

# Interspecific Differential Expression Analysis of RNA-Seq Data Yields Insight into Life Cycle Variation in Hydractiniid Hydrozoans

Steven M. Sanders<sup>1,\*</sup> and Paulyn Cartwright

Department of Ecology and Evolutionary Biology, University of Kansas

<sup>1</sup>Present address: Department of Surgery, Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA

\*Corresponding author: E-mail: sanderssm@upmc.edu.

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## Abstract

Hydrozoans are known for their complex life cycles, which can alternate between an asexually reproducing polyp stage and a sexually reproducing medusa stage. Most hydrozoan species, however, lack a free-living medusa stage and instead display a developmentally truncated form, called a medusoid or sporosac, which generally remains attached to the polyp. Although evolutionary transitions in medusa truncation and loss have been investigated phylogenetically, little is known about the genes involved in the development and loss of this life cycle stage. Here, we present a new workflow for evaluating differential expression (DE) between two species using short read Illumina RNA-seq data. Through interspecific DE analyses between two hydractiniid hydrozoans, *Hydractinia symbiolongicarpus* and *Podocoryna carnea*, we identified genes potentially involved in the developmental, functional, and morphological differences between the fully developed medusa of *P. carnea* and reduced sporosac of *H. symbiolongicarpus*. A total of 10,909 putative orthologs of *H. symbiolongicarpus* and *P. carnea* were identified from de novo assemblies of short read Illumina data. DE analysis revealed 938 of these are differentially expressed between *P. carnea* developing and adult medusa, when compared with *H. symbiolongicarpus* sporosacs, the majority of which have not been previously characterized in cnidarians. In addition, several genes with no corresponding ortholog in *H. symbiolongicarpus* were expressed in developing medusa of *P. carnea*. Results presented here show interspecific DE analyses of RNA-seq data to be a sensitive and reliable method for identifying genes and gene pathways potentially involved in morphological and life cycle differences between species.

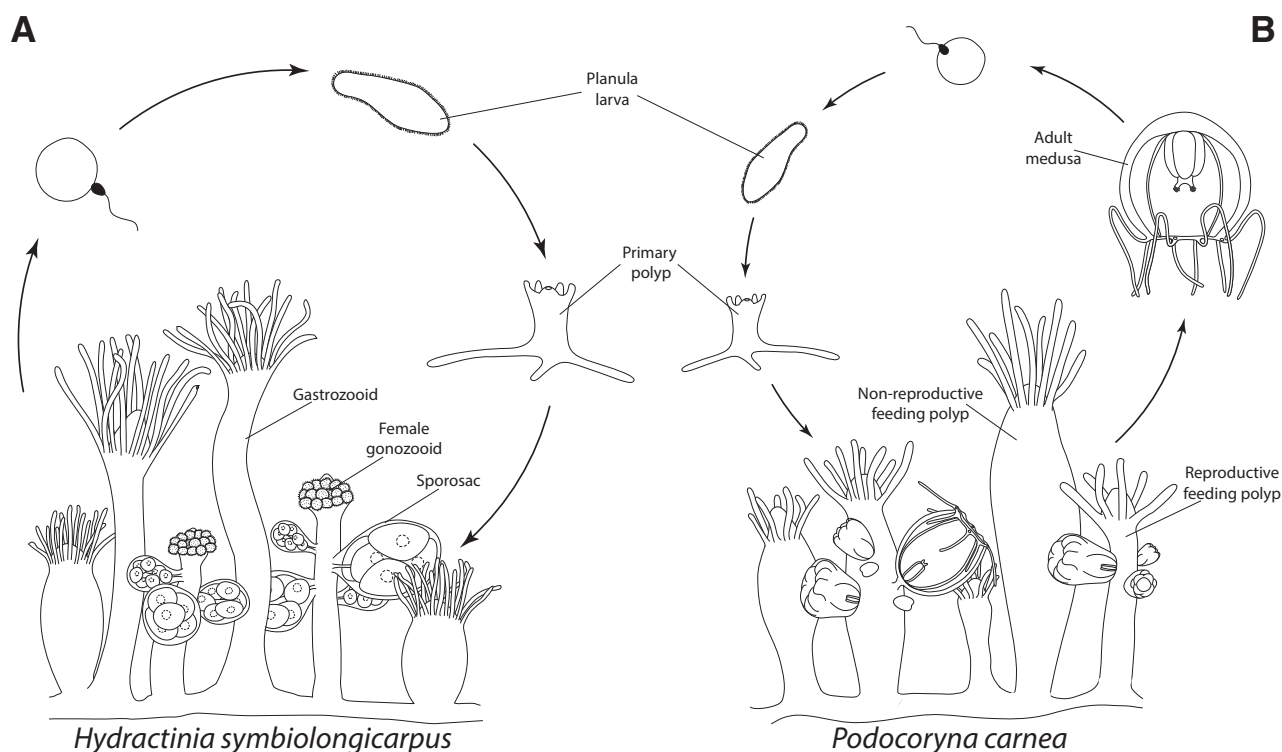
**Key words:** RNA-Seq, transcriptomics, differential expression, comparative expression, Cnidaria, Hydrozoa.

## Introduction

In Hydrozoa (phylum Cnidaria), many species exhibit an alternation of generations, where asexually reproducing polyps give rise to sexually reproducing jellyfish (medusae). However, across hydrozoans, there is much variation in this sexually reproducing life cycle stage. In most hydrozoan species (~70%), development of the medusa bud (gonophore) is truncated to some degree or entirely absent (Leclère et al. 2009; Cartwright and Nawrocki 2010; Gibbons et al. 2010). In these taxa, sexual maturity is reached in a gonophore that resembles an early ontogenetic stage of medusae development. The degree of gonophore development ranges from completely reduced structures called sporosacs that lack any resemblance of the medusa (fig. 1A), to more developed

forms called medusoids, that may or may not detach and swim, but lack the ability to feed (not shown), to the fully developed medusa stage that detaches from the hydroid polyp and can feed, swim, and sexually reproduce in the water column (fig. 1B).

The evolution of this structure and its reduced forms has been a topic of investigation for the last 150 years (Allman 1864; Cornelius 1992; Cunningham and Buss 1993; Marques and Migotto 2001; Leclère et al. 2007, 2009; Miglietta et al. 2009, 2010; Cartwright and Nawrocki 2010; Miglietta and Cunningham 2012). Phylogenetic studies have revealed multiple independent losses of medusae (Cunningham and Buss 1993; Leclère et al. 2007, 2009; Cartwright and Nawrocki 2010; Miglietta and Cunningham 2012) and possibly even



**FIG. 1.**—Illustration of hydrozoan life cycles. (A) In the life cycle of *Hydractinia symbiolongicarpus*, gonophores develop into sporosacs that lack all medusa features and remain attached to the colony on specialized reproductive polyps called gonozooids. Sexual reproduction occurs in the water column after the sporosacs release their gametes. Sexual reproduction results in a planula larva that eventually settles onto a suitable substrate and metamorphoses into a primary polyp. This polyp will asexually produce other polyps to form a colony and the cycle repeats. (B) *Podocoryna carnea*'s life cycle is similar to that of *H. symbiolongicarpus* except that medusae asexually bud from reproductive polyps and detach from the colony to sexually reproduce in the water column.

re-revolution (Leclère et al. 2009; Cartwright and Nawrocki 2010; Miglietta and Cunningham 2012). Although phylogenies are important for recognizing evolutionary patterns of character transitions, understanding complex patterns of character loss and possible re-gain will come from insight about their development. Specifically, maintenance of developmental regulatory pathways underlying medusae ontogeny in reduced forms could add support to arguments for medusae re-evolving in the Hydrozoa. The hydrozoan family Hydractiniidae provides an excellent system for identifying key components in medusa development and truncation, as the entire spectrum of gonophore development is exhibited within this group (Schuchert 2008). The hydractiniid species *H. symbiolongicarpus* and *P. carnea* exhibit either ends of this developmental spectrum, possessing a sporosac (fig. 1A) and medusa (fig. 1B), respectively.

Now that transcriptomes of nontraditional model systems can be readily obtained and characterized in different stages or parts of an organism (Hao et al. 2011; Siebert et al. 2011; Helm et al. 2013; Sanders et al. 2014; Schunter et al. 2014), comparing transcriptomes between species is the obvious next step. Dunn, Luo, and Wi (2013) extensively reviewed the utility of comparative expression across multiple species,

as well as its challenges. Although not as abundant as intra-specific transcriptomic studies, interspecific analyses have proved illuminating on a diversity of topics (Yang and Wang 2013; Boyle et al. 2014; Pankey et al. 2014). These studies took a general approach to comparing whole transcriptomes but did not apply interspecific differential expression (DE) in an unbiased approach to identify genes potentially involved in differences between species.

Here, we present a workflow for performing DE analyses between two species from short read Illumina RNA-Seq data. Specifically, we use previously published RNA-Seq data from *H. symbiolongicarpus* (Sanders et al. 2014) and *P. carnea* (Sanders and Cartwright 2015), to identify genes and gene pathways that are potentially involved in the life cycle differences between truncated and fully developed medusae in the Hydractiniidae.

## Materials and Methods

### Animal Care

Transplanted colonies of *P. carnea* and *H. symbiolongicarpus* were kept on microscope slides, placed in slide racks, and kept

in seawater (REEF CRYSTALS, Aquarium Systems) aquaria at room temperature (~21 °C) with a salinity of 29 and 32 ppt, respectively. Colonies were fed 2–3-day-old nauplii of *Artemia* three times a week.

### Transcriptome Assembly and Annotation

Figure 2 is a schematic of our bioinformatic pipeline for identifying differentially expressed orthologs. Raw Illumina RNAseq data for *H. symbiolongicarpus* and *P. carnea* were downloaded from the SRA archive (SRP038762 and SRP041583, respectively). *Hydractinia symbiolongicarpus* libraries included four replicated libraries of feeding (nonreproductive) polyps (gastrozooids), two replicated libraries of female reproductive polyps (gonozooids), two replicated libraries of male reproductive polyps (gonozooids), and four replicated libraries of defensive (nonreproductive) polyps (dactylozooids). *Podocoryna carnea* libraries included three replicated libraries of nonreproductive gastrozooids, four replicated libraries of female reproductive gastrozooids, and three replicated libraries of female adult medusa. These reads were pooled by species and then assembled with Trinity (Grabherr et al. 2011) through the automated bioinformatics pipeline, Agalma (Dunn, Howison, and Zapata 2013), under default settings.

To perform gene ontology (GO) analyses, transcripts were blasted against the nr protein database using BLASTx with the “-outfmt 5” flag for xml formatted output. BLAST output was imported into BLAST2Go (Conesa et al. 2005; Götz et al. 2008) where GO mapping and annotations were performed. Conserved protein domains were also identified using with the PFAM (Punta et al. 2012) and TIGR (<http://blast.jcvi.org/web-hmm/>) databases using HMMER (<http://hmm.org/>). The enriched GO terms and protein domains were assessed with the Fisher’s exact test and corrected for a false discovery rate (FDR) < 0.05 in R.

### Ortholog Identification and DE Analysis

To identify putative orthologs, assemblies were filtered based on a minimum fragments per kilobase of transcript per million mapped reads (FPKM) value ( $\geq 1.0$ ) and default Transdecoder (<http://transdecoder.sourceforge.net/>) reading frame criteria (fig. 2). An FPKM value was calculated for each transcript across all libraries used in the assemblies and were used as a means of assessing the relative coverage of each transcript. Transcripts that met both filtering criteria (filtered transcriptome) were then translated by their longest reading frame and blasted against the other filtered transcriptome using the BLASTp algorithm. One-to-one reciprocal best BLAST hits (RBBHs) with both *e* values > 1e-03 were treated as orthologous genes. As our study involved two closely related taxa, reciprocal BLAST best hits is an adequate means of establishing orthology and is a commonly used method (Yang and Wang 2013; Pankey et al. 2014). As the number of taxa

considered increases, tree-based methods become necessary to identify orthologous genes.

Expression of orthologs was calculated with RSEM (Li and Dewey 2011) by remapping the raw reads from the individual libraries to the filtered transcriptome of the corresponding species, excluding only libraries specific to the *H. symbiolongicarpus* dactylozooids. RSEM calculates expression levels and computes three different expression values: expected counts, transcripts per million (TPM), and FPKM. Because fully annotated genomes were not available for both species, DE analyses were conducted between clusters of transcripts (as inferred by RSEM) as a proxy for a gene-level assessment. Separate DE analyses were performed with EBSeq (Leng et al. 2013) using the TPM and FPKM data sets. DE was not assessed using expected counts, as these do not include any normalization for library size. Results were filtered based on the inferred posterior probability that a gene was differentially expressed (PPDE; equal to one minus the FDR:  $1 - \text{FDR}$ ) for a particular expression pattern.

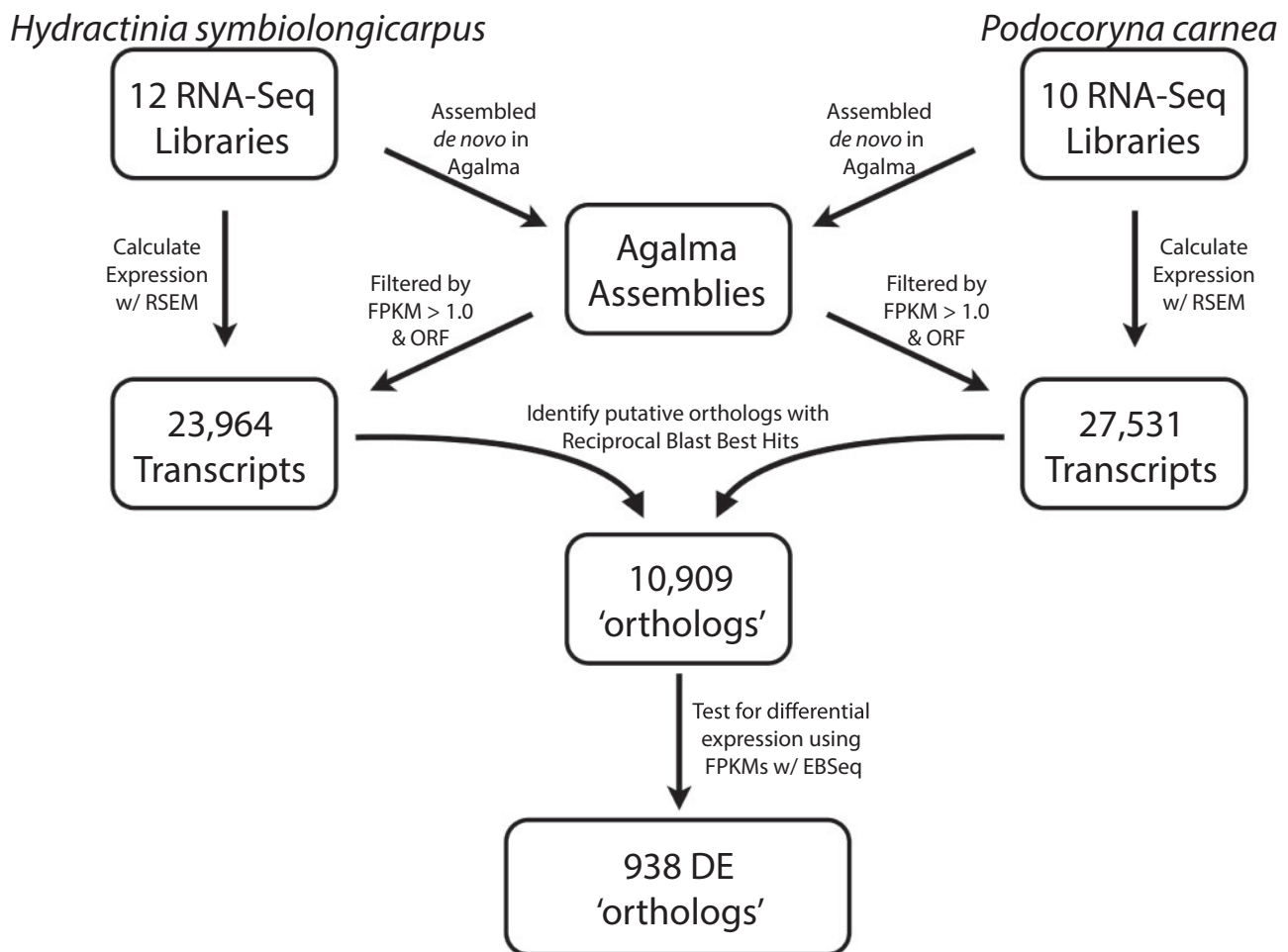
As the number of conditions increases, so do the number of possible expression patterns. With six conditions, there are 203 possible patterns (supplementary table S3, Supplementary Material online). To limit the results to those informative to our question, we identified 44 potentially informative expression patterns that are gonophore- and medusa specific. Transcripts marked by the remaining 159 expression patterns were ignored. Transcripts identified with a PPDE  $\geq 0.95$  along one of these 44 patterns (supplementary table S4, Supplementary Material online), with the highest expression observed in one of the gonophore/medusa containing conditions, were selected as candidates for further study in medusa evolution (supplementary table S5, Supplementary Material online).

### Probe Synthesis and In Situ Hybridization

Sequences of transcripts listed in table 4 were identified from each assembly. The reading frames of each species copy were aligned, and primers (supplementary table S6, Supplementary Material online) were selected to encompass homologous regions of each transcript. These fragments were then amplified from cDNA, cloned using the Invitrogen TOPO-TA Cloning Kit, and DIG labeled riboprobes were synthesized from clones using the Invitrogen T7/T3 Megascript kit. In situ hybridization (ISH) protocol was adapted from Gajewsky et al. (1996). Hybridization was carried out at 50 °C for 16–18 h with a probe concentration of 0.1 ng/μl. Hybridization was detected by immunostaining with anti-DIG-Fab-AP (ROCHE) and NBT/BCIP.

### Molecular Phylogenetic Analyses

Cnidarian sequences belonging to homeobox, helix-loop-helix, and *PDGF/VEGF* gene families were mined from the nr NCBI database and subject to phylogenetic analysis as a



**Fig. 2.**—Schematic of bioinformatics workflow. Initial transcriptomes for *Hydractinia symbiolongicarpus* and *Podocoryna carnea* are assembled from 12 and 10,100 bp paired-end Illumina libraries, respectively, using the program Agalma. Each transcriptome is filtered for transcripts that meet the Transdecoder reading frame criteria (as implemented in Agalma) and have an FPKM  $\geq 1.0$ . Expression values are estimated for these remaining transcripts from each library independently using RSEM. Orthologs are identified using one-to-one reciprocal BLAST best hits between the transdecoder protein translations of the subsetted transcriptome using BLASTp under default setting. DE analyses are performed with EBSeq using FPKM and TPM expression normalizations.

means to quickly establish orthology with those genes in our data set. *Podocoryna carnea* and *H. symbiolongicarpus* amino acid sequences belonging to the gene families of interest were identified using the HMMER annotations, extracted from the assemblies, and subject to a clustering analysis using CD-HIT (Li and Godzik 2006; Fu et al. 2012) (under a 90% sequence similarity threshold) to remove redundant gene copies. Alignments were conducted with Mafft (Kato et al. 2005) under the L-insi alignment algorithm. Maximum-likelihood estimates of the gene trees were then inferred using RAxML (Stamatakis et al. 2008) on the CIPRES portal (Miller et al. 2010) using the rapid bootstrapping (-f a) algorithm with 100 bootstrap replicates under the PROTGAMMA+WAG model (supplementary figs. S3 and S5, Supplementary Material online).

## Results and Discussion

### Transcriptome Assembly and Annotation, Enrichment Analyses, and Orthology Prediction

Raw Illumina RNA-Seq data for *H. symbiolongicarpus* and *P. carnea* were downloaded from NCBI (SRA archive no. SRP038762 and SRP041583, respectively). For *H. symbiolongicarpus*, these data were generated from four separate tissue sources: gastrozooids (feeding polyps), dactylozooids (defensive polyps), gonozooids (reproductive polyps) bearing male gonophores, and gonozooids bearing female gonophores (table 1). For *P. carnea*, data were generated from the following tissues: nonreproductive feeding polyps (gastrozooids), reproductive (medusa-budding) feeding polyps, and free-living medusae. All *P. carnea* data were generated from female

tissues. Final assemblies, which combined data from all libraries of that species, consisted of 127,716 and 178,396 transcripts for *H. symbiolongicarpus* and *P. carnea*, respectively (table 2).

GO analyses identified 11,196 unique GO terms and 16,386 hidden Markov model (HMM) domains from at least one transcriptome. As a means of identifying candidate “medusa” genes, enrichment analyses (Fisher’s exact test) were performed on the abundance of each GO term and HMM domain in either assembly. GO enrichment analyses did not identify any over abundant GO terms in *P. carnea*’s transcriptome, when compared with the total number of GO terms for each species combined. Similarly, enrichment analyses of HMM domains did not identify any overrepresented domains in the *P. carnea*’s transcriptome. In contrast, 110 GO terms and 27 HMM domains were overrepresented in *H. symbiolongicarpus*’ transcriptome. This is most likely due to the inclusion of dactylozooids and both male and female gametic tissues in the assembly for *H. symbiolongicarpus*, whereas *P. carnea* does not have dactylozooids, and male gametic tissue were not sampled. Because neither sets of enrichment analyses yielded insight into gene and/or signaling pathways involved in life cycle differences between these two taxa, we performed interspecific DE analyses to detect quantitative differences in gene expression levels associated with the phenotypic differences between these species’ gonophores.

**Table 1**  
RNA-Seq Illumina Libraries

Condition	No. Replicates
<i>Hydractinia symbiolongicarpus</i> <sup>a</sup>	
gastrozooid	4
Female gonozooid <sup>b</sup>	2
Male gonozooid <sup>b</sup>	2
<i>Podocoryna carnea</i>	
Nonreproductive polyp	3
Medusae-budding polyp <sup>b</sup>	4
Adult medusae <sup>b</sup>	3

<sup>a</sup>The other condition, dactylozooid, was included in the assembly but not in the DE analysis.

<sup>b</sup>Conditions of interest.

**Table 2**  
Assembly Statistics Summary

	<i>Hydractinia symbiolongicarpus</i>			<i>Podocoryna carnea</i>		
	Initial	Filtered	RBBH	Initial	Filtered	RBBH
No. transcripts	127,716	23,964	10,909	178,396	27,531	10,909
N25 (bp)	3,960	4,389	4,538	3,342	3,906	4,341
N50 (bp)	2,459	2,895	2,991	1,977	2,626	2,882
N75 (bp)	1,290	1,929	2,016	945	1,796	1,959
GC content	35.40%	36.49%	36.71%	38.60%	38.12%	36.56%

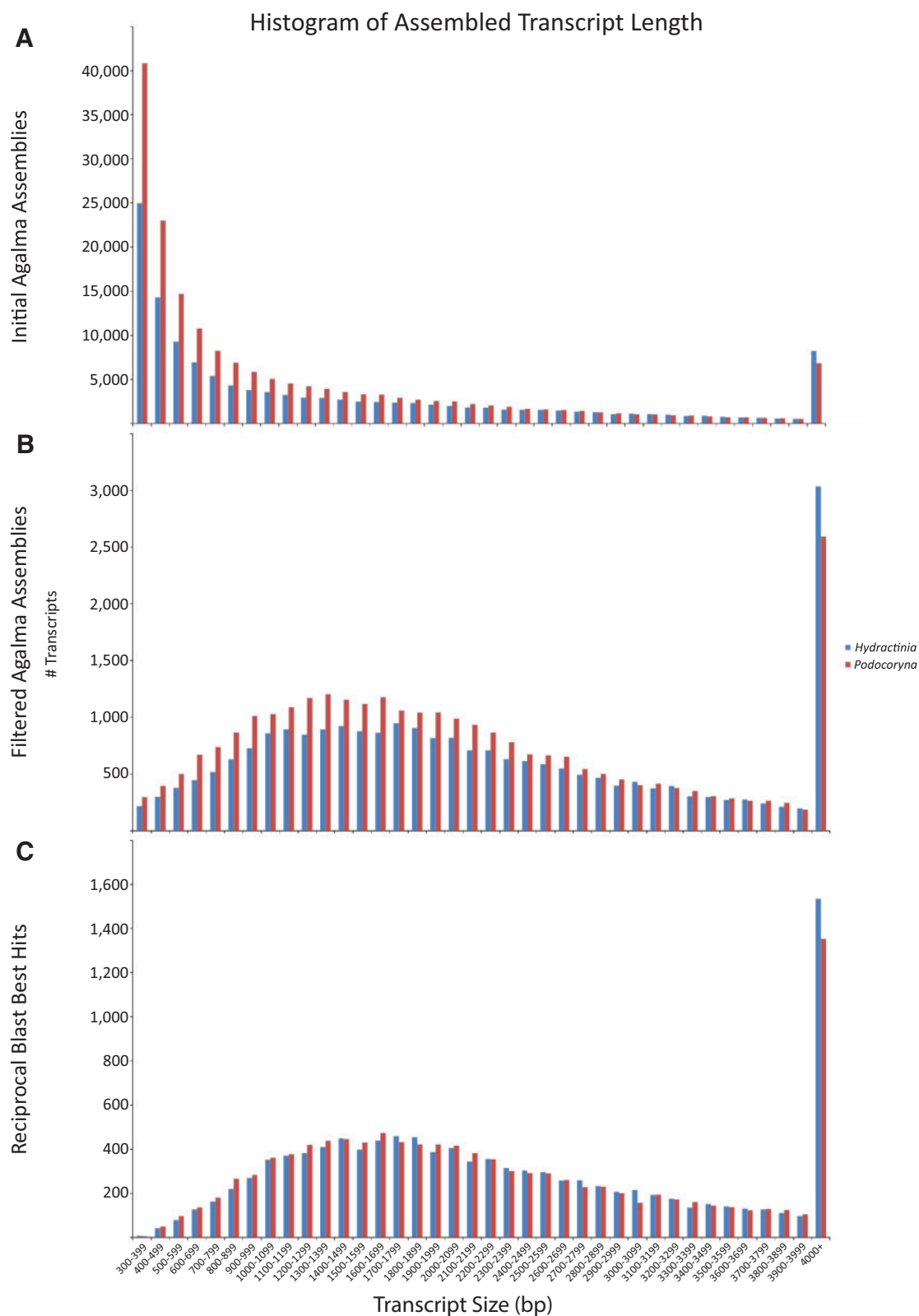
NOTE.—Initial, transcriptomes assembled with Agalma; filtered, transcripts remaining after transcriptomes were filtered by FPKM and transdecoder reading frame criteria; RBBH, transcripts with a one-to-one RBBH match between the two filtered transcriptomes.

When comparing gene expression between species, the first critical step is to establish robust orthology assignments. To avoid artifactual differences due to different assembly methods, each transcriptome was assembled de novo by an automated bioinformatics pipeline, Agalma (Dunn, Howison, and Zapata 2013), under identical settings, as opposed to using a previously published genome-guided transcriptome for *H. symbiolongicarpus* (Sanders et al. 2014) and a de novo transcriptome for *P. carnea* (Sanders and Cartwright 2015). Of further concern is the effect of polymorphisms on transcript/gene redundancy in the assembly. Polymorphisms (common in data collected from noninbred lines) can lead to an increase in the number of paths to reconcile during the assembly process, thus increasing the number of fragmented and rare variants of a transcript/gene. To minimize the number of fragmented and redundant transcripts, each assembly was filtered for transcripts with a minimum FPKM expression value ( $\geq 1.0$ ) and reading frame criteria prior to orthology prediction.

After initial filtering, approximately 24K and 27.5K transcripts remained (referred to from here on as the filtered transcriptomes) in the *H. symbiolongicarpus* (GCHW00000000) and *P. carnea* (GCHV00000000) assemblies, respectively (fig. 2, table 2). This reduction in transcript number greatly reduced the differences between each transcriptome assemblage characteristics, including the distribution of transcript size, N50, and GC content (table 2, fig. 3). Most importantly, removing incomplete transcripts and underrepresented variants increases our confidence in the transcripts remaining for orthology prediction and subsequently, the reliability of the inferred relative expression of each predicted ortholog. A total of 10,909 putative orthologs were identified (fig. 2, table 2; supplementary tables S1 and S2, Supplementary Material online) between our filtered assemblies. Not surprisingly, the resulting orthologous gene data set further decreased the disparity between the summary statistics for each species (table 2, fig. 3).

### DE Analyses

Two separate expression matrices, FPKM and TPM, were generated for the 10,909 orthologs using RSEM (Li and



**FIG. 3.**—Distributions of transcript size. Histograms of the (A) initial Agalma-assembled transcriptomes. (B) Assemblies filtered by FPKMs and reading frame criteria. (C) Transcripts with a one-to-one reciprocal BLAST best hit (orthologs). Red, *Podocoryna carnea*; blue, *Hydractinia symbiolongicarpus*. x axis is constant. y axis changes with each assembly.

Dewey 2011) and analyzed with EBSeq (Leng et al. 2013). EBSeq is a well-suited software for assessing DE between species as EBSeq's FDR and statistical power have been shown to be less sensitive to overdispersion of expression values between conditions, when compared with other DE software (Leng et al. 2013). Furthermore, EBSeq allows one to test for DE between multiple conditions (i.e., tissue types) simultaneously, whereas most other DE software only allow for individual pairwise comparisons between two conditions. EBSeq simultaneously assesses DE between multiple conditions by assigning a posterior probability to each possible expression pattern in an enumerated list of all possible expression patterns, given a set of conditions. These patterns are defined as the unique combination of significant differences in expression values between a given number of conditions. As more conditions are present, the number of possible patterns increases.

To take advantage of this feature, we included nonreproductive gastrozooids from each species (see description above) in addition to conditions that have a gonophore stage present (male and female gonozooids of *H. symbiolongicarpus*, medusa-budding polyps of *P. carnea*, and the fully developed medusae stage of *P. carnea*) (table 1). Inclusion of the nonreproductive tissue types increases the complexity of the expression landscape within and between each species (i.e., more patterns), effectively increasing the power of the DE analysis. With six conditions in the analyses (table 1), EBSeq identified a total of 203 possible expression patterns, although not all of which are informative to our question (fig. 4A; [supplementary table S3, Supplementary Material](#) online). This is another advantage of EBSeq, as the researcher can insert biologically relevant constraints on expression patterns a priori, retaining only those patterns that are specific to the conditions of interest for that particular study (fig. 4B).

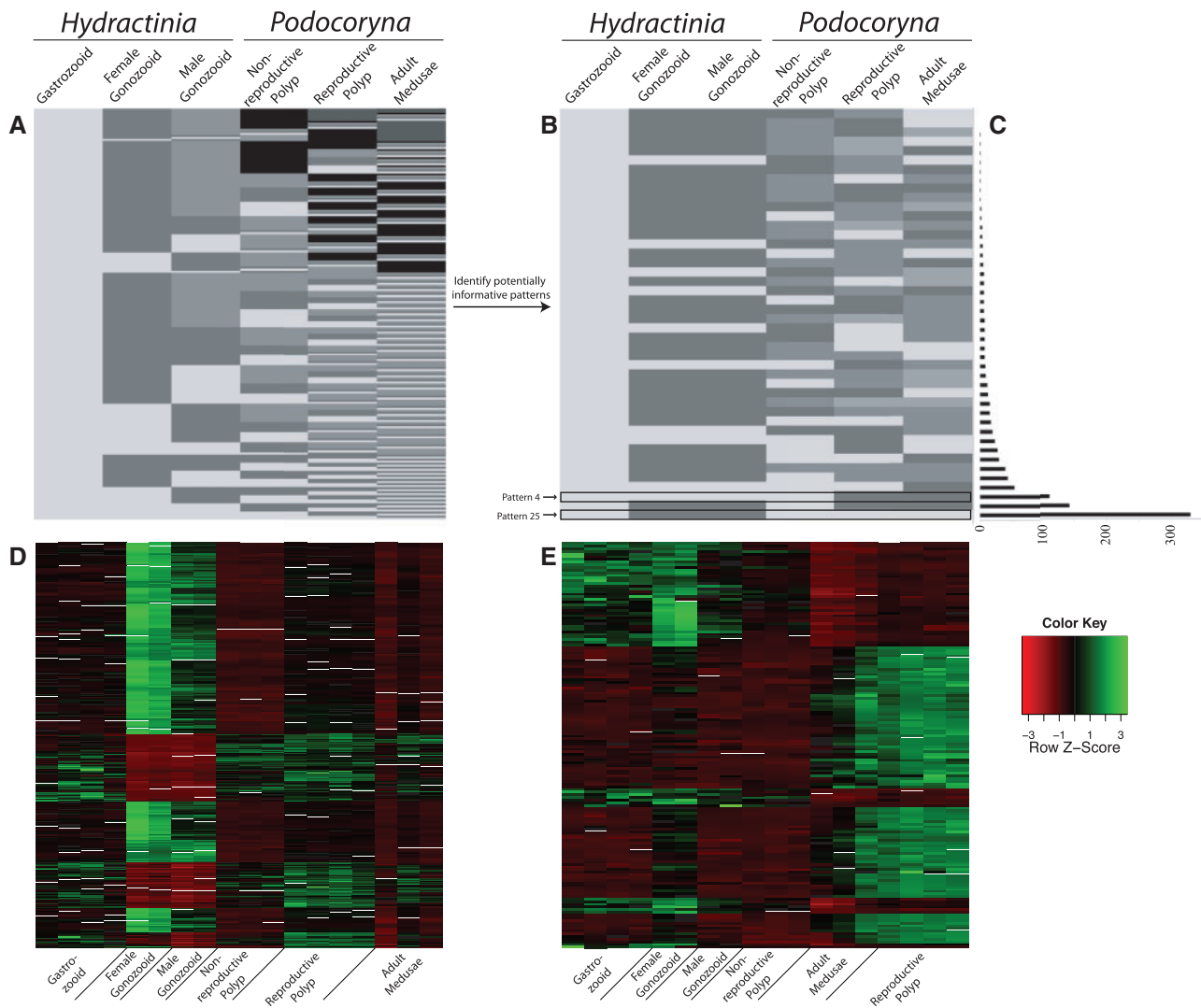
Capitalizing on this aspect, we identified 44 expression patterns that were potentially informative, greatly reducing the number of potential results (fig. 4B; [supplementary table S4, Supplementary Material](#) online). These patterns were selected with an initial constraint that *Hydractinia* male and female gonozooid expressions are statistically equivalent. Following assumptions made by Sanders et al. (2014), transcripts differentially expressed between male and female gonozooids can be attributed to differences in gametogenesis (either spermatogenesis or oogenesis). Because only female gametic tissues were sampled in *P. carnea*, gene expression driven by maternal transcript generation during oogenesis will be highly expressed in the budding and adult medusae, potentially skewing the DE results. Assuming maternally loaded genes are conserved between closely related species, patterns where expression of *H. symbiolongicarpus* male and female sporosacs are not statistically equivalent were removed, thus reducing the number of patterns to 52. Further patterns that were not relevant to life cycle differences between species were also removed to increase the chance of finding differentially expressed genes in developing gonophore and/or adult

medusae. For example, patterns where expression is statistically equivalent between nonhomologous, interspecific tissue samples (i.e., polyp tissue in *Hydractinia* and medusae tissue in *Podocoryna*) but are differentially expressed between intraspecific tissue samples (i.e., nonreproductive polyps and medusae tissue of *Podocoryna*) were removed from the analysis, resulting in a total of 44 potentially informative patterns (fig. 4B; [supplementary table S4, Supplementary Material](#) online).

Approximately 75% (8,210) of the putative orthologs are recovered as significantly differentially expressed in at least one of the data sets along one of the 203 expression patterns at a PPDE > 0.95 (table 3; [supplementary table S2, Supplementary Material](#) online). This high proportion of DE genes is not entirely surprising as only one condition needs to significantly vary from any of the others to be recovered as such (fig. 4). In either case, both data sets perform similarly, with only 686 and 858 of those DE transcripts unique to the FPKM and TPM data sets at this significance threshold, respectively. The percentage of transcripts identified as differentially expressed in both data sets remains roughly constant (between 79–81%), until the FDR decreases to 0.00 (PPDE = 1.00). At this threshold, FPKM performs more conservatively than TPM, with only 5.2% (266) of the total DE orthologs (5,120) specific to the FPKM data set, whereas 23.2% (1,187) are unique to the TPM data set (table 3; [supplementary fig. S1, Supplementary Material](#) online). This increased conservation can be attributed to the added scaling by transcript length for FPKM expression values.

A total of 2,439 potentially informative transcripts (*PIT*; transcripts whose expression is consistent with one of the 44 potentially informative patterns) were identified in at least one of the two data sets (table 3). Of those 1,611 were found significantly differentially expressed in both data sets, whereas an additional 359 and 368 were specific to the FPKM and TPM sets, respectively (FDR < 0.05; table 3). Although 2,338 *PIT* are differentially expressed between the test conditions in at least one of the data sets, they are not necessarily expressed in a gonophore/medusa-specific manner since the predefined expression patterns do not contain information about the magnitude of expression for each condition (fig. 4D and E). Further examination of these DE *PIT* revealed that 938 are upregulated in one of the gonophore/medusa-containing conditions, of which 605 were significant in both (FDR < 0.05; table 3; fig. 4C; [supplementary tables S2 and S5, Supplementary Material](#) online).

Discrepancies between the different normalization methods on which putative orthologs are differentially expressed can be seen across all levels of the DE analysis and are explained by differences in the expression pattern assigned the highest posterior probability. This is largely due to disagreement on constraints imposed to identify potentially informative patterns such as scaling by transcript length in FPKM. Although Li and Dewey (2011) suggest that TPM is better expression measure for comparisons between species, there



**FIG. 4.**—Pattern reduction to informative patterns. (A) With six conditions (columns) present in the DE analysis, EBSeq identifies 203 possible expression patterns (rows). (B) Using biologically relevant constraints on expression in an attempt to reduce the noise in the DE signal, the number of patterns is reduced to 44 potentially informative patterns. Colors in this schematic do not indicate magnitude of expression, just nondirectional levels of expression to show statistically equivalent, and nonequivalent levels of expression between conditions in the analysis. (C) Bar graph of the number of DE genes (FDR < 0.05) specific to each 44 of the potentially informative patterns. (D) Z-normalized heatmap of all orthologs whose expression is consistent with “Pattern 25” in the FPKM data set, an expression that should contain sporosac-specific orthologs. (E) Z-normalized heatmap of all orthologs whose expression is consistent with “Pattern 4” in the FPKM data set, an expression that should contain genes specific to *Podocoryna carnea* reproductive polyps and adult medusae.

**Table 3**

Number of Differentially Expressed Transcripts

	FDR < 0.05				FDR = 0.00			
	FPKM	TPM	Shared	Total	FPKM	TPM	Shared	Total
Tot. DE	686	858	6,666	8,210	266	1,187	3,667	5,120
Tot. <i>PIT</i>	402	349	1,688	2,439	–	–	–	–
Tot. DE <i>PIT</i>	359	368	1,611	2,338	173	403	847	1,423
Upreg. DE <i>PIT</i>	181	152	605	938	80	168	244	492

NOTE.—FPKM and TPM columns correspond to the number of transcripts unique to that data set. Upreg. DE *PIT*, counts for transcripts specific to one of the conditions of interest shown in footnote b in table 1.



has been no comprehensive evaluation of normalization methods for RNA-Seq in an interspecific DE framework. Therefore, to minimize the effect that either normalization method has on the DE results, any putative ortholog identified in either data set are considered candidates for future study and ones shown to be significant in both data sets can be considered most reliable.

### Spatial Expression of Differentially Expressed Orthologs

To further validate our unbiased interspecific DE analyses, several of the 492 transcripts identified as significantly upregulated in either *Hydractinia* sporosacs or *Podocoryna* developing and/or adult medusae in at least one data set (FDR=0.00; table 3) were selected for spatial expression analysis by whole mount ISH (table 4; fig. 5; supplementary table S6, Supplementary Material online). Selection was based on relatively high levels of expression that could be detected with this method and because of potential biological interest. None of the candidates discussed below have been previously characterized in cnidarians. Spatial expression of each gene was examined with ISH in each of the tissues sampled for DE analyses. Medusa buds were examined across all 10 stages of medusa development as defined by Frey (1968) (fig. 5). Expression of the candidates discussed below was primarily restricted to tissues in the gonophores and adult medusae (fig. 5), except for *APLP*, which also exhibited expression in polyp tissues (supplementary fig. S2, Supplementary Material online).

Two of the candidates surveyed with ISH, *IF2B2* and *TF3C6* exhibited similar endodermal expression patterns in *P. carnea* gonophores. *IF2B2* was recovered as significantly upregulated in all gonophore stages (including *H. symbiolongicarpus* sporosacs and *P. carnea* medusa buds and adult medusa), whereas *TF3C6* was recovered as specific to *P. carnea* medusa tissues. ISH of both genes shows strong endodermal expression from stages 1 to 6 of medusa development in *P. carnea*, which then ceases by stage 7. After liberation, *IF2B2* is also expressed the proximal portion of the tentacle bulbs of the adult medusae (fig. 5), a region corresponding to early stages of

nematogenesis in hydrozoan medusae (Denker et al. 2008). Expression patterns here suggest that these genes might have a role inducing cell proliferation as they are expressed in highly proliferative regions of the developing and/or adult medusa (Spring et al. 2000; Seipel, Yanze, et al. 2004; Denker et al. 2008). These spatially restricted expression patterns were not observed in *H. symbiolongicarpus* sporosacs (fig. 5).

Furthermore, ISH of *IF2B2* revealed expression consistent with a general role in gametogenesis in both *H. symbiolongicarpus* and *P. carnea* (fig. 5). *IF2B2* is expressed around early- and late-stage oocytes in *P. carnea*. In *H. symbiolongicarpus*, *IF2B2* is expressed around oocytes in the germinal zone (body column) of female gonozooids (not shown), where oogenesis begins (Berrill 1953; Müller 1964; Bunting 1894), and expression continues in the endoderm surrounding oocytes after it moves into the gonophores, where oocyte differentiation continues (Berrill 1953; Müller 1964; Bunting 1894). In males, expression is specific to the gametic tissues of mature sporosacs (fig. 5) (Bunting 1894; Berrill 1953). This expression pattern suggests that *IF2B2* is only operating in gametogenesis and plays no role in patterning the sporosac. This is different from ISH results of *TF3C6* where no expression of *TF3C6* was seen in either male or female sporosacs, while expression was observed around early and late stage oocytes in *P. carnea*.

ISH of three other genes recovered as upregulated in *P. carnea* medusa libraries (*Notch*-like, *KLF12*, and *PLST3*) revealed similar spatially restricted expression patterns at the distal tip of the developing axes of medusa buds of *P. carnea*. For each of these genes, ISH shows minor endodermal expression at various stages of medusa development, but in each case, the prominent expression is seen at the distal end of the developing bell axis by stages 5 and 6. *Notch*-like expression precedes the expression of both *KLF12* and *PLST3* and is strongly expressed at the distal end of the gonophore prior to opening of the bell margin in stage four. Past stage 6, expression for all genes is specific to maturing oocytes, although after liberation, *KLF12* is also expressed in the tentacle bulbs and along the manubrium (the structure bearing the gonads and mouth at its distal end). Similar to *TF3C6*

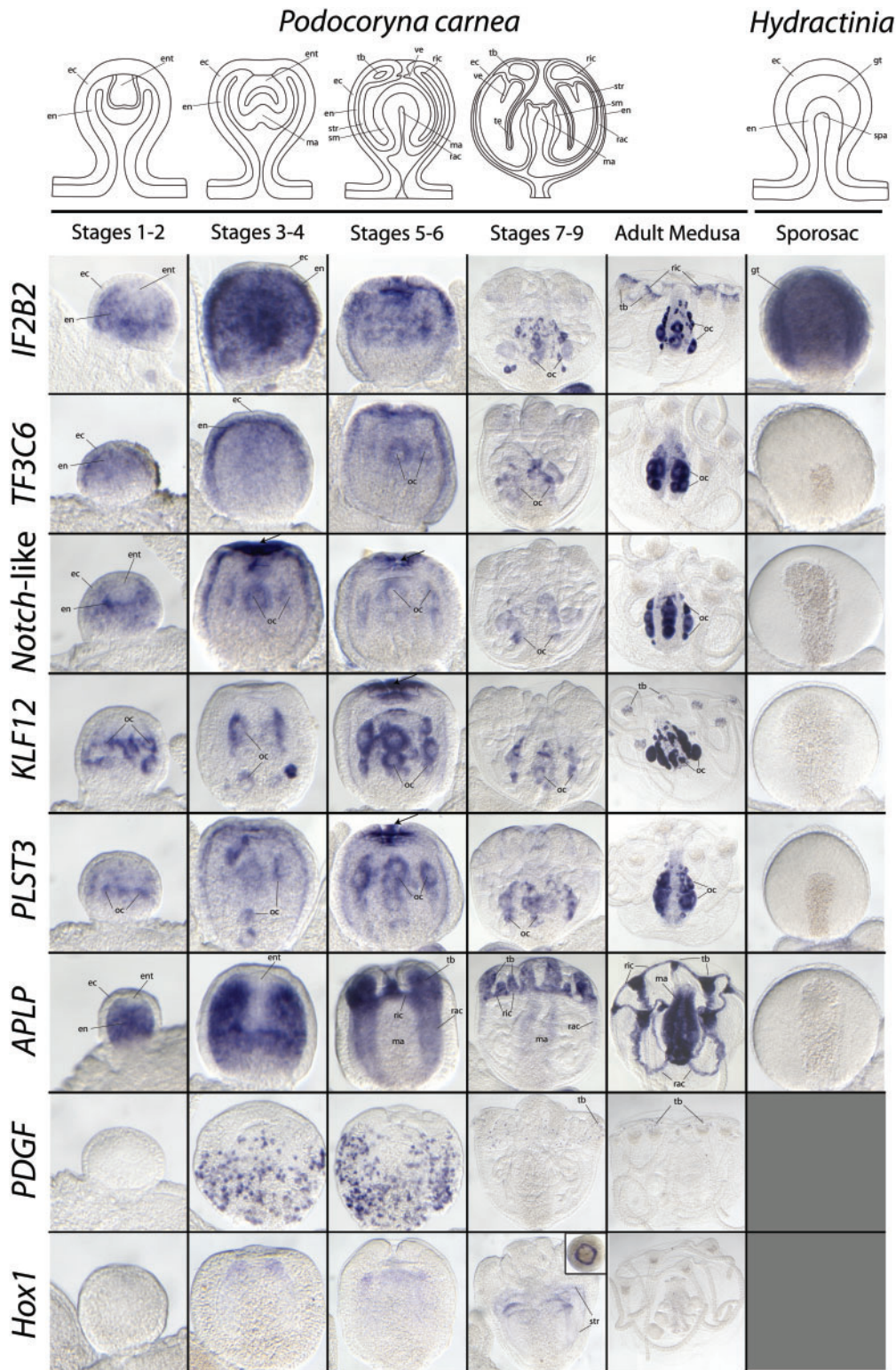
**Table 4**

Differentially Expressed Orthologs and *Podocoryna carnea*-Specific Genes Validated with ISH

RBBH ID	Name	BLAST Hit	FPKM	TPM
RBBH_6358	<i>IF2B2</i>	Insulin-like growth factor 2 mRNA-binding protein 2	**	NS
RBBH_7273	<i>TF3C6</i>	General transcription factor 3C polypeptide 6	**	NS
RBBH_608	<i>Notch</i> -like	Neurogenic locus notch homolog protein 1	**	**
RBBH_8405	<i>KLF12</i>	Krueppel-like factor 12	**	**
RBBH_2122	<i>PLST</i>	Plastin-3	**	**
RBBH_3474	<i>APLP</i>	Apolipoporphins	**	**
None	<i>PDGF</i>	Platelet-derived growth factor subunit A	NA	NA
None	<i>Hox1</i>	Homeobox protein Hox-B1	NA	NA

NOTE.—NS, not significant; NA, not subject to DE analyses; none, ortholog not present in *Hydractinia symbiolongicarpus*.

\*\*Orthologs that are significant in either data set at a PPDE=1.00.



**Fig. 5.**—Whole mount ISH results. Images position the oral end of the gonophores/medusa toward the top. Arrows mark regions of concentrated expression at the distal end of the bell axis or the oral end of the developing manubrium. Only male *Hydractinia symbiolongicarpus* sporosacs are shown as eggs in females block the view of the spadix (manubrium anlage). Inset in *Hox1*, stages 7–9 pane is a view from the oral end of the gonophore looking down. ec, ectoderm; en, endoderm; ent, entocodon; gt, gametic tissue; ma, manubrium, oc, oocytes; rac, radial canal; ric, ring canal; sm, smooth muscle; spa, spadix; str, striated muscle; tb, tentacle bulb; ve, velum.

expression, ISH did not detect expression of these genes in the sporosacs of *H. symbiolongicarpus* (fig. 5), except for minor expression around early stage oocytes (not shown).

Although the top BLAST hit was a “neurogenic notch” gene, the ortholog, *Notch*-like, examined in this study are not orthologous to those notch genes examined in *Hydra* and *Nematostella* (Käsbauer et al. 2007; Marlow et al. 2012). Sequence analysis of *Notch*-like reveals that it lacks the LNR and NOD domains that characterizes “notch” genes but does possess the EGF domains which is present in, but not specific to notch genes (Käsbauer et al. 2007; Marlow et al. 2012). In each of the genes examined here, ISH reveals spatially restricted expression patterns in highly proliferative somatic regions during *P. carnea* development. These expression patterns, together with the lack of somatic expression of these genes in *H. symbiolongicarpus* sporosacs suggest a potential role of these genes in medusae morphogenesis and evolution. Future functional studies will need to be performed to confirm these results.

*APLP* is the only candidate selected for ISH that does not exhibit expression consistent with any role in oogenesis in *P. carnea*. Throughout gonophore development, ISH reveals *APLP* expression to be specific to the endodermal tissues that give rise to gastric structures in the adult medusa. Starting at stage one, strong *APLP* expression is detected in the endoderm of the newly formed gonophore. As development proceeds, *APLP* expression remains specific to the endodermal tissue beginning to form the radial canals in stages 3–6. *APLP* expression is excluded from and clearly outlines the entocodon, which is medusa-specific tissue layer formed through evagination of the distal ectoderm of the gonophore that gives rise to striated muscle (Avset 1961). By stages 5 and 6, strong expression is noted in the newly formed ring canal and tentacle bulbs but is excluded from the developing manubrium. This pattern continues through the later stage buds but seems to decrease in the strength of expression (especially in the radial canals) until the medusa is liberated from the colony, where expression strongly reappears in all digestive tissues; including the fully developed manubrium, radial and ring canals, and tentacle bulbs (fig. 5). *APLP* expression was not observed in the sporosacs of *H. symbiolongicarpus*.

Throughout medusa development, *APLP* appears to be expressed in a manner consistent with the patterning and development of the digestive tract of the medusa, whereas after liberation, it remains expressed in digestive tissues. This is consistent with *APLP*'s role in other animals, where it functions not only as a lipid trafficking molecule but also plays a critical role in patterning, through regulating *hedgehog* and Wnt signaling during wing development in *Drosophila* (Panáková et al. 2005). Previous studies have implicated canonical Wnt signaling in medusae evolution (Duffy et al. 2010; Duffy 2011; Nawrocki and Cartwright 2013; Sanders and Cartwright 2015), yet, given the dual role of the Wnt pathway as both a maternal effect for larval development and in adult

patterning (Plickert et al. 2006; Teo et al. 2006; Momose and Houlston 2007; Müller et al. 2007; Momose et al. 2008; Amiel and Houlston 2009; Duffy et al. 2010), DE expression of Wnt signaling genes in medusa would likely be obscured by high expression in female gametic tissue due to maternal loading. Yet even without many of the key Wnt signaling components recovered by our DE analyses, recovery of *APLP* further implicates the role of Wnt signaling in medusa development and evolution (Nawrocki and Cartwright 2013; Sanders and Cartwright 2015). Further ties between *APLP* and Wnt signaling come from the expression patterns of *APLP* in both *P. carnea* and *H. symbiolongicarpus* polyps (supplementary fig. S2, Supplementary Material online). In the feeding polyps of both species, *APLP* is expressed in a ring around the distal tip of the hypostome and in the endoderm at the tip of the tentacles (supplementary fig. S2, Supplementary Material online), consistent with observed *Wnt3* expression in *Hydra* (Guder et al. 2005; Lengfeld et al. 2009; Gee et al. 2010), *Hydractinia* (Plickert et al. 2006; Müller et al. 2007; Duffy et al. 2010), *Podocoryna* (Sanders and Cartwright 2015), and *Ectopleura* (Nawrocki and Cartwright 2013). Similarly, ISH revealed *APLP* expression at the distal tip of *H. symbiolongicarpus* gonozooids (supplementary fig. S2, Supplementary Material online), consistent with *Wnt3* expression in *H. echinata* (Müller et al. 2007; Duffy et al. 2010) and *H. symbiolongicarpus* (S. Sanders, unpublished data).

#### Previously Published Medusae-Specific Genes

Several previous studies, using a candidate gene approach, identified genes specific to medusae development in *P. carnea* (Schuchert et al. 1993; Aerne et al. 1995; Gröger et al. 1999; Masuda-Nakagawa et al. 2000; Müller et al. 1999; Yanze et al. 1999; Spring et al. 2000; Spring et al. 2002; Müller et al. 2003; Seipel, Yanze, et al. 2004; Seipel et al. 2004a–c; Stierwald et al. 2004; Torras et al. 2004; Galle et al. 2005; Reber-Müller et al. 2006). As additional validation of our DE results, we determined whether any of these genes were present in our pool of 938 candidates that were identified as significantly upregulated in developing gonophores and/or adult medusa (table 3). In our DE analysis, some, but not all, previously reported medusa-specific genes are differentially upregulated in developing and/or adult medusa stages of *P. carnea* relative to *H. symbiolongicarpus* sporosacs (table 5). These include orthologs of striated muscle-specific homeobox genes *msx* (Galle et al. 2005) and *orthodenticle* (*otx*) (Müller et al. 1999), a myosin heavy chain, *myo1* (Schuchert et al. 1993), a tropomyosin, *tpm2* (Gröger et al. 1999), and a zinc finger transcription factor *snail* (Spring et al. 2002).

Moreover, several of the previously reported medusae-specific genes appear to be absent in the *H. symbiolongicarpus* transcriptome examined here. These include three homeobox genes, *Hox1* (Aerne et al. 1995), *six1/2*

**Table 5**Differentially Expressed Orthologs Consistent with Previously Published Studies in *Podocoryna carnea*

RBBH ID	Name	Source	FPKM	TPM	Specificity
RBBH_4080	<i>Myo1</i>	Schuchert et al. (1993)	**	**	All medusa stages
RBBH_3250	<i>Tpm2</i>	Gröger et al. (1999)	**	**	All medusa stages
RBBH_5585	<i>Otx</i>	Müller et al. (1999)	NS	*	Adult medusa
RBBH_6387	<i>Snail</i>	Spring et al. (2002)	*	**	All medusa stages
RBBH_540	<i>Msx</i>	Galle et al. (2005)	*	*	Adult medusa

NOTE.—NS, not significant.

\*PPDE &gt; 0.99.

\*\*Orthologs significant in either data set at a PPDE = 1.00.

(Stierwald et al. 2004), and *cnox2-Pc* (Masuda-Nakagawa et al. 2000) (not orthologous to *cnox-2* in *H. symbiolongicarpus*; Cartwright et al. 1999) (supplementary fig. S3, Supplementary Material online), as well as a helix-loop-helix transcription factor, *jellyD1* (Müller et al. 2003). Interestingly, these genes have previously been shown to be expressed in medusa-specific structures, including striated muscle, the manubrium, the entocodon, and/or tentacle bulbs (Aerne et al. 1995; Masuda-Nakagawa et al. 2000; Stierwald et al. 2004). Gene tree analyses and searching unpublished genomic scaffolds of *H. symbiolongicarpus* reveal that *Hox1* and *jellyD1* lack an ortholog in *H. symbiolongicarpus* (supplementary figs. S3 and S4, Supplementary Material online), whereas the absence of *six1/2* and *cnox2-Pc* in this *H. symbiolongicarpus* transcriptome appear to be instances of downregulated expression of these genes in *H. symbiolongicarpus* sporosacs.

#### Podocoryna carnea Genes that Lack a Corresponding Ortholog in *H. symbiolongicarpus*

Although differential regulation of orthologous genes does explain differences in homologous structures between species, evolutionary shifts between phenotypes are likely accompanied by gene gain or loss as well. To further explore the role of novel gene gain and loss in hydractiniid life cycle differences, we selected two *P. carnea* genes without a *H. symbiolongicarpus* ortholog (table 4; supplementary figs. S3 and S5, Supplementary Material online) that are also significantly upregulated in developing and adult medusae of *P. carnea* (Sanders and Cartwright 2015) for further study with ISH. These genes were, an unpublished growth factor, *PDGF*, and the previously published homeobox gene, *Hox1* (Aerne et al. 1995) (table 4). Phylogenetic analysis of the platelet-derived/vascular endothelial growth factor family (*PDGF/VEGF*) (supplementary fig. S5, Supplementary Material online) further suggested the absence of a *PDGF* ortholog in *H. symbiolongicarpus* and was confirmed by its absence in the previously mentioned unpublished genomic scaffolds. Although a member of the same gene family, *PDGF* is not homologous to the *VEGF* gene previously characterized in *P. carnea* (Seipel et al. 2004c) (supplementary fig. S5, Supplementary Material online) whose expression is restricted

to the endoderm of the medusae buds and its resulting tissues during medusae development. ISH of *PDGF* confirmed the specificity of this gene to developing and adult medusa stages with no expression detected in the polyp stage of *P. carnea* (not shown). *PDGF* expression begins at bud stages 3 and 4 of medusa development, revealing a speckled expression pattern (fig. 5). This pattern shifts as medusa development continues, and *PDGF*-positive cells appear to be evenly distributed except at the most distal tip of the gonophore by stages 5 and 6. By stages 7 and 8 of medusa development, expression is limited to just a few cells in the tentacle bulbs and this continues through the adult medusa. These cells most notably resemble differentiating stem cells called nematoblasts (Denker et al. 2008), most likely in some very early stage of nematogenesis as they appear to migrate toward the tentacle bulb, although further research is necessary to confirm this. Since this gene is not expressed in the known stem cell populations of hydrozoan polyps (Teo et al. 2006; Müller et al. 2007; Millane et al. 2011; Duffy et al. 2012; Hemmrich et al. 2012), it suggests a potential medusa-specific stem cell lineage.

Although members of the same gene family, *PDGF* and *VEGF* (Seipel et al. 2004c) display very different expression patterns throughout medusae development. Seipel et al. (2004c) showed *VEGF* expression to be consistent with morphogenesis in *P. carnea*, particularly during tube formation (both in the tentacles or the canal system of the medusae) and suggested that as the ancestral metazoan function of *VEGF* genes. This is consistent with the role of *VEGF* signaling in vasculogenesis and angiogenesis in vertebrates (Ferrara and Davis-Smyth 1997; Nasevicius et al. 2000). However, in *Drosophila*, *VEGF* signaling is involved in blood cell migration and differentiation (Duchek et al. 2001; Heino et al. 2001; Cho et al. 2002). Our expression patterns suggest that *PDGF* expression is more consistent with this role in cell migration and differentiation. These results would suggest that at least two different (and potentially conserved) *PDGF/VEGF* signaling pathways are operating during medusae development and that the alteration of these pathways has implications in medusae evolution.

ISH showed relatively little expression of *Hox1* through medusa development with no expression detected in the polyp. Noticeable (although very faint) expression begins by

stage 3 and continues through the later stages of gonophore development, until the medusa is fully developed, where expression ceases. At the earlier stages, *Hox1* expression is seen as a ring-like pattern around the distal region of the differentiating entocodon (fig. 5). This pattern is maintained as gonophore development progresses, broadening the expression ring as the gonophore grows. By stages 7 and 8, the strongest expression is seen at the distal end of the expression domain yet with more minor expression dispersed more proximally along the striated muscle tissue of the developing medusa.

ISH of *Hox1* is consistent with expression reported by Aerne et al. (1995), where expression was detected in bud stages with developing striated muscle. Later, Yanze et al. (2001) showed *Hox1* expression throughout embryonic development and in the aboral end of the planula, consistent with expression of *Hox1* orthologs (78% BS support; [supplementary fig. S3, Supplementary Material](#) online) reported in *Clytia* (Chiori et al. 2009) and *Eleutheria* (Jakob and Schierwater 2007), where it appears to play a role in the oral-aboral patterning. Two of three taxa (*Clytia* and *Eleutheria*) with a documented ortholog of *Hox1* possess fully developed medusa and in each case *Hox1* expression is not observed in the striated muscle. In *Clytia hemisphaerica* medusa, *Hox1* expression is specific to the balancing organ (statocyst; not present in *P. carnea* medusa) (Chiori et al. 2009), whereas *Eleutheria dichotoma* benthic medusae exhibit no *Hox1* expression (Jakob and Schierwater 2007).

Although results from the phylogenetic analyses suggest that *Hox1* evolved earlier in Hydrozoa and was subsequently lost in *H. symbiolongicarpus* ([supplementary fig. S3, Supplementary Material](#) online), *jellyD1* and *PDGF* appear to have been the result of duplication events in *P. carnea* ([supplementary figs. S4 and S5, Supplementary Material](#) online); however, this could be an artifact of limited sampling of these gene families in other cnidarians. The lack of an orthologous *Hox1* gene in *H. symbiolongicarpus* is consistent with the loss of striated muscle during truncation of the medusa following a *Hox1* deletion, this, however, does not explain the observed variability of *Hox1* expression in the medusa of more distantly related hydrozoans. This variable expression of *Hox1* across distantly related taxa suggests a potential evolutionary scenario where *Hox1* was co-opted to be involved in striated muscle development during a transition toward fully developed medusa in *P. carnea* as no other hydrozoan medusa exhibits *Hox1* expression in their striated muscle tissues (Jakob and Schierwater 2007; Chiori et al. 2009). Future areas of research should focus on sampling more intermediate levels of medusa truncation to determine whether changes in expression correlate with the development of striated muscle tissue.

## Conclusion

These results show interspecific DE analyses to be a more sensitive method for identifying candidate genes and/or

gene networks involved in the evolutionary transitions between different life history forms than more common comparative methods such as enrichment analyses. Our DE analysis revealed new candidate genes that may be involved in the evolutionary transition in medusae loss or re-evolution that have not been previously characterized in hydrozoa. Albeit more powerful, our method is reliant on identifiable orthologs. Further analyses of the genes *PDGF* and *Hox1*, which were absent in *H. symbiolongicarpus*, revealed expression consistent with an important role in medusa development in *P. carnea*. Thus, both up- and downregulation of orthologous genes and novel gene gain and loss appear important for life cycle differences between these two species and may play a role in reduction and possible re-evolution of the medusa life cycle stage in the Hydractiniidae. With nearly 100 million years of divergence between these two species (Miglietta and Cunningham 2012), which exhibit the “book-end” phenotypes of gonophore development, the differential regulation of orthologs, and gene duplication and loss, most likely accompanied the transitions between the fully developed and fully truncated medusa.

Addressing questions of parallel incidences of medusa loss, and even re-gain, requires increased taxonomic sampling. Increasing the number of taxa sampled adds a new layer of complexity to ensure the validity of the DE analysis. Dunn, Luo, and Wi (2013) extensively reviewed not only the utility of comparative expression across multiple species but also the numerous challenges it presents. As with any phylogenetic statistical analysis, the nonindependent nature of the data can have large effects on the results (Felsenstein 1985). Future studies sampling more than two species will need to expand current DE software to utilize independent contrasts.

Here, we have provided a new workflow with which one can effectively quantify cross-species differences in expression using short read Illumina data. DE results between these two hydractiniid species reveal 938 candidate orthologs correlated with hydractiniid life cycle variation. These can serve as a useful guide for future studies in spatial gene expression analyses and can potentially be combined with high-throughput functional assays (e.g., Varshney et al. 2015). Moreover, orthology assignments and phylogenetic analyses suggest multiple instances of novel gene loss and gain correlated with phenotypic differences of the gonophore in *P. carnea* and *H. symbiolongicarpus*. In addition, expanding this method to include more taxa and utilizing independent contrasts should provide significant insight into the role of these genes in medusa evolution in Hydrozoa.

## Supplementary Material

Supplementary tables S1–S6 and figures S1–S5 are available at *Genome Biology and Evolution* online (<http://www.gbe.oxfordjournals.org/>).

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