AFRL-AFOSR-VA-TR-2020-0159

Optical, biochemical, and molecular characterization of new light producing systems from marine and terrestrial organisms, with emphasis on violet/blue

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06/15/2020
Final Report

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Air Force Research Laboratory
AF Office Of Scientific Research (AFOSR)/ RTB2
Arlington, Virginia 22203
Air Force Materiel Command

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*
31-08-2020

2. REPORT TYPE
Final Performance

3. DATES COVERED *(From - To)*
15 Mar 2017 to 14 Mar 2020

4. TITLE AND SUBTITLE
Optical, biochemical, and molecular characterization of new light producing systems from marine and terrestrial organisms, with emphasis on violet/blue

5a. CONTRACT NUMBER

5b. GRANT NUMBER
FA9550-17-1-0189

5c. PROGRAM ELEMENT NUMBER
61102F

6. AUTHOR(S)
Dimitri Deheyn

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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LA JOLLA, CA 92093-1500 US

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AF Office of Scientific Research
875 N. Randolph St. Room 3112
Arlington, VA 22203

10. SPONSOR/MONITOR'S ACRONYM(S)
AFRL/AFOSR RTB2

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AFRL-AFOSR-VA-TR-2020-0159

12. DISTRIBUTION/AVAILABILITY STATEMENT
A DISTRIBUTION UNLIMITED: PB Public Release

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This report compiles data about the mechanisms of light production in the marine worm Chaetopterus, as well as some preliminary data on the extraction and purification of biochromophores with interesting UV absorbance and Stokes shift. The data on the marine worm identified the pathway by which sustained energy is provided to the chromophore to make long glows (lasting >72hrs). This process involves a very efficient ferritin that is able to perform reduction that can be induced by blue light (including the blue bioluminescence of the mucus), making this system a self-powered. We were able to identified the residues in the worm ferritin that makes it so efficient, and able to increase efficiency of human ferritin. The blue remote

15. SUBJECT TERMS
light producing, bioluminesence, photoprotein

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			GIMM, JUNG-HWA
Unclassified	Unclassified	Unclassified	UU		19b. TELEPHONE NUMBER *(Include area code)* 703-696-9542

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

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FINAL TECHNICAL REPORT

(3/15/2017-3/14/2020)

1. COVER SHEET

Program: AFOSR, Natural Materials and Systems Program

Project title: Optical, biochemical, and molecular characterization of new light producing systems from marine and terrestrial organisms, with emphasis on violet/blue and red/near IR emissions and large Stokes shift

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Lab Assistant:
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Agreement: FA95550-17-1-0189

Start date: Mar. 15, 2017
End date: Mar. 14, 2020

Changes in research objectives, if any: None

Changes in AFOSR program manager, if any: Yes
Yr1: Dr. Hugh De Long/ Dr. Sofi Bin Salamon
Yr2-Yr3: Dr. Jung-Hwa (Aura) Gimm

Extensions granted or milestones slipped, if any: None

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2. OBJECTIVES

This research program aims at finding **novel biochromophores and light-producing compounds**, identify **new chemistries leading to light production**, with the end goal to facilitate the use of light in applied biotechnology and (bio)engineering. This research program specifically addressed the biochemical pathways leading to light production in luminous organisms for which the luminous biochemistry remains unknown, and to extract biochromophores that show large Stokes shifts in fluorescence (thus not necessarily luminous species). The research has focused on species known to have unique light production and spectral characteristics that were identified in the previous AFOSR funded project.

In this particular effort, we have focused our work on the luminous marine worm *Chaetopterus dewysee*, which is characterized by a long-lasting light production associated with secreted mucus, through a process that is independent of oxygen. This combined several characteristics that clearly defy the conventional mechanisms of light production described thus far. Another focus was on organisms that were shown to have large Stokes shift when observed under fluorescence. Having a large Stokes shift (or band gap) is attractive to increase the means of multiplex detection in spectral applications.

More specifically, the key objectives of this program were to:

(1) Identify the source of renewed energy in the luminous mucus of *Chaetopterus*;

(2) Identify the photoprotein/biochromophore responsible for light production in *Chaetopterus*;

(3) Characterize the large Stokes-shift fluorescence of marine and terrestrial samples and work on extracting, isolating, and purifying these chromophores.

Overall, the objectives of this program have been reached for all the various aspects of the proposed research: we have characterized the likely mechanism that is responsible for the long-glow in *Chaetopterus* mucus, we have established a methodology that allowed us to extract a protein that, when added to the mucus stimulates the light production (thus a promising biochromophore or photoprotein), and last, we have made good progress on extracting chromophores with high UV absorbance (and stable coloration in some cases) from some deep-sea invertebrates but also plant seeds.

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3. EXECUTIVE SUMMARY

This research program had very well directed goals but very challenging ones, because addressing molecular and biochemical processes from complex and especially unknown mechanisms from species that are not very well studied and for which no genome and no standard procedure has been established, for which specimen availability is scarce and standard extraction or analysis methods are often ineffective. Despite these challenges, we have made good research progress and have reached a good level of understanding of some of these unknown biochemistries.

The research was to specifically target the glowing luminous mucus secreted by the worm *Chaetopterus*, because of its long-lasting property that is also independent of oxygen (the only exception of such mechanism discovered thus far). Two clear questions were about (1) what is the sustained energy supply mechanism that delivers electrons to the light producing chromophore since the mucus is secreted in the open sea water with no metabolic support from a cellular reaction, and about (2) what is the molecular entity (chromophore or photoprotein) that is involved for producing the photons.

From previous AFOSR funded research, we demonstrated that the luminous mucus relies on iron as a co-factor. Interestingly though, the mucus was shown to contain both reduced and oxidized forms of iron, when it is well known that the most abundant form of iron is the oxidized one since the more stable one in seawater; however, only the reduced form of iron has a biological use, hence there must always be a reduction pathway for iron to play a molecular function in biological systems. Such reduction pathway must take place in the mucus, and during this most recent research period, we demonstrated that the mucus contains a **ferritin that is very efficient** despite being secreted in the surrounding environment (why would an organism otherwise get rid of an efficient enzyme?). We described which residues of the worm ferritin make it so efficient (8x greater efficiency than human ferritin) and demonstrated using **genetic manipulation of the worm and human ferritins to alter their efficiency**, that we were able to “turn worm ferritin into human ferritin” (diminished efficiency) while also able to “turn human ferritins into worm ferritin” and improve their efficiency about 4x. One of the mutations also made the worm ferritin even faster by another ~40%, all of which can be a valuable resource towards various biomedical and biotechnological applications.

Aside from the efficiency and investigation of it through mutants, we demonstrated using voltammetry that the **worm ferritin is able to perform reduction on its own, via the ferroxidase site.** Like with the previous part of characterization, we compared with HuHF and found that the mechanism of reduction, and possibly oxidation, too, must be different in both species. Ferritins are known to be able to reduce and release Fe(II) in a reducing environment, which can be induced by the presence of traditional redox cycling elements such as flavins (flavin mononucleotide, FMN) and NAD (nicotinamide adenine dinucleotide), which are naturally found in the worm mucus.

Where it gets really interesting, is that worm ferritin in the mucus may still be able to reduce Fe through the ferroxidase center, while small reducing agents are presumed to directly reduce off the mineral core inside the ferritin, skipping the ferroxidase center. This reaction is typically low in efficiency, but with the help of the flavins and the blue light, may become much more efficient. We have outstanding proof that the reduction process of the ferritin (critical for the redox potential and long energy supply) is facilitated by blue light. Indeed, when exposing the purified worm ferritin to exogenous blue light, the reduction process of iron is accelerated (more reduced iron is released). Such **photoreduction of ferritins** has been reported in the literature in the past, but with

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no particular biological function associated with it. Here, because the mucus produces blue light, one can conceive the fact that blue light stimulates the release of reduced iron, which in turn allows more blue light to be produced, and so on. This is a good example of autocatalytic process at its best! In addition to the demonstration of photoreduction in ferritin with blue light, we showed that exposure of the mucus to exogenous blue light indeed triggers increase of bioluminescence from the mucus, thus validating *ex vivo* for the complex system that makes mucus what we described *in vitro* with recombinant ferritins.

We therefore consider that the ferritin, because of its unique characteristics of efficiency and ability to perform reduction, especially under the influence of blue light, is the **"engine" that provides the long-lasting energy to the light production process**.

With the energy source characterized, we also made good progress in isolating a new protein from the worm mucus (not a ferritin) that shows affinity to iron and that, when isolated and added back to worm mucus, triggers increase of light production. The combination of these observations is encouraging to believe that this protein is involved in the mechanism of light production. The exact identity of this protein remains to be determined through further sequencing.

During this program we also worked on extracting novel biochromophores that show interesting Stokes shifts, and although this aspect did not lead to transformative data yet, it made good progress towards our goal of characterization and identification of such chromophores. We performed extraction and purification of the molecule that provides strong photostability to the seeds of *Magnolia* trees. The honokiol compound we isolated has capacity to absorb UV >100x times that of conventional UV absorbers currently used as standards in the marketplace. We were also successful in extracting the chromophores from the blue arils of the *Ravenala* traveller's palm tree. These arils ("skin" that covers the seeds) are extremely stable which might be related to the fact that the vivid blue color was found mixed with wax material. We now have made significant progress in extracting and purifying these chromophores, which we are analyzing with NMR. We also performed characterization of the light production and fluorescence process from the deepsea sea anemone *Umbellula magniflora*, which was collected at 900m depth during a deepsea marine biology student excursion. The emission of bioluminescence is blue yet the fluorescence spans various colors up into the NIR. The research has mainly characterized the light production process which interestingly resembles the one of the intertidal sea pansy *Renilla reniformis*, and distant cousin of this deepsea organism. No significant progress on the chromophore identification could be made because not enough of these organisms were successfully collected during the grant period. So, at this stage we keep remaining samples frozen to accumulate material for future research, and focus on the more easily accessible samples (such as plant seeds).

In addition to the research developed to target the specific objectives of this program, some side projects using the technology or recent findings from AFOSR funded research have also been pursued. The data then are not presented in this report because they are not directly relevant to reaching the objectives of this program; these data are available as publications in which the AFOSR funding is acknowledged (see Section 6 - Publications).

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4. FINDINGS

This research program has generated a large amount of data summarized below for each goal separately, yet with deeper interest for the luminous worm *Chaetopterus* which was subject to the main research effort and generated the most exciting accomplishments.

The text below provides a summary of the main data when these have been published (publications are provided in Appendix section). It provides complimentary information (not published) and also preliminary research output that is still on-going, if the case.

4.1. Marine worm *Chaetopterus dewysee: model for a unique bioluminescence reaction*

The project allowed us to make good progress with regard to understanding the light production process in the marine worm *Chaetopterus* (Fig. 1). Based on previous AFOSR funding, we started by building a mechanistic hypothesis on how the light production is sustained for such a long time

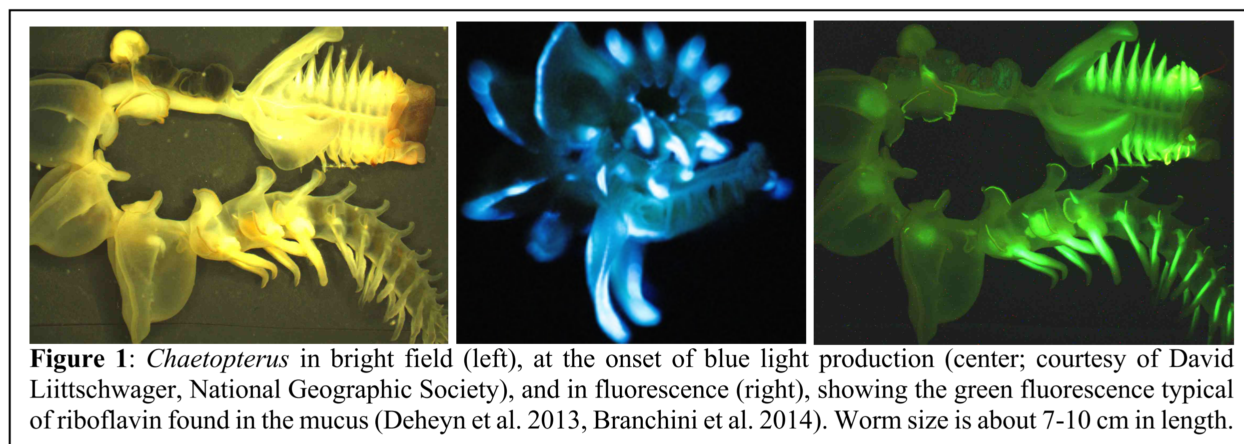

Figure 1: *Chaetopterus* in bright field (left), at the onset of blue light production (center; courtesy of David Liittschwager, National Geographic Society), and in fluorescence (right), showing the green fluorescence typical of riboflavin found in the mucus (Deheyn et al. 2013, Branchini et al. 2014). Worm size is about 7-10 cm in length.

in the mucus. As published earlier (Rawat and Deheyn 2016), we have a mechanistic model that shows that ferritin is at the core of the light production process in the mucus (Fig. 2), which is a conclusion we reached then because the mucus contains ferritin in abundance but also both reduced and oxidized iron; in addition, chromatography fractions with ferritins were also shown to exhibit bioluminescence property, thus clearly associated ferritin with the light production (Rawat and Deheyn 2016). The key questions from this mechanistic model were then the following:

- How is reduced iron (Fe(II)) made available in the mucus? Is it extracted out of the ferritin by coupling with FMN and NAD redox coupling, like in human system? Or is the ferritin itself responsible for driving the reduction, since in a secreted mucus, the availability and proximity of FMN-NAD might not be easy to control. Being able to highlight the redox coupling between the ferritin and FMN-NAD would provide the framework to propose that ferritin is the “engine” of the light production, by providing Fe(II) for building a redox potential.

- The other question is of course about the light production itself. What is the light-producing biochromophore? Is it flavin/FMN, as is the case for bacteria? Or is it a new chromophore or photoprotein, which might (or not) interact with Fe(II) (known to be a co-factor of the light production) for facilitating the production of photons.

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These two questions are the two fundamental goals we have had for this program. They are based on research from previous AFOSR funded research (Deheyn *et al*. 2013, Branchini *et al*. 2014, Rawat and Deheyn 2016).

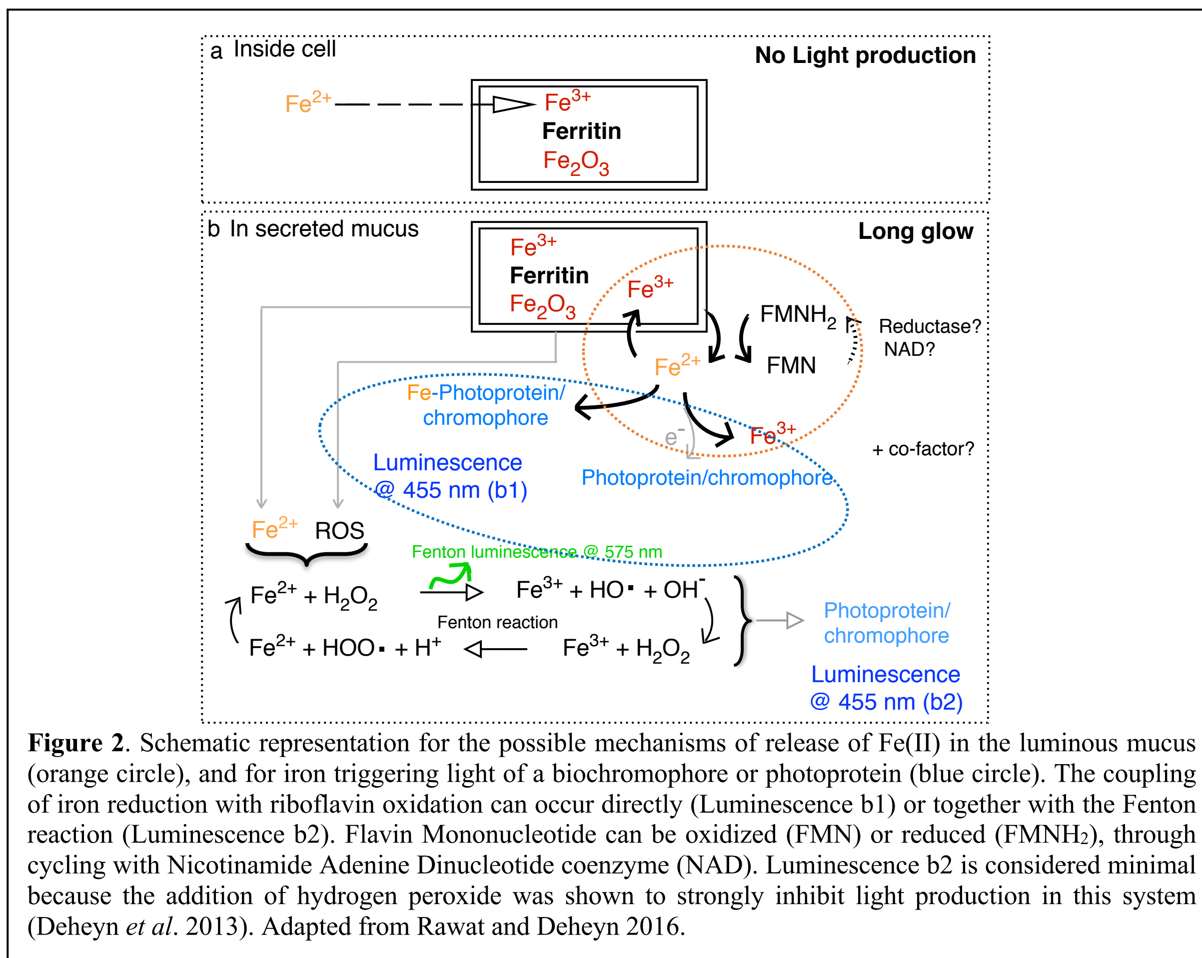

Figure 2. Schematic representation for the possible mechanisms of release of Fe(II) in the luminous mucus (orange circle), and for iron triggering light of a biochromophore or photoprotein (blue circle). The coupling of iron reduction with riboflavin oxidation can occur directly (Luminescence b1) or together with the Fenton reaction (Luminescence b2). Flavin Mononucleotide can be oxidized (FMN) or reduced ($FMNH_2$), through cycling with Nicotinamide Adenine Dinucleotide coenzyme (NAD). Luminescence b2 is considered minimal because the addition of hydrogen peroxide was shown to strongly inhibit light production in this system (Deheyn *et al*. 2013). Adapted from Rawat and Deheyn 2016.

Goal (1). Identify the source of renewed energy in the luminous mucus of *Chaetopterus*

This goal was achieved under 4 different sections that are complementary to one another, and all are part of or aiming to be part of peer-reviewed publications.

1.1. Chaetopterus ferritin ChF is more efficient than human ferritin. [see Appendix #1]

A first paper was dedicated to the physical and functional characterization of the protein found in the worm mucus so we could transition from a "hypothetical" ferritin to a well characterized ferritin. We demonstrated here that the ferritin is, in fact, very performant, about 8x better at

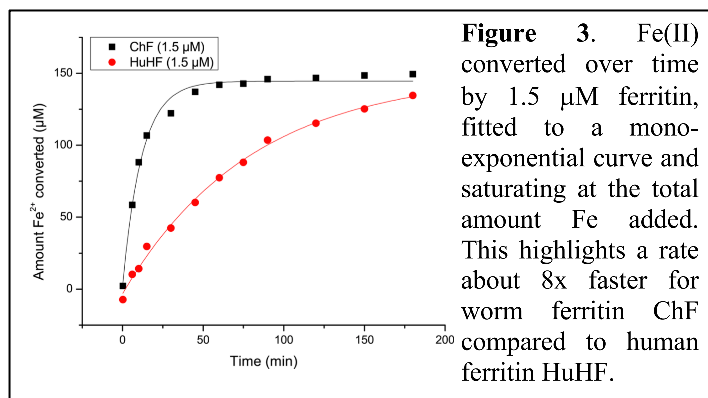

Figure 3. Fe(II) converted over time by 1.5 μM ferritin, fitted to a mono-exponential curve and saturating at the total amount Fe added. This highlights a rate about 8x faster for worm ferritin ChF compared to human ferritin HuHF.

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performing ferroxidation than the recombinant human ferritin HuHF (Fig. 3). This clearly indicated that the iron chemistry was critical for the ferritin to perform in the mucus. We performed crystallographic analyses of the ChF and HuHF and proposed mechanisms that could explain why the ChF was better at performing ferroxidation. We identified just a few key residues close to the active sites that could play a role in transferring iron through the ferritin channels. Other residues caused a wider opening in the 3-fold channel of ChF or had the capability of increasing flexibility near this important entrance for Fe(II) into the ferritin cage. The paper describing these details was published as the cover story of the journal issue (Fig. 4) and also received extensive press considering that this was the first ferritin crystallized from an invertebrate, and thoroughly assessed for its biochemical performances (see Section #6 for popular materials).

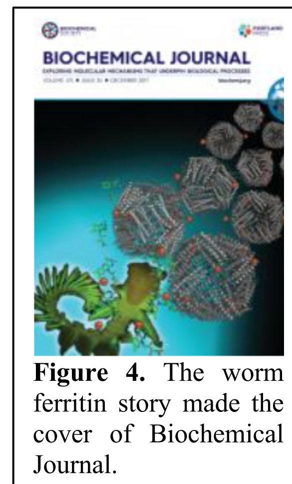

Figure 4. The worm ferritin story made the cover of Biochemical Journal.

1.2. How to optimize ferroxidase activity in ferritin unveiled. [see Appendix #2]

A natural follow-up study was to further investigate the effect of the proposed key residues on the performance of the ferritin. We generated relevant mutants of the recombinant proteins that we then tested for biochemical performances. Our data were exciting! We were nearly able to transform worm ferritins into human ferritins with regards to their performances, and vice versa. This means that we have been able to pinpoint key residues involved in the efficiency of native worm ferritins, and are now able to increase efficiency of human ferritin by only one or two residue substitution. These are attributes that can clearly have some important biotechnological and biomedical applications. The paper on this story is finalized and will be submitted soon to a peer-review journal.

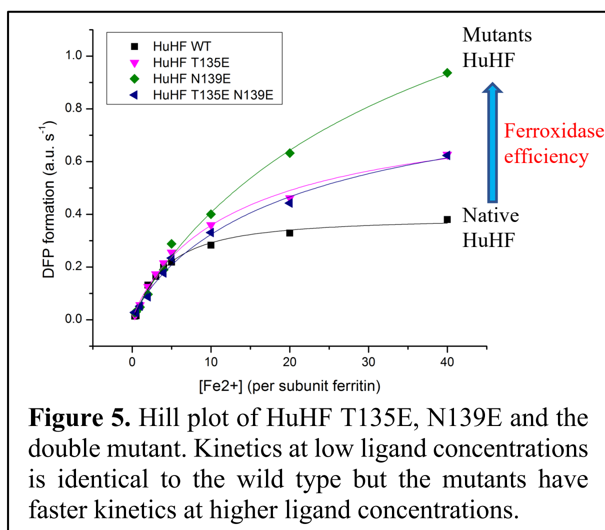

Figure 5. Hill plot of HuHF T135E, N139E and the double mutant. Kinetics at low ligand concentrations is identical to the wild type but the mutants have faster kinetics at higher ligand concentrations.

1.3. Chaetopterus ferritin is able to perform ferroreduction. [see Appendix #3]

Through the process of understanding the structural features involved in the efficiency of ferritin, the question naturally came to us about other properties and functions the worm ferritin could perform. A few years back, we had performed some crude preliminary experiments in which we had placed mucus in a luminometer setup, with two electrodes in the mucus for passing current. We had found that there was some ability to trigger bioluminescence of the worm using electrical impulse (this data was presented at the AFOSR review meeting in 2014). The idea was then to follow up on this study and experiment with some electrochemistry and voltammetry. Before being able to interpret measurements on mucus, we needed to establish a baseline for ferritin and determine whether ferritin can perform iron reduction. With that knowledge, we can test whether the reduction of iron is associated with a flash of light, either through ferroreduction by ferritin or *via* electronic transfer into photons. An additional challenge was therefore to develop the technique for voltammetric analyses to work with gooey mucus. After an initial collaboration with Prof. Cliff Kubiak (UCSD, Chemistry) we ended up working with engineering Prof. Prabhakar Bandaru (USCD) who works on photo- and electrochemistry combined.

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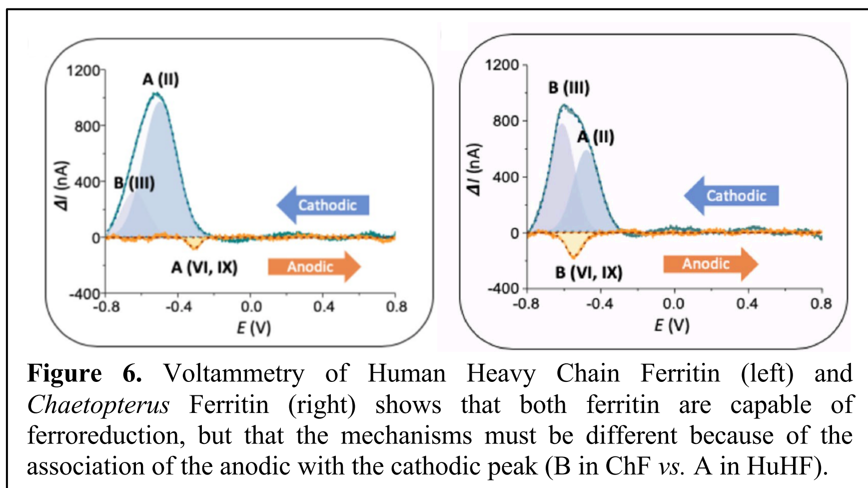

Figure 6. Voltammetry of Human Heavy Chain Ferritin (left) and *Chaetopterus* Ferritin (right) shows that both ferritin are capable of ferroreduction, but that the mechanisms must be different because of the association of the anodic with the cathodic peak (B in ChF *vs.* A in HuHF).

Our collaborative effort materialized in a paper that shows that the worm ferritin is able to perform reduction following a different mechanism than for the human ferritin (Fig. 6). This provided the key demonstration that ChF can clearly be efficient at building a redox potential with flavins and NAD present in the mucus. Our follow-up study with Prof. Andrea Tao on this topic is to assess whether the redox chemistry of the worm ferritin is associated with electronic transfer to a receiving chromophore from where the light production would take place.

1.4. Photoreduction processes can be induced on Chaetopterus ferritin to facilitate long lasting light production.

While the exact detailed process of iron release from ferritin is still not much short of a mystery, both in nature and in the lab, it is often overlooked and forgotten that ferritin is susceptible to photoreduction (Aubailly *et al*. 1991, Ohishi *et al*. 2005, Wolszczak and Gajda 2010). This means that ferritin can reduce (and release) iron under the influence of light. The studies on this property of ferritin are rare and not exhaustive, to the point where it is not really known how this photoreduction process work, nor whether it is depended on color (spectra) of the incident light, exposure time and duration of exposure. These aspects are in fact in our interest for future studies, in order to perform an exhaustive radiometrically calibrated assessment of photo-control of ferritin function. Until then, we performed our studies to the best possible control of the properties of interest, paving the field for more targeted studies further down the line.

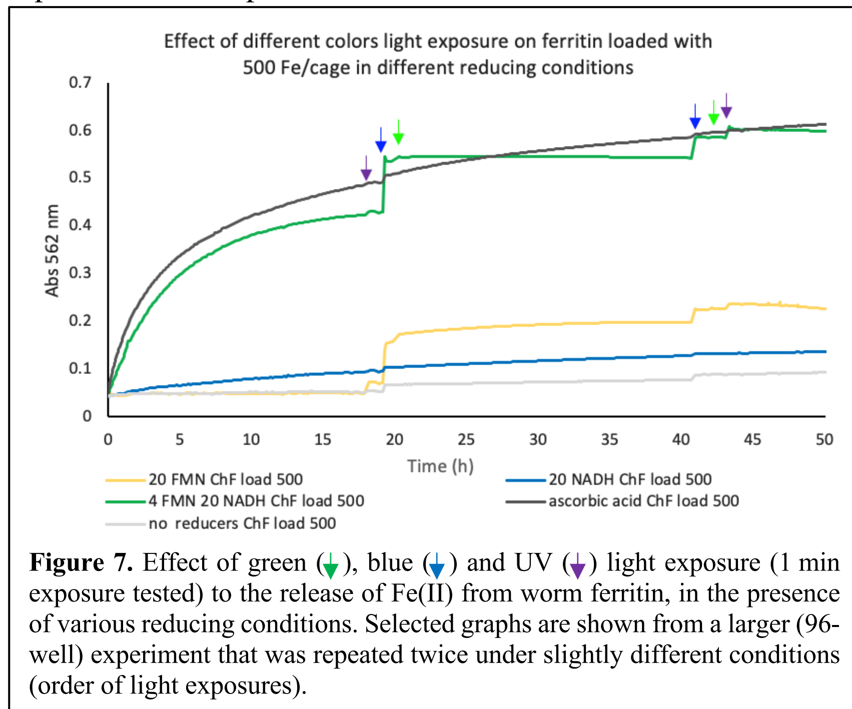

Figure 7. Effect of green (↓), blue (↓) and UV (↓) light exposure (1 min exposure tested) to the release of Fe(II) from worm ferritin, in the presence of various reducing conditions. Selected graphs are shown from a larger (96-well) experiment that was repeated twice under slightly different conditions (order of light exposures).

Since we did not truly know the color at which the ferritin responds to light, we tried exposure of ferritin to green (510-540 nm), blue (440-460 nm), “UV long” (360 nm, UV-A), and “UV short” light (254 nm, UV-C) measured the reduced iron

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being released by a ferrozine colorimetric assay (Fig. 7). In terms of intensity, the blue and green light are comparable, and the UV sources are mutually comparable since they come from the same source, respectively.

What we see first is that not all colors of light affect the photoreduction identically. Green light seems to have no effect at all, while UV can have limited effect, and blue light triggers the most intense response (see various colored arrows in Fig. 7, and the corresponding jumps in the graphs for each light exposure). Interestingly, this effect is still there when the reducing power of added reducers like FMN and NAD is already worked out completely (most curves have reached "plateau" prior to exposure). Only the sample with ascorbic acid is showing what the maximum amount of Fe(II) released would eventually be.

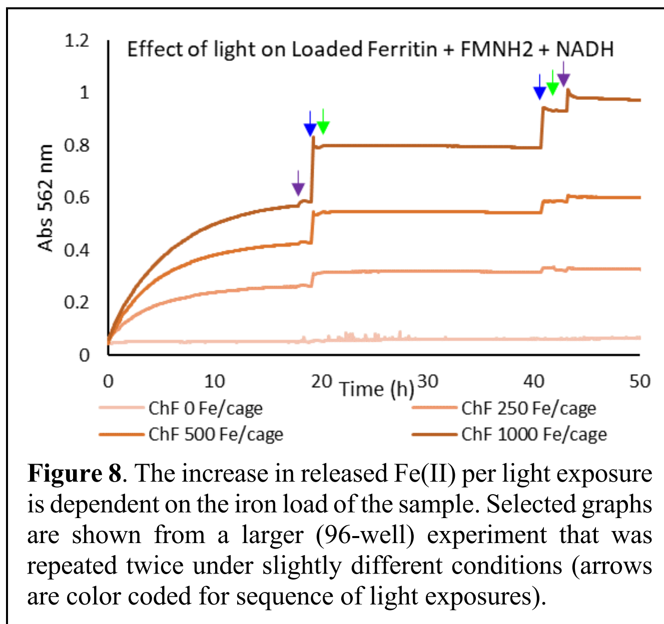

Figure 8. The increase in released Fe(II) per light exposure is dependent on the iron load of the sample. Selected graphs are shown from a larger (96-well) experiment that was repeated twice under slightly different conditions (arrows are color coded for sequence of light exposures).

From this selection of samples shown in Fig. 7, we also learn that the presence of FMN is crucial for the enhanced sensitivity of the ferroreduction reaction to light. The samples with only NAD or ascorbic acid show very small response to the light exposures, while all reactions with FMN in it (even the one without NAD) release a large amount of Fe upon exposure to blue light, and to lesser extent, to UV light. As shown in Fig. 8, we were also able to show that this effect is concentration-dependent, with reference to the ligand, which for the reduction reaction is the load of iron mineral in the ferritin core. The increase in released Fe(II) per light exposure is dependent on the iron load of the sample.

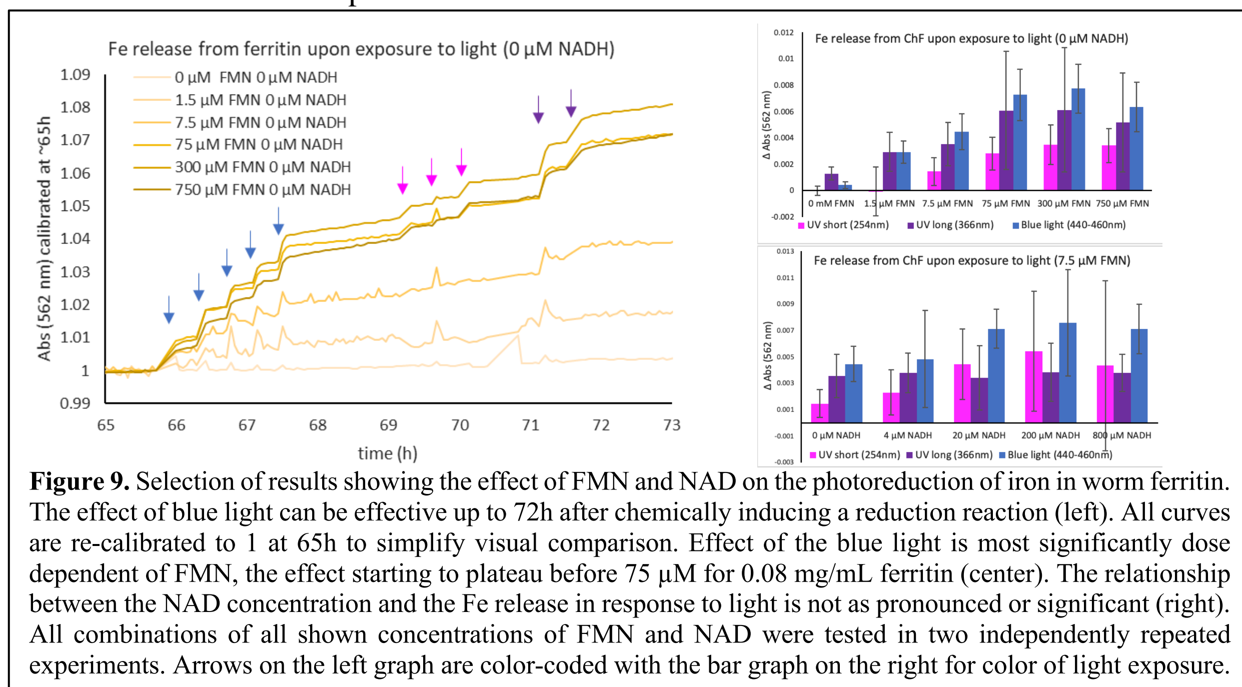

Figure 9. Selection of results showing the effect of FMN and NAD on the photoreduction of iron in worm ferritin. The effect of blue light can be effective up to 72h after chemically inducing a reduction reaction (left). All curves are re-calibrated to 1 at 65h to simplify visual comparison. Effect of the blue light is most significantly dose dependent of FMN, the effect starting to plateau before 75 µM for 0.08 mg/mL ferritin (center). The relationship between the NAD concentration and the Fe release in response to light is not as pronounced or significant (right). All combinations of all shown concentrations of FMN and NAD were tested in two independently repeated experiments. Arrows on the left graph are color-coded with the bar graph on the right for color of light exposure.

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Because FMN and NAD often co-occur in biological system, the mucus being no exception, we wanted to address in more details the relationship between these redox cyclers when exposed to UV and blue light only (since green did not show any effect). We showed that the effect of FMN on the photoreduction is dose-dependent and can last for up to 3 days (Fig. 9). The effect of exposure by "UV long" light is more pronounced in these experiments, which can potentially be related to a more oxidized state of the FMN after 66 hours since this affects the absorption spectrum (Shibata *et al*., 2013), but the dose response was in all cases only significant for flavin, not NAD.

The role of FMN in absorbance of blue light mainly, and in comparison to NAD, is no surprise considering spectral absorbance data (Fig. 10). Indeed, FMN shows strong absorbance in the UV region (254 and 360nm) but also the blue (440-460nm), while no absorbance is reported for green (510-540nm). From these peaks, only the peaks in the 300-500 nm region change when the oxidation state of FMN changes. These absorbance data are consistent with our data interpretation that FMN is able to absorb the spectral energy and that out of these, the "UV long" and Blue light could have the potential to affect the photoreduction process in ferritin. How this process exactly happens would be purely speculative at this stage.

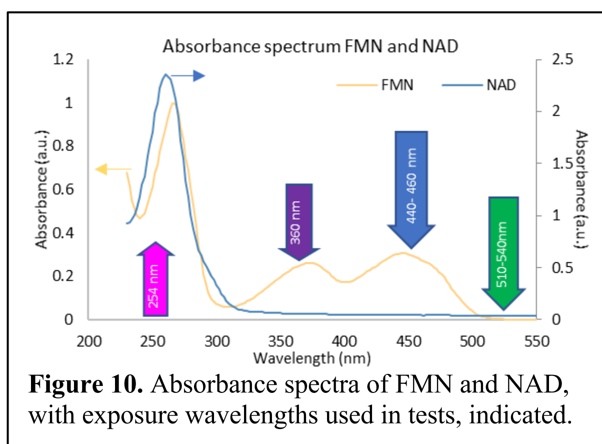

Figure 10. Absorbance spectra of FMN and NAD, with exposure wavelengths used in tests, indicated.

Overall, this data on the ferritin shows that the ferritin is capable of reducing iron (as suggested by our electrochemical study), yet that this process is strongly enhanced by the combination of reducing agents (flavin especially, considering spectral absorbance) and blue light. Interestingly, the mucus also contains in abundance flavin, and produces blue light at around 455-460nm.

It was therefore natural for us to test the photoreduction also on mucus samples, using "extra" exogenous light (thus on top of the native bioluminescence). The basic experiment we performed was to test photoreduction on mucus samples left in the dark versus exposed to blue light. Because we knew the importance of FMN and NAD on the redox cycling, and especially FMN on the photoreduction, we started the investigation with experiments where we introduced FMN and/or NAD in the mucus prior to exposing to blue light (Fig. 11).

What Fig. 11 shows overall is that indeed, the exposure of mucus to exogenous blue light generates a burst of light production that would last several minutes after just one minute of exogenous blue light, which was clearly distinct and significant compared to the dark control, that only showed native bioluminescence. Of interest to note was that the injection of FMN actually inhibited the bioluminescence. Such an inhibition on the light production is interesting, showing that FMN clearly plays a critical role in the processes leading to light production. We interpret this effect as if FMN added exogenously (probably in excess) to the system had been able to shunt electrons for its own redox cycling, thus preventing such electrons to be available to chromophores. To verify this, a next experiment would be to determine whether this inhibition is dose dependent, and/or possibly counter balanced with the addition of Fe(II) to the system. Adding recombinant worm ferritins could also be of interest and in fact such experiments would progressively allow us to reconstruct in vitro the light production process from the worm mucus.

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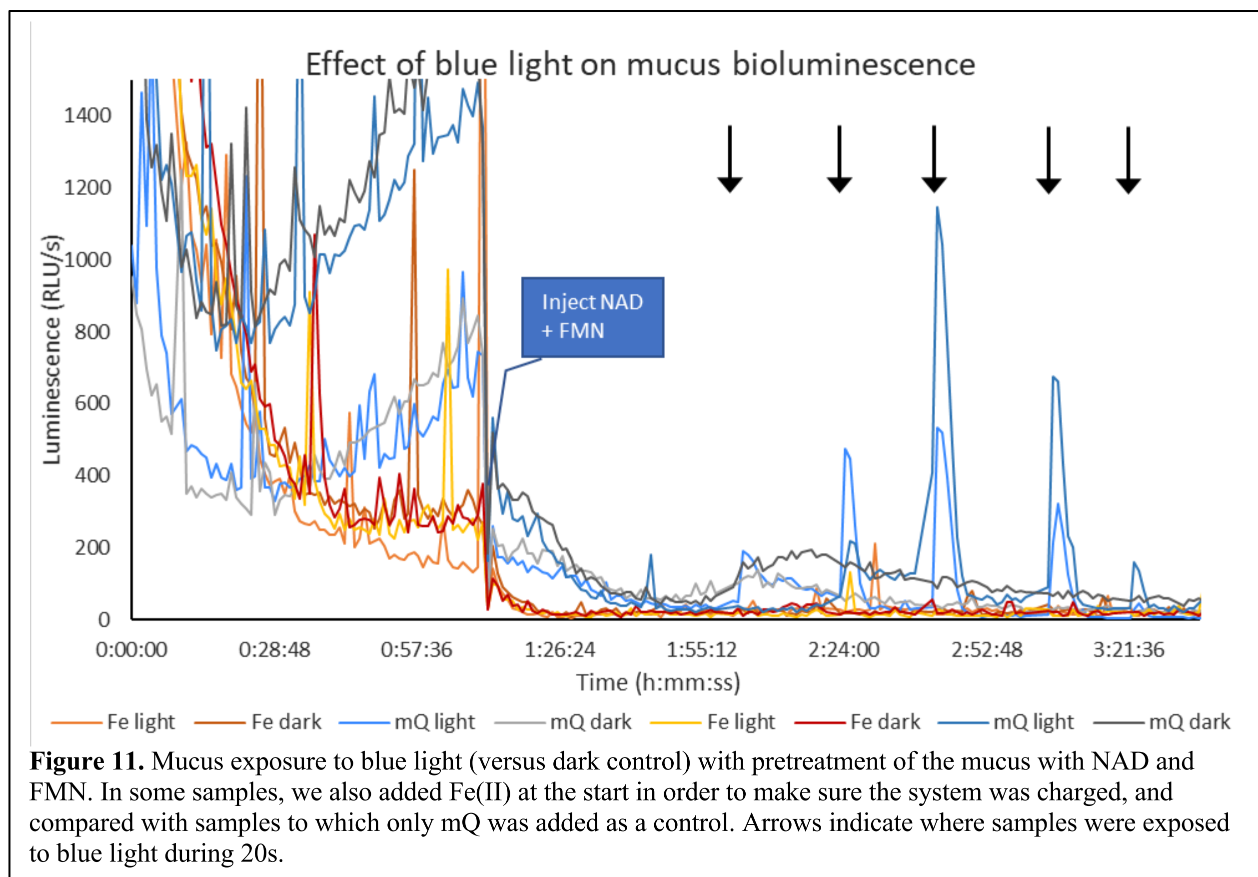

Figure 11. Mucus exposure to blue light (versus dark control) with pretreatment of the mucus with NAD and FMN. In some samples, we also added Fe(II) at the start in order to make sure the system was charged, and compared with samples to which only mQ was added as a control. Arrows indicate where samples were exposed to blue light during 20s.

Since we know that there is (blue) light present when iron is required from ferritin for bioluminescence in the mucus of *Chaetopterus*, we were also wondering whether photoreduction could be affected by exogenous iron. Once mucus is secreted from the worm, it is difficult to imagine an incentive for ferritin to release iron. Ferritin is typically found inside the cell, or also in blood, and the uptake *vs*. release is likely well regulated by the cell metabolism. This is not possible in a secreted mass in sea water. Our initial tests in which we exposed mucus to (blue) light to investigate if there is a chance that the mucus is maintaining its own bioluminescence, showed some small peaks after 1-2 min long exposures to light (hours after addition of FMN and NAD; Fig. 11). Other experiments showed a much stronger effect, in successive exposures to light, on native mucus, as well as in mucus with $FeCl_2$ added to it, regardless even of whether the bioluminescence had already died out naturally or not (Fig. 12).

We learned that when the mucus is not exposed to exogenous light, the bioluminescence dies out faster than when it is exposed to light. Also, when the bioluminescence seems almost fully spent (at around 4 hrs after harvest in this case), exposure to blue light can revive bioluminescence to levels that of samples that have been exposed to exogenous light in the meantime. (Fig. 12). These data also indicate that mucus does not need additional FMN to be responsive to blue light, but that samples with added Fe(II) show systematically greater responses compared to control samples with only MilliQ added (see Fig. 12). The original addition of MilliQ water stimulated light to some extent, as opposed to the inhibition by Fe(II). The effect of Fe(II) on bioluminescence is dependent on the concentration of Fe(II), and is described in Appendix #4. The bioluminescence was restored after exposure to exogenous blue light.

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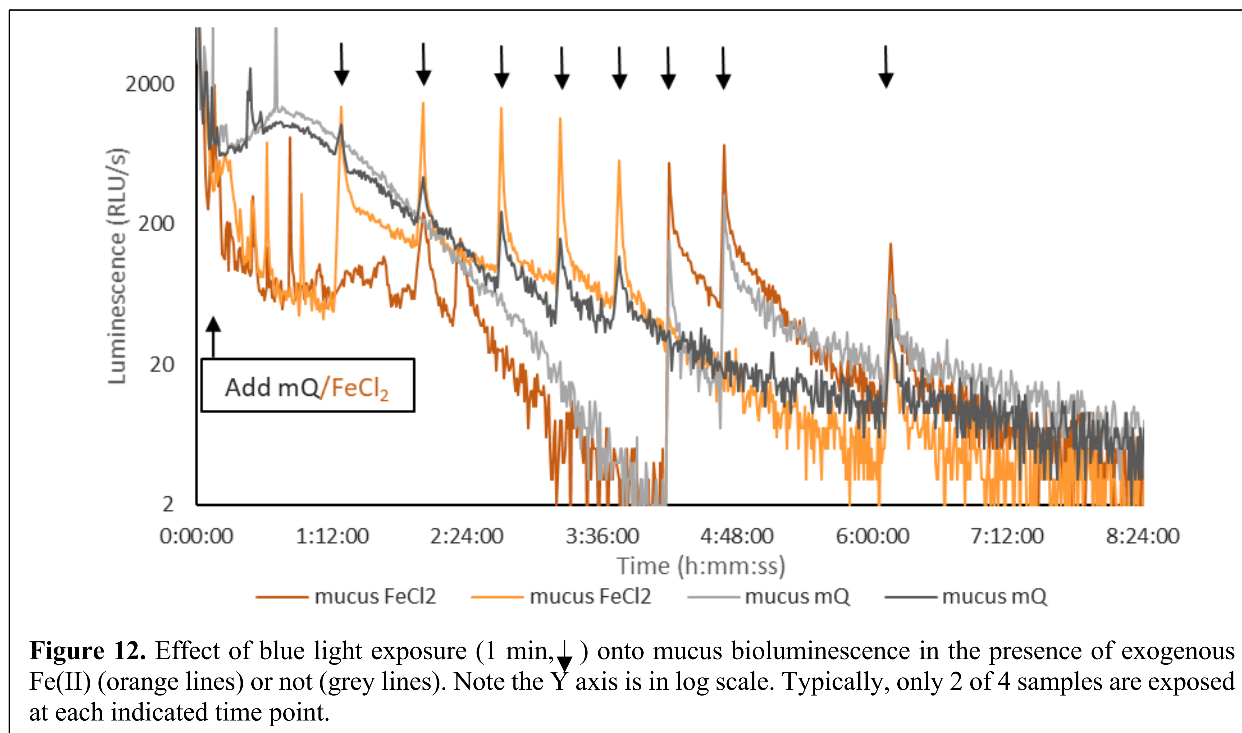

Figure 12. Effect of blue light exposure (1 min, ↓) onto mucus bioluminescence in the presence of exogenous Fe(II) (orange lines) or not (grey lines). Note the Y axis is in log scale. Typically, only 2 of 4 samples are exposed at each indicated time point.

To test the "need for Fe(II)", we conducted a similar experiment but added Fe(II) after a few hours and blue light exposures, to all samples. We found that the extra addition of Fe(II) generated even stronger light production, even after 7h of experimental monitoring (Fig. 13). Note the effect of the concentration of Fe(II) added throughout the entire experiment (lightest orange is lower Fe(II) concentration added). Interestingly, after the 2nd addition of $FeCl_2$, the MilliQ and buffer control samples respond more strongly to the light exposures than the samples that already had $FeCl_2$ added hours prior to exposure. This particular aspect could clearly be the subject of further investigation to help us better understand the balance between iron and blue light on regulation of the light production.

In order to relate the importance of the ferroreduction in ferritin with the bioluminescence in worm mucus, we wanted to verify that the same light sensitivity was present in the mucus. We therefore performed exposure to various spectra of light as the mucus was monitored for light production over time. On average, the data were as expected and concurrent with our ferritin equivalent experiment, thus confirming that the drivers are related to the ability to flavin (possibly depending on the ratio of $FMN/FMNH_2$), which is naturally present in the mucus to interact with light (Fig. 14).

Just like in the ferritin experiments, on average, the largest response is seen followed to exposure to blue light and the smallest to green light. Considerable effect is seen in response to UV Long, and the response to UV short is also visible (Fig. 14). Note, however, that the bioluminescence in the mucus is never regular, and we don't know what else is happening naturally during the period of exposure. For example, in the first exposure of the UV short, the mucus is naturally producing an increase in bioluminescence, exactly like the peaks that are happening randomly in between the exposure times.

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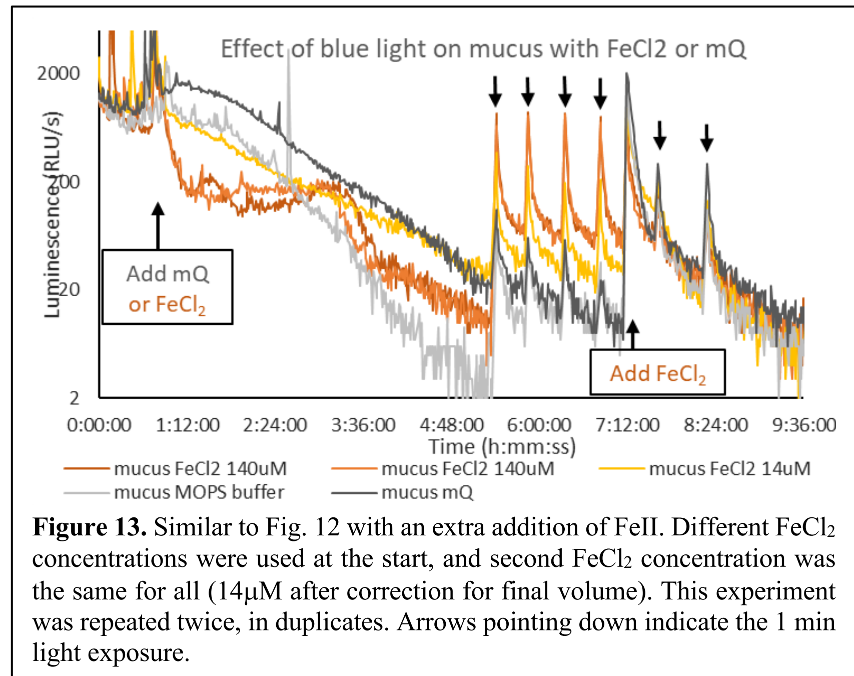

Figure 13. Similar to Fig. 12 with an extra addition of FeII. Different $FeCl_2$ concentrations were used at the start, and second $FeCl_2$ concentration was the same for all (14µM after correction for final volume). This experiment was repeated twice, in duplicates. Arrows pointing down indicate the 1 min light exposure.

This variability is observed before any treatment and reported in earlier publications (Deheyn *et al.*, 2013), which is a reason why our studies, although based on dozens of replicates, usually only show a few representative curves. The trends of the data are kept throughout the many replicates we do, but the intensities and differences can vary so greatly that this presentation of data is needed to obtain clarity. Along with the overall bioluminescence mechanism, it would be interesting to understand the source of this variability, which has been a questions posed in our lab several times, yet without addressing the light sensitive aspect of the mucus, which now could open the door to a new approach and methodology, by working in the dark and under red light.

As for UV and blue light, the mucus bioluminescence responded sharply after exposure to exogenous light (20 sec exposure), increasing in intensity sometimes 4-5x following the exposure, restoring back to close to the original light level within the next several minutes (Fig. 14). This is

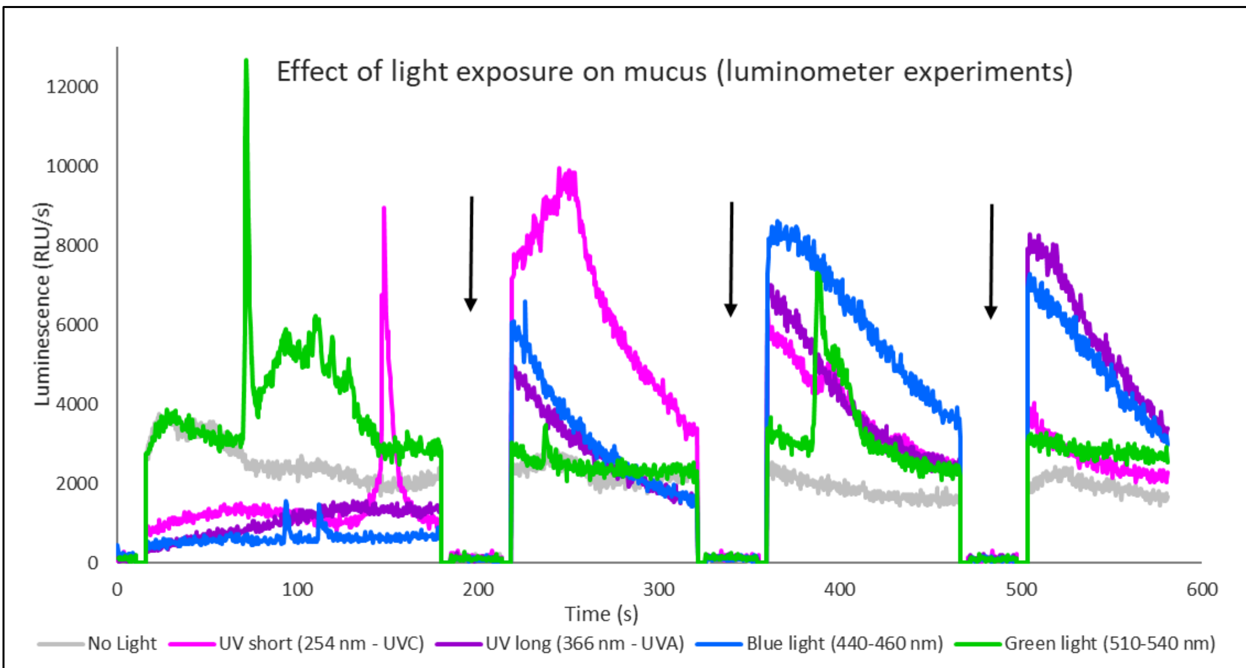

Figure 14. Effect of exogenous light of different wavelengths on the worm mucus bioluminescence. Light exposures lasted 20 seconds for each exposure (↓). Note that the bioluminescence signal is naturally unpredictable and we clearly caught the mucus in a spontaneous upward trend in the first peak of the UV short test.

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in good agreement with the observations in ferritin, where a short, fast burst of Fe(II) is set free from the storage core upon exposure to the said light sources (see Figs 7-9). This clearly indicates that the mechanism of photoreduction we observed with recombinant ferritin *in vitro* is also occurring in the mucus, and that the scenario in which the blue light of the bioluminescence can also trigger iron release from the secreted ferritin *in vivo*. A paper on this topic is in early stage of preparation based on the data presented above, which were presented at the international conference Experimental Biology 2020, and selected as a highlight of the conference (see Appendix #5 for published abstract in FASEB).

Anecdotally, we must report that the mucus has been reported before for its seemingly unpredictable burst of light, which now might find explanation in the photoreduction process naturally occurring in the mucus. In other words, the bursts of light might result from blue bioluminescence of the mucus that induces release of reduced iron from ferritin, with the bursts representing when blue photons are successfully reaching the ferritin (keeping in mind that the mucus is a dynamic, viscous and inhomogeneous environment).

Overall, this first goal of the program, which focused on identifying the energy process sustaining the long-lasting light production has made good progress in identifying the key role of the ferritin ability to release reduced iron “easily on its own”, by this release process to be facilitated by exposure to blue light, which is the native color of the bioluminescence, and that the flavin has a key role in the light absorbance to facilitate the iron reduction from the ferritin. Even though some of these steps are still not very well understood from the mechanistic and molecular aspect, they allow us to have a solid demonstration of such events driving the long-lasting light in the mucus. We believe that the availability of reduced iron is key for the light production to take place. At the onset of the mucus secretion, the release of reduced iron could take place directly from the ferritin solely, maybe with interaction with flavin. Yet as soon as some light is being produced, the process becomes self-powered to some extent, since the blue light of the bioluminescence will stimulate the release of reduced iron, which in turn will stimulate more blue light. There is also a recent publication postulating that the worm creates an intracellular bioluminescence based on the classical luciferin/luciferase system, while the mucus bioluminescence is a different mechanism (Mirza *et al*. 2020). This intracellular bioluminescence could also be the starting light source. Either way, the cycle can then go on and on until the molecular actors of this process start to degrade. In the lab, we have observed this “autocatalytic” process go on for days!! This is an impressive and ingenious system to sustain a chemistry outside a metabolic support a cell can offer. Eventually though, the ferritin runs out of Fe.

Goal (2). Identify the photoprotein/biochromophore) responsible for light production in *Chaetopterus*

A major challenge has been to identify the biochromophore or photoprotein actually responsible for the emission of photons. Our original search for it led us to the ferritin (Rawat and Deheyn 2016), which appears to be the engine of the light production. Because of the mucus stickiness and rheological complexity (Weigand et al. 2017), we have thus far been challenged to isolate good candidates of biochromophore or photoprotein.

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To resolve this problem, we looked into performing specific ion affinity chromatography. We thought of this approach based on the fact that trace elements are known to interfere with the light production (Rawat and Deheyn 2016), while also known to interfere with the ferritin function. We retained three elements for this purpose: iron, zinc and cobalt. Iron of course would show affinity to ferritin, but also possibly to a putative photoprotein if Fe(II) is indeed an allosteric co-factor for the photoprotein. Zinc has been chosen because it is a competitive inhibitor element for the ferroxidase function of ferritin, while Cobalt in contrast is an alternative source to iron that ferritin can use for oxidation.

Based on the work from a MS thesis done in the lab (see Appendix #4), we found some interesting data that can guide us on our quest towards the chromophore (Fig. 15). Our hypothesis and experiments were based on the interaction of Cobalt and Zinc with ferritin being the primary expected effect, while our observations showed differently. First, our assumption that Zn would stop the light production by halting the ferroxidase activity was not found to materialize. This could come from the fact that Zn is a competitive inhibitor for oxidation only, and not for reduction. Second, we though that Co would still allow energy and electron flow, and would allow bioluminescence to still happen, which was not the case. Instead, we believe that Co acts directly as an inhibitor on the co-factor site of the photoprotein, as opposed to work on the ferritin (reduced iron was still found in mucus with Co). Because of this, there is a good possibility that Co has adequate affinity for the binding site of the photoprotein to use test in affinity chromatography. We indeed performed Fe-affinity and Co-affinity column, and compared the output.

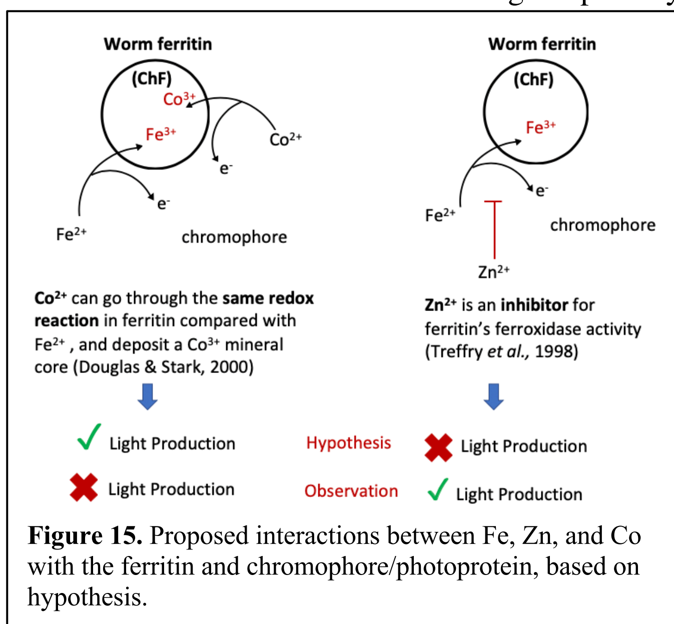

Figure 15. Proposed interactions between Fe, Zn, and Co with the ferritin and chromophore/photoprotein, based on hypothesis.

In this continued attempt to find the chromophore or photoprotein, we used Fe(II)-affinity chromatography of the mucus to select for proteins that may interact with Fe(II). Because of the instability of Fe(II) in most buffers, Fe(II)-affinity chromatography is rather challenging and time-sensitive. None of the proteins that eluted from this column were found to induce the bioluminescence when recombined with mucus. A possible explanation for this is that we were working at low pH (~4.5) to keep Fe(II) in the reduced state as long as possible, either denaturing the proteins or interfering with the bioluminescence reaction in a different pH-related way. Based on common properties with Fe(II) and knowledge from literature that Co(II) can act as a ligand for ferritin, where it undergoes the same oxidation reaction and deposit a Co(III) mineral core on the inside (Douglas and Stark, 2000), we tried the same methods but loaded the HiTrap Chelating HP column with Co(II) instead of Fe(II) for Co(II) affinity chromatography. The result looked almost identical on SDS-PAGE, although the most prominent band turned from a double band into a single band (at the same molecular weight) (Fig. 16). When we tested the bioluminescence activity of the fractions from this purification, a consistent increase in light production was observed upon addition to the mucus (Fig 17).

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In order to evaluate the effects of elements such as Fe, Zn and Co on the light production, and more specifically at what stages or steps of the mechanisms leading to the light production, we repeated some of our experiments with blue light on mucus treated or not with these ions. The idea behind the experiment was to identify whether the elements could be associated with specific targets in the mechanisms, whether the ferritin, the FMN-ferritin interaction, and/or the biochromophore/photoprotein.

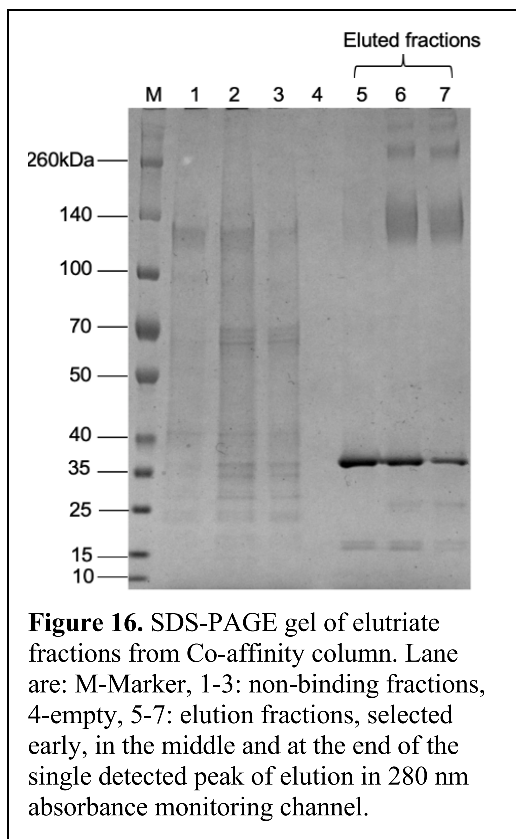

Figure 16. SDS-PAGE gel of elutriate fractions from Co-affinity column. Lane are: M-Marker, 1-3: non-binding fractions, 4-empty, 5-7: elution fractions, selected early, in the middle and at the end of the single detected peak of elution in 280 nm absorbance monitoring channel.

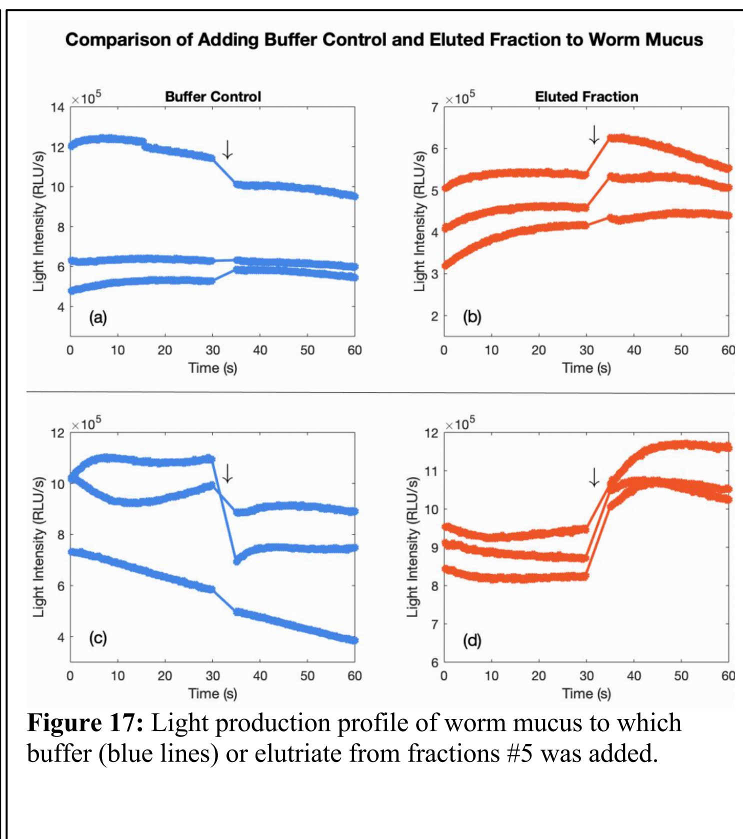

Figure 17: Light production profile of worm mucus to which buffer (blue lines) or elutriate from fractions #5 was added.

We already showed that that adding iron to the mucus increased the response to light exposures (Fig. 12-13 + Fig. 18, orange lines). When adding Zn(II), the luminous mucus became only mildly responsive to the blue light exposure, until more Fe(II) was added on the mucus (which contained Zn(II) already). The response then was even greater than that of Fe-treated mucus (Fig. 18, green lines). Knowing that Zn(II) is an inhibitor for Fe-uptake in ferritin, we now know that the bioluminescence process does not use electrons from the oxidation process in ferritin for its light production. Zn(II) may however act as a competitive inhibitor to Fe(II) on the photoprotein, but clearly has weaker affinity than iron to its active site(s). When adding Co(II), the response to light died down completely to background noise (Fig. 18, purple lines), indicating that the Co(II) may have stronger affinity to the active sites otherwise occupied by Fe(II). Knowing that Co(II) is not an inhibitor for ferritin and seeing barely any response to blue light in these samples (despite the fact that reduced iron is still likely found in the mucus) suggest that Co(II) might act at the level of the chromophore/photoprotein rather than the ferritin, and have significant affinity to the active site. This observation confirms our finding made using the Co-affinity column chromatography.

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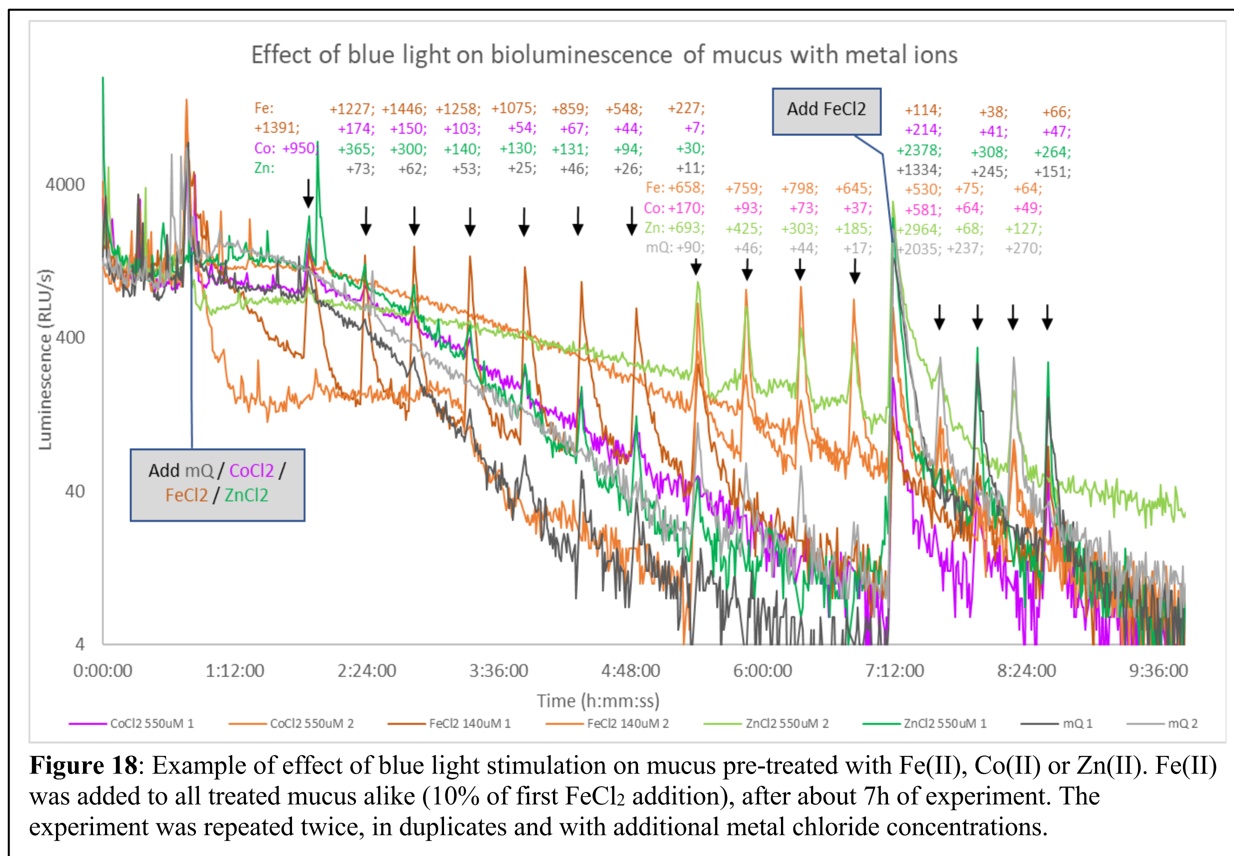

Figure 18: Example of effect of blue light stimulation on mucus pre-treated with Fe(II), Co(II) or Zn(II). Fe(II) was added to all treated mucus alike (10% of first $FeCl_2$ addition), after about 7h of experiment. The experiment was repeated twice, in duplicates and with additional metal chloride concentrations.

Future directions (*Chaetopterus* research)

Future efforts will be taken to further characterize the blue light photoreduction process of the ferritin, which seems to be at the core of the long-lasting supply of electrons to a photoprotein/biochromophore that transforms them into photons. Such characterization will be the demonstration of a passive, self-driven reaction in biology with the potential of (self-) regeneration in technological applications. Applications would also have the ability to be controlled remotely using blue light (think viz., remote activation of a redox potential that is linked to an induction process or even just the activatable glow by itself as a fully biodegradable application).

The second aspect would be to focus on the identification of the photoprotein, especially the sequencing of the protein isolated in Cobalt-affinity chromatography that stimulates light production when added back to the mucus. Further research is needed to identify what it is, but also how it links to the ferritin and/or to the Fe(II) co-factor (how are electrons transferred to chromophore, which also means how is electric energy transformed into photonic energy). The ultimate goal is to connect the worm ferritin to the light production process, and ultimately be able to reproduce the process of light emission *in vitro*, using recombinant proteins and co-factors.

4.2. Various species as *models for unique biofluorescent chromophores*

A second axis of this research program was to search for biochromophores with interesting Stokes shift properties, or interesting spectral properties regarding stability or efficiency in absorbance.

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This research axis encompasses the third goal of this research. We worked on several separate projects for this goal, all depending on availability of specimens, and found some significant results that are summarized below and more detailed in appendices.

Goal (3). Characterize the large Stokes-shift fluorescence of marine and terrestrial samples and work on extracting, isolating, and purifying these chromophores.

3.1. Deepsea sea anemone *Umbellula magniflora*. *[see Appendix #6]*

Umbellula magniflora is a deepsea (280 - 6275 m, Dolan 2008) sea pen (Pennatulid) characterized by a long stalk, easily reaching 20 cm, anchoring each individual in the sandy ocean floor and a branched polyp. Like many species in the order Pennatulacea, *Umbellula* is bioluminescent, and blue light is observed in both parts of this sea pen. Previous research in our lab has given clues towards a luciferin-luciferase based bioluminescence mechanism.

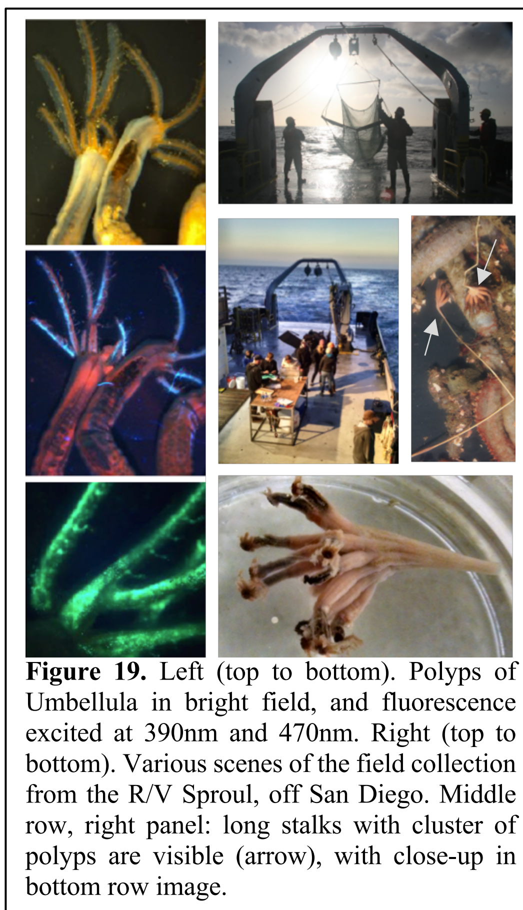

Figure 19. Left (top to bottom). Polyps of Umbellula in bright field, and fluorescence excited at 390nm and 470nm. Right (top to bottom). Various scenes of the field collection from the R/V Sproul, off San Diego. Middle row, right panel: long stalks with cluster of polyps are visible (arrow), with close-up in bottom row image.

More recent research in our lab (see Appendix # 6) has studied the compatibility of the chemistry of *Umbellula* with that of *Renilla reniformis* (or Sea pansy), a Pennatulid that lives in shallow water (2.3-80m) and has a well-characterized luciferin-luciferase system, that besides coelenterazine as its luciferin also uses a luciferin binding protein that releases the luciferin upon binding with Ca(II) (Anderson *et al*. 1974).

A variant of the classical "hot and cold" experiments were performed to test the potential of the luciferin of *Renilla* to (cross-)react with the protein moiety of *Umbelulla* and vice versa. The results presented themselves as successful although the presence of the luciferin binding protein complicated the interpretation of the controls.

Purification of the protein moieties was attempted through size exclusion chromatography, from which 2 interesting peaks were identified that caused bioluminescence when combined with coelenterazine and Ca(II). The *Umbellula* stalk sample gave us a protein with a molecular weight around 111 kDa in native conditions, while the *Umbellula* polyp sample gave us a sample around 20.6 kDa.

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3.2. Brittlestar *Ophiopsila californica [see Appendix #7]*

The brittlestar *Ophiopsila californica* is one of the larger brittlestar species (arm length <200 mm, disk diameter <15 mm) (Figure 20). It has bright orange arms that produce intense luminescence (495-515 nm) when mechanically stimulated, but also when chemically stimulated (in the lab). The luminescence occurs as trains of flashes that travel up and down the arms, closely following nervous impulses (Brehm 1977; Brehm and Morin 1977) and a disconnected, autonomously flashing arm can be used to deter predators by "blinding" them, or as a decoy to lure them away from the main disk (which contains the vital organs) (Brehm and Morin 1977). Based on morphological similarities and neuro-electrochemical studies showing that the light and nerve pulse follow exactly the same pattern along the arms (Brehm *et al*. 1973; Brehm 1977), it is believed that the light-producing cells, or photocytes, are neuro-derived. The light-producing reaction uses Ca(II) as a cofactor and and an unknown photoprotein.

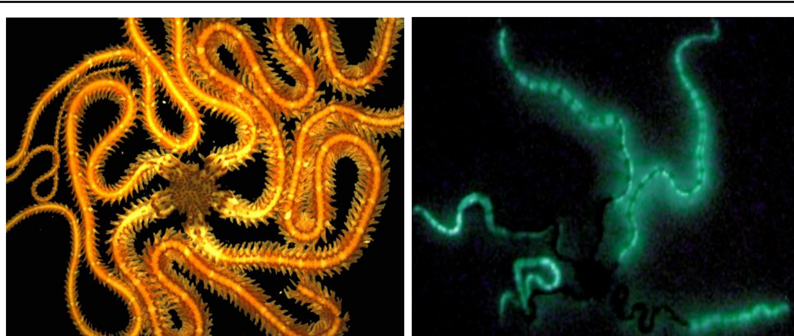

Figure 20. The brittlestar *O. californica* under white light (left) and with arms producing bioluminescence (right). The central disk is not luminescent.

After the emission of light, the photoprotein in *O. californica* becomes fluorescent with an emission peak of 518-526 nm when excited at 380 nm (Brehm and Morin 1977). Both the bioluminescence and fluorescence originate from vesicles inside the photocytes where the photoprotein and Ca(II) are found (Shimomura, 1986). The goal of this research was to extract, isolate, and identify the photoprotein responsible for light production in *O. californica*. Our first challenge was to extract the photoprotein in solution while still preserving its activity, and then use its ability to produce light (either in bioluminescence or fluorescence) to track the photoprotein through the isolation and purification process using various techniques of chromatography. Bioluminescence activity was measured by integration of the total amount of light produced by a sample for 30s, after addition of co-factor calcium (using a luminometer equipped with injectors).

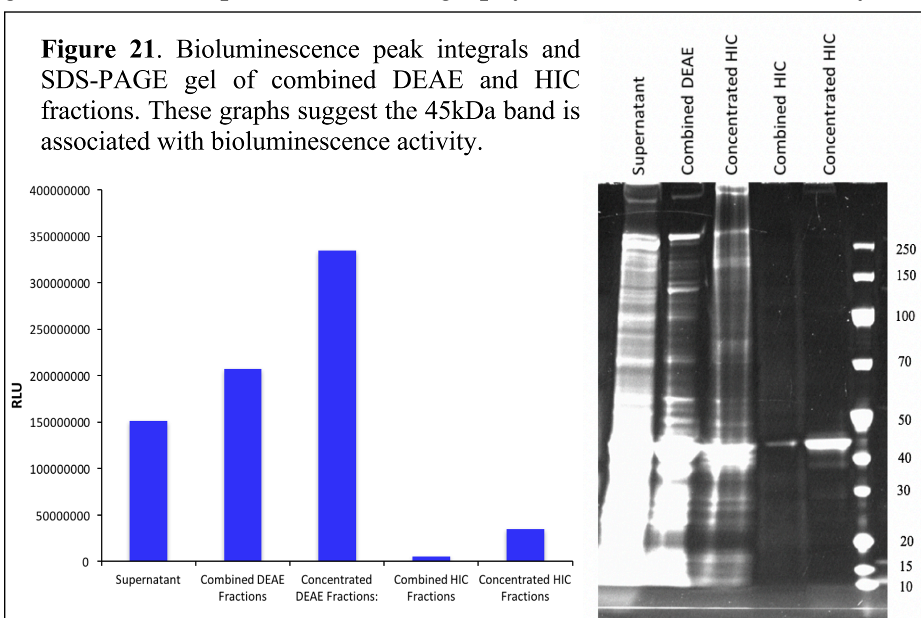

Figure 21. Bioluminescence peak integrals and SDS-PAGE gel of combined DEAE and HIC fractions. These graphs suggest the 45kDa band is associated with bioluminescence activity.

Extraction and chromatography. In a first stage, the light producing protein was extracted from entire arms, grinded and lysed in Ca-free

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artificial sea-water. After consecutive purification steps of the supernatant on anion exchange using diethylaminoethyl cellulose (DEAE) chromatography, size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC), a relatively unstable membrane protein with a molecular weight of ~45 kDa was found as a good candidate for the photoprotein (Fig. 21). For better results, we decided to switch from entire arms as starting material, to isolated photocytes instead, extracted from the arms using a protocol established earlier (Deheyn *et al.* 2000).

After the isolation of the photocytes proved successful, the cells were physically lysed in attempt to extract the light producing protein. After lysis (by mechanical agitation or sonication), the sample was then centrifuged to remove all insoluble cell material. The remaining sample (supernatant) failed to produce light with addition of calcium. The pellet was then re-suspended in buffer and activity returned, equal to that before centrifugation. This suggests that the photoprotein has a transmembrane domain as suggested previously by results published in Brehm, 1977. Several non-ionic detergents were then used to separate the protein from the membrane. The addition of these detergents at initial levels was not sufficient to solubilize the protein and allow it to be detected in the supernatant. Higher levels of the detergents resulted in no activity in the pellet or supernatant. This might imply that when the protein is disassociated from the membrane it can no long function and produce light. A current working hypothesis is that there is a calcium channel (with evidence of it possibly being ryanodine in nature) closely associated to the photoprotein within the membrane. Removal from the membrane may disrupt this association and inhibit the protein from being activated by calcium.

Future work in this aspect of the research will evolve additional investigation into separating the photoprotein from the membrane while maintaining activity. This will involve the studying of the calcium channel's involvement in the light production process. If we can characterize this relationship, it could be possible to design better methods to maintain activity during the purification process. In addition, the best possible candidate to be the photoprotein is still the 45 kDa protein band, which will be sent out for MS analysis. In addition, the above tests have been used using the bioluminescence capacity of the photoprotein to track activity. Now we will try to use fluorescence as an alternative property of the photoprotein to track it down, once dissociated from the membrane. Work is already in progress in that direction.

3.3. Terrestrial samples: blue seeds from Traveler's Palm *Ravenala*. *[see Appendix #8]*

The seeds of the *Ravenala* is covered with arils that have a bright blue and very photostable color (Fig. 22). The arils can be rubbed onto paper (or another surface), with a blue smear remaining, indicating that the color is not associated with structural coloration, but to a pigmentary chromophore embedded to what looks like a waxy material.

Our preliminary analysis has led to testing solvents with different polarities to extract the blue pigment. We found that the BRIJ-35 (a non-ionic solvent best used for extraction of membrane proteins) was best for extracting a blue pigment. However, we also discovered that the pigment would remain stable only for 7-12 hrs, after which it oxidizes and becomes invisible (the blue color then cannot be used to track the pigment during extraction and purification). We have performed spectral analysis of different arils extracts and found that in addition to the blue pigment, our extracts contained large amount of a very strong UV absorbing compound. We have tried to purify both the UV-absorbing and blue compounds, and have performed LC-MS analysis which showed clean signals, but were indicative of new a compound that needs further investigations for chemical structure identification. We are planning on performing NMR analysis next, which can

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be done straight on the UV-absorbing compound, because extracted in abundance; the blue pigment in contrast requires further concentrating and purifying steps.

We are also interested however in further characterizing the blue pigment, because of its large Stokes shift (Fig. 22), which might also need to address protecting the pigment from oxidation. Whether the blue pigment and the UV-absorbing compound are chemically related is also a question we would like to address in future investigation, as well as how *Ravenala* keeps the blue color stable.

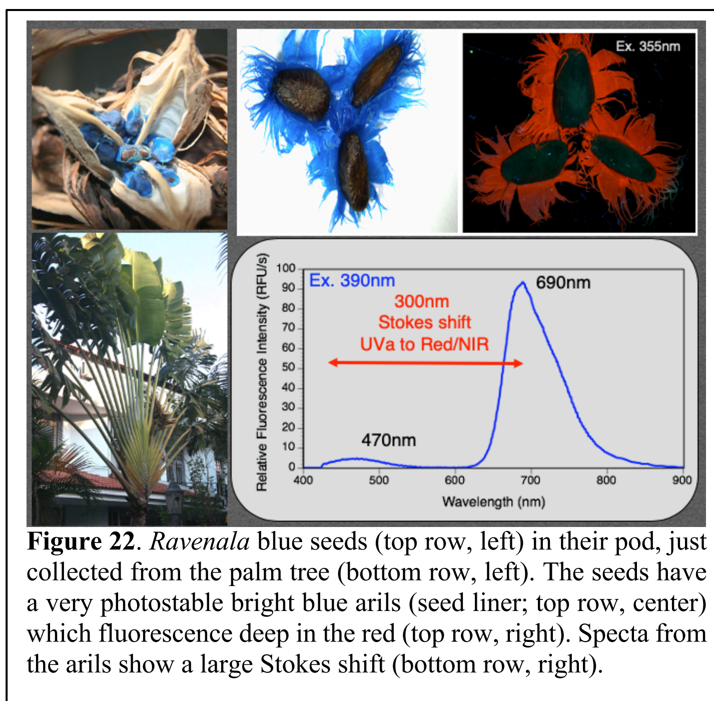

Figure 22. *Ravenala* blue seeds (top row, left) in their pod, just collected from the palm tree (bottom row, left). The seeds have a very photostable bright blue arils (seed liner; top row, center) which fluorescence deep in the red (top row, right). Specta from the arils show a large Stokes shift (bottom row, right).

This seed therefore offers a few avenues for interesting research to be continued with regards to the interplay between photostability to UV and oxidation processes affecting color.

3.4 Terrestrial samples: *Magnolia* seeds. *[see Appendix #9]*

The research on the *Magnolia* red seeds follow the same approach as for the blue seeds of *Ravenala*. In the case of the red seeds, we were able to extract the red pigment with regular solvents (MeOH, EtOH, as well as DCM) and able to measure spectral properties of absorbance (Fig. 23), as well as identify chemical structure of the main compound from the extract.

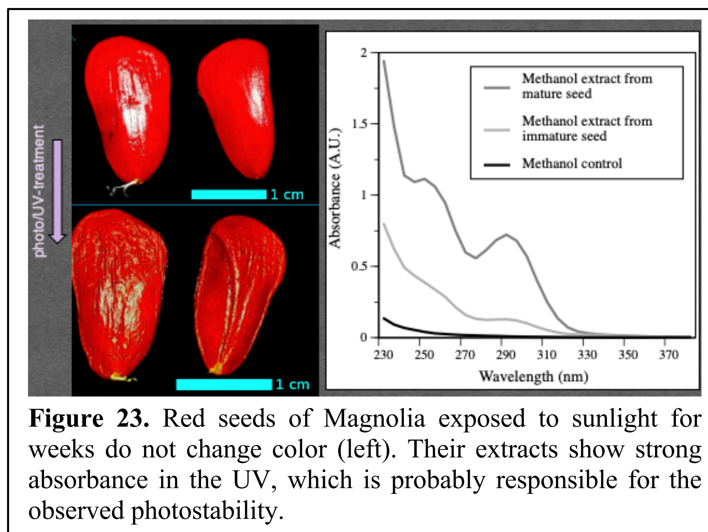

Figure 23. Red seeds of Magnolia exposed to sunlight for weeks do not change color (left). Their extracts show strong absorbance in the UV, which is probably responsible for the observed photostability.

We found that the red compound had a very strong stability in the UV, which was a couple orders of magnitude greater than the conventional references used in this field (Fig. 24). The details of these data, and the chemical biosynthesis of the UV-absorbing compound have been disclosed during our 2019 AFOSR review but not disclosed here since we are trying to develop a product from this compound, to provide increased UV-stability to materials.

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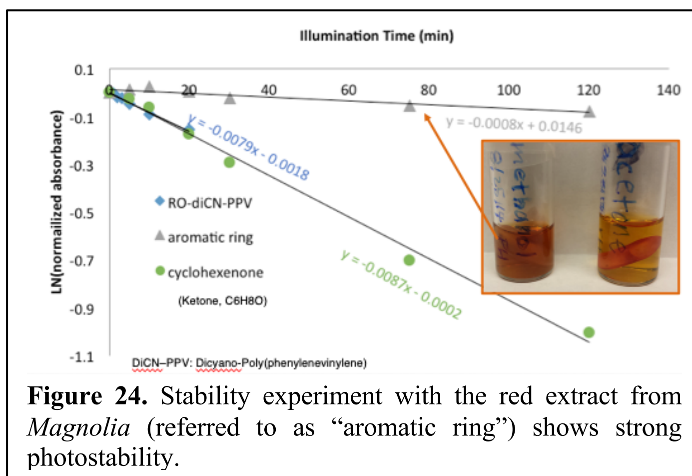

Figure 24. Stability experiment with the red extract from *Magnolia* (referred to as "aromatic ring") shows strong photostability.

Meanwhile, a first publication on the red seeds is in the final stages of preparation for submission in a peer-review journal. This publication relates our research on the photostability of the red coloration, which is unusual in Nature (red-looking objects usually absorb high-energy blue-shifted light, which usually comes with high degradability to the absorbing chromophore). We also describe the fact that the red seeds display a glossy coloration, that is homogeneous throughout the entire surface of the seed. We describe then some ultrastructure in the epithelium that could be involved in scattering light around, and thus contribute to homogeneous light and coloration through the skin of the seed.

This is the first time such structure is described in a plant seed, and research on the use of nano-structures to promote homogeneity of light will be further considered in future studies.

Conclusion

This research program has been successful and productive, making significant progress for the marine worm *Chaetopterus*, in which we identified a pathway of sustained energy supply for the light-producing chemical reaction. We also have good leads for a putative photoprotein, in *Chaetopterus* as well as in other organisms. We have also made some good progress in extracting and providing the preliminary analysis of stable compounds with interesting UV-absorbing properties, which seems to be associated with a long Stokes shift as well. We have identified a number of compounds from nature and are now looking at expanding our research towards the red and blue colored pigments of these same organisms. Overall, the progress made in this research puts AFOSR in a leading position for the discovery of new light producing systems.

Future direction of the research program

The research program has identified critical routes to pursue in order to complete the description of unique light producing chemistries, and unique light producing or UV-absorbing compounds. Continued collaboration with DoD labs (AFRL, NRL) will facilitate the process on directing the research for various applications of interest to the DoD, with regards to UV protection, and the use of light (whether fluorescence or bioluminescence) as reporters showing long lasting signals and high stability, and possibly interesting Stokes shift. This is particularly important to allow usage in harsh environments (directly in the field) where light can be used for specific visual signaling.

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5. PERSONNEL SUPPORTED

Dr. Dimitri Deheyn (PI), Associate Research Scientist, supported at 10%.

Dr. Evelien De Meulenaere, Postdoctoral Researcher, supported at 100%.

Michael C. Allen (Lab Assistant at max. 25%)

Collaborators

- Dr. Bruce Branchini, Connecticut College, CT USA
- Dr. Nancy Kelley-Loughnane, Air Force Research Laboratories, OH USA
- Dr. Jorge Chavez Benavides, Air Force Research Laboratories, OH USA
- Dr. Bennett Ibey, Air Force Research Laboratories, Fort Sam Houston, TX USA
- Dr. David Gruber, City University of New York, NY USA
- Dr. David Kisailus, University of California Irvine, CA USA
- Dr. Rae Anderson, USD, USA
- Dr. Andrea Tao, UCSD, USA
- Dr. Prabhakar Bandaru, UCSD, USA

Student/interns (26 total)

-UCSD-SIO graduate student Taylor Hernandez (2019-present)
-UCSD undergraduate student Mara Casebeer (2019-present)
-UCSD Master student Mohammad Sedarat (2019-present)
-UCSD-SIO graduate student Kara Wiggin (2019-present)
-UCSD undergraduate student Edward Banuelos (2019-present)
-UCSD undergraduate student Christina Puzzanghera (2019-present)
-UCSD Master student Laura (Ze) Gong (2019-present)
-MS student (La Rochelle, France) Carla Costa (spring 2019)
-UCSD (NanoEng) undergraduate student Aidan Lucas (2018-present)
-UCSD Master student Zida (Kotachi) Liu (2018-present)
-UCSD undergraduate student Sarah Wolfe (2018-present)
-UCSD Master student Kelly Govenar (2018-present)
-Postdoctoral researcher Dr. Evelien De Meulenaere (2014-present)
-MS student (La Rochelle, France) Klara Peumery (spring 2020)
-MS student (La Rochelle, France) Julie Van Ferneij (spring 2019)
-UCSD Master student Tianyun (Esther) Hua (2018-2020)
-UCSD undergraduate student Sarah Romero (2018-2020)
-MS student (La Rochelle, France) Mathilde Cadu (spring 2018)
-MS student (La Rochelle, France) Elina Ramus (spring 2018)
-UCSD Master student Kira Price LaFace (2017-2019)
-UCSD undergraduate student Kelly Govenar (2017-2018)
-UCSD (MAE) undergraduate student Zida (Kotachi) Liu (2017-2018)
-MS student (La Rochelle, France) Luvina Carbonnier (spring 2017)
-MS student (La Rochelle, France) Florent Cornec (spring 2017)
-UCSD undergraduate student Cameron Rogers (2016-2018)
-Unpaid volunteer Andre Briscoe (2016-2019)

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6. PUBLICATIONS (acknowledging AFOSR funding; 10 total including the ones in review)

This AFOSR project assesses light production but also chromophores with interesting spectral properties (Stokes shift). Hence, some side projects have also addressed the topic of transparency and coloration, under the topic of biophotonics. The mindset of these publications is also to used changes in color, fluorescence and/or transparency as a reporter for some material property and/or physiological state of the organisms, while also considering chemical composition of the environment that can be interrogated remotely.

Lowder KB, Deheyn DD, Allen MC, Day JMD, Taylor JRA (2017). Grass shrimp cryptic colouration under ocean acidification and warming conditions. ICES Journal of Marine Science: pp. 1-9. DOI:10.1093/icesjms/fsw246.

Weigand WJ, Messmore A, Deheyn DD, Morales A, Blair DL, Urbach JS, Robertson-Anderson RM (2017). Active microrheology of *Chaetopterus* mucus determines three intrinsic lengthscales that govern material properties. PLoSOne 12(5): e0176732. DOI: 10.1371/journal.pone.0176732.

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Verdes A, Alvarez-Campos P, Nygren A, San Martin G, Rouse G, Deheyn DD, Holford M. (2020) Molecular phylogeny of *Odontosyllis* (Annelida, Syllidae): A recent and rapid radiation of marine bioluminescent worms. (Available on BioRXiv since 2018; now being finished and updated for publication in a peer-review journal).

Velasco-Hogan A, Deheyn DD, Koch M, Heiland B, Arzt E, Meyers MA (2019). Exploration of the deep-sea dragonfish: a structural understanding of their hollow transparent teeth. *Matter*. https://doi.org/10.1016/j.matt.2019.05.010.

Chen P, De Meulenaere E, Deheyn DD, Bandaru PR (2020). New iron redox pathway revealed in ferritin via electron transfer analysis. *Scientific Reports* 10:4033 https://doi.org/10.1038/s41598-020-60640-z.

De Meulenaere E, Deheyn DD (2020). Speeding up Fe^{2+} transportation inside human ferritin cage: inspiration from the marine worm *Chaetopterus* ferritin. (In prep., close to submission). [see appendix #2].

Briscoe A, Deheyn DD (2020). Photostability of the glossy red seeds from the *Magnolia grandiflora tree.* (In prep., close to submission). [see appendix #9]

Cerullo A, Allam B, Baer A, Barnes WJP, Barrientos Z, Deheyn DD, Fudge DS, Gould J, Harrington MJ, Holford M, Hung C-S, Jain G, Lai TY, Mayer G, Medina M, Monge-Nájera J, Napolitano T, Espinosa EP, Schmidt S, Thompson1 EM, Braunschweig AB (2020). Comparative animal mucomics: Inspiration for functional materials from a ubiquitous and understudied biopolymer. (In review). [see appendix #10]

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Popular materials about my research under this AFOSR area of interest (9 total)

WagTV – UK science program (2016); “What on Earth”. http://www.wagtv.com/shows/detail/showid/62

Jackson Hole wildlife film festival (2017). El Niño: A shifting normal. Short movie by Kyle McBurnie (with contribution of Deheyn using fluorescence as a health reporter). https://vimeo.com/217735964

SIO Press Release (2017). Bioluminescent Worm Found to Have Iron Superpowers. https://scripps.ucsd.edu/news/bioluminescent-worm-found-have-iron-superpowers

NBC news San Diego (2018) 'Bloom' of Bioluminescence Lights Up San Diego Beaches. https://www.nbcsandiego.com/news/local/Blue-Glow-Ocean-San-Diego-Beaches-Bioluminescence-482163161.html

BlueTech SeaHead Innovation (2019) Biomimicry and the Blue Economy: Sustainable Innovation Inspired by Nature. https://sea-ahead.com/news/2018/12/20/innovation-inspired-by-nature

New York Times (2019). Meet the deep-sea dragonfish. Its transparent teeth are stronger than a Piranha’s. https://www.nytimes.com/2019/06/05/science/dragonfish-teeth-transparent.html.

UCSD Press Release (2019). Researchers discover what makes deep-sea dragonfish teeth transparent. http://jacobsschool.ucsd.edu/news/news_releases/release.sfe?id=2803.

UCSD Press Release (2020). Coral-Inspired Biomaterials Could Lead to Highly Efficient Biofuel Production. https://scripps.ucsd.edu/news/coral-inspired-biomaterials-could-lead-highly-efficient-biofuel-production.

SIO Research Highlight (2020). Marine organism’s bioluminescence could inspire new eco-friendly, long-lasting light sources. (Experimental Biology and FASEB highlighted abstract)
https://scripps.ucsd.edu/news/tube-worm-slime-displays-long-lasting-self-powered-glow

7. INTERACTIONS/TRANSITIONS

a. *Conferences/seminars.*

Conferences (19 total):

-2020 Apr., Experimental Biology, San Diego, CA USA

-2020 Feb., Ocean Sciences Meeting, San Diego, CA USA

-2020 Feb., NOVA Int Conference on Cellulose Fibers, Cologne, Germany

-2019 Sep., Global Dornbirn Congress, Dornbirn, Austria

-2019 Sep., Unveil Fashion, Washington, DC USA

-2019 Jun., Sustainable Brands, Detroit, MI, USA

-2019 May, SoCal Soc. Environmental Toxicology And Chemistry, San Diego, CA USA

-2019 Apr., Experimental Biology, Orlando, FL USA

-2019 Apr., Surface Ocean Lower Atmosphere Study, Sapporo, Japan

-2019 Feb., SPIE, San Francisco, CA USA

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-2018 Nov., The Maritime Alliance BlueTech week Intl meeting, San Diego, CA USA
-2018 Sep., SIO Students Symposium, La Jolla, CA USA
-2018 Apr., Living Light, Cambridge, United Kingdom
-2018 Feb., Ocean Sciences Meeting, Portland, OR USA
-2017 Dec., Intl Conference on Mechanics of Biomaterials and Tissues, Waikoloa, HI USA
-2017 Oct., Energy-Water-Food Nexus International Summit, Orlando, FL USA
-2017 Mar., UCSD-Natl Sun Yat-Sen Univ. Bilateral Research Symposium, La Jolla, CA USA
-2017 Feb., American Society of Limnology and Oceanography, Honolulu, HI USA
-2017 Feb., Oceanology International, La Jolla, CA USA

Seminars (42 total):
-2020 Feb. Nature to support green and sustainable innovation. The Explorers Club – Northern Europe Chapter, Oslo, Norway
-2020 Feb. Biomimicry: A topic that fosters STEM training and research, industry interactions, and disruptive innovations. US Naval Staff College visit to SIO, La Jolla CA, USA.
-2020 Jan. Microfiber pollution. The Waitt Foundation. Fort Lauderdale, FL, USA
-2020 Jan. Scripps Oceanography Researcher protecting paradise: A look at microplastic and microfibers. The Belgian Consulate, Los Angeles, CA, USA
-2019 Nov. Biomimicry: A realistic solution to human-induced problems? AAAS DoSer program, SIO, La Jolla CA USA
-2019 Nov. Biomimicry: A realistic solution to reach realistic SDG's? The Maritime Alliance, BlueTech week, San Diego, CA USA
-2019 Nov. Microfibers pollution: Global monitoring initiative. Global Leadership Initiative, UCSD, La Jolla, CA USA
-2019 Nov. Molecular photonics in marine biology. Katholieke Univ. Leuven (KUL), Belgium
-2019 Oct. Biomimicry: Innovating using Nature's toolbox. The Birch Aquarium at Scripps, Perspectives in Ocean Sciences, La Jolla, CA, USA
-2019 Oct. Biomimicry: A topic that fosters training and research, industry interactions and disruptive innovations. The Princeton Club, NYC, USA
-2019 June Assessing the goods and the bads of sunscreens. The Explorers Club (Oceans' Week, joined session with United Nations), NYC, USA.
-2019 June To be or not to be a marine biologist? Grove Hill School (5th graders), The Explorers Club (Oceans' Week, joined session with United Nations), NYC, USA.
-2019 June Light production and light manipulation in living organisms: from magical displays to bioinspired applications. Torrey Pines Docent Society Guest Speaker series. Del Mar, CA, USA.
-2019 June Biomimicry at SIO: A topic that fosters STEM training and research, industry interactions, and disruptive innovations. Chinese University of Hong Kong visit at SIO. La Jolla, CA, USA.
-2019 May Living light: from magical displays with ecological functions to biotechnological applications. The Ocean Institute seminar series. Dana Point, CA, USA.
-2019 Apr. Impacts of ocean plastic and microfibers on air quality and climate. Surface Ocean Lower Atmosphere Study – SOLAS. Hokkaido, Japan
-2019 Apr. Living light and living colors: A source of inspiration for novel biotechnological

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applications. SIO visit from the 30 members of the NICE lab – Nature Inspires Creativity Engineers laboratory, University of Sofia-Antipolis, France. SIO, La Jolla, CA, USA.

-2019 Mar. Towards understanding the fundamental mechanisms of color and light production in Nature. Seminar series. Advanced Science Research Center (ASRC) at CUNY. New York City, NY, USA.

-2018 Nov. Biomimicry: A topic that fosters STEM training and research, industry interactions, and disruptive innovations. The Maritime Alliance gala dinner, BlueTech week, keynote address, San Diego, CA USA.

-2018 Nov. Assessing microfibers pollution in the oceans: An industry partnership. The Maritime Alliance annual meeting, BlueTech week at SIO, La Jolla, CA USA.

-2018 Oct. Living light and living colors: A source of inspiration for novel biotechnological applications. Work meeting with PEPSI innovation team. La Jolla, CA USA.

-2018 Aug. Light production and light manipulation in living organisms: from magical displays to bioinspired applications. Lenzing Inc, Lenzing, Austria.

-2018 July. About the misconceptions of oceans plasticity in relation to plastics. Paul Ricard Foundation, Paris, France.

-2018 June. Microfibers and microplastics: the invisible threats. The Explorer Club and United Nation Oceans Week, New York City, NY USA

-2018 May. Living light: A source of inspiration for novel biotechnological applications. FADEX joint meeting, La Jolla, CA USA

-2018 May. Living light: A source of inspiration for novel biotechnological applications. Join meeting with BASF at SIO, La Jolla, CA USA

-2018 Apr. Light production and manipulation in Nature: from magical displays to bioinspired applications. University of Ghent, Belgium.

-2018 Apr. Understanding adaptations of organisms to inspire technological innovation: The case of living light. UC Administrative Management Professionals 50th Annual conference. San Diego, CA USA

-2017 Dec. Living light: from spectacular displays with ecological functions to biotechnological applications. Holidays Light event, Birch Aquarium, La Jolla, CA USA

-2017 Oct. Light production and manipulation in Nature: from magical displays to bio-inspired applications. Sea and Learn: Enhance your Environmental Awareness. Windwardside, Saba Island, The Caribbean.

-2017 Oct. Light and color produced by organisms: Why, and so what? Saba Sacred Heart School (6th graders). St John's, Saba Island, The Caribbean.

-2017 Oct. Water, water, water… How to make some following inspiration from Nature. EnergyWaterFoodNexus Intl Summit, Orlando, FL USA

-2017 June. To be (seen) or not to be: Exploring lessons from Nature. The Explorer Club, New York City, NY USA

-2017 Mar. Biomimicry at SIO: A topic that fosters STEM training and research, industry interactions, and disruptive innovations. SIO Development team, La Jolla, CA USA

-2017 Mar. Light production and light manipulation in living organisms: from magical displays associated with biological functions to bioinspired applications. UC San Diego-National Sun Yat-Sen University Bilateral Research Symposium, UCSD, La Jolla, CA USA

-2017 Mar. Light producing organisms: Why? French Cultural Center, San Diego, CA USA

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-2017 Feb. Biomimicry: A topic that fosters STEM training and research, industry interactions, and disruptive innovations. La Jolla, CA USA
-2017 Feb. Fundamental aspects of bioluminescence: how to work with it, and deal with it. Space and Naval Warfare Systems Command (SPAWAR), San Diego, CA USA
-2017 Jan. light producing organisms: Natural beauty to scientific application, Explorers Club, Birch Aquarium, La Jolla, CA USA
-2016 Dec. Biomimicry: Understanding life adaptations to advance technology. PiEnergy Inc., La Jolla, CA USA
-2016 Dec. Living light: from magical displays to visual ecology. Linköping University, Sweden
-2016 Dec. Living light: from spectacular displays with ecological functions to biotechnological applications. Living Light (biofluorescence) event at the Birch Aquarium, La Jolla, CA USA

b. *Consultancy*.

Since 2014, Deheyn has been an Academic Scientific Advisor for PyroFarm Inc (https://pyrofarms.com, former Biopop Inc), which promotes the use of light production in toys and art, but also in innovative use of biological light.

c. *Transitions*.

Invention disclosure request is in process in relation to the new biochemistry of the light production in the marine worm *Chaetopterus sp*., and in consideration with the new patenting rights of proteomic/genetic material from Nature. The worm ferritin will be made available to DoD labs (already sent to the AFRL labs in OH), but also through Addgene.com (where plasmids can be requested for scientific use).

From earlier stages of this program (which focused on fluorescent proteins), we still have transitions, which are combined with new opportunities coming out of the current research.

-Dr. Nancy Kelley-Loughnane research group (AFRL, OH) is using the bright bfloGFPa1 from Amphioxus as a reporter, including for riboswitch. Many academics are now using this bfloGFPa1 as well, aside from DoD labs.

-Dr. Bennett Ibey (AFRL, TX) is using the luminous brittlestar to assess effect on the nervous system of high wave radio frequency (using changes in light production as a reporter). Dr. Ibey and Dr. Ron Barnes from his teams came to the Deheyn lab twice for a period of about 2 weeks to perform experiments on the feasibility of using luminous organisms to assess effects of radio frequencies on nervous system or cell functions. A joint paper is in progress on that aspect, focusing on luminous dinoflagellates stimulated using electric fields.

-Dr. Jorge Chavez Benavides (AFRL, OH) is using the luminous brittlestar as a potential source for luminous cell culture. A Student Research Assistant (Andrea Poole) was supposed to perform a summer internship between the labs of Kelley-Loughnane (AFRL, OH) and Deheyn (SIO, CA) in 2020, which was cancelled because of Covid-19 pandemic.

-Dr. Joe Slocik (AFRL, OH) has received the new worm ferritin to assess its use with regards to the greater performances of this ferritin in biotechnological and DoD applications.

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8. NEW DISCOVERIES

Results on biochemistry and molecular biology of the luminous system of the marine worm *Chaetopterus* are new discoveries that could be used by various entities of the DoD, from biochemical engineering to biotechnology and molecular science. Both the ferritin from the worm mucus (the "engine" of the light production able to perform both oxidation and reduction), and the chromophore, reflect a totally novel light producing system.

Other proteins from the luminous mucus of *Chaetopterus* have significant similarities with proteins from the deep-sea worm *Alvinella pompejana*, which is the iconic species associated with hydrothermal vents. The fact to have only similarity with this worm is curious and of great interest considering that *Chaetopterus* is found mainly in shallow water and not in association with hydrothermal venting system (anymore at least). We hypothesize that *Chaetopterus* lives in symbiosis with bacteria that allows chemolithoautotrophy, and that by-products of this symbiosis are involved in the light production process. This association and resemblance with the deep-sea Pompeii worm is a new discovery that will offer new insights on the biology of organisms from extreme environments while helping understand the unique light production process in *Chaetopterus*.

Extraction of the chromophores with large Stokes shift are also novel discoveries. None of the model samples studied (from deepsea invertebrates to plant seed) are the current subject of investigation (to our knowledge). This might be related to the difficulty to access some of these samples, but also the difficulty in extracting and purifying some of the chromophores of interests. Nonetheless, this is what makes the research attractive to us and positioning our lab (and the AFOSR) on the forefront of cutting-edge science in the search for novel compounds with attractive spectral properties.

9. HONORS/AWARDS

- 2017 Nominated finalist to the Christoffel Plantin Prize for research excellence, Belgium.

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Appendix

Final Report 2020 - FA95550-17-1-0189

Deheyn, UCSD-SIO

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