Luiza de Oliveira Saad

Da luz à escuridão: História evolutiva do sistema fotosensorial em planárias (Platyhelminthes, Tricladida)

From light to darkness: Evolutionary history of the photosensory system in planarians (Platyhelminthes, Tricladida)

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências Biológicas, na Área de Zoologia.

Orientador: Prof. Dr. Federico David Brown Almeida

Coorientador: Prof. Dr. Peter W. Reddien

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Dedicatória / Dedication

Dedico esta tese aos meus pais,

Meu porto seguro e maior exemplo

Epígrafe / Preface

Ciência é como mergulhar na costa de uma praia tropical Brasileira.

Ao voltar seus olhos para o oceano, a vasta imensidão azul te assusta, o desconhecido te apavora, pois, seus olhos são incapazes de mensurar tal magnitude.

Porém, ao voltar seus olhos para a costa, é possível captar pequenos movimentos de uma concha se escondendo na areia, o colorido dos corais te encanta, a gentileza do nado dos lambaris te hipnotiza, os detalhes que importam, e o desconhecido te fascina.

Naquele universo embaixo d'água, tudo está conectado, tudo se interage, se comunica, e gera vida.

Você é uma parte insignificante dessa profundeza de vidas, elas já estavam aqui antes de você chegar, e vão continuar depois que você se for, mas você se sente único de poder estar ali, de compreender minimamente essa complexidade, de estar diante da extraordinariedade do desconhecido.

Por mim, eu mergulharia todos os dias.

—Luiza Saad

"Se você sabe que está no caminho certo, se você tem esse conhecimento interiormente, então ninguém pode tirar isso de você... não importa o que eles digam"

— Barbara McClintock

Science is like diving on a tropical Brazilian beach.

When you turn your eyes to the ocean, the vast blue immensity scares you, the unknown is terrifying, because your eyes are incapable of measuring such magnitude.

However, when you turn your eyes to the shore, it is possible to capture small movements of a shell hiding in the sand, the color of the corals enchants you, the kindness of the lambaris swimming is hypnotizing, the details that matter, and the unknown fascinates.

In this underwater universe, everything is connected, everything interacts, communicates, and generates life.

You are just an insignificant part of this life infinity, they were already here before your arrival, and they will continue be after you are gone, but there is an indescribable uniqueness to be able to be there, to minimally understand this complexity, to be faced with the extraordinariness of this unknown world.

For me, I would dive every day.

—Luiza Saad

"If you know you are on the right track, if you have this inner knowledge, then nobody can turn you off... no matter what they say."

— Barbara McClintock

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about science and life. Thank you for your friendship and for pushing me forward to improve myself as a researcher and as a person, I am so lucky to had you as a mentor.

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General Introduction

Evolutionary relationships of photosensory systems

Many species rely on their photosensory system to move, find food, reproduce, or avoid predation (Cariani, 2001; Nilsson & Bok, 2017). The photosensory organs differ across animals, some are composed of simple photoreceptor-cells detecting only the presence or absence of light, whereas other can be complex camera eyes capable of processing color images (Gehring, 2005; Kozmik, 2008). The association of specialized photoreceptors cells, which can sense light by biochemical reactions needed for its specific function, is defined as an eye (Land & Nilsson, 2012). Eyes are present in many species across Metazoa, with extensive variation in morphology, physiology, and development resulting in a disparity of structures and degrees of perception of light in each animal group (Gehring, 2005; Kozmik, 2008). How did these structures evolve and diversify? To understand the evolution of form and diversification in the photosensory systems of animals is necessary to study the function and aspects of eye cell morphology, the processes involved phototransduction physiology and eye development across different species (Land & Nilsson, 2012; Nilsson & Arendt, 2008)

Comparative studies of flatworms in the order Tricladida represent a good candidate animal group to help answer how eye structures evolved and diversified due to: i) the simple yet diverse eyes in the group, ii) vast histological descriptions of eye cell types in the different species, iii) as well as a deep understanding on the development of eyes in a few model species planarians. The comprehension of eye cell evolution and diversification in planarians may allow us to understand the evolution of more complex eye cell types in other animals. Therefore, the review of the principal aspect of morphology, physiology, development and evolution of eyes, including eye loss, within Tricladia will be highlighted here.

Photosensory system morphology

The morphological disparity between eye structures in animals suggests the occurrence of 40 to 60 independent events of eye evolution (Salvini-Plawen & Mayr, 1977; Shubin et al., 2009). Among these occurrences, we can highlight the following examples: i. single-celled ocellus in the jellyfish larvae in contrast to lensed eyes in the adult medusae; ii. simple pigmented epithelium connected with photoreceptor cells in platyhelminths; iii. morpho-functional variation of compound eyes of insects with intriguing visual capacities (Salamanca

& Brown, 2018; Warrant, 2015); iv. camera eyes in vertebrates, cephalopods, arthropods, and polychaetas (Fernald, 2000; Land & Nilsson, 2012).

Eyes can also display morphological differences even between close related species, which is the case of the planarians from the order Tricladida (Platyhelminth). Planarian eyes, also called ocelli, are generally assumed to have a very simple organization. Each eye is usually composed of only two cell types, photoreceptor neurons/cells and optic/pigment cup cells (Paskin et al., 2014). Photoreceptor neurons are located within a pigment cup epithelium on one side and connected to a bipolar brain on the other side (Deochand et al., 2016; Okamoto et al., 2005). However, a careful comparison of eyes across different Tricladida species reveal morphological variation in eye forms that have been extensively documented (Fig. I1, Table I1). Tricladida representatives eyes morphology have been used for taxonomy differentiation and it structural and anatomical variations include: (1) number of eyes, where some species may only have two eyes or others that may have many small eyes generally concentrated anteriorly or spread throughout the entire length of the body; (2) number of cells that make up the pigmented epithelium, either a single cell (unicellular) or several cells (multicellular); (3) number of photoreceptor cells, where some species may display few or several photoreceptor cells; (4) overall eye morphology and structure, where species may display either lensed eyes, or eyes embedded in a ganglionic mass that extend from the brain (Alvarez-Presas et al., 2014; Carbayo et al., 2013; Rossi et al., 2016; Sluys, 1986, 1989a; Sluys et al., 2009) (detailed information in Fig. I1 and Table I1).

Whereas structurally and morphologically, photosensory organs in animals have convergently evolved, a similar genetic regulatory circuitry controls eye formation in metazoans (Erclik et al., 2009; McCune & Schimenti, 2012). Additionally, if we focus on the molecular mechanisms of light transduction, the photosensory systems of animals are underlined by certain shared ancestral characteristics (Randel & Jékely, 2016). Thus, it becomes clear that photosensory systems in animals albeit structurally and morphologically diverse share similar genetic regulatory circuitry and photoreceptor homology.

Light phototransduction

The association of cells and other structures to form an organ called an eye is unique to animals, but photoreceptor cells were already present in plants, eukaryotes, and prokaryotes before eye appeared (Fernald, 2004). Photoreceptor cells are sensitive to light and composed of the transmembrane protein rhodopsin. Rhodopsin is a G protein-coupled receptor and it is

composed of two parts: (1) a vitamin-A-derived chromophore (retinal), and (2) the integral membrane protein opsin. Opsins are members of the seven-transmembrane receptor family, also known as G protein-coupled receptors (GPCRs), which are covalently linked to a chromophore responsible for absorbing photons (Shichida & Matsuyama, 2009). Light absorption by the chromophore produces a photochemical reaction inducing changes in the opsin conformation, activating the rhodopsin. Subsequently, the rhodopsin activates a G protein signal-transduction cascade that generates an electrical response in the photoreceptor cell producing a physiological response (Shichida & Matsuyama, 2009; Wensel, 2012).

There are two types of photoreceptor cells: rhabdomeric and ciliary. Rhabdomeric photoreceptor cells have folds in their apical cell surface that store photopigments, are composed of r-opsins, and use a phospholipase C (PLC) motif for phototransduction (Nilsson, 2004; Randel & Jékely, 2016; Yau & Hardie, 2009). In this case, the G-coupled opsin-based pigments stimulates phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DG), and inositol 1,4,5-triphosphate (IP3) to initiate the phosphoinositol cascade. This cascade leads to a depolarizing response of the photoreceptor cells (Koyanagi & Terakita, 2014; Terakita et al., 2012). On the other hand, ciliary photoreceptors present a ciliated membrane and are composed of c-opsins and uses a cyclic-nucleotide motif for phototransduction (Nilsson, 2004; Randel & Jékely, 2016; Yau & Hardie, 2009). In this case, the G-coupled opsin-based pigments, activate transducin (Gt), which in turn activates phosphodiesterase, that will later hydrolyze cGMP to 5′GMP. The decrease in cGMP concentration results in closure of the cyclic nucleotide (cGMP)-gated cation (CNG) channel, leading to a hyperpolarizing response of the cells (Koyanagi & Terakita, 2014; Terakita et al., 2012).

Although the opsin activated signal-transduction cascade display these highlight differences, all visual systems analyzed so far share homologous opsins, use rhodopsin for photoreception, and trigger a G protein-mediated signaling cascade when activated by light (Arendt, 2003; Randel & Jékely, 2016), arguably suggesting a monophyletic origin of photoreceptor cells (Halder et al., 1995a; Land & Fernald, 1992). Rhabdomeric and ciliary photoreceptor cells are present in both deuterostome and protostomes and their origins are unclear. One hypothesis suggests that rhabdomeric and ciliary photoreceptor cell types evolved independently multiple times from an intermediate photoreceptor cell type present in the Urbilateria ancestor (Arendt, 2003), whereas another hypothesis suggests that the two distinct photoreceptor cell types co-existed already in Urbilateria (Arendt & Wittbrodt, 2001; Arendt,

2003; Morshedian & Fain, 2017; Nilsson, 2005). However, in both cases an ancient simple photoreceptor cell presumably differentiated into distinct cell morphologies during the evolution of more complex visual systems of the different phyla.

Planarians rely on an R-opsin cascade-like phototransduction mechanism and many phototransduction-related genes are known to be express in planarian eyes (Lapan & Reddien, 2012). Work on planarian eye-related genes demonstrate expression in photoreceptor neurons for two R-opsin orthologs, two β-arrestin orthologs, Gα-q, IP3-receptors, two Trp channel-encoding genes, and also encoding enzymes of the phosphoinositide cycle which replenishes PIP2 after its hydrolysis by PLC (Agata et al., 1998; Fain et al., 2010; Lapan & Reddien, 2012; Sánchez-Alvarado & Newmark, 1999). Surprisingly, transcriptome analysis has also shown that planarian eyes express genes that are typically associated with the phototransduction pathway found in ciliary photoreceptors, such as genes encoding the cGMP pathway components guanylate cyclase, cGMP-dependent phosphodiesterase, cGMP-gated ion channel hyperpolarization-activated cyclic nucleotide-gated channel (Lapan & Reddien, 2012). Although the roles of these genes in planarian vision are not currently known, in Tricladida, all species eyes are composed of homologous rhabdomeric photoreceptor cells as the basic unit for phototransduction, and comparison within member of this order suggest that although different morphological components on eye structure, the species share homologous phototransduction elements (Alvarez-Presas et al., 2008; Riutort et al., 2012; Sluys et al., 2009)

Genes underlying eye development

Animals display different developmental processes of eye formation during embryogenesis (Chow & Lang, 2001). For example, vertebrate eye formation begins with an expansion of the diencephalon, forming the optic vesicle, which continues to expand and contacts the head ectoderm (Graw, 1996). This contact initiates a signaling process that will induce the proliferation and formation of the lens placode, which will give rise to the lens. This signaling process activates the optic vesicle to fold in on itself, forming the optic cup surrounding the lens. The optic cup will eventually differentiate into the neural and pigmented layers of the adult retina (Gilbert, 2000; Lamb et al., 2007). As a second example, cephalopod eye morphogenesis is characterized by four successive ectodermal folds. The first fold forms the primary optic vesicle that will give rise to the retina, the second fold will form the lens, a third ectodermal fold form a secondary cornea, and the fourth fold forms the eyelid (Imarazene et al., 2017; Sivak & Sivak, 2019). As a third example, during the development of insect

compound eyes, hundreds of ommatidia –each containing eight photoreceptor cells– differentiate along the morphogenetic furrow of the eye imaginal discs in the larvae, which undergo a wave of differentiation recruiting adjacent unspecified cells for ommatidium formation. The first cell (R8) differentiates into a photoreceptor cell and subsequently induces surrounding cells to differentiate and connect to each other, forming the ommatidial cluster (Carthew, 2007; Hiesinger & Meinertzhagen, 2009; Wawersik, 2000).

Although morphogenetic processes differ in eye development across animals, the underlying developmental gene pathways that specify eye cell types appear to be conserved (Gehring & Ikeo, 1999; Nilsson, 2009; Pichaud & Desplan, 2002; Treisman, 2004). Studies have shown that Pax6 transcription factors, including one vertebrate Pax6 ortholog or two Pax6 orthologs in flies (*ey* and *toy*), activate a progressive induction of downstream genes that control eye cell type specification and differentiation, such as *sine oculis* (*so*), *eyes absent* (*eya*), *dachshund* (*dach*), *six*, and *otx* in different species (Cvekl & Callaerts, 2017; Gehring, 1996; Halder et al., 1995b; Heavner & Pevny, 2012; Hiesinger & Meinertzhagen, 2009). Mutations *Pax6* orthologs in different animals disrupt eye formation altogether suggesting that these genes are necessary for eye development, and have been suggested to act as 'master regulators' of eye formation (Kozmik, 2005; Wawersik, 2000). Pax6 orthologs have been found to display a high protein sequence and function conservation in cnidarians (Kozmik et al., 2003), mollusks (Imarazene et al., 2017; Tomarev et al., 1997), tunicates (Glardon et al., 1997), annelids (Arendt et al., 2002; Klann & Seaver, 2019) and cephalochordates (Glardon et al., 1998). Also, loss of function of either Pax6 orthologs or other eye encoding transcription factors, such as *so*, *eya*, *dach*, *six*, and *otx* results in reduced eyes or no eyes at all in different fila across the metazoans (Bando et al., 2020; Chow & Lang, 2001; Cvekl & Callaerts, 2017; Gehring & Ikeo, 1999; Halder et al., 1995a, 1995b; Lapan & Reddien, 2012; Nilsson, 2004; Prosser & Van-Heyningen, 1998). Thus, similar genetic cascades pattern is found in analogous anatomical eye structures in evolutionarily distant organisms.

In summary, while morphological differences are suggestive of numerous evolutionary origins of eyes, shared developmental genes and homologous opsin activated signal-transduction cascade suggest that animal eyes likely evolved from an ancestral "proto-eye" made up of a rhodopsin signaling-based photoreceptor cell (Cvekl & Callaerts, 2017; Gehring, 2014). The development of the proto-eye may have been under the control of *Pax6* and the associated downstream regulatory gene cascade. Presumably, the proto-eye latter evolved into the other cell types of more complex animal eyes (Gehring & Ikeo, 1999). However, in several

species, *Pax6* homologues are not directly involved in regulating eye development (Backfisch et al., 2013; Cvekl & Callaerts, 2017; Pineda et al., 2002), raising questions about the extent in which developmental regulatory pathways must be shared to support the hypothesis of a monophyletic origin of eyes (Cvekl & Callaerts, 2017). While the origins of eyes are still not fully understood, there are traces of eye evolutionary history in the morphological, ontogenetic, and molecular processes of each species to help us answer how eyes evolved (Nilsson, 2004). Exploring these aspects in different animals will contribute to a broader understanding on eye macroevolution, and for this reason I focus on uncovering molecular process of eye development in planarians.

Planarians are usually capable of developing eyes during embryogenesis, asexual reproduction after fission, or during regeneration in response to injury (Reddien & Sánchez-Alvarado, 2004). Planarians develop eyes within a week after decapitation/fission. A fast development of eyes is possible due to the presence of proliferative cells, called neoblasts, which are found in the embryo and in adult tissues (Reddien, 2013). Developmental studies in planarians have shown that both of these processes use similar molecular and cellular pathways that are conserved evolutionarily, in which the same genes involved in eye development in other metazoans species (i.e., *so*, *eya*, *six*, and otx) are also responsible for embryonic eye development plus adult regeneration in the different triclad species (Lapan & Reddien, 2011, 2012; Martín-Durán et al., 2012; Wenemoser et al., 2012).

Planarian holds pluripotent stem cells called cNeoblasts and specialized neoblasts that replace lost cells resulting from normal physiological turnover, and thus maintain general tissue homeostasis in the planarian body (Reddien, 2018). Position-control genes are expressed to regulate regional tissue identity and to determine the medial-lateral, antero-posterior and dorso-ventral axes (Li et al., 2019; Reddien, 2022; Yazawa et al., 2009). These genes are important to positionally determine the fate of specialized neoblasts as they continuously replace particular cell types of the body maintaining general tissue homeostasis (Chen et al., 2013; Wurtzel et al., 2015). When planarians are bisected, neoblasts also play a major role in regeneration. After decapitation (0 to 16 hours after), a generic wound signaling initiates, the muscle surrounding the site of the wound contracts to close the injury, neoblasts start to proliferate and accumulate at the wound site at the same time as cell death is intensified reorganizing the regenerating body of the planarian by gene induction (Reddien, 2018; Wenemoser & Reddien, 2010; Wurtzel et al., 2015). Within 36-48 hours after decapitation the position-control genes are up-regulated establishing of the new planarian axes and giving

identity to the tissues that will regenerate (Li et al., 2019; Reddien, 2022; Yazawa et al., 2009). At this time, anterior neoblasts proliferate and are induced to develop into precursors of head cell types (Reddien, 2018; Wenemoser & Reddien, 2010). After 48-72 hours of decapitation, neoblasts accumulate at the site of injury and active cell proliferation begins forming an unpigmented region called the blastema. Blastema cells are mainly composed of specialized neoblasts that proliferate and migrate to specific target locations. In the head blastema they will begin to differentiate and form all new head tissues (Reddien & Sánchez-Alvarado, 2004; Scimone et al., 2014). The specialized neoblasts express different transcription factors as they differentiate, for example, eye-specialized neoblasts express eye-associated transcription factors *six-1/2*, *eya*, and *ovo*. These eye progenitors form aggregates at the eye primordium site oriented by positional-control genes too. These eye progenitors sequentially follow a program to either differentiate into rhabdomeric photoreceptor neurons (PRNs) by expression of *otxA*, or alternatively follow a program to differentiate into optic pigment cells (PCs) by the expression of *sp6-9* and *dlx* (Lapan & Reddien, 2011, 2012; Martín-Durán et al., 2012). After 72 hours of decapitation the spatial relationships and proportions of tissues and organs of the planarian body are reorganized, and growth-related gene expression and cell proliferation continue until all structures are fully formed and the individual returns to the maintenance of homeostasis (Reddien, 2018).

Early in eye development, *six-1/2*, *eya*, and *ovo* are expressed in specialized neoblasts of eye progenitors. Orthologs of these genes have been implicated in eye defects in flies and vertebrates, supporting homology between regulatory networks and pathways that direct eye development and function in different phyla (Chow & Lang, 2001; Fernald, 2000; Mathers et al., 1997; Pichaud & Desplan, 2002; Pignoni et al., 1997). However, in planarians the 'master regulator' of animal eye development *Pax6* has drifted from its highly conserved determinative role, and has instead been expressed only in planarian neural development (Pineda et al., 2002). The absence in planarians of this important transcription factor of animal eye development has raised questions the degree of developmental genetic systems drift affecting highly conserved developmental determinants across animals. Such detailed descriptions of eye development are only known in a few members of the Duguesiidae family, other Tricladida species are still lacking information on the gene expression patterns during eye development. However, it is reasonable to assume that the eye development pathway is conserved in planarians and other more distantly related groups in the phylum.

Environmental influences on eye evolution

Each environment displays unique light intensity distribution, for example, during the day light cycle illumination can be very intensive, intermediate, and very low. Another example is the dramatic effect on light intensity related with depth in water, in which light intensity decrease for every 100 m (Nilsson, 2009). Animals' sensory system will be subject to specific subset of environment light intensity and evolution will select structures that works towards extracting the sensory information from the environment (Nilsson, 2009). Eyes or any other sensory structure evolutionary process start by genetic variation that causes modification in sensory structure and function, in which allows for variation in the environment exploration, that relies on the sensory information, which ultimately causes the change in fitness that selection can act on (Nilsson, 2013). For this reason, selection to environmental conditions has generated an intriguing phenotypic diversity of sensory structure in animals, often well-adapted to the ecological niche of each species (Endler & Basolo, 1998; Feldmann et al., 2010; Land & Fernald, 1992; Nilsson, 2013). These adaptations result in modifications during eye development that allow animals to evolve distinct eye phenotypes according to resolution or wavelength sensitivity requirements to extract sensory information from their respective habitats (Escobar-Camacho et al., 2019; Fernald, 2000). For example, lens to improve focus and other eye specializations have evolved in terrestrial and aquatic environments in tune with the fluid medium (i.e. air or water) animals live in (Land & Nilsson, 2012). Eye evolution is also influenced by animal activity patterns and light-guided behaviors even within small species groups. For example, relative eye size in anuran visual systems has been associated with mating habitat and activity patterns, presenting a notable decreases in eye investment among fossorial, subfossorial and aquatic species (Thomas et al., 2020); relative cornea size of mammalian species has been associated to levels of light intensity of their habitats, in which diurnal and cathemeral mammals from forested habitats exhibited relatively larger corneas (Veilleux & Lewis, 2011); and eye morphology of shallow and deep-water inhabiting bivalves have been shown to be associated to faunal or epifaunal behaviors, in which transitions to crevice-dwelling habit appear associated with convergent gains of eyespots in epifaunal lineages, on the other hand multiple losses occurred in lineages that shift to burrowing lifestyles and deep-sea habitats (Audino et al., 2019; Malkowsky & Götze, 2014). The results indicate a taxon-/clade-specific evolution of visual characters associated with the different habitats that species occupy.

Species of the order Tricladida inhabit marine, terrestrial, and fresh water environments, and display diverse eye phenotypes accommodated to their ecological demands (Riutort et al., 2012; Sluys, 1989a). Originally the Tricladida order was classified according to the different habitat species lived in, for example the orders Maricola and Cavernicola corresponded to the species groups that inhabited marine or cave environments respectively (Sluys, 1989b). Although recent phylogeny classification sowed that not all groups fall under these categories, Tricladida phylogenetic relationships suggest several monophyletic groups in which their evolutionary history is tied with the different environment that species occupy, whereas it is reasonable to assume that the habitat shaped the evolutionary trajectory of the group (Sluys & Riutort, 2018). For this reason, functionally adaptive characters, such as planarian eyes, are ecologically constrained and are often used as diagnostic features of species groups (Sluys, 1989a, 1989b). In here I attempt to summarized the most update phylogenetic relationships within Tricladida groups, according to the current higher classification (Table I2), and discuss their lifestyle evolution related by plotting their eye structure diversification in the most recent group phylogeny (Fig. I2). Although Tricladida eye are usually considered simple because are composed of only two cell types (photoreceptor neurons and optic/pigment cup cells), different groups reveal morphological variation in eye forms often associated with their life style, and this association will be discussed (Fig. I1,2, Table I1).

Species in the Suborder Maricola, are the only Tricladida to inhabit marine or brackish water environments, and present only a pair of eyes that are characterized by unicellular eye cups with few (2-7) retinal cells (photoreceptor cells) (Chen et al., 2019; Sluys & Ball, 1988; Sluys, 1989a). In addition to the regular planarian eyes, several Maricola species display of lensed eyes (e.g. *Sabussowia ronaldi*, *Paucumara mentulalacertosa*, *Nerpa fistulate*) (Sluys, 1989a). Lens are located in the opening of the pigment cup and usually extends beyond the outline of the cup, as an extension of the pigment cup epithelium (Sluys, 1989a). Maricola suborder is divided in four families (Cercyridae, Uteriporidae, Bdellouridae, and Procerodidae) and except for Procerodidae, all other families present species with lensed eyes (Chen et al., 2019; Li et al., 2021; Li, Sluys, et al., 2019 Sluys & Kawakatsu, 1995). Because the phylogenetic relationships between the families is not fully resolved, it is not possible to determine the character state of lensed eyes, but its presence is found exclusively in the marine Tricladida, suggesting an association between the marine environment and development of lensed eyes (Fig. I2).

Many Tricladida species living in freshwater are eyed and are manly distributed in three families: Planariidae, Dendrocoelidae and Dugesiidae. But are also found in other families like Dimarcusidae and Kenkiidae. Planariidae, Dendrocoelidae and Kenkiidae belong to the Planarioidea superfamily and are closely related, whereas Dugesiidae is grouped with the land planarians Geoplanidae constituting the Geoplanoidea superfamily (Riutort et al., 2012; Sluys & Riutort, 2018). Whereas Planarioidea eyes are very diverse, Dugesiidae show more commonalities. In Planarioidea, eyes can vary according to: i) number and position, which can vary from no eyes, two, or several eyes, positioned in the anterior part of the head, along the lateral margins of the body, or sometimes positioned in row in the middle of the head. *Dendrocoelum* is a genus that contains species with the most notable differences, including species with a pair of eyes, multiple eyes, or no eyes at all (Stocchino et al., 2013; Stocchino et al., 2017a); ii) number of cells that make up the pigmented epithelium, which can be unicellular or multicellular; iii) number of photoreceptor cell with few (3 to 10) or several (>10) (Table 2). Unfortunately, Planarioidea worms are still under-represented in molecular phylogenies, making it difficult to infer the phylogenetic history of eye evolution and diversity in the group. Kenkiidae position itself is inferred solely on morphology, due to lack of gene sequencing. On the other hand, the fresh-water planarians from Dugesiidae family eyes are consistently the same between the species and are characterized by the presence of a pair of eyes, containing a multicellular pigment cup, and numerous photoreceptor cells (Ball, 1974; Sluys, 1989a). These results suggest that the freshwater environment is followed by eye diversification, in which light sensitive selected different eye complexity within, sometimes, invidious from the same genus, like *Dendrocoelum* example (Fig. I2).

Dugesiidae family is close related with land planarians from Geoplanidae family. Molecular study of the superfamily Geoplanoidea, that group the two families, showed that a single transition occurred from freshwater to the terrestrial habitat (from a common ancestor with the Dugesiidae) (Alvarez-Presas et al., 2008). The similarity between the two families is supported by molecular inferences, but also it has been previously suggested that eyes (containing a multicellular pigment cup, and numerous photoreceptor cells) to be a synapomorphy of the Geoplanoidea (Alvarez-Presas et al., 2008; Alvarez-Presas & Riutort, 2014). However, more recent studies have been suggesting inconsistences with this shared character. First the presence of multicellular pigment cup, and numerous photoreceptor cells is also found in dendrocoelids species (Family Dendrocoelidae), thus disputing on the validity of this character as a Geoplanoidea synapomorphy (Sluys & Kawakatsu, 2006). Second, eyes

between Dugesiidae and land planarians present a small difference, in the latter the photoreceptor cells face the opening of the pigment cup and the eye dendrites penetrate between the pigment cells, whereas in the Dugesiidae the photoreceptor cells face the pigment cup and the dendrites enter the eye cup via its opening (Carranza et al., 1998; Sluys, 2019; Sluys et al., 2009; Sluys, 1989a). Third, land planarians of the subfamily Bipaliinae eyes diverge from the others land planarians, because they are the only group presenting unicellular pigment cells with few (1 to 8) photoreceptor cells (Alvarez-Presas et al., 2008; Sluys, 1989a), similar to those of the Maricola or some members of the Planariidae family, raising questions on the eye synapomorphy between Dugesiidae + Geoplanidae. Finally, land planarian eyes present a large diversity related to number of eyes, their distribution and format, which will be detailed described next (Fig. I2).

Geoplanidae family is composed by four subfamily (Bipaliinae, Microplaninae, Rhynchodeminae, and Geoplaninae) and the various kinds of multi-cellular eyes of land flatworms was been associated with the facilitation of terrestrialization and adaptive radiation (Sluys, 2019). Molecular study strong support Bipaliidae to a basal position for the family (Alvarez-Presas et al., 2008). Bipaliidae species are characterized by the presence of a spatulate head and multiple eyes crowded located in the head and sometimes neck region, but sparsely in staggered submarginal row along body, they are simple pigment-cup type (unicellular), and present only 1-4 photorreceptor cells (Abdel-Haleem et al., 2014; Bhakat, 2020; Winsor, 1983). However, all other terrestrial planarias (Microplaninae, Rhynchodeminae, Geoplaninae) are known to present a multicellular pigment cup and several photoreceptor cell, although unfortunately recent descriptions have not evaluated these features in other terrestrial eyes to confirm the if the plesiomorphic character (unicellular pigment cup and few photoreceptor cells) is present only in Bipaliidae (Sluys, 1989a; Sluys & Riutort, 2018). In this case it is hard to know if Bipaliidae presents an independent case in which the most plesiomorphic eye character is still present, retaining the ancestral form or a secondary loss of the multicellular pigment cell cup. Species still to be described will contribute to a better understanding.

The phylogenetic relationship between Microplaninae, Rhynchodeminae, Geoplaninae is not fully resolved yet, which may be due to the presumably old age and subsequent fast radiation of the group (Carranza et al., 1998). For this reason, eye evolutionary history could not be traced, but each group present very particular eye morphology (Fig. I2). Majority of the Microplaninae species present only two eyes, in which sometimes are situated in pigment-free patches presenting a "fried egg" aspect, in which can only be found in member of this clade.

Surprisely, two Microplaninae species (*Microplana plurioculata* and *Microplana polyposis*) have multiple eyes located in the anterior end of the body and can vary between 2-4 eyes on either side (Mateos et al., 2017; Sluys et al., 2016). However, *Microplana plurioculata* and *Microplana polyposis* are cluster together as a sister group of the other Microplaninae species, suggesting a unique common ancestor and multiple eyes as a group synapomorphy (Sluys et al., 2016); The Subfamily Rhynchodeminae is divided in two tribes, Caenoplanini and Rhynchodemini. Caenoplanini species present numerous eyes, but they are usually arranged in a single row across the body, but do not extend dorsally, on the other hand, Rhynchodemini species display of only two large eyes located little distant from the anterior tip (Alvarez-Presas & Riutort, 2014; Breugelmans et al., 2012; Hu et al., 2019; Justine et al., 2018; Mateos et al., 1998; Sluys, 1989a). Geoplaninae species also display numerous eyes, but different from Rhynchodeminae, their eyes are located in multiple rows and often extending dorsally, sometimes eyes extend only marginally. Usually, eyes encircle the head in a single row but become more numerous on the sides of the body, where they may extend along the whole body or may extend only to about mid-body (Ogren & Kawakatsu, 1991; Sluys & Riutort, 2018). Also, Geoplaninae species eyes are described as two types, a single-cup (monolobulated), or three optical cells in each pigmented cup (trilobulated), this description is related with the epithelium format and not necessarily with the epithelium cell number (Carbayo et al., 2013; Marcus, 1951). Some species of *Obama, Paraba* genus, and the *Winsoria bipatria* species eyes located anteriorly are monolobated, and the posterior are trilobated. Suggesting a diversification of eyes types in Geoplaninae species (Amaral et al., 2012; Carbayo et al., 2013; Negrete et al., 2020; Rossi et al., 2016).

Overall, the variation of eye morphology and organizaition in the Tricladida suggest a complex evolutionary history. The plesiomorphic eyes of the Tricladida are presumably composed of unicellular eye cups containing few photoreceptor cells, as this type of eyes are generally also found in the sister-group of Tricladida (i.e. Rhabdocoela), as well as in many other flatworms (representants from Proseriata Unguiphora, Prolecithophora, Trematoda, and Monogenea order), suggesting a secondary diversification of eyes in the Tricladida (Sluys & Kawakatsu, 2006; Sopott-Ehlers et al., 2001). Although the majority of Maricola and some Planarioidea species (e.g. *Polycelis feline*) also present unicellular eye cups containing few photoreceptor cells, which can be considered a plesiomorphic character, some species in these groups have evolved exceptions. For example, in Maricola lens have evolved (Delogu & Galletti, 2011; Yang et al., 2018) or in Planarioidea (e.g species from the genus

Dendrocoelum, Phagocata, and Baikalobia) multicellular eye cups containing several photoreceptor cells have evolved (Stocchino, 2017a, 2017b). As for other representatives of Tricladia order eyes display large diversification. For example, the presence of multicellular eye cups containing several photoreceptor cells can be found in several species (from the superfamily Geoplanoidea and Planarioidea) and likely gained independently multiple times in the course of Tricladida evolution. Also, numerous eyes tend to follow the same pattern of independent gain, since they are found in species of such distant groups like Geoplanoidea and Planarioidea (Fig. I2). On the other hand, unique eye structures, like lens (found only in Maricola species), trilobulated eyes (found only in Geoplaninae), and numerous eyes across the whole body (found only in terrestrial species) are exclusive from species of the same habitat, suggesting that eye morphology is probably associated with the life history (Fig. I2).

Tricladida species were able to diversify in marine, freshwater, and terrestrial environments (Sluys, 2019). Species lifestyle determines key innovations required to produce adaptive behaviors, that will be selected for species survival (Nilsson, 2013). It is important to explore the association between phenotypes characteristic and the evolutionary dynamics of the group in different landscapes as evidences to understand evolution itself (Losos, 2011). Here I attempted to summarize the main key innovation found in Tricladida eyes and highlight the importance of future detail studies on different species eye, which can be used as a model for trait evolution to comprehend how diversification interact with the environment. Each day new studies pointed to fascinating discoveries of unique species that challenges the classification of the group, making the history even more interesting and complex.

Eye loss

Animals living in habitats with low light, such as the deep-sea or caves, have specialized morphological, behavioral, and physiological traits that differ from their ancestral forms residing outside the caves (Krishnan & Rohner, 2017). These animals often display an increase in eye size with tubular eyes and large lens, which allow them to perceive low intensities of light (Douglas et al., 1998). However, absence of light allows characters of the visual system to become dispensable leading to partial or complete eye regression in only a few generations (Jeffery, 2009; Protas & Jeffery, 2012). Evolutionary eye regression and loss has been documented to occur multiple times across distant groups of cave-dwelling animals (Jeffery, 2005).

Troglobites or troglobiont species correspond to organisms that live exclusively in hypogean habitats, i.e., caves (Trajano, 2012). Several evolutionary mechanisms have been documented to be responsible for the loss of eyes in troglobiont species. First, direct natural selection for eye-reduction or eye-loss can be explained by a trade-off between the energetic cost of developing eyes vs. the cost of developing or enhancing other systems, e.g., enhancement of chemoreceptors (Moran et al., 2015; Rétaux & Casane, 2013). Thus, eyes in dark environment would not only be energetically advantageous for the organisms that lose them, but may also increase survival and fitness by allocating this energy to other mor dark-advantageous systems related with better sensorial response, for example (Krishnan & Rohner, 2017; Niven & Laughlin, 2008). Second, eye-loss may also result from indirect selection, i.e., selection for another trait may negatively impact eye development, resulting in a pleiotropic effect (Jeffery, 2010; Yamamoto et al., 2009). For example, in embryos of surface fish, during eye development expression of sonic hedgehog (*shh*) signals from the ventral midline of the forebrain to upregulate *Pax2* and *Vax1* and downregulate *Pax6* expression in the optic stalk (Hallonet et al., 1999; Krishnan & Rohner, 2017). In the other hand, cavefish express relatively higher levels of *shh* in the midline of the forebrain than surface fish, resulting in an upregulation of *Pax2* and *Vax1* in the optic stalk that negatively interfere with the expression of *Pax6*, resulting in a pair of reduced optic cups, which consequently results in the degeneration of eye structures (Rétaux & Casane, 2013; Yamamoto et al., 2009). Surprisingly, overexpression of *shh* in cave fishes also results in pleiotropic developmental effects on other phenotypes, such as larger jaws, more teeth, enhancement of oral and taste bud development, and a larger hypothalamus. These effects suggest a developmental "compensation" between the regressive and constructive traits mediated by pleiotropy due to indirect selection (Jeffery, 2010; Rétaux & Casane, 2013; Torres-Paz et al., 2018; Yamamoto et al., 2009). Third, eye-loss may result from relaxed selection due to the removal of a previously important source of selection associated to light environments, leading to the accumulation of deleterious genes (i.e. genetic drift) and increasing the probability of trait loss (Lahti et al., 2009). However, the mechanisms involved in eye-loss of different species may vary considerably (Aspiras et al., 2012; Protas & Jeffery, 2012; Stemmer et al., 2015). Therefore, the study of troglomorphic traits of cave and surface sister taxa offers a great opportunity to understand the mechanisms of convergent evolution of regressive traits, and may help explain how selection acts.

Several Tricladida species independently colonized different subterranean habitats, and often display troglobiont features (e.g., lack of eyes and pigmentation) and its species are

reported on Table I1 (Benítez-Álvarez et al., 2020). Majority of cave-dwelling tricladid lineages are found in fresh-water environment and belongs to the Cavernicola suborder, Planarioidea superfamily, and Dugesiidae family (Benítez-Álvarez et al., 2020). However, recently studies reported interesting finding, species of terrestrial and Maricola living in cave locations. Between the cave terrestrial species majority of them do not present evidences for troglobitic traits, suggesting the use of caves as shelter or temporary habitat (Amaral et al., 2019; Leal-Zanchet & Marques, 2018a, 2018b). However, two other species actually display some troglobiont features, i) *Geoplana subterranea* is the only terrestrial species that is albine and eyeless, and can be found underground feeding on earthworms (Carbayo et al. 2013; Ogren and Kawakatsu 1991); ii) The land species *Microplana astricta,* although it does not live underground, is suggested to present no eyes, it anteriormost tip of the body is unpigmented making hard to visualize eyes in live animal, displaying a level of pigment loss (Mateos et al., 2017). On the other hand, the cave Maricola species *Sluysia triapertura* was actually found in a brackish water and present no eyes and no pigmentation, suggesting first evidence of true troglobiont features (Souza et al., 2018).

Cavernicola is close related with the Maricola suborder, but their species are manly eyeless and found in cave freshwater environment. Although, interestingly, there is few records of species living in a humid terrestrial habitat, in which troglobiont features are still present, suggesting a secondary event of terrestrial habitat conquer and retained of troglobiont characteristics (Sluys & Laumer, 2019; Stocchino et al., 2021). Contradicting the others Cavernicula suborder species, the ones from the genus *Rhodax* are not found in caves and also present two eyes (Benítez-Álvarez et al., 2020). Recent ancestral states analysis of Cavernicola suggest that, although their close phylogenetic relationship to the marine triclads, they originated in an epigean freshwater habitat with a subsequently independent colonization of hypogean and terrestrial environments, in which *Rhodax* species retain the two eyes ancestral character (Benítez-Álvarez et al., 2020). Contrastingly, (Harrath et al., 2012) hypothesizes that due to oceanic regressions, marine ancestors may have become isolated inland, living underground, and evolved into an aquatic hypogean fauna, in this case, *Rhodax* species reacquired eyes that were lost in its underground ancestors and recolonized surface regions. The report of *Sluysia triapertura*, a Maricola brackish water cave inhabitant, corroborate with the hypotheses of a marine transgressions and regressions leading to the isolation of this species inside a cave (Souza et al., 2018).

Similarly, several species from the freshwater superfamily Planarioidea also are found in subterranean environment, and often display lack of eyes. All described species of the Kenkiidae family are devoid of eyes, probably an adaptation to their subterranean habitat, unfortunately this family is understudied and there is no molecular data available to infer their relationships with other cave planarians (Glasgow & Pierce, 2015; Sluys & Kawakatsu, 2006). Species from the Dendrocoelidae family are often endemic and inhabit subterranean water, although in majority of the species the body is unpigmented, the two pigmented eyes can be frequently found, suggesting different mechanisms of loss between pigmentation and eyes in troglobiont animals. However, some species are eyeless, and curiously the species *Dendrocoelum vesiculosum* present two different morphotypes within the population, a morphotype characterized by the absence of eyes, another morphotype with two-eyed individuals, suggesting an in-progress process of adaptation to the subterranean environment (Stocchino, 2018; Stocchino et al., 2013; Stocchino et al., 2017a, 2017b).

Several freshwater Dugesiidae species are also found in cave environment, presenting a great variation of troglobitic traits (lack of eyes and pigmentation). Some cave *Girardia* species display interesting combinations of body pigment and eye loss, for example, in *G. arenicola* eyes are present but body pigmentation is absent, in *G. corumbataiensis* both eyes and body pigmentation are present, and in *G. spelaea* eyes and body pigmentation are absent (Morais et al., 2021). Suggesting independent events of cave lineages and consequently different morphological features, unfortunately these species are still under-represented in molecular phylogenies to be able to infer precise evolutionary history between eye and pigmentation lost.

Within the Tricladida evolutionary history cave habitats offer an ideal scenario for study of regressive phenotypes mechanisms evolution, in which each cave is a particular case, because of characteristics that favor high endemism, with species being restricted even to a single cave. At the same time, different species present similar troglobitic traits, wherein many independent times species loss their eyes and/or body pigmentation (Morais et al., 2021; Souza et al., 2016). The evolutionary mechanisms (e.g., natural selection, indirect selection, neutral selection) underlying cave trait loss are still under discussion, it is reasonable to assume that each event of cave conquest may present unique evolutionary case. Towards the course of evolution probably different Tricladida species end up trapped inside caves, using cave as a shelter, or to find food, or sometimes due to water transgressions and regressions leading these animals to inhabit dark environments (Amaral et al., 2019; Leal-Zanchet & Marques, 2018a,

2018b; Souza et al., 2018). Selection pressures acted on these species, leading to changes related to troglobiont features, and the mechanisms involved in the convergent evolution of regressive traits are not fully comprehended (Aspiras et al., 2012; Krishnan & Rohner, 2017; Stemmer et al., 2015). Specific studies exploring cave species dynamics, molecular cues, light response, cell movement, morphogenic gradients, local control of cell proliferation and apoptosis, eye morphogenesis, and gene expression help to understand the molecular and evolutionary mechanisms driving the evolution of troglobitic traits (Wilkens, 2020; Wilkens & Strecker, 2017).

Based on the above considerations, during my PhD I introduced the obligate cave-dwelling planarian *Girardia multidiverticulata* (Souza et al., 2015), an eyeless and non-pigmented animal, as a new research model to understand the mechanism involved in cave adaptation. At the beginning of my doctorate, I discovered that this species –originally described as eyeless– actually presented two morphotypes among siblings: one with a small rudimentary pigmented eye, and another that presumably showed a complete absence of eyes. This finding represented a unique opportunity to study the genetics and plasticity of eye developmental disruption in planarians. Also, the close phylogenetic position and the eye morphology similarity with other Dugesiidae species allows a comparison associate with differences in expression patterns of eye-related genes, once the developmental signaling pathway for eye formation is well described in members of this family (Lapan & Reddien, 2011, 2012; Martín-Durán et al., 2012). I was able to examine the main molecular players that control or regulate the development of the cave planarian visual system, and increase our understanding of the level of robustness in developmental processes during the evolution of cave specific adaptations.

I was able to conclude that i) both cave planarian morphotypes actually have small eyes when compared with surface planarians; ii) the small eyes of cave planarians are related to a lower occurrence stem cell fate specification into eye cells, and not necessary to eye cell loss related to cell death eye homeostasis; iii) cave planarians are able to perceive light, specifically choosing darker regions in a light gradient; iv) cave planarians express conserved transcription factors of eye development with some exceptions during late differentiation of eye cells; v) *Girardia multidiverticulata* exhibit different expression levels of eye-related genes involved in light transduction, transmembrane receptors regulation related with G protein-coupled, and pigment formation when compared with surface species; vi) aside for the external visual differences between the two *Girardia multidiverticulata* morphotypes they also display of

different eye enriched genes, related with eye differentiation, eye phototransduction, and pigment regulation, between two different stage of eye regression within the same population, suggesting an in-progress process of adaptation to the subterranean environment.

The thesis presented here encompasses all the results I have obtained throughout my period as a doctoral student in the Postgraduate Program of Zoology at the Instituto de Biociências at USP, and during an 18-month research internship in Dr. Reddien laboratory at the Whitehead Institute in Cambridge, MA, USA. All results are presented here in a single chapter, in manuscript format, aims to elucidate the developmental and molecular changes that presumably took place during the evolution of the rudimentary eyes found in a cave planarian. Different research approaches –including comparative morphology, developmental studies and functional analyses using behavior– are used to reveal some of the changes that have occurred during one macroevolutionary event of eye regression. In this manuscript I propose a novel mechanism where the effects on homeostasis and progenitor cell availability may affect the regression of certain traits during major evolutionary transitions.
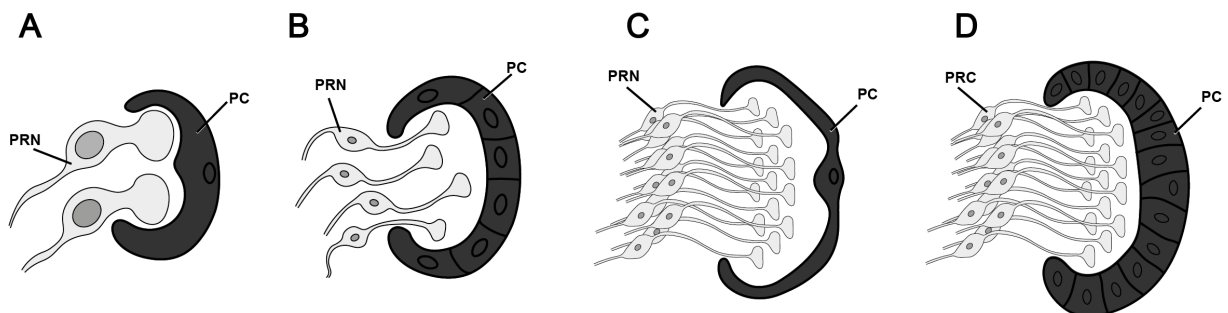
Figure I1 – Tricladida eye types schematic drawing. A) unicellular pigment cup eye with few photoreceptor cells; B) multicellular pigment cup eye with few photoreceptor cells; C) unicellular pigment cup eye with several photoreceptor cells; D) multicellular pigment cup eye with several photoreceptor cells. Abbreviations: PRN, photoreceptor neurons; PC, pigment cup. Images based on previous description found in Sluys (1989a)
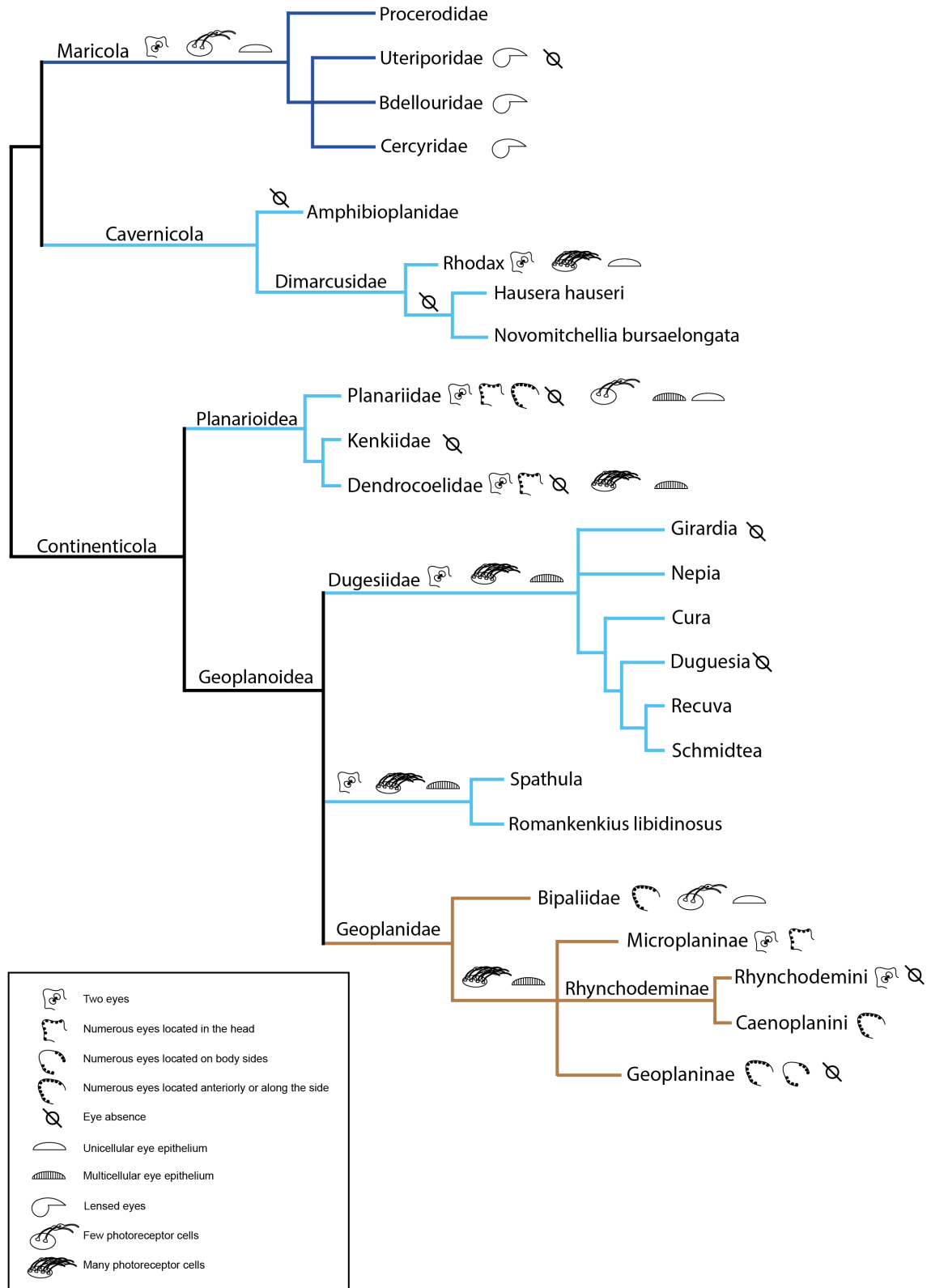
Figure I2 - Consensus phylogeny of Tricladida representing current understanding of major group relationships based on molecular data (Benítez-Álvarez et al., 2020; Sluys & Riutort, 2018; Stocchino et al., 2021), with the exception of the Kenkiidae that was inferred by morphology. Eye characters were plotted in the tree based on published species descriptions summarized in Table I1. Characters on branches represent the ancestral states. Characters next to the group names are representatives for the group, but do not necessarily mean that all species

in the group present that trait. Color codes: deep blue, marine flatworms; light blue, freshwater flatworms; brown, terrestrial flatworms.

Table I1 - Principal characteristics of the photosensory systems in Tricladida species, their habitats, GenBank accession numbers of genes used in phylogenetic analyses with their respective references.

https://www.dropbox.com/scl/fi/9e2tp0vrm22ddxem6m79t/Table-I1_general-introduction.xlsx?dl=0&rlkey=dfoa0yl1f7r53wfifhba1dm87

Table I2 - Current classification of Tricladida (Lang, 1884), reviewed by Sluys & Riutort (2018)

Suborder Maricola Hallez, 1892
    Superfamily Cercyroidea Böhmig, 1906
        Family Centrovarioplanidae Westblad, 1952
        Family Cercyridae Böhmig, 1906
        Family Meixnerididae Westblad, 1952
    Superfamily Bdellouroidea Diesing, 1862
        Family Uteriporidae Böhmig, 1906
            Subfamily Uteriporinae Böhmig, 1906
            Subfamily Ectoplaninae Bresslau, 1933
        Family Bdellouridae Diesing, 1862
            Subfamily Bdellourinae Diesing, 1862
            Subfamily Palombiellinae Sluys, 1989
    Superfamily Procerodoidea Diesing, 1862
        Family Procerodidae Diesing, 1862
Suborder Cavernicola Sluys, 1990
        Family Dimarcusidae Mitchell and Kawakatsu, 1972
Suborder Continenticola Carranza and al, 1998
    Superfamily Planarioidea Stimpson, 1857
        Family Planariidae Stimpson, 1857
        Family Dendrocoelidae Hallez, 1892
        Family Kenkiidae Hyman, 1937
Superfamily Geoplanoidea Stimpson, 1857
        Family Dugesiidae Ball, 1974
        Family Geoplanidae Stimpson, 1857
            Subfamily Bipaliinae Von Graff, 1896
            Subfamily Microplaninae Pantin, 1953
            Subfamily Rhynchodeminae Von Graff, 1896
            Subfamily Geoplaninae Stimpson, 1857

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Final Considerations

How photosensory system structures evolve and diversify in animals was the main research question I wanted to address in my doctoral thesis. However, large evolutionary trait changes within different species can sometimes take place over long time-scales and thus making it hard to be directly studied (Revell, 2013). For this reason, I decided to focus my research in a smaller group of animals (Tricladida: Platyhelmminthes) and explore aspects of eye phenotypic development and evolution to infer the processes involved in trait diversity evolution. Disentangling the processes that generate eye phenotypes resulted in a far more complex use of research tools that allowed me to interconnection many new aspects of phenotypic development and evolution than I could have ever originally anticipated. Trait diversity evolution is ultimately driven by mutations, and fixation of mutations results from the action of drift and/or selection. Selection, in turn, depends on the interactions of species with their environment. As a result, evolutionary modification of traits involves a dynamic process, in which selective pressures and fitness, as well as environmental factors interplay (Edwards et al., 2018; Nilsson, 2013). Therefore, to understand eye evolution and diversification of eye cell morphology in Tricladida it was necessary to comprehend the function and molecular aspects of the processes involved in eye phototransduction physiology and development across different species, and to relate these processes with the environmental characteristics where the different species live.

Tricladida eyes share several characteristics, for example the presence of rhabdomeric photoreceptor cells as the basic unit for phototransduction, a conserved eye developmental gene pathway across different species, and eyes composed by two main cells types, i.e., pigment cup cells and photoreceptor cell (Rink, 2018; Sluys, 1989). Those similarities together suggest that Tricladida eyes are homologous. The eye morphology literature reviewed in this thesis reveals a larger diversification of eyes frequently associated with evolutionary radiations in different environments. Tricladida species were able to diversify in marine, freshwater, and terrestrial habitats, and each event presents a set of eye innovations (Sluys, 2019). For example, lensed eyes are only found in marine species, or numerous eyes located along the body are only present in terrestrial species (Carbayo et al., 2013; Sluys, 1989). Plotting eye characters in a phylogenetic tree highlights the potential of Tricldada to help understand how many times photoreceptor innovations have evolved and whether lifestyles are associated with these transitions. Future Tricladida phylogenetic reconstructions together with ancestral state

estimation inferences and using appropriate statistical methods will help reveal changes in trait evolution to comprehend how diversification of traits interacts with the environment.

Another common characteristic found in several Tricladida groups was the presence of troglobitic traits on cave species. This order displayed several independent events of cave colonization in distant groups, yet species convergently shared loss of eyes and/or pigmentation (Morais et al., 2021). These occurrences allowed me to raise the main question of this thesis: what molecular and evolutionary mechanisms are underlying eye loss? To answer this question, the cave planarian *Girardia multidiverticulata* was introduced as a new potential laboratory animal model. In order to study and describe the processes controlling eye loss in this species, this study employed different methodological approaches, including behavioral experiments, comparative cell quantifications, animal regeneration assays, gene expression analyses by Fluorescence *In Situ* Hybridization and immmunocytochemistry, differential gene expression analysis using transcriptome data, cell proliferation and cell death assays, among others.

Results found in the main chapter of this thesis (Chapter 1) describes cellular and molecular changes associated with a cave adaptation found in *Girardia multidiverticulata*. Main findings include the retainment of small and functional rudimentary eyes; the presence of two morphotypes within the population presenting differences in expression of genes related with eye differentiation, phototransduction and pigmentation; similar expression of conserved transcription factors of eye development when compared with surface species, but with some differences in expression levels of eye-related genes involved in light transduction and pigment formation; and the formulation of a novel evolutionary mechanism associated with evolutionary size trait change in cave environment, in which modifications in homeostasis or regenerative stem cell dynamics led to the reduction of adult eyes. Specifically, eye reduction was associated with a lower occurrence and incorporation of adult eye specific stem cells. These results further our understanding of the different mechanisms responsible for trait loss or reduction, which help expand previous studies on the evolution of eye loss.

This is the first study to shed light on evolutionary eye reduction mechanisms in a Spiralian species. Studying evolutionary trait loss in diverse species is necessary to uncover distinct and common underlying mechanisms, revealing the cellular and molecular processes evolution uses for trait change. In this sense, there are still many gaps that need to be filled in future studies. Among them, we highlight the importance of increasing studies that carefully

describe the molecular mechanisms of trait loss in other invertebrate representatives, in order to clarify the evolutionary history of eye loss in a broader context.

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Resumo

Só é possível compreender a diversificação dos sistemas fotossensoriais dos animais uma vez que os aspectos morfológicos, fisiológicos, do desenvolvimento e seu contexto ambiental são abordados em diversas espécies em um nível comparativo. O estudo da diversificação ocular em animais da ordem Tricladida nos permite rastrear a seleção associada às diferentes condições ambientais, uma vez que esses animais constantemente exibem caracteres visuais associados aos distintos habitats que ocupam. De fato, nossos resultados demonstraram que espécies da ordem Tricladida habitam diferentes nichos e apresentam diversos fenótipos oculares adaptados às suas demandas ecológicas, como por exemplo a presença de olhos com lentes encontradas apenas em espécies marinhas ou olhos trilobados presentes apenas em espécies terrestres. Um outro exemplo é a constante perda de olhos em diferentes espécies localizadas em ambientes de cavernas, que permite investigar a evolução de caracteres convergentes relacionados à escuridão. A fim de elucidar as mudanças moleculares que ocorreram durante a evolução dos olhos rudimentares encontrados em uma planária de caverna, detalhamos o desenvolvimento ocular na planária de caverna *Girardia multidiverticulata*. Nossos resultados revelaram que *G. multidiverticulata*, inicialmente descrita como uma espécie sem olhos, na verdade retém pequenos olhos funcionais. Essas planárias expressam os mesmos fatores de transcrição conhecidos na formação do olho em planárias de superfície, com pequenas exceções durante o estágio final de diferenciação celular. No entanto, a análises de expressão diferencial revelou alterações nos níveis de expressão de genes envolvidos na função ocular e na formação de pigmentos quando comparados com espécies de superfície. Descobrimos que a redução no número de células oculares nessa espécie é causada por uma diminuição da taxa de especificação de progenitores de olhos, levando a uma menor taxa de diferenciação de novas células oculares durante a homeostase e também durante a regeneração. Por outro lado, a disponibilidade de progenitores para a formação de outros tecidos, como por exemplo o cérebro, foi semelhante entre as espécies da caverna e da superfície. Além disso, a população de *G. multidiverticulada* encontrada na caverna apresentou dois morfotipos diferentes, um apresentando olhos pequenos e pigmentados e o outro olhos pequenos não pigmentados. Além das diferenças de pigmentação entre os dois morfotipos seus níveis de expressão genética também são diferentes. A existência de dois estágios diferentes de regressão de olhos dentro da mesma população, combinados com a diminuição de progenitores específicos de olhos, juntamente com a modificação da expressão genética sugerem que esta espécie representa um estágio intermediário de adaptação ao ambiente subterrâneo. *Girardia multidiverticulata* representa um novo modelo para a de perda de olhos, em que a mudança na taxa de diferenciação de progenitores em olhos resulta em redução no tamanho do órgão. Esses resultados ampliam nossa compreensão dos diferentes mecanismos responsáveis pela perda de caracteres, o que é essencial para a compreensão das perspectivas evolutivas sobre a perda de olhos.

Abstract

To understand the evolution of form and diversification in the photosensory systems it is necessary to study the function, development, cell morphology, and environment context of eyes in different animals. Comparative studies of flatworms in the order Tricladida represent a good animal group to address questions about how eye structures evolved and diversified because Tricladida can occupy different environments, and display visual characters associated with it, offering an opportunity to study adaptations to the different environmental conditions. Indeed, our comparative review studies demonstrate that species of the order Tricladida inhabit different environments, and display diverse eye phenotypes accommodated to their ecological demands. Several eye-related key innovations found in Tricladida can be used to study how the environment shapes the diversification and evolution of traits, such as the presence of lensed eyes only found in marine species, or trilobated eyes only present in terrestrial species. Furthermore, Tricladida eyes have been repeatedly lost in different cave species, and therefore allow comparative studies related with the evolution of convergent traits associated to living in the dark. In order to elucidate the developmental and molecular changes that took place during the evolution of the rudimentary eyes found in a cave planarian, we carried detailed analyses of eye development in the cave planarian *Girardia multidiverticulata*. We found that *G. multidiverticulata*, which were initially described to be an eyeless species, actually retain small and functional, rudimentary eyes. With some exceptions during late differentiation of eye cells, these cave planarians expressed orthologs of conserved transcription factors known to be important for eye formation in surface planarians. However, differential expression analyses revealed changes in expression levels of genes involved eye function and pigment formation when compared with surface species. We found that the reduction in eye-cell number in cave planarians is caused by a decreased rate of stem cell fate specification to eye progenitors. This led to a lower rate of new eye cell differentiation during homeostasis, and also during regeneration. By contrast, formation of other tissues, including other neurons in the brain, was comparable between the cave and surface species. Additionally, two different morphotypes within the cave population exist and show heritable differences in eyes: one has small, pigmented rudimentary eyes (discernible eyes) and the other has unpigmented small eyes that are non-discernible by light microscopy. Aside for the pigmentation differences between the two *Girardia multidiverticulata* morphotypes presented changes in expression levels of genes. The existence of two different stages of eye trait regression within the same population, combined with reduced but not absent stem cell fate specification into eye cells, and altered eye gene expression suggest this species represents an intermediate stage of trait adaptation to the subterranean environment. We conclude that *Girardia multidiverticulata* represents a novel mode of evolutionary trait loss, in which change to the rate of stem cell fate selection in an adult setting result in reduction in organ size. These results further our understanding of the different mechanisms responsible for trait loss, which are essential to understand the evolution of eye loss.