The association of a novel *Decadidymus* species (Temnocephalida) with Australian redclaw crayfish (*Cherax quadricarinatus*); impacts and management

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ABSTRACT

There are growing concerns surrounding aquaculture sustainability as the industry grows and production surpasses that of wild-caught fisheries. Disease and parasite emergence are serious threats to aquaculture, hence management action to reduce these threats is a high priority. This study describes a new species within the *Decadidymus* genus (Temnocephalida) using morphometrics and molecular tools, examines its impact on developing redclaw eggs, and explores possible management solutions. This new temnocephalid species, for which the name *Decadidymus valverdi* sp. nov. is proposed, is highly prevalent and abundant on berried redclaw, feeds on the yolk of developing eggs, and acts as a pathogen vector for *Aeromonas* spp., potentially increasing disease emergence. These worms can be easily removed from broodstock by treating adult redclaw with a 30-minute salt bath at a salinity of 15, followed by a 90-minute fresh-water bath in a holding tank, before entering broodstock. Whilst future efforts could refine this process, in its current state, this rapid and low-cost method will remove these detrimental worms from broodstock and hatcheries, increasing production and sustainability in redclaw hatcheries.

Disclaimer: Some work described in this dissertation was completed by others (Dr. Graham Burgess, Alicia Maclaine, Dr. Chris Hauton), which is indicated by *[name of collaborator] in the appropriate methodology sections.

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Introduction

Fish production plays a crucial role in global food security, significantly contributing to nutritional requirements, accounting for approximately 17% of the global population's intake of animal protein (FAO, 2016). In 2014, aquaculture fish production for human consumption surpassed that of capture fisheries (FAO, 2016). The rapid growth of the aquaculture industry and its increasing importance as a food source is driving the need for resource efficiency and environmental responsibility of production (Boyd *et al*. 2007), both of which are crucial if the industry is to contribute to the nutritional requirements of our increasing global population.

Aquaculture and wild-caught crustaceans are a considerable proportion of total seafood production (Stentiford *et al*. 2012), with high nutritional and economic value. Crustaceans are a low-fat, high-protein food source that provides a valuable source of nutrition, particularly in coastal regions (FAO, 2016). In 2014, crustaceans accounted for 9% of total aquaculture production by weight but 23% by value; conversely, molluscs accounted for 22% of production by weight but only 12% by value (FAO, 2016). As such, there is growing interest in crustacean aquaculture, particularly with freshwater crayfish (Figure 1), with yabby (*Cherax destructor*), marron (*Cherax tenuimanus*), and redclaw (*Cherax quadricarinatus*) as the dominant species. Redclaw has several physical, biological and commercial characteristics ideal for aquaculture; it has rapid growth, reaching sexual maturity within 12 months, and its non-aggressive nature maintains high survival rates at high stocking density (Jones, 1989; Holdich, 1993; Masser and Rouse, 1997). Redclaw is also a hardy species with broad physiological tolerances particularly with respect to temperature, salinity and dissolved

oxygen (Holdich, 1993; Masser and Rouse, 1997), allowing for variable culture conditions without stock mortality.

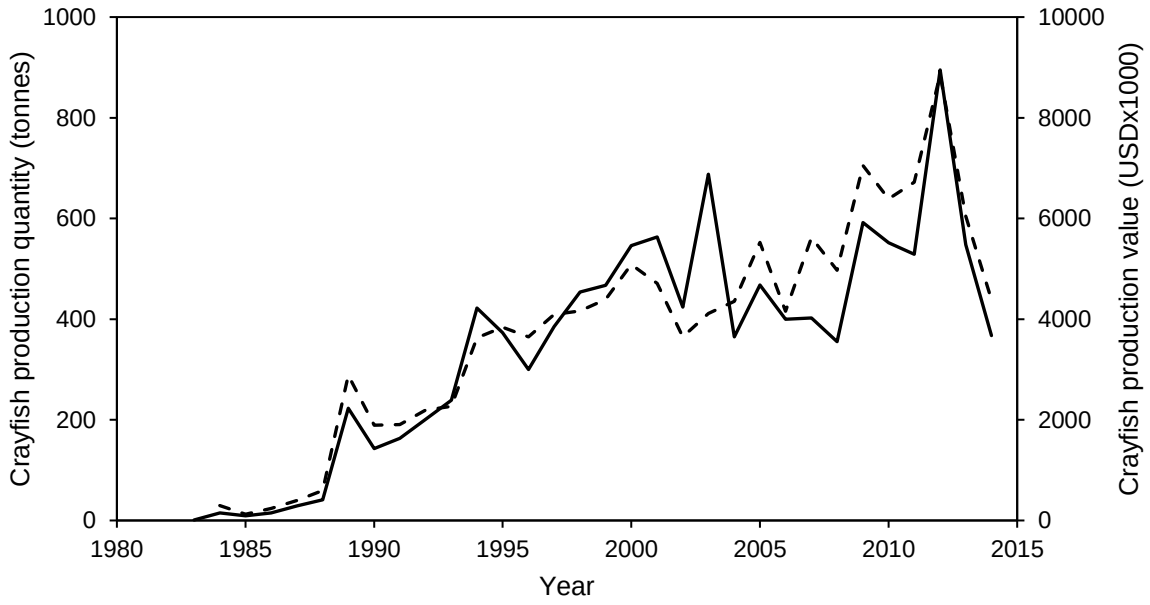

Figure 1: Aquaculture production quantity (solid line) and value (dashed line) of freshwater crayfish species listed in FAO database (Global aquaculture production – Quantity 1950-2015 and Value 1984-2015. Species included: Danube crayfish, Marron, Noble crayfish, Redclaw, Signal crayfish, Yabby, Euro-American crayfishes not elsewhere included)

The aquaculture industry in Australia has historically focused on the production of salmonids, edible oysters and pearl oysters (O'Sullivan, 1991). However, freshwater crayfish production is growing; in 1988, Australian crayfish aquaculture produced A$0.4m (0.3% of total Australian aquaculture), compared to A$2.6m from 1989-90 (1.3% of total), of which A$0.8 million was from hatcheries, and A$1.8 million from market (Holdich, 1993). Although significant production in Australia has not yet been reached, the potential for a successful redclaw aquaculture industry is great (Jones *et al*. 1994); the challenge is to maximise and sustain production to meet export demands.

Hatchery development

Hatchery and nursery technology has been developed for most farmed aquatic species (FAO, 2014), including crustaceans such as shrimp, lobster, freshwater prawn, crab and some freshwater crayfish (Nelson and Dendy, 1979; Malecha, 1983; Charmantier-Daures and Charmantier, 1991; Jackson *et al*. 1992; Jones, 1995; Kittaka, 1997). Implementing hatcheries in crayfish aquaculture addresses the key limiting factors on production; seed stock availability (Villarreal and Pelaez, 2000) and the intensity and inconsistency of pond rearing, hence the interest in the development of economical hatchery and nursery protocols is growing.

However, constraints on the success of hatchery and nursery facilities exist, most significantly of which is frequent and seemingly sporadic crop failures, thought to be caused by disease. Disease has caused substantial animal and economic losses in hatcheries and the industry (Walker and Winton, 2010), and the occurrence of outbreaks is expected to increase as the industry grows (Edgerton *et al*. 2002). Therefore, to improve global food security in the crustacean aquaculture industry and reduce production losses, a greater understanding of crayfish diseases and parasites is required, coupled with management efforts towards their eradication (Murray and Peeler, 2005; Ghanawi and Saoud, 2012; Stentiford *et al*. 2012).

Crustacean disease and parasites in aquaculture

The most significant threat to the continued expansion of aquaculture is disease and parasite emergence (Meyer, 1991; Bondad-Reantaso *et al*. 2005), exacerbated by aquaculture providing a conducive environment for disease emergence, establishment and transmission (Murray and Peeler, 2005). Decapod crustaceans at all life stages are susceptible to a variety of pathogens and parasites, including viral, bacterial,

fungal and metazoan (Longshaw, 2011; Stentiford *et al*. 2012). Whilst a variety of metazoan parasites are found on crustaceans (such as trematodes, cestodes, turbellaria and small parasitic crustaceans), the most severe losses to production are attributable to viruses and bacteria (Bower *et al*. 1994; Stentiford *et al*. 2012).

Several reviews have examined diseases specific to crayfish (Edgerton, 1999; Edgerton *et al*. 2002; Longshaw, 2011; Saoud *et al*. 2013), although much greater understanding of redclaw diseases is needed (Ghanawi and Saoud, 2012). Disease and parasite reviews often include fouling organisms such as Branchiobdellida and Temnocephalida, although they receive little attention in comparison to disease. This is due to their classification as ectocommensals or ectosymbiotes, rather than parasitoids or predators, which would cause harm to hosts and reduce production.

This study aims to investigate the ecological relationship between a *Decadidymus* temnocephalid and redclaw crayfish (*Cherax quadricarinatus*). The identity of this species is discussed, with regards to morphological characteristics and phylogeny. The epidemiology of this species is examined, in terms of its prevalence, abundance and impact on hatchery productivity, as a potential egg predator and pathogen vector. Lastly, this research explores possible management solutions for the control of this species.

Chapter 1: Species Characterisation

1.1 Introduction

Temnocephalids (phylum Platyhelminthes) are small, active flatworms found on fresh water crustaceans such as redclaw (Volonterio, 2009). They are characterised by the presence of eyespots, a posterior sucker and anterior processes used for attachment and movement around the host (Sewell and Whittington, 1995; Edgerton *et al*. 2002). Australia is considered the global centre of temnocephalan diversity, with 91 named temnocephalid species within 13 genera (Table 1, Sewell, 2013). Some temnocephalids have been examined in detail, such as *Diceratocephala boschmai, Temnocephala minor* and *Craspedella spenceri*, however most remain understudied.

One somewhat understudied group within the Temnocephalida is the genus *Decadidymus* (Family Diceratocephalidae). The only species currently listed within this genus is *Decadidymus gulosus* (Cannon, 1991), with a description of its gross morphology and key anatomical features such as reproductive systems, mouth, pharynx and gut. Since this description, the apomorphies of the Diceratocephalidae have been identified (Joffe *et al*. 1998). However, there is no published data on the molecular characterisation of *Decadidymus* species; suggested phylogenetic placement of *Decadidymus* within the Diceratocephalidae is based on morphological data (Figure 2, Joffe *et al*. 1998).

This investigation aims to identify a temnocephalid that occurs in redclaw crayfish broodstock. For the first time, a genetic sequence of a *Decadidymus* species was provided. Combining molecular tools with morphological characteristics of the species in question, the possibility of a novel species is discussed.

Table 1. Taxonomic diversity of Australian Temnocephalida (from Sewell, 2013).

Family	**Subfamily**	**Genera**	**Number of species**
Didymorchiidae	-	*Didymorchis*	2
Actinodactylellidae	-	*Actinodactylella*	1
Diceratocephalidae	-	*Diceratocephala*	1
Diceratocephalidae	-	*Decadidymus*	1
Temnocephalidae	-	*Achenella*	2
Temnocephalidae	-	*Temnomonticellia*	5
Temnocephalidae	-	*Temnosewellia*	52
Temnocephalidae	-	*Temnohaswellia*	12
Temnocephalidae	-	*Notodatylus*	1
Temnocephalidae	Craspedellinae	*Craspedella*	9
Temnocephalidae	Craspedellinae	*Gelasinella*	1
Temnocephalidae	Craspedellinae	*Heptacraspedella*	1
Temnocephalidae	Craspedellinae	*Zygopella*	3

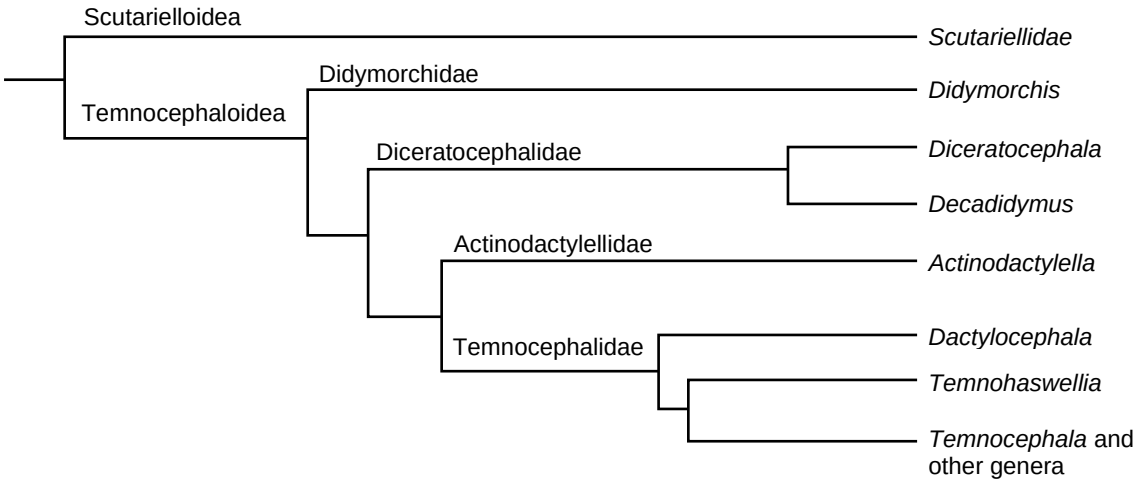

Figure 2: Cladogram representing phylogeny of Temnocephalida based on apomorphies (from Joffe et al. 1998).

1.2 Sampling, materials and methods

Redclaw were obtained from indoor, climate controlled broodstock tanks from a hatchery in Atherton, North Queensland during the wet season (Jan-Mar 2016). *Decadidymus* specimens were separated from adult crayfish and their eggs following egg-stripping of berried females. The abdomen and cephalothorax of the crayfish were examined and any remaining worms were removed with fine forceps.

1.2.1 Morphology

Decadidymus specimens were held alive in 40ml sterile physiologically-buffered saline (0.9% NaCl solution) (PBS) for no longer than an hour before subsequent treatments. Worms required anaesthetising before exposure to fixative to prevent body distortion. Anaesthetisation trials were conducted to determine the most effective method (Appendix A); the selected method was dropwise addition of room-temperature 95% ethanol to a specimen jar containing worms and 20ml of sterile water, until activity ceased and worms became fully relaxed. All morphological characteristics identified were compared to Temnocephalida morphology (Table 2) and dorsal facies (Figure 3) to identify the species to genus level.

Whole mounts and staining

Following anaesthetisation, worms were washed in PBS three times. Smaller worms were fixed in a specimen jar containing 40ml 10% neutral-buffered formalin (NBF). Larger worms were positioned between two glass slides separated by Vaseline™ so as not to destroy the specimens, and placed inside a screw-cap Coplin Jar containing 10% NBF, producing slightly flattened fixed specimens for whole mounting of larger specimens. Specimens were transported to laboratories in 10% NBF, for staining and mounting. Worms were washed three times in PBS to remove traces of NBF. Some

were dehydrated through an ethanol series and mounted (Appendix B) without staining, whilst others were stained prior to dehydration and mounting. To determine the most effective method for staining specimens, staining trials were conducted (Appendix B); the chosen protocol was staining with Mayer's Paracarmine (Gray, 1954).

Histology

Histological preparation and analysis was conducted according to (Bancroft and Gamble, 2008). Briefly, fixed worms were placed in cassettes in a fluid-transfer tissue processor (Thermo Scientific Excelsior ES Tissue Processor) to infiltrate worms with paraffin wax during automated cycles of dehydration, clearing and wax infiltration. Specimens were then embedded in paraffin wax (Microm Modular Tissue Embedding Center EC350-1 and Cryo Console EC350-2), with careful specimen orientation. Paraffin blocks containing specimens were hardened on ice and sectioned at 5µm (Manually-Operated Rotary Microtome CUT 4060). Sections were transferred to a flotation bath held at 60^{o}C to manipulate sections onto glass slides, which were then fixed in a dry oven at 60^{o}C. Fixed slides were exposed to a series of xylene, ethanol and stains (Appendix C). Stained slides were mounted by adding a few drops of DPX mountant and a coverslip (removing any bubbles), and air dried for 48 hours.

Scanning electron microscopy (SEM)

Reagents used for SEM sample preparation must be handled in controlled laboratory conditions under fume hoods, hence live *Decadidymus* specimens were transported to laboratories. Reagents were prepared (Appendix D) prior to anaesthetisation and fixation. Approximately 25 worms were anesthetised, and then fixed in 2.5% glutaraldehyde in a sodium cacodylate buffer (0.2M, pH7.2), before conducting

standard SEM sample preparation (Appendix D). A JEOL Scanning electron microscope (JSM-5410LV) was used to capture images, adjusting the position and orientation of the specimen within the vacuum by moving, tilting and rotating the specimen stage, and adjusting the quality of the image using resolution, magnification, and contrast and brightness settings.

Table 2. Morphological characteristics to identify Temnocephalida to genus (Sewell, 2013).

Genus	Locomo-tory cilia	Number of tentacles	Medial tentacle bulb-shaped	Dorsal scales	Number of dorsal papillate ridges	Ciliated papillae in rows on tentacles	Number of pairs of testes
Didymorchis	Y	0	-	N	0	N	1
Diceratocephala	Y	2	-	N	0	N	1
Decadidymus	N	2	-	N	0	N	10
Actinodactylella	N	12	-	N	0	Y	2
Temnohaswellia	N	6	N	N	0	N	2
Temnomonticellia	N	5	Y	N	0	N	2
Temnosewellia	N	5	N	N	0	N	2
Achenella	N	5	N	N	0	N	1
Notodactylus	N	5	N	Y	0	N	2
Zygopella	N	5	N	N	1	Y	2
Gellasinella	N	5	N	N	2	Y	2
Craspedella	N	5	N	N	3	Y	2
Heptacraspedella	N	5	N	N	7	Y	2

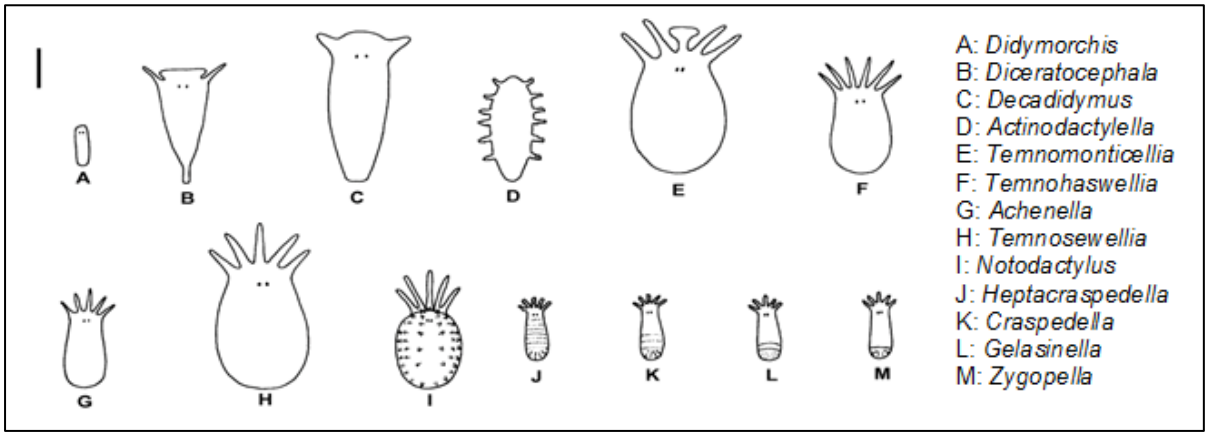

Figure 3: Dorsal facies of Australian Temnocephalida. Scale bar = ~1 mm (Sewell, 2013).

1.2.2 Molecular characterisation *[Dr. Graham Burgess and Alicia Maclaine]

Once separated from crayfish eggs, specimens were immediately washed in sterile PBS five times and preserved in 80% ethanol (no anaesthetisation required). DNA was extracted (Bioline ISOLATE II Genomic DNA Kit), and primers chosen (Table 3) produced amplicons of 28S and 18S ribosomal RNA sequences. PCR products were sequenced (Macrogen Inc. South Korea), and sequences were assembled using Geneious 9.1 (Biomatters Ltd New Zealand). A search on NCBI BLAST returned the sequences with greatest homology to the sequence of this study species.

Table 3: Primers used to sequence Decadidymus valverdi sp. nov.

Primer name	Primer sequence	Sequence amplified	Expected size (bp)	Purpose	Reference
U178	GCACCCGCTGAAYTTAAG	28s	1525	PCR	Lockyer *et al.* 2003
L1642	CCAGCGCCATCCATTTTCA				
U1148	GACCCGAAAGATGGTGAA	28s	1391	PCR	Lockyer *et al.* 2003
L2450	GCTTTGTTTTAATTAGACAGTCGGA				
FW-28s-1322-F	AGCAGGTCTCCAAGGTTA	28s	1332	PCR	This study
FW-28s-1322-R	ACTTAGAGGCGTTCAGTCT				
FW-28s-401-F	AGTAACGCAGGTGTCCAA	28s	401	Sequencing	This study
FW-28s-401-R	CTCTCGTACTGAGCAGGATTA				
FW-18s-1786 F	GTCTCAAAGATTAAGCCATGC	18s	1786	PCR	This study
FW-18s-1786 R	CGGAAACCTTGTTACGACTT				
FW-28s-570-F	AGAACTGGCACGGACAAG	18s	570	Sequencing	This study
FW-28s-570-R	GCTCACCTTTGGACACCT				

1.3 Results

1.3.1 Morphology

This study species belongs to the *Decadidymus* genus; specimens possess the three apomorphies of the Diceratocephalidae family (Joffe *et al*. 1998), as well as the morphological characteristics of *Decadidymus* (Table 2 and Figure 3). Features of these study specimens consistent with the *Decadidymus gulosus* description (Cannon, 1991) include; heavy-bodied worms, 3-4mm in length, a posterior, muscular adhesive disk, and excretory pores on each side on the anterolateral margin (Figures 4 and 5). The presence of two prominent anterior eyespots, sensory papillae covering the body (particularly on the tentacles and posterior sucker), 10 pairs of testes and a stylet (Figures 4 and 5) are also features consistent with Cannon's description of *D. gulosus*.

However, there are notable differences between Cannon's description of *D. gulosus* (1991) and these study specimens; the location and size of the mouth, the relative size of the pharynx compared to total body length, the size and location of the gonopore, the location of the excretory pores and the size of the stylet (Figures 4, 5 and 6, Table 4). These features are consistent across all worms that were examined morphologically (approximately 80 worms across wholemount, histology and SEM examinations). Furthermore, SEM images of *D. gulosus* (Cannon, 1991) showing overall body plan and position of the mouth appear vastly different to the SEM images produced in this study (Figure 6). As such, the name *Decadidymus valverdi* sp. nov. is proposed.

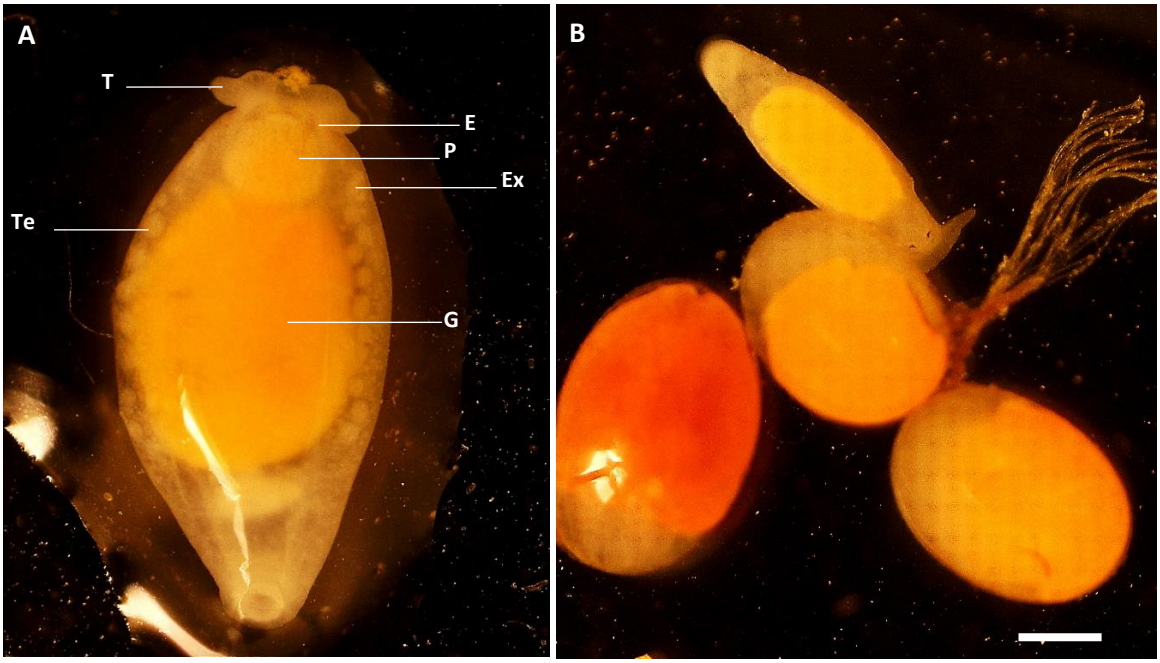

Figure 4: Live Decadidymus valverdi sp. nov. under dissection microscope A) Key morphological characteristics; E, eyespot; Ex, excretory organ; G, gut; P, pharynx; T, tentacle; Te, testes. B) With crayfish eggs from host. Scale bars approximately 1mm.

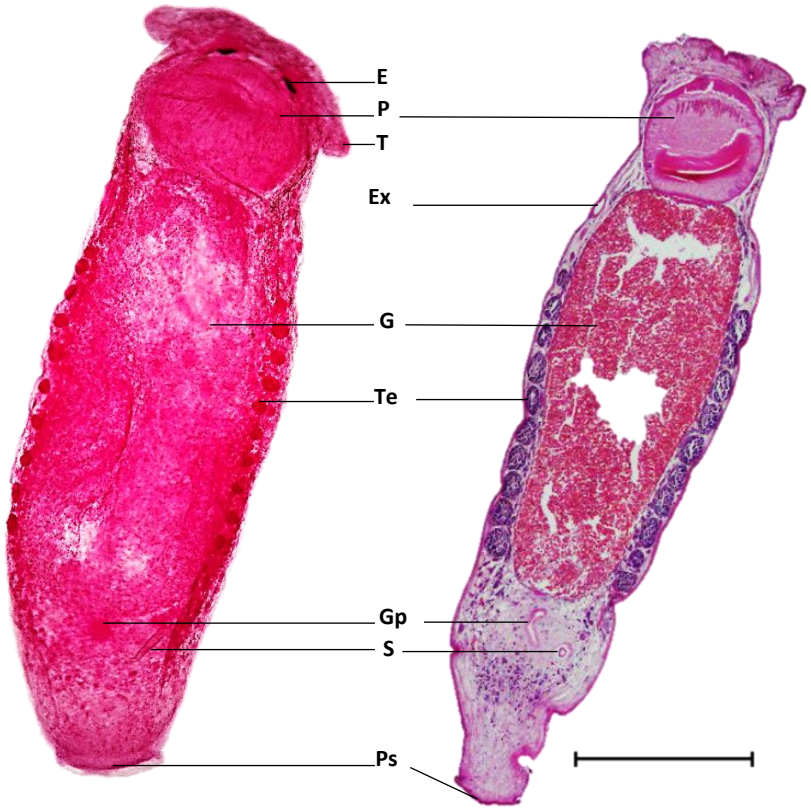

Figure 5: Left) Decadidymus valverdi sp. nov. whole mount stained with Mayer's paracarmine. Right) 5µm section stained with Hematoxylin and Eosin. E, eyespot; Ex, excretory organ; G, gut; Gp, gonopore; P, pharynx; Ps, posterior sucker; S, stylet; T, tentacle; Te, testes. Scale bar approximately 1mm.

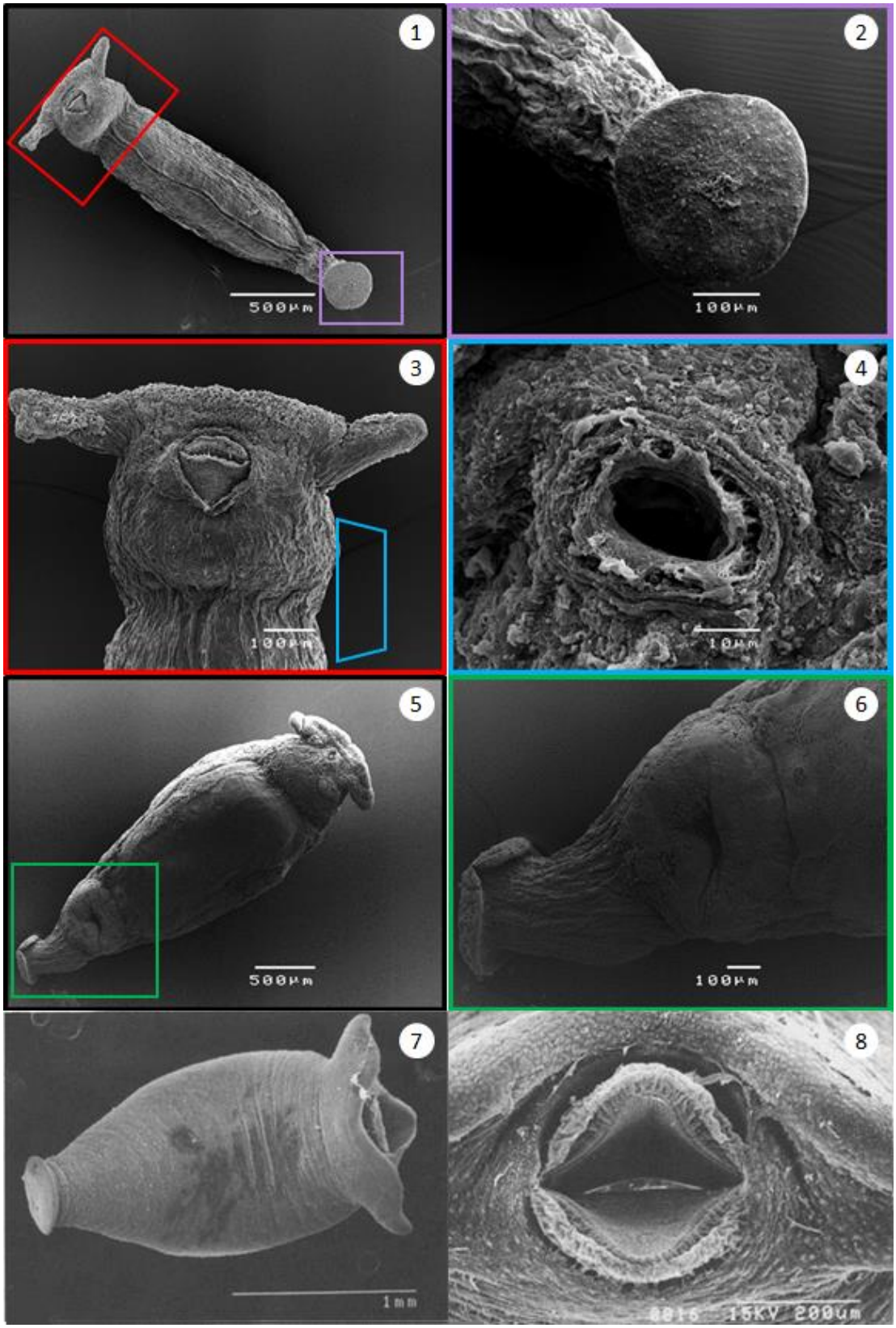

Figure 6: SEM images of Decadidymus valverdi sp. nov. (1-6) and Decadidymus gulosus (7-8, Cannon, 1991). 1) Whole worm, ventral surface, 2) posterior sucker 2, 3) ventral surface of head, 4) excretory pore, 5) whole worm, ventral surface, 6) gonopore. 7) Whole worm (D. gulosus) dorsal surface, 8) detail of pharynx and anterior end showing non-cellular 'lip' and small papillae especially dense on anterior margin.

Table 4: Morphological differences between Decadidymus valverdi sp. nov and D. gulosus.

Feature	***Decadidymus gulosus* (Cannon, 1991)**	***Decadidymus valverdi sp. nov.* (This study)**
Excretory pore	"Just posterior to level of eyespots"	Level with posterior pharynx margin
Gonopore	"180µm, one-third from posterior end"	150µm, one-fifth from posterior end
Mouth	"Subterminal, large opening slightly below anterior margin that joins tentacles. About 300µm wide, opens below frontal margin, pharynx projects from it"	Ventral surface of head, comparatively small opening (inner lips about 150µm wide), opens much further below frontal margin. Appears to have inner and outer lips.
Pharynx	"Large, about one-third of body, consists of 2 large muscle blocks"	Comparatively much smaller, about one-sixth of body, 700µm long.
Stylet	"About 270µm long, curves at about 180µm from its base"	About 450µm long, curves at about 250µm from its base

1.3.2 Phylogeny

18S primers successfully amplified a sequence of 1700bp (Appendix G), which showed highest homology with *Diceratocephala*, *Temnosewellia* and *Didymorchis* (Table 5). 28S primers successfully amplified a sequence of 3363bp (Appendix G), which showed highest homology with *Temnosewellia* and *Didymorchis* (no 28S sequence for *Diceratocephala* available) (Table 6). Sequences will be published on Genbank (available at: https://www.ncbi.nlm.nih.gov/genbank), however submissions are not yet complete (hence Accession 'TBD' in Tables 5 and 6).

Table 5: Sequence homology of the 18S small subunit ribosomal RNA gene - partial sequences of Decadidymus valverdi sp. nov. with 10 closest sequence matches on BLAST.

Accession	**Species**	**% Identity**
TBD	***Decadidymus valverdi sp. nov.* Australia 2016**	**100.0**
KC517073	*Diceratocephala boschmai* Host crayfish Thailand 2013	93.1
AJ012520	*Temnocephala* sp. Host freshwater crustacean 1999	92.1
AY157183	*Temnosewellia minor* Host *Cherax destructor* Australia 2003	91.7
AY157182	*Didymorchis* sp. Australia-DTJL-2002	90.6
AF051332	*Temnocephala* sp. Spain 1998	90.1
KC529481	*Carcharodopharynx* sp. NVS-2013 isolate UH157.4	83.8
KC529500	*Rhynchomesostoma rostratum* isolate UH77.15	83.8
KC529499	*Rhynchomesostoma rostratum* isolate UH96.3	83.7
AY775777	*Castrella truncata* 2004	83.7
KC529486	*Strongylostoma devleeschouweri* isolate UH77.2	83.4

Table 6: Sequence homology of the 28S small subunit ribosomal RNA gene - partial sequences of Decadidymus valverdi sp. nov. with 10 closest sequence matches on BLAST.

Accession	**Species**	**% Identity**
TBD	***Decadidymus valverdi sp. nov.* Australia 2016**	**100.0**
AY157164	*Temnosewellia minor* UK 2002	87.8
AY157163	*Didymorchis* sp. Australia-DTJL-2002	85.6
KC869887	*Castrella truncata* USA 2013	76.3
KC869884	*Promesostoma cochleare* USA 2013	74.5
KC869882	*Kytorhynchus* sp. n. CEL-2014 USA 2013	74.1
AY157160	*Nematoplana* sp. Shelly River	74.1
KC869870	*Lithophora* gen. n. sp. n. CEL-2014	73.8
KC869868	*Polystyliphora karlingi*	73.6
KC869850	*Hoploplana californica*	72.9
AF342800	*Stylochus zebra*	72.9

1.4 Discussion

The morphology of this study species (Table 4) exhibits clear differences from that of *Decadidymus gulosus* (Cannon, 1991), hence the name *Decadidymus valverdi* sp. nov. is proposed. Whilst a complete taxonomic description of this proposed new species is required, the characteristics noted here are sufficient to justify this name. The distinct morphometric differences of this new species (compared with *Decadidymus gulosus*) are: 1) the ventral location and smaller opening of the mouth, which possesses inner and outer lips, 2) the large stylet, 3) the small pharynx relative to body size, 4) the excretory pores located further from the anterior of worm, and 5) the smaller gonopore located closer to the posterior of the worm.

A detailed taxonomic description of the only other species within the *Decadidymus* genus is available (*D. gulosus* Cannon, 1991), however there is no information on the features of *Decadidymus* that may differ at species level as there are no other previously described species. However, descriptions of new species within the *Temnocephala* genus exist, and are based on various morphological characteristics. New species are principally characterised by the reproductive complex, in particular the penile stylet (Volonterio, 2007), as seen in descriptions of *Temnocephala curvicirri* sp. nov. (Amato and Amato, 2005), *Temnocephala mertoni* n. sp. (Volonterio, 2007), *Temnocephala colombiensis* n. sp. (Garcés *et al*. 2013), *Temnocephala pereirai* n. sp. and *Temnocephala cuocoloi* n. sp. (Volonterio, 2010). However, other distinctive characteristics have also been used to describe new species within the *Temnocephala*, including; dorsolateral excretory syncytial plates, paranephrocytes, intestinal partitioning, and the position of excretory pores (Amato *et al*. 2003; Amato and Amato, 2006; Amato and Amato, 2007; Damborenea and Brusa, 2008; Garcés *et*

al. 2013). These other characteristics have significant taxonomic weight and will aid species identification (Volonterio, 2007), demonstrating that a variety of morphological characteristics are important in species descriptions within the Temnocephalida. Therefore, the features of this *Decadidymus* study species that have been described are considered sufficient to propose the novel species *Decadidymus valverdi* sp. nov., and to justify a future taxonomic description of this species.

The genetic sequence obtained from this study species shows greatest homology with other temnocephalids and confirms its position within the Temnocephalida (Tables 5 and 6). Sequence homology comparisons cannot be made between *D. gulosus* and *D. valverdi* sp. nov., as there are no published sequences for *D. gulosus*; originally described by Cannon (1991), type species are held at the Museum of Queensland only as wholemounts and serial sections (none preserved in alcohol), hence sequencing cannot be undertaken without prohibited destructive sampling. Nonetheless, a new species, *Decadidymus valverdi* sp. nov., is proposed based on the significant morphological characteristics, and a genetic sequence is provided, aiding future research in resolving the molecular phylogeny within the Temnocephalida.

Chapter 2: Impacts

2.1 Introduction

Temnocephalids, universally described as ectocommensals, occupy specific sites on host crayfish, such as the carapace or gill chamber (Edgerton *et al*. 2002). Most commonly found feeding on fouling organisms including other temnocephalids (Longshaw, 2011), there has been no evidence of parasitism within the Temnocephalida (Jennings, 1971). The only exception to this is *Scutariella didactyla*, which feeds on host body fluids (Jennings, 1971). Whilst another temnocephalid species (*Diceratocephala boschmai*) has been observed feeding on damaged crayfish eggs, it was not considered to play a significant role in crayfish egg mortality (Jones and Lester, 1993).

A few studies have examined impacts of temnocephalids on crayfish, including egg predation by *D. boschmai* (Jones and Lester, 1993), host asphyxiation when inhabiting the gill cavity (Sammy, 1988; Edgerton *et al*. 2002), and the impact on crayfish aesthetics and marketability (Herbert 1987). The presence of temnocephalids may also provide a niche for bacteria and other epibiota (Jennings, 1971), which can affect ventilation of crayfish eggs, as well as increase the emergence of disease in the system. However, stock mortalities from temnocephalid infestations have never been reported, despite heavy infestations (Herbert 1987; Edgerton *et al*. 2002). This suggests that either these temnocephalid impacts are not significantly reducing production, or the causative agent of crop failure has been previously misidentified.

These rare studies on temnocephalid impacts typically focus on individual species, leading to the potential to overlook significant impacts of the lesser-known

Temnocephalida. The impacts of *Decadidymus* spp. on crayfish health and aquaculture production remain unstudied. Since its description, few publications have investigated this species, with one study on its ultrastructure and spermiogenesis (Watson *et al*. 1995) and one on the phylogeny within the Temnocephalida including *Decadidymus* (Joffe *et al*. 1998). It is essential to understand the impacts of *Decadidymus vavlerdi* sp. nov. in hatcheries, due to their suggested egg predation and pathogen transmission. To assess its potential impact on redclaw juvenile survival and hatchery productivity, this study examined its prevalence, abundance and infestation intensity, its feeding behaviour, and its bacterial load hence its potential to act as a pathogen vector between facilities and individuals.

2.2 Materials and methods

2.2.1 Prevalence, abundance and intensity

Most worms were attached to crayfish eggs by their posterior sucker and were therefore removed from the females as the eggs were stripped. Worms and crayfish eggs were placed in a labelled container for counting. The crayfish abdomen and cephalothorax were subsequently examined for any remaining worms, which were removed with fine forceps and placed into the container. When collecting *Decadidymus valverdi* sp. nov. specimens, their location on host crayfish was noted. The colour of the worm gut was also photographed and described in comparison to crayfish egg yolk. The sex and weight of each crayfish, the number of worms and the number of crayfish eggs per brood were recorded. The prevalence, abundance and intensity of worm infections was analysed relative to these factors.

Mean worm burden (zeros included) and mean worm intensity (zeros excluded) were calculated from abundance data for berried and unberried crayfish. All other statistical analysis was performed using transformed worm abundance data (SQRT+1), which produced a normally-distributed data set (Anderson-Darling test, AD=7.976, $P < 0.005$, $\alpha=0.05$). To compare transformed worm abundance data against crayfish (berried female, unberried female or male), Kruskal-Wallis One Way Analysis of Variance on Ranks was required (data failed test for equal variance; F-test $P < 0.05$, $\alpha=0.05$), and pairwise comparisons used Dunn's Method. Transformed abundance data was compared against the weight of crayfish and number of eggs per crayfish using Pearson's Correlation (normally-distributed data).

2.2.2 Feeding behaviour

In vitro observations

A feed trial was conducted with 12 live worms to monitor their activity, interaction with and predation on crayfish eggs. Each specimen jar contained one worm and three redclaw eggs in approximately 30ml ozone-treated freshwater held at 26^{o}C. Four worms were incubated with live eggs, four with live eggs that were punctured just before the trial began, and four with intact dead eggs; eggs were determined as live or dead by observing movement under a dissection microscope. Worms and eggs were monitored and photographed every 24 hours under a dissection microscope to determine if eggs were damaged or lost volume, and if any changes to worm gut (size or colour) were visible. A full water exchange was completed for each specimen jar after observations.

Gut content analysis

To determine if these worms feed on bacteria amongst the egg mass, sections of paraffin blocks from histological morphology studies were stained for the presence of bacteria (Gram-Twort); dark blue or pink stained gut contents would indicate the presence of bacteria. Sections were cut to 5µm, heat fixed onto glass slides, dehydrated, cleared, stained, infiltrated with xylene and mounted (Appendix C). An unstained slide, with tissue containing Gram positive and Gram negative bacteria was taken through the above protocol, providing a positive control for the staining procedure. In this protocol, Gram-positive bacteria are stained dark blue, Gram negative are stained pink, nuclei are stained red and cytoplasm is stained light green.

To determine if these worms feed on crayfish egg yolk, cryosections were stained for the presence of lipids (Herxheimer's). Approximately 20 worms and 20 crayfish eggs were transported back to laboratories alive. Specimens were anaesthetised and

transferred to a cryostat (Leica CM1850 Cryostat). Worms were embedded in Jung Tissue freezing medium, sections were cut to 5µm and transferred to glass slides, all within the cryostat. Sections were fixed with FAA (formaldehyde–acetic acid–ethanol) for two minutes before staining (Appendix C) and mounting in aqueous mounting media (Grey and Weisse). Lipids are stained bright red, nuclei are stained blue. This process was repeated for crayfish eggs, acting as a positive control by confirming the presence of lipids within the egg.

Molecular analysis *[Dr. Chris Hauton]

Gut contents were removed from 10 worms under a dissection-microscope and collected in a single sterile microcentrifuge tube for DNA extraction (Qiagen DNeasy Blood & Tissue Kit) according to the manufacturer's recommendations without modification. PCR was undertaken using 18S ribosomal RNA gene nested primers (Table 7). PCR conditions were optimised and amplicons were gel extracted (Qiagen QIAquick Gel Extraction Kit) and cloned (Promega pGEM®-T Easy Vector Systems). DNA plasmids from positive colonies (determined by blue-white screening) were extracted (Qiagen QIAprep® Miniprep) and the size of the cloned inserts was tested by standard colony PCR methods using M13 primers and size fractionation on an agarose gel. Extracted plasmids were sequenced (SourceBioscience Lifescience, Nottingham), and the returned sequence was searched in NCBI BLAST, returning the sequences with highest homology to that isolated in this study.

Table 7: Primers used for gut contents molecular analysis and size of target amplicon.

Primer Name	Primer Sequence	Expected size (bp)
23F – primary	AAATTACCCACTCCCGGCAC	901
23R – primary	GTTGGTGGAGTGATTTGTCTGG	
25F – nested	TGCTTACTGTCACGCTCCGAAC	279
25R – nested	GTGAAATTCTTGGACCGTCGCA	

2.2.3 Bacteriology

It is important to understand the bacterial load of these worms with respect to its role in pathogen transmission between broodstock and hatcheries. Bacterial isolates from these worms were identified to species level (where possible), to determine if these worms could act as a pathogen vector for strains thought to cause frequent and sporadic batch failures in hatcheries. If these worms do possess such pathogens, their removal from aquaculture facilities is required to reduce production losses.

Ten worms were removed from each of two crayfish (20 worms total), and were washed three times with sterile physiological saline (PBS) to remove most bacteria on the epidermis originating from the environment or crayfish host. Primary plates were prepared on site; the ten worms from each crayfish were homogenized in a sterile environment, swabbed and plated onto a tryptone soy agar (TSA) plate. Inoculated primary plates were incubated at 30^{o}C for 24 hours, and bacterial growth on primary plates was quantified according to Drew (1997); 3+ characterised by heavy bacterial growth on primary and secondary streaks; 2+ by heavy bacterial growth only on primary streak; 1+ has 10 to 60 colonies on the entire plate; 1 has less than 10 colonies on the entire plate; and 0 has no bacterial growth on any area of plate. After 24 hours of incubation at 30^{o}C, primary plates were incubated at 4^{o}C and transported to laboratories within 24 hours for subculturing.

Primary plates were examined and subcultures of all distinct colony types were prepared under sterile conditions. A single colony of each type was removed from the primary plate and streaked onto separate sterile TSA plates which were incubated for 24 hours at 30^{o}C, producing monocultures that could be identified.

All monocultures were identified to species level where possible. Initial characterisation of cultures was achieved by Gram staining (Gram, 1884) (Appendix E), identifying cultures as Gram positive or negative, and characterising the shape of the bacteria. Oxidase and catalase tests were also conducted, before biochemical tests could be used. Biochemical tests (bioMérieux API 20E and 20NE) were used to identify Gram negative bacterial isolates down to species level; Gram negative, oxidase positive isolates required API 20NE tests, and Gram negative, oxidase negative isolates required API 20E tests (Appendix E). Results were entered into an online database for identification, and species identification was accepted if the confidence level was above 95%. Gram positive isolates cannot be identified using the above biochemical tests, so were identified by molecular techniques **[Dr. Graham Burgess and Alicia Maclaine]*. Once identified, bacterial isolates were preserved immediately by freezing each of the strains on polypropylene beads at -80^{o}C (Appendix E).

2.3 Results

2.3.1 Prevalence, abundance and intensity of *Decadidymus valverdi* sp. nov.

No *Decadidymus valverdi* sp. nov. specimens were found anterior of the abdomen; all were located amongst the egg mass of berried crayfish, often attached to the eggs, and occasionally to the abdomen of berried and unberried crayfish. The prevalence of worms on unberried crayfish (male and female) was 16% (n=37), whereas prevalence reached 80% on berried crayfish (n=97). The severity of infestations (mean worm intensity) also differed between these groups, with mean intensity of 1.2 worms on unberried crayfish (n=6), compared to 15.6 worms (n=78) on berried crayfish. Although one berried female was found with 103 worms, the most frequent worm abundance grouping on berried females was 20-39 worms (Figure 7).

Mean worm burden was significantly higher (H_2 = 49.479, P < 0.001, α=0.05) on berried females than unberried female and male crayfish (which showed no significant difference between the two) (Figure 8). Worm abundance showed a significant positive correlation with the number of eggs per crayfish (r = 0.282, n = 97, P = 0.048) (Figure 9). There was no significant correlation between worm abundance and crayfish weight (r = -0.064, n = 97, P = 0.536).

It should be noted that whilst examining the crayfish for *D. valverdi* sp. nov., other temnocephalid species were present, including *Diceratocephala boschmai, Temnohaswellia* spp. and *Notodactylus handschini*, although these were all in much lower abundances (< 10 per crayfish) than *D. valverdi* sp. nov..

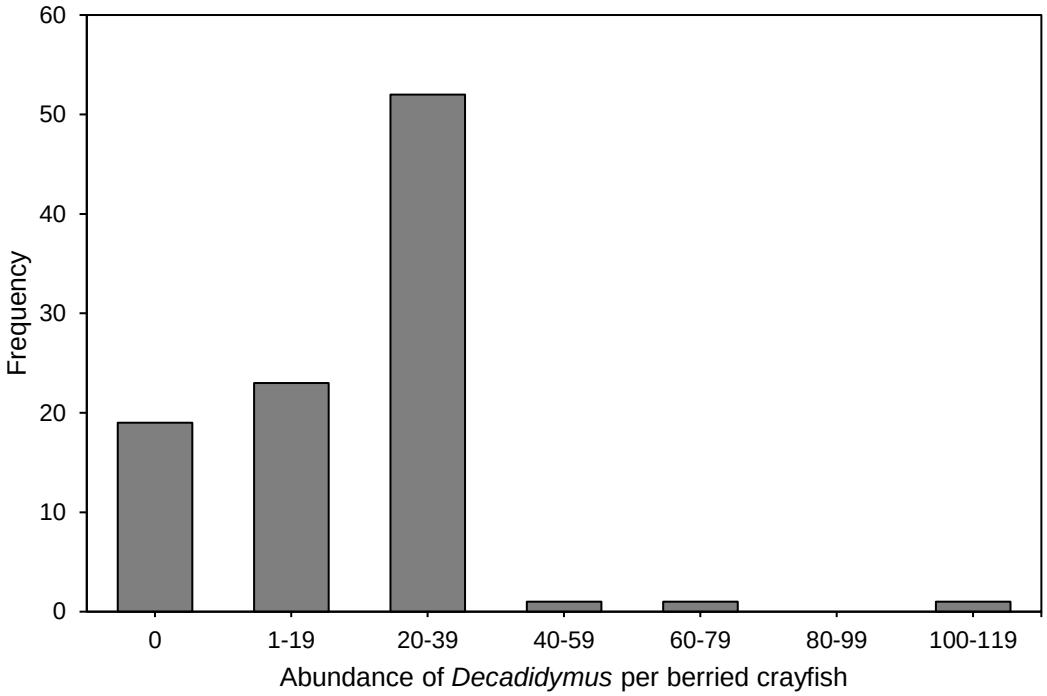

Figure 7: Decadidymus valverdi sp. nov. abundance (grouped) per berried female redclaw (n=97).

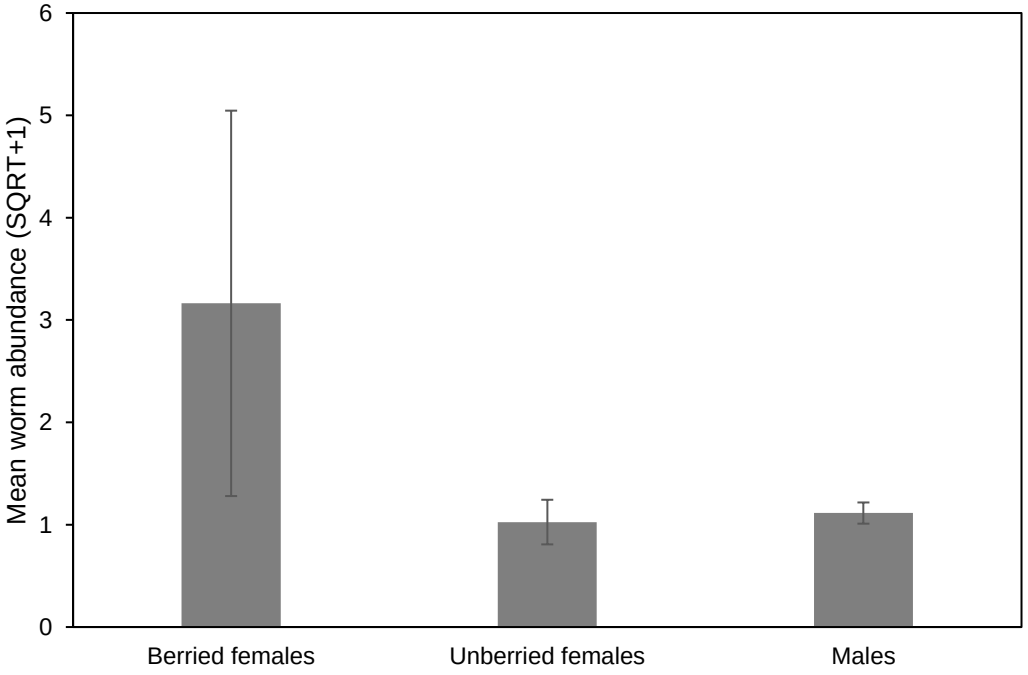

Figure 8: Mean Decadidymus valverdi sp. nov. abundance on berried female (n=97), unberried female (n=16) and male (n=21) redclaw. Error bars represent standard deviation.

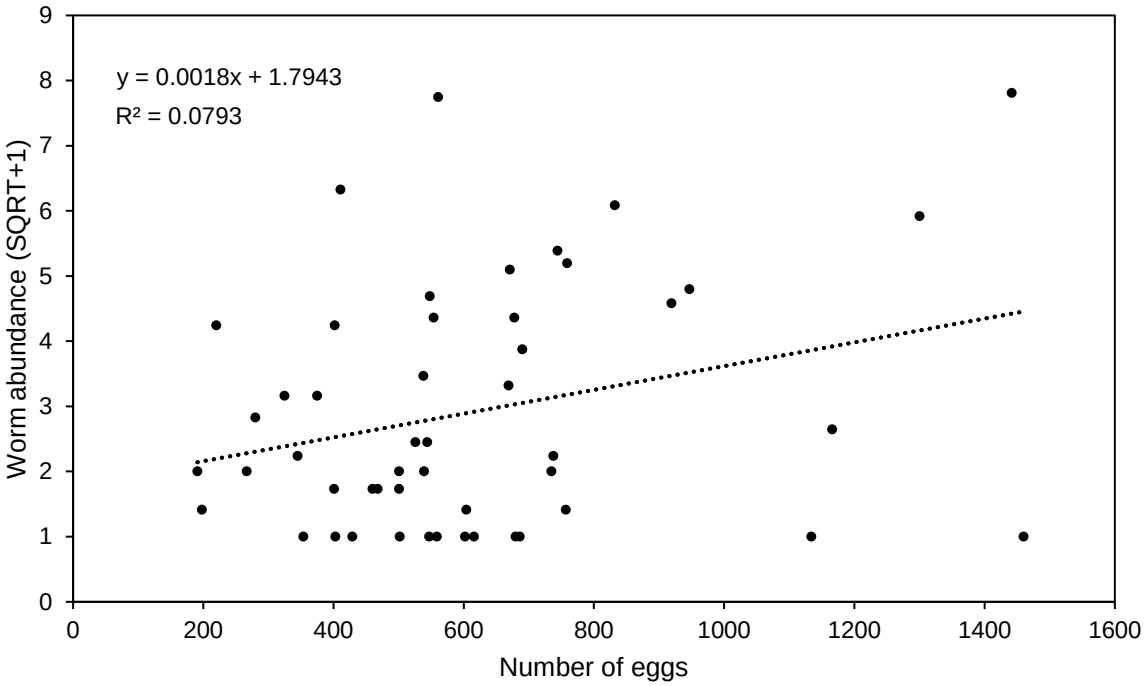

Figure 9: Decadidymus valverdi sp. nov. abundance (SQRT+1) as a function of the number of crayfish eggs per host brood, with correlation equation and R2 value (r = 0.282, n = 97, P = 0.048).

2.3.2 Egg predation

As previously stated, *Decadidymus valverdi* sp. nov. specimens were consistently located amongst the egg mass of berried crayfish, often attached to the eggs. The gut contents of the worms had the same colouration as redclaw eggs (Figure 4, Chapter 1). The worms did not appear to feed on eggs during the feed trial, regardless of state (live, dead or punctured). Most worms interacted with the eggs by attaching to the egg surface with their posterior sucker, however none of the eggs visibly lost volume or showed signs of perforation under the dissection microscope. All worms in the punctured and dead egg treatment groups died within 2 and 4 days of the trial respectively, possibly due to poor water quality (even with regular water exchanges) (Table 8). None of the worms incubated with live eggs died during the trial (Table 8).

Gram-Twort stained sections show no bacteria present in the gut contents of these worms. Positive stains for bacteria on the control remove the possibility of a false negative. Lipid stained sections (Figure 10) confirm the ubiquitous presence of lipids in the gut of these worms, and in the crayfish egg control sections. Molecular analysis of the worms gut contents was unsuccessful in isolating crayfish DNA. Primary PCR produced putative amplicons which were cloned and purified for sequencing. Nested PCR did not produce amplicons of the target size; an indication of spurious primary PCR amplicons. The closet match of these amplicons in BLAST is for *Ostrea edulis* (European Flat Oyster) (99% identity, E = 0.0), and the top 30 matches were all in order Ostreoida (*Ostrea, Saccostrea, Crassostrea, Dendostrea*). This result is likely due to contamination.

Table 8: Worm mortality (number dead) in each treatment (n=4 per treatment) during the in vitro feeding trial.

Egg condition	**DAY 1**	**DAY 2**	**DAY 3**	**DAY 4**
Live	0	0	0	0
Punctured	0	1	3	4
Dead	0	4	4	4

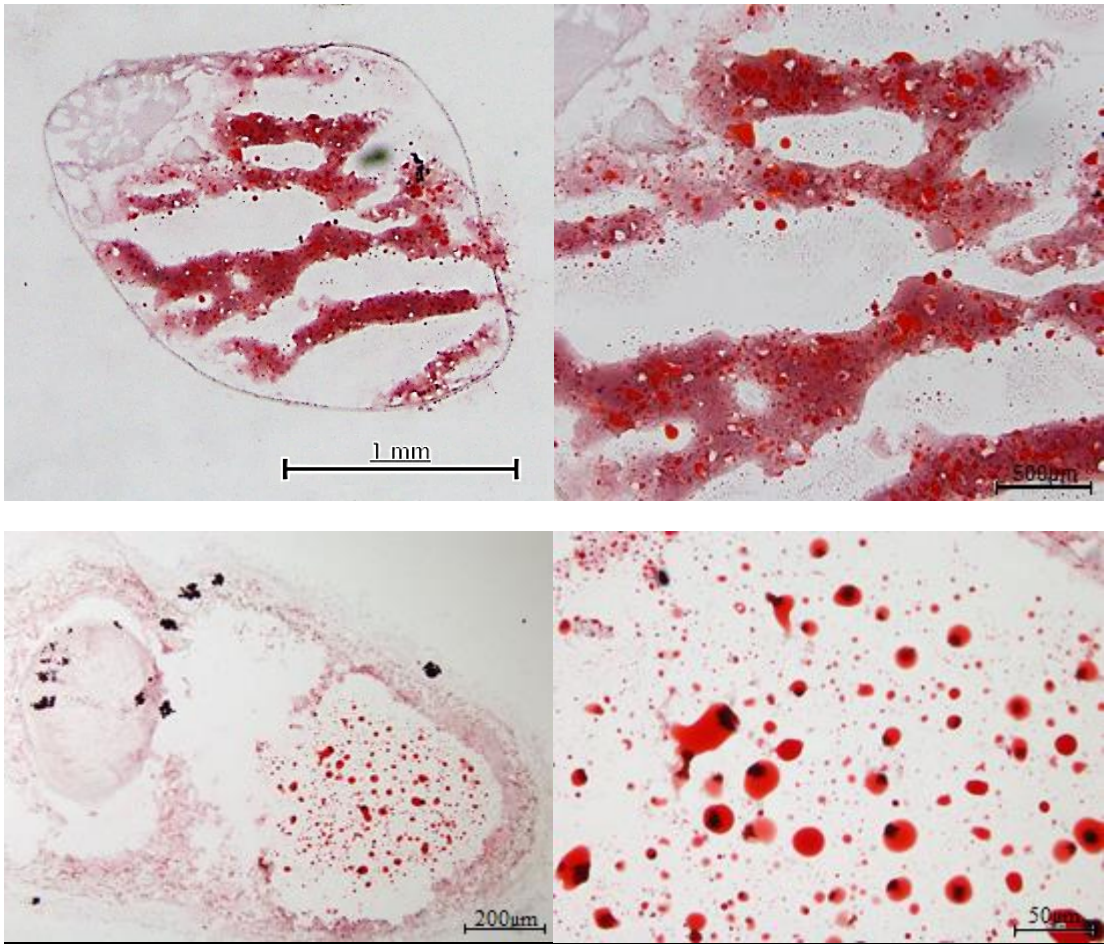

Figure 10: Herxheimer's lipid stained cryosections of (top) crayfish eggs and (bottom) worm gut contents. Left) overview of organism showing location of high concentrations of lipids. Right) magnified view of lipids.

2.3.3 Bacteriology

Decadidymus valverdi sp. nov. isolated from crayfish were found to carry high bacterial loads, with growth on primary plates characterised as 2+ on one plate and 3+ on the other. The following bacterial strains were isolated from primary plates and identified; *Aeromonas hydrophila*, *Aeromonas sobria, Paenibacillus* sp., *Lactococcus* sp., and *Shewanella putrefaciens*.

2.4 Discussion

2.4.1 Prevalence, abundance and intensity

A substantial ecological relationship between *Decadidymus valverdi sp. nov.* and redclaw eggs was found; identified by the worms' consistent location amongst the egg mass, the high prevalence of these worms on berried females (80% of broodstock were infested), and a significantly larger number of worms on berried redclaw than on unberried redclaw, male or female (Figure 8). The correlation between the infestation intensity and the number of eggs per crayfish brood (Figure 9) further supports this association between these worms and redclaw eggs. Additionally, worm infestation intensity is unaffected by the host crayfish weight, indicating that these worms are not directly dependent on adult crayfish resources, concurrent with their previous description as ectocommensals with regards to the adult host. It has been previously recorded that host grooming behaviours and molting can affect temnocephalid (*Diceratocephala boschmai*) populations on the host (Jones and Lester, 1996). This may account for the large variation of infestation intensities on berried females (Figure 8), but does not nullify the evident association between the presence and number of crayfish eggs and the infestation intensity of *D. valverdi* sp. nov.. This association is unlikely to harm the adult crayfish directly; whilst some temnocephalids may damage the host, such as by asphyxiation (Sammy, 1988; Edgerton *et al*. 2002), the consistent location of this species amongst the egg mass suggests they are unable to harm the adult host, consistent with findings of similar temnocephalid species (*D. boschmai*, Herbert, 1987).

Whilst the most common infestation intensity per crayfish was 20-39 (Figure 7), intensity surpassed 100 on one individual. Although host grooming behaviour reduces

the number of temnocephalids on the crayfish (Saoud *et al*. 2013), the efficacy of such behaviours could decline as infestation intensity increases. Moreover, infestations may harm developing crayfish eggs, as ventilation of the egg mass will reduce, and a niche for opportunistic pathogens is provided (Jennings, 1971). Furthermore, other impacts of this worm (as discussed below) will be exacerbated at high infestation intensities.

2.4.2 Feeding behaviour

Clear evidence for the consumption of redclaw egg yolk by *D. valverdi* sp. nov. is demonstrated in the lipid-stained cryosections of the worm gut and crayfish egg contents, confirming high lipid concentrations in both (Figure 10). Crustacean egg yolk is known to contain mostly lipids, proteins and carbohydrate (Adiyodi, 1985), supporting these findings. Whilst most temnocephalids feed on other fouling organisms (Longshaw, 2011), the high concentration of lipids found in the guts of *D. valverdi* sp. nov. indicate that this is not true of this species, as the gut contents would not be almost exclusively lipids if they were feeding on whole bodies of other ectocommensals. The high lipid concentration of the yolk likely resulted in molecular methods failing to isolate redclaw sequences from the gut contents of the worm; the scarcity of redclaw 18S target amplicon in the yolk consumed by these worms led to the production of spurious amplicons later identified as *Ostrea edulis*, a known contaminant in the facilities used. Future research should explore lipid profiling techniques to confirm the origin of the lipids present in the gut of these worms.

Additionally, the aforementioned association between these worms and redclaw eggs, the location of the worms amongst the egg mass (rather than the dorsal carapace which most temnocephalids inhabit), and the remarkable similarity between the gut colour of these worms and the crayfish egg yolk further suggests that these worms rely on redclaw eggs as a food source. Herbert (1987) noted a similar resemblance between host crayfish eggs and the gut colour of *D. boschmai*, which was later found feeding on damaged crayfish eggs (Jones and Lester, 1993), substantiating the link between gut colour and yolk ingestion.

During the *in vitro* feed trial, *D. valverdi* sp. nov. interacted with redclaw eggs, but did not feed on the yolk. Reasons for this (when other evidence points towards yolk ingestion) may include; the worms were not starved for long enough prior to trial commencement, the artificial environment deterred usual feeding behaviours, or the rapid mortality in punctured and dead egg treatments due to poor water quality led to early mortality before the need to feed arose. Despite the failure of the feed trial to witness the consumption of egg yolk by these worms, the above evidence is sufficient to determine that these worms feed on redclaw egg yolk. If future research can monitor these worms feeding on crayfish eggs *in situ*, the extent to which this affects redclaw juvenile survival and hatchery productivity should be examined, to quantify the magnitude of the impact this *Decadidymus* species has on redclaw crayfish hatcheries. Nonetheless, the evidence provided highlights the importance of investigating management techniques to eradicate this species from broodstock and hatchery facilities.

2.4.3 Bacteriology

Quantification of bacterial growth on primary plates exhibits the high bacterial load of these worms. Most bacteria isolated from these worms are commonly found in soil and water and pose no significant threats to hatchery production (*Paenibacillus* sp., *Lactococcus* sp., and *Shewanella putrefaciens*). It is likely that these worms carry a variety of environmental bacteria, of which this study has isolated a small sample. This was not examined further as the focus of this study was to determine if these worms possess pathogens and aid their transmission between broodstock and hatchery environments.

The isolation of *Aeromonas hydrophila* and *Aeromonas sobria* confirms that these worms contain pathogens and will transmit these between broodstock and hatcheries if transferred to the hatchery amongst redclaw eggs. *Aeromonas* spp. are opportunistic pathogens that are especially damaging and of high concern in culture conditions, since stressed animals are predisposed to disease (Sung *et al*. 2000; Nielsen *et al*. 2001; Quaglio *et al*. 2006). Stress is often caused by high stocking density, heavy parasite infestations, and previous infections or disease, all of which frequently occur in crayfish aquaculture. *A. hydrophila* is a highly virulent pathogen in crayfish aquaculture, causing rapid and severe stock mortality after infection (Jiravanichpaisal *et al*. 2009); *A. sobria* may have similar effects, although this is untested. Nonetheless, these worms do possess *Aeromonas* spp. and will act as a vector for these pathogens. Therefore, to prevent disease transmission from broodstock to hatchery, either the pathogens inside the worms must be eradicated, or the worms must be removed from crayfish eggs prior to transferral to the hatchery.

Chapter 3: Management

3.1 Introduction

These worms pose two key threats to the production of redclaw hatcheries; the transferral of pathogens into hatcheries, and the predation on crayfish eggs, both of which are exacerbated with high prevalence and infestation intensity.

To minimise pathogen transmission to the hatchery, crayfish eggs (once stripped from females) are routinely treated with antifungal and antimicrobial chemicals before being transferred into the hatchery. Formaldehyde (formalin) treatments are effective and the most common (Celada *et al*. 2004; Melendre *et al*. 2006; Sáez-Royuela *et al*. 2009; Kouba *et al*. 2010), although others such as peracetic acid, copper hydroxide and sodium chloride have also proven successful (van West, 2006; Carral *et al* 2009; Jussila *et al*. 2011; Kouba *et al*. 2012). It is time consuming to remove worms by hand from stripped redclaw eggs as the worms are small, abundant, and similar in appearance to redclaw eggs. Chemical treatments will kill temnocephalids present in the egg mass (O'Donoghue *et al*. 1990), but still result in worm bodies and any viable pathogens inside being transferred to the hatchery, potentially causing crop failure. Therefore, for chemical treatment to sufficiently control the impacts of these worms, it must kill the worms and all pathogens they contain. However, whilst chemical treatments address the key concern of pathogen transmission, they do not address the potentially severe egg predation which is likely to occur both prior to egg-stripping and in the hatchery.

Chemical treatments of diseases such as the crayfish plague (*Aphanomyces astaci*) and *Saprolegnia* infections are relatively well-understood, however treatments for

ectoparasites and ectocommensals remain vastly understudied, due to their perceived lower pathogenicity. However, with the findings that *Decadidymus valverdi* sp. nov. is likely to impact hatchery productivity, it is more important to consider treatments that will remove these worms from crayfish broodstock. Small doses of chemical treatments such as peracetic acid have been suggested to remove ectocommensals from broodstock (Jusilla *et al*. 2011), however exposing adult crayfish to chemical treatments may have significant adverse effects. Salt solutions have been previously suggested as a method to control ectocommensals, with evidence supporting its ability to remove temnocephalids from crayfish hosts (Jones *et al*. 1994; Soleng *et al*. 1998).

This research aims to test the efficacy of two suggested control methods for this temnocephalid species; formalin baths to remove pathogens carried by *D. valverdi* sp. nov., and exploiting salinity tolerances of redclaw and *D. valverdi* sp. nov. to remove these worms from infested adult crayfish. In doing so, this study pinpoints the production stage where control is required; with worms on berried crayfish or amongst the stripped egg mass.

3.2 Materials and methods

3.2.1 Formalin treatment – removing pathogens inside worms

To examine the effect of formalin treatments on the bacterial load of *Decadidymus valverdi* sp. nov., 60 randomly selected worms were exposed to each of the following treatments in 70ml specimen jars; 1) five washes with sterile PBS (control), 2) incubation with 25ml 1000mg/L formalin for 15 minutes, and 3) incubation with 25ml 1500mg/L formalin for 15 minutes. 20 worms were exposed to each treatment, with ten worms per treatment specimen jar (2 replicates). After treatment, worms were washed twice in PBS to remove any traces of formalin left on the outside of the worms which would hinder bacterial growth when incubated. Bacteriology for each treatment group of ten worms was conducted (Chapter 2 and Appendix E) to determine if worm bacteriology was affected by formalin treatments.

3.2.2 Exploiting salinity tolerances – removing worms from adult crayfish

To determine the salinity tolerance of *D. valverdi* sp. nov., worms were exposed to salinities between 0 and 30 in specimen jars for a total of 150 minutes, and mortality was recorded every 15 minutes for one hour, with a final assessment after 150 minutes. Specimens were classed as dead when no movement was observed when pushed with forceps and the posterior sucker was detached from the jar. Ten worms were placed in each 70ml specimen jar containing 40ml of sterile water at the appropriate salinity. Due to a limited number of specimens available, for the control group (0 salinity) only one jar (ten worms) was used. For salinities 2-30, three jars (30 worms) were used and mean mortality per treatment (n=3) was calculated. Results were normally-distributed (AD = 7.976, $P < 0.005$, $\alpha=0.05$) and had equal variance ($P = 0.656$, $\alpha=0.05$), so the effect of salinity and treatment exposure time on mean

accumulated mortality was analysed using a two-way repeated measures ANOVA with pairwise comparisons (Holm-Sidak).

Salinity treatments were then tested on worms still attached to crayfish, to examine the viability of this treatment in removing worms from adult host crayfish. 20 berried crayfish in individual containers were exposed to salinity treatments (five crayfish per treatment), with enough water to submerse crayfish (approximately 2 litres). Salinities tested were 0 (control), 10, 15 and 20, all followed by a 90-minute fresh water bath. The number of worms that had fallen off the crayfish was recorded after the 30-minute salt water treatment, and subsequent 90-minute fresh water bath. Crayfish were then stripped and the number of worms remaining on each female and amongst the egg mass was recorded. The percentage of total worms per crayfish that had fallen off was calculated (hereafter referred to as 'worm drop-off') for each salinity treatment and subsequent fresh water bath, and arcsine transformed. After transformation, data was tested for normality (AD = 1.198, $P < 0.005$, $\alpha=0.05$) and equal variance ($P = 0.635$, $\alpha=0.05$). A two-way repeated-measures ANOVA with pairwise comparisons (Holm-Sidak) was undertaken to compare results across different treatments.

Behaviour of crayfish curling of tail underneath abdomen) was monitored throughout the trial. Mortality of crayfish used in this trial was observed for seven days following the trial, to determine if salinity treatments increased stress of broodstock and increased mortality.

3.3 Results

3.3.1 Formalin treatment – bacterial control

Bacterial growth on primary plates decreased as the concentration of formalin in treatments increased. In the control group, both plates were characterised as 2+ (heavy bacterial growth on primary streak only), and six isolates were identified; *Aeromonas hydrophila*, *A. sobria*, *Paenibacillus* sp., *Lactococcus* sp., *Shewanella putrefaciens* and *Citrobacter youngae*. When treated with 1000mg/L formalin, one plate was characterised as 1+ (10 - 60 colonies on entire plate), the other as 2+, and six isolates were identified; *A. hydrophila*, *A. sobria*, *Chromobacterium violaceum, S. putrefaciens, Kocuria* sp. and *Lactococcus* sp. When treated with 15000mg/L formalin, both plates had bacterial growth characterised as 1 (< 10 colonies on entire plate), and 2 isolates were identified; *A. sobria* and *Microbacterium* sp..

As with the initial bacteriology study (Section 2.4.3), most isolates identified are of environmental origin and considered harmless, and *A. hydrophila* and *A. sobria* were isolated again, confirming that these worms contain pathogens. A formalin concentration of 1000mg/L is not enough to reduce the bacterial load carried by these worms, whereas 1500mg/L will greatly reduce bacterial load. However, even at the highest formalin concentration tested (higher than that commonly used in crayfish aquaculture industry), *Aeromonas sobria* was still present and viable.

3.3.2 Salinity treatment – worm removal

Initial salinity trials showed that increasing salinity and exposure time increased accumulated worm mortality (Figure 11). As salinity increased, the time required for

mean accumulated mortality to reach 100% (LT_{100}) decreased; 15 minutes in salinity of 30, 30 minutes in salinities of 20 and 25, and 150 minutes for salinities of 10 and 15 (Figure 11). LT_{100} was not reached during the 150 minutes of this trial for salinities between 0 and 8. This highlights the significant interaction between salinity and exposure time on the mean accumulated mortality of these worms ($F_{36,139}$=5.913, P <0.001). Due to this interaction, main effects of salinity and exposure could not be independently examined, hence pairwise comparisons were required. After 15 minutes of exposure, there was a significant increase in the mean accumulated mortalities between treatment salinities of 6, 20, 25 and 30 from the control, whereas salinities of 2, 4, 8, 10 and 15 showed no significant difference from the control (Figure 11). At 30, 45 and 60 minutes of exposure, salinities of 15 and above showed significant differences from the control, whereas salinities of 2-10 showed no significant difference. After 150 minutes, salinities of 10 and above showed significant differences from the control, whereas salinities of 2-8 showed no significant difference from the control (Figure 11).

Salinity trials undertaken with worms on host crayfish found a significant interaction between treatment salinity and the treatments used (30-minute salt water and subsequent 90-minute fresh water) on worm drop-off ($F_{3,16}$=17.41, P < 0.001) (Figure 12). There are no significant differences in worm drop-off between the 30-minute salt bath and subsequent 90-minute fresh water bath in salinities of 0 (P = 1.000) and 10 (P = 0.134), but there are significant differences in this when treated with initial salinities of 15 (P < 0.01) and 20 (P < 0.01) (Figure 12). After the 30-minute salt-bath, mean worm drop-off was 0% in the control group, 38% at a salinity of 10, 87% at a salinity of 15, and 88% at a salinity of 20. Pairwise comparisons showed there were

significant differences in the worm drop-off at all salinities tested ($P < 0.01$), except between 15 and 20 ($P = 0.834$) (Figure 12; 0 < 10 < 15, 20). Variation within the treatment group at a salinity of 10 was much greater than within groups at salinities of 15 and 20 (Figure 12).

After the 90-minute fresh water bath following salinity exposure, the same trend was observed amongst the treatment groups; there were significant differences in the worm drop-off at all salinities tested ($P < 0.01$), except between 15 and 20 ($P = 0.393$) (Figure 12; 0 < 10 < 15, 20). Mean worm drop-off remained at 0% in the control group, but rose to 41% in subjects initially treated with a salinity of 10, 94% in subjects initially treated with a salinity of 15, and 97% in subjects initially treated with a salinity of 20. Variation within the treatment group at a salinity of 10 remained much greater than within groups at salinities of 15 and 20 after the fresh water treatment (Figure 12).

In the salt-bath treatment at salinity of 20, all crayfish curled their tails underneath their body, which was not seen in any of the other treatment groups. Mortality was monitored for seven days following the trial; one individual died from the treatment salinity of 10 (on day 1), and one individual died from the treatment salinity of 15 (on day 2). No individuals from salinities of 0 or 20 died. Other temnocephalid species inhabiting the redclaw were also removed at high salinity treatments, although this was not quantified.

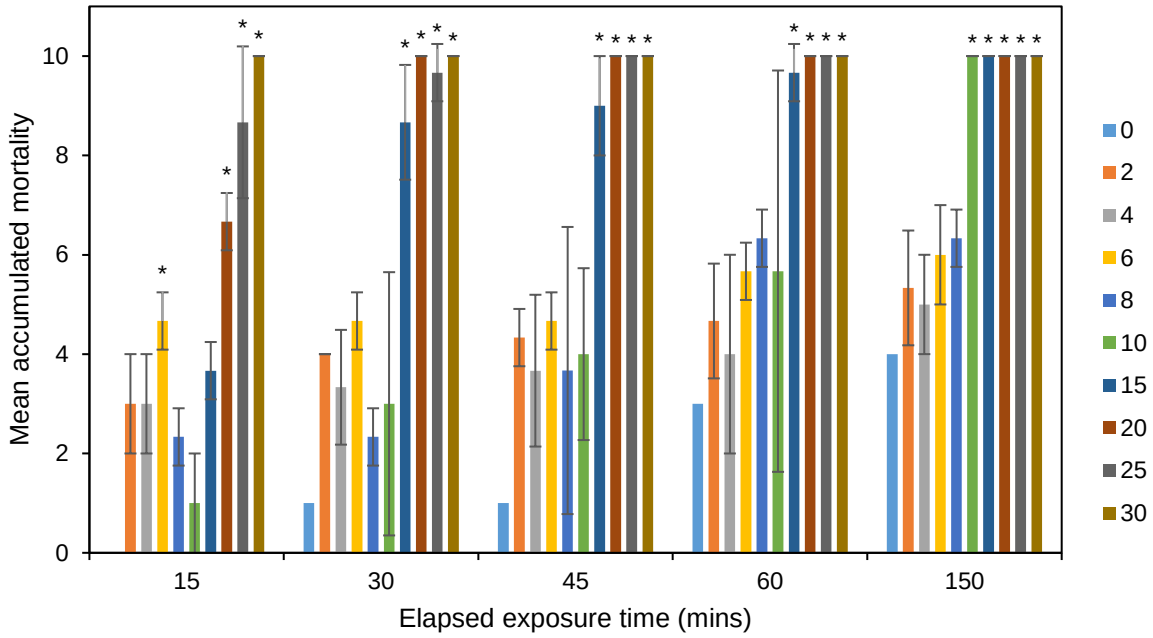

*Figure 11: Mean accumulated mortality of Decadidymus valverdi sp. nov. when exposed to a range of salinities (0-30) at 5 time intervals during a 150-minute period. * indicates a significant difference from the control (0 salinity) within each time period.*

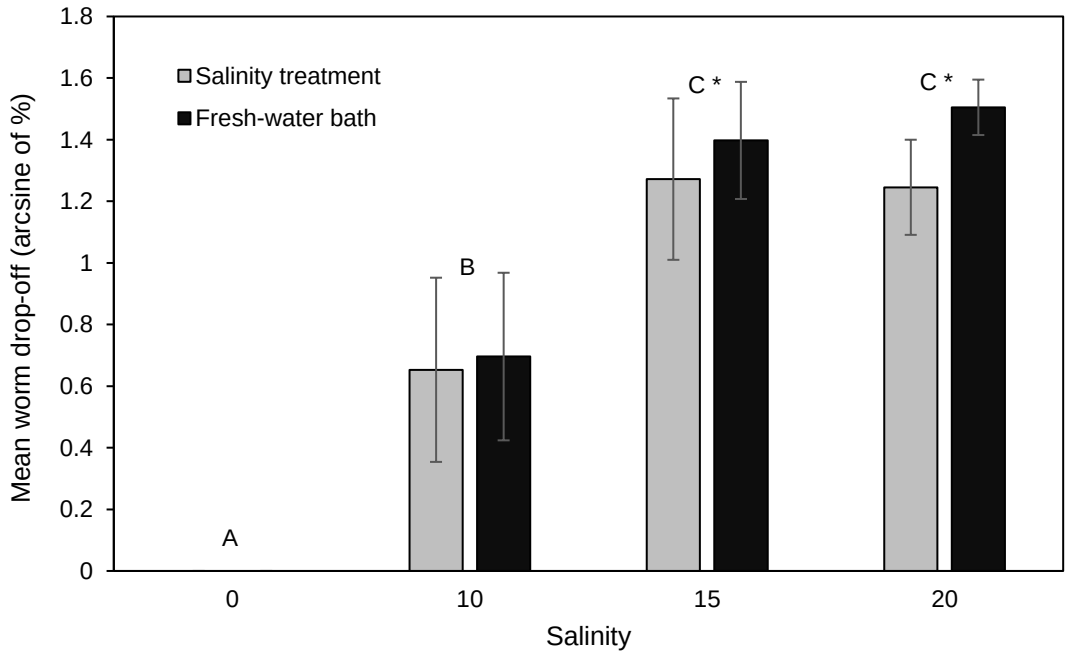

*Figure 12: Mean worm drop-off when exposed to a 30-minute salt-water bath at a range of salinities (0, 10, 15, 20) and a subsequent 90-minute fresh-water bath. Letters represent significant differences between different salinities for both salt-water and subsequent fresh water bath (same significance results), * indicate significant differences within each salinity treatment between salt-bath and fresh-water bath.*

3.4. Discussion

The bacterial load and number of environmental strains of bacteria in these worms reduced when treated with formalin, but *Aeromonas* spp. (*A. hydrophila* and *A. sobria*) remained viable 1000mg/L and 1500mg/L formalin treatments. This demonstrates that these treatments are not sufficient in eradicating these pathogens inside *Decadidymus valverdi* sp. nov.. Additionally, chemical treatments to remove these worms do not address the damage caused by the feeding behaviour of these worms prior to egg stripping. Furthermore, there are significant environmental and health and safety risks associated with the use of formalin (Arndt *et al*. 2001; Gieseker *et al*. 2006). As a result of the viability of pathogens inside worms after high concentration chemical treatments, and associated environmental and health concerns of such treatments, a protocol to remove or kill these worms before eggs are stripped from berried crayfish is required to prevent pathogen transmission into the hatchery.

Salinity treatments were effective, as worms dropped off crayfish when exposed to high salinities due to osmotic stress. Salinity tolerance studies on *D. valverdi* sp. nov. revealed an exposure time of at least 30 minutes was required, as results from exposure times less than this showed high variation across results (Figure 11). The time required to reach 100% mortality (LT_{100}) was 30 minutes in salinity treatments of 20, 25 and 30. At a salinity of 15, mean mortality reached 87% after 30 minutes, a significant increase from mortalities in salinities 0-10. An exposure time of 30 minutes is therefore concluded to achieve consistently high mortalities of *Decadidymus* specimens at salinities of 15 and above.

Results from trials with worms on crayfish hosts found that whilst there is a significant increase in worm drop-off at a salinity of 10 in comparison to the control, over half of the worms remained on the crayfish (Figure 12). Furthermore, the results were variable between individuals treated with a salinity of 10, hence salinities above 10 are required if enough worms are to be consistently removed from individuals in broodstock. There was no significant difference in worm drop-off between salinity treatments of 15 and 20 (Figure 12), thus a 30-minute bath in a salinity of 15 is considered the best practise method for worm removal, as lower salinities are less stressful for redclaw adults and juveniles.

The crayfish mortality observed in these trials was likely due to handling stress rather than salinity exposure, as no mortality occurred in redclaw exposed to the highest salinity treatments. Furthermore, redclaw are known to tolerate salinities and exposure times used in this study; Prymaczok *et al*. (2008) found growth performance and adult redclaw survival were unaffected by long exposure (3 weeks) to salinities up to 15g/L. Similarly, although increased salinity decreases juvenile hatching rate (Anson and Rouse 1994), there is no significant difference in juvenile growth when reared in salinities between 0 and 14g/L for 12 weeks (Austin 1995). This indicates that salinity treatments recommended in this study will have no significant detrimental effect on the growth and survival of both adult and juvenile redclaw.

Treatment groups with salinities of 15 and 20 showed a significant increase in worm drop-off after the additional 90-minute fresh water bath than after the initial salt bath (Figure 12). This is likely due to salinity exposure and resultant behaviour of crayfish; individuals in the salinity of 20 treatment tucked their tail underneath their abdomen

and around the egg mass, trapping some worms that would have otherwise fallen off due to osmotic stress. When these crayfish are transferred to the fresh water bath, a less stressful environment, their tails uncurled allowing for the worms that had died from salinity exposure to drop out of the egg mass. This resulted in a significantly higher worm removal when the salt water treatment is combined with a subsequent fresh water bath than in the salt water bath alone.

Salinity treatments will effectively remove *D. valverdi* sp. nov. from redclaw, with the potential to also act as a disease and antifungal treatment (Marking *et al*. 1994; Schreier *et al*. 1996; Mifsud and Rowland, 2008; Kozák *et al*. 2009; Policar *et al*. 2011). Therefore, a 30-minute salt bath with a salinity of 15, followed by a fresh water bath for 90 minutes in a holding tank is considered the best practice method for eradicating *D. valverdi* sp. nov. from broodstock and preventing reinfestation. This will remove worms from the broodstock before eggs are stripped and transferred to the hatchery, preventing egg mortality from predation and the transferral of pathogens between the environments. This recommended protocol is effective, rapid, low-cost and simple (with regards to facility requirements and training), hence has already been adopted by the aquaculture facility used for this research. The industry would benefit from future research refining this protocol, with particular focus on the time required for the subsequent fresh water bath, as only one time was tested in this study. Furthermore, it would be beneficial to examine any potential long term effects of this treatment, both in terms of how the eradication of these worms affects juvenile survival and hatchery production, and any potential negative effects of this recommended treatment on juvenile growth, survival and hatching rate.

Conclusions

The name *Decadidymus valverdi* sp. nov. is proposed for this species. Identifying morphological features of this species (compared to *Decadidymus gulosus*) are; 1) the ventral location and smaller opening of the mouth, which possesses inner and outer lips, 2) the large stylet, 3) the small pharynx, relative to overall body size, 4) the excretory pores located further from the anterior of worm, and 5) the smaller gonopore located closer to the posterior of the worm. These features are sufficient to justify the proposal of a new species, hence genetic sequencing was undertaken to further describe this new species. Sequence homology confirms the presence of this new species within the Temnocephalida, however cannot be compared to *D. gulosus* as no sequence for this species was obtained during its description. The phylogeny within the Temnocephalida requires more attention in future studies, as does a detailed taxonomic description of this proposed new species.

This new species is highly prevalent and abundant on berried redclaw and exhibits a strong association with host egg masses. Whilst adult redclaw are likely to be unaffected by infestations, the presence and intensity of infestation of this temnocephalid species will cause significant harm to developing juveniles. These worms feed on the yolk of developing eggs, which is predicted to severely hinder juvenile development and survival. *D. valverdi* sp. nov. is also capable of transmitting pathogens into the hatchery, potentially leading to crop failure and therefore a reduction in hatchery productivity and sustainability. Future research should quantify the extent to which *D. valverdi* sp. nov. affects juvenile survival and hatchery output. Nonetheless, the strong association with redclaw eggs, feeding behaviour and pathogens present in this worm signify a severe detrimental impact to production,

highlighting the need for the management and eradication of this *Decadidymus* species in aquaculture facilities.

Salinity treatments are commonly used in redclaw aquaculture as a rapid, low-cost solution to ectoparasites and disease, due to their high salinity tolerance. Salt baths are effective for removing these worms from adult crayfish; a 30-minute salt bath with a salinity of 15 is recommended, followed by a 90-minute fresh water bath in a holding tank before entering broodstock. This is the more effective management protocol to reduce the impacts of this *Decadidymus* species, as formalin treatments will not remove pathogens inside these worms and do not address the issue of yolk consumption by these worms prior to egg stripping.

This recommended protocol has already been implemented at the study site facility, although long term benefits to production are yet to be quantified. Future assessments of this technique should test the efficacy of shorter fresh water baths after salinity treatment, as the full 90 minutes used in this study may not be required. Another aspect to consider in future development of this protocol is the possibility of long-term effects of salinity exposure on juvenile growth, survival and hatching rate; the advantages of increased survival and production by removing these worms with salinity treatments, against the disadvantages of potentially reduced growth, survival and hatching rates of juveniles should be compared.

References

Adiyodi, R. G. (1985). Reproduction and its control. *The biology of Crustacea*, *9*, 147-215.

Amato, J. F. R., Amato, S. B., & Daudt, L. C. C. (2003). New species of *Temnocephala* Blanchard (Platyhelminthes, Temnocephalida) ectosymbiont on *Aegla serrana* Buckup & Rossi (Crustacea, Anomura) from southern Brazil. *Revista Brasileira de Zoologia*, *20*(3), 493-500.

Amato, J. F., & Amato, S. B. (2005). New species of *Temnocephala* Blanchard (Platyhelminthes, Temnocephalida) ectosymbiont on giant water bugs, *Belostoma* spp. (Hemiptera, Belostomatidae) from southern Brazil. *Revista Brasileira de Zoologia*, *22*(1), 107-118.

Amato, J. F., Amato, S. B., & Seixas, S. A. (2006). A new species of *Temnocephala* Blanchard (Platyhelminthes, Temnocephalida) ectosymbiont on *Trichodactylus fluviatilis* Latreille (Crustacea, Decapoda, Trichodactylidae) from southern Brazil. *Revista Brasileira de Zoologia*, *23*(3), 796-806.

Amato, J. F., Seixas, S. A., & Amato, S. B. (2007). A new species of *Temnocephala* Blanchard (Platyhelminthes, Temnocephalida) ectosymbiont on creeping water bugs, *Cryphocricos granulosus* De Carlo (Hemiptera, Naucoridae) from southern Brazil. *Revista Brasileira de Zoologia*, *24*(4), 1043-1051.

Andersen, W. C., Turnipseed, S. B., & Roybal, J. E. (2006). Quantitative and confirmatory analyses of malachite green and leucomalachite green residues in fish and shrimp. *Journal of agricultural and food chemistry*, *54*(13), 4517-4523.

Anson, K. J., & Rouse, D. B. (1994). Effects of Salinity on Hatching and Post-Hatch Survival of the Australian Red Claw Crayfish *Cherax quadricarinatus*. *Journal of the World Aquaculture Society*, *25*(2), 277-280.

Arndt, R. E., Wagner, E. J., & Routledge, M. D. (2001). Reducing or withholding hydrogen peroxide treatment during a critical stage of rainbow trout development: effects on eyed eggs, hatch, deformities, and fungal control. *North American Journal of Aquaculture*, *63*(2), 161-166.

Austin, C. M. (1995). Effect of temperature and salinity on the survival and growth of juvenile redclaw (*Cherax quadricarinatus*). *Freshwater Crayfish*, *10*, 419-426.

Bancroft, J. D., & Gamble, M. (2008). *Theory and practice of histological techniques*. (6th edition) Churchill Livingstone, Elsevier, Philadelphia, USA.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z. & Shariff, M. (2005). Disease and health management in Asian aquaculture. *Veterinary parasitology*, *132*(3), 249-272.

Bower, S. M., McGladdery, S. E., & Price, I. M. (1994). Synopsis of infectious diseases and parasites of commercially exploited shellfish. *Annual Review of Fish Diseases*, *4*, 1-199.

Boyd, C. E., Tucker, C., McNevin, A., Bostick, K., & Clay, J. (2007). Indicators of resource use efficiency and environmental performance in fish and crustacean aquaculture. *Reviews in Fisheries science*, *15*(4), 327-360.

Cannon, L. R. (1991). Temnocephalan symbionts of the freshwater crayfish *Cherax quadricarinatus* from northern Australia. *Hydrobiologia*, *227*(1), 341-347.

Carral, J. M., González, A., Celada, J. D., Sáez-Royuela, M., Melendre, P. M., González, R., & García, V. (2009). Antifungal treatments in artificial incubation of crayfish eggs (*Pacifastacus leniusculus*, Astacidae): searching for alternatives to formaldehyde. *Knowledge and Management of Aquatic Ecosystems*, (394-395), 16.

Celada, J. D., Carral, J. M., Sáez-Royuela, M., Melendre, P. M., & Aguilera, A. (2004). Effects of different antifungal treatments on artificial incubation of the astacid crayfish (*Pacifastacus leniusculus* Dana) eggs. *Aquaculture*, *239*(1), 249-259.

Charmantier-Daures, M., & Charmantier, G. (1991). Mass-culture of *Cancer irroratus* larvae (Crustacea, Decapoda): adaptation of a flow-through sea-water system. *Aquaculture*, *97*(1), 25-39.

Damborenea, C., & Brusa, F. (2008). A new species of *Temnocephala* (Platyhelminthes, Temnocephalida) commensal of *Pomella megastoma* (Mollusca, Ampullariidae) from Misiones, Argentina. *Revista mexicana de biodiversidad*, *79*, 1-7.

Drew, W. L. (1977). Value of sputum culture in diagnosis of pneumococcal pneumonia. *Journal of clinical microbiology*, *6*(1), 62-65.

Edgerton, B. F. (1999). A review of freshwater crayfish viruses. *Freshwater Crayfish*, *12*, 261–278.

Edgerton, B. F., Evans, L. H., Stephens, F. J., & Overstreet, R. M. (2002). Synopsis of freshwater crayfish diseases and commensal organisms. *Aquaculture*, *206*(1), 57-135.

FAO (Food and Agriculture Organisation) (2014). The State of World Fisheries and Aquaculture 2014. Opportunities and Challenges. Rome. 223 pp.

FAO (Food and Agriculture Organisation) (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp.

Garcés, A. C., Puerta, L., Tabares, Y., Lenis, C., & Velásquez, L. E. (2013). *Temnocephala colombiensis* n. sp. (Platyhelminthes: Temnocephalidae) from Antioquia, Colombia. *Revista mexicana de biodiversidad*, *84*(4), 1090-1099.

Ghanawi, J., & Saoud, I. P. (2012). Molting, reproductive biology, and hatchery management of redclaw crayfish *Cherax quadricarinatus* (von Martens 1868). *Aquaculture*, *358*, 183-195.

Gieseker, C. M., Serfling, S. G., & Reimschuessel, R. (2006). Formalin treatment to reduce mortality associated with *Saprolegnia parasitica* in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, *253*(1), 120-129.

Gram, C. (1884). The differential staining of Schizomycetes in tissue sections and in dried preparations. *Fortschritte der Medizin*, *2*, 185-9.

Herbert, B. (1987). Notes on diseases and epibionts of *Cherax quadricarinatus* and *C. tenuimanus* (Decapoda: Parastacidae). *Aquaculture*, *64*(3), 165-173.

Holdich, D. M. (1993). A review of astaciculture: freshwater crayfish farming. *Aquatic Living Resources*, *6*(4), 307-317.

Jackson, C. J., Pendrey, R. C., & Rothlisberg, P. C. (1992). The Larvatron: a computer-controlled apparatus for rearing planktonic animals under experimental conditions. *Marine Ecology Progress Series*, *86*(2), 189-199.

Jennings, J. B. (1971). Parasitism and commensalism in the Turbellaria. *Advances in parasitology*, *9*, 1-32.

Jiravanichpaisal, P., Roos, S., Edsman, L., Liu, H., & Söderhäll, K. (2009). A highly virulent pathogen, *Aeromonas hydrophila*, from the freshwater crayfish *Pacifastacus leniusculus*. *Journal of invertebrate pathology*, *101*(1), 56-66.

Joffe, B. I., Cannon, L. R., & Schockaert, E. R. (1998). On the phylogeny of families and genera within the Temnocephalida. *Hydrobiologia*, *383*(1), 263-268.

Jones, C. M. (1995). Production of juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae) I. Development of hatchery and nursery procedures. *Aquaculture*, *138*(1), 221-238.

Jones, C. M., Medley, P. B., & Avault Jr, J. W. (1994). Redclaw crayfish, *Cherax quadricarinatus*: production, economics and marketing. *World Aquaculture*, *25*, 4.

Jones, T. C., & Lester, R. G. J. (1993). Aspects of the biology and pathogenicity of *Diceratocephala boschmai* (Platyhelminthes: Temnocephalida), an ectosymbiont on the redclaw crayfish, *Cherax quadricarinatus*. *Marine and Freshwater Research*, *44*(6), 927-933.

Jones, T. C., & Lester, R. J. (1996). Factors influencing populations of the ectosymbiont *Diceratocephala boschmai* (Platyhelminthes; Temnocephalida), on the redclaw crayfish *Cherax quadricarinatus* maintained under laboratory conditions. *Aquaculture*, *143*(3-4), 233-243.

Jussila, J., Makkonen, J., & Kokko, H. (2011). Peracetic acid (PAA) treatment is an effective disinfectant against crayfish plague (*Aphanomyces astaci*) spores in aquaculture. *Aquaculture*, *320*(1), 37-42.

Kittaka, J. (1998). Culture of larval spiny lobsters: a review of work done in northern Japan. *Marine and Freshwater Research*, *48*(8), 923-930.

Kouba, A., Carral, J. M., Buřič, M., Mráz, J., Policar, T., & Kozák, P. (2010). Artificial incubation of noble crayfish (*Astacus astacus*) eggs in a partially recirculating system using formaldehyde as an antifungal treatment. *Aquaculture Research*, *41*(10), e618-e623.

Kouba, A., Kuklina, I., Niksirat, H., Máchová, J., & Kozák, P. (2012). Tolerance of signal crayfish (*Pacifastacus leniusculus*) to Persteril 36 supports use of peracetic acid in astaciculture. *Aquaculture*, 350, 71-74.

Kozák, P., Policar, T., Fedotov, V. P., Kuznetsova, T. V., Buřič, M., & Kholodkevich, S. V. (2009). Effect of chloride content in water on heart rate in narrow-clawed crayfish (*Astacus leptodactylus*). *Knowledge and Management of Aquatic Ecosystems*, (394-395), 08.

Lockyer, A. E., Olson, P. D., & Littlewood, D. T. J. (2003). Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biological Journal of the Linnean Society*, *78*(2), 155-171.

Longshaw, M. (2011). Diseases of crayfish: a review. *Journal of Invertebrate Pathology*, *106*(1), 54-70.

Marking, L. L., Rach, J. J., & Schreier, T. M. (1994). American fisheries society evaluation of antifungal agents for fish culture. *The Progressive Fish-Culturist*, *56*(4), 225-231.

Masser, M. P., & Rouse, D. (1997). Australian red claw crayfish. *Southern Regional Aquaculture Center,* 224.

Melecha, S. (1983). Commercial seed production of the freshwater prawn, *Macrobrachium rosenbergii* in Hawaii. *CRS Handbook of Mariculture*, *1*, 203-205.

Melendre, P. M., Celada, J. D., Carral, J. M., Sáez-Royuela, M., & Aguilera, A. (2006). Effectiveness of antifungal treatments during artificial incubation of the signal crayfish eggs (*Pacifastacus leniusculus* Dana. Astacidae). *Aquaculture*, *257*(1), 257-265.

Meyer, F. P. (1991). Aquaculture disease and health management. *Journal of animal science*, *69*(10), 4201-4208.

Mifsud, C., & Rowland, S. J. (2008). Use of salt to control ichthyophthiriosis and prevent saprolegniosis in silver perch, *Bidyanus bidyanus*. *Aquaculture Research*, *39*(11), 1175-1180.

Murray, A. G., & Peeler, E. J. (2005). A framework for understanding the potential for emerging diseases in aquaculture. *Preventive veterinary medicine*, *67*(2), 223-235.

Nelson, R. G., & Dendy, J. S. (1979). Conditions for holding and propagating crawfish brood stock (*Procambarus clarkii*). In *Proceedings of the World Mariculture Society, 10*(1-4), 503-509. Blackwell Publishing Ltd.

Nielsen, M. E., Høi, L., Schmidt, A. S., Qian, D., Shimada, T., Shen, J. Y., & Larsen, J. L. (2001). Is *Aeromonas hydrophila* the dominant motile Aeromonas species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China?. *Diseases of aquatic organisms*, *46*(1), 23-29.

O'Donoghue, P., Beveridge, I., & Phillips, P. (1990). Parasites and ectocommensals of yabbies and marron in South Australia. *Central Veterinary Laboratories (VETLAB), South Australian Department of Agriculture, Adelaide*.

O'Sullivan, D. (1991). Status of Australian aquaculture 1989-90. *Austasia Aquaculture*, 2-13.

Policar, T., Smyth, J., Flanigan, M., Kouba, A., & Kozák, P. (2011). Sodium chloride as effective antifungal treatment for artificial egg incubation in *Austropotamobius pallipes*. *Knowledge and Management of Aquatic Ecosystems*, (401), 13.

Prymaczok, N. C., Medesani, D. A., & Rodríguez, E. M. (2008). Levels of ions and organic metabolites in the adult freshwater crayfish, *Cherax quadricarinatus*, exposed to different salinities. *Marine and Freshwater Behaviour and Physiology*, *41*(2), 121-130.

Quaglio, F., Morolli, C., Galuppi, R., Bonoli, C., Marcer, F., Nobile, L., De Luise, G. & Tampieri, M. P. (2006). Preliminary investigations of disease-causing organisms in the white-clawed crayfish *Austropotamobius pallipes* complex from streams of northern Italy. *Bulletin Francais de la Peche et de la Pisciculture*, (380-381), 1271-1290.

Sáez-Royuela, M., Melendre, P. M., Celada, J. D., Carral, J. M., González, A., González, R., & García, V. (2009). Possibilities of artificial incubation of signal crayfish (*Pacifastacus leniusculus* Dana) eggs at high densities and reduced flow rate using formaldehyde as antifungal treatment. *Aquaculture*, *288*(1), 65-68.

Sammy, N. (1988). Breeding biology of Cherax quadricarinatus in the Northern Territory. *In Proceedings of the First Australian Shellfish Aquaculture Conference*, Perth. (Eds L. H. Evans and D. O'Sullivan.) pp. 79-88.

Saoud, I. P., Ghanawi, J., Thompson, K. R., & Webster, C. D. (2013). A review of the culture and diseases of redclaw crayfish *Cherax quadricarinatus* (von Martens 1868). *Journal of the World Aquaculture Society*, *44*(1), 1-29.

Sapkota, A., Sapkota, A. R., Kucharski, M., Burke, J., McKenzie, S., Walker, P., & Lawrence, R. (2008). Aquaculture practices and potential human health risks: current knowledge and future priorities. *Environment international*, *34*(8), 1215-1226.

Schreier, T. M., Rach, J. J., & Howe, G. E. (1996). Efficacy of formalin, hydrogen peroxide, and sodium chloride on fungal-infected rainbow trout eggs. *Aquaculture*, *140*(4), 323-331.

Sewell, K. B. (2013). Key to the genera and checklist of species of Australian temnocephalans (Temnocephalida). *Museum Victoria Science Reports*, *17*, 1-13.

Sewell, K. B., & Whittington, I. D. (1995). A light microscope study of the attachment organs and their role in locomotion of *Craspedella* sp. (Platyhelminthes: Rhabdocoela: Temnocephalidae), an ectosymbiont from the branchial chamber of *Cherax quadricarinatus* (Crustacea: Parastacidae) in Queensland, Australia. *Journal of natural history*, *29*(5), 1121-1141.

Soleng, A., Bakke, T. A., & Hansen, L. P. (1998). Potential for dispersal of *Gyrodactylus salaris* (Platyhelminthes, Monogenea) by sea-running stages of the Atlantic salmon (Salmo salar): field and laboratory studies. *Canadian Journal of Fisheries and Aquatic Sciences*, *55*(2), 507-514.

Stentiford, G. D., Neil, D. M., Peeler, E. J., Shields, J. D., Small, H. J., Flegel, T. W., Vlak, J. M., Jones, B., Morado, F., Moss, S., Lotz, J., Bartholomay, L., Behringer, D. C., Hauton, C. & Lightner, D. V. (2012). Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. *Journal of invertebrate pathology*, *110*(2), 141-157.

Sung, H. H., Hwang, S. F., & Tasi, F. M. (2000). Responses of giant freshwater prawn (*Macrobrachium rosenbergii*) to challenge by two strains of *Aeromonas* spp. *Journal of invertebrate pathology*, *76*(4), 278-284.

van West, P. (2006). *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. *Mycologist*, 20(3), 99-104.

Villarreal, H., & Pelaez, A. (2000). Commercial semi intensive production in clay ponds of the freshwater red claw crayfish *Cherax quadricarinatus*. In *Proceedings of the World Aquaculture America 2000 symposium,* New Orleans, USA (p. 271).

Volonterio, O. (2007). A new species of *Temnocephala* (Platyhelminthes, Temnocephalida) and a description of *T. axenos* from Uruguay. *Journal of Natural History*, *41*(21-24), 1245-1257.

Volonterio, O. (2009). First report of the introduction of an Australian temnocephalidan into the New World. *Journal of Parasitology*, *95*(1), 120-123.

Volonterio, O. (2010). Two new species of *Temnocephala* (Platyhelminthes, Temnocephalida) from the South American snake-necked turtle *Hydromedusa tectifera* (Testudines, Chelidae). *Zoological science*, *27*(12), 965-970.

Walker, P. J., & Winton, J. R. (2010). Emerging viral diseases of fish and shrimp. *Veterinary research*, *41*(6), 51-75.

Watson, N. A., Rohde, K., & Sewell, K. B. (1995). Ultrastructure of spermiogenesis and spermatozoa of *Decadidymus gulosus*, *Temnocephala dendyi*, *T. minor*, *Craspedella* sp., *C. spenceri* and *Diceratocephala boschmai* (Platyhelminthes, Temnocephalida, Temnocephalidae), with emphasis on the intercentriolar body and zone of differentiation. *Invertebrate reproduction & development*, *27*(2), 131-143.

Appendices

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A: Anaesthetisation trials

Small, soft bodied organisms often curl up in unfavourable environments, hence there has been research into the anaesthetisation of these organisms before exposure to fixatives such as NBF (Klemm, 1982; Sewell and Cannon, 1995). Therefore, to examine the morphology of these worms without distortion of body shape, a successful method for anaesthetisation must be determined.

Materials and methods

Anesthetisation trials included four methods; 1) incubating worms at 4^{o}C, 2) exposure to soda water, 3) adding menthol crystals, and 4) adding 95% ethanol dropwise. Worms were observed for activity (wriggling or moving to new areas of the jar) and if their sucker was attached to the jar. Successful anaesthetisation was determined by the worms' reaction to 10%NBF; if the body distorts they are not anaesthetised, if they remain elongated they are sufficiently anaesthetised.

Fridge trial: three 70ml specimen jars each containing 20ml of hatchery water (26^{o}C) and ten worms of varying sizes (3-10mm length) were placed in the same area of a fridge. 10%NBF was placed in the fridge in the same quantities (20ml in 70ml specimen jars), to test relaxation of worms that appear anaesthetised by their reaction to formalin of the same temperature they have been incubated in. Jars were left for half an hour to reach 4^{o}C before time trial began. Once started, worm activity in every jar was examined every hour until signs of relaxation began. When this occurred, anaesthetisation was tested for one jar by exposing specimens to cold 10%NBF. If no body distortion in any of the worms in the jar was observed, the trial finished. If some worms curled up, the remaining jars continued incubation for at least another hour, until anaesthetisation was complete.

Soda water trial: three 70ml specimen jars each containing 20ml of hatchery water (26^{o}C) and ten worms of varying sizes (3-10mm length) were prepared. Hatchery water was quickly removed and replaced with soda water, and activity was observed before testing anaesthetisation with 10%NBF.

Menthol crystals trial: three 70ml specimen jars each containing 20ml of hatchery water (26^{o}C) and ten worms of varying sizes (3-10mm length) were prepared. Menthol crystals were added to the surface of the water until the entire surface was covered, and activity was observed before testing anaesthetisation with 10% NBF.

Dropwise 95% ethanol trial: three 70ml specimen jars each containing 20ml of hatchery water (26^{o}C) and ten worms of varying sizes (3-10mm length) were prepared. Room-temperature 95% ethanol was added dropwise using a pipette and the jar was swirled to ensure the ethanol diffused evenly throughout the water. This continued until activity reduced and anaesthetisation was tested with 10% NBF.

Results

Fridge: In all jars, no narcotisation was observed until after 6 hours. Effective, but slow.

Soda: almost instantaneous anaesthetisation of larger specimens, but smaller specimens showed some body distortion upon exposure to soda water (before NBF).

Menthol crystals: effective but slow, lots of crystals required.

Dropwise ethanol: Initial activity of worms increased after first few drops added, but as more ethanol was added activity decreased. Smaller worms were narcotised with a lower concentration of ethanol than larger worms. This method worked quickly on all worm sizes once enough ethanol was added (approximately 5-10ml).

Chosen method of anaesthetisation was dropwise ethanol until observed anaesthetisation, as provided most rapid and effective treatment.

B: Staining trials, dehydration and mounting protocols

Five stains were selected for trials; Mayer's paracarmine, Harris' hematoxylin, Solphenyl blue, Toluidine blue and Kirkpatrick's carmine.

Stain preparation

Mayer's parcarmine:

Stain	
Aluminium chloride	0.5g
70% ethanol (heated)	100ml
Calcium chloride	4.0g
Carmine	1.0g
Once cooled, mixture is filtered	
Differentiator	
70% ethanol	99ml
Glacial acetic acid	1ml

Kirkpatrick's carmine:

Stain	
Carmine	2.5g
Glacial acetic acid	2.5ml
Potassium aluminium sulphate	2.5g
Distilled water	100ml
Differentiator	
Distilled water	

Solphenyl blue:

Stain; JCU stock solution
Differentiator; Distilled water

Toluidine blue:

Stain	
Toluidine blue	1.0g
Borax	1.0g
Boric acid	1.0g
Distilled water	100ml
Differentiator	
Formaldehyde	5ml
Acetic acid	5ml
80% ethanol	90ml

Harris' Hematoxylin:

Stain	
Stock Harris Hematoxylin (JCU)	100ml
Glacial acetic acid	4ml
Differentiator	
70% ethanol	99ml
Glacial acetic acid	1ml

Protocols

Mayer's paracarmine and Harris' hematoxylin: cover specimens in cavity block with stain solution for 24 hours. Destain with acidic alcohol (1%HCl-70% ethanol) until differentiation of tissues is visible. Neutralise with 1%NH4-70% ethanol.

Solphenyl blue: cover specimens in cavity block with solphenyl blue (approximately 3 drops), leave for 1 minute. Fill cavity block with water, check stain for differentiation (empty water and refill if differentiation is unclear). Remove water when differentiation visible and mount immediately without dehydrating (see below).

Toluidine blue: half fill cavity block with distilled water and specimens for staining. Add five drops of toluidine blue and leave for 2 minutes. Wash briefly with water, remove with pipette. Add differentiator (FAA – formaldehyde, acetic acid, ethanol) and watch for differentiation of tissues. Remove FAA with pipette when differentiation is visible.

Kirkpatrick's carmine; cover specimens in cavity block with stain solution (approximately 3 drops) leave for 3 minutes. Fill cavity block with distilled water and remove with pipette, repeat until water is clear, check for differentiation of tissues, stop rinsing when differentiation is visible.

Results

Left to right: Mayer's paracarmine, Harris' hematoxylin, Solphenyl blue, Toluidine blue, Kirkpatrick's carmine. Scale bar approximately 1mm.

Chosen method of staining was with Mayer's paracarmine, as it produced the most distinguishable features within the specimen.

Dehydration

Leave specimens in 50% ethanol for 2 minutes, followed by 2 minutes each in 60%, 70%, 80% and 90% ethanol solutions. Transfer to 100% ethanol and leave for 5 minutes, then to another 100% ethanol for another 5 minutes. Remove half of the ethanol with a pipette and replace with xylene by trickling it slowly down the side of the cavity block with a pipette. Leave for 5 minutes before removing the supernatant and flooding the cavity block with xylene.

Mounting

Add a few drops of mountant to cavity slide (DPX for all specimens except those stained with solphenyl blue which were mounted with Grey and Weiss mountant). Arrange specimen(s) in desired position and orientation in mountant. Add another drop of mountant over the top of the specimen(s) and lower a coverslip over the top from one edge to reduce bubble formation. Push any remaining bubbles out by applying light pressure to the coverslip with a toothpick. Leave to dry for 48 hours before examining specimens under a microscope.

C: Paraffin wax histology slides: dehydrating, clearing, staining, mounting

Dehydrating and clearing of fixed glass slides:

- Xylene 2 minutes
- Xylene 2 minutes
- Ethanol 1 minute
- Ethanol 1 minute
- Ethanol 1 minute
- Water wash 1 minute

Stains:

Haematoxylin and Eosin:

- Mayer's haematoxylin 8 minutes
- Water Wash 30 seconds
- Scott's tap water substitute 30 seconds
- Water wash 2 minutes
- Young's Eosin 4 minutes

Differentiate in water wash ~20 seconds

Gram-Twort:

- 2% crystal violet 2 minutes
- Water wash 5 seconds
- Lugol's iodine 3 minutes
- Rinse with water 5 seconds
- Decolourise with acetone 5 seconds
- Twort's solution (1:3 water) 10 minutes

Rinse with water 5 seconds

Herxheimer's:

- Herxheimer's 8 minutes
- Rinse with 70% ethanol 5 seconds
- Rinse with water 5 seconds
- Mayer's Haematoxylin 2 minutes
- Rinse with water 5 seconds
- Scott's tap water substitute 30 seconds

Rinse with water 5 seconds

Xylene infiltration:

- Ethanol 1 minute
- Ethanol 1 minute
- Ethanol 2 minutes
- Xylene 2 minutes
- Xylene 2 minutes
- Xylene until mounting

Mounting:

Remove stained slides from xylene bath and stand up to allow xylene to drip off (without drying out slides). Add a few drops of DPX mountant (or Grey and Weisse if aqueous mountant is required) to slide and lower a coverslip over the top from one edge to reduce bubble formation. Push any remaining bubbles out by applying light pressure to the coverslip with a toothpick. Leave to dry for 48 hours before examining specimens under a microscope.

D: SEM reagent and sample preparation protocol

Note: using SEM also required completing JCU AAC risk assessments

- Nitrile rubber gloves, safety glasses and a lab coat worn
- Risk assessments and COSHH completed and MSDS consulted (JCU) before carrying out this protocol
- Completed under a fume hood

Hazardous chemicals used in this procedure:

1. **Glutaraldehyde solution**. Toxic if swallowed or inhaled, causes severe skin burns and eye damage, may cause an allergic skin reaction, may cause allergy or asthma symptoms or breathing difficulties if inhaled, may cause respiratory irritation, very toxic to aquatic life with long lasting effects, corrosive to the respiratory tract.
2. **Sodium cacodylate trihydrate**. Toxic if swallowed or inhaled, suspected of causing cancer, very toxic to aquatic life with long lasting effects.
3. **HMDS (Hexamethyldisilazane)**. Highly flammable liquid and vapour, harmful if swallowed or if inhaled, toxic in contact with skin, harmful to aquatic life with long lasting effects.

Protocol:

- Dissolve sodium cacodylate in distilled water, correct pH with HCl to obtain a sodium cacodylate buffer of 0.2M and pH7.2
- Mix with glutaraldehyde to produce 2.5% glutaraldehyde in sodium cacodylate buffer
- Incubate anaesthetised worms in this fixative buffer solution for 24 hours
- Transfer sample into fresh buffer solution (no fixative) using forceps
- Mix well and all of buffer removed, leaving sample behind
- Add fresh buffer, repeated twice
- Removed as much buffer as possible from sample, replace with 50% ethanol in deionized water solution, leave for 15 minutes
- Repeat using increasing ethanol concentrations (60%, 70%, 80%, 90% and 100%)
- Repeat 100% ethanol step twice
- Replace 100% ethanol with a 1:1 solution of 100% ethanol and HMDS, leave for 15 minutes
- Replace ethanol/HMDS solution with 100% HMDS, leave for 15 minutes, repeat twice
- At third 100% HMDS stage, leave sample immersed in solution to evaporate off in fume hood, loosely covered by lid (overnight)
- Carefully orientated samples onto SEM stub (with dissecting scope if needed), mount using carbon based, electrically conductive, double sided adhesive discs (Leit tabs)
- Gold-coat stubs (conducted by JCU Advanced Analytical Centre staff)

COSHH Summary: Glutaraldehyde solution, Sigma-Aldrich 340855

MSDS supplier and revision date: Sigma-Aldrich, 24.02.17	
Task description: Glutaraldehyde mixed with sodium cacodylate buffer of 0.2M and pH7.2, to produce 2.5% glutaraldehyde in sodium cacodylate buffer; used to fix specimens for SEM.	
Hazard statements:	**Precautionary statements:**
H301 + H331 Toxic if swallowed or inhaled	P260 Do not breathe dust/ fume/ gas/ mist/ vapours/ spray
H314 Causes severe skin burns and eye damage	P280 Wear protective gloves/ protective clothing/ eye protection/ face protection
H317 May cause an allergic skin reaction	P301 + P330 + P331 + P310 IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. Immediately call a POISON CENTER/doctor
H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled	
H335 May cause respiratory irritation	P304 + P340 + P310 IF INHALED: Remove person to fresh air and keep comfortable for breathing. Immediately call a POISON CENTER/doctor
H410 Very toxic to aquatic life with long lasting effects	
EUH071 Corrosive to the respiratory tract	P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
	P403 + P233 Store in a well-ventilated place. Keep container tightly closed

Could a less hazardous substance be used instead? No

Control measures

Practice: Handle in accordance with good laboratory hygiene and safety practice. Wash hands before leaving laboratory. Avoid contact with skin and eyes. Avoid inhalation of vapour or mist, handle in a fume hood. Ensure adequate ventilation.
Personal protective equipment: Safety glasses and face shield, nitrile rubber gloves, lab coat.
Storage: Store in cool place. Keep container tightly closed in a dry and well-ventilated place, keep upright to prevent leakage.

Emergency procedure

General advice:	Consult a physician. Show MSDS (attached) to doctor in attendance.
Inhalation:	Move person into fresh air. If not breathing, give artificial respiration. Consult a physician.
Skin contact:	Take off contaminated clothing and shoes immediately. Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.
Eye contact:	Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.
Ingestion	Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.
Spill procedure:	Prevent further leakage or spillage if safe to do so. Do not let product enter drains, discharge into the environment must be avoided. Soak up with inert absorbent material, keep in closed container and dispose of as hazardous waste. Follow hazardous waste disposal protocol for laboratory.

Fire-fighting measures:	Use water spray, alcohol resistant foam, dry chemical or carbon dioxide. Fire-fighters to wear self-contained breathing apparatus where possible

Health surveillance **not required**.
Specific training **not required**.
Hazards and risks are suitably controlled using above measures.

COSHH Summary: Sodium cacodylate trihydrate, Sigma-Aldrich C0250

MSDS supplier and revision date: Sigma-Aldrich, 28.12.15	
Task description: Dissolved in sterile water to produce buffer of 0.2M and pH7.2, then mixed with glutaraldehyde to produce 2.5% glutaraldehyde in sodium cacodylate buffer; used to fix specimens for SEM.	
Hazard statements: H301 + H331 Toxic if swallowed or inhaled H351 Suspected of causing cancer H410 Very toxic to aquatic life with long lasting effects	**Precautionary statements:** P261 Avoid breathing dust P273 Avoid release to the environment P281 Use personal protective equipment as required. P301 + P310 IF SWALLOWED: Immediately call a POISON CENTER or doctor/ physician. P311 Call a POISON CENTER or doctor/ physician P501 Dispose of contents/ container to an approved waste disposal plant.

Could a less hazardous substance be used instead? No

Control measures
Practice: Handle in accordance with good laboratory hygiene and safety practice. Wash hands before leaving laboratory. Avoid formation of dust and aerosols. Avoid eye and skin contact, and inhalation of dust or vapours. Handle in a fume hood. Ensure adequate ventilation.
Personal protective equipment: Safety glasses, nitrile rubber gloves, lab coat.
Storage: Store in cool place. Keep container tightly closed in a dry and well-ventilated place, keep upright.

Emergency procedure

General advice:	Consult a physician. Show MSDS (attached) to doctor in attendance.
Inhalation:	Move person into fresh air. If not breathing, give artificial respiration. Consult a physician.
Skin contact:	Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.
Eye contact:	Flush eyes with water as a precaution.
Ingestion	Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.
Spill procedure:	Prevent further leakage or spillage if safe to do so. Do not let product enter drains, discharge into the environment must be avoided. Pick up and arrange

disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal. Follow hazardous waste disposal protocol for laboratory.

Fire-fighting measures: Use water spray, alcohol resistant foam, dry chemical or carbon dioxide. Fire-fighters to wear self-contained breathing apparatus if necessary

Health surveillance **not required**.
Specific training **not required**.
Hazards and risks are suitably controlled using above measures.

COSHH Summary: Hexamethyldisilazane (HMDS), Sigma-Aldrich H4875

MSDS supplier and revision date: Sigma-Aldrich, 28.07.15	
Task description: Samples incubated in 1:1 HMDS and ethanol, and 100% HMDS. 100% HMDS left to evaporate under fume hood	
Hazard statements:	**Precautionary statements:**
H225 Highly flammable liquid and vapour.	P210 Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking
H302 + H332 Harmful if swallowed or if inhaled	P261 Avoid breathing dust
H311 Toxic in contact with skin	P273 Avoid release to the environment
H412 Harmful to aquatic life with long lasting effects	P280 Wear protective gloves/ protective clothing/ eye protection/ face protection
	P302 + P352 + P312 IF ON SKIN: Wash with plenty of water. Call a POISON CENTER or doctor/ physician if you feel unwell
	P304 + P340 + P312 IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER or doctor/ physician if you feel unwell

Could a less hazardous substance be used instead? No

Control measures
Practice: Handle in accordance with good laboratory hygiene and safety practice. Wash hands before leaving laboratory. Avoid contact with skin and eyes. Avoid breathing vapours, mist or gas. Ensure adequate ventilation. Keep away from sources of ignition - no smoking. Take measures to prevent the build-up of electrostatic charge. Handle in a fume hood.
Personal protective equipment: Safety glasses and face shield, nitrile rubber gloves, lab coat.
Storage: Store in cool place. Keep container tightly closed in a dry and well-ventilated place. Containers which are opened must be carefully resealed and kept upright to prevent leakage.

Emergency procedure

General advice: Consult a physician. Show MSDS (attached) to doctor in attendance.

Inhalation: Move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

Skin contact: Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.

Eye contact:	Flush eyes with water as a precaution.
Ingestion	Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.
Spill procedure:	Prevent further leakage or spillage if safe to do so. Do not let product enter drains, discharge into the environment must be avoided. Contain spillage, and then collect with an electrically protected vacuum cleaner or by wet-brushing and place in suitable, closed containers for disposal. Follow hazardous waste disposal protocol for laboratory.
Fire-fighting measures:	Use water spray, alcohol resistant foam, dry chemical or carbon dioxide. Fire-fighters to wear self-contained breathing apparatus if necessary

Health surveillance **not required**.
Specific training **not required**.
Hazards and risks suitably controlled using above measures.

E: Bacteria identification and preservation

Initial characterisation

Gram staining

Small sample removed from monoculture plate using a sterile inoculating loop, smeared onto a glass slide with a drop of PBS and heat fixed. Fixed culture covered with crystal violet for 30 seconds, rinsed briefly with water, and covered with iodine for 30 seconds. Iodine rinsed off very briefly with water followed by decolouriser (acetone) (excess washing with acetone will remove all of crystal violet!). Counterstained by covering fixed culture with safranin for 30 seconds, briefly rinsed with water and left to air dry. Once dry, slides were examined under oil immersion microscope to determine stain colour (Gram positive or negative) and shape.

If colour of Gram stain was uncertain, a small sample from the monoculture was added to a drop of 3% KOH and mixed well with a sterile inoculating loop. If a mucoid string formed, confirmed to be Gram negative (as breakdown of outer cell lipopolysaccharide layer releases intracellular viscous or emulsified material). If no stringy substance formed, it was identified as Gram positive.

Oxidase test

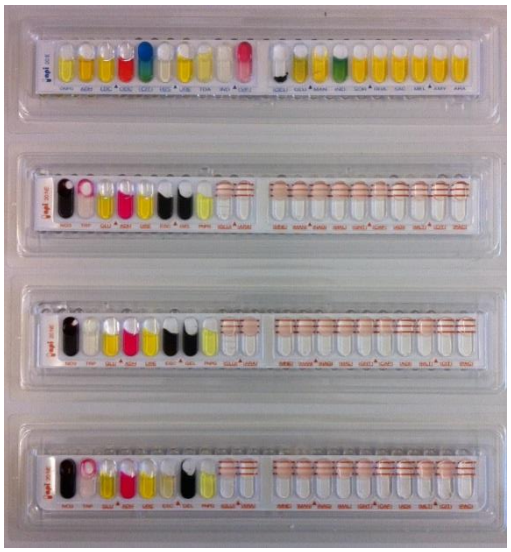
Oxidase tests were conducted using 1% Kovac's oxidase reagent; a drop of reagent was added to sterile filter paper and a small sample of bacteria was added. Colour changes were noted; turning from colourless to blue/black indicates oxidase positive bacteria, and no colour change indicates oxidase negative bacteria.

Catalase test

Catalase tests were conducted by observing the decomposition of hydrogen peroxide through the formation of bubbles. Bacteria samples were added to a drop of hydrogen peroxide; if bubbles form, the bacteria are catalase positive, if no bubbles form they are catalase negative.

Biochemical tests – API 20E and 20NE

Carried out according to manufacturer's guidelines (bioMérieux API 20E and API 20NE). Briefly, test strips were prepared and inoculated with strain to be identified in a sterile environment. Strips were incubated at 30^{o}C for 18-24 hours and read according to instructions (colour changes and positive/negative results). A numerical profile of each strain was determined according to results, and entered into database to obtain species identity



Examples of results from inoculation strips (1: API 20E, 2-4: API 20NE) showing colour changes/opacity of test cupules.

Molecular sequencing *[Dr. Graham Burgess and Alicia Maclaine]

Sequencing of 16S RNA was undertaken to provide identification to genus level.

Results

CHAPTER 2

Isolate ref. code	Gram	Oxidase	Shape	Catalase	ID Method	ID
1	Neg	Pos	R	Neg	20NE	*Aeromonas hydrophila*
2	Neg	Pos	R	Neg	20NE	*Aeromonas sobria*
3	Pos	Neg	C	Pos	PCR	*Paenibacillus sp*
4	Pos	Neg	C	Neg	PCR	*Lactococcus sp*
5	Neg	Pos	R	Pos	20NE	*Shewanella putrefaciens*

CHAPTER 3

	Isolate ref. code	Gram	Oxidase	Shape	Catalase	ID Method	ID
PBS (Control)	A	Neg	Pos	R	Neg	20NE	*Aeromonas sobria*
	B	Neg	Pos	R	Neg	20NE	*Aeromonas hydrophila*
	C	Pos	Neg	C	Pos	PCR	*Paenibacillus sp.*
	D	Pos	Neg	C	Neg	PCR	*Lactococcus sp.*
	E	Neg	Pos	R	Pos	20NE	*Shewanella putrefaciens*
	F	Neg	Neg	R	Pos	20E	*Citrobacter youngae*
1000mg/L Formalin	G	-	-	-	-	-	*Chromobacterium violaceum*
	H	Neg	Pos	R	Neg	20NE	*Shewanella putrefaciens*
	I	Pos	Neg	C	Pos	PCR	*Kocuria sp.*
	J	Neg	Pos	R	Neg	20NE	*Aeromonas hydrophila*
	K	Neg	Pos	R	Neg	20NE	*Aeromonas sobria*
	L	Pos	Neg	C	Neg	PCR	*Lactococcus sp.*
1500mg/L Formalin	M	Neg	Pos	R	Neg	20NE	*Aeromonas sobria*
	N	Pos	Neg	C	Pos	PCR	*Microbacterium sp.*

Strain preservation

Under sterile conditions, an individual colony from the monoculture plate for one strain was transferred to a labelled preservation vial containing the polypropylene beads, glycerol and culture broth (Brucella broth). Once the colony has been transferred and most of the bacteria is suspended in the broth, the loop was removed, the screw cap replaced and the vial inverted 10 times to ensure the bacteria is evenly distributed throughout the broth. The vial was left for approximately 15 minutes at room temperature, before removing and discarding the remaining vial solution (what has not been absorbed by the beads) with a sterile pipette and transferring to a -80^{o}C freezer. This process was repeated for every bacterial isolate that was identified.

F: Use of formalin; Protocol and COSHH Summaries

- Nitrile rubber gloves, safety glasses and a lab coat worn
- Risk assessments and COSHH completed and MSDS consulted (JCU) before carrying out this protocol
- Completed under fume hood (laboratories) or in well-ventilated area (study site)

Hazardous chemicals used in these procedures:

1. **Formalin.** Harmful if swallowed, may cause an allergic skin reaction, suspected of causing genetic defects, may cause cancer

Protocol summaries:

1. Fixation of specimens for morphological studies

- Specimens held in 70ml screw cap specimen jar with no more than 40ml of formalin (10% NBF) per jar, specimens added to jar in a well-ventilated area
- Jars transported from study site to laboratories in secondary container to avoid spillage. No waste disposal of formalin on site
- In laboratories, all work with formalin completed under fume hood. Specimens washed prior to subsequent treatments. Formalin waste disposed of according to laboratory procedures

2. Chemical treatment (disinfectant)

- Dilutions required (100mg/L and 1500mg/L) prepared in laboratories under fume hood prior to use in field
- Specimens held in 70ml screw cap specimen jar for 15 minutes, with no more than 40ml of formalin per jar, completed in a well-ventilated area
- Specimens washed before completing bacteriology studies. Formalin waste retained and transported to laboratories and disposed of according to standard procedures

3. Anaesthetisation trials

- After anaesthetisation method completed, tested by adding specimens to formalin (10% NBF), in 70ml screw cap specimen jar with no more than 40ml of formalin per jar, specimens added to jar in a well-ventilated area
- Worms assessed for activity and body distortion. Specimens washed prior to subsequent treatments. Formalin waste retained and transported to laboratories and disposed of according to standard procedures

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COSHH Summary: Formalin solution, neutral buffered, 10%, Sigma-Aldrich HT501128

MSDS supplier and revision date: Sigma-Aldrich, 26.03.15
Task description: Specimens incubated in formalin in screw-cap specimen jars for fixation or for disinfectant trials (specimens incubated in formalin up to 1500mg/L).

Hazard statements:		**Precautionary statements:**	
H302	Harmful if swallowed.	P201	Obtain special instructions before use.
H317	May cause an allergic skin reaction.	P280	Wear protective gloves/ protective clothing/ eye protection/ face protection
H341	Suspected of causing genetic defects.	P301 + P312 + P330	IF SWALLOWED: Call a POISON CENTER or doctor/ physician if you feel unwell. Rinse mouth.
H350	May cause cancer.	P308 + P313	IF exposed or concerned: Get medical advice/ attention.
		P333 + P313	If skin irritation or rash occurs: Get medical advice/ attention.

Could a less hazardous substance be used instead? No

Control measures
Practice: Handle in accordance with good laboratory hygiene and safety practice. Wash hands before leaving laboratory. Avoid contact with skin and eyes. Avoid ingestion and inhalation of vapour, handle in a fume hood. Ensure adequate ventilation. Keep away from sources of ignition - no smoking. Take measures to prevent the build-up of electrostatic charge.
Personal protective equipment: Safety glasses, nitrile rubber gloves, lab coat.
Storage: Store in cool place. Keep container tightly closed in a dry and well-ventilated place. Containers which are opened must be carefully resealed and kept upright to prevent leakage.

Emergency procedure

General advice:	Consult a physician. Show MSDS (attached) to doctor in attendance.
Inhalation:	Move person into fresh air. If not breathing, give artificial respiration. Consult a physician.
Skin contact:	Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.
Eye contact:	Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.
Ingestion	Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.
Spill procedure:	Prevent further leakage or spillage if safe to do so. Do not let product enter drains, discharge into the environment must be avoided. Contain spillage, and then collect with an electrically protected vacuum cleaner or by wet-brushing and place in closed container for disposal. Follow hazardous waste disposal protocol for laboratory.
Fire-fighting measures:	Use water spray, alcohol resistant foam, dry chemical or carbon dioxide. Fire-fighters to wear self-contained breathing apparatus if necessary

Health surveillance **not required**.
Specific training **not required**.
Hazards and risks suitably controlled using above measures.

G: *Decadidymus valverdi sp. nov.*, Australia 2016; 18S and 28S partial sequences

18S (small subunit) ribosomal RNA gene – partial sequence, 1700bp

TTCACACCACTTGATGGTGAACCGCGAATGGCTCATTAAATCAGCTTTTGTTTCTTAGACTTTACCCATTACTTGGATAACTA
TAGTAATTCTAGAGCTAATACATGCCAATGTGCCGTTGCGTTTTTGCATCGGCGGTTTGATTAGATCAAAGCCAACCGGTCC
GTTTGGACTGTTAGCTTGGTGACTCTAGATGACCTACCTAATCGCACGATCTTTGAATCGGCGATGTATCTTTCAAGTGTCT
GACCTATCAACTTTCGATGGTGAGAGATATGCTCACCATGGTGATAACGGGTAACGGGGAATCAGTGTTCGATTCCGGAGA
GGGAGCCTTAAAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTGCCCACTCTCAGTTAGAGGAGGCAGT
GACGATAAATAACAATGCAAGACTCAAATTGAGGCCTTGCAATTGGAATGAGAACAATTTAAATCCTTTATCGAGGATCAAT
TGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTGCTGCAGTTGAAAAGCT
CGTAGTTGGATATGAGGTTTGAGGCGTCGGTTACGTTTTATAGCATATACTGTACGCTTCGGCCTTAAACAGCCGTTAGTT
GCTTTGCGTGCTCTTCACTGAGTGCTGCAGGGTGCTCGGCAATTTTACTTTGAACAAATTAAAGTGCTCAAAGCAGGCTAC
CAAGAGCTTGCATAGTTTTGCATGGAATAATGAAATAGGACTTTGGTTCTATTTGTTGGTTTTCGGTATCAAAGTAATGATTA
AAAGAGACAAACGGGGGCTTATGTATGGCAGAGGGAGAGGTGGAATTCTAGGATCTTTGCCAGACAGCCTACTGCGAAAG
CATTTGCCAAGGATGCCTTCATTAATCAAGAACGAAAGTGTGAGGTTCGAAGACGATCAGATACCGTCCTAATTCACACCC
CAAACGATGCCGACTAACAATCGAGTATGGCTTTATTTTATAAGCCTACTTGGCTGTCCCTGGGAAACCTTAAGTAAAGGTT
CTGGGGGGAATATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTA
ATTTGACTCAACACGGGGAAACTCACCCGAACCGGACTCTGTAAGGATAGACAGATTGATAGCTCTTTCTTGATTCAGAGG
TTGGTGGTGCATGGCCGTTCTTAGTTCGTGGACTGATTTGTCTGCCCAATCGCGATAACGAACGAGACTCTATTCTGCTAA
CTAGTATACAGGCGCCGGTACGTTTGTGCCACAGTCTGTACAGACTTCTTAGAGAGATGGGCGAACTTAAATCGCAAGAAA
GAGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCCGCACGCGCGCTACAATGATAGCGTCAGCAAGTAATAA
CACCTGGTCCGAAAGGATTGGGAAATCTTTGCATTGCTATCTCATCAGGGATTGAGACTTGTAATTATTTCTCATGAACGAG
GAATTCCTAGTAGATGCATGTCATCAGCATGCGTCGATTATGTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGA
TTGAATGGTTTAGTGAGGTCATCGGATTGATCTAGAGTGGGGGTAACTTCGCTCTGGTGTCGAGAAGACGATCGAACT

28S (large subunit) ribosomal RNA gene – partial sequence, 3363bp

TAACAAGGATTCCCCTAGTAACAGCGAGCGAACAGGGAACAGCCCAAAACTGAATCCCCTGGCCTAGCGGTCAGTGGGAA
ATGTAGTTTTAGGGTGATCTCTTGCTTTGGTGACGCCGGTCTGAAGTTCGCACGATTGCGGCCACTTCTCAGAGAGGGTGT
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GGTAAACTCCATCCAAGGCTAAATATAACACTAGACCGATAGTCAACAAGTACCGTGAGGGAAAGTTGAAAAGAACTTTGA
AGAGAGAGTTAATAGTACGTGAAATCACCCAGAGGTAAACGGGTAAAGCCGAAATTGGTGAGGGGGTTGATACCTTTCGG
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CGCCGAGACCATGATCGACGATCGGGTCGTCTGTGGCAGGAGAAGGTAGCGGTTGGGTCGTAAGACTTGATCGTGTTATA
GCTCCTGTCAGTTCAGCGGACTGACTTGGTTGTTGGGCAGTTGCGATGTAAGCCCTTTGAGGCGCACACATGCCTCTACG
GCTGGCCGGCGCTTGGCTCACTTGTTTTAGAGTTTGCCGAGTGCAGGGCTGGTCGATGGAGGTAGCATGCTGTGCATTAC
GTCCACAATCTATGGTTCAATGTAGGCCCTTTACCTGTCCCGTCTTGAAACACGGACCAAGGAGTCTAACATGTTTGCGAG
TCATTGGGCGATACGAACCCCAAAGGCGAAGTGAAAGTGAAGGCCTGTGTTGGCAGGCTTAGGCAGGATCGTTGTGCCCT
AGGGTGCGATGCGCACTGCCGACTCATTGGGTACTCCTAGTGAAGTCAGAGCAAACATGTTGTAACCCGAAAGATGGTGA
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AAACTTGAAATGGGTGAGAAGCCGAGCTCACTCAACTGGAGCTCTGGCCGCTCGAATATGAGAGCAAAGTGGGCCATTTT
TGGTAAGCAGAACTGGCGCTGCGGGATGAACCGAACGAGGTGGTTTAGGTGCCAAACATTTCGCTCATGAGATACCACAA
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CGAATCAACCAGCCCTGAAAATGGATGGCGCTTGAGCGATCGACCTAGACCGCGCTGTTACCGCGATTGTGCATGGATGC
GGTAACAAGTAGAAGGGCGACATGGTTGCTTTGAAGCTGCGGCCGTGAGGCCGGGTGGAGCGGCTATGTGTGCAGATCT
TGGTGGTAGTAGCAAGTATTCAAGTGAGAACCTTGAAGACTGATGTGGAGAAGGGTTCCATGTGAACAGCAGTTGAACATG
GGTTAGCCGGTCCTAAGTGAAACGGTAACTCGTTGCGCAGCGAGGGGTGTATGATATTGCGGTGGCTCAGCTCGGCAGTA
TCCAAATACGGTGGCTAGAGAGCTACCCCTCAGAGCGAAAGGGTGTTGGGTTAATATTCCCAAGCCAGTCTGTGGAAATTT
ACCTTCGGGTAAAGTGCGGTAACGCAATCGAGCCTGGAGACGATAGCGAGAGTACTTGGAAGAGTTCTCTTTTCTTTGTTA
GAAAGTATTTTCCCTGGAAAGTTGTTGTAACGAGATAGGGAAAGCGCTTTCGTAAAGCACTGCGGTTTCTGTAGTGTCAAG
TTTACTCTTGTTGTCCCTTGAAAATCCAGGTGAGAGACAATTTCCAGACTGTCCGTACCCATATCCGCAGCAGGTCTCCAA
GGTTAAAAGCCTTTAGTCTTTGAACAATGTAGGTAAGGGAAGTCGGCAAATTAGATTCGTACCTTCGGTAGAAGGATTGGC
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GGGTCTAAAAGGCTGTTAACATTTCGTGATTTCTGCTCAGTGCTCTGAATGTCAAAGTGAAGAGATTCAATGAAGCTCGAGT
AAACAGCGGGAGTAACTATGACTCTCTTAAGGTAGCCAAATGCCTCGTCCTCTAATTAGGGACGCGCACGAATGGATCAAC
GAGATTCCCACTGTCCCTATCTACTATCTAGCGAAACCACAGCCAAGGGAACGGGCTTGGAAAAATCAGCGGGGAAAGAA
GACCCTGTTGAGCTTGACTCTAGTCCGATTTTGTGAAGAGACATAAGAGGTGTAGTATAGATGGGAGACGCAAGTCGAATT
TGAAATACCATTACTTTTATCGTTTCTTTACTTATTCAGTGATACAGAACACGGATGAATTTCCATGTTTTATTGATTTGAAGT
GTGCAACCACGAGTTGTGCATGACCTGTGCTGAAGACAATTTCAGGCAGGGAGTTTGACTGGGGCGGTACATCTGTCAAA
AAGTAACGCAGGTGTCCAAAGGTGAGCTCAGCCAGGACAGAAACCTGGTGTAGAGTAAAAGGACAAAAGCTTGCTTGATT
TTGATTTTCAGTATGAATACAGACCGCGAAAGCGGGGCCTATCGATCCTTTTGAATTTCAACATTCAGAGTTTGAAGCAAGA
GGTGTCAGAAAAGTTACCACAGGGATAACTGGCTTGTGGCGGCCAAGCGTTCATAGCGACGTCGCTTTTTGATCCTTCGAT
GTCGGCTCTTCCTATCATTATGAAGCAGAATTCATCAAGCGTTGGATTGTTCACCCACTAATAGGGAACGTGAGCTGGGTT
TAGACCGTCGTGAGACAGGTTAGTTTTACCCTACTGATGATGACCGTCGTTGTGATGGTAATCCTGCTCAGTACGAGAGGA
ACAGCAGGTTCAGGCATTTGGTTTATGTAGCTGGTCGAAAGGCCAATGCTGCGAA

H: Molecular Protocols, COSHH and Risk Assessment

Polymerase chain reaction (PCR, or qPCR) of cDNA or plasmid samples using DNA polymerase enzymes: Hauton 006

- ***This protocol should be carried out in room 454-07, 454-01, 454-11, 454-15***
- ***Laboratory gloves should be worn when using this protocol***
- ***All* E. coli *contaminated material should disposed of in the biohazard waste bins in 454-01 and 454-07 and should then be autoclaved before ultimate disposal***
- ***This standard protocol can be performed outside of normal working hours.***

There are no hazardous chemicals used in this protocol for which COSHH assessments are necessary. All chemical waste can be disposed of as normal lab waste.

Procedure (will vary depending on polymerase and purpose of the PCR):

1. Add the following reagents to a thin-walled 200 µl or 500 µl PCR tube in the order listed below.

Amount	Component	Final Concentration
5 µl	10X Taq PCR Reaction Buffer	1 X
1 µl	Deoxynucleotide Mix	200 µM (each dNTP)
x µl	Forward primer	0.1 - 5 µM[+]
y µl	Reverse primer	0.1 - 5 µM
2.5 µl	Taq DNA Polymerase	0.05 unit/µl
- ml	Template DNA (typically 10 ng)*	200 pg/ml
q.s.	Water	
50 µl	*Total reaction*	

** if using PCR to test for colony inserts then simply transfer a small amount of the colony to the PCR tube using a sterile toothpick.*

[+] *Volume of primers required must be determined empirically*

***Note**: A master mix is highly recommended when setting up multiple reactions.*

2. Mix gently by vortex and briefly centrifuge to collect all components to the bottom of the tube.
3. Optimum cycling parameters vary with PCR composition (i.e. primer sequences, template, $MgCl_2$ concentration etc.) and thermal cycler. It may be necessary to optimize the cycling parameters to achieve maximum product yield and/or quality. Common cycling parameters are given in the following table.
4. Place the tubes into the thermal cycler, taking care not to touch the heated block of the cycler or the heated lid. If necessary use tweezers to add and remove tubes.

For cycles 1-30

Denaturation 94 °C 1 min
Annealing temp. 30 seconds
Extension 72 °C 30 sec - 3 min
Note: 25-30 cycles of amplification are recommended.

1. The amplified DNA can be evaluated by agarose gel electrophoresis

DNA gel electrophoresis: Hauton 011

- ***Laboratory gloves should be worn when using this protocol***
- ***A separate protocol should be consulted for the preparation of aliquots of ethidium bromide stock (see Hauton 020)***
- ***Ethidium bromide stock solutions (10mg ml^{-1}) should be handled in the fume cupboard in room 454-07***
- ***All ethidium bromide contaminated waste should be disposed of in the 'ethidium bromide' designated waste bin in 454-07***
- ***This standard protocol can be performed outside of normal working hours***

Hazardous chemicals used in this procedure (as identified in the attached COSHH forms):
1. Ethidium bromide solution (10 mg ml^{-1}). H302-H330-H341. Harmful if swallowed, fatal if inhaled, suspected of causing genetic defects.

Procedure:

1. 0.5ml aliquots of stock ethidium bromide (10mg ml^{-1}) are stored in the fridge in 454-07.
2. Select a gel kit and rinse with de-ionised water.
3. Make up a 1% agarose gel using 1x TAE buffer. Typically 100-200ml volumes of agarose can be prepared and stored in the fume cupboard in 454-07. Melt the agarose suspension using the microwave in 454-07. Select high power on the microwave and heat for 30 second intervals until completely dissolved. WEARING THERMALLY INSULATED GLOVES open the microwave and mix the agarose regularly during this period to aid dissolution and to prevent the agarose boiling over. DO NOT cover the agarose will foil or cling film during the melt or melt agarose in a screw topped bottle - this is an explosion risk.
4. WEARING THERMALLY INSULATED GLOVES remove the melted agarose from the microwave and pour out 30ml volumes into a conical flask in the fume cupboard, allow to cool.
5. Add 1 µl of stock ethidium bromide per 10 ml of gel to the cooled agarose in a fume cupboard. Discard the contaminated tip into the designated ethidium bromide waste container in 454-07.
6. Set up the gel kit and pour in the cooled agarose
7. Place the comb into the gel and remove any bubbles with the end of a pipette tip
8. Leave the gel to cool and go opaque
9. Once set, remove the comb and cover the gel in 1 X TAE
10. Load the DNA samples using the appropriate loading buffer, remember to include a lane for the DNA ladder.
11. Run the gel at 70V for one hour. Ensure that the power leads are fully secured and the gel kit lid is in place before switching on the power
12. Check the gel using the GelDoc imaging system in lab 454-07. Always wear gloves when using the GelDoc system but do not touch the computer keyboard whilst wearing gloves.

CHEMICAL RISK ASSESSMENT FORM

Version Sept 2013

Department:	OES	**Location of use:**	454/07	**Persons involved:**	Assessor, personnel working on the task, staff and students working in the location
Lab procedure ref:	DNA gel electrophoresis (Hauton 011)			**MSDS supplier and revision date:**	Sigma-Aldrich 24.04.2012
Describe the task:	Agarose gel is mixed and dissolved by warming in microwave oven. After partial cooling, ethidium bromide is added and gel is poured into gel block. When fully cooled, gel is submerged in TAE buffer in a gel kit and DNA samples and standards are loaded. Electrical current is applied to the submerged gel using a power pack. Gel is visualised using a GelDoc imaging system.				

HAZARD IDENTIFICATION AND CONTROL

Chemical(s) or Product Name	Risk Phrases/Hazard Statements	Hazard Group	Exposure Potential	Exposure Control Approach (ECA)
Ethidium bromide	H302- Harmful if swallowed. H330- Fatal if inhaled. H341- Suspected of causing genetic defects.	E	Low	EC3

For multiply chemicals what is the highest ECA required for this task?	EC3
Will you be using a lower level ECA (only allowed for those denoted by*)?	N/A

SPECIAL CONSIDERATIONS	
Could a less hazardous substance be used instead?	No
Does the substance present additional risks to certain groups or individuals?	Expectant mothers.
Do your chemicals have risk phrases or hazard statements that require a DSEAR assessment?	No

PERSONAL PROTECTIVE EQUIPMENT (PPE)			
Eye protection:	Safety glasses	**Hand protection:**	Nitrile examination gloves
Face protection:		**Special clothing:**	No, standard lab coat
Respiratory protection:		**Any others:**	

EMERGENCY PROCEDURES	
Eye contact:	Flush eyes with water as a precaution.
Inhalation:	Mover person into fresh air. If not breathing, give artificial respiration. Consult a physician.
Skin contact:	Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.
Ingestion:	Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician.
Spill procedure:	Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

HEALTH MONITORING

Is health surveillance required for the protection of the health of employees?
This is required when: (a) there is a disease associated with the substance in use (eg Asthma, Dermatitis, Cancers); (b) it is possible to detect the disease or adverse change and reduce the risk of further harm; (c) the conditions in the workplace make it likely that the disease will appear. Please refer to Guidance for COSHH Health Surveillance on the H&S Website.

No. Expectant mothers will not be permitted to perform this protocol

SPECIAL TRAINING REQUIREMENTS

No. Appropriate individual training will be provided before this protocol is used for the first time.

INSTRUCTIONS FOR SAFE STORAGE
How should the substance be stored? (e.g. locked cupboard which is correctly labelled, away from other substances, etc.)
Is there any other substance that this substance must not come into contact with?

Substance should be stored and used only in designated ethidium bromide areas.

DISPOSAL PROCEDURES *Detail fully how the chemical waste is to be disposed of (down sink, by specialist contractor, etc)*

Are chemicals with Risk Phrases R50-R59 (environmental hazards) involved?

Place all ethidium bromide contaminated waste in designated hazardous waste stream.

ASSESSMENT OF RISK USING CONTROLS DETAILED ABOVE

Are the hazards/risks suitably controlled, using the control measures detailed above? If not, state the further actions required, e.g. Requirement for a standard operating procedure (SOP), restricting access, prohibiting lone working, specifying supervision, etc in the box below.

Standard protocol (Hauton 011) exists and specific individual training will be provided.

FNES / Ocean & Earth Science: General Risk Assessment Form

This form must be used in conjunction with the Risk Assessment Guidance Notes and Hazard Checklist *available on NOCSNET H&S section Version 1.3 (Jan 2013)

Faculty / Service / Academic Unit / Team / Department: *(see Note 1)*	FNES/ERE/OES/Hauton MIPI-LAB	**Location / Room Number / Map Reference:**	454/07, 454/01, 454-11, 454-15
RISK ASSESSMENT TITLE *MAIN ACTIVITY*	PCR and quantitative PCR (Hauton 006)		
Briefly describe the 'tasks' being assessed:	Preparation of running of polymerase chain reactions using template DNA and a thermocycler		
Other assessments, documents or considerations which might also be required:	Protocol: Hauton006.		

IF THE RISKS FOR ANY OF THE HAZARDS IN THE TABLE IS DEEMED TO BE HIGH, WORK MUST NOT PROCEED UNTIL FURTHER CONTROLS ARE PUT IN PLACE.

IDENTIFICATION OF REASONABLY FORESEEABLE HAZARDS *(see Notes 2i & 2ii)*				**INDICATE CONTROL MEASURES IN PLACE & EVALUATE THE INHERENT OR RESIDUAL RISKS** *(see Notes 3i & 3ii)*							
Reference (a)	***Task / Aspect of Work.*** *(b)*	***What are the hazards?*** *Refer to checklist* *(c)*	***Who might be harmed and how could that harm arise?*** *(i.e. Who, how and nature of harm)* *Any special considerations? (d)*	**SEVERITY 1-5**	**LIKELIHOOD 1-5**	**INHERENT RISK** *(e)*	***What are you already doing? List existing measures to control risk.*** *(f)*	**SEVERITY 1-5**	**LIKELIHOOD 1-5**	**RESIDUAL RISK** *(g)*	**Further Controls Required? (YES/NO)**
	INCUBATION OF REACTION STEPS IN A THERMOCYCLER	HEATED LID OF THERMOCYCLER	USER, BURNS TO SKIN IF TOUCHING THE HEATED LID OR BLOCK	**2**	**1**	**LOW**	ENSURE THE LID IS FULLY RAISED BEFORE PLACING TUBES ONTO THE HEATED BLOCK. TUBES SHOULD BE PLACED INTO THE BLOCK AND REMOVED FROM THE BLOCK USING FORCEPS	**2**	**1**	**LOW**	**NO**
	BIOHAZARD RISK FROM E.COLI CONTAMINATED MATERIAL WHEN CONDUCTING PCR WITH PLASMID TEMPLATES	GASTROINTESTINAL DISORDER	USER	**2**	**1**	**LOW**	WEAR LATEX-FREE NITRILE EXAMINATION GLOVES. GMO BIOHAZARD CLASS 1 PROCEDURE - APPROVED BY UOS GMBSC. WEARING LABORATORY GLOVES, GLP. USING E.COLI THAT ARE WEAKENED LABORATORY STRAINS THAT DO NOT SURVIVE OUTSIDE OF THE LABORATORY ON SELECTIVE MEDIA. VIABLE E.COLI ARE GMO BIOHAZARD CLASS 1 PROCEDURE - APPROVED BY UOS GMBSC. WEARING LABORATORY GLOVES, GLP. USING E.COLI THAT ARE WEAKENED LABORATORY STRAINS THAT DO NOT SURVIVE OUTSIDE OF THE LABORATORY ON SELECTIVE MEDIA. VIABLE E.COLI ARE USED IN VERY SMALL QUANTITIES. E.COLI CONTAMINATED WASTE (TOOTHPICKS) IS DISCARDED IN THE DESIGNATED BIOHAZARD WASTE BINS IN 454-01 AND 454-07. AFTER THE PCR REACTION IS COMPLETE THE E.COLI IS DENATURED AND RENDERED SAFE	**2**	**1**	**LOW**	**NO**

FNES / Ocean & Earth Science: General Risk Assessment Form

Faculty / Service / Academic Unit / Team / Department: *(see Note 1)*	FNES/OES/Hauton MIPI-LAB	**Location / Room Number / Map Reference:**	454/07
RISK ASSESSMENT TITLE *MAIN ACTIVITY*	DNA gel electrophoresis (Hauton 011)		
Briefly describe the 'tasks' being assessed:	Agarose gel is mixed and dissolved by warming in microwave oven. After partial cooling, ethidium bromide is added and gel is poured into gel casting tray. When fully cooled, gel is submerged in 1 x TAE buffer in a gel kit and DNA samples and standards are loaded. Electrical current is applied to the submerged gel using a power pack. Gel is visualised using a GelDoc imaging system.		
Other assessments, documents or considerations which might also be required:	COSHH assessment and MSDS for ethidium bromide. Protocol Hauton011		

IF THE RISKS FOR ANY OF THE HAZARDS IN THE TABLE IS DEEMED TO BE HIGH, WORK MUST NOT PROCEED UNTIL FURTHER CONTROLS ARE PUT IN PLACE.

IDENTIFICATION OF REASONABLY FORESEEABLE HAZARDS *(see Notes 2i & 2ii)*				**INDICATE CONTROL MEASURES IN PLACE & EVALUATE THE INHERENT OR RESIDUAL RISKS** *(see Notes 3i & 3ii)*							
Reference *(a)*	***Task / Aspect of Work.*** *(b)*	***What are the hazards?*** *Refer to checklist* *(c)*	***Who might be harmed and how could that harm arise?*** *(i.e. Who, how and nature of harm)* *Any special considerations? (d)*	**SEVERITY 1-5**	**LIKELIHOOD 1-5**	**INHERENT RISK** *(e)*	***What are you already doing? List existing measures to control risk.*** *(f)*	**SEVERITY 1-5**	**LIKELIHOOD 1-5**	**RESIDUAL RISK** *(g)*	**Further Controls Required? (YES/NO)**
1	MAKING, LOADING AND READING GEL	TOXIC IF SWALLOWED, FATAL IF INHALED. SUSPECTED OF CAUSING GENETIC. INHALATION OR SKIN CONTACT WITH ETHIDIUM BROMIDE	ASSESSOR, PERSONNEL WORKING ON THE TASK, STAFF AND STUDENTS WORKING IN THE LOCATION	**4**	**3**	**HIGH**	VERY SMALL QUANTITIES AT LOW CONCENTRATION (10 MG/ML) LIQUID ARE HANDLED IN FUME CUPBOARD. AGAROSE IS HEATED IN MICROWAVE AND THEN PLACED IN FUME HOOD PRIOR TO ADDING ETHIDIUM BROMIDE. LATEX FREE SURGICAL GLOVES WORN AT ALL TIMES DURING THE PROCEDURE. DESIGNATED WASTE DISPOSAL IN 454/07.	**4**	**2**	**MED**	**NO**
2	LOADING AND REMOVING GEL FROM GEL KIT	ELECTROCUTION FROM GEL KIT POWER PACK	ASSESSOR, PERSONNEL WORKING ON THE TASK, STAFF AND STUDENTS IN DIRECT CONTACT WITH INSTRUMENT	**4**	**2**	**MED**	GEL KITS AND POWER PACKS SHOULD ONLY BE OPERATED ACCORDING TO OPERATING MANUAL. POWER LEADS ALWAYS CONNECTED BEFORE POWER IS TURNED ON. GEL KIT DESIGN ENSURES THAT LIDS ARE SECURELY IN PLACE BEFORE CURRENT CAN BE APPLIED	**4**	**2**	**MED**	**NO**
3	MAKING GEL	BURNS FROM HANDLING HOT AGAROSE	ASSESSOR, PERSONNEL WORKING ON THE TASK	**2**	**4**	**MED**	HOT AGAROSE IN THE MICROWAVE SHOULD ONLY BE HANDLED WITH THERMALLY PROTECTIVE GLOVES. AGAROSE IS ALLOWED TO COOL IN FUME CUPBOARD BEFORE ETHIDIUM BROMIDE IS ADDED AND GEL IS POURED.	**2**	**2**	**LOW**	**NO**
4	MAKING GEL	EXPLOSION RISK USING MICROWAVE	ASSESSOR, PERSONNEL WORKING ON THE TASK, STAFF AND STUDENTS WORKING IN THE LOCATION	**3**	**3**	**MED**	AGAROSE GELS IN MICROWAVE ARE NEVER COVERED OR STOPPERED DURING MELTING. MICROWAVE RADIATION IS ONLY APPLIED IN INTERMITTENT (30 SECOND) PERIODS. AGAROSE IS REGULARLY MIXED DURING THE MELT PROCESS.	**3**	**2**	**LOW**	**NO**