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De novo assembly and annotation of the European abalone *Haliotis tuberculata* transcriptome

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Abstract :

The European abalone *Haliotis tuberculata* is a delicacy and consequently a commercially valuable gastropod species. Aquaculture production and wild populations are subjected to multiple climate-associated stressors and anthropogenic pressures, including rising sea-surface temperatures, ocean acidification and an emerging pathogenic *Vibrio* infection. Transcript expression data provides a valuable resource for understanding abalone responses to variation in the biotic and abiotic environment. To generate an extensive transcriptome, we performed next-generation sequencing of RNA on larvae exposed to temperature and pH variation and on haemolymph of adults from two wild populations after experimental infection with *Vibrio harveyi*. We obtained more than 1.5 billion raw paired-end reads, which were assembled into 328,519 contigs. Filtration and clustering produced a transcriptome of 41,099 transcripts, of which 10,626 (25.85%) were annotated with Blast hits, and 7380 of these were annotated with Gene Ontology (GO) terms in Blast2Go. A differential expression analysis comparing all samples from the two life stages identified 5690 and 10,759 transcripts with significantly higher expression in larvae and adult haemolymph respectively. This is the greatest sequencing effort yet in the *Haliotis* genus, and provides the first high-throughput transcriptomic resource for *H. tuberculata*.

Keywords : RNA-Seq, Mollusca, Gastropoda, Development, *Vibrio harveyi*, Climate change

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1. Introduction

Coastal marine organisms are increasingly being impacted by global climate change and anthropogenic pressures (Harley et al., 2006). The study of their responses to these stressors is vital for understanding how marine populations and species are able to acclimatize or adapt to future change and for devising management strategies for species of economic interest. The European abalone *Haliotis tuberculata* is a commercially valuable gastropod, with

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48 production based on fisheries of wild populations or hatchery-based aquaculture. As is the
49 case with many calcifying species, rising pCO_2 and subsequent ocean acidification increase
50 the energetic cost of calcification (Hofmann and Todgham, 2010). The concurrent effects of
51 rising sea surface temperatures and declining pH have deleterious consequences for the
52 development of calcifying organisms (Parker et al., 2010), with abalone being particularly
53 sensitive to changes in pH and temperature (Byrne, 2011). In addition, the emergence of
54 abalone disease due to *Vibrio harveyi* has been associated with the warming of coastal waters
55 (Nicolas et al., 2002). In order to better understand how the European abalone responds to
56 abiotic and biotic stressors, global transcript expression (RNA-Seq) was examined as part of
57 two ongoing experiments: one that is investigating the effects of pH and temperature on
58 larval development; the other investigating the immune response of adults to infection with *V.*
59 *harveyi*. The aim of this work was to provide a detailed transcriptomic data set for *H.*
60 *tuberculata*. Rather than carrying out separate transcriptome assemblies for each life stage,
61 Illumina sequences from both experiments were combined and assembled into a single
62 transcriptome, allowing generation of a more complete transcriptomic resource, and direct
63 comparison of larvae and adults. This data provides an invaluable resource for identifying
64 transcripts involved in the response of *H. tuberculata* to global change and other
65 anthropogenic stresses, and taken with other recent high throughput transcriptomic data for
66 *Haliotis* species (De Wit and Palumbi, 2013; Franchini et al., 2011; Huang et al., 2012;
67 Picone et al., 2015; Shiel et al., 2015; Valenzuela-Muñoz et al., 2014) enriches the genomic
68 resources available for this commercially important genus.

69

70 **2. Data Description**

71 *2.1 Sampling and Sequencing*

72 Biological samples used for generating the *H. tuberculata* transcriptome were collected as
73 part of two ongoing studies in our group. Combining the sequences from two life stages
74 (larvae and adults) and two tissues (whole organism and haemolymph) enables the assembly
75 of a more complete transcriptome which reflects a broader range of functions. Here we
76 present the bioinformatic analysis used to assemble the *de novo* transcriptome, and provide a
77 differential expression analysis of whole larvae versus adult haemolymph. Detailed
78 differential expression analysis dealing with the experimental treatments performed for each
79 study will be examined in two companion papers.

80 *H. tuberculata* larvae were produced following controlled fertilizations carried out at a
81 commercial hatchery (France Haliotis, Plouguerneau, Finistère, France). Larvae were
82 transferred to experimental facilities at Ifremer (Plouzané, Finistère, France) and reared in
83 one of four treatments (control = 20°C pH 8.1; acidified = 20°C pH 7.9; warmed = 22°C pH
84 8.1; interaction = 22°C pH 7.9), RNA samples deriving from approximately 10,000 pooled
85 larvae were collected by filtration from two replicated experimental tanks for each treatment
86 at 14, 24 and 38 hours post fertilization. These 24 samples were flash frozen in liquid
87 nitrogen and stored at -80°C until RNA extraction. Adult haemolymph RNA was collected
88 during successive infection experiments on abalone from two different natural populations in
89 Brittany (Molène and Saint-Malo, average size of individuals = 74 ± 15 mm) with the
90 bacteria *Vibrio harveyi* (strain ORM4), carried out during the spawning period. Haemolymph
91 was sampled 72h post-infection, following the first exposure for abalone from Molène and
92 after the third exposure for abalone from Saint-Malo. Uninfected control abalone were
93 sampled at the same time point as infected individuals for both populations. Three replicates
94 were sampled per treatment for a total of 12 samples. Two mL of haemolymph were
95 withdrawn with a 5 mL syringe from each abalone, and spun at 200 rcf for 10 minutes. The

96 serum was removed, 1 mL of TRIzol reagent (Life Technologies) was added to the pellet, and
97 then the samples were frozen and stored at -80°C until RNA extraction.

98 All samples were homogenised by bead beating and total RNA was extracted using TRIzol
99 reagent (Life Technologies) according to manufacturer's instructions. RNA quality and
100 concentration were determined using an Agilent 2100 RNA Bioanalyser (Agilent, Santa
101 Clara, CA, USA) and NanoDrop 8000 Spectrophotometer (NanoDrop Technologies,
102 Wilmington, DE, USA) respectively. Library preparation and sequencing of larval samples
103 was carried out at the Institute of Clinical Molecular Biology, Christian-Albrechts-University
104 Kiel, Germany, with Illumina HiSeq (101 bp paired-end, non-strand-specific), with 8 samples
105 multiplexed per lane (two samples were resequenced later with 126 bp paired-end reads).
106 Library preparation and sequencing of adult haemolymph RNA samples was performed by
107 The GenePool, University of Edinburgh, Scotland, on Illumina HiSeq (100 bp paired-end,
108 strand-specific). For adult haemolymph RNA, two runs were performed with lanes of 12
109 multiplexed samples. Raw sequence data is available from the NCBI sequence read archive
110 (SRA) under the accession SRA303338.

111

112 *2.2* De Novo *transcriptome assembly*

113 A total of 849,307,722 and 668,045,828 paired-end reads were generated for larvae and adult
114 haemolymph respectively. Adapters and low quality bases were trimmed using the
115 Trimmomatic tool, with a 4 bp sliding window, cutting when the average quality score
116 dropped below Q = 20 (Bolger et al., 2014), leaving 790,015,978 paired-end reads for larvae
117 (mean length = 99.6 bp) and 544,556,316 paired-end reads for adult haemolymph (mean
118 length = 88.9 bp). Separate *in silico* normalisation of kmers was carried out for larvae and
119 adult haemolymph (Haas et al., 2013), and the transcriptome was assembled from the

120 combined normalised reads using Trinity ver. 2.0.6 (Grabherr et al., 2011) with default
121 parameters. Initial assembly produced 514,943 contigs of between 224 and 32,662 bp length.
122 Removal of short contigs (≤ 300 bp) reduced this to 328,519 contigs with an average length
123 of 825 bp and an N50 of 1,103 bp. This Transcriptome Shotgun Assembly project has been
124 deposited at DDBJ/EMBL/GenBank under the accession GEAU00000000. The version
125 described in this paper is the first version, GEAU01000000. The relatively large number of
126 contigs was in part a result of sequencing effort, which was an order of magnitude greater
127 than in many comparable molluscan *de novo* transcriptome assemblies (Table 1). Therefore
128 to maximise confidence in our transcriptome, we carried out a number of filtrations. Initially,
129 lowly expressed contigs were removed by applying a FPKM cut-off of 1. This filtration
130 produced 79,374 contigs, with an average length of 1022 bp and N50 of 1509 bp. Contigs
131 with high similarity, which likely represent different spliced isoforms or duplicated genes
132 were grouped by Trinity into 41,099 non-redundant 'unigenes' (hereafter referred to as
133 transcripts), with an average length of 1015 bp and an N50 of 1529 bp. This filtered
134 transcriptome is used in subsequent stages of annotation and analysis, and is available in the
135 supplementary material (Appendix 1).

136 **Table 1.** Comparison of molluscan transcriptomes assembled *de novo* from Illumina sequences using Trinity and other bioinformatic assembly

137 tools.

Species	Raw reads (millions)	Assembler	Total contigs	Filtered and/or non-redundant contigs	Mean contig length (bp)*	N50 (bp)*	Number of annot. contigs (percent)*	Reference
Haliotis tuberculata	1517	Trinity	328,519	41,099	1,033	1,544	10,626 (25.9)	This study
H. laevigata	38	Trinity	222,172	97,420	-	1313	20,702 (21.3)	Shiel et al., 2015
H. midae	25	CLC	22,761	-	260	356	3,841 (16.9)	Franchini et al., 2011
H. rufescens	355.68	CLC	162,928	-	522	653	48,004 (29.5)	De Wit et al., 2013
Anadara trapezia	27	Trinity	75,024	63,778	505	-	29,013 (38.7)	Prentis et al., 2014
Clio pyramidata	400	Trinity	45,739	30,800	618	852	9,280 (20.3)	Maas et al., 2015
Conus tribblei	33.54	Trinity	163,513	-	513	614	21,069 (12.9)	Barghi et al., 2014
Mytilus galloprovincialis	393	Trinity	-	151,320	570	-	50,998 (33.7)	Moreira et al., 2015
Nucella lapillus	42.08	Trinity	90,674	-	-	413	20,922 (23.1)	Chu et al., 2014
Patinopecten yessoensis	55.88	Trinity	135,963	86,521	733	1,266	28,228 (20.8)	Sun et al., 2015
Reishia clavigera	110.77	Trinity	197,324	151,684	499	582	28,948 (14.7)	Ho et al., 2014
Trintonia diomedia	133.1	Trinity	185,546	123,154	74	1,353	18,246 (14.8)	Senatore et al., 2015
Corbicula fluminea	62.25	Oases	-	134,684	791	1,264	38,985 (28.9)	Chen et al., 2013
Crassostrea gigas	2204	Oases	-	55,651	1,659	-	44,912 (80.7)	Riviere et al., 2015
Cr. virginica	52.86	Oases	48,562	-	874	-	11,237 (23.1)	Zhang et al., 2014
Elliptio complanata	361.03	CLC	136,000	-	-	982	26,689 (19.6)	Cornman et al., 2014
Pinctada fucata	26.51	SOAP	723,797	92,964	434	-	33,882 (36.4)	Huang et al., 2013

* Contig stats and annotation reported either from total or non-redundant contigs, depending on cited example

138

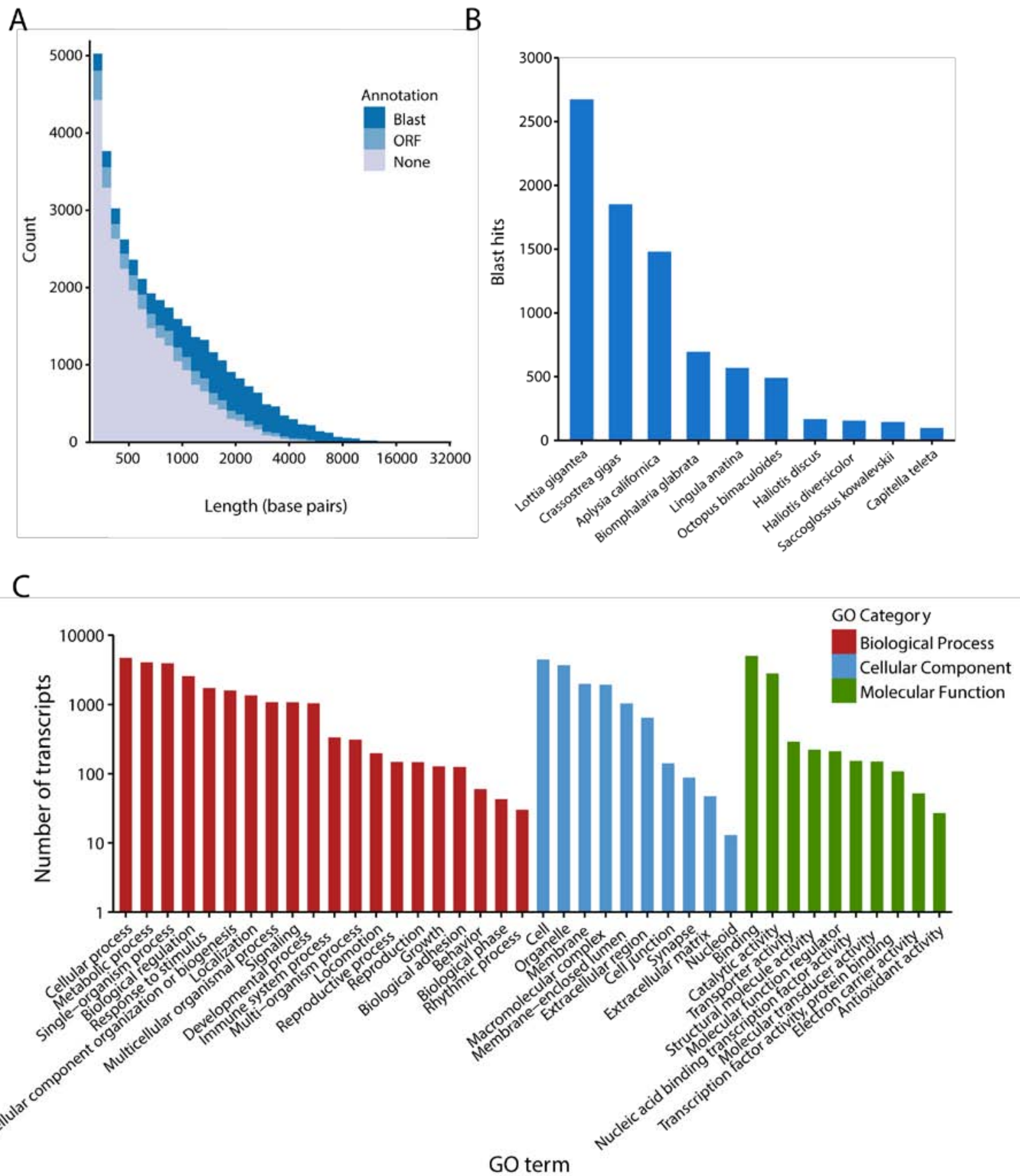
139 *2.3 Functional annotation*

140 Coding sequence detection was carried out with TransDecoder (Haas et al., 2013). Among
141 the 41,099 transcripts in the filtered transcriptome were 12,587 (30.6%) that contained at
142 least one open reading frame (ORF) of ≥ 100 amino acids length. Translated ORFs were used
143 as Blastp queries against the NR and Swiss-Prot databases, and non-translated nucleotide
144 sequences from all transcripts were used as Blastx queries against the same databases. All
145 blasts were carried out using an e-value cutoff of 1 e^{-5}.

146 A total of 10,626 transcripts (25.85%) were annotated following Blast analysis: 10,603
147 transcripts were associated with blast hits in the NR database, and 9,347 transcripts were
148 associated with blast hits in the Swiss-Prot database. Longer transcripts were more likely to
149 contain an ORF and be associated with blast hits (Fig. 1A); however, our total of 10,626
150 annotated transcripts is comparable in terms of percentage annotation (25.9%) with other
151 Illumina transcriptome assemblies of non-model marine molluscs without a reference genome
152 (Table 1). When blast results from the NR database were given precedence over those from
153 the Swiss-Prot database, the majority of hits among the 10,626 annotated transcripts derived
154 from mollusc species, with four species accounting for over 63% of results: *Lottia gigantea*
155 (25.22%), *Crassostrea gigas* (17.46%), *Aplysia californica* (13.96%), and *Biomphalaria*
156 *glabrata* (6.55%). Furthermore, the congeners *H. discus* and *H. diversicolor* were also among
157 the top 10 most represented species, accounting for 1.56% and 1.46% of hits respectively
158 (Fig.1B). To estimate the influence of fragmentation on the transcriptome, non-duplicated
159 accession numbers among blast annotated transcripts were counted (with NR results again
160 given priority over Swiss-Prot results). A total of 9,378 different accession numbers were
161 present among the 10,626 annotated transcripts, suggesting that the large majority (88.25%)
162 of annotated transcripts had unique identities.

163 Nevertheless, the number of transcripts presented here is higher than the number of genes
164 commonly reported in marine mollusc genomes (e.g. 21,013 for *Aplysia californica*, 24,676
165 for *Lottia gigantea* and 32,250 for *Crassostrea gigas*), and the majority remain unannotated.
166 Sequencing of expressed transcripts is expected to identify many novel contigs that do not
167 align to known gene models: up to 30-40% in organisms with reference genomes (Wang et
168 al., 2009). Unidentified transcripts may derive from non-coding RNA (Eddy, 2001), which
169 can represent a large part of the transcriptome of many organisms, and play an important
170 functional role in regulation (Guttman and Rinn, 2012). A recent study on transcript
171 expression in *C. gigas* by Riviere et al. (2015) demonstrates that *de novo* transcriptome
172 assembly will often produce many novel contigs with unknown functions; in that case, over
173 56,000 contigs were identified including 11,817 contigs that did not match any known region
174 of the reference genome or RefSeq protein records.

175 Finally, Blast2GO (Götz et al., 2008) was used to retrieve gene ontology (GO) terms among
176 blast-annotated transcripts. For this analysis, blast results from the Swiss-Prot database took
177 precedence over those from the NR database, as they often include more functional
178 information. As part of the annotation procedure, the InterProScan plugin of Blast2GO was
179 used to search for functional domains (Jones et al., 2014). This step was applied to all 41,099
180 transcripts, revealing 16,305 transcripts that contained at least one InterPro domain (including
181 8,265 that had not been annotated following blast analysis). For Blast2GO annotation, default
182 parameters were applied, and subsequent GO terms were merged with InterProScan results;
183 furthermore, the Annex function of Blast2GO was used to augment GO term annotation
184 among the transcripts. A total of 46,336 GO terms were annotated to 7,380 transcripts
185 (17.96% of total transcripts). The number of transcripts with second level GO terms is
186 provided for each of the three GO categories of biological process, molecular function and
187 cellular component in (Fig. 1C).



188

189 **Figure 1**. A) Sequence length distribution of 41,099 filtered transcripts showing those with

190 Open Reading Frames (ORFs) and Blastp hits in the NR database; B) Species distribution of

191 blast hits among the 10,626 annotated transcripts. Results from NR take precedence over

192 Swiss-Prot; C) Gene ontology (GO) classification of 7,380 *H. tuberculata* Blast2GO

193 annotated transcripts split by category. Results from Swiss-Prot take precedence over NR.

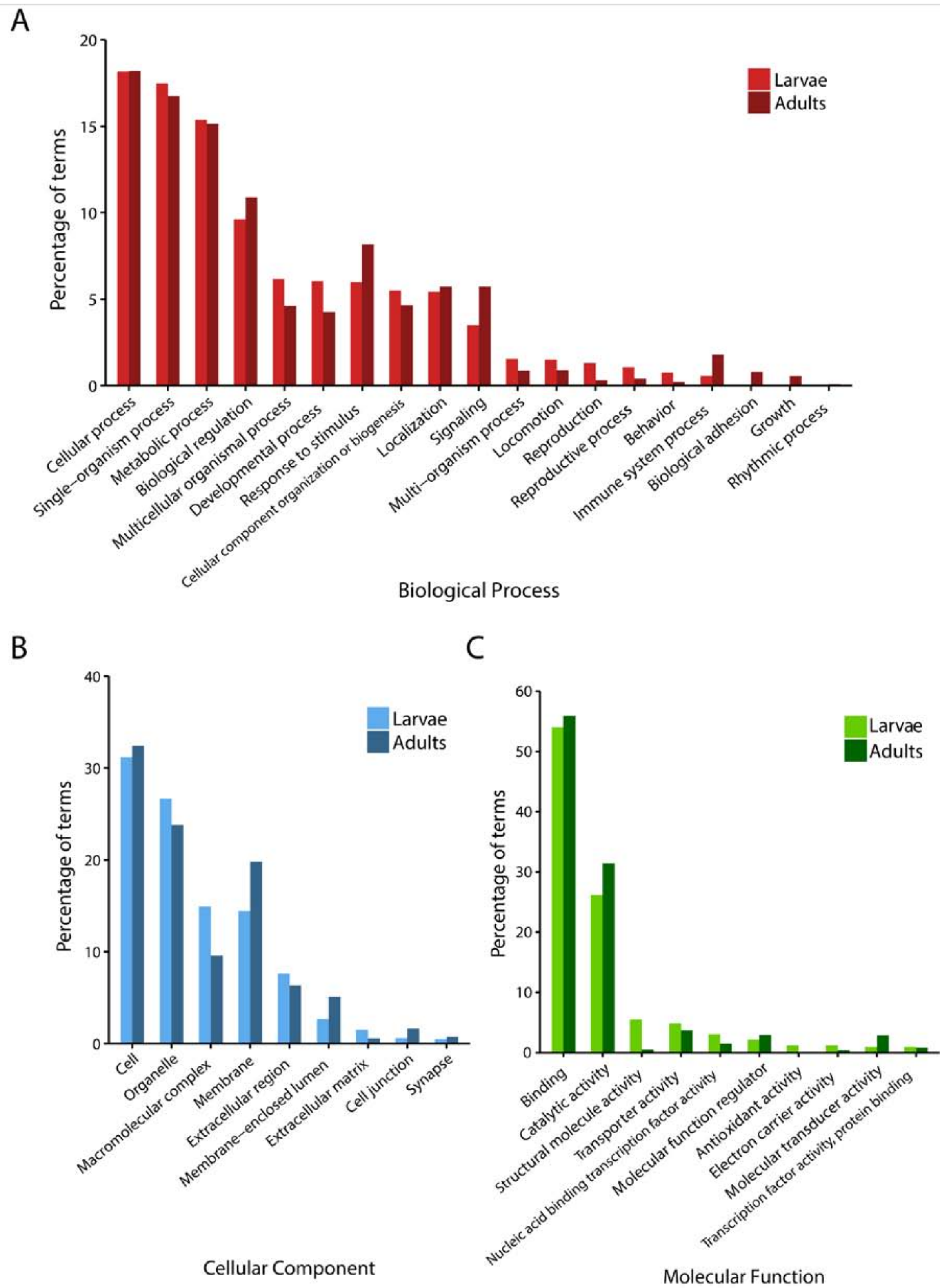
194

2.4 Comparison of GO terms in larvae and adults

Broad differences in function between larvae and adult haemolymph were visualised by comparing relative contributions of second level GO terms using contigs differentially expressed between the life stages. Differential expression analysis was carried out in R using the package *edgeR* (McCarthy et al., 2012), and considered normalised expression values from all adult haemolymph libraries (across both populations and both treatments) against normalised expression from all larval libraries (across all stages and all treatments). Using all the libraries increased statistical replication for each life stage, and thus confidence in the identification of differentially expressed transcripts. Of the 41,099 transcripts considered, 16,449 were significantly differentially expressed between adult haemolymph and larvae (Benjamini-Hochberg FDR <0.01, log-fold change > 5). Specifically, 5,690 transcripts were significantly more expressed in larvae, and 10,759 were significantly more expressed in adult haemolymph. Among these transcripts, 1,185 and 3,537 were blast-annotated in larvae and adult haemolymph respectively, of which 566 and 2,416 also had Blast2go annotation. Full lists of differentially expressed transcripts and their annotations are included in the supplementary material (appendix 2). For the three GO categories of biological process, cellular component and molecular function, second level GO terms for larvae and adult haemolymph were expressed as a percentage of the total number of GO terms in that category (Fig. 2). Amongst the biological processes, '*multicellular organismal process*' and '*developmental process*' had higher percentages in larvae compared to adults; while '*response to stimulus*', '*signalling*', '*biological regulation*' and '*immune system process*' had higher percentages in adults compared to larvae (Fig. 2A). Similarly, the cellular component '*macromolecular complex*' and molecular function '*structural molecule activity*' had higher percentages in larvae, while the cellular component '*membrane*' and molecular function '*catalytic activity*' had higher percentages in adults (Fig. 2B and 2C). These results provide a

220 first glimpse into life stage specific transcript expression in *H. tuberculata*. Further analysis
221 of function and differential expression between treatments in larvae and adult haemolymph
222 will follow in companion papers.
223

224



225 **Figure 2.** Comparison of second level GO terms (percentage contribution towards the total)

226 in differentially expressed larval and adult haemolymph transcripts, split according to GO

227 categories of A) biological process; B) cellular component and c) molecular function.

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