RESEARCH PLAN

A. Project Title

Long title: Regulation of diapause in *Neocalanus*
Short title: Copepod diapause

B. Proposal Summary

Neocalanus is the dominant calanoid copepod in the Northeast Pacific during spring and early summer. These copepods are a major grazer of phytoplankton stocks, and represent a critical food source for many important fisheries. The life history of *Neocalanus* includes a period of diapause that is associated with the transport of this important prey item from deep waters to the continental shelf in the Gulf of Alaska and Bering Sea. Multiple abiotic and biotic factors are involved in this process that are not completely understood. The purpose of this research is to elucidate the biological mechanisms involved in diapause and examine the role of diet and temperature. Samples of active and diapause *Neocalanus* will be collected from the field. Two separate experimental trials will be conducted that focus on the initiation and the termination of diapause, respectively. Gene expression analysis will be carried out to identify genes involved in initiating and terminating diapause. In addition, lipid analysis will be performed to examine the role of internal lipid stores in diapause. Upon the completion of the proposed project we will have valuable information on internal and external factors controlling behavior that will aid in predicting transport events on and off the continental shelf in the Gulf of Alaska. Similarly, the food density and temperature trials will allow modelers to predict *Neocalanus* response to food availability and better understand system productivity in the future.

C. Project Responsiveness to NPRB Research Priorities or Identified Project Needs

This research project directly addresses the priority area: 1.a.iii. Oceanic zooplankton species in shelf food webs: on-shelf transport processes.

This project will use *Neocalanus plumchrus* from the Strait of Georgia. This is the same species found in the Gulf of Alaska and the results from this research will greatly enhance our understanding of the transport of zooplankton in all Northern Pacific Large Marine Ecosytems. Furthermore, the feeding and migration behavior of *Neocalanus plumchrus* does not differ between the population in the Gulf of Alaska and the population in the Strait of Georgia (Dagg and Walser, 1987). Using samples collected from the Strait of Georgia will also facilitate the experimental trials and teacher workshops to be conducted at the Friday Harbor Marine Labs in the San Juan Islands, WA. The shorter distance to laboratory facilities where the gene expression analysis will be carried out will protect against sample degradation. The information gained as part of this project will be applicable to predicting transport of *Neocalanus* across its range.

D. Soundness of Project Design and Conceptual Approach

Background and Rationale

Neocalanus plumchrus is the dominant calanoid copepod in the Northeast Pacific during spring and early summer. *Neocalanus* is a major grazer of phytoplankton stocks, and represents a critical food source for many fish, bird, and whale species, including several species of juvenile salmon, auklets and bowhead whales. Seasonal abundance of *Neocalanus* can have significant system-wide effects. It has recently emerged that the timing of the peak abundance of *Neocalanus* in the surface waters has shifted back by about 60 days in the NE Pacific and by about 30 days in the Strait of Georgia, relative to

historical trends (J.Dower, personal communication). There is little known about underlying causes of this shift. Coinciding with this shift there has been a dramatic decline in the survival of many, once productive, salmon stocks in the Strait of Georgia (SoG), and the shift in timing could have implications for the survival of fish species in the Gulf of Alaska region.

Like many calanoids, *Neocalanus plumchrus* (*Neocalanus* hereafter) spends part of the year at depth in diapause. Adult *Neocalanus* spawn at depth in late January - early February and release their eggs into the water column. The eggs hatch as they rise, and the copepods progress through six stages of early ontogenetic development referred to as nauplii (numbered N1 to N6) and the first four of the juvenile, or copepodid, stages (numbered C1 to C4) as they rise through the water column during late winter/early spring (Conover, 1988). The later developmental stages (e.g. C2s through C5s) occupy the surface layers of the NE Pacific and the Strait of Georgia each summer.

During the late spring and summer, the C5s will consume phytoplankton and store lipids in preparation for an overwintering period. This grazing period is when the copepods are the most available to predators. Near the end of the summer, the C5s will cease development, and sink to a depth below 300 meters, where they will overwinter for several months before emerging from this resting stage, known as diapause, and continue development into adulthood. *Neocalanus*, unlike other key oceanic calanoid copepods such as *Calanus finmarchicus* in the Northern Atlantic, reproduces at the bottom of the water column. In addition, the adult females do not have functioning mouthparts, which indicates that all reproduction depends on the energy reserves from the previous summer's grazing.

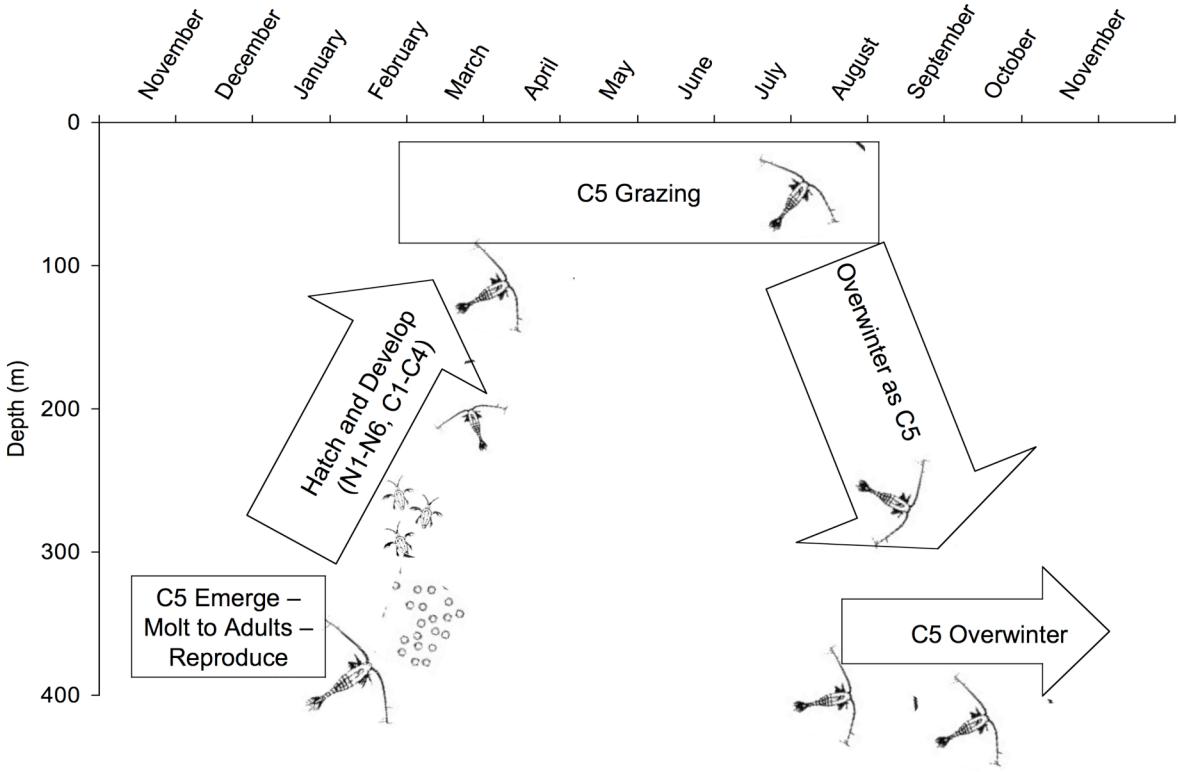


Figure 1. Annual life cycle of *Neocalanus* species in relation to water column depth. Adults reproduce at depth and release their eggs into the water column. *Neocalanus* early larvae (nauplii 1 through 6) are denoted as N1-N6, and the early juvenile stages (copepodid 1 through 4) are denoted as C1-C4. The final juvenile stage is C5, which sinks and overwinters at depth. Timing of life stages in this figure coincides with 2003-2005 surveys in the targeted sampling region.

Recent efforts to link environmental parameters to the timing of diapause in oceanic plankton calanoid copepod species in the Northwest Atlantic have met with mixed results. No single environmental cue definitively explains the timing of entry into and/or emergence from diapause (Johnson et al., in press, Dahms 1995). Some of the proposed triggers tested include: photoperiod, surface temperature and the average concentration of surface layer chlorophyll *a*. Although temperature, as a singular variable, cannot explain the entry into and/or emergence from diapause, it is clear that temperature has an effect upon *Neocalanus* development. A temperature increase from 2^{o}C to 4^{o}C has been shown to shorten development times of *Neocalanus plumchrus* through the naupliar and copepodid stages from 91 to 75 days (Saito and Tsuda 2000). This suggests that changes in temperature may play a role in other aspects of *Neocalanus* life stages, such as influencing the initiation of entry into and/or emergence from diapause.

Because no single environmental cue appears to be solely responsible for triggering entry into or exit from diapause, Johnson et al (in press) has now proposed two alternative hypotheses to explain both the timing of entry into dormancy and the emergence from diapause. The first of these, the "lipid accumulation window" hypothesis, suggests that individuals can only enter dormancy if their food and temperature history has allowed them to accumulate sufficient lipid stores to endure overwintering, molting and gonad maturation upon emergence.

Phytoplankton availability during the period of maximum grazing activity can affect the ability of *Neocalanus* to reproduce in the following year. Recent data collected by University of Victoria scientists during the summers of 2003 to 2005 suggests that (i) juvenile *Neocalanus* were growing much slower than usual in 2005, and (ii) they also had extremely low levels of DHA, a fatty acid required for both growth and reproduction (Dower et al., 2006). When natural phytoplankton densities were supplemented by adding *Isochrysis* and *Pavlova*, algal species rich in the essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), a 25 fold increase in food concentration resulted in a 16% increase in development. A 52-fold increase in food concentration increased development rate by as much as 28% (Liu and Hopcroft 2006).

Johnson et al (in press0 also links lipid storage to emergence, and has proposed that oceanic calanoid copepods posses a "lipid-modulated endogenous timer", which is in agreement with observations of *Neocalanus*. Campbell et al. (2004) and Fulton (1973) observed that samples of *Neocalanus* collected early in the season and reared in the laboratory did not molt into adults as readily as the populations collected later in the overwintering season, when endogenous lipid reserves were similarly depleted in both groups of copepods.

While there is a general understanding and sound hypothesis on abiotic and biotic factors involved in diapause in *Neocalanus* there is no information on the biological mechanisms regulating this process. The purpose of this research is to elucidate the genes involved in diapause and examine the role of diet and temperature. A more thorough understanding of how dormancy and emergence are controlled will provide essential information for predicting how factors will influence transport.

The specific objectives of the current proposal are to:

1) Characterize genes regulating diapause in *Neocalanus*
2) Examine the role of diet and temperature in initiating diapause
3) Characterize genes involved in the termination of diapause
4) Examine the role of temperature in termination of diapause

Detailed Materials and Methods

Sampling

During the course of the project, we will sample three times. We will collect *Neocalanus* samples in the Strait of Georgia in order to obtain active and diapause copepods for gene expression analysis, collect pre-diapause C5s for the food density trial, and collect overwintering C5s for emergence trials. Based on recent field surveys and the life history of this species in the SoG, we know that pre-diapause C5 will be in the upper levels of the water column during the summer months. We plan to take our first sampling cruise during the month of July, to capture pre-diapause C5 for the food density diet trial. It is possible that copepodids that reached the upper levels of the water column in the spring may have had sufficient time to reach diapause, so in addition to sampling the 30m at the surface (sample 1a in Figure 2), we will also sample the mid-depth (200 m) (sample 1b in Figure 2) and bottom of the water column (400 m) (sample 1c in Figure 2) using and open-close net at this time to capture any C5s that may have entered diapause early.

On these cruises and at home base at the Friday Harbor Labs, we will bring trained undergraduate workers and our teacher workshop assistants to assist in the sorting of the copepodids into their respective stages. *Neocalanus* is a relatively large calanoid, and the morphology has been well-described. Using size and distinctive morphometric characteristics, we will sort out sufficient quantities of pre-diapause C5s. Specifically, 50 individual grazing C5s will be placed in a sterile 1.5 ml vial and frozen in liquid nitrogen for later gene expression analysis. If samples are collected from other depths then those samples (n=50) will also be collected. At least two hundred C5s will be selected for the food density trials. The food density trials will take place during and immediately after the first cruise of the summer.

At the end of the summer, the C5 copepodids will begin to sink to the bottom and undergo changes related to entering the diapause stage. This overwintering migration will occur during late August through early October, so our second sampling cruise will take place in September. Again, we will sample at the surface (2a in Figure 2), mid-depth (2b in Figure 2) and bottom (2c in Figure 2) at the same station as the summer sampling. We anticipate finding copepodids in stage C5 at all levels of the water column, which will be the primary source for our comparisons of gene expression. Copepodids near the top of the water column will be close to entering diapause, while those at the mid and bottom depths will be in diapause. This second cruise will be the most labor intensive of the three, so we will bring along at least two undergraduate students pre-trained in the sorting of C5 copepodids. From each of the 3 water collections (e.g. different depths) 50 C5s will be placed in sterile 1.5 ml vials and frozen in liquid nitrogen for later gene expression analysis.

To ensure that we have captured C5 copepodids at their maximum level of dormancy, we will sample again at all three depths (3a,3b, and 3c in Figure 2) during the overwintering period from November through early January. Our third sampling cruise is slated for mid-November, during the middle of the overwintering phase. It is possible that some of the early overwintering members of the population will also begin to emerge at this time (observed by Campbell et al. 2004). The male and female forms of *Neocalanus* are very easy to distinguish from the copepodids, so we will again enlist our undergraduate helpers to separate out the overwintering copepodids from the adults (found at the bottom in sample 3c). During this cruise we will collect 50 C5s from the deep water and they will be placed in a sterile 1.5 ml vial and frozen in liquid nitrogen for later gene expression analysis. Additionally, another 200 C5s will be used for the emergence incubation trials. Upon completion of this third cruise we will have a minimum of 150 (3 groups) to be used in gene expression analysis.

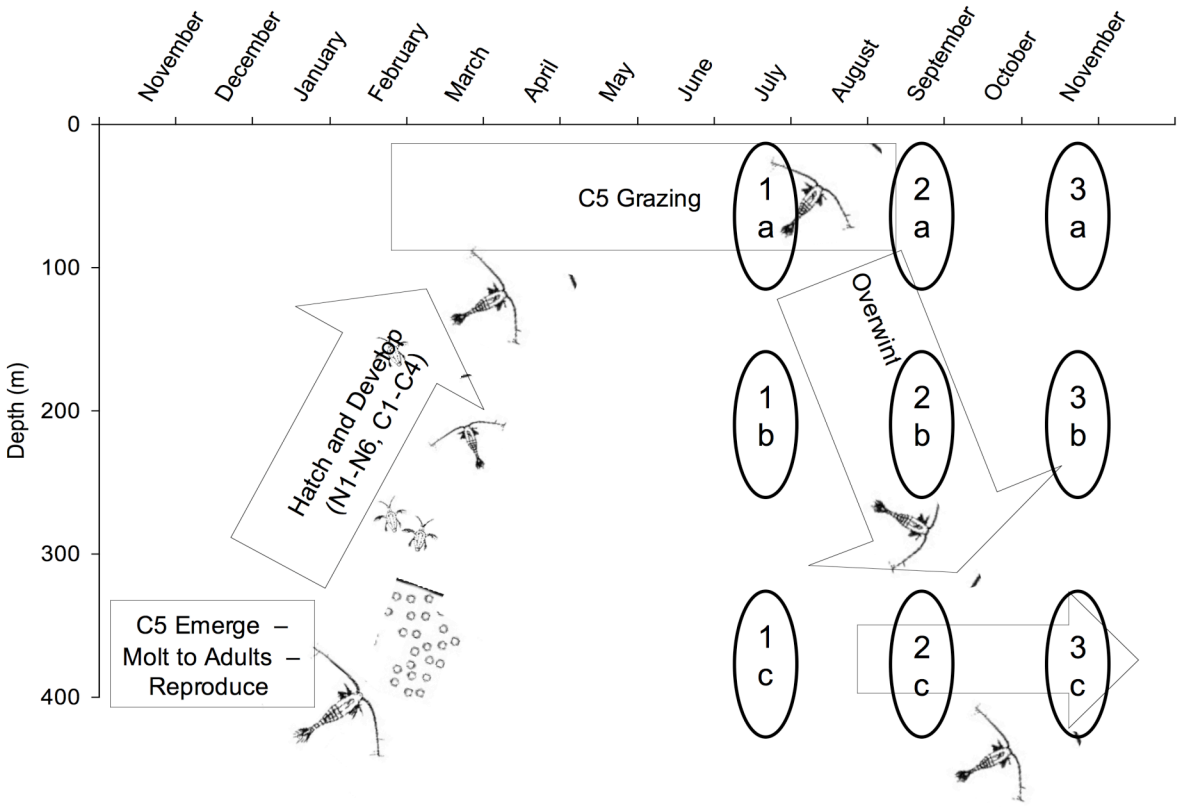


Figure 2. Sampling scheme superimposed on life cycle of *Neocalanus plumchrus*. The time and depth of samples are indicated by open ovals. Samples with the same number will be conducted on the same day. Samples with the same letter will be conducted at the same depth (depths are adjusted for SoG conditions: *a* indicates the upper 30 m of the water column, *b* indicates 200 m and *c* indicates >380 m).

Our field sampling and a portion of the teacher workshop will be conducted on board the R/V Centennial based out of Friday Harbor Labs. The R/V Centennial is a 58' vessel built in 1990 as a commercial trawler and seine fishing vessel and was subsequently modified to also participate in the longline and pot fisheries. It is equipped with a Marine hydrowire winch with levelwind and 800 m of Amagraph .322 conducting cable that can be used with equipment needing to be controlled or monitored from shipboard. This hydrowinch is used in conjunction with an oceanographic carousel consisting of 12 Niskin bottles and a CTD (Seacat 19, with added dissolved oxygen and fluorescence sensors). This winch can also be used with opening-closing plankton nets, such as the one required for the deep and mid water samples. The lifting capacity of the winch is more than sufficient: 3000 lb. The R/V Centennial can accommodate up to 36 people, even though our class size will be limited to ten teacher apprentices for our summer workshop. The 160 square feet of interior dry (and electronics) lab has sinks, counters, cabinets, water supply and power hookups.

Food density and temperature diet trial

Diet is important for the onset of diapause, particularly as it relates to lipid bioaccumulation. It has been proposed that individuals can only enter dormancy if their food and temperature history allows them to accumulate sufficient lipid to endure overwintering, the final molt into adulthood and undergo early stages of gonad maturation.

In order to directly look at the influence of food density on diapause initiation, assess lipid uptake, and characterize associated physiological changes in *Neocalanus*, a controlled laboratory experiment will be carried out. Two of the hypotheses that we will test on pre-diapause copepods are:

A.) *Neocalanus plumchrus* C5s provided with a higher density of phytoplankton (5.0 mg C m^{-3}) will enter diapause earlier than *Neocalanus plumchrus* C5s provided with a low density of phytoplankton (0.25 mg C m^{-3}); and

B.) *Neocalanus plumchrus* C5s populations reared at 2°C will enter diapause earlier than *Neocalanus plumchrus* C5s reared at 5°C when both populations are provided the same diet.

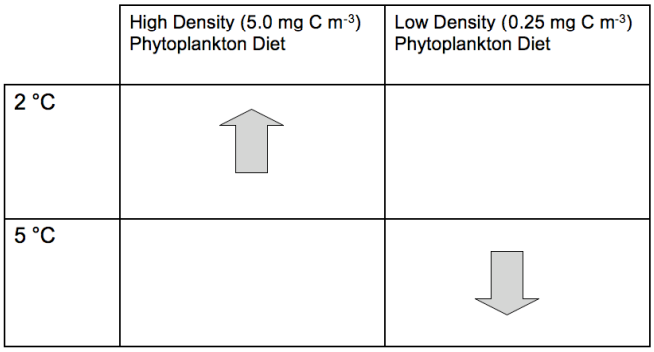


Figure 3. Sampling schematic for copepods in the diet and temperature laboratory tests. Samples will be collected at time 0, 2 weeks, 4 weeks and at first signs of diapause in any of the treatments (or 6 weeks past collection if diapause is not detected.) The up arrow indicates the treatment with the most favorable conditions for lipid bioaccumulation and possible diapause, and the down arrow indicates the treatment with the least favorable conditions.

The premise for hypothesis A is that an increased diet will provide essential lipid stores necessary to initiate diapause. The premise for hypothesis B is that increased temperature will increase lipid metabolism, thereby reducing lipid stores and hence delaying diapause. The ambient water temperature where these overwintering *Neocalanus* reside is 2°C. To test these hypotheses, we will pick out active and undamaged C5 copepodids from our plankton tows in the middle of the summer grazing season (Sample Cruise One - surface collection) and transport them on ice to the nearby Friday Harbor labs for maintenance in a stock culture system. As indicated in Figure 3, two variables will be altered, food density and temperature. The low density diet corresponds to the equivalent diet they would receive in the wild whereas the high density diet corresponds to a 50 fold increase. The diet will consist of 50% by weight *Pavlova* and 50% by weight *Isochrysis*. This is a standard, optimal diet used for copepod rearing (Liu and Hopcroft, 2006). Food density will be adjusted on a daily basis by partial (50%) water changes and introduction of fresh phytoplankton. The culture vessels will be cleaned by siphoning the fecal pellets from the bottom of the containers when conducting water changes.

Samples will be collected at time 0, 2 weeks, 4 weeks and at first signs of diapause in any of the treatments (or 6 weeks past collection if diapause is not detected.) For gene expression analysis, 30 individuals from each condition will be placed in sterile 1.5 ml vials and frozen in liquid nitrogen. At the end of 2 weeks, 4 week, and at first signs of diapause (or 6 weeks), copepods will be taken from each replicate and analyzed for lipid content and storage by classes using HPLC. Two-way ANOVA will be conducted on the effects of temperature and food density on lipid content at weeks 2, 4, and at first signs of diapause (or 6 weeks). Qualitative comparisons of the differences in the genetic regulation of our genes of interest will be prepared for each sampling period of the experiment.

Teacher workshops

As part of our outreach efforts, we will conduct a summer workshop open to ten marine science high school or middle school teachers who would like to serve as apprentices to our project. The ten instructors will be provided with housing and food for the duration of the one week workshop at Friday Harbor Labs. The course content will include an introduction to copepod biology, instruction on basic oceanographic and field sampling techniques (e.g. chlorophyll measurement and CTD sampling), and a field day conducted in concert with our first research cruise. The objective of the course is to provide a basic understanding of the complex food web dynamics that drive biological production in the Northern Pacific. The participants will be encouraged to take up an individual project during the cruise portion of the class and present their findings to the workshop as a whole (e.g. co-occurring zooplankton or phytoplankton species, plankton biomass measured in Niskin bottles, etc.)

Dr. Rhodes will be responsible for the teacher workshops and coordination of activities. The undergraduate assistants will accompany the researchers on the cruises and be responsible for some of the genetic analysis on the UW campus. The undergraduates who participate will be expected to produce a poster for the university-wide Undergraduate Research Symposium.

Diapause emergence incubation

We will collect overwintering copepodids at the bottom of the water column during sampling cruises two and three (samples 2c and 3c in Figure 2) and allow them to emerge in the laboratory. Incubating the overwintering copepodids into adulthood is simpler than the other experiments, as *Neocalanus* does not feed during this time. All energy is supplied by endogenous resources. We will bring overwintering C5s back into the lab and maintain them under controlled conditions. C5 copepodids will be sorted and set up in triplicate containers in two incubators at 2^{o}C and 4^{o}C.

Copepodids will be analyzed for lipid content and class composition at the time of collection and monthly thereafter until December when samples will be taken twice a month. Samples will be analyzed for gene expression difference and lipid composition. One-way ANOVA will be conducted on the lipid content of the copepodids reared at the two temperatures at each sampling period. Qualitative comparisons will be made concerning the gene expression as the copepodids complete the overwintering phase.

	September	October	November	December	January	At Termination (when > 50% adults are visible in any one of the replicate cultures)
Over wintering Group 1 (OWG1)	X	X	X	X X	X X	All replicates in both incubators will be terminated at the same time for OWG1.
Over wintering Group 2 (OWG2)			X	X X	X X	All replicates in both incubators will be terminated at the same time for OWG2.

Figure 4: Schematic of sampling for diapause emergence incubation. Two groups will be incubated, one from collection in September (OWG1), the other from collection in November (OWG2). Each group will be incubated at 2°C and 4°C. The letter X indicates sampling

Gene expression analysis

One major component of this proposal is to determine which genes regulate significant life history events that directly influence the availability of *Neocalanus* as a prey item for important North Pacific fisheries. Specifically, we will characterize genes associated with diapause initiation (or the process of entering into dormancy) and genes controlling the emergence from diapause. This will be done by gene expression analysis of: a) grazing, migrating and overwintering *Neocalanus* collected in the field, b) temporal sampling of grazing *Neocalanus* used in food density trials, and c) temporal sampling of dormant *Neocalanus* emerging under controlled conditions. The methodology that will be used to carry out this work is provided here.

In order to examine which genes regulate diapause in *Neocalanus*, two approaches will be carried out. First, a candidate gene approach will be used. Based on prior research in other taxa, there is a general understanding of what controls these metamorphoses. For example, insect diapause involves insulin signaling, neuropeptide F, cGMP-kinase, AMP-activated protein kinase, and adipokinetic hormone (recent review by Hahn and Denlinger 2007). There is hardly anything known about the *Neocalanus plumchrus* genome, let alone homologus of genes regulating diapause in other species. Currently, there are only nine sequences in NCBI's Genbank database for the organism *Neocalanus plumchrus*. All of these sequences are either mitochondrial or ribosomal RNA. For the current proposal we will use degenerative primers to obtain candidate genes (e.g. internal factors that are involved in diapause and emergence). Designing degenerative primers relies on identifying conserved regions in complementary DNA (cDNA) sequence to increase the likelihood of PCR amplification. This technique has be successfully used by the PI on numerous occasions. PCR primers will be designed in conserved regions using Geneious v3.5 Software; Biomatters. PCR reactions will be performed on genomic DNA and cDNA in order to amplify and isolate candidate gene homologs in *Neocalanus*. The numerous Expressed Sequence Tags (ESTs) from the taxa Copepoda will contribute to this effort. At the time of this proposal submission there were over 17,000 ESTs from the taxa Copepoda in NCBI's GenBank. These sequences will be screened based on sequence similarity searches to determine candidate genes that might be present. If candidate genes are present, this will facilitate the gene discovery efforts. Furthermore, the investigators of the current proposal have used sequence similarity analysis on these same 17,000+ Copepoda ESTs to identify putative functionality using Gene Ontology

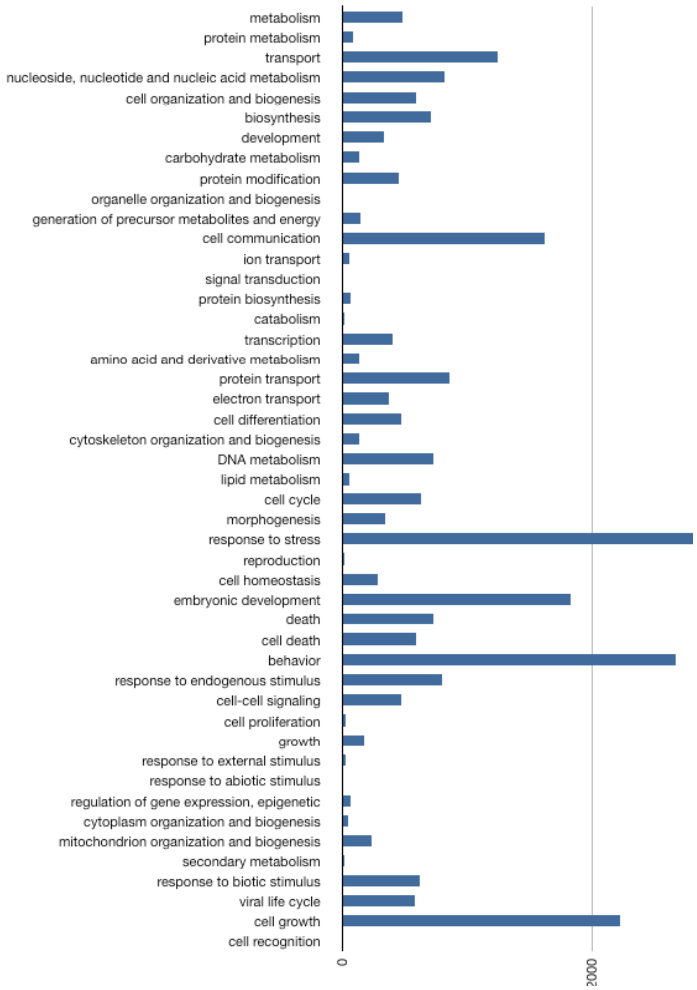


Figure 5. Primary biological processes in which the 17,000 expressed sequence tags for Copepoda fall into based on Gene Ontology Consortium Definitions. The x-axis represents the number of individual expressed sequence tags that are associated with a given biological process.

Consortium definitions (Figure 5). These preliminary data will help identify transcripts that would likely be involved in physiological and morphological changes associated with diapause and dormancy. For example, some of the categories defined by our ESTs analysis include; *lipid metabolism*, *behavior*, *cytoskeleton organization and biogenesis*, and *response to abiotic stimulus* (see Figure 5)

The second approach that we will use to identify genes involved in diapause does not require a pre-conceived concept of the genes (e.g. mechanisms) involved in this physiological process. Differential display analysis will be used according to the GeneFishing DEG System (Seegene) and uses a large number of random primers to identify differentially expressed genes. Genes differentially expressed at a specific developmental time (i.e. just entering diapause) are more likely to be involved in internal regulation processes. Using this approach we will not only be identifying novel genes in *Neocalanus*, but we will also have data indicating expression patterns. This differential display system employs Annealing Control Primers (ACP) technology. The reason this approach is being used is that it is economical, fast, and enables comparison of a large number of different samples. The principle of ACP technology is based on the tripartite structure of a specific oligonucleotide primer (ACP) having 3'- and 5'- end distinct portions separated by a regulator and the interaction of each portion during two-stage PCR. The resulting PCR products will be separated on an agarose gel, and differentially expressed bands removed and stored for DNA sequencing. The PI on the current proposal has used this technique successfully on several occasions including identifying genes involved in bay scallop metamorphosis (Figure 6). PCR products will be cloned into TOPO TA pCR 2.1 (Invitrogen) and colonies of transformed bacteria will be grown for extraction of plasmid DNA. Templates will be prepared in a Rev Prep Orbit (GeneMachines) and the resulting cDNAs sequenced using a modified dideoxy chain termination method with Big Dye Terminator (Applied Biosystems). Sequencing reactions will be precipitated and pellets resuspended in Hi-Di Formamide with EDTA (Applied Biosystems) and analyzed using a 3730 Sequencer (Applied Biosystems). All sequences will be analyzed with NCBI Blast programs for similarity to known genes (Altschul, 1997). ClustalW (MacVector 7.2) analysis will be used for sequence pair-wise and multiple protein alignments. Previous use of the this technique by the PI indicates one differentially expressed gene will be identified for ~10 primer sets used. We will screen up to 150 primer pairs. To identify genes involved in the initiation of diapause, we will examine pooled whole organism RNA samples from *Neocalanus* taken at 1) shallow water (grazing C5s), 2) mid-water (migrating C5s) and 3) deep water (overwintering C5s). .

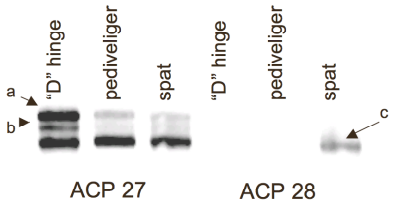


Figure 6. Example of differential display analysis used to identify genes associated with bay scallop (*Argopecten irradians*) metamorphosis. ACP 27 and ACP 28 are two different random primer sets and lower case letters (a, b, & c) with arrows indicate differentially expressed genes.

Once genes have been isolated from *Neocalanus* by using both the candidate gene and differential display techniques, 4-6 genes will be selected to be used in quantitative RT-PCR on the samples of grazing, migrating, and overwintering *Neocalanus.* Once gene products have been sequenced, quantitative RT-PCR will be used to confirm differential expression by analyzing gene expression levels in multiple samples. For quantitative PCR (qPCR), analysis will be conducted on individual *Neocalanus*. While we will have a minimum of 50 organisms at each of the three water depths, qPCR will be carried out on 20 samples. RT-PCR reactions (Brilliant SYBR Green QRT-PCR Master Mix Kit, 1-Step, Stratagene) will be carried out in an Opticon Continuous Fluorescence Detection System (Bio-Rad). For all real-time assays, melting curves will be analyzed to verify that no primer dimers are formed and that CT values represent the desired amplicon. CT values will then be converted to relative abundance levels based on their respective standard curves and normalized to the corresponding 18S RNA values. For data analysis t-tests will be used where appropriate, relationships between gene expression and lipid levels will be

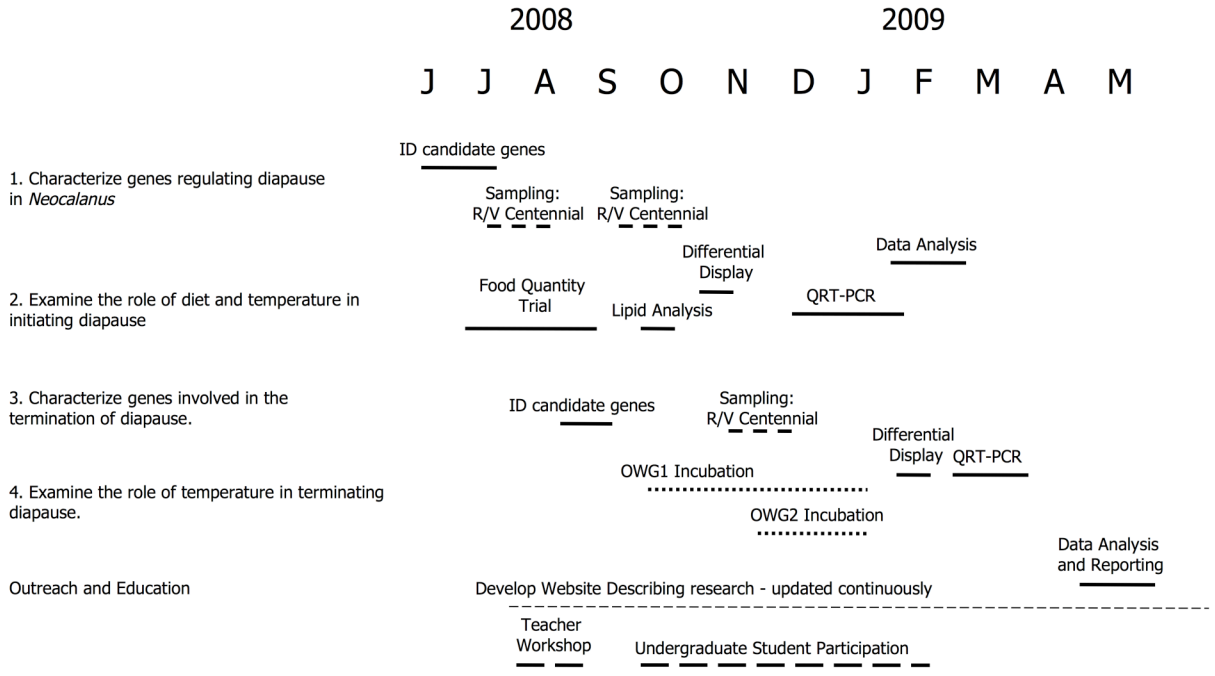
examined with correlation analysis, and patterns of gene expression will be analyzed using principal components analysis.

The second research objective of this proposal is to characterize the effects of food quantity and temperature on the timing of diapause. Sampling from the food quantity and temperature diet trial (described above) will occur at time 0, 2 weeks, 4 weeks, and the first sign of diapause or six weeks past field collection. Thirty individual copepods will be collected at each of the four sampling events. RNA will be extracted and quantitative RT-PCR carried out using the same 4-6 genes identified from the candidate gene and differential display techniques described above. Regardless or whether the diet trial induces diapause, the gene expression analysis (e.g. quantitative RT-PCR) will elucidate the physiological processes underway during the four treatments (high vs. low food density x high vs. low temperature). Similarities and differences of gene expression patterns among the laboratory cultures and field samples will also be examined. In this manner, in the event that complete dormancy is not achieved, inferences could be made from the gene expression patterns in comparison with wild samples.

The third research objective of this proposal is to identify what genes are involved in the termination of diapause. Once again, the rationale for this is that if we have a better understanding of the physiological mechanism involved in this process, we will be able to better predict transport events. For this objective, gene expression patterns will be assessed in *Neocalanus* emerging from dormancy under closely monitored conditions. As described above, deep-water samples containing overwintering C5s will be brought back to the lab, and incubated in environmentally controlled chambers. We will select a subset of genes based on the candidate gene and differential display approaches carried out in 2008 in order to perform quantitative RT-PCR on the emergent samples. As there will clearly be other physiological mechanisms involved in emergence as compared to entering into dormancy, degenerative primer based PCR and the differential display system will also be used to identify genes unique to emergence from dormancy. For example, we will target ecdysteroid and molt-inhibiting genes described for premetamorphic insects. For the differential display analysis, pooled samples will be taken from early, mid, and late time points. For example, for the overwintering group we collect and establish in September (OWG1), RNA would be pooled and sampled monthly until we collect and establish an overwintering population from November (OWG2). After the second group has been established, we will increase the frequency of sampling to biweekly until more than 50% of the C5s in culture have emerged from dormancy, as indicated by the final molt into adulthood. We will conduct qPCR on individuals (n=20) utlizing 4- 6 candidate genes unique to the emergent phase of diapause in *Neocalanus*.

E. Timeline and Milestones

As described above, we have already completed some initial bioinformatics analysis of copepod gene sequences which will assist in developing a list of candidate genes to isolate from *Neocalanus* at the initiation of this project. Currently, in GenBank, there are only 264 nucleotide sequences for the entire genus *Neocalanus*, all of which are primarily sequences for population genetics (i.e. COI). In the first couple of months of this project we will submit multiple sequences to GenBank. These sequences will be made available to the public and will be valuable for other scientists studying *Neocalanus sp*. In the late summer of 2008, the first research cruise will take place to collect grazing C5s for the food quantity trials. We will have data regarding the morphometric effects on diet and temperature parameters by Fall 2008. At this time, we will be preparing for the second set of research cruises in which we will collect samples throughout the water column (3 depths). Samples will be brought back to Friday Harbor and sorted with the assistance of our teacher apprentices from our teacher workshop. By the end of 2008, we will have completed quantitative RT-PCR analysis on wild caught samples and on organisms in the food quantity trials. In early winter of 2009, the third series of cruises will take place to collect over wintering *Neocalanus* for the diapause emergence incubations. By April 2009, we will have completed the quantitative RT-PCR analysis on samples at the termination of diapause. Primary milestones will occur at the halfway point and near the end of the project when we will have characterized the gene expression patterns associated with the *Neocalanus* diapause initiation and termination, respectively. The combined data from this project will immediately provide tools for researchers to study *Neocalanus* throughout the North Pacific and better predict environmental impacts on zooplankton biomass.



F. Project Management

The proposed project will be managed by the PI, Dr. Roberts. Dr. Roberts will be responsible for directing molecular approaches and coordinating sampling. The research program of Dr. Roberts focuses on understanding physiological differences in aquatic organisms by characterizing genes and their respective expression pattern. As similar activities are ongoing in his lab, access to state of the art equipment and related resources will be made easy. Dr. Roberts will also be responsible for developing and hosting the website, similar to the one describing another project- http://fish.washington.edu/research/genefish/robertslab/dermo.html. The post-doctoral fellow, Dr. Rhodes will be working full-time on this project and will be responsible for carrying out the sampling, diet trials, and molecular analysis. Dr. Rhodes has almost ten years experience working with zooplankton cultures. She has experience with triggering diapause egg production in *Centropages hamatus* and *Acartia tonsa* using temperature and light cues. These trials will take place at Friday Harbor where Dr. Rhodes will reside for approximately two months in the early fall. During this time the teacher workshops will take place. Dr. Rhodes will run these workshops. Dr. Rhodes has trained marine science professionals in the maintenance of zooplankton cultures as a guest instructor at Harbor Branch Oceanographic Institution in Ft. Pierce, FL and Seahorse Ireland in Carna, Ireland. She also recently taught an upper level class on marine plankton at UW Tacoma.

G. References

Campbell, R.W., Boutillier, P., and J.F. Dower. 2004. Ecophysiology of overwintering in the copepod *Neocalanus plumchrus*: changes in lipid and protein contents over a seasonal cycle. Marine Ecology Progress Series 280: 211-226.

Conover, R.J. 1988. Comparative life histories in the genera *Calanus* and *Neocalanus* in high latitudes of the northern hemisphere. Hydrobiologia, 167-168: 127-142.

Dagg, M.J. and W.E. Walser. 1987. Ingestion, gut passage, and egestion by the copepod *Neocalanus plumchrus* in the laboratory and in the subarctic Pacific Ocean. Limnology And Oceanography 32 (1): 178-188.

Dahms, H. 1995 Dormancy in the Copepoda—an overview. Hydrobiologia 306:199–211

Dower, J.F. pers. comm. Assistant Professor, Biology Department and School of Earth & Ocean Sciences, University of Victoria http://web.uvic.ca/~dower/dowerlab/research.html

Dower, J.F., Sastri, A. and R. El-Sabaawi. 2006. Collapse of *Neocalanus plumchrus* population in Strait of Georgia. *In* State of the Pacific Ocean 2005. Fisheries and Oceans Canada – Pacific Region.

Fulton, J. 1973. Some aspects of the life history of *Calanus plumchrus* in the Strait of Georgia. J Fish Res Board Can 30:811–815.

Hahn, D.A. and D.L. Denlinger. 2007. Meeting the energetic demands of insect diapause: Nutrient storage and utilization. Journal of Insect Physiology 53 (8): 760-773.

Johnson, C.L., Leising, A.W., Runge, A., Head, E.J.S., Pepin, P., Plourde, S. and E.G. Durbin. *In press*. Characteristics of *Calanus finmarchicus* dormancy patterns in the northwest Atlantic. Marine Ecology Progress Series.

Liu, H. and R.R. Hopcroft. 2006. Growth and development of *Neocalanus flemingeri/plumchrus* in the northern Gulf of Alaska: validation of the artificial-cohort method in cold waters. Journal of Plankton Research 28 (1): 87-101.

Saito, H. and Tsuda, A. Egg production and early development of the subarctic copepods *Neocalanus cristatus, N. plumchrus* and *N. flemingeri*. 2000. Deep-Sea Research Part I-Oceanographic Research Papers 47 (11): 2141-2158.

Tarrant, A.M., Baumgartner, M.F., Verslycke, T. and C.L. Johnson. *In press.* Differential gene expression in diapausing and active *Calanus finmarchicus* (Copepoda). Marine Ecology Progress Series.