a chemical suppression event occurred, similar to the one mentioned earlier in this chapter. Unfortunately, our study did not provide any clarification as to whether a post-mating – pre-zygotic barrier is present and further studies would be required.

Our mate choice studies did not produce any L6 female x L4 male hybrids and produced few L6 female x L6 male events compared with the number of combined L4 female reproductive events. The lack of hybrids is consistent with expectations based on studies conducted as part of the translocation event in 1993 (Hughes, et al., 2003). There have been no recorded instances of L6 female and L4 male hybrids in any studies of these two populations over the past 27 years which, along with our studies, indicate that the reason for the lack of hybridisation is likely due to pre-zygotic barriers preventing fertilisation. However, the L6 females in our study did not produce enough reproductive events to statistically determine whether the lack of hybridisation is due to a partial or complete breakdown in reproduction or whether the reproductive barrier is a pre-mating barrier or a post-mating – pre-zygotic barrier.

The results of our mate choice experiments indicate that although L4 females appear to be able to produce viable offspring with L6 males, there is positive assortative mating with other L4 males. This breakdown in random mating is a strong indicator of speciation with the breakdown developing as an evolutionary advantage (positive reinforcement *sensu* BSC), driven usually by ecology-based preferences (Thilbert-Plante & Gavrilets, 2013). Although both populations occur in a connected river system, ecological preferences and rheotactic behaviour appears to prevent natural contact between populations (Hancock & Hughes, 1999). Pfennig (1998) proposed that mate choice primarily serves two key functions, species recognition to ensure individuals are genetically compatible and mate-quality recognition to ensure offspring fitness

is maximised. In regard to the L4 female preferences towards L4 males, the latter function would be expected to occur in its natural range where L4 males have adapted to the higher altitudes and lower temperatures of the headwaters of Kilcoy Creek and would help explain the lack of persistence of the L6 genotype when introduced to Kilcoy Creek. However, such a preference would be counter intuitive when L4 samples were translocated to Branch Creek. If mate choice were based on mate-quality then surely the introduced L4 females would gravitate toward the better adapted, resident L6 males. However, allozyme studies conducted after the translocation event indicate the con-lineage preference was maintained indicating that the mate-quality is not the key driver. Instead, based on Pfennig's assessment, the preference would be driven by genetic recognition. Regardless of the driver, a significant con-lineage preference appears present in L4 females providing additional evidence of reproductive breakdown between the two populations.

The number of unsuccessful breeding events in the L4 female x mixed tank experiments could also point towards a partial breakdown in post-mating-prezygotic process where the L4 female recognises the L6 male, but fertilisation is restricted. However, the success rate appears relatively consistent with other Caridean mate choice experiments (Wickins & Beard, 1974; Ganeswaran, 1989) so would likely be attributed to natural attrition.

Although not investigated during our study, prior studies have identified potential hybrid breakdown as an additional, post-zygotic reproductive barrier between the populations. While our study produced viable L6 female and L4 male hybrid offspring, multi-generational studies were not conducted to assess hybrid sterility, F1 hybrid mate choice or hybrid breakdown. Fawcett et al. (2010) studied the population structure of Branch Creek after the 1993 translocation event and found that over the two year period of 2001 and 2002, the translocated L4 genotype had greater reproductive success producing more pure L4 and hybrid offspring than the resident L6 genotype. Both the pure L4 and hybrid offspring had low survivability producing a population ratchet effect typical of populations with high reproductive success but low fitness. The results of this study discard the occurrence of hybrid sterility but support the presence of hybrid breakdown though a reduction of fitness in F_n hybrids across generations.

Multiple reproductive barriers appear to exist between these two populations. Pre-zygotic barriers between L6 females and L4 males, con-lineage preferences by L4 females and apparent hybrid breakdown for L4 female and L6 male hybrids all appear to contribute to the displacement of the L6 genotype during the translocation events of 1993. Similar hybrid breakdown has been observed in two closely related crayfish, the invasive species *Orconectes*

rusticus and the resident species *Orconectes propinquus* (Arcella, et al., 2014). During secondary contact as a result of an invasion event, these two species produce hybrid offspring that initially show increased fitness in the form of higher growth rates and survivorship. However, backcross offspring show a rapid reduction in fitness, particularly when backcrossing with the resident parental species. This rapid reduction in fitness puts pressure on the resident species population, ultimately resulting in displacement.

Coyne and Orr (2004) highlight that successful reproduction in laboratory conditions does not necessarily reflect the outcome that would occur between two populations in natural sympatry. An example is the African lion and leopard. Both species occupy the same regions, however no records of natural hybridisation have been noted. The species have been successfully crossed in zoos and laboratories and have produced fertile offspring. With *P. australiensis* however, the introduction of L6 into pools containing L4 conducted by Hughes, et al. (2003), demonstrate that these lineages will hybridise under "natural" conditions and the results demonstrated in this report support those found naturally.

5.2   SPECIES DELIMITATION

The two populations included in this study are fascinating from a species definition perspective. Here we have two highly divergent populations of the same species living in apparent sympatry through a connected stream system. Despite this connectedness, populations do not naturally appear in the same pool and when placed in contact artificially, demonstrate reproductive breakdown in the form of three reproductive barriers with two of the reproductive barriers allowing semi-permeable gene flow resulting in partial interbreeding. These partial breakdowns in reproduction allowing semi-permeable gene flow have been considered an incongruence for a number of species concepts, in particular the BSC (Frankham, et al., 2012).

Using the combined results of our study and previous studies on these two populations, we can estimate the extent in which this species has advanced through the speciation process. A theoretical depiction of the speciation process is illustrated in Figure 18. While the differentiation methods listed are not an exhaustive list of criteria, they can provide guidance as to how various differentiation methods change along the continuum.
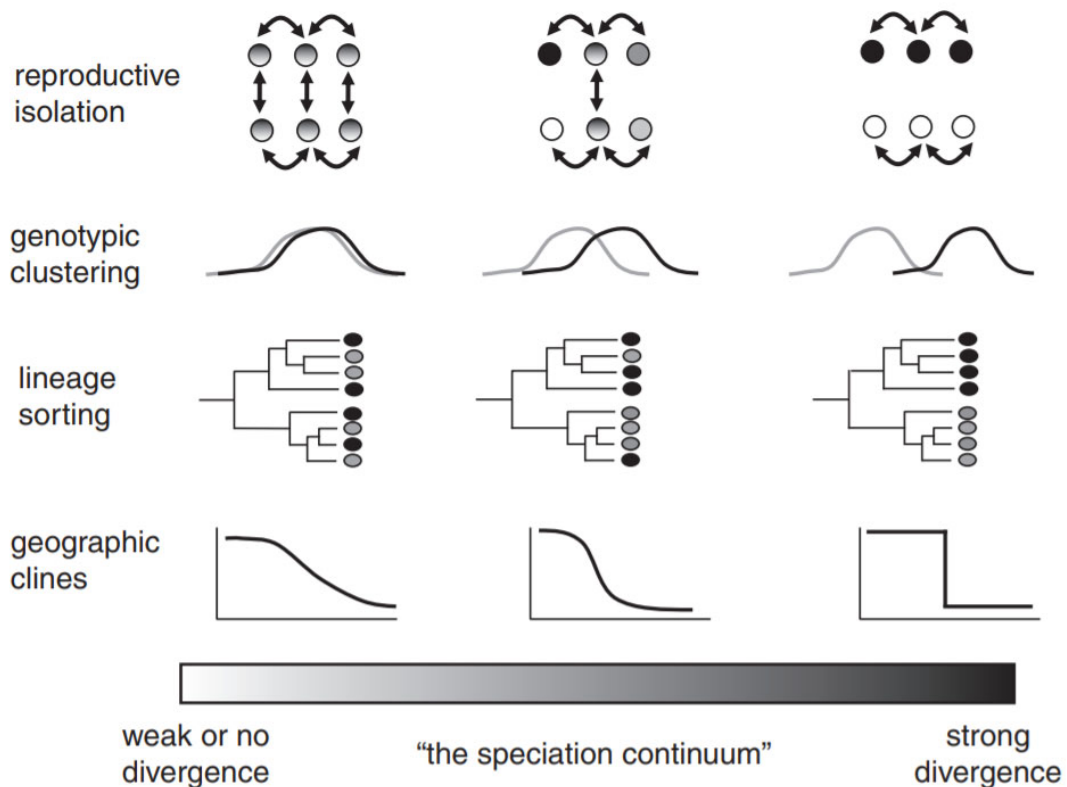
Figure 18: Schematic illustration of the continuous nature of divergence during speciation. Four different types of differentiation across three arbitrary points along the speciation continuum have been depicted. Two-headed arrows represent mating between individuals (Nosil, 2015)

Our study demonstrates significant reproductive breakdown between the two populations, prior allozyme and mtDNA studies have shown fixed differences at multiple loci indicating the populations are highly divergent (Hurwood, et al., 2003), a phylogenetic analysis across the species range has identified nine equally divergent lineages (Cook, et al., 2006); and hybrid zone studies have produced results consistent with the step cline estimation model indicating slightly different biogeographic preferences between populations (Wilson, et al., 2016). The combination of these results indicate that these two populations are at the later stages of the speciation process tending towards strong divergence, and based on the speciation continuum illustration proposed by De Queiroz and Weins (2007) discussed earlier in this paper (see Figure 3), these populations would meet the criteria for being recognised as different species in a greater number of species concepts compared to concepts that would classify them as a single species.

The recognition species concept (RSC) predicts that a species that separates from the main population for a period of time and then is reintroduced will result in one of two possible outcomes. One – random mating will continue (i.e. individuals from both populations still represent the same 'good' species and can continue to freely interbreed) or two – one of the

two populations will have a competitive edge and the other will eventually become extinct (i.e. this will occur before any positive reinforcement can lead to speciation *sensu* the BSC). The translocation studies indicate that the latter is occurring, and our mate choice experiments demonstrate that the lack of random mating could be due to recognition issues. These reproductive barriers are also criteria for separating species under the speciation with gene flow model where reproductive isolation occurs in sympatry as a result of assortative mating and hybrid back-crossing.

The phylogenetic analysis conducted by Cook, et al. (2006) appears to satisfy the separation of species from a systematics approach in accordance with the PSCs as well as meeting the corollaries of the ESC.

Habitat preference between the two populations and hybrid zone analysis conducted by Wilson et al. (2016), support separation under the BSC and recent studies conducted by Rogl (2020) into candidate speciation genes support separation under the GSC.

The species concepts that do not support the separation of these two populations into distinct species include the BSC which requires a complete reproductive isolation for species separation and the PhSC which requires sufficient morphological variation to separate species. The method in which *P. australiensis* was re-synonomised in to one species by Williams and Smith (1979) was largely through a phenetic approach, therefore it is not surprising that the PhSC supports the unification of the species.

Unfortunately, the call to either split the species or maintain a single synonomised species is not unanimously supported by all species concepts despite the populations appearing to have progressed a long way through the speciation process. From a practical point of view, maintaning a single species could have a significant impact on the maintenance of genetic diversity throughout the population. While the species is widespread and has a conservation status of 'least concerned', there are four lineages that are only found in single river systems (see Figure 19). These isolated lineages may be vulnerable to anthropogenic and natural disturbances. Indeed the threat of climate change may have an significant impact on populations through the contraction of suitable habitat ranges as a result of increased water temperatures and decreased water flow throughout south-east Australia (van Vliet, et al., 2013) as well as through a shift in ranges of potential predatory fish into headwater streams (Bond, et al., 2011).

Figure 19: Distribution of the 9 distinct lineages identified through COI mtDNA analysis (Cook, et al., 2006). Colours represent lineages as identified in Figure 1.

If the reproductive barriers described between the two populations of our study are present in these other lineages, genetic rescue may not be possible in the event of population reduction. In fact, the current classification as a single species could lead to rapid displacement of resident lineages in the event that populations are relocated across river systems in an attempt to repopulate weakened systems. In the event of anthropogenic or environmental pressures, classifying these lineages as separate species may allow for earlier conservation action which would ultimately assist with maintaining genetic diversity (Frankham, et al., 2012).

Chapter 6: Conclusions

Substantial reproductive barriers have been observed between two highly divergent lineages of *P. australiensis* occurring in apparent sympatry. Our mate choice experiments have demonstrated significant mate choice preferences and asymmetrical hybridisation between the populations. Hybridisation between L4 females and L6 males is possible, however there is a significant preference for the L4 x L4 reproductive events. Where hybridisation has occurred, prior studies have shown evidence for hybrid breakdown suggesting additional reproductive isolation in the form of post-zygotic barriers.

Our study found no instances of hybridisation between L6 females and L4 males demonstrating a reproductive breakdown likely in the form of post-mating – pre-zygotic barriers, a partial recognition breakdown or a complete lack of recognition between lineages. Additional research in the form of courtship and behavioural observation and testing the ability to detect the chemical cues would provide clarity as to the extent of the breakdown.

Extending similar mate choice experiments across the range of this species to other river systems where multiple lineages occur in apparent sympatry (i.e. multiple lineages occurring in the same catchment), will provide more information as to the extent of the reproductive isolation across the entire population.

Rogl (2020) identified 50 potential candidate speciation genes considered to be responsible for the divergence of these two lineages. Research into the expression of the genes responsible for mate recognition when the two lineages are placed in contact could provide insight as to the drivers of the non-random mating preferences.

The combined results of this study and prior research provides compelling evidence to support arguments for a reclassification of this species not only from a theoretical point of view where evidence suggests that the species has progressed a significant way through the speciation process, but also from a practical sense where, in order to maintain genetic diversity, reclassification may be advantageous to allow for more appropriate management of isolated populations.

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Appendices

Allele summary for microsatellite analysis

Table 11: Allele summary for microsatellite analysis for both loci. Blue cells indicate L4 type alleles, Green cells indicate L6 type alleles.

	ION44					ION09				
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 2_1	1	116	SC	120	SC	1	210	SC	210	SC
Juv Type 1	7	110	SC	116	SC	24	210	SC	210	SC
Juv Type 2	6	116	SC	126	SC					
Juv Type 3	5	120	SC	126	SC					
Juv Type 4	2	110	SC	120	SC					
Ineffective runs	4									
Total Samples	25					25				
Likely Male		110	SC	126	SC		210	SC	210	SC
	ION44					ION09				
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 3_2	N/a									
Juv Type 1	11	114	KC	114	KC	10	202	KC	202	KC
Juv Type 2						1	200	KC	202	KC
Ineffective runs	1					1				
Total Samples	12					12				
Likely Male		114	KC	114	KC					
	ION44					ION09				
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage

	ION44					ION09				
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 3_3	1	114	KC	114	KC	N/a				
Juv Type 1	12	114	KC	114	KC	5	200	KC	202	KC
Juv Type 2						7	202	KC	202	KC
Ineffective runs						1				
Total Samples	13					13				
Likely Male		114	KC	114	KC					

	ION44					ION09				
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 3_4	1	114	KC	114	KC	1	200	KC	202	KC
Juv Type 1	11	114	KC	114	KC	3	200	KC	202	KC
Juv Type 2						8	202	KC	202	KC
Ineffective runs	1					1				
Total Samples	13					13				
Likely Male		114	KC	114	KC		202	KC	202	KC

	ION44					ION09				
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 3_5	1	114	KC	114	KC	1	200	KC	202	KC
Juv Type 1	11	114	KC	114	KC	6	202	KC	202	KC
Juv Type 2						3	200	KC	202	KC
Ineffective runs	1					3				
Total Samples	13					13				
Likely Male		114	KC	114	KC		202	KC	202	KC

	ION44					ION09				
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 3_6	1	114	KC	114	KC	1	202	KC	202	KC
Juv Type 1	12	114	KC	114	KC	4	200	KC	202	KC
Ineffective runs	0					8				
Total Samples	13					13				
Likely Male		114	KC	114	KC		200	KC	200	KC

		ION44					ION09			
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 4_1	1	114	KC	114	KC	1	202	KC	202	KC
Juv Type 1	12	114	KC	114	KC	12	202	KC	202	KC
Ineffective runs										
Total Samples	13					13				
Likely Male		114	KC	114	KC		202	KC	202	KC
		ION44					ION09			
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 4_2	1	114	KC	114	KC	1	202	KC	202	KC
Juv Type 1	11	114	KC	114	KC	12	202	KC	202	KC
Ineffective runs	1									
Total Samples	13					13				
Likely Male		114	KC	114	KC		202	KC	202	KC
		ION44					ION09			
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 4_3	1	114	KC	114	KC	1	202	KC	202	KC
Juv Type 1	4	110	SC	114	KC	12	202	KC	210	SC
Juv Type 2	7	114	KC	120	SC					
Ineffective runs	1									
Total Samples	13					13				
Likely Male		110	SC	120	SC		210	SC	210	SC
		ION44					ION09			
	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Female 4_4	N/a									
Juv Type 1	22	114	KC	114	KC	6	202	KC	202	KC
Juv Type 2						12	200	KC	202	KC
Juv Type 3						4	200	KC	200	KC
Juv Type 4						1	202	KC	210	SC

	# of Samples	Allele 1	Lineage	Allele 2	Lineage	# of Samples	Allele 1	Lineage	Allele 2	Lineage
Ineffective runs	2					1				
Total Samples	24					24				
Likely Male		114	KC	114	KC					
			ION44					**ION09**		
	# of Samples	**Allele 1**	**Lineage**	**Allele 2**	**Lineage**	**# of Samples**	**Allele 1**	**Lineage**	**Allele 2**	**Lineage**
Female 4_5	1	114	KC	114	KC	1	202	KC	202	KC
Juv Type 1	11	114	KC	114	KC	10	202	KC	202	KC
Ineffective runs	1					2				
Total Samples	13					13				
Likely Male		114	KC	114	KC		202	KC	202	KC
			ION44					**ION09**		
	# of Samples	**Allele 1**	**Lineage**	**Allele 2**	**Lineage**	**# of Samples**	**Allele 1**	**Lineage**	**Allele 2**	**Lineage**
Female 4_6	1	114	KC	114	KC	1	200	KC	200	KC
Juv Type 1	12	114	KC	114	KC	12	200	KC	202	KC
Ineffective runs										
Total Samples	13					13				
Likely Male		114	KC	114	KC		202	KC	202	KC
			ION44					**ION09**		
	# of Samples	**Allele 1**	**Lineage**	**Allele 2**	**Lineage**	**# of Samples**	**Allele 1**	**Lineage**	**Allele 2**	**Lineage**
Female 4_7	1	114	KC	114	KC	1	202	KC	202	KC
Juv Type 1	12	110	SC	114	KC	23	202	KC	210	SC
Juv Type 2	9	114	KC	124	SC					
Juv Type 3	1	120	SC	120	SC					
Ineffective runs	2					1				
Total Samples	25					25				
Likely Male		110	SC	124	SC		210	SC	210	SC
			ION44					**ION09**		
	# of Samples	**Allele 1**	**Lineage**	**Allele 2**	**Lineage**	**# of Samples**	**Allele 1**	**Lineage**	**Allele 2**	**Lineage**
Female 5_1	1	126	SC	126	SC	1	210	SC	210	SC

Juv Type 1	6	120	SC	126	SC	12	210	SC	210	SC
Juv Type 2	4	120	SC	120	SC					
Ineffective runs	2									
Total Samples	13					13				
Likely Male		120	SC	120	SC		210	SC	210	SC

Appendix B

Pilot Studies

Xenoplus Recirculating System

The original project design involved the use of the sophisticated Techniplast Xenoplus Multi-linking Water Treatment System (see Figure 20) for mate choice experiments. The system was cleaned and rinsed thoroughly prior to use to remove any residues from prior experiments.

Figure 20: A - Techniplast Xenoplus Multi-linking Water Treatment System. B - Internal Mechanical filter, Carbon Filter, pH and Conductivity Probes and UV Steriliser. C - Example of a 27L tank used in the system with the inbuilt overflow drain.

The system was then filled with conditioned tap water and calibrated for the parameters set out in Table 1. The automatic water top-off was connected to a reverse-osmosis water source and the system was turned on and allowed to cycle for approximately three to four weeks. During this period regular water tests were conducted using API freshwater Ammonia, Nitrite and Nitrate kit to monitor the progress of the nitrogen cycle. Once cycled a small number of shrimp were collected from the Stony Creek collection site (see Figure 8) and added to the system using the methodology detailed below. Unfortunately, the shrimp perished during the first night despite the water quality test results showing suitable system conditions. A Techniplast technician was engaged to investigate and after a few minor adjustments, the system was drained and re-filled, filter media replaced, and the cycling process was repeated. After another three to four weeks, more shrimp were collected and added to the system. These shrimp also

died within a few hours of introduction resulting in the decision to abandon the Xenoplus system.

Isolated Tanks

Prior to adopting a new approach for the mate choice experiment, a pilot study was performed using stand-alone 10L tanks with a small sponge filter attached to a central air pump. This pilot study was used to test the suitability of the set up for the mate choice experiments using three different water sources; conditioned tap water, reverse osmosis (RO) water with conductivity solution added and natural creek water. Twelve tanks, four of each treatment, were set up with a single sponge filter connected to an air compressor. For half of the tanks, a layer of gravel substrate collected from the Stony Creek site was added to determine if substrate had an impact on maintaining water quality.
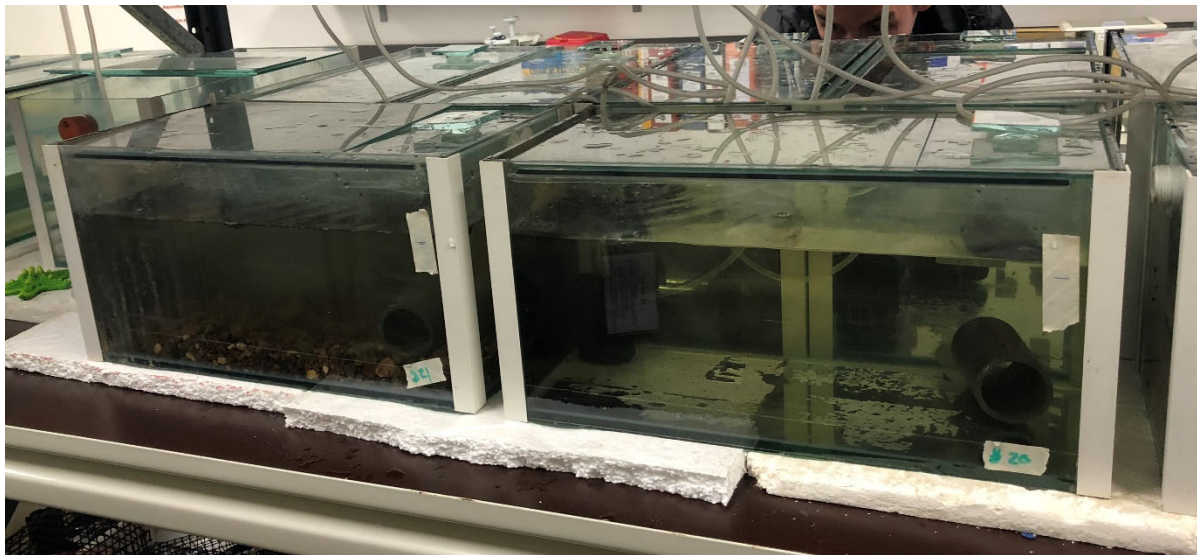

Figure 21: Pilot Study Tank Set Up. Left hand tank contains creek water and substrate. Right hand tank contains creek water with no substrate.

The water for the tap water tanks was conditioned using Seachem Prime water ager to remove chlorine and other heavy metals. Conductivity salts were added to the RO water tanks to bring conductivity levels to between 150 - 300μS. Both the Tap water and RO water treatment tanks were established two weeks prior to adding shrimp to allow the water to cycle. Creek water was collected from the Bellthorpe National Park picnic area in the lower branches of the Stony Creek catchment using 10L plastic containers. Final tests were performed prior to adding the shrimp with results shown in Table 12.

Table 12: Pilot study initial water parameters

Tank	Treatment	NH3 (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)	EC (µS)	pH	Temp (°C)	DO
1	RO - Substrate	0.25	0	0	281	6.848	19	9.25
2	RO - No substrate	0.25	0	0	299	6.993	19	9.39
3	RO - No substrate	0.25	0	0	254	7.02	19	9.37
4	RO - Substrate	0.25	0	0	281	7.195	19.5	9.18
5	Tap - No Substrate	0.25	0	0	424	7.896	19.3	9.31
6	Tap - Substrate	0.5	0	0	436	7.693	19.7	9.11
7	Tap - Substrate	0.25	0	0	498	7.681	19.9	9.16
8	Tap - No Substrate	0.25	0	0	633	7.087	19.7	9.16
18	Creek - No Substrate	0.25	0	0	179	7.401	21.2	9.04
19	Creek - Substrate	0	0	0	215	7.116	21.3	8.9
20	Creek - Substrate	0	0	0	169	7.174	21.6	8.82
21	Creek - No Substrate	0	0	0	170.6	7.179	21.6	8.77

Shrimp were collected from the same site as those used in the Xenoplus system pilot study and were added to the tanks and observed over a three week period. The results presented in Table 13 found that although shrimp were successfully maintained in all treatments, there was a higher survival rate in creek water (97%) when compared with RO water (44%) and treated tap water (51%). Although tanks without substrate had a lower survival rate (43%) when compared with substrate (81%), water quality maintenance, fry collection and sample observation were significantly easier in tanks without substrate and as a result all experimental tanks were set up without substrate.

Table 13: Shrimp survival rates for each treatment type

Treatment	Total Shrimp Added	No of Survivors	Survival Rate
RO Water	50	22	44%
Treated Tap Water	41	21	51%
Creek Water	29	28	97%
Substrate	53	43	81%
No Substrate	67	29	43%