Molecular aspects of eye development and regeneration in the Australian redclaw crayfish, *Cherax quadricarinatus*

Tomer Ventura a,*, Michael J. Stewart a, Jennifer C. Chandler a, Bronwyn Rotgans a, Abigail Elizur a,1, Alex W. Hewitt b, c,1

a GeneCology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Sunshine Coast, Queensland 4556, Australia
b Lions Eye Institute, University of Western Australia, Perth, Western Australia 6330, Australia
c School of Medicine, Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania 7005, Australia

ARTICLE INFO

Keywords:
Ocular development
Regeneration
Compound eye
Crustacean
*Cherax quadricarinatus*
Transcriptome

ABSTRACT

The compound eye evolved over 500 million years ago and enables mosaic vision in most arthropod species. The molecular regulation of the development of the compound eye has been primarily studied in the fruit fly *Drosophila melanogaster*. However, due to the nature of holometabolous insects halting growth after their terminal metamorphosis into the adult form, they lack the capacity to regenerate. Crustaceans, unlike holometabolous insects, continue to grow during adulthood, achieved through regular shedding of their exoskeleton, in a cyclic process known as molting. This therefore offers crustaceans as a highly suitable model to study ocular regeneration in the adult arthropod eye. We have assessed the regenerative capacity of the retinal section of the *Cherax quadricarinatus* (red-claw crayfish) eye, following ablation and successive post-metamorphic molts. This work then provides a transcriptomic description of the outer, pigmented retinal tissue (the ommatidia and lamina ganglionaris) and the basal, non-pigmented neuroendocrine ocular tissue (the X-organ Sinus Gland complex, hemiellipsoid body and optic nerve). Using comparative analysis, we identified all the transcripts in the *C. quadricarinatus* ocular transcriptome that are known to function in compound eye development in *D. melanogaster*. Differentially and uniquely transcribed genes of the retina are described, suggesting proposed mechanisms that may regulate ocular regeneration in decapod Crustacea. This research exemplifies the application *C. quadricarinatus* holds as an optimal model to study the regulation of ocular regeneration. Further in-depth transcriptomic analyses are now required, sampled throughout the regeneration process to better define the regulatory mechanism.

1. Introduction

The compound eye evolved an estimated 520 million years ago, generating a complex tissue that enables mosaic vision in most arthropod species, which account for most animal species on earth (Clarkson, Levi-Setti, & Horvath, 2006). The compound eye consists of several similar anatomical units known as ommatidia, which are encased on their exposed surface by a faceted, transparent, and multi-layered cornea (Meyer-Rochow, 2001). Each ommatidium is composed of a cluster of photoreceptor and accessory cells, which together form the structural unit of the compound eye and the reflective layer of the retina (Cronin & Marshall, 2001; Meyer-Rochow, 2001). Aside from some select insect groups, where an intermediate form is found, the compound eye can be divided into two functional-structural arrangements known as the apposition and superposition eye (Nilsson, 1983). While appositional eyes enable better resolution, the superposition eye has evolved in nocturnal and burrowing species as an adaptation to low light intensity (Meyer-Rochow, 2015). In decapod crustacean species which often comprise planktonic larval stages and benthic, nocturnal post-metamorphic stages, the eye changes from appositional to superposition as an adaptive response to the changes in environment and life style; an example being the spiny lobster, *Jasus edwardsii* (Mishra, Jeffs, & Meyer-Rochow, 2006). To date, much of the research into the development of the compound eye has focused on the apposition eye (Friedrich,
2003), employing the fruit fly Drosophila melanogaster as the model organism, with investigations devoted to studying the underlying molecular pathways that orchestrate its initial development (Silver & Rebay, 2005).

The cell fate of the primordial eye in D. melanogaster is set by the time an individual reaches the second instar larva, through a biochemical cascade which progressively defines the entire head region. Competing transcription factors that are produced at different levels by different cells in the head region, define the boundaries of the eye from the antenna and head cuticle, leading to differential expression in the region that will become the adult eye (Treisman, 2013). Autoregulatory loops in the cells committed to become the retina progressively drive photoreceptors differentiation. The genes responsible for ocular development in D. melanogaster include master control genes which are considered to function as a “transcriptional unit”, with the loss of any one resulting in malformation (Kumar, 2001). The genes include eyeless (ey), twin of eyeless (toy), sine oculis (so), eyes absent (eya), as well as dachshund (dac) and optx which were shown to be sufficient to induce ectopic eye (Kumar, 2001). In addition, there is an array of secondary genes that integrate into the complex regulatory network that coordinates ocular development. This work has highlighted the role of lineage-committed progenitor cells, suggesting a conserved mode of muscle regeneration with the vertebrates (Konstantinides & Averof, 2014). However, while the regulation of regeneration itself has been investigated, the role of cellular reprogramming as part of the regeneration process has received little attention. In the context of ocular regeneration, data is limited to a handful of observational studies from over a century ago (Steele, 1907) and more recently in a case described in the commercially important black tiger prawn Peneaus monodon, in the context of inducing gonad maturation (Desai and Achuthankutty 2000a).

Although there is a paucity of knowledge regarding the biochemical machinery that regulates regeneration in invertebrates, the regenerative capabilities of the eye have been explored in detail in the vertebrates (Goldman, 2014). In particular, the cellular reprogramming that leads to the functional replacement of damaged retinal cells has been studied in several teleost fish (Lindsey & Powers, 2007; Lindsey & Powers, 2007; Sherpa et al., 2008). In these species, the regenerative Müller glial cells, are capable of genetic reprogramming to develop as any of the retinal cells, allowing for the specific migration, proliferation and differentiation of each cell type, ultimately enabling complete restored vision in the damaged eye (Lindsey & Powers, 2007; Sherpa et al., 2008).

The first step, cellular reprogramming, was found to be initiated through the secretion of several factors, some of which appear to be specific to the regeneration process. In contrast, the secondary stages of cellular proliferation, migration and differentiation tend to be regulated through the ubiquitous pathways that are responsible for the regulation of primary eye development (Goldman, 2014). The specific factors involved in cellular reprogramming in Chordata include a heparin-binding EGF-like growth factor (Hbegf) and a tumor necrosis factor-alpha (Tnf-α). Other transcription factors with a conserved function in retinal neurogenesis include the basic helix-loop-helix (bHLH) genes, aka Atonal (ato) in D. melanogaster, Math5 in Mus musculus and Ath5 in Xenopus laevis; although their exact function varies across species (Brown et al., 1998).

We have therefore harnessed the unique regenerative capacity of the crustacean eye, to expand our understanding of ocular regeneration. To do so, we have turned to the aquaculture species, Cherax quadricarinatus (red-claw crayfish). As an aquaculture species, C. quadricarinatus is readily abundant and has previously well-established research pipelines through which to synergistically tackle wider-reaching questions. This offers the species (as well as many other cultured Crustacean species) as a commercially and logistically viable research model. Herein, we report the regenerative capacity of the retinal section of the C. quadricarinatus eye, following eye-ablation through to full regeneration, through a series of successive post-metamorphic molts. In an attempt to elucidate the molecular mechanism coordinating this regeneration, we then report a transcriptomic analysis of the C. quadricarinatus ocular tissue, comparing the differentially and uniquely transcribed genes defining the outer (pigmented) retinal section (comprising the ommatidia and lamina ganglionaris) (Meyer-Rochow, 2015) and the basal (non-pigmented) ocular tissue (comprising XO-SG, the hemiellipsoid body, optic nerve and 4 neural cell clusters) (Skinner & Graham, 1970). Finally, we consider the mechanism through which ocular regeneration is regulated in this crustacean.

2. Materials and methods

2.1. Animals

C. quadricarinatus juveniles (weighting 0.86–2.59 g) were purchased locally and placed in separate fenestrated containers (16 cm × 8 cm × 7.5 cm), floated in a 300 L aquarium with constant
2.2. Histological analysis

Post the regeneration period, untreated and regenerated eyes were dissected as previously described. Eyes were rinsed in PBS and fixed in Bouin’s solution (Sigma-Aldrich) for 16–24 h, then transferred to 70% Ethanol and stored at 4 ºC, until dehydrated and processed for sectioning and hematoxylin and eosin staining, as described in Ventura, Aflalo, Weil, Kashkush, and Sagi (2011).

2.3. Sample preparation and sequencing

Male and female eyestalks were separately pooled, then placed in RNA-later (Ambion) for 24 h at room temperature, followed by storage at −20 ºC. From each pool, at least 20 eyes were taken for dissecting the retina (pigmented region) from the ganglionic region (non-pigmented region), generating a total of four samples (male and female; pigmented and non-pigmented regions) for sequencing. Dissection was performed based on visual observation of the pigmented region. Total RNA was isolated with the RNAzol RT Reagent (MRC), according to the manufacturer’s instructions. Samples were sequenced by BGI (HongKong Co. Ltd) as per manufacturer’s protocol (Illumina, San Diego, CA). Briefly, poly (A) mRNA was isolated using oligo (dT) beads and the addition of fragmentation buffer for shearing mRNA into short fragments (200–700 nt). This was followed by cDNA synthesis using random hexamer-primers in order to prevent priming bias. The short cDNA fragments were further purified using QiaQuick PCR extraction kit and resolved with EB buffer for ligation with Illumina paired-end adapters. This was followed by size selection (~200 bp), PCR amplification and Illumina sequencing using an Illumina Genome Analyzer (HighSeq 2000, Illumina, San Diego, CA), performing 90 bp–paired end sequencing. The sequence reads were stored as FASTQ files, generating at least 4 GB of cleaned data (at least 45 million reads for each of the four samples).

2.4. Bioinformatics analyses

Cleaning of low quality reads, followed by assembly and mapping were conducted using the CLC Genomics Workbench (CLC Bio, version 7.0.3) under default parameters (with the exception of similarity fraction elevated to 0.9 in the mapping stage). From each library, 69.3%–73.5% of the reads mapped to the complete de novo assembled C. quadricarinatus ocular transcriptome. BAM files were then uploaded onto Partek Genomics Suite (Partek GS) to quantify expression, defined as reads per kilobase of the transcript, per million reads of the total library (RPKM). The RPKM values were filtered to include only transcripts where RPKM ≥1 in at least one sample. This subset of data was normalized to include a minimum of 0.05 instead of 0 RPKM and the normalized RPKMs were subjected to ANOVA, performed in Partek GS, to compare between the pigmented and non-pigmented samples. The threshold for statistical significance was set to P < 0.005 and a fold-change of at least two was considered as significant.

To assess the level of conservancy with other arthropod eye development, we utilized the curated list of proteins annotated as functioning in D. melanogaster compound eye morphogenesis (Gene Ontology, GO identifier 0001745). To identify protein homologues in our C. quadricarinatus ocular transcriptome, we conducted a BLAST search of this transcriptome (as predicted by ORF-Predictor http://proteomics.ysu.edu/tools/OrfPredictor.html), using CLC (v 7.5.1). Where homologous transcripts were identified, the amino acid sequences were aligned using the LaTeX TextShade package (Beitz, 2000) and ORF and PFAM domain schematics were constructed using simple modular architecture research tool (SMART) (Schultz, Milpetz, Bork, & Ponting, 1998). All reference sequences for phylogenetic analyses were retrieved from GenBank and trees constructed using MEGA (v 7.0) maximum likelihood. Confidence levels for the groups defined in the topology were assessed by bootstrap and interior branch tests (with 1000 replicates).

3. Results and discussion

3.1. Eye regeneration assessment

Ten of the 22 individuals (45%; from which one eye was removed at the base, including both pigmented and non-pigmented tissue and the other eye, which was dissected to remove the pigmented retina only), survived for at least twelve weeks and completed three successive molts. Of these ten individuals, only those eyes where the retina alone was removed, were capable of partial regeneration in four individuals (Fig. 1C, left eye and Fig. 1D). None of the eyes that were removed at the base showed any evidence of regeneration over the three molts (Fig. 1C, right eye). Interestingly, these observations differ slightly from that described in the penaeid prawn, P. monodon, where the authors describe the complete regeneration of fully ablated eyes, when both regions (pigmented and non-pigmented) were removed (Desai and Achuthanukthy 2000b). However, as this observation was not a controlled experiment and rather a by-product of commercial eyestalk ablation, it may be that the entirety of the non-pigmented ocular tissue was not removed with complete accuracy.

Regeneration in C. quadricarinatus occurred as follows: by the first molt, a melanised scab developed around the removed retina, as has previously been documented in both ocular (Steele, 1907) and limb (Das & Durica, 2013; Hopkins, 2001; Konstantinides & Averof, 2014) regeneration in several crustacean species, suggesting a shared physiological regeneration response. By the second molt, the eye either failed to regenerate (n = 4; 40%), partially regenerated (n = 4; 40%; Figs. 1 and 2), or regenerated in the form of an antenna (n = 2; 20%; Fig. 2B). In those individuals that showed partial ocular regeneration at the second molt, by the third molt a seemingly completely regenerated eye was present. In these cases, although complete regeneration had occurred, comparative to untreated eyes (Fig. 1A and B) the regenerated eyes were smaller (Fig. 1C), with irregularities in the cornea surface (Fig. 1D). These developmental abnormalities appear to be a common feature of ocular regeneration, as has been described in the zebrafish, where ~50% of regenerated eyes showed defects and abnormalities and in some cases, lacked the ability to determine when regeneration was complete, resulting in over-proliferation (Sherpa et al., 2008). This suggests that even in those species that can achieve full ocular regeneration, the regenerative process does not achieve the optimal conditions of initial development.

One well-documented case of defective regeneration, is antennal regeneration in replacement of a damaged eye in D. melanogaster larvae. This was initially hypothesized to be due to the hyper-activation of the antennal-producing EGRF pathway, relative to the suppression of the ocular-inducing Notch pathway, stimulating a biochemical transition in development (Kumar, 2001; Kumar & Moses, 2001). It has been suggested that the specific effects of each pathway are, to an extent, shaped by the genetic background in which they function (Kumar, 2001; Kumar & Moses, 2001). Although this model of Notch-EGRF eye-antenna specification has not been gaining support, considering the examples of antennal regeneration observed here (with complete development of all antennal substructures; Fig. 2B), the “background” expression profile of an adult regenerating tissue, would probably differ quite dramatically from that of primary differentiating embryonic tissue and may well...
explain the emergence of developmental irregularities like that observed here, as well as that described in the zebrafish (Sherpa et al., 2008). A more likely mechanism for double-antenna discs generation in D. melanogaster showed that down-regulation of the Notch pathway results in a duplication of the antenna, rather than a switch in fate, as a consequence of a severe reduction in proliferation (Kenyon, Ranade, Curtiss, Mlodzik, & Pignoni, 2003). With that, it is noteworthy that crustaceans show continual growth and in this study, we witnessed a fully differentiated eye which regenerated as an antenna, rather than the case in the holometabolous insect D. melanogaster where the disc which is
in the process of differentiation has re-differentiated. More recently, a study by Wang and Sun (2012) has shown specific antagonistic pathways which define the eye and the antenna regions during the mid-second instar larval stage. A follow-up study examining the change in expression of these specific antagonistic factors in re-differentiating eyes to antennae in crustaceans could better explain the mechanism which enables a fully differentiated eye to regenerate as an antenna.

In the regenerated eyes, regeneration appeared to originate from the periphery of the retinal tissue. This is suggestive of two putative regenerative origins: either that these regions contain remaining retinal cells as the source of regeneration, or the adjacent non-pigmented tissue contains the necessary progenitor cell clusters. The first case would suggest a regenerative mechanism in keeping with vertebrate studies, where the regeneration of all dioptric layers of the retina develop from the re-differentiation and proliferation of remaining retinal cells (Hitchcock, Lindsey Myhr, Easter, Mangione-Smith, & Jones, 1992). The second case suggests a mechanism similar to limb regeneration studies described in the amphipod crustacean P. hawaiensis, where regeneration is achieved through lineage-committed progenitor cells, local to the amputated region (Konstantinides & Averof, 2014). Considering the lack of regeneration with complete ocular ablation, it may be that the adjacent non-pigmented ocular region of the eye houses these locally sourced progenitor cells (or alternatively, cells which produce the molecular signal/s required for retinal cell reprogramming or retinal stem cell differentiation), which when removed, result in the lost regenerative capacity observed here (Fig. 2C). We therefore sought to characterize the transcriptomic patterns that distinguish the pigmented and non-pigmented regions of the eye, in order to identify any key factors whose differential expression might implicate them in regulating the eye regeneration.

3.2. Transcriptome characteristics

Eye tissue from male (n = 23) and female (n = 27) C. quadricarinatus were separated into pigmented and non-pigmented regions and sequenced as such, generating over 180 million reads, which were de novo assembled to generate a total of 89,626 transcripts (mean length = 706, N50 = 771). A total of 56,920 transcripts had an RPKM (reads per kilobase per million reads) of ≥1 in at least one sample.

3.3. Conservation with Drosophila eye development

Of 469 proteins annotated to be involved in compound eye development in Drosophila, 89% (418 proteins) were identified with an e-value ≤ 10−3 in the C. quadricarinatus ocular transcriptome (Table S1). The majority (50 out of 54) of proteins characterised as having eye-development associated expression profiles in Drosophila (selected from the interactive fly, http://www.sdbonline.org/sites/fly/aimorph/eye.htm), were also present in the C. quadricarinatus transcriptome; the four exceptions being roughex, outstretched, Chl and Phyllopod. Of the 50 C. quadricarinatus homologues, most transcripts (when blasted at NCBI) shared highest similarity with proteins from the branchiopod crustacean, Daphnia pulex (29/50 factors) and the arthropod beetle, Tribolium castaneum (15/50 factors); Table 1. These annotations reflect the availability of annotated genomes for D. pulex, T. castaneum and D. melanogaster and the phylogenetic distance each share with C. quadricarinatus. In summary, it is apparent that the vast majority of functional regulators characterised in eye morphogenesis in D. melanogaster, are present in the C. quadricarinatus ocular transcriptome.

Although the genetic basis of eye development is most extensively characterised in D. melanogaster, studies in other invertebrates have begun to extend this knowledge (Lapan & Reddien, 2012; Tessmar-Raible & Arendt, 2003). It appears that while some genes such as D. melanogaster’s eyeless (ey) and twin of eyeless (toy), aka pax6 in vertebrates, are highly conserved in eye development across the nemertines, cephalopods, Dugesia, and Platynereis (although interestingly not in the planarian, Schmidtea mediterranea (Lapan & Reddien, 2012)) many of the other genes are not so conserved (Tessmar-Raible & Arendt, 2003). This highlights the importance of species-specific characterisation. Planarians, as members of the Lophotrochozoa, the sister group to the Ecdysozoa (comprising all Arthropoda), are significant to understanding the molecular basis of eye evolution; furthermore, they are able to show full ocular regeneration capacity through adulthood (Lapan & Reddien, 2012). RNA sequencing studies in the planarian, S. mediterranea, highlighted that while the (otherwise) highly conserved toy/pax6 gene family does not play a role in eye development, ocular development is instead regulated by a zinc finger transcription factor termed Ovo (Lapan & Reddien, 2012). An ovo-like homologue is present in D. melanogaster (termed shavenbaby) but has only been studied in the context of epidermal morphogenesis, not eye development. Interestingly, the coordination of regeneration in S. mediterranea employs the same suite of genes (ovo, Six-1/2 and eye) involved in initial embryonic development (Lapan & Reddien, 2012). This suggests that regeneration occurs through re-establishing the genetic environment of embryogenesis.

3.4. Eye regeneration pathways

Other than that described in the planarian, the majority of studies regarding ocular regeneration have been conducted in the vertebrates. In vertebrates, eye regeneration is initiated and mediated by Müller glial cells. Although coordinated through different genes, regeneration to full visual capacity is achieved through the reactivation of the embryonic “developmental niche” (Sherpa et al., 2008), similar to that described in S. mediterranea (Lapan & Reddien, 2012). The genes responsible in the vertebrates are hbegf and tnf-α, which have a specific function in the cellular reprogramming and trans-differentiation of the Müller glia cells (Goldman, 2014), rendering them pluripotent and thus recapitulating the developmental conditions of embryonic development (Sherpa et al., 2008). Although we were able to identify most components of the key Wnt and Notch regulatory pathways in the C. quadricarinatus ocular transcriptome, the master regulatory factors, tfα and hbegf, were not identified. Indeed, homologues of these genes could not be found in any Achordate when screened at NCBI, with the exception of a tumor necrosis factor (TNF) found in the shrimp species Litopenaeus vannamei. However, as this TNF is not a Tnf-α, it is unlikely to be functionally associated with retinal-cell reprogramming (Wang et al., 2012). This suggests that the same genetic coordination of cellular reprogramming as described in the vertebrates, is not conserved in Crustacea.

3.5. Pigmented and non-pigmented regions differential expression

Thus, in order to identify, as yet, uncharacterized factors with putative involvement in ocular regeneration in Crustacea, we conducted a transcriptomic comparison of the expression profiles of the pigmented (retinal) and non-pigmented (neuroendocrine) regions of the eye. Those transcripts with an RPKM ≥1 in at least one of the four samples were subject to ANOVA. A total of 1325 transcripts had ≥2 fold-change in expression between the two tissue types (P 0.005), of which 162 (12.2%) were up-regulated in the pigmented (retinal) region (Fig. 3A). Among the 50 key factors associated with compound eye development (Table 1), 29 were up-regulated in the non-pigmented (neuroendocrine) tissue and only 3 were up-regulated in the pigmented (retinal) tissue (boxed in Table 1).

This expression pattern may reflect the diverse functional role of the non-pigmented region of the eye, which comprises musculature and connective tissues but more significantly, the major neuroendocrine XO-SG complex (Skinner, 1985). Furthermore, considering that this region must remain intact for retinal regeneration to occur, the non-retinal tissue may be that responsible for expressing the key genes involved in stimulating regeneration. This may be achieved as previously suggested, with this region housing the localised progenitor cells that serve as the source of regeneration (as described in P. hawaiensis (Konstantinides &
Table 1

| Gene Name                  | Red Claw Contig | Reference Species (Accession) | Coverage   | E-value | F Pigmented | M Pigmented | F Non-pigmented | M Non-pigmented |
|----------------------------|-----------------|------------------------------|------------|---------|-------------|-------------|-----------------|-----------------|
| Scabrous                  | contig12586     | Daphnia pulex (EFX84903.1)   | 72.30%     | 2.00E-38| 0.2 0.5     | 1.1 1.3     |                  |                 |
| Sevenless                 | contig16208     | Daphnia pulex (EFX62423.1)   | 9.80%      | 4.00E-11| -1.5 -0.4    | 0.2 0.2     |                  |                 |
| frizzled                  | contig19669     | Daphnia pulex (EFX86266.1)   | 100.00%    | 2.00E-145| 4.6 6.2     | 0.3 0.4     |                  |                 |
| Retinoblastoma-family protein, RBF | contig20405 | Daphnia pulex (EFX71662.1)   | 91.90%     | 0.00E-00| 0.4 0.5     | 0.2 0.2     |                  |                 |
| Calmodulin                | contig22088     | Tribolium castaneum (XP_965708.1) | 100.00%    | 3.00E-70| 1.3 1.1     | 0.9 0.8     |                  |                 |
| atonal                    | contig22098     | Daphnia pulex (EFX77712.1)   | 14.90%     | 2.00E-10| 0.7 0.6     | 1.0 0.9     |                  |                 |
| Karst (βHeavy Spectrin)   | contig23736     | Daphnia pulex (EFX84686.1)   | 31.00%     | 0.00E-00| 0.9 -0.3    | 0.2 0.1     |                  |                 |
| gi-X2 (AKA Tubulin/tuberosous sclerosis) | contig27978 | Tribolium castaneum (XP_968197389.1) | 49.80%     | 3.00E-101| -0.9 0.0     | 0.4 0.4     |                  |                 |
| Seven in absentia (sina)  | contig28695     | Bombyx mori (XP_004921575.1) | 100.00%    | 2.00E-166| 0.1 0.1     | 0.5 0.4     |                  |                 |
| spitz                     | contig30170     | Daphnia pulex (EFX61975.1)   | 67.20%     | 2.00E-17| -0.6 -0.6    | -0.6 -0.4   |                  |                 |
| argosse                   | contig30538     | Tribolium castaneum (EFA11599.1) | 44.60%     | 9.00E-51| -1.3 -0.4    | -0.2 -0.7   |                  |                 |
| glass                     | contig3091      | Tribolium castaneum (AAP46162.1) | 41.10%     | 4.00E-85| 1.2 1.3     | -0.1 0.1    |                  |                 |
| Hedgehog                  | contig43073     | Daphnia pulex (EFX56363.1)   | 71.90%     | 6.00E-14| -1.5 -0.7    | 0.2 0.0     |                  |                 |
| strabismus                | contig45526     | Daphnia pulex (EFX57935.1)   | 78.60%     | 0.00E-00| -0.8 -0.7    | -0.1 0.1    |                  |                 |
| Protein tyrosine-phosphatase 69D | contig48432 | Daphnia pulex (EFX66834.1)   | 17.30%     | 1.00E-56| -1.5 -0.9    | -0.3 -0.5   |                  |                 |
| Seven-up                  | contig48455     | Daphnia pulex (EFX88423.1)   | 28.80%     | 3.00E-49| -1.3 -1.2    | 0.0 -0.1    |                  |                 |
| Pannier                   | contig4881      | Tribolium castaneum (EFA04531.1) | 83.50%     | 3.00E-55| 0.7 0.7     | 0.1 0.1     |                  |                 |
| cyclin A                  | contig51470     | Daphnia pulex (EFX71416.1)   | 53.50%     | 3.00E-95| 0.9 -0.8    | -0.1 -0.5   |                  |                 |
| Trait                | Contig   | Species               | Identity  | Score   | E-value | Log-odds | ORF1 | ORF2 | ORF3 | ORF4 | ORF5 |
|----------------------|----------|-----------------------|-----------|---------|---------|----------|------|------|------|------|------|
| Small wing           | contig5017| *Tribolium castaneum* (EPA123319.1) | 27.10%    | 3.00E-95 | -4.6    | -4.3     | 0.1  | 0.1  | 0.1  | 0.1  | 0.1  |
| Eyes absent, cliff   | contig55986| *Daphnia pulex* (EEX89861.1) | 58.10%    | 8.00E-86 | 0.0     | 0.0      | 0.0  | 0.0  | 0.0  | 0.0  | 0.0  |
| Orthodonticle        | contig56037| *Tribolium castaneum* (NP_001034513.1) | 40.20%    | 3.00E-28 | -0.1    | -0.1     | 0.1  | 0.1  | 0.1  | 0.1  | 0.1  |
| Yan                  | contig5619| *Aedes aegypti* (XP_001654606.1) | 74.90%    | 6.00E-58 | 0.1     | 0.1      | 1.1  | 1.1  | 1.2  | 1.2  | 1.2  |
| Sine oculis/absent   | contig58413| *Tribolium castaneum* (EPA404748.1) | 93.70%    | 3.00E-129 | 0.0    | 0.0      | 0.2  | 0.2  | 0.3  | 0.3  | 0.3  |
| Minded/medusa        | contig60649| *Bombus terrestris* (XP_003195437.1) | 28.50%    | 0.00E-00 | 0.6     | 0.7      | 0.4  | 0.4  | 0.3  | 0.3  | 0.3  |
| Ultraspiracle        | contig62341| *Daphnia pulex* (EFX64341.1) | 56.50%    | 2.00E-77 | 0.1     | 0.1      | 0.2  | 0.2  | 0.2  | 0.2  | 0.2  |
| Hopscotch            | contig64933| *Daphnia pulex* (EFX87846.1) | 10.40%    | 3.00E-33 | -0.1    | -0.1     | -0.3 | -0.3 | -0.6 | -0.6 | -0.6 |
| Star                 | contig64988| *Daphnia pulex* (EFX80626.1) | 98.80%    | 8.00E-56 | 0.5     | 0.5      | -0.1 | -0.1 | -0.1 | -0.1 | -0.1 |
| Corkscrew            | contig6543| *Tribolium castaneum* (AXP_003002233.1) | 45.00%    | 9.00E-103 | -0.5    | -0.5     | -0.5 | -0.5 | -0.2 | -0.2 | -0.2 |
| Mirror               | contig67606| *Tribolium castaneum* (EPA01337.1) | 50.30%    | 5.00E-51 | -1.1    | -1.0     | 0.2  | 0.2  | 0.2  | 0.2  | 0.2  |
| Rolled               | contig6862| *Penaeus monodon* (ADT80936.1) | 100.00%   | 0.00E-00 | 0.2     | 0.2      | 0.3  | 0.3  | 0.2  | 0.2  | 0.2  |
| Transtrack           | contig69264| *Tribolium castaneum* (NP_001157610.1) | 64.90%    | 7.00E-44 | -1.3    | -0.9     | -0.7 | -0.7 | -0.2 | -0.2 | -0.2 |
| Leonardo             | contig69456| *Daphnia pulex* (AHJ25961.1) | 57.90%    | 5.00E-66 | -0.7    | -0.9     | -0.1 | -0.1 | -0.6 | -0.6 | -0.6 |
| Fringe               | contig70325| *Daphnia pulex* (EEX89391.1) | 100.00%   | 3.00E-106 | -4.8    | -4.2     | 0.4  | 0.4  | 0.5  | 0.5  | 0.5  |
| Eyeless              | contig70485| *Daphnia pulex* (EFX75784.1) | 82.50%    | 1.00E-160 | -1.3    | -1.3     | -0.2 | -0.2 | 0.0  | 0.0  | 0.0  |
| Eyeless              | contig70485| *Daphnia pulex* (EFX75784.1) | 82.20%    | 5.00E-154 | -1.3    | -1.3     | -0.2 | -0.2 | 0.0  | 0.0  | 0.0  |
| Sparkling            | contig70485| *Tribolium castaneum* (EPA01154.1) | 91.30%    | 6.00E-60 | -1.3    | -1.3     | -0.2 | -0.2 | 0.0  | 0.0  | 0.0  |
| Wingless             | contig70941| *Daphnia pulex* (EFX86386.1) | 55.90%    | 8.00E-54 | -1.3    | -1.4     | -0.5 | -0.5 | -0.1 | -0.1 | -0.1 |
Averof, 2014), as well as the XO-SG, responsible for the secretion of a suite of neuropeptides which govern a wide array of functions in crustaceans (Nguyen, Cummins, Elizur, & Ventura, 2016), including the MIH and thus the more systemic regulation of molting and consequential ability to regenerate (Das & Durica, 2013).

In contrast, if it is that regeneration originates from remaining retinal cells, still present after retinal amputation (in a mechanism similar to the vertebrate Müller glial cells), the factors responsible for the cellular reprogramming would be present and up-regulated in the pigmented region. Of the 162 transcripts identified as up-regulated in the pigmented region, only 28 had a predicted open reading frame (ORF) of 100 amino acids (aa) or more. This suggests either that most transcripts are truncated, or perhaps indicates the presence of long non-coding RNAs, known to be key players in cellular regulation (Mercer, Dinger, & Mattick, 2009) (Supplementary file S1). Of those 28 transcripts with a predicted ORF, only nine (32.1%) had identifiable PFAM domains (Fig. 3B table inset).

Of these nine transcripts, one (contig 7074), was identified as sharing homology with the SOX transcription factor family, specifically Sox14 (e-value = 0.0, 381/428, 89% identical amino acids, without gaps, from Scylla paramamosain) and SoxC (e-value = 0.0, 339/433, 78% identical amino acids, without gaps, from Macrobrachium nipponense). The C. quadricarinatus homologue possesses the High Mobility Group box (HMGB) protein domain (residue positions 48–116 aa), which defines the sox gene family and is essential for DNA binding (Stros, Launholt, & Grasser, 2007) (Fig. 3C). Our phylogenetic analyses of the putative C. quadricarinatus Sox14, with a range of other annotated sox factors, indicate clear clustering of the transcript within the invertebrate clade, sharing closest homology with other crustacean species.

In vertebrates there are estimated to be over 20 sox genes, involved in a myriad of developmental processes (Stros et al., 2007). Many SOX-domain containing genes have also been reported in the invertebrates, with some known to have important developmental functions in invertebrate model systems (Phochanukul & Russell, 2010). In the context of retinal development and regeneration, a SoxB transcription factor has been shown to have an eye-determining role in the planarian, S. mediterranea (Lapan & Reddien, 2012). Indeed, the authors suggests that the SoxB family encodes a group of genes with an ancestral role in eye biology. SoxB genes in Drosophila, specifically sox-neuro and fish-hook, are expressed in the eye disc (Mukherjee, Shan, Mutsuddi, Ma, & Nambu, 2000). Whilst in the vertebrates, sox2 has been shown to be involved in the maintenance of neural progenitor cells and differentiation of retinal ganglion cells (Matsushima, Heavner, & Pevny, 2011), while sox15 is involved in the regeneration of skeletal muscle tissue (Lee et al., 2004). Given the lack of functional assessment, we suggest this up-regulated retinal transcription factor, as a C. quadricarinatus sox14 homologue, which warrants further functional investigation in the context of the regenerative capacity of the crustacean eye.

### 4. Conclusions

This work is, to our knowledge, the first to provide a transcriptomic description of the ocular tissue in a crustacean species, highlighting notable conservation with the genes functioning in ocular development in Drosophila. Interestingly, re-differentiation from eye to antennae observed in this study is also in keeping with other arthropod mechanisms, indicating a conserved evolution within Arthropoda. We then consider the regenerative capacity of the Crustacea in the context of ocular regeneration, describing the phenotypic progression of retinal

---

| STAT       | contig | Species                        | P-value  | FDR   | log2 fold change | Valid |
|------------|--------|--------------------------------|----------|-------|-----------------|-------|
| STAT       | contig7151 | Tribolium castanuwy (EFA04581.1) | 59.60%   | 0.00E-00 | -0.2             | 0.3   | 0.7   | 0.7 |
| rough      | contig71985 | Daphnia pulex (EFX86406.1)       | 37.00%   | 8.00E-19 | -1.3             | -1.4  | -0.5  | 0.0 |
| Homoborax  | contig76655 | Daphnia pulex (EFX75946.1)       | 30.70%   | 2.00E-06 | -1.3             | -1.2  | -0.3  | 0.0 |
| Sprouty    | contig82283 | Aedes aegypti (XP_001640010.1)   | 41.60%   | 1.00E-08 | -1.3             | -1.2  | -0.1  | 0.1 |
| Eyelid (os) | contig834 | Daphnia pulex (EFX70974.1)       | 53.80%   | 6.00E-15 | 0.8              | 1.1   | 1.0   | 1.1 |
| Furrowed   | contig83915 | Daphnia pulex (EFX85039.1)       | 12.00%   | 7.00E-53 | -1.1             | -1.1  | -0.8  | -0.5 |
| EGF-Receptor | contig85149 | Daphnia pulex (EFX470758.1)     | 30.00%   | 2.00E-117| -1.3            | -1.2  | -0.3  | -0.3 |
| Tyrosine   | contig86020 | Daphnia pulex (EFX66834.1)       | 19.30%   | 3.00E-57 | -1.3             | -1.2  | -0.1  | -0.4 |
| lozenge    | contig86915 | Daphnia pulex (EFX86301.1)       | 37.30%   | 4.00E-54 | -2.3             | -2.2  | -0.4  | 0.1 |
| Ras oncogene at 85D | contig8856 | Daphnia pulex (EFX62142.1)       | 78.40%   | 2.00E-75 | -0.9             | -0.9  | 0.2   | 0.2 |
| twins      | contig9128 | Daphnia pulex (EFX77026.1)       | 100.00%  | 0.00E-00 | 0.6              | 0.7   | 0.9   | 0.9 |
| BOSS (Bridle of sevenless) | contig9646 | Tribolium castanuwy (EFA12175.1) | 45.00%   | 1.00E-42 | 0.3              | 0.5   | -0.8  | -0.7 |
| Jun        | contig9894 | Tribolium castanuwy (NP_001164127.1) | 100.00%  | 3.00E-47 | 0.8              | 0.8   | 1.1   | 1.0 |

roughhex not found
outrstretched not found
Cbl not found
Phyllopod not found
regeneration and evaluating the putative modes through which this may be regulated. These modes being (1) a regenerative mechanism similar to that described for limb regeneration, initiated through local progenitor cells, putatively present in the adjacent non-pigmented ocular tissue or (2) through the re-differentiation of the remaining retinal cells themselves, achieved by reconfiguring the molecular environment present during primary development (referred to as the “developmental niche”), similar to the mechanism that has been described in teleost vertebrates, with support from the Planaria. We have identified a putative Sox14 transcription factor in *C. quadricarinatus*, which warrants further functional investigation in this context. These investigations could validate the overall mode through which regeneration is mediated, confirming if it is indeed through the re-differentiation of the retinal cells; RNAi mediated knock-down of sox14 in a (retina) ablated eye and subsequent comparison of regenerative success would prove a powerful tool in tackling this question.

In addition, this work provides a ready example of the significance of non-model species in gaining a more representative understanding of key developmental processes, which are often limited to a handful of model species. We advocate for the application of aquaculture species, such as *C. quadricarinatus*, as optimal models to study these processes, such as that described here. The growing availability and relative affordability of transcriptomic analyses offer the opportunity for aquaculture species research to achieve further reach and impact than that directly relating to aquaculture alone, an opportunity not to be missed.

Acknowledgments

This work was supported by funding from the BrightFocus Foundation, and a Ramaciotti Establishment Grant to AWH.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aaf.2018.04.001.

References

Beitz, E. (2000). TEXshade: Shading and labeling of multiple sequence alignments using LATEX2 epsilon. Bioinformatics (Oxford, England), 16, 135–139.
Brown, N. L., Kanekar, S., Vetter, M. L., Tucker, P. K., Gemza, B. L., & Glaser, T. (1998). Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Development, 125, 4821–4833.
Chang, E. S., & Mykles, D. L. (2011). Regulation of crustacean molting: A review and our perspectives. General and Comparative Endocrinology, 172, 323–330.
Clarkson, E., Levi-Setti, R., & Horvath, G. (2006). The eyes of trilobites: The oldest preserved visual system. Arthropod Structure & Development, 35, 247–259.
Cronin, T. W., & Marshall, J. (2001). Parallel processing and image analysis in the eyes of mantis shrimps. The Biological bulletin, 200, 177–183.
Das, S., & Durica, D. S. (2013). Ecdysteroid receptor signaling disruption obstructs blastemal cell proliferation during limb regeneration in the fiddler crab, Uca pugilator. *Molecular and Cellular Endocrinology, 365*, 249–259.

Desai, U. M., & Achuthankutty, C. T. (2000). Complete regeneration of ablated eyestalk in penaeid prawn, Penaeus monodon. *Research Communications, 79*.

Desai, U. M., & Achuthankutty, C. T. (2000). Complete regeneration of ablated eyestalk in penaeid prawn, Penaeus monodon. *Current Science, 79*, 1602–1603.

Friedrich, M. (2003). Evolution of insect eye development: First insights from fruit fly, grasshopper and flour beetle. *Integrative and Comparative Biology, 43*, 508–521.

Goldman, D. (2014). Muller glial cell reprogramming and retina regeneration. *Nature Reviews Neuroscience, 15*, 431–442.

Hitchcock, P. F., Lindsey Myhr, K. J., Easter, S. S., Jr., Mangione-Smith, R., & Jones, D. D. (2002). Local regeneration in the retina of the goldfish. *Journal of Neurobiology, 23*, 187–203.

Hopkins, P. M. (2001). Limb regeneration in the Fiddler crab, Uca pugilator: Hormonal and growth factor control. *American Zoologist, 41*, 389–398.

Kenyon, K. L., Ranade, S. S., Curtiss, J., Mlodzik, M., & Pignoni, F. (2003). Coordinating proliferation and tissue specification to promote regional identity in the Drosophila head. *Developmental Cell, 5*, 403–414.

Konstantinides, N., & Averof, M. (2014). A common cellular basis for muscle regeneration in arthropods and vertebrates. *Science, 343*, 798–791.

Kumar, J. P. (2001). Signalling pathways in Drosophila and vertebrate retinal development. *Nature Reviews Genetics, 2*, 846–857.

Kumar, J. P., & Moses, K. (2001). EGF Receptor and Notch signaling act upstream of Eyeless/Pax6 to control eye specification. *Cell, 104*, 687–697.

Lapan, S. W., & Reddien, P. W. (2012). Transcriptome analysis of the planarian eye identifies ovo as a specific regulator of eye regeneration. *Cell Reports, 2*, 294–307.

Lee, H. J., Goring, W., Ochs, M., Muhlfeld, C., Steding, G., Paprotta, I., et al. (2004). Sox15 gene, 217, 91–106.

Mukherjee, A., Shan, X., Mattussi, M., Ma, Y., & Nambu, J. R. (2000). The Drosophila sox factor receptor in the prawn Macrobrachium rosenbergii: Function and putative signaling cascade. *Endocrinology, 154*, 3188–3196.

Sharabi, O., Ventura, T., Manos, R., Affalo, E. D., & Sagit, A. (2013). Epidermal growth factor receptor in the prawn Macrobrachium rosenbergii: Invertebrate Sox functions. *Nature Reviews Genetics, 10*, 155.

Steele, M. I. (1907). Regeneration in compound eyes of Crustacea: Integument, pigments, and hormonal processes. *Academic Press.*

Tessmar-Raible, K., & Arendt, D. (2003). Emerging systems: Between vertebrates and invertebrates. *Science, 302*, 8436.

Ventura, T., Affalo, E. D., Weil, S., Kashkush, K., & Sagit, A. (2011). Isolation and characterization of a female-specific DNA marker in the giant freshwater prawn Macrobrachium rosenbergii. *Heredity, 107*, 456–461.

Wang, C.-W., & Sun, Y. H. (2012). Segregation of eye and antenna fates maintained by Eyeless/Pax6 to control eye speciation. *Developmental Cell, 5*, 1175–1197.

Wang, P. H., Wan, D. H., Pang, L. R., Gu, Z. H., Qiu, W., Weng, S. P., et al. (2012). Molecular cloning, characterization and expression analysis of the tumor necrosis factor (TNF) superfamily gene, TNF receptor superfamily gene and lipopolysaccharide-induced TNF-alpha factor (LITAF) gene from Litopenaeus vannamei. *Development & Comparative Immunology, 36*, 39–50.