1 **Applying the power of transcriptomics: Understanding male sexual development in**
2 **decapod *Crustacea***

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Abstract

The decapod *Crustacea* are the most species-rich order of the crustacean classes and include some of the most charismatic and highly-valued commercial species. Thus, the decapods draw a significant research interest, in relation to aquaculture as well as gaining a broader understanding of these species' biology. However, the diverse physiology of the group considered with the lack of a model species have presented an obstacle for comparative analyses. In reflection of this, the recent integration of comparative transcriptomics has rapidly advanced our understanding of key regulatory pathways and developmental phenomena, an example being our understanding of sexual development. We discuss our work in the Eastern spiny lobster, *Sagmariasus verreauxi*, in the context of what is currently known about male sexual development in the decapods, highlighting the importance of transcriptomic techniques in achieving our recent advancements.

Firstly we describe male sexual differentiation and maturation, as mediated by the insulin-like androgenic gland hormone (IAG), integrating the role of regulatory binding proteins (IGFBPs), a tyrosine kinase insulin receptor (TKIR), as well as the upstream effect of neuroendocrine hormones (GIH and MIH). We then consider the less well understood mechanism of male sex determination, with an emphasis on what we believe to be the key regulatory factors, the Dsx- and mab-3-related transcription factors (Dmrts). Finally we discuss the function of the antennal gland (AnG) in sexual development, relating to the emergence of male-biased up-regulation in the AnG in later sexual maturation and the sexually dimorphic expression of two key genes *Sv-TKIR* and *Sv-Dmrt1*. We then present the AnG as a case study to illustrate how comparative transcriptomic techniques can be applied to guide preliminary analyses, like the hypothesis that the AnG may function in pheromone biosynthesis.

In summary we describe the power of transcriptomics in facilitating the progress made in our understanding of male sexual development, as illustrated by the commercial decapod species, *S. verreauxi*. Considering future directions, we suggest that the integration of multiple omics-based techniques offers the most powerful tool to ensure we continue to piece together the biology of the important group of decapod *Crustacea*.

59 ***Definitions***

60 ***Sexual development:*** The integrated cascade that stems from an initial genetic/environmental
61 signal and eventuates with (1) sex determination, leading to activation of the downstream
62 mediators that govern (2) sex-specific differentiation and (3) ongoing maturation.

63 ***1. Sex determination:*** The genetic (or environmental) mechanism that coverts a
64 chromosomal signal to the onset of a sex-specific developmental biochemical network
65 that triggers male or female phenotypic differentiation.
66 ***2. Sexual differentiation:*** The subsequent manifestation of sex determination, resulting
67 in sex-specific phenotypic development and consequential sexual dimorphism
68 between the sexes.
69 ***3. Sexual maturation:*** The ongoing regulation of a sexually differentiated individual to
70 reach and maintain reproductive maturity and enable reproductive functionality
71 (behavioural, physiological, morphological and anatomical).

72

73 ***Introduction***

74 The group *Crustacea* includes around 50,000 species, occupying an abundance of ecological
75 niches and adopting a diversity of life history strategies (Porter et al. 2005). The decapods
76 comprise the most species-rich order of the *Crustacea* including the commercially significant
77 species of the *Palaemonidae* (prawns), *Palinuridae* (spiny lobsters), *Parastacidae* (crayfish),
78 *Penaeidae* (shrimps) and *Portunidae* (crabs) (Porter et al. 2005). Global crustacean
79 aquaculture exceeded value of US$30 billion in 2012 (FAO 2014), however, despite the
80 commercial importance of this group, the limited knowledge concerning decapod crustacean
81 physiology, considered with the lack of genomic data (Mykles and Hui 2015) is a significant
82 obstacle to our understanding of these species' biology. In reflection of this, comparative
83 transcriptomics has become the primary tool to facilitate the rapid identification of genes,
84 allowing us to develop a mechanistic understanding of key regulatory pathways from sexual
85 development (Leelatanawit et al. 2009; Zhang et al. 2011; He et al. 2012; Gao et al. 2014;
86 Jiang et al. 2014; Liu et al. 2015; Powell et al. 2015; Chandler et al. in press) to
87 metamorphosis (Ventura et al. 2015).

88 In the context of aquaculture, an understanding of sexual development and reproduction is
89 central to successful and sustainable culture (Nagaraju 2011). In addition, an insight into the

90 regulation of sexual development provides scope for the use of novel biotechnologies to
91 induce sex-change, benefitting productivity like that seen with the giant freshwater prawn,
92 *Macrobrachium rosenbergii* (Ventura et al. 2012; Sagi et al. 2013). Despite the latter
93 achievement, our broader understanding of the genetic regulation of sexual development is
94 still poor amongst the decapods. In addition, the diversity and complexities of sex
95 determination mechanisms across *Animalia* (Stothard and Pilgrim 2003; Suzuki 2010; Kopp
96 2012; Matson and Zarkower 2012; Beukeboom and Perrin 2014) severely limit what can be
97 learnt from the closest model species in the branchiopods (*Daphnia*) and arthropods.

98 Our model species, the Eastern spiny lobster, *Sagmariasus verreauxi*, is an example of one
99 such commercially valued decapod species, where a high demand and high value (Phillips
100 2006; Jeffs et al. 2013) offer significant economic gains through successful culture
101 (Fitzgibbon and Battaglene 2012a). In recent years a dramatic advancement in hatchery
102 technologies (Fitzgibbon and Battaglene 2012a; Fitzgibbon and Battaglene 2012b; Jensen et
103 al. 2013; Fitzgibbon et al. 2014) has enabled the life cycle of *S. verreauxi* to be successfully
104 closed in culture. One of the next advancements called for is to integrate a molecular
105 understanding of the species' biology to enhance current culture practices.

106

107 ***Male sexual development in Decapoda: an overview***

108 <u>*Sexual differentiation*</u>

109 Although our understanding of sexual development amongst the decapods is limited there is
110 an element of the developmental pathway that unifies our knowledge of the group. As
111 members of *Malacostraca,* the effect of the male-specific androgenic gland (AG) is
112 fundamental to male sexual differentiation. The AG is located along the sperm duct or testes
113 (Charniaux-Cotton 1954; Charniaux-Cotton 1958; Charniaux-Cotton 1956; Sagi et al. 1997)
114 and is responsible for the expression of the insulin-like androgenic gland hormone (IAG)
115 (Martin et al. 1999; Okuno et al. 1999; Manor et al. 2007). It is this IAG hormone that is
116 central to male sexual differentiation, directly governing testicular development right through
117 to the emergence of secondary-sexual characteristics (Ventura et al. 2009; Rosen et al. 2010),
118 with further evidence of its role in sexual behaviour mediated through the AG (Barki et al.
119 2003; Karplus et al., 2003; Barki et al. 2006).

120 The central function of IAG has been explicitly demonstrated by the full, functional sex-
121 reversal of male *M. rosenbergii*, to generate homogametic (ZZ) neo-females, ultimately
122 facilitating the production of an all-male population within two generations. First *via* AG
123 removal (Sagi et al. 1990; Aflalo et al. 2006) and later by specifically silencing *IAG* using
124 RNAi (Ventura et al. 2009; Rosen et al. 2010; Ventura et al. 2012), employing genetic sex
125 markers (Ventura et al. 2011a) to facilitate the rapid and accurate identification of sex-
126 changed individuals. Sex change of females into neo-males has also been achieved in
127 *M. rosenbergii,* through the use of AG-grafting techniques (Malecha et al. 1992). More
128 recently the production of active recombinant IAGs might provide a more elegant and
129 efficient method to achieve the same "masculinising" effects (Katayama et al. 2014; Aizen et
130 al. under review).

131 The X-organ-Sinus-gland complex (XO-SG), acting as the primary neuroendocrine gland in
132 the eyestalk, also has a role in sexual development, acting through the XO-SG – AG – Gonad
133 axis (Rodriguez et al. 2007). Removal of the XO-SG through eyestalk ablation leads to
134 hypertrophy and hyperplasia of the AG cells (Khalaila et al. 2002) with the increased IAG
135 production resulting in accelerated testicular development (Nagaraju 2011; Zhang et al.
136 2014a). The secreted neurohormones, gonad inhibiting hormone (GIH) aka VIH
137 (vitellogeneis inhibiting hormone in females) and molt-inhibiting hormone (MIH) are thought
138 to be those directly regulating *IAG* expression (Li et al. 2015a), having an ongoing inhibitory
139 effect throughout sexual development and reproduction (Suwansa-ard et al. 2015).

140 A visual summary of our current understanding of sexual development in *Decapoda* can be
141 seen in Figure 1A. In Figure 1B and C, we have placed this regulatory cascade of *Decapoda*,
142 in the context of the sexual development of our model species, *S. verreauxi*. Figure 1B
143 describes *S. verreauxi* development in culture, subdivided into an embryonic phase, lasting
144 66 - 68 days at 18°C, followed by hatching and a short nauplius stage lasting under an hour.
145 Seventeen phyllosoma instar then follow (Kittaka et al. 1997), lasting for a period of 5.5 - 8
146 months at 21 - 23°C (Q. Fitzgibbon. pers com); reduced from the 8 - 12 months seen in the
147 wild (Montgomery and Craig 2005). The oceanic phyllosoma then molt into a lecithotrophic,
148 non-feeding puerulus phase (a nektonic stage that swims towards the shore to seek a suitable
149 habitat). The puerulus exists for a period of 19 - 28 days at 21°C (Fitzgibbon et al. 2014),
150 over which time a digestive system re-develops, demonstrated by the emergence of the
151 hepatopancreas during the H-phase, followed by cuticular pigmentation (Ventura et al. 2015).
152 The metamorphic molt follows as the puerulus transitions into a juvenile. Within a few molts

153 the gonopores can be identified at the base of the fifth walking leg; the first morphological
154 emergence of sexual differentiation (Q. Fitzgibbon. pers com). Full sexual maturation then
155 occurs over a period of 4.5 -5 years at 18°C.

156 Considering the pivotal role of IAG during sexual differentiation, Figure 1C contextualises
157 the temporal expression profile of *Sv-IAG* alongside the morphological development of
158 *S. verreauxi*. In-depth temporal sequencing is presented (as reads per kilobase per million
159 reads, RPKM) for the phyllosoma instar 17, which can be accurately classified into intermolt,
160 early and late premolt (defined by retraction of the hepatopancreas from the cephalic shield
161 (Ventura et al. 2015)), as well as two puerulus stages taken at postmolt and at H-phase.
162 Expression was also quantified from an AG taken from an immature 400g male, the age at
163 which the AG is first visually identifiable and finally an AG taken from a sexually mature
164 3kg male. The dramatic increase in *Sv-IAG* expression over the period of later sexual
165 differentiation and maturation is readily apparent, falling below 0.5 RPKM through
166 immaturity (< 400g) but reaching nearly 1500 RPKM at sexual maturity (Ventura et al.
167 2014).

168 These data are true demonstration of the depth of sequencing achieved by next-generation
169 techniques, quantifying expression < 1 RPKM, which would otherwise go un-noticed using
170 PCR technologies. Indeed, when one considers that the first evidence of sexual differentiation
171 occurs during this phase of extremely low *IAG* expression, these data raise the debate over
172 what level of expression is to be considered biologically relevant.

173 Although the *IAG* gene has now been identified in well over fifteen decapod species
174 (reviewed in Ventura et al. 2011b; with the subsequent additions of Banzai et al. 2011;
175 Mareddy et al. 2011; Li et al. 2012a; Ma et al. 2013; Huang et al. 2014; Savaya-Alkalay et al.
176 2014; Ventura et al. 2014; Liu et al. 2015) including all those key commercial groups
177 mentioned above, there is still a limited understanding of the regulation and endocrinology of
178 the hormone. Thus, our work has used the power of comparative transcriptomics to gain a
179 more comprehensive understanding of the molecular regulation of male sexual development
180 in *S. verreauxi* as a representative for *Decapoda*.

181 In doing so, the use of high-throughput sequencing technologies has rapidly added to the
182 understanding of IAG's insulin-like endocrinology. An example of this is the recent
183 discovery of the first non-IAG insulin-like peptide (ILP), identified in *S. verreauxi* (Chandler
184 et al. 2015). *Sv-ILP1* was the first ILP of its kind to be identified amongst the decapods,

185 adding to the evidence of a broader insulin-like endocrinology. Further targeted screening
186 aided the identification of an insulin-like growth factor binding protein (*Sv-IGFBP*)
187 (Chandler et al. 2015) as well as four other putative chaperone proteins orthologous to Alpha-
188 2 macroglobulins, which were identified due to their AG-biased expression (Chandler et al.,
189 in press). The conserved function of these proteins in binding and chaperoning insulin-like
190 peptides in vertebrates (Borth 1992; Hwa et al. 1999) and decapods (Rosen et al. 2013a; Li et
191 al. 2015b) is an indication that secondary regulatory-proteins must be considered to
192 understand the bioavailability and cellular activity of IAG.

193 The identification of orthologous genes is demonstration of the power of transcriptomics,
194 where comparative analyses can improve understanding of gene function. This was the case
195 with *Sv-MAG*, the *S. verreauxi* orthologue of the AG-specific membrane-anchored protein
196 (*Cq-MAG*) identified in *Cherax quadricarintus* (Rosen et al. 2013b). Unlike in
197 *C. quadricarinatus*, *Sv-MAG* was not AG-specific but AG-biased, due to weak expression in
198 the gonads (Chandler et al. in press), suggesting that it might facilitate the function of other
199 ILPs or that IAG is expressed in the gonads. Nevertheless these data provide supportive
200 evidence for the function of the MAG protein in sexual differentiation and maturation as
201 mediated through the AG.

202 Perhaps the most significant gap in our understanding of IAG endocrinology was relating to
203 the identification of the hormone's insulin-like receptor. Using transcriptomic *in silico*
204 techniques, we were able to assemble a complete 7081-nucleotide transcript encoding a
205 conserved tyrosine kinase insulin receptor (TKIR), which (after molecular validation) we
206 termed *Sv-TKIR*. This in turn facilitated further *in vitro* studies, which provided for the first
207 time proof-of-activation of IAG with its receptor, as demonstrated through a COS-7
208 luciferase assay (Aizen et al. under review). The latter work has strong support in the recent
209 findings of Sharabi et al. (2015), who have also identified a highly homologous TKIR in
210 *M. rosenbergii* (*Mr-IR*) and demonstrated its receptor-ligand interaction with IAG, as well as
211 its explicit function in sexual development through gene silencing.

212

213 <u>*Sex determination*</u>

214 Thus far only the terminal effectors of male sexual development, regulating sexual
215 differentiation, have been discussed. The initiation of sexual development occurs with a sex-
216 specific genetic cascade mediated through a chromosomal mechanism of sex determination.

217 Unlike the unifying role of IAG, the decapods do not show a conserved mechanism of sex
218 determination. In groups such as the penaeid shrimp, a general ZW/ZZ mechanism seems to
219 exist (Benzie et al. 2001; Li et al. 2003; Preston et al. 2004; Preechaphol et al. 2007; Zhang et
220 al. 2007; Coman et al. 2008; Gopal et al. 2010), however in other groups such as the crabs
221 and freshwater crayfish, both ZW/ZZ (*Eriocheir sinensis* (Cui et al. 2015) and
222 *C. quadricantus* (Parnes et al. 2003)) and XX/XY (*Charybdis feriatus* (Trino et al. 1999),
223 *Austropotamobius pallipes* and *Austropotamobius torrentium* (Mlinarec et al. 2016)
224 mechanisms exist. All that is known in the spiny lobsters is a suggested XX/XY mechanism
225 in the Hawaiian spiny lobster *Panulirus marginatus* (Shaklee 1983). This disparity illustrates
226 the complexity of sex determination mechanisms in the decapods and elucidates to why we
227 currently lack a mechanistic understanding of the process.

228 Following this, there is no knowledge of the master sex-regulator that triggers the onset of
229 sex determination. A range of sex determination linked orthologues have been identified in
230 the decapods, mainly guided by genes characterised in the arthropods: these include
231 orthologues of the master sex-determinant in *Drosophila, Sxl* (Zhang et al. 2013a; Shen et al.,
232 2014; Powell et al. 2015; Chandler et al. in press) and downstream mediators *TRA* and *TRA-2*
233 (Leelatanawit et al. 2009; Li et al. 2012b; Zhang et al. 2013b; Cui et al. 2015; Liu et al. 2015;
234 Chandler et al. in press) as well as genes from *C. elegans* such as *FEM-1* (Ma et al. 2012; Jin
235 et al. 2013; Goa et al. 2014; Robinson et al. 2014; Cui et al. 2015; Liu et al. 2015; Powell et
236 al. 2015; Song et al. 2015). However, due to the rapid and repetitive divergence of sex
237 determination systems (Stothard and Pilgrim 2003; Suzuki 2010; Kopp 2012; Matson and
238 Zarkower 2012) the functional significance of these genes in decapods is questionable.

239 Instead, we believe that the emphasis should be placed on a family of zinc-finger, DNA-
240 binding transcriptional regulators, termed the DM-domain DNA binding motif, also known as
241 *Dsx-* and *mab-3-related transcription factor* (*Dmrt*). This gene family has a diverse and
242 conserved role in sex determination and sexual differentiation across *Animalia* (Haag and
243 Doty 2005; Hong et al. 2007; Kopp 2012; Wexler 2014). We recently identified three DM-
244 and one DMA-domain containing *Dmrts* in *S. verreauxi* (Chandler et al. in press), one of
245 which was found to be a *Dmrt11E* orthologue, also identified in *M. rosenbergii* (Yu et al.
246 2014), *Fenneropenaeus merguiensis* (Powell et al. 2015) and *E. sinensis* (Cui et al. 2015). In
247 *M. rosenbergii*, *Mro-Dmrt11E* was shown to regulate *Mr-IAG*, as silencing of the gene
248 caused a near 50% reduction in *IAG* expression (Yu et al. 2014). Again employing
249 comparative analyses, the identification of *Dmrt11E* across multiple decapod species, the

250 functional data from *M. rosenberrgii* and the AG-biased expression of *Sv-Dmrt11E* in
251 *S. verreauxi* (Chandler et al. in press)*,* strongly suggest that this *Dmrt* has a regulatory effect
252 over the AG, acting upstream of IAG. Considering the other *Dmrts* identified in *S. verreauxi*
253 and other decapods, it is our belief that this gene family should be the focus of future studies
254 regarding the regulation of sex determination and development.

255 Together, each of these advances demonstrates how comparative transcriptomics has
256 facilitated the identification and integration of novel genes into the regulatory pathway of
257 male sexual development in decapods.

258

259 ***The power of the transcriptome***

260 As well as facilitating the targeted identification of specific genes and functional pathways,
261 transcriptomic datasets also allow for broader expression analyses. Differential expression
262 analyses (DEA) provide a comparative tool to visualise large-scale patterns of transcriptomic
263 regulation. DEA have been repeatedly applied across the animal kingdom highlighting the
264 central role of sex-biased gene expression in the regulation of sexual dimorphism (Ingleby et
265 al. 2014). This is owing to the fact that sex-specific development is primarily regulated by the
266 sexually-dimorphic differential expression of shared genes, which it appears, are most
267 commonly upregulated in males (male-biased) (Ellengren and Parsch 2007; Ingleby et al.
268 2014).

269 Thus, in addition to the targeted identification of specific genes, we have applied
270 transcriptomics to visualise the broader patterns of sexual dimorphism in *S. verreauxi*.
271 Through the comparison of multiple tissues (taken from sexually mature males and females at
272 3kg) it was readily apparent that the most striking differential expression was found between
273 the gonads (12.04% of transcripts), which conformed to the general phenomenon of male-
274 biased expression (87.8% of differentially expressed transcripts were found in the testis)
275 (Chandler et al. in press). This data is hardly surprising, as the mature testis and ovary are two
276 highly dimorphic tissues reflective of their sex-specific function. What is more interesting are
277 the patterns of differential expression observed in the morphologically, and (seemingly) also
278 functionally homogenous antennal gland (AnG). Although a far lower level of differential
279 expression existed between the sexes (1.42%), this was the only other tissue to display a sex-
280 bias in differential expression, with 73.5% of transcripts again over-represented in males.

281 Placing the functional genes discussed earlier in the context of this broader-scale observation,
282 both *Sv-TKIR* and the *Sv-Dmrts* (most significantly *Sv-Dmrt1*) also show considerable
283 differential expression between male and female AnGs; measured by both RPKM and
284 semiquantitative RT-PCR; both techniques show strong correlation (Fig. 2A-B and D-E).
285 Interestingly however, these two genes appear to show a female-bias in their differential
286 expression. Although somewhat contradictory, taken together these data provide evidence
287 that the AnG appears to show sexually dimorphic expression patterns in mature individuals,
288 including genes considered to function in the regulation of sexual development. This presents
289 the idea that the AnG should be considered alongside the gonad and AG as a tissue with a
290 function in sexual development. A research outcome attained through a synergy of
291 transcriptomic data: both the targeted identification of candidate genes and broader
292 expression analyses.

293

294 ***A case study: the antennal gland***

295 Considering the biological significance of the antennal gland (AnG), herein we present an
296 investigative case study of the AnG to demonstrate the strengths of comparative
297 transcriptomics in the research of a non-model species such as *S. verreauxi*.

298 <u>*Physiology and function*</u>

299 The AnG, also known as the green or maxillary gland, is found in all decapod *Crustacea*. The
300 bilateral glands are located at the base of each antenna and have a single nephropore which
301 opens at the underside of the antennal coxae (Vogt 2002). The AnG functions somewhat like
302 a mammalian kidney (Behnke et al. 1990; Dove 2005) and is responsible for osmotic and
303 ionic regulation along with excretion of metabolic waste products as urine (Vogt 2002). In
304 addition to its excretory function, this urine also provides a means for chemical
305 communication and is the source of pheromones (Shabani et al. 2009; Aggio and Derby
306 2011). It is thought that these pheromones are either general metabolic bi-products within the
307 urine or unique substances mixed into the urine (Bushmann and Atema 1993). This suggests
308 that these chemical cues are produced within the AnG (Dunham 1978), however another
309 source gland may also be involved such as the gonads (McLeese et al. 1977) or a distinct
310 secretory tissue such as rosette glands, found at the base of the nephropore in the clawed
311 lobster *Homarus americanus* (Bushmann and Atema 1993; Bushmann and Atema 1996).

312 There is an abundance of behavioural data demonstrating the use of chemical communication
313 amongst the decapods (Wyatt 2011). In the spiny lobsters there is evidence for urine-borne
314 sexual communication, with females using male urine to guide mate-selection (Raethke et al.
315 2004); social recognition, to establish and maintain dominance hierarchies (Shabani et al.
316 2009); and aggregation and avoidance, mediating communal living (Horner et al. 2006;
317 Horner et al. 2008; Briones-Fourzán et al. 2008) amongst other social interactions (Aggio and
318 Derby 2011). Thus if the AnG is indeed the source of these chemical cues, this may well
319 explain the sexually dimorphic expression observed in mature individuals.

320 Considering this we chose to investigate the sexually dimorphic role of the mature AnG
321 relative to AnGs from immature individuals. To do so, we compared our mature data with
322 sequencing data from AnGs taken from sexually immature (~700g) males and females for
323 evidence of a similar sex-biased differential expression.

324

325 *The immature antennal gland*

326 Paired reads from three immature male AnGs mapped to an average of 90.0% of the entire
327 reference transcriptome (described in Chandler et al. (in press)), with three female AnGs
328 mapping to 88.8%. This is fitting with the data from Chandler et al. (in press), which
329 determined that of the entire reference transcriptome 89.4% of Unigenes and Contigs were
330 present in the mature AnGs and therefore provides good evidence for a thorough sequencing
331 coverage across the immature AnGs. Interestingly when analysed in immature AnGs, there
332 was no evidence of any significant differential expression or sex-bias between males and
333 females ($P \leq 0.05$ with FDR, fold change of 2). What is more, considering the specific genes
334 discussed previously, neither *Sv-TKIR* nor *Sv-Dmrt1* showed any evidence of differential
335 expression between immature male and female AnGs when analysed by qPCR (Fig. 2C and
336 2F). This suggests that the AnG develops dimorphic expression patterns later during sexual
337 maturation, including the expression of key genes considered to be involved in sexual
338 development.

339 Considering this result and our knowledge of the biology of the AnG, we chose to investigate
340 the male-biased expression seen in mature AnGs in the context of reproductive-related
341 pheromone production. To do this, we merged our two data sets (both mature and immature)
342 and performed a preliminary annotation scan to identify genes with known function in the
343 pheromone biosynthesis (guided by that characterised in arthropods (Tillman et al. 1999;

344 Zhang et al. 2014b; Xia et al. 2015). In addition, for a more in-depth analysis of broader
345 spatial expression, we included the RPKM expression values for all tissues comprising the
346 reference transcriptome. This allows for the comparative evaluation of broader tissue
347 expression, highlighting any tissue-specificity or bias. When considered together these data
348 provide improved guidance for candidate gene selection for further analyses.

349 This simple exercise highlighted over fifty genes (defined with an E -value < 1.00^{-20}) which
350 show a > 2-fold male-biased differential expression in mature AnG with no evidence of
351 differential expression in immature glands (see Sup. Material 1). After providing simplistic
352 criteria for rapid gene identification, annotation scanning also guides further comparative
353 analyses. Consider CL8012.Contig1 identified using our approach (Sup. Material 1), a
354 "Elongation of very long chain fatty acids protein" ($6.0e^{-70}$), an orthologue of which, *Bond*,
355 has been characterised in *Drosophila* as essential for the biosynthesis of the male sex-
356 pheromone and spermatogenesis, thus central to male fertility (Ng et al. 2015). In *Drosophila*
357 the gene is expressed in females, but shows male-biased up-regulation in the ejaculatory bulb
358 (the site of pheromone synthesis) and gonad (Ng et al. 2015); a trend also seen in *S. verreauxi*
359 (showing a male-biased expression of 2-fold in AnG and 3-fold in gonad). In conducting
360 these preliminary analyses, we are not suggesting that all genes listed in Sup. Material 1 have
361 a definite function in pheromone biosynthesis in *S. verreauxi*. Instead we are aiming to
362 demonstrate the power of transcriptomics in guiding preliminary analyses, highlighting
363 candidate genes that stimulate future research and functional validation.

364

365 ***Conclusions***

366 In summary, we present this research in *S. verreauxi,* as evidence for the advances made in
367 our understanding of decapod sexual development through the application of comparative
368 transcriptomics. We aim to demonstrate how transcriptomic data sets like these can be
369 applied to tackle long-standing research challenges like that regarding the need for molecular
370 data to advance our understanding of chemical communication in *Crustacea* (as described by
371 Thiel and Breithaupt (2011): Research challenges for Twenty-First Century). Although each
372 of us specialises with regard to species or question, there is an over-arching theme that unifies
373 twenty-first century research: an aim to understand the underlying mechanisms that govern a
374 certain phenotype or developmental phenomenon. It is this common feature that allows the

broader application of the transcriptomic techniques described to enhance our research capability amongst these non-model species.

However, there is no doubt that these advances could be improved further, through the use of integrated ‘omics-based techniques. Genome data would provide clarity in cases where transcriptomics is lacking, providing definitive evidence of gene omissions and emergence, allowing for full comparative evolutionary analyses (Hardison 2003). In addition, considering that our research questions tend to lie at the phenotypic end of the Central Dogma cascade, the integration of proteomics and metabolomics would provide a front-line functional relevance that can be masked when inferring from the genetic level alone. Hence although not detracting from the power and application of transcriptomics, we believe that the integration and synergy of multiple ‘omics-based techniques offers the most “powerful” progression forward.

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831 ***Figure legends***

832 **Figure 1: An integrated illustration of male sexual development in a decapod**

833 **1A)** A representation of what is currently known of male sexual development in the
834 *Decapoda*, highlighting the three main phases of sex determination, sexual differentiation and
835 sexual maturation. **1B)** A developmental trajectory of male development in *S. verreauxi* in
836 culture. The representative images show (from left to right): an early stage blastula; a late-
837 stage embryo; stage 1 instar; stage 17 intermolt instar; H-phase puerulus; recently emerged
838 juvenile; 400g immature male; and 2.5kg mature male.* indicates the first evidence of sexual
839 differentiation: male-specific gonopores emerge within the first 2-3 juvenile instars, at 1-3g.
840 **1C)** A contextualised expression profile of *IAG* in *S. verreauxi*, as quantified by RPKM,
841 individual values are presented in red for: phyllosoma instar 17, intermolt (0 RPKM), early
842 (0.07 RPKM) and late (0.17 RPKM) molt; postmolt puerulus (0.31 RPKM) and H-phase
843 puerulus (0.23 RPKM); AG from an immature 400g male (0.40 RPKM); and an AG from a
844 sexually mature 3kg male (1495 RPKM); the line break indicates the dramatic increase in
845 IAG expression seen in later development.

846

847 **Figure 2: Three methodologies testing expression patterns of *Sv-TKIR* (Figure 2A - C)**
848 **and *Sv-Dmrt1* (Figure 2D - F)**

849 ***Mature:*** **2A)** Transcriptomic expression profile of *Sv-TKIR* and **2D)** *Sv-Dmrt1* quantified as
850 RPKM across all mature transcript libraries, namely the male and female brain (BR), eyestalk
851 (ES), gonads (TS and OV), antennal gland (AnG) and fifth walking leg (5WL) and the
852 mature androgenic glands (AG1 and AG2*) where * indicates a hypertrophied gland. As the
853 full *Sv-TKIR* gene was present as several transcript fragments, an average RPKM across
854 transcripts is shown; error bars indicate the standard error. **2B)** Semiquantitative RT-PCR
855 expression profile of *Sv-TKIR* and **2E)** *Sv-Dmrt1* including all the mature tissues used for
856 transcriptomic analyses with the addition of male and female hepatopancreas (HP); negative
857 control (nc) in the fifteenth lane, with 16S acting as a positive control. ***Immature:*** **2C)** qPCR
858 quantification of *Sv-TKIR* and **2F)** *Sv-Dmrt1* across testis (TS), ovary (OV) and antennal
859 glands (AnG); n=8, standardised against *Sv-18S*. Figure 2A and B are adapted from Aizen et
860 al. (under review).

861 **Supplementary Material 1:** List of transcripts, annotated with an E -value < 1.00^{-20}, with
862 putative involvement in a male-related pheromone biosynthesis pathway in the antennal
863 gland (AnG).