UNIVERSITY OF CALIFORNIA SAN DIEGO

The Potential Role of Ryanodine Receptors on the Bioluminescence in the Brittlestar *Ophiopsila californica*

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Marine Biology

by

Ze Gong

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2022

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University of California San Diego

2022

DEDICATION

This thesis is dedicated to my dream of being a marine biologist since primary school.

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ACKNOWLEDGEMENTS

I would like to thank my parents who always support me financially and mentally.

I would also like to thank my boyfriend Shuai Han who spent a lot of time with me when I was upset and his family who supported me during COVID-19 when I could not go back home in China.

I also thank my friends Youran Li, Tianyun Hua, and Liu Yang who are great friends who helped both my mental and physical health during my master's study.

I would like to thank my advisor Dimitri Deheyn, Martin Tresguerres, Evelien De Meulenaere, and Eric Esteve for their intellectual and all other support through this challenging project.

I would like to thank Nicholas Holland and Horst Felbeck for giving me feedback on my thesis.

I would like to thank Phil Zerofski for collecting brittlestars for this research.

Finally, I want to give a huge thank all my lab mates in the Deheyn lab
both past and present especially Kara Wiggin and Kelley Govenar for supporting me.

This thesis contains unpublished material coauthored with Deheyn, Dimitri D., and De Meulenaere, Evelien. The thesis author was the primary author of this thesis.

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ABSTRACT OF THE THESIS

The Potential Role of Ryanodine Receptors on the Bioluminescence in the Brittlestar *Ophiopsila californica*

by

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Master of Science in Marine Biology

University of California San Diego, 2022

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Bioluminescence, the production and emission of visible light from a chemical reaction in living organisms, is found in almost every major phylum in the marine ecosystem, but only a few bioluminescent mechanisms are well understood today. The luminous brittlestar *Ophiopsila californica* produces intense green luminescence from its long arms when mechanically stimulated. Distinctively, the light producing cells (photocytes) of *O. californica* appear to be of neural origin. Understanding the mechanism of light production in this species could identify novel chemistry and cellular biology for biological light production, which could have additional

applied uses in a wide range of fields including neuroscience, other biomedical research, and commercial usages. *O. californica* bioluminescence is produced by a photoprotein that requires calcium ions as a cofactor. However, previous studies in the Deheyn lab showed that bioluminescence can also be triggered in the absence of calcium in the extracellular environment, thus indicating the possibility that intracellular calcium is somehow involved in the light production mechanism. The goal of my thesis was to investigate whether ryanodine receptors (a type of calcium channel) are involved in the bioluminescence mechanism of *O. californica* photocytes. I applied pharmacological agents known to activate or inhibit ryanodine receptors from other species on isolated cell suspensions enriched in photocytes and assessed their effect on bioluminescence. My results suggested that ryanodine receptors are not involved in the process. Further research is needed to unveil the cellular mechanisms underlying bioluminescence production by *O. californica*.

INTRODUCTION

1. Bioluminescence in Ocean

Bioluminescence is the production of visible light from a chemical reaction in living organisms. Bioluminescent species are present in almost every major phylum in marine ecosystems, and very prevalently in cnidarians, echinoderms, bryozoans, and annelids (Haddock et al., 2010). These organisms are widely distributed across the ocean both geographically and vertically from the surface to the deep sea (Haddock et al., 2010). Unlike the terrestrial environment where the sun is the primary light source, the primary source of light in most of the ocean is bioluminescence, since the aphotic zone comprises the majority of the ocean volume (Haddock et al., 2010). It is widely accepted that the ability to produce light has evolved at least 40 independent times; however, the exact number is unknown due to the difficulty of defining independent origin (Haddock et al., 2010). For example, while bioluminescence may have evolved only once in bacteria, each animal lineage that hosts symbiotic bioluminescent bacteria has independently developed specialized organs to maintain the bacteria (Haddock et al., 2010).

The widespread use of bioluminescence indicates its importance to individual fitness and its ecological impact through at least three functions: defense (startle, counterillumination, smoke screen, distractive body parts, burglar alarm, sacrificial tag, and warning coloration) (Clarke, 1963; Hasting, 1971; Young & Roper, 1976; Grober, 1988), offense (lure prey, lure with external light, stun or confuse prey, and illuminate prey) (Morin, 1983), and mate recognition and attraction (Haddock et al., 2010). Moreover, a given organism can use this ability for multiple functions (Haddock et al., 2010). But despite the importance of bioluminescence in the ocean, many unknowns remain regarding its mechanisms and functions.

2. Mechanisms of Bioluminescence

Bioluminescence is produced as energy is released during a chemical reaction (Shimomura, 2012), which often involves the oxidation of small organic light-emitting molecules called “luciferins" (Haddock et al., 2010). The light producing moiety of luciferin is known as the chromophore (Stojanovic & Kishi, 1994). There are five main known luciferin families among all the marine bioluminescent organisms including bacterial luciferin ($FMNH_2$), dinoflagellate luciferin, vargulin (also known as cypridina-type luciferin), coelenterazine, and firefly luciferin (Hastings, 1983).

In addition to luciferins, light production requires an enzyme to catalyze the oxidation reaction (Shimomura, 2012). The two main categories of these enzymes are luciferase and photoprotein. The major difference between them is that luciferase requires exogenous oxygen during the reaction, but the photoprotein is a combination of an apo-protein, luciferin and pre-existing oxygen, and thus additional oxygen is not required (Haddock et al., 2010). In the luciferase mechanism, binding to luciferase results in luciferin becoming negatively charged, which allows the oxygen to oxidize the luciferin (Figure 1A) (Shimomura, 2012). The oxidation of luciferin excites the electrons in the chromophore to an excited state, and the energy is released as photons when they return to the ground state (Stojanovic & Kishi, 1994). In the photoprotein mechanism, bioluminescence is triggered by a cofactor ion, namely Ca^{2+} or Fe^{2+} (Shimomura, 2012). As the cofactor binds to the photoprotein, the chromophore excites the electrons of the chromophore, and when these return to their ground state, they release energy that produces light (Figure 1B) (Shimomura, 2012).

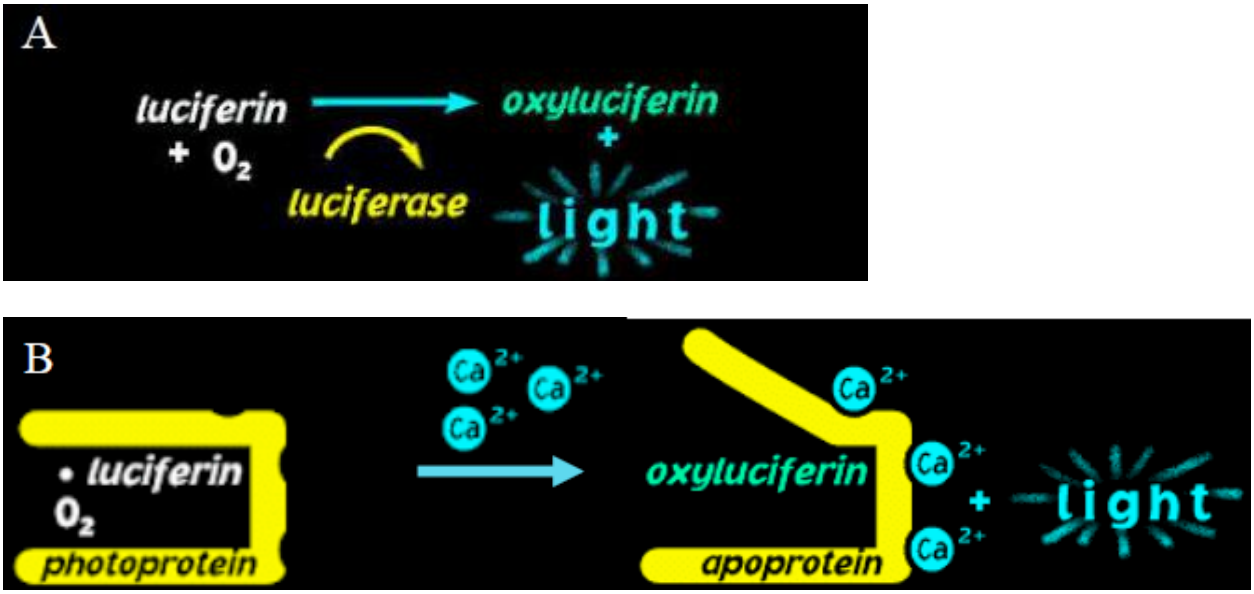

Figure 1. The two light production mechanisms (A) Luciferin-Luciferase reaction (B) Photoprotein reaction (Shimomura, 2012).

Although luciferins are conserved throughout all lineages, luciferases and photoproteins are very diverse, probably reflecting their independent origins (Haddock et al., 2010). The great diversity of luciferases and photoproteins indicates a potential for uncovering novel light production proteins, which could lead to novel applications for biological research as well as for biotechnological purposes.

3. Bioluminescence in Brittlestars

Bioluminescence seems to be ubiquitous among echinoderms except for sea urchins. Ophiuroidea (brittlestars) have numerous species that can produce bioluminescence: out of 222 brittlestar species that have been tested, 77 (or 34%) were found to be bioluminescent (Dubois et al., 2013). Brittlestar bioluminescence has been proposed to serve in multiple defense strategies against predators. One way is to emit intense light from all five arms to startle the predator away (Basch, 1988). If the predator is not startled, the brittlestar will break one of the light-producing arms in a behavior known as self-autonomy, confusing the predator by luring it away from the

main body so the brittlestar can crawl away and escape (Basch, 1988). Another way that brittlestars avoid predation is a burglar alarm strategy, which involves illuminating the predator to make it visible to a higher-level predator (Basch, 1988).

The mechanisms of bioluminescence in Ophiuroidea vary from species to species. *Amphiura squamata* and *A. fliliformis* have been extensively studied; these species use the luciferin-luciferase system and therefore need environmental oxygen during the process (Harvey, 1952). The species of interest in my thesis is *Ophiopsila californica*, which is commonly found off the coast of California (Clark, 1921). It is relatively large, with a disk diameter <15 mm and an arm length of about 100 mm (Shimomura, 2012). Their arms produce intense flashes of green luminescence (λ_{max} 510 nm) when mechanically stimulated (Shimomura, 2012). As in many other bioluminescent organisms, the light is produced in specialized cells called photocytes (Hastings & Morin, 1991). A distinguishing feature of *O. californica* is the association of bioluminescence with its radial nerve cord. In fact, commonalities with nervous tissue suggest that photocytes may even be of neural origin, leading to the hypothesis that the photocytes are under direct neuronal control (Brehm, 1977). This idea is supported by the fact that the propagation rate of bioluminescence along the arm of *O. californica* is similar to that of neurological signals along the radial nerve cord (Brehm, 1977). Furthermore, the light flashes are sensitive to drugs that affect excitable cells, thus giving this luminous system an attractive potential to report neurological and cardiac health.

However, the cellular mechanisms underlying light production in *O. californica* remain nearly unknown beyond the fact that the light production is associated with the luciferin-photoprotein system (Brehm, 1977). The cofactor of this photoprotein is still a topic of debate. Brehm (1977) proposed that the bioluminescence relies on Ca^{2+} as a cofactor. His research

showed that when the organism was placed in Ca^{2+}-free artificial sea water (ASW), it did not produce any light, and electrical activity in the radial nerve cord was only detected after the addition of Ca^{2+} (Brehm, 1977). However, another study showed that the purified photoprotein and the cell extract of *O. californica* did not produce light after the addition of Ca^{2+}, suggesting that Ca^{2+} is not directly involved in the bioluminescence activity (Shimomura, 1986). However, whether the purified protein was the actual photoprotein was not confirmed.

A recent Master's thesis from the Deheyn Lab reported that an isolated cell suspension enriched in photocytes (hereafter referred to as "isolated photocytes") was able to produce light in the absence of extracellular Ca^{2+}, provided that the Ca^{2+} ionophore A12387 was added to the incubation medium (Alferness, 2017). This result suggested that extracellular Ca^{2+} is not required for bioluminescence; however, the cellular mechanisms underlying light production in *O. californica* remain unknown, and specifically, whether the source of Ca^{2+} is from intracellular stores.

4. Ca^{2+} and Bioluminescence

Ca^{2+} is involved in the bioluminescence mechanism of several marine organisms by binding to the photoprotein as a cofactor. The first photoprotein found to be Ca^{2+}-activated was aequorin from the jellyfish *Aequorea* (Shimomura et al., 1962). Subsequently, other Ca^{2+}-activated photoproteins were discovered including obelin from the hydrozoan *Obelia longissima* (Campbell, 1974), thalassicolin from the protozoan *Thalassicola sp.* (Herring, 1979), mitrocomin from the jellyfish *Mitrocoma cellularia* (Shimomura et al., 1963), clytin from the jellyfish *Phialidium gregarium* (Levine & Ward, 1982), mnemiopsin from the ctenophores *Mnemiopsis*

sp. and berovin from the ctenophores *Beroe ovata* (Ward & Seliger, 1974a; Ward & Seliger, 1974b).

In addition to being a cofactor for photoproteins, Ca^{2+} can stimulate bioluminescence in dinoflagellates by depolarizing the membrane in response to mechanical stimuli (Dassow & Latz, 2002). The mechanotransduction cascade leads to the entry of Ca^{2+} into the cell, which in turn triggers release of Ca^{2+} from intracellular stores (Dassow & Latz, 2002). Thus, bioluminescence mechanism in dinoflagellates might be similar to excitation-contraction coupling in skeletal muscles, which also involves the coupling between mechanotransduction and mobilization of intracellular Ca^{2+} stores (Dassow & Latz, 2002). Previous research has shown that lowering the Ca^{2+} concentration in the external media inhibited the KCl-induced luminescence of three species of brittlestars, indicating that Ca^{2+} plays an important role in the bioluminescence mechanism (unpublished data from Deheyn Lab).

The fact that *O. californica* can produce light in a Ca^{2+}-free medium suggests the involvement of Ca^{2+} release from intracellular stores in the bioluminescence mechanism (Alferness, 2017). There are two main Ca^{2+} release channels families that mediate the release of intracellular stores: the ryanodine receptors (RyRs) (Meissner, 1994) and the inositol 1,4,5-trisphosphate receptors (IP3R) (Patterson et al., 2004). In mammalian myocytes, both types of Ca^{2+} channels can be present in the endoplasmic reticulum (ER), or sarcoplasmic reticulum (SR) in muscle, membranes (Meissner, 2017). An observation from Deheyn's lab showed that the propagation of bioluminescence in *O. californica* is similar to the beating pattern of cardiac muscle cells, which is mediated by RyRs. Interestingly, RyRs-like coding genes are present in several species of Asteroidea (starfish), Echinoidea (sea urchins), and Crinoidea (crinoid) (NCBI

Database). Thus, it is almost certain that brittlestars have RyRs-like channels as well. RyRs receptors are the main subject of my thesis.

The name RyRs originates ryanodine, an alkaloid from an insecticidal plant *Ryania speciosa* (Rogers et al., 1948). In mammals, RyRs are found in many cell types including myocytes, neurons, exocrine cells, and epithelial cells (Lanner, 2010). There are three RyR isoforms (RyR1–3), of which RyR1 is primarily found in skeletal muscle, RyR2 in the heart, and RyR3 in the brain (Takeshima et al. 1989; Zorzato et al. 1990; Otsu et al. 1990; Nakai et al. 1990; Hakamata et al. 1992). RyRs activity can be regulated by many factors which inhibit or stimulate Ca^{2+} release from intracellular stores, such as ryanodine, Ca^{2+}, Mg^{2+}, ATP, calmodulin (CaM), protein kinases, caffeine, and Ruthenium Red. Of these, ryanodine, Ca^{2+}, and Mg^{2+} have a biphasic effect on RyRs in muscle cells (Meissner, 2017), which means they are stimulatory up to a certain concentration, but higher concentrations are inhibitory. Specifically, RyRs are activated by micromolar ryanodine and Ca^{2+} concentrations and millimolar ATP concentrations, and are inhibited by millimolar ryanodine, Ca^{2+}, and Mg^{2+} concentrations (Meissner, 2017; Xu et al., 1998).

Caffeine (1,3,7-trimethylxanthine) can activate RyRs without affecting its sensitivity to regulation by Ca^{2+}, Mg^{2+}, and ATP (Rousseau et al., 1988; Meissner, 2017), and thus it has been commonly used as a RyR agonist (Sitsapesan & Williams, 1990; McPherson & Campbell, 1993). In sea urchin eggs, caffeine increases cyclic ADP-ribose (cADPR)-induced Ca^{2+} release, which is mediated by RyRs (Tanaka & Tashjian, 1994). Therefore, my study used caffeine to test whether RyRs are involved in the bioluminescence of *O. californica*. However, caffeine can potentially induce multiple effects in addition to stimulating RyR-dependent Ca^{2+} release from intracellular stores. For example, caffeine can also inhibit phosphodiesterase activity, leading to

intracellular increases in the levels of the signaling molecule cyclic adenosine monophosphate (cAMP) (Butcher & Sutherland, 1962). In addition, caffeine can inhibit adenosine receptors and some voltage gated Ca^{2+} channels (Fredholm, 1979; Hughes et al., 1990).

In contrast, ruthenium red is an antagonist of RyRs (Zucchi & Ronca-Testoni, 1997) and it was used in my study to test whether RyRs were involved in the bioluminescence. However, ruthenium red can interact with many proteins such as calmodulin, transient receptor potential ion channels (i.e., TRPV1-6), and Ca-ATPase (Dreses-Werringloer et al, 2013; Hajnóczky et al., 2006).

5. Project Prospects and Goals

In this study, I tested the hypothesis that bioluminescence by photocytes of *O. californica* can be triggered by Ca^{2+} from both extracellular and intracellular origin. Furthermore, I hypothesized that the latter is mediated by RyRs in intracellular Ca^{2+} stores. To test these hypotheses, I generated bioluminescence-Ca^{2+} dose-response curves by isolated photocytes. In addition, I tested the effect of the RyRs agonist caffeine, and of the antagonist ruthenium red, on photocyte bioluminescence production. I expected that bioluminescence would be stimulated by increasing concentrations of Ca^{2+} and caffeine, and that it would decrease after exposure to ruthenium red.

MATERIALS AND METHODS

1. Photocyte Isolation

1.1 Specimen Collection

O. californica specimens were dug out of the sand with a shovel by a SCUBA diver; they were transported to the Marine Biology Research Division experimental aquarium at Scripps Institution of Oceanography (University of California, San Diego) and kept in a running seawater tank with natural ambient temperature around 12 ˚C. Brittlstars were fed every other week with approximately 3g of Larval AP-100 Dry Larval Diet (Zeigler). Before each experiment, a brittlestar was randomly chosen, one arm was dissected, and the brittlestar was placed back in a holding tank. In total, five brittlestars were used during my thesis; different arms from the same brittlestar were used in different experiments but I did not keep track of their origin.

1.2 Cell Dissociation

Cell dissociation and photocyte isolation were based on the methods developed by Deheyn et al. 2000b (Figure 2). To dissociate the cells, a brittlestar was pulled out from the holding tank and placed in a jar with ~3 cm deep of seawater. The jar was put into a freezer at -20℃ for 30 minutes to anesthetize the brittlestar (but ensuring that it was not frozen to death), which was assessed by the lack of arm movement. This method was used because the traditional anesthetizing method with 3.5% $MgCl_2$ could inhibit RyRs (Meissner, 2017). After anesthesia and with the brittlestar still in the jar, about 10 cm of an arm was cut using a blade or a pair of scissors and placed into a mortar on ice. The rest of the brittlestar was left in the jar to let it recover from the freezing, which took about 10 minutes. Once the brittlestar moved its arms normally, it was placed back into its holding tank.

The dissected arm was gently ground up using a mortar and pestle until the arm was segmented into <2mm pieces. The ground tissue was placed into a 1.5 mL Eppendorf tube; if the mixture was greater than 0.5 mL it was divided into two tubes. 1 mL of buffer (0.5% protease (P5147 SIGMA) dissolved in 25 mM TRIS-HCl, 500 mM NaCl in Milli-Q water, pH 8.1) was added to each Eppendorf tube. This buffer was chosen instead of artificial seawater to prevent the potential interference of Ca^{2+} and Mg^{2+}on the photocytes and RyRs. Also, the buffer had similar osmolarity as seawater to prevent cell swelling and bursting or shrinking. The tubes containing the tissue mixture were covered with aluminum foil to keep them from light and placed on their sides on an oscillating incubator at 28˚C for 35 minutes at 200 RPM to pellet down large debris. After incubation, the mixture was taken out and placed vertically on a rack. The supernatant was pipetted into another Eppendorf tube and centrifuged for 10 minutes at 2,000 RPM at 4℃ to pellet down the dissociated cells. The supernatant was removed, and the cell pellet was resuspended in 200-300 L buffer and stored on ice to maintain cell viability. The process was repeated twice more on the debris-containing pellet a, resulting in ~1 mL resuspended cells that were stored in the buffer on ice.

1.3 Photocyte Isolation

After dissociation, photocytes were enriched relative to other cell types by using a Percoll gradient in a Beckman L8–70 ultracentrifuge (rotor SW 41). A 63% Percoll solution was diluted with buffer resulting in an ~ 11 mL mixture that was placed in an ultracentrifuge tube. 1 mL of the dissociated cell mixture was gently added on top of the Percoll solution and centrifuged for 30 minutes at 20,500 RPM at 4℃ with acceleration level at 1 (which accelerates the rotor at the fastest speed), and level 0 deceleration (which allows the rotor to stop freely without applying

any friction so the solution would not be disturbed by the sudden stop of the rotor). It took about 10 minutes for the rotor to stop completely in this action.

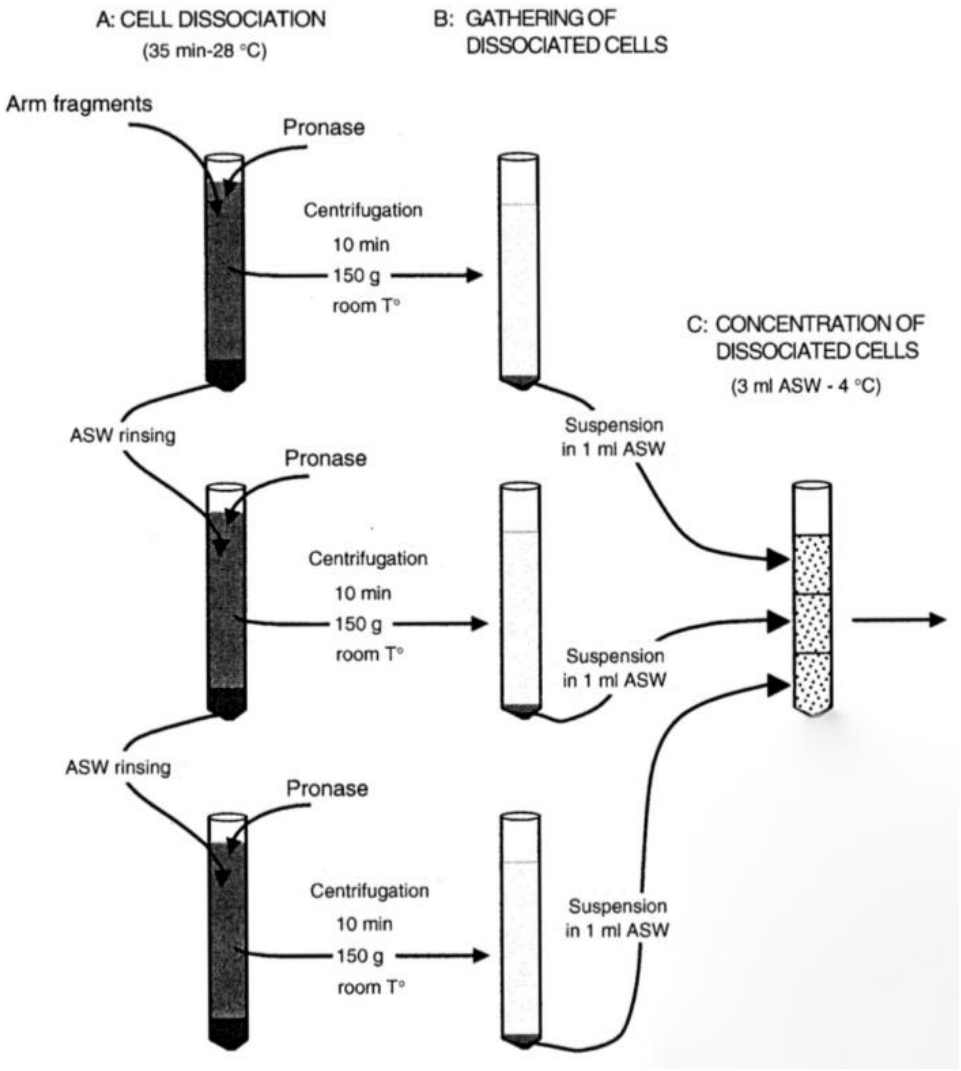

Figure 2. Procedure for dissociating photocytes from brittlestar arms (ASW = artificial seawater) (taken from Deheyn et al. 2000b).

After ultracentrifugation, there was a visible thin brown band of cells in the upper 1/5 portion of the tube, which has the most robust light production and therefore is enriched in photocytes (Deheyn, 2000b) (Figure 3). This cell-containing band was transferred to a fresh Eppendorf tube using a glass pipet, taking great care to not collect other portions of the solution. The consistency of the band was oily, and the cells were attached together, which made removal easy. The volume of this fraction typically was ~200 μL.

The remaining Percoll solution was washed from the photocytes by adding 1 mL buffer and centrifugating for 10 minutes at 2,000 RPM at 4℃. The photocyte-containing pellet was resuspended in buffer, the exact volume depended on the specific experiment. The photocyte-containing mixture was divided into aliquots of equal volume, and each aliquot was placed in a luminometer-measuring tube and stored on ice to maintain the cell viability until the experiments started, which was normally within 1 h and always less than 24 h.

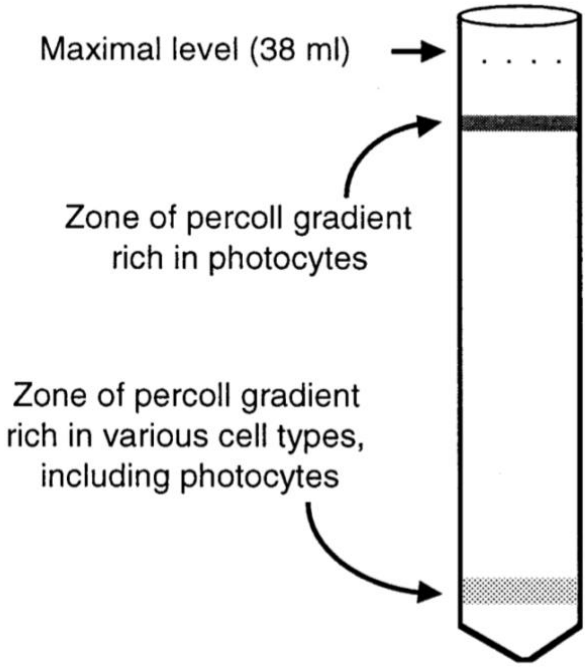

Figure 3. Distribution of photocytes along the Percoll gradient after ultracentrifuge (taken from Deheyn 2000b).

2. Luminescence Kinetics

Bioluminescence was measured as the kinetics of the intensity of light produced within a certain time period using the Sirius single-tube luminometer (Berthold Detection Systems). The luminometer was programmed to inject two solutions automatically and sequentially while the photocytes remained inside the luminometer. Each injection was added in equal volume-to-volume ratio relative to the photocyte sample to ensure good mixing. The sample volume was 20

μL, 30 μL, or 50 μL depending on the experiment. The first injection volume was the same as the sample. The second injection volume was double (i.e., 40 μL, or 60 μL, or 100 μL, respectively). The first injection was set at the 31st second and the second injection was set at the 61st second. The initial 30 seconds of measurements served to determine the background bioluminescence of each sample. During 31st60th seconds, the effect of the first injection was measured. During 61st90th seconds, the effect of the second injection was measured. All the experiments in this study used this kinetics measurement method.

3. Ca^{2+} Dose Effect on Bioluminescence of *O. californica*

The goal of this experiment was to determine the concentration of external Ca^{2+} needed to trigger bioluminescence by *O. californica* photocytes, and to establish the optimal Ca^{2+} concentration for subsequent experiments. A previous study at the Deheyn Lab used 400 mM $CaCl_2$ in the buffer resulting in a consistent stimulation of bioluminescence (Alferness, 2017). In my experiment, a series of concentrations of $CaCl_2$ was added to 20 μL photocyte-enriched suspension: 0.4 μM, 4 μM, 40 μM, 400 μM, 4 mM, 40 mM, 400 mM dissolved in buffer. After measuring the background bioluminescence of each sample, the first injection was either the buffer or one of the $CaCl_2$ concentrations (Figure 4).

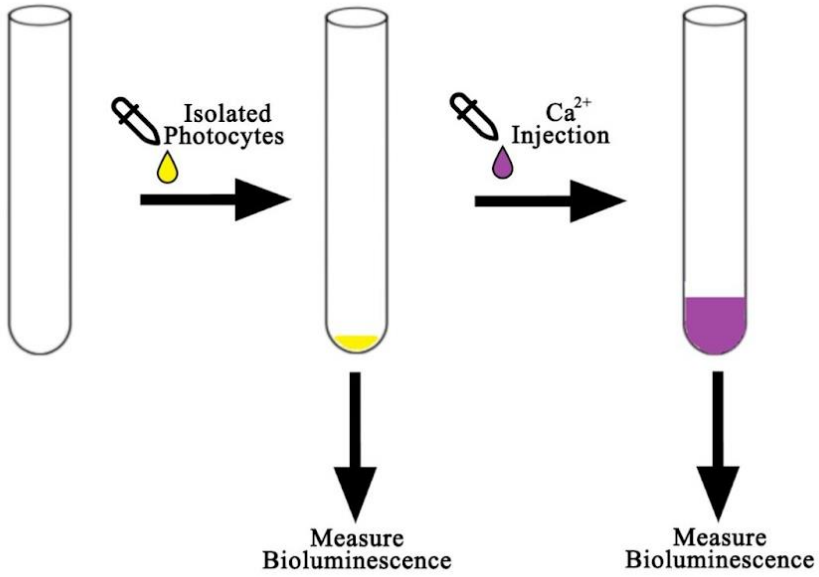

Figure 4. The process of the Ca2+ Dose Effect experiment. The isolated photocytes solution was hand-injected. The first and second injections were injected by the luminometer, which were added on a volume-to-volume ratio to the existing volume.

4. Effect of Caffeine on Bioluminescence of *O. californica*

The effect of caffeine on the light production of *O. californica* was tested by adding 0.2 mM, 2 mM, and 20 mM; these concentrations were chosen based on previous studies on mammalian cells (Meissner, 2017). After measuring background luminescence, caffeine was injected, and luminescence kinetics was measured for 30 seconds. Then, the second injection of 400 mM $CaCl_2$ (dissolved in buffer) was added as a positive control to ensure that the photocytes were capable of producing light, and to determine the full bioluminescence capacity and standardize the effect of caffeine (Figure 4).

In another experiment, I used Milli-Q water as the solvent for caffeine and tested its effect on bioluminescence. This experiment was performed as described above, but caffeine was dissolved in Milli-Q water instead of buffer.

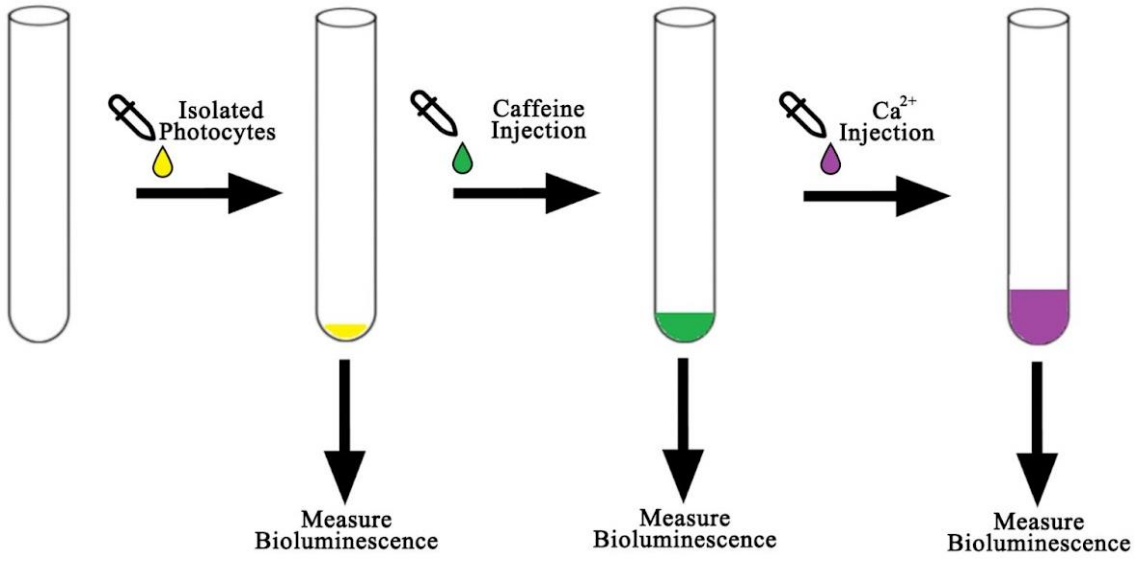

Figure 5. The process of the Effect of Caffeine experiment.

5. The Effect of Shaking on Bioluminescence of *O. californica*

During my initial experiments., I observed that luminescence intensity seemed to be much higher if the tubes containing the isolated photocytes were shaken during handling. To test the effect of shaking on light production, the tubes were carefully transported to the luminometer keeping as minimal agitation as possible. After the initial 30 seconds to measure background bioluminescence, the tube was taken out of the luminometer and vigorously shaken 10 times but ensuring that no liquid was spilled out, which took about 20 seconds. Then the tube was put into the luminometer again and bioluminescence was measured for 30 seconds.

6. The Effect of Ruthenium Red on Bioluminescence of *O. californica*

I hypothesized that if RyRs were involved in the photocyte bioluminescence mechanism, addition of the RyR antagonist, ruthenium red, would decrease bioluminescence. Testing this hypothesis required to stimulate bioluminescence after the addition of ruthenium red. However, the stimulation could not be done by adding Ca^{2+} because this would by-pass the putative Ca^{2+}

release from intracellular stores, even if ruthenium red was effective. Thus, I used shaking to stimulate bioluminescence. To my knowledge, the effect of ruthenium red on brittlestar photocytes was never tested, so I conducted a dose-response curve with 0.02 μM, 2 μM, 20 μM, and 200 μM ruthenium red. After measuring background bioluminescence of a 50 μL sample, 50 μL of either buffer or ruthenium red was injected into the luminometer and bioluminescence was measured for 30 seconds. The tube was removed from the luminometer and incubated for 10 minutes at room temperature to allow ruthenium red to enter the cells. Subsequently, the tube was carefully placed into another luminometer, and background bioluminescence was measured for 10 seconds. Then, the tube was vigorously shaken 10 times as described above, placed back into the luminometer, and bioluminescence was measured for another minute (Figure 5). In the end, 400 mM $CaCl_2$ was injected into the solution as a positive control to ensure that every sample was still viable to produce light.

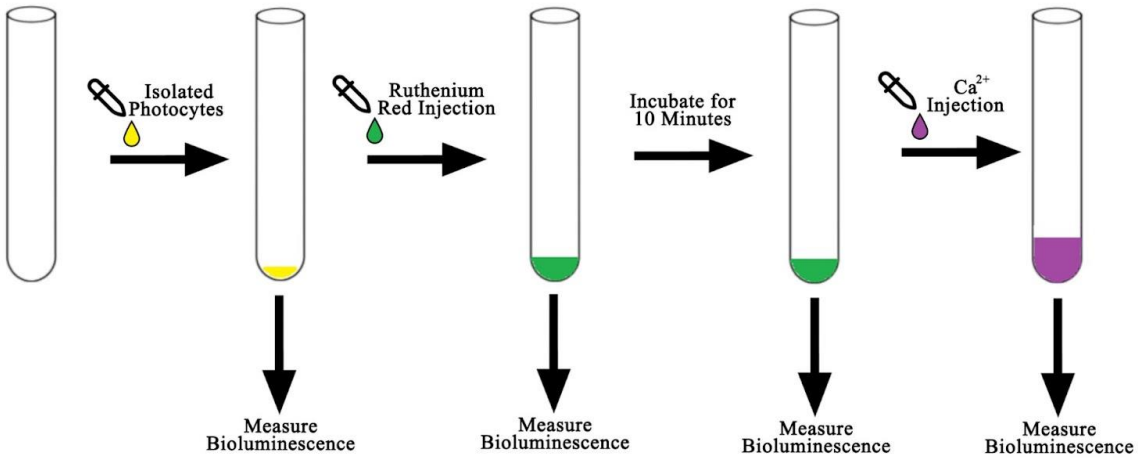

Figure 6. The process of the Effect of Ruthenium Red experiment.

7. Bioluminescence Analysis

Photocyte bioluminescence was estimated from the luminescence kinetics data provided by the luminometer, which collects light every 0.2 second and is integrated over the measuring period (30, 60, or 90 seconds). To account for the innate variability of the samples, all measurements were standardized over the background luminescence signal obtained during the initial 30 seconds of each experiment. Importantly, many of the previous studies did not correct for the background, so my method is likely to produce more accurate and reproducible results.

RESULTS

1. Ca^{2+} Dose Response on Bioluminescence of *Ophiopsila californica*

This experiment tested whether external Ca^{2+} can trigger bioluminescence and to determine the optimal Ca^{2+} concentration for subsequent experiments. Eight different solutions were added to the photocytes: buffer only, and 0.4 μM, 4 μM, 40 μM, 400 μM, 4 mM, 40 mM, 400 mM Ca^{2+} so that the cells were exposed in 0, 0.2 μM, 2 μM, 20 μM, 200 μM, 2 mM, 20 mM, 200 mM $CaCl_2$ final solution. After measuring the background bioluminescence of each sample, either the buffer or one of the $CaCl_2$ concentrations was injected into the luminometer. As shown in Figure 6, here were significant difference in bioluminescence between the addition of different Ca^{2+} concentrations ($F = 2.542$, p-value = 0.0361).

The photocyte isolation protocol for this experiment was slightly different from other experiments as this was the very first experiment performed. During the process, the deceleration speed of the ultracentrifuge was not set to 0 (no deceleration) but to 1 (max deceleration), which could result in a significant loss of photocytes during this step, leading to very few photocytes for the Ca^{2+} Dose Response Experiment. This might affect the activity of the photocytes. Therefore, this experiment should be redone to confirm the dose response of Ca^{2+}.

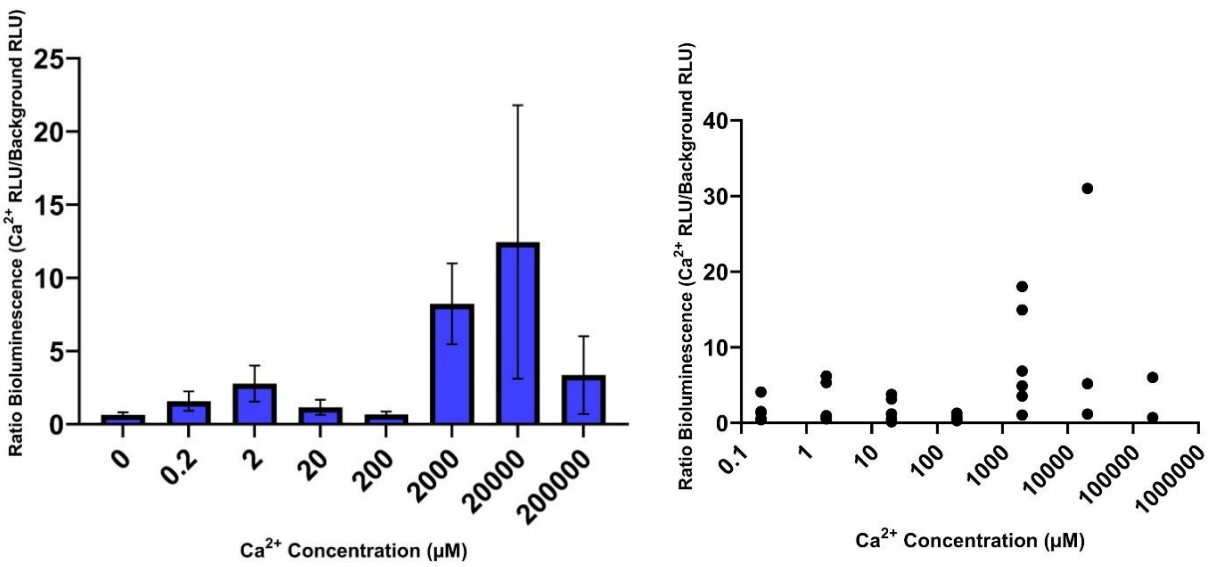

Figure 7. The result of the Ca2+ Dose Effect experiment. The scatter plot showed the ratio between the average of the integral bioluminescence after being injected with different concentrations of CaCl2 and that of the background.

2. Effect of Caffeine on Bioluminescence of *O. californica*

To test whether RyR are involved in the bioluminescence of *O. californica*, the RyR agonist caffeine was added to photocyte preparations. I hypothesized that caffeine would trigger bioluminescence. To test this hypothesis, I added caffeine at three concentrations (0.1 mM, 1 mM, and 10 mM) and buffer alone to photocytes and measure bioluminescence. The first 30 seconds measured background bioluminescence, and the next 30 seconds measured the bioluminescence after being injected with either caffeine or the buffer. The third 30 second-period measured the bioluminescence after the injection of 400 mM $CaCl_2$ into the mixture, which was a positive control to see whether the photocytes were viable to produce light.

As shown in Figure 7, there were no significant differences in bioluminescence between the background measurements and after the injection of caffeine in the absence of external Ca^{2+} (n = 5, Kruskal-Wallis statistic = 0.3371, p-value = 0.9529). Isolated photocytes demonstrated higher bioluminescence in the presence of 200 $CaCl_2$, and addition of higher concentration of

caffeine induced a significant decrease (n = 5, p = 0.0063). This result suggests that RyR-dependent release of Ca^{2+} from intracellular stores do not stimulate light production by *O. californica* photocytes, and instead may inhibit Ca^{2+} entry into the cell. Alternatively, it is possible that caffeine does not stimulate the RyR of *O. californica*.

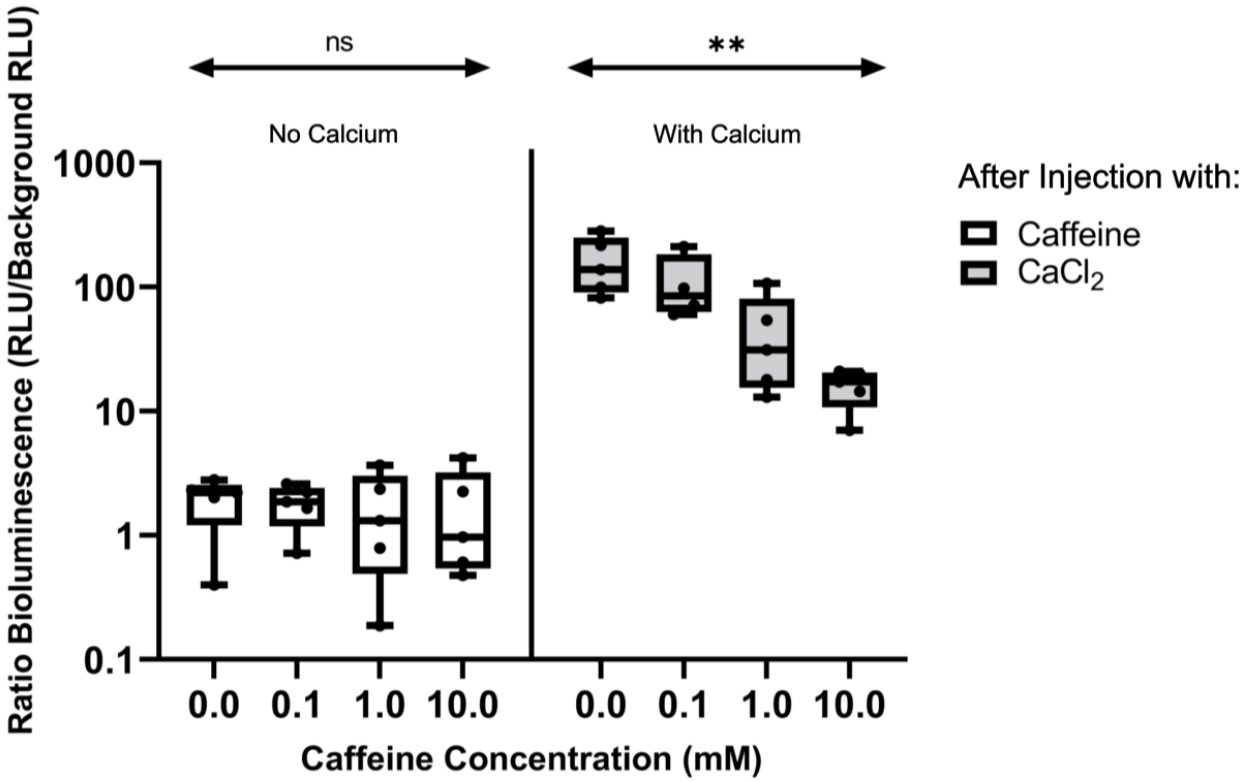

Figure 8. The result of the Effect of caffeine experiment. The boxplots were the ratio between the bioluminescence after injection of caffeine and that of the background.

3. Effect of Agitation on Light Production

After I observed an increase in bioluminescence in samples that were accidentally shaken during transport into the luminometer, I decided to test whether mechanical stimulation or sudden oxygenation would affect bioluminescence production.

Although there was no statistical significance after shaking the samples (t = -1.1752, df = 4.7358, p = 0.2956) (probably due to high variance among samples), there was a trend for

increased mean bioluminescence after shaking (Figure 8). This result suggested that the bioluminescence mechanism in *O.* californica photocytes might be activated by mechanical stimulation, sudden oxygenation, or both.

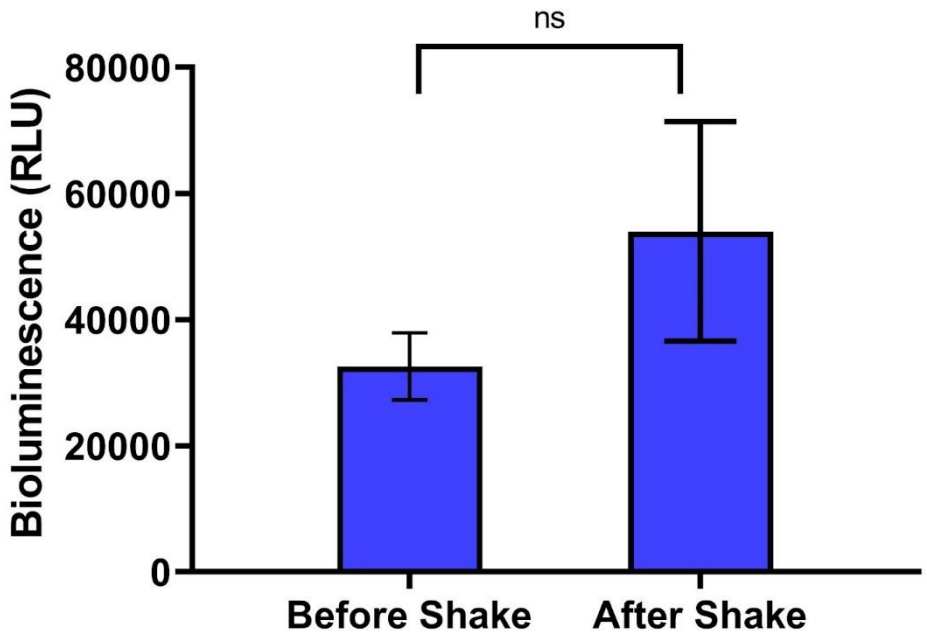

Figure 9. Effect of shaking on shaking-induced bioluminescence in *O. californica.* The barplots were the bioluminescence before shaking and after shaking.

4. Effect of Ruthenium Red on Bioluminescence of *O. californica*

Since ruthenium red has an inhibitory effect on Ca^{2+} release from intracellular stores through RyRs, an effect on *O. californica*'s bioluminescence would only be evident following stimulation *via* an extracellular Ca^{2+}-independent pathway, such as shaking.

The addition of different concentrations of ruthenium red had a significant effect on the bioluminescence of *O. californica* after shaking ($t = 2.965$, $df = 4.7358$, $p = 0.04130$); however, the correlation coefficient between the light intensity and the concentration of ruthenium red was not significant ($r^2 = 0.002939$, $F = 0.08253$, $p = 0.7760$). This result meant that blocking the release of calcium from ryanodine receptors did not always lead to a decrease of

bioluminescence after shaking, which indicated that when the bioluminescent pathway was mechanically activated, the role of ryanodine receptors was not clear and needs further research.

The result of the addition of $CaCl_2$ had a similar pattern. There was a significant difference of bioluminescence for different concentrations of ruthenium red (t test: t = 6.196, df = 4, p = 0.0035), but the correlation between the bioluminescence and concentrations of ruthenium red was not statistically significant (r^2= 0.008374, F = 0.2365, p = 0.6306). The pattern between the ruthenium red concentrations and bioluminescence was unclear, which needs further research to find their relationship and the reason for the difference.

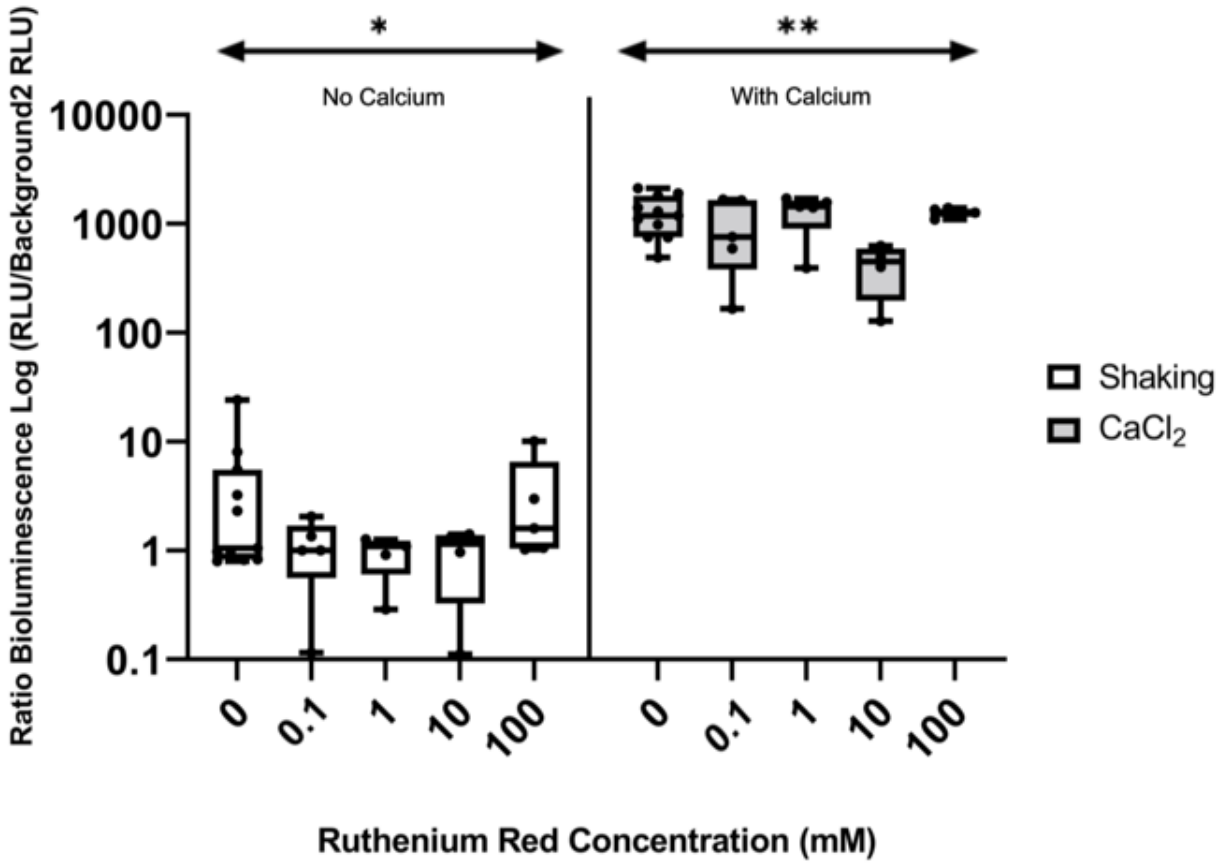

Figure 10. The result of the Effect of Ruthenium Red experiment. The boxplots are the ratio between the bioluminescence after shaking followed by the injection of different concentrations of caffeine and that of the second background.

5. Methods Optimization: Compare the Effect of Milli-Q and buffer as the Solvent for Caffeine

Initially, Milli-Q water induced a larger increase in bioluminescence compared to buffer However, photocytes treated with Milli-Q had dramatically decreased bioluminescence after the subsequent injection of Ca^{2+}. The total amount of bioluminescence produced for using buffer as solvent was significantly different than using Milli-Q water as solvent (ANOVA test: F = 9.65, p = 0.0037). This result suggests that Milli-Q water has a large negative effect on cell function, perhaps inducing swelling and lysing. These results align from previous reports indicating that photocyte integrity is essential for bioluminescence (Alferness, 2017).

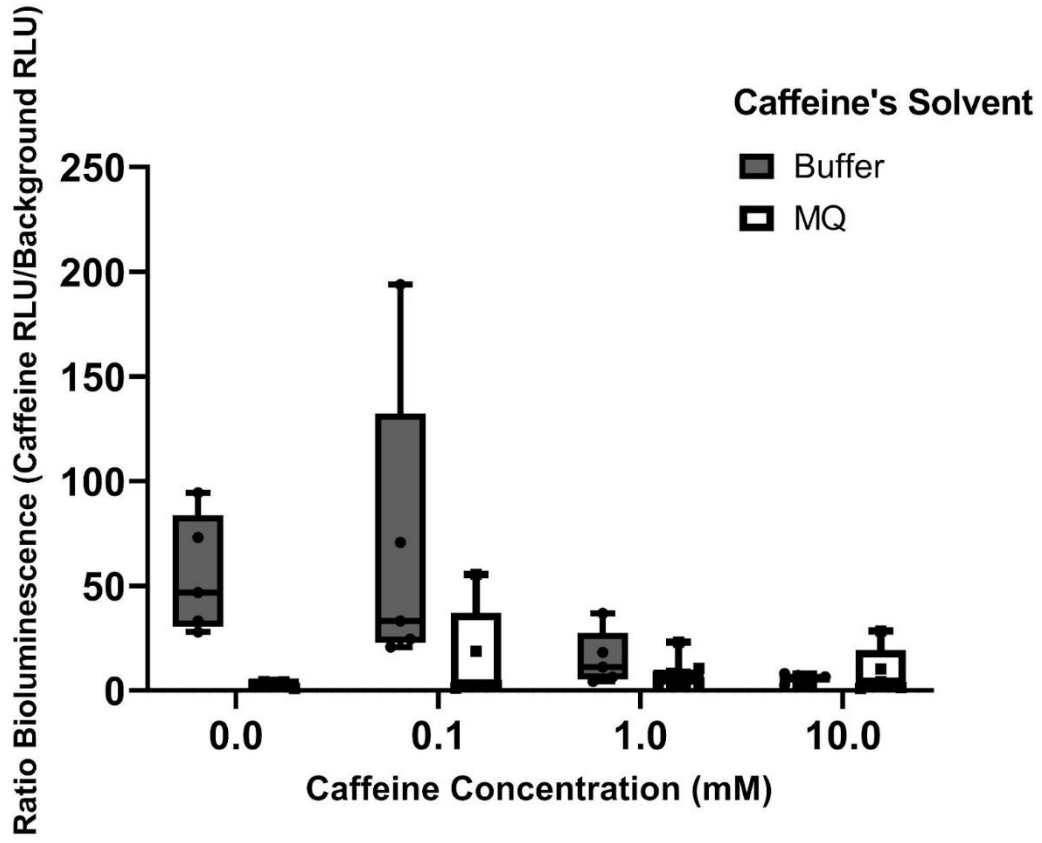

Figure 11. Comparison of using the buffer and Milli-Q water as solvents for caffeine on the total amount of Ca2+-induced bioluminescence in *O. californica.*

DISCUSSION

1. Extracellular Ca^{2+}

My results show that addition of extracellular Ca^2 can trigger bioluminescence of *O. californica*; however, it did not induce a clear dose-response. It is possible that when there is high concentration of Ca^2 in the cytosol, ATPases move Ca^2 into the intracellular stores and block the entry of Ca^2 to ensure the homeostasis of Ca^2 in the cytosol.

Naturally, extracellular Ca^{2+} can be imported into the cells by store-operated calcium entry (SOCE), which is the mechanism in maintaining cellular Ca^{2+} homeostasis and signaling (Hogan & Rao, 2015). When cells are affected by some physiological agonists, intracellular Ca^{2+} is released from intracellular stores and mobilize out of cells, resulting in depletion of Ca^{2+} in cisternae of ER (Hogan & Rao, 2015). This depletion triggers the import of extracellular Ca^{2+} into the cells to refill cellular Ca^{2+} stores (Hogan & Rao, 2015). Moreover, Ca^{2+} can also induce Ca^{2+} release from intracellular stores via IP3R and RyR, which known as calcium-induced calcium release (CICR) (Bootman et al., 2002). In the Calcium Dose Response experiment, it was possible that the high concentrations of extracellular calcium diffused into the cells, inducing CICR and making more Ca^{2+} available in cytosol for photoproteins to produce light.

2. Two Possible Bioluminescence Mechanisms

The experiments with caffeine and ruthenium red were performed as an indirect way to test whether RyRs are involved in the bioluminescence mechanism of *O. californica*. The main idea was that the RyR agonist caffeine would induce bioluminescence production, and that the RyR antagonist ruthenium red would inhibit it. However, caffeine not only did not induce

bioluminescence under Ca^{2+}-free conditions, suggesting that RyR may not be involved in releasing Ca^{2+} from intracellular stores. However, it unexpectedly inhibited it during Ca^{2+}-containing conditions. These results could be explained by caffeine blocking the entry of Ca^{2+} into the cells (for example, by inhibiting Ca^{2+} channels in the cell membrane of the photocytes), or somehow affecting bioluminescence otherwise. Although caffeine has been used as an agonist for RyRs for many studies, other research has shown that caffeine could block the RyRs channels (Xu et al., 1998; Meissner, 2017). However, my dose response would have captured both possible responses. Further studies are needed to understand the mechanism of the effect of caffeine on brittlestar bioluminescence.

Additionally, my experiments showed that mechanical stimulation can trigger bioluminescence by brittlestar photocytes in the absence of external Ca^{2+}. This is similar to dinoflagellates, which produce bioluminescence in response to shear force (Latz et al., 1987). In addition, Brehm (1977) has found that the luminescence in the radial nerve cord of intact brittlestar could be stimulated by either mechanical activation or electrical activation. My study suggests that isolated photocytes respond to similar mechanical stimuli as photocytes *in situ* in the living animal. A possible pathway is that the mechanosensing receptors on the plasma membrane experience the shear or stretch of cells, sending a signal to a secondary messenger such as IP3, which binds to IP3R, triggering intracellular Ca2+ release and produce bioluminescence. However, the shaking experiment may also because shaking allows more oxygen get into the cells, which may fuel the photoprotein.

3. Cell integrity is important for light production

Photocytes treated with Milli-Q water could barely produce light. This result aligns with a previous study that lysed the cells using four different methods including physical shearing,

sonication, bead beating, and homogenization, and found that the resulting supernatants had decreased the bioluminescence activity to less than 1% of the original activity (Alferness, 2017). Both studies suggest that cell integrity is required for the bioluminescence reaction. One possibility is that the photocyte plasma membrane creates a voltage potential difference between the cytoplasm and the external environment that is important for light production. A similar concept could apply to intracellular Ca^{2+} stores, which could also be disrupted by lysing. Another, perhaps more plausible, possibility is that the bioluminescence requires the photoprotein and other components in proximity. After rupturing the cells, the components are too diluted to react with each other. This could explain the initial exhaustion of bioluminescence that later cannot be restored.

4. Future research

The results from my experiments can be used to design future experiments and generate additional hypotheses. First, it is important to confirm whether the photocytes do have RyRs. This could initially be tested by examining the effect of ryanodine addition on photocyte bioluminescence and confirmed by studies to clone RyR and establish their cellular and subcellular localizations in brittlestars. For example, if RyR are indeed present in the photocytes, they could be present on the plasma membrane or in the ER or SR membranes. In these later cases, then other Ca^{2+} channels must be involved in the influx of Ca^{2+} into the cell and subsequent effect on bioluminescence. This could be explored using Ca^{2+} imaging experiments in live isolated photocytes. This technique could also be used to test whether caffeine blocks the entry of Ca^{2+}. Thirdly, IP3R may be involved in the bioluminescence. To see whether IP3R is involved in the bioluminescence, similar methods for this thesis can be used by first applying IP3 to the photocytes to confirm the existing of IP3R. Then, using IP3R specific agonist and

antagonist to see how they affect bioluminescence. Finally, it would also be interesting to look at whether Ca^{2+}-induced bioluminescence and mechanical-induced bioluminescence can interact with each other.

This thesis contains unpublished material coauthored with Deheyn, Dimitri D., De Meulenaere, Evelien. The thesis author was the primary author of this thesis.

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