A nationwide collaborative to maximize the benefits of reproductive sterility in shellfish aquaculture

1. Introduction/background/justification

The need for a topical hub

The United States aquaculture industry, valued at over $1.4 billion, is a critical component for domestic food security. Shellfish farming is one of the most sustainable protein production systems in the United States (Hilborn et al. 2018), and contributes significantly to domestic aquaculture production, helping to offset the substantial US seafood trade deficit (Kite-Powell, Rubino, and Morehead 2013).

Tremendous potential exists to increase productivity in shellfish aquaculture, whether it be through improved culture techniques, broadened growing areas, addition of new species, expanded marketing, or genetic improvement. For shellfish genetics, a number of genetic improvement programs have come online around the globe (Kube et al. 2011; Dove et al. 2013; Dégremont et al. 2015) and across the US (Langdon et al. 2003; Guo et al. 2008; Frank-Lawale et al. 2014), for the two major oyster species, *Crassostrea gigas* and *C. virginica*. Selective breeding continues to be a major opportunity to increase productivity in shellfish farming, especially with new developments such as genomic selection (Houston 2017). ***The most impactful genetic technology in shellfish aquaculture to date, however, has been the ability to create sterile oysters.*** This is primarily accomplished by creating triploids (three sets of chromosomes) (Stanley et al. 1981), which itself was optimized by the development of tetraploids (four sets of chromosomes) (Guo and Allen 1994). Triploid production is possible wherever there is hatchery technology with access to tetraploids; triploid oysters are now produced world-wide (Guo et al. 2009).

Sterility has clear advantages in shellfish aquaculture including the ability to i) increase growth and flesh quality for marketability as energy reserves are redirected to somatic tissue (Garnier-Géré et al. 2002, Wadsworth et al. 2019a), ii) prevent accidental introduction of non-native species into the environment (e.g., Calvo et al., 2001), and iii) preclude genetic contamination of wild, native bivalve populations by farmed conspecifics (e.g., Vadopalas and Davis, 2004). Despite these advantages, barriers to environmentally sustainable production and economic viability of using sterile oysters remain.

Marketability

Triploids have significant benefits on the farm that have made them popular wherever hatcheries are involved with commercial seed production. In France, for example, despite the widespread availability of wild-caught seed, there is a substantial

market for triploid *C. gigas* seed, making France the largest producer of triploids in the world (~3 billion units in 2012: Degremont et al. 2015). Approximately 50% of Pacific oysters grown in Washington State are triploid, owing to the reduction of less palatable gonad tissue compared to diploids. The proportion of triploid Eastern oysters grown in Chesapeake Bay is even higher, hovering between 80-95% (Hudson, 2018; chesapeakebay.noaa.gov/fish-facts/oysters). In the Gulf of Mexico, production of sterile triploid Eastern oysters has likewise increased since the adoption of this technology. The shellfish aquaculture industry produces these reproductively impaired triploid oysters for the benefits of year-round marketability and improved performance traits (Allen and Downing 1986). **However*, considerable losses to the shellfish industry still exist due to poor survival and performance of sterile triploid oysters in subpar environments and/or at specific developmental stages (described below)*.**

Nonnative biosecurity

Sterility also confers an advantage to the shellfish industry by enabling the farming of non-native species by substantially reducing the risks of unintentional introductions. In locales where ecological conditions (e.g. disease) hinder the recovery and farming of the native *C. virginica*, one of the considered solutions has been the use of a nonnative species as a viable replacement. Calvo et al (2001) describe the relative disease resistance and enhanced performance of the non-native *C. ariakensis*, rendered sterile via the induction of triploidy, in Chesapeake Bay. For non-native production, fidelity, stability, and sterility of triploids need to be ascertained to ensure ecological sustainability (National Research Council, 2004)**.**

Conservation of native species

Farming native shellfish has become increasingly attractive as farming of non-native species is often disallowed to reduce the ecological risk of invasive species. Here too, producing sterile native shellfish is beneficial because with sterility, farmed-wild interbreeding of conspecifics cannot occur, thus eliminating genetic risks (loss of within-population diversity, loss of among-population diversity, and loss of fitness) to wild populations (Camara and Vadopalas 2009; Waples, Hindar, and Hard 2012) including the potential to disrupt naturally occurring patterns of adaptation (Heare et al. 2017; Heare et al. 2018). With the goal of sustainability, proposals to regulatory agencies for new native species for aquaculture are often denied unless provision for production of sterile outplants has been made (see letter of support from Washington Department of Fish and Wildlife), yet adapting currently available sterility induction technology (triploidy) to new species for aquaculture remains a substantial challenge.

Limitations to maximizing the benefits of sterility

Sterile or non-reproductive shellfish are both a market-driven need and an ecologically sustainable approach to increasing food production. ***Despite the widespread use of triploidy as a genetic tool to increase productivity, and the need for reproductive impairment to enable diversification of the shellfish aquaculture industry, there are significant limitations to maximizing the benefits of sterility***. One principal limitation is that mated triploid technology (diploid x tetraploid crosses) has thus far been limited to *Crassostrea,* despite attempts to create tetraploids in other species (*c.f.*, Guo et al. 2009). This limitation may be related to overall viability of other species in the tetraploid state. Another limitation of triploid technology, our focus in this proposal, is an apparent "sensitivity" of triploids to stressful environments such as high temperature or low food conditions. Sensitivity may be related to elevated triploid metabolism. Clearly, advancing our understanding about creating sterile shellfish and maximizing their performance are warranted for the "technology of sterility."

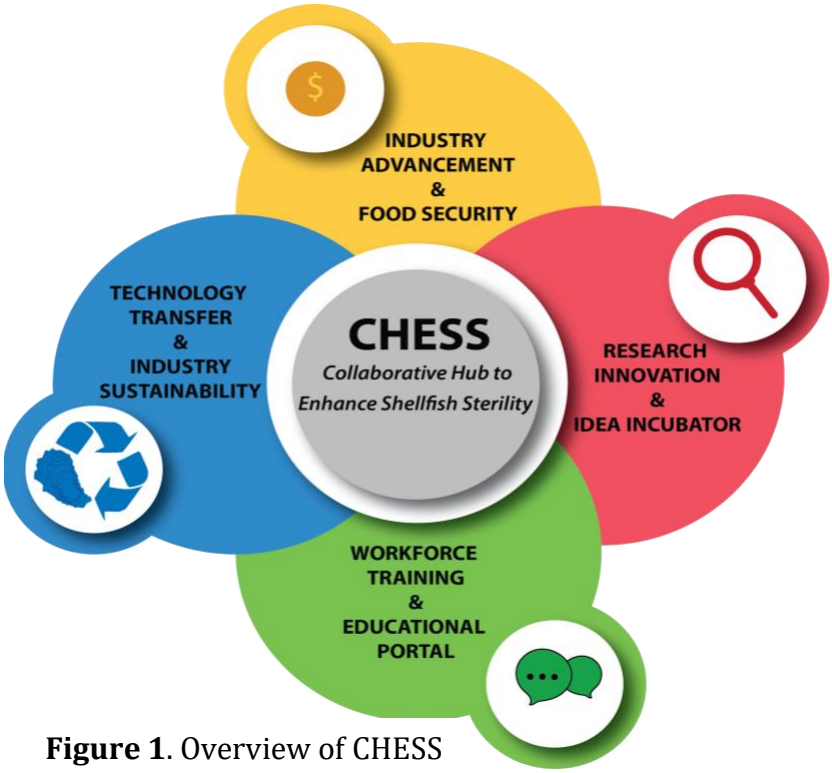

Figure 1. Overview of CHESS

The collaborative approach

Technology for the control of reproduction is rapidly advancing in biological discovery, selective breeding, and genetic manipulation. For example, the search for quantifiable phenotypes to enable targeted breeding can be accelerated by coupling molecular and organismal physiology to, for example, provide brood stock for better performing triploids Many of these advances are now within reach of the aquaculture sector. ***We propose developing a topical hub (CHESS - Collaborative Hub to Enhance Shellfish Sterility) focused on sterility in shellfish that will leverage existing field trials with the application of new expertise.*** The proposed CHESS project (Figure 1) would provide an integrated, interjurisdictional, and multidisciplinary initiative that would enable new capacity to address barriers and support evidence-based decision making for the shellfish aquaculture industry. The CHESS project not only will improve the state of

knowledge for shellfish reproductive control, but also has the potential for knowledge transfer and integration with other fields of marine aquaculture.

Our overall project objectives include:

A. Establish a national, industry-driven Hub serving as a biological material repository, idea incubator, and education resource.

B. Tackle major issues hampering the efficiency and efficacy of aquaculture (e.g., mortality associated with triploid oysters).

C. Improve aquaculture workforce training through curricula development and industry technology transfer

We envision that CHESS will provide a stepwise approach, with a long-term vision. While initially we will build the hub infrastructure and focus on a prominent issue (triploid mortality in *C. virginica*), in subsequent years of CHESS, we envision re-assembling expertise to address triploid performance in *C. gigas*, develop sterility induction techniques for other species, and develop new technologies that to maximize the benefits of reproductive sterility in shellfish aquaculture. The CHESS project responds directly to the needs of the shellfish industry (see letters of support) by addressing critical barriers to the production of sterile shellfish.

Context for Establishing the Collaborative Hub

Washington Sea Grant is an ideal center for CHESS because the West Coast has the richest array of commercial shellfish species in the U.S. and one of the most advanced shellfish aquaculture industries in the world. This proposed research is well matched to NOAA aims to "*Support critical extension/technology transfer capacity to enhance the synthesis and transfer of past research as well as inform next generation efforts; and Advance the topic or geography of focus to the next appropriate phase of development.*" Specifically, the CHESS project addresses NOAA goals of "*Prioritizing and supporting research to remove production barriers or bottlenecks related to disease, genetics and genomics, hatchery seed stock, feed availability, engineering limitations and/or other topics*". This will be accomplished through a focus on shellfish reproduction. Here we proposed to initiate this hub using the case of triploid mortality assessed through leveraging pedigreed families for structured grow-out trials and physiological characterization.

Collaborative Hub Expertise

This collaborative project brings together a team of researchers with complementary expertise to address the specific problem of triploid mortality in *C. virginica*. Washington Sea Grant (WSG) under Callender (WSG Director) will lead the project and work directly with Roberts (Chew Endowed Professor in Aquaculture at the University of Washington, UW) and Vadopalas (Aquaculture Specialist for Washington Sea Grant). This leadership group collectively has over 40 years of experience in shellfish biology, aquaculture, and molecular biology, including the use of cutting-edge genomic technologies. Allen (Virginia Institute of Marine Science, VIMS), one of the inventors of both triploid and tetraploid oysters, has conducted collaborative studies in molecular genetics; cytogenetics and gametogenesis in polyploid shellfish; selection and breeding in aquaculture; genetic conservation in fisheries; and shellfish culture techniques. Walton (Mississippi-Alabama Sea Grant Consortium; Auburn University, AU) conducts applied research with local shellfish farmers, commercial and recreational harvesters of wild stocks, national and local organizations, and works closely with industry to answer pressing questions, including recent experimental fieldwork examining the question of triploid mortality. In particular, Walton focuses on development of oyster mariculture in the southern U.S. as a sustainable, environmentally friendly industry that creates jobs, increases incomes, and preserves a traditional way of life on the Gulf coast. Putnam (University of Rhode Island, URI) has valuable expertise in physiological ecology of marine invertebrates including oysters, clams, and corals. Her focus is on how the immediate abiotic environment and biotic interactions drive organism phenotype and the mechanisms involved in responses across levels of biological complexity that include gene, protein, metabolite, and organismal responses. Palm-Flawd (Bellingham Technical College, BTC) manages shellfish and finfish teaching hatchery facilities, leads the college's aquaculture training program, and teaches aquaculture subjects to her students. Palm-Flawd is leading the charge to build education capacity in her aquaculture training program, broaden their shellfish aquaculture curriculum, and conduct ongoing public outreach on environmentally sustainable food production via aquaculture. The CHESS team will work directly with the shellfish industry and natural resource managers to address impediments to sustainable shellfish aquaculture.

Importantly, these researchers and extension specialists have demonstrated experience in the use of advanced technologies and application (i.e., technology transfer) to improve U.S. aquaculture production. The CHESS team integrates with the shellfish aquaculture industry through their direct participation Sea Grant marine advisory services (Walton, Vadopalas), through ongoing research and development (Allen, Walton, Roberts, Putnam, Vadopalas), and through workforce development and aquaculture education (Palm-Flawd). The track record of the CHESS team indicates a long-term investment in improvements for the aquaculture industry. Through close collaboration, our team will combine recent advances in physiological analyses with state-of-the-art breeding programs

to advance the state of knowledge in the control of shellfish reproduction. We will leverage the substantial genomic resources now available for cultured shellfish (e.g. Eastern oyster, Pacific oyster, Pacific geoduck), and integrate our collective expertise in genetics, reproductive development, physiology, and bioinformatics to solve a contemporary issue associated with triploid sterility in *C. virginica*. The research will be done while also enhancing scientific literacy and hands-on training. This CHESS project will lay the necessary foundation and springboard for addressing issues of state, national, and international importance to aquaculture and food security.

The Transformative Advancement of the Hub

The CHESS hub has the capacity to thrive and expand beyond an initial investment. The CHESS project will set up a framework to support a national agenda in food resource security through sustainable fisheries and aquaculture. The innovation of this hub comes through its overarching umbrella of a goal to enhance and extend shellfish sterility across species and geographic boundaries. By doing so, the CHESS hub has the capacity to increase sustainable protein production and enhance the economic resilience of the aquaculture industry.

The CHESS initiative is a creative approach to address a broad range of technological issues on shellfish sterility facing aquaculture. Focused, collaborative, cross-disciplinary efforts will have a greater chance of adequately addressing specific problems facing shellfish aquaculture. We believe that these specific problems can be prioritized and have chosen a contemporary problem as the first project to establish through CHESS. Other priorities for future work include, but are not limited to: a) prevention of the differentiation of primordial germ cell precursors during embryogenesis to achieve sterility across multiple species of shellfish, b) development of tetraploid technology for other species such as purple hinge rock scallops or geoducks by addressing the viability of polyploids, and c) uncovering biomarkers in triploid oysters (both *C. virginica* and *C. gigas*) indicative of improved performance for use in breeding.

Triploid mortality in *C. virginica* will be the first priority to be addressed by CHESS because i) triploid mortality is well-documented (Wadsworth 2018; Guévelou et al. 2019), ii) a pedigreed breeding program for triploid Eastern oysters is already underway, and iii) a high-quality genome for *C. virginica* is available. With a relatively modest investment, we can leverage the pedigreed triploid families and combined expertise in physiology to define the trait(s) behind triploid mortality and incorporate the(se) trait(s) into selection programs.

Background on Triploid Oyster Mortality

After creation and adoption of hatchery technology to overcome production issues, triploid induction techniques were developed in the 1980s to enhance both growth and marketability of oysters (Stanley et al. 1981; Downing and Allen 1987). By virtue of an extra set of chromosomes, reproductive impairment was observed (Allen and Downing 1986). The effective sterility of triploids meant that oysters could be successfully marketed year-round as a valuable alternative to the less palatable gonad-rich oysters during the summer months. In addition, the shift in energy allocation from reproduction to somatic growth was thought to lead to decreased time to market. While the marketability of triploid oysters is irrefutable, their performance is variable; in conditions generally suitable for oyster culture, triploids excel (Allen and Downing 1986; Nell 2002; Dégremont, Garcia, and Allen 2015). ***However, when conditions are not optimal, it is increasingly evident triploid oysters can underperform diploid oysters with reports of triploid oyster mortality on the rise.***

The occasional poor performance of triploids is a substantial issue across the United States. The Pacific Coast Shellfish Growers Association's Research Committee lists triploid performance among its top 2019 priorities: "There is strong interest to utilize breeding approaches to decrease mortalities in both diploid and triploid oysters grown on intertidal beds exposed to high summer temperatures and warmer mean seawater temperatures." In a recent "white paper" written by the East Coast Shellfish Breeding Consortium entitled *Research Priorities to address productivity of shellfish aquaculture on the East and Gulf Coasts*, the group highlights the issue of triploid mortality stating (emphasis added), "It seems clear that further investigations into the physiological differences between susceptible triploids and resistant diploids is warranted and would benefit the entire region accordingly. *Understanding the mechanism responsible for triploid mortality is the first step in defining the trait that will be used to breed for triploid survival*."

Recent work in the northern Gulf of Mexico paired diploid and triploid oysters for grow-out and found significantly greater cumulative mortality in triploids at all four sites (Wadsworth 2019b, **Figure 2**), but no clear cause of these mortalities was evident with different patterns of timing and magnitude. In a suite of grow-out experiments in the Gulf of Mexico carried out by the co-PI Walton (Bodenstein & Walton, Submitted), triploid oysters exposed to over 24 hours of desiccation experienced higher mortality than diploid oysters at one of three sites. Guévelou et al. (2019) investigated triploid mortality in the Chesapeake Bay, following reports of large mortality events on commercial farms in 2012. No clear smoking gun appeared linked to the mortality events; the authors concluded that future research should address the physiology and energetics of triploids during suspected windows of mortality. We propose to investigate these factors in the experiments below.

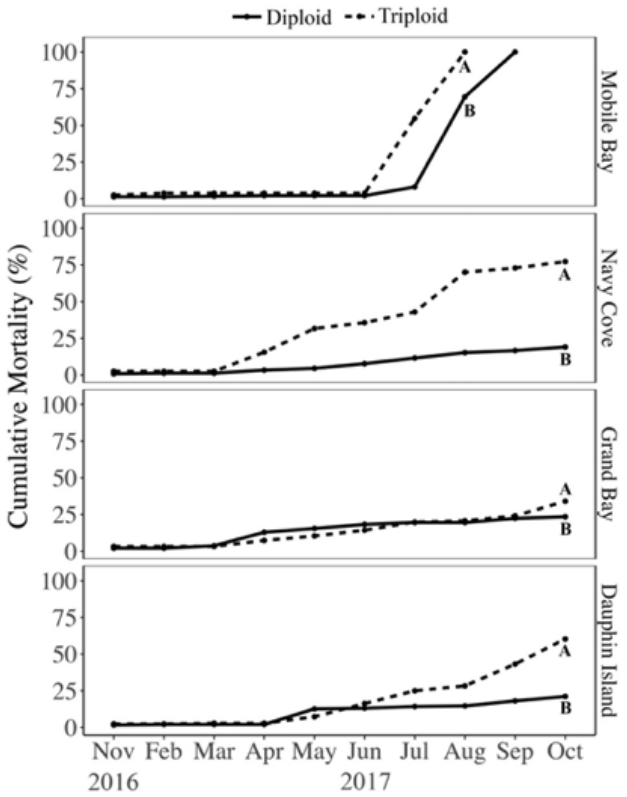

Figure 2. Modified from (Wadsworth 2018): Cumulative mortality of diploid (solid line) and triploid (dashed line) oysters at the Mobile Bay, Navy Cove, Grand Bay, and Dauphin Island sites. Different letters annotating lines indicate statistical differences ($P < 0.05$) between ploidy groups

On the United States West Coast, although poor performance of triploid *C. gigas* (Pacific oyster) is regularly observed and results in economic losses (Tim Morris, Pacific Seafood; Benoit Eudeline, Taylor Shellfish; Kurt Grinnell, Jamestown S'Klallam Tribe; Joth Davis, Baywater Inc. personal communication), no clear explanatory pattern, nor phenotype to target for selection, has emerged. Improving performance in triploid oysters is a research priority identified by the Pacific Coast Shellfish Growers Association (see letter of support). Environmental conditions implicated in differential mortality of triploid *C. gigas* include high water temperature, hypoxia, poor nutrition, low salinity, and high air temperature during exposure during summer low tides (Cheney 2000). How these specific factors directly relate to ploidy and reproductive status has yet to be determined. Moreover, it is unknown whether there is a common or specific physiological response to each stress factor. It should be noted that there is not necessarily a relationship between the etiology of mortality for *C. gigas* on the West Coast (where *C. gigas* is an introduced species) and the etiology of mortality for the native *C. virginica*. We are treating triploid mortality of *C. virginica* as a specific case, but will be looking for links to mortality among *C. gigas* populations.

In order to make genetic improvements in triploids, their constituent parent lines (both diploid and tetraploid) must be genetically improved, for which there needs to be a quantifiable phenotype to measure. ***Characterizing the physiological mechanisms underlying performance (i.e., mortality) is a critical step in this process.***

2. Project Objectives

The **goal** of the CHESS project is to establish a nationally networked hub to address bottlenecks associated with maturation control in shellfish and to maximize its potential across shellfish species. The specific **objectives** for the first phase of the CHESS initiative (Figure 3) are as follows:

A. Establish a national, industry-driven Hub serving as a biological material repository, idea incubator, and education resource.

B. Tackle major issues hampering the efficiency and efficacy of aquaculture (e.g., mortality associated with triploid oysters).

C. Improve aquaculture workforce training through curricula development and industry technology transfer.

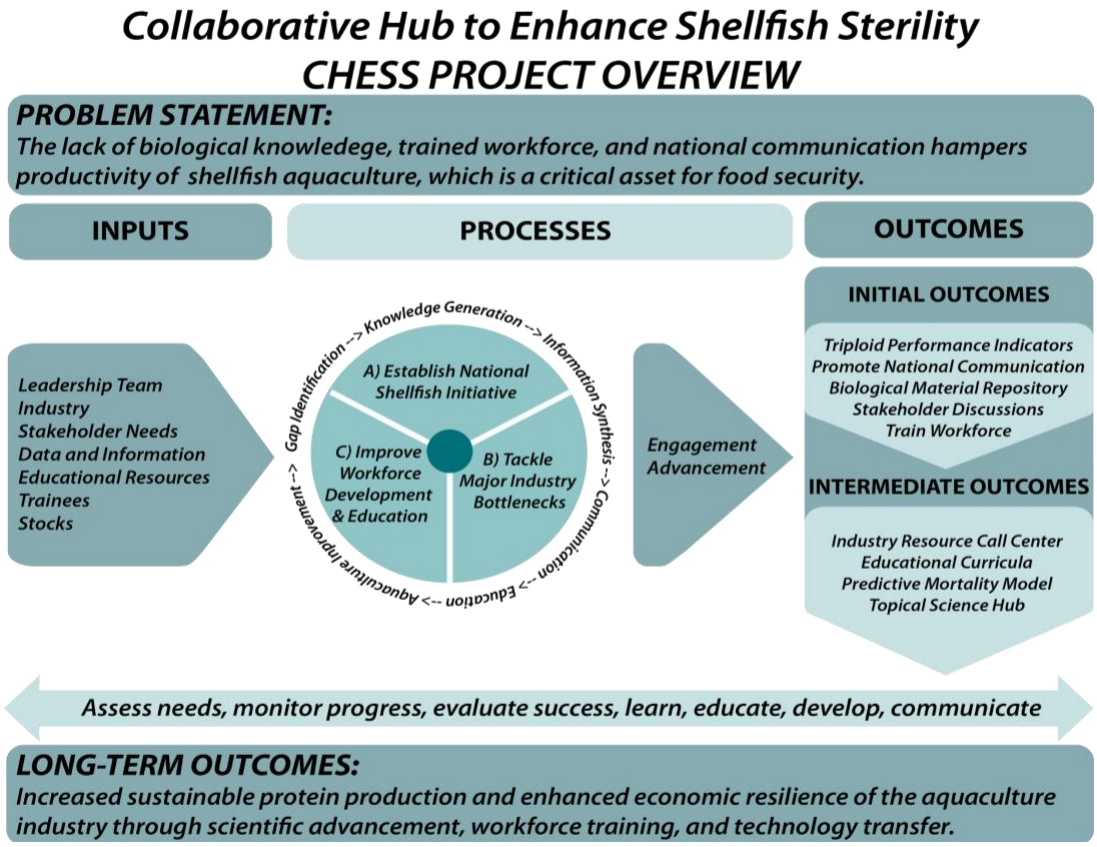

Figure 3. Overview of the industry issue and the proposed CHESS topical hub objectives and outcomes to increase aquaculture production and sustainability.

2.A *Establish an industry-driven Hub*

CHESS is meant to provide a platform for a concerted approach for improving methods to obtain and sustain reproductive sterility in shellfish, thus eliminating duplicative efforts, integrating expertise, and creating synergies among different disciplines (Figs. 1, 3). Our team is also well positioned to coordinate the research, education, and outreach components described below, and has strong connections to the industry.

2.B *Identify factors responsible for triploid performance*

The identification of physiological factors and environmental conditions responsible for superior performance in triploid oysters is key to maximizing the benefits of reproductive sterility in shellfish aquaculture. To this end, we will leverage current knowledge and an experimental design using an array of triploid families grown in key locations, backed up by state-of-the-art investigation of phenotypes associated with mortality across the genetic groups. This collaborative, integrated approach will provide the power to identify distinct physiological drivers of improved performance. The details are included below on the Plan of Work.

Preliminary results on triploid performance

To begin exploring the molecular underpinnings of poor performance in triploid oyster, we have carried out preliminary trials to evaluate differences in the stress response of diploid and triploid *C. gigas* to increased temperature and desiccation. Oysters were subjected to a) control conditions (seawater 13℃), b) air exposure at 27℃ for 24 hours, c) air exposure at 45℃ for 1 hour, and d) air exposure at 27℃ for 24 hours followed by 45℃ for 1 hour.

To date, we have performed gene expression on a small subset of samples. These preliminary data indicate differences in stress related expression profiles. This can be seen for heat shock protein 90 (HSP90)

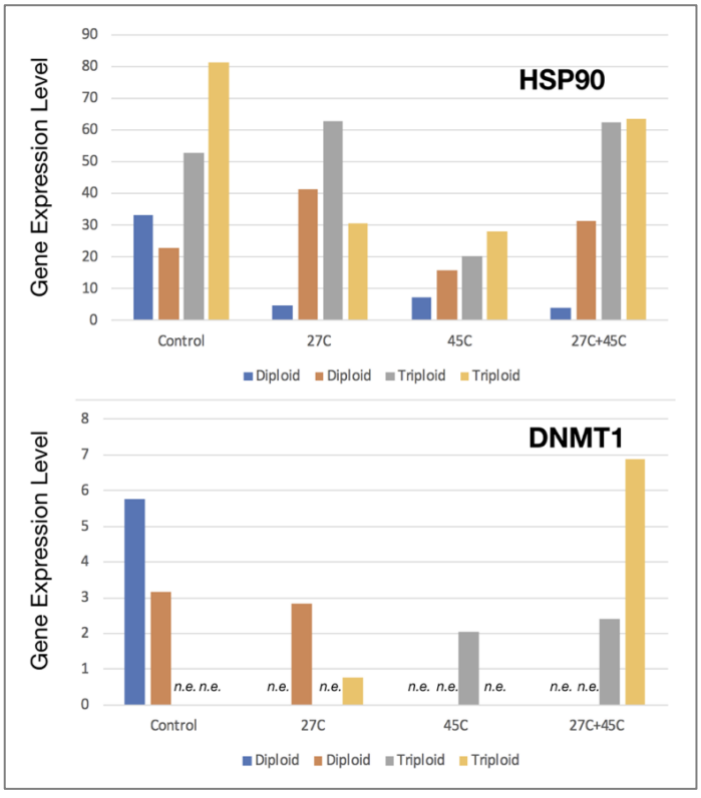

Figure 4. Relative gene expression levels of heat shock protein 90 (HSP90) in diploid (blue and orange; left) and triploid (grey and yellow; right) oysters. *n.e.* indicates no expression detected.

and DNA methyltransferase 1 (DNMT1) (Figure 4) where expression levels under 'ambient' conditions are different between two diploid oysters compared to two triploid oysters (*n.e.* = no expression). Likewise, under combined stress conditions (27℃ for 24 hours followed immediately with 45℃ for 1 hour) gene expression differs between triploids and diploids. While these preliminary data suggest a fundamental physiological difference in stress response between triploid and diploid oysters, a proper and comprehensive characterization is critical to better defining a phenotype.

2.C *Improve aquaculture workforce training*

Improved and expanded training opportunities for an aquaculture workforce is our third objective. Workforce training is a critical need nationwide, as the shellfish industry recently indicated (Walton, pers. comm). For example, according to a recent survey of the US west coast industry (n = 22), only 10% of hired technicians had any formal training in shellfish production. Of respondents who were able to make predictions of future workforce needs, 93% indicated that increased workforce training for shellfish technicians would benefit their organization.

Despite the great need for trained shellfish technicians, there are very few colleges and universities that offer specialized training in shellfish aquaculture on the US west coast. Bellingham Technical College (BTC) is one of these, and it is the only accredited program on the west coast that offers a two-year Associate in Applied Science degree in hands-on technical and scientific training for shellfish aquaculture. BTC graduates provide a much-needed stream of trained technicians who enter the shellfish aquaculture industry across the country as farm managers, hatchery technicians, algae culturists, and nursery (e.g. FLUPSY) operators. See Section 3 for detailed work plan.

3. General Work Plan and Milestones

This section provides the work plans associated with the three primary objectives of the proposal:

A. Establish a national, industry-driven Hub serving as a biological material repository, idea incubator, and education resource.

B. Tackle major issues hampering the efficiency and efficacy of aquaculture (e.g., mortality associated with triploid oysters).

C. Improve aquaculture workforce training through curricula development and industry technology transfer.

3.A *Establishment of CHESS*

Foremost with respect to this proposal is the establishment of a topical hub designed to address challenges in reproductive control in shellfish (Figure 3). This hub (CHESS) will be developed for long-term sustainability and proactive, strategic

prioritization of research needs, with the immediate challenge addressed is that of triploid mortality. The specific team assembled around this bottleneck includes expertise from the Virgina Institute of Marine Sciences, Auburn University, University of Rhode Island, and University of Washington. Team coordination will be managed through Washington Sea Grant (Callender/Vadopalas) with quarterly virtual meetings for coordination of effort, development of adaptive approaches, and discussing emerging technologies and areas of focus. Within the larger group, focused teams will coordinate on a more regular basis on topical areas including field grow-out, physiology, curricula development, and industry engagement. Beyond the integration of expertise from across the US, there are several tangible aspects of the CHESS including serving as a (A.1) biological material repository, (A.2) idea incubator, and (A.3) education resource.

3.A.1: *Biological Material Repository*

As part of the proposed work, samples from populations of oyster undergoing mortality events in controlled grow-out experiments, on industry farms, and from controlled lab-based seed trials (all detailed below) will be collected. A majority of these samples will be processed as part of Objective B; however, we expect to archive all samples to lay the foundation for future research efforts. All samples will be shipped to Washington Sea Grant (Vadopalas) where they will be split and maintained at the University of Washington (Roberts) and the University of Rhode Island (Putnam). Samples will comprise ctenidia, mantle, and gonad stored in appropriate preservatives. Upon arrival to Washington Sea Grant, a public sample database will be updated. The repository will be fully implemented by the end of Year 1.

3.A.2: *Idea Incubator*

Annual working groups will be implemented on reproductive barriers in marine aquaculture for knowledge, data transfer, prioritization of knowledge gaps, and designing experiments and studies to solve problems. CHESS partners will identify areas for future research, adapt the team to tackle priority areas, and bring in additional expertise as needed to address barriers to ecologically sustainable and economically viable shellfish aquaculture. These working groups will be coordinated with national aquaculture meetings and will be facilitated in part by the number of other research efforts the Co-PIs are involved in. We also will hold virtual working group meetings. Any virtual working groups will be advertised, with targeted effort to industry members, and available for participation by anyone interested. Details of the working group outcomes will be made publicly available on the dedicated online portal.

3.A.3: *Education resource*

Washington Sea Grant will provide information on the progress in near real-time on a dedicated online portal. Information will include data related to outplant trials and experimental trials as well as the database of samples we collect. Co-PIs (Roberts and Putnam) have labs that use online electronic notebooks which will be featured on the portal. This will include access to data and code so other would be able to reproduce the work or interrogate datasets for answers to questions that the research groups are not pursuing. The online portal will also feature a means for the public to directly inquire about research activities. Educational resources (paper and electronic) will be distributed to Sea Grant Extension Specialists and at regional shellfish growers conferences. The online portal will be established in the first month of the project and updated regularly. We expect other educational resources to be published on an annual basis.

3.B *Identify factors responsible for triploid performance*

The core research objective of the proposed work is to identify the physiological factors and environmental conditions responsible for contributing to mortality of *C. virginica*. This will be done by examining the diversity of phenotype among pedigreed triploid families (B.1), describing the physiological characteristics associated with triploid morbidity and mortality in the field trials and the physiological cascade leading to triploid morbidity and mortality in stressor experiments (B.2), and generating a phenotypic predictive capacity for triploid morbidity and mortality (B.3).

3.B.1: *Characterize the diversity in phenotype in triploid families in structured field trials*

Families of triploid *C. virginica* will be used as the basis of structured field trials designed to characterize the diversity of phenotypic responses associated with triploid mortality. The Aquaculture Genetics and Breeding Technology Center (ABC) at the Virginia Institute of Marine Science (VIMS) now routinely runs progeny tests of triploids for determining heritability of traits in its constituent diploid (female) and tetraploid (male) parents. Triploid families are produced by crossing a tetraploid male with a diploid female, both of which have a pedigree association in the breeding program. Families produced by Allen will be provided to the Auburn University Shellfish Lab (AUSL) for grow-out in Alabama (pending regulatory approval – see below for concerns and alternatives). Spawning will occur April-May in years 1 and 2, creating YC2020 (year class) and YC2121. For VIMS only, there will be an opportunity to follow cohorts from the YC2019 because the spawns are currently already scheduled. Nested pair matings of triploid families will be accomplished using at least 10 male and female parents, creating at least 20 family groups for this project (Figure 5). All families will be grown in a nursery in the York River prior to deployment for the progeny test. Prior to deployment, 15 samples from each triploid family will be verified for ploidy via flow cytometry by staining cells in DAPI/DMSO.

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Figure 5: Nested design for creating triploid families for controlled field trials. As a matter of course, tetraploid half-sibs will also be produced for possible use in breeding for resistance to triploid mortality.

Families produced during the spawning season will be overwintered on the ABC longline system in the York River in front of VIMS. In early spring, triploid families will be deployed to three sites in the Chesapeake Bay: the York River, the Choptank River, and Nandua Creek. The York River (15-25 ppt) and Choptank River (5-12 ppt) have been the primary sites for ABC's family breeding program. Nandua Creek is a commercial site with regular episodes of triploid mortality. All families will be deployed in three replicate baskets and reared on an adjustable long line system at York River and Choptank River. For Nandua Creek, triploid families will be deployed in three replicate bags in bottom cages. In our experience, three replicates are adequate to obtain a reasonable variance on the measure of mortality. All replicates will start with 125 oysters. Triploid mortality in Virginia first manifests in late May/ June. We will sample all 20 triploid families before the expected mortality event (early May) and archive them. After the mortality window, we will identify the top three and bottom three families for physiological analysis, pre- and post-mortality.

In the Gulf of Mexico, pending approval by regulatory authorities, AUSL will receive certified disease-free eyed larvae of each family produced at ABC to set on site. This design allows the greatest scientific advance. If approval is not obtained, AUSL will replicate the breeding design implemented by ABC, using local broodstock, including Gulf tetraploids.

All Gulf families will be grown in a nursery at AUSL research farm (either Grand Bay or Portersville Bay) prior to deployment for the progeny test. Prior to deployment, 15 samples from each triploid family will be verified for ploidy via flow cytometry (FCM) by staining cells in DAPI/DMSO. Usually, families produced during the spawning season are overwintered on at the AUSL nursery sites. In late winter/early spring, triploid families will be deployed to three sites in Alabama: Grand Bay Oyster Park (GBOP, 15-25 ppt), Bayou Sullivan Oyster Park (BSOP, 5-15 ppt) and Navy Cove Oyster Company (NCOC,

10-20 ppt). NCOC is a commercial site with periodic episodes of triploid mortality. All families will be deployed in three replicate bags and reared in a floating cage system for all sites, and all replicates will start with 125 oysters. Triploid mortality in Alabama first manifests in March/April. We will sample all 20 triploid families before the expected mortality event (February) and archive them. After the mortality window, we will identify the top three and bottom three families for physiological analysis, pre- and post-mortality.

At the pre- and post-sampling dates, triploid families from all sites experiencing mortality will be measured for traits relevant to the commercial industry: survival, shell morphometry, whole weight, and meat weight. For survival (i.e., triploid mortality), the number of live oysters will be counted from each replicate bag or basket. All other traits will be measured from a sample of 10 individuals from each replicate. Whole weight is the total weight of the live oyster and meat weight will be measured after removing the shell and draining residual liquid. Ctenidia, mantle, and gonad tissue will be divided, snap frozen, and shipped to UW and URI.

3.B.2: *Describe the physiological characteristics associated with triploid mortality in the field outplants*

The structured field trials in both the Chesapeake Bay and in the Gulf of Mexico will be used to identify the "winning" and "losing" families with respect to performance. We will characterize their physiological trait, both to understand the mechanistic basis for triploid mortality, and to identify potential molecular, cellular, or organismal biomarkers that can be used to select triploid lines for better survival. In addition, these biomarkers will be tested on juveniles in the stressor trials to explore possible concordance between reproductive and juvenile stage responses.

In order to describe the physiological underpinnings of differential performance in triploid oysters we will use the complementary approach of coupling indicators of fundamental metabolism and energetics with gene expression. Physiological assays will be conducted at the whole organism level via controlled lab stressor experiments described below, and at the cellular and molecular levels via field outplant and controlled lab stressor experiments. At the organismal level, metabolic rate will be assessed as oxygen uptake in the dark (accounting for a blank chamber of filtered seawater) in sealed chambers containing one individual, measured by Presens fiber optic oxygen electrodes with temperature compensation and normalized to mass. To further assess energetic state, samples will be analyzed for total lipids using a phenol-chloroform method (Bligh and Dyer 1959), carbohydrate content using the phenol-sulfuric acid method (Dubois et al. 1956), and protein content using the Pierce BCA assay (Jeung et al. 2016)), as well as ash free dry weight (AFDW) (Byrne et al. 2018). At the cellular level metabolic rates will be compared to measurements of metabolic function via citrate synthase enzyme activity

(Farrell et al. 2015) and antioxidant capacity via catalase activity. For gene expression analysis, QuantSeq 3' mRNA-Seq (Moll et al. 2014) will be used, as this will provide a significant increase in cost-efficiency compared to traditional RNA-seq. This sequencing approach will identify cellular pathways associated with family, family susceptibility, and environment in connection to morbidity and mortality.

Physiological variables will be analyzed initially with PCA to characterize multivariate physiology and its separation by environmental, family, and family susceptibility and tested with PERMANOVA. Further univariate analyses will be conducted for each response variable individually using the above independent variables. Gene expression will be analyzed for differentially expressed genes by independent variables, as well as weighted gene coexpression network analysis (WGCNA (Langfelder and Horvath 2008)), which identifies gene modules showing similar expression patterns and the linkages between these gene modules and traits. Ultimately, metrics identified to predict improved performance will be used in broodstock selection programs.

3.B.3: *Conduct a series of controlled lab stressor trials to describe the physiological cascade leading to morbidity or mortality*

Oysters seed (juveniles) generated by ABC/VIMS will be sent to Washington State (BTC) for controlled stress trials. In addition, juvenile triploid *C. virginica* grown in Washington will be used. These lab stressor trials will complement field trials by providing the ability to provide biomarkers in juvenile triploid oysters that are predictive of performance. Trials will be conducted under quarantine at the Perry Center, which has wet lab space available to accommodate the recirculating saltwater systems for the triploid stressor trials. Stressors will include extended exposure to air at elevated temperature, low algal ration, and low salinity. Trials will be performed with both single and multiple stressors at such a level determined to be near-lethal. Trials will be conducted for 1 month durations with samples at the beginning, mid-point, and termination of the trials. These trials will provide information on fundamental differences in the physiological performance of triploid oysters at an early life stage, outside of the window of the triploid mortality observed in adult oysters on the farm. We expect that there will be physiological signatures of performance in the juvenile oysters that will be predictive of adult phenotypes. Physiological metrics used to characterize structured field outplant oyster will also be used for seed with combined results used to generate phenotypic predictive capacity.

3.B.4: *Generate a phenotypic predictive capacity for triploid mortality through the integration of mortality associated phenotypes in the field and lab*

Integration of the data from field and lab experiments is needed to provide capacity to predict triploid morbidity and mortality prior to loss, and to inform breeding approaches to minimize morbidity and mortality. We will use a hierarchical approach of ordinal logistic regression (i.e. a multivariate extension of a generalized linear modeling approach) and AIC model selection (Safaie et al. 2018) to determine how the relative log odds of morbidity or mortality depends on the physiological, cellular, and molecular variables and their interactions. First, we will generate three models at the molecular, cellular, and organismal levels. The Molecular model includes gene expression modules of co-expression generated by WGCNA from the QuantSeq 3' mRNA-Seq. The Cellular model includes citrate synthase activity, catalase activity, lipids, proteins, carbohydrates, and AFDW. The Organismal model includes shell morphometry, whole weight, and meat weight. This modeling approach will be tested to rank the dominant biological predictors of mortality and morbidity using a stepwise model selection approach, based on AIC weights at each biological level. We will check for correlation among predictor models using variance inflation factors. Parameters that are correlated will be reciprocally removed for all possible comparisons. Second, the reduced models (at each biological level) identified by model selection will then be tested against each other. The selection approach will compare the three models for the greatest predictive capacity for mortality and morbidity. Together this will provide a mechanistic understanding at the three model levels as well as identifying the best fit predictive model that could explain the dominant factors associated with triploid mortality and together provide biomarkers for early testing and detection.

3.C *Improve aquaculture workforce training through curricula development and industry technology transfer*

As part of the long-term sustainability of CHESS we propose a third objective focused on developing educational curricula and establishing industry partnerships.

3.C.1 *: Create and implement shellfish aquaculture curriculum and outreach materials.*

CHESS will play a strong role in the generation and improvement of shellfish aquaculture curriculum and outreach materials. The existing shellfish aquaculture curriculum at BTC, VIMS, Auburn, and UW will be expanded to include triploid oyster production, algae culturing, broodstock management, biosecurity measures, and recirculating aquaculture systems. BTC Fisheries and Aquaculture Sciences instructors will organize hub meetings with industry and research experts to inform novel design of cutting edge curriculum and current gaps. The curriculum development will be initiated in Year 1. In Year 2 the hub partners will implement a trial run of the new curriculum and

incorporate industry feedback and changes into a finalized curriculum, which will be taught, and improved upon, in Years 2 and 3.

BTC will develop and incorporate curriculum to include biosecurity measures in shellfish aquaculture and hands-on experience conducting stress trials for triploid Eastern oysters. The biosecurity module will include aquatic disease, pest, and invasive species prevention measures for both hatchery and field operations. For example, case histories of breached security will be presented to the students who will be tasked with finding alternative solutions and preventative measures.

Additionally, the hub will host K-12 and community outreach events to strengthen public knowledge of shellfish aquaculture and to increase ocean literacy in the community. This will occur in conjunction with all of the partnering institutions and supported in part through the direct Sea Grant relationships. For instance, the Perry Center associated with BTC is conveniently located in downtown Bellingham and regularly hosts K-12 and community tours. As part of this project, BTC will develop new outreach materials (e.g., brochures, hand-outs, activities) to incorporate shellfish aquaculture into the existing community education framework. K-12 and community members attending Perry Center tours will also see the triploid oyster trials firsthand and learn more about shellfish hatchery production, current challenges and benefits in oyster cultivation, and NOAA's work to support the shellfish aquaculture industry.

As the hub leads effort to develop improved aquaculture curricula, the content will be integrated into courses (e.g. *Integrative Environmental Physiology* at UW, *Marine Environmental Physiology* and *Risking our Reefs* at URI, and *Shellfish Aquaculture in the Gulf of Mexico* at AU) at the CHESS partner institutions. Hub partners will also contact and visit other institutions to present and provide these resources. To provide very broad reach, Washington Sea Grant will consolidate and host these materials in a centralized online portal as part of their web presence. This will include K-12 curriculum, aquaculture training guides, genetics tutorials, etc. CHESS will also engage students at UW, URI, VIMS and AU by providing a distributed undergraduate and graduate seminar class taught by the team and other aquaculture experts. In this way we can engage future CHESS hub members and enhance the sustainability of this topic and project.

***3.C.2**: Implement industry sampling program that fosters technology transfer*

We will establish a system for growers to submit samples of their triploids during mortality events, using three 'call centers': UW for the Pacific Northwest, VIMS for the east coast and AUSL for the Gulf coast. Industry members will make initial contact with the respective point of contact, where team members will assure that standardized questions are asked and basic data are collected. Industry members will be given directions about sample collection and all samples will be sent to the University of Washington where they will be dissected and essentially split, for gene expression analyses (Roberts Lab) and

physiological assays (Putnam Lab). Samples will be analyzed as described above and results and interpretation will be made available to the submitter and added to our sample database. When samples are analyzed as described above, we will develop educational resources available in both hard copy documents and as interactive online modules. Those sending samples will be provided concise summaries of the results obtained. Collectively, this response network will also help increase our understanding of the timing and extent of the geographic range for triploid mortality. To the extent possible, we will try to incorporate samples from *C. gigas* mortality events on key west coast farms, to at least archive samples for later comparison with *C. virginica* samples.

While recommendations on broodstock selection and preferred farming protocols will be made publicly available, the "call center" adds considerable value for both CHESS and the industry. Looking beyond the current work, the more relationships the hub has with industry will ensure industry needs and challenges are addressed. For the team working on the current proposal, this will allow greater diversity of samples to improve predictive capacity. Finally, we predict these partnerships will improve technology transfer. In this case, we are specifically referring to our ability to efficiently provide recommendations on how to improve triploid performance. This information will include details on which stocks are most likely to have desired performance traits and which environmental conditions will limit mortality events.

Milestones and Timelines

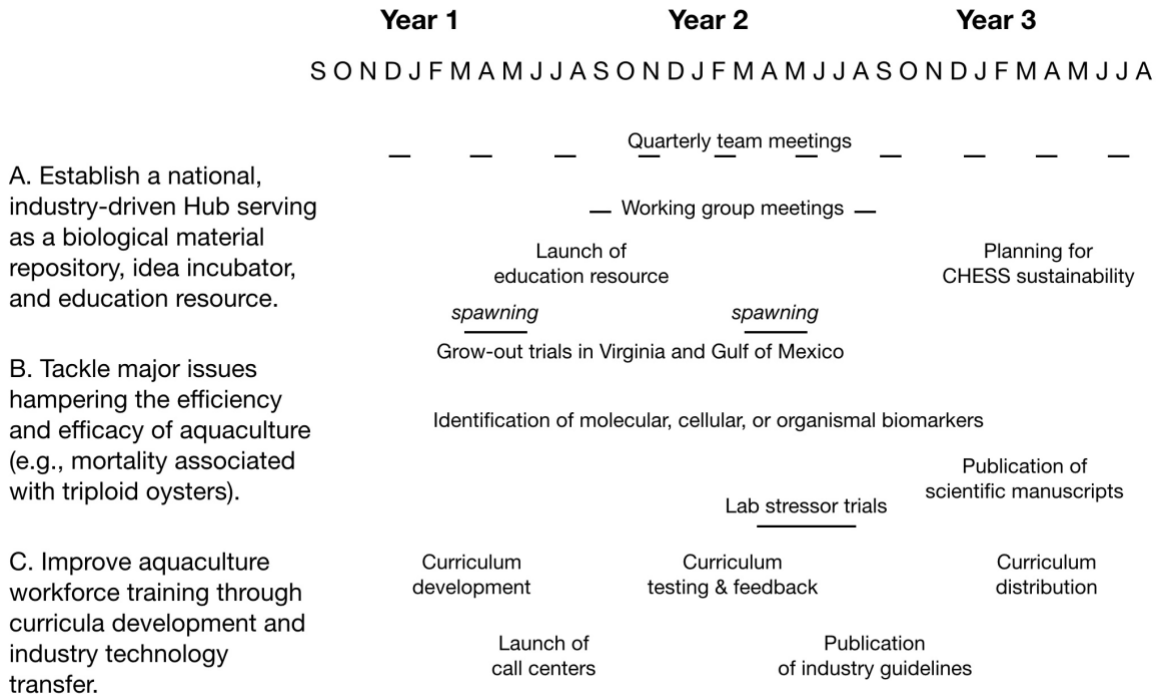

Figure 6. Project objectives along with major milestones and anticipated timelines

Assessment of Work Plan

The CHESS project endeavors to advance the topical hub of shellfish sterility to continually target the next appropriate phase for development. This will ensure we are using adaptive planning for both current and future industry needs. In order to do this we will directly communicate our results and potential solutions to production barriers with the commercial shellfish aquaculture industry, use this information to assess the success of our plan of work, continue to work with industry to identify knowledge gaps, and course-correct as needed. The progress towards goals and success of objectives will be assessed as follows:

- Objective A will be assessed as electronic communications and quarterly team meetings of the PIs, number of interactions with stakeholders, samples received and archived in the repository, virtual presentations to stakeholders for gaps and idea generations, and establishment of a centralized resource portal.

- Objective B will be assessed as the completion of field grow-out trials, experimental treatments, the collection of samples, the completion of the physiological assays, the analysis, archival, and publication of datasets and the training of the team and workforce. Direct progress indicators will include numbers quantified from: protocols, sample databases, sequence files, data presentations, peer reviewed manuscripts, and trainee advancement through career stages.

- Objective C will be assessed as completion of lesson plans and curricula development, stakeholder interactions, protocol transfers to industry, number of students/workforce members trained in aquaculture skills, and completion of a curriculum distribution portal.

4. Anticipated Outcomes

The CHESS project provides an approach to overcoming barriers to maximize the benefits of reproductive sterility for sustainable shellfish aquaculture. CHESS provides a functional network of professionals to facilitate integrated data analysis focused on an industry-driven problem that limits sustainability in shellfish aquaculture. The hub will develop an archive of biological material for future research, identify physiological processes associated with triploid mortality and performance, prioritize future research foci for ecological and economical sustainability, and facilitate an educational portal focused on reproductive impairment in shellfish for the aquaculture industry, resource managers, and the research community.

The potential for breeding constituent diploid and tetraploid parent families for the purpose of creating commercial triploid oysters that exhibit better survival under non-ideal

growing conditions is another anticipated outcome of the proposed work. By describing physiological and phenotypic diversity, we expect to describe physiological biomarkers that can be implemented in new breeding schema to optimize shellfish performance and production. With Washington Sea Grant leading the current effort there are significant resources available to support an education and outreach portal for industry, researchers, and the general public.

An outcome that will have the greatest short-term (nearly real-time) impact on the aquaculture industry will be guidelines on how to minimize oyster mortality. This will be attainable through data generated by replicated grow-out trials in Virginia and Alabama, complemented with industry submitted data. The collaborative nationwide network of CHESS facilitates the integrated data analysis that will allow us to provide recommendations. These guidelines will be made available a) directly to all industry participants, b) on our web portal, c) at regional shellfish industry meetings, and d) through local Sea Grant Aquaculture and Outreach Specialists.

We also anticipate the significant expansion of shellfish aquaculture training, including workforce training and development. This expansion will take the form of valuable hands-on experiential learning opportunities for students interested in shellfish aquaculture, through enhancement of wet lab capabilities. In addition, the shellfish aquaculture curriculum will be broadened to include a biosecurity module, science literacy via direct experience conducting experiments, as well as integration of the wet lab enhancements. Curricula will be implemented in all partnering institutions and made available for general use.

5. Coordination with Sea Grant program elements

As the Project Lead, Washington Sea Grant will centralize the efforts for research, outreach, and education. here will be considerable synergy among Washington Sea Grant, Rhode Island Sea Grant and the Mississippi-Alabama Sea Grant Consortium through participation in quarterly CHESS meetings to coordinate effort, develop adaptive approaches, discuss emerging technologies and areas of focus. Sea Grant programs will coordinate on a more regular basis on specific research topics, outreach endeavors, and provision of learning opportunities for industry, students, and resource managers. Vadopalas (Washington Sea Grant - CoPI) will be coordinating all aspects of biological material repository infrastructure as well as outreach and education. Washington Sea Grant has a staff of 25 with expertise in communication, outreach, and research. Walton (Auburn University and Mississippi-Alabama Sea Grant Oyster Aquaculture Specialist - CoPI) will facilitate extension in the Gulf of Mexico (including logistics of industry sample collection) as well as develop a mobile phone application for data collection from shellfish farmers.

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