

ALMA MATER STUDIORUM

UNIVERSITA' DI BOLOGNA

FACOLTA' DI SCIENZE MATEMATICHE FISICHE E NATURALI

Corso di Laurea Magistrale in BIOLOGIA MARINA

**INVESTIGATIONS ON THE G-PROTEIN INVOLVED IN
CNIDARIAN PHOTOTRANSDUCTION**

Tesi di Laurea in EVOLUZIONE ed ADATTAMENTI DEGLI
INVERTEBRATI MARINI

Relatore

Prof. Fausto Tinti

Correlatore

Prof. Todd Oakley

Presentata da

Federico Gaiti

(III sessione)

Anno Accademico 2010/2011

TABLE OF CONTENTS

<u>1. ABSTRACT</u>	5
<u>2. INTRODUCTION</u>	7
2.1 Phylum Cnidaria	7
<i>2.1.1 Systematics, fossil record, life history, general biology, and ecology</i>	
2.2 Cnidocysts	9
<i>2.2.1 The different types and functions</i>	
<i>2.2.2 Nematocysts structure</i>	
<i>2.2.3 Nematocysts discharge mechanism</i>	
<i>2.2.4 How is the prey detected?</i>	
<i>2.2.4.1 Chemo- and mechano-sensitivity</i>	
2.3 Photosensitivity	14
2.4 Project Goals	17
<u>3. MATERIALS AND METHODS</u>	19
3.1 Materials	19
3.2 Experimental animals	19
3.3 Photobehaviour assay using pertussis toxin	
(G_i alpha subunit inhibitor)	20
3.4 Nematocysts capture assay	21
3.5 Transgenic <i>Hydra</i>	23
3.6 <i>Hydra</i> gene gun	27
3.7 Data processing	28

<u>4. RESULTS</u>	31
4.1 Photobehaviour assay using pertussis toxin (G_i alpha subunit inhibitor)	31
<i>4.1.1 No evidence that pertussis toxin ablates Hydra photobehaviour</i>	
4.2 Nematocysts capture assay	33
<i>4.2.1 Influence of pertussis toxin in the presence of NANA and Proline</i>	
4.3 Transgenic Hydra	38
<i>4.3.1 Hydropsin5 promoter construct development</i>	
4.4 Hydra gene gun	39
<i>4.4.1 A promising result of hydropsin5 expression</i>	
<u>5. DISCUSSIONS</u>	47
5.1 No evidence that pertussis toxin ablates Hydra photobehaviour	47
<i>5.1.1 Preliminary suggestion that G_i alpha subunit is not involved in light sensitivity</i>	
5.2 Influence of pertussis toxin on nematocysts firing in the presence of NANA and Proline	48
<i>5.2.1 Additional suggestions that G_i alpha subunit is not involved in light sensitivity</i>	
5.3 Hydropsin5 promoter construct development	53
<i>5.3.1 A promising result of hydropsin5 expression</i>	
5.4 Conclusion	54
<u>6. REFERENCES</u>	55

1. ABSTRACT

This study aimed to investigate which genes Cnidaria use for photoreception and test whether G_i alpha subunit protein is involved in the phototransduction cascade, giving additional tools to investigate light-mediated behaviors. Here, I engineered an opsin gene promoter construct useful to test whether nematocyte sensory cells express opsin gene. By determining the expression of one of the unique EST opsin genes of the eyeless hydrozoan *Hydra magnipapillata* genome in nematocyte sensory cells, we will be able to investigate whether light modulation is an ancestral feature in Cnidaria, and whether regulation of nematocyte discharge by opsin-mediated phototransduction predated this pathway's function in cnidarian eyes. Nematocytes, the cnidarians stinging cells, discharge nematocysts to capture prey. As nematocysts are energetically expensive, the discharge is tightly regulated and occurs after proper chemical and mechanical stimulation. Cnidarians are also known to display a rich corpus of photobehaviors, which are often associated with activities that involve nematocytes. Previous experiments on nematocyst firing modulation show that light decreases nematocyte firing. This study contributed to confirm that bright light decreases the tendency for nematocytes to discharge in *Haliplanella luciae*. Similar findings in cubozoan and hydrozoan lead us to believe that light modulation of cnidocytes may be an ancestral feature of Cnidaria. Experimentally, I found no evidence that pertussis toxin, a G_i alpha subunit protein inhibitor, ablates *Hydra magnipapillata* photobehaviour, preliminary suggesting that G_i alpha subunit protein is not involved in photoresponse. I found no significant association between pertussis toxin and nematocyte firing in *Haliplanella luciae* both in conditions of dim and bright light, suggesting that G_i alpha subunit protein is not involved in photoresponse. We have preliminary evidence for a prevalence of photoreception over chemoreception, tending toward conditions of bright light. This finding may suggest the involvement of a G_s alpha subunit protein in *Haliplanella luciae* phototransduction pathway. While nematocyte chemo- and mechano-sensitivity have been extensively studied, further research is necessary to better understand what an ancestral phototransduction cascade looked like, and how opsin-based phototransduction acts to regulate nematocyte discharge.

2. INTRODUCTION

2.1 Phylum Cnidaria

2.1.1 Systematics, fossil record, life history, general biology, and ecology

Cnidarians represent a primordial group of animals, whose evolutionary divergence with bilaterians probably occurred in the late Precambrian period, over 600 million years ago (Ma), as reconstructed with the Ediacaran fauna (Scrutton, 1979). New molecular evidence suggests that cnidarians originated approximately 741 Ma, and the major taxa diversified prior to the Cambrian period (543 Ma) (Park *et al.*, 2012). Many of the best cnidarian fossils date back to the Vendian period, the time when animals first appear in the fossil records. The fossil record of soft-bodied cnidarians is very sparse while cnidarians with hard skeletons, in particular corals, have left a huge number of fossils since their first appearance in the Triassic period (Stanley, 2003; Veron *et al.*, 1996; Veron, 1995). Despite this, cnidarians present particular problems for systematics.

A recent cladistics analysis (Schuchert, 1993) and molecular data suggest that the Anthozoa are the most primitive, the only group of living cnidarians that lacks a medusoid stage in the life cycle (Bridge *et al.*, 1995) (Fig. 1). The phylum Cnidaria is divided into four major living classes: Scyphozoa (“true jellyfishes”), Cubozoa (“box jellyfishes”, with complex eyes and potent toxins), Hydrozoa (e.g. *Hydra*, *Physalia*, *Obelia*, etc.), and Anthozoa (sea pens, corals and sea anemones).



Fig. 1 Tree diagram showing the relationship between the four classes of the phylum Cnidaria and the extinct group Conulata. The basal branching point in the tree represents the ancestor of the other groups in the tree. This ancestor diversified over time into several descendent subgroups, which are represented as internal nodes and terminal taxa to the right. Tree following Werner (1973) and Bridge *et al.* (1995), taken from TREE OF LIFE web project.

This highly diverse phylum contains over 10,000 species found exclusively in aquatic and mostly marine environments. Within the hydrozoans, perhaps the best-known genus is *Hydra* (family: Hydridae, order: Anthomedusae).

A part of this study was conducted on *Hydra magnipapillata*, which is a small freshwater polyp that has been well known for over 200 years for its remarkable regenerative capacity. *Hydra*'s body has a radial symmetry and a tubular shape, secured by a simple adhesive foot called the basal disc. At the apical part of the body, the mouth is surrounded by five or six mobile tentacles. This organism has only two germ layers, ectoderm and endoderm that constitute epidermis and gastrodermis of the animal, respectively. Mesoglea, a layer of jellylike amorphous substance which contains scattered cells and collagen fibers, divides the two layers. The nervous system resembles a net and lacks central structures but allows for coordinated movements and it is necessary for feeding behavior on small aquatic invertebrates. *Hydra* does not have special respiratory and circulatory systems, in fact the entire body surface participate in gas exchange. Gases and nutrients are transported directly from cell to cell or by diffusion through the mesoglea. *Hydra* polyps are generally sessile but when hunting, they occasionally move. *Hydra* shows extreme regeneration capability, when polyps are cut to pieces they regenerate the missing structures completely (Gierer *et al.*, 1972). This indicates that *Hydra* can create patterning signals and developmental gradients along its body *de novo*. *Hydra* has a reduced life cycle, not having a medusa or colonial polyp stage. When in poor feeding conditions sexual reproduction occurs, while when food is plentiful, many polyps reproduce asexually by producing buds in the body wall which break away when mature.

Haliplanella luciae, commonly named the Orange-striped sea anemone, on which the second part of this study was conducted, belongs to the class Anthozoa, order Actinaria, which includes the commonly known sea anemones. Within the anthozoans, there are two contemporary subclasses, Octocorallia and Hexacorallia, the latter being divided into six contemporary orders even if with several uncertainties. Anthozoa lacks a medusoid stage in the life cycle, distinguishing themselves from most other cnidarians. They spend their whole life as a polyp and tend to stay attached to the same spot until conditions become harsh. Their diet is mainly composed of small fish and zooplankton that they capture, even though many species also depend on a mutual relationship with

Zooxanthellae algae contained within their cells. The captured prey is typically moved by the tentacles from the mouth into the gastrovascular cavity, where digestion occurs. Cells in the outer layer (epidermis) and the inner layer (gastrodermis) have microfilaments that group into contractile fibers. The tentacle motion, as well as the body motion, is realized through the contraction of these fibers, which are longitudinal fibers in the tentacles and oral disc, and circular fibers around the body wall. *H. luciae*, as all the others anthozoans, has a relatively simple nervous system, without central structures.

The characteristic feature of both *H. magnipapillata* and *H. luciae*, as of all the members of the phylum, is the presence of a type of cells called cnidocytes/nematocytes, of which all the cnidarians are armed. In fact, the name Cnidaria comes from the Greek word “*cnidos*”, which means stinging nettle.

2.2 Cnidocysts

2.2.1 The different types and functions

Cnidocysts are sub-cellular organelles ejected from single-use cnidocyte cells. Depending on their function and structure, three different typologies can be identified.

The first type, with the main function of penetration for prey capture and with a harpoon-like structure, are the so called nematocysts, which are common in all cnidarians, even if very morphologically diverse (Fautin, 2009). Not all nematocysts release toxic content, as is commonly thought; the discovery of solvent and glutinant nematocysts, used for defense and locomotion, respectively, is an indication of this (Purcell, 1984). The consequence of the elevate numbers of different types of nematocysts is that no cnidarian class displays all types. Only two types of nematocysts were found in sea anemones, microbasic p-magistophore and basitrichous isorhiza, while 17 unique types were found in hydrozoans (Fautin, 2009; Kravesky *et al.*, 2010).

Ptychocysts, the second type, are the most taxonomically restricted in distribution, occurring only in the anthozoan order Ceriantharia; their function is to entangle bits of mud among their robust tubules to form the feltwork that constitutes the tube of these burrowing animals (Mariscal *et al.*, 1977).

Spyrocysts, the last type, are unique to Anthozoa and are volvent, used like a rope that wraps around the prey (Krayesky *et al.*, 2010).

2.2.2 Nematocysts structure

Nematocysts are organelles, which include a bulb-shaped capsule containing a long coiled hollow tubule, enclosed in a vesicular membrane composed of disulfide-linked collagen-like molecules, called mini-collagens (Golz, 1994; Nüchter *et al.*, 2006).

An *operculum* acts as a lid of the capsule and at the externally-oriented side of the nematocyte, a *cnidocil* acts as a “hair-like trigger” device. Nematocysts are surrounded by nematocyte cytoplasm (Fig. 2). A net of microtubules and intermediate filaments connect the cell membrane to the nematocyst vesicular membrane.

Within the epidermis, nematocyte cells form various class specific complexes with supporting cells. In *Haliplanella luciae*, two different types of ciliated complexes are observed.

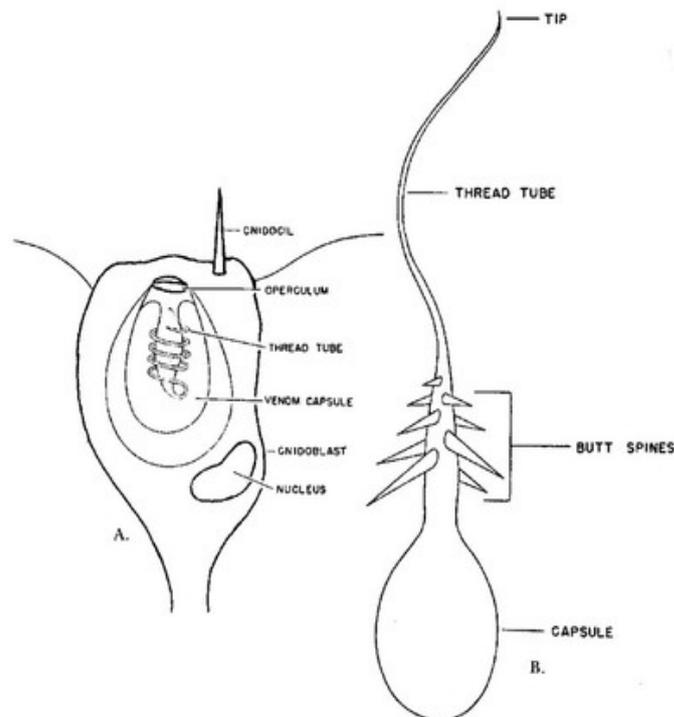


Fig. 2 Principal morphological features of the a) undischarged and b) discharged nematocysts. This represents only a single type of nematocyst, but portrays the basics of function. Taken from Halstead, 1988.

One complex is called the cnidocyte/supporting cell complex (CSCC) and the other is called the sensory cell/supporting cell complex (SNSC). In both these complexes, hair bundles are observed as prominences outside the epithelium. Hair bundles, arising from SNSC, tune nematocyst discharge responding to mechanical stimuli (Mire-Thibodeaux and Watson, 1994; Watson and Hessinger, 1989).

2.2.3 Nematocysts discharge mechanism

The mechanism of discharge is still debated and not all the steps have been clearly identified. The main driving force of the discharge is thought to be the high pressure inside the capsule. The nematocyst capsule stores a large concentration of calcium ions, which are released from the capsule into the cytoplasm of the nematocyte when the trigger is activated. The stimulation of the cnidocil induces a depolarization of the nematocyte membrane through the opening of Ca^{2+} ion channels (Gitter *et al.*, 1994). This influx of calcium causes a large concentration gradient of calcium across the nematocyte plasma membrane and the fusion of the nematocyst capsule with the nematocyte membrane (Özbek *et al.*, 2009). The resulting osmotic pressure causes a rapid influx of water into the cell. Discharge is initiated by the opening of the capsule operculum. The pressure of the water flowing into the nematocyte forces the water into the tubular nematocyst. Immediately the tubule begins to evert out with a twisting motion. The twisting acts to drill the tubule into the prey. The tubule then separates from the capsule and remains imbedded in the prey epidermis. Research by Nüchter and colleagues (Nüchter *et al.*, 2006) measured the escape velocity and kinetics of nematocyst discharge in the freshwater hydrozoan, *Hydra*. The steps above took place during 700 nanoseconds, creating an acceleration of 5,410,000 g (Fig. 3).

Nematocysts can discharge independently of each other in certain cases, or be influenced by interactions with surrounding cells or even the simple nerve net system. In fact, an electrical coupling between nematocytes has been described to increase the probability of discharge (Thurm *et al.*, 2004).

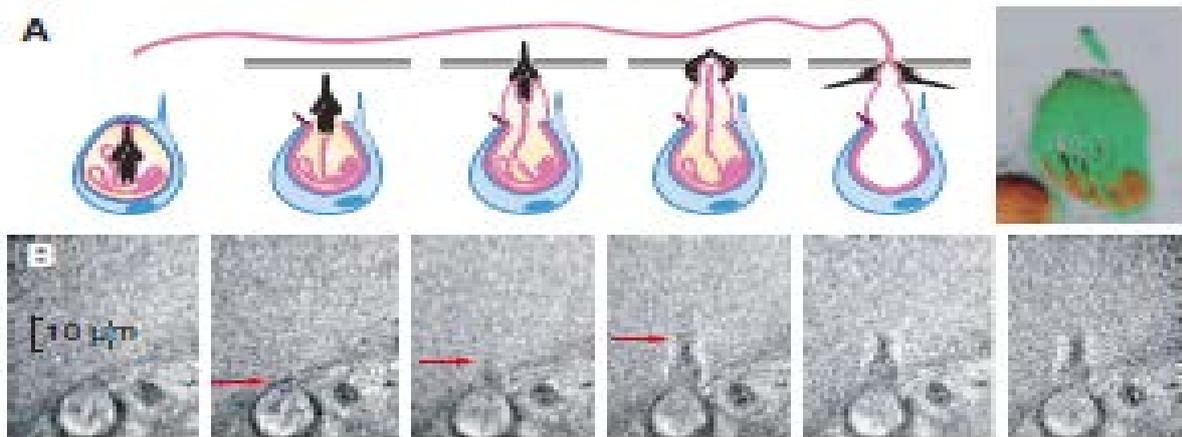


Fig. 3 Discharge of a nematocyst shown schematically (A) and recorded with a high-speed camera in the framing mode. (A) Nematocytes (blue; cell and vesicle membrane in dark blue) harbor one cyst (pink; minicollagen wall, tubule, and operculum) with stylets (black) punching a hole into prey. The inset shows microtubules anchoring capsule (tubulin antibody, green; DAPI-stained nucleus, red). (B) Sequential images; arrows indicate progress of discharge. Taken from Nüchter *et al.*, 2006.

2.2.4 How is the prey detected?

2.2.4.1 Chemo- and mechano-sensitivity

Nematocysts are continuously produced by the Golgi apparatus of cells, known as nematoblasts (Watson, 1988). Since they are “single-use” cells (i.e. they are not reused following discharge) and it is energetically costly to produce them, nematocysts are discharged only when the right combinations of stimuli are present.

Both mechanical and chemical stimuli may act to trigger nematocyst firing. Watson and Hessinger discovered that in *Haliplanella*, two types of mechanoreceptors are involved in the discharge mechanism: a contact-sensitive mechanoreceptor (CSM) and a vibration-sensitive mechanoreceptor (VSM) (Watson and Hessinger, 1992). CSMs are responsible of the discharge only upon direct tactile stimulation of the tentacle. Nematocysts are preferentially discharged into targets vibrating at specific frequencies and VSMs are involved in the frequency-tuning of nematocyst discharge. Hair bundles of the sensory cell/supporting cell complex detect vibrations and communicate the information to the cnidocyte. This detection makes the hair bundles oscillate causing a change in the membrane polarization in the supporting cells. The signal is transmitted to the

sensory cells, which by integrating the information, communicate with a ganglion cell. The pathway ends with a chemical synapses with the nematocyte cell (Westfall, 2004).

Thorington and Hessinger (1988) described two classes of chemoreceptors: the first is largely specific to a variety of amino and imino compounds and it is inhibited by histamine antagonist, e.g. proline. The second is specific for *N*-acetylated sugars, e.g. *N*-acetylneuraminic acid (NANA). These specific substances are detected by these two classes of chemoreceptors present at the surface of the supporting cells. The detection of these substances sensitizes the CSMs to initiate discharge into static targets and tune the VSMs, lowering the threshold of mechanical stimuli required to trigger the firing. The level of nematocysts discharged is maximized at 10^{-7} M for *N*-acetylated sugars and 10^{-8} M for proline (Watson and Hessinger, 1989). *N*-acetylated sugars shift the VSMs tuning-frequency to lower frequencies with an elongation of the hair bundle of SNSC, while proline shortens the hair bundle, in association with sugars. It seems that proline alone does not affect VSMs.

N-acetylated sugars are present on the prey coating and proline is present in the hemolymph of crustaceans. The approaching prey generates a local increase of *N*-acetylated sugar causing a sensitization of the mechanoreceptors to the corresponding lower frequencies. Once the prey is captured and wounded, proline is released shifting the VSMs tuning-frequency to higher frequencies than those generated by the prey, preventing additional discharge of nematocysts into a prey already netted (Watson and Hessinger, 1994). Some aspects of this model are still debated, but this version seems to maximize the efficiency of the prey capture, making its biological interpretation quite reasonable.

Watson and Hessinger suggest that the transduction of the chemo-signal from the receptor is achieved via a G-protein, adenylate cyclase, and cAMP pathway (Watson and Hessinger, 1994). Classically, a ligand binds its surface chemoreceptor which activates the associated G-protein. Watson and Hessinger found that caged GTP- γ -S, which produces sustained activation of G-protein coupled signaling pathways, tunes VSMs both to higher and lower frequencies (Watson and Hessinger, 1992), hypothesizing the presence of two G-proteins with antagonist action. G_s alpha subunit is a heterotrimeric G-protein subunit that activates the cAMP-dependent pathway by activating adenylate cyclase (AC).

G_i alpha subunit is a heterotrimeric G-protein subunit which mainly inhibits the cAMP dependent pathway by inhibiting AC activity, decreasing the production of cAMP from ATP.

By stimulating chemoreceptors for *N*-acetylated sugars, frequency responses of nematocyst discharge shift to lower frequencies as the hair bundles elongate. By stimulating chemoreceptors for proline after stimulating receptors for *N*-acetylated sugars, frequency responses of discharge shift to higher frequencies as the hair bundles shorten. G_s alpha subunit protein agonists induce the same effect as *N*-acetylated sugars, while pertussis toxin (Ptx) blocks the effect of proline on vibration-dependent discharge. Pertussis toxin is the major toxin produced by virulent strains of *Bordetella pertussis*, the bacterium that causes whooping cough, which has been found to catalyze the ADP-ribosylation of the G_i regulatory component of adenylate cyclase (Bokoch *et al.*, 1983; Codina *et al.*, 1983). Thus, pertussis toxin has become a valuable tool in the study of the regulation of adenylate cyclase.

After these considerations, it can be presupposed that *N*-acetylated sugars receptors may stimulate G_s alpha subunit while proline receptors stimulate G_i alpha subunit, modulating AC levels in opposite directions. This suggests that the frequency-specificity of hair bundles varies with intracellular levels of cAMP (Watson and Hessinger, 1994), which are in turn related to cAMP-dependent protein kinase.

2.3 Photosensitivity

In addition to chemo- and mechano-sensory behaviors, cnidarians are known to display a rich corpus of photobehaviors that include diurnal migration, phototaxis, and contraction responses to light. These behaviors are often associated with feeding or defense, two activities that involve cnidocytes. These observations raised the question: could light information from the environment also play a role in regulating cnidocyte function?

David Plachetzki discovered that light sensitivity genes (opsins) are expressed in sensory cells associated with cnidocytes in *Hydra magnipapillata* (Plachetzki *et al.*, 2010) and that opsin-based phototransduction acts to regulate

cnidocyte discharge by integrating information from the light environment into a behavioral outcome (Plachetzki *et al.*, unpublished).

Plachetzki *et al.* (unpublished) found that different light levels modulate firing rates, in *Hydra magnipapillata*. A significant difference in firing rate was observed between dim-light adapted animals and bright-light adapted animals, with significantly more nematocysts fired under dim-light adaptation. Gold and Fong investigated the same aspects on *Anthopleura* (class Anthozoa) and *Aurelia aurita* (class Scyphozoa), respectively. Both these unpublished experiments showed higher firing rates in dim-light adapted animals compared to bright-light adapted animals. Jindrich conducted firing experiments in *Haliplanella luciae*, showing the same pattern as above, a higher firing rate in dim blue light than bright light. These series of experiments showed that four species from three cnidarian classes have a similar behavioral pattern, suggesting that light modulation of cnidocytes may be an ancestral feature of Cnidaria.

A biological explanation could be associated with nematocyte photosensitivity. Since cnidarian diet is mostly composed of zooplankton, which are most active at dusk and dawn, dim light could be a useful indicator of when the preys is likely to be most active. This would also maximize the discharge efficiency, since it is really energetically expensive to produce nematocysts, avoiding to fire when the probability of catching a prey is low. Considering this hypothesis, bright light could instead favor transition to rely on photosynthetic symbionts. Another interesting hypothesis could be that a prey, in direct contact or a few centimeters from the tentacle, makes a localized shadow which disposes the surrounding cells to discharge nematocysts (Katia Jindrich's master's thesis). As an alternative, it could be that chemosensitivity and light sensitivity signal through similar molecular pathways, and that in this hypothesis the light response would be just a side effect of the chemoresponse.

An additional exciting observation is that chemo and photoreception seem to share the same molecular components as G-protein, adenylate cyclase, and cAMP signaling pathway. All known examples of visual perception in animals are accomplished, at the physiological level, by an opsin-mediated phototransduction cascade. Animal phototransduction is a canonical G-protein coupled receptor (GPCR) signaling pathway that results universally in a shift in the electro-chemical potential of photoreceptor neurons by the opening or closing of ion channels.

The cnidarian phototransduction cascade is similar to the vertebrate “ciliary” mode of phototransduction in that it utilizes a cyclic nucleotide gated (CNG) ion channel and other components of the vertebrate visual cycle. Koyagani (2008) found CNG cDNA transcripts from the eye of the cubozoan *Carybdea rastonii*. Opsin and CNG mRNA are co-expressed in *Hydra magnipapillata* (Plachetzki *et al.*, 2010), and a CNG inhibitor, cis-diltiazem, ablates a specific photoresponse, suggesting a functional relationship between opsin and CNG ion channel in *Hydra*. However, knowledge about the molecular basis of phototransduction in cnidarians is very recent and little work has been done to understand the origin of photosensitivity in animals. To investigate this matter, an accurate depiction of what an ancestral phototransduction cascade looked like is necessary.

Opsin is present within all the metazoan phyla that have been sequenced that followed sponges, suggesting that this novelty arose between Porifera and Cnidaria. Eyes are found only in some derived cnidarian lineages but numerous opsins are present in the two cnidarian genomes sequenced to date, *Hydra magnipapillata* and *Nematostella vectensis*, which both lack eyes or ocelli (Plachetzki *et al.*, 2007). By examining the genomic areas that surround opsins in *Hydra magnipapillata*, a putative PAX6 binding site was observed, indicating that the developmental gene was present before complex eyes evolved (Matt Harms’ undergraduate research thesis). PAX6 is a member of the PAX gene family, which provides instructions for making proteins that attach to specific areas of DNA and helps control the activity (expression) of particular genes. On the basis of this action, PAX proteins are called transcription factors and they play a critical role for the development of eyes and other sensory organs, certain neural and epidermal tissues, as well as other homologous structures. Thus, it seems that the regulation of cnidocyte discharge by opsin-mediated phototransduction predated this pathway’s function in cnidarian eyes.

It is known that in bilaterians, different opsins activate specifically a G-protein belonging to one of the three classes known: G_i, G_q, or G_s. Koyanagi *et al.* (2008) identified most of the messengers involved in the phototransduction in the cubozoan *Carybdea rastonii*. They found a G_s alpha subunit protein, which co-localizes with the opsin expressed specifically in visual cells. G_s alpha subunit is present in the *Hydra* genome but whether it is involved in photosensitivity has not yet been tested in *Hydra*. The inhibitory G_i alpha subunit protein is also present in

Hydra genome and seems to co-localize with opsin (Plachetzki *et al.*, unpublished). In bilaterians, the activation of the G_s alpha subunit protein triggers an increase of the level of AC, which synthesizes cAMP. In a cubozoan, Koyagani reported specific abundance of AC in visual cells (Koyanagi *et al.*, 2008). Adenylate cyclase is responsible for cAMP synthesis, and in cells expressing the cubozoan opsin gene, a light-dependent cAMP increase was observed.

Supposing that the phototransduction cascade is conserved among cnidarians, these findings might show indication for a G_s and/or G_i alpha subunit protein, adenylate cyclase, cAMP, and CNG phototransduction pathway in Cnidaria.

2.4 Project Goals

The over-reaching goal is to understand how phototransduction and vision originated in animals.

Where did phototransduction genes come from? Were new genes required, new combinations, or slight modification of old genes required? How does this novelty arise? Clearly, to investigate all these questions, a smaller scale approach is required.

This study aimed to investigate which genes Cnidaria use for photoreception and test which G alpha subunit protein is involved in the phototransduction cascade, giving additional tools to investigate light-mediated behaviors, as nematocyte firing.

This study addressed two main questions.

The first one was: do nematocyte sensory cells express opsin gene?

There are several good reasons, described in the previous section, to believe that they do and, using transgenic techniques, the goal was to test this hypothesis. By identifying where opsin gene is expressed in the eyeless hydrozoan *Hydra magnipapillata*, we will be able to test whether nematocyte sensory cells express opsin gene. This finding will shed light on whether the opsin-based phototransduction acts to regulate nematocyte discharge by integrating information from the light environment into a behavioral outcome. By determining the expression of one of the unique EST (Expressed Sequence Tag) opsin genes of *H. magnipapillata* genome in nematocyte sensory cells, we will be able to

investigate whether light modulation is an ancestral feature in Cnidaria, and whether regulation of nematocyte discharge by opsin-mediated phototransduction predated this pathway's function in cnidarian eyes.

The second question was: is G_i alpha subunit protein involved in phototransduction cascade in Cnidaria?

This second part of the project was driven by the evidence that chemo- and photo-reception share the same molecular components, as mentioned in the previous section. Previous knowledge on nematocyst firing modulation suggests that light decreases nematocyte firing. Thus, perhaps G_i alpha subunit is involved in the light response. Combining behavioral assays and pharmacology, the goal was to test this hypothesis and determine whether G_i alpha subunit is involved in light sensitivity. This finding will help to have a more accurate depiction of what an ancestral phototransduction cascade looked like helping to suggest how the novelty arose.

3. MATERIALS AND METHODS

3.1 Materials

Pertussis toxin (Ptx) was obtained from List Biological Laboratories, Campbell, CA. *N*-acetylneuraminic acid (NANA) type VI and proline were obtained from Sigma. In order to be activated, Ptx was incubated for 1 h at 25°C in 500 µL of 100 mM DTT and 500 µL of Milli-Q water.

3.2 Experimental animals

Specimens of the fresh water polyp *Hydra magnipapillata* were cultured, according to standard procedures, in Pyrex® dishes containing hydra medium (1 mM CaCl₂, 0.1 mM MgCl₂, 0.1 mM KCl, 1 mM NaH₂CO₃, pH 7.8) and held at room temperature (22°C ± 1°C) (Fig. 4). Specimens of the sea anemone *Haliplanella luciae* were obtained from Glen Watson, University of Louisiana at Lafayette, LA, USA. Animals were cultured in Pyrex® dishes containing natural seawater and held at room temperature (22°C ± 1°C). Since the culture dishes were held in the laboratory, the animals were exposed to a natural day light cycle. They were fed every three days with Selcon® enriched *Artemia* nauplii. Both hydra medium and natural seawater were changed at least three hours after each feeding.

Sea anemones were tested in artificial sea water (ASW; Marine Biological Laboratory, Woods Hole) consisting (in mM) of NaCl, 423; KCl, 9; MgCl₂, 22.94; MgSO₄, 25.50; CaCl₂, 9.27 and NaH₂CO₃, 2.14.



Fig. 4 Time-lapse photograph of a *Hydra magnipapillata* polyp, cultured in hydra medium. Photo by Cat Fong and Todd Oakley.

3.3 Photobehaviour assay using pertussis toxin (G_i alpha subunit inhibitor)

Work plan outline of the experiment

1. Incubation of *H. magnipapillata* polyps in hydra medium + pertussis toxin at a certain concentration, for a certain amount of time
 2. 20 min darkness adaptation
 3. Exposure to bright light of 3500 lux
 4. Contraction response assay
 - a) Control + pharmacological treatment for each experiment
 - b) Count of contracted and uncontracted polyps
 5. Mechanical response assay
 - a) Count of unresponsive polyps
-

For each trial, ten *Hydra* were tested in 5 mL of hydra medium in small Pyrex® dishes. The photobehaviour assay was performed following Plachetzki *et al.*'s protocol (Plachetzki *et al.*, 2010). Subjects were left for a minimum of 20 min in total darkness to allow dark-adaptation and recovery from any transportation shock. After 20 min, subjects were exposed to blue light of 3500 lux. Light of a sharply defined spectrum peaking at 470 nm was emitted from a light-emitting diode (LED) array (SuperBright LEDs). Light intensity, as given in lux, was measured using a Smart Lux meter (Milwaukee Instruments, SM 700).

Hydra polyps were tested for the contraction response (Passano & McCullough 1962, 1965) in small Pyrex® dishes. Ten individuals were tested at a time, facilitating the observation of the contraction response. The time until first complete contraction was recorded, and experiments were terminated after 20 min if the animal did not contract. For each experiment, both pharmacological treatment and control were performed. Pertussis toxin, after being activated as described in the previous section, was used in hydra medium at different concentrations: 62.5 ng/mL, 125 ng/mL, 250 ng/mL, 300 ng/mL, 350ng/mL, 400 ng/mL, 500 ng/mL, and 1 µg/mL. The Pyrex® dishes, containing different pertussis toxin concentrations each trial, were then incubated for different time to test the

minimum time of incubation (i.e. the time did not kill or damage the animals). For this purpose the incubation times were: 1 h, 2 h, 3 h, 4 h, 6 h, 12 h, and 24 h. After the incubation, photobehaviour assays were performed, as described above. In order to test whether the amount of toxin or the incubation time would kill the polyps, the animals were tested for the mechanical response. The animals were classified as “unresponsive” if, after having touched them, no contraction response was observed.

All the photobehaviour assays were performed on the third day after the last feeding, approximately at the same hour, in order to minimize the variables that could influence the contraction response.

3.4 Nematocysts capture assay

Work plan outline of the experiment

1. Transfer of animals from culture dish to a new one filled with artificial sea water (ASW)
 2. Acclimation of animals for 4 h in two different light conditions: dim and bright
 3. Replacement of ASW with test substances in ASW at specified concentrations
 - a) 10^{-7} M NANA and/or 10^{-8} M proline + 10^{-11} , 10^{-13} , 10^{-15} , or 10^{-17} M pertussis toxin
 4. After 15 min, touch of the distal tip of a single tentacle of each animal using a gelatin-coated probe
 5. Count of the microbasic p-mastigophore nematocysts discharged on the probe under light microscopy (40X)
-

In order to investigate whether light sensitivity in Cnidaria involves G_i alpha subunit protein, nematocysts discharge in *Haliplanella luciae* was tested using pertussis toxin.

The assay was conducted under two diverse light conditions (bright or dim light), following Watson and Hessinger (1994)'s protocol. The light source used was a blue LED (SuperBright LEDs) with a spectrum peak at 470 nm.

Light intensity, as given in lux, was measured using a Smart Lux meter (Milwaukee Instruments, SM 700).

For each assay, animals were transferred from culture Pyrex® dishes to other Pyrex® dishes filled with ASW (about 12 animals per dish), and they were acclimatized for approximately 4 h to light of different intensities: approximately 50 lux for the dim light condition and about 3500 lux for the bright light condition. These illuminance levels correspond to those measured in a fully lighted street at night and on a sunny day, respectively.

The physical disruption of being detached from the culture dish surface and placed in a new Pyrex® dish might have some influence on the firing. Scratching sea anemones off their attachment point causes stress and therefore may also affect the animal's reactivity. To minimize this possible impact, the polyps were placed in the new Pyrex® dishes at least one hour before the acclimation process.

After the acclimation phase, ASW was replaced with test substances in ASW at specified concentrations. 10^{-7} M NANA and/or 10^{-8} M proline were used because each of these ligand concentrations induces maximal discharge of nematocysts into test probes (Watson and Hessinger, 1994). In addition, pertussis toxin at different concentrations was used, precisely 10^{-11} , 10^{-13} , 10^{-15} , or 10^{-17} M. After exactly 15 minutes, the distal tip of a single tentacle of each animal was touched using a gelatin-coated probe, to trigger nematocysts discharge. This probe consisted of a 10 cm 1812 g fishing line segment, dipped three times into a 10% (w/v) gelatin mix (preheated to 70°C) and allowed to dry for 30 min before use. The probes were then placed on wet mounts with glycerol and the microbasic p-mastigophore (mpm) nematocysts, discharged into the gelatin, were counted under light microscopy (40X). The number of nematocysts discharged into the whole probe was counted, by searching the full length and the width of the probe by adjusting the focal plane of the microscope accordingly.

Data for nematocysts counts excluded the other cnidocyst types (spirocysts and basitrich nematocysts) discharged into the probes.

3.5 Transgenic *Hydra*

Work plan outline of the promoter construct development

1. Purification of *H. magnipapillata* total DNA from polyps' tissues
 2. Amplification of 1148 base pairs of sequence upstream hydropsin5 gene (promoter region), using a high fidelity polymerase
 3. Cloning of the product using TOPO-blunt to confirm that it was correct and error-free
 4. Re-amplification of the promoter to add Sall restriction site to 5' end and PstI restriction site to 3' end
 5. Cloning of the product using TOPO-blunt to confirm that the correct 5' and 3' ends have been added
 6. Double digestion of promoter region with the two restriction enzymes mentioned above, and cloning into pHyVec13 vector cut with these two restriction enzymes
 7. Sequencing of the resulting plasmid with the GFP-R primer (TTC CGT ATG TTG CAT CAC CTT C) to confirm that the fusion between the promoter and GFP was correct
 8. Transient transfection of *Hydra* by particle bombardment
-

A promoter construct was engineered to drive the expression of a *Hydra*-opsin gene in cells.

In a previous experiment by Matt Harms (undergraduate research thesis), that was carried out to determine the number of unique opsin loci within the *H. magnipapillata* genome, a sequence with typical features of opsin gene was searched in *Hydra* genome using the tblastn function. Addressing the issue of which opsins were also expressed, 8 opsin loci were found to be Expressed Sequence Tags (ESTs) (Matt Harms' undergraduate thesis). In this previous study, it had been determined that the region of high similarities between these opsin loci had characteristics of a PAX6 regulatory binding site, which could be involved in directing opsin expression. This hypothesis, however, was not tested but just suggested based on sequence similarity. For our purpose, since there could be other regulatory regions involved, the longest sequence of promoter region had to be amplified from the *H. magnipapillata* genome.

The opsin gene used in this study was hydroopsin5 (GenBank: AB050602.1), located in the contig37884 of *H. magnipapillata* genome from position 49,843 to 50,444 base pairs (bp) (<http://hydrazome.metazome.net>). The upstream region of hydroopsin5, from position 50,402 to 51,588 bp in the same contiguous, was used to develop the promoter construct.

Total DNA was purified from polyps' tissues following Spin-Column Protocol by QIAGEN (DNeasy Blood & Tissue Handbook). Primers were designed to amplify the longest sequence of hydroopsin5 promoter region (Tab. 1). 1148 bp of sequence upstream hydroopsin5 gene were amplified setting a reaction in 10 μ L volume consisting of: 0.5 μ L Fw primer GRF_3 + 0.5 μ L Rv primer GF_R2+ATG, 2 μ L 10X Herculase II Fusion DNA Polymerase Buffer, 0.3 μ L 10 mM dNTPs, 0.2 μ L Herculase II fusion DNA Polymerase, 2 μ L *H. magnipapillata* DNA genome, and 4.5 μ L Milli-Q water. The cycle used was: 94/5m [94/30s 51.8/30s 72/2:15m] x30 72/10m. The promoter sequence was successfully introduced into an *E. coli* plasmid, through the use of the Zero Blunt® TOPO® PCR Cloning protocol. After selecting the colonies that possessed the promoter, the bacteria were grown in Luria-Bertani (LB) medium. Plasmids were then extracted using FastPlasmid Mini Kit. Sequencing of the isolated promoter was done to confirm that it was correct and error-free.

The fragment was then re-amplified to add the restriction sites, Sall for the 5' end and PstI for the 3' end. New primers were built and for the 3' end, the sequence encoding the amino acids that are upstream of the **PstI site** in GFP (ATG GCC GAT GAT GAA GTT GCC GCC CTC **GCT GCA G**) was included (Tab. 1). 1189 bp of sequence upstream hydroopsin5 gene were then amplified with these new primers setting a reaction in 10 μ L volume consisting of: 1.5 μ L Fw primer GF_F4+Sall + 1.5 μ L Rv primer PstI-R2, 1 μ L 10X Herculase II Fusion DNA Polymerase Buffer, 1 μ L 25 mM MgCl₂, 0.3 μ L 10 mM dNTPs, 0.2 μ L Herculase II fusion DNA Polymerase, 1 μ L of the plasmid diluted 1:100, and 3.5 μ L Milli-Q water. The cycle used was: 94/5m [94/30s 52/30s 72/2:15m] x30 72/10m. Through the use of the Zero Blunt® TOPO® PCR Cloning protocol, the promoter sequence with restriction sites was successfully introduced into an *E. coli* plasmid. After selecting the colonies that possessed the promoter, the bacteria were grown in LB medium. Plasmids were then extracted using FastPlasmid Mini Kit.

Sequencing of the isolated promoter was done to confirm that the correct 5' and 3' ends had been added.

The plasmid was then double digested and cut with the two restriction enzymes, Sall and PstI, in order to ligate the promoter region in pHyVec13, a vector developed by Rob Steele at UC Irvine (Addgene plasmid 34796). The vector, derived from pBluescript II SK+, has GFP and actin promoter (Fig. 5). The vector is 5204 bp in length, and when cut with Sall + PstI, two fragments were obtained, one of 1408 bp and one of 3796 bp. The 1.4 kb fragment was the actin promoter. The hydropsin5 promoter region was cloned into the ~3.8 kb fragment.

In order to cut the promoter out from the *E. coli* plasmid, a digestion reaction was set using Promega reagents: 10 μ L 10X Buffer D, 4 μ L Sall, 4 μ L PstI, 10 μ L of plasmid containing the promoter region with restriction sites, and Milli-Q water to a final volume of 100 μ L. The reaction was incubated at 37°C overnight. A sequential digestion was performed to cut pHyVec13 vector. The first digestion reaction was set using Promega reagents: 2 μ L 10X Buffer H, 0.5 μ L PstI, 0.25 μ L pHyVec13, and Milli-Q water to a final volume of 20 μ L. The reaction was incubated at 37°C overnight. The reaction was deactivated at 65°C for 15 min and subsequently cleaned-up and re-suspended in Milli-Q water using NucleoSpin® Extract II.

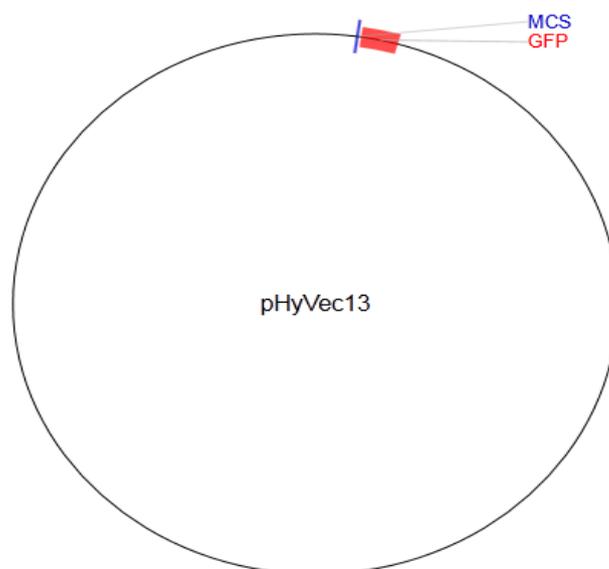


Fig. 5 pHyVec13 (Addgene plasmid 34796) map with GFP and Multiple Cloning Site (MCS). The sequence of the MCS is: GGA TCC AAG GTA CCT TCT CGA GTT ACT AGT AGT CTA GAC TAT CGA TAG GAT ATC TCA GAT CTA GGT CGA C.

The second digestion reaction was performed incubating the previous digestion product with 2 μL of 10X Buffer D, 0.5 μL Sall, and Milli-Q water to a final volume of 20 μL at 37°C overnight. The reaction was deactivated at 65°C for 15 min and subsequently cleaned-up and re-suspended in Milli-Q water using NucleoSpin® Extract II. DNA fragments, correspondent to 3796 bp for pHyVec13 and 1189 bp for the promoter region, were extracted from 1% w/v agarose gels using NucleoSpin® Gel Clean-up and eluted in 50 μL of pre-heated 50°C Milli-Q water. A 1% w/v agarose gel confirmed the digestions were both right.

A ligation reaction was performed in order to insert the promoter region into pHyVec13 vector. Using Promega reagents the subsequent reaction was set: 2 μL ligation buffer, 2 μL T4 DNA Ligase, 8 μL digested pHyVec13, and 8 μL digested hydropsin5 promoter. The reaction was incubated at room temperature overnight. 5 μL of the ligation reaction was then transformed in 50 μL of One Shot® TOP10 Chemically Competent *E. coli* cells following Invitrogen™ protocol. The pHyVec13 vector was successfully introduced into an *E. coli* plasmid. After selecting the colonies that possessed the vector, the bacteria were grown in LB medium. Plasmids were then extracted using FastPlasmid Mini Kit and glycerol stocks were made as backup.

To confirm the ligation was successful, both PCR and digestion reactions were performed on the plasmids. 15 μL of each plasmid were incubated at 37°C overnight with 2 μL Buffer 10X NEB, 2 μL 10X BSA, 0.5 μL Sall, and 0.5 μL PstI. A 1% w/v agarose gel confirmed the presence of the promoter region in the plasmids. Eight colonies of *E. coli* were taken from the LB + Ampicillin backup plates and were grown individually in LB + Ampicillin medium. 1 μL of the medium was added to 9 μL of Milli-Q water to a final volume of 10 μL , used as template in the following PCR reaction. 1189 bp of sequence upstream hydropsin5 gene were then amplified setting a reaction in 10 μL volume consisting of: 1.5 μL Fw primer GF_F4+Sall + 1.5 μL Rv primer PstI-R2, 1 μL 10X Herculase II Fusion DNA Polymerase Buffer, 1 μL 25 mM MgCl_2 , 0.3 μL dNTPs 10 mM, 0.2 μL Herculase II fusion DNA Polymerase, 1 μL of template, and 3.5 μL Milli-Q water. The cycle used was: 94/5m [94/30s 51.8/30s 72/2:15m] x30 72/10m. A 1% w/v agarose gel confirmed the amplification and, therefore, the presence of hydropsin5 promoter region in the plasmids.

The next step consisted in the usage of a gene gun in order to preliminary test the construct and subsequently investigate whether nematocyte sensory cells express hydropsin5. The plasmids needed to be re-suspended in Milli-Q water at a concentration of 1 mg/mL or greater for use with the gene gun. For this purpose, *E. coli* cells from glycerol stocks were grown on fresh LB plates and after selecting the colonies that possessed the vector, the bacteria were grown in LB medium. Plasmids were then extracted using FastPlasmid Mini Kit and combined together to reach the required concentration. Sequencing of the resulting plasmid was done to confirm that the fusion between the promoter region and GFP was correct. GFP-R primer was built, following Rob Steele's suggestions, as reverse primer, and a new primer upstream of the Sall site in pHyVec13, was designed to use for forward sequencing (Tab. 1).

Sequencing was performed by UC Berkeley DNA Sequencing Facility. Sequences were checked using Sequencher 5.0.

3.6 *Hydra* gene gun

Professor Todd Oakley used *Hydra* gene gun technique at Irvine to preliminary test the construct.

Materials needed were:

- 1.0 M Spermidine free base (Sigma-Aldrich S0266), made up in Milli-Q water, aliquoted into 1.5 mL tubes, and stored at -20°C.
- 2.5 M CaCl₂ made up in Milli-Q water, filtered sterilize, and stored at 4°C.
- Plasmid DNA prepared as described in the previous section and stored at -20°C.
- Gold particles prepared at a concentration of 1.0 M, according to Bio-Rad instructions (Page 21 in Users' Manual). After re-suspension at 60 mg/mL in 50% glycerol, aliquoted in 1.5 mL tubes and stored at -20°C.

The protocol followed to perform the transient transfection of *Hydra* by particle bombardment, was from Rob Steele's lab.

3.7 Data processing

For the nematocysts capture assays, the probe was not considered in the final dataset if the tested sea anemone wrapped its tentacle around the probe, touched the probe with another tentacle, or it was impossible to distinguish all the nematocysts.

The remaining data were compiled and a subset, including NANA + proline and Ptx treatments, was subjected to a Two-Way ANOVA to test for interaction between light and pertussis toxin. A generalized linear model regression analysis, to assess a hypothetical association of pertussis toxin and/or light with firing rate, was performed. A One-Way ANOVA was performed to test for an effect of pertussis toxin on firing rate in both the two conditions of light. A generalized linear model regression analysis was then performed, to test whether there was association between pertussis toxin and firing rate, in both the two conditions of light.

P-values of less than 0.05 were considered significant. All data, for both photobehaviour assays and nematocysts capture, were analyzed using R software.

Table 1. Primers used in PCR reactions and sequencing. All the primers were built using Integrated DNA Technologies OligoAnalyzer 3.1. Name, sequence, G-C content, and melting temperature, are listed for each primer. GRF_3 and GF_R2+ATG were used to amplify the promoter region of hydropsin5 gene. GF_F4+Sall and PstI-R2 were used to add Sall and PstI restriction sites to hydropsin5 promoter region. Sallup-F2 and GFP-R were used to sequence pHyVec13 after having performed the ligation reaction, to confirm the fusion between hydropsin5 promoter region and GFP. Hydropsin5 gene within *H. magnipapillata* genome was searched using blastn function (<http://hydrazome.metazome.net>) (GenBank Access N°: AB050602.1).

Primer Name	Sequence	GC content	Tm (°C)
GRF_3	5'- ACC TGC CAA ATG TGA GAA GAA CGT -3'	45.8 %	59.0
GF_R2+ATG	5'- CAT ACT TTT CTG TTG GTT TAG ATA AAT CAT GGT CCT G -3'	32.5 %	58.8
GF_F4+Sall	5'- AGG CGG GTC ACC TGC CAA ATG TGA GAA GAA CGT -3'	55.6 %	69.6
PstI-R2	5'- CGC CCT GCA GCG AGG GCG GCA ACT TCA TCA TCG GCC ATG ATT TAT TAT CTA AAC CAA CAG -3'	51.7 %	71.2
Sallup-F2	5'- GCC GTA AAG CAC TAA ATC GGA ACC -3'	50.0 %	58.3
GFP-R	5'- TTC CGT ATG TTG CAT CAC CTT C -3'	45.5 %	55.4

4. RESULTS

4.1 Photobehaviour assay using pertussis toxin (G_i alpha subunit inhibitor)

4.1.1 No evidence that pertussis toxin ablates Hydra photobehaviour

After having performed photobehaviour assays, as described in §Materials & Methods, it has been observed that certain combinations of pertussis toxin concentration and duration of exposure have caused *Hydra* polyps to be unresponsive, which means, after having touched them, no contraction response was observed (Fig. 6). Usually, the polyps scrunch into a ball upon probing, in a behavior that looks the same as when exposed to bright light, as described by Passano & McCullough (1963, 1964).

A total number of 181 polyps were tested using pertussis toxin. The number of unresponsive animals was 44. As shown in figure 6, an incubation of 12 h using pertussis toxin at a concentration of 1000 ng/mL, caused all the ten *Hydra* tested in the trial to be unresponsive. Halving the Ptx concentration (500 ng/mL), an incubation of 3 h caused the same response. A lower proportion of unresponsive *Hydra* was observed after incubating the polyps, using Ptx at a concentration of 250 ng/mL, for 24 and 12 h. In these trials, 8 and 4 polyps were unresponsive, respectively. Two other trials have shown just one unresponsive polyp (350 ng/mL for 12 h; 500 ng/mL for 6 h), and in all the others, no unresponsive polyps were observed.

Since no similar previous studies had ever used pertussis toxin to investigate the photobehaviour in *Hydra*, the goal was to find both the right concentration of pertussis toxin and duration of exposure that would have caused the inhibition of G_i alpha subunit by the toxin, and the subsequent ablation of typical contraction response in *Hydra*, showing the involvement of G_i alpha subunit protein in light sensitivity.

Pharmacological treatment and a control were performed for each trial. For each trial, behavioral assays were conducted under conditions of blue bright light (3500 lux, 470 nm).

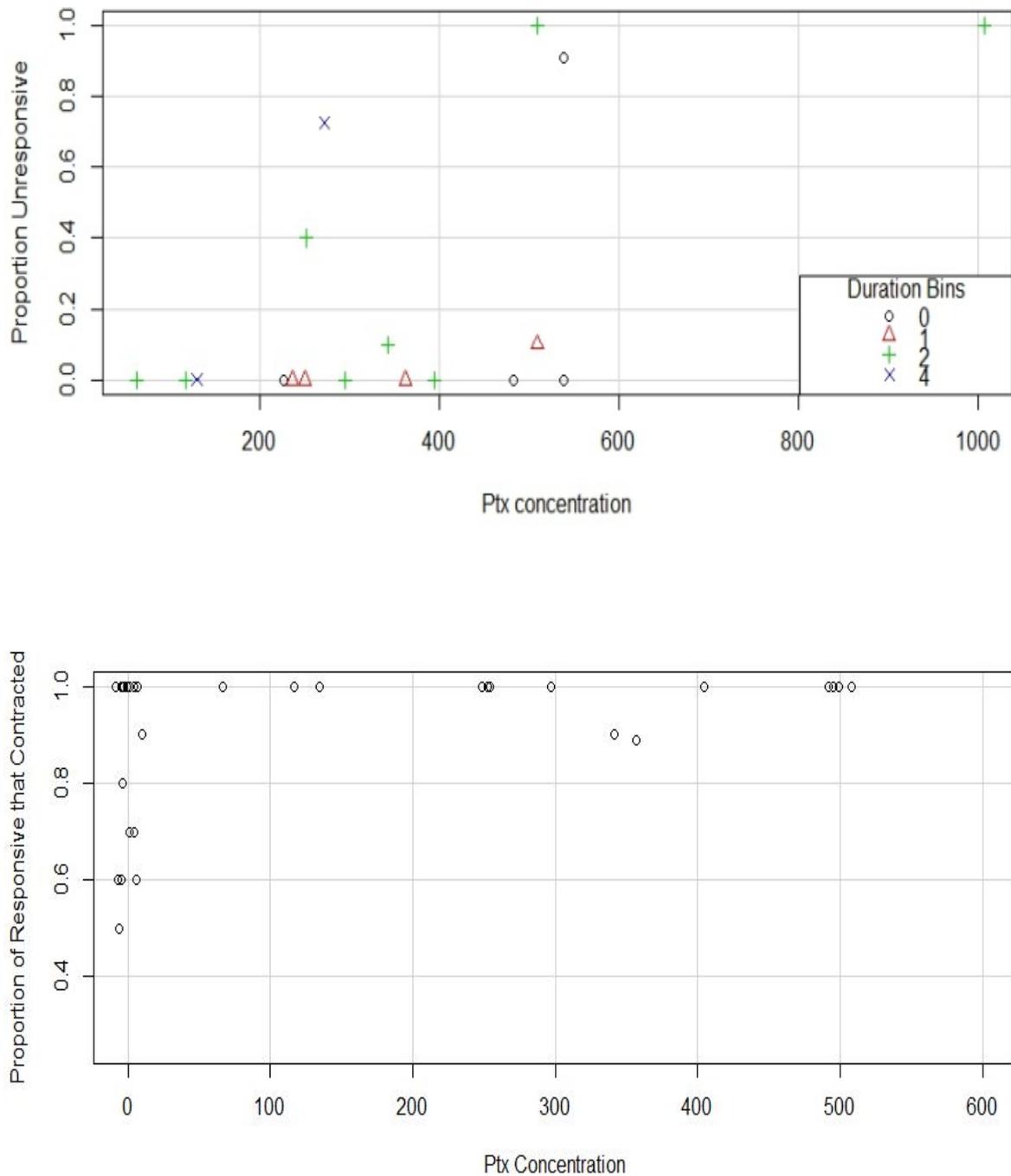


Fig. 6 Plots of the unresponsive *Hydra* polyps, and of the proportion of responsive polyps that contracted, as a function of Ptx concentration, respectively, after having performed photobehaviour assays. The animals were classified as “unresponsive” if, after having touched them, no contraction response was observed. Responsive *Hydra* are the sum of contracted and uncontracted polyps. The contraction response observed was the typical behaviour of *Hydra*, as described by Passano & McCullough (1963, 1964). Pertussis toxin concentration is given in ng/mL. Duration Bins are given in multiples and divisors of 6 h. “Cross”: incubation time of 24 h, “Plus”: incubation time of 12 h, “Triangle”: incubation time of 6 h, “Circle”: incubation time of <6 h. Ptx concentration equal to zero corresponds to the control conditions.

Figure 6 shows that the proportion of responsive polyps is high, no matter the pertussis toxin concentration, suggesting that G_i alpha subunit protein is not involved in light sensitivity. In all trials, across various Ptx concentrations, where the *Hydra* remained responsive to mechanical stimuli, they also remained responsive to bright light. Under control conditions, 152 polyps showed the typical contraction response as they were exposed to bright light, while 30 polyps did not contract. Under treatment with the G_i alpha subunit inhibitor, the number of responsive polyps that contracted was 135, while just 2 polyps were classified as uncontracted. These results suggest that G_i alpha subunit protein is not involved in light sensitivity.

4.2 Nematocysts capture assay

4.2.1 Influence of pertussis toxin in the presence of NANA and Proline

In order to study whether light sensitivity in Cnidaria involves G_i alpha subunit protein, nematocysts discharge in *Haliplanella luciae* was tested using pertussis toxin.

When the animals were subjected to bright light (± 3500 lux), test probes had a mean of 4.17 ± 2.88 (mean \pm SE) microbasic p-mastigophore (mpm) nematocysts adhering to them, based on $n = 12$ probes. When the animals were subjected to dim light (± 50 lux), test probes had a mean of 8.00 ± 3.21 (mean \pm SE) mpm nematocysts adhering to them, based on $n = 20$ probes. This result is in accord with previous results obtained by Katia Jindrich in her experiments on *Haliplanella luciae* (Katia Jindrich's master's thesis). Here, the same experimental protocol had been followed, and the same pattern had been found, i.e. a higher firing rate in dim blue light than bright light.

As shown in figure 7, the number of nematocysts captured per probe is higher in proline treatment (B_proline) if only compared to artificial seawater (ASW_control). When animals were exposed to bright light and 10^{-8} M proline, test probes had a mean of 13.56 ± 4.00 (mean \pm SE) mpm nematocysts discharged based on 9 test probes, while under conditions of dim light and 10^{-8} M proline, test probes had a mean of 18.33 ± 7.54 (mean \pm SE) ($n = 9$ probes) of mpm nematocysts adhering to them. Watson and Hessinger proposed that proline acts

in opposition to *N*-acetylneuraminic acid (NANA), shortening hair bundles and tuning nematocyte firing to higher frequency vibrations (Watson & Hessinger, 1994), suggesting that proline inhibits firing through G_i alpha subunit protein. The result obtained in this study rows against this suggestion, and it does not explain how proline increases firing rate compared to control condition.

When animals were subjected to bright light and 10⁻⁷ M NANA, test probes had a mean of 31.88 ± 6.97 (mean ± SE) (n= 8 probes) mpm nematocysts discharged based on 8 test probes, while under conditions of dim light and 10⁻⁷ M NANA test probes had a mean of 51.57 ± 9.25 (mean ± SE) (n= 7 probes) mpm nematocysts adhering to them. In *Haliplanella*, NANA may stimulate adenylyl cyclase, via G_s alpha subunit protein, to decrease cAMP, which lengthens hair bundles to tune nematocyte firing to lower frequency vibrations (Watson & Hessinger, 1994). This result is consistent with this suggestion, confirming a higher firing rate in presence of NANA than that with proline.

When animals were exposed to bright light and 10⁻⁷ M NANA + 10⁻⁸ M proline (NP), test probes had a mean of 24.00 ± 8.25 (mean ± SE) mpm nematocysts adhering to them, based on n= 6 probes, while under conditions of dim light and 10⁻⁷ M NANA + 10⁻⁸ M proline test probes had a mean of 22.14 ± 11.12 (mean ± SE) mpm nematocysts adhering to them (n= 7 probes). Activating receptors for NANA induces hair bundles to elongate while tuning discharge of mpm nematocysts to lower frequencies. Subsequently, activating receptors for proline induces hair bundles to shorten while tuning discharge to higher frequencies. The result obtained here seems to be coherent with the Watson and Hessinger (1994)'s suggestion that receptors for NANA and proline exhibit antagonistic activities.

Animals exposed to bright light and NP + 10⁻¹⁷ M Ptx, had a mean of 15.29 ± 5.15 (mean ± SE) mpm nematocysts discharged on test probes, while under conditions of dim light and same substances, test probes had a mean of 21.00 ± 8.46 (mean ± SE) of mpm nematocysts adhering to them, both based on n= 7 probes. When animals were exposed to bright light and NP + 10⁻¹⁵ M Ptx, they discharged a mean of 23.88 ± 5.25 (mean ± SE) mpm nematocysts on 8 test probes, while under conditions of dim light and same substances, test probes had a mean of 36.29 ± 6.80 (mean ± SE) (n= 7 probes) mpm nematocysts adhering to them.

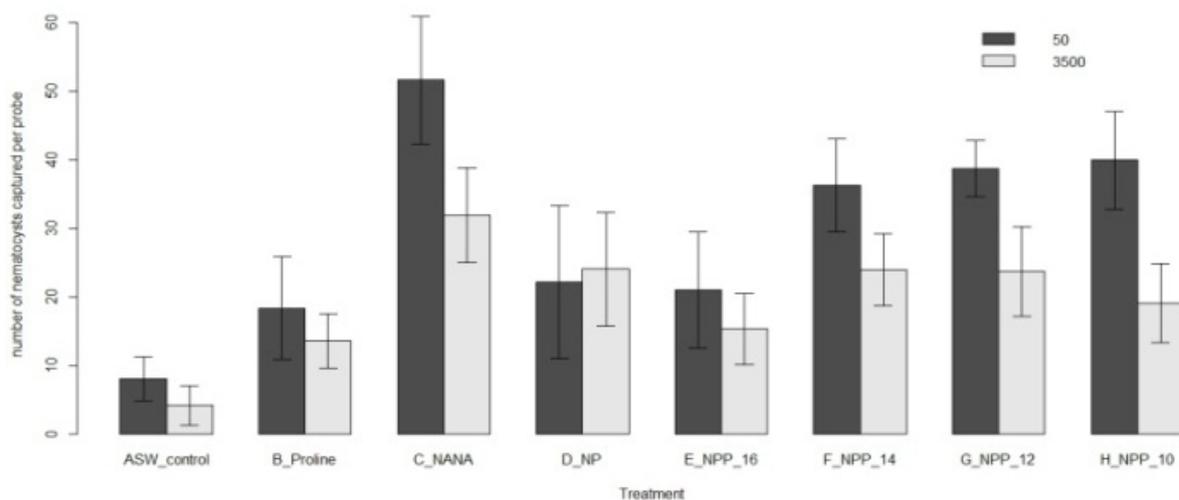


Fig. 7 Bar graph of microbasic p-mastigophore (mpm) nematocysts discharge in *Haliplanella luciae*, in response to pertussis toxin (Ptx), for two different light conditions: bright light (3500 lux) and dim light (50 lux). Y-axis: number of nematocysts captured per probe; x-axis: treatment. Dark grey bar: mean \pm SE of mpm nematocysts discharged in condition of dim light (50 lux). Light grey bar: mean \pm SE of mpm nematocysts discharged in condition of bright light (3500 lux). Experiments were performed as described in §Materials & Methods. Test probes were touched to tentacles after 15 min in ASW alone (designated ASW_control), after 15 min in ASW containing 10^{-8} M proline (B_Proline), after 15 min in ASW containing 10^{-7} M *N*-acetylneuraminic acid (C_NANA), or after 5 min in ASW containing 10^{-7} M NANA followed by 10 min in 10^{-7} M NANA and 10^{-8} M proline (D_NP). The remaining treatments indicate data for specimens exposed for 5 min to 10^{-7} M NANA followed by 10 min to 10^{-7} M NANA, 10^{-8} M proline, and Ptx at a concentration of respectively, 10^{-17} , 10^{-15} , 10^{-13} , and 10^{-11} M.

Animals subjected to bright light and NP + 10^{-13} M Ptx discharged in average 23.67 ± 6.56 (mean \pm SE) mpm nematocysts on test probes (n= 9 probes), while under conditions of dim light and same substances, test probes had a mean of 38.67 ± 4.06 (mean \pm SE)(n= 9 probes) mpm nematocysts. As for the last trial, when animals were exposed to bright light and NP + 10^{-11} M Ptx, test probes had a mean of 19.10 ± 5.76 (mean \pm SE) (n= 10 probes) mpm nematocysts adhering to them, while an incubation under conditions of dim light and same substances, caused the discharged of a mean of 39.89 ± 7.18 (mean \pm SE) mpm nematocysts on 9 test probes. As seen in figure 7, in bright light (and NANA-proline), pertussis toxin seems to have no effect while, in dim light (and NANA-proline), the higher the concentration of pertussis toxin, the higher the firing rate. In this case, the G_i alpha subunit inhibitor seems to affect firing rate. Also this would be consistent with Watson & Hessinger (1994)'s results.

Considering NANA + proline (D_NP in figure 7) and Ptx treatments, a Two-Way ANOVA was performed in order to assess whether an interaction between light and Ptx would have been present. Since the interaction was not significant (p-value: 0.22179, F-statistic: 1.5179 on 1 DF), this term was dropped and a Two-Way ANOVA was performed to test for light and Ptx main effects on nematocyte firing. A highly significant difference was found among light marginal means (p-value: 0.01217, F-statistics: 6.5976 on 1 DF) but not among Ptx marginal means (p-value: 0.51767, F-statistics: 0.4225 on 1 DF). This result suggests that the difference in number of nematocysts discharged by *Haliplanella luciae* is explained by the two levels of light, dim and bright, but that there is no influence of the pertussis toxin (Fig. 8).

Testing for an association between the dependent variable (number of nematocysts discharged), and the explanatory variables (light and pertussis toxin), a multiple regression analysis was performed. Using a quasi-Poisson generalized linear model (GLM), that deals with over-dispersion in count data, the analysis showed that pertussis toxin is not associated with the number of nematocysts released (p-value: 0.5159, t-value: 0.653). Instead, the number of mpm nematocysts discharged is significantly associated with light (p-value: 0.0122, t-value: -2.568). Dispersion parameter for quasi-Poisson family was equal to 14.09534, confirming the over-dispersion in the data.

A One-Way ANOVA was performed to test whether pertussis toxin had effect on nematocysts discharge in both the two different light conditions. In dim light, pertussis toxin had no effect on the firing rate (p-value: 0.1564, F-statistics: 2.0953 on 1 DF). In bright light, since the assumption of normality was not respected, a non-parametric Kruskal-Wallis rank sum test was performed. As expected, no effect of pertussis toxin was found (p-value: 0.8333, chi-squared: 1.4622 on 4 DF).

A regression analysis was then performed to test whether pertussis toxin was associated with firing rate in both the two different light conditions. In bright light, no association between firing rate and Ptx was observed (p-value: 0.669, t-value: -0.431, quasi-Poisson GLM regression analysis), suggesting that the number of nematocysts discharged is not associated with the G_i alpha subunit protein inhibitor. Dispersion parameter for quasi-Poisson family was equal to 13.98232.

As figure 7 shows, the higher the pertussis toxin concentration used in the experiments, the higher the firing rate, in dim light. This would be consistent with the involvement of G_i alpha subunit in light sensitivity, but no association between nematocyte firing rate and pertussis toxin was found (p-value: 0.239, t-value: 1.198, quasi-Poisson GLM regression analysis). Dispersion parameter for quasi-Poisson family was equal to 14.41666.

These results suggest that G_i alpha subunit protein is not involved in light sensitivity.

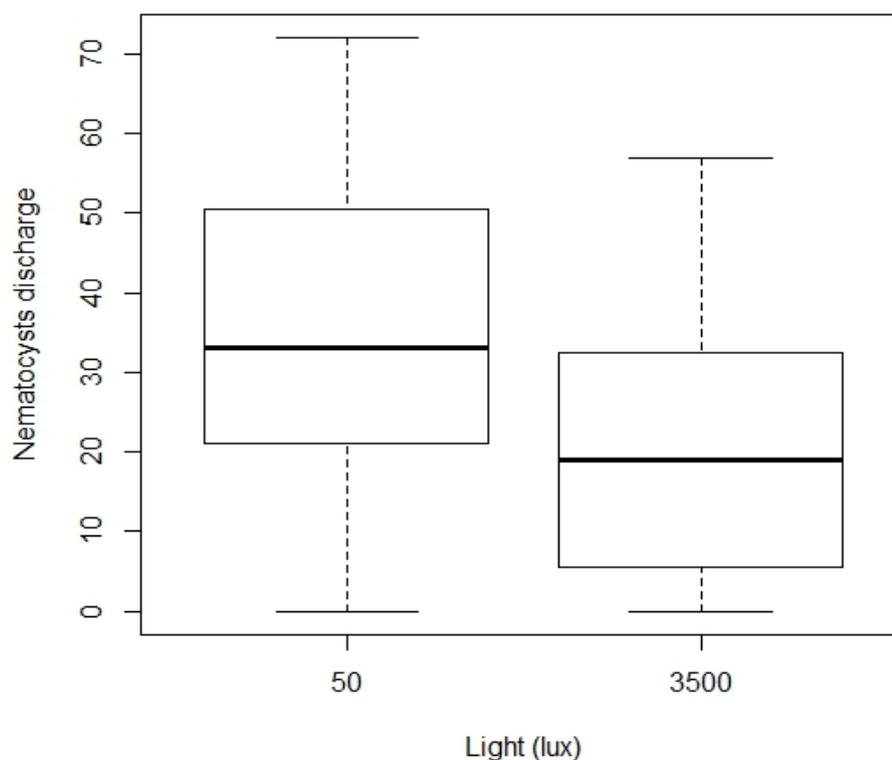


Fig. 8 Boxplot of microbasic p-mastigophore nematocysts discharge, in response to pertussis toxin (Ptx), for two different light conditions: bright light (3500 Lux) and dim light (50 Lux). The experiments were performed as described in §Materials & Methods. Only NP and Ptx treatments were included in the data. Test probes were touched to tentacles after 5 min in ASW containing 10^{-7} M NANA followed by 10 min in 10^{-7} M NANA and 10^{-8} M proline for NP treatment. For the Ptx treatments specimens were exposed for 5 min to 10^{-7} M NANA followed by 10 min to 10^{-7} M NANA, 10^{-8} M proline, and Ptx at a concentration of respectively, 10^{-17} , 10^{-15} , 10^{-13} , and 10^{-11} M. The number of mpm nematocysts discharged was significantly different between dim and bright light (p-value: 0.01217, F-statistics: 6.5976 on 1 DF, Two-Way ANOVA) but no significant effect of pertussis toxin was observed.

4.3 Transgenic *Hydra*

4.3.1 *Hydropsin5* promoter construct development

The amplification of the longest sequence upstream *hydropsin5* gene, including PAX6 domain and probably other regulatory regions, was the first step of the promoter construct development. Several primers were built and many different PCR parameters and cycles were tried, in order to obtain a unique and intense target DNA band, putatively correspondent to the promoter region, in the agarose gel electrophoresis analysis.

The amplified longest sequence, on which all the next work was done, was 1148 bp long. In order to add restriction sites to both promoter 5' and 3' ends, several primers were built, many attempts were done, and numerous PCR reactions were tried. Primer dimers, multiple bands, and the amplification of non-target bands were some of the troubles encountered during this step of construct development.

To solve these problems, optimizing the PCR reaction, temperature gradient PCRs were done, different dilutions of template, and different amounts of primers were used. When finally the right PCR cycle was found and the reaction was correctly set, the promoter region was successfully cloned and sequenced to confirm the presence of the restriction sites at both 5' and 3' ends (Fig. 10).

The ligation of the promoter region into pHyVec13 vector was not straightforward, since it was difficult to completely digest the vector and, at the same time, obtain a high concentration to make the ligation reaction doable. After several attempts, the double digestion of pHyVec13 was performed in a sequential step to improve the quality of digestion. To maximize the yield and obtain a higher concentration, the digested vector, excised from a 1% w/v agarose gel, was then eluted in pre-heated water. Several combinations of temperature and incubation time were tried to perform the ligation reaction. It was discovered that a ligation reaction overnight at room temperature worked best. After having transformed *E. coli* cells, a double digestion and a PCR reaction were performed on the plasmids to have a raw confirmation of the successful ligation. Only after having sequenced the resulting plasmids with both Sallup-F2 & GFP-R primers, to confirm that the fusion between the promoter and GFP of pHyVec13 was correct, the successful

development of the construct was confirmed. Plasmid sequences were cleaned-up and were searched for the following GFP sequence: ATG GCC GAT GAT GAA GTT GCC GCC CTC GCT GCA GCC CCG GTA GAA AAA ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG GGT GAA GGT GAT GCA ACA TAC GGA AAA (yellow: PstI restriction site; green: site where the GFP-R primer binds).

In the plasmid sequences, PstI site correctly added by means of PstI-R2 reverse primer, sequence encoding the amino acids upstream of the PstI site in GFP (ATG GCC GAT GAT GAA GTT GCC GCC CTC G), and sequence encoding the amino acids downstream of the PstI site in GFP, were all present. Figure 11 shows that GFP of pHyVec13 was correctly fused with hydropsin5 promoter region, confirming that the promoter construct was successfully engineered.

4.4 Hydra gene gun

4.4.1 A promising result of hydropsin5 expression

Do nematocyte sensory cells express hydropsin5 gene?

The promoter construct should allow us to test this hypothesis and answer this question.

After having amplified the upstream region of hydropsin5, a gene gun was used to transiently transfect *Hydra* polyps by particle bombardment.

Professor Todd Oakley used *Hydra* gene gun technique at Irvine to preliminarily test the plasmid. The gene gun shoots tiny gold particles into the polyps. If they stick in a cell that expresses the transcription factors that guide expression of the insert, then GFP will be made. The gene gun does not show everywhere hydropsin5 gene is expressed, it just shows that the construct works. There was a promising result in one animal, with GFP expression appearing as a bright spot at the base of a small polyp (Fig. 9).

However, the dsRed positive control never showed up, so this test was inconclusive and needs to be repeated.



Fig. 9 GFP expression (bright spot) in one small polyp of *Hydra magnipapillata* after having performed the transient transfection of *Hydra* by particle bombardment. Photo by Todd Oakley.

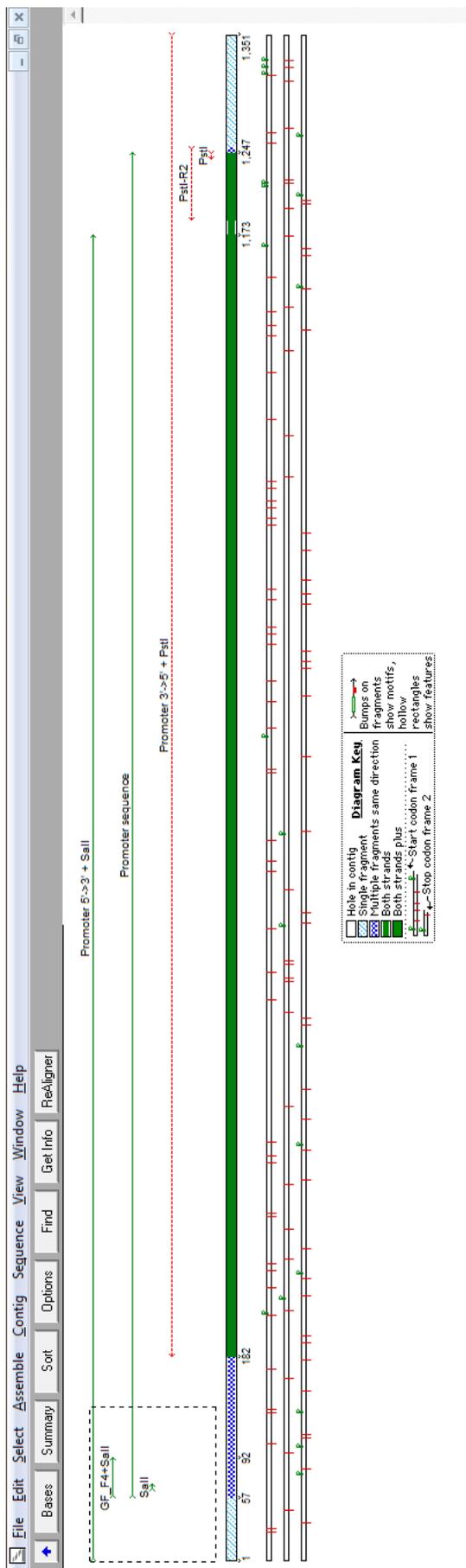


Fig. 10a Overview map showing contiguous of hydropsin5 regulatory region sequence, amplified using both GF_F4+Sall & PstI-R2 primers in order to add Sall and PstI restriction site. Sequencing was performed by UC Berkeley DNA Sequencing Facility using Sallup-F2 & GFP-R primers (see Table 1 in \$Materials & Methods). Sequences were assembled using Sequencher 5.0 (Parameters: Min overlap= 6, Min match= 90%).

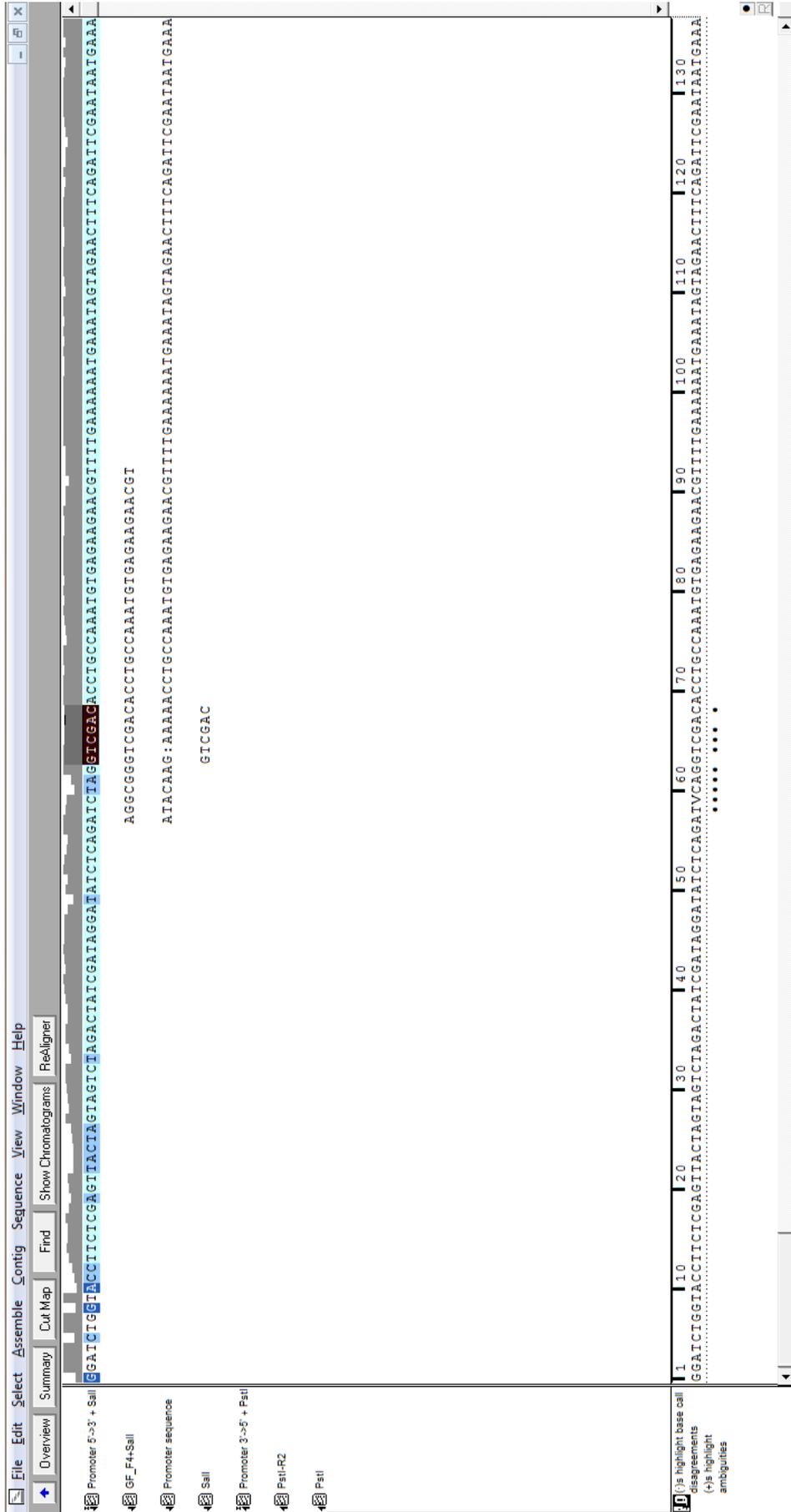


Fig. 10b Hydropsin5 regulatory region sequence showing the addition of Sall restriction site to 5' end by means of GF_F4+Sall forward primer (see Table 1 in §Materials & Methods). Sall site correctly added to the promoter region is highlighted in black. Sequencing was performed by UC Berkeley DNA Sequencing Facility using Sailup-F2 & GFP-R primers (see Table 1 in §Materials & Methods). Sequences were assembled using Sequencher 5.0 (Parameters: Min overlap= 6, Min match= 90%).

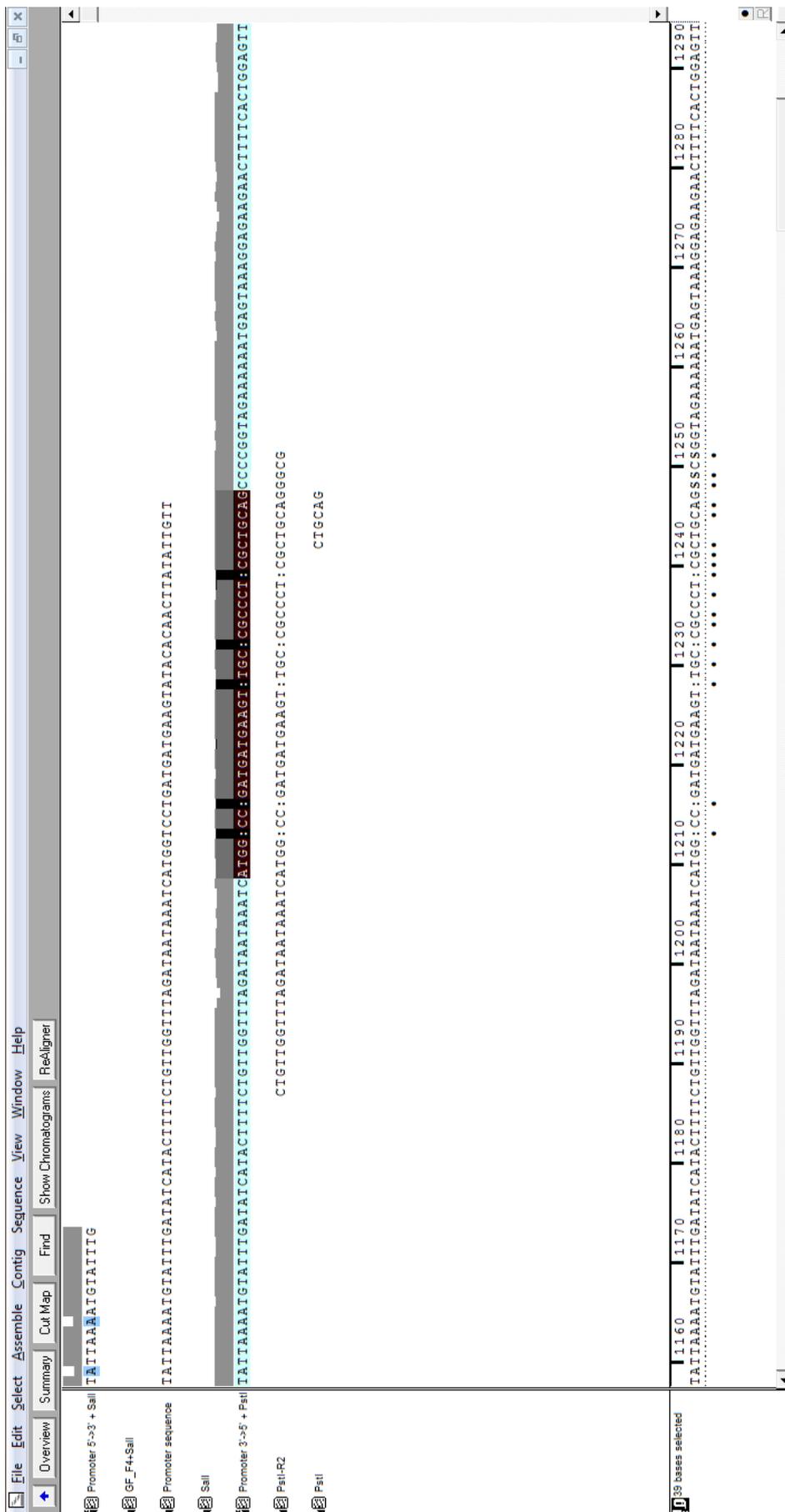


Fig. 10c Hydropsin5 regulatory region sequence showing the addition of both PstI restriction site and sequence encoding the amino acids upstream of the PstI site in GFP to 3' end, by means of PstI-R2 reverse primer (see Table 1 in §Materials & Methods). PstI site and sequence encoding the amino acids upstream of the PstI site in GFP are highlighted in black. Sequencing was performed by UC Berkeley DNA Sequencing Facility using Sallup-F2 & GFP-R primers (see Table 1 in §Materials & Methods). Sequences were assembled using Sequencher 5.0 (Parameters: Min overlap= 6, Min match= 90%).

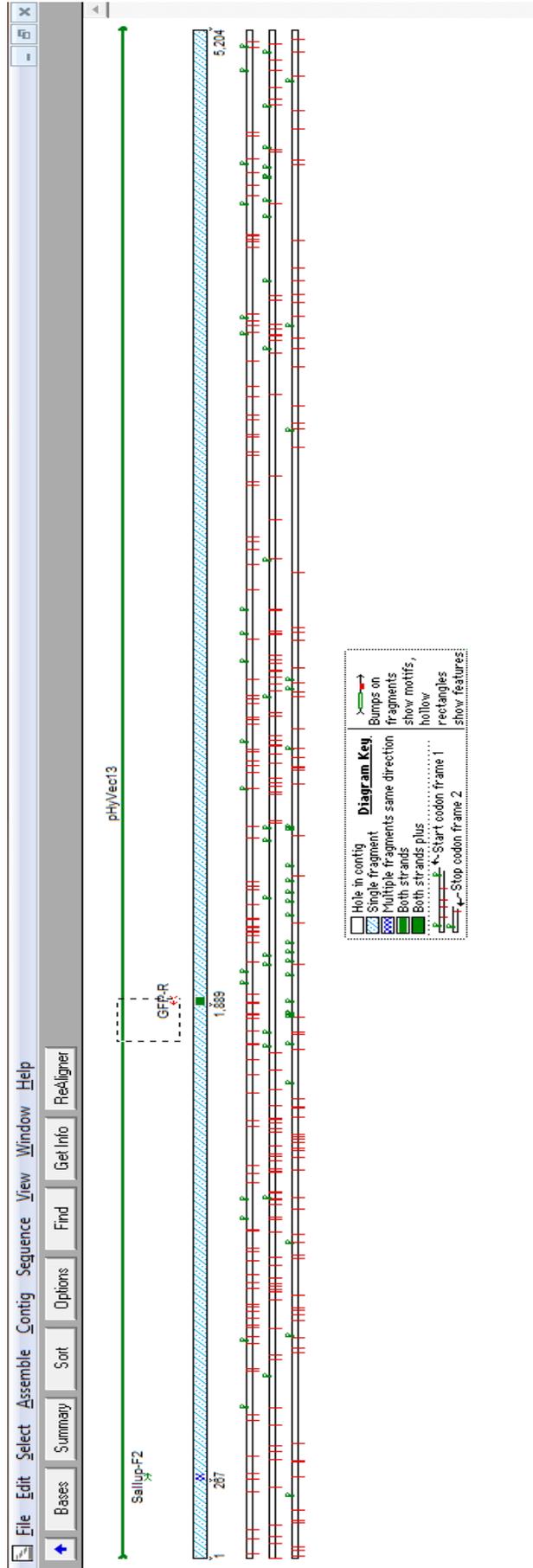


Fig. 11a Overview map of pHyVec13 vector sequence with both Sallup-F2 and GFP-R primers used for sequencing (see Table 1 in \$Materials & Methods). Sequences were assembled using Sequencher 5.0 (Parameters: Min overlap= 20, Min match= 90%).

5. DISCUSSIONS

5.1 No evidence that pertussis toxin ablates *Hydra* photobehaviour

5.1.1 Preliminary suggestion that G_i alpha subunit is not involved in light sensitivity

The main question in this part of the study was whether the G_i alpha subunit inhibitor, pertussis toxin, could have an effect on light sensitivity, demonstrating a possible involvement of the G_i alpha subunit protein in the phototransduction pathway of *Hydra magnipapillata*.

Pertussis toxin has been widely used as a reagent to characterize the involvement of heterotrimeric G-proteins in signaling. This toxin catalyses the ADP-ribosylation of specific G alpha subunits of the G_i proteins family, and this modification prevents the occurrence of the receptor-G-protein interaction.

I have shown here that, across various Ptx concentrations, where the *Hydra* polyps remained responsive to mechanical stimuli, they also remained responsive to bright light, showing the typical contraction response observed and described by Passano & McCullough (1963, 1964), suggesting that G_i alpha subunit protein is not involved in photoresponse. Therefore, possibly, there is no light inhibition with pertussis toxin, unless the concentration used in the experiments was too high or the duration of exposure too long as to kill the *Hydra* polyps tested. Since no similar previous studies had ever used pertussis toxin to test the photobehaviour in *Hydra*, there were no baselines available as starting points for the experiments. Consequently, the result obtained in this study can be only considered as a preliminary inspection, since we had no way to really know if we used the right concentration of pertussis toxin.

This result clears some space for other potential explanations. First, despite having tried a wide range of pertussis toxin concentrations and durations of exposure, there could likely have been one particular concentration that was not tried in the experiments, which would have ablated *Hydra* photobehaviour, without interrupting responsiveness.

The choice of the initial Ptx concentration of 1000 ng/mL was dictated by the results obtained in the study by New *et al.* (1998). In this study the sea

anemone *Actinia* spp. was examined for the presence of heterotrimeric G-proteins. The bacterial toxin produced by *Bordetella pertussis* was able to adenosine diphosphate (ADP)-ribosylate a membrane-associated protein with a molecular mass of approximately 39 kDa, suggesting the presence of G alpha subunits. In support of this finding, mastoparan, a direct activator of G-proteins, induced a rapid tentacle contraction in a dose-dependent manner. Given that Ptx can ADP-ribosylate a membrane associated protein in sea anemones, they attempted to abolish the effect of mastoparan by pretreating the animals with the toxin. Results from one or two animals indicated that the response to mastoparan had been abolished by treating the animal with a Ptx concentration of 1000 ng/mL (New *et al.*, 1998).

Second, G_i alpha subunit could be involved in both mechano and light sensitivity, so that all responses were knocked out by the G_i alpha subunit inhibitor, preventing us to distinguish between the two types of sensitivity, and infer whether G_i protein is involved in light sensitivity.

These were the main reasons for which we decided to perform more analysis, moving to *Haliplanella luciae* on which numerous significant studies had already been done.

5.2 Influence of pertussis toxin on nematocysts firing in the presence of NANA and Proline

5.2.1 Additional suggestions that G_i alpha subunit is not involved in light sensitivity

As settled in the §introduction, *N*-acetylated sugars, like NANA, tend to increase the number of nematocysts fired in *Haliplanella luciae*, whereas proline decreases firing. Supposing that *N*-acetylated sugars receptors may stimulate G_s alpha subunit while proline receptors stimulate G_i alpha subunit, it can be stated that they modulate adenylate cyclase in opposite directions. Since pertussis toxin is a G_i protein inhibitor, it blocks the effect of proline on vibration-dependent nematocysts discharge, making proline less and less detectable by the sea anemone. This finding by Watson and Hessinger (1994) acted as our baseline for this part of the study.

In this study we tested whether light sensitivity involves G_i alpha subunit protein, using pertussis toxin. We first found that there is no interaction between light and pertussis toxin in the regulation of nematocyte firing, which means that the effect of pertussis toxin on firing rate does not change as the two levels of light vary. We found that the difference in number of nematocysts discharged by *Haliplanella* is explained by the two levels of light, dim and bright. On the other hand, there is no significant association with and influence of pertussis toxin. We already know, from a previous study by Katia Jindrich, that *Halipanella* shows a significant difference in firing rate between bright and dim light, but this result includes the additional information that Ptx does not seem to influence nematocyte firing.

This result, if coupled with previous studies in which the same result had been obtained, would suggest that light modulation of cnidocytes could be an ancestral feature of Cnidaria. If we consider that chemo and photoreception share the same molecular components as G-protein, adenylate cyclase, and cAMP signaling pathway, this result allows us to hypothesize that G_i alpha subunit is not involved in photo response.

If NANA acts as a stimulant through a G_s alpha subunit pathway and proline acts as an inhibitor through G_i alpha subunit pathway, as suggested by Watson and Hessinger (1994), the higher number of nematocysts in NANA trial and the counteracting effect in NANA + Proline trial, would confirm Watson and Hessinger (1994)'s results. Possibly NANA and proline can cancel each other out, but it is unclear how nematocyte firing was higher in bright light than in dim light (see Fig. 7, D_NP treatment). This result casts doubts on the common finding of a higher firing rate in dim light than in bright light, as observed by D. Plachetzki, C. Fong, D. Gold, and K. Jindrich in their unpublished experiments on Cnidaria. On the other hand, since all the other trials of this study support this common finding, we supposed this anomaly was caused by the low number of replications of the experimental condition and by low light levels in the experimental room, making distance between probe and tentacle harder to assess (see §Materials & Methods for nematocysts capture assay protocol).

In dim light we hypothesized that the higher the concentration of pertussis toxin used in the trials, the higher the firing rate. This would suggest that pertussis toxin affects firing rate, blocking the effect of proline on vibration-dependent

nematocysts discharge. This would also be consistent with Watson & Hessinger (1994)'s results, confirming the idea that Ptx inhibits proline detection by the sea anemone. The result obtained here follows the pattern described by Watson and Hessinger (1994), the more Ptx used in the trials, the more the results look like the trial with NANA alone, even though there was proline acting in the opposite direction. However, no significant association between nematocyte firing rate and pertussis toxin was found in dim light. This suggests that G_i alpha subunit could not be involved in the photoresponse.

In bright light we would have expected that the higher the concentration of pertussis toxin used in the trials, the higher the firing rate but with a lower number of nematocysts discharged, if compared with dim light trials. No significant association was found between number of nematocysts discharged and pertussis toxin. Moreover, looking at figure 7, it seems that bright light could overwhelm the NANA + Proline signal overriding Ptx effects, since the number of nematocysts discharged did not show an increase as shown in dim light. Perhaps this is an indication of the involvement of a G_s alpha subunit protein in the photoresponse.

Since NANA acts through G_s alpha subunit pathway, it seems that high illuminance could exhaust the pathway making NANA less detectable by the animal (Fig. 12). At the organismal level, both *N*-acetylated sugars and light modulate the propensity for nematocysts to discharge. Now, from a molecular perspective, light and sugar also seem to signal through similar pathways. Considering that there is no association between nematocysts discharge and pertussis toxin both in dim and bright light, and that in bright light the number of nematocysts discharged remains unvaried as the pertussis toxin increases, we could hypothesize that both NANA and light act through G_s alpha subunit protein pathway, acting both as stimulant. This would explain the higher number of nematocysts discharged in dim light in comparison with bright light. A high illuminance could exhaust the pathway taking advantage of the chemoresponse, making NANA less detectable, and consequently making the sea anemone fires a lower number of nematocysts. Therefore, the different levels of light would modulate nematocyte firing, with a predominance of photosensory as the environmental conditions become brighter.

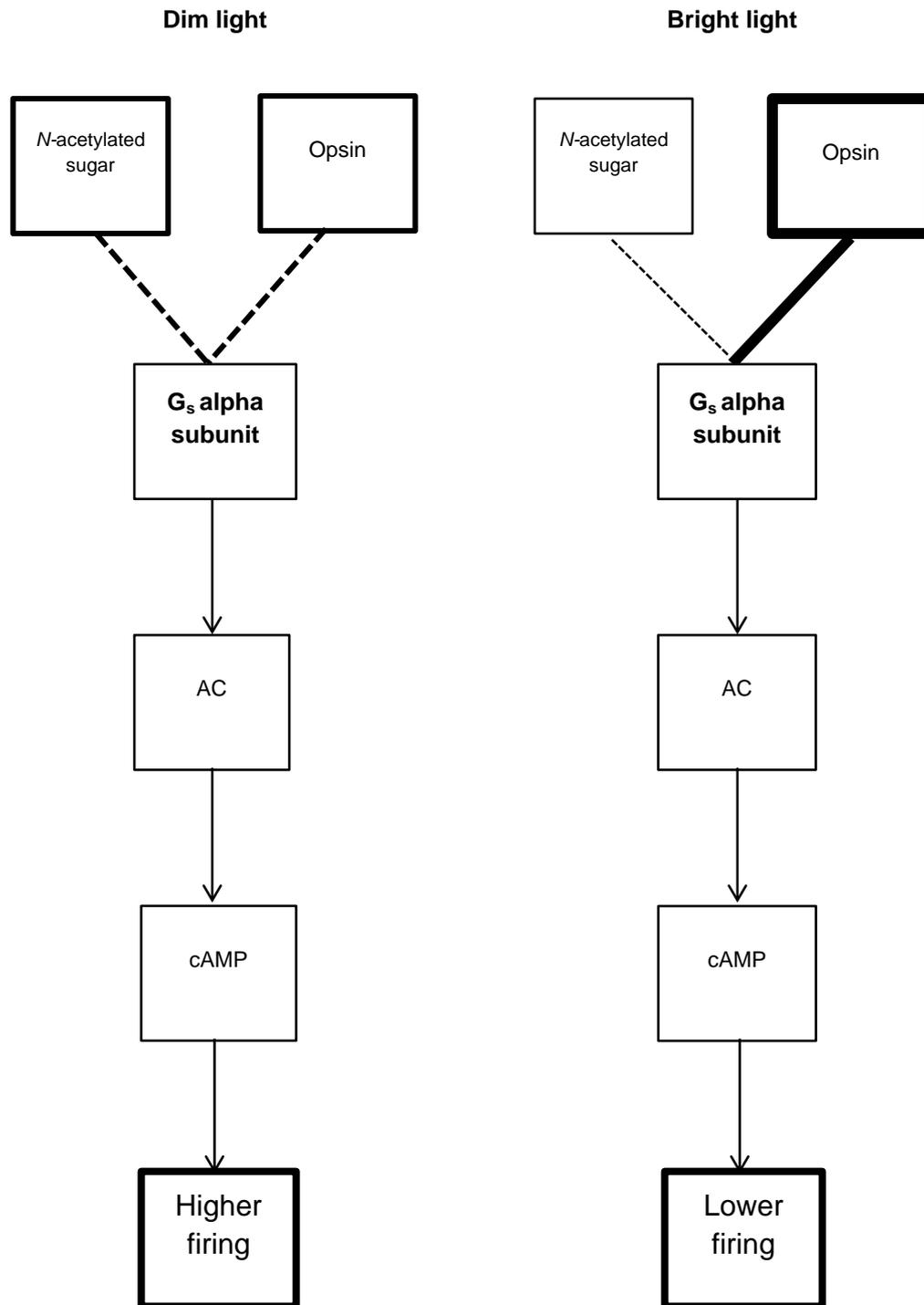


Fig. 12 Probable molecular mechanism for the influence of light and sugar on the discharge level. The general function of G_s alpha subunit is to activate adenylate cyclase (AC), which, in turn, produces cAMP, which, in turn activates cAMP-dependent protein kinase. The different levels of light would modulate nematocyte firing, with a predominance of photosensory over chemosensory as the environmental condition become brighter. We hypothesized that both *N*-acetylated sugars and light modulate the tendency for nematocysts to discharge binding to the same G-protein. A high stimulation by bright light would strongly reduce the effect of the sugar since G_s protein is already sequestered by the high stimulating light. This would make *N*-acetylated sugar less detectable, consequently making the sea anemone fires a lower number of nematocysts in comparison to dim light. Thus, in dim light, the chemoreception would not be overwhelmed by the luminous stimulus.

The interactions receptor-G-protein can have various levels of specificity; a situation of greatest specificity in which the receptor couples only with a certain G-protein, or a situation in which a receptor can bind to two different G-proteins, or, as hypothesized in this study, two or more receptors bind to the same G-protein. In this case the high stimulation by bright light would strongly reduce the effect of NANA since G_s alpha subunit protein is already sequestered by the high stimulating light, while the low stimulation by dim light would leave more G_s alpha subunit protein available for NANA effect, without overwhelming the chemoreception. This would be the same mechanism that protects neurons from excessive inhibitory or stimulatory stimuli.

As described in the §Introduction, *N*-acetylated sugars are present on the prey coating and proline is present in the hemolymph of crustaceans. The approaching prey generates a local increase of *N*-acetylated sugar causing a sensitization of the mechanoreceptors to the corresponding lower frequencies. Once the prey is captured and wounded, proline is released shifting the VSMs tuning-frequency to higher frequencies than those generated by the prey, preventing additional discharge of nematocysts into a prey already netted (Watson and Hessinger, 1994). A biological explanation could be also associated with nematocyte photosensitivity. Since cnidarian diet is mostly composed of zooplankton, which are most active at dusk and dawn, dim light could be a useful indicator of when prey is likely to be most active. This would also maximize the efficiency of discharge, since it is really energetically expensive to produce nematocysts, avoiding to fire when the probability of catching a prey is low.

Thus, dim light can be considered as an excitation signal that, acting through a G_s alpha subunit pathway, indicates to the sea anemone when it is convenient to discharge more nematocysts. Considering this hypothesis, bright light could instead favor transition to rely on photosynthetic symbionts. The high luminous intensity could exhaust the G_s alpha subunit protein pathway, causing less detection of the *N*-acetylated sugar by the animal, acting as an indicator for the sea anemone that it is not the right moment to have a higher nematocysts discharge since prey is likely to be most active at dusk and dawn. This would be consistent with a lower amount of nematocysts discharged, as observed in this study, independently of the inhibition of the G_i alpha subunit protein by pertussis toxin.

After these considerations, coupling these results with those previously obtained with *Hydra*, it can be concluded that G_i alpha subunit protein is not involved in photoresponse, suggesting a possible involvement of a G_s alpha subunit protein in photoresponse. This last hypothesis is supported by the involvement of G_s alpha subunit protein in box jellyfish phototransduction cascade but it would be rejected by the co-localization of G_i alpha subunit and opsin in *Hydra* sensory cells, as Plachetzki and collaborators found (Plachetzki *et al.*, unpublished). Moreover, it is not impossible that different opsins use different G-proteins, since there are more than one opsin, and that these opsins are not all expressed in the same location.

Further studies are required to better understand how these complex mechanisms work.

5.3 Hydropsin5 promoter construct development

5.3.1 A promising result of hydropsin5 expression

The main question for this first part of the study was whether nematocyte sensory cells express opsin gene.

Owing to time constraints, my work was limited to the development of the hydropsin5 promoter construct. Professor Todd Oakley preliminary tested the construct obtaining a promising result of opsin expression. This raises hopes for the useful utilization of the construct to answer the question we addressed at the beginning of the study.

Plachetzki and coworkers already discovered that cells expressing a cnidarian opsin were distributed in neurons throughout *Hydra magnipapillata*, and they were especially concentrated in the oral hypostome and in cells associated with nematocytes (Plachetzki *et al.*, 2007). Once this construct will be confirmed, it will be injected into *Hydra magnipapillata* embryos. The embryos will be allowed to develop, and the mature organisms will show the green fluorescent protein anywhere hydropsin5 is expressed.

This outcome will allow us to investigate whether nematocyte sensory cells express hydropsin5 gene used in the promoter construct.

5.4 Conclusion

In conclusion, I engineered a hydroopsin5 gene promoter construct useful to test whether nematocyte sensory cells express opsin gene.

This study contributed to confirm that bright light decreases the tendency for nematocytes to discharge in *Haliplanella luciae*, and probably in all Cnidarians, as obtained in previous studies, suggesting that light modulation of cnidocytes may be an ancestral feature of Cnidaria.

Experimentally, I found no evidence that pertussis toxin ablates *Hydra magnipapillata* photobehaviour, suggesting that G_i alpha subunit protein is not involved in photoresponse. I found also no significant association between pertussis toxin and nematocyte firing in *Haliplanella luciae* both in conditions of dim and bright light, suggesting that, if coupled with *Hydra* results, G_i alpha subunit protein is not involved in photoresponse.

I found that bright light seems to overwhelm NANA + proline signal. Thus, there is an evidence for a prevalence of photoreception over chemoreception tending toward conditions of bright light. This finding may suggest the involvement of a stimulatory G-protein in *Haliplanella luciae* phototransduction pathway.

While nematocyte chemo- and mechano-sensitivity have been extensively studied, further research is necessary to better understand what an ancestral phototransduction cascade looked like, and how opsin-based phototransduction acts to regulate nematocyte discharge.

6. REFERENCES

- Boetgger A, Alexandrova O, Cihala M, Schade M, Herold M, David CN. (2002).** GFP expression in *Hydra*: lessons from the particle gun. *Dev Genes Evol*, **212**: 302–305.
- Bokoch, G.M., Katada, T., Nortup, J.K., Hewlett, E.L. and Gilman, A.G. (1983).** *J. Biol. Chem.*, **258**: 2072-2075.
- Bridge, D., C. W. Cunningham, R. DeSalle, and L. W. Buss. (1995).** Class-level relationships in the phylum Cnidaria: Molecular and morphological evidence. *Molec. Biol. Evol.*, **12**: 679-689.
- Codina, J., Hildebrandt, J., Iyengar, R., Birnbaumer, L., Sekura, R.D. and Manclark, C.R. (1983).** *Proc. Natl. Acad. Sci. U.S.A.*, **80**: 4276-4280.
- D.C. New, N.J. McGaughey, Y.H. Wong, J.T.Y. Wong. (1998).** Evidence for heterotrimeric G proteins in sea anemones. *Molecular Marine Biology and Biotechnology*, **7(1)**: 31-38. doi: 10.1016/j.ympbev.2011.10.008.
- Fautin D.G. (2009).** Structural diversity, systemic, and evolution of cnidae. *Toxicon*, **54**: 1054-1064.
- Gierer A, Berking S, Bode H, David CN, Flick K, Hansmann G, Schaller H, Trenkner E. (1972).** Regeneration of *Hydra* from reaggregated cells. *Nat New Biol*, **239**: 98–101.
- Gitter, A.H., Oliver, D., Thurm, U. (1994).** Calcium- and voltage-dependence of nematocyst discharge in *Hydra vulgaris*. *J. Comp. Physiol.*, **A 175**: 115-122.
- Golz, R. (1994).** Apical surface of hydrozoan nematocytes: structural adaptations to mechanosensory and exocytotic functions. *J. Morphol.*, **222**: 49-59.
- Halstead, BW. (1988).** Poisonous and Venomous Marine Animals of the World. *2nd Ed. The Darwin Press, Inc. Princeton, NJ.*
- Harms, M.** Undergraduate research thesis.

- Jindrich, K.** (2011). Master's degree research thesis.
- Koyanagi M., Takano K., Tsukamoto H., Ohtsu K., Tokunaga F., Terakita A.** (2008). Jellyfish vision starts with cAMP signaling mediated by opsin-G_s cascade. *Proc. Natl Acad. Sci. USA*, **105**: 15 576–15 580.
- Krayesky, S.L., Mahoney, J.L., Kinler, K.M., Peltier, S., Calais, W., Allaire, K. and Watson, G.M.** (2010). Regulation of spirocyst discharge in the model sea anemone *Nematostella vectensis*. *Mar. Biol.*, **157**: 1041-1047.
- Mariscal, R. N., Conklin, E.J. and Bigger, C.H.** (1977). The ptychocyst, a major new category of cnida used in tube construction by a cerianthid anemone. *Biol. Bull.*, **152**: 392-405.
- Mire-Thibodeaux, P. and Watson, G.M.** (1994). Morphodynamic hair bundles arising from sensory cell/ supporting cell complexes frequency-tune nematocyst discharge in sea anemones. *J. Exp. Zool.*, **268**: 282–292.
- Nütcher, T., et al.** (2006). Nanosecond-scale kinetics of nematocyst discharge. *Curr. Biol.*, **16**: R316-R318.
- Özbek, S., Balasubramanian, P.G. and Holstein, T.W.** (2009). Cnidocyst structure and the biomechanics of discharge. *Toxicon*, **54**: 1038-1045.
- Park Eunji, Hwang Dae-Sik, Lee Jae-Seong, Song Jun-Im, Seo Tae-Kun, Won Yong-Jin.** (2012). Estimation of divergence times in cnidarian evolution based on mitochondrial protein-coding genes and the fossil record. *Molecular Phylogenetics and Evolution*, **62 (1)**: 329-345.
- Passano, L. M. & McCullough, C. B.** (1962). The light response and the rhythmic potentials of *Hydra*. *Proc. Natl Acad. Sci. USA*, **48**: 1376–1382.
- Passano, L. M. & McCullough, C. B.** (1963). Pacemaker hierarchies controlling the behaviour of hydras. *Nature*, **199**: 1174–1175.
- Passano, L. M. & McCullough, C. B.** (1965). Co-ordinating systems and behaviour in hydra. II. The rhythmic potential system. *J. Exp. Biol.*, **42**: 205–231.

- Plachetzki, D. & Oakley, T. H. (2007).** Key transitions during the evolution of animal phototransduction: novelty, 'tree-thinking,' co-option, and co-duplication. *Integr. Comp. Biol.*, **47**: 759–769.
- Plachetzki, D. C., Degnan, B. M. & Oakley, T. H. (2007).** The origins of novel protein interactions during animal opsin evolution. *PLoS ONE*, **2**: e1054.
- Plachetzki, D.C., Fong, C.R. and Oakley, T.H. (2010).** The evolution of phototransduction from an ancestral cyclic nucleotide gated pathway. *Proc. Roy. Soc. B. published online March 15, 2010.*
- Purcell, J.E. (1984).** The functions of nematocysts in prey capture by epipelagic siphonophores (Coelenterate, Hydrozoa). *Biol. Bull.*, **166**: 310-327.
- Schuchert, P. (1993).** Phylogenetic analysis of the Cnidaria. *Z. Zool. Syst. Evolut.-forsch.*, **31**: 161-173.
- Scrutton, C. T. (1979).** Early Fossil Cnidarians. Pp. 161-207 in M. R. House (ed.) *The Origin of Major Invertebrate Groups. Academic Press, London.*
- Stanley, G.D. (2003).** The evolution of modern corals and their early history. *Earth-Sci. Rev.*, **60**: 195-225.
- Terakita A. (2005).** The opsins. *Genome Biol.*, **6**: 213.
- Thorington, G.U. and Hessinger, D.A. (1988).** Control of discharge: I. Evidence for two classes of chemoreceptor. *Biol. Bull.*, **174**: 163–171.
- Thurm, U., Brinkmann, M., Golz, R., Holtmann, M., Oliver, D. and Sieger, T. (2004).** Mechanoreception and synaptic transmission of hydrozoan nematocytes. *Hydrobiologia*, **530/531**: 97–105.
- Veron, J., Odorico, D., Chen, C., Miller, D. (1996).** Reassessing evolutionary relationships of scleractinian corals. *Coral Reefs*, **15**: 1–9.
- Veron, J.E.N. (1995).** Corals in Space and Time: The Biogeography and Evolution of the Scleractinia. *Cornell Univ. Press, Ithaca.*

- Watson, G. M.** (1988). Ultrastructure and cytochemistry of developing nematocysts. Pp. 143-164 in D. A. Hessinger and H. M. Lenhoff (eds.) *The Biology of Nematocysts*. Academic Press, San Diego and other cities.
- Watson, G.M. and Hessinger, D.A.** (1989). Cnidocytes and adjacent supporting cells form receptor- effector complexes in anemone tentacles. *Tissue&Cell*, **21(1)**: 17-24.
- Watson, G.M. and Hessinger, D.A.** (1992). Receptors for N-Acetylated sugars may stimulate adenylate cyclase to sensitize and tune mechanoreceptors involved in triggering nematocyst discharge. *Exp. Cell. Res.*, **198**: 8-16.
- Watson, G.M. and Hessinger, D.A.** (1994). Antagonistic frequency tuning of hair bundles by different chemoreceptor regulates nematocyst discharge. *J. exp. Biol.*, **187**: 57–73.
- Werner, B.** (1973). New investigations on systematics and evolution of the class Scyphozoa and the phylum Cnidaria. *Publ. Seto Mar. Biol. Lab. (Proceedings of the Second International Symposium on Cnidaria)*, **20**: 35-61.
- Westfall, J.A.** (2004). Neural pathways and innervation of cnidocytes in tentacles of sea anemones. *Hydrobiologia*, **530/531**: 117-121.