Regressive Evolution of Vision and Speciation in the Subterranean Diving Beetles from Western Australia

Barbara Langille

A thesis submitted for the Degree of Doctor of Philosophy
Department of Ecology and Evolutionary Biology
School of Biological Sciences, Faculty of Sciences
The University of Adelaide

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Abstract

Subterranean animals are highly specialized for life underground, having converged on regressive traits such as on loss of eyes/vision and pigmentation. Despite centuries of study, understanding the evolutionary processes and genetic basis for regressive characters is still the subject of considerable debate, with two main evolutionary drivers at the forefront: natural selection and neutral evolution. An assemblage of independently-evolved beetle species (Dytiscidae), from a subterranean archipelago in Western Australia, converged on eye/vision loss, providing a powerful system to explore the genetic basis of adaptive and regressive evolution in parallel. I conducted a behavioural light-dark study of six subterranean beetle species in the genera *Paroster* and *Limbodessus*, and revealed evidence for one light avoiding species. This study suggested that highly troglomorphic beetles may have evolved from an ancestor that exhibited negative phototaxis as a pre-adaptation to living in permanent darkness. To investigate whether genes specifically involved in vision showed patterns of neutral evolution, I carried out exon capture analyses on a suite of phototransduction genes, from a total of 36 beetle species (32 stygobionts and 4 surface beetles). I found evidence for pseudogenisation of six genes in multiple species, supporting the neutral theory. Finally, an 18 base pair deletion and a shared stop codon were found in the long wavelength opsin gene of a phylogenetic sister triplet of beetle species from one calcrete. I sequenced long wavelength opsin in other *Paroster* species and mapped the mutations to a robust multi-gene phylogeny, to show that the mutation was unique to these three sister species. These analyses provide strong evidence that the three species evolved underground from a common

ancestor that was already adapted to living underground. My studies add to the growing body of evidence supporting the neutral theory as the mode of eye regression and the potential for speciation underground, and further highlight that subterranean dytiscids provide a unique model system for exploring fundamental questions on the evolution of subterranean animals.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in text. In addition, I certify that no part of this work will, in future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Full citations of publications that appear in this thesis:

Langille BL, Tierney SM, Austin AD, Cooper SJB (2019) How blind are they? Phototactic responses in stygobiont diving beetles (Coleoptera: Dytiscidae) from calcrete aquifers of Western Australia. *Austral Entomology*. doi: 10.1111/aen.12330

Tierney, SM, Langille BL, Humphreys WF, Austin AD, Cooper SJB (2018) Massive parallel regression: A précis of genetic mechanisms for vision loss in diving beetles. *Integrative and Comparative Biology*, **58**, 465-479. doi: 10.1093/icb/icy035

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Signed: Barbara Langille Date: 16/01/2020

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“It is not the strongest of the species that survives,

not the most intelligent that survives.

It is the one that is the most adaptable to change.”

~Charles Darwin

Chapter 1: The evolution of subterranean diving beetles (Dytiscidae) from Western Australia

Photo: Karl Jones and Mark Haase

Highly perplexing, subterranean animals have endlessly interested researchers in many fields of study, such as taxonomy, evolution, genetics, behaviour, development, and physiology, to name a few. Convergent trait regression, such as eye and pigmentation loss, has particularly generated a lot of attention, however, no clear mechanism of how regression occurs has yet to be agreed on. This introductory chapter outlines the current views in regressive evolution studies, focussing on subterranean species with regressed eyes, and highlights studies which support the various theories. Background studies of phototaxis (light perception), the process of vision, and the genes involved in this specific trait are presented. Information on our study group, the subterranean diving beetles of Western Australia, and speculation on possible modes of speciation in this group are presented, and finally the aims of the project are outlined.

REGRESSIVE EVOLUTION

The loss or simplification of a trait(s), now referred to as regressive evolution, has played a role in the evolution of many organisms (Fong *et al*. 1995; Jeffery 2009). However, the mechanisms behind the regression of traits has remained unclear and highly debated. Beginning in 1859, Darwin (in the *Origin of Species,* 1859) described trait loss (e.g. loss of eyes in cave animals) using Lamarckian theory of use and disuse, as a driver of their evolution. Given the lack of knowledge of genetics at the time, Darwin could provide no better explanation for both eye and pigmentation loss in cave animals, which perplexed him. However, as the genetic basis for natural selection was developed in the 20th century (Fisher 1930), the Lamarckian theory

was discarded, and replaced with revised Darwinian theories (Espinasa and Espinasa 2008). Subsequently, the field divided into those supporting neutral evolution theory (Wilkens 2010 and references therein) and those supporting natural selection (Jeffery 2009 and references therein), as mechanisms of regressive evolution (Fig. 1). Additionally, some researchers hypothesize that both neutral theory and natural selection are likely acting together on the regression of traits (Borowsky 2013), however, this view is not widely shared.

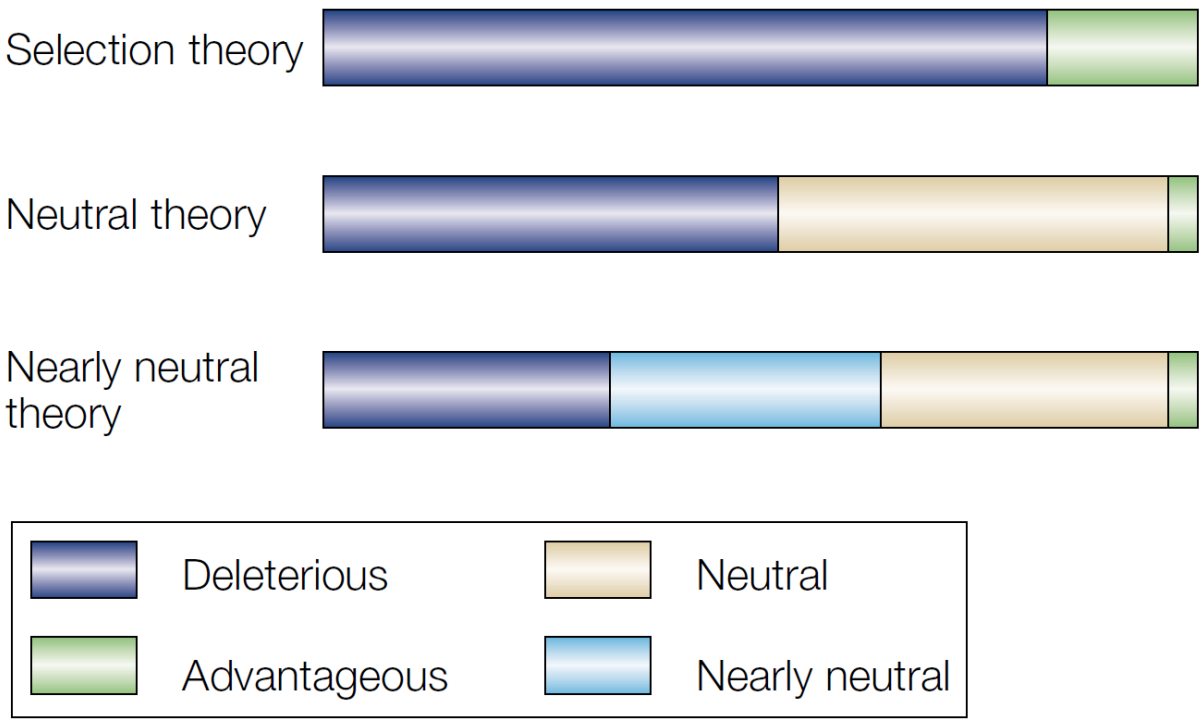

Figure 1: Selectionist, neutral and nearly neutral theories, where selection theory assumes that all mutations will affect fitness and be advantageous or deleterious. Neutral theory considers that neutral mutations exceed those that are advantageous, and therefore, neutral sites influence the rate of molecular evolution. Not considered here, the nearly neutral theory is a more recent extension of neutral theory, where some mutations will have a slight positive or negative effect on fitness (from Bromham and Penny 2003).

Neutral mutation theory

Neutral theory of evolution has remained a popular theory for explaining regressive evolution, as its principle mechanism relies on the accumulation of random mutations in genes specifically associated with the regressed trait. Under relaxed selection, it is theorised that alleles will accumulate random mutations through genetic drift (Kimura 1984). These mutations (e.g. insertions, nonsense mutations, and nonsynonymous changes) will accumulate in protein coding genes that are not under functional constraints, altering the amino acid sequence of the encoded protein. Thus over time, the gene(s) will encode non-functional protein(s), with mutations that prevent proper folding and interactions with other pathways or functions (Leys *et al*. 2005). Therefore, neutral evolution can potentially be identified by the presence of pseudogenes (non-functional genes through the accumulation of amino acid changing, nonsense and frameshift mutations) or pseudo-like genes (genes that are under neutral evolution, but have had insufficient time for mutations to be fixed) in the place of once functional genes (Tierney *et al*. 2015).

Research into the genes involved in a particular regressed trait, have become instrumental in studying how regressive evolution occurs, on which neutral evolution studies rely heavily upon. Several important factors can be used to provide evidence for neutral evolution: relaxation of selection based on ratios of nonsynonymous to synonymous mutations in related genes, variability of regressed traits both genetically and phenotypically, and loss of function mutations/pseudogenes (Li *et al*. 1981; Li *et al*. 1985; Yang and Bielawshi 2000; Hurst

2002; Wilkens 2010). Neutral evolution, as a basis for regressive evolution, has gained support mainly through research on the loss of pigmentation in cave animals, using the highly studied model, *Astyanax* cavefish; with over 40 years of detailed research on the ecology, genetics, and evolution of this group. In general, pigmentation genes in the cavefish were found to contain numerous function-altering mutations, likely due to a relaxation of selection (Jeffery 2009 and references therein). Quantitative trait loci (QTL) for melanophore abundance in *Astyanax mexicanus* display high levels of variability, supporting recurrent genetic drift and neutral mutation (Protas *et al*. 2007). Loss of function mutations were first found in globin genes in the 1980s (Proudfoot and Maniatis 1980; Liebhaber *et al*. 1985). The first loss of function mutations in subterranean vision genes (three frameshifts and numerous stop codons) were found in an interphotoreceptor retinoid binding protein gene (vision gene) in the blind marsupial mole, leading to the conclusion that molecular changes in genes under relaxed selective constraints, influenced the regressed trait of vision (Springer *et al*. 1997). In another study on various subterranean mole species, researchers found several phototransduction (long wavelength opsin and short wavelength sensitive 1) pseudogenes, which were significantly correlated with lower levels of light dependence, and an increased number of retinal pseudogenes (Emerling and Springer 2014), implying a relaxation of selection on the unneeded vision genes. Uniquely, non-functional *rhodopsin* has been found in at least three amblyopsid cavefish lineages (Teleostei: Amblyopsidae), with several other lineages displaying a functional *rhodopsin* but with increased rates of nonsynonymous mutations when compared to surface lineages, suggesting a repeated loss of selective constraint in this gene (Niemiller *et*

al. 2012). Research conducted on 11 subterranean diving beetle (Dytiscidae) species from Western Australia, identified increased sequence evolution and missense mutations, including frameshifts and stop codons, in a pigmentation-specific (*cinnabar*) gene, when compared to the functional gene copies from surface relatives, indicative of pseudogenes (Leys *et al*. 2005). Finally, a general lack of opsin gene (phototransduction specific gene) transcription was recently uncovered among the subterranean Dytiscidae when compared to surface relatives, providing evidence that the genes had been inactivated and lending support for the neutral evolution hypothesis (Tierney *et al*. 2015).

Natural selection

In 1858, Charles Darwin and Alfred Russel Wallace unveiled the theory of natural selection, which has remained the foundation concept in evolutionary studies. Natural selection is the inheritance by offspring, of a trait or traits that are either advantageous, or the removal of negative traits that are detrimental to fitness and survival. It is most likely that natural selection has driven the evolution of adaptive traits required by cave animals (Yoshizawa *et al*. 2012), however, the mechanism behind the regression of traits conflicts even among the Selectionists. Consequently, two main hypotheses have emerged from the natural selection theory, each with their own set of supporting research: direct natural selection and indirect natural selection.

Direct natural selection: positive and negative

Direct selection refers to the selection on genes that directly affect a particular trait, consequently inferring a survival or fitness benefit (Lande and Arnold 1983; Jeffery 2009). Initially observed by Darwin in the wing-loss of island beetles (1859) and again by means of eye loss in cavefish (Sadoglu 1967), direct natural selection was used to describe the survival advantage that these animals may have gained by losing unneeded traits. Two alternative hypotheses have been proposed to explain trait loss under direct selection; positive and negative selection. Positive selection describes an inherent benefit due to the loss of a trait (e.g. energy gain; Protas *et al*. 2007), while in negative selection the trait is not beneficial to the individual and can impede reproduction or reduce fitness (e.g. eyes can get damaged and possibly infected in lightless environments; Jeffery 2009 and references therein). However, studies advocating direct selection as a basis for loss of eyes in subterranean animals have been widely rejected based on flaws in the methods, reasoning, and conflicting results from other studies (Wilkens 2010; Protas *et al*. 2007; Dufton *et al*. 2012; Yoshizawa *et al*. 2015; Wilkens 2016 and references therein). The most popular theory surrounds the idea of energy conservation as an explanation for the reduction of the eye in *Astyanax* cavefish (Moran *et al*. 2015). The difficulty in accepting this theory is that eyeless cave animals are also found in some energy rich environments, so it does not seem to be an adequate theory to explain eye loss generally (Wilkens 2016; Wilkens and Strecker 2017). It has been found that juvenile *Astyanax* cavefish begin developing an eye before losing it as adults, however, this has been linked with development in the forebrain (Jeffery 2005; Pottin *et al*. 2011), and therefore is likely not to result from direct selection.

Indirect natural selection: pleiotropy and linkage

Indirect natural selection involves cases where the genes involved in a regressed trait are directly involved in, or closely linked to genes associated with, unrelated traits that are beneficial for survival or reproduction (Lande and Arnold 1983; Rétaux and Casane 2013; Pechmann *et al*. 2015). Additionally, pleiotropy is when one gene affects several traits, while linkage is when multiple genes are inherited together because they are close together on a chromosome. Therefore, the direct increase in frequency of alleles at one gene may occur simply because the gene is closely linked to beneficial alleles at a second gene (Plate 1910; Barr 1968; Stearns 2010). The indirect natural selection hypothesis has recently gained favour in eye regression studies, following developmental genetic analyses of the model cavefish *Astyanax fasciatus* (Jeffery 2008 and references therein). Many studies on the regression of eyes in *Astyanax* cavefish, point towards a suite of pleiotropic and linked interactions. Specifically, it has been suggested that eye regression could be involved in or linked with an increase in taste buds (Jeffery *et al*. 2000), an enlargement of the olfactory pit (Yamamoto *et al*. 2004), brain alterations, including increased darkness competence (Menuet *et al*. 2007), and cave-adapted metabolism modifications (Borowsky and Wilkens 2002), among others. However, most of these cases have been argued against for a variety of reasons. A study by Yamamoto *et al*. (2009) which involved a classical crossing of *Astyanax* cavefish and surface fish, eventually produced F_3 hybrids that showed an inverse relationship between eye size and taste buds/jaw size, leading to the conclusion that constructive traits were likely involved in the regression of eyes. As explained by Wilkens (2016), F_3 hybrids were inappropriate to use, as they are derived from a single F_2 hybrid, which only

represents a small portion of the overall variability. The F_2 hybrids would have been the proper specimens to analyse for such an association study (Wilkens 2016). Additionally, the taste bud and eye traits sort independently during meiosis (i.e. the developmental genes involved operate in separate pathways and so selection acting on one trait is unlikely to affect both traits simultaneously; Wilkens 2016). Subsequent study into the connection of constructive and regressive traits revealed little to no correlation in: lens ablation studies (Dufton *et al*. 2012), Quantitative Trait Loci (QTL) analyses of taste buds (Schemmel 1967; Protas *et al*. 2007), crossing analyses of number of teeth (Protas *et al*. 2007), jaw size, nose pit size, or mouth width (Wilkens 2010), to name a few (See Wilkens 2016 for review).

Difficulties distinguishing the neutral and selection hypothesis for regressive evolution

Based on the literature to date, no clear evidence has been generated to conclusively support either neutral or selective processes as the main evolutionary force in the regression of traits. The major problem with attempting to explain the regression of traits lies in the conflicting results generated for different genes, among different traits, and between different species. In a recent study on marine snails (family Solariellidae; Sumner-Rooney *et al.* 2016), evidence was found to support at least seven eye degeneration pathways. Therefore, the path to eye reduction for closely related species is not predictable. Evolutionary developmental analyses may provide insights into how eyes and vision are being lost in the early stages of regressive evolution in specific recently-evolved cases. However, they are not likely

to be helpful when investigating the actual mode of evolution (i.e. neutral evolution or purifying selection) acting specifically on different genes involved in regressed traits. Such genes may remain intact for substantial periods of time, until chance deleterious mutations occur and become fixed in populations by genetic drift. On average, nucleotide substitutions in multicellular organisms occur at an approximate rate of 10^{-9} to 10^{-8} base substitutions/site/generation (Lynch 2010). Therefore, in the case of subterranean animals, hundreds of thousands to millions of years may be required to generate the chance mutations required to assess whether genes associated with a regressed trait, such as vision, are subject to neutral evolution or purifying selection.

Pseudogenisation, the process by which a functional gene evolves into a non-functional gene through neutral processes, may be essential to regressive evolution of traits, however, pseudogenes can be difficult to identify for a multitude of reasons (see Podlaha and Zhang 2010 for a review). First, pseudogenes are difficult to define in some cases; some pseudogenes can be adaptive. The human cysteine-aspartic protease 12 (*CASPASE12*) is involved in the suppression of immune response to endotoxins, and most humans (~90%) have a mutation that inserts a premature stop codon (Wang *et al*. 2006). Through epidemiological studies, it was determined that this mutation is associated with a reduced incidence of severe sepsis (Saleh *et al*. 2004), suggesting positive selection. Additionally, pseudogenes may have typical mutations (e.g. indels and frameshifts) expected of a non-functional gene, but show unusual patterns of evolution consistent with chimeric function (i.e. exon shuffling following RNA processing may create a functional isoform; Podlaha and Zhang 2010). The alcohol dehydrogenase gene in *Drosophila*

has been duplicated, with the pseudogene copy containing many protein-altering mutations (Begun 1997). However, it also contains a lower rate of nucleotide substitutions in exons than introns, and the silent substitution rate is higher than the replacement rate, consistent with chimeric genes (Begun 1997). Finally, technical issues can arise in attempting to isolate an 'old' pseudogene as the mutations it has accumulated may make it very difficult to both PCR amplify and sequence or to confidently assign the sequences to the correct gene. It is possible that both selective and neutral processes could be involved in the regression of traits in subterranean animals, however, additional research is required to provide insight into this perplexing evolutionary phenomenon.

PHOTOPHOBIC BEHAVIOUR IN SUBTERRANEAN ANIMALS

Photophobic behaviour, the act of avoiding light, was speculated to have promoted many subterranean animals to seek out and even thrive in darkness (Timmermann and Plath 2009; Borowsky 2011). Subterranean lineages of animals residing in close proximity to ambient surface light may use negative phototaxis (light avoidance), as a way to reduce entering the light habitat where they may be exposed to increased predation and/or competition for resources (Langecker 2000; Borowsky 2011). Subterranean animals with highly reduced or even absent eyes have been found to have strong negative phototactic responses, such as in a cave beetle (*Ptomaphagus hirtus*; Friedrich *et al*. 2011), crustaceans (Borowsky 2011; Fišer *et al*. 2016), a roundworm (*Caenorhabditis elegans*; Edwards *et al*. 2008), salamanders

(Taylor 1972), cavefish (Tarttelin *et al*. 2012, and references therein) and mole rats (Kott *et al*. 2010).

Extraocular photoreceptors, light sensitive structures found outside the eye, are likely responsible for the majority of negative phototactic responses in the eyeless subterranean animals. A study on the eyeless crayfish, *Orconectes australis packardi*, found that they likely perceive light through the caudal ganglion of the brain (Wilkens and Larimer 1976). Similarly, the blind larvae of *D. melanogaster* have light responses that have been linked to simple neurons (Xiang *et al*. 2010). In some arthropods, the optic lobes and the ventral nerve cord may be the most important locations for extraocular photoreceptors (Fleissner and Fleissner 2003), which was found to be the case in eyeless cave amphipods, *Niphargus frasassianus*, *N. ictus* (Borowsky 2011), and other *Niphargus* species (Fišer *et al*. 2016). Not much data exist on this interesting ability, however, an improved understanding of what genes are involved in eye regression will help to elucidate the mechanism behind phototaxis.

THE EVOLUTION OF EYES IN SUBTERRANEAN ANIMALS

Central to the regressive evolution debate, eye reduction, or loss, in subterranean animals has been at the forefront. Eyes remain an exceptional trait for regressive evolutionary studies as they have been well characterized at the biochemical, physiological, and structural level, and they are evolutionarily conserved (Speiser *et al*. 2014). A massive body of research on eyes has been generated (Freund *et al*. 1996), making it an ideal trait for comparative studies.

The general mechanism behind sight

The eyes and process of vision of most animals can be divided into two main categories: "non-compound", as found in vertebrates and some invertebrates, and "compound", as can be found in insects and some other arthropods. The familiar non-compound eye, or simple eye, is composed of one surface (most commonly a refractive cornea; Land and Fernald 1992). The singular surface directs light into the back of the eye where a signal is directed to the suprachiasmatic nuclei. Ultimately, the nuclei control the pupillary light reflex, and relay information on to the brain. Simple eyes, contrary to their name, are not actually simple; at least five different types of eye forms have evolved independently (Nilsson 1989). In general, the retina contains thousands of photoreceptors called cone cells (responsible for day vision) and rod cells (responsible for peripheral and night vision), which are the photosensitive areas of the eye.

Structurally different from the simple eye, the convex compound eye is composed of many ommatidia, each with its own ability to refract light. Thousands of ommatidia may be present in one eye, all providing input from a slightly different angle. The brain is then able to piece together all the input to form an image. The compound eye can also be subdivided into several different eye forms, however, the same few cellular components of the ommatidia have been conserved from type to type: the cornea, a cone, a rhabdom, and a pigment screen (Hardie and Stavenga 1989). In general, one ommatidia can be made up of four cone cells: ~eight retinula cells, one rhabdom, which is the photosensitive structure, and the cornea that sits on top as the protective layer (Land and Nilsson 2002).

The vision of animals, in both compound and non-compound eyes, is enabled by the transduction of photons into neural impulses. Light photons of specific wavelengths are absorbed by a visual pigment and, through complicated chemical signalling, the photon eventually results in an electrical signal to the brain (Fig. 2; Montel 2012 for review of visual transduction). These visual pigments are responsible for the absorption of light and convert the light into an electrical response, which is essential in the visual signalling pathway (Hardie and Stavenga 1989; Henze *et al*. 2012).

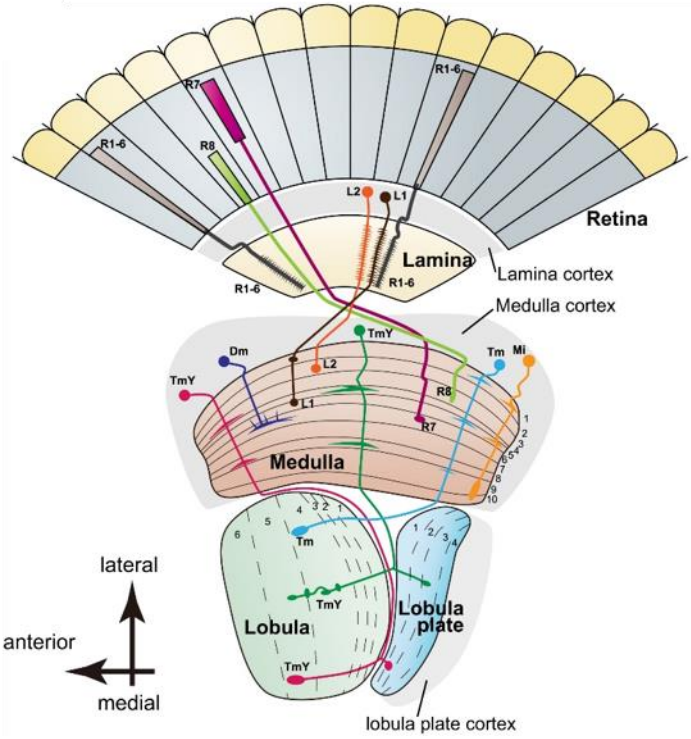

Figure 2: Schematic drawing of a horizontal section of the adult *Drosophila* visual system, including the retina, lamina, lobula and lobula plate (amended from Sato *et al*. 2013).

Genes involved in eye regression

Genes involved in eye development and function have been extensively studied (Jeffery 2008 and references therein; Jeffery 2009 and references therein), providing candidate genes for regressive evolutionary research. Functional eyes are mediated

by a variety of genes encoding proteins that detect light (opsins and cryptochromes), absorb light (pigment synthesis enzymes), refract light (lens crystallins), as well as various transcription factors required for development of eyes and light interacting structures (Zattara *et al*. 2017). Developmental genes are likely important in the loss of eyes in some species (e.g., *Astyanax fasciatus*; Jeffery 2009 and references therein), therefore, it is possible that modified expression of these genes could switch off eye development without negative consequences to other genes. Therefore, the main focus of my project is to study the genes specifically involved in the process of vision, rather than the developmental genes associated with building an eye (see Friedrich *et al*. 2011 for a list of important phototransduction genes).

Light detecting opsin genes

Opsin genes are central to the process of vision. They are G-protein coupled transmembrane receptors usually located in the photoreceptor cells in both the non-compound eye of vertebrates and compound eye of arthropods. Although both are referred to as opsins, those found in the compound eye of insects can be quite distinct (Figure 1 from Terakita 2005 for molecular phylogeny of the opsin family) from those found in the non-compound eye (Hill *et al*. 2002). It is thought that there are, at minimum, two main lineages of opsin genes in the evolutionary history of vertebrates and invertebrates, where the diversification of one ancestral opsin gene led to the distinct opsin genes now found in the compound eye (Velarde *et al*. 2005). There are five main visual and non-visual opsins in invertebrates: long wavelength opsin (LWO), ultraviolet wavelength opsin (UVO), blue opsin (also known as short

wavelength blue; SWB), ciliary opsins, and rhabdomeric opsins such as rhodopsin. Visual opsins are directly involved in vision, while non-visual opsins are required for light perception (Delroisse *et al*. 2014) and aid in the regulatory function of light-mediated processes such as circadian rhythm (Cavallari *et al*. 2011).

Research into opsins in subterranean animals has revealed variable results over the many years of study. The first group to study the evolution of opsins in subterranean animals was Crandall and Hills (1997) who found functional rhodopsin in three genera of subterranean invertebrate crayfish, with no apparent differences in substitution rate between surface and subterranean species. Two distinct paralogs of middle wavelength opsin were discovered in surface and cave populations of the fish *Gammarus minus*, however, there were low levels of sequence variation and no relaxation of selection, based on the ratio of nonsynonymous to synonymous mutations (Carlini *et al*. 2013). Carlini *et al*. (2013) also performed expression analyses and found that levels of expression were significantly reduced in cave populations relative to surface populations, implying a possible pleiotropic function of middle wavelength opsin, unrelated to vision. In a separate study on freshwater crayfish, Stern and Crandall (2018) found a lowered expression of 17 genes related to phototransduction (such as opsins and arrestins) in 10 subterranean species, compared to four surface species, however, all genes were intact (Stern and Crandall 2018). In the head transcriptome of the subterranean beetle, *Ptomaphagus hirtus*, transcripts of phototransduction opsins were detected in their highly reduced eyes (Friedrich *et al*. 2011). These results suggested that there could be a functional role for retaining opsin proteins for light detection, circadian rhythm regulation or other similar processes (Friedrich *et al*. 2011). The embryos of the

recently evolved *Astyanax fasciatus* cavefish (Fumey *et al*. 2018) revealed continued expression of opsin genes as eye degeneration occurred, suggesting developmental or regulatory genes rather than structural genes, were the cause of eye regression (Langecker *et al*. 1993). Recently, the independent loss of functional *rhodopsin* was found in three anciently-evolved amblyopsid cavefish species, with several other cavefish lineages having functional copies, but with increased rates of nonsynonymous mutations (therefore relaxation of selection), suggesting neutral selection in all species (Niemiller *et al*. 2012). The loss of opsin transcripts was found in three subterranean beetle species (revised by Langille *et al*. unpublished) when compared to surface relatives, suggesting neutral regressive evolution (Tierney *et al*. 2015). Over time, opsin research has produced variable results based on specific genes, the system being studied, level of light dependence, and time of colonization underground, of which the final point might be the most important in terms of understanding the mechanisms involved with regressive evolution. It is theorised that a significant amount of time is required for opsin genes, and other vision genes, to accrue mutations under neutral evolution, that render their encoded proteins non-functional in subterranean species (Podlaha and Zhang 2010).

Light screening genes: pigmentation of the eye

The colour pigments of eyes are mediated by a large number of specific pigmentation genes. Pigment granules (small masses of pigment) can be found in the photoreceptors and, in the case of compound eyes, between ommatidia acting as insulation, and as carriers of ommochrome pigments, which are responsible for

eye colour and the regulation of light influx. In cave adapted animals, a reduction or loss of pigment of the eyes (if present) has been attributed to many different metabolic and ABC transporter complex genes (Ambegaokar and Jackson 2010; Khan *et al*. 2017; Mackenzie *et al*. 1999; Osanai-Futahashi *et al*. 2012). Metabolic genes are essential for biological functions, and in the case of eye pigmentation, are responsible for the production of pigment granules (Lloyd *et al*. 1998). Eye pigment compounds are transported by ABC transporter proteins which are encoded by ABC transporter complex genes. These proteins are essential for the movement of pigments within the compound eye (Jones and George 2004).

An investigation into the eye regression of *Phomaphagus hirtus*, identified over 20 intact metabolic genes involved in the pigmentation of the eye (Friedrich *et al*. 2011). However, all of the ABC transporter complex genes (three were examined in his study) were not detected, leaving the authors to conclude that this species had likely lost the ability to produce eye pigmentation (Friedrich *et al*. 2011). In insects, *cinnabar* (metabolic gene) has also been studied in subterranean species (Leys *et al*. 2005; Reed and Nagy 2005). This gene plays a role in pigmentation of the eye by encoding for kynurenine 3-monooxygenase, an enzyme important for the synthesis of ommochromes (Wittkopp and Beldade 2009). Preliminary evidence suggested that *cinnabar* was a pseudogene in the subterranean Dytiscidae based on missense mutations and the rate of sequence evolution, when compared to surface relatives (Leys *et al*. 2005). Well studied in *D. melanogaster* and *Tribolium castaneum* (Lorenzen *et al*. 2002), the single copy cinnabar gene is unlikely to be involved in additional non-visual developmental pathways (Lorenzen *et al*. 2002), and therefore, could be an important gene in regressive evolutionary studies.

EYE REGRESSION IN CAVEFISH *ASTYANAX*

The most popular study system, to date, for the study of eye regression has been the cavefish, *A. fasciatus* (Fig. 3). This species has been described as both a great 'lab rat' and the 'fruit fly of cave animals' (Jeffery 2009). They are easily kept in laboratories, have a simple diet, spawn frequently and abundantly, embryos are large and clear, and many cave populations exist that have evolved independently from surface ancestors (Jeffery 2001, 2009). As a consequence of being the same species, the surface and cave forms are inter-fertile (Sadoglu 1957), allowing for hybrid cross experiments. *Astyanax* also shares a relatively close phylogenetic history with the zebrafish (*Danio rerio*), allowing for the vast information available on zebrafish, such as the genome and mapped chromosomes, to be utilized in *Astyanax* studies (Jeffery 2009).

Figure 3: *Astyanax fasciatus* (*mexicanus*) surface fish on top and cavefish on bottom. Photographs by Yoshiyuki Yamamoto (from Ghysen *et al*. 2010).

Despite all the benefits to using this very popular model fish in trait regression studies, there are some limitations to its use. Cave populations have evolved relatively recently (Fumey *et al*. 2018) and it is possible that intact genes may only reflect that there has been insufficient time to accumulate mutations that become fixed by genetic drift. Despite intensive study, the systematics of *Astyanax* remains confusing, as a wide variety of diploid chromosome number exists (Morelli *et al*. 1983), different types of B chromosomes can be found among the cells of different species, and *Astyanax* species have different distributions of repetitive DNAs (Fernandes and Martins-Santos 2005; Mantovani *et al*. 2005; Hashimoto *et al*. 2008; Vicari *et al*. 2008; Daniel *et al*. 2012; Santos *et al*. 2013; Silva *et al*. 2013). The 150+ species of *Astyanax* do not form a monophyletic group based on morphological and molecular markers (Javonillo *et al*. 2010; Mirande 2010; Oliveira *et al*. 2011), as there exists species complexes (Moreira-Filho and Bertollo 1991; Artoni *et al*. 2006; Castro *et al*. 2015) and putative cryptic species (Pansonato-Alves *et al*. 2013). However, understanding the regression of traits relies on first understanding the inter-relationships of different cave populations, whereby hybridization, commonly found in *Astyanax*, can affect how populations are grouped based on their shared allelic backgrounds (Panaram *et al*. 2005). Some populations of cavefish have individuals with eyes, intermediate forms, or eyeless forms (Avise and Selander 1972; Mitchell *et al*. 1977; Romero 1985), and it is known that migration of surface forms into cave systems does occur (Bradic *et al*. 2013), although it is unclear if there has been gene flow between cave and surface forms (Wilkens and Strecker 2017). Finally, there are other excellent subterranean systems such as the isopod *Asellus aquaticus* (Verovnik *et al*. 2004; Protas *et al*. 2011; Konec *et al*. 2015), which could aid

in the neutral vs. selection debate of regressed traits, and these need to be considered and studied.

SUBTERRANEAN ECOSYSTEMS IN AUSTRALIA

Unlike the Northern Hemisphere, Australia did not experience significant glaciation, but rather became more arid and cool, and therefore it was thought that the biodiversity of subterranean fauna in Australia would be low (Moore 1964; Hamilton-Smith 1967; Barr 1973; Humphreys 2004). However, once researchers began delving into groundwater systems, a plethora of stygofauna and troglofauna (subterranean aquatic and terrestrial species, respectively) were discovered (Humphreys 2006, 2008 and references therein, 2009; Boulton 2009). This subterranean fauna appears to be both extensive and novel in its diversity, with subterranean environments likely containing thousands of undescribed species. A previous assessment of Western Australian fauna estimated a total of ~4100 species of which only about ~19% are described or known (Guzik *et al*. 2011).

Arid Australia and calcrete aquifers

Under drying conditions, possibly of the Late Eocene to Early Oligocene (37-30 million years ago (mya)), groundwater evaporated along palaeodrainages (Bowler 1976), leading to the precipitation of a carbonate rock known as calcrete (Morgan 1993). Individual calcrete bodies are proposed to have undergone karstification during a wet phase in the Miocene (30-10 mya), providing a suitable habitat for

subterranean fauna (Morgan 1993). Australia began the shift to an arid climate ~ 15 mya during the Mid-Late Miocene (Morgan 1993; Sniderman *et al*. 2016) at which point the surface water dried up in central Western Australia. It is likely that ~3-6 mya, Australia returned briefly to warm, and wet conditions (Byrne *et al*. 2008; Sniderman *et al*. 2016). However, this period was quickly followed by continued desiccation (Byrne *et al*. 2008). Within the Yilgarn region of Western Australia, over 200 major (> 100km^2) and hundreds of minor (< 100km^2) calcretes are known, the whole region resembling a subterranean archipelago (Cooper *et al*. 2007).

Individual calcrete bodies have been shown to represent closed island environments, as most of them are physically separated from each other by fine sediments, clays, and sand, as well as each containing endemic taxa (Guzik *et al*. 2009). A combination of stygobiontic species and surface species, pre-adapted to life underground, are likely to have independently colonized calcretes (Leijs *et al*. 2012). Subsequent evolution within each separate calcrete led to speciation, resulting in the unique assemblages of species in each calcrete observed today (Cooper *et al*. 2007). Molecular clock analysis suggest that there has been a lack of gene flow between stygobiontic species of separate calcretes and surface species from approximately 3-10 mya, although some groups may have colonized as early as the Mid-late Miocene (~15-24 mya) (Leys *et al*. 2003; Cooper *et al*. 2008; Guzik *et al*. 2008). This colonization time loosely coincided with the major period of aridity of the Australian continent (Bowler 1976; Sniderman *et al*. 2016).

Stygofauna and troglofauna in calcretes

The stygofauna of the Yilgarn calcretes, comprises obligate subterranean groundwater invertebrates such as crustaceans and water beetles (Humphreys 2008). These calcrete habitats have been described as biodiversity hotspots (Bradford *et al*. 2014). The current stygofauna and troglofauna (the latter not treated here) assemblages include various species of Amphipoda (Williams and Barnard 1988; Bradbury and Williams 1997; King *et al*. 2012), Isopoda (Taiti and Humphreys 2001; Wilson 2003; Cooper *et al*. 2008; Guzik *et al*. in press), Coleoptera (Eberhard *et al*. 2016; Watts and Humphreys 2009 and references therein), Copepoda (Karanovic and Cooper 2012 and references therein), Diplura (Koch 2009), Myriapoda (Edgecombe 2005), and Arachnida (Guzik *et al*. 2011 and references therein; Harrison *et al*. 2014).

Calcrete habitats generally provide similar ecological conditions that remain relatively stable (Humphreys 2009), despite being unconnected to each other. In the Yilgarn calcretes there are two major groups of amphipods found in the calcrete aquifers: Paramelitidae (Williams and Barnard 1988), and Chiltoniidae (originally treated as Hyalidae; King *et al*. 2012). Different species of Chiltoniidae are located in the more southern, saline areas, while species of Paramelitidae are located in more northern, less saline areas, as they originated from a freshwater lineage (Williams and Barnard 1988). Distinct assemblages of stygobitic isopods have been discovered in ~25 calcretes to date with most, if not all, species falling into the genus *Haloniscus* (undescribed species; Cooper *et al*. 2008). Investigations into copepods have intrigued researchers as they appear to be quite diverse in the calcrete system,

though not well studied taxonomically. A total of 12 species, one subspecies, and three cryptic species have been described from only a few calcretes to date (Karanovic 2004; Karanovic and Cooper 2012; Karanovic *et al*. 2015). Approximately 100 species of dytiscids from the tribes Bidessini and Hydroporini, have been identified (Fig. 4; Leys *et al*. 2003), with usually between one to three endemic and different-sized species per calcrete (Watts and Humphreys 2009 and references therein). The wide variety of families found within a single calcrete (Humphreys 2004, 2008), illustrate how diverse underground Australia really is.

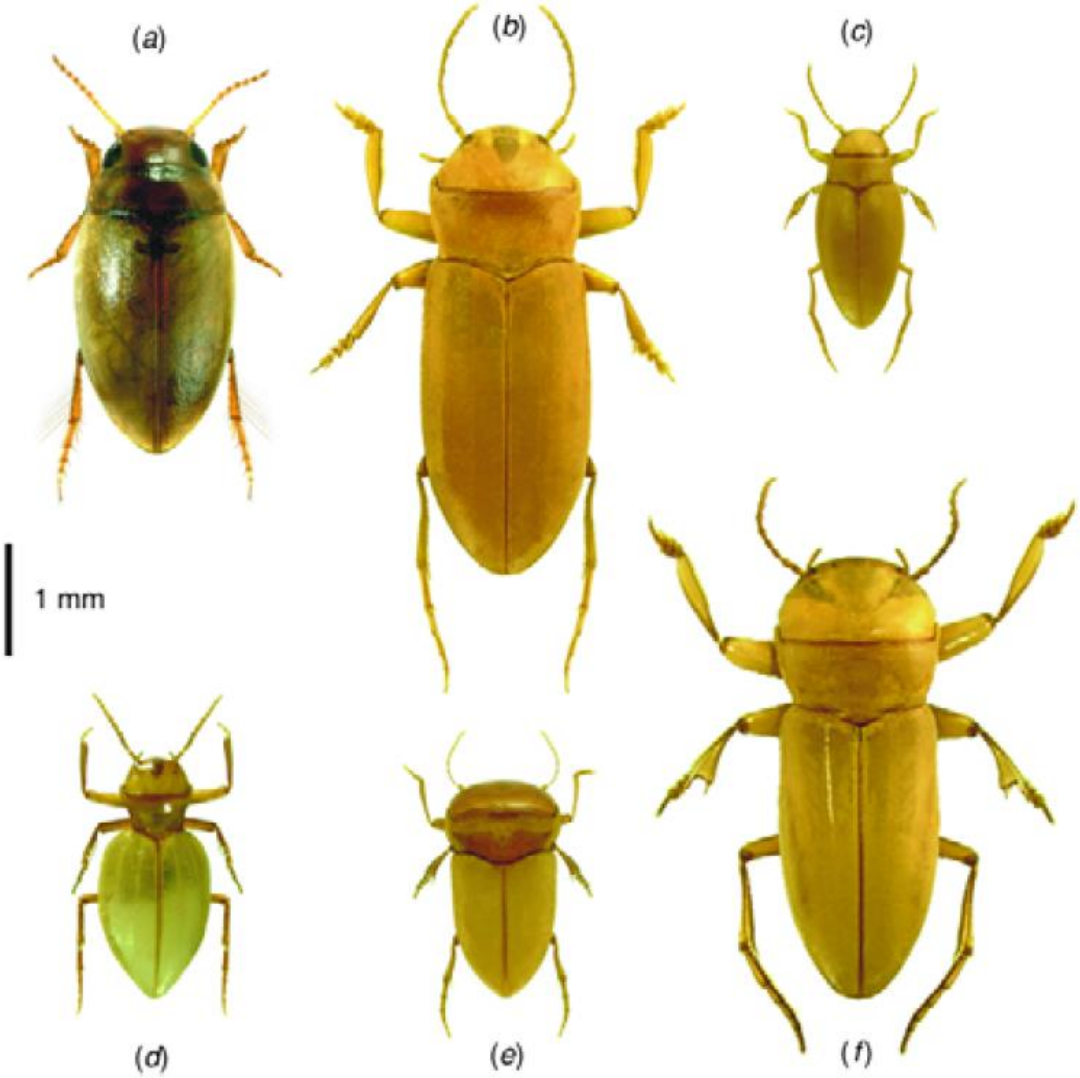

Figure 4: Examples of morphology and size variation in *Paroster* where species *b* and *c* are sympatric sister species. (*a*) *P. couragei*; (*b*) *P. macrosturtensis*; (*c*) *P. microsturtensis*; (*d*) *P. arachnoides*; (*e*) *P. macrocephalus*; and (*f*) *P. byroensis* (amended from Leys and Watts 2008).

Subterranean diving beetles: An excellent candidate system for future regressive evolutionary studies

Of the subterranean stygofauna, the predatory diving beetles (Dytiscidae) have been found in more than 45 calcretes, with over 100 beetle species described to date (Watts and Humphreys 1999, 2000, 2001, 2003, 2004, 2006). This makes these beetles, the most diverse known assemblage of subterranean dytiscids in the world (Balke *et al.* 2004; Guzik *et al*. 2009). In general, subterranean dytiscid species have typical arthropod cave troglomorphy: reduced or absent eyes, vestigial or fused wings, and reduced pigment in the body (Watts and Humphreys 2009 and references therein). The diving beetles are short range endemics, with over 75% of species having evolved-independently from surface ancestors. The remaining 25% of species are sympatric species pairs or triplets, as identified through phylogenetic analysis (Fig. 5; Leijs *et al*. 2012). Based on molecular clock estimates, the beetles likely became isolated in the calcretes between 3 and 10 mya (Leys *et al*. 2003; Leys and Watts 2008).

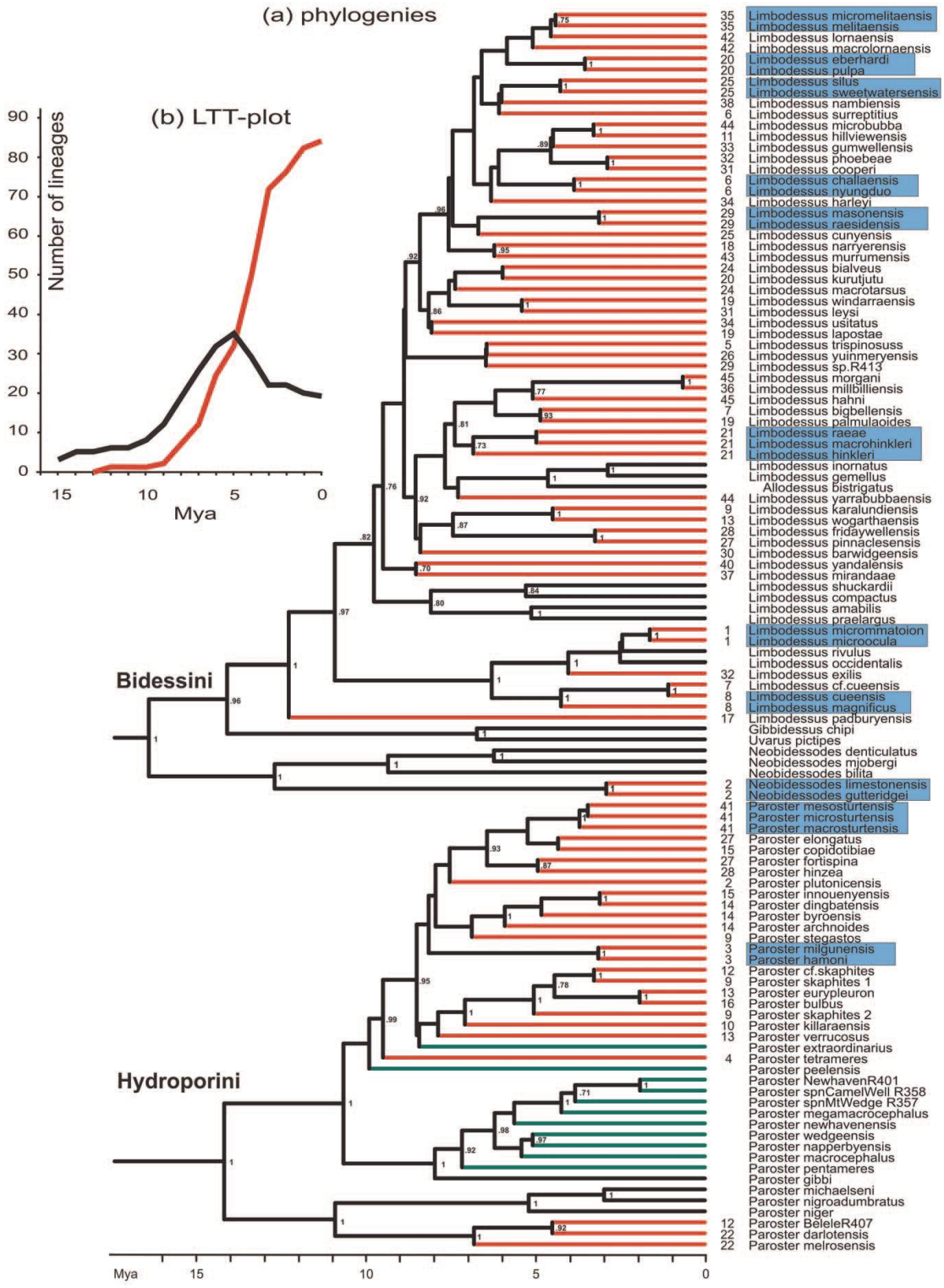

Figure 5: Mitochondrial molecular phylogeny and lineage through time plot of subterranean diving beetles. (a): Molecular phylogeny with sympatric sister pairs (blue boxes) shown, and posterior probabilities near branches. Red lines indicate terminal branches leading to a subterranean species, black lines indicate surface lineages, and green lines indicate subterranean lineages from aquifers outside of the Yilgarn region. (b): Lineage-through-time plot for surface (black) and subterranean (red) lineages showing the high number of ancestral species (20+) during the major radiation of diving beetles ~3-7 Mya (from Leijs *et al*. 2012).

The sympatric sister species, are highly important as they likely give a time point of when the species were actually underground and how they may have speciated. It has been theorized that a common ancestor to the sister species in Western Australia, either speciated underground or colonized the same calcrete several times at different time periods. Mathematical modelling supported underground speciation (Leys *et al*. 2012), although in practice these are difficult hypotheses to distinguish between. For example, sympatric troglobiont spider (*Dysdera*) species of the Canary Islands have segregation of body types (i.e. small, medium, and large body size), cheliceral modifications, and genetic and morphological similarity leading to the suggestion that they evolved underground in sympatry (Arnedo *et al*. 2007). Additionally, monophyletic lineages of cave beetle taxa (Leiodidae and Trechinae; Faille *et al*. 2010; Ribera *et al*. 2010) were found from the Western Mediterranean, suggesting that they speciated underground. However, it is difficult to confirm speciation using phylogenetic methods due to the potential extinction of surface ancestors during major climatic events (e.g. glaciations) on the surface. For this reason, no studies have robustly confirmed post-colonisation speciation occurring within subterranean systems.

The subterranean dytiscid beetles of the Yilgarn region in Western Australia represent an ideal system for regressive evolutionary studies for many reasons. First, molecular clock analyses suggest that most beetles in this system have been isolated from the surface and other calcretes for over three million years (Leys *et al*. 2003. The temporal and spatial isolation of calcretes (Poulson and White 1969), provides simple, stable populations (Humphreys 2009), however, water fluctuations and the structure of the calcretes may make them quite dynamic at the

population level (Bradford *et al*. 2013; Humphreys 2008). The large number of independently-evolved subterranean beetle species and closely related surface lineages persistent in coastal regions of Australia (original surface ancestors most likely extinct; Cooper *et al*. 2007), allow for large comparative genomic-level investigations into trait regression and other evolutionary ecology/history questions. In 2015, the transcriptomes of three subterranean beetle species (representing the three known genera, *Paroster, Neobidessodes and Limbodessus*) and two surface beetle species were generated and opsins (*UV*, *long-wavelength*, and *ciliary-type*) were identified (Tierney *et al*. 2015). Two subterranean species showed a parallel loss of these genes (Fig. 6), as might be expected for neutrally evolving pseudogenes. However, the absence of transcription, does not necessarily imply the absence of a functional gene and, therefore, a more extensive investigation is required to determine the pseudogene status of these genes.

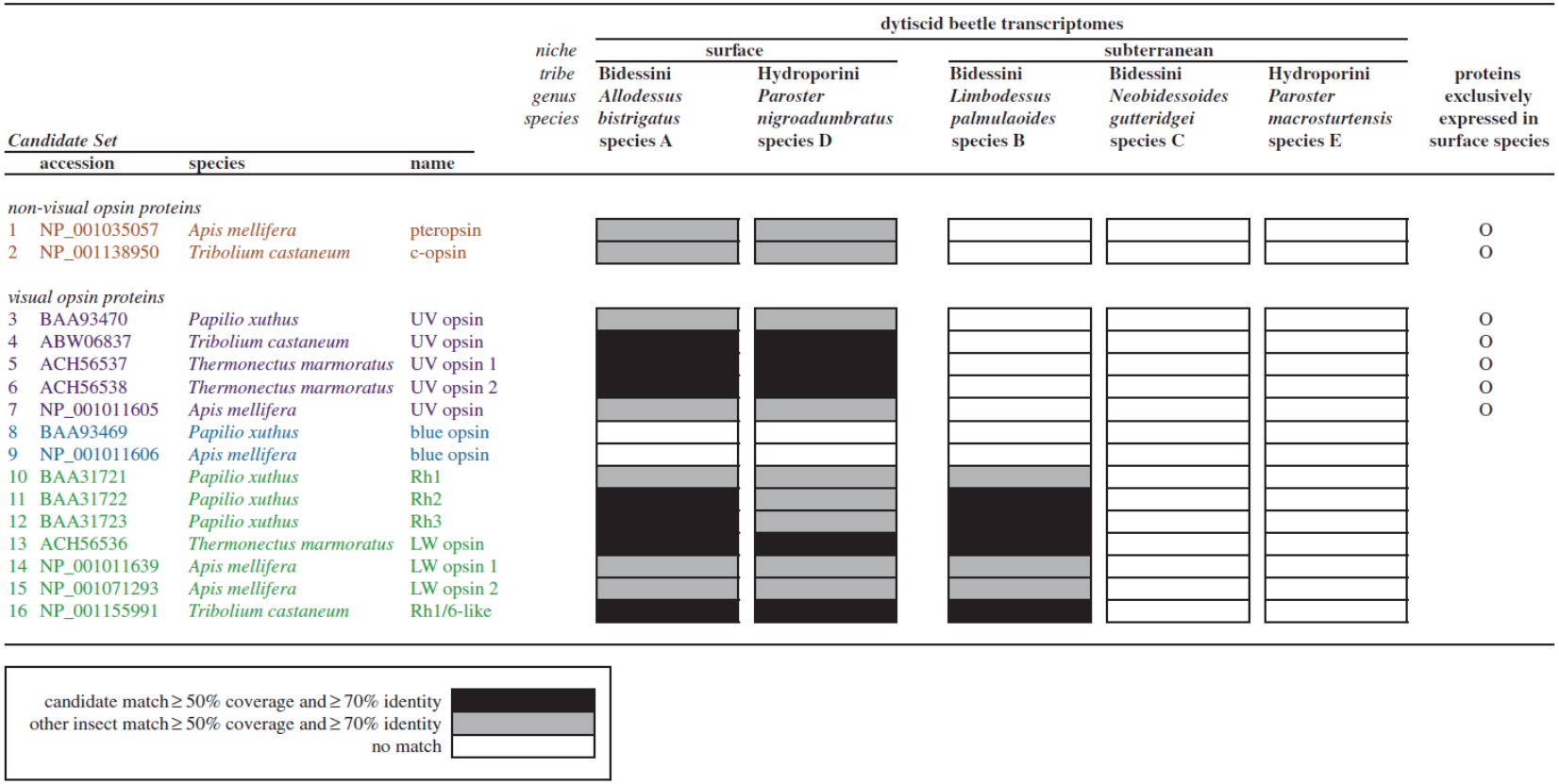

Figure 6: Transcriptome data BLAST search for opsin genes in subterranean species against a database of known opsin genes. Proteins only expressed by both surface species, but absent from all subterranean species are identified by an open circle (from Tierney *et al*. 2015).

AIMS

Subterranean diving beetles (Dytiscidae) endemic to groundwater calcretes of Western Australia exhibit convergent traits typical of troglomorphic arthropods, including loss of eyes, pigmentation and wings. The aims of this project were threefold: 1) to test the phototactic responses of subterranean diving beetles from two calcretes in the Yilgarn, 2) to sequence vision-specific genes from a large assemblage of diving beetles and test the hypothesis that they are evolving under neutral evolution, and 3) to utilise vision-specific genes to investigate how subterranean beetles speciated (i.e. underground or via multiple colonisations by surface species).

I carried out the first aim by exposing six different subterranean beetle species to a light-dark choice test, to identify if they have a preference for light, dark, or neither. As they are eyeless, they were expected to have zero preference and, therefore, be found in the light and dark equally. The second aim involved investigating the molecular evolution of photo-transduction genes from a wide sampling of surface and subterranean dytiscid beetles, including sister species pairs and triplets, to test whether neutral gene evolution is associated with the loss of vision in blind cave animals. As beetles have been underground, isolated from the light for millions of years, a neutral evolution hypothesis leads to the prediction that random mutations should accumulate and vary from species to species, and only be present in genes specifically associated with the regressed trait of vision. The final aim utilised the ~25% of subterranean beetle species that may not have evolved independently from surface species (as evidenced by the existence of sympatric sister species). Therefore, following the discovery of opsin pseudogenes

in the subterranean beetles, I investigated whether these genes could be used to test the hypothesis of speciation underground. I also attempted to clarify the phylogenetic placement of subterranean beetle species from the genus *Paroster* in a robust multigene phylogeny, with the purpose of confirming the presence of sympatric sister species, and ultimately deducing how they speciated (i.e. speciation underground or multiple colonisations from the same or related surface species). Overall, my research highlights how the dytiscid beetles and calcrete system provides a unique model system for exploring the highly debated topics of trait regression and modes of speciation in subterranean animals.

The following empirical research chapters are presented in manuscript format and will, therefore, contain some repetition within the introduction and discussion sections. Each chapter also contains differing formats as required by the journals in which I intend to submit. However, I aimed to create a thesis with as much continuity as possible, while still presenting adequate information in each section for each chapter to be stand alone manuscripts. The final chapter is a discussion of all research chapters together, with implications for furture research.

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Chapter 2: How blind are they? Phototactic responses in stygobiont diving beetles (Coleoptera: Dytiscidae) from calcrete aquifers of Western Australia

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Steven Cooper		
Contribution to the Paper	Collection of samples, co-manuscript planning, editing the manuscript.		
Signature		Date	24/7/2019

Name of Co-Author	Simon Tierney		
Contribution to the Paper	Data analysis, interpretation of data, editing the manuscript.		
Signature		Date	18 JULY 2019

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Andrew Austin			
Contribution to the Paper	Editing the manuscript.			
Signature			Date	12/8/19

Name of Co-Author	William Humphreys			
Contribution to the Paper	Editing the manuscript.			
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How blind are they? Phototactic responses in stygobiont diving beetles (Coleoptera: Dytiscidae) from calcrete aquifers of Western Australia

Barbara Langille,[1]* Simon M Tierney,[4] Andrew D Austin,[1] William F Humphreys[1,3] and Steven J B Cooper[1,2]

[1]*Australian Centre for Evolutionary Biology and Biodiversity, Department of Ecology and Evolutionary Biology, School of Biological Sciences, University of Adelaide, Adelaide, SA 5005, Australia.*
[2]*Evolutionary Biology Unit South Australian Museum, North Terrace, Adelaide, SA 5000, Australia.*
[3]*Western Australian Museum, Locked Bag 40, Welshpool DC, WA 6986, Australia. School of Animal Biology, University of Western Australia, Nedlands, WA 6009, Australia.*
[4]*Hawkesbury Institute for the Environment, Western Sydney University, Locked Bag 1797, Penrith, NSW 2751, Australia.*

Abstract Subterranean water beetles endemic to groundwater calcretes of Western Australia exhibit convergent traits typical of troglomorphic arthropods, including loss of eyes, pigmentation and wings. As these dytiscid species are estimated to have been isolated underground in permanent darkness for over three million years, it is predicted that they will completely lack phototactic responses. We tested this hypothesis by analysing the behaviour of six subterranean beetle species within an observational arena with dark and light hemispheres. Scan samples at 1 min intervals and total time spent on each hemisphere were recorded over a 20 min period, testing at least 15 individuals per species. We quantified behaviour as an index (dark ratio) so that individual species in this, and future, studies can be consistently compared. Results analysed as both categorical and absolute proportion of time spent in each hemisphere suggest negative phototaxis in *Paroster macrosturtensis*. The remaining five species did not display any preference for either light or dark hemispheres. These results raise the possibility that some ancestral *Paroster* species may have exhibited negative phototactic behaviour prior to subterranean colonization. The retention of such a behavioural trait in lightless environments could represent the maintenance for some unknown pleiotropic function. Alternatively, it is possible that insufficient time has passed for neutral processes to render photoreception genes and phototactic behaviours non-functional. Our study adds to a growing body of evidence that implies highly troglomorphic animals may have evolved from ancestral species that exhibited negative phototaxis as a preadaptation to living in permanent darkness.

Key words calcrete aquifer, cave animals, negative phototaxis, *Paroster macrosturtensis*, trait modification.

INTRODUCTION

Subterranean habitats and deep cave environments lack light and may have a restricted energy input (Friedrich *et al.* 2011). Populations within these habitats are generally isolated or have limited gene flow, forming relatively simple community structures (Juan *et al.* 2010; Friedrich 2013). In spite of these environmental conditions and limitations, cave animals have become highly adapted for these dark underground environments and have convergently evolved a shared suite of traits known as troglomorphies (Christiansen 2005; Culver & Pipan 2009; Tierney *et al.* 2017). The most conspicuous and widespread of these traits are the reduction or loss of eyes and cuticular pigmentation, and wings in the case of insects (Christiansen 2005; Sket 2008; Rétaux & Casane 2013). Recently, there has also been increased investigation into regressed behavioural traits (Parzefall & Trajano 2010), in particular, the reaction to light – phototaxis.

Visual capability can be organised into three main functional abilities: light detection, recognition of light directionality and image formation (Borowsky 2011). These abilities do not all require a functional eye, therefore, it is possible for subterranean animals to retain the ability to both sense of light and its direction, despite highly reduced or even apparent absence of eyes. The initial development of photophobic behaviour was speculated to have promoted invertebrates to seek out and even thrive in darkness (Timmermann & Plath 2009; Borowsky 2011). In the cave beetle, *Ptomaphagus hirtus*, strong negative phototactic responses were observed, despite their highly reduced eyes (Friedrich *et al.* 2011). Within the first 2 min of exposure to a light/dark choice test, over 70% of animals were recorded in the dark side, suggesting this species was using peripheral photoreception (Friedrich *et al.* 2011). Similar, albeit less pronounced, trends were also observed in the apparently eyeless amphipods, *Niphargus frasassianus* and *N. ictus*, leading to the conclusion that they had the ability to detect light (Borowsky 2011). Additionally, light facilitated responses have been established in various subterranean animals including crustaceans (Borowsky 2011, and references therein; Fišer *et al.* 2016), a roundworm (*Caenorhabditis elegans*; Edwards *et al.* 2008), salamanders (Taylor 1972), cavefish (Tarttelin *et al.* 2012, and references therein) and mole rats (Kott *et al.* 2010).

*barbara.langille@adelaide.edu.au

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The calcrete aquifers of Western Australia contain the most diverse assemblages of subterranean diving beetles (Dytiscidae) known worldwide. The fauna, all of which have highly regressed or absent eyes, comprises approximately 100 known species found within 45 separate calcrete bodies (Leys *et al.* 2003; Watts & Humphreys 2009 and references therein). Calcrete aquifers are shallow (~10 m thick) carbonate deposits, formed by the evaporation of groundwater, and they share many characteristics with the uppermost zone of epikarst habitats (Culver & Pipan 2009). Molecular clock analyses suggest that ancestral dytiscid species likely became isolated in these calcretes during the Late Miocene to Early Pliocene (8–3 million years (Myr) ago; Cooper *et al.* 2002; Leys *et al.* 2003). Most subterranean dytiscid species have independently evolved from surface ancestor species, with the exception of at least 13 cases in which a single ancestral species likely speciated within individual calcrete bodies (Leys *et al.* 2003; Leijs *et al.* 2012) (See Leijs *et al.* 2012 for a recent phylogeny).

Facultative subterranean lineages of animals residing in close proximity to ambient surface light are thought to have evolved negative phototactic behaviour, to prevent them from entering the light habitat, where they may be exposed to increased predation and/or competition for resources (Langecker 2000; Borowsky 2011). However, obligate subterranean animals occupying lightless environments are hypothesised to have lost all perception capabilities (Langecker 2000), although orientation to directional light has recently been reported in a suite of stygobiont amphipod and other species (Borowsky 2011 and references therein).

In the following study, we asked the question: is it possible for eyeless, subterranean dytiscid beetles from lightless calcretes to exhibit phototactic behaviour? We tested six subterranean dytiscid beetle species from two calcretes in the Yilgarn region of Western Australia and observed behavioural responses to a light/dark experimental arena. The null hypothesis was that beetles would display no bias in the time spent in either the light or dark hemisphere of the arena. Our assumed expectation that beetles will have no phototactic capabilities, consistent with Langecker's theory (2000), is based on phylogenetic evidence that suggests photic isolation for at least 3 Myr (Leijs *et al.* 2012).

MATERIALS AND METHODS

Dytiscidae sampling

Live subterranean beetle species were collected in October 2015 using a weighted net (mesh 250 μm) from mineral exploration bore holes in the Yilgarn region of Western Australia. Three subterranean species of *Paroster* (*Paroster macrosturtensis, P. mesosturtensis* and *P. microsturtensis*) were collected from Sturt Meadows calcrete, WA (28.7155° S, 120.8931° E), and three species of *Limbodessus* (*Limbodessus palmulaoides, L. windarraensis* and *L. lapostaae*) from the Laverton Downs calcrete, WA (28.3983° S, 122.2038° E) (Fig. 1). These six

Fig. 1. Paroster beetles displayed form left to right: *P. macrosturtensis*, *P. mesosturtensis*, and *P. microsturtensis*. Photo provided by Chris Watts of the South Australia Museum. [Colour figure can be viewed at wileyonlinelibrary.com]

species represent at least four independent evolutionary transitions from surface to subterranean environments: phylogenetic and modelling analyses suggest the Sturt Meadows species most likely evolved within the calcrete from a single common stygobitic ancestor that in turn evolved from a surface/interstitial species; the Laverton species each evolved independently from separate interstitial/surface species (Leys *et al.* 2003; Leijs *et al.* 2012). Various numbers of each species were collected and 20 individuals were selected from each group for the behavioural experiments, with the exception of *L. palmulaoides* as there were only 15 individuals available. We added extra individuals of *P. macrosturtensis*, as observations of this species were quite different from the other five species. Dytiscids were sorted into species using a stereomicroscope, and stored in plastic jars containing calcrete groundwater. All animals were maintained in darkness at ~25°C, which is the temperature of the water in the calcretes (SJBC, pers. comm.). All jars were provided with a food source of freshly crushed amphipods (*Austrochiltonia australis*) once a week for the 10 week duration of experimental procedures. Experiments with distantly related interstitial (partial eyed) dytiscid beetles would be desirable for outgroup comparison, but currently remain beyond immediate feasibility due to the heavy field work required for collecting and the associated cost.

Experimental design

All experiments were conducted in a temperature controlled room (25°C) devoid of ambient light, between 9 am and 5 pm from November 2nd to January 25th. An LED white light bulb was the only source of light: General Electric, 40 W equivalent output, 240 V 50 Hz; 2750 lux across a spectrum of 456 to 644 nm. The lamp head was positioned 24 cm above the test arena perpendicular to the lid (Fig. 2). A circular observational arena (15 cm diameter, 1.5 cm deep plastic/glass Petri dish) was filled with 25°C calcrete water. Kitchen-grade aluminium foil was used to (1) cover half of the arena lid and (2) direct light vertically from the lamp head away from the foil covered side of the arena, creating a dark hemisphere and a light hemisphere. Additionally, we used a black marker to black out the dark half to minimise reflected light from entering the dark hemisphere of the arena. This set-up was generally modelled based on a behavioural arena previously developed by Friedrich *et al.* (2011).

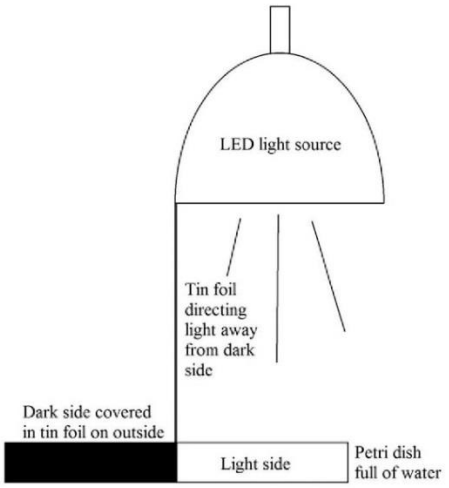

Fig. 2. The experimental set up showing how the light source was positioned to the petri dish in relation to the dark and light sides (hemispheres).

All animals were kept in darkness prior to exposure to experimental conditions and were transferred into the arena using a pastette to the same position each time (middle of light zone and ~1–2 cm from dark zone). Each experimental test comprised of a single beetle observed in isolation for a 20 min period. Data were collected by a single observer (B. L.) recording the position of an individual in the arena (observations in the light and dark) using two methods: (1) scan samples at 1 min intervals so that categorical data could be used to test deviations from the null assumption; and (2) continuous observation logging the time that a subject crossed hemispheres enabling the calculation of absolute time spent on each hemisphere. A minimum of 15 different specimens of each species were subjected to the experimental procedure once.

Statistical analyses

Analyses were carried out using SPSS v24. We expected an equal probability (bimodal distribution) of finding beetles on either hemisphere of the arena (light and dark) or analysed categorical scan samples and absolute time (seconds) data formats separately. Raw data were converted to proportional values in order to compare data from alternate formats (scans and absolute time) and undertake parametric analyses where data conformed to tests of normality and homoscedasticity. We calculated an index of behaviour so that time spent on either hemisphere of the arena was comparable between species, namely *dark ratios* of scans (r_s) and time (r_t) for each species: r_s = number of scans recorded on the dark hemisphere / total scan samples; r_t = seconds spent on the dark-hemisphere / total observation seconds. All ratio proportions were subsequently arcsine transformed to normalise the data (*per* Sokal & Rohlf 1981).

Animals were not acclimated to the arena prior to experimental light-treatment, however, we did use post-hoc statistics to explore whether behaviour had changed over observation time. We did this by excluding the first 10 min of results (as an acclimation-proxy), repeating analyses only using data from the last 10 min of the observation period.

RESULTS

Baseline activity

A total of 20 individuals were tested for each species, with the exception of 15 for *Limbodessus palmulaoides* and 22 for *Paroster macrosturtensis* (*see* Appendices S1 & S2 for raw data). All individuals were observed moving randomly and freely about the petri dish (travelling along the edge, crossing

through the centre and additional sporadic movements). We first assessed relative activity within the observation arena for each species by scoring the number of times each individual crossed the hemispheric border (dark:light boundary) as a proxy for general activity. A Kruskal-Wallis (non-parametric one-way ANOVA) showed that movement around the arena (number of hemisphere changes) was not significantly different among species ($H_5 = 10.6$, $P = 0.6$).

Dark ratios

A longitudinal line graph of r_s at each scan sample period is presented for each species in Figure 3, which plots the categorical data as arcsine transformed dark ratios. *P. macrosturtensis* is the only species to exhibit r_s values >0.5 throughout most of the treatment period.

Plots of mean dark ratios (r_s and r_t) for each species are presented in Figures 4 (full 20 min) and 5 (last 10 min) wherein the red stippled line indicates the null assumption of no hemispheric preference ($r = 0.5$). All three figures show that *P. macrosturtensis* is the only species to consistently exhibit a preference for the dark hemisphere.

We first compared mean dark ratios over the full 20 min observation period. A one-way ANOVA indicated a significant difference in dark hemisphere scan sample observations (r_s) between species ($F_{5,111} = 4.15$, $P = 0.02$). Post-hoc pairwise comparisons (Bonferroni) indicate that r_s values of *P. macrosturtensis* significantly differ from all other species (all $P \leq 0.05$), but no other species differ from each other (all $P > 0.99$). We then analysed dark ratios of absolute time (r_t) in the same manner, which also returned a significant result ($F_{5,111} = 4.915$, $P \leq 0.01$) with equivalent significance values for post-hoc pairwise comparisons among species.

To examine possible effects of acclimation to the arena, we repeated the ANOVA but excluded the first 10 min from the data set (Fig. 5). Subsequent analyses indicated no significant differences between species when the scan sample data were employed ($r_s - F_{5,111} = 1.253$, $P = 0.29$); but did return a significant difference for the absolute time format ($r_t - F_{5,111} = 2.745$, $P = 0.022$). However, the only significant pairwise difference in r_t over the last 10 min of observations was between *P. macrosturtensis* and *L. palmulaoides* (Bonferroni $P = 0.009$).

DISCUSSION

The phototactic responses observed by five of the six subterranean species follow a pattern of no differentiation between the light and dark hemispheres, a behaviour that is expected for species living in permanent darkness, that completely lack vision. In contrast, the subterranean species *Paroster macrosturtensis* exhibited negative phototaxis. This result is

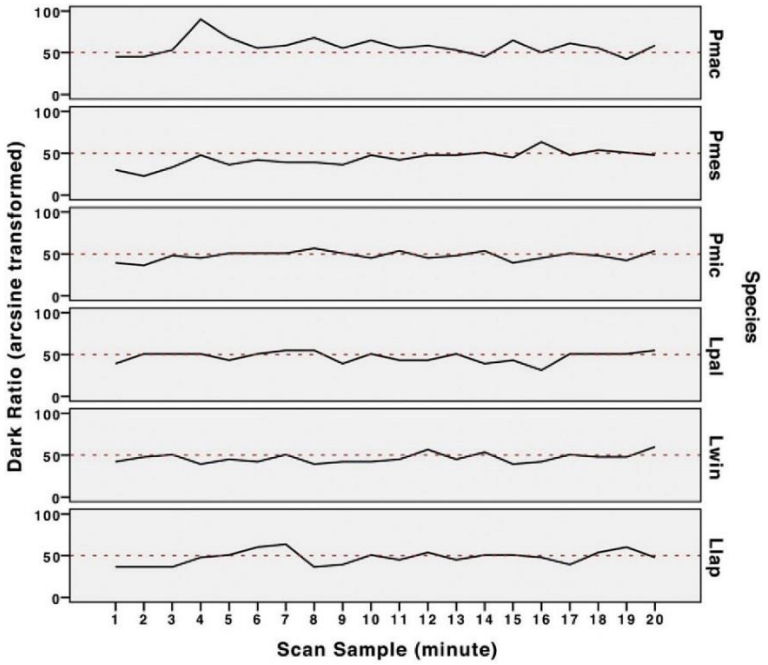

Fig. 3. Longitudinal plot of dark ratio at each scan sample (r_s). The null assumption of no hemispheric preference ($r = 0.5$) is indicated by a red stippled line. Abbreviations as follows: *Paroster macrosturtensis*, Pmac; *P. mesosturtensis*, Pmes; *P. microsturtensis*, Pmic; *Limbodessus palmulaoides*, Lpal; *L. windarraensis*, Lwin; *L. lapostaae*, Llap. [Colour figure can be viewed at wileyonlinelibrary.com]

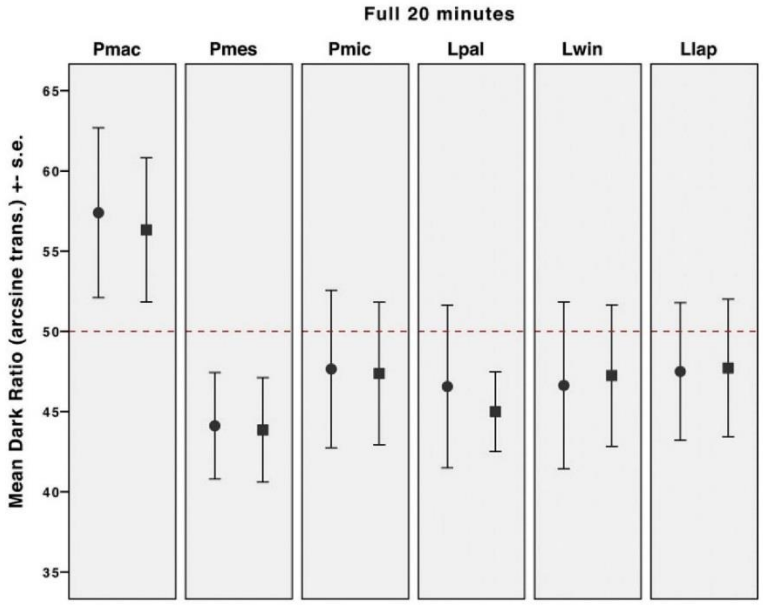

Fig. 4. Mean dark ratios over the full observation period (20 min) for each species. The null assumption of no hemispheric preference ($r = 0.5$) is indicated by a red stippled line; r_s denoted by circles; r_t denoted by squares; error bars represent standard error of the mean. Name abbreviations found in Figure 3. [Colour figure can be viewed at wileyonlinelibrary.com]

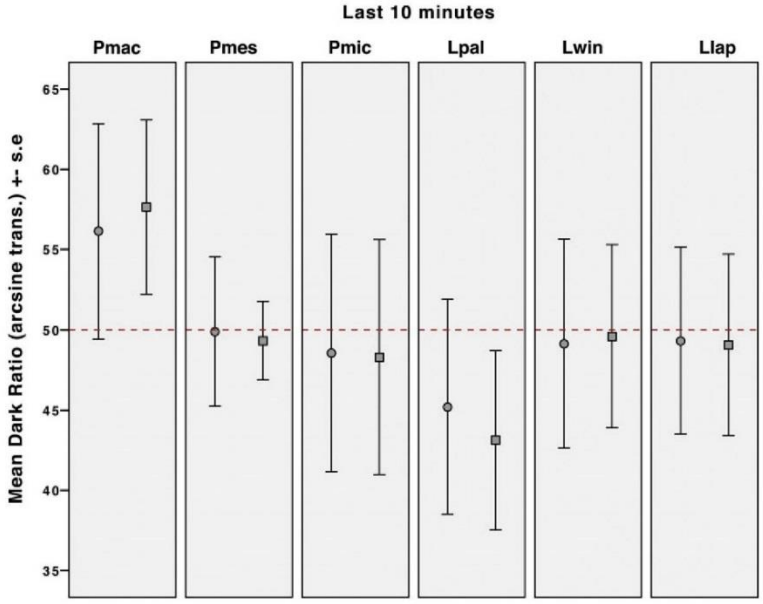

Fig. 5. Mean dark ratios over the last half of observation period (last 10 min) for each species. The null assumption of no hemispheric preference ($r = 0.5$) is indicated by a red stippled line; r_s denoted by circles; r_t denoted by squares; error bars represent the standard error of the mean. Name abbreviations found in Figure 3. [Colour figure can be viewed at wileyonlinelibrary.com]

curious as *P. macrosturtensis* appears to be completely eyeless, although histological investigations of the presence or absence of internal eye structures has not been carried out to date.

Eyes are not the only structures able to perceive light, and it is possible that extraocular photoreceptors could be responsible for light detection in *P. macrosturtensis*. The latter was the case in a

study on the eyeless crayfish, *Orconectes australis packardi*, which like marine decapods can perceive light through the caudal ganglion of the brain (Wilkens & Larimer 1976). Similarly, the larvae of *Drosophila melanogaster* have also displayed light mediated responses that have been linked to simple neurons (Xiang *et al.* 2010). The brain is thought to be the source of phototactic perception in the eyeless cave amphipods, *Niphargus frasassianus*, *N. ictus* (Borowsky 2011) and other *Niphargus* species (Fišer *et al.* 2016). However, the location of photoreception has yet to be examined in these groups. The cave beetle, *Ptomaphagus hirtus*, has negative phototactic responses that may be linked to their very small (~120 μm width), highly regressed visual system (Friedrich *et al.* 2011). Support for this link is based on the presence of phototransduction protein machinery transcripts in the head of *P. hirtus* (Friedrich *et al.* 2011). Extraocular photoreceptors have been uncovered in the optic lobes and within the ventral nerve cord of arthropods (Fleissner & Fleissner 2003) and may be the location(s) of light perception in the eyeless dytiscids from Western Australia.

Paroster macrosturtensis has a significant but not strong reaction to light. On average, individuals spend more time in the dark, but they do not have the extreme reactions that some cave species have that possess highly regressed eyes. Cave beetle individuals of *P. hirtus* were found to retreat into the dark after 2 min and not return to the light in 70% of cases (translated to 17% of the overall time spent in the light; Friedrich *et al.* 2011). Cavefish *Phreatichthys andruzzii* (among many other cavefish; Ercolini & Berti 1975 and references therein) have strong negative phototaxis behaviour, residing in the light only 12.5% of the overall time (Ercolini & Berti 1975). The degree of reaction to light is notably less severe in *P. macrosturtensis* and may represent a transition zone to completely losing the negative phototaxis behaviour.

The data presented here mostly agree, but still conflict overall with the theory stated earlier, that species with no access to light are expected to exhibit no phototactic ability (Langecker 2000). The results for *P. macrosturtensis* imply evidence of maintained phototaxis after considerable periods of evolutionary time in an aphotic habitat, because these dytiscids are estimated to have been isolated within the subterranean calcretes for at least 3 Myr (Leys *et al.* 2003). In comparison, amblyopsid cavefish have been isolated from surface lineages for ~2.7 Myr (Niemiller *et al.* 2012) and *Astyanax* cavefish diverged somewhere between 1.8 and 4.5 Mya (Gross 2012). However, a notable environmental difference between the Australian dytiscid beetles and North American cave fish is that the latter live in open cave systems. Surface morphs of a type of cavefish exhibited the expected negative phototaxis response when exposed to a dark and light zone and for a variety of light intensities (Timmermann & Plath 2009). Additionally, a study on cave beetle phototaxis included a eutroglophile species (species able to sustain stable subterranean populations) with a full visual system, *P. cavernicola*, with strong evidence supporting their preference for the dark hemisphere over the light (Friedrich *et al.* 2011).

We posit that an ancestor to the three *Paroster* species in the Sturt Meadows calcrete may have resided in interstitial spaces in river gravel (Watts *et al.* 2016), where it may have developed negative phototaxis, prior to their colonization of the subterranean groundwater within the calcrete. The aridification of the Australian continent, which deepened during the Late Pliocene following a wet–warm period in the early Pliocene (Sniderman *et al.* 2016), may have favoured the evolutionary survival of aquatic beetle lineages with negative phototactic behaviour. Following the evolution of the three stygobitic *Paroster* species within the calcrete (Leijs *et al.* 2012), we suggest that two of the species lost phototaxis, while it was retained in *P. macrosturtensis*. Similarly, the three *Limbodessus* species studied here have also likely lost phototaxis, conforming to the expected subterranean adaptations.

What is the role of maintained phototactic perception in a lineage that has been absent from the light potentially for over 3 Myr? There may be previously unknown benefits to maintaining phototaxis, or a pleiotropic function that is maintained by selection. However, it may also be possible that behaviour has remained unchanged purely due to chance (i.e. the genetic pathway responsible for a phototactic behaviour has not been lost through the accumulation of new mutations). A neutral theory explanation posits that the loss of traits that are no longer required result from the accumulation of random mutations in genes that are no longer under purifying selection (Kimura 1984; for a review, see Wilkens 2010). Additional investigations are required to deduce how *P. macrosturtensis* is physiologically able to perceive light, possibly through some unknown extraocular structure, which may shed light on the retention of light perception in a completely lightless environment. Additionally, molecular studies into the functionality of eye and vision genes, such as opsins, arrestins and G-proteins, from various locations in the body could indicate the pathways involved in retaining the perception of light. We are currently undertaking this project using a hybrid enrichment method to capture and sequence genes involved in eye transduction, which will provide additional evidence from a functional genetic perspective. The subterranean calcrete system is ideal for these studies because we can test theoretical predictions in an integrative and comparative manner.

CONCLUSIONS

Behavioural experiments for five subterranean species inhabiting two isolated calcretes of Western Australia did not display any preference for the light or dark, as expected, in contrast to *Paroster macrosturtensis*, which surprisingly showed negative phototaxis. The results for *P. macrosturtensis* contradict the hypothesis put forth by Langecker (2000) that animals separated from the light will have no phototactic ability. An ancestor to *P. macrosturtensis* likely evolved the trait prior to entering the calcretes, as an adaptation to living in an interstitial environment. Future studies investigating different light intensities and also wavelengths may provide a better understanding of how *P. macrosturtensis* perceive light (e.g. as per Fišer *et al.* 2016), and we have now provided a behavioural index (dark ratio) so that any subsequent data can be directly compared to the current study. In addition, a comprehensive investigation into dytiscid

eye anatomy (i.e. to determine whether they have any eye remnants under their apparently eyeless exterior) may also aid in understanding how phototaxis occurs in this ancient subterranean lineage.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Appendix S1 Raw data table where species 1 is *P. macrosturtensis*, 2 is *P. mesosturtensis*, 3 is *P. microsturtensis*, 4 is *L. palmulaoides*, 5 is *L. windarraensis*, and 6 is *L. lapostaae*.
Appendix S2 Raw data table solely containing the final 10 minutes of observation where species 1 is *P. macrosturtensis*, 2 is *P. mesosturtensis*, 3 is *P. microsturtensis*, 4 is *L. palmulaoides*, 5 is *L. windarraensis*, and 6 is *L. lapostaae*.

Due to its size, Appendix 1 and 2 can be found at the end of this thesis in Appendix 3, for easier reading.

Chapter 3: Evidence for speciation underground in diving beetles (Dytiscidae) from a subterranean archipelago

Statement of Authorship

Title of Paper	Evidence for speciation underground in diving beetles (Dytiscidae) from a subterranean archipelago
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Name of Principal Author (Candidate)	Barbara Langille
Contribution to the Paper	Collection of samples, bulk of laboratory work, editing of data, data analysis, interpretation of the data, writing and editing the manuscript.
Overall percentage (%)	80
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: July 15, 2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Steven Cooper
Contribution to the Paper	Collection of samples, co-manuscript planning, editing the manuscript.
Signature	Date: 24/7/2019

Name of Co-Author	Simon Tierney
Contribution to the Paper	Strategic planning for targeted genes, data analysis, interpretation of data, editing the manuscript.
Signature	Date: 18 JULY 2019

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Josephine Hyde		
Contribution to the Paper	Shotgun sequencing		
Signature		Date	9/8/2019

Name of Co-Author	Kathleen Saint		
Contribution to the Paper	Laboratory work		
Signature		Date	9/8/2019

Name of Co-Author	Andrew Austin		
Contribution to the Paper	Editing the manuscript.		
Signature		Date	12/8/19

Name of Co-Author	William Humphreys		
Contribution to the Paper	Editing the manuscript.		
Signature		Date	8/8/2019

Letter

Evidence for speciation underground in diving beetles (Dytiscidae) from a subterranean archipelago

Barbara L. Langille[1], Josephine Hyde[1,2], Kathleen M. Saint[1], Simon M. Tierney[3], William F. Humphreys[4], Andrew D. Austin[1], Steven J.B. Cooper[1,5]

[1]Australian Centre for Evolutionary Biology and Biodiversity, Department of Ecology and Evolutionary Biology, School of Biological Sciences, University of Adelaide, South Australia 5005, Australia.
[2] Department of Environmental Sciences, The Connecticut Agricultural Experiment Station, New Haven 06511 CT, USA.
[3]Hawkesbury Institute for the Environment, Western Sydney University, Locked Bag 1797 Penrith NSW 2751, Australia.
[4]Western Australian Museum, Locked Bag 40, Welshpool DC, WA 6986, Australia.
School of Animal Biology, University of Western Australia, Nedlands, Western Australia.
[5]Evolutionary Biology Unit, South Australian Museum, North Terrace, Adelaide, South Australia 5000, Australia.

Short running title: Underground speciation of subterranean beetles

Keywords: subterranean animals, radiation underground, climate relict, adaptive shift, assortative mating, repeated colonization, neutral evolution, long wavelength opsin, Australian diving beetles, sympatric speciation

Abstract

Most subterranean species are assumed to have evolved from surface ancestors following colonisation of a cave system, and there are few confirmed cases of speciation in underground habitats or sympatric speciation within a cave. Numerous endemic subterranean diving beetle species, from the calcrete archipelago in Western Australia have independently evolved following colonisation by surface ancestors. However, the presence of sympatric sister species raises the possibility of speciation underground within a single calcrete aquifer. We explored this hypothesis by using the neutrally evolved gene, long wavelength opsin (*lwop*) from 32 subterranean and surface species in the genus *Paroster*. We identified a unique 18 bp deletion and a missense mutation leading to a stop codon in the long wavelength opsin gene (*lwop*) that was shared between a subterranean sister-species triplet, and a 2 bp insertion in *lwop* shared by a pair of species from adjacent calcretes. In both these cases, a common ancestor likely had these vision-gene altering mutations, implying that their ancestor was already adapted to living underground. The analyses of genes undergoing pseudogenisation, such as opsin genes in blind cave animals, provides a unique way of testing modes of speciation in subterranean animals.

Introduction

The Climate-relict (CRH) and adaptive-shift hypotheses (ASH) are the two most common hypotheses used to explain speciation of subterranean animals (Howarth 1973; Holsinger 2000; Rivera et al. 2002; Wessel et al. 2007). CRH was first used to describe the evolution of cave species from continental temperate ecosystems (Holsinger 1988, 2000; Peck and Finston 1993), whereby surface species colonized cave environments and became isolated from surface populations when climatic fluctuations, such as glaciation or aridification, rendered surface populations extinct, a process of allopatric speciation. ASH was first used to describe a diverse tropical cave fauna, where cave species were found alongside closely related surface species (Howarth 1987; Rouch and Danielopol 1987; Desutter-Grandcolas and Grandcolas 1996). Under the ASH, cave species evolved by active colonisation of cave environments and divergent natural selection in parapatry with surface populations, ultimately resulting in reduced gene flow and parapatric speciation. CRH and ASH explain many cases of cave species evolution, however, the presence of sympatric sister species in some cave taxonomic groups raises the question of whether speciation may potentially occur underground from cave ancestors (Guzik et al. 2009; Faille et al. 2010; Ribera et al. 2010; Leijs et al. 2012).

Previous research suggested cave species would be unlikely to undergo adaptive evolution underground because of reduced genetic and phenotypic variation, and, therefore, were unlikely to speciate inside cave systems (Poulson and White 1969). However, since the advancement of molecular research, it has been shown that cave species may have considerable genetic diversity as a consequence of range and population expansion (Barr 1968; Stepien et al. 2001; Buhay and Crandall 2005; Finlay et al. 2006; Lejeusne and Chevaldonné 2006; Guzik et al. 2009). Few studies have examined the post-colonisation speciation process within subterranean systems. Sympatric cave spider

(*Dysdera*) species of the Canary Islands have segregation of body types (i.e. small, medium, and large body size) and cheliceral modifications, leading authors to conclude prey specialization (Arnedo et al. 2007). This prey specialization along with genetic and morphological similarity (in the specific case of *D. hernandezi* and *D. esquiveli*), suggest they evolved underground in sympatry (Arnedo et al. 2007). Studies based on beetles (Leiodidae and Trechinae; Faille et al. 2010; Ribera et al. 2010) have uncovered large monophyletic lineages of cave taxa, implying speciation underground rather than multiple colonisations by surface ancestors, the latter being the traditionally accepted theory. The problem with this, and other such studies, is that it is difficult to confirm speciation underground using only phylogenetic methods due to the possibility that the apparent monophyly of subterranean taxa resulted from extinction of their surface ancestors (e.g. resulting from climatic events such as glaciations; Juan et al. 2010).

In Western Australia, a huge network of groundwater calcretes can be found, which are ~10m thick carbonate deposits formed by the evaporation of water within palaeodrainage channels (Humphreys 2001). These calcretes contain a diverse assemblage of subterranean invertebrates (Humphreys 2006, 2008 and references therein; Boulton 2009; Humphreys et al. 2009), including the world's most diverse group of subterranean diving beetles (Coleoptera, Dytiscidae) (Balke et al. 2004). The subterranean diving beetles are short range endemics with over 100 described species in more than 45 individual calcretes (Watts and Humphreys 2009 and references herein). Each isolated groundwater system contains beetle species which have evolved distinct size variation (small, medium and/or large), with one to five beetle species per calcrete (Watts and Humphreys 2009 and references therein). Molecular clock analyses suggest that beetle species have been isolated underground for 3 to 10 million years (Leijs et al. 2012), likely following a period of aridity in Australia (Sniderman et al. 2016). Each beetle species has evolved typical cave traits observed in many subterranean systems; notably eye and

pigmentation loss, and they respire directly from the water (Watts and Humphreys 2009; Jones et al. 2019).

The subterranean beetle system provides an interesting case for study, because while ~75% of species evolved independently from surface ancestors, likely via the climate-relict hypothesis, ~25% did not (Cooper et al. 2002; Leys et al. 2003; Leijs et al. 2012); there are 13 known cases of sympatric sister species (Leijs et al. 2012). These sister species have segregation of body types, which suggests niche partitioning within calcretes, and the possibility that they evolved underground via sympatric speciation (Cooper et al. 2002; Leys et al. 2003; Leys and Watts 2008). However, it is also possible that they evolved following multiple colonisation events from the same surface ancestor or a related species. Mathematical modelling suggests evolution underground to be more plausible than the latter hypothesis, particularly for the case of sympatric sister triplets (Table 1 for sister species groups; Leijs et al. 2012). However, the mathematical model relied upon the assumption that surface ancestors were widespread, which may not be the case if species evolved from an interstitial ancestor that remained in the vicinity of the calcrete after each colonisation event. It is also possible that mitochondrial DNA (mtDNA) introgression among related species may account for the apparent monophyly of species within a calcrete (Hubbs 1955; Taylor and McPhail 2000; McDonald et al. 2008; Langille 2014).

Here we apply a phylogenetic and gene discovery approach to investigate the hypothesis that sympatric sister species in the genus *Paroster* speciated underground from a stygobiont ancestor. Specifically, we aim to: 1) confirm the sister species status of these taxa using phylogenetic analyses of nuclear gene markers, and 2) investigate the molecular evolution of a predicted neutral gene marker (long wavelength opsin gene) to determine whether there are shared mutations indicative of a relaxation of selection in the common ancestor of sympatric sister species.

Methods

Taxon collection

DNA samples of 28 subterranean diving beetle species and four surface species from the genus *Paroster* (Coleoptera; Dytiscidae), were obtained from South Australia Museum (SAM) and Western Australia Museum (WAM) collections (Table 1). DNA from the subterranean dytiscid species *Limbodessus palmulaoides* was used as an outgroup for phylogenetic analyses. The subterranean species represented in these analyses were originally collected from 15 different calcretes representing six separate palaeodrainages (Table 1; Fig. 1).

Table 1: List of all subterranean species used in this study with location information

Drainage	Palaeovalley	Calcrete	Species	Field number	ABTC
Western	Gascoyne	Milgun Station	*P. hamoni*	R055	78583
			P. milgunensis	R054	78582
		Three Rivers Station	*P. plutonicensis*	R402	78975
	Lyons	Mount Augustus	*P. tetrameres*	R211	78765
	Murchison	Bryo West	*P. arachnoides*	R106	78660
			P. byroensis	R124	78678
			P. dingbatensis	R121	78675
		Innouendy	*P. copidotibae*	R120	78674
			P. innouendyensis	R118	78672
		Karalundi	*P. skaphites*	R116	78670
			P. stegastos	R148	78702
		Moorarie	*P. verucosus*	R128	78682
		Moorarie Bin Bin	*P. bulbus*	R105	78659
Inland	Carey	Melrose Station	*P. darlotensis*	R345	78918
			P. melrosensis	R346	78919
	Ngalia Basin: N.T.	Central Mount Wedge	*P. spnMtWedge*	R357	78930
			P. wedgeensis	R233	78787
		Napperby	*P. macrocephalus*	R038	78566
			P. napperbyensis	R064	78592
		Newhaven Camel Well	*P. pentameres*	R224	78778
			P. spnCamelWell	R358	78931
		Newhaven Homestead	*P. newhavenR401*	R401	78974
			P. newhavenensis	R063	78591
	Raeside	Pinnacles Station	*P. elongates*	R026	78554
			P. fortispina	R179	78733
		Sturt Meadows	*P. macrosturtensis*	R271	78844
			P. mesosturtensis	R354	78927
			P. microsturtensis	R352	78925
Surface	VIC	18km W Casterton	*P. gibbi*	R061	78589
	WA	Camel Stock	*P. michaelseni*	R135	78689
	WA	6km S Pinjarra	*P. niger*	R372	78945
	SA	12km N Forreston	*P. nigroadumbratus*	-	78584
Inland	Carey	Mount Windarra	*L. palmulaoides*	-	-

*Sympatric sister species, based on mtDNA analyses, are highlighted in grey; the outgroup, *L. palmulaoides* sample is from Hyde et al. 2018. ABTC refers to the Australian Biological Tissue Collection at the South Australian Museum.

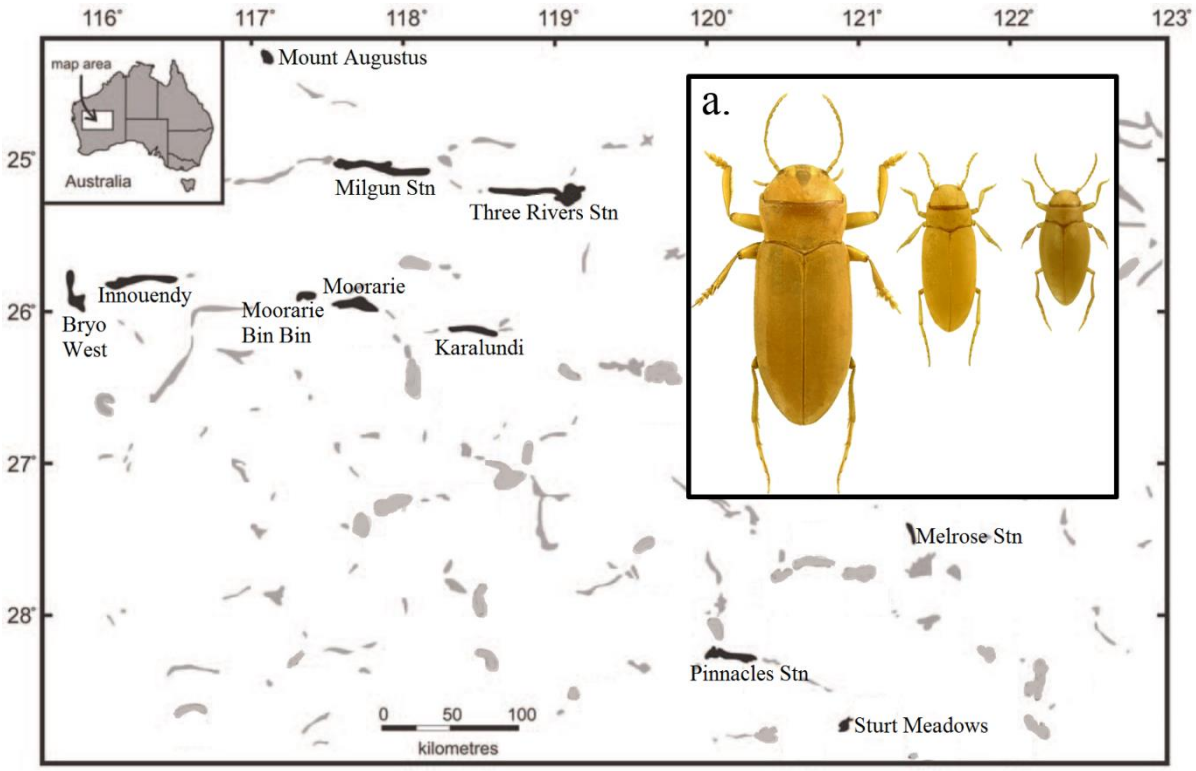

Figure 1: All calcrete locations in Western Australia used in this study highlighted in black with inset a. representing a sympatric sister triplet of species from the Sturt Meadows calcrete: *Paroster macrosturtensis*, *P. mesosturtensis*, and *P. microsturtensis*, in descending order.

Shotgun sequence data for primer design

Whole genomic shotgun libraries generated by Hyde et al. (2018), produced 12.2 million sequences from *L. palmulaoides*, 14.6 million from *P. macrosturtensis*, 1.0 million from *P. mesosturtensis*, and 4.6 million from *P. microsturtensis*. The raw data from each species were BLASTn analysed for long wavelength opsin (*lwop*) using full length *lwop* cDNA sequence data derived from the surface species *Paroster nigroadumbratus* (Tierney et al. 2015). We chose *lwop* as it is a photoreceptor gene specifically involved in the regressed vision trait in subterranean beetles and therefore, it is predicted to be evolving neutrally (i.e. it may have protein code altering missense and/or nonsense mutations). *Lwop* genomic sequences were identified from *P. macrosturtensis* (one sequence of length 476 bp) and *P. microsturtensis* (four overlapping sequences with a total length of 476 bp). These *Paroster lwop* sequences were aligned with cDNA sequence data from *P. nigroadumbratus* in

Geneious v.10.2.6 (Kearse et al. 2012) using default settings in the plugin ClustalW (Larkin et al. 2007). Assembled *lwop* fragments revealed a protein altering 18 base pair deletion and stop codon shared by the sister species *P. macrosturtensis* and *P. microsturtensis*, not found in the surface species. Primers were designed from conserved *lwop* exon regions of each species: forward (labelled G2743) 5'-GAAGAATATGCGAGAACAGG-3' and reverse (labelled G2744) 5'-GGCAAGRGGAGTGATGTTC-3', with a melting temperature of 50^{O}C and 52^{O}C respectively (Table 2).

Table 2: All primers for each gene used in this study. SAM – South Australian Museum

Locus name	Forward (5' to 3') followed by Reverse (3' to 5')	Primer symbol	Ta (^{o}C)	Reference
argk	F-GATTCTGGAGTCGGNATYTAYGCNCCYGAYGC R-GCCNCCYTCRGCYTCRGTGTGYTC	AK183F AK939R	53	Wild & Maddison 2008
cn	F-AAYTAYYTNCAYATHTGGCC R-RTARTTRTACATNGC	- -	48.8	Lorentzen et al. 2002
	F-ACNTTYATGATGATHGC R-TCCAYRTAATTRTACAT5GCCAR5TC	- -	48.8	Lorentzen et al. 2002
COI	F-CAACATTTATTTTGATTTTTTGG R-TCCAATGCACTAATCTGCCATATTA	PatCOIF PatCOIR	48	Simon et al. 1994
lwop	F-GAAGAATATGCGAGAACAGG R-GGCAAGRGGAGTGATGTTC	G2743 G2744	65x10, 55x20	Kathy Saint (SAM)
topo	F-GAGGACCAAGCNGAYACNGTDGGTTGTTG R-GGWCCDGCATCDATDGCCCA	TP675F TP932R	55	Wild & Maddison 2008
wg	F-ATGCGTCAGGARTGYAARTGYCAYGGYATGTC R-CACTTNACYTCRCARCACCARTG	Wg550F WgAbR	53	Wild & Maddison 2008

Ta = annealing temperature

Laboratory methods, sequence editing and alignment

The following gene regions were chosen for amplification: wingless (*wg*), topoisomerase (*topo*), arginine kinase (*argk*), cinnabar (*cn*), cytochrome c oxidase I (*COI*), and long wavelength opsin (*lwop*) (see Table 2 for primer information). Standard PCR amplification included 1x PCR buffer (Applied Biosystems), 0.2 mM of each dNTP, 6 pM of each primer, and 0.5 U of immolase enzyme (Bioline Reagents Ltd) in a 25 µL reaction volume. PCRs were carried out on an Eppendorf Thermal Cycler for (every gene except *lwop*) 1

cycle of 95ºC for 10 minutes (min) (5 min for *COI*), followed by 35 cycles of 95ºC for 30 seconds (s), primer specific temperature (Table 2) for 30 s, and 72ºC for 90 s (45 s for *COI*). Each profile ended with a final incubation step at 72ºC for 10 min (5 min for *wg* and *topo*) and 25ºC for 1 min. The *lwop* gene consisted of a touchdown profile, with 1 cycle of 95ºC for 10 min, followed by 10 cycles of 95ºC for 30 s, 65ºC for 30 s, 70ºC for 45 s, and 25 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 45 s, with a final extension of 72ºC for 10 min and 25ºC for 1 min. PCR products were purified using a Multiscreen 384 vacuum well PCR plate (Millipore Sigma). Sequencing was performed using the ABI Prism BigDye Terminator Cycle sequencing kit (PE Applied Biosystems) with 10 μL reaction volumes according to manufacturer's protocol, and subsequently followed by a clean-up using a Multiscreen 384 vacuum well SEQ plate (Millipore Sigma). Sequencing reactions were sent to Australian Genome Research Facility (AGRF, Adelaide, Australia) who used capillary separation and AB GeneMapper software to generate sequence data.

All sequences were edited and aligned, by gene, in Geneious v.10.2.6 using default settings in the plugins Muscle (Edgar 2004) and ClustalW. All sequences were verified using NCBI's (https://www.ncbi.nlm.nih.gov/) BLASTn program. Additional gene regions of *COI* and *lwop* were sourced from NCBI and included in all further analyses (Supplementary Table 1). Sequence data from the outgroup, *L. palmulaoides*, were sourced from Genbank and genomic data (Hyde et al. 2018; Hyde et al. unpublished; Supplementary Table 1) for all six genes used in this study.

Phylogenetic analyses

Bayesian phylogenies were constructed using MrBayes v.3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) for each gene, for all genes concatenated, and all nuclear genes concatenated. The two concatenated gene alignments were analysed using PartitionFinder2 v. 2.1.1 (Guindon et al. 2010; Lanfear et al. 2012; Lanfear et al. 2016) in

Anaconda v.2.0 – Python v.2.7 (https://anaconda.com; https://docs.python.org/release/2.7/) to find an optimum partitioning scheme (Table 3). We used these partitions and models (General Time Reversable- GTR) in MrBayes to construct a 50% posterior probability tree, derived from two independent runs of four chains (one cold) and using a burn-in of 25%. Noninformative priors, unlinked parameters, and variable rates were all selected. The convergence of runs was assessed by identifying values of effective sample size (ESS), viewing likelihood plots, and by assessing the average standard deviation of split frequencies. TreeAnnotator v.2.4.7 was used to compile all trees and derive a maximum clade credibility tree. Final gene trees were viewed and prepared for publication in FigTree v.1.4.3 (Rambaut 2012).

Table 3: Optimum partitioning scheme for concatenated gene alignments

Type of data	Full dataset	Nuclear dataset
Number of partitions	3	4
Ideal model	**1** GTR+I+G **2** GTR+G **3** GTR+I+G	**1** GTR+I+G **2** GTR+G **3** GTR+I+G **4** GTR+G
Partitions composed of	**1** *topo*-pos1, 2/*argk*-pos1, 2 **2** *topo*-pos3/*argk*-pos3/*lwop*-pos2, 3/*cn*-pos2, 3 **3** *wn*-pos1, 2, 3/*lwop*-pos1/*cn*-pos1	**1** *COI*-pos1, 3/*argk*-pos2 **2** *COI*-pos2 **3** *topo*-pos1, 2/*wn*-pos1, 2, 3/*argk*-pos1/*lwop*-pos1, 2/*cn*-pos-1, 2 **4** *topo*-pos3/*argk*-pos3/*lwop*-pos3/*cn*-pos3

pos = reading frame position

Lwop

An alignment of *lwop* was generated by assembling all available sequences using Geneious and default settings in the plugins Muscle (Edgar 2004) and ClustalW. The *lwop* alignment was used to visually identify indels (insertions and deletions) and stop codons shared between different species. We mapped all shared *lwop* indels and nonsense

mutations between sister species to both phylogenies (nuclear genes only and all genes), which included all species used in this study.

Results

Phylogenetic analyses

A total of 137 new nuclear gene sequences of 3407 bp (1135 amino acids) total, was produced for 32 species of *Paroster* diving beetles (Supplementary Table 2). A total of eight trees were generated, one per each gene, and a concatenated data set with and without *CO1*, which contained most subterranean *Paroster* species from calcretes in Western Australia (Fig. 2; Fig. 3; Sup. 1).

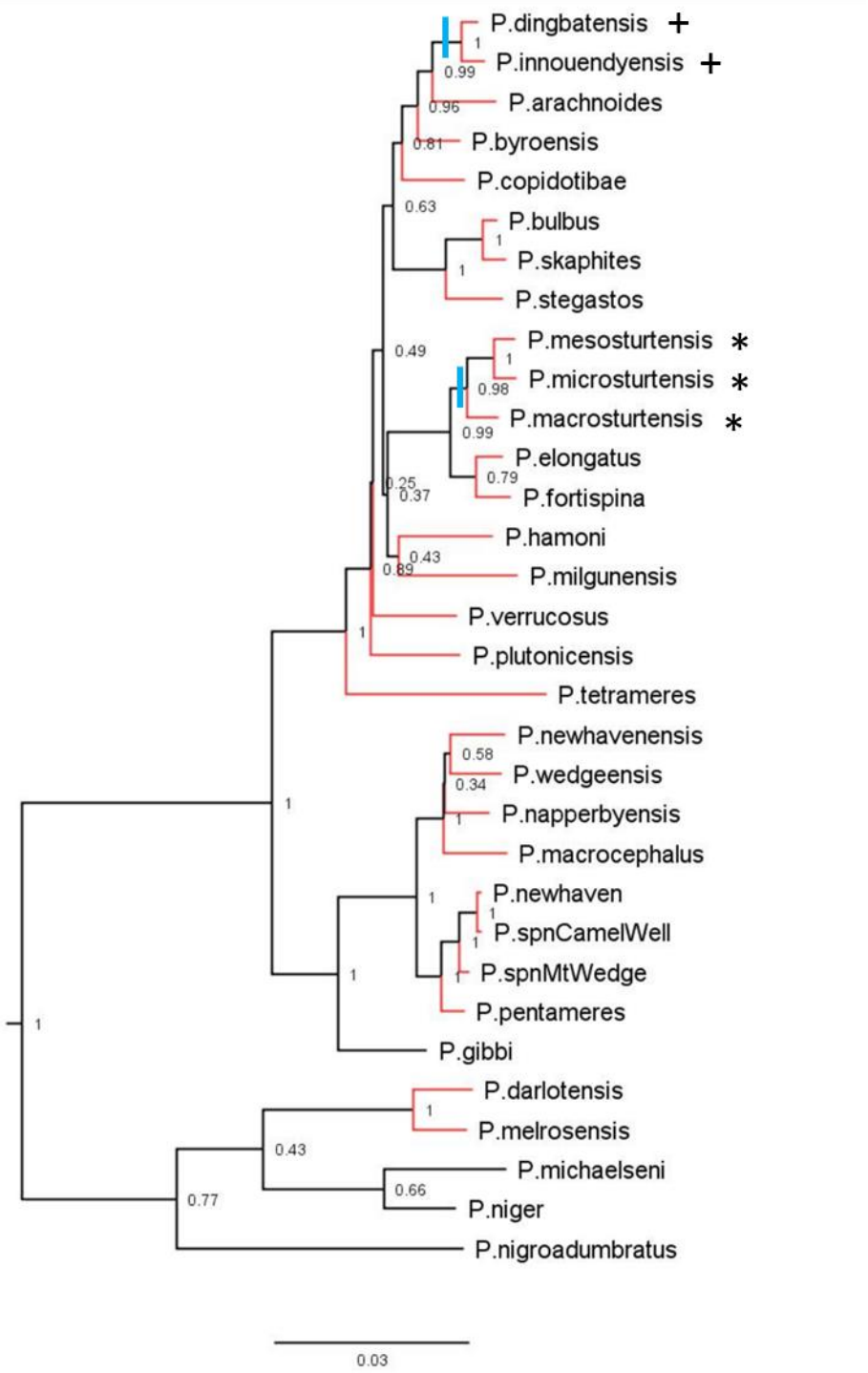

Figure 2: Bayesian phylogenetic tree based on all concatenated nuclear genes with posterior probability support values on nodes. * indicates species with a shared 18 bp deletion and stop codon mutation in their *lwop* genes (see Fig. 4), while + indicates species with a shared 2 bp insertion (GC). A blue mark indicates the branch where the mutation must have occurred, based on analyses of related taxa. Red branches indicate a subterranean species, while black indicate a surface species. Tree was rooted with the outgroup *L. palmulaoides*. Species with calcrete information found in Table 1 and Figure 1.

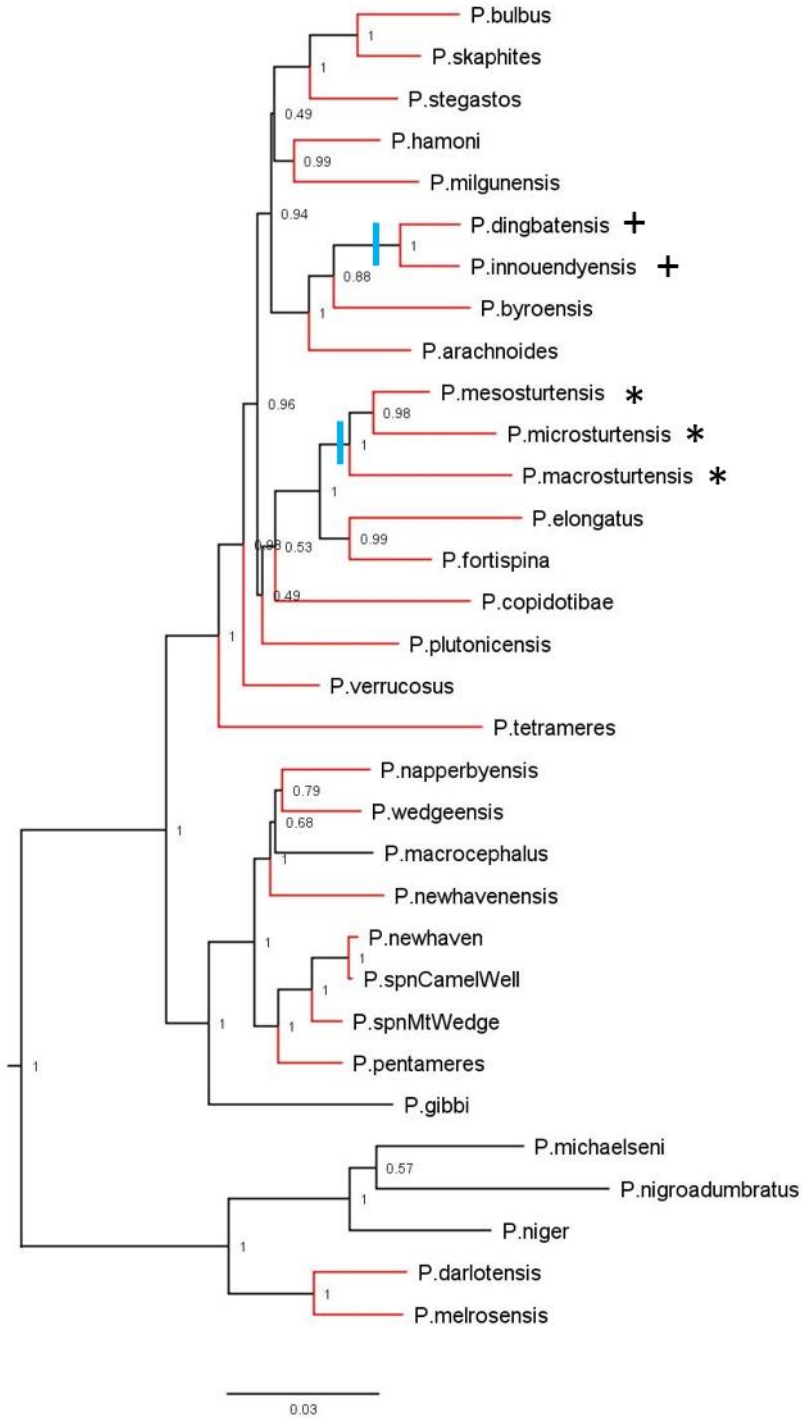

Figure 3: Bayesian phylogenetic tree based on all concatenated genes, including *COI*, with posterior support on nodes. ***** indicates locations with 18 bp deletion and stop codon mutation, while + indicates locations with 2 bp insertion (GC). A red mark indicates the branch where the mutation must have occurred. Red branches indicate a subterranean species, while black indicate a surface species. Tree was rooted with the outgroup *L. palmulaoides*. Species with calcrete information found in Table 1 and Figure 1.

Only one group of sympatric sister species were strongly supported phylogenetically on all phylogenies. The three species from Sturt Meadows (*P. macrosturtensis*, *P. mesosturtensis*, and *P. microsturtensis*) were shown to be monophyletic with a posterior probability of > 0.80. The two species from Milgun Station (*P. hamoni* and *P. milgunensis*), which were shown to be monophyletic in a previous study (Leijs et al. 2012) were supported in most single gene phylograms where we had data for both species (Supplementary Table 2) with a posterior probability of > 0.58. *Paroster hamoni* and *P. milgunensis* were also supported in both phylograms based on concatenated datasets (Figure 2, 3) with a posterior probability of 0.43 (nuclear gene phylogeny) and 0.99 (full concatenated gene phylogeny).

lwop analyses

We sequenced a fragment of *lwop* (204 bp) from a total of 13 different subterranean species and two surface species (Fig. 4). In addition to the sympatric sister species triplet from Sturt Meadows, we acquired *lwop* data for the phylogenetically close *P. fortispina*, *P. copidotibae*, *P. verrucosus, P. plutonicensis*, and *P. stegastos*, as well as the monophyletic *P. dingbatensis* and *P. innouendyensis*, and more phylogenetically distant species *P. wedgensis, P.darlotensis*, and *P.melrosensis*. Upon visual inspection of the *lwop* gene alignment, several shared mutations were revealed (Fig. 4). The sympatric sister species triplet *P. macrosturtensis*, *P. mesosturtensis*, and *P. microsturtensis* shared an 18 bp deletion at position 97 in the alignment as well as a mutation at position 124 that resulted in a stop codon in *P. macrosturtensis* and *P. microsturtensis* and a loss of 88 amino acids (*P. mesosturtensis* had a deletion of 11 bp at position 59, resulting in a frameshift mutation that changed the reading frame of the protein, and removed the stop codon). *Paroster dingbatensis* and *P. innouendyensis* also shared a 2 bp insertion (GC) at position 121, the latter leading to a frameshift in the encoded protein and a stop codon at position 152. All

shared mutations were mapped to the nuclear phylogeny (Fig. 2). Both sets of mutations were not found in other related species outside the monophyletic groups and, therefore, mapped to the most recent common ancestor of each group. We also found significant changes in *lwop* of other subterranean species (Fig. 4). In *P. verrucosus*, we found an insertion of 3 bp at position 34 and a deletion of 9 bp at position 76. In *P. copidotibae*, we found a mutation from G to A (second nucleotide in codon) at position 125 that resulted in a stop codon, and a deletion of 1 bp at position 180. Finally, we found a deletion of 30 bp at position 143 in *P. stegastos*, and a deletion of 1 bp at position 88 in *P. plutonicensis*. A total of seven species had stop codons in this small 204 bp region of *lwop*, resulting in a truncation of the encoded protein. It was difficult to PCR-amplify *lwop* for many of the subterranean species, with only 13 out of 28 successful (Supplementary Table 2). A total of nine subterranean species had major indels and nonsense mutations in *lwop* sequences (see Fig. 4), which we attributed to relaxed selection. It is, therefore, likely that the low success rate for *lwop* PCR-amplifications resulted from primer sites having too much sequence variation to allow annealing of the primers.

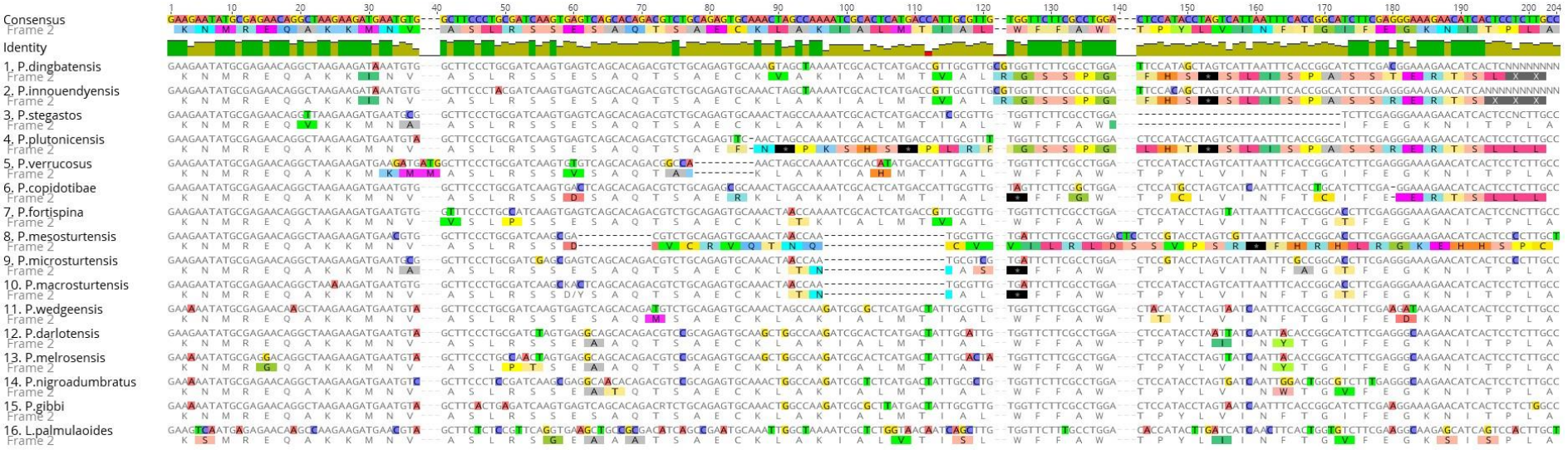

Figure 4: Alignment of long wavelength opsin with *L. palmulaoides* reference from Geneious.

Discussion

The climate-relict and adaptive shift hypotheses, are the generally accepted theories for the evolution of cave species, and it is widely accepted that most subterranean species evolved directly from surface ancestors (Mayr 1963; Holsinger 1988, 2000; Coyne 1992; Rice and Hostert 1993; Peck and Finston 1993; Leys et al. 2003; Juan et al. 2010). Our study provides strong evidence to support the hypothesis that at least five subterranean diving beetle species evolved from a stygobiont ancestor that speciated underground. Using phylogenetic analyses of nuclear gene data, we confirmed the previously designated sympatric sister species in the genus *Paroster* (Leijs et al. 2012), a sister triplet at the Sturt Meadows calcrete and, in part, a sister pair from the Milgun Station calcrete (Fig. 2, 3). We also used a novel approach, analysing a phototransduction gene, *lwop*, that is evolving under neutral evolution (see chapter 4), and identified a unique deletion and nonsense mutation that were shared only by a sister species triplet from the Sturt Meadows calcrete. This finding provides strong evidence that their common ancestor was a stygobiont, living in darkness within the calcrete, and hence they evolved by speciation underground. Additionally, a shared two bp deletion in *lwop*, resulting in a frameshift mutation in the encoded protein, was shared by a pair of species from adjacent calcretes, providing additional support that their common ancestor was also a stygobiont.

Our study also raises the possibility that the sister species triplet from the Sturt Meadows calcrete evolved by a process of sympatric speciation. As proposed by Coyne and Orr (2004), a series of four criteria can be used to decide if species are likely products of sympatric speciation: 1) the species must be largely or completely sympatric, and, crucially, that they were sympatric at the time of speciation, 2) the species must have substantial reproductive isolation, 3) the taxa must be sister groups, but not from hybridisation, and 4) the biogeographic and evolutionary history of the groups must make the existence of an allopatric phase very unlikely. Previous studies have presented

evidence for support of the second criterion, with strong evidence from both genetic and morphological analyses for reproductive isolation of the Sturt Meadows species (Watts and Humphreys 2006; Guzik et al. 2009). The first criterion is also strongly supported here. The shared deletion and nonsense mutation suggest their ancestor was a stygobiont, living within the confines of the Sturt Meadows calcrete, and that the three species were sympatric at the time of speciation (Fig. 4). Phylogenetic analyses of nuclear gene data also support the third criterion, that the three species form a sister group, that is unlikely to have resulted from hybridisation and introgression of mtDNA among related species, as there was phylogenetic concordance for nuclear and mitochondrial data.

The final criterion for sympatric speciation, that the biogeographic and evolutionary history of the groups must make the existence of an allopatric phase very unlikely, is more difficult to support. Previous research showed evidence for fine-scale population structure in beetle species and amphipods within a 3.5 km^2 section of the Sturt Meadows calcrete, possibly due to fluctuating water levels and the heterogeneious nature of the calcrete, and therefore, the possibility of micro-allopatric speciation can not be entirely ruled out (Guzik et al. 2009; Bradford et al. 2013). However, these periods of allopatry are likely to have been relatively short, with initially wet conditions in the early Pliocene, followed by 20,000 year cycles of wet and dry phases to the Late Pliocene when speciation most likely occurred (Byrne et al. 2008; Leijs et al. 2012; Sniderman et al. 2016). Therefore, speciation with gene flow (parapatric or sympatric speciation) seems a more likely scenario. We suggest that divergent or disruptive selection likely drove size differences among beetles that simultaneously led to reproductive incompatibilities and assortative mating. Recent stable isotope analyses of the three Sturt Meadows beetles suggest that there are differences in their trophic niches (M. Sacco, Pers. Comm.), which may be associated with the disruptive selection.

Contrary to the results described above, we also found evidence of allopatric speciation underground for the common ancestor of *P. dingbatensis* and *P. innouendyensis*, each found in adjacent calcretes of the Murchison palaeodrainage system. Both species share protein altering mutations in *lwop* (Fig. 4), suggesting that their ancestor was a stygobiont. It is likely that past gene flow between the two calcretes (or fragmentation of a single calcrete into two calcretes) led to these two species having a common stygobiontic ancestor. This pattern of allopatric speciation is similar to that proposed for the evolution of numerous troglobiont taxa in the Leptodirini group of beetles (Faille et al. 2010; Ribera et al. 2010). Although it is difficult to entirely rule out the possibility that surface ancestors went extinct during glacial periods from the phylogenetic analyses conducted in these studies, our results suggest that speciation underground in these Pyrenean cave systems is entirely feasible. However, as explained by Leijs et al. (2012), despite speciation underground from a stygobiontic ancestor as the likely mode of speciation for the cases outlined above, we are unable to generalise this evolutionary mode for all other sympatric sister species groups of dytiscid beetles that are currently known.

Conclusions

Our study provides strong evidence that at least five subterranean beetle species have evolved underground from stygobiontic common ancestors. Our approach, using genetic markers that are subject to pseudogenisation through regressive evolution, provides a potentially powerful tool to unravel the nature of speciation in subterranean ecosystems. It also has the potential to enhance our understanding of evolutionary relationships and the biogeographic history of cave animals.

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Author contributions B.L.L. was responsible for majority of laboratory work, data editing, writing the manuscript, and incorporating all edits. J.H compiled shotgun data and found *lwop* sequences in the data. K.S. provided guidance with laboratory methods. S.M.T., W.F.H, and A.D.A provided edits to the manuscript. S.J.B.C. aligned and viewed shotgun *lwop* data, provided guidance with paper direction and scope, and edited the manuscript.

Data accessibility All data generated from this project can be found on NCBI's Genbank (www.ncbi.nlm.nih.gov) with the accession numbers available upon publication.

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Supplementary Data can be found in Appendix 4

Chapter 4: Darwin's dilemma: Neutral evolution drives vision-gene loss in blind beetles

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Name of Principal Author (Candidate)	Barbara Langille		
Contribution to the Paper	Collected samples, laboratory work (exon capture), edited data, analysed data, interpreted the data, wrote and edited the manuscript.		
Overall percentage (%)	60		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	July 15, 2019

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Steven Cooper		
Contribution to the Paper	Collected samples, co-planned manuscript, edited the manuscript.		
Signature		Date	24/7/2019

Name of Co-Author	Simon Tierney		
Contribution to the Paper	Generated transcriptomes, developed Hybrid-Capture (RNA baits) technique, edited the manuscript.		
Signature		Date	18 JULY 2019

Name of Co-Author	Terry Bertozzi		
Contribution to the Paper	Bioinformatics guidance and re-assembled transcriptome data, edited the manuscript.		
Signature		Date	9 August 2019

Name of Co-Author	Andrew Austin		
Contribution to the Paper	Edited the manuscript.		
Signature		Date	12/8/19

Name of Co-Author	William Humphreys		
Contribution to the Paper	Collected samples, edited the manuscript.		
Signature		Date	8/8/2019

Darwin's dilemma: Neutral evolution drives vision-gene loss in blind beetles

Barbara L. Langille [1*], Simon M. Tierney [1, 2], Terry Bertozzi [1, 3], William F. Humphreys [1, 4], Andrew D. Austin [1], Steven J. B. Cooper [1, 3]

[1]Australian Centre for Evolutionary Biology and Biodiversity, Department of Genetics and Evolution, School of Biological Sciences, University of Adelaide, South Australia 5005, Australia.
[2]Hawkesbury Institute for the Environment, Western Sydney University, Locked Bag 1797 Penrith NSW 2751, Australia.
[3]Evolutionary Biology Unit, South Australian Museum, North Terrace, Adelaide, South Australia 5000, Australia.
[4]Western Australian Museum, Locked Bag 40, Welshpool DC, WA 6986, Australia.
School of Animal Biology, University of Western Australia, Nedlands, Western Australia.

Summary

Neutral evolution theory predicts that genes specific to the development/function of eyes in subterranean animals, living in permanent darkness, will evolve under relaxed selection, ultimately becoming pseudogenes. However, evidence for the role of neutral processes in the evolutionary loss of vision remains controversial. An assemblage of independently-evolved beetle (Dytiscidae) species, from a subterranean archipelago in Western Australia converged on eye/vision loss, providing a powerful system to explore changes to the genome that accompany evolution in the dark. We provide evidence for the independent and parallel loss of key phototransduction genes from subterranean beetle species, proving that convergent regressive evolution can act on a common suite of genes. These genes, including arrestins, opsins, and trp-like, either contained loss of function mutations or elevated rates of evolution in their encoded proteins, indicative of pseudogenes. Our results provide strong evidence to support neutral evolution of phototransduction genes as a major contributing factor to the loss of vision in subterranean animals, re-igniting the centuries old debate.

MAIN

Regressive evolution, the evolutionary process driving the loss of phenotypic traits, such as limbs in snakes, wings in birds and insects, eyes and pigment in subterranean animals, has long intrigued evolutionary biologists (Dawin 1857; Kimura 1983; Jeffery 2009). Charles Darwin, revered for his insight and intellect, struggled with the idea of regressed features, particularly in subterranean animals; "…*As it is difficult to imagine that eyes, though useless, could be in any way injurious to animals living in darkness, their loss may be attributed to disuse*…" (On the origin of species, 1857); an unexpected statement largely accepting Lamarckian theory. Incredibly, 161 years later, there is still considerable debate surrounding the evolutionary mode of trait loss, particularly eye/vision loss, in subterranean animals, with major theories largely based on NeoDarwinian selection or neutral evolution (Culver & Wilkens 2000; Espinasa & Espinasa 2008; Retaux & Casane 2013).

Selection theory, proposes that an advantage (positive or negative, direct or indirect)(Breder 1942; Sadoglu 1967; Jeffery et al. 2000; Yamamoto et al. 2003; Menuet et al. 2007; Protas et al. 2007; Jeffery 2009 and references herein) is gained by the species due to the loss of the trait or character. Conversely, neutral evolution theory suggests that traits are lost through random mutations and genetic drift in genes that are specifically associated with the regressed trait, as there is no longer directional or purifying selection acting upon them (Kimura 1968; Yokoyama et al. 1995; Wilkens 2004). Despite widespread acceptance that many parts of the genome are under the influence of neutral evolution (Lynch 2007; Ho et al. 2017; Kumar & Patel 2018; Yoder et al. 2018; Zhang 2018), selection theory remains the most widely accepted theory in the context of eye regression in subterranean animals.

The majority of eye regression research has focused upon the model cavefish *Astyanax fasciatus*, where a wealth of studies have provided highly informative insights

from a relatively recently evolved vertebrate lineage (Langecker et al. 1993; Wilkens et al. 2003; Jeffery 2009; Fumey et al. 2018; Herman et al. 2018). However, these and other studies of recently-evolved cave animals may have failed to detect the evolutionary forces operating on 'eye genes' due to an insufficient time for the accumulation and fixation of deleterious mutations in these genes. Here we investigate a more ancient (> 3 million years) invertebrate system; the numerous (~100) and independently evolved (Cooper et al. 2002; Leys et al. 2003; Leijs et al. 2012) subterranean diving beetles (Dytiscidae) from calcrete (carbonate) aquifers in Western Australia (Watts & Humphreys 2009 and references herein). These calcretes represent closed island-like systems, with more than 200 existing calcrete bodies resembling a subterranean archipelago (Cooper et al. 2002). Each calcrete hosts a unique suite of aquatic subterranean taxa (stygobionts), including between one and three diving beetle species (Leys et al. 2003). The majority (71%) of species have independently evolved typical cave troglomorphies (i.e. loss of vision, wings and pigment), which collectively, provide an unrivalled opportunity for comparative genomic scale analyses of regressive trait evolution (Tierney et al. 2018). The additional 29% of species, which form sister pairs or triplets with sympatric subterranean species, have potentially evolved underground from a stygobiontic ancestor (Leijs et al. 2012; Chapter 3).

This study aims to investigate the molecular evolution of phototransduction genes from a wide sampling of surface and subterranean dytiscid beetles to test whether neutral gene evolution is associated with the loss of vision in blind cave animals. For genes under neutral evolution, our predictions are: 1) they should show evidence for loss of function mutations in the encoded proteins, through insertions or deletions (indels) and stop codons, and/or increases in the rate of evolution of amino acid changes, 2) there should be evidence for parallel neutral evolution, associated with different loss of function mutations, in phototransduction genes from phylogenetically independent subterranean lineages, 3)

neutral evolution should only occur in genes that are specific to the regressed trait (i.e. genes involved in other essential/developmental pathways would remain under purifying selection), and 4) recently evolved subterranean species may show no evidence for loss-of-function mutations in their phototransduction genes, compared to anciently evolved subterranean species, due to an insufficient time to accumulate and fix mutations by genetic drift.

Transcriptome analyses

Transcriptome data were generated from five diving beetle species, including two surface (*Allodessus bistrigatus* and *Paroster nigroadumbratus*) and three subterranean (*Limbodessus palmulaoides*, *Neobidessoides gutteridgei*, and *Paroster macrosturtensis*) species and used to identify 19 specific phototransduction genes (Table 1; see Tierney et al. 2015 for transcriptome generation and assembly). An assessment of functionality revealed 10 genes showing either no detectable transcription or evidence for pseudogenisation (indels leading to stop codons in the encoded sequence) in subterranean species relative to surface species (Table 1): *arrestin 1* (*arr1*), *arrestin 2* (*arr2*), *inactivation no afterpotential D* (*inaD*), *neither inactivation nor afterpotential C* (*ninaC*), invertebrate *c-opsin* (*c-opsin*), *long wavelength opsin* (*lwop*), *ultraviolet opsin* (*uvop*), *prominin* (*prom*), *transient receptor potential* (*trp*), and *transient receptor potential-like* (*trpl*). The remaining nine phototransduction genes had open reading frames in both subterranean and surface species, each encoding highly conserved amino acid sequences, indicating that they are under purifying selection. Based on gene expression studies in *Drosophila melanogaster* (http://flybase.org/), we determined that these 9 genes are likely to be pleiotropic in the beetles (i.e. they are required for other biological functions, in addition to phototransduction; Supplementary Information: SI1) and hence they would not be likely candidates for neutral evolution.

Table 1: A functionality evaluation of key phototransduction genes from transcriptome data.

Photoreceptor genes	Surface species		Subterranean species		
	Allodessus bistrigatus	*Paroster nigroadumbratus*	*Limbodessus palmulaoides*	*Neobidessodes gutteridgei*	*Paroster macrosturtensis*
Arrestin 1			*	*	
Arrestin 2			*		*
Chaoptin	*		*		*
G protein gamma 30A			*		*
G protein alpha 49B					*
G protein beta 76C					
G protein-coupled receptor kinase 1		*	*	*	
inaC					*
inaD	*	*	*		*
ninaC	*	*	*		*
No receptor potential A, type I		*	*		*
Opsin c-opsin				*	
Opsin ultraviolet					
Opsin long-wavelength	*	*			
Prominin	*	*	*		
Rab-protein 6a					
Spacemaker (eyes shut)			*	*	
Transient receptor potential	*	*			
Transient receptor potential-like	*	*			*

Gene present with an open reading frame; Gene present but no open reading frame due to stop codons or indels; No transcript was detected; ***** indicates only a partial coding sequence was present; Numeric values correspond with a particular transcriptome sequence from the original data; ninaC = Neither inactivation nor afterpotential C; ina* = Inactivation no afterpotential. Data generated and adapted from Tierney et al. (2015) and unpublished data of authors SMT, SJBC, KMS, TB, JH, WFH & ADA.

Targetted capture of candidate phototransduction genes

We further evaluated the evolution of the phototransduction genes displaying evidence of pseudogenisation or an absence of transcription in subterranean species using targeted exon capture (Gnirke et al. 2009). We obtained exon sequence data for a total of 32 distinct subterranean *Limbodessus* (22) and *Paroster* (10) species, including five groups of sympatric sister species from 20 unique calcretes, and eight genes: *arr1*, *arr2*, *inaD*, *c-opsin*, *lwop*, *uvop*, *trp*, and *trpl* (Fig. 1; Table 2). Capture success was high in the surface species, however, it was varied in the subterranean species (Table 2). The levels of missing data were not consistent across all eight genes for one species, or across one gene for all 32 species, which implies that the capture success was mostly influenced by the presence of highly mutated genes and not the quality of the baits or the initial DNA. However, the absence of *trp* in all *Paroster* species and *uvop* in all *Limbodessus* species implies the baits may not have worked in these cases. Orthology with functional surface beetle copies of each gene was confirmed using BLAST and phylogenetic analyses (see Tierney at al. 2015

for methods unless otherwise modified and Supplementary Information: SI2, SI3, and SI4 for results). We were unable to make an assessment on *ninaC* or *prominin* as no quality sequences were captured.

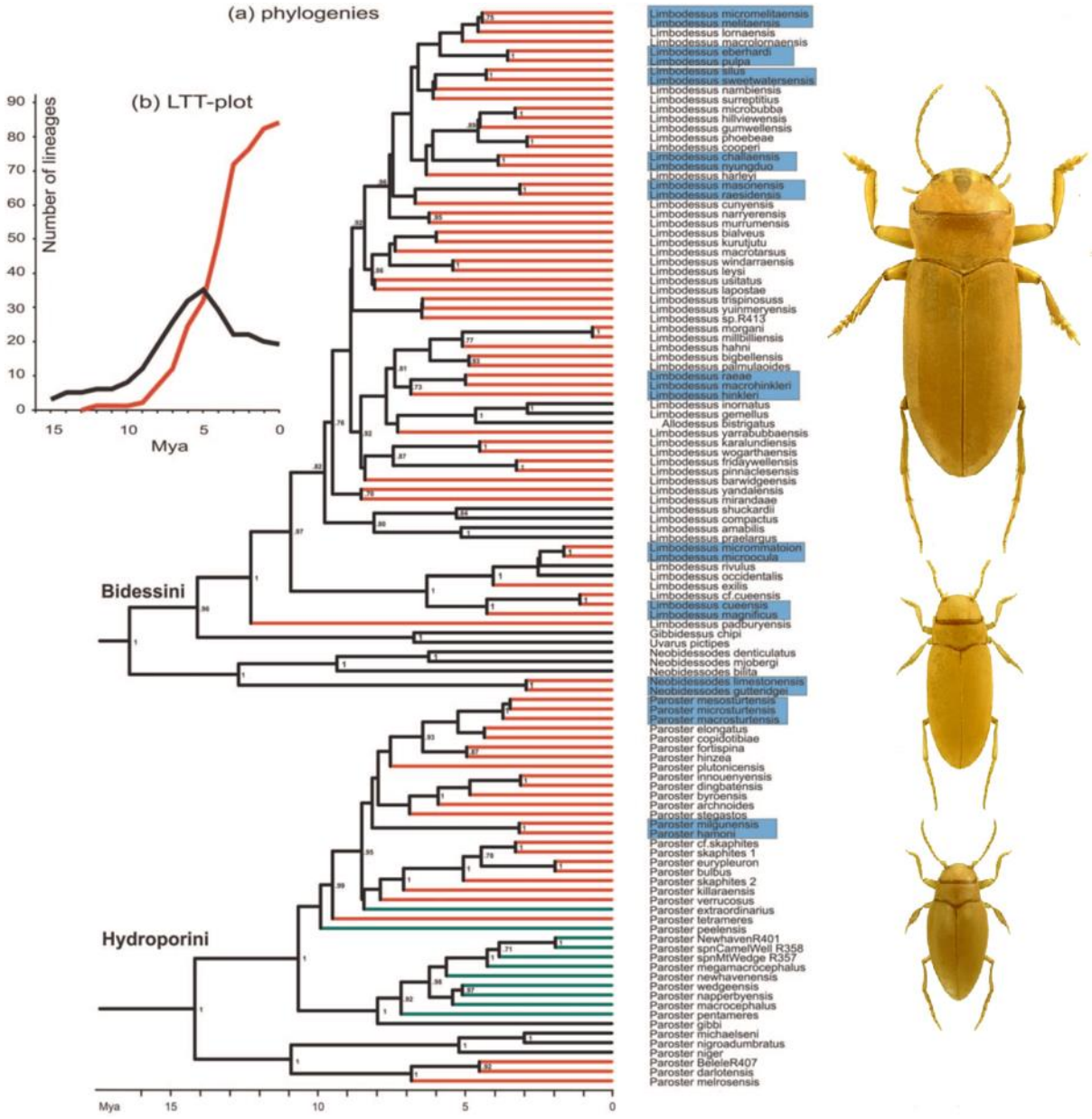

Figure 1: Phylogenetic relationships and habitat distribution of subterranean beetles. **a.** Current phylogeny of the subterranean beetles from Leys *et al*. 2012, with blue boxes representing sympatric sister species groups, and coloured branches representing: subterranean species found inside the Yilgarn (red), surface species (black) and subterranean species found outside the Yilgarn (green). An example of a sympatric sister triplet of species (*P. macrosturtnesis*, *P. mesosturtensis*, and *P. microsturtensis*) endemic to the Sturt Meadows calcrete along the right hand side.

Table 2: **A functionality evaluation of key phototransduction genes from exon capture data.**

	Calcrete	Species	Genes							
			arr1	*arr2*	*inaD*	*c-opsin*	*lwop*	*uvop*	*trp*	*trpl*
Surface species	N/A	*P. nigroadumbratus*								
	N/A	*P. gibbi*	**16**	**15**	**29**	**25**		**34**		**28**
	N/A	*L. rivulus*								**47**
	N/A	*L. compactus*								**39**
Subterranean *Limbodessus*	Laverton Downs	*L. palmulaoides*				**33**	**12**		**11**	**45**
		L. windarraensis				**43**			**13**	**47**
		L. lapostaae	**74**	**20**		**43**	**21**		**12**	**61**
	Paroo	*L. eberhardi**		**68**		**18**	**55**			**67**
		*L. pulpa**				**31**			**27**	**68**
	Mt Morgan	*L. cooperi*	**74**				**54**		**14**	**59**
		L. leysi	**74**			**39**			**18**	**45**
	Barwidgee	*L. barwidgeensis*				**20**	**29**			**42**
	Uramurdah Lake	*L. hahni*		**14**		**67**	**23**		**14**	**43**
	Hinkler Well	*L. hinkleri*					**72**			**61**
	Lake Violet	*L. millbilliensis*	**40**	**11**		**54**	**55**			**67**
	Miranda West	*L. mirandaae*					**40**		**12**	**53**
	Cunyu: Sweetwaters	*L. cunyuensis*	**19**	**88**	**11**	**14**	**60**		**18**	**69**
		*L. silus**		**15**		**30**			**15**	**36**
		*L. sweetwatersensis**		**73**			**56**			**70**
	Cunyu: SBF	*L. bialveus*				**27**			**14**	**47**
		L. macrotarsus				**57**	**14**		**21**	**50**
	Melita	*L. melitaensis**	**21**	**88**		**23**	**65**		**36**	**50**
		*L. micromelitaensis**	**88**	**86**			**63**		**14**	**45**
	Bunnawarra	*L. microocular**								**59**
		*L. micrommatoion**								**64**
	Cue	*L. cueensis*	**40**			**23**	**66**		**12**	**55**
Subterranean *Paroster*	Sturt Meadows	*P. macrosturtensis**	**15**	**38**	**13**	**82**	**66**	**58**		
		*P. mesosturtensis**		**51**			**59**			
		*P. microsturtensis**	**22**	**45**		**85**	**38**			
	Melrose Station	*P. darlotensis*					**18**	**90**		
		P. melrosensis								
	Central Mt Wedge	*P. wedgeensis*	**31**		**13**		**42**	**63**		
	Moorarie	*P. verrucosus*			**28**	**61**	**66**			
	Innouendy	*P. copidotibae*		**56**	**13**	**81**	**75**			
	Milgun Station	*P. hamoni*	**43**	**87**						
	Three Rivers Station	*P. plutonicensis*		**34**			**58**			

Gene present with an open reading frame; Gene present but no open reading frame due to stop codons or indels; No orthologous sequence was detected; ***** indicates sister species; Numeric values correspond to the percentage of missing data in that gene, with missing data < 10% not recorded

Pseudogene assessment

When we compared the eight targeted orthologous genes from the subterranean species to that of the surface species (Table 2), we detected mutations indicative of pseudogenes; indels leading to frameshift mutations and stop codons, resulted in a truncation of the encoded proteins, in six genes: *arr1, arr2, c-opsin, lwop, uvop*, and *trpl* (Supplementary Information: SI5 for an example alignment). Importantly, each of these pseudogenes from each subterranean species contained its own unique combination of protein altering

mutations, with pseudogenisation occurring in parallel across multiple species (*arr1* (20 pseudogenes/ 32 different subterranean species sequenced), *arr2* (17/29), *c-opsin* (17/28), *lwop* (22/31), *uvop* (2/3) and *trpl* (10/22); Table 2). Conversely, *inaD* and *trp* had full open reading frames (ORF) for all species in which the genes were detected: 32 and 22 subterranean species, respectively (Table 2). Due to the missing data and poor capture success of *uvop*, we were unable to use it in any further analyses, although the data obtained suggest *uvop* has degraded to a significant extent in subterranean species.

Tests of purifying selection and neutral evolution

Given that a proportion of the phototransduction genes had open reading frames, we tested whether these genes had elevated rates of nonsynonymous nucleotide substitutions, or amino acid substitutions in the subterranean lineages, relative to surface lineages, indicative of neutral evolution. Relative rates of molecular evolution were examined in eight genes across five independent comparisons of subterranean species to surface species. A likelihood ratio test (LRT) was used to test the null hypothesis that two sequences evolve at equal rates when compared to an outgroup (Muse & Weir 1992). For analyses of nucleotide variation, 13 comparisons showed a faster rate in subterranean lineages than surface ones, with 6 being significantly faster (Table 3). LRT analyses of the genes *lwop* and *trp* were significant (i.e. the rate of evolution was faster in the subterranean lineage relative to the surface) in every case, except between *L. rivulus* and *L. microocular*, which were not significant for any gene. Similarly, amino acid pairwise overall relative rates between *L. microocular* and *L. rivulus* showed no significant differences in the rate of evolution of any gene, however, all other *lwop* and *arr2* comparisons were significant. Additionally, higher rates of amino acid evolution in subterranean lineages compared to surface ones, were found in 15 comparisons, with 11 being significantly faster (Table 3).

Table 3: Phylogenetically independent tests of variation in overall substitution rate of subterranean lineages compared to surface lineages.

Gene	Lineage 1 (surface)	Lineage 2 (subter.)	Outgroup (surface)	Overall nucleotide LR	dN LR	Amino acid LR	Amino acid p-value
arr1	*L. rivulus*	*L. microocular*	*L. compactus*	0.071	1.018	>0.001	0.999
	L. compactus	*L. macrotarsus*	*L. rivulus*	0.509	10.49*	21.47	3.58e-6*
	P. nigroadumbratus	*P. melrosensis*	*P. gibbi*	0.450	0.617	31.11	2.43e-8*
	P. gibbi	*P. microsturtensis*	*P. nigroadumbratus*	10.79*	8.856*	1.464	0.226
arr2	*L. rivulus*	*L. microocular*	*L. compactus*	1.001	0.607	>0.001	0.999
	L. compactus	*L. macrotarsus*	*L. rivulus*	2.992	0.436	44.49	2.55e-11*
	P. nigroadumbratus	*P. melrosensis*	*P. gibbi*	-	-	-	-
	P. gibbi	*P. microsturtensis*	*P. nigroadumbratus*	7.669*	6.637*	36.65	1.42e-9*
inaD	*L. rivulus*	*L. microocular*	*L. compactus*	0.139	0.359	1.361	0.243
	L. compactus	*L. macrotarsus*	*L. rivulus*	2.481	15.53*	0.002	0.961
	P. nigroadumbratus	*P. melrosensis*	*P. gibbi*	0.007	0.495	0.188	0.665
	P. gibbi	*P. microsturtensis*	*P. nigroadumbratus*	2.058	0.031	4.099	0.043*
c-opsin	*L. rivulus*	*L. microocular*	*L. compactus*	1.032	0.163	2.718	0.099
	L. compactus	*L. macrotarsus*	*L. rivulus*	2.035	0.104	1.106	0.293
	P. nigroadumbratus	*P. melrosensis*	*P. gibbi*	2.038	0.121	0.616	0.432
	P. gibbi	*P. microsturtensis*	*P. nigroadumbratus*	0.926	0.608	7.973	0.005*
lwop	*L. rivulus*	*L. microocular*	*L. compactus*	0.129	0.547	0.009	0.924
	L. compactus	*L. macrotarsus*	*L. rivulus*	5.631*	0.871	9.578	0.002*
	P. nigroadumbratus	*P. melrosensis*	*P. gibbi*	3.863*	3.796*	25.80	3.78e-7*
	P. gibbi	*P. microsturtensis*	*P. nigroadumbratus*	13.37*	0.871	15.92	6.59e-5*
trp	*L. rivulus*	*L. microocular*	*L. compactus*	0.787	0.132	>0.001	0.999
	L. compactus	*L. macrotarsus*	*L. rivulus*	6.748*	21.83*	4.796	0.029*
trpl	*L. rivulus*	*L. microocular*	*L. compactus*	0.109	0.011	0.023	0.881
	L. compactus	*L. macrotarsus*	*L. rivulus*	0.430	0.967	6.012	0.014*

subter. = subterranean; LR = Likelihood ratio, which tests the likelihood of the data fitting into a particular model in comparison to another; * denotes a significant p-value of < 0.05

Site by site analyses in HyPhy (Fixed Effects Likelihood (FEL) and Single Likelihood Ancestor Counting (SLAC); Pond & Frost 2005) were used to infer nonsynonymous (dN) and sysnonymous (dS) substitution rates (collectively known as ω). Overall, FEL analyses showed that most genes (except for *inaD* and *trp*) in the subterranean lineages had an ω value close to 1 (ranging from 0.687 to 1.28; average 0.924), while most surface lineages had an ω value close to 0.1 (ranging from 0.033 to 0.252; average 0.129). Combined, *inaD* and *trp* had an average of ω = 0.316 (ranging from 0.228 to 0.360) in subterranean lineages, and ω = 0.101 (ranging from 0.051 to 0.132) in surface species (Table 4). It is unclear where along the branch leading to a subterranean species it actually colonised the calcrete and evolved in darkness and, hence, ratios of non-synonymous to synonymous substitutions (omega: ω) would be predicted to show values <1 (i.e. indicative of purifying selection). To overcome this issue we utilised the sympatric

sister species, where we predict that the entire tip branch leading to these taxa represents evolution in the dark, with ω values approaching 1 under neutral evolution. Using sympatric species only, we found similar results to the overall values of ω, however, they were generally slightly higher, ranging from 0.431 to 4.34 (average 1.33).

Table 4: Independent site by site analyses of nonsynonymous to synonymous mutations (ω) at each codon for all species, and ω branch rate inferred from sympatric sister species lineages only.

Genera	Gene	Reference ω	Test ω	Sympatric Sister species ω
Limbodessus	***arr1***	0.0334	0.687	1.65
	arr2	0.0753	0.762	0.431
	inaD	0.132	0.360	0.458
	c-opsin	0.252	1.13	1.07
	lwop	0.149	0.959	0.913
	trp	0.0507	0.361	0.684
	trpl	0.156	0.804	0.551
Paroster	***arr1***	0.0964	0.814	4.34
	arr2	0.162	0.748	0.783
	inaD	0.120	0.228	0.308
	c-opsin	0.132	1.28	1.36
	lwop	0.105	1.13	0.912

Reference ω = Surface species nonsynonymous/synonymous rate ratio using Bayesian phylogeny and alignments; Test ω = Subterranean species nonsynonymous/synonymous rate ratio using Bayesian phylogeny and alignments; Sympatric sister species ω = Subterranean sympatric sister species nonsynonymous/synonymous rate ratio using Bayesian phylogeny and alignments

We ran a branch model test using HyPhy (RELAX; Kosakovsky et al. 2015) in Datamonkey (datamonkey.org), which identifies the level of selection intensity (*K*) that invariably influences the overall estimation of ω (i.e. ω is estimated for each branch of the tree). Under these parameters, a value of $K > 1$ is indicative of purifying selection, while $K < 1$ is indicative of relaxed selection. Both the null and alternative model were used to estimate ω for each branch of the tree, however, the null model does not transform the branches, whereas the alternative model estimates *K* which transforms ω for two different branch classes. We only used lineages associated with sympatric sister species for this analysis and compared to the surface lineages. In all cases, the alternative model fitted the

data better, based on likelihood ratio values (Table 5). Most genes had a K value of less than one (range 0.00 to 0.92), indicating a relaxation of selection in the branches associated with subterranean species. The exceptions were *Limbodessus trp* and *trpl* which were suggestive of significant purifying selection along branches (i.e. significant *K* value over 1), and *Limbodessus inaD, lwop*, and *Paroster inaD,* which did not have a significant value of *K* (i.e. all values were not significantly different from 1; Table 5).

Table 5: Branch corrected (RELAX), independent comparisons of surface and subterranean sympatric sister species branches for determination of selection strength (*K*).

Species	Gene	Model	*log*L	AICc	np	*K*	p-value	LR
Limbodessus	*arr1*	null	-4685.6	9514.3	71	1.00	-	-
		alternative	-4670.9	9486.8	72	0.02	< 0.001	29.52
	arr2	null	-4680.8	9504.6	71	1.00	-	-
		alternative	-4671.0	9487.1	72	0.16	< 0.001	19.46
	inaD	null	-4321.5	8785.9	71	1.00	-	-
		alternative	-4320.7	8786.3	72	2.48	0.205	1.61
	c-opsin	null	-4533.4	9209.7	71	1.00	-	-
		alternative	-4533.3	9211.6	72	0.92	0.699	0.15
	lwop	null	-4820.8	9784.7	71	1.00	-	-
		alternative	-4820.8	9786.8	72	1.16	0.999	0.01
	trp	null	-6037.6	12217.8	71	1.00	-	-
		alternative	-6033.3	12211.3	72	3.78	0.003	8.55
	trpl	null	-5221.3	10579.4	68	1.00	-	-
		alternative	-5217.9	10574.6	69	3.82	0.009	6.75
Paroster	*arr1*	null	-3593.7	7278.4	45	1.00	-	-
		alternative	-3575.8	7244.6	46	0.00	< 0.001	35.89
	arr2	null	-1987.3	4050.0	37	1.00	-	-
		alternative	-1987.3	4052.1	38	0.02	< 0.001	27.77
	inaD	null	-4087.0	8264.6	45	1.00	-	-
		alternative	-4086.1	8264.8	46	0.47	0.178	1.81
	c-opsin	null	-2464.6	5004.1	37	1.00	-	-
		alternative	-2462.9	5002.9	38	0.00	0.071	3.27
	lwop	null	-2562.1	5211.4	43	1.00	-	-
		alternative	-2556.1	5201.4	44	0.40	0.001	12.10

np = number of parameters; *K* = selection intensity parameter, where a significant $K > 1$ indicates intensification of selection and a significant $K < 1$ indicates a relaxation of selection; p-value, where $p < 0.05$ indicates significance; LR = likelihood ratio

Discussion

Regressive evolution has played a crucial role in the evolution of traits, such as the loss of limbs in snakes (Bejder & Hall 2002 for a review and references therein), loss of eye and pigment in subterranean animals (Jeffery 2009; Juan et al. 2010), and loss of teeth in birds (Louchart & Viriot 2011 for a review and references therein), to name a few. However,

how trait regression occurs, either via selection or neutral evolutionary processes, is a matter of great debate. In this study, we show that of a suite of 19 phototransduction genes, six have clear evidence of pseudogenisation and parallel neutral evolution in multiple *Paroster* and *Limbodessus* diving beetles species. Specifically, we found unique mutations leading to a loss of function in the encoded protein of genes *arr1*, *arr2*, *c-opsin*, *lwop*, *uvop*, and *trpl*, for multiple species, with all ω values approaching one. In many study systems, the ability to detect the mode of evolution operating on genes specifically associated with regressed traits is hampered by the problem that there has been insufficient time for the accumulation and fixation of mutations that result in pseudogenisation (Podlaha & Zhang 2010). An example of this problem in the beetle system is *L. microocular* and *L. microomatoion*, which have only recently evolved from a surface ancestor, and show no evidence of pseudogenisation in any of the vision genes studied here. Clear evidence of all the genes that are evolving neutrally may not become apparent for millions of years.

Most genetic studies on eye regression in subterranean animals have tended to focus on opsin genes, with limited evidence of pseudogenisation found to date. In 1995, Yokoyama *et al*. found an increased rate of C → T transversions and nucleotide substitutions in red and green opsin gene sequences of *Astyanax fasciatus* from Pichon and Micos caves, indicative of pseudogenisation. The melanopsin and rhodopsin of the Somalian cavefish, *Phreatichthys andruzzii*, were found mutated and non-functional, likely from a relaxation of selection (Calderoni et al. 2016), with a similar result found in the rhodopsin of amblyopsid cavefishes (Neimiller et al. 2012), supporting neutral evolution. *Opsins* are directly involved in vision (photoreception), as are the *arrestins*, *ninaC*, and *prominin*, and are unlikely to have a function outside of the visual network. The additional phototransduction genes detected using transciptome data (Table 1) have a multitude of functions not related to vision, including non-vision sensory systems, channel activity, and

involvement in other organ functions (flybase; SI1), and should all remain under purifying selection because of these pleiotropic roles. The diving beetles are mostly ancient relicts that have been underground for millions of years, have multiple (75+ known) species that have independently evolved from surface species, and some sympatric sister species that have most likely evolved underground (Cooper et al. 2002; Leys et al. 2003; Leijs et al. 2012). Therefore, this study system is ideal for comparative genomics, and understanding the evolutionary forces that are operating on key genes in the genome, during evolution in the dark.

METHODS

Calcrete sampling. Subterranean diving beetle species from the genera *Limbodessus* and *Paroster* were collected from calcretes in the Yilgarn region of Western Australia utilising pre-drilled bore holes. A total of 32 subterranean beetle species were sampled from 20 calcretes and stored in 100% ethanol (Table 2). Five surface species (*Allodessus bistrigatus, Limbodessus compactus, L. rivulus, Paroster nigroadumbratus,* and *P. gibbi),* that are closely related to the subterranean species, were also sampled from surface pools, and stored in 100% ethanol.

Sequence capture probe design. *De novo* assemblies of putative transcripts for five diving beetles (two surface and three subterranean) were used to identify and annotate 19 phototransduction genes (Table 1) (method from Tierney et al. 2015). We selected a subset of 10 genes, where transcripts were present in the surface species, but either absent or showed evidence of non-functionality in the subterranean species. Sequence capture probes were developed from the orthologous transcript sequences of these genes from the two surface and three subterranean species used in Tierney et al. (2015) and synthesized by Arbor Biosciences (Ann Arbor, MI).

Library preparation and Hybridization enrichment. DNA was extracted from whole beetle specimens using the Gentra protocol for small quantities of DNA (Gentra Systems, Inc.), with minor modifications (SI6). Starting material for sonication ranged from 100 ng to 500 ng as verified by fluorometry. We constructed sequencing libraries using the Meyer and Kircher protocol (Meyer & Kircher 2010), using double indexing primers (Hugall et al., 2015; Glenn et al., 2016). We assessed the success of library preparation by qPCR, using a DNA quantification kit with the standard protocol in a LightCycler 96 Real-Time PCR System.

We performed the enrichment following the Arbor Biosciences MYbaits user manual v2 (formerly Microarray), with minor modifications (SI6). Following the enrichment, all samples were pooled in equal concentrations and subsequently dried down to 30 μL. The first MiSeq run contained six pooled samples (four different species), the second contained eight pooled samples (eight different species), the third contained 22 pooled samples (18 different species) and the final Miseq run contained 15 pooled samples (12 different species). Each pooled set was run on its own lane on the Illumina MiSeq platform (AGRF facility in Adelaide, Australia), obtaining 300 bp paired end reads for Miseq run one, two and four, and 150 bp paired end reads for Miseq run three.

Bioinformatics. Raw sequencing reads for each species were assessed using FASTQC v.0.11.3 (Babraham Institute). Using a shell script, all sequences were then cleaned, trimmed, mapped, and indexed (Supplimentary Information: SI8) on a 12-core virtual machine on the NeCTAR research cloud (National Research Infrastructure for Australia) under an Ubuntu 16.04 LTS image. Resulting files were viewed in IGV v.2.3.92 (Robinson et al. 2011; Thorvaldsdóttir et al. 2013) and exon-intron junctions were manually separated and subsequently re-mapped.

Cleaned reads for each species were *de novo* assembled using various assemblers (see SI6) and subsequently compared to a personalized BLASTn database (Tierney et al. 2015)

containing the vision genes from transcriptome data (Table 1). The alignment function of Geneious v.10.2.6 (Kearse et al. 2012) was used to map cleaned reads to the reference sequences as mentioned above, in order to verify mapping quality, but also to extend final sequences if possible.

Orthology of genes. We used the BLASTn feature within Genbank to compare our nucleotide sequences to the database of available genes, with the top three hits recorded (SI2). An orthologous match was considered positive when identities were greater than or equal to 70% with at least 50% of the gene covered by the match (Tommaso et al. 2011; Tierney et al. 2015).

Each gene group, arrestin (*arr1* and *arr2*), opsin (*c-opsin*, *lwop*, and *uvop*), trp (*trp* and *trpl*) and *inaD*, were aligned with CLUSTALW in Geneious. We were unable to capture any reads for *ninaC* or *prominin*, possibly due to problems with the transcript assembly and bait design.

Subsequently, Bayesian phylogenies were constructed using BEAUTi v.2.4.7 (Bouckaert et al. 2014) and BEAST v.1.7.5 (Drummond et al. 2012) for each gene group with outgroup references from other species from Genbank for each gene (SI3), in order to verify the identity and orthology of genes. Two independent runs using a General Time Reversible (GTR) model of sequence evolution (Tavaré 1986), were carried out with 50 million generations and trees sampled every 5000 generations. We used a burn-in of 25% (12.5 million) generations per run. The convergence of runs was assessed using Tracer v.1.5 (Rambaut & Drummond 2009), ensuring effective sample sizes > 200. Final gene trees were viewed and edited in FigTree v.1.4.3 (Rambaut 2012).

Pseudogene assessment. All sequences were aligned and assessed for ORFs to determine whether the sequences were likely to code for functional proteins. We took note of sequences that contained indels (insertions or deletions) and pre-mature stop-codons and assessed the read quality of these sites for sequencing errors by mapping raw reads onto

the sites using Geneious. For genes under neutral evolution, we would expect them to either contain translational stop codons and/or frameshift mutations leading to altered protein, or increased rates of nonsynonymous substitutions (d*N)* relative to synonymous substitutions (d*S)* (i.e. $\omega = dN/dS \Rightarrow 1$).

Tests of Selection. We used HyPhy to determine the pairwise relative rates of independent comparisons of surface and subterranean species, using a GTR model of sequence evolution (Tavaré 1986). First, we tested for variation in overall and nonsynonymous substitution rates and predicted that there will be elevated rates of evolution of exon sequences of genes under neutral evolution in subterranean species when compared to surface species. We compared overall rates of nucleotide substitution rate by comparing the likelihood scores of a shared substitution rate between taxa. Branch lengths were estimated independently with global (i.e. shared) model parameters. Rate parameters were calculated using maximum likelihood, and equilibrium (nucleotide) frequencies were 'observed'. We repeated overall pairwise relative rates on inferred amino acid sequences following the same parameters as above. Additionally, we performed pairwise relative rate tests of nonsynonymous subtitutions, using the same options as above, except using local (i.e independent) model parameters instead of global.

We then employed Datamonkey v.2.0 (datamonkey.org; Weaver et al. 2018) for phylogenetic hypothesis testing, which required Bayesian inferred trees (constructed in BEAST using the main seven nuclear genes from the exon capture (*arr1*, *arr2*, *lwop*, *c-opsin*, *inaD*, *trp*, and *trpl*, but excluded *uvop* as there was too much missing data) and mitochondrial genes *COI*, *16S*, and *ND1* from Genbank (accession numbers found in SI9), following specifications listed above). In Datamonkey, we used two site-specific methods that calculate ω independently at each codon: single-likelihood ancestor counts (SLAC) which are simplistic, and fixed effect likelihood (FEL), which are less susceptable to Type 1 errors (Pond & Frost 2005). We expected a higher value of ω in the subterranean species

(close to 1) than the surface species that have genes under purifying selection. For this analysis and the following, we chose to focus on sympatric sister species, as these species are likely to have speciated underground (Leijs et al. 2012; Chapter 3), and hence the branch tip for these taxa would not comprise any period of evolution on the surface.

We carried out branch by branch analyses (RELAX in Datamonkey; Wertheim et al. 2015) comparing surface branches to subterranean sympatric sister species tip branches. RELAX estimates a value of ω along each branch by a model of branch site-random effects likelihood (BS-REL) and then fits the selection intensity (*K*) which quantifies the level by which ω diverges from neutrality along each branch. Under this model, $K > 1$ is indicative of purifying selection, while a $K < 1$ is indicative of relaxed selection. RELAX requires an open reading frame, therefore, all insertions that were not a multiple of three were removed, an 'N' was added to the third position of stop codons, and deletions were filled with N's until the sequence was back in the correct reading frame.

General methods. A full detailed description of the laboratory methods and references can be found in Appendix 5: Supplementary Information: SI6 and SI7.

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Supplementary Information is found in Appendix 5

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manuscript. A.D.A. provided edits to the manuscript. S.J.B.C. provided guidance with genetic software, paper direction and scope, and edited the manuscript.

Author Information Genbank accession numbers for all genes will be available upon acceptance of this manuscript. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to B.L.L (barbara.langille@adelaide.edu.au).

Chapter 5: General discussion

Photo by Chris Watts

Paroster macrosturtensis

Subterranean animals are perplexing and highly understudied, as they are usually expensive and difficult to find or locate, however, they are immensely important in elucidating many evolutionary and biological questions (Page *et al*. 2008; Juan *et al*. 2010). The overarching aim of this project was to improve our understanding and knowledge of the vision-related and evolutionary processes associated with the massive assemblage of subterranean diving beetles found in Western Australia by: examining their behavioural reactions to light, determining how the genes of the regressed vision phenotype evolved, and elucidating how they may have speciated (i.e. is there any evidence for speciation underground). This study has identified one beetle species (of six) that has retained negative phototactic responses, likely an ability beneficial to its interstitial ancestor; next generation sequencing has been used to successfully target vision related genes from a variety of subterranean beetle taxa, and found six photoreceptor-specific genes (out of 19) to be evolving neutrally due to the high number of unique mutations and pseudogenes; and specific protein-altering mutations in a neutrally evolving long wavelength opsin gene were found shared among sympatric sister groups, indicating a common ancestor of these species was already living underground prior to speciation.

Retained negative phototaxis and the importance of this discovery

Visual capability can be subdivided into categories of light detection, recognition of light directionality, and image formation (Borowsky 2011), which do not all require a functional eye. Therefore, it is possible for subterranean animals to sense light and its direction, despite having highly reduced or absent eyes. It was hypothesized that

animals living in darkness will have lost all ability to perceive light (i.e. phototactic responses), given enough time (Langecker 2000). However, little information exists on eyeless subterranean animals, with amphipods representing the main body of current existing work (Borowsky 2011; Fišer *et al*. 2016). The eyeless diving beetles in Western Australian calcretes live in permanent darkness, and have done so for millions of years (Leys *et al*. 2003) and therefore could be used to test Langecker's theory. Therefore, given the choice between light and dark, we would expect the eyeless beetles to have zero preference as they should not be able to perceive light.

Based on a standard light-dark choice test, *Paroster macrosturtensis* was found in the dark significantly more often than the light, suggesting negative phototaxis. The remaining five species did not display any preference for either light or dark hemispheres (Chapter 2). The genes inaD and trp, which are both important in the visual cascade (Appendix 5: Table SI1), had open reading frames in all subterranean and surface species used in this study, suggesting they are functional (Chapter 4). At present, we have only 20% of the entire c-opsin gene in *P. macrosturtensis*, and therefore, the protein could still be functional (Chapter 4). Furthermore, it is possible that additional opsins could be present that we have yet to detect and/or identify. Further work is clearly needed to be done in this area to determine how *P. macrosturtensis* has the ability to detect and behaviourally respond to light. Although *P. macrosturtensis* appears to be completely eyeless, histological investigations of the presence or absence of internal eye structures have yet to be undertaken. It is also possible that extraocular photoreceptors could be responsible for light detection in *P. macrosturtensis*. In other studies, the brain was thought to be the source of phototactic perception in some cave species (Wilkens and Larimer

1976; Fleissner and Fleissner 2003; Xiang *et al*. 2010; Borowsky 2011; Fišer *et al*. 2016), and could be involved in this case as well. This study is one of a very few to find phototactic responses in eyeless animals, demonstrating the importance of this subterranean beetle group in vision studies on a more complex behavioural and genetic level.

Modes of speciation in subterranean beetles: evidence for speciation underground from a neutrally evolving gene

Speciation modes of animals in cave systems have been relatively well studied (Howarth 1987; Rouch and Danielopol 1987; Holsinger 1988, 2000; Peck and Finston 1993; Desutter-Grandcolas and Grandcolas 1996; Rivera *et al*. 2002; Wessel *et al*. 2007), as underground systems offer unique features ideal for evolutionary study (Poulson and White 1969; Cooper *et al*. 2007; Page *et al*. 2008). Most subterranean species, including the majority of subterranean beetles in this study, evolved independently by allopatric speciation following colonisation by different surface ancestor species (Mayr 1963; Holsinger 1988, 2000; Coyne 1992; Rice and Hostert 1993; Peck and Finston 1993; Leys *et al*. 2003). However, sympatric sister species in the beetle system (Leijs *et al*. 2012) raised the possibility of alternative speciation modes. Mathematical modelling suggests that the sister species likely evolved underground in the calcretes (Leijs *et al*. 2012), however, at the time, the lack of data made it difficult to rule out evolution by multiple colonisations from the same ancestral species. Living underground from 3 to 10 million years ago (Leys *et al*. 2003; Leys and Watts 2008), these beetles are of sufficient paleo-age to

find major mutations in the neutrally evolving genes specifically associated with regressed traits (Chapter 4). These same genes should potentially also be helpful in determining how sympatric sister species speciated, as shared deleterious mutations in photoreceptor genes of sympatric sister species could provide strong evidence that speciation took place underground.

A sympatric sister triplet of species from the Sturt Meadows calcrete were found to share several major mutations in *lwop* (an 18 base pair deletion and a mutation leading to a stop codon in two of the three species; Chapter 3). A two base pair insertion was also found in *lwop* of a sister pair located in adjacent calcretes, Innouendy and Byro West. These specific mutations were mapped to the common ancestral branch of each sister species triplet/pair in the beetle phylogeny, therefore, suggesting the ancestor was already adapted to living underground prior to the further divergence of each taxon. There was no evidence of multiple copies of *lwop* in any of the analyses we conducted using exon capture analyses, including the data we obtained from the three Sturt Meadows species. However, we can not entirely rule out the possibility that *lwop* duplicated in the ancestor of the three Sturt Meadows species. It is also possible that the common ancestor had a non-functional *lwop* prior to entering calcretes, perhaps during a period of evolution in interstitial habitats. However, we identified a functional *lwop* in an interstitial species, *L. rivulus* (Chapter 4), suggesting that the loss of *lwop* may not be associated with an interstitial lifestyle and most likely occurred during evolution underground within the calcretes. However, it would be important to study additional interstitial taxa to confirm this finding.

The analyses provide evidence that the triplet found within the Sturt Meadows calcrete evolved underground (Chapter 3), potentially via sympatric speciation underground through disruptive selection (following criteria by Coyne and Orr 2004), a theory also supported in other subterranean studies (Barr and Holsinger 1985; Barr 1960; Morton *et al*. 1998; Buhay and Crandall 2005; Christman *et al*. 2005; Faille *et al*. 2010; Ribera *et al*. 2010). However, a limitation in many of these studies is they are reliant upon phylogenies, and so cannot rule out the possibility that ancestral surface species have gone extinct. Additionally, it is difficult to rule out allopatric processes occurring within the calcrete (e.g. by isolation of populations due to water table fluctuations). However, one sister species pair (*L. melitaensis* and *L. micromelitaensis*), not examined in Chapter 3, are found in a tiny calcrete and therefore, opportunities for micro-allopatric speciation may have been limited. Periods of isolation may have been relatively short, meaning parapatric or sympatric speciation is likely in this case. The shared *lwop* mutation in two sister species from adjacent calcretes (Innouendy and Byro West) help support the hypothesis for speciation underground, however, in this case by allopatric speciation possibly due to physical separation of a once continuous calcrete body in the region.

Comparative genomic study reveals neutrally evolving vision genes

Regressive evolution, such as in the loss of limbs in snakes, teeth in birds, and eyes and pigment in subterranean animals, is commonly found in nature, but it

has long intrigued evolutionary biologists (Darwin 1859; Jeffery 2009; Kimura 1984). Despite centuries of study, the evolutionary mechanism behind trait regression is still highly debated. A large assemblage (100+ species) of subterranean diving beetles that independently evolved and converged on eye/vision loss over millions of years (Leys *et al*. 2003; Watts and Humphreys 2006 and references therein; Watts and Humphreys 2009; Leijs *et al*. 2012), provides an unparalleled comparative system to explore changes to the genome. Using transcriptome data and exon capture methods to enrich and sequence genes involved in vision, our study was able to successfully generate sequence data from 19 photoreceptor genes, including arrestins and opsins, from 32 different subterranean species (Chapter 4).

We found unique loss of function mutations or elevated rates of evolution in the encoded protein of six genes, *arr1*, *arr2*, *c-opsin*, *lwop*, *uvop*, and *trpl*, for subterranean species of *Paroster* and *Limbodessus*. These analyses lend support to the neutral theory, which has operated in parallel on genes specifically involved in photoreception. Despite selection being the more popular theory in studies of the regressive evolution of eyes in cave animals (Breder 1942; Sadoglu 1967; Jeffery *et al*. 2000; Yamamoto *et al*. 2003; Menuet *et al*. 2007; Protas *et al*. 2007; Jeffery 2009 and references therein), neutral evolution and pseudogenisation of photoreceptor genes in subterranean species has been identified in other systems (Yokoyama *et al*. 1995; Kim *et al*. 2011; Niemiller *et al*. 2013; Calderoni *et al*. 2016). Our study adds additional support for the neutral evolution of vision genes, but also highlights the random nature of neutral evolution, as not all of the same genes were found with protein altering mutational changes (Chapter 4). It is possible that some

species have not had enough time to accumulate and fix mutations in these photoreceptor genes, especially for the more recently evolved species. Additional genes, *inaD* and *trp*, although they were not detected in the transcriptomes of subterranean species, had open reading frames, suggesting they encoded functional proteins, likely connected to their multiple roles within the sensory system (flybase; SI1 from Chapter 4). We also found what appears to be fully functional photoreceptor genes, with open reading frames, in a recently evolved subterranean sister species pair, *L. microocular* and *L. microomatoion*, that have maintained small eye remnants, despite these species living in complete darkness within a calcrete. Recently evolved groups allow for an assessment of whether selection is potentially involved in driving the pseudogenisation process, as we would expect that under selection, the fixation of mutations would proceed more quickly when compared to fixation under neutral evolution. However, in this case, selection appears unlikely given the results for *L. microocular* and *L. microomatoion* (Chapter 4).

Regressive evolution and the importance of the subterranean diving beetle system

We were able to successfully sequence extremely mutated and highly variable photoreceptor genes, highlighting the value of the gene capture approach for studies of the regressive evolution of genes. Previously, we were restricted by genetic techniques (i.e. single gene methods such as Sanger sequencing) and limited to sequencing a standard suite of genes for which primers could be designed for

PCR-amplification. For example, *cinnabar* was sequenced in the diving beetles by using existing genes and primers found from *Drosophila melanogaster* (flybase) and *Tribolium castaneum* (Lorentzen *et al*. 2002), with a nested PCR approach, which was necessary due to the difficulty in PCR-amplifying this nuclear gene (Leys et al. 2005). However, next generation sequencing techniques and, specifically, hybridisation capture methods were optimized, and were crucial in the success of this study, as they allowed for the simultaneous study of multiple genes involved in vision. Evidence for pseudogenisation and neutral evolution in six vision-specific genes was found in parallel (Chapter 4), highlighting the value of using many independently evolved subterranean species. As previously stated, these species have been evolving underground for millions of years (Leijs *et al*. 2012), allowing random mutations to accumulate and become fixed within the species. In contrast, the *Astyanax* cavefish system has been extensively used to elucidate the mechanism behind eye regression (Fumey *et al*. 2018; Jeffery 2009 and references therein). However, eye genes in this system have been found functional thus far, which may solely reflect that not enough time has passed to allow the accumulation of mutations in vision-related genes, as the *Astyanax* system is relatively young (Fumey *et al*. 2018). The recovery of these vision genes in non-model organisms through next generation sequencing techniques is a promising first step for exploring genomic changes that accompany evolution underground.

Using neutrally evolved genes associated with regressed traits, we can find patterns of evolution that we would otherwise have missed in investigations of how subterranean animals have speciated. These diving beetles are useful for long term studies of evolution underground, and the sympatric sister species allow one to

look at the mutations that are occurring early in their evolution. However, despite the benefits of this system, there are some limitations. The subterranean beetles are not ideal for evolutionary developmental studies as we have yet to establish a breeding program and there is no surface conspecific with which to make hybrid crosses. In this respect, the *Astyanax* cavefish system is far more appropriate. *Astyanax* has been deemed both the 'fruit fly' and 'lab rat' of the regressive evolution field, as they have surface conspecifics that are interfertile and are easy to keep in a laboratory with a well-established breeding routine (Jeffery 2009). In addition, *Astyanax* and zebrafish share a close phylogenetic relationship, thereby allowing a transmission of usable techniques from the extensively studied zebrafish to *Astyanax* (see Jeffery 2009 for a review); an invaluable resource.

Future directions

There are many significant challenges when studying these subterranean beetles; the calcretes are expensive/complicated to get to (i.e. remote and/or privately owned land, e.g. under mining leases) and there is very little access to the groundwater, as we are entirely reliant on existing bore holes and wells. In addition, we know very little about the full life cycle and biology of the beetles as we have yet to establish stable lab colonies. A major limitation in our knowledge of these beetles stems from the fact that we do not currently have a robust phylogeny of the entire group. Phylogenetic studies have been undertaken (Leys and Watts 2008; Leijs *et al*. 2012; Chapter 4), however, they only included mitochondrial data, which are not always correct at discerning species relationships (Hurst and Jiggins 2005

and references therein), or, in our case, only include a small subset of all the subterranean beetle species. Additionally, another study found discordance with the placement of *Allodessus bistrigatus* (Balke and Ribera 2004). Instead of falling within the subterranean *Limbodessus,* as was found in later work (Leijs *et al*. 2012), it formed a sister group to all the *Limbodessus*. A robust phylogeny is the scientific basis for a plethora of biological applications such as classification, speciation, refugial re-colonisation and dating, biogeographic history, and molecular evolutionary analyses (Baum and Smith 2013), to name a few.

In this study, we were able to show that one subterranean beetle species out of six was able to perceive light (Chapter 2). This result is curious as these beetles are devoid of obvious eye structures. Therefore, it will be important to determine, through internal and external imaging, if there is any underlying eye structure or even an additional structure for vision, as seen in *Drosophila* (Helfrich-Förster *et al*. 2002), which could explain light perception. *Drosophila* have three specialized simple eyes found in the middle of their head, called ocelli, which are responsible for light perception and estimating day-night length, among others (Helfrich-Förster *et al*. 2002; Berry *et al*. 2007). Ocelli are more sensitive to light (specifically ultraviolet light) than the compound eye and therefore, are highly important in perceiving the contrast between sky and ground (Chappell and DeVoe 1975). A mixture of transcriptome studies based on specific body locations (e.g. head or leg, etc.), along with quantitative PCR (quantifies abundance of each gene) has been instrumental in identifying unique vision-related functions in animals with poor to no vision (Avivi *et al*. 2002; Friedrich *et al*. 2011; Aspiras *et al*. 2012; Crowe-Riddell *et al*. 2019). Recently, multiple roles have been found for some opsin genes in

Drosophila (Leung and Montell 2017), which may be important in light perception being retained. It is also possible that negative phototaxis has been retained in other subterranean beetle species, therefore, it would be advantageous to know if/which other species could also perceive light. A newly discovered interstitial beetle species from northern Australia was found with typical morphological troglomorphic adaptations, indicative of a possible transition state between surface and subterranean life (Watts *et al*. 2016). Interstitial species in Australia are relatively unknown, however, they may be highly important in elucidating whether the ancestral species that colonised the calcretes had phototactic behaviour as might be expected for species living in interstitial environments.

In this thesis, we chose to focus on a small subset of genes specifically involved in vision, due to time and budget constraints. However, there are many other genes with vision-related functions such as those involved in eye pigmentation and circadian rhythms. Using the beetle system we can explore these genes and compare them to the genes of other cave animals. In a previous study by Friedrich *et al*. (2011), on *Ptomaphagus hirtus* cave beetles, they found 25 genes involved in eye pigmentation, and an additional 16 genes involved in circadian rhythm. Most pigmentation genes were found based on *Drosophila* gene orthologs, however, they failed to find any ABC transporter genes (Friedrich *et al*. 2011), which is significant since the ABC transporter proteins are responsible for the movement of eye pigments across the cellular membrane of the granule in which the pigments are stored, all within the compound eye (Mackenzie *et al*. 2000). All the circadian clock genes were found functional, which was curious as *P. hirtus* has very reduced eyes (Friedrich *et al*. 2011). In a study on the ground beetle, *T. stolzi* (Bartkowiak *et*

al. 1991), which is thought to have a similar level of eye reduction as *P. hirtus*, it exhibited weak light entrainment (Lamprecht and Weber 1983, 1992), which could explain how *P. hirtus* has maintained functional circadian genes. Therefore, it would be interesting to compare our completely eyeless and interstitial partial-eyed beetles to these well-known partial-eyed cave beetle systems.

Recently, it was determined that the subterranean diving beetles in Western Australia do not go to the surface for air, but instead live continuously underwater, likely diffusing oxygen straight from the water through their thin cuticle (Jones *et al*. 2019). Jones et al. (2019) proposed that this process limits beetle size to approximately 5 cm, as this keeps their cuticle thin enough to allow oxygen to diffuse. This fascinating result opens up an entire new line of investigation as adaptations such as cuticle diffusion could help us to understand how subterranean animals have evolved to fit environmental constraints. These incredible ancient diving beetles have many adaptations and regressed features that could contribute to resolving current evolutionary debates about regressive evolution and elucidating evolutionary questions such as climate relict vs. ecological/parapatric modes of speciation by adaptive shift (Howarth 1973; Holsinger 2000; Rivera et al. 2002; Wessel et al. 2007), dispersal vs. vicariance (Porter 2007), and regressive evolution theories of selection (Yamamoto and Jeffery 2000; Jeffery 2005; Romero and Green 2005) vs. neutrality (Kosswig 1960; Culver and Wilkens 2000; Leys et al. 2005), to name a few.

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Appendix 1: Other publications relating to this project

Langille, B.L., Bertozzi, T., Tierney, S., Austin, A., Humphreys, W., and Cooper, S.J.B. (2018). Molecular evolution of 'eye genes' in blind beetles from the calcrete archipelago of Western Australia: evidence for neutral evolution of phototransduction genes. *ARPHA Conference Abstracts* doi.org/10.3897/aca.1.e30237

Cooper, S.J.B., **Langille, B.L**., Hyde, J., Humphreys, W., and Austin, A. (2018). Speciation underground in desert aquifers or just another case of hybridisation by blind beetles? *ARPHA Conference Abstracts* doi.org/10.3897/aca.1.e29830

Tierney, S.M., **Langille, B.L**., Humphreys, W.F., Austin, A.D., and Cooper, S.J.B. (2018). Massive parallel regression: A precis of genetic mechnisms for vision loss in diving beetles. *Integreative and Comparative Biology* **58**, 465-479.

Appendix 2: Conference presentations relating to this project

Langille, B.L., Bertozzi, T., Tierney, S.M., Austin, A.D., Humphreys, W.F., and Cooper, S.J.B. International Symposium on Animal Functional Genomics, Adelaide Australia, November 2018. Bertozzi orally presented: Functional genomics without a genome: the phototransduction pathway of blind beetles.

Langille, B.L., Bertozzi, T., Tierney, S.M., Austin, A.D., Humphreys, W.F., and Cooper, S.J.B. International Society of Subterranean Biology, Aveiro, Portugal, August 2018. Oral presentation: Regressive evolution of eyes in subterranean diving beetles (Dytiscidae) from Western Australia.

Langille, B.L., Tierney, S.M., Austin, A.D., and Cooper, S.J.B. School of Biological Sciences Symposium, Adelaide Australia, July 2018. Oral presentation: Regressive evolution of eyes in subterranean diving beetles.

Langille, B.L., Tierney, S.M., Austin, A.D., and Cooper, S.J.B. Society of Systematic Biologists Conference**,** Adelaide Australia, November 2017. Oral Presentation: Regressive evolution of eyes in subterranean diving beetles (Dytiscidae) from Western Australia.

Langille, B.L., Tierney, S.M., Austin, A.D., and Cooper, S.J.B. South Australian Museum – Railroad tour, August 2017. Oral presentation: Eye loss in subterranean animals.

Langille, B.L., Tierney, S.M., Austin, A.D., and Cooper, S.J.B. School of Biological Sciences Symposium, Adelaide Australia, July 2017. Poster presentation: How blind are they: Phototactic responses in subterranean diving beetles.

Langille, B.L., Tierney, S.M., Austin, A.D., and Cooper, S.J.B. Society of Systematic Biologists Workshop**,** Adelaide Australia, July 2016. Oral Presentation: Regressive evolution of eyes in subterranean diving beetles (Dytiscidae) from Western Australia.

Appendix 3:

Chapter 2: Appendix 1: Raw data table where species 1 is *P. macrosturtensis*, 2 is *P. mesosturtensis*, 3 is *P. microsturtensis*, 4 is *L. palmulaoides*, 5 is *L. windarraensis*, and 6 is *L. lapostaae*.

					Scan samples where 0 is light and 1 is dark																			
Species	**Individual**	**TimeL (sec)**	**TimeD (sec)**	**Hemisphere Change**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
1	1	647	553	45	1	1	0	0	0	0	1	1	1	1	0	1	1	0	1	0	0	0	1	1
1	2	328	872	5	0	0	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
1	3	501	699	30	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0
1	4	439	761	11	0	0	0	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1
1	5	740	460	33	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1
1	6	631	569	8	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0
1	7	153	1047	25	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1
1	8	185	1015	18	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	0	0
1	9	182	1018	13	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
1	10	363	837	23	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	1	0	0	1
1	11	555	645	48	0	0	1	1	1	1	1	1	0	0	1	0	0	0	1	1	1	1	1	0
1	12	317	883	36	1	1	1	1	1	1	1	1	0	1	1	0	0	0	1	0	1	1	0	0
1	13	352	848	33	0	0	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	1	0	1

1	14	304	896	21	1	0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	1	1
1	15	145	1055	13	1	1	1	1	1	1	0	1	1	1	0	1	0	1	1	1	1	1	1	1
1	16	543	657	53	1	0	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	1	0	1
1	17	444	756	23	0	1	1	1	1	0	1	1	0	1	0	1	1	1	1	0	1	1	1	1
1	18	453	747	4	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0
1	19	103	1097	7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1
1	20	503	697	21	0	0	0	1	1	0	1	1	1	0	1	1	1	0	1	1	0	0	0	1
1	21	57	1143	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	22	523	677	37	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1
2	1	577	623	41	0	1	1	1	0	0	0	0	1	0	0	1	0	1	1	1	1	0	0	1
2	2	598	602	25	0	1	0	1	0	0	0	0	1	1	1	1	0	1	1	1	0	0	1	1
2	3	403	797	14	0	1	1	1	0	1	1	1	1	0	0	0	1	1	0	1	1	1	1	0
2	4	518	682	27	0	1	1	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	1	1
2	5	573	627	38	0	0	1	1	0	0	0	1	1	0	1	0	0	1	0	1	0	1	1	0
2	6	858	342	7	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0	1

2	7	438	762	24	1	1	0	1	0	0	1	0	0	1	1	0	1	1	1	1	1	1	1	0
2	8	754	446	16	0	0	0	1	0	0	0	1	0	0	1	0	1	1	1	1	1	0	0	0
2	9	915	285	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0
2	10	664	536	31	0	0	0	0	1	1	1	0	1	0	0	1	1	0	0	0	0	1	0	1
2	11	606	594	37	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0	1	1	1	1	1
2	12	635	565	21	0	0	0	0	0	1	0	1	0	1	0	1	0	1	0	1	1	1	1	1
2	13	434	766	41	0	0	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1
2	14	579	621	29	0	0	0	0	0	1	0	0	1	1	1	1	0	1	1	1	0	1	1	1
2	15	928	272	22	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0
2	16	662	538	10	0	0	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	1	0	0
2	17	582	618	48	1	1	1	1	1	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0
2	18	652	548	46	1	0	0	1	0	1	1	0	0	1	1	0	1	0	1	1	0	0	1	0
2	19	569	631	23	1	0	0	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	0	1
2	20	494	706	47	1	0	0	1	0	0	1	1	0	1	1	1	0	1	1	1	1	0	0	1
3	1	546	654	14	1	1	1	1	1	1	0	1	0	0	1	0	0	1	0	0	0	1	0	0

3	2	672	528	28	0	0	1	0	0	0	0	1	1	0	1	1	0	1	0	0	0	0	0	0
3	3	615	585	48	1	0	0	0	0	0	1	1	0	1	1	0	1	0	0	1	1	0	1	0
3	4	854	346	28	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0
3	5	985	215	6	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
3	6	626	574	23	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0	1	1	0	0	1
3	7	375	825	15	1	1	1	1	1	0	1	1	1	0	0	1	1	1	1	0	0	1	1	1
3	8	285	915	24	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	0	1	1	0
3	9	310	890	19	0	0	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	1
3	10	466	734	1	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
3	11	650	550	23	0	0	0	0	0	1	0	0	0	1	1	1	0	1	0	0	1	1	0	1
3	12	472	728	19	1	1	1	1	0	1	1	0	1	0	1	1	0	0	0	0	0	1	1	1
3	13	417	783	15	0	0	1	0	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1
3	14	436	764	19	0	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1
3	15	624	576	21	0	0	0	1	1	0	1	0	1	1	1	0	1	0	0	1	1	0	1	1
3	16	482	718	27	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	1

3	17	585	615	9	0	0	0	0	0	1	1	1	1	1	0	0	1	1	0	1	1	0	0	1
3	18	630	570	17	0	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0	1	1	0	1
3	19	816	384	11	0	0	0	1	1	0	1	0	1	1	1	0	1	0	0	0	0	0	1	1
3	20	195	1005	10	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1	1	0
4	1	585	615	38	0	1	0	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0
4	2	594	606	37	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	1
4	3	600	600	31	1	1	1	0	0	1	1	1	0	0	1	0	0	1	1	0	1	1	1	1
4	4	529	671	6	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	0	0
4	5	632	568	41	0	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1
4	6	548	652	76	1	1	0	0	1	1	1	1	1	1	0	0	1	0	0	0	0	1	1	0
4	7	695	505	35	0	0	0	0	1	0	1	0	0	1	0	1	0	0	0	1	1	1	1	1
4	8	400	800	19	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1
4	9	638	562	18	0	1	1	1	1	0	1	1	1	0	0	0	1	1	0	0	0	1	1	0
4	10	610	590	31	0	0	1	0	0	1	1	1	0	1	1	0	0	1	1	0	1	0	0	1
4	11	655	545	19	0	0	0	0	1	0	1	1	0	0	0	1	1	1	1	0	1	0	1	1

4	12	561	639	19	1	1	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	1
4	13	853	347	12	0	0	0	1	0	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0
4	14	515	685	9	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	1	0	1
4	15	581	619	7	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1
5	1	383	817	51	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	0	0	1
5	2	502	698	59	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	1
5	3	554	646	39	1	1	1	0	1	0	0	0	1	0	0	1	0	0	1	0	1	0	0	1
5	4	481	719	44	1	0	1	1	0	0	1	0	0	0	1	1	0	0	0	1	1	1	1	0
5	5	503	697	41	0	1	1	0	0	0	1	1	0	1	1	1	1	1	1	0	0	1	0	1
5	6	252	948	7	1	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1
5	7	747	453	29	0	0	0	1	0	0	0	1	0	1	0	1	1	1	0	0	1	1	1	1
5	8	535	665	42	0	1	0	1	1	1	1	1	0	0	1	0	0	1	0	1	0	1	0	0
5	9	514	686	23	1	1	0	0	1	0	0	0	1	0	1	1	1	1	1	0	1	1	1	1
5	10	447	753	48	0	0	1	1	0	1	0	0	0	0	1	1	0	1	1	0	0	0	0	0
5	11	226	974	13	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1

5	12	505	695	43	1	0	0	0	0	1	0	0	0	1	1	0	1	1	1	0	0	0	1	1
5	13	758	442	31	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	1	0	0	0	1
5	14	497	703	27	0	1	1	0	0	0	0	0	1	1	0	1	1	1	0	0	1	0	0	1
5	15	610	590	32	0	0	1	1	0	1	1	1	0	1	0	0	0	1	0	1	0	0	1	0
5	16	906	294	22	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
5	17	930	270	9	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1
5	18	726	474	25	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	1	0	1	1
5	19	293	907	21	0	1	1	0	1	0	1	1	1	1	1	0	1	1	1	0	0	1	1	1
5	20	730	470	25	0	1	1	0	1	0	1	0	1	0	0	1	1	1	0	0	1	0	0	1
6	1	137	1063	8	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	0
6	2	537	663	34	0	1	0	0	0	1	0	1	0	0	1	1	1	1	1	0	0	1	1	0
6	3	534	666	41	0	0	1	1	0	1	1	0	0	0	0	1	1	1	0	1	0	1	1	1
6	4	424	776	42	1	0	0	1	1	0	1	0	0	1	0	1	1	0	1	1	0	1	1	0
6	5	559	641	17	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1
6	6	733	467	13	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	1	1	1	1	1

6	7	566	634	22	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1	0	0	0	0
6	8	623	577	19	0	0	0	1	1	1	1	0	1	0	1	0	0	1	1	0	1	0	0	1
6	9	910	290	8	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0
6	10	316	884	16	0	0	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	0
6	11	907	293	19	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	1
6	12	525	675	26	1	0	0	0	0	1	0	1	0	1	0	1	1	0	1	0	1	0	1	0
6	13	457	743	37	0	1	1	1	0	1	1	0	0	1	0	0	0	0	1	0	1	0	1	1
6	14	340	860	34	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1	0	1	1	0
6	15	734	466	11	0	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1
6	16	606	594	15	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1
6	17	620	580	35	0	0	0	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1
6	18	389	811	15	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	1	1	1
6	19	450	750	22	0	1	0	0	1	1	1	0	0	1	0	0	1	1	1	0	1	1	1	0
6	20	557	643	23	1	0	1	0	1	1	1	0	0	1	0	0	0	1	0	1	0	1	1	1

Chapter 2: Appendix 2: Raw data table solely containing the final 10 minutes of observation where species 1 is *P. macrosturtensis*, 2 is *P. mesosturtensis*, 3 is *P. microsturtensis*, 4 is *L. palmulaoides*, 5 is *L. windarraensis*, and 6 is *L. lapostaae*.

					Scan samples where 0 is light and 1 is dark									
Species	**Individual**	**TimeL (sec)**	**TimeD (sec)**	**Hemisphere Change**	11	12	13	14	15	16	17	18	19	20
1	1	358	242	22	0	1	1	0	1	0	0	0	1	1
1	2	63	537	4	1	1	1	0	1	1	1	1	1	1
1	3	254	346	25	1	1	0	0	1	1	0	0	1	0
1	4	133	467	6	1	1	1	0	1	1	1	1	1	1
1	5	297	303	26	0	0	0	0	0	0	1	1	0	1
1	6	457	143	6	0	0	0	0	0	0	1	1	0	0
1	7	68	532	10	1	1	1	1	1	0	1	1	0	1
1	8	118	482	7	1	1	1	0	1	1	1	0	0	0
1	9	62	536	2	1	1	1	1	1	1	1	1	1	1
1	10	114	486	12	1	1	1	1	1	0	1	0	0	1
1	11	253	347	28	1	0	0	0	1	1	1	1	1	0
1	12	184	416	23	1	0	0	0	1	0	1	1	0	0
1	13	186	414	16	1	1	0	1	1	1	0	1	0	1
1	14	219	381	10	0	0	1	1	0	0	1	1	1	1
1	15	99	501	8	0	1	0	1	1	1	1	1	1	1
1	16	256	344	23	0	0	0	0	1	1	1	1	0	1
1	17	166	434	8	0	1	1	1	1	0	1	1	1	1
1	18	192	408	1	1	1	1	1	1	1	0	0	0	0
1	19	98	502	4	1	1	1	1	1	0	1	1	0	1
1	20	254	346	13	1	1	1	0	1	1	0	0	0	1
1	21	0	600	0	1	1	1	1	1	1	1	1	1	1
1	22	217	383	18	1	1	1	1	0	1	1	0	0	1
2	1	288	312	17	0	1	0	1	1	1	1	0	0	1
2	2	227	373	14	1	1	0	1	1	1	0	0	1	1

2	3	198	402	6	0	0	1	1	0	1	1	1	1	0
2	4	243	357	12	1	1	0	0	0	1	0	0	1	1
2	5	246	354	16	1	0	0	1	0	1	0	1	1	0
2	6	334	266	5	0	0	1	1	0	0	1	1	0	1
2	7	168	432	9	1	0	1	1	1	1	1	1	1	0
2	8	264	336	10	1	0	1	1	1	1	1	0	0	0
2	9	322	278	2	0	0	0	0	1	1	1	1	1	0
2	10	251	349	17	0	1	1	0	0	0	0	1	0	1
2	11	310	290	18	0	1	1	0	0	1	1	1	1	1
2	12	256	344	10	0	1	0	1	0	1	1	1	1	1
2	13	142	458	24	0	1	1	1	1	1	1	1	1	1
2	14	206	394	16	1	1	0	1	1	1	0	1	1	1
2	15	333	267	20	0	0	1	1	0	1	1	1	1	0
2	16	327	273	4	1	1	1	0	0	0	0	1	0	0
2	17	288	312	21	0	1	1	0	0	0	0	0	0	0
2	18	258	342	21	1	0	1	0	1	1	0	0	1	0
2	19	260	340	14	0	0	0	0	1	1	0	1	0	1
2	20	195	405	26	1	1	0	1	1	1	1	0	0	1
3	1	460	140	8	1	0	0	1	0	0	0	1	0	0
3	2	334	266	12	1	1	0	1	0	0	0	0	0	0
3	3	410	190	12	1	0	1	0	0	1	1	0	1	0
3	4	474	126	14	0	0	0	0	0	0	1	0	0	0
3	5	561	39	2	0	0	0	0	1	0	0	0	0	0
3	6	302	298	13	0	0	0	1	0	1	1	0	0	1
3	7	260	340	11	0	1	1	1	1	0	0	1	1	1
3	8	145	455	13	0	0	1	1	1	1	0	1	1	0

3	9	115	485	7	1	1	0	1	1	1	1	1	0	1
3	10	0	600	0	1	1	1	1	1	1	1	1	1	1
3	11	218	382	10	1	1	0	1	0	0	1	1	0	1
3	12	267	333	11	1	1	0	0	0	0	0	1	1	1
3	13	113	487	8	1	1	1	1	1	1	0	1	0	1
3	14	158	442	7	1	0	1	1	1	1	1	1	0	1
3	15	268	332	12	1	0	1	0	0	1	1	0	1	1
3	16	215	385	12	1	1	1	1	0	1	1	0	1	1
3	17	282	318	8	0	0	1	1	0	1	1	0	0	1
3	18	270	330	9	1	1	0	0	1	0	1	1	0	1
3	19	424	176	4	1	0	1	0	0	0	0	0	1	1
3	20	172	428	7	0	1	1	1	0	0	1	1	1	0
4	1	301	299	19	1	1	1	0	0	0	0	0	0	0
4	2	241	359	20	0	0	0	0	1	1	0	0	0	1
4	3	339	261	19	1	0	0	1	1	0	1	1	1	1
4	4	452	148	4	0	0	0	0	0	1	1	1	0	0
4	5	324	276	22	0	1	1	1	1	0	0	0	1	1
4	6	295	305	47	0	0	1	0	0	0	0	1	1	0
4	7	248	352	24	0	1	0	0	0	1	1	1	1	1
4	8	325	275	14	1	1	1	0	1	0	1	1	1	1
4	9	451	149	12	0	0	1	1	0	0	0	1	1	0
4	10	345	255	18	1	0	0	1	1	0	1	0	0	1
4	11	272	328	11	0	1	1	1	1	0	1	0	1	1
4	12	237	363	9	1	1	1	0	0	0	1	0	0	1
4	13	503	97	6	1	0	0	0	0	0	0	1	0	0
4	14	379	221	5	0	0	0	0	0	1	1	1	0	1

4	15	77	523	2	1	1	1	1	1	0	1	1	1	1
5	1	184	416	24	1	1	1	0	1	1	1	0	0	1
5	2	203	397	27	1	1	0	1	0	1	1	1	1	1
5	3	291	309	11	0	1	0	0	1	0	1	0	0	1
5	4	178	422	18	1	1	0	0	0	1	1	1	1	0
5	5	210	390	20	1	1	1	1	1	0	0	1	0	1
5	6	117	483	3	0	1	1	1	1	1	1	1	1	1
5	7	305	295	20	0	1	1	1	0	0	1	1	1	1
5	8	325	275	26	1	0	0	1	0	1	0	1	0	0
5	9	140	460	11	1	1	1	1	1	0	1	1	1	1
5	10	198	402	15	1	1	0	1	1	0	0	0	0	0
5	11	74	526	4	1	1	1	1	0	1	1	1	1	1
5	12	158	442	26	1	0	1	1	1	0	0	0	1	1
5	13	385	215	14	0	0	0	0	0	1	0	0	0	1
5	14	187	413	14	0	1	1	1	0	0	1	0	0	1
5	15	380	220	23	0	0	0	1	0	1	0	0	1	0
5	16	523	77	6	0	0	0	0	0	0	0	1	0	0
5	17	375	225	5	0	1	0	0	0	0	1	1	1	1
5	18	372	228	9	0	1	0	0	0	1	1	0	1	1
5	19	86	514	8	1	0	1	1	1	0	0	1	1	1
5	20	409	191	9	0	1	1	1	0	0	1	0	0	1
6	1	135	465	7	1	1	0	1	1	1	0	1	0	0
6	2	152	448	16	1	1	1	1	1	0	0	1	1	0
6	3	211	389	23	0	1	1	1	0	1	0	1	1	1
6	4	255	345	25	0	1	1	0	1	1	0	1	1	0
6	5	253	347	8	1	1	1	0	0	1	0	1	1	1

6	6	324	276	9	0	0	0	1	0	1	1	1	1	1
6	7	282	318	7	1	1	1	1	1	1	0	0	0	0
6	8	312	288	13	1	0	0	1	1	0	1	0	0	1
6	9	470	130	5	1	0	0	0	0	0	0	0	0	0
6	10	161	439	5	0	1	0	0	1	1	1	1	1	0
6	11	307	293	19	1	1	0	0	0	0	1	0	1	1
6	12	174	426	15	0	1	1	0	1	0	1	0	1	0
6	13	231	369	20	0	0	0	0	1	0	1	0	1	1
6	14	131	469	16	1	1	1	1	1	1	0	1	1	0
6	15	584	16	1	0	0	0	0	0	0	0	0	0	1
6	16	133	467	7	0	1	1	1	1	1	1	1	1	1
6	17	311	289	16	1	1	0	1	0	1	0	1	1	1
6	18	171	429	8	1	1	1	1	1	0	0	1	1	1
6	19	239	361	13	0	0	1	1	1	0	1	1	1	0
6	20	269	331	10	0	0	0	1	0	1	0	1	1	1

Appendix 4:

Chapter 3: Supplementary Table 1: Genbank submission numbers of all sequences sourced for this study.

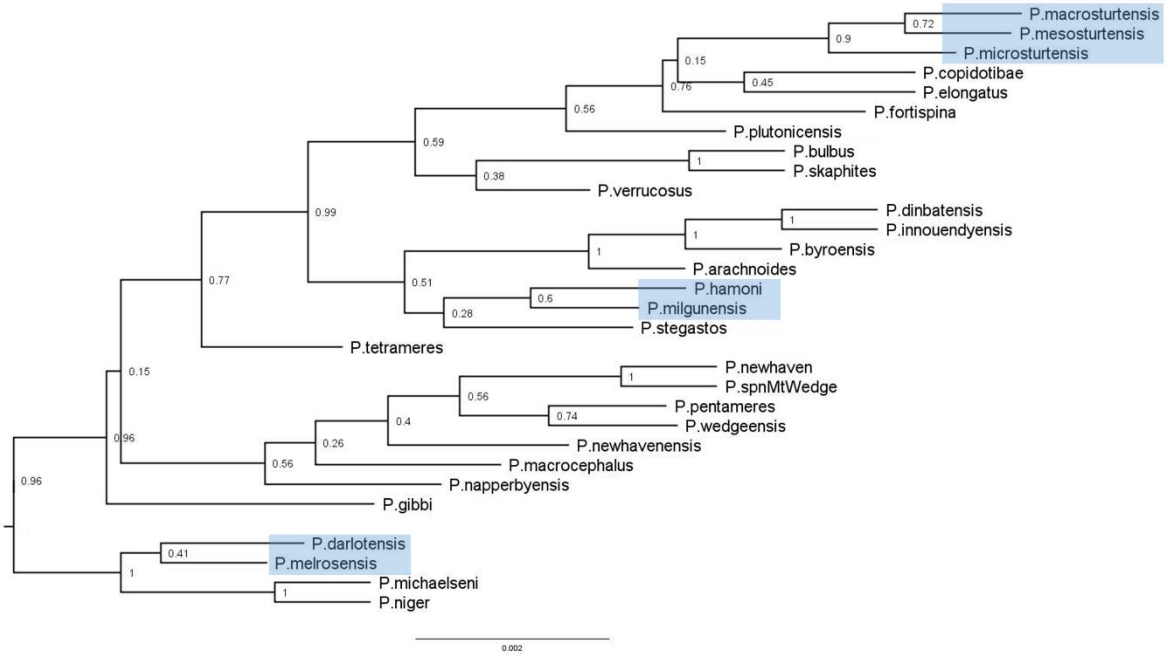
Species	Gene	Accession number
P. bulbus	*CO1*	EU616973.1
P. dingbatensis	*CO1*	EU616966.1
P. fortispina	*CO1*	AY350900.1
P. gibbi	*CO1*	AJ850568.1
P. macrocephalus	*CO1*	AY350886.1
P. macrosturtensis	*CO1*	FJ647871.1
P. mesosturtensis	*CO1*	FJ647985.1
P. microsturtensis	*CO1*	FJ648069.1
P. newhaven	*CO1*	JQ745788.1
P. newhavenensis	*CO1*	AY350897.1
P. niger	*CO1*	EU616989.1
P. pentameres	*CO1*	EU616991.1
P. tetrameres	*CO1*	EU616985.1
L. palmulaoides	*argk*	Hyde et al. unpublished
L. palmulaoides	*cin*	Hyde et al. unpublished
L. palmulaoides	*CO1*	JQ745762.1
L. palmulaoides	*lwop*	KP219382.1
L. palmulaoides	*topo*	Hyde et al. unpublished
L. palmulaoides	*wg*	Hyde et al. unpublished

Chapter 3: Supplementary Table 2: All sequences generated from this study, where grey blocks represent a successfully sequenced gene and white blocks represent no sequence data. Data sourced for this study were not included here (see Supplementary Table 1). Genbank numbers available upon publication.

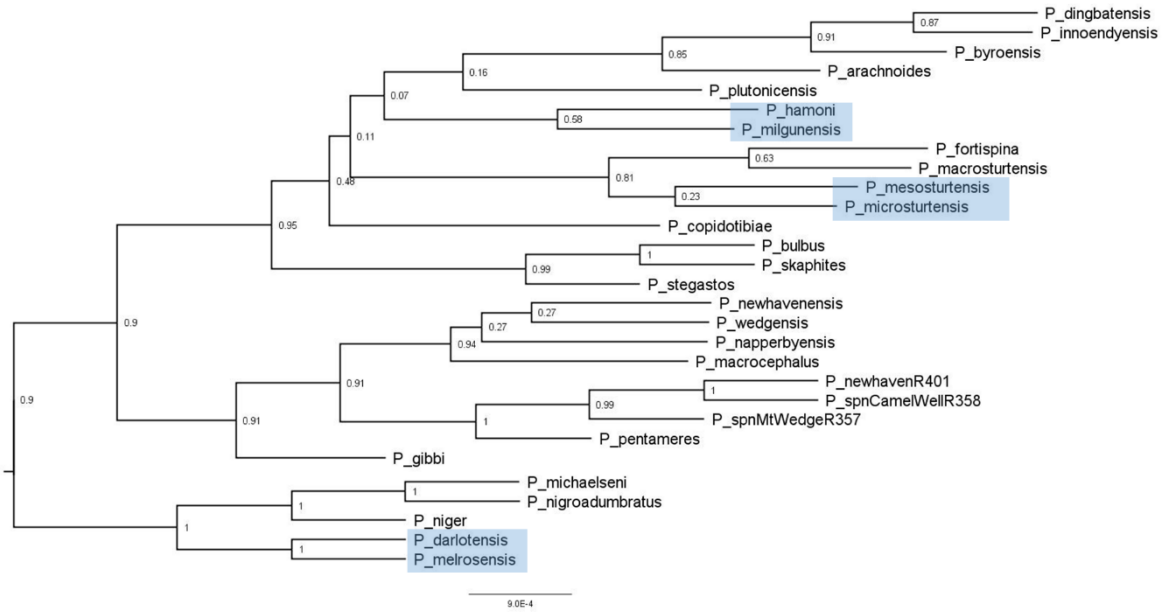
Species	Genes					
	argk	*cin*	*CO1*	*lwop*	*topo*	*wn*
P. arachnoides						
P. bulbus						
P. byroensis						
P. copidotibae						
P. darlotensis						
P. dingbatensis						
P. elongatus						
P. fortispina						
P. gibbi						
P. hamoni						
P. innouendyensis						
P. macrocephalus						
P. macrosturtensis						
P. melrosensis						
P. mesosturtensis						
P. michaelseni						
P. microsturtensis						
P. milgunensis						
P. napperbyensis						
P. newhaven						
P. newhavenensis						
P. niger						
P. nigroadumbratus						
P. pentameres						
P. plutonicensis						
P. skaphites						
P. spnCamelWell						
P. spnMtWedge						
P. stegastos						
P. tetramers						
P. verrucosus						
P. wedgeensis						

Chapter 3: Supplementary Figure 1: Bayesian phylograms from individual genes with previously identified and putative sister species highlighted in blue, where a) is COI, b) is argenine kinase, c) is cinnabar, d) is long wavelength opsin, and e) is topoisomerase. All trees were rooted with *L. palmulaoides*.

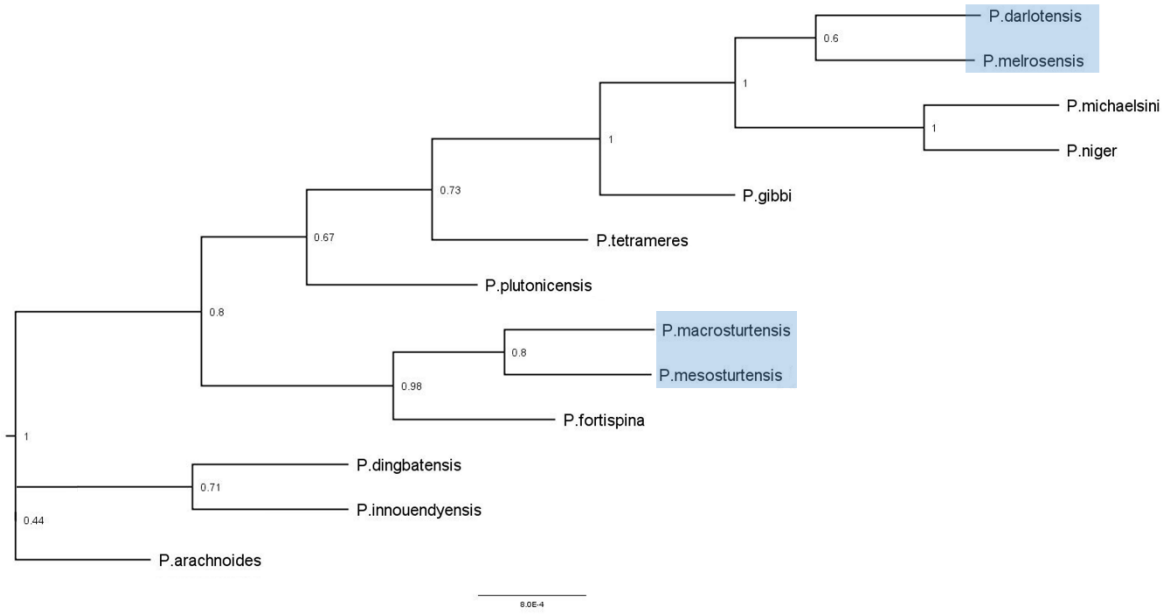
a)



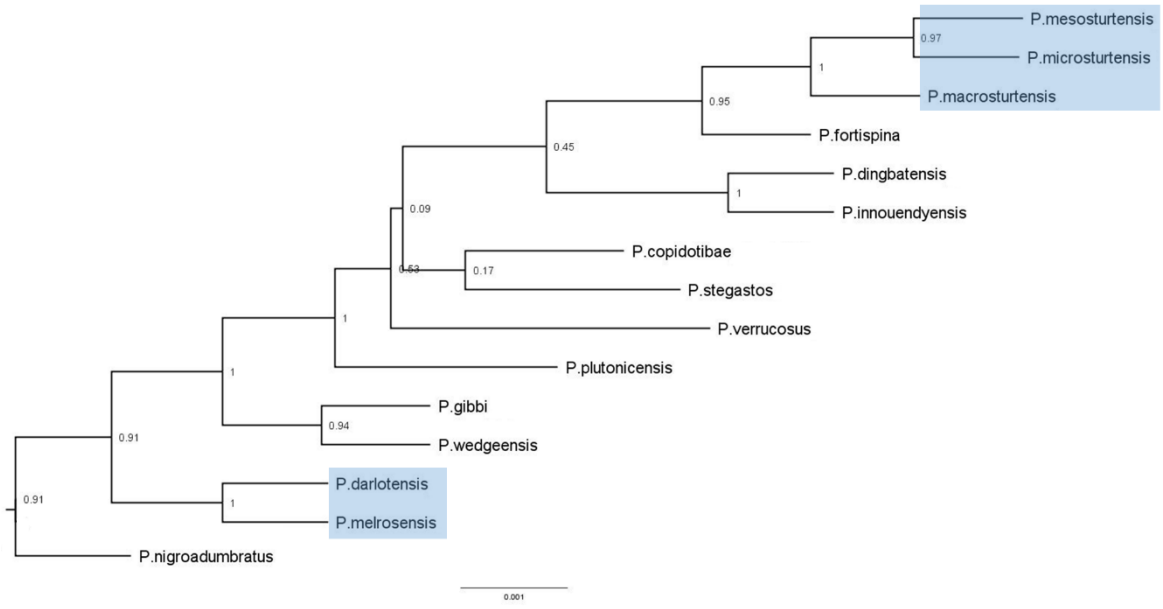
b)



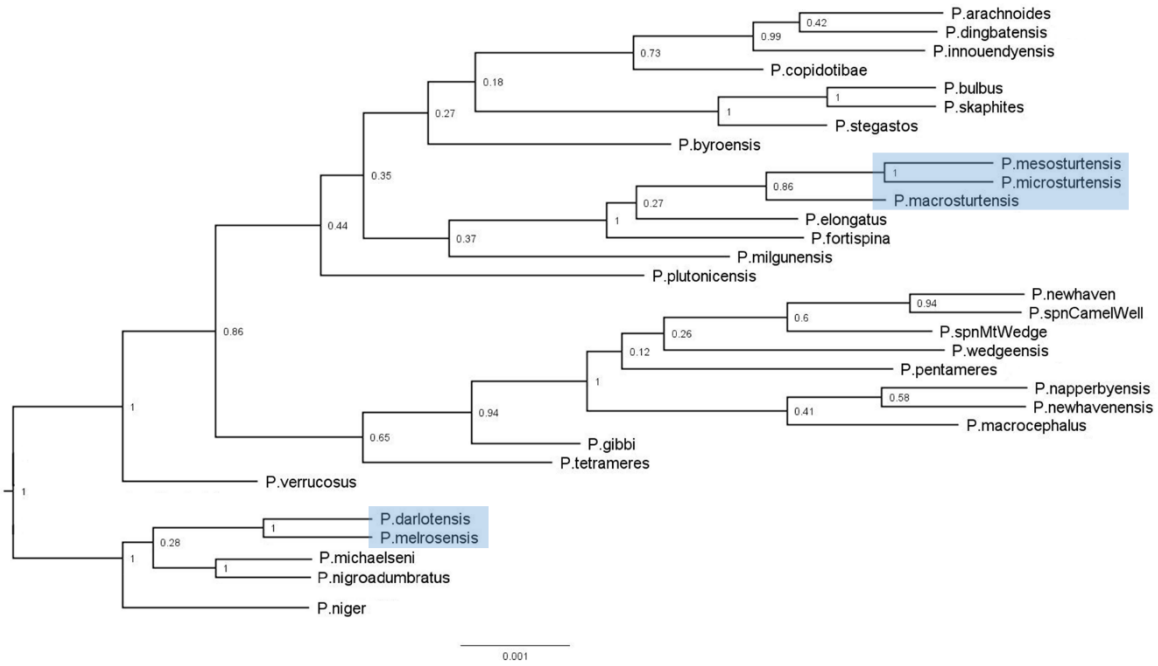
c)


P.darlotensis
P.melrosensis
P.michaelsini
P.niger
P.gibbi
P.tetrameres
P.plutonicensis
P.macrosturtensis
P.mesosturtensis
P.fortispina
P.dingbatensis
P.innouendyensis
P.arachnoides
0.6
1
1
1
0.73
0.67
0.8
0.8
0.98
1
0.71
0.44
8.0E-4

d)


P.mesosturtensis
P.microsturtensis
P.macrosturtensis
P.fortispina
P.dingbatensis
P.innouendyensis
P.copidotibae
P.stegastos
P.verrucosus
P.plutonicensis
P.gibbi
P.wedgeensis
P.darlotensis
P.melrosensis
P.nigroadumbratus
0.97
1
0.95
0.45
1
0.09
0.63
0.17
1
1
0.91
0.94
0.91
1
0.001

e)


P.arachnoides
P.dingbatensis
P.innouendyensis
P.copidotibae
P.bulbus
P.skaphites
P.stegastos
P.byroensis
P.mesosturtensis
P.microsturtensis
P.macrosturtensis
P.elongatus
P.fortispina
P.milgunensis
P.plutonicensis
P.newhaven
P.spnCamelWell
P.spnMtWedge
P.wedgeensis
P.pentameres
P.napperbyensis
P.newhavenensis
P.macrocephalus
P.gibbi
P.tetrameres
P.verrucosus
P.darlotensis
P.melrosensis
P.michaelseni
P.nigroadumbratus
P.niger
0.42
0.99
0.73
0.18
0.27
1
1
1
0.35
0.86
1
0.27
0.44
1
0.37
0.94
0.6
0.26
0.12
1
0.58
0.41
0.65
0.94
1
1
0.28
1
1
0.001

Appendix 5:

Chapter 4: Supplementary Information: Table SI1: Flybase information for all photoreceptor genes identified by transcriptome analyses of diving beetles.

Gene	Molecular function	Biological processes	Phenotypes manifest in
Arrestin 1	opsin binding	sensory perception of smell; metarhodopsin inactivation; desensitization of G protein-coupled receptor signaling pathway by arrestin; deactivation of rhodopsin mediated signaling; endocytosis; photoreceptor cell maintenance	eye photoreceptor cell; rhabdomere
Arrestin 2	protein binding; opsin binding	adaptation of rhodopsin mediated signaling; sensory perception of sound; deactivation of rhodopsin mediated signaling; photoreceptor cell maintenance; sensory perception of smell; metarhodopsin inactivation; desensitization of G protein-coupled receptor signaling pathway by arrestin	eye photoreceptor cell; ommatidium; photoreceptor cell; rhabdomere; retina; eye
Chaoptin	unknown	rhabdomere development; microvillus organization; homophillic cell adhesion via plasma membrane adhesion molecules	mesothoracic tergum; rhabdomere of eye photoreceptor cell; rhabdomere R7; photoreceptor cell; rhabdomere; rhabdomere microvillus
G protein gamma 30A	GTPase activity, protein heterodimerization activity	phototransduction; sensory perception of taste; cellular response to carbon dioxide; phospholipase C-activating G-protein coupled receptor signalling pathway; G-protein coupled receptor signalling pathway	adult olfactory receptor neuron Gr21a/63a
G protein alpha 49B aka G protein alpha q	G-protein beta/gamma-subunit complex binding; GTPase activity; G-protein coupled receptor binding; guanyl nucleotide binding	regulation of biological quality; regulation of phospholipase C activity; regulation of anatomical structure morphogenesis; immune system process; vesicle-mediated transport; thermotaxis; behavior; nervous system process; homeostatic process; cellular response to oxygen-containing compound; neuron differentiation	tract neuropil; cell part; mesothoracic segment; rhabdomere; multi-cell-component structure; axon; thorax; embryonic/larval nervous system; adult thorax; midline crossing tract; neuron part; neuromuscular junction
G protein beta 76C	protein heterodimerization activity	negative regulation of smoothened signaling pathway; activation of phospholipase C activity; G-protein coupled receptor signaling pathway; deactivation of rhodopsin mediated signaling; phototransduction; rhodopsin mediated signaling pathway	photoreceptor cell
G protein-coupled receptor kinase 1	rhodopsin kinase activity; ATP binding; G-protein coupled receptor kinase activity; protein serine/threonine kinase activity; G-protein coupled receptor binding; phosphatidylinositol binding	phototransduction, visible light; metarhodopsin inactivation; protein phosphorylation; positive regulation of smoothened signaling pathway	vision; wing
Inactivation no afterpotential C	ATP binding; protein kinase C activity; zinc ion binding; protein serine/threonine kinase activity; protein binding	development of primary female sexual characteristics; response to light intensity; nervous system process; locomotion; response to chemical; establishment of localization; multicellular organism development; organic	germarium; egg chamber; retina; eye; photoreceptor cell; gustatory receptor neuron; photoreceptor; ovary; ovarian sheath

		substance metabolic process; response to ethanol; localization	
Inactivation no acterpotential D	photoreceptor activity; myosin binding; structural molecule activity; myosin III binding; protein binding; receptor signaling complex scaffold activity; calmodulin binding	phototransduction; deactivation of rhodopsin mediated signaling; protein localization; cellular response to light stimulus; detection of light stimulus involved in sensory perception; sensory perception of sound	photoreceptor; retina; photoreceptor cell; eye photoreceptor cell
Neither inactivation nor afterpotential C	motor activity; phosphatidylinositol binding; protein tyrosine kinase activity; protein serine/threonine kinase activity; ATPase activity, coupled; calmodulin binding; protein kinase activity; ATP binding; protein binding	protein localization; localization; amide transport; protein metabolic process; cellular component organization or biogenesis; regulation of biological quality; cellular protein metabolic process; multicellular organismal homeostasis; cellular localization; cellular protein localization	retina; eye; ommatidium; photoreceptor cell; male genitalia; eye photoreceptor cell; rhabdomere
No receptor potential A, type I	phosphatidylinositol phospholipase C activity; phospholipase C activity; GTPase activator activity; protein binding; calcium ion binding	locomotion; locomotory behavior; positive regulation of cellular component organization; sensory perception of bitter taste; organ or tissue specific immune response; adult behavior; diacylglycerol metabolic process; positive regulation of receptor-mediated endocytosis; sensory organ morphogenesis; immune system process; retina homeostasis; taxis; detection of chemical stimulus involved in sensory perception of bitter taste	cell; cell projection; rhabdomere R5; dendrite; membrane-bounded organelle; cell part; cytoplasm; intracellular part; supraesophageal ganglion; intracellular; somatodendritic compartment; rhabdomere
Prominin	-	rhabdomere development	rhabdomere of eye photoreceptor cell; photoreceptor cell; tergite; rhabdomere; ommatidium; eye
Rab-protein 6a	GTP binding; GTPase activity; protein binding	localization; biological regulation; establishment of localization; compound eye morphogenesis; Golgi vesicle transport; signal transduction; endosomal transport; response to other organism; regulation of membrane potential; detection of abiotic stimulus	ovariole; gonad; hemocyte; female germline cyst; photoreceptor cell R7; female organism; immaterial anatomical entity; adult mesothoracic segment; circulatory system; sclerite
Rhodopsin 2	G protein-coupled receptor activity; G protein-coupled photoreceptor activity	phototransduction; visual perception; G protein-coupled receptor signaling pathway	eye photoreceptor cell
Rhodopsin 3 (Ultraviolet opsin)	G protein-coupled photoreceptor activity	visual perception; cellular response to light stimulus; phototransduction, UV; G protein-coupled receptor signaling pathway; phototransduction; detection of UV; absorption of UV light	eye photoreceptor cell; ommatidium
Rhodopsin 7 (Blue opsin)	peptide binding; G protein-coupled receptor activity; G protein-coupled photoreceptor activity	rhodopsin mediated signaling pathway; circadian behavior; entrainment of circadian clock by photoperiod; cellular response to light stimulus; G protein-coupled receptor signaling pathway; response to photoperiod, blue light; visual perception	l-LNv neuron
Spacemaker (eyes shut)	calcium ion binding; extracellular matrix structural constituent; protein binding	temperature compensation of the circadian clock; cell morphogenesis; rhabdomere development	rhabdomere; Johnston organ; scolopidium; plasma membrane bounded cell projection; head segment; cell part; internal sensillum; cell; antenna; cell projection
Transient receptor potential	inositol 1,4,5 trisphosphate binding; store-operated calcium channel activity; light-activated ion channel activity; protein heterodimerization activity; calcium channel activity; identical protein binding; protein	divalent inorganic cation transport; localization; divalent metal ion transport; cation transport; cognition; multicellular organismal homeostasis; protein localization; mitochondrion organization; cellular response to	mitochondrion; adult antennal segment; cytoplasm; rhabdomere; photoreceptor cell R6; antenna; region of integument; membrane-bounded organelle; photoreceptor cell R3; intracellular part; plasma

	binding; protein homodimerization activity	anoxia; response to oxygen levels; sequestering of calcium ion; manganese ion transport	membrane bounded cell projection
Transient receptor potential like	protein heterodimerization activity; calcium channel activity; identical protein binding; protein binding; cation channel activity; light-activated ion channel activity; inositol 1,4,5 trisphosphate binding; ion transmembrane transporter activity; store-operated calcium channel activity; calmodulin binding	transport; regulation of biological process; divalent metal ion transport; detection of visible light; localization; response to abiotic stimulus; regulation of biological quality; response to radiation; cellular response to decreased oxygen levels; calcium ion homeostasis; cellular process; sensory perception of mechanical stimulus	photoreceptor cell; eye photoreceptor cell; Malpighian tubule; L-type sensillum L2; S-type sensillum S6; photoreceptor

Chapter 4: Supplementary Information: Table SI2: BLASTn results for each gene, containing the top three hits from the Genbank database.

SI2.1: BLASTn table for *arr1*.

Species	1st hit Accession no.	% Pairwise identity	% Query coverage	2nd hit Accession no.	% Pairwise identity	% Query coverage	3rd hit Accession no.	% Pairwise identity	% Query coverage
L. barwidgeensis	AB291229.1	73.01	71	XM_019043448.1	70.98	98	XM_018481085.2	70.10	98
L. bialveus	XM_018718729.1	71.80	100	XM_019043448.1	73.29	90	XM_018481085.2	71.67	100
L. compactus	XM_020024243.1	73.84	99	XM_020019156.1	73.84	99	XM_019043448.1	73.32	99
L. cooperi	AB291229.1	75.00	94	XM_018481085.2	73.64	94	XM_018481084.2	73.64	94
L. cueensis	XM_023048639.1	77.40	97	AB291229.1	76.40	92	XM_018481085.2	74.94	97
L. cunyenesis	XM_023048639.1	76.39	76	XM_018718729.1	76.08	77	XM_018718728.1	76.08	77
L. eberhardi	XM_018718729.1	69.80	100	XM_018718728.1	69.80	100	AJ303080.1	69.03	97
L. hahni	XM_019043448.1	73.30	100	XM_018718729.1	72.68	99	XM_018718728.1	72.68	99
L. hinkleri	XM_019043448.1	74.08	100	XM_018481085.2	73.49	99	AJ303080.1	72.41	99
L. lapostaae	XM_018718729.1	73.50	100	XM_018718728.1	73.50	100	XM_023048639.1	74.34	95
L. leysi	XM_017928836.1	75.00	95	XM_018718729.1	72.65	100	XM_018718728.1	72.65	100
L. macrotarsus	XM_019043448.1	72.63	99	XM_018718729.1	72.29	99	XM_018718728.1	72.29	99
L. melitaensis	XM_023048639.1	76.20	97	XM_019043449.1	75.06	98	XM_019043448.1	75.06	98
L. micromelitaensis	AJ303080.1	82.69	95	XM_019043448.1	80.37	98	XM_018481085.2	80.77	95
L. microocular	XM_019043448.1	73.99	99	XM_018481085.2	73.26	99	XM_020024243.1	72.96	100
L. microomatoion	XM_019043448.1	73.21	99	XM_018481085.2	73.03	99	AJ303080.1	71.98	99
L. millbilliensis	XM_023048639.1	78.66	91	XM_018718729.1	76.46	93	XM_018718728.1	76.46	93
L. mirandaae	XM_019043448.1	73.81	99	XM_018481085.2	72.80	99	XM_020024243.1	72.69	100
L. pulpa	XM_019043448.1	73.27	99	XM_018481085.2	72.98	99	AJ303080.1	72.46	99
L. rivulus	XM_019043448.1	72.98	99	XM_018481085.2	72.80	99	XM_020024243.1	72.45	100
L. silus	XM_019043448.1	70.21	99	XM_020024243.1	69.84	100	XM_018481084.2	74.58	89
L. sweetwatersensis	XM_018718729.1	73.64	100	XM_018718728.1	73.64	100	XM_019043448.1	73.09	99
L. windarraensis	XM_019043448.1	71.06	99	XM_020024243.1	71.20	98	XM_020019156.1	71.20	98

P. copidotibae	XM_020024243.1	72.80	89	XM_020019156.1	72.80	89	AJ303080.1	70.76	90
P. darlotensis	XM_020024243.1	73.19	96	XM_020019156.1	73.19	96	AB291229.1	71.21	98
P. gibbi	XM_020024243.1	72.22	98	XM_020019156.1	72.22	98	XM_018718729.1	71.34	98
P. hamoni	XM_020024243.1	72.74	95	XM_020019156.1	72.74		AB291229.1	72.16	91
P. macrosturtensis	XM_020024243.1	70.69	91	XM_020019156.1	70.69	91	AB291229.1	69.72	86
P. melrosensis	XM_020024243.1	72.55	98	XM_020019156.1	72.55	98	XM_018718729.1	70.85	98
P. mesosturtensis	XM_020024243.1	72.32	98	XM_020019156.1	72.32	98	XM_018718729.1	71.06	98
P. microsturtensis	XM_020024243.1	72.47	98	XM_020019156.1	72.47	98	AB291229.1	72.06	96
P. nigroadumbratus	XM_020024243.1	74.07	98	XM_020019156.1	74.07	98	AB291229.1	72.31	98
P. plutonicensis	XM_020024243.1	72.23	93	XM_020019156.1	72.23	93	AB291229.1	71.09	94
P. verrucosus	XM_020024243.1	72.91	96	XM_020019156.1	72.91	96	AJ303080.1	71.16	98
P. wedgeensis	XM_020024243.1	73.53	96	XM_020019156.1	73.53	96	XM_018718729.1	72.90	92
A. bistrigatus	XM_018718729.1	74.07	100	XM_018718728.1	74.07	100	AJ303080.1	72.97	99

SI2.2: BLASTn table for *arr2*.

Species	1st hit Accession no.	% Pairwise identity	% Query coverage	2nd hit Accession no.	% Pairwise identity	% Query coverage	3rd hit Accession no.	% Pairwise identity	% Query coverage
L. barwidgeensis	XR_001537349.2	68.37	99	XM_015736319.2	68.37	99	XM_018479179.2	67.61	99
L. bialveus	XR_001537349.2	67.00	99	XR_001537349.2	69.23	83	XM_015736319.2	69.23	83
L. compactus	XR_001537349.2	72.65	99	XM_015736319.2	72.65	99	XM_018479179.2	72.31	99
L. cooperi	XR_001537349.2	71.30	99	XM_015736319.2	71.30	99	XM_015335890.1	70.44	99
L. cueensis	XM_018479179.2	70.98	99	XR_001537349.2	70.72	99	XM_015736319.2	70.72	99
L. cunyenesis	XM_026895327.1	76.64	73	XM_018479179.2	73.33	93	XM_012431149.2	80.77	54
L. eberhardi	LC384837.1	75.07	98	XR_001537349.2	74.35	98	XM_015736319.2	74.35	98
L. hahni	XR_001537349.2	69.49	99	XM_015736319.2	69.49	99	XM_022077053.1	72.22	47
L. hinkleri	XR_001537349.2	71.53	96	XM_015736319.2	71.53	96	XM_018479179.2	70.61	99
L. lapostaae	XM_015335890.1	70.92	96	XM_014756084.1	69.75	96	NM_001170613.1	69.36	97
L. leysi	XM_018479179.2	69.28	100	XM_025976367.1	69.28	100	XR_001537349.2	68.82	99
L. macrotarsus	XM_018479179.2	70.31	88	XR_001537349.2	68.59	99	XM_015736319.2	68.59	99
L. melitaensis	XM_020024486.1	76.43	100	XM_023454090.1	74.29	100	XM_018707507.2	74.29	100
L. micromelitaensis	XM_020024486.1	76.73	95	XM_022342128.1	77.33	90	XM_023454090.1	75.47	95
L. microocular	XR_001537349.2	71.81	99	XM_015736319.2	71.81	99	XM_018479179.2	71.64	99
L. microomatoion	XR_001537349.2	71.90	99	XM_015736319.2	71.90	99	XM_018479179.2	71.73	99
L. millbilliensis	XM_015335890.1	70.57	95	XR_001537349.2	68.95	90	XM_015736319.2	68.95	90
L. mirandaae	XR_001537349.2	70.10	99	XM_015736319.2	70.10	99	XM_015335890.1	69.57	99
L. palmulaoides	XR_001537349.2	71.49	97	XM_015736319.2	71.49	97	XM_018479179.2	70.52	99
L. pulpa	XR_001537349.2	71.70	98	XM_015736319.2	71.70	98	XM_015335890.1	70.69	98
L. rivulus	XM_018479179.2	71.64	99	XR_001537349.2	71.90	99	XM_015736319.2	71.90	99
L. silus	XR_001537349.2	70.07	98	XM_015736319.2	70.07	98	XM_018479179.2	69.27	99
L. sweetwatersensis	XR_001537349.2	76.27	97	XM_015736319.2	76.27	97	XM_022342128.1	78.06	85
L. windarraensis	XR_001537349.2	74.22	96	XM_015736319.2	74.22	96	LC384837.1	72.71	73
P. copidotibae	XM_019695368.1	77.05	66	XM_019673482.1	75.96	66	XM_026429038.1	73.49	78
P. darlotensis	XM_017912320.1	78.79	92	XM_018479179.2	76.81	96	XM_012431149.2	73.72	95
P. gibbi	LC384837.1	73.56	99	JN871509.1	72.22	99	XM_028315724.1	72.24	97
P. hamoni	XM_022979832.1	84.81	100	XM_026873822.1	81.65	100	XM_028170561.1	81.01	100

P. macrosturtensis	XM_012675850.2	76.50	86	XM_012280175.1	66.06	99	XM_003699141.2	66.06	99
P. mesosturtensis	XM_012675850.2	74.25	74	XM_011302215.1	74.11	72	XM_018020234.2	72.80	80
P. microsturtensis	XM_012675850.2	74.25	60	XM_011302215.1	74.11	58	XM_018020234.2	72.80	65
P. nigroadumbratus	XM_012280175.1	71.02	99	XM_003699141.2	71.02	99	XM_012397606.2	71.39	99
P. plutonicensis	NM_001170613.1	72.98	99	LC384837.1	71.90	97	JN871509.1	71.25	96
A. bistrigatus	XM_018479179.2	71.89	99	XR_001537349.2	71.73	99	XM_015736319.2	71.73	99

SI2.3: BLASTn table for *inaD*.

Species	1st hit Accession no.	% Pairwise identity	% Query coverage	2nd hit Accession no.	% Pairwise identity	% Query coverage	3rd hit Accession no.	% Pairwise identity	% Query coverage
L. barwidgeensis	XM_023163081.1	70.75	78	XM_018707301.2	70.11	80	XM_023163080.1	70.62	78
L. bialveus	XM_025974435.1	70.32	53	XM_018707301.2	69.86	80	XM_023163081.1	70.10	75
L. compactus	XM_018707301.2	70.45	72	XM_018707303.2	70.14	64	XM_018707300.2	70.14	72
L. cooperi	XM_018707301.2	70.11	73	XM_018707303.2	69.79	64	XM_018707300.2	69.79	73
L. cueensis	XM_018707301.2	71.62	73	XM_017919024.1	70.95	98	XM_018707303.2	71.21	73
L. cunyenesis	XM_018707301.2	77.12	58	XM_018707300.2	76.86	57	XM_025974435.1	74.33	49
L. eberhardi	XM_025974435.1	70.65	53	XM_018707301.2	70.12	72	XM_017919024.1	69.71	55
L. hahni	XM_025974435.1	72.95	43	XM_017919024.1	71.02	48	XM_018707301.2	70.20	77
L. hinkleri	XM_025974435.1	69.88	56	XM_018707301.2	70.98	66	XM_018707300.2	70.62	66
L. lapostaae	XM_020016880.1	69.59	77	XM_018707301.2	70.47	72	XM_023163081.1	71.08	75
L. leysi	XM_025974435.1	72.01	45	XM_018707301.2	70.17	78	XM_018707303.2	69.85	69
L. macrotarsus	XM_018707301.2	70.78	78	XM_018707303.2	70.45	53	XM_018707300.2	70.45	78
L. melitaensis	XM_018707301.2	70.32	74	XM_025974435.1	70.45	53	XM_018707300.2	70.00	74
L. micromelitaensis	XM_025974435.1	70.59	53	XM_018707301.2	70.62	74	XM_018707300.2	70.30	74
L. microocular	XM_018707301.2	70.47	72	XM_017919024.1	70.64	70	XM_017919023.1	70.64	70
L. microomatoion	XM_018707301.2	70.32	72	XM_002172196.1	69.66	62	XM_017919024.1	70.76	70
L. millbilliensis	XM_025974435.1	70.15	56	XM_018707301.2	70.32	71	XM_018707303.2	70.00	63
L. mirandaae	XM_025974435.1	70.60	43	XM_018707301.2	70.47	72	XM_018707303.2	70.15	64
L. palmulaoides	XM_018707301.2	70.64	72	XM_018707303.2	70.27	63	XM_018707300.2	70.27	72
L. pulpa	XM_018707301.2	71.85	66	XM_020016880.1	68.90	78	XM_018707300.2	71.48	66
L. rivulus	XM_017919024.1	70.78	70	XM_018707301.2	70.62	72	XM_018707303.2	70.30	64
L. silus	XM_018707301.2	70.47	72	XM_023163081.1	70.59	71	XM_018707303.2	70.15	70
L. sweetwatersensis	XM_018707301.2	70.78	78	XM_018707303.2	70.45	53	XM_018707300.2	70.45	78
L. windarraensis	XM_025974435.1	73.06	69	XM_018707301.2	70.85	68	XM_018707303.2	70.32	60
P. copidotibae	XM_017919024.1	70.68	64	XM_018707301.2	69.56	59	XM_018707300.2	69.19	59
P. darlotensis	XM_017919024.1	71.35	58	XM_017919023.1	71.35	58	XM_008183551.2	71.35	58
P. gibbi	XM_025974435.1	69.20	56	XM_023163081.1	68.56	64	XM_017919024.1	69.15	57
P. hamoni	XM_017919024.1	70.82	58	XM_017919023.1	70.82	58	XM_015981813.1	72.73	47

P. macrosturtensis	XM_017919024.1	70.75	67	XM_017919023.1	70.75	67	XM_018707301.2	69.38	61
P. melrosensis	XM_017919024.1	70.82	58	XM_017919024.1	70.82	58	XM_015981813.1	72.51	47
P. mesosturtensis	XM_017919024.1	70.42	65	XM_018707301.2	69.38	60	XM_018707303.2	69.01	60
P. microsturtensis	XM_017919024.1	70.75	66	XM_018707301.2	69.73	60	XM_018707303.2	69.37	60
P. nigroadumbratus	XM_015981810.1	72.80	58	XM_018707301.2	69.53	61	XM_017919024.1	71.48	43
P. plutonicensis	XM_017919024.1	70.21	64	XM_017919023.1	70.21	64	XM_018707301.2	70.44	59
P. verrucosus	XM_018707301.2	68.71	62	XM_018707303.2	68.64	62	XM_018707300.2	68.64	62
P. wedgeensis	XM_024227640.1	67.19	46	XM_018707301.2	70.16	45	XM_023057169.1	70.62	45
A. bistrigatus	XM_025974435.1	71.99	58	XM_020016880.1	69.32	85	XM_002431236.1	70.56	47

SI2.4: BLASTn table for *c-opsin*.

Species	1st hit Accession no.	% Pairwise identity	% Query coverage	2nd hit Accession no.	% Pairwise identity	% Query coverage	3rd hit Accession no.	% Pairwise identity	% Query coverage
L. barwidgeensis	KP219386.1	94.78	99	KP219385.1	76.12	96	XM_023165964.1	69.36	98
L. bialveus	KP219386.1	92.29	50	KP219385.1	70.72	50	XM_023165964.1	66.11	49
L. compactus	KP219386.1	94.57	70	KP219385.1	75.96	68	XM_023165964.1	68.29	69
L. cooperi	KP219386.1	93.46	80	KP219385.1	74.88	78	XM_023165964.1	68.63	78
L. cueensis	KP219386.1	94.46	99	KP219385.1	76.90	96	XM_023165964.1	70.39	98
L. cunyenesis	KP219386.1	93.95	73	KP219385.1	73.08	73	XM_023165964.1	66.38	76
L. eberhardi	KP219386.1	94.01	64	KP219385.1	71.86	64	XM_023165964.1	66.06	68
L. hahni	KP219386.1	96.94	99	KP219385.1	76.53	99	XM_022343113.1	71.64	99
L. hinkleri	KP219386.1	93.82	80	KP219385.1	74.50	78	XM_023165964.1	68.18	78
L. lapostaae	KP219386.1	93.18	99	KP219385.1	76.03	99	XM_026897038.1	69.12	97
L. leysi	KP219386.1	93.19	51	KP219385.1	71.31	50	XM_023165964.1	67.10	53
L. macrotarsus	KP219386.1	93.18	100	KP219385.1	71.91	99	LC009258.1	69.70	64
L. melitaensis	KP219386.1	94.49	61	KP219385.1	73.13	61	XM_026885747.1	74.23	28
L. micromelitaensis	KP219386.1	93.10	80	KP219385.1	74.23	78	XM_023165964.1	67.82	81
L. microocular	KP219386.1	94.69	70	KP219385.1	75.46	68	XM_023165964.1	68.18	71
L. microomatoion	KP219386.1	94.81	70	KP219385.1	75.96	68	XM_023165964.1	68.18	70
L. millbilliensis	KP219386.1	76.29	34	KP219385.1	78.87	12	XM_023165964.1	83.93	10
L. mirandaae	KP219386.1	93.10	79	KP219385.1	74.23	79	XM_023165964.1	67.47	80
L. palmulaoides	KP219386.1	96.06	100	KP219385.1	76.67	100	XM_026885747.1	71.60	98
L. pulpa	KP219386.1	93.44	64	KP219385.1	71.23	64	XM_023165964.1	65.45	68
L. rivulus	KP219386.1	95.17	80	KP219385.1	76.20	78	XM_023165964.1	68.41	81
L. silus	KP219386.1	92.68	65	KP219385.1	70.92	64	XM_023165964.1	65.14	65
L. sweetwatersensis	KP219386.1	92.98	80	KP219385.1	74.26	78	XM_023165964.1	67.89	81
L. windarraensis	KP219386.1	94.55	77	KP219385.1	72.40	77	XM_023165964.1	67.39	77
P. darlotensis	KP219385.1	93.98	74	KP219386.1	72.50	72	XM_022258904.1	69.10	44
P. gibbi	KP219385.1	90.32	95	KP219386.1	76.58	93	XM_013306536.1	68.97	75
P. melrosensis	KP219385.1	90.07	77	KP219386.1	69.09	77	XM_014514264.1	64.32	50
P. microsturtensis	KP219385.1	86.67	100	KP219386.1	80.00	98	XM_028176172.1	75.00	96

P. nigroadumbratus	KP219385.1	100.00	74	KP219386.1	73.89	74	XM_013306536.1	68.84	46
P. verrucosus	KP219385.1	86.02	54	KP219386.1	80.00	17	XM_023863785.1	80.00	17
A. bistrigatus	KP219386.1	100.00	70	KP219385.1	76.45	68	XM_023165964.1	69.05	69

SI2.5: BLASTn table for *lwop*.

Species	1st hit Accession no.	% Pairwise identity	% Query coverage	2nd hit Accession no.	% Pairwise identity	% Query coverage	3rd hit Accession no.	% Pairwise identity	% Query coverage
L. barwidgeensis	KP219381.1	81.91	89	KP219382.1	81.77	89	KY368375.1	71.50	99
L. bialveus	KP219382.1	90.08	84	KP219381.1	89.98	84	KY368375.1	78.42	84
L. compactus	KP219382.1	92.58	85	KP219381.1	92.58	85	KY368375.1	79.75	84
L. cooperi	KP219382.1	89.94	86	KP219381.1	89.94	86	KY368375.1	80.58	99
L. cueensis	KP219382.1	87.61	82	KP219381.1	87.92	82	KY368375.1	81.73	99
L. cunyenesis	KP219382.1	82.53	80	KP219381.1	82.53	80	KY368375.1	73.40	82
L. eberhardi	KP219382.1	92.41	74	KP219381.1	92.41	74	KY368283.1	80.13	76
L. hahni	KP219382.1	90.79	86	KP219381.1	90.79	86	KY368375.1	79.89	99
L. hinkleri	KP219382.1	92.70	99	KP219381.1	92.13	99	KY368375.1	85.39	99
L. lapostaae	KP219382.1	90.66	86	KP219381.1	90.66	86	KY368375.1	80.69	99
L. leysi	KP219382.1	91.87	85	KP219381.1	91.87	85	KY368375.1	79.53	84
L. macrotarsus	KP219382.1	91.86	81	KP219381.1	92.09	81	KY368283.1	77.88	82
L. melitaensis	KP219381.1	88.15	100	KP219382.1	87.93	100	KY368375.1	72.25	99
L. micromelitaensis	KP219381.1	93.85	100	KP219382.1	93.62	100	KY368375.1	77.29	99
L. microocular	KP219381.1	91.46	85	KP219382.1	91.06	85	KY368375.1	77.96	84
L. microomatoion	KP219381.1	91.57	85	KP219382.1	91.16	85	KY368375.1	77.96	84
L. millbilliensis	KP219382.1	91.78	76	KP219381.1	91.78	76	KY368283.1	78.64	76
L. mirandaae	KP219382.1	92.44	91	KP219381.1	92.28	91	KY219380.1	75.23	91
L. palmulaoides	KP219381.1	91.13	87	KP219382.1	90.83	87	KY368283.1	74.29	84
L. pulpa	KP219382.1	88.11	85	KP219381.1	88.11	85	KY368375.1	78.66	77
L. rivulus	KP219382.1	92.38	85	KP219381.1	92.78	85	KY368375.1	79.49	84
L. silus	KP219382.1	88.72	85	KP219381.1	88.72	85	KY368375.1	76.74	84
L. sweetwatersensis	KP219382.1	82.53	74	KP219381.1	82.53	74	KY368283.1	73.40	75
L. windarraensis	KP219382.1	92.07	85	KP219381.1	92.07	85	KY368375.1	79.63	84
P. copidotibae	KY219380.1	88.21	100	EU921225.1	78.21	100	KF539443.1	76.36	98
P. darlotensis	KY219380.1	92.71	86	KY368375.1	77.92	96	EU921225.1	77.27	91
P. gibbi	KY219380.1	91.25	90	KY368375.1	79.46	98	EU921225.1	78.46	95

P. macrosturtensis	KY219380.1	82.53	82	KY368375.1	71.73	92	EU921225.1	71.63	88
P. melrosensis	KY219380.1	90.82	92	KY368375.1	75.36	92	EU921225.1	74.45	94
P. mesosturtensis	KY219380.1	75.62	100	KY368298.1	68.28	94	KP219381.1	67.84	100
P. microsturtensis	KY219380.1	81.63	82	KY368375.1	72.00	92	KY368298.1	71.43	99
P. nigroadumbratus	KY219380.1	100.00	87	KY368375.1	78.74	94	EU921225.1	77.08	91
P. plutonicensis	KY219381.1	88.21	100	EU921225.1	76.79	100	KY368375.1	75.36	100
P. verrucosus	KY219381.1	80.47	83	KY368375.1	71.08	100	EU921225.1	71.43	89
P. wedgeensis	KY219381.1	87.58	80	KP219382.1	74.32	80	KP219381.1	74.32	80
A. bistrigatus	KY219380.1	100.00	85	KP219382.1	99.39	85	KY368375.1	78.70	84

SI2.6: BLASTn table for *uvop*.

Species	1st hit Accession no.	% Pairwise identity	% Query coverage	2nd hit Accession no.	% Pairwise identity	% Query coverage	3rd hit Accession no.	% Pairwise identity	% Query coverage
P. darlotensis	EU921226.1	78.31	76	KP219383.1	96.77	28	CP000806.1	90.62	29
P. gibbi	KP219383.1	89.91	100	EU921226.1	76.15	100	KY368350.1	75.93	99
P. macrosturtensis	KP219383.1	86.58	82	KY368309.1	73.53	85	EU921227.1	72.08	95
P. nigroadumbratus	KP219383.1	100.00	84	EU921226.1	74.54	90	KY368262.1	72.02	99
P. wedgeensis	KP219383.1	83.87	99	KY368350.1	71.99	99	KY368345.1	70.97	99

SI2.7: BLASTn table for *trp*.

Species	1st hit Accession no.	% Pairwise identity	% Query coverage	2nd hit Accession no.	% Pairwise identity	% Query coverage	3rd hit Accession no.	% Pairwise identity	% Query coverage
L. barwidgeensis	XM_015325802.1	72.38	99	XM_015325801.1	72.38	99	XM_023455053.1	71.94	99
L. bialveus	XM_015325801.1	69.90	93	XM_014757067.1	69.32	93	XM_015741511.2	69.18	93
L. compactus	XM_015325801.1	69.88	93	XM_023455053.1	70.05	93	XM_014757067.1	69.36	93
L. cooperi	XM_015325801.1	70.38	93	XM_023455053.1	70.06	92	XM_015741511.2	69.74	93
L. cueensis	XM_015741511.2	72.63	84	XM_023455053.1	72.23	85	XM_015325803.1	72.10	84
L. cunyenesis	XM_015325803.1	70.39	90	XM_015325801.1	70.39	90	XM_023455053.1	69.84	90
L. eberhardi	XM_015325803.1	70.40	60	XM_015741511.2	69.83	89	XM_023455053.1	69.48	95
L. hahni	XM_015325801.1	70.14	90	XM_015741511.2	69.84	90	XM_014757067.1	69.79	90
L. hinkleri	XM_015325801.1	69.39	94	XM_023455053.1	69.82	89	XM_015741511.2	69.74	90
L. lapostaae	XM_015325803.1	70.37	87	XM_015325801.1	70.37	87	XM_015265646.1	71.25	83
L. leysi	XM_015325801.1	69.46	93	XM_015741511.2	69.87	92	XM_023455053.1	69.54	93
L. macrotarsus	XM_015325803.1	71.65	87	XM_015325801.1	71.65	87	XM_023157474.1	71.06	92
L. melitaensis	XM_015325803.1	73.80	66	XM_015325801.1	73.80	66	XM_008562061.1	72.58	73
L. micromelitaensis	XM_015325801.1	69.54	93	XM_023455053.1	70.28	87	XM_015741511.2	69.94	91
L. microocular	XM_014757067.1	71.16	88	XM_015325803.1	71.11	62	XM_023455053.1	69.48	94
L. microomatoion	XM_015741511.2	72.13	86	XM_023455053.1	72.07	87	XM_015325803.1	71.91	87
L. millbilliensis	XM_015325801.1	70.52	90	XM_015741511.2	70.37	90	XM_023455053.1	70.64	87
L. mirandaae	XM_023157474.1	71.34	91	XM_015325803.1	70.60	87	XM_015325801.1	70.60	87
L. palmulaoides	XM_015325803.1	70.84	87	XM_015325801.1	70.84	87	XM_023157474.1	72.87	83
L. pulpa	XM_015325803.1	71.21	90	XM_015325801.1	71.21	90	XM_023157474.1	71.70	91
L. rivulus	XM_015325803.1	70.44	94	XM_015325801.1	69.34	94	XM_015741511.2	69.36	93
L. silus	XM_015741511.2	71.16	83	XM_015325803.1	70.83	83	XM_023157474.1	71.54	84
L. sweetwatersensis	XM_023157474.1	69.00	94	XM_008562061.1	68.09	94	XM_015741511.2	69.27	93
L. windarraensis	XM_015741511.2	70.84	83	XM_015325803.1	69.74	87	XM_023157474.1	72.06	84
A. bistrigatus	XM_015325801.1	73.12	94	XM_023157474.1	72.56	94	XM_015741511.2	72.44	94

SI2.8: BLASTn table for *trpl*.

Species	1st hit Accession no.	% Pairwise identity	% Query coverage	2nd hit Accession no.	% Pairwise identity	% Query coverage	3rd hit Accession no.	% Pairwise identity	% Query coverage
L. barwidgeensis	XM_023157464.1	70.73	78	XM_023863548.1	70.21	79	XM_023455051.1	70.06	78
L. bialveus	XM_023455051.1	71.80	94	XM_023157464.1	70.75	94	XM_023056957.1	71.35	85
L. compactus	XM_023455051.1	73.58	94	XM_023157464.1	71.46	94	XM_023056957.1	71.35	85
L. cooperi	XM_026616242.1	68.87	98	XM_026616240.1	68.87	98	XM_024095357.1	70.22	80
L. cueensis	XM_014384982.2	69.37	97	XM_014384981.2	69.37	97	XM_026616242.1	68.82	98
L. eberhardi	XM_023455051.1	73.22	97	XM_023157464.1	72.88	74	XM_026902616.1	70.19	49
L. hahni	XM_023863548.1	67.57	87	XM_023157464.1	66.86	86	XM_014432617.1	67.04	87
L. hinkleri	XM_023455051.1	72.09	91	XM_023157464.1	71.46	94	XM_023056957.1	72.40	85
L. lapostaae	XM_026616242.1	69.40	97	XM_026616240.1	69.40	97	XM_014384982.2	68.99	97
L. leysi	XM_026902616.1	72.52	76	XM_024095357.1	70.05	98	XM_024095356.1	70.05	98
L. macrotarsus	XM_023455051.1	74.35	59	XM_023157464.1	70.35	78	XM_014432617.1	68.90	78
L. melitaensis	XM_026616242.1	69.05	98	XM_026616240.1	69.05	98	XM_024095357.1	70.99	80
L. micromelitaensis	XM_014384982.2	67.54	97	XM_014384981.2	67.54	97	XM_026616242.1	66.83	98
L. microocular	XM_014384982.2	68.29	97	XM_014384981.2	68.29	97	XM_026616242.1	67.33	98
L. microomatoion	XM_023157464.1	72.88	74	XM_014384982.2	67.48	96	XM_014384981.2	67.48	96
L. millbilliensis	XM_023455051.1	71.46	95	XM_023056957.1	71.54	91	XM_023157464.1	71.00	95
L. mirandaae	XM_024095357.1	71.50	81	XM_024095356.1	71.50	81	XM_024095355.1	71.50	81
L. palmulaoides	XM_026616242.1	69.04	98	XM_026616240.1	69.04	98	XM_024095357.1	70.88	81
L. pulpa	XM_023455051.1	74.41	96	XM_014384982.2	68.94	94	XM_014384981.2	68.94	94
L. rivulus	XM_014384982.2	69.71	97	XM_014384981.2	69.71	97	XM_026616242.1	68.33	98
L. silus	XM_026616242.1	67.71	98	XM_026616240.1	67.71	98	XM_014504999.1	68.16	86
L. windarraensis	XM_023455051.1	73.46	94	XM_023157464.1	72.88	95	XM_023056957.1	73.18	86
A. bistrigatus	XM_026616242.1	71.79	95	XM_026616240.1	71.79	95	XM_023455051.1	71.01	99

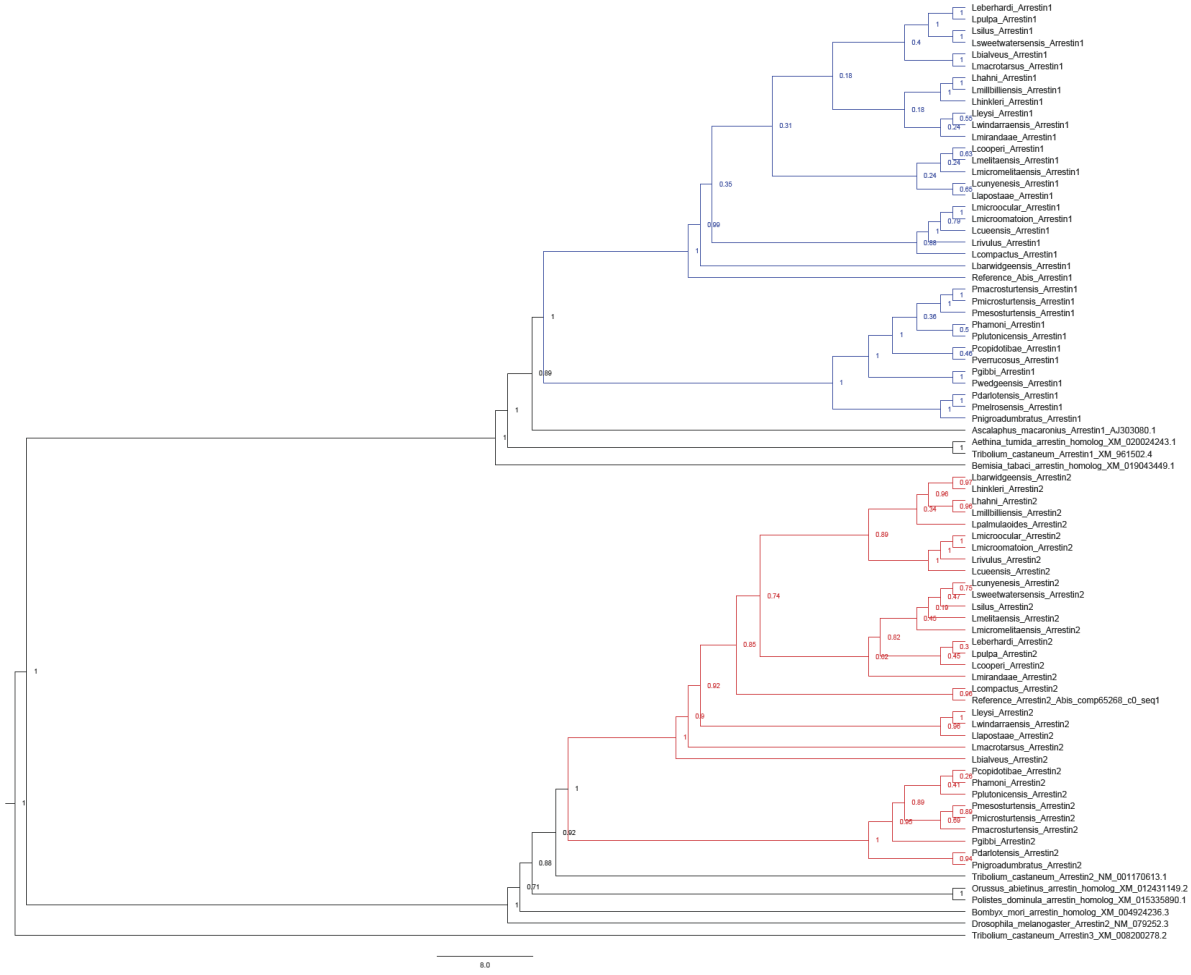
Chapter 4: Supplementary Information: Table SI3: Various references for phylogenetic (orthology) analyses.

Gene class and taxon name	Order	Common name	Gene	Accession	Reference
ARRESTIN 1					
Ascalaphus macaronius	Neuroptera	Owlfly	Arrestin 1	AJ303080.1	Bentrop et al. 2001
Tribolium castaneum	Coleoptera	Red flour beetle	Arrestin 1	XM_961502.4	NCBI 2016
Aethina tumida	Coleoptera	Small hive beetle	Arrestin homolog	XM_020024243.1	NCBI 2017
Bombyx mori	Lepidoptera	Silkworm	Arrestin homolog	XM_004924236	NCBI 2017
Bemisia tabaci	Hemiptera	Silverleaf whitefly	Arrestin homolog	XM_019043449	NCBI 2016
ARRESTIN 2					
Drosophila melanogaster	Diptera	Common fruit fly	Arrestin 2	NM_079252.3	Adams et al. 2000
Tribolium castaneum	Coleoptera	Red flour beetle	Arrestin 2	NM_001170613.1	Richards et al. 2008
Tribolium castaneum	Coleoptera	Red flour beetle	Arrestin 3	XM_008200278.2	Kim et al. 2010
Orussus abietinus	Hymenoptera	Parasitic wood wasp	Arrestin 2	XM_012431149.2	Misof et al. 2014
Polistes dominula	Hymenoptera	European paper wasp	Arrestin homolog	XM_015335890	NCBI 2016
INACTIVATION NO AFTERPOTENTIAL D					
Drosophila melanogaster	Diptera	Common fruit fly	inaD	NT_033778.4	Adams et al. 2000
Drosophila melanogaster	Diptera	Common fruit fly	inaC	NM_057515.3	Adams et al. 2014
Anoplophora glabripennis	Coleoptera	Asian long-horned beetle	inaD	XM_018707301.2	NCBI 2018
Lepitinotarsa decemlineata	Coleoptera	Colorado potato beetle	inaD-like	XM_023163085.1	NCBI 2017
NON-VISUAL OPSIN					
Takifugu rubripes	Tetraodontiformes	Pufferfish	Multiple tissue opsin	AF402774	Moutsaki et al. 2003
Danio rerio	Cypriniformes	Zebrafish	Multiple tissue opsin	AF349947	Moutsaki et al. 2003
Apis mellifera	Hymenoptera	European honey bee	Pteropsin	NM_001039968	Velarde et al. 2005
Tribolium castaneum	Coleoptera	Red flour beetle	C-opsin	NM_001145478	NCBI 2013
Bombyx mori	Lepidoptera	Domestic silkworm	Parapinopsin-like	XM_004928326	NCBI 2013
UV OPSIN					
Papilio xuthus	Lepidoptera	Swallowtail butterfly	UV opsin	AB028218	Kitamoto et al. unpub.
Apis mellifera	Hymenoptera	European honey bee	UV opsin	NM_001011605	Townson et al. 1998

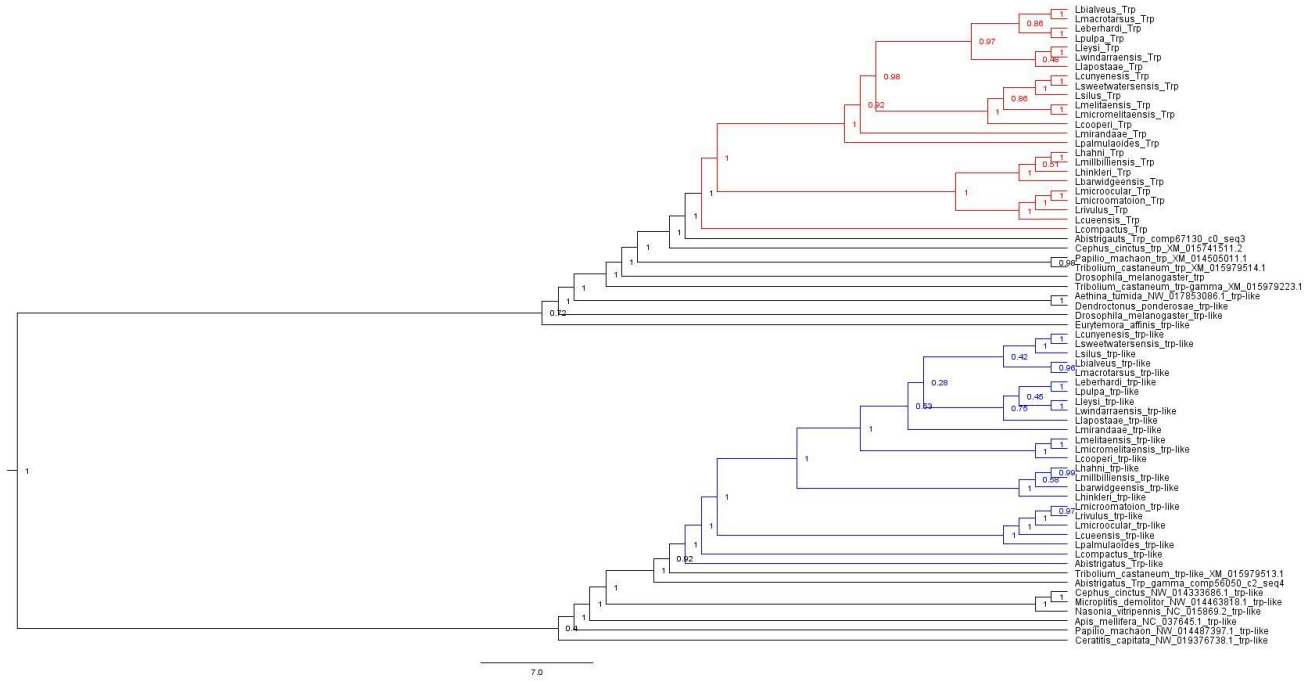
Tribolium castaneum	Coleoptera	Red flour beetle	UV opsin-like	XM_965251	NCBI 2008
Thermonectus marmoratus	Coleoptera	Sunburst diving beetle	UV opsin 1	EU921226	Maksimovic et al. 2009
Thermonectus marmoratus	Coleoptera	Sunburt diving beetle	UV opsin 2	EU921227	Maksimovic et al. 2009
LONG WAVELENGTH OPSIN					
Tribolium castaneum	Coleoptera	Red flour beetle	Rhodopsin 1/6-like	NM_001162519	Park et al. 2008
Thermonectus marmoratus	Coleoptera	Sunburst diving beetle	Lwop	EU921225	Maksimovic et al. 2009
Apis mellifera	Hymenoptera	European honey bee	Lwop 1	NM_001011639	NCBI 2014
Apis mellifera	Hymenotpera	European honey bee	Lwop 2	NM_001077825	Velarde et al. 2005
Papilio xuthus	Lepidoptera	Swallowtail butterfly	Rh1	AB007423	Kitamoto et al. 1998
Papilio xuthus	Lepidoptera	Swallowtail butterfly	Rh2	AB007424	Kitamoto et al. 1998
Papilio xuthus	Lepidoptera	Swallowtail butterfly	Rh3	AB007425	Kitamoto et al. 1998
TRANSIENT RECEPTOR POTENTIAL (AND –LIKE)					
Drosophila melanogaster	Diptera	Common fruit fly	Trp	NT_033777.3	Adams et al. 2000
Papilio machaon	Lepidoptera	Swallowtail butterfly	Trp	XM_014505011	NCBI 2015
Cephus cinctus	Hymenopterans	Wheat stem sawfly	Trp	XM_015741511	NCBI 2018
Tribolium castaneum	Coleoptera	Red flour beetle	Trp	XM_015979514.1	NCBI 2016
Tribolium castaneum	Coleoptera	Red flour beetle	Trp-gamma	XM_015979223.1	NCBI 2016
Aethina tumida	Coleoptera	Small hive beetle	Trp-like	NW_017853550.1	NCBI 2016
Apis mellifera	Hymenoptera	Western honey bee	Trp-like	NC_037645.1	Wallberg et al. 2018
Cephus cinctus	Hymenopterans	Wheat stem sawfly	Trp-like	NW_014333686.1	Robertson et al. 2012
Ceratitis capitata	Diptera	Mediterranean fruit fly	Trp-like	NW_019376738.1	Murali et al. unpublished
Dendroctonus ponderosae	Coleoptera	Mountain pine beetle	Trp-like	NW_017850257	Keeling et al. 2013
Drosophila melanogaster	Diptera	Common fruit fly	Trp-like	NT_033778	Matthews et al. 2015
Eurytemora affinis	Calanoida	Na	Trp-like	NW_019396480	Murali et al. unpublished
Microplitis demolitor	Hymenoptera	Na	Trp-like	NW_014463818.1	Burke et al. 2014
Nasonia vitripennis	Hymenoptera	Na	Trp-like	NC_015869.2	Werren et al. 2010
Papilio machaon	Lepidoptera	Swallowtail butterfly	Trp-like	NW_014487397.1	Li et al. 2015
Tribolium castaneum	Coleoptera	Red flour beetle	Trp-like	XM_015979513.1	NCBI 2016

Chapter 4: Supplementary Information: Figure SI4: Bayesian phylogenies supporting orthology of all genes. Each gene type was found in monophyletic groups with reference genes of the same type. a. is *arr1* (blue), *arr2* (red). b. is *trp* (red), and *trpl* (blue). c. is *inaD* (red), and d. is *lwop* (red), *uvop* (green), and *c-opsin* (blue).

a.



b.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Lleysi_Trp
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Llapostaae_Trp
Lcunyenesis_Trp
Lsweetwatersensis_Trp
Lsilus_Trp
Lmelitaensis_Trp
Lmicromelitaensis_Trp
Lcooperi_Trp
Lmirandaae_Trp
Lpalmulaoides_Trp
Lhahni_Trp
Lmillbilliensis_Trp
Lhinkleri_Trp
Lbarwidgeensis_Trp
Lmicroocular_Trp
Lmicroomatoion_Trp
Lrivulus_Trp
Lcueensis_Trp
Lcompactus_Trp
Abistrigatus_Trp_comp67130_c0_seq3
Cephus_cinctus_trp_XM_015741511.2
Papilio_machaon_trp_XM_014505011.1
Tribolium_castaneum_trp_XM_015979514.1
Drosophila_melanogaster_trp
Tribolium_castaneum_trp-gamma_XM_015979223.1
Aethina_tumida_NW_017853086.1_trp-like
Dendroctonus_ponderosae_trp-like
Drosophila_melanogaster_trp-like
Eurytemora_affinis_trp-like
Lcunyenesis_trp-like
Lsweetwatersensis_trp-like
Lsilus_trp-like
Lbialveus_trp-like
Lmacrotarsus_trp-like
Leberhardi_trp-like
Lpulpa_trp-like
Lleysi_trp-like
Lwindarraensis_trp-like
Llapostaae_trp-like
Lmirandaae_trp-like
Lmelitaensis_trp-like
Lmicromelitaensis_trp-like
Lcooperi_trp-like
Lhahni_trp-like
Lmillbilliensis_trp-like
Lbarwidgeensis_trp-like
Lhinkleri_trp-like
Lmicroomatoion_trp-like
Lrivulus_trp-like
Lmicroocular_trp-like
Lcueensis_trp-like
Lpalmulaoides_trp-like
Lcompactus_trp-like
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Cephus_cinctus_NW_014333686.1_trp-like
Microplitis_demolitor_NW_014463878.1_trp-like
Nasonia_vitripennis_NC_015869.2_trp-like
Apis_mellifera_NC_037645.1_trp-like
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Ceratitis_capitata_NW_019376738.1_trp-like
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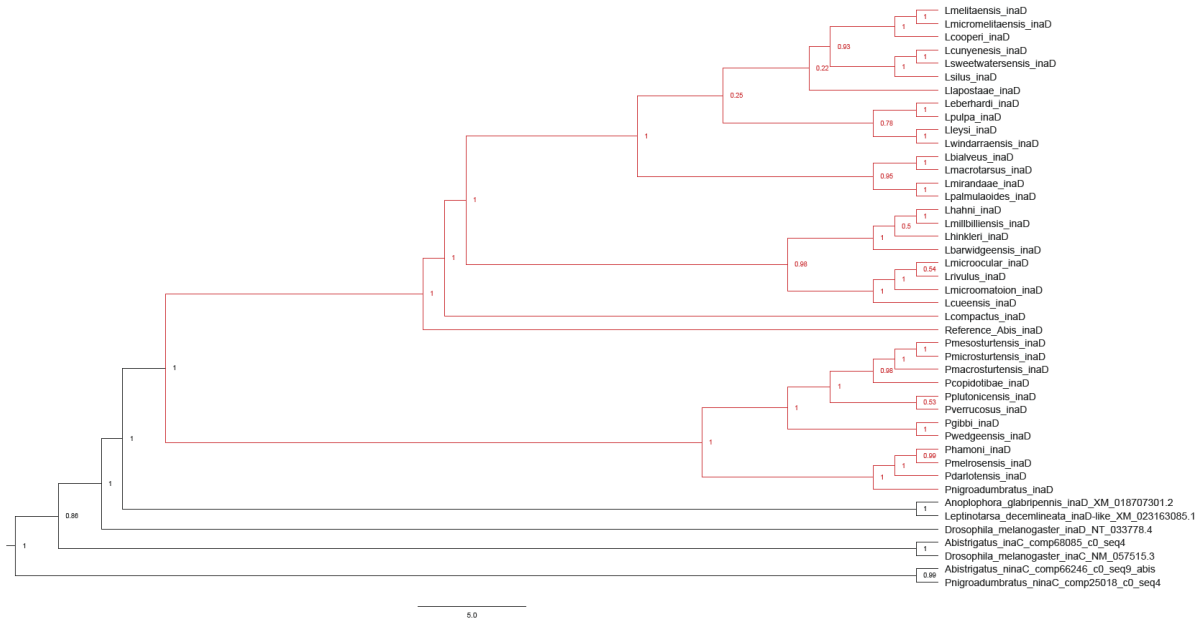
c.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Leberhardi_inaD
Lpulpa_inaD
Lleysi_inaD
Lwindarraensis_inaD
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Lcueensis_inaD
Lcompactus_inaD
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Pmesosturtensis_inaD
Pmicrosturtensis_inaD
Pmacrosturtensis_inaD
Pcopidotibae_inaD
Pplutonicensis_inaD
Pverrucosus_inaD
Pgibbi_inaD
Pwedgeensis_inaD
Phamoni_inaD
Pmelrosensis_inaD
Pdarlotensis_inaD
Pnigroadumbratus_inaD
Anoplophora_glabripennis_inaD_XM_018707301.2
Leptinotarsa_decemlineata_inaD-like_XM_023163085.1
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Abistrigatus_inaC_comp68085_c0_seq4
Drosophila_melanogaster_inaC_NM_057515.3
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Pnigroadumbratus_ninaC_comp25018_c0_seq4
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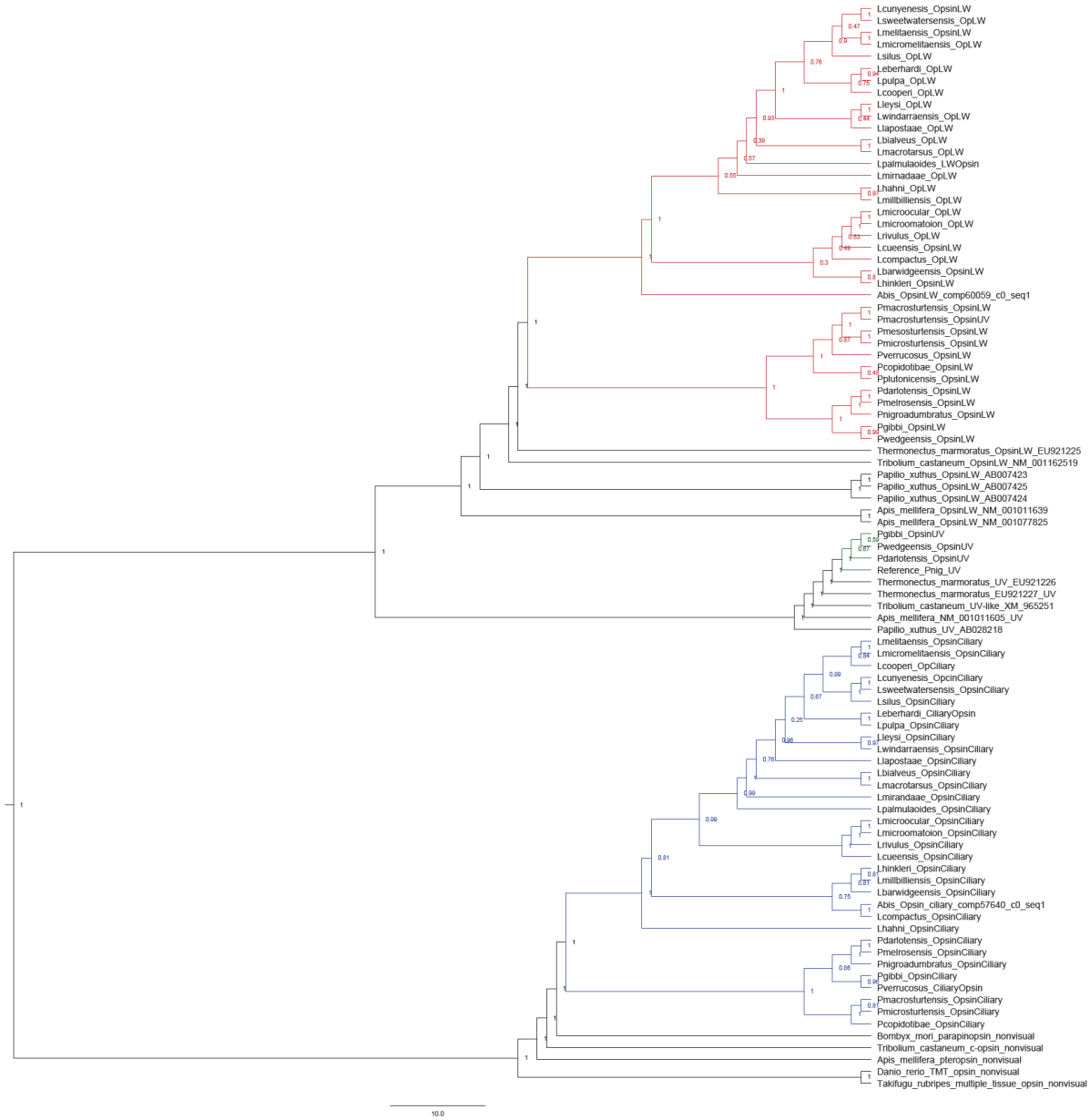
d.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Lsilus_OpLW
Leberhardi_OpLW
Lpulpa_OpLW
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Pmesosturtensis_OpsinLW
Pmicrosturtensis_OpsinLW
Pverrucosus_OpsinLW
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Pplutonicensis_OpsinLW
Pdarlotensis_OpsinLW
Pmelrosensis_OpsinLW
Pnigroadumbratus_OpsinLW
Pgibbi_OpsinLW
Pwedgeensis_OpsinLW
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Pgibbi_OpsinUV
Pwedgeensis_OpsinUV
Pdarlotensis_OpsinUV
Reference_Pnig_UV
Thermonectus_marmoratus_UV_EU921226
Thermonectus_marmoratus_EU921227_UV
Tribolium_castaneum_UV-like_XM_965251
Apis_mellifera_NM_001011605_UV
Papilio_xuthus_UV_AB028218
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Lmicromelitaensis_OpsinCiliary
Lcooperi_OpCiliary
Lcunyenesis_OpcinCiliary
Lsweetwatersensis_OpsinCiliary
Lsilus_OpsinCiliary
Leberhardi_CiliaryOpsin
Lpulpa_OpsinCiliary
Lleysi_OpsinCiliary
Lwindarraensis_OpsinCiliary
Llapostaae_OpsinCiliary
Lbialveus_OpsinCiliary
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Lpalmulaoides_OpsinCiliary
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Lmicroomatoion_OpsinCiliary
Lrivulus_OpsinCiliary
Lcueensis_OpsinCiliary
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Apis_mellifera_pteropsin_nonvisual
Danio_rerio_TMT_opsin_nonvisual
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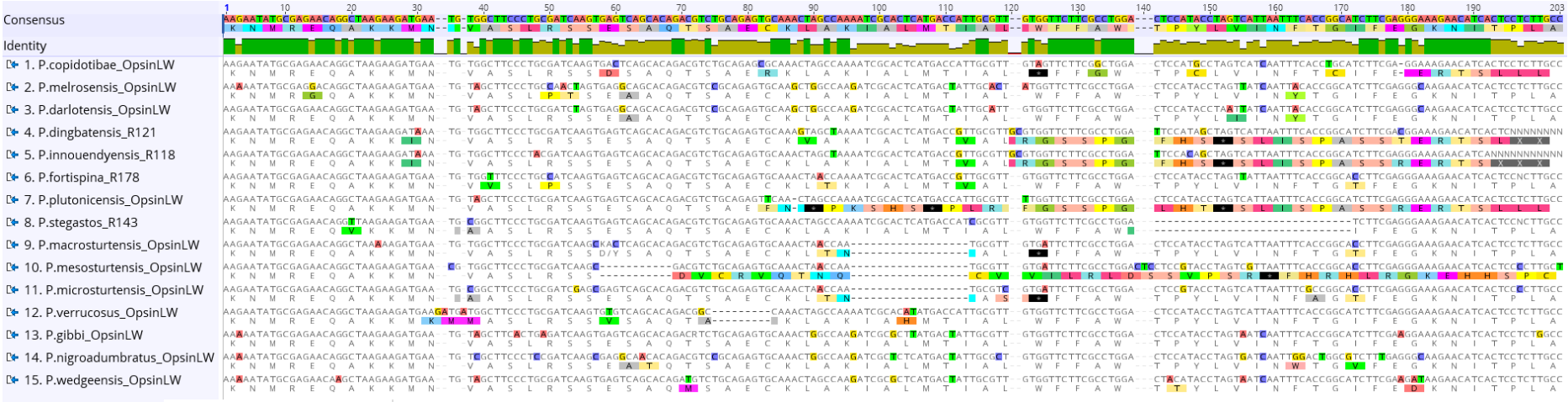
10.0

Chapter 4: Supplementary Information: Figure SI5: *Paroster lwop* data alignment showing mutational variation in coding genes.



Chapter 4: Supplementary Information: SI6: Expanded details of laboratory and bioinformatics methods.

Calcrete sampling. Subterranean diving beetle species from the genera *Limbodessus* and *Paroster* were collected from calcretes in the Yilgarn region of Western Australia utilising pre-drilled bore holes ~10 meters deep. A total of 32 subterranean beetle species were sampled from 20 calcretes representing seven different paleodrainages (Table 2). A plankton net with 250 μm mesh was lowered to the bottom and pulled through the groundwater to concentrate the stygofauna into a collecition tube at the bottom of the net (Allford et al. 2008). Sampled diving beetles were stored in tubes of RNAlater or 100% ethanol. A total of five surface species were used as comparison: *Allodessus bistrigatus*, *L. compactus, L. rivulus, P. nigroadumbratus,* and *P. gibbi*, which were collected from surface pools and stored in 100% ethanol until ready to use.

Transcriptome assembly and bait design

De novo assemblies of putative transcripts for five diving beetles (two surface and three subterranean) were used to find and annotate 19 photoreceptor genes (Table 1) from Tierney et al (2015), following the method outlined by the authors. These 19 genes were then assessed for functionality We selected a subset of 10 genes, where transcripts were present in the surface species, but either absent or showed evidence of non-functionality in the subterranean species. Sequence capture probes were developed from the orthologous transcript sequences of these genes and synthesized by Arbor Biosciences (formally MYcroarray, Ann Arbor, MI).

Library preparation. We extracted DNA from whole beetles following the Gentra protocol (Gentra Systems, Inc.) with the following modifications; 0.5 uL of glycogen was used instead of 2 μL and precipitated DNA was centrifuged for 15 minutes instead of 10 minutes at 4°C. We measured DNA concentrations using a Qubit 2.0 fluorometer (Life Technologies) using a dsDNA quantification kit, and we pooled multiple samples when less than 1 ng/μL. For *P. nigroadumbratus*, we used previously extracted DNA, which had been stored at -80°C. Starting material for sonication ranged from 100 ng to 500 ng. We sheared DNA to an average fragment distribution of 400-600 base pairs (bp) using an on/off setting of 30/30 for 2 cycles, in a Diagenode Bioruptor sonicator. DNA fragment sizes were subsequently verified using a High Sensitivity D1000 screen tape following the accompanying kit protocol for an Agilent 4200 TapeStation System.

Following sonication, we constructed sequencing libraries using the Meyer and Kircher protocol (Meyer and Kircher 2010), using double indexing primers (Hugall et al., 2015; Glenn et al., 2016). We assessed the success of library preparation by measuring DNA concentration using a Library Quantification kit, following the manufacturer's protocol. A DNA quantification kit and a standard qPCR run in a LightCyler 96 Real-Time PCR System was used. All samples underwent a 0.9-1.8X Ampure XP bead clean-up after each step and as a final step following Meyer and Kircher (2010).

Hybridization of baits to libraries and sequencing. Prior to capture we divided the target baits into ¼ capture reactions. We enriched each species in its own ¼ capture or added a maximum of two species together in ¼ capture to ensure maximum targeting of each species. We performed the enrichment following the Arbor Biosciences MYbaits user manual v2 (formerly Microarray). The enrichment was performed at 65 ºC for a period of

44 hours for the first enrichment which contained *L. palmulaoides*, and 18 hours for all other enrichments, as we determined that 18 hours was sufficient for hybridisation.

We verified enrichment success with qPCR as previously mentioned, and using a tapestation for visualization of fragments to confirm they were within the correct size range for sequencing. Size selection was performed using Ampure XP beads in order to reduce the small bp size fragments, following the Meyer & Kircher protocol (2010). All samples were pooled in equal concentrations and subsequently concentrated to 30 μL. The first MiSeq run contained six pooled samples (four different species), the second contained eight pooled samples (eight different species), the third contained 22 pooled samples (18 different species) and the final MiSeq run contained 15 pooled samples (12 different species). Each pool was run on its own lane on the Illumina MiSeq platform (AGRF facility in Adelaide, Australia), obtaining 300 bp paired end reads for MiSeq run one, two and four, and 150 bp paired end reads for Miseq run three. We chose the smaller read return for capture three because starting material was fragmented into sizes too low for 300 bp paired end reads.

Bioinformatics. *Quality assessment and mapping*. Raw sequencing reads for each species were assessed using FASTQC v.0.11.3 (Babraham Institute). Poor quality bases and Illumina adapter sequences were then trimmed using BBDuk v.2 (Bushnell 2015) with the following parameters; `literal=AGATCGGAAGAGCAC,AGATCGGAAGAGCGT ktrim=r k=15 mink=15 hdist=0 tbo qtrim=rl trimq=20 minlength=30 threads=10`. Cleaned reads were mapped to either *L. palmulaoides*, *A. bistrigatus*, or *P. nigroadumbratus* transcriptome data gene sequences, using BWA v.0.7.12 (Li and Durbin 2009) with default parameters and the resulting alignments converted to BAM format, only retaining mapped reads, using SAMTools v.1.3.1 (Li et al. 2009). Trimming and mapping steps were implemented in a unix shell script (SI8) on a 12-core virtual machine on the NeCTAR research cloud (National Research Infrastructure for Australia, developed by FULLER) under an Ubuntu 16.04 LTS image. BAM files were viewed in IGV v.2.3.92 (Robinson et al. 2011; Thorvaldsdóttir et al. 2013) to visually identify exon-intron junctions (i.e. presence of softclips to the left in some sequences and to the right in others of a particular position), which were manually separated in a FASTA file and subsequently re-mapped following the above protocol.

Cleaned reads for each species were *de novo* assembled using four different assemblers to optimize successful coverage of exons across genes and to verify the mapping approach detected all sequences: IDBA v.1.1.1 (Peng 2009), RAY (Boisvert et al. 2010), SPAdes v.3.13.0 (Bankevich et al. 2012) and Celera v.8.3 (WGS; Denisov et al. 2008). All assemblies were viewed in Geneious and subsequently compared to a personalized BLASTn database containing the photoreceptor genes from the transcriptome data (Table 1). Geneious was also used to map cleaned reads to the same reference sequences as mentioned above, as a comparison to our BAM files, in order to verify mapping quality, but also to extend final sequences if possible. The general coverage of exons was higher in the first and second capture as it only included six and eight samples, respectively, resulting in hundreds to thousands of sequence coverage. The general coverage was consistently lower in the remaining two captures, as we would expect due to the pooling of samples for sequencing, resulting in less than a hundred sequence coverage. The coverage was consistently lower near the edges of the exons, due to an issue termed

the ‘edge effect’ (Bi et al 2012), consistent with other studies (Bragg et al 2015; Portik et al 2016; Puritz & Lotterhos 2018).

Orthology of genes. We used the BLASTn feature within Genbank to compare our nucleotide sequences to the database of available genes, with the top two hits recorded (SI2). An orthologous match was considered positive when identities were greater than or equal to 70% with at least 50% of the gene covered by the match (Tommaso et al, 2011).

Each gene group, arrestin (*arr1* and *arr2*), opsin (*c-opsin*, *lwop*, and *uvop*), trp (*trp* and *trpl*) and *inaD*, were aligned with CLUSTALW in Geneious. No sequences were captured for the gene *ninaC* or *prominin*, most likely, because the transcriptome assembly led to an erroneous bait design.

Subsequently, Bayesian phylogenies were constructed using BEAUTi v.2.4.7 (Bouckaert et al. 2014) and BEAST v.1.7.5 (Drummond et al. 2012) for each gene group with references from Genbank (SI3). Two independent runs were carried out with 50 million generations and trees sampled every 5000 generations. We used a burn-in of 25% (12.5 million) generations per run. The convergence of runs was assessed using Tracer v.1.5 (Rambaut and Drummond 2009) and by identifying values of the effective sample size (ESS). Final gene trees were viewed and edited in FigTree v.1.4.3 (Rambaut 2012).

Pseudogene assessment. All sequences were aligned and assessed for ORFs to determine whether the sequences were likely to code for functional proteins. We took note of sequences that contained indels (insertions or deletions) and pre-mature stop-codons and assessed the read quality of these sites for sequencing errors by mapping raw reads onto the sites using Geneious. As genes approach neutrality and pseudogenization, we would expect genes to either contain translational stop codons and/or frameshift mutations leading to altered protein, or increased rates of nonsynonymous substitutions (d*N)* relative to synonymous substitutions (d*S)* (i.e. $\omega = dN/dS \Rightarrow 1$).

Tests of Selection. We used HyPhy to determine the pairwise relative rates of independent comparisons of surface and subterranean species, using a General Time Reversible (GTR) model of sequence evolution (Tavaré 1986). First, we tested for variation in overall and nonsynonymous substitution rate and predict that there will be elevated rates of evolution in subterranean species when compared to surface species. We compared overall rates of nucleotide substitution by comparing the likelihood scores of a shared substitution rate between taxa. Branch lengths were estimated independently with global (i.e. shared) model parameters. Rate parameters were calculated using maximum likelihood, and equilibrium (nucleotide) frequencies were ‘observed’. We repeated overall pairwise relative rates on inferred amino acid sequences following the same parameters as above. Additionally, we performed pairwise relative rate analyses of nonsynonymous subtitutions, using the same options as above, except using local (i.e independent) model parameters instead of global.

We then employed Datamonkey v.2.0 (datamonkey.org; Weaver et al. 2018) for phylogenetic hypothesis testing, which required Bayesian inferred trees (constructed in BEAST using the main seven nuclear genes from the exon capture (*arr1*, *arr2*, *lwop*, *c-opsin*, *inaD*, *trp*, and *trpl*, but excluded *uvop* as there was too much missing data) and mitochondrial genes *COI, 16S*, and *ND1* from Genbank (accession numbers found in SI9), following specifications listed above). In Datamonkey, we used two site-specific methods that calculate ω independently at each codon: single-likelihood ancestor counts (SLAC) which are simplistic, and fixed effect likelihood (FEL), which are less susceptible to Type

1 errors (Pond and Frost 2005). It is unclear where along the branch leading to a subterranean species it actually went underground. However, using the branches associated with sympatric sister species, which most likely speciated underground (Leijs et al. 2012; Langille et al. unpublished), for these and subsequent analyses ensures that omega (ω) will be approaching 1 under neutral evolution. We expected a higher value of ω in the subterranean lineages (close to 1) than the surface lineages, the latter expected to show genes under purifying selection.

We also carried out branch by branch analyses using RELAX (in HyPhy; Wertheim et al. 2015) comparing surface lineages to subterranean ones. RELAX estimates a value of ω along each branch by a model of branch site-random effects likelihood (BS-REL) and then fits the selection intensity, *K*, to it (ω^K). Under these parameters, a $K > 1$ is indicative of purifying selection, while a $K < 1$ is indicative of relaxed selection. RELAX requires proper codon structure therefore all insertions that were not a multiple of three were removed, an 'N' was added to the third position of stop codons, and deletions were filled with N's until the expected reading frame was obtained.

Chapter 4: Supplementary Information: SI7: References from the methods section in text and from supplementary information.

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Chapter 4: Supplementary Information: SI8: BASH script containing commands designed to clean raw data, create FASTqc reports, map cleaned data to references, and generate final files for viewing in IGV.

```
#!/bin/bash
#
#
# usage: program.sh <ref.fa> <path to raw data>
#
# Automated NGS mapping
# Barbara Langille
# November 2016



function error_exit
{
    # Exit function due to fatal error
    # Accepts 1 arg:
    # string - descriptive error message

    echo "${PROGNAME}: ${1:-"Unknown error"}" 1>&2
    exit 1
}


#------adjust these for your run-----
BBMDIR="/home/blangille/src/bbmap/bbduk.sh"
THREADS=10
#-----------------------------------



# go to the working directory
cd $2

for file in *_R1.fastq.gz
do

        FILESTEM=${file%_*}

        # run all paired data through bbmap
        $BBMDIR in=$file in2=$FILESTEM"_R2.fastq.gz"
out=../clean/$FILESTEM"_R1_clean.fq.gz"
out2=../clean/$FILESTEM"_R2_clean.fq.gz"
outs=../clean/$FILESTEM"_singletons.fq.gz"
literal=AGATCGGAAGAGCAC,AGATCGGAAGAGCGT ktrim=r k=15 mink=15 hdist=0 tbo
qtrim=rl trimq=20 minlength=30 threads=$THREADS || error_exit "$LINENO:
Error cleaning R1 or R2"

        # run cleaned data through fastqc
        mkdir -p ../clean/fastqc
        fastqc --noextract --threads $THREADS -o ../clean/fastqc
../clean/$FILESTEM"_R1_clean.fq.gz" ../clean/$FILESTEM"_R2_clean.fq.gz"
../clean/$FILESTEM"_singletons.fq.gz"

        # index reference *need to add an if clause here because only
need one
        #if <*.fai> file exists move on
        bwa index ../refs/LpalREF_concat.fa

        # mapping of PE and singletons to reference
        mkdir -p ../clean/files
        bwa mem -t $THREADS -R '@RG\tID:PE\tSM:'
../refs/LpalREF_concat.fa <(zcat ../clean/$FILESTEM"_R1_clean.fq.gz)
<(zcat ../clean/$FILESTEM"_R2_clean.fq.gz) | samtools view -hu -q 1 -@
$THREADS - | samtools sort -o ../clean/files/$FILESTEM"_PE.sorted.bam" -
T temp.sort -@ $THREADS - || error_exit "$LINENO: Error mapping PE"
```

```
        bwa mem -t $THREADS -R '@RG\tID:Singleton\tSM:'
../refs/LpalREF_concat.fa <(zcat ../clean/$FILESTEM"_singletons.fq.gz")
| samtools view -hu -q 1 -@ $THREADS - | samtools sort -o
../clean/files/$FILESTEM"_singletons.sorted.bam" -T temp.sort -@
$THREADS - || error_exit "$LINENO: Error mapping singletons"

        # merge PE and singletons sorted.bam together
        samtools merge ../clean/files/$FILESTEM"_PEandSingletons.bam"
../clean/files/$FILESTEM"_PE.sorted.bam"
../clean/files/$FILESTEM"_singletons.sorted.bam"

        # index PEandSingletons.bam
        samtools index ../clean/files/$FILESTEM"_PEandSingletons.bam"


done
```

“Freedom of thought is best promoted by the gradual illumination of [women’s and] men’s minds which follows from the advance of science.”

~Charles Darwin