극지유전체 101 프로젝트: 극지생물 유전체 정보 분석 및 활용기반 구축 (국외위탁과제)

Polar Genome 101 Project

Biochemical regulation of dormancy and resurrection from dormancy in the Antarctic copepod, *Boeckella poppei*

2020. 03. 30.

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Specifications and Formats of Final Report

1. Cover
2. Submission
3. Summary
4. Table of Contents
5. Report Body
6. Back Cover

Submission

To : Chief of Korea Polar Research Institute

This report is submitted as the final report (Report title:" PE18070-18090_final_report") of entrusted research "Biochemical regulation of dormancy and resurrection from dormancy in the Antarctic copepod, *Boeckella poppei*" project of "Polar Genomics 101 Project: Genome analysis of polar organisms and establishment of application platform" project.

2020 . 01 . 28

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Summary

I. Title: Biochemical regulation of dormancy and resurrection from dormancy in the Antarctic copepod, *Boekella poppei*.

II. Purpose and Necessity of R&D

Climate change, anthropogenic chemicals, and anthropogenic activities are altering the physical and chemical structure of Antarctic lakes. Unfortunately, this is occurring before we fully understand these diverse lacustrine environments. Zooplankton are positioned at the apex of a simple food web in Antarctic lakes, because macrofauna are not present. Their position as an apex organism makes them ideal indicator species. The first step to using zooplankton as indicator species is to study their physiology in an ecological context, which is the purpose of this project.

III. Contents and Extent of R&D

This project is the first to study mechanisms of dormancy and biochemical events in post-dormancy development of the Antarctic zooplankton, *Boeckella poppei*. Storage and culture conditions were optimized. Anoxia tolerance and freeze tolerance of the dormant embryo were then tested. Ultrastructure, phosphagens and intracellular pH during post-dormancy development are also assessed. To provide ecological context and relevance for this study and future research, physical, chemical and biological variables in Antarctic lakes were added to the study. Because lipophilic toxicants were found in lake sediments, the permeability of embryos to lipophilic chemicals was tested.

IV. R&D Results

The data generated in this project demonstrates that the Antarctic zooplankton, *Boeckella poppei*, has at least three overwintering mechanisms available that are ecologically relevant: diapause, anoxia-induced quiescence, and freeze tolerance by partial dehydration. Dormancy under non-freezing conditions is associated with an

acidification of the intracellular environment similar to that observed in the brine shrimp, *Artemia franciscana*. Early development after exit from dormancy differs from *A. franciscana* in that cell proliferation and utilization of lipid stores occurs prior to hatching of the free-swimming larval stage. The embryos are permeable to lipophilic toxicants, and mobilization of lipid stores during early development would release lipophilic toxicants that passively partition to lipid stores during prolonged dormancy. This is important, because this study also demonstrates that lipophilic toxicants are present in Antarctic sediments where the embryos are deposited. A surprising result of the field studies is that B. poppei is present at high abundance in only a small number of Antarctic lakes, despite close proximity of the lakes. No physical, chemical or biological variables explain this distribution.

V. Application Plans of R&D Results

The purpose of this project was to provide a biochemical and developmental foundation for assessments of anthropogenic impacts on zooplankton in Antarctic freshwater lakes that will be actionable for research and military base authorities on King George Island. Future research is needed to elucidate the mechanisms underlying dormancy in embryos of the copepod, *B. poppei*, and to evaluate the susceptibility of those mechanisms to climate change and anthropogenic chemicals.

Table of Contents

Chapter 1 Introduction (Purpose and Necessity)

As a strategy to maintain the population in a harsh environment, zooplankton species on all seven continents of the world produce embryos capable of surviving decades to centuries in a cryptobiotic state [1-7]. While evolutionary biologists and ecologists have explored the impacts of cryptobiotic "egg banks" on lacustrine communities for decades [1, 2, 8-10], the implications of anthropogenic influence on these important storehouses of genetic diversity remain largely unstudied [5]. The list of lipophilic chemicals of anthropogenic origin in Antarctic waters, organisms, and ice is expanding rapidly, but almost nothing is known about the fate or impact of these chemicals in freshwater lakes. Furthermore, the effect of these chemicals on lake biota is complicated by simultaneous and complex changes in thermal challenges resulting from the loss of lake ice and meltwater input from catchment snowpack during the austral summer. We propose that emerging chemical and thermal challenges jeopardize lake ecosystems by endangering zooplankton that comprise the top of simple trophic cascades. We further propose that the most serious potential impact is a loss of decades to centuries of stored genetic diversity in the form of decreased recruitment from sediment "egg banks" (dormant embryos). Recent work on daphnids demonstrates that embryonic and maternally-derived cuticular structures fail to protect dormant embryos from lipophilic compounds like pesticides and PCBs [5, 11]. Both the abundance and hatchability of embryos for estuarine calanoid copepods decrease following brief exposures to the lipophilic pesticide, rotenone [12]. By contrast, the embryos of the anostracan, *Artemia franciscana*, are protected from lipophilic chemicals by a proteinaceous shell of maternal origin [13]. A similar proteinaceous shell surrounds the Antarctic and subantarctic anostracan, *Branchinecta* spp. [14]. However, embryos of the Antarctic copepod, *Boeckella poppei* (Mrázek, 1901), appear to lack a proteinaceous shell, and, like other calanoid copepods, are susceptible to the lipophilic pesticide, rotenone (Covi, Lee and Park, unpublished observations). Thus, the impacts of lipophilic chemicals on embryos of *B. poppei*, is highly likely. Likewise, the loss of meltwater input before the end of the austral summer makes it likely that lake temperatures will increase during and after females produce the diapause embryos that carry the population through the austral winter. It is only a matter of time until these impacts manifest on *B. poppei* populations.

Chapter 2 Current R&D Status in Korea and Other Nations

The recognition and mitigation of human influence in Antarctica is one of the six thematic priorities identified in 2014 by the Scientific Committee on Antarctic Research [19], and it is critical that we understand the physiology of key species in all ecosystems in Antarctica in order to assess the degree of threat these systems face from anthropogenic activities and climate change. Freshwater zooplankton occupy apex positions in simple trophic webs that will be severely altered if they are lost, because low diversity ecosystems are especially vulnerable to global climate change [20]. Populations of Antarctic zooplankton are also uniquely isolated; while unsupported arguments have been made for rapid colonization of lakes by *Boeckella poppei* [21], freshwater calanoid copepods do not disperse efficiently, and it is probable that this species survived the last glacial maxima in Antarctic refugia rather than recolonizing the continent from subantarctic sites [22]. There are 21 confirmed species of crustacean zooplankton found in Antarctic lakes [22, 23]. Of these, five freshwater species and one saline species are found on the South Shetland Islands or Antarctic Peninsula [22], which is at the forefront of impacts from human activity and warming trends. It is therefore surprising that no research has been conducted on the dormant embryonic stages that facilitate overwintering of freshwater zooplankton. This is very concerning, because ancient and relatively isolated populations of zooplankton in freshwater lakes are under increased pressure from the loss of cold water input from snowpack due to climate change, the deposition of anthropogenic chemicals, and the introduction of non-native species. Because there is an almost complete lack of the most basic information about dormancy and development in freshwater zooplankton endemic to Antarctic lakes, it is critical that we study both development and the physiology of dormancy in the context of realistic short-term anthropogenic stressors.

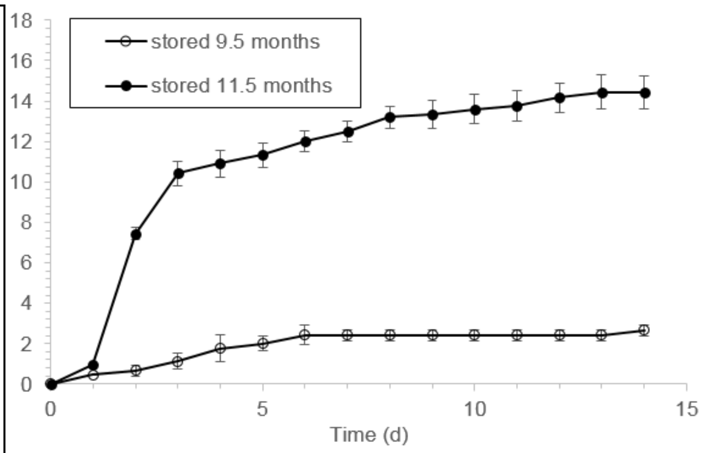
The most profound examples of dormancy in zooplankton are presented by copepods. Embryos of multiple species of calanoid copepod from North America remain viable and dormant in bottom sediments of lakes and estuaries for 60 to 332 years [2, 24], and embryos of the calanoid copepod, *B. poppei*, were recently isolated from 196 year old sediments in freshwater lakes on King George Island in the South Shetland Islands, Antarctica [3]. The metabolic status of dormant zooplankton embryos is, without a doubt, most thoroughly studied in the brine shrimp, *Artemia franciscana*; metabolic suppression is so complete in hydrated dormant embryos of this anostracan that heat production and substrate utilization are nearly undetectable [7, 25-27].

Two distinct states of embryonic dormancy are recognized in zooplankton: diapause and quiescence. Diapause is induced in the gastrula stage as part of a developmental program, and must be broken for development to continue [28]. By contrast, quiescence is environmentally controlled, and prevents the embryo from developing under non-permissive conditions, including anoxia [28, 29]. When environmental conditions are permissive, and the diapause state is broken, embryos resurrect from their dormant state, and rapidly emerge as motile larvae [2, 30-32]. Thus, there are two periods of environmental challenge to consider for zooplankton in Antarctic freshwater lakes: (1) challenge during embryonic dormancy and (2) challenge during early development after resurrection from dormancy. The study proposed here is entirely unique for Antarctic research, because it will provide a timely biological foundation for predicting anthropogenic impacts on zooplankton. To the best of our knowledge, this will be the first study to assess the role of intracellular pH, adenylate status, guanylate status and glycolytic enzyme activity in establishing and recovering from a reversible state of dormancy in a copepod species.

B. poppei: an ideal model for the study of anthropogenic influence in Antarctic freshwater lakes

The vast majority of *B. poppei* embryos are in the diapause state in Antarctic lake sediments, and storage at 4°C followed by light exposure breaks diapause. When embryos from a freshwater lake on King George Island were separated from natural lake sediment 9.5 months after collection, 100% of the embryos appeared intact under 1,000x magnification (Covi, Lee and Park, unpublished observations), but only 2.7% hatched during the first six days of incubation at 4°C under constant lighting (Fig. 1). No additional embryos hatched during the subsequent week. Recent work on *Daphnia* spp. demonstrates that prolonged storage in natural sediments, with subsequent light exposure, breaks the diapause state [5]. A similar phenomenon is observed for *Artemia* spp., in which prolonged dark storage followed by light exposure synchronizes hatching [33]. Breakage of diapause in *B. poppei* appears to require treatments similar to that of *Daphnia* and *Artemia* spp.; 12.3% of *B. poppei* hatched after six days at 4°C with constant lighting if the embryos were stored for 11.5 months at 4°C in natural sediments (Fig. 1). This is 4.3 fold greater than that observed after 9.5 months of storage ($p<0.0009$). Furthermore, hatching in the population was more synchronous after 11.5 months of storage (Fig. 1). Based on these data, we predict that between one third and one half of the embryos collected in 2015 will hatch within 3 days of light exposure when proposed project begins in January of 2017. This will provide a large number of both diapause and post-diapause embryos. That said, it is important to note that there may be variability within sediment grab samples, because it is challenging to thoroughly mix the sediment before making aliquots (Covi and Lee, personal observations) Samples collected in 2016 will provide an opportunity to conduct experiments with a larger number of diapause embryos, or to repeat experiments at a later date without storage time as a potential confounding factor.

Figure 1. Prolonged storage of *B. poppei* embryos in native sediment at 4°C increases the proportion that develop immediately when removed from sediment and exposed to light (Covi, Lee and Park, unpublished observations). The number of embryos that develop and hatch was significantly greater after 11.5 months of storage (n=12) than 9.5 months of storage (n=4) on days 2-14 of incubation in sterile artificial fresh water under constant lighting ($p<0.001$; two-tailed Student's t test at each time point used in order to relate to proposed future end-point assays).



Zooplankton represent the top of a simple tropic web in Antarctic freshwater lakes. Based on published data [3, 34], and the PI's own personal observations, only two species of crustacean zooplankton are found on King George Island, *B. poppei* and the anostracan, *Branchinecta gaini*. Embryos of *B. gaini* tend to aggregate in mud and the spaces between rocks that remain frozen into mid-February, making them challenging to collect [34](Covi, personal observations). Embryos of this species are also surrounded by a thick shell that presents a formidable barrier to disruption by freezing [34], and, based on studies with *A. franciscana*, it is likely to also present a barrier to penetration by lipophilic chemicals [13] and metals [35]. In short, *B. gaini* embryos are resilient and difficult to obtain. By comparison, diapause embryos of *B. poppei* are found in greater densities in soft sediments at the center of lakes where freezing is less likely; during a 2015 expedition to King George Island, the PI recorded shoreline embryo densities of 5±0.3 embryos per cm^3 sediment and central lake densities in excess of 500 embryos per cm^3. Like most copepods, the embryos of *B. poppei* are transparent and lack a thick shell, which should provide less protection than that of *B. gaini* (Fig. 2).

An analysis of embryonic structural features in diverse zooplankton species suggests that lipophilic compounds will bioaccumulate in embryos if a specialized barrier to these compounds is not present. To the best of our knowledge, the only embryonic structure demonstrated to be impermeable to lipophilic compounds is the proteinaceous chorion of the anostracan, *A. franciscana* [13]. After removal of the maternally-derived chorion, via dechorionation with a solution of bleach and NaOH, *A. franciscana* embryos are only surrounded by the embryonic cuticle [15]. The shell of *B. poppei* is transparent without chemical treatment, and closely resembles partially dechorionated embryos of *A. franciscana* as the shell and embryonic cuticle are shed during early post-diapause development (Fig. 2). Lipophilic chemicals readily pass through arthropod cuticles [36], including those of diapausing freshwater cladocerans, marine copepods and dechorionated *A. franciscana* [5, 11-13, 16, 17]. Invertebrate embryos, including those of calanoid copepods [37], also possess rich intracellular lipid stores where lipophilic chemicals could accumulate by passive partitioning. Importantly, dormant embryos may also lack the metabolic capacity required to breakdown or export such toxicants, and these embryos often lie in anoxic sediments devoid of light and oxygen that would otherwise facilitate chemical breakdown by oxidative processes. Thus, without a specialized barrier to lipophilic compounds, dormant embryos of *B. poppei* should bioaccumulate lipophilic compounds identified in sediment or sediment pore water.

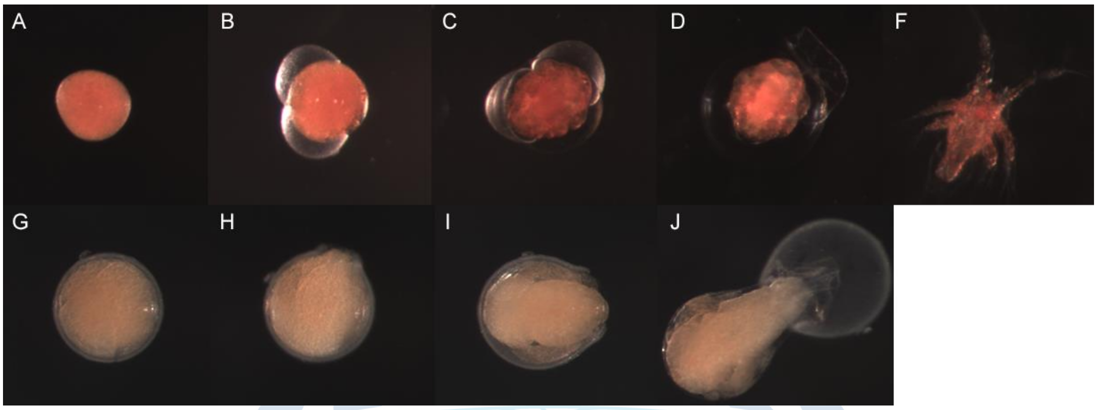
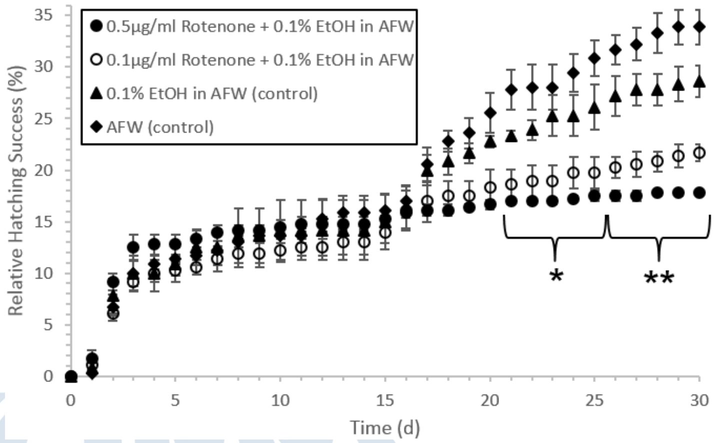


Figure 2. Early development in *B. poppei* (A-F; Covi, Lee and Park unpublished observations) and partially dechorionated embryos of *A. franciscana* (G-J; modified from Neumeyer et al. 2015). The appearance of the shell is similar in both species when shed (c.f. C and J).

To determine whether the embryos of *B. poppei* are permeable to lipophilic compounds we exposed them to the pesticide, rotenone, after separation from the sediment. Rotenone is a moderately lipophilic (logP = 4.01) inhibitor of NADH:ubiquinone oxidoreductase activity [38]. By blocking electron transport required for oxidative phosphorylation, rotenoids essentially mimic hypoxia at low concentrations, and anoxia at higher concentrations. Not surprisingly, the active life-stages of most zooplankton in the water column are extirpated within hours of rotenone treatment [39-43]. This effect presumably occurs because adults and larvae in the water column lack anoxia tolerance. Embryos in the sediment are expected to have greater tolerance of the chemical, because sediments are often hypoxic or anoxic. However, at least two studies demonstrate that hatching success for embryos of calanoid copepods and the anostracan, *A. franciscana*, decreases after brief rotenone exposures [12, 16]. In *B. poppei*, the initial hatching event that occurs during the first 5 days of culturing in artificial freshwater (AFW) at 4°C with constant light exposure was unaffected by the presence of rotenone, but hatching of embryos was inhibited by rotenone in a concentration-dependent manner beginning with day 21 of exposure (Fig. 3). All nauplii died less than 24 h after hatching in both rotenone treatments for the duration of the experiment (30 days) while nauplii in controls without rotenone often survived for the duration of the experiment (Covi, Lee and Park, unpublished observations). These data indicate that moderately lipophilic compounds will penetrate the embryonic cuticle/shell in *B. poppei*, but that this penetration will be slower than that observed in fully dechorionated *A. franciscana* embryos [16]. Interestingly, this comparative result is predicted by microscopy, which demonstrate that the shell of diapausing *B. poppei* resembles that of *A. franciscana* that are only partially dechorionated (Fig. 2). An ultrustructural assessment of the cyst shell and emergence hatching membranes are needed to verify the presence of a thin maternally-derived shell, homologous to that of *A. franciscana*.

Figure 3. Rotenone penetrates diapausing *B. poppei* cultured in AFW. Ethanol did not change hatching rates at any time point ($p>0.06$); 0.5 µg/ml rotenone differed from controls starting at day 21 ($p<0.05$); both rotenone treatments differed from controls starting on day 26 ($p<0.001$); mean±s.e.m.; ANOVA + Tukey's post hoc for each time point used to demonstrate effectiveness of end-point assays proposed later; n=3 (Covi Lee and Park, unpublished observations)



The biological effects of anthropogenic chemicals on zooplankton development and resurrection from dormancy are largely unknown. Tests have generally been conducted on sediment from heavily contaminated sites, including San Francisco Bay where chemical mixtures, rather than individual chemical pollutants, are associated with toxicity to invertebrates. The growth and survival of amphipod and bivalve embryos is negatively impacted by a complex mixture of heavy metals and persistent organics in sediments of San Francisco Bay [44]. However, a principle components analysis of toxicity test on fourteen of 127 monitored sites in the bay indicates that the synergistic effects of these pollutants is responsible for toxicity, not an individual chemical [44]. For this reason, it is absolutely essential that any study on dormant life-stages of invertebrates include a thorough assessment of both putative organic and heavy metal contaminants. The complementary proposal submitted to NSF-PLR was designed with this in mind.

Lipophilic organic chemicals as potential toxicants in Antarctic freshwater lakes

Persistent organic pollutants (POPs) are ubiquitous, and air transport combined with cold precipitation leads to deposition in the arctic, mountainous regions like the Himalayas and Antarctica. Dioxins, organochlorines and DDT are found in arctic lakes [45], but this is not surprising given their proximity to industrial and agricultural activity in the northern hemisphere. It is primarily atmospheric transport that brings anthropogenic chemicals to sites in the arctic including a diverse array of organochlorines (CBzs, CL-VERs, Dioxins, Furans, HCB, OCSTYR, PCAs, PeCB, PCBs, aldrin, dieldrin, entrin, CHBs, toxaphene, chlordane, DDT, DDE, endosulfan, HCHs, methoxychlor, mirex, trifluralin), polycyclic aromatic hydrocarbons (over 16 forms) and Mercury [46]. Similar atmospheric deposition is occurring with HCB, HCH and DDT in the Antarctic [47-49], and organochlorines, PCBs and PAHs in remote regions of the Himalayas [50]. Cycling of volatile POPs between aquatic, terrestrial, snow and ice deposition sites is continual [51], so transport from the marine environment is important to consider for maritime lakes in Antarctica. Recent studies identified organochlorines (including PCBs), HCB, PeCB, PDBE and PAHs in terrestrial soil, mosses, marine sediment, seawater, benthic marine organisms, fish and seabirds in Antarctica [52-55]. Fish and seabirds act as biovectors, transferring POPs like PCBs to terrestrial trophic cascades [56], so these compounds will eventually appear in freshwater lakes that terrestrial sites drain into. However, despite the large number of studies that test for POPs in Antarctica, the topic of pollutants in freshwater lakes is not addressed in review articles on the subject [e.g. 57].

Relatively high levels of PCBs [58, 59] and perfluorinated compounds (Park, Personal Communication) were recently identified in terrestrial soil, lichen, marine sediment, marine fish, and seabirds from the Barton Peninsula, King George Island, which is the site of origin for the embryos and sediments used in the present study. There is still a chance to effect positive change if these chemicals are found to be present and detrimental to invertebrate development in Antarctic freshwater lakes. Some can be directly addressed by changing human activities in the Antarctic and sub-Antarctic regions. Polycyclic aromatic hydrocarbons in Antarctic snow, for example, originate primarily in Antarctica from combustion processes, not intercontinental air transport [60]. It is especially important that studies focus on areas with a high incidence of combustion processes. This is exactly what the complementary NSF-PLR study does. Lakes on King George Island were selected, because human activity on the Island is among the highest for any site in Antarctica.

We predict that Antarctic lakes currently have low levels of anthropogenic organics, but that these chemicals will increase over time. In other words, sediment taken from these lakes will provide a source of zooplankton embryos that are relatively free of the background impacts of anthropogenic chemicals. Therefore, they represent one of the most ideal populations in the world to use in an assessment of the biochemical basis of dormancy in freshwater zooplankton.

Metals as potential toxicants in Antarctic freshwater lakes

An overwhelming amount of evidence demonstrates that low levels of individual elements such as cadmium, selenium, lead, zinc, mercury and arsenic block hatching or induce developmental abnormalities in copepods, rotifers and branchiopods [61-72]. Research on point-source contamination also demonstrates that sediments harbor higher levels of heavy metals that surficial waters in freshwater lakes [73]. Because *B. poppei* must hatch within the sediment, or at the sediment/water interface, they are likely to be exposed to higher levels of metals than adults or larvae in the water column. A total of 33 elements, including cadmium, arsenic, lead and zinc, were identified in one of the relatively rare studies of metals freshwater lakes in Antarctica [74]. The primary metal of concern is organic mercury, because its lipophilic nature will allow it to pass across the cuticle of zooplankton embryos and partition to intracellular lipid stores. Field evidence demonstrates that mercury levels in terrestrial Antarctic environments are on the rise, and suggest that air transport combined with cold precipitation could make Antarctica a global deposition site for the metal [75]. The primary study lakes are also located just above ASPA zone 171, and it is common to find the remains of penguins along the shorelines these lakes. This provides a path for mercury entry in addition to atmospheric deposition. The dynamics of mercury exchange between biota, sediments, water, snow, ice and air are complex, but it is relevant to note that mercury (especially methyl-mercury) deposited into freshwater arctic lakes is rapidly taken up by lake biota, including zooplankton, and mobility of the metal is decreased as a result [76].

The bioavailability of metals must be considered when making predictions about metal toxicity [77-79], and the only way to truly test bioavailability is test toxicity in the presence of the natural sediment matrix. Attempting to determine both the bioavailability and relative abundance of metals across just one lake-bed is prohibitively expensive, and is only valid for one time-point. Furthermore, methods like Equilibrium Partitioning (EqP), which are used to assess sediment toxicity for a defined mixture of metals [80], still fail to reproduce the incredibly broad array of potential synergistic effects that are likely occur in natural environments. Rather than attempt to generate a benchmark with limited applicability by testing the effect of individual metals, the complementary NSF-PLR study tests the effects of thermal stressors in the context of metals that are already associated with a natural sediment matrix by preincubating and hatching embryos in the lake sediments they were collected in. Data generated by this study will provide a foundation for future research that could address targeted topics, like the development of resistance to metals toxicity in zooplankton, and rates of change in community structure over time in lakes that are subject to high metal loads from Antarctic stations. However, the NSF-PLR study does not investigate the biochemical regulation of dormancy. The study described in the present proposal complements the NSF-PLR proposed study by providing a biochemical foundation that can be used to generate hypotheses regarding the impact of anthropogenic chemicals on Antarctic lakes.

<u>Temperature of Antarctic freshwater lakes</u>

According to the Antarctic Climate Change Report, elevated temperatures will have complex detrimental effects on limnetic ecosystems in Antarctica [81]. Increases in yearly mean temperatures will change lake structure by melting previously permanent ice rafts and increasing solute deposition in meltwater from catchments. Elevated temperatures could also eliminate snowpack that cools freshwater lakes during the austral summer. Direct effects of temperatures on biota in these lakes will be difficult to predict, because the lack of thermal stability usually provided by surface ice and catchment snowmelt will result in complex thermal profiles that are likely to include both an increase in the rate of temperature change, frequency of temperature cycling and maximum temperature.

Since the late 1940's mean air temperatures on King George Island have increased by as much as 1.2°C [82, 83]. Between 1947 and 1996, the mean annual temperature increased at a rate of 0.022-0.028°C per year [84, 85]. When this record was extended to 2011, the mean rate of increase was 0.019°C per year with the greatest increase occurring during winter months [82]. While yet unconfirmed by testing of the recording equipment, temperatures at Argentina's Esperanza Base on the Antarctic Peninsula reached a record high 15.5°C on March 24, 2015. On a clear day, high air temperatures like this, in combination with heating from solar irradiance, would very likely raise the temperature above the thermal limit for some zooplankton adults. It is relevant to note that adult *B. poppei* reared at temperatures similar to that of minimum summer lake water temperatures (2°C) experience close to 50% mortality at 10°C and 100% mortality at 15°C (Lee, personal communication). It is unknown what effect elevated sediment temperatures will have on stored embryos, because this has never been tested.

Chapter 3 R&D Implementation Contents and Results

- ***Obj. 1:*** Use images captured with light and electron microscopy to describe early development in post-diapause embryos of *B. poppei* in order to provide a temporal context for biochemical studies.
- ***Obj. 2:*** Expose post-diapause and diapause embryos of *B. poppei* to anoxia, and describe the effects on viability during dormancy and subsequent aerobic development.
- ***Obj. S1:*** Assess freeze tolerance of *B. poppei* embryos by examining distribution of embryos in Antarctic lakes and exposing embryos to temperatures between 4°C and -80°C in a controlled environment. Viability and the use of cryoprotectants will be investigated. (This supplemental objective was added to study at the end of year 1.)
- ***Obj. S2:*** Assess physical and chemical environmental factors in lakes with and without populations of *B. poppei*. (This supplemental objective was added to study in 2018.)
- ***Obj. 3:*** Use ^{31}P-NMR to determine the role of intracellular pH transitions in establishing and breaking diapause and anoxia-induced quiescent states in *B. poppei*.
- ***Obj. 4:*** Use HPLC to assess adenylate and guanylate status in diapause and anoxic post-diapause embryos of *B. poppei*.
- ***Obj. 5:*** Test effects of pH on hexokinase, phosphofructokinase and pyruvate kinase isolated from diapause and post-diapause *B. poppei*.

- ***<u>Report on Experimental Objective 1</u>:***
- ***Objective:*** Use images captured with light and electron microscopy to describe early development in post-diapause embryos of *B. poppei* in order to provide a temporal context for biochemical studies.
- **Hypothesis:** Early normal and abnormal developmental events in *B. poppei* are the same as those published for post-diapause embryos of the brine shrimp, *A. franciscana*.
- **Summary:** We demonstrate that embryos of *B. poppei* shed two embryonic cuticles by osmotic swelling of the extraembryonic space between the innermost embryonic cuticle and the developing nauplius larva. This follows predictions based on the development of *A. franciscana*. Like *A. franciscana* [15], naupliar development and emergence from the embryonic cuticle are not perfectly coordinated in *B. poppei.* Both species can experience either early emergence or complete failure to emerge from the first embryonic cuticle. However, unlike *A. franciscana*, early emergence from the first embryonic cuticle in *B. poppei* is 100% lethal. Nuclear count in dormant embryos is less than that of brine shrimp, and, unlike the brine shrimp, nuclear count

increases during early preemergence development. Like the brine shrimp, yolk stores provide energy to support early development prior to hatching, but yolk stores are almost completely used before hatching of a swimming nauplius. This is unlike the brine shrimp. In addition to testing the primary hypothesis, we also tested culture conditions, and selected a set of standard conditions for biochemical and molecular studies to take place in year 2 of this grant.

* **Significance:** The current project demonstrates that embryo storage conditions approximating deep sediment during the austral summer in lakes on Barton Peninsula, King George Island, Antarctica, (dark; 2-4ºC) increases the number of embryos that emerge early in development and die as a consequence. Based on this, we predict that early development in *B. poppei* will be highly susceptible to disruption by anthropogenic chemicals and climate change. Furthermore, the use of yolk stores before hatching makes it likely that feeding after hatching is crucial, and hatching must coincide with an abundance suspended food resource.
* **Results:**
* Four distinct categories of normal early developmental morphology were identified using a combination of light (Fig. 1) and electron microscopy (Figs. 2 and 3): Early Development (ED), Intermediate Development 1 (ED1), Intermediate Development 2 (ED2) and Pre-nauplius (PN). These stages will facilitate developmental studies in year 2 and 3 of the proposal. The ED stage is characterized by a diffuse appearance under light microscopy (Fig. 1A). Electron micrographs demonstrate that the diffuse appearance is due to a lack of differentiation within the cell mass; large electron-dense granules and clear droplets are the predominant structures found throughout the embryo (Fig. 2). The electron dense granules and clear droplets are similar in appearance to proteinaceous yolk platelets and lipid droplets, respectively, found in diapause embryos of *A. franciscana*. No complete cell membranes appear in micrographs of the ED embryo, suggesting that the diapause embryo consists of a partial syncytial cell mass. The ED stage also possesses a relatively simple embryonic cuticle with a thin outer membrane, thick lamellar layer and thin inner electron-dense layer (Fig. 2). By contrast, the PN stage has cellular structure visible with a dissecting light microscope (Fig. 1), and electron micrographs reveal that both the lipid droplets and electron dense granules of the syncytial ED stage cells are replaced by differentiated cells possessing mature mitochondria, myofibril protein arrays and complete plasma membranes (Fig.3). Twitching is occasionally observed in the PN stage if emergence is delayed.

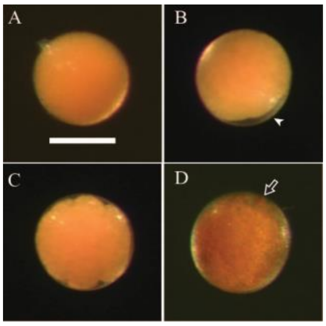


Figure 1. Light micrographs of *B. poppei* development prior to emergence and hatching; (A) ED stage, (B) ID1 stage, (C) ID2 stage and (D) PN stage. White arrowhead indicates beginning of apolysis as the first naupliar exoskeleton is formed under the first embryonic cuticle, and black arrow indicates appearance of x-shaped ocellus. White bar = 100 µm.

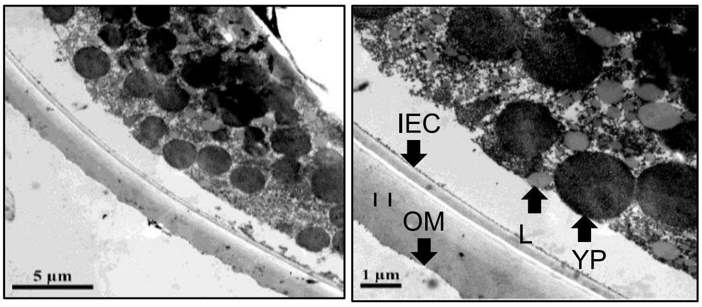


Figure 2. Transmission electron micrographs of *B. poppei* in ED stage. Embryo fixed immediately after isolation from sediment. No complete plasma membranes were observed in radial orientation. Lipid droplets (LD) and proteinaceous yolk platelets (YP) are dominant structures.

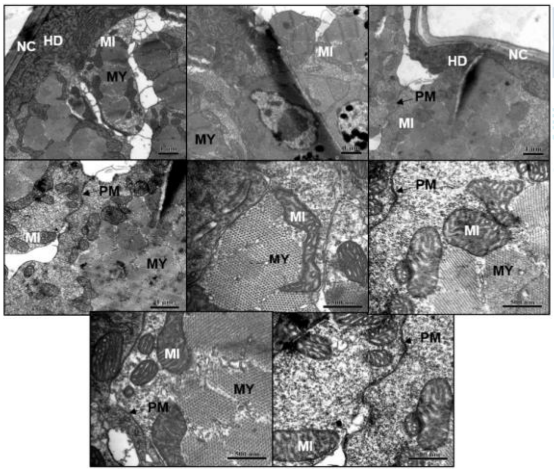


Figure 3. Representative transmission electron micrographs of *B. poppei* in PN stage. Complete plasma membranes (PM) mature mitochondria (MI), myofibril arrays (MY), hypodermis (HD) and a simple naupliar exoskeleton (NC) are visible.

* Data generated with light microscopy and digital videography confirms the presence of multiple embryonic cuticular layers that are shed sequentially during the hatching process (Fig 4). The space between the nauplius larva and the embryonic cuticle clearly expands during the process of emergence and hatching (Fig. 4B-E), and rupture of the outer embryonic cuticle always occurs in a single linear plane (Fig. 4C). As osmotic expansion continues, a second layer is shed (Fig. 4D,E). Hatching of a free-swimming individual occurs when the nauplius larva (Fig. 4F) breaks through the innermost of the three embryonic cuticle layers with swimming motions (see video sample: https://youtu.be/ZRnCH0qxBBY).

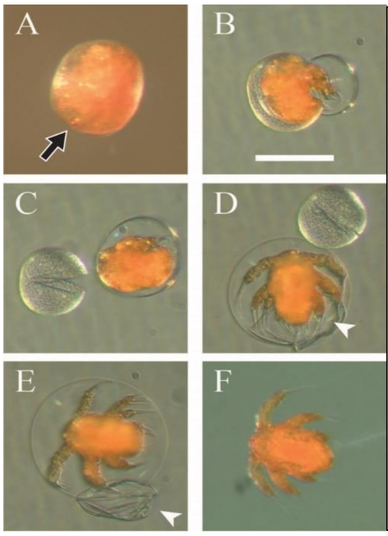


Figure 4. Light micrographs of *B. poppei* nauplius during emergence and hatching; (A) fully formed nauplius larva with eyespot (indicated by black arrow) inside the embryonic cuticle, (B) first embryonic cuticle splits open and expanding second cuticle protrudes, and (C) swelling eventually pushes first cuticle off. The second embryonic cuticle expands (D and E) until weakened enough for nauplius to break free (F). A translucent layer (indicated by white arrowhead) is shed during expansion, but before hatching; 25 min elapsed between images B and F; White bar = 100 μm.

* Emergence from the first embryonic cuticle while the embryo is still in the ED stage is lethal (Fig. 5), and occurs in 10-20% of embryos isolated from native Antarctic sediments by the sugar floatation method. In brief, for the sugar floatation method, sediments are mixed 1:9 with 80% sucrose and centrifuged at low speed to separate sediment particles from *B. poppei* embryos. The embryos are then rinsed with artificial freshwater made from artificial sea salts (Instant Ocean®) at a final concentration of 0.35 parts per thousand (ppt). It is possible that this form of premature emergence is caused by osmotic cycling during the isolation and rinsing steps. Regardless, the sugar floatation method provides a sufficient number of embryos for biochemical and toxicological studies without optimization to decrease the probability of premature emergence. The fact that the embryos are unable to continue developing when exposed to the surrounding medium suggests that they lack the capacity to regulate ionic composition in the ED stage. This will be investigated in year 2 of the proposed study by evaluating the expression of Vacuolar (V-type) proton-ATPase and sodium-potassium ATPase expression at the mRNA level. Based on studies for *A. franciscana*, including those conducted by the PI [13], we predict that the *B. poppei* ED embryo will not express sodium-potassium ATPase, and will only express the V-ATPase for energization of heavy membranes.
* In order to make sure that optimal culture conditions were used for biochemical and toxicological experiments planned for years 2 and 3 of the study, we examined the sensitivity of hatching to light, total salinity, and ionic composition. Optimal hatching was observed under continuous lighting with 0.35 ppt to 3.5 ppt Instant Ocean® (Fig. 6A). Salinities of 35 ppt and 0.035 ppt did not support early development or hatching (Fig. 6A). Commercially available artificial freshwater and freshwater with a composition based on analyses of the lakes of origin on Kind George Island both provided hatching rates similar to that of 0.35 ppt Instant Ocean®

(Fig. 6). In order to standardize hatching medium preparation, 0.35 ppt Instant Ocean® was adopted for all developmental, biochemical and toxicological studies.

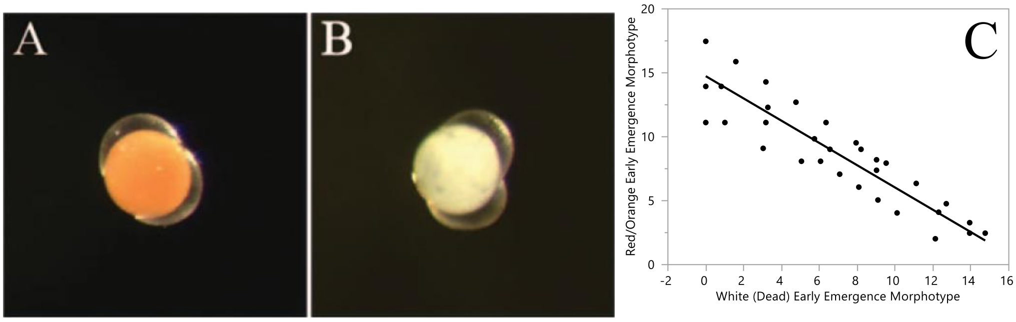
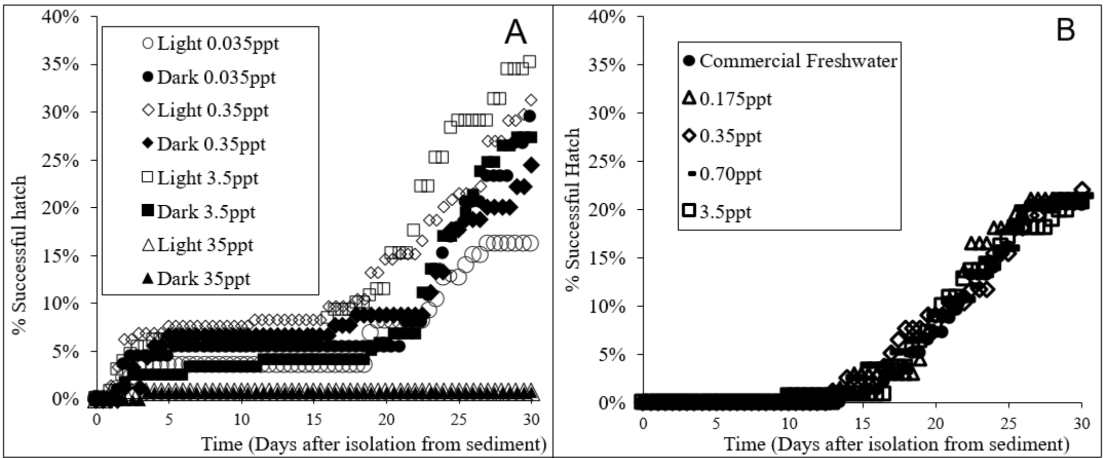


Figure 5. Emergence from the first embryonic cuticle during the ED stage (A) leads to mortality visible as embryo bleaching (B). The orange morph appears first, and is replaced by the white morph. The white morph then decays. Regression analysis of two color morphs (C), starting from when bleaching is first observed, demonstrates that embryos emerging during the ED stage will die before further embryonic development is observed (r^2=0.829, p<0.0001).



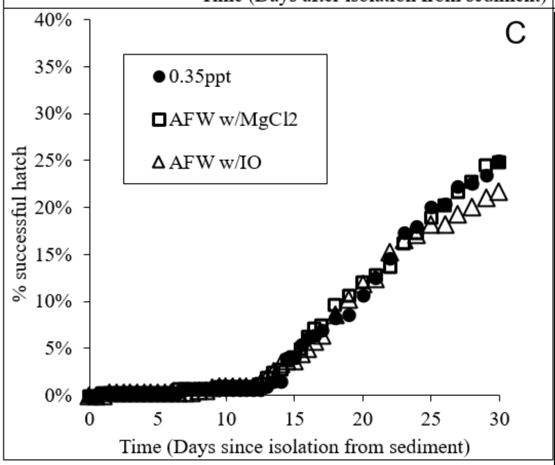


Figure 6. (A) Continuous lighting mildly enhances hatching of *B. poppei,* but only at 0.35 and 3.5 ppt artificial sea salts (IO, Instant Ocean®); *B. poppei* develop and hatch successfully in water with artificial sea salts brought up to a total salinity of 3.5 ppt to 0.175 ppt (A and B, respectively); no hatching is observed when salinity is decreased to 0.035 ppt. (B) Use of sterile commercial freshwater did not enhance hatching. (C) Artificial freshwater solutions (AFW) based on chemical analysis of water samples from native *B. poppei* populated lakes on Barton Peninsula, King George Island, Antarctica, did not enhance hatching over that obtained with IO salts, even if additional $MgCl_2$ or IO was added.

* In order to assess cell proliferation and energy utilization during early development, nuclei and yolk platelets were reconstructed using transmission electron micrographs of serial sections of *B. poppei* at three developmental stages. Nuclear count increases from just over 500 nuclei per embryo during early post-dormancy development (ED stage) to 2000 nuclei per embryo in the prenauplius just before hatching (Figure 7). The large increase in nuclear count is concomitant with a dramatic decrease in yolk energy stores, indicating that yolk stores support cell proliferation during preemergence development.

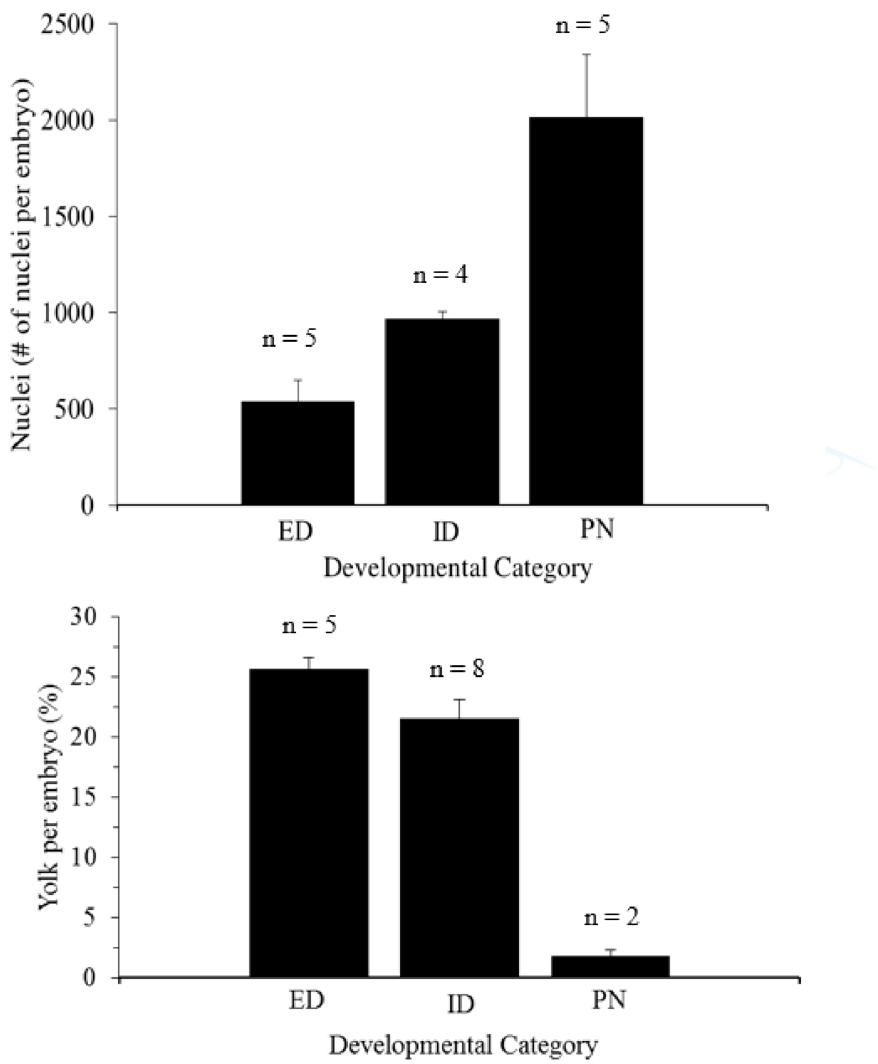


Figure 7. Assessment of yolk energy stores at three developmental stages: Early Development embryo (ED), Intermediate Development embryo (ID) and prenauplius (PN). Increased nuclear count in the prenaupliar stage is concomitant with a large decrease in yolk energy stores.

* ***Report on Experimental Objective 2*:**

* ***Objective:*** Expose post-diapause and diapause embryos of *B. poppei* to anoxia, and describe the effects on viability during dormancy and subsequent aerobic development.

* **Hypothesis:** Embryos of the copepod, *B. poppei*, survive in sediments for prolonged periods in two forms of metabolic and developmental arrest: (1) developmentally programmed diapause and (2) anoxia-induced quiescence.

* **Summary:** *B. poppei* embryos display a diapause state that can be broken while the embryo is stored in the dark at 4℃. Prolonged storage leads to longer hatching observations, because hatching is delayed, and embryos in shallow diapause appear to activate and die while in storage. Many *B. poppei* embryos exposed to severe hypoxia/anoxia continued to develop to an early or intermediate developmental stage, regardless of whether or not *A. franciscana* was used as an oxygen scrubber.

* **Significance:** Embryos in a shallow state of diapause are subject to activation, and severe hypoxic conditions will not prevent these embryos from trying to develop, despite the fact that they will succumb during the process of development. This suggests that the *B. poppei* egg banks are likely to be very susceptible to depletion during warm austral summers.

* **Results:**

* An analysis of hatch tests on embryos collected with lake sediment from Barton Peninsula, King George Island, Antarctica, in February 2015 and January 2016 indicates that embryos in

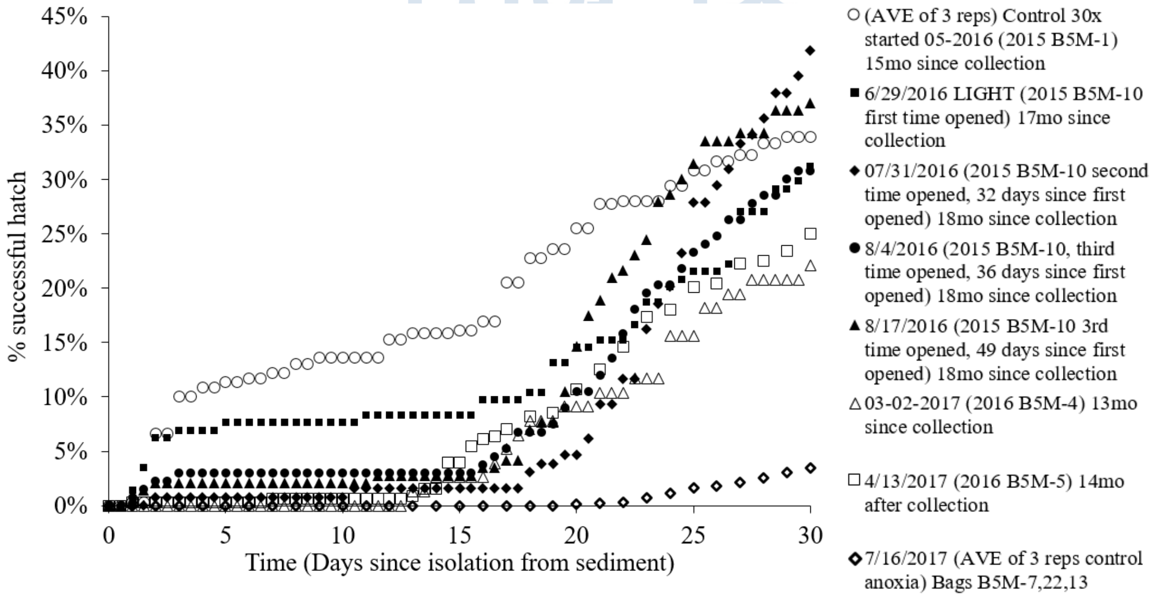


Figure 8. Hatching profiles for embryos collected in 2015 and 2016, and tested over a one year period, suggests that older embryos in sediment are in a deeper state of diapause that requires more time to break in order for development to begin.

the sediment are in a state of diapause that requires prolonged incubation under aerobic conditions in the presence of light to break (Fig. 8). Samples collected in 2015 were collected at a time of the austral summer when females were producing diapause embryos. Early hatch tests with these demonstrated a biphasic hatching profile for the population; 5-10% of the population was in a shallow form of diapause that was broken in 2-3 days while the remainder took two or more weeks of light exposure to being development (Fig. 8). The longer these samples were stored in the dark at 4ºC, the longer it took for embryos to initiate development (Fig. 8). This is consistent with observations from samples collected in 2016. During the 2016 field collection, we noticed that females were not releasing diapause embryos. Consequently the 2016 samples differ from the 2015 samples in that the youngest embryos were actually 1 year old at the time of collection. Our laboratory studies suggest that embryos in a shallow state of diapause are likely to continue developing into an intermediate stage in sediments even if the sediment is severely hypoxic or anoxic (Fig. 9). Not surprisingly, the samples collected in 2016 did not contain any embryos in a shallow form of diapause that could be broken quickly. Total hatch rates for 2016 samples demonstrate a hatching trajectory that will allow them to eventually reach levels comparable to those observed for 2015 samples.

* A delay in activation from a dormant state is well documented for *A. franciscana*, and thus is not surprising to find for *B. poppei*. Based on these observations, we conclude that future sample collection would yield the highest quality material if the collection occurs when females are producing diapause embryos (i.e. February). It is possible, however, that part of the reason for the loss of the early hatching individuals does appear to be due to the initiation of hatching by some embryos while in storage at 4ºC.
* Anoxia induces a form of dormancy in *A. franciscana* that is just as profound as diapause. The capacity of *B. poppei* embryos to enter dormancy under anoxia was tested by placing ED embryos into tubes continuing 0.35 ppt artificial seawater purged with 99.5% nitrogen (Fig. 9). *Artemia franciscana* embryos were added to one set of anoxic tubes in order to act as oxygen scrubbers. Surprisingly, *B. poppei* embryos continued to develop in all preparations, despite being oxygen limited (Fig. 9). This suggests that these *B. poppei* embryos will not use anoxia-induces quiescence as a means of maintaining dormancy if the diapause state is broken while they reside in hypoxic or anoxic sediments. That said, anoxic treatment does increase hatching success in those that do not die as a result of premature emergence (Fig. 9).

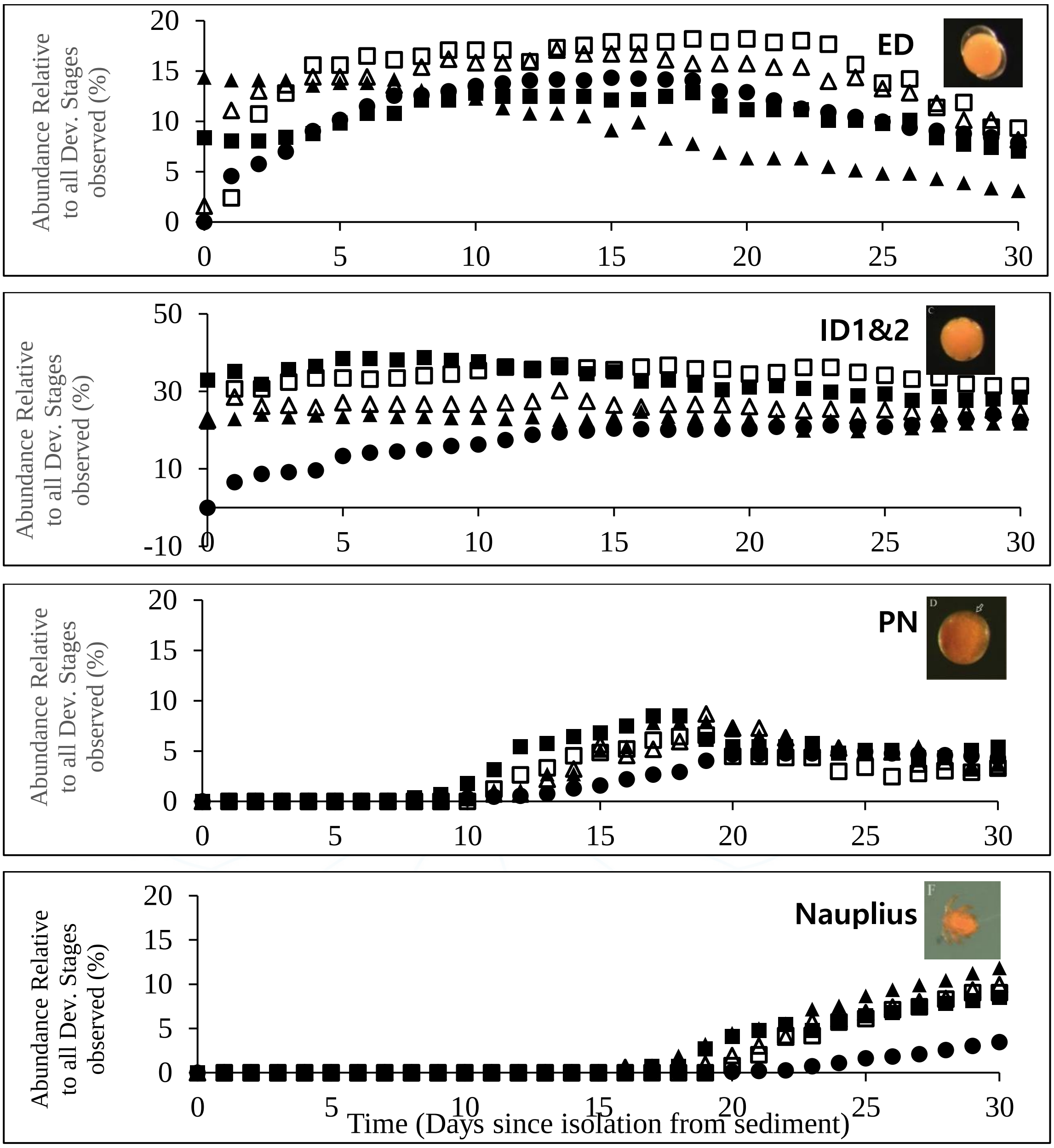


Figure 9. Emergence and hatching profiles during early development of *B. poppei* following anoxic treatment. All embryos were incubated in 0.35ppt following appropriate anoxic treatment. Treatments included control (normal aerobic incubation, closed circles), anoxia for 14 days with Artemia as oxygen scrubber (open squares) or with *B. poppei* alone (closed squares), anoxia for 1 month with Artemia as oxygen scrubber (open triangles) or with *B. poppei* alone (closed triangles). Each data point is the average of 3 separate replicates. Statistics and error bars not shown.

* ***<u>Report on Supplemental Objective S1</u>:***
* **Objective:** Assess freeze tolerance of *B. poppei* embryos by examining distribution of embryos in Antarctic lakes and exposing embryos to temperatures between 4°C and -80°C in a controlled environment. (This supplemental objective was added at the end of year 1.)

* **Hypothesis:** Embryos of *B. poppei* are most abundant in lakes where the sediment does not freeze, because freezing increases mortality in a temperature-dependent manner.
* **Summary:** This objective is almost completed. We determined that embryos of *B. poppei* are sensitive to freezing temperatures. Tissue damage and increased mortality was observed when embryos in native sediment were exposed to temperatures of -12°C to -80°C for 30 days. Hatching rates decreased as temperature decreased, and no embryos hatched after 30 d at -80°C. Investigating the use of cryoprotectants to survive freezing is currently underway.
* **Significance:** These novel data demonstrate that limited freeze tolerance in the embryonic stage is a major environmental factor limiting the distribution of this species in Antarctica. Based on these data, we predict that the range of *B. poppei* in the Antarctic will increase as a consequence of global climate change. The data also demonstrate that sample shipping and long-term sample storage must be at 4°C to maximize embryo quality. Because lake water temperatures reached the freezing point at the sediment water interface, but temperatures did not decrease enough to freeze living cells of any organism, it is clear that embryos of *B. poppei* must remain in diapause or anoxia-induced quiescence for the duration of the winter or they will die immediately after hatching in sediments under frozen waters.
* **Results:** Preliminary tests of freeze tolerance in year 1 of this study suggested that some embryos tolerate freezing, and have the capacity to tolerate simultaneous partial dehydration during freezing. In the first half of year 2 we incubated *B. poppei* embryos for 30 d at -12°C, -24°C, -80°C. A 4°C treatment was used as a control for the freezing treatments. Four embryo

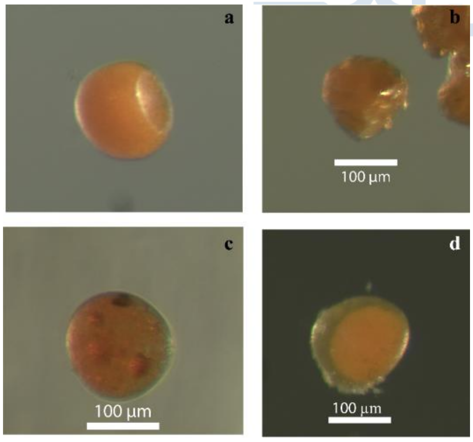


Figure 10. Four types of morphology unique to embryos of *B. poppei* after one controlled freeze-thaw event in native sediments. (a) slightly dehydrated with single indentation, (b) severely dehydrated with wrinkled surface, (c) speckled cell mass suggestive of tissue damage, and (d) embryo mass shrinks away from cuticle.

morphologies were observed immediately upon thawing of frozen sediments (Fig. 10). None of these four morphologies were observed in the 4°C control treatment. Following thawing, the embryos were cultured in artificial freshwater for 30 d to assess viability. The four abnormal embryo morphologies were counted once per day (Fig. 11a-d). Normal developmental stages were also counted once per day (Fig. 11e-h). Less than 10% of embryos initiated development after 30 d at -80°C (Fig. 11e), and only 6% hatched as swimming nauplii (Fig. 11h). By contrast, the initiation of development and hatching after 30 d at -12°C was identical to the 4°C control

(Fig. 11e,h). Incubation at -24°C for 30 d had an effect that was intermediate between the effects of -12°C and -80°C treatments.

* The decreased survivorship and hatching of *B. poppei* after the -80°C and -24°C treatments may be because the embryos did not have time to dehydrate. We hypothesize that the process of dehydration is protective, because removing water would limit ice crystal development in the embryo. In support of this, more embryos were dehydrated in the -12°C treatment as compared to the -24°C and -80°C treatments (Fig. 11a).

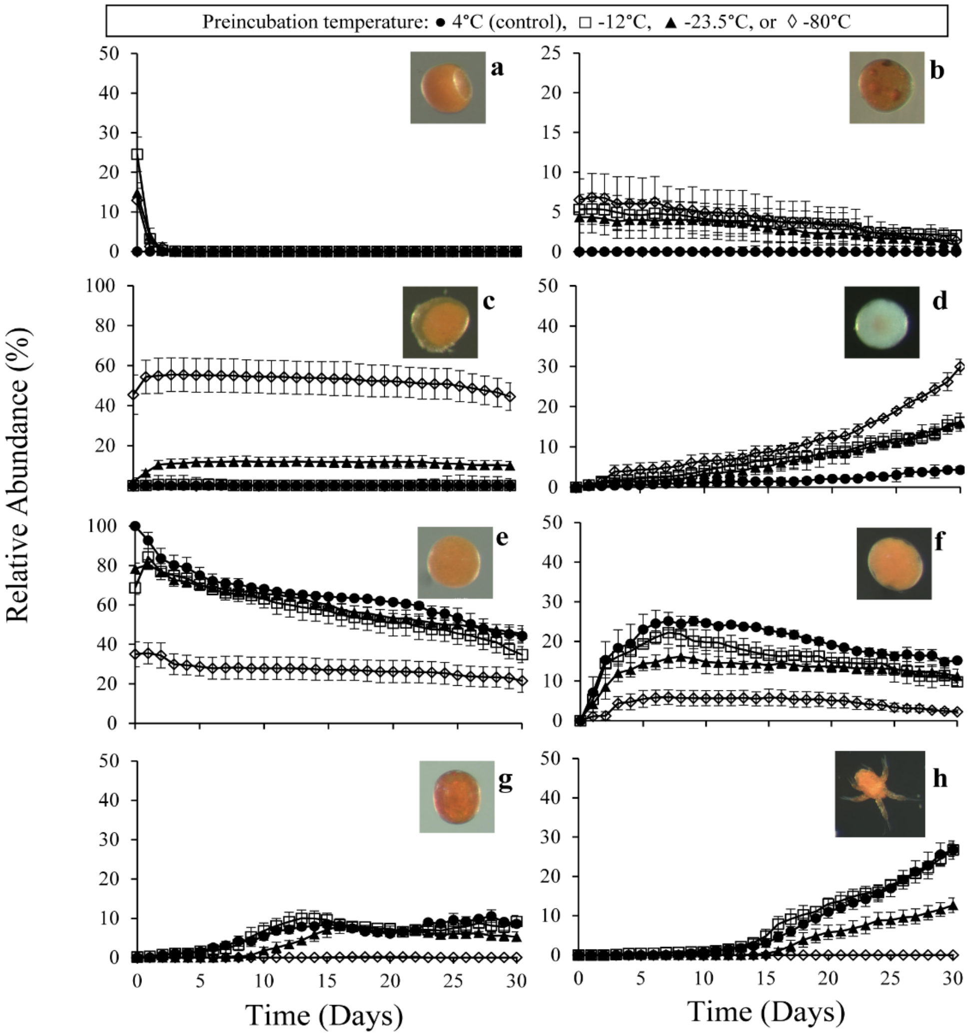


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Figure 11. Developmental progression in B. poppei following 30 d pre-exposure to temperatures of -12°C, -24°C or -80°C. A 4°C pretreatment was used as a control, and development is considered normal for this treatment. The 30 d pre-exposure to freezing temperatures terminated at day zero in the plots. Developmental progression was monitored for 30 d after thawing. Images of the following stages are provided in the plot fields.

a: Dehydrated embryo **b**: Speckled embryo
c: Embryo pulling away from cuticle **d**: White embryo (dead/decaying)
e: Early Development (ED) embryo**f**: Intermediate Development (ID) embryo
g: Pre-nauplius (PN) **h**: Nauplius

*

* We determined the abundance of *B. poppei* embryos in the sediments of 29 lakes on King George Island, Antarctica, that contained adults in the water column (Fig. 12). The highest abundance of embryos was associated with rich organic sediment in lakes with sediment that is unlikely to freeze in winter. Low abundance was generally associated with sandy soils containing small black particulates. Most lakes (16 of 29) did not contain dormant embryos in

the sediment, and had few adults in the water column (Fig. 12). It is possible that these lakes are recolonized by embryos carried by birds each spring. Only 1 of 29 lakes contained more than 10 embryos per cm^3 of sediment (Fig. 12).

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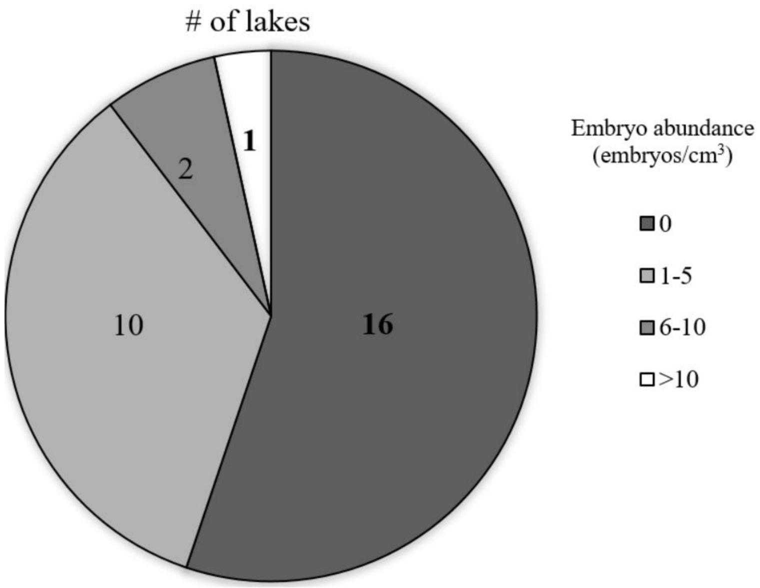

Figure 12. Number of lakes on King George Island, Antarctica, that contain no embryos, 1-5 embryos/cm^3, 6-10 embryos/cm^3 or 158 embryos/cm^3.

＊ To test the hypothesis that embryo abundance is highest in sediments that do not freeze, temperature sensors were deployed at the sediment/water interface in three locations of three lakes with varying abundance of *B. poppei* embryos . The data demonstrate that the temperature remained at or above 0˚C in all three lakes over the course of the Austral winter (Figure 13). Because of colligative properties, it is not possible for the embryos of any zooplankton species to freeze at 0˚C. Consequently, freeze tolerance does not explain the variation in embryo abundance among the three lakes. Importantly, it is clear that the lakes with highest abundance of embryos on King Barton Peninsula, King George Island, did not experience freezing during the winter months when the water column may be completely frozen. This indicates that embryos must remain in dormancy via diapaus or anoxia-induced quiescence.

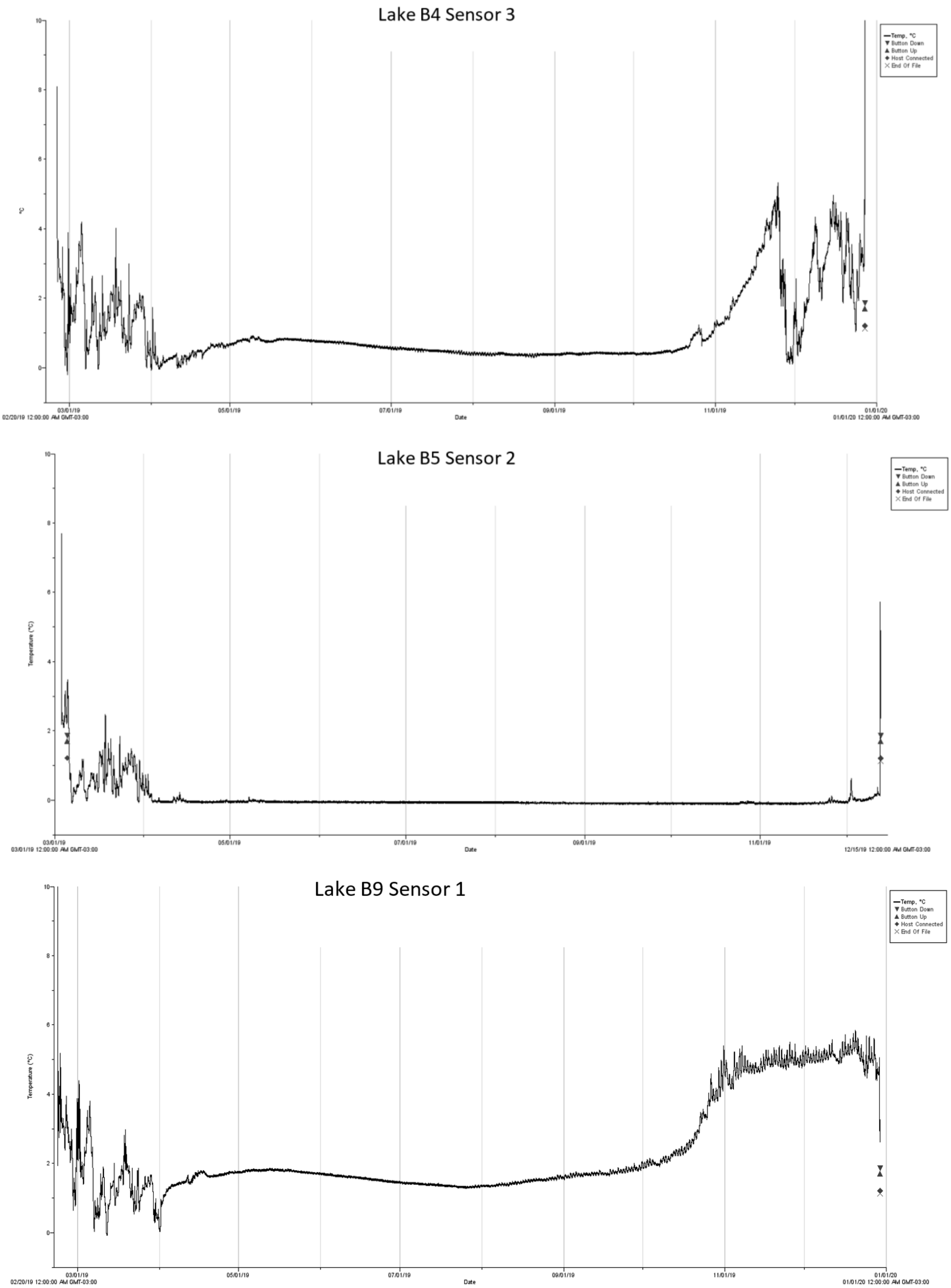

Figure 13. Three representative temperature profiles at the sediment/water interface of three freshwater lakes with *B. poppei* populations on Barton Peninsula, King George Island, Antarctica. Three temperature sensors were deployed in each of three lakes, providing 9 independent recordings. Each recording begins in March 2019 and ends in December 2019.

* ***Report on Supplemental Objective S2:***
* **Objective:** Assess physical and chemical environmental factors in lakes with and without populations of *B. poppei* (This supplemental objective was added to the study in 2018 as a foundation for biochemical laboratory studies.)
* **Hypothesis:** Embryo distribution in Antarctic lakes is determined by water chemistry and the presence of toxic metals in the sediment. (NOTE: Objective 2 complete in year 1 of the study demonstrated that *B. poppei* embryos are permeable to lipophilic chemicals, which would include organic metals.)
* **Summary:** Depth, Temperature, pH, dissolved oxygen, redox status, conductivity, and photosynthetic pigments were measured in lakes with and without *B. poppei* populations in 2018 and 2019. Elements in the sediment were also analyzed. At least two distinct forms of pyrite were observed near lakes that contain *B. poppei*, one that oxidized in a manner consistent with iron content and one that was brass in color. Samples of lake sediment were collected from nine lakes and shipped to a new collaborator at the Universidade de Evora, Portugal, for a complete analysis of element composition. These data demonstrate that of all measured physical and chemical variables during the austral summer, only Ni content in the sediment can potentially explain embryo abundance in sediments for *B. poppei*.
* **Significance:** In order to conduct properly controlled biochemical studies, we must know more about water chemistry and susceptibility to potentially toxic trace elements. If elements that are toxic when converted to organic form by microbial activity differ among lakes, then elemental composition of the lake sediment could affect the distribution of *B. poppei* in Antarctic lakes.
* **Results:** During the 2018 expedition, we gathered samples of sediment from 29 lakes that varied in the abundance of *B. poppei*. We also took pictures of basins that clearly differed in the type and amount of pyrite. We established a new collaboration with a researcher from Universidade de Evora, Portugal. This new collaborator conducted an analysis of 43 elements in sediments from nine of the lakes. Five elements (chromium, manganese, iron, nickel and copper) are present in concentrations that exceed United States Environmental Protection Agency benchmarks for water quality (Table 1). Two additional potentially toxic elements (arsenic and lead) are present in some lakes, but below USEPA benchmarks for water quality (Table 1). These data suggest that, like the brine shrimp, *B. poppei* possess a resistance to putative toxic elements. The concentration of one metal, nickel, could potentially explain differences in embryo abundance in sediments of Antarctic lakes.

Table 1: Metal and zooplankton embryo abundance in sediments of nine lakes on King George Island, Antarctica.

	Cr (PPM)	Mn (PPM)	Fe (PPM)	Ni (PPM)	Cu (PPM)	As (PPM)	Pb (PPM)	*B. poppei* embryos per cm^3 sediment
Lake Barton 4	17.4	937.2	53825.3	0.0000	81.6	2.6116	14.4054	138
Lake Barton 5	26.2	1066.1	60391.4	1.4639	179.9	1.1177	6.9427	4
Lake Barton 9	40.5	1307.1	71522.8	2.4826	58.2	1.9315	0.0000	0
Lake Fildes 1	46.1	1317.9	75022.1	0.7979	140.8	3.1831	0.0000	0
Lake Fildes 2	95.6	1335.5	66084.3	48.1343	92.6	2.1955	6.4214	0
Lake Fildes 5	40.1	1200.1	65530.9	0.0000	48.5	0.2048	0.0000	27
Lake Fildes 6	39.3	1167.7	63616.5	5.7772	43.4	0.0000	2.0844	1
Lake Weaver 5	52.1	1302.2	72246.4	3.7037	67.7	1.5442	0.0000	0
Lake Weaver 6	57.7	1347.4	72377.4	6.1145	42.8	1.8880	0.0000	2
Bioaccumulating (yes/no)	No	No	No	Yes	Yes	Yes	Yes	
EPA Benchmark (PPM)	43.4	460.0	20000.0	22.700	31.6	9.800	35.800	

Note: grey boxes indicate values greater than the U.S. Environmental Protection Agency benchmark for water quality.

* In addition to sediment analyses, physical variables and water chemistry were monitored using a YSI multi-parameter meter. Measurements of conductivity, total dissolved solids, temperature, pH, redox status and dissolve oxygen were taken in the water column of lake on King George Island at multiple depths and locations in 23 lakes in March 2018, 14 lakes in March 2019 and 5 lakes in December 2019. Maximum depth of measurement was 2.5 m, and measurements were made every 0.5 m to provide a vertical profile of each variable. Temperature of the lakes ranged from -0.06 to 5.39˚C. Specific conductivity and salinity ranged from 29.6 to 224.9 μS cm^{-1} and 0.01 to 0.11 PSU, respectively. Dissolve oxygen ranged from 72 to 120.8 % saturation. pH varied from 5.96 to 8.02. Redox potential ranted from 21.9 to 381.1. Chlorophyll ranged from 0 to 6.09 μg L^{-1}. None of these physical or chemical variables were positively or negatively correlated with embryo abundance (data not shown; $p>0.05$).

* ***<u>Report on Objective 3</u>:***
* **Objective:** Use ^{31}P-NMR to determine the role of intracellular pH transitions in establishing and breaking diapause and anoxia-induced quiescent states in *B. poppei*.
* **Hypothesis:** Anoxia-induced quiescence in *B. poppei* is initiated by intracellular acidification, but intracellular pH will acidify to a lesser extent under diapause. (Year 1 and 2)
* **Summary:** We used ^{31}P-NMR to evaluate intracellular pH by monitoring the chemical shift of inorganic phosphate. This technique also allows us to evaluate changes in other phosphor-compounds during early development.
* **Significance:** These are the first data to reveal intracellular pH in an Antarctic zooplankton, and only the second data to demonstrate a transition in intracellular pH as a dormant zooplankton is activated and resumes metabolic and developmental processes.

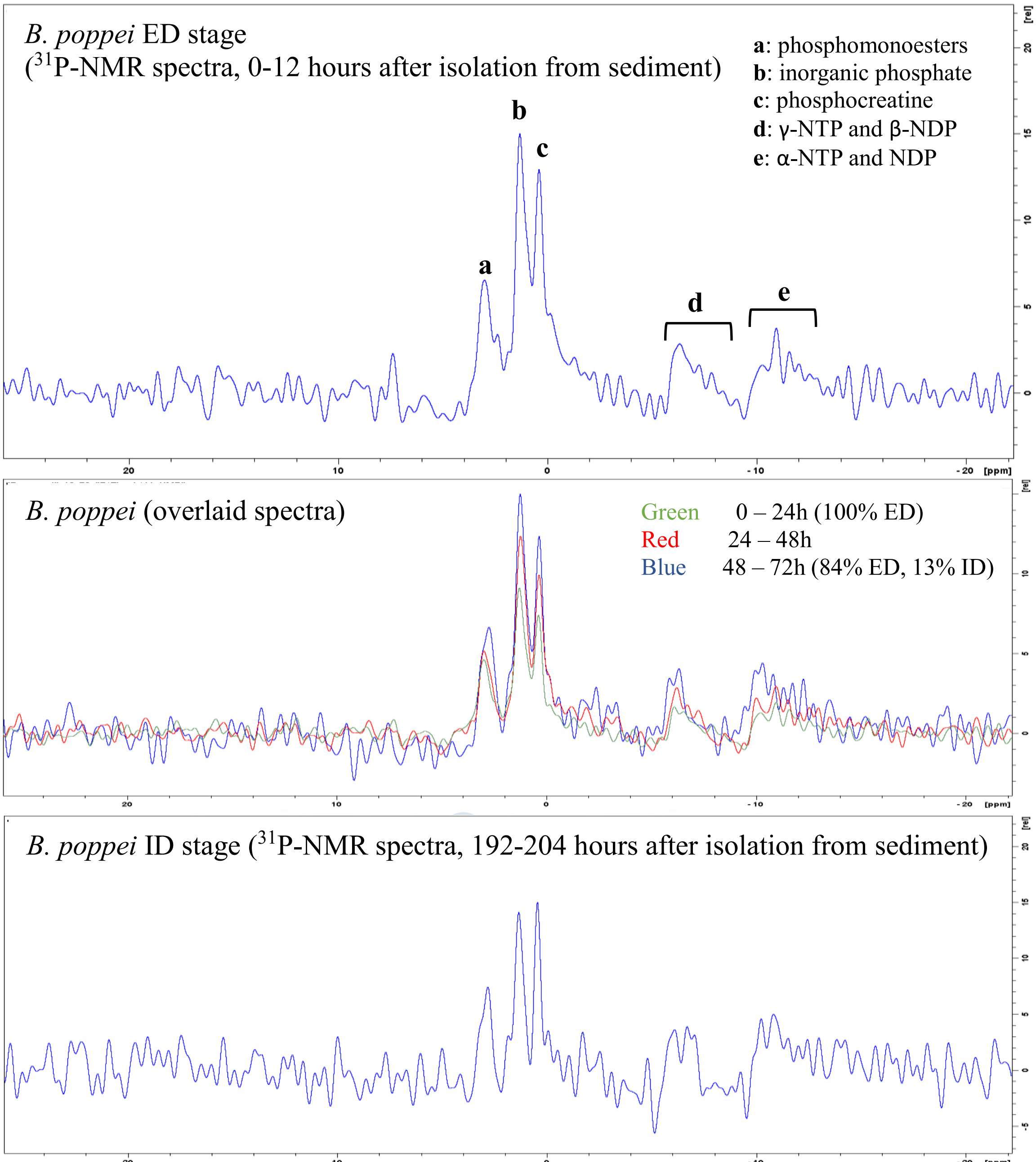

Figure 14. ^{31}P-NMR spectra for *B. poppei*; ED, early development; ID, intermediate development. Phospho-compounds identified in top spectrum; development in lower spectra.

* Phosphate containing compounds are detectable immediately upon isolation of B. poppei from Antarctic lake sediments (Fig. 14). As is observed in the brine shrimp, *Artemia franciscana*, NTP and NDP peaks increase during early development, and the inorganic phosphate peak becomes more pronounced (Fig. 14, three overlaid spectra). Putative phosphocreatine is surprisingly high during early development (Fig. 14). Interestingly, phosphocreatine is not observed in the brine shrimp, *Artemia franciscana* (Covi et al. 2005). ^{31}P-NMR analysis of

anoxia was disrupted by Hurricane Florence. Similar to embryos of *Artemia franciscana*, embryos of B. poppei experience intracellular alkalization of a phosphate containing compartment during early post-diapause development (Fig. 15). A single acidified compartment present during 0-12 h of development alkalinizes to the same intracellular pH (pH = 7.9) observed in developing *A. franciscana* (c.s. Covi et al. 2005 and Fig. 15). Also similar to *A. franciscana*, a more acidic compartment appears as a second peak in the phosphate chemical shift range, indicating the presence of two compartments with differing pH during early development (Fig. 15). The pH of these compartments is unaltered as development continues, and the putative phosphocreatine peak remains as well (Fig. 16.)

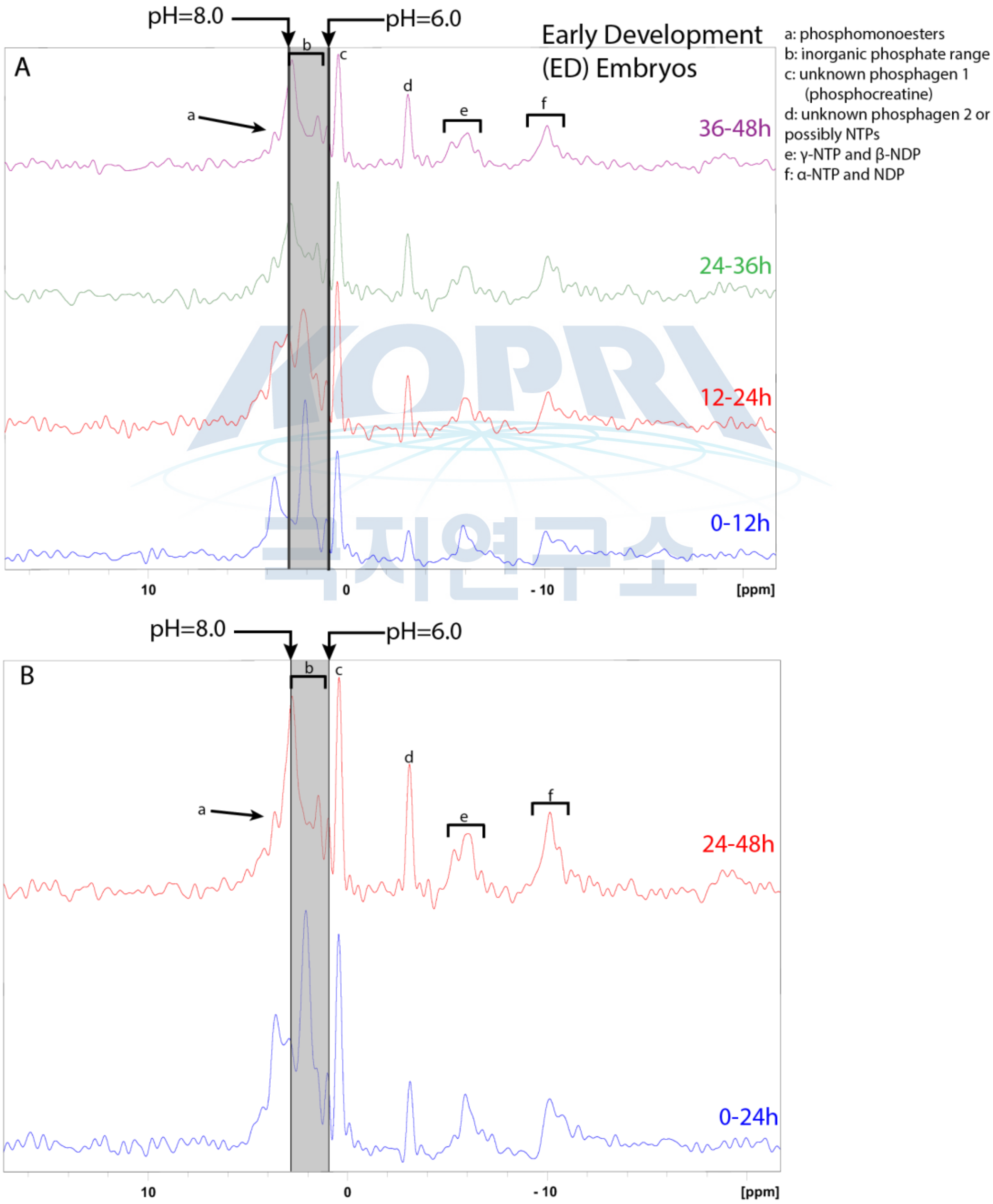

Figure 15. ^{31}P-NMR spectra for *B. poppei*. pH range identified in grey box was determined with a standard curve generated using inorganic phosphate in a crustacean ringers saline solution. Spectra were summed with over 12 hours (top panel) or 24 hours (lower panel).

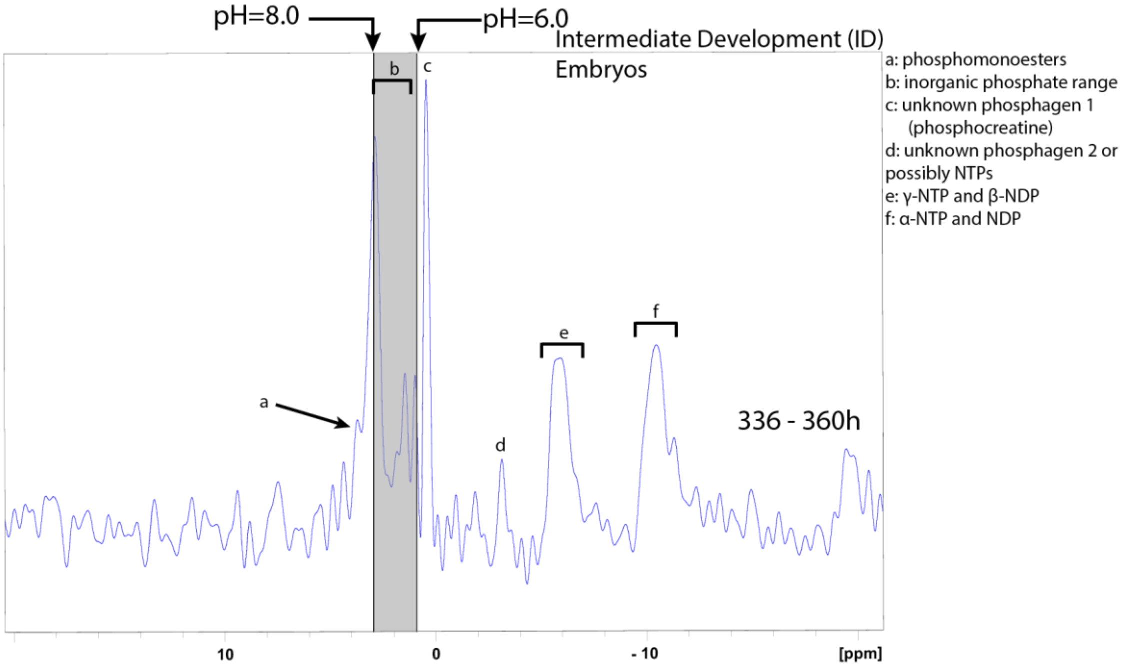

Figure 16. ^{31}P-NMR spectra for embryos of *B. poppei* in an intermediate stage of development. pH range identified in grey box was determined with a standard curve generated using inorganic phosphate in a crustacean ringers saline solution. Embryos were pulled out of culture vessel between 336 and 360 h after isolation from sediment.

* ***Report on Objective 4:***
* **Objective:** Use HPLC to assess adenylate and guanylate status in diapause and anoxic post-diapause embryos of *B. poppei*.
* **Hypothesis:** Both quiescence and diapause in *B. poppei* are accompanied by a decrease in guanylate and adenylate energy charge, AMP:ATP ratio and ATP:ADP ratio. (Year 2)
* **Summary:** Samples for these experiments were destroyed during Hurricane Florence. New sediment containing *B. poppei* were collected in 2019, but the compressed timeline prevented rerunning of experiments to complete the HPLC project.

* ***Report on Objective 5:***
* **Objective:** Test effects of pH on hexokinase, phosphofructokinase and pyruvate kinase isolated from diapause and post-diapause *B. poppei*.
* **Hypothesis:** Acidic pH inhibits the glycolytic enzymes: hexokinase, phosphofructokinase and pyruvate kinase in embryos of *B. poppei*. (Year 2)
* **Summary:** Samples for these experiments were destroyed during Hurricane Florence.

New sediment containing *B. poppei* were collected in 2019, but the compressed timeline prevented rerunning of experiments to complete the enzyme activity project.

Chapter 4 Degree of R&D Goal Achievement and Degree of Contribution to Outside Research Institute

The PI, Joseph A. Covi, has a long-standing record of commitment to broader impacts associated with applied physiology and basic research. PI Covi has a strong relationship with teachers and administrators at local K-12 schools in the Wilmington, NC, area. This connection is rooted in his regular involvement in science fair judging, human physiology outreach programs and international communication outreach programs. PI Covi has over nine years of experience in leading K-12 physiology outreach programs that establish supervised learning relationships between K-12 students and college undergraduates and is already using the zooplankton, *A. franciscana*, as a model system for K-12 outreach programs in ecotoxicology studies. This program is based on micrographic and diagrammatic developmental profiles published in 2015 [15]. This tested program structure will be used as a foundation for an outreach program that will allow elementary, middle school and high school students to monitor the effects of anthropogenic household chemicals and temperature on the development of a common zooplankton embryo while they learn about university student work on the uncommon Antarctic zooplankton, *B. poppei*. As part of this effort, an educational toxicology kit was developed by PI Covi and a local high school teacher in 2018. This kit is being provided to local schools at cost this year. In addition to conducting safe experiments with model zooplankton in their classrooms, 5th grade students will view online images and video of undergraduates conducting research on *B. poppei* in cold room facilities at UNCW and at King Sejong Station, Antarctica. Now that this work has been submitted for publication, information from this project will also be included in a senior seminar course on the adaptations of Antarctic organisms taught by the PI. Students from this course will monitor the progress of the expedition to King George Island, Antarctica, in the fall 2018 semester, and will relate this work to individualized critical evaluations of data on how organisms are adapted to specific environmental variables in Antarctica.

Chapter 5 Application Plans of R&D Results

The data produced by this study in accomplishes three of five primary objectives proposed in the original proposal plus two supplemental objectives added to the study as it progressed. The first supplemental objective assesses freeze tolerance and the second supplemental objective assesses the chemical, elemental and physical environment where dormant embryos of *B. poppei* reside. Completion of the objectives was affected by Hurricane Florence in September, 2018. This hurricane is the most damaging storm in the history of the state of North Carolina, and is estimated to be one of the costliest storms in the history of the United States.

The purpose of this project was to provide a biochemical and developmental foundation for assessments of anthropogenic impacts on zooplankton in Antarctic freshwater lakes that will be actionable for research and military base authorities on King George Island. Future research is needed to elucidate the mechanisms underlying dormancy in embryos of the copepod, *B. poppei*, and to evaluate the susceptibility of those mechanisms to climate change and anthropogenic chemicals. These embryos can remain dormant for nearly 200 years. Thus, it is possible that the embryonic stage is passively exposed to toxicants for periods far in excess of any other life-stage of this species.

Chapter 6 References

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